Transcriptionally driven cruciform formation in vivo

Andrey Dayn, Sergei Malkhosyan[1] and Sergei M.Mirkin*
Department of Genetics, University of Illinois at Chicago, 808 S.Wood Street, Chicago, IL 60612 and
[1]California Institute of Biological Research, La Jolla, CA 92037, USA

ABSTRACT

We studied the formation of $d(A-T)_n$ cruciforms in E.coli cells by probing intracellular plasmid DNA with chloroacetaldehyde followed by fine analysis of modified DNA bases. $d(A-T)_{16}$ sequences were inserted into specifically designed plasmids either upstream of a single trc promoter, or between two divergent trc promoters. We found that in both cases, induction of transcription by IPTG leads to the transition of the $d(A-T)_{16}$ stretch into a cruciform state. In the case of two divergent promoters, we observed cruciform formation even without IPTG. Enhanced cruciform formation correlates with the elevation in promoter activity as defined by the opening of the promoter at the −10 to +2 positions. We conclude that transcriptionally driven negative supercoiling provokes cruciform formation in vivo.

INTRODUCTION

Cruciform structures formed by invertedly repeated DNA sequences have been detected in practically all circular DNA isolated from different living cells (reviewed in 1). The formation and possible functional role of these structures in vivo are less well understood. Recently, we and others provided direct evidence for cruciform formation in E.coli cells by probing intracellular DNA with chloroacetaldehyde and osmium tetroxide (2,3). There are also some indirect indications of cruciform formation in vivo (4,5).

In the course of these studies, several important conclusions have been made. First, all detected in vivo cruciforms were formed by AT-rich inverted repeats, in particular $d(A-T)_n$ sequences. GC-rich inverted repeats have slow kinetics of cruciform formation that lead to a kinetic barrier for their formation in vivo (6). Second, the mean DNA superhelicity in E.coli cells is usually insufficient for cruciform extrusion (2,3,7,8). Therefore, cruciforms were observed in cells undergoing stresses that increase torsional tension in vivo, e.g., chloramphenicol treatment, osmotic shock, anaerobiosis (2,3). Thus, the combination of rapid relaxation kinetics and an elevated level of DNA supercoiling leads to cruciform formation in vivo.

A traditional view of DNA supercoiling in E.coli cells is that it is regulated through the counteraction of DNA gyrase and DNA topoisomerase I (reviewed in 9,10). Another potentially powerful mechanism for inducing local changes in DNA supercoiling is transcription (11). It was suggested that plasmid DNA containing two divergently working promoters may be separated in vivo into two supercoiling domains: the upstream DNA segment accumulates extra negative supercoils, while the downstream one acquires extra positive supercoils (11,12). This 'dynamic' DNA supercoiling was detected in E.coli cells after selective blockage of either DNA gyrase (13) or topo I (14). In normal cells, where all topoisomerases are functional, the actual value of transcriptionally driven DNA supercoiling is not yet clear. This value may strongly depend on the relative orientation and strength of the promoters (12), efficiency of transcription-translation coupling (11,15), membrane association of protein products (15), relative orientation of other regulatory elements (e.g. replication origins) (16), etc.

If transcription does elevate DNA supercoiling upstream of a promoter in wild type E.coli cells, it could provoke the formation of non-B DNA structures in vivo. Indeed, quite recently this was found to be true for Z-DNA forming sequences inserted between divergent promoters (17,18). Here we studied cruciform extrusion for a $d(A-T)_{16}$ sequence cloned in a plasmid either upstream of a single trc promoter or upstream of two divergent trc promoters. Transcription from the trc promoter, in our case, was repressed due to an excess of lac-repressor encoded by the $lacI^q$ gene located in the same plasmid. It can be induced by the addition of IPTG into the growth media. Using direct chemical probing of intracellular DNA, we found that under IPTG treatment cruciforms were indeed observed. Surprisingly, we didn't see any significant difference between single and divergent promoters, or between membrane associated and cytoplasmic proteins encoded by reference genes.

In balance, we believe that transcriptionally driven negative DNA supercoiling upstream of a promoter may cause cruciform formation. The supercoiling density required for the $d(A-T)_{16}$ cruciform extrusion under physiological ionic strength in vitro is −0.05. Thus, we suggest that the actual torsional tension in vivo upstream of the trc promoter may reach this value at least in some plasmids. However the lack of any difference between the single and divergent promoters indicates that this occurs only transiently, and we do not observe the appearance of highly supercoiled domains, most probably due to the action of DNA topoisomerases.
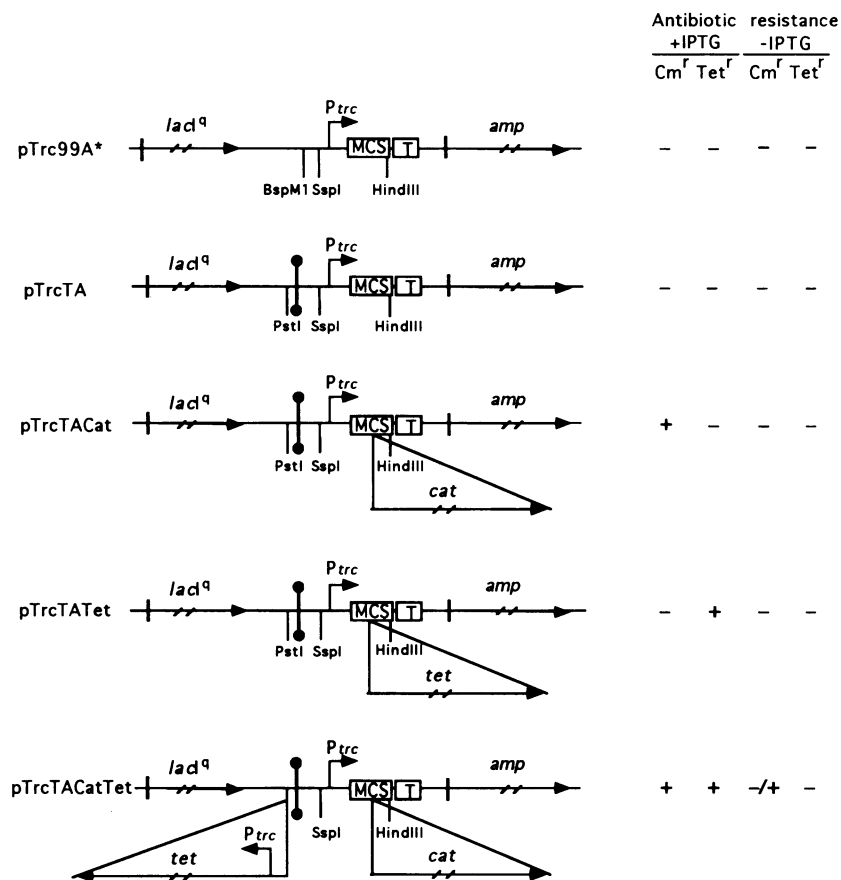
* To whom correspondence should be addressed

Figure 1. Plasmid construction. pTrc99A* is a derivative of pTrc99A with the PstI-BspMI sequence deleted from the multiple cloning site. MCS—multiple cloning site; T—T1 and T2 terminators of the *rrnB* gene; vertical dumb bell shows the location of d(A-T)$_{16}$ insert. Antibiotic resistance was estimated by colony formation on agarized LB with either chloramphenicol or tetracycline.

MATERIALS AND METHODS

Plasmid DNA

Plasmid pTrc99A (19) was obtained from Pharmacia. Plasmid pAT32, containing the d(A-T)$_{16}$ insert in the SmaI site of pUC19 polylinker, was described in (20). Plasmid construction is described in Results. All plasmids were maintained in the *E. coli* strain DH5α (21). Plasmid DNA was isolated by the alkali lysis protocol, followed by equilibrium centrifugation in cesium chloride—ethidium bromide gradients (22).

Chemical modification of intracellular DNA

Overnight culture of DH5α carrying a plasmid of interest was 100-fold diluted in 2,000 ml of fresh LB broth with 100 μg/ml of ampicillin and grown for 2 hr at 37°C with vigorous aeration. Then the culture was split into 2 halves, IPTG was added to one half up to 1 mM, and subcultures were incubated for 60 min. at 37°C. Cells were then centrifuged and resuspended in 10 ml of potassium phosphate buffer (50 mM KH$_2$PO$_4$, pH 7.5; 150 mM NaCl), followed by the addition of 650 μl of a 50% solution of chloroacetaldehyde in water (Fluka). 1 mM IPTG was added to the sample that was previously incubated with an inducer. The modification continued for 20 min. at 37°C with shaking. Then cells were 10-fold diluted in a cold potassium phosphate buffer,

centrifuged, washed by TES buffer, and plasmids DNA was immediately isolated.

Location of modified DNA bases at a sequence level

(A) Maxam – Gilbert technique. For single promoter constructs, 2 μg of plasmid DNA were digested by XbaI, labeled with α-^{32}P-dCTP by Klenow enzyme, then digested by NcoI. The resulting 253 bp-long end-labeled fragment was isolated from a low melting agarose gel. In the case of the divergent promoter construct, 2 μg of plasmid DNA were digested by BspMI and EcoRV, labeled by α-^{32}P-dATP and Klenow enzyme on the 3' end, followed by purification of the resulting 320 bp-long end-labeled fragment from low melting agarose gel. End-labeled samples were treated with hydrazine in high salt, according to the standard Maxam – Gilbert protocol (C reaction) (23). DNA samples were then cleaved by 1M piperidine and run on a 10% sequencing gel.

(B) Sanger technique. Two primers: 5'-GCGCCTTGAGCGA-CACGA-3', and 5'-GTGCCTGACTGCGTTAGCAA-3' were synthesized using an ABI 394 RNA/DNA Synthesizer. Primer extension was performed with the Sequenase Version 2.0 sequencing kit (US Biochemical) according to the manufacturer's protocol, but instead of dideoxyNTP termination, 1 μl of a dNTP mixture of 2.5 mM each was added to the labeling reaction for 5 min. at 37°C.

2-dimensional gel-electrophoresis

A mixture of DNA topoisomers of pAT32 was obtained by treatment of plasmid DNA with DNA Topoisomerase I (BRL) in the presence of varying concentrations of EtBr as described in (20). 2-D electrophoresis was performed in a 1.5% agarose gel as previously described (2). The separation in the first dimension was carried out in 0.2M sodium phosphate buffer (pH 7.0).

RESULTS

Plasmid construction

To study the influence of transcription on cruciform formation, we inserted the cruciform forming $d(A-T)_{16}$ sequence upstream a tightly regulated promoter, and looked for changes in DNA structure *in vivo* when transcription is either induced or repressed. As starting material, we used plasmid pTrc99A (19). This vector contains a strong *trc* promoter (a hybrid of *trp* and *lac* promoters), a *lacZ* ribosome binding site, a multiple cloning site, and the transcriptional terminator of the *rrnB* gene. To provide the repression of *trc* promoter, it also contains the lacI^q repressor gene (Fig.1). The plasmid pAT32 containing a $d(A-T)_{16}$ insert in the SmaI site of the pUC19 polylinker was described by us before (20).

We first removed the PstI and BspMI sites from the multiple cloning site of pTrc99 for convenience during further cloning. Then we cloned an EcoR1-HindIII fragment from pAT32, which included pUC19 polylinker and the $d(A-T)_{16}$ stretch into the BspMI site of pTrc99 which is located 174 bp upstream from the transcriptional start site. Next we inserted either a *cat* promoterless cartridge (EcoR1-Ban1 fragment from the pCAT plasmid) or a *tet* promoterless cartridge (HindIII-Ava1 fragment from the pBR322) into the SmaI site of the pTrc polylinker (Fig.1). As result we obtained two plasmids where the $d(A-T)_{16}$ sequence was located 174 bp upstream of the same P*trc* which determined transcription of genes coding either a cytoplasmic (Cat) or a membrane attached (TetA) protein.

Finally, we cloned the P*trc-tet* cartridge (an Ssp1-HindIII fragment from the pTrcTATet plasmid) into the unique blunt-ended PstI site of the pTrcTACat plasmid, which is located immediately upstream of the $d(A-T)_{16}$ stretch (Fig.1). As a result we obtained a plasmid with two divergent *trc* promoters. The $d(A-T)_{16}$ stretch is located in a 279 bp-long promoter-bounded segment, 174 bp upstream of the P*trc-cat* start site and 73 bp upstream of the P*trc-tet* start site. This segment may accumulate extra negative supercoils after the induction of transcription.

To check the efficiency of transcriptional repression we followed antibiotic resistance of *E. coli* cells containing the above plasmids in the presence or absence of the transcriptional inducer IPTG. As demonstrated in Fig.1, plasmids containing single *cat* or *tet* genes provide resistance to chloramphenicol or tetracycline, respectively, only in the presence of IPTG. The plasmid with divergent *cat* and *tet* genes provides resistance to both antibiotics in the presence of IPTG, though minor chloramphenicol resistance was observed even without the inducer. Generally, in all cases lacI^q effectively represses P*trc* unless IPTG is added to cells. Thus, comparing DNA peculiarities *in vivo* in the presence or absence of IPTG, one can estimate the influence of transcriptional status on DNA structure.
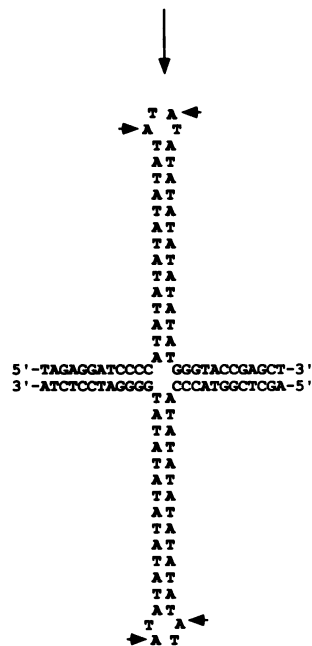

Figure 2. Cruciform formation by the $d(A-T)_{16}$ sequence. Arrows indicate adenines modified by chloroacetaldehyde.

Influence of transcription on cruciform formation

As a tool for detection of cruciforms *in vivo*, we used the chemical carcinogen chloroacetaldehyde (CAA). CAA interacts specifically with bases in DNA, forming imidazole rings between the N3 and N4 positions of cytosine or the N1 and N6 positions of adenine (24,25). It can also react with guanines forming imidazole rings between the N1 and N2 or the N2 and N3 positions (26). As a result, the stability of phosphodiester bonds decreases, and after Maxam—Gilbert DNA sequencing one can see additional bands corresponding to CAA-modified cytosines on the purine ladder and to CAA-modified purines on the cytosine ladder (27). In double stranded DNA, these positions are involved in hydrogen bonding and are not reactive to CAA. Distortions in a regular double helix could lead to the accessibility of those base-pairing positions to CAA. This is why CAA was used for the detection of altered DNA conformations including H DNA (27), Z DNA (28), and cruciforms (2). We have previously shown (2) that cruciform formation by $d(A-T)_n$ sequences in supercoiled DNA leads to CAA reactivity at two central adenine residues which correspond to the looped out bases in cruciforms (Fig.2). Thus, one can estimate the efficiency of cruciform formation following CAA modification of the central adenines. CAA penetrates bacterial cells (2,29) which make it possible to study cruciform formation *in vivo* .

For chemical modification of intracellular DNA, exponentially growing *E. coli* cells containing plasmids described above were first incubated for 60 min. with or without IPTG, followed by 20 min. CAA treatment as described in Materials and Methods. CAA was then washed from the cells, plasmid DNA was isolated, and modified DNA bases were defined at a sequence resolution by the Maxam—Gilbert protocol. The results for plasmids with
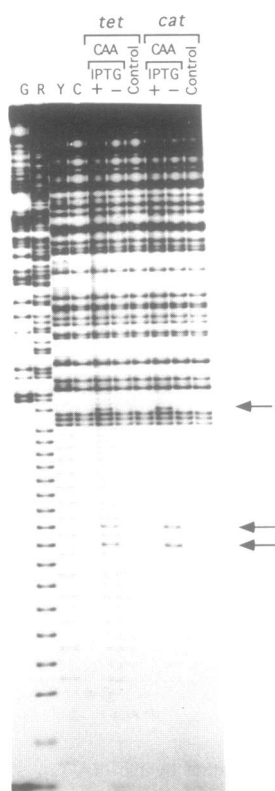
Figure 3. Cruciform formation *in vivo* in plasmids with a single *trc* promoter. *tet*—pTrcTATet plasmid; *cat*—pTrcTACat plasmid; control—DNA from unmodified cells, CAA—DNA from chloroacetaldehyde-modified cells. G, R, Y, C—standard Maxam–Gilbert sequencing reactions. In the rest of the samples, end-labeled plasmid DNAs were treated by hydrazine in high salt (C reaction) followed by piperidine treatment. Arrows indicate adenines modified by chloroacetaldehyde.

Figure 4. Cruciform formation *in vivo* in the plasmid with divergent *trc* promoters, pTrcTACatTet. Control—DNA from untreated cells, CAA—DNA from chloroacetaldehyde-treated cells. G, R, Y, C—standard Maxam–Gilbert sequencing reactions. In all other samples, end-labeled plasmid DNAs were treated by hydrazine in high salt (C reaction) followed by piperidine treatment. Arrows indicate adenines modified by chloroacetaldehyde.

a single *trc* promoter are presented in Fig. 3. One can see that in the absence of IPTG, there is no modification within the d(A-T)$_{16}$ stretch. Thus under these conditions we do not detect cruciform formation *in vivo*, which fits precisely with our earlier observations (2). In contrast, after the addition of IPTG one can clearly see the modification of the two central adenines within the d(A-T)$_{16}$ stretch, as well as an adenine located three bases away from the stretch. We believe that this modification pattern shows the loop of a cruciform and some distortions at the B-cruciform junctions. These results directly demonstrate that the transcriptional inducer provokes cruciform formation *in vivo*.

It is important to note that the efficiency of cruciform induction was virtually the same for the P*trc-tet* and P*trc-cat* containing plasmids. TetA protein is known to bind the cell membrane, while Cat protein is a soluble component of the cytoplasm. It was suggested before (15) that the anchoring of synthesizing TetA on the membrane prevents RNA polymerase from rotating along DNA, resulting in an accumulation of supercoils in the template. If it is true that cruciform formation reflects transcriptionally driven torsion tension, then the anchoring protein product is not a crucial factor in this process, according to our data.

To rule out the possibility that IPTG-induced cruciform formation is not due to the direct effect of the inducer on the overall plasmid DNA topology, we provided a control experiment with the derivative of the pTrcTACat plasmid where the d(A-
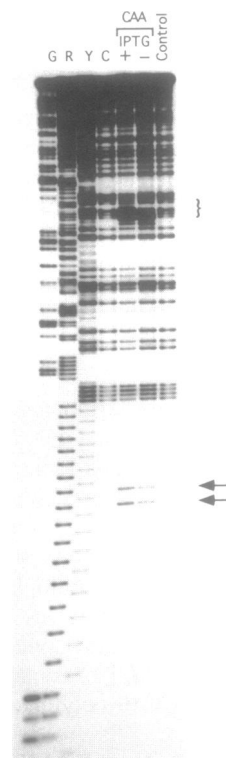
T)$_{16}$ sequence was inserted into the ScaI site inside the *amp* gene in place of the BspMI site upstream of the *trc* promoter. In this case we didn't observe any cruciform formation after the addition of IPTG (data not shown). Thus, we believe that IPTG may change DNA supercoiling locally upstream of P*trc*, rather than in the whole plasmid DNA.

The situation for the plasmid with two divergent *trc* promoters is somewhat different (Fig.4). In this case we see modification of central adenines even in the absence of IPTG, reflecting cruciform formation. Addition of an inducer causes a 1.5 to 2 fold increase in the modification intensity. Thus, activation of transcription again induces cruciform formation. The appearance of cruciform without IPTG may be due to incomplete transcriptional repression in this case. This suggestion is supported by the partial antibiotic resistance caused by this plasmid even in the conditions of repression (Fig.1).

Comparison of the data in Fig. 3 and 4 shows no differences in the rate of cruciform formation in the presence of IPTG between single and divergent promoters. Thus, in our case the relative orientation of transcribing units does influence the rate of cruciform induction (see also Discussion). It should be noticed, however, that we don't know whether both promoters are active in most plasmids in the case of divergent promoters. Additional experiments with independently regulated divergent promoters may clarify this issue.

Our data clearly indicate that d(A-T)$_{16}$ insert may adopt a cruciform conformation at least on a subset of the plasmid
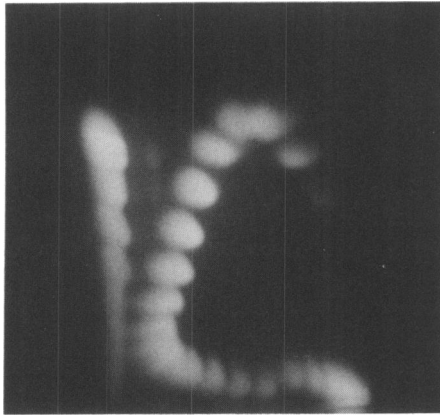
Figure 5. 2-dimensional gel-electrophoresis of pAT32 DNA at physiological ionic strength. First dimension—from the top to the bottom, second dimension—from left to right.

Figure 6. *trc* promoter reactivity to chloroacetaldehyde *in vivo*. **A.** Detection of modified DNA bases in the pTrcTACatTet plasmid was provided by the primer extension technique. Control—DNA from unmodified cells, CAA—DNA from chloroacetaldehyde-modified cells; G, A, T, C—standard Sanger sequencing reactions; in all other ladders, primers were annealed and extended as described in Materials and Methods. Template and partner strands indicate corresponding DNA strand, that served as a template for sequencing and primer extension. Note the DNA polymerase stops opposite modified DNA bases. Thus, a stop site corresponding to G or T in the sequencing ladder is reflected by a modification of C or A, respectively, in the template strand. **B.** Graphical representation of the modification data. Arrows indicate DNA bases where modification was observed in the absence of IPTG, and was elevated by the addition of IPTG. Black boxes indicate DNA bases where we observed modification only in the presence of IPTG.

population. Thus, we may roughly estimate the actual supercoiling density that may be achieved upstream of the *trc* promoter as the superhelical density required for the d(A-T)$_{16}$ transition into the cruciform state. To measure this value we provided 2-dimensional gel-electrophoresis of a topoisomer mixture of a plasmid containing the d(A-T)$_{16}$ insert. We have previously found that the superhelical density of the transition depends dramatically on the ionic strength (2). Thus, we carried out the first dimension of the electrophoresis at 200 mM of Na$^+$, which mimics physiological conditions. As one can see from Fig.5, the d(A-T)$_{16}$ sequence undergoes structural transition at the -13.5 topoisomer, corresponding to $\sigma_{tr} = -0.05$.

P*trc* opening *in vivo*

To study if there exists a correlation between the changes in promoter structure and cruciform extrusion, we studied the pattern of chemical modification of the P*trc* in the presence or absence of the transcriptional inducer. Chemical modification of intracellular plasmid DNA by CAA was described above. Because of the lack of appropriate restriction sites in the vicinity of the *trc* promoter, we used the primer extension technique to locate modification sites. CAA modifies base pairing positions of several DNA bases, thus leading to the termination of DNA polymerases opposite modified bases (2).

The modification results of the P*trc* from the plasmid with divergent promoters are presented in Fig. 6. We didn't observe any difference in the pattern of modification for the plasmids with single promoters (data not shown). The addition of IPTG causes dramatic changes in the CAA modification of the promoter DNA. As in Fig.6B, an opening of the -10 to $+2$ area of the promoter is observed. It should be noted that chemical modification in the same area was previously observed in the case of purified RNA polymerase opening the *lac* promoter (30,31). Though we see the most prominent modifications in the template DNA strand, this does not necessarily reflect strand asymmetry of an open promoter complex. Indeed, the template strand contains numerous C and A residues which are the best targets for CAA. Conversely, the coding DNA strand contains thymines, which are not modified by CAA, and guanines, which are poor CAA targets (27).
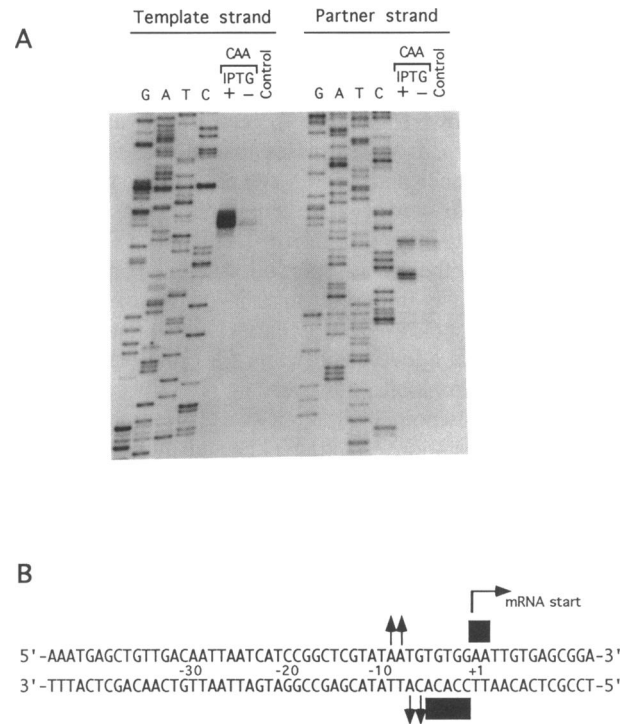
Experiments with other chemicals may clarify this point. Thus, according to our data, IPTG triggers the formation of an open promoter complex.

It is noteworthy that even in the absence of IPTG we detect moderate CAA reactivity at the positions -9 to -6. This may reflect either promoter opening in a portion of plasmids that didn't bind repressor or the unwinding of the promoter by RNA polymerase at a TATA box even in the presence of repressor. The latter explanation seems more likely. Indeed, one can clearly see that, in the coding strand for example, IPTG changes the pattern of modification rather than simply increasing the modification intensity.

Comparison of data in Figs. 3, 4 and 6 clearly shows that although there is a correlation between promoter opening and cruciform extrusion, the effect on promoter structure is much more pronounced. Since, in all cases, CAA treatment of cells was always the rather long period of 20 min., this may reflect either the very slow rate of CAA modification of cruciforms *in vivo*, or the relative inefficiency of cruciform formation. The latter seems to be the case because we didn't observe significant differences in cruciform modification when varying CAA treatment from 5 to 30 min. (data not shown).

DISCUSSION

We found that induction of transcription of the *trc* promoter causes the formation of $d(A-T)_{16}$ cruciforms in *E.coli* cells. Previously we showed that $d(A-T)_n$ cruciforms are formed in *E.coli* cells under different stresses that increase the torsional tension of intracellular DNA. Thus, we believe that transcriptionally driven negative supercoiling upstream of the promoter leads to the cruciform formation. These results generally support the 'twin-supercoiled-domain' model of Liu and Wang (11). It is important that, in our case, transcription overcame DNA topoisomerase activities, which tend to maintain torsional tension at a level insufficient for cruciform formation.

Two of our observations, however, are not fully consistent with the common viewpoint. First, it was discussed that both transcription-translation coupling (11) and attachments of the protein product to the cell membrane (15) are essential for the accumulation of extra supercoils. In our case we detected practically the same level of cruciform formation notwithstanding whether a membrane-attached or a soluble protein was expressed under the P*trc* promoter (Fig.3). Moreover, the incorporation of a translation stop-codon into the *cat* gene didn't affect cruciform formation (data not shown).

Second, we observed similar rates of cruciform induction when the $d(A-T)_{16}$ sequence was placed upstream of single or divergent *trc* promoters. Meanwhile, it was found that transcriptionally driven DNA supercoiling strongly depends on the relative orientation of transcriptional units in the plasmid (12–14). The reversing of the orientation of the transcribing genes (for example *tet* and *amp* in pBR322) caused a dramatic, though not all-or-none, effect (12). The residual transcriptionally driven supercoiling was attributed to the activity of other promoters present in the same plasmid (12).

It should be noted, however, that experiments proving transcriptional DNA supercoiling were performed under blockage of one or another DNA topoisomerase. In those cases, topological problems caused by the movement of the transcriptional-translational complexes anchored on membrane along DNA templates and the divergent orientation of promoter units cannot be resolved, which finally leads to dramatic changes in the steady-state DNA supercoiling level. Apparently, active DNA topoisomerases can quickly overcome these constraints (32). We suggest that transcription can change the level of DNA supercoiling (as reflected by our data on cruciform formation) in a transient manner. In other words, transcriptional waves of supercoiling may exist in normal cells only temporarily, i.e., there exists an equilibrium between the supercoils induced by RNA polymerase and those relaxed by topoisomerases. Therefore, the area of transient supercoiling would immediately follow the transcribing RNA polymerase (Droge and Nordheim (33) recently demonstrated this phenomenon studying the induction of the Z DNA formation by purified T7 RNA polymerase *in vitro*). The topoisomerases may mask our ability to detect any possible effect of transcription-translation and protein-membrane coupling, as well as the inter-orientation of the promoters. The transient character of structural changes upstream of the promoter may also explain the low level of modification within the $d(A-T)_{16}$ sequence relative to the modification within the *trc* promoter after induction.

Quite recently, Rahmouni and Wells (18) have found transcriptionally driven Z DNA formation in *E.coli* cells by *in vivo* footprinting. A $d(C-G)_n$ sequence was inserted between the

two divergent promoters (*tac* and *lacI*q), and Z DNA formation was observed after induction of the *tac* promoter. However, the insertion of an 800 bp promoterless DNA piece between these promoters abolished Z DNA formation. This was interpreted as a requirement of transcription of closely located divergent promoters for Z DNA extrusion. This interpretation contradicts our data, as well as the above explanation. First, we observe cruciform formation in plasmids with a single *trc* promoter, where the cruciform-forming insert is located between an antibiotic resistance gene and the *lacI*q gene transcribing in the same direction (Fig.1). Second, we don't see serious differences between plasmids with single or divergent *trc* promoters. The reasons of this controversy are not yet clear. One explanation is that the insertion of an 800 bp exogenous DNA segment may not only mechanically increase the distance between promoters but also change the high-order structure of the whole intervening DNA region (by changing the binding pattern of chromatin proteins, topoisomerases, *etc.*). This, in turn, may prevent Z DNA extrusion. Further experiments are required to clarify this point.

According to our data, torsional tension upstream of the *trc* promoter in at least some portion of plasmid population may achieve the superhelical density required for the $d(A-T)_{16}$ extrusion into the cruciform, which corresponds to -0.05. Recently, studying the cruciform formation for $d(A-T)_n$ sequences of varying lengths, we estimated the steady-state level of superhelicity in *E.coli* cells as -0.04 (2), which fits with the values obtained by several other approaches (3,34). Thus, our data demonstrate that transcription may transiently increase the negative superhelical density in the upstream areas up to -0.01. Though transient, this 20% increase may have biological implications, since a number of sequences that form supercoil-dependent non-B DNA structures *in vitro* are located upstream of eukaryotic promoters (reviewed in 35).

Our data show a correlation between promoter opening after the addition of IPTG and cruciform formation. In contrast, it was previously described (31) that the addition of IPTG does not influence the pattern of chemical modification of the *lac* promoter *in vivo*. However, these results were obtained for a multicopy plasmid containing the *lac* promoter but lacking the *lacI*q repressor gene. Because the amount of repressor under such circumstances is insufficient, a significant fraction of promoters are active even in the absence of the inducer. Thus, the moderate effect of IPTG on the promoter structure is not surprising.

Several mechanisms of transcriptional repression of the *lac*-promoter have been discussed. The established facts are that the repressor of the *lac*-operon (LacR) binds to the operator sequence which overlaps transcriptional start site (36,37), and the repression could be reversed by the addition of the inducer IPTG (38). According to the classical viewpoint, LacR simply prevents binding of RNA polymerase to the promoter (39). It was later found that RNA polymerase and LacR may coexist on the promoter and that the repressor prevents the open complex formation (40). Quite recently, however, it was suggested that the primary effect of LacR is the blockage of the initiation-to-elongation transition of RNA polymerase rather than the open complex formation (41).

Our data on chemical modification of the promoter *in vivo* allow us to distinguish between these hypotheses. We found that even in the absence of the inducer the -9 to -6 part of the promoter is unwound. The addition of IPTG causes a prominent modification of the $-10-+2$ area of the promoter. We believe,

therefore, that RNA polymerase may bind the promoter and unwind the TATA box even in the presence of LacR. The repressor, however, prevents the formation of a normal open complex in which a bigger portion of DNA between the TATA box and the mRNA start site is unwound. LacR leaves DNA in the presence of IPTG, triggering promoter opening.

ACKNOWLEDGEMENTS

REFERENCES

1. Lilley, D. M., and Hallam, L. R. (1983) *J. Biomolec. Struct. Dynam.* **1**, 169–182.
2. Dayn, A., Malkhosyan, S., Duzhii, D., Lyamichev, V., Panchenko, Yu., and Mirkin, S. (1991) *J. Bacteriol.* **173**, 2658–2664.
3. McClellan, J. A., Boublikova, P., Palecek, E., and Lilley, D. M. J. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 8373–8377.
4. Panayotatos, N., and Fontaine, A. (1987) *J. Biol. Chem.* **262**, 11364–11368.
5. Horwitz, M. S. Z., and Loeb, L. A. (1988) *Science* **241**, 703–705.
6. Courey, A. J., and Wang, J. C. (1983) *Cell* **33**, 817–829.
7. Sinden, R. R., Broyles, S. S., and Pettijohn, D. E. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 1797–1801.
8. Lyamichev, V., Panyutin, I., and Mirkin, S. (1984) *J. Biomolec. Struct. Dynam.* **2**, 291–301.
9. Gellert, M. (1981) *Annu. Rev. Biochem.* **50**, 879–910.
10. Wang, J. C. (1985) *Annu. Rev. Biochem.* **54**, 665–697.
11. Liu, L. F., and Wang, J. C. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 7024–7027.
12. Wu, H.-Y., Shyy, S., Wang, J. C., and Liu, L. F. (1988) *Cell* **53**, 433–440.
13. Lockshon, D., and Morris, D. R. (1983) *Nucleic Acids Res.* **11**, 2999–3017.
14. Pruss, G. L., and Drlica, K. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 8952–8956.
15. Lodge, J. K., Kazic, T., and Berg, D. E. (1989) *J. Bacteriol.* **171**, 2181–2187.
16. Yang, L., Jessee, C. B., Lau, K., Zhang, H., and Liu, L. F. (1989) *Proc. Natl. Acad. Sci. USA.* **86**, 6121–6125.
17. Rahmouni, A. R., and Wells, R. D. (1989) *Science* **246**, 358–363.
18. Rahmouni, A. R., and Wells, R. D. (1992) *J. Mol. Biol.* **223**, 131–144.
19. Amann, E., Ochs, B., and Abel, K.-J. (1988) *Gene* **69**, 301–315.
20. Panyutin, I., Lyamichev, V., and Mirkin, S. (1985) *J. Biomolec. Struct. Dynam.* **2**, 1221–1234.
21. Raleigh, E. A., Lech, K., and Brent, R. (1989) in: *Current Protocols in Molecular Biology* (Ausubel, F. M. et. al., eds.), Unit 1.4. Publishing Associates and Wiley Interscience, New York.
22. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual.* CSH Laboratory Press, CSH
23. Maxam, A. M., and Gilbert, W. (1980) *Methods Enzymol.* **65**, 499–560.
24. Kochetkov, N. K., Shibaev, V. N., and Kost, A. A. (1971) *Tetrahedron Lett.* **22**, 1993–1996.
25. Barrio, J. R., Secrist, J. A., and Leonard, N. J. (1972) Biochem. Biophys. Res. Comm. **46**, 597–604.
26. Sattsangi, P. D., Leonard, N. J., and Frihart, C. R. (1977) *J. Org. Chem.* **42**, 3292–3296.
27. Kohwi, Y., and Kohwi-Shigematsu, T. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 3781–3785.
28. Vogt, N., Marrot, L., Rousseau, N., and Leng, M. (1988) *J. Mol. Biol.* **201**, 773–776.
29. Kohwi, Y., Malkhosyan, S. R., and Kohwi-Shigematsu, T. (1992) *J. Mol. Biol.* **223**, 817–822.
30. Kirkegaard, K., Buc, H., Spassky, A., and Wang, J. C. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 2544–2548.
31. Sasse-Dwight, S., and Gralla, J. D. (1989) *J. Biol. Chem.* **264**, 8074–8081.
32. Cook, D. N., Ma, D., Pon, N. G., and Hearst, J. E. (1992) *Proc. Natl. Acad. Sci. USA*, in press.
33. Droge, P., and Nordheim, A. (1991) *Nucleic Acids Res.* **19**, 2941–2946.
34. Sinden, R. R., Carlson, J. O., and Pettijohn, D. E. (1980) *Cell* **21**, 773–783.
35. Wells, R. D., Collier, D. A., Hanvey, J. C., Shimizu, M. & Wohlrab, F. (1988) *FASEB J.* **2**, 2939–2949.
36. Gilbert, W., and Muller-Hill, B. (1966) *Proc. Natl. Acad. Sci. USA* **56**, 1891–1898.
37. Schmidt, M. C., and Galas, D. J. (1979) *Nucleic Acids Res.* **6**, 111–137.
38. Chen, B., de Croumbrugghe, B., Anderson, W., Gottesman, M., Pastan, I., and Perlman, R. (1971) *Nature* **233**, 67–70.
39. Majors, J. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 4394–4398.
40. Straney, S. B., and Crothers, D. M. (1987) *Cell* **51**, 699–707.
41. Lee, J., and Goldfarb, A. (1991) *Cell* **66**, 793–798.