Effect of Magnesium on Cruciform Extrusion in Supercoiled DNA

ARTICLE *in* JOURNAL OF MOLECULAR BIOLOGY · JULY 1999

Impact Factor: 4.33 · DOI: 10.1006/jmbi.1999.2811 · Source: PubMed

2 AUTHORS:

Maria Vologodskaia
The Rockefeller University
12 PUBLICATIONS **2,050** CITATIONS
SEE PROFILE

Alexander Vologodskii
126 PUBLICATIONS **5,368** CITATIONS
SEE PROFILE

Article No. jmbi.1999.2811 available online at http://www.idealibrary.com on IDEAL® J. Mol. Biol. (1999) 289, 851–859

JMB

Effect of Magnesium on Cruciform Extrusion in Supercoiled DNA

Maria Y. Vologodskaia and Alexander V. Vologodskii*

Department of Chemistry
New York University
31 Washington Place, New York, NY 10003, USA

Recently, it was reported that Mg^{2+} greatly facilitates cruciform extrusion in the short palindromes of supercoiled DNA, thereby allowing the formation of cruciform structures *in vivo*. Because of the potential biological importance of this phenomenon, we undertook a broader study of the effect of Mg^{2+} on a cruciform extrusion in supercoiled DNA. The method of two-dimensional gel electrophoresis was used to detect the cruciform extrusion both in the absence and in the presence of these ions. Our results show that Mg^{2+} shifts the cruciform extrusion in the d(CCC$(AT)_{16}$GGG) palindrome to a higher, rather than to a lower level of supercoiling. In order to study possible sequence-specific properties of the short palindromes for which the unusual cruciform extrusion in the presence Mg^{2+} was reported, we constructed a plasmid with a longer palindromic region. This region bears the same sequences in the hairpin loops and four-arm junction as the short palindrome, except that the short stems of the hairpins are extended. The extension allowed us to overcome the limitation of our experimental approach which cannot be used for very short palindromes. Our results show that Mg^{2+} also shifts the cruciform extrusion in this palindrome to a higher level of supercoiling. These data suggest that cruciform extrusion in the short palindromes at low supercoiling is highly improbable. We performed a thermodynamic analysis of the effect of Mg^{2+} on cruciform extrusion. The treatment accounted for the effect of Mg^{2+} on both free energy of supercoiling and the free energy of cruciform structure *per se*. Our analysis showed that although the level of supercoiling required for the cruciform extrusion is not reduced by Mg^{2+}, the ions reduce the free energy of the cruciform structure.

Keywords: DNA cruciform; inverted repeat; DNA supercoiling; DNA structure; supercoiling free energy

*Corresponding author

Introduction

Since the formation of cruciform structures in supercoiled DNA was discovered in 1980 (Lilley, 1980; Panayotatos & Wells, 1981), the phenomenon has been studied extensively (for reviews, see Murchie & Lilley, 1992; Vologodskii, 1992). The cruciform structures are formed at inverted repeats by two hairpins and the four-arm junction. They interrupt the regular structure of double-stranded DNA and therefore increase the free energy of DNA molecules. However, the cruciform extrusion decreases the free energy of negative supercoiling, since complementary strands are not interwound in that structure, thereby allowing its appearance in supercoiled DNA. Although the cruciform structures can be easily detected in natural palindromic regions of DNA extracted from cells, attempts to find these structures in the same DNA molecules inside cells have repeatedly failed (Borst *et al.*, 1984; Courey & Wang, 1983; Lyamichev *et al.*, 1984; Sinden *et al.*, 1983). Palindromic regions of 24-32 bp in length, which can be found in many phage and plasmid DNAs, form the cruciform structures at a level of the superhelical stress that does not exist in cells under normal conditions (Murchie & Lilley, 1992; Vologodskii, 1992). Longer palindromic sequences may form cruciforms under the lower stress found in prokaryotic cells, but the extrusion is repressed kinetically because its rate is

Present address: M. Y. Vologodskaia, The Rockefeller University, New York, NY 10021, USA.

Abbreviations used: EtBr, ethidium bromide.

E-mail address of the corresponding author: alex.vologodskii@nyu.edu

0022-2836/99/240851–9 $30.00/0

reduced dramatically when torsional stress diminishes (Borst *et al*., 1984; Courey & Wang, 1983; Gellert *et al*., 1983; Panyutin *et al*., 1984; Sinden & Pettijohn, 1984; Vologodskii & Frank-Kamenetskii, 1983). The only cruciform structures detected inside the cell were formed by $d(AT)_n$ palindromes ($n \geqslant 15$; Dayn *et al*., 1991; Haniford & Pulleyblank, 1985; McClellan *et al*., 1990). The extrusion of cruciforms in such palindromes occurs at relatively low superhelical stress with relatively fast kinetics. Such sequences are not stable in genomes, however (Courey & Wang, 1983; Haniford & Pulleyblank, 1985; McClellan *et al*., 1990; Panyutin *et al*., 1985). Thus, it is generally accepted that the cruciform structures do not exist in living cells, at least under normal conditions.

This conclusion was called into question by reports that very short palindromic sequences, 13-17 bp in length, are able to form the cruciform structures in the presence of Mg^{2+} (Dai *et al*., 1997, 1998; Iacono-Connors & Kowalski, 1986). Dai *et al*. (1997) concluded that the extrusion occurs at a superhelix density, σ, as low as -0.027. Taking into account that Mg^{2+} is present inside cells and that, inside cells, the torsional stress of DNA corresponds to a σ of $(-0.04) \div (-0.025)$ (see reviews by Drlica, 1992; Vologodskii, 1992), one has to conclude that these palindromic regions can adopt cruciform structures inside the cell. If this is the case, the finding may have very important biological consequences. Although these reports do not fit our current knowledge of cruciform properties, there is insufficient information about the effects of Mg^{2+} on cruciform extrusion. It was shown that Mg^{2+} changes the conformation of the four-arm junction (For reviews, see Lilley & Clegg, 1993; Seeman & Kallenbach, 1994), an important element of the cruciforms, and dramatically lowers the rate of migration of the four-arm junction (Panyutin & Hsieh, 1994; Sinden & Pettijohn, 1984). Mg^{2+} also lowers the rate of the cruciform extrusion (Sinden & Pettijohn, 1984). Since Mg^{2+} strongly affects the conformations of supercoiled DNA (Adrian *et al*., 1990; Rybenkov *et al*., 1997b,c), it should also change the free energy of supercoiling, the driving force of the cruciform extrusion. Thus we can expect that Mg^{2+} exerts a critical influence on the cruciform extrusion. So far the only systematic study of the influence of Mg^{2+} on the formation of cruciforms in supercoiled DNA was published by Singleton (1983), who did not observe that Mg^{2+} significantly decreases the degree of supercoiling at which the extrusion occurred. His data, however, do not exclude the possibility that Mg^{2+} shifts the equilibrium transition to lower values of $|\sigma|$, but the extrusion is too slow to be observed at this values of σ. Palindromic sequences studied by Dai *et al*. (1997, 1998) are able to form unusually stable hairpins; thus, we cannot exclude the possibility that their extrusion occurs faster, allowing one to observe the cruciforms at lower $|\sigma|$ values. This consideration prompted us to study the effect of Mg^{2+} on cruciform extrusion. Several issues are addressed here. We studied the effect of Mg^{2+} on the cruciform extrusion in supercoiled DNA in general, and also tested the reported effect of the specific palindromic sequence on the extrusion in the presence of Mg^{2+}. Although the effect of Mg^{2+} on the cruciform extrusion in two different palindromes was qualitatively the same, a quantitative difference was found. We analyzed the possible reasons for this difference. Finally, we performed a thermodynamic analysis of the effect of Mg^{2+} on cruciform extrusion in supercoiled DNA.

Among various techniques that allow detection of cruciform extrusion, the method of two-dimensional gel electrophoresis is probably the most reliable one (Wang *et al*., 1983). If DNA with a palindromic region reveals a sharp decrease in its electrophoretic mobility at a given value of σ, and the same DNA without this palindromic region does not show any change in the mobility, a local conformational transition certainly occurs in the region. With the exception of special sequences that can also adopt a *Z* form, the transition corresponds to the cruciform extrusion. Two-dimensional gel electrophoresis allows, as a result of a single experiment, the measurement of σ at which the transition occurs. We used this method here to study cruciform extrusion in two different palindromic regions. The method does not allow for the study of local transitions that could take place in short palindromic sequences, because the probability of the cruciform formation would increase gradually in the broader interval of σ for shorter palindromes, and the total effect of the transition on the DNA electrophoretic mobility would be smaller (Vologodskii, 1992). Thus, the DNA mobility would not decrease abruptly as a result of the transition. To apply the method to one of the short palindromes studied by Dai *et al*. (1997, 1998), we extended the original 15 bp palindrome by two six base-pair inserts. The cruciform structure formed by our palindrome has the same sequences in the four-arm junction and hairpin loops as the cruciform structure formed by the shorter palindrome. Therefore, our extended palindrome is likely to contain the sequence specificity of the palindrome studied by Dai *et al*. (1997).

We found that both palindromic regions studied here adopt the cruciform conformation in the presence of Mg^{2+} at higher $|\sigma|$ than in the absence of these ions. We did not observe that the specific sequences of the four-arm junction or hairpin loops facilitate the cruciform extrusion in the presence of Mg^{2+}. Using these data, and the results of computations of the supercoiling free energy, we analyzed the effect of Mg^{2+} on the thermodynamics of the cruciform extrusion. The analysis revealed that Mg^{2+} reduces the free energy of the cruciform structures. The ions, however, also reduce the free energy of supercoiling, and it turns out that the last factor has the larger effect on cruciform extrusion. As a result, Mg^{2+} increases $|\sigma|$ of the cruciform extrusion in supercoiled DNA.

Results

Cruciform formation in the d(CCC(AT)$_{16}$GGG) palindrome

We first studied the effect of Mg^{2+} on the cruciform extrusion in pAT32 plasmid, formed by inserting the palindromic region d(CCC(AT)$_{16}$GGG) into the pUC19 plasmid. This palindrome is very convenient for our purpose, since it is sufficiently long to adopt the cruciform structure at relatively low supercoiling, without interference with other possible structural transitions. Relatively fast kinetics of the cruciform extrusion in this palindrome (Panyutin *et al*., 1985) also facilitate the study of the equilibrium transition.

Two-dimensional gel electrophoresis allows one to detect local structural transitions resulting from the torsional stress in supercoiled DNA (Wang *et al*., 1983). An observable transition takes place under the ionic conditions of the electrophoresis experiment in the first dimension, while separation in the second dimension helps to identify the topoisomer spots obtained during the electrophoresis in the first dimension. Therefore, to study the effect of Mg^{2+} on the cruciform extrusion, we performed the electrophoresis in the first dimension both with and without Mg^{2+}.

A pAT32 DNA sample, characterized by a broad topoisomer distribution, $\sigma = -0.07 \div 0.012$, was subjected to electrophoresis (the distribution was shifted by -0.010 in the Mg^{2+}-containing buffer, in agreement with Rybenkov *et al*. (1997a)). When the separation in the first dimension was performed in TBE buffer, a structural transition was revealed as an abrupt decrease in the topoisomer mobility at $\sigma_{tr} = -0.041$ (Figure 1(a)), in agreement with Panyutin *et al*. (1985)). Since no transition was observed in a control experiment with pUC19 DNA (Figure 1(b)), we concluded that the transition took place in the palindromic region and corresponded to the cruciform extrusion.

Next, the electrophoresis in the first dimension was performed in the presence of Mg^{2+}, in 50 mM Tris-acetate, 20 mM magnesium-acetate, 20 mM ammonium-acetate, for both pAT32 and pUC19 DNAs (Figure 2). Again, an abrupt decrease of the topoisomer mobility was observed for pAT32 DNA only. In this case, it took place at $\sigma = -0.048$. Thus, contrary to our expectations, the cruciform extrusion in the presence of Mg^{2+} was observed at higher negative supercoiling that in its absence.

Two-dimensional gel electrophoresis of the topoisomers of pAT32 DNA in TBE buffer confirmed that the cruciform extrusion is fast in comparison with the time-scale of the electrophoresis experiment. Indeed, the topoisomer 15, which corresponds to the middle of the transition, migrates as a single spot (Figure 1(a)). This means that many transitions between the linear and cruciform states of each DNA molecule occur during the electrophoresis. In Mg^{2+}-containing buffer, however, exchange between two states of the palindromic regions occurs on the time-scale of the electrophoresis: the topoisomer 14 is seen as a diffused spot in Figure 2(a). To ensure that the measured values of σ_{tr} corresponded to equilibrium values and were not affected by the slow kinetics of the cruciform extrusion in Mg^{2+}-containing buffer, the mixture of

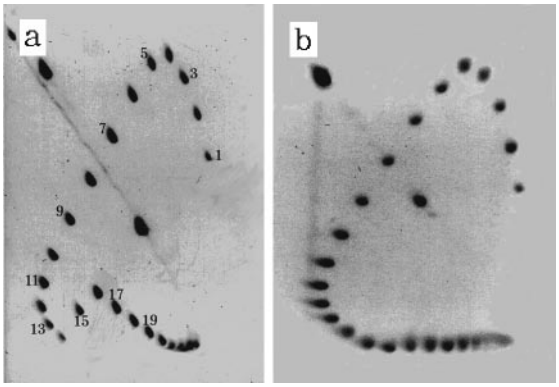

Figure 1. Two-dimensional gel electrophoresis of pAT32 and pUC19 DNAs in TBE buffer. (a) A mixture of topoisomers of pAT32 DNA covering a wide range of superhelix density was electrophoresed from the top to the bottom at room temperature in TBE buffer. The gel was then soaked for eight hours in TBE buffer containing 1.5 μg/ml of chloroquine before electrophoresis in the second dimension, from left to right. The cruciform extrusion in pAT32 DNA causes a decrease in the topoisomer mobility between topoisomers 14 and 16, at the linking number difference, ΔLk, of -11. (b) The same experiment with pUC19 DNA showed no structural transition in this range of supercoiling.

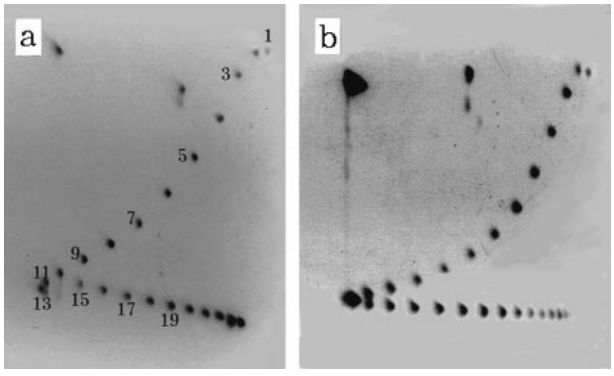

Figure 2. Two-dimensional gel electrophoresis of pAT32 and pUC19 DNAs in Mg^{2+}-containing buffer. (a) Mixture of topoisomers of pAT32 was electrophoresed from the top to the bottom at 4° in 50 mM Tris-acetate, 20 mM magnesium acetate, 20 mM ammonium acetate. The gels were then soaked for eight hours in TBE buffer containing 1.5 μg/ml of chloroquine and subjected to electrophoresis in the second dimension, from the left to the right. The cruciform extrusion in pAT32 DNA occurs between topoisomers 13 and 15 ($\Delta Lk = -12.5$). (b) The same experiment with pUC19 DNA showed no structural transition in this range of supercoiling.

the topoisomers was heated to 60 °C and then cooled over one hour to room temperature, prior to electrophoresis. It was shown that this procedure greatly increases the rate of cruciform extrusion in supercoiled DNA (Courey & Wang, 1983; Panyutin *et al*., 1984). Two-dimensional gel electrophoresis of this sample gave the same result as presented in Figures 2(a) (data not shown). We concluded that the increase of $|\sigma_{tr}|$ observed in the presence of Mg^{2+} is not a result of slow kinetics of the cruciform extrusion in the Mg^{2+}-containing buffer.

To investigate to what extent the effect of Mg^{2+} on cruciform extrusion depends on the sequences of inverted repeats, we studied the transition in another DNA with a palindromic insert.

Cruciform formation in the extended P2 palindromic region

One of the goals of this study was to test for unusual cruciform extrusion in the presence of Mg^{2+} reported for the bacteriophage N4 vRNAP P2 promoter region (Dai *et al*., 1997). To this end, we designed a palindromic region consisting of this inverted repeat extended by two six base-pair inserts (Figure 3) and cloned the construct into pUC19 (plasmid pMS1). The extension allowed us to use two-dimensional gel electrophoresis to register the cruciform extrusion in the region, while retaining the original sequences of the cruciform loops and the four-arm junction which, in turn, could define the sequence-specific properties of the palindromic region.

Two-dimensional gel electrophoresis of topoisomers of pMS1 DNA in TBE buffer showed that the cruciform extrusion occurs at $\sigma_{tr} = -0.046$ (Figure 4(a)). Again, transferring this DNA to Mg^{2+}-containing buffer shifted the transition to higher value of negative supercoiling, $\sigma_{tr} = -0.074$ (Figure 4(b)). In this case, the shift of σ_{tr} caused by Mg^{2+} was even larger. Heating the topoisomer mixture prior to electrophoresis did not change the electrophoretic patterns (data not shown). Thus, the observed effect of Mg^{2+} on σ_{tr} reflects the equilibrium conformational properties of the plasmid DNA.

Sequence dependence of the effect of Mg^{2+} on cruciform extrusion

Our data show that Mg^{2+} increases rather than decreases the value of negative supercoiling at which the cruciform extrusion takes place. We found, however, that the shift in σ_{tr} depends on the sequence of the palindromic region. There are two possible explanations for this effect. First, Mg^{2+} can change the free energy of cruciform formation in a sequence-specific manner. We prefer another explanation of the effect, however. It is well known that different conformational transitions in supercoiled DNA absorb the same free energy of supercoiling and thus compete with one another (see, for example, Vologodskii, 1992). In particular, cruciform extrusion competes with the formation of short segments of *Z*-DNA. Although segments of Z-DNA are formed first in $d(CG)_n$ sequences, as much as 30 % of DNA can adopt a *Z* form under a large torsional stress (Brahms *et al*., 1982; Pohl *et al*., 1982). Formation of short *Z*-DNA segments starts at σ of $(-0.05) \div (-0.07)$ in typical plasmid and phage DNA molecules (Brahms *et al*., 1989; Di Capua *et al*., 1983; Hagen *et al*., 1985; Revet *et al*., 1984).

```
5'-ctagaagaacttaaggctccgccttaagttctttgctgca-3'
   3'-ttcttgaattccgaggcggaattcaagaaaacg-5'
```

Figure 3. Palindromic insert cloned in pUC19 DNA between *Xba*I and *Pst*I sites. Sequence of the insert corresponds to bacteriophage N4 vRNAP P2 promoter region extended by adding two sites of *Afl*II (bold letters). The inverted repeat is underlined.

The issue can be illustrated quantitatively, since formation of the *Z* form in supercoiled DNA with different sequences has been studied intensively both experimentally and theoretically (data summarized by Ho *et al*., 1986; Mirkin *et al*., 1987). We can calculate the probability of *B-Z* transition for each base-pair of a particular circular DNA, for a given value of σ (Anshelevich *et al*., 1988). The calculation is based on the statistical-mechanical model of *B-Z* equilibrium that contains six parameters (Mirkin *et al*., 1987). These parameters depend on ionic conditions and have been determined for TBE buffer only (Ellison *et al*., 1985; Mirkin *et al*., 1987; Peck & Wang, 1983). Using this approach, we calculated the probability of *Z*-DNA segment formation in pUC19 for TBE buffer (Figure 5(a)). Figure 5(b) shows that the effective value of the superhelix density, σ_{eff}, which specifies the actual torsional stress in the supercoiled DNA, changes very slowly for $\sigma < -0.05$ because of the increase in the total length of *Z*-DNA

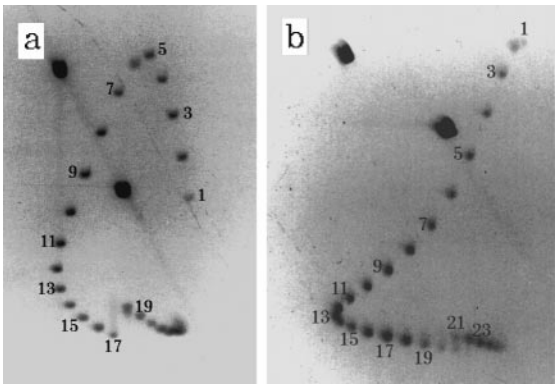

Figure 4. Formation of the cruciform structure in pMS1 DNA. A mixture of topoisomers of this DNA was electrophoresed as described. Electrophoresis in the first dimension was performed in (a) TBE buffer and in (b) Mg^{2+}-containing buffer. The ΔLk value at which the cruciform extrusion occurs is equal to −12 for TBE buffer and −19 for Mg^{2+}-containing buffer.

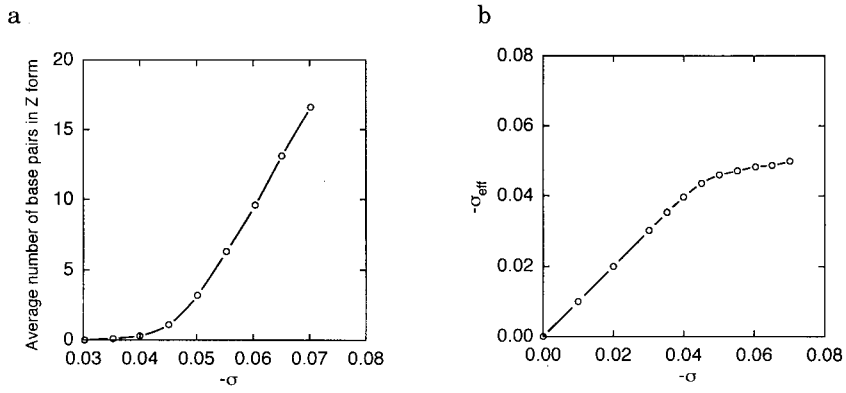

Figure 5. Formation of the *Z* form in pUC19 DNA under the action of supercoiling. (a) The average number of base-pairs in the *Z* conformation, $\langle N_Z \rangle$, was calculated as described by Anshelevich *et al.* (1988) as a function of σ. Parameters of the *B-Z* transition that were used in the calculation correspond to the ionic composition of TBE buffer. About five or six short segments of this DNA make a comparable contribution to $\langle N_Z \rangle$, although the probability of the *Z* form does not exceed 0.25 for any of these segments. (b) Formation of the *Z* form reduces the elastic deformation in the supercoiled DNA which is now specified by the effective superhelic density, $\sigma_{eff} = \sigma + 1.8\langle N_Z \rangle / N$, rather than by σ ($N$ is the DNA length in base-pairs). For this DNA and under the ionic conditions used, σ_{eff} does not change significantly for $\sigma < -0.05$.

segments. Formation of short *Z*-DNA segments (six to eight base-pairs in length in the case of pUC19 DNA) occurs over a broad range of σ, and thus cannot be detected by an increase in the electrophoretic mobility of DNA topoisomers with an increase in $|\sigma|$. It follows from Figure 5(b) that a cruciform extrusion taking place at $\sigma < -0.05$ would depend both on its own properties and on the entire sequence of this DNA. We do not know the parameters of the *B-Z* transition for our Mg^{2+}-containing buffer to make similar calculations for these ionic conditions, but it is known that even a low concentration of Mg^{2+} facilitates formation of *Z*-DNA (Behe & Felsenfeld, 1981). Thus, formation of *Z*-DNA segments should also affect the cruciform extrusion in the presence of Mg^{2+}. The effect of these competing transitions on the cruciform extrusion will be more pronounced, in general, for the cruciform extrusion in shorter palindromes when $|\sigma_{tr}|$ is higher.

Discussion

The data obtained in this study of the effect of Mg^{2+} on the cruciform extrusion in supercoiled DNA clearly show that Mg^{2+} does not facilitate the transition. This is the case for both the $d(AT)_n$ type palindrome and for the palindromic region of bacteriophage N4 vRNAP P2 promoter extended by two six base-pair inserts (Figure 3). Our results are in agreement with the data reported by Singleton (1983), who also found that adding 10 mM Mg^{2+} only increases $|\sigma_{tr}|$ of cruciform extrusion. However, this conclusion is not consistent with the reports that Mg^{2+} greatly facilitates cruciform formation in certain short palindromic sequences (Dai *et al.*, 1997, 1998; Iacono-Connors & Kowalski, 1986). One of the palindromic regions in this study was specially designed to test the ability of the regulatory palindromic sequence of N4 bacteriophage to adopt the cruciform structure at very low values of negative supercoiling ($\sigma_{tr} = -0.027$) reported by Dai *et al.* (1997). We extended the sequence in the middle of the cruciform hairpins, retaining the sequences of the hairpin loops and four-arm junction. The extension allowed us to use two-dimensional gel electrophoresis to study the cruciform extrusion in the constructed plasmid. It is unlikely that this extension affected the sequence-specific features of the original palindrome (Dai *et al.*, 1997). We found that our longer palindrome adopts the cruciform structure only at high value of negative supercoiling, $\sigma_{tr} = -0.074$. Increasing the length of a palindromic region does not change the free energy of the cruciform structure, but increases the gain in the free energy of supercoiling from the extrusion. Thus, the extension of the palindromic region has the effect of reducing the value of $|\sigma_{tr}|$ only. These theoretical arguments are in agreement with the experimental data obtained for $d(AT)_n$ palindromes (Panyutin *et al.*, 1985). Thus, our result makes highly improbable that cruciform extrusion occurs at low supercoiling in the short palindromes studied by Dai *et al.* (1997, 1998).

What could be the reasons for the different conclusions drawn by us based on this work and the work by Singleton (1983), on the one hand, and by Iacono-Connors & Kowalski (1986) and Dai *et al.* (1997, 1998), on the other? The difference seems especially striking because all the studies except ours used the same endonuclease technique to detect the cruciform structures. The method was used in different ways, however. Singleton (1983) applied the original procedure by Lilley (1980) and Panayotatos & Wells (1981). Using restriction analysis, Singleton (1983) showed that a large fraction of circular DNA molecules was digested by single-strand-specific endonuclease at a particular palindromic region. Thus, the palindromic regions were the major targets for the enzyme in the entire DNA. Iacono-Connors & Kowalski (1986) and Dai *et al.* (1997, 1998) found only that Mg^{2+} dramatically increases endonuclease cleavage in the palindromic regions. They did not show that the palindromes became the major targets for the endonucleases in the entire circular DNA molecules, i.e. that most of the circles were digested in these regions. Their data do not exclude the possi-

bility that there are many more sites in these DNAs that are cleaved efficiently by the enzymes in the presence of Mg^{2+}. A study of the relative sensitivity of various regions of the entire plasmids to endonuclease cleavage could be the first step to understanding the nature of the conformational changes in the palindromic regions observed by Iacono-Connors & Kowalski (1986) and Dai *et al.* (1997, 1998). Although these changes are not likely to be so dramatic as the cruciform extrusion, they may be very important for transcription regulation.

The fact that Mg^{2+} does not reduce $|\sigma_{tr}|$ for the cruciform extrusion seems unexpected, given that this divalent ion greatly stabilizes the structure of the four-arm junction (for reviews, see Lilley & Clegg, 1993; Seeman & Kallenbach, 1994). However, the analysis described below shows that the free energy difference between cruciform and linear structure of a palindromic region, G_c, does decline in the presence of Mg^{2+}.

The equilibrium value of σ_{tr} is defined by the condition that the reduction in the free energy of supercoiling resulting from a cruciform extrusion, ΔG_s, is equal to G_c (see, for example, Vologodskii, 1992):

$$G_c = -\Delta G_s \quad (1)$$

It is accepted that for circular DNA of N base-pairs in length ($N > 2500$):

$$G_s = 10RTN\sigma^2 \quad (2)$$

where R is the gas constant and T is the absolute temperature (Wang *et al.*, 1983). The value of the coefficient in equation (2) was obtained for solutions containing a few millimol of Mg^{2+} or 0.2 M Na^+ (Depew & Wang, 1975; Horowitz & Wang, 1984; Pulleyblank *et al.*, 1975), conditions under which the electrostatic repulsion between segments of supercoiled DNA is efficiently screened. This repulsion becomes very important at low concentrations of monovalent ions, substantially changing the conformations of supercoiled DNA (Adrian *et al.*, 1990; Bednar *et al.*, 1994; Lyubchenko & Shlyakhtenko, 1997; Rybenkov *et al.*, 1997b,c; Vologodskii & Cozzarelli, 1994a; Vologodskii *et al.*, 1992). The repulsion also changes the free energy of supercoiling. The dependence of G_s on ionic conditions was studied in detail by Monte Carlo computations (Klenin *et al.*, 1991; Vologodskii & Cozzarelli, 1994a; Vologodskii *et al.*, 1992). The computed results are in very good agreement with available experimental data (Rybenkov *et al.*, 1997b,c). The computations confirmed (Vologodskii & Cozzarelli, 1994a) that equation (2) is valid for $-0.06 \leqslant \sigma \leqslant 0$ for solutions containing 10 mM of Mg^{2+} or 0.2 M Na^+, although it was established experimentally only for very low values of supercoiling (Figure 6). The computed data show, however, that under ionic conditions of TBE buffer, G_s does not follow equation (2) (Vologodskii & Cozzarelli, 1994a). In this case, G_s can be approximated as (see Figure 6):

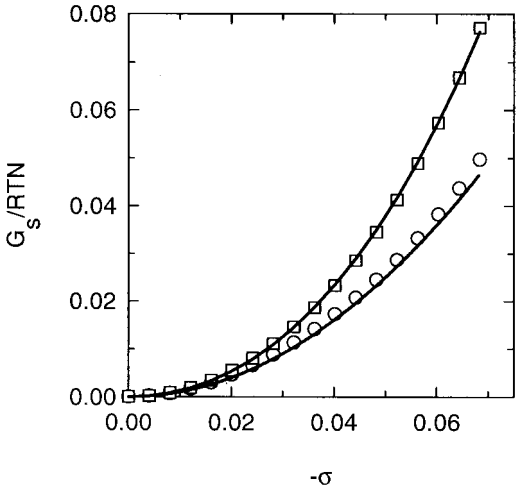

Figure 6. The free energy of supercoiling under different ionic conditions. Points correspond to Monte Carlo computations for 10 mM of Mg^{2+} or 0.2 M of Na^+ (○) and the ionic composition of TBE buffer (□) (data from Vologodskii & Cozzarelli, 1994b). The computed data are well approximated by equation (2) (lower curve) and equation (3) (upper curve).

$$G_s = 12RTN(\sigma^2 - 5.25\sigma^3), \quad -0.06 \leqslant \sigma \leqslant 0 \quad (3)$$

Although equation (3) has not been confirmed experimentally for the entire range of σ, $-0.06 \leqslant \sigma \leqslant 0$, recent experimental measurements of the variance of the equilibrium topoisomer distribution, $\langle(\Delta\sigma)^2\rangle$, for TBE buffer showed very good agreement with these computations (Rybenkov *et al.*, 1997a). We used equation (3) to analyze the cruciform extrusion in TBE buffer and equation (2) for solutions containing a few millimol of Mg^{2+}, or 0.2 M Na^+.

The value of σ specifies the elastic deformation of the double helix caused by the supercoiling in the absence of cruciforms or other alternative structures. Formation of a cruciform of m base-pairs in length reduces the elastic deformation of the double helix, since the complementary strands are not interwound in the cruciform region. The ΔG_s value is specified in this case by the effective superhelix density, $\sigma + m/N$ (Vologodskii *et al.*, 1979). The value of ΔG_s related to cruciform extrusion, can be expressed as:

$$\Delta G_s = \frac{dG_s}{d\sigma}\delta\sigma \quad (4)$$

where $\delta\sigma = m/N$ is the change in the effective superhelix density and $dG_s/d\sigma$ is taken at $\sigma = \sigma_{tr}$. We used the data obtained for the palindromic region $d(CCC(AT)_{16}GGG)$, where the cruciform extrusion occurs at rather low supercoiling, and should not be affected by the formation of Z-DNA segments. For this palindrome $\sigma_{tr} = -0.41$ (see Results) in TBE buffer, and from equations (1), (3)

and (4) we calculated that $G_c = 29$ kcal/mol. It is noteworthy that this value of G_c exceeds by factor of 1.6 the corresponding estimate based on equation (2) which was, in most cases, used in the analysis under ionic conditions of TBE buffer. For the Mg^{2+}-containing solution $\sigma_{tr} = -0.048$ for the same palindrome, and equations (1), (2) and (4) give $G_c = 22$ kcal/mol. Thus, we conclude that the energetic cost of cruciform formation is reduced by Mg^{2+}, in accord with structural stabilization of the four-arm junction by Mg^{2+} (Lilley & Clegg, 1993; Seeman & Kallenbach, 1994). However, the ions also reduce the free energy of supercoiling, the driving force for cruciform formation (see equation (1)). It appears that the reduction of the free energy of cruciform formation in the presence of Mg^{2+} is not sufficient to diminish the value of DNA supercoiling at which the cruciform extrusion occurs.

Materials and Methods

DNAs

The oligonucleotide shown in Figure 3 was inserted in the polylinker of pUC19 plasmid between *Xba*I and *Pst*I sites. The insert corresponds to bacteriophage N4 vRNAP P2 promoter region extended by adding two sites of *Afl*II. The sites were used during selection of the clones. The construct was transformed into *Escherichia coli* DH-5α cells. The exact sequence of the insert of the designed plasmid, pMS1, was verified by sequencing both strands. Plasmid pAT32, containing $d(AT)_{16} \cdot d(AT)_{16}$ insert at the *Sma*I site of the pUC19 polylinker, was a generous gift from Dr S. M. Mirkin (University of Illinois at Chicago, IL). This plasmid and pUC19 were also transformed into the *E. coli* DH-5α cells.

The QIAGEN Plasmid Maxi Purification kit was used to extract DNA from *E. coli* cells. Purified DNAs were linearized by *Pst*I restriction endonuclease and then religated into circles in the presence of $0.0 \div 0.75$ μg/ml of ethidium bromide (EtBr) to obtain topoisomers with superhelix densities varying over a wide range. After religation with EtBr, DNA was extracted twice with phenol, once with phenol/chloroform, and once with chloroform, precipitated overnight with 2 volumes of ethanol and 0.2 M NaCl to remove EtBr. Air-dried DNA was resuspended in TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). A mixture of eight samples, ligated at different EtBr concentrations was used for the gel electrophoresis.

Two-dimensional gel electrophoresis

Electrophoresis in TBE buffer

Approximately 2.7 μg of the topoisomer mixture was loaded into a single well in the corner of a 1.5% agarose gel (23 cm × 13 cm). Electrophoresis in the first dimension was carried out at 3 V/cm for 24 hours in 90 mM Tris-borate (pH 8.3), 1 mM EDTA (TBE) which was recirculated at 10 ml/minute. Separation was carried out at room temperature. The gel was then soaked overnight in TBE buffer containing $0.9 \div 1.5$ μg/ml of chloroquine. After rotating the gel by 90°, it was electrophoresed in the same buffer with chloroquine for 20 hours at 3 V/cm. The gel was stained in an EtBr solution for 40 minutes, and then destained extensively (at least one hour) in water. Photography of the gels was performed under ultraviolet illumination with the orange filter to reduce the background.

Electrophoresis in the presence of magnesium ions

Mg^{2+} essentially decreases the solution resistance, and therefore increases Joule heating during the electrophoresis. To eliminate this effect the electrophoresis in the first dimension was carried out in a strip 1.5% agarose gel (23 cm × 1 cm) with a dielectric insert (23 cm × 12 cm), which filled up the rest of the electrophoresis chamber. The entire circulating buffer, 50 mM Tris-acetate, 20 mM magnesium-acetate, 20 mM ammonium-acetate, was replaced by new sterilized solution every six hours. Electrophoresis was carried out at 3 V/cm for 42 hours at 4 °C. After washing the strip gel in TBE buffer with chloroquine for two hours, the 13 cm segment containing all topoisomers of the supercoiled DNA was used to prepare the 1.5% slab agarose gel for the second dimension. All other procedures were performed as described earlier for the TBE buffer gels.

Acknowledgments

We thank Dr N. Kallenbach who generously provided experimental facilities for this work. The study was supported by National Institutes of Health grant GM54215 to A.V. Editorial assistance was provided by Dr Birgit An der Lan, Bethesda, MD 20814.

References

Adrian, M. B., ten Heggeler-Bordier, B., Wahli, W., Stasiak, A. Z., Stasiak, A. & Dubochet, J. (1990). Direct visualization of supercoiled DNA molecules in solution. *EMBO J.* **9**, 4551-4554.

Anshelevich, V. V., Vologodskii, A. V. & Frank-Kamenetskii, M. D. (1988). A theoretical study of formation of DNA noncanonical structures under negative superhelical stress. *J. Biomol. Struct. Dynam.* **6**, 247-259.

Bednar, J., Furrer, P., Stasiak, A., Dubochet, J., Egelman, E. H. & Bates, A. D. (1994). The twist, writhe and overall shape of supercoiled DNA change during counterion-induced transition from a loosely to a tightly interwound superhelix. *J. Mol. Biol.* **235**, 825-847.

Behe, M. & Felsenfeld, G. (1981). Effects of methylation on a synthetic polynucleotide: the B-Z transition in poly(dG-m^5dC)·poly(dG-m^5dC). *Proc. Natl Acad. Sci. USA*, **78**, 1619-1623.

Borst, P., Overdulve, J. P., Weijers, P. J., Fase-Fowler, F. & Van den Berg, M. (1984). DNA circles with cruciforms from *Isospora (Toxoplasma) gondii*. *Biochim. Biophys. Acta*, **781**, 100-111.

Brahms, S., Vergne, J. & Brahms, J. G. (1982). Natural DNA sequences form left-handed helices in low salt solution under conditions of topological constraint. *J. Mol. Biol.* **162**, 473-493.

Brahms, S., Nakasu, S., Kikuchi, A. & Brams, J. G. (1989). Structural changes in positively and negatively supercoiled DNA. *Eur. J. Biochem.* **184**, 297-303.

Courey, A. J. & Wang, J. C. (1983). Cruciform formation in a negatively supercoiled DNA may be kinetically forbidden under physiological conditions. *Cell,* **33**, 817-829.

Dai, X., Greizerstein, M. B., Nadas-Chinni, K. & Rothman-Denes, L. B. (1997). Supercoiled-induced extrusion of a regulatory DNA hairpin. *Proc. Natl Acad. Sci. USA,* **94**, 2174-2179.

Dai, X., Kloster, M. & Rothman-Denes, L. B. (1998). Sequence-dependent extrusion of a small DNA hairpin at the N4 virion RNA polymerase promoters. *J. Mol. Biol.* **283**, 43-58.

Dayn, A., Malkhosyan, S., Duzhy, D., Lyamichev, V., Panchenko, Y. & Mirkin, S. (1991). Formation of $(dA\text{-}dT)_n$ cruciform in *E. coli* cells under different environmental conditions. *J. Bacteriol.* **173**, 2658-2664.

Depew, R. E. & Wang, J. C. (1975). Conformational fluctuations of DNA helix. *Proc. Natl Acad. Sci. USA,* **72**, 4275-4279.

Di Capua, E., Stasiak, A., Koller, T., Brahms, S., Thomae, R. & Pohl, F. M. (1983). Torsional stress induced left-handed helical stretches in DNA of natural base sequence: circular dichroism and antibody binding. *EMBO J.* **2**, 1531-1535.

Drlica, K. (1992). Control of bacterial DNA supercoiling. *Mol. Microbiol.* **6**, 425-433.

Ellison, M. J., Kelleher, R. J., III, Wang, A. H., Habener, J. F. & Rich, A. (1985). Sequence-dependent energetics of the *B-Z* transition in supercoiled DNA containing nonalternating purine-pyrimidine sequences. *Proc. Natl Acad. Sci. USA,* **82**, 8320-8324.

Gellert, M., O'Dea, M. H. & Mizuuchi, K. (1983). Slow cruciform transitions in palindromic DNA. *Proc. Natl Acad. Sci. USA,* **80**, 5545-5549.

Hagen, F. K., Zarling, D. A. & Jovin, T. M. (1985). Electron microscopy of SV40 DNA cross-linked by anti-Z DNA IgG. *EMBO J.* **4**, 837-844.

Haniford, D. B. & Pulleyblank, D. E. (1985). Transition of a cloned $d(AT)_n$-$d(AT)_n$ tract to a cruciform *in vivo*. *Nucl. Acids Res.* **13**, 4343-4363.

Ho, P. S., Ellison, M. J., Quigley, G. J. & Rich, A. (1986). A computer aided thermodynamic approach for predicting the formation of *Z*-DNA in naturally occurring sequences. *EMBO J.* **5**, 2737-2744.

Horowitz, D. S. & Wang, J. C. (1984). Torsional rigidity of DNA and length dependence of the free energy of DNA supercoiling. *J. Mol. Biol.* **173**, 75-91.

Iacono-Connord, L. & Kowalski, D. (1986). Altered DNA conformations in the regulatory region of torsionally-stressed SV40 DNA. *Nucl. Acids Res.* **14**, 8949-8962.

Klenin, K. V., Vologodskii, A. V., Anshelevich, V. V., Dykhne, A. M. & Frank-Kamenetskii, M. D. (1991). Computer simulation of DNA supercoiling. *J. Mol. Biol.* **217**, 413-419.

Lilley, D. M. (1980). The inverted repeat as a recognizable structural feature in supercoiled DNA molecules. *Proc. Natl Acad. Sci. USA,* **77**, 6468-6472.

Lilley, D. M. J. & Clegg, R. M. (1993). The structure of the four-way junction in DNA. *Annu. Rev. Biophys. Biomol. Struct.* **22**, 299-328.

Lyamichev, V., Panyutin, I. & Mirkin, S. (1984). The absence of cruciform structures from pAO3 plasmid DNA *in vivo*. *J. Biomol. Struct. Dynam.* **2**, 291-301.

Lyubchenko, Y. L. & Shlyakhtenko, L. S. (1997). Visualization of supercoiled DNA with atomic force microscopy in situ. *Proc. Natl Acad. Sci. USA,* **94**, 496-501.

McClellan, J., Boublikova, P., Palecek, E. & Lilley, D. (1990). Superhelical torsion in cellular DNA responds directly to environmental and genetic factors. *Proc. Natl Acad. Sci. USA,* **87**, 8373-8377.

Mirkin, S. M., Lyamichev, V. I., Kumarev, V. P., Kobzev, V. F., Nosikov, V. V. & Vologodskii, A. V. (1987). The energetics of the B-Z transition in DNA. *J. Biomol. Struct. Dynam.* **5**, 79-88.

Murchie, A. I. H. & Lilley, D. M. J. (1992). Supercoiled DNA and cruciform structures. *Methods Enzymol.* **211**, 158-180.

Panayotatos, N. & Wells, R. D. (1981). Cruciform structures in supercoiled DNA. *Nature,* **289**, 466-470.

Panyutin, I. G. & Hsieh, P. (1994). The kinetics of spontaneous DNA branch migration. *Proc. Natl Acad. Sci. USA,* **91**, 2021-2025.

Panyutin, I., Klishko, V. & Lyamichev, V. (1984). Kinetics of cruciform formation and stability o cruciform structure in superhelical DNA. *J. Biomol. Struct. Dynam.* **1**, 1311-1324.

Pantyutin, I., Lyamichev, V. & Mirkinm, S. (1985). A structural transition in $d(AT)_n \cdot d(AT)_n$ inserts within superhelical DNA. *J. Biomol. Struct. Dynam.* **2**, 1221-1234.

Peck, L. J. & Wang, J. C. (1983). Energetics of B-to-Z transition in DNA. *Proc. Natl Acad. Sci. USA,* **80**, 6206-6210.

Pohl, F. M., Thomae, R. & Di Capua, E. (1982). Antibodies to Z-DNA interact with form V DNA. *Nature,* **300**, 545-546.

Pulleyblank, D. E., Shure, M., Tang, D., Vinograd, J. & Vosberg, H. P. (1975). Action of nicking-closing enzyme on supercoiled and nonsupercoiled closed circular DNA: formation of a Boltzmann distribution of topological isomers. *Proc. Natl Acad. Sci. USA,* **72**, 4280-4284.

Revet, B., Zarling, D. A., Jovin, T. M. & Delain, E. (1984). Different Z DNA forming sequences are revealed in FX174 RFI by high resolution darkfield immuno-electron microscopy. *EMBO J.* **3**, 3353-3358.

Rybenkov, V. V., Vologodskii, A. V. & Cozzarelli, N. R. (1997a). The effect of ionic conditions on DNA helical repeat, effective diameter, and free energy of supercoiling. *Nucl. Acids Res.* **25**, 1412-1418.

Rybenkov, V. V., Vologodskii, A. V. & Cozzarelli, N. R. (1997b). The effect of ionic conditions on the conformations of supercoiled DNA. II. Equilibrium catenation. *J. Mol. Biol.* **267**, 312-323.

Rybenkov, V. V., Vologoskii, A. V. & Cozzarelli, N. R. (1997c). The effect of ionic conditions on the conformations of supercoiled DNA. I. Sedimentation analysis. *J. Mol. Biol.* **267**, 299-311.

Seeman, N. C. & Kallenbach, N. R. (1994). DNA branched junctions. *Annu. Rev. Biophys. Biomol. Struct.* **23**, 53-86.

Sinden, R. R. & Pettijohn, D. E. (1984). Cruciform transition in DNA. *Biol. Chem.* **259**, 6593-6600.

Sinden, R. R., Broyles, S. S. & Pettijohn, D. E. (1983). Perfect palindromic lac operator DNa sequence exists as a stable cruciform structure in supercoiled DNA *in vitro* but not *in vivo*. *Proc. Natl Acad. Sci. USA,* **80**, 1797-1801.

Singleton, C. K. (1983). Effects of salts, temperature, and stem length on supercoil-induced formation of cruciforms. *J. Biol. Chem.* **258**, 7661-7668.

Vologodskii, A. V. (1992). *Topology and Physics of Circular DNA*, CRC Press, Boca Raton, FL.

Vologodskii, A. V. & Cozzarelli, N. R. (1994a). Conformational and thermodynamic properties of super-

coiled DNA. *Annu. Rev. Biophys. Biomol. Struct.* **23**, 609-643.

Vologodskii, A. V. & Cozzarelli, N. R. (1994b). Supercoiling, knotting, looping, and other large-scale conformational properties of DNA. *Curr. Opin. Struct. Biol.* **4**, 372-375.

Vologodskii, A. V. & Frank-Kamenetskii, M. D. (1983). The relaxation time for a cruciform structure in superhelical DNA. *FEBS Letters,* **160**, 173-176.

Vologodskii, A. V., Lukashin, A. V., Anshelevich, V. V. & Frank-Kamenetskii, M. D. (1979). Fluctuations in superhelical DNA. *Nucl. Acids Res.* **6**, 967-982.

Vologodskii, A. V., Levene, S. D., Klenin, K. V., Frank-Kamenetskii, M. D. & Cozzarelli, N. R. (1992). Conformational and thermodynamic properties of supercoiled DNA. *J. Mol. Biol.* **227**, 1224-1243.

Wang, J. C., Peck, L. J. & Becherer, K. (1983). DNA supercoiling and its effects on DNA structure and function. *Cold Spring Harbor Symp. Quant. Biol.* **47**, 85-91.

Edited by I. Tinoco

(Received 12 January 1999; received in revised form 8 April 1999; accepted 19 April 1999)