Evidence of cruciform structures in superhelical DNA provided by two-dimensional gel electrophoresis

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Received 13 January 1983

Abstract and keywords not received

1. INTRODUCTION

Two unusual DNA structures have recently been in the focus of attention. They are the cruciform states forming in palindromic sequences and the Z structure forming in alternating $(dG–dC)_n$ sequences. Both are highly unstable in linear DNA with the ordinary chemical composition under physiological conditions. However, negative superhelicity, which is inherent in native DNA, should favour the formation of these structures. The first theoretical estimate of the possibility of occurrence of cruciform structures in superhelical DNA was published by Hsieh and Wang [1]. A thorough statistical mechanical treatment of the problem has been undertaken [2,3]. This treatment allowed for the competition between cruciform states and open regions for the superhelix energy. This led to the conclusion that in short palindromic regions, such as the recognition sites for restriction endonucleases, cruciform structures could not occur at any superhelix density. At the same time in all DNAs with known sequences for which calculations were performed, at least one palindromic region was found where the probability of cruciform occurrence was about unity within the physiological range of superhelix densities [3].

The first experimental evidence of cruciform structures in natural DNA was obtained by [4] and [5].

They discovered that the cleavage site of the single-strand-specific endonuclease in superhelical DNA was the center of the main palindrome. However, as has been argued most recently [6], the enzyme itself might shift the equilibrium in favour of the cruciform structure.

Therefore an alternative approach is needed for studying the occurrence of cruciform states in palindromic regions. This approach may consist of measuring the reduction of DNA mobility in gel due to formation of a cruciform structure. Indeed, authors in [6] and [7] have demonstrated such a reduction as a result of the B–Z transition in artificially inserted $(dG–dC)_n$ regions. The technique of 2-dimensional electrophoresis used in [6] proved to be especially powerful.

However, authors in [6] were unable to detect the formation of a cruciform structure in pBR322 DNA by this method. The reason for this might have been the saturation of DNA mobility *vs* superhelix density at low superhelix density values where the cruciform probability remained negligible.

We have reported evidence to the effect that the mobility of pAO3 DNA topoisomers drops after a certain critical superhelix density is reached [8] and interpreted this result as evidence of the transition of the largest palindrome in this plasmid into the cruciform state. However, since we used one-dimensional electrophoresis, other explanations could not be completely excluded.

Here, we use 2-dimensional electrophoresis to prove beyond any doubt the formation of a cruciform structure in superhelical DNA.

2. MATERIALS AND METHODS

2.1. *Preparations*

The strain *Escherichia coli* C600 carrying plasmid pA03 was kindly supplied by Dr A. Oka.

Published by Elsevier Biomedical Press

00145793/83/0000–0000/$3.00

The pA03 DNA is a quarter of the ColE1 plasmid. It is 1683 bp long and has been sequenced [9]. The DNA was extracted and purified in our laboratory as in [10]. To obtain a broad distribution of topoisomers over the number of superhelical turns we used a mixture of independently extracted DNA preparations and DNA treated with topoisomerase I with ethidium as in [8]. Topoisomerase I from mouse ascid tumors was kindly supplied by E.S. Bogdanova. The *Msp*I and *Bsp*I endonucleases and phage T7 DNA were received from the Institute of Organic Chemistry, USSR Academy of Sciences (Siberian Branch). The *Eco*RI endonuclease was kindly supplied by S.L. Mekhedov. Lyophilized S1 endonuclease (Boehringer Mannheim) was dissolved in a buffer containing 50 mM NaCl, 10^{-4} M $ZnSO_4$, 5 mM Tris (pH 7.5) and 50% glycerol. The digestion of pA03 DNA with the S1 endonuclease was performed as in [8].

2.2. *Gel electrophoresis*

A polyacrylamide–agarose gel containing 2% acrylamide and 0.5% agarose was prepared using a buffer of 36 mM Tris (pH 7.7), 30 mM NaH_2PO_4 and 1 mM EDTA by the method in [11]. This gel was used for separating DNA topoisomers in the 'first dimension' as in [8]. After the separation was completed a 5 mm wide strip of the resulting gel was cut out and placed in a low-ionic strength buffer.

This was done to improve the resolution in the second dimension. The remaining slab was stained with ethidium bromide and used for controlling the quality of separation in the first dimension.

For separation in the second dimension we used Keller's gel [12] containing 1.9% agarose in a buffer of 40 mM Tris (pH 8.0), 5 mM CH_3COONa, 1 mM EDTA supplemented with 0.02 μg/ml of ethidium bromide. Electrophoresis was performed in this gel using a horizontal apparatus in 130 × 120 × 6 mm slabs.

A groove 5 mm deep and 2.5 mm wide was made along one edge of the slab. The polyacrylamide–agarose gel strip cut out after the first electrophoresis run was placed in this groove.

Electrophoresis was performed at room temperature with an electric field of 3 V/cm for 16–18 h. The gel was stained with a fresh solution of ethidium bromide (1 μg/ml) for 30–60 min and then photographed.

Electrophoresis in 4% and 8% polyacrylamide gel was performed as in [8].

3. RESULTS AND DISCUSSION

Fig.1a shows the separation in the first dimension. We have avoided the early saturation of mobility with increasing superhelix density by using the composite polyacrylamide–agarose gel. In the second dimension (fig.1b) we used an agarose gel with ethidium bromide.

The presence of the intercalating dye reduced the twisting number for all topoisomers. This diminished the stress caused by negative superhelicity, so that any region that might have turned into an unusual structure, e.g., cruciform or Z-form, under stress now reverted to the ordinary B-form (for discussion and other applications of 2-dimensional electrophoresis see [6,13]).

Thus in the second dimension we observe the usual picture where mobility is proportional to the

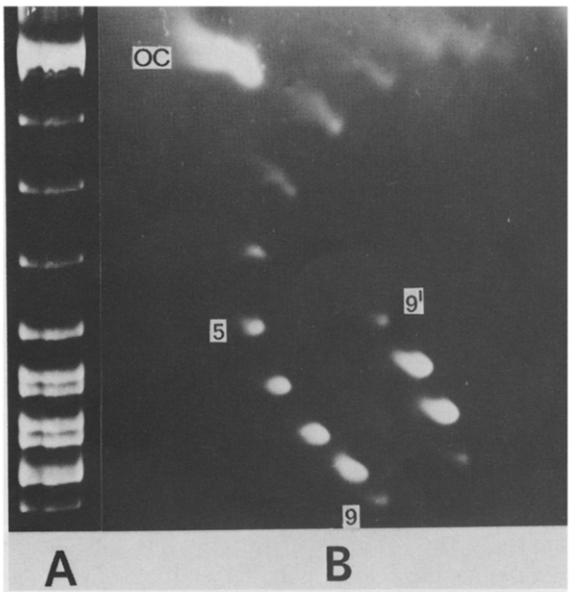


Fig.1. Two-dimensional gel electrophoresis of native pA03 DNA: (a) Electrophoretic separation of pA03 DNA topoisomers in the first dimension; (b) Electrophoretic separation of pA03 DNA in the second dimension in the presence of ethidium bromide. Figures show the number of titratable negative superhelical turns in a topoisomer under the conditions of the first electrophoretic separation. OC, open circular molecules.

(absolute) number of titratable superhelical turns (for the conditions of the second electrophoresis run). One can see 13 topoisomers in total. The topoisomers having 0,1,2,3 and 4 titratable superhelical turns under the conditions of the first electrophoresis run are positively supercoiled in the presence of ethidium. The 5th topoisomer is relaxed in ethidium and moves like open circular DNA. The other 7 topoisomers are negatively supercoiled and their mobility increases progressively with the number of superhelical turns.

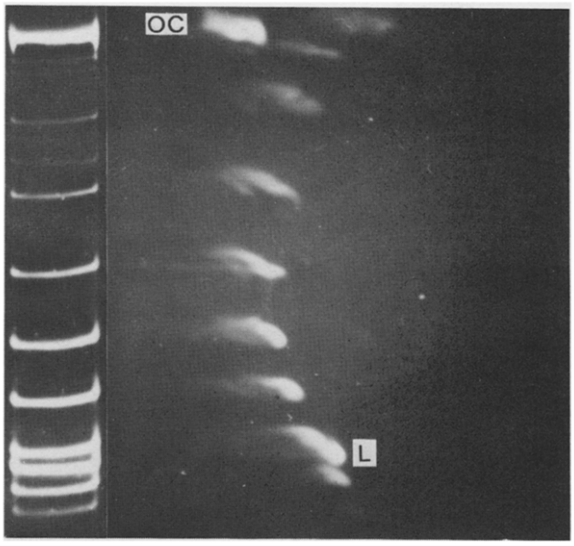
This is not the case in the first dimension. One can clearly see from the 2-dimensional pattern (fig.1b) that in the first dimension mobility increases progressively only up to the 9th topoisomer. Then mobility drops and the 10th topoisomer moves in the first dimension at almost the same rate as the 6th topoisomer. As the number of turns increases, mobility starts growing again.

The pattern shown in fig.1b is remarkably similar to the one observed in [6] for a plasmid carrying the $(dG–dC)_n$ insertion. But our pA03 plasmid contains no artificial insertions and we cannot attribute the drop in mobility to a B–Z transition. Therefore we conclude that what we observed was a transition of the main palindrome of pA03 DNA (which is the same as in ColE1 plasmid) into the cruciform state as assumed in [8].

Let us now compare our data with the results of the S1 endonuclease test for the cruciform structure. We have treated our preparation with the S1 endonuclease and then obtained the 2-dimensional pattern (fig.2). As expected, all topoisomers starting from the 9th have disappeared.

Using restriction endonucleases *Msp*I and *Eco*RI we have shown that the site for the S1 endonuclease is located, to within several nucleotides, in the middle of the main palindrome of the pA03 DNA. The results proved to be basically the same as in [8] (fig.3,4). Special experiments have shown the S1 endonuclease to be non-specific in a preparation containing only topoisomers < 9.

The above results make it quite certain that lower mobility of the 10th topoisomer compared with the 9th one is the result of a cruciform structure forming in the longest palindrome. They also show that the S1 endonuclease attacks only molecules that carry cruciform structure and practically does not shift the equilibrium between the cruciform and the double-helical states.



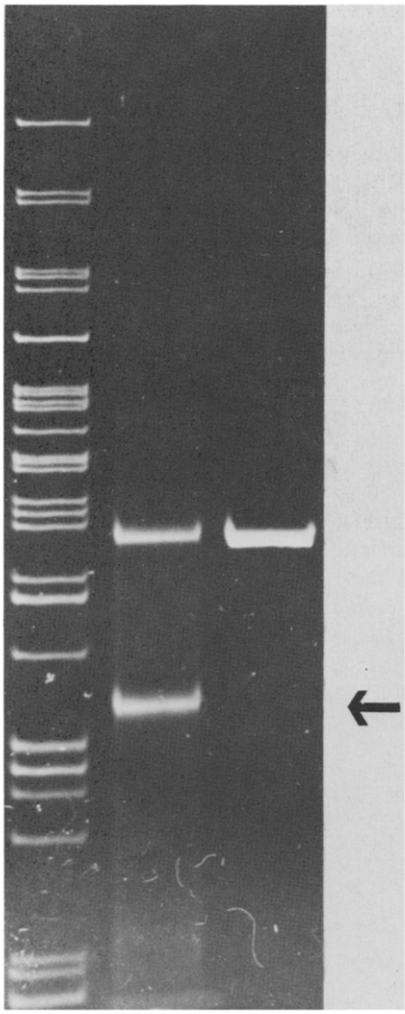
a b

Fig.2. Two-dimensional electrophoresis of pA03 DNA after S1 endonuclease treatment: (a) electrophoretic separation in the first dimension; (b) electrophoretic separation in the second dimension; L, linear molecules.

There are two features of the 2-dimensional pattern that deserve a special discussion:

(1) The 9th topoisomer shows two spots, the second one (9′) coinciding with the 5th topoisomer in the first dimension. The coincidence is so exact that the 5th and 9′th spots are not resolved in the first dimension (fig.1a). What is the nature of the 9′th spot? We could not help assuming that the 9′th spot is the 9th topoisomer in which the cruciform structure has been formed. In the 9th spot the cruciform is obviously not formed. This explanation, however, is valid only if one assumes that the equilibrium between the cruciform and the double-helical states of the palindromic region is not reached in the course of electrophoresis in the first dimension; i.e., for ~20 h. This conclusion has been confirmed by the fact that when electrophoresis was performed at a higher temperature where the relaxation rates should be higher, we observed no such doublets.

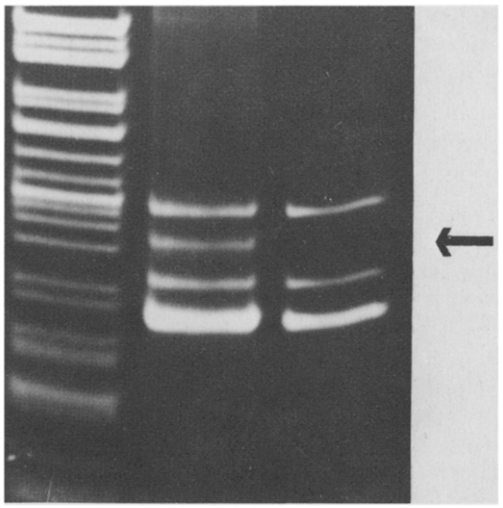
(2) The change in mobility due to cruciform formation proved to be greater than we had expected.



a b c

Fig.3. Mapping of the site for the S1 endonuclease. Electrophoresis was performed in a 4% polyacrylamide gel: (a) the reference pattern obtained as a result of *Bsp*I digestion of T7 DNA [16]; (b) pA03 DNA after treatment with the S1 endonuclease and then the *Msp*I restriction endonuclease; (c) native pA03 DNA treated with the *Msp*I restriction endonuclease. The additional fragment which appeared after the S1 endonuclease treatment is shown by the arrow. The second fragment is too short to be detected under these conditions (see fig.4).

Indeed, the largest palindrome in A03 DNA is 31 bp long and one could expect a cruciform-induced drop in mobility corresponding to 3 superhelical turns. In reality it proved to correspond to exactly 4 turns. In this respect the situation is different from the case of the B–Z transition, where the drop in mobility is almost as expected [6,7]. One could speculate that larger than expected retardation in the case of the cruciform structure is due to its direct influence on DNA mobility in gel. Indeed, even small open regions in DNA are known to affect DNA mobility in polyacrylamide gel [14].



a b c

Fig.4. Mapping of the site for the S1 endonuclease. Electrophoresis was performed in a 8% polyacrylamide gel: (a) the reference pattern obtained as a result of *Bsp*I digestion of T7 DN [16]; (b) pA03 DNA after treatment with the S1 endonuclease and then with the *Msp*I restriction endonuclease; (c) native pA03 DNA treated with the *Msp*I restriction endonuclease. The additional fragment which appeared after the treatment with the S1 endonuclease is shown by the arrow. For details concerning the mapping of the cleavage site see [8].

4. CONCLUSION

Two-dimensional electrophoresis demonstrates the formation of a cruciform structure in pA03 DNA when the number of superhelical turns in the molecule is $\geqslant 9$. This corresponds to the superhelix density $\sigma = -0.05$ and correlates very well with the theoretical prediction [3]. The S1 endonuclease does not shift the equilibrium between the cruciform and double helical states. So one can use this enzyme as a reliable probe of the cruciform state [4,5,15].

ACKNOWLEDGEMENTS

We are grateful to Professor James Wang (Harvard University) for sending us a preprint of his paper [6] which was our main stimulus in conducting this work. We also thank Professor Yu.S. Lazurkin, Dr Yu.L. Lyubchenko and Dr A.V. Vologodskii for helpful discussions and encouragement.

REFERENCES

[1] Hsieh, T.S. and Wang, J.C. (1975) Biochemistry 14, 527–535.
[2] Vologodskii, A.V., Lukashin, A.V., Anshelevich, V.V. and Frank-Kamenetskii, M.D. (1979) Nucleic Acids Res. 6, 967–982.
[3] Vologodskii, A.V. and Frank-Kamenetskii, M.D. (1982) FEBS Lett. 143, 257–260.
[4] Lilley, D.M. (1980) Proc. Natl. Acad. Sci. USA 77, 6468–6472.
[5] Panayotatos, N. and Wells, R.D. (1981) Nature 289, 466–470.
[6] Wang, J.C., Peck, L.T. and Becharer, K. (1983) Cold Spring Harbor Symposium on Quantitative Biology, in press.
[7] Singleton, C.K., Klysik, J., Stirdivant, S.M. and Wells, R.D. (1982) Nature 299, 312–316.
[8] Panyutin, I.G., Lyamichev, V.I. and Lyubchenko, Y.L. (1982) FEBS Lett. 148, 297–301.
[9] Oka, A., Nomura, N., Morita, M., Sugisaki, H., Sugimoto, K. and Takanami, M. (1979) Mol. Gen. Genetics 172, 151–159.
[10] Borovik, A.S., Kalambet, Y.A., Lyubchenko, Y.L., Shitov, V.T. and Golovanov, E.I. (1980) Nucleic Acids Res. 8, 4165–4184.
[11] Germond, G.E., Hirt, B., Oudet, D., Gross-Bellard, H. and Chambon, P. (1975) Proc. Natl. Acad. Sci. USA 72, 1843–1847.
[12] Keller, W. (1975) Proc. Natl. Acad. Sci. USA 72, 4878–4880.
[13] Lee, C.H., Mazusawa, H. and Kakefuda (1981) Proc. Natl. Acad. Sci. USA 78, 2838–2842.
[14] Lyamichev, V.I., Panyutin, I.G. and Lyubchenko, Y.L. (1982) Nucleic Acids Res. 10, 4813–4826.
[15] Singleton, C.K. and Wells, R.D. (1982) J. Biol. Chem. 257, 6292–6295.
[16] Gordon, R.L., Humphries, P. and McConnel, O.J. (1978) Mol. Gen. Genet. 162, 329–339.