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Early melting of supercoiled DNA

Yu.L.Lyubchenko and L.S.Shlyakhtenko

Institute of Molecular Genetics, USSR Academy of Sciences, Kurchatov Sq. 46, Moscow 123182, USSR

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ABSTRACT

Denaturing gradient gel electrophoresis (formamide with urea) has been used to study the melting of supercoiled DNA. A linear gradient of denaturant concentration proportional to a 25°C linear increase of temperature (T_{eff}) from the left to the righ edge of the gel was created perpendicular to DNA migration. The mobility of supercoiled DNA molecules was shown to drop to the level of relaxed molecules a long way (5-30°C) before linear DNA began to melt. The futher increase of T_{eff}, including the melting range for linear molecules, caused no appreciable changes in the mobility of relaxed molecules. The transition curves are S-shaped for all the topoisomers, and an increase of superhelicity shifts the transition towards lower T_{eff} values. The analysis of the results indicates that the observed relaxation of superhelical molecules is due to denatured region forming in them, their size increasing with the topoisomer number.

INTRODUCTION

Negative superhelicity of circular DNA molecules causes a sharp increase of their conformational mobility. As a result natural supercoiled covalenly closed (cc) DNA can form a number of alternative structures under near-physiological conditions, viz. the Z form [1,2], the cruciform [3-6], H-form [7] and denatured segments [8,9]. The fact that these structures may have a part to play in the major molecular biological processes stimulates intensive studies of their structure and of the thermodynamic and kinetic characteristics of their formation in cc DNA. Considerable progress has been made in the study of transitions to Z, H and cruciform conformations within individual segments of cc DNA (e.g. see [10]). This was stimulated by the development of various immunological, enzymic and physico-chemical techniques sensitive to the formation of

such structures. Meanwhile very little is known about the formation of the melted regions in cc DNA. The traditional approaches to the study of linear DNA melting proved ineffective in this case [8]. The first clear evidence of early denaturation in cc DNA was obtained in [8] using an unconventional approach: equilibrium centrifugation in a solution of denaturing salt. In [9] two-dimentional gel electrophoresis was used in an attempt to examine early ccDNA melting more closely. However, the analysis of early melting was hampered, presumably by the formation of alternative, possibly cruciform, structures [9].

In the present work we have used denaturating gradient gel electrophoresis [11-14] to look at the early melting of ccDNA. The marked differences in electrophoretic mobility between supercoiled and relaxed DNA molecules made it possible to observe the denaturation of each pAO3 DNA topoisomer leading to topological relaxation. Such transitions occur in the 5-30^{o}C range, before the melting of linear molecules; the transition width varies from 6^{o}C to 10^{o}C for different topoisomers. The temperature-dependent relaxation occurs the earlier the larger the topoisomer s number.

MATERIALS AND METHODS

1. DNA preparations: pAO3 and pUC19 plasmid DNAs were obtained by the alkali method [14] modified for large volumes, with a subsequent purification of the supercoiled molecules by equilibrium centrifugation in a CsCl density gradient with ethidium bromide. Extraction by butanol followed by chromatography on a column with Sephacryl S-300 was used to obtain preparations free of RNA and ethidium bromide in a 0.1xSSC buffer.

2. Topoisomerase 1 from calf thymus was kindly supplied by Dr. I.B.Bronstein from Institute of Molecular Biology, USSR Academy of Sciences. To obtain DNA preparations with different sets of topoisomers, native DNA was treated with the topoisomerase (1-2 enzyme units to 5 ug of DNA) in the presence of ethidium bromide in different concentrations. The reaction was carried out at room temperature, in darkness, for 18-20 hours. The DNA was

purified of enzyme and ethidium bromide by phenol, precipitated by ethanol and dissolved in a 0.1xSSC buffer.

3. Denaturing gradient gel electrophoresis was carried out at different temperatures in a specially designed apparatus (see detailed description in [14]). A prescribed denaturant concentration gradient was created by gradually mixing two different volumes of solutions required for the preparation of a polyacrylamide gel with different amounts of denaturing agents (formamide with urea). After polymerization the gel slab was turned by 90 and placed in the temperature-controlled apparatus, then after 30 min 10 ug of DNA was layered on the top edge of the slab. Electrophoresis was performed in TAE buffer containing 40 mM Tris, 20 mM sodium acetate, 2mM EDTA, pH 7.8. Electrophoresis lasted for 18 hours at a field voltage of 3.6 v/cm.

4. Two-dimensional gel electrophoresis was performed as described in [5]. The separation in the first direction was carried out in a gel with 2% acrylamide (acrylamide to bisacrylamide ratio

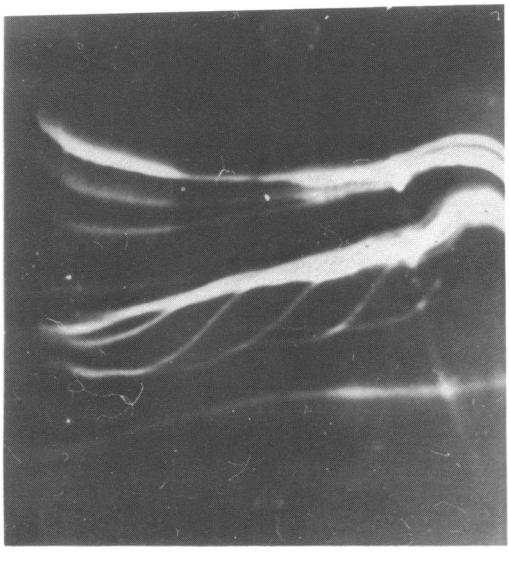


FIGURE 1. Separation of pUC19 DNA topoisomers by denaturing gradient gel electrophoresis. Denaturant concentration increases from left to right. DNA molecules move from top to bottom. The temperature of the gel controlled at 35°C.

20:1) and 0.5% agarose. In several experiments gel was polymerised in the presence of denaturants (5-45%). Electrophoresis in the first direction lasted for 18 hours at a field voltage of 4.6 v/cm. Then the gel was removed from the glassplates and soaked for 5 hours in a solution containing 10 ug/ml chloroquine whereupon electrophoresis was performed in the second direction, perpendicular to the first, for 18 hours at a field voltage of 4.6 v/cm in a buffer containing chloroquine.

RESULTS

Figure 1 presents data on the separation of pUC19 DNA topoisomers in a denaturant concentration gradient. The experiment was carried out at T=35°C. DNA moves from top to bottom, denaturant concentration increase from left to righ. The left edge of the gel corresponds to a situation in which all molecules are native and the separation of topoisomers proceeds

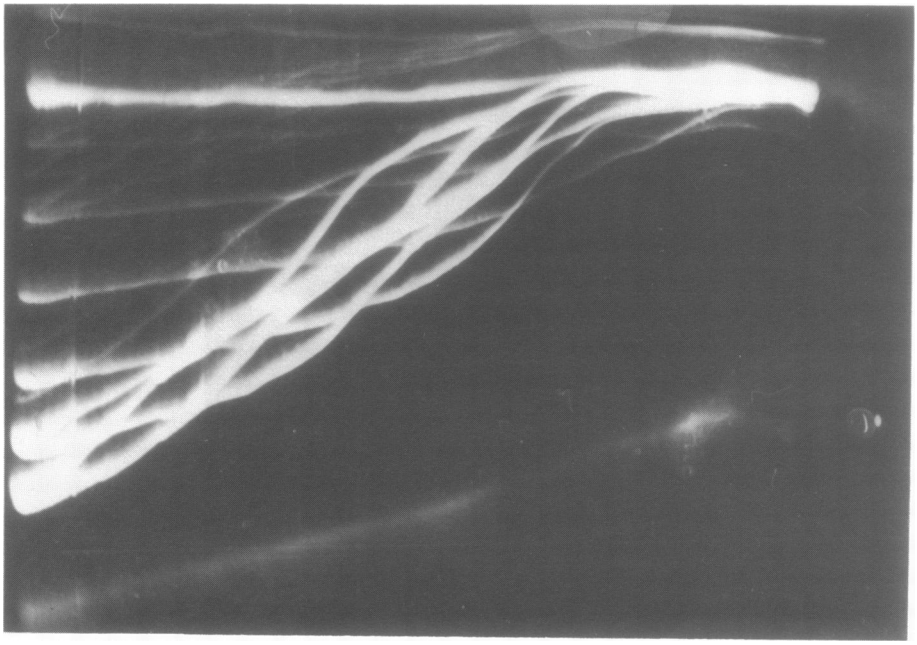


FIGURE 2. Separation of pAO3 DNA topoisomers by denaturing gradient gel electrophoresis. The conditions were the same as in Figure 1 except that the temperature was 43°C.

according to the number of supercoils. Then, as the denaturant concentration increases, the mobility of all topoisomers decreases to that of open circular DNA. Such curves of topological relaxation caused by denaturation are S-shaped, and each topoisomer has its own transition curve. Under these conditions the denaturation of linear molecules occurs well beyond the right edge of the gel. Hence what we observed here is an early denaturational transition in pUC19 DNA causing the topological relaxation of molecules.

We undertook a more detailed investigation of the early denaturation in pAO3 ccDNA. It is about 1.5 shorter than pUC19 DNA, allowing a better mobility-wise resolution of topoisomers in the same kind of gel. Besides, this DNA is well studied both in terms of melting [15,16] and in terms of forming in it cruciform structures [5,7,10].

The gel electrophoresis of pAO3 DNA is presented in Figure 2. Clearly one gets a much better separation of topoisomers. As in the case of pUC19, we observe clear-cut transitions, but the transition curve for some of the topoisomers intersect, making it difficult to unambiguously identify the transition curves for all topoisomers. To get rid of the ambiguity, we performed analogous experiments with pAO3 DNA preparations containing fewer topoisomers. We obtained such preparations by treating the original pAO3 DNA with topoisomerase 1 in the presence of different amounts of ethidium bromide [5].

Figure 3a presents the results of gel electrophoresis for a DNA preparation that contains only 5 topoisomers, as established by two-dimentional electrophoresis (data not shown). In this situation the mobility of topoisomer with one superhelical turn (t1) is no different from that of relaxed molecules or open circular DNA. The experiment was performed at T=52^{o}C. The transitions for topoisomers t3, t4, t5 are very clear. As in the case of pUC19 DNA (see Figure 1), the curves are S-shaped, and the transition ends in a virtually complete topological relaxation. One can see that topoisomer t5 is the first to melt; it is followed by t4, then t3. Note that under these conditions (gel temperature 52^{o}C) linear molecules melt somewhat

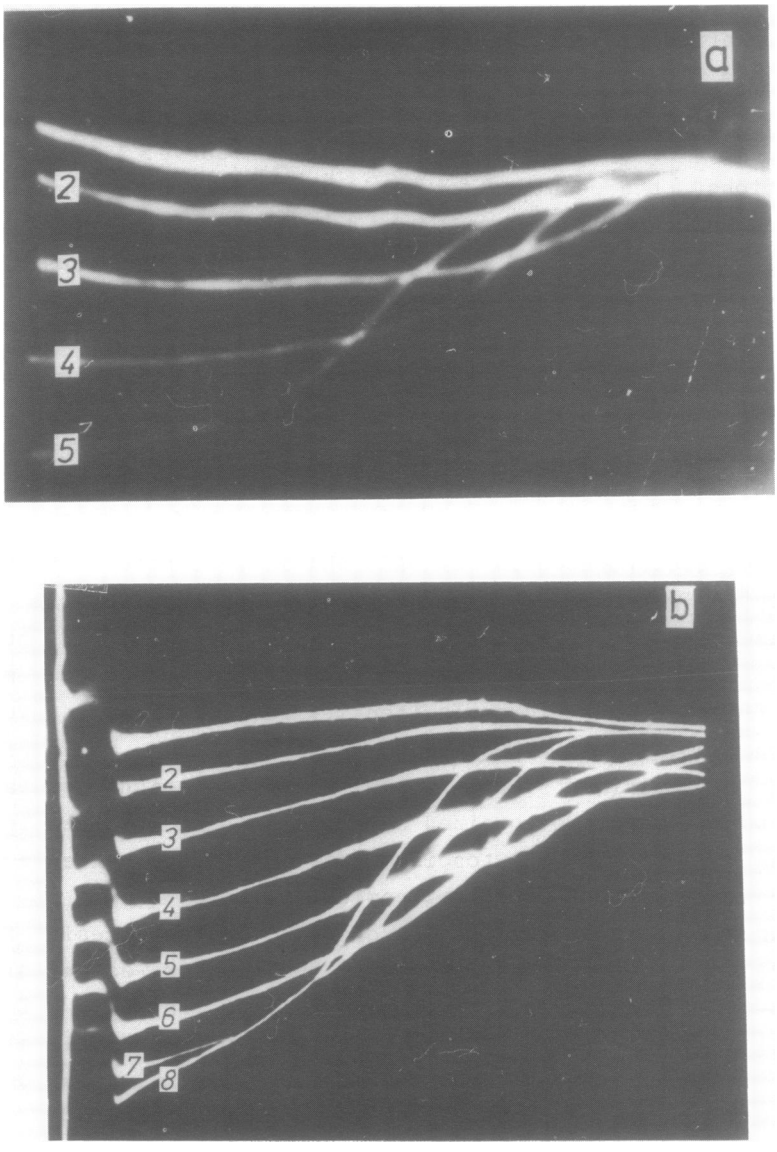
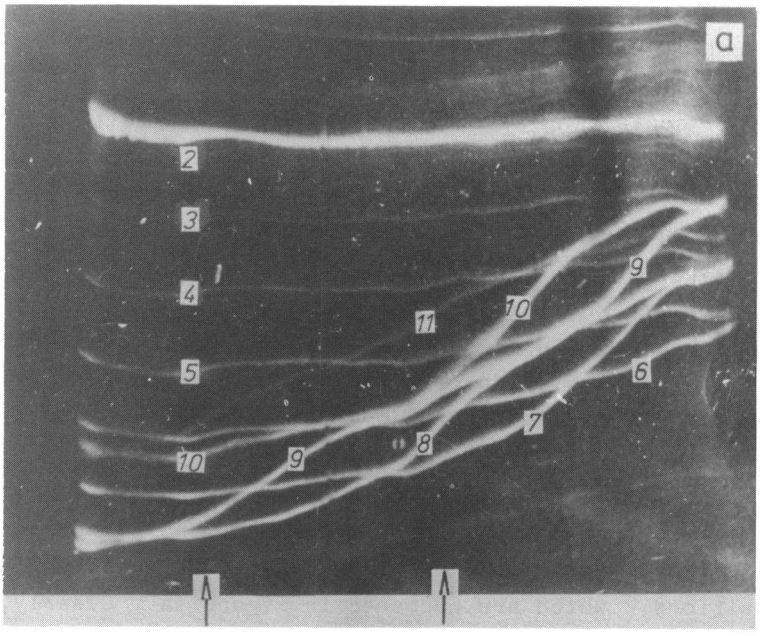
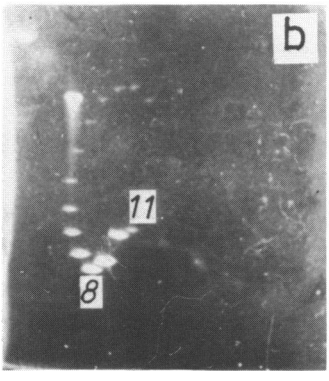


FIGURE 3. Separation of pAO3 DNA topoisomers in the temperature controlled gel: a) 1 to 5 superhelical turns (52°C); b) 1 to 8 superhelical turns (43°C).





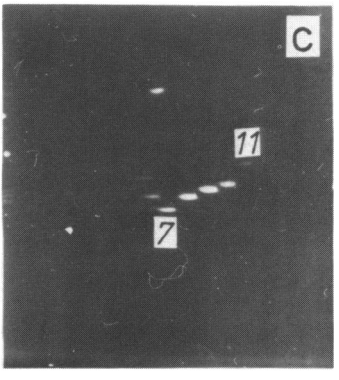


FIGURE 4. a) Separation of pAO3 DNA topoisomers at 35°C. Arrows indicate points at which two-dimensional gel electrophoresis was performed. b) Two-dimensional gel electrophoresis of pAO3 DNA at 35°C in the presence of 20% denaturant. c) Two-dimensional gel electrophoresis of pAO3 DNA at 35°C in the presence of 45% denaturant.

to the right of topoislmer t3. We did not observe the melting of topoisomer t2.

Figure 3b shows similar data obtained for a pAO3 DNA preparation containing all the topoisomers, t1 to t8. As in Figure 3a, the greater a topoisomer s superhelicity the earlier we observe its denaturation.

Apart from topoisomers t1-t8, whose melting is clearly recorded in Figure 3a,b, the initial pAO3 DNA preparation (see Figure 2) contains more supercoiled topoisomers, including those carryng cruciform sructures. To identify all the transitions in Figure 2, we compared the results of gel electrophoresis under denaturing conditions with the results of two-dimensional electrophoresis. Figure 4a presents the denaturing gradient gel electrophoresis at 35°C, and Figure 4b,c presents the two-dimensional gel electrophoresis for two denaturant concentrations (indicated by arrows in Figure 4a). This comparison enabled us to identify all the transitions, which are numbered in Figure 4a. Clearly, all the transitions occur the earlier the larger the number of the topoisomer concerned, and topoisomers t10 and t11, which carry cruciform structures, are not exception.

DISCUSSION

The data presented above demonstrate that denaturing gradient gel electrophoresis makes it possible to observe the melting of ccDNA caused by negative supercoiling. Since it is possible to observe this transition for individual topoisomers, one can conclusively prove that the previously observed early melting of ccDNA leads to the topological relaxation of supercoiled molecules. Our parallel studies of the melting of linear pAO3 DNA and its restriction fragments by means of high-resolution spectrophotometry and denaturing gradient gel electrophoresis made it possible to turn our estimation of ccDNA melting in terms of denaturant concentration into an estimation in terms of effective temperature (T_{eff}). Thus it appears that the denaturant concentration range corresponds to a T_{eff} range of 25 °C. This means that the data in Figure 2 correspond to a temperature change from 43°C to 68°C. For our ionic conditions (TAE buffer, see MATERIALS AND METHODS) the melting of linear

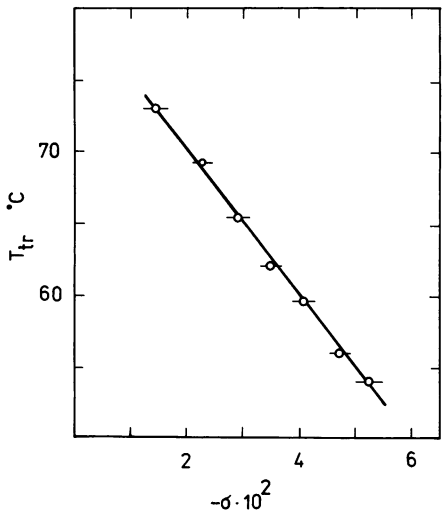


FIGURE 5. Relation between superhelix density (σ) and temperature transition (T_{tr}) for individual topoisomers.

pAO3 DNA molecules starts at 70°C (the first sharp peak in the melting profile [15,16] corresponds to 74°C).

Consequently, the transition for all the topoisomers in Figure 2 end before the melting of linear DNA begins, hence what we observe is the early melting of ccDNA. It is also possible to estimate the transition width for the various topoisomers. It amounts to 6°C for t4 and t5, and 8-10° C for the others; topoisomer t9 has the largest transition width. We also used these data to establish the transition temperature for the various topoisomers: we assumed this to be the temperature corresponding to that point on the transition curve in which the mobility of the given topoisomer coincided with that of a topoisomer having half as many supercoils. These data, based on the results of different experiments, are presented in Figure 5. One can see that for all the topoisomers that do not contain cruciform structures, i.e. for t3-t9, the transition temperature falls with increasing superhelicity.

Let us analyse these data using the description of ccDNA melting described in [17]. That study deals with the melting of

TABLE 1. The melted regions size (n) of pAO3 DNA calculated by formulae (4) for topoisomers with different number of superhelical turns (t).

t	9	8	7	6	5	4	3
n	87±30	88±20	60±15	59±15	52±15	39±10	35±10

a ccDNA region of n base pairs with uniform stability. In this case the free energy change ΔF can be written as

$$\Delta F=(\Delta H-T\Delta S)\cdot n + F_s + \Delta G \qquad (1)$$

where ΔH, ΔS are, respectively, the change in melting enthalpy and entropy per bp, F_s is free energy of the boundary, ΔG is the change of the free energy of supercoiling upon the melting of the n-base-pair-long region. The expression for ΔG one can obtain using the equation for free energy of supercoiling G which account for formation of denatured regions in supercoiled DNA [17]:

$$G(\sigma,\theta) = 10\ RTN[(1-b)\cdot(\sigma+\theta)^2 + b\sigma^2] \qquad (2)$$

here $\theta=n/N$ is degree of denaturation, b - is a theoretical parameter; σ - is the supercoiled density, which is (by definition) equal to 10 t/N, where t is the number of superhelical turns and N is the number of base pairs in the DNA. Then for the change of free energy of supercoiling upon melting of n base-pairs-long region ΔG, we obtain the following equation:

$$\Delta G = \left|(\partial G/\partial\theta)\right|_{\theta=0} \cdot n/N = 20RT(1-b)\cdot n\cdot\sigma \qquad (3)$$

Then $\Delta F=0$ at the transition point (T_{tr}), and by substituting (3) into (1) we get the following expression for n:

$$n=F_s[20RT_{tr}(1-b)(-\sigma)-\Delta H(1-T_{tr}/T_m)]^{-1} \qquad (4)$$

where T_m is the region's melting temperature in linear DNA.

We calculated n for the following values: F_s=6000 kcal/mol, ΔH=8800 kcal/mol (this value corresponds to the melting enthalpy

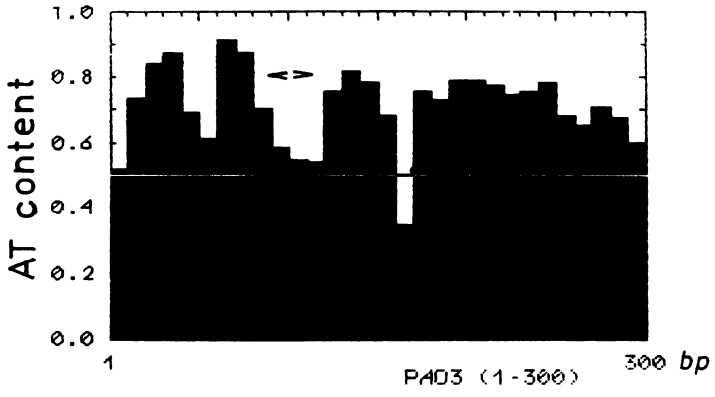


FIGURE 6. Histogram of AT distribution in the most AT-enriched fragment of pA03 DNA (window size is 10 bp). The arrows above indicate the location of the major palindrome of this DNA.

of the lowest-melting regions of pA03 DNA containing 80% AT base pairs [16], b=0.2 [17]. Table 1 presents the data for n corresponding to topoisomers t3 to t9. Assuming that complete relaxation occurs upon the melting of a DNA region of n=10t, we can see that the results are close to the expected values. Of course, the above analysis, which is based on the "all-none" melting model for a ccDNA region, should be regarded only as a qualitative consideration of early ccDNA melting, especially since the values of all the thermodynamic parameters were taken for linear DNA, while they may be different for ccDNA. Still, the resulting reasonable values of n show that our interpretation of the data is on the whole correct: the observed transitions in ccDNA amount to a partial denaturation caused by the excessive energy of supercoiling.

In Figure 2 and 4a one notice the complexity of the transition curves for topoisomers t8 and t9.They contain several inflection points and, by analogy with the melting of linear DNA [19], one can suppose that the early ccDNA melting profile has a fine structure. It manifests itself most clearly for the transition curves of topoisomers t8 and t9. This effect may be due to the highly uneven distribution of AT pairs in the AT-enriched region of pA03 DNA. Figure 6 shows the AT content histogram for a

300bp-long fragment of pAO3 DNA. It is the most AT-enriched part of pAO3 DNA, which extends to the right of the EcoR1 site and includes the major palindrome of this DNA [5,20] (indicated by arrows in the top part of the histogram in Figure 6). In this region one notice two especially AT-rich segments, each some 20-30 bp long, spaced by relatively GC-enriched stretch. It seems natural to suppose that it is the denaturation of this part of the molecule that causes the temperature-dependent relaxation of pAO3 ccDNA, and then the highly uneven distribution of AT pairs may lead to the stepwise melting of the entire AT-enriched region.

Here we should note an important distinction between the formaton of a melted region and the formation of other alternative structures. Once a denatured region is formed, it can, by virtue of its flexibility, serve as a kind of swivel reducing the overall superhelical stress.

The above analysis referred to cruciform-free topoisomers. However, the data in Figures 2 and 4a make it possible to examine the melting of topoisomers t10 and t11 which contain cruciform structures. The distinctive feature of these topoisomers is that, apart from the formation of melted regions, the structure of the cruciform itself may change. This would exacerbate the complexity of the melting process, which seems to be, reflected in the melting profiles of these topoisomers.

Certainly the relation between the early melting of ccDNA and changes of cruciform structure is the most interesting question and now the experiments on this problem are in progress.

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