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Direct visualization of supercoiled DNA molecules in solution

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The shape of supercoiled DNA molecules in solution is directly visualized by cryo-electron microscopy of vitrified samples. We observe that: (i) supercoiled DNA molecules in solution adopt an interwound rather than a toroidal form, (ii) the diameter of the interwound superhelix changes from about 12 nm to 4 nm upon addition of magnesium salt to the solution and (iii) the partition of the linking deficit between twist and writhe can be quantitatively determined for individual molecules.

Key words: cryo-electron microscopy/DNA structure/DNA topology/interwound supercoiling/supercoiled DNA

Introduction

Studies of the shape of supercoiled DNA in solution are important for the precise determination of the mechanical properties of DNA and for understanding the biological mechanisms which involve bending, twisting and looping of DNA. Previous work on the shape of supercoiled double-stranded (ds) DNA in solution has led to controversial interpretations which have supported both a toroidal (Brady *et al.*, 1976, Brady *et al.*, 1987) and an interwound model of DNA supercoiling (Langowski, 1987; Torbet and DiCapua, 1989). Most electron microscopy data favor the interwound model (Rhoades and Thomas, 1968; Sperrazza *et al.*, 1984; Boles *et al.*, 1990). However, these routine electron microscopy preparation procedures can be misleading; it cannot be ruled out that supercoiled DNA molecules undergo a transition from a toroidal to an interwound form as a result of the adsorption and drying on the supporting film. Analysis of DNA knots and catenanes produced in solution by phage lambda integrase (Int) provided the first clear-cut evidence that supercoiled DNA molecules in solution can adopt an interwound form (Spengler *et al.*, 1984, 1985), but again, this form of supercoiling could have been induced by the interaction of supercoiled DNA molecules with the Int protein. The present observations made by cryo-electron microscopy of vitrified specimens (Dubochet *et al.*, 1985, 1988) demonstrate that in a solution typical for *in vitro* experimentation the natural state of protein-free supercoiled DNA is an interwound superhelix.

Results

Naturally supercoiled DNA appears in low ionic strength solution as interwound superhelix with a diameter of ~12 nm

Many of the physico-chemical studies of the shape of supercoiled DNA molecules take as a reference value the data obtained from a DNA sample in low ionic strength solution and compare it with a sample in high ionic strength solution (Brady *et al.*, 1987; Langowski, 1987; Torbet and DiCapua, 1989). We decided to follow this approach.

Figure 1 shows a vitrified specimen of naturally supercoiled plasmid pUC18 DNA molecules (2686 bp) in 10 mM Tris–HCl, 1 mM EDTA, pH 7.5. The molecules are in a ~50 nm thick unsupported layer of vitrified buffer spanning a hole in a perforated supporting film; they are neither stained nor chemically fixed. Most of the molecules are supercoiled DNA molecules that form unbranched interwound superhelices. Only one branched supercoiled DNA molecule is visible in the field (b); another DNA molecule is probably nicked because it appears as a relaxed circle (r). As expected, the width of the dsDNA is ~2nm. The diameter of the superhelix is ~12 nm in this buffer.

Interwound superhelix decreases its diameter upon addition of magnesium salt to the solution

Previous studies of superhelical DNA did not lead to consensus about the effect of rising ionic strength of solution on the superhelix configuration (compare Brady *et al.*, 1987 with Langowski, 1987). The lack of consensus results in part from a choice of different calculation procedures which match very indirect data (like the X-ray scattering profiles) with a chosen model of DNA molecules.

By observing DNA directly in solution we are in a position to verify previous estimations of the effect of rising ionic strength on the shape of superhelical DNA. We increased the ionic strength of the DNA solution by adding $MgCl_2$ to a final concentration of 10 mM. Influence of Mg ions on DNA is particularly interesting as the low salt buffers containing about 10 mM $MgCl_2$ are solutions which support protein–DNA interactions in typical *in vitro* reactions, like restriction endonuclease digestion or RNA polymerase binding to DNA.

Figure 2 shows the same DNA as that analyzed in Figure 1 but it was vitrified in the buffer supplemented with 10 mM $MgCl_2$. The striking difference between the two samples is that in the low salt buffer (without added $MgCl_2$) the diameter of the interwound superhelix is ~12 nm but it decreases to ~4 nm in the presence of 10 mM $MgCl_2$. In the latter case, circular supercoiled DNA molecules look like linear filaments as the two interwound double-stranded segments are difficult to follow individually, except at the end-loops.

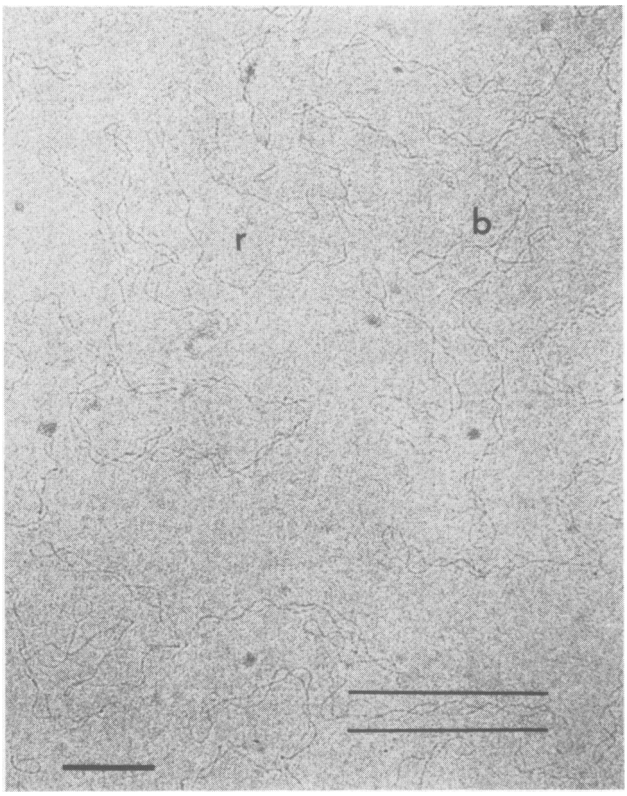

Fig. 1. Sample of naturally supercoiled pUC18 DNA (2686 bp) in a thin layer of vitrified solution (10 mM Tris−HCl, 1 mM EDTA, pH 7.5); the specimen is observed in its hydrated state by cryo-electron microscopy. Most molecules form unbranched interwound superhelices. Two exceptions are marked: r) relaxed DNA molecule. b) branched interwound molecule. A straight region of an interwound molecule is framed between two parallel lines. Magnification bar: 100 nm.

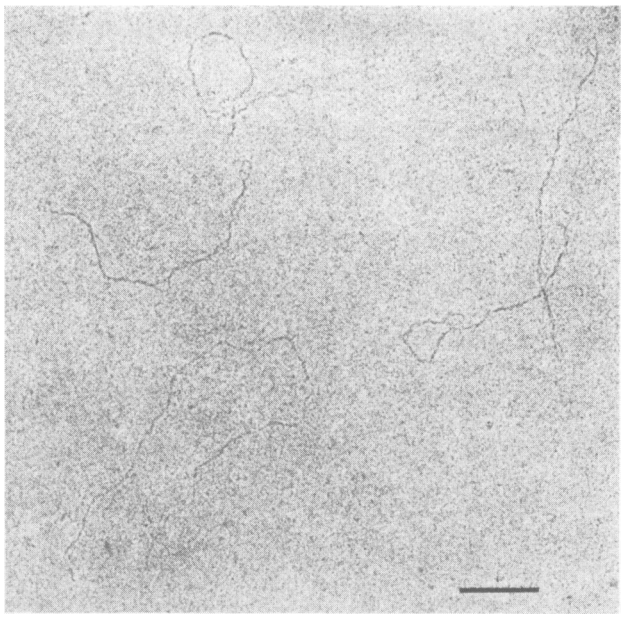

Fig. 2. Supercoiled pUC18 DNA molecules in a thin layer of vitrified buffer containing divalent cations (10 mM, Tris−HCl, 1 mM EDTA, 10 mM $MgCl_2$, pH 7.5). Four naturally supercoiled and one relaxed molecules are seen. The diameter of the interwound superhelix is about 4 nm in the magnesium salt containing buffer while the relaxed DNA remains in the form of an unfolded circle (compare with Figure 1). The relaxed molecule is in the left lower part of the micrograph. The two supercoiled DNA molecules on the right side overlap each other at one point, the supercoiled molecule on the upper left side is folded and its axis of supercoiling forms a loop. Magnification bar: 100 nm.

DNA molecules in vitreous layer are suspended freely in the available volume and are not preferably adsorbed onto the air−liquid interface

The power of cryo-electron microscopy of vitrified layers rests on the possibility of observing bio-molecules which are freely suspended in solution and are not flattened down by interaction with a supporting surface (Dubochet *et al.*, 1985, 1988). However, it has to be demonstrated that DNA molecules remain in solution and are not preferably adsorbed to the air−liquid interface before vitrification. If this type of adsorption did take place, supercoiled DNA molecules could get distorted in similar way to specimens prepared by conventional methods. This could lead to quantitative or even qualitative changes in the structure of supercoiled DNA molecules (such as the change from toroidal to interwound supercoiling).

In order to determine the position of DNA molecules in vitrified layer we have obtained stereo images of vitrified samples by taking pairs of pictures at different tilt angles with respect to the electron beam. Such a stereo pair is shown in Figure 3. The pair of pictures shows one of the tightly supercoiled DNA molecules, prepared in a buffer containing magnesium. This molecule adopted a kind of super-super helix configuration with one and a half turns. For those who cannot see the stereo representation, the direct comparison of the two micrographs also demonstrates that the molecule is not planar but that it extends in the third dimension. These pictures and many others obtained (not shown) demonstrate that the DNA molecules are suspended freely in the aqueous layer rather than being adsorbed onto the air−liquid interface. The thickness of the vitrified film (50−100 nm) is smaller than the total length of the interwound superhelix. The supercoiled DNA molecules are therefore confined to this layer of vitrified solution. In the electron microscope the vitrified layer is perpendicular or slightly tilted towards the imaging beam, therefore the suspended molecules are seen in lateral projections. Head-on views are not observed.

Partition of linking deficit into change of twist and writhe can be calculated for the individual observed molecules

The supercoiling of a dsDNA molecule reflects how the linking deficit (ΔLk) is partitioned between the change of writhe (ΔWr) and the change of twist (ΔTw). Many experimental (Horowitz and Wang, 1984; Spengler *et al.*, 1985; Brady *et al.*, 1987; Langowski, 1987; Torbet and DiCapua 1989; Boles *et al.*, 1990) and theoretical (M. Frank-Kamenetskii, personal communication) approaches have been used to determine the writhe of supercoiled DNA in solution, but direct measurements have not been possible up to now.

A rigorous determination of writhe requires that the 3-D shape of the molecule is reconstructed from its projections (E.H.Egelman and J.Dubochet, in preparation) but an approximate value can already be deduced from a single projection as, for example, the one under- and overlined in Figure 1. In particular, the two superimposed undulations indicate that the two DNA segments wind helically around a common axis. The supercoiled molecule can therefore be

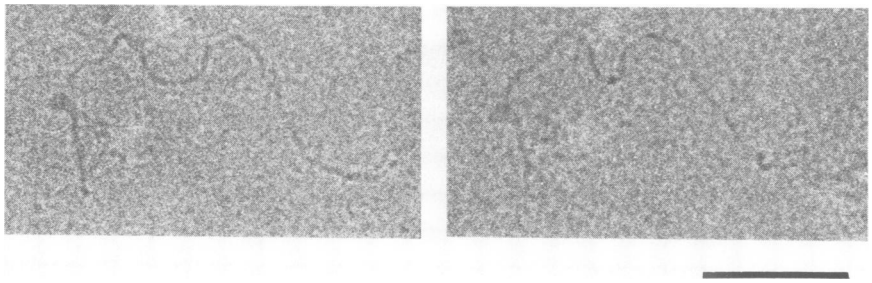

Fig. 3. Stereo pair of a supercoiled pUC9 DNA molecule vitrified in buffer containing 10 mM $MgCl_2$. The tilt axis is vertical and the tilt angle is 20°. Magnification bar: 100 nm.

approximated by two interwound helices wrapped symmetrically around the surface of a cylinder and connected at both ends by semi-circular links. For this geometry, the writhe (Wr) is related to the pitch angle α of the superhelix and to the number of times (n) the DNA is wrapped around the virtual cylinder by the relation (1): $Wr = -n.\sin\alpha$ (Bauer *et al.*, 1980). For a supercoiled topoisomer of pUC18 DNA isolated by gel electrophoresis (Pough *et al.*, 1989) with $\Delta Lk = -12$, we observed that the vitrified molecules show 11 or 12 intersections which correspond approximately to 11.5 wrappings around the surface of the virtual cylinder. The pitch angle α of the superhelix was $55 \pm 6°$ when measured in regions where the axis of the supercoiled molecule is approximately straight, as in the molecule framed in Figure 1. From these data using equation (2), $\Delta Lk = \Delta Wr + \Delta Tw$ (Bauer *et al.*, 1980), it is calculated how the linking deficit, $\Delta Lk = -12$, is partitioned into a change of twist, $\Delta Tw = -2.6$ and a change of writhe, $\Delta Wr = -9.4$. However, we would like to stress that the procedure used in this work does not yet allow a reliable determination of the measurement error. Nevertheless, the partition of linking deficit between change of twist and writhe can be estimated to be between 1:3 and 1:4. The increase of the DNA helical repeat in this supercoiled plasmid of 2686 bp is therefore only of the order of 0.1 bp/turn, though the torsional stress is about twice as high as that *in vivo* (Bliska and Cozzarelli, 1987). We have checked that Wr remains unaffected during specimen preparation by observing that most of the molecules constructed with $\Delta Lk = 0$ (circular double-stranded DNA molecules ligated from the relaxed nicked state) appeared as relaxed, not self-intersecting, although irregular circles (not shown).

Discussion

Our study demonstrated that in solutions used for physico-chemical or biochemical experimentations, supercoiled DNA molecules adopt an interwound rather than a toroidal form. (Figures 1 and 2). We observed that the shape of supercoiled DNA molecules is strongly affected by the presence of magnesium ions in the solution (compare Figures 1 and 2). With this method we can try to answer what factors make important contribution in determining the shape of supercoiled DNA molecules. In the supercoiled DNA, torsional tension tends to bring interwound segments together but at the same time the two opposing, interwound segments are kept at a distance by mutual repulsion and by entropic effects (M.Frank-Kamenetskii, personal communication). The interplay between these factors defines the diameter of the virtual cylinder on which the interwound helices are wrapped. We demonstrated here that the diameter of supercoiled DNA molecules is ~12 nm in 10 mM Tris buffer and it is reduced to 4 nm when 10 mM $MgCl_2$ is added to the solution, therefore approaching direct contact between DNA segments. In the former case, one may be surprised by the apparent long range repulsion under conditions where the Debye length is only ~3 nm. This result, however, is in agreement with a dynamic simulation (M.Frank-Kamenetskii, personal communication), thus pointing to the important role of the entropic effect in defining the shape of DNA in solution. The compaction of supercoiled DNA induced by Mg ions might be specific for these ions and other ions could have less pronounced effects. It remains, however, very significant as changes in inter-cellular concentration of counter-ions could modulate the structure of torsionally stressed DNA. Whether the magnesium induced compaction of a supercoiled DNA is due to a reduction of the effective diameter of the DNA, to a decrease of the number of base pairs per turn (increasing torsional stress) or to a change in DNA torsional and bending resistance remains to be determined.

Materials and methods

Cryo-electron microscopy

Samples were prepared and photographed according to method described previously (Adrian *et al.*, 1984; Dubochet *et al.*, 1988): a 3 μl droplet of DNA solution (200 μg/ml) was put on a hydrophobic perforated supporting film; most of the solution was removed with an automatic blotting device (Cyrklaff *et al.*, in press) and the grid was plunged into liquid ethane. Rapid freezing leads to specimen vitrification without induction of ice crystal formation. The thickness of the vitrified layers was between 50–100 nm. After vitrification, specimens were quickly mounted in a liquid nitrogen precooled Gatan 626 cryo-holder and introduced into a Philips EM 12 electron microscope. Images were recorded at a magnification of 45–60 000× with minimal electron dose (dose on the specimen was ~2000 electrons/nm^2). Though there is no beam induced bubbling on clean DNA solution, higher dose leads to a reduction of the contrast of the molecule and to more severe beam induced drift. SO163 Kodak films were developed for 12 min in D19, full strength (speed ~2 mm^2/electron) leading to an optical density of 1. Magnification was determined by a cross-grating replica.

DNA preparation

Naturally supercoiled pUC18 DNA was prepared following standard plasmid isolation procedure (Sambrook *et al.*, 1989) which includes lysis by alkali, CsCl–ethidium bromide equilibrium gradients and isoamyl alcohol extraction to remove ethidium bromide from DNA. Individual topoisomers were isolated from the agarose gels after two dimensional electrophoresis following the procedure described by Pough *et al.*, (1989).

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