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Winding of the DNA helix by divalent metal ions

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ABSTRACT

When supercoiled pBR322 DNA was relaxed at 0 or 22°C by topoisomerase I in the presence of the divalent cations Ca^{2+}, Mn^{2+} or Co^{2+}, the resulting distributions of topoisomers observed at 22°C had positive supercoils, up to an average ΔLk value of +8.6 (for Ca^{2+} at 0°C), corresponding to an overwinding of the helix by 0.7°/bp. An increase of the divalent cation concentration in the reaction mixture above 50 mM completely reversed the effect. When such ions were present in agarose electrophoresis gels, they caused a relaxation of positively supercoiled DNA molecules, and thus allowed a separation of strongly positively supercoiled topoisomers. The effect of divalent cations on DNA adds a useful tool for the study of DNA topoisomers, for the generation as well as separation of positively supercoiled DNA molecules.

INTRODUCTION

Double-stranded circular DNA molecules can exist in several isomeric forms that are distinguished by their linking number, *Lk* (number of helical turns per circular DNA molecule). Different topoisomers of a plasmid differing by unit *Lk* can be separated by their electrophoretic mobility in an agarose gel (1–3). If the topoisomer with the electrophoretic mobility of the open circular ('nicked') form of the plasmid is given a ΔLk value of zero, then positive and negative ΔLk values produce a similar number of positive or negative supercoils of the plasmid, respectively.

Because of its biological importance, DNA supercoiling has often been studied with bacterial plasmids or other circular DNA molecules. For example, the extent of supercoiling of plasmids isolated from bacteria has been used to obtain information about the function of gyrases (4); different topoisomers of plasmid DNA have been used as substrates for RNA polymerase (5,6) or recombination enzymes (7,8). In such studies different topoisomers are generally identified by their electrophoretic mobility. However, positively and negatively supercoiled plasmids may have identical electrophoretic mobilities, and strongly positively or negatively supercoiled topoisomers show a limit of electrophoretic mobility that does not allow a distinction of different topoisomers. For negatively supercoiled DNA, this limit can be overcome by including intercalating agents, such as chloroquine or ethidium bromide, in the electrophoresis gel (1,2). However, there has been no agent available that would overwind DNA and allow a similar analysis in the case of positively supercoiled DNA.

Until the discovery of reverse gyrase (9), it was difficult to produce positively supercoiled DNA *in vitro*. Plasmids isolated from bacteria treated with gyrase inhibitors are positively supercoiled (4), presumably by the combined action of RNA polymerase and topoisomerases, which produces overwound DNA upstream of the transcription complex (10–12). Positively supercoiled DNA has also been produced by the combined action of DNA ligase and DNA gyrase on open circular DNA in the absence of ATP (13), and by treating circular DNA with topoisomerase I in the presence of the minor groove binders netropsin or distamycin A (14). Here it is shown that positively supercoiled pBR322 DNA can be readily produced if plasmids are relaxed by topoisomerase I in the presence of divalent metal ions, and such positively supercoiled DNA may be analysed by including divalent cations in the electrophoresis gel.

Monovalent cations have previously been found to overwind DNA (15). With the exception of electron microscopic studies of supercoiled DNA molecules in the presence of either NaCl or $MgCl_2$ (16,17), divalent ions have not been systematically checked in this regard. In those studies, specific ion effects exert themselves through long-range electrostatic effects on persistence length and effective diameter (i.e., the closest approach of two parallel DNA strands) that contribute to the variance of the linking number distribution, whereas we show here a specific effect of divalent metal ions on the mean value of the linking number. The analyses of cryo-electron microscopic images of circular DNA showed that twist, writhe, effective diameter and overall shape of supercoiled DNA all change as a result of cation interactions (16,17). For example, at 10 mM concentration, $MgCl_2$ reduces the effective diameter of DNA 3-fold from 12 to 4 nm (16). DNA parameters have also been determined from the extent of knot formation during cyclization of linear DNA molecules that have complementary single-stranded ends like those of phage lambda. Using this method, NaCl was found to reduce the effective diameter of DNA like $MgCl_2$, but only at 10-fold higher concentrations (18). These effects were thought to result from the neutralization of electrostatic repulsion between interacting DNA segments (19). Again using knot formation, a direct comparison of NaCl and $MgCl_2$ indicated that Mg^{2+} not only shields the negatively charged DNA more effectively than Na^+, but may also introduce an attractive potential between DNA segments at concentrations >50 mM

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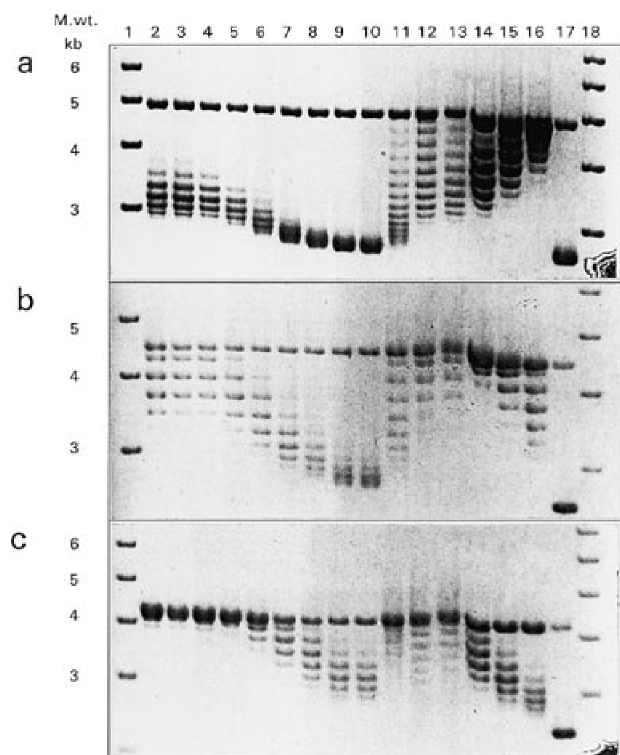

Figure 1. Analysis by agarose gel electrophoresis of pBR322 topoisomer distributions generated during relaxation of pBR322 DNA with topoisomerase I. Lanes 1 and 18, 1 kb ladder (see scale at left); lanes 2–13, relaxation at 0°C in the presence of increasing concentrations of $CaCl_2$ (0, 0.1, 0.5, 1, 2, 5, 10, 20, 40, 60, 100 and 400 mM); lanes 14–16, relaxation at 30°C in the presence of increasing concentrations of ethidium bromide (0, 0.2 and 0.4 µg/ml); lane 17, untreated plasmid DNA. Electrophoresis was carried out at 22°C. (**a**) Gel contained 0.75 µg/ml chloroquine; (**b**) normal gel without additions; (**c**) gel contained 1.5 mM $CaCl_2$.

(20). Based on the observation that 100 mM NaCl causes the same reduction in effective DNA diameter as 10 mM $MgCl_2$, it had been concluded (17) that the tightening of supercoiled DNA by $MgCl_2$ does not involve the formation of magnesium bridges (21). However, such bridge formation has not been ruled out at >50 mM $MgCl_2$ concentrations. The effective DNA diameter and helical repeat have also been determined from the variance of the equilibrium distribution of the linking number of circular DNA molecules: the lower the effective diameter, the greater the variance of topoisomers with a given average linking number (22). In that work, nicked DNA circles were either ligated with T4 ligase for $MgCl_2$-containing solutions, or supercoiled DNA was relaxed with wheatgerm topoisomerase I for NaCl solutions. Again, magnesium ions were found to be much more effective than sodium ions. Finally, DNA aggregates at higher temperatures in the presence of divalent cations; for example, at 100 mM concentrations of manganese, calcium or strontium, 50% of linear DNA molecules aggregate when the temperature is raised to 40, 70 and 85°C, respectively (23). The relevance of these observations for our current data on DNA winding will be discussed below.

MATERIALS AND METHODS

Chemicals and enzymes

Chloroquine (diphosphate) was purchased from Sigma Chemical Co. Agarose (electrophoresis grade, low EEO) was from Fisher Scientific. DNA topoisomerase I (from wheatgerm) was a gift from S.Levene.

Preparation of supercoiled pBR322 DNA

A culture of the *Escherichia coli* strain HB101 (24) harboring a pBR322 monomer was grown in a rotary shaker bath to an OD_{600} of 0.8 at 37°C in LB medium with 30 µg/ml tetracycline and 50 µg/ml ampicillin. The plasmid was amplified by further incubation overnight in the presence of 20 µg/ml chloramphenicol (25). Plasmid DNA was prepared by the alkaline lysis method, followed by isopycnic CsCl centrifugation in the presence of ethidium bromide (26). The fraction of supercoiled DNA was extracted with butanol, dialysed against TE buffer and stored at –20°C.

Relaxation of DNA

The supercoiled plasmid DNA was relaxed with wheatgerm topoisomerase I, either at 0°C (Fig. 1) or 22°C (Table 1) overnight in 50 mM Tris–HCl pH 7.5, 0.1 mM EDTA, 1 mM DTT, and different concentrations of metal ions as indicated. Under our conditions (Tris buffer), the relaxation reaction did not require NaCl, contrary to a previous report (22) where phosphate buffer had been used. For the experiments in Figure 1, however, all reaction mixtures also contained 50 mM NaCl.

Table 1. Change in positive supercoiling of pBR322 DNA by different metal ions at 22°C[a]

Ion	ΔLk	°/bp
None	0.00	0.00
Na^+	0.35	0.03
K^+	1.35	0.11
Cs^+	1.79	0.15
Li^+	2.40	0.20
NH_4^+	2.31	0.19
Ba^{2+}	1.81	0.15
Sr^{2+}	3.41	0.28
Mg^{2+}	3.63	0.30
Ca^{2+}	5.38	0.44
Mn^{2+}	5.86	0.48[b]
Co^{2+}	5.93	0.49

[a]0.3 µg pBR322 DNA was relaxed with wheatgerm topoisomerase I in the presence of 40 mM metal chloride, as indicated. The reaction mixture contained 50 mM Tris–HCl pH 7.5, 0.1 mM EDTA, 1 mM DTT, 80 U enzyme and 40 mM of metal chloride as indicated, in a total volume of 100 µl. The reaction was incubated at 22°C for 26 h. Samples of the reaction mixtures were analysed on normal (TAE) gels and on gels containing 0.5 µg/ml chloroquine, and evaluated as described in the text.

[b]In another series of reaction mixtures at 22°C with different concentrations of $MnCl_2$ between 0 and 60 mM, the ΔLk value at 60 mM $MnCl_2$ was +6.6 (0.55 °/bp).

Agarose gel electrophoresis

Electrophoresis was performed in horizontal slab gels (15 cm × 16 cm × 0.4 cm) submerged in Tris-acetate–EDTA (TAE) buffer (26). All gels contained 0.7% agarose. The electrophoresis was carried out at 1.1 V/cm for 22 h with constant circulation of the buffer at room temperature (22°C). After electrophoresis, gels were stained with 1 µg/ml ethidium bromide for 1 h and destained for 30 min. Photographs were taken with Polaroid type 55 film on a near UV light box. The lanes on the negatives were scanned with the LKB Ultrascan XL. Peak areas of the scan were measured by tracing on a graphics board (Apple Corp.) linked to a computer.

RESULTS

Relaxation of circular DNA with topoisomerase I in the presence of $CaCl_2$

Purified DNA of plasmid pBR322 was treated with topoisomerase I at 0°C in the presence of $CaCl_2$ at different concentrations between 0 and 400 mM (Fig. 1, lanes 2–13); the resulting plasmid topoisomers were then analysed by electrophoresis at 22°C on three different agarose gels containing Tris-acetate–EDTA buffer with either 0.75 µg/ml chloroquine (Fig. 1a), no additions (Fig. 1b), or 1.5 mM $CaCl_2$ (Fig. 1c). The top band in each lane of the gels contains open circular DNA. In the normal gel (Fig. 1b), the band immediately below the top band corresponds to an absolute linking number difference, $|\Delta Lk|$, of 1; the following band has a $|\Delta Lk|$ value of 2, and so forth, corresponding to increasing numbers of supercoils. With increasing calcium concentrations in the reaction mixture, the extent of positive supercoiling increased up to a concentration of 40 mM $CaCl_2$ (lane 10). At higher Ca^{2+} concentrations (60–400 mM, lanes 11–13) the extent of positive supercoiling decreased again. The intercalating agent chloroquine reduces DNA twist and hence decreases the number of negative supercoils in negatively supercoiled DNA. In the case of positively supercoiled DNA, a decrease in negative supercoiling is equivalent to an increase in positive supercoiling, as seen in Figure 1a. Conversely, the presence of $CaCl_2$ reduces the mechanical stress in positively supercoiled DNA and thereby relaxes the structure, i.e., decreases the number of positive supercoils (Fig. 1c). 1.5 mM $CaCl_2$ in the electrophoresis buffer (Fig. 1c) removed about two positive supercoils, which is most clearly seen by comparing the patterns in Figure 1b and c for lane 7 (relaxation at 5 mM $CaCl_2$). The value of two removed supercoils by 1.5 mM $CaCl_2$ during electrophoresis agrees with the increase in ΔLk obtained by relaxation in the presence of 1.5 mM $CaCl_2$ (interpolation between the ΔLk values observed with 1 and 2 mM $CaCl_2$, respectively; Fig. 1b, lanes 5 and 6, compared to lane 2; see also evaluation in Fig. 4 below).

The distributions in Figure 1b and c show an additional difference that reflects the slower electrophoretic movement in the presence of calcium ions, presumably due to the charge reduction as a result of calcium binding to the DNA (see Discussion).

As controls, Figure 1 also shows the products of a topoisomerase I reaction at 30°C containing different concentrations of ethidium bromide (lanes 14–16). This produces negative ΔLk values (27). In this case chloroquine in the electrophoresis gel makes the circular DNA more positively supercoiled (Fig. 1a, lanes 14–16), whereas calcium in the gel increases the negative supercoiling (Fig. 1c, lanes 14–16). The difference in the DNA distributions in lanes 2 and 14, both obtained in the absence of calcium chloride, reflects the different temperature during the relaxation reaction, i.e. 0 and 30°C, respectively.

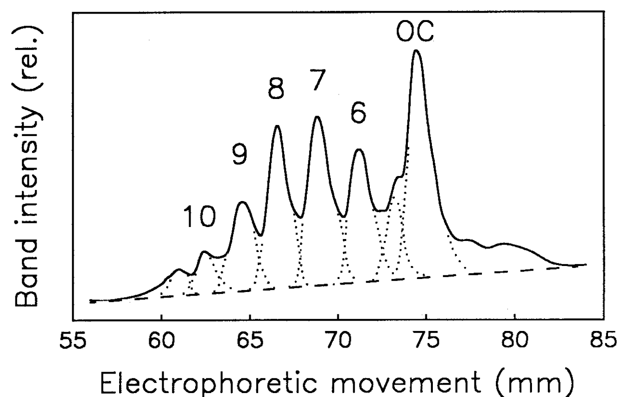

Figure 2. Example of a scan of the pBR322 topoisomer distribution, obtained from lane 8 in Figure 1c (relaxation in the presence of 10 mM $CaCl_2$; electrophoresis in the presence of 1.5 mM $CaCl_2$). OC, open circular (relaxed) DNA; numbers above peaks represent ΔLk values. Dashed line, assumed base line; dotted lines, assumed peak areas, lines drawn so that the sum of the overlapping peaks results in the observed distribution, given by the solid line.

After scanning photographic negatives of the ethidium bromide-stained gels with a densitometer and measuring the peak areas in every lane (example in Fig. 2 corresponds to lane 8 of Fig. 1c: relaxation in the presence of 10 mM $CaCl_2$; analysed with 1.5 mM $CaCl_2$ in the gel), frequency distributions of topoisomers were obtained as a function of ΔLk (Fig. 3), and the average ΔLk values from the distributions were calculated and plotted as a function of the $CaCl_2$ concentration used during the topoisomerase reaction (Fig. 4). Up to a concentration of ~50 mM (i.e., value between the observed points at 40 and 60 mM) during the reaction, ΔLk increased from +2.0 to a maximum average of +8.6; at higher calcium concentrations it decreased again to a lower limit value of about –0.25 (Fig. 1). The slightly positive supercoiling in the absence of calcium in the topoisomerase reaction is the result of the low (0°C) temperature during the reaction (28–30). The same effects were observed with another plasmid of similar size, pACYC184 (data not shown).

Effects of other ions

The effects of other mono- and divalent cations were examined similarly (Fig. 5; Table 1). To minimize the effect of temperature on the topoisomer distribution (28), both the relaxation and agarose gel analysis were carried out at 22°C. In these experiments, NaCl was omitted from the reaction mixtures. The presence of divalent ions in the relaxation mixture led to significantly higher positive supercoil values than monovalent ions. Ca^{2+}, Co^{2+} and Mn^{2+} had the strongest effects; for divalent cations, the effects were reversed at concentrations >50 mM (at 0°C; Figs 4 and 5, and other experiments not shown).

Because of the temperature dependence of the average helix rotation angle (28), a 22°C difference in the relaxation temperature (experiments of Fig. 1 and Table 1) was expected to produce different ΔLk values. Assuming a temperature coefficient of –0.012°/°C/bp (28) and 4361 bp per pBR322 plasmid, the expected difference would be –3.2. The observed difference at

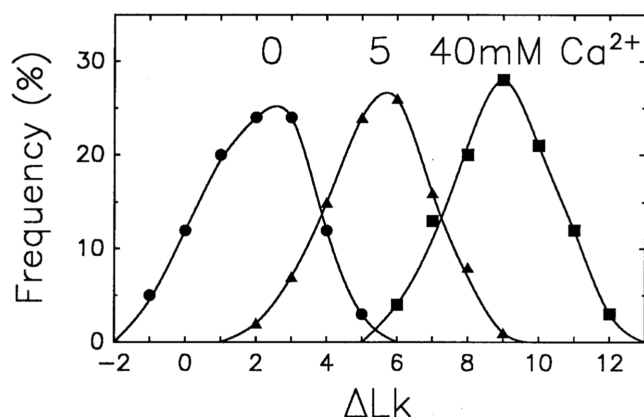

Figure 3. Examples of three pBR322 topoisomer distributions obtained by relaxation in the presence of different concentrations of $CaCl_2$ (0, 5 and 40 mM, as indicated) The distributions were obtained from the scans of selected lanes in Figure 1: 0 mM Ca^{2+} (Fig. 1b, lane 3); 5 mM Ca^{2+} (Fig. 1b, lane 7); 40 mM Ca^{2+} (Fig. 1c, lane 10). The curves drawn represent smoothed fits to observed points, not fits to Gaussian distributions.

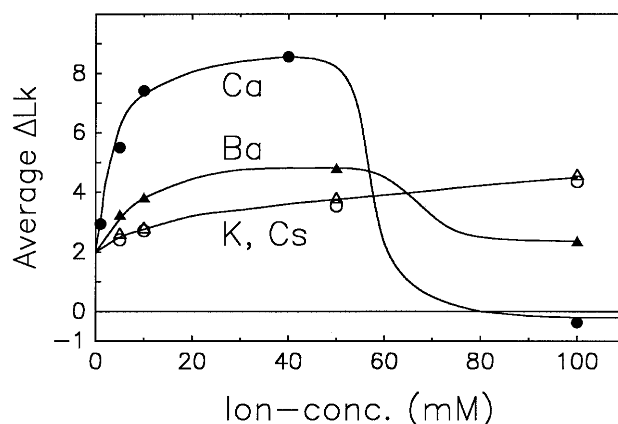

Figure 5. Effects of Ca^{2+}, Ba^{2+}, K^+ or Cs^+ on the relaxation of pBR322 DNA with topoisomerase I: average ΔLk value of the resulting topoisomer distributions. The data for the calcium curve are from Figures 1 and 4; the data for the other curves are from similarly analysed reactions. For the monovalent ions (open symbols), circles and triangles refer to Cs and K, respectively.

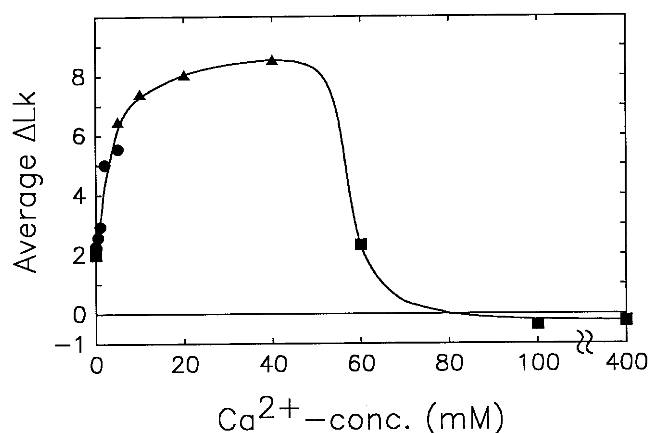

Figure 4. Average ΔLk values of pBR322 topoisomer distributions observed in the experiments of Figure 1 as a function of $CaCl_2$ concentration during the relaxation reaction with topoisomerase I. ●, from Figure 1b, lanes 2–7; ▲, from Figure 1c, lanes 7–10; ■, from Figure 1a, lanes 2 and 11–13.

40 mM Ca^{2+}, i.e., ΔLk = 8.6 for the reaction at 0°C (Fig. 1; evaluation in Fig. 3) and ΔLk = 5.4 for the reaction at 22°C (Table 1), agrees with this expectation (5.4 – 8.6 = –3.2).

The type of anion used had no measurable effect, since no differences were seen for $MgCl_2$ or $MgSO_4$, or for KCl, KBr, KI, KNO_3, KNO_2, K_2CO_3, K_2SO_4, KH_2PO_4, K-acetate, K_2-tartrate or K_3-EDTA, in agreement with previous observations (15,31)

DISCUSSION

As mentioned above (Introduction), monovalent cations have previously been found to overwind DNA; in those studies Na^+ had the least (i.e., non-measurable) and NH_4^+ the strongest effect (15). We have confirmed these results (Table 1). In addition, Ca, Co and Mn were found to overwind DNA more than twice as much to ~0.5°/bp at 22°C (Table 1) and more at 0°C. This extent of overwinding is as much as has been obtained with reverse gyrase (eight positive supercoils of pBR322 DNA at 25°C; ref. 9) or by relaxation in the presence of netropsin (14). For all divalent cations, the effect approached a maximum plateau at concentrations between 10 and 50 mM; at higher concentrations the effect was reversed (Figs 4 and 5). This is in contrast to monovalent ions, for which the effect continued to increase at higher concentrations (Fig. 5) up to ~0.2 M (15).

Different cations stimulate the extrusion of cruciform DNA from circular plasmids (31). Since cruciform extrusion is favored by negative supercoiling which is increased by cations, it might seem that the effects of salts on cruciform extrusion are related to the supercoil effects observed here. However, the stimulation of cruciform extrusion was correlated with the radius rather than valence of ions. For example, Ba stimulated cruciform extrusion more than Ca, and K stimulated as much as Ca, in contrast to the effects observed here (Table 1).

It is likely that the stronger effect of divalent cations on overwinding of the helix in comparison to monovalent ions is related to a stronger binding of divalent cations to DNA. To assess the binding of metal ions to DNA, we have observed the effect of these ions on the electrophoretic mobility of DNA (data not shown). For example, the presence of 25 mM $CaCl_2$ in the electrophoresis buffer reduced the maximum electrophoretic mobility of short, linear DNA molecules by 40%. The maximum electrophoretic mobility was determined by extrapolation to either zero molecular weight of DNA or zero agarose concentration; both extrapolations gave the same maximum mobility of 11 mm/h per V/cm in the absence of metal ions. At the same (25 mM) concentration, monovalent ions had no measurable effect on the electrophoretic mobility, either alone, or in competition with divalent ions such as Ca^{2+}. The curve describing the reduction in electrophoretic mobility of DNA as a function of calcium concentration in the electrophoresis buffer mirrored the effect on DNA overwinding seen in Figure 4. Although the relationship between ion binding to DNA and its electrophoretic mobility is complex (32,33), these observations suggest that divalent cations bind much more strongly to DNA than monovalent cations. This is consistent with previous conclusions based on the reduction of the effective diameter of DNA

molecules by NaCl and $MgCl_2$ (see Introduction), and on NMR studies of the binding competition between Na^+ and $Co(NH_3)_6^{3+}$ (34). Since the repulsive forces within the DNA helix are expected to stretch and thereby unwind the helix, charge neutralization by metal ions might be responsible for at least part of the overwinding of DNA. This is suggested by the fact that the extent of overwinding with different monovalent cations (15; Table 1) reflects the different binding affinities of these ions to DNA, as measured by NMR (35). The higher extent of overwinding achieved with divalent cations in comparison to monovalent cations (Table 1) might have additional reasons. Perhaps divalent cations can form two ionic bonds with the phosphate residues of adjacent nucleotides and thereby cause an additional overwinding of the DNA helix. The distance between adjacent phosphate residues in B-DNA (6.7 Å) appears to be suitable to hold a divalent cation by Coulomb forces (calcium ion radius 0.99 Å) in addition to the H_2O molecules bound to the non-esterified phosphate oxygen (36).

For technical reasons, it was not possible to observe the effect of higher calcium concentrations (>25 mM) on the electrophoretic mobility of DNA. [The cathode would get covered with insulating $Ca(OH)_2$, which drastically reduced the voltage gradient within the gel, and the platinum anode was oxidized and dissolved, presumably to hexachloroplatinate, which was reduced again to platinum black within the buffer.] Therefore, we have no further observations that might shed light on the reversal of the overwinding effect at higher divalent cation concentrations. With increasing concentrations of monovalent cations, DNA should approach the state of a neutral salt, but for divalent cations, this would not be expected. In general, divalent cations, especially when they are crowding DNA, should form only single ionic bonds with DNA and leave a free positive charge associated with the cation. This could explain the previous finding mentioned above (Introduction) that, in the presence of >50 mM $MgCl_2$, interactions between DNA segments appear to be attractive rather than repulsive (19); i.e., when DNA segments contain both negative and positive charges. This might also cause the aggregation of DNA at high concentrations of divalent cations (see Introduction). Therefore, the reversal of DNA overwinding at high divalent cation concentrations might be related to a change in the charge distribution along the DNA helix, i.e., when this distribution involves both positive and negative charges.

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