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Large-scale opening of A+T rich regions within supercoiled DNA molecules is suppressed by salt

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ABSTRACT

Large-scale cooperative helix opening has been previously observed in A+T rich sequences contained in supercoiled DNA molecules at elevated temperatures. Since it is well known that helix melting of linear DNA is suppressed by addition of salt, we have investigated the effects of added salts on opening transitions in negatively supercoiled DNA circles. We have found that localised large-scale stable melting in supercoiled DNA is strongly suppressed by modest elevation of salt concentration, in the range 10 to 30 mM sodium. This has been shown in a number of independent ways: 1. The temperature required to promote cruciform extrusion by the pathway that proceeds via the coordinate large-scale opening of an A+T rich region surrounding the inverted repeat (the C-type pathway, first observed in the extrusion of the ColE1 inverted repeat) is elevated by addition of salt. The temperature required for extrusion was increased by about 4 deg for an addition of 10 mM NaCl. 2. A+T rich regions in supercoiled DNA exhibit hyperreactivity towards osmium tetroxide as the temperature is raised; this reactivity is strongly suppressed by the addition of salt. At low salt concentrations of added NaCl (10 mM) we observe that there is an approximate equivalence between reducing the salt concentration, and the elevation of temperature. Above 30 mM NaCl the reactivity of the ColE1 sequences is completely supressed at normal temperatures. 3. Stable helix opening transitions in A+T rich sequences may be observed with elevated temperature, using two-dimensional gel electrophoresis; these transitions become progressively harder to demonstrate with the addition of salt. With the addition of low concentrations of salt, the onset of opening transitions shifts to higher superhelix density, and by 30 mM NaCl or more, no transitions are visible up to a temperature of 50°C. Statistical mechanical simulation of the data indicate that the cooperativity free energy for the transition is unaltered by addition of salt, but that the free energy cost for opening each basepair is increased. These results demonstrate that addition of even relatively low concentrations of salt strongly suppress the large-scale helix opening of A+T rich regions, even at high levels of negative supercoiling. While the opening at low salt concentrations may reveal a propensity for such transitions, spontaneous opening is very unlikely under physiological conditions of salt, temperature and superhelicity, and we conclude that proteins will therefore be required to facilitate opening transitions in cellular DNA.

INTRODUCTION

DNA strand separation is required for a number of important genetic processes, including the initiation of transcription (1) and DNA replication (2). Potentially unstable regions might also be more prone to recombination or mutagenesis. Unwinding of DNA will be facilitated by negative superhelix density, and DNA topology plays an important role in both transcription (3) and DNA replication (4).

Cooperative destabilisation of significant stretches of linear DNA was first suggested on the basis of thermal denaturation experiments (5). Localised opening of A+T rich regions of supercoiled circular DNA at elevated temperature has been observed by electrophoretic and enzyme and chemical probing methods (6–11), leading to a number of suggestions of a biological role for large-scale opening. Opening transitions have been observed in yeast replication origins (12) by nuclease cleavage and two-dimensional gel electrophoresis, and it was suggested that stable unwinding in A+T rich sequences adjacent to the 11 bp core consensus sequence was required for function (13). Similar unwinding was observed when sequences related to nuclear matrix attachment regions were cloned into supercoiled plasmids (14). Recently Benham has used statistical mechanical calculations to predict superhelical stress-induced melting occurring in a number of prokaryotic and eukaryotic regulatory regions (15).

The collective effect of these studies may have left the impression that temperature and superhelicity may alone effect the opening of particular regions of DNA for biological function.

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However, it should be stressed that all the observations of thermally-induced melting transitions in supercoiled DNA have been made under conditions of very low ionic strength. Many studies of the thermal melting of double-stranded DNA polymers have demonstrated that helix opening is suppressed by the addition of salt (16–21), and in general the melting temperature of DNA increases with the addition of salts (18). Thus elevation of the ionic strength should tend to suppress local opening of A+T rich sequences within the supercoiled DNA, and the opening might become insignificant under physiological conditions. However, the great majority of studies of the effect of salt on DNA melting have been carried out on linear species; it is possible that negative supercoiling might reduce the effect of salt in some circumstances. Negative superhelicity can be treated as an intensive thermodynamic variable rather like temperature (22), and thus local melting within a supercoiled DNA circle might be facilitated even in the presence of salt. We have therefore sought to investigate the effect of salt on the local melting of A+T rich sequences under conditions of negative superhelical stress.

In a recent study (10), we have applied the techniques of two-dimensional gel electrophoresis and probing with single-strand selective chemical reagents to the study of local helix melting of plasmid-borne A+T-rich sequences derived from ColE1. These sequences promote cruciform extrusion in supercoiled DNA at low ionic strength, by the C-type mechanism, involving the unpairing of significant stretches of DNA (23–25). At low ionic strength, these A+T rich sequences were observed to undergo stable melting at around 25°C and above, and to be chemically reactive to probes such as osmium tetroxide. In the present study we have reexamined these properties as a function of added salt concentration. We find that addition of low concentrations of salt is approximately equivalent to lowering the temperature, and that moderate increases in ionic strength lead to a strong suppression of large-scale helix opening. We therefore conclude that helix opening in the cell will not occur unaided, and will therefore require the intervention of proteins.

EXPERIMENTAL PROCEDURES

Preparation of cruciform-free supecoiled plasmid DNA

Cruciform-free supercoiled plasmid DNA was prepared from *Escherichia coli* as previously described (23). In brief, DNA was extracted from chloramphenicol-amplified *E.coli* HB101 by lysis with lysozyme, SDS and EDTA, and applied to an isopycnic CsCl/ethidium bromide gradient. Supercoiled plasmid DNA recovered from a second caesium chloride gradient was extracted eight times with butan-1-ol at 0°C to remove the ethidium bromide. This DNA was dialysed extensively at 7°C against 10 mM Tris–HCl, pH 7.5, 0.1 mM EDTA, and then stored at −20°C. Thawing of the DNA was performed on ice to prevent perturbation of the plasmid secondary structure.

Osmium tetroxide reactions

Approximately 1 μg of supercoiled DNA was reacted at various temperatures with 1 mM osmium tetroxide, 1 mM 2,2′-bipyridine. The reactions were performed with varying final concentrations of NaCl in 45 mM Tris–borate, pH 8.3, 0.5 mM EDTA (TBE/2). Analysis of the modifications at the sequence level required 'single-hit' conditions which were obtained by using relatively short incubation times. Preliminary experiments showed that the extent of reaction was not greatly dependent on ionic strength under these conditions, and the incubation times used were 4 min at 30°C and 3 min at 40°C.

The sites of modification on pColIRΔxba were observed at the nucleotide level after cleavage at the *Eco*RI site and radioactive [3′-^{32}P] labelling of the DNA with Klenow DNA polymerase. The DNA was ethanol precipitated, cleaved at the *Bam*HI site (in the presence of excess non-radioactive dATP), and the approximately 420 bp band was purified from a 1% agarose gel. The DNA was incubated with 1 M piperidine at 90°C for 30 min to cleave at the sites of modification. After extensive lyophilisation, the DNA was electrophoresed in 6–8% denaturing polyacrylamide gels containing 7 M urea in 90 mM Tris–borate, pH 8.3, 10 mM EDTA. Each gel also contained corresponding asymmetrically [3′-^{32}P] labelled fragments subjected to sequencing by chemical degradation (26). Radioactive DNA fragments were observed by autoradiography of dried gels at −70°C with intensifier screens, or with storage phosphor screens and a 400S phosphorimager (Molecular Dynamics). Quantitation of the gels was performed directly on the phosphorimage.

Measurements of the extent of cruciform extrusion

The amount of cruciform extrusion of plasmid pColIR315 was found for ionic conditions equivalent to the osmium tetroxide reactions. Supercoiled DNA (approximately 1 μg) was buffered by TBE/2, in the presence of various concentrations of NaCl. Samples were incubated in a thermostatted water bath for 5 minutes and then removed to ice. After digestion with 10 U S1 nuclease in 15 μl of 50 mM Na acetate, pH 4.6, 50 mM NaCl, 1 mM $ZnCl_2$ at 15°C for 30 min, and complete cleavage at the *Bam*HI site, the samples were electrophoresed on 1% agarose gels to reveal the relative degree of cruciform extrusion. Quantification of the amount of extrusion was as described previously (24).

Preparation of plasmid topoisomers and two-dimensional gel electrophoresis

Distributions of topoisomers were obtained by incubating native supercoiled plasmid DNA with rat liver topoisomerase I in the presence of various concentrations of ethidium bromide. The DNA was recovered by ethanol precipitation and dissolved in 30 μl of 10 mM Tris–HCl, pH 7.5, 0.1 mM EDTA, and the different distributions were stored separately at −20°C. Each set of distributions was analysed in chloroquine-containing agarose gels. Topoisomer distributions were mixed to give spots of equivalent intensities over the complete range of superhelical density (27).

Two-dimensional gel electrophoresis (28) was performed as described previously (10). Briefly, a 1% agarose gel (24.5×22 cm) was assembled in an apparatus with serpentine thermostatted water circulation, with preincubation to ensure that the required temperature was achieved (monitored with a platinum wire thermocouple). Differences in temperature across the gel were found to be ± 0.5°C at all of the temperatures used. Approximately 3 μg of a mixture of topoisomers in 8 μl of electrophoresis buffer were loaded into a single well in the corner of the agarose gel. The buffer for the first dimension was TBE/2 with various concentrations of NaCl added; this was recirculated at 30 ml/min during the electrophoresis. The electrophoresis was carried out at 85 V for 25–17 hours at various temperatures from 20–50°C. For gels that had contained NaCl in the first dimension, the gel was given two washes for 1 hour in 90 mM

Tris–borate, pH 8.3, 1 mM EDTA (TBE) plus chloroquine to remove any residual salt. The gel was then soaked for 6 hours in TBE containing the indicated concentration of chloroquine (usually 20 μg/ml). After rotating the gel by 90° it was electrophoresed in the same TBE/chloroquine solution for 17 hours at 85 V. The gel was stained in 1 μg/ml ethidium bromide solution for at least 2 hours and then soaked extensively in water at 7°C to reduce non-specific staining. Photography of the gels was performed under uv illumination, using red and green filters to reduce background. Negatives were densitometrically scanned to produce dark spots on a light background.

Statistical mechanical simulation of helix–coil transitions in supercoiled DNA

The statistical mechanical calculations used to simulate the initial unwinding observed by two-dimensional gel electrophoresis have been described previously (10). The model assumes that a segment of the plasmid of defined length is capable of melting, with the same average value assigned to thermodynamic parameters for melting for each base pair within the tract (ΔG°_{bp}). Loop entropy was ignored in the first approximation, thus the model is analogous to that used by Peck and Wang (29) to describe the B–Z transition. The partition function for the helix–coil transition is thus:

$$Z = 1 + \sum_{n=1} \omega(n) \qquad (1)$$

$$= 1 + \left\{ \sum_{n=1} (M_0 + 1 - n)\cdot\sigma_{hc} + \sum_{n=1} P(2, n.M_0)\cdot\sigma_{hc}^2 \right\} \cdot \exp\left\{ -\left[\Delta G^\circ_{sc} + n \cdot \Delta G^\circ_{bp} \right] / RT \right\} \qquad (2)$$

where $\omega(n)$ is proportional to the probability of opening n basepairs within a total meltable domain of M_0 basepairs. σ_{hc} is the cooperativity factor given by $\exp(-2\Delta G^\circ_j/RT)$, where ΔG°_j is the free energy for forming one mole of helix–coil boundary and ΔG°_{bp} is the average free energy needed for melting one mole of base pairs. ΔG°_{sc} is the free energy of supercoiling due to the unwinding of n bp

$$\Delta G^\circ_{sc} = K\left\{ (\Delta Lk - n.a)^2 - \Delta Lk^2 \right\} \qquad (3)$$

where the unwinding factor, a, is assumed to be 1/10.5 and ΔLk is the linking difference of a given topoisomer. In our earlier calculations we used a value for K of 1050RT/N (10), where N is the total plasmid size in bp, since this is close to that observed from a number of different experimental conditions and protocols (30–32). However, these earlier measurements used in the estimation of K were performed at salt concentrations of at least 0.2 M. This estimate for K is, therefore, a lower limit for the environmental conditions used here since the torsional and bending stiffnesses of DNA increase as the salt concentration is lowered. Recently it has been estimated that K is approximately 1175RT/N at a monovalent cation concentration of 0.01 M (33), and thus we have used this value in the calculations reported here. A comparison of these two values of K showed that the higher value increased the amount of helix-opening at each $-\Delta Lk$ by approximately 0.5 helical turns at 40°C. $P(2, n_{hc}, M_0)$ is the number of possible ways of forming two distinct open regions within a tract of M_0 bp (34). It was found that the inclusion of a second melted region had only a very small effect on the simulations, and thus higher order terms corresponding to the simultaneous existence of three or more distinct open regions were ignored. The average unwinding resulting from helix opening was calculated from the average number of basepairs opened (m_{av}), which was computed from

$$m_{av} = \sum_{n=1} n \cdot \omega(n) / Z \qquad (4)$$

RESULTS

We have employed two plasmids in these studies (Figure 1): 1. pColIR315 contains a 440 bp sequence from ColE1, containing the 31 bp inverted repeat embedded in its A+T rich flanking sequence (35). The inverted repeat can extrude a cruciform by the C-type mechanism (24), influenced by the flanking DNA. 2. pColIRΔxba was derived from pColIR315 by deletion of the inverted repeat and its replacement by an *Xba*I linker sequence

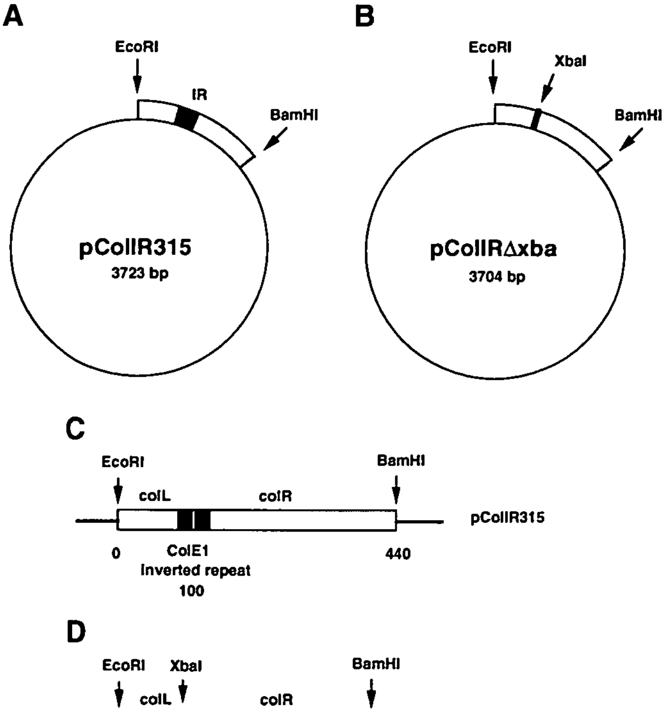

Figure 1. Maps of the plasmids used in these studies. **A** and **B** show circular maps of pColIR315 and pColIRΔxba respectively, with detailed maps of the ColE1-derived regions shown in **C** and **D**. These plasmids are based on pAT153 (43), with the ColE1 DNA (stippled regions) inserted between the *Eco*RI and *Bam*HI restriction sites. The complete ColE1 inverted repeat (IR, dark stippled region) is contained in pColIR315, which may undergo cruciform extrusion. The only difference between the two plasmids is the deletion of the inverted repeat in pColIRΔxba, which has been replaced with a linker sequence containing an *Xba*I site (shown in black) (24). The ColE1 A+T rich sequences that flank the inverted repeat have sizes of approximately 90 bp (colL, adjacent to *Eco*RI) and 300 bp (colR, adjacent to *Bam*HI) respectively.

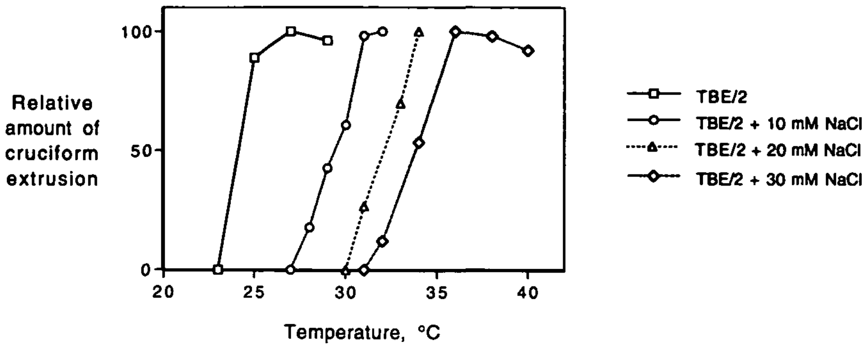

Figure 2. Cruciform extrusion profiles for pColIR315 at various concentrations of NaCl. The extent of extrusion of the ColE1 inverted repeat was measured after 5 minutes incubation at the indicated temperature in TBE/2 buffer containing 0 (□), 10 (○), 20 (△) and 30 (◇) mM NaCl. For each set of environmental conditions, the amount of extrusion at each temperature is plotted as a percentage of the maximal amount of extrusion observed for that particular experiment.

(24); this plasmid cannot undergo cruciform formation, but the sequences flanking the inverted repeat in pColIR315 are unaltered.

In the following studies, we have examined the influence of added salt on the large-scale helical opening by three independent methods, *viz* by the effect on the ability of an inverted repeat to adopt the cruciform conformation, by the chemical reactivity of thymine bases in their unpaired state, and by the unwinding-induced relaxation of the supercoiled DNA observed by two dimensional gel electrophoresis.

The influence of ionic environment upon C-type cruciform extrusion

Inverted repeats in very A+T rich sequence contexts, such as the ColE1 sequences, extrude cruciforms by the C-type mechanism. This pathway appears to involve the formation of a large and stable region of helix opening in which the single strands can adopt hairpin structures in the region of two-fold symmetry. The facility with which the cruciform may extrude by this process therefore reflects the ability of the surrounding sequences to undergo large-scale helix opening and we have demonstrated a correlation between the kinetics of cruciform extrusion and the propensity of the flanking sequences to undergo cooperative melting calculated using a statistical mechanical model of denaturation (36).We have previously shown that increasing concentrations of NaCl reduced the extent of extrusion of pColIR315 in a specific time period (23). We may ask to what extent increase in temperature might counteract the effect of elevated ionic strength.

The extent of cruciform formation occurring during a five minute incubation was studied as a function of both salt concentration and temperature (Figure 2). Elevation of prevailing NaCl concentration in the range 0 to 30 mM raised the temperature required to achieve the maximal extrusion observed at each ionic strength, by approximately 4 deg for each 10 mM increase in NaCl concentration. We conclude that the helix opening that leads to C-type cruciform extrusion is suppressed by increased salt concentration, but that increased temperature can overcome this effect in the range studied. With further increase in salt concentration we have noted previously that the mechanism of extrusion changes to the S-type pathway that does not depend on the opening of the flanking DNA (37).

Chemical probing of the A+T rich sequences as a function of salt concentration

We have previously shown that the supercoiled A+T rich sequences that are responsible for promoting C-type cruciform extrusion are chemically reactive at low salt concentrations to single-strand selective agents such as osmium tetroxide, consistent with their propensity to undergo large-scale melting (8, 10). We have therefore examined the reactivity as a function of salt concentrations in the range studied above. We chose to perform these experiments on pColIRΔxba (which lacks an inverted repeat) for two reasons. First, the presence of the inverted repeat in pColIR315 complicates the analysis of chemical modification experiments (10), and second, we wished to remove any additional destabilising effects due to the presence of the inverted repeat. Helix-opening in pColIRΔxba is fully reversible with temperature (10).

pColIRΔxba was reacted with 1 mM osmium tetroxide, 1 mM 2,2′-bipyridine at various temperatures and NaCl concentrations. After restriction cleavage and radioactive [3′-^{32}P] labelling, any thymine cis-diester adducts were cleaved with hot base, and the DNA was examined at single-base resolution by sequencing gel electrophoresis (Figure 3A). Reactivities of individual bases were estimated, and are shown schematically on the sequence in Figure 3B.

As we have observed previously (10), at temperatures above 20°C, the A+T rich ColE1 DNA exhibits a pronounced chemical reactivity over more than a hundred basepairs. An increase in temperature from 30 to 40°C produced an expansion of the reactive region, particularly into the ColR region up to the C+G rich block 50 bp from the *Xba*I site. However, addition of moderate concentrations of NaCl to the reaction mixture substantially reduced the amount of modification at all temperatures. This is seen most clearly at 30°C, where all reactivity within the ColE1-derived DNA was abolished by 10 mM NaCl. In the presence of 5 mM NaCl there was a weak reactivity of thymines within colL at this temperature. At 40°C addition of 10 mM NaCl did not affect the modification within colL, but it greatly reduced the chemical reactivity within colR. Addition of 20 mM NaCl totally suppressed the reactivity in colR, but colL remained weakly reactive. The residual reactivity in colL was uniform along the length of the sequence, suggesting that the entire region was either open or closed in a given topoisomer, without leading to the formation of a significant population of partially closed species.

The reactivity of pColIRΔxba towards 1 mM osmium tetroxide/bipyridine was slightly greater at 40°C in the presence of 10 mM NaCl than at 30°C in the absence of added salt. Thus the inhibitory effect of 10 mM NaCl can be overcome by raising the temperature about 10°C. This effect is not due to direct inhibition of the osmium tetroxide–thymine chemical reaction, since the the addition of 50 mM NaCl to pColIR315 does not alter reactivity at the loop of the extruded cruciform (Bowater and Lilley, unpublished results). The results show that at a particular temperature the addition of NaCl acts to reduce severely the extent of chemical modification, i.e. to suppress large-scale helix-opening.

Direct observation of stable helical unwinding by two-dimensional gel electrophoresis

Formation of a melted region of DNA in a supercoiled molecule is topologically equivalent to an unwinding in the DNA, and stable large-scale unpairings within plasmids can be detected by two-dimensional gel electrophoresis (6, 7, 10). Previously we used this technique to show that the extent of unwinding for pColIRΔxba and pColIR315 at different temperatures was in good agreement with that expected from the chemical modification experiments (10). In these studies we have subjected topoisomer distributions of pColIRΔxba to two-dimensional gel electrophoresis as a function of temperature (in the range 20–50°C) and salt concentration. The conditions were equivalent to those of the chemical modification experiments. Some representative results are shown in Figure 4. As we have shown previously (10), at temperatures above 25°C we observe transitions in the supercoiled DNA at low salt concentrations, corresponding to the formation of stably unwound regions, with increasing unwinding as the linking difference becomes more negative. As the salt concentration present in the first dimension was increased, these transitions were suppressed, and beyond 30 mM NaCl no transitions remained detectable below at least 50°C.

We have quantified the extent of unwinding in pColIRΔxba from the transitions observed in the two-dimensional gels, and these are presented graphically in Figure 5. For the higher salt concentrations, the structural transitions occur within an area of the gel where there is relatively poor resolution, close to the point of saturation of mobility in the first dimension. Thus we have only quantified the extent of unwinding for linking numbers that can be clearly seen to undergo a transition. Figure 5A presents the extent of unwinding as a function of temperatures at low ionic strength, while Figure 5B shows the extent of unwinding as a function of added salt concentration at constant temperature (40°C).

The unwinding measurements show that gradual changes take place continuously once a threshold level of supercoiling has been reached. Thus, once the structural transition has begun, an increase in negative supercoiling produces an approximately constant increase in helix-opening. The residual linking number of such plasmids will therefore be approximately constant over the range of the transition, and this has been predicted theoretically and observed experimentally for other DNA sequences (33, 34). Examination of gels obtained under different temperatures and salt concentrations in the first dimension shows that the pattern of unwinding is qualitatively very similar for each.

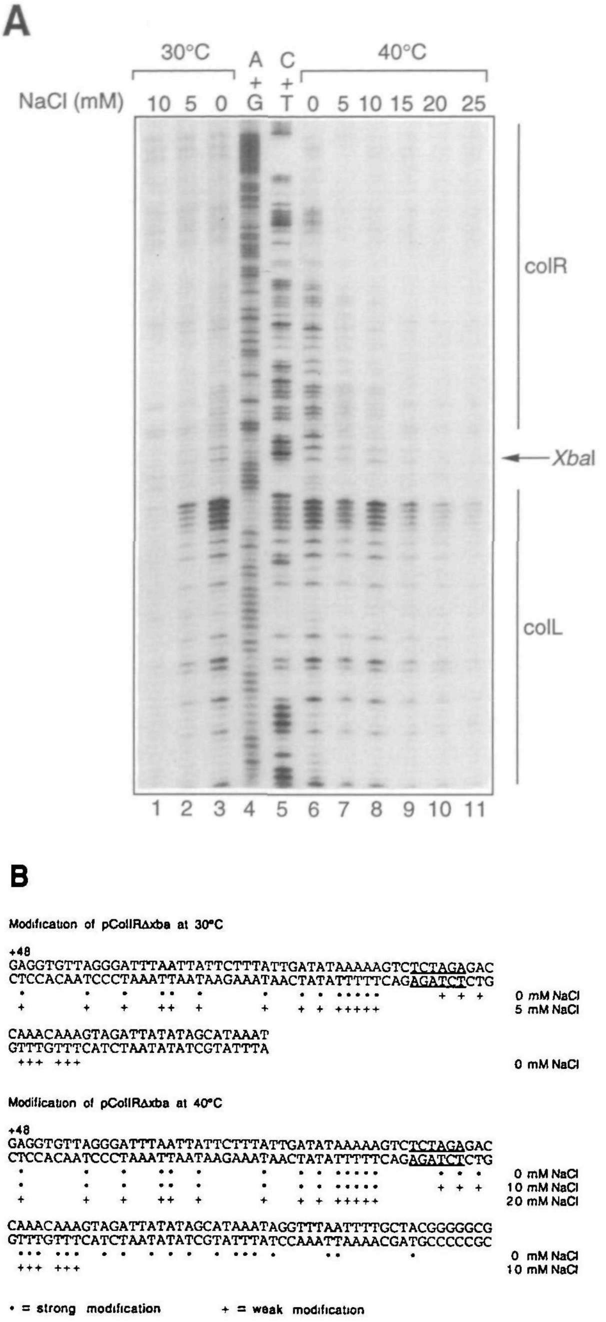

Figure 3. Osmium tetroxide modification of pColIRΔxba as a function of ionic strength and incubation temperature. **A.** Reaction of pColIRΔxba with osmium tetroxide/2,2′-bipyridine at 30 and 40°C, at various concentrations of NaCl. Aliquots of supercoiled pColIRΔxba were preincubated at the required temperature in TBE/2 plus the indicated concentration of NaCl, and then reacted with 1 mM osmium tetroxide/1 mM 2,2′-bipyridine (for 4 and 3 minutes at 30 and 40°C respectively). After ethanol precipitation to stop the reaction, the modified DNA was digested with *Eco*RI and [3′- ^{32}P] labelled. The DNA was then cleaved with *Bam*HI, purified from an agarose gel, incubated with hot piperidine to cleave at the sites of modification, and electrophoresed on a 6% sequencing gel. The dried gel was subjected to autoradiography. Tracks 4 and 5 contain purine and pyrimidine sequence markers respectively, generated by chemical degradation of the equivalent unmodified fragment of plasmid (26). **B.** Summary of the chemical modifications of pColIRΔxba at 30 and 40°C. The sequence of the ColE1 A+T rich DNA flanking the XbaI site (underlined) is given, beginning at +48 relative to the first nucleotide of the EcoRI site. The relative extent of osmium tetroxide modification in TBE/2 plus the indicated concentration of NaCl is shown; ● and + indicate a base with strong or relatively weak reactivity respectively.

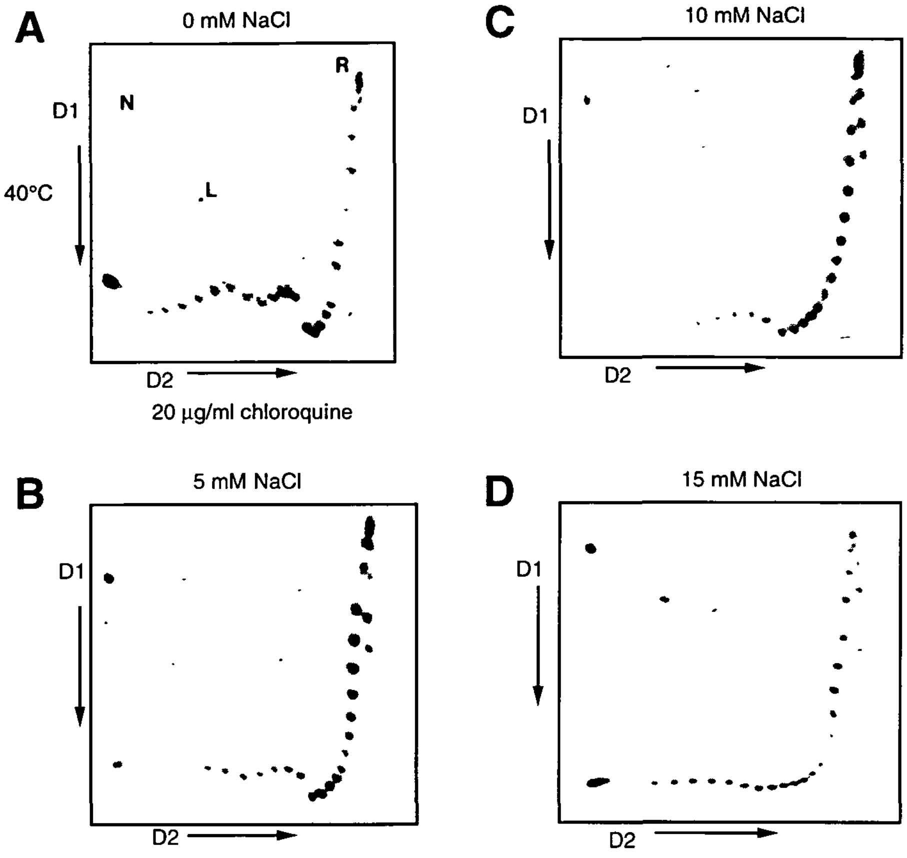

Figure 4. Two-dimensional gel electrophoresis of pColIRΔxba as a function of ionic strength. A distribution of topoisomers of pColIRΔxba were electrophoresed in the first dimension (D1) at 40°C in TBE/2 plus the indicated concentration of NaCl. The second dimension of electrophoresis (D2) was carried out in TBE containing 20 µg/ml chloroquine; under these conditions, all of the topoisomers in the distribution become positively supercoiled. The position of nicked (N) and linear DNA (L), and topoisomers that are relaxed in the first dimension (R), are indicated in gel A. Topoisomers were assigned values of linking difference by counting from position R. Topoisomers with a lower mobility than R in the second dimension were originally negatively supercoiled in the absence of chloroquine, while topoisomers faster than R were positively supercoiled. Each gel is labelled with the final concentration of NaCl in the buffer used for the first dimension: (A) no added NaCl; (B) 5 mM NaCl; (C) 10 mM NaCl; (D) 15 mM NaCl.

The only differences were found at the level of supercoiling at which the particular extent of unwinding took place.

Examination of Figure 5A shows that raising the temperature from 30°C to 40°C reduces the $-\Delta Lk$ for the onset of the helix-opening from 14 to 12; in Figure 5B it can be seen that at 40°C the addition of 5 mM NaCl raises the $-\Delta Lk$ for the same transition from 12 to 14. Thus, in these experiments, it would seem that an increase in temperature of 10 deg can overcome the inhibition of the helix-openings that is caused by the addition of 5 mM NaCl.

Statistical mechanical simulations of the initial helix-opening events

We have previously used statistical mechanical calculations to analyse the data from our two-dimensional gel electrophoresis experiments, whereby we obtained a reasonable fit for the initial helix-opening events in TBE/2 for the plasmid pColIRΔxba (10) and for other plasmids which contained potential Z-DNA forming sequences at various positions relative to the ColE1 A+T-rich DNA (38). In these calculations we assume that a segment of defined length within the plasmid is able to undergo melting, with each base pair within the tract having the same parameters for melting. The free energy of formation (ΔG°_f) of a melted region of n bp within the susceptible region is the sum of the free energies for altering the conformation of each affected basepair in the tract (ΔG°_{bp}), plus the cooperativity free energy arising from the formation of two junctions between B-DNA and the open regions (ΔG°_j), i.e.

$$\Delta G^\circ_f = n \cdot \Delta G^\circ_{bp} + 2 \cdot \Delta G^\circ_j \qquad (5)$$

We have used the same analysis to examine the effect of NaCl on the initial helix-opening within pColIRΔxba. In Figure 6 we show examples of the predicted openings from the calculations when only one of these parameters is varied, while the other is kept constant. Figure 6A shows that altering the junction energy at constant ΔG°_j changes the threshold level for the onset of the helix-coil transition, but the progression of the opening is similar for each irrespective of the variation of the junction energies. Figure 6B shows that if the junction energy is kept constant and ΔG°_{bp} is increased, then the curves have a similar shape but they are shifted towards a requirement of higher negative supercoiling to achieve a similar opening. Although such simple calculations cannot be expected to simulate the experimental data perfectly, it is apparent that a significantly better fit to the

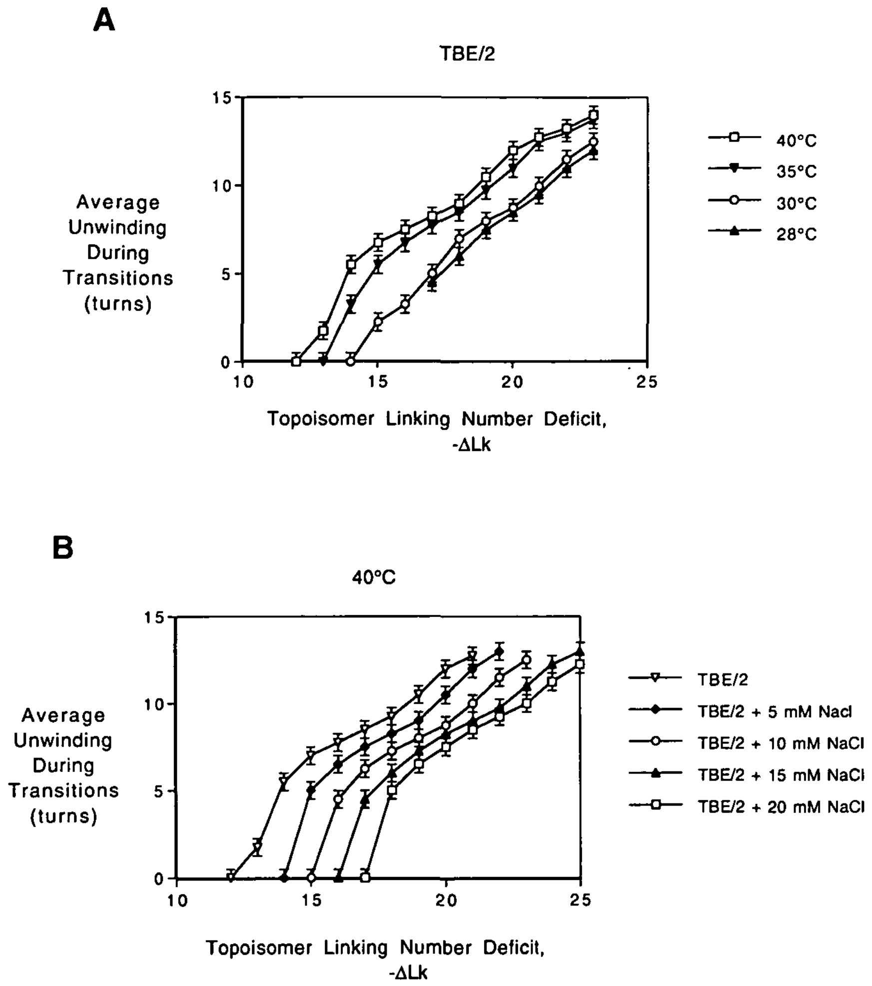

Figure 5. Quantitation of the stable unwinding observed in two-dimensional gel electrophoresis of pColIRΔxba at various temperatures and ionic strengths. The unwinding occurring at each linking number is quantified for topoisomers with retarded migration in the first dimension. The error bars indicate the width of the topoisomer spots. **A.** The average unwinding measured as a function of temperature in TBE/2. First dimension electrophoresis was performed at 28°C (▲), 30°C (○), 35°C (▼) and 40°C (□). **B.** The average unwinding measured as a function of NaCl concentration at 40°C. First dimension electrophoresis was performed in TBE/2 plus 0 (▽), 5 (◆), 10 (○), 15 (▲) and 20 mM NaCl (□).

experimental data is obtained when the junction energy is kept constant (Figure 6B).

Comparison of the experimental data and computer simulation shows that at 40°C a relatively close fit to the data in TBE/2 can be obtained with ΔG°_j equal to 5 kcal/mol junction and a value of ΔG°_{bp} of 0.3 kcal/mol. These values are similar to those previously calculated from data obtained at 34°C (10). The experimental data from the two-dimensional gel electrophoresis in TBE/2 + 10 mM NaCl at 40°C can be reasonably well fitted if the ΔG°_j is kept constant at 5 kcal/mol junction, but ΔG°_{bp} is raised to 0.4 kcal/mol.

DISCUSSION

These results have demonstrated that the large-scale stable melting of A+T rich supercoiled DNA is strongly suppressed by modest elevation of salt concentration. This has been shown in three independent ways: 1. as an increase in the temperature required to promote cruciform extrusion by the C-type mechanism, i.e. the pathway that proceeds via the coordinate opening of a large region; 2. as a strongly reduced reactivity of A+T rich regions to attack by the single-strand selective probe osmium tetroxide and 3. by the suppression of transitions indicating stable melting in A+T rich regions upon examination of topoisomer distributions by two-dimensional gel electrophoresis.

These results are also in good agreement with those of Blake and Delacourt (39), who used high resolution thermal melting analysis to study the local denaturation of oligoadenine sequences cloned into supercoiled pBR322, as a function of counterion concentration. They found that the gradient of the melting temperature as a function of $\log_{10}[Na^+]$ was in the range of 17–20 for $d(A\cdot T)_n$ tracts of different length. The different experimental approaches used here give relatively good agreement for the influence of low concentrations of NaCl on helix-opening events in supercoiled plasmids. Under these conditions we observed an inverse relationship between temperature and salt concentration. Thus the inhibitory effect of 5–10 mM NaCl on the unwinding observed using two-dimensional gel electrophoresis

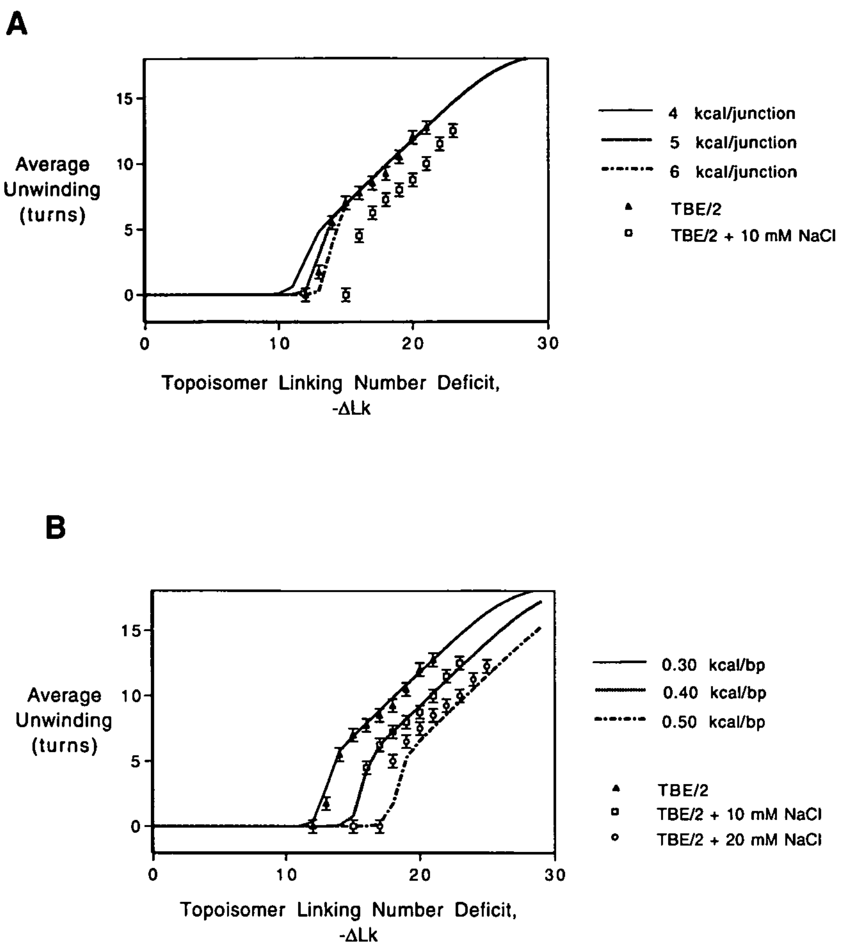

Figure 6. Statistical mechanical modelling of helix opening in pColIRΔxba as a function of the superhelicity. The statistical mechanical model discussed in the text was used to simulate the unwinding within the A+T rich ColE1 sequences at 40°C, with variation in the parameters used for the average free energy required to melt 1 mole of basepairs (ΔG°_{bp}), and the free energy for forming 1 mole of helix-coil boundary (ΔG°_j). **A.** ΔG°_{bp} was kept constant at 0.3 kcal/mol, and the calculations were performed with ΔG°_j equal to 4 (——), 5 (······) or 6 (–·–·–) kcal/mol. **B.** ΔG°_j was kept constant at 5 kcal/mol, and ΔG°_{bp} was varied between 0.3 (——), 0.4 (······) and 0.5 (–·–·–) kcal/mol of basepairs. In both (A) and (B) the experimental data from Figure 5B at zero (▲) and 10 mM NaCl (☐) is plotted; (B) also contains the experimental data at 20 mM NaCl (○). The error bars indicate the width of the topoisomer spots.

and modification with 1 mM osmium tetroxide/2, 2′-bipyridine could be overcome by raising the temperature by 10 deg. There is some discrepancy with the extent of extrusion observed in pColIR315, since a 10 deg increase in temperature could apparently overcome the effect of the addition of 30 mM NaCl. However, this could be explained if the extent of helix-opening at low salt concentrations was greater than that required for extrusion of the cruciform; although addition of 10 mM NaCl would reduce the amount of opening in the DNA, the opening might still encompass the inverted repeat and so extrusion could still take place. The suppression of C-type cruciform extrusion by elevated salt concentrations supports the model for extrusion based on the formation of hairpin loops within a large unpaired region in the supercoiled DNA (23–25). As salt concentrations were raised beyond 30 mM NaCl, complete suppression of opening transitions was observed at 40°C or below.

Quantitative analysis of earlier chemical modification and two-dimensional gel electrophoresis experiments showed a good agreement between these two fundamentally different approaches (10). Under conditions which resulted in very large-scale helix-opening, the data agreed only in a qualitative manner, but it would be unreasonable to expect better agreement from such a simple model. For example, loose winding of the single strands was not taken into account. In this study, data from chemical modification experiments and two-dimensional gel electrophoresis give good agreement for the extent of the helix-opening at various temperatures and concentrations of NaCl. Over the range of concentrations of NaCl from 0–20 mM, the initial helix-opening events can be well simulated by simple statistical mechanical calculations in which the free energy for melting the base-pairs is the major controlling factor in determining the onset of denaturation. Under the moderate concentrations of NaCl used in these experiments, the addition of 10 mM NaCl raised the free energy for the opening of each basepair in the melted region (ΔG°_{bp}) by approximately 0.1 kcal/mol. By contrast, the cooperativity free energy required in the formation of the junctions between the double- and single-stranded regions of DNA (ΔG°_j) was not affected by the ionic strength.

The results presented here demonstrate that the spontaneous, cooperative large-scale opening of A+T rich sequences in supercoiled DNA is strongly suppressed by even quite modest concentrations of added salt, and are therefore unlikely to be important at physiological salt concentrations, temperature and superhelix densities. Biological roles have been ascribed to opening transitions seen in regulatory sequences under superhelical stress (9, 13, 40), but such large-scale opening seems unlikely to occur in a cellular environment without the aid of other factors, such as the mediation of helicases or other proteins. An example of this may be drawn from studies on the operation of the replication origin in *Escherichia coli*. Bramhill and Kornberg (41) showed that the three repeats of a 13 bp sequence in the A+T rich region of the replication origin are essential for function. These repeats are selectively unwound on negatively supercoiled plasmids at low salt concentration (40). Upon the addition of Mg^{2+}, however, the unpairing of the 13 bp repeats only occurred in the presence of DnaA protein that is also required *in vivo* (42). In each case, though, the same region of DNA was opened. Thus experiments at low ionic strength may reveal the propensity of certain sequences to undergo denaturation, but it seems almost certain that proteins will be required to facilitate helix opening in the cell.

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