Articles

Large-Scale Stable Opening of Supercoiled DNA in Response to Temperature and Supercoiling in (A + T)-Rich Regions That Promote Low-Salt Cruciform Extrusion†

Richard Bowater, Fareed Aboul-ela, and David M. J. Lilley*
Department of Biochemistry, The University, Dundee DD1 4HN, U.K.
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ABSTRACT: We have studied the properties of (A + T)-rich sequences derived from ColE1 that promote cruciform extrusion at low ionic strength in supercoiled plasmids. We compared the chemical reactivity of the sequences in negatively supercoiled DNA (using osmium tetroxide and bromoacetaldehyde) with the results of two-dimensional gel electrophoresis performed under the same conditions. Taken together, the results indicate the occurrence of cooperative helix–coil transitions in the (A + T)-rich DNA at low ionic strength, to form stable, denatured regions. The extent of the open region is a function of temperature and superhelix density, with an additional local destabilization brought about by the presence of cruciform structures. We present a simple statistical mechanical model of the helix–coil transition in the (A + T)-rich DNA, from which we have obtained estimates of the free energy for average base-pair opening of 0.31 kcal mol^{-1} and that for the formation of a helix–coil junction of 4.9 kcal mol^{-1}, in 45 mM Tris–borate, pH 8.3, 0.5 mM EDTA. The results offer a model for the C-type mechanism of cruciform extrusion. Inverted repeats that are incorporated into the melted region undergo hairpin loop formation below 50 °C, and upon closure of the melted region, by reduction of temperature or increased ionic strength, they remain as a fully extruded cruciform structure.

The strands of DNA must be locally separated for many important biological processes. Yet, NMR studies of imino proton exchange rates have shown that in short, linear DNA molecules unpairing of bases is both infrequent and transient (Guéron et al., 1987). DNA supercoiling adds another dimension to this behavior (Vinograd et al., 1965) because the topology of constrained circular molecules is coupled to structural transitions resulting in changes of helical twist. The energy of negative supercoiling (typically around 100 kcal mol^{-1} for a native preparation of a plasmid the size of pBR322) may offset that of a perturbed local structure, such as a cruciform (Gellert et al., 1979; Lilley, 1980; Panayotatos & Wells, 1981) or a section of left-handed Z-DNA (Haniford & Pulleyblank, 1983; Nordheim & Rich, 1983; Peck et al., 1982; Singleton et al., 1982; Wang et al., 1979). In principle, this energy should stabilize unpairing of regions of DNA, and the least thermally stable sequences would be expected to be most readily melted in negatively supercoiled DNA.

Our studies of cruciform extrusion in supercoiled DNA molecules have suggested that large-scale helix opening may occur in (A + T)-rich regions. We observed that (A + T)-rich DNA promotes cruciform extrusion from nearby inverted repeats with kinetic properties that are quite different from normal sequences; the extrusion proceeds at low salt concentrations, at relatively low temperatures, and with very high enthalpy and entropy of activation (Lilley, 1985; Sullivan & Lilley, 1986). Cruciform extrusion of this kind (termed C-type extrusion) is dependent upon the presence of neighboring (A + T)-rich sequences. We interpreted these properties in terms of a kinetic intermediate that was closely related to a large open region and suggested that it was the relatively low thermal stability of the (A + T)-rich sequence that was responsible for the formation of the open region.

The proposed C-type extrusion mechanism was consistent with a number of observations. First, the kinetic properties of C-type extrusion could be approximated in DNA of normal base composition in the presence of helix-destabilizing agents, and conversely, the effect of the (A + T)-rich sequences could be removed by addition of low concentrations of distamycin which is known to bind to and stabilize (A + T)-rich DNA (Sullivan & Lilley, 1988). Second, we observed that the (A + T)-rich regions were particularly reactive toward certain single-strand-selective chemical agents, under similar conditions to those that maximize C-type cruciform extrusion (Furlong et al., 1989). In addition, statistical thermodynamical helix–coil transition calculations indicated that the sequences that promoted the unusual kinetic effects would have a high propensity to undergo cooperative unpairing (Schaeffer et al., 1989).

Such a large-scale opening of (A + T)-rich DNA could be of potential biological significance, providing helix opening required for important genetic processes, including the initiation of transcription and replication. Such unstable regions might also prove to be recombinogenic. However, the exact nature of the open region was not clear from our earlier experiments. The major question concerned the lifetime of the open region: could a stable unwinding exist in the supercoiled DNA under some conditions, or was C-type extrusion promoted by low-frequency, large-amplitude openings that existed transiently in the supercoiled DNA? Chemical probes cannot distinguish between these two possibilities.

Observations in other laboratories have indicated that stable unwinding could occur in supercoiled DNA molecules. Lee and Bauer (1985) carried out two-dimensional gel electro-

† This work was supported by the Cancer Research Campaign and SERC.
* To whom correspondence should be addressed.

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phoresis on circular plasmids as a function of temperature, and observed marked structural transitions that were interpreted in terms of helix opening in the supercoiled molecules. More recently, Kowalski and colleagues (Kowalski et al., 1988) have observed similar transitions in supercoiled pBR322 and plasmids containing sequences of yeast origin; these plasmids were also found to be susceptible to nuclease probes with single-strand specificity.

We have therefore applied the gel electrophoretic methods to plasmids containing the (A + T)-rich sequences that promote C-type cruciform extrusion and compared the data with a detailed analysis of temperature-dependent chemical reactivity in the same molecules. The two approaches are complementary. Gel electrophoresis reveals topology-dependent structural transitions that occur within supercoiled DNA molecules in a way that is nonperturbing, and, moreover, can provide an indication of the duration of the open state—in order to observe a clear transition the perturbed state must be relatively stable and must occur in a high proportion of the molecules of a given topoisomeric state. However, no indication is given concerning which sequences in the molecule are participating in the transition. Probing experiments localize the sequences undergoing rearrangement but require the intervention of an enzyme or chemical; chemical probing methods have been extensively applied to the study of nonstandard DNA structures (Lilley, 1983; Lilley & Palecek, 1984; Barton & Raphael, 1985; Herr, 1985; Johnston & Rich, 1985; Kohwi-Shigematsu & Kohwi, 1985; Nejedly et al., 1985; Furlong & Lilley, 1986; Galazka et al., 1986; Gough et al., 1986; Scholten & Nordheim, 1986; Millgram-Burkoff & Tullius, 1987; Hanvey et al., 1988; Htun & Dahlberg, 1988; Johnston, 1988; McLean et al., 1988; Voloshin et al., 1988; Sen & Gilbert, 1988; Sundquist & Klug, 1989; Wani et al., 1989; Williamson et al., 1989; Klysik et al., 1990; McCarthy et al., 1990; Sullivan & Lebowitz, 1991).

We find that the (A + T)-rich sequences that promote C-type cruciform extrusion undergo transitions in the supercoiled DNA that may be interpreted in terms of a stable unpairing that is initiated at around 24 °C under our conditions. There is excellent agreement between the results obtained by gel electrophoresis and chemical probing. The open state is responsible for the onset of chemical reactivity and the formation of hairpin structures that lead, on cooling, to the formation of the cruciform structure. The (A + T)-rich sequences should therefore be regarded as regions of low helix stability, with a propensity to undergo cooperative unpairing.

Materials and Methods

Preparation of Cruciform-Free Supercoiled Plasmid DNA. Cruciform-free plasmid DNA was prepared by lysozyme, SDS, and EDTA lysis of *Escherichia coli* HB101 cells as previously described (Lilley, 1985). The supercoiled DNA recovered from the second cesium chloride gradient was extracted eight times with butan-1-ol at 0 °C to remove ethidium bromide. This DNA was dialyzed extensively against 10 mM Tris-HCl, pH 7.5/0.1 mM EDTA at 7 °C, and stored at −20 °C. DNA was thawed on ice to prevent extrusion of inverted repeats.

Osmium Tetroxide Reactions. A total of 1 μg of supercoiled DNA was reacted at temperatures between 0 and 50 °C with 1 mM osmium tetroxide and either 1% pyridine or 1 mM 2,2′-bipyridine as the basic ligand. Higher concentrations used in preliminary experiments are indicated in the text where appropriate. In order to facilitate comparison with the results from the two-dimensional gel electrophoresis experiments, reactions were performed 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 temperature under these conditions, with optimal times of 4 min at 0 °C and 2 min at 50 °C.

Sequence Mapping of Osmium Tetroxide Modification Sites. Chemically modified DNA was cleaved with *Eco*RI, and the resulting termini were radioactively labeled. DNA was 3′-^{32}P labeled with Klenow DNA polymerase and [α-^{32}P]dATP. DNA was 5′-^{32}P labeled after dephosphorylation with calf intestinal alkaline phosphatase using polynucleotide kinase and [γ-^{32}P]ATP. After cleavage with *Bam*HI, the approximately 440-bp band was purified from a 1% agarose gel by electroelution and was reacted with 1 M piperidine at 90 °C for 30 min to cleave at the sites of modification. After extensive lyophilization, the DNA was electrophoresed in 6–8% polyacrylamide sequencing gels containing 7 M urea in 90 mM Tris–borate, pH 8.3/10 mM EDTA (TBE). Each gel also contained equivalent asymmetrically ^{32}P-labeled fragments subjected to chemical degradation sequencing reactions (Maxam & Gilbert, 1980). 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).

Bromoacetaldehyde Preparation. Bromoacetaldehyde (BAA) was prepared by hydrolysis of the diethyl acetal (Fluka) according to the method of McLean et al. (1987). The residual ether was evaporated under an air stream, and aliquots were stored at −70 °C. The stock solution contained BAA at a concentration of approximately 2.5 M.

Bromoacetaldehyde Reactions. A total of 1 μg of supercoiled DNA was incubated with 2% (v/v) BAA, in a total volume of 100 μL in TBE/2. Reaction times varied between 120 min at 37 °C and 210 min at 20 °C. After reaction, BAA was removed by means of two extractions with diethyl ether, and the DNA was ethanol precipitated. The DNA was cleaved with *Eco*RI, 3′-^{32}P or 5′-^{32}P labeled, and cleaved with *Bam*HI as outlined above. Sites of BAA modification were determined at sequence resolution by treating the isolated fragment with either hydrazine or formic acid and reacting with piperidine (Kohwi & Kohwi-Shigematsu, 1988). The enhanced reactivities of unpaired bases was observed by quantitative analysis of the gel tracks in the phosphorimage.

Kinetic Measurements of Cruciform Extrusion. Kinetic parameters of cruciform extrusion were obtained for the variety of conditions related to those used for the osmium tetroxide reactions. Cruciform-free supercoiled pColIR315 (1 μg) was diluted in TBE/2, in the presence of the indicated concentrations of pyridine or 2,2′-bipyridine. Samples were incubated in a thermostated water bath for 5 min at various temperatures and then removed to ice. The relative extent of cruciform extrusion that had occurred during the incubation was assayed using cleavage of the cruciform loop with S1 nuclease (Lilley, 1985; Sullivan & Lilley, 1986). Concentrated reaction buffer was added to the DNA aliquot to bring the final ionic conditions to 50 mM sodium acetate, pH 4.6/50 mM NaCl/1 mM $ZnCl_2$, and the mixture was incubated at 15 °C for 20 min with 10 units of S1 nuclease (BRL). DNA was ethanol precipitated and cleaved to completion with *Bam*HI, and the products were analyzed by gel electrophoresis in 1% agarose in TBE. Ethidium bromide stained gels were photographed under UV illumination using Kodak Tri-X Pan film. The relative extent of cruciform extrusion in each sample was determined by laser densitometry of the photographic negatives and plotted as a function of temperature. The temperature

at which the relative extent of cruciform extrusion during the 5-min incubation reached 50% maximal was taken as the T° (half extrusion).

Preparation of Plasmid Topoisomers. 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. To check that the reactions had reached completion, each set of distributions was analyzed in agarose gels containing various concentrations of chloroquine. Typically, 10 concentrations of ethidium bromide were used (Bowater et al., 1991) such that a mixture from each sample gave spots of equivalent intensities over the range of superhelical density from slightly positively to very negatively supercoiled.

Two-Dimensional Gel Electrophoresis (Wang et al., 1983). A 1% agarose gel (24.5 × 22 cm) in TBE/2 was assembled in a horizontal electrophoresis apparatus in which the gel was supported on a plate through which water was circulated at a constant temperature. When the gel had reached a stable, specified temperature, electrophoresis was begun. The temperature was monitored with a platinum wire thermocouple. Below 30 °C the accuracy was ±1 deg; at temperatures up to 50 °C the deviation from that required was ±4 deg. Differences in temperature across the gel were found to be ±0.5 deg at all of the temperatures used. A total of 3 μg of a mixture of the topoisomers was precipitated from ethanol, dissolved in 8 μL of TBE/2 containing Ficoll dye, and loaded into a single circular well in the corner of the agarose gel. The buffer for the first dimension was TBE/2, and it was recirculated at 30 mL/min during the electrophoresis. The electrophoresis was carried out at 85 V for 25–17 h at various temperatures from 20 to 50 °C. The gel was then soaked for 6 h in the dark in TBE containing the indicated concentrations of chloroquine (usually 20 μg/mL). After the gel was rotated by 90°, it was electrophoresed in the same buffer for 17 h at 85 V. The gel was stained in 1 μg/mL ethidium bromide for up to 2 h and then destained extensively (at least overnight) in water at 7 °C to reduce nonspecific staining. Photography of the gels was performed under ultraviolet illumination with red and green filters to reduce background. Negatives were densitometrically scanned to produce dark spots on a light background. Topoisomers were counted from the relaxed position in the first dimension, making allowance for the non-integer value of Lk°. However, the lower resolution of the topoisomer spots around the relaxed position gives the determination of $-\Delta Lk$ an uncertainty of ±0.5.

Statistical Mechanical Model of Helix–Coil Transition in Supercoiled DNA. A statistical mechanical model was used to fit initial unwinding measured from two-dimensional gels for plasmids in TBE/2. 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 ($\Delta G^{\circ}_{bp,hc}$). Loop entropy was ignored in the first approximation, and thus the model is analogous to that used by Peck and Wang (1983) to describe the B–Z transition. The partition function for the helix–coil transition is thus

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

$$Z = 1 + \left(\sum_{n=1} (M_0 + 1 - n)\sigma_{hc} + \sum_{n=1} P(2)\sigma_{hc}^2\right) \exp[-(\Delta G_{sc} + n\Delta G^{\circ}_{bp,hc})/RT] \quad (2)$$

where $\omega(n)$ is proportional to the probability of opening n base pairs within a total meltable domain of M_0 base pairs. σ_{hc} is the cooperativity factor given by $\exp(-2\Delta G^{\circ}_{j,hc}/RT)$, where $\Delta G^{\circ}_{j,hc}$ is the free energy for forming 1 mol of helix–coil boundary; $\Delta G^{\circ}_{bp,hc}$ is the average free energy required to melt 1 mol of base pairs. ΔG°_{sc} is the change in the free energy of supercoiling due to the unwinding of n bp given by

$$\Delta G^{\circ}_{sc} = K[(\Delta Lk - na)^2 - \Delta Lk^2] \quad (3)$$

where ΔLk is the linking difference of a given topoisomer and K is given by $1050RT/N$, where N is the size of the plasmid (in base pairs). a is 1/10.5 if the possibility of twisting within the open region is ignored. $P(2, n, M_0)$ is the number of possible ways of forming two distinct open regions within a tract of M_0 bp given by

$$P(2, n, M_0) = (M_0 - n - 0.5)(n - 1)(M_0 - n) \qquad (n < M_0/2) \quad (4)$$

$$P(2, n, M_0) = (M_0 - n - 0.5)(M_0 - n - 1)(M_0 - 1) \qquad (n > M_0/2) \quad (5)$$

Higher order terms corresponding to the simultaneous existence of three or more distinct open regions were ignored. The average number of base pairs opened (m_{av}) was computed from

$$m_{av} = \sum_{n=1} n\,\omega(n)/Z \quad (6)$$

where the degree of unwinding due to helix opening was obtained by dividing by the assumed helical repeat of 10.5 bp/turn.

Results

(A + T)-Rich Sequences Flanking the ColE1 Inverted Repeat Are Chemically Reactive. We have demonstrated that the (A + T)-rich DNA sequences that flank the ColE1 inverted repeat are responsible for cruciform extrusion with unusual C-type kinetic properties (Sullivan & Lilley, 1986). These kinetic properties led us to propose that the extrusion was mediated by the formation of a large region of opened helix, formed by the relatively low helical stability of the (A + T)-rich sequences. This was consistent with the reactivity of these sequences to single-strand-selective chemical agents (Furlong et al., 1989). In this study we have explored the chemical reactivity of the sequences flanking the inverted repeat of ColE1 as a function of temperature in more detail and compared the results with those of two-dimensional gel electrophoresis.

We have employed two plasmids in these studies, both of which contain sequences derived from the natural *E. coli* plasmid ColE1 (Figure 1). pColIR315 contains a 440-bp sequence from ColE1 (Lilley, 1981), containing the 31-bp inverted repeat that lies close to the 3′ ends of the *colE1* and *imm* genes. The inverted repeat is embedded in its very (A + T)-rich flanking sequence and can undergo cruciform formation. pColIRΔxba was derived from pColIR315 by deletion of the inverted repeat and its replacement by an XbaI linker sequence. It therefore contains the (A + T)-rich ColE1 flanking sequences without the inverted repeat and cannot undergo cruciform formation as a consequence.

Chemical Reactivity of the (A + T)-Rich ColE1 Sequences Depends on Temperature and the Presence of the Inverted Repeat. In the presence of basic ligands (typically 1–5% pyridine), osmium tetroxide may react with thymine bases to generate cis esters at the 5,6 positions. However, the required stereochemistry of attack is normally hindered by stacking into the double helix, and therefore thymines that are conven-

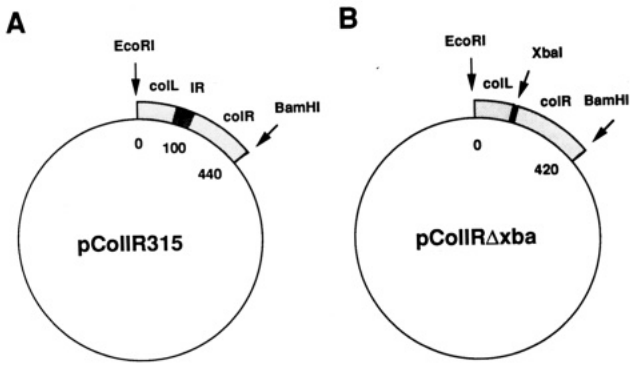

FIGURE 1: Maps of the plasmids used in these studies. Parts A and B show circular maps of pColIR315 and pColIRΔxba, respectively. The (A + T)-rich sequences derived from ColE1 are indicated by the stippled regions, flanked by the *Eco*RI and *Bam*HI sites used in the analysis of chemical modification. The ColE1 inverted repeat (IR) that undergoes cruciform formation is flanked on both sides by (A + T)-rich sequences. On the *Eco*RI side is an approximately 90-bp region (colL), while on the *Bam*HI side is an approximately 300-bp region (colR). The only difference between pColIR315 and pColIRΔxba is that the inverted repeat has been deleted in pColIRΔxba, and replaced with an *Xba* linker sequence (shown in black).

tionally base-paired are usually unreactive. Unpairing of the A·T base pair brings about a large increase in reactivity, as can be seen in the modification of thymine bases that are present in the unpaired loops of cruciform structures for example (Lilley & Palecek, 1984). The positions of reactive thymine bases in the sequence is readily revealed, since the adduct is unstable to alkaline cleavage. Thus, following chemical modification, the DNA is cleaved with restriction enzymes, uniquely radioactively end-labeled with ^{32}P, cleaved with piperidine at 90 °C, and analyzed by gel electrophoresis and autoradiography.

We have reacted both pColIR315 and pColIRΔxba with osmium tetroxide in 1% pyridine, as a function of temperature, at low ionic strength (TBE/2 buffer; 45 mM Tris–borate, pH 8.3, 0.5 mM EDTA). Plasmids of native superhelix density were prepared from *E. coli* under conditions known to remove all cruciform structures (Lilley, 1985) and were kept on ice. These were then treated in one of two ways. The DNA either was left on ice for a further period or was incubated in TBE/2 buffer for 5 min at 45 °C—conditions previously established to promote cruciform formation in C-type molecules. Both types of DNA sample were then reacted with osmium tetroxide at a temperature between 0 and 40 °C and analyzed as above. We were careful to ensure that the osmium tetroxide reactions were performed under single-hit conditions, and therefore relatively low concentrations of osmium tetroxide were used and the reaction times were kept short. The results are shown in Figure 2.

Preincubation of pColIR315 markedly affected the sites of modification by 1 mM osmium tetroxide/1% pyridine (Figure 2B). At temperatures of 20 °C and lower, modification was only observed if the plasmid had already been subjected to preincubation at 45 °C. For preincubated DNA there was modification in the center of the inverted repeat, corresponding to the hairpin loop of a cruciform, even at the lowest temperatures. At 20 °C and above, the sequences immediately adjacent to the inverted repeat became reactive, and by 30 °C this reactivity was seen even in the DNA that was not preincubated. At still higher temperatures (Figure 2C) there was extensive reactivity throughout the flanking regions, and by 50 °C the entire inverted repeat was reactive. It is interesting to note that the last region to become reactive was the potential stem formed by the inverted repeat. These results suggest that preincubation of supercoiled pColIRΔxba creates a large opening in the (A + T)-rich DNA, whereupon the inverted repeat forms a hairpin loop structure in the single-stranded DNA below 50 °C.

By contrast, preincubation of pColIRΔxba DNA did not affect the pattern of chemical reactivity (Figure 2D). Nevertheless, extensive reactivity of the (A + T)-rich ColE1 sequences was observed above 20 °C. Moreover, the extent of the reactive region appeared to depend on the reaction temperature, the reactive region becoming longer as the temperature was increased. Thus, the chemical reactivity data suggest large-scale opening in the equivalent sequences of both plasmids with increased temperature.

Threshold Temperatures for Chemical Reactivity. The above results for pColIR315 indicate that in the temperature range 10–20 °C, the presence of the inverted repeat appears to influence the reactivity following preincubation. The reactivity of both plasmids toward 1 mM osmium tetroxide/1% pyridine was therefore studied over a narrower temperature range. For pColIR315 the reactivity was again studied with and without preextrusion of the cruciform (Figure 3A); for pColIRΔxba the reaction was limited to samples stored on ice, since preincubation at high temperatures did not affect the sites of reactivity (Figure 3B).

In the absence of preincubation, modification of both plasmids began at about 16–18 °C. Below this temperature there was no reactivity of DNA that had not been exposed to the higher temperature. However, pColIR315 that had been preincubated at 45 °C was reactive in the sequences flanking the inverted repeat. Furthermore, the reaction temperature altered the extent of modification of the DNA. At 4 °C and below, there was reactivity only within the cruciform loop. By 8 °C, thymines within the 10 bp immediately adjacent to the stems of the cruciform were reactive. Increasing the temperature brought about a further expansion of the reactive region, summarized in Figure 3C. At the onset of reactivity in the flanking regions we observed modification within 2–3 base pairs of the stem, or in bases 85–87 (relative to the *Eco*RI site of ColE1) for pColIRΔxba. At higher temperatures there were distinct stages for the spread of reactivity, and this expansion appeared to stall when G·C base pairs were encountered; two or three consecutive G·C base pairs presented a barrier that could only be passed by further increase in temperature. For pColIR315 the distinct regions can be assigned as first, 78–127; second, 59–139; and third, <16–158. For pColIRΔxba, there seem to be two distinct regions which can be similarly assigned from the sequence: first, 42–129; and second, <36–139.

Further comparison of parts A and B of Figure 3 reveals that sequences in colL and colR that were unmodified below 18 °C in pColIRΔxba were reactive in pColIR315 provided that the DNA had been preincubated at a higher temperature. Indeed, these sequences were reactive in pColIR315 even if the DNA was restored to 0 °C for a period after the preincubation (data not shown). This suggests that the presence of the hairpin structures in the preincubated pColIR315 can itself exert a significant destabilization on sequences flanking the inverted repeat.

Chemical Reactivity of the ColE1 Sequences as a Function of Preincubation Temperature. The differences in chemical reactivity observed in pColIR315 with and without preincubation at 45 °C indicate that a structural alteration occurs as a result of the elevated temperature and that some aspect of this is preserved in the supercoiled DNA that subsequently

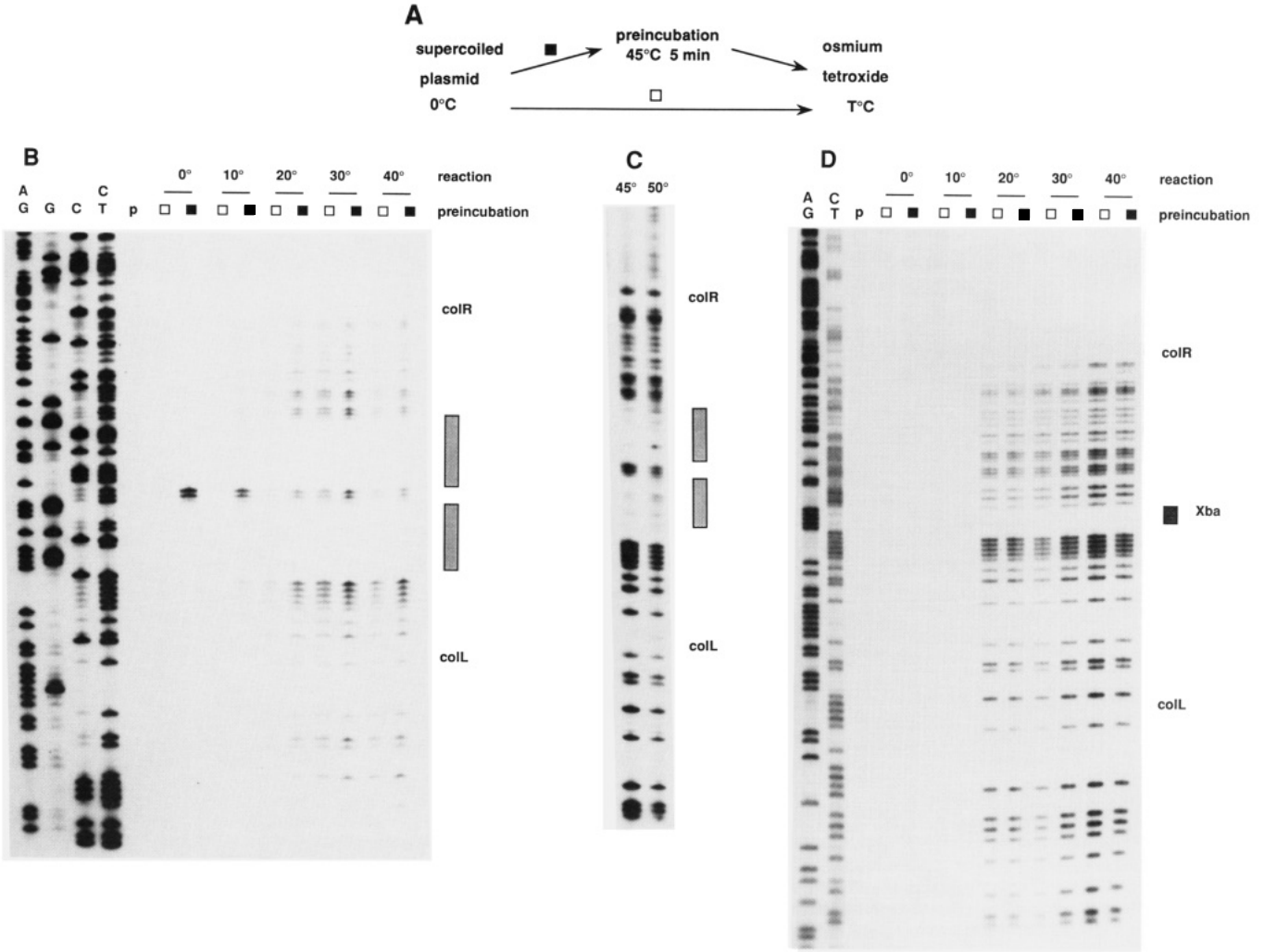

FIGURE 2: Osmium tetroxide modification of supercoiled (A + T)-rich ColE1 sequences (with and without the ColE1 inverted repeat) as a function of reaction temperature and preincubation at 45 °C. (A) Experimental scheme. Supercoiled cruciform-free DNA was taken from storage at a low temperature, and a sample was preincubated at 45 °C in TBE/2 for 5 min. Osmium tetroxide modification reactions were then performed under identical conditions for pairs of samples that either were preincubated or were taken directly from storage. The reactions were performed at a variety of temperatures as indicated. The modified DNA was restriction cleaved and radioactively labeled, cleaved with piperidine, electrophoresed on a sequencing gel, and autoradiographed. (B) pColIR315 reacted at temperatures between 0 and 40 °C. An autoradiograph of the sequencing gel is shown. Lanes containing preincubated DNA are indicated by the filled squares; DNA taken directly from the low temperature is indicated by the open squares. The temperatures for the osmium tetroxide reactions are given above the squares. The left four lanes contain sequence markers generated by chemical degradation of the equivalent unmodified fragment from pColIR315. The location of the inverted repeat is indicated by the stippled boxes (right), with the location of colR and colL for orientation. (C) pColIR315 reacted at the high temperature. Supercoiled pColIR315 was reacted with osmium tetroxide at 45 and 50 °C. (D) pColIRΔxba reacted at temperatures between 0 and 40 °C. Samples of pColIRΔxba preincubated at 45 °C (filled squares) or taken directly from the low temperature (open squares) were reacted with osmium tetroxide at the indicated temperatures. The left two lanes contain sequence markers generated by chemical degradation of the equivalent unmodified fragment from pColIRΔxba. The locations of the *Xba*I linker, colL, and colR are indicated on the right.

affects the chemical reactivity at a lower temperature. Does this depend on the temperature chosen for the preincubation?

We performed a number of osmium tetroxide reactions that only differed in the temperature (15–45 °C) of their 5-min preincubation. The subsequent chemical reactions were performed at 12 °C. Figure 4 shows that no modification was seen following a preincubation at 15 °C. However, preincubation at 25 °C or higher was sufficient to generate subsequent chemical reactivity of thymines at the lower temperature, and in each case an identical pattern of modification was observed, corresponding to the center of the inverted repeat and the immediate flanking region.

More detailed studies showed that the critical preincubation temperature for the generation of chemical reactivity was 24 °C under these conditions (data not shown). The patterns of modification indicate that a similar structure is preserved in the preincubated DNA when it is returned to 12 °C and reacted with osmium tetroxide. The results suggest that there is a large-scale opening of the (A + T)-rich sequences in the supercoiled DNA above the threshold temperature and that an inverted repeat may adopt hairpin loop geometry under these conditions. The temperature 24 °C is very close to the temperature at which cruciform extrusion begins to occur in this plasmid, and this suggests that this helix opening may be the structural change that is rate limiting in the mechanism of C-type cruciform extrusion.

Chemical Modification by Osmium Tetroxide Is Dependent on the Ligand. In the experiments discussed so far we have used 1 mM osmium tetroxide in the presence of 1% pyridine. Most of the earlier studies on this and similar systems have used higher concentrations of chemicals: 2 mM osmium tetroxide with 3% pyridine has probably been the most widely used combination. However, we suspected that pyridine might destabilize the helix in supercoiled DNA, although this has

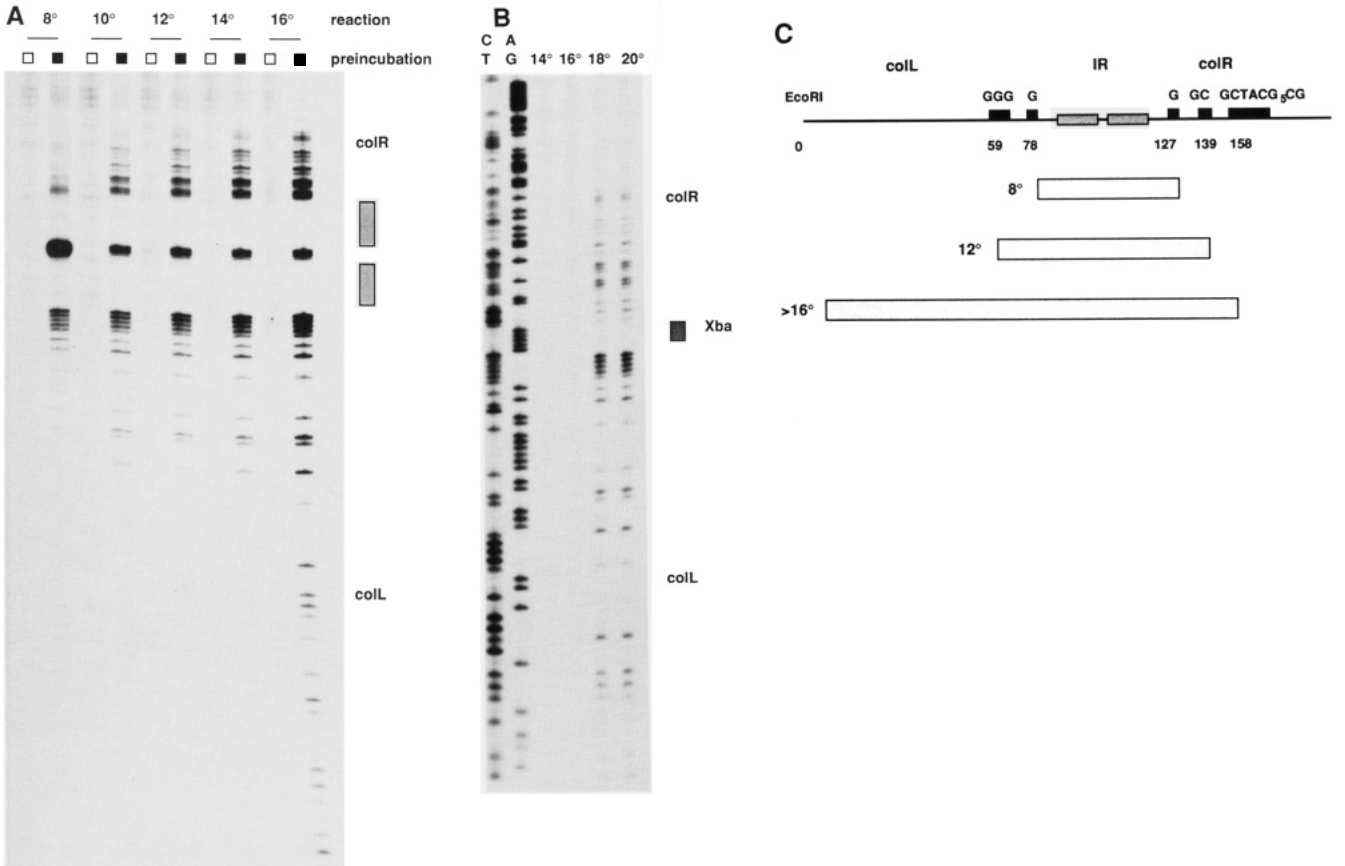

FIGURE 3: Chemical reactivity of the ColE1 sequences over a narrow temperature range. (A) Supercoiled pColIR315 was reacted over the temperature range between 0 and 20 °C, with (filled squares) and without (open squares) preincubation at 45 °C. The modified DNA was restriction cleaved and radioactively labeled, cleaved with piperidine, electrophoresed on a sequencing gel, and autoradiographed. The locations of the CoE1 inverted repeat, colL, and colR are indicated on the right. (B) pColIRΔxba reacted over the temperature range 14–20 °C. Supercoiled pColIRΔxba was reacted with osmium tetroxide at the indicated temperatures, followed by analysis as in (A). The first two lanes on the autoradiograph show sequence markers generated by chemical degradation of the equivalent unmodified fragment from pColIRΔxba. The locations of the *Xba*I linker, colL, and colR are indicated on the right. (C) Summary of the regions of pColIR315 that become chemically reactive in TBE/2 as a function of temperature. The reactive regions are indicated by the stippled boxes. Sequences found at the ends of the open region are indicated by the darker boxes.

only been thought to be of importance at high concentrations (>5%). Palecek et al. (1989) have examined a number of potential ligands for osmium tetroxide, and we decided to use 2,2′-bipyridine, since this could be used at a concentration an order of magnitude lower than pyridine. It has been successfully used to study cruciform extrusion both in vitro and inside the cell (McClellan et al., 1990).

Experiments were performed over various temperature ranges, on both pColIR315 and pColIRΔxba. An example of the modification sites on pColIR315 with 1 mM osmium tetroxide/1 mM 2,2′-bipyridine over small increases in reaction temperature, with and without preincubation at 45 °C, is shown in Figure 5. In the presence of bipyridine, osmium tetroxide reacted with the ColE1 DNA sequences at the same locations as before, but a higher reaction temperature was required for a given pattern of reactivity. The minimum temperature for observation of reactivity in pColIR315 that had not been preincubated was 24 °C, compared to 16–18 °C in the presence of 1% pyridine. Similarly, osmium tetroxide/1 mM 2,2′-bipyridine reacted with pColIRΔxba (data not shown) only at temperatures above 24 °C. The sites of modification were similar to those observed using 1% pyridine as ligand, although there was a more intense reaction at the center of the modified region.

We note that the minimum temperature at which chemical reactivity in the presence of bipyridine is observed in pColIRΔxba and unpreincubated pColIR315 is very similar to the minimum preincubation temperature, and this suggests that in each case we are observing the same phenomenon, i.e., the initial thermal opening of the (A + T)-rich DNA.

Table I: Cruciform Extrusion Temperatures for pColIR315 as a Function of Added Heterocyclic Bases

added base	$T°$ (half-extrusion) (°C)
none	22
0.1% pyridine	18
0.5% pyridine	16
1% pyridine	13
1 mM 2,2′-bipyridine	22

These results indicate that the 1% pyridine used in osmium tetroxide reactions may act as a helix-destabilizing agent, reducing the temperature required to observe chemical modification in the supercoiled DNA by approximately 10 deg. We have previously demonstrated that other helix-destabilizing agents, such as dimethylformamide, reduce the temperature required for the extrusion of C-type cruciforms (Sullivan & Lilley, 1988), and so we examined the effect of pyridine on the temperature dependence of cruciform extrusion by pColIR315. Table I presents the temperatures required for 50% maximal extrusion during a 5-min interval in the presence of pyridine and other bases. The inclusion of 1% pyridine in the incubation mixture lowered the temperature required for extrusion by about 9 °C relative to that without ligand or with 2,2′-bipyridine. This is in good agreement with the differences

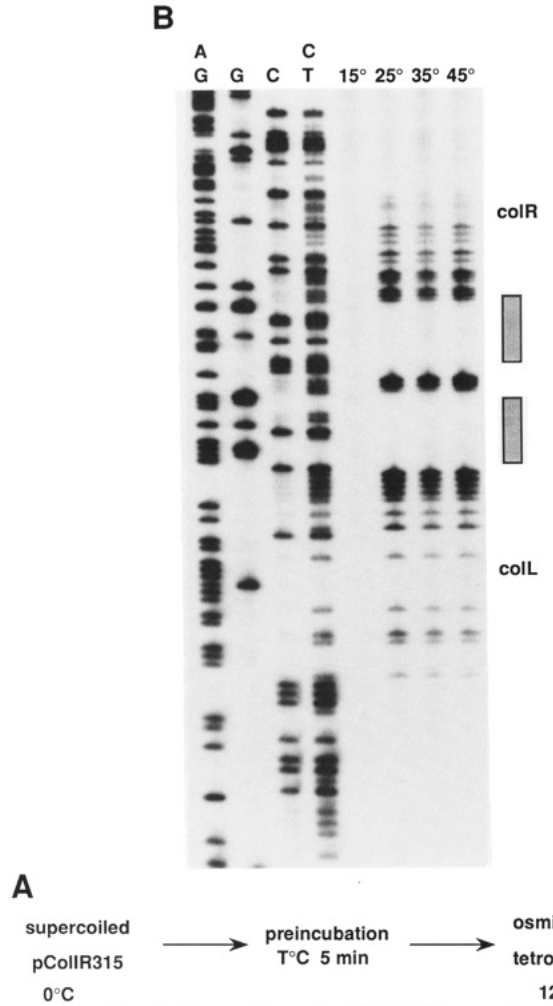

FIGURE 4: Chemical reactivity of pColIR315 as a function of preincubation temperature. (A) Experimental scheme. Samples of supercoiled, cruciform-free pColIR315 were preincubated in TBE/2 for 5 min at the indicated temperatures, before modification with osmium tetroxide at 12 °C. The modified DNA was restriction cleaved and radioactively labeled, cleaved with piperidine, electrophoresed on a sequencing gel, and autoradiographed. (B) The autoradiograph of the sequencing gel. The first four lanes contain sequence markers generated by chemical degradation of the equivalent unmodified fragment from pColIR315, followed by the supercoiled DNA preincubated at the indicated temperatures. The locations of the CoE1 inverted repeat, colL, and colR are indicated on the right.

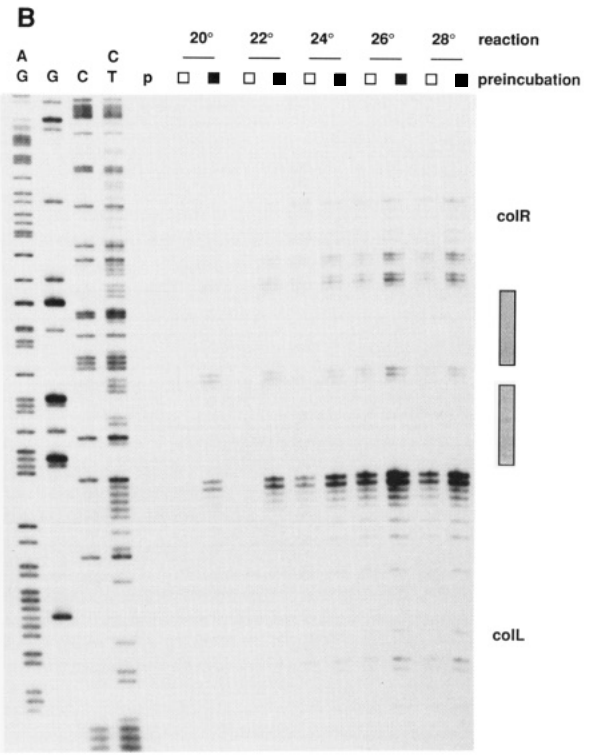

FIGURE 5: Chemical reactivity of ColE1 sequences of pColIR315 to osmium tetroxide/2,2′-bipyridine. (A) Experimental scheme. Supercoiled cruciform-free pColIR315 was taken from storage at the low temperature, and a sample was preincubated at 45 °C in TBE/2 for 5 min. Osmium tetroxide modification reactions were then performed under identical conditions for pairs of samples that either were preincubated or were taken directly from storage. The reactions were performed in 1 mM bipyridine, at a variety of temperatures as indicated. The modified DNA was restriction cleaved and radioactively labeled, cleaved with piperidine, electrophoresed on a sequencing gel, and autoradiographed. (B) Autoradiograph of the sequencing gel. Lanes containing preincubated DNA are indicated by the filled squares; DNA taken directly from low temperature is indicated by the open squares. The temperatures for the osmium tetroxide/bipyridine reactions are given above the squares. The left four lanes contain sequence markers generated by chemical degradation of the equivalent unmodified fragment from pColIR315. The locations of the CoE1 inverted repeat, colL, and colR are indicated on the right.

observed in the osmium tetroxide reaction and shows that the major explanation for the differences between the two ligands was due to destabilization of supercoiled DNA by pyridine.

Chemical Modification of pColIR315 by Bromoacetaldehyde. Since chemical probes must interact with their targets, there is always a possibility that the interaction perturbs the structure that is studied. The helical destabilization by pyridine is an example of this. For this reason it is good practice to broaden the source of probing data with the use of different chemical reagents, and it is preferable to compare probes that attack DNA in different ways. We have therefore extended the studies of pColIR315 using bromoacetaldehyde (BAA), which reacts mainly with adenines and cytosines (Sechrist et al., 1972 Kayasuga-Mikado et al., 1980; Kohwi-Shigematsu et al., 1983; Lilley, 1983). In these reactions, etheno adducts are formed between the N1 and N6 positions of adenine, and with N3 and N4 positions of cytosine. Since adduct formation must disrupt base-pair formation, this suggests that any base must be fully unpaired if it is to be modified. We analyzed the modifications at sequence level following the procedure of Kohwi and Kohwi-Shigematsu (1988); modification of bases is revealed by the appearance of new or enhanced bands when the DNA is sequenced by using Maxam and Gilbert (1980) chemistry. Since we were particularly interested in the reactivity of adenine bases, the BAA-modified DNA was reacted with hydrazine in the presence of NaCl followed by cleavage with piperidine (i.e., the Maxam–Gilbert C-reaction).

Equivalent samples of pColIR315 were reacted with BAA or osmium tetroxide/1 mM 2,2′-bipyridine at temperatures between 15 and 30 °C. After cleavage of the DNA at the *Eco*RI site, the samples treated with osmium tetroxide were 3′-^{32}P radioactively labeled, and those reacted with BAA were 5′-^{32}P labeled. Thus, the BAA and osmium tetroxide modifications were observed on the top and bottom strands, respectively, thereby directly comparing adenine and thymine reactivities within the same base pairs. When pColIR315 was reacted with BAA at 20 °C, no reaction greater than the background level was observed. However, above 25 °C we observed modification at all adenines and cytosines in the

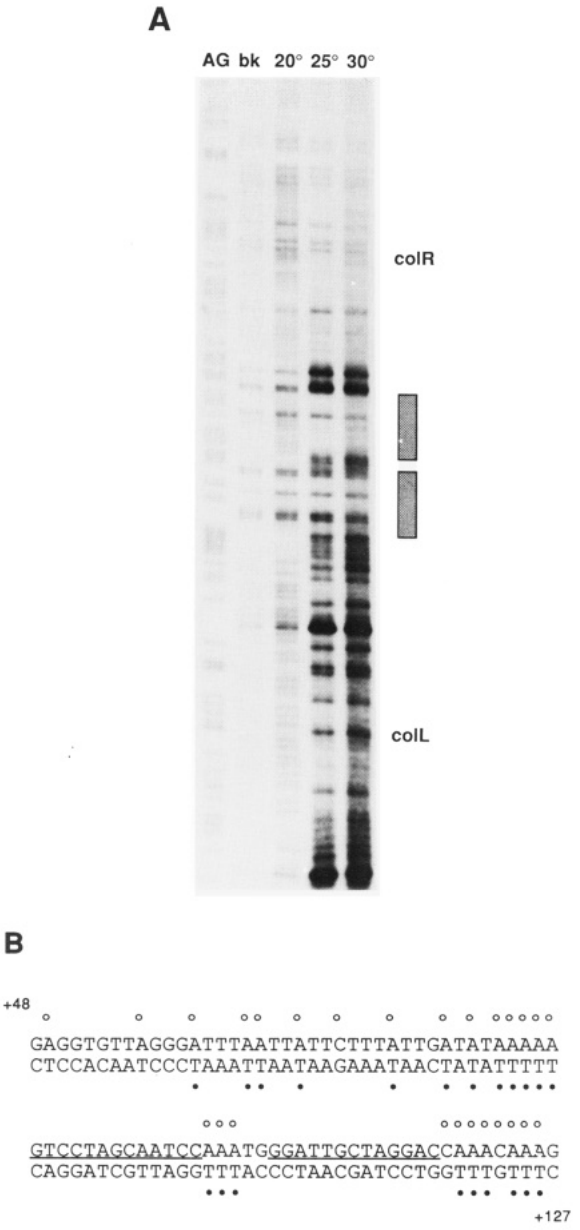

FIGURE 6: Comparison of the reactivity of the ColE1 sequences of pColIR315 to bromoacetaldehyde and osmium tetroxide. (A) Supercoiled pColIR315 was modified with bromoacetaldehyde in TBE/2 at the indicated temperature. The DNA was restriction cleaved and radioactively labeled before carrying out a secondary modification using hydrazine in NaCl, followed by cleavage with piperidine. The DNA was electrophoresed on a sequencing gel followed by autoradiography. The first lane contains a sequence marker generated by chemical degradation of the equivalent unmodified fragment from pColIR315. The locations of the CoE1 inverted repeat, colL, and colR are indicated on the right. At 20 °C, the only bands seen arise from hydrazine modification of cytosine bases, but as the temperature is increased additional bands due to BAA-adducted adenine become evident. (B) Summary of the results of comparing the BAA modification of adenine bases on the top strand (open circles) with osmium tetroxide modification of thymine bases on the lower strand (closed circles).

ColE1 sequences from base 40 in colL through to base 158 in colR (Figure 6A). Figure 6B shows the sites of modification at sequence resolution; modified adenine and cytosine bases are deduced from new or intensified bands in the ladder (determined by laser densitometry of autoradiographs). All adenines paired with reactive thymine bases were modified by BAA, and cytosines were only attacked by BAA when they fell in the same region; cytosines lying beyond base 158, or in the putative stem regions, were not modified. These results confirm that we are observing a complete opening of the DNA helix within the (A + T)-rich ColE1 sequences, and the agreement between the two probes suggests that a direct participation by the probes to promote the opening process is unlikely. This is further supported by the observation of reactivity in the ColE1 sequences to another chemical probe, carbodiimide (Wani et al., 1989).

Direct Observation of Stable Helical Unwinding by Two-Dimensional Electrophoresis. The above studies show that the (A + T)-rich regions that are responsible for cruciform extrusion occurring at low ionic strength are chemically reactive above 24 °C. We have suggested previously that the (A + T)-rich sequences are easily denatured, and the observed chemical reactivity is consistent with this hypothesis. However, some outstanding questions remain. In particular, if the (A + T)-rich sequences are unstable to denaturation in supercoiled DNA, does this correspond to the stable formation of an open region, or do the chemical probes react with bases that become transiently exposed during enhanced breathing of the helix? A subsidiary question concerns whether or not the chemical probes may actually participate in the unwinding process. In order to answer these questions, we have analyzed the topology of pColIR315 and pColIRΔxba using two-dimensional gel electrophoresis as a function of temperature.

Two-dimensional gel electrophoresis (Wang et al., 1983) reveals topology-dependent structural transitions in supercoiled molecules. The transition gives rise to a change in mobility in agarose that results from the global alteration in the shape of the supercoiled molecule as a consequence of a local change in helical twist and is seen as a "jump" in the position of topoisomer spots in the second dimension [see Bowater et al. (1991)]. Formation of a melted region of DNA in a supercoiled molecule would be equivalent to an unwinding, in a similar way to the other structures such as cruciforms, Z-DNA, and triplexes, and might be detectable by two-dimensional gel electrophoresis. We therefore performed two-dimensional gel electrophoresis on pColIR315 and pColIRΔxba at many temperatures over the range 20–50 °C, and some representative examples are shown in Figure 7. The first dimension of these gels was electrophoresed in 45 mM Tris–borate, pH 8.3/0.5 mM EDTA, in order to be equivalent to the chemical modification experiments. This also provided excellent buffering capacity throughout the long electrophoresis times.

When the first dimension was run at 20 °C, neither plasmid revealed any unusual mobility changes with DNA supercoiling. By 24 °C for pColIR315 and 26 °C for pColIRΔxba, it was just possible to see a decrease in mobility as the negative linking difference ($-\Delta Lk$) passed 21. Progressive increases of the temperature in the first dimension by increments of 2 °C made it easier to observe this mobility decrease, and at temperatures in excess of 30 °C a number of structural transitions may be clearly seen.

For both pColIR315 and pColIRΔxba the first indication of a transition at 34 °C occurred at topoisomer $-\Delta Lk = 13$. We note the extent of unwinding for specific linking numbers was always higher for pColIR315 than for pColIRΔxba. This would be expected if the unwinding occurred in a region that included the boundary of colL and colR, because pColIR315 contains an extra 19 base pairs in this region. The observed transitions were correlated with the regions of DNA that undergo chemical modification by subjecting the topoisomer mixture of pColIRΔxba to reaction with osmium tetroxide, followed by two-dimensional gel electrophoresis (data not shown). While the topoisomer spots of $-\Delta Lk < 13$ remained

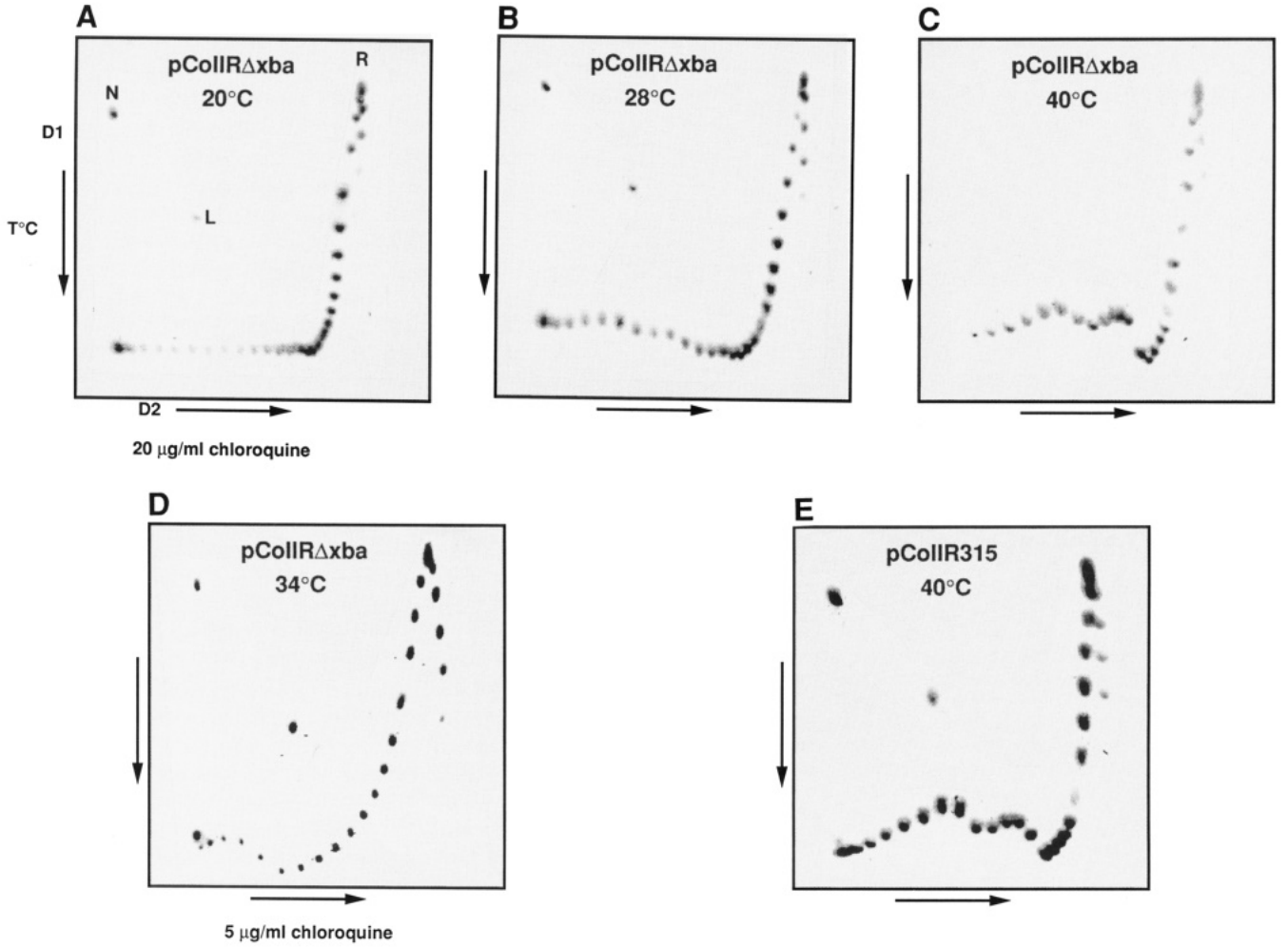

FIGURE 7: Two-dimensional gel electrophoresis of pColIRΔxba and pColIR315 topoisomers as a function of temperature. The first dimension (D1) was electrophoresed in TBE/2 at a controlled temperature. The second dimension (D2) was carried out in TBE containing chloroquine. In gels A, B, C, and E, the second dimension was electrophoresed in the presence of 20 μg/mL chloroquine; under these conditions all the topoisomers are positively supercoiled. In gel D, the second dimension was electrophoresed in the presence of 5 μg/mL chloroquine, giving a better resolution of the topoisomers of smaller $-\Delta Lk$. The positions of nicked (N) and linear (L) DNA and topoisomers that are relaxed in the first dimension (R) are indicated in gel A. Topoisomers spots were assigned values of linking difference by counting from position R. Each gel is labeled with the temperature for the first dimension: (A) pColIRΔxba at 20 °C; (B) pColIRΔxba at 28 °C; (C) pColIRΔxba at 40 °C; (D) pColIRΔxba at 34 °C (5 μg/mL chloroquine); (E) pColIR315 at 40 °C.

sharp, those that were more supercoiled formed retarded smears in the gel, indicative of chemical modification. This experiment shows the equivalence of the two techniques: the threshold levels of supercoiling required to observe mobility changes in the gel and chemical reactivity are identical. Both methods indicate that the initial structural change requires a level of DNA supercoiling equivalent to $-\Delta Lk \geq 14$ at 34 °C.

We have employed a simple statistical mechanical model to analyze the data from the two-dimensional gel electrophoresis in terms of helix–coil (melting) transitions in the supercoiled DNA, in order to obtain estimates of the thermodynamic parameters for the initial opening transition. In this model we assume that a segment of defined length within the plasmid can undergo melting, with the same parameters for melting each base pair within this tract. We ignore loop entropy. The model is described in greater detail in the Materials and Methods section. An example of the fit to the experimental data for pColIRΔxba at 34 °C is shown in Figure 8, in which the points are the experimental data and the line shows the fit using the statistical mechanical model. The first transition is fitted very well using this simple model. This was obtained using a free energy for average base-pair opening (ΔG°_{hc}) of 0.31 kcal mol^{-1}, that for a junction between B-DNA

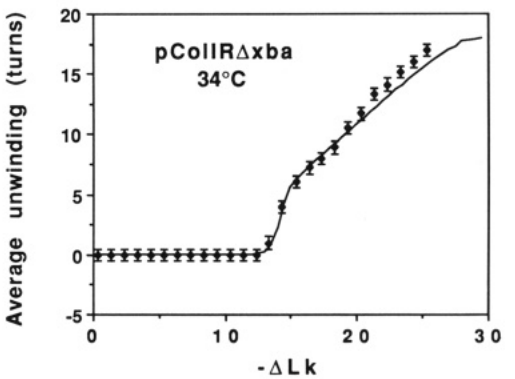

FIGURE 8: Statistical mechanical modeling of helix opening in pColIRΔxba at 34 °C as a function of superhelicity. The points are experimental data taken from two-dimensional gel electrophoresis (with an error of ±0.5 in the unwinding measurement indicated), and the continuous line was generated by the model, described in the text.

and a melted region ($\Delta G^\circ_{j,hc}$) of 4.9 kcal mol^{-1}, and a potential melting region (M_0) of 200 bp in length. The shape of the simulated curve was not significantly affected by increasing M_0 above 200 bp. Similar values of ΔG°_{hc} and $\Delta G^\circ_{j,hc}$ were required to fit data at other temperatures, although the value of ΔG°_{hc} was found to decrease with temperature, indicating

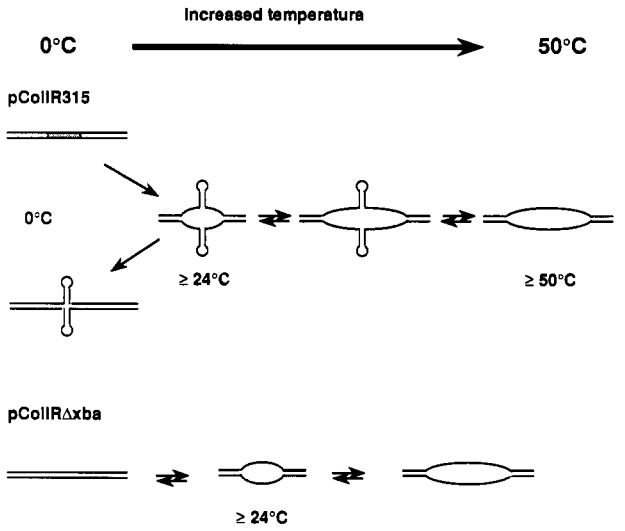

FIGURE 9: Model for the formation of open regions in supercoiled (A + T)-rich DNA at elevated temperature, and the proposed mechanism for C-type cruciform extrusion. Under the conditions employed in these experiments, increased temperature and superhelicity result in a local melting of the (A + T)-rich ColE1 sequences, observed by chemical modification and two-dimensional gel electrophoresis. In the absence of an inverted repeat contained in the melted region (pColIRΔxba), the opening is fully reversible with temperature. However, the presence of an inverted repeat results in the formation of hairpin loops within the melted region at temperatures below 50 °C; subsequent reduction in temperature and/or increased ionic strength results in closure of the open region leaving the hairpin loops "trapped" as a cruciform structure. This we propose to be the mechanism of formation of cruciform structures by the C-type pathway.

a positive (i.e., favorable) entropy for melting. All the experimental data were fitted for values in the range of $0.25 < \Delta G^{\circ}_{hc} < 0.35$ and $4.5 < \Delta G^{\circ}_{j,hc} < 6$ kcal mol^{-1}.

DISCUSSION

All the available evidence indicates that the (A + T)-rich sequences that were previously proposed to induce cruciform extrusion by the C-type mechanism undergo large-scale helix opening as a function of temperature, superhelicity, and ionic strength. Helix opening is suggested by the following: (a) Chemical reactivity that is localized to the (A + T)-rich sequences. The size of the modified region is dependent on temperature. (b) Transitions are seen by two-dimensional gel electrophoresis, consistent with stable opening of the (A + T)-rich sequences as a function of temperature and superhelix density. (c) These (A + T)-rich sequences are the same regions of DNA that are responsible for cruciform extrusion with kinetic properties that suggest large-scale helix opening (Lilley, 1985; Sullivan & Lilley, 1986; Sullivan et al., 1988). Statistical mechanical calculations indicated that these sequences have a propensity to undergo cooperative denaturation at elevated temperatures (Schaeffer et al., 1989).

Evidence for localized unwinding of (A + T)-rich sequences in supercoiled DNA has been obtained for different sequences in other laboratories (Lee & Bauer, 1985; Kowalski et al., 1988).

Large-Scale Helix Denaturation and the Mechanism of C-Type Cruciform Extrusion. A model describing the behavior of the (A + T)-rich sequences, which incorporates all the available data, is presented in Figure 9. This is based on the localized unpairing of the sequences at elevated temperature, beginning at a minimum temperature of 24 °C at a superhelix density of −0.056 and the ionic conditions described above. The detailed behavior of the molecules depends on whether or not an inverted repeat is present. (a) (A + T)-rich sequences in isolation from an inverted repeat (pColIRΔxba): There is a completely reversible helix opening beginning at 24 °C, with an expansion as a function of increased temperature and superhelicity. (b) (A + T)-rich sequences adjacent to an inverted repeat (pColIR315): The inverted repeat adds an additional complexity to the behavior of the DNA. The initial opening occurs at the same temperature, but the single-stranded region can undergo hairpin formation due to the sequences of 2-fold symmetry, and the hairpin structures are stable up to a temperature of 50 °C. On reducing the temperature, the hairpin loops remain in the individual strands, as the open region closes around them. Ultimately, a cruciform is left flanked by fully base-paired supercoiled DNA. At intermediate temperatures between 4 and 24 °C, the cruciform itself exerts a local destabilizing effect, inducing chemical reactivity in the immediately adjacent DNA; this is consistent with the open structure of the four-way junction in the absence of metal ion binding (Duckett et al., 1988; McClellan & Lilley, 1987).

We note that there is a coincidence of a number of temperature thresholds observed for these molecules, in the region of 22–25 °C. These are as follows: (a) The minimum reaction temperature for observation of chemical reactivity of the (A + T)-rich sequences in pColIRΔxba or unpreincubated pColIR315. This is true for osmium tetroxide/bipyridine, or bromoacetaldehyde under our standard conditions. (b) The minimum preincubation temperature for pColIR315, in order to observe subsequent reactivity at lower temperatures. (c) The minimum temperature at which transitions are discernible by two-dimensional gel electrophoresis. (d) The minimum temperature at which cruciform extrusion proceeds at a measurable rate in the same sequence context (Sullivan & Lilley, 1986).

This suggests that the same physical process is being studied in each case, which we would interpret to be the initial formation of an opened region in the (A + T)-rich DNA. This cooperative opening (the transition in the two-dimensional gels) of a section of helix generates DNA of single-stranded character that is chemically reactive and facilitates the formation of hairpin loops where the sequence symmetry permits. It is this formation of hairpin loops in the single-stranded open region that is the basis of the formation of cruciform structures by the C-type mechanism.

This gives a simple explanation of cruciform extrusion at low ionic strength by the C-type mechanism and the role of the (A + T)-rich flanking sequences. In this view, the (A + T)-rich sequences promote cruciform extrusion by forming a large denatured region of DNA that includes the inverted repeat. Individual hairpin structures are formed by the inverted repeat, and on renaturation of the region, either by reduction of temperature or by increased ionic strength, these are trapped in the DNA as a cruciform structure. The (A + T)-rich C-type inducing sequences thus operate in a very straightforward way; there is no need to invoke long-range transient fluctuations in DNA structure, or similar concepts. Moreover, the majority of the properties of C-type cruciform extrusion are essentially those of the formation of large-scale cooperative helix opening at low ionic strength and therefore provide a means for studying such denaturation in supercoiled DNA.

The Formation of Open Regions in Superhelical DNA. The size of the region of (A + T)-rich DNA that is opened in response to elevated temperature and supercoiling may be estimated from the change in twist measured by electrophoresis and the extent of chemical modification for a given set of

conditions. Once again, we find good agreement between these fundamentally different approaches. Comparing the results obtained using pColIR315, two-dimensional gel electrophoresis reveals two broad transitions, with mean twist changes at 40 °C of approximately 8 and 13 turns. The chemical reactivity studies revealed progressive expansion of the reactive region with temperature, and stages of opening corresponding to 5, 8, and 14 turns were observed.

The mean superhelical density of our plasmid preparations was about −0.056, i.e., the Boltzmann distribution of topoisomers was centered on $-\Delta Lk = 21$. At 40 °C, most of the DNA would therefore have an unwinding of at least 13 turns. All of the chemical probes suggested that at this temperature the number of exposed bases was greater than 100. There is, therefore, good qualitative agreement between the extent of opening estimated from chemical modification studies and that measured from two-dimensional gel electrophoresis experiments. It would be difficult to expect more since we do not know what the structure of the DNA was in the area being unwound. For example, single-stranded DNA in a large unpaired region is likely to undergo loose left-handed winding in response to the negative supercoiling, but the extent cannot be estimated. Thus, it is not possible to interpret the measured unwinding directly in terms of a particular size of opened DNA.

We have been able to model the DNA opening process as a function of superhelical density using a very simple statistical mechanical model. The average parameters calculated for the helix–coil transition in the (A + T)-rich DNA in TBE/2 buffer differ slightly from those obtained in previous studies using polymers (Crothers & Zimm, 1964; Wartell & Benight, 1985). These differences may reflect several factors, including the influence of the ionic conditions, the effect of topological constraints on the helix, the loop and the helix–coil junction, and perhaps the unique properties of the ColE1 sequences themselves. The model employed in this study gives a good description of the initial opening transition but is probably inadequate beyond this point, as the deviation between calculated and experimental data indicates. At higher levels of supercoiling, a number of potentially important factors would need to be included, such as twisting in the open region and competition with other transitions elsewhere in the molecule (Benham, 1990); however, we lack much of the experimental data required for the parametrization of these processes.

General Significance of Large-Scale Cooperative Opening of DNA. These studies demonstrate the (A + T)-rich DNA sequences may undergo cooperative helix opening over significant regions, in response to elevated temperature and negative supercoiling. This suggests that such sections of DNA might exert a destabilizing effect on nearby DNA sequences, as our earlier studies of cruciform extrusion have suggested. Moreover, our earlier studies of cruciform extrusion show that in addition to the local helical destabilization brought about by the (A + T)-rich ColE1 sequences, they can also exert a significant influence on the kinetics of structural transitions occurring in the same region of DNA.

Transitions of the type described here have been linked with both gene transcription and DNA replication, as both processes require strand opening as a fundamental event (Bramhill & Kornberg, 1988a,b; Siebenlist, 1979). Kohwi-Shigematsu and Kohwi (1990) have recently shown that supercoiled (A + T)-rich sequences associated with an immunoglobulin enhancer are both reactive to chloroacetaldehyde and exhibit opening transitions on two-dimensional gels. Kowalski and Eddy (1989) showed that the *E. coli* replication origin, *oriC*, exhibits opening transitions and is cleaved by single-strand-specific nuclease when supercoiled. A similar opening was found to occur in a yeast ARS element (Umek & Kowalski, 1990). These transitions, like those analyzed in the studies presented here, were observed at low ionic strength. However, the helix opening at low salt concentration probably reflects a propensity for unwinding by the same sequences at more physiological conditions; some chemical reactivity was found to remain in the immunoglobulin enhancer sequences even at elevated ionic strength, for example. It seems probable that the transitions occurring in the DNA alone at low salt concentration are closely related to those mediated by proteins in the cells, such as the opening of the *oriC* sequences by the DnaA protein (Gille & Messer, 1991).

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