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Localized Chemical Hyperreactivity in Supercoiled DNA: Evidence for Base Unpairing in Sequences That Induce Low-Salt Cruciform Extrusion†

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ABSTRACT: Certain A+T-rich DNA sequences (C-type inducing sequences) cause adjacent inverted repeats to undergo cruciform extrusion by a particular pathway (C-type extrusion), which is characterized by large activation energies and extrusion at low salt concentrations and relatively low temperatures. When they are supercoiled, these sequences become reactive toward the normally single-strand-selective reagents bromoacetaldehyde, glyoxal, osmium tetraoxide, and sodium bisulfite. The following evidence is presented: (1) The most reactive sequences are those to the left of the inverted repeat. (2) Chemical reactivity is suppressed by either sodium chloride or micromolar concentrations of distamycin. The suppression of reactivity closely parallels that of C-type cruciform extrusion. (3) Chemical reactivity requires a threshold level of negative supercoiling. The threshold superhelix density depends on the prevailing salt concentration. (4) Analysis of temperature dependences suggests that reaction with osmium tetraoxide involves transient unstacking events, while bromoacetaldehyde requires larger scale helix opening. Thus a variety of opening events may occur in the supercoiled A+T-rich sequences, from small-amplitude breathing to low-frequency, large-amplitude openings. The latter appear to be responsible for C-type cruciform extrusion.

Contextual effects on cruciform extrusion provide a striking example of the transmission of structural effects in supercoiled DNA (Sullivan & Lilley, 1986). C-type inducing sequences are very A+T-rich regions of DNA which act upon nearby inverted repeats, changing the entire character of the process by which the molecule isomerizes to form a cruciform structure. All available evidence (Lilley, 1985; Sullivan & Lilley, 1986, 1987, 1988; Sullivan et al., 1988) suggests that the inducing sequences are responsible for a change in the mechanistic pathway for the extrusion process. The C-type pathway is beleved to proceed via a transition state which is well represented by a large melted "bubble" in the DNA, which may then undergo intrastrand base pairing to form a fully extruded cruciform in a single step. It is characterized kinetically by a very large activation energy (in the region of 200 kcal mol^{-1}) and extrusion in the absence of added salts. The C-type mechanism involves large-scale unpairing of bases in the DNA. The properties of the inducing sequences may be summarized as follows:

(1) The sequences are very A+T rich (≥70%). Application of statistical thermodynamic DNA melting theory (Schaeffer et al., 1988) indicates that the inducing sequences have a high propensity to cooperative melting. This is further substantiated by the observation that sequences of normal base sequence become quasi-C-type in character if helical stability is reduced by addition of solvents such as formamide (Sullivan & Lilley, 1988).

(2) Inducing sequences operate on neighboring inverted repeats in cis, without regard to polarity (Sullivan & Lilley, 1986). The nature of sequences (termed transmitting sequences) in between the inducing sequence and the inverted repeat is important (Sullivan et al., 1988).

(3) A single C-type inducing sequence is sufficient to confer C-type character on a nearby inverted repeat in 0 mM sodium chloride (Sullivan & Lilley, 1986).

(4) The effect of a C-type inducing sequence may be modulated by changes in sequence at least 100 bp distant from

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the inverted repeat (Sullivan & Lilley, 1986).

(5) The true inducing sequence may be relatively short. The crucial sequence in the left-side ColE1 sequence is a 30-bp sequence we have called col30, which may act in isolation (Sullivan et al., 1988). The effect of such sequences may be augmented by an appropriate transmitting sequence (Sullivan et al., 1988).

(6) The effect of the C-type inducing sequences is strongly suppressed by addition of sodium chloride (75 mM) or low concentrations of the minor groove binding drug distamycin (5 μM) (Sullivan & Lilley, 1988).

All the known properties of these elements are consistent with the concept that they are the source of DNA instability and unpairing. This could then perturb the stability of the inverted repeat and thus prejudice the selection of mechanistic pathway for the cruciform extrusion transition. This might occur via long-range telestability (Burd et al., 1975) effects, i.e., equilibrium processes, mobile transient structural fluctuations such as solitons (Englander et al., 1980), or perhaps a combination of these. In general, we think that the telestability effects are probably sufficient to explain all the observations, as calculations based on equilibrium properties give excellent agreement with our experimental data (Schaeffer et al., 1988), but we cannot exclude other models at this time.

If the inducing sequences are a source of DNA unpairing, can we observed this experimentally? In this study we have probed the C-type inducing sequences using a series of chemicals which selectively react with single-stranded DNA. We find that they are indeed hyperreactive when supercoiled and that a number of parallels may be drawn between chemical reactivity and effects on cruciform extrusion. However, the temperature dependence of the chemical reactions suggests that there is a preponderence of small openings arising transiently in the inducing sequences. Thus, the combination of chemical reactivity and extrusion properties indicates that these sequences have a very dynamic nature when supercoiled, covering a range of sizes of openings.

Experimental Procedures

Plasmid DNA. Escherichia coli HB101 transformed by the required plasmid was grown in M9 salts minimum medium, with amplification for 14–16 h by 150 μg/mL chloramphenicol. DNA was isolated by lysis in lysozyme, SDS, and EDTA and purified by two rounds of cesium chloride density gradient centrifugation in the presence of ethidium bromide (Clewell & Helinski, 1972). Supercoiled plasmid recovered by side puncture was extracted with cold butan-1-ol and dialyzed against 10 mM Tris-HCl, pH 7.5, and 0.1 mM EDTA at 7 °C.

Topoisomer Distributions. Native supercoiled plasmid DNA was partially relaxed by incubation with rat liver topoisomerase I in the presence of various concentrations of ethidium bromide. Individual topoisomer distributions were purified by small-scale (1 mL) cesium chloride density ultracentrifugation in a Beckman TL100 ultracentrifuge. Superhelical density was determined by band counting methods, using agarose gel electrophoresis in the presence of chloroquine (Keller, 1975).

Bromoacetaldehyde Reactions. Bromoacetaldehyde was prepared by hydrolysis of the diethyl acetal (Fluka) according to the method of McLean et al. (1987). In standard reaction conditions, approximately 2 μg of supercoiled DNA was incubated with 2% (approximately 50 mM) bromoacetaldehyde at 37 °C for 120 min in 10 mM Tris-HCl, pH 7.5, and 0.1 mM EDTA in a total volume of 100 μL. Changes to this protocol are indicated in the text where relevent. After incubation the bromoacetaldehyde was removed from the aqueous phase by two ether extractions followed by two ethanol precipitations from 0.3 M sodium acetate. Chloroacetaldehyde was obtained from Fluka and used under the same conditions as bromoacetaldehyde.

Osmium Tetraoxide Reactions. Approximately 2 μg of supercoiled DNA was reacted with 2 mM OsO_4 (Sigma) at 20 °C for 15 min, in 3% pyridine, 5 mM Tris-HCl, pH 8, and 0.5 mM EDTA. DNA was recovered by two ethanol precipitations from 0.3 M sodium acetate. Changes to this protocol are indicated in the text where relevent.

Glyoxal Reactions. Approximately 2 μg of supercoiled DNA was reacted with 220 mM glyoxal (trimeric dihydrate, Sigma) at 37 °C for 50 min in 18 mM Tris–borate, pH 7.8, and 0.5 mM EDTA.

Analysis of Chemical Adducts. Following chemical reaction, DNA was digested to completion with an appropriate restriction enzyme.

For lowest resolution of the products of these reactions, the linearized DNA was digested with 5 units of S1 nuclease for 20 min at 15 °C in 50 mM sodium acetate, pH 4.6, 50 mM NaCl, and 1 mM $ZnCl_2$. The DNA was electrophoresed in 1% agarose gels in 90 mM Tris–borate, pH 8.3, and 10 mM EDTA. The gels were stained with ethidium bromide and photographed with UV back-illumination. Quantitation was performed by laser densitometry of photographic negatives using an LKB Ultroscan 2202 densitometer. Extent of modification was calculated as the intensity of the band corresponding to site-selective modification, as a fraction of the total intensity. This was then expressed on a scale of 0–4. These relative extents of chemical modification cannot be compared between different experiments, since different chemicals, or batches of chemicals, may have been used. These values cannot be placed on an absolute scale as the S1 nuclease cleavage step is deliberately mild, and therefore incomplete, and the values are thus only lower estimates of the true degree of modification. In most experiments the degree of chemical reaction of A+T tracts is between 10 and 50% of molecules modified.

For intermediate resolution, chemically modified DNA was digested by restriction enzymes and S1 nuclease as above. The DNA was radioactively 3′-^{32}P labeled with DNA polymerase (Klenow fragment, Bethesda Research Laboratories) (Jacobson et al., 1974). DNA was electrophoresed in 5% polyacrylamide gels in 90 mM Tris–borate, pH 8.3, and 10 mM EDTA. For resolution of the products of OsO_4 modification at the nucleoside level, DNA was 5′-^{32}P labeled by the kinase exchange reaction (Berkner & Folk, 1977) or 3′-^{32}P labeled with Klenow DNA polymerase (Jacobson et al., 1974). Following a second restriction digestion, the resulting chemically modified, asymmetrically labeled fragments were isolated from 5% polyacrylamide gels and subjected to cleavage by 1 M piperidine at 90 °C for 30 min and extensive lyophilization. DNA was electrophoresed in 8% denaturing polyacrylamide gels containing 7 M urea in 90 mM Tris–borate, pH 8.3, and 10 mM EDTA. Radioactive DNA was visualized by autoradiography of dried gels at −70 °C with intensifier screens.

Chemical Sequencing of DNA. Asymmetrically ^{32}P-labeled DNA fragments were subjected to sequencing by chemical cleavages as described by Maxam and Gilbert (1980).

Bisulfite Modification of DNA. A total of 5 μg of supercoiled pColIR315 was incubated with 1.4 M sodium metabisulfite (Sigma), 0.84 M sodium sulfite, 2 mM hydroquinone, 3.5 mM NaCl, and 0.35 mM sodium citrate, pH 7.0, for 24 h at 37 °C. The DNA was transformed (Cohen et al., 1972)

into *E. coli* BD10 (Duncan et al., 1978). Transformants were pooled, and a preparation of DNA was cleaved with *Eco*RI and *Bam*HI. The 440-bp mixed-population fragments were isolated from a 5% polyacrylamide gel by electroelution. These were ligated into *Eco*RI- and *Bam*HI-cleaved double-stranded phage M13mp9 RF (Messing & Vieira, 1982) and transfected into *E. coli* JM109. Phages were prepared from individual plaques by precipitation in 20% poly(ethylene glycol) and 2.5 M NaCl on ice, and single-stranded phage DNA was obtained by phenol extraction and ethanol precipitation. DNA was sequenced by primer extension (Sanger et al., 1977).

Results

Sequences Which Flank the ColE1 Inverted Repeat Are Chemically Reactive. We wished to study the reactivity of the ColE1 sequences which flank the inverted repeat, avoiding any possible confusion which might arise due to the presence of the cruciform itself. For this reason we chose to employ the plasmid pColIRΔxba, from which the inverted repeat had been previously deleted (Sullivan & Lilley, 1986) and which therefore could not possibly extrude a cruciform in this region.

We decided to examine possible site-selective reactivity in pColIRΔxba toward a number of single-strand-selective chemical reagents which we have employed previously in our studies of cruciform structures, including bromoacetaldehyde (Kayasuga-Mikado et al., 1980; Kochetkov et al., 1981; Secrist et al., 1982; Kohwi-Shingematsu et al., 1983; Lilley, 1983; McClean et al., 1987), osmium tetraoxide (Beer et al., 1966; Chang et al., 1977; Hodgson, 1977; Marzilli, 1977; Glikin et al., 1984; Lilley & Palecek, 1984; Lukasova et al., 1984; Johnson & Rich, 1985; Nejedly et al., 1985), and glyoxal (Broude & Bodowsky, 1981; Gough, 1986; Lilley, 1986). These probes differ in their chemistry, reaction conditions, and target bases, as summarized in Table I.

Initially we studied the modification of supercoiled pColIRΔxba at low resolution. Samples of DNA were reacted with a reagent and cut at a unique restriction site, and the resulting linear species were cleaved at the positions of adduct formation with S1 nuclease. The DNA samples were then examined by electrophoresis in agarose gels, site-selective reactivity being revealed by bands of DNA of greater mobility than the full-length linear plasmid. The advantage of beginning at this resolution is that information is obtained about relative chemical reactivity in the plasmid as a whole, which can be seen on a single gel.

The results of these studies are presented in Figure 1. It is very clear that both bromoacetaldehyde and osmium tetraoxide react with the supercoiled plasmid at a single major location, resulting in one predominant band (labeled mod) which migrates faster than the full-length linear plasmid. Measurement of the sizes of these bands, together with data obtained with other restriction enzymes, shows that the region of pronounced chemical reactivity is that of the ColE1 sequences. Site-selective reactivity was not seen when linear DNA was used as substrate for any of the chemicals. Thus, negative supercoiling in pColIRΔxba generates a region of localized chemical reactivity in the plasmid.

Sequences within colL Are Especially Reactive. Having shown that the ColE1 flanking sequences are chemically reactive, we examined the pattern of reactivity more closely to see how uniform the chemical modification might be.

Samples of supercoiled pColIRΔxba were reacted with either bromoacetaldehyde or glyoxal and digested to completion with *Bam*HI, modified nucleotides were cleaved with S1 nuclease, and finally the DNA was 5′-^{32}P labeled. To provide an appropriate marker fragment, supercoiled

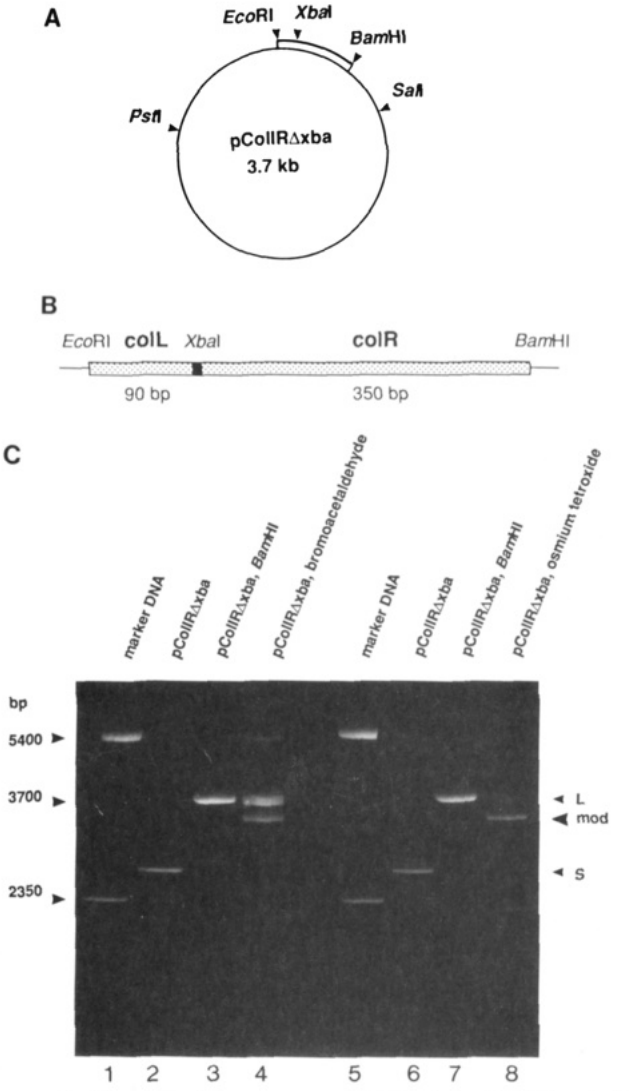

FIGURE 1: The A+T-rich sequences which flank the ColE1 inverted repeat are chemically hyperreactive. (A) Map of pColIRΔxba, a plasmid containing the A+T-rich flanking sequences from ColE1, where the inverted repeat has been replaced by an *Xba*I site (Sullivan & Lilley, 1986). In the circular map the ColE1 sequences are indicated by the open region. (B) ColE1 region of pColIRΔxba shown expanded. Restriction sites used in these studies are indicated. The ColE1 sequences are approximately 440 bp in length, extending from the *Eco*RI to *Bam*HI sites. They may be divided into the 90-bp left-side sequence (colL) and the longer right-side sequence (colR). (C) Agarose gel of results of chemical modification of pColIRΔxba. Tracks (left to right): 1, phage PM2 cleaved with *Hin*dIII as size markers; 2, supercoiled pColIRΔxba; 3, pColIRΔxba cleaved with *Bam*HI; 4, pColIRΔxba modified with bromoacetaldehyde, cleaved with *Bam*HI, and incubated with S1 nuclease; 5, PM2, *Hin*dIII markers; 6, supercoiled pColIRΔxba; 7, pColIRΔxba, *Bam*HI; 8, pColIRΔxba modified with osmium tetraoxide, cleaved with *Bam*HI, and incubated with S1 nuclease. Fragment sizes (bp) are indicated on the left. The arrows on the right denote the positions of linear (L) and supercoiled (S) pColIRΔxba and the band arising from modification at the ColE1 sequences (mod).

pColIR315 was cleaved with S1 nuclease, which cuts predominantly at the cruciform loop, followed by *Bam*HI restriction cleavage and radioactive labeling, resulting in a ^{32}P-labeled 350-bp fragment. The fragments arising from these experiments were electrophoresed in 5% polyacrylamide gels which were then autoradiographed. The results are shown in Figure 2.

These gels confirm that the A+T-rich ColE1 sequences are the major target for these chemicals. In contrast to the S1 nuclease cleavage of the ColE1 cruciform, which is tightly

Table I: Summary of the Chemical Probes Employed in These Studies[a]

chemical	target bases (major)	chemistry	conditions	analysis procedure
bromoacetaldehyde ($BrCH_2CHO$)	A, C	etheno adducts, 1,N^6-ϵA and 3,N^4-ϵC	50 mM BAA, 10 mM Tris, pH 7.5, 0.1 mM EDTA	S1, AG or PAG
osmium tetraoxide (OsO_4)	T	cis ester adduct, C5-C6	1 mM OsO_4, 5 mM Tris, pH 8.0, 0.5 mM EDTA, 3% pyridine	S1, AG or piperidine, 90 °C, sequence gel
glyoxal (CHO-CHO)	G	cyclic diol adduct, 1,N^2	220 mM glyoxal, 18 mM Tris–borate, pH 7.8, 0.5 mM EDTA	S1, AG or PAG
bisulfite (HSO_3^-)	C	deamination to dU	1.4 M sodium metabisulfite, 0.84 M sodium sulfite, 2 mM hydroquinone, 3.5 mM NaCl/citrate, pH 7.0	transformation into *E. coli* sequencing C-T transitions

[a] S1, cleavage with a restriction enzyme followed by digestion of the adduct with S1 nuclease; AG, agarose gel electrophoresis; PAG, polyacrylamide gel electrophoresis; BAA, bromoacetaldehyde.

localized to the loop, the modification of pColIRΔxba covers a much wider area, spreading over at least 100 bp, centered on the region from which the inverted repeat was deleted (i.e., the *Xba*I site). The region of strongest reactivity appears to be that to the left of the *Xba*I site (this is particularly clear for glyoxal, both with and without the ColE1 inverted repeat), and this has been confirmed by use of *Eco*RI in place of *Bam*HI, i.e., examining fragments arising from the opposite side of the *Xba*I site. These sequences are also chemically reactive in pColL, which contains only the 100-bp left-side ColE1 sequence (Sullivan & Lilley, 1986) (data not shown).

We have been able to study the location of the sequences of greatest reactivity in further detail using osmium tetraoxide. Thymine–osmium tetraoxide–pyridine adducts may be cleaved with hot piperidine, and hence chemical reactivity studied at single-nucleotide resolution on sequencing gels. The result of osmium tetraoxide probing of supercoiled pColIRΔxba is shown in Figure 3. The strong reactivity of thymine bases in the colL sequence is very clear, while no modification at all is discernible in the sample of linear DNA which was identically treated. The *Xba*I proximal end of the sequence exhibits high reactivity; this is the sequence which we have called col30, which acts independently as a C-type inducing sequence (Sullivan et al., 1988). Equivalent experiments mapping osmium tetraoxide modification on the opposite strand (not shown) indicate that the col30 sequences are equally hyperreactive on both strands.

ColE1 Flanking Sequences Are also Modified by Bisulfite. We have employed a fourth probe of single-stranded character in these studies. Bisulfite deaminates single-stranded cytosine bases, resulting in C–T transition mutations on transformation into *E. coli* (Hayatsu et al., 1970; Shapiro et al., 1970, 1973; Goddard & Schulman, 1972; Hayatsu, 1976). Supercoiled pColIR315 was reacted with bisulfite, according to procedures employed previously for studying cruciform geometry (Gough et al., 1986), and the plasmid was then transformed into the repair-deficient *E. coli* BD10. All the transformants were pooled, and a mixed preparation of DNA was made. The *Eco*RI to *Bam*HI ColE1 fragment was recloned into M13mp9 and transfected into *E. coli* JM109 cells, and phage DNA was prepared from plaques. These were sequenced by primer extension techniques using dideoxynucleotides (Sanger et al., 1977). From 28 mutant sequences found, 17 were in or immediately adjacent to the ColE1 cruciform loop. However, seven were found in the middle of the cruciform stem regions, and four more were discovered within the regions flanking the inverted repeat for 60 bp on either side. By comparison, in our earlier studies of bisulfite mutagenesis on S-type plasmids (Gough et al., 1986), *no* mutations were detected outside the inverted repeats—sequencing of bisulfite mutants of pIRbph13 revealed no base changes outside the hexanucleotide center

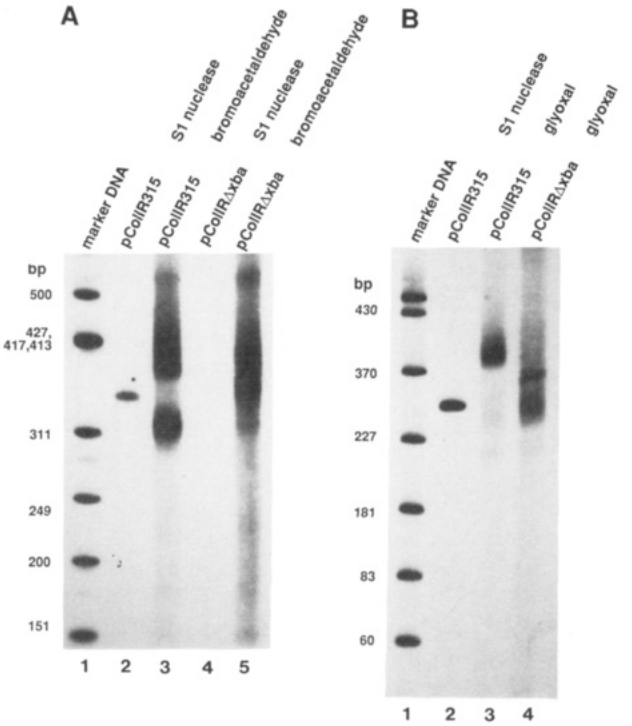

FIGURE 2: The left-hand side ColE1 flanking sequences (colL) are particularly reactive toward bromoacetaldehyde and glyoxal. Supercoiled pColIR315 (containing cruciform) or pColIRΔxba (no cruciform) was cleaved with S1 nuclease or reacted with bromoacetaldehyde or glyoxal followed by complete digestion with *Bam*HI. Chemically modified 3′-^{32}P-labeled DNA samples were then digested with S1 nuclease at 15 °C to cleave the DNA at positions rendered permanently single stranded due to adduct formation, electrophoresed in 5% polyacrylamide gels, and autoradiographed. (A) Bromoacetaldehyde modification. Tracks (left to right): 1, ϕX174 cleaved with *Hin*fI as fragment size markers; 2, pColIR315 cleaved with S1 nuclease; 3, pColIR315 modified by bromoacetaldehyde; 4, pColIRΔxba incubated with S1 nuclease; 5, pColIRΔxba modified by bromoacetaldehyde. (B) Glyoxal modification. Tracks (left to right): 1, pBR322 cleaved with *Hae*II as fragment size markers; 2, pColIR315 cleaved with S1 nuclease; 3, pColIR315 modified by glyoxal; 4, pColIRΔxba modified by glyoxal. Fragment sizes (bp) are indicated on the left of each gel.

of the inverted repeat. Just as with the other chemical probes, bisulfite appears to detect an unusually reactive aspect of the ColE1 sequences when they are supercoiled.

C-Type Inducing Sequence from Drosophila Is Chemically Reactive. Is chemical reactivity toward reagents like bromoacetaldehyde and osmium tetraoxide a general property of C-type inducing sequences? The plasmid pIRbke/DsAT contains a 200-bp A+T-rich sequence cloned from *Drosophila melanogaster* and which exhibits C-type cruciform extrusion by the bke inverted repeat in the absence of added salt

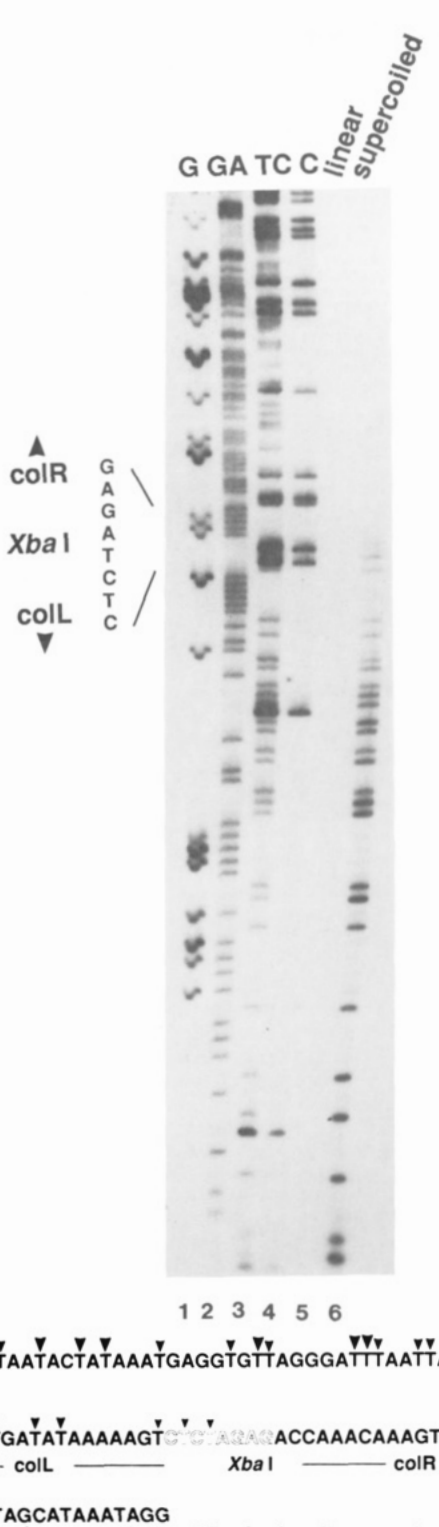

FIGURE 3: Osmium tetraoxide modification in colL—mapping reactive thymines at nucleotide resolution. Supercoiled and linear pColIRΔxba were modified with osmium tetraoxide and asymmetrically labeled *Eco*RI 5′-^{32}P-labeled *Bam*HI fragments isolated. After piperidine cleavage, these were electrophoresed in a 5% polyacrylamide sequencing gel and autoradiographed. (A) Sequencing gel showing typical results of modification of left-side ColE1 sequences. Tracks (left to right): 1–4, sequencing ladder derived by chemical cleavage reactions of *Eco*RI 5′-^{32}P-labeled *Bam*HI pColIRΔxba; 5, linear pColIRΔxba modified with osmium tetraoxide; 6, supercoiled pColIRΔxba modified with osmium tetraoxide. The position of the *Xba*I linker sequence which replaces the ColE1 inverted repeat in this plasmid is indicated on the left. Left-side ColE1 sequences extend below this sequence in this gel. (B) Base sequence of the relevent region of pColIRΔxba, showing the positions of reactive thymine bases. The sizes of these arrows reflect the intensity of osmium tetraoxide modification.

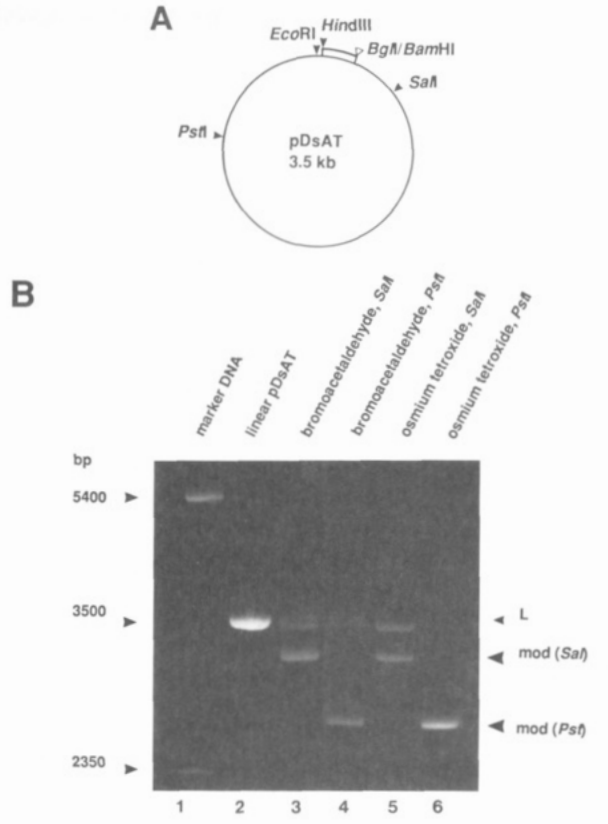

FIGURE 4: Chemical reactivity of an A+T-rich *Drosophila* sequence which acts as a C-type inducing sequence. (A) Map of pDsAT, a plasmid containing a 200-bp A+T-rich sequence (open region) cloned from *Drosophila* (Sullivan et al., 1988). Restriction sites used in these studies are indicated, and a *Bgl*I to *Bam*HI site created in the construction of pDsAT is shown by the open arrow. (B) Agarose gel of results of chemical modification of pDsST. Tracks (left to right): 1, phage PM2 cleaved with *Hin*dIII as size markers; 2, pDsAT linearized with *Sal*I; 3, pDsAT modified with bromoacetaldehyde, cleaved with *Sal*I, and digested with S1 nuclease; 4, pDsAT modified with bromoacetaldehyde, cleaved with *Pst*I, and digested with S1 nuclease; 5, linear pDsAT; 6, pDsAT modified with osmium tetraoxide, cleaved with *Pst*I, and digested with S1 nuclease. Fragment sizes (bp) are indicated on the left. The arrows on the right indicate the positions of linear pDsAT (L) and the bands arising from modification at the A+T-rich *Drosophila* tract when cleaved with *Sal*I and *Pst*I (mod).

(Sullivan et al., 1988). We have performed low-resolution mapping studies of bromoacetaldehyde and osmium tetraoxide modification of supercoiled pDsAT, the progenitor plasmid to pIRbke/DsAT, which lacks the inverted repeat. The result is shown in Figure 4. As with the ColE1 sequence, specific bands are seen on the agarose gel, indicating that there is strong chemical reactivity in the region of the A+T-rich *Drosophila* sequence.

Chemical Reactivity Is Reduced by Increasing Salt Concentration. Up to this point all of our experiments have been performed in buffer without added salt. We therefore examined the modification of supercoiled pColIRΔxba by bromoacetaldehyde and osmium tetraoxide as a function of added sodium chloride concentration. Samples of the plasmid were reacted for fixed times in the presence of varying concentrations of salt, followed by restriction cleavage and S1 nuclease digestion as before. Following agarose gel electrophoresis, the extent of reaction was assessed as the relative intensity of the band arising from site-selective modification. The results are presented graphically in Figure 5. The profiles for the two reagents are very similar—in each case there is marked reduction in reactivity of the ColE1 sequences as the salt concentration increases.

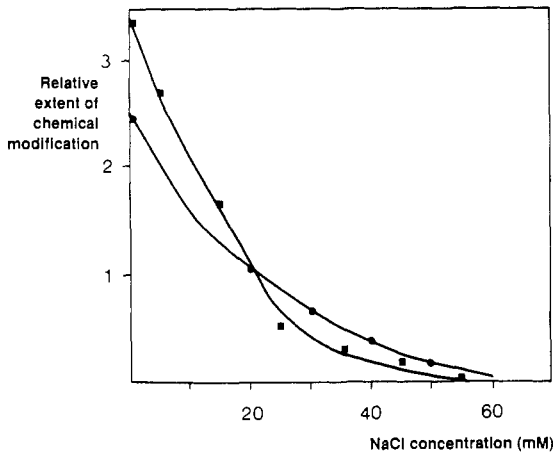

FIGURE 5: Chemical reactivity of ColE1 sequences is suppressed by addition of salt. Supercoiled pColIRΔxba was reacted with either bromoacetaldehyde or osmium tetraoxide in the presence of various concentrations of added sodium chloride, followed by complete cleavage with *Bam*HI and then incubation with S1 nuclease. All samples were electrophoresed in 1% agarose gels and the relative extents of chemical modification estimated by densitometry. (●) Bromoacetaldehyde modification; (■) osmium tetraoxide modification.

Chemical Reactivity Requires a Threshold Level of Negative Supercoiling. We have shown that site-specific chemical reactivity of the ColE1 sequences requires a supercoiled plasmid; a linear molecule cannot react in the same way. This strongly suggests that negative supercoiling is an essential prerequisite to reactivity, and we decided to investigate this in greater detail. We prepared topoisomer distributions of pColIRΔxba of different mean superhelix density (σ) using topoisomerase relaxation of supercoiled plasmid in the presence of various amounts of ethidium bromide. The mean linking difference of these preparations was measured by electrophoresis in agarose gels containing chloroquine, as we have employed previously in studies of cruciform thermodynamics (Lilley & Hallam, 1984).

These topoisomer distributions were each reacted with bromoacetaldehyde or osmium tetraoxide for fixed times, followed by restriction cleavage, S1 nuclease digestion, and agarose gel electrophoresis. The extent of chemical reaction was assessed as the relative intensity of the band arising from site-selective modification.

The results are presented in Figure 6. For bromoacetaldehyde and osmium tetraoxide the profiles are closely similar. Each gives a sigmoidal curve, with a threshold level of supercoiling for chemical reactivity at about $\sigma = -0.02$. We have examined the effect of added sodium chloride on these profiles. The major effect is a shift of the curves to higher superhelix densities, thus raising the threshold for chemical reactivity. There is also some reduction in the maximum level of reactivity attainable. These effects explain the reduction in the reactivity of native supercoiled plasmid with the addition of salt.

Distamycin Binding Reduces Chemical Reactivity in the ColE1 Flanking Sequences. Distamycin (Zimmer et al., 1971), like its close relative netropsin (Finlay et al., 1951), binds in the minor groove of DNA at A+T-rich sequences (Kopka et al., 1985; Zimmer & Wahnert, 1986; Coll et al., 1987) and stabilizes the helix (Reinart et al., 1979). We have shown that very low concentrations of distamycin, in the 3–5 μM range, can almost totally suppress the extrusion of C-type cruciforms (Sullivan & Lilley, 1988) and have presumed this to be a result of binding to the C-type inducing sequences adjacent to the inverted repeat. We therefore decided to examine the effect of similar concentrations of distamycin on the chemical reactivity of supercoiled pColIRΔxba.

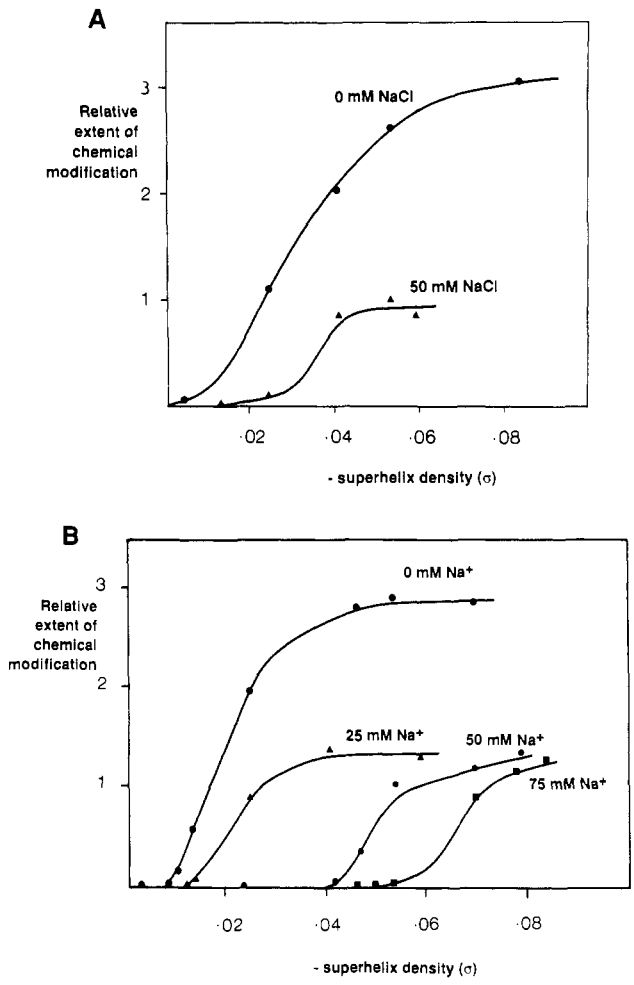

FIGURE 6: Supercoiling is essential for chemical reactivity of the ColE1 sequences. Topoisomer distributions of different mean superhelix density were prepared from pColIRΔxba. Each was reacted with either bromoacetaldehyde or osmium tetraoxide in the presence of indicated concentrations of added sodium chloride, followed by complete cleavage with *Bam*HI and then incubation with S1 nuclease. All samples were electrophoresed in 1% agarose gels and the relative extents of chemical modification estimated by densitometry. (A) Graph of bromoacetaldehyde modification as a function of superhelix density with 0 or 50 mM NaCl added. (B) Osmium tetraoxide modification as a function of superhelix density with 0, 25, 50, or 75 mM NaCl added.

Supercoiled pColIRΔxba was reacted for fixed times with osmium tetraoxide in the presence of various concentrations of distamycin in the range 0–10 μM. The extent of chemical reaction was assessed as before, with subsequent restriction cleavage, S1 nuclease digestion, and agarose gel electrophoresis. We observed a strong suppression of the chemical reactivity by low concentrations of distamycin, which was virtually linear with distamycin concentration to 3 μM, at which point suppression was almost total. Thus, distamycin binding does indeed stabilize the ColE1 sequences to attack by the chemical reagent, and at the same concentration as it exerts its effects on cruciform extrusion.

Temperature Dependence of Chemical Reactivity. We have studied the chemical reactivity of native supercoiled pColIRΔxba toward osmium tetraoxide and bromoacetaldehyde as a function of temperature, the results of which are shown in Figure 7A. It may be seen that the temperature dependences are quite different. Bromoacetaldehyde requires a temperature of excess of 25–30 °C for measurable reaction, while osmium tetraoxide is reactive almost down to 0 °C. Additional experiments (data not shown) have revealed that

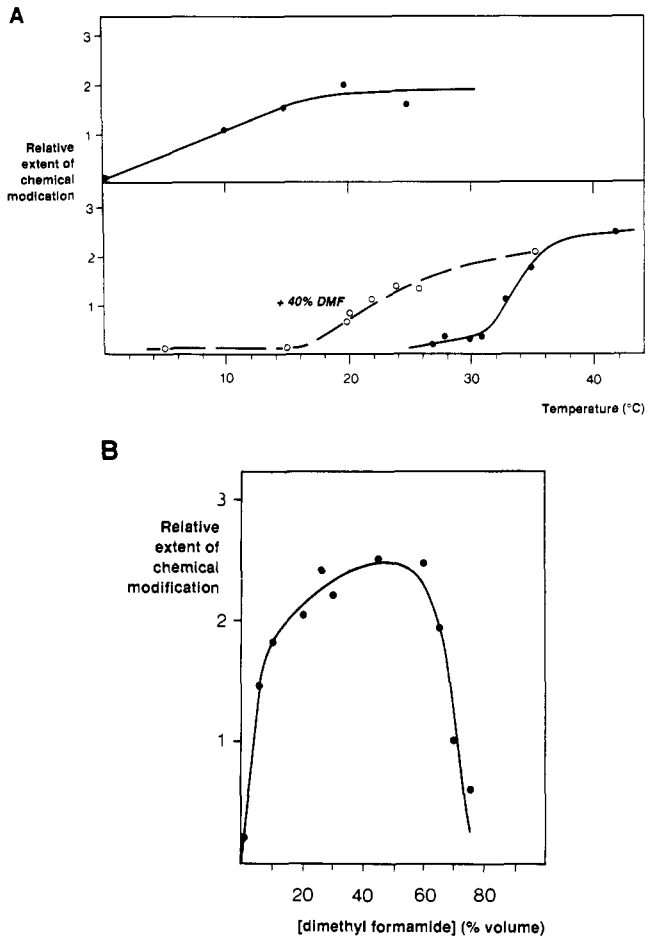

FIGURE 7: Osmium tetraoxide and bromoacetaldehyde exhibit different temperature dependences for reaction with the ColE1 sequences. Supercoiled pColIRΔxba was reacted with either bromoacetaldehyde or osmium tetraoxide at various temperatures, followed by complete cleavage with *Bam*HI and then incubation with S1 nuclease. All samples were electrophoresed in 1% agarose gels and the relative extents of chemical modification estimated by densitometry. (A) Graphs of osmium tetraoxide (upper) or bromoacetaldehyde (lower) modifications as a function of temperature in the absence (solid lines) or presence (broken line) of 40% dimethyl formamide. (B) Reactivity of pColIRΔxba to bromoacetaldehyde at 22 °C as a function of added dimethyl formamide concentration. Supercoiled DNA was reacted with bromoacetaldehyde at 22 °C in the presence of various concentrations of dimethyl formamide, and the extent of modification was measured as in (A).

the temperature dependence for reaction with osmium tetraoxide is approximately linear between 0 and 10 °C. The less reactive chloroacetaldehyde has a temperature dependence identical with that of bromoacetaldehyde (data not shown). These results suggest that the haloacetaldehydes and osmium tetraoxide may be reactive toward different states of the DNA.

Suspecting that the higher temperature threshold might reflect a cooperative opening of the helix, we reasoned that addition of helix-destabilizing solvents might reduce the point at which bromoacetaldehyde reacts with the DNA. We have shown previously (Sullivan & Lilley, 1988) that solvents such as formamide and dimethyl formamide (DMF) permit normally S-type cruciforms to extrude in the absence of added salt, by an apparently C-type mechanism, and cruciforms which are already C-type to extrude at reduced temperatures. In the experiment shown in Figure 7B, pColIRΔxba was reacted with bromoacetaldehyde at 22 °C, i.e., well below the temperature threshold shown in Figure 7A, with the addition of various concentrations of DMF. While there is no significant modification at this temperature in the absence of solvent, addition of DMF facilitates the attack by bromoacetaldehyde, with optimal reactivity at about 50% by volume of DMF. Additionally, this result shows that the temperature threshold in the absence of DMF is not due to the intrinsic temperature dependence of the bromoacetaldehyde reaction, which is confirmed by preliminary experiments showning that single-stranded DNA is reactive to modification at lower temperatures. Figure 7A includes a temperature profile for supercoiled pColIRΔxba reacting with bromoacetaldehyde in the presence of 40% DMF (broken line). It may be seen that the effect of the solvent is to reduce the threshold temperature for reaction by about 10 deg. Thus, destabilizing these sequences further with helix-destabilizing agents leads to an increase in their chemical reactivity.

Discussion

We have demonstrated that the inducing sequences which confer C-type cruciform extrusion kinetics on adjacent inverted repeats are chemically reactive toward a number of single-strand-selective probes, provided that they are sufficiently supercoiled. These sequences are by far the most reactive of any present in the plasmids in which they are studied. By comparison, S-type flanking sequences exhibit no reactivity above the background for the plasmid overall. The chemical reactivity for the inducing sequences may be directly compared with their effects on cruciform extrusion in a number of respects:

(1) All the sequences which act as C-type inducing sequences are chemically reactive in isolation. This includes subfragments of both the right- and left-hand sides of the ColE1 sequences and a *Drosophila* sequence.

(2) The most reactive sequences in the ColE1 DNA are centered around the region we have called col30. col30 is the basic C-type inducing sequence within colL, since its removal abolishes all C-type extrusion, and it has C-type inducing activity in isolation.

(3) The effects of salt on C-type extrusion and chemical reactivity are very similar. The profiles of extrusion rate of pIRxke/col as a function of salt concentration and osmium tetraoxide modification of pColIRΔxba as a function of salt concentration are virtually superimposable.

(4) The effects of distamycin on C-type extrusion and chemical reactivity are also extremely similar. Both cruciform extrusion (in pIRxke/col) and chemical reactivity (in pColIRΔxba) are strongly suppressed by 3 μM distamycin.

(5) The effect of the helix-destabilizing solvent DMF is to reduce the required temperature for both C-type extrusion and modification of the ColE1 sequences by bromoacetaldehyde.

Processes leading to greater helix stability result in reduced chemical reactivity and C-type extrusion, while reduction in stability increases both reactivity and C-type extrusion. Clearly there is an intimate relationship between chemical reactivity, helix stability, and the mechanism of cruciform extrusion. These results are fully consistent with the idea that the C-type inducing sequences are the source of helical instability in the regions forming the context of the inverted repeats which extrude via the C-type mechanism and that the lowered helix stability is the fundamental basis for the selection of this extrusion mechanism. DNA stability will be reduced by increased supercoiling, and we have demonstrated that the local chemical reactivity requires a threshold level of supercoiling. It has been demonstrated that rates of cruciform extrusion in pAO3, a deletant of the C-type plasmid ColE1, become markedly faster as supercoiling is increased (Panyutin et al., 1984). Since DNA structure is stabilized by cations, supercoiling and salt concentration are mutually antagonistic,

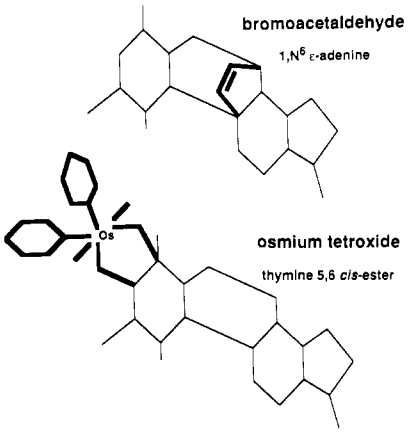

FIGURE 8: Base adducts formed by bromoacetaldehyde and osmium tetraoxide are very different in character. The schematic illustrates the difference between the base adducts formed by bromoacetaldehyde and osmium tetraoxide in relation to the thymine–adenine base pair. While the bromoacetaldehyde etheno adduct is formed with the adenine functionalities involved in base-pair hydrogen bonding, the thymine osmate ester does not directly interfere with base pairing. The normal single-strand selectivity of osmium tetraoxide is likely to be mainly a consequence of hindered out-of-plane attack on the π-orbitals of the C5–C6 bond, and thus, thymine reactivity is sensitive to base stacking in the first instance.

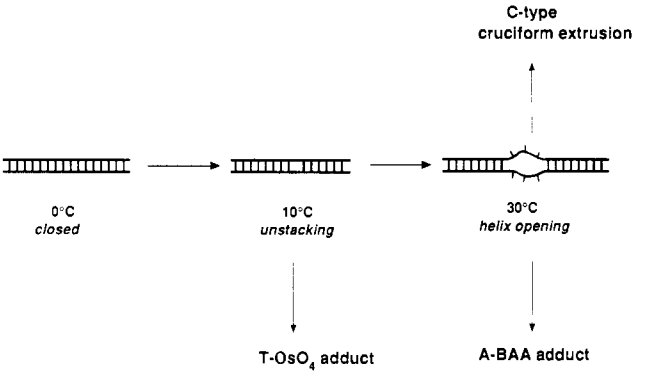

FIGURE 9: Dissection of the helix opening process as probed with chemical attack and cruciform extrusion. At the lowest temperatures the DNA appears to be unreactive to chemical attack and is therefore effectively closed. As the temperature is raised above freezing noncooperative unstacking events expose thymines in the ColE1 sequences to attack by osmium tetraoxide, but the DNA remains quite unreactive to modification by bromoacetaldehyde. Only at temperatures of around 30 °C do the adenines of these same sequences react with bromoacetaldehyde, implying a cooperative opening of the helix which exposes the bases to attack at the normally hydrogen-bonded positions. This cooperative opening is probably closely related to the state reponsible for C-type cruciform extrusion. This scheme is consistent with all our data on chemical reactivity and cruciform extrusion.

leading to the observed higher thresholds of superhelix density with sodium chloride concentration.

Results obtained with different chemical probes are in most respects very similar, but interesting differences may be revealed by examining the temperature dependences. While bromoacetaldehyde requires a temperature of 30 °C for measurable reaction, osmium tetraoxide reacts with supercoiled pColIRΔxba even below 10 °C. It seems most probable that these differences reflect quite different requirements for reactivity between the chemicals, resulting from their different modes of adduct formation, as summarized in Figure 8. Bromoacetaldehyde attacks adenine at N1 and N^6 forming an etheno adduct. Except in the case of Hoogsteen pairing, which seems to be excluded in the present case, these positions are both involved in hydrogen bonding to thymine, and hence, this reaction cannot proceed unless the base pair is broken. Thus the reagent is a reliable probe of base opening. Osmium tetraoxide is very different however. It reacts with the 5,6 double bond of thymine, which lies on the opposite side of the pyrimidine ring from the hydrogen bonding to adenine. However, adduct formation will require out-of-plane attack by the electrophile and will be sterically hindered by stacking of neighboring base pairs. If this stacking is disrupted, then the osmium tetraoxide may possibly react, and thus, it is possible that this reagent is really a probe of *stacking*. We have previously successfully explained hyperreactivity of alternating adenine–thymine tracts to osmium tetraoxide in similar terms (McClellan et al., 1986; McClellan & Lilley, 1987).

On this basis we may dissect the opening process as a function of temperature. Above 0 °C the A+T-rich inducing sequences undergo unstacking events, which allow reaction with osmium tetraoxide to proceed, while below this temperature the supercoiled DNA is fairly inert toward all the chemical probes. Around 30 °C the chemical reactivity changes again, the bases becoming reactive toward chemicals which react with opened base pairs. The temperature dependences of the two events are quite different. Over the range 0–20 °C there is a gradual increase in osmium tetraoxide reaction, corresponding to an Arrhenius activation energy (not shown) of 11 kcal mol^{-1}, while the transition to bromoacetaldehyde reactivity occurs over a much narrower temperature interval, suggesting a more cooperative process with an activation energy of around 80 kcal mol^{-1}. In a study of supercoiled pBR322 probed with mung bean nuclease at low ionic strength, Sheflin and Kowalski (1985) observed marked cleavage of an A+T-rich sequence centered at 3240 bp at 37 °C, which was refractory to cleavage at 27 °C. Taken together these results suggest that at the lower temperatures the inducing sequences undergo small-scale breathing motions, probably unstacking single dinucleotide steps, while at higher temperatures cooperative helix opening events of larger amplitude become possible. This is shown schematically in Figure 9. The temperature at which the latter events occur can be reduced by lowering helix stability with DMF. This is closely similar to the effect of this solvent on C-type extrusion (Sullivan & Lilley, 1988), and we think that this higher temperature open state is probably that which is responsible for initiating extrusion by the C-type mechanism and that it will be closely related to the cooperative opening predicted by our statistical thermodynamic calculations (Schaeffer et al., 1988). While the chemical data imply the existence of helical openings, these are likely to be transient however. Large-scale permanent unpairing of bases would be detected by alteration in gel mobility of the supercoiled plasmids, which is not the case, and would result in virtually instantaneous cruciform extrusion.

Thus we believe that cruciform extrusion processes and the different chemical probes are providing a variety of "windows" on the dynamic nature of the A+T-rich C-type inducing sequences under the imposed torsional stress of negative supercoiling. An entire spectrum of opening events is probably present in these sequences. These will range from relatively probable small openings through to much more infrequent large openings. Thus events which only require a small helix opening, such as proton exchange, will be dominated by the higher frequency openings of smaller amplitude, while C-type cruciform extrusion which requires a large bubble to be opened selects the lower frequency larger amplitude events. This description contrasts with that of linear DNA fragments, where proton exchange studied by NMR (Guéron et al., 1987) reveals base opening to be rare, short lived, and noncooperative and

illustrates the role of negative supercoiling in enhancing the dynamic character of DNA.

The dynamic properties of the DNA must therefore be regarded as a function of both local (sequence) and global (supercoiling) influences, which may radically change the nature of the DNA.

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