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Transition of a cloned d(AT)n-d(AT)n tract to a cruciform *in vivo*

David B.Haniford and David E.Pulleyblank

Department of Biochemistry, University of Toronto, Toronto, Ontario M5S-1A8, Canada

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ABSTRACT

A 34 base pair tract of the simple repeating dinucleotide d(AT)n-d(AT)n cloned into a 2.4 kb polylinker plasmid vector undergoes a structural transition in response to negative superhelical coiling. The transition has been characterized by 2 dimensional gel electrophoresis, mapping of S1, P1 and T7 endonuclease 1 sensitive sites, and mapping of sites that are sensitive to modification by bromoacetaldehyde. After S1 nuclease treatment it is possible to trap supercoiled species that are nicked on one or both strands near the center of the palindrome. These data show that the alternate state adopted by the d(AT)n-dAT)n insert is a cruciform rather than a Z conformation.

Unlike other B-cruciform transitions the transition in d(AT)n-d(AT)n has a low activation energy and the transition is facilitated by the presence of magnesium ions. Evidence from in-vivo topoisomer distributions is presented which shows that under conditions of blocked protein synthesis the d(AT)n-d(AT)n insert will spontaneously adopt the cruciform state in-vivo in E. coli.

INTRODUCTION

The mechanisms by which gene expression can be influenced by superhelical torsion are only partially understood (1-3). Superhelical torsion has been shown to cause a variety of DNA sequences to spontaneously undergo conformational transitions. These transitions may serve as sensitive regulatory switches. For example: it has been reported that a Z-DNA tract in a cloned gene blocks transcription by E. coli RNA polymerase (4). Other structural transitions which could serve regulatory functions include transitions from the B form to a cruciform, to a melted helix and to a novel structure associated with anomalous protonation in polypurine-/polypyrimidine sequences (5). Each of these transitions results in a reduction of the interstrand twisting number and the exposure of phosphodiester bonds to cleavage by nucleases that are usually regarded as being specific for single stranded polynucleotides.

In the course of the present work we have analysed the capacity of the major pBR322 and ΦX174 inverted repeats and an insert of the self-complementary alternating purine/pyrimidine sequence d(AT)n-d(AT)n within

supercoiled plasmids to adopt stable cruciform structures in-vitro and in-vivo. Among these, a 34 bp d(AT)n-d(AT)n (n=17) insert in a supercoiled plasmid undergoes a rapid transition to a cruciform state in-vitro. Similar transitions in other palindromic sequences have previously been shown to be kinetically slow (6-8). Since DNA sequences that are co-palindromic with the d(AT)n-d(AT)n tract also enter the cruciform state, the presence of d(AT)n-d(AT)n within a palindromic sequence facilitates the transition in the adjoining sequence.

Evidence has previously been presented (9) showing that a B-Z transition can occur in d(GC)n-d(GC)n tracts in-vivo in E. coli in response to chloramphenicol treatment. There has been no direct evidence for other types of torsionally induced structural transitions in-vivo. In the present work examination of in-vivo topoisomer distributions has provided evidence that a B-cruciform transition can occur in d(AT)n-d(AT)n tracts in-vivo under conditions similar to those previously shown to cause a B-Z transition in d(GC)n-d(GC)n tracts. The pBR322 and ØX174 palindromes do not appear to undergo a transition to a cruciform in-vivo under these conditions. d(AT)n-d(AT)n is the first example of a sequence able to adopt a cruciform structure within an intracellular environment.

RESULTS

Helical unwinding associated with a transition in d(AT)n-d(AT)n.

Topoisomers of plasmid DNA molecules that contain a DNA sequence in which a local, torsionally induced structural transition has occurred exhibit reduced mobility in electrophoretic gels relative to corresponding topoisomers of control plasmids (5-7,9-13). The reductions in mobility result from the release of torsional and bending stresses in the remainder of the plasmid. Quantitative analysis of the mobility shifts permit the degree of unwinding and thermodynamic parameters associated with the transition to be calculated. Figure 1A illustrates a two dimensional electrophoretic gel in which topoisomers of pDPL13 (parent vector) and pAT34 (pDPL13 with a 34 bp d(AT)n-d(AT)n insert) were electrophoresed side by side. The first dimension, run in the absence of intercalative ligands, resolved the topoisomers on the basis of torsion in the backbone. Since structural transitions due to unwinding torsion are reversed in the presence of chloroquin phosphate, a second dimension run in the presence of this ligand resolved the topoisomers on the basis of linking difference. The mobilities of control (pDPL13) topoisomers followed a smooth progression in the first dimension from completely relaxed to highly negatively supercoiled. The mobilities of topoisomers of pAT34 paralleled those of pDPL13 until the topoisomer with a linking difference of -8. Past

this point mobility shifts were apparent relative to the pDPL13 topoisomer ladder. Molecules of pAT34 with a linking difference of -10 had the same mobility in the first dimension of electrophoresis as molecules with the (theoretical) linking difference of -5.5. A torsionally induced transition in the d(AT)n-d(AT)n insert therefore resulted in the release of 4.5 negative superhelical turns. On the assumption that the transition involved only the d(AT)n-d(AT)n insert and that the starting conformation of the d(AT)n-d(AT)n helix is right handed with 10.7 bp per turn of helix (14,15) this degree of unwinding could indicate that the insert was adopting a left handed conformation with 1 left handed helical turn per 26 residues. As shown below, other data indicate that six base pairs on either side of the d(AT)n-d(AT)n insert which form part of the palindromic sequence also participate in the structural transition. Since the transition is spread over a total of 46 bp the degree of unwinding corresponds to a transition from a right handed 10.7 bp/turn helix to an unwound state within the affected sequence. For a B to Z transition confined to the alternating purine-pyrimidine sequence the expected helical unwinding would be (34/10.7 + 34/12)=6.0 right handed helical turns.

Figure 1B shows a two dimensional gel containing samples of pAT34 which were either heated for one hour at 60°C in electrophoresis buffer, or incubated at room temperature in the same buffer. This thermal treatment altered neither the magnitude of the unwinding nor the critical superhelical torsion required to initiate the transition. Since the method used for the preparation of the topoisomer series in these experiments did not expose the DNA to destabilizing conditions (6) the result indicated that a thermal treatment was not required to overcome the activation barrier for the transition of the d(AT)n-d(AT)n sequence. It is apparent from figures 1A and B that the d(AT)n-d(AT)n transition was cooperative and occurred under the conditions of electrophoresis at a superhelix density of -.039.

The addition of magnesium ions to the first dimension gel and electrophoresis buffer facilitated the transition in the d(AT)n-d(AT)n insert of pAT34. In the presence of 3mM magnesium chloride (left hand topoisomer series in Figure 1C) the transition rate in the d(AT)n-d(AT)n insert was increased so that the time averaged electrophoretic mobility of molecules with a linking difference of -8 reflected a time averaged partial transition. Molecules with a linking difference of -9 had completely undergone the transition. In the absence of magnesium ions, molecules with the same linking difference did not undergo the transition. The extent of helical unwinding associated with the transition was not altered by the presence of magnesium ions. In other work (unpublished) we have shown that slow interconversion between two structural states of a polymer insert

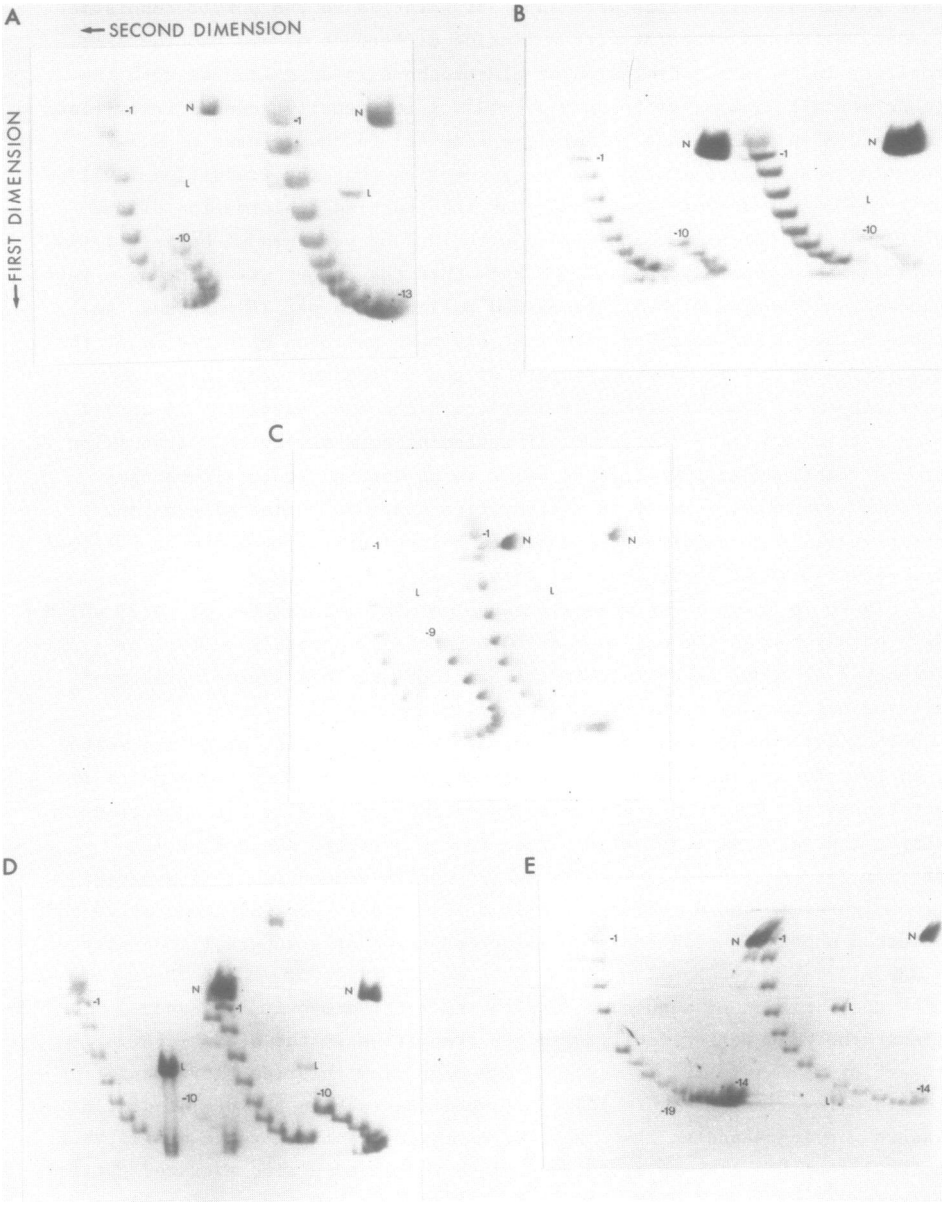


Figure 1 Two dimensional gel electrophoresis of plasmid DNAs. Plasmid DNA samples of differing superhelix densities were prepared by standard techniques (6). The first and second dimensions of electrophoresis are as indicated in (A). For all gels the first dimension electrophoresis buffer contained 40mM Tris,20mM Acetic acid,5mM NaAc,1mM EDTA. The second dimension buffer is the same as the first dimension buffer except for the

addition of 8μg/ml chloroquine phosphate. The time of electrophoresis in both dimensions was 18 hours at 3V/cm. (A) pDPL13 (right) vs pAT34 (left). (B) pAT34 heated (right) vs pAT34 non-heated (left). (C) pDHX4 (pDPL13 containing the insert of the major ϕX174 inverted repeat, right) vs pAT34 (left). In this experiment MgAc was added to a final concentration of 3mM to the first dimension buffer. (D) pAT34 untreated (right) vs pAT34 S1 treated (left-see figure 4 for description of S1 digestion conditions). (E) pDHX4 S1 treated (right) vs pDHX4 untreated (left). N=nicked, L=linear.

results in smearing of topoisomer bands in which the interconversion is occurring under near equilibrium conditions. In the present instance the relatively sharp band formed by topoisomer -8 indicated that interconversion between the two structural states of the polymer insert was sufficiently rapid, on the time scale of electrophoresis, to prevent band smearing. This observation provides further evidence for a low activation energy pathway for the transition of d(AT)n-d(AT)n to its unwound conformation.

Nuclease cleavage of inverted repeat sequences

Although the degree of unwinding associated with the torsionally induced structural transition in pAT34 was consistent with a B-cruciform transition there are other possible underwound structures that the insert could adopt. The possibility that B-Z junctions might preferentially form in d(AT)n-d(AT)n was considered because of the relatively large energy input required for the formation of B-Z junctions in random sequence DNA (16-20). In this case only part of the insert might form a left handed helix. A third possibility: a model in which the strands of the d(AT)n-d(AT)n insert simply unpair, was ruled out by thermodynamic considerations. The free energy of the transition calculated from the mobility shift of the topoisomers shown in figure 1 is 18 kcal/mole (6,9,16). This is sufficient energy to break only 2-3 base pairs (21).

A transition of a palindromic sequence to a cruciform exposes residues near the center to attack by "single strand specific" (SS) endonucleases. The right hand topoisomer series in Figure 1C shows a topoisomer ladder of a pDPL13 clone containing an insert of the Tha 1 fragment which includes the major inverted repeat from ϕX174 (pDHX4). As shown in the right hand topoisomer series of figure 1E a transition in this relatively short palindromic sequence was detected by selective S1 nuclease nicking of pDHX4 topoisomers containing exposed residues (22). The sensitive phosphodiester bonds mapped to the center of this palindrome as discussed below. Two dimensional gel electrophoresis (right hand topoisomer series figure 1C and left hand topoisomer series figure 1E) showed that the structural transition in pDHX4 occurs at sufficiently high linking difference that the

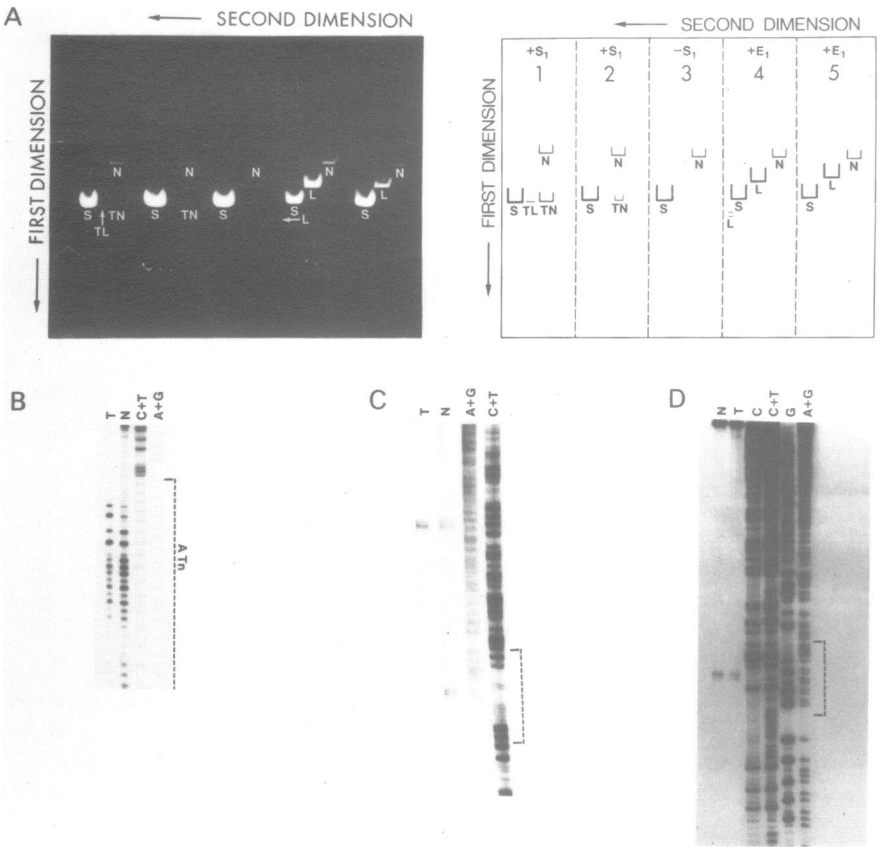


Figure 2 Trapping experiments.
(A) pAT34 was treated with either S1 nuclease or T7 gene 3 product endonuclease 1. S1 digestion conditions are similar to those described in figure 4, and E1 digestion conditions are described in figure 7. After the digestions were terminated the samples were loaded onto a 1.5% agarose gel (same buffer as for first dimension electrophoresis in figure 1) and run at 4V/cm for 2 hours. The gel was then soaked for 4 hours in the first dimension buffer containing 1µg/ml chloroquine. The gel was then turned 90 degrees and electrophoresed for 2 hours at 4V/cm in a second dimension. The illustration accompanying the photograph indicates the lanes for each sample. (1) S1 digest-15% nicked, (2) S1 digest-5% nicked, (3) untreated control, (4) E1 digest-50% nicked, (5) E1 digest-40% nicked. N=nicked, L=linear, S=supercoiled, TN=trapped-nicked, TL=trapped-linear. Nicked and trapped-nicked molecules were eluted from agarose gels, digested with an appropriate restriction enzyme and end-labeled. After removal of the second end-label samples were electrophoresed on DNA sequencing gels with the appropriate standards. For each autoradiogram the lanes for trapped (T) and untrapped (or nicked) molecules (N) are indicated. (B) pAT34, (C) pDHX4, contains the ϕX174 major inverted repeat, (D) pDPL13, containing the major pBR322 inverted repeat. Approximately equal numbers of counts were loaded in N and T channels for the respective sequencing gels.

associated mobility shift in the first dimension is too small to be detected.

The analogous experiment with pAT34 (figure 1D) showed that S1 nuclease selectively cleaved topoisomers of this plasmid which have undergone the torsionally induced transition. Since the strand cleavage by S1 nuclease occurs at the same critical superhelix density required to initiate the transition under electrophoresis conditions, the critical features of this transition were not altered by the low pH conditions of the S1 nuclease reaction. Similar results have recently been reported for another palindromic sequence (23).

The experiment shown in figure 1D reveals another characteristic of the transition in pAT34 which is consistent with the transition being to a cruciform rather than to some other structure. Molecules nicked on one or both strands migrated as though supercoiled in the first dimension. Reversal of the transition by chloroquine (second dimension) revealed the nick(s) in these molecules. This phenomenon is discussed below.

Trapping Experiments.

The high resolution experiment shown in figure 1D presents a complex picture that is most easily interpreted through reference to the experiment shown in figure 2A. Supercoiled pAT34 was either treated or not treated with S1 nuclease (pH 5). After the reaction was terminated samples were directly loaded onto a 1.5% agarose gel (no intercalator present) where nicked, linear, and supercoiled molecules were resolved. A second dimension run in the presence of chloroquine at a concentration of 1μg/ml resolved three species at the position where supercoiled molecules migrate in the first dimension. The intense band (S) was composed of supercoiled molecules and had the highest mobility in the second dimension. The less intense band (TN) had a very limited mobility in the second dimension and was only observed as a result of S1 treatment. An even fainter species (TL-see illustration) was detected between S and TN. Elution of these three bands and subsequent electrophoresis with standards showed that TN was a population of nicked molecules and that TL was a population of linear molecules, each of which migrated with supercoiled molecules (S) in the first dimension.

The experiments shown in figures 2A and 1D demonstrated that even in the absence of magnesium it is possible to trap torsionally constrained circular pAT34 DNA molecules which have been nicked on one or both strands. The nicked sites in the trapped molecules were mapped to the center of the d(AT)n-d(AT)n insert. Unwinding of the plasmid by chloroquine phosphate reversed this trapping, converting the supercoiled singly-nicked molecules to unsupercoiled nicked molecules and the supercoiled doubly-nicked molecules to linear. Torsional strain in the plasmid therefore stabilized

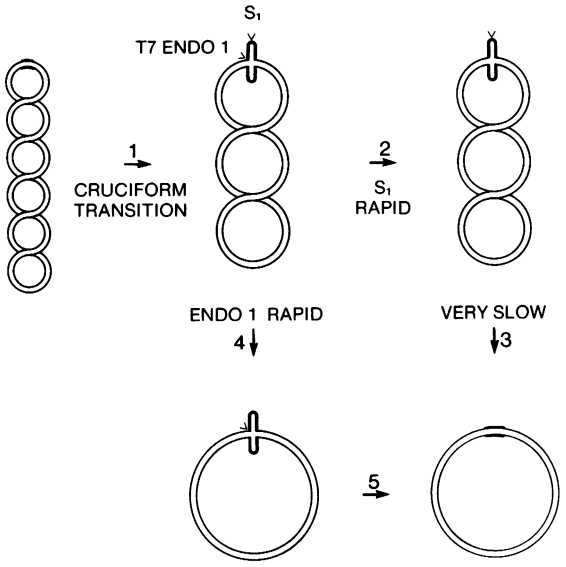


Figure 3 Trapping of nicked cruciforms in a supercoiled state.
(1) An inverted repeat sequence (dark lines) undergoes a torsionally induced transition to a cruciform.
(2) Limited S1 treatment introduces a nick (v) into the central region of the cruciform.
(3) The conversion of the S1-treated molecule to a relaxed circular or linear state is very slow providing the nick(s) is/are within the torsionally isolated part of the cruciform loop.
(4) Limited treatment of the supercoiled substrate with T7 endo1 rapidly causes the release of torsion in the backbone of the molecule since the cleavage site is located outside the cruciform arms.
(5) The rate of resolution of the cruciform back into a duplex state (in the absence of torsion) is rapid at 23°C in the absence of Mg^{++}.

the nicked secondary structure. Detailed examination of figure 1D also shows that trapping requires additional superhelical torsion beyond that required to initiate the structural transition. Figure 3 illustrates diagramatically the torsional separation between the termini of a cruciform and the remainder of the plasmid that results in this type of trapping. A change in helix handedness in the insert of pAT34 would not enable this type of trapping, while the formation of a loop structure would.

Although nicking of a supercoiled plasmid at the termini of a cruciform loop would not immediately result in release of torsional constraint, nicking of the plasmid outside the palindrome would be expected to result in an immediate release of torsion as illustrated in figure 3. Cleavage of pAT34 by T7 endonuclease 1 adjacent to the insert region (see below) did not

enable the type of torsion dependent trapping seen after S1 nuclease digestion (Figure 2A).

Analysis of nuclease cleavage sites.

The sites of S1 nuclease cleavage in supercoiled samples of pAT34, pDHX4 and pDPL13 were analysed by treating samples having native superhelix density with S1 nuclease such that 10-40% of the molecules were nicked as determined by a fluorimetric assay (24). Nicked molecules were purified electrophoretically to ensure that the nuclease cleavage sites observed did not result from second strand cleavage opposite an initial nick. The predominantly singly nicked molecules were opened with an appropriate restriction endonuclease and end-labeled to permit fine resolution mapping of nuclease cleavage sites within the palindromic region. Figure 4 presents the cleavage data for each of the S1 sensitive regions. The patterns of S1 nuclease cleavage for the pBR322 and ϕX174 major inverted repeat sequences were essentially confined to the center of the sequence where base pairing is interrupted. In contrast the cleavage patterns within the d(AT)n-d(AT)n insert were anomalously broad and the most prominent cleavage sites were displaced by 4 residues on opposite strands with respect to the center of the insert. As shown in figure 5 the nuclease cleavage pattern observed within the d(AT)n-d(AT)n insert was not dependent on pH or S1 nuclease, since P1 nuclease used at pH 7 yielded a similar cleavage pattern. These enzymes depend upon similar conformational characteristics of phosphodiester bonds for their cleavage specificity (5). It has been reported that S1 nuclease sensitivity in a double stranded DNA is not a simple consequence of unpairing of the bases and is more likely to depend upon a specific conformation of the phosphodiester bond that is accessible to single stranded nucleic acids but which occurrs rarely in double stranded DNA (25,5). In the case of the d(AT)n-d(AT)n insert of pAT34 the residues that were detected as being unpaired by chemical modification (see below) were not those that were most sensitive to S1 nuclease.

Nuclease cleavage sites have been mapped in both trapped and untrapped molecules eluted from two dimensional preparative gels. In the case of pAT34, molecules containing a nick within the d(AT)n-d(AT)n insert could be trapped. As shown in figure 2B approximately equivalent cleavage intensities were observed within the insert of trapped and untrapped molecules. Although it has been possible to trap supercoiled molecules containing a nick at the center of the 25 base pair inverted repeat of pBR322 we have not been able to trap plasmids nicked in the 28bp ϕX174 inverted repeat. Since the sizes of the latter inverted repeat sequences are similar, our inability to trap the ϕX174 cruciform is probably a result of its relatively high AT content. Although the AT content of the pAT34 palindrome is equally high, the greater length of this palindrome (46

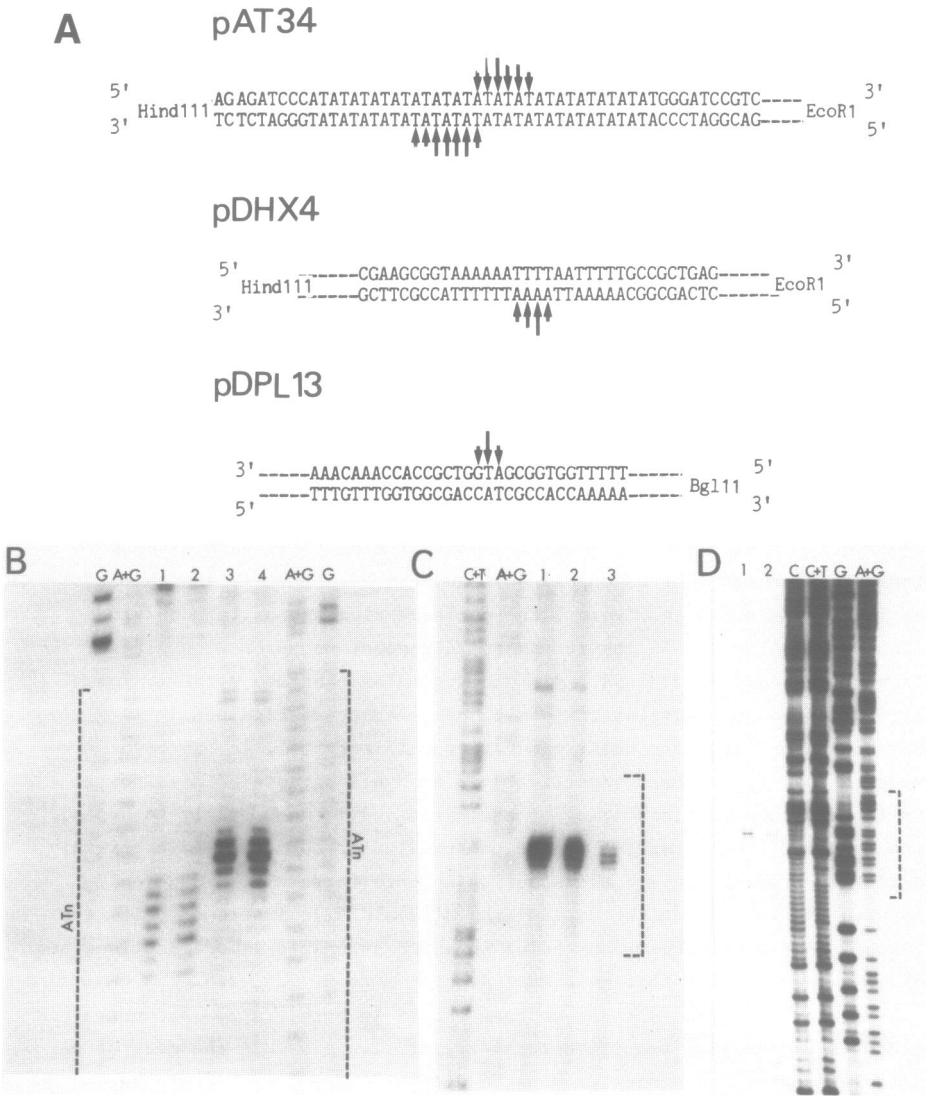


Figure 4 Mapping S1 Cleavage Sites.
(A) Sequence maps of the pAT34 d(AT)n-d(AT)n inverted repeat (note that 6 bp on either side of the polymer insert retain inverted repeat motif), the ϕX174 major inverted repeat (present in pDHX4), and the pBR322 major inverted repeat (present in pDPL13). Arrows depict relative cleavage intensities. In each experiment 10μg aliquots of supercoiled plasmid DNA were digested with .5U of S1 nuclease (BRL) in .05M NaAc,.2mM $ZnCl_2$ pH5. The reactions were terminated by the addition of EDTA (to 1mM) and SDS (to 1%) final concentrations. The percentage of nicked molecules was calculated by an alkali fluorescence assay (24). Samples were electrophoresed on low melting

agarose gels and the nicked material was recovered. pAT34 and pDHX4 were digested with either HindIII or EcoRI and end-labeled. In pDPL13 a BglII site is located approximately 150 bp away from the major pBR322 inverted repeat sequence and this site was used for end-labelling this plasmid. After the second end-label was removed samples were applied to 8% denaturing acrylamide gels with DNA sequencing standards. (B) S1 digested pAT34, (1) HindIII-labeled, S1 digested-20% nicked (2) HindIII-labeled, S1 digested-35% nicked (3) EcoRI-labeled, S1 digested-35% nicked (4) EcoRI-labeled, S1 digested-40% nicked. (C) S1 digested pDHX4 (all samples were HindIII-labeled), (1) 35%-nicked (2) 30%-nicked (3) 15%-nicked. (D) S1 digested pDPL13, (1) 30%-nicked (2) 15%-nicked. Inverted repeat regions in each plasmid are outlined on the autoradiograms. Note that fragments generated by S1 cleavage are one residue longer than the corresponding fragments produced by chemical cleavage.

residues) may confer sufficient additional kinetic stability to permit the nicked intermediates to be trapped.

Exposure of dA residues in pAT34 to modification by bromoacetaldehyde

Bromoacetaldehyde is a reagent that selectively modifies adenine residues in which the N1 atom is exposed and dC residues in which N3 is exposed (26). Bromoacetaldehyde modified dA residues in the d(AT)n-d(AT)n insert were mapped through the enhanced sensitivity of the positively charged ϵ-A residues to acid induced depurination. As shown in figure 6 only the central dA residues within the d(AT)n-d(AT)n insert were modified by this reagent. This result shows that despite the relative insensitivity of these particular residues to S1 nuclease the bases of these residues were the only ones exposed to the medium. This result is not compatible with a model for the structural transition in the d(AT)n-d(AT)n insert of pAT34 in which the insert is either a completely unpaired structure or is in a partial Z-conformation. Since reaction of this tract with bromoacetaldehyde was independent of pH in the range from 5-7 the structural transition in the d(AT)n-d(AT)n tract is not facilitated by anomalous protonation of this sequence.

In other unpublished work we have shown that cytosine residues modified by bromoacetaldehyde are directly sensitive to cleavage by piperidine. The lack of enhanced chemical cleavage at either dA residues or dC residues at the point at which the arms of the pAT34 cruciform joins the helix demonstrated that there is a discrete structure at this point in which the bases are protected against modification by single strand specific chemical agents.

Mapping resolvase cleavage sites.

The point at which the arms of a cruciform join the main helix is topographically equivalent to a Holliday intermediate (27). In all cruciforms that have been investigated these junctions were insensitive to

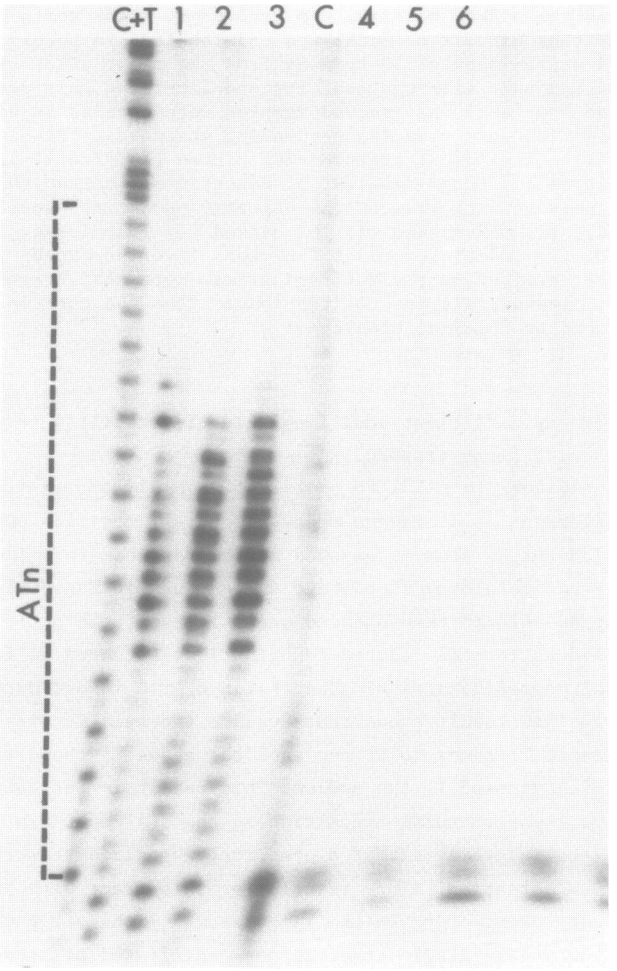


Figure 5 P1 Treatment of pAT34 and pDPL13.
10ug aliquots of pAT34 and pDPL13 were treated with either S1 nuclease or P1 nuclease. S1 digestion conditions are as indicated in Figure 2. P1 digests were carried out in S1 buffer at either pH5 or pH7. For the pH5 digests .001μg of P1 nuclease (P-L Biochemicals) per 10ug of supercoiled DNA was used. For the pH7 digests .006μg of P1 nuclease per 10ug of supercoiled DNA was used. (1) S1 digested pAT34, (2) P1 digested pAT34, pH5, (3) P1 digested pAT34, pH7, (4) S1 digested pDPL13, (5) P1 digested pDPL13, pH5, (6) P1 digested pDPL13, pH7.

S1 nuclease. Bacteriophage enzymes (resolvases) capable of resolving Holliday intermediates have recently been characterized (28,29). Lilley et al. have mapped the cleavage sites of the T4 resolvase for a number of inverted repeat sequences in supercoiled plasmids (23,30). The finding that

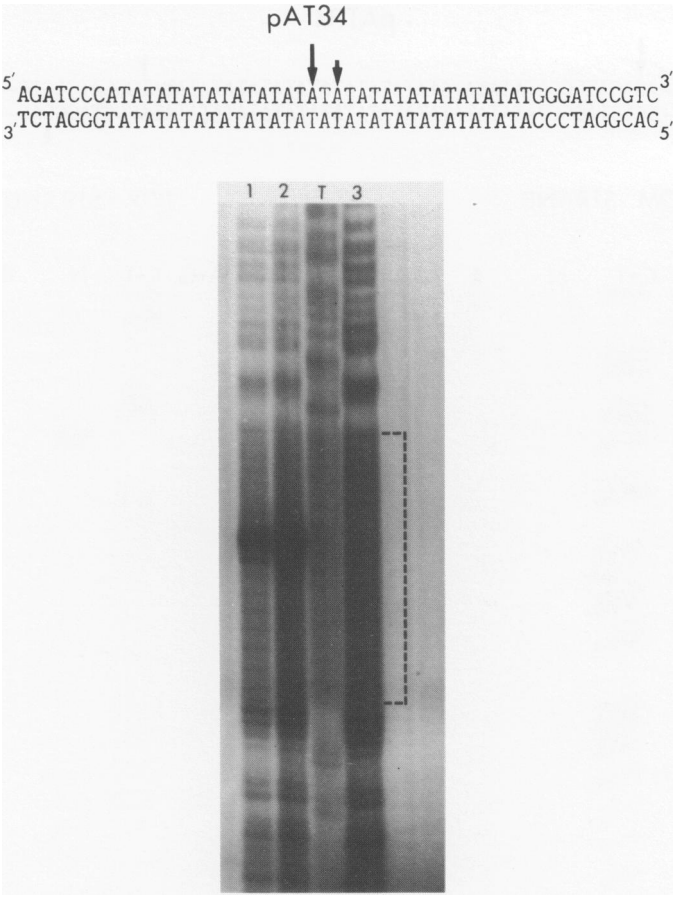
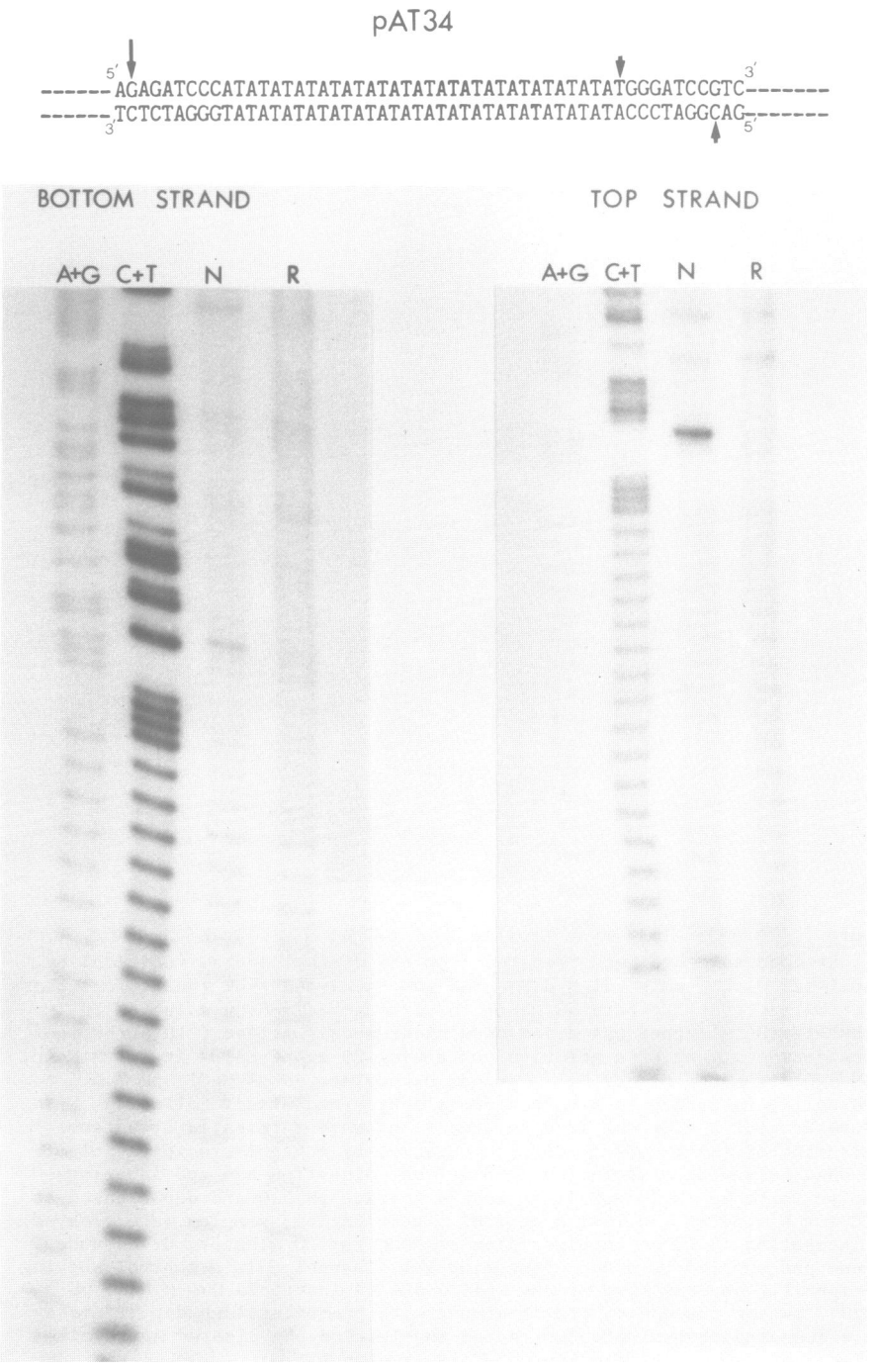


Figure 6 Bromoacetaldehyde treatment of pAT34.

Bromoacetaldehyde was prepared from the diethyl acetal (Aldrich) by hydrolysis with 2 vols 0.1 M H_2SO_4 and was extracted from the aqueous phase by methyl-tertbutyl ether. 50 µL of the Bromoacetaldehyde solution in methyl-tertbutyl ether was dried down under an air stream (~10 µL bromoacetaldehyde). 1 mL of a solution containing 20 µg of pAT34 in 0.1 M NaAc, 5 mM MgCl2 (channel 1) or 0.1 M sodium cacodylate, 5 mM $MgCl_2$ pH 7.0 (channel 2) was added to the bromoacetaldehyde and mixed. Following incubation for 2 hours at 23°C the reactions were terminated by butanol extraction of the bromoacetaldehyde followed by ethanol precipitation of the DNA. Following restriction endonuclease digestion and end labeling samples taken up in 275 µL 0.1 M sodium acetate pH 5.0 and depurinated at 90°C for 4 minutes. Following ethanol precipitation, samples were cleaved by incubation in 20 µL 1M piperidine at 90°C for 30 minutes, dried under vacuum and applied to a DNA sequencing gel. Channel 3 is a control showing non-specific depurination of the d(AT)n-d(AT)n insert in 0.1 M NaAc pH 5.0, at 90°C in the absence of pre-treatment with bromoacetaldehyde, "T" is a DNA sequencing channel of non-treated end-labeled DNA cleaved at thymine residues by osmium tetroxide/piperidine treatment.


pAT34
5′
3′
AGAGATCCCATATATATATATATATATATATATATATATATATGGGATCCGTC
TCTCTAGGGTATATATATATATATATATATATATATATATATACCCTAGGCAG
3′
5′
BOTTOM STRAND
TOP STRAND
A+G C+T N R
A+G C+T N R

the cleavage sites were located in the stem-base region of a cruciform provided an elegant confirmation of the structural specificity of this enzyme. The T7 gene 3 product, endonuclease 1, also has the properties of a resolvase (29). We have used the latter enzyme (kind gift of Dr. P. D. Sadowski) to probe the transition structure of the d(AT)n-d(AT)n sequence. Figure 7 shows the results of mapping both strands of pAT34. Two cleavage sites were evident on the top strand. The cleavage site at the 5′ end was located 2 bp from the end of the inverted repeat sequence. At the 3′ end a weaker cleavage site was identified within the dAT portion of the inverted repeat. Due to a lack of suitable restriction sites for mapping only one cleavage site was identified on the opposite strand. Since these cleavage sites are not recognized in the relaxed plasmid, the cleavage specificity depends upon the structural transition. These observations support the conclusion that the altered structure of d(AT)n-d(AT)n is a type of cruciform.

In vivo transition in pAT34.

The assay to detect a structural transition involving helical unwinding in vivo, relies upon the tight regulation of native DNA superhelix density found in bacteria (3,31,32). When a portion of a molecule undergoes an unwinding transition, the net number of negative supercoils in the molecule is reduced without a corresponding change in the linking number. Because the state of supercoiling is important in a number of cellular processes the regulatory system designed to buffer such changes reduces the linking number of such molecules so that the level of supercoiling preceding the transition is reestablished (9). This regulatory system requires the activities of DNA gyrase and topoisomerase 1 (33,34). The B-Z transition in d(GC)n-d(GC)n and the cruciform transition in d(AT)n-d(AT)n are highly cooperative and go to completion once initiated in any particular plasmid molecule. In an intracellular plasmid population in which some of the molecules has undergone a transition, the resulting family of topoisomers is bimodal.

Figure 7 T7 endonuclease 1 digests of supercoiled and relaxed pAT34.
50ug of pAT34 was completely relaxed by treatment with topoisomerase 1 isolated from chicken erythrocytes. Native and relaxed pAT34 aliquots were then digested with T7 endo 1 in .05M Tris-HCl, 3mM MgCl, 5mM β-mercaptoethanol (pH 8). 20μg of DNA was incubated with .5 units of T7 endo 1 for 10 min. at room temperature. (1 unit of T7 endo 1 converts 10 μg of form I DNA to form II in 10 min at 23°C). Digested samples of relaxed (R) and native (N) pAT34 were divided into aliquots such that endo 1 cleavage sites on both strands could be mapped. Top strand mapping was performed by labeling at the EcoR1 site. Bottom strand mapping was carried out by labeling at the Hindlll site. Fragments produced by endo 1 digestion are one residue longer than the corresponding fragments produced by chemical cleavage.

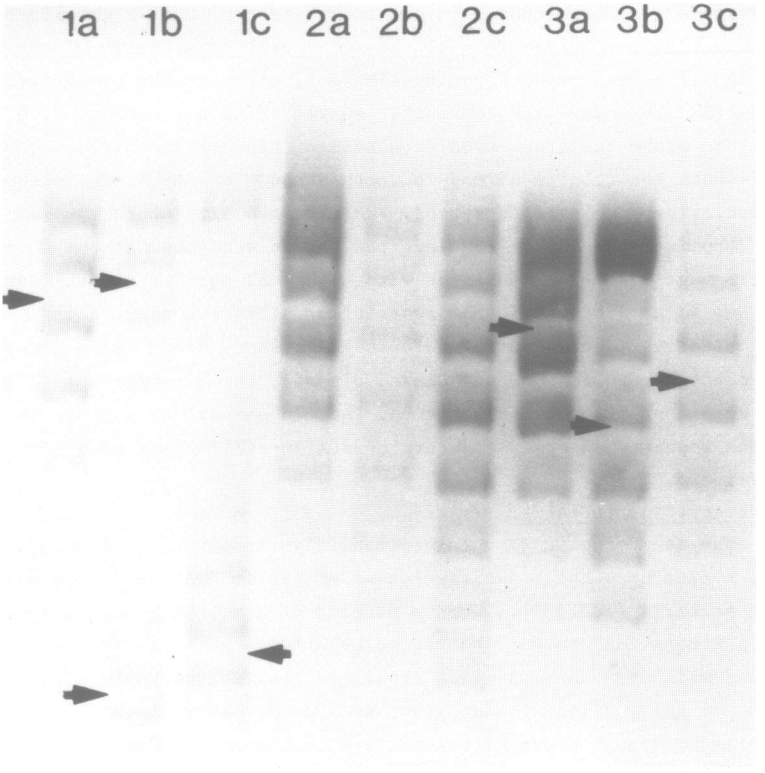


Figure 8 In vivo transition in pAT34.
Cultures of E.coli (HB101) harboring pAT34, pDPL13, or pAT34 plus pDPL13 were grown under two sets of conditions. In one case the cells were grown in M9 medium (+ .4% glucose + .5% casamino acids) at 37 degrees C and harvested in mid-log phase (lane a). In the second case the remaining cells in the cultures above were treated with chloramphenicol (100µg/ml) and harvested either one (lane b) or 18 hours (lane c) after the addition of the drug. Plasmid DNA was purified (33) and approximately one µg of DNA from each culture was loaded onto a 1.5% agarose gel containing 10µg/ml chloroquine phosphate. Electrophoresis was carried out for 18 hours at 3V/cm. (1) pAT34, (2) pAT34 + pDPL13, (3) pDPL13. Densitometric traces were made in order to determine the Gaussian center(s) of each native topoisomer distribution (arrows).

The plasmid samples in each lane A of figure 8 were prepared by homogeneous lysis (35) from cultures harvested while in logarithmic growth phase (not chloramphenicol treated). In experiments of this type, the choice of a plasmid preparation method that does not depend upon the preliminary preparation of lysosyme spheroplasts is critical, since it has previously been shown the superhelix density of plasmids changes in response to altered intracellular conditions (36). The agarose gel system used to resolve the native topoisomers contained 10μg/ml chloroquine phosphate. In this gel system molecules with lower linking numbers have the highest mobilities. The plasmid samples in channels B and C were from cultures harvested one or 18 hours respectively after the addition of chloramphenicol (100μg/ml). As shown in figure 8 (channels 1B and C) the bimodal distribution of topoisomers expected for an *in vivo* transition involving approximately half of the molecules was observed when pAT34 was isolated from cells in which protein synthesis was inhibited. A comparable bimodal distribution has previously been observed in plasmids containing d(GC)n-d(GC)n tracts (9). The molecules which comprise the less supercoiled part of the distribution possessed the degree of supercoiling characteristic of the control plasmid (pDPL13) which does not contain the insert capable of undergoing the transition. The second part of the pAT34 distribution is shifted to a higher linking difference. The bimodal distribution observed for pAT34 was not observed in the control plasmid, but was observed in mixed *E. coli* cultures harboring both pAT34 and the control plasmid.

Measuring the linking difference between the Gaussian centers of the bimodal pAT34 distribution of topoisomers allows a quantitation of the *in vivo* transition. One hour after the growth conditions were altered to allow the transition to occur the linking difference between the Gaussian centers corresponds to 7.5 superhelical turns. After 18 hours, when equilibrium was attained, the linking difference decreased to approximately 5 superhelical turns. This amount of helical unwinding corresponds closely to that observed in the *in vitro* pAT34 transition.

DISCUSSION

Structure of d(AT)n.d(AT)n

A number of structural models have been proposed for the different forms of the simple repeating DNA polymer d(AT)n-d(AT)n. Early fibre diffraction studies of ammonium or sodium salts of this polymer revealed that this it is capable of undergoing a transition to a novel (D) eight-fold helix that is probably right-handed in conditions of low relative humidity (37). The high resolution NMR spectra of this polymer in solution

have been interpreted in terms of an equilibrium blend of right and left handed helices where the base pairing is Watson-Crick. The "left-handed" NMR spectrum which predominates in concentrated salt solutions is distinct from that of the left handed Z form of d(GC)n-d(GC)n (38). The present study indicates that at low ionic strength d(AT)n.d(AT)n is right handed and responds to unwinding torsion by entering a cruciform rather than a left handed structure. The in-vivo occurrence of the cruciform indicates that it may be a physiologically important conformation of this sequence. Since the adjacent polylinker sequence of pAT34 also becomes part of the cruciform, the presence of d(AT)n-d(AT)n within other palindromes may facilitate their transition to cruciform states. The present work does not address the question of why d(AT)n.d(AT)n can undergo a transition to a cruciform with such facility. Since the sequence has the minimum alternating purine/pyrimidine requirement for the formation of a Z-DNA helix it is possible that transient appearance of a Z conformation within the insert might serve to promote the transition. Another possibility is that the transition in this simple self complementary sequence might be initiated by a strand slip process.

Inhibition of protein synthesis causes increased superhelical coiling in-vivo

Uncoupling of protein synthesis from DNA replication has previously been shown to induce a B-Z transition in cloned d(GC)n-d(GC)n tracts in-vivo (9). It appears that only after the addition of chloramphenicol is the superhelical torsion in either plasmid sufficient to drive the transition. Under these conditions the superhelix densities of control plasmids show only a minor increase which is too small to explain the all or none effect of chloramphenicol addition. It is possible that under normal conditions of growth there is an active system inhibiting the appearance of altered conformations of the DNA helix which is turned off in response to inhibition of protein synthesis. The simplest version of this interpretation: - that the uncoupled plasmid synthesis following inhibition of protein synthesis dilutes out a protein component that inhibits these transitions - was ruled out by time course experiments such as that shown in figure 8. Shifts in the Gaussian center of the pAT34 distribution (and pGC23), indicative of the structural transitions occurred as soon as 20 minutes after the addition of chloramphenicol. There is only a minor increase in plasmid copy number during this time.

The results of experiments designed to estimate the extent of unconstrained topological stress in eukaryotic nuclei have not been decisive (39-41). It has been suggested that eukaryotic cells contain a type II topoisomerase which is capable of introducing negative supercoils in DNA (42-44). If torsion introduced by this activity is not constrained

within a protein complex it could be sufficient to drive a variety of structural transitions. Recent studies have demonstrated that S1 nuclease sensitive sites identified in naked plasmid DNAs correspond to sites recognized in the same sequences incorporated in nuclear chromatin, an observation which can be interpreted as suggesting that such structural transitions occur in chromatin (45). three inverted repeat sequences in pBR322, including that discussed here, mapped as S1 sensitive sequences in chromatin when the plasmid is inserted into eukaryotic genomes. S1 nuclease is probably not the best single strand-specific nuclease for this type of analysis because acid induced alterations of the chromatin structure could facilitate cruciform extrusion in the sequences identified as S1 sensitive. As shown in the present study, P1 nuclease recognizes the same structure as S1 nuclease and may represent a better choice of probe for studies of chromatin structure since it can be used at neutral pH.

Conformation of SS nuclease sensitive bonds.

In the regions of the d(AT)n.d(AT)n insert of pAT34 that were most sensitive to S1 nuclease the dA residues were not exposed to modification by bromoacetaldehyde. This result argues that the S1 sensitive region of the palindrome was not single stranded. The conformation of the phosphodiester backbone in the sensitive region probably alternates since the strongest sites of cleavage of pAT34 by S1 nuclease lie to the 5′ side of dA residues. Asymmetric cleavage that alternates between adjacent residues has also been observed in B-Z junctions (46) and in a protonated form of d(TC)n-d(GA)n (5). Consideration of the model proposed for the protonated form of d(TC)n-d(GA)n (5) has suggested the possibility that S1 and other SS endonucleases selectively cleave phosphodiester bonds in which the conformation of the C4′-C5′ bond is (gauche,trans) and where the 3′sugar residue is not inverted as in the Z conformation. Although a model has been proposed for d(AT)n.d(AT)n in which the conformation of the C4′-C5′ bonds alternate between (gauche,gauche) and (gauche,trans) (47) crystallographic data indicate that this conformation does not occur in torsionally unconstrained d(AT)n-d(AT)n fibres (48).

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