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Nuclease recognition of an alternating structure in a $d(AT)_{14}$ plasmid insert

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ABSTRACT

The nuclease reactivity and specificity of a cloned tract of poly·(dA-dT)·poly(dA-dT) has been explored. Digestion with DNAse I, Mung Bean nuclease, S1 nuclease, DNAse II, and copper $(1,10\text{-phenanthroline})_2$ on a 256 base pair restriction fragment containing $d(AT)_{14}A$ revealed a dinucleotide repeat structure for the alternating sequence. Furthermore, conditions which wind or unwind the linear DNA had little effect on the reactivity of the AT insert. These preferred cleavages offer insights to structural alterations within the DNA helix which differ from A, B, or Z-DNA. Nucleation into flanking sequences by this structural alteration was not observed.

INTRODUCTION

Evidence has been accumulating for some time that the solution structure of the alternating DNA polymer poly(dA-dT)·poly(dA-dT) differs from that of a classic B-DNA helix. NMR studies (1-3), melting studies (4) and the results of nuclease digestions (5) all point to a dinucleotide repeat structure for this polymer. The conformational details of this helix (which has been designated an "alternating B" helix) are not yet known. It is apparently a right-handed helix with a helical repeat of approximately 10.6 base pairs (6) and with significantly different phosphodiester torsion angles at the d(ApT) and d(TpA) base pair steps. In the presence of CsF it can change over to still another non-B DNA conformation which has been named X-DNA (7-8). A quantitative description of the torsion angles which characterize alternating B DNA has not yet been generally accepted, although several models have been advanced (9-10).

The biological role, if any, of alternating B-DNA is also unknown. Tracts of $d(AT)_n$ ranging from $d(AT)_8$-$d(AT)_{14}$ (11-14) have shown up in several genes (in particular in mitochondrial and chloroplast genes) and recently interspersed repeats of $d(AT)_{34}$ and $d(AT)_{23}$ have been detected in Xenopus (15). AT-rich DNA sequences have been shown to bind several

different kinds of proteins, including histone H1 (16), lac repressor (17) and proteins of unknown function from Drosophila (18) and Dictyostelium (19). Since $d(AT)_n$ can form nucleosomes in the presence of histones, and given the dAT preference of histone H1, it has been suggested that d(AT) sequences could be involved in nucleosome phasing (12). The recent discovery that d(AT) sequences can form cruciforms under conditions of moderate negative supercoil density with a very low activation barrier raises the possibility that alternating d(AT) sequences could be sites of duplex to cruciform transitions in vivo (20,21).

Since most of the studies which have detected a dinucleotide repeat structure for $d(AT)_n$ sequences have been carried out on the $d(AT)_n$ polymer itself, we decided to investigate the effect of neighboring B-DNA sequences on nuclease recognition of this conformation. Analogous studies on the B-Z junction (Z-DNA being not only a left-handed helix, but a dinucleotide repeat helix as well) have shown that Z-DNA can, under some conditions, nucleate into adjacent non-alternating purine-pyrimidine sequences (22) and that the B-Z junction can be recognized by some nucleases (23). By preparing a restriction fragment with a d(AT) insert (specifically a 256 base pair HindIII-HaeIII restriction fragment with a $d(AT)_{14}A$ sequence cloned into the SmaI site of pUC8) we have found nucleases which recognize different aspects of alternating B DNA.

MATERIALS AND METHODS

Plasmids. The plasmid pRWAT14.1 is a derivative of pUC8 (24) containing a $d(AT)_{14}A$ insert at the SmaI site. This was prepared by sonicating poly(dA-dT) to give fragments 200 base pairs and below then cloning following the procedure of Deininger (25). The plasmids were transformed into the E. Coli strain K12F$^-$ZΔm15 recA$^-$ and screened for inserts in the usual way (26). Of 20 white colonies 15 had lost the SmaI site. pRWAT14.1 was isolated by the large scale boiling method (27) using CsCl purification.

Sequencing. Plasmid pRWAT14.1 was digested with HindIII and end-labelled with dATP(γ-^{32}P) (New England Nuclear) and T4 polynucleotide kinase (Pharmacia). The DNA was then cut with HaeIII to produce a 256 base pair fragment with the d(AT) insert which was isolated from a 5% - polyacrylamide gel. The fragment was sequenced using standard Maxam-Gilbert conditions (28). For some reason there was a small amount of a

broken fragment which appeared after isolation. It corresponded to breakage at a site 150 base pairs from the 5'-end.

Nucleases. Digestions were carried out at 37°C in 10µl volumes unless otherwise stated. Typically 20,000-30,000 cpm (Cerenkov) of labelled DNA were digested in the following mixture: 2µl of labelled DNA, 1µl 10x buffer, 2µl calf-thymus DNA (1µg), 1µl enzyme solution and 4µl water. Reactions proceeded for 10 min upon addition of enzyme and were stopped by addition of 10µl of sequencing loading buffer. Samples were stored at -78°C and were heated at 90°C for 2 min followed by chilling to 0°C and loading onto a sequencing gel.

DNAse I experiments included 0.4 units/ml enzyme (Pharmacia), 100mM Tris-Cl, 10mM $MgCl_2$, 1mM DTT, pH 7.6. DNAse II experiments included 0.4 units/ml enzyme (Sigma), 50mM NH_4OAc, 1mM EDTA, pH 5.4. Mung Bean nuclease digestion included 8 units/ml enzyme (Pharmacia), 30mM NaOAc, 1mM $ZnCl_2$, pH 5.4. Several experiments were run using this enzyme in the presence of salt or formamide. For the NaCl study, reaction mixtures were supplemented with 50, 100, 150 and 200 mM NaCl. For the formamide study, the reactions were supplemented to make the final formamide concentrations 10, 20, 30 and 40% (v/v). Reactions were also run at different temperatures: 4° (18 hr), 23° (20 min), 50° (5 min), 60° (6 min) and 70° (6 min), with the standard buffer. S_1 nuclease conditions used 20 units/ml (Pharmacia), 30mM NaOAc, 1mM $ZnCl_2$, pH 5.4. Copper-phenanthroline reactions contained 20µM $CuSO_4$, 200µM 1,10-phenanthroline (Aldrich), 5mM mercaptopropionic acid (Sigma) and 50mM Tris-Cl, pH 7.6. The copper reactions were mixed with an equal volume of 5M NH_4OAc and twice ethanol precipated to remove the reagent and finally redissolved in loading buffer.

The amounts of enzyme to be used were determined by excising the full-length ^{32}P-labelled fragment from the gel and Cerenkov counting it relative to an undigested control. Conditions were chosen so that 70±5% of full length DNA remained after digestion. This leads to approximately 80% single-hit kinetics assuming a Poisson distribution.

Densitometry. A Hoeffer Scientific GS300 densitiometer equipped with a Hewlett-Packard 3390A integrator was used to quantify the intensities of gel bands. Relative cutting rates were assigned following Lutter (29). Bands were assigned using the standard correction factors for nuclease mapping (30). This means, for example, that the first DNAse I cut in the d(AT) region generates a $5'p(N)_{28}OH3'$ fragment which migrates on a gel

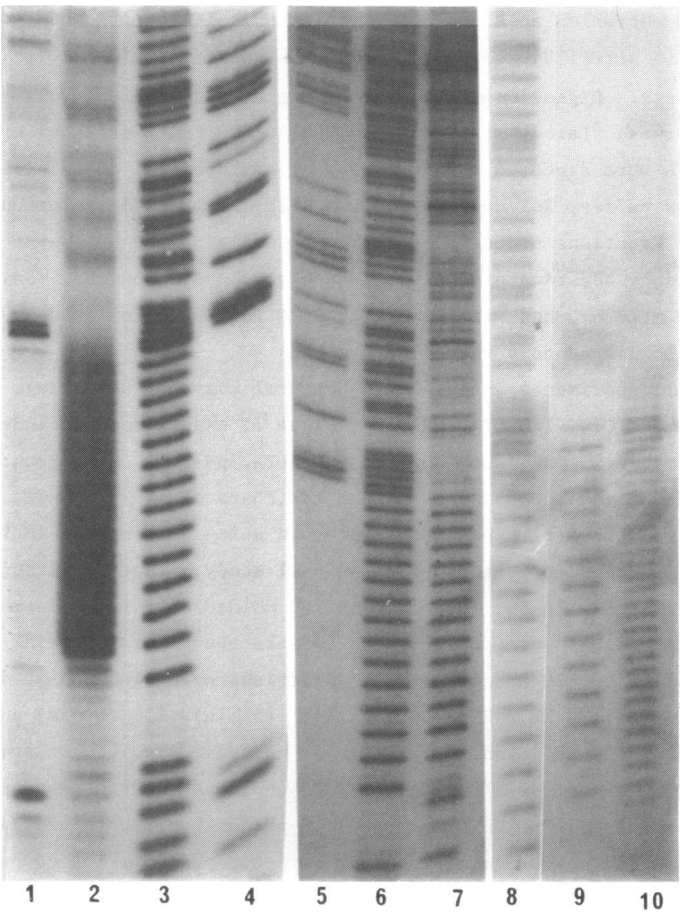

Figure 1. **Autoradiograms of nuclease digestion experiments within the $(AT)_{14}A$ region of the HindIII/HaeIII fragment of pRWAT14.1.** Lanes 3,6, and 8 are Maxam-Gilbert "A+G" lanes. Lanes 4 and 5 are Maxam-Gilbert "G" lanes. The following are nuclease digestions of the fragment: lane 1, DNAse II; lane 2, copper-phenanthroline; lane 7, DNAse I (in the presence of 2.5 mM $CaCl_2$); lane 9, Mung Bean nuclease (37°C); lane 10, S_1 nuclease. See methods section for reaction conditions.

the same distance as the 5'p$(N)_{29}$p3' Maxam-Gilbert fragment resulting from cleavage at the second dA residue in the insert.

RESULTS

DNAse I. DNAse I has been used in several studies on the influence of sequence on DNA conformation (31-33). It is a Mg^{+2} dependent enzyme which cleaves the DNA backbone at the 03'-P bond (34). DNAse I exhibits

a 100-500 fold preference for doubled-stranded DNA over single-stranded, and it makes essentially no double-strand cuts (35). A correlation was suggested between twist angle at a base pair step and rate of cutting (36). More recently, it has been proposed that the width of the DNA minor groove can affect the rate of hydrolysis, with groove widths in the B DNA range of 12Å being optimal (37).

Figure I shows the results of treating the 05'-^{32}P-labelled 256 base HindIII/HaeIII restriction fragment with DNAse I at 37°. As can be seen, the dinucleotide repeat which DNAse I recognizes in poly d(AT) is retained when a d(AT) sequence is embedded in random sequence DNA. To place the cutting probabilities on a quantitative footing, the gel autoradiograph was subjected to densitometry. A 41 base region was scanned, centered about the 29 base $d(AT)_{14}A$ repeat. Probabilities of cleavage were calculated following the procedure of Lutter (29). The results are plotted in figure 2, where the heights of the line above each base pair step represent the natural log of the probability of cutting at that bond. DNAse I recognizes the dinucleotide repeat across the entire $d(AT)_{14}A$ sequence, with little of an end effect. The dinucleotide repeat is not (as far as DNAse I can detect) transmitted into the adjacent sequence. From the densitometry results the probability of cutting at a d(ApT) step is at least 80 times that of cutting at a d(TpA) step. This is due to the special conformation of the alternating B conformation rather than any intrinsic reactivity of a d(ApT) step as shown by the lack of reactivity of the d(ApT) phophodiester bond in the nearby EcoRl site. Supplementing the digestion buffer with 2.5mM $CaCl_2$ increased the selectivity of DNAse I for the d(AT) region 20-fold, with the selective cutting at d(ApT) steps being retained. The presence of Ca^{+2} has been reported to enhance DNAse I's hydrolysis of AT-rich sequences (38).

Mung Bean Nuclease. A nuclease that gives results similar to DNAse I is Mung Bean nuclease. This is an endonuclease which degrades single-stranded DNA to mono- or oligonucleotides with 5'-phosphates (39). It is frequently used to convert protruding DNA overlaps to blunt-ended fragments (40). Unlike other duplex DNAs, poly d(AT) is rapidly hydrolyzed by Mung Bean nuclease (41). At 37° in the absence of Mg^{+2}, single-stranded DNA is digested only twice as fast as poly d(AT). Because of this selectivity it has been called a region-specific nuclease. Recently, under the strongly denaturing conditions of 30-45% formamide at 50°, this nuclease was reported to cleave Plasmodium genomic DNA at sites before

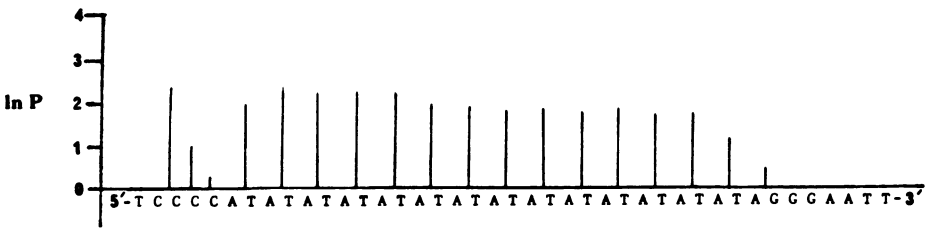

Figure 2. ln [probability of DNAase I cleavage] in and about the d(AT) tract of the HindIII/HaeIII fragment of pRWAT14.1 in the presence of 2.5 mM $CaCl_2$.

and after genes (42). Since this cleavage was not associated with any specific DNA sequence, but rather appeared to be related to structural features of the DNA, we wished to discover how it would recognize the alternating B structure. Mung Bean nuclease is a robust enzyme which allowed us to vary temperature, salt concentration, and solvent composition in order to see how these variables affected the dinucleotide repeat.

Figure 3 records the probability of hydrolysis of the phosphodiester bonds by Mung Bean nuclease in the region surrounding the $d(AT)_{14}$ insert under a variety of conditions. This region accounted for over 80% of the cuts in the 256 base pair fragment. As is the case with DNAse I, the d(ApT) steps are more reactive toward Mung Bean nuclease than the d(TpA) steps within the alternating B region. There is more of a variability in the rate of cutting within the insert than was the case with DNAse I. This is seen most clearly under standard conditions (37°, normal buffer) where cutting at the 5' end of the $d(AT)_{14}$ sequence is pronounced. This probably reflects the nearby (28 base pairs from the first A) 5' end to which the nuclease could bind. One also notes that in figure 3 the central d(ApT) steps are the most reactive. This could arise from cleavage of a minor equilibrium amount of a hairpin loop (perhaps stabilized by protein binding). However, from the results of Sheflin and Kowalski (43) one would expect the d(TpA) step in a loop to be cleaved also.

Conditions which either stabilize (increased $[Na^+]$) or destabilize (40% formamide) the duplex state tend to level out the probability for Mung Bean nuclease to cut a particular d(ApT) in the alternating B region. In the presence of 40% formamide there is the beginning of cutting at d(TpA) steps at the 5' end of the insert. Given the cooperative nature of melting, strand separation would not be expected to begin next to

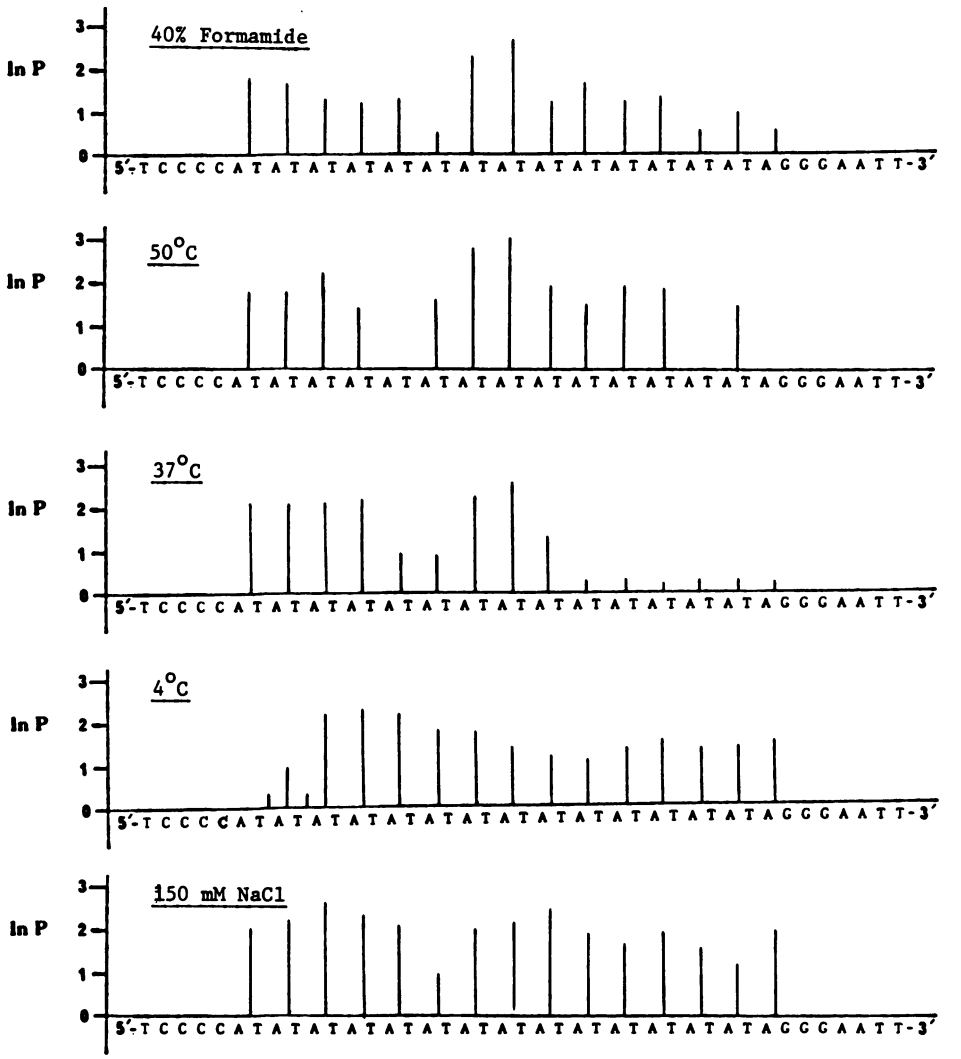

Figure 3. ln [probability of Mung Bean nuclease cleavage] using varied helix stabilizing and destabilizing conditions.

a G-C rich region (44), so that this breakdown in the dinucleotide repeat pattern is probably not due to a single-strand region at this site. It could instead represent the intrusion of B-DNA (or at least a non-alternating structure) into the alternating B region. Because organic solvents unwind the helix (45), and since the d(TpA) steps are predicted

by Dickerson's sum functions to be unwound (46), increasing $HCONH_2$ concentration possibly would begin to unwind the d(ApT) steps. This should lessen the conformation alternation along the DNA backbone and perhaps bring the d(TpA) phosphodiester bond into a conformation more easily hydrolyzed by the enzyme.

S_1 Nuclease. A nuclease that is often considered to have analogous reactivity to Mung Bean nuclease is S_1 nuclease. This is a single-strand specific enzyme which cleaves 03'-P phosphodiester bonds in the presence of Zn^{+2} at low pH (pH 5.4) (47). It frequently has been used to detect regions of perturbed DNA structure, in particular, junctions between right-handed and left-handed DNA (48). S_1 shows no base recognition pattern, seeming to need only several adjacent phosphodiester bonds which are not part of a double helix (47).

Figure 4 gives the relative rates of cleavage of the $d(AT)_{14}A$ insert and its surrounding region by S_1 nuclease. The d(AT) region is more reactive toward S_1 than the adjacent sequences, as would be expected from the T_m of poly d(AT). Comparing the Mung Bean nuclease results at 50^o (where there should be significant opening and closing of the d(AT) duplex region) with the S_1 results, for example, shows that these two normally single-strand selective nucleases appear to recognize different structural features. Alternatively, S_1 nuclease could be more efficient at stabilizing the d(AT) insert in a single-strand state, compared to Mung Bean nuclease. A second possible reason for the observed differences could result from the ability of S_1 nuclease to cleave DNA strands opposite nicks, generating gaps. Mung Bean nuclease has a lower level of exonuclease activity than S_1 (49,50).

DNAse II. The last enzyme whose reactivity toward alternating B DNA we have screened is DNAse II. This nuclease cleaves the DNA backbone at the 05'-P bond. It may recognize regions of stacked single-

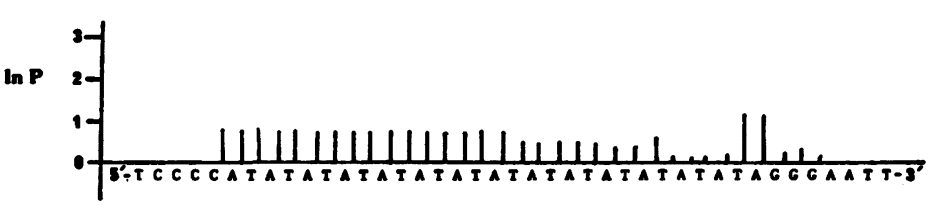

Figure 4. ln [probability of S1 nuclease cleavage] within the d(AT) and surrounding regions of the restriction fragment at 37^oC.

stranded bases which would explain its preference for purine-rich sequences (51). Figure 1 shows the autoradiograph of DNAse II digestion of the 256 base pair restriction fragment and figure 5 summarizes the cutting probability around the $d(AT)_{14}$ region. Unlike the other nucleases we have used, DNAse II avoids cutting in the $d(AT)_{14}$ region, instead cutting at the boundaries of the insert. At least in part this may be due to the insertion of the $d(AT)_{14}A$ fragment into the SmaI site, since DNAse II has a preference for cutting at $d(G)_n$ sites and will make doubled-strand as well as single-strand cuts. There is a $d(G)_4$ site near the 3' end of the fragment starting at base 157 from the 5' end which is also strongly cut by DNAse II. Whether there are special features of the alternating B-B junction which DNAse II recognizes must await construction of plasmids with $d(AT)_n$ inserts at other sites.

DNAse II has been shown to cleave at junctions of $d(A)_n(G)_n$, regions in which the minor groove width changes discontinously from narrow to wide (52). A similar sort of cutting is observed in this study. It has also been noted that the reactivity of DNA sequences toward DNAse I and DNAse II are anticorrelated (37). This is true, on the average, for our 256 base pair fragment since DNAse II does not cleave to any great extent within the $d(AT)_{14}$ insert. The anticorrelation does not, however, extend to the individual nucleotide level since d(TpA) bonds are not preferentially cleaved in the alternating B region by DNAse II. This may reflect either an unfavorable conformation of the phosphodiester linkage or a lessened binding constant of DNAse II toward DNA in the alternating B form.

Copper-phenanthroline. In addition to enzymatic nucleases, we investigated the ability of a small molecule to recognize the alternating B structure. Several amine complexes of redox-active metals have been shown to cause backbone cleavage in DNA (53). One of the simplest to prepare and use is the copper-$(1,10\text{-phenantholine})_2$ complex (54,55). In the presence of a reducing agent and oxygen (or equivalent conditions) this complex generates a species (possibly hydroxyl radical) capable of oxidative destruction of the sugar ring. This reagent is selective for B DNA and degrades A DNA (in the form of poly(rA)·poly(dT) duplex) almost an order of magnitude more slowly. DNA in the Z conformation, as well as single stranded DNA, is unaffected by this reagent (56).

An inspection of figure 1 indicates that $Cu(1,10\text{-phenanthroline})_2$ shows a marked preference for the $d(AT)_{14}$ region of the 256 base pair

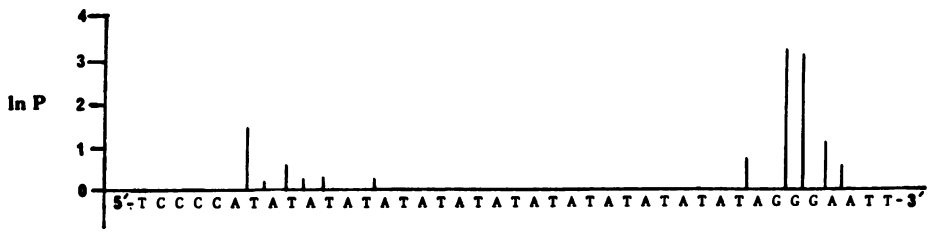

Figure 5. ln [probability of DNAse II cleavage] within the d(AT) and surrounding regions of the restriction fragment at 37°C.

restriction fragment. Greater than 90% of the cleavages were in this region. In fact, of all the nucleases we have investigated, this small molecule was the most selective for cleaving within the $d(AT)_{14}$ insert. At shorter exposure times the autoradiograph exhibited a band structure with apparent cleavage at each bond. However, since the copper reagent is reported to produce both 3' and 5' phosphate residues, we cannot yet say if there is a preference for cutting at d(ApT) vs. d(TpA) steps. One would not expect much of a selectivity for individual base pair steps since Cu(1,10-phenanthroline)$_2$ produces a diffusible intermediate (probably hydroxyl radical) which attacks the deoxyribose ring. With Dervan's EDTA-Fe^{+2} compounds there is a normal distribution of phosphodiester bond cleavages 4-6 base pair steps wide around the iron binding site, because cleavage is caused by a diffusible species (57).

It is not known if Cu(1,10-phenanthroline)$_2$ is an intercalator or a minor groove binder. Intercalators prefer G-C rich sequences while groove binders are selective for A-T regions (58). Based on that generalization the copper complex would be expected to be a groove binder. However, if one of the characteristics of the alternating B conformation is a strong d(ApT) stack and a loose d(TpA) stack, then the helix is pre-distorted toward an intercalative geometry (59). Thus, the alternating B geometry of a $d(AT)_n$ region could be an exception to the rule that intercalators bind more weakly at A-T regions.

DISCUSSION

It is interesting to compare the reaction of DNAse I and copper (1,10-phenanthroline)$_2$ on the 256 base pair fragment with digestion studies of Drew and Travers using these same nucleases on a 160 base pair fragment containing the tyrT promoter (37). In the tyrT study

Drew and Travers found that regions of A,T-rich DNA (at least those without d(TpA) steps), which included sequences such as dAAAATTA and dATTTTT, were resistant to DNAse I and the copper reagent. Since d(ApT), d(TpT) and d(ApA) steps can close the minor groove (due to the propeller twist of AT base pairs) (60) it was suggested that minor groove width plays a major role in the rate at which these two nucleases hydrolyze DNA. The optimum minor groove width for DNAse I and Cu(1,10-phenanthroline)$_2$, it was proposed, would be in the vicinity of 12Å (characteristic of B DNA) with widths above or below that value slowing down backbone cleavage.

The high degree of reactivity of the alternating B d(AT)$_{14}$ insert toward these nucleases which we have seen, compared to the other AT-rich sequences mentioned earlier, would arise therefore from the presence of d(TpA) steps in the d(AT)$_{14}$ sequence. These steps prevent narrowing of the minor groove since, as Calladine showed (61), purine-purine steric clashes in the minor groove oppose propeller twisting of d(TpA) steps, and propeller twisting closes the minor groove. This does not mean that the minor groove width in the alternating B insert is necessarily the same as in B DNA. The d(TpA) step can still propeller twist if it avoids a purine-purine clash by some combination of base pair roll, decrease in helical twist (unwinding) and base pair slide. Since each dA is part of a d(ApT) and d(TpA) step in a d(AT)$_n$ sequence, the d(AT)$_{14}$ region could exhibit normal propeller twist at the d(ApT) step bringing the phosphate into the minor groove at that step, but at the d(TpA) step a compensatory motion to allow propeller twisting (probably a decrease in the helical twist) would leave the d(TpA) phosphate near a B DNA-like position. Because of the helical nature of the DNA backbone, the phosphate opposite a d(ApT) phosphodiester is a d(TpA) phosphodiester. Thus, if this alternation in phosphate position really takes place, the backbone of alternating d(AT)$_n$ sequences would have a herringbone (or zigzag) appearance. The groove width would be essentially constant due to one phosphate residing at a B DNA position and the opposing phosphate displaced inward.

Whatever the conformational details of the alternating d(AT) structure turn out to be, our results clearly show that regions of alternating d(AT) (which are found in natural DNAs) persist in adopting a dinucleotide repeat structure even when surrounded by random sequence DNA. The alternating structure exists at physiological pH and temperature in the absence of torsional strain. It is maintained over a range of temperatures,

ionic strengths and solvent compositions. The B-alternating B junction (at least for the SmaI site) shows no evidence of conformational stress in its reactions with nucleases and the dinucleotide repeat is damped out as soon as the $d(AT)_{14}$ sequence ends. If sequence-dependent DNA structural variations are important elements in some biological control systems, then the alternating B structure, being so easily recognized even by relatively non-specific nucleases, should be one of the elements of such control systems.

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