Detection of an unusual distortion in A-tract DNA using KMnO₄: effect of temperature and distamycin on the altered conformation

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ABSTRACT

The chemical probes potassium permanganate (KMnO₄) and diethylpyrocarbonate (DEPC) can be used to study the conformational flexibility of short tracts of adenine (A-tracts) present in DNA. With these probes, we demonstrate that a novel distortion is induced in a 5 base pair A-tract at low temperature. Formation of this distorted A-tract structure, which occurs in a DNA fragment from the promoter region of the plasmid pBR322, is distinguished by a dramatic increase in the KMnO₄ reactivity of the central thymines in this tract at 12°C. This alteration occurs in the absence of any detectable rearrangement in the conformation of the adenines in the complementary strand. Induction of this low temperature A-tract structure is blocked by the minor groove binding drug distamycin. Hydroxyl radical footprinting of distamycin binding to the fragment containing the d(A)₅ tract at 12°C suggests that this drug has two different modes of binding to DNA in agreement with recent NMR data. These experiments show that short A-tracts are capable of forming more than one structural variant of B DNA in solution. The possible relationship between the intrinsic bending of DNA containing short phased A-tracts and the low temperature A-tract conformation is discussed.

INTRODUCTION

Short tracts of poly d(A)·poly d(T) (A-tracts) embedded in fragments of random sequence B DNA appear to form at least two distinct variants of the ideal B DNA structure (1−6). One of these variant B DNA conformations has been found in single crystals of dodecamers containing short A-tracts. The A-tracts in these crystals have a high propellor twist between the A.T base pairs, cross strand bifurcated hydrogen bonds, and a narrow minor groove (1−3). The existence of a second A-tract specific conformation has been inferred from proton NMR studies of A-tract containing oligonucleotides in solution (4,6). Although these studies have yet to yield a detailed structure, some features of the second A-tract specific conformation are becoming apparent.

This latter A-tract structure forms detectable junctions with the flanking DNA, and has a narrow minor groove (4,6). The A-tract specific conformation detected in solution by NMR does not appear to have any significant propellor twist, but may have a relatively high A·T base pair tilt (6).

We have recently demonstrated that two probes of DNA structure, diethylpyrocarbonate (DEPC, A > G) and potassium permanganate (KMnO₄, T > > C), react uniquely with many short A-tracts (3−6 bp) present in various DNA sequences (7). The probe DEPC produces two related patterns of reactivity with A-tract adenines, suggesting that at least two different conformations can occur in solution (7). One pattern of DEPC reactivity is recognized by a high level of DEPC modification at all of the A-tract adenines, except the 3' terminal adenine, which appears to be protected from DEPC attack. A distinctive feature of this class of A-tract reactivity is the elevated DEPC reactivity of the penultimate 3' end adenine. The second A-tract specific pattern of DEPC reactivity is distinguished by a lower and more equal DEPC reactivity at each adenine of the tract, except at the 3' terminal adenine which is again relatively insensitive to attack. In the majority of the 28 short A-tracts examined previously, only the 3' terminal thymine is sensitive to KMnO₄ attack (7).

The DEPC and KMnO₄ reactivity patterns of the short A-tracts examined previously were only altered to a minor extent as the reaction temperature was lowered (7). These results suggested that the conformation of the bases in these A-tracts varied little between 0−43°C. However, during a recent examination of temperature variations on the DEPC and KMnO₄ reactivities of additional short A-tracts, we discovered a particular A-tract which displays an anomalous reactivity pattern with KMnO₄ at low temperature. In this paper, we show that the poly d(T) rich strand of this A-tract becomes significantly changed at low temperature, while the poly d(A) rich strand appears to be unaltered. We also demonstrate that distamycin, a drug which binds in the minor groove of A+T rich sequences, can inhibit the transition to the low temperature conformation. Finally, evidence is presented which indicates some degree of flexibility in the A+T rich DNA immediately flanking the A-tract is required for the formation of this low temperature conformation.

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METHODS

Plasmid DNA. The plasmid pDPL6 is a derivative of pBR322, which has nucleotides 21-651 (Hind III−Sal 1), 778−2352 (Hae II−Hae II), and the internal 4 bp of the EcoR1 site removed (David Pulleyblank), and it contains a small polylinker fragment with Hind III, Xba I, Sma I, BamH I, and Sal I sites inserted in place of the deleted Hind III-Sal I fragment. Plasmid DNA was purified as described (7). DNA fragments were 5′ end labeled with [^{32}P] ATP by polynucleotide kinase and gel purified as described previously (7). The appropriate fragments for the experiment in Figure 5 were prepared as follows. Plasmid pDPL 6 DNA was 5′ end labeled at the Cla I site and then recut with ApaL I, and the 349 bp ApaL I−Cla I fragment containing the d(A)5 tract of interest was gel purified. The purified fragment was then cut with either Ssp I, Aat II, or BspH I, phenol extracted and ethanol precipitated.

KMnO$_4$ Reactions. 60 µl (Figure 2) or 100 µl (Figure 4) reactions containing 5′ end labeled DNA in 50mM sodium cacodylate pH 7.0, and 2mM EDTA were preincubated at various temperatures for either 5 (Figure 2) or 15 minutes (Figure 4 and 5). Then either 1.5 µl of 62.5 mM KMnO$_4$ (Figure 2), or 4 µL of a 50 mM KMnO$_4$ (Figure 4 and 5) was reacted with the DNA at the preincubation temperature for 4 minutes. Reactions were stopped by adding 300 µl ice cold mix of 294 µL ethanol, 3 µL beta-mercaptoethanol, and 3 µL of 2 µg/µL tRNA. Ten µL 3 M sodium acetate was then added, and the modified DNA was immediately precipitated at −70°C for 1 hour. Precipitated DNA was resuspended in 200 µL H$_2$O on ice, then reprecipitated with 20 µL 3M sodium acetate and 500 µL ethanol at −70°C for one hour. The DNA was pelleted, washed with 750 µL 70% ethanol, and dried. Modified DNA was resuspended in 40 µL 1M piperidine, and heated to 92°C for 30 minutes (8). Piperidine was removed by a butanol precipitation procedure (9), with some modifications (7). The DNA cleavage products obtained were resuspended in 80% formamide, and TBE buffer (89 mM Tris, 89 mM Boric acid, and 2 mM EDTA) containing bromophenyl blue dye, and run on DNA sequencing gels. Control lanes containing modified DNA (but without piperidine treatment) were frequently run as controls, and did not show significant cleavage of the DNA (Figure 5, lane 7, and data not shown).

For the experiment presented in Figure 6, the 102 bp Xba I-Dde I and 349 bp Cla I-ApaL I fragments of pDPL6, 5′ end labeled at the Xba I and Cla I sites respectively, were preincubated at the indicated reaction temperatures in 100 µL of the reaction buffer described above. After 15 minutes, the DNA was reacted at the indicated temperatures with 1 µL of 2.5 mM KMnO$_4$ (Xba I-Dde I fragment) or 2 µL of 5 mM KMnO$_4$ (Cla I-ApaLI fragment). After incubations of 2 minutes for the short fragment, and 5 minutes for the long fragment, the reactions were terminated. The modified DNA was then processed and run on DNA sequencing gels as described above.

DEPC Reactions. 100 µl reactions containing 5′ end labeled DNA in 50 mM sodium cacodylate pH 7.0, and 2mM EDTA were preincubated at appropriate temperatures for 5 minutes (Figure 3) or 15 minutes (Figure 4). Then 6 µl of DEPC was added and incubated for 15 minutes at appropriate temperatures. Reaction mixes were vortexed vigorously at the start of the DEPC reaction and after 7 minutes as previously described (10). The DEPC reaction on denatured DNA is identical to that described above

except that the reaction mix was boiled for 2 minutes, quick chilled, and then preincubated at 23°C for 5 minutes prior to chemical treatment. All reactions were terminated and precipitated as for the KMnO$_4$ reactions, except the beta-mercaptoethanol was not added. Piperidine treatment, and analysis of the cleavage products was similar to that described above.

DNA sequencing. G specific chemical sequencing was done by setting up 100 µl reactions as described for the DEPC reactions, preincubated at room temperature for 15 minutes, then 0.5 ul dimethyl sulphate was added and the mixture incubated for 2−3 minutes at room temperature. Reactions were terminated, and subsequently processed as in the DEPC reactions above. A/C specific sequencing was carried out as described previously (9)

Hydroyl Radical Cleavage. 100 µl reactions were set up as described for the DEPC reactions, and preincubated at the appropriate reaction temperatures for 15 minutes. Then the reactions were made 0, 0.1, and 1mM in distamycin, and further incubated at the appropriate temperatures for 45 minutes prior to the chemical treatment reaction. The hydroxyl radical cleavage reactions were initiated by adding 25 µL of a cocktail containing 5 µL 0.2 mM (NH$_4$)$_2$FeSO$_4$, 10 µL 0.3% hydrogen peroxide, and 10 mM L-ascorbic acid (11). Note that the EDTA required for hydroxyl radical production (11) is already in the reaction mix. Cleavage reactions were carried out at the appropriate temperatures for 1.5 minutes. The reactions were terminated, and the DNA was precipitated twice as described previously (11). The precipitated DNA was washed three times with 70% ethanol, and resuspended in DNA sequencing gel loading buffer. We have noted that better resolution of the hydroxyl radical cleavage products result when small aliquots (< 25% total) of the products are run per lane.

Densitometric Scanning. Scanning laser densitometry of autoradiographs was performed using a LKB Ultroscan 2202 densitometer.

RESULTS

The Induction of a Major Distortion in the Conformation of a Five Base Pair A-tract by Low Temperature. We have recently shown that short A-tracts react distinctly with two chemical probes of DNA structure (7). These chemical probes, DEPC and KMnO$_4$ (DEPC is purine specific, A > G; KMnO$_4$ is pyrimidine specific, T > > C), produce a number of related patterns of reactivity with different short A-tracts. One of the primary differences between the various A-tract reactivity patterns occur at the A-tract bases which abut the flanking DNA, suggesting
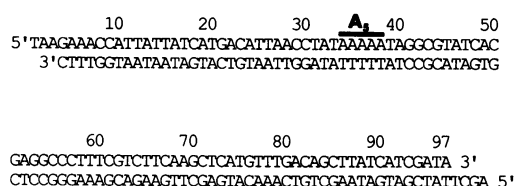
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              10        20        30    A₁  40        50
5'TAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGTATCAC
3'CTTTGGTAATAATAGTACTGTAATTGGATATTTTTATCCGCATAGTG
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           60        70        80        90     97
GAGGCCCTTTCGTCTTCAAGCTCATGTTTGACAGCTTATCATCGATA 3'
CTCCGGGAAAGCAGAAGTTCGAGTACAAACTGTCGAATAGTAGCTATTCGA 5'
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Figure 1. Sequence of the 97 bp DdeI-HindIII Fragment of pDPL6 Containing a 5 Base Pair A-Tract. This fragment corresponds to nucleotides 4290−30 in pBR322, except that the internal 4 bp of the EcoR1 site is deleted. The Cla 1 site is located at nucleotide 92.
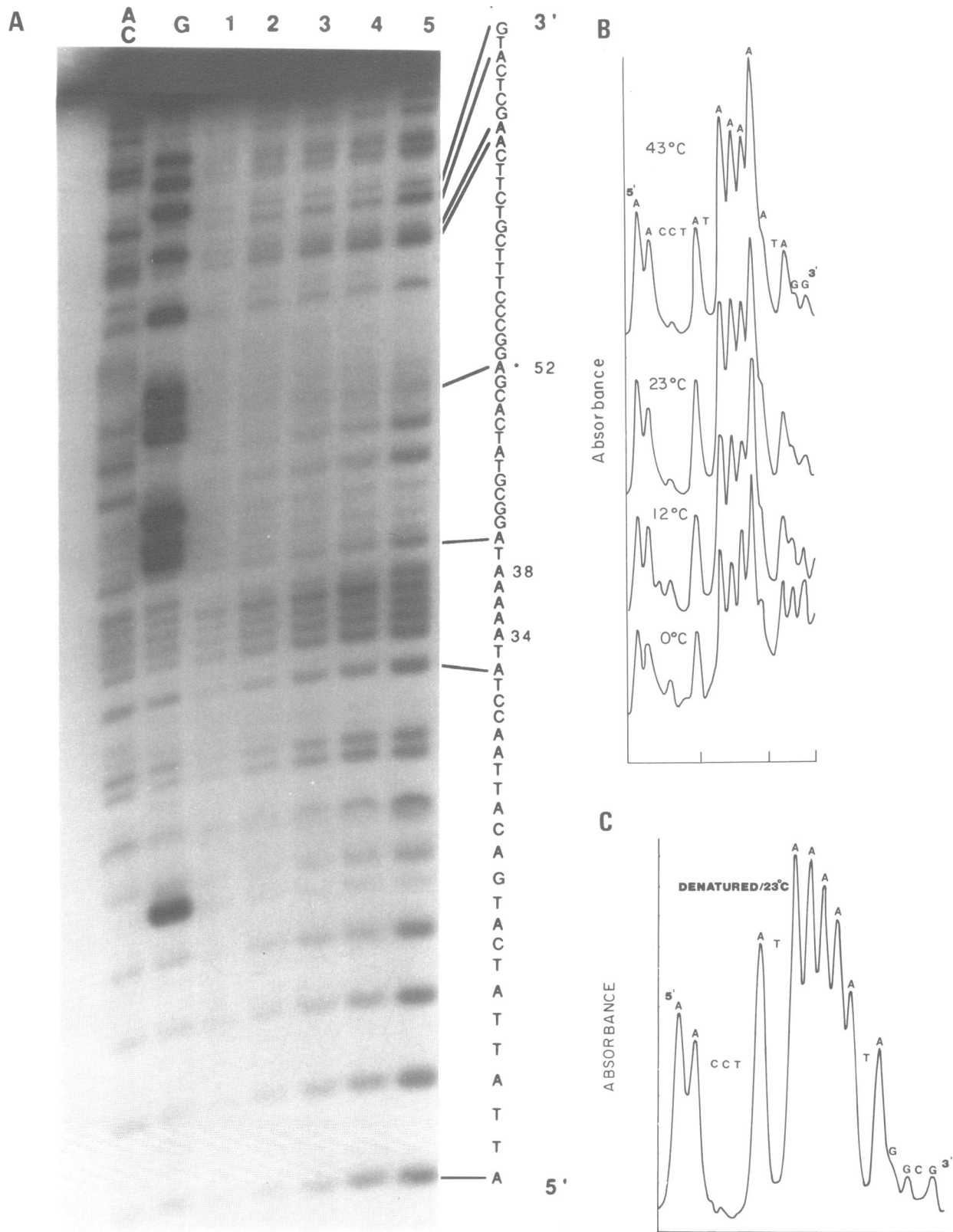
that different types of junctions can exist between the B DNA conformation(s) of short A-tracts and the conformation of the flanking DNA. We did not find major changes in the DEPC or KMnO$_4$ reactivity within any of the A-tracts examined as the temperature was lowered, although we did note that the KMnO$_4$ reactivity at the 3' terminal thymine of some tracts was lost at 4°C (7). The former result was unexpected because earlier evidence (12,13) had implied that lower temperature can significantly increase the DNA bending associated with certain fragments containing short A-tracts, and suggested that a structural transition may be stabilized by low temperature in some short A-tracts.

While studying the effect of temperature on the reactivity of other short A-tracts with these probes, we identified a five base pair A-tract which undergoes a major conformational transition at low temperature. Figure 1 shows the sequence of the 97 bp Dde I—Hind III fragment of pBR 322 containing this A-tract. The effect of temperature on the KMnO$_4$ reactivity of thymines in the 5 base pair A-tract of this fragment is shown in Figure 2A,

and densitometer scans of the autoradiographs generated from this experiment are shown in Figure 2B. At 43°C (Figure 2A, lane 4), all of the thymines in the d(T)$_5$ stretch are protected from KMnO$_4$ attack, except the 3' terminal thymine which reacts weakly with this probe. This pattern of KMnO$_4$ reactivity is similar to that seen with the thymines in the majority of the 28 A-tracts examined previously from 0—43°C (7). It should be noted that the DNA treated at 43°C is somewhat over reacted, and therefore the intensity of the larger cleavage products is somewhat lower than those seen at 23°C. However, when the reaction temperature was lowered, the thymines present in the A-tract of the 97 bp fragment that were normally protected from KMnO$_4$ became very sensitive to this probe (Figure 2, lanes 1—3). At both 12 and 0°C, the KMnO$_4$ reactivity peaked near the center of the d(T)$_5$ tract (T 36 and T 37). None of the other thymines in this fragment show any increased KMnO$_4$ reactivity as the temperature is lowered below 43°C. Thus, the high KMnO$_4$ reactivity of the d(T)$_5$ stretch at 12°C is probably due to a significant conformational change that is localized to the 5bp

Figure 2. KMnO$_4$ Reactivity of Thymines in the 5 bp A-Tract of the DdeI-HindIII Fragment at Different Temperatures. Panel A: The gel purified Dde I—Hind III fragment, 5' end labeled at the Hind III site, was reacted with KMnO$_4$ at the appropriate temperatures, and treated with piperidine as described in the methods. The DNA cleavage products were resolved on a DNA sequencing gel and visualized by autoradiography. Lanes 1—4, KMnO$_4$ modifications performed at 0,12,23, and 43°C; G and A/C are marker chemical sequencing lanes. An asterisk denote thymines which display very low KMnO$_4$ reactivity at all temperatures. The nucleotide numbering corresponds to that shown in Figure 1. **Inset:** magnification of the KMnO$_4$ reactivity seen in the d(T)$_5$ region. Panel B: Densitometer tracings corresponding to d(T)$_5$ region of lanes 1—4 from panel A. The arrow denotes position of the 5' adenine.

Figure 3. DEPC Reactivity of Adenines in the 5bp A-tract of the Dde1-ClaI Fragment at Different Temperatures. Panel A: Gel purified Dde I—Cla I fragment, 5' end labeled at the Dde I site, was reacted with DEPC at the appropriate temperatures. The DNA was subsequently treated with piperidine and analyzed as in Figure 2A. Lanes 1—4; DEPC modifications performed at 0,12,23, and 43°C; Lane 5, 23°C DEPC modification of denatured DNA. G and A/C are sequencing markers. Asterisk denotes an adenine (A 52) which reacts only marginally with DEPC in double and single strand DNA. Panel B: Densitometer tracings corresponding to d(T)₅ region of lanes 1—4 from panel A. Panel C. Densitometer tracing obtained from lane 5 of panel A (denatured DNA, reacted at 23°C).

A-tract. Recently, we also observed a similar elevated KMnO$_4$ reactivity in two other short A-tracts, both of which are found in kinetoplast DNA from *Crithidia fasciculata* (J. McCarthy and A. Rich, unpublished experiments).

The reactivity of thymines with KMnO$_4$ is primarily the result of attack by this probe at the 5−6 double bond from above or below the plane of the base (14). Therefore, the extent of KMnO$_4$ reactivity with a thymine in double strand DNA is directly related to the level of base stacking of that thymine (15−17). The unusually high KMnO$_4$ reactivity of the A-tract thymines at 12°C (Figure 2) implies that a structure possessing considerable unstacking of the central thymines has formed in the A-tract at low temperature. Osmium tetroxide, another chemical probe that detects unstacking of thymines in DNA (18), also detects a low temperature distortion of the thymines in this A-tract (data not shown). However, osmium tetroxide reactivity at the central A-tract thymines was only detected at 0°C, and not at 12°C. This implies that the unstacking which occurs in the d(T)$_5$ strand of the A-tract increases between 12°C and 0°C, and that the unstacking within the d(T)$_5$ sequence at 12°C cannot be detected by the larger osmium tetroxide probe.

At 23°C, KMnO$_4$ reacts moderately with all but one (T 35) of the A-tract thymines. The pattern of A-tract KMnO$_4$ reactivity at 23°C (Figure 2A) appears to be a composite of the reactivities at 43°C and 12°C, and suggests that both types of reactivity can occur at this temperature. When the reaction temperature approaches 53°C, the KMnO$_4$ reactivity of thymines T 38−T 35 increases slightly, and nearly reaches the level of reactivity that occurs at the 3′ terminal thymine (data not shown). Thus, either the A-tract unwinds slightly between 43−53°C, increasing the KMnO$_4$ reactivity of the thymines, or the 3′ junction may becomes less distinct at higher temperatures, thereby reducing the reactivity of the 3′ thymine relative to the other thymines in the tract. As described previously (7), KMnO$_4$ also displays a variable reactivity at thymines not present in A-tracts, with some thymines being protected from KMnO$_4$ attack (T 49) at all temperatures, and other thymines always reacting significantly with this probe (T 81).

The Low Temperature A-Tract Distortion is Primarily Limited to the Poly d(T) Strand. The effect of temperature on the DEPC reactivity of adenines in the d(A)$_5$ tract is shown in Figure 3A,
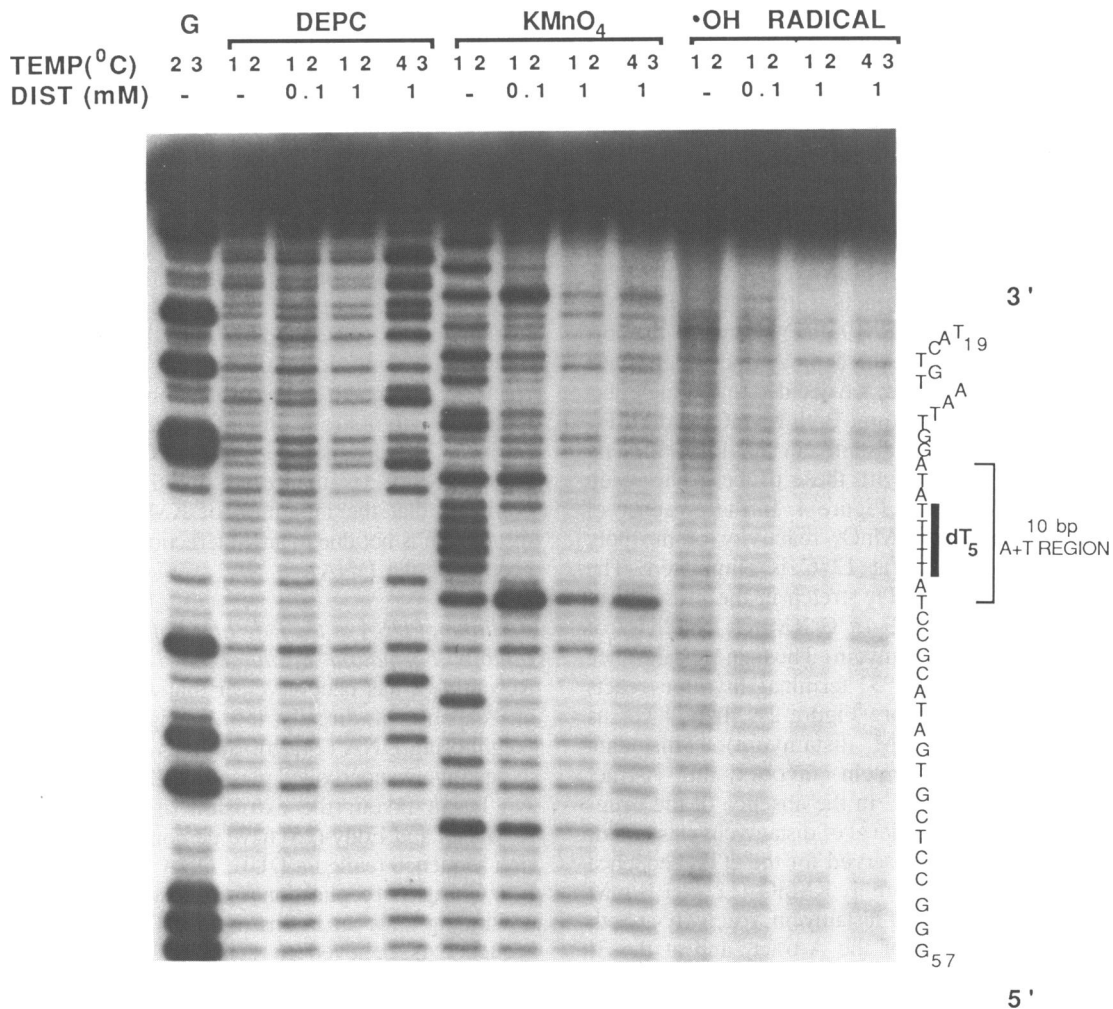
Figure 4. Effect of Distamycin Binding on the Reactivity of the 5 bp A-tract with KMnO$_4$ and Hydoxyl Radical at 12°C. Gel purified Dde I−Cla I fragment, 5′ labeled at the Cla I site, was reacted at the appropriate temperature with either KMnO$_4$, DEPC or hydroxyl radical in the presence of 0, 0.1, and 1 mM distamycin. The piperidine cleavage products of the KMnO$_4$ and DEPC modified DNA, and the hydroxyl radical cleavage products were resolved on a DNA sequencing gel and visualized by autoradiography.

and densitometer scanning of this data is shown in Figure 3B. In contrast to the KMnO$_4$ reactivity of thymines in this tract, the pattern of DEPC reactivity of the A-tract adenines does not change between 0−43°C, although modification of the susceptible adenines does decrease at the lower temperatures (Figure 3A, lanes 1−4). At each temperature, DEPC reacts to a relatively high level with all the adenines of the A-tract, except with the 3' terminal adenine. In addition, the adenine next to the 3' terminal adenine of this A-tract displays a particularly high DEPC reactivity relative to other adenines in this fragment. The DEPC reactivity pattern observed for this d(A)$_5$ tract falls into one of the classes of A-tract specific DEPC reactivity described previously (7). When the fragment is denatured prior to reaction with DEPC, the 3' terminal adenine becomes sensitive to DEPC attack (Figure 3A, lane 5, and Figure 3C). This result implies that the resistance of the 3' terminal adenine to DEPC attack results from the specific conformation of this adenine at the junction between the A-tract and the flanking DNA. It is interesting to note that the 3' terminal A-tract adenine displays a slightly higher dimethyl sulphate reactivity than other A-tract adenines (Figure 3A, G marker lane). The relationship of this observation to the conformation of the 3' terminal A-tract adenine is not known. The reactivity of DEPC with other adenines in this fragment is variable, some adenines react to a similar extent as guanines (A 52), while other adenines display a much higher DEPC reactivity (A 32). We have proposed (7) that differential DEPC reactivity of adenines, and KMnO$_4$ reactivity of thymines (Figure 2A) in double strand DNA reflects sequence specific differences in the conformations of these bases in the B-DNA helix.

Distamycin Binding Inhibits The Formation of the Low Temperature A-tract Structure. Distamycin is an oligopeptide antibiotic which forms a tight interaction in the minor groove of A+T rich regions of DNA (1, 19−23). Because the low temperature induced distortion of the 5 bp A-tract might have some alteration in the minor groove, we decided to test the effect of distamycin binding on this structure. Both KMnO$_4$ and DEPC react in the major groove and thus distamycin should not directly block the reactivity of the bases with these probes. The result of this experiment is presented in Figure 4. In the presence of 0.1 mM distamycin, the high KMnO$_4$ reactivity of the poly d(T)$_5$ stretch normally observed at 12°C is eliminated. The 12°C KMnO$_4$ reactivity at the d(T)$_5$ stretch in the presence of 0.1 mM distamycin resembles the 43°C KMnO$_4$ reactivity of this region in the absence of distamycin. Thus, in the presence of 0.1 mM distamycin only the 3' terminal thymine reacts significantly with KMnO$_4$ (compare Figure 2A, lane 4 versus Figure 4, KMnO$_4$/12°C/0.1 mM distamycin). This data indicates that 0.1 mM distamycin favours the A-tract conformation which exists at 43°C in the absence of the drug. When the effect of a 10 fold higher level of distamycin was tested, the pattern of KMnO$_4$ reactivity observed for the d(T)$_5$ sequence at 43°C was also eliminated (Figure 4, KMnO$_4$/12°C/1.0 mM distamycin). At this concentration of distamycin, reactivity at both the 3' terminal thymine (T 34) of the A-tract, and at the 3' neighboring thymine (T 32) was diminished substantially. When the same KMnO$_4$ reaction was performed at 43°C in the presence of 1 mM distamycin, a pattern of KMnO$_4$ reactivity similar to that found at 12°C and 1 mM distamycin was observed. Similarly, we have recently observed that distamycin eliminates the elevated KMnO$_4$ reactivity that occurs in two of the A-tracts

of the *C. fasciculata* kinetoplast DNA bending sequence (unpublished experiments).

Distamycin binding in the minor groove of DNA is believed to afford protection from hydroxyl radical attack at the ribose-phosphate backbone (20,21). Hydroxyl radical footprinting of distamycin binding to the 5 bp A-tract at 12°C results in only a weak protection from hydroxyl radical at concentrations of antibiotic which inhibit the low temperature transition (Figure 4, ·OH/12°C/0.1 mM distamycin). However, when this experiment is carried out with a 10 fold higher concentration of distamycin, decreased reactivity with hydroxyl radical is seen for at least 7 bp of the A+T sequence that includes the d(A)$_5$ tract (Figure 4, ·OH/12°C/1 mM distamycin). This result suggests that distamycin may have a significant on/off rate for DNA binding at 12°C in the buffer conditions used in this experiment, and thus produces a very weak footprint at the lower level of distamycin. It is also possible that distamycin may have at least two different modes of binding to DNA. One mode of binding may not afford significant protection of the labeled backbone from hydroxyl radical attack, but is capable of inhibiting the transition to the low temperature A-tract structure. The second mode, which appears to be favoured by high concentrations of distamycin, affords both protection from hydroxyl radical and inhibits the formation of the low temperature A-tract conformation. Evidence for the existence of two different modes of distamycin binding to DNA has recently been presented (1,22,23).

The distamycin induced loss of KMnO$_4$ reactivity at the thymines of the A+T region is not accompanied by any significant loss of DEPC reactivity at neighboring adenines A 39, A 33, and A 31 (Figure 4, DEPC/12°C/0.1 and 1 mM distamycin). Also, the DEPC reactivity pattern of the adenines in the d(A)$_5$ stretch is not altered by the presence of either 0.1 mM or 1 mM distamycin, although the reactivity of each A-tract adenine does decrease somewhat as the concentration of distamycin is raised (data not shown). Thus, the helical alteration(s) induced by distamycin binding to the d(A)$_5$. d(T)$_5$ tract at 12°C result in the protection of thymines from KMnO$_4$ attack, but do not radically affect the A>G DEPC reactivity of purines. The observation that DEPC attack at purines, via the major groove, is unaffected by the presence of distamycin confirms that the inhibition of KMnO$_4$ reactivity induced by distamycin is not due to any interaction of the drug with the major groove of the DNA.

Addition of DNA at the Dde I End of the Dde I−Cla I Fragment Inhibits Formation of the Low Temperature Conformation. Recently, it has become clear that A+T rich regions of DNA are capable of directly influencing the conformation of the neighboring DNA (24,25). Sullivan et al. (24) have shown that in negatively supercoiled plasmids, DNA sequences with >65% A+T can favour the extrusion of a neighboring cruciform. Kowalski and Eddy (25) have demonstrated that a specific A+T rich sequence (called a DUE, *D*NA *U*nwinding *E*lement) in the E. coli origin of replication (ori) allows negative supercoil induced unwinding to occur in this region. The helical flexibility of this sequence is believed to facilitate the protein induced DNA unwinding of the ori region which is associated with the the start of DNA replication in E. coli. The sequence between the Dde I site and the d(A)$_5$ tract is 71% A+T rich (Figure 1). We speculate that some intrinsic helical flexibility of this A+T rich sequence may contribute to the formation of the low temperature A-tract structure. To test this, we generated labeled fragments
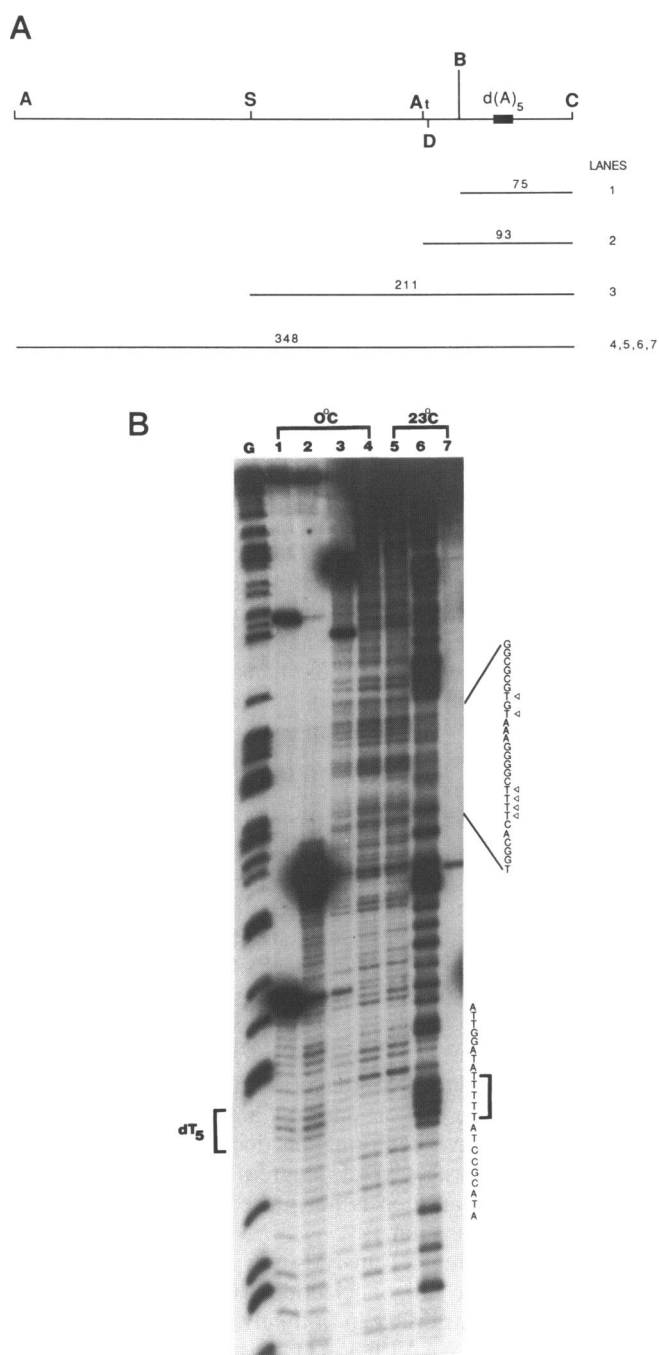
which were 5' end labeled at the Cla I site, and had different lengths of DNA sequence beyond the Dde I site (Figure 5A). These isolated fragments were treated with $KMnO_4$ at 0°C, and the reactivity of $d(T)_5$ thymines were analyzed. The result of this experiment is presented in Figure 5B. It demonstrates that the presence of additional DNA 5' to the Dde I site interferes with the transition to the low temperature conformation of the $d(A)_5$ tract. Equivalent results were obtained when related fragments were 5' end labeled at the Sal 1 site, gel isolated and reacted with $KMnO_4$ at 0°C, that is, high $d(T)_5$ $KMnO_4$ reactivity in the 122 bp Sal 1-Dde fragment and normal reactivity at $d(T)_5$ in the 244 bp Sal 1-Ssp 1 fragment (data not shown). The extra DNA added (Figure 5A) lowers the A+T content of DNA at the Dde I end of the A-Tract to 64% A+T. Sullivan et al., 1988 (24) have shown that when a short G/C rich sequence is placed adjacent to an A+T helix destabilizing sequence, the destabilizing effect of the A+T sequence can be compromised. Thus, the fact that the first 40 bp of the DNA added to Dde I end of the 97 bp fragment is only 40% A+T rich suggests that the high G/C content of this region may contribute to the elimination of the low temperature conformational transition in the $d(A)_5$ tract seen in Figure 5B.

If the induction of the altered structure which occurs at low temperature in the 5 bp A-tract of shorter fragments, but not of longer fragments, is a consequence of some intrinsic difference
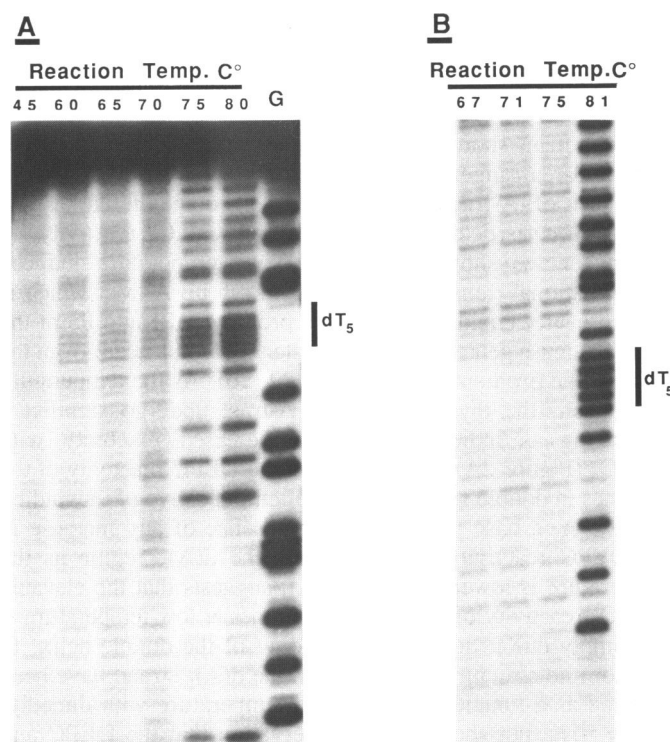

Figure 5. Effect of Flanking DNA on the Low Temperature Conformation in the 5 bp A-Tract. Panel A: Position of the restriction sites used to produce variously sized fragments that are each 5' end labeled at the Cla I site. The position of the Dde I site is included for reference. A, ApaL I; S, Ssp I; At, Aat II; D, Dde I; B, BspH I; C, Cla I. The lengths of the fragments are shown above each fragment. Panel B: The fragments described in panel A were preincubated at the indicated temperatures, and then reacted with $KMnO_4$ at these temperatures as described in the methods. The piperidine cleavage products of the modified DNA were then separated on a DNA sequencing gel, and visualized by autoradiography. Lanes: 1, BspH I−Cla I; 2, Aat II−Cla I; 3, Ssp I−Cla I; 4 and 5, ApaL I−Cla I; 6, ApaL I−Cla I fragment was denatured in reaction buffer by heating at 93°C prior to reaction with $KMnO_4$; 7, control lane with same $KMnO_4$ modified DNA as in lane 5 except the piperidine treatment was omitted, this indicates that the majority of the DNA cleavage seen in lane 5 is the result of piperidine induced cleavage of $KMnO_4$ modified bases. The sequence around the A-tract of interest is indicated. The other sequence demarcates a region of the labeled ApaL I−Cla I single strand which has a significantly reduced level of $KMnO_4$ reactivity, presumably due to secondary structure in this DNA.

Figure 6. Short and Long Fragments Containing the 5 bp A-Tract Also Exhibit Different Properties At High Temperature. Panel A: The 102 bp Xba I-Dde I fragment was reacted with a low concentration of KMnO4 at various temperatures, and the piperidine cleavage products were run on a DNA sequencing gel as described in the methods; G indicates guanine specific sequencing marker. Panel B: The 349 bp Apal I-Cla I fragment was reacted with a low concentration of KMnO4 at various temperatures, and the piperidine cleavage products were run on a DNA sequencing gel. For clarity, only the portion of the fragment containing the 5 bp A-tract is shown, but the remainder of this strand is also hyperreactive at 81°C.

in the helical stabilities of these fragments, we speculate that these fragments may also differ in their response to elevated temperature. It has been shown previously that thymines in double stranded DNA do not react with very low concentrations of KMnO₄, but most thymines react strongly with this reagent when the DNA has been denatured prior to the treatment (17). Here, we utilized the inability of 25−50 uM KMnO₄ to react with thymines in double stranded DNA to test whether any differences existed between the response of a short and a long fragment containing the 5 bp A-tract to high temperatures. The results obtained for the short 102 bp Xba I-Dde I fragment are shown in Figure 6 (panel A). As predicted, at 45°C the thymines in this fragment did not react with KMnO₄. However, between 60°C and 70°C, a low level of KMnO₄ reactivity was detected at most of the thymines. At 75°C, and above, a high level of KMnO₄ reactivity was detected at all the thymines in this fragment, a reactivity similar to that expected if the DNA was single stranded. In striking contrast, when the longer 349 bp ApaL I-Cla I fragment was treated in the same way, no moderate KMnO₄ reactivity was found before the sharp transition (of the whole molecule) to the KMnO₄ hyperreactive nucleic acid structure which occurs between 76°C and 81°C (Figure 6; panel B). Although more work is necessary to determine whether the transition to the KMnO₄ hyperreactive conformation detected in Figure 6 results from the formation of a partially melted, or fully melted DNA structure, these experiments clearly demonstrate that different helical constraints are placed on the sequence containing the short 5 bp tract by adding DNA over 30 bp away.

DISCUSSION

Although many short A-tracts do not appear to undergo any major structural rearrangements as the temperature is lowered (7), a small set of A-tracts do appear to become altered at low temperature, as detected by an elevated KMnO₄ reactivity. In this paper we show that such a low temperature induced conformational transition can occur in a 5 bp A-tract which is located nearly equidistant from the promoters P1 and P3 of the beta-lactamase gene, and approximately 65 bp upsteam from the tetracycline promoter (P2) of pBR322 (26). As the temperature is reduced, the thymines of this A-tract undergo significant unstacking as indicated by their increased reactivity with KMnO₄ (Figure 2A). An increase in the DEPC reactivity of the adenines in double strand DNA is often indicative of unwinding at those A·T base pairs (27). The absence of any increase in the DEPC reactivity of adenines in the d(A)₅ region as the temperature is lowered (Figure 3A), suggests that the elevated KMnO₄ reactivity of the A-tract thymines at low temperature is not simply due to localized unwinding of the double helix. In addition, no structural alterations in the d(A)₅ strand have been detected as a function of decreased temperature with dimethyl sulfate (data not shown). The observation that the DEPC and DMS reactivity patterns of the d(A)₅ strand are not altered as the temperature is decreased (Figure 3B), while the KMnO₄ reactivity of the d(T)₅ strand is significantly altered (Figure 2B), suggests that the distortion of this A-tract at low temperature is primarily limited to the d(T)₅ strand of the tract.

A number of different alterations in the conformation of the thymines in this A-tract could explain the loss of d(T)₅ stacking interactions at low temperature. For example, there may be an increase in the tilt and/or roll of the A-tract base pairs (centered at T 36−T 37), leading to different levels of A·T base pair

opening into the major groove and a unusually high, but differential accessibility of the thymine 5−6 double bonds to KMnO₄ attack. However, any model of the thymine strand A-tract alteration must accommodate the observation that this distortion does not significantly affect the DEPC reactivity at the N7 position of the A-tract adenines (Figure 3B).

Some interesting parallels exist between properties of the low temperature A-tract conformation, and particular features of the high resolution 434 repressor/DNA co-crystal structure. The DNA binding domain of the 434 repressor protein induces a large distortion at the 3 bp A-tract present in the center of the protein binding site (28). The DNA binding domain of the 434 repressor makes direct contacts at both sides of the d(A)₃ tract, and causes significant unstacking of the thymines in this short A-tract. This protein induced distortion of the central d(T)₃ occurs without any detectable rearrangement at the complementary d(A)₃ strand. Overwinding, high propellor twist and narrowing of the minor groove at the central A·T base pairs accompanies formation of the d(T)₃ distortion (28). Furthermore, although the central A-tract region is straight, overall the DNA oligonucleotide is bent. Stress induced by the d(T)₃ distortion in the 434 repressor binding site appears to be accommodated within the helix by diffusion to the flanking sequences (28). It is possible that a similar distortion of A-tract DNA can occur in a linear fragment when aided in cis by a potential A+T rich helix destabilizing sequence (24,25), and by low temperature. Our observation that adding a sequence with a G/C rich region to one end of the 97 bp A-tract containing fragment inhibits formation of the low temperature conformation (Figure 5B) is consistent with this proposal.

The data presented in Figure 6 strengthens the argument that neighboring DNA sequences can significantly influence the constraints placed on the conformation of the DNA around the 5bp A-tract being studied here. In this experiment, a short fragment containing the 5 bp A-tract exhibits a low KMnO₄ reactivity over at least 10°C before the transition to the KMnO₄ hyperreactive premelt/melted structure occurs at 75°C. However, when this sequence is contained in a longer fragment, the KMnO₄ hyperreactive conformation does not occur until the temperature is least several degrees higher, and more significantly, this transition is not preceded by any detectable intermediate conformation.

X-ray crystallographic analysis of a complex between distamycin and the oligonucleotide d(CGCA₃T₃GCG) has demonstrated that this drug can fit tightly into the minor groove of the sequence 5'-AAATT-3' (1). Distamycin is a crescent shaped molecule which has three essentially planar pyrrole rings and four planar amide linkages. In the co-crystal structure, a slight twist occurs between each pyrrole ring such that the drug follows the normal curvature of the minor groove. Distamycin forms bifurcated hydrogen bonds to both adenines (at N3) and thymines (at O2) on the floor of the minor groove, in addition to making van der Waals contacts with both the floor and walls of the minor groove (1). A similar group of contacts between distamycin and the oligonucleotide d(CGCGAATTCGCG) have been shown to occur in solution by NMR (22). More recently, Pelton and Wemmer 1989 (23) have proposed that a second type of distamycin/DNA complex can form in solution involving two distamycins stacked on each other lying in an expanded minor groove. It is possible that decreased hydroxyl radical attack on the DNA at the higher concentration of distamycin (Figure 4, ·OH/12°C/1 mM distamycin, and ·OH/43°C/1 mM distamycin)

is due to new distamycin-DNA backbone interactions, and/or widening of the minor groove with the concomitant alterations in the conformation of the sugar phosphate backbone, which result from formation of the 2:1 complex.

It has been shown previously that distamycin can eliminate the DNA bending associated with phased A-tracts (29). Since distamycin also eliminates the low temperature distortion of the $d(A)_5$ tract (Figure 4, KMnO$_4$/12°C/0.1 mM distamycin) it is possible that the low temperature structure may be associated with changes in the curvature of DNA. It has been shown previously that the reaction of hydroxyl radical with A-tracts involved in DNA bending decreases in the 5'−3' direction (30). This directional decrease in ·OH reactivity is believed to be due to the narrowing of the minor groove 5'−3' in A-tracts which form a bend in DNA (30,31). Thus, it is interesting to note that the minor groove of the 5 bp A-tract studied here has previously been shown to narrow substantially in the 5'−3' direction as the temperature is lowered from 43−0°C (30). Densitometer scanning of the lane corresponding to hydroxyl radical treatment at 12°C in Figure 4 (·OH/12°C/no distamycin) also indicates that the deoxyribose of the 5' thymine in this $d(A)_5$ tract is the most sensitive to hydroxy radical attack (data not shown). These correlations, together with the involvement low temperature in accentuating DNA bending (12,13), suggest that the low temperature A-tract structure might be involved in some type of DNA bending. However, the low temperature conformation detected by KMnO$_4$ only forms in a very limited number of A-tracts (7), and the majority of the A-tracts in the bent *C. fasciculata* kinetoplast DNA do not form the low temperature structure (J. McCarthy and A. Rich, unpublished experiments).

A-tracts are a common feature of DNA. It is clear that they are able to adopt a number of conformations, some of which are likely to be utilized in biological systems. More experiments will be necessary to fully understand them.

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