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A structural analysis of the bent kinetoplast DNA from *Crithidia fasciculata* by high resolution chemical probing

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ABSTRACT

The chemical probes potassium permanganate ($KMnO_4$) and diethylpyrocarbonate (DEPC) have been used to study the conformation of bent kinetoplast DNA from *Crithidia fasciculata* at different temperatures. Chemical reactivity data shows that the numerous short A-tracts of this bent DNA adopt a similar structure at 43°C. This conformation appears to be very similar to the conformation of A-tracts in DNA exhibiting normal gel mobility. The A-tract structure detected by chemical probing is characterized by a high degree of base stacking on the thymine strand, and by an abrupt conformational change at the 3′ end of the adenine strand. In general, no major alteration of this A-tract specific structure was detected between 4–53°C. However, probing with $KMnO_4$ revealed two unusual features of the *C. fasciculata* sequence that may contribute to the highly aberrant gel mobility of this DNA: 1) the B DNA/A-tract junction 5′ dC/A_{3-6} 3′. 5′ dT_{3-6}/G 3′ is disproportionately represented and is conformationally distinct from other 5′ end junctions, and 2) low temperature favors a novel strand-specific conformational distortion over a 20 base pair region of the bent kinetoplast DNA. Presence of the minor groove binding drug distamycin had little detectable effect on the A-tract conformation. However, distamycin did inhibit formation of the novel $KMnO_4$ sensitive low temperature structure and partially eliminated the anomalous gel mobility of the kinetoplast DNA. Finally, we describe a simple and reproducible procedure for the production of an adenine-specific chemical DNA sequence ladder.

INTRODUCTION

Several years ago, it was proposed that a single short A-tract (4–6 base pairs of poly d(A). poly d(T)) can cause a small but distinct bend in DNA, and that molecules of DNA containing several A-tracts in phase with the helical repeat of B DNA have intrinsic curvature (1–6). Curvature of a DNA fragment is usually associated with the aberrant mobility of such DNA on polyacrylamide gels (7 and references therein). According to one model, sequence-directed DNA curvature (bending) is generated by a change in trajectory of the long axis of the DNA helix at A-tract/flanking sequence junctions (5, 8). Such changes in helical direction are believed to result from the clash between a unique A-tract conformation (B′) and the more 'normal' B-DNA conformation of the flanking sequences (reviewed in 7). Consistent with this model, several groups have presented evidence suggesting that short A-tracts are indeed capable of forming at least two distinct B-DNA like conformations (9–13). At present however, the A-tract conformation(s) involved in A-tract directed DNA bending is not known precisely.

The majority of the studies concerning sequence-directed DNA bending have used ligation products of synthetic oligonucleotides which contain short A-tracts (7). Therefore, fragments of curved DNA produced in this manner contain multiple repeats of short, simple sequences and probably lack certain features of more complex naturally occurring bent DNA. A number of genomic sequences have been found which display altered electrophoretic mobility on polyacrylamide gels (7), and thus are believed to contain bent DNA. Most natural bending sequences are considerably more complex than model bent DNA, and can be frequently found in functionally important regions such as replication origins (14), transcription control regions (15, 16), scaffold attachment sites (17), and sequences involved in site specific recombination (18). However, despite evidence for the involvement of DNA bending in processes such as transcription initiation (19), few detailed structural studies have been carried out directly on naturally occurring bending sequences.

One complex bending sequence that has been studied in some detail is a 211 bp region of *Crithidia fasciculata* kinetoplast DNA which contains 18 short A-tracts (20). Fragments containing this sequence have a particularly anomalous mobility on polyacrylamide gels, and under some conditions, they comigrate with fragments at least three times their actual size (20). Using the hydroxyl radical as a probe, Burkoff and Tullius (21) have shown that the minor groove of A-tracts in the *C. fasciculata* kinetoplast DNA narrow in the 5′–3′ direction, and their results suggest that all the A-tracts in this fragment adopt a similar conformation in solution. More recently, Krishnamurthy et al.

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(22) have used ethidium bromide associated photo-cleavage to demonstrate that ethidium bromide binds poorly to A-tracts in the *C. fasciculata* bending sequence, supporting the position that these tracts have a unique conformation.

To obtain additional information concerning the structure of the bending sequence from *C. fasciculata*, we have probed this DNA with two base specific probes of DNA structure. These probes, $KMnO_4$ and DEPC, have previously been shown to be useful for analyzing the conformation of B DNA, and especially the conformation of A-tracts (9). We have also examined the influence of distamycin on the conformation and gel mobility of the *C. fasciculata* bending sequence. The results obtained indicate that the conformation of most A-tracts in the bent *C. fasciculata* kinetoplast DNA is similar to that of A-tracts in DNA with normal gel mobility. However, a large number of the A-tracts in kinetoplast DNA lack the distinctive junction normally detected between the 3′ end of the thymine strand (of an A-tract) and the flanking sequence. Furthermore, one region of the kinetoplast sequence adopts a novel $KMnO_4$ sensitive conformation on the thymine strand at ambient/low temperature. The minor groove binding drug distamycin inhibits formation of this $KMnO_4$ sensitive conformation, in addition to partially reducing the aberrant gel mobility associated with this kinetoplast DNA. The two novel conformational features of the bent *C. fasciculata* DNA described here may contribute to the highly aberrant gel mobility of this DNA.

MATERIALS AND METHODS

Plasmid DNA

Plasmid pPK201/CAT contains the 211 bp Stu I-Acc I bending fragment from *Crithidia fasciculata* kinetoplast DNA (20) cloned into the BamHI site of the vector pSP65 (Promega). pPK201/CAT plasmid DNA was purified through two successive CsCl gradients as described previously (9). Plasmid pPE103 (1) contains the 414 bp Sau 3AI fragment from *Leishmania tarentolae* cloned into the Bam HI site of pBR 322 (1, 23). pPK201/CAT and pPE103 were kind gifts of Dr P.T. Englund. The fragments used for chemical probing and native gel electrophoresis experiments (+/− distamycin) were 3′ or 5′ end labeled on one strand and isolated from native gels as described previously (9). The gel purified labeled DNA fragments were resuspended in TE (10 mM Tris−HCl pH 7.0, 1 mM EDTA).

Chemical probing reactions

DNA fragments, 3′ or 5′ end labeled on one strand, were resuspended in 100 microliters (μL) of 50 mM sodium cacodylate, (pH 7.0), 2 mM EDTA, and the samples were preincubated for 15 minutes at the appropriate temperature. For $KMnO_4$ reactions, 4 μL of 50 mM $KMnO_4$ (Aldrich) was then added to the samples. After 4 minutes, reactions were terminated by the addition of an ice cold solution containing 294 μL of ethanol, 3 μL beta-mercaptoethanol, and 3 μL of 2 μg/μL *E.coli* tRNA. Finally, 10 μL 3 M sodium acetate was added, and the nucleic acids were precipitated at −70°C for one hour. $KMnO_4$ probing of the distamycin/DNA complex (Figure 5) was carried out in a similar manner, except that the DNA was preincubated for 15 minutes at 23°C with distamycin (Sigma) prior to addition of the chemical probe. For DEPC reactions, 6 μL of DEPC (Sigma) was added to the samples after preincubation at different temperatures. The samples were mixed at the beginning and at 7 minutes. The reactions were terminated after 15 minutes with the stop solution used for the $KMnO_4$ reactions, except the beta-mercaptoethanol was omitted, and the nucleic acids were precipitated at −70°C for one hour.

Modified DNA was recovered by centrifugation, resuspended in 200 μL of H_2O on ice, and reprecipitated at −70°C in the presence of 20 μL 3M sodium acetate and 500 μL ethanol. The DNA was recovered by centrifugation, washed with 750 μL of 70% ethanol and dried. To cleave the DNA at sites of $KMnO_4$ or DEPC modification, the precipitated DNA was then treated with piperidine and processed as previously described (9). The piperidine cleavage products were separated on DNA sequencing gels and detected by autoradiography. Because of the very low $KMnO_4$ and DEPC reactivity of B-DNA, even a small number of imperfections in the substrate DNA will ultimately lead to inaccurate results. Therefore, it is important to note that these probing reactions cannot usually be performed on synthetic oligonucleotides because such DNA harbors a low amount of piperidine sensitive lesions. Furthermore, if $KMnO_4$ probing of B DNA is performed in certain buffers, such as Tris−HCl, a side reaction occurs between the buffer and the probe producing a new reactive species. This species apparently generates an extra set of piperidine sensitive lesions (as yet uncharacterized) which obscure the specific reaction between $KMnO_4$ and susceptible thymines (J.McC. and H.Buc, unpublished observations).

Chemical sequencing

Guanine specific reaction: labeled DNA was preincubated in 100 μL 50 mM sodium cacodylate (pH 7.0), 2 mM EDTA at 23°C for fifteen minutes. Then 0.5 μL of dimethyl sulfate was added. After 2−3 minutes, the modification reaction was terminated and the modified DNA was processed as described above for the DEPC reactions.

Thymine-specific reaction: this reaction has been described previously (24).

Adenine specific reaction: labeled DNA was preincubated in 100 μL 50 mM sodium cacodylate (pH 7.0), 2 mM EDTA at 90°C for two minutes. 1 μL DEPC was then added, the mix was vortexed, and the reaction was incubated at 90°C for a further 2−3 minutes. The reaction was subsequently quenched on ice, and the modified DNA was processed as described above for the DEPC reaction. Because the single stranded substrate contains no secondary structure at 90°C, the conditions described here produce a very homogeneous purine reactivity; with adenines consistently exhibiting greater DEPC reactivity than guanines (DEPC/90°C; Figure 2). The new adenine reaction conditions presented here should improve the utility of DEPC for genomic sequencing and carboxyethylation interference studies.

Native polyacrylamide gel electrophoresis of kinetoplast DNA

The plasmid pPK201/CAT was digested with Xba I and Sma I, phenol-chloroform-isoamyl alcohol (25:24:1) extracted, and ethanol precipitated. This digest yields a 236 bp *C. fasciculata* bending fragment plus the 3.0 kb vector fragment. The DNA was recovered by centrifugation, washed twice with 70% ethanol, dried, and resuspended in 120 μL TE. Drug binding reactions of 20 μL containing 50 mM Tris−HCl (pH 8.0), 50 mM NaCl, 10 mM $MgCl_2$, and 1 μg of the digested plasmid DNA with 1−5 ng of gel purified 5′ end labeled Xba I-Sma I fragment, were set up at room temperature (18−22°C). Various amounts of distamycin were subsequently added, and the reactions were incubated at room temperature for 10 minutes. Six microliters of gel loading solution (40% sucrose containing bromophenol

blue dye) was added, and the samples were loaded on a 5% native polyacrylamide gel (19:1, acrylamide:bisacrylamide; 1×TBE buffer). Prior to loading, the gel was prerun for approximately 2 hours. The loaded gel was run 14 hours at room temperature (18−22°C) with 0.5×TBE and at 75 volts, it was then stained with ethidium bromide, and photographed. The gel was dried, and the labeled DNA was detected by autoradiography.

RESULTS

Mini circles from the mitochondria of *Crithidia fasciculata* kinetoplasts contain a 211 base pair sequence that has 18 short A-tracts (4−6 bp; 20). Many of the A-tracts are phased with respect to the helical repeat of B DNA, and they are believed to be responsible for the significant curvature of this DNA (20, 25). To study the conformation of this DNA in solution, we have reacted the kinetoplast DNA with two sensitive chemical probes of DNA structure. The first, potassium permanganate ($KMnO_4$), oxidizes the 5-6 double bond of pyrimidines to produce a modified base that is sensitive to piperidine cleavage (T>>C; 26, 27). As base stacking protects pyrimidines from attack by this reagent, $KMnO_4$ can be utilized as a sensitive probe for thymine stacking in B DNA (9). The second probe, diethylpyrocarbonate (DEPC), reacts with double stranded DNA primarily at the N-7 position of purines, and generates a piperidine sensitive carboxyethylated adduct (A>G; 28, 29). The purines of several unusual DNA conformations exhibit elevated levels of reactivity with DEPC, making this reagent one of the most commonly used chemical probes to detect structures such as Z DNA, triple helical DNA, and cruciforms (30−32). Chemical probing with DEPC can also be used to detect subtle sequence specific variations in B DNA structure (9).

$KMnO_4$ and DEPC reactivity towards the thymine rich strand of the *C.fasciculata* bending sequence

The $KMnO_4$ reactivity of thymines in several A-tracts is illustrated in Figure 1A. We consider a thymine sensitive to $KMnO_4$ attack if its reactivity is similar to, or greater than, the piperidine induced cleavage observed at neighboring guanines. Figure 1A shows that, in general, A-tract thymines do not react significantly with $KMnO_4$; although 3′ terminal thymines sometimes react with this probe. This result demonstrates that A-tract thymines have strong, and perhaps equivalent, stacking interactions throughout the tract. The same pattern of $KMnO_4$ reactivity has also been found for short A-tracts in DNA that has no detectable curvature (9), indicating that the thymine strand

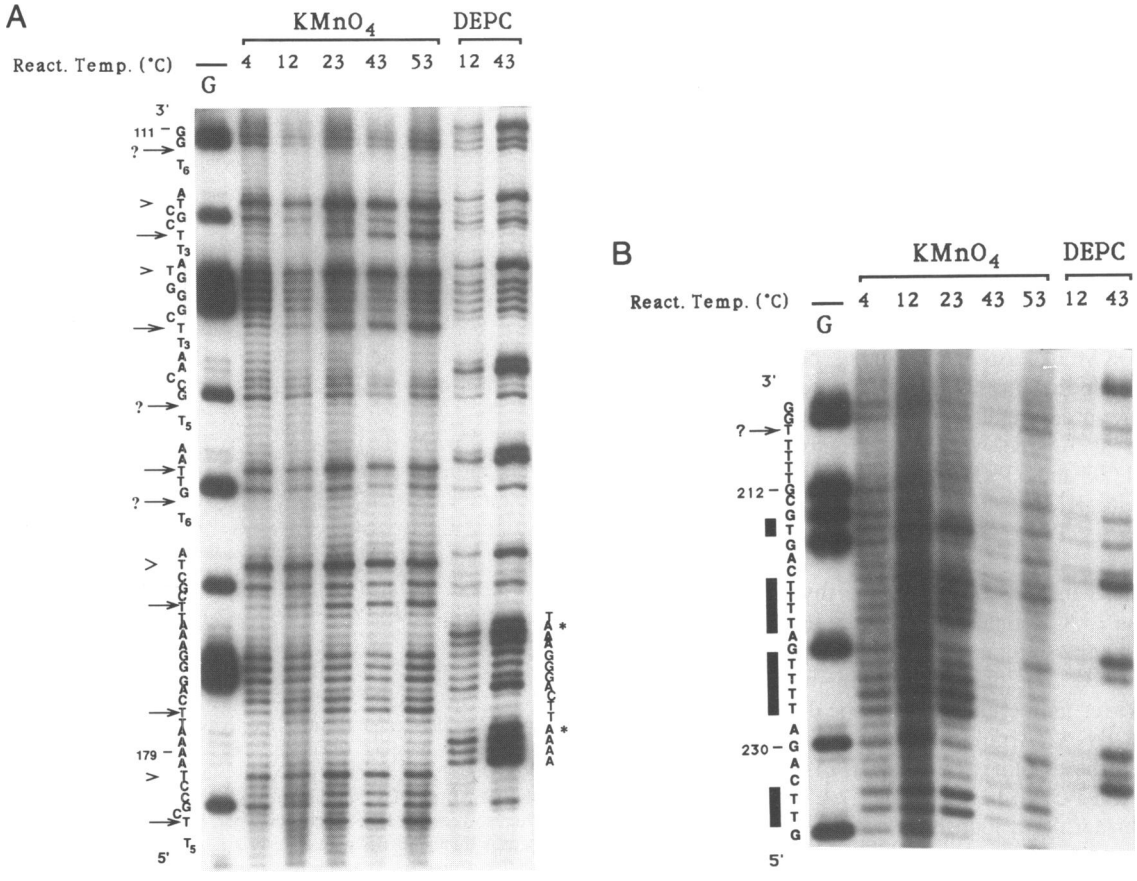

Figure 1. $KMnO_4$ and DEPC reactivity on the thymine rich strand of the *C.fasciculata* bending sequence. The 236 bp Xba I−Sma I fragment of pPK201/CAT, 5′ end labeled at the Xba I site, was reacted with the indicated probes at various temperatures and then treated with piperidine as described in the Methods. Cleavage products were separated on a 7% DNA sequencing gel, and then visualized by autoradiography. Panel A and B are different electrophoretic separations of the same DNA samples. Symbols. Arrowheads indicate single thymines which react with $KMnO_4$; arrows indicate 3′ terminal thymines which react with $KMnO_4$; arrows with (?) indicate the absence of $KMnO_4$ reactivity at 3′ terminal thymines; an asterix marks 3′ terminal adenines displaying reduced DEPC reactivity. Black bars in panel B indicate thymines displaying high reactivity with $KMnO_4$ between 4°C and 23°C.

of most short A-tracts adopts a similar conformation in solution. Note that the $KMnO_4$ reactivity of thymines in double stranded DNA is at least 5−10 fold lower than that seen for 'exposed' thymines in certain DNA structures such as the 'open' complex formed by *E.coli* RNA polymerase at the lac L8UV5 promoter (J.McC and H. Buc, unpublished experiments).

Although earlier experiments had indicated the 3′ terminal thymine of an A-tract usually reacts with $KMnO_4$ (9), the data shown in Figure 1A demonstrates that many of the A-tracts of the kinetoplast DNA exhibit no $KMnO_4$ reactivity from 4°C−53°C at this position (see tracts T 113−T 118 and T 144−T 148). In each case, a guanine flanks the unreactive 3′ terminal A-tract thymine. Therefore, the thymines in these A-tracts apparently form a continuous stacking arrangement with the 3′ flanking base at each of the temperatures examined. Conversely, when other bases flank the 3′ terminal thymine of an A-tract, base stacking across the 3′ junction is generally less stable, as revealed by the accessibility of these thymines to $KMnO_4$ attack at temperatures above 23°C (T 124 and T 135). The contention that the base step between the 3′ terminal thymine and its flanking base is the only point of flexure on the thymine strand is consistent with data presented by Lyamichev (33). This author showed that a 3′ terminal thymine (flanked by an adenine) was the most flexible A-tract thymine.

Attack at $KMnO_4$ sensitive 3′ terminal thymines is often dramatically reduced at the lower temperatures (see for example; T 124−T 127). This observation implies that—in the context of certain sequences—the stacking interaction between a 3′ terminal A-tract thymine and its flanking base can increase significantly as the temperature is lowered. Figure 1A also shows that the $KMnO_4$ reactivity of thymines not present in A-tracts varies considerably, and suggests that large differences exist in the base stacking of these thymines. All of the single thymines in Figure 1A react with $KMnO_4$, although some (T 181) react significantly from 4°C to 53°C, while others (T 129) are much more reactive at the higher temperatures.

One region of the thymine rich strand has an abnormal reactivity with $KMnO_4$. All the thymines between base pairs 214 and 235, including the thymines located in two A-tracts, display an elevated reactivity with $KMnO_4$ when the temperature is at, or below 23°C (Figure 1B). At higher temperatures (43°C and 53°C), only two of these thymines react significantly with $KMnO_4$ (T 219 and T 233). Neighboring thymines, such as T 211−T 207, remain unreactive at all temperatures.

The elevated $KMnO_4$ reactivity of the thymines between T 214 and T 235 at 23°C demonstrates that the stacking interactions between these thymines are reduced as the temperature is lowered from 43°C to 23°C. The base unstacking, which is evident in this region at lower temperatures, could be due to formation of single stranded DNA. Arguing against this explanation is the observation that the adenines between T 214 and T 235 react normally with DEPC at 12°C (A 217, A 229, and A 231; Figure 1B). Furthermore, there was no increase in $KMnO_4$ or DEPC reactivity towards the opposite (adenine rich) strand of this region below 23°C (data not shown). The increased $KMnO_4$ reactivity of thymines between base pairs 214 and 235 is more likely to be due to the induction of a minor strand-specific distortion in this region at temperatures below 23°C. We expect this putative conformational distortion to be quite small because thymines on only one strand became reactive (weakly) with $KMnO_4$, and because the DEPC reactivity of the adenines in this region (on both strands) was unchanged at low temperature. A potentially related low temperature induced distortion of an A.T rich sequence has been reported recently (34).

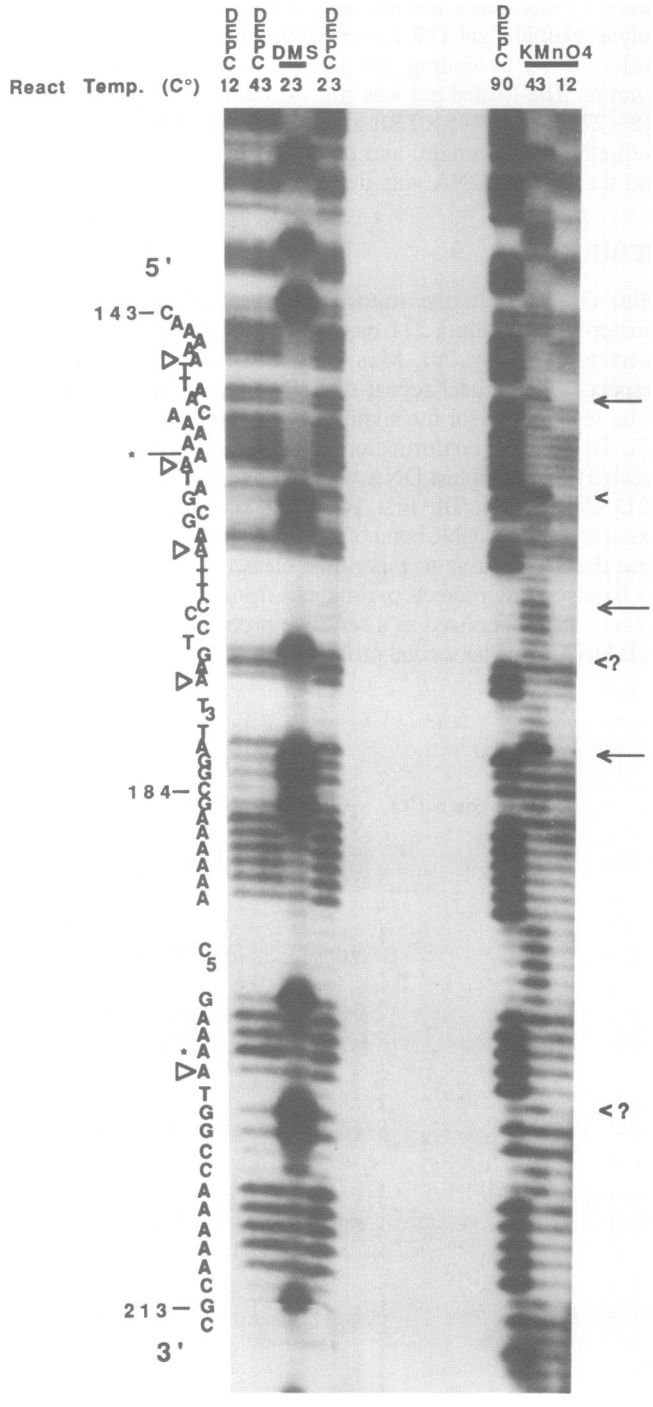

Figure 2. DEPC and $KMnO_4$ reactivity on the adenine rich strand of the *C.fasciculata* bending sequence. The 236 bp Xba I-Sma I fragment of pPK201/CAT, 3′ labeled at the Xba I site, was reacted at various temperatures with $KMnO_4$ and DEPC, then treated with piperidine. The cleavage products were resolved on a DNA sequencing gel. Symbols are the same as for Figure 1, except A-tract adenines with an asterix have a higher DEPC reactivity and open triangles demarcate 3′ terminal A-tract adenines with reduced DEPC reactivity at all temperatures examined. Arrowheads with (?) indicate single thymines that are protected from $KMnO_4$ attack.

DEPC and $KMnO_4$ reactivity towards the adenine rich strand of the *C.fasciculata* bending sequence

The DEPC and $KMnO_4$ reactivity of the adenine rich strand is illustrated in Figure 2. All the A-tract adenines, with the exception of the 3′ terminal adenine, react significantly with DEPC. In general, the A-tracts exhibit two slightly different patterns of DEPC reactivity (9). These two patterns are distinguished by the DEPC reactivity at the penultimate 3′ terminal adenine. In one pattern (class I), all the adenines, except the 3′ terminal adenine, have a slightly elevated DEPC reactivity (A 186−A 191). The second reactivity pattern (class II), is identified by the relatively high level of DEPC attack at the adenine flanking the unreactive 3′ terminal adenine (such as A 158 in the A-tract A 154−A 159). High DEPC reactivity at this 3′ penultimate adenine suggests that A-tracts with class II DEPC reactivity form a more pronounced 3′ end junction with the flanking sequence. This is consistent with the results obtained from an NMR study of a short oligonucleotide with an A-tract which has indicated that the penultimate adenine can participate in the 3′ end A-tract junction (35). Identical patterns of DEPC reactivity (class I and II) have been found for short A-tracts in DNA displaying no aberrant mobility on polyacrylamide gels (9).

Two short stretches of poly d(T) exist on the adenine rich strand of the kinetoplast DNA, and they react normally with $KMnO_4$ (Figure 2). The thymines of these two A-tracts have a high degree of base stacking; only their 3′ terminal thymines react significantly with $KMnO_4$ at 43°C (T 180 and T 169; 3′ flanked by a cytosine and a thymine respectively). One of the three single thymines in Figure 2 (T 160; ATA) exhibits a relatively high reactivity with $KMnO_4$ at 43°C. The other two single thymines on the adenine strand (T 173 and T 202), both of which are flanked on the 3′ side by guanines, display no significant $KMnO_4$ reactivity at 43°C. In agreement with previous results (9), the data in Figure 2 demonstrates that cytosines in short regions of poly d(C). poly d(G) can adopt an unusual conformation as the temperature increases above 23°C. All the cytosines in these short tracts, except the 3′ terminal cytosine, have an elevated $KMnO_4$ reactivity at 43°C (C 170−C 172 and C 192−C 196). No $KMnO_4$ reactivity was observed at cytosines below 23°C. Because short stretches of poly d(C) have no unusual reactivity with $KMnO_4$ when they are present in single stranded DNA (J. McCarthy, unpublished results), the $KMnO_4$ reactivity of cytosines in short poly d(C). poly d(G) tracts illustrated here may be due to a structure induced change in the electronic configuration of these cytosines at 43°C (36). It remains to be determined whether the conformation responsible for the abnormal $KMnO_4$ reactivity found at 43°C for cytosines in short poly d(C). poly d(G) tracts has any relationship to intermediates that may participate in the transition to the non-B structure(s) adopted by long tracts of poly d(C). poly d(G) (37).

Distamycin only partially inhibits the aberrant gel mobility associated with the *C.fasciculata* kinetoplast DNA

In general, the binding of distamycin to fragments of DNA results in a decreased electrophoretic mobility of the DNA (3). However, when this drug is complexed with the highly bent fragment of *Leishmania tarentolae* kinetoplast DNA, the drug-DNA complex migrates with the mobility expected for this fragment (3). To learn more about the mechanism by which distamycin normalizes the mobility of such natural bending sequences, we investigated the effect of this drug on the gel mobility of the bent kinetoplast DNA from *Crithidia fasciculata*.

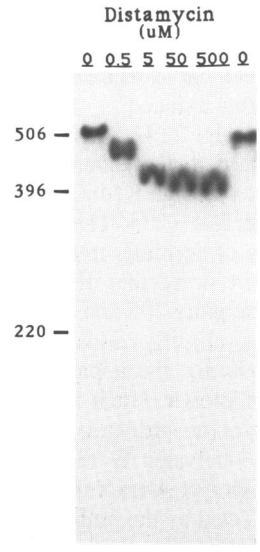

Figure 3. Distamycin partially eliminates the aberrant gel mobility of a fragment containing the *C.fasciculata* bending sequence. Gel pure 236 bp Xba I−Sma I fragment of pPK 201/CAT, 5′ end labeled at the Xba I site, was mixed with 1 μg of Xba 1−Sma 1 digested pPK201/CAT plasmid DNA in binding buffer (see Methods), and then incubated with different amounts of distamycin for 15 minutes. Subsequently, the DNA was run on a native 5% polyacrylamide gel and visualized by autoradiography. The numbers on the left correspond to the base pair sizes of marker fragments.

The influence of distamycin on the gel mobility of a fragment containing the bent DNA of *C. fasciculata* is illustrated in Figure 3. In the absence of drug, this fragment migrated very anomalously, exhibiting a relative length of 2.08 on a 5% gel. Unexpectedly, addition of distamycin only partially decreased the aberrant mobility of this DNA. Even in the presence of a high level of distamycin (0.5 mM), the mobility of the 236 bp *C. fasciculata* fragment was still significantly retarded (relative length of 1.65). In fact, we have yet to find conditions under which distamycin eliminates more than 40% of the anomalous behavior of the *C. fasciculata* fragment. Control experiments run under identical conditions have demonstrated that several other similarly sized fragments of DNA were all significantly retarded in the presence of 0.5 mM distamycin (data not shown).

To confirm that distamycin completely eliminates the aberrant gel mobility of the *L. tarentolae* kinetoplast DNA fragment (3), but only partially eliminates the aberrant mobility of the *C. fasciculata* kinetoplast DNA, we directly compared the effect of this drug on the mobility of these two bent DNA fragments under identical electrophoretic conditions. As predicted, 50 μM distamycin restored normal mobility to the bent *L. tarentolae* fragment, but only partially restored the expected mobility for the bent *C. fasciculata* fragment (data not shown). Therefore, while distamycin is capable of eliminating the aberrant mobility of some naturally occurring bent DNA fragments at ambient temperature (3), the data presented indicates that this effect is not universal for all bent fragments.

Distamycin eliminates the high $KMnO_4$ reactivity associated with two A-tracts of the *C.fasciculata* DNA at lower temperatures

The results presented above demonstrate that distamycin partially reduces the aberrant gel mobility of the bent *C. fasciculata*

kinetoplast DNA. To investigate the structural basis for this effect in more detail, we used $KMnO_4$ to probe the complex(es) formed at 23°C between the *C. fasciculata* DNA and distamycin. The most striking drug induced conformational alteration was found between base pairs 214−235 (Figure 4A). Even at the lowest concentration of distamycin tested (50 μM), the drug completely eliminated the high $KMnO_4$ reactivity normally seen at 23°C for base pairs 214−235. The drug had no influence on the $KMnO_4$ reactivity of thymines in the neighboring A-tract (T 198−T 201). We have noted that distamycin appears to cause guanines between base pairs 205 and 230 (especially G 230 and G 224) to become slightly sensitive to $KMnO_4$ attack (Figure 4A). The basis for the apparent $KMnO_4$ reactivity of these guanines is not known, but it is specific for this region. Distamycin had little or no influence on the $KMnO_4$ reactivity of the many guanines between G 184 and G 88 (Figure 4B).

The $KMnO_4$ reactivity of A-tract thymines between T 83−T 198 was largely unaffected by the presence of 50 μM distamycin. However, we have already shown that distamycin at this concentration produces the maximum reduction of DNA bending (Figure 3). Taken together, these results imply that the loss of bending in the presence of 50 μM distamycin is not due to a major drug induced change in the conformation of the A-tracts. Furthermore, the absence of an alteration in the $KMnO_4$ reactivity of A-tract junctions at 50 μM distamycin suggests that this drug does not distort these junctions. However, the experiments presented here are unable to rule out the possibility that 50 μM distamycin significantly alters the conformation of the phosphate backbone−either within A-tracts, at A-tract/flanking sequence junctions, or between the A-tracts—and thereby influences the gel mobility of the bent kinetoplast DNA.

Higher concentrations of distamycin (0.25 and 1.0 mM) produced a few minor changes in the $KMnO_4$ reactivity between T 83−T 198 (Figure 5B). For example, the 3′ terminal thymines in A-tracts T 113−T 118 and T 144−T 148—which are situated in normally unreactive 5′ TG 3′ junctions—became susceptible to $KMnO_4$ attack upon addition of 0.25 and 1.0 mM distamycin. This effect, however, was not observed at all 5′ TG 3′ junctions. For instance, T 154, which is also within a 5′ TG 3′ A-tract junction, only exhibited a slightly higher level of $KMnO_4$ reactivity at 0.25 and 1.0 mM distamycin (Figure 4B). The relationship of the latter conformational changes to the distamycin induced reduction of DNA bending remains to be determined.

Outside the A-tracts, one major conformational change was detected at all the concentrations of distamycin studied. Figure 4B illustrates that addition of distamycin increased the $KMnO_4$

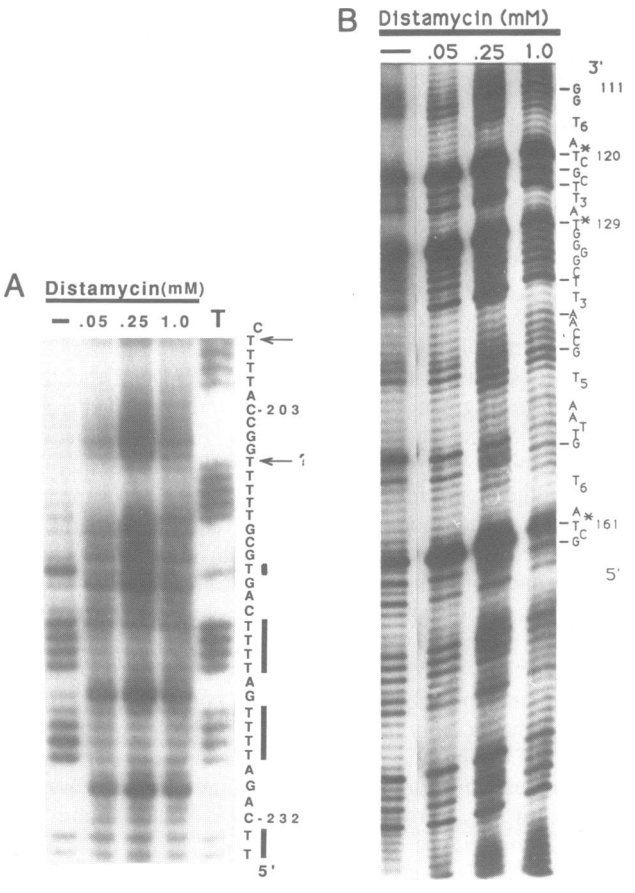

Figure 4. Distamycin bocks frmation of the low temperature induced conformation. The 236 bp Xba I−Sma I fragment of pPK201/CAT, 5′ end labeled at the Xba I site, was incubated with the indicated amounts of distamycin at 23°C and then reacted with $KMnO_4$ at this temperature. The reactions were stopped, treated with piperidine, and the cleavage products were run on a DNA sequencing gel. Panels A and B represent different electrophoretic separations of the same samples. Symbols are the same as in Figure 1, except that the asterisks in panel B indicate thymines at 5′ApT3′ base steps that have some $KMnO_4$ hyperreactivity in the presence of distamycin.

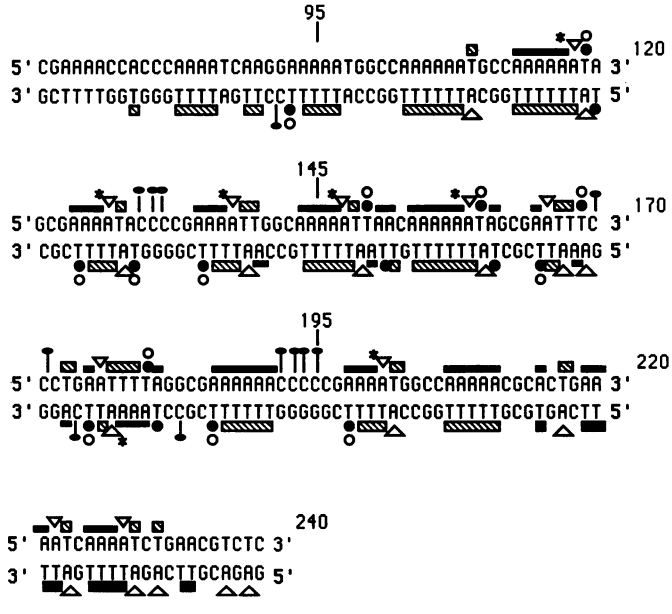

Figure 5. Summary of the $KMnO_4$ and DEPC reactivity of the *C.fasciculata* bending sequence. Symbols for the $KMnO_4$ reactivity of both strands at 12, 23 and 43°C: hatched bars indicate thymines that react poorly with $KMnO_4$ at all temperatures; solid bars indicate thymines that react significantly with $KMnO_4$ at 12°C and 23°C, but not at 43°C; closed circles indicate thymines that react significantly at all temperatures examined; double circles (open and closed) indicate thymines that react poorly with $KMnO_4$ at 4°C but react significantly with this probe at higher temperatures. Symbols for the DEPC reactivity of the top strand at 23°C and the bottom stand at 12°C: speckled bars identify adenines with DEPC reactivities greater than that seen at guanines; an asterix identifies the elevated DEPC reactivity at a penultimate 3′ terminal adenine; triangles identify adenines with DEPC reactivities similar to, or less than, the DEPC reactivity of guanines; the stick figures demarcate cytosines with an unusually high $KMnO_4$ reactivity at 43°C.

reactivity at the single thymines T 120, T 129, and T 161 (each of which are present in a 5'TA3' step). Since these conformational alterations are detected at 50 μM distamycin, it is plausible that changes in base stacking at these single thymines may contribute to the 40% reduction of bending observed for the distamycin/*C.fasciculata* DNA complex. This conclusion is consistent with earlier data showing that the aberrant mobility of a naturally occurring bent fragment can be significantly altered by a single base substitution in the DNA separating the A-tracts (38).

DISCUSSION

The chemical reactivity patterns observed for A-tracts in the bent kinetoplast DNA are closely related to those found earlier for short A-tracts in DNA that migrates normally on polyacrylamide gels (9). This result implies that most A-tracts form one 'general' A-tract specific conformation (often referred to as B'). A distinctive feature of this conformation is the absence of $KMnO_4$ reactivity with thymines, although the 3' terminal A-tract thymine often displays a weak reactivity with $KMnO_4$ (Figure 5; also see ref. 9). The $KMnO_4$ reactivity data presented here, and earlier (9), demonstrate that A-tract thymines, excluding the 3' terminal thymine, have strong, and probably equivalent stacking interactions with one another. The absence of $KMnO_4$ reactivity at 5' terminal A-tract thymines implies that these thymines also have a strong stacking interaction with their respective 5' flanking bases (A,C, or G).

The $KMnO_4$ reactivity detected at 3' terminal thymines is probably due to attack perpendicular to the 3' face of the 5-6 double bond: $KMnO_4$ attack at the 5' face of this terminal thymine is likely to be blocked via its strong stacking interaction with the adjacent (5' flanking) thymine. However, the data presented in Figure 1A clearly demonstrates that when a 3' terminal A-tract thymine is flanked by a guanine, no $KMnO_4$ attack is detected at that thymine (at any temperature). This implies that the 5' TG 3' step favors a highly stacked configuration at each of the temperatures studied. In fact, reexamination of earlier experiments documenting the $KMnO_4$ reactivity of more than 15 A-tracts (9) also shows that when a guanine flanks a 3' terminal A-tract thymine, no $KMnO_4$ reactivity occurs at that thymine. It is interesting to note that two different bent kinetoplast DNA both possess an unusually large number of A-tracts with a 5' TG 3' junction; 8 of 19 A-tracts in the *C. fasciculata* sequence (Figure 5), and 14 of 27 A-tracts >3 bp in the *L. tarentolae* bending sequence (39) have this junction. Unfortunately, because the function of the kinetoplast bending sequence is not known, the biological significance of the numerous 5' TG 3' A-tract junctions in kinetoplast DNA remains to be elucidated.

Among a set of A-tract containing oligonucleotides studied by Koo et al. (5), the oligonucleotide with the greatest bending has a 5' TG 3' base step at its 5' A-tract junction. Similarly, experiments carried out by Abagan et al. (40) imply that the presence of a 5' TG 3' step at the 5' junction exerts a strong influence on DNA bending. This group has shown that ligation products of the oligonucleotide 5' d(CCAAAAAAGG). 5' d(CCTTTTTTGG) migrate much more aberrantly than ligation products of the closely related fragment 5' d(GGAAAAAAGG). 5' d(CCTTTTTTCC). Such observations, together with the data presented here, prompt us to propose that the A-tract junction 5' TG 3' acts to accentuate the overall bend of an A-tract. It appears plausible that stable base stacking between the 3' terminal A-tract thymine and a flanking guanine generates a unique deflection of the DNA helix at the 5' end of an A-tract, and that the net effect of this deflection is to amplify the bending intrinsic to the A-tract sequence itself. The base step 5' TG 3' is conserved in a number of important sequences such as the yeast telomere repeat ($C_{1-3}A$; 41), the consensus recognition sequence of helix-loop-helix proteins (CANNNGT; 42), and the CAAT box of eucaryotic promoters (43). The stable base stacking associated with the base step 5' TG 3' may be a unique and important element of these protein-DNA interactions.

Only one significant conformational change has been detected in the A-tracts as the temperature was lowered. That is, $KMnO_4$ reactivity with 3' terminal A-tract thymines frequently disappeared at lower temperatures (Figure 1A). In relation to the model proposed above, this latter observation suggests that lower temperatures stabilize the stacking interaction between the 3' terminal thymine and its 5' flanking base, and thereby increases DNA bending. This interpretation is supported by the fact that A-tract directed DNA bending is dramatically increased by lowering the temperature (7). Two recent reports further support our contention that changing the stacking interaction between the 3' terminal thymine and its flanking base results in an alteration of DNA bending. Hagerman has shown that replacing a 3' terminal thymine with uracil can increase DNA bending as much as replacing all the A-tract thymines with uracil (44). Using a related base substitution approach, Diekmann et al. (45) have demonstrated that base stacking on the thymine strand, especially at the 3' end of this strand, impacts significantly on A-tract directed DNA curvature.

Another characteristic feature of the 'general' A-tract conformation is the 3' end junction it forms (on the adenine strand) with the flanking sequence (9). This 3' end junction, which is manifest by the weak DEPC reactivity at the 3' terminal adenine, is also present in A-tracts of the *C. fasciculata* bending sequence (Figure 2, Figure 5). Poor reactivity of the 3' terminal adenine is apparently due to the inability of DEPC to approach the N-7 position of this adenine. DEPC also displays an increased reactivity towards the penultimate 3' terminal adenine of some A-tracts (Figure 5; 9), possibly signifying the existence of a higher roll angle at this position. In fact, the 3 angstrom resolution structure of the CAP–DNA complex demonstrates that the 3' penultimate base pair of an A-tract in the 30 base pair fragment has a relatively high roll angle (46). The high level of roll at this position is associated with an 8° bend. Whether this bend exists when the 30 mer is free in solution is not yet known.

Variable DEPC reactivity at the penultimate 3' terminal adenine may also reflect the existence of two different types of junction between the 3' end of short A-tracts and their flanking sequences. Because the majority of the bending of an A-tract is thought to be produced at the 3' end junction (5), these two postulated 3' end junctions could be associated with different levels of DNA bending. Chemical probing experiments and gel mobility measurements using appropriately designed plasmid sequences would address this issue.

The most striking structural anomaly associated with the bent *C.fasciculata* DNA is the low temperature induced distortion found between base pairs 214–235. At 23°C and below, this region assumes a novel conformation which is characterized by the increased $KMnO_4$ reactivity of the thymine rich strand. This low temperature induced conformational distortion is specific for the thymine rich strand because no temperature induced changes

have been detected on the opposite strand by chemical probing. Because the $KMnO_4$ reactivity of thymines in double stranded DNA is probably sensitive to small conformational changes, the relatively small increase in the $KMnO_4$ reactivity between bp 214–235 is likely to be due to a small change in the roll and/or tilt of the reactive thymines. The absence of detectable changes in the DEPC reactivity of adenines in the 214–235 bp region between 12°C and 43°C (Figure 1B) supports the proposal that this region undergoes only a minor conformational distortion.

Addition of 50 μM distamycin resulted in the loss of the low temperature induced conformational distortion at base pairs 214–235 (Figure 4A). This was the only structural change specifically detected at the A-tracts in the presence of 50 μM distamycin. This observation, together with the fact that 50 μM distamycin caused the maximum reduction of bending (40%; Figure 3), suggests that the low temperature conformation may contribute to the highly aberrant gel mobility of the bent *C.fasciculata* kinetoplast DNA. How might the low temperature conformation influence the electrophoretic mobility of DNA? The unusual conformation adopted between base pairs 214–235 may retard the mobility of DNA by being asymmetrically under-wound on one strand, thereby generating curvature. This additional curvature would thus augment the intrinsic DNA bending associated with the helically repeated A-tracts. Or, the unusual structure between base pairs 214–235 may effect the mobility of the *C.fasciculata* DNA by exhibiting a partially melted character. Meyers et al. (47) have invoked a similar proposal to explain the reduced gel mobility of DNA containing mismatches.

To date, only one published report has directly shown that distamycin can reduce the bending associated with a simple (repetitive) bending sequence at 4°C (48). However, the maximum reduction of bending by this model sequence was only 70–80% (A. Minskey, personal communication). Because these workers have not studied the effect of distamycin on the bending of such oligonucleotides at ambient temperature, it is not known whether the drug mediated inhibition of bending in these synthetic sequences would be larger or smaller at the higher temperatures studied here. Therefore, it is difficult to compare the influence of distamycin on the gel mobility of this model sequence at 4°C (48) with our results obtained for the *C. fasciculata* kinetoplast DNA at ambient temperature. Nevertheless, although distamycin has been reported to completely eliminate the aberrant gel mobility associated with naturally occurring bent DNA (3, 17, 25), our results clearly demonstrate that this effect is not universal.

Finally, can the minor distortion of the A+T rich DNA between base pairs 214–235 be biologically significant? Scaffold attachment regions (SARs) are A+T rich sequences which interact specifically with the nuclear scaffold, and are thought to be involved in the organization of DNA loops and higher order chromatin structures in eucaryotic nuclei (49). Kas et al. (17) have shown that histone H1 binds preferentially to SAR sequences and they have suggested that specific binding of H1 and other nuclear scaffold binding proteins to SARs may be directed by a specific feature of the A-tracts in SAR sequences. Histone H1 may preferentially bind to A+T rich segment(s) of SAR sequences via the ability of this DNA to adopt an asymmetrically under-wound structure related to the one described here. In fact, Bode et al. (50) have recently shown that a potential SAR sequence (or MAR sequence; matrix attachment region) can adopt an under-wound conformation when subject to negative superhelical stress. The proposal that an asymmetrically under-wound conformation of A+T rich DNA may facilitate the binding of certain DNA binding proteins, such as histone H1, is also supported by the observation that relatively low concentrations of distamycin (20–50 μM) can both inhibit the aberrant mobility of DNA containing a SAR sequence and eliminate the preferential binding of H1 to a SAR sequence (17). $KMnO_4$ probing of various SAR sequences, in the presence and absence of histone H1, could shed light on the intrinsic flexibility of this important class of structural/regulatory sequences.

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