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Chemical Reactivity of Potassium Permanganate and Diethyl Pyrocarbonate with B DNA: Specific Reactivity with Short A-Tracts†

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ABSTRACT: We have examined the reactivity of B DNA with two chemical probes of DNA structure, potassium permanganate ($KMnO_4$; thymine specific) and diethyl pyrocarbonate (DEPC; purine specific, A > G). The DNA probed is from the β-lactamase promoter region of the vector pBR322, and from the 3′ noncoding region of a chicken embryonic myosin heavy chain gene. The chemical probes display variable reactivity with the susceptible bases in these fragments, suggesting that modification of these bases by $KMnO_4$ and DEPC is quite sequence dependent. In contrast, these probes react with the short A-tracts present in these DNA fragments in a reproducible fashion, generating two related patterns of reactivity. In the majority of the A-tracts, all but the 3′-terminal thymine are protected from $KMnO_4$ attack, while DEPC reacts significantly with all but the 3′-terminal adenine of the A-tracts. Some A-tracts also display a very high DEPC reactivity at the adenine adjacent to the 3′-terminal unreactive adenine. Little qualitative difference in the $KMnO_4$ reactivity of the A-tracts was found between 12 and 43 °C. However, at lower temperatures the elevated $KMnO_4$ reactivity at the 3′-terminal A-tract thymine is sometimes lost. Raising the temperature of the $KMnO_4$ reaction can cause relatively large increases in the reactivity of some single thymines, suggesting that significant local changes in stacking occur at these thymines at elevated temperatures. The data presented suggest that many short A-tracts embedded in long fragments of DNA can assume a number of related structures in solution, each of which possess distinct junctions with the flanking DNA. This result is consistent with high-resolution structural studies on oligonucleotides containing short A-tracts. The relevance of these results to current models of A-tract structure and DNA bending is discussed. Our data also indicate that $KMnO_4$ and DEPC are potentially useful reagents for the study of sequence-dependent variations in B DNA structure.

Over the last 10 years, high-resolution X-ray crystallographic analysis of B DNA in single crystals (Wing et al., 1980; Dickerson & Drew, 1981; Fratini et al., 1982; Coll et al., 1987; Nelson et al., 1987; Yoon et al., 1988) has clearly demonstrated that B DNA can possess a remarkably high degree of sequence-dependent structural heterogeneity. Sequence-dependent variations in B DNA structure are manifested by rather small changes in the positions of the bases relative to their positions in idealized B DNA, and by subtle changes in the conformation of the ribose–phosphate backbone. Evidence for structural heterogeneity of B DNA in solution has been obtained from a number of different experimental approaches, such as Raman and NMR spectroscopies (Leroy

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et al., 1988; Taillandier et al., 1987; Kintanar et al., 1987; Katahira et al., 1988; Nadeau & Crothers, 1989).

One of the most distinct and extensively studied examples of the structural heterogeneity of B DNA is the altered conformation assumed by poly(dA)·poly(dT). This synthetic polymer displays a number of anomalous properties, such as an unexpectedly high melting temperature (Arnott et al., 1976) and exclusion from nucleosome assembly (Rhodes, 1979). The bending associated with short, phased runs of poly(dA)·poly(dT) (A-tracts) is also believed to be due to the altered B DNA helix assumed by short A-tracts (Marini et al., 1982; Wu & Crothers, 1984; Hagerman, 1985; Nadeau & Crothers, 1989). Using X-ray diffraction data from sodium salt fibers of poly(dA)·poly(dT), Arnott et al. (1983) proposed that the most striking difference between poly(dA)·poly(dT) and idealized B DNA was the presence of different sugar puckers for each strand of the duplex. In this heteronomous model of poly(dA)·poly(dT), the conformation of the two strands are different, with the poly(dA) strand assuming an A-type conformation and the poly(dT) strand assuming a B-type conformation. More recently, others have reinterpreted these sodium fiber diffraction data and proposed other models for poly(dA)·poly(dT). The model of Alexeev et al. (1987), subsequently modified by Lipanov and Chuprina (1987), proposes that both strands of poly(dA)·poly(dT) have the same sugar pucker (C2′-endo, as in idealized B DNA). This model of the polymer also has an unusually narrow minor groove and a significant negative tilt (−7°) of the bases. Aymami et al. (1989) have proposed another structure, called the Bp DNA model of poly(dA)·poly(dT). The Bp model incorporates recent crystallographic data which have demonstrated that A·T base pairs within short regions of poly(dA)·poly(dT) can possess a very high propellor twist and form bifurcated hydrogen bonds (Nelson et al., 1987; Coll et al., 1987; DiGabriele et al., 1989). In the Bp DNA model, poly(dA)·poly(dT) has a very narrow minor groove, high propeller twist, and a system of cross strand bifurcated hydrogen bonds (Aymami et al., 1989).

The structure of oligonucleotides containing short tracts of poly(dA)·poly(dT) in solution has also been investigated by NMR spectroscopy. The study of d(CGA$_5$TCGG)·d(CCGAT$_5$CG) by Kintanar et al. (1987) revealed that the 5′- and 3′-terminal A·T base pairs of the A-tract exhibit unusual structural deviations, indicating that significant junctions exist between the A-tract and the flanking DNA. In addition, the second to last A·T base pair appears to participate in the 3′ junction, suggesting that this junction is more distorted than the 5′ junction. They also noted that the 5′-terminal A·T base pair may have a significant propeller twist and that the A-tract had a narrow minor groove. Katahira et al. (1988) have reported that the A-tract structure in d(CGCA$_6$GCG)·d(CGCT$_6$GCG) has a high propeller twist of the A·T base pairs, progressive narrowing of the minor groove in the 5′–3′ direction of the A-tract, and distinct junctions with the G/C flanking sequences. Recently, Nadeau and Crothers (1989) have examined the structure(s) formed by A-tracts of various lengths in the oligonucleotides d(GGCA$_n$CGG)·d(CCGT$_n$GCC) (n = 3–5, 7, 9) by NMR. In agreement with the previous results (Kintanar et al., 1987; Katahira et al., 1988) and those from hydroxyl radical cleavage experiments with DNA containing short A-tracts (Milgram-Burkhoff & Tullius, 1987), they found that the minor groove of A-tracts (>3 bp long) narrows in the 5′–3′ direction and that these tracts possess distinct junctions with the flanking G/C-rich DNA. However, in contrast to the two previous reports, Nadeau and Crothers' (1989) study of A-tract-containing oligonucleotides found no significant propeller twist in these A-tracts, although they did find evidence for considerable base pair tilt at the center of the longer tracts. Thus, while these studies clearly demonstrate that the conformation of A-tracts in solution deviates significantly from that of idealized B DNA, the precise structure(s) assumed by an A-tract in solution is (are) still uncertain.

Many of the investigations on the detailed structure of B DNA, and on the structural heterogeneity inherent in this particular polymer, have employed short oligonucleotides. However, it is also important to relate the structural information obtained from these studies on oligonucleotides to the structure of longer fragments of DNA. In the last few years, various chemical probes of DNA structure have been successfully used to detect large alterations of DNA structure such as the B–Z DNA transition (Herr, 1985; Johnston & Rich, 1985), the induction of triple-stranded DNA (Voloshin et al., 1988; Hanvey et al., 1988; Kowhi & Kowhi-Shigematsu, 1988; Htun & Dahlberg, 1988), formation of cruciforms (Lilly & Palecek, 1984; McClellan et al., 1986; Furlong & Lilley, 1986; Scholten & Nordheim, 1986), and the narrowing of the minor groove associated with short A-tracts (Milgram-Burkhoff & Tullius, 1987). If short A-tracts embedded in longer fragments of B DNA possess a distinctive conformation, is it possible that chemical probes could be used to find evidence for the presence of this (these) unique A-tract structure(s)? To address these issues, we have reacted two DNA fragments with the structural probes diethyl pyrocarbonate (DEPC) and potassium permanganate ($KMnO_4$). We find that these probes have unusual reactivities with A-tracts, particularly near the junctions between the A-tracts and the flanking DNA. We also show that these probes generally react with B DNA in a sequence-specific fashion and that one of these probes ($KMnO_4$) is a useful reagent to detect subtle temperature-induced alterations in the B DNA helix. The relevance of the unique reactivity of $KMnO_4$ and DEPC with short A-tracts to various models of the A-tract DNA structure is discussed.

Experimental Procedures

Plasmid DNA. The plasmids pDPL6 (David Pulleyblank) and pXH-N were isolated from *Escherichia coli* by lysis with lysozyme as described (Maniatis et al., 1982), except that 10% sarcosyl was substituted for SDS, and further purified by two rounds of cesium chloride density centrifugation in the presence of ethidium bromide (Maniatis et al., 1982) to obtain plasmid that was >75% supercoiled. The plasmid pDPL6 is a derivative of pBR322 which has nucleotides 21–651 (*Hin*dIII–*Sal*I), 778–2352 (*Hae*II–*Hae*II), and the internal 4 bp of the *Eco*RI site removed. A small polylinker fragment containing *Hin*dIII, *Xba*I, *Sma*I, *Bam*HI, and *Sal*I sites was then inserted into the *Hin*dIII and *Sal*I sites. The plasmid pXH-N has the 217 base pair *Xba*I–*Hin*dIII fragment of pCEMH 1.3 (McCarthy & Heywood, 1987) subcloned into these sites of the vector pDPL6. Fragments of pDPL6 labeled with ^{32}P at one end were obtained by digestion of 2–4 μg of plasmid DNA with the appropriate restriction endonuclease. The restriction sites used to generate the labeled fragments are shown in Figure 1A. For 5′ end labeling reactions, completed restriction digests were made 100 mM in EDTA, and 200 units of bacterial alkaline phosphatase was added. This mixture was incubated for 1–2 h at 60 °C, then exhaustively phenol extracted, and ethanol precipitated. The dephosphorylated DNA was labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP, followed by one phenol–chloroform–isoamyl alcohol extraction and ethanol precipitation. 3′ end labeled DNA was obtained by filling in a 3′ recessed end with the large fragment of *E. coli* DNA

polymerase 1 and the appropriate [α-^{32}P]dNTP. 5′ or 3′ end labeled DNA was then recut with a second restriction enzyme to produce a unique fragment with label at one end which contains the sequence of interest. This second digest was run on a native 5% polyacrylamide gel. The appropriate gel slice was excised, and the DNA was subsequently eluted from the gel by diffusion into TE buffer (Maniatis et al., 1982).

$KMnO_4$ Reactions. One hundred microliter reactions containing 3′ or 5′ end labeled DNA in 50 mM sodium cacodylate, pH 7.0, and 2 mM EDTA were preincubated at the appropriate temperature for 15 min. Then 4 μL of a 50 mM solution of $KMnO_4$ (Aldrich) was added (final concentration 2.0 mM). Reactions were allowed to proceed at appropriate temperatures for 4 min. For reactions carried out with denatured DNA, the DNA reaction mix was boiled for 2 min, quick chilled, and then preincubated at 23 °C for 15 min prior to chemical treatment. The reactions were stopped by adding 300 μL of an ice-cold solution containing 294 μL of ethanol, 3 μL of β-mercaptoethanol, and 3 μL of 2 μg/μL tRNA. Finally, 10 μL of 3 M sodium acetate was added and the modified DNA immediately precipitated at −70 °C for 1 h. Precipitated DNA was pelleted in a microcentrifuge, resuspended in 200 μL of H_2O on ice, and reprecipitated with 20 μL of sodium acetate and 500 μL of ethanol at −70 °C for 1 h. The DNA was repelleted, washed with 750 μL of 70% ethanol, and dried. (Note: DNA with radioactive label on a 5′ overhanging thymine is not a suitable substrate for $KMnO_4$ reactions due to the extreme sensitivity of this base to $KMnO_4$ attack relative to internal thymines.)

DEPC Reactions. One hundred microliter reactions containing 3′ or 5′ end labeled DNA in 50 mM sodium cacodylate, pH 7.0, and 2 mM EDTA were preincubated at appropriate temperatures for 15 min. Then 6 μL of DEPC was added and incubated for 15 min at appropriate temperatures. Reaction mixes were vortexed vigorously at the start of the DEPC reaction and after 7 min as previously described (Herr, 1985). Identical reactions were carried out on denatured DNA (obtained as described above). DEPC reactions were terminated and precipitated as above, except the β-mercaptoethanol was omitted.

Chemical Sequencing. Guanine-Specific Reactions. One hundred microliter reactions were set up as described for the DEPC reactions and preincubated at room temperature for 15 min, then 0.5 μL of dimethyl sulfate was added, and the mixture incubated for 2–3 min at room temperature. Reactions were terminated and subsequently processed as in the DEPC reactions above. A+C reactions were carried out essentially as described previously (Bencini et al., 1984).

Piperidine Cleavage and Analysis of the Modified DNA. The dried, modified DNA was resuspended in 40 μL of 1 M piperidine and heated to 92 °C for 30 min (Maxam & Gilbert, 1980). Piperidine was removed by a butanol precipitation procedure (Bencini et al., 1984) with some modifications. Briefly, 900 μL of butanol was added to the piperidine cleavage reactions, and after 5 min on ice, the DNA was pelleted by a 5-min spin in a microcentrifuge at 4 °C. The DNA pellet was resuspended in 100 μL of 0.1% SDS on ice by vortexing and reprecipitated with 100 μL of 70% ethanol and 800 μL of butanol as above. The DNA pellet was then resuspended in 100 μL of H_2O and 30 μL of 3 M sodium acetate, followed by an ethanol precipitation at −70 °C for 1 h. The DNA pellet was washed twice with 750 μL of 70% ethanol and dried. The DNA was then resuspended in 50 μL of H_2O and lyophilized for 2 h. Finally, the DNA cleavage products obtained were resuspended in 80% formamide and 1 × TBE (Maniatis et al., 1982) containing bromophenol blue dye and run on a DNA sequencing gel. Control lanes containing $KMnO_4$ or DEPC-modified DNA (without piperidine treatment) were also often run on the sequencing gels. The absence of significant cleavage in such lanes clearly demonstrated that the vast majority of the cleavage detected after piperidine treatment was due to the specific modifications introduced by prior chemical treatments described above (data not shown). Autoradiographs of dried gels were obtained by using Kodak XAR-5 film. Scanning densitometry of the autoradiograms was done on an Apple scanner, and the data obtained were analyzed by using the scan analysis program from Biosoft.

Chemical Treatment and Analysis of the Fragment from pXH-N Containing Multiple A-Tracts. The 217 base pair *Xba*I–*Hin*dIII fragment of pXH-N was 5′ end labeled on both strands as described above and was subsequently gel isolated. The gel-purified, labeled DNA was then reacted with DEPC and $KMnO_4$ at the appropriate temperatures. After the chemicals were removed, the modified samples of DNA were denatured by heating to 90 °C in the presence of 80% formamide and run on a native 6% polyacrylamide gel to separate the strands. The purified single strands were eluted from the gel into TE buffer. The gel-pure, modified single-stranded DNA was subsequently treated with piperidine, and the cleavage products were run on a DNA sequencing gel as described above.

Results

To study small variations in the structure of relatively long stretches of B DNA at nucleotide resolution, we have investigated the reactivity of B DNA with chemical probes of DNA structure (Lilley & Palecek, 1984; Herr, 1985; Borowiec et al., 1987). We have chosen small chemical probes which react (i) with well-characterized chemistry, (ii) with at least some base specificity, and (iii) in the major groove of B DNA where many sequence-specific interactions with biopolymers take place (Seeman et al., 1976). Because the structure of short A-tracts is widely believed to deviate significantly from that of idealized B DNA, one of our criteria for selecting a chemical probe capable of recognizing structural variations within B DNA is that it should display some distinct reactivity pattern(s) with short tracts of poly(dA)·poly(dT). Here we demonstrate that two chemical probes of DNA structure, $KMnO_4$ and DEPC, satisfy these requirements.

$KMnO_4$ Reactivity of B DNA at Different Temperatures. $KMnO_4$ reacts with DNA primarily via oxidation of the 5–6 double bond of pyrimidines (T ≫ C; Howgate et al., 1968; Iida & Hayatsu, 1971). Because the 5–6 double bond of thymine is attacked from above or below the plane of the base, stacking affords protection from this reaction (Hayatsu & Ukita, 1967). When base stacking is disrupted (as in the transition from double-stranded DNA to single-stranded DNA), the susceptibility of thymines to attack by $KMnO_4$ increases significantly (Hayatsu & Ukita, 1967; Rubin & Schmid, 1980; J. McCarthy, unpublished results). Hence, $KMnO_4$ has been utilized for chemical sequencing of single-stranded DNA (dT specific; Rubin & Schmid, 1980; McCarthy, 1989), and recently this probe has also been employed to detect regions of B DNA that have become either severely distorted, or denatured, as a result of protein binding (Borowiec et al., 1987; Borowiec & Hurwitz, 1988; O'Halloran et al., 1989).

To detect small variations in the stacking of thymines in B DNA, 5′ or 3′ end labeled fragments of plasmid DNA were reacted with $KMnO_4$. Modification of the bases was detected by cleavage with piperidine and resolution of the products on

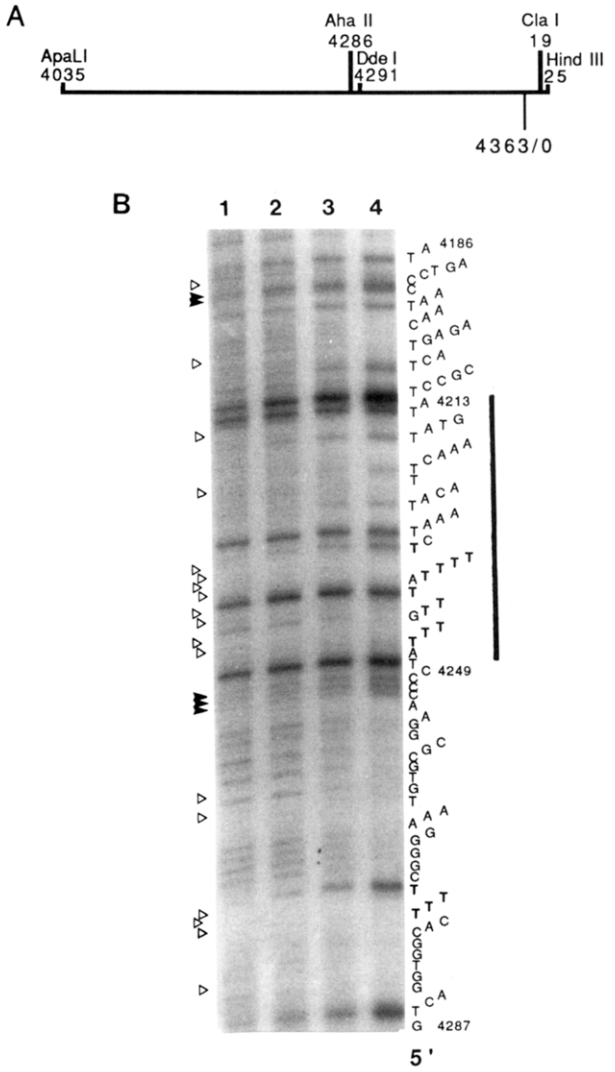

FIGURE 1: $KMnO_4$ reactivity at various temperatures of the 347 base pair *Apa*LI–*Cla*I fragment of pDPL6, 5′ end labeled at the *Cla*I site. Panel A: Map positions of the restriction sites used to generate 5′ or 3′ ^{32}P end labeled fragments from the plasmid pDPL6. For comparative purposes, the nucleotide positions given correspond to the analogous sequence in the parent plasmid pBR322. Panel B: Lanes 1–4, piperidine cleavage products of DNA modified by $KMnO_4$ at 0, 12, 23, and 43 °C, respectively. Open triangles indicate thymines with $KMnO_4$ reactivity below that of the average $KMnO_4$ reactivity of guanines; closed arrowheads indicate cytosines in tracts of poly(dC) which show moderate $KMnO_4$ reactivity at higher temperatures. The 5′ end of the labeled fragment is at the bottom of the figure. Solid black bar indicates region analyzed by densitometry.

DNA sequencing gels. $KMnO_4$ modification reactions were also carried out at a number of different temperatures to study the effect of temperature on local stacking interactions. An example of $KMnO_4$ reactivity with a fragment of double-stranded DNA from the β-lactamase promoter region of pDPL6 (a pBR322 derivative) at 0, 12, 23, and 43 °C is shown in Figure 1B. This probe reacts significantly with some thymines, but not others. $KMnO_4$ also reacts weakly with the other bases, with guanine being most sensitive to $KMnO_4$ attack. The $KMnO_4$ reactivity of guanine increases very gradually with temperature and is therefore a useful standard for measuring the relative $KMnO_4$ reactivities of thymines (see Figure 5). In general, reaction temperature from 0 to 43 °C has a relatively small affect on specific $KMnO_4$ attack at the most reactive thymines (such as T 4248, and T 4214). One clear exception is T 4212, which becomes hyperreactive at 43 °C. However, attack at a number of thymines increases dramatically as the temperature is raised (Figure 1B; T 4286 and T 4272), suggesting that distinct temperature-induced changes in base stacking interactions occur at these thymines. Many of the thymines which are protected from $KMnO_4$ attack at all temperatures are single thymines contained in runs of purines or located between two guanines. This result indicates that these unreactive thymines have rather strong stacking interactions and that their arrangement in the B DNA helix is largely unaffected by increasing temperature. Another interesting feature of the reactivity of $KMnO_4$ with B DNA is demonstrated in Figure 1B. While cytosines usually react very poorly with this probe, cytosines within stretches of poly(dC) react significantly with $KMnO_4$ at higher temperatures. There are two tracts of cytosines in Figure 1B [$d(C)_4$, C 4249–C 4252; $d(C)_3$, C 4191–C 4193], and in both cases all but the 3′-terminal cytosine reacts significantly with $KMnO_4$ at 43 °C (lanes 3 and 4). At present, the basis of this enhanced reactivity of cytosines in short tracts of poly(dG)·poly(dC) is not known, although it may be related to one of the structural alterations in poly(dG)·poly(dC) tracts described recently by Kowhi and Kowhi-Shigamatsu (1988).

The $KMnO_4$ reactivity of the thymines in the *Apa*LI–*Aha*II fragment, 3′ ^{32}P labeled at the *Aha*II site, is presented in Figure 2A,B ($KMnO_4$; lanes 1–3). As demonstrated above, the susceptibility of thymines to $KMnO_4$ attack is sequence specific, with some thymines being modified significantly more than guanines, particularly as the temperature increased (e.g., T 4227; Figure 2A), and other thymines apparently protected from $KMnO_4$ attack at all temperatures (and less reactive than guanines, e.g., T 4225; Figure 2A). As mentioned previously, guanines display a low, but significant, level of reactivity with $KMnO_4$. However, in contrast to the reactivity of many of the thymines, the reactivity of guanines appears to increase relatively little from 0 to 43 °C.

$KMnO_4$ Reactivity of the Thymines in Short A-Tracts. When the $KMnO_4$ reactivity with stretches of poly(dT) in A-tracts is examined, an easily discernible pattern emerges. There are four tracts of thymines (3–5 nucleotides) shown in Figure 1B. In each case, only the thymine at one end of the tract displays any significant reactivity. The reactive thymine in three of these tracts is located at the 3′-terminal thymine. In the fourth tract, the only marginally reactive thymine is located at the 5′ end of the tract (T 4246). There are four examples of short stretches of poly(dT) longer than 2 nucleotides in Figure 2A,B. One of these tracts (T 4219–T 4221) is unusual in that none of these thymines react significantly with $KMnO_4$ at any temperature, although the complementary poly(dA) tract reacts with DEPC in the expected fashion (see below; Figure 5). Interestingly, this run of thymines is centrally located in the −35 region of the β-lactamase promoter (Brosius et al., 1982; Figure 5). In contrast, the second poly(dT) tract shown in Figure 2A (T 4229–T 4231) and the two stretches of poly(dT) longer than 2 nucleotides in Figure 2B all react to produce the unique pattern of the poly(dT) tracts described for Figure 1B; that is, only the 3′-terminal thymine displays significant $KMnO_4$ reactivity. However, it is important to note that the elevated $KMnO_4$ reactivity at the 3′ thymine of some A-tracts is essentially lost at the lowest temperature (Figure 1B; T 4234–T 4238 vs T 4240–T 4242). The level of $KMnO_4$ attack at the 3′-terminal thymine in the A-tract T 4229–T 4231 (Figure 2A) is rather high compared to that seen in other A-tracts. This result suggests that an unusually pronounced junction exists at the 3′ end of this tract and may be related to the presence of the short 3′ flanking polypurine sequence. Thus, many of the poly(dT) stretches

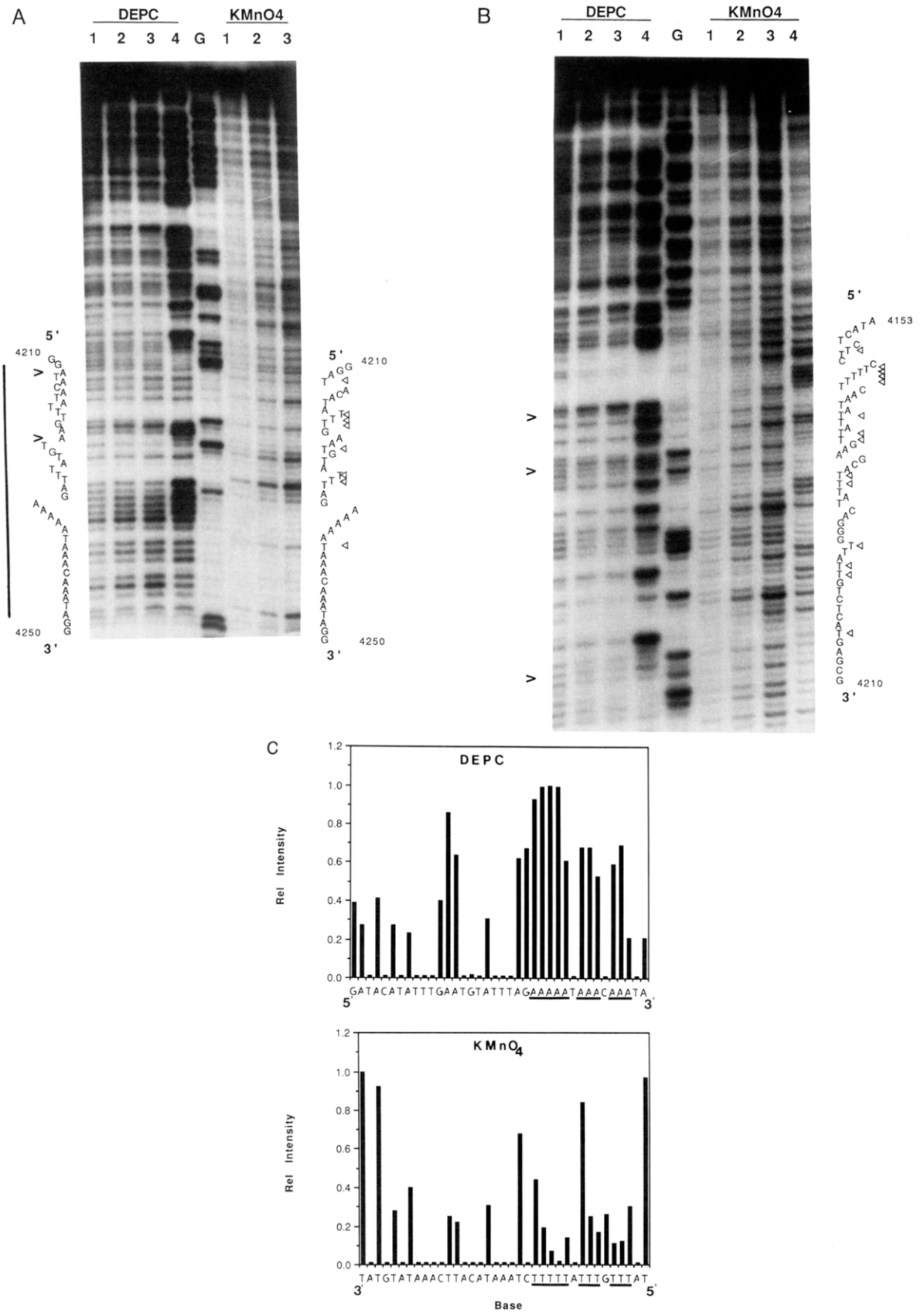

FIGURE 2: $KMnO_4$ and DEPC reactivity at various temperatures of the 251 base pair *Aha*II–*Apa*LI fragment from pDPL6, 3′ end labeled at the *Aha*II site. Panels A and B are different electrophoretic separations of the same modification and piperidine cleavage reaction products. Temperatures of the modification reactions in this experiment were as follows: lane 1, 0 °C; lane 2, 23 °C; lane 3, 43 °C; lane 4, modification of denatured DNA at 23 °C. G is the marker lane showing the products of a guanine-specific Maxam–Gilbert sequencing reaction. Large arrow heads indicate adenines with DEPC reactivity equivalent to guanines. Open triangles indicate thymines with $KMnO_4$ reactivity below that of the average $KMnO_4$ reactivity of guanines at all temperatures. Solid black bar in panel A indicates region analyzed by densitometry. Panel C: Relative $KMnO_4$ and DEPC reactivities of the bases in the −35 region of the β-lactamase promoter at 43 °C were determined by densitometry. The $KMnO_4$ reactivity data were obtained from the autoradiograph shown in Figure 1B. It is noted that the highest reactivities are probably underestimates because these bands are overexposed in the autoradiographs, and the reactivity of the 3′-terminal adenines may in some cases be overestimated because of spillover from the very high reactivity of the neighboring adenine.

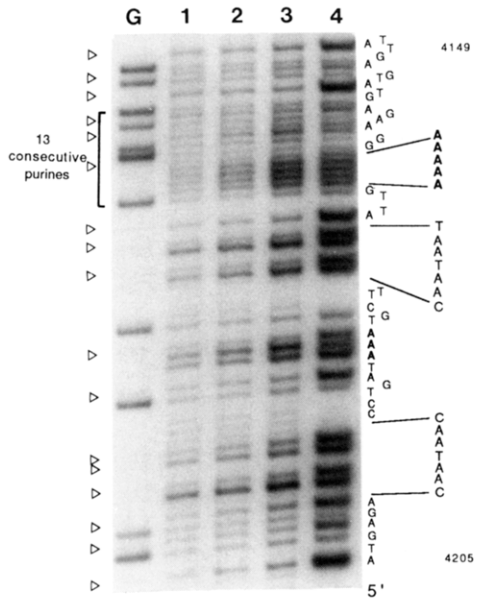

FIGURE 3: DEPC reactivity at various temperatures of the 256 bp *Apa*LI–*Dde*I fragment of pDPL6, 5′ end labeled at the *Dde*I site. Lanes 1–3, cleavage products from DEPC modifications at 0, 23, and 43 °C, respectively; lane 4, cleavage products from DEPC modification of denatured DNA at 23 °C. G is the marker lane shown the products of a guanine-specific Maxam–Gilbert sequencing reaction. Open triangles indicate adenines with low DEPC reactivity.

in short A-tracts display a similar pattern of $KMnO_4$ reactivity at the higher temperatures, with only the 3′ thymine displaying significant reactivity (Figures 1B and 2A,B). Nonetheless, occasionally a few A-tracts display different patterns of $KMnO_4$ reactivity such as that seen in Figure 2A (T 4219–T 4221), and a rare low temperature induced pattern (J. McCarthy, unpublished results).

DEPC Reactivity of B DNA at Different Temperatures. DEPC has been extensively used to detect a number of unusual DNA structures such as Z DNA, cruciforms, and H DNA/triple helices (Herr, 1985; Johnston & Rich, 1985; Furlong & Lilley, 1986; Scholten & Nordheim, 1986; Voloshin et al., 1988; Hanvey et al., 1988). However, although it is known that DEPC attacks double-stranded DNA primarily at the N-7 position of purines (A > G; Leonard et al., 1971; Herr et al., 1982), the reactivity of DEPC with long fragments of B DNA has not been described in detail. To study this process, we have reacted DEPC with end-labeled DNA fragments containing the promotor region of the β-lactamase gene. In addition, we have tested the ability of DEPC to detect temperature-induced effects on B DNA structure by carrying out the DEPC modification reactions at different temperatures.

Figure 3 illustrates both the reactivity of double-stranded DNA with DEPC and the effect of temperature on this reaction. The DEPC reactivity of guanines is uniformly low, while the reactivity of adenines varies considerably. Of the 27 A's in Figure 3 (lanes 1–3), 17 display a low (G-like) reactivity with DEPC, and the remainder display higher levels of reactivity. However, the A > G specificity of DEPC attack is significantly less distinct at 0 °C than at 23 and 43 °C (Figure 3, lane 1 versus lanes 2 and 3). DEPC modification of reactive adenines increases with temperature, while the modification of the unreactive adenines and guanines remains largely unchanged. This result suggests that, unlike $KMnO_4$, DEPC is not a particularly sensitive reagent for the detection of subtle temperature-induced changes in DNA structure. When the fragment of DNA was denatured prior to treatment with DEPC (at 23 °C), the majority of the adenines became substantially more reactive with this probe (Figure 3, lane 4). However, the DEPC reactivity with certain adenines did not increase significantly after the DNA was denatured. It is likely that many of these unreactive adenines reside in regions of the single-stranded DNA which are involved in the secondary structure of this denatured DNA. However, we have noted that each of the adenines in Figure 3 which remain weakly reactive with DEPC after denaturation are flanked by guanines. Because it is unlikely that all of these unreactive adenines participate in the Watson–Crick base paired secondary structures of the denatured DNA, it is quite plausible that residual stacking interactions between some purines in single-stranded DNA interfere with the DEPC modification reaction. If this interpretation is correct, then low DEPC reactivity at adenines is not, as routinely assumed, always synonymous with the presence of a B DNA conformation (Voloshin et al., 1988; Hanvey et al., 1988; Htun & Dahlberg, 1988).

DNA sequences which display DEPC hyperreactivity are often described as being single stranded. However, Jeppesen and Nielsen (1988) have recently shown that compounds such as ethidium bromide, which intercalate between the bases and unwind the B DNA double helix, also induce DEPC (and $KMnO_4$) hyperreactivity. In fact, the DEPC and $KMnO_4$ hyperreactivity of DNA containing intercalated ethidium bromide is even more striking and base specific when such experiments are carried out at higher ethidium concentrations (J. McCarthy, unpublished results). These important observations suggest that DEPC hyperreactivity induced by negative superhelical stress (Hanvey et al., 1988; Voloshin et al., 1988), or $KMnO_4$ and DEPC hyperreactivity induced by protein binding (Borowiec & Hurwitz, 1988; O'Halloran, et al., 1989; Buckle & Buc, 1989), might be a manifestation of unwinding of the double helix rather than the induction of more complex structures containing single-stranded DNA.

A striking example of the ability of DEPC to detect subtle differences in DNA conformation is indicated by a variation in the reactivity of two identical sequences (Figure 3). DEPC reacts to a similar extent with three of the four adenines in the two CAATAA sequences seen in Figure 3 (lanes 1–3). In contrast, one of the adenines (underlined) exhibits a markedly different DEPC reactivity at these two sites. The variation in the DEPC reactivity of this particular adenine in these two elements presumably reflects an alternate configuration of this adenine at each site and indicates that the overall structure of this element is affected by the presence of different flanking sequences.

DEPC Reactivity of the Adenines in Short A-Tracts. One of the most consistent observations from limited DEPC modification of double-stranded DNA is the pattern of DEPC reactivity that occurs with adenines present in short A-tracts. Each short tract of adenines displays a relatively high level of DEPC reactivity at all but the 3′-terminal adenine of the tract. The fragment probed in Figure 3 contains two A-tracts longer that 2 base pairs [$d(A)_3$ and $d(A)_5$]. Although both tracts react strongly with DEPC, the latter tract is less reactive, perhaps because it exists within a 13-base string of purines. In double-stranded B DNA the reactivity of the 3′-terminal adenines of both A-tracts in Figure 3 is low. However, both of these adenines display a significant increase in DEPC reactivity when the DNA is denatured. This result suggests that DEPC attack at the 3′-terminal adenines of A-tracts may be blocked.

The DEPC reactivity of three other A-tracts (>2 bp) is illustrated in Figure 2A (DEPC, lanes 1–3). The relatively

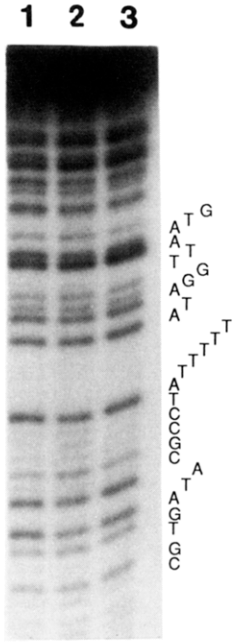

FIGURE 4: DEPC modification reaction as a function of time. The *Dde*I–*Cla*I fragment of pDPL6, 5′ end labeled at the *Cla*I site, was reacted with DEPC at 23 °C as described under Experimental Procedures for various lengths of time. Lanes 1–3, piperidine cleavage products of DNA modified with DEPC for 5, 15, and 60 min, respectively.

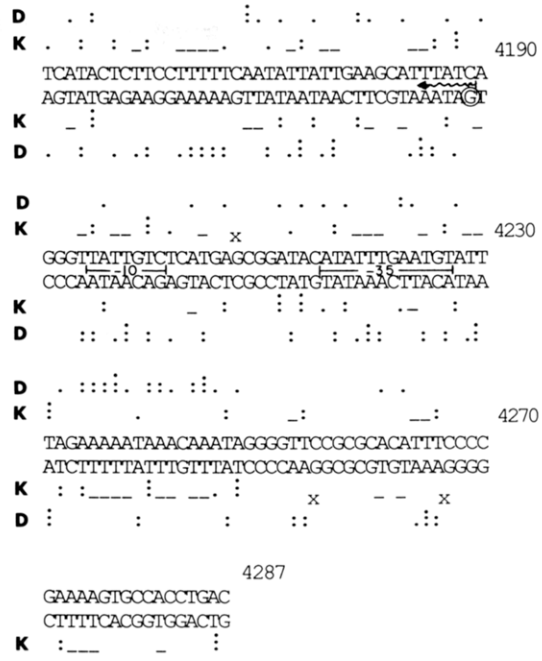

FIGURE 5: Schematic representation of the relative extent of $KMnO_4$ and DEPC modifications occurring at 43 °C within the β-lactamase promoter region of pDPL6. The relative levels of chemical modification occurring at the bases have been visually determined from various electrophoretic separations of the 43 °C samples used to generate Figures 1–3. The −35 and −10 regions of the β-lactamase promoter are marked, the starting point for transcription is circled, and the initiator methionine codon starts at nucleotide 4154 (Brosius et al., 1982). Symbols: D, DEPC; K, $KMnO_4$. (−) indicates below guanine reactivity; (.) indicates DEPC and $KMnO_4$ reactivites which are at, or near, that of neighboring guanines; (:) and (⁝) indicate increasingly significant levels of DEPC and $KMnO_4$ reactivity above that of neighboring guanines.

strong DEPC reactivity of all but the 3′-terminal adenines in A-tracts at 23 and 43 °C is again obvious. However, when this DNA was denatured prior to DEPC modification at 23 °C, the reactivity of the two $d(A)_3$ tracts was only modestly increased, while the reactivity of four adenines in $d(A)_5$ increased more dramatically (Figure 2A; DEPC, lane 4). As discussed above, a low reactivity of adenines in denatured DNA is suggestive of the involvement of these adenines in the secondary structure interactions of this single-stranded DNA. However, because the DEPC reactivity patterns of the two $d(A)_3$ tracts in denatured DNA (Figure 2A, DEPC, lane 4) is substantially different from the DEPC reactivity of these tracts in double-stranded DNA, it is likely that the secondary structure in which these stretches of poly(dA) are involved is rather different from their normal duplex structure.

There is one variable feature of the reactivity of DEPC with short A-tracts. Some tracts, such as A 4163–A 4167 (Figure 3), react with DEPC to a moderate but uniform extent at all of the adenines except the 3′-terminal adenine of the tract. In contrast, many other tracts such as A 4234–A 4238 (Figure 2A) display a higher reactivity with DEPC and exhibit an even greater reactivity at the second to last 3′-terminal adenine of the tract. This observation suggests that the poly(dA) strand of A-tracts can assume two related conformations. The high reactivity of the second to last 3′-terminal adenine exhibited by some tracts also suggests that the more DEPC-reactive A-tracts may form a different and more extensive junction with the 3′ flanking DNA than the less DEPC reactive A-tracts. The involvement of the last two adenines in the 3′ end junction of the poly(dA) strand has also been observed in some A-tract-containing oligonucleotides in solution (Kintanar et al., 1987). Thus, it appears that there are at least two broad classes of A-tract structure which can be defined by the DEPC reactivity data presented here. Class I has a moderate and uniform DEPC reactivity of the adenines, while class II has a generally higher reactivity with DEPC and displays hyperreactivity at the second to last adenine from the 3′ end. Both classes of DEPC reactivity have an unreactive 3′-terminal adenine.

Effect of Reaction Time on the Chemical Reactivity Pattern. The effect of reaction time on the chemical reactivity patterns was determined by varying the time of the modification reactions. The result of one such experiment with DEPC is shown in Figure 4. The data presented in this figure demonstrate that varying reaction times from 5 to 60 min has little qualitative effect on the pattern of reactivity, although there are some quantitative differences. When the $KMnO_4$ reactivity of this fragment was examined at reaction times of 5, 15, and 45 min, similar results were obtained (data not shown). Thus, the patterns of DEPC and $KMnO_4$ reactivity change little over this time period, indicating that the probe does not alter the DNA conformation over time. The results obtained from these experiments also suggest that the chemical probes are reacting with a conformation of the nucleic acid that is relatively stable over the time period tested.

Summary of the DEPC and $KMnO_4$ Reactivity at the Promoter Region of the β-Lactamase Gene. A compilation of the DEPC and $KMnO_4$ reactivity with the β-lactamase promoter at 43 °C is presented in Figure 5. This figure illustrates that these probes can be employed to detect small local variations in the B DNA helix. For example, $KMnO_4$ can detect thymines which become partially unstacked at 43 °C (T 4248; Figure 5), in addition to those thymines that maintain significant stacking interactions between 0 and 43 °C (T 4225; Figure 5). The usefulness of these probes to detect variations in B DNA structure is seen by examining their overall reactivity with short A-tracts. The data represented in Figure 5 clearly demonstrate that many A-tract

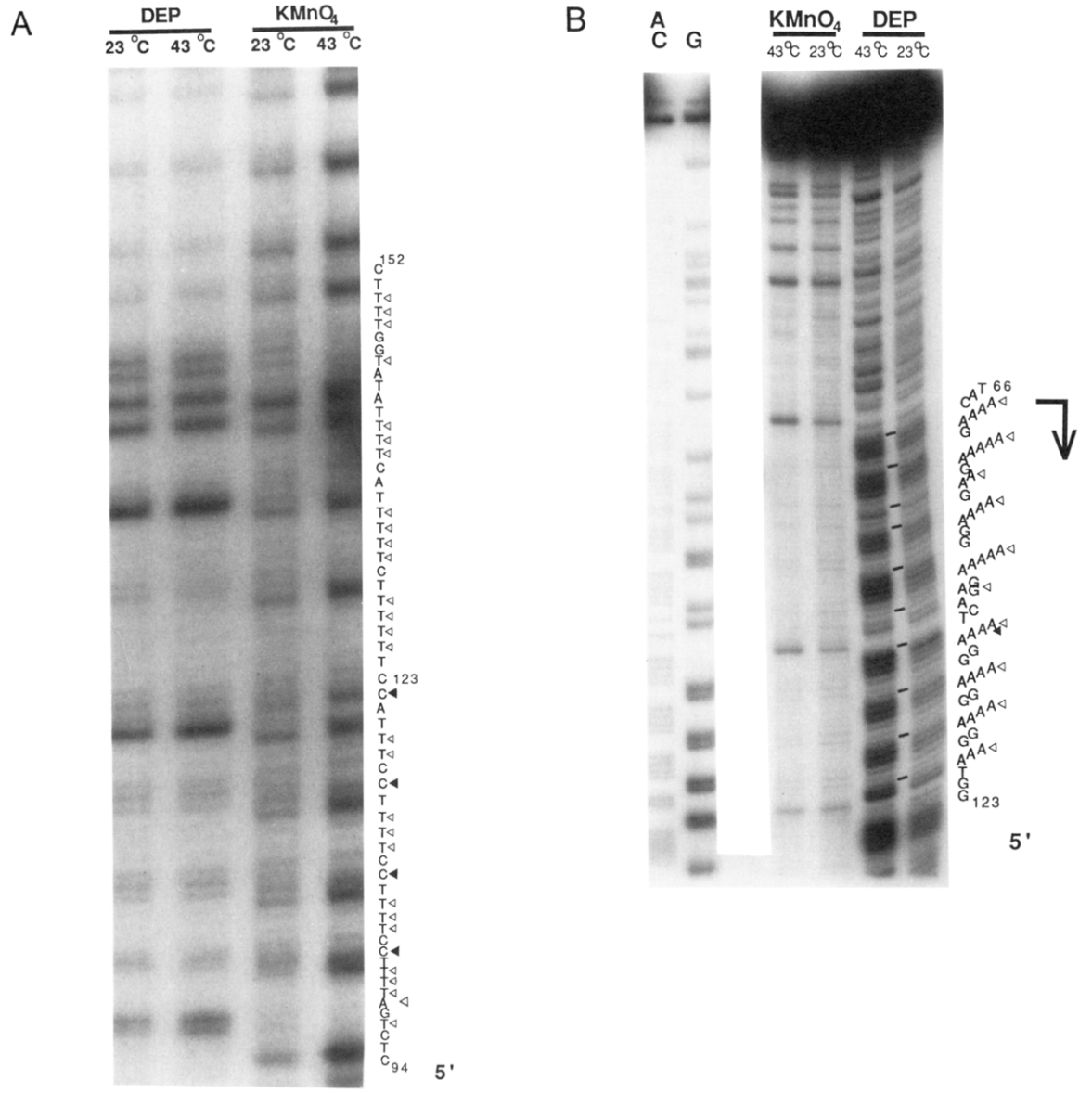

FIGURE 6: $KMnO_4$ and DEPC reactivity of the multiple short A-tracts in an A+T rich polypurine/polypyrimidine sequence. The 217 base pair *Xba*I–*Hin*dIII fragment of pXH-N, 5′ end labeled on both strands, was reacted with either DEPC or $KMnO_4$. The DNA was then strand separated, and the purified single strands were cleaved at the modified bases with piperidine. Cleavage products were subsequently run on a 7% DNA sequencing gel. Panel A: Polypyrimidine strand. Open triangles indicate thymines with low $KMnO_4$ reactivity; closed triangles indicate cytosines displaying unusual $KMnO_4$ reactivity. Panel B: Polypurine strand. Open triangles indicate adenines with low DEPC reactivity; the closed triangle indicates an adenine with very high DEPC reactivity. The black dashes between the 23 and 43 °C DEPC lanes demarkate the position of the 3′ unreactive adenines of each A-tract. The large arrow marks the starting point and direction of the polypurine tract.

adenines (all eight tracts in this figure) react with DEPC to a similar extent, displaying a high reactivity with this probe at all but the 3′-terminal adenine. However, a subset of the A-tracts do have rather low DEPC reactivity (e.g., A 4163–A 4167). Figure 5 also graphically demonstrates that many short regions of poly(dA)·poly(dT) also have a similar pattern of $KMnO_4$ reactivity, with the majority of the A-tracts displaying significant reactivity only at the 3′-terminal thymine (six of eight tracts). Overall, the A-tract reactivity data illustrated in Figure 5 indicate that short A-tracts embedded in long fragments of DNA form a number of closely related variants of B DNA in solution. The qualitative observations of the chemical reactivities presented in Figure 5 are drawn from visual inspections of the autoradiographs. A more quantitative measurement of the relative reactivities was also possible for some of the A-tracts by densitometer scanning of the autoradiographs. Examination of the data generated by densitometry (Figure 2C) generally follows the visual estimates of the relative base reactivities shown in Figure 5.

$KMnO_4$ and DEPC Reactivity of the Multiple Short A-Tracts in an A+T Rich Polypurine/Polypyrimidine Sequence. To further define the specificity of the $KMnO_4$ and DEPC reactions with short A-tracts, a 217 bp fragment containing the long A+T rich polypurine/polypyrimidine (pur/pyr) tract from the immediate 3′ noncoding region of an embryonic chicken myosin heavy gene (McCarthy & Heywood, 1987) was reacted with these probes. This pur/pyr tract contains 20 short (3–6 bp) A-tracts, which are separated from one another by a few nucleotides, but fragments containing this pur/pyr tract do not display any unusual mobility in polyacrylamide gels. The reactivity of a portion of the pyrimidine strand of this fragment is shown in Figure 6A. In agreement with the data presented earlier, only the 3′-terminal thymine in each of the eight stretches of poly(dT) reacts significantly with $KMnO_4$, and this reactivity increases with temperature. A similar pattern of $KMnO_4$ reactivity is seen for all of the

A-tracts in this fragment (data not shown). At lower temperatures (below 17 °C) the $KMnO_4$ reactivity at all the 3′-terminal thymines becomes greatly reduced (data not shown). It is also interesting to note that each of the 5′ cytosines in the CC doublets present in this fragment possess unusually high $KMnO_4$ reactivity (Figure 6A). All but one of the pyrimidine strand adenines in Figure 6A react moderately (A > G) with DEPC.

The reactivity of a portion of the polypurine strand is shown in Figure 6B. The isolated thymines present on this purine-rich strand display a moderately high (>G) $KMnO_4$ reactivity. The majority of the adenine stretches on this strand display the class I DEPC reactivity described above; that is, each poly(dA) stretch has a moderate and uniform rectivity at all the adenines except for the suppressed reactivity at the 3′ adenine. This result suggests that the A-tract conformation associated with class I reactivity is favored by the significant cumulative stacking interactions which probably occur in the polypurine strand. One tract [$d(A)_4$, A 100–A 103] possesses a class II reactivity pattern, displaying a somewhat hyperreactive second to last 3′ adenine. The different DEPC reactivity of this tract (relative to the reactivity of the majority of the tracts in this pur/pyr sequence) may be related to the presence of the two 3′ flanking pyrimidines. The data presented in Figure 6 clearly show that short A-tracts react specifically and reproducibly with $KMnO_4$ and DEPC. The demonstration that the majority of the multiple A-tracts in this pur/pyr sequence have nearly identical reactivities with both $KMnO_4$ and DEPC (class I reactivity) further suggests that sequence context has some contribution to the particular conformation adopted by these A-tracts.

Discussion

Small chemical probes which react with the bases of DNA have been used by many groups to detect non-B DNA structures such as Z DNA, cruciforms, and the postulated triple helices of polypurine/polypyrimidine tracts in supercoiled plasmids (Herr, 1985; Johnston & Rich, 1985; Lilley & Palecek, 1984; McClellan et al., 1986; Voloshin et al., 1988; Hanvey et al., 1988; Kowhi & Kowhi-Shigamatsu, 1988). Here, we have investigated the potential of two commonly used probes of DNA structure, $KMnO_4$ and DEPC, to study small variations in B DNA structure. The data presented here clearly show that attack by $KMnO_4$ or DEPC at potentially susceptible bases in B DNA is dependent on the position of these bases in the DNA sequence (Figure 5). This contrasts with chemical probes like dimethyl sulfate (DMS), which generally react uniformly with their susceptible base(s) irrespective of the position of that base(s) in the DNA sequence (Maxam & Gilbert, 1980). However, it is well-known that even the DMS reactivity of guanines in DNA is occasionally affected by sequence context. One example of the poor DMS reactivity of a particular guanine is seen here in Figure 2A (G lane; G 4208).

The data obtained by reacting B DNA with $KMnO_4$ and DEPC demonstrate that these probes can recognize distinct junctions between the flanking DNA and the majority of the A-tracts examined. This result, together with the specific chemical reactivity patterns found for these A-tracts, implies that an altered B DNA helix is formed by this sequence. Thus, the data presented are in agreement with previous NMR studies which show that A-tracts form an unusual B DNA conformation in solution (Kintanar et al., 1987; Katahira et al., 1988; Nadeau & Crothers, 1989).

Examination of the $KMnO_4$ reactivity of thymines in different A-tracts indicates that these bases are generally protected from $KMnO_4$ attack, except for the 3′-terminal thymine, which usually reacts moderately with this probe at the higher temperatures. Because $KMnO_4$ can only attack the susceptible 5–6 double bond of thymine from above or below the plane of the base (Howgate et al., 1968; Iida & Hayatsu, 1971), these results suggest that the thymines of A-tracts usually exist in a uniform arrangement within the A-tract-specific helix and probably have considerable stacking interactions with one another. This is in contrast with the thymines present in random sequence DNA, which display significant variability in their reactivity with $KMnO_4$ (Figures 1B and 5). Such differential reactivity is likely to be a good measure of the relative degree of stacking between a thymine and both flanking bases, which is in turn related to the helical parameters tilt, twist, and roll. Therefore, probing with $KMnO_4$ can yield useful information on the position of local helical distortions present within B DNA and the effects of environmental factors such as temperature on these distortions.

The observation that the 5′-terminal thymine and all the internal A-tract thymines are equally protected from $KMnO_4$ attack (Figures 1B, 2A,B, and 6A) suggests that there is probably no significant variation in the roll and/or tilt between the individual A·T base pairs over a temperature range of 0–43 °C within the A-tracts examined. This observation also suggests these thymines have relatively equivalent stacking interactions. Variation in the extent of $KMnO_4$ attack at the 3′ thymines of A-tracts in and around the β-lactamase promoter implies that these thymines are unstacked to different extents in the DNA. This unstacking presumably occurs primarily to the 3′ side of the poly(dT) strand where the altered A-tract structure clashes with the flanking B DNA helix. The similarity of the 3′ thymine reactivities of the A-tracts in Figure 6A suggests that the poly(dT) stretches of these tracts form rather uniform 3′ end junctions with the flanking DNA. The properties of the A-tract reactivity with DEPC and $KMnO_4$ documented here are inconsistent with certain aspects of the wedge model for A-tract DNA (Ulanovsky & Trivanov, 1987) but can be accommodated by both the high tilt and high propeller twist models for A-tract DNA (Lipanov & Chuprina, 1987; Nadeau & Crothers, 1989; Nelson et al., 1987; Coll et al., 1987; Aymami et al., 1989).

Two of the eight A-tracts in the β-lactamase promoter region react with $KMnO_4$ in an unusual fashion. All of the thymines in the poly(dT) stretches T 4219–T 4221 and T 4244–T 4246 are essentially protected from $KMnO_4$ attack (Figure 5). This result suggests that no significant 3′ end junctions are formed on the poly(dT) strand of these A-tracts, although detectable 3′ junctions are formed on the poly(dA) strands of these tracts (Figure 5). Yet another pattern of A-tract $KMnO_4$ reactivity has also been detected in a small number of A-tracts. This unique A-tract reactivity, favored at low temperature, will be described elsewhere. The A-tracts which possess the unusual poly(dT) strand variants just described have class II DEPC reactivity on the poly(dA) strand. These observations, together with the two patterns of DEPC reactivity that occur with A-tracts (discussed below), extend the observations of others (Sheflin & Kowalski, 1984; McCarthy & Heywood, 1986; Nelson et al., 1987; Coll et al., 1987; Kintanar et al., 1987; Aggarwal et al., 1988; Nadeau & Crothers, 1989) that A·T rich DNA possesses significant structural flexibility.

As noted earlier, there appear to be two distinct classes of A-tract reactivity with DEPC. Class I reactivity is represented by the $d(A)_5$ tract (A 4163–A 4167) in Figure 3. In this case adenines A 4164–A 4167 react to a moderate, but relatively

similar extent with DEPC, while the 3′-terminal adenine (A 4163) reacts poorly with DEPC. It is probably significant that this A-tract which displays class I DEPC reactivity is located in a 13 bp polypurine/polypyrimidine region, because the majority of the short A-tracts present in a long A+T rich polypurine/polypyrimidine tract (Figure 6A) also display a class I pattern of DEPC reactivity. This latter observation strongly implies that the DNA sequence surrounding a short A-tract can influence the structure formed within that tract. The class II pattern of DEPC reactivity is somewhat more common in nonrepetitive DNA and is represented by the $d(A)_5$ tract (A 4234–A 4238) in Figure 2A. In this tract A 4234–A 4236 react quite strongly with DEPC, the second to last adenine (A 4237) reacts very strongly, and the 3′-terminal adenine (A 4238) reacts poorly with DEPC. The very high reactivity of the second to last adenine suggests that this adenine may also participate in an extensive 3′ end A-tract/B DNA junction.

The molecular basis for the DEPC hyperreactivity of adenines, but not guanines, in single-stranded DNA is not understood (Figures 2A,B and 3). The decreased DEPC reactivity of adenines in double-stranded DNA versus single-stranded DNA may simply be caused by the reduced accessibility of the N-7 position of adenine to DEPC attack as proposed previously (Herr, 1985; Johnston & Rich, 1985). However, it is possible that some other conformational constraint(s) on the adenines in double-stranded DNA may also contribute to their low DEPC reactivity.

It is interesting to note that models have suggested that DEPC reaction intermediates could be stabilized by proper geometric arrangement of amino groups and nucleophilic N-7 positions (L. Williams, unpublished results). Hydrogen bond donating amino groups of DNA have the potential to acid-catalyze DEPC attack on the N-7 positions. The reactivity of a specific adenine N-7 with DEPC could partially depend on the ability of an N^6 amino group to rotate to attain the optimum geometry. The N^6 amino group of adenine should be relatively free to rotate in single-stranded DNA compared to double-stranded DNA, thus assuming a more favorable geometry for enhancing DEPC hydrolysis. Consistent with such a proton catalysis of DEPC attack is the observation that the N-3 amino group of cytosine appears to be well situated relative the N-7 of an adjacent guanine in Z DNA and could contribute to the unusually high level of DEPC attack which occurs at guanines in the Z DNA form of poly[dGC)].

In the absence of a well-described reaction mechanism for DEPC attack at adenines, it is not possible to predict what structural feature(s) of short A-tracts cause their relatively high reactivity with this probe. However, because the poly(dT) strand of A-tracts exhibits such a high degree of base stacking ($KMnO_4$ insensitivity), it is unlikely that the poly(dA) strand of these A-tracts is substantially underwound. Other more plausible causes of the elevated DEPC reactivity of A-tract adenines include a high tilt (Wu & Crothers, 1984) or some combination of increased roll and tilt which raises solvent/probe accessibility near the N-7, but not at the 5–6 position the thymines. Alternatively, if the N^6 amino group of adenine catalyzes DEPC attack, perhaps the N^6 group of A-tract adenines exists in a conformation that favors DEPC reactivity. Another notable feature of the reactivity of A-tracts with DEPC is the consistently low reactivity of the 3′-terminal adenine. Again, either the accessibility of the N-7 position is sharply reduced at this adenine or there is some other strong conformational restraint on DEPC attack here. The absence of reactivity at 3′ adenines in both classes of A-tracts described above may also be related to the sharp brake in the 5′–3′ narrowing of the minor groove of A-tracts which occurs in this region (Milgram-Burkhoff & Tullius, 1987).

The relationship of either of the two A-tract structures classified above on the basis of DEPC reactivity to DNA bending has yet to be determined. However, if these structures are involved in bending, the observation that detectable junctions can be found at both the 5′ and 3′ ends of some A-tracts, and only at the 3′ ends of others, would be significant. If any such correlations are found, it would favor models of A-tract-dependent DNA bending which involve junctions between an A-tract-specific conformation and the flanking B DNA to produce a significant alteration of the helical axis (Koo et al., 1986; Koo & Crothers, 1988; Nadeau & Crothers, 1989; Nelson et al., 1987; Coll et al., 1987). However, any correlation between the $KMnO_4$ and DEPC reactivities of A-tracts and the DNA bending associated with A-tracts will require the chemical probing of well-characteized fragments which are bent. In such experiments, bent DNA oligonucleotides which are ligated (Hagerman, 1985; Koo et al., 1986; Koo & Crothers, 1988) are preferable to naturally occurring bent DNA (Marini et al., 1982), because the data presented here suggest the chemical reactivity of A-tracts, and hence their structure, can be affected by the flanking sequences.

The ability of $KMnO_4$ and DEPC to detect minor conformational differences in B DNA suggests a number of potential applications. For example, many homeodomain transcriptional regulatory proteins have been shown to possess an unexpectedly high degree of promiscuity in their DNA binding capabilities (Desplan et al., 1988; Baumruker et al., 1988; Garcio-Blanco et al., 1989). This ability of homeodomain proteins to bind a set of closely related sequences implies that the specificity of binding is affected not only by the sequence of a DNA binding site but also by the conformation and/or ability to adopt a conformation at that site. In view of the results presented here, it will be interesting to determine if some common structural component, or particular conformational flexibility, exists in one set of A+T rich homeodomain protein binding sites by probing with $KMnO_4$ and DEPC.

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