Cytosine Methylation Can Induce Local Distortions in the Structure of Duplex DNA†

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ABSTRACT: Methyl groups at the C5 position of pyrimidines located within oligopurine–oligopyrimidine tracts in DNA have been shown previously to modulate curvature generated by those tracts. However, it was not known whether the influence of such methyl groups is consequent to the altered helical structure within the tracts themselves. In the current study, it is demonstrated that methylation of cytosines up to three base pairs away from a $(dA)_5$·$(dT)_5$ tract (A-tract) can still result in alterations of the net curvature of the A-tract-containing DNA, as measured by alterations in electrophoretic mobility. This latter effect depends strongly on both the sequence of the non-A-tract DNA and the positions of the methylated C residues. The current results lend further support to the notion that the biological consequences of cytosine methylation may be effected through local alterations in DNA structure as well as through direct protein–DNA interactions.

Methylation of DNA is generally regarded as an important mechanism for the control of gene expression, particularly at the level of the regulation of transcriptional activity (Cedar, 1988; Dörfler, 1983; Razin, 1980), and as a means of gene dosage compensation through chromosomal inactivation (Lock, 1987; Cedar, 1988). Despite the common belief that the influence of the methyl groups is through direct methyl group–protein interactions, there is little direct evidence that the only means by which methyl groups modulate protein–DNA interactions is through such direct contacts. In fact, on the basis of their investigation of the effects of A-N6-methylation on the binding of the *Eco*RI restriction endonuclease, Diekmann and McLaughlin (1988) have suggested that methyl groups may regulate, in part, by altering the conformation of the DNA within the binding domain of the protein.

In previous work on the effects of methyl substituents on pyrimidines within $(dA)_n$·$(dT/dU)_n$ and $(dI)_n$·$(dC/d5^{me}C)_n$ tracts (Hagerman, 1990b), it was observed that pyrimidine methylations induced structural changes within the oligopurine–oligopyrimidine tracts, as evidenced by alterations in the net curvature of the DNA molecules containing those tracts (for a general review of DNA curvature, see Hagerman, 1990a). However, it was not determined whether the observed structural alterations are a consequence of methyl groups being located *within* the A- and I-tracts or whether such effects are more general. Therefore, in order to assess the generality of the structural distortions induced by pyrimidine methyl groups, we have endeavored to test the effects on DNA conformation of cytosine methyl groups, either in DNA molecules containing A-tracts, where the 5meC residues are placed outside of the A-tracts, or in DNA molecules lacking A-tracts. As in the previous study (Hagerman, 1990b), electrophoretic mobility is used as the experimental measure of the effects of methylation.

MATERIALS AND METHODS

Synthesis and Purification of Oligonucleotides. All oligodeoxyribonucleotides employed in the current study (Table I) were synthesized using a Biosearch Model 8750 automated oligonucleotide synthesizer. C-to-5meC substitutions were carried out by substitution of the deoxycytidine phosphoramidite by the 5-methyldeoxycytidine phosphoramidite. All phosphoramidites were purchased from Milligen/Biosearch. Cleavage of the oligonucleotides from the support, base deprotection, and subsequent purifications were performed as described previously (Hagerman, 1985). The single-stranded DNA oligomers were phosphorylated with polynucleotide kinase (New England Biolabs) at 37 °C in 0.5 mM ATP, 50 mM Tris-HCl, pH 8.0, and 10 mM $MgCl_2$ (KL buffer). Following the phosphorylation reaction, appropriate oligomers were mixed, heated to 68 °C for 15 min, and allowed to cool to room temperature.

Construction of DNA Molecules Containing Direct Repeats of Duplex Oligomers. The duplex oligomers were ligated at 16 °C in KL buffer, with ligase concentration and reaction times being adjusted to produce suitable ligation ladders. Aliquots were withdrawn from the ligation reaction mix at various times and were quenched with equal volumes of 25 mM NaEDTA, 25% glycerol, 0.01% bromophenol blue, and 0.01% xylene cyanol. The aliquots were then combined, and a portion of the mixture was run on polyacrylamide gels.

Gel Electrophoresis. Polyacrylamide gels were poured and run at room temperature with buffer circulation. The current was kept below 25 mA in order to avoid heating of the gels. The gel dimensions were 0.8 mm (thickness) × 14 cm (width) × 24 cm (length). The gel running buffer was TEM (40 mM Tris-acetate, pH 8.0, 1 mM EDTA, 5 mM $MgCl_2$). All gels were 9%, unless specified otherwise, with a monomer-to-bis ratio of 37:1. Following the electrophoresis runs, the gels were stained with ethidium bromide and photographed, and the photographic negatives were scanned using an ImageQuant computing densitometer from Molecular Dynamics.

RESULTS

The Curvature of DNA Containing $(dA)_5$·$(dT)_5$ Tracts Is Modulated by Methylation of Cytosine Residues That Are Not Adjacent to the Tracts. In an effort to determine whether C5-methylation of pyrimidine residues outside of oligopurine–oligopyrimidine tracts has an effect on the overall conformation

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Table I: Sequences of the Synthetic Oligodeoxynucleotides Used in the Current Study

oligomer designation[a]	sequence[b]	oligomer designation[a]	sequence[b]
A-Tract		Non-A-Tract	
1047	CAAAAACACA	1168	TACCGATGGATGCCGATGGA
1058	TTTTGTGTGT	1169	GGCTACCTACGGCTACCTAT
1049	^{me}CAAAAACACA	1166	TA^{me}C^{me}CGATGGATG^{me}C^{me}CGATGGA
1058	TTTTGTGTGT	1169	G GCTACCTAC G GCTACCTAT
1052	CAAAAA^{me}CACA	1168	TACCGAT G GATGCCGAT G GA
1058	TTTT GTGTGT	1167	GGCTA^{me}C^{me}CTACGGCTA^{me}C^{me}CTAT
1053	CAAAAACA^{me}CA	1166	TA^{me}C^{me}CGAT G GATC^{me}C^{me}CGAT G GA
1058	TTTTGT GTGT	1167	G GCTA^{me}C^{me}CTAC G GCTA^{me}C^{me}CTAT
1054	^{me}CAAAAA^{me}CA^{me}CA	1424	TACAT G GATGCAT G GA
1058	TTTT GT GTGT	1425	GTA^{me}C^{me}CTACGTA^{me}C^{me}CTAT
1135	CAAAAACGCG	1198	GGCCACGTGACCTGACGTAC
1134	TTTTGCGCGT	1196	GGTGCACTGGACTGCATGCC
1136	^{me}CAAAAACGCG	1197	GGCCA^{me}CGTGACCTGA^{me}CGTAC
1134	TTTTGCGCGT	1196	GGT GCACTGGACT GCATGCC
1137	CAAAAACG^{me}CG	1513	GGCCTCTTGACCTGTCTTAC
1134	TTTTGC GCGT	1189	GGAGAACTGGACAGAATGCC
1138	CAAAAA^{me}CGCG	1190	GGCCT^{me}CTTGACCTGT^{me}CTTAC
1134	TTTT GCGCGT	1189	GGA GAACTGGACA GAATGCC
1139	^{me}CAAAAA^{me}CG^{me}CG	1514	GGCCACATGACCTGACATAC
1134	TTTT GC GCGT	1194	GGTGTACTGGACTGTATGCC
1159	CAAAAAGCCG	1195	GGCCA^{me}CATGACCTGA^{me}CATAC
1158	TTTTCGGCGT	1194	GGT GTACTGGACT GTATGCC
1426	CAAAAAG^{me}CCG	1515	GGCCTCATGACCTGTCATAC
1158	TTTTC GGCGT	1191	GGAGTACTGGACAGTATGCC
1160	CAAAAAG^{me}C^{me}CG	1192	GGCCT^{me}CATGACCTGT^{me}CATAC
1158	TTTTC G GCGT	1191	GGA GTACTGGACA GTATGCC
1423	CAAAAAGC^{me}CG		
1158	TTTTCG GCGT		

[a] Numerical designations represent oligomer reference numbers. [b] Sequences of the top strand of the oligomers are written in a 5′ to 3′ direction. The bottom strand of each duplex is written in a 3′ to 5′ (left to right) direction as it pairs with its complement. ^{me}C refers to 5-methyl cytosine.

of the DNA helix, a series of DNA polymers containing phased $(dA)_5$·$(dT)_5$ tracts was constructed (Table I). The polymers containing A-tracts were all based on a decamer repeat, the only differences among them being the sequence and positions of 5-methyl C residues within the remaining non-A-tract sequence. For this set of molecules, the A-tracts should serve as an "amplifier" of what are anticipated to be relatively small structural perturbations due to the methyl groups on the cytosine residues.

The general strategy of these experiments is to compare the electrophoretic mobilities of an A-tract-containing molecule, with and without cytosine methylation, as a qualitative indicator of changes in net curvature. Therefore, the mobilities of the methylated fragments are reported relative to those of the unmethylated molecules having the same base sequence. The electrophoretic mobilities of the various unmethylated controls, $\mu_{(-)}$, relative to electrophoretically normal standards, μ_{std}, are displayed in Figure 1. It should be noted that ratios of mobilities per se, not the ratios of apparent to actual number of base pairs, are reported in the current analysis. It is felt that mobilities more directly reflect the properties of the DNA molecules on gels than do their apparent sizes. However, for purposes of comparison, the 180-bp molecule comprising 18 copies of the decameric repeat CA_5GCCG (1159/1158; Table I), which has a relative electrophoretic mobility of 0.57, has a ratio of apparent size to actual size of 2.55. This latter number compares favorably with the results of Koo et al. (1986) for various A_5N_5 sequences, when one takes into consideration the slightly lower polyacrylamide concentration used by those authors.

One other aspect of the data presented in Figure 1 is worth noting. It is clear that alterations in base sequence two base pairs away from the A-tracts can influence the net curvature of the A-tract-containing molecules. Specifically, the conversion of the N_5 sequence in $(A_5N_5)_n$ from CGCGC (1135/1134) to CACAC (1047/1058) results in a small but detectable increase in mobility of the $(A_5N_5)_n$ polymers. Moreover, the simple reversal of two G × C base pairs in the N_5 sequence, from CGCGC (1135/1134) to GCCGC (1159/1158), results in a significant reduction in electrophoretic mobility.

The electrophoretic behaviors of 11 A-tract polymers $[(A_5N_5)_n]$ containing methylated C residues in the N_5 region are displayed in Figure 2. As demonstrated in Figure 2 (a and b), C5 methylation, either as single or multiple methy-

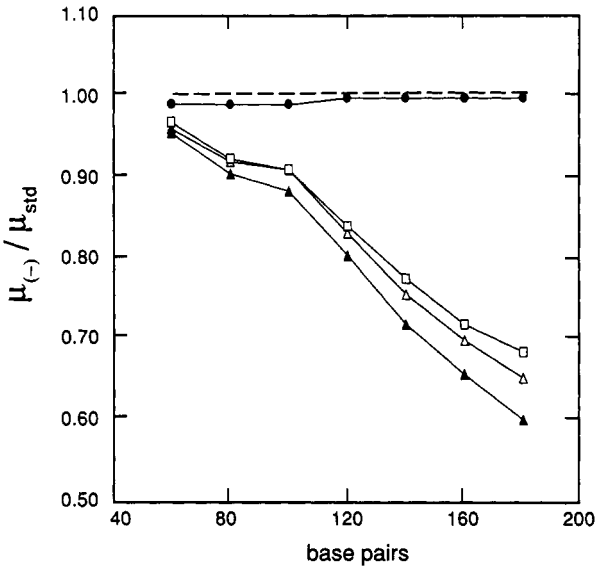

FIGURE 1: Plot of the electrophoretic mobilities, $\mu_{(-)}$, of the various nonmethylated DNA molecules employed in this study, relative to electrophoretically normal control molecules (μ_{std}). For all duplex sequences, the second sequence is written 3′ → 5′, as in Table I. (—) TACCGATGGATGCCGATGGA·GGCTACCTACGGCTACC-TAT (1168/1169) (control); (●) GGCCACGTGACCTGACG-TAC·GGTGCACTGGACTGCATGCC (1198/1196); (△) CAAAAACGCG·TTTTGCGCGT (1135/1134); (▲) CAAAAAGCCG·TTTTCGGCGT (1159/1158); (□) CAAAAACACA·TTTTGTGTGT (1047/1058).

lation events, has only a modest influence on the net curvature of molecules containing the N_5 sequences, CGCGC and CACAC. However, methylations within the N_5 sequence, GCCGC, all appear to reduce the net curvature of their corresponding $(A_5N_5)_n$ species from that of the unmethylated control (Figure 2c). Moreover, for the N_5 sequence GCCGC, the effects of the methyl groups on net curvature would appear to be nearly additive, although the significance of this last observation remains to be determined. Finally, for the N_5 sequence GCCGC, a single methylation event at the central C residue results in a clear reduction in the net curvature (increased mobility) of the $(A_5N_5)_n$ species (Figure 2c; 1423/1158), even though the methylated C residue is three base pairs from the A-tracts. This last observation provides evidence that cytosine methylation results in structural alterations of DNA within non-A-tract sequences.

The altered mobilities of the ligated, methylated species are not a result of nicks in the ligated species. A control experiment using duplex oligomers 1160/1158, in which oligo 1158 was left unphosphorylated, showed a greater increase in mobility than the completely phosphorylated species, with distinct band positions. Furthermore, a denaturing gel showed that greater than 90% of an excised 40-mer, produced by ligation of a 10-mer, contained full-length single strands.

Cytosine Methylation Does Not Induce Significant Distortions of the Helix Axis in Non-A-Tract DNA. The results presented thus far (Figure 2, above; Hagerman, 1990b) indicate that cytosine methylation can alter the net curvature of DNA molecules containing oligopurine–oligopyrimidine tracts. However, it is not clear whether the effects of methylation act only through the tracts themselves, or whether axial distortions secondary to cytosine methylation can occur in the absence of such tracts. Therefore, the effects of cytosine methylation were investigated by constructing ligation ladders from 13 additional methylated (or unmethylated) oligomer duplexes lacking A-tracts. The sequences of these oli-

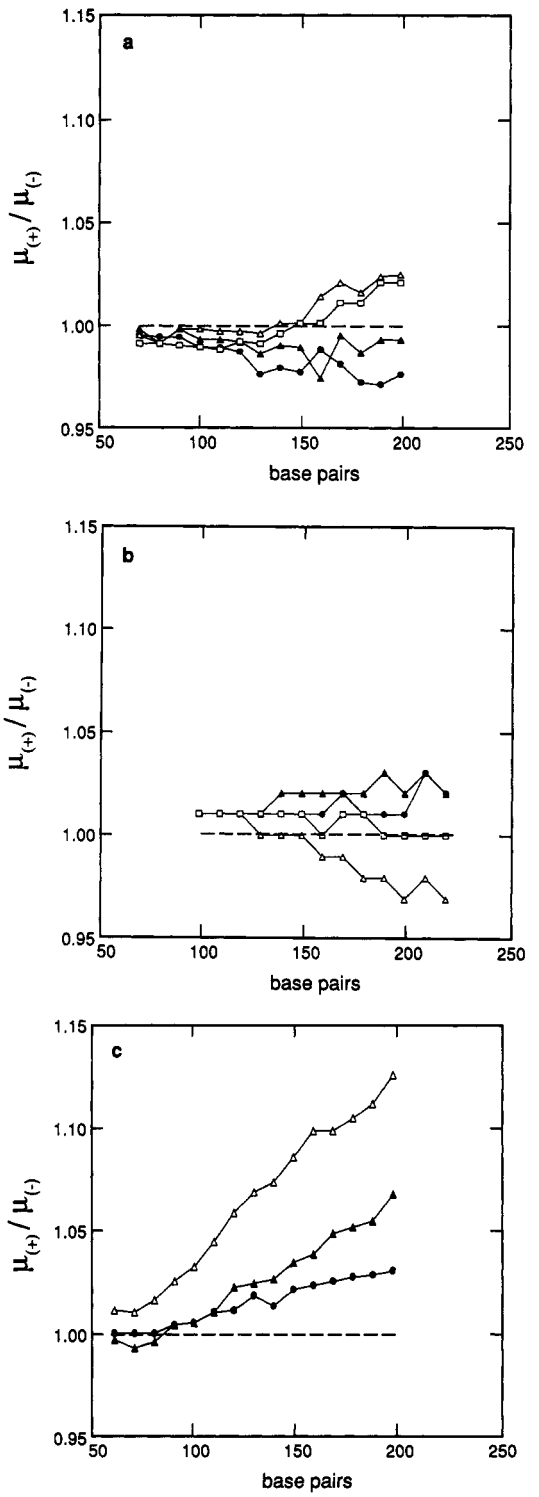

FIGURE 2: Plots of the differential mobilities of various A-tract DNA molecules consequent to cytosine methylation. Differential mobilities are plotted as $\mu_{(+)}/\mu_{(-)}$, the ratio of the mobilities of the methylated (+) species to their nonmethylated (–) counterparts. Panel a: (●) ^{me}CAAAAACGCG·TTTTGCGCGT (1136/1134); (△) CAAAAACG^{me}CG·TTTTGCGCGT (1137/1134); (▲) CAAAAA^{me}-CGCG·TTTTGCGCGT (1138/1134); (□) ^{me}CAAAAA^{me}CG^{me}-CG·TTTTGCGCGT (1139/1134); (—) CAAAAACGCG·CAAAAACGCG (1135/1134, nonmethylated reference). Panel b: (●) ^{me}CAAAAACACA·TTTTGTGTGT (1049/1058); (△) CAAAAA^{me}CACA·TTTTGTGTGT (1052/1058); (▲) CAAAAACA^{me}CA·TTTTGTGTGT (1053/1058); (□) ^{me}CAA-AAA^{me}CA^{me}CA·TTTTGTGTGT (1054/1058); (—) CAAA-AACACA·TTTTGTGTGT (1047/1058, nonmethylated reference). Panel c: (●) CAAAAAG^{me}CCG·TTTTCGGCGT (1426/1158); (△) CAAAAAG^{me}C^{me}CG·TTTTCGGCGT (1160/1158); (▲) CAA-AAAGC^{me}CG·TTTTCGGCGT (1423/1158); (—) CAAAAA-GCCG·TTTTCGGCGT (1159/1158, nonmethylated reference).

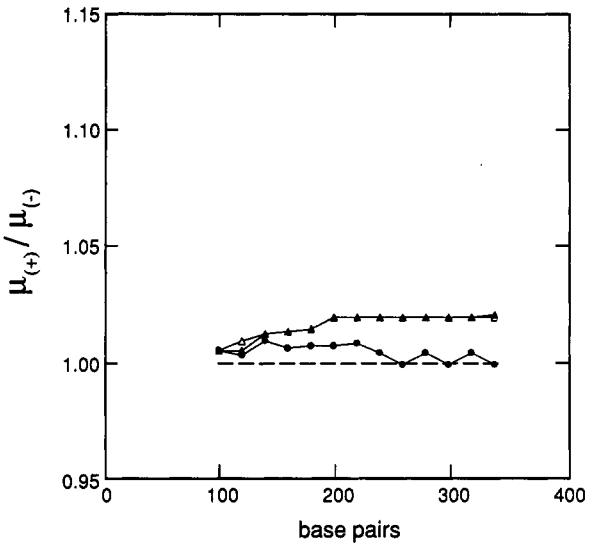

FIGURE 3: $\mu_{(+)}/\mu_{(-)}$ plots for non-A-tract molecules containing ^{me}C-^{me}C methylated species. (—) TACCGATGGATGCCGATGGA·GGCTACCTACGGCTACCTAT (1168/1169, nonmethylated reference); (●) TA^{me}C^{me}CGATGGATG^{me}C^{me}CGATGGA·GGCTACCTACGGCTACCTAT (1166/1169); (△) TACCGATGGATGCCGATGGA·GGCTA^{me}C^{me}CTACGGCTA^{me}C^{me}CTAT (1168/1167); (▲) TA^{me}C^{me}CGATGGATC^{me}C^{me}CGATGGA·GGCTA^{me}C^{me}CTACGGCTA^{me}C^{me}CTAT (1166/1167).

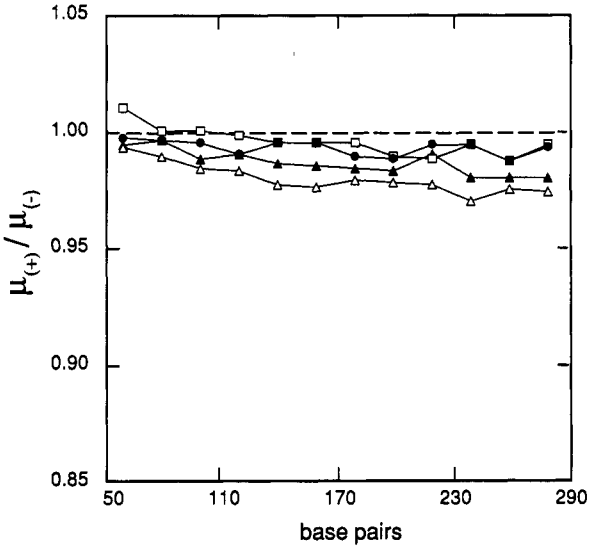

FIGURE 4: $\mu_{(+)}/\mu_{(-)}$ plots for a series of non-A-tract molecules which represent variants of the sequence of the E_{USF} box (1198/1196; see text), 9% polyacrylamide gel. (●) GGCCT^{me}CTTGACCTGT^{me}CTTAC·GGAGAACTGGACAGAATGCC (1190/1189); (△) GGCCA^{me}CATGACCTGA^{me}CATAC·GGTGTACTGGACTGTATGCC (1195/1194); (▲) GGCCT^{me}CATGACCTGT^{me}CATAC·GGAGTACTGGACAGTATGCC (1192/1191); (□) GGCCA^{me}CGTGACCTGA^{me}CGTAC·GGTGCACTGGACTGCATGCC (1197/1196).

gomers are listed in Table I, and the results of electrophoresis experiments are presented in Figures 3 and 4.

Inspection of Figure 3 reveals that phased methylations of the form $^{me}C^{me}C$ appear to produce at most only a slight alteration of the electrophoretic mobilities of *n*-mers of the reference non-A-tract sequence 1168/1169 (Table I). The slight increase in electrophoretic mobility observed for 1168/1167 and 1166/1167 is reproducible, but is probably not due to simple curvature, since DNA molecules possessing an 8-bp $^{me}C^{me}C$ repeat (1424/1425) demonstrate the same slight increase in mobility as does their counterpart, 1168/1167, the latter possessing a 10-bp $^{me}C^{me}C$ repeat (data not shown). The origin of this latter effect is unknown, but could arise either from a slight axial compression of the TCCA sequence upon methylation or from a small increase in the net axial charge density of the methylated DNA.

The measurements presented in Figure 3 were stimulated by the observation (Figure 2c) that pairs of contiguous, methylated C residues are capable of significantly altering the net curvature of A-tract-containing molecules. Therefore, it is somewhat surprising that methylation of contiguous C residues in molecules lacking A-tracts appears to have only a minor effect on mobility. One possible explanation for the latter observation is that the effects of the methyl groups are transmitted to the A-tract region, resulting in an alteration in the conformation of the tract. However, it is also possible that the methyl groups exert their effects primarily through alterations in helix twist, which would not be observable in helices otherwise lacking regions of curvature. In this latter regard, Nikol and Felsenfeld (1983) demonstrated that C5-methylation of *Hpa*II sites in a circular plasmid could not have altered local twist by more than 1.7° per site. Although their results tend to argue against the influence of twist, methylation-dependent twist deformations may be sensitive to flanking base pairs. The resolution of this issue awaits further investigation.

In order to examine further the effects of cytosine methylation on the structure of DNA lacking A-tracts, a second set of DNA molecules was constructed. The sequences of this latter set consist of phased repeats, GGCCACGTGACCTGACGTAC (1198/1196, Table I), of the upstream stimulatory factor (USF) E box site from the adenovirus major late promoter, that promoter being recognized by the c-Myc basic region (Prendergast & Ziff, 1991), and sequence variants thereof (Table I). The choice of the E box sequence is based on the observation by Prendergast and Ziff that binding of the c-Myc basic motif is specific for the E_{USF} box containing an unmethylated CpG. The additional sequences were generated by varying the bases flanking the C residues at positions 6 and 16 of sequence 1198 (Table I). For example, in oligomer 1515, ACG (1198) has been changed to TCA. The results of this gel analysis are presented in Figure 4. Methylation appears to cause very little distortion of the helix axis for this latter set of molecules, suggesting either that the methyl effect is not due to a structural distortion in this case or that the effects are too subtle to be detected with our mobility shift assay.

DISCUSSION

In a previous work (Hagerman, 1990b), it was demonstrated that methylation of pyrimidine residues within oligo(dA)·oligo(dU) or oligo(dI)·oligo(dC) tracts influences the magnitude of DNA curvature generated by those tracts, and therefore, that the methyl groups were capable of altering the local structure of the DNA helix. However, the observed influence on curvature of the methyl groups within the A- or I-tracts may be a specific consequence of the unusual environment of the methyl groups within those tracts. In the current work, we have demonstrated that the influence of pyrimidine methyl groups on DNA structure is somewhat more general. In particular, for the case of cytosine methylation, we have demonstrated that methylation-dependent perturbations in the local helix structure do not require the methyl groups to reside within an oligopurine–oligopyrimidine tract. Moreover, we have demonstrated that the conformational perturbations induced by cytosine methylation depend on the sequence of the bases that flank the sites of methylation.

Behe and Felsenfeld (1981) provided the first evidence that methylation of cytosine residues could influence the relative stabilities of DNA conformers. In particular, those authors demonstrated that methylation of the cytosine residues in the alternating copolymer poly(dG–dC)·poly(dG–dC) has a profound influence on the salt-dependence of the B–Z transition. More recently, Wu and Behe (1985) demonstrated that methylation of the pyrimidines in poly(dA–dU)·poly(dA–dU) facilitates its transition to an alternating B conformation of the form originally proposed by Klug et al. (1979). The results of the current work therefore provide further support for the suggestion of Wu and Behe (1985) that pyrimidine methylation may influence the action of transcriptional proteins through alterations in DNA conformation, in addition to direct protein–methyl group interactions. It should be noted that Diekmann and McLaughlin (1988) have made a similar proposal for the role of N6 methyl groups, namely, that such methyl groups might influence biochemical reactions through their alteration of local DNA structure.

The nature of the conformational alterations introduced by the methyl groups is not known at present. The observations presented in Figure 2 indicate that methyl groups could exert their influence on A-tracts positioned up to three base pairs away from the sites of methylation. While such an influence at a distance is plausible, it is also possible that the methyl-group effects are the result of local alterations in twist and/or direction of the helix axis, with a consequent alteration in the net curvature of the DNA molecule as a whole. The absence of a significant modulation of electrophoretic mobility by C5 methylation in non-A-tract DNA would tend to argue for the former model; however, subtle alterations in the direction of the helix axis may not be detectable on gels in the absence of an A-tract amplifier sequence.

It is hoped that the current work will stimulate two types of investigation. First, high-resolution structural studies (e.g., X-ray crystal diffraction, NMR) should be performed in order to elucidate both the primary influence of the methyl group and the reason for the influence of the flanking base pairs. Second, it is hoped that the current results will stimulate the further refinement of theoretical models for the energetics of DNA conformation, since current models do not predict a dependence of conformation on the presence of the pyrimidine methyl group (Srinivasan et al., 1987; Olson et al., 1990; Hausheer et al., 1989).

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Registry No. Cytosine, 71-30-7; poly(dA), 25191-20-2.