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Methylated Pyrimidines Stabilize an Alternating Conformation of Poly(dA-dU)·Poly(dA-dU)†

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ABSTRACT: We have investigated the effect of increasing percentages of methylated pyrimidines on the structure of poly(dA-dU)·poly(dA-dU). This was done by synthesizing analogous polynucleotides that contained deoxythymidine residues as well as deoxyuridine residues and observing their ^{31}P NMR spectra in increasing amounts of CsF. The results show that methylated pyrimidines play a large role in the stabilization of the "alternating B" conformation of DNA.

The effect of DNA methylation on the expression of genes in eukaryotic cells has been reviewed several times in the past few years (Bird, 1984; Felsenfeld & McGhee, 1982). Extensive methylation often is associated with gene inactivation, while undermethylation is sometimes required for a gene to be expressed. The methylated sites in chromosomal DNA are most often at the C5 position of cytosine in a -CG- dinucleotide sequence. There has been speculation that the effect of methylation is to alter DNA conformation since methylation of cytosines in a polymer of that sequence, poly(dG-m^{5}dC)·poly(dG-m^{5}dC), was shown (Behe & Felsenfeld, 1981) to greatly facilitate the B to Z transition. However, no B–Z transition was seen when a plasmid containing the chicken adult β-globin gene was methylated at its -CCGG- sites by *Hpa*II methylase (Nickol & Felsenfeld, 1983).

The presence of a methylated pyrimidine can cause other conformational variations besides the B–Z transition, however. It has been observed (Patel et al., 1982; Chen et al., 1983) that the methylated synthetic polymer poly(dG-m^{5}dC)·poly(dG-m^{5}dC) is in an "alternating B" conformation in solutions of moderate NaCl concentration but the analogous unmethylated polymer is not. The alternating B conformation was first proposed by Klug et al. (1979) for poly(dA-dT)·poly(dA-dT), another polymer containing methylated pyrimidines, and the appearance of a closely spaced doublet (0.2–0.3 ppm separation) in its ^{31}P NMR spectrum greatly supported the alternating model (Shindo et al., 1979; Chen & Cohen, 1983; Patel et al., 1981). Klug et al. (1979) speculated that the alternating structure was stabilized by a stacking interaction involving the methyl group of the pyrimidine base.

In order to elucidate the dependence of the alternating B conformation on methylated pyrimidines, we have examined the ^{31}P NMR spectra of several synthetic polynucleotides. The polymers studied include poly(dA-dU)·poly(dA-dU), poly(dA-dT)·poly(dA-dT), and analogous polynucleotides con-

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taining various proportions of dT and dU. We represent these polymers by an expression such as poly(dA-dU,T)·poly(dA-dU,T)–60% dT for an alternating purine–pyrimidine polymer in which all of the purine bases are adenosines, 60% of the pyrimidine bases are thymine, and 40% of the pyrimidine bases are uridine. We have observed that the separation of peaks in the ^{31}P NMR spectra of the polymers increases with an increasing proportion of methylated pyrimidines. The results suggest that increasing the percentage of methylated pyrimidines gradually shifts the conformation from a regular to an alternating structure.

Materials and Methods

Synthesis of the Polymers. Poly(dA-dT)·poly(dA-dT), dATP, and dTTP were purchased from P-L Biochemicals. dUTP was obtained from Sigma. The Klenow fragment of DNA polymerase I was isolated from *Escherichia coli* strain CJ155 (Joyce & Grindley, 1983), which was the gift of Dr. Catherine Joyce.

Poly(dA-dU)·poly(dA-dU) was synthesized as follows: In 100 μL of reaction media were contained 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.6, 6 mM $MgCl_2$, 1 mM mercaptoethanol, 10 μg of poly(dA-dT)·poly(dA-dT), 3 mM dATP, 3 mM dUTP, and 2 units of Klenow fragment. After 16-h incubation at 37 °C, the entire mixture was added to 1 mL of reaction media containing the same reactants in the same concentrations except that no poly(dA-dT)·poly(dA-dT) was added. After a second 16-h incubation at 37 °C, the entire mixture was added to 10 mL of reaction media, again with no added poly(dA-dT)·poly(dA-dT). The mixture was incubated for 16 h at 37 °C. The synthesized polymer was precipitated with ethanol, redissolved in 0.4 mL of 10 mM Tris-HCl, pH 7.5, extracted twice with phenol and then chloroform, and passed through a column of Sephadex G-50 equilibrated with 5 mM Tris-HCl, pH 7.5, and 0.1 mM ethylenediaminetetraacetic acid (EDTA). The total yield of polymer was about 3 mg.

Polymers containing different percentages of dT and dU were synthesized by the same procedure except that dTTP was also added to the reaction media. The total amount of dTTP plus dUTP was kept constant at 3 mM.

The percentages of nucleotide residues of the synthetic polymers were determined by reversed-phase high-pressure liquid chromatography (HPLC). Prior to injection, samples were digested into nucleotides by the method of Kuo et al. (1980). Each injection contained 2.5 μg of digested DNA, the column was a Bondapak C18 (Waters), and the mobile phase was a linear gradient of 5–15% acetonitrile/1.0% ammonium acetate, pH 5.8.

NMR Spectroscopic Studies. ^{31}P NMR spectra were obtained with an IBM-Bruker Model WP200SY NMR set at 81.01 MHz and were recorded at 25–30 °C in the deuterium lock mode with 4K data points and a spectral window of 4000 Hz. Typically 40 000–50 000 scans were acquired.

Prior to an NMR experiment, the solution, containing 3–4 mg of synthetic DNA polymer, was sonicated (Cohen et al., 1981) for 3 h by using a Heat Systems–Ultrasonics Model W-375 sonicator on pulsed mode, 50% duty cycle, with the output control set at 6. The temperature was kept at 3–8 °C. Polyacrylamide gel electrophoresis showed that the size of the polymers was reduced to 50–250 base pairs by this treatment. After sonication, the polymer was precipitated with ethanol and redissolved in 5 mM Tris, pH 7.4, 0.1 mM EDTA, 0.1 M NaCl, and 33% D_2O (low-salt buffer). One microliter of trimethyl phosphate was added as an internal standard, and all chemical shifts are measured upfield from it. After the NMR spectrum was recorded in this solution, solid CsF was added to the desired concentration, and more scans were acquired.

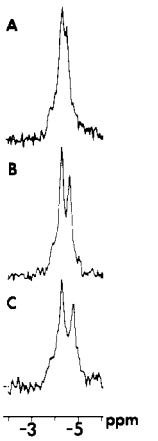

FIGURE 1: ^{31}P NMR spectra of poly(dA-dT)·poly(dA-dT) in low-salt buffer plus (A) 0 M CsF, (B) 1 M CsF, and (C) 3 M CsF.

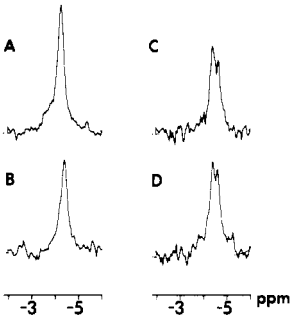

FIGURE 2: ^{31}P NMR spectra of poly(dA-dU)·poly(dA-dU) in low-salt buffer plus (A) 0 M CsF, (B) 1 M CsF, (C) 2 M CsF, and (D) 3 M CsF.

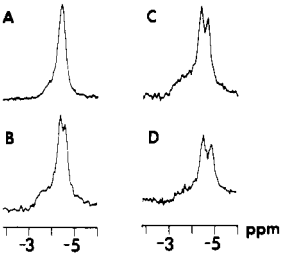

FIGURE 3: ^{31}P NMR spectra of poly(dA-dU,T)·poly(dA-dU,T)–40% dT in low-salt buffer plus (A) 0 M CsF, (B) 1 M CsF, (C) 2 M CsF, and (D) 3 M CsF.

Results

Poly(dA-dT)·poly(dA-dT) has been seen to give two closely spaced peaks in its ^{31}P NMR spectrum (Shindo et al., 1979; Chen & Cohen, 1983) at moderate salt concentrations, and this has been interpreted as strong evidence for an alternating B conformation (Klug et al., 1979). The separation of the peaks can be increased by the addition of high concentrations of CsF (Kypr et al., 1981; Patel et al., 1981). In our hands, this behavior of poly(dA-dT)·poly(dA-dT) is reproduced, as seen in Figure 1A–C. The separation of the peaks is 0.19 ppm in 0.1 M NaCl and increases to 0.51 ppm in 3 M CsF. In the case of the totally unmethylated polymer, poly(dA-dU)·poly(dA-dU), a single peak, is seen at moderate salt concentrations (Cohen et al., 1981; Figure 2A,B) and resolves into two peaks only at very high CsF levels (Figure 2C,D). For polymers containing both dU and dT, the separation of the two peaks increases with increasing percent dT (Figures 3–5).

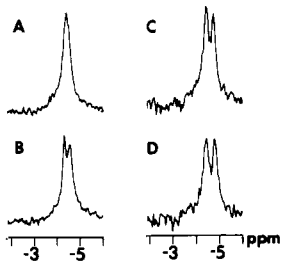

FIGURE 4: ^{31}P NMR spectra of poly(dA-dU,T)·poly(dA-dU,T)–49% dT in low-salt buffer plus (A) 0 M CsF, (B) 1 M CsF, (C) 2 M CsF, and (D) 3 M CsF.

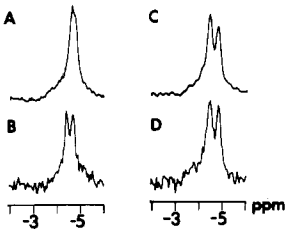

FIGURE 5: ^{31}P NMR spectra of poly(dA-dU,T)·poly(dA-dU,T)–68% dT in low-salt buffer plus (A) 0 M CsF, (B) 1 M CsF, (C) 2 M CsF, and (D) 3 M CsF.

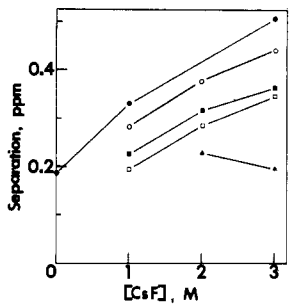

FIGURE 6: Separation of peaks as a function of [CsF] in the ^{31}P NMR spectra of (●) poly(dA-dT)·poly(dA-dT), (○) poly(dA-dU,T)·poly(dA-dU,T)–68% dT, (■) poly(dA-dU,T)·poly(dA-dU,T)–49% dT, (□) poly(dA-dU,T)·poly(dA-dU,T)–40% dT, and (▲) poly(dA-dU)·poly(dA-dU).

The separation of the peaks for the various polymers is plotted vs. CsF concentration in Figure 6. It can be seen that for all polymers except poly(dA-dU)·poly(dA-dU), the separation of the two phosphorus resonances continuously increases with increasing CsF concentration. Additionally, separation of peaks at any particular CsF concentration increases with increasing percent dT of the polymer. In Figure 7, with the CsF concentration held constant at 3 M, the chemical shift of the downfield and upfield peaks of each polymer is plotted vs. percent dT of the polymer. As the percentage of methylated pyrimidines increases from 0% to 100%, the downfield peak gradually shifts by 0.1 ppm to lower negative values, and the upfield peak gradually shifts by 0.2 ppm to higher negative values.

Discussion

Methylated sites in eukaryotic DNA are thought to have a role in the regulation of gene function (Bird, 1984; Felsenfeld & McGhee, 1982). There are several ways that methylation can be thought to regulate genes. The first is by a regulatory protein recognizing the methylated sequence directly. Presumably, the DNA is in a more or less normal B conformation, and the extra contacts provided by the methyl groups are sufficient for the protein to distinguish it from the unmethylated sequence. A second possibility is that methylation of DNA causes a change in the conformation of the DNA itself. The altered conformation can then either be bound by

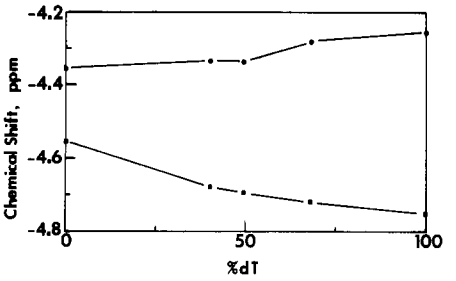

FIGURE 7: Chemical shift of the downfield and upfield ^{31}P NMR peaks of the polymers in 3 M CsF vs. percent dT.

a regulatory protein or is itself a better or worse template for a polymerase.

The second alternative has received much attention in the past few years since it was shown that methylation can sometimes affect the conformation of DNA quite dramatically. Behe & Felsenfeld (1981) demonstrated that methylation of the synthetic polynucleotide poly(dG-dC)·poly(dG-dC) at the 5-position of cytosine, which is often the site of eukaryotic methylation, can lower the concentration of counterions required to flip the polymer into the Z conformation, so that the methylated polymer could exist in the Z form at physiological ionic strength.

However, the methylation of DNA can apparently have other effects on structure. Klug et al. (1979) proposed a model for the structure of poly(dA-dT)·poly(dA-dT) which they dubbed the alternating B conformation. In this model, the two sequences A_pT and T_pA differed in base stacking and phosphodiester conformation. It was suggested (Klug et al., 1979) that the altered structure occurred to maximize the stacking of thymine's methyl group on the base below it. Physical evidence for the model was provided by ^{31}P NMR studies (Shindo et al., 1979; Chen & Cohen, 1983; Patel et al., 1981) of poly(dA-dT)·poly(dA-dT) which showed two closely spaced, but distinct, phosphorus resonances, reflecting the two phosphorus environments of the polymer. Eckstein & Jovin (1983) demonstrated that the downfield peak was due to T_pA while the upfield peak was due to A_pT. Patel et al. (1981) and Kypr et al. (1981) showed that the separation of the peaks could be increased by large concentrations of CsF.

The alternating purine–pyrimidine polymer poly(dG-dC)·poly(dG-dC) did not give two phosphorus resonances (Cohen et al., 1981) except at very high salt concentration (Patel et al., 1979). However, the analogous methylated polymer poly(dG-m^{5}dC)·poly(dG-m^{5}dC) did give two closely spaced peaks even at low salt concentration (Patel et al., 1982; Chen et al., 1983), which was interpreted as reflecting an alternating B conformation for the polymer. Recently we have synthesized the two mixed ribo–deoxyribo copolymers poly(rG-dC)·poly(rG-dC) (Wu & Behe, 1984) and poly(rG-m^{5}dC)·poly(rG-m^{5}dC) (Wu & Behe, 1985). Both of these polymers are in the A conformation at moderate salt concentrations. Although poly(rG-dC)·poly(rG-dC) gives a single ^{31}P NMR peak, the analogous methylated polymer gives two closely spaced peaks. We have interpreted this as evidence for an "alternating A" conformation. Thus, it appeared to us, as Klug et al. (1979) speculated, that a methylated pyrimidine was a promoter of an alternating conformation. We then decided to investigate the effect of methyl groups on structure by making a series of polymers containing varying ratios of methylated to unmethylated pyrimidines.

We anticipated three possible situations before we began. The first was that the occurrence of the alternating structure was a very localized phenomenon, with methylated base pairs

locked into the alternating form, but that the alternating structure did not extend further into the rest of the helix. The second case was that the regular to alternating transition was highly cooperative. Third, increased methylation might gradually change the helix from a regular to an alternating structure. In the first situation, one would expect to see a third ^{31}P NMR peak, corresponding to the regular B conformation, increasing in intensity and the two original peaks decreasing as the percentage of methyl groups was decreased. In the second case, the highly cooperative situation, one would expect two peaks of equal intensity for all polymers down to a critical percentage of methylated residues and a single peak after that. (This is what is seen in the B–Z transition as a function of salt concentration.) As seen in Figures 1–5, neither of these postulated cases occurs. Instead, as seen in Figures 6 and 7, at any one CsF concentration, as the percentage of methylated bases decreases the separation between the phosphrus resonances decreases. This suggests that the conformation of the polymer is gradually progressing from a regular helix to an alternating helix as the percentage of methylated pyrimidines increases from 0% to 100%. This further implies that a continuum of conformations, stabilized by an increasing percentage of methylated pyrimidines, is stable between a regular and an alternating helix. This interpretation is supported by studies of the effect of CsF (Chen & Cohen, 1983; Patel et al., 1981; Kypr et al., 1981) on peak separation in an alternating structure. As CsF is continuously increased, the separation of phosphorus resonances of poly(dA-dT)·poly(dA-dT) and the polymers reported here continuously increases, strongly suggesting the gradual shift to a more pronounced alternating structure, as Chen & Cohen (1983) argued in the case of poly(dA-dT)·poly(dA-dT).

This study was conducted on polymers of the general form poly(dA-dU,T)·poly(dA-dU,T) because, among other reasons, they do not have a propensity to flip to a Z form at high salt concentrations like poly(dG-dC)·poly(dG-dC). Discussion of the results obtained here, then, should be limited to sequences of dA-dT residues. Nonetheless, it is tempting to speculate that methylation of -CG- sequences in vivo is having a similar effect as on the synthetic polymers, shifting a region of DNA toward a more alternating structure, and that regulatory proteins are recognizing such subtle conformational changes. It should be noted, however, that methylation is not always required for a right-handed alternating structure. Both poly(dI-dC)·poly(dI-dC) in the B form (Cohen et al., 1981) and poly(rG-rC)·poly(rG-rC) in the A form (Hall et al., 1984) give two closely spaced ^{31}P NMR peaks at lower salt concentrations, indicative of dinucleotide repeats.

We have observed that another factor besides methylation that a priori might have been thought to favor the alternating structure is insufficient to do so: the sugar conformations of the polynucleotide. In the alternating B model of Klug et al. (1979) for poly(dA-dT)·poly(dA-dT), the deoxyribose of dA is in the C3′-endo conformation and the sugar of dT is C2′-endo. As mentioned above, poly(rG-dC)·poly(rG-dC) does not show an alternating structure (Wu & Behe, 1984) even though the purine residues are expected to be in the C3′-endo conformation since ribonucleotides favor that form. The analogous methylated polymer, however, is in an alternating conformation at moderate salt concentration (Wu & Behe, 1985).

Registry No. Poly(dA-dU), 34607-75-5; poly(dA-dT), 26966-61-0.

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