Effects of methylation on a synthetic polynucleotide: The B–Z transition in poly(dG-m⁵dC)·poly(dG-m⁵dC)

(left-handed helical DNA/circular dichroism/polyelectrolyte)

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ABSTRACT   We have compared the behavior in solution of the synthetic polynucleotide poly(dG-m⁵dC)·poly(dG-m⁵dC) with that of the unmethylated polynucleotide poly(dG-dC)·poly(dG-dC). In solutions containing high concentrations of salt, poly(dG-dC)·poly(dG-dC) is known to exhibit altered circular dichroic and absorption spectra correlated with formation of a left-handed Z DNA structure. Poly(dG-m⁵dC)·poly(dG-m⁵dC) behaves similarly, but the spectral transition from the B to the Z form occurs at much lower salt concentrations, close to usual physiological conditions. Divalent and polyvalent ions are particularly effective: The B–Z transition of poly(dG-m⁵dC)·(dG-m⁵dC) can be induced at a Mg²⁺ concentration three orders of magnitude lower than that required for the unmethylated polymer. We have also studied mixed copolymers containing both dC and m⁵dC. Our results suggest that the sequence m⁵dC-dG, which occurs in eukaryotic DNA, can have a disproportionately large effect on the B–Z transition.

It is now accepted that DNA of appropriate nucleotide sequence is capable of forming left-handed double helical structures with Watson–Crick base pairing, as well as the more familiar right-handed structures. X-ray crystallographic studies of the synthetic oligonucleotide d(CpGpCpGpCpG), demonstrated the left-handed, or Z-DNA, conformation (1). Subsequent studies of the smaller oligonucleotide d(CpGpCpG) suggested that there are at least two related Z conformations (2, 3). Diffraction patterns consistent with the Z form have also been observed (4) with fibers of the high molecular weight alternating copolymer poly(dG-dC)·poly(dG-dC) and with poly(dA-dC)·poly(dG-dT).

The solution properties of poly(dG-dC)·poly(dG-dC) were described and analyzed by Pohl and Jovin some years ago (5). They showed that at high salt concentrations (above 2.5 M NaCl or 0.7 M MgCl₂) the polynucleotide is converted to a new form by a cooperative, intramolecular process. This new form displays a circular dichroic spectrum that is inverted compared to that of the B form, and an ultraviolet absorption spectrum with increased molar extinction compared to the B form in the range 280–300 nm. The recent x-ray diffraction results, as well as nuclear magnetic resonance studies in solution (6), provide strong evidence that the unusual optical properties are associated with the Z form.

In this paper we examine the B–Z transition in another synthetic polynucleotide, poly(dG-m⁵dC)·poly(dG-m⁵dC). The dinucleotide sequence m⁵dC-dG occurs quite frequently in eukaryotic DNA; in many organisms it composes more than half of all "d(CpG)" sequences and in all cases it is the major site of methylation (7). Furthermore, the extent of methylation at sites within a structural gene appears to be correlated with the

transcriptional activity of the gene (7). For these reasons, we wished to compare the properties of the methylated C-G synthetic polymer with the unmethylated compound.

We have found that, as judged by circular dichroism and ultraviolet absorption, poly(dG-m⁵dC)·poly(dG-m⁵dC) also undergoes a transition from the B to the Z form, but at much lower salt concentrations, so that the Z form is stable under typical physiological conditions. Furthermore, in mixed alternating copolymers of dG-dC and dG-m⁵dC, the presence of the latter sequence has a disproportionate effect in lowering the amount of divalent ion required to stabilize the Z form. Our results provide some information about the forces that govern the B–Z transition and suggest that there are circumstances in which the Z form might be observed *in vivo*.

MATERIALS AND METHODS

Chemicals and Enzymes. *Escherichia coli* DNA polymerase large fragment was purchased from Boehringer Mannheim, the restriction endonuclease *Hha* I was purchased from Bethesda Research Laboratories, and micrococcal nuclease was obtained from P-L Biochemicals, as were poly(dI-dC)·poly(dI-dC), poly(dI-[2-¹⁴C]dC)·poly(dI-[2-¹⁴C]dC), poly(dG-dC)·poly(dG-dC), and most deoxynucleotides. Labeled deoxynucleotides were purchased from New England Nuclear. 5-Methyl-dCMP, spermine, and spermidine were obtained from Sigma. The diaminoalkanes were obtained from Calbiochem. Co(NH₃)₆Cl₃ was the gift of Jon Widom.

5-Methyl-dCTP was prepared by the reaction of pyrophosphate with the 5′-phosphoromorpholidate of 5-methyl-dCMP (8). The purified product ran as a single spot on polyethyleneimine-cellulose thin-layer chromatography in 0.25 M sodium phosphate, pH 7.0.

Preparation of the Methylated Polymer. Poly(dG-m⁵dC)·poly(dG-m⁵dC) was synthesized in a manner similar to that used for the preparation of poly(dG-dC)·poly(dG-dC) by Wells *et al.* (9). The reaction mix contained the following: 50 mM Tris·HCl (pH 8.0), 5 mM MgCl₂, 1 mM 2-mercaptoethanol, 0.4 mM dGTP and 5-methyl-dCTP, poly(dI-dC)·poly(dI-dC) at 50 μg/ml, and *E. coli* DNA polymerase large fragment at 5 units/ml. After 16 hr at 37°C, EDTA was added to 10 mM. Low molecular weight material was removed by passage through a Sephadex G-50 column, and the polymers were precipitated with ethanol and redissolved in 0.5 ml of 50 mM Tris·HCl (pH 8.0)/5 mM MgCl₂/5 mM 2-mercaptoethanol. *Hha* I was added to give 30 units/ml of original reaction mixture and the solution was incubated at 37°C for 16 hr. *Hha* I, whose naturally occurring restriction site is d(G-C-G-C), can digest the poly(dI-dC) template but not the methylated polymer. The reaction was stopped by addition of EDTA to 10 mM and the low molecular

weight oligonucleotides were removed by another passage through a Sephadex G-50 column. More than 90% of the template was removed by this treatment, as determined by using ^{14}C-labeled poly(dI-dC)·poly(dI-dC) as template. Protein was removed from the poly(dG-m^{5}dC)·poly(dG-m^{5}dC) by digestion with proteinase K and extraction with phenol and chloroform. The ultimate yield of methylated polymer was about 100 μg/ml of original reaction mixture, which is a 2-fold increase over the added template.

Polymers with mixed dC and m^{5}dC content were prepared by adding various amounts of [^{3}H]dCTP and 5-methyl-dCTP to the original reaction mixture. The proportion of dC to m^{5}dC in the polymer product was determined from its specific radioactivity.

Characterization of the Methylated Polymer. Nearest-neighbor frequency analysis of poly(dG-m^{5}dC)·poly(dG-m^{5}dC) was performed with [α-^{32}P]dGTP (10). Nucleoside monophosphates were separated by polyethyleneimine-cellulose thin-layer chromatography. Greater than 95% of the ^{32}P activity was associated with 5-methyl-dCMP.

Sedimentation velocity and equilibrium values of the methylated polymer were determined by using a Beckman model E analytical ultracentrifuge. The polymer had a sedimentation coefficient of 7.1 S in 50 mM sodium phosphate, pH 7.0, and sedimentation equilibrium yielded a weight-average molecular weight of 3×10^5. A sedimentation equilibrium experiment was also done on a sample of the methylated polymer that had been melted and annealed. The sample gave a molecular weight of 1.3×10^5, strongly suggesting that the polymer was originally double-stranded and formed a hairpin structure after melting and annealing.

Thermal denaturation experiments yielded a melting temperature of 76°C for poly(dG-m^{5}dC)·poly(dG-m^{5}dC) in 0.3 mM sodium phosphate, pH 7.0 (0.5 mM Na$^+$), compared to a literature value of 80°C in 1.0 mM KCl (11). Poly(dG-m^{5}dC)·poly(dG-m^{5}dC) in the phosphate buffer plus 1.0 mM MgCl$_2$ (to induce the formation of the Z structure) failed to melt at temperatures up to 98°C.

Spectral Measurements. Absorption measurements were performed with a Cary 219 spectrophotometer equipped with microcell adaptors to permit measurements on 0.1-ml samples. Circular dichroism was measured with a Jasco J-500A high-sensitivity spectropolarimeter. Samples for spectroscopy were prepared by diluting stock concentrations of polymer 1:100 into various solvents. It was found that heating the polymer to 60°C for 10 min allows it to achieve conformational equilibrium (approached from either side of the B–Z transition) and that there is no measureable temperature dependence of the transition for these polymers (see also ref. 5). Therefore most samples were heated and then cooled to ambient temperature for measurement.

RESULTS

Effect of cations

Gill *et al.* (11) have reported that poly(dG-m^{5}dC)·poly(dG-m^{5}dC) at high ionic strength shows unusual optical properties like those of poly(dG-dC)·poly(dG-dC) under similar conditions. The circular dichroic spectrum and absorption spectrum of the methylated polymer in 3 M NaCl confirm this conclusion (Fig. 1). We will refer to the conformation giving rise to these spectra as the "Z form." However, identification with a left-handed helical structure must be made with caution until the transition can be demonstrated in similar solvents by means of x-ray diffraction of the polymer fiber.

Although both the (dG-dC) and the (dG-m^{5}dC) polymers are in the Z form at high ionic strength, their behaviors with chang-
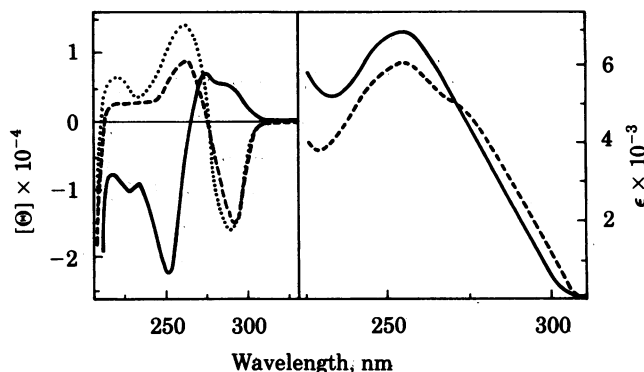

FIG. 1. Circular dichroic spectra (*Left*) and absorbance spectra (*Right*) of poly(dG-m^{5}dC)·poly(dG-m^{5}dC) in 0.1 M NaCl (——) and 3.0 M NaCl (-----). Also shown (*Left*, ····) is the circular dichroic spectrum of poly(dG-dC)·poly(dG-dC) in 3 M NaCl. In Figs. 1–4 the units for the molar ellipticity [Θ] are degrees·cm^2·dmol^{-1} and those for molar absorptivity ε are M^{-1}·cm^{-1}.

ing salt concentration are strikingly different. At very low ionic strength, both polymers have the B conformation. As reported by Pohl and Jovin (5), the midpoint of the B–Z transition in poly(dG-dC)·poly(dG-dC) occurs at 2.5 M NaCl or 0.7 M MgCl$_2$. The transition midpoint in poly(dG-m^{5}dC)·poly(dG-m^{5}dC), however, occurs at 0.7 M NaCl or 0.6 mM MgCl$_2$ (in the presence of 50 mM NaCl). Spectra on either side of the Mg^{2+}-induced transition are shown in Fig. 2. The concentration of Mg^{2+} required to stabilize the Z form of the methylated polymer is thus three orders of magnitude smaller than that required for the unmethylated one.

Because Mg^{2+} has such a potent effect on the poly(dG-m^{5}dC)·poly(dG-m^{5}dC) transition, we next examined the effects of a variety of divalent and polyvalent ions. All of these experiments were carried out in the presence of 50 mM NaCl, using the change in the absorption spectrum to monitor the transition. As shown in Table 1, Ca^{2+} and Ba^{2+} are as effective as Mg^{2+} in inducing the B–Z transition in poly(dG-m^{5}dC)·poly(dG-m^{5}dC). Ethylene- and propylenediamine, which also carry two positive charges, are slightly less effective than the alkaline earth ions. Diamines with longer aliphatic chains separating the charges require higher concentrations to induce the transition, but the pentanediamine produces an anomalous circular dichroic spectrum.

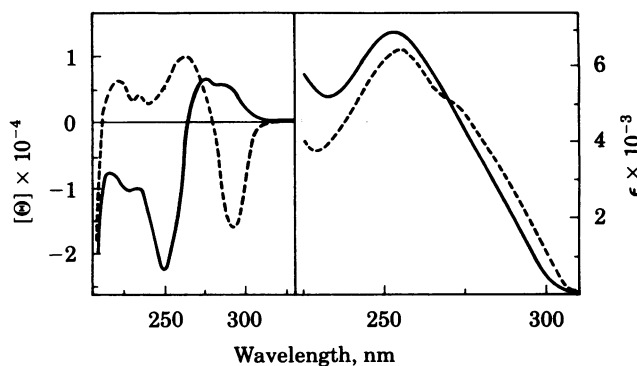Ions with more than two positive charges are even more effective. When the complex ion hexamminecobalt,

FIG. 2. Circular dichroic spectra (*Left*) and absorbance spectra (*Right*) of poly(dG-m^{5}dC)·poly(dG-m^{5}dC) in 0.2 mM Mg^{2+} (——) and 2.0 mM Mg^{2+} (-----). All solvents contained 50 mM NaCl, 5 mM Tris·HCl, pH 8.0. Mg^{2+} was added as the chloride.

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Table 1. Concentrations of cations or ethanol at the midpoint of the B–Z transition

Ion	poly(dG-dC)·poly(dG-dC)	poly(dG-m⁵dC)·poly(dG-m⁵dC)
Na^+	2500	700
Mg^{2+}	700	0.6
Ca^{2+}	100*	0.6
Ba^{2+}	40*	0.6
$NH_3(CH_2)_2NH_3^{2+}$	†	1.0
$NH_3(CH_2)_3NH_3^{2+}$	†	1.0
$NH_3(CH_2)_4NH_3^{2+}$	†	2.0
$NH_3(CH_2)_5NH_3^{2+}$	†	30*
$Co(NH_3)_6^{3+}$	0.02	0.005
Spermidine³⁺	‡	0.05
Spermine⁴⁺	§	0.002
Ethanol	60%	20%

Ion concentrations are given as mM; ethanol concentration is vol/vol. All ions were added as the chlorides. All solutions contain 50 mM NaCl, 5 mM Tris·HCl, pH 8.0.
* Transition produces anomalous circular dichroic spectrum (see text).
† No change in optical properties is observed up to 100 mM.
‡ Produces B form absorption spectrum up to 1 mM spermidine and then aggregates.
§ Produces B form absorption spectrum up to 0.05 mM spermine and then aggregates.

$Co(NH_3)_6^{3+}$, is added, the midpoint of the transition in poly(dG-m⁵dC)·poly(dG-m⁵dC) is reached at a concentration of 5 µM (in 50 mM NaCl), and the transition is complete at 7 µM. The midpoint for the tetrapositively charged ion spermine occurs at 2 µM, and the reaction is complete at 4 µM. At the DNA concentrations used in these experiments, the endpoints correspond to 2 positive charges per phosphodiester group in the case of hexamminecobalt, and 0.3 in the case of spermine.

In order to be certain that the change in absorption spectrum observed in the above titrations reflects a transition to the Z form, we measured in each case the circular dichroic spectrum of the sample on either side of the transition point. In every instance but one the appropriate circular dichroic spectrum was observed. The spectrum of the Z form induced by any of the polyvalent ions is quite similar to that observed in the presence of 3.0 M NaCl (Fig. 3).

The presence of certain concentrations of ethanol is known to favor transition to the Z form (12). The data reported in Table 1 show that a lower concentration of ethanol is required to induce the transition of poly(dG-m⁵dC)·poly(dG-m⁵dC) than of poly(dG-dC)·poly(dG-dC).

In order to complete the comparison of poly(dG-m⁵dC)·poly(dG-m⁵dC) with poly(dG-dC)·poly(dG-dC), we examined the effects of all cations mentioned above on the B–Z transition of the latter polymer. As shown in Table 1, most of the cations are much less effective with poly(dG-dC)·poly(dG-dC). A surprising exception is hexamminecobalt, which exhibits a potent effect on the transition, though at higher concentrations than required for the methylated polymer (Fig. 4). The effect on poly(dG-dC)·poly(dG-dC) of divalent ions other than Mg^{2+} is more difficult to interpret. The absorption spectrum shows a shift to longer wavelengths at concentrations of Ca^{2+} and Ba^{2+} about 1/7th and 1/17th, respectively, of those required to induce the B–Z conformational change with Mg^{2+}. However, the circular dichroic spectrum in the presence of Ca^{2+} or Ba^{2+} is unlike that of the Z form in 0.7 M Mg^{2+} or 3 M NaCl. The negative band at 290 nm is small, and the positive band is shifted to longer wavelengths relative to the spectrum of the canonical Z form. Still another kind of spectrum is seen when 1,5-diaminopentane is added to poly(dG-m⁵dC)·poly(dG-
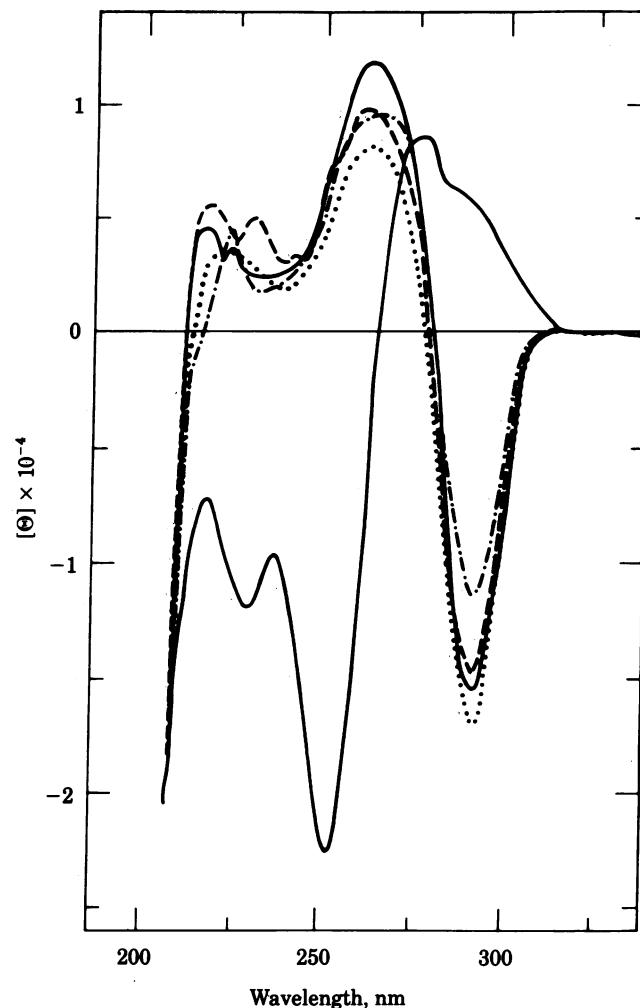

FIG. 3. Circular dichroic spectra of poly(dG-m⁵dC)·poly(dG-m⁵dC) in 5 mM Tris·HCl, pH 8.0, plus the following: buffer only (——, minimum at 250 nm); 1 mM $BaCl_2$ (----); 0.02 mM hexamminecobalt (-·-); 5 mM $NH_3(CH_2)_4NH_3^{2+}$ (····); 30% (vol/vol) ethanol (——).

m⁵dC) (data not shown). This is the only anomalous spectrum we have observed with the methylated polymer; all of the shorter diamines (Table 1) produce the usual spectrum of the Z form.

In order to learn more about the role of ions such as hexamminecobalt in stabilizing the Z form, we have studied the B–Z transition in solvents containing mixtures of hexamminecobalt and NaCl. The effect of hexamminecobalt on the poly(dG-dC)·poly(dG-dC) transition is a function of the NaCl concentration, as shown in Fig. 5. The critical concentration of hexamminecobalt varies approximately as $[Na^+]^{2.8}$.

Hexamminecobalt is known to be capable of inducing the condensation of native DNA (i.e., its collapse into a more compact form), though usually at critical concentrations higher than those used here to induce the B–Z transition (14). At higher DNA concentrations, aggregation may occur. However, the sedimentation velocity behavior of the polymers used in these experiments shows that collapse does not occur under the solution conditions we employ. In the absence of hexamminecobalt the sedimentation coefficients of poly(dG-dC)·poly(dG-dC) and poly(dG-m⁵dC)·poly(dG-m⁵dC) are 10.0 S and 7.0 S, respectively. In the presence of 10 µM hexamminecobalt the coefficients are 8.9 S and 6.5 S. Similar results are obtained for the methylated polymer in 1 mM $MgCl_2$ (all solvents contained
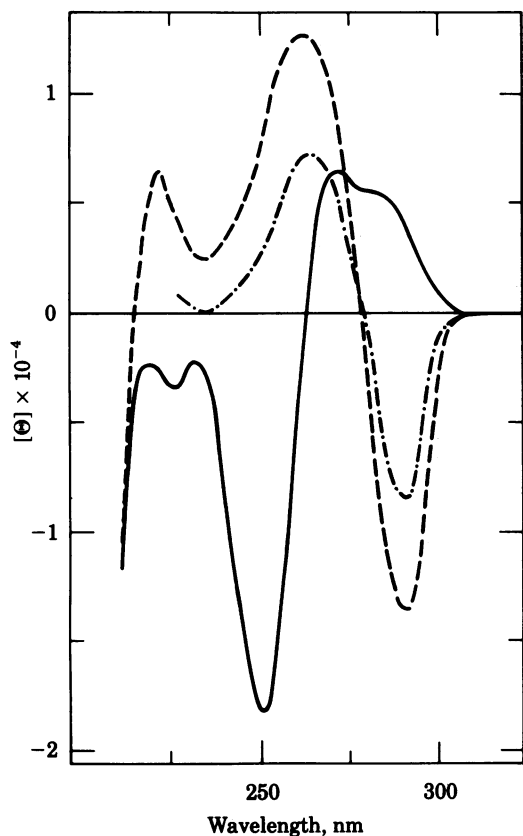
FIG. 4. Circular dichroic spectra of poly(dG-dC)·poly(dG-dC) in 5 mM Tris·HCl, pH 8.0, plus the following: buffer only (——); 0.02 mM hexamminecobalt (-----); 1 M MgCl$_2$ (–·–).

50 mM NaCl and 5 mM Tris·HCl, pH 8). The sedimentation coefficient of the Z form is slightly smaller than that of the B form; no condensation or aggregation occurs under these conditions.

Mixed copolymers

We have synthesized alternating copolymers of dG with various proportions of dC and m^{5}dC. The B–Z transition curve as a
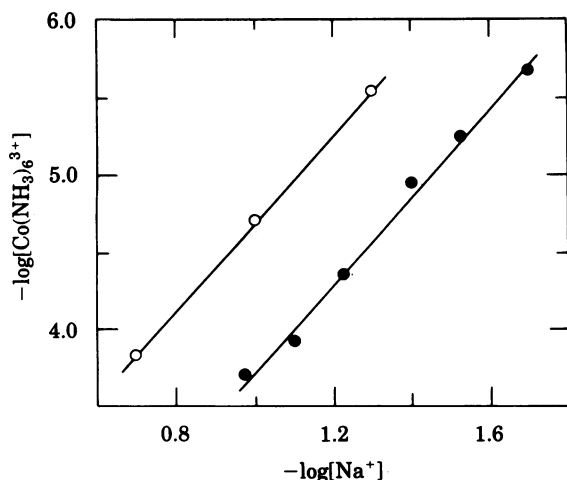

FIG. 5. Concentrations of hexamminecobalt and sodium at the midpoint of the B–Z transition. ●, Poly(dG-dC)·poly(dG-dC); ○, poly-(dG-m^{5}dC)·poly(dG-m^{5}dC). The fractional neutralization of charge on poly(dG-dC)·poly(dG-dC) along the line of transition, calculated by the method of Wilson and Bloomfield (13), varied between 0.847 and 0.83. For the methylated polymer the fractional neutralization was 0.82–0.78.

function of Mg^{2+} concentration (in the presence of 50 mM NaCl) was measured for a polymer containing 70% m^{5}dC, and for another containing 30% m^{5}dC. The transition midpoint occurred at 1 mM Mg^{2+} and 20 mM Mg^{2+}, respectively, for these two polymers, both higher than that for the fully methylated polymer (0.6 mM Mg^{2+}), but much lower than for the fully unmethylated polymer (700 mM).

DISCUSSION

The data presented here show that the alternating polymer poly(dG-m^{5}dC)·poly(dG-m^{5}dC) undergoes the transition from the B to the Z form at salt concentrations much lower than those required to induce the transition in poly(dG-dC)·poly(dG-dC). In solvents containing 50 mM NaCl, the methylated polymer is converted from B to Z form at divalent ion concentrations in the millimolar range. In contrast, conversion of poly(dG-dC)·poly(dG-dC) requires divalent ion concentrations between one and three orders of magnitude greater. In every solvent we have tested, including those containing polyamines or ethanol, it is easier to induce the B–Z transition in the methylated polymer than in the unmethylated one.

We can only speculate on the mechanism by which methylation of cytosine increases the relative stability of the left-handed helix. Let us assume for the moment that the Z form of the methylated polymer in solution is similar to that suggested for poly(dG-dC)·poly(dG-dC) on the basis of x-ray diffraction studies of the crystalline hexamer (1). In the hexamer, each cytosine residue shows strong stacking interactions with the cytosine residue on one of its two neighboring base pairs. It is reasonable to suppose that an alteration in the dipole moment and polarizability produced by methylation could alter the stacking free energy of both the Z form and the B form. It is also possible that interactions of water with the methylated polymer are different from those with the unmethylated polymer.

The x-ray diffraction studies of the hexamer also reveal that the distribution of backbone negative charge is quite different in the left-handed helix and in the right-handed B form. The average negative charge density (phosphodiester groups per unit contour length) is lower in the Z form than·in the B form, but in the Z form half of the distances between phosphodiester groups on opposing strands are unusually short ($\approx$0.8 nm), while the other half are considerably larger ($\approx$1.2 nm) (3). Presumably the effects of polyvalent cations on the B–Z transition can be explained in terms of interactions with this charge distribution.

Manning (15) and Record et al. (16) have used an ion-atmosphere model to calculate the contributions to free energy arising from electrostatic interactions between polyelectrolytes and their counterions. This model, which replaces the discrete charge distribution on the polyelectrolyte by a uniform linear charge density, explains many of the properties of DNA quite well, but·it excludes consideration of site-specific or counterion-specific effects. Widom and Baldwin (13) have pointed out that in the case of DNA condensation the theory gives a good approximate explanation of the phenomenon, but cannot account for the sometimes considerably different effects on condensation among cations of the same charge.

The behavior of the B–Z transition in both the methylated and unmethylated dG-dC polymers presents an even more serious challenge to a simple ion-atmosphere model. If the Z forms of these polymers in solution have the linear charge density inferred from the x-ray diffraction data, then raising the salt concentration should, according to the model, stabilize the B form relative to the Z form, because the former has the higher

linear charge density. As the data in Table 1 show, the opposite is true. Furthermore, at least in the case of the unmethylated polymer, there is no simple correlation between cationic charge and the ability to induce the transition. [In certain mixtures of cations, however, the transition points correspond to approximately constant fractional neutralization of the polymer, as required by the Manning theory (see Fig. 5 legend).]

To account for our results, we must suppose that the chemistry of the cation plays an important role, and that the details of spacing between the negative charges of the DNA backbone are also important (see refs. 1–3). The cation may be involved in formation of stabilizing bridges between the close pairs of phosphodiester groups on the Z DNA backbone or between a phosphodiester group and a base. A structure in which Mg^{2+} forms a bridge to N7 of guanine, and through water to a phosphodiester oxygen, has been described in crystals of the dG-dC hexamer crystallized with Mg^{2+} (3).

The primary purpose of this study was to determine the effect of methylation of cytosine on the properties of a simple DNA containing repeated d(CpG) sequences. Our results show that substitution of a methyl group at the 5 position of cytosine allows the polymer to adopt the Z conformation under physiological conditions. Furthermore, the experiments with mixed copolymers of dC and m^5dC suggest that the introduction of methylated cytosine has a disproportionate effect in lowering the transition point: even when only 30% of the cytosine residues are methylated, the transition from B to Z form occurs at a Mg^{2+} concentration 1/35th of that required for the completely unmethylated polymer. A small number of methylcytosines may thus have a relatively long-range effect on the alternating G-C structure.

The sequence d(m^5CpG) occurs often in eukaryotic cells. As noted in the Introduction, it has been suggested that this methylation is somehow related to control of eukaryotic gene expression. Our data show that methylation of d(CpG) can have a major effect on the conformation of a double-stranded DNA composed entirely of that sequence. Whether similar or related effects can be demonstrated in naturally occurring DNA remains to be determined.

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