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**A one- and two-dimensional NMR study of the B to Z transition of (m<sup>5</sup>dC-dG)<sub>3</sub> in methanolic solution**

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**ABSTRACT**

The deoxyribose hexanucleoside pentaphosphate (m<sup>5</sup>dC-dG)<sub>3</sub> has been studied by 500 MHz <sup>1</sup>H NMR in D<sub>2</sub>O (0.1 M NaCl) and in D<sub>2</sub>O/deuterated methanol mixtures. Two conformations, in slow equilibrium on the NMR time scale, were detected in methanolic solution. Two-dimensional nuclear Overhauser effect (NOE) experiments were used to assign the base and many of the sugar resonances as well as to determine structural features for both conformations. The results were consistent with the an equilibrium in solution between B-DNA and Z-DNA. The majority of the molecules have a B-DNA structure in low-salt D<sub>2</sub>O and a Z-DNA structure at high methanol concentrations. A cross-strand NOE between methyl groups on adjacent cytosines is observed for Z-DNA but not B-DNA. The B-DNA conformation predominates at low methanol concentrations and is stabilized by increasing temperature, while the Z-DNA conformation predominates at high methanol concentrations and low temperatures. <sup>31</sup>P NMR spectra gave results consistent with those obtained by <sup>1</sup>H NMR. Comparison of the <sup>31</sup>P spectra with those obtained on poly(dG-m<sup>5</sup>dC) allow assignment of the lower field resonances to GpC in the Z conformation.

**INTRODUCTION**

Conformational studies of DNA have become of increasing interest since the discovery that (dC-dG)<sub>3</sub> crystallized into a left-handed double helical structure, termed Z-DNA after the alternating zig-zag structure of its sugar-phosphate backbone.<sup>1,2</sup> This structure has been shown to correspond to a form of poly(dG-dC) previously observed by circular dichroism (CD) in high salt solution.<sup>3</sup> In the Z-DNA structure the guanine bases are syn and the cytosine bases are anti to the sugars which are in C3' endo and C2' endo respectively. This contrasts with B-DNA where all bases are anti and the sugars adopt primarily a C2' endo conformation.

Experimental evidence for a biological role of Z-DNA has now begun to accumulate.<sup>4-7</sup> Alternating G-C or G-T sequences in supercoiled plasmids have been shown to exist in the Z-form at near physiological superhelical densities, as judged by antibody binding<sup>8,9</sup> or 2-D gel electrophoresis.<sup>9</sup> Preliminary studies indicate the existence of proteins which bind specifically

to Z-DNA.<sup>11</sup>

A variety of factors have been shown to stabilize the Z conformation, including chemical modification of cytosine at the 5 position,<sup>12</sup> ions,<sup>13-14</sup> and alcohol.<sup>15</sup> Behe and Felsenfeld demonstrated, using CD, that poly(dG-m<sup>5</sup>dC) adopts the Z conformation at near physiological salt solutions.<sup>12</sup> This is of particular interest since there is some evidence that methylation of C at the 5 position in CpG sequences is inversely correlated with gene expression in eukaryotes.<sup>17</sup> The hexamer, (m<sup>5</sup>dC-dG)<sub>3</sub>, has been crystallized and has been found to adopt a Z-DNA helix which differs only slightly from that of (dC-dG)<sub>3</sub>.<sup>18</sup>

Although all of the alternating pyrimidine-purine sequences which have been crystallized thus far have a Z-DNA structure, it has been difficult to study the Z-form by solution NMR. This is because the high salt conditions generally used to promote Z formation in solution make it difficult to obtain good NMR spectra due to aggregation, precipitation, and reduced solubility of the samples. Most of the NMR studies to date on Z-DNA have been on polymers of (dG-dC)<sup>19,20</sup> and (dG-m<sup>5</sup>dC)<sup>21,22</sup> and have focused primarily on <sup>31</sup>P NMR. The observation of a large downfield shift of half of the phosphate residues in poly(dG-dC) in high salt is considered indicative of the Z conformation, and has been used to characterize Z formation in other polymers.<sup>21,22,23</sup> Proton NMR studies of the non-exchangeable proton resonances in (dG-dC)<sub>8</sub> and poly(dG-dC)<sup>19</sup> as well as poly(dG-m<sup>5</sup>dC)<sup>21</sup> have also been reported. In the latter study,<sup>21</sup> the observation of a strong NOE between the GH8 protons and the H1' protons, expected for the syn conformation of the G bases in Z-DNA, was taken as evidence for the Z DNA conformation of poly(dG-dC) and poly(dG-m<sup>5</sup>dC) in 4M and 1.5 M NaCl respectively.

Because of the cooperative nature of the B to Z transition,<sup>3</sup> complete conversion of short oligomers to the Z form in solution is more difficult than for the polymers. Additionally, line-broadening due to solution viscosity and sample aggregation as well as precipitation have hampered attempts at detailed <sup>1</sup>H NMR structural studies. Proton NMR spectra of the hexamer d(m<sup>5</sup>C-G-C-G-m<sup>5</sup>C-G) in 0.1 M and 2M NaCl were reported in a recent communication,<sup>24</sup> but the resonances in the putative Z conformation were not assigned and no structural analysis was attempted.

In this work, we have studied the conformational transition between the B and Z forms of the hexamer (m<sup>5</sup>dC-dG)<sub>3</sub> as a function of methanol concentration in 0.1 M NaCl. Two-dimensional NOE (nuclear Overhauser effect) experiments were used to assign all of the base, H1', and many of the other sugar

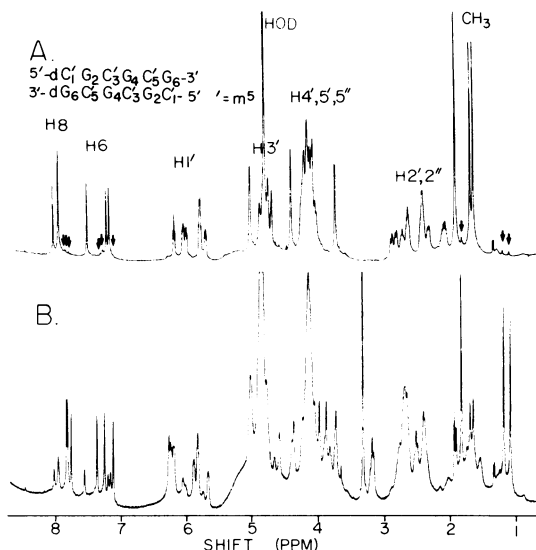
resonances for both conformations. The results of the 2D NOE experiments are consistent with a B-DNA conformation predominating at low salt in low methanol concentrations and a Z-DNA conformation predominating at high methanol concentrations.

#### MATERIALS AND METHODS

The synthetic DNA hexamer ( $m^5dC-dG$ )<sub>3</sub> was prepared by an improved phosphotriester method in liquid phase as previously described.<sup>25</sup> The sodium form of the molecules was obtained by cation exchange on a Dowex 50W-X8 cation exchange resin column. The DNA containing fractions were lyophilized and the resulting material was redissolved in 10 mM Na phosphate, pH 7.0, 0.1 M NaCl at a concentration of 2mM in duplex. Samples were prepared by lyophilization at least twice in 99.996% D<sub>2</sub>O (Stohler Isotopes) and were contained in Wilmad 528pp NMR tubes. Most of the experiments described in this paper were done with one 240 $\mu$ l sample. Water/methanol solutions were prepared by drying the sample under a stream of N<sub>2</sub> gas, adding the desired amount of D<sub>2</sub>O to dissolve the sample and then adding methanol-d<sub>4</sub> (Stohler Isotopes) to the desired percent (v/v). A vortex plug was then inserted and the NMR tube cap was sealed with parafilm after purging the tube with N<sub>2</sub>(g). If water and alcohol were added together, instead of first dissolving the sample in D<sub>2</sub>O, the samples tended to precipitate. Some precipitation also occurred with the higher percent alcohol solutions and at low temperatures. Most of the sample could be quickly redissolved by placing the NMR tube briefly in a hot water bath.

All spectra were obtained at the Francis Bitter National Magnet Laboratory at MIT. Proton spectra were taken on the home-built 500MHz spectrometer. Proton chemical shifts for samples in D<sub>2</sub>O were determined relative to the chemical shift of the residual HDO peak which had been carefully referenced to 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as a function of temperature. Because of the problems associated with comparing the chemical shifts of protons in molecules in different solvent systems, we chose to reference the spectra obtained in D<sub>2</sub>O/methanol mixtures to the chemical shift of the methyl protons of methanol and extrapolated to 0% methanol for the samples in D<sub>2</sub>O (relative to DSS).

Two dimensional NOE (NOESY)<sup>26,27</sup> data were collected using the pulse sequence  $[90^\circ-t_1-90^\circ-\tau_m-90^\circ-t_2]$  and the phase cycling described by States et al.<sup>28</sup> to obtain pure absorption phase spectra. Typically, 1024 data points with a sweep width of 8000 Hz were collected in  $t_2$  and 512 points were



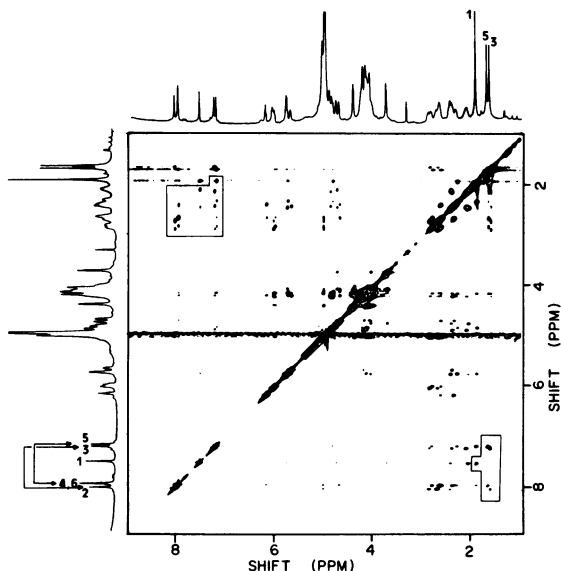
**Figure 1** 500MHz  $^1\text{H}$  NMR spectra of  $(m^5\text{dC-dG})_3$  in (A)  $\text{D}_2\text{O}$  and (B) 40% methanol at  $23^\circ\text{C}$ . Both samples contain 10mM phosphate, pH 7.0, 0.1M NaCl and are 2mM in duplex. Assignments to proton type are given in the figure. The arrows in (A) indicate base proton resonances arising from the small percentage (2-4%) of Z-DNA in the sample. The numbering system used for the hexamer is indicated at the top of the figure.

extracted; approximately 350  $t_1$  values were obtained and zero filled to 512. Spectra were line-broadened (exponential multiplication) by 4 Hz in both dimensions.

The 109 MHz  $^{31}\text{P}$  NMR spectra were obtained on a Bruker HX-270 MHz multi-nuclear instrument. The spectrum of the sample in  $\text{D}_2\text{O}$  is referenced relative to internal standard trimethyl phosphate. The spectrum of the sample in methanol was arbitrarily referenced to correspond as closely as possible with the  $\text{D}_2\text{O}$  spectrum, since the same sample was used for proton experiments and because of the known solvent effects on  $^{31}\text{P}$  chemical shifts.<sup>29</sup>

## RESULTS AND DISCUSSION

Spectra of the non-exchangeable proton resonances for  $(m^5\text{dC-dG})_3$  in  $\text{D}_2\text{O}$ , 10 mM phosphate, pH 7.0, 0.1 M NaCl (henceforth referred to as  $\text{D}_2\text{O}$ ), and in 40% methanol- $\text{d}_4$ /60%  $\text{D}_2\text{O}$  (henceforth referred to as 40% methanol) at  $23^\circ\text{C}$  are shown in Figure 1. Dramatic differences can be seen in the spectra of the molecule under the two different conditions. The spectrum in  $\text{D}_2\text{O}$  is assigned to a right handed B-DNA helix as discussed below. The changes observed in the

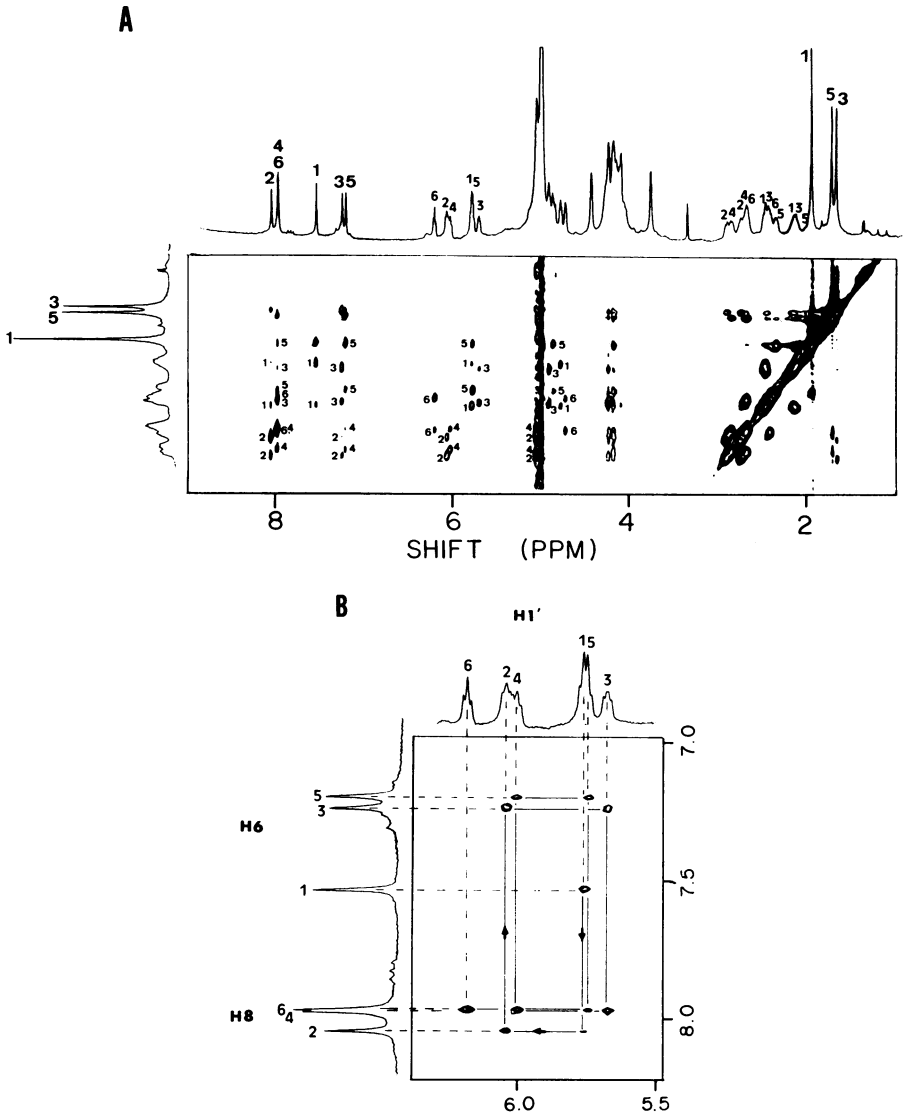


**Figure 2** Contour plot of NOESY spectrum of  $(m^5dC-dG)_3$  in 10% methanol at  $11^\circ C$ . The B-DNA conformation predominates under these conditions. The mixing time  $\tau_m$  was 300 msec and the recycle delay was 2 seconds. Cross-peaks between the aromatic and H2', H2'' resonances are indicated by the irregular rectangle at upper left and cross-peaks between the cytosine methyl and the guanine H8 and the cytosine H6 resonances are indicated by the irregular rectangle at lower right. These cross-peaks allow assignment of the terminal base proton resonances as well as pair-wise assignment of the interior base proton resonances.

spectrum on going from 100% D<sub>2</sub>O to 40% methanol reflect a conformational transition for most of the molecules to a new form. However, this new form is in slow equilibrium on the NMR time scale with the B-DNA conformation. The predominant conformation (~80%) in 40% methanol is assigned to a left-handed Z-DNA structure (see below).

#### Two-Dimensional NOE of $(m^5dC-dG)_3$ in 10% Methanol

A contour plot of a NOESY spectrum of  $d(m^5C-dG)_3$  in 10% methanol is shown in Figure 2. The guanine H8 and cytosine H6 proton resonances show strong NOE crosspeaks to the sugar H2' (H2'') resonances (boxed, upper left in Figure 2) and only very weak ones to the H1' sugar resonances. This indicates that all of the bases are in the *anti* conformation,<sup>30</sup> as expected for B-DNA. Analysis of the cross-peaks observed in the NOESY plot allows the assignment of all of the base, H1', and H2', H2'' resonances, as recently discussed,<sup>31-36</sup> if a double-helical A, B, C-type structure is assumed. The procedure is



**Figure 3** A) Portion of the NOESY spectrum given in Figure 2 showing the H2', H2'' diagonal peaks and area of cross-peaks between the methyl and H2', H2'' resonances and the full spectrum. The spectrum shown is a transpose of that in Figure 2. Nucleotide assignments of the G88, CH6, H1', H2', H2'' and CMe resonances are indicated on the 1-D spectra. The numbers next to the cross-peaks indicate the H2' or H2'' resonance with which they are associated. The primes are omitted for clarity, but except for the terminal G<sub>6</sub> all H2' resonate downfield of their respective H2''. The H2'' from nucleoside 5 is obscured beneath the C<sub>1</sub>Me. B) Portion of the NOESY spectrum given in Figure 2 showing cross-peaks between the

aromatic CH6 and GH8 resonances and the H1' sugar resonances. All bases except the terminal C<sub>1</sub> show both an intra-nucleotide and an inter-nucleotide NOE. Arrows indicate the first steps in the assignment of the aromatic and H1' protons, i.e. C<sub>1</sub>H6→C<sub>1</sub>H1'→G<sub>2</sub>H8→G<sub>2</sub>H1'→C<sub>3</sub>H8.

straightforward for this simple hexamer. The base protons CH6, GH8, and CMe all resonate in well-defined spectral regions and can be assigned to proton type by inspection. Connectivities between the CMe and CH6 protons are manifested by strong intrabase NOEs. In addition, for either a right-handed B or a left-handed Z-DNA molecule, examination of models indicates that the methyl protons at the 5 position of m<sup>5</sup>C are close to the GH8 on its 5' side but not on its 3' side. For (m<sup>5</sup>dC-dG)<sub>3</sub>, this means that two GH8-CMe NOE cross-peaks are expected (C<sub>3</sub>-G<sub>2</sub> and C<sub>5</sub>-G<sub>4</sub>), while G<sub>6</sub>H8 will have no cross-peak to the methyl region (boxed, lower right in Fig. 2). Thus the terminal base proton resonances are immediately assigned, while the interior base proton resonances are grouped into pairs.

Portions of the contour plot shown in Figure 2 are given in Figure 3 A and B. Complete assignment of the base as well as all of the H1' resonances is accomplished by inspection of the small NOE cross-peaks appearing between the aromatic and H1' proton resonances, as illustrated in Figure 3B. These relatively very small cross-peaks arise from higher order NOEs from the H6 or H8 protons to the H1' protons via the H2', H2" protons.<sup>33</sup> Examination of models of A,B,C-DNA type helices indicates that the base H6 or H8 protons will be close to sugar protons from their own nucleotide and from their 5' neighboring nucleotides, but are quite far from any sugar protons on the 3' neighboring nucleotide. Thus, all but the C<sub>1</sub>H6 could have NOEs to two sugar H1' resonances, and all but the 5' terminal H1' could have NOEs to two bases (H6 and H8 protons). If we start with the C<sub>1</sub>H6 we can see (Fig. 3B) that it has only one cross-peak to the H1' region, which identifies that sugar as nucleotide 1, but that same sugar also shows an NOE cross-peak to one of the GH8 resonances. This identifies that GH8 as nucleotide 2. The G<sub>2</sub>H8 has a second NOE cross-peak to a different H1' sugar which is identified as nucleotide 3. This H1' sugar resonance also has a cross-peak to one of the CH6 resonances which must therefore be C<sub>3</sub>H6. This procedure is repeated until all of the base and H1' resonances are assigned. Note that the assignments made in this way are consistent with the partial assignments of the base proton resonances discussed previously. This is especially important since those assignments should be correct regardless of whether the molecule was in a right-handed B or left-handed Z conformation.

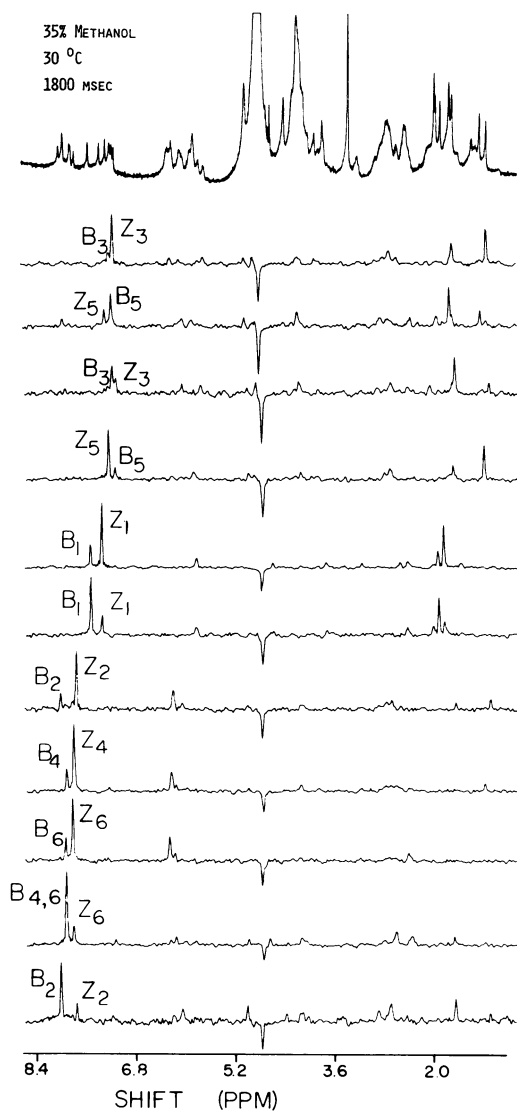
Once the H1' resonances are assigned, the H2', H2'' sugar resonances can be assigned on the basis of the NOESY (or COSY,<sup>33,35</sup> data not shown) cross-peaks between the two sets of resonances, which will only arise from intra-sugar interactions. The assignments are indicated in Fig. 3A. Intra-sugar cross-peaks between the H2' and H2'' resonances confirm the pair-wise assignments. If the B-DNA conformation is assumed, then the more intense cross-peak in each pair (seen at shorter mixing times or higher contours) can be assigned to the H2'. Assignments of the NOE crosspeaks between the H2', H2'' and the H3' sugars are also indicated in the figure.

#### Assignment of the Z-Form by 2D Chemical Exchange

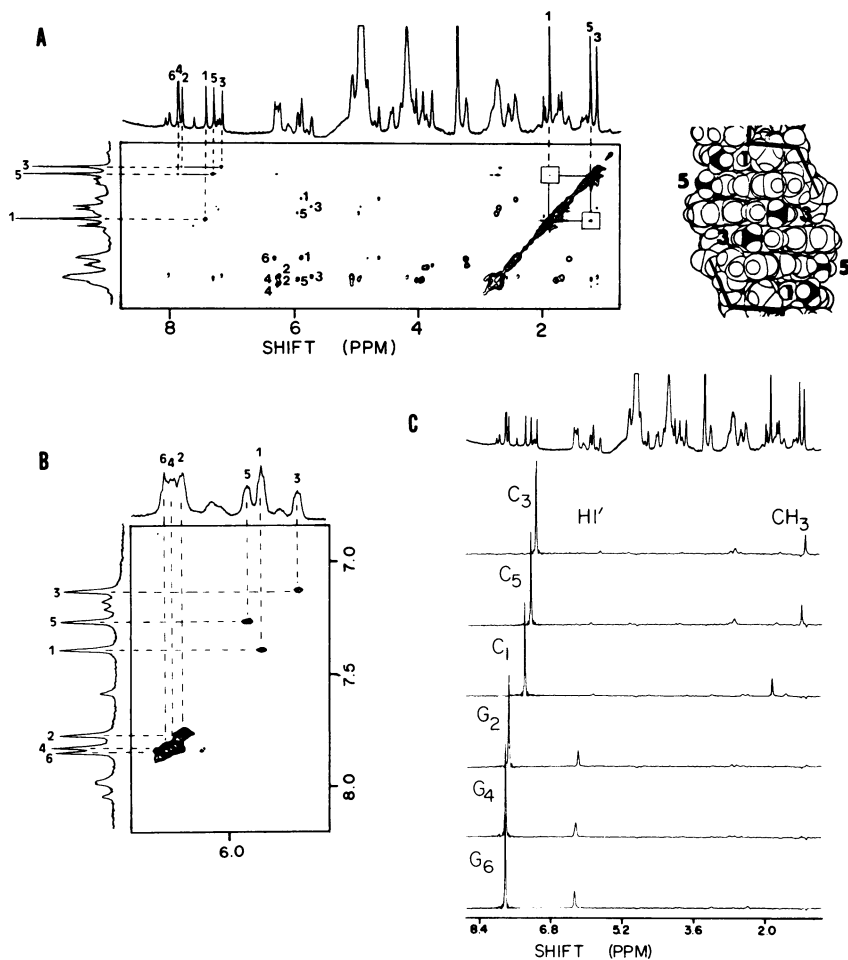
Once the resonance peaks are assigned for the conformation of (m<sup>5</sup>dC-dG)<sub>3</sub> that predominates in low salt D<sub>2</sub>O (low methanol) solution, it is possible to assign all of the base proton resonances in the conformation that predominates at high methanol concentration and low temperatures without making any assumptions about this alternate conformation. The method that was used takes advantage of the slow equilibrium that exists between the two conformations. By using the NOESY experiment in its original application, i.e. to study chemical exchange,<sup>37</sup> it is possible to see chemical exchange cross-peaks between the same protons (different resonance positions) in the two conformations. At 35% methanol and 30°C, approximately equal populations of the two DNA conformations are observed. Results of a NOESY experiment on the hexamer under these conditions with the long mixing time,  $\tau_m$ , of 1800 msec are illustrated in Fig. 4. The figure shows cross-sections taken through the aromatic resonances of the 2D spectrum. Each aromatic resonance in one conformation has a chemical exchange cross-peak to the corresponding aromatic resonance arising from the other conformation. By observing the pattern of cross-peaks, the aromatic proton resonances in the high methanol conformation were assigned. There is an ambiguity in the G<sub>4</sub>H8 and G<sub>6</sub>H8 assignments due to the overlap of the two in the B-form under these conditions; however, this is resolved by NOE from the CMe resonances as shown below. The same method was used to assign the methyl proton resonances.

The sugar resonances are more difficult to assign by this method, since they are not as well resolved in the two forms as are the base protons. Furthermore, the very large NOE between the GH8 and the H1' resonances in the high methanol form dominates over the chemical exchange pathway (vide infra). The H1' and other sugar resonances were therefore assigned by 2D-NOE as discussed below.





**Figure 4** Cross-sections through the aromatic resonances of  $(m^5dC-dG)_3$  in 35% methanol at 30°C showing chemical exchange cross-peaks between the two DNA conformations. The experiment is the same as the NOESY but a very long mixing time,  $\tau_m$ , of 1800 msec was used. Approximately equal populations of the two conformations are present under these conditions. The GH8 and CH6 resonances from the conformation predominating in low salt,  $D_2O$  are indicated by B and resonances from the conformation predominating at high methanol concentrations are indicated by Z; the subscripts refer to the nucleotide number. Note that the corresponding C methyl resonances can also be identified.



**Figure 5** A) Portion of a NOESY spectrum (contour plot) of  $(m^5dC-dG)_3$  in 40% methanol at 21°C. A mixing time of 300 msec and recycle delay of 2 seconds were used. Assignments of the base (GH8, CH6, CMe<sub>3</sub>) proton resonances are indicated in the 1-D spectra. Connectivities between the CMe and the GH8 and CH6 resonances are indicated in the upper left. The numbers beside the cross-peaks arising between the H1' and H2', H2'' proton resonances indicate the nucleoside with which they are associated. An interstrand NOE between C<sub>1</sub>Me and C<sub>5</sub>Me is indicated by the boxed cross-peaks in the upper right of the figure. The inset shows that in the crystal structure of  $(m^5dC-dG)_3$  the methyl protons in the CpG steps on opposite strands will be near each other (4.6Å C-C distance). B) Another portion of the NOESY spectrum given in Figure 5A, showing the cross-peaks between the aromatic GH8 and CH6 resonances and the H1' sugar resonances. Under these conditions, only one (intranucleotide) crosspeak is seen between each aromatic and H1' resonance. C) Cross-sections through the aromatic (GH8 and CH6) resonances of the NOESY spectrum shown in Fig. 5A and B, showing the relative intensities of the NOEs. The

very large NOE crosspeak between the GH8 and the H1' proton resonances is comparable in intensity only to the NOEs between CH6 and CMe and is indicative of the syn conformation.

#### Two-Dimensional NOE of (m<sup>5</sup>dC-dG)<sub>3</sub> in 40% Methanol

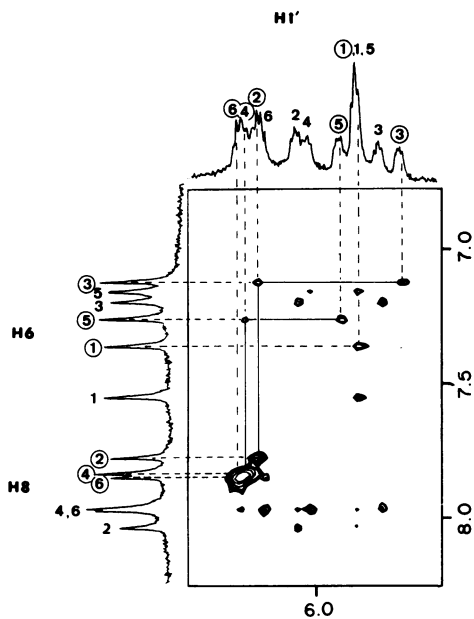
Portions of a NOESY spectrum of the hexamer in 40% methanol are shown in Figure 5A,B (contour plots) and C (cross-sections). The same pattern of cross-peaks between the CMe, CH6, and GH8 protons that was observed in the 10% methanol spectrum is observed here (Fig. 5B, upper left). We are thus able to corroborate the assignments made by 2D chemical exchange discussed above.

In contrast to the aromatic-methyl interactions, the pattern of cross-peaks observed between the aromatic and H1' sugar proton resonances are quite different for the two DNA structures. For the experiment shown in Figure 5A, only one cross-peak is observed between each aromatic proton and a sugar H1' proton resonance. Thus, the methods heretofore discussed to assign all of the base and H1' protons by 2D-NOE clearly cannot be used for all DNA structures. The very strong NOE crosspeak observed between each GH8 proton resonance and H1' sugar (Fig. 5B and C) indicates that these bases are in the syn conformation expected for Z-DNA and is discussed further below. Since we can independently assign the aromatic proton resonances by chemical exchange, we are able to assign the H1' sugar resonance which interacts with each GH8. By elimination, the remaining cross-peaks can be used to assign the respective cytosine H1' resonances. Cross-peaks between the H1' and the H2', H2'' resonances were used to assign the latter, and these are indicated in the figure.

Figure 6 shows the cross-peaks between the H1' and the aromatic protons for the sample in 35% methanol. The spectrum was taken at 26°C with a mixing time of 1400 msec. Approximately equal populations of the two forms are present under these conditions. At this mixing time and temperature, the chemical exchange cross-peaks are much smaller than for the experiment shown in Fig. 4, but all of the base-H1' NOEs for both conformations are seen. This figure illustrates that the cross-peaks for the two forms are well resolved and can be followed separately. At this long mixing time, the interior CH6 resonances show cross-peaks both to their own sugar and to the 5' neighboring H1' sugar, but the GH8s only interact with their own sugar.

#### Conformational Features of the High Methanol Form

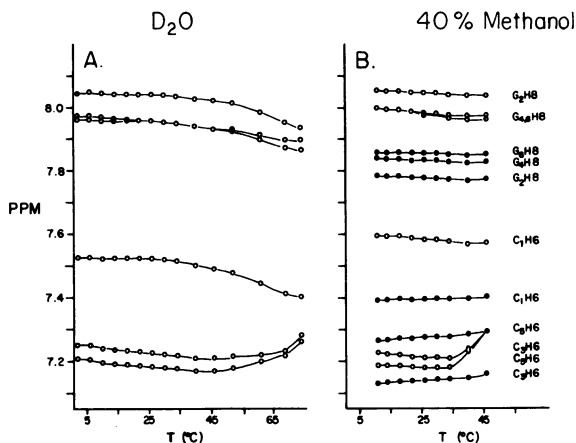
The strong NOE between the GH8 and the H1' is one of the dominant features of the NOESY spectrum of (m<sup>5</sup>dC-dG)<sub>3</sub> at high methanol concentrations. This is illustrated in Fig. 5C, where cross-sections taken through the six



**Figure 6** Portion of a NOESY spectrum (contour plot) of  $(m^5dC-dG)_3$  in 35% methanol at  $26^\circ C$  with a mixing time of 1400 msec. Nearly equal populations of the two conformations are present under these conditions. The assignments are given by the 1-D spectra, with circles indicating Z-DNA resonances. All of the intra- and inter-nucleotide crosspeaks for the DNA in both conformations can be separately resolved in this spectrum. The connectivities for the Z-DNA form are shown by dashed and solid lines. Two additional cross-peaks ( $C_3H6-G_2H8$ ) and ( $C_5H6-G_4H8$ ) can be seen which were not observed at the shorter mixing time shown in Fig. 5B.

aromatic resonances are shown. In contrast, the NOE cross-peaks between the CH6 and the nearest H2' are relatively small. The only cross-peaks of comparable intensity are between the CH6 and CMe resonances. Since the CMe-CH6 distance is fixed at  $\sim 2.5\text{\AA}$  (distance of closest approach, assuming this will dominate the NOE), this gives us an approximate internal calibration of distances between protons. Although precise distances cannot be obtained without a careful study of the build-up of NOEs,<sup>38</sup> reasonably good approximations can be made. The comparable intensity of the GH8-H1' cross-peaks to the CH6-CMe cross-peaks indicates that the distances between GH8 and H1' for the Z-form is also approximately  $2.5\text{\AA}$ , and this is consistent with the results of the crystallographic studies.

Another interesting feature of the NOESY spectrum of the high methanol form of  $(m^5dC-dG)_3$  is the observation of a cross-peak between  $C_1Me$  and  $C_5Me$  (Fig. 5A, upper right). This cross-peak indicates an interaction between methyl groups on opposite strands in the CpG steps. The analogous interaction expected between the two  $C_3Me$  groups obviously cannot be detected due to symmetry. The molecular model of  $(m^5dC-dG)_3$  derived from the crystal structure is illustrated in the inset of Fig. 5A, showing the spatial relationship of the methyl groups in the CpG steps (see also Fig. 2 of ref. 18). The observed carbon-carbon distance in the crystal structure was  $4.6\text{\AA}$ ,

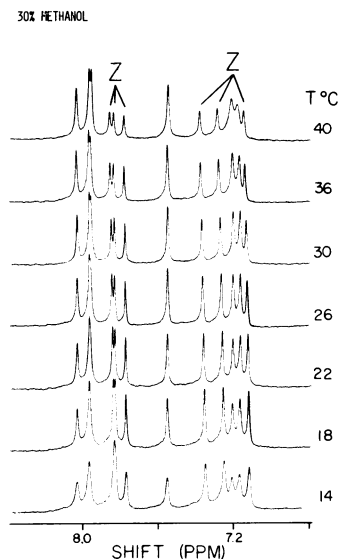


**Figure 7** Chemical shifts of the aromatic resonances as a function of temperature in (A)  $D_2O$  and (B) 40% methanol. The open circles represent the B-DNA chemical shifts and the closed circles represent the Z-DNA chemical shifts.

so the protons would have a distance of closest approach of  $\sim 3 \text{ \AA}$ . From an examination of the molecular model of the Z-form ( $m^5dC-dG$ )<sub>3</sub> it is clear that the observed cross-peaks between the two methyl resonances must arise from a first order NOE, since there are no other closer protons which might give rise to the cross-peaks via an indirect pathway. This could be confirmed experimentally by measuring the intensity of the NOE as a function of mixing time.

#### Effect of Temperature and Methanol Concentration on the B/Z Equilibrium

The temperature dependence of the chemical shifts of the aromatic proton resonances for ( $m^5dC-dG$ )<sub>3</sub> in  $D_2O$  is plotted in Fig. 7. The chemical shifts of the resonances as a function of temperature are used to monitor the cooperative duplex to strand transition. Because it was not possible to obtain spectra above  $72^\circ C$  in the instrument used, we are able only to estimate the midpoint of the melting transition at  $\sim 60^\circ C$ . In any case, the molecule remains >95% in duplex form until at least  $30^\circ C$ . The resonances shift as average peaks with little observable line broadening through the duplex to strand transition, indicating that the equilibrium between duplex and strand is near fast exchange on the NMR time scale, at least at temperatures where significant amounts of both duplex and strand would be present. This contrasts with the equilibrium between the B and Z-DNA conformations of the DNA observed in methanolic solution, in which two separate sets of resonances



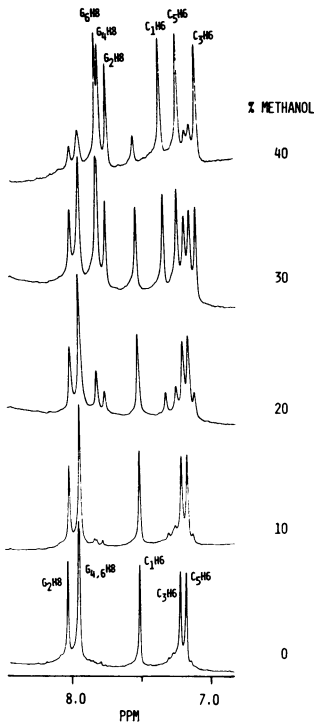
**Figure 8** 500 MHz  $^1\text{H}$  NMR spectra of the aromatic region of  $(\text{m}^5\text{dC-dG})_3$  as a function of temperature in 30% methanol. The resonances arising from the Z-DNA conformation are indicated. 200 FIDs were collected at each temperature and spectra are line broadened by 0.5 Hz.

are observed, indicating a slow equilibrium between the two conformations on the NMR time scale.

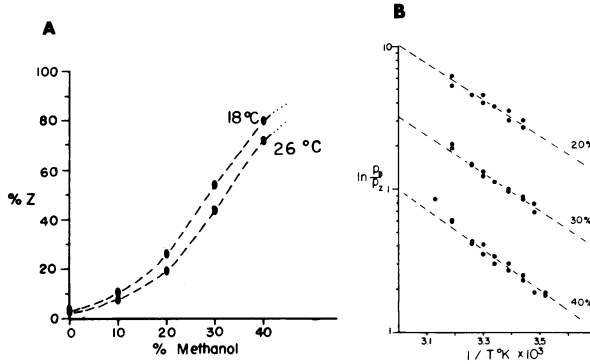
Figure 7B shows the temperature dependence of the chemical shifts of the aromatic resonances for both the B and Z forms in 40% methanol. The Z form is the predominant species at all but the highest temperature studied under these conditions (data not shown). The temperature range studied was limited at the low end by sample precipitation and at the high end by the solvent. (A room temperature spectrum was obtained after the highest temperature run in order to confirm that no solvent had evaporated.) The chemical shifts of the aromatic resonances for the Z-DNA conformation change little if at all throughout the temperature range studied, indicating that the molecule remains double-stranded throughout this temperature range.

Figure 8 illustrates the effect of temperature on the equilibrium between the two conformations of the hexamer in 30% methanol. The equilibrium shifts toward the B-form as the temperature is raised. This is in contrast to other studies on polymers of dG-dC and dG-m<sup>5</sup>dC in which high salt concentrations were used to effect the B to Z transition, and the Z form was found to be stabilized at higher temperature.<sup>21</sup>

Spectra of the aromatic resonances of  $(\text{m}^5\text{dC-dG})_3$  as a function of methanol concentration at 17°C are shown in Figure 9. The equilibrium shifts toward the Z-form as the methanol concentration increases. Unfortunately,



**Figure 9** 500 MHz  $^1\text{H}$  NMR spectra of the aromatic regions of  $(m^5dC-dG)_3$  as a function of percent methanol at  $18^\circ\text{C}$ . Assignments of the B-DNA resonances are indicated at the bottom; assignments of the Z-DNA resonances are indicated at the top. 200-300 FIDs were collected and spectra are line-broadened by 1-2 Hz.



**Figure 10** A) Plot of the percentage of Z-DNA vs. B-DNA as a function of percent methanol at  $18^\circ\text{C}$  and  $26^\circ\text{C}$ . The relative amounts of B and Z-DNA were determined from the intensities of the best resolved aromatic resonances ( $G_2H_8$  and  $C_1H_6$ ). The average of the two values obtained is plotted. B) Plot of  $P_B/P_Z$  vs.  $T(^{\circ}\text{K})^{-1}$ , where  $P_B/P_Z$  is the fractions of B vs. Z-DNA. Data are plotted for 20, 30, and 40% methanol. Relative proportions of B and Z-DNA were determined as in Fig. 10A, and both values are shown to indicate the precision of the measurement.

sample precipitation precluded the study of higher methanol concentrations than those shown here. Even at the lower methanol concentrations some sample aggregation occurred. Note that even in pure  $D_2O$  (0.1 M NaCl) there is still about 2-4% Z-DNA. Although this may be difficult to see in the aromatic spectrum shown, it can be seen quite clearly in the methyl region of the  $D_2O$  spectrum shown in Figure 1.

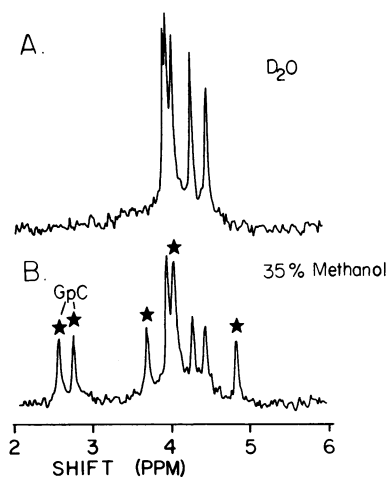
A plot of % Z vs. % methanol is given in Figure 10A for two different temperatures. The sigmoidal shape of the curves indicates that the transition from B to Z is cooperative, although for this short molecule it occurs over a much wider range than would be expected for a polymer.

Arrhenius plots of  $\ln P_B/P_Z$  vs.  $1/T$ , where  $P_B/P_Z$  is the fraction of B vs. Z are given in Fig. 10B for 20, 30 and 40% methanol. Linear least square fits to the data give enthalpies,  $\Delta H$ , of 6, 8, and 7 kcal/mole duplex respectively, and entropies,  $\Delta S$ , of 23, 27, and 21 cal/mole  $^{\circ}K$  respectively. This gives an average  $\Delta H$  of  $\sim 1.2$  kcal/mole base pair and  $\Delta S$  of  $\sim 4$  cal/mole  $^{\circ}K$  base pair. Only the data below  $35^{\circ}C$  was used in this analysis since above that temperature the spectral changes indicate a conformational change in the B-conformation or a change in the B-Z-strand kinetics in methanol/water mixtures, especially at the higher methanol concentrations (see Fig. 7). Although the error in these calculations is fairly large due to difficulties in getting accurate integrated peak intensities, the data do indicate that in this system the enthalpy term favors the Z-DNA conformation, while the entropy term favors B-DNA conformation. Spectra were also obtained on the sample in 30% ethanol- $d_6$  (results not shown). The spectral changes observed were similar to those for the sample in methanol, except that a greater proportion of Z-DNA was present than for the same concentration of methanol.

### $^{31}P$ NMR

Phosphorus NMR spectra of the hexamer in  $D_2O$  and in 35% methanol- $d_4$  are shown in Figure 11. Five resonances are observed in the spectrum of the hexamer in  $D_2O$  at  $\sim 4$  ppm, corresponding to the five non-equivalent phosphates (each resonance arises from two equivalent phosphates). Three of these resonances are clustered with .1 ppm of each other, while the remaining two appear about 0.3 and 0.5 ppm further upfield respectively. Five additional resonances appear in the  $^{31}P$  spectrum of the hexamer in 35% methanol at  $26^{\circ}C$ . The proton NMR results on the identical sample at  $26^{\circ}C$  indicate that under these conditions there are approximately equal populations of the two DNA conformations (B and Z) in slow exchange on the NMR time scale. The phosphorous spectra likewise indicate that there are two conformations of the





**Figure 11** 109 MHz  $^{31}\text{P}$  NMR spectra of  $(\text{m}^5\text{dC-dG})_3$  at approximately  $26^\circ\text{C}$  in (A)  $\text{D}_2\text{O}$  and (B) 35% methanol. Approximately equal populations of B and Z-DNA are present in (B) in slow equilibrium on the NMR time scale. The Z-DNA peaks are indicated by stars. Tentative assignment of the two GpC resonances in the Z-form are indicated.

DNA in slow equilibrium even at this lower frequency. Five of the resonances are nearly superimposable with the spectrum obtained in  $\text{D}_2\text{O}$ . Of the remaining five resonances, two are observed  $\sim 1$  ppm downfield from the main cluster. The assignments of the resonance signals to the Z-DNA peaks indicated in the figure were confirmed by comparing these spectra to those obtained at lower and higher temperatures (data not shown). The changes in the relative populations of the two conformations with temperature were consistent with those observed in the proton spectra.

Phosphorus NMR studies on poly(dG-dC)<sup>19,20</sup> and poly(dG-m<sup>5</sup>dC)<sup>21,22</sup> in low and high salt have shown that a downfield shift of half of the phosphate resonances (centered at  $\sim 4.2$  ppm in B-DNA) to  $\sim 3$  ppm is indicative of the Z conformation for these molecules. The downfield shift has been attributed to different environments around the GpC vs. CpG phosphates in the alternating backbone of the structure, but the factors affecting phosphorus chemical shifts are not well understood and the resonances could not be assigned. If the downfield shift is indeed due to a difference between GpC and CpG, then we can assign the two resonances in the hexamer that shift to lower field to the two GpCs while the three higher field resonances would be from the three CpGs (Fig. 11b). While this paper was being written, similar results on  $(\text{m}^5\text{dC-dG})_3$  in concentrated  $\text{NaClO}_4$  solutions were reported.<sup>39</sup> The assignment of the lower field resonances in the Z-DNA form to GpC are also consistent with results obtained with thiol-substituted poly(dG-dC).<sup>23</sup> It is worth noting, however, that the largest chemical shift difference of the higher field  $^{31}\text{P}$  resonances for the Z conformation of the hexamer is of the same magnitude (1.1 ppm) as

that between the averaged lower and higher field resonances.

In the  $^{31}\text{P}$  NMR spectrum of poly(dG-m<sup>5</sup>dC) in low salt, the phosphate resonances appear as a partially resolved doublet split by  $\sim 0.15$  ppm.<sup>21,22</sup> The presence of the doublet has been attributed to an alternating phosphodiester backbone conformation in the right-handed B-DNA by Chen *et al.*<sup>22</sup> Again, if this line of reasoning is correct, then the two higher field resonances in the  $^{31}\text{P}$  spectrum of the hexamer in D<sub>2</sub>O may correspond to the GpC phosphates, although this is speculative due to chemical shift dispersion from end effects in this small molecule.

The B and Z conformations of the DNA are in slow equilibrium on the NMR time scale for the  $^{31}\text{P}$  NMR experiments. The lower field (109 MHz) for these experiments compared to the 500 MHz  $^1\text{H}$  NMR experiments could provide a shorter kinetic time window, depending on the chemical shift differences in the two forms. However, because we have not yet assigned these resonances to specific phosphates, the chemical shift differences between the B and Z resonances cannot be used to place a lower limit on the exchange rate between the two forms.

#### Equilibrium Kinetics of the B to Z Transition

Since the aromatic resonances were assigned to specific bases in both the B and Z-DNA forms, we can estimate the upper limit of the exchange rate between the two DNA conformations.<sup>40</sup> The smallest chemical shift difference between the same aromatic proton resonances in the two forms is  $\sim 40$  Hz (below 35°C), which corresponds to  $k \ll 100 \text{ sec}^{-1}$  for equal populations of the two conformations. A more accurate estimate of the exchange rates is theoretically obtainable from an analysis of the 2D chemical exchange data at several mixing times.<sup>37</sup> However, the analysis is complicated by magnetization transfer via NOE as well as chemical exchange. We note that the methyl protons act as relaxation sinks<sup>41</sup> for this molecule, as is evident from the unusual observation of these off-diagonal cross-peaks being as large or larger than the diagonal peaks in some cases at very long mixing times (see Fig. 4). In addition, since non-selective spin-lattice relaxation of these protons are of the same order of magnitude as the mixing times used (data not shown), the chemical exchange experiments are very time-consuming due to the decrease in signal-to-noise. We do observe a substantial increase in the magnitudes of the chemical exchange cross-peaks relative to their diagonal peaks for several mixing times studied when the temperature was changed from 26°C to 31°C (e.g. average of 0.13 vs. 0.35 at  $\tau_m = 1400$  msec). This is consistent with a large temperature effect on the kinetics.

## CONCLUSIONS

The NMR results reported here indicate that two different conformations of the deoxyribose hexamer ( $m^5dC-dG$ )<sub>3</sub> exist in slow equilibrium on the NMR time scale in methanolic solution. Using a minimum of assumptions about conformation, we are able to assign all of the base, H1', and many of the other sugar resonances by NOESY and 2D-chemical exchange. We note that the 2D-NOE assignment strategies currently being used to assign resonances in DNA oligomers are not valid for the Z-DNA conformation.

The NOESY spectra clearly indicate that the low methanol form of the DNA has all the bases in the anti conformation, as expected for B-DNA, while in the high methanol form the guanines are syn and the cytosines are anti as expected for Z-DNA. Further experiments analyzing the build-up of NOEs as a function of mixing time to determine more precise distances,<sup>38</sup> combined with a detailed analysis of the sugar conformations,<sup>42</sup> should allow much more complete structural analysis of the two conformations. However, the data presented, including the <sup>31</sup>P spectra, are completely consistent with the B and Z-DNA conformations as expected.

An inter-strand NOE observed between methyl groups on adjacent base pairs in the  $m^5CpG$  step indicates a close inter-proton distance ( $\sim 3\text{\AA}$ ). An unexpectedly close interaction between these two methyl groups was also seen in the crystal structure of the molecule.

The effect of methanol on the B to Z equilibrium was studied as a function of methanol concentration and temperature. A small amount of Z-DNA (2-4%) is observed even in the D<sub>2</sub>O spectrum (0.1M NaCl). The percentage of Z-DNA increases in a cooperative manner as a function of methanol concentration and decreases with increasing temperature.

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