Volume 14 Number 3 1986 Nucleic Acids Research

The tetraribonucleotide rCpGpCpG forms a left-handed Z-RNA double-helix

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Received 13 November 1985; Accepted 27 December 1985

ABSTRACT

NMR and circular dichroism studies show that the RNA tetranucleotide rCpGpCpG can form a Z-RNA left-handed double-helix. In 1.0 M $NaClO_4$, circular dichroism measurements indicate that the tetramer is in the A-form. In 6.0 M $NaClO_4$, there is a characteristic change in the circular dichroism, indicating that the tetramer adopts a left-handed Z-form. This conformation is verified by phosphorus and proton NMR studies. The ^{31}P spectrum shows a large downfield shift in one of the resonances upon an increase in salt concentration. Proton nuclear Overhauser effect (NOE) experiments indicate that the guanosines are in the syn conformation. These results are consistent with the formation of a Z-form double-helix.

INTRODUCTION

Recently it has been shown that a self-complementary RNA polynucleotide, poly [r(C-G)], can form a left-handed double-helix, called Z-RNA (1). As shown by NMR and circular dichroism studies, in 6.0 M $NaClO_4$ at 20°C the polymer is in the A-form, but when the temperature is increased to 45°C it adopts a Z-RNA structure. A downfield shift in one of the ^{31}P resonances shows that there has been a dramatic change in the phosphate-sugar backbone conformation. NOE experiments indicate that the guanosines switch from the anti- to the syn-conformation. Characteristic changes in the circular dichroism spectra are consistent with a change from a right-handed to a left-handed structure (1,2). These results are very similar to those seen in the B to Z transition of the DNA polynucleotide poly[d(C-G)] with increasing NaCl concentration (3,4).

The discovery of a Z-conformation in a polyribonucleotide immediately suggests a search for the transition in an oligoribonucleotide. For an RNA A-to-Z transition to have any biological utility, the transition must occur in short lengths of C-G sequences, since long stretches of alternating C-G are unlikely in natural RNA's. Therefore, it was necessary to find a short

length of alternating C-G that would adopt a Z-form. The DNA tetranucleotide dCpGpCpG assumes a Z-conformation (5,6), but tetranucleotides with other G-C sequences do not (7). Also, the limited resolution of the polynucleotide NMR spectra makes it impossible to determine many of the finer details of the Z conformation. For these reasons we chose to study the oligoribonucleotide rCpGpCpG in low and high $NaClO_4$ solutions to determine its ability to form a left-handed double helix.

MATERIALS AND METHODS

The tetranucleotide triphosphate rCpGpCpG was synthesized by a phosphotriester method (8,9). NMR samples containing RNA, $NaClO_4$, 10 mM sodium phosphate pH 7 and 0.2 mM EDTA were lyophilized 3 times from 99.8% D_2O before being taken up into 99.99% D_2O.

^{31}P NMR spectra were recorded at 121 MHz with a Cryomagnet Systems 300/50 7.05T magnet equipped with a Nicolet 1280 computer. Spectra were taken in both 1.0 M and 6.0 M $NaClO_4$ at temperatures from 0°C to 40°C, and were referenced to trimethyl phosphate. Proton NMR spectra were taken at 500 MHz on a Bruker AM 500 over the same temperature range, and referenced to an internal standard of TSP. RNA concentrations were approximately 0.6 mM in a volume of 0.5 ml.

Ultraviolet absorbance measurements were taken with a Gilford 250 spectrophotometer. The temperature was controlled by a Gilford 2527 thermoelectric temperature programer. Data were obtained by either a Commodore PET model 2001 computer, or by an Apple IIe computer. The concentration of the tetranucleotide was determined using the extinction coefficient of Uesugi et al.(10), who calculated it to be 8.7×10^3 at 260 nm in 0.1 mM NaCl and 10 mM sodium phosphate. Samples contained RNA, $NaClO_4$, and 1.0 mM EDTA pH 7.

Circular dichroism (CD) spectra of the same samples were recorded on a Jasco J 500 C spectropolarimeter in both 1.0 M and 6.0 M $NaClO_4$. Temperature was controlled with a Carl Zeiss model P/N 93 00 80 thermoelectric cell block.

RESULTS AND DISCUSSION

U. V. Absorbance

The melting point of the tetramer was expected to be low, so the absorbance spectra were measured at 0°C in order to maximize the double-

strand formation. The spectra of the duplex forms in 1.0 M and 6.0 M $NaClO_4$ are different: the duplex in 6.0 M $NaClO_4$ has a small shoulder on the main absorption band. This shoulder near 295 nm is characteristic of the Z-form absorption spectra of both ribo- and deoxy-polynucleotides. The effect of the shoulder is to change the ratio of absorbances at 256 nm and 295 nm in the A and Z forms, so that the absorption at 295 nm can be used as an indication of the presence of Z-form (3). For rCpGpCpG this ratio is 4.4 in 1.0 M $NaClO_4$ and 3.4 in 6.0 M.

U. V. melting curves of the tetramer were used to determine the melting temperatures of rCpGpCpG in the different salt concentrations. As shown by the absorbance vs. temperature plots in Figure 1, the melting curves are very broad. The T_m for the tetramer in 1 M $NaClO_4$ cannot be obtained accurately, but it lies between 20 and 30°C in the concentration range from 0.14 to 0.59 mM. In 6.0 M salt the T_m is depressed to near 0 °C, thus the tetramer is only 50% double-stranded at this temperature. This lowering of T_m is partly due to the denaturing effect of $NaClO_4$. It is one of several chaotropic salts (11) which are all denaturants of various degrees. The chaotropic effect in fact may be linked to a salt's ability to stabilize Z-RNA (12). The lower stability of a Z-helix relative to the right-handed helix may also contribute to the decrease in T_m. The deoxytetranucleotide dCpGpCpG is known to have a lower melting temperature in the Z-form than the B-form (5).

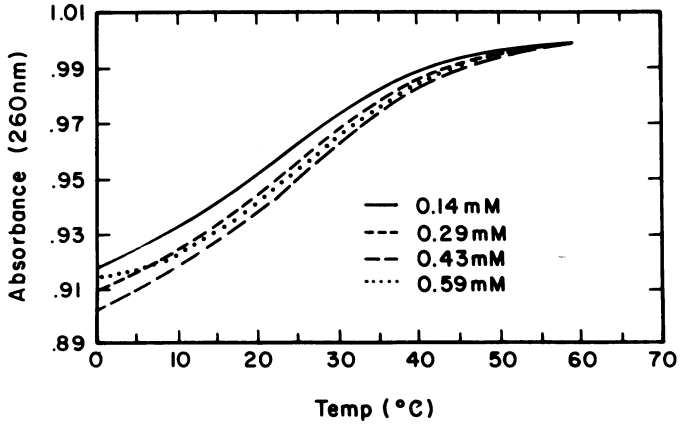

Figure 1. Optical melting curves at 260 nm for rCpGpCpG in 1.0 M $NaClO_4$ at various oligomer concentrations. Curves are normalized to a single-strand absorbance of 1.0 at 260 nm. All samples contain 1.0 mM EDTA, pH 7.

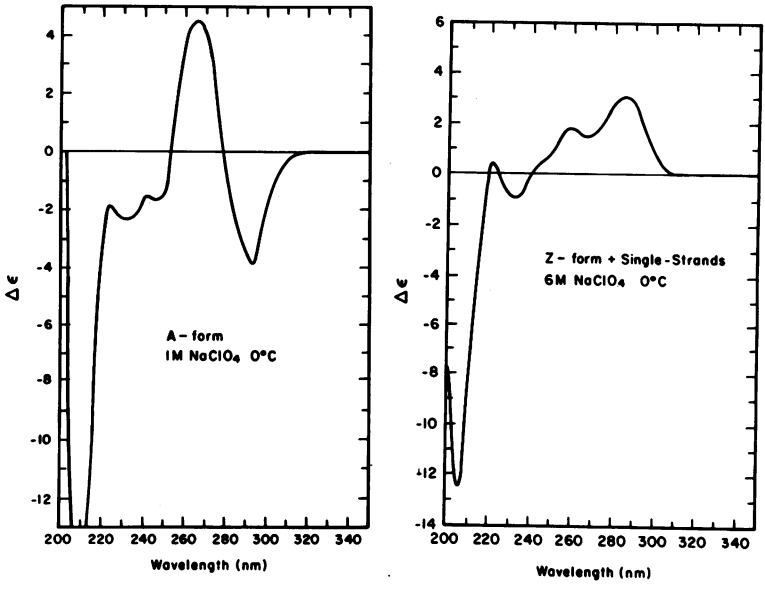

Figure 2. Circular dichroism spectra of rCpGpCpG at 0°C. In 1.0 M $NaClO_4$ the tetranucleotide is A-form. In 6.0 M $NaClO_4$ it is approximately half single-strands and half double-stranded Z-form. Samples contained 1.0 mM EDTA, pH 7.

Circular Dichroism

A stronger indication that rCpGpCpG can adopt Z-conformation comes from CD spectra in 1.0 M and 6.0 M $NaClO_4$. CD has been used extensively to monitor structural changes in both DNA and RNA (1,2,13). Characteristic changes in the CD spectrum occur with the transition between right- and left-handed RNA and DNA. The CD of rCpGpCpG in high and low salt show two dramatically different spectra which are similar to those seen with poly[r(C-G)] in A- and Z-forms, respectively. Figure 2 shows CD spectra of the tetranucleotide in both 1.0 M and 6.0 M $NaClO_4$ at 0°C. The CD spectrum of the tetranucleotide in 1.0 M $NaClO_4$ is almost identical to that of poly[r(C-G)] in A-conformation. The trough is blue-shifted 2 nm and the positive band is red-shifted 3 nm in the tetranucleotide spectrum relative to the polynucleotide spectrum. The ratio of A266/A292 = 1.21 for the tetranucleotide, and A_{263}/A_{294} = 1.85 for the polynucleotide. In contrast, the 6.0 M perchlorate CD spectrum of the tetranucleotide is not quite so similar to that of the Z-form polynucleotide, but it shares many features. The negative CD band at 295 nm in the Z-form polynucleotide

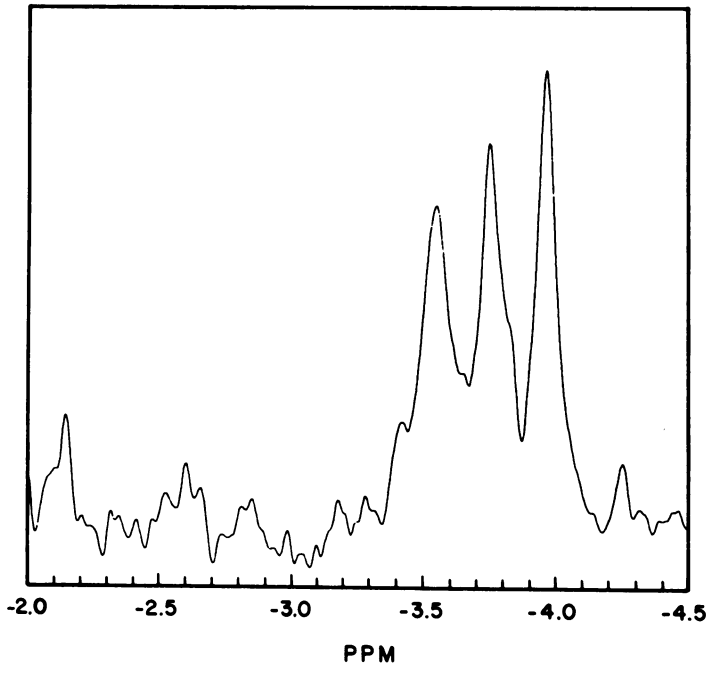

Figure 3. ^{31}P spectrum of rCpGpCpG in 1.0 M $NaClO_4$ at 0°C (A-form). Proton broad-band decoupling is used during each acquisition. ^{31}P FID's are multiplied by an 8 Hz exponential line-broadening contribution prior to Fourier transformation to improve the signal-to-noise ratio. This spectrum is a sum of 512 scans. ^{31}P chemical shifts are referenced to trimethyl phosphate. All NMR samples contain 10 mM sodium phosphate, pH 7, and 0.2 mM EDTA.

spectrum becomes positive and blue-shifted in the 6.0 M spectrum for the tetranucleotide. There is also a dramatic decrease in the large band near 265 nm, a change which is more pronounced for the tetranucleotide, and the small positive band near 220 nm is much larger in the polynucleotide. These differences are understandable since the tetranucleotide is only partly double-stranded, so the CD reflects a mixture of single- and double-strands.

^{31}P NMR

In 1.0 M salt, the ^{31}P spectrum at 40°C has 3 peaks corresponding to the three distinct phosphate linkages of rCpGpCpG (Fig. 3). At 0°C, the molecule is in the double-stranded state, as shown by uv absorbance spectroscopy. There are still only three phosphorous peaks for the double-strands, due to the 2-fold symmetry axis of the double-helix. The range of

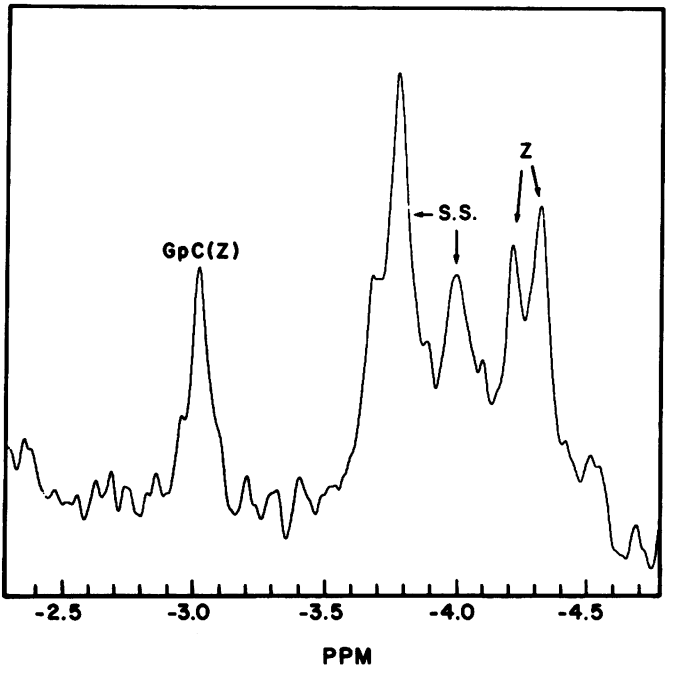

Figure 4. ^{31}P spectrum of rCpGpCpG in 6.0 M $NaClO_4$ at 0°C. Resonances corresponding to both the single-strands and the double-stranded Z-form are observed due to the slow exchange rate between the two forms. Peaks were assigned by observing the increase in intensity of the double-stranded resonances as the sample temperature was lowered. Assignment of the low-field peak as the GpC phosphate is discussed in the text. The spectrum is the sum of 2048 scans.

chemical shifts is rather small, about 0.5 ppm, which indicates that the phosphates are structurally nearly equivalent. This result is consistent with the A-form, since nucleotides in an A-double-helix have approximately the same conformation: each of the phosphate linkages is in a gauche-gauche conformation (14).

In 6.0 M salt ^{31}P NMR spectra show a much different behavior of the tetranucleotide. At 30°C it is in the single-stranded state, and there is one peak corresponding to the three overlapping phosphate resonances. As the temperature is lowered, two changes take place. First, the peak begins to separate into two, the one downfield being twice as large as the other. Second, and more interesting, is that three new peaks appear: two upfield from the single strand peaks, and one downfield, as seen in Figure 4. The range of chemical shifts is now nearly 1.3 ppm. This result indicates that there has been a significant change in the conformation of

the backbone, since ^{31}P chemical shifts are quite sensitive to conformational changes (15). In a DNA Z-helix CpG and GpC linkages are conformationally quite different. CpG linkages are gauche-gauche, while the GpC is gauche-trans, so one observes a significant difference in chemical shifts of the respective 31P signals (4). The presence of only one GpC linkage vs. two CpG linkages in rCpGpCpG suggests that the downfield resonance corresponds to the GpC, while the two upfield correspond to the two CpG's. This assignment is consistent with the assignment of ^{31}P resonances of Z-DNA (16).

The NMR also shows that the transition between the single-strands and double-strands is very different for the A- and Z-duplexes. In the 1.0 M spectra, only one set of resonances appears, corresponding to the weighted average of the chemical shifts of the single-strands and A form: the transition kinetics are fast. The single-strand-to-Z transition, however, is slow on the NMR timescale (with a life time greater than 10 ms), so that two sets of resonances, for both the single-strand and Z-form, are observed simultaneously. The ability to observe signals of both forms should allow determination of kinetics of transformations for individual nuclei. Unfortunately, this phenomenon also reduces the signal-to-noise ratio, and increases the complexity of the spectrum.

Proton NMR

There are four distinct non-exchanging aromatic protons in the tetranucleotide. The chemical shifts of these protons are sensitive to changes in stacking interactions, such as in the helix to coil transition, as well as changes in helical conformation, such as in the A-to-Z transition of RNA. In the 1.0 M spectra, as the temperature is lowered, one of the CH6 doublets and one of the GH8 singlets both broaden and change their chemical shift as the equilibrium shifts toward the double-stranded state. The A-form assignments shown in Figure 5 were made by observing the spectrum at 40°C, where the GH8 singlets and CH6 doublets are resolvable, then following the change in chemical shift as the temperature was lowered. As with the ^{31}P spectra, one only observes a single resonance for each nucleus, due to the moderately fast exchange rate between the two forms. At 0°C, the two broadened, shifting peaks have stopped moving and have sharpened considerably, which indicates that only double-stranded A-form is present. One CH6 is now downfield of the stationary GH8, while the other GH8 is upfield of the stationary CH6. These assignments are consistent with those made by Sinclair et al. (8) and Uesugi et al., (10) for

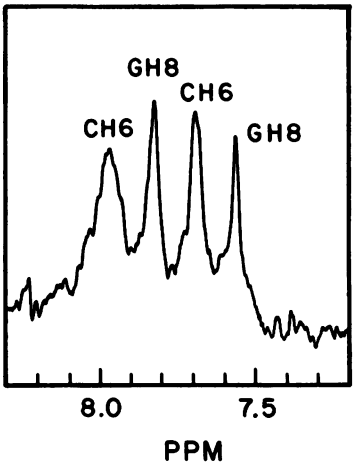

Figure 5. Aromatic region of the proton NMR spectrum of rCpGpCpG in 1.0 M $NaClO_4$ at 0°C (A-form). Four peaks are present, corresponding to the two unique GH8 protons and the two unique CH6 protons in the double-helix. The spectrum is the sum of 512 scans, and 2 Hz line-broadening was used to improve signal-to-noise.

rCpGpCpG in NaCl concentrations from 0.1 M to 4.0 M and temperatures below 20°C. Small differences in actual chemical shift values are presumably due to the difference in salt conditions, as well as temperature.

In the 6.0 M salt, a dramatic difference is observed in the spectra as the temperature is decreased. Near 20°C one singlet (two overlapped GH8's) and two doublets are observed in the aromatic region between 7 and 8 ppm (Fig. 6), corresponding to the single-stranded state. As the temperature is lowered, new peaks appear in addition to those belonging to the single-strands, as with the ^{31}P spectra. At 0°C, these are resolved into two doublets upfield near 7.2 ppm and two singlets near 7.7 ppm, corresponding to the double-strands. Unlike the 1.0 M A-form spectra, both CH6's are shifted far upfield, and both GH8's are positioned downfield. This marked change from the A-form spectrum is similar to that seen in the proton NMR spectra of poly[r(C-G)] with the transition from A-form to Z-form (1). In the Z-form poly[r(C-G)] spectrum, the CH6 peak is upfield at about 7.1 ppm, while the GH8 is downfield near 7.8 ppm. This similarity of the two spectra is consistent with rCpGpCpG forming a left-handed double-helix.

Proton NMR can also be used to determine much more conclusive evidence of structure. One characteristic of the Z-form double-helix is that the guanosines adopt a syn-conformation, while in the A-form they are in an

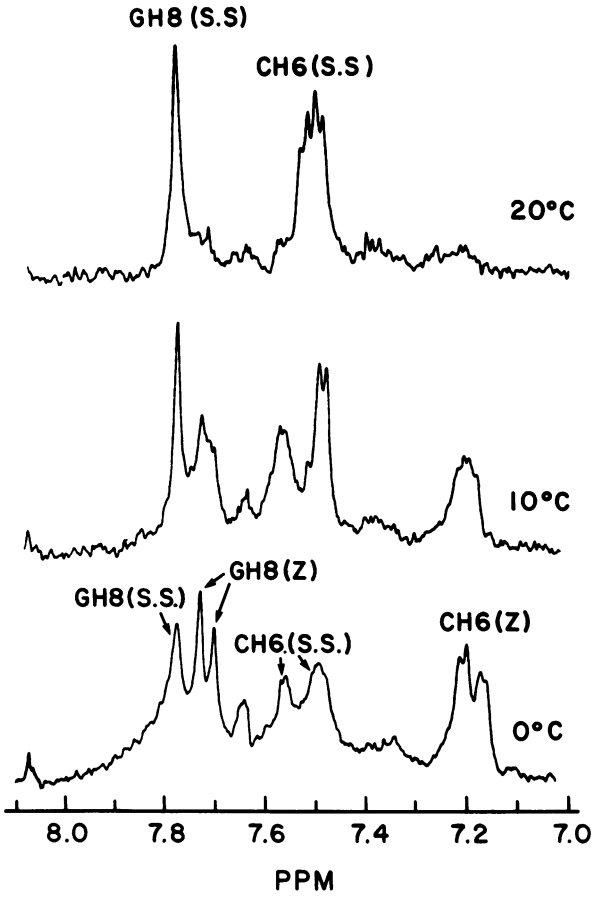

Figure 6. Aromatic region of the proton NMR spectrum of rCpGpCpG in 6.0 M $NaClO_4$. At 20°C the tetranucleotide is nearly all single-strands. At 10°C a significant amount of double-strands are present. At 0°C the tetranucleotide is nearly 50% double-stranded. In comparison with the 10°C spectrum, the broad peak upfield near 7.2 ppm has resolved into two CH6 doublets, while the broad peak downfield near 7.7 ppm has resolved into two GH8 singlets. Each spectrum is the sum of 1024 scans. 2 Hz line-broadening was used on the 20°C and 10°C spectra, and 4 Hz line-broadening was used on the 0°C spectrum.

anti-conformation. The two can be distinguished by using nuclear Overhauser effect (NOE) experiments. In the syn conformation, a significant NOE can be observed between the ribose H1' and the purine H8 protons. In the anti-conformation this NOE is much less pronounced, due to the increase in distance between the two protons. This technique has been used to characterize the Z-form in both DNA and RNA (1,4).

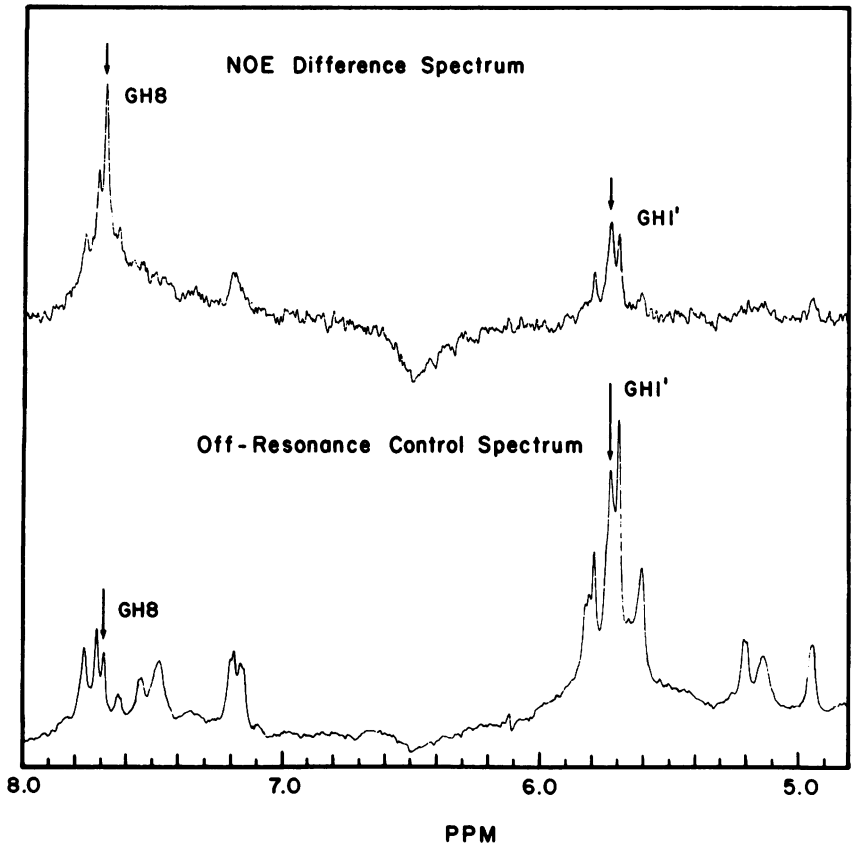

Figure 7. Proton NMR experiment showing the NOE between one Z-form GH8 and the neighboring GH1' protons. Shown are the difference spectrum of off-resonance and on-resonance irradiation, and the off-resonance control spectrum. Arrows indicate which of the GH8 resonances was irradiated, as well as the position of the corresponding GH1' resonance. On- and off-resonance spectra were collected in the interleave mode, with 64 scans taken per set for a total of 1024 scans per spectrum. The difference spectrum was obtained by subtracting FID's prior to Fourier transformation, and 4 Hz line-broadening was used on the difference FID.

An NOE experiment was done on rCpGpCpG in 6.0 M $NaClO_4$ determine whether the guanosine is in the syn conformation. The two downfield singlets that appear in the aromatic region at lower temperature correspond to the double-stranded GH8's, so one of these was chosen to be irradiated. The NOE difference spectrum (Fig. 7) shows that there is a significant NOE between this GH8 and a resonance in the H1' region of the spectrum, corresponding to a GH1' proton. This demonstrates that this

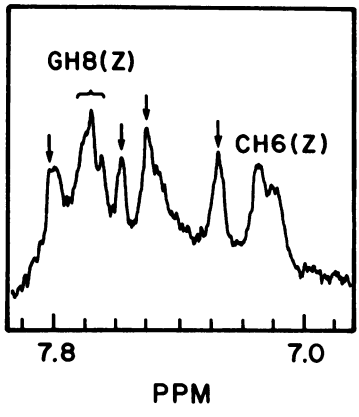

Figure 8. Aromatic region of the proton NMR spectrum of rCpGpCpG in 6.0 M $NaClO_4$ at -15°C. Arrows indicate the peaks which have become prominent at this temperature. There is still a large fraction of the Z-form present where indicated. The spectrum is the sum of 2048 scans, and 2 Hz line-broadening was used.

guanosine is indeed in the syn conformation. The NOE from the other GH8 to its H1' is substantial, but less pronounced than the first one. This result suggests that one of the guanosines, most likely the terminal one, is more conformationally labile than the other, but that both are probably in syn-conformations.

It is known that the A-to-Z transition is temperature dependent for the poly[r(C-G)] (1). In 5.0 M NaClO4 at 45°C, the polynucleotide is in the Z-form, while at 20°C it reverts back to the A-form. The 6.0 M salt concentration depresses the freezing point of the solution to less than -20°C, making it possible to study the tetranucleotide at lower temperatures. As the temperature is decreased below 0°C, aromatic resonances corresponding to the single strands decrease, while a set of four new peaks become prominent beside those of the proposed Z-form. At -15°C, the mixture is approximately half Z-form and half new form (Fig. 8). Comparison of this result to the behavior of the polynucleotide suggests that the -15°C form may be the A-form. These four new peaks resemble the A-form spectrum. The limited resolution, however, makes it difficult to assign the resonances, making conclusive identification of this form impossible at this time.

CONCLUSION

The results shown here are substantial evidence that the RNA tetra-

nucleotide rCpGpCpG forms a left-handed double-helix in 6.0 M $NaClO_4$ that is similar to left-handed Z-form poly[r(C-G)]. NOE experiments indicate that a guanosine adopts a syn-conformation. ^{31}P NMR data is consistent with two conformationally different phosphodiester linkages in the sugar-phosphate backbone, as found in the Z-form of poly[r(C-G)]. CD spectra suggest that the molecule is adopting a double-helical form similar to Z-RNA. All of these data and conclusions are consistent with similar results obtained for the DNA B-to-Z transition.

Uesugi et al. (17) have shown that the ribo-tetramer r(C-Br8G-C-Br8G) adopts a Z-like structure in low salt solution. They also noted that the unsubstituted tetramer rCpGpCpG was unable to adopt a Z-form. Westerink et al. (18) reached the same conclusion using a different solvent system. This apparent inability to induce a Z-conformation was due to the choice of solvents, however, and not to an inherent property of the tetramer. There are many solvents that facilitate the DNA B-to-Z transition (13), but few have been found for the RNA A-to-Z transition (12). For this reason, $NaClO_4$ was critical in inducing the formation of a Z-conformation in the tetramer rCpGpCpG.

The discovery that the different forms of rCpGpCpG are in slow exchange may lead to some very useful and important information about the exchange process. Tran-Dinh et al. (19) and Cavailles et al. (20) found that DNA hexamers of alternating C-G sequences containing 5-methyl-C residues can exist in slow exchange between B and Z forms. Using polarization transfer experiments and line-width calculations they were able to determine that the B-to-single-strand transition is a direct conversion and occurs fast on the NMR timescale, while the Z-to-single-strand transition must pass through the B-form as an intermediate and takes place at a much slower rate. In comparison, the A-to-single-strand transition is fast for rCpGpCpG, while the Z-to-single-strand transition is significantly slower. This suggests that the Z-to-single-strand transition may require an A-form intermediate, which is similar to the mechanism proposed for the DNA oligomers. Saturation transfer and temperature-jump experiments should reveal more about the mechanism and kinetics of the transition.

As yet there is no evidence for a biological role for Z-RNA. The diverse structures and activities of RNA, however, leave many possibilities open. For example, there are several regions of alternating purine-pyrimidine base sequences in ribosomal RNA's (21). The discovery that the

tetranucleotide rCpGpCpG can adopt a left-handed structure is an important step in determining whether such a structure might occur in natural RNA's. Subsequence studies with different sequences, hairpins, etc. may give better clues as to where to search for Z-RNA *in vivo*.

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