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Temperature-Dependent CD and NMR Studies on a Synthetic Oligonucleotide Containing a B-Z Junction at High Salt†

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ABSTRACT: It is now accepted that two or more conformations may exist within the same DNA molecule, thereby generating conformational junctions. The presence of B-Z junctions between right- and left-handed DNA conformations has been detected in plasmids by a number of techniques. Preliminary characterization of the first example of a B-Z junction is a short DNA oligonucleotide has recently been reported [Sheardy, R. D. (1988) *Nucleic Acids Res. 16*, 1153-1167]. We report additional CD and NMR data that support the existence of the junction in this model oligomer. These studies indicate that only three base pairs are involved in the junction and only one of these is dramatically distorted. Furthermore, the NMR saturation-transfer experiments suggest the junction's internal motion is temperature dependent.

It is now recognized that a segment of DNA may possess contiguous left- and right-handed conformations, mandating the existence of a conformational junction within that segment. Most of the studies to date on these B-Z junctions are concerned with junctions contained within plasmids [for example, Singleton et al. (1982) and Johnston and Rich (1985)]. In these systems, (dG-dC) oligomers are cloned into plasmids that are then subjected to conditions conducive to Z formation of the inserts, thereby generating B-Z junctions at either end of the insert. Although these types of studies can lead to broad generalizations about the nature of B-Z junctions, they give little detailed structural information at the molecular level.

We have recently reported the preparation and initial characterization of the synthetic DNA hexadecanucleotide (Sheardy, 1988):

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5'-C*--G--C*--G--C*--G--C*--G--A--C--T--G--A--C--T--G-3'

3'-G--C*--G--C*--G--C*--G--C*--T--G--A--C--T--G--A--C-5'

   1  2  3  4  5  6  7  8  9  10 11 12 13 14 15 16
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(C* is 5-methyldeoxycytidine.[1] The numbering is for exchangeable proton chemical shift assignments.) The sequence of this duplex was designed such that the first eight base pairs (as written) could assume a left-handed conformation under dehydrating conditions, while the last eight base pairs should remain right-handed under all conditions. The existence of both conformations within the molecule would mandate a conformational junction between the two (i.e., a B-Z junction). Such a model could provide insight into the biological significance of conformational junctions in terms of their roles in gene expression and regulation.

The initial CD, UV, and NMR studies reported indicate that this molecule assumes a right-handed conformation at low salt. However, under conditions of high salt (5.0 M NaCl), the duplex contains regions of both left- and right-handedness and thus must contain a B-Z junction. The preliminary ^{31}P NMR studies also indicated that the span of the junction is on the order of four to six base pairs.

We report additional CD and NMR data that support the existence of the B-Z junction in the model oligomer. These studies indicate that only three base pairs are involved in the junction, with one of these dramatically distorted. Furthermore, data from both ^{1}H NMR and saturation-transfer experiments suggest that the internal motion of the junction is temperature dependent.

Experimental Procedures

Materials. The oligonucleotide was synthesized and purified as previously described (Sheardy, 1988). Phosphate buffer (0.01 M sodium phosphate, 1 mM EDTA, pH adjusted to 7.0 with NaOH) was used in all experiments. The low-salt buffer had no added NaCl; the high-salt buffer had NaCl added to a final concentration of 5.0 M. Stock oligonucleotide duplex solutions were prepared by combining solutions of the individual strands to give 1:1 mixtures, followed by heating to 90

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[1] Abbreviations: C*, 5-methyl-2'-deoxycytidine; C, 2'-deoxycytidine; G, 2'-deoxyguanosine; T, 2'-deoxythymidine; A, 2'-deoxyadenosine; CD, circular dichroism; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancement.

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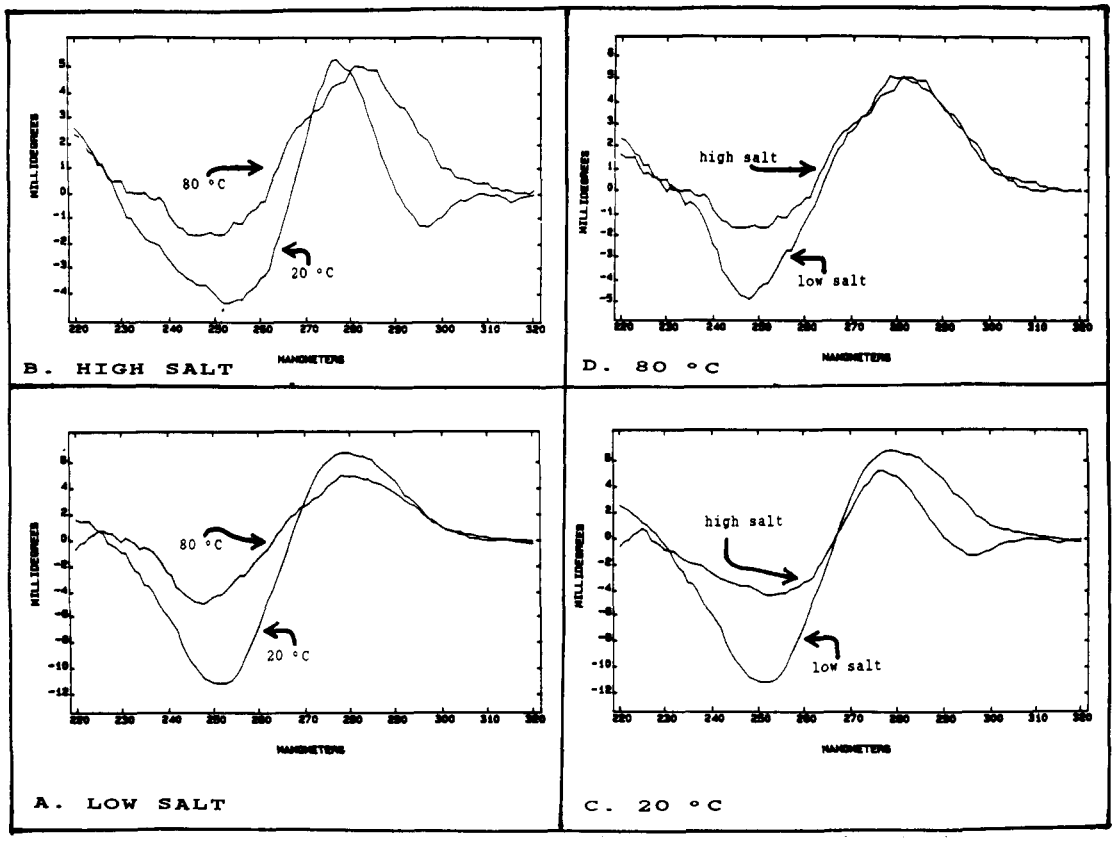

FIGURE 1: Comparisons of the CD spectra for the oligomer discussed in this paper: (A) The oligomer in low-salt buffer at 20 and 80 °C; (B) the oligomer in high-salt buffer at 20 and 80 °C; (C) the oligomer at 20 °C under low- and high-salt conditions; (D) the oligomer at 80 °C under low- and high-salt conditions.

°C and subsequent slow cooling. NMR solutions were made by combining sufficient oligonucleotide stock solution to give concentrations between 5×10^{-4} M and 2×10^{-3} M in residues with 0.6 mL of the desired phosphate buffer. These solutions were frozen, lyophilized to dryness, and then reconstituted with H_2O/D_2O to give 0.6-mL samples containing 15% D_2O.

CD Studies. The CD spectra of the duplex in both low- and high-salt buffers were recorded every 5 °C from 20 to 80 °C with a Model 60DS AVIV CD spectropolarimeter equipped with a thermoelectrically controlled cell holder.

^1H NMR Studies. Proton NMR spectra of the exchangeable oligonucleotide resonances in H_2O/D_2O solution were obtained at various temperatures at 400 MHz by using a Varian XL-400 NMR spectrometer. A jump and return pulse sequence described by Wang and Pardi (1987) was employed for water suppression. The carrier frequency was offset 3160 Hz downfield of the water signal to optimize solvent suppression. Typical experimental conditions were as follows: 10000 scans, a 1-s acquisition time with a 1–2-s pulse delay, 20480 data points, and a spectral width of 10000 Hz with reference to dioxane. Assignments of the resonances were made by one-dimensional NOE experiments and thermal denaturation experiments in a manner similar to that of Wilson et al. (1986). Experimental conditions for the NOE experiments were the same as above with the following modification: a 250–300-ms irradiation of the signal of interest with the decoupler channel (using the low-power decoupler set at 18–20 dB). Decoupler frequencies were arrayed, and the spectra were interleaved. The reference spectrum was produced by using a decoupler frequency upfield of the exchangeable proton resonance frequencies and downfield of the nonexchangeable proton resonance frequencies. A 2-Hz line broadening was applied prior to transforming *or* after subtracting the FIDs and before transforming the resultant difference spectra.

Saturation-transfer experiments (Johnston & Redfield, 1977; Wüthrich, 1986) were performed in a similar manner with the following changes: the decoupler frequency was that of the residual water signal, the mixing time was 1 s, and the decoupler power was 58 dB with a 1-s delay.

^{31}P NMR Studies. Phosphorus NMR spectra were obtained at 161.9 MHz on a Varian XL-400 NMR spectrometer using the following experimental conditions: typically, 10000 scans, 70° flip angle, 0.800-s acquisition time, 16000 data points, 10000-Hz sweep width, proton WALTZ decoupled. A 3–5-Hz line broadening was applied prior to transforming. Samples were referenced to the inorganic phosphate signal. The chemical shifts of the inorganic phosphate resonance at various temperatures in both low-salt and high-salt conditions were determined relative to the signal for H_3PO_4 (85% H_3PO_4 in a capillary tube) in separate samples.

It should be noted that both phosphorus and proton NMR spectra were determined at a variety of DNA concentrations. No concentration effects were observed in the concentration range reported in this study.

Results

The overlays of the CD spectra at 20 and 80 °C of the duplex at low and high salt are shown in parts A and B, respectively, of Figure 1. For the low-salt form, CD spectra were identical up to 35 °C, with a trough at 252 nm (λ_1) and a peak at 279 nm (λ_2). As the temperature is increased to 80 °C, there is a 63% increase in the molar ellipticity at 252 nm and a 28% decrease in the ellipticity at 279 nm with an isoelliptic point at 269 nm. Finally, there is a shift in λ_1 from 252 nm at 20 °C to 248 nm at 80 °C and a shift in λ_2 from 279 nm at 20 °C to 280 nm at 80 °C. The observed melting behavior is typical of right-handed double-helical DNA (Hartmann et al., 1983).

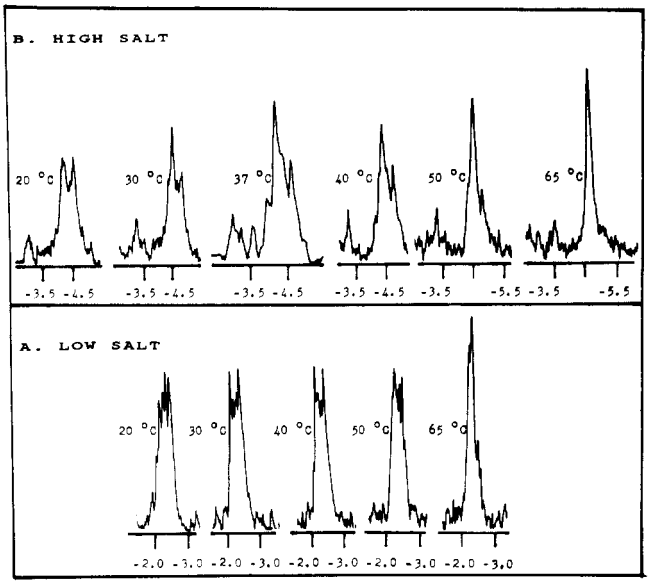

FIGURE 2: 162-MHz ^{31}P NMR spectra of the oligomer at the temperature indicated: (A) The oligomer in 10 mM phosphate buffer, 15% D_2O, pH 7.0 (low-salt buffer); (B) the oligomer in 10 mM phosphate buffer, 5.0 M NaCl, 15% D_2O, pH 7.0 (high-salt buffer). The number of transients for the inset 37 °C spectrum (which is presented on an expanded plot) was 20 000 with a line broadening of 10 Hz. For the other spectra, the number of transients was 10 000 with a line broadening of 3 Hz.

The high-salt spectra are identical from 20 to 45 °C. These spectra are characterized by a trough at 252 nm (λ_1), a peak at 277 nm (λ_2), and a shallow trough at 297 nm (λ_3). As the temperature is increased to 80 °C, there is a 63% increase in the molar ellipticity at 252 nm, a 19% decrease in the ellipticity at 277 nm, and an inversion of the ellipticity at 297 nm. The decrease in ellipticity at 277 nm and the inversion of 297 nm begin to occur at temperatures above 65 °C. There are also differences in both λ_1 and λ_2 at the lower and upper temperatures.

For comparison, the overlay of the low- and high-salt spectra at 20 °C is shown in Figure 1C, and the overlay at 80 °C is shown in Figure 1D. The 20 °C spectra are identical with those previously reported (Sheardy, 1988). It should be noted that the concentration of DNA is identical for both salt conditions. What is very interesting is the overlay of the low- and high-salt forms at 80 °C. Although the ellipticity around 280 nm is similar, there is a substantial difference around 248 nm. This suggests that the species present have different structures at low and high salt at 80 °C.

The phosphorus NMR spectra of the duplex at low and high salt at a variety of temperatures are shown in parts A and B, respectively, of Figure 2. Examination of the low-salt, low-temperature spectra indicates that there is some dispersion in the ^{31}P chemical shifts, suggesting that there is some heterogeneity in the backbone structure. As the temperature is raised, there are subtle changes in the phosphorus spectra up to 55 °C. At temperatures above the optically determined T_m (60 °C), the spectra change markedly, as would be expected for a molecule undergoing a helix-to-coil transition.

The spectra for the oligomer at high salt and temperatures below 30 °C exhibit several differences from the spectra of the oligomer at low salt at these temperatures. The resonance at −3.2 ppm has previously been used to demonstrate the existence of Z-DNA (Patel et al., 1979) and has been assigned, for other oligomers, to the phosphates in GpC linkages (Patel et al., 1979; Hartmann et al., 1983; Cheng et al., 1982; Jovin et al., 1983; Gorenstein et al., 1982). Between −4.4 and −5.0

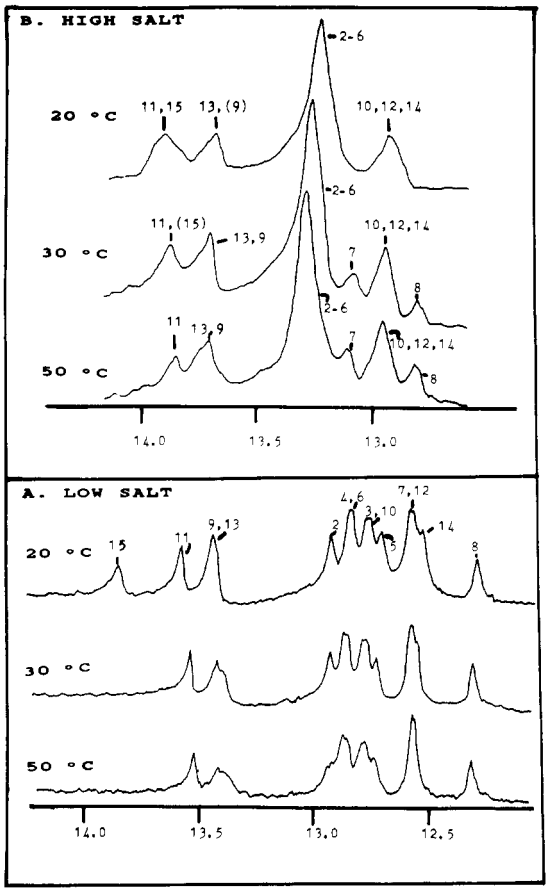

FIGURE 3: 400-MHz ^1H NMR spectra of the exchangeable resonances of the oligomer at the temperatures indicated: (A) The oligomer in 10 mM phosphate buffer, 15% D_2O, pH 7.0 (low-salt buffer); (B) the oligomer in 10 mM phosphate buffer, 5.0 M NaCl, 15% D_2O, pH 7.0 (high-salt buffer).

ppm, there is a collection of resonances whose appearance changes as the temperature is raised from 30 °C to temperatures well below the optical T_m of 70 °C. As the temperature is increased, the low-field resonance at −2.3 ppm also changes. From 20 to 35 °C, this resonance gains a narrower line width. At 37 °C, it decreases in intensity and two new resonances at −3.4 and −3.6 ppm appear. At 40 °C, these new resonances have disappeared and the resonance at −3.2 ppm has regained its intensity. From 40 to 60 °C, the resonance at −3.2 ppm has the same intensity and line shape. At 65 °C (5° C below the optical T_m), it begins to broaden and lose intensity.

The proton NMR spectra of the "exchangeable" resonances at various temperatures under low-salt conditions are presented in Figure 3A. Assignments of the resonances to the exchangeable protons of the oligomer are given in this figure. Resonances are not observed for the exchangeable protons of base pairs 1 and 16. As the temperature is increased, the oligomer begins to fray and resonances disappear (Crothers et al., 1974). Resonances corresponding to exchangeable protons of base pairs 14 and 15 disappear at temperatures below the T_m. The resonance for the exchangeable protons of base pair 13 also broadens and begins to exchange out at 40 °C.

Proton NMR spectra of the exchangeable resonances at various temperatures under high-salt conditions are presented in Figure 3B. The assignments for each spectrum are given. At 20 °C, certain of the exchangeable protons observed at low salt are not observed, at least fully, in the high-salt buffer. Integration of the two A:T signals suggests a 1.5:1 ratio for the downfield–upfield signals. Assignments of these signals

to base pairs 11 and 15 for the downfield resonances and to 13 and 9 (with 9 only partially observed) are based upon the assignments of the oligomer under low-salt conditions. The A:T end of the oligomer maintains a relatively B-like conformation under both salt conditions, and thus the chemical shifts of the A:T exchangeable proton resonances would be similar in both buffers. Integration of the two G:C signals suggests a 5:3 ratio for the downfield–upfield signals. The large downfield G:C resonance may be assigned to base pairs 2–6, which are in a Z-like conformation. For a particular sequence, the exchangeable protons residing in a Z conformation exhibit ^{1}H NMR resonances downfield from their resonances when they reside in a B conformation (Mirau et al., 1985). The upfield G:C resonance is for base pairs 10, 12, and 14, which are in a B-like conformation. While NOEs were observed between both A:T resonances and the upfield G:C resonances, no NOEs were observed between the downfield G:C resonances and the other resonances at 20 °C.

At 30 °C, two new resonances appear in the G:C region of the spectrum, the downfield A:T resonance has lost intensity, and the upfield A:T resonance has gained intensity. The two G:C resonances observed at 20 °C have the same relative intensities at 30 °C. In addition to the NOEs noted above (at 20 °C), NOEs are observed between the new upfield G:C resonance and the new downfield G:C resonance and between the new upfield G:C resonance and the upfield A:T resonance (corresponding to A:T base pairs 13 and 9). Additionally, NOEs are observed between the new downfield G:C resonance and the G:C resonances corresponding to base pairs 2–6. Thus, the two new G:C resonances are assigned to exchangeable protons for base pairs 8 (upfield resonance) and 7 (downfield resonance) as indicated in Figure 3B at 30 °C. The increase in intensity of the signal for the upfield A:T resonance is due to the presence of a signal for base pair 9 at 30 °C. The loss in intensity for the downfield A:T signal comes from fraying and the exchange of base pair 15 with water (Crothers et al., 1974). At 50 °C, the resonance for the A:T base pair 15 is lost while the resonances for base pairs 7–9, which appear at 30 °C, are still observed.

A saturation-transfer experiment was conducted to obtain qualitative information on which of the exchangeable protons of the oligomer might be more susceptible to interaction with water under high-salt conditions. The results of the experiment are given in Figure 4. At 20 °C, the signal for A:T base pairs 11 and 15 have virtually disappeared, the signal for A:T base pairs 13 and 9 has diminished significantly in intensity, and those for all the G:C base pairs remain (for those G:C resonances normally observed at 20 °C). At 30 °C, the resonances for all A:T base pairs have disappeared, while the resonances for G:C base pairs 10, 12, and 14 (in the B region), G:C* base pairs 2–6 (in the Z region), and G:C* base pairs 7 and 8 are still observed. At 40 °C, the signal for G:C* base pair 7 has disappeared, the signals for G:C* base pairs 2–6 and G:C base pairs 10, 12, and 14 are still observed, and there is a slight decrease in the intensity for the signal from G:C* base pair 8 (relative to the other two signals). Finally, at 50 °C, only the signal for G:C* base pairs 2–6 and, somewhat surprisingly, the signal for G:C* base pair 8 are still observed.

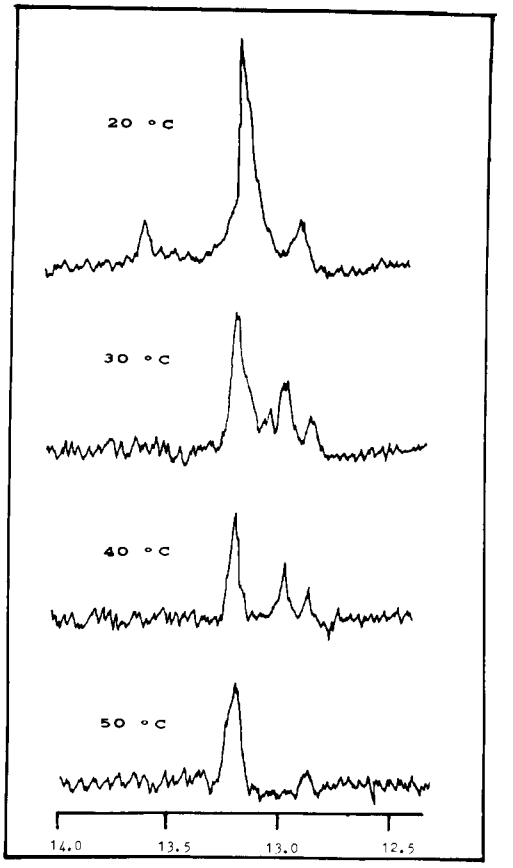

FIGURE 4: 400-MHz ^{1}H NMR saturation-transfer spectra of the exchangeable resonances of the oligomer in 10 mM phosphate buffer, 5.0 M NaCl, 15% D_2O, pH 7.0 (high-salt buffer) at the temperatures indicated. (Note: The vertical scale of the 20 °C spectrum is 75% of the vertical scale of the other spectra.)

Discussion

The CD, ^{31}P NMR, and ^{1}H NMR studies reported here support the conclusion of previous studies on this oligomer (Sheardy, 1988) that while the oligomer is in a B-form conformation under low-salt conditions, the oligomer contains both B-form and Z-form conformations in 5 M NaCl. Thus, this molecule must contain a B–Z junction at high salt. Furthermore, there are subtle, temperature-dependent conformational fluctuations in the junction region at temperatures below the T_m.

The CD data presented here indicate that the oligomer in low salt behaves like other oligomers previously shown to be in a B-like conformation in low salt [for example, Hartmann et al. (1983)] throughout the melting process. The CD data for the oligomer under high-salt conditions indicate that as the temperature is raised, the "B end" of the duplex starts to melt before the "Z end" of the molecule. This is based on the observation that the trough at 252 nm (corresponding to the B end of the duplex) starts to lose amplitude at temperatures well below that for which the trough at 297 nm (corresponding to the Z end of the duplex) starts to lose amplitude.

In 5 M NaCl, ^{31}P NMR signals corresponding to phosphates in a Z conformation are observed. Specifically, a resonance integrating to six phosphates is observed downfield of the other phosphate resonances. Previously, this resonance has been assigned to 5′-GpC-3′ phosphates in Z conformations (Patel et al., 1979; Hartmann et al., 1983; Cheng et al., 1982; Jovin et al., 1983; Gorenstein et al., 1982). This indicates that a portion of the molecule under discussion is in a Z-like conformation. Additionally, ^{31}P phosphate resonances in two groups are observed further upfield. The middle ^{31}P group is found in a relative position consistent with phosphates in a B-like conformation, and the upfield ^{31}P resonance group occurs in a relative position consistent with 5′-CpG-3′ phosphates in a Z conformation (Gorenstein et al., 1982; Chen & Cohen, 1984).

At temperatures below the T_m, the ^{31}P NMR spectrum of the oligomer in 5 M salt is quite temperature dependent. The

^{31}P NMR spectrum of the oligomer in low-salt conditions also shows some temperature dependence. However, on the basis of the ^{31}P and ^{1}H NMR spectra of the exchangeable protons, this oligomer behaves at low salt like other B-form oligomers previously investigated [for example, Patel et al. (1982)]. Thus, the marked changes in the ^{31}P NMR spectra in 5 M NaCl as the temperature is raised must arise from the conformations present in high salt.

The exchangeable proton NMR spectrum at high salt also indicates that the oligomer contains multiple conformations. The two G:C resonances, corresponding to five and three protons, are observed at 20 °C. The relative chemical shifts of these signals allow them to be assigned respectively to a Z conformation and a B conformation. Since terminal base pair protons are rarely observed at temperatures ca. 20 °C, the signal with the relative intensity of five can be assigned to base pairs 2–6 *in a Z conformation.* The signal with the intensity of three protons can then be assigned to base pairs 10, 12, and 14, which are in the portion of the oligomer that might be expected to maintain a B conformation.

These findings are consistent with the conclusion that the oligomer under discussions contains both a Z conformation (most likely between base pairs 1 and 6) and a B conformation (at least between base pairs 10 and 16). There must be a region, or junction, connecting the Z end and the B end of the molecule. This junction most likely encompasses base pairs 7–9.

As has been shown in crystallographic studies (Wang et al., 1979) and NMR studies (Skelnar et al., 1987) and, for a review, Chen and Cohen (1984), B- and Z-type DNAs show distinctive structural differences. Numerous studies have also pointed to the differences in flexibility of B- and Z-type DNAs (Mirau et al., 1985; Thomas & Bloomfield, 1983; Genest et al., 1987). Furthermore, the existence of both Z- and B-form DNA conformations within the same molecule has also been observed in plasmids (Singleton et al., 1982; Stirdivant et al., 1982; Kilpatrick et al., 1983; Johnston & Rich, 1985; Palecek et al., 1987). It has been suggested that the energy required to overcome the barrier to Z-DNA formation (and, thus, junction formation) is provided by the negative supercoiling of the plasmid (Singleton et al., 1983; Ellison et al., 1985; Johnston & Rich, 1985). However, the detection of B–Z junctions in a 153 base pair DNA fragment with GC segments on either end (Porschke et al., 1987) and the formation of a B–Z junction in the molecule studied in this paper suggest that B–Z junctions can form in linear systems. A significant aspect of the work reported here is that the junction can form in a relatively short piece of DNA.

The data presented here also give further insight into the molecular nature of the B–Z junction for the oligomer under study. What is particularly interesting is that base pairs 7–9 have properties distinctly different from either the Z region (base pairs 1–6) or the B region (base pairs 10–16). One notable difference is observed in the exchangeable proton NMR spectrum of this molecule. The resonances for base pairs 7, 8, and possibly 9 only become observable at temperatures greater than or equal to ca. 30 °C. A possible explanation is that at temperatures below 30 °C these base pairs are in an orientation that makes them readily accessible to exchange with solvent. As the temperature is raised, the conformation in this region of the molecule is altered to one in which such accessibility to exchange is diminished. This conformational change may be due to changes in the flexibility of the DNA backbone as the temperature is raised, as has been observed with other oligomers in the Z conformation (Hartmann et al., 1983; Genest et al., 1987; Taboury et al., 1985). The changes with temperature observed in the ^{31}P NMR spectra are consistent with this notion. It is tempting to suggest that the changes observed (most notably at 37 °C) are due to alterations in the junction region. Thus, the NMR results suggest that the junction may possess flexibility properties different from either the B or Z regions.

The oligonucleotide discussed in this paper is the first example for which a B–Z junction has been detected in a short oligonucleotide. The advantage to this approach is that we have a definable model from which we can gain discrete structural information. This allows us to address some of the previously reported ideas about the nature of these B–Z junctions. For example, model-building studies show that at least one base pair at the junction must be substantially different from either B-form or Z-form conformations (Arnott & Chandrasekaran, 1981; Arnott et al., 1982; Hingerty & Broyde, 1983; Harvey, 1983). Our results are completely consistent with the above. The ^{1}H NMR studies show that G:C base pair 8 is dramatically different from all other base pairs.

Other studies have been concerned with the span of the junction, with estimates from 3 to 12 base pairs being involved in the junction (Wartell et al., 1982; Singleton et al., 1982; Kilpatrick et al., 1983; Azorin et al., 1984; Pochet et al., 1986; Porschke et al., 1987; Sheardy, 1988). Our results indicate that, for this model, only three base pairs are involved in the junction.

Finally, it has also been suggested that the junction may be partially single stranded (Singleton et al., 1982; Stirdivant et al., 1982; Kilpatrick et al., 1983; Palecek et al., 1987). Our data indicate that at temperatures between 30 and 50 °C, all internal hydrogen bonds (base pairs 2–14) are intact. The saturation-transfer experiments described here also give us the opportunity to investigate the dynamical properties of the hydrogen bonds (Wüthrich, 1986). The exchangeability of the hydrogen-bonded protons must be related to the internal motion of the molecule in a particular region. At all temperatures studied, the resonance for G:C* base pairs 2–6 is visible, suggesting that they are not readily exchanging with water. Furthermore, and somewhat surprisingly, G:C* base pair 8 seems to show only some sign of exchange at 50 °C. The resonances for G:C base pairs 10, 12, and 14 are visible at 40 °C but not at 50 °C, suggesting that these base pairs are somewhat more susceptible to exchange. The data for G:C* base pair 7 suggest that this base pair begins to exchange readily above 40 °C. At temperatures above 30 °C, all A:T base pair resonances have disappeared, suggesting that these base pairs are readily exchanging with water. The relative observed ease of exchange for the A:T base pairs and G:C base pairs 10, 12, and 14 is consistent with the CD results which indicate that the B end of the molecule melts before the Z end. These saturation-transfer data suggest the accessibility of the various hydrogen-bonded protons for exchange with water is both temperature *and* conformationally dependent.

In summary, the NMR and CD results presented here indicate that the molecule possesses both B and Z conformations in 5 M NaCl. A junction region spanning three base pairs connects the B and Z regions. Finally, this junction has properties distinctive from those of either the B or Z forms.

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