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THE UNPAIRED CYTOSINE IN THE DEOXYOLIGONUCLEOTIDE DUPLEX

dCA₃CA₃G·dCT₆G IS OUTSIDE OF THE HELIX

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ABBREVIATIONS

NMR: nuclear magnetic resonance; NOE: nuclear Overhauser effect; TSP: sodium 3-(trimethylsilyl)propionate-2,2,3,3-d₄; EDTA: ethylene diamine tetraacetic acid.
ABSTRACT

The conformation and thermodynamics were determined for the oligonucleotide duplex dCA₃CA₃G + dCT₆G which contains an extra, unpaired cytosine residue. The conformation was investigated using nuclear magnetic resonance to observe both the aromatic base protons and base pairing imino protons. The nuclear Overhauser effects between imino protons on either side of the unpaired cytosine show that the cytosine is extrahelical; the base is not stacked in the helix. The temperature dependence of the chemical shifts of the aromatic base protons support this conclusion. The observed nuclear Overhauser effects and a large upfield shift in the imino proton resonance of A·T base pair 4, adjacent to the extrahelical cytosine indicate there is a perturbation in the base pair structure of this A·T base pair as well as a change in the overlap with adjacent base pairs. Thermodynamic parameters were determined for this duplex as well as for dCA₆G + dCT₆G. The extrahelical cytosine causes a decrease in the duplex melting temperature of 15°C, at a concentration of 200μM per single strand. The standard free energy for duplex formation at 25°C is 2.9 kcal mol⁻¹ more positive for the duplex with the extra cytosine. This decrease in stability for the perturbed helix is caused by an unfavorable change in enthalpy and a possibly favorable change in entropy for duplex formation.
INTRODUCTION

The ability to predict the structure of RNA or DNA is based on an understanding of the energetics involved in forming that structure. Although there are data available on the thermodynamics of double strand formation in RNA (Borer et al., 1974) there are limited data for DNA. Predictions for the thermodynamics of duplex formation in DNA helices must, presently, be done using the RNA values. Relatively little is known about the thermodynamics for the formation of secondary structures in RNA, such as hairpins (Uhlenbeck et al., 1973; Gralla & Crothers, 1973), mismatched bases, or base bulges (Fink & Crothers, 1972), and even less is known for DNA. Previous investigations have probed the effects of base pair mismatches in synthetic polynucleotide helices (for a review see Lomant and Fresco, 1974). These studies used optical properties to determine if the polynucleotides formed helices containing noncomplementary base oppositions, or if the bases, either stacked or not stacked in the helix, had no base opposition on the cross strand. Evidence for looped out bases in polynucleotides has been obtained using circular dichroism (Gray et al., 1980) and photodimerization (Lomant and Fresco, 1973; Evans and Morgan, 1982). The free energy for a bulge defect has been measured for poly(A,A*) + poly(U), where A* is the N-1 oxide of adenine (Fink and Crothers, 1972). The stabilities for base bulges may be valuable in investigating mechanisms of mutagenesis, in particular frameshift mutagenesis (Streisinger et al., 1966).

Nuclear magnetic resonance (NMR) has been used to study the conformation of a deoxyoligonucleotide containing an extra adenine and the base was found to be stacked in the helix (Patel et al., 1982). In this paper we demonstrate the use of nuclear magnetic resonance to study the
deoxyoligonucleotide duplex, dCA3CA3G + dCT6G, in comparison with the parent duplex, dCA6G + dCT6G. By analyzing the temperature dependence of the chemical shifts of aromatic base protons and the nuclear Overhauser effects (NOE) between imino protons, we have determined that the unpaired cytosine is not stacked in the helix. UV absorption melting curves have been measured and analyzed, for these helices, to obtain the standard free energies, enthalpies and entropies of duplex formation.

MATERIALS AND METHODS

All of the oligonucleotides were synthesized by one of two techniques; dCT6G, dCA5G and dCT5G were synthesized using diester solution techniques (Khorana, 1968); dCA3CA3G and dCA6G were synthesized using phosphoramidite solid support techniques (Beaucage & Caruthers, 1981; Matteucci & Caruthers, 1981). They were desalted several times using Biogel P-2 columns and then lyophilized for storage. Extinction coefficients were estimated from the coefficients for mononucleotides and dinucleotide phosphates (Handbook of Biochem. & Mol. Biol, 1975). The extinction coefficients per mole of strand at 260 nm and 25°C for dCA3CA3G, dCA6G, dCA5G, dCT5G and dCT6G are 98 x 10³, 91 x 10³, 79 x 10³, 58 x 10³, 66 x 10³, 1 M⁻¹cm⁻¹, respectively. Samples used for thermodynamic studies were lyophilized from H2O and then dissolved in a 0.01M phosphate buffer, pH=7, containing 0.1mM EDTA and 1M NaCl. The optical melting curves were obtained using a procedure described previously (Nelson et al., 1981).

NMR samples were prepared with buffer containing 28 mM phosphate, 0.1 mM EDTA and 0.2 M NaCl. D2O samples, containing oligonucleotide and buffer, were lyophilized 3 or 4 times from 99.98% D2O (Biorad) and finally dissolved in 100% D2O (Biorad) in a glove bag under N2 atmosphere. H2O samples were
prepared following the same procedure, but were lyophilized from H$_2$O and prepared outside the glove bag. The water samples contained 10-15% D$_2$O. All H$_2$O samples were adjusted to be pH = 7 ± 0.1 and D$_2$O samples were adjusted to pD = 7 ± 0.1 (measured as 6.6 on a pH meter). Spectra taken in H$_2$O were obtained using a Redfield 21412 pulse sequence (Redfield et al., 1975). NOE difference spectra were obtained with an irradiation time of 0.5 seconds and a repetition rate of ~0.8 seconds, using a Bruker WMS500 instrument at the University of Washington, Seattle. Other NMR spectra were obtained using the 360 MHz instrument at the Stanford Magnetic Resonance Laboratory or the 500 MHz instrument at the UC, Davis, NMR Facility, as indicated. All spectra were referenced to the internal standard TSP.

THERMODYNAMIC RESULTS

The optical melting curves of dCA$_3$CA$_3$G + dCT$_6$G taken at seven different concentrations are shown in Figure 1. The curves are normalized at 60°C to an absorbance of 1. The upper curve is the single-strand melting curve, determined from a weighted average of melting curves from the two individual strands. The curves show an increase in hypochromicity as the concentration increases; this has been attributed to aggregation of the double strands (Nelson et al., 1981). The lower baseline of the double strand melt is difficult to determine, because this duplex melts at approximately room temperature. A non-linear least squares program based on a two-state model was used to fit $\Delta H^\circ$, $\Delta S^\circ$, and the lower baseline slope to the normalized melting curve. The slope for the most concentrated sample was determined to be $1.04 \times 10^{-3}$ per °C. This slope was used to determine low temperature baselines for the other concentrations. The melting curves were analyzed to determine the melting temperature using the slope of the lower baseline.
previously discussed and the experimental single strand melting curve. Figure 2 shows the $1/T_m$ vs. $\log C_0$ plot used to determine $\Delta H^o$ and $\Delta S^o$, where $C_0$ is the total concentration of each of the oligomer strands (Nelson et al., 1981). These results are given in Table I. The same procedure was used for dCA$_6$G + dCT$_6$G with the exception that the slope of the low temperature baseline for the second most concentrated sample was used to obtain low temperature baselines for the other curves. This slope was used because it gave baselines that fit the curves better than those determined from the slope of the most concentrated sample. The slope used was $1.55 \times 10^{-3}$ per °C. Values obtained using the low temperature baseline slope of the most concentrated sample were within 3% of the values reported here. The $1/T_m$ vs. $\log C_0$ plot from these data is also shown in Figure 2. Thermodynamic parameters are reported in Table I for dCA$_6$G + dCT$_6$G and for dCA$_5$G + dCT$_5$G (Nelson et al., 1981).

NMR RESULTS AND DISCUSSION

Before discussing the thermodynamic parameters it is helpful to know more about the conformation of the oligonucleotide duplex with the extra base. The conformation has been studied by NMR, both by using NOE to probe proton-proton distances in the duplex and by investigating the temperature dependence of the chemical shifts of aromatic protons.

Spectra of the base pairing imino protons from dCA$_5$G + dCT$_5$G, dCA$_3$CA$_3$G + dCT$_6$G, and dCA$_6$G + dCT$_6$G are shown in Figure 3. The assignments of the resonances from base pairs 1, 7, and 8 in the dCA$_3$CA$_3$G + dCT$_6$G duplex were made in analogy to the assignments for the dCA$_5$G + dCT$_5$G duplex determined by Pardi, et al. (1981). The assignments of the other imino resonances were determined by imino-imino NOE, as discussed below.
The nuclear Overhauser effect is a process where spin can be transferred from one nucleus to another through space (Noggle and Schirmer, 1971). The process depends inversely on the sixth power of the distances between the nuclei. The NOE is also affected by several other factors. The intensity is dependent on the correlation time of the molecule, which depends on the length of the duplex. The NOE can also be in competition with other possible relaxation processes, such as exchange with water. We have used this technique to assign resonances and to obtain information about the conformation around the extra cytosine. In a regular helix the distance between imino protons on neighboring base pairs is less than 4Å, a reasonable distance to see NOE's. In A·T base pairs there is also an NOE from the imino proton to the adenine(H2) on the same base pair and this NOE has been used to assign imino resonances to A·T (or A·U) vs. G·C base pairs (Sanchez et al., 1980; Hare and Reid, 1982). The NOE difference spectra for dCA₃CA₃G + dCT₅G are shown in Figure 4 along with a Redfield spectrum showing all the resonances for reference. In spectra b, c, d, e and f, NOE to a sharp line in the aromatic region, upfield from the imino region, indicates the saturated resonances are due to A·T base pairs. Given the above information we can now interpret the NOE spectra shown in Figure 4.

The assignment procedure consists of selectively saturating the imino resonances and detecting the NOE. We expect to see an NOE from an imino proton to the imino protons on the neighboring base pairs. In Figure 4b saturating the imino resonance from base pair 2 should show an NOE to resonances from base pairs 1 and 3. Base pair 1 is one of the terminal G·C base pairs and an inversion recovery experiment shows the recovery time of this resonance is less than 0.05 seconds. Thus, observation of NOE to this resonance is not likely. As expected, only a peak from base pair 3 is
observed in the difference spectrum in Figure 4b. It should be noted that this resonance could also be due to partial saturation as it is close to the resonance for base pairs 2 and 5. Saturation of the resonance from base pair 5 shows NOE to base pairs 4 and 6, as expected. NOE from base pair 3 to base pair 4 and possibly 2 are shown in Figure 4c. The peak from base pairs 2 or 5 may again be due to partial saturation. NOE is observed from the imino resonance for base pair 4 to base pairs 3 and 5 in Figure 4d. In Figure 4e, NOE is observed to base pairs 5 and 7 when saturating the imino resonance from base pair 6. In Figure 4f, NOE is observed from base pair 7 to base pair 6. No NOE is observed to base pair 8, because this resonance has a rapid relaxation rate. This is also a problem when trying to observe NOE from the imino resonance of base pair 8 and, as shown in Figure 4g, none are observed in the lowfield region. From the observation of imino-imino NOE, described above, we have assigned the lowfield resonances as given in Figure 4a.

All the A(H2) resonances can be assigned knowing the assignment of the imino resonance from base pair 7 and the fact that there is a strong NOE between the imino proton and the A(H2) resonance in an A·T base pair. The assignments for the A(H2) resonances are in good agreement with the assignments for the imino resonances as shown by the following. In Figure 4b we observe NOE from the imino proton on base pair 2 to its own H2 proton, A2(H2), and to A3(H2) on the adjacent base pair. In the same spectrum we observe NOE from the imino proton in base pair 5 to its own H2 and to the H2 on base pair 6. This general trend is seen in other A·T base pairs in this duplex. Saturation of the imino proton resonance, T(H3), gives NOE to the A(H2) it is base paired with and to the A(H2) in the 3' direction from the first A(H2). This is the trend we expect to see, based on distances
calculated for a regular DNA helix. Thus we observe, in Figure 4e, NOE from the imino proton on base pair 6 to the H2 on that base pair and the H2 on base pair 7. In the spectrum shown in Figure 4f NOE is observed only from the imino proton on base pair 7 to its own H2 because, base pair 8 is a G·C base pair and does not contain an H2 proton. In Figure 4c saturation of the imino resonance from base pair 3 gives NOE to A3(H2) and A4(H2), as expected. We also observe NOE to A2(H2) and A5(H2) due to partial saturation of imino resonances from base pairs 2 and 5, respectively. The only example that does not follow the trend is shown in Figure 4d. Saturation of the imino resonance from base pair 4, one of the base pairs adjacent to the unpaired C, shows NOE to A4(H2) and A5(H2), as expected, but in addition we observe NOE to the A3(H2) which is the neighboring base pair on the 5' side. This implies that the distance between the imino proton from base pair 4 and the H2 proton on base pair 3 is less than the analogous distance in other parts of the helix, where similar NOE is not observed. This decrease in distance could be achieved by a decrease in winding angle between these two base pairs (3 and 4) or a translation of one base pair relative to the other.

The observation of NOE from base pair 4 to base pair 5 characterizes the conformation of the unpaired cytosine. If the extra cytosine were stacked in the helix, the distance between base pairs 4 and 5 would be almost 7 Å and no imino-imino NOE would be observed. However, as shown in Figure 4d, we do observe NOE from the imino proton on base pair 4 to the imino proton on base pair 5. Thus, the unpaired cytosine can not be stacked in the helix. This is corroborated by the observation of NOE from the imino proton on base pair 4 to the A5(H2), as shown in Figure 4d.

There are several other potential assignment schemes for the imino
protons. However, these can be ruled out, either because of inconsistencies in the imino-imino NOE's or inconsistencies in the imino-A(H2) NOE's. There is one assignment scheme for the imino protons, other than the one presented, that can not be ruled out by any obvious inconsistencies. This alternate assignment consists of exchanging the assignments for the imino resonances from base pairs 3 and 4, compared to those shown in Figure 4. Assignment of the A(H2) resonances can be made consistent with this imino assignment by exchanging the A(H2) assignments for base pair 3 and 4 as well as base pair 2 and 5. With this alternate assignment scheme, NOE is observed from the imino protons of base pair 3, 4, and 5 to their own A(H2) and to the A(H2)'s of the base pairs on both sides of it. This is not consistent with the expected NOE from a regular DNA helix of this sequence, but the assignment can not be ruled out completely on this basis. There is NOE observed from the imino proton of base pair 4 to the A5(H2) and from the imino proton of base pair 5 to the A4(H2), so this alternate assignment scheme is also consistent with the extra cytosine being out of the helix.

More evidence for the cytosine being extrahelical is obtained by monitoring the aromatic proton resonances and their temperature dependence. When the aromatic bases are placed in a magnetic field there is a local field induced, thus, a proton directly above or below the base will be shielded from the applied field and a proton in the plane of the base will be deshielded. For an aromatic proton in a double strand, such ring current effects will be mainly due to the base pairs above and below the proton and from the base it is base paired to on the cross strand. The equilibrium between double strand and single strand formation changes as a function of the temperature, thus the environment around a proton changes as the temperature changes. This is observed in the NMR spectrum as a change
in the chemical shift and can be seen in Figure 5. At low temperature the resonances are broad due to exchange between the single strand and double strand environment. This broadening makes assignment of the resonances more difficult. Assignment of the cytosine doublets at low temperature was accomplished using a double quantum pulse sequence (spectrum shown in top of Figure 6). This pulse sequence creates double quantum coherence and allows observation of only those resonances which have double quantum transitions (Hore et al., 1982a,b). The region of the spectrum between 6.5 - 8.5 ppm contains only singlets and doublets and becomes simplified to contain only doublets when the double quantum pulse sequence is used. It is characteristic of this pulse sequence that the two peaks in the doublet will be $180^\circ$ out of phase, as seen in the top of Figure 6. The low temperature chemical shifts of the cytosine doublets were obtained from the spectrum in Figure 6. The high temperature chemical shifts of the cytosine doublets in dCA$_3$CA$_3$G + dCT$_6$G can be assigned by comparison with dCA$_5$G + dCT$_5$G (Pardi et al., 1981) and with dCA$_6$G + dCT$_6$G. In Figure 7 the chemical shifts as a function of temperature are compared for dCA$_3$CA$_3$G + dCT$_6$G and dCA$_6$G + dCT$_6$G. The only major difference between the curves for the two terminal cytosines is that the curve for the dCA$_6$G + dCT$_6$G resonances shift to higher temperature due to the increased melting temperature of this duplex. The curve at the bottom of Figure 7 can now be assigned to the extra cytosine in dCA$_3$CA$_3$G + dCT$_6$G. Because this duplex has non-self-complementary stands, the single strand chemical shifts as a function of temperature can also be obtained. The chemical shift versus temperature of dCA$_3$CA$_3$G, dCT$_6$G, and the double strand are shown in Figure 8. As duplex formation occurs, with decreasing temperature, both the C1(H6) and the C8(H6) show an increase in chemical shift, a downfield shift, due to the deshielding effect of the
bases on the cross strand. This deshielding will not be present for the extra cytosine, regardless of whether or not it is stacked in the helix, because there is no base directly across from it on the cross strand. Chemical shift calculations done for the extra cytosine, assuming it is in the base stack and all bases are stacked in a B-form geometry around it, predict an upfield shift on going from high to low temperature. If the cytosine were stacked in the helix, it would be shielded by the A·T base pairs around it. However, what is observed in Figure 8 is a large (0.3 ppm) increase in the chemical shift for this resonance, relative to the chemical shift in the single strand. We also note that the chemical shift of the extra cytosine at high temperature, where the bases are partially unstacked, is almost identical to the shift at low temperature, where the bases should be stacked. If the unpaired cytosine were extrahelical then the environment around that base could be similar to the environment in the unstacked or single strand state. This chemical shift behavior suggests that the extra cytosine is not stacked in the helix.

With the imino proton resonances assigned, the spectra of the imino protons for dCA₃CA₃G + dCT₆G and dCA₆G + dCT₆G can be compared as shown in Figure 3. The largest change in chemical shift is for base pair 4 which has shifted upfield by at least 0.4 ppm. Some of this shift could be due to the increased overlap with A·T base pair 3, as discussed previously. However, most of the ring current effect due to this A·T base pair is already present in dCA₆G + dCT₆G so 0.4 ppm is a large shift to be explained by only an improved overlap. We propose that some of the upfield shift is due to a weakened or lengthened hydrogen bond in A·T base pair 4. Work done by Wagner et al. (1983) on proteins indicates a strong correlation between H-bond length and chemical shift. Also the resonance from A·T base pair 4
is broader than the other A·T resonances and may indicate faster exchange with water. This increase in exchange rate would be expected if the hydrogen bond were weaker. Saturation recovery experiments to determine these exchange rates are currently being done in our laboratory.

DISCUSSION OF THERMODYNAMICS

We can now discuss the thermodynamic results in view of the result that the cytosine is extrahelical. Table I compares the thermodynamic parameters for all three helices. The melting temperature, at 200μM per single strand, decreases by 15°C when we introduce the perturbation of the extra cytosine. It is decreased 10°C over the duplex with one less A·T base pair and no perturbation. Another indication of the destabilization is that the ΔG° (25°C) of double strand formation is not as energetically favorable for the duplex with the unpaired cytosine: a 2.9 kcal mol⁻¹ destabilization from the parent duplex and 1.7 kcal mol⁻¹ destabilization compared to the duplex with one less A·T base pair. In RNA there is a favorable enthalpy and unfavorable entropy change on going from a single strand to a double strand state (Borer et al., 1974). This trend is observed when comparing dCA₆G + dCT₆G to dCA₃G + dCT₃G. In this case we have one more A·T base pair, which decreases the enthalpy by 12 ± 5 kcal mol⁻¹ and decreases entropy by 36 ± 13 e.u. When comparing dCA₃CA₃G + dCT₆G with dCA₆G + dCT₆G there is an unfavorable change in enthalpy of 6 ± 4 kcal mol⁻¹, which characterizes the loss of stacking of the cytosine and the deformation of the double helix around the unpaired cytosine. There is a small, probably favorable, change in the entropy, 11 ± 13 e.u., presumably explained by the freedom of the extra cytosine which is not stacked in the helix.

Several other groups have studied the effects of an extra base on the
conformation and thermodynamics (Lomant & Fresco, 1974; Evans & Morgan, 1982; Gray et al., 1980; Fink & Crothers, 1972; Patel et al., 1982). Several of these studies have shown that these bases are extrahelical. Work done by Fink and Crothers on the polyribonucleotide poly(A,A*) + poly(U), where A* is the N-1 oxide of adenine, shows a destabilization in the standard free energy at 25°C of 2.8 kcal mol⁻¹ due to this perturbation. This is in good agreement with the value of 2.9 kcal mol⁻¹ obtained in this paper for the deoxyribo-oligonucleotide. Work done by Patel et al. (1982) on the nearly self-complementary tridecamer dC-G-C-A-G-A-A-T-T-C-G-C-G indicates that the extra adenine is stacked in the helix. There is no difference in enthalpy, within experimental error, for forming the duplex with the extra adenine compared to the completely self-complementary dodecamer, dC-G-C-G-A-A-T-T-C-G-C-G. The extra base and the sequence of the duplex around that base apparently determine whether the base remains in the helix or is bulged out. As adenine is the base with the strongest stacking tendency, it is reasonable that an extra adenine remains in the helix, but that a cytosine surrounded by adenines is forced out of the helix. Our understanding of the sequence dependence of conformation will become clearer as different sequences containing perturbations are studied.

CONCLUSION

We have studied the conformation of an extra cytosine in the deoxyribonucleotide duplex dCA₃CA₃G + dCT₆G. Using the nuclear Overhauser effect, which depends on the inverse sixth power of the interproton distances, we find that the A·T base pairs on either side of the extra cytosine are stacked on each other. Thus the cytosine must be extrahelical; that is, it is not stacked with neighboring base pairs in the helix. These experiments
also demonstrate that the extrahelical cytosine causes local perturbations in the conformation of these adjacent A·T base pairs. The temperature dependence of the chemical shifts of the cytosine H6 doublets in this duplex also indicates that the unpaired cytosine is extrahelical.

The thermodynamic parameters were determined for this duplex and compared to parameters for dCA6G + dCT6G. The extrahelical cytosine causes a 2.9 kcal mol\(^{-1}\) decrease in the standard free energy at 25°C and a decrease in the enthalpy of double strand formation. Comparing the results we obtain for this sequence with results obtained on other sequences (Patel et al., 1982) indicates that stacking interactions may determine whether a base will be stacked or not stacked in the helix.

The effects of an extra base on conformation and energetics of the duplex has implication on the mechanisms of mutagenesis. Models have been proposed for stacking interactions between frameshift mutagens and an extrahelical base (Streisinger et al., 1966; Drake & Baltz, 1976). Several studies have demonstrated the possibility of this type of interaction (Lee & Tinoco, 1978; Helfgott & Kallenbach, 1979). The work presented here has shown how dCA\(_3\)CA\(_3\)G + dCT\(_6\)G in solution forms a stable duplex with the unpaired cytosine not stacked on the neighboring base pairs in the helix and thus this duplex would be ideal for studying the interaction between frameshift mutagens and extrahelical bases.
ACKNOWLEDGMENTS

We are grateful to Dr. Arthur Pardi for useful discussion as well as for generously providing data prior to publication. We thank Dr. Jerry Matson at the U.C., Davis, NMR facility for assistance in obtaining the double quantum spectra. We also thank Dr. Dave Wemmer for taking the NOE spectra and for giving helpful advice. Support for the Bruker WM 500 NMR at the University of Washington, Seattle, is given by the Murdock Charitable Trust.
REFERENCES


Table I: Thermodynamic Parameters from $1/T$ vs. log $C_o$ Plot

<table>
<thead>
<tr>
<th>Compound</th>
<th>$T_m$ (°C, 200μM)</th>
<th>$\Delta G^\circ$ (kcal mol$^{-1}$)</th>
<th>$\Delta H^\circ$ (kcal mol$^{-1}$)</th>
<th>$\Delta S^\circ$ (e.u.)</th>
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<td>dCA$_3$CA$_3$G + dCT$_6$G</td>
<td>22</td>
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<td>-161</td>
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<td>dCA$_8$G + dCT$_6$G</td>
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<td>-7.8</td>
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<td>dCA$_3$G + dCT$_5$G$^a$</td>
<td>32</td>
<td>-6.6</td>
<td>-47</td>
<td>-136</td>
</tr>
</tbody>
</table>

a. Data from Nelson et al., 1981.
FIGURE CAPTIONS

Fig. 1. Melting curves of dCA₃CA₃G + dCT₆G in 1M NaCl with concentrations ranging from 0.01mM-0.9mM in single strand. The curves are normalized to 1 at 60°C. The upper curve is the single strand melting curve as described in the text.

Fig. 2. The 1/Tₘ vs. log C₀ plot for (○) dCA₃CA₃G + dCT₆G and (□) dCA₆G + dCT₆G.

Fig. 3. The 360 MHz ¹H NMR spectra of the base pairing imino protons from dCA₅G + dCT₅G, dCA₃CA₃G + dCT₆G, and dCA₆G + dCT₆G. Samples were 1mM per single strand in H₂O. Spectra are the sum of 400-600 accumulations.

Fig. 4. The 500 MHz ¹H NOE difference spectra from dCA₃CA₃G + dCT₆G. Spectra b-g are the sum of 12,000 accumulations (6,000 with irradiation on resonance and 6,000 off resonance). Spectrum a was obtained using a Redfield pulse with no irradiation. The sample was 1mM per single strand in 90% H₂O.

Fig. 5. The 360 MHz ¹H NMR spectrum of dCA₃CA₃G + dCT₆G as a function of temperature. The sample is 1mM per single strand in D₂O.
Fig. 6. The 500 MHz $^1$H NMR spectrum of dCA$_3$CA$_3$G + dCT$_6$G. The top spectrum is the sum of 10,400 scans using the double quantum pulse sequence, as discussed in the text. The bottom spectrum is the single quantum spectrum of the same sample. The sample was 0.5mM per single strand in D$_2$O.

Fig. 7. Chemical Shift vs. Temperature curves for the C(H6) protons in (x) dCA$_3$CA$_3$G + dCT$_6$G and (Δ) dCA$_6$G + dCT$_6$G. The samples were 1mM per single strand in D$_2$O.

Fig. 8. Chemical Shift vs. Temperature curves for the C(H6) protons in dCA$_3$CA$_3$G + dCT$_6$G. Both the (x) double strand and the (○) single strand curves are shown. The sample was 1mM per single strand in D$_2$O.
Figure 1.
Figure 2.

\[ \frac{1}{T_m} \times 10^3 \text{ (°K}^{-1}) \]

\[ \log(\text{concentration}) \]
Figure 3.

1 2 3 4 5 6 7
\[dC\text{-}X\text{-}X\text{-}d\]
\[G\text{-}T\text{-}T\text{-}T\text{-}T\text{-}T\text{-}d\], 5°C

6 (2,3,4,5) 7 1

1 2 3 4 5
\[dC\text{-}C\text{-}X\text{-}X\text{-}d\]
\[G\text{-}T\text{-}T\text{-}T\text{-}T\text{-}T\text{-}C\], 1°C

7 6 2,5 3 8 4 1

1 2 3 4 5 6 7 8
\[dC\text{-}X\text{-}X\text{-}X\text{-}X\text{-}X\text{-}X\text{-}d\]
\[G\text{-}T\text{-}T\text{-}T\text{-}T\text{-}T\text{-}T\text{-}C\], 1°C

7 (2,3,4,5,6) 8 1

PPM
Figure 5.

- 55°C -

- 35°C -

- 25°C -

- 1°C -
Figure 7.

Chemical Shift (ppm) vs. Temp. (°C)

- C8(H6)
- CI(H6)
- Bulge C(H6)

Sequences:

```
G-T-T-T-T-T-T-Cd
1 2 3 4 5 6 7 8
```

```
G-T-T-T-T-T-T-Cd
1 2 3 4 5 6 7 8
```
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