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# Structure and Energetics of a Hexanucleotide Duplex with Stacked Trinucleotide Ends Formed by the Sequence d(GAATTCGCG)†

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ABSTRACT: Nuclear magnetic resonance (NMR) and differential scanning calorimetry (DSC) have been used to investigate structural and energetic features of the helix-to-coil transition of the duplex formed by the partially self-complementary sequence d(GAATTCGCG) (henceforth called 9-mer). These results are compared with those obtained from a corresponding study on the helix-to-coil transition of the duplex formed by the fully self-complementary sequence d-(GGAATTCC) (henceforth called 8-mer). The two sequences contain a common GAATTC hexanucleotide duplex with this core flanked by d(GCG) trinucleotide ends in the 9-mer and by dG·dC base pairs in the 8-mer duplex. The NMR parameters for the 9-mer reveal formation of the hexanucleotide core duplex and stacking of the unpaired bases at the trinucleotide ends. The imino proton line widths suggest that under the conditions of the NMR experiment and at low temperature the 9-mer duplexes aggregate through pairing of the complementary ends. DSC on both the 8-mer and 9-mer duplex in 1 M NaCl reveals that the calorimetric transition enthalpies are essentially equal [~60 kcal (mol of double strand)$^{-1}$] despite a 6.2 °C higher melting temperature, $T_m$, for the 8-mer relative to the 9-mer duplex. From a comparison of the model-dependent van't Hoff and model-independent calorimetric enthalpies we conclude that the helix-to-coil transition of the 8-mer approaches two-state behavior while the corresponding 9-mer transition involves intermediate states. We assign the cooperative component of the 9-mer transition to disruption of the hexanucleotide duplex core with the remaining noncooperative component being associated with the disruption of the stacked ends.

The early research on the helix-coil transition of DNA and RNA oligonucleotide sequences focused on fully base-paired duplexes (Cross & Crothers, 1971; Patel, 1974; Breslauer et al., 1975; Borer et al., 1975; Kallenbach et al., 1976; Breslauer & Sturtevant, 1977; Early et al., 1977, 1980; Patel & Canuel, 1979; Miller et al., 1980; Pardi et al., 1981; Albergo et al., 1981; Marky et al., 1981; Patel et al., 1981a). More recent efforts have extended these investigations to oligonucleotides containing mismatched base pairs and extra bases on one of the strands in the interior of duplex regions (Haasnoot et al., 1980; Cornelis et al., 1979; Patel et al., 1981b,c).

By contrast, there is limited information on the structure and energetics of oligonucleotide duplexes containing single-stranded ends (Uhlenbeck et al., 1973; Romaniuk et al., 1978; Alkema et al., 1981). We report here a nuclear magnetic

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Chart I

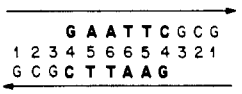
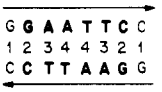



Chart II




resonance (NMR) and calorimetric investigation of the partially self-complementary nonanucleotide sequence d-(GAATTCGCG) that can form a hexanucleotide duplex with trinucleotide ends (Chart I). The NMR and calorimetric parameters for d(GAATTCGCG) are compared with the related parameters for the self-complementary sequence d-(GGAATTCC) (Chart II). This comparison allows us to evaluate the relative effect of having a common hexanucleotide duplex flanked at both ends by either d(GCG) trinucleotide strands or dG·dC base pairs.

## Experimental Procedures

*Synthesis.* The d(GGAATTCC) 8-mer was purchased from Collaborative Research, Waltham, MA. The preparation of the protected DM–GAATTCGCG–OBz was reported previously (Patel et al., 1981a). The protecting groups were first removed by overnight treatment with 1:1 concentrated ammonium hydroxide/pyridine at 50 °C and then by treatment with 80% acetic acid for 20 min. The workup procedures after base and acid treatment were similar to those reported previously. The deprotected 9-mer was passed through a millipore filter, taken up in 7 M urea and Tris[1] buffer, and chromatographed on a DE-23 ion-exchange column with a KCl salt gradient. This procedure gave 300 OD units of d-(GAATTCGCG). The urea and salts were removed as described previously (Patel et al., 1981a).

*NMR.* The 9-mer exchangeable imino proton NMR spectra in $H_2O$ and nonexchangeable proton NMR spectra in $^2H_2O$ were recorded as a function of temperature on Bruker 360-MHz spectrometers in the correlation and Fourier transform modes, respectively. The 9-mer phosphorus NMR spectra were recorded with broad band proton noise decoupling on a Varian XL-200 spectrometer. The $T_1$ and nuclear Overhauser effect (NOE) experiments were undertaken as described previously (Patel et al., 1981a).

*Calorimetry.* The differential scanning calorimetry was carried out on a Microcal-1 instrument as previously described (Patel et al., 1981a). The data are obtained as excess heat capacity values vs. temperature. The strand concentrations of the 8-mer and 9-mer were calculated with extinction coefficients of $8.3 \times 10^4$ at 70 °C and $9.1 \times 10^4$ at 70 °C, respectively.

Buffer solutions (10 mM phosphate and 0.1 mM EDTA, pH 7.0) containing 1.0 M NaCl were used for the calorimetric experiments so that the relatively short 8-mer and 9-mer duplexes would begin their broad melts at temperatures high enough to allow convenient and accurate determinations of the pretransition base lines.

## Results

The partially self-complementary nonanucleotide d-($G_4A_5A_6T_6T_5C_4G_3C_2G_1$) is assigned the same numbering system used for the self-complementary dodecanucleotide d($C_1G_2C_3G_4A_5A_6T_6T_5C_4G_3C_2G_1$) previously reported (Patel et al., 1981a). As shown in Chart I, the base-paired regions occur at positions 4–6 while the strands extend over positions 1–3.

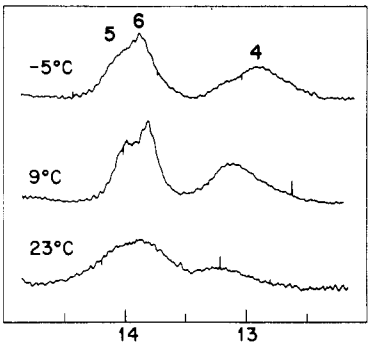



FIGURE 1: The 360-MHz correlation proton NMR spectra (12.5–14.5 ppm) of the 9-mer in 0.1 M phosphate, 2.5 mM EDTA, and 4:1 $H_2O/^2H_2O$, pH 7.58, at −5, 9, and 23 °C. The signal to noise of the spectra was improved by applying a 5-Hz exponential line broadening contribution.

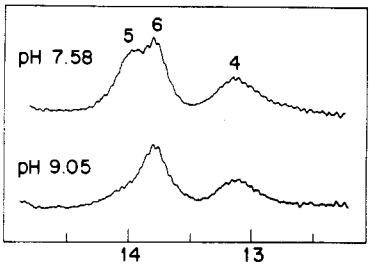



FIGURE 2: The 360-MHz correlation proton NMR spectra (12.5–14.5 ppm) of the 9-mer in 0.1 M phosphate, 2.5 mM EDTA, and 4:1 $H_2O/^2H_2O$, 16 °C, at pH 7.58 and 9.05. The signal to noise of the spectra was improved by applying a 5-Hz exponential line broadening contribution.

*NMR Studies.* (*A*) *Exchangeable Protons.* The thymidine H-3 and guanosine H-1 exchangeable imino protons are sensitive probes of hydrogen-bonding interactions in nucleic acids (Kearns et al., 1971; Patel & Tonelli, 1974). The 360-MHz correlation proton NMR spectra of d(GAATTCGCG) in 0.1 M phosphate, pH 7.58, between −5 and 23 °C are presented in Figure 1. The thymidine imino protons are observed at ~14 ppm while the guanosine imino protons are observed at ~13 ppm (Hilbers, 1979).

We observe an area of ~1.5 guanosine imino protons relative to an area of 2 thymidine imino protons in the 9-mer spectrum recorded at −5 °C (Figure 1). The area of the 9-mer guanosine imino protons reduces to ~1 on raising the temperature to 16 °C. The two thymidine imino protons of the 9-mer shift to high field with increasing temperature between −5 and 16 °C in the premelting region. The guanosine imino proton envelope undergoes a large downfield shift in the 9-mer duplex in this temperature range.

We have investigated the pH dependence of the imino proton line widths of the 9-mer duplex in 0.1 M phosphate solution. The 16 °C imino proton spectra of the 9-mer at pH 7.58 and 9.05 are presented in Figure 2. It is readily apparent that the downfield thymidine imino proton in the 9-mer duplex broadens significantly with increasing pH compared to the upfield thymidine imino proton (Figure 2).

(*B*) *Nonexchangeable Protons.* We have recorded the nonexchangeable base and sugar proton spectra of the 9-mer in 0.1 M phosphate and $^2H_2O$ solution as a function of temperature. The resonances are partially resolved in the duplex state at low temperatures and are well resolved at higher temperature.

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; OD, optical density; EDTA, ethylenediaminetetraacetic acid.

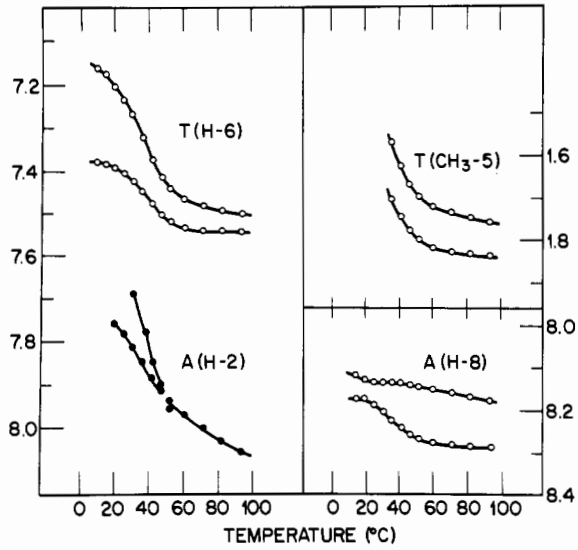



FIGURE 3: The temperature-dependent 360-MHz chemical shifts of the adenosine H-8 and H-2 and the thymidine H-6 and $CH_3$-5 resonances of the 9-mer duplex in 0.1 M phosphate, 2.5 mM EDTA, and $^2H_2O$.

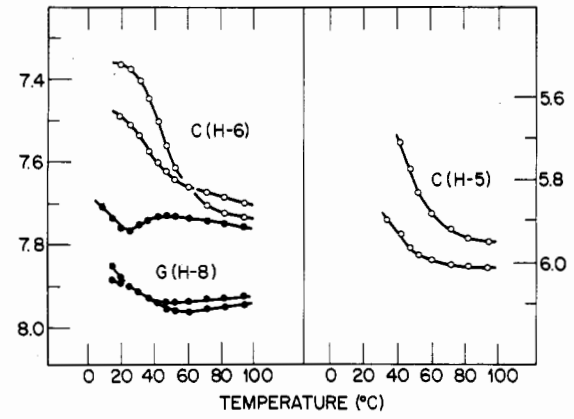



FIGURE 4: The temperature-dependent 360-MHz chemical shifts of the cytidine H-5 and H-6 and guanosine H-8 resonances of the 9-mer duplex in 0.1 M phosphate, 2.5 mM EDTA, and $^2H_2O$.

The temperature dependence of the thymidine and adenosine chemical shifts in the 9-mer are plotted in Figure 3. We can follow both adenosine H-8 and both thymidine H-6 resonances through the melting transition but are unable to monitor the adenosine H-2 resonances and the thymidine $CH_3$-5 resonances at low temperature (Figure 3). A transition midpoint of 37 °C for the 9-mer in 0.1 M phosphate solution is measured.

We are able to monitor the two cytidine H-6 and three guanosine H-8 resonances through the melting transition of the 9-mer in 0.1 M phosphate solution (Figure 4). By contrast, the cytidine H-5 resonances in the 9-mer cannot be followed at low temperature in solution (Figure 4).

We have compared the temperature-dependent chemical shifts of the upfield thymidine H-6 proton in the d(GAATTCGCG) 9-mer in 0.1 M phosphate solution with the corresponding resonance in the d(GGAATTCC) 8-mer in 0.1 M NaCl solution (Patel & Canuel, 1979). Both sequences exhibit similar chemical shifts for this resonance in the duplex and strand states with the self-complementary eight base-paired 8-mer melting at a higher temperature than the six base-paired 9-mer sequence.

(*C*) *Phosphodiester Backbone.* The proton noise decoupled 81-MHz $^{31}P$ spectra of the 9-mer in the duplex state (7.5 °C), near the transition midpoint (34.7 °C), and in the strand state (62.5 °C) in 20 mM phosphate are presented in Figure 5. At

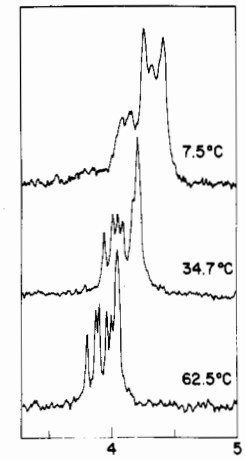



FIGURE 5: The proton noise decoupled 80.996-MHz Fourier transform phosphorus NMR spectra (3–5 ppm upfield from standard trimethyl phosphate) of the 9-mer in 20 mM phosphate, 0.5 mM EDTA, and $^2H_2O$, pH 8.0, at 7.5, 34.7, and 62.5 °C. The signal to noise of the spectra was improved by applying an 0.5-Hz exponential line broadening contribution. The chemical shifts are not corrected for the temperature dependence of the standard.

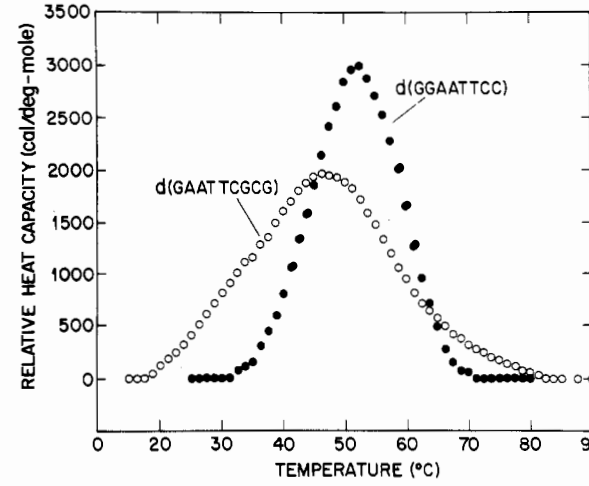



FIGURE 6: Calorimetric heat capacity vs. temperature curves for the 9-mer (strand concentration 0.70 mM) and the 8-mer (strand concentration 0.70 mM) in 1 M NaCl, 10 mM phosphate, and 0.1 mM EDTA, pH 7.0. The temperature was scanned from 10 to 95 °C at a rate of ~1 °C/min.

7.5 °C the phosphodiesters are dispersed between 4.0 and 4.5 ppm upfield from trimethyl phosphate in the 9-mer spectrum. With increasing temperature, these resonances shift downfield with partial resolution of five of the eight phosphodiesters.

The partially resolved phosphodiester resonances exhibit spin–lattice relaxation times ($T_1$) in the range 1.36 ± 0.06 s and nuclear Overhauser effect ($1 + \eta$) values in the range 1.13 ± 0.04 for the 9-mer at 20 °C. The magnitude of these values increase with temperature with $T_1$ values in the range 1.80 ± 0.30 s and ($1 + \eta$) values in the range 1.37 ± 0.06 for the 9-mer at 45 °C. This trend in the relaxation parameters with temperature is similar to previous measurements on oligonucleotides (Davanloo et al., 1979) and polynucleotides (Neumann & Tran-Dinh, 1981) in aqueous solution.

*Calorimetric Studies.* The calorimetric heat capacity curves for the 9-mer and 8-mer in 1 M NaCl are shown in Figure 6. The transition midpoints at comparable concentrations are 46.3 °C for the 9-mer and 52.5 °C for the 8-mer duplex.

(*A*) *Transition Enthalpy.* From the areas under the heat capacity curves, calorimetric transition enthalpies are obtained for the 8-mer and 9-mer in 1.0 M NaCl solution. Specifically, we calculate values of 59 and 60 kcal (mol of double strand)$^{-1}$

Table I: Calorimetric and van't Hoff Enthalpies for the Melting Transition of the 9-mer and 8-mer Duplexes in 1 M NaCl Solution[a]

| | $\Delta H_{cal}$ (kcal) | $\Delta H_{V.H.}$ (kcal) | $T_m$ (°C) |
|---|---|---|---|
| 9-mer | 60 | 35 | 46.3 |
| 8-mer | 59 | 59 | 52.5 |

[a] Buffer is 1.0 M NaCl, 10 mM phosphate, 1 mM EDTA, and $H_2O$, pH 7.0. Calorimetric data represent averages of at least three independent determinations.

for the 8-mer and 9-mer, respectively.

*(B) van't Hoff Enthalpy.* The van't Hoff enthalpies can be calculated from the shapes of the calorimetric heat capacity curves as previously described (Gralla & Crothers, 1973; Patel et al., 1981a). This treatment yields the van't Hoff enthalpies of 59 and 35 kcal for the 8-mer and 9-mer, respectively. These data along with the calorimetrically obtained values are listed in Table I.

## Discussion

*Sticky Ends.* We can resolve the two thymidine imino protons at ~14 ppm in the 9-mer duplex at −5 °C (Figure 1), which demonstrates that dA·dT base pairs 5 and 6 are intact in the duplex state. We also observe guanosine imino proton resonance(s) at ~13 ppm in the 9-mer duplex at −5 °C (Figure 1) with an area of ~1.5 protons relative to 2 protons for the thymidine imino proton resonances. This demonstrates that dG·dC base pair 4 is intact in the duplex state with the additional area reflecting partial contributions from the remaining dG residues in the sequence.

The d(GAATTCGCG) sequence can self-aggregate by hydrogen bond formation at positions 1 and 2 between adjacent 9-mer duplexes, so that the additional area may originate in the guanosine imino proton at position 1 participating in end-to-end aggregation at low temperature. In this connection, the upfield thymidine imino proton exhibits a line width of ~70 Hz in d(GAATTCGCG) compared to a much narrower line width of 28 Hz for the same resonance in d(CGCGAATTCGCG) at −5 °C (Patel et al., 1981a).

*Fraying.* We note that the downfield thymidine imino proton is broader (Figure 1) and its line width increases at basic pH (Figure 2) compared to the upfield thymidine imino proton in the 9-mer duplex. This pH-dependent broadening is indicative of fraying at the ends of the duplex (Patel, 1974; Patel & Hilbers, 1975; Kan et al., 1975) and permits assignment of the imino protons of dA·dT base pairs 5 and 6 in the 9-mer sequence. We assign the pH-sensitive downfield thymidine imino proton to dA·dT base pair 5, which is closer to the ends of the 9-mer duplex than dA·dT base pair 6.

*Stacked $d(G_3C_2G_1)$ Segment.* We have previously reported on the observation of large upfield shifts at the three nonterminal cytidine H-5 and H-6 resonances on formation of the d(CGCGAATTCGCG) duplex (Patel et al., 1981a). By contrast, the chemical shifts of the guanosine H-8 resonances are essentially insensitive to the dodecanucleotide duplex formation.

The two cytidine H-6 and the two cytidine H-5 resonances shift upfield on formation of the 9-mer duplex (Figure 4). We cannot differentiate between positions 2 and 4 for the dC resonances at this time. However, the upfield shifts demonstrate that both cytidines are stacked on formation of the duplex state. This requires that cytidine 2 be stacked with adjacent guanosine(s) in the d(GCG) strands extending from the 9-mer hexanucleotide duplex regions.

*Calorimetry.* The 8-mer duplex has two terminal dG·dC base pairs that flank the common hexameric duplex core while in the 9-mer duplex two d(GCG) ends flank the common core. The high-temperature, single-stranded forms of both duplexes show very similar spectroscopic properties thereby suggesting that the final, random-coil states are equivalent. Consequently, comparison of the melting temperatures associated with the helix-to-coil transitions of these two duplexes allows us to define the stabilizing or destabilizing influence of two d(GCG) ends relative to two terminal dG·dC base pairs. The calorimetric data allow us to define the thermodynamic origins of any structurally induced differential stability between the two duplexes. In addition, the calorimetric data allow us to characterize the influence of the structural differences on the nature of the transitions.

*Overall Stability.* Inspection of the melting temperature data in Table I reveals that at comparable oligomer concentrations the 8-mer duplex is thermally more stable than the 9-mer duplex by 6.2 °C. Thus, the two terminal dG·dC base pairs stabilize the central hexameric duplex core more than the two d(GCG) ends.

*Transition Enthalpies.* The calorimetric enthalpy changes associated with the thermally induced helix-to-coil transitions of the 8-mer and 9-mer duplexes are listed in Table I. The significant observation is that the calorimetric enthalpy values do not substantially differ despite the major structural differences between the two duplexes as manifested in their different $T_m$ values. Apparently, the additional single-stranded base stacking interactions contributed by the ends on either side of the hexameric duplex core in the 9-mer about equals that which results from extending the core duplex by two additional dG-dC base pairs to form the 8-mer. This observation is consistent with the NMR data, which reveal that the bases in the ends stack with the duplex core and with each other.

*Nature of the Transition.* Inspection of the data in Table I reveals that for the 8-mer duplex $\Delta H_{cal} = \Delta H_{V.H.}$. Therefore, we can conclude that the 8-mer duplex undergoes the double- to single-strand transition in essentially a two-state manner. By contrast, for the 9-mer duplex $\Delta H_{cal} > \Delta H_{V.H.}$, thereby allowing us to conclude that the transition of this duplex involves a significant population of intermediate states (Tsong et al., 1970). Thus, although the two different terminal regions flanking the central hexameric core [dG·dC base pairs vs. d(GCG) ends] do not substantially alter the transition enthalpies, they do differentially affect the nature of the transition. Inspection of the transition widths in Figure 6 reveals the large difference in cooperativity for the helix-to-coil transitions of these two duplexes. Specifically, the 8-mer duplex melts much more cooperatively than the hexameric duplex formed by the 9-mer sequence.

More quantitative insight into the detailed nature of the transition can be gleaned by using the thermodynamic data to calculate the fraction of the helix that melts in a cooperative manner. By dividing each of the van't Hoff enthalpies by the corresponding calorimetric enthalpies, a measure of the size of the cooperative unit is obtained. For the 8-mer duplex we conclude that 100% of the structure melts cooperatively since $\Delta H_{cal} \sim \Delta H_{V.H.}$. However, for the 9-mer duplex only about 60% of the structure melts in a cooperative manner. We suggest that the cooperative component of the transition for the 9-mer corresponds to the central, hexameric duplex core. Thus, we assign 35 kcal ($\Delta H_{V.H.}$) to the disruption of the central core duplex with the balance of 25 kcal resulting from stacking effects associated with the single-stranded ends. Significantly, the 35 kcal is approximately what we would predict for the transition enthalpy of an isolated d(GAATTC)

hexameric duplex, based upon nearest-neighbor interactions.

The NMR data suggest that one of the effects associated with the single-stranded ends is end-to-end aggregation at low temperature. If this effect prevails under the conditions of the calorimetric experiment, then part of the noncooperative 25 kcal would result from disruption of the aggregates. However, the calorimetric transitions begin at temperatures above which aggregates are believed to exist. Furthermore, if significant intermolecular association were influencing our thermodynamic data, then, contrary to fact, we would expect to observe that $\Delta H_{V.H.} > \Delta H_{cal}$. For these reasons we believe that our calorimetric data are not influenced by aggregate formation and that the interpretation presented above is valid.

Acknowledgments

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