Evidence from CD spectra and melting temperatures for stable Hoogsteen-paired oligomer duplexes derived from DNA and hybrid triplexes

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ABSTRACT

The pyr*pur-pyr type of nucleic acid triplex has a purine strand that is Hoogsteen-paired with a parallel pyrimidine strand (pyr*pur pair) and that is Watson–Crick-paired with an antiparallel pyrimidine strand (pur·pyr pair). In most cases, the Watson–Crick pair is more stable than the Hoogsteen pair, although stable formation of DNA Hoogsteen-paired duplexes has been reported. Using oligomer triplexes of repeating d(AG)6 or d(CT)6 or r(CU)12 sequences that were 24 nt long, we found that hybrid RNA*DNA as well as DNA*DNA Hoogsteen-paired strands of triplexes can be more stable than the Watson–Crick-paired strands at low pH. The structures and relative stabilities of these duplexes and triplexes were evaluated by circular dichroism (CD) spectroscopy and UV absorption melting studies of triplexes as a function of pH. The CD contributions of Hoogsteen-paired RNA*DNA and DNA*DNA duplexes were found to dominate the CD spectra of the corresponding pyr*pur-pyr triplexes.

INTRODUCTION

The two major nucleic acid triple-helix motifs with Hoogsteen or reverse Hoogsteen pairing of a third strand to a DNA duplex and the numerous ways in which a third strand might inhibit transcription or mediate mutagenesis and recombination in antigenic drug therapies have been reviewed (1–3). There is evidence that transcription or mediate mutagenesis and recombination in antigene and the numerous ways in which a third strand might inhibit transcription or reverse Hoogsteen pairing of a third strand to a DNA duplex could potentially be used to control biochemical processes that encompass mRNA, RNA-DNA hybrids or RNA hairpins. In the pyr*pur-pyr motif, the subject of this paper, the strands are restricted to being all homopurine or homopyrimidine. As defined by others (13–16), '*' and '·' respectively denote pairing between parallel Hoogsteen-paired and antiparallel Watson–Crick-paired strands. The pyr*pur-pyr motif contains protonated C·G·C base triples, diagrammed at the top of Figure 1, with which T/U*A·T/U base triples are isostructural. The requirement for protonation of Hoogsteen-paired C+ bases leads to enhanced triplex stability at low pH (17,18).

There are eight possible combinations of DNA and RNA strands in a triplex. Roberts and Crothers (19) explored the effects of backbone composition on pyr*pur-pyr triplex stability using four Watson–Crick-paired hairpin duplexes (D–D, D–R, R–D and R–R) having 12 bp, including eight G·C pairs, in the stems. These were mixed with homopyrimidine DNA (D) or RNA (R) 12mers of the proper sequences to form Hoogsteen pairs. At pH 5.5, the D·R·D and D·R·R triplexes did not form. In general, a triplex was formed with a Hoogsteen DNA pyrimidine strand only when it could pair with a DNA purine strand. Melting was by Scheme I or II of Figure 1. Four triplexes (D·R·D, D·R·R, R·R·D and R·R·R) melted with two transitions, with the lower Tm values corresponding to the separation of the third Hoogsteen-paired strand. Only one, or two very close, transitions were found for the R·D·R and R·D·R triplexes, indicating that the Hoogsteen-paired strand was stable until the hairpin duplex melted. Noronha and Damha (20,21) confirmed and extended the Roberts and Crothers (19) results with the same hairpin duplex sequences. Other workers used relatively long Watson–Crick duplexes to make partial triplexes with short Hoogsteen-paired third strands (22–24). Again it was found that the D·R·D and D·R·R triplexes did not form, R·D·D and R·D·R were among the most stable triplexes and the shorter Hoogsteen-paired oligomers always melted at a lower temperature than the Watson–Crick duplexes.

Many studies of triplexes, including NMR and thermodynamic studies, have made use of constructs in which two or all three strands are covalently linked (13,25–37). However, the relative stability of the Hoogsteen-paired strand is more certain when mixtures are made of separated oligomers. Liu et al. (38) recognized that unlinked strands can form a Hoogsteen-paired DNA duplex at pH 5.1. The strand sequences were constructed so that an anti-parallel Watson–Crick alignment would not be stable. One interesting finding was that CD and IR bands were similar for the separate Hoogsteen-paired duplex and the corresponding triplex. The authors explained the similarities as resulting from the coincident helix axes and isostructural nature of the Hoogsteen-paired strands in the two cases, which was later confirmed by crystal structures of two DNA triplexes (39). Whether Watson–Crick or Hoogsteen duplexes are more stable in mixtures of separate strands depends on the sequences and conditions involved (15,18,40). Lavelle and Fresco (18) inferred from optical and thermodynamic studies that the two melting transitions of a d(C·T)6·d(AG)6·d(CT)6 triplex formed from separate strands at pH 4.2 represented (i) a dissociation at lower temperatures of the.

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Watson–Crick pair; (ii) the dissociation of a more stable Hoogsteen-paired duplex at higher temperatures. That is, dissociation occurred by Scheme III of Figure 1. At higher pH values, the Hoogsteen-paired strand was less stable and dissociated first. Our work adds new information that D*D·D and R*D·R triplexes formed from separate d(AG)12 and d(CT)12 or r(CU)12 strands melt with two transitions at low pH. For these cases, where the same pyrimidine sequences can pair by either Watson–Crick or Hoogsteen pairing, our data support the conclusion by Lavelle and Fresco (18) that Hoogsteen-paired strands may be more stable than Watson–Crick-paired strands at low pH. (In the present paper, sequences for all three strands will be written 5'→3'.)

MATERIALS AND METHODS

Oligonucleotides

The three oligomers, d(AG)12, d(CT)12 and r(CU)12, used in this study were purchased from Oligos Etc, Inc. (Wilsonville, OR). The oligomers were single bands by gel electrophoresis prepared by the manufacturer. To inhibit RNase degradation, the oligomers were dissolved in water treated with diethyl pyrocarbonate (41) and were stored in 200 µl aliquots at −20°C. For a mixing experiment, an aliquot of 200 µl was thawed and diluted into 2 mM NaCl (phosphate buffer, pH 7.0). Concentrations were determined from absorbance measurements and extinction coefficients at 260 nm (ε260). The ε260 values for d(CT)12 and r(CU)12 at 20°C were calculated as described by Gray et al. (42). For d(AG)12, which possessed intrastrand structure at 20°C, the oligomer concentration was determined from equimolar nucleotide concentrations of ~6 × 10−5 M (~2.5 µM in strands) in 0.05 M NaCl (phosphate buffer, pH 7.0). For pH values of 8, 6.5, 6, 5.6 and 5, the pH was adjusted by adding small aliquots of 1 M NaOH or 1 M HCl before the strands were mixed. The pH was measured to ±0.03 with a Beckman meter and AgCl electrode.

Mixtures

Complementary oligomers [d(CT)12 and d(AG)12 or r(CU)12 and d(AG)12] were mixed at defined pyrimidine:purine molar ratios of 100:0, 80:20, 67:33, 60:40, 50:50, 40:60, 33:67, 20:80 and 0:100. Mixtures at pH 8 and 7 were allowed to equilibrate at room temperature for 2 h or at 4°C for 24 h, with equivalent results. Acidic mixtures of d(CT)12 and d(AG)12 or of r(CU)12 and d(AG)12 were momentarily heated at 90°C for 120 or 90 s, respectively, to melt any intrastrand structures and then were allowed to equilibrate at room temperature for 48 h.

Instrumentation

Absorbance and CD measurements were performed as previously described (41).

Melting profiles

The OD260 was monitored as the temperature was increased from 20°C in increments of 1°C. Samples were incubated for 3 min at each 1°C interval to allow for equilibration before taking OD readings. Tm values were obtained from the first derivatives of the melting profiles as in previous work (41). Temperatures were reproducible to ±0.1°C and were calibrated to be accurate to ±0.5°C. Samples were tightly capped with teflon stoppers and were weighed before and after each melting profile to determine the amount of evaporation, which was usually <1%. A sample of poly[d(AT)] was routinely included as a control and had Tm values that were reproducible to ±0.2°C. Spectral values at increased temperatures were all corrected for volume expansion.

Melting and annealing

In one type of annealing experiment, absorbance melting profiles for the 2:1 mixtures of [d(CT)12]:d(AG)12 and [r(CU)12]:d(AG)12 at pH 5.6 were obtained as described above and were followed by annealing profiles using the same protocol. A second type of experiment involved taking CD and absorption spectra at 11–14°C fixed, increasing temperature intervals of 5 or 10°C from 20 to 90 or 95°C for the 2:1 (pyr:pur) mixtures of [d(CT)12]:d(AG)12 and [r(CU)12]:d(AG)12 at pH 5.6 and 5. Samples were incubated for 7 min after reaching each set temperature before beginning a spectral scan. After reaching the highest temperature, samples were annealed by decreasing the temperature to the same fixed intervals in a reverse order and CD and absorption spectra were again acquired.

Figure 1. Three paths for the dissociation of a pyr*pur*pyr triple helix. The C*G·C base triple at the top of the figure is isostructural with T/U*A·T/U base triples. The strands can be of different allowed combinations of DNA and RNA. The strands containing Hoogsteen C*G pairs are parallel and the strands containing Watson–Crick G·C pairs are antiparallel. Scheme I: strands with Hoogsteen pairs are less stable and dissociate at lower temperatures than the strands with Watson–Crick pairs. Scheme II: the three strands simultaneously dissociate. Scheme III: the strands with Hoogsteen pairs are more stable and dissociate at higher temperatures than the strands with Watson–Crick pairs.

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Gel electrophoresis

To determine the extent of acid depurination of d(AG)₁₂ under the above melting/annealing protocols, d(AG)₁₂ was 5'-end-labeled with ³²P in a reaction with T4 polynucleotide kinase. Samples of d(AG)₁₂ in 0.05 M Na⁺ (phosphate), pH 5.6 or 5, were subjected to pertinent heating/cooling protocols in a PCR thermal cycler and electrophoresed in non-denaturing gels at pH 8.3 (see below). Results were digitized using a Molecular Dynamics PhosphorImager and ImageQuant 3.3 software.

To investigate the possible existence of unexpected base paired structures, mixtures of d(AG)₁₂ (labeled) plus d(CT)₁₂ or r(CU)₁₂ were electrophoresed in 15% polyacrylamide/bisacryl-amide (19:1) non-denaturing gels with buffers of either 0.09 M Tris–boric acid, 0.02 M EDTA, pH 8.3, or 0.2 M Na₂HPO₄–citric acid, pH 5.5, at 300 V for 7.5 h, following the work of Liu et al. (38). The mixtures were made as described above for spectral measurements, in 0.05 M Na⁺ (phosphate), pH 8 or 5. Results were visualized by autoradiography.

RESULTS

Duplex and triplex formation

Mixtures of the d(CT)₁₂ plus d(AG)₁₂ strands and the r(CU)₁₂ plus d(AG)₁₂ strands yielded either duplexes or triplexes (at 0.05 M Na⁺), depending on the pH. The stoichiometry of the reaction was evaluated using CD and OD mixing curves. Examples of CD mixing curves obtained at the pH extremes of 5 and 8 are shown in Figure 2. Mixing curves at a single wavelength may not reveal all of the stoichiometric states that are present in a mixture. However, full CD spectra were obtained for seven mixtures plus the single strands for each set of oligomers and each pH. At the pH extremes the spectra fell into two spectral groupings, above and below 50% pyrimidine strand (at pH 7 or 8) or above and below 67% pyrimidine strand (at pH 5 or 5.6). Each group or family of spectra consisted of the spectrum of a free strand, the spectrum of a stoichiometric mixture and combinations of the two. That is, at the pH extremes there was only one obvious breakpoint and there was no evidence for the presence of more than one complex, the duplex or the triplex. Illustrative families of such CD spectra are included as Supplementary Material Figures S1 and S2.

According to the spectral data, the duplex was formed in mixtures of d(CT)₁₂ plus d(AG)₁₂ at pH 8 and 7, while the triplex was formed at pH 5.6 and 5. At the intermediate pH values of 6 and 6.5, CD spectra provided evidence that both the duplex and triplex could form in mixtures of d(CT)₁₂ plus d(AG)₁₂ (not shown). For r(CU)₁₂ plus d(AG)₁₂, the duplex was formed at pH 8 and 7, both the duplex and triplex were present at pH 6.5, while only the triplex was formed at pH 6, 5.6 and 5.

As an alternative test as to whether only the triplex form was present at lower pH in 1:1 as well as 2:1 (pyr:pur) mixtures, we compared the measured spectra of 1:1 mixtures at pH 5 with spectra calculated as 3/4 of the spectrum of the single-stranded d(AG)₁₂ at both pH 5 and 5.6. These comparisons are included as Supplementary Material Figure S3A and C. For both sets of complementary strands, the measured spectra of 1:1 mixtures at pH 5 were intermediate between the calculated spectra at pH 5 and 5.6 and implied that these samples contained mixtures of triplex plus free purine strand. Reciprocal calculations were done in which the measured spectra of 2:1 mixtures at pH 8 were compared with spectra calculated as 2/3 of the duplex spectrum (spectrum of the 1:1 mixture) plus 1/3 of the spectrum of single-stranded d(AG)₁₂ at the same pH (shown in Fig. S3B and D). These latter calculations showed that the spectra of 2:1 mixtures at pH 8 were consistent with the presence of only duplex plus free pyrimidine strand.

The CD spectra measured for the d(AG)₁₂·d(CT)₁₂ and d(AG)₁₂·r(CU)₁₂ duplexes and the d(C·T)₆*d(AG)₁₂·d(CT)₁₂ and r(C·U)₆*d(AG)₁₂·r(CU)₁₂ triplexes are shown in Figure 3. By comparing the measured spectra with the weighted averages of the spectra of the single strands, one can see the CD changes that were induced by base pairing. Generally, the CD band at 210–220 nm became less positive upon forming base pairs and became quite negative with triplex formation. A negative band at 210–220 nm is characteristic of the A conformation, as suggested in other studies of hybrids and triplexes (41–43).

Gel electrophoresis

Gel electrophoresis experiments were performed to (i) assay for the loss of intact d(AG)₁₂ that might take place due to acid depurination during a melting experiment; (ii) verify the structures present in stoichiometric mixtures. To assay for acid depurination, end-labeled d(AG)₁₂, before and after heat treatment at pH 5 or 5.6, was electrophoresed at pH 8.3 in non-denaturing gels (Materials and Methods). Lane A4 of Figure 4 shows an example of a 1:1 mixture at pH 8.3 that has undergone acid treatment.
of a sample of d(AG)₁₂ at pH 5.6 treated to approximate heating during a melting profile. Ninety-five percent of the material (±1%, range of duplicate samples), relative to the control (lane A1), remained in the full-length band. Lanes A2 and A3 show samples of d(AG)₁₂ at pH 5 and 5.6 that were subjected to a melting/annealing procedure to approximate the treatment of samples during the acquisition of multiple CD and absorption spectra. Under these conditions, the remaining full-length d(AG)₁₂ was reduced to 65 ± 2 and 82 ± 2%, respectively.

One-to-one mixtures of labeled d(AG)₁₂ and either d(CT)₁₂ or r(CU)₁₂, made at pH 8 with the same protocol as mixtures for the spectral measurements, electrophoresed at pH 8.3 essentially as single bands (lanes B2 and B3, respectively). These bands were of lower mobility than the free d(AG)₁₂ (lane B1). The results were in agreement with spectral data that only duplexes d(AG)₁₂·d(CT)₁₂ and d(AG)₁₂·r(CU)₁₂ were present in 1:1 mixtures at high pH.

One-to-one mixtures containing the Watson–Crick duplexes made at pH 8 also electrophoresed as single bands in gels at pH 5.5 (lanes C2 and C5) at lower mobility than d(AG)₁₂ alone (lane C1), in agreement with results by Liu et al. (38). When the pH during mixing with d(CT)₁₂ or r(CU)₁₂ was reduced to 5 and the pyr:pur ratio was increased to 2:1, all or part of the d(AG)₁₂ electrophoresed in one or two closely spaced bands of lower mobility (lanes C3 and C6). These bands of lower mobility are most likely dominated by d(C'T)₁₂·d(AG)₁₂·d(CT)₁₂ and r(C'U)₁₂·d(AG)₁₂·r(CU)₁₂ triplexes, with possibly some fraction of the Hoogsteen duplexes. Both the triplex and Hoogsteen duplex have lower mobility than the duplex due to the presence of protonated C⁺ (38). The lower stability of the DNA triplex relative to the hybrid triplex resulted in its partial dissociation to give some Watson–Crick duplex under the electrophoresis conditions. (It is reasonable to expect that a Watson–Crick DNA duplex would be more stable than a Hoogsteen duplex at the 0.4 M Na⁺ concentration in the gel; 38.) However, if the pyr:pur ratio was further increased to 3:1 to overcome any
dissociation, all the label appeared at lower mobility (lanes C4 and C7). We conclude that the distinct structures that could be separated by gel electrophoresis were consistent with their being duplexes and triplexes and there was no evidence for unexpected base paired structures, such as concatemers, that these repetitive sequences might have formed.

Absorbance melting profiles

Figure 5 shows representative melting profiles for mixtures of d(CT)12 plus d(AG)12 (Fig. 5A) and of r(CU)12 plus d(AG)12 (Fig. 5B) at the various pH values. Melting temperatures are summarized in Table 1.

The 1:1 (pyr:pur) mixtures of d(CT)12 plus d(AG)12 at the highest pH values melted with one transition at 54.5–55.2°C that represented dissociation of the duplex. The 2:1 mixture at pH 6.5 also dissociated with a T_m characteristic of the duplex. At pH 6, there was some evidence for the influence of a triplex component in the 2:1 mixture because the T_m of this sample was higher (58.6°C) than that of the duplex. At pH 5.6 and 5, the 2:1 mixtures of d(CT)12 plus d(AG)12 melted with two clear transitions, both of which were cooperative. The first transition was close to that of the duplex, while the second transition was at a much higher temperature, which increased from 72 to 78°C as the pH decreased from 5.6 to 5.

An even more dramatic effect of pH was evident on the melting temperatures of mixtures of the RNA, r(CU)12, and the DNA, d(AG)12. At pH 8 and 7, the 1:1 mixtures containing duplexes melted with one transition at 45.5–45.7°C (Fig. 5B and Table 1). The melting profiles of the 2:1 mixtures at acidic pH values exhibited two cooperative transitions and the T_m values of both transitions increased as the pH decreased. In this case, the stabilities of the Watson–Crick- and Hoogsteen-paired duplexes were both apparently increased by cytosine protonation.

In control melting profiles of the isolated single strands at pH 5, the r(CU)12 and d(CT)12 acid self-structures melted below 30°C and d(AG)12 showed only a non-cooperative increase in absorbance (not shown).

### Table 1. Melting temperatures for 1:1 and 2:1 mixtures of d(CT)12 plus d(AG)12 and of r(CU)12 plus d(AG)12 in 0.05 mM Na+ (phosphate) at various pH values

<table>
<thead>
<tr>
<th>Strands mixed</th>
<th>pH</th>
<th>Ratio (pyr:pur)</th>
<th>1st T_m (°C)</th>
<th>2nd T_m (°C)</th>
<th>Full-length d(AG)12 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>d(CT)12 plus d(AG)12</td>
<td>8</td>
<td>1:1</td>
<td>55.2 ± 0.3a</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>1:1</td>
<td>54.5 ± 0.6</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>2:1</td>
<td>54.1 ± 0.4</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>2:1</td>
<td>58.6 ± 1.3</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.6</td>
<td>2:1</td>
<td>55.4 ± 0.5</td>
<td>71.8 ± 0.8</td>
<td>95 ± 1</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2:1</td>
<td>56.9 ± 0.9</td>
<td>77.6 ± 0.6</td>
<td>84 ± 3</td>
</tr>
<tr>
<td>r(CU)12 plus d(AG)12</td>
<td>8</td>
<td>1:1</td>
<td>45.7 ± 0.4</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>1:1</td>
<td>45.5 ± 0.5</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>2:1</td>
<td>46.9 ± 0.8</td>
<td>62.4 ± 0.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
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<td>52.5 ± 1.3</td>
<td>66.1 ± 0.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.6</td>
<td>2:1</td>
<td>57.2 ± 1.7</td>
<td>75.2 ± 1.8</td>
<td>95 ± 1</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2:1</td>
<td>62.8 ± 0.7</td>
<td>80.8 ± 0.7</td>
<td>84 ± 3</td>
</tr>
</tbody>
</table>

a Determined as the end-labeled material remaining in a single band, relative to untreated d(AG)12, after heating to 90°C at the same pH and at the same rate as used for the melting profile (see Materials and Methods and lane A4 in Fig. 4). Errors are ranges from duplicate measurements.

b T_m values and errors are the averages and standard deviations from at least six independent melting profiles.

Figure 5. (A) Example melting profiles of d(CT)12 plus d(AG)12 mixtures at different pH values. One-to-one mixtures at pH 8 (—) and pH 7 (—–); 2:1 mixtures at pH 6.5 (—––), pH 6 (circle), pH 5.6 (open triangle) and pH 5 (filled triangle). (B) Example melting profiles of r(CU)12 plus d(AG)12 mixtures at different pH values. One-to-one mixtures at pH 8 (—) and pH 7 (—–); 2:1 mixtures at pH 6.5 (—––), pH 6 (circle), pH 5.6 (open triangle) and pH 5 (filled triangle). Absorption values were at 260 nm, with each profile scaled to 1.0 by dividing the OD_{260} at each temperature by the starting OD_{260} value. Data for Figures 5 and 6 were obtained with total strand concentrations of ~2.5 µM and at a [Na+] of 0.05 M.

In summary, the 2:1 mixtures of [d(CT)12]2:d(AG)12 and [r(CU)12]2:d(AG)12 at pH 5.6 and 5 melted with two cooperative
transitions, the second of which was at an unusually high temperature and was pH-dependent. Depurination during heating was minimal (Table I), there were no unexpected structures in the mixtures and strands separated in the first melting transition did not reform stable structures. Finally, the transitions were characteristically different for the DNA and hybrid triplexes. In agreement with conclusions drawn by Lavelle and Fresco (18) from their study of the shorter triplex d(C+T)6·d(AG)6·d(CT)6 and with results by Liu et al. (38) on a Hoogsteen-paired DNA duplex, our explanation is that at low pH (at 0.05 M Na+) the higher \( T_m \) values for the DNA and hybrid 2:1 mixtures were from melting of the Hoogsteen duplexes and that the triplexes melted by Scheme III of Figure 1. The pH dependence of the second transition is in accord with the involvement of a protonated cytosine in a Hoogsteen C+·G base pair. For the shorter 12mer triplex sequence studied by Lavelle and Fresco (18), the increase in \( T_m \) of the second transition occurred between pH 5 and 4.2, while with our 24mers the \( T_m \) of the second transition progressively increased as the pH dropped below 6.5. The influence of pH on the first transition (of the Watson–Crick duplex) indicated that the third Hoogsteen-paired strand also stabilized the Watson–Crick base pair, especially in the case of the hybrid triplex. Although at pH 7 or 8 the hybrid duplex had a lower \( T_m \) than did the DNA duplex, at low pH both transitions of the hybrid triplex were actually increased to be at higher temperatures than those of the DNA triplex. That is, at low pH both types of base pairing were relatively more stable in the A conformation of the hybrid triplex (compared with those of the DNA triplex).

**CD spectral changes during melting**

CD spectra generally provide a particularly sensitive measure of nucleic acid secondary structural changes and were used to help characterize the melting transitions for the two triplexes at pH 5.6 and 5. As described in Materials and Methods, CD and absorbance spectra were taken at 11–14 discrete intervals as the sample temperature was increased from 20 to 90 or 95°C and again at the same intervals as the temperature was decreased back to 20°C. Spectra at each temperature were averaged from three separate experiments for each triplex. These CD and absorption spectra at representative temperatures for the triplexes at pH 5 are shown as Supplementary Material (Figs S4 and S5). Absorption spectra of the same samples showed two melting transitions consistent with the melting data of Table I. Two stages of melting were also seen in the CD spectra, especially in the decrease of the negative bands at 210–220 nm. The CD melting profiles at 216 nm [for the d(C+T)12·d(AG)12·d(CT)12 triplex] or at 208 nm [for the r(C+U)12·r(AG)12·r(CU)12 triplex] at pH 5.6 and 5 are shown in Figure 6.

The CD spectral changes during the melting transitions were analyzed by singular value decomposition (SVD) of the sets of 11–14 spectra taken at increasing temperatures and again at decreasing temperatures. SVD is an unbiased means of deriving the minimum number of orthonormal basis vector spectral components that are needed to account for the temperature dependence of the CD spectra (41,44,45). We expected that there might be more than two significant basis vector spectral components, especially during melting, because there were two transitions that involved at least three states (triplex, one-separated strand plus two base paired strands and three separated strands). This was clearly not the case, however. Only two significant basis vectors were needed to describe the melting transitions of either triplex at either pH. Likewise, only two significant basis vectors were needed to describe the annealing transitions (discussed in the next section). The third basis vectors were essentially at the baseline level. The basis vectors derived from the CD spectra during the melting transitions at pH 5 are shown in Figure 7A and C. The basis vectors from the CD spectra at pH 5.6 were essentially identical to those obtained from CD data at pH 5. Depurination during melting should have been no greater than indicated in Table I. Note that the major basis vectors in Figure 7A and C are similar in shape to the actual triplex spectra in Figure 3B and D (solid lines). It was the loss of this spectral shape that characterized both melting transitions for a given triplex.

Our CD experiments confirmed and amplified the results of Liu et al. (38), who found that a Hoogsteen-paired parallel-stranded duplex had a CD spectrum similar in shape to that of the corresponding pyr·pur·pyr triplex sequence. Their sequence was a 20mer containing 50% G·C+ pairs and a majority of nearest neighbors (12 of 19) that were alternating C-T. We interpret our data as showing that the loss of a Watson–Crick strand from the
The essence of our results is that the second transition was not reversible at either pH 5.6 or 5 and the Hoogsteen pairs did not appear to form until after the temperature dropped below that of the Watson–Crick pair, suggesting that the pairing of the third strand depended on the presence of the Watson–Crick duplex. In one set of experiments, annealing temperatures were investigated for the triplexes at pH 5.6 while monitoring the absorbance at identical melting and annealing rates. Values that are averages of three separate experiments on different samples are given in Table 2. During annealing there was no hypochromicity until a temperature was reached that corresponded to the first \( T_{m} \), which we ascribe to be that of the duplex. 

### Table 2. Melting and annealing temperatures for 2:1 mixtures of d(CT)\(_{12}\) plus d(AG)\(_{12}\) and of r(CU)\(_{12}\) plus d(AG)\(_{12}\) in 0.05 mM Na\(^+\) (phosphate) at pH 5.6

<table>
<thead>
<tr>
<th>Strands mixed</th>
<th>1st ( T_{m} (\degree C) )</th>
<th>2nd ( T_{m} (\degree C) )</th>
<th>Annealing temperature (\degree C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>d(CT)(<em>{12}) plus d(AG)(</em>{12})</td>
<td>55.4 ± 0.1</td>
<td>71.8 ± 1.2</td>
<td>52.0 ± 0.6</td>
</tr>
<tr>
<td>r(CU)(<em>{12}) plus d(AG)(</em>{12})</td>
<td>57.7 ± 0.5</td>
<td>74.6 ± 0.5</td>
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</tr>
</tbody>
</table>

\( T_{m} \) values and errors are the averages and standard deviations from three to four separate experiments. These data were not included in Table 1.

CD annealing profiles shown in Figure 6 were in agreement with the absorbance annealing profiles and melting data of Table 2 in that there was no change until the temperature dropped below ~60\(^\circ\)C and then the annealing temperature was at or below the first melting temperature. Moreover, the major CD basis vectors were similar for the annealing as for the melting transitions, as may be seen by comparing the basis vectors in the top and bottom panels of Figure 7. This was true for basis vectors derived from data acquired at both pH 5 and 5.6.

The full-length d(AG)\(_{12}\) that remained after equivalent heating plus cooling protocols was estimated to be 65 ± 2\% at pH 5 and 82 ± 2\% at pH 5.6. Given the reversibility of the CD spectral shapes (Fig. 6 and Supplementary Material Figs S4 and S5) and that of the Watson–Crick pair, suggesting that the pairing of the third strand depended on the presence of the Watson–Crick duplex. In one set of experiments, annealing temperatures were investigated for the triplexes at pH 5.6 while monitoring the absorbance at identical melting and annealing rates. Values that are averages of three separate experiments on different samples are given in Table 2. During annealing there was no hypochromicity until a temperature was reached that corresponded to the first \( T_{m} \), which we ascribe to be that of the duplex. 

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and C + *G·C base triples are more stable than the Watson–Crick and R*D·R triplexes indicated significant association of the third strand during annealing. The apparent annealing temperature, which was at or below the $T_m$ of the Watson–Crick duplex and below the $T_m$ of the Hoogsteen duplex, probably consisted of the simultaneous pairing of both pyrimidine strands to form a triplex. There was no CD evidence for the existence of an isolated Watson–Crick duplex during either the melting or annealing transitions of either triplex and there was no evidence for formation from separate strands of the Hoogsteen-paired duplex alone at a temperature above that at which the Watson–Crick duplex reformed, on the time scale of these experiments.

**DISCUSSION**

Hoogsteen-paired hybrid and DNA duplexes can be stable at low pH

CD mixing spectra and gel electrophoresis demonstrated that mixtures of 24mers having simple, repeating AG and CT or CU sequences contain discrete duplexes at pH 7 and 8 and triplexes at pH 5 and 5.6. From melting profiles as a function of pH, we conclude that the Hoogsteen-paired strands of D*D·D and R*D·R triplexes containing alternating T*A·T or U*A·U are more stable than the Watson–Crick-paired strands, so that melting at low pH (0.05 M Na+) is by Scheme III of Figure 1. Annealing appears to involve coordinate pairing of all three strands by the reverse of Scheme II. Melting profiles at low pH were accompanied by relatively minor loss of the full-length d(AG)$_{12}$ strand, presumably from depurination. The loss was greater at pH 5 than at 5.6 and was increased for samples held at higher temperatures for longer times. However, loss of intact d(AG)$_{12}$ during combined melting and annealing experiments appeared to only affect the extent of annealing under our conditions.

The third strand of the triplexes with identical 24mer repeating pyrimidine strands has to have an overhang of at least one base when binding is by Hoogsteen pairing. Under acidic conditions the arrangement with the maximal number of stable C*G·C triples in the D*D·D triplex would be:

- 5' C (TC)$_{11}$ T 3'
- 5' AG (AG)$_{11}$ 3'
- 3' TC (TC)$_{11}$ 5',

where the C residues in the top strand are protonated and are Hoogsteen-paired with the purine strand. An analogous arrangement would hold for the R*D·R triplex. For the shorter 12mer repeating DNA sequences studied by Lavelle and Fresco (18), the number of protons released upon triplex disruption was consistent with an equilibrium between this arrangement with a 3' overhang and the alternative register with a 5' overhang of the Hoogsteen-paired strand. We assume that differences due to the two possible registers of the third strand, either of which has 23 of 24 bases paired, were not detectable in our melting profiles and CD spectra.

Others have recognized that a Hoogsteen-paired DNA duplex can be stable and can increase the $T_m$ of a Watson–Crick-paired strand (18,27,35). An increase in the stability of the Watson–Crick duplex when bound to a reverse-Hoogsteen-paired strand in a pur-motif DNA triplex has also been observed (48). Dagneaux et al. (49), in an FTIR study of R*D·D versus D*D·D pyr motif triplexes formed from separate 13mer strands in which the RNA contained methylated 2'-O-riboses, found that the binding of the methylated RNA third strand increased the $T_m$ of the duplex (at pH 5, 0.1 M NaCl) so that all three strands melted simultaneously. To our knowledge, it has not been recognized that the use of unmodified RNA as the Hoogsteen-paired third strand can stabilize a Watson–Crick D-R hybrid pair (Fig. 5 and Tables 1 and 2). The first (duplex) $T_m$ increases 17.1°C as the pH is decreased from pH 8 to 5 in the case of the R*D·R triplex, compared with an increase of only 1.7°C in the case of the D*D·D triplex (Tables 1 and 2). This suggests the possibility of using an R*D parallel-stranded duplex to form Watson–Crick pairs with a target site RNA. The use of an RNA as the Hoogsteen-paired third strand of a pyr*pur-pyr triplex would have an additional advantage over the use of a DNA third strand in that a competing C+C base paired i-motif is less favored for C-containing RNA at low pH (14).

**CD spectra of triplexes are dominated by Hoogsteen pairing**

We showed that only two major CD basis vector spectral components are needed to characterize the melting transitions between three states (triplex, Hoogsteen duplex and single strands) of D*D·D and R*D·R triplexes. The major spectral components are, in fact, very similar (in shape) to the spectra of the respective triplexes. (Compare the first basis vectors in Fig. 7A and C with solid lines in Fig. 3B and D, respectively.) These results are in agreement with the similarity of CD spectra of a triplex and the isolated Hoogsteen D*D duplex reported by Liu et al. (38). Given that the two pyrimidine strands of a triplex are related by dyad symmetry (39), a possible explanation is that the CD spectra of triplexes have a large component from intrastand base–base interactions in the two pyrimidine strands, which are qualitatively similar. Thus, dissociation of each pyrimidine strand could affect the CD spectrum in the same qualitative (but not necessarily quantitative) manner, as long as the Watson–Crick pair is the first dissociated so that the remaining pyrimidine base stacking interactions are relatively unaltered.

CD spectra have been an important means of characterizing nucleic acid structures, including triplexes, as a function of solution conditions or nucleotide modifications (18,19,21,27,32,38,42,50–53). CD spectra are generally very sensitive to slight differences in nucleic acid structures. Our data add a caution that CD spectra of different structures may be qualitatively similar if the stacking interactions in the constituent nucleic acid strands of the structures being compared are similar.

**SUPPLEMENTARY MATERIAL**

Five figures show CD spectra of mixtures of d(CT)$_{12}$ plus d(AG)$_{12}$ (Fig. S1), CD spectra of mixtures of r(CU)$_{12}$ plus d(AG)$_{12}$ (Fig. S2), CD spectra of 1:1 and 2:1 mixtures compared with calculated spectra (Fig. S3), CD and absorption spectra during melting and annealing of the DNA triplex (Fig. S4) and CD and absorption spectra during melting and annealing of the hybrid triplex (Fig. S5).

See Supplementary Material available in NAR Online.
ACKNOWLEDGEMENTS

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REFERENCES