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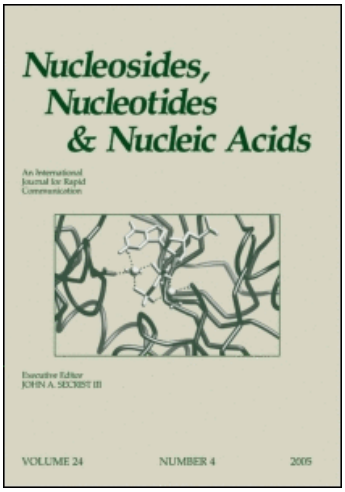

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Interaction of Oligodeoxyribonucleotides Through Formation of Chimeric Duplex/Triplex Complexes

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INTERACTION OF OLIGODEOXYRIBONUCLEOTIDES THROUGH FORMATION OF CHIMERIC DUPLEX/TRIPLEX COMPLEXES

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Abstract: An intramolecular duplex/triplex chimera is formed between d-CTTCCT-$(T)_4$-TCCTTCAGCACA and target d-GTTTGTGCTGAAGGACACAC. The high Tm and hypochromicity of the triplex part of the chimera may be due to additional interactions outside the underlined binding site of the 20-mer.

Oligodeoxyribonucleotides are capable of forming triple-stranded complexes. In addition to intermolecular triplex formation involving three oligonucleotides[1-15] or intramolecular triplex formation by a single oligonucleotide[16-22], triple-stranded complexes involving two oligonucleotides have also been characterized. For example, oligopyrimidines can form triplexes with the double helical stems of hairpin oligonucleotides[23-25]. Oligomer systems in which the target sequence consists of a homopurine tract and the binding oligomer contains two homopyrimdine sequences which interact with this tract *via* both Watson-Crick and Hoogsteen hydrogen-bonding schemes have also been studied. Bimolecular triplex formation of this type has been observed with a variety of binding oligonucleotides and oligonucleotide analogs. These binding oligomers include: circular DNAs[26-28]; stem-loop DNA oligonucleotides[29]; DNA oligonucleotides in which the homopyrimidine binding tracts are connected by a short oligonucleotide sequence[30,31] or by a non-nucleotide linker[32,33].

In the bimolecular systems described above, the binding site of the target oligomer is completely engaged in triplex formation with

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sequences of the binding oligomer. Here we describe complex formation between a binding and target oligodeoxyribonucleotide which involves formation of a duplex/triplex chimera. A sequence at the 3'-end of the binding oligomer is complementary to and forms a duplex with a target sequence within the target oligomer. This target sequence contains a shorter homopurine tract which, under appropriate conditions, serves as a site for formation of an unusually stable triplex with a short homopyrimidine sequence at the 5'-end of the binding oligomer.

MATERIALS AND METHODS

Oligodeoxyribonucleotides Oligodeoxyribonucleotides, whose sequences are shown in Figure 1A, were prepared using commercially available protected nucleoside 3'-(β-cyanoethyl-*N*,*N*-diisopropylphosphoramidites). The oligomers were assembled on commercially available controlled pore supports (CPG) using a Biosearch Model 8700 DNA synthesizer and standard phosphoramidite chemistry[34]. The oligonucleotide containing 8-oxo-2'-deoxyadenosine was prepared as previously described[35]. The 5'-terminal dimethoxytrityl group was removed by the synthesizer and the oligomers were then removed from the support and deprotected by treatment of the CPG with a solution containing concentrated ammonium hydroxide/pyridine (1:1 v/v) at 55°C for 6 hr. The deprotected oligomers were purified by reversed phase high performance liquid chromatography (HPLC) on a Rannin C-18 Microsorb column using a linear gradient of 2% to 15% or 2% to 20% acetonitrile in 50 mM sodium phosphate buffer, pH 5.8. The oligomers were desalted using a Waters SEP PAK reversed phase cartridge and were eluted from the cartridge with 50% aqueous acetonitrile. The purity of the oligomers was checked by analytical reversed phase HPLC and/or by polyacrylamide gel electrophoresis after phosphorylation using polynucleotide kinase and [γ-^{32}P]ATP. The extinction coefficients of the oligomers were determined by recording their UV absorption spectra prior to and after hydrolysis with snake venom phosphodiesterase[35].

Melting Experiments Complexes were formed by mixing equal volumes of 2.0 μM or 4.0 μM stock solutions of the target oligomer and the binding oligomer at room temperature. The final strand concentrations of the complexes in these experiments was either 1.0 μM or 2.0 μM. The solutions were then stored at 4°C overnight. The buffers contained 140 mM potassium chloride, 10 mM sodium chloride, 10 mM magnesium chloride, and 10 mM 2-(N-morpholino)ethane sulfonic acid (MES), pH 5.8; 10 M piperazine-N,N'-bis(2-ethane)sulfonic acid (PIPES), pH 6.2 or 6.9; or 10 mM 3-(N-morpholino)propane sulfonic acid (MOPS), pH 7.2 or 7.9. The pH of the buffers were determined at 22°C. The A_{260} versus temperature profiles were recorded on a Cary 3E UV/Vis spectrophotometer fitted with a 6-sample thermostatable cell block and a temperature controller. Up to five melting curves to be recorded simultaneously. In experiments in

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which the effect of pH on melting temperature was being investigated, the melting curves for a particular complex at the different pH values were run simultaneously. The oligonucleotide solutions were loaded into the sample cuvettes at 0°C. Both the sample and reference cuvettes were bathed in dry nitrogen to prevent moisture condensation at low temperatures. The samples were heated at a rate of 0.5°C/min over a temperature range of 0-70°C and the melting temperatures were determined from the midpoint of the transition curves. Identical melting curves and melting transitions were obtained for duplicate samples.

RESULTS AND DISCUSSION

The sequences of target oligonucleotides and binding oligonucleotides are shown in Figure 1a. The twelve nucleotides at the 3'-end of binding oligomer B-1 are complementary to nucleotides 4-15 of target oligomer T-1. Part of the target sequence of T-1 contains a homopurine tract, -GAAGGA-, which is a potential site for triplex formation with the d-CTTCCT- sequence at the 5'-end of B-1. The duplex and triplex forming sequences of B-1 are linked together by four thymidine nucleotides.

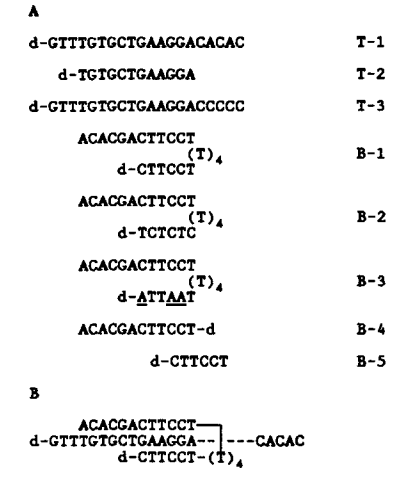

Figure 1 Binding and target oligonucleotides. A is 8-oxo-2'-deoxyadenosine.

The interaction between B-1 and T-1 was studied by UV melting experiments. The A_{260} *vs* temperature profiles for 1:1 mixtures of the two oligomers as a function of pH are shown in Figure 2. At pH 6.9 (curve #2), two distinct transitions are observed at 28°C and 51°C. The temperature at which the first transition occurs and the hypochromicity of this transition both decrease as the pH of the solution increases. The first transition essentially disappears at pH 7.7 (curve #4). Conversely, at lower pH, 6.2 (curve #1), the temperature of the first transition is shifted to 40°C and the hypochromicity increases. Changes in pH do not affect the temperature at which the second transition occurs.

These results are consistent with formation of the duplex/triplex chimeric structure shown schematically in Figure 1b. The first transition is assigned to melting of the triplex portion of the chimera. This assignment is based upon the changes in this transition as the pH of the solution is varied. The stability of the triplex portion of the

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chimera would be expected to be sensitive to pH because its formation involves C⁺•G•C triads in which the Hoogsteen hydrogen bonded cytosines are protonated. Such protonation is favored at low pH and leads to stabilization of triplexes which include C bases[36-38]. The second transition is assigned to melting of the duplex portion of the chimera. The melting temperature of the duplex would be expected to be independent of pH over the pH range studied.

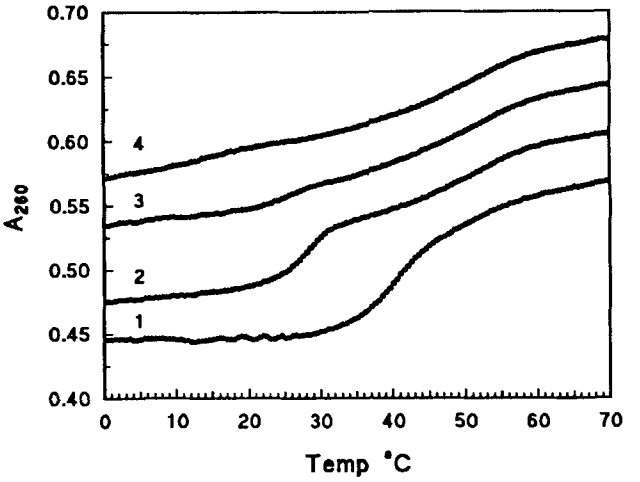

Figure 2 Melting profiles of B-1/T-1 at pH 6.2 (1), pH 6.9 (2), pH 7.2 (3) and pH 7.7 (4). The curves are offset for clarity and conditions are described in MATERIALS AND METHODS.

The assignment of the first melting transition to the triplex portion of the chimera was further confirmed by studying the interaction of target oligomer T-1 with binding oligomer B-2. The sequence at the 5'-end of B-2 is d-TCTCTC-, which contains the same number of C's and T's as found at the 5'-end of B-1, but in an order which is not compatible with triplex formation. As shown in Figure 3, a single transition at Tm 51°C is seen for a 1:1 mixture of T-1 and B-2 at pH 6.9 which corresponds to melting of the duplex formed between these two oligomers. When the melting curves of T-1/B-2 and T-1/B-1 are superimposed, the curves in the temperature region in which the duplex melts are identical. A sigmoidal transition is not seen in the temperature range 0 to 35°C for T-1/B-2, because the 5'-

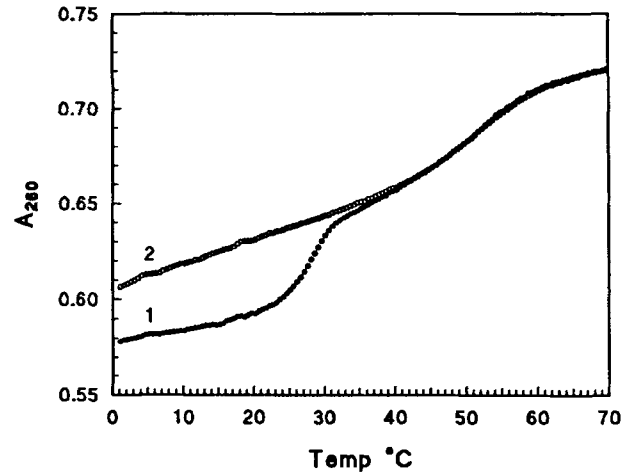

Figure 3 Superimposed melting curves of B-1/T-1 (1) and B-2/T-1 (2)at pH 6.9. The conditions are described in MATERIALS AND METHODS.

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Table 1. Melting temperatures of the triplex portions of duplex/triplex chimeras[a].

Oligomers	Tm°C pH 6.9	pH 7.9
B-1/T-1	28	<0
B-3/T-1	28	28

(a) Melts were carried out in a buffer containing 140 mM potassium chloride, 10 mM magnesium chloride and 10 mM PIPES (pH 6.9) or MOPS (pH 7.9) at an oligomer concentration of 2 μM per strand.

homopyrimidine sequence of the binding oligomer cannot participate in triplex formation. Instead, the A_{260} *vs* temperature profile in this region of the melting curve appears to be similar to that observed for T-1/B-1 at pH 7.7 (see Figure 2, curve 4).

This assignment is further supported by melting experiments with a binding oligomer which contains 8-oxo-2'-deoxyadenosine, A. Recent studies from several laboratories have shown that A can form base triads with G•C base pairs of duplex oligo DNA[35, 39-41]. The 8-oxo-adenine base of this nucleoside is in the *syn* conformation and keto tautomeric form. This unusual conformation allows the base to donate two hydrogen bonds from the N-7 and 6-amino positions of A to the N-7 and O-6 groups of G. Unlike C⁺•G•C triad formation, formation of the A•G•C triad does not require protonation and is thus independent of pH over the range pH 6 to pH 8. The melting profile for the complex formed between binding oligomer B-3, which contains three 8-oxo-2'-deoxyadenosines in place of C residues at the 5-end of the oligomer, and target oligomer T-1 showed two transitions. The second transition occurred at 51°C and corresponds to melting of the duplex portion of the chimera. The first transition, which corresponds to melting of the 8-oxo-A containing triplex portion of the chimera, occurred at 28°C. As shown in Table 1, this same Tm was observed when the melting experiments were carried out at either pH 6.9 or at pH 7.9. This behavior is in contrast to that observed for melting of the triplex portion of the chimera formed by B-1 and T-1. In this case the Tm of the triplex decreased from 28°C at pH 6.9 to less than 0°C at pH 7.9.

At pH 6.9, the stability of the triplex containing 8-oxo A is comparable to that formed by the C-containing oligomer, B-1, whereas at pH 6.2 the triplex portion of B-1/T-1 melts at 40°C, which is 12°C higher than that observed for the triplex portion of B-3/T-1. In both cases, the G•C base pairs of the duplex are contacted by two hydrogen bonds. The additional stabilization observed for B-1/T-1 most likely results from the partial neutralization of negatively charged

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phosphodiester backbone of B-1 due to the presence of protonated C's in the oligomer B-1. This partial neutralization would be expected to reduce the overall charge repulsion between the phosphodiester backbones in the triplex and thus lead to enhanced stability of the triplex. Because protonation of C occurs more readily at lower pH, the effect on stability would be expected to be more noticeable at lower pH. Base protonation, on the other hand, is not involved in binding of oligomer B-3 to T-1 and consequently one would expect no effect on electrostatic repulsion between the negatively charged phosphodiester backbones of the oligomers.

The third, Hoogsteen strand of a three-stranded intermolecular triplex containing twelve T•A•A triads, two C⁺•G•C triads, where C is 5-methylcytosine, and one C⁺•G•C triad had a Tm of 32°C at pH 7.0 in a buffer containing 0.1 M sodium chloride, 20 mM magnesium chloride, 50 mM Tris[35]. The Tm and hypochromicity of the triplex transition of the duplex/triplex chimera formed by B-1 and T-1 is quite high considering it consists of only six base triads. This stability most likely arises in part from the intramolecular nature of the triplex. Thus the A_{260} *vs* temperature profile of a 1:1:1 stoichiometric mixture of oligomers T-1, B-4 and B-5 at pH 6.9 showed a single transition at 48°C, which corresponded to the Tm of the duplex formed between T-1 and B-4. The 6-mer, B-5 did not participate in triplex formation under these conditions. Results similar to these were reported by Salunkhe *et al*[33] for the interaction of d-(TTTTTT-X-TTTTTT) with d-(AAAAAA) where X is a terphthalamide linker. The bimolecular complex melted with a single transition at 32°C in 1.0 M sodium chloride at pH 7.0, whereas the separated oligomers, d-(TTTTTT) and d-(AAAAAA) at a stoichiometry of 2:1 melted at <2°C under the same conditions.

Interactions outside the Watson-Crick/Hoogsteen binding region also appear to play a role in stabilizing the duplex/triplex chimera and may account for the unusually high hypochromicity of the first transition. A 1:1 mixture of B-1 and target T-2, gave a melting profile with a single transition at 52°C at pH 6.9. The target oligomer consists only of the binding sites for the duplex and triplex forming regions of binding oligomer B-1. This result suggests that interactions between the T_4 loop of B-1 and nucleotides at the 3'-end of T-1 in the B-1/T-1 complex may contribute to formation and stabilization of the triplex portion of the chimera. This idea receives additional support from the observation that the complex formed by B-1 and target oligomer T-3, whose 3'-terminal sequence consists of five C residues, shows a single melting transition at 51°C. Thus it appears that nucleotides in the T_4 loop may interact with one or more of the A residues in -CACAC sequence at the 3'-end of T-1. Disruption of the ordered structure of the loop could lead to additional hypochromicity upon melting of the third strand.

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Binding oligonucleotides which can form duplex/triplex chimeras of the type reported here may also be useful in targeting regions in mRNA which contain short tracts of purines. This binding motif might be used to position reactive functional groups which can further modify the targeted nucleic acid. Targeting nucleic acids in this way could provide novel strategies for designing antisense oligonucleotides.

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