Single Strand Targeted Triplex Formation: Parallel-Stranded DNA Hairpin Duplexes for Targeting Pyrimidine Strands

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Triplex formation by oligodeoxyribonucleotides with double helical DNA through T•A:T, C^+•G:C (Py•Pu:Py moti,f) or G•G:C (Pu•Pu:Py motif) triplets provided an opportunity to develop sequence-specific agents that act at the level of transcription that could be useful as research tools and therapeutic agents.[1,2] In recent years, several groups have reported the formation of foldback or circular triplexes at purine single-stranded sites using linear,[3] circular,[4] or ligand-conjugated linear[5] pyrimidine oligonucleotides that might be useful as agents to target m-RNA for control of gene expression at the level of translation. The application of the triple helix approach for *in vivo* studies was largely precluded because of the limited base recognition and because it requires nonphysiological acidic pH conditions. Several groups have developed modified bases and alternate designs[2] to overcome these problems, but with limited success. Recently, an approach for targeting single-stranded pyrimidine sites using circular oligonucleotides in the Pu•Pu:Py motif has been proposed.[6]

We have developed a new approach to target pyrimidine DNA and RNA single strands by triple helix formation in the Py•Pu:Py motif. This approach uses non-Watson–Crick T•A and C•G base-paired DNA hairpins in which the two strands are aligned in a parallel fashion for complexing with the pyrimidine strands through formation of a triple helix (Figure 1). The complementary purine and pyrimidine sequences are attached[7] through either a 3′–3′ or a 5′–5′ linkage using a nucleotide loop to facilitate parallel orientation of the two strands. These sequences (**3** and **4**) form stable parallel-stranded (ps) hairpin duplexes.[8] The purine strands of oligonucleotides **3** and **4** form a Watson–Crick duplex with a single-stranded DNA or RNA pyrimidine target sequence (**2**); therefore, the parallel pyrimidine strand lies in the major groove via Hoogsteen hydrogen bonding, resulting in the formation of a triple helix. The conformational and stereochemical feasibility of ps structures to form a triplex with the pyrimidine target is studied by molecular modeling.

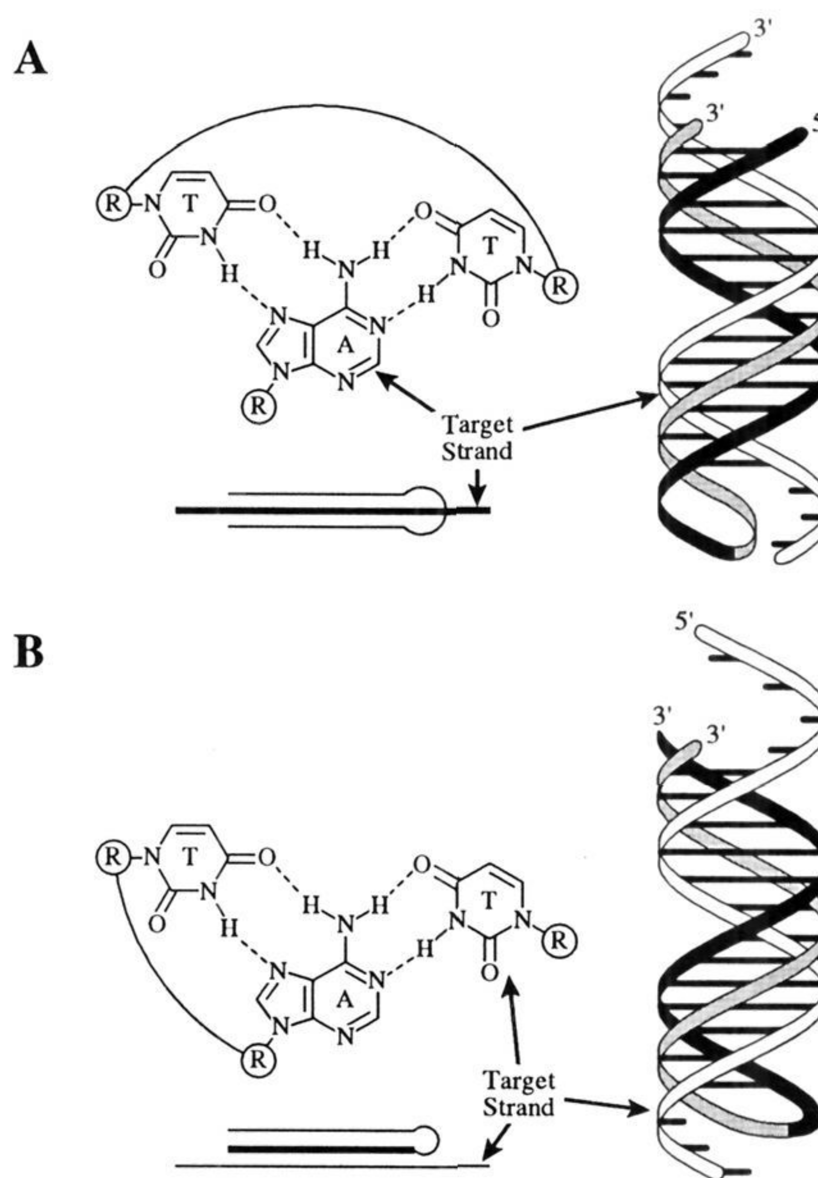

Figure 1. Models showing purine and pyrimidine single-strand targeted foldback triplexes. (A) Previously reported strategy for binding purine (A, G) single strands.[3,4b] (B) The new strategy described herein for targeting pyrimidine (C, T, U) single strands. Hydrogen bonding pattern of the T•A:T triplet is shown, and the other triplet C^+•G:C is not shown. The two strands joined covalently are shown. Covalent attachment of two pyrimidine strands (5′–3′ attachment) in (A) does not result in hairpin duplex formation. In (B), however, the purine–pyrimidine strands covalently attached through either 3′–3′ or 5′–5′ linkage form a ps hairpin duplex. Thick and thin lines represent purine and pyrimidine strands, respectively.

In UV thermal melting studies, oligonucleotides **3** and **4** showed T_m values of 47.8 and 45.3 °C, respectively, due to the formation of ps duplexes (Table 1). CD studies confirmed formation of Hoogsteen hydrogen-bonded ps hairpin duplexes[8] below pH 6.0 by **3** and **4**. Native polyacrylamide gel electrophoresis experiments revealed the difference between ps and aps (antiparallel-stranded) hairpin duplexes.[8] The linear ps and aps duplexes formed by control oligonucleotides **1** and **2** with the complementary strand have T_m values of 41.0 and 48.6 °C, respectively (Table 1).

In the presence of the DNA target strand (**2**), oligonucleotides **3** and **4** formed stable triplexes with considerably higher stability than the ps or aps duplexes (Table 1). The resulting triplexes showed single, cooperative melting transitions with T_m values of 63.1 and 63.4 °C (pH 5.0) for **3** and **4**, respectively (Table 1). Both oligonucleotides formed complexes with 1:1 stoichiometry as determined by electrophoretic mobility shift assay on native polyacrylamide gels, similar to the foldback triplexes formed with control oligonucleotides **5** and **6** (data not shown).[3a] The foldback triplexes of **5** and **6** with their single-stranded DNA–purine target (**2**) showed T_m values of 62.5 and 63.3 °C, respectively. Oligonucleotides **3** and **4** formed triplexes at pH

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Table 1. Oligonucleotide Sequences and Thermal Melting Data[a]

Oligonucleotide	Sequence[b]	Tm, °C[c]			
		pH 5.0		pH 7.6	
		DNA	RNA	DNA	RNA
1	5'-TCC TTC TTT CTT TTT T-3' 5' 3'	41.0	----	45.3	----
2[d]	5' 3' 3'-TCC TTC TTT CTT TTT T-5'	48.6	32.7 [51.5][e]	52.0	32.1 [56.2]
3	5'-TCC TTC TTT C TT TTT T 5'-AGG AAG AAA GAA AAA A-3'-3'	47.8	----	48.6	----
4	TCC TTC TTT CTT TTT T-3' 5'-5'-AGG AAG AAA GAA AAA A-3'	45.3	----	46.9	----
3	5'-TCC TTC TTT C TT TTT T 5'-AGG AAG AAA GAA AAA A-3'-3' 3' 5'	63.1 (14.5)[f]	53.3 (20.6)	54.6 (2.6)	41.4 (9.3)
4	TCC TTC TTT CTT TTT T-3' 5'-5'-AGG AAG AAA GAA AAA A-3' 3' 5'	63.4 (14.8)	53.7 (21.0)	53.3 (1.3)	43.1 (11.0)
5	5'-TCC TTC TTT CTT TTT T 5' 3' 3'-TCC TTC TTT CTT TTT T	62.5 (13.9)	50.0 (-1.5)	54.2 (2.2)	54.6 (-1.6)
6	TCC TTC TTT CTT TTT T-3' 5' 3' TCC TTC TTT CTT TTT T-5'	63.3 (14.7)	50.2 (-1.3)	54.8 (2.8)	54.3 (-1.9)

[a] Thermal melting curves were recorded by measuring absorbance at 260 nm as a function of temperature at a heating rate of 0.5 °C/min in a buffer solution. The concentration of each strand was 1.2 μM. Melting transition midpoints were determined by plotting dA/dT vs T. Uncertainty in the T_m values is about 1 °C. [b] Loop sequence in oligonucleotides **3** and **4** is -GGAGG- and in **5** and **6** is -CTCTC-. Thick and thin lines represent purine and pyrimidine complementary strands, respectively. [c] pH 5.0 buffer: 100 mM sodium acetate, 10 mM MgCl2. pH 7.6 buffer: 100 mM sodium acetate, 10 mM MgCl2, and 1 mM spemine. [d] DNA sequence is shown. In RNA sequence, U replaces T. [e] T_m values in brackets are for control aps duplex of DNA oligonucleotide **2** and its complementary RNA purine strand. [f] Numbers in parentheses are ΔT_m values relative to the Watson–Crick aps duplex of oligonucleotide **2**.

7.6 in the presence of spermine,[9] although the triplexes are 10 °C less stable[10] than those formed at pH 5.0 (Table 1). Similar results were obtained with oligonucleotides **5** and **6** at pH 7.6 (Table 1). Comparison of the melting profiles and CD spectra (data not shown) of the triplexes of **3** and **4** to those of **5** and **6** revealed similar characteristics, suggesting the formation of highly stable triplexes in both the cases.[3a,4b]

Oligonucleotides **3** and **4** exhibited strong binding to the single-stranded RNA target (**2**), with T_m values of 53.3 and 53.7 °C, respectively, at pH 5.0. Under the same experimental conditions, the heteroduplex of RNA **2** and its complementary strand showed a T_m of 32.7 °C.[11a] Triple helices of **3** and **4** with the RNA target sequence have 20 °C (ΔT_m) higher thermal stability than the Watson–Crick heteroduplex. The ΔT_m calculated for RNA triplex is about 6 °C higher than that calculated for the DNA triplex[11] (Table 1). This difference in ΔT_m is about 10 °C higher for the RNA triplex at pH 7.6 than at pH 5.0 (Table 1), suggesting a more stable triplex formation with the RNA target at physiological pH. The complexes of control oligonucleotides **5** and **6** with RNA purine target showed T_m values that are comparable to duplex T_m values of oligonucleotide **2** and the same RNA purine target (Table 1), indicating that oligonucleotides **5** and **6** do not form stable triplexes with RNA as reported earlier.[3a,c,11] Further studies of the formation of triple helices under different temperature and pH conditions revealed stable triplex formation even when the ps hairpin (**3** and **4**) and target DNA or RNA strand (**2**) were incubated at 4 °C, confirming that the triplex forms readily and does not require conformational changes.

Molecular modeling studies revealed that oligonucleotides **3** and **4** can form Watson–Crick base pairs with the pyrimidine target strand without affecting conformation of the Hoogsteen hydrogen-bonded pyrimidine parallel strand.[12] Energy minimization of the loop led to a structure where the bases were oriented in random directions with no stacking. Minimization with hydrogen bonding and base stacking constraints, however, resulted in a G–G bonded structure that could contribute to additional stability of hairpin duplexes.

Oligonucleotides **3** and **4** showed high stability against exonucleases (snake venom or spleen phosphodiesterases), single- and double-strand-specific endonucleases (S1 nuclease and DNase I), and 10% fetal calf serum (non-heat-inactivated) due to unusual 3′–3′ or 5′–5′ attachment and the formation of a stable duplex structure[13] (data not shown).

The new strategy described here for targeting single-stranded DNA and RNA pyrimidine sequences by formation of triple helices using ps hairpin oligonucleotides not only significantly increases the number of sequences that can be targeted by triplex formation but also decreases the susceptibility of oligonucleotides to degradation by cellular nucleases without chemical modification. The latter characteristic is significant in the development of therapeutic agents for *in vivo* uses and treatment of diseases.

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Supplementary Material Available: Description of the thermal melting method; four figures showing plots of dA/dT vs T from thermal melting data of duplexes and DNA and RNA target triplexes at pH 5.0 and 7.6; CD spectra of oligonucleotides; and stereodrawings of energy-minimized structures of **3** and its triplex with complementary single-stranded DNA pyrimidine target **2** (8 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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