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SELECTIVE RECOGNITION OF A C-G BASE-PAIR IN THE PARALLEL DNA TRIPLE-HELICAL BINDING MOTIF

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Abstract: Selective recognition of a C-G base-pair within the parallel DNA triple-helical binding motif was achieved by a third strand containing the base 5-methyl pyrimidin-2-one. The third strand affinities (K_D) for a representative 15-mer duplex sequence containing all four Watson-Crick base pairs (X-Y) in the center are C-G (26 nM) >> A-T (270 nM) ~ T-A (350 nM) > G-C (ca. 700 nM).

Strong and sequence specific triple-helix formation of oligonucleotides with genomic DNA can selectively interfere with gene expression on the level of transcription and, therefore, is of interest in medicinal chemistry and biotechnology.[1] Molecular recognition of a DNA-duplex in the major groove via a third strand oligonucleotide in either the parallel [2,3] or the antiparallel [4,5] binding motif, however, is restricted to homopurine–homopyrimidine DNA-sequence tracts. Despite considerable efforts over the past decade, a general recognition motif by third strands for any given DNA-sequence is still elusive.[6]

Approaches to overcome this sequence limitation in the past included the preparation and evaluation of new, unnatural bases that are designed to either target a pyrimidine base via one hydrogen-bond, [7] or complete pyrimidine-purine base-pairs.[8,9] The use of abasic sites or aromatic heterocycles, that unselectively contribute to stacking without any base-reading capability via hydrogen bonding was also evaluated, however, with limited success.[10]

We recently reported on the selective recognition of G-C base-pairs in DNA duplexes by parallel complementary oligodeoxynucleotides containing the unnatural nucleoside 7-(2'-deoxy-β-D-ribofuranosyl)hypoxanthine (^{7}H).[11] Although only one H-bond between ^{7}H and G can be formed, the ^{7}H•G-C base triple (*Figure 1*) equals the stability of the canonical $\underline{C}^+$•G-C base-triple ($\underline{C}$ = 5-methyl deoxycytidine) at pH 7.0. Empirically we attributed the remarkable stability of the ^{7}H-G base-pair to the assistance of two additional C-H···O hydrogen-bonds, flanking the conventional one (*Figure 1*). As a consequence of this result, we explored whether the pyrimidine base uracil can be recognized by hypoxanthine in a similar way.[12] We were able to show that up to three uracil units within a 15 bp triplex target sequence (amounting to a 20% pyrimidine content) can be recognized, although with lower efficiency compared to a canonical triplex structure. We have now expanded on that theme and show here,

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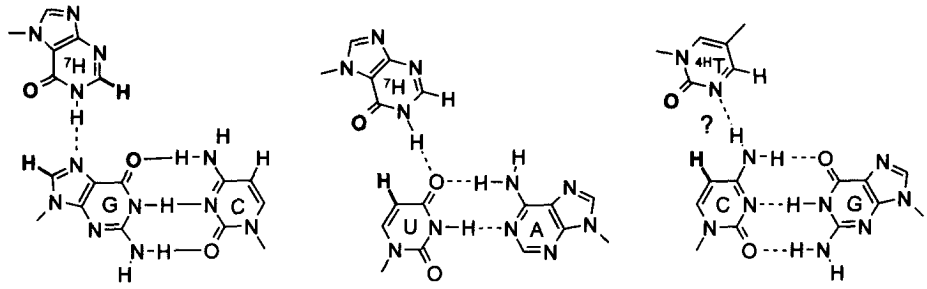
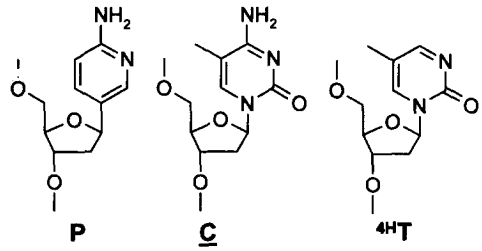
that the base cytosine can specifically be recognized with the double hydrogen-bond acceptor base 5-methyl-pyrimidine-2-one in the third strand.

Figure 1: *Structure of the ^{7}H•G-C, the ^{7}H•U-A and the ^{4H}T•C-G base-triples.*



The DNA target duplexes **1a-d**, as well as the third-strand oligonucleotides **2-4**, containing either 5-methyl-deoxycytidine (C) or 5-methyl-1-(2'-deoxy-β-D-ribofuranosyl)pyrimidin-2-one (^{4H}**T**), opposite the recognition site (X-Y) of **1** (*Figure 2*), were synthesized by standard phosphoramidite chemistry from commercially available or known (^{4H}T)[13] building blocks.

Figure2: *DNA-duplex target sequences **1**, X-Y = A-T (**a**), T-A (**b**), C-G (**c**) and G-C (**d**), and deoxy-oligonucleotide third strands **2-4**. All oligonucleotides were synthesized according to standard cyanoethyl phosphoramidite chemistry, purified by DEAE-anion exchange HPLC and analyzed by MALDI-ToF-MS.*



1a-d 5'-GCTAAAAAGA**X**AGAGAGATCG
CGATTTTTCT**Y**TCTCTCTAGC-5'

2 5'-TTTTTCT^{4H}**T**TCTCTCT
3 5'-TTTTTPT^{4H}**T**PTPTPT
4 5'-TTTTTCT**C**TCTCTCT

In a first series of experiments, the binding efficiency of the third strands to the target duplexes was assessed by UV-melting curve analysis. The melting temperatures for dissociation of the third strands **2-4** using typical buffer conditions (10mM Na-cacodylate, 100 mM NaCl, 0.25mM spermine, pH 6.5) are summarized in *Table 1*.

Inspection of the data clearly reveal that the third strand **2**, containing C for G-C recognition, and the base ^{4H}T opposite X-Y, forms the thermally most stable triplex with the C-G containing duplex **1c**. Against all other arrangements X-Y, a decrease in Tm of 4-11°C is observed. If the base C for recognition of the regular G-C base-pair is replaced by the recently developed more basic nucleoside P, [14,15] as in **3**, a slight increase in affinity to the C-G target (ΔTm=3°C) rel. to **3**, and a considerable increase in selectivity

to X-Y = C-G relative to the arrangements X-Y = A-T, T-A and G-C (ΔTm=11-16°C) is encountered. Not unexpectedly, third strand **4**, presenting the base C opposite to X-Y of the duplex also recognizes the C-G base-pair as its second choice (primary binding site is G-C) with almost the same efficiency as ^{4H}T. From these experiments we conclude, that the amino function of C plays no critical (repulsive) role in the recognition of the C-G base-pair. But the loss of the amino function in ^{4H}T relative to C suppresses the recognition of the G-C site. This selectivity, however, is traded in for affinity since binding of **2** and **3** to the C-G target is considerably weaker than binding of **4** to the G-C target (canonical triplex structure).

Table 1: *Tm data of third strand dissociation from UV-melting curves (260 nm). c(triplex)= 1.6 μM, in 10 mM Na-cacodylate, 100mM NaCl, 0.25 mM spermine, pH 6.5. UV melting curves were recorded with a temperature gradient of 0.5°C/min. Tm values were determined from the first derivative of the dissociation curves and were shown to be reproducible within ±0.5°C.*

	5'-GCTAAAAAGA**X**AGAGAGATCG CGATTTTTCT**Y**TCTCTCTAGC-5'			
1a-d X/Y	A/T	T/A	C/G	G/C
2	17.2	17.0	**28.3**	24.0
3	15.0	20.3	**31.2**	19.6
4	20.3	20.4	**27.1**	45.2

In order to confirm the results of the Tm measurements, we used non-denaturing gel electrophoresis as an alternative analytical tool for the detection of triplex formation. For these experiments the third strand **3** was radiolabeled with T4 kinase and γ^{32}P-ATP. A constant amount of third strand **3** (50 nmol) was then incubated with increasing concentrations of duplex **1a-d** (0-100 μmol). *Figure 3* shows the autoradiogram after gel electrophoresis.

From the fractions of bound vs. unbound third strand, dissociation constants (K_D) were calculated.[16] Strongest binding was observed to C-G (26 nM) followed by A-T (270 nM), T-A (350 nM) and G-C (ca. 700 nM). In essence, these data are consistent with the data from Tm-measurements.

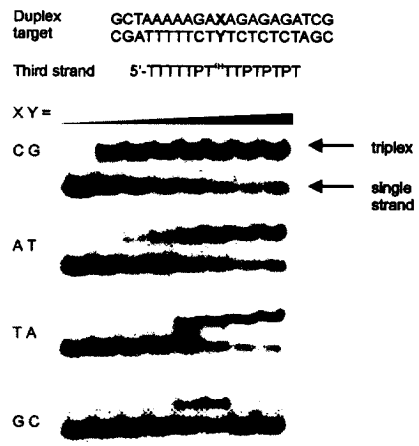


Figure 3: *Autoradiography of non-denaturing 20% (19:1) polyacrylamide gels of triplex forming oligonucleotide 3 (50 nM) with duplex **1a-d** (top to bottom) in increasing concentrations (left to right [μM]: 0, 0.025, 0.05, 0.1, 0.5, 1, 5, 10) in 10mM $MgCl_2$, 50 mM PIPES, pH 6.5. Samples of 3 were incubated with the respective amount of duplex overnight at 25°C. Electrophoresis was carried out with constant power of 7 W for 6h at 25-27°C. Quantification was performed on a phosphor-imager using the program imagequant.*

The fact that relatively stable interactions within the parallel binding motif exist between a third strand cytosine and a duplex C-G base-pair has already been recognized already earlier.[17,18] Previous attempts to improve cytosine recognition either in the antiparallel or parallel triple helical binding motifs via base design were mainly compromised by loss of selectivity of base-pair formation due to ambiguous H-bonding patterns[9] or unselective intercalation. [8] Our results demonstrate that cytosine can be recognized with the base ^{4H}T as efficiently as with C or T, but without the compromise in selectivity inherent to the latter two bases, which preferentially recognize a G-C or A-T base-pair. While ^{4H}T fullfills the criteria of selectivity it certainly does not in terms of affinity. However, given the selectivity, affinity of third strand binding is tunable by using third strand bases that show enhanced binding to the purine bases, as shown here with the analog **P** (more efficient G-C base-pair recognition than **C**), and/or by using triplex specific intercalators. Whether also in this case, a positive contribution to binding arises from a dipole interaction between H-C(5) of cytosine and the carbonyl group of the third strand base, however, needs further experimental investigations.

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