Cooperative Binding of 8-mer Oligonucleotides Containing 5-(1-Propynyl)-2′-deoxyuridine to Adjacent DNA Sites by Triple-Helix Formation

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The design of cooperative domains between DNA-binding ligands for modulation of the kinetics and thermodynamics of ligand–nucleic acid interactions is at an early stage of development.[1-4] Pyrimidine oligodeoxyribonucleotides 11 nucleotides (nt) in length are known to bind cooperatively to abutting sites on double-helical DNA by triple-helix formation, resulting in a 20-fold increase in the association constant for one oligonucleotide in the presence of the neighboring oligonucleotide.[3] This 1.8 kcal·mol^{-1} interaction energy may arise from π-stacking between the bases at the triple-helical junction.[3] The incorporation of discrete dimerization domains to oligonucleotides such as those capable of forming a short Watson–Crick helix produces a cooperative interaction and results in a 44-fold enhancement in the association constant of one oligonucleotide in the presence of a neighbor.[2]

An alternative approach to increasing cooperativity by the addition of dimerization domains is the use of modified bases, which could allow greater interactions between bases at the triple-helical junction. It is known that replacing 2′-deoxycytidines in the third strand with 5-methyl-2′-deoxycytidines, and replacing 2′-deoxyuridines with thymidines, 5-ethynyl-2′-deoxyuridines, or 5-(1-propynyl)-2′-deoxyuridines, increases the stability of triple-helical complexes.[5,6] If this enhanced stability is due to increased stacking in the third strand, one might expect similar effects at triple-helix junctions. We report here that two short oligodeoxyribonucleotides containing 5-(1-propynyl)-substituted 2′-deoxyuridine (^{P}U) and 5-methyl-2′-deoxycytidine (^{Me}C) bind cooperatively to adjacent sites on double-helical DNA at micromolar concentrations (10 mM Bis-Tris·HCl at pH 7.0, 10 mM NaCl, 1 mM spermine, 24 °C) (Figure 1). The equilibrium constant of an 8-mer binding in the presence of a neighboring bound 8-mer is enhanced by a factor of *at least 40* (relative to the 8-mer alone) under the conditions used.

A system was designed in which two 8-nt oligodeoxyribonucleotides, **1** and **2**, bind site-specifically to adjacent sites on an 850-bp 3′-^{32}P-end-labeled duplex DNA fragment via specific Hoogsteen hydrogen bonding (^{P}U·AT and ^{Me}C + GC) (Figure 1).[7] Thymidine–EDTA (T*) was incorporated at the 5′-terminus of oligonucleotide **1** to allow thermodynamic analysis of site-specific binding using the quantitative affinity cleavage titration method.[9,10] To determine the interaction energy between oligonucleotides **1** and **2**, the equilibrium association constants K_1 for **1** binding to site A alone and $K_{1,2}$ for **1** binding to site A in the presence of 1.0 μM **2** bound to neighboring site B were measured.[3,10] Analysis of cleavage data yielded equilibrium association constants of $<2 \times 10^4$ M^{-1} for **1** binding alone (K_1) and 8.0 ($\pm$2.1) $\times 10^5$ M^{-1} for **1** in the presence of 1.0 μM **2** ($K_{1,2}$), resulting in a dramatic binding enhancement, $K_{1,2}/K_1 > 40$ (Table 1).[11] This corresponds to a cooperative interaction energy of >4.5 kcal·mol^{-1}.[12]

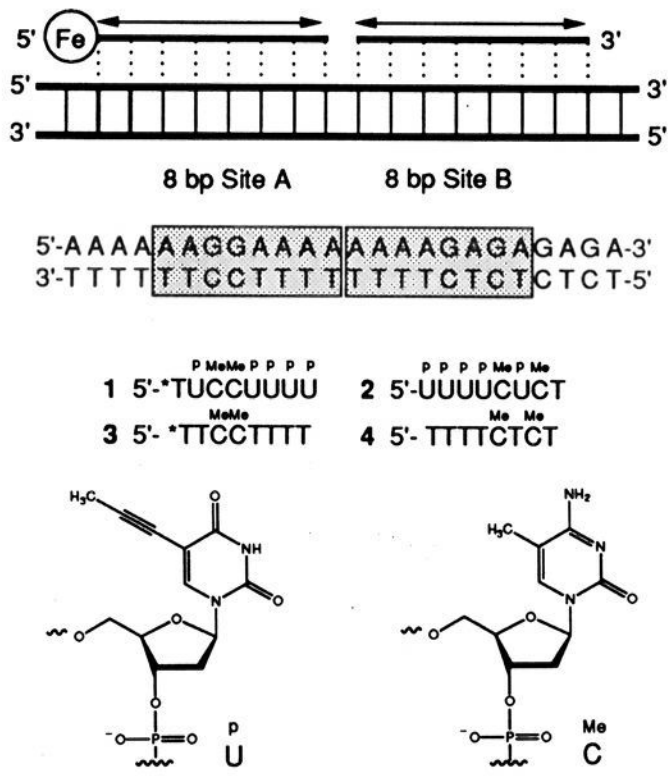

Figure 1. Schematic representation of a complex composed of two triple-helix-forming oligonucleotides binding at adjacent sites on double-helical DNA. Thick solid lines represent the DNA backbone of the target site and associated oligonucleotides. Thin solid lines represent Watson–Crick hydrogen bonds while dashed lines indicate Hoogsteen hydrogen bonds. Binding of the oligonucleotides is assessed by affinity cleavage using T*. ^{P}U and ^{Me}C represent the C5-propynyl-substituted 2′-deoxyuridine and 5-methyl-2′-deoxycytidine nucleotides, respectively.

For comparison, binding of the corresponding unmodified oligonucleotides **3** and **4** containing a methyl (thymidine) instead of a propynyl group at the 5-position of 2′-deoxyuridine, was analyzed under the same conditions (Figure 1). Oligonucleotide **3** at $\leq$100 μM both in the absence and in the presence of 1.0 μM

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(11) (a) Under the conditions chosen, oligonucleotide **1** alone at concentrations up to 100 μM produced only minimal cleavage. It was therefore assumed that K_1 is $<2 \times 10^4$ M^{-1}. Oligonucleotide 5′-^{P}U^{P}U^{P}U^{P}U^{Me}C^{P}U^{Me}-CT*-3′ with the same sequence as **2** but containing T* at the 3′-end, also produced minimal cleavage at $\leq$100 μM, and its equilibrium constant as well as that of **2** were also assumed to be $<2 \times 10^4$ M^{-1}. (b) Similar binding constants were obtained for oligonucleotide **1** binding to site A in the presence of 5.0 and 10 μM **2**.
(12) For the definition of and details on the determination of the cooperative interaction energy E_{coop} between two different ligands binding to two distinct sites in a heterodimeric cooperative system, see ref 3 and references cited therein.

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Table 1. Equilibrium Association Constants for 8-mer Modified Triple Helix Forming Oligonucleotides Binding at Adjacent Sites on DNA[a]

oligonucleotide	K (M^{-1})
1	$<2 \times 10^4$
1 + **2** (1.0 μM)	$8.0 (\pm 2.1) \times 10^5$
1 + **4** (1.0 μM)	$<2 \times 10^4$
3	$<2 \times 10^4$
3 + **2** (1.0 μM)	$9.7 (\pm 0.6) \times 10^4$

[a] Values reported in the table are mean values measured from affinity cleavage titration experiments performed in Association buffer (10 mM Bis-Tris·HCl, 10 mM NaCl, 1 mM spermine, pH 7.0, 24 °C).

4 did not produce any observable cleavage, and therefore no cooperativity could be measured.[13,14]

Possible sources for the cooperative interaction between oligonucleotides containing 5-(1-propynyl)-2′-deoxyuridines include a structural transition between adjacent sites on DNA and increased base stacking between the propyne-substituted 2′-deoxyuridines.[15,16] The right-handed nature of the triplex allows stacking of the propyne group of the base on the 3′-side of the junction onto the 5′-base across the junction, but not the stacking of the propyne group at the 5′-side of the junction onto the 3′-adjacent base (Figure 2). It is reasonable to believe that a 5′-T^{p}U-3′ stack (Figure 2A) is more favorable than a 5′-^{p}UT-3′ stack (Figure 2B) due to greater interactions between delocalized π-orbitals in the former rather than in the latter.[17]

To test this model, experiments were carried out to determine the interaction energies observed when 5-(1-propynyl)-modified oligonucleotide **1** binds to the 5′-side of the triple helical junction (site A, Figure 1) in the presence of 1.0 μM unmodified oligonucleotide **4** bound to the 3′-side (site B, Figure 1), and when unmodified oligonucleotide **3** binds to the 5′-side in the presence of 1.0 μM modified oligonucleotide **2** bound to the 3′-side (Figure 1). In both cases, site B should be <2% occupied by 1.0 μM oligonucleotide **2** or **4** in the absence of bound **1** or

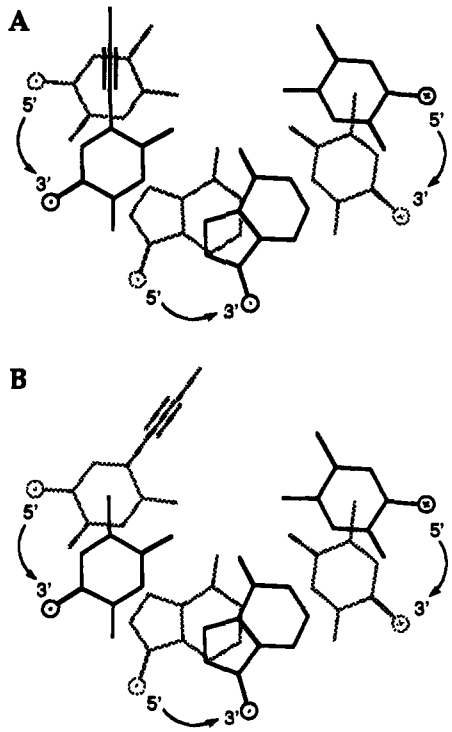

Figure 2. Base-stacking configuration of two ^{p}U·AT triples drawn on the basis of the structure proposed for DNA triple-helix $(T{\cdot}AT)_n$.[18] The C1′ atoms of the deoxyribose sugars are represented by the open circles. The 5′ → 3′ polarity of the strands is indicated by the ⊗ symbols (into the plane of the page) and ⊙ (out of the plane of the page).

3. Under the titration conditions, modified oligonucleotide **1** at ≤ 20 μM in the presence of 1.0 μM unmodified **4** produced minimal cleavage, and its equilibrium constant for binding to site A could not be measured. In contrast, unmodified oligonucleotide **3** bound to site A in the presence of 1.0 μM modified **2** with an increased association constant ($K_{3,2}$ = 9.7 (±0.6) × 10^4 M^{-1}) (Table 1). These results are consistent with a model wherein cooperativity between a modified and an unmodified oligonucleotide is dramatically affected by the position (3′ or 5′) of the modified oligonucleotide with respect to the triple-helical junction due to two stacking arrangements (Figure 2). This suggests that some significant contribution to the interaction energy between **1** and **2** originates from stacking between the modified bases.[19] We conclude that cooperativity can be used to allow modified oligonucleotides as small as 8 nt in the molecular weight range of 2500 to bind to adjacent sites covering a specific 16-bp region on double-helical DNA at micromolar concentrations.

Acknowledgment. We are grateful to the National Institutes of Health for grant support (HG 00329) and for a National Research Service Award (Predoctoral Training in Genome Analysis HG00021-03) to N.C.

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(14) The equilibrium constant of **3** (K_3) was assumed to be $<2 \times 10^4$ M^{-1}.

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(17) It is known that the presence of a large mobile π-electron system favors stacking between aromatic molecules. For examples, see: *Molecular Associations in Biology*; Pullman, B., Ed; Academic Press Inc.: New York, 1968.

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(19) Consistent with this, separation of sites A and B by a one-base-pair gap abolishes the binding enhancement of oligonucleotide **1** in the presence of oligonucleotide **2**.