Cooperative Oligonucleotide-Directed Triple Helix Formation at Adjacent DNA Sites

Natalia Colocci, Mark D. Distefano, and Peter B. Dervan*

Contribution from the Arnold and Mabel Beckman Laboratories of Chemical Synthesis, Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California 91125

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Abstract: The changes in binding free energies due to cooperativity for oligodeoxyribonucleotides bound to adjacent sites by triple helix formation have been determined by quantitative affinity cleavage titrations. A 20-fold enhancement in equilibrium association constant is realized for an 11mer pyrimidine oligonucleotide binding in the presence of a neighboring bound site at 24 °C and pH 7.0 (25 mM Tris–OAc, 10 mM NaCl, 1 mM spermine). This corresponds to an increase in binding free energy of 1.8 ± 0.2 kcal mol^{-1}. This cooperativity is not observed when the two binding sites are separated by one base pair.

Cooperative interactions between proteins bound to DNA are essential for the regulation of gene expression.[1] These cooperative interactions serve to increase the sequence specificity of DNA-binding proteins as well as the sensitivity of the binding equilibrium to concentration changes.[2] When cooperative interactions occur between two (or more) ligands bound at neighboring sites on DNA, the specific binding of each ligand is enhanced.

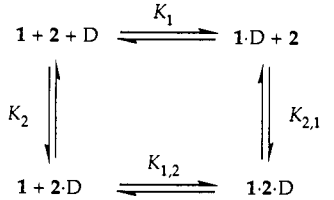
Oligodeoxyribonucleotide-directed triple helix formation is a powerful method for the sequence-specific recognition of double-helical DNA.[3] To date, at least two classes of triple-helix-forming oligonucleotides have been identified. In one class, pyrimidine oligonucleotides bind in the major groove of double-helical DNA parallel to purine tracts by Hoogsteen hydrogen bond formation.[3a] Specificity is derived from thymine (T) recognition of adenine–thymine base pairs (T·AT base triplet) and protonated cytosine (C+) recognition of guanine–cytosine base pairs (C+GC base triplet).[4] In another class of triple-helical structures, purine-rich oligonucleotides bind in the major groove antiparallel to the Watson–Crick purine strand.[3c,e] In this case, specificity is derived from G recognition of GC base pairs (G·GC base triplet) and A or T recognition of AT base pairs (A·AT and T·AT base triplets).[5]

It has been demonstrated that oligonucleotides can bind *cooperatively* by triple helix formation to adjacent purine sites on double-helical DNA.[6] For two 9-nt oligonucleotides bound to adjacent 9-bp sites on an 18-bp purine tract, the binding enhancement at micromolar concentration was observed to be a factor of 3.5.[6] Likely the adjacent sites were not fully occupied, and this qualitative observation is a minimum value. Cooperativity in oligonucleotide-directed sequence-specific recognition of DNA by triple helix formation can be dramatically enhanced by the addition of discrete dimerization domains to oligonucleotides.[7] However, the maximum enhancement possible for the simplest case, involving *directly abutting* sites occupied by triple-helix-forming oligonucleotides, has yet to be determined.

We report here the energetics of cooperative binding of pyrimidine oligodeoxyribonucleotides to adjacent sites on DNA by triple helix formation measured using quantitative affinity cleavage titrations.[8] We find that pyrimidine oligonucleotides bind cooperatively to abutting purine sites on double-helical DNA. The equilibrium association constant of an oligonucleotide bound to an 11-bp site is measured in the absence and presence of a second oligonucleotide occupying an adjacent, directly abutting site. The binding affinity of an 11mer in the presence of a neighboring bound pyrimidine oligonucleotide is enhanced by a factor of 20 at 24 °C and pH 7.0 (25 mM Tris–OAc, 10 mM NaCl, 1 mM spermine).

Results

The binding of oligonucleotides **1** and **2** to adjacent sites A and B on double-helical DNA (D) forms the binary complexes **1**·D and **2**·D, respectively, and the binding of either **1** to **2**·D or **2** to **1**·D forms the ternary complex **1**·**2**·D (Figure 1):



Experiments were designed to measure K_1, the binding of oligonucleotide **1** to site A alone, and $K_{1,2}$, the binding of the same oligonucleotide **1** to site A in the presence of oligonucleotide **2** bound to neighboring site B. This allowed us to determine the

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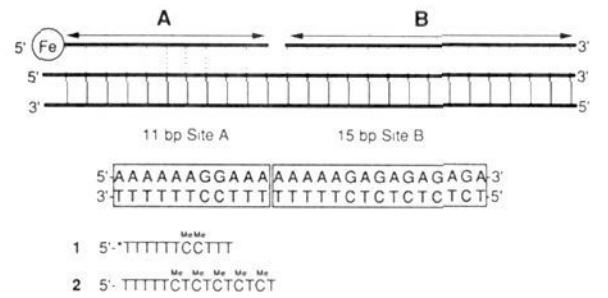

Figure 1. Schematic representation of a complex composed of two triple-helix-forming oligonucleotides binding at adjacent sites on double-helical DNA. Thick solid lines represent the DNA backbone of the target site and associated oligonucleotides. Thin solid lines represent Watson–Crick hydrogen bonds, while dashed lines indicate Hoogsteen hydrogen bonds. Binding of the oligonucleotides is assessed by affinity cleavage using T*.

energetics of binding as a function of the occupancy of a neighboring site.

Affinity Cleavage Reactions. Oligodeoxyribonucleotide **1**, equipped with thymidine–EDTA at the 5′-position,[9] was allowed to equilibrate with an 850-bp 3′-^{32}P-end-labeled duplex DNA fragment at 24 °C and pH 7.0 (25 mM Tris–OAc, 10 mM NaCl, 1 mM spermine) for 24 h followed by addition of dithiothreitol (DTT) to initiate cleavage. After 6 h the reactions were stopped by ethanol precipitation, and the products were separated by denaturing polyacrylamide gel electrophoresis (Figure 2). The small amount of cleavage in a reaction containing **1** alone at 100 nM concentration indicates that **1** binds to the 11-bp target site weakly under these conditions (Figure 2, lane 2). However, oligonucleotide **1** at the same concentration (100 nM) in the presence of 1.0 μM concentration of oligonucleotide **2** produces a dramatic increase in cleavage (Figure 2, lane 3).

Cooperative Binding of 11mer + 15mer at Directly Abutting Sites. To measure the magnitude of the cooperative enhancement, quantitative affinity cleavage titrations were performed over an extensive concentration range (Figure 3). The extent of cleavage for each reaction is determined by phosphorimaging analysis,[10] and an equilibrium association constant, K, is obtained by fitting a Langmuir binding isotherm to the experimental data (Figure 4).[8] Quantitative affinity cleavage titrations were carried out with **1** alone and with **1** in the presence of oligonucleotide **2** at a concentration sufficient to saturate the nearby 15-bp site (24 °C, pH 7.0). The equilibrium constant for **3**, an oligonucleotide with the same sequence as **2** but containing T* targeted to the 15-bp site B, was found to be $(1.3 \pm 0.3) \times 10^7$ M^{-1}. As the substitution of T* for T has a negligible influence on the binding affinity,[8] we estimate that the 15-bp site should be 93% occupied by oligonucleotide **2** at 1 μM concentration.

Analysis of cleavage data yielded equilibrium association constants of $(1.2 \pm 0.3) \times 10^6$ M^{-1} for **1** binding alone (K_1) and $(2.3 \pm 0.5) \times 10^7$ M^{-1} for **1** in the presence of 1.0 μM **2** ($K_{1,2}$) (Table I, Figure 4). The binding enhancement $K_{1,2}/K_1$ is a factor of 20. The free energies of binding (ΔG) at the 11-bp site for oligonucleotide **1** in the absence and presence of oligonucleotide **2** are -8.2 ± 0.2 and -10.0 ± 0.2 kcal mol^{-1}, respectively, and the minimum free energy of interaction, $\Delta G_{1,2} - \Delta G_1$, is -1.8 ± 0.3 kcal mol^{-1}.

Effect of a One Base Pair Gap Between Adjacent Sites. Quantitative affinity cleavage titrations were used to obtain isotherms for **1** binding in the presence and absence of **2** when the binding sites A and B are separated by one base pair (Figures 5 and 6). The association constants for the binding of **1** in the absence and presence of oligonucleotide **2** are $K_1 = (2.9 \pm 0.2) \times 10^6$ M^{-1} and $K_{1,2} = (2.5 \pm 0.3) \times 10^6$ M^{-1} (Table II). When the binding sites for the two oligonucleotides are separated by one base pair there is no enhancement of the binding of **1** in the presence of **2**.

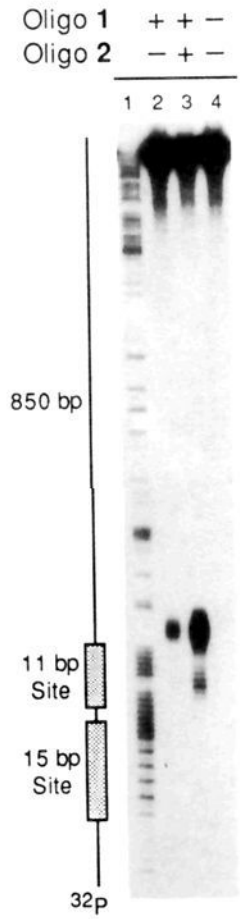

Figure 2. Autoradiogram of an 8% denaturing polyacrylamide gel of reaction products from affinity cleavage reactions with oligonucleotides **1** and **2** and a ^{32}P-labeled restriction fragment from pMD5152 containing the target site shown in Figure 1. The reactions were performed at 24 °C in 10 mM NaCl, 1 mM spermine, 25 mM Tris–acetate, pH 7.0. Oligonucleotides **1** and **2** are present at 100 nM and 1 μM concentrations, respectively. Lane 1, A-specific sequencing reaction performed on ^{32}P-labeled restriction fragment; lane 2, oligonucleotide **1**; lane 3, oligonucleotides **1** and **2**; lane 4, no oligonucleotide.

Cooperative Binding of 11mer + 11mer at Directly Abutting Sites. The equilibrium binding constants for the binding of oligonucleotide **1** in the absence and presence of the 11mer oligonucleotide **4** at 1.0 μM concentration to DNA are $K_1 = (1.2 \pm 0.3) \times 10^6$ M^{-1} and $K_{1,4} = (2.4 \pm 0.2) \times 10^7$ M^{-1} (Figures 7 and 8). This is a 20-fold increase in the binding of oligonucleotide **1** in the presence of the 11mer oligonucleotide **4** (Table I). The equilibrium binding constant of **5**, an oligonucleotide with the same sequence as **4** but containing T* at the 3′-end, to the 11-bp site was shown to be $(1.4 \pm 0.5) \times 10^6$ M^{-1}. Therefore, at 1 μM concentration, the 11-bp site should be 58% occupied by oligonucleotide **4** in the absence of bound **1**.[8]

Discussion

There is a 20-fold difference in the association constants for the binding of **1** in the absence versus the presence of oligonucleotide **2** at 1.0 μM concentration (Table I). In the presence of 1 μM concentration of **2**, the 15-bp site is at least 93% saturated with the adjacent 11-bp site being unoccupied. The observed 1.8

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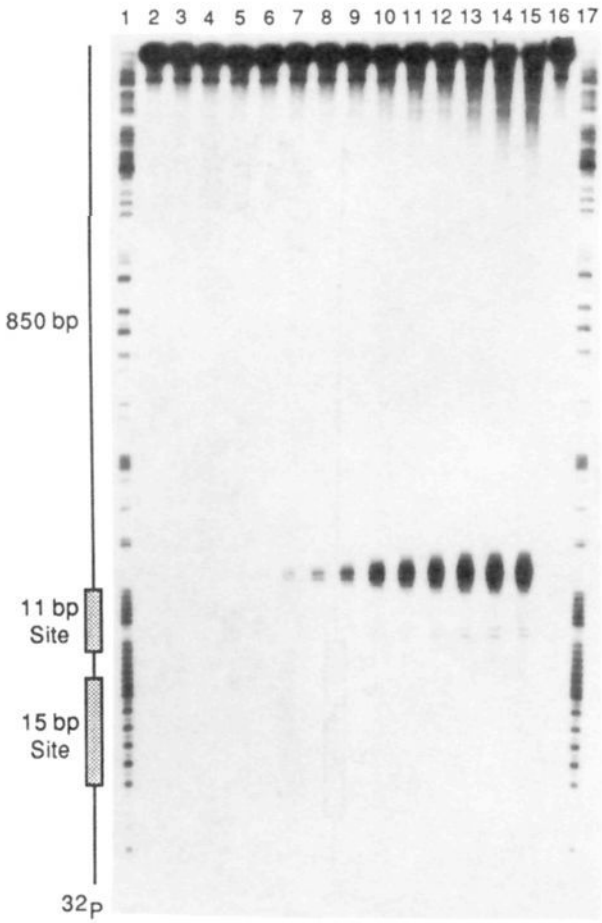

Figure 3. Autoradiogram of an 8% denaturing polyacrylamide gel of reaction products from affinity cleavage reactions with oligonucleotide **1** and a ^{32}P-labeled restriction fragment from pMD5152 containing the target site shown in Figure 1. The reactions were performed at 24 °C in 10 mM NaCl, 1 mM spermine, 25 mM Tris–acetate, pH 7.0. Lanes 1 and 17, A-specific sequencing reaction performed on ^{32}P-labeled restriction fragment; lanes 2–15, DNA affinity cleavage reaction products produced by **1** at various concentrations, 1 nM (lane 2); 2 nM (lane 3); 5 nM (lane 4); 10 nM (lane 5); 20 nM (lane 6); 50 nM (lane 7); 100 nM (lane 8); 200 nM (lane 9); 500 nM (lane 10); 1 μM (lane 11); 2 μM (lane 12); 5 μM (lane 13); 10 μM (lane 14); 20 μM (lane 15); no oligonucleotide (lane 16).

kcal mol^{-1} energy difference represents the minimum free energy of interaction between **1** and **2**. To determine the full magnitude of cooperativity between **1** and **2**, a model which takes into account the extent of occupation of the adjacent 15-bp site allows calculation of the total cooperative interaction energy.

Heterodimeric Cooperative Systems. Hill has derived a grand partition function (ξ) for a heterodimeric cooperative system in which two different ligands, L_A and L_B, bind uniquely to two distinct sites and each ligand perturbs the binding equilibrium at the noncognate site:[2b]

$$\xi = 1 + K_A[L_A] + K_B[L_B] + yK_A[L_A]K_B[L_B] \quad (1)$$

where K_A and K_B are equilibrium association constants for L_A and L_B binding in the absence of each other, and y is related to the interaction energy, E_{coop}, between L_A and L_B by:

$$y = \exp(-E_{coop}/RT) \quad (2)$$

The fractional occupancy of site A by L_A is given by:

$$\theta_A = [L_A]\frac{\partial \ln \xi}{\partial [L_A]} = \frac{\Phi K_A[L_A]}{1 + \Phi K_A[L_A]} \quad (3)$$

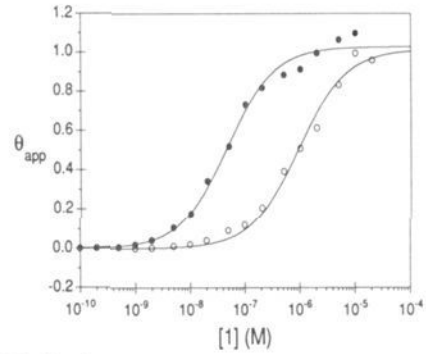

Figure 4. Binding isotherms obtained for oligonucleotide **1** alone (○) and in the presence of 1.0 μM oligonucleotide **2** (●), using quantitative affinity cleavage titration. A theoretical binding curve represented by the Langmuir equation (7) (see Experimental Section) was used to fit the experimental data using I_{sat} and K as adjustable parameters. Five (in the case of oligonucleotide **1**) to six (in the case of oligonucleotide **1** in the presence of oligonucleotide **2**) complete data sets were used to determine each association constant. The data points were divided by I_{sat} to obtain θ_{app}. Each data point represents the average of 5–6 individual experiments.

Table I. Equilibrium Association Constants for Two Triple-Helix-Forming Oligonucleotides Binding at Adjacent Sites on DNA[a]

oligonucleotide	K (M^{-1})	ΔG (kcal mol^{-1})
1	$(1.2 \pm 0.3) \times 10^6$	-8.2 ± 0.2
1 + **2** (1 μM)	$(2.3 \pm 0.5) \times 10^7$	-10.0 ± 0.2
1 + **4** (1 μM)	$(2.4 \pm 0.2) \times 10^7$	-10.0 ± 0.1

[a] Values reported in the table are mean values measured from affinity cleavage titration experiments performed in association buffer (10 mM NaCl, 1 mM spermine, 25 mM Tris–acetate, pH 7.0, 24 °C).

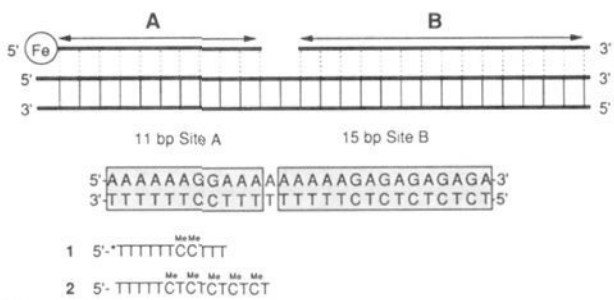

Figure 5. Schematic representation of a complex composed of two triple-helix-forming oligonucleotides binding on double-helical DNA where the two target sites are separated by one base pair. Thick solid lines represent the DNA backbone of the target site and associated oligonucleotides. Thin solid lines represent Watson–Crick hydrogen bonds, while dashed lines indicate Hoogsteen hydrogen bonds.

where

$$\Phi = \frac{1 + yK_B[L_B]}{1 + K_B[L_B]} \quad (4)$$

Equations 2 and 4 can be combined and rearranged to give the following expression for the interaction energy:

$$E_{coop} \approx -RT \ln \frac{\Phi(1 + K_B[L_B]) - 1}{K_B[L_B]} \quad (5)$$

Energetics of Cooperatively Binding Oligonucleotides: 11mer + 15mer. This simple model requires that two conditions be satisfied: (1) the ligands do not interact in solution and (2) under experimental conditions chosen, L_A does not bind to site B and L_B does not bind to site A. The sequences of the 11- and 15-bp sites were chosen to maximize the binding specificities of oligonucleotides **1** and **2** for their designated sites. For oligo-

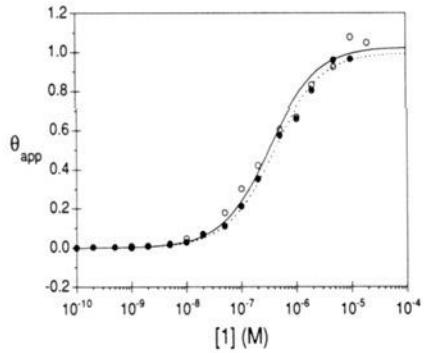

Figure 6. Binding isotherms obtained for oligonucleotide **1** alone (○) and in the presence of 1.0 μM oligonucleotide **2** (●) bound to DNA where the two target sites are separated by one base pair. The solid sigmoidal curve represents the titration binding isotherm obtained for oligonucleotide **1** alone; the dashed sigmoidal curve represents the titration binding isotherm obtained for oligonucleotide **1** in the presence of oligonucleotide **2**. The data shown were obtained using the quantitative affinity cleavage titration method as described above (see Figure 4).

Table II. Equilibrium Association Constants for Two Triple-Helix-Forming Oligonucleotides Binding on DNA where the Target Sites are Separated by One Base Pair[a]

oligonucleotide	K (M^{-1})	ΔG (kcal mol^{-1})
1	$(2.9 \pm 0.2) \times 10^6$	-8.8 ± 0.1
1 + **2** (1 μM)	$(2.5 \pm 0.3) \times 10^6$	-8.7 ± 0.1

[a] Values reported in the table are mean values measured from affinity cleavage titration experiments performed in association buffer (10 mM NaCl, 1 mM spermine, 25 mM Tris–acetate, pH 7.0, 24 °C).

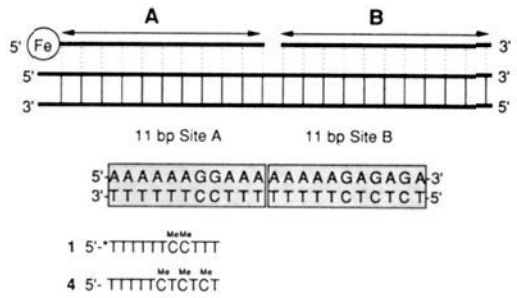

Figure 7. Schematic representation of a complex composed of two 11mer oligonucleotides binding at adjacent sites on double-helical DNA. Thick solid lines represent the DNA backbone of the target site and associated oligonucleotides. Thin solid lines represent Watson–Crick hydrogen bonds, while dashed lines indicate Hoogsteen hydrogen bonds.

nucleotide **1** at ≤20 μM concentrations, there was no detectable cleavage in the 15-bp target site region, evidence that **1** has low affinity for the 15-bp site. For oligonucleotide **2**, at ≤1.0 μM, there was no detectable binding in the 11-bp target site region.

Since **1** and **2** do not dimerize significantly in solution and since neither oligonucleotide shows any detectable affinity for its noncognate site under the conditions chosen,[11,12] the model described above can be reliably used to determine E_{coop}. For the cooperatively binding oligonucleotides described here, L_A is oligonucleotide **1**, site A is the 11-bp site, and K_A is K_1. Similarly, L_B is oligonucleotide **2**, site B is the 15-bp site, and K_B is K_2. Comparison of the Langmuir expression (see Experimental Section, eq 7) and eq 3 shows that $K_{1,2}$ in the former equals ΦK_1 in the latter. Thus, $\Phi_{1.0} = K_{1,2}/K_1 = 20$ where $\Phi_{1.0}$ is the value of Φ when [**2**] = 1.0 μM. Substitution of the values 20 ± 6, $(1.3 \pm 0.3) \times 10^7$ M^{-1}, and $(1.0 \pm 0.1) \times 10^{-6}$ M for Φ, K_2, and [**2**], respectively, in eq 5 affords a value of -1.8 ± 0.2 kcal mol^{-1} for E_{coop} between the 11mer **1** and the 15mer **2**. The correspondence of this value with $\Delta G_{1,2} - \Delta G_1 = -1.8$ kcal mol^{-1} can be explained by the fact that the 15-bp site is likely fully saturated (>93% occupied) by oligonucleotide **2** in the presence of **1**.

(11) In a heterodimeric cooperative system, intersite competition may reduce the apparent magnitude of cooperative binding. In order to achieve the maximum cooperative interaction, it is necessary to choose an oligonucleotide concentration that is sufficiently high to allow saturation at the designated target site and sufficiently low to prevent competitive binding at noncognate sites. An increase in oligonucleotide **2** concentration from 1.0 to 50 μM caused a 5.5-fold decrease in the binding of the **1**–**2** complex. Similarly, an increase in the concentration of oligonucleotide **4** from 1.0 to 50 μM caused a 2.7-fold decrease in the binding of the **1**–**4** complex. These results are consistent with oligonucleotides **2** and **4** occupying site A when present at very high concentrations.

(12) An approximate binding constant of 4.2×10^5 M^{-1} was determined by quantitative affinity cleavage titration for **3** binding to site A. This value suggests that at 1.0 μM, site A is ca. 30% occupied by **2**.

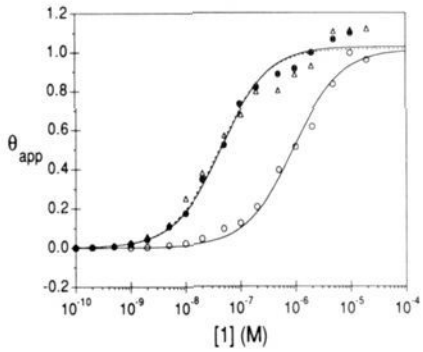

Figure 8. Binding isotherms obtained for oligonucleotide **1** alone (○), in the presence of 1.0 μM oligonucleotide **2** (●), and in the presence of 1.0 μM oligonucleotide **4** (Δ). The solid sigmoidal curves represent the titration binding isotherm obtained for oligonucleotide **1** alone and for oligonucleotide **1** in the presence of oligonucleotide **2**; the dashed sigmoidal curve represents the titration binding isotherm obtained for oligonucleotide **1** in the presence of oligonucleotide **4**.

Energetics of Cooperatively Binding Oligonucleotides: 11mer + 11mer. Under the conditions used for quantitative affinity cleavage titrations and in the absence of any cooperative energetic benefit, oligonucleotide **4** at 1 μM concentration occupies the 11-bp site 58%. For the cooperatively binding oligonucleotides described here, L_A is oligonucleotide **1**, site A is the 11-bp site, and K_A is K_1. Similarly, L_B is oligonucleotide **4**, site B is the designated 11-bp site, and K_B is K_5. Substitution of the values 20 ± 5, $(1.4 \pm 0.5) \times 10^6$ M^{-1}, and $(1.0 \pm 0.1) \times 10^{-6}$ M for Φ, K_5, and [**4**], respectively, in eq 5 affords a value of -2.1 ± 0.2 kcal mol^{-1} for E_{coop} between the 11mers **1** and **4**. This value is the same within experimental uncertainty as that obtained when 11mer **1** and and 15mer **2** fully saturate the adjacent sites on DNA. This suggests that the binding site of oligonucleotide **4** is more than 58% occupied at 1 μM concentration and likely saturated due to the binding of oligonucleotide **1** to its designated site A. From quantitative affinity cleavage titrations, we found that the binding of 11mer **1** to its site is increased 20-fold in the presence of directly abutting oligonucleotide **4**. In the presence of 1 μM **1**, the occupancy level of oligonucleotide **4** at site B is calculated to be 97%.

Origin of Cooperative Binding Forces. Two oligonucleotides bind cooperatively to duplex DNA with an interaction energy of 1.8–2.1 kcal mol^{-1}. Potential sources for this cooperative interaction energy include propagation of a conformational transition between adjacent sites and base stacking of the terminal thymines. Early fiber diffraction studies[13a] and chemical footprinting experiments[13b,c] are consistent with a B- to A′-form conformational change upon formation of a triple-helical complex. However, IR, CD, and NMR studies suggest that DNA triplexes

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contain structural features of both A and B conformations.[14] One would anticipate that triplex formation at site A would be facilitated by a structural transition at site B. Because the cooperative enhancement was not observed when triple helix binding sites were separated by one base pair, our data suggest that either there are no significant propagated structural changes beyond the immediately neighboring base pair or that a one base pair gap creates a junction that prevents the propagation.

The free energy of base stacking, which arises from induced dipole interactions,[15] and hydrophobic forces[16] has been shown to range from –0.5 to –3 kcal mol^{-1} for monomer nucleosides associating in aqueous solution, depending on solution, salt conditions, nucleoside identity, concentration, and temperature.[15] To date, no value for the free energy change associated with stacking interactions between contiguous bases of a triple-helix-forming oligonucleotide has been reported. The structure at the interface of oligonucleotides **1** and **2** bound on neighboring sites is likely similar to that of the corresponding contiguous triplex. The 1.8–2.1 kcal mol^{-1} interaction energy between oligonucleotides **1** and **2** when binding to adjacent sites on DNA at 24 °C and pH 7.0 (25 mM Tris–OAc, 10 mM NaCl, 1 mM spermine) may reflect a significant contribution from a T-T stack in the third strand of triple helices.[17] Under these conditions, if this is true, we would anticipate that the 1.8–2.1 kcal mol^{-1} cooperative effect is sequence dependent. The energetics of a CT, TC, and CC stack at the junction will be necessary to answer this question.

Implications. Pyrimidine oligodeoxyribonucleotides can bind cooperatively to abutting sites on double-helical DNA with an interaction energy of 1.8–2.1 kcal mol^{-1} for TAT triplets at the triple helix junction (Figure 9). Thus, for oligonucleotide-directed sequence-specific recognition of DNA, cooperative interactions may allow for significant reduction in the length of oligonucleotides with little decrease in the binding affinity or specificity.

Experimental Section

General. *Escherichia coli* JM110 was obtained from Stratagene. Qiagen plasmid kits were purchased from Qiagen Inc. Sonicated, deproteinized calf thymus DNA was purchased from Pharmacia. Nucleotide triphosphates were obtained from Pharmacia. Nucleotide triphosphates labeled with ^{32}P (≥3000) Ci/mmol) were purchased from Amersham. Restriction endonucleases were purchased from New England Biolabs and used according to the suggested protocol in the provided buffer. Thymidine and 2′-deoxy-5-methylcytidine phosphoramidites were obtained from ABI and Cruachem, respectively. Sephadex resins were obtained from Pharmacia. Sequenase Version 2.0 was obtained from United States Biochemical.

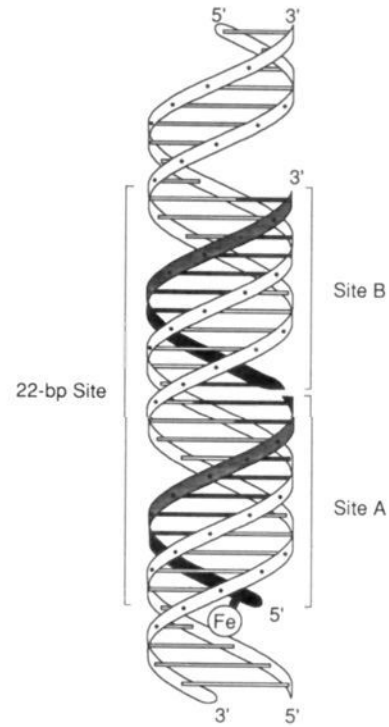

Figure 9. Ribbon model of oligonucleotides **1** and **4** bound at adjacent sites on duplex DNA by triple helix formation. The Watson–Crick duplex strand are shown as white ribbons, and the triple-helix-forming oligonucleotides are shown as dark ribbons.

Construction of pMD5152 and pMD5354. These plasmids were prepared by standard methods.[18] Briefly, pMD5152 was prepared by hybridization of two synthetic oligonucleotides, 5′-ACGTTC-CTAAAAAAGGAAAAAAAAGAGAGAGAGATCTG and 5′-GATC-CAGATCTCTCTCTCTTTTTTTTCCTTTTTTAGGA, followed by ligation of the resulting duplex with pUC18 DNA previously digested with *Hind*III and *Bam*HI; this ligation mixture was used to transform *E. coli* JM110 (Stratagene). Plasmid DNA from ampicillin resistant white colonies was isolated, and the presence of the desired insert was confirmed by restriction analysis and Sanger sequencing. Preparative isolation of plasmid DNA was performed using a Qiagen plasmid kit. Plasmid pMD5354 was prepared using a similar procedure with the oligonucleotides 5′-AGCTTCCTAAAAAAGGAAAAAAAAAGAGA-GAGAGATCTG and 5′-GATCCAGATCTCTCTCTCTTTTTTTTTC-CTTTTTTAGGA.

Oligonucleotide Preparation. Oligonucleotides were synthesized by standard automated solid-support chemistry on an Applied Biosystems Model 380B DNA synthesizer using *O*-cyanoethyl-*N*,*N*-diisopropyl-phosphoramidites.[19] The phosphoramidite of thymidine–EDTA (T*) was prepared according to published procedures.[9] Controlled pore glass derivatized with T* was prepared as described by Strobel.[20] Oligonucleotides **1**, **3**, and **5** containing T* were deprotected with 0.1 N NaOH at 55 °C for 24 h, neutralized with glacial acetic acid, desalted on Pharmacia Sephadex (G-10-120) spin columns, and dried *in vacuo*. Oligonucleotides **2** and **4** were deprotected with concentrated ammonium hydroxide at 55 °C for 24 h and dried *in vacuo*. The crude 5′-terminal dimethoxytrityl-protected oligonucleotides were purified by reverse-phase FPLC on a ProRPC 10/10 (C_2–C_8) column (Pharmacia LKB) and a gradient of 0–40% CH_3CN in 0.1 M triethylammonium acetate, pH 7.0, detritylated in 80% AcOH, and chromatographed a second time. Triethylammonium acetate was removed via repeated resuspensions of the oligonucleotides in water followed by lyophilization. Oligonucleotide concentrations were determined by UV absorbance at 260 nm using extinction coefficients (M^{-1} cm^{-1}) of 8800 (T and T*) and 5700 (mC).

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Preparation of Labeled Restriction Fragment. The 3′-^{32}P-labeled duplex was prepared by digestion of the plasmid pMD5152 (or pMD5354) with *Eco*RI, followed by treatment with α-^{32}P-dATP and α-^{32}P-TTP in the presence of Sequenase. To remove nonincorporated radioactivity, the fragment was passed through a Pharmacia NICK column. The DNA was then digested with *Xmn*I, followed by separation of the resulting products on a 5% nondenaturing polyacrylamide gel (19:1, monomer/bis). The gel band corresponding to the desired 850-bp fragment (851-bp for pMD5354) was visualized by autoradiography, excised, crushed, and soaked in 10 mM Tris, pH 8.0, and 20 mM EDTA at 37 °C for 12 h. The resulting suspension was filtered through a 0.45-μm filter, and the eluted DNA present in the supernate was precipitated with NaOAc/EtOH. The DNA pellet was washed with 70% EtOH, dried *in vacuo*, resuspended in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA), extracted five times with phenol and twice with 24:1 $CHCl_3$/isoamyl alcohol, and reprecipitated with NaOAc/EtOH. The DNA pellet was washed with 70% EtOH, dried *in vacuo*, and resuspended in 25 mM Tris–acetate, 100 mM NaCl, pH 7.0 at a final activity of 100 000 cpm/μL, and stored at -20 °C.

Quantitative Affinity Cleavage Titrations. In a typical experiment, a 5-nmol aliquot of the desired oligonucleotide–EDTA was dissolved in 50 μL of aqueous 200 μM $Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$ to produce a solution that was 100 μM in oligonucleotide. This solution was then diluted serially to yield 100 μM, 10 μM, 1 μM, 100 nM, 10 nM, and 1 nM solutions. The appropriate volume of these solutions was then distributed among 15–17 1.5-mL microcentrifuge tubes at the appropriate concentrations. A stock solution containing labeled target DNA, Tris–acetate buffer, NaCl, calf thymus DNA, the second oligonucleotide where needed, and water was distributed to each reaction tube in 12-μL aliquots, and the appropriate volume of water and 4 μL of 10 mM spermine were added to each tube to bring the total volume to 36 μL. The oligonucleotide–EDTA·Fe(II) and the DNA were allowed to equilibrate for 24 h at 24 °C. The cleavage reactions were initiated by the addition of 4 μL of a 40 mM aqueous DTT solution to each tube. Final reaction conditions in 40 μL of association buffer were 25 mM Tris–OAc at pH 7.0, 10 mM NaCl, 1 mM spermine, 100 μM bp calf thymus DNA, 4 mM DTT, and approximately 10 000 cpm labeled DNA. The reactions were incubated for 6 h at 24 °C. Cleavage reactions were terminated by NaOAc/EtOH precipitation, and the reaction tubes were then stored at –20 °C for 30 min before centrifugation. The DNA cleavage products were washed with 70% aqueous EtOH, dissolved in 50 μL of water, and dried *in vacuo*. The DNA was then resuspended in 10 μL of formamide–TBE loading buffer, heated at 55 °C for 10 min to effect dissolution, and transferred to a new tube. The Cerenkov radioactivity of the solutions was measured with a scintillation counter, followed by dilution to the same cpm/μL with more formamide–TBE buffer. The DNA was denatured at 95 °C for 3 min, and 5 μL from each sample were loaded onto an 8% denaturing polyacrylamide gel.

Construction of Titration Binding Isotherms. Gels were exposed to photostimulable storage phosphor imaging plates (Kodak Storage Phosphor Screen S0230 obtained from Molecular Dynamics) in the dark at 24 °C for 5–12 h.[12] A Molecular Dynamics 400S PhosphorImager was used to obtain data from the phosphorimaging screens. Rectangles of the same dimensions were drawn around the cleavage bands at the target and at the reference sites. The ImageQuant v. 3.0 program running on an AST Premium 386/33 computer was used to integrate the volume of each rectangle.

Affinity Cleavage Titration Fitting Procedure. A detailed description of the affinity cleavage titration procedure has been published.[8] The relative cleavage efficiencies at the target site for each oligonucleotide concentration were determined by using the following equation:

$$I_{site} = I_{tot} - \lambda I_{ref} \tag{6}$$

where I_{tot} and I_{ref} are the intensities of the cleavage bands at the target site and at the reference site, respectively, and λ is a scaling parameter defined as the ratio I_{tot}/I_{ref} at the lowest oligonucleotide concentration. A theoretical binding curve, represented by eq 7 where I_{sat} is the apparent maximum cleavage, K_i the equilibrium association constant for oligonucleotide i, and $[O]_{tot}$ the oligonucleotide–EDTA concentration, was used to fit the experimental data using I_{sat} and K_i as adjustable parameters:

$$I_{fit} = I_{sat}\frac{K_i[O]_{tot}}{1 + K_i[O]_{tot}} \tag{7}$$

KaleidaGraph software (version 2.1, Abelweck Software) running on a Macintosh IIfx computer was used to minimize the difference between I_{fit} and I_{site} for all data points. All values reported in the text are the means of 3–6 experimental observations ± SEM, unless otherwise indicated. For graphical representation and comparison, I_{site} values were converted to apparent fractional occupancies (θ_{app}) by dividing I_{site} by I_{sat}.

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