Thermodynamics of Oligodeoxyribonucleotide-Directed Triple Helix Formation: An Analysis Using Quantitative Affinity Cleavage Titration

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Abstract: The free energy for oligodeoxyribonucleotide-directed triple helix formation at a single site on a DNA plasmid fragment has been analyzed using quantitative affinity cleavage titration. Measurement of the amount of site-specific cleavage of a 339-bp radiolabeled DNA duplex produced at 24 °C (100 mM Na^+, 1 mM spermine·4HCl, 50 mM Tris-acetate, pH 7.0) over a concentration change of four orders of magnitude for oligodeoxyribonucleotide–EDTA·Fe **1**·Fe, (5′-T*TTTTCTCTCTCTCT-3′) yields an equilibrium binding constant, $K_T = 3.7 \pm 1.1 \times 10^6\ M^{-1}$ ($\Delta G_T = -9.0 \pm 0.2$ kcal·mol^{-1}). Quantitative affinity cleavage titration affords association constants that are identical within experimental uncertainty with those obtained from quantitative DNase I footprint titration of the same oligonucleotide with and without EDTA·In (**1**·In and **6**, respectively). Removal of one thymidine and one cytidine residue from the 3′ end of **1**·Fe reduces the free energy of binding by 0.5 kcal·mol^{-1}, and removal of two thymidine and two cytidine residues from its 3′ terminus decreases the binding free energy by 1.1 kcal·mol^{-1} at pH 7.0. Single internal base triplet mismatches result in a destabilization of the local triple-helical structure by 2.5–3.0 kcal·mol^{-1}. Quantitative affinity cleavage titration is a general method which should allow for the measurement of equilibrium constants for the association of many DNA-binding molecules to single sites on relatively large DNA under a broad range of solution conditions.

Introduction

Oligonucleotide-directed triple helix formation is one of the most versatile methods for the sequence-specific recognition of double-helical DNA.[1-5] The ability to target a broad range of DNA sequences, the high stabilities of the triple-helical complexes, and the apparent sensitivity to single base mismatches make this a powerful technique for binding single sites within large segments of double-helical DNA.[1-5] This approach to DNA recognition has been used to mediate single site-specific cleavage of human chromosomal DNA[2e] as well as to inhibit transcription in vitro.[2f,5a]

At least two classes of DNA triple helices exist which differ in the sequence compositions of the third strand, the relative orientations and positions of the backbones of the three strands, and the base triplet interactions. Pyrimidine oligonucleotides bind purine tracts in the major groove of DNA parallel to the purine Watson–Crick strand, through the formation of specific Hoogsteen-type hydrogen bonds to the purine Watson–Crick bases.[1a,6] Specificity is derived from thymine (T) recognition of adenine·thymine (AT) base pairs (T·AT triplet; Figure 1) and N3-protonated cytosine (C+) recognition of guanine·cytosine (GC) base pairs (C+GC triplet; Figure 1). An additional family of triple-helical structures consists of purine-rich oligonucleotides bound in the major groove to purine tracts of DNA antiparallel to the Watson–Crick purine strand.[5c,7] Sequence specificity is derived from G recognition of GC base pairs (G·GC triplet) and from A or T recognition of AT base pairs (A·AT and T·AT triplets). The identification of other natural base triplets,[1e] alternate strand triple helix formation,[8] and the design of non-natural base triplets[1h,i] have demonstrated the generalizability of oligonucleotide-directed triple helix formation for the recognition of mixed sequences containing all four base pairs.

The stability of triple helices is dependent on the length, sequence composition, and functional modification of the oligonucleotide as well as solution conditions, including pH and cation concentrations. A full understanding of the factors contributing to the stability and specificity of the binding of oligonucleotides to double-helical DNA will require a complete characterization of the thermodynamics of complex formation. The measurement of binding constants at individual DNA sites represents the first step of this process. While some thermodynamic data has been reported for homopolymeric[9] and, more recently, oligonucleotide

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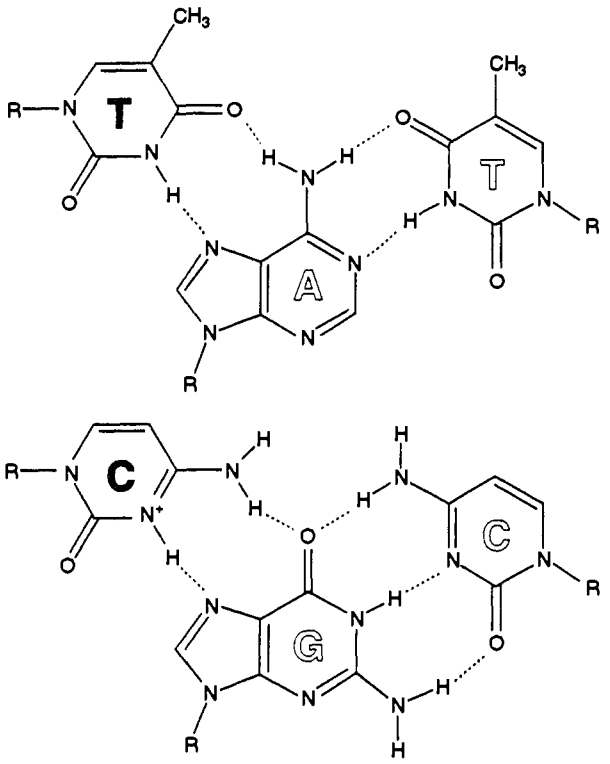

Figure 1. Two-dimensional models depicting the T·AT and C+GC base triplets formed by Hoogsteen hydrogen bonding of T to a Watson–Crick AT base pair (top) and by Hoogsteen-type hydrogen bonding of N3-protonated C to a Watson–Crick GC base pair (bottom), respectively. The bases of the third strand are labeled with shaded type and the bases of the Watson–Crick duplex are labeled with outline type.

triplexes at acidic pH,[10,11] we wish to measure thermodynamic parameters for oligonucleotide-directed triple helix formation at single sites on relatively large DNA (>200 bp) near pH 7.

The affinity cleaving technique, which has proven useful for studying DNA recognition, relies on the covalent attachment of a DNA cleaving agent to a DNA binding molecule and the subsequent generation of a nonspecific, diffusible oxidant near a bound ligand-DNA complex.[12] The affinity cleaving assay has been used previously to evaluate the relative binding strengths of oligonucleotide–EDTA·Fe conjugates.[1,5c,8a,c] These analyses relied on the assumption that the cleavage efficiency produced by an oligonucleotide–EDTA·Fe is proportional to the fraction of labeled duplex DNA bound by the oligonucleotide–EDTA·Fe. We report the extension of the affinity cleaving technique to the quantitative measurement of equilibrium association constants for the binding of oligodeoxyribonucleotide–EDTA conjugates to single sites on large DNA duplexes at neutral pH. By measuring the amounts of site-specific cleavage produced over four orders of magnitude of oligonucleotide–EDTA·Fe concentration, we can construct empirical titration binding curves and determine equilibrium association constants for oligodeoxyribonucleotide-directed triple helix formation. As an independent check of the method, we demonstrate that quantitative affinity cleavage titration affords association constants that are identical with those obtained from quantitative DNase I footprint titration. The affinity cleavage method has been used to measure the effects of covalent modification of an oligodeoxyribonucleotide with EDTA·Fe, oligonucleotide length, and internal base triplet mismatches on the stability of a local triple helical complex at a single site in large DNA.

Theory

Like the quantitative titration of DNase I footprints,[13,14] titration of an affinity cleaving molecule should allow for binding constant measurements at independent DNA sites. Moreover, quantitative affinity cleavage titration may offer a number of advantages over a footprint titration. The use of affinity cleavage eliminates the possibility of direct interactions between a complex and a footprinting agent that can reduce or eliminate a footprint. Furthermore, the use of a cleaving functionality that shows virtually sequence-independent cleavage, such as EDTA·Fe, allows all potential binding sites to be measured with similar accuracy. The fact that affinity cleavage experiments involving a tethered EDTA·Fe have been performed between pH 5.5 and pH 10, at temperatures between 0 and 45 °C, and in the presence of a variety of cations and cation concentrations, indicates that affinity cleavage titration may be possible under conditions where enzymatic or chemical footprinting is not feasible.[1,12] The intensities of densitometric bands from an affinity cleavage experiment constitute positive signals and, hence, substantially increase the signal-to-noise ratio in the primary data when compared to the observation of signal dimunition for a footprint.

A potential disadvantage of using affinity cleavage is that the EDTA·Fe moiety must be covalently attached to the ligand, and the affinity constants measured by this technique may be somewhat altered from those of the parent molecule. It should be possible to calibrate the effect of the EDTA·Fe group on the binding affinity by comparison with a footprint titration. While in the case of DNA affinity cleavage the ligand could, in principle, be any DNA affinity cleaving molecule, we will discuss only oligonucleotide–EDTA·Fe affinity cleavage here.

The theoretical development of the quantitative affinity cleavage titration technique is based on the formulation, by Ackers and co-workers,[13a] of the quantitative DNase I footprinting experiment. Using the definition of the fraction of DNA bound, these authors demonstrated that accurate binding constants could be measured when only the total ligand concentration and fractional DNA occupation were known quantities. We wished to adapt their ideas to affinity cleavage.

The equilibrium association constant for the one-to-one binding of a DNA duplex, D, by an oligonucleotide, O, to form a complex, T, containing a local triple-helical structure is defined by

$$O + D \rightleftharpoons T$$

$$K_T = \frac{[T]_{eq}}{[O]_{eq}[D]_{eq}} \qquad (1)$$

where $[T]_{eq}$, $[D]_{eq}$, and $[O]_{eq}$ are the equilibrium concentrations of the triple-helical complex, the unbound DNA duplex, and the unbound oligonucleotide, respectively. One can redefine the association constant based on the fraction of target DNA sites occupied by an oligonucleotide–EDTA, $\theta = [T]_{eq}/\{[T]_{eq} + [D]_{eq}\}$:

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$$K_T = \frac{\theta}{1 - \theta}\frac{1}{[O]_{eq}} \quad (2)$$

This variable, θ, may be related to an experimentally observable value in the following way. The amount of DNA cleavage products resulting from an oligonucleotide–EDTA·Fe bound to a radiolabeled DNA fragment is dependent on the amount of triple helix formed and the kinetics of the cleavage reaction. This relation is expressed by

$$[P_{cl}]_t = [T]_{eq}(1 - e^{-k_{cl}t}) \quad (3)$$

where $[P_{cl}]_t$ is the concentration of the DNA cleavage product at time t, and k_{cl} is the unimolecular rate constant for formation of the product from the bound complex. If the amount of this radiolabeled product is measured by autoradiography, then, as shown in the following equation, the intensity of the densitometric signal at the target site (I_{site}) will be proportional to the amount of cleavage product present ($|P_{cl}|$) and the specific activity of the radiolabel (A), where k_{dens} is the proportionality constant for the densitometric analysis

$$I_{site} = k_{dens}A|P_{cl}| \quad (4)$$

Equations 3 and 4 may be combined to express I_{site} as a function of $[T]_{eq}$:

$$I_{site} = k_{dens}AV_{rxn}(1 - e^{-k_{cl}t})[T]_{eq} \quad (5)$$

where V_{rxn} is the reaction volume. Using the definition of θ, eq 5 may be rewritten as

$$I_{site} = \{k_{dens}AV_{rxn}(1 - e^{-k_{cl}t})[D]_0\}\theta \quad (6)$$

where $[D]_0$ is the concentration of radiolabeled duplex DNA prior to initiation of the cleavage reaction. Thus, for a series of affinity cleaving reactions where the reaction time, reaction volume, and concentration of labeled duplex DNA are constant, θ will be proportional to the densitometric intensity at the binding site (I_{site}). Upon definition of the signal intensity resulting from cleavage at saturation binding as the constant I_{sat}, the affinity constant may be rewritten as a function of the cleavage signal intensity using eq 6

$$K_T = \frac{I_{site}}{I_{sat} - I_{site}}\frac{1}{[O]_{eq}} \quad (7)$$

Equation 7 leads to the important conclusion that an accurate determination of the affinity constant requires only that the intensity of the cleavage signal and the concentration of unbound ligand be measured. The amount of cleavage can be measured from the optical densities of bands on an autoradiogram or from the intensity of luminescence from photostimulable storage phosphor screens.[15] A reduction in the total amount of radiolabeled duplex DNA present in the reaction volume to a concentration of less than 5% of the lowest total oligonucleotide–EDTA concentration allows for the approximation that the concentration of unbound oligonucleotide is equal to the total concentration of oligonucleotide used in the reaction. Furthermore, because K_T is independent of the concentration of unbound DNA, the exact concentration of DNA does not need to be measured. This fact allows the use of radiolabeled DNA because it is not necessary to know the specific activity.

The amount of cleavage in a band at the recognition site (I_{tot}) has a specific component (I_{site}), which is produced by the affinity cleaving agent bound at the target site, and a nonspecific component (I_{uns}), which is produced by unbound oligonucleotide–EDTA

$$I_{tot} = I_{site} + I_{uns} \quad (8)$$

Although both components should vary with the concentration of the affinity cleaving reagent added, eq 7 treats only the specific component. I_{site} can be determined by rearranging eq 8, but, in general, I_{uns} is not directly measurable; however, I_{uns} should be proportional to the cleavage at a reference site (I_{ref}), which is spatially removed from the target sequence and has no specific component of cleavage. Thus, the specific component of cleavage will be given by

$$I_{site} = I_{tot} - \lambda I_{ref} \quad (9)$$

where λ is a scaling parameter, given by the ratio I_{tot}/I_{ref} at $\theta = 0$, that accounts for any intrinsic differences between the target and reference sites (e.g., number of bands quantitated). Rearrangement of eq 7, followed by substitution with the relationship for the specific component of cleavage and the approximation $[O]_{eq} = [O]_{tot}$ yields the form described by eq 10

$$I_{tot} - \lambda I_{ref} = I_{sat}\frac{K_T[O]_{tot}}{1 + K_T[O]_{tot}} \quad (10)$$

Hence, we can measure K_T by performing a series of affinity cleaving reactions in which only the concentrations of oligonucleotide–EDTA·Fe is varied, measuring the amount of cleavage products generated at the target site and at a reference site, generating a semilogarithmic plot of I_{site} versus $[O]_{tot}$ and fitting the data using a standard nonlinear least squares algorithm.

Results and Discussion

Oligodeoxyribonucleotide–EDTA·Fe Association Constants by Quantitative Affinity Cleavage Titration. To assess the association constants for oligonucleotides binding to a 339-bp DNA duplex, 5′-^{32}P-end labeled DNA (<100 pM) and various concentrations of an oligodeoxyribonucleotide–EDTA·Fe (1 nM–10 μM) were mixed in association buffer (100 mM Na^+, 1 mM spermine tetrahydrochloride, 50 mM Tris-acetate, pH 7.0) and incubated at 24 °C. The association reactions were allowed to equilibrate over 24 h. In fact, we observed no changes in the shapes of best-fit binding titration isotherms or measured values of K_T for experiments in which the equilibration time was greater than 18 h. The length of time required for the binding reaction to reach equilibrium is consistent with the saturation of a target duplex to 95% of its equilibrium occupancy within 3 min and 34 h in the presence of 10 μM and 10 nM, respectively, of a 21-mer oligonucleotide at 37 °C and pH 6.8.[2b] Only after the reactions had reached equilibrium was DTT added to initiate the EDTA·Fe-mediated cleavage chemistry. The duration of the cleavage reactions was constrained to allow for a maximum site-specific cleavage yield of about 5%. This constraint was enforced to maximize the statistical probability that specific cleavage of the target resulted from single-hit binding and cleaving and to ensure that a majority of the nonspecific cleavage products had lengths greater than those products from specific cleavage.[16] The products were separated by polyacrylamide gel electrophoresis under strand denaturing conditions. The results of a typical experiment performed using the Fe complex of oligodeoxyribonucleotide–EDTA **1** (**1**·Fe, Figure 2) are shown in Figure 3. The amounts of radiolabeled DNA in the bands at the target cleavage site (Figures 3 and 4) and at a reference site (Figure 3) were measured from a photostimulable storage phosphor autoradiogram, and the ([**1**·Fe], I_{site}) data points were plotted semilogarithmically along with the Langmuir titration binding isotherm, which was obtained by performing a nonlinear least squares fit (see eq 10 and the paragraph under the Affinity Cleavage Titration Fitting Procedure heading in the Experimental Section), in Figure 5. The mean value of the equilibrium association constant extracted from three such experiments is equal to $3.7 \pm 1.1 \times 10^6$ M^{-1} (Table I).

In order to validate the affinity cleavage titration method, we compared the association constant measured by affinity cleavage with that obtained from quantitative DNase I footprint titration,

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(16) Additional quantitative affinity cleavage titration experiments have revealed no changes in the shapes of binding isotherms or measured values of K_T when the cleavage reactions are allowed to proceed to yields of 20–25%. This independence likely arises from a combination of the relatively long triplex lifetimes (see ref 2b) and the high specificity of the oligonucleotide–EDTAs for the target sequence.

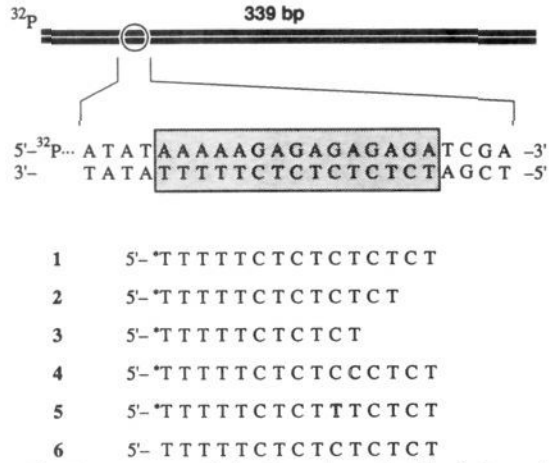

Figure 2. The sequences of oligodeoxyribonucleotides **1**–**6** are shown, where T* indicates the position of thymidine–EDTA. The sequence of the 15-bp target site (shaded box) is shown at the top, along with the *relative position of the target site within the radiolabeled duplex.* The nucleotides represented by outline type in oligonucleotides **4** and **5** indicate the positions where C·AT and T·GC mismatched triplets, respectively, are expected to be formed upon triple helix formation.

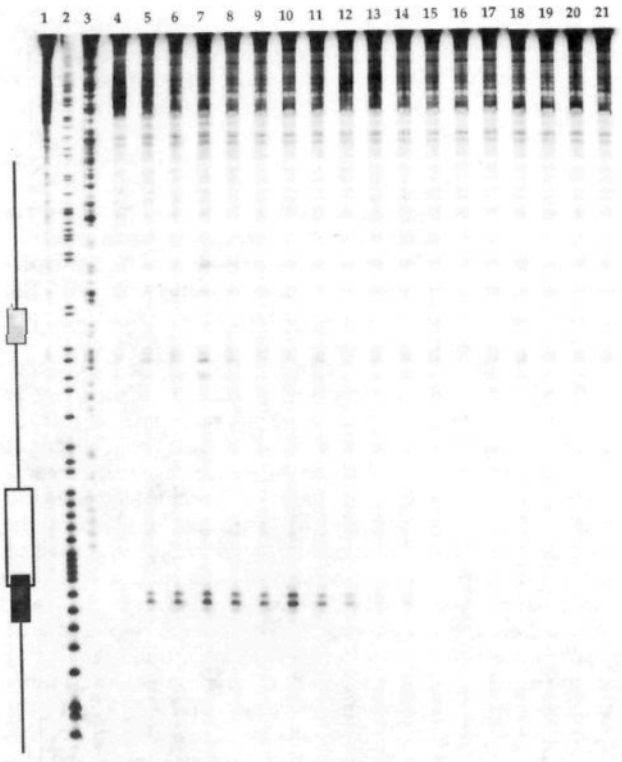

Figure 3. Autoradiogram of an 8% denaturing polyacrylamide gel used to separate the cleavage products generated by **1**·Fe during a quantitative affinity cleavage titration experiment performed in association buffer (100 mM Na^+, 1 mM spermine tetrahydrochloride, 50 mM Tris-acetate, pH 7.0) at 24 °C. The bar drawn on the left of the autoradiogram indicates the position of the 15-bp duplex target site (white box), the bands used to measure I_{tot} (black box), and the bands used to measure I_{ref} (grey box). (Lane 1) Intact 5′ labeled duplex obtained after incubation in association buffer at 4 °C in the absence of oligonucleotide–EDTA·Fe and DTT. (Lane 2) Products of an adenine specific sequencing reaction. (Lane 3) Products of a guanine specific sequencing reaction. (Lanes 4–21) DNA affinity cleavage products produced by **1**·Fe at various concentrations: no oligonucleotide (lane 4); 10.0 μM (lane 5); 7.5 μM (lane 6); 5.0 μM (lane 7); 2.5 μM (lane 8); 1.0 μM (lane 9); 750 nM (lane 10); 500 nM (lane 11); 250 nM (lane 12); 100 nM (lane 13); 75 nM (lane 14); 50 nM (lane 15); 25 nM (lane 16); 10 nM (lane 17); 7.5 nM (lane 18); 5.0 nM (lane 19); 2.5 nM (lane 20); 1.0 nM (lane 21).

a technique which has been previously used to examine the energetics of protein–DNA interactions and has given results that

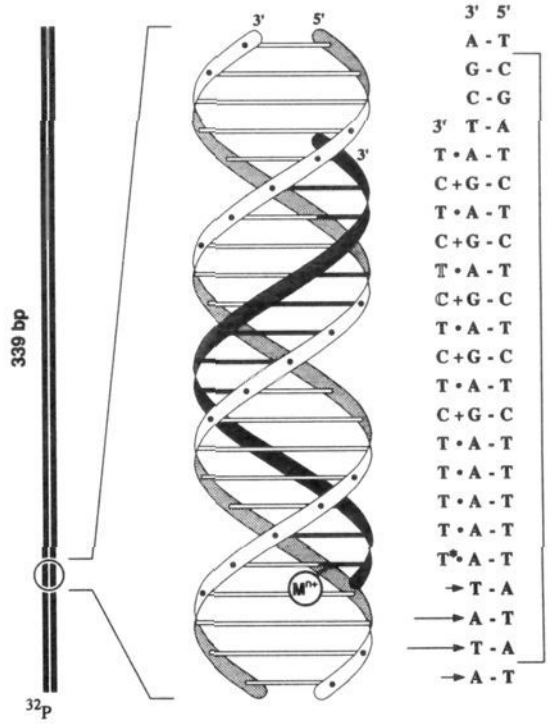

Figure 4. Ribbon model of the local triple-helical structure formed by the binding of 1·M^{n+} (M^{n+} = Fe^{2+} for affinity cleaving and In^{3+} for footprinting experiments) to the 15-bp target sequence within the 339-bp end labeled duplex. The Watson–Crick duplex strands are depicted as white ribbons, while the oligonucleotide–EDTA is depicted as a dark ribbon. The sequences modelled by the ribbons are shown at right. The nucleotide positions represented by outline type indicate the positions where mismatched base triplets will form when oligonucleotides **4** and **5** bind to the target duplex. The bracket to the right of the sequences indicates the nucleotide positions of the duplex protected from DNase I digestion when **1**·In or **6** is bound. *The four arrows indicate the four* nucleotide positions which are cleaved most efficiently by oligodeoxyribonucleotides **1**·Fe, **2**·Fe, **3**·Fe, **4**·Fe, and **5**·Fe, and the lengths of the arrows are proportional to the relative cleavage efficiency produced by **1**·Fe at the designated position.

are consistent with other physical methods.[13a] We performed the footprint titration essentially according to the published protocol. Briefly, oligodeoxyribonucleotide–EDTA **1** was allowed to chelate In^{3+}, which is redox inactive, and then various concentrations of the complex were mixed with end-labeled duplex in footprint buffer (10 mM Na^+, 20 mM Mg^{2+}, 20 mM Ca^{2+}, 1 mM spermine tetrahydrochloride, 50 mM Tris-acetate, pH 7.0)[17] and incubated at 24 °C. After 24 h, DNase I was added to each solution, and the digestion products were separated by polyacrylamide gel electrophoresis under denaturing conditions. The results of such a footprint titration are shown in Figure 6, and the region of the duplex footprinted is indicated on the model in Figure 4. The amounts of DNase I digestion at both the 15-bp target site and at a reference site (Figure 6) were measured by integrating the bands in a storage phosphor autoradiogram and the ([**1**·In], θ_{app}) data points were fit to the published equation for a titration binding isotherm (see ref 13a and the text under the Footprint Titration Fitting Procedure heading in the Experimental Section). The data points and the best fit curve for the titration shown in Figure 6 are plotted in Figure 7A. The mean value of the binding constant for association of **1**·In with the duplex target, as measured by footprint titration, is $2.8 \pm 0.5 \times 10^6$ M^{-1} (Table I). This is within the experimental uncertainty of the value $3.7 \pm 1.1 \times 10^6$ M^{-1}

(17) Quantitative affinity cleavage titrations performed in footprint buffer (10 mM Na^+, 20 mM Mg^{2+}, 20 mM Ca^{2+}, 1 mM spermine·4HCl, 50 mM Tris·OAc, pH 7.0) yielded apparent association constants identical to those obtained from experiments using association buffer (100 mM Na^+, 1 mM spermine·4HCl, 50 mM Tris·OAc, pH 7.0).

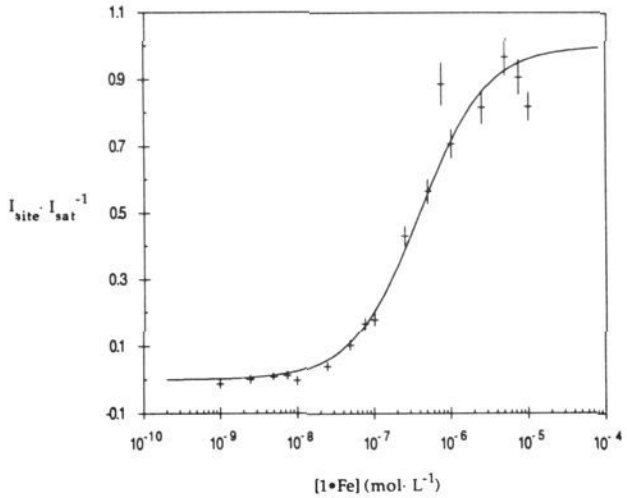

Figure 5. Data from the quantitative affinity cleavage titration experiment whose autoradiogram is shown in Figure 3. The I_{site} data were obtained using photostimulable storage phosphor autoradiography and eq 9 and have been normalized to a maximum value of 1 using the fit value of I_{sat}. The data points are represented by crosses whose horizontal and vertical bars indicate the ranges covered by the estimated uncertainties $[\mathbf{1}\cdot Fe]_{tot}$ and I_{site} (eq 14), respectively. The solid curve is the best fit Langmuir binding titration isotherm obtained from a nonlinear least squares algorithm using eq 11 and a value of 1 for I_{sat}.

Table I. Comparison of Footprint and Affinity Cleavage Titration Measurements of K_T at 24 °C and pH 7.0[a]

oligonucleotide	method[b]	K_T (M^{-1})
1•Fe	AC	3.7 (±1.1) × 10^6
1•In	F	2.8 (±0.5) × 10^6
6	F	3.7 (±0.4) × 10^6

[a] Affinity cleavage (AC) and footprint (F) titration experiments were performed at 24 °C in association buffer (100 mM Na^+, 1 mM spermine•4HCl, 50 mM Tris-acetate, pH 7.0) and footprint buffer (10 mM Na^+, 20 mM Mg^{2+}, 20 mM Ca^{2+}, 1 mM spermine•4HCl, 50 mM Tris-acetate, pH 7.0), respectively.[17] [b] The K_T value reported in row one was measured by quantitative affinity cleavage titration (AC), and the K_T values reported in rows two and three were measured by quantitative DNase I footprint titration (F).

obtained from affinity cleavage titration.

In a similar manner, we measured the association constant for oligodeoxyribonucleotide **6** (Figure 2), which lacks the metal-chelator functionality (i.e., the natural thymidine nucleoside has been substituted for T* at the 5′-end of the oligonucleotide), using quantitative DNase I footprint titration (Figure 7B). We obtained a mean value of $K_T = 3.7 \pm 0.4 \times 10^6$ M^{-1} (Table I) for the equilibrium binding of **6** to the duplex target site.

The results of the three types of experiments described in this section are summarized in Table I. The fact that the values of K_T measured by affinity cleavage titration and the established footprint titration method agree within the experimental uncertainty indicates that quantitative affinity cleavage will be useful for the reliable measurement of equilibrium binding constants for oligodeoxyribonucleotide-directed triple helix formation. Furthermore, a comparison of the measured K_T values from rows one and two of Table I with that from row three suggests that the covalent attachment of the EDTA moiety to a thymine heterocycle at the 5′-end of an oligodeoxyribonucleotide has little effect (i.e., one that is not measurable within experimental limitations) on the association of the oligonucleotide with its duplex target at 24 °C and pH 7.0.

The Influence of Oligodeoxyribonucleotide Length. Comparison of the K_T values for the equilibrium binding of **1**•Fe, **2**•Fe, and **3**•Fe (Figure 2) to the duplex target in association buffer at 24 °C reveals quantitative information concerning the effect of oligodeoxyribonucleotide length on binding affinity. The binding isotherms measured in association buffer are plotted in Figure 8A, and the K_T values are reported in the first three rows of Table

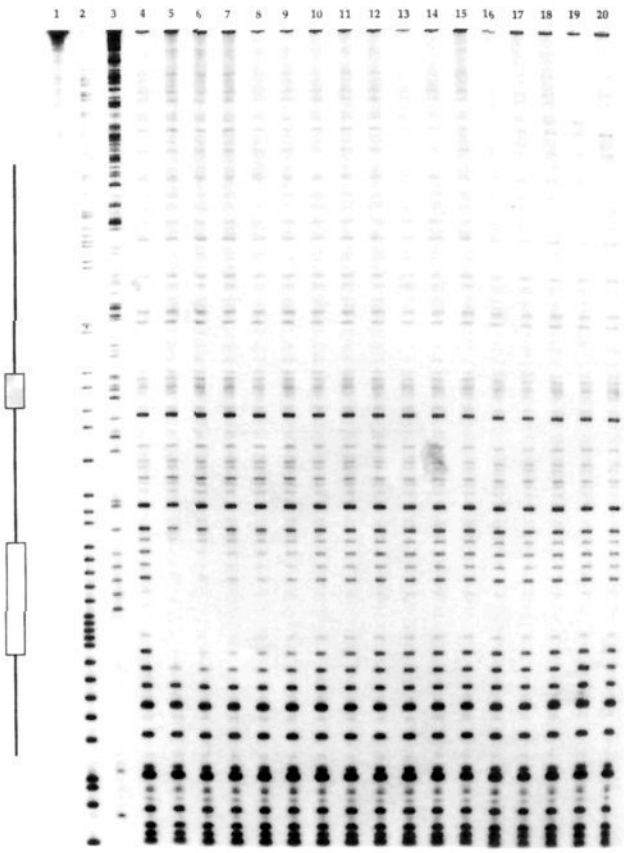

Figure 6. Autoradiogram of an 8% denaturing polyacrylamide gel used to separate the products generated by DNase I digestion during a quantitative footprint titration experiment using **1**•In and performed in footprint buffer (10 mM Na^+, 20 mM Mg^{2+}, 20 mM Ca^{2+}, 1 mM spermine tetrahydrochloride, 50 mM Tris-acetate, pH 7.0) at 24 °C. The bar drawn on the left of the autoradiogram indicates the bands used to measure I_{tot} within the 15-bp duplex target site (white box) and the bands used to measure I_{ref} (grey box). (Lane 1) Intact 5′ labeled duplex obtained after incubation in footprint buffer at 4 °C in the absence of oligonucleotide–EDTA•In and DNase I. (Lane 2) Products of an adenine specific sequencing reaction. (Lane 3) Products of a guanine specific sequencing reaction. (Lanes 4–20) DNase I digestion products produced in the presence of various concentrations of **1**•In: no oligonucleotide (lane 4); 8.0 μM (lane 5); 4.0 μM (lane 6); 2.0 μM (lane 7); 1.0 μM (lane 8); 800 nM (lane 9); 400 nM (lane 10); 200 nM (lane 11); 100 nM (lane 12); 80 nM (lane 13); 40 nM (lane 14); 20 nM (lane 15); 10 nM (lane 16); 8.0 nM (lane 17)l 4.0 nM (lane 18); 2.0 nM (lane 19); 1.0 nM (lane 20).

II. The association constants for binding of the 13-mer **2**•Fe and the 11-mer **3**•Fe are clearly reduced from that of **1**•Fe, a 15-mer. The quantitative difference in the binding constants for **1**•Fe, **2**•Fe, and **3**•Fe corroborate the relative ranking of the affinities of these three modified oligodeoxyribonucleotides that was reported earlier and was based on the amount of specific cleavage generated at a single concentration of each oligonucleotide.[1a]

An analysis of the energetics of binding for **1**•Fe, **2**•Fe, and **3**•Fe (Table II) reveals that removal of one thymidine residue and one cytidine residue from **1**•Fe reduces K_T by a factor of 2 ($\Delta G_2 - \Delta G_1 = 0.5$ kcal·mol^{-1}), and removal of two thymidine and two cytidine residues from **1**•Fe reduces K_T by a factor of 4 ($\Delta G_3 - \Delta G_1 = 1.1$ kcal·mol^{-1}). Surprisingly, the changes in the free energy of binding with decreasing strand length are 6-fold smaller than the changes in the free energy of duplex formation predicted from the sequences of **1**, **2**, and **3**.[18] The relatively modest dependence of triple helix stability on third-strand length likely arises from several sources. One probable factor is a reduced role in triple helix stabilization played by the cytidine residues in the third strand

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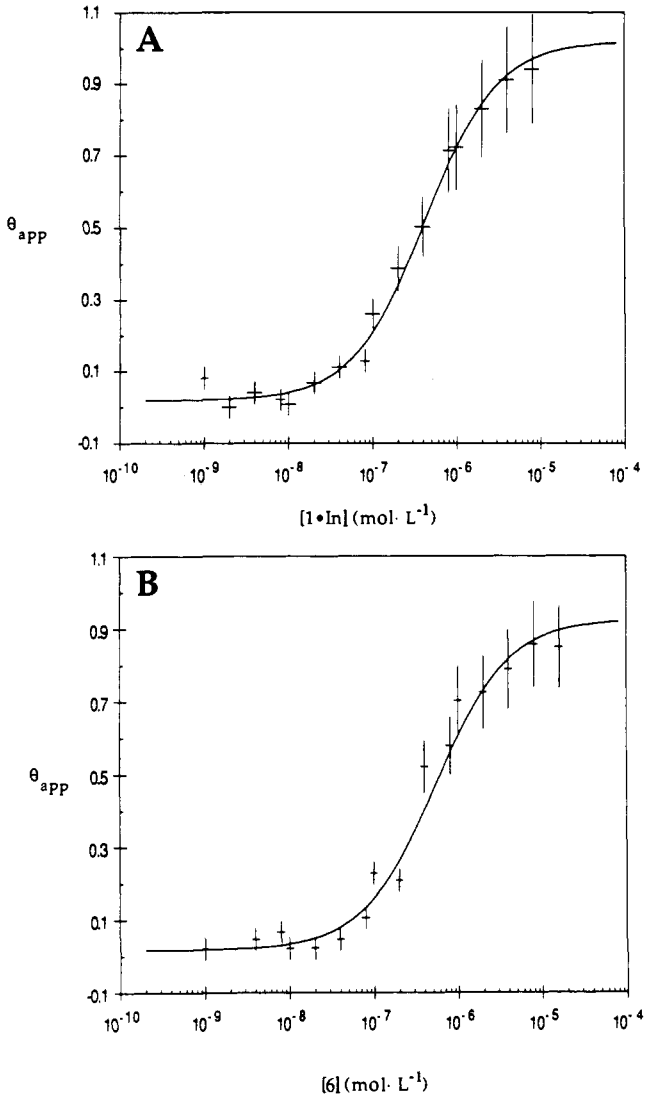

Figure 7. (A) Data from the quantitative DNase I footprint titration experiment whose autoradiogram is shown in Figure 6. The θ_{app} data were obtained using photostimulable storage phosphor autoradiography and eq 12. The data points are represented by crosses whose horizontal and vertical bars indicate the ranges covered by the estimated uncertainties in $[\mathbf{1}\cdot\mathrm{In}]_{tot}$ and θ_{app} (eq 15), respectively. The solid curve is the best-fit Langmuir binding titration isotherm obtained from a nonlinear least squares algorithm using eq 13. (B) Data from a quantitative DNase I footprint titration experiment using **6**. The experiment was performed exactly as the one whose results are presented in Figures 6 and 7A. The data are plotted as described for Figure 7A.

at pH 7.0 where these bases may not be protonated. In fact, preliminary evidence indicates that removing two thymidine residues from the 5′-terminus of **1** reduces K_T by a factor of nearly 10, suggesting that T·AT triplets confer higher energetic stabilization to the triplex than do C+GC triplets at pH 7.0.[19] Clearly, the actual energetic contributions to triple helix stability by single T·AT and C+GC base triplets will be distinct and will depend on the identity of nearest neighbor triplets[1i] as well as the pH of the buffer.

Influence of Base Sequence Mismatches. A comparison of the equilibrium binding constants for **4**·Fe and **5**·Fe, which contain single, internal C·AT and T·GC base triplet mismatches,[1a,c] respectively (Figures 2 and 4), with that for **1**·Fe provides information on the effect of sequence mismatches between an oligodeoxyribonucleotide and its target duplex. The data obtained from reactions in association buffer at 24 °C and the best-fit titration binding isotherms are plotted in Figure 8B. The measured K_T

(19) Singleton, S. F.; Dervan, P. B., unpublished observations.

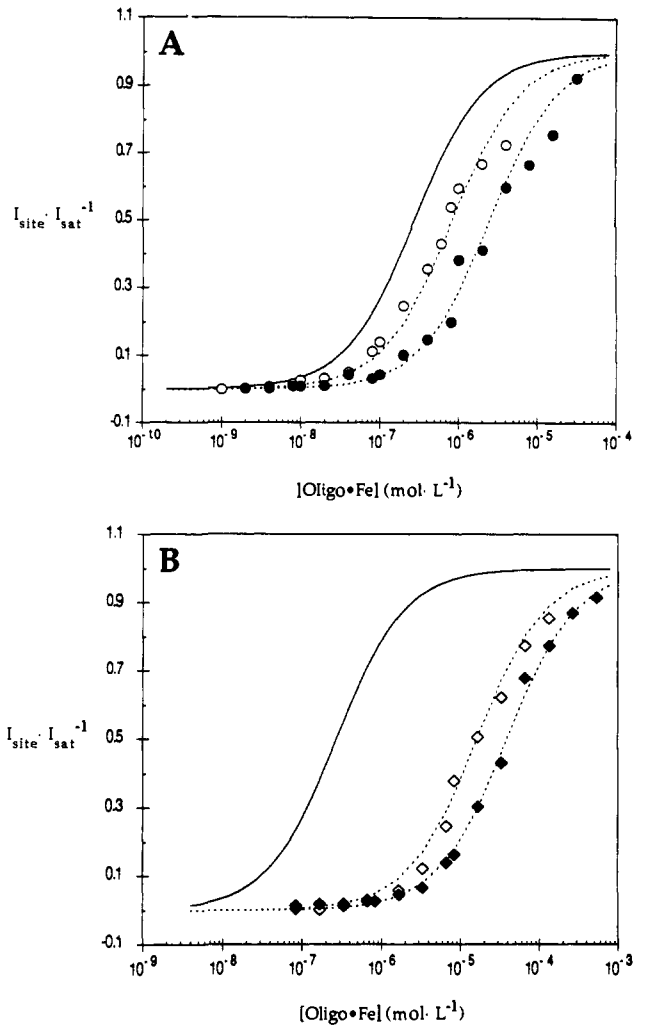

Figure 8. (A) Data for quantitative affinity cleavage experiments involving oligodeoxyribonucleotides **2**·Fe (○) and **3**·Fe (●) in association buffer at 24 °C. The data points represent the average site-specific cleavage signal intensities from three experiments. The dotted sigmoidal curves show the titration binding isotherms plotted using the mean values of K_T for **2**·Fe and **3**·Fe (Table II) and eq 11. The data points were normalized using I_{sat} from each experiment, and the binding curves were subsequently normalized using $I_{sat} = 1$ for eq 11. The solid curve shows the normalized titration binding isotherm obtained using the mean value of K_T for **1**·Fe. (B) Data for quantitative affinity cleavage experiments involving oligodeoxyribonucleotides **4**·Fe (◇) and **5**·Fe (◆) in association buffer at 24 °C. The data points represent the average site-specific cleavage signal intensities from three experiments. The dotted sigmoidal curves show the titration binding isotherms plotted using the mean values of K_T for **4**·Fe and **5**·Fe (Table II) and eq 11. The data points and fit binding curves were normalized as above. The solid curve shows the normalized titration binding isotherm obtained using the mean value of K_T for **1**·Fe.

values for **1**·Fe, **4**·Fe, and **5**·Fe are reported in rows one, four, and five of Table II, respectively. The results show that a single, internal base triplet mismatch can reduce the equilibrium binding constant for an oligodeoxyribonucleotide by two orders of magnitude, a result that is consistent with the relative amounts of cleavage generated by these three oligonucleotides at a single concentration.[1a]

An analysis of the differences in binding free energy between **1**·Fe and **4**·Fe and between **1**·Fe and **5**·Fe (Table II) indicates that a single internal C·AT mismatch results in a 2.5 kcal·mol^{-1} loss of stability for the triple helical complex, while a single internal T·GC mismatch results in a 3.0 kcal·mol^{-1} stability loss. Although we expect that the energetic penalties for a mismatch will be affected by nearest neighbor base triplets, the energetic changes observed here are consistent with those recently derived from equilibrium competition experiments and shape analysis of UV

Table II. Length and Sequence Mismatch Effects on K_T at pH 7.0 and 24 °C

oligo-nucleotide	length (nts)	no. T's (nts)	no. C's (nts)	base mismatch	K_T (M^{-1})	ΔG_T (kcal·mol^{-1})
1	15	10	5		3.7 (±1.1) × 10^6	−9.0 (±0.2)
2	13	9	4		1.8 (±0.4) × 10^6	−8.5 (±0.1)
3	11	8	3		6.2 (±3.8) × 10^5	−7.9 (±0.6)
4	15	9	6	C·AT	6.1 (±2.3) × 10^4	−6.5 (±0.2)
5	15	11	4	T·GC	2.5 (±0.1) × 10^4	−6.0 (±0.1)

[a] Values reported in the table are mean values measured from affinity cleavage titration experiments performed in association buffer (100 mM Na^+, 1 mM spermine tetrahydrochloride, 50 mM Tris-acetate, pH 7.0).

melting curves for oligonucleotide triplexes at pH 5.0[10g] and with the energetic destabilization range estimated by two-dimensional gel electrophoresis studies of H-DNA triplexes.[20] Moreover, thermal denaturation studies at pH 5.6[21] and NMR spectroscopic investigations at pH 5.2[6c] are consistent with a large degree of destabilization resulting from mismatched C·AT and T·GC base triplets. Perhaps of most interest is the observation that our measured free energies for triplex destabilization are near those reported for the destabilization of DNA[22] and RNA[23] duplexes with mismatched base pairs. The high specificity for a designated duplex target sequence afforded by oligonucleotide-directed triple helix formation demonstrates clearly the potential functional application of oligonucleotides or their analogues to problems of DNA recognition in biology and human medicine.[24]

Conclusion

A method for the determination of equilibrium constants for the formation of local triple-helical complexes at single sites within large DNA using affinity cleavage titration experiments has been presented. Equilibrium constants for oligodeoxyribonucleotide–EDTA binding measured using this method are identical within experimental uncertainty with those measured by DNase I footprint titration and indicate that covalent attachment of EDTA·Fe to the 5′-terminal base of an oligodeoxyribonucleotide does not have a measurable effect on the binding constant. The order of thermodynamic stabilities obtained by comparing the magnitudes of the oligonucleotide binding constants is consistent with earlier qualitative rankings obtained from comparison of the amounts of site-specific cleavage produced at a single oligonucleotide–EDTA·Fe concentration.[1a] An analysis of the thermodynamics of oligodeoxyribonucleotide binding at pH 7.0 revealed only a modest energetic dependence on oligonucleotide length but a substantial penalty (2.5–3.0 kcal·mol^{-1}) for a single internal base triplet mismatch. Moreover, prior energetic analyses of triple helices using short oligonucleotide duplex targets and acidic solution conditions[10,18] can now be compared with the affinity cleavage thermodynamic data for single-site triple helix formation on large DNA at pH 7.0.

Because the synthesis of affinity cleaving molecules has proven to be general[12] and because affinity cleavage experiments can be performed under a wide range of conditions,[1,12] the use of quantitative affinity cleavage titration should allow further thermodynamic analyses of DNA binding by oligonucleotides or other ligands under solution conditions where experiments involving oligonucleotide duplexes or competitive footprinting agents may not be successful.

Experimental Section

General Methods and Materials. Sonicated, deproteinized calf thymus DNA (Pharmacia) was dissolved in H_2O to a final concentration of 2.0 mM in base pairs and was stored at 0 °C. Glycogen was obtained from Boehringer-Manheim as a 20 mg/mL aqueous solution. Nucleotide triphosphates were Pharmacia Ultra-Pure grade and were used as supplied. Nucleoside triphosphates labeled with ^{32}P (≥5000 Ci/mmol) were obtained from Amersham. Cerenkov radioactivity was measured with a Beckman LS 2801 scintillation counter. Restriction endonucleases were purchased from Boehringer Manheim or New England Biolabs and were used according to the supplier's recommended protocol in the activity buffer provided. Vent polymerase and T4 polynucleotide kinase were obtained from New England Biolabs. DNase I was purchased from Pharmacia. Phosphoramidites were purchased from ABI. A pH of 7.0 during equilibration was maintained with a Tris-acetate buffer system. The pH of the equilibration solutions was measured as follows: a 1-mL solution containing all buffer and salt components (except radiolabeled DNA) at concentrations identical to those used in the reactions was prepared and equilibrated at the reaction temperature, and the pH of the buffer–salt solution was recorded using a digital pH/millivolt meter (Model no. 611, Orion Research) and a ROSS semimicro combination pH electrode (Model no. 81-15, Orion Research). General manipulations of duplex DNA[25] and oligonucleotides[26] were performed according to established procedures.

Oligodeoxyribonucleotide–EDTA Synthesis. Oligodeoxyribonucleotides (Figure 2) were synthesized by standard automated solid-support chemistry using an Applied Biosystems Model 380B DNA synthesizer and *O*-cyanoethyl-*N*,*N*-diisopropyl phosphoramidites. The conjugate thymidine-EDTA (T*) phosphoramidite was prepared as described[27] and incorporated at the 5′ end of oligodeoxyribonucleotides **1–5** with the EDTA carboxylates protected as ethyl esters. Deprotection was carried out in 0.1 N NaOH solution at 55 °C for 24 h. Crude oligodeoxyribonucleotide products containing the 5′-terminal dimethoxytrityl protecting group were purified by reverse phase FPLC using 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% aqueous acetic acid, and chromatographed a second time. Purified oligodeoxyribonucleotides were desalted on Pharmacia NAP-5 Sephadex columns followed by extensive dialysis against H_2O. The concentrations of single-stranded oligodeoxyribonucleotides were determined by UV absorbance at 260 nm using extinction coefficients calculated by the nearest-neighbor method from the monomer and dimer values (values for T were used in place of T* in the calculation).[28] Oligodeoxyribonucleotide solutions were lyophilized to dryness for storage at −20 °C.

DNA Preparation. The 5′-^{32}P-labeled duplex was prepared by PCR amplification of a 438-bp segment from the plasmid pDMAG10,[1a] using a "Vector PCR" procedure.[29] The primers used for amplification, 5′-GAGGCCCTTTCGTCTTCAAG-3′ (5′pDM-EcoRI) and 5′-TGCGGCGACGATAGTCATGC-3′ (3′pDM-SalI), were synthesized and purified as described above. The primer 5′pDM-EcoRI was 5′-end labeled using γ-^{32}P-ATP and T4 polynucleotide kinase and was subsequently purified on a Pharmacia NICK column.

PCR-based amplifications were performed in a 0.6-mL microcentrifuge tube as follows. To the tube were added 25 nmol each dNTP, 50

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pmol primer 3'pDM-SalI, approximately 50 pmol 5'-^{32}P-labeled primer 5'pDM-EcoRI, acetylated BSA, and an AmpliWax PCR Gem (Perkin-Elmer Cetus) to a volume of 70 μL in 1× Vent Buffer (supplied by New England Biolabs). This solution was heated to 80 °C for 5 min and then cooled to 4 °C. To the tube were added 2 units of Vent DNA polymerase and 10 ng of pDMAG10 in a volume of 30 μL 1× Vent buffer. The cycling reactions were performed in a Perkin-Elmer Cetus DNA Thermal Cycler, with each cycle consisting of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min, and extension at 72 °C for 1 min. The extension time during the last cycle was 10 min to ensure that all single stranded DNA had been copied or reannealed to form duplex. Thirty cycles were performed. The completed reactions were extracted twice with equal volumes of phenol and desalted on a NICK column. The PCR products were digested with Eco NI restriction endonuclease, and the products were separated on an 8% polyacrylamide gel. The gel band corresponding to the labeled 339-bp fragment was excised, crushed, and soaked in elution buffer (25 mM Tris·HCl, 250 mM NaCl, 5 mM EDTA, 0.1% SDS, pH 8.0) at 37 °C for 18 h. This suspension was filtered through a Millipore UltraFree DuraPore filter, and the DNA was precipitated with ethanol. Following washing with 70% ethanol and vacuum drying of the precipitate, the labeled DNA was resuspended in 0.5-× TE buffer at a final activity of 40000 cpm per μL. A typical yield was 2 μg of the desired fragment with a total Cerenkov radioactivity of 6×10^6 cpm.

Quantitative Affinity Cleavage Titrations. In a typical quantitative affinity cleaving experiment, involving 15 data lanes and one control lane, a stock solution containing labeled target DNA in association buffer was prepared by mixing 0.5 M Tris-acetate buffer at pH 7.0, 2.5 M NaCl, calf thymus DNA at a concentration of 2.0 mM in bp, 10 mM spermine·4HCl at pH 7.0, approximately 300000 cpm 5'-end labeled target DNA, and enough H_2O to bring the total volume to 1.36 mL. The stock solution was allowed to equilibrate at room temperature for 15 min and was then distributed among 16 0.5-mL microcentrifuge tubes in 80-μL aliquots. A dried pellet of the oligodeoxyribonucleotide–EDTA was dissolved in a solution of aqueous $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ to produce a solution that was 80 μM in oligodeoxyribonucleotide and 88 μM in Fe(II). The oligodeoxyribonucleotide–EDTA·Fe(II) solution was allowed to equilibrate for 15 min at room temperature and was then diluted serially. To each reaction tube was added 10 μL of oligodeoxyribonucleotide–EDTA·Fe(II) at the appropriate concentration. The oligodeoxyribonucleotide–EDTA·Fe and the DNA were allowed to equilibrate for 24 h at 24 °C. The cleavage reactions were initiated by the addition of 10 μL of a 40 mM aqueous DTT solution to each tube. The reactions were incubated for 6 h at 24 °C. Final reaction conditions in 100 μL association buffer were 50 mM Tris-acetate at pH 7.0, 100 mM NaCl, 1 mM spermine, 0.1 mM-bp calf thymus DNA, 4 mM DTT, and approximately 17500 cpm labeled duplex (the specific activity of the DNA varied slightly from experiment to experiment but was the same for each reaction within a given experiment and always resulted in a final target site concentration of less than 0.1 nM). The cleavage was quenched by the addition of EDTA, glycogen, NaOAc (pH 5.2), and $MgCl_2$ to final concentrations of 1.0 mM, 70 μg/mL, 280 mM, and 10 mM, respectively. The DNA was precipitated with 2.5 volumes of ethanol and isolated by centrifugation. The precipitate was washed once with 70% aqueous ethanol and was immediately dissolved in 20 μL of H_2O. The solutions were frozen, and the water was removed by lyophilization. The DNA in each tube was resuspended in 5 μL of formamide-TBE loading buffer and transferred to a new tube. The DNA solutions were assayed for Cerenkov radioactivity by scintillation counting and diluted to 2500 cpm/μL with more formamide-TBE loading buffer. The DNA was denatured at 90 °C for 5 min, and 5 μL of each sample were loaded onto an 8% denaturing polyacrylamide gel prepared with a special comb consisting of 20 6-mm teeth separated by 6 mm and surrounded by seven 6-mm teeth separated by 3 mm. The DNA cleavage products were electrophoresed in 1× TBE buffer at 50 V·cm^{-1}. The gel was dried on a slab dryer and then exposed to a storage phosphor screen at 24 °C.

Quantitative DNase I Footprint Titrations. DNase I footprinting experiments were run essentially as described,[13a] in a manner analogous to the quantitative affinity cleaving experiment. Only the differences are described below. Oligodeoxyribonucleotide–EDTAs were reconstituted with $InCl_3$ rather than $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$. The stock solution of radiolabeled DNA in footprint buffer was prepared from 0.5 M Tris-acetate buffer at pH 7.0, 2.5 M NaCl, 1.5 M $MgCl_2$, 1.5 M $CaCl_2$, calf thymus DNA at a concentration of 2.0 mM in bp, 10 mM spermine·4HCl at pH 7.0, approximately 300000 cpm 5'-end labeled target DNA, and enough H_2O to bring the total volume to 1.36 mL. Following equilibration of the oligodeoxyribonucleotide with the target duplex, DNase I and the nonspecific oligonucleotide $(dA)_{10}$, which was used to maintain uniform DNase I reactivity, were added and the digestion allowed to proceed at 24 °C for 10 min. Final reaction conditions in 100 μL footprint buffer were 50 mM Tris-acetate at pH 7.0, 20 mM NaCl, 10 mM $MgCl_2$, 10 mM $CaCl_2$, 1 mM spermine, 0.12 mM bps calf thymus DNA, 1 μM $(dA)_{10}$, approximately 17500 cpm labeled duplex, and 0.5 units DNase I. Reaction workup and electrophoresis were performed as above.

Quantitation by Storage Phosphor Technology Autoradiography. Photostimulable storage phosphor imaging plates (Kodak Storage Phosphor Screen S0230 obtained from Molecular Dynamics) were pressed flat against dried gel samples and exposed in the dark at 24 °C for 12–24 h. A Molecular Dynamics 400S PhosphorImager was used to obtain all data from the storage screens. The data were analyzed by performing volume integrations of the target site and reference blocks using the ImageQuant v.3.0 software running on an AST Premium 386/33 computer. A description of these phosphor imaging plates and a characterization of their behavior has been presented.[15]

Affinity Cleavage Titration Fitting Procedure. The site-specific cleavage for all oligodeoxyribonucleotide–EDTA concentrations used were calculated using eq 9, where I_{tot} and I_{ref}, the cleavage intensities in the site and reference blocks, respectively, were determined from integration of storage phosphor images as described above. For each experiment, λ was calculated from the average of the minimum I_{tot}/I_{ref} ratios near $\theta = 0$. For example, for the experiment whose results are shown in Figures 3 and 5, λ was calculated using the average of the ratios of I_{tot} to I_{ref} from each of the three lowest oligonucleotide concentrations (Figure 3, lanes 19–21) and the control reaction containing no oligonucleotide (Figure 3, lane 4). A theoretical binding curve was fit to the experimental data using the apparent maximum cleavage (I_{sat}) and the K_T as adjustable parameters:

$$I_{fit} = I_{sat}\frac{K_T[O]_{tot}}{1 + K_T[O]_{tot}} \tag{11}$$

The difference between I_{fit} and I_{site} for all data points was minimized using the nonlinear least squares fitting procedure of KaleidaGraph software (version 2.1; Abelbeck Software) running on a Macintosh IIfx computer. All data points were included in the fitting procedure unless visual inspection of the computer image from a storage phosphor screen revealed a flaw at either the target site or reference blocks, or the I_{site} value for a single lane was greater than two standard errors away from both values from the neighboring lanes. Data from experiments for which fewer than 80% of the data lanes were usable were discarded. Typically, four-fifths of the gels resulted in usable data, each with 13 acceptable data lanes out of 15 total. The goodness of fit of the binding curve to the data points was judged by the χ^2 criterion, and fits were judged acceptable for $\chi^2 \leq 1.0$. Correlation coefficients reported for acceptable fits were ≥0.95.

Footprint Titration Fitting Procedure. The analysis of DNase I footprint titrations was performed according to the previously described method.[13a] Briefly, θ_{app} was determined using the following equation:

$$\theta_{app} = 1 - \frac{I_{tot}/I_{ref}}{I_{tot}^{\circ}/I_{ref}^{\circ}} \tag{12}$$

where I_{tot} and I_{ref} are defined as above, and I_{tot}° and I_{ref}° indicate the amount of digestion at the target site and at the reference site in a DNase I control to which no oligodeoxyribonucleotide was added, respectively. The $([O]_{tot}, \theta_{app})$ data points were fit by minimizing the difference between θ_{app} and θ_{fit}, using eq 13, where θ_{min} and θ_{max} represent the experimentally observed values of the degree of saturation when $\theta = 0$ and $\theta = 1$, respectively:

$$\theta_{app} = \theta_{min} + (\theta_{max} - \theta_{min})\cdot\frac{K_T[O]_{tot}}{1 + K_T[O]_{tot}} \tag{13}$$

Error Analysis. All fits described in this text were performed without weighting the data points. For quantitative affinity cleavage and footprint titrations, the error in the observed intensity was proportional to the uncertainty in the radioactivity of the DNA sample loaded onto the lane in question. By making use of the formula for propagation of errors, we can derive the following equation describing the uncertainty in I_{site}, where ρ is the fractional error in the loading:

$$\epsilon(I_{site}) = \rho[I_{tot}^2 + 3(\lambda I_{ref})^2]^{1/2} \tag{14}$$

Similarly, we were able to obtain an equation expressing the uncertainty in θ_{app} obtained from footprint titration:

$$\epsilon(\theta_{app}) = 2\rho\theta_{app} \tag{15}$$

There are two main sources of error in the oligodeoxyribonucleotide concentrations, one being the initial uncertainty from employing the additive nucleoside extinction coefficients for calculating the oligodeoxyribonucleotide molar extinction coefficient, and the second from

pipetting errors during the serial dilution. By using relatively large volumes (i.e., ≥ 10 μL) and well-calibrated pipetors, one can minimize the error resulting from the latter. However, the approximately 10% error resulting from the former source is propagated throughout. A fit of data points using statistical weighting and the described uncertainties results in uncertainties in the fitting parameter K_T that were typically 20–30% of the value of K_T.

Repeat experiments using a particular oligodeoxyribonucleotide–EDTA·Fe were performed using different serial dilutions of the oligonucleotide and different preparations of 5′-end labeled duplex DNA. Standard deviations among different experimental measurements of K_T were typically 30–50% of the mean K_T. All K_Ts reported in the text or tables are means of three to five experimental observations ±SEM.

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