A Fluorescent Intercalator Displacement Assay for Establishing DNA Binding Selectivity and Affinity

WINSTON C. TSE AND DALE L. BOGER*
Department of Chemistry and the Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037

Received May 13, 2003

ABSTRACT
A summary of the qualitative and quantitative elements of a fluorescent intercalator displacement (FID) assay useful for establishing the DNA binding selectivity, affinity, stoichiometry, and binding site size and distinguishing modes of DNA binding is provided.

Introduction

The regulation of gene expression is based on the sequence-selective recognition of nucleic acids by repressor, activator, and enhancer proteins. A full understanding of the proteins involved, the delineation of the sequences to which they bind, and the discovery of the genes that they regulate holds significant promise in therapeutic medicine.[1−6] Thus, extensive efforts continue to be directed at understanding the transcriptional process and are being increasingly directed at the discovery of small molecules that selectively bind DNA and activate (block a repressor) or inhibit (block an activator) gene expression.[7−11]

Of the techniques commonly used to establish the DNA binding properties of small molecules and proteins, most are technically challenging, require the knowledge of specialized biochemical procedures, and are time and labor intensive. The most widely used methods are footprinting[12] and affinity cleavage.[13] Because of the power of the technique, a number of such methods have been introduced including DNase I,[14] exonuclease III,[15] MPE−Fe(II),[16] 1,10-phenanthroline−Cu(I),[17] and EDTA−Fe(II)[18] footprinting. Complementary approaches for disrupting binding (interference footprinting)[19] by specific base or phosphate modifications have also been introduced. Less general techniques that capitalize on a compound's intrinsic DNA cleavage (e.g., bleomycin, endiynes), alkylation/thermal cleavage[20] (e.g., CC-1065, duocarmycins), alkylation (inhibition of in vitro transcription), or cross-linking[21,22] (e.g., mitomycin) properties have been applied to selected classes of molecules. Recently, techniques for expanding the sequence coverage of footprinting[23] and the use of electrophoretic mobility shift assays (EMSA, gel retardation assay)[24] and DNase I footprinting[25,26] for the iterative deconvolution of mixture libraries have been disclosed, expanding their applications. Inherent in these methods is the characterization of the highest affinity sites within a size-limited segment of DNA. Similarly, the DNA binding properties of proteins[27] are typically assessed by selection screening,[28−30] footprinting,[14] or EMSA.[31] The former provides exhaustive sequence coverage for deducing the preferred site(s), but it selects only the highest affinity sites and does not provide quantitative binding information. Footprinting and EMSA[31] have been used to define, or at least refine, a protein's binding selectivity, but their most frequent uses have been to provide qualitative distinctions and quantitative comparisons among candidate binding sites or those constructed to assess single base pair (bp) substitutions.

Herein, we review a complementary technique, a fluorescent intercalator displacement (FID) assay,[32,33] for establishing DNA binding affinity, sequence selectivity, and binding stoichiometry. The assay is nondestructive, technically nondemanding, and amenable to high-throughput screening. The former feature would permit DNA immobilization onto reusable supports for repetitive use and expansion of the sequence space beyond that presently exemplified (all 5-bp sites). For a single compound, the technique permits establishment of a rank order binding profile for all possible 5-bp sites, comprehensively defining the sequence selectivity in a single experiment. For a defined sequence, it permits the high-throughput identification of binding agents from a library of compounds or quantitative titrations for establishment of binding constants. The assay is not limited to small molecule assessments and has been used with a variety of ligands, including proteins and triplex-forming oligonucleotides.

The FID Assay

The assay utilizes the displacement of ethidium bromide (or thiazole orange)[34,35] from hairpin deoxyoligonucleotides (Figure 1). Hairpin DNAs are treated with the intercalator, yielding a fluorescence increase upon binding. Addition of a DNA binding compound results in a decrease in fluorescence due to displacement of the bound intercalator, where the percent fluorescence decrease is directly related to the extent of binding. For a panel of DNA sequences displayed in a 96-well format, the resulting profile of percent fluorescence decrease provides relative binding affinities and comprehensively defines a compound's sequence selectivity. With individual sequences, quantitative titration of a compound against a hairpin prebound with ethidium provides reliable binding constants and the stoichiometry of binding. These two systems may be used in a complementary fashion, one intended for a high-throughput screen

Dale L. Boger is the Richard and Alice Cramer Professor of Chemistry at The Scripps Research Institute and a member of the Skaggs Institute for Chemical Biology.

Winston C. Tse received a B.A. from Northwestern University, where he worked in the laboratory of Professor Joseph B. Lambert, and his Ph.D. from The Scripps Research Institute with Professor Boger. He joined Gilead Sciences in 2003.

* Corresponding author. Tel.: (858) 784-7522. Fax: (858) 784-7550. E-mail: boger@scripps.edu.

10.1021/ar030113y CCC: $27.50
Published on Web 11/18/2003

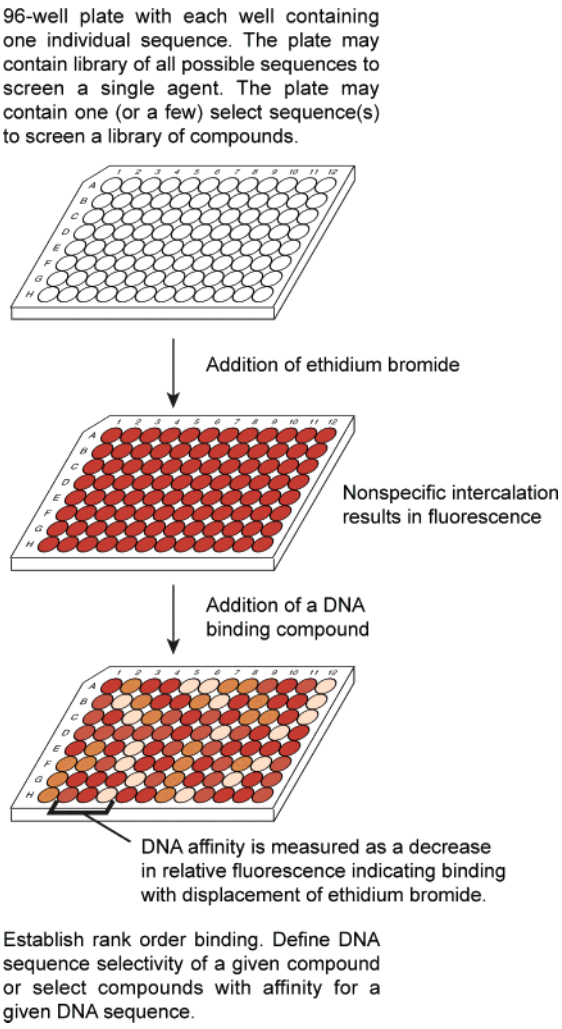


FIGURE 1. The FID assay.

capable of handling large compound libraries or providing comprehensive sequence preference data, and one intended for quantitative elucidation of DNA−ligand interactions and establishing binding constants.

Following the characterization of the intrinsic fluorescent increase that accompanies DNA intercalation,[36] the displacement of DNA-bound ethidium bromide has been widely used to establish DNA binding.[37] Ethidium's nonselective,[38] rapidly equilibrating, and low binding affinity ($\sim 10^5$ M^{-1})[36] allows assessment of compounds with low binding affinity or assessment of low affinity sites, aspects not easily addressed by other techniques. For tight binding sequences, the ethidium displacement proceeds in a virtually noncompetitive manner, permitting quantitative binding assessments.

Thiazole orange[34,35] is an effective alternative intercalator that addresses three issues: (1) its excitation and emission maxima are distinct from those of ethidium, (2) its fluorescent enhancement upon intercalation exceeds that of ethidium (ca. 3000-fold vs 20-fold), and (3) it displays less sequence-dependent DNA binding, albeit with a higher affinity.[39] This permits the examination of systems where there would be ethidium fluorescence interference and screening at lower concentrations or with greater sensitivities than achievable with ethidium, and

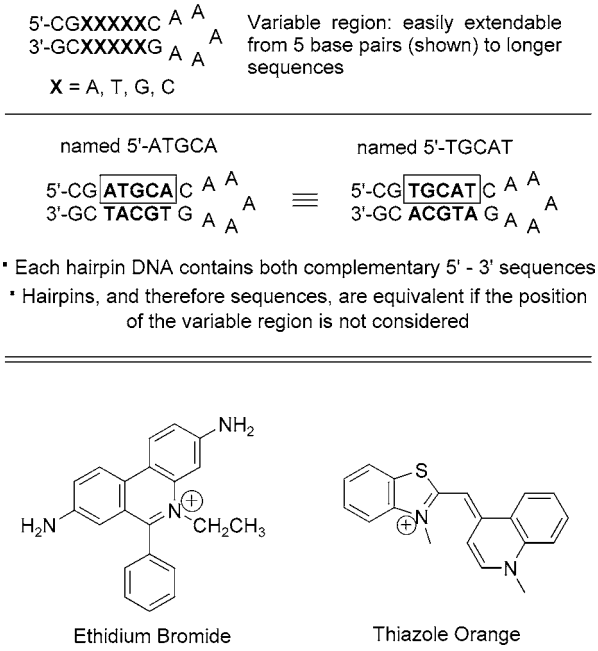


FIGURE 2. Structures of hairpin deoxyoligonucleotides, ethidium bromide, and thiazole orange.

with less sequence variation and greater consistency in the absolute fluorescence readings. However, the binding constants established with thiazole orange are slightly lower than those established with ethidium.[32,39] This reflects the higher association constant and greater competitive binding of thiazole orange, reducing the measured apparent binding constant.

Hairpin deoxyoligonucleotides[40] (Figure 2) proved especially useful in the FID assay. Embedded in the hairpin are two complementary 5′-to-3′ sequences, connected by a loop, avoiding the requirement for two separate strands and the associated additional quantitation and mixing. The number of hairpins required to create a library of sequences is half the number of sequences. For example, 512 hairpins are required for a library of all 1024 possible 5-bp sequences (Figure 2). Moreover, the hairpins were established to provide stable duplexes at working temperatures (25 °C), independent of the sequence.[32,33] The variable most critical to the success of the assay, and most likely to be responsible for avoidable errors, is the quality of the hairpins. In addition to the concern about their constitution and purity, their concentration is critical and may be determined by measuring the UV absorption (260 nm) of the denatured, single-stranded DNA at 80−95 °C. Since the hairpins exist in a construct representing a combination of double- (stem) and single-strand (loop) DNA at 25 °C, calculations based on the UV absorption at 25 °C, utilizing the standard coefficients useful for single-stranded deoxyoligonucleotides, underestimate the concentration by as much as 25%.[41]

Binding Constants and Stoichiometry

Quantitative displacement of ethidium from a hairpin deoxyoligonucleotide provides a well-defined titration curve that is useful for establishing binding constants and

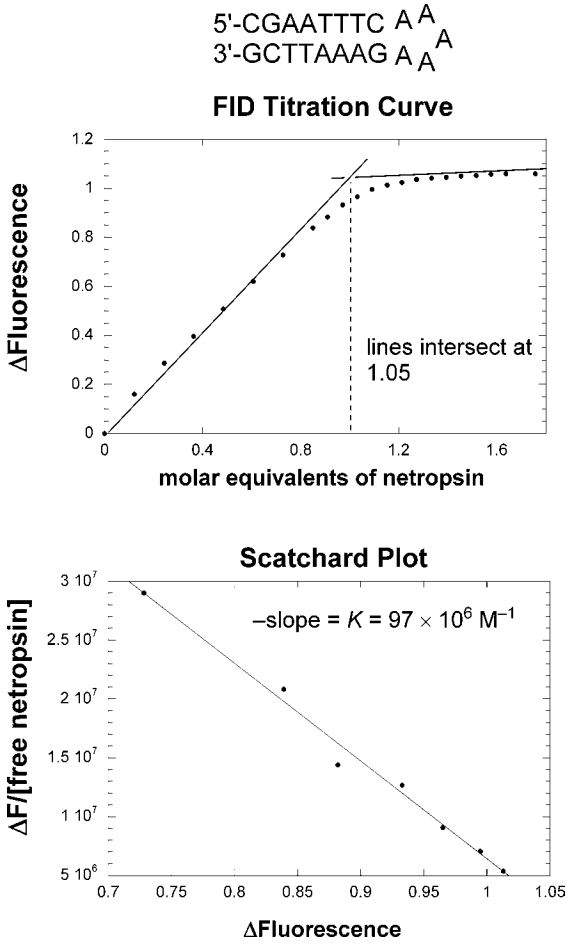


FIGURE 3. FID titration curve of netropsin against a hairpin deoxyoligonucleotide containing the sequence 5′-AATTT, and Scatchard plot for K_a (−slope) determination.

the stoichiometry of binding. A plot of the change in fluorescence versus equivalents of compound provides a titration curve from which the stoichiometry of binding may be derived as the intersection of the pre- and postsaturation portions of the curve (Figure 3). This method, analogous to that introduced by Bruice with Hoechst 33258,[42,43] is easily extended to analyzing higher order 2:1 and 3:1 complexes. For 1:1 binding, binding constants are established by Scatchard analysis[44] of the equilibrium portion of the titration curve, generating a plot of ΔF/[free agent] versus ΔF, yielding a linear section where the slope provides K_a. Binding constants produced by this indirect technique involving the displacement of ethidium yielded results comparable to those obtained directly by monitoring the fluorescent increase of selected fluorescent DNA binding compounds (e.g., DAPI, Table 1).

DNA Binding Sequence Selectivity

Minor Groove Binding Compounds. In developmental stages of the assay, distamycin, netropsin, DAPI, Hoechst 33258, and berenil were examined (Figure 4).[32] DAPI, Hoechst 33258, and berenil are fluorescent dyes, enabling a direct assessment of binding. Notably, the fluorescence enhancement characteristic of their binding did not interfere with the measurement of the fluorescence decrease derived from ethidium displacement (e.g., for EB, ex. 545 nm, em. 595 nm; for DAPI, ex. 372 nm, em. 454 nm).

Table 1. DAPI Binding Constants

DNA sequence	K ($\times 10^6$ M^{-1})[a]	K ($\times 10^6$ M^{-1})[b]
5′-AATTT	110	120
5′-AATAA	59	87
5′-ATTAA	52	77
5′-AAAAA	50	65

[a] Scatchard analysis of ethidium displacement titration. [b] Direct titration using fluorescence enhancement of DAPI.

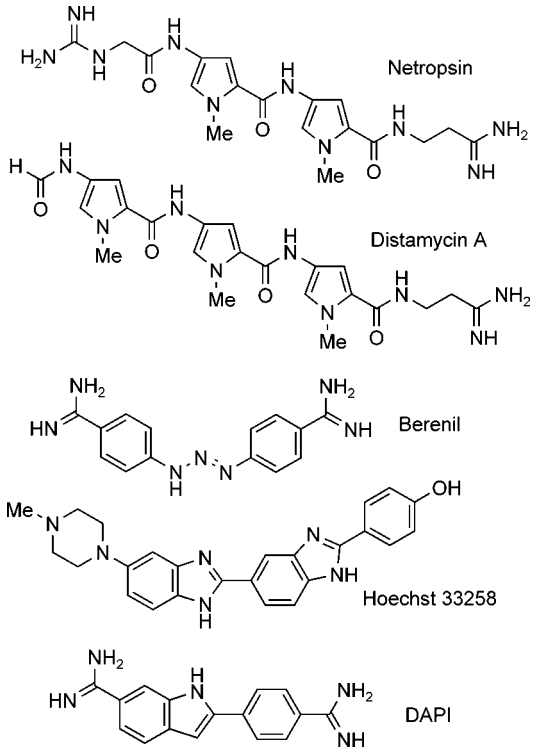


FIGURE 4.

Each compound was screened against a library of 512 DNA hairpins (1.5 μM) containing all possible 5-bp sites in a 96-well format, enlisting three compound concentrations (1.0, 1.5, and 2.0 μM) in duplicate and a Gemini SpectraMax plate reader.[32] On this scale and at these concentrations, the cost of the purchased hairpins is approximately $100/assay. This provided a rank order binding profile for all possible 5-bp sites that is represented as a merged bar graph in Figure 5 for distamycin. In addition to rapidly providing a comprehensive definition of each compound's sequence selectivity, the comparisons provided insights not previously easily recognized. While all compounds displayed the expected A/T binding selectivity, netropsin was the most A/T selective and, by some accounts, distamycin was the least. All exhibit tight binding to 5-bp > 4-bp > 3-bp A/T sites (Figure 6). This preference is related in part to the conformational characteristics of DNA, where longer A/T sites possess a narrower, deeper minor groove known to contribute to the selective binding. Interestingly, distamycin displayed an affinity for GC bp interrupted 5-bp A/T sites (2 × 2-bp) that exceeded even that of 3-bp A/T

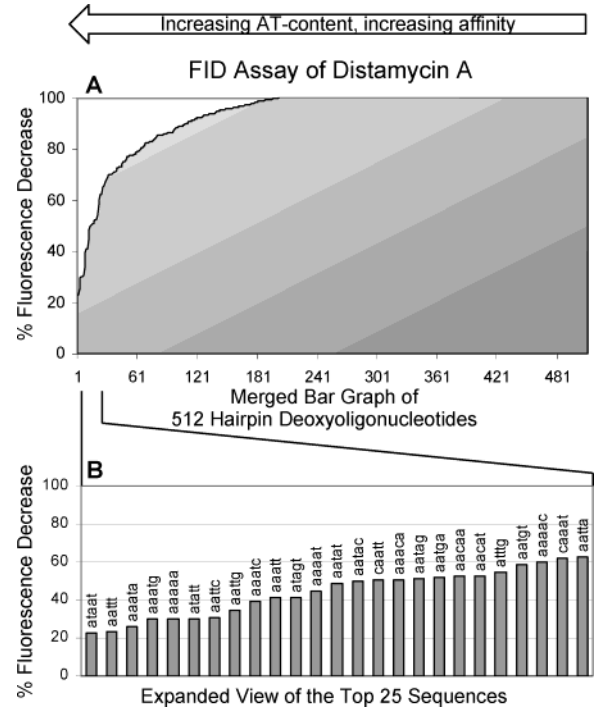


FIGURE 5. FID assay of distamycin A against a library of 512 hairpin deoxyoligonucleotides: (A) merged bar graph; (B) the top (highest affinity) 25 sequences.

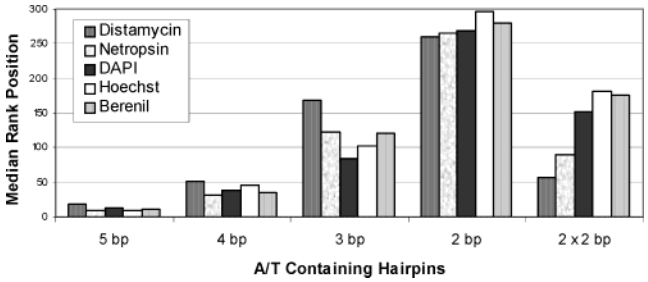


FIGURE 6. Median rank positions of hairpin DNAs containing five, four, three, two, and two × two contiguous A/T bp for the five compounds surveyed.

sites and was only slightly weaker that that of 4-bp A/T sites. Netropsin exhibited a weaker preference for such 2 × 2-bp A/T sites, reduced from its affinity to 4-bp A/T sites. In constrast, DAPI, Hoechst 33258, and berenil exhibited a clear preference for a 3-bp A/T site over a 2 × 2-bp A/T site. Combined, this was suggested to reflect the larger 5-bp binding site requirement for distamycin and its unique compensating ability to bind selected GC bp interrupted 5-bp A/T sites. For each compound, binding constants were determined by ethidium displacement titrations with selected sequences. These were found to be comparable to those established by footprinting or calorimetry, and to be nearly identical (within 2-fold) to those obtained by directly monitoring the fluorescence increase of DAPI and Hoechst 33258 upon DNA binding (see Table 1).[32]

A similar screen of netropsin against a library of all possible 4-bp sites was disclosed, and binding constants for all 10 possible 4-bp A/T sites were determined by titrations (Table 2).[39] With binding constants for all 10

Table 2. Comparative Titration Binding Constants

titrant	DNA sequence	K ($\times 10^6$ M^{-1})	binding stoichiometry
EB	5′-AATT	2.7	2.92
TO	5′-AATT	15	3.05

		K ($\times 10^6$ M^{-1})	
titrant	DNA sequence	ethidium bromide	thiazole orange
netropsin	5′-AAAT	127	113
	5′-AAAA	92	71
	5′-AATT	65	54
	5′-AATA	64	44
	5′-ATTA	45	35
	5′-ATAT	41	33
	5′-ATAA	34	18
	5′-TAAA	26	15
	5′-TTAA	11	8
	5′-TATA	11	8

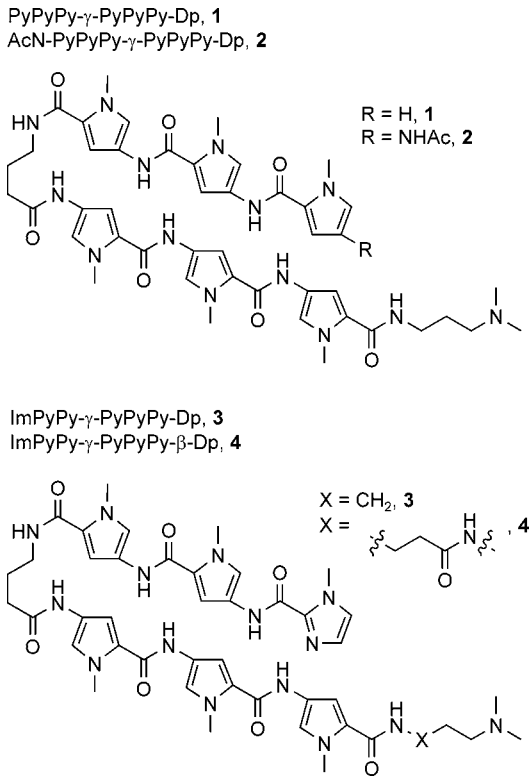


FIGURE 7.

possible A/T sites, an explicit rank order binding was established that was more comprehensive than that provided in all prior combined footprinting studies. Highlighted in these studies was the fact that the assay allows the rapid identification of the preferred ensemble of sequences for a compound, but that substantive conclusions drawn about adjacent sequences in the 96-well screen should be reserved for quantitative titrations.

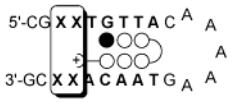
Hairpin Polyamides. An analysis of four pyrrole (Py)/imidazole (Im) hairpin polyamides (Figure 7) was conducted that not only served to establish the utility of the assay for examining minor groove binding polyamides, but also underscored its comprehensive capabilities. The polyamides were chosen to examine the subtle effects of N- and C-terminal functionalization on the DNA binding selectivity, representing a challenging test of the assay

Table 3. Sequence Selectivity of ImPyPy-γ-PyPyPy-β-Dp

A. FID Assay Analysis

Sequence[a]	1.5 μM agent		2.0 μM agent	
	Avg Rank	Avg Score	Avg Rank	Avg Score
5′-WGWWW	55	0.47	54	0.47
5′-WWGWW	82	0.44	36	0.55
5′-WCWWW	97	0.32	105	0.28
5′-WWWWW	131	0.27	111	0.27

B. Binding Constants by FID Titration

Binding Mode[b]	Sequence	K_a (M^{-1})
	1. 5′-TAACAAT[c]	2.39×10^8
	2. 5′-TAACAAC	1.73×10^8
	3. 5′-TAACACC	1.33×10^7
	4. 5′-GAACAAT	3.31×10^7
	5. 5′-GAACAAC	2.84×10^7
	6. 5′-ATTGTTA	9.87×10^7
	7. 5′-GTTGTTA	9.89×10^7
	8. 5′-GGTGTTA	2.57×10^7
	9. 5′-GTTGTTC	3.30×10^7

[a] W = A or T. [b] ○, Py; ●, Im. [c] Lit.[48] $K = 2.9 \times 10^8$ M^{-1}, footprinting.

capabilities. The complete binding profiles of the four molecules to all possible 5-bp sites were established using the FID assay.[45]

The comparison of **1** and **2** revealed the detrimental effect of the *N*-acetyl substitution with **2**,[46,47] and the hairpin polyamide ImPyPy-γ-PyPyPy-Dp (**3**), like **1**, displayed a straightforward correlation with its expected selectivity for 5′-WGWWW. For ImPyPy-γ-PyPyPy-β-Dp (**4**), the ability to screen complete sequence space resulted in the discovery of an unexpected 5′-WWCWW sequence specificity (Table 3A). Although detailed studies have probed the ligand−DNA interactions of this molecule and its sequence selectivity was established (5′-WGWWW),[48] its explicit binding to 5′-WWCWW had not been described. Use of the FID assay to comprehensively screen DNA provided a complete picture of its selectivity. Strikingly, the quantity and quality of the data generated by the assay also provided the basis for a binding model. This model established that **4** has an optimal binding site of 6 bp (not the expected 5 bp) of the form 5′-WWGWWW, and that the β/Dp tail combination requires two (not one) degenerate A/T base pairs. FID titrations confirmed the model by providing quantitative binding constants (Table 3B).

Also disclosed in this study was the analysis of sequence sets and two scoring methods for their comparison (Table 3A). The first simply calculates the average rank order position of the constituent members of a sequence set, whereas the second calculates an average %*F* decrease of the sequence set relative to the highest affinity sequence. This latter, less obvious scoring procedure may better reflect the relative importance of binding to a sequence set.

Selection of Binding Agents for a Defined Sequence(s) from Compound Libraries

Most of the current screening technologies are sufficiently technically demanding that they are most often used to investigate a few individual compounds. A true attribute of the FID assay is its capabilities for screening libraries of compounds (mixtures or individual).[33,49]

The rapid screening of individual sequences was illustrated with two hairpin deoxyoligonucleotides containing two sequences of the androgen response element (ARE), the 14-bp ARE-consensus, and PSA-ARE-3.[33,49] The emergence of hormone-independent, constituently active androgen receptor dimer is responsible for prostrate cancer relapse resistant to chemotherapeutic treatment. At this stage, competitive inhibition of the androgen receptor dimer DNA binding has therapeutic potential and could arise from small molecules with selective affinity toward the ARE-consensus and PSA-ARE-3. Libraries of distamycin-like compounds were prepared using 11 heteroaromatic subunits in addition to the *N*-methylpyrrole native to distamcyin (Figure 8). Libraries were produced such that the first two subunits (B and C) were fixed, producing 132 mixtures of 10 compounds with variations at the last position (A). Screening the library against the two hairpins using the FID assay revealed that the mixture containing the pyrrole subunit at both the second (B) and third (C) positions gave the largest decrease in fluorescence with the PSA-ARE-3 sequence which contains a 5-bp A/T site. The affinity dropped for the ARE-consensus containing a GC bp at the center of the 5-bp A/T site of this sequence. Deconvolution by screening individual compounds of this mixture afforded the distamycin analogue (**5**) as having the highest affinity, followed closely by an analogue containing a thiophene subunit at the position A (Figure 9). Both compounds exhibited diminished affinity for the ARE-consensus sequence resulting from the intervening GC bp.[33,49]

Two other 10-compound mixtures also bound the PSA-ARE-3 sequence effectively. Deconvolution to individual compounds identified **6** and **7** as tight binders comparable to **5**. Notably, **6** showed a loss of affinity for the ARE-consensus analogous to **5**, but **7** retained equal affinity, making it ideal for binding both the PSA-ARE-3 and ARE-consensus sequences (Figure 9). Impressively, **7** exhibited potent ($IC_{50} = 8$ nM) and selective (ca. 40-fold) inhibition of androgen receptor-mediated gene transcription in a cell-based reporter assay, albeit requiring liposome delivery of the compound for cell penetration and observation of activity.

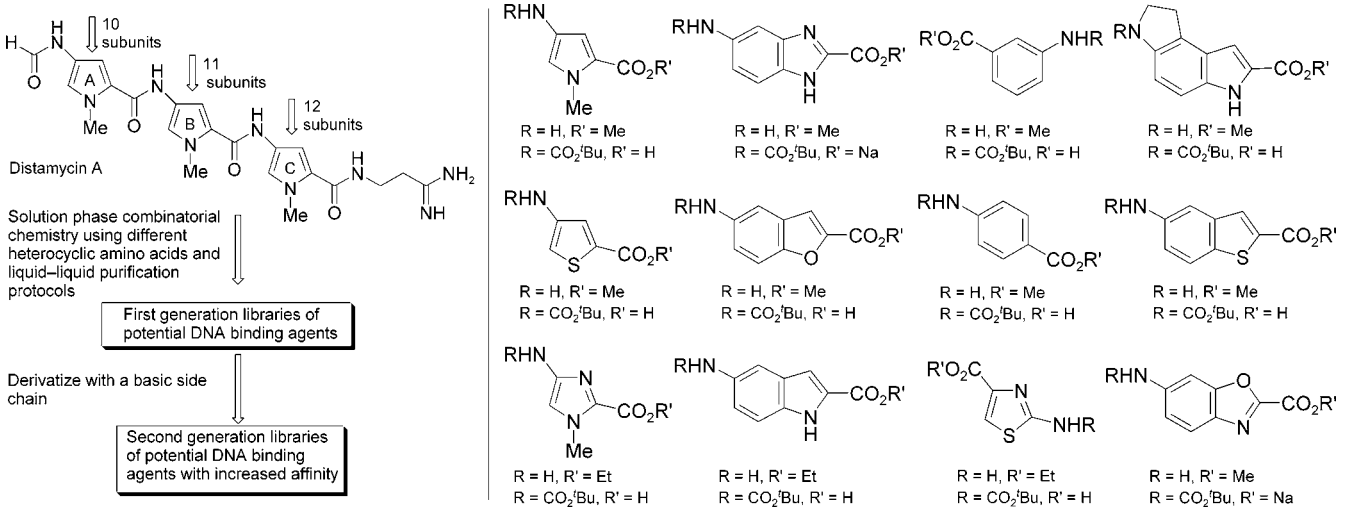


FIGURE 8. Solution-phase strategy for libraries and the subunits used.

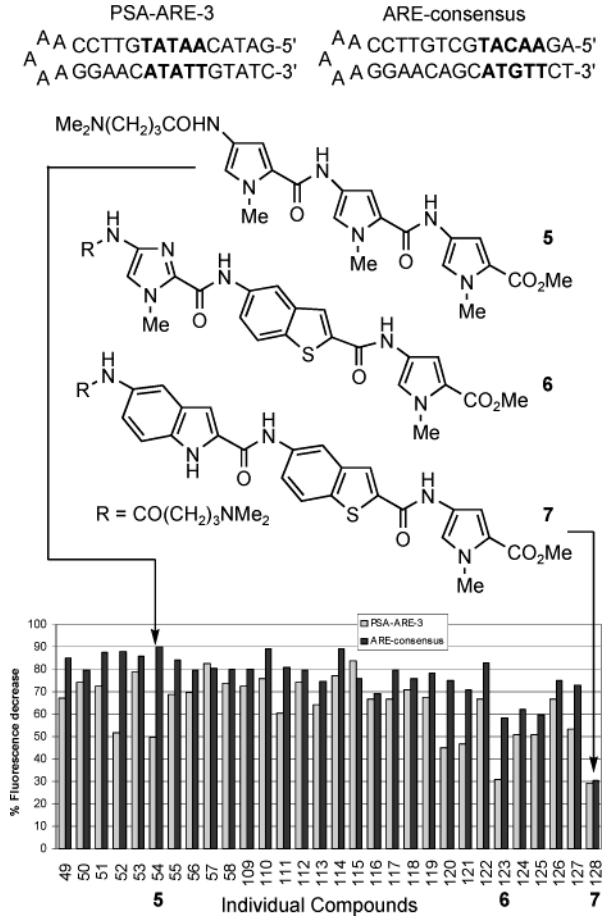


FIGURE 9. FID assay for selecting members of a library with affinity for the ARE-consensus and PSA-ARE-3.

Establishing Subtle Modes of Binding: Hairpin versus Extended Binding of Polyamides

The linkage of polyamides with γ-aminobutyric acid (γ) provides hairpin-bound polyamides that mimic the 2:1 side-by-side antiparallel binding of unlinked polyamides, enhances the binding affinity 10^2-10^4-fold, and improves the binding selectivity. In constrast, polyamides incorporating a one-carbon shorter head-to-tail linker, β-alanine (β), bind in an extended conformation, forming 1:1 or side-by-side antiparallel 2:1 complexes.[50] A variant of the FID titrations was used to study the DNA binding properties of α-substituted β-alanine-linked polyamides and a series of novel iminodiacetic acid (IDA)-linked polyamides (Figure 10).[51,52] In these two series, the bound conformation (hairpin versus extended binding and parallel versus antiparallel binding) could be established by analysis of FID titrations of hairpin deoxyoligonucleotides containing a systematically varied A/T binding site size. Complementary assessments using a combination of footprinting and affinity cleavage techniques[12,14–18] are technically more demanding, require the separate preparation of the linked Fe−EDTA affinity cleavage derivatives, and do not as easily distinguish between such alternative binding modes.

In the first of the two studies, a series of Py polyamides were linked using α-substituted β-alanines to probe their impact on binding.[51] Polyamide binding was assessed by using a series of hairpin deoxyoligonucleotides containing a systematically varied length of the A/T binding site (5−12 bp). The (*R*)-OMe-substituted derivative **9** bound with moderately high affinity, whereas the parent β-alanine **8** bound with intermediate affinity to the shortest hairpin (Figure 10). Very little change in the behaviors of **8** and **9** was seen until the length of the binding site reached 8 bp, where **8** dramatically changed. The binding constant increased by 2 orders of magnitude, and the stoichiometry of binding increased to 2. This corresponds to the expected behavior of extended binding over a 8−9-bp site as an antiparallel 2:1 side-by-side dimer.[53–55] In contrast-,the binding stoichiometry of **9** remained constant at 1:1, displaying a binding constant of $\sim 8 \times 10^7$ M^{-1} throughout the range of 5−10 bp until a binding site length of 11 bp was reached, where a second binding event was observed. The behavior seen with the 11- and 12-bp A/T sites represents two sequential binding events of **9** adopting a hairpin conformation, each requiring nonoverlapping 5-bp A/T sites (Figure 10).

The studies illustrated that a generalizable variant of the FID titrations may be utilized to distinguish hairpin versus extended binding, provide information on the

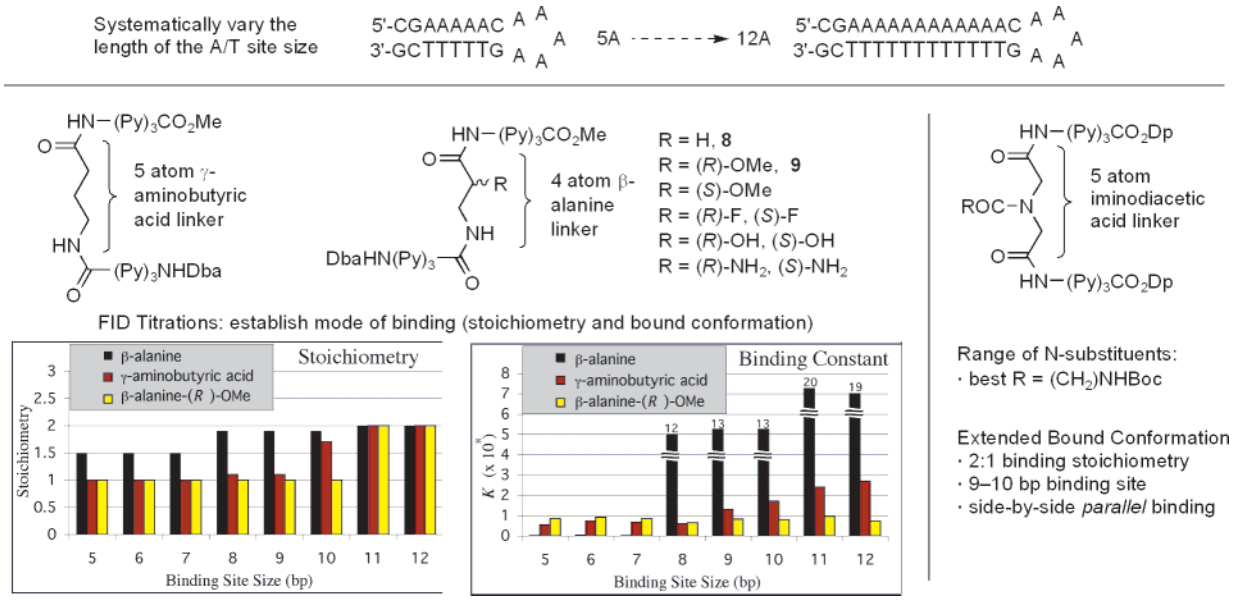


FIGURE 10. Establishment of DNA binding mode for γ-aminobutyric acid-linked, β-alanine-linked, and iminodiacetic acid-linked polyamides. Hairpin deoxyoligonucleotides containing varying A/T site sizes are utilized to determine binding constants and stoichiometry.

binding site size and stoichiometry, and establish absolute affinity. Its use resulted in the discovery that, while most substituents on a β-alanine linker disrupt DNA binding favoring an extended binding mode, (*R*)-α-methoxy-β-alanine maintains strong binding affinity and prefentially adopts a hairpin versus extended binding mode.[51]

In a second disclosure, the DNA binding properties of iminodiacetic acid-linked polyamides were examined (Figure 10). Utilizing the same series of hairpin deoxyoligonucleotides containing a systematically varied A/T binding site size, the binding affinity, stoichiometry, and binding site size were determined. Collectively it was determined that the IDA linker has a unique effect on the ability of the polyamides to bind DNA, providing what appears to be the first well-characterized example of cooperative parallel extended 2:1 side-by-side binding.[52]

DNA Binding Properties of Proteins

The DNA binding affinity and selectivity of proteins[27] are typically assessed by selection screening,[28−30] footprinting and affinity cleavage,[14] or EMSA.[31] Each technique possesses unique strengths, and the FID assay presents a complementary, technically nondemanding method for qualitative or quantitative assessment of DNA bindingwith concurrent establishment of the stoichiometry of binding.

Lymphoid enhancer-binding factor[56] (LEF-1, also referred to as TCF-1α[57,58]) and closely related T-cell factors (TCF-1[59]) are cell type-specific DNA binding proteins that play important regulatory roles.[60,61] The majority of colorectal tumors contain mutations that result in accumulation of β-catenin. β-Catenin binds to and activates transcription factors including LEF-1, which binds the DNA minor groove through a high-mobility-group (HMG) domain, recognizing a consensus sequence 5′-CTTTGWW (W = A or T).[56−63] A recent NMR structure of the complex of the LEF-1 HMG domain and a 15-bp deoxyoligonucleotide[64] revealed that the protein makes extensive and continuous contacts in the DNA minor groove, encompassing the entire region implicated by chemical footprinting and mutagenesis.[56−63]

FID titration of the HMG domain of LEF-1 against a hairpin deoxyoligonucleotide containing 5′-CTTTGAAG provided a well-defined titration curve (Figure 11). Scatchard analysis provided a K_a of 1.5×10^9 M^{-1}, virtually identical to that established by EMSA.[63] Once the viability of monitoring protein−DNA binding with the FID assay was confirmed, several other sequences were rapidly evaluated, including those implicated in initial studies, but not subsequently examined in detail. The third base of the consensus sequence, 5′-CTTTGWW, was permuted to each base variation and revealed that the C, G, and A substitutions bind the LEF-1 HMG domain effectively and almost indistinguishably, exhibiting K_a's less than 2-fold lower than that of the reported consensus sequence (Table 4). As such, the consensus sequence of 5′-CTTTGWW is accurate but could easily by refined to 5′-CTNTGWW (N = G, C, A or T) to reflect this nearly indiscriminant third site.[65]

Although not demonstrated in this work, the FID assay could easily be extended to the examination of a library of proteins or mutants against such hairpins.

Summary and Outlook

The fluorescent intercalator displacement (FID) assay is a rapid, high-resolution, and technically nondemanding technique for establishing DNA binding selectivity and affinity for small molecules, proteins, and oligonucleotides.[66] In a 96-well format, the assay provides for the high-throughput evaluation of a single compound against a library of DNA sequences (establish sequence selectivity) or for the high-throughput selection of high-affinity binders for a defined sequence from a library of compounds.

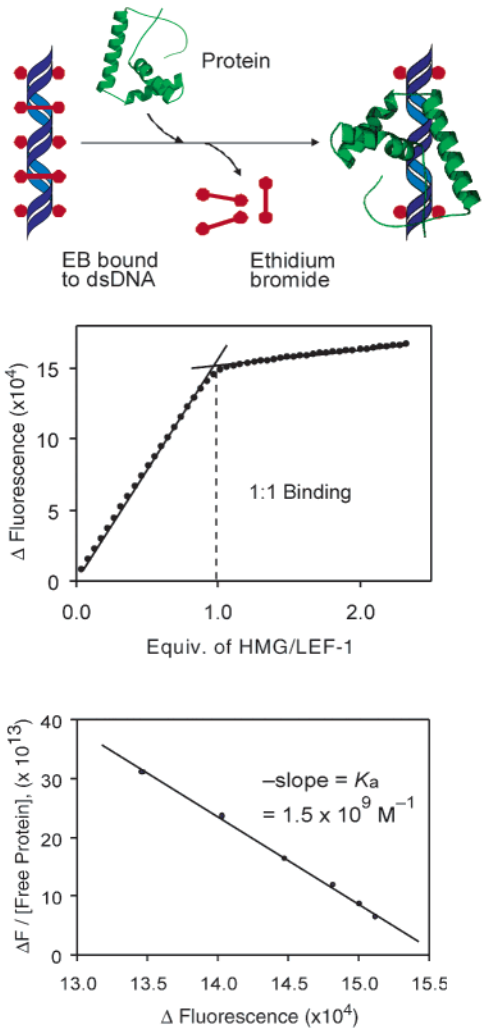


FIGURE 11. Titration of the HMG domain of LEF-1 versus a hairpin DNA containing 5′-CTTTGAA.

Table 4. HMG/LEF-1 Binding Constants

DNA sequence	K_a (M^{-1})
5′-CTTTGAAG[a]	1.5×10^9
5′-CTTTGTTG	1.0×10^9
5′-CCCCGAAG	$\leq 1.0 \times 10^8$
5′-CTCTGAAG	9.4×10^8
5′-CTGTGAAG	8.9×10^8
5′-CTATGAAG	8.3×10^8

[a] Lit.[63] $K_d = 1.0 \times 10^{-9}$ M, EMSA.

Alternatively, FID titrations provide detailed information on single compounds and their binding to individual sequences, including binding constants, stoichiometry of binding, and binding site size. This latter application, as well as the selection screening against a single sequence, is amenable to examination of any sequence length. Its use in screening against a complete library of individual sequences has been exemplified with sequence sizes of ≤5-bp variable regions requiring a library of up to 512 hairpin deoxyoligonucleotides. Longer sequences require increasingly larger numbers of hairpins (e.g., 6 bp =1024, 7 bp = 2048) or a pooling strategy for simple laboratory implementation. Unlike complementary techniques, the FID assay is nondestructive, providing the opportunity for hairpin immobilization onto reusable supports (chips, beads, or glass slides), thus removing the barrier to comprehensive and repeated screening of sequences longer than that presently exemplified.

We gratefully acknowledge the financial support of the National Institutes of Health (CA41986, CA78045), the Department of Defense/Army (DAMD17-02-1-0135), the Skaggs Institute for Chemical Biology, and the award of an ACS Division of Medicinal Chemistry Graduate Fellowship sponsored by Wyeth-Ayerst (W.C.T., 2002−2003). W.C.T. is a Skaggs Fellow.

References

(1) Browne, M. J.; Thurlbey, P. L. *Genomes, Molecular Biology and Drug Discovery*; Academic: London, 1996.
(2) Matteucci, M. D.; Wagner, R. W. In pursuit of antisense. *Nature* **1996**, *384*, 20−22.
(3) Neidle, S.; Thurston, D. E. In *New Targets for Cancer Chemotherapy*; Kerr, D. J., Workman, P., Eds.; CRC: Boca Raton, FL, 1994.
(4) Thurston, D. E. Nucleic acid targeting: therapeutic strategies for the 21st century. *Br. J. Cancer* **1999**, *80* (Suppl. 1), 65−85.
(5) Choo, Y.; Sanchez-Garcia, I.; Klug, A. In vivo repression by a site-specific DNA-binding protein designed against an oncogenic sequence. *Nature* **1994**, *372*, 642−645.
(6) Neidle, S. Recent developments in triple-helix regulation of gene expression. *Anticancer Drug Des.* **1997**, *12*, 433−442.
(7) Trauger, J. W.; Baird, E. E.; Dervan, P. B. Recognition of DNA by designed ligands at subnanomolar concentrations. *Nature* **1996**, *382*, 559−561.
(8) Knudsen, H.; Nielsen, P. E. Antisense properties of duplex- and triplex-forming PNAs. *Nucleic Acids Res.* **1996**, *24*, 494−500.
(9) Werstuck, G.; Green, M. R. Controlling gene expression in living cells through small molecule−RNA interactions. *Science* **1998**, *282*, 296−298.
(10) Chiang, S. Y.; Azizkhan, J. C.; Beerman, T. A. A comparison of DNA-binding drugs as inhibitors of E2F1- and Sp1-DNA complexes and associated gene expression. *Biochemistry* **1998**, *37*, 3109−3115.
(11) Gottesfeld, J. M.; Neely, L.; Trauger, J. W.; Baird, E. E.; Dervan, P. B. Regulation of gene expression by small molecules. *Nature* **1997**, *387*, 202−205.
(12) Dervan, P. B. Design of sequence-specific DNA-binding molecules. *Science* **1986**, *232*, 464−471.
(13) Taylor, J. S.; Schultz, P. G.; Dervan, P. B. DNA affinity cleaving. Sequence specific cleavage of DNA by distamycin−EDTA−iron(II) and EDTA−distamycin−iron(II). *Tetrahedron* **1984**, *40*, 457−465.
(14) Galas, D. J.; Schmitz, A. DNase footprinting: a simple method for the detection of protein−DNA binding specificity. *Nucleic Acids Res.* **1978**, *5*, 3157−3170.
(15) Royer-Pokora, B.; Gordon, L. K.; Haseltine, W. A. Use of exonuclease III to determine the site of stable lesions in defined sequences of DNA: the cyclobutane pyrimidine dimer and cis and trans dichlorodiammine platinum II examples. *Nucleic Acids Res.* **1981**, *9*, 4595−4609.
(16) Van Dyke, M. W.; Hertzberg, R. P.; Dervan, P. B. Map of distamycin, netropsin, and actinomycin binding sites on heterogeneous DNA: DNA cleavage-inhibition patterns with methidiumpropyl-EDTA-Fe(II). *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 5470−5474.
(17) Kuwabara, M. D.; Sigman, D. S. Footprinting DNA−protein complexes in situ following gel retardation assays using 1,10-phenanthroline-copper ion: *Escherichia coli* RNA polymerase-lac promoter complexes. *Biochemistry* **1987**, *26*, 7234−7238.
(18) Tullius, T. D.; Dombroski, B. A.; Churchill, M. E.; Kam, L. Hydroxyl radical footprinting: a high-resolution method for mapping protein−DNA contacts. *Methods Enzymol.* **1987**, *155*, 537−558.
(19) Hayashibara, K. C.; Verdine, G. L. Template-directed interference footprinting of cytosine contacts in a protein−DNA complex: potent interference by 5-aza-2′-deoxycytidine. *Biochemistry* **1992**, *31*, 11265−11273.
(20) Boger, D. L.; Johnson, D. S. CC-1065 and the duocarmycins: Understanding their biological function through mechanistic studies. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 1438−1474.
(21) Rajski, S. R.; Williams, R. M. DNA Cross-linking agents as antitumor drugs. *Chem. Rev.* **1998**, *98*, 2723−2796.
(22) Tao, Z. F.; Saito, I.; Sugiyama, H. Highly cooperative DNA dialkylation by the homodimer of imidazole-pyrrole diamide−CPI conjugate with vinyl linker. *J. Am. Chem. Soc.* **2000**, *122*, 1602−1608.

(23) Hardenbol, P.; Wang, J. C.; Van Dyke, M. W. Identification of preferred distamycin−DNA binding sites by the combinatorial method REPSA. *Bioconjugate Chem.* **1997**, *8*, 617−620.
(24) Chaltin, P.; Borgions, F.; Van Aerschot, A.; Herdewijn, P. Comparison of library screening techniques used in the development of dsDNA ligands. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 47−50.
(25) Guelev, V. M.; Harting, M. T.; Lokey, R. S.; Iverson, B. L. Altered sequence specificity identified from a library of DNA-binding small molecules. *Chem. Biol.* **2000**, *7*, 1−8.
(26) Hamy, F.; Albrecht, G.; Florsheimer, A.; Bailly, C. An ARE-selective DNA minor groove binder from a combinatorial approach. *Biochem. Biophys. Res. Commun.* **2000**, *270*, 393−399.
(27) Larson, C. J.; Verdine, G. L. In *Bioorganic Chemistry: Nucleic Acids*; Hecht, S. M., Ed.; Oxford University: Oxford, 1996; pp 324−346.
(28) Blackwell, T. K.; Kretzner, L.; Blackwood, E. M.; Eisenman, R. N.; Weintraub, H. Sequence-specific DNA binding by the c-Myc protein. *Science* **1990**, *250*, 1149−1151.
(29) Blackwell, T. K.; Weintraub, H. Differences and similarities in DNA-binding preferences of MyoD and E2A protein complexes revealed by binding site selection. *Science* **1990**, *250*, 1104−1110.
(30) Thiesen, H. J.; Bach, C. Target Detection Assay (TDA): a versatile procedure to determine DNA binding sites as demonstrated on SP1 protein. *Nucleic Acids Res.* **1990**, *18*, 3203−3209.
(31) Chodosh, L. A.; Carthew, R. W.; Sharp, P. A. A single polypeptide possesses the binding and transcription activities of the adenovirus major late transcription factor. *Mol. Cell Biol.* **1986**, *6*, 4723−4733.
(32) Boger, D. L.; Fink, B. E.; Brunette, S. R.; Tse, W. C.; Hedrick, M. P. A simple, high-resolution method for establishing DNA binding affinity and sequence selectivity. *J. Am. Chem. Soc.* **2001**, *123*, 5878−5891.
(33) Boger, D. L.; Fink, B. E.; Hedrick, M. P. Total synthesis of distamycin A and 2640 analogs: A solution-phase combinatorial approach to the discovery of new, bioactive DNA binding agents and development of a rapid, high-throughput screen for determining relative DNA binding affinity or DNA binding sequence selectivity. *J. Am. Chem. Soc.* **2000**, *122*, 6382−6394.
(34) Lee, L. G.; Chen, C. H.; Chiu, L. A. Thiazole orange: a new dye for reticulocyte analysis. *Cytometry* **1986**, *7*, 508−517.
(35) Nygren, J.; Svanvik, N.; Kubista, M. The interactions between the fluorescent dye thiazole orange and DNA. *Biopolymers* **1998**, *46*, 39−51.
(36) LePecq, J. B.; Paoletti, C. A fluorescent complex between ethidium bromide and nucleic acids. Physical−chemical characterization. *J. Mol. Biol.* **1967**, *27*, 87−106. Reviews: Morgan, A. R.; Lee, J. S.; Pulleyblank, D. E.; Murray, N. L.; Evans, D. H. Ethidium fluorescence assays. Part I. Physiochemical studies. *Nucleic Acids Res.* **1979**, *7*, 547−569. Morgan, A. R.; Evans, D. H.; Lee, J. S.; Pulleyblank, D. E. Ethidium fluorescence assay. Part II. Ezymatic studies and DNA−protein interactions. *Nucleic Acids Res.* **1979**, *7*, 571−594.
(37) For a complete list of references, see ref 32, footnote 9.
(38) Ethidium possesses relatively little sequence preference, although it displays a slight bias toward binding GC-rich DNA tracts. Each hairpin is normalized to its own 100% fluorescence to ensure that any bias in ethidium binding does not affect assay results, and accurate results may be obtained with any sequence composition.
(39) Boger, D. L.; Tse, W. C. Thiazole orange as the fluorescent intercalator in a high-resolution FID assay for determining DNA binding affinity and sequence selectivity of small molecules. *Bioorg. Med. Chem.* **2001**, *9*, 2511−2518.
(40) Broude, N. E. Stem−loop oligonucleotides: a robust tool for molecular biology and biotechnology. *Trends Biotechnol.* **2002**, *20*, 249−256.
(41) For the 5-bp variable region hairpin, the UV A_{260} at 25 °C may be converted to an accurate concentration by adjusting the millimolar extinction coefficients published by Invitrogen Corp. for ssDNA. Correction factors relating A_{260} at 90 °C/25 °C were determined to be 1.18, 1.13, 1.12, 1.12, 1.10, and 1.06 for the six combinations of 5-bp A/T sites contained in the 512 library: 5-bp AT, 4-bp AT/1 bp G/C, 3-bp AT/2-bp GC, 2-bp AT/3-bp GC, 1-bp AT/4-bp GC, and 5-bp GC, respectively.
(42) Browne, K. A.; He, G. X.; Bruice, T. C. Microgonotropens and their interactions with DNA. 2. Quantitative evaluation of equilibrium constants for 1:1 and 2:1 binding of dien-microgonotropen-a, -b, and -c as well as distamycin and Hoechst-33258 to d(GGCGCAAATTTGGCGG)/d(CCGCCAAATTTGCGCC). *J. Am. Chem. Soc.* **1993**, *115*, 7072−7079.
(43) Satz, A. L.; Bruice, T. C. Synthesis of fluorescent microgonotropens (FMGTs) and their interactions with dsDNA. *Bioorg. Med. Chem.* **2000**, *8*, 1871−1880.
(44) Scatchard, G. *Ann. N.Y. Acad. Sci.* **1949**, *51*, 660.
(45) Tse, W. C.; Ishii, T.; Boger, D. L. Comprehensive high-resolution analysis of hairpin polyamides utilizing a fluorescent intercalator displacement (FID) assay. *Bioorg. Med. Chem.* **2003**, *11*, 4479−4486.
(46) Hawkins, C. A.; Pelaez de Clairac, R.; Dominey, R. N.; Baird, E. E.; White, S.; Dervan, P. B.; Wemmer, D. E. Controlling binding orientation in hairpin polyamide DNA complexes. *J. Am. Chem. Soc.* **2000**, *122*, 5235−5243.
(47) Lacy, E. R.; Le, N. M.; Price, C. A.; Lee, M.; Wilson, W. D. Influence of a terminal formamido group on the sequence recognition of DNA by polyamides. *J. Am. Chem. Soc.* **2002**, *124*, 2153-2163.
(48) Parks, M. E.; Baird, E. E.; Dervan, P. B. Optimization of the hairpin polyamide design for recognition of the minor groove of DNA. *J. Am. Chem. Soc.* **1996**, *118*, 6147−6152.
(49) Boger, D. L.; Dechantsreiter, M. A.; Ishii, T.; Fink, B. E.; Hedrick, M. P. Assessment of solution-phase positional scanning libraries based on distamycin A for the discovery of new DNA binding agents. *Bioorg. Med. Chem.* **2000**, *8*, 2049−2057.
(50) Dervan, P. B. Molecular recognition of DNA by small molecules. *Bioorg. Med. Chem.* **2001**, *9*, 2215−2235.
(51) Woods, C. R.; Ishii, T.; Wu, B.; Bair, K. W.; Boger, D. L. Hairpin versus extended DNA binding of a substituted β-alanine linked polyamide. *J. Am. Chem. Soc.* **2002**, *124*, 2148−2152.
(52) Woods, C. R.; Ishii, T.; Boger, D. L. Synthesis and DNA binding properties of iminodiacetic acid-linked polyamides: characterization of cooperative extended 2:1 side-by-side parallel binding. *J. Am. Chem. Soc.* **2002**, *124*, 10676−10682.
(53) Wade, W. S.; Mrksich, M.; Dervan, P. B. Design of peptides that bind in the minor groove of DNA at 5′-(A,T)G(A,T)C(A,T)-3′ sequences by a dimeric side-by-side motif. *J. Am. Chem. Soc.* **1992**, *114*, 8783−8794.
(54) Mrksich, M.; Wade, W. S.; Dwyer, T. J.; Geierstanger, B. H.; Wemmer, D. E.; Dervan, P. B. Antiparallel side-by-side dimeric motif for sequence-specific recognition in the minor groove of DNA by the designed peptide 1-methylimidazole-2-carboxamide netropsin. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 7586−7590.
(55) Wade, W. S.; Mrksich, M.; Dervan, P. B. Binding affinities of synthetic peptides, pyridine-2-carboxamidonetropsin and 1-methylimidazole-2-carboxamidonetropsin, that form 2:1 complexes in the minor groove of double-helical DNA. *Biochemistry* **1993**, *32*, 11385−11389.
(56) Travis, A.; Amsterdam, A.; Belanger, C.; Grosschedl, R. LEF-1, a gene encoding a lymphoid-specific protein with an HMG domain, regulates T-cell receptor alpha enhancer function. *Genes Dev.* **1991**, *5*, 880−894.
(57) Waterman, M. L.; Jones, K. A. Purification of TCF-1 alpha, a T-cell-specific transcription factor that activates the T-cell receptor C alpha gene enhancer in a context-dependent manner. *New Biol.* **1990**, *2*, 621−636.
(58) Waterman, M. L.; Fischer, W. H.; Jones, K. A. A thymus-specific member of the HMG protein family regulates the human T cell receptor C alpha enhancer. *Genes Dev.* **1991**, *5*, 656−669.
(59) van de Wetering, M.; Oosterwegel, M.; Dooijes, D.; Clevers, H. Identification and cloning of TCF-1, a T lymphocyte-specific transcription factor containing a sequence-specific HMG box. *Embo J.* **1991**, *10*, 123−132.
(60) Grosschedl, R.; Giese, K.; Pagel, J. HMG domain proteins−Architectural elements in the assembly of nucleoprotein structures. *Trends Genet.* **1994**, *10*, 94−100.
(61) Eastman, Q.; Grosschedl, R. Regulation of LEF-1/TCF transcription factors by Wnt and other signals. *Curr. Opin. Cell. Biol.* **1999**, *11*, 233−240.
(62) Aoki, M.; Hecht, A.; Kruse, U.; Kemler, R.; Vogt, P. K. Nuclear endpoint of Wnt signaling: Neoplastic transformation induced by transactivating lymphoid-enhancing factor 1. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 139−144.
(63) Giese, K.; Amsterdam, A.; Grosschedl, R. DNA-binding properties of the HMG domain of the lymphoid-specific transcriptional regulator LEF-1. *Genes Dev.* **1991**, *5*, 2567−2578.
(64) Love, J. J.; Li, X. A.; Case, D. A.; Giese, K.; Grosschedl, R.; Wright, P. E. Structural basis for DNA bending by the architectural transcription factor LEF-1. *Nature* **1995**, *376*, 791−795.
(65) Ham, Y. W.; Tse, W. C.; Boger, D. L. High-resolution assessment of protein DNA binding affinity and selectivity utilizing a fluorescent intercalator displacement (FID) assay. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 3805−3807.
(66) Yeung, B. K. S.; Tse, W. C.; Boger, D. L. Determination of binding affinities of triplex forming oligonucleotides using a fluorescent intercalator displacement (FID) assay, *Bioorg. Med. Chem. Lett.* **2003**, *13*, 3801−3804.

AR030113Y