[22] Footprinting Methods for Analysis of Pyrrole–Imidazole Polyamide/DNA Complexes

By JOHN W. TRAUGER and PETER B. DERVAN

Introduction

The design of synthetic ligands that read the information stored in the DNA double helix has been a long-standing goal at the interface of chemistry and biology. Cell-permeable small molecules that target predetermined DNA sequences enable a potential approach for the regulation of gene expression. The development of "pairing rules" for minor groove-binding polyamides offers a code to control sequence specificity.[1,2] Crescent-shaped polyamides containing three aromatic amino acids, N-methylimidazole (Im), N-methylpyrrole (Py), and N-methyl-3-hydroxypyrrole (Hp), bind double-helical DNA with affinities and specificities comparable to those of natural DNA-binding proteins.[3–6] Sequence specificity is controlled by side-by-side pairs of rings that afford unique set(s) of sequence-specific hydrogen bonds with the minor groove edges of the intact Watson–Crick base pairs.[7,8] The pair Im/Py specifies a G · C base pair while Py/Im targets C · G. The Py/Py pair binds both A · T and T · A. Hp/Py and Py/Hp pairs discriminate T · A from A · T.[1–8] Although the generality of these pairing rules has been demonstrated by synthesizing a large number of polyamides that bind many different sequences, we find that there are limitations with regard to some sequence contexts, which are probably due to the sequence-dependent microstructure of the DNA helix. Remarkably, eight-ring polyamides have been shown to permeate cells and, in a few encouraging examples, inhibit the expression of target genes in cell culture experiments.[9,10]

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A pivotal step in the discovery–evaluation process of new polyamide motifs is the characterization of the affinity and specificity of next-generation molecules following the design–sythesis phase (i.e., structure–function relationships). In the design phase, it is often the case that the sequence preference, as well as the energetics of any new molecule binding at each potential site, are not perfectly understood and it is crucial to scan "libraries" of many potential DNA-binding sites in order to identify true high-affinity binding sites. Characterization of equilibrium–association constants must guide the choice of sequence context for DNA : ligand complexes selected for subsequent structure elucidation by nuclear magnetic resonance (NMR) or X-ray methods.

A DNA restriction fragment, typically a few hundred base pairs in length, provides a library of contiguous small molecule-binding sites on a string addressed by a ^{32}P end label. The high-resolution separation of nucleic fragments by gel electrophoresis underpinning "footprinting" methods has revolutionized the field of small molecule–DNA discovery. This chapter describes three complementary footprinting methods and the protocols used for analysis of polyamide : DNA complexes: (1) MPE–Fe(II) footprinting,[11–14] (2) affinity cleavage,[15–17] and (3) quantitative DNase I footprint titration[18–21] (Fig. 1). Footprinting with methidiumpropyl–EDTA–Fe(II) [MPE–Fe(II)] is used to identify high-affinity polyamide-binding sites to near nucleotide resolution (Fig. 1A). Affinity cleavage is used to determine the orientation of the bound polyamide in the minor groove of DNA (Fig. 1A). Quantitative DNase I footprinting is used to determine equilibrium association constants (K_a) for polyamide–DNA complexes at previously identified match and mismatch sites (Fig. 1B).

Description of Methods

Footprinting was first described for analysis of protein–DNA complexes using the enzyme DNase I.[22] DNase I has been used to footprint small molecules as

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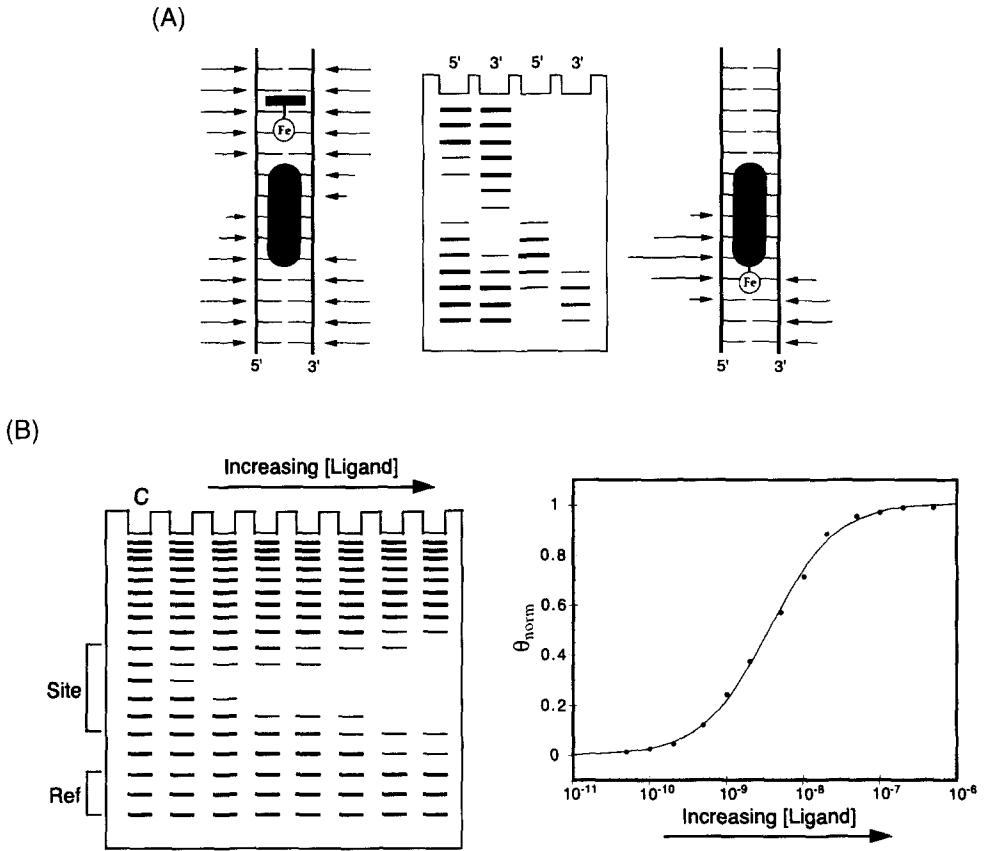
(A)

(B)

FIG. 1. (A) Schematic of (*left*) MPE–Fe(II) footprinting and (*right*) affinity-cleaving techniques. Cleavage products obtained on a denaturing gel with DNA end labeled either on the 5' or 3' strand are shown. (B) *Left:* Cleavage pattern generated by quantitative DNase I footprint titration, on a 3' end-labeled DNA fragment in the presence of increasing ligand concentrations. *Right:* Langmuir binding titration isotherm obtained from DNase I data.

well as proteins.[13,23] However, DNase I does not cleave all sequences equally and footprints can appear significantly larger than the actual base pairs occupied by the DNA-binding ligand.[13] Both natural product DNA-binding ligands, as well as designed polyamides, typically cover only five or six base pairs and accurate assignment of the binding site sequence becomes important. The chemical foot-printing reagent, MPE–Fe(II), based on the nonspecific intercalator methidium with EDTA–Fe attached, cleaves DNA[24,25] with minimal sequence specificity in

[23] K. R. Fox and M. J. Waring, *Nucleic Acids Res.* **12**, 9271 (1984).

the presence of a reducing agent and O_2, and allows a higher resolution footprint. By directly attaching the EDTA–Fe moiety to the DNA-binding ligand, the orientation of a ligand in its complex with DNA can be determined in a technique called affinity cleavage.[15–17] In addition, affinity cleavage experiments are useful for determining the groove location of a ligand, because distinct 5'- or 3'-shifted cleavage patterns are observed for cleavage in the major versus the minor groove. Minor groove-binding ligands produce 3'-shifted cleavage patterns[17] (Fig. 1A).

Quantitative DNase I footprinting procedures were developed by Ackers and co-workers, and are the foundation of the protocol presented here.[18–21] For quantitative footprinting experiments, equilibrium mixtures of ^{32}P end-labeled DNA and a range of polyamide concentrations are subjected to partial digestion with DNase I. The resulting cleavage products are separated by gel electrophoresis and visualized by autoradiography. DNA sites bound by a polyamide are protected from cleavage, resulting in a gap in the ladder of bands on the gel. The fractional protection of a binding site as a function of polyamide concentration can be determined by densitometric analysis of the gel, and these data can be fitted to a theoretical binding isotherm to determine the equilibrium association constant (K_a) (Fig. 1B). The data analysis for this assay assumes that the polyamide concentration is much higher than the DNA concentration, allowing the approximation $[L]_{free} = [L]_{tot}$, where $[L]_{free}$ is the concentration of polyamide free in solution and $[L]_{tot}$ is the total polyamide concentration. The total equilibration volume specified in this protocol is 400 μl and the DNA concentration is ~5 pM. As a consequence, apparent association constants greater than ~2 × 10^{10} M^{-1} should be regarded as lower limits. In the protocol described here, polyamide and radiolabeled DNA are equilibrated in the absence of any unlabeled carrier DNA (such as calf thymus DNA) to avoid underestimation of association constants due to polyamide binding to sites on the carrier DNA.

Materials

DNase I [fast protein liquid chromatography (FPLC) pure], calf thymus DNA (sonicated, deproteinized), and NICK columns are purchased from Pharmacia (Piscataway, NJ) Restriction enzymes, polynucleotide kinase, *Taq* DNA polymerase, Klenow DNA polymerase, and glycogen are obtained from Boehringer Mannheim (Indianapolis, IN). Sequenase (version 2.0) is purchased from United States Biochemicals (Cleveland, OH). [α-^{32}P]Thymidine 5'-triphosphate ($\geq$3000 Ci/mmol) and [α-^{32}P]deoxyadenosine 5'-triphosphate ($\geq$6000 Ci/mmol) are purchased from Du Pont-NEN (Boston, MA). [γ-^{32}P]adenosine triphosphate (end-labeling grade) is from ICN (Costa Mesa, CA). Denaturing polyacrylamide gel

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[25] R. P. Hertzberg and P. B. Dervan, *Biochemistry* **23,** 3934 (1984).

mix, Tris–borate–EDTA (TBE) packets, and gel plates, spacers, and stands are obtained from GIBCO-BRL (Gaithersburg, MD). Presiliconized 1.5-ml tubes are obtained from Sorenson Biosciences, Inc.

Preparation of ^{32}P End-Labeled DNA

End-labeled DNA is prepared from plasmid DNA containing the binding site(s) of interest. The desired DNA fragment is generally prepared by restriction digest and 3' fill-in, using a DNA polymerase and [α-^{32}P]dNTPs (Procedures 1 and 2, below), but can also be prepared by polymerase chain reaction (PCR), using a 5' ^{32}P-labeled primer (Procedure 3, below). When labeling by 3' fill-in, it is best to cut the end to be labeled with an enzyme whose 5' overhang contains only two different nucleotides because the overhang can then be completely filled in with two labeled dNTPs. The DNA-binding sites should ideally be located 40–100 base pairs from the labeled end of the DNA fragment, although sites of up to ~200 base pairs from the label can usually be resolved. The size of the DNA fragment can range from 100 base pairs to more than 500 base pairs.

Procedure 1

Procedure 1 is the most convenient labeling procedure, and can be used if (1) both enzymes cut efficiently in the same buffer, and (2) the enzyme that cuts at the unlabeled end leaves either a blunt end or a 3' overhang. Enzyme combinations that work well are EcoRI*/PvuII (this example) and AflII*/FspI (the asterisk denotes the labeled end).

1. Combine plasmid DNA (4 μg) and water in a total volume of 55 μl. Add 7 μl of H buffer (Boehringer Mannheim), 4 μl of EcoRI (20 units/μl), and 4 μl of PvuII (20 units/μl). Incubate for 2–4 hr at 37°. Add 8 μl of water, 5 μl of H buffer, 10 μl of [α-^{32}P]dATP, 10 μl of [α-^{32}P]dTTP, and 10 μl of Klenow DNA polymerase (2 units/μl). Allow the reaction to proceed for 25 min at room temperature. Add 5 μl of dATP/dTTP mix (each at 5 mM) and allow the reaction to proceed for 5 min.

2. Add 30 μl of 5× Ficoll loading buffer, mix, and load on a nondenaturing 7% (w/v) polyacrylamide gel (two lanes, each ~75 μl). Run the gel and isolate the DNA as described below.

Procedure 2

In this example (EcoRI*/HindIII), the second cutter, HindIII, produces a 5' overhang and requires a buffer different from that required by EcoRI. For these reasons, the second cut is done after the first cut and fill-in.

1. Combine plasmid DNA (4 μg) and water in a final volume of 46 μl. Add 8 μl of H buffer (Boehringer Mannheim), 10 μl of [α-^{32}P]dATP, 10 μl of [α-^{32}P]dTTP, 4 μl of *Eco*RI (20 units/ml), and 2 μl of Sequenase (8–25 units/ml). Incubate at 37° for 4 hr. Add 5 μl of dATP/dTTP mix (each at 5 mM) and 0.5 μl of Sequenase, and incubate at 37° for 20 min. Pass through a NICK column equilibrated with water to remove unincorporated nucleotides. To the 400 μl of eluate add 25 μl of 4 M NaCl, 1 μl of glycogen (20 mg/ml), and 860 μl of absolute ethanol. Centrifuge at 14,000 rpm for 30 min in a cold room and decant the ethanol solution. Wash the pellet with 70% (v/v) ethanol (100 μl), decant, and dry briefly in a Speed-Vac (Savant Instruments, Hicksville, NY).

2. Dissolve the DNA pellet in 42 μl of water, 5 μl of buffer B (Boehringer-Mannheim), and add 3 μl of *Hin*dIII. Incubate at 37° for 2–4 hr. Add 15 μl of 5× Ficoll loading buffer and load on a 7% (w/v) nondenaturing gel. Run the gel and isolate the DNA as described below.

Procedure 3

When suitable restriction sites are not present, a 5′ ^{32}P end-labeled DNA fragment can be prepared by PCR.

1. To 60 pmol of primer A (the primer to be labeled) add 80 μl of water, 10 μl of 10× kinase buffer, 4 μl of [γ-^{32}P]ATP (~250 μCi), and 6 μl of polynucleotide kinase. Incubate at 37° for 30 min. Add 5 μl of 0.5 M EDTA and extract with 25 : 24 : 1 (v/v/v) phenol–chloroform–isoamyl alcohol (four times, 100 μl each). Purify the DNA with a NICK column equilibrated with water to remove unincorporated ATP. To the 400 μl from the NICK column add 24 μl of 4 M NaCl, 1 μl of glycogen (20 mg/ml), and 860 μl of absolute ethanol, mix, and centrifuge (14,000 rpm) for 30 min in a cold room. Decant, wash with 70% (v/v) ethanol (100 μl), decant, and dry briefly in a Speed-Vac.

2. Dissolve 60 pmol of primer B in 60 μl of water. To the pellet from step 1, add 50 μl of the primer B solution, 33 μl of water, 10 μl of PCR buffer (Boehringer Mannheim), 3.7 μl of plasmid DNA (0.003 μg/ml), 2 μl of dNTP mix (each at 10 mM), and 1 μl of 100× bovine serum albumin (New England BioLabs, Beverly, MA). Transfer the solution to a PCR tube.

3. Heat the tube at 70° in a thermocycler for 5 min (hot start PCR). Carefully add 4 units (0.8 μl) of *Taq* DNA polymerase, mix, and cover the solution with mineral oil.

4. Thermocycle as follows: 30 cycles of (1) 94° for 1 min, (2) 54° for 1 min, (3) 72° for 1.5 min, and then 72° for 10 min.

5. Carefully transfer the aqueous phase to a new tube. For each 100 μl of solution, add 30 μl of 5× Ficoll loading buffer. Load onto a 7% (w/v) nondenaturing gel and purify as described below.

Gel Purification of End-Labeled DNA

1. Prepare 7% (w/v) nondenaturing gel mix as follows: to a 125-ml Erlenmeyer flask add 8.1 g of acrylamide (*caution*: acrylamide is toxic!), 290 mg of bis-acrylamide, and 24 ml of 5× TBE, and dilute to 120 ml with water and stir until homogeneous. Initiate polymerization by adding 700 μl of 10% (w/v) ammonium persulfate (APS) and 42 μl of N,N,N',N'-tetramethyl ethylene diamine (TEMED), and pour the gel. Prepare 1 liter of 1× TBE gel running buffer. Run the gel at ~200 V for ~2 hr.

2. Carefully take the gel down and cover it with Saran Wrap. Expose the gel to X-Omat film (Kodak, Rochester, NY) for 30–60 sec. Place the developed film under the gel and cut out the desired band, using a razor blade. Transfer the gel slice to a 1.5-ml tube.

3. Crush the gel slice with a pipette tip and add 700 μl of elution buffer (20 mM Tris-HCl, 250 mM NaCl, pH 8). Place the tube in a shielded container and soak overnight (8–16 hr) in a 37° shaker. Filter the DNA, using a plastic centrifugal filter (Quiksep; Isolab, Singapore) into a 15-ml Falcon 2059 tube, and transfer the eluate to a 1.5-ml tube. Add 1.5 volumes of 2-propanol, mix by inversion, and precipitate by spinning in a microcentrifuge at 14,000 rpm for 30 min in a cold room. Decant, and then add 100 μl of 75% (v/v) ethanol, spin briefly (30–90 sec), decant, and dry the DNA pellet briefly in a Speed-Vac.

4. Dissolve the DNA in 100 μl of water and pass it through a NICK column equilibrated with water. Divide the 400-μl eluate into aliquots, which are then counted in a scintillation counter and stored at −80° until use.

Preparation of Polyamide Serial Dilutions

Solid-phase methods for the synthesis of polyamide have been described.[26] Polyamide stock solutions are conveniently prepared from dry 25-nmol aliquots. Concentrations are determined by UV absorption, using an experimentally determined extinction coefficient. Extinction coefficients can be estimated on the basis of the number of aromatic rings, using the relation 8690 M^{-1} cm^{-1}/aromatic ring for the absorption maximum between 290 and 315 nm (e.g., for an eight-ring polyamide, $\varepsilon \approx 69,500$ M^{-1} cm^{-1} at the maximum between 290 and 315 nM).

Polyamides having at least one positive charge per eight rings are generally freely soluble up to a concentration of at least 500 μM. The presence of multiple β-alanines can reduce solubility. It is good practice, especially for polyamides expected to have relatively low solubility, to spin the initial stock solution in

[26] E. E. Baird and P. B. Dervan, *J. Am. Chem. Soc.* **118**, 6141 (1996).

a microcentrifuge for several minutes at high speed (14,000 rpm) to pellet any insoluble material and transfer the supernatant to a new tube before measuring the concentration.

For quantitative footprinting experiments, the goal is to prepare binding reactions over a range of polyamide concentrations that produce from 0 to 100% fractional saturation of the DNA-binding site(s) of interest. This generally requires at a minimum a three order of magnitude concentration range. In a typical titration, 10–13 data reactions, 1 control reaction (with no polyamide), and 1 undigested DNA control should be prepared.

Prepare polyamide stock solutions as follows: Dissolve 25 nmol of polyamide in water to give an approximately 10 μM solution. Determine the concentration of this solution by UV absorption, and then prepare 10× polyamide stock solutions with nearly equal spacing on a log scale (e.g., 1, 2, 5, and 10 nM).

Quantitative DNase I Footprinting Procedure

1. Prepare a 1.14× DNA solution: Combine 2.06 ml of 5× TKMC, pH 7.0 (50 mM Tris-HCl, 50 mM KCl, 50 mM MgCl$_2$, 25 mM CaCl$_2$, pH 7.0), 7.0 ml of water, and ^{32}P-labeled DNA (400,000 cpm). The amount of DNA used is calculated to give a final loading of $\sim$15,000 cpm per lane.

2. Prepare equilibrium binding mixtures: To a 1.5-ml presiliconized Eppendorf tube add 40 μl of 10× polyamide solution (or water for the control lanes) and 350 μl of 1.14× DNA solution. Allow the mixtures to equilibrate at room temperature for 4–24 hr. Polyamides with association constants over $10^9\ M^{-1}$ should be allowed to equilibrate for at least 12 hr.

3. Prepare DNase I stop buffer: Combine 40 μl of glycogen (20 mg/ml), 40 μl of 1 mM bp calf thymus DNA, 107 μl of water, 788 μl of 4 M NaCl, and 425 μl of 0.5 M EDTA, pH 8.0. The EDTA in this buffer quenches DNase I activity by chelating the essential Mg^{2+} and Ca^{2+} ions. The other ingredients provide for good ethanol precipitation and subsequent resuspension.

4. Prepare a DNase I stock solution: Combine 975 μl of water and 20 μl of 50 mM dithiothreitol (DTT), and chill the solution on ice. Add 5 μl of DNase I (Pharmacia FPLCPure, 7500 units/ml) and mix the resulting 38 units/ml-solution by inverting the tube several times. Prepare the final stock solution by adding 15–70 μl of DNase I (38 units/ml) to a prechilled mixture of 20 μl of 50 mM DTT and 930–965 μl of water (the total final volume should be 1000 μl). Keep the DNase I solution on ice throughout the experiment (prepare this solution freshly from the 7500-units/ml stock and use within 1 hr). The exact amount of DNase I to use depends on the batch of DNase I and the restriction fragment being used. The goal is to achieve $\sim$50% digestion of the restriction fragment. Although the exact amount of DNase I to use can be determined by running a titration, it is

usually possible to estimate on the basis of the length of the restriction fragment. For a 220-bp fragment, dilute ~50 μl of the 38-units/ml stock solution to 1 ml. Shorter restriction fragments require more DNase I, longer fragments less (e.g., for a 440-bp fragment, use ~25 μl of the 38-units/ml solution).

5. Digest the DNA: To each tube (except the intact DNA control) add 10 μl of the final DNase I solution and mix by vortexing. Allow the reaction to proceed for 7 min at room temperature, and then add 50 μl of "stop buffer" and mix by vortexing. Add 975 μl of room temperature absolute ethanol and mix by inversion.

Carry out this step while using a stopwatch: First, open the tops of all tubes, then start the stopwatch. Every 15 sec, add DNase I to a tube, close the top, mix by vortexing, and place the tube in a microcentrifuge. When all the tubes are in the microcentrifuge, spin them briefly (~2 sec), remove from the centrifuge, and open all the tops. When 7 min has elapsed, reset the stopwatch. Add stop buffer every 15 sec and vortex. Finally, add ethanol and mix.

6. Precipitate the DNA: Spin the tubes in a microcentrifuge in a cold room at 14,000 rpm for 25–30 min (start the samples spinning right away, and do not spin for longer than 30 min: spinning longer may make resuspension of the DNA more difficult). After precipitation the pellets should be visible. Carefully decant the supernatant, and then add 300 μl of 70% (v/v) ethanol. Vortex the tube briefly to thoroughly wash the pellet. Spin the tubes briefly (10–20 sec) in a microcentrifuge, and then carefully decant the supernatant.

7. Resuspend the DNA: To each tube add 15 μl of water and vortex (5–10 sec) to resuspend the DNA (dissolving the samples in water and reconcentrating them increases the chances that the DNA will resuspend properly and not hang in the wells). Freeze the samples by placing them in liquid nitrogen, or in a −80° freezer. The samples may be stored overnight at this point at −80°. Concentrate the solution to dryness in a Speed-Vac (to reduce the chances of hanging lanes, do not overdry; it is best to take the samples out as soon as they are dry). Next, add 7 μl of 80% (v/v) formamide–1× TBE loading buffer (prepared by mixing 8 ml of formamide with 2 ml of 5× TBE). The loading buffer should be stored at 4° and discarded after 1–2 months (use of old loading buffer can result in smearing of bands on the gel). Thoroughly vortex each tube (~30 sec) to resuspend the DNA.

8. Denature and load the gel: Denature the DNA by heating for 10 min at 85–90°, and then immediately place the samples on ice. Load 5 μl per lane on a prerun 8% (w/v) [19 : 1 (w/w) acrylamide–bisacrylamide] denaturing polyacrylamide gel (prerun the gel for 15–40 min until the temperature of the front gel plate reaches 50–55°). Load one or more chemical sequencing reaction lanes.[27,28] Run the gel at

[27] A. M. Maxam and W. S. Gilbert, *Methods Enzymol.* **65,** 499 (1980).
[28] B. L. Iverson and P. B. Dervan, *Nucleic Acids Res.* **15,** 7823 (1987).

~2000 V (running bromphenol blue to the bottom of the gel is good for resolving DNA sites 40–80 base pairs from the labeled end). To help ensure that the gel sticks to only one glass plate when the plates are separated, one of the gel plates may be treated with a silanizing reagent (e.g., SigmaCote; Sigma, St. Louis, MO) occasionally (once every 10 runs or so).

9. Transfer the gel to drying paper, cover with plastic wrap, and dry on a gel dryer for 60 min at 80°. Expose the gel to a storage phosphor screen (Molecular Dynamics, Sunnyvale, CA) for 8–16 hr.

Data Analysis

Image the gel with a phosphorImager such as the Molecular Dynamics 400S PhosphorImager. Intensity data are obtained from the gel image by quantitation using ImageQuant software (Molecular Dynamics). Background-corrected (i.e., quantitate the intact DNA control lane and subtract this background value) volume integration of rectangles encompassing the footprint sites and a reference site at which DNase I reactivity is invariant across the titration provides values for the site intensities (I_{site}) and the reference intensities (I_{ref}), respectively. The apparent fractional occupancy (θ_{app}) of the sites is then calculated using Eq. (1):

$$\theta_{app} = 1 - (I_{site}/I_{ref})/\left(I^0_{site}/I^0_{ref}\right) \tag{1}$$

where I^0_{site} and I^0_{ref} are the site and reference intensities, respectively, from a control lane to which no polyamide was added. The ([L], θ_{app}) data points are then fitted to a general Hill equation [(Eq. (2)] by minimizing the difference between θ_{app} and θ_{fit}:

$$\theta_{fit} = \theta_{min} + (\theta_{max} - \theta_{min})\left(K^n_a[L]^n/1 + K^n_a[L]^n\right) \tag{2}$$

where [L] is the total polyamide concentration, K_a is the equilibrium association constant, and θ_{min} and θ_{max} are the experimentally determined site saturation values when the site is unoccupied or saturated, respectively. The data are fitted by a nonlinear least-squares fitting procedure (such as KaleidaGraph software; Synergy, Reading, PA) with K_a, θ_{min}, and θ_{max} as the adjustable parameters, and with n fixed at either 1 or 2. A good fit to a Langmuir isotherm [Eq. (2), $n = 1$] is consistent with formation of a 1 : 1 polyamide–DNA complex, whereas a good fit to a cooperative isotherm (Eq. (2), $n = 2$] is consistent with cooperative dimeric binding.[29] The data are normalized by Eq. (3):

$$\theta_{norm} = (\theta_{app} - \theta_{min})/(\theta_{max} - \theta_{min}) \tag{3}$$

[29] C. R. Cantor and P. R. Schimmel, "Biophysical Chemistry, Part III: The Behavior of Biological Macromolecules," p. 863. W. H. Freeman, New York, 1980.

Reported association constants are the average values obtained from three independent footprinting experiments.

Methidiumpropyl–EDTA–Fe(II) Footprinting Protocol and Affinity Cleavage Protocols

The protocol reported here avoids the use of carrier DNA and gives apparent affinities that are the same as those obtained using the DNase I footprinting assay described above. The final solution conditions are 20 mM HEPES, 200 mM NaCl glycogen (50 μg/ml), 5 mM DTT, pH 7.3, and either (1) 0.5 μM MPE–Fe(II) for footprinting experiments or (2) 1 μM Fe(II) for affinity cleavage experiments.

1. Prepare a 1.43× DNA solution: To a 15-ml Falcon 2059 tube add 1286 μl of 5× cleavage buffer (100 mM HEPES, 300 mM NaCl, pH 7.3), 225 μl of 4 M NaCl, 3 ml of water, and labeled DNA (250,000 cpm).

2. Prepare 10× polyamide (or EDTA–polyamide) stock solutions.

3. Set up equilibrations: To a 1.5-ml Eppendorf tube add 280 μl of 1.43× DNA solution, 40 μl of 10× polyamide, and 40 μl of glycogen (0.5 mg/ml). Allow the solution to equilibrate for 1–18 hr.

4. Prepare a precipitation buffer by combining 35 μl of glycogen (20 mg/ml), 35 μl of calf thymus DNA (1 mM bp), and 180 μl of water.

5a. For MPE footprinting experiments: Prepare 5 μM MPE–Fe(II) by combining equal volumes of 10 μM MPE and freshly prepared 10 μM Fe(NH$_4$)$_2$(SO$_4$)$_2$. To the polyamide–DNA solution add 40 μl of 5 μM MPE–Fe(II); allow to equilibrate for 10 min. Add 10 μl of 200 mM DTT (use a freshly thawed DTT aliquot). Allow cleavage to proceed for 30 min. Add 1 ml of ethanol, mix the tubes by inversion, and spin briefly.

5b. For affinity cleavage experiments: To the EDTA–polyamide/DNA solution add 20 μl of freshly prepared Fe(NH$_4$)$_2$(SO$_4$)$_2$ and equilibrate for 10–30 min. Add 40 μl of DTT (use a freshly thawed aliquot) and allow cleavage to proceed for 30 min. Add 1 ml of ethanol, mix the tubes by inversion, and spin briefly.

6. Precipitate the DNA and run the gel: Add 10 μl of precipitation buffer to each tube and mix by inversion. Spin the tubes at 14,000 rpm in a cold room for 30 min. Decant, wash with 75% (v/v) ethanol (350 μl), and decant. Resuspend the DNA in 16 μl of water, freeze, and Speed-Vac dry. Resuspend in 7 μl of 80% (v/v) formamide–1× TBE loading buffer, heat denature (10 min at 85–90°, then place on ice), and load 5 μl per lane on a denaturing 8% (w/v) gel and subject to electrophoresis.

7. Transfer the gel to paper, cover with plastic wrap, and dry on a gel dryer (45–60 min) at 80°. Expose the gel to a storage phosphor screen for 8–16 hr and image with a PhosphorImager.

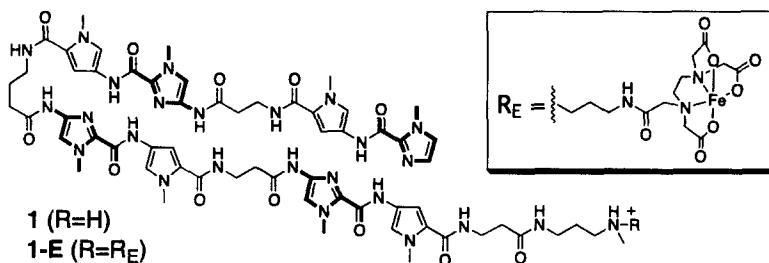FIG. 2. Structures of polyamide ImPy-β-ImPy-γ-ImPy-β-ImPy-β-Dp (**1**) and EDTA–polyamide ImPy-β-ImPy-γ-ImPy-β-ImPy-β-Dp (**1–E**) (β, β-alanine; γ, γ-aminobutyric acid; Dp, dimethylaminopropyl).

Data Analysis

The extent of cleavage protection [for MPE–Fe(II) footprinting] and cleavage intensities (for affinity cleavage experiments) are determined by quantitation of the gel, using ImageQuant software. For MPE–Fe(II) footprinting experiments, θ_{app} values are calculated for each band, using Eq. (1). For affinity cleavage experiments, quantitation of cleavage products provides the cleavage efficiency directly.

Characterization of ImPy-β-ImPy-γ-ImPy-β-ImPy-β-Dp

We reported previously that the eight-ring polyamide ImPy-β-ImPy-γ-ImPy-β-ImPy-β-Dp (**1**) (Fig. 2) binds to 5′-(A,T)GC(A,T)GC(A,T)-3′ target sequences within the human immunodeficiency virus type 1 (HIV-1) promoter region that are adjacent to binding sites for the cellular transcription factors TBP and Ets-1, blocks HIV-1 transcription *in vitro,* and inhibits HIV-1 replication in cell culture.[10] As an example application of the quantitative DNase I footprinting protocol described here, we present results of experiments that (1) assessed the specificity of **1** for a match site versus several mismatch sites, and (2) compared equilibrium association constants determined with TKMC buffer (10 mM Tris-HCl, 10 mM KCl, 10 mM MgCl$_2$, 5 mM CaCl$_2$, pH 7.0), which provides optimal DNase I activity, with those obtained with solution conditions approximating those expected to prevail within a living cell.

Complexes of **1** with binding sites on the 282-bp *Eco*RI/*Pvu*II restriction fragment from pJT2B2 were characterized by quantitative DNase I footprinting, MPE–Fe(II) footprinting, and affinity cleavage experiments. Plasmid pJT2B2 was prepared by hybridizing the complementary oligonucleotides 5′-CCGGCTTAAGTTC GTGGGCCATGCTGCATTCGTGGGCCATGGTGGATTCGTGGGCCATGTTA CATTCG-3′ and 5′-TCGACGAATGTAACATGGCCCACGAATCCACCATGGC
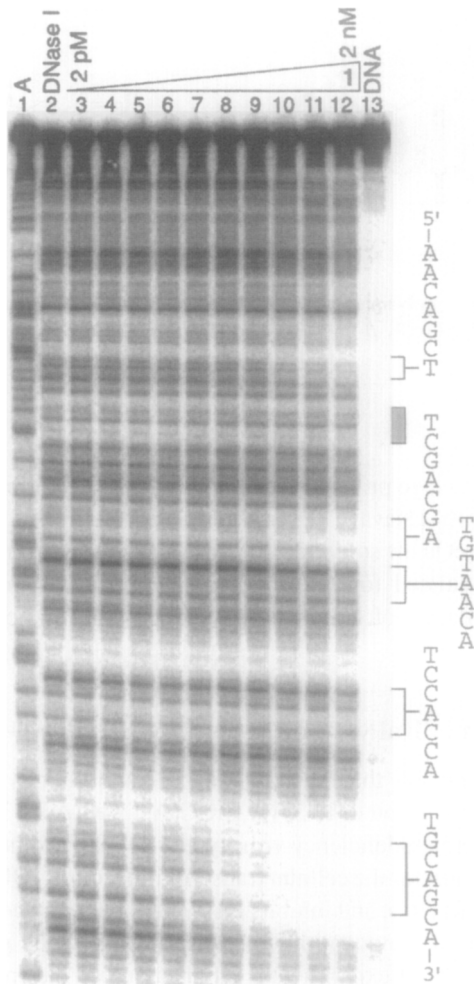
FIG. 3. Storage phosphor autoradiogram of a denaturing 8% (w/v) polyacrylamide gel used to separate the products generated by DNase I digestion in a quantitative footprinting experiment with polyamide **1**. Lane 1, A-specific sequencing lane (see Dervan[17]); lane 2, DNase I digestion products obtained in the absence of polymide; lanes 3–12, DNase I digestion products obtained in the presence of 0.002, 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, and 2 nM polyamide **1**, respectively; lane 13, intact DNA. Polyamide-binding sites and the reference site (shaded bracket) are indicated along the right side of the autoradiogram. All reactions contained 15 kcpm of ^{32}P end-labeled restriction fragment, 10 mM Tris-HCl, 10 mM KCl, 10 mM MgCl$_2$ and 5 mM CaCl$_2$ (pH 7.0, 24°).
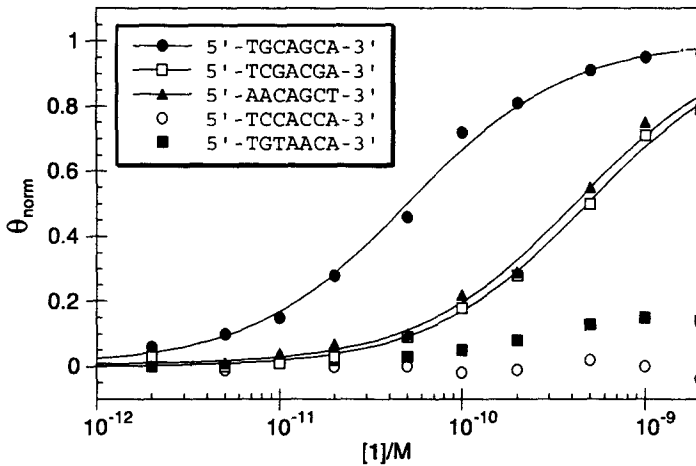
FIG. 4. Binding isotherms generated from the gel shown in Fig. 3. The θ_{norm} values were calculated and fit by Eq. (2) ($n = 1$) as described in text.

CCACGAATGCAG CATGGCCCACGAACTTAAG-3', and ligating the resulting duplexes to the large pUC19 *Ava*I/*Sal*I fragment. The plasmid was transformed into *Escherichia coli,* plasmid DNA was isolated by standard methods, and the sequence of the *Eco*RI/*Pvu*II segment was confirmed by dideoxy sequencing.

Quantitative DNase I footprinting (Figs. 3–5) revealed that **1** binds the match sequence 5'-TGCTGCA-3' with subnanomolar affinity ($K_a = 1.5 \times 10^{10}\,M^{-1}$) and is $\sim$10-fold specific for this site relative to both the single-base pair mismatch site
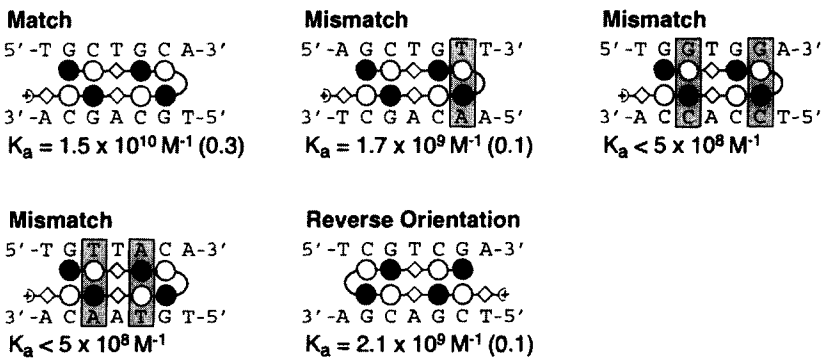

FIG. 5. Binding models and experimentally determined equilibrium association constants (M^{-1}) for polyamide **1**. The reported equilibrium association constants are the average values determined from three footprint titration experiments. The standard deviation for each value is indicated in parentheses.

TABLE I

EQUILIBRIUM ASSOCIATION CONSTANTS OF
POLYAMIDE 1 FOR ITS MATCH SITE
5′-TGCTGCA-3′[a]

Buffer[b]	Temperature(°C)	K_a (M^{-1})
TKMC	24	1.5×10^{10} (0.3)
	37	8.4×10^{9} (2.3)
Intracellular	24	1.9×10^{10} (0.6)
	37	1.1×10^{10} (0.2)

[a] Values reported are the mean values from at least
three DNase I footprint titration experiments.
The standard deviation for each value is indi-
cated in parentheses.
[b] TKMC buffer: 10 mM Tris-HCl, 10 mM KCl,
10 mM MgCl$_2$, and 5 mM CaCl$_2$, pH 7.0 at
24°. Intracellular buffer: 10 mM HEPES-HCl,
140 mM KCl, 10 mM NaCl, 1 mM MgCl$_2$, 1 mM
spermine, pH 7.2.

5′-AGCTG$\underline{T}$$\underline{T}$-3′ and the "reverse orientation" site[26] 5′-TCGTCGA-3′. The (θ_{norm}, [L]) data points for 1 binding to these sites were well fit by Langmuir binding isotherms [Eq. (2), $n = 1$], consistent with formation of the expected 1 : 1 "hairpin" polyamide–DNA complexes. Polyamide 1 is >50-fold specific for its match site 5′-TGCTGCA-3′ relative to the double-base pair mismatch sites 5′-T$\underline{C}$CA$\underline{C}$CA-3′ and 5′-TG$\underline{T}$A$\underline{A}$CA-3′. Additional footprinting experiments indicate that increasing the equilibration temperature from 24 to 37°, and changing the solution conditions from standard polyamide assay conditions (i.e., TKMC buffer: 10 mM Tris-HCl, 10 mM KCl, 10 mM MgCl$_2$, 5 mM CaCl$_2$, pH 7.0 at 24° to conditions modeling those encountered within a typical mammalian cell[30] (140 mM KCl, 10 mM NaCl, 1 mM MgCl$_2$, 1 mM spermine, pH 7.2) has little effect on the affinity of polyamide 1 for its match site 5′-TGCTGCA-3′ (Table I). We note that DNase I activity is nearly 100-fold lower in the model intracellular buffer compared with TKMC.

MPE–Fe(II) footprinting and affinity cleavage experiments confirm that 1 binds to its match site 5′-TGCTGCA-3′ (Fig. 6). The observed affinity cleavage pattern, which consists of roughly equal cleavage patterns at both ends of the binding site, is expected because the ligand can bind its pseudosymmetric match site in two orientations. Cleavage patterns are shifted toward the 3′ end of each strand at the binding site, consistent with binding of the ligand in the minor groove of DNA.

[30] R. J. Jones, K. Y. Lin, J. F. Milligan, S. Wadwani, and M. D. Matteucci, *J. Org. Chem.* **58**, 2983 (1993).
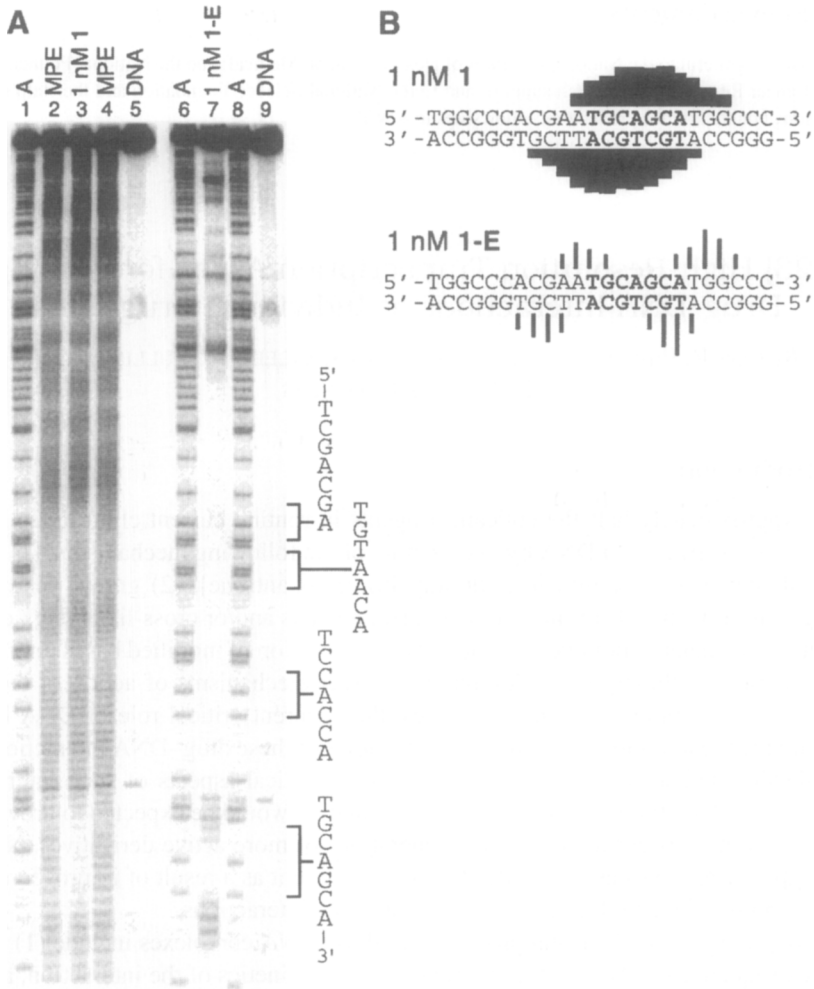
FIG. 6. (A) Storage phosphor autoradiogram of a denaturing 8% (w/v) polyacrylamide gel used to separate the products generated by an MPE–Fe(II) footprinting experiment with polyamide **1** and by an affinity cleavage experiment with polyamide **1-E.** Lanes 1, 6, and 8, A sequencing lanes; lanes 2 and 4, MPE–Fe(II) cleavage products obtained in the absence of **1;** lane 3, MPE–Fe (II) cleavage products obtained in the presence of 1 n*M* **1;** lane 5, intact DNA [no MPE–Fe(II)]; lane 7, cleavage products obtained in the presence of 1 n*M* **1-E;** lane 9, intact DNA (no **1-E**). The reactions contained 15 kcpm of restriction fragment, 20 m*M* HEPES, 200 m*M* NaCl, glycogen (50 μg/ml), and 5 m*M* DTT at pH 7.0, and 0.5 μ*M* MPE–Fe(II) [for MPE–Fe(II) footprinting, lanes 2–4] or 1 μ*M* Fe(II) (for affinity cleavage, lanes 7 and 9). (B) Results of MPE–Fe(II) footprinting (*top*) and affinity cleavage experiments (*bottom*) with polyamides **1** and **1-E,** respectively. Horizontal and vertical bar heights are proportional to the amount of cleavage protection and cleavage, respectively, at the indicated base.

[23] High-Resolution Transcription Assay for Probing Drug–DNA Interactions at Individual Drug Sites

By Don R. Phillips, Suzanne M. Cutts, Carleen M. Cullinane, and Donald M. Crothers

Introduction

Approximately half the anticancer agents in routine current clinical use are known to interact with DNA by one or more of the following mechanisms: (1) intercalation [e.g., doxorubicin (Adriamycin), mitoxantrone], (2) groove binding (e.g., distamycin), (3) formation of covalent adducts and/or cross-links (e.g., cisplatin, melphalan, mitomycin C), and (4) incorporation of modified bases (e.g., 5-fluorouracil, 6-thioguanine). Although the exact mechanisms of action of these agents remain unresolved in some cases, the apparent critical role of DNA has prompted the initiation of a wide range of studies of these drug–DNA interactions. A detailed understanding of the chemical/biochemical aspects of such interactions with DNA (particularly categories 1–3 above) would be expected to provide the necessary insight to design new generations of more active derivatives. New therapeutic approaches have indeed become apparent as a result of improved understanding of the molecular detail of drug–DNA interactions.

Some of the fundamental properties of drug–DNA complexes include (1) the DNA sequence involved in the interaction, (2) the kinetics of the interaction, and (3) the affinity of the interaction. There have been two distinct phases in the development of experimental approaches to determine these parameters. Early procedures relied on a variety of physicochemical methods such as detergent sequestration, equilibrium dialysis, and spectrophotometric/spectrofluorimetic binding studies. Although these procedures all yield useful and valuable information concerning the overall drug–DNA interaction, a general limitation is that they usually yield only average binding parameters resulting from the multiple equilibria occurring at a multitude of binding sites on heterogeneous DNA and do not provide details of the drug–DNA interaction at individual sites on the DNA. To overcome this limitation several new approaches were subsequently developed that relied on the use of identical sequences of DNA (usually from plasmids), rather