Short Technical Reports

Footprinting with an Automated Capillary DNA Sequencer

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ABSTRACT

*Footprinting is a valuable tool for studying DNA-protein contacts. However, it usually involves expensive, tedious and hazardous steps such as radioactive labeling and analyses on polyacrylamide sequencing gels. We have developed an easy four-step footprinting method involving (*i*) the generation and purification of a PCR fragment that is fluorescently labeled at one end with 6-carboxyfluorescein; (*ii*) brief exposure of the fragment to a DNA-binding protein and then DNase I; (*iii*) spin-column purification; and (*iv*) analysis of partial digestion products on the ABI PRISM™ 310 capillary DNA sequencer/genetic analyzer. Very detailed and sensitive footprints of large (> 400 bp) DNA fragments can be easily obtained, as illustrated by our use of this method to characterize binding of PhcA, a LysR-type activator, to two sites greater than 100 bp apart in the 5′ untranslated region of* xpsR*, one of its regulated target genes. The advantages of this new method are that it (*i*) uses long-lived, safe and easy-to-make fluorescently labeled target fragments; (*ii*) uses sensitive, robust and highly reproducible fragment analysis using an automated DNA sequencer, instead of gel electrophoresis and autoradiography; and (*iii*) is cost effective.*

INTRODUCTION

Galas and Schmitz (1) were the first to describe DNA footprinting, a now widely used technique that maps physical contacts between a DNA molecule and a protein to the nucleotide level (2,4,9). Usually target DNA fragments are labeled at a single 5′ end with ^{32}P, incubated with the DNA-binding protein and then partially digested with DNase I. The resultant randomly cleaved fragments are size-separated on polyacrylamide sequencing gels. After autoradiography, the fragmentation pattern is compared to that of an identical fragment that was not exposed to the protein. Fragments generated by DNase I cleavage within the region bound by the protein are reduced in amount because the protein sterically restricts access of DNase I to the DNA backbone in the vicinity of its binding site. Exonuclease III can be substituted for DNase I but only reveals the 3′ ends of binding sites (10). Partial chemical cleavage of the DNA backbone using hydroxyl radicals (8), bis 1, 10-phenanthroline-Cu (7) or piperidine [after methylation by dimethylsulfate (2)] has also been used because these smaller cleavage reagents can more closely access DNA near the bound protein, yielding higher resolution footprints.

A major disadvantage of most footprinting methods is the time-consuming, problematic and hazardous preparation of target fragments (or primers) that are radioactively labeled at only one end. The same can be said for analysis of radioactive fragments on thin sequencing gels. Footprinting using infrared dye-labeled fragments eliminates radioactivity and is very sensitive (5), but preparation of these fragments is very expensive, and the instruments that analyze infrared dye-labeled fragments still utilize sequencing gels and are not yet widely available.

For a laboratory inexperienced in manual DNA sequencing, setting up gel/radioactivity-based footprinting is a formidable and expensive undertaking. Automated DNA sequencers, which detect fluorescently labeled DNA, provide an opportunity for safer and easier footprinting (6). However, most use sequencing gels and are not economical or easy to adapt for analysis of smaller numbers of footprinting reactions. Newer, single capillary-based DNA sequencer/genetic analyzers have none of these drawbacks. These instruments can analyze and quantify DNA fragment patterns in individual samples with very high sensitivity and ease, such as in microsatellite analysis and AFLP analysis. Here, we describe a convenient and sensitive footprinting method using stable and easy-to-make fluorescently labeled DNA fragments and a capillary DNA sequencer.

MATERIALS AND METHODS

Primers and Standards

A derivative of an M13 forward primer with eight additional nucleotides (*M13L: 5′-CACGACGTTGTAAAACGACGGCCAGT-3′) and labeled with 6-carboxyfluorescein (6-FAM*) at the 5′ end was obtained from Applied Biosystems (Foster City, CA, USA), as were GeneScan®-500-ROX (500-ROX) molecular weight standards, a mixture of 35–500 bp DNA fragments labeled with ROX (carboxy-X-rhodamine) and the 6-FAM, ROX, NED and JOE matrix standards. Unlabeled T7 primer (T7: 5′-TAATACGACTCACTATAGGG-3′) and M13 forward primer (M13F: 5′-TGTAAAACGACGGCCAGT-3′) were from the Molecular Genetics Instrumentation Facility at the University of Georgia.

Preparation of DNA Fragments Labeled at One End with 6-FAM

DNA fragments for footprinting were prepared by PCR using pTZRLZ1 [a pTZ18U derivative with a 374-bp insert containing the *xpsR* promoter and upstream sequences between -338 and +36 (3)] as template and primer pairs T7 and *M13L. Reactions (100 µL) contained: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM each primer, 10 ng pTZRLZ1 and 1.5 U *Taq* DNA polymerase (Sigma, St. Louis, MO, USA). After denaturation at 95°C for 2 min, 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min were used, followed by a final incubation at 72°C for 10 min. PCR products were electrophoresed on a 1% agarose gel in TAE buffer; the approximately 400-bp 6-FAM-labeled fragment was excised from the gel and purified using a QIAquick™ Gel Extraction Kit (Qiagen, Valencia, CA, USA). Purified PCR products were quantified with a DyNAQuant™ 200 (Hoefer Pharmacia Biotech, San Francisco, CA, USA).

Footprinting Reactions

A modification of the method of Kullik et al. (4) was used. Each 10-µL reaction contained: 10 mM Tris-HCl,

pH 7.5. 5 mM KCl, 1 mM EDTA, 2 mM dithiothreitol (DTT), 0.1 mg/mL bovine serum albumin (BSA), 8% glycerol, 40 ng (approximately 10 nM final concentration) of 6-FAM-labeled DNA fragment and 0.02–1.0 µg (final concentration 13–650 nM) of purified, tetrameric PhcA protein prepared as described previously (3). BSA was added to a final total protein concentration (including PhcA) of 4.5 µg/10 µL. After incubation at 30°C for 30 min, samples were transferred to a 26°C water bath, and 5 µL DNase I (1.2×10^{-5} U/µL), freshly prepared by diluting the 10 U/µL stock of RNase-free DNase I (Roche Molecular Biochemicals, Indianapolis, IN, USA) into D buffer (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM $CaCl_2$ and 0.1 mg/mL BSA), were added. After 4 min at 26°C, digestion was stopped by chilling on ice and adding 15 µL 0.5 M EDTA, pH 8.0. Reactions were extracted with phenol-chloroform-isoamyl alcohol (25:24:1), and DNA fragments were further purified using a CENTRI-SEP™ column (Princeton Separations, Adelphia, NJ, USA) as specified by the manufacturer. Eluted samples were vacuum-dried and dissolved in 12 µL deionized formamide. After adding 0.2 µL (1.6 fmol) of 500-ROX size standards, samples were denatured at 95°C for 5 min, briefly centrifuged and then transferred to 0.5-mL sample tubes (Applied Biosystems).

Optimum DNase I fragmentation conditions were determined as follows: the 10 U/µL DNase I stock was diluted 1:10 000 at 4°C in D buffer and then serially diluted 1:3 four times to final concentrations of approximately 3×10^{-4}, 1×10^{-4}, 3×10^{-5} and 1×10^{-5} U/µL. Footprinting reactions with 6-FAM-labeled fragments were assembled as above, and after 30 min at 30°C, 5 µL each DNase I dilution was added. Digestions were performed for 2, 3 and 4 min at 26°C, stopped, electrophoresed on a 6% polyacrylamide 8 M urea gel and fragmentation visualized with a model 575 FluorImager™ with ImageQuant™ software (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The DNase I concentration that gave a moderately smeared pattern with approximately 60% fragment remaining intact gave optimum fragment size distribution.

Fragment Analysis on the ABI PRISM310

Fragments were separated on an ABI PRISM 310 DNA Sequencer/Genetic Analyzer equipped with 61 cm × 50 µm uncoated capillary, 1-mL syringe, ABI PRISM 310 GeneScan version 3.1 and ABI PRISM 310 Genetic Analyzer Data Collection version 1.0.2 software using the GeneScan application (all from Applied Biosystems). Run parameters

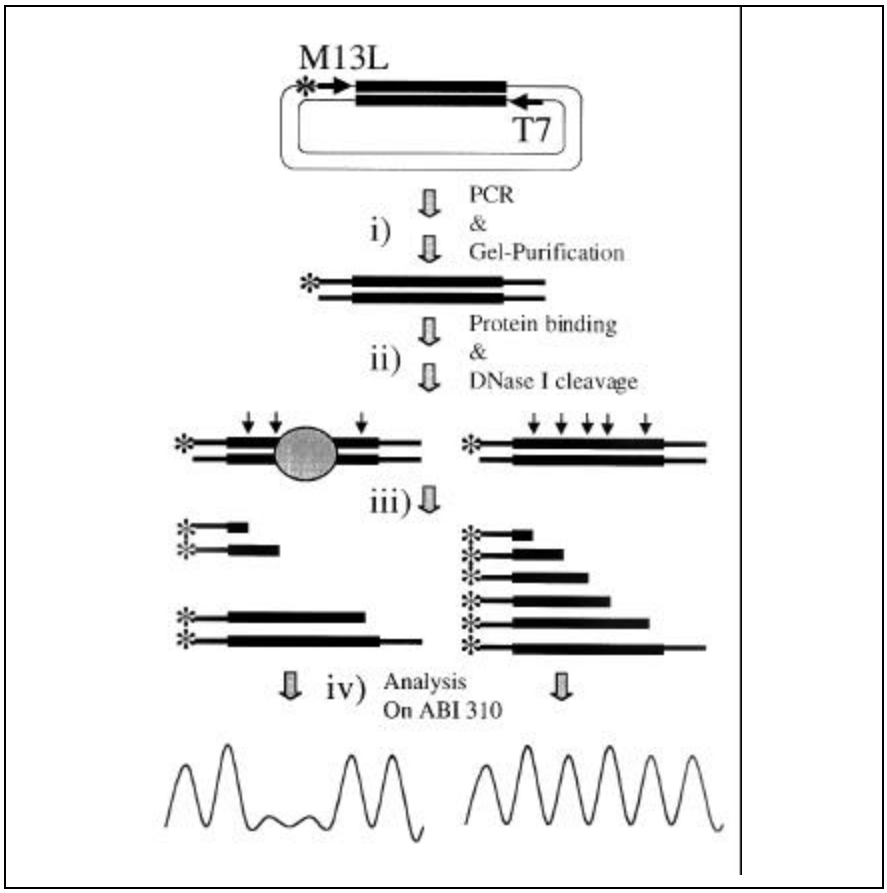

Figure 1. Outline of the footprinting method. (i) The fluorescently labeled target fragment is generated by PCR using universal primers (T7 and M13L), only one of which is 5′ end labeled with 6-FAM (*) and a plasmid with a cloned target fragment as template. (ii) The gel-purified target fragment is incubated with purified DNA binding protein (oval) and DNase I (solid arrows represent potential DNase I cleavage sites). (iii) Partial digestion products are purified with a spin column and (iv) analyzed with the ABI PRISM310 and its software.

were SEQPOP6 (1 mL); Filter set, A; Run Time, 90 min; Injection Time, 40 s. When samples were analyzed with the GeneScan run module (*GS_POP4_D_1ml*), Run Time was set at approximately 40 min. To generate a Matrix File that corrects for spectral overlap of fluorescent dyes, 1 µL each ROX, 6-FAM, NED and JOE matrix standards in 12 µL deionized formamide were heated at 90°C for 2 min, chilled and individually run on the ABI PRISM 310 as above. DNA sequencing was performed on pTZRLZ1 using an M13F primer and ABI PRISM BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems). Before running the sequencing reactions on the ABI PRISM 310, 0.2 µL 500-ROX standard was added

Sample files with output data from each run were automatically created by the ABI PRISM310 and then analyzed with GeneScan Analysis software using Default parameters (e.g., Data Processing, check Baseline, and Multicomponent; Min. Peak Half Width, 3 Pts.; Size Call, All). To determine sizes of fragments, a standard curve was applied to each electropherogram using the migration positions of the internally added 500-ROX size standards and Local Southern Method. Results were analyzed and viewed as electropherograms in the Results Control window. More detailed running and analysis information can be found in the User's Manuals for the ABI PRISM 310 and GeneScan Analysis software.

RESULTS AND DISCUSSION

To eliminate many shortcomings of current footprinting protocols, we developed a simple four-step method (Figure 1). After cloning the target fragment into a pUC-type vector, the insert was PCR amplified using two universal primers, only one of which was fluorescently labeled at the 5′ end with 6-FAM. To footprint both strands, two reactions are performed, alternating which primer is fluorescently labeled. This yields a pair of identical fragments labeled at either end. After gel purification, approximately 1 µg amplified target fragment was obtained, enough for 25 footprinting reactions; labeled fragments were stable for more than 6 months when stored at -20°C. The unit cost of the fluorescent primer to make 1 µg fragment is less than $1.

Footprinting reactions contained 40 ng 6-FAM-labeled target fragment and 0.02–1.0 µg purified DNA binding protein (13–650 nM final concentration). Here, we used PhcA, a LysR-type transcriptional activator (3), and a 400-bp fragment containing the promoter and further upstream regions of the *xpsR* gene, which, according to previous gel shift assays (3), contained a PhcA binding site. After protein binding, reactions were digested with approximately 5×10^{-5} U DNase I for 4 min (see Materials and Methods). Digestion products were purified by spin column and spiked with 500-ROX molecular weight stan-

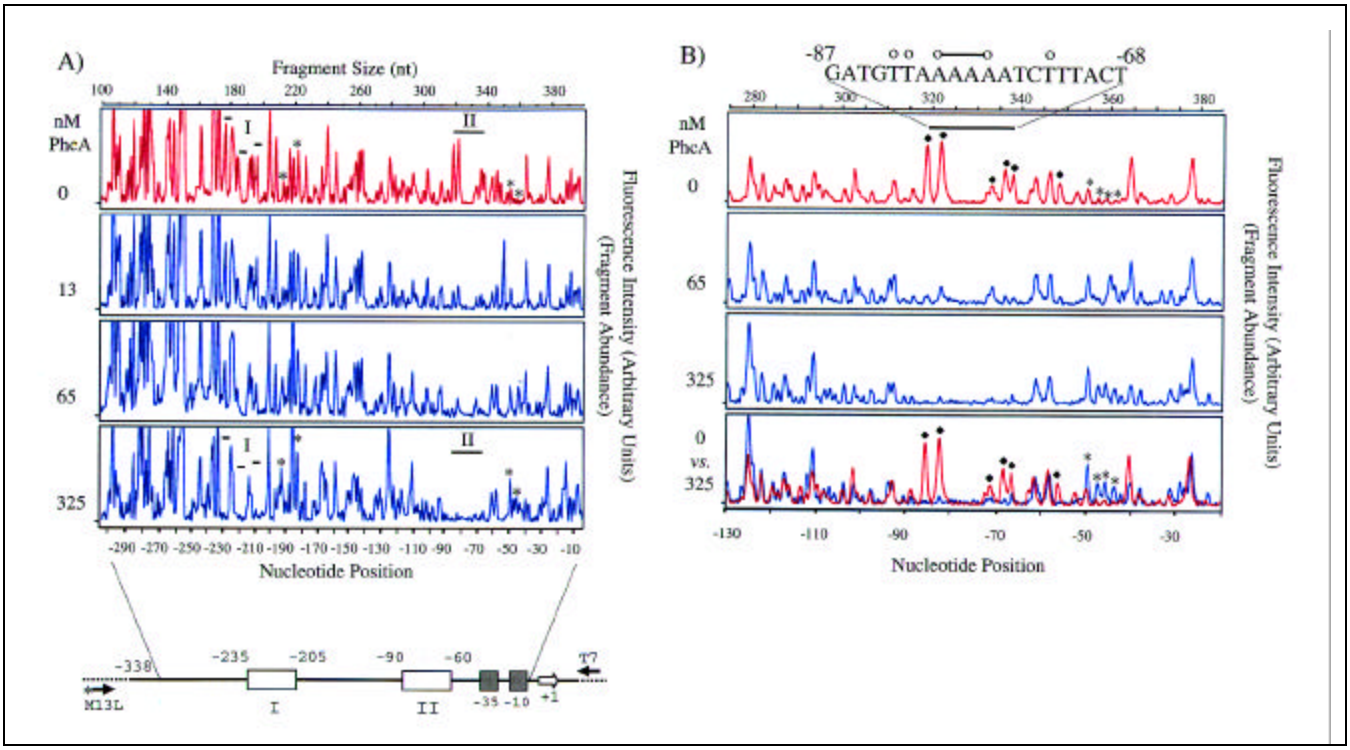

Figure 2. Output data from analysis of PhcA footprinting reactions on the ABI PRISM310. Reactions were set up, processed and analyzed as described in the text. (A) Electropherograms from reactions with increasing amounts of PhcA. Solid bars (I and II) mark the upstream regions of *xpsR* that are protected from DNase I digestion by PhcA. At the bottom is a scale that gives nucleotide position relative to the *xpsR* transcription start site and a schematic diagram of the *xpsR* promoter target fragment showing the location of primer sites (M13L, T7), transcription start site (+1), -35/-10 consensus hexamers and PhcA binding sites (I and II). (B) Expanded views of the region II-containing portion of selected electropherograms from panel A that were generated using the GeneScan Zoom command. Specific sites that are protected (◆) or made hypersensitive (*) by PhcA are marked. The DNA sequence of region II is shown above; nucleotides identified by mutagenesis as critical for transcription activation and binding by PhcA are marked (O). Bottom panel shows the superimposition of the top and the third panels. nt, nucleotides.

dards before separation and analysis by capillary electrophoresis on the ABI PRISM 310. Typical run outputs from footprinting reactions displayed as electropherograms are shown in Figure 2. The y-axis gives fluorescence intensity (i.e., fragment abundance), while the x-axis gives elution position, which is proportional to size. The nucleotide size scale on the top was generated from the elution positions of the internally added molecular weight standards. Running a dideoxy sequencing reaction with the same fragment/primer combination and spiked with the same 500-ROX standards demonstrated that this nucleotide size calibration method was accurate to within 2 bp (not shown).

Inspection of these electropherograms clearly reveals two regions upstream of the *xpsR* transcription start site where the abundance of specific fragments decreases with increasing amounts of PhcA (Figure 2A), which is consistent with specific binding of PhcA to these two regions. As observed with other footprinting methods, some positions in the fragment also become hypersensitive to DNase I cleavage in the presence of PhcA. From the size of these affected fragments, the position of nucleotides in the two regions protected from digestion by PhcA was determined to be between 175 and 200 bp and between 315 and 340 bp from the 6-FAM-labeled end. This places the protected regions between -205 and -230 (Figure 2A, region I) and between -60 and -90 (Figure 2A, region II) relative to the *xpsR* transcription start site. Similarly, prominent hypersensitive sites were found between -195 and -186, and between -50 and -46. It can also be seen in Figure 2A that higher amounts of PhcA were required for marked protection of region I as compared to region II, suggesting that PhcA has a lower affinity for region I. Similar PhcA-mediated protection of both regions I and II was observed in footprinting reactions with the complementary strand [i.e., using PhcA and the same *xpsR* fragment, except that it was PCR amplified using a fluorescently labeled T7 primer and unlabeled M13 primer (not shown)]. Footprinting analyses of the *xpsR* promoter fragment using similar amounts of mock-purified PhcA (prepared the same as purified PhcA, except using cells lacking *phcA*) or using 3 µg purified VsrC [a DNA binding protein of *Ralstonia solanacearum* that does not control *xpsR* (3)] gave fragmentation patterns identical to those with no added protein (not shown). Finally, footprinting reactions using up to 2 µg purified PhcA and a fragment with an internal coding sequence of the *egl* gene of *R. solanacearum* showed no protected regions.

When portions of these electropherograms were expanded and further analyzed with ABI PRISM 310 software,

the PhcA-protected region II could be more accurately located and defined to the segment between -68 and -87 (Figure 2B). Moreover, superimposing this expanded output from runs of reactions with PhcA onto those from runs that lacked PhcA enhanced the ability to detect smaller changes in fragmentation patterns, revealing less prominent protected and hypersensitive sites (Figure 2B, bottom panel). Besides presentation as an electropherogram, ABI PRISM310 run data can be displayed by Genotyper software (Applied Biosystems) as a false gel image, a series of bands whose darkness is proportional to the abundance of each fragment and whose position is proportional to the size of the fragment (not shown).

The conclusion that region II is a bona fide PhcA binding site is supported by the previous observations that nucleotide substitution mutations at -83, -82 and -73 of the *xpsR* promoter dramatically reduce its transcriptional activation by PhcA (3). Moreover, these mutations were also reported to reduce the binding of PhcA to *xpsR* promoter fragments when analyzed by gel shift assays. This previous study also presented circumstantial evidence for a very weak PhcA binding site located between -239 and -183; this region contains the weakly protected region I detected by our PhcA footprinting analysis.

Thus, our new footprinting method can accurately and sensitively detect both strong and weak protein binding sites with relative ease and high specificity. Detection and quantification can be enhanced because the digital output produced by the ABI PRISM 310 is amenable to computerized analysis and manipulation. In less than one day, our new method allowed detailed and sensitive detection of two protected regions on a 400-bp fragment separated by greater than 100 bp, as compared to using a polyacrylamide gel that would probably require two separate loadings and prolonged autoradiography to get comparable detail. It is reasonable to assume that with appropriate modifications, larger fragments with more distantly separated binding sites could be readily detected by our method. Although here we used DNase I, there is no obvious theoretical reason why the higher resolution chemical cleavage reagent dimethylsulfate/piperidine (2) could not be used. This new method is very sensitive since only 100 fmol fragment gives reasonable signals, similar to the sensitivity of radioisotope/gel footprinting methods. Unlike gel methods, though, if signals are too low or too high, injection time and/or sample concentration can be quickly adjusted and the sample immediately rerun on the ABI PRISM 310. There were many more clearly resolved peaks in the electropherograms than would be expected for discrete bands obtained from analysis of ^{32}P-labeled target fragments on sequencing gels, attesting to the higher resolution of our method. Analysis on the ABI PRISM 310 was also highly reproducible because duplicate runs on the same sample gave almost identical profiles, as did duplicate footprinting reactions, even when runs were performed weeks apart or after storage at -20°C. The reagent cost for each ABI PRISM run is less than $10. With the looming obsolescence of equipment and reagents for manual DNA sequencing, this method can provide an improved alternative to gel-based footprinting.

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