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On the cover:

Fluorescent-labeled NGF mRNA produced by RT-PCR in adult male mouse salivary gland. Image was collected and processed quantitatively using an MRC 600 confocal microscope. Image provided by Thomas R. Van De Water. For further information see *BioTechniques* 16:76-80, 1994.

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INFORMATION FOR AUTHORS

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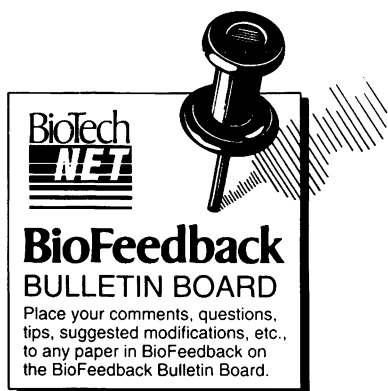
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Short Technical Reports

SHORT TECHNICAL REPORTS

Manuscripts published in the Short Technical Reports section are shorter and less comprehensive in scope than full Research Reports.



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Nonradioactive, Solid-Phase DNase I Footprints Analyzed on an A.L.F. DNA Sequencer™

ABSTRACT

Solid-phase DNase I footprinting provides a powerful tool for analyzing the sequence-specific interactions of DNA binding proteins. Classically this type of assay requires radioactively labeled DNA molecules. Substitution of the isotope by fluorescein labeling of the DNA fragments enables the analysis of footprint patterns on a standard automated laser fluorescent (A.L.F.) DNA Sequencer™. The combination of solid-phase footprinting technology and fluorescence-based nonradioactive detection of fragments has unique advantages over established footprinting technologies.

INTRODUCTION

One of the advances in molecular biology technology in the recent years is the development of automated DNA sequencers (1,5) which use on-line detection of fluorescently labeled DNA fragments in high resolution polyacrylamide gels and direct data storage in a computer. Automated DNA sequencing technology is widely distributed in large-scale DNA sequencing laboratories and is becoming increasingly popular also in small-scale sequencing facilities. It has been shown previously, that such devices cannot only be used as DNA sequencers, but as DNA fragment analyzers in general, e.g., for analyses of RFLPs, primer extension experiments, restriction fingerprinting or microsatellite PCR products (6). However, the technology has not yet been adapted to the analysis of DNA-protein interactions by DNase I footprinting that also requires high-resolution analysis of DNA fragments (3). Recently, Sandaltzopoulos and Becker (4) described an adaptation of the standard DNase I footprinting procedure using ³²P-labeled fragments to solid-phase technology that minimizes the duration of the assay and endows it with versatility.

Here we describe that solid-phase DNase I footprinting using fluorescein-labeled DNA combined with fragment analysis on an A.L.F. DNA Sequencer™ (Pharmacia Biotech, Uppsala, Sweden) provides a powerful tool for DNA-protein interaction studies with several advantages over the established procedure that requires radioactive DNA.

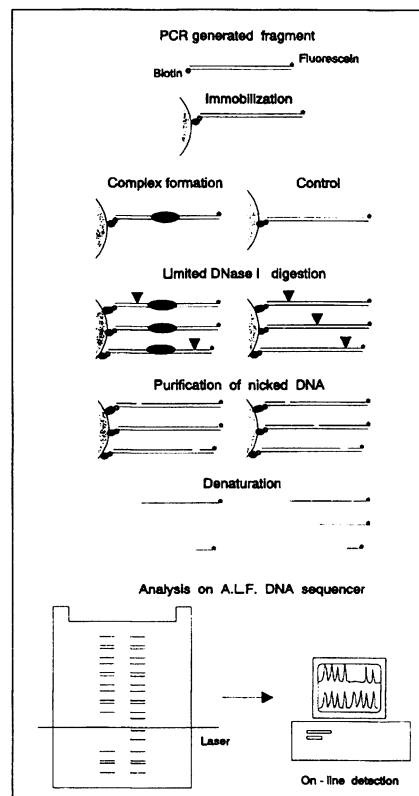


Figure 1. Outline of nonradioactive, solid-phase footprinting. A PCR fragment containing the hsp70 promoter was generated as described (4) with 100 pmol of biotinylated primer A and 100 pmol of fluorescein-labeled primer B. To the completed PCR, an equal volume of 4 M NaCl was added, and the unpurified biotinylated PCR product was immobilized on 5 mg of streptavidin-coated paramagnetic beads (4). The DNA beads were washed and finally resuspended in 100 μ L 2 M NaCl/10 mM Tris-HCl, pH 7.5, 1 mM EDTA. 10 μ L of DNA beads were used for a solid-phase footprinting reaction (4) which provided material for 10 gel runs. The footprinting reactions require incubation of immobilized DNA with the sequence-specific binding protein (here: HSF) to allow complex formation, partial DNase I digestion and purification of the nicked DNA fragment by high-salt buffer washes while still attached to the beads. The reaction products were heat-denatured, 10% were electrophoresed on a 6.5% (29:1) denaturing polyacrylamide gel under standard DNA sequencing electrophoresis conditions and analyzed on an A.L.F. DNA Sequencer (Figure 2).

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RESULTS AND DISCUSSION

The combination of nonradioactive, solid-phase DNase I footprinting and DNA fragment analysis on an A.L.F. DNA Sequencer is schematically described in Figure 1. Suitable DNA fragments harboring the presumed protein binding site are prepared by polymerase chain reaction (PCR) using a combination of a biotinylated primer and a fluorescein-labeled oligonucleotide. It is also possible to generate the biotinylated and fluorescent fragments by isolating suitable restriction fragments and filling in the ends with the corresponding nucleotide derivatives and Klenow polymerase (6). As previously described (4), the labeled, biotinylated fragments are immobilized on streptavidin-coated paramagnetic beads (Dynabeads® M-280 Streptavidin; Dynal, Oslo, Norway) and purified from unincorporated primers and free nucleotides during subsequent washes. The protein binding reaction, as well as the DNase I digestion and washing steps, is performed with the immobilized DNA fragments in respective buffer systems as described (4). Finally, the fluorescently labeled fragments are released from the beads by the addition of loading buffer containing 96% formamide and dextran blue (6 mg/mL), heat denatured and analyzed on a denaturing high resolution (6.5%)

polyacrylamide gel as described (1). Beads do not interfere with electrophoresis and automated detection. Figure 2 shows an exemplary result of the fragment pattern obtained from a footprinting reaction assaying the binding of *Drosophila melanogaster* heat shock factor [HSF, (2)] in crude extracts from HSF-expressing *Escherichia coli* to heat shock elements in the hsp70 promoter. Binding of HSF to the heat shock elements is deduced from the absence of fluorescent fragments (peaks) in a window (shaded bar) corresponding to sequences that are protected from DNase cleavage by HSF as compared to the control reaction without the protein. Flanking the binding site, a hyper-reactive phosphodiester bond (HS) is also observed that presumably reflects a distortion of the DNA helix upon protein binding.

The combination of nonradioactive, solid-phase DNase I footprinting with fragment analysis on an A.L.F. DNA Sequencer offers several advantages over the standard procedure using radioactive DNA. The radiation risks are eliminated, and no wastes that need special disposal are produced. Since the fluorescence label does not decay, immobilized template and standards can be stored for years until needed. Moreover, since the label is not a substrate for phosphatases, the described protocol is the method of choice when work-

ing with crude extracts that contain phosphatase activity. The pattern of fluorescent fragments generated by DNase I digestion are processed on-line and can be analyzed directly using appropriate software (e.g., GenSkipper, developed at EMBL, Heidelberg, FRG, available from C. Schwager, EMBL). Raw data need not be evaluated immediately but can be stored indefinitely. The option of peak quantitation that will become available in the future with the development of appropriate software might be important in cases where partial occupancy at a protein binding site necessitates precise evaluation. Analysis is cost-effective, no film is needed to visualize the footprint and no gel scanning is required. Hands-on time is reduced significantly, and hence the total time required until the result is known is in the order of from 3 to 4 hours. The use of 60-cm long high-resolution gels allowing separation of up to 1000 bases offers the possibility to analyze even longer regions containing several sites of DNA-protein interactions (7).

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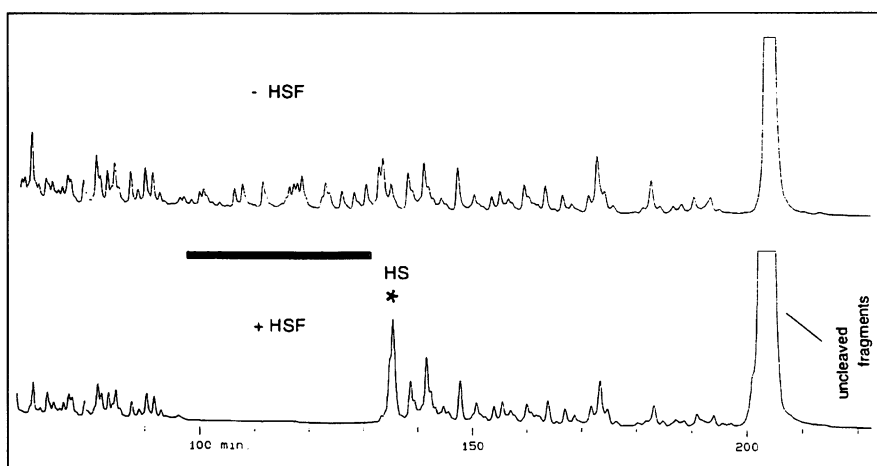


Figure 2. Raw data output of a footprinting reaction assaying the binding of *D. melanogaster* (HSF) in crude extracts from HSF expressing *E. coli* to heat shock elements in the hsp70 promoter. The fluorescein-labeled fragments from the footprinting reaction were analyzed on-line after laser beam excitation on an A.L.F. DNA Sequencer. Upper lane: Control reaction with fragment pattern obtained in the absence of HSF protein. Lower lane: Fragment pattern obtained in the presence of HSF protein. The extent of the HSF "footprint" is indicated by the horizontal bar. A hyperreactive site (HS) is labeled with an asterisk.

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