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Mapping Small DNA Ligand Hydroxyl Radical Footprinting and Affinity Cleavage Products for Capillary Electrophoresis

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Abstract

The mapping of DNA footprints and affinity cleavage sites for small DNA ligands is affected by the choice of sequencing chemistry and end label, and the potential for indexing errors can be significant when mapping small ligand-DNA interactions. Described here is a mechanism for avoiding such errors based on a summary of standard labeling, cleavage and indexing chemistries and a comparison among them for analysis of these interactions by capillary electrophoresis. The length dependence of the difference between Sanger and Maxam-Gilbert indexing is examined for a number of duplexes of mixed sequence.

Keywords

DNA; footprinting; capillary electrophoresis; sequencing; ligand-DNA interactions

Footprinting [1, 2] and affinity cleavage [3] are powerful tools for mapping the binding sites of DNA ligands. Precision in establishing these sites to the nucleotide level is critical to many projects and is especially challenging for small DNA ligands. While many classic small DNA ligands have been studied using footprinting [4, 5], newer molecules continue to emerge, and the details of their DNA binding behaviors are of interest [6, 7].

DNase I footprinting is preferred by many for its convenience, and works well when studying large ligands (e.g. proteins or large synthetic molecules) [1] (**Fig. 1**). Hydroxyl radical chemistry [8] is preferred when detailed information about the solvent-accessible surface of the DNA-ligand complex is needed, when footprinting small DNA ligands and/or when footprinting any ligands that bind AT rich sequences [2], which are particularly poor DNase I substrates [9]. Hydroxyl radical footprinting can give highly detailed pictures of nucleic acid structure [10]. EDTA-iron affinity cleavage chemistry is hydroxyl radical chemistry, but by design it is constrained to occur only at the binding site(s) of the ligand modified with the EDTA group [3].

With footprinting and affinity cleavage, the results are mapped using reference sequence information. In traditional polyacrylamide gel electrophoresis (PAGE), either the 5' or 3' end of the DNA is radiolabeled, sometimes side-by-side. The availability of high throughput capillary electrophoresis (CE)-based DNA sequencing machines offers a throughput

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advantage over manually-poured sequencing gels. Accompanying elimination of radioactive labels and exposure to acrylamide are eliminated, making CE a most effective means of modern footprinting analysis [11].

In capillary electrophoresis [12], mobility of DNAs is influenced by both the nature of the end group and the presence of dyes [13, 14]. Dyes are typically positioned on the 5' end when footprinting results are analyzed, and the mobility shift caused by the dyes used in CE is much more significant than for normal PAGE/slab gels [13-15]. The choice of sequencing chemistry and endlabeling method affects the mapping of footprints and affinity cleavage sites. When DNA-binding ligands are of low molecular weight, the potential for mapping errors is significant. The shifts in cleavage patterns for major and minor groove binders adds additional complications [3]. To aid the adoption of CE-based footprinting methods regardless of cleavage chemistry, we summarize the labeling, cleavage and indexing chemistries and compare them according to a protocol that aids analysis by CE.

Sanger dideoxy chain termination [16] is the most robust and convenient sequencing chemistry. As illustrated in **Fig. 2A**, the 3'-ends are neutral (having been elongated from the 5' terminus of a template-bound primer using a mix of "normal" dNTPs and 2',3'-dideoxynucleotide triphosphates) and are similar in mobility to that of the corresponding 5'-labeled DNase I footprint products. Due to the effects of dye labeling on DNA mobility [13, 14], more accurate indexing for a footprinting experiment is achieved by performing four separate reactions (G, A, T, and C; e.g. using Sequenase) using the same dye endlabel employed for footprinting and/or affinity cleavage. If sequencing utilizes DNAs labeled at the terminal dideoxy nucleotide with different dyes for each nucleotide (as with the ABI Big Dye method), the differential influences of the dyes will alter DNA mobility during CE, leading to ambiguities in mapping the ligand-DNA binding site.

Sanger chemistry has been applied to index fragments generated by other, usually radical, chemistries [12, 17]. However, using these reactions to index and map hydroxyl radical chemistry products leads to an artificial 5' shift when working with 5' fluorescently labeled DNA. This is principally due to the chemical destruction of the terminal base being identified in a hydroxyl radical reaction [8], resulting in a fragment one nt shorter than would appear when indexing with Sanger chemistry, which leaves the terminal base intact (**Fig. 2A**). Furthermore, hydroxyl radical reactions yield negatively charged phosphate termini (both 5' and 3') and/or side products such as the anionic 3'-phosphoglycolate [8, 18, 19] (**Fig. 2A**). These products are not resolved from one another in low percentage PAGE gels [20] or by CE (data not shown). Maxam Gilbert (MG) reactions [21] modify the bases, open the ribose ring, and render the phosphate backbone subject to base-catalyzed hydrolysis. This leads to destruction of the attacked nucleotide in a manner related to hydroxyl radical cleavage. Since MG and radical mechanisms both yield phosphate ends, more accurate indexing of footprinting and affinity cleavage is achieved [20] by direct comparison of footprinting/affinity cleavage results with MG reaction products than with Sanger products. While the potential for incorrect indexing with Sanger methods may be of no consequence in some studies [12, 17], it is critical when studying small ligand-DNA interactions.

To understand the mobility difference between Sanger and Maxam-Gilbert products as analyzed by CE, these reactions were conducted for five separate 200-300 bp 5'-Fam or Hex-labeled sequences from the genome of HPV 16 (see Experimental). Fragment lengths were assigned to the nearest integral nucleotide position and the data plotted as the difference (Sanger minus Maxam-Gilbert) vs. real position (**Fig. 2B**). The pattern observed is consistent among different sequences. When DNA fragments are less than 100 nt in size, the difference between the two sequencing methods is quite large (up to several nt). This is

attributed to the greater influence of varying end-group chemistry or labeling for smaller DNAs. As DNA length increases, this difference decreases exponentially to about 0 nt near 200 nt in length. This occurs because, as the length increases, the relative influence of any difference in charge decreases, regardless of where the charge is located. Since many experimenters work with DNAs that are smaller than 200 bp and the sensitivity of fragmentation product analysis by CE typically decreases with length after about 200–300 nt, it is recommended that Maxam-Gilbert reactions be performed for every different DNA sequence. Applying Sanger sequencing to hydroxyl radical cleavage products and using a simple subtraction algorithm for the entire length of the DNA is prone to indexing errors.

Experimental

The regions 7662–122, 7348–7798, and 3538–3888 of the HPV16 genome were generated by PCR using a 5' Fam (fluorescein) or Hex (hexachlorofluorescein)-labeled primer on one strand, purified by agarose gel electrophoresis and a Qiagen gel extraction kit (Valencia, CA). One or both strands of these fragments were sequenced using both Sanger chemistry (USB, Affymetrix, Santa Clara, CA) and Maxam-Gilbert reactions [21]. The samples were analyzed using an ABI 3730XL capillary electrophoresis instrument at the Univ. Missouri DNA Core Facility with GeneScan 600 LIZ size standards (ABI). POP 7 polymer and ABI 3730 buffer were used. Data were processed using Genemarker V1.97 software (Softgenetics LLC, State College, PA) and used to correlate sequence information from both methods. Plots in **Fig. 2B** were generated using Kaleidagraph 4.1.3 software (Reading, PA).

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Fig. 1.

Schematic of footprinting using a 5' Fam-labeled duplex generated by PCR. Black lines indicate fragments that are detectable by capillary electrophoresis; gray lines represent fragments that are not detectable. A Fam-labeled primer is used on the top strand (horizontal arrow left) and an unlabeled primer is used on the bottom strand (horizontal arrow gray right). Cleavage is indicated with vertical arrows. Major and minor groove binding ligands have different effects on the location of cleavage on top vs. bottom strand ([3]; omitted for clarity). Fragments 1a and 3a are detected by CE. Fragment 2a is not present when the ligand protects the DNA from cleavage. The portion of the top strand on the 3' side of the ligand site and the bottom strand are also cleaved, but without a label, they are undetected (1b, 1d, 2b, 2d, 3b, 3d). The bottom strand cleavage fragments 1d and 3d can be visualized simultaneously with 1a and 3a by 5'-labeling the top and bottom strands with different dyes (omitted for clarity).

Fig. 2.

A: Summary of chemical ends generated by various footprinting and sequencing chemistries assuming a 5' dye label and targeting the Nth base. Approximate relative lengths are deliberately implied. B: Examples of the difference between Sanger and Maxam-Gilbert positions vs. real position for five different sequence regions of the HPV 16 genome. In the top two panels, the dye is Fam; in the bottom three, the dye is Hex.