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Footprinting: A method for determining the sequence selectivity, affinity and kinetics of DNA-binding ligands

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Abstract

Footprinting is a simple method for assessing the sequence selectivity of DNA-binding ligands. The method is based on the ability of the ligand to protect DNA from cleavage at its binding site. This review describes the use of DNase I and hydroxyl radicals, the most commonly used footprinting probes, in footprinting experiments. The success of a footprinting experiment depends on using an appropriate DNA substrate and we describe how these can best be chosen or designed. Although footprinting was originally developed for assessing a ligand's sequence selectivity, it can also be employed to estimate the binding strength (quantitative footprinting) and to assess the association and dissociation rate constants for slow binding reactions.

Keywords: Footprinting; DNase I; Hydroxyl radicals; Sequence selectivity; DNA binding

1. Introduction

Footprinting was developed in 1978 for studying the interaction of DNA-binding proteins with their target sites [1]. This was easily adapted as a method for assessing the sequence specific interaction of ligands (small molecules) with DNA and can be used to provide information on the sequence selectivity, affinity and binding kinetics [2–5]. Soon after its development it had been successfully used to discover the sequence specificity of a number of well known ligands, such as actinomycin, echinomycin and related compounds, distamycin and other minor groove binders, mithramycin and a wide range of other ligands [6–13]. The technique continues to be widely used for assessing the sequence specificity of novel ligands including polyamides [5], triplex-forming oligonucleotides [14,15] and minor groove binding ligands [16,17].

Footprinting is essentially a protection assay in which the digestion of double-stranded DNA by a cleavage agent such as DNase I or hydroxyl radicals is locally inhibited by the binding of a ligand at specific binding sites within a DNA fragment. The main principle of the method is illustrated in Fig. 1. A double-stranded DNA fragment, which is labelled at one end of one strand, is cut by a chemical or enzymatic cleavage agent so that on average each DNA molecule is only cleaved once (*i.e.* single-hit kinetics). If the cleavage agent does not possess any sequence selectivity, then it will produce a random distribution of products which can be resolved on a denaturing polyacrylamide gel. When the digestion is repeated in the presence of a sequence selective ligand this will protect from cleavage at the regions to which it is bound, and these products will therefore be missing from the reaction and will be evident as a gap (''footprint'') in the gel. By running control and ligand-treated digestion alongside suitable markers (*e.g.* a Maxam–Gilbert chemical sequencing reaction) the exact binding sites for the ligand can be easily determined. By performing the footprinting reaction over a range of ligand concentrations its binding affinity can be estimated and, if the kinetics are slow, the time dependence of the appearance of the footprint can be used to assess the reaction

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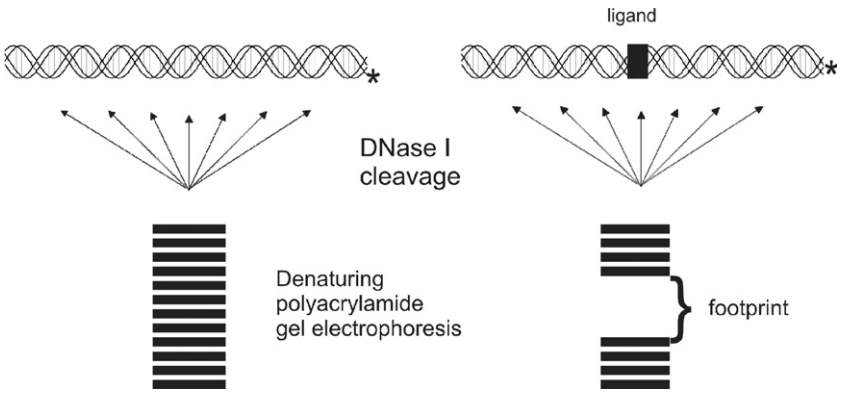

Fig. 1. Schematic representation of the footprinting experiment. The fragment is labelled (asterisk) at one end of one strand and digested with a cleavage agent such as DNase I under conditions of single-hit kinetics. The ligand protects from cleavage at its binding site and this is evident as a footprint when the products of digestion are resolved on a denaturing polyacrylamide gel.

kinetics. The practical aspects for determining each of these parameters are considered below.

1.1. Choice of cleavage agent

The ideal cleavage agent should be sequence neutral (*i.e.* generate an even ladder of bands in the ligand-free control), quick, cheap and easy to use and should provide high resolution information about the ligand's preferred binding site(s). No single agent fulfils all these criteria and a number of enzymic and chemical probes have been employed in different studies. The most widely used are DNase I and hydroxyl radicals, which are considered below, though others such as MPE [6,8,18] are also popular.

1.1.1. DNase I

DNase I is a double-strand-specific endonuclease (m.w. 30,400) which acts by introducing single strand nicks into the phosphodiester backbone, cleaving the O3′–P bond. It requires divalent cations for activity, and magnesium or calcium are the usual ions of choice [19,20]. The reaction can therefore be easily stopped by chelating these ions with EDTA. Although early studies suggested that the enzyme requires both these ions [19,20] we find that either can be used alone, though it is about 10-fold more efficient with magnesium than calcium. Manganese enhances the cleavage efficiency and we usually include this in the reaction buffers. Some studies have suggested that manganese might affect the DNase I cleavage pattern [21]; we have never observed such an effect. Although DNase I is not sequence specific, it produces a very uneven ladder of cleavage products, which are determined by the local DNA structure [22,23]. The most important factors affecting the cleavage are the local minor groove width and the flexibility of the DNA; $A_n{\cdot}T_n$ tracts and GC-rich regions are generally poor cleavage sites. DNase I binds to its substrate by inserting an exposed loop into the minor groove [21,24,25] and it therefore cannot bind to sequences which possess narrow minor grooves (such as $A_n{\cdot}T_n$ tracts). Crystal structures have shown that DNase I distorts its substrate [24,25], bending the DNA towards the major groove, and this is thought to be a necessary part of the catalytic mechanism. Rigid DNA sequences (such as GC-rich regions) are therefore poor substrates. DNase I footprinting may be less successful if the ligand under investigation binds to one of these poorly cut regions.

DNase I is a large glycoprotein and it binds to about 10 base pairs (one turn of the DNA helix). This limits the accuracy for estimating the preferred binding sites and the enzyme overestimates the ligand site size by about 3 base pairs.

DNase I binds across the DNA minor groove and, since the phosphodiester backbone is inclined relative to the helical axis, the region protected from cleavage is staggered across the two strands. As a result the footprint is staggered in the 3′-direction by about 2–3 bases. A similar effect is seen with hydroxyl radical cleavage patterns, and is most easily resolved by comparing the protection patterns on each strand of the duplex in separate experiments.

Despite these limitations DNase I is cheap and simple to use and it is the most popular footprinting probe. The reaction is easy to control and it can be stopped by addition of EDTA. The enzyme functions over a wide range of pHs (we have used between pH 5.0 and 9.0), temperatures (we have used between 4 and 70 °C) and ionic strengths (up to 1 M NaCl). Depending on the enzyme concentration short incubation times can be employed and there is usually no need to remove the enzyme before loading the reaction products onto a denaturing polyacrylamide gel. Although DNase I cuts from the DNA minor groove it is able to detect the ligands that bind from the major groove (such as triplex-forming oligonucleotides) [14,15], presumably because these alter the DNA structure and/or flexibility.

1.1.2. Hydroxyl radicals

Hydroxyl radicals are generated by the Fenton reaction between Fe^{2+} and H_2O_2 (Eq. 1) and are highly reactive freely diffusible species. As a result of their small size they are able to cleave close to the bound ligand and so more