COMMENTARY

Methylation interference was used initially by Siebenlist and Gilbert (1980) to study the interaction of RNA polymerase and the T7 promoter. This technique utilized the chemistry developed for the G reaction in DNA sequencing (Maxam and Gilbert, 1980). Recently methylation interference has been coupled with the mobility shift DNA-binding assay (UNIT 12.2), resulting in relatively rapid analyses of the contact sites for DNA-binding proteins.

The two procedures typically used to map the region of DNA-protein interaction are methylation interference and DNase I protection. Methylation interference has several advantages over DNase I protection. First, methylation interference analyses are not hindered by incomplete binding because all of the probe in a retarded complex is bound by protein. In contrast, DNase I protection experiments require protein titrations to fully saturate probe DNA. Another disadvantage of DNase I digestions is that protein exchange from the binding site during the digestion can lead to cutting within the binding site. Finally, methylation interference allows the determination of specific nucleotides that are in relatively close contact with the DNA-binding protein. As a result, much more information is obtained about the binding site than from the comparatively large protected region produced by a DNase I footprint. For example, methylation interference has determined single nucleotide differences in binding site specificities that could not be determined by DNase I protection (Baldwin and Sharp, 1988).

Although methylation interference does not measure direct protein contacts with nucleotides, it permits detection of nucleotides that are closely apposed to the proteins. DMS methylates guanine residues at the N-7 position that protrudes into the major groove, and also at the N-3 position of adenines that protrudes into the minor groove. Adenines can be detected in this assay but are much weaker than the G reactions (Maxam and Gilbert, 1980).

A logical extension of methylation interference has been described recently (Brunelle and Schleif, 1987). In this procedure, depurinated and depyrimidated DNA probes are substrates in the binding reaction. Bound and free probes are separated by native gel electrophoresis and cleaved by piperidine, allowing detection of proteins closely apposed to T and C, as well as G and A, residues.

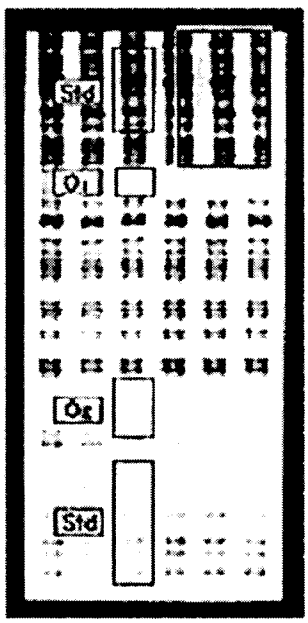
DNase I Footprint Analysis of Protein-DNA Binding

Deoxyribonuclease I (DNase I) protection mapping, or footprinting, is a valuable technique for locating the specific binding sites of proteins on DNA. The basis of this assay is that bound protein protects the phosphodiester backbone of DNA from DNase I--catalyzed hydrolysis. Binding sites are visualized by autoradiography of the DNA fragments that result from hydrolysis, following separation by electrophoresis on denaturing DNA sequencing gels. Footprinting has been developed further as a quantitative technique to determine separate binding curves for each individual protein-binding site on the DNA. For each binding site, the total energy of binding is determined directly from that site's binding curve. For sites that interact cooperatively, simultaneous numerical analysis of all the binding curves can be used to resolve both the intrinsic binding and cooperative components of these energies.

The unit is divided into three parts. The basic protocol covers DNase I footprint titration and involves (1) preparation of a singly end-labeled DNA restriction fragment, (2) equilibration of the protein with DNA, (3) exposure of the equilibrium mixture to DNase I, and (4) electrophoretic separation on gels of the denatured hydrolysis products, followed by autoradiography. The support protocol describes (1) densitometric analysis of the autoradiograms to obtain binding data and (2) numerical analysis of the binding data to yield binding curves and equilibrium constants for the interactions at each of the separate sites. An alternate protocol describes the qualitative use of footprinting to identify DNA-binding proteins in crude extracts.

ALTERNATE PROTOCOL: DNASE FOOTPRINTING IN CRUDE FRACTIONS

DNase footprinting is frequently used to locate proteins in crude fractions, thus providing an assay for use during purification. The purpose of this type of footprinting is usually to locate a particular binding activity, not to characterize the strength of the interaction. Thus one first establishes conditions under which a complete footprint can be observed, and then assays fractions of a crude extract for that activity.



Portion of a footprint titration autoradiogram showing the different blocks that need to be defined for analysis. "Std." denotes a standard block used to correct for variations in the DNA loaded onto each lane. OI and OE denote protein-binding sites 1 and 2, respectively. The insert shows regions for a standard block for which background determinations will be made.

COMMENTARY

In both prokaryotes and eukaryotes the binding of proteins to specific DNA sequences is critical to the regulation of many cellular processes. In addition, cooperative interactions among bound proteins (perhaps also with non-DNA--binding proteins) are known to be critical to the regulation of transcription, replication, and recombination. A key to understanding the molecular mechanism by which these proteins regulate cellular processes is the measurement of the free energy of binding of these proteins to DNA and the free energies that describe the cooperative interactions occurring among them.

The DNase I footprint titration method, with subsequent quantitative analysis, has been shown to yield thermodynamically valid individual site--binding curves for site-specific protein-DNA interactions. In systems containing multiple sites, these curves can be used to resolve intrinsic binding constants, describing the binding of a protein to a site in the absence of other sites, and constants that describe the cooperative interactions among proteins bound to multiple sites. The power of the method is that it is, in principle, not limited by the number of sites or the number of proteins.

The method is based on the fact that DNase I probes, without perturbing, the equilibrium distribution of binding protein with DNA. Control experiments show that DNase I does not perturb the equilibrium between cI-and Gal-repressors and their respective binding sites under the solution conditions we have studied. This conclusion may not be valid, however, for all systems under all experimental conditions. Thus, control experiments must be conducted for new systems and drastically new conditions. Some general considerations are discussed in critical parameters.

DNase I protection mapping, or footprinting, was developed as a qualitative technique to locate the specific protein-binding sites on DNA (Galas and Schmitz, 1978). Subsequently, it was used to compare the relative affinities of cI repressor to multiple binding sites (Johnson et al., 1979). Using the data of Johnson et al., Ackers et al. (1982) resolved the intrinsic binding and cooperative free energies for the binding of cI repressor to the OR operator of the bacteriophage l. A general theory for individual site--binding curves has been developed (Ackers et al., 1983). The fact that quantitative DNase I footprint titrations yield thermodynamically valid individual site--binding curves for site-specific protein-DNA interactions was demonstrated by Brenowitz et al. (1986a,b). A discussion of the use of the footprint titration method (and other popular binding methods) to determine the intrinsic binding and cooperativity constants for systems of multiple, interacting sites has been presented (Senear et al., 1986). Quantitative footprint titration methods for studying drug-DNA interactions have been developed in parallel with the work on proteins presented in this protocol (Dabrowiak and Goodisman, 1989).

Other probes. As a probe of the occupancy of protein-binding sites, DNase I has several advantages over high-resolution probes such as Fe-EDTA (Tullius et al., 1987) or methidiumpropyl-EDTA (MPE) (Hertzberg and Dervan, 1982). First, the uniform footprint produced by DNase I yields large density changes on the autoradiogram that can be accurately quantitated. This facilitates the precise determination of binding curves. Second, divalent cations are known to bind tightly to DNA, are frequently involved in protein structure and self-assembly, and are physiologically important. Unlike MPE and Fe-EDTA, DNase I can be used conveniently with (and requires) divalent cation--containing buffers. Third, the fact that DNase I is an enzyme specific for DNA precludes the possibility of degradation of the binding protein by promiscuous free radicals. Thus, DNase I may be particularly valuable for the analysis of binding proteins sensitive to degradation.

The single base-pair resolution of Fe-EDTA and MPE make these reagents important tools if detailed structural information is desired. There is no a priori reason that they cannot be used to monitor protein or drug titrations with single basepair resolution.

Other binding methods. A virtue of footprinting for quantitative analysis is that protein binding is determined at equilibrium. Unlike other methods, such as the mobility shift assay (UNIT 12.2), the manipulations required to separate and visualize liganded and unliganded DNA are performed after the binding and DNase I--catalyzed reactions are quenched. This feature of the method provides for its application over a wide range of precisely controlled experimental conditions.

In contrast, the use of the mobility shift assay to obtain titration curves that separately represent each protein-DNA complex with exactly n bound proteins requires that the protein-DNA complexes do not dissociate in the gel during electrophoresis and that their distribution remains unchanged. It has been noted that quantitation of the unbound DNA band is less affected by dissociation of protein-DNA complexes in the gel. If only the unbound DNA is quantitated, however, all of the information concerning potential cooperative free energies is lost. For a theoretical discussion of the determination of cooperativity constants from the mobility shift assay, see Senear et al. (1986).

UV Crosslinking of Proteins to Nucleic Acids

Irradiation of protein-nucleic acid complexes with ultraviolet light causes covalent bonds to form between the nucleic acid and proteins that are in close contact with the nucleic acid. Thus, UV crosslinking may be used to selectively label DNA-binding proteins based on their specific interaction with a DNA recognition site. As a consequence of label transfer, the molecular weight of a DNA-binding protein in a crude mixture can be rapidly and reliably determined.

The procedure can be divided into 3 stages: (1) Extract containing the protein of interest is incubated with a radioactive, uniformly labeled DNA fragment that contains a high-affinity binding site for the protein; (2) protein-DNA complexes are crosslinked with UV irradiation and digested with nuclease, leaving only those labeled DNA fragments that are crosslinked and in close contact with the DNA-binding protein; and (3) the molecular weights of the crosslinked proteins are determined by SDS-polyacrylamide gel electrophoresis followed by autoradiography. One of the advantages of this technique is that proteins bound specifically to the DNA probe can be easily distinguished from those bound nonspecifically.

BASIC PROTOCOL: UV CROSSLINKING USING A BROMODEOXYURIDINE-SUBSTITUTED PROBE

DNA molecules containing halogenated analogs of thymidine, such as bromodeoxyuridine (BrdU), are considerably more sensitive to UV-induced crosslinking compared to unsubstituted DNA. Although use of BrdU-substituted probes is not essential for detecting protein-DNA crosslinking by UV light, in many cases it is helpful.

COMMENTARY

Crosslinking proteins to nucleic acids with UV light is a simple method for rapidly and accurately determining the molecular weight of a DNA-binding protein in a crude extract. Moreover, the specificity of the photoadduct can be rigorously determined by measuring the ability of an excess of unlabeled competitor DNA to compete for binding sites on the protein.

The goal of the UV crosslinking method is to specifically transfer a radioactive label from a