Distinguishing specific from nonspecific complexes on Southwestern blots by a rapid DMS protection assay

Maria Polycarpou-Schwarz¹ and Athanasios G. Papavassiliou²,*
European Molecular Biology Laboratory, ¹Gene Expression and ²Differentiation Programmes,
Postfach 10.2209, Meyerhofstrasse 1, D-6900 Heidelberg, Germany

Received March 8, 1993; Revised and Accepted April 15, 1993

Southwestern blotting has been extensively used in studies aiming to the identification of sequence-specific DNA-binding activities in cellular extracts (1). While the sensitivity of this methodology aids to the detection of factors with low, specific DNA-binding affinities, the odd equilibrium and kinetics of the interaction of a DNA segment with a matrix of immobilized protein molecules, allow the simultaneous detection of additional proteins with high, nonspecific DNA-binding affinities, that are present in excess to the probing DNA sequence. Binding to these latter proteins is sometimes abrogated by competition with unlabelled specific DNA (and/or is not affected by unspecific DNA) and manipulating parameters such as ionic strength and pH may not enable the differentiation between specific and nonspecific interactions. In this report we utilized dimethyl sulfate (DMS) in a rapid in situ protection assay (DNA elution/cleavage/purification performed in a single step) to distinguish specific from superfluous DNA—protein complexes on blotting membranes. Apart from providing complete information about a particular DNA—protein interaction (molecular mass of the protein component and protein—contact positions on the DNA), the small size and hydrophobic nature of this chemical reagent render it perfectly suitable for an assay of this kind because of the distinct microenvironments existing in the binding interface of specific and nonspecific complexes. Nonspecific DNA binding to immobilized targets is driven electrostatically (multiple, strong salt bridges with the polyamionic backbone of phosphate and sugar groups, lacking directionality) and by the release of counterions and water bound loosely to it. This transfer of ordered water and hydrated cations to bulk solvent strengthens the ionic DNA—protein interactions by making a favorable contribution to the reaction entropy, and creates an extended, virtually hydrophobic interface readily penetrable by DMS, which will methylate all accessible reaction sites. On the other hand, specific DNA binding involves primarily gradual formation of oriented hydrogen bonds and van der Waals contacts; as water is recruited to bulk solvent to correct ‘imperfections’ in complementarity, it builds a three-dimensional network linking DNA and protein which increases the number of direct and water-mediated hydrogen bonds, hence reinforcing binding and further driving the association. A ‘compact’ hydrophilic interface is thus generated which will exclude DMS by severely impairing its diffusional freedom, limiting its activity to only distorted and exposed base nitrogens. Conceivably, the methylation/cleavage pattern of the interface in DNA derived from nonspecific complexes will be markedly enhanced compared to its unbound counterpart, whereas that from specific complexes will exhibit protections and enhancements due to the specific contacts and specific binding-triggered perturbations, respectively. Although DNase I can in principle substitute for DMS as a probe in this assay (2), its bulky dimensions, mode of target searching and binding to cleave (2), and the requirement for Mg²⁺ (which often stabilizes both specific and nonspecific complexes) highly reduce its potential to ‘sense’ the aforementioned differences.

Total or partially purified nuclear proteins (100—200 μg) are resolved in a preparative SDS polyacrylamide gel and electrotransferred on to a supported hydrophilic membrane [i.e., Hybond-N (Amersham); see below]. The filter-immobilized proteins are renatured under established conditions (3, 4, 5) and probed with 10—20 μg (2—4x10⁶ cpm) of an asymmetrically ³²P-labelled restriction fragment or oligonucleotide bearing the putative binding site. The membrane sheet is exposed (wet) to X-ray film to locate regions of radioactive signal which are then cut with a razor blade and immediately immersed in 500 μl of 20 mM Tris—HCl, pH 8.0. After a 10’ equilibration at 25°C, the radioactivity retained on the strips is measured for Cherenkov counts and a comparable amount of cpm of the same probe in solution is added to 250 μl of 20 mM Tris—HCl, pH 8.0 in a separate tube and put aside awaiting treatment. The membrane strips are transferred with forceps into 500 μl of freshly made 0.18% (v/v) DMS (reagent grade) solution in 20 mM Tris—HCl, pH 8.0 and incubated for 25—40’ at 25°C (to avoid variations in the exposure of samples to DMS, during the DMS addition step no more than one sample should be handled at a time). Although the longer the DNA probe the shorter the length of incubation to achieve the desirable single modification per molecule, we have found (employing probes of various lengths) that the above combination of reaction time and DMS concentration ensures that single-hit kinetics is followed (the percentage of uncut DNA in the final analysis is greater than 70%, Figure 1) and sufficient cleavage for a good signal to noise ratio is generated. Due to the increased kinetic stability of a filter-immobilized DNA—protein complex (reversible binding to even low-affinity proteins is enhanced because the excess unbound DNA fraction has been washed out and hence is not exchangeable), these reaction parameters are not determined by the dissociation rate of the complex, which interferes with DMS protection assays performed in solution. Methylation are quenched by quickly removing the strip (with forceps) and immersing it in a tube

* To whom correspondence should be addressed
containing 1 ml of freshly prepared DMS stop solution A (20 mM Tris—HCl, pH 7.3, 0.5 M β-mercaptoethanol, 15 mM EDTA) prewarmed to 30°C. After 5' at 30°C (inactivation of DMS is faster at this temperature) the filter is transferred into a new tube, rinsed twice (by gentle vortexing) with 1 ml of 20 mM Tris—HCl, pH 7.3 to remove all traces of reagents, and subsequently submerged in 250 μl of 5% (v/v) freshly-diluted 0.2 M NaCl, and 5 mM EDTA in a silanized 0.5-ml tube. The tube is capped using conformable needle and discarded; 10 μg of glycogen is added as carrier, and centrifugation the strip is carefully lifted with the aid of a syringe (Figure 1, SC lane) suggests protein binding-induced conformational alterations of the DNA leading to more exposed (Figure 1, NSC lane corresponds to a base located in the synthetic linker region, outside of the relevant K sequence). The increased reactivity observed at the boundaries of the protected bases (Figure 1, SC lane) suggests protein binding-induced conformational alterations of the DNA leading to more exposed reaction sites, or formation of lipopholic pockets by the protein on the DNA ‘tunnelling’ the reagent and augmenting its local concentration.

ACKNOWLEDGEMENT

We thank Roberto Di Lauro (Stazione Zoologica ‘A. Dohrn’, Napoli) for providing the extracts and the oligonucleotides used in this work and for helpful comments on the manuscript.

REFERENCES