PROTECTION AGAINST EXONUCLEASE III DIGESTION

A new way to investigate protein–DNA interactions

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1. Introduction

Interactions between proteins and DNA molecules form the basis of many biological control mechanisms and of the central processes of molecular biology, replication and transcription. Virtually everything that happens to DNA molecules involves some protein binding to it in a particular way before beginning to act. Control proteins and proteins involved in site-specific recombination do not just recognise general features of a DNA molecule but bind specifically to a particular DNA sequence. In such cases the first step in an investigation of the DNA–protein interaction is to locate the binding site. Clearly every tool that helps us study these interactions is useful, and we report here another way of approaching this problem, which we have used in our studies on the binding of λ integrase to the phage attachment site. A substrate for exonuclease III digestion is synthesised enzymatically in vitro so that the double-stranded section contains the presumed binding region, and the results of digesting this substrate in the presence or absence of the DNA binding protein are compared.

2. Materials and methods

Exonuclease III was purchased from New England Biolabs, and the Klenow fragment of DNA polymerase I from Boehringer, Mannheim.

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2.1. *Preparation of DNA substrate*

DNA substrate was prepared by primed incorporation on a purified λ r-stand as in [1]. A 50 basepair long restriction fragment known to map just outside the region of interest [1] was mixed (40 ng) with 2 μl purified λ r-strand (1 mg/ml) and 0.5 μl '10 × *Hin*' buffer consisting of 66 mM Tris–HCl (pH 7.4), 66 mM $MgCl_2$, 500 mM NaCl and 10 mM DTT. After mixing and boiling in a sealed capillary tube for 3 min the primer and template were annealed for 30 min at 65°C. Sterile H_2O (10 μl) and 4 μl of a nucleotide mix were added. The nucleotide mix contained equal volumes of 0.5 mM dCTP, dTTP, dGTP and 10 × *Hin* buffer. The whole mixture was added to 2 μCi dried d[α-^{32}P]ATP (400 Ci/mmol, Amersham), 0.2 units of the Klenow fragment of DNA polymerase I (Boehringer) were added and the solution was incubated for 10 min at 22°C. The reaction was stopped by adding 2.5 μl 0.25 M EDTA and heating for 10 min at 70°C. Under these conditions 300–500 nucleotides are incorporated. The DNA was separated from salts and unincorporated d[α-^{32}P]ATP by passing it over a 1 ml Sephadex G-100 column equilibrated with 2.5 mM Tris–HCl (pH 7.4) and 0.05 mM EDTA. The DNA peak was lyophilised to dryness and the DNA dissolved in the required buffer.

2.2. *Purification of λ integrase*

Integrase was purified as detailed in [2,3].

2.3. *Exonuclease III digestions*

Exonuclease III digestions were carried out in a buffer in which the integrase remained stably bound (see section 3); 10 mM Tris–HCl (pH 7.4), 10 mM

$MgCl_2$, 0.2 mM EDTA, 70 mM KCl, 1 mM DTT. These conditions are not ideal for exonuclease III, but it is a simple matter to compensate for this by raising the enzyme concentration as far as is necessary. The amount of DNA used in each experiment was a molar equivalent of 5 ng of a 1000 basepair double-stranded region, and amounts of the DNA-binding protein integrase were added which had been shown in separate filter binding experiments [4] to give specific binding of the attachment site region. Integrase was allowed to bind to DNA at 0°C for 5 min, and then the solution was placed in a 22°C or 37°C waterbath for 1 min before adding exonuclease III. An amount of exonuclease III that, in the absence of integrase, gave complete digestion of the labelled strand in this buffer in 15 min at 22°C or 5 min at 37°C was added, and the reaction was allowed to proceed for the times indicated. Eight units of Biolabs exonuclease III was usually sufficient in a 50 μl reaction volume but we have used up to 24 units without losing protection. It is, of course, necessary to determine these quantities empirically in each binding buffer for each DNA-binding protein. Exonuclease III digestion was stopped by the addition of 5 μl 0.25 M EDTA, the DNA ethanol-precipitated in the presence of 50 μg/ml *E. coli* tRNA, the precipitate washed twice with prechilled 95% ethanol and dried. Best results were obtained if this precipitate was dissolved in 10 μl sterile H_2O, passed over a 1 ml Sephadex G-100 column in 2.5 mM Tris (pH 7.4), 0.05 mM EDTA, then lyophilised to dryness.

2.4. *Preparation of DNA sequence standards*

All DNA sequence standards were prepared by primed DNA synthesis on a single-stranded template using the chain termination procedure. Any available system suffices; the standards used in these experiments are from primings on an exonuclease III-treated [6] linearised plasmid clone of human mitochondrial DNA.

2.5. *Gel electrophoresis and autoradiography*

The dry DNA was taken up in 5 μl sterile H_2O, mixed with an equal volume of deionised 98% formamide containing 0.03% bromphenol blue and 0.03% xylene cyanol, boiled for 3 min and electrophoresed in a 7 M urea, 8% polyacrylamide thin gel [7] using an LKB 2103 power pack at 30 mA. The DNA sequencing standards were treated identically and run alongside the experimental samples. The gels were fixed for 5 min in 10% acetic acid, washed in deionised water, dried, wrapped in Saran wrap and autoradiographed.

3. Results and discussion

Exonuclease III has the distinctive feature of being relatively specific for duplex DNA [8], digesting the 3′-OH terminated strand at an end or nick in a 3′ → 5′ direction and leaving a protruding 5′-ended chain. Thus when a small primer DNA fragment is annealed to a single-stranded template and the Klenow fragment of DNA polymerase I is used to incorporated [α-^{32}P]-nucleotide into newly synthesised DNA starting at the 3′-OH terminus of the primer, a population of labelled molecules is produced which are ideal substrates for exonuclease III. If the approximate locatin of a binding site of a DNA-binding protein is known, a primer–template combination and times of incubation can be chosen so that the newly-synthesised labelled molecules mostly contain the binding site in the duplex region and have 3′-OH ends beyond the binding site. Exonuclease III will digest the labelled strand (and the primer) completely, leaving the template untouched because of the long overhanging single-stranded ends. However, if the DNA binding protein is firmly bound to its binding site, some proportion of the exonuclease III molecules will be obstructed and will be prevented from digesting the labelled strand further. This can subsequently be visualised as a concentration of labelled primed strands of a length corresponding to the distance from the 5′-end of the primer to the point of obstruction, giving rise to a strongly radioactive band in a polyacrylamide–urea gel. The length of the DNA fragment in this band can be determined by comparison with DNA sequence standards, so that the position in the DNA molecule at which exonuclease III digestion has been obstructed can be determined. This would then mark the outer boundary of the binding site, or more likely the position of the surface of the DNA-binding protein itself.

We have tested this procedure using λ integrase and phage attachment-site DNA substrate made as above and detailed in section 2. Figure 1 is a diagrammatic representation of the procedure using the particular example of the integrase-attachment site interaction.

Under the conditions for exonuclease III diges-

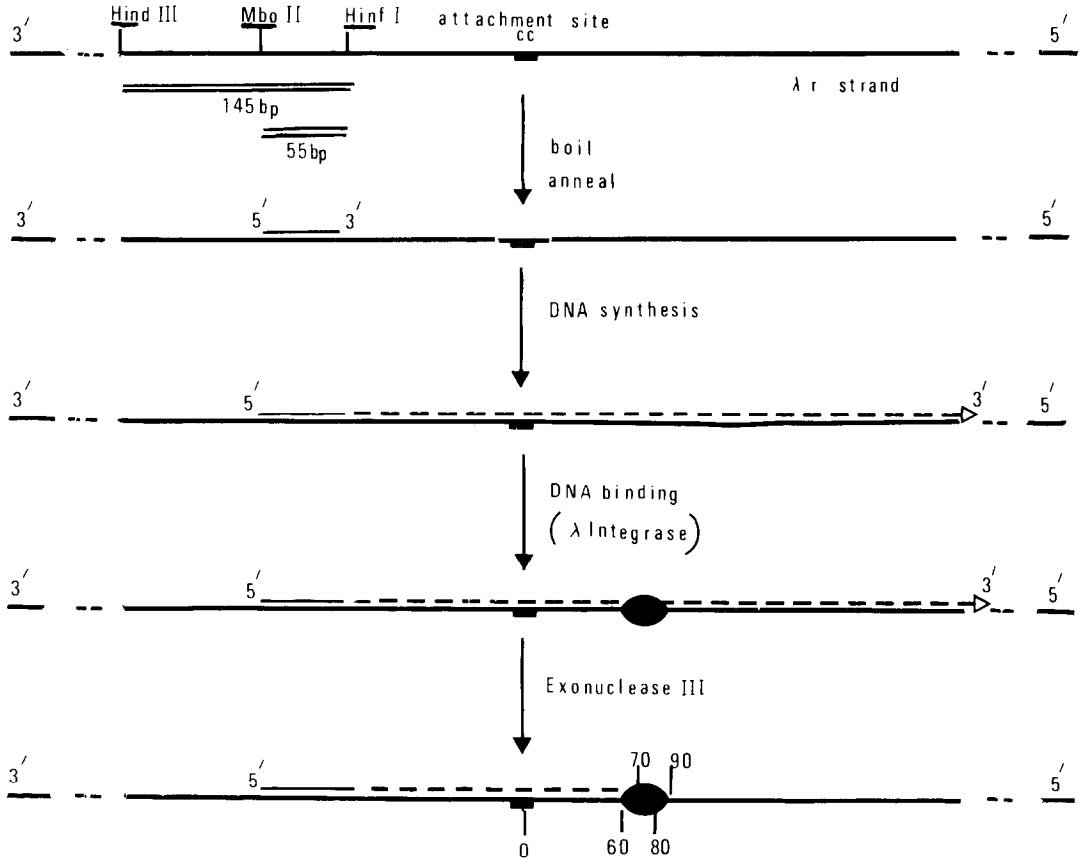

Fig.1. Diagrammatic representation of the exonuclease III protection procedure, using the λ integrase–attachment site interaction as an example. The primers used to synthesise the DNA substrates as in section 2 were the 55 basepairs *Mbo* II–*Hin*fI fragment or the 145 basepairs *Hin*dIII–*Hin*fI fragment shown in this figure. The identity and isolation of these fragments is described in [1]. The symbol cc refers to the common core sequence known to reach the centre of the attachment site, and the numbers on the last line give the distances of the exonuclease III stop points seen in fig.2a,b from the middle basepair of the common core, marked 0.

tion used by Smith ([6]; 70 mM Tris–HCl (pH 8), 1 mM $MgCl_2$, 10 mM DTT) or Roberts (personal communication; 10 mM Tris–HCl (pH 7.5), 2 mM $MgCl_2$, 1 mM 2-mercaptoethanol) we did not observe any protection by a preparation of λ integrase known to be active and specific by the filter binding test. This is presumably due to the absence of KCl. We, therefore, used the standard integrase binding buffer in all experiments and worked out empirically the amount of exonuclease III and time of digestion required to digest unprotected strands. Using these conditions (⩾8 units Biolabs exonuclease III for 5 ng equivalents of a 1000 basepair duplex region in 50 μl reaction volume for 15 min at 22°C or 5 min at 37°C) we were able to demonstrate an integrase-dependent protection effect (fig.2(a,b)). In this way we could identify a high-affinity binding region in the attachment site whose position is amply corroborated by other kinds of experiments ([4], S. G. Minter, R. W. D., P. H. S., M. L. K., H. Echols, in preparation).

While this procedure is not a substitute for protection against DNase I [9–11] or the more precise footprinting [12] and DMS-protection [13], it can usefully augment the information obtained by these techniques. In the simplest case of a DNA-binding protein binding to a single site and obstructing the passage of exonuclease III, information is obtained about the shape of the protein molecule and its positioning on the DNA duplex under conditions far milder than those used for protection against

DNase I. In the experiments presented here, only one direction of DNA synthesis was used. Clearly it is simple to prime DNA synthesis in both directions across the region of interest. In this way both ends of a protein-obstruction (or DNA-deformation, see below) region may be defined. The fact that exonuclease III can thus be used to probe the state of one strand of the DNA duplex at a time enables one to obtain more information than with complete digestion using DNase I. If multiple binding sites are present in a region of DNA, this will show up immediately if both kinds of substrate are used, since the first obstruction points will be relatively far apart. The situation could then be further investigated by taking aliquots of the DNA synthesis reaction mixture at various time points, producing a population of labelled strands with ends distributed at random through the region of interest. Exonuclease III digestion in the presence of the DNA-binding protein would then give rise to a series of bands corresponding to the obstruction points of each of the multiple binding sites. The only comparable approach of which we are aware is unpublished work of M. Ptashne and coworkers mentioned in [9], where λ exonuclease and S1 nuclease were used to show that RNA polymerase protects a 65 basepair region of λ PR against λ exonuclease, compared to the 45 basepairs it protects against DNase I.

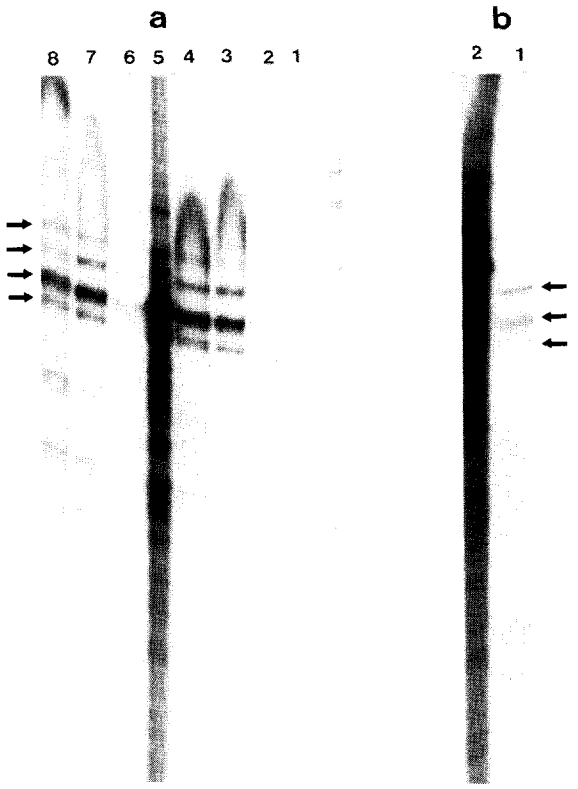

Fig.2. Autoradiographs of 7 M urea 8% polyacrylamide gels of exonuclease III-treated Int–DNA complexes. Experiments were carried out as in section 2. Int and DNA being mixed at 0°C, brought to digestion temperature and exonuclease III added. In 2a 1 μl exonuclease III was used (8 units), and in 2b 3 μl (24 units) were used. In tracks a1, a2, a3 and a4, 0, 1, 5 or 10 μl Int were used, respectively, exonuclease III digestion being for 15 min at 22°C. Track a5 is a human mitochondrial DNA sequence standard. Track a6; 1 μl Int added at the beginning, and at 5 and 10 min during a 15 min digestion with exonuclease III at 22°C. Track 7 is the same as track 6, but 5 μl Int was added at each time. Track 8; 5 μl Int added at the start, and after 1 and 2.5 min of a 5 min exonuclease III digestion at 37°C. In (b) track 2 is a standard DNA sequence, and in the sample in track 1 conditions as in 2a track were used, except that the DNA was subsequently purified over a Sephadex G-100 column to prevent the salt effects seen at the top of 2a. The main Int-dependent bands produced by exonuclease III digestion are shown by arrows. Note that in the experiment shown in (b) with low levels of Int, the top band is not seen, as is the case under similar conditions in 2a; track 2. Int, integrate.

Figure 2 shows that the case of integrase is not so simple, since 4 bands of differing intensities are produced rather than a single band and some background. This is discussed further in [4], but it is clear from other experiments that these bands do occur in a region containing a high-affinity binding site for λ integrase. One interpretation of this effect would suggest that exonuclease III digestion can be used as a probe for the structure of the protein–DNA complex. Thus, banding patterns with a similar spacing are produced when DNA in nucleosomes is digested by exonuclease III [14], so that we may be able to use this method to detect wrapping of a DNA duplex around a protein or protein complex.

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