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Detection of transcription factor binding *in vivo* using lambda exonuclease

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In vivo footprinting techniques have revealed the complex interplay between transacting proteins and DNA in the context of chromatin (1). One simple yet elegant approach to detect such interactions utilizes Exonuclease III (Exo III) to map transcription factor boundaries (2). Exo III is introduced *in vivo* onto the promoter of interest via an adjacent restriction endonuclease site and proceeds to digest the DNA in a 3′ to 5′ direction (Figure 1). Digestion continues until the exonuclease encounters a bound factor that impedes or blocks digestion. Following digestion, the DNA is purified, single stranded (ss) overhangs are trimmed with either S1(2,3) or Mung Bean (4) nucleases, then analyzed by Southern blotting (2,3) or more recently by reiterative Taq polymerase based techniques (4,5).

In this report, we demonstrate that Lambda (λ) exonuclease can be successfully used as an alternative for mapping the boundaries of protein–DNA interactions *in vivo* (Figure 2). The choice of λ exonuclease offers several distinct advantages in these experiments. Firstly, λ exonuclease hydrolyses DNA in a 5′ to 3′ fashion, which allows mapping of protein–DNA interactions on the strand complementary to that analyzed by Exo III (Figure 1). Secondly, unlike Exo III, the rate of digestion by λ exonuclease is highly processive and independent of the base composition of the substrate DNA (6). Presumably this will allow more uniform and reproducible analysis. Finally, as depicted in Figure 1, an additional advantage of digestion with λ exonuclease over Exo III is that analysis using Taq polymerase is simplified by eliminating the need for removal of ss DNA overhangs. In addition to the examination of chromatin templates shown here, this technique could be applied to study protein–DNA interactions on transiently introduced templates, as well as replicating viral templates.

The data in Figure 2 shows the application of the λ exonuclease footprinting strategy to detect transcription factor interactions on the Mouse Mammary Tumour Virus Long Terminal Repeat (MMTV LTR) stably maintained in human T47D breast cancer cells (7). Using digestion conditions optimized for λ exonuclease activity (8), prominent stops were detected on the MMTV LTR (lanes 3 and 4). The stop at position −82 corresponds to that for Nuclear Factor 1 (NF1) detected previously by Exo III (3). Using λ exonuclease, an additional stop at −49 that was not detected using Exo III was observed that maps to the octamer binding sites (cf. lanes 4 and 6). The intensity of the specific stops increased with increasing amounts of λ exonuclease (cf. lanes 3 and 4) and decreased substantially when the digestions were performed under pH and salt conditions optimal for Exo III (lane 5). As we have previously shown for Exo III (5), detection of these stops was dependent upon both a restriction enzyme generated entry site and the addition of λ exonuclease, as no stops were observed in samples digested with either alone (lanes 1 and 2 respectively).

In summary, we have demonstrated that λ exonuclease provides an alternative, effective and simplified means for examining transcription factor–DNA interactions *in vivo*. This enzyme not only provides new information on protein–DNA contacts on the strand complementary to that analyzed by Exo III, but also detects additional interactions not observed with Exo III (cf. lanes 4 and 6).

METHODS

Cell line 2963.1 was derived from human T47D breast cancer cells by stable transfection of the chimeric bovine papilloma based construct pJ83d, carrying the MMTV LTR attached to the

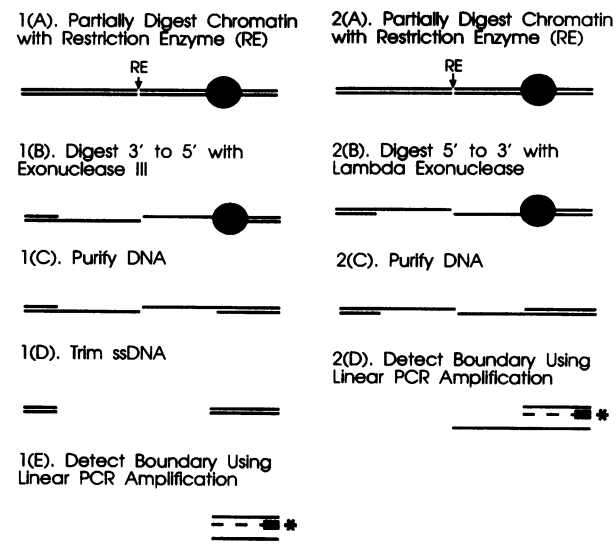

Figure 1. Schematic of methods to detect transcription factor loading *in vivo* using Exo III (left) or λ exonuclease (right). Note the extra step to remove ss DNA in the Exo III protocol.

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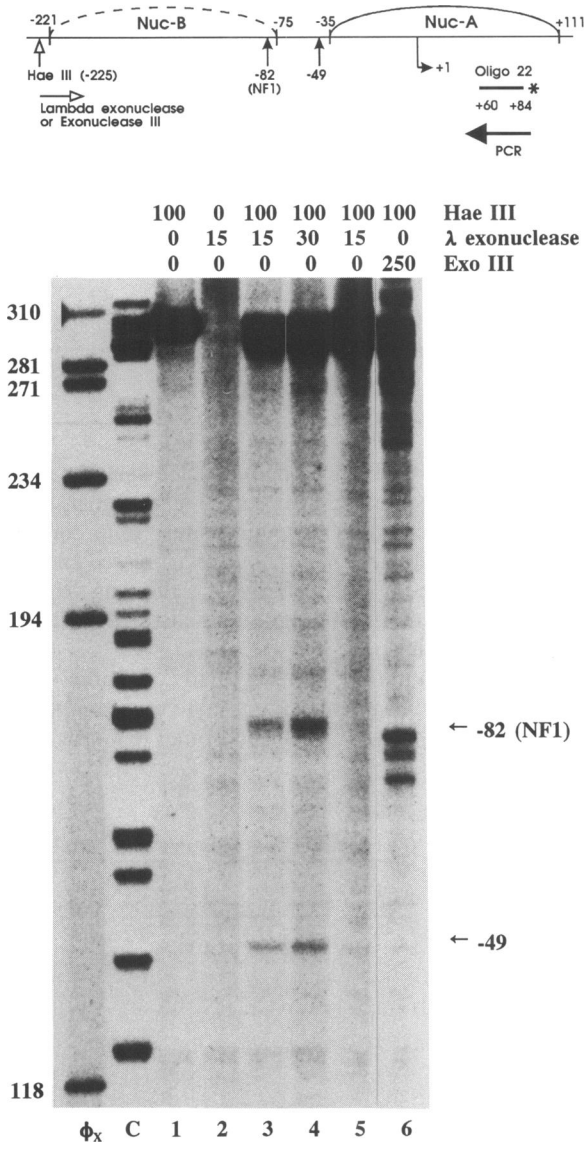

Figure 2. TOP: A schematic representation of a segment of the MMTV LTR, chromatin structure, restriction enzyme entry site (Hae III) and exonuclease stop sites (−82, −49). **BOTTOM:** Linear Taq polymerase analysis of protein−DNA interactions on the MMTV LTR detected using λ exonuclease or Exo III. Specific exonuclease stops are indicated by arrows on the right. The units of enzyme added to individual samples are indicated at the top of each lane. Lanes 1−4 digestion buffer B (λ); lanes 5 and 6 digestion buffer A (Exo III). ϕ_x-PhiX174 RF DNA digested with Hae III, C-C sequencing track.

bacterial CAT gene and contains approximately 10 copies/cell (7). Cells were grown in Dulbecco's modified eagle medium containing 10% fetal bovine serum. $1-2\times10^7$ cells were treated with the synthetic progestin R5020 (generously provided by Roussel Uclaf) at 10^{-8} M for 60 minutes. Nuclei were isolated as previously described with some modifications (3). Unless otherwise noted, all procedures were conducted at 4°C. Cells were rinsed with PBS, detached from the plates and collected by low speed centrifugation. The cell pellet was resuspended in 5 ml of homogenization buffer (10 mM Tris−HCl pH 7.4, 15 mM NaCl, 60 mM KCl, 1 mM EDTA, 0.1 mM EGTA, 0.1% NP-40, 0.15 mM spermine, 0.5 mM spermidine, 5% sucrose), transferred to a 7 ml Dounce homogenizer and lysed by 3 strokes with pestle A. Lysates were overlaid on 1 ml of sucrose pad (10 mM Tris−HCl pH 7.4, 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 10% sucrose) in conical centrifuge tubes. Nuclei were pelleted by 20 minutes of centrifugation at 1400×g, resuspended in 4 ml of wash buffer (10 mM Tris−HCl pH 7.4, 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine) and centrifuged at 700×g for 5 minutes. Nuclei were resuspended in 0.4 ml of digestion buffer A (10 mM Tris−HCl pH 7.4, 15 mM NaCl, 60 mM KCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 5% glycerol, 1 mM DTT; optimized for Exo III) or digestion buffer B (50 mM Tris−HCl pH 9.0, 10 mM NaCl, 2.5 mM $MgCl_2$, 5% glycerol, 1 mM DTT; optimized for λ exonuclease). 0.1 ml aliquots were supplemented with 100 units of Hae III (New England Biolabs), 15 or 30 units of λ exonuclease (Gibco-BRL) or 250 units of Exo III (Sybtrel Biotechnologies). Digestions were performed at 30°C for 15 minutes and terminated by the addition of 0.9 ml of stop buffer (10 mM Tris−HCl pH 7.6, 10 mM EDTA, 0.5% sodium dodecylsulfate, 0.2 mg/ml proteinase K). Samples were incubated at 37°C for at least 4 hours, phenol/chloroform extracted until no interface was visible and ethanol precipitated. Precipitated genomic DNA was digested to completion with Pst I (New England Biolabs). This reduced sample viscosity, provided an internal standard for accessing the extent of *in vivo* cleavage and confirmed that equivalent amounts of DNA were used for the linear Taq polymerase analysis. Exo III treated DNA was further digested with 45 units of Mung Bean nuclease at 30°C for 30 minutes in 1×Mung Bean digestion buffer (50 mM sodium acetate pH 5, 30 mM NaCl) to remove single stranded overhangs, repurified and reprecipitated. 10 μg of each sample was analyzed using linear Taq polymerase amplification in 30μl 1×Taq buffer (10 mM Tris−HCl pH 8.3, 50 mM KCl, 5 mM $MgCl_2$, 0.5% Tween 20) with ^{32}P-labelled primer #22 (5′-TCTGGAAAGT-GAAGGATAAGTGACG-3′) specific for the MMTV LTR. After an initial cycle of 4 minutes at 94°C, 2 minutes at 55°C, and 3 minutes at 72°C, 29 additional cycles were performed at 2 minutes at 94°C, 2 minutes at 55°C, and 3 minutes at 72°C, followed by a 15 minute extension at 72°C. 0.1 ml of stop buffer (200 mM sodium acetate pH 7, 10 mM Tris−HCl pH 7.5, 5 mM EDTA, 0.1 μg/μl yeast tRNA) was then added to each reaction and samples were purified by phenol/chloroform extraction and ethanol precipitation. Extended products were analyzed on 7% polyacrylamide denaturing gels and autoradiographed.

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