This protocol has advantages over other methods in that no ultracentrifugation is required, the virus does not aggregate appreciably during concentration, and the method can be readily scaled-up.

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Improved LM-PCR Procedure for In Vivo Footprinting Analysis of GC-Rich Promoters

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The mechanisms that lead to transcriptional control of promoters by DNA-binding proteins are usually determined by in vitro techniques such as electrophoretic mobility shift analysis (EMSAs), in vitro footprinting and mutational analyses of reporter constructs. However, in many cases, these analyses do not accurately reproduce the true in vivo situation of endogenous promoters in the context of the cellular chromatin structure.

In vivo footprinting analysis of genomic DNA has been developed to address this issue. This type of analysis visualizes, at single-nucleotide resolution, DNA-protein contacts occurring in living cells, thus providing critical information on the presence of transcription factors on endogenous promoters. Currently, several protocols are available to perform this analysis (for examples, see References 1,3,5-7,9). All of them involve two steps: (i) in situ modifications of genomic DNA performed on cell cultures, tissues, whole embryos etc. with chemical, enzymatic or physical agents (e.g., methylating agents, DNase I or UV irradiation, respectively) whose action on DNA is disturbed by the presence of DNA-bound proteins; and (ii) visualization of the DNA residues protected by proteins within a single-copy gene of interest. So far, the most sensitive technique used for step 2 involves a combination of ligation-mediated polymerase chain reaction (LM-PCR) amplification and genomic sequencing procedures that are performed with sets of primers specific for the studied gene (3,5,6). However, the success of this long and difficult technique is highly dependent on the GC-composition of the gene under study. Indeed, GC-rich, single-copy sequences are difficult to amplify by conventional PCR techniques, possibly due to rapid renaturation of the template and very stable secondary structures which cause elongation blocks during amplification by

DNA polymerase. We repeatedly faced this problem during the study of cell cycle-regulated genes, which have promoters that are often highly GC-rich. To resolve this issue, we modified several steps of conventional in vivo footprinting protocols (1,3,5-7,9). Notably, we introduced dimethyl sulfoxide (DMSO) all along the procedure since this compound had been previously shown to improve PCR amplification of GC-rich DNA sequences by decreasing the rapid renaturation of GC-rich templates (8,10). Moreover, to reduce the duration of an experiment that usually requires two to three days with conventional protocols, we also used a quick-ligation step (4) and a RoboCycler® Gradient Temperature Cycler (Stratagene, La Jolla, CA, USA) allowing us to optimize and perform a complete experiment in 1.5 days.

To assess the efficiency of this protocol for analyzing GC-rich sequences, we used it to visualize in vivo DNA-protein contacts on the promoter of the human cyclin E gene (2) in exponentially growing MCF7 breast cells and in human IMR 90 fibroblasts. The investigated sequence is located -384/-175 nucleotides (nt) relative to the transcription initiation site, is 83% GC-rich (Figure 1A) and is "resistant" to analysis by standard genomic footprinting procedures (e.g., Figure 1B, lanes 1 and 2).

Living cells and control naked genomic DNA were treated with the guanosine methylating agent dimethyl sulfate (DMS; Sigma, St. Louis, MO, USA) (3,5). 2×10^6 cells per 14-cm-diameter dish were treated with DMS at 0.2% for 5 min at room temperature in their cell culture medium (Dulbecco's modified Eagle medium [DMEM]/fetal calf serum [FCS]) buffered with HEPES (20 mM final), pH 7.4. After DMS treatment, cells were washed 3× with cold PBS/2% β-mercaptoethanol and then collected in 1 mL of lysis buffer (50 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1% sodium dodecyl sulfate [SDS], 2% β-mercaptoethanol). Genomic DNA was isolated by three gentle extractions with phenol (pH 8.0) followed by two precipitations in 4 M ammonium acetate with 3 vol of absolute ethanol (with 70% EtOH washes). DNA was then redissolved in 1 mL of water. As a reference, genomic DNA

isolated from non-treated cells (1 mg/mL in water) was methylated in vitro with 0.5% DMS for 4 min at room temperature. Piperidine cleavage at methylated bases was performed in 1 N piperidine at 95°C for 30 min. Chemically cleaved samples were precipitated in ethanol, evaporated 2× and finally resuspended to 0.4 mg/mL in water. Samples were analyzed on agarose gels to monitor their cleavage (smears should be below 500 bp). Two micrograms of

these samples were used for LM-PCR.

These modified DNAs were then used for both LM-PCR and genomic sequencing that was performed in either the presence or absence of DMSO. PCR primers ASCE7 (5'-GGAC-AGGGCGGGGCCAAGTGACAG-3'), ASCE8 (5'-GCCAAGTGACAGGGC-CTGCGCG-3') and ASCE9 (5'-AAGT-GACAGGGCCTGCGCGCCGAG-3') were designed to analyze the human cyclin E promoter (Figure 1A). Given

Figure 1. Comparative analysis of a GC-rich promoter sequence by a conventional DMS-LM-PCR genomic footprinting procedure vs. by our modified method. (A) An 83% GC-rich region of the human cyclin E promoter (nt -384/-175 relative to the transcription initiation site) (9) was analyzed by in vivo DMS-LM-PCR genomic footprinting (2). (B) LM-PCR was carried out with ASCE7, -8, and -9 primers on methylated genomic DNA prepared from DMS-treated human MCF7 cells (lanes 1 and 3) or from naked genomic DNA treated in vitro with DMS (lanes 2 and 4). Amplified DNA ladders that are visible correspond to guanosine of the antisense strand. A strong elongation block cannot be overcome using conventional protocols (without DMSO) (lanes 1 and 2 show representative examples of the block whose position is indicated by a black arrow in Panel A). This block was not overcome even when PCR annealing temperatures were increased to maximal values (i.e., 76°C) (data not shown). By contrast, using our protocol, the elongation block is overcome and protected residues between in vivo (lane 3) and in vitro (lane 4)-methylated DNAs could be detected (black dots). This analysis shows that a putative binding site for SP1 is occupied in vivo. Experiments were carried out either with (lanes 3 and 4) or without DMSO (lanes 1 and 2) using the following cycling temperatures: $T_{an/el} = 64^{\circ}\text{C}$, $T_{an/amp} = 68^{\circ}\text{C}$ and $T_{an/lab} = 68^{\circ}\text{C}$.

Benchmarks

the high GC content of the sequence, the melting temperatures (T_m s) of ASCE 7, 8 and 9 is very high, i.e., 70°, 69° and 72°C, respectively. All reactions were performed with a Robocycler Gradient 96 Temperature Cycler.

A 2 µg amount of piperidine-cleaved genomic DNA and 0.3 pmol of the ASCE7 oligonucleotide in 25 µL of first-strand buffer (10 mM Tris-HCl, pH 8.9, 5 mM MgSO₄, 40 mM NaCl, 20 µg/mL bovine serum albumin [BSA] and 10% DMSO) were denatured at 95°C for 5 min and then annealed for 30 min at various temperatures to determine the optimum temperature of annealing of the elongation step ($T_{an/el}$). Five $T_{an/el}$ temperatures are usually screened simultaneously with the Robocycler and are determined as follows: (i) T_m of ASCE7, (ii) $T_m - 6^\circ\text{C}$, (iii) $T_m - 3^\circ\text{C}$, (iv) $T_m + 3^\circ\text{C}$ and (v) $T_m + 6^\circ\text{C}$. Freshly prepared dNTPs (up to a 0.25 mM final concentration) and 0.5 U of

Vent® *exo*⁻ DNA Polymerase (New England Biolabs, Beverly, MA, USA) were then added to the annealed DNA/primer mixture, and elongation was carried out for 15 min at 76°C. A 45-µL vol of ligation mixture (30 mM Tris-HCl, pH 7.5, 25 mM MgCl₂, 20 mM dithiothreitol [DTT], 1 mM ATP, pH 7.0, 50 µg/mL BSA, 10% polyethylene glycol [PEG] 6000), 4 U of T4 DNA ligase (Boehringer Mannheim GmbH, Mannheim, Germany) and 25 pmol of annealed linkers LINK1/LINK2 (plus strand LINK1-5'-GAATTCAGATC-3', minus strand LINK2-5'-GCGGTGACCCGAGAGATCTGAATTC-3' as previously described; Reference 3) were then added to the elongation reaction mixture. This asymmetric LINK1/LINK2-annealed linker was used to promote a unidirectional ligation using a quick ligation procedure (4). After a 1-h incubation at 17°C, ligated DNA was then precipitat-

ed with cold absolute ethanol, washed with 70% ethanol and resuspended in 100 µL of amplification buffer (65 mM Tris-HCl, pH 8.8, 40 mM NaCl, 10 mM β-mercaptoethanol, 3 mM MgCl₂, 10% DMSO, 0.4 mM dNTPs). PCR amplification was next performed upon addition of 10 pmol of ASCE 8 primer and 1 U of *Taq* DNA Polymerase (Perkin-Elmer International, Rotkreuz, Switzerland). PCR conditions were as follows: 4 min at 95°C followed by 30 cycles of 1 min at 95°C, 2 min at temperature of annealing of the amplification step ($T_{an/amp}$), 2 min at 76°C and 7 min at 76°C ($T_{an/amp}$, as described for $T_{an/el}$, we simultaneously screened five temperatures around the T_m of ASCE8). A 15-µL aliquot of this amplification reaction was then mixed with 5 µL of a labeling buffer (65 mM Tris-HCl, pH 8.8, 40 mM NaCl, 10 mM β-mercaptoethanol, 2 mM MgCl₂, 3.75 mM dNTPs, 10% DMSO, 0.5 U of Vent *exo*⁻ DNA poly-

merase containing 0.15 pmol of ^{32}P -end-labeled ASCE9 primer (3×10^6 cpm/pmol). PCR/labeling conditions follow: 4 min at 95°C followed by nine cycles: 1 min at 95°C , 2 min at temperature of annealing of the labeling step ($T_{\text{an/lab}}$) and 2 min at 76°C ($T_{\text{an/lab}}$). As for $T_{\text{an/el}}$ and $T_{\text{an/amp}}$, five temperatures around the T_{m} of ASCE9 were tested. The reaction was stopped by adding 80 μL of 0.3 M sodium acetate pH 5.5 and 10 μg of transfer RNA (tRNA). Labeled DNAs were extracted with phenol once and precipitated with two volumes of cold ethanol. Pellets were washed with 70% ethanol, resuspended in 10 μL of sample loading buffer (95% formamide, 10 mM EDTA, 20 mM NaOH, 0.025% bromophenol blue, 0.025% xylene cyanol) and denatured 3 min at 95°C . A 5- μL volume of each sample was loaded onto a 5% sequencing gel and electrophoresed at 50 W. Dried gels were analyzed with a PhosphorImager® (Molecular Dynamics, Sunnyvale, CA, USA).

When using either this protocol in absence of DMSO or several other standard protocols previously described for DMS-LM-PCR genomic footprinting analysis (1,3,5–7,9), we were unable to generate DNA ladders encompassing more than 40 bp upstream of the designed oligonucleotides (Figure 1B, the dark arrow in Figure 1A indicates the position of this DNA elongation block). This block was not overcome by varying all three annealing temperatures ($T_{\text{an/el}}$, $T_{\text{an/amp}}$ and $T_{\text{an/lab}}$) neither individually nor simultaneously. In addition, the highest possible temperature tested (76°C) led to a strong decrease of the ladder signal without overcoming the elongation block (data not shown).

By contrast, in the presence of DMSO, this block completely disappeared, allowing us to detect the *in vivo* protection of a sequence identified as a putative SP1 binding site (Figure 1B). In conclusion, in one day, the LM-PCR procedure described in this study allows the *in vivo* footprinting analysis of GC-rich genomic sequences that are otherwise difficult or impossible to analyze by this very informative technique.

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