Methyltransferase-based single-promoter analysis assay (PROT38)

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Introduction

Nucleosomal positioning [reviewed in (1-3)] plays a pivotal role in the regulation of transcriptional initiation. Transcriptional co-activator complexes interact with nucleosomes (4) to induce nucleosomal rearrangements. Nucleosomes often have to unfold completely (5) or be disassembled (6) at the transcription start site, to allow for transcriptional initiation (7, 8).

Most of the studies done on the nucleosomal rearrangements of histones use conventional footprinting techniques, which rely on nuclease digestion and primer extension. However, promoters are molecular 'modules', which are controlled as individual entities. When analyzed by conventional methodologies this modularity is destroyed. Our lab has modified a previously described footprinting strategy (9,10), which now allows us to study the chromatin structure of individual molecules. MSPA (methylation-sensitive promoter analysis) allows for the study of unmethylated CpG islands by treatment of nuclei with the CpG-specific DNA methyltransferase SssI (M.SssI), followed by genomic bisulfite sequencing of individual progeny DNA molecules (Fig. 1) (11-13) (see comment 1). This gives single molecule resolution over the promoter and allows for the physical linkage between binding sites on individual promoter molecules to be maintained.

Our lab has successfully used this method to study the difference in nucleosomal positioning in the p16 promoters in two human cell lines (11), to identify transcription factor binding sites and their combinatorial organization during endoplasmic reticulum stress (12), and to study the changes in nucleosome occupancy silencing of the three transcription start sites in the bidirectional MLH1 promoter CpG island in cancer cells (13).

Procedure

Treatment of Nuclei with M.SssI

Nuclei are first purified from cells. M.SssI is then added to the purified nuclei. The M.SssI then enters the nuclei and methylates the chromatin (comment 2).

Nuclei extraction

1. Actively growing cells are trypsinized and washed once with cold phosphate buffer saline (PBS). It is recommended to start with at least 10^7
   cells, however, this procedure has been done successfully with 200,000 cells (variations below).
2. Cells are then resuspended in 1 ml of ice cold RSB Buffer and kept on ice for 10 min.

The following steps are all done at 4°C:

3. Following the 10 min incubation, 0.1 ml of 10% Nonidet P-40 (NP-40) detergent is added and the cells are homogenized with 15 strokes of the tight pestle of a Dounce homogenizer. Homogenized cells are then put in eppendorf tube and centrifuged for 5 min at 5000 rpm at 4°C. The supernatant is discarded.

4. Nuclei are then resuspended in 1 ml of RSB Buffer. At this time a small aliquot can be checked for intact nuclei and complete lysis of the cellular membrane using a microscope. Samples are then centrifuged for 5 min at 5000 rpm at 4°C. The supernatant is discarded.
5. Nuclei are then washed again with either RSB Buffer or with 1x M.SssI buffer. (It should be noted that epithelial nuclei tend to lyse during centrifugation if washed with 1X M.SssI buffer, however fibroblast nuclei stay intact with the 1X M.SssI buffer wash) (comment 3). Samples are then centrifuged for 5 min at 5000 rpm at 4°C. The supernatant is discarded.
6. The nuclei are then resuspended in 1X M.SssI buffer so that there are 10^6 nuclei per 74.25µl.

M.SssI treatments

M.SssI treatments of nuclei are done immediately after nuclei are prepared.

M.SssI is purchased from New England Biolabs. A 10X M.SssI buffer and AdoMet (SAM) is included. (Note: We actually buy fresh M.SssI
every time we perform the assay to ensure that the footprints we see are due to protection of the DNA by nucleosomes and not by the inefficiency of an old enzyme. At a minimum fresh AdoMet should be used.)

1. For M.SssI treatment of 10^6 nuclei add the following to an eppendorf tube:

10X M.SssI buffer	15 µl
32 mM SAM	0.75 µl
1 M Sucrose	45 µl
Nuclei 10^6 (6 µg DNA)	74.25 µl
M.SssI (NEB)	15 µl (60 U) + (comment 3)
H_2O	to make volume up to 150 µl

   Incubate at 37°C for 15 mins
2. Reactions are stopped by the addition of an equal volume of stop solution (150 µl).
3. Samples are then incubated with 200 µg/ml proteinase K at 55°C for 16 h.
4. DNA is purified by phenol/chloroform extraction and ethanol precipitation.
5. The DNA is then subjected to genomic bisulfite sequencing which was first reported by Frommer et al (14) and described in detail below.

Bisulfite Conversion

Before bisulfite conversion the genomic DNA should be digested with restriction enzymes which cut outside the sequence to be cloned. (Note: Bisulfite conversion can be done without cleavage of the DNA, but this may lead to insufficient conversion of some sequences)

Bisulfite conversion can be done using different methods. (All methods described below have been done successfully in our lab).

Jones Lab Method

1. 2-4 µg of DNA is digested with restriction enzymes in a total volume of 20 µl (comment 4).
2. DNA is then denatured at 90 °C for 20 min (comment 5).
3. 5 µl of 3M NaOH is then added to the denatured DNA and incubated at 45 °C for 20 min. (Note: The 3M NaOH is made fresh)
4. To each sample add 12 µl of Hydroquinone
   (0.11 g in water with a final volume of 10 mL) and 208 µl of Sodium Bisulfite, pH 5.0 (3.76 g in water with a final volume of 10 mL) (comment 6).
5. Samples are incubated for 16 h at 55 °C (comment 7).
6. DNA is then separated from the bisulfite solution using the Promega Wizard kit (Note: DNA is eluted from the columns using 50 µl of 80 °C de-ionized water.)
7. Samples are desulfonated by adding 5ul of 3M NaOH to the eluted DNA and incubated at 40 °C for 15 min.
8. 50 µl 5M NaOAc, 300 µl ethanol and 1 µl glycogen are added to the desulfonated DNA and the solution is incubated at -80 °C for 1 h or -20 °C overnight (comment 8).
9. Samples are centrifuged in a microcentrifuge at 14,000 X g for 20 min at 4 °C.
10. Supernatant is discarded and the pellet is washed once with 70% ethanol.
11. Pellet is allowed to dry and is then resuspended in 40 µl de-ionized water.

Qiagen Bisulfite Kit

Qiagen makes a nice kit which protects the DNA from being degraded during the bisulfite conversion (comment 9). Follow manufacturers instructions.

PCR and Cloning

PCR reactions are performed using bisulfite specific primers. These specific primers are designed so that they contain within their sequence converted C's. These primers must not contain CpG sites in their sequence (comment 10).

1-2 µl of bisulfite converted DNA is usually used per PCR reaction and each PCR is performed for 40 cycles.

PCR products are then cloned using Invitrogen's TOPO TA cloning kit following manufacturer's instructions (comment 11).

Individual clones are sequenced (comment 12).

Materials & Reagents

RSB Buffer	10 mM Tris-HCl, pH 7.4 10 mM NaCl 3 mM $MgCl_2$
1X SssI Buffer	10 mM Tris-HCl, pH 7.9, 50 mM NaCl 10 mM $MgCl_2$ 1 mM dithiothreitol 0.3 M sucrose (**comment 3**)
Stop Solution (2X lysis buffer)	20 mM Tris-HCl, pH 7.9 600 mM NaCl 1% SDS 10 mM EDTA
M.SssI	New England Biolabs
1 M Sucrose	
Sodium Bisulfite Solution, pH 5.0	3.76 g in water with a final volume of 10 mL (pH to 5.0 with NaOH) (**comment 6**)
Hydroquinone	0.11g in water with a final volume of 10 mL
3M NaOH	1.2 g in 10 mL of water

Author Notes

1. Although we recommend starting out with 10 X 10^6 cells, this procedure can be performed with as little as 200,000 cells. When the procedure is done with a small number of cells all the nuclei are treated with M.SssI (amount of M.SssI is adjusted proportionally, but the reaction volume is kept at 150 µL)
2. To remove tight binding transcription factors that may be interfering with the nucleosome footprint, nuclei can be treated with RSB Buffer
   containing 400 mM NaCl after Step 4 of the nuclei extraction. Nuclei are then centrifuged down at 5,000 X g for 5 min and washed once with the normal RSB Buffer.
   Some nuclei are more fragile than others and may lyse during the high salt treatment (this can be checked by looking at a small aliquot under the microscope). Sometimes lysis of the nuclei during this treatment can be overcome by incubating the nuclei in the high salt buffer for a couple of minutes and then diluting the sample 10-20 fold with RSB Buffer
   before spinning. Nuclei can also be centrifuged at a lower speed for a longer amount of time. In addition nuclei can be resuspended in RSB Buffer containing 200 mM NaCl and then an equal amount of RSB Buffer containing 600 mM NaCl can be carefully added.
3. The Jones Lab bisulfite conversion method can be done with as little as 100 ng of DNA. Yeast tRNA can be used as a carrier during the purification of the DNA using the Promega wizard kit. After ethanol precipitation DNA is resuspended in 10 µl of water. 1 µl of the sample is then used for PCR. When such low amounts of DNA are being used for PCR, we recommend performing the PCR in triplicate to eliminate PCR bias.
4. If multiple products or primer dimers occur during PCR amplification of the bisulfite converted DNA, we recommend gel purification of the product before cloning.

Reviewer Comments

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1. We have also taken advantage of the power of bisulfite genomic sequencing to provide single molecule analysis of chromatin structure, which we termed MAP-IT (methyltransferase accessibility protocol-individual templates; Jessen,

W.J., Hoose, S.A., Kilgore, J.A., and Kladde, M.P. (2006) Active *PHO5* chromatin encompasses variable numbers of nucleosomes at individual promoters. *Nat. Struct. Mol. Biol*. **13**:256-263). Our study was performed *in vivo* and used M.HhaI; however, in principle, any DNA methyltransferase could be used as the chromatin probe.

2. As M.SssI has the same CpG specificity as native vertebrate DNA methyltransferases, only regions that have no or very low levels of endogenous CpG methylation can be probed with the enzyme. With the caveat that it uses a selection, regions lacking CpG methylation can also be enriched for by methylation-specific PCR (see ref. 13). Alternatively, the GpC methyltransferase M.CviPI can be used as the chromatin probe, which only overlaps endogenous CpG methylation at GpCpG sites (Kilgore, J.A., Hoose, S.A., Gustafson, T.L., Porter, W., and Kladde, M.P. (2007) Single-molecule and population probing of chromatin structure using DNA methyltransferases. *Methods* **41**:320-332).
3. One should consider reducing the concentration of Mg^{2+}, as exceeding 1 mM can cause compaction and precipitation of chromatin (Ausio, J., Sasi, R., and Fasman, G.D. (1986) Biochemical and physiochemical characterization of chromatin fractions with different degrees of solubility isolated from chicken erythrocyte nuclei. *Biochemistry* **25**:1981-1988; Hansen, J.C., and Wolffe, A.P. (1992) Influence of chromatin folding on transcription initiation and elongation by RNA polymerase III. *Biochemistry* **31**:7977-7988). Our 1X buffer for probing nuclei is: 20 mM HEPES, pH 7.5, 70 mM NaCl, 0.5% glycerol, 0.5 mM EGTA, 0.25 mM EDTA, 1 mM DTT, 0.25 mM PMSF, supplemented with 160 µM SAM. It is important to freshly add DTT, PMSF and SAM. Note that decreasing the concentration of the divalent cation increases the affinity of M.SssI for DNA and its methylation processivity (Matsuo, K., Silke, J., Gramatikoff, K., and Schaffner, W. (1994) The CpG-specific methylase SssI has topoisomerase activity in the presence of Mg^{2+}. *Nucleic Acids Res*. **22**:5354-5359).
4. We usually omit the restriction digestion and obtain excellent bisulfite conversion frequencies with our protocol.
5. We add 10 µL sample denaturation buffer (make fresh shortly before use) mixed as a cocktail from the following components in the ratio of: 3.0 µL 3 N NaOH (make fresh from pellets), 0.5 µL 0.5 M EDTA, pH 8.0, 6.5 µL degassed, distilled H_2O. Samples are vortexed and denatured at 98 °C for 5 min to limit thermal scission of DNA.
6. We freshly make the 100 mM hydriquone solution in degassed, distilled H_2O. A fresh, saturated solution of sodium metabisulfite is made as follows. A 5 g vial is filled to the brim with sodium metabisulfite crystals (weighing is not necessary as the solution will be saturated). We transfer the ~5 g sodium metabisulfite to a 20-mL scintillation vial containing a stir bar and 100 µL 100 mM hydriquone, add 7 ml degassed, distilled H_2O, vortex and then 1 mL fresh 3 N NaOH and more as needed to titrate the pH to 5.0 while stirring. Prewarm the saturated sodium metabisulfite solution to 50 °C, then add 200 µL to each denatured sample (while it is held at 98 °C), quickly cap each tube and vortex.
7. Using the saturated sodium metabisulfite solution, incubation for 4-6 h at 50 °C in the dark routinely achieves conversion frequencies of C to T (after PCR amplification) of at least 98%. The shorter incubation time decreases the amount of DNA degradation.
8. After desulfonation, we add 18 µL 10 M NH_4OAc, 200 µL ethanol and 1 µL molecular biology grade glycogen and incubate at -20 °C overnight.
9. We have also obtained excellent results with either the Qiagen Epitect or Zymo Research bisulfite conversion kit.
10. If CpG sites cannot be avoided, it is preferably to position them as close as possible to the 5' end of the primer and include a degenerate C/T or G/A base (depending on the strand being analyzed and if it is the forward or reverse primer). The methylation status of such CpG sites cannot be scored.
11. When TA cloning is problematic, we have also used pairs of bisulfite primers with different restriction sites at their 5' ends to allow directional cloning into a suitable plasmid. We screen for recombinants with full-length inserts by colony PCR.
12. We do not purify plasmid DNA for sequencing and instead sequence single-stranded DNA template generated by TempliPhi rolling circle amplification.

Figures

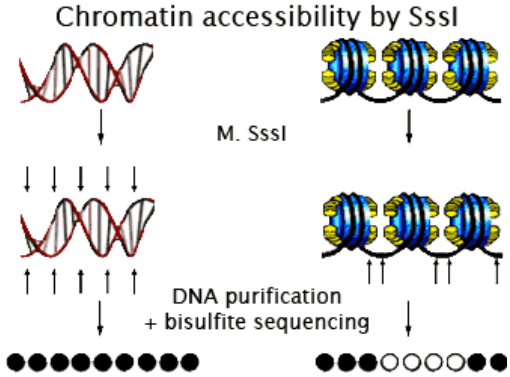

Figure 1. Schematic of M.SssI footprinting. First DNA or nucleosomes are treated with M.SssI. This enzyme methylates all CpG sites in purified DNA, but it cannot methylate the same sites when they are assembled into nucleosomes or are associated with tight-binding factors. Next the DNA is purified, the sequences are bisulfite converted and individual molecules are cloned. Patches which are inaccessible to M.SssI are revealed. Black circles indicate CpG sites that are methylated and white circles indicate sites that are unmethylated.

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