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Single-molecule and population probing of chromatin structure using DNA methyltransferases

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Abstract

Probing chromatin structure with DNA methyltransferases offers advantages over more commonly used nuclease-based and chromatin immunoprecipitation methods for detection of nucleosomes and non-histone protein–DNA interactions. Here, we describe two related methods in which the readout of MTase accessibility is obtained by assaying 5-methylcytosine in DNA through the PCR-based technique of bisulfite genomic sequencing. The methyltransferase accessibility protocol (MAP) determines the relative frequency at which the enzyme accesses each of its target sites over an entire population of PCR amplified product. While MAP yields much quantitative information about relative accessibility of a region of chromatin, a complementary single-molecule view of methyltransferase accessibility, termed MAP for individual templates (MAP-IT), is provided by analysis of cloned PCR products. Absolute rather than relative methylation frequencies in a region are obtained by summing the methylation status at each site over a cohort of clones. Moreover, as the integrity of individual molecules is maintained in MAP-IT, unique information about the distribution of multiple footprints along continuous regions is gleaned. In principle, the population MAP and single-molecule MAP-IT strategies can be used to analyze chromatin structure in a variety of model systems. Here, we describe the application of MAP in living *Saccharomyces cerevisiae* cells and MAP-IT in the analysis of a mammalian tumor suppressor gene in nuclei. This application of MAP-IT provides the first means to simultaneously determine CpG methylation of mammalian genes and their overlying chromatin structure in the same single DNA molecule.

Keywords: Bisulfite; Chromatin; *GAL1*; MAP; MAP-IT; Methylation; Methyltransferases; Nucleosomes; *Saccharomyces cerevisiae*; *SIM2*

1. Introduction

DNA–protein interactions are crucial to proper genome function in all living organisms. Effective methods for the detection and quantification of these interactions are therefore needed to facilitate our understanding of both normal and disease states. Classical techniques for footprinting nucleosome positions or sites of protein interaction often assess the accessibility of DNA to chemical or enzymatic probes, such as DNase I or micrococcal nuclease. The resultant DNA damage is invasive and, in the case of strand scission, *a priori* destroys the integrity of a DNA molecule, which precludes detection of multiple independent cleavages along a single DNA strand.

DNA methyltransferases (MTases) offer an alternative and highly effective means to map chromatin architecture in both population-ensemble and, more recently, single-molecule approaches [1–9]. MTase probing can be performed in vivo by expressing MTase genes in cells, ex vivo in nuclei or in vitro. Cytosine-5 MTases are particularly attractive probes, as a relatively non-intrusive methyl group is introduced at the C5 position of accessible cytosines that can be detected by the powerful PCR-based technique of bisulfite genomic sequencing (BGS) [10,11]. In BGS, 5-methylcytosine (m^5C) resists bisulfite deamination and thus

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is stably replicated as cytosine during PCR amplification. Sequencing in the presence of 2′,3′-dideoxyguanosine triphosphate (ddGTP) positively displays on a sequencing gel residues that were methylated, and hence accessible, in vivo. The overall assay, termed MAP, has successfully detected positioned nucleosomes and a range of non-histone protein–DNA interactions in cell populations [3–6]. In fact, we are unaware of any instance where a known protein-DNA interaction was not footprinted by MAP when MTase sites were within about 8–10 bp of the factor binding site. Additionally, adaptation of MAP for in vitro footprinting was effective in mapping the association of several mammalian factors (aryl hydrocarbon receptor, Sp1 and estrogen receptor α/Sp1 complex) using nuclear extracts, when conventional DNase I footprinting proved ineffective [12–15]. Two MTases, M.SssI (CpG) [16] and M.CviPI (GpC) [17], currently afford the highest resolution for MAP, as they are the only known enzymes with dinucleotide site specificity.

MAP is readily extended to provide a unique single-molecule view of MTase accessibility called MAP for individual templates (MAP-IT) [9]. As in the original BGS procedure of Frommer et al. [10,11], the cloning and sequencing of individual molecules from a PCR amplicon allows assignment of m^5C or non-methylation at each potential MTase target in single molecules. Advantages of the single-molecule MAP-IT platform over MAP include: (1) obtaining absolute methylation frequencies by summing the number of m^5C residues at each MTase site among a set of cloned molecules; (2) not being subject to the constraints of single-hit kinetic levels of probe modification (as governed by the Poisson distribution); and (3) detection of factor co-occupancy (or loss of two or more footprints) along a single continuous molecule of DNA. For example, we recently used MAP-IT to show that a variable number of nucleosomes were remodeled in single cells during transcriptional induction of the yeast *PHO5* promoter [9]. Moreover, disruption of two or more adjacent nucleosomes in single promoters was disproportionately high at the sites of activator interaction, indicating that chromatin remodeling is localized and possibly spreads from a focus of bound activators. Patches of protection against methylation by M.SssI in cloned molecules have also been suggested to represent positions of individual nucleosomes at an unmethylated CpG island in mammalian nuclei [7]. The use of M.SssI as a chromatin probe in vertebrate systems is restricted to regions lacking m^5CpG, such as CpG islands. The GpC specificity of M.CviPI circumvents this limitation, allowing vertebrate chromatin structure to be probed via MAP and MAP-IT at regions with a high density of m^5CpG.

2. Methods

2.1. MAP and MAP-IT in Saccharomyces cerevisiae

Here, we primarily focus on new developments and applications of DNA MTases as chromatin probes. The reader is recommended to consult Jessen et al. [6] and Hoose and Kladde [8] for more detailed descriptions of several procedures, as needed. Vectors for expressing M.CviPI and M.SssI have been described in these manuscripts and are available upon request. Procedures common to MAP and MAP-IT are discussed in Sections 2.1.1, 2.1.2, 2.1.3, 2.1.4, 2.1.5. Detection of m^5C in populations (MAP) and individually cloned molecules (MAP-IT) are the subject of Sections 2.1.6 and 2.1.7, respectively.

2.1.1. Preparation of plasmids expressing MTases

Escherichia coli strains used for subcloning and propagation of plasmids containing genes for foreign DNA MTases must be completely deficient for the methylation-dependent restriction systems, Mrr and McrBC, e.g. ER1821 (New England Biolabs), DH5α and XL1-Blue. Propagation of M.SssI, in particular, is apparently toxic to these hosts, presumably due to leaky expression. Extreme care must thus be exercised when subcloning the MTases to new plasmids or growing cells to purify plasmid DNA. It is recommended that *E. coli* colonies on plates and liquid cultures be grown for as short a time as possible (8–12 h) to avoid accumulation of mutations that inactivate MTase activity. As an additional precaution, a strongly transcribed, constitutive *E. coli* promoter is incorporated downstream and in the opposite orientation of the MTase gene, which does not interfere with expression of the enzyme in eukaryotic cells [18]. It is important to sequence all newly constructed recombinant plasmids.

2.1.2. Expression and screening for activity of C5 DNA MTases in budding yeast

Saccharomyces cerevisiae lacks detectable endogenous DNA methylation [19], essentially making it a blank canvas for detection of de novo methylation at accessible sites following MTase expression. Probing in living cells allows experiments to be performed under conditions that maintain physiology and avoid loss of weakly associated chromatin components. In keeping with these goals, we commonly employ a system that expresses the MTases from an estrogen-inducible promoter that does not affect normal yeast physiology. Control experiments have demonstrated that the MTase expression levels typically employed in the actual footprinting studies do not affect growth or induction of specific genes. Jessen et al. [6] can be consulted for detailed descriptions of this MTase expression system and methods for its integration in yeast. Integration is preferred over episomal expression to ensure that all cells contain a copy of the transgene, which is of increased importance in the single-molecule approach of MAP-IT. If desired, strains can be tested for correct integration of the MTase expression vector by colony PCR. MTase activity can be ascertained through isolation of genomic DNA, described below, and its subsequent digestion with the McrBC endonuclease complex [6]. Positive strains should be frozen as glycerol stocks as soon as possible.