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Novel method to ascertain chromatin accessibility at specific genomic loci from frozen brain homogenates and laser capture microdissected defined cells

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ABSTRACT

We describe a novel method for assessing the "open" or "closed" state of chromatin at selected locations within the genome. This method combines the use of Benzonase, which can digest DNA in the presence of actin, with quantitative polymerase chain reaction to define digested regions. We demonstrate the application of this method in brain homogenates and laser captured cells. We also demonstrate application to selected sites within more than 1 gene and multiple sites within 1 gene. We demonstrate the validity of the method by treating cells with valproate, known to render chromatin more permissive, and by comparison with classical digestion with DNase I in an in vitro preparation. Although we demonstrate the use of this method in brain tissue, we also recognize its applicability to other tissue types.

1. Introduction

The epigenetic regulation of chromatin structure is a major component of the gene expression regulatory machinery, and this regulation is accomplished by multiple mechanisms including DNA methylation and hydroxymethylation, multiple histone modifications, binding molecules, and complex interactions among all these elements (Jaenisch and Bird, 2003; Mastroeni et al., 2011; Chouliaras et al., 2013). These complex interactions converge to modulate chromatin structure, much as multiple inputs converge on the final common path of the anterior horn cells (Sherrington, 1906). By analogy, we posit chromatin structure as the final common path of multiple epigenetic mechanisms.

Chromatin structure determines whether any selected segment of DNA is accessible to transcription factors, DNA binding proteins, or other modifiers which govern a gene's availability for transcription (Quina et al., 2006). There are multiple classes of methods for examining chromatin structure. Broadly speaking, these are methods that use DNase I digestion, chromatin precipitation, matrix-assisted reader chromatin capture, and others, which are typically followed by methods to determine location of permissive or repressive chromatin structure.

In the past, permissive, or open, chromatin has been identified by DNase I nuclease sensitivity analysis (Krebs and Peterson, 2000), and more recently, open chromatin data generated from DNase-seq have been able to predict cell-type–specific gene expression (Natarajan et al., 2012).

An overview of current methods available to ascertain chromatin structure yields a broad range of approaches, many of which are variations of chromatin immunoprecipitation (ChIP), an antibody-based methodology first described by Gilmour and Lis (1984, 1985). Fast carrier ChIP (Fast CChIP) can be used to study transcription factor binding in small amounts of fresh tissue (Hao et al., 2008). Another ChIP-based technique uses genomewide chromatin immunoprecipitation in concert with tiling microarrays (ChIP-chip). ChIP-chip, along with ChIP–quantitative polymerase chain reaction (qPCR), showed that transcription factor FoxA1 is differentially recruited to cell-type–specific enhancers (Lupien et al., 2008). ChIA-PET combines ChIP with paired-end DNA sequencing to determine global de novo chromatin interactions (Fullwood et al., 2009). PAT-ChIP, also a ChIP-directed technique, was used to isolate, extract, and sequence chromatin from formalin-fixed, paraffin-embedded tissue samples (Fanelli et al., 2010; Fanelli et al., 2011; Amatori et al., 2014). A common method used to determine chromatin structure is DNase I digestion of DNA with subsequent sequencing of resulting fragments (DNase-seq).

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The sites of cleavage are called *DNase I hypersensitive sites* (DHSs), suggesting areas of the chromatin accessible to DNase digestion to be "open" and poised for transcription. A recent international epigenetic collaborative study, Encyclopedia of DNA Elements (ENCODE), created a genomewide map of DHSs in 125 human cell lines and tissue types and correlated those sites with ChIP-seq data (Thurman et al., 2012). In addition to genomewide mapping of DHS, DNase I sensitivity and sequencing can be used to analyze receptor binding influences on chromatin structure at specific gene loci in vitro (Tewari, et al., 2012). More recently, a modification to ChIP-Seq, called *Bar-ChIP*, allowed multiple DNA-protein interactions to be simultaneously profiled by attaching molecular barcodes to chromatin fragments from *Saccharomyces cerevisiae* (Chabbert et al., 2015).

Chromosome conformation capture (3C) technologies have been used for more than a decade. First described in Dekker et al. (2002), 3C delivers 3-dimensional chromatin structure information based on interaction frequencies between genomic loci. Some 3C technologies, such as e4C, use ChIP within the protocol (Sexton et al., 2012). Capture-C, the most recent derivative of the 3C technique, modifies the 3C method into a multiplexed, high-throughput approach to analyzing *cis*-acting elements that control gene expression (Hughes et al., 2014).

A ChIP-less technique to examine the epigenome, called *MARCC* (matrix-assisted reader chromatin capture), is a platform that probes combinatorial histone modification patterns to determine functional chromatin conformation (Su et al., 2014). Finally, direct chromatin PCR is a method for gene-specific chromatin analysis performed on cultured cells using properties of standard PCR buffers and thermal cycling temperatures to amplify open regions of chromatin (Vatolin, et al., 2012).

Although ChIP and non–ChIP-based techniques have been broadly used in elucidating chromatin structure and its impact on gene regulation, caveats to their use exist. The ChIP-based techniques rely on antibodies, which involve problems with antibody specificity and optimization (Orlando, 2000). Moreover, most ChIP and non–ChIP-based methods need large amounts of cultured cells or fresh tissue to start with. In addition, these complicated protocols take several days to complete and can be expensive with labor, reagents, equipment, and per-sample core costs (Sheffield and Furey, 2012).

A major limitation of DNase I–based methods is the susceptibility of DNase I to inhibition by actin, a component of many cells and tissues. Recently, a protocol was developed for determining chromatin accessibility in frozen tissue homogenates using Benzonase, a robust nuclease whose efficacy is not affected by the presence of actin (Grøntved et al., 2012). Here we describe a protocol to assess chromatin structure at specific loci using Benzonase digestion and qPCR of DNA extracted from frozen tissue homogenates and laser capture dissected defined cells. This protocol is relatively quick, inexpensive, and achievable in any research laboratory outfitted with standard real-time PCR equipment. This novel method will allow a more focused examination of chromatin structure, its influence on gene expression, and the impact it may have on gene expression in normal and diseased brain as well as other tissues.

2. Materials and methods

2.1. Tissue (samples)

All frozen brain tissue samples and frozen brain sections were obtained from the Brain Bank at Banner Sun Health Research Institute. Average postmortem delay at this Brain Bank is 2.6 hours. Homogenate studies used approximately 1 g of middle temporal gyrus (MTG) and cerebellum (CBL) from each of 3 nondemented controls (NDs) and 3 Alzheimer's disease (AD) cases. Laser capture microdissection (LCM) studies used 10-μm serial sections of MTG (area 22) from 1 ND and 1 AD case and 10-μm serial sections of hippocampus from 5 ND and 5 AD cases.

2.2. Primers

2.2.1. Primer design for chromatin accessibility analysis

Primers for KAT6B, APP, and RSP28 were designed to cover the area 300 base pairs (bp) upstream of the transcription start site and 100 bp downstream of the transcription start site. The primer sequences for GAPDH, CTCF4, and CTCF10 were taken from He et al. (2014) and chosen to represent areas of the genome known to be constitutively open (GAPDH) and/or uniform in openness across sample types (CTCF4, CTCF10) (Li et al., 2013). All primers were checked for specificity via the National Center for Biotechnology Information's BLAST search.

To examine chromatin structure in multiple regions of 1 gene, primers for ARC were designed based on ENCODE data (Thurman et al., 2012). ARC1 was located in the promoter region, an area identified with high significance as open chromatin and validated by DNase I hypersensitivity and formaldehyde-assisted isolation of regulatory elements (FAIRE) assays. This area contained DNase I hypersensitivity in 113 of 125 cell lines/types. Transcription factor ChIP-seq data show it to be densely populated with DNA binding proteins (transcription factors, transcription machinery components, chromatin factors) with varying degrees of occupancy. ARC2 was located adjacent to ARC1, breaching the start of the coding sequence. This area was validated by both DNase I hypersensitivity and FAIRE assays with DNase I hypersensitivity in 16 of 125 cell lines/types. ChIP-seq data identified several DNA binding proteins with varying degrees of occupancy. ARC3 was located near the end of the gene in the third exon. This area contained DNase I hypersensitivity in 10 of 125 cell lines/types. This region was considered high significance as open chromatin but not validated by both DNase I and FAIRE assays. Transcription factor CTCF occupancy is shown to be weak. ARC4 was designed in an area that could be considered a "control," an intronic region between exons 2 and 3.

To determine the optimal annealing/extension temperatures for all primer sets designed, a gradient PCR with melting curve was run. Based on the results of the gradient, a standard curve PCR was performed to test the PCR efficiency. Efficiency greater than 90% was acceptable. After optimizing PCR protocols, the samples were run within those parameters. Primer sequences are found in Table 1.

2.3. Brain homogenates

2.3.1. Benzonase digestion (adapted from Grøntved et al., 2012)

Approximately 600 mg of frozen brain tissue was pulverized with a mortar and pestle that had been prechilled on dry ice. The pulverized tissue was transferred to a prechilled 15-mL tube and then suspended in 4 mL of lysis buffer (30 mmol/L Tris-HCL [pH 8.0], 2 mmol/L EDTA, 2 mmol/L EGTA, 20 mmol/L sodium butyrate, 2 mmol/L sodium orthovanadate, 4 mmol/L sodium fluoride, protease

Table 1
Primer sequences used for qPCR to determine chromatin accessibility

Gene	Forward	Reverse
KAT6B	CCCCAATGGGCTGCAGAGTGGTTAG	CGCTGCCGCTGCCTGAGAAACT
APP	CTCCCACTGTTCACGAAGCCCAGGT	CTACCGCTGCCGAGGAAACTGACG
ARC1	GGGCCTCGCTGGCTGCATAAAGAG	GTCCGGTGGTCCAGCTCCATCTGT
ARC2	ACTCGCAGCGCTGGAAGAAGTCCAT	GCGTAGGGGCTGACGGTGTAGTCGT
ARC3	TGGTCCTTCACTGCCCACTCTCCTG	CTAAGCTGGGGCTCCTGCCCTCTG
ARC4	ATGAGCTCCTCCCCAGACCCCAGAC	GCAGTGAAGGACCACGCAGGACAGT
RPS28	AGAGCGAAGGGTCCCGGCTTAGGAG	AGAGGAGTCACGTGCTTCGGGGAGA
GAPDH	AAAAGCGGGGAGAAAGTAGG	AGAGGAGTCACGTGCTTCGGGGAGA
CTCF4	CCCCAGAGAGTAGGGAACAG	GGCACGCAAAGACATACTGA
CTCF10	AGAGCACCCCCTACTGGCTAA	TAAGAAGCTGTGCGCGATGAC