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Estimating the total mouse DNA methylation according to the B1 repetitive elements

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Abstract

Measuring the degree of methylation of the B1 element in mouse may represent the global DNA methylation status because about 30,000 copies of the B1 element are randomly dispersed in the total mouse genome. Six CpG dinucleotides are located within each 163 bp size of B1 element, and each CpG dinucleotide was partially methylated. We quantitated the DNA methylation of the B1 repetitive elements by performing PCR for the methylation specific PCR (MSP) and also by the pyrosequencing. Each CpG dinucleotide was methylated at an average of 9% in the mouse genome by the pyrosequencing and MSP. Especially, we checked whether CpG methylation of the B1 element could respond to a treatment of the DNA methylation inhibitor, 5-azacytidine (5-AzaC). Consequently, the calibration graph resulting from measuring the relative CpG methylation percentage of the B1 element is linearly decreased with the increasing amount of 5-AzaC (up to 50 ng/ml concentration) in the NIH3T3 cells with a standard deviation of only 1.73% between three independent assays. Our methods can be applied to the routine analysis of the global DNA methylation changes in mouse in vivo and in vitro in pharmaceuticals and basic epigenetic research with efforts being less labor-intensive.

Keywords: DNA methylation; MSP; B1 element; Pyrosequencing; 5-Azacytidine

DNA methylation is one of the most common epigenetic events in the mammalian genome, and this process could regulate the expression of the genes containing the CpG islands [1–4]. The regulation of DNA methylation in a gene-specific promoter has been associated with various human diseases such as aging and the development of cancer [5–7]. The methods for analyzing DNA methylation have been extensively developed [8], and these methods include methylation specific PCR (MSP) [9], amplified methylation polymorphism (AMP) [10], pyrosequencing [11], and the matrix-assisted laser desorption ionization mass spectrometric (MALDI) methods [12]. These methods can detect a sequence change of cytosine to uracil in the DNA after a bisulfite treatment, and these methods have usually focused gene-specific analysis [13]. Therefore, they cannot provide a global picture of DNA methylation throughout the total genome.

Yang et al. [14] have established a measurement for global DNA methylation with the simultaneous PCR amplification of the Alu and LINE elements, which was based on the bisulfite treatment of genomic DNA in humans. The Alu family of retrotransposons represents the most typical short interspersed element (SINE) seen in primates [15–17], and the CpG dinucleotide repeats make up about 20% of Alu sequences. Furthermore, there are more than one million copies of the Alu repetitive elements in the mammalian genome. Thus, analyzing the methylation status of the repetitive

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elements could serve as a barometer for the global DNA methylation. Recently, the global variation of DNA methylation has been noted to occur during implantation as part of the genome reprogramming processes in mammals [8,18], and so this is useful in animal experiments to develop DNA methylation inhibitors [19,20].

For measuring the global DNA methylation in a mouse, we selected the B1 repetitive element because there are about 30,000 copies of it, and it is the most common DNA repetitive element in the mouse genome [21]. The B1 element contains 6 CpG dinucleotides within the 163 bp of each repeat. In this experiment, we studied the global DNA methylation measurement of the mouse genome by performing B1 element analysis. We established using both the MSP and pyrosequencing methods to measure the DNA methylation of the B1 element in the mouse genome. We then confirmed that both methods are sufficient to measure the global methylation changes in the mouse genome by using a treatment with DNA methylation inhibitor. The B1 element methylation may represent the global DNA methylation in the mouse genome.

Materials and methods

Tissue and cell line. ICR male mice (8 weeks) were purchased from the HANLYM LAB (ANIMALCO, Kyunggido, Korea) for isolation of their testes. NIH3T3 cells, the mouse fibroblast cell line, were cultured in DMEM containing 10% FBS and 1% antibiotic/antimyotic under a 5% CO_2 atmosphere at 37 °C. The cells were treated with 5, 7.5, 15, 30, and 50 ng/ml of 5-AzaC (Sigma, Steinheim, Germany) for 24 h prior to being harvested [22,23].

DNA isolation and bisulfite modification. We isolated the genomic DNA (gDNA) from the testis and the NIH3T3 cells by using a PUREGENE Cell and Tissue kit (Gentra, Minneapolis, USA). Sodium bisulfite treatment of the gDNA (1 μg) was performed with the CpGenome DNA modification kit. All reactions were done as recommended by the manufacturer's instructions (CHEMICON, Temecula, USA). After isolation, the bisulfite-treated DNA was stored at −20 °C until further use.

MSP. PCR amplification of the B1 element was performed in a 25 μl reaction mixture containing AmpliTaq Gold (Applied Biosystems, Foster City, California, USA). The PCR conditions were 94 °C for 1 min, 53 °C for 1 min, and 72 °C for 1 min for 30 cycles. All the primers were used at concentrations of 0.1 μM, and primer sequences used for PCR were as follows; the unmethyl forward primer was 5′-TAA CCTCAAACTCAAAAATCCACC-3′ and the unmethyl reverse primer was 5′-GTTGGGTGTAGTGGTATATATTTTTAATTTTA-3′. The methyl forward primer was 5′-CTCGAACTCAAAAATCCGCC-3′ and the methyl reverse primer was 5′-GTCGGGCGTAGTGGTATAT ATTTTT-3′, which are also indicated in Fig. 1A. The intensity of the MSP products on the agarose gel was measured through the Bio-1d program (Vilber Lourmat, GI-070AP).

Pyrosequencing. PCRs of the B1 element were performed for the pyrosequencing, and the PCR cycling conditions were 95 °C for 2 min, 56 °C for 90 s, and 72 °C for 2 min for 40 cycles. The sequences for the unmodified and modified PCR primer for the B1 element are indicated in Fig. 1A, and the unmodified sequences were as follows; the forward primer was 5′-TGGTGGTGGTTGAGACAGC-3′ (0.1 μM), and the reverse primer was 5′-TAGTGGCACACACCTTTAATCC-3′ (0.1 μM), and the modified sequences were as follows; the forward primer was 5′-GGTGGTGGTGGTTGAGATAG-3′ (0.1 μM) and the biotinylated reverse primer was 5′-AATAACACACACCTTTA ATCCCAACACT-3′. The sequencing primer for CpG-1 of the B1 element was 5′-TGGTGGTGGTTGAGA-3′ and for CpG-2 it was 5′-TTTGTAGATTAGGTTGGTTT-3′. The biotinylated PCR product (40 μl) was purified by using streptavidin–Sepharose beads (Amersham Biosciences, Uppsala, Sweden). The single strand PCR product acted as a template in a pyrosequencing reaction [24,25], and all the reactions were constructed as recommended by the manufacturer's instruction (Pyrosequencing, Westborough, MA). The pyrosequencing reaction was performed automatically by using a PSQ 96MA system (Pyrosequencing AB, Uppsala, Sweden) along with a SNP Reagent Kit (Biotage AB, Uppsala, Sweden).

Results and Discussion

B1 element for measuring DNA methylation in mouse genome

The B1 element is the most common SINE with more than 30,000 copies being present in the mouse genome. The full sequence of the B1 element is shown in Fig. 1A, and the B1 element has a full length of 163 bp with a total of 6 CpG dinucleotide repeats. We designed the PCR primers for performing B1-PCR, pyrosequencing, and MSP, and we can specifically amplify all B1 elements by these techniques within the total mouse genome. The size of each primer used for unmethyl and methyl MSP was adjusted to allow using the same melting temperature because the unmethyl MSP products have a complex secondary structure because of the sequencing changes after the gDNA sodium bisulfite treatment. The PCRs for the unmethyl and methyl MSP were performed under the same conditions but using only the different primers shown in Fig. 1A. We tested the PCR condition for B1 element amplification using four different primer sets with unmodified DNA or modified (sodium bisulfite treated) DNA (Fig. 1B). We, respectively, performed PCR with unmodified and modified B1-PCR primers. Actually, B1-PCR amplification was working only at modified B1-PCR primers with modified gDNA and also at unmodified B1-PCR primers with unmodified gDNA. However, in case of MSP, both unmethyl and methyl MSP primers were just working only on modified gDNA (Fig. 1B). B1-PCR and MSP reactions were specifically performed at total mouse genome. Our results showed that the sequences of all primer sets were specific, and our condition for sodium bisulfite treatment of mouse gDNA was a standard method for estimating DNA methylation on mouse total genome.

Substantial method for measuring DNA methylation through the MSP and pyrosequencing with B1 element

Quantitation of the B1 methylation was performed by MSP in the mouse testis DNA. The methyl and unmethyl