Biochemical and Biophysical Research Communications 434 (2013) 873–878

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Analysis of DNA methylation change induced by Dnmt3b in mouse hepatocytes

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ARTICLE INFO

Article history:
Received 30 March 2013
Available online 20 April 2013

Keywords:
DNA methylation
DNA methyltransferase
Epigenetics
Hepatocytes
Gene expression

ABSTRACT

DNA methylation is a key epigenetic contributor to gene regulation in mammals. We have recently found that in the mouse liver, the promoter region of glycerol-3-phosphate acyltransferase 1, a rate-limiting enzyme of *de novo* lipogenesis, is regulated by DNA methylation, which is mediated by Dnmt3b, an enzyme required for the initiation of *de novo* methylation. In this study, using primary cultures of mouse hepatocytes with adenoviral overexpression of Dnmt3b, we characterized Dnmt3b-dependent DNA methylation on a genome-wide basis. A genome-wide DNA methylation analysis, called microarray-based integrated analysis of methylation by isoschizomers, identified 108 genes with Dnmt3b dependent DNA methylation. In DNA expression array analysis, expression of some genes with Dnmt3b-dependent DNA methylation was suppressed. Studies with primary mouse hepatocytes overexpressing Dnmt3b or Dnmt3a revealed that many genes with Dnmt3b-dependent methylation are not methylated by Dnmt3a, whereas those methylated by Dnmt3a are mostly methylated by Dnmt3b. Bioinformatic analysis showed that the CANAGCTG and CCGGWNCSC (N denotes A, T, G, or C; W denotes A or T; and S denotes C or G) sequences are enriched in genes methylated by overexpression of Dnmt3b and Dnmt3a, respectively. We also observed a large number of genes with Dnmt3b-dependent DNA methylation in primary cultures of mouse hepatocytes with adenoviral overexpression of Dnmt3, suggesting that Dnmt3b is an important DNA methyltransferase in primary mouse hepatocytes, targets specific genes, and potentially plays a role *in vivo*.

1. Introduction

Covalent modification of DNA by methylation of cytosine residues in CpG is a heritable and reversible epigenetic process involved in the regulation of a diverse range of biological processes in mammals [1]. DNA methylation is essential for normal embryonic development, regulation of gene expression, and genomic imprinting, and altered DNA methylation patterns have been implicated in tumorigenesis. In spatiotemporal regulation of gene expression, DNA methylation participates in determining the chromatin structure [1]. In mammals, three CpG DNA methyltransferases, Dnmt1, Dnmt3a, and Dnmt3b, coordinately regulate DNA methylation in the genome. Dnmt1 is known to promote DNA methylation after DNA replication and plays a major role in the maintenance of methylation [2]. Dnmt3a and Dnmt3b are required for the initiation of *de novo* methylation [2]. They appear to have different roles in development and gene regulation; Dnmt3a and Dnmt3b knockout mice are both embryonic lethal, and Dnmt3b has a more severe phenotype than Dnmt3a [3]. Transgenic overexpression of Dnmt3b but not Dnmt3a in throughout the body using the *ROSA26* promoter has caused colon cancer in adenomatous polyposis coli multipule intenstinal neoplasia ($Apc^{min/+}$) mice, which tend to develop carcinomas, with massive methylation of the *SFRP2* gene [4]. We previously created transgenic mice overexpressing

Abbreviations: Dnmt, DNA methyltransferase; *Gpam*, glycerol-3-phosphate acyltransferase 1; MIAMI, microarray-based integrated analysis of methylation by isoschizomers.

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0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.04.041

Dnmt3a in the adipose tissue, where we observed no marked change in DNA methylation or gene expression [5]. On the other hand, we recently reported that Dnmt3b, but not Dnmt3a, is recruited to the promoter region of glycerol-3-phosphate acyltransferase 1 (*Gpam*), a rate-limiting enzyme of *de novo* lipogenesis in the neonatal mouse liver [6]. We also observed increased DNA methylation of the promoter region of *Gpam* in primary cultures of mouse hepatocytes overexpressing Dnmt3b. Thus, Dnmt3b may be a major *de novo* DNA methylase in *Gpam* regulation. However, the role of Dnmt3b and its target genes in the liver are largely unknown.

Hatada et al. developed a genome-wide DNA methylation analysis called microarray-based integrated analysis of methylation by isoschizomers (MIAMI) [7]. This microarray method detects DNA methylation using the methylation-sensitive restriction enzyme *Hpa*II and its methylation-insensitive isoschizomer *Msp*I. *Hpa*II cleavage differences are usually associated with methylation differences between two samples. This method has been applied for genome-wide profiling of lung cancer [8], neural differentiation, and adipocyte differentiation [9]. In this study, the MIAMI method was applied to genome-wide DNA methylation analysis to identify DNA methylation target genes of Dnmt3b in mouse hepatocytes.

2. Materials and methods

2.1. Primary cultures of mouse hepatocytes

Primary hepatocytes were isolated from 10-week-old male C57BL/6 mice, as described previously [6].

2.2. Preparation of recombinant adenoviruses

The full-length mouse Dnmt3b and Dnmt3a cDNAs were subcloned into the pShuttle vector provided in the BD Adeno-X Expression System (BD Biosciences, Franklin Lakes, NJ, USA). BD Adeno-X-enhanced green fluorescent protein (GFP) was used as a control (Ad-GFP; BD Biosciences). Each recombinant adenovirus (Ad-Dnmt3b, Ad-Dnmt3a, or Ad-GFP) was added to a medium of primary cultured mouse hepatocytes (1.8×10^7 infection-forming units in 500 μl) [6].

2.3. Western blotting analysis

Cell culture dishes were washed with phosphate-buffered saline (PBS), and cells were scraped off into cell lysate buffer [10 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% TritonX-100, 1 mM PMSF, and protease inhibitor cocktail]. The lysate was gently agitated for 1 h at 4 °C and centrifuged. The supernatant was used for Western blotting analysis [6]. The following primary antibodies were used: anti-Dnmt3b (IMG184A; Imgenex San Diego, CA, USA) and anti-Dnmt3a (IMG268A; Imgenex, San Diego, CA, USA).

2.4. Methylation profiling by the MIAMI method

The MIAMI method was performed using 1 μg of genomic DNA, as described previously [7]. The complete experimental procedure is available at http://www.grc.dept.med.gunma-u.ac.jp/~gene/image/MIAMI20Protocol20V4.pdf. In brief, to examine the changes in DNA methylation, we analyzed the sample differences between methylation-sensitive *Hpa*II cleavage and methylation-insensitive *Msp*I cleavage. Adaptors were ligated and PCR-amplified, and the products were hybridized with microarrays containing 41,332 probes [7]. Transcriptional start sites for the genes were characterized on the basis of the Ensembl database annotation (MGSCv37, dated Sept. 17, 2011) using the BioMart program [10]. The MIAMI probes were mapped on MGSCv37 using Bowtie 0.12.5 [11] to yield their chromosomal positions, and the distances to the MIAMI probes from the transcription start sites of the nearest genes were calculated. These are shown in Supplementary Tables 1, 2, and 3.

2.5. Functional annotation analysis in DNA methylated genes caused by Dnmt3b overexpression

Gene ontology functional annotations, pathway analysis using the KEGG (Kyoto Encyclopedia of Genes and Genomes) database resource, and classification of protein families using the Pfam database were performed with DAVID v6.7 [12], which is a web application providing a comprehensive set of functional annotation tools to understand the biological meaning of a large list of genes. The list of gene symbols that showed increased DNA methylation by Dnmt3b overexpression was submitted and detected a significantly overrepresented GO biological process (GOTERM BP-FAT), the KEGG pathway, or Pfam protein motifs/domains.

2.6. Systematic identification of specific sequences enriched in DNA methylated genes

We performed *de novo* motif prediction using the Dispom algorithm [13] for systematic identification of specific sequences enriched in the DNA of genes whose methylation was induced by Dnmt3b or Dnmt3a. Dispom is a tool that can predict differentially abundant motifs and their positional preferences [13]. Thus, the mouse genome was investigated in the region of −0.5 kb to +0.5 kb relative to the MIAMI probe of an individual gene. In this study, the enrichment of genomic sequences was estimated for methylation induced by Dnmt3b overexpression vs. Dnmt3a overexpression and vice versa. Statistical hypothesis testing was performed between opposite sets of genes (genes with increased methylation by Dnmt3b were contrasted with those by Dnmt3a). Sequence logos in Fig. 4 were produced following the method described by Crooks et al. [14], and the IUPAC codes of the motifs were generated following the method described by Cavener et al. [15].

2.7. Bisulfite DNA methylation analysis

Genomic DNA was isolated by a standard procedure with proteinase K treatment. Sodium bisulfite treatment of the DNA was performed using a BisulFast DNA Modification Kit (Toyobo, Osaka, Japan), according to the manufacturer's instructions. Sequential PCR amplification of the genes of interest was performed using specific primers: *Dpp4* forward: AGGGTTTTTATGAGTGAGGGATAAG, reverse: ACAAAACAAATAAACATTCCCCAAA; *Lrp5* forward: GTAGGGGGAGGGGATTTATATTAGG, reverse: CAAAAAACCATTACA ATTACAACCCATAA. The reaction profiles were 38 cycles of 96 °C for 15 s, 57 °C for 30 s, and 72 °C for 60 s (*Dpp4*), and 35 cycles of 96 °C for 15 s, 58 °C for 30 s, and 72 °C for 60 s (*Lrp5*). The amplified fragments were ligated into the vector pGEM-T easy (Promega, Madison, WI, USA), and more than 10 clones were sequenced per reaction. QUMA (a web-based quantification tool for methylation analysis) (http://www.quma.cdb.riken.jp/) [16] was used for bisulfite sequencing analysis of CpG methylation.

2.8. cDNA microarray analysis

RNA was isolated from primary cultured mouse hepatocytes overexpressing Dnmt3b, Dnmt3a, or GFP. Each sample was labeled with a cyanine 3-CTP using the Low Input Quick Amp Labeling Kit (Agilent Technologies, Inc., Santa Clara, CA, USA) and hybridized to the Agilent whole mouse genome (4 × 44 K) microarray, which

contains 41,534 genes including expressed sequence tags. Signal detection and data analysis were performed according to the manufacturer's instructions.

2.9. Quantitative RT-PCR analysis

Total RNA was prepared using TRIzol (Life Technologies, Carlsbad, CA, USA). cDNA was synthesized from 250 ng of total RNA using the QuantiTect Rev. Transcription Kit (QIAGEN K.K, Tokyo, Japan). Gene expression levels were measured with an ABI PRISM 7000 using SYBR Green PCR Master Mix (Life Technologies) designed to detect cDNAs. The primers used are shown in Supplementary Table 4.

3. Results and discussion

3.1. Identification of DNA methylation changes in Dnmt3b-overexpressing hepatocytes

To detect methylation changes induced by Dnmt3b overexpression, we isolated genomic DNA from primary cultured mouse hepatocytes expressing Dnmt3b or GFP, which were subjected to the MIAMI analysis [7]. Dnmt3b protein expression was confirmed by Western blotting analysis (Fig. 1A). In this experiment, increased *Gpam* DNA methylation by Dnmt3b was confirmed, as reported previously [6] (Supplementary Fig. 1). Comparison of the sensitivity of the methylation-sensitive enzyme *Hpa*II between samples with Dnmt3b overexpression revealed a large number of differences in DNA methylation (Fig. 1B). We observed 108 genes that gained and 0 genes that lost methylation for Dnmt3b- vs. GFP-overexpressing hepatocytes (Supplementary Table 1). Not all genomic regions showed increased DNA methylation, suggesting that targeted DNA methylation was induced by Dnmt3b overexpression.

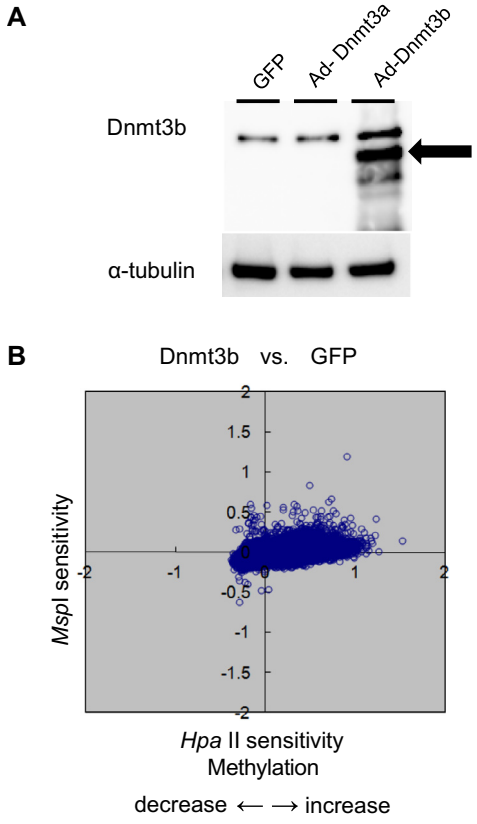

Fig. 1. Western blotting analysis and genome-wide analysis of DNA methylation in Dnmt3b-overexpressing hepatocytes. (A) Primary hepatocytes were infected with adenoviral Dnmt3b, Dnmt3a, or GFP. Cell lysate was analyzed by Western blotting analysis using an antibody against Dnmt3b, showing protein overexpression. The molecular size of detected Dnmt3b was 110 kDa. Alpha-tubulin antibody was used as the loading control. (B) MIAMI analysis of Dnmt3b- versus GFP-overexpressing hepatocytes. Plots of *Hpa*II (methylation sensitive, horizontal axis) and *Msp*I (methylation insensitive, vertical axis); spreading horizontal axis indicates larger differences in DNA methylation between samples. Thus, an increasing horizontal axis denotes increased DNA methylation, and a number <0 denotes decreased DNA methylation in the sample with overexpression of Dnmt3b (subject) compared with the sample with overexpression of GFP (control). Numbers denote the fold in log2 ratio (Supplementary Table 1 for details).

3.2. Change in expression of genes whose DNA is methylated by Dnmt3b

To assess the relationship between DNA methylation and gene expression, DNA microarray analysis was performed using hepatocytes overexpressing Dnmt3b or GFP. Differences in gene expression between Dnmt3b- and GFP-overexpressing hepatocytes are listed in Supplementary Table 1. Expression of 17 out of 108 methylated genes was downregulated by <0.5-fold, and 33 genes were downregulated by <0.8-fold, whereas 2 genes were upregulated by >2-fold. The data of microarray analysis were confirmed by quantitative real-time RT-PCR analysis of representative genes (Supplementary Fig. 2). Thus, Dnmt3b-dependent DNA methylation appears to influence the expression of some genes. DNA methylation of the promoter and gene body regions tends to suppress and facilitate gene expression, respectively [17,18]. The probes used in the MIAMI analysis are located in both the promoter and gene body regions, neither of which was markedly affected, suggesting that DNA methylation alone is not sufficient for modifying gene expression. Under our culture conditions, other stimuli besides DNA methylation may be required for changes in gene expression.

3.3. Functional classification of products of genes whose DNA is methylated by Dnmt3b

We performed gene ontology analysis of the 108 genes with increased methylation to identify potential functional similarities in

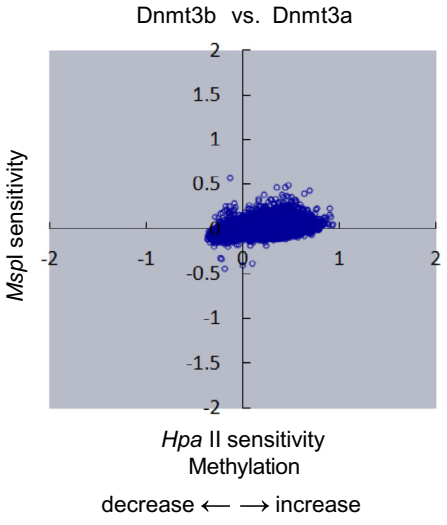

Fig. 2. Genome-wide analysis of DNA methylation in Dnmt3b- vs. Dnmt3a -overexpressing hepatocytes. MIAMI analysis of Dnmt3b- versus Dnmt3a-overexpressing hepatocytes. Plots of *Hpa*II (methylation sensitive, horizontal axis) and *Msp*I (methylation insensitive, vertical axis); spreading along the horizontal axis indicates larger differences in DNA methylation between samples. Thus, an increase along horizontal axis denotes increased DNA methylation, and a number <0 denotes decreased DNA methylation in the sample with overexpression of Dnmt3b (subject) compared with the sample with overexpression of Dnmt3a (control). Numbers denote the fold-change in log2 ratio (Supplementary Table 3 for detail).

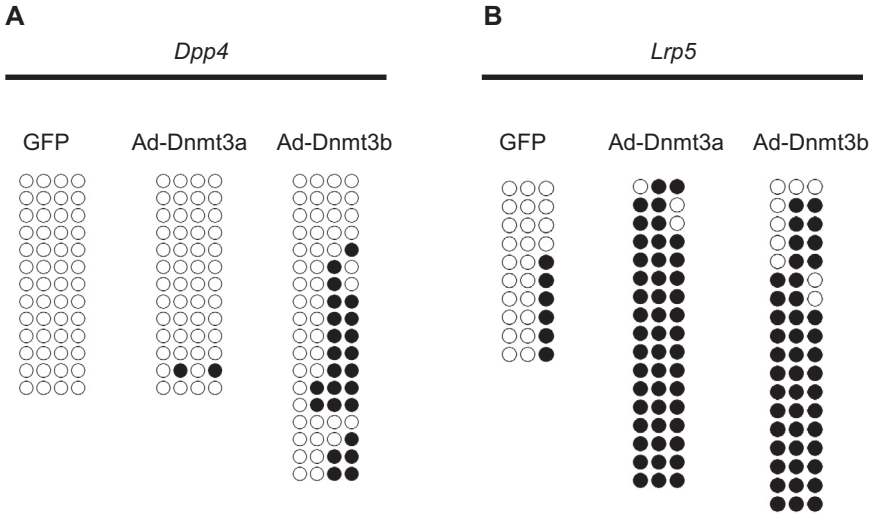

Fig. 3. Bisulfite analysis: DNA methylation of representative genes. Bisulfite analysis of (A) *Dpp4* and (B) *Lrp5* in Dnmt3b- and Dnmt3a-overexpressing hepatocytes. Representative bisulfite data with open and filled circles are shown. Filled circles indicate methylated and open circles unmethylated CpGs.

gene products. We found four GO categories: ion transport (11 genes), cytoskeleton organization (6 genes), negative regulation of transcription from RNA polymerase II promoter (5 genes) and tissue morphogenesis (5 genes), out of 108 genes. Gene names belonging to each category are shown in Supplementary Table 5 and might be potentially involved in Dnmt3b-mediated cellular functional change. Dnmt3b-mediated increased DNA methylation in a specific set of genes may be related to a later phenotype of gene expression and cellular differentiation. For example, Zhx2 is a repressor known to repress the alpha phetoprotein gene, a marker of inmature hepatocytes [19], whose gene expression was up-regulated (5-fold in our microarray). The gene body of Zhx2 was methylated in our analysis, which may contribute to increased gene expression. But, as described above, the expressions of methylated genes were not changed in our culture condition. Analysis of the condition with changed expression of these genes should be examined in the future. In addition to GO analysis, we also performed KEGG database-based pathway analysis and a Pfam protein motif/specific domain search in the gene product of 108 genes. We observed no significant KEGG pathway (data not shown), and a small number of motifs (2 motifs, each containing 2 out of 108 genes) in the Pfam search (Supplementary Table 6), which we did not think a remarkable change as the numbers were small.

3.4. Comparison of DNA methylation in Dnmt3b- and Dnmt3a-overexpressing hepatocytes

We also compared by the MIAMI analysis DNA methylation patterns in Dnmt3b- and Dnmt3a-overexpressing hepatocytes [7]. In this study, Dnmt3a protein expression was confirmed by Western blotting analysis (Supplementary Fig. 3). To confirm that Dnmt3a increases specific DNA methylation, we compared Dnmt3a- and GFP-overexpressing hepatocytes. This comparison showed that 100 genes gained methylation and that no gene lost methylation (Supplementary Table 2), indicating that Dnmt3a methylates DNA in hepatocytes.

Comparison of sensitivity to the methylation-sensitive enzyme *Hpa*II revealed a large number of changes in DNA methylation with overexpression of Dnmt3b compared with that of Dnmt3a (Fig. 2 and Supplementary Table 3). As the MIAMI analysis is a method to observe the difference of DNA methylation between two samples (showing relative value, but not absolute value), it is difficult to detect signals in gene regions when both Dnmt3b and Dnmt3a show increased DNA methylation at similar levels. For detailed analysis, we included the DNA methylation fold-change data (Dnmt3b/Dnmt3a: the ratio of values of Dnmt3b-/Dnmt3a-overexpressing hepatocytes, Dnmt3a/Dnmt3b: the ratio of values of Dnmt3a-/Dnmt3b-overexpressing hepatocytes) (Supplementary Tables 1 & 2). Bisulfite analysis was also performed using representative differentially methylated genes; *Dpp4* and *Lrp5* [20,21] (Fig. 3 and Supplementary Tables 1 & 2). Increased DNA methylation was confirmed at the *Hpa*II sites corresponding to the probes of MIAMI analysis and neighboring CpG sites in *Dpp4* only by Dnmt3b, and in *Lrp5* by both Dnmt3b and Dnmt3a (Fig. 3). In Supplementary Table 1, the DNA methylation change (Dnmt3b/Dnmt3a) for *Dpp4* was 2.47. The number of genes with values higher than 2.47 was 54 out of 83 genes (65%) (25 of 108 genes methylated by Dnmt3b overexpression did not produce any signal). Only a single gene showed higher DNA methylation in Dnmt3a-overexpressing hepatocytes than in Dnmt3b-overexpressing hepatocytes (the DNA methylation change of Dnmt3b/Dnmt3a is <1). Thus, many genes with Dnmt3b-dependent methylation were not methylated by Dnmt3a. In contrast, the DNA methylation change (Dnmt3a/Dnmt3b) for *Lrp5* was 1.74 and only 7 out of 95 genes (7%) showed the methylation change of >1.74. Thus, genes methylated by Dnmt3a were mostly methylated by Dnmt3b (Supplementary Table 2).

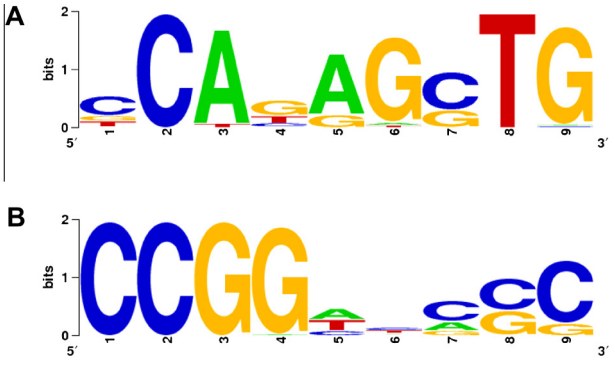

Fig. 4. Sequence motifs enriched in genes methylated by Dnmt3b- or Dnmt3a-overexpression. (A, B) Sequence obtained from the prediction made using Dispom. It was enriched in genes methylated by (A) Dnmt3b overexpression (preferentially methylated by Dnmt3b) and (B) Dnmt3a overexpression (methylated by both Dnmt3b and Dnmt3a).

There is *in vitro* evidence that Dnmt3b shows higher methylation preference than Dnmt3a for DNA within the nucleosome core region, whereas Dnmt3a shows higher DNA methylation activity than Dnmt3b on the naked DNA and naked part of nucleosomal DNA [22,23]. If this trend holds *in vivo*, the chromatin structure of genes methylated preferentially by Dnmt3b may be different from those by both Dnmt3b and Dnmt3a in hepatocytes. Further *in vivo* studies of the genomic chromatin structure, such as by chromatin immunoprecipitation and DNA methylation analysis, are required.

3.5. Presumed DNA motifs rich in DNA methylated genes

For systematic identification of specific sequences enriched by the presence of Dnmt3b, we predicted a *de novo* motif in genes with differential methylation using the Dispom algorithm. In genes methylated preferentially by Dnmt3b, the CANAGCTG sequence was enriched (Fig. 4A). In genes methylated by both Dnmt3b and Dnmt3a, the CCGGWNCSC sequence (W denotes A or T, N denotes A, T, G, or C; and S denotes C or G) was enriched (Fig. 4B). Thus, the CANAGCTG sequence may be recognized preferentially by Dnmt3b. Dnmt3b and Dnmt3a share similar functional protein domains, including their catalytic domains, but their overall amino acid sequence similarity is relatively low. They have been shown to interact with different proteins, whereby they may be recruited to genomic DNA [24]. A recent study showed that approximately 1 kb of genomic DNA autonomously determines the status of DNA methylation, which is affected by the presence of motifs for DNA-binding factors in the DNA region [25], supporting the idea that Dnmt3b and Dnmt3a can be recruited by transcription factors. In this study, we found that CANAGCTG and CCGGWNCSC sequences are enriched in genes methylated preferentially by Dnmt3b and in those methylated by both Dnmt3b and Dnmt3a, respectively. Thus, Dnmt3b may be recruited to genomic DNA by interaction with the proteins recognizing the CANAGCTG sequence, although the sequences do not appear to resemble known transcription factor-binding motifs.

3.6. CG content in DNA methylated genes

Given that genes methylated by both Dnmt3b and Dnmt3a contain the CCGGWNCSC sequence, which is relatively rich in CG, the CG contents of DNA methylated genes were examined. Distribution graphs of the frequency of CG content in the sequences were drawn. The frequency distribution had a peak at approximately 20 CGs per 1000 bp for all probe sequences used in the MIAMI array (Supplementary Fig. 4A). A peak at a similar position appeared in the frequency distribution of genes methylated preferentially by Dnmt3b (Supplementary Fig. 4B). In contrast, the frequency of CG content was highest at approximately 60 CGs per 1000 bp for genes methylated by both Dnmt3b and Dnmt3a, in addition to the peak near 20 CGs (Supplementary Fig. 4C). The data suggest an association of Dnmt3b and Dnmt3a with CG-rich sequences and preferential methylation of CG-rich genes.

4. Conclusions

We have recently found that in the mouse liver, the promoter region of *Gpam*, a rate-limiting enzyme of *de novo* lipogenesis, is regulated by DNA methylation, which is mediated by Dnmt3b. In this study, we observed a large number of genes with Dnmt3b-dependent DNA methylation in mouse hepatocytes with adenoviral overexpression of Dnmt3, suggesting that Dnmt3b is a major DNA methyltransferase in hepatocytes. The data of this study provides a clue to the better understanding of the mechanism of Dnmt3b-dependent DNA methylation and its functional significance in the liver *in vivo*.

Acknowledgments

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, The Ministry of Health, Labour and Welfare of Japan, and research grants from Novo Nordisk Study Award for Growth and Development, Novo Nordisk Insulin Study Award, Ono Medical Research Foundation, The Morinaga Foundation for Health and Nutrition, and The Nutrition and Food Science Fund of the Japan Society of Nutrition and Food Science.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.04.041.

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