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Undetectable levels of N6-methyl adenine in mouse DNA: Cloning and analysis of PRED28, a gene coding for a putative mammalian DNA adenine methyltransferase

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Abstract **Three methylated bases, 5-methylcytosine, N4-methylcytosine and N6-methyladenine (m6A), can be found in DNA. However, to date, only 5-methylcytosine has been detected in mammalian genomes. To reinvestigate the presence of m6A in mammalian DNA, we used a highly sensitive method capable of detecting one N6-methyldeoxyadenosine per million nucleosides. Our results suggest that the total mouse genome contains, if any, less than 10^3 m6A. Experiments were next performed on PRED28, a putative mammalian N6-DNA methyltransferase. The murine PRED28 encodes two alternatively spliced RNA. However, although recombinant PRED28 proteins are found in the nucleus, no evidence for an adenine-methyltransferase activity was detected.**

Keywords: Adenine methylation; DNA methylation; N6-methyladenine; Down syndrome

1. Introduction

In mammalian DNA, the crucial function of cytosine methylation in chromatin structure and gene functions has led to the consideration of 5-methylcytosine (m5C) as the fifth base of DNA [1,2]. In addition to m5C, there are two other known methylated bases, N6-methyladenine (m6A) and N4-methylcytosine (m4C). However, m6A and m4C are generally considered to be specific to bacterial or protist DNA [3], and have not yet been identified in mammalian DNA. In prokaryotes, the function of m4C and m5C seems to be limited to restriction-modification systems [4], although m5C is also part of the very short-patch repair system [5]. On the contrary, the biological role of m6A in bacteria is more widespread, and includes postreplicative DNA mismatch repair, chromosome compaction and regulation of gene expression [4]. Moreover, adenine methylation is essential for the virulence or viability of several pathogenic bacterial strains [6–8]. This suggests that bacterial N6-DNA methyltransferase is a potential target for the design of new antimicrobial agents [9]. The interest for this new generation of antibiotics highlights the necessity for determining the existence of N6-DNA methyltransferases in higher eukaryotes.

The current assumption that m5C is the only methylated base present in animal DNA relies on experiments performed about thirty years ago with a detection limit of around 0.01% [10–12]. However, even if analytical methods failed to detect m6A in mammalian DNA, indirect evidence based on restriction enzyme analyses has suggested the presence of m6A in the mouse myogenic and the rat type 2 steroid 5α-reductase genes [13,14]. Hence, current analytical methods are sufficient to detect m5C in mammalian DNA (approximately 1–6%), but may be inadequate to detect the possible presence of a few hundred m6A in the 3.3×10^9 bases of the *Homo sapiens* genome [15]. This suggests that the methylation status of adenine in mammalian DNA could be similar to the situation encountered a few years ago with cytosine methylation in *Drosophila melanogaster*. Indeed, it had been well-accepted that DNA from *D. melanogaster* was not methylated, a belief recently challenged by the presence of m5C in this species [16]. In this regard, the recent characterization of a N6-adenine DNA methyltransferase from wheat coleoptiles suggests the occurrence of adenine methylation in higher eukaryotes [17].

A prerequisite for the presence of m6A in mammalian DNA is the expression of a N6-DNA methyltransferase. In silico analyses of mouse and human genomes have identified the existence of the PRED28 gene that encodes a putative N6-DNA methyltransferase (http://harvester.embl.de/harvester/Q9Y5/Q9Y5N5.htm; GenBank gene ID: 29104). This gene is located on human chromosome 21 or mouse chromosome 16, two chromosomes syntenic for this region. This situation prompted us to reexamine the methylation status of adenine in mammalian DNA.

2. Materials and methods

2.1. Bacterial strains

Escherichia coli TOP10 (Invitrogen) was used for transformation procedures and maintenance of plasmids. *E. coli* GM 272 (*dam*$^-$, *dcm*$^-$, *hsdS*$^-$) [18] was used for expression studies. This strain has mutations in genes encoding the adenine DNA methyltransferases and is therefore expected to have very little m6A in its DNA.

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2.2. DNA purification and analysis

DNA analysis: Genomic DNA was extracted using the Genomic DNA buffer set and Qiagen Genomic Tip from Qiagen (Qiagen, Courtaboeuf, France). Human placental mitochondria were purified as described [19]. Mitochondrial DNA was extracted using the QIAamp DNA kit from Qiagen (Qiagen, Courtaboeuf, France). Subsequently, genomic or mitochondrial DNA was incubated with a mixture of endo- and exonucleases, and alkaline phosphatase that quantitatively liberate N6-methyldeoxyadenosine (MedAdo) [20]. This was demonstrated using plasmid DNA that contained a known amount of MedAdo. A HPLC-electrospray ionization tandem mass spectrometry (HPLC–MS/MS) method has been developed for the detection of MedAdo, as previously described for the measurement of oxidized nucleosides [21,22]. The HPLC-MS/MS system used for the detection of MedAdo has been previously described [23]. The limits of sensitivity ($S/N = 3$) for the assay were close to 20 fmol of injected MedAdo (corresponding to a concentration of 0.5 nM). Quantification was performed by external calibration, and the amount of DNA was determined by UV absorption using the peak of deoxyguanosine [22].

2.3. RNA purification and reverse transcription (RT)-PCR

Total RNA extraction and RT-PCR were performed using the Rneasy and Qiagen OneStep RT-PCR kits (Qiagen, Courtaboeuf, France). Oligonucleotide primers were designed based on the mouse PRED28 cDNA sequence available in the GenBank™ data base under Accession AY456393. The oligonucleotides used were PRED1a 5′-GTGCGTACTCAACGCCCGCCCATAGG-3′ (forward) and PRED1b 5′-GCTGCCGGCAGCTCTTGTTTACTTCTGTGG (reverse). Genomic DNA contamination was controlled by PCR of non-reverse transcribed RNA.

2.4. Cloning of PRED28 splice variants

The coding regions of PRED28α and PRED28β were amplified by RT-PCR from embryonic mouse brain mRNA and cloned into pcDNA3.1/V5His-TOPO (Invitrogen) to generate pcDNA3/PRED28α and pcDNA3/PRED28β. Primers used for PRED28α amplification are 5′-GCGGAAGGGAAAAGATGGCGGCGCC-3′ (forward) and 5′-GAGGGACTTGCTGAACCTGAGGACTGACAGG-3′ (reverse). Primers used for PRED28β are 5′-GCGGAAGGGAAAAGATGG-CGGCGCC-3′ (forward) and 5′-CAATTGTTTTAAAGATTTCCT-CTCAG-3′ (reverse). The cDNA sequence of PRED28α corresponds to the sequence of the *Mus musculus* N6-DNA methyltransferase mRNA in GenBank under Accession AY456393, whereas the cDNA sequence of PRED28β corresponds to a new transcript variant deposited under Accession AY536887. The full-length coding regions of pcDNA3/PRED28α and pcDNA3/PRED28β were amplified with the primers 5′-GGGGTACCGAGCTCGGATCCACTAG-3′ (forward) and 5′-CACGGGGGATCCGCAAACAACAGATGGCTGGCAAC-3′ (reverse) and subcloned in the *Bam*HI site of the eukaryotic expression vector pcDNA4/TO (Invitrogen) to generate pcDNA4/PRED28α and pcDNA4/PRED28β. To express PRED28 isoforms in *E. coli*, the coding regions of the two isoforms of PRED28 were amplified and cloned under the control of the *araBAD* promoter in the bacterial expression vector pBAD-TOPO (Invitrogen) to generate pBAD/PRED28α and pBAD/PRED28β. The primers used for PRED28α amplification were 5′-GCGGCGCCGAGTGTCCCCACGCCG-3′ (forward) and 5′-GAGGAATAATAAATGGCGGCGCCGAGTGTCCCCAC-GCCG-3′ (reverse), and the primers used for PRED28β are 5′-GCGGCGCCGAGTGTCCCCACGCCG-3′ (forward) and 5′-TAATAAATGGCGGCGCCGAGTGTCCCCACG-3′ (reverse). All the resulting recombinant proteins contain a C-terminal V5 epitope tag and a C-terminal 6 × His tag. All the constructs were verified by DNA sequencing.

2.5. Cell culture and transfection

C2C12 cells were grown to approximately 80% confluence, and transfection was performed by incubating C2C12 cells with either pcDNA4/PRED28α or pcDNA4/PRED28β vector using the Lipofectamine 2000™ reagent according to the manufacturer's instructions (Invitrogen). For selection of stable recombinant clones, zeocin (100 μg/ml) was added 72 h after transfection. Expression was verified by immunofluorescence staining and Western blot using a V5 antibody (Invitrogen).

2.6. Bacterial expression of recombinant PRED28

For the bacterial expression of recombinant proteins, *E. coli* GM272 clones harboring either pBAD/PRED28α or pBAD/PRED28β were grown at 37 °C in LB broth containing Amp to an OD_{600} of ~0.25. At this point the cultures were split, and L-arabinose (0.1% final concentration) was added to one of the cultures to induce protein production. After 1 h, the bacteria were centrifuged and processed for either DNA extraction or for Western blot analysis. For Western blot analysis, cell pellets were resuspended and lysed in CelLytic™ buffer (Sigma). Then, cell lysates were centrifugated at $18000 \times g$ for 15 min to pellet the insoluble material. Western blot analyses were performed on both the supernatant and insoluble fractions using a V5 antibody (Invitrogen). For DNA analysis, DNA was extracted using the DNA genomic buffer set from Qiagen according to the manufacturer's instructions.

3. Results

3.1. Determination of m6A content in mammalian and mitochondrial DNA

Previous base analyses of mammalian DNA have failed to detect the presence of m6A. However, these analyses were performed over twenty years ago with a detection threshold around 0.01%. Therefore, in an attempt to reexamine the problem of adenine methylation in mammalian DNA, we have developed an extremely sensitive assay for MedAdo with a detection threshold of one MedAdo per 10^6 nucleosides. However, even using this sensitive assay we were unable to detect adenine methylation in the nuclear DNA of several mouse tissues (Table 1). According to the size of the mouse genome (2.5 Gb), these results suggest that the total mouse genome contains less than 2.5×10^3 m6A. In addition, since it has been suggested that mitochondria could be the degenerate descendants of endosymbiotic bacteria, and since bacterial DNA is known to contain m6A, mitochondrial DNA from human placenta was also included in this study. In contrast to bacterial DNA, which contains MedAdo, no MedAdo was detected in mitochondrial DNA (Table 1).

3.2. Tissue expression profile of PRED28/N6AM1, a putative N6-DNA methyltransferase

The presence of less than 10^3 m6A in the whole mouse genome could be biologically significant if adenine methylation occurs in rare specific gene regulatory elements. However, it is difficult to demonstrate the existence of a base which is

Table 1
Content of MedAdo in the DNA of different mouse tissues or cells

Source of DNA	MedAdo/10^6 nucleosides
Heart	<1
Muscle	<1
Liver	<1
Brain	<1
Testis	<1
Brain	<1
Mitochondria	<1
C2C12	<1
C2C12-Pred α	<1
C2C12-Pred β	<1
E. coli GM272-PREDα non-induced ($n = 3$)	38.4 ± 10.5
E. coli GM272-PREDα induced ($n = 3$)	35.1 ± 9.6
E. coli GM272-PREDβ non-induced ($n = 2$)	44.1 ± 3.9
E. coli GM272-PREDβ induced ($n = 3$)	35.2 ± 4.2