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Dnmt2 is not required for *de novo* and maintenance methylation of viral DNA in embryonic stem cells

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ABSTRACT

We have shown previously that *de novo* methylation activities persist in mouse embryonic stem (ES) cells homozygous for a null mutation of *Dnmt1* that encodes the major DNA cytosine methyltransferase. In this study, we have cloned a putative mammalian DNA methyltransferase gene, termed *Dnmt2*, that is homologous to *pmt1* of fission yeast. Different from *pmt1* in which the catalytic Pro-Pro-Cys (PPC) motif is 'mutated' to Pro-Ser-Cys, *Dnmt2* contains all the conserved methyltransferase motifs, thus likely encoding a functional cytosine methyltransferase. However, baculovirus-expressed Dnmt2 protein failed to methylate DNA *in vitro*. To investigate whether Dnmt2 functions as a DNA methyltransferase *in vivo*, we inactivated the *Dnmt2* gene by targeted deletion of the putative catalytic PPC motif in ES cells. We showed that endogenous virus was fully methylated in *Dnmt2*-deficient mutant ES cells. Furthermore, newly integrated retrovirus DNA was methylated *de novo* in infected mutant ES cells as efficiently as in wild-type cells. These results indicate that Dnmt2 is not essential for global *de novo* or maintenance methylation of DNA in ES cells.

INTRODUCTION

DNA methylation at the C-5 position of cytosine in CpG dinucleotides is the major form of DNA modification in vertebrate animals. DNA methylation has been shown to be essential for mammalian development as inactivation of Dnmt1, a major maintenance DNA cytosine methyltransferase, results in genome-wide demethylation and embryonic lethality (1,2). The function of DNA methylation has been implicated in a diverse range of biological processes. Molecular and genetic studies have demonstrated that DNA methylation plays critical roles in regulation of parent-origin-specific expression of imprinted genes (3–5) and X chromosome inactivation (6,7). Recently, growing evidence also suggests that DNA methylation is involved in carcinogenesis (8–10).

While the function of DNA methylation has been studied extensively, mechanisms by which DNA methylation is regulated and tissue specific DNA methylation patterns are established during development are poorly understood. The major obstacle has been the lack of information about the enzymes that catalyze *de novo* methylation and demethylation (11). The enzyme encoded by *Dnmt1* functions primarily as a maintenance methyltransferase which transfers methyl groups to cytosine in hemi-methylated CpG sites after DNA replication (12). Although this enzyme can also methylate unmethylated DNA *in vitro*, no evidence has been established so far for its role as a *de novo* methyltransferase *in vivo*. Recently, we showed that ES cells homozygous for a null mutation of *Dnmt1* contained residual levels of methyl cytosine and retained the ability to methylate provirus DNA *de novo* (2). This result provides the first genetic evidence for the existence of an independently encoded *de novo* DNA methyltransferase in mammalian cells.

In this study, we report the cloning of a mammalian gene *Dnmt2* that shares homology with the *pmt1* gene of fission yeast (13), and encodes a protein which contains all the conserved methyltransferase motifs. We provide genetic evidence that Dnmt2 is not essential for maintenance methylation nor for *de novo* methylation of viral DNA in ES cells.

MATERIALS AND METHODS

Cloning of the mammalian *Dnmt2* gene

A search of the dbEST database was performed with the TBLASTN program (14) using bacterial cytosine methyltransferases as queries. Two human EST clones (GenBank accession nos N31314 and R95731) were found to match the *M.HgiGI* sequences. The clones were obtained from American Type Culture Collection (ATCC, MD) and sequenced by the MGH sequencing core facility. The deduced amino acid sequences of the clones share a significant homology with the yeast *pmt1* (13). The insert DNA of these clones were cut out by *Eco*RI/*Not*I digestion and used as probes for screening cDNA libraries.

Nine positive clones were obtained by screening a mouse ES cell cDNA library (Clontech, CA) and sequenced. Two of them contained uninterrupted ORFs corresponding to the entire ORF of *pmt1*, but lacked a stop codon upstream of the first ATG. The human cDNA clones were obtained by screening a human heart cDNA library (Clontech, CA). Of 14 positive clones, one showed

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a continuous ORF with a stop codon upstream of the putative initiation codon.

RNA preparation and northern analysis

Total RNA was prepared from ES cells, ovary and testis using the GTC-$CsCl_2$ centrifugation method (15), fractionated on a formaldehyde denatured 1% agarose gel by electrophoresis, and transferred to nylon membranes. A poly A+ RNA blot of mouse tissues was obtained from Clontech, CA. All blots were hybridized with a random-primed 540 bp *Eco*RI–*Pst*I cDNA fragment of the mouse *Dnmt2* in a standard hybridization solution containing 50% formamide at 42°C, washed with 0.2× SSC, 0.1% SDS at 65°C, and exposed to X-ray film.

Construction of the gene targeting vector

A 14 kb *Sma*I–*Xho*I genomic DNA fragment of the *Dnmt2* gene was isolated from a 129/Sv genomic DNA library and subcloned into the pBluescript vector. A 1 kb *Stu*I–*Sna*BI genomic DNA fragment containing exons encoding the putative catalytic domain (the PPC motif) was removed and replaced by the IRES-βgeo cassette with a splicing accept site (16). The resulting gene targeting vector contains a 9.2 kb fragment upstream and a 3.7 kb fragment downstream of the IRES-βgeo cassette (Fig. 3).

Generation of Dnmt2-deficient mutant ES cell lines

Transfection of J1 ES cells with linearized targeting vector DNA and subsequent G418 selection and cloning of drug-resistant colonies were carried out as described previously (1). Genomic DNA from G418-resistant clones was digested with *Bam*HI and analyzed by Southern blot hybridization using the probe pXhR5 (Fig. 3). To generate ES cell lines homozygous for the mutation, cells of a heterozygous clone were subject to selection in medium containing a high concentration of G418 (0.5 mg/ml of pure form) (17).

Analysis of *de novo* and maintenance methylation of provirus DNA

Infection of wild-type and *Dnmt2*-deficient ES cells with the MoMuLV^{sup}-1 virus, DNA preparation from infected ES cells, analysis of *de novo* and maintenance methylation of endogenous or newly integrated viral DNA by Southern analysis of *Hpa*II/*Msp*I digested DNA were carried out as described previously (2).

RESULTS AND DISCUSSION

Cloning of the mammalian homologs of the yeast *pmt1* gene

One of the approaches that we took in search of a *de novo* DNA methyltransferase in mammalian cells was to screen the dbEST database using the amino acid sequences of different prokaryotic methyltransferases as query sequences (14). When sequences of the bacterial restriction methyltransferase *M.HgiGI* were used as query sequences, two EST clones of the same gene (GenBank accession nos N31314 and R95731) were found to give significant matches. Sequencing analysis of the EST clones revealed that they contained three of the highly conserved methyltransferase motifs. Multiple cDNA clones of the gene were isolated subsequently by screening human and mouse

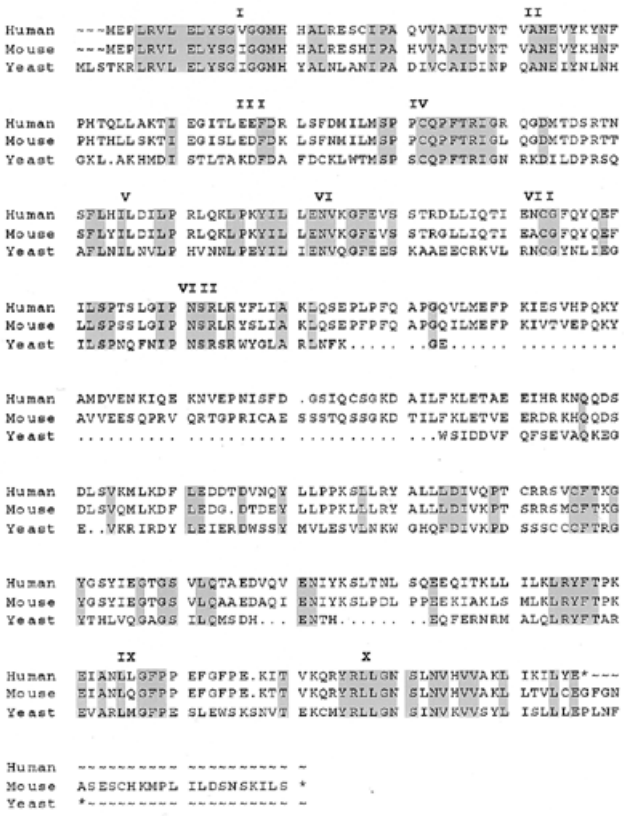

Figure 1. The comparison of the deduced amino acid sequences between the mammalian *Dnmt2* and the yeast *pmt1*. The identical amino acids are shadowed. The conserved DNA methyltransferase motifs (I–X) are marked with roman numerals. The stop codons are indicated with asterisks (*) and gaps with dots (...).

cDNA libraries using the EST clones as probes, and a full length cDNA was constructed with overlapping cDNA fragments after DNA sequencing. The deduced amino acid sequences of the human and mouse cDNA showed 81% identity and revealed that both genes contained all the conserved cytosine methyltransferase motifs (Fig. 1) (18). The same gene was independently cloned by Yoder and Bestor, and was named Dnmt2 (19). A BLAST search of GenBank with human and mouse cDNA sequences identified *pmt1* of fission yeast *Schizosaccharomyces pombe* as the most closely related sequences, sharing 42% identity at the amino acid level. The yeast *pmt1* contains all other conserved methyltransferase motifs except that the catalytic Pro-Pro-Cys motif was 'mutated' to Pro-Ser-Cys (13).

To determine whether Dnmt2 has methyltransferase activity, the mouse cDNA was expressed in *Escherichia coli* or in insect cells using the baculovirus expression system. Methyltransferase activity assay was carried out using either poly(dI-dC) or λ phage DNA as substrates under a standard assay condition which could detect residual levels of enzyme activity in protein extracts prepared from the *Dnmt1* null mutant ES cells (2). Despite the presence of large amounts of Dnmt2 protein in both bacterial and insect cell extracts, no methyltransferase activities were detected so far (data not shown). At the moment, it is not clear why the recombinant proteins have no detectable activities. The following two possibilities are considered: (i) the recombinant Dnmt2

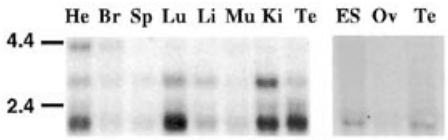

Figure 2. *Dnmt2* expression in organs and ES cells. A blot with 2 µg of poly A+ RNA from mouse tissues (Clontech, CA) is shown on the left, and a blot with 20 µg of total RNA from ES cells, ovary and testis is shown on the right. He, heart; Br, brain; Sp, spleen; Lu, lung; Li, liver; Mu, skeletal muscle; Ki, kidney; Te, testis; ES, ES cells; and Ov, ovary. Note that three *Dnmt2* transcripts of sizes 1.6, 2.6 and 4.0 kb were detected in mouse tissues, and the 1.6 kb transcript was the most abundant one in the organs examined.

protein may lack the natural conformation or modification, or is very unstable *in vitro*; (ii) Dnmt2 may require cofactors to catalyze methylation reaction. Further studies are necessary to investigate these possibilities.

Dnmt2 expression in mouse organs and ES cells

Dnmt2 expression in mouse ES cell lines and various organs were analyzed by northern hybridization using a full length cDNA fragment as probes. We showed that three *Dnmt2* transcripts of 1.6, 2.6 and 4.0 kb were detected in mouse tissues, and the 1.6 kb transcript was the most abundant one in most tissues examined (Fig. 2). *Dnmt2* appeared to express ubiquitously but at very low levels in mouse tissues, with relatively high levels in the heart, lung, kidney and testis (Fig. 2). *Dnmt2* expression was also detected in mouse ES cells (Fig. 2), suggesting that *Dnmt2* might be responsible for the residual methyltransferase activity detected in *Dnmt1* null ES cells.

Dnmt2-deficient ES cells are viable

To investigate the role of *Dnmt2* in development, we generated a putative null allele of *Dnmt2*, termed *Dnmt2*^{m1}, by deletion of the exons encoding the putative catalytic PPC motif through homologous recombination in ES cells (Fig. 3A). Of 85 G418-resistant colonies analyzed by Southern blot hybridization, six were positive for homologous recombination (Fig. 3B). To generate ES cell lines homozygous for the mutation, cells of a heterozygous ES cell line were cultured in medium containing a high concentration of G418 (0.5 mg/ml) for 14 days. Of 29 colonies analyzed, two were homozygous for the mutant allele (Fig. 3C). The Dnmt2 homozygous ES cells appeared to be normal in growth and morphology after consecutive passaging for more than 20 generations (data not shown), suggesting that Dnmt2 function is not essential.

De novo and maintenance methylation of provirus DNA in *Dnmt2*^{m1}/*Dnmt2*^{m1} ES cells

Since *Dnmt2* transcripts were detected in ES cells, we speculated that Dnmt2 might be required for *de novo* methylation. We showed previously that ES cells homozygous for a *Dnmt1* null mutation were able to methylate provirus DNA *de novo*. We carried out a similar analysis of *de novo* methylation of integrated provirus DNA in infected *Dnmt2* mutant ES cells.

First, we examined methylation status of endogenous virus in *Dnmt2*^{m1}/*Dnmt2*^{m1} ES. DNA isolated from wild-type and *Dnmt2*^{m1}/*Dnmt2*^{m1} ES cells was digested with the methylation-sensitive restriction enzyme *Hpa*II or its isoschizomer *Msp*I that cuts CCGG sequences regardless of whether CpG sites are methylated or not, and was then subject to Southern blot hybridization with a MoMuLV cDNA probe that hybridizes with

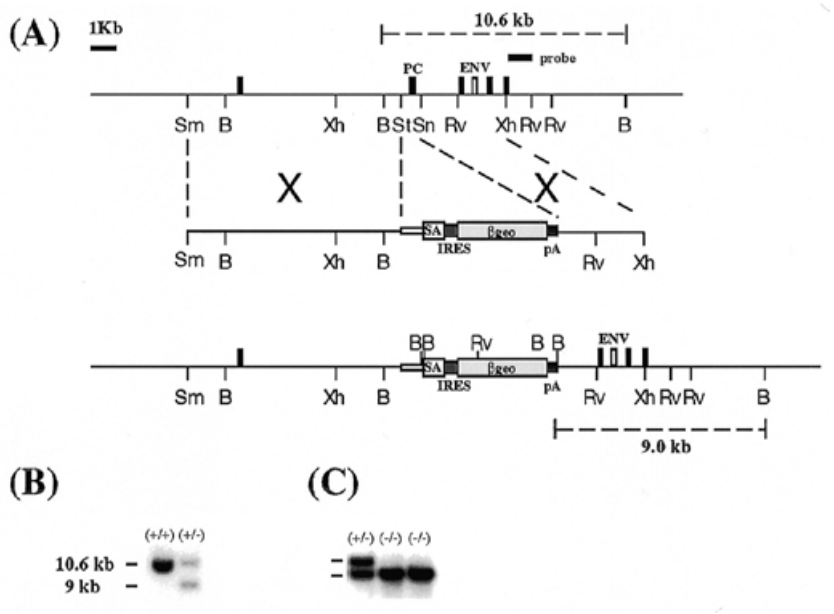

Figure 3. Targeted disruption of the *Dnmt2* gene. (**A**) The wild-type *Dnmt2* genomic locus (top), the targeting vector (middle), and the targeted allele (bottom). The location of the exons (solid bars), PC motif, ENV motif and the IRES-βgeo cassette are shown. The 1.3 kb *Xho*I–*Eco*RV genomic fragment was used as a probe for Southern analysis, and the 10.6 and 9.0 kb *Bam*HI fragments from wild-type and targeted alleles, respectively, are indicated as dashed lines. Sm, *Sma*I; B, *Bam*HI; Xh, *Xho*I; St, *Stu*I; Sn, *Sna*BI; Rv, *Eco*RV; SA, splicing acceptor; and pA, poly (A) signal. (**B**) Southern blot hybridization of genomic DNA from wild-type and targeted ES cell clones. DNA was digested with *Bam*HI, blotted and hybridized to the probe shown in (A). (**C**) Southern analysis of genomic DNA from a heterozygous and two homozygous mutant ES cell clones.

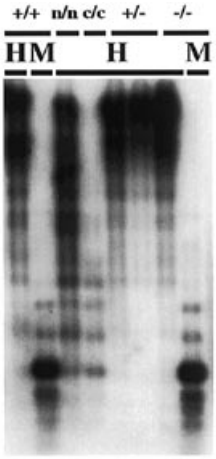

Figure 4. Methylation of endogenous provirus DNA in the *Dnmt2* null mutant ES cells. Genomic DNA was isolated from ES cells, digested with *Hpa*II (H) or *Msp*I (M), blotted and hybridized to the MoMuLV cDNA probe (1). +/– and –/– are *Dnmt2*^{m1}/+ and *Dnmt2*^{m1}/*Dnmt2*^{m1} cells while n/n and c/c are *Dnmt1*^{n}/*Dnmt1*^{n} and *Dnmt1*^{c}/*Dnmt1*^{c} ES cells, respectively (2).

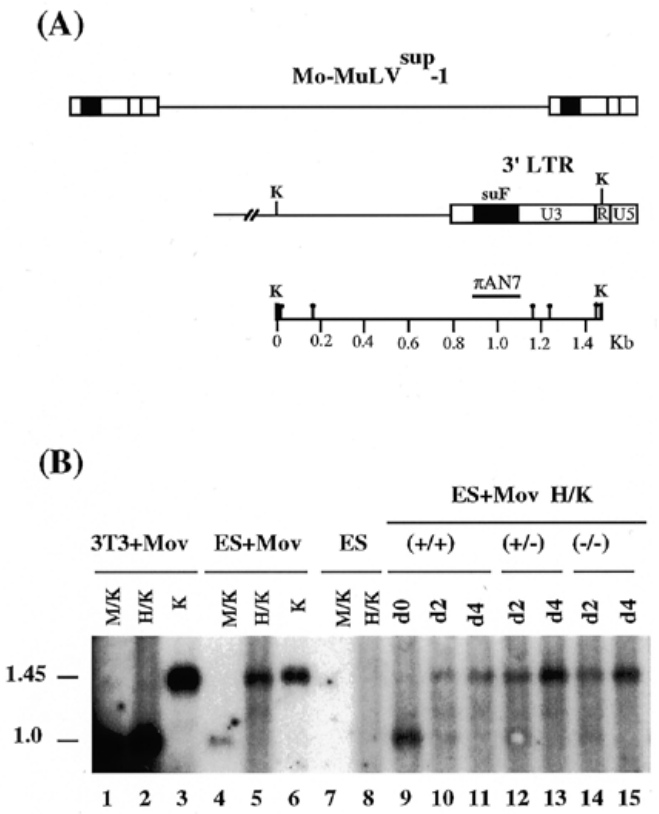

Figure 5. *De novo* methylation of provirus DNA in *Dnmt2* mutant ES cells. (**A**) Schematic diagrams of the MoMuLV^{sup}-1 provirus genome (top), the 3′ LTR region (middle), the size marker, the location of the πAN7 probe and the five *Hpa*II/*Msp*I sites (bottom) (2). (**B**) Genomic DNA was isolated from infected 3T3 cells (lanes 1–3), infected wild-type (lanes 4–6 and 9–11), uninfected wild-type (lanes 7 and 8), infected heterozygous mutant (lanes 12 and 13) and infected homozygous mutant (lanes 14 and 15) at day 0 (lane 9), day 2 (lanes 10, 12 and 14) and day 4 (lanes 4–6, 11, 13 and 15) post-infection. DNA was digested with *Msp*I/*Kpn*I (lanes 1, 4 and 7), *Hpa*II/*Kpn*I (lanes 2, 5 and 8–15), or *Kpn*I alone (lanes 3 and 6), blotted and hybridized to the πAN7 probe. Mov, MoMuLV^{sup}-1 virus infected; M, *Msp*I; H, *Hpa*II; K, *Kpn*I.

endogenous provirus DNA (1). We showed that endogenous virus DNA in *Dnmt2*^{m1}/*Dnmt2*^{m1} ES cells was methylated to the same levels as in wild-type cells (Fig. 4). This result indicates that Dnmt2 is not required for the maintenance methylation of genomic DNA.

To examine whether *Dnmt2*^{m1}/*Dnmt2*^{m1} ES cells were able to methylate foreign DNA such as newly integrated provirus DNA, we infected *Dnmt2* mutant ES cells with the MoMuLV^{sup}-1 retrovirus and analyzed the methylation status of newly integrated provirus DNA 2–4 days after infection. DNA was digested with *Kpn*I and *Hpa*II, or with *Kpn*I and *Msp*I as controls, and analyzed by Southern blot hybridization using the πAN7 probe that would recognize a 1.45 kb *Kpn*I fragment of infected viral DNA but not the endogenous proviruses (Fig. 5A). We found that the newly integrated virus DNA was methylated in *Dnmt2*^{m1}/*Dnmt2*^{m1} ES cells as efficiently as in wild-type cells as shown by the presence of an *Hpa*II-resistant 1.45 kb fragment (Fig. 5B), indicating that Dnmt2 is not an essential component of the *de novo* methyltransferases.

The lack of detectable methyltransferase activities *in vitro* and *in vivo* raises interesting possibilities that *Dnmt2* might encode a sequence-specific DNA methyltransferase which methylates only a small number of target sequences in the genome, or it may methylate cytosine in non-CpG sequences such as CpNpG. It is also possible that Dnmt2 is simply not a functional cytosine DNA methyltransferase, despite having all the conserved DNA methyltransferase motifs. Dnmt2 may be involved in cellular processes other than DNA methylation, such as DNA repair by binding to mismatched nucleotides as the bacterial cytosine methyltransferases (21–23), DNA recombination and carcinogenesis.

Since *Dnmt2* is not essential for *de novo* methylation in ES cells, additional DNA methyltransferases that catalyze *de novo* methylation are predicted to be present in mammalian cells. It is formally possible that both Dnmt1 and Dnmt2 are *de novo* methyltransferases and can functionally compensate each other. Recently, a gene known as *masc1* was cloned through homology-based screening using a PCR amplification method, and genetic analysis has revealed that *masc1* is involved in *de novo* methylation in *Ascobolus* (24). The protein encoded by *masc1* contains all the conserved methyltransferase motifs except that motif VI has an EET sequence rather than the ENV sequence that is conserved in almost all the known DNA cytosine methyltransferases. The methyltransferase activity of *masc1* encoded proteins has not been reported. Sequence analysis indicates that *masc1* is distantly related to *Dnmt1* and *Dnmt2* (data not shown). It remains to be seen whether a mammalian homologue of *masc1* exists, and whether it functions as a *de novo* DNA methyltransferase.

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