The Eukaryotic DNMT2 Genes Encode a New Class of Cytosine-5 DNA Methyltransferases*

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DNMT2 is a subgroup of the eukaryotic cytosine-5 DNA methyltransferase gene family. Unlike the other family members, proteins encoded by DNMT2 genes were not known before to possess DNA methyltransferase activities. Most recently, we have shown that the genome of Drosophila S2 cells stably expressing an exogenous Drosophila dDNMT2 cDNA became anomalously methylated at the 5′-positions of cytosines (Reddy, M. N., Tang, L. Y., Lee, T. L., and Shen, C.-K. J. (2003) Oncogene, in press). We present evidence here that the genomes of transgenic flies overexpressing the dDNmt2 protein also became hypermethylated at specific regions. Furthermore, transient transfection studies in combination with sodium bisulfite sequencing demonstrated that dDNmt2 as well as its mouse ortholog, mDNmt2, are capable of methylating a cotransfected plasmid DNA. These data provide solid evidence that the fly and mouse DNMT2 gene products are genuine cytosine-5 DNA methyltransferases.

Methylation of the vertebrate genomes at cytosines is known to be accomplished by the combined actions of proteins encoded by different cytosine-5 DNA methyltransferases or DNA MTases. These proteins include the de novo DNA MTases Dnmt3a and Dnmt3b, the maintenance DNA MTase Dnmt1, and their isomers (1–4). There are also other DNA MTase-like proteins expressed in the eukaryotic cells, but they are without well documented DNA methylation activities. These include the Dnmt3L (5) and Dnmt2 proteins.

The Dnmt2 proteins are relatively shorter than Dnmt3a, Dnmt3b, or Dnmt1, and structurally they are similar to the bacteria dcm enzyme (6). The eukaryotic Dnmt2 protein family consists of the yeast pmt1 (7), the mammalian Dnmt2 including mouse mDnmt2 and human hDnmt2 (8, 9), and Drosophila dDnmt2 (10, 11). Of the Dnmt2 proteins known, pmt1 has been demonstrated to be enzymatically inactive due to amino acid change at a potential catalytic site (12). Sequence analysis showed that mDnmt2, hDnmt2, and dDnmt2 all contain the conserved DNA MTase motifs (8–11). DNA methylation analysis of ES cells with homozygous knock-out of the mouse mDnmt2 genes suggested that mDnmt2 protein might also be an inactive DNA MTase (8). Finally, there has been no report on the DNA methylation activity of the Drosophila dDnmt2 until very recently. By overexpression of dDnmt2 in Drosophila S2 cells and subsequent analysis of the S2 cell genome with the sodium bisulfite sequencing approach, it was shown that specific regions were anomalously methylated in comparison to S2 cells without overexpression of the dDnmt2 protein (13).

To avoid potential side effects resulting from use of the long term-selected S2 cell culture in the above study, we have now examined the genome of transgenic Drosophila flies stably overexpressing dDnmt2. Interestingly, specific genomic regions of the transgenic flies were also found to be anomalously hypermethylated. To complement the fly analysis, we further carried out DNA transfection experiments to transiently express fly dDnmt2 or mouse mDnmt2 in S2 cells. As shown below, dDnmt2 as well as mDnmt2 are capable of methylating cytosines of a cotransfected plasmid. The conservation of the enzymatically active Dnmt2 proteins from mammals to flies suggests that this DNA MTase subfamily likely carries out important and to-be-identified function(s).

EXPERIMENTAL PROCEDURES

Plasmid Constructs—For transgenic fly work, the dDNMT2 cDNA was released from pGEM(1)-dDNMT2 with PacI-NcoI and blunt ended. This dDNMT2 fragment, which contains bp 6984–7024 linked to 7074–8051 of the dDNMT2 gene, was cloned at the EcoRI site of the polylinker of pUAST (14), and the resulting plasmid, pUAST-dDNMT2, was used for germ line transformation. For transient transfection, pAC5.5V5-HisA vector (Invitrogen) containing Drosophila actin promoter was digested with KpnI, blunt ended, and regested with EcoRI. pSG424 plasmid (15) was cut with BglIII, blunt ended, and the GAL4 DNA binding domain-containing fragment was released by EcoRI digestion. This fragment was ligated to the KpnI-EcoRI-digested pAC5.5V5-HisA, resulting in pAC-GAL. For construction of pAC-GAL-dDNMT2 fusion plasmid, pGEM(2)-dDNMT2 was digested with Apol, blunt ended, and the dDNMT2 fragment was released by EcoRI digestion. The fragment was ligated to pAC-GAL digested with EcoRI-SmaI. For construction of pAC-GAL-mDNMT2, full-length mDNMT2 cDNA was amplified from total RNA of mouse MEL cells by reverse transcriptase-PCR and cloned into pC DNA-3.1 HisC vector (Invitrogen), resulting in pC DNA-mDNMT2. The mDNMT2 fragment was released from pC DNA-mDNMT2 by EcoRI-NolI digestion and ligated to EcoRI-NolI digested pAC-GAL. The target plasmid, pGEM-CS5347-p, contains the Drosophila CS5347 promoter region cloned into pGEM-T vector (Promega) following the manufacturer's guidelines. The details of construction of pGEM(1)-dDNMT2 and pGEM(2)-dDNMT2 are available upon request.

Antibodies—Anti-dDnmt2 antibody was prepared as described in Reddy et al. (13). dDnmt2-specific antibody was purified by passing the serum through dDnmt2-coupled CNBr-Sepharose column (Amersham Biosciences). The anti-gal mouse IgG monoclonal antibody, anti-mouse IgG, and anti-rabbit IgG secondary antibodies were from Santa Cruz Biotechnology Inc.

Fly Stocks and Transgenic Flies Overexpressing dDNMT2—Oregon R (OR) was used as the wild-type standard. The maintenance and manipulation of the Drosophila stocks were performed at 25 °C with standard cornmeal-yeast-agar food. For overexpression experiments, pUAST-dDNMT2 transgenic lines were established and crossed with daughterless-Gal4 (da-GAL4) driver that provides ubiquitous overexpression of the dDNMT2 protein.

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S2 Cell Culture and Cotransfection—Drosophila S2 cells were maintained in monolayers at 24 °C in Schneider’s medium (Sigma), pH 6.9, supplemented with CaCl₂ (0.6 mg/ml), NaHCO₃ (0.4 mg/ml), 10% heat-inactivated fetal bovine serum (Invitrogen), and antibiotics (25 units/ml penicillin and 25 μg/ml streptomycin, Invitrogen). Transient transfection of S2 cells was performed by the CaPO₄ precipitation method. S2 cells at a density of 1 × 10⁶ cells/ml in 5 ml were seeded into T 25-cm² flasks (Nunclon) and allowed to adhere to the surface for 12 h. 3 h before transfection, the medium was replenished. The calcium phosphate-DNA precipitate was prepared by mixing 5 μg of the expression plasmids (pAC-GAL, pAC-GAL-dDNMT2, or pAC-GAL-mDNMT2) and 5 μg of the target plasmid (pGEM-CG8547-p) in 225 μl of 0.1× TE (1 mm Tris-HCl, pH 8.0, and 0.1 mm EDTA), 25 μl of 2.5× CaCl₂, and 250 μl of 2× HEPES-buffered saline, pH 7.1. The precipitate was added dropwise to the cultures. After 12 h of incubation, the medium was replaced, and incubation continued for another 48 h.

Protein Isolation and Western Blot—The Drosophila protein extracts were prepared by homogenizing embryos or adult flies in 0.4 ml of 50 mm Tris-HCl, pH 8.0, 100 mm NaCl, 5 mm EDTA, 0.1 mm dithiothreitol, 0.5% Triton X-100, 0.2% protease inhibitor mixture (Sigma), and 1 mm phenylmethylsulfonyl fluoride. The lysates from S2 cells transiently transfected with pAC-GAL, pAC-GAL-dDNMT2, or pAC-GAL-mDNMT2 were prepared by lysis in the same buffer as above but with three freeze-thaw cycles. The lysates were centrifuged at 10,000 × g for 30 min. The protein concentrations in the supernatants were determined using Bio-Rad protein assay kit. 50 μg per lane of the extracts were loaded on SDS-PAGE gel and transferred onto nitrocellulose membrane by semidy transfer method. The membranes were probed with anti-Dnmt2 antibody or with anti-gal mouse monoclonal antibody. The hybridizing bands were developed with the enhanced chemiluminescence Western blotting detection system (PerkinElmer Life Sciences).

DNA Isolation for Sodium Bisulfite Genomic Sequencing—Drosophila embryos or adult flies were homogenized in 20 ml Tris-HCl, pH 8.0, 100 mm NaCl, 10 mm EDTA, 8.5% SDS, and 10 μg/ml proteinase K, and incubated at 50 °C overnight. The DNA was extracted with phenol: chloroform:isoamyl alcohol and then precipitated with isopropanol. After resuspension in H₂O containing 1 μg/ml RNase A, the samples were incubated at 50 °C for 30 min and then stored overnight at 4 °C before use.

To prepare nuclear DNA from transfected S2 cells, the cells were incubated in 10 ml Tris-HCl, pH 8.0, 140 mm NaCl, 1.5 mm MgCl₂, and 0.5% Nonidet P-40 (v/v) on ice for 10 min. The lysed cells were centrifuged at 1600 × g for 5 min. The pelleted nuclei were washed twice with phosphate-buffered saline and lysed in 0.5 ml of TRIzol (Invitrogen). The lysates were mixed with 0.2 ml of chloroform and incubated at room temperature for 10 min. The aqueous phase was separated by centrifugation at 10,000 × g for 10 min. The DNA was then precipitated with ethanol, dissolved in 1× TE, pH 8.0, and used for bisulfite sequencing.

Bisulfite Genomic Sequencing—The sodium bisulfite genomic sequencing followed the procedures used by Warnecke et al. (16) with modifications as described in Reddy et al. (13). The PCR products from the second round of amplification were gel-purified, subcloned into pGEM-T (Promega), and subjected to automated sequencing in ABI 3730 DNA analyzer (Applied Biosystems).

RESULTS AND DISCUSSION

Anomalous Genome Hypermethylation of dDnmt2-Overexpressing Flies—dDnmt2 gene is located on Drosophila chromosome 2L and cytogenetically mapped to 33C4. After splicing, its mRNA encodes a protein 345 amino acids long (Fig. 1A). To examine whether overexpression of the dDnmt2 protein leads to deregulated methylation or hypermethylation of the fly genome, pUAST-dDNMT2 transgenic fly lines were established and crossed with da-GAL4 as described under “Experimental
The overexpression of dDnmt2 protein was determined by immunoblotting with anti-dDnmt2. Under the conditions used, little dDnmt2 could be detected in the extracts from embryos and adults of the wild-type flies (lanes 1 and 3, Fig. 1B). On the other hand, the embryos and adult flies of the dDnmt2 transgenic line OR(dDNMT2) contain apparently overexpressed dDnmt2 protein (lanes 2 and 4, Fig. 1B).

We isolated genomic DNAs from embryos and adult flies of both the wild-type and OR(dDNMT2). The DNA samples were then subjected to bisulfite genomic sequencing. Typical fluorograms of the sequencing results are exemplified in Fig. 1C, and the data of the methylation status of two of the genomic regions analyzed, CG8547 and CG8553, are summarized in Table I.

It has been shown previously that 0.1–0.6% of the cytosines of the fly genome are methylated, with the methylation level highest in the early embryonic stage (17, 18). As shown in Table I, overexpression of dDnmt2 in the flies resulted in deregulated hypermethylation of both CG8547 and CG8553. In particular, the sequencing data of 20 clones derived from the 6 h embryos of the wild type indicate that the region is void of m5C. However, the sequences of 10 out of the 24 clones from the same region of the 6 h embryos of OR(dDNMT2) showed hypermethylated in the genome of this dDnmt2-overexpressing line (Table I). Similarly, CG8553 of the 2-h embryos of OR(dDNMT2) is hypermethylated in comparison with the wild type embryos. Both the embryos and adults of OR(dDNMT2) appear to have more m5C within this region than the wild type (Table I). In particular, 7 out of 20 clones from the 2-h embryos of OR(dDNMT2) have identical hypermethylated pattern as shown partly in Fig. 1C.

Both dDnmt2 and mDnmt2 Generated m5C on Cotransfected Plasmid DNA in Drosophila Cells—The Drosophila S2 cell line has been used recently in a stable transfection assay to demonstrate methylation of the fly genome by dDnmt2 (13). To double check potential side effects that might be caused by long-term cell culturing in the above assay, we carried out transient transfection studies with S2 cells. A series of plasmids, including pAC-GAL, pAC-GAL-dDNMT2, and pAC-GAL-mDNMT2, were constructed previously for the test of potential repressor activities of dDnmt2 and mDnmt2. We have used these plasmids to see whether transient expression of gal-dDmnt2 or gal-mDnmt2 fusion would methylate a cotransfected plasmid.
As described under “Experimental Procedures,” the three expression plasmids were individually cotransfected with the target plasmid pGEM-CG8547-p. The expression of either gal-dDnmt2 or gal-mDnmt2 could be easily detected by Western blot (Fig. 2A). At 60 h post-transfection, the nuclei of the transfected cells were purified. The nuclear DNA samples containing mainly the plasmids transported into the nuclei were isolated and subjected to sodium bisulfite sequencing using appropriate primers specific for the insert of pGEM-CG8547-p (Fig. 2B). As exemplified in Fig. 2C, none of the 15 DNA clones from the nuclei expressing gal contained m5C-derived C bases (panel I, Fig. 2C). In contrast, the DNA sequences of 6 out of 21 and 6 out of 24 clones derived from the nuclei expressing gal-dDnmt2 and gal-mDnmt2, respectively, indicated that the DNA insert of the target plasmid was methylated by these two proteins in transiently transfected S2 cells. As exemplified in panels II–IV and V–VII, respectively, of Fig. 2C, dDnmt2 and mDnmt2 caused similar and overlapping of C-methylation of the target plasmid. The data demonstrated that both dDnmt2 and mDnmt2 possess C-5 methyltransferase activities.

The Dnmt2 Proteins of Mammals and Flies Are Cytosine-5 DNA MTases—In this study, we have provided further evidence supporting that Drosophila dDnmt2 protein is indeed a DNA MTase. It not only generated deregulated hypermethylation of the genome of stably transfected Drosophila cell line (13), but it is also active, albeit as a gal fusion polypeptide, in the current transient transfection assay. In addition, it caused deregulated hypermethylation of specific regions of the genomes of transgenic flies overexpressing the protein. Most interestingly, the mouse ortholog of the fly enzyme, mDnmt2, also methylates DNA in the transient transfection assay. It should be noted here that CG dinucleotide is not the primary target of methylation by the Dnmt2 proteins, as in the case of the other eukaryotic DNA MTases.

Our data are consistent with several previous studies implicating that, except for yeast pmt1 (7, 12), the Dnmt2 protein family members might be active DNA MTases. First, the functional motifs conserved among the well known DNA MTases such as Dnmt1 are also conserved in dDnmt2 and mammalian Dnmt2 (8–11). Second, the human Dnmt2 protein binds tightly to DNA, and in crystals, its complex with DNA adopts a conformation similar to the protein-DNA complex of a bacterial C-methylating enzyme HhaI (19). Third, the mammalian Dnmt2 proteins, as well as other known active DNA MTases, are attached to 5′-azacytidine-incorporated DNA (20).

While proposing that the eukaryotic DNMT2 gene family encodes active DNA MTases, we note here that mouse with homozygous knock-out of the mDnmt2 gene has no obvious phenotype. Similarly, we have not yet noticed obvious phenotype of the dDnmt2-overexpressing flies. Thus, the DNMT2 gene is not essential for the growth and development of the flies and mice. Identification of the target genes regulated by the Dnmt2 proteins could be the first step toward understanding the biological functions of this new class of eukaryotic DNA MTases.

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Note Added in Proof—The human Dnmt2 protein has recently been shown to methylate DNA in vitro (Hermann, A., Schmitt, S., and Jeltsch, A. (2003) J. Biol. Chem., in press).

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