Characterization of the Human DNA Methyltransferase Splice Variant Dnmt1b* 

Claire Bonfils‡, Normand Beaulieu‡, Eric Chan, Judith Cotton-Montpetit, and A. Robert MacLeod§

From MethylGene Inc., Montreal, Quebec H4S 2A1, Canada

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In mammals, modification of the 5′-position of cytosine by methylation is the only known naturally occurring covalent modification of the genome. DNA methylation patterns correlate inversely with gene expression (1). Therefore, DNA methylation has been suggested to be an epigenetic determinant of gene expression. DNA methylation has been correlated with several other cellular processes, including chromatin structuring (2, 3), genomic imprinting (4, 5), somatic X-chromosome inactivation in females (6), and timing of DNA replication (7). The enzyme DNA 5-cytosine methyltransferase (DNA MeTase)1 catalyzes the transfer of a methyl group from S-adenosyl methionine to the 5′-position of cytosines residing in the dinucleotide sequence CpG (8). Thus far three DNA MeTases have been identified in somatic tissues of vertebrates. Dnmt1 is the most abundant DNA MeTase in mammalian cells (9). Dnmt1 preferentially methylates hemimethylated DNA as its substrate and therefore is believed to be primarily responsible for maintaining methylation patterns established in development (10). The recently identified DNA MeTase enzymes, Dnmt3a and Dnmt3b, have been suggested to encode the long sought de novo methylation activities responsible for methylating previously unmethylated DNA, to generate new patterns of DNA methylation (11).

DNA methylation patterns are highly plastic throughout development and involve both global demethylation and de novo methylation events (see Ref. 12 for review). Genetic experiments have demonstrated that proper regulation of DNA methylation is essential for normal mammalian development. Mice homozygous for the targeted disruption of Dnmt1 (Dnmt1+/– mice) fail to maintain established DNA methylation patterns and do not survive past mid gestation (13). Similarly, the Dnmt3b+/– genotype produces embryonic lethality in mice, whereas Dnmt3a+/– mice develop to term but become runted and die at approximately 4 weeks of age (14).

In addition to the role the DNA methylation plays in development, it is also implicated in tumorigenesis (see Ref. 15 for review). Abnormal methylation patterns are observed in malignant cells and may contribute to tumorigenesis by improper silencing of tumor suppressor genes or growth-regulatory genes (16). Elevated levels of Dnmt1 mRNA and DNA MeTase activity have been observed in many cancer cells in vitro (17) and tumors in vivo (18, 19). Activation of the oncogenic ras signal transduction pathway has been shown to induce Dnmt1 expression (20, 21). In addition, elevated Dnmt1 levels are required to maintain the phenotype of these ras-transformed cells, suggesting that Dnmt1 is an important downstream target of these pathways (22). This is supported by the finding that increased Dnmt1 levels are required to maintain the phenotype of fibroblasts transformed with the fos oncogene (23). Significant overexpression of Dnmt3b has also recently been demonstrated in human tumors suggesting that deregulation of enzymes modulating distinct aspects of DNA methylation may lead to tumorigenesis (11).

The DNA MeTase enzymes, Dnmt1, Dnmt3a, and Dnmt3b

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1 The abbreviations used are: DNA MeTase, DNA cytosine-5 methyltransferase; RPA, RNAse protection analysis; PBMCs, peripheral blood mononuclear cells; RT-PCR, reverse transcriptase-polymerase chain reaction; NOS1, nitric oxide synthase; VEGF, Vascular endothelial growth factor; kb, kilobase(s); bp, base pair(s).

† Contributed equally to this work.
‡ To whom correspondence should be addressed. MethylGene Inc., 7220 Frederick Banting, Montreal, Quebec H4S 2A1, Canada. Tel.: 514-337-3333 (ext. 241); Fax: 514-337-3550; E-mail: macleod@ methylgene.com.
are encoded by independent genes (24). Diversity of mammalian DNA MeTases has been shown to result from alternative translation initiation in the case of the murine Dnmt1 (25). Additional DNA MeTase diversity has been suggested to arise from alternative splicing of the Dnmt1 mRNA. Dnmt1 mRNA splice variants have been observed in rat (26) and more recently in human cells (27). Alternative splicing of human the Dnmt1 gene could potentially produce a large number of DNA MeTase protein isoforms because it is composed of at least 40 exons and 39 introns spanning 60 kilobases (kb) (28). The Dnmt1 catalytic domain alone is composed of 11 exons (28). It has been suggested that alternative splicing in this region could generate DNA MeTase isoforms with altered sequence specificity (26). However, in both cases that reported alternative splicing of the Dnmt1 gene, characterization of the splice variants was limited to the RNA level and as such the existence of Dnmt1 protein isoforms has not been demonstrated.

During the course of determining Dnmt1 levels in human peripheral blood mononuclear cells (PBMCs), we documented the presence of a splice variant of the primary Dnmt1 transcript. The existence of this splice variant in human cells has since been reported by another group (27).

Here we report the quantification of this novel Dnmt1b transcript in human cells as determined by RNase protection analysis (RPA), the characterization of kinetic properties and substrate specificities of the baculovirus-expressed Dnmt1b protein, the sensitivity of this transcript to antisense inhibitors targeting the 5'- and 3'-positions of the primary Dnmt1 transcript, and the quantification of Dnmt1b protein levels in human cells. The data presented in this study demonstrate that Dnmt1b represents a fairly abundant Dnmt1 splice variant at the mRNA level yet encodes a DNA MeTase protein isoform that represents a minor component of cellular DNA MeTase activity.

**EXPERIMENTAL PROCEDURES**

**Reverse Transcriptase-Polymerase Chain Reaction Analysis—**Reverse transcriptase (RT) reactions were performed with SuperScript (Life Technologies, Inc.) according to the manufacturer's instructions, using 5 µg of total RNA and a specific downstream primer. For polymerase chain reactions (PCRs), one tenth of the reverse transcription reaction volume was used in a 100-µL reaction containing 400 nm of each primer, 200 µM of each nucleotide, and 5 U of Pfu polymerase (Stratagene). Reactions were carried out for 30 cycles of denaturing (94 °C for 45 s), annealing (55 °C for 45 s), and extension (72 °C for 4.5 min). Several pairs of primers, scanning the whole open reading frame, have been used. Only one pair revealed a doublet; primer 81, 5'-CAG-GAACTCACCACATACTT3', which anneals from base 1785 to 1765 of the sequence X63692, combined with primer 80, 5'TGGCTAAAGT-CAAATCCCTTTT3', which anneals from base 473 to 494. Each band of the doublet has been separately cloned into pC RosScript (Stratagene) and sequenced by BioS&T (Pointe-Claire, Quebec).

**RNAse Protection Analysis—**Sequence from nucleotides 538 to 752, containing the 48 nucleotides specific to Dnmt1b, was subcloned into pC RosScript (Stratagene), in antisense orientation with respect to the Dnmt1 gene, characterization of the splice variants was limited to the RNA level and as such the existence of Dnmt1 protein isoforms has not been demonstrated.

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**RESULTS AND DISCUSSION**

**Identification and Quantification of a Dnmt1 Splice Variant in Human Cells—**Dnmt1 has been shown to regulate the expression of tumor suppressor genes in cancer (31) and as such is suggested to be a novel target for the treatment of cancer (32). Treatment of human cancer cells with nanomolar concentrations of second generation Dnmt1 antisense molecules inhibits the expression of both Dnmt1 mRNA and protein, induces the expression of the cell cycle regulators p16Ink4a and p21Waf1/Cip1, and dramatically affects proliferation of human cancer cells (31). The human DNA MeTase inhibitor MG98 is a potent second generation (2'-O-methyl-modified phosphorothioate) antisense oligonucleotide currently undergoing phase I clinical evaluation for treatment of cancer in humans. As part of the clinical evaluation of MG98, we wished to determine DNA MeTase mRNA levels in peripheral blood mononuclear cells isolated from treated patients. To this goal, we developed a quantitative RT-PCR assay to monitor Dnmt1 mRNA levels in the freshly isolated PBMCs from patients. The Dnmt1 gene codes for a protein with many conserved motifs (Fig. 1A). During the development of our RT-PCR assay, we tested primers spanning several regions of the Dnmt1 gene. To control for potential contamination of genomic DNA, we chose primers spanning exons. Amplification with primers 80 and 81 (see “Experimental Procedures”) consistently produced two products of 1.31 and 1.36 kb in length, respectively, when resolved on agarose gels (Fig. 1B). Both RT-PCR products were present in RNA prepared from various cell lines (Fig. 1B, left) and from freshly isolated human PBMCs (Fig. 1B, right). Both products were derived from mRNA and not from genomic DNA templates, because omission of the reverse transcriptase or RNA template from the amplification reaction did not produce either band (Fig. 1B). These two RT-PCR products were cloned into plasmid vectors for sequencing. The sequence of the smaller 1312-base pair (bp) product corresponded perfectly to the published Dnmt1 cDNA (GenBank™ accession no. X63692). However, the larger 1360-bp product was found to
contain an extra 48 bp between bases 682 and 683 of the known Dnmt1 sequence. This position corresponds to a splice acceptor site between exons 4 and 5 (28). This insert was determined to be in-frame and to theoretically code for an alternate exon to generate a DNA MeTase with an additional 16 amino acids in this region (Fig. 1C). Hsu et al. (27) independently reported the identification of this novel Dnmt1 splice variant and named it Dnmt1b. Amplification with other primer pairs flanking introns 17 through 32 resulted in only one PCR product of the size expected from the Dnmt1 sequence, consistent with previous findings (data not shown) that suggested that alternative splicing between exons 4 and 5 was a unique event. The two target mRNA molecules Dnmt1 and DNmt1b were amplified by the same primers and the products were of similar size; therefore, they compete with each other during the amplification process. Under such circumstances, quantification is only reliable when competitor (Dnmt1 in this case) and driver (Dnmt1b in this case) are of nearly equal abundance (33, 34). Therefore, to verify that the identification of the Dnmt1 splice variant was not a consequence of a flaw in our PCR-based assay, and to quantify the relative abundance of Dnmt1b mRNA to Dnmt1 in human cells, we designed an RNase protection analysis strategy capable of simultaneously detecting Dnmt1 mRNA and the Dnmt1 splice variant Dnmt1b. As shown schematically in Fig. 2A, the region of the RNA probe complementary to the Dnmt1b sequence was completely protected by endogenous Dnmt1b transcripts yielding a protected radiolabeled RNA molecule 263 nucleotides in length. However, Dnmt1 mRNA could not completely protect the RNA probe and could be cleaved by the RNase treatment into two products 145 and 70 nucleotides in length. To validate this approach we generated baculovirus expression constructs de-
signed to produce either Dnmt1 or Dnmt1b in High Five (Hi5) insect cells. We extracted total cellular RNA from Hi5 cells infected with baculovirus constructs expressing either Dnmt1 or Dnmt1b and used these as controls in our Dnmt1/Dnmt1b RNase protection analysis (Fig. 2B). RNA from Hi5 cells expressing Dnmt1b completely protected the RNA probe, resulting in the protected product of 263 bases, with only the region corresponding to vector sequences being digested (Fig. 2B). Conversely, RNA from Hi5 cells infected with Dnmt1 expression constructs produced the two predicted fragments of the RNA probe with the expected sizes of 145 and 70 bases (Fig. 2B).

The relative abundance and tissue specificity of Dnmt1b in human cells was determined by employing our RNase protection analysis on total cellular RNA isolated from a variety of human cancer cell lines, including colon, lung, kidney, breast, and bladder cancers; the normal skin fibroblast line MRHF; and RNA from freshly isolated human PBMCs. All the human cell lines tested and the PBMCs had detectable levels of Dnmt1b mRNA (Fig. 3A). The relative abundance of Dnmt1b to Dnmt1 was determined by densitometric quantification of the signal corresponding to the upper Dnmt1b RNase-protected band (D1) relative to the densitometric signal for the 145-base Dnmt1-specific RNase-protected band (D2). All signals were normalized to account for size of the molecular species giving rise to the band by the formula: (D1/263)/(D2/145). The relative abundance of Dnmt1b mRNA ranged from 6% of the level of Dnmt1 in T24 human bladder cancer cells to between 20% and 30% in the breast cancer cell line MDA-MB-231 (Fig. 3A, middle). These results differ significantly from those obtained by Hsu et al. (27), where Dnmt1b expression levels were estimated by RT-PCR to range from 40% to 70% of Dnmt1 mRNA levels in human cells (27). The finding that Dnmt1b is present in human cancer lines, normal cell lines, and primary cells strongly suggests that it represents a ubiquitously expressed transcript.

To determine whether the Dnmt1b signal could have resulted from hybridization with a splicing intermediate and not from a mature transcript, we compared the levels of Dnmt1b transcript in nuclear extracts to those obtained from cytosolic extracts from the highest expressing cell line MDA-MB231 (Fig. 3B). Enrichment of Dnmt1b in the nuclear fraction would suggest that it may represent an immature splicing intermediate. The level of Dnmt1b relative to Dnmt1 was similar in both cytoplasmic and nuclear fractions (Fig. 3B), demonstrating that the Dnmt1b signal does not arise from prespliced heteronuclear RNA and that it represents a mature DNA Metase transcript.

**Dnmt1b Transcript Is Inhibited by Antisense Oligonucleo-"
Fig. 3. A, Dnmt1/Dnmt1b RNase protection analysis of human cell lines (cancer and normal, middle), freshly isolated human PBMCs (right), and recombinant baculovirus-expressed controls (left). B, nuclear versus cytoplasmic distribution of Dnmt1 and Dnmt1b mRNA. C: top, dose-dependent inhibition of both Dnmt1 and Dnmt1b by the Dnmt1 antisense inhibitors MG88 and MG98 but not mismatch controls MG208 and MG207; bottom, densitometric quantification of Dnmt1b RNA levels relative to Dnmt1.
tides Targeting Both 5’ and 3’ of the Dnmt1 mRNA—As mentioned above, we have developed the potent Dnmt1 second generation antisense inhibitors, MG98 and MG88. These chemically modified 20-mer oligonucleotides target the 5’ and 3’ extremities of the Dnmt1 mRNA, respectively. To determine the effect of specific reduction of Dnmt1 levels on Dnmt1b extremities of the Dnmt1 mRNA, respectively. To determine the effect of specific reduction of Dnmt1 levels on Dnmt1b extremities of the Dnmt1 mRNA, respectively. To determine the effect of specific reduction of Dnmt1 levels on Dnmt1b extremities of the Dnmt1 mRNA, respectively. To determine the effect of specific reduction of Dnmt1 levels on Dnmt1b extremities of the Dnmt1 mRNA, respectively. To determine the effect of specific reduction of Dnmt1 levels on Dnmt1b extremities of the Dnmt1 mRNA, respectively. To determine the effect of specific reduction of Dnmt1 levels on Dnmt1b extremities of the Dnmt1 mRNA, respectively. To determine the effect of specific reduction of Dnmt1 levels on Dnmt1b extremities of the Dnmt1 mRNA, respectively.

MDA-MB231, with MG88, MG98, or equal concentrations of mismatch control oligonucleotides (MG208 and MG207) (six mismatches). 24 h after exposure to Dnmt1 antisense inhibitors at concentrations ranging from 25 to 100 μM, total RNA was isolated from cells and Dnmt1/Dnmt1b RNA protection analysis performed (Fig. 3C). Both MG88 and MG98 produced a dose-dependent reduction in Dnmt1 mRNA levels as expected (Fig. 3C). Dnmt1b RNA levels were also reduced by MG88 or MG98 treatment (Fig. 3C). This suggests that MG88 and MG98 antisense target sequences present in Dnmt1 mRNA are conserved in Dnmt1b mRNA. MG98 is targeted to the 3’-untranslated region of Dnmt1 mRNA, and MG88 is targeted to a region near the translation initiation site of Dnmt1. Therefore, Dnmt1b appears to contain the same antisense target sequences as Dnmt1 at both 5’ and 3’ extremities. In addition, because the terminating mechanism by which second generation antisense oligonucleotides eliminate their target mRNA involves degradation of the target mRNA molecule by the enzyme RNase H (35), the accessibility to this enzyme at the target sequence must be similar in both Dnmt1 and Dnmt1b. Alternatively, Dnmt1b levels may be coordinately regulated with Dnmt1 protein levels. However, short term inhibition (24 h) of Dnmt1 RNA results in modest inhibition of Dnmt1 protein levels due to the long half-life of Dnmt1 protein (data not shown); therefore, transcriptional regulation of Dnmt1b in response to reduced Dnmt1 protein levels should not account for the inhibition seen after 24 h. These results, coupled with our finding that RT-PCR with primers flanking other introns yield only the product of the expected size for the published Dnmt1...
Dnmt1b mRNA suggests that this transcript represents a fairly abundant DNA methyltransferase in human cells. To determine whether this novel transcript encodes an abundant C-5 DNA methyltransferase protein isoform in human cells and to investigate potential tissue-specific expression of this splice variant at the protein level, we raised antibodies against a protein sequence unique to Dnmt1b. The specificity and relative affinities of both the Dnmt1 antibody (α-Dnmt1-NH₂) or the Dnmt1b protein isoform-specific antibody (α-Dnmt1b-exon 4) were determined by their ability to detect the recombinant baculovirus-expressed Dnmt1 or Dnmt1b proteins, respectively. α-Dnmt1b polyclonal antibody recognized both Dnmt1b and Dnmt1 proteins (lanes 1–3 and lane 4, respectively) as expected, because these proteins share the NH₂ terminus to which the antibody was raised. Western blot analysis revealed that the α-Dnmt1b polyclonal antibody recognizes baculovirus-expressed Dnmt1b (Fig. 4A, lanes 5–7) as expected, but does not cross-react with Dnmt1 protein (Fig. 4A, lane 8). Dilution of the recombinant protein used for Western analysis demonstrates that, under the assay conditions used, α-Dnmt1 and α-Dnmt1b antibodies detect either Dnmt1 or Dnmt1b with similar sensitivity (Fig. 4A). Having determined that the antibodies α-Dnmt1 and α-Dnmt1b are both selective and of similar potency for their respective DNA MeTase isoforms, we ran duplicate Western blot analyses on total cellular extracts from a variety of human cancer cell lines to determine the relative levels of the DNA MeTase isoforms (Fig. 4B). The major DNA methyltransferase Dnmt1b was highly expressed at the protein level in all the cancer cell lines tested (Fig. 4B, left). As expected, Dnmt1 protein is present at a much lower level in the human normal skin fibroblast line, MRHF, compared with transformed cells. Dnmt1b protein levels, however, were very low compared with those of Dnmt1 (Fig. 4B, right). The human breast cancer cell line MDA-MB231 expressed the highest level of Dnmt1b mRNA (Fig. 3A). MDA-MB231 also demonstrated the highest amount of Dnmt1b protein in the panel of cell lines tested. However, this amount of protein represented only 2% of the amount of Dnmt1 protein. Thus, although the DNA methyltransferase Dnmt1b splice variant accumulates to significant levels as a mature transcript in all human cells tested, the corresponding Dnmt1b protein isoform does not contribute significantly to cellular DNA methyltransferase protein levels. Differential regulation of alternatively spliced transcripts at the level of translation or cellular localization is not without precedence. The neuronal nitric oxide synthase (NOS1) mRNA has recently been found to be a family of highly diverse transcripts of at least nine unique exon 1 splice variants (37). Differential splicing in the 5′-untranslated region of NOS1 has profound effects on translation efficiency and processing of the respective mRNAs (37). Likewise, four alternatively spliced transcripts have been identified for the endothelin-specific angiogenic factor vascular endothelial growth factor (VEGF) in breast cancer tissues. However, VEGF protein corresponding to only one of these splice variants has been detected in these tissues (38). In the case of the DNA methyltransferase splice variant, Dnmt1b, the mechanisms controlling the expression of this mRNA remain to be investigated. Moreover, although we have investigated the enzymatic activity, substrate specificity, and cellular level of Dnmt1b protein in human cells, additional amino acid sequences could potentially alter interactions of Dnmt1b with other proteins, thus affecting its function. Further studies will be required to identify protein binding partners for Dnmt1b and to elucidate potential roles of Dnmt1b in development and disease.

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