The human DNA methyltransferases (DNMTs) 1, 3a and 3b: coordinate mRNA expression in normal tissues and overexpression in tumors

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ABSTRACT

DNA methylation in mammals is required for embryonic development, X chromosome inactivation and imprinting. Previous studies have shown that methylation patterns become abnormal in malignant cells and may contribute to tumorigenesis by improper de novo methylation and silencing of the promoters for growth-regulatory genes. RNA and protein levels of the DNA methyltransferase DNMT1 have been shown to be elevated in tumors, however murine stem cells lacking Dnmt1 are still able to de novo methylate viral DNA. The recent cloning of a new family of DNA methyltransferases (Dnmt3a and Dnmt3b) in mouse which methylate hemimethylated and unmethylated templates with equal efficiencies make them candidates for the long sought de novo methyltransferases. We have investigated the expression of human DNMT1, 3a and 3b and found widespread, coordinate expression of all three transcripts in most normal tissues. Chromosomal mapping placed DNMT3a on chromosome 2p23 and DNMT3b on chromosome 20q11.2. Significant overexpression of DNMT3b was seen in tumors while DNMT1 and DNMT3a were only modestly overexpressed and with lower frequency. Lastly, several novel alternatively spliced forms of DNMT3b, which may have altered enzymatic activity, were found to be expressed in a tissue-specific manner.

INTRODUCTION

Mammalian cells possess the capacity to epigenetically modify their genomes via DNA methylation. Methylation occurs at the 5 position of the cytosine ring within the context of the CpG dinucleotide (1). Approximately 70% of the CpG residues in the mammalian genome are methylated, however the distribution of CpG is not random and the majority of the genome is CpG-poor (2). Certain regions of the genome which are often, but not always, clustered at the 5'-ends of genes possess the expected CpG frequency and have been termed CpG islands (3). CpG islands are not normally methylated in cells and the mechanism preventing islands from becoming de novo methylated may involve transcription factor binding (4,5). The effects of DNA methylation on cells include transcriptional repression by methylation of promoter regions (6), formation of compact chromatin structures (7), X chromosome inactivation (8) and imprinting control (9).

Until recently only one DNA methyltransferase, DNMT1, had been cloned from human and mouse cells. DNMT1 is a large enzyme (193.5 kDa) composed of a C-terminal catalytic domain with homology to bacterial cytosine 5-methylases and a large N-terminal regulatory domain with several functions, including targeting to replication foci (10–13). Disruption of Dnmt1 in mice results in abnormal imprinting (9), embryonic lethality, greatly reduced levels of DNA methylation (14) and derepression of endogenous retroviruses (15). Dnmt1−/− embryonic stem (ES) cells are viable and still possess the ability to de novo methylate viral DNA, suggesting the existence of an independently encoded de novo DNA methyltransferase (16).

Several forms of DNMT1 have been detected which differ in their translation start sites and their preferences for hemimethylated versus unmethylated substrates (17). Targeting of DNMT1 to replication foci via the N-terminal domain is believed to allow for copying of methylation patterns from the parental to the newly synthesized daughter DNA strand. Forced overexpression of DNMT1 or cleavage between the N-terminal regulatory domain and C-terminal catalytic domain has been shown to result in increased de novo methylation activity (18,19) and cellular transformation (20). DNMT1 RNA and activity levels were shown to be elevated in colon cancer relative to adjacent normal mucosa and this was proposed to be responsible for the abnormal methylation patterns observed in this and other tumor types (21–23). Another study, however, showed only very small increases in DNMT1 RNA when the proliferative status of the tumor and normal tissue was taken into account, calling into

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question the exact role of DNMT1 overexpression in tumor genesis (24).

Since de novo methylation activity remains in Dnmt1 knockout ES cells and because the exact role of DNMT1 in tumor-specific methylation abnormalities remains unclear, a search for additional DNA methyltransferases was carried out by several groups. A second potential DNA methyltransferase, Dnmt2, was isolated by two groups but has not been shown to possess methylation ability (25,26). Recently another group of DNA methyltransferases, DNMT3a and 3b, was isolated by database search. These related enzymes were shown to be expressed at increased levels in undifferentiated ES cells and were down-regulated in differentiating ES cells and adult murine tissues. Furthermore, both Dnmt3a and 3b methylated hemimethylated and unmethylated DNA with equal efficiencies, making them potential candidates for the long sought de novo methyltransferases (27). In the present work we have investigated the expression patterns of human DNMT1, DNMT3a and DNMT3b in normal and tumor tissues. We found high level expression of all three transcripts in most fetal tissues examined and lower level, but still widespread, expression in most adult tissues. Analysis of expression levels in tumor and adjacent normal tissues showed that DNMT1 and 3a were overexpressed (at >2-fold) at low frequency, while DNMT3b was overexpressed at higher frequency even when the proliferative status of the tumor was taken into account. Furthermore, several novel splice variants of DNMT3b were detected and characterized which have the potential to alter its catalytic activity.

MATERIALS AND METHODS

GenBank accession numbers

The cDNA sequences of the human DNMT3b splice variants have been deposited in GenBank under accession nos AF129267 for DNMT3b1, AF129268 for DNMT3b4 and AF129269 for DNMT3b5.

Human tissues and RNA preparation

Normal human tissue cDNA preparations were purchased from Clontech. Bladder, colon, kidney and pancreas tumor and adjacent normal tissues were collected at the USC/Norris Comprehensive Cancer Hospital in accordance with institutional guidelines and immediately frozen in liquid nitrogen. RNA was extracted as described previously (28).

Reverse transcriptase (RT)–PCR

Reverse transcription was carried out with random hexamers (Pharmacia), Superscript II reverse transcriptase (Life Technologies) and 2.5 µg of total RNA as recommended by the manufacturer and as described previously (28) in a total volume of 50 µl. One microliter of RT reaction was used for subsequent PCR amplification for each of the desired transcripts with dNTPs (Boehringer Mannheim) and Taq DNA polymerase (Sigma) as described (29). Primer sequences used were as follows: DNMT3a sense, 5'-GGG GAC GTC CGC AGC GTC ACA C-3'; DNMT3a antisense, 5'-CAG GGT TGG ACT CGA GAA ACC AAT CAG GC-3'; DNMT3b sense, 5'-CCT GCT GAA TTA TTA CTC ACG CCC C-3'; DNMT3b antisense, 5'-GTC TGT GTA GTG CAT GCC GGA AAA GCC-3'; DNMT1 sense, 5'-GAT CGA ATT CAT GCC GGC GGC TAC CGC CCC AG-3'; DNMT1 antisense, 5'-ATG GTG GTT TGC CTG GTG C-3'; β-actin sense, 5'-GGA GTG TGG TGG CAT CCA CG-3'; β-actin antisense, 5'-CTA GTA GAA GCA TTT GGC GTG GA-3'. The proliferating cell nuclear antigen (PCNA) primers, probe and amplification conditions have been described previously (29). Amplification conditions were: 94°C for 2.0 min, 1 cycle, 94°C for 0.5 min, transcript-specific annealing temperature for 1.0 min, and 72°C for 1.0 min for a cycle number optimized for each set of primers and RNA sample to keep amplification within the linear range. Annealing temperatures were 65°C for DNMT3a and 3b, 58°C for DNMT1 and 60°C for β-actin. Cycle numbers varied between 18 and 25 for β-actin, 25 and 30 for PCNA and 30 and 35 for the DNMT transcripts. Generally the RNA derived from the tumor and adjacent normal tissues required a higher number of cycles compared to the commercially available normal tissue samples. PCR reactions were electrophoresed on 1.5% agarose gels, transferred to nylon membrane (Genescreen Plus; NEN) and hybridized to end-labeled oligonucleotide probes at 48°C as described previously (29). Probe sequences were as follows: DNMT3a, 5'-CCG CGC GTC ATG ATG CAG GAG GGG GTA GAA CTC-3'; DNMT3b, 5'-CAG TGG ATT ACA CTA CCT GCA GAA CCG TGA GAT G-3'; DNMT1, 5'-ATG GCA GAT GCC AAC AGC CC-3'; β-actin, 5'-ACA TCC GCA AAG ACC TGT ACG CCA ACA CAG-3'.

Northern blot analysis

The fetal tissue northern blot was purchased from Invitrogen and the human adult northern blot was purchased from Clontech. Probes were labeled with the Random-Prime DNA Label Kit (Boehringer Mannheim) and hybridized at 65°C in ExpressHyb hybridization buffer (Clontech) according to the manufacturer’s instructions. The DNMT3a cDNA probe corresponded to the EcoRI–NcoI fragment (376 bp) derived from the EST plasmid containing the DNMT3a cDNA (GenBank accession no. W76111) and the DNMT3b cDNA probe corresponded to the HindIII–SmaI fragment (324 bp) derived from the EST plasmid containing the DNMT3b cDNA (GenBank accession no. T66356) (27). The DNMT1 probe corresponded to the EcoRI–Xbal fragment (330 bp) derived from plasmid pKR11D-6. pKR11D-6 was created by RT–PCR with Raji cell line RNA as template and primers sense, 5'-GAT CGA ATT CAT GCC GGC GGC TAC CGC CCC AG-3'; and antisense, 5'-ACA CAG GTG ACC GTG CTT ACA GTA CAC-3', and PCR conditions identical to those described above for DNMT1, except an extension time of 3 min was used. The 1249 bp band generated (corresponding to bp 1–1249 of human DNMT1; 30) was cloned with the T7Blue-Blunt cloning system (Novagen) and sequenced. For β-actin, an end-labeled oligonucleotide probe was used (5'-CTG TGG TGG CTT ACA GGG TTG TGC GGA TGT-3') and the hybridization conditions were identical except the temperature was lowered to 48°C.

Cloning and analysis of DNMT3b splice variants

The four RT–PCR products generated after amplification of human testis cDNA with DNMT3b primers were separated on a 1.5% agarose gel; then each fragment was gel purified using the Qiaex II gel extraction kit (Qiagen) according to the manufacturer’s instructions and cloned using the T7Blue-Blunt cloning kit (Novagen). At least two independent clones corresponding to
each transcript were isolated and sequenced at the USC Microchemical Core Facility and aligned with the EST sequence for human DNMT3b3 (GenBank accession no. T66356) and murine Dnmt3b1 cDNA (GenBank accession no. AF068626) (27). Human testis cDNA was used as template because it was the only tissue we examined which expressed significant levels of all four splice variants.

Chromosomal mapping

Fluorescence in situ hybridization (FISH) was carried out on normal female lymphocyte chromosome spreads as described previously (31). Probes for DNMT3a and DNMT3b, which corresponded to the entire cDNA inserts derived from the respective EST plasmids (GenBank accession nos W76111 and T66356 for DNMT3a and 3b, respectively) were labeled with biotin, hybridized to chromosome spreads, r-banded and then detected with fluorescein-avidin.

RESULTS

DNMT1, 3a and 3b are coordinately expressed in most human tissues

Northern blotting with poly(A)-selected RNA was initially employed to quantitatively examine DNMT expression levels in a panel of fetal and adult human tissues. Transcripts for DNMT3a (Fig. 1A) and DNMT1 (Fig. 1C) were present at high levels in all fetal tissues examined while DNMT3b (Fig. 1B) was expressed at high levels only in fetal liver. DNMT3b transcripts were detectable after long exposure in all fetal tissues (not shown). It is interesting to note that fetal liver expressed extremely high levels of all DNMTs, which may be due to residual hematopoiesis in this tissue (32). Transcripts for DNMT3a and 3b were readily detectable in adult heart, skeletal muscle, thymus, kidney, liver, placenta and peripheral blood mononuclear cells (PBMC) and appeared to be coordinately expressed in many cases (Fig. 1). Bands were detectable in all tissues after long exposure (not shown). DNMT3a and 3b transcripts were slightly larger than the previously reported murine homologs (27). DNMT1 was expressed at detectable levels in all adult tissues except small intestine. It is also notable that multiple bands hybridized in many tissues for all three transcripts, but especially intestine. It is also notable that multiple bands hybridized in many tissues following synchronization and release showed readily detectable differences in the expression of DNMT3a and 3b (data not shown). To further examine the degree of coordinate expression of the three DNMTs, semi-quantitative RT–PCR with independently prepared RNA samples representing a subset of the tissues analyzed in Figure 1 followed by hybridization and quantitation was carried out. Primers employed for this analysis were 100% specific for each DNMT transcript (data not shown). Figure 2 shows that the three DNMTs were coordinatedly expressed in most tissues, with the exception of skeletal muscle which showed similar differences in the northern blot analysis as well. The absolute levels of each DNMT by RT–PCR analysis parallel the Northern hybridization intensities in many cases (brain, heart, skeletal muscle, colon, spleen and placenta for example) but not all (liver and PBMC for example) and is most likely due to the semi-quantitative nature of the RT–PCR analysis and differences in origin and preparation of the RNA samples.

Alternative splicing in the 3′-end of DNMT3b

Several splice variants of murine DNMT3b have been previously reported. One of these regions was within the C-terminal catalytic domain and thus had the potential to alter catalytic activity (27). Such splice variants, some of which delete the catalytic site (conserved methyltransferase motif IV) and alter the spacing between motifs, have been reported for DNMT1 (33). We used semi-quantitative RT–PCR to examine potential DNMT3b splice variants in a panel of 13 normal human tissues followed by Southern hybridization with an internal oligonucleotide probe. Figure 3A shows that expression of multiple DNMT3b variants could be detected in all tissues examined. Amplification of PCNA (Fig. 3B) and β-actin (Fig. 3C) served as controls for proliferative state and RNA integrity, respectively. PCNA levels were elevated in ‘proliferating tissues’ such as the small intestine, testis, placenta and colon (Fig. 3B). A total of four DNMT3b bands could be detected in human testis DNA and each band was cloned and sequenced. The structures of the four splice variants are shown schematically in Figure 4A (top) with their migration in the agarose gel shown in Figure 4A (bottom). The predicted protein products are aligned with the murine Dnmt3b1 sequence (27) in Figure 4B. Two of the four transcripts correspond to previously described splice variants of murine Dnmt3b3 (Dnmt3b1 and 3b3) while two others are novel (termed Dnmt3b4 and 3b5 in Fig. 4). These latter two transcripts give rise to truncated protein products due to frameshifts after splicing resulting in loss of the conserved methyltransferase motifs IX and X (11). DNMT3b4 terminates one amino acid after the splice junction (a deletion of 108 amino acids relative to DNMT3b1), while DNMT3b5 encodes a novel 45 amino acid region followed by a stop codon (Fig. 4B). It can be seen in Figure 3A that DNMT3b3 (lowest band) was ubiquitously expressed in normal tissues while DNMT3b1 (top band) was expressed in all tissues except brain, skeletal muscle and PBMC. DNMT3b4 (upper middle band) was expressed in all tissues except brain, skeletal muscle, lung and prostate while DNMT3b5 (lower middle band) was detectable...
Figure 1. Northern analysis of expression levels of DNMTs in normal human tissues. Approximately 2 µg of poly(A)-selected RNA derived from fetal tissues (left), with age given in weeks at the top, or adult tissues (right) was hybridized to cDNA probes specific for (A) DNMT3a, (B) DNMT3b and (C) DNMT1. β-Actin (D) served as a control for RNA integrity and indicated that samples were equally loaded. Molecular weights in kilobases (kb) are indicated at the left of each panel. PBMC, peripheral blood mononuclear cell; Sm. int., small intestine; Sk. muscle, skeletal muscle. (E) Summary of the relative levels of each DNMT in fetal and adult liver derived from phosphorimager quantitation of results in (A)–(C) and exposure times. This tissue was used since it expressed relatively high levels of all DNMTs making quantitation more accurate and because it was a tissue present on both the fetal and adult northern blots. Results are expressed relative to the DNMT1 level on each blot (set at 1.0). Note, however, that expression levels in fetal liver were nearly 20-fold higher than in adult liver.

Chromosomal mapping of DNMT3a and 3b

Given that DNMT3a and 3b appeared to be coordinately expressed in many tissues we wished to map their chromosomal location. FISH using cDNA probes for each methyltransferase was carried out on normal lymphocyte chromosome spreads and

only in testis and at a very low level in brain and prostate (Fig. 3A). It should also be noted that the additional Dnmt3b splice variant (Dnmt3b2) described in murine tissues could not be distinguished from DNMT3b1 in our analysis because the Dnmt3b2 alternatively spliced region was located 5' to our RT–PCR primers (27).
Figure 2. Analysis of DNMT expression levels by semi-quantitative RT–PCR. Primers specific for each DNMT were used with cDNA derived from each of the normal human tissues (Clontech) listed below the graph. These cDNA samples were prepared from different RNA sources than those used for the northern analysis in Figure 1. PCR products were hybridized with internal oligonucleotide probes (not shown), quantitated and expressed relative to β-actin, as a control for RNA integrity. Abbreviations are as in Figure 1.

Figure 3. Alternative splicing in the catalytic domain of DNMT3b (A) was monitored by RT–PCR using cDNA derived from the normal human tissues (Clontech) indicated above the figure. Amplification of PCNA (B) and β-actin (C) served as controls for proliferative state and RNA integrity, respectively. PCR products were then hybridized to internal oligonucleotide probes specific for each transcript. Abbreviations are as in Figure 1.

indicated that DNMT3a was located at chromosome 2p23 and DNMT3b was located at chromosome 20q11.2 (data not shown). DNMT1 has been previously mapped to human chromosome 19p13.2–p13.3 (30).

Overexpression of DNMTs in tumor tissues

Previous studies have revealed significant overexpression of DNMT1 in tumor tissues (21,22) while another study found very little increase (24). We examined the expression levels of all three methyltransferase genes by RT–PCR from 10 normal–tumor matched sets (five bladder, three colon, one kidney and one pancreas) and related this to the proliferation-associated marker PCNA. PCNA is a component of the DNA replication apparatus and is also known as the polymerase processivity factor or sliding clamp (35). While its levels vary modestly (3–7-fold depending on the study) during the G1 to S transition, it is nearly undetectable in G0 cells by northern blot and its synthesis has been shown to correlate directly with the proliferative state of the cell (36,37). A representative subset of the RT–PCR data from the 10 normal–tumor matched sets for DNMT1, 3a and 3b is shown in Figure 5. Amplification of PCNA and β-actin served as controls for cell proliferation and sample quality, respectively (Fig. 5). Figure 6 summarizes the fold change (tumor/normal) for the 10 normal–tumor matched sets relative to PCNA for DNMT3a (top), DNMT3b (middle) and DNMT1 (bottom). A level of overexpression ≥2-fold was observed in 5 of 10 samples for DNMT3a, 6 of 10 samples for DNMT1 and 8 of 10 samples for DNMT3b. DNMT3b clearly showed the largest fold increases (an average of 7.5-fold) of all three enzymes and there were several tumors which overexpressed all three enzymes simultaneously, such as bladder sample 16. Levels of overexpression of DNMT3a (an average of 3.1-fold) and DNMT1 (an average of 4.0-fold) were more modest in the majority of the samples, in keeping with a previous study of DNMT1 (24).

DISCUSSION

In the present paper we have described the expression patterns of the three known, catalytically active DNA methyltransferases in a large number of human fetal and adult tissues and mapped their chromosomal locations. Expression of DNMT1, 3a and 3b mRNA was widespread in a coordinate manner in most tissues.
Figure 4. Analysis of 3′-end splice variants of DNMT3b. Four splice variants were detected during RT–PCR for DNMT3b in normal human testis and a subset of these four were detected in all tissues examined (Fig. 3A). (A) RNA structure (top line, with shaded box indicating the alternatively spliced region) and protein structure (bottom boxes, with white representing unaltered reading frame and shading representing frameshifts) of the four products after cloning and sequence analysis. DNMT3b1 and 3b3 were similar to the previously reported murine homologs (27), while DNMT3b4 and 3b5 have not been previously reported. Approximate positions of the most conserved DNA methyltransferase motifs are boxed (11). Arrows represent the locations of the RT–PCR primers. The bottom panel is a representative ethidium bromide stained 1.5% agarose gel showing the migration of each of the splice variants after RT–PCR amplification from human testis RNA. (B) Amino acid sequence alignment of the 3′-ends of human DNMT3b1 and 3b3 were similar to the previously reported murine homologs (27), while DNMT3b4 and 3b5 have not been previously reported. Approximate positions of the most conserved DNA methyltransferase motifs are boxed (11). Arrows represent the locations of the RT–PCR primers.
DNMT3a and 3b relevant to cancer? This is a complicated question but our results suggest that they may indeed play a role. While overexpression levels of DNMT1 and 3a were modest, DNMT3b was significantly overexpressed in most tumors we examined. That DNMT3a and 3b may be involved in the abnormal de novo methylation observed in tumors is strengthened by the previous work in ES cells. Undifferentiated ES cells clearly have the capacity to de novo methylate DNA (16), like tumor cells, and levels of both DNMT3a and 3b are very high in ES cells (27). Furthermore, amplifications of human chromosome 2p23, the location of DNMT3a, are frequent in certain B cell neoplasms (39) and neuroblastomas (40). This region also contains the N-myc proto-oncogene (39) and the anaplastic lymphoma kinase gene ALK (41). Deletions of portions of the long arm of human chromosome 20, which include the DNMT3b locus at 20q11.2, have been reported in a wide spectrum of myeloid disorders (42,43). These tumors will be particularly interesting to examine in terms of their methylation patterns and might provide information as to the exact role of DNMT3a and 3b in cancer.

An interesting aspect of DNMT3b was the finding of multiple alternatively spliced forms. Four forms, arising from alternative splicing near the 3′-end, were expressed in a tissue-specific manner and several of these forms were up-regulated in tumors more frequently than others. DNMT3b3 was ubiquitously expressed in normal tissues and most tumors and possesses the conserved methyltransferase motifs. It is therefore likely that this form is enzymatically active in vitro, however the altered spacing between motifs could affect activity. The enzymatic activity of murine Dnmt3b3 in vitro was not addressed in a previous study (27). Preliminary experiments in which the murine Dnmt3a, 3b1 and 3b2 cDNAs were transfected into insect cells have confirmed the results of Okano et al. that the expressed proteins are enzymatically active using a poly(dI·dC:dI·dC) substrate incubated with cell extracts (P.Vollmayr and N.Reich, personal communication). This, combined with the very high level of homology between the human and murine forms (∼95% identity at the amino acid level for DNMT3a for example; data not shown) makes it likely that the human forms will possess enzymatic activities similar to the murine homologs. The finding that two of the human splice variants (DNMT3b4 and 3b5) result in frameshifts that truncate the protein and delete conserved methyltransferase motifs IX and X may indicate that these forms have differential methylation activities. Interestingly, motifs IX and X, which may be involved in cofactor binding, are not conserved in all known cytosine 5-methyltransferases (11,44).

Alternative splicing and differential activity of DNMT3b variants could have important implications for cancer. In general it has been observed that bulk DNA becomes hypomethylated in cancer while CpG islands associated with promoters become abnormally de novo methylated (6,23). Different forms of the same enzyme, in this case DNMT3b, may have enhanced or reduced catalytic activity or may have altered target site specificity (11). An alternatively spliced form with greater affinity for CpG islands or a form which lacks a region preventing its association with CpG islands could help explain the methylation abnormalities associated with cancer. In vitro functional studies of the human splice variants will be the subject of future study.
Relating DNMT levels to proliferative state has been an important issue to those studying the role of DNA methylation in cancer. Our studies on the levels of expression of the three DNMT transcripts in normal tissues is, to our knowledge, the most extensive to date and raises several interesting issues. The first relates to the relative levels of the three DNMTs. It was clear, based on exposure times of the northern blots, that DNMT1 was expressed at the highest level of all three DNMTs in all the normal tissues we examined. The second issue is that, although some relationship between DNMT mRNA levels and proliferative state may be operational. Sequestration of Dnmt1 in the oocyte cytoplasm has been observed previously (34). Such studies of DNMT3α and 3β will be carried out once antibodies become available and will be an important issue when considering their involvement in cancer as well.

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