Alternative Splicing Yields Protein Arginine Methyltransferase 1 Isoforms with Distinct Activity, Substrate Specificity, and Subcellular Localization

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PRMT1 is the predominant member of a family of protein arginine methyltransferases (PRMTs) that have been implicated in various cellular processes, including transcription, RNA processing, and signal transduction. It was previously reported that the human PRMT1 pre-mRNA was alternatively spliced to yield three isoforms with distinct N-terminal sequences. Close inspection of the genomic organization in the 5’-end of the PRMT1 gene revealed that it can produce up to seven protein isoforms, all varying in their N-terminal domain. A detailed biochemical characterization of these variants revealed that unique N-terminal sequences can influence catalytic activity as well as substrate specificity. In addition, our results uncovered the presence of a functional nuclear export sequence in PRMT1v2. Finally, we find that the relative balance of PRMT1 isoforms is altered in breast cancer.

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The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1 and 2.

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3 The abbreviations used are: PRMT, protein arginine methyltransferase; hhnRNP, heterogeneous nuclear ribonucleoprotein; NES, nuclear export sequence; GFP, green fluorescent protein; EGFP, enhanced GFP; GST, glutathione S-transferase; RT, reverse transcription; siRNA, small interfering RNA; PBS, phosphate-buffered saline; EST, Expressed Sequence Tag; ss, splice site; nt, nucleotides; TSS, transcriptional start site; AdoMet, S-adenosyl-L-methionine; ES, embryonic stem.
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protein isoforms, themselves differing at their N terminus (23, 24). We describe here the complex genomic organization in the 5′-end of the PRMT1 gene that can produce up to seven protein isoforms expressed in a tissue-specific manner. Biochemical characterization of these seven isoforms (designated as v1 to v7) revealed that they are all active, except for v7, and that their unique N-terminal sequences can confer distinct substrate specificity. Moreover, we demonstrate that the amino acid sequence unique to v2 (encoded by exon 2) contains a CRM1-dependent nuclear export sequence (NES) that regulates its subcellular localization. Finally, we find that the relative balance of these PRMT1 splicing variants is altered in breast cancer cell lines.

EXPERIMENTAL PROCEDURES

Cells, Reagents, and Antibodies—The human HeLa cervical carcinoma cell line was purchased from ATCC (Manassas, Virginia) and grown as a monolayer in Dulbecco’s modified Eagle’s medium supplemented with 1 mm sodium pyruvate, 50 IU/ml penicillin, 50 mg/ml streptomycin, and 10% fetal calf serum (Wisent). The human normal breast cell line HS 578 Bst and the human breast cancer cell lines HS 578T, BT-20, BT-549, MCF7, MDA-MB-231, and T-47D were purchased from ATCC and grown as monolayers in complete Dulbecco’s modified Eagle’s medium supplemented with minimum Eagle’s medium nonessential amino acids and 10% fetal bovine serum (Wisent). Mouse wild type and PRMT1+/− ES cells were kindly provided by Dr. David Lohnes (University of Ottawa, ON, Canada) and Dr. H. Earl Ruley (Vanderbilt University Medical Center, Nashville, TN), respectively, and were maintained as previously described (25). Transfections were performed using the Lipofectamine Plus transfection reagent (Invitrogen) according to manufacturer’s instructions.

Polyclonal anti-GFP antibodies were purchased from Sigma. Rabbit polyclonal antibodies against human PRMT1, Sam68, and asymmetric dimethylarginine (ASYM24–25) were described previously (4, 26, 27) and were a kind gift from Dr. Stephane Richard (McGill University, Montreal, QC, Canada). Monoclonal antibodies against β-actin and glyceraldehyde-3-phosphate dehydrogenase were purchased from Sigma and Covance, respectively.

DNA Constructs—Total RNA was extracted from HeLa cells using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. RNA concentration was measured with a spectrophotometer, and RNA quality was determined by agarose gel electrophoresis. First strand cDNA synthesis was performed using 5 μg of total RNA and the avian myeloblastosis virus reverse transcriptase (Promega) with an oligo-dT primer. cDNAs were amplified using PRMT1 isoform-specific oligonucleotides (v1–7) designed to introduce the amplified fragments in frame with a hexahistidine tag in the EcoRI and XhoI sites of the pET-20b expression vector (Novagen). PRMT1v4 was amplified from an EST clone (IMAGE: 387210) using v4-specific primers, and PRMT1v7 was isolated using the v1- and v2-specific primers on HeLa cDNA. The resulting plasmids were digested with BamHI and XhoI, and the released full-length inserts were subsequently cloned in-frame with EGFP into the BglII and XhoI sites of the pEGFP-N1 vector (Clonetech). The PRMT1v2-EGFP NES mutant, in which Val-15 and Leu-18 were replaced by alanines, was generated by using the Stratagene QuikChange mutagenesis kit. The pGFP-Rev-NES vector was generously provided by Dr. Stephen Lee (University of Ottawa, ON, Canada).

The expression vectors encoding GST-fused RG motif of colin (Val-385 to Val-423) and Sam68 (full-length or P3 (Gly-285 to Pro-308) proteins were kindly provided by Dr. Stephane Richard (McGill University). The expression vectors encoding GST-fused RG motifs of MRE11 (Phe-554 to Arg-680), SmB (Psp-206 to Leu-231), and SmB’ (Pro-206 to Pro-240) have been described (28, 29). Full-length fibrillarin and hnRNP A1 were amplified from HeLa CDNA and introduced in-frame with a GST tag in the BamHI/XhoI restriction sites of the pGEX-4T2 vector (GE Healthcare). The DNA sequence of all constructs were verified by sequencing (StemCore Laboratories, Ottawa, ON, Canada). Purified ASF/SF2 protein was kindly provided by Dr. Martin Pelchat (University of Ottawa).

RT-PCR Analysis—Total human tissue cDNAs were obtained from BioChain (Hayward, CA). PCR were performed in 25-μl reaction mixture using TaqDNA polymerase (Qiagen). cDNAs were amplified using the PF/PR primers (24) specific for PRMT1v1 to -3 or using either a forward PRMT1v4-specific primer (5′-aaatcttccagcggggtcgcg-3′), PRMT1v5-specific primer (5′-actgagagatggttctggt-3′), PRMT1v6-specific primer (5′-aagtgcagacaagacaggg-3′), or a PRMT1v6-specific primer (5′-tctatgagagatgctcaagg-3′) with the reverse PR primer. Primers specific for actin (24) were used to show that an equal amount of total CDNA was used for each reaction. An initial incubation at 98 °C for 2 min was followed by 35 cycles consisting of a 95 °C denaturation step (30 s), a 65 °C annealing step (30 s), and a 72 °C extension step (30 s). A final extension step at 72 °C was included for 10 min. For PRMT1 expression analysis in breast cancer cells, total RNA extraction and first strand cDNA synthesis were performed as described above. PCR reactions were also performed as described above but using 1 μl of a 1/10 cDNA dilution. Cycle number was determined empirically for each primer pair to stay within the linear range of amplification (see supplemental Fig. 1). All PCR products were electrophoresed on 2.5% agarose gels and visualized by ethidium bromide staining.

RNA Silencing—The small interfering RNA (siRNA) specific to PRMT1 exon 2 was designed using Gene Link RNAi Explorer, and the siRNA-annealed oligonucleotide duplex was purchased from Invitrogen. Concomitantly, a PRMT1 exon 2-specific short hairpin RNA expression plasmid was generated according to Brummelkamp et al. (30). Briefly, 60-bp synthetic DNA oligonucleotides containing PRMT1 exon 2-specific sense and corresponding antisense sequences flanking a 7-base hairpin were inserted into the BamHI and Hind III restriction sites of the pRS vector (OriGene). The siRNA duplex and the short hairpin RNA expression plasmid were concurrently transfected in HeLa cells using the X-tremeGENE siRNA transfection reagent (Roche Applied Science).

Protein Purification—His6 fusion proteins were overexpressed in Escherichia coli BL-21 cells (Strategene) by induction with a final concentration of 1 mm isopropyl-β-D-thiogalactopyranoside. After induction, cells were spun down, resus-
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Results

Genomic Structure of the Human Prmt1 Gene—Upon initial identification of the Prmt1 genomic locus, it was observed that three transcripts could be produced through alternative splicing of exons 2 and 3 (named v1–v3; Fig. 1A), and expression of these variants at the RNA level was later confirmed by an independent study (23, 24). Zhang and Cheng (32) then predicted the existence of three additional isoforms (named v4–v6; Fig. 1A) by comparing available ESTs with human PRMT1 genomic sequence but did not investigate further how these mRNAs were produced. Mapping of the unique sequences present at the 5′-end of v4 and v6 mRNAs onto the human PRMT1 genomic locus (human Mar. 2006 hg18 assembly; NCBI Build 36.1) using the UCSC Genome Bioinformatics BLAT alignment tool (33), suggested the existence of at least two additional exons upstream of the previously identified first exon. Further examination of splicing junctions in the genomic sequence using UCSC Genome Browser (34) revealed the presence of four alternative 5′-exons in the Prmt1 gene that we have renamed e1a–d, e1d being the exon previously labeled exon 1 (Fig. 1A). e1c and e1d do not seem to have functional 3′ splice sites (3′ss) and, hence, are never joined together and are utilized as alternate first exons. In contrast, exon 1b has two alternative 3′ss that can be paired with the 5′ss of e1a. In addition, the intron between these exons is sometimes retained in the mRNA (Fig. 1A). These splicing events would take place ~1 kilobase away from the previously identified exon 1 and support the presence of a transcriptional start site (TSS1) upstream of e1a. Indeed, a promoter (genomic coordinates 54,870,654–54,870,904) and a putative TSS (54,871,225) are predicted in this region using the “Promoter Scan” and “DBTSS” algorithms (35, 36). However, the highest score obtained for a TSS using the Neural Network Promoter Prediction algorithm (37) maps to a site just upstream of e1d (54,872,277), which is compatible with this exon being the most represented first exon among ESTs. Preliminary results from a 5′-rapid amplification of cDNA ends analysis of PRMT1 mRNAs confirms the existence of this TSS2 (data not shown and Fig. 1A). Thus, these observations are consistent with a model where alternative promoters would dictate at least in part the choice of 5′-exon usage. The splicing patterns of alternative exons 2, 3, and 4 are also more complex than initially anticipated from previous reports (23, 24) and involve the use of multiple alternative 5′ss and 3′ss. For example, the intron between e2 and e3 includes minor spliceosome AT+AC splice sites (38–41) in addition to a non-canonical GT-cc pair. In this case the 5′ss used determines the reading frame irrespective of the 3′ss to which it is paired and, thus, will dictate AUG start codon choice; GT results in the use of a start codon in e3 and AT in e4, yielding protein isoforms v3
and v6, respectively. Finally, protein isoform v5 results from pairing of e1d-e2-e3 (GT-cc) but with usage of an alternative 5’-ss downstream of e3 (Fig. 1A).

The predicted protein-coding regions (excluding 5’ and 3’-untranslated regions) of the various Prmt1 transcripts are composed of 1059 nt (v1), 1113 nt (v2), 1041 nt (v3), 1026 nt (v4), 975 nt (v6), or 960 nt (v7) and would encode 7 deduced polypeptides with a predicted molecular mass of 40.5, 42.5, 39.9, 40.1, 39.4, 37.7, and 36.7 kDa, respectively, excluding any posttranslational modifications (Fig. 1A).

**Expression Profile of PRMT1 Isoforms in Normal Human Tissues**—Experiments investigating the expression pattern of PRMT1v1, -v2, and -v3 in different human tissues have demonstrated that these variants are ubiquitously expressed (23, 24). To address the expression profile of the newly isolated isoforms, we analyzed the relative expression levels of the seven PRMT1 isoforms in human normal tissues by semiquantitative RT-PCR using the expression level of the actin gene as an internal control (Fig. 1B). PRMT1v1 was mostly expressed in the kidney, liver, lung, skeletal muscle, and spleen, whereas PRMT1v2 was predominantly detectable in the kidney, liver, and pancreas. The PRMT1v3 isoform was present at comparable, but low levels in all tested tissues. In contrast, PRMT1v4 to v7 showed a higher degree of tissue specificity in their expression patterns. For example, v4 was detectable only in the heart, whereas v5 was mostly expressed in the pancreas, and v7 was predominantly present in the heart and skeletal muscles (Fig. 1B). Finally, the predicted PRMT1v6 mRNA was not detectable in any normal tissues tested under the experimental conditions used here. These results show that the expression level of the PRMT1 variants varies significantly between human tissues.

**PRMT1 Expression in Breast Cancer Cells**—It has been previously reported that the relative prevalence of PRMT1 isoforms 1–3 is different between normal and cancerous breast tissues (24). In addition, a large proportion of ESTs for PRMT1v6 were obtained from cancer cells libraries, suggesting that the balance of specific alternative isoforms might be altered in transformed cells. To assess whether the expression of PRMT1 N-terminal variants is distinct in breast cancer cells, we examined the expression of the Prmt1 gene in normal and cancerous breast cell lines using semiquantitative RT-PCR. Actin mRNA was used as a control for the amount of cDNA used (Fig. 1C, bottom panel, and supplemental Fig. 1). We find that PRMT1 expression level is on average 14-fold higher among the breast cancer cell lines tested (Fig. 1C). Further-
more, the relative expression ratio of the isoforms also varied between normal and breast cancer cell lines. Specifically, the ratio of v2 over v1 mRNAs was increased on average 3.5-fold in breast cancer cell lines (Fig. 1F), suggesting that the v2 isoform is selectively increased relative to v1. Among the other isoforms, PRMT1v5 and -v6 were detected uniquely in specific breast cancer cell lines but not in normal breast cells (Fig. 1C, compare lanes 3, 6, and 7, with lane 1). The newly identified v7 isoform expression level was also on average 3-fold higher in the breast cancer lines tested (Fig. 1C, compare lane 1 with lanes 2–7). Finally, an overall 9.5-fold increase in PRMT1 expression was detected in a human breast tumor sample as compared with the adjacent normal breast tissue (Fig. 1D), confirming the observations made in breast cancer cell lines.

To evaluate whether the observed PRMT1 isoforms mRNA profiles correlated with protein levels, we immunoblotted normal and cancerous breast cell protein extracts with our anti-PRMT1 antibody (4). Because this antibody is directed against the common C-terminal domain, it is predicted that it should recognize all PRMT1 isoforms. Three major bands can be detected/resolved using this antibody in HeLa cell extracts (4). Based on its predicted molecular mass, the upper band should correspond to PRMT1v2 (42.5 kDa), and this was confirmed by detecting this protein using a v2-specific siRNA duplex (see supplemental Fig. 2). Based on the predicted molecular mass, the upper band should correspond to PRMT1v2 (42.5 kDa), and this was confirmed by detecting this protein using a v2-specific siRNA duplex (see supplemental Fig. 2). PRMT1v2 (40.5 kDa) migrates with the stronger middle band (Fig. 1E, top panel, lane 2), and because of their very small difference in predicted sizes, it is likely that isoforms v3–5 cannot be resolved from each other and also migrate as part of this band. The identity of the lower band is more difficult to predict, but it could correspond to PRMT1v6 (37.7 kDa) and/or -v7 (36.7 kDa). Using this antibody, the major middle band (labeled v1 here for simplicity) as well as a weak faster-migrating species, were observed in normal breast fibroblasts (Fig. 1E, top panel, lane 1). Unexpectedly, this band does not change significantly in breast cancer cells, although we cannot rule out the possibility that an increase in PRMT1v1 could be masked by co-migration with other isoforms. In contrast, PRMT1v2 was clearly present at higher levels in the Hs578T, BT-549, and MCF-7 breast cancer cells (Fig. 1E, lanes 2, 4, and 5, respectively), which is consistent with the profile observed for mRNA levels (Fig. 1F). The intensity of the faster migrating protein species also varies slightly between the various samples, but these changes did not follow the profile observed for PRMT1v6 or -v7 mRNAs. Hence, the identity of that band remains unclear.

PRMT1 is the predominant Type I PRMT in cells, and we have found that the expression profile of at least some of its splicing variants are altered in breast cancer. Thus, we hypothesized that the overall state of type I arginine methylation should also be affected in these cancer cells. To test this hypothesis, total protein extracts were immunoblotted with asymmetric dimethylarginine-specific antibodies ASYM24 and ASYM25 (27). Strikingly, an overall increase of arginine-methylated protein levels was observed in the breast cancer cell lines as compared with the normal breast cell line (Fig. 1E, middle panel). Moreover, the asymmetric dimethylarginine-specific antibodies immunoreacted with similar but distinct proteins between normal and cancerous breast cells as well as between the different breast cancer cell types. Taken together, our results show that an altered PRMT1 isoform expression profile correlates with a differential pattern of arginine methylation in breast cancer cell lines. This, in turn, suggests that misregulation of arginine methylation could contribute to the onset of breast cancer.

Unique N-terminal Sequences Can Influence PRMT1 Enzymatic Activity and Substrate Specificity—To assess the contribution of the alternatively spliced N terminus in PRMT1 function, the methyltransferase activity of each recombinant PRMT1 protein isoform (v1 to v7) was tested in vitro. The PRMT1 variants were expressed as C-terminal His6-tagged proteins, purified, and incubated with a selection of known arginine methylation substrates (Fig. 2A). After incubation in the presence of S-adenosyl[3H]-l-methionine as a methyl donor, the reactions were resolved by SDS-PAGE, and the methylated proteins were visualized by fluorography. In these experiments PRMT1 variants 1–6 were active toward all substrates tested, with v3 and v4 being the less active isoforms overall (Fig. 2, B–E). Remarkably, each active isoform preferentially methylated a distinct subset of protein substrates. Sam68 and SmB, for example, were better substrates for PRMT1v1 and -v2, whereas hnRNP A1 was more effectively methylated by v5 and v6 (see the respective bar graphs in Fig. 2C). Among the substrates tested, Coilin is a proposed substrate of PRMT5 and was shown to harbor ω-Nε,Nε-dimethylarginines in vivo (42, 43). Similarly, Sm proteins B and B’ are targets of PRMT5 and CARM1 and, accordingly, were shown to contain both ω-Nε,Nε- and ω-Nε,Nε-dimethylarginines (44–48). Surprisingly, we have detected methyltransferase activity of specific PRMT1 isoforms toward these substrates (Fig. 2, C–E). Finally, ASF/SF2 is a well known general and alternative splicing factor and was previously identified in two distinct proteomic screens for arginine-methylated proteins (27, 49), although the specific methylase responsible for these modifications remained unknown. We report here for the first time that ASF/SF2 is a substrate of PRMT1 in vitro (Fig. 2C).

In contrast, PRMT1v7, which lacks exons 2 to 4, was completely inactive when incubated with the same selection of substrates (Fig. 2B and data not shown). This observation corroborated previous results suggesting important roles for helix αX (encoded by exon 4) both in cofactor binding and catalysis (32). Because PRMT1v7 is the only inactive variant and PRMT1 dimerization is essential for its activity (32), we speculated that PRMT1v7 might regulate the activity of other PRMT1 isoforms by acting as a “natural” dominant negative. To address this possibility, a competitive methylation assay between PRMT1 variant 1 and 7 was performed (Fig. 3A). Specifically, methylation assays were performed as described above with constant amounts of GST-Sam68 substrate and PRMT1v1 but in the presence of increasing concentrations of PRMT1v7. Again, PRMT1v7 resulted in no detectable methylation activity, and PRMT1v1 methylated efficiently GST-P3, when each was used alone (Fig. 3A, lane 1 and 6, respectively). However, the addition of increasing amounts of PRMT1v7 did not influence the PRMT1v1 methyltransferase activity toward Sam68 in these assays (Fig. 3A, lanes 2–5). The same result was obtained when the competition assay was performed using v7 with v6 or any of the other PRMT1 isoforms (Fig. 3B and data not shown, respec-
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FIGURE 2. PRMT1 isoforms enzymatic activity and substrate specificity in vitro. A, amino acid sequences of the known RG-rich containing methylation substrates used in our in vitro methylation assays. B, in vitro methylation assays were performed by incubating the recombinant substrates with equal amounts of purified His$_6$-tagged PRMT1 protein isoforms v1 to v7 in the presence of [³H]AdoMet as a methyl donor. Methylation reactions were resolved by SDS-PAGE, gels were stained with Coomassie Brilliant Blue to visualize protein bands, and ³H-labeled proteins were visualized by fluorography. All fluorographs were exposed at $-80^\circ\text{C}$ for 1 week. In vitro methylation of GST-Sam68 and MRE11 are shown as representative examples (left and middle panels). Methylation reactions were performed with purified PRMT1v1 and v7 using the P3 (Gly-285 to Pro-308) fragment of Sam68 (right panel). Fluorography was performed as described above using the same conditions. C, mean methylation activity ± S.E. of each isoform is expressed as an arbitrary unit derived from the band intensity normalized to the amount of isoform and substrate seen on the Coomassie stain, as determined by densitometry. These values were then plotted and are grouped by substrates. Each methylation assay has been reproduced at least three times, and one-way analysis of variance statistical analysis indicated that differences in means were significant ($\alpha < 0.0001$; 95% confidence intervals). D, the same data are represented normalized relative to the mean methylation activity of v1 toward Sam68 (set as 1; dotted line) and grouped by PRMT1 isoforms. E, table summarizing the relative in vitro methyltransferase activity of each PRMT1 variant toward the RG-rich substrates. $+$ and $-$ signs are representative of the relative activity of each isoform for each substrate following the scale shown on the right.

FIGURE 3. PRMT1v7 does not act as a dominant negative for the activity of other isoforms. A, methylation assays were performed as described above with constant amounts of the GST-P3 substrate and PRMT1v1 but in the presence of increasing concentrations of PRMT1v7. Coomassie staining is shown below the fluorograph to confirm the relative abundance of v1 and v7. B, the same experiment was performed with the PRMT1v6 and -v7 pair.

Hence, the functional significance of the v7 variant remains unknown.

To determine whether these observations can be generalized to additional endogenous substrates, hypomethylated cellular extracts prepared from PRMT1$^{-/-}$ ES cells were labeled in vitro using the His$_6$-tagged PRMT1 isoforms in the presence of [³H]S-adenosyl-L-methionine ([³H]AdoMet). Consistent with our observations on purified substrates, PRMT1v1 to -v6 efficiently methylated cellular proteins, whereas PRMT1v7 showed no activity (Fig. 4A). As was observed with purified substrates, PRMT1v3 and -v4 exhibited lower enzymatic activity toward endogenous proteins (Fig. 4A, lanes 3 and 4). However, all PRMT1 isozymes methylated an apparently similar set of cellular proteins, and differences in substrate specificity were observed mainly between PRMT1v1 and -v2 (marked with a dot in Fig. 4A; compare lanes 1 and 2). The absence of more drastic differences in the methylation pattern was not due to the activity of endogenous methyltransferases in the lysates, since PRMT1 ES cell lysates were heat-inactivated before the methylation assays and lacked detectable methyltransferase activity (data not shown).

We reasoned that reducing the complexity of the protein lysates used as substrates would result in an increased PRMT/substrates ratio, which in turn might help reveal differences in specificity between the isoforms. PRMT1 ES cells were fractionated into cytoplasmic, nuclear, and insoluble fractions (using the QProteome nuclear protein kit from Qiagen). Quality of cellular fractionation was assessed by Western blotting using antibodies against the nuclear...
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FIGURE 4. PRMT1 isoforms have distinct substrate specificity toward endogenous substrates. A, 15 μg of endogenous protein substrates from mouse PRMT1-/- ES cells were incubated with equal amounts of purified His<sub>6</sub>-tagged PRMT1 protein isoform v1 to v7 in the presence of [<sup>3</sup>H]AdoMet. All methylation reactions were resolved by SDS-PAGE, gels were stained in Coomassie to visualize protein bands, and [<sup>3</sup>H]-labeled proteins were revealed by fluorography. Fluorographs were exposed at –80 °C for 1 week. Bands that show differences between isoforms are indicated with a dot. B, cellular fractionation was assessed by Western blot (IB) with antibodies against the nuclear protein Sam68 and the cytoplasmic protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH). C, mouse PRMT1-/- ES cell extracts were fractionated using the QProteome nuclear protein kit (Qiagen). All obtained fractions were then dialyzed against 50 mM sodium phosphate buffer, pH 7.5. In vitro methylation assays were performed as described in A with 10 μg of total proteins. Fluorographs for total (T), cytoplasmic (C), nuclear (N), and nuclear insoluble (I) fractions are shown. Bands that show differences between isoforms are indicated with an asterisk.

protein Sam68 and the cytoplasmic protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Fig. 4B). All obtained fractions were dialyzed against sodium phosphate buffer and used for in vitro methylation assays as above, with purified PRMT1v1 and -v6 enzymes. Again, no major differences in the profile of methylated bands were observed in the total (T) and cytoplasmic (C) cell extract fractions for each isoform (Fig. 4C, compare lanes 1–2 with lanes 5–6, respectively). Strikingly, drastic differences were found in the methylation profile of PRMT1v6 when incubated with the nuclear and insoluble fractions (Fig. 4C, compare lanes 3–4 with lanes 7–8). Taken together, these results show that the unique sequences at the N terminus of PRMT1 isoforms can influence substrate specificity.

N-terminal Sequences of PRMT1 Can Influence Subcellular Localization—Because cellular fractionation has uncovered differences in substrate specificity between certain PRMT1 isoforms, we next wanted to investigate the role of unique N-terminal sequences on PRMT1 intracellular distribution. Plasmid constructs expressing the different PRMT1 isoforms fused to a C-terminal EGFP tag were generated and used to transiently transfect HeLa cells grown on glass coverslips. Intracellular distribution of each PRMT1-GFP isoform was assessed 36 h post-transfection using fluorescence microscopy (Fig. 5A). Equal expression of each PRMT1-GFP fusion was confirmed by anti-GFP immunoblotting (Fig. 5B). Cells expressing PRMT1v3, -v4, -v5, and -v6 showed an even distribution of the GFP fusion proteins between the nucleus and the cytoplasm (Fig. 5A, respective panels). Cells expressing PRMT1v1 and -v7, however, presented a more intense nuclear staining (Fig. 5A, respective panels). Strikingly, PRMT1v2 expression resulted in a predominantly cytoplasmic staining concentrated around the nuclear compartment (Fig. 5A). These observations were quantified by plotting the fluorescence intensity distribution of representative PRMT1-GFP fusions signals relative to the exclusively nuclear signal distribution of 4',6-diamidino-2-phenylindole (Fig. 5A, graphs next to each series of panels). Intracellular localization was also confirmed using confocal microscopy (data not shown).

To confirm that the observed differential subcellular localization of PRMT1 isoforms was not due to overexpression and/or the GFP tag, we used cellular fractionation to assess the distribution of the endogenous PRMT1 variants 1 and 2. HeLa cells were fractionated as above, and an equal proportion of each fraction was resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted using the polyclonal anti-PRMT1 antibodies described above. Consistent with the distribution observed for transfected PRMT1v2-GFP, endogenous PRMT1v2 is detected at higher levels in the cytoplasmic fractions of HeLa and HS578T cells (Fig. 5C, left and right panel, respectively). Taken together, these results show that the alternatively spliced N terminus of PRMT1 can direct its intracellular localization.

The Alternative PRMT1 Exon 2 Encodes a Leucine-rich Nuclear Export Signal—Because PRMT1v2 is the only isoform predominantly located in the cytoplasm, we sought to identify the amino acid sequence responsible for this effect. We focused our attention on the sequences encoded by exon 2 since PRMT1v2 is the only isoform harboring this exon (Fig. 6A). The amino acid sequence encoded by exon 2 was submitted to the
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A

EGFP  DAPI  Merge  Quant. Intensity Profile

PRMT1 v1

PRMT1 v2

PRMT1 v3

PRMT1 v4

PRMT1 v5

PRMT1 v6

PRMT1 v7

B

C

HeLa

HeLa

T  C  N

Hs578T

T  C  N

IB  eEGFP

MW (kDa)

0

50

32

1 2 3 4 5 6 7 8 9

1 2

PRMT1

V2

V1

PRMT1

V2

V1

Sam68

GAPDH

Sam68
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Complex Genomic Structure at the 5’-End of the PRMT1 Gene Leads to the Expression of Multiple PRMT1 Isoforms—
The human PRMT1 gene locus was originally named Hrpm112 because of its high sequence similarity with yeast Saccharomyces cerevisiae Hmt1 (23). This early study had identified three different mRNAs (termed v1–v3) varying at their 5’ end, likely arising from the same gene through alternative splicing. The existence of v1–v3 at the RNA level was further confirmed in a later study where the genomic locus was more precisely mapped and sequenced (24). Close inspection of available ESTs and comparison with complete genomic sequences strongly suggested the existence of previously unidentified exons upstream from the exon harboring the AUG start codon for v1–v3 (32). We reported here the detailed genomic organization of ~1 kilobase of sequence upstream from that previously identified AUG that can yield, through a complex pattern of alternative splicing, at least seven different protein isoforms (Fig. 1A). Among these novel isoforms, v4 is the only mRNA where translation initiation actually starts at a new AUG located in the upstream 5’ exon. For isoforms v3 and v5, inclusion of alternative exons e2 and e3 in the message introduces an in-frame STOP codon (UGA) and results in translation initiating at the next AUG in exon 3. The occurrence of in-frame premature STOP codons can sometimes result in mRNAs getting targeted and degraded through the so-called “nonsense-mediated mRNA decay” pathway (54). This pathway is thought to be triggered when such an in-frame STOP codon is located ≥50–55 nt upstream of an exon-exon junction (54). Hence, following this rule, the mRNAs for v3 and v5 should not trigger the nonsense-mediated mRNA decay pathway since the in-frame STOP codon is located at 48 and 33 nt, upstream of the closest exon-exon junction, respectively. However, recent developments suggest that this rule does not necessarily apply to every mRNA and whether or not an mRNA is subjected to nonsense-mediated mRNA decay needs to be determined experimentally (55–57).

According to EST databases, a number of different mRNAs can lead to the production of the v5 and v6 proteins from various possible pairing combinations of exons e1a, e1b, e2, and e3 (Fig. 1A). The presence of these alternative 5’ exons in mRNAs, as detected by RT-PCR (Fig. 1, B and C, and data not shown) strongly argues for the existence of at least one additional TSS upstream of exon 1a. However, we cannot rule out the existence of TSSs associated with each individual alternative 5’ exon, and further studies will be required to determine precisely the contribution of alternative promoters and/or alternative splicing of 5’ exons in the regulation of PRMT1 gene expression. Interestingly, these newly identified alternative splicing patterns would lead to the production of mRNAs with distinct 5’-untranslated

DISCUSSION

In this study we describe the complex genomic organization in the 5’-end of the PRMT1 gene that can produce up to seven protein isoforms expressed in a tissue-specific manner. Biochemical characterization of these seven isoforms revealed that they are all active, except for v7, and that their unique N-terminal sequences can confer distinct substrate specificity. Moreover, we demonstrate that the amino acid sequence unique to v2 contains a CRM1-dependent NES that regulates its subcellular localization. Finally, we report the differential expression of these PRMT1 splicing variants in breast cancer cell lines.
regions (e.g. v3, v5, and v6). These unique 5'-untranslated regions might contribute to regulating the expression of these mRNAs post-transcriptionally, by affecting their stability and/or translation efficiency, a phenomenon that looks to be more common than previously appreciated (58).

PRMT1v1–v3 represent the vast majority of human ESTs with 251, 31, and 113 representative clones listed in the most recent Aceview data base. As was reported previously (24), these three major isoforms are present in every tissue we have tested, although the relative amount of each isoform does vary between tissues (Fig. 1B). In our hands the expression of all PRMT1 isoforms was very low in brain, although we have been able to amplify the major isoforms by increasing the PCR cycle numbers or the amount of input cDNA (data not shown). This observation is consistent with the original Northern blot results of Scott et al. (23), which showed strong expression of PRMT1 in fetal brain but weak expression in the adult tissue. This profile has also recently been confirmed at the protein level in mice, where PRMT1 expression was found to be high during mouse embryonic development but then decreased rapidly after birth (59).

Expression of the minor isoforms is more restricted to specific tissues, with PRMT1v4 found only in heart and v5 found only in pancreas, whereas v7 was expressed mainly in heart and skeletal muscles (Fig. 1B). Interestingly, Scott et al. (23) noted the presence of smaller bands in adult heart and skeletal muscles on Northern blots; these likely correspond to v4 and/or v7. Finally, we did not detect the presence of PRMT1v6 in any normal tissues tested, even at higher cycle numbers, but did detect expression in specific breast cancer cell lines (Fig. 1C and see below).

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served in mouse, and it was previously shown that they can methylate different, but overlapping sets of proteins in PRMT1−/− ES cell extracts (31). Intriguingly, the addition of an N-terminal His tag to these variant enzymes abolished the differences in substrate specificity (31). Using a similar assay, we now report that differences in substrate specificity can also be observed between human PRMT1v1 and -v2, and in addition, PRMT1v3 and -v4 display reduced enzymatic activity (Fig. 4A). However, the differences were not as drastic as those observed with the mouse isoforms (31), which could be a consequence of using mouse ES cell extracts as a source of substrates in combination with human PRMT1 isoforms. In addition, Pawlak et al. (31) were producing the PRMT1 isoforms in insect cells, which could reflect the need for post-translational modifications for the full spectrum of differences to be observed. Nevertheless, significant differences were revealed between PRMT1v1 and -v6 upon fractionation of protein extracts used for the methylation assays (Fig. 4C). For one, this could be explained by a lower complexity of the protein extracts, which likely reflects more accurately the enzyme:substrate ratio found in vivo. Alternatively, it is possible that the fractionation protocol permits the extraction and solubilization of specific substrates that were not present in the sonicated extracts used in Fig. 4A. This would be consistent with the fact that PRMT1 is known to methylate many proteins associated with the nuclear matrix (e.g. hnRNPs) and chromatin.

Alignment of known mammalian PRMTs revealed that the N-terminal domains are the more divergent between various PRMTs. Although the function of these PRMT-specific sequences remains unclear in most cases, it was suggested that they may contribute to enzymatic activity and/or substrate specificity (e.g. see Refs. 20 and 21). According to the PRMT1 three-dimensional crystallographic structure (32), the N-terminal sequences would be juxtaposed to an already fairly exposed α helix (αY) located in the space between the AdoMet and substrate binding pockets (see Fig. 6A and Ref. 32). In a structural analysis of the PRMT3 enzyme, an additional α helix (termed αX) was observed N-terminal to αY and shown to be important for cofactor binding and catalysis (60). Furthermore, deletion of the N-terminal part of Hmt1, the yeast PRMT1 homologue, significantly impairs its oligomerization and reduces its methyltransferase activity (22). This is consistent with PRMT1v7 showing no detectable activity in our hands (Figs. 2 and 3), since this isoform does not harbor the αY helix and the invariant YFXYX motif (32, 60). Because our in vitro competition assays suggest that PRMT1v7 does not act as a dominant negative (Fig. 3 and data not shown), the functional significance of having a methylase-dead version of PRMT1 in cells remains unclear. Nevertheless, we cannot rule out the possibility that v7 might behave differently in cells or could be highly specific for a substrate(s) not present in mouse ES cell extracts.

In contrast, the αX and αY helices as well as the actual AdoMet binding domain are all present in PRMT1v1-v6, which suggests that at least according to existing structural information, these isoforms should all interact with the AdoMet cofactor to a similar extent. Interestingly, no x-ray diffraction data were obtained for the extreme N-terminal domain of the protein crystallized by Zhang and Cheng (32), which is usually an indication that this portion of the structure is highly mobile in solution. Hence, we envisage a model where the extreme N-terminal sequences would fold back and make crucial contacts with the substrate during catalysis. This could in turn provide a molecular mechanism for the differences in overall activity and substrate specificity observed between the various PRMT1 N-terminal variants. Because PRMT1 is known to interact relatively stably with its substrates, this model would predict that PRMT1 N-terminal variants should interact with different subsets of proteins in cells. We have performed a large scale protein-protein interaction screen with purified His-tagged PRMT1v1 and -v2 using the Invitrogen ProtoArray® Human Protein Microarray Version 3.0. In support of the above hypothesis, a number of proteins interacted with both isoforms, but a subset showed reproducible preference for either v1 or v2. Hence, this approach, which looks at thousands of interactions in one experiment, supports our in vitro methylation assay data and strongly suggests that N-terminal unique sequences can influence PRMT1 protein-protein interactions.

**Identification of a Functional NES in PRMT1 Alternative Isoform v2**—The current study uncovered a functional CRM1-dependent NES in PRMT1 isoform v2 (Figs. 5 and 6). This means that by regulating the alternative splicing of PRMT1 exon 2, cells can modulate the nuclear versus cytoplasmic pools of PRMT1 isoforms that possess distinct protein-protein interaction profiles and substrate specificity. We have mapped this NES to amino acids 15–24 of PRMT1v2 (15VATLANGMSL24), which matches the consensus derived from the sequence of multiple, experimentally tested, functional NESs (Ref. 53 and Fig. 6C). The use of leptomycin B or the expression of NES mutants (V15A/L18A) resulted in v2 being localized in both the nucleus and cytoplasm (Fig. 6D), suggesting that PRMT1v2 normally enters the nucleus and is then efficiently exported to the cytoplasm through its CRM1-dependent NES. This is in agreement with a recent study showing that PRMT1 is a highly mobile protein in both the nucleus and cytoplasm and can relocalize between compartments upon general inhibition of methylation (18). Our results might also provide an explanation for some of the discrepancy observed in the literature regarding the intracellular localization of PRMT1 (4, 18, 19, 25, 61, 62). For instance, depending on the antibodies used to detect the endogenous PRMT1, only a specific isoform(s) may be recognized. Alternatively, the use of constructs with N-terminal tags or with truncated N-terminal domains might also result in differences in localization. Most strikingly, it was recently reported that PRMT1 localized to nuclear splicing speckles, a subnuclear compartment enriched in splicing factors (19). However, we have not observed the localization of any of the PRMT1 isoforms to nuclear speckles, and we have found the localization of PRMT1 isoforms to be actinomycin D-independent (data not shown). Arginine methylation by PRMT1 was shown to regulate the intracellular localization of many substrates (e.g. see Ref. 4, 63, and 64 and references therein), and, at least for RIP140, this effect seems to be mediated by PRMT1 directly.

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4 J. Côté, unpublished data.
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modulating interaction with the nuclear export receptor CRM1 (64). This suggests the possible existence of a regulatory loop involving PRMT1, and it will be interesting to investigate how the various isoforms (and in particular, v2) respond to changes in arginine methylation in cells.

Arginine Methylation and Cancer?—A number of studies have provided evidence linking arginine methylation with anti-proliferative signaling or with a protective role against cancer (13, 17, 65–72). However, these observations may not be applicable to all PRMTs and all cancer types. For example, the fact that PRMT1 and CARM1 can act as coactivators of nuclear receptors makes them likely candidates to be overexpressed in hormone-dependent cancers, including breast cancer (2). Indeed, it has been found that CARM1 overexpression correlates with androgen independence in human prostate carcinomas (73, 74). It has also been observed that increased methylation of a PRMT6 substrate correlated with breast tumors of higher metastatic potential (75, 76). Consistent with these predictions, we observed that overall PRMT1 mRNA levels are increased in a panel of breast cancer cell lines and in a human breast tumor (Fig. 1 C and D, respectively). Moreover, the relative balance of alternatively spliced PRMT1 isoforms is altered in these cell lines when compared with immortalized normal breast cells. Among the major isoforms, the PRMT1v2 mRNA, which encodes for an enzyme that is mostly localized to the cytoplasm, is selectively increased relative to v1 (Fig. 1F). Most strikingly, among the minor variants, PRMT1v7 was overexpressed in all of the breast cancer lines tested, and PRMT1v6 was detected in BT-20 and MDA-MB-231 (Fig. 1C). A second RT-PCR product of slightly higher size was also reproducibly detected in BT-20 cells using v6-specific primers (i.e. a forward primer specific for exon 1a). The size of that band would be compatible with an EST-predicted mRNA containing alternative exons e2 and e3 in addition to 1a and 1b (see Fig. 1A), but further experiments will be required to confirm the identity of this product.

At the protein level, immunoblotting with our polyclonal antibody against PRMT1 did not reveal major differences in the level of v1 between normal and breast cancer cell line extracts. This suggests that translational and/or post-translational mechanisms (like protein stability) may be involved in regulating PRMT1v1 protein levels in cancer cells. However, we cannot rule out the possibility that differences in PRMT1v1 level could be masked by the fact that our polyclonal antibody also recognizes other isoforms that likely co-migrate with v1 because of their very similar molecular weights. In contrast, an increase in the relative abundance of PRMT1v2 is clearly observed in breast cancer cell lines (Fig. 1, E and F). Although seen in every breast cancer cell lines tested, this increase in the v2 protein relative to v1 is most prominent for the Hs578T, BT-549, and MCF-7 cell lines (see Fig. 1F). This correlates well with the relative mRNA expression profiles observed for these cell lines as well as for the BT-20 cell line, which shows a smaller increase both at the level of mRNA and protein. In contrast, the levels of the v2 mRNA and protein do not correlate well in the MDA-MB-231 and T-47D cell lines, suggesting the contribution from additional regulatory mechanisms at the translational or post-translational level in these breast cancer cell lines.

Finally, overall levels of \(\omega-N^G,N^G\)-dimethylarginine-containing proteins were increased in all the breast cancer cell lines tested (Fig. 1F). Although this profile does not correlate with the cell line-specific overexpression of PRMT1v2, other type I PRMTs could also contribute to this increase in \(\omega-N^G,N^G\)-dimethylarginine methylation. Indeed, as mentioned above, CARM1 and PRMT6 were found to be overexpressed in certain cancers, and we have also found these PRMTs to be expressed at higher levels in the panel of breast cancer cell lines used in this study.\(^5\) Further work will be required to address the functional significance of PRMT overexpression in various cancers as well as the general role(s) that arginine methylation pathways might play in cellular transformation.

In addition to PRMT1, alternative splicing was also reported for CARM1 and PRMT7 pre-mRNAs (77, 78), although extensive biochemical characterization of the respective PRMT isoforms generated is still lacking. As we have shown in the current study, alternative splicing can lead to the production of PRMT variants with a wide array of different biochemical and regulatory properties. It will be important to explore this additional level of complexity in the expression of other PRMTs in future studies.

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