Journal of Cellular Biochemistry 95:902–917 (2005)

Physical and Functional Interactions Between the Human DNMT3L Protein and Members of the De Novo Methyltransferase Family

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Abstract The de novo methyltransferase-like protein, DNMT3L, is required for methylation of imprinted genes in germ cells. Although enzymatically inactive, human DNMT3L was shown to act as a general stimulatory factor for de novo methylation by murine Dnmt3a. Several isoforms of DNMT3A and DNMT3B with development-stage and tissue-specific expression patterns have been described in mouse and human, thus bringing into question the identity of the physiological partner(s) for stimulation by DNMT3L. Here, we used an episome-based in vivo methyltransferase assay to systematically analyze five isoforms of human DNMT3A and DNMT3B for activity and stimulation by human DNMT3L. Our results show that human DNMT3A, DNMT3A2, DNMT3B1, and DNMT3B2 are catalytically competent, while DNMT3B3 is inactive in our assay. We also report that the activity of all four active isoforms is significantly increased upon co-expression with DNMT3L, albeit to varying extents. This is the first comprehensive description of the in vivo activities of the poorly characterized human DNMT3A and DNMT3B isoforms and of their functional interactions with DNMT3L. To further elucidate the mechanism by which DNMT3L stimulates DNA methylation, we have mapped in detail the domains that mediate interaction of human DNMT3L with human DNMT3A and DNMT3B. Our results show that the C-terminus of DNMT3L is the only region required for interaction with DNMT3A and DNMT3B and that interaction takes place through the C-terminal catalytic domain of DNMT3A and DNMT3B. The implications of these findings for the regulation of de novo methyltransferases and genomic imprinting are discussed. This article contains Supplementary Material available at http://www.mrw.interscience.wiley.com/suppmat/0730-2312/suppmat/2005/95/chen.html. J. Cell. Biochem. 95: 902–917, 2005.

Key words: DNA methyltransferase; genomic imprinting; epigenetics; DNMT3L; DNMT3A; DNMT3B

Mammalian DNA methyltransferases (DNMTs) catalyze the addition of methyl groups to cytosines usually located within CpG dinucleotides. DNA methylation, particularly in CpG-rich promoters, has been shown to silence gene expression in a heritable manner. The transcriptional silencing associated with 5-methylcytosine is required for fundamental physiological processes such as embryonic development, protection against intragenomic parasites, X-chromosome inactivation and genomic imprinting [Bestor, 2000; Reik and Walter, 2001; Li, 2002]. In addition, aberrant promoter methylation and inappropriate silencing of tumor suppressor genes has recently emerged as a major cause leading to cancer [Robertson, 2001; Jones and Baylin, 2002; Laird, 2003].

Four mammalian active DNA methyltransferases have been identified and genetic and biochemical studies have indicated functional differences between these enzymes. DNMT3A and DNMT3B are considered to be de novo DNA

Grant sponsor: National Institutes of Health (to JRM); Grant number: GM64378-01; Grant sponsor: National Institutes of Health (to ADR); Grant number: GM50575; Grant sponsor: California Cancer Research Coordinating Committee (to FC); Grant number: 3-441038-34384; Grant sponsor: IRG-American Cancer Society (to F.C.); Grant number: 95-125-07.

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Received 24 September 2004; Accepted 20 December 2004
DOI 10.1002/jcb.20447

methyltransferases, since they show equal activity on unmethylated and hemi-methylated DNA in vitro [Okano et al., 1998; Gowher and Jeltsch, 2001] and are responsible for de novo methylation during early embryonic development [Okano et al., 1999]. DNMT1, on the other hand, shows preference for hemi-methylated DNA substrates, is associated with replication foci, and is therefore, considered a maintenance DNA methyltransferase [Leonhardt et al., 1992; Chuang et al., 1997; Pradhan et al., 1999]. Together, these enzymes can generate new DNA methylation patterns and maintain them through cell divisions [Chen et al., 2003; Riggs and Xiong, 2004]. Recently, the DNMT2 protein was shown to possess a weak DNA methyltransferase activity, although its biological role remains unclear [Hermann et al., 2003; Kunert et al., 2003]. The DNMT3L protein was assigned to the DNMT3 family on the basis of the high conservation of its N-terminal PHD-like zinc finger domain with the corresponding domains of DNMT3A and DNMT3B [Aapola et al., 2000]. The C-terminus of DNMT3L also shows partial homology to the C-terminal regions of DNMT3A and DNMT3B, although key residues involved in catalysis are not conserved in DNMT3L. This raised the possibility that DNMT3L might not function as a DNA methyltransferase proper. Targeted mutagenesis of the *Dnmt3L* gene in mouse, however, revealed that Dnmt3L is required for the methylation of most imprinted loci in male and female germ cells [Bourc'his et al., 2001; Hata et al., 2002; Kaneda et al., 2004]. Since Dnmt3L was shown to co-localize and co-immunoprecipitate with Dnmt3a and Dnmt3b, it was suggested that it could contribute to the regulation of these de novo DNA methyltransferases [Hata et al., 2002].

Previous work showed that while DNMT3L is inactive on its own, co-expression of human DNMT3L and mouse Dnmt3a results in much more robust methylation of target sequences than is observed when Dnmt3a is expressed alone. The enhancement of methyltransferase activity was dependent upon the functional catalytic site of Dnmt3a and appeared to not change target-sequence specificity. This led to the suggestion that DNMT3L is a general stimulatory factor for de novo methylation by Dnmt3a [Chedin et al., 2002]. While this study suggested a role for DNMT3L, the identity of the biological target(s) of DNMT3L remained uncertain. Dnmt3a is expressed at low levels

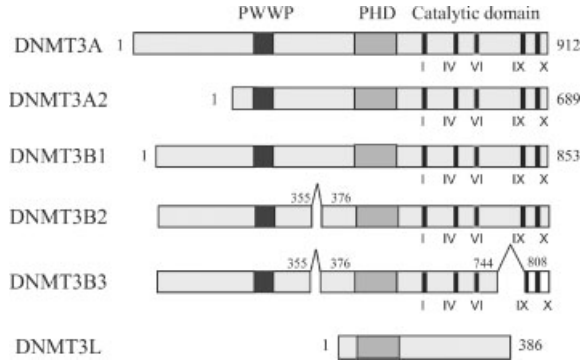

Fig. 1. Schematic diagrams of human DNMT3A, DNMT3A2, DNMT3B1, DNMT3B2, DNMT3B3, and DNMT3L. Three conserved regions (PWWP, PHD, and catalytic domain) are indicated along with the total number of amino acids in each protein. Conserved methyltransferase motifs are indicated by roman numerals.

in almost all analyzed somatic tissues [Chen et al., 2002], suggesting it may have a housekeeping function. In contrast, a shorter isoform of Dnmt3a, named Dnmt3a2 (Fig. 1), is highly expressed in ES cells and shows restricted expression in tissues that undergo active de novo methylation, including ovary and testis [Chen et al., 2002]. Therefore, it has been suggested that Dnmt3a2 is more likely to play a major role in carrying out de novo methylation during early development and in the regulation of genomic imprinting [Chen et al., 2002; Chen and Li, 2004]. In addition, several isoforms of mouse and human DNMT3B have been described [Okano et al., 1998; Robertson et al., 1999; Xie et al., 1999; Chen et al., 2002], and it is possible that some of these isoforms could interact with and be stimulated by DNMT3L.

While the enzymatic properties of several mouse Dnmt3a and Dnmt3b isoforms have been well studied in vitro and in vivo [Hsieh, 1999; Aoki et al., 2001; Chen et al., 2002, 2003], only little is known about the enzymatic activities of human DNMT3A and DNMT3B isoforms [Kim et al., 2002]. We report here the systematic study of five isoforms of the human DNMT3A and DNMT3B proteins for activity and their ability to undergo stimulation by human DNMT3L in vivo. Our results show that the human DNMT3A, DNMT3A2, DNMT3B1, and DNMT3B2 isoforms are catalytically active and are all stimulated upon co-expression with DNMT3L. Human DNMT3B3, however, had no detectable activity in our system and was not stimulated by DNMT3L. We have also mapped

in detail the physical interaction domains between DNMT3L, DNMT3A, and DNMT3B1. Our results show that interaction involves the C-terminal catalytic domain of DNMT3A and DNMT3B and the C-terminus of DNMT3L. This is consistent with the fact that DNMT3L can stimulate DNMT3A2, which lacks the N-terminal 223 aa of DNMT3A.

MATERIALS AND METHODS

Expression Vectors and Episomes

The human *DNMT3A* and *DNMT3A2* coding regions were amplified from plasmid pKH2020 (a generous gift from Dr. En Li, Massachusetts General Hospital) by PCR using a forward primer containing an EcoRI site and a reverse primer containing a BamHI site (the sequences of all primers used in this study are available upon request). The amplified *DNMT3A* and *DNMT3A2* PCR fragments were cloned into EcoRI and BamHI sites of pGADT7 and pBluescript SK, respectively. The resulting constructs were called pGADT7/DNMT3A and pBluescript SK/DNMT3A2. Full-length cDNAs of human *DNMT3B2* and *DNMT3B3* were amplified by RT-PCR from total RNA of human HepG2 cells and cloned into the EcoRI–BamHI sites of pBluescript KS, generating plasmids pBluescript KS/DNMT3B2 and pBluescript KS/DNMT3B3, respectively, and their identity was verified by DNA sequencing. Full-length *DNMT3B1* cDNA was constructed in multiple steps. First, *DNMT3B2* cDNA was excised from pBluescript KS/DNMT3B2 as an EcoRI–BamHI fragment and was subsequently cloned into the corresponding sites of pGEX-6P-1 (Amersham Pharmacia Biotech, Piscataway, NJ) to generate pGEX-6p-1/DNMT3B2. The I.M.A.G.E. Consortium (LLNL) expressed sequence tags (EST) clone 3504148 (GenBank accession number BE279495) [Lennon et al., 1996] corresponding to *DNMT3B1* was purchased from Incyte Genomics (St. Louis, MO) and was sequenced to confirm the presence of sequences encoding aa 356–375 of DNMT3B1, which are absent in DNMT3B2 due to alternative splicing. The *DNMT3B2* sequences in pGEX-6P-1/DNMT3B2 between the unique SphI and HindIII sites were then replaced by the corresponding SphI–HindIII fragment isolated from the I.M.A.G.E. clone, resulting in pGEX-6P-1/DNMT3B1. A human EST clone containing the full-length human *DNMT3L* sequence (I.M.A.G.E. cDNA clone 3138514; GenBank accession number BE281379) [Lennon et al., 1996] was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Sequencing revealed that this EST clone encoded a variant of DNMT3L that is 1 aa shorter than the longer isoform (386 vs. 387 aa, respectively). Additionally, this clone carries an arginine to glycine substitution at position 278 compared with the curated *DNMT3L* cDNA sequence (NM_013369). Here, we changed glycine 278 back to arginine using a PCR-based megaprimer approach [Sarkar and Sommer, 1990] and cloned the full-length cDNA into the EcoRI and SalI sites of pBluescript KS. Details of constructions are available upon request. The resulting plasmid, pBluescript KS/DNMT3L, was sequenced to confirm the introduction of AGG for arginine at position 278 and the absence of any additional mutation.

For transient expression of N-terminal Myc-tagged proteins in mammalian cells, we recloned the coding regions of the DNA methyltransferases into a modified version of the pcDNA3 expression vector (Invitrogen, Carlsbad, CA), referred to as pcDNA3/Myc. In the modified vector, the region of the multiple cloning site located between the HindIII and NotI sites was replaced by a DNA segment containing a Kozak consensus sequence, an ATG start site and the Myc epitope sequence followed by EcoRI and BamHI restriction sites. The full-length cDNA for DNMT3A, DNMT3A2, DNMT3B1, DNMT3B2, and DNMT3B3 were excised as EcoRI–BamHI fragments from corresponding pBluescript KS, pGEX-6P-1, or pGADT7 vectors and subcloned into the EcoRI and BamHI sites of pcDNA3/Myc. For construction of pcDNA3/Myc-DNMT3L, the full-length *DNMT3L* sequence was excised from pBluescript KS/DNMT3L as an EcoRI–XhoI fragment and cloned into EcoRI–XhoI sites of pcDNA3/Myc. Mutants with cysteine-to-alanine substitution at the active site (DNMT3A2 C487A and DNMT3B1 C651A) were created using a PCR-based megaprimer method [Sarkar and Sommer, 1990], and cloned into the EcoRI–BamHI sites of pcDNA3/Myc. Details of constructions are available upon request. For transient expression of N-terminal FLAG-tagged proteins in mammalian cells, a modified version of pcDNA3, pcDNA3/FLAG, was constructed as described above for pcDNA3/Myc, except that the Myc epitope was exchanged

for a FLAG epitope. The full-length cDNA for *DNMT3A2* and *DNMT3L* were excised from corresponding pcDNA3/Myc constructs as EcoRI–BamHI and EcoRI–XhoI fragments, respectively, and cloned into pcDNA3/FLAG to generate plasmids pcDNA3/FLAG-DNMT3A2 and pcDNA3/FLAG-DNMT3L.

For the yeast two-hybrid assays, we used pGBKT7 for creating bait constructs and pGADT7 for creating prey constructs (Clontech, Palo Alto, CA). The two-hybrid constructs for full-length *DNMT3A*, *DNMT3A2*, *DNMT3B1*, *DNMT3B2*, and *DNMT3L* were made as follows: pGADT7/DNMT3A was created by direct cloning of a PCR product into pGADT7, as described above. pGBKT7/DNMT3A was constructed by ligating the EcoRI–BamHI fragment from pGADT7/DNMT3A into the corresponding sites of pGBKT7. For full-length DNMT3A2, DNMT3B1, and DNMT3B2, the EcoRI–BamHI fragment was first excised from pcDNA3/Myc-DNMT3A2, pGEX-6P-1/DNMT3B1, and pBluescript KS/DNMT3B2, respectively. These fragments were subsequently cloned into the EcoRI and BamHI sites of pGBKT7 or pGADT7. The full-length *DNMT3L* two-hybrid constructs were made by ligating the EcoRI–SalI fragment from pBluescript KS/DNMT3L into the same sites of pGBKT7 or a modified pGADT7, in which a SalI restriction site was introduced into the multiple cloning site. Deletion constructs for mapping the interaction domains between DNMT3L and DNMT3B1 were created as follows: DNA fragments encoding aa 1–172, 1–303, 47–386, and 173–386 of DNMT3L were amplified by PCR using primers containing EcoRI and SalI restriction sites at the 5′ and 3′ end, respectively. The PCR fragments were ligated into EcoRI and SalI sites of a modified pGADT7, in which a SalI restriction site was introduced into the multiple cloning site. DNA fragments encoding aa 1–558, 383–853, 559–853, and 596–853 of DNMT3B1 were amplified by PCR using primers containing EcoRI and BamHI restriction sites at the 5′ and 3′ end, respectively. The PCR fragments were ligated into EcoRI and BamHI sites of pGADT7. All PCR amplified sequences were confirmed by DNA sequencing after cloning.

For expressing glutathione S-transferase (GST) fusion proteins in *E. coli*, the pET-41a vector (Novagen, Madison, WI) was first modified by replacing the sequence between the unique SpeI and HindIII sites with a linker containing the recognition sequences for EcoRI, KpnI, SacII, and BamHI restriction sites. The resulting expression vector, pET-GST, provides an EcoRI site immediately downstream of the GST coding sequence. To generate GST-DNMT3A deletion mutants, DNA fragments corresponding to aa 1–617, 224–912, and 618–912 of DNMT3A were amplified by PCR and cloned into the EcoRI–BamHI sites of pET-GST. Expression plasmids for GST-DNMT3L and GST-DNMT3L deletion mutants (aa 1–303 and 173–386) were constructed by cloning the EcoRI–XhoI fragments from the respective pGADT/DNMT3L vectors into pET-GST.

Episomes used as methylation targets have been described previously [Chedin et al., 2002]. All episomes contain the EBNA1/OriP replication system derived from the Epstein–Barr virus and can be stably maintained. pCLH22 has been described previously and carries a luciferase gene [Hsieh, 1994]; pFC19 contains a 940 bp fragment corresponding to the differentially methylated CpG island of the human *SNRPN* imprinting center (IC) in place of the luciferase gene [Chedin et al., 2002]. pFC60 contains a 1.2 kb region of the mouse *Air*/*Igf2r* IC [Chedin et al., 2002].

Cell Lines and Transfections

The HEK293 EBNA1 cell line (293c18, ATCC) was used in all experiments and transfections were performed using the calcium phosphate method in a 6-well plate, as described previously [Wigler et al., 1979; Hsieh, 1994]. Briefly, 500 ng of each desired expression vector and episome target was used for each 35 mm well. Cells were allowed to grow for 2–3 days before being transferred in a 100-mm diameter plate. Upon reaching confluence, cells were harvested for episomal DNA extraction according to the Hirt method [Hirt, 1967]. No selection was applied during cell culture. All transfections were repeated independently. Expression of DNMT3A, DNMT3A2, DNMT3B1, DNMT3B2, DNMT3B3, and DNMT3L after transient transfection into 293c18 cells was verified by Western blots using anti-Myc antibody. No significant change in the expression of DNMT3A, DNMT3A2, DNMT3B1, or DNMT3B2 could be detected upon co-transfection of DNMT3L, thus allowing comparison of methylation efficiencies in the presence or absence of DNMT3L (data not shown).

Analysis of DNA Methylation

Two independent methods were used to assess DNA methylation. DNA samples recovered from transfections were digested with excess methylation-sensitive restriction enzymes such as HpaII and HhaI (New England Biolabs, Beverly, MA) overnight. The resulting DNA fragments were then resolved by agarose gel electrophoresis and transferred onto Nylon membranes by capillary transfer. The membranes were then probed with ^{32}P-radiolabeled DNA fragments, as indicated. After appropriate washes, the membranes were exposed to PhosphorImager screens and scanned using a Molecular Dynamics Typhoon running ImageQuant version 5.2. Genomic bisulfite sequencing [Clark et al., 1994] was used as an alternative and more quantitative method to measure DNA methylation. In this case, the DNA samples were first digested by PstI and sodium bisulfite treatment was performed. A 287 bp fragment of the human *SNRPN* IC containing 23 CpG sites was chosen for analysis, as described earlier [Chedin et al., 2002]. The overall efficiency of bisulfite conversion was superior to 99% as estimated from control samples in which no exogenous DNA methyltransferase was expressed.

Yeast Two-Hybrid Analysis

The Matchmaker GAL4 two-hybrid system 3 (Clontech) was used. The *Saccharomyces cerevisiae* strain AH109, which contains the *HIS3* and *ADE2* reporter genes, was co-transformed with appropriate pairs of bait and prey constructs and selected on synthetic dropout medium lacking tryptophan and leucine (SD/-Trp/-Leu). The interaction was evaluated by growth of resulting transformants on selective medium lacking adenine, histidine, tryptophan and leucine (SD/-Ade/-His/-Trp/-Leu) using the following procedures. Individual transformant colonies were grown to log-phase at 30°C in synthetic dropout medium (SD/-Trp/-Leu). The cultures were normalized based on optical density at 600 nm to 1 optical density U/ml and serially diluted in water in tenfold steps. Four different dilutions of each transformant were spotted on non-selective and selective plates, respectively (SD/-Trp/-Leu and SD/-Ade/-His/-Trp/-Leu). The plates were incubated at 30°C and pictures were taken after 2, 3, 4, and 6 days.

Co-Immunoprecipitation Experiments

HEK293 cells were transiently cotransfected in 10 cm dishes with pcDNA3/FLAG-DNMT3L, or an empty vector, together with expression constructs for the indicated Myc-tagged DNMT3 isoforms using FuGENE 6 transfection reagent according to manufacturer's instructions (Roche, Indianapolis, IN). Cells were harvested 36 h after transfection and lysed for 30 min at 4°C in 1 ml lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) containing complete mini EDTA-free protease inhibitor cocktail (Roche). Cell lysates were clarified by centrifugation for 10 min at 14,000 rpm at 4°C. Cleared lysates were then incubated with 30 μl of anti-FLAG M2 affinity gel (Sigma, St. Louis, MO) for 3 h at 4°C with rotation. The affinity gels were then washed five times with washing buffer (same as the lysis buffer but with 0.1% Triton X-100). Proteins bound to the affinity gel were eluted by competition with FLAG peptide, separated by SDS–PAGE and transferred to PVDF membranes. Western blot analyses were then performed with an anti-FLAG M2 monoclonal antibody (Sigma) and anti-Myc antibody (9E10, Roche), respectively.

GST Pull-Down Assays

GST and GST fusion proteins were expressed in *E. coli* Rosetta (DE3)pLysS (Novagen). When the OD_{600} reached 0.6, 1 mM isopropyl-β-D-thiogalactopyranoside was added to the cultures to induce protein expression and cultures were grown for an additional 3 h. The harvested cells were lysed by sonication and soluble GST fusion proteins were purified using glutathione-agarose beads (Sigma). For pull-down assays, human HEK293 cells transiently transfected with expression plasmids encoding FLAG-tagged DNMT3A2 or FLAG-tagged DNMT3L were lysed for 30 min at 4°C in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol (DTT), 1% Triton X-100) containing complete mini EDTA-free protease inhibitor cocktail (Roche). Equal amounts of cleared cell lysates were incubated with approximately 8 μg of GST alone or GST fusion proteins bound to 25 μl of glutathione-agarose beads for 2–3 h at 4°C with rotation. The beads were then washed five times with washing buffer (same as the lysis buffer but with 0.1% Triton X-100). Proteins bound to the

beads were eluted with SDS loading buffer for 5 min at 95°C, separated by SDS–PAGE and transferred to PVDF membranes. Western blots were then performed with an anti-FLAG M2 monoclonal antibody (Sigma).

RESULTS

DNMT3L Enhances De Novo Methylation by DNMT3A and DNMT3B Isoforms

We constructed expression vectors for the human DNMT3A, DNMT3A2, DNMT3B1, DNMT3B2, and DNMT3B3 proteins and measured their de novo methylation activity, as well as the effect of co-expression of DNMT3L on their catalytic activity in vivo. For this, we transfected 293c18 cells with various expression vectors together with a replicating Epstein–Barr virus-based episome carrying sequences of interest that were used as methylation targets. Cells were cultured for 7–8 days after transfection and the episomal DNA was recovered by the Hirt method [Hirt, 1967]. DNA methylation was then analyzed using methylation-sensitive restriction enzymes such as HpaII or HhaI, followed by agarose gel electrophoresis, Southern blotting and hybridization with a radiolabeled probe complementary to the region of interest. In the case of pFC19, which contains a ~1 kb region of the human *SNRPN* IC [Chedin et al., 2002], larger DNA fragments indicative of de novo DNA methylation activity were readily observed when DNMT3A was expressed (Fig. 2A, compare lanes 1 and 2). Co-expression of DNMT3A with DNMT3L resulted in an increased proportion of methylated DNA molecules (compare lanes 2 and 3). This increase was particularly visible for high-molecular weight species (indicated by an arrow), which correspond to molecules for which most, or all, the restriction sites on the target episome were methylated. DNMT3L, when expressed alone, did not result in any significant methylation activity. DNMT3A2, a shorter isoform of DNMT3A, when expressed alone resulted in robust methylation of the target molecules (lane 4). Co-expression of DNMT3A2 with DNMT3L resulted in a significant increase of the proportion of methylated molecules and in the appearance of very highly methylated molecules (Fig. 2A, compare lanes 5 and 4). DNMT3B1, which corresponds to the longest DNMT3B isoform, was able to methylate the target molecules when expressed alone. Co-expression of DNMT3B1 with DNMT3L resulted in a clear increase in the proportion of

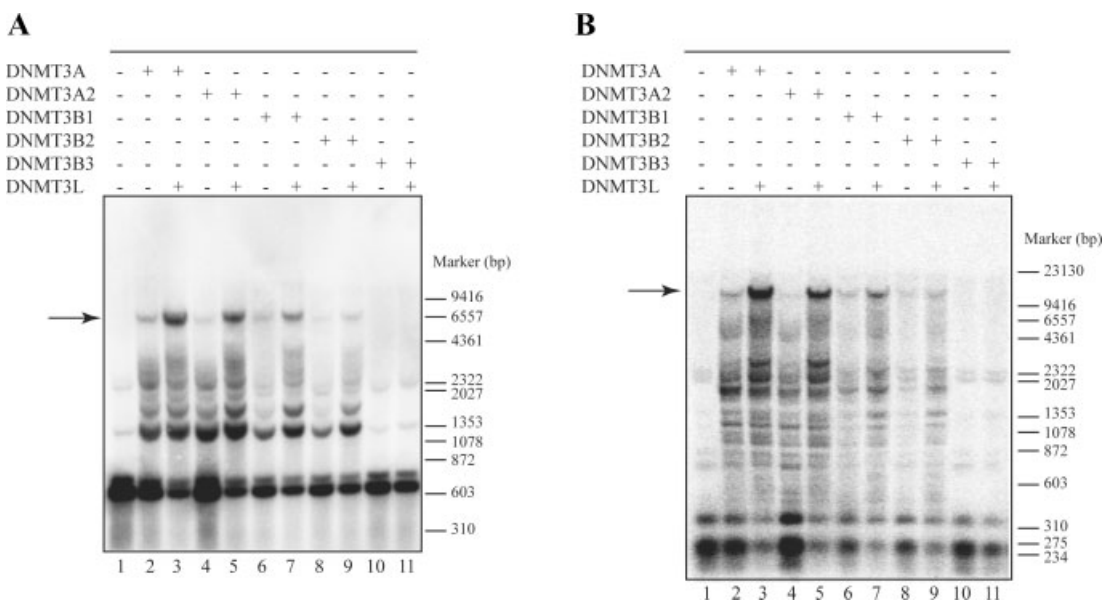

Fig. 2. DNMT3L stimulates de novo methylation. **A**: Episomes carrying a portion of the *SNRPN* IC were co-transfected with various expression vectors, as indicated by a (+) sign. DNA samples harvested from these cells were digested with the methylation-sensitive enzyme HpaII and analyzed by Southern blot. A probe complementary to the *SNRPN* region was used. DNA methylation can be detected as fragments of increased size. High-molecular weight species indicative of very high level of methylation are indicated by an arrow. **B**: As above except that pCLH22, which carries the luciferase gene instead of *SNRPN*, was used. The membrane was probed with a fragment complementary to the luciferase gene. Molecular weight markers are indicated on the side of the gels.

methylated DNA molecules and in the production of highly methylated DNA species (Fig. 2A, compare lanes 7 and 6). DNMT3B2, which lacks exon 10 due to alternative splicing, showed a consistent and reproducible methylation activity when expressed alone (lane 8) and its co-expression with DNMT3L resulted in an increase of DNA methylation (Fig. 2A, compare lanes 9 and 8). Expression of DNMT3B3 alone, which lacks the C-terminal exons 21 and 22, did not result in any appreciable methylation of the target molecules, suggesting that this isoform has no detectable activity in our system (lane 10). This is consistent with previous findings that mouse Dnmt3b3 does not have DNA methylation activity [Aoki et al., 2001; Chen et al., 2003]. Also, its co-expression with DNMT3L did not result in any increased methylation (lane 11). In all cases, no high-molecular weight bands were observed in control digests by MspI (data not shown).

To test whether DNMT3L can also stimulate methylation by human DNMT3 isoforms at non-imprinted sequences, plasmid pCLH22, which carries a luciferase gene instead of the *SNRPN* IC, was used for similar cotransfection experiments. Results described for the human *SNRPN*-containing episome (Fig. 2A) also held true for this target (Fig. 2B). In all cases, co-expression of DNMT3L resulted in an increase in the proportion of methylated molecules and in the production of highly methylated species (indicated by an arrow). DNMT3B3, as was observed above, showed no activity when expressed alone or when co-expressed with DNMT3L. Similar data was obtained for a third target molecule (pFC60) for which the human *SNRPN* IC had been replaced by a portion of the murine *Igf2r/Air* IC (data not shown). Altogether, our data show that DNMT3L enhanced DNA methylation activity by DNMT3A, DNMT3A2, DNMT3B1, and DNMT3B2, irrespective of DNA sequence.

Enhancement of De Novo Methylation Activity Is Due to Stimulation of the Active Partner

To demonstrate whether the stimulation of isoforms of human DNMT3A and DNMT3B is dependent on their catalytic activity, we created mutants of DNMT3A2 and DNMT3B1 by mutating the conserved cysteine residue of the PC motif to an alanine residue using a PCR-based megaprimer approach [Sarkar and Sommer, 1990]. The substitution of cysteine with alanine removes the critical SH-containing side chain of cysteine, which is known to be important for catalysis by mouse Dnmt3a and Dnmt3b [Kimura et al., 2002]. The expression vectors for mutant proteins were then transfected into 293c18 cells either alone or together with the DNMT3L expression vector and their resulting activity was compared to the one observed with the corresponding wild-type expression vectors (Fig. 3). The episome target used here was pFC19. Methylation by the DNMT3A2 C-A mutant was drastically reduced compared to its wild-type counterpart (compare lanes 3–2). Similarly, the enhanced activity observed in the presence of DNMT3A2 and DNMT3L (lane 4) was drastically reduced when the mutant DNMT3A2 was expressed with DNMT3L (lane 5). These results are similar to the ones reported for the murine Dnmt3a protein, using a cysteine to serine mutation [Chedin et al., 2002]. The DNMT3B1 C-A mutant also displayed a much reduced activity compared to its wild-type counterpart (compare lanes 8–7). Expression of DNMT3L, while it enhanced the activity of the wild-type enzyme, did not restore any activity to the mutant protein (compare lanes 10 and 9). Enhancement of DNA methylation by DNMT3L, therefore, requires the presence of a functional catalytic site in the partner protein. These results strongly suggest that DNMT3L functions by

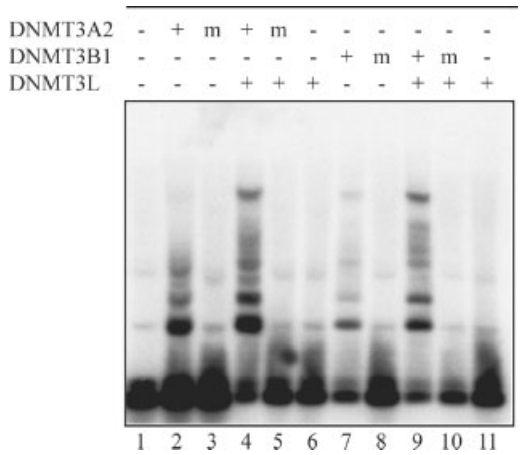

Fig. 3. Enhancement of DNA methylation by DNMT3L is due to a stimulation of de novo methylation. pFC19 was co-transfected with expression vectors for DNMT3A2, DNMT3B1, and DNMT3L, as indicated. ''m'' indicates that the corresponding vector carried a mutation in the catalytic cysteine, which was mutated to an alanine. Methylation was assessed by Southern blot after HpaII digestion using a probe complementary to the SNRPN IC, as described in Figure 2.

TABLE I. Summary of Bisulfite Methylation Sequencing Data

	DNMT3A		DNMT3A2		DNMT3B1		DNMT3B2	
DNMT3L	−	+	−	+	−	+	−	+
^{me}CpG	178	294	126	333	99	244	117	139
n	25	19	31	24	37	24	36	20
% Methylation	30.9	67.3	17.7	60.3	11.6	44.2	14.1	30.2
Fold stimulation	2.2		3.4		3.8		2.1	
P value (t-test)	0.003		<0.0001		<0.0001		0.03	

^{me}CpG corresponds to the number of methylated CpG sites observed for a 287 bp region from the *SNRPN* fragment carried by pFC19 in each sample. The sample size (n) corresponds to the number of independent DNA molecules analyzed. The efficiency of CpG methylation (% methylation) is calculated by dividing the number of methylated sites observed by the total number of CpG sites in each sample. The fold stimulation corresponds to the ratio of the efficiency observed in the added presence of DNMT3L relative to the efficiency observed in the absence of DNMT3L. The corresponding P values from unpaired t-tests comparing sample sets are indicated.

stimulating the activity of its de novo methyltransferases partners.

Quantification of the Stimulation Effect Using Genomic Bisulfite Sequencing

To obtain more detailed information about the stimulation provided by DNMT3L, we analyzed DNA methylation levels by genomic bisulfite sequencing. For this, a 287 bp fragment corresponding to the human SNRPN IC and containing 23 CpG sites was analyzed [Chedin et al., 2002]. For each condition, two independent samples were treated independently with sodium bisulfite and two independent PCR reactions were performed for each sample. The PCR products were cloned and individual DNA molecules sequenced. Results are summarized in Table I and are expressed as a percentage of total methylation in each sample (number of methylated CpGs/total number of CpG sites). In the absence of any exogenous DNA methyltransferase, a low-methylation activity (1.2%) was detected (data not shown). This probably corresponds to a low endogenous methylation activity for this particular cell line, since faint methylated bands were also observed for the corresponding samples by Southern blot analysis (Fig. 2A). In agreement with the Southern blot data, human DNMT3A showed significant methylation activity, giving about 30% methylation of all CpG sites. DNMT3A-catalyzed methylation was increased a further 2.2-fold in the added presence of DNMT3L. Note however, that the extent of stimulation for DNMT3A might be underestimated since the resulting methylation activity in the presence of DNMT3L probably approaches a plateau representing a maximal value. Stimulation was observed at all CpG sites (see supplemental Fig. 1. All supplementary material for this article is available at http://www.mrw.interscience.wiley.com/suppmat/0730-2312/suppmat/2005/95/chen.html). For DNMT3A2, addition of DNMT3L resulted in a statistically highly significant 3.4-fold stimulation of DNA methylation ($P < 0.0001$, t-test). As seen in the individual methylation profiles, this stimulation was observed at all CpG sites, and resulted in the generation of a large percentage of highly methylated DNA fragments (Fig. 4A). The efficiency of methylation in the presence of DNMT3B1 alone was 11.6%. In the added presence of DNMT3L, methylation by DNMT3B1 showed a strong, statistically highly significant, 3.8-fold stimulation (Fig. 4B and Table I). DNMT3B2 alone resulted in a methylation efficiency of about 14% (Table I, see supplemental Fig. 1). However, the extent of stimulation by DNMT3L observed for DNMT3B2 was markedly lower (2.1-fold). Although the difference between these two samples was statistically significant when analyzed by an unpaired t-test, it became non-significant when the non-parametric Mann–Whitney was used (data not shown). Altogether, these data show that DNMT3L stimulates de novo methylation by four active isoforms of DNMT3A and DNMT3B, but to various extents.

Human DNMT3L Physically Interacts With Active Isoforms of Human DNMT3A and DNMT3B

Previously, it was shown that the mouse Dnmt3L protein co-localizes with the Dnmt3a and Dnmt3b methyltransferases in transiently transfected COS cells [Hata et al., 2002]. This raised the possibility that stimulation of de novo methylation by DNMT3L might be achieved through direct physical interaction. To gain further insights into the mechanism of stimulation, we characterized in detail the interactions between human DNMT3L and two human de novo methyltransferases. We first used yeast

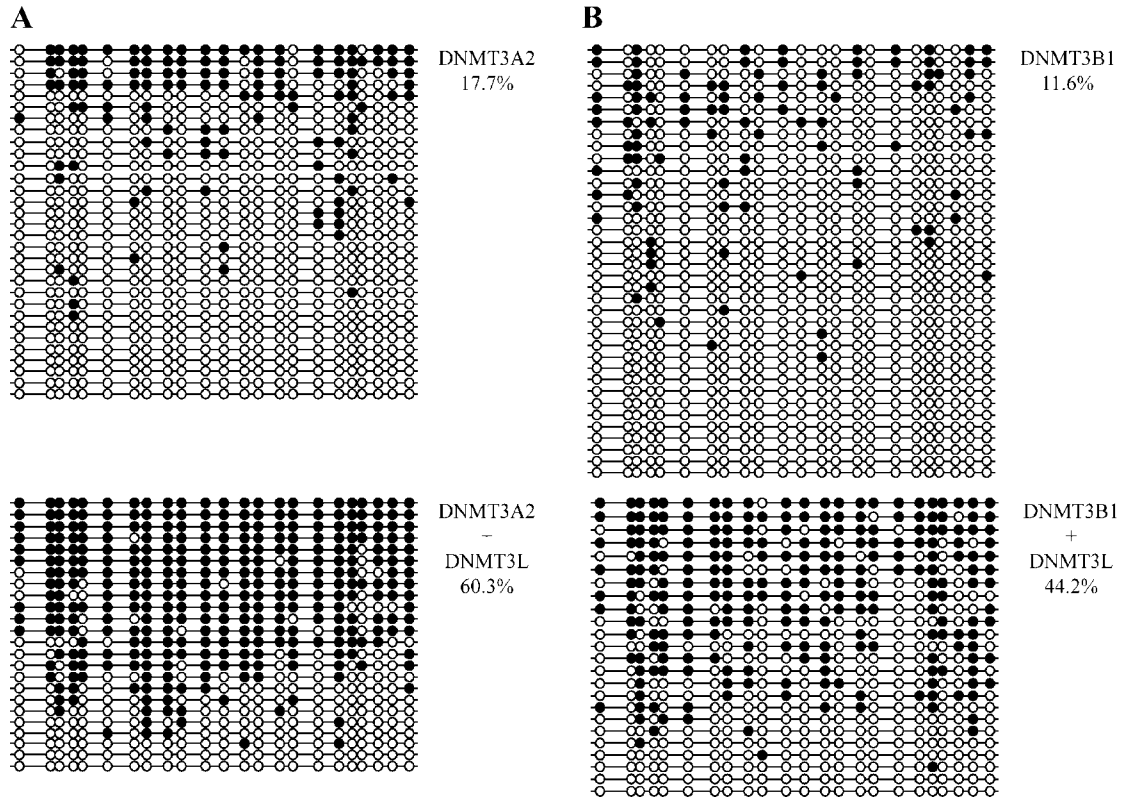

Fig. 4. Genomic bisulfite sequencing analysis of DNA methylation. A 287 bp fragment of the *SNRPN* IC containing 23 CpG sites was analyzed by bisulfite sequencing. Each line represents an individual DNA molecule. Closed circles represent methylated CpG sites; open circles represent unmethylated CpG sites. **A**: DNA was harvested from transfections in which the DNMT3A2 enzyme was expressed alone (**top**) or together with DNMT3L (**bottom**). The overall percentage of methylation in each sample is indicated on the right. **B**: As above except DNMT3B1 was expressed either alone (**top**) or together with DNMT3L (**bottom**).

two-hybrid assay to test whether human DNMT3L, like its mouse counterpart, is able to interact with the four active isoforms of human DNMT3A and DNMT3B proteins. The DNMT3L protein and DNMT3A, DNMT3A2, DNMT3B1, and DNMT3B2 were expressed as fusions with the GAL4 DNA binding domain (Gal4DB) or the GAL4 activation domain (Gal4AD). Expression vectors for these fusion proteins were introduced as appropriate pairs into the yeast strain AH109. Growth on selective plates lacking histidine and adenine indicated physical interaction between the two fusion proteins. As shown in Figure 5A, when Gal4DB-DNMT3L and Gal4AD-DNMT3A fusion proteins were co-expressed, the *HIS3* and *ADE2* reporter genes were both activated, allowing growth on selective medium and indicating a physical interaction. This interaction was specific, since no interaction could be detected when Gal4DB-DNMT3L fusion protein was co-expressed with the Gal4 activation domain alone (Gal4AD), or Gal4AD-DNMT3A was co-expressed with the Gal4 DNA binding domain alone (Gal4DB) (Fig. 5A). Strong interaction was also observed in a reciprocal two-hybrid test where DNMT3L was fused to Gal4AD and DNMT3A was fused to Gal4DB, further confirming the results (data not shown). Similar data were observed for DNMT3A2, DNMT3B1 and DNMT3B2 (Fig. 5A). We conclude that human DNMT3L strongly interacts with all four active isoforms of human DNMT3A and DNMT3B in a yeast two-hybrid assay.

To further determine whether these interactions can occur in mammalian cells, we transiently expressed Myc-tagged DNMT3A, DNMT3A2, DNMT3B1, or DNMT3B2 proteins in human HEK293 cells with or without FLAG-tagged DNMT3L protein. Whole-cell extracts were then subjected to immunoprecipitation using anti-FLAG M2 affinity gel. As shown in the upper panel of Figure 5B, immunoprecipitation of the FLAG-tagged DNMT3L resulted in the co-precipitation of Myc-tagged DNMT3A, DNMT3A2, DNMT3B1, or DNMT3B2 proteins,

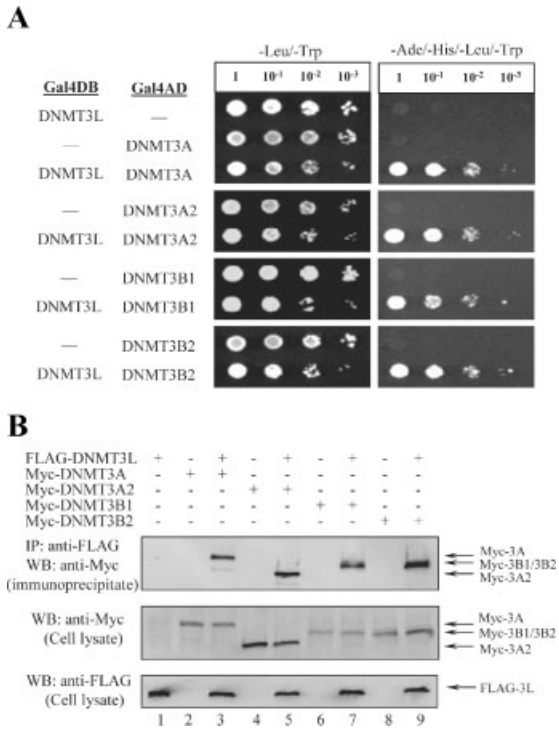

Fig. 5. Human DNMT3L physically interacts with active isoforms of human DNMT3A and DNMT3B. **A**: Yeast two-hybrid analysis. DNMT3L was fused with the GAL4 DNA-binding domain (Gal4DB or bait). DNMT3A, DNMT3A2, DNMT3B1, and DNMT3B2 were fused to the GAL4 activation domain (Gal4AD or prey). ''—'' represents empty vectors containing only Gal4DB or Gal4AD, which were used as negative controls. The yeast strain AH109 was co-transformed with the indicated pair of bait and prey plasmids. Tenfold serial dilutions of the transformants were spotted onto a plate containing synthetic medium lacking tryptophan and leucine (-Leu/-Trp) and a plate containing synthetic medium lacking adenine, histidine, tryptophan, and leucine (-Ade/-His/-Leu/-Trp). Growth of yeast cells on the -Ade/-His/-Leu/-Trp plate represents positive interaction, while growth on the -Leu/-Trp plate confirms the presence of both fusion constructs. Photographs were taken after 3 days of growth at 30°C. **B**: Co-immunoprecipitation experiments. HEK293 cells in 10-cm dishes were co-transfected with either pcDNA3/FLAG-DNMT3L (3 μg) or empty vector (3 μg) together with expression constructs for Myc-DNMT3A (5 μg), Myc-DNMT3A2 (5 μg), Myc-DNMT3B1 (10 μg), or Myc-DNMT3B2 (10 μg), as indicated. Whole cell extracts were immunoprecipitated (IP) with anti-Flag M2 affinity gel, and the immunoprecipitated proteins were analyzed by Western blot (WB) using anti-Myc antibody (**upper panel**). WB analysis of cell lysates confirmed the expression of FLAG-DNMT3L (**lower panel**) and Myc-tagged DNMT3 isoforms (**middle panel**). The positions of the various DNMT3A and DNMT3B isoforms are indicated by arrows on the right side. Myc-3B1/3B2 refers to the position of the closely migrating DNMT3B1 (**lanes 6** and **7**) and DNMT3B2 (**lanes 8** and **9**) isoforms.

respectively (lanes 3, 5, 7, and 9). The Myc-tagged DNMT3 isoforms could not be detected in the absence of FLAG-DNMT3L, suggesting that the observed co-immunoprecipitations were specific (lanes 2, 4, 6, and 8). These results demonstrate that DNMT3L associates with active isoforms of DNMT3A and DNMT3B in vivo when they are co-expressed in HEK293 cells.

The C-Terminal Catalytic Domains of DNMT3A and DNMT3B1 Interact With DNMT3L

In addition to their conserved C-terminal catalytic domain, DNMT3A and DNMT3B each contain a large N-terminal region, which has been thought to play regulatory roles. The N-terminal region consists of a conserved PWWP domain, a conserved PHD-like zinc finger domain, and a variable region that shows little sequence similarity between DNMT3A and DNMT3B. To determine which domain(s) is responsible for the interaction with DNMT3L, various truncation mutants of DNMT3B1 were constructed in frame with the GAL4 activation domain. Each truncation mutant was tested for interaction with full-length DNMT3L fused to the GAL4 DNA binding domain in the yeast two-hybrid assay. N-terminal truncations of DNMT3B1 up to position 383, which removes the conserved PWWP domain, or to position 559, which removes both the PWWP domain and the PHD domain, did not affect the interaction with DNMT3L (Fig. 6A). Accordingly, the entire N-terminal region (aa 1–558) did not promote any interaction with DNMT3L. By contrast, the C-terminal catalytic domain alone (aa 559–853) fully retains the ability to interact with DNMT3L. This is consistent with the observation that the C-terminal domain of mouse Dnmt3b interacts with human DNMT3L [Margot et al., 2003]. Interestingly, deletion of an additional 37 aa (construct 596–853), which includes the conserved DNA methyltransferase motif I, resulted in complete loss of interaction with DNMT3L. These results show that the C-terminal catalytic domain of DNMT3B1 is necessary and sufficient for interaction with DNMT3L.

Deletion analysis using a yeast two-hybrid assay also revealed that the C-terminal domain of DNMT3A mediates the binding to DNMT3L (see supplemental Fig. 2). This result is consistent with our observation that DNMT3L stimulated both DNMT3A and DNMT3A2, suggesting that the interaction of the two proteins did not involve the first 223 aa of DNMT3A. To confirm this result biochemically, we also carried out in vitro GST pull-down experiments. Three bacterially expressed and

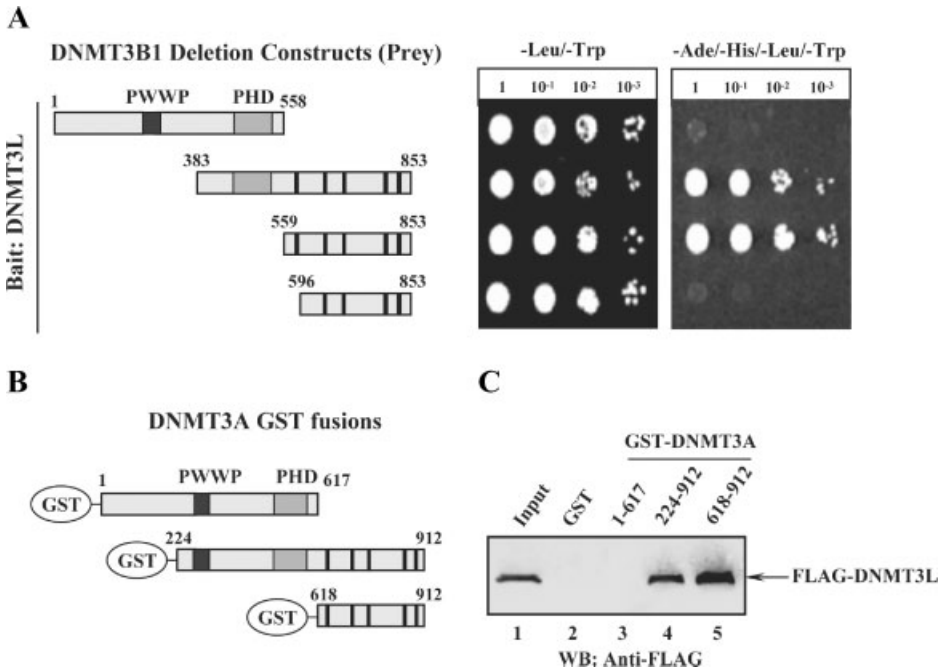

Fig. 6. The C-terminal catalytic domains of DNMT3A and DNMT3B1 interact with DNMT3L. **A**: Yeast two-hybrid analysis. DNMT3B1 deletion mutants used for the deletion analysis are depicted with start and end coordinates relative to DNMT3B1. Yeast AH109 cells were co-transformed with plasmids encoding for full-length DNMT3L fused to Gal4DB (Bait) and the indicated DNMT3B1 deletion mutants fused to Gal4AD (Prey). The growth of yeast cells on plates lacking adenine, histidine, tryptophan and leucine (-Ade/-His/-Leu/-Trp) represents positive interaction. No interactions were observed when DNMT3B1 deletion constructs were co-transformed with plasmids expressing Gal4DB alone (data not shown). Photographs were taken after 3 days of growth at 30°C. **B**: Schematic representations of DNMT3A deletion mutants fused to GST. The coordinates refer to the position of amino acids in DNMT3A. **C**: GST pull-down experiments. Whole cell extracts from HEK293 cells expressing FLAG-DNMT3L were incubated with immobilized GST-DNMT3A deletion fusion proteins or GST alone. Bound proteins were analyzed by WB using anti-FLAG antibody. **Lane 1** represents 10% of the cell extracts used for GST pull-downs.

purified GST-DNMT3A deletion fusion proteins (Fig. 6B) were tested for interaction with FLAG-tagged DNMT3L transiently expressed in human HEK293 cells. In agreement with the yeast two-hybrid results, GST fusion proteins containing the C-terminal catalytic domain of DNMT3A (618–912, Fig. 6C, lane 5) efficiently pulled down FLAG-DNMT3L, whereas GST fusion with the entire N-terminal region (1–617, Fig. 6C, lane 3) did not. We also confirmed that GST-DNMT3A (224–912) (Fig. 6C, lane 4), which corresponds to the full-length DNMT3A2 protein, readily pulled down FLAG-DNMT3L. We conclude that both DNMT3A and DNMT3B1 interact with DNMT3L through their C-terminal catalytic domains.

The C-Terminal Region of DNMT3L Binds to DNMT3A and DNMT3B

The interaction region in DNMT3L was also mapped by yeast two-hybrid and GST pull-down analyses. Various DNMT3L truncation mutants fused to the GAL4 activation domain were constructed and tested independently for interaction with full-length DNMT3B1 fused to the GAL4 DNA binding domain. As shown in Figure 7A, deletion of the N-terminal 172 aa, which removes the entire conserved PHD-like domain, had no effect on the binding of DNMT3L to DNMT3B1. However, deletion of the C-terminal 83 aa eliminated the interaction, indicating the C-terminal region of DNMT3L (173–386) is necessary and sufficient for the DNMT3L-DNMT3B1 interaction. Similar two-hybrid data was also obtained for DNMT3A2 (see supplemental Fig. 3) and was further confirmed by GST pull-down analysis. As shown in Figure 7C, a GST fusion to the C-terminus of DNMT3L alone (173–386, lane 5) bound to FLAG-DNMT3A2 as efficiently as a GST fusion with the full-length DNMT3L protein (lane 3).

DISCUSSION

Proper control of cytosine methylation patterns is critical for mammalian development,

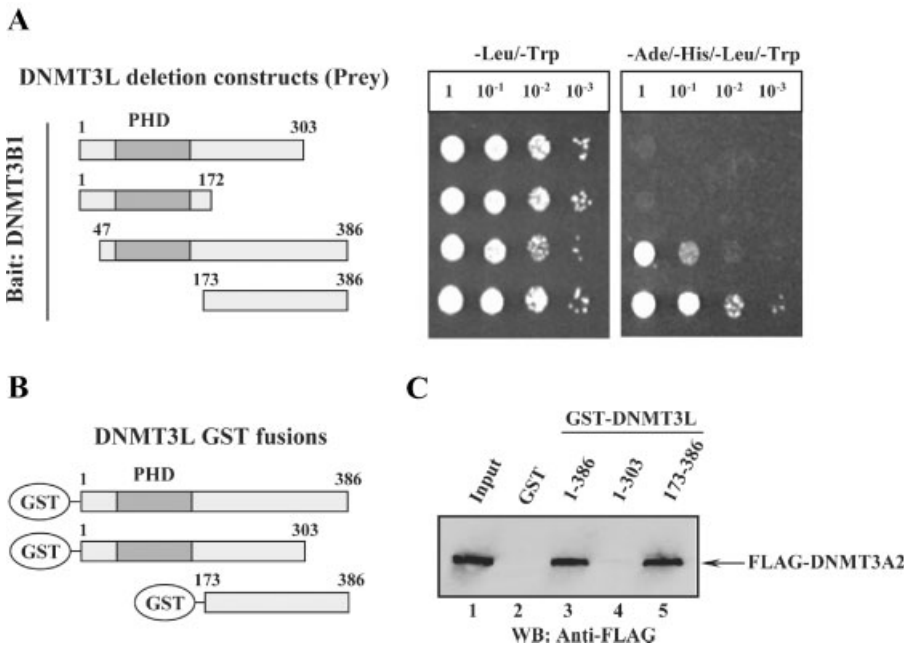

Fig. 7. The C-terminal region of DNMT3L binds to DNMT3A and DNMT3B. **A**: Yeast two-hybrid analysis. DNMT3L deletion mutants used for deletion analysis are depicted with start and end coordinates relative to DNMT3L. Yeast AH109 cells were co-transformed with plasmids encoding for full-length DNMT3B1 fused to Gal4BD and the indicated DNMT3L deletion mutants fused to Gal4AD. Positive interaction is scored by growth on -Ade/-His/-Leu/-Trp plates. No interactions were observed when DNMT3L deletion constructs were co-transformed with plasmids expressing Gal4BD alone (data not shown). Photographs were taken after 3 days of growth at 30°C. **B**: Schematic representation of DNMT3L deletion mutants fused to GST. The coordinates refer to the position of amino acids in DNMT3L. **C**: GST pull-down experiments. Whole cell extracts from HEK293 cells expressing FLAG-DNMT3A2 were incubated with immobilized GST-DNMT3L deletion fusion proteins or GST alone. Bound proteins were analyzed by WB using anti-FLAG antibody. **Lane 1** represents 10% of the cell extracts used for GST pull-downs.

and at least three active mammalian DNA methyltransferases, DNMT1, DNMT3A, and DNMT3B, are required for the precise establishment and maintenance of methylation patterns. Given the complexity and dynamics of methylation patterns and the limited number of DNA methyltransferases, it is likely that the control of methylation patterns is at least partly achieved through interaction with accessory factors. Dnmt3L is expressed during gametogenesis at a time when parental methylation imprints are acquired and mouse knockout studies have demonstrated that Dnmt3L participates in genomic imprinting [Bourc'his et al., 2001; Hata et al., 2002; Kaneda et al., 2004]. A recent study also showed that Dnmt3L is involved in the methylation of dispersed retrotransposons repeats in mouse male germ cells [Bourc'his and Bestor, 2004]. However, since murine and human DNMT3L do not possess intrinsic de novo methyltransferase activity, DNMT3L was thought to play a regulatory role. The data presented here, together with our previous studies [Chedin et al., 2002], suggest that DNMT3L is directly involved in setting up methylation patterns by physically interacting with DNMT3A and DNMT3B and stimulating their de novo methylation activity, irrespective of DNA sequence.

Our results show that all active isoforms of human DNMT3A and DNMT3B are stimulated by DNMT3L, although to different extents (Figs. 2 and 4, Table I). This suggests that all active isoforms are potential biological targets for stimulation by DNMT3L, provided they are expressed with the same tissue- and development stage-specificity. Mouse *Dnmt3L* is expressed in ES cells, in the chorion, as well as in growing oocytes in adult females and in perinatal prospermatogonia in males [Bourc'his et al., 2001; Hata et al., 2002; La Salle et al., 2004]. Human *DNMT3L* has been shown to be expressed in testis, ovary and thymus [Aapola et al., 2000], and a recent study found that it is also expressed in early embryo, but may not be expressed in oocytes. [Huntriss et al., 2004]. The DNMT3A and DNMT3B isoforms exhibit very different expression patterns during development. In mouse, *Dnmt3a* is expressed at low levels in almost all analyzed somatic tissues,

while the shorter isoform, *Dnmt3a2*, is highly expressed in ES cells and shows restricted expression in testis, ovary and thymus [Chen et al., 2002]. Mouse *Dnmt3b1* is highly expressed in ES cells and early embryos but is almost undetectable in most somatic tissues examined [Chen et al., 2002; Watanabe et al., 2002]. It has thus been postulated that Dnmt3a2 and Dnmt3b1 might carry out de novo methylation in the early embryo and serve to establish methylation patterns, while Dnmt3a probably plays a housekeeping function that may be involved in maintaining tissue-specific methylation patterns [Chen et al., 2003; Chen and Li, 2004]. In addition, human *DNMT3B1* was recently reported to be the only isoform of *DNMT3B* to be expressed in early embryo at a time when *DNMT3L* is also expressed [Huntriss et al., 2004]. Therefore, taken together with the expression data, our results raise the possibility that mouse and human DNMT3A2 and DNMT3B1 are potentially the major biological targets for stimulation by DNMT3L. A recent mouse knockout study revealed that *Dnmt3a*, but not *Dnmt3b*, was required for the methylation of imprinted genes in germ cells [Kaneda et al., 2004]. This suggests that Dnmt3a, or more likely the Dnmt3a2 isoform, functions together with Dnmt3L in setting methylation imprints in germ cells. Future studies using isoform-specific targeted deletions in mice might provide a definitive answer as to whether Dnmt3a2 is the only Dnmt3 isoform required for methylation imprinting. It remains possible that mouse and human DNMT3B represents a biological target for stimulation by DNMT3L in other as yet unidentified DNMT3L-regulated processes.

During the preparation of this manuscript, Suetake et al. [2004] reported that human DNMT3L stimulates the methylation activity of both mouse Dnmt3a and Dnmt3b in an in vitro system using purified recombinant proteins. This is in agreement with our in vivo data for human DNMT3A and DNMT3B, suggesting that the stimulatory effect of DNMT3L on the methylation activity of DNMT3A and DNMT3B is highly conserved between human and mouse. A previous study which found no significant stimulation of DNMT3B2 by DNMT3L [Chedin et al., 2002] can be explained on the basis that the expression vector used carried a version of DNMT3B2 with an extra 12 aa (MEPSPEPPSLES) at the N-terminus and that the expressed protein carried a Myc-tag at its C-terminus. In contrast, all proteins in the present study possessed short N-terminal Myc tags only. Since interaction of DNMT3L with DNMT3A and DNMT3B occurs through their respective C-termini, it is possible that these C-terminal tags interfered with the formation of the complex and resulted in a weaker stimulation. Nevertheless, even in the present study, the stimulation of DNMT3B2 by DNMT3L was the weakest, and its statistical significance is in question (Table I). Analysis of the DNA methylation patterns created by DNMT3A2 and DNMT3B1 using genomic bisulfite sequencing (Fig. 4) revealed that individual CpG sites were methylated at various efficiencies for both proteins. The range of methylation efficiencies at individual CpG sites was broad, varying from 3.2 to 32.2% for DNMT3A2 and from 0 to 32.4% for DNMT3B1. The high- and low-methylation sites differed between DNMT3A2 and DNMT3B1, suggesting that the variation in range might reflect sequence preference by these enzymes, as noted previously [Hsieh, 1999]. The limited size of the sample, however, does not allow the actual determination of putative sequence preferences. In the presence of DNMT3L, methylation was increased at all CpG sites irrespective of the sequence of the target DNA fragment. Interestingly, the methylation patterns observed in the presence of DNMT3L were more uniform than when DNMT3A2 or DNMT3B1 were expressed alone. For instance, the distribution of methylation levels at individual CpG sites was much narrower, ranging from 37.5 to 87.5% for DNMT3A2 and from 20.8 to 66.7% for DNMT3B1. This suggests that methylation in the presence of DNMT3L results in a "smoother" pattern of methylation where each CpG site has a high likelihood of becoming methylated.

In this study, we also characterized in detail the physical interaction of human DNMT3L with human DNMT3 methyltransferases. Our co-immunoprecipitation results demonstrate that DNMT3L physically associates with active isoforms of DNMT3A and DNMT3B when they are co-expressed in human HEK293 cells. This suggests that the observed stimulation of methylation activity by DNMT3L in our in vivo study might be the result of its interaction with the de novo methyltransferases. Domain mapping analysis reveals that the carboxy-terminal catalytic domain of DNMT3A and DNMT3B is

responsible for interaction with the carboxy-terminal half of DNMT3L (Figs. 6 and 7). Similar results have recently been reported for mouse Dnmt3a and Dnmt3b [Margot et al., 2003; Suetake et al., 2004], suggesting that the physical interactions of DNMT3L with DNMT3A and DNMT3B are conserved between human and mouse. Interestingly, all other cellular factors known to associate with mammalian DNA methyltransferases, with the exception of p23 binding to DNMT1, do so by binding to their N-terminal regulatory region [Robertson, 2002]. While p23 was reported to interact with the C-terminal catalytic domain of DNMT1, no effect on the catalytic activity of DNMT1 could be demonstrated and the functional role, if any, of this interaction remains elusive [Zhang and Verdine, 1996]. DNMT3L is, therefore, the first accessory factor able to physically interact with the catalytic domains of mammalian DNA methyltransferases, as well as to functionally stimulate their catalytic activity. The exclusive C-terminal binding of DNMT3L to DNMT3A and DNMT3B also suggests that DNMT3L may directly modulate the catalytic activity of target DNA methyltransferases. The precise mechanism of the stimulation remains to be determined. It was previously reported that the N-terminal PHD-like domain of DNMT3L directly interacts with the histone deacetylase HDAC1, resulting in transcriptional repression independently of the C-terminal region [Aapola et al., 2002; Deplus et al., 2002]. Our finding that DNMT3L binds to DNMT3A and DNMT3B solely by its C-terminal region suggests that this region is structurally and functionally distinguishable from the N-terminal transcription repression domain. Recent data indicate that the C-terminal region of DNMT3L on its own is capable of stimulating the activity of Dnmt3a and Dnmt3b in vitro [Suetake et al., 2004].

While the precise mechanism by which DNMT3L contributes to the establishment of genomic imprinting still needs to be elucidated, we suggest that its biological role as an imprinting regulator is exerted at least partly through its physical and functional interaction with its target de novo DNA methyltransferases. Stimulation of de novo methylation, as documented here, leads to the formation of extensively methylated patterns, which are a well-known characteristic of ICs. The requirement for maximal methylation levels at these loci probably stems from the need to escape the genome-wide demethylation wave that ensues shortly after fertilization. The degree of methylation of a given DNA fragment indeed directly correlates with its ability to be transmitted to the next generation [Howell et al., 1998; Lorincz et al., 2002]. In this way, germ cell-specific methylation marks can be inherited by the zygote as a parent-of-origin specific mark. However, several outstanding questions remain concerning the manner by which parental-specific methylation marks are acquired at ICs. In mouse, it is unclear if Dnmt3L contributes only to the enhancement of DNA methylation at specific loci, such as ICs, or alternatively, if it is involved in setting genome-wide methylation patterns. In the system used here, DNMT3L does not change sequence specificity. This suggests that if IC-specific targeting of DNA methylation occurs, an additional as yet unknown targeting factor may be required. It was recently reported that maternally methylated imprinted genes such as *Snrpn* could not be remethylated by expression of murine Dnmt3a, Dnmt3a2, or Dnmt3b1 in *Dnmt3a*$^{-/-}$ *Dnmt3b*$^{-/-}$ ES cells [Chen et al., 2003], while other regions of the genome could be. Considering that *Dnmt3L* is highly expressed in ES cells [Hata et al., 2002], this would indicate that maternally methylated regions might be protected from de novo methylation and/or that their methylation might require specific accessory factors only expressed in female germ cells. Finally, it remains to be established whether human DNMT3L also plays a role in the post-fertilization zygote, as suggested by recent expression data [Huntriss et al., 2004] and analysis of imprinting patterns [El-Maarri et al., 2001].

ACKNOWLEDGMENTS

We thank Dr. En Li for the generous gift of the DNMT3A cDNA clone.

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