Reconstitution and Mechanism of the Stimulation of de Novo Methylation by Human DNMT3L

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The DNMT3-like protein, DNMT3L, is required for germ line DNA methylation, although it is inactive as a DNA methyltransferase per se. Previous studies have shown that DNMT3L physically associates with the active de novo DNA methyltransferases, DNMT3A and DNMT3B, and stimulates their catalytic activities in a cell culture system. However, the mechanism by which DNMT3L stimulates de novo methylation remains unclear. Here, we have purified the full-length human DNMT3A2 and DNMT3L proteins and determined unique conditions that allow for the proper reconstitution of the stimulation of DNMT3A2 de novo methyltransferase activity by DNMT3L. These conditions include the use of buffers resembling physiological conditions and the preincubation of the two proteins. Under these conditions, maximal stimulation is reached at equimolar amounts of DNMT3L and DNMT3A2 proteins, and the catalytic efficiency of DNMT3A2 is increased up to 20-fold. Biochemical analysis revealed that whereas DNMT3L on its own does not significantly bind to the methyl group donor, S-adenosyl-L-methionine (SAM), it strongly increases the binding of SAM to DNMT3A2. DNA binding, on the contrary, was not appreciably improved. Analysis of DNA methyltransferase complexes in solution using size exclusion chromatography revealed that DNMT3A2 forms large structures of heterogeneous sizes, whereas DNMT3L appears as a monomer. Binding of DNMT3L to DNMT3A2 promotes a dramatic reorganization of DNMT3A2 subunits and leads to the formation of specific complexes with enhanced DNA methyltransferase activity and increased SAM binding.

In mammals, the methylation of cytosines represents the only known form of covalent DNA modification that has a clear biological function, through its association with stable transcriptional silencing (1). Such silencing is critical for proper embryonic development, genome stability, X chromosome inactivation, genomic imprinting, and the silencing of retrotransposons (2–4). In addition, aberrant DNA methylation underlies many human diseases, including cancer, which is often associated with a genome-wide loss of DNA methylation and inappropriate silencing of tumor suppressor genes (5, 6). DNA methyltransferases (DNMTs) are essential enzymes that catalyze the transfer of a methyl group from a donor molecule, S-adenosyl-L-methionine (SAM), to a cytosine ring, usually located within the context of a symmetrical CpG dinucleotide (7). Mammalian genomes carry three distinct active DNMT genes. Two de novo DNMTs, encoded by the DNMT3A and DNMT3B genes, have been identified (8). These proteins show a high similarity to each other and have several domains in common, including a highly conserved C-terminal domain containing catalytic DNMT motifs that are homologous to those originally defined for bacterial type II cytosine DNMTs (9). DNMT3A and DNMT3B are considered to be de novo DNMTs, since they are responsible for initiating DNA methylation during early embryonic development (10), and show a preference for unmethylated DNA in vitro (8, 11). Specific isoforms of DNMT3A and DNMT3B are expressed during germ cell development and early embryogenesis, which correspond to the two most active phases of methylation reprogramming. These isoforms are generated through the use of an alternative promoter in the case of DNMT3A and of alternative splicing for DNMT3B. The isoforms and their expression patterns are conserved from mice to humans, indicating that they may carry out specific biological functions, although little is known about these potential roles. The DNMT1 enzyme, contrary to DNMT3A and DNMT3B, shows a preference for hemimethylated DNA substrates, is associated with replication foci, and is therefore considered a maintenance DNA methyltransferase (12–14), although it is also capable of de novo activity, in particular in the context of cancer (15). Together, these enzymes can generate new DNA methylation patterns and maintain them through cellular divisions, therefore forming the basis of a stable epigenetic transcriptional memory.

The DNMT3-like protein, DNMT3L, was identified as a third member of the DNMT3 family based on the high conservation of its N-terminal cysteine-rich domain with the corresponding domains in DNMT3A and DNMT3B (16). The C terminus of DNMT3L also shows partial homology to the catalytic C-terminal regions of DNMT3A and DNMT3B, although the domain is truncated and key residues involved in catalysis are not conserved. As a result, DNMT3L is inactive as a DNMT proper (17, 18). However, targeted mutagenesis of the DNmt3L
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gene in mouse revealed that Dnmt3L is essential for the establishment of maternal methylation imprints during oogenesis [(17, 19)]. In addition, Dnmt3l<sup>−/−</sup> males are sterile and show loss of methylation and reactivation of normally silent retrotransposon elements (20–22). DNMT3L is therefore critical for establishing methylation patterns in the germ line. Using an episomal system in a human cell culture model, we have shown that DNMT3L is a general stimulatory factor for de novo methylation mediated by all active isoforms of DNMT3A and DNMT3B (18, 23). Stimulation by DNMT3L was observed at all sequences tested, suggesting that it does not function as a targeting factor. Instead, DNMT3L promotes the acquisition of extensive DNA methylation patterns that are characteristic of gametic methylation marks found at imprinting centers. This stimulation is mediated by a direct protein-protein interaction between the C-terminal, catalytic-like, region of DNMT3L and the active catalytic domains of DNMT3 isoforms (23–25). Whereas DNMT3L can stimulate all active isoforms of DNMT3A and DNMT3B, genetic studies strongly suggest that members of the DNMT3A family are the most likely functional partners of DNMT3L. Indeed, germ cell-specific disruption of the Dnmt3a locus recapitulates many of the phenotypes observed in Dnmt3l<sup>−/−</sup> animals, suggesting that the two gene products functionally interact to establish DNA methylation marks in germ cells. On the contrary, disruption of the Dnmt3b locus had no detectable effect (26). Expression studies confirm these results and further indicate that DNMT3A2 might be the most physiologically relevant methyltransferase to associate with DNMT3L in germ cells. Murine Dnmt3L expression is highly up-regulated at specific stages of germ cell development, coinciding with the acquisition of gametic methylation in the male and female germ lines (27, 28). In males, Dnmt3a, particularly the Dnmt3a2 isoform, is also highly overexpressed at the same stage in gonocytes (28, 29). Altogether, these data strongly indicate that DNMT3A2 is an important physiological target for stimulation by DNMT3L. In this work, we have purified full-length human DNMT3A2 and DNMT3L proteins, reconstituted the stimulation of de novo methylation in vitro, and analyzed the biochemical mechanism of this stimulation.

**MATERIALS AND METHODS**

**Chemicals, Enzymes, and Chromatography Media**—Unless indicated, chemical reagents were purchased from Sigma. Proteinase K was from Roche Applied Science. Restriction endonucleases and T4 polynucleotide kinase were from New England Biolabs. Shrimp alkaline phosphatase and [γ<sup>32</sup>P]ATP were purchased from U. S. Biochemical Corp. and PerkinElmer Life Sciences, respectively. Pfu DNA polymerase was from Stratagene. S-Adenosyl-L-[methyl<sup>3</sup>H]methionine was purchased from GE Healthcare (specific activity 150 Ci/mmol). Chromatography media and prepacked HisTrap and HiTrap DEAE columns were from GE Healthcare (Amersham Biosciences), with the exception of chitin resin (New England Biolabs).

**Generation of Expression Vectors for Human DNMT3A2 and DNMT3L Proteins**—The human DNMT3A2 and DNMT3L coding regions were from previously published expression vectors (23). The DNMT3A2 coding region was excised from pcDNA3/MYC-DNMT3A2 using EcoRI and BamHI sites and recloned into a modified version of pGEX-6p-2 (GE Healthcare), leading to pGEX*-hDNMT3A2. In this modified version, the multiple cloning site of pGEX-6p-2 was changed to include an in-frame His<sub>6</sub> tag immediately followed by EcoRI, HindIII, XbaI, BamHI, and NotI sites. This vector allows for affinity purification using the cleavable GST tag with the cleaved protein still carrying an N-terminal His<sub>6</sub> tag suitable for nickel affinity chromatography. The DNMT3L coding sequence was first recloned into the modified pGEX vector using EcoRI and XbaI sites (generating pGEX*-hDNMT3L) and reamplified by PCR using primers introducing unique Nhel and SapI sites 5′ to the His<sub>6</sub> tag and immediately 3′ to the end of the coding region, respectively. The PCR fragment was introduced into pT7B1 (New England Biolabs) using the Nhel and SapI sites, generating a fusion protein between hDNMT3L and the self-cleavable intein-CBD tag (generating pT7B1-hDNMT3L). Using this vector, DNMT3L can be purified through nickel and chitin affinity chromatography.

**Protein Purification**—Human DNMT3A2 protein was purified from freshly transformed *Escherichia coli* Rosetta (DE3) cells (Novagen) grown in LB broth supplemented with ampicillin (100 μg/ml) and 0.2% glucose. After induction of protein expression for 2 h with 0.5 mM isopropyl 1-thio-β-d-galactopyranoside at 37 °C, cells were harvested and lysed by a French press in pT7B1-hDNMT3L lysis buffer (phosphate-buffered saline supplemented with 1 mM EDTA, 250 mM NaCl, 0.1 mM dithiothreitol, 0.1% Triton X-100, 25 μg/ml RNase A, 1 mM phenylmethylsulfonyl fluoride, and 50 μg/ml pepstatin A). The lysate was spun for 30 min at 30,000 × g to remove any cellular debris, and the supernatant was subsequently loaded onto a 5-ml HiTrap DEAE FF column. The DEAE flow-through was immediately applied to a 20-ml GST column and washed with 3 column volumes of lysis buffer, and the fusion protein was eluted using GST elution buffer (50 mM Tris-HCl, pH 8.5, 250 mM NaCl, 0.1 mM dithiothreitol, 0.1% Triton X-100, 40 μM reduced glutathione). After dialysis against Prescission protease buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1 mM EDTA), the GST tag was cleaved by Prescission protease (GE Healthcare) overnight at 4 °C. Cleavage efficiency was typically greater than 95%. The cleaved DNMT3A2 protein was dialyzed against nickel buffer (20 mM sodium phosphate, pH 7.4, 500 mM NaCl, 40 μM imidazole), loaded onto a 1-ml HisTrap HP column, and eluted using nickel buffer supplemented with 250 mM imidazole. This elution was dialyzed against S-200 buffer (25 mM Tris-HCl, pH 7.5, 1.5 mM NaCl, and 0.5 mM EDTA) and run on a 16/60 Superdex-200 preparation grade gel filtration column. Fractions corresponding to full-length DNMT3A2 protein were combined, concentrated, and frozen at −80 °C in storage buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.1 mM dithiothreitol, and 20% glycerol).

The human DNMT3L protein was purified from *E. coli* BL21 (DE3) cells (Stratagene) grown in LB broth supplemented with ampicillin (100 μg/ml) and 0.2% glucose. After induction of protein expression for 2 h with 0.15 mM isopropyl 1-thio-β-d-galactopyranoside at 37 °C, cells were lysed by a French press in nickel buffer supplemented with 0.2% Triton X-100, 10% glycerol, 25 μg/ml RNase A, 1 mM phenylmethylsulfonyl fluoride, and 50 μg/ml pepstatin A. The lysate was spun as...
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described above, and the supernatant was applied to a 5-ml HisTrap FF column. The fusion protein was eluted using nickel buffer supplemented with 10% glycerol, 0.1% Triton X-100, and 500 mM imidazole. The eluted protein was dialyzed against chitin binding buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.2% Triton X-100, and 10% glycerol), and bound to chitin resin. DNMT3L was cleaved from the bound chitin binding protein using chitin cleavage buffer (20 mM Tris-HCl, pH 8.5, 500 mM NaCl, 100 mM dithiothreitol, 0.2% Triton X-100, and 10% glycerol). The eluted DNMT3L was concentrated and frozen in storage buffer supplemented with 0.1% Triton X-100.

Protein concentration was determined by their absorbance at 280 nm using theoretical extinction coefficients (ε = 122,320 M⁻¹ cm⁻¹ for DNMT3A2 and 68,610 M⁻¹ cm⁻¹ for DNMT3L) and by Bradford assays (Bio-Rad). The protein preparations were over 93% pure for DNMT3A2 and 99% pure for DNMT3L, as calculated using band densitometry (Fig. 1) and devoid of any contaminating exonucleases (data not shown). Yields of 0.25–0.5 mg of purified protein/g of cells (wt mass) were typically obtained.

Activity Assays—DNA methyltransferase activity was monitored by the transfer of tritiated methyl groups from S-adenosyl-[methyl-3H]methionine ([3H]-SAM) onto double-stranded poly(dl-dc) DNA substrates, as described (13) with a few modifications. The concentration of poly(dl-dc) is reported as the concentration of CI dinucleotides (assuming an average length of 7000 bp). The reactions were performed in either “standard” buffer (25 mM Tris acetate, pH 7.5, 5 mM EDTA, 10 mM NaOAc, 100 μg/ml BSA, and 1 mM dithiothreitol), or activity buffer (25 mM Tris-HCl, pH 7.5, 50 mM KCl, 0.5 mM MgCl₂, 100 μg/ml BSA, and 1 mM dithiothreitol). Unless otherwise indicated, purified DNMTs were combined with [3H]-SAM in buffer and preincubated at 37 °C for 1 h before the reaction was initiated by the addition of DNA. Reactions were stopped by rapid freezing and inactivation of the DNMTs by the addition of proteinase K to 450 μg/ml followed by incubation at 55 °C for 10 min. Reactions were spotted onto 2.4-cm DE81 filters (Whatman), and the filters were washed five times with 1 ml of cold 0.3 M ammonium bicarbonate, three times with 1 ml of nanopure H₂O, and three times with 1 ml of 100% EtOH. Filters were then air-dried and counted in 4 ml of Ecolite Scintillation Mixture (Beckman-Coulter). Background SAM binding to the filters was measured by subtracting the counts obtained for a no protein control (typically 100–300 cpm). Nonlinear regression was performed using Prism version 4.0 (GraphPad Software).

Electrophoretic Mobility Shift Assays—Binding reactions were set up in activity buffer lacking BSA. Varying concentrations of purified DNMTs were preincubated at 37 °C for 1 h before the addition of 0.5 μM [3H]-SAM. SAM binding was allowed to reach equilibrium over 20 min at 37 °C, at which time the binding reactions were partially passed through YM-30 Microcon centrifugal filters (Millipore). The radioactivity present in the filtrate and retentate was then measured by scintillation counting, as described above. The concentration of SAM in the filtrate corresponds to the concentration of free SAM, [SAM], whereas the concentration of SAM in the retentate corresponds to the sum of the concentrations of free SAM plus bound SAM, [SAM]ᵣ + [SAM]ᵢ. Nonspecific retention of SAM in the upper chamber in the absence of any protein was observed and measured using multiple independent controls. The average value for this nonspecific binding was subtracted from the total bound SAM value in each binding reaction, therefore allowing us to measure specific binding of SAM to DNMTs. No significant binding of SAM to the membrane during centrifugation was observed, as indicated by the fact that the total SAM amounts in the system were not different before and after centrifugation and did not show any protein-dependent change (data not shown). The concentration of bound SAM was plotted as a function of protein concentration, and Kᵦ was determined by nonlinear regression to the quadratic solution for a single-site binding mechanism as shown in Equation 1,

\[
[SAM]ᵣ = \frac{B - [\beta]² - 4[DNMT][SAM]₀}{2}
\] (Eq. 1)

where \( B = [DNMT]ᵣ + [SAM]ᵣ + K_D \).

Gel Filtration—Purified DNMTs were dialyzed into activity buffer lacking BSA and preincubated at 37 °C for 1 h. 100 μg of protein at 1 mg/ml was loaded onto a Superose 6 HR 10/30 column controlled by an AKTA FPLC system run by Unicorn version 4.00.16 software (GE Healthcare). The flow rate was kept constant at 0.3 ml/min. Elution volumes (\( V_e \)) were determined using the Unicorn software. To create the standard curve, 100 μg of the five protein components of the Sigma non-denatured protein standard kit (urease, BSA, chicken egg albumin, carbonic anhydrase, and α-lactalbumin) as well as apofer-
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RESULTS

Reconstitution of the Stimulation of de Novo Methylation by DNMT3L in Vitro—In order to analyze the mechanism by which the DNMT3L protein stimulates de novo methylation by the DNMT3A2 DNA methyltransferase, we purified both full-length proteins using recombinant expression vectors in E. coli. The resulting proteins were 93% pure for DNMT3A2 and 99% pure for DNMT3L (Fig. 1) and were devoid of any exonuclease activity that could otherwise affect the nature and concentration of the DNA substrate (data not shown). We first assayed DNA methyltransferase activity using DNMT3A2 alone in order to verify that the protein was active. For this, the protein (0.5 mM), the DNA substrate (poly(dI–dC); 100 mM measured in CI dinucleotides), and radiolabeled \(^3\)H-SAM (2 mM) were mixed, and methylation activity was followed over 2 h. DNMT3A2 showed robust, time-dependent methyltransferase activity, as shown in Fig. 2. Methyltransferase activity was strictly dependent on the DNMT3A2 protein concentration (data not shown) and was linear with time for the first 15 min of the reaction, after which the velocity of the reaction slowly decreased (Fig. 2A). This nonlinear shape is, at least in part, due to a build-up of S-adenosyl-L-homocysteine levels, the breakdown product of SAM and an inhibitor of enzyme activity. As shown in Fig. 2A, DNMT3A2 activity resulted in the transfer of over a 0.1 mM concentration of methyl groups in the first 30 min, which is within range of the \(K_i\) for S-adenosyl-L-homocysteine (data not shown). The addition of DNMT3L protein in a 1:1 molar stoichiometry did not result in a significant increase in the initial rate of the reaction (Fig. 2, A and B), although the methyltransferase activity was progressively increased with time to reach a total stimulation of 1.6-fold after 2 h. Since DNMT3A2 and DNMT3L physically interact with each other through their C-terminal domains (23), we investigated whether preincubating the two proteins together prior to the initiation of the reaction would result in improved stimulation by allowing time for the two proteins to form physical complexes. For this, DNMT3A2 was preincubated either alone or together with an equimolar amount of DNMT3L, in the presence of \(^3\)H-SAM for 1 h at 37 °C, and the reaction was initiated by the addition of DNA and followed over time. Fig. 2 shows that whereas DNMT3A2 was not significantly affected by preincubation, the methyltransferase activity of DNMT3A2 observed in the presence of DNMT3L was strongly enhanced at early time points, leading to a 2.5-fold stimulation of the initial rate. Over time, however, the level of stimulation observed with and without preincubation was similar, presumably because the two proteins have ample time to interact during the course of the 2-h reaction. Thus, preincubation of the two proteins is required for observing maximal stimulation in vitro, particularly at early time points. Preincubating the proteins for 2 h did not change the level of stimulation; however, a 30-min preincubation was not sufficient to reach maximal stimulation (data not shown).

In the previous experiments, we employed a “standard” buffer, which, with minor variations, is often used in the field and is characterized by low ionic strength, low monovalent salt concentrations, high EDTA concentrations, and the absence of divalent salts. In order to determine optimal conditions for the reconstitution of the stimulation of DNA methyltransferase activity, we investigated whether buffer conditions could affect the process. Specifically, we investigated whether stimulation by DNMT3L could be reconstituted under more physiological buffer conditions, since human cells contain both monovalent and divalent salts. We first removed EDTA and increased the monovalent salt concentration to 50 mM KCl and observed that the stimulatory effect of DNMT3L on the initial velocity of the reaction was increased (Fig. 2C). We then titrated in 0.5, 1, or 2 mM of MgCl\(_2\) and observed an increase in the relative stimulation by DNMT3L both at the level of the initial rate and extent at 2 h (Fig. 2C and data not shown). Strikingly, the initial rate of the reaction was stimulated nearly 20-fold at 1 mM MgCl\(_2\), as compared with a modest 1.7-fold in “standard” buffer conditions. The stimulatory effect of DNMT3L is therefore only fully unmasked in buffers containing nearly physiological levels of free magnesium ions (0.5–1 mM (30, 31)) and monovalent salts. It should be noted that the addition of MgCl\(_2\) actually inhibited DNMT3A2 methyltransferase activity, in agreement with a previous report (32). DNMT3L relieved the inhibitory effect of magnesium ions on DNMT3A2 (supplemental Fig. 1).

In addition to preincubation and buffer conditions, we investigated the requirements in terms of the molar stoichiometric ratio of DNMT3A2 to DNMT3L required for optimal stimula-
For this, DNMT3A2 was kept constant at 0.5 μM, DNMT3L was varied, and the relative fold stimulation afforded by DNMT3L was measured (a buffer containing 0.5 mM MgCl₂ was used together with preincubation of the proteins for 1 h). Fig. 2D clearly shows that maximal stimulation by DNMT3L is observed at a 1:1 molar stoichiometric ratio. Increasing the ratio in favor of DNMT3L does not lead to any further increase above this maximal level (data not shown). At a 1:1 ratio, under our optimized conditions, the initial velocity of the reaction is increased 7-fold, and the reaction extent at 2 h is increased about 3-fold. Note that the reduced level of stimulation at 2 h relative to that of the initial rate probably reflects the fact that S-adenosyl-L-homocysteine is accumulated faster, and thus inhibits the reaction sooner, in the presence of DNMT3L. Thus, initial rates are a better measure of true stimulation than reaction extents at later time points. For the rest of the study, an activity buffer containing 0.5 mM MgCl₂ and 50 mM KCl was used in conjunction with preincubation of the proteins mixed in an equimolar ratio for 1 h before initiation of the reaction. This buffer allows good, although not maximal, stimulation by DNMT3L yet corresponds to physiological concentrations of magnesium ions and allows DNMT3A2 to maintain robust activity.

**Mechanism of Stimulation of de Novo Methylation by DNMT3L**

In order to analyze the mechanism by which DNMT3L stimulates de novo methylation by DNMT3A2, we first determined how DNMT3L affects the steady-state kinetic parameter \( K_m \), for both SAM and DNA substrates, using double reciprocal, substrate titrations (see supplemental Fig. 2). For this, we used 0.5 μM protein in activity buffer and measured the initial velocity of the reaction at 5 min. Each measurement was performed at least in triplicate. SAM was varied from 0.0625 to 0.75 mM, and DNA was varied from 0.625 to 35 mM. For determining \( K_m^{DNA} \), the initial rates obtained at different DNA concentrations were plotted as a function of SAM concentration, and the data were fit to the Michaelis-Menten equation. The apparent \( V_{max} \) values derived from fitting were then replotted.
as a function of DNA concentration, and the true $V_{\text{max}}$ and $K_{m}^{DNA}$ data at theoretically infinite concentrations of $5\text{AM}$ and DNA were obtained by fitting to the Michaelis-Menten equation (all fits gave an $R^2$ value greater than 0.90). Reciprocal plots of the data were used to determine the true $K_{m}^{SAM}$ values. Table 1 summarizes our results. $K_{m}^{SAM}$ for DNMT3A2 (0.23 ± 0.01 μM) on its own was in close agreement with previously published values for murine Dnmt3a protein on the same DNA substrate (33, 34). $K_{m}^{DNA}$ for DNMT3A2 (6.25 ± 0.59 μM) was higher than previously determined, which might reflect the fact that the shorter DNMT3A2 isoform interacts with DNA slightly differently from the full-length murine Dnmt3a protein. In the presence of DNMT3L, the $K_{m}^{SAM}$ value was unchanged (0.25 ± 0.01 μM), whereas the $K_{m}^{DNA}$ value was increased by almost 2-fold (12.18 ± 0.27 μM), a statistically significant increase. These measurements were repeated at a 10-fold lower enzyme concentration (50 nM), under which condition the reaction is linear for the first 60 min, and the newly derived $K_{m}$ values were found to be in close agreement with the ones measured at higher enzyme concentration (data not shown). The true $V_{\text{max}}$ value observed for DNMT3A2 on its own (~0.08 μmol of CH3/h) was increased 9-fold in the presence of DNMT3L (~0.76 μmol of CH3/h), highlighting the strong stimulatory effect of DNMT3L on de novo methylation by DNMT3A2. We then measured the catalytic rates ($k_{\text{cat}}$) for DNMT3A2 either on its own or together with DNMT3L, under conditions in which SAM and DNA are in a large excess (2 and 100 μM, respectively, or at least 8 times above their respective $K_{m}$ values). For this, we measured the initial velocity of the reaction after 1 min for various protein concentrations in five independent experiments. Initial rates were linear with protein concentrations, and the $k_{\text{cat}}$ was determined from the slope of the linear regression. In agreement with our previous results (Fig. 2D), we found that DNMT3L stimulated the catalytic activity of DNMT3A2 by close to 6-fold (Table 1) from 0.52 ± 0.07 to 2.96 ± 0.29 h⁻¹ for DNMT3A2 and DNMT3A2 with DNMT3L, respectively. Using the $k_{\text{cat}}/K_{m}$ ratio as a rough measure of catalytic efficiency, we can therefore conclude that the catalytic efficiency at which SAM is used is increased 5.2-fold in the presence of DNMT3L, whereas the usage of DNA is only increased 2.9-fold (Table 1).

**DNMT3L Does Not Significantly Improve DNA Binding by DNMT3A2**—In order to determine how DNMT3L achieves stimulation of de novo methylation by DNMT3A2, we first investigated whether DNMT3L can enhance DNA binding by DNMT3A2. For this, we analyzed DNA binding using electrophoretic mobility shift assays using a radioactively end-labeled 488-bp DNA fragment from the human SNRPN imprinting center as a probe. Binding was conducted at a range of protein concentrations, and bound and unbound species were separated by agarose gel electrophoresis. The amount of the unbound species was quantified and used to determine apparent $K_{D}^{DNA}$ values by Bjerrum plot analysis (35). Fig. 3A shows the result of such experiments. DNMT3A2 or both DNMT3A2 and DNMT3L readily bind to DNA as judged by the disappearance of the unbound species. Binding probably led to the formation of multiple bound bands of varying mass, thus explaining the upward smears observed on the gels. Quantification of the binding revealed that DNMT3A2 binds to this DNA fragment with a $K_{D}^{DNA}$ of 0.10 ± 0.04 μM. With the addition of DNMT3L, the $K_{D}^{DNA}$ value was increased to 0.13 ± 0.04 μM, which does not represent a significant change but nonetheless reflects the fact that DNMT3L does not seem to enhance the binding affinity of DNMT3A2 for DNA. The values for the apparent $K_{D}^{DNA}$ were also not significantly changed upon binding to an SsI-methylated DNA substrate, or in the presence of 500 μM S-adenosyl-L-homocysteine (data not shown). On its own, DNMT3L was capable of binding to DNA, albeit with a low affinity, reflected in a $K_{D}^{DNA}$ value of 1.28 ± 0.49 μM. Similar results were obtained using 20- or 30-mer oligonucleotide substrates (data not shown).

Since visual inspection of the electrophoretic mobility shift assay gels did not allow us to determine whether DNMT3L was still part of stable DNMT3A2-DNMT3L complexes when bound to DNA, we used a DNA pull-down assay to directly observe which proteins were bound to the DNA. For this, we used a biotinylated PCR fragment corresponding to the same 488-bp fragment and performed binding reactions either with DNMT3A2 alone, DNMT3A2 and DNMT3L together, or DNMT3L alone. The DNA and associated proteins were pulled down using streptavidin-coated agarose beads, and the beads were extensively washed with binding buffer. The pulled down material was then loaded onto an 8% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue. As shown in Fig. 3B, minor amounts of DNMT3A2 were present in the pulled down material even in the absence of DNA (lane 1). However, significantly more DNMT3A2 was pulled down in the presence of DNA (lanes 2 and 4), whereas binding by DNMT3L on its own was weak (lane 3). In the presence of both DNMT3A2 and DNMT3L (lane 4), both proteins were clearly detected above background levels in the pulled down material, and the ratio of intensities after Coomassie staining is indicative of a 1:1 stoichiometry when compared with a control lane in which equimolar amounts of DNMT3A2 and DNMT3L were loaded (lane 5). This shows that whereas DNMT3L on its own binds to DNA only weakly, it is readily found as part of DNA-bound...
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complexes in the presence of DNMT3A2. This suggests that most DNA-bound protein species correspond to equimolar DNMT3A2-DNMT3L complexes.

DNMT3L Significantly Increases SAM Binding by DNMT3A2—We next assessed whether DNMT3L enhances binding of the methyl group donor SAM to DNMT3A2 by directly measuring SAM binding using centrifugal ultrafiltration (36). For this, varying concentrations of proteins were incubated with a fixed amount of $^3$H-SAM (0.5 μM). The reactions were allowed to reach equilibrium and a small portion of the volume was then spun through a filter coated with streptavidin-coated agarose beads. The proteins were separated on an 8% SDS-polyacrylamide gel that was stained with Coomassie Brilliant Blue. The proteins included in the binding reactions are indicated on top. The identity of each band is indicated on the right, and a molecular weight marker is shown on the left. An equimolar amount of DNMT3A2 and DNMT3L is shown to serve as a control for staining (lane 5).

DNMT3L increases binding of SAM to DNMT3A2. The amount of $^3$H-SAM bound to DNMT3L (A), DNMT3A2 (B), and DNMT3A2-DNMT3L (C) is indicated on the y-axis as a function of protein concentration (the concentration of each subunit was used in the case of DNMT3A2-DNMT3L). Data were measured using centrifugal ultrafiltration from at least triplicate experiments. Error bars are indicated. The results were fit to the quadratic solution for single-site binding, as indicated under "Materials and Methods."

and Methods”), and the $K_D^{SAM}$ values were obtained from the fit. An attempt to fit the binding data to the quadratic solution for two binding sites was unsuccessful, suggesting that each DNMT3A2 monomer or DNMT3A2-DNMT3L heterodimer can only bind to a single molecule of SAM (data not shown). DNMT3A2 readily bound to SAM with a measured $K_D^{SAM}$ of 2.95 ± 0.32 μM (Table 1). DNMT3L showed very little, if any, binding to SAM (Fig. 4), leading to an estimated $K_D^{SAM}$ of 157 μM, which effectively precludes any possibility for SAM binding by DNMT3L under physiological conditions. Interestingly, however, DNMT3A2 and DNMT3L, when assayed together, showed a significantly increased binding to SAM (Fig. 4), leading to a 4-fold lower $K_D^{SAM}$ at 0.69 ± 0.11 μM. Thus, although DNMT3L on its own is unable to bind to SAM, it significantly enhances the affinity of DNMT3A2 for SAM when both proteins are in a complex.

DNMT3L Reorganizes DNMT3A2 Complexes, Leading to the Formation of Specific, Higher Order Complexes in Solution—Our data indicate that preincubation of DNMT3A2 and DNMT3L significantly enhances stimulation of de novo methylation. DNMT3L and DNMT3A2 physically interact (23–25), and this suggests that the preincubation time is required for the two proteins to form a complex. In order to detect complex formation and determine its approximate mass, we employed analytical gel filtration chromatography. For this, DNMT3A2, DNMT3L, and the two proteins together were preincubated for 1 h at 37 °C in activity buffer in the absence of DNA and were loaded onto a Superose 6 fast protein liquid chromatography column. Elution volumes, which are roughly inversely correlated to the mass of the complexes, were then followed. Standards of known molecular weights were used to draw a standard curve to which the sample elution volumes were compared. Our data show that DNMT3L appears as a well-defined peak corresponding closely to a monomer in solution (Fig. 5). To our surprise, DNMT3A2 eluted in a broad peak, the average mass of which would correspond roughly to the one expected for 10 monomers of DNMT3A2 (~ 827 kDa) with a considerable distribution on both ends. This suggests that
DNMT3A2 is not a monomer in solution but rather that it forms large complexes of heterogeneous sizes. These complexes were also visualized using native PAGE, which also confirmed that DNMT3L is a monomer (data not shown). DNMT3A2 and DNMT3L, after preincubation together in a 1:1 molar ratio, eluted as a unique and specific peak, whereas both original peaks completely disappeared, suggesting that complex formation was nearly 100% efficient under these conditions (Fig. 5 and data not shown). The DNMT3A2-DNMT3L peak eluted around an average molecular mass of 497 kDa, which is closest to the mass expected for a complex composed of 4 units of DNMT3A2 and DNMT3L in a 1:1 stoichiometry (expected mass ~520 kDa). Whereas the precise subunit composition of this complex cannot be inferred from these experiments, the fact that both original DNMT3A2 and DNMT3L peaks disappeared upon complex formation supports the notion that the two proteins are present in an equimolar ratio. Interestingly, we also observed that complex formation is time-dependent, with a 30-min incubation being insufficient for complete assembly, and condition-dependent, with complete assembly being only observed in activity buffer containing magnesium ions (data not shown). These observations precisely reflect the conditions required for optimal stimulation by DNMT3L, as described above (Fig. 2).

**DISCUSSION**

Using a cell culture model system, we have previously shown that DNMT3L functionally stimulates the activity of all active isoforms of DNMT3A and DNMT3B (18, 23). This stimulation is mediated by a direct physical interaction between the C-terminal half of DNMT3L and the catalytic domain of DNMT3A and DNMT3B (17, 23–25), suggesting that DNMT3L might function by directly enhancing the catalytic activity of its partner de novo methyltransferases. Here, using purified recombinant proteins, we have successfully reconstituted the stimulation of de novo methylation by DNMT3L, using the DNMT3A2 protein as an active and physiologically relevant partner. Importantly, we have established novel conditions that allow for the optimal reconstitution of the stimulatory effect by DNMT3L. Our data clearly indicate that DNMT3L exerts maximal stimulation using buffers that resemble physiological conditions and include 0.5–1 mM magnesium ions and monovalent salts (Fig. 2C). Furthermore, our data clearly show that the two proteins need to be preincubated for a significant amount of time (~1 h) before initiation of the reaction in order to achieve maximal stimulation (Fig. 2, A and B). Both preincubation and the addition of magnesium ions favor the formation of specific complexes between DNMT3L and DNMT3A2 (see below). Under these conditions, maximal stimulation is readily achieved at a 1:1 molar stoichiometric ratio of DNMT3L to DNMT3A2 (Fig. 2D) and results in a striking stimulation of the reaction's initial rate, which can reach up to 20-fold at 1 mM MgCl₂. These results are in contrast to two previously published studies that claim to have reconstituted the stimulation of de novo methylation by DNMT3L in vitro (24, 37). In both studies, buffers analogous to the often-used “standard” buffer described here (i.e. containing low amounts or no monovalent salts, devoid of magnesium ions, and instead containing millimolar concentrations of EDTA) were used. Furthermore, no preincubulation of the proteins was carried out. As a result, the stimulation observed by these authors was weak. One study reported an overall 1.5-fold stimulation for Dnmt3a, using a heterologous combination of mouse Dnmt3a and human, GST-fused DNMT3L (24). Using murine proteins, another study claimed to observe a 5-fold stimulation of the activity of full-length Dnmt3a by Dnmt3L, although the catalytic activity (k_{cat}) of the truncated catalytic domain of Dnmt3a was only stimulated 1.2-fold under the same conditions (37). Furthermore, large molar excesses of Dnmt3L (from 8- to 40-fold) were used in order to detect any stimulation. In light of our data, it is likely that the conditions used in these studies did not allow for proper reconstitution of the stimulation of de novo methylation by DNMT3L, thus rendering any further comparison with our data difficult. Under the conditions described here, which underestimate the extent of stimulation by DNMT3L, equimolar amounts of DNMT3L stimulate the k_{cat} of DNMT3A2 by ~5.7-fold (Table 1), which is reflected by a corresponding increase in the reaction's V_{max} of 9-fold.

Increased binding to one or both substrates of the reaction, SAM and DNA, would provide a simple and attractive explanation for the stimulatory effect of DNMT3L on de novo methylation. In order to investigate this possibility, we used double-reciprocal substrate titrations to determine K_m values as well as direct binding assays to determine substrate dissociation constants, K_D. Our data strongly suggest that DNMT3L primarily exerts its effect by increasing binding of DNMT3A2 to the methyl group donor, SAM. This conclusion is supported by two independent lines of evidence. First, we showed that whereas the reaction maximal velocity (V_{max}) is increased by 9-fold in the presence of DNMT3L, the K_{cat}^{SAM} value remains unchanged, thus suggesting that the efficiency at which SAM is used is strongly increased (k_{cat}^{cat}/K_m^{SAM} is increased by 5.2-fold). Second, direct measurement of SAM binding using centrifugal ultrafiltration revealed that whereas binding to DNMT3L on its own could not be detected, binding of SAM to DNMT3A2 was
strongly increased in the presence of DNMT3L (Fig. 4), resulting in a 4.3-fold decreased dissociation constant for SAM, $K_D^{\text{SAM}}$ (Table 1). The lack of SAM binding to DNMT3L is consistent with the fact that the residues thought to be responsible for SAM binding are missing from DNMT3L. However, the fact that DNMT3L can enhance the binding of SAM to DNMT3A2 suggests that DNMT3L can induce a change in DNMT3A2 that allows for improved binding. We believe that the increased ability of DNMT3A2 to bind SAM is a plausible and physiologically relevant mechanism underlying the ability of DNMT3L to stimulate DNA methylation. SAM is indeed the sole methyl group donor for multiple cellular pathways leading to the methylation of substrates as varied as proteins, RNA, lipids, and multiple other small molecules, in addition to DNA (38).

All of the corresponding methyltransferases compete with and multiple other small molecules, in addition to DNA (38). The decrease in $K_D^{\text{SAM}}$ afforded by DNMT3L is therefore significant in that it is expected to enhance the ability of DNMT3A2-DNMT3L complexes to compete for SAM binding and perform DNA methylation. Further supporting a role for SAM availability in the efficiency of DNA methylation is the well documented link between diet, particularly folate intake, and epigenetic gene regulation at imprinted genes and retrotransposons (48, 49).

On the other hand, our data suggest that increased DNA binding is unlikely to be a significant contributor to the mechanism by which DNMT3L stimulates de novo methylation. Indeed, we showed that whereas binding of DNMT3L to DNA could be detected, it was weak, and under physiological conditions, DNMT3L only appears to access DNA through binding to DNMT3A2 (Fig. 3). Furthermore, electrophoretic mobility shift assays showed that binding to DNA is not improved in the presence of DNMT3L; instead, the $K_D^{\text{DNA}}$ value was slightly increased (Table 1), suggesting, if anything, a lower affinity for DNA by DNMT3A2-DNMT3L complexes. We cannot, however, rule out a kinetic effect on the binding of DNMT3A2 to DNA. Our data also suggest that once bound to DNA, the complexes formed by DNMT3A2 and DNMT3L are relatively stable, since DNA pull-down experiments allowed recovery of nearly stoichiometric amounts of DNMT3A2 and DNMT3L proteins even after a 30-min incubation followed by a series of washes (Fig. 3B). Similar results were obtained in a previous study (24).

Our study also reveals an important and unexpected role for the formation of specific DNA methyltransferase complexes in solution. Our data show that DNMT3A2 primarily exists in solution as large complexes of heterogeneous sizes (Fig. 5 and data not shown). The mass of these complexes varied from the one expected for complexes of 4–30 DNMT3A2 monomers, with an average mass centered on a decamer. These complexes were detected using analytical gel filtration chromatography and native PAGE and appeared to be stable even in 1.5 M NaCl. We believe that formation of DNMT3A2 complexes is not an artifact of recombinant protein preparations. Interaction between independent DNMT3A molecules can be readily identified in human cells in culture by co-immunoprecipitation experiments using differently tagged expression vectors. Furthermore, deletion analysis reveals that oligomerization of DNMT3A molecules in human cells is mediated by interactions through a portion of the C-terminal catalytic domain.3 It is therefore likely that DNMT3A exists in vivo as protein oligomers, not as isolated monomers. DNMT3L, however, appears as a monomer in solution and has the unique ability to reorganize the large DNMT3A2 complexes into specific DNMT3A2-DNMT3L complexes (Fig. 5). The fact that DNMT3L also physically interacts with DNMT3A through the C-terminal catalytic domain suggests that DNMT3L might be able to disrupt DNMT3A2 self-interactions and trigger the redistribution of DNMT3A2 into heterodimeric units. Our findings that DNMT3A2 and DNMT3L need to be preincubated in order to observe maximal stimulation of de novo methylation probably reflect the time needed for this reorganization to occur. Consistent with our observation that stimulation of DNA methylation is maximal at a 1:1 molar stoichiometric ratio of DNMT3A2 to DNMT3L, we observe that preincubation of equimolar amounts of the two proteins leads to the complete disappearance of both original peaks in gel filtration chromatography, suggesting that the reorganized complexes correspond to oligomers of heterodimeric units. In agreement, the average mass derived from gel filtration chromatography indicates that DNMT3A2-DNMT3L complexes are most consistent with the formation of tetramers of DNMT3A2-DNMT3L heterodimers. The exact subunit composition and mass of these complexes remains to be further characterized using more sensitive methods. These results lead to the notion that human DNA methyltransferases might function as complexes in vivo and that the activity of a given DNMT may be influenced by the presence of interacting partners within the complex, even if these partners are catalytically inactive as is the case for DNMT3L. Here, we show that whereas DNMT3A2 forms large structures of heterogeneous sizes in solution, binding of DNMT3L to DNMT3A2 promotes a dramatic reorganization of DNMT3A2 molecules to form specific complexes with enhanced DNA methyltransferase activity. Altogether, our data suggest that DNMT3L functions first by mediating specific contacts with DNMT3A2 complexes in solution that lead to the redistribution of DNMT3A2 molecules into complexes composed of an equimolar amount of each protein. Formation of these complexes is accompanied by a change in DNMT3A2 that allows for improved binding to the methyl group donor, SAM, and for a more efficient catalysis of methyl group transfer. Once bound to DNA, these complexes are proposed to be stable and lead to increased methylation activity. This model does not exclude the possibility that DNMT3L might possess additional roles that might affect the reaction’s putative catalytic order, processivity, or dissociation rates. These parameters are currently under investigation.

3 C. Viet and F. Chédin, unpublished observations.
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