Dnmt3L has been identified as a stimulator of the catalytic activity of de novo DNA methyltransferases. It is essential in the development of germ cells in mammals. We show here that Dnmt3L stimulates the catalytic activity of the Dnmt3A and Dnmt3B enzymes by directly binding to their respective catalytic domains via its own C-terminal domain. The catalytic activity of Dnmt3A and -3B was stimulated ~15-fold, and Dnmt3L directly binds to DNA but not to S-adenosyl-L-methionine (AdoMet). Complex formation between Dnmt3A and Dnmt3L accelerates DNA binding by Dnmt3A 20-fold and lowers its $K_d$ for DNA. Interaction of Dnmt3L with Dnmt3A increases the binding of the coenzyme AdoMet to Dnmt3A, and it lowers the $K_m$ of Dnmt3A for AdoMet. On the basis of our data we propose a model in which the interaction of Dnmt3A with Dnmt3L induces a conformational change of Dnmt3A that opens the active site of the enzyme and promotes binding of DNA and the AdoMet. We demonstrate that the interaction of Dnmt3A and Dnmt3L is transient, and after DNA binding to Dnmt3A, Dnmt3L dissociates from the complex. Following dissociation of Dnmt3L, Dnmt3A adopts a closed conformation leading to slow rates of DNA release. Therefore, Dnmt3L acts as a substrate exchange factor that accelerates DNA and AdoMet binding to de novo DNA methyltransferases.

In vertebrates DNA is methylated at the 5-position of cytosine residues within CG dinucleotides, which are methylated to 70–80% in a tissue- and cell-specific pattern (1–3). Methylation is involved in epigenetic regulation of gene expression, X-chromosome inactivation, genomic imprinting, and development (4, 5); aberrant methylation contributes to aging and cancer (6, 7). The methyl group of the coenzyme S-adenosyl-L-methionine (AdoMet)$^1$ is transferred to cytosine residues of the DNA by DNA methyltransferases (MTases) three families of which have been identified to date in mammals: Dnmt1, Dnmt2, and Dnmt3 (8). All these enzymes contain a domain of ~400–500 amino acid residues, which is characterized by the presence of 10 conserved amino acid motifs, shared between prokaryotic and eukaryotic enzymes of DNA-(cytosine-C5)-methyltransferases (8, 9). In addition, the Dnmt1 and the Dnmt3 enzymes harbor large N-terminal regulatory parts (8, 10). Dnmt1 has a strong preference for hemimethylated CG sites implicating a function in maintenance of the methylation pattern of the DNA after replication (11–15). Dnmt2 is the smallest enzyme among the eukaryotic methyltransferases comprising only the catalytic domain. It has been shown to be an active methyltransferase, with a very slow turnover (16–19).

The Dnmt3 family consists of three different proteins, Dnmt3A, Dnmt3B, and Dnmt3L (see Fig. 1) (20, 21). Dnmt3A and -3B have a regulatory N-terminal domain that in contrast to Dnmt1 is not essential for catalysis (22, 23) but is involved in enzyme targeting (24–27). The N-terminal parts of Dnmt3A and -3B contain an ATRX-like Cys-rich domain (also called PHD domain) and a PWWP domain, which are involved in interactions of the enzymes with other proteins and in targeting to heterochromatin (24–28). Dnmt3A and -3B also methylate cytosines in a non-CG context, however the biological function of this activity is not known (29–32). Both enzymes do not distinguish between unmethylated and hemimethylated substrates and are involved in de novo methylation in vivo (20, 30, 33). Despite these biochemical similarities the Dnmt3A and -3B enzymes have distinct biological roles. Dnmt3B is responsible for methylation of pericentromeric satellite regions (33–35). Dnmt3B$^-/-$ knock-out mice die during late embryonic stage and the embryos lack methylation in pericentromeric repeat region (33). Loss of Dnmt3B activity in human leads to the ICF syndrome, a genetic disorder that is accompanied by low methylation in pericentromeric satellite region of chromosomes 1, 9, and 16 (36). Dnmt3A knock-out mice show developmental abnormalities and die few weeks after birth (33). This enzyme has been associated with the methylation of single copy genes and retrotransposons (37–39), and it is required for the establishment of the genomic imprint during germ cell development (40).

The third member of the Dnmt3 family, Dnmt3L, shows clear homology to the Dnmt3A and -3B enzymes (21). Its N-terminal part contains the PHD domain, but not the PWWP domain, and the C-terminal part extends up to conserved motif VIII (Fig. 1). Strikingly, Dnmt3L carries mutations within all conserved motifs that contain the catalytic residues of DNA-(cytosine-C5)-methyltransferases, suggesting that Dnmt3L adopts the typical MTase fold, but it does not have catalytic activity. Co-transfection experiments Dnmt3L has been shown to stimulate DNA methylation by Dnmt3A in human cell lines (41). Stimulation of Dnmt3B was not detectable in these experiments.
Mechanism of Stimulation of Dnmt3A and -3B by Dnmt3L

**Proteins**—Dnmt1, Dnmt3A, Dnmt3B, and their catalytic domains (42 amino acid residues) were cloned into pET28a (+) by reverse transcription–PCR using RNA isolated from mouse testis, and its sequence was confirmed. A truncated version of Dnmt3L (CD-Dnmt3L comprising amino acids 208–421) was prepared by PCR mutagenesis as described previously (44). Dnmt3L and CD-Dnmt3L were purified as 208–421) was prepared by PCR mutagenesis as described previously (44). Dnmt3L and CD-Dnmt3L were purified as

**Oligonucleotide Substrates**—The following oligodeoxynucleotide substrates were used in this study (written in 5′ → 3′ direction): CG30: Br-GAG CTT GCC GGG AGG AGG GTG CAAATG CAC TCT CCT CCC GGA AGT CCC AGT TTCA; CA30: Bt-GAG ACT TCG GCC GGG AGG AGT CCT CCC GGA AGT CC; AG 12: Br-AGT GCC GGG AGG AGA GTG CAAATG CAC TCT CCT CCC GGA AGT CCC AGT TTCA; CT30: Bt-GAG ACT TCG GCC GGG AGG AGA GTG CAAATG CAC TCT CCT CCC GGA AGT CCC AGT TTCA; TG2: Bt-GAG ACT TCG GCC GGG AGG AGA GTG CAAATG CAC TCT CCT CCC GGA AGT CCC AGT TTCA; GG2: Bt-GAG ACT TCG GCC GGG AGG AGA GTG CAAATG CAC TCT CCT CCC GGA AGT CCC AGT TTCA; GC2: Bt-GAG ACT TCG GCC GGG AGG AGA GTG CAAATG CAC TCT CCT CCC GGA AGT CCC AGT TTCA; GG2: Bt-GAG ACT TCG GCC GGG AGG AGA GTG CAAATG CAC TCT CCT CCC GGA AGT CCC AGT TTCA.

**Experimental Procedures**

**Proteins**—Dnmt1, Dnmt3A, Dnmt3B, and their catalytic domains were expressed and purified as described (14, 23, 30). Murine Dnmt3L (421 amino acid residues) was cloned into pET22a (+) by reverse transcription–PCR using RNA isolated from mouse testis, and its sequence was confirmed. A truncated version of Dnmt3L (CD-Dnmt3L comprising amino acids 208–421) was prepared by PCR mutagenesis as described previously (44). Dnmt3L and CD-Dnmt3L were purified as described for Dnmt3A. All proteins were >90% pure as determined from Coomassie-stained SDS-gels.

**Oligonucleotide Substrates**—The following oligodeoxynucleotide substrates were used in this study (written in 5′ → 3′ direction): CG30: 5′-GGAG AGG AGA GTG CAAATG CAC TCT CCT CCC GGA AGT CCC AGG T.TCA, 3′-B-GAG CTT GCC GGG AGG AGG GTG CAAATG CAC TCT CCT CCC GGA AGT CCC AGG T.TCA; CA30: 5′-B-GAG CTT GCC GGG AGG AGA GTG CAAATG CAC TCT CCT CCC GGA AGT CCC AGG T.TCA, 3′-B-GAG CTT GCC GGG AGG AGA GTG CAAATG CAC TCT CCT CCC GGA AGT CCC AGG T.TCA; AG 12: 5′-B-GAG CTT GCC GGG AGG AGA GTG CAAATG CAC TCT CCT CCC GGA AGT CCC AGG T.TCA, 3′-B-GAG CTT GCC GGG AGG AGA GTG CAAATG CAC TCT CCT CCC GGA AGT CCC AGG T.TCA; CT30: 5′-B-GAG CTT GCC GGG AGG AGA GTG CAAATG CAC TCT CCT CCC GGA AGT CCC AGG T.TCA, 3′-B-GAG CTT GCC GGG AGG AGA GTG CAAATG CAC TCT CCT CCC GGA AGT CCC AGG T.TCA; TG2: 5′-B-GAG CTT GCC GGG AGG AGA GTG CAAATG CAC TCT CCT CCC GGA AGT CCC AGG T.TCA, 3′-B-GAG CTT GCC GGG AGG AGA GTG CAAATG CAC TCT CCT CCC GGA AGT CCC AGG T.TCA; GG2: 5′-B-GAG CTT GCC GGG AGG AGA GTG CAAATG CAC TCT CCT CCC GGA AGT CCC AGG T.TCA, 3′-B-GAG CTT GCC GGG AGG AGA GTG CAAATG CAC TCT CCT CCC GGA AGT CCC AGG T.TCA; GC2: 5′-B-GAG CTT GCC GGG AGG AGA GTG CAAATG CAC TCT CCT CCC GGA AGT CCC AGG T.TCA, 3′-B-GAG CTT GCC GGG AGG AGA GTG CAAATG CAC TCT CCT CCC GGA AGT CCC AGG T.TCA; GG2: 5′-B-GAG CTT GCC GGG AGG AGA GTG CAAATG CAC TCT CCT CCC GGA AGT CCC AGG T.TCA, 3′-B-GAG CTT GCC GGG AGG AGA GTG CAAATG CAC TCT CCT CCC GGA AGT CCC AGG T.TCA.

**Surface Plasmon Resonance Experiments**—Biophysical interaction analysis was performed by surface plasmon resonance (SPR) experiments using a Biacore X instrument in buffer HBS-E (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20) as recommended by the supplier. Experiments were performed at a flow rate of 30 μl/min. Ammonium thiocyanate and PEG was added to the buffer to reach a final concentration of 150 mM and 2% (w/v) PEG, respectively. The mixture was incubated on ice for 5 min and then exposed to UV in a UV cross-linker (Hoefer UV-500) for 5 min at 320 nm, followed by incubation for 1 h at 37 °C.

**Determination of Activation and Find that Binding to Dnmt3L Induces a Conformational Change of Dnmt3A That Facilitates DNA and AdoMet Binding**

The interaction of Dnmt3A and Dnmt3L is transient and Dnmt3L dissociates from Dnmt3A–DNA complexes. Therefore, Dnmt3L functions as a substrate exchange factor for Dnmt3A and -3B.

**RESULTS**

**Activation of Dnmt3A and -3B by Dnmt3L**—It has been shown previously that Dnmt3L stimulates DNA methylation by Dnmt3A and -3B in 1.5- to 3-fold in vitro (43). As an initial step in investigating the mechanism of this process we purified Dnmt3A, -3B, -3L, and -3L and studied the stimulation of DNA methylation by Dnmt3L using synthetic oligonucleotide substrates. As shown in Fig. 2A, in the presence of 4 μM CD-Dnmt3L, the initial rate of the enzymatic reaction of Dnmt3A was stimulated about 5-fold. We confirmed the absence of DNA methylation activity of Dnmt3L (data not shown), which is in agreement with the observation that all the essential DNA-(cytosine-C5)-MTase motifs (U-X) are indicated by the black squares in Dnmt3A and Dnmt3B. In Dnmt3L, the corresponding regions are gray.
Dnmt3L requires the interaction of the N-terminal part of Dnmt3A, we have investigated the stimulation of the catalytic domain of Dnmt3A (CD-Dnmt3A), which is active even in the absence of the N-terminal domain (22, 23). The results demonstrate a similar level of stimulation of CD-Dnmt3A and Dnmt3L as observed for the full-length Dnmt3A protein (Fig. 2B). For test for an involvement of the PHD domain of Dnmt3L in the stimulation process, we deleted the N-terminal part of Dnmt3L and studied stimulation of Dnmt3A by the resulting C-terminal fragment of Dnmt3L. Again a strong increase in methylation activity was observed (Fig. 2D), indicating that the stimulation is caused by an interaction of the catalytic domain of Dnmt3A with the C-terminal domain of Dnmt3L. Titration experiments using the same amounts of Dnmt3L and Cd-Dnmt3L demonstrated a comparable degree of stimulation. Similar experiments were also performed with Dnmt3B and its isolated catalytic domain, which was activated to a similar degree as Dnmt3A (Fig. 2, C and E). In summary, addition of Dnmt3L or CD-Dnmt3L caused a 5- to 10-fold stimulation of the DNA methylation activity of Dnmt3A, Dnmt3B, CD-Dnmt3A, and CD-Dnmt3B but not of Dnmt1.

**Determination of the Maximum Level of Stimulation**—We were interested to determine the maximum level of stimulation of Dnmt3A and -3B by Dnmt3L. Because this process involves a functional interaction of Dnmt3A and Dnmt3L, the degree of stimulation should be dependent on the concentration of Dnmt3L and the ratio of Dnmt3L and active methyltransferase (Dnmt3A or -3B). Higher amounts of Dnmt3L will increase the level of stimulation until saturation is reached. Therefore, we performed methylation experiments with CD-Dnmt3A and CD-Dnmt3B in the presence of increasing amounts of Dnmt3L (Fig. 3A). The stimulation effects were analyzed by binary binding experiments to estimate the apparent binding affinity of both proteins and the intrinsic level of stimulation of Dnmt3L on CD-Dnmt3A and CD-Dnmt3B. With CD-Dnmt3A and CD-Dnmt3B apparent binding constants of Dnmt3L were in the lower micromolar range indicating a low affinity interaction: $K_d$(CD-Dnmt3A) = 8 x 10$^{-4}$ M$^{-1}$ and $K_d$(CD-Dnmt3B) = 9 x 10$^{-4}$ M$^{-1}$. A binary binding model was chosen for analysis, because it is the simplest model that allowed fitting the activity curves. Given that a more complicated reaction mechanism is likely (see below), these numbers can only be interpreted as phenomenological values that do not reflect the real binding affinities of the two proteins. These titration experiments allow extrapolation to the stimulation level that would be observed at saturating concentrations of Dnmt3L. This stimulation level reflects the absolute difference between the catalytic activities of free Dnmt3A or -3B and the Dnmt3A- or -3B-Dnmt3L complexes. On the basis of our data the molecular activation level of CD-Dnmt3A and -3B by Dnmt3L is estimated 15-fold in the case of CD-Dnmt3A and 13-fold in the case of CD-Dnmt3B. We conclude that saturating amounts of Dnmt3L induce a considerable (~15-fold) stimulation of the catalytic activity of the catalytic domains of Dnmt3A and -3B.

**Biochemical Analysis of the Stimulation of CD-Dnmt3A**—Next we attempted to understand the mechanistic basis of the activation of the de novo DNA MTases by Dnmt3L. For technical reasons, we performed these experiments with the catalytic domain of Dnmt3A. First, we have determined the rates of DNA methylation at different concentrations of DNA and AdoMet (Fig. 3B). A global fit of the data to the Michaelis-Menten model yielded $K_m$(AdoMet) = 2.55 μM, $K_m$(DNA) = 0.76 μM, and $k_{cat}$ = 0.65 min$^{-1}$ in the absence of Dnmt3L. In the presence of Dnmt3L these values were determined as $K_m$(AdoMet) = 0.96 μM, $K_m$(DNA) = 0.25 μM, and $k_{cat}$ = 0.78 min$^{-1}$. Simulations revealed $K_m$ values were valid within ±20%, and the accuracy of $k_{cat}$ values was ±30%. These results demonstrate that the apparent $K_m$ values of CD-Dnmt3A for DNA and AdoMet were improved 3.0- and 2.7-fold, respectively, whereas $k_{cat}$ was only marginally changed (1.2-fold).

Because the Michaelis-Menten analysis suggested an influence of Dnmt3L on DNA and AdoMet binding of Dnmt3A, we directly tested the effect of Dnmt3L on AdoMet binding using a UV-cross-linking assay. In this assay, 3H-labeled AdoMet was incubated with Dnmt3A, cross-linked to the protein by UV-irradiation, and the binding was analyzed by autoradiography of SDS-polyacrylamide gels. As shown in Fig. 3C, AdoMet binding by Dnmt3A was improved in the presence of Dnmt3L. Free Dnmt3L did not bind AdoMet, which is in agreement with the absence of a complete AdoMet binding site in Dnmt3L, which is conserved in active DNA MTases.

We also tested the stimulation of Dnmt3A by Dnmt3L using substrates bearing the target cytosine in a non-CG environment. Dnmt3A methylates such non-CG substrates with reduced but clearly detectable activity (30). Our data show that methylation of non-CG sites is stimulated to a lesser degree by addition of Dnmt3L than methylation of CG sites (Fig. 4B). We have also studied the effect of the length of the oligonucleotide substrate on the level of stimulation of CD-Dnmt3A by Dnmt3L using a series of oligonucleotides that range in size from 12 to 45 bps and contain the CG site in the same sequence context.
Mechanism of Stimulation of Dnmt3A and -3B by Dnmt3L

The results shown in Fig. 4A indicate that on short oligonucleotide substrates a 2.5-fold stimulation was observed that increased if the length of the substrate was raised from 12 to 45 bps. A reduced level of activation was also observed with a Dnmt3A variant, in which the active site cysteine residue is replaced by alanine. We have recently shown that this variant is catalytically active albeit at a reduced level (22, 23). Addition of Dnmt3L caused only a weak (about 1.5-fold) activation of this variant (data not shown). These observations are discussed in the context of the mechanistic model for stimulation of Dnmt3A and -3B by Dnmt3L under “Discussion” (see below).

**DNA Binding by Dnmt3L**—Next we wanted to follow up on our observation of the improved $K_m$ of Dnmt3A for DNA in the presence of Dnmt3L. As a first step, we investigated if purified Dnmt3L itself binds to DNA. As determined by nitrocellulose-filter binding experiments, Dnmt3L binds to 30-mer oligonucleotides containing a hemimethylated or unmethylated CG site with binding constants of $K_a = 6.3(\pm 1.9) \times 10^8 \text{ M}^{-1}$ (Fig. 5A). The CA substrate without CG site was bound with $K_a = 7.9(\pm 2) \times 10^8 \text{ M}^{-1}$ indicating that Dnmt3L binds to DNA without detectable preference for CG sites or methylation state. Because Suetake et al. (2004) could not detect DNA binding by Dnmt3L (43), we verified this result by surface plasmon resonance (SPR) experiments using CD-Dnmt3L binding to biotinylated CG30 oligonucleotide immobilized on avidin-coated sensor chips (Sensor Chip SA, Biacore). As shown in Fig. 5B, DNA binding was detectable in the SPR experiment as well. The SPR signal was dependent on the concentration of Dnmt3L used. DNA binding of Dnmt3L was determined at three different concentrations (1000, 500, and 250 nM). Fitting the data to a simple binding equilibrium was not possible. However, the binding curves could be fitted by a biphatic process comprising DNA binding and a conformational change of the Dnmt3L-DNA complex (Fig. 5B),

\[
\text{DNA} + 3L \rightleftharpoons \text{DNA-3L} \rightleftharpoons \text{DNA-3L}^* \\
\]

**REACTION 1**

yielding the following rate constants for $k_1$, $k_{-1}$, $k_2$, and $k_{-2}$:

- $2.3 \times 10^7 \text{ s}^{-1} \text{ M}^{-1}$, $0.059 \text{ s}^{-1}$, $3.5 \times 10^{-3} \text{ s}^{-1}$, and $1.9 \times 10^{-3} \text{ s}^{-1}$ (for the error margins of these numbers see Supplemental Table S1). The equilibrium binding constants of CD-Dnmt3L to DNA calculated using these numbers are $3.9 \times 10^8 \text{ M}^{-1}$ for the first binding step and $7.4 \times 10^6 \text{ M}^{-1}$ for both steps combined,
which is in excellent agreement to the results of the nitrocellulose filter binding experiments.

**Kinetics of DNA Binding by CD-Dnmt3A**—We studied the kinetics of DNA binding by CD-Dnmt3A using the SPR assay as well. DNA binding and release was slow as indicated by the slow approach of the signal to the equilibrium level after adding CD-Dnmt3A and the very slow decline of the signal after withdrawal of CD-Dnmt3A (Fig. 5C). After each binding experiment, the residual amounts of bound protein were released from the surface by application of 2.5 M NaCl for 2 min, a procedure that proved effective and led the SPR signal return to the original level observed before application of Dnmt3A (data not shown). DNA binding by CD-Dnmt3A could be fitted to a simple binding equilibrium with $k_1$ of $5.0 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$ and $k_{-1}$ of $2.3 \times 10^{-4} \text{ s}^{-1}$ (for the error margins of these numbers see Supplemental Table S1). The high affinity of the DNA binding reaction ($K_a = 2.2 \times 10^9 \text{ M}^{-1}$) is reflected by the much higher SPR signal observed at similar enzyme concentrations as compared with the Dnmt3L results.

The slow rate of DNA binding to Dnmt3A suggested that this step might be rate-limiting for enzymatic turnover. Therefore, we studied DNA binding to CD-Dnmt3A in the presence of CD-Dnmt3L. Experiments were carried out using 1 μM Dnmt3L and 50–300 nM Dnmt3A. We used excessive amounts of Dnmt3L to saturate Dnmt3A with Dnmt3L. Given the binding constant of Dnmt3A to Dnmt3L (see below) under these conditions Dnmt3A exists almost completely as Dnmt3A-Dnmt3L complex. However, the excess free Dnmt3L does not strongly affect the binding curves, because DNA binding by Dnmt3L is much weaker than by Dnmt3A. As shown in Fig. 5C, application of a mixture of Dnmt3A and Dnmt3L to the DNA chip resulted in a roughly 2-fold increase in the SPR signal as compared with the signal observed with Dnmt3A alone. This difference was expected, because the molecular mass of Dnmt3A-Dnmt3L complexes is ~2-fold higher than Dnmt3A, and the SPR signal is directly proportional to the mass of the ligand bound to the surface. Binding experiments were performed at different concentrations of Dnmt3A and analyzed in a global fitting procedure. The binding curves determined in the presence of Dnmt3A and Dnmt3L could not be fitted to a simple binding equilibrium or a model assuming a conformational change after initial DNA binding (data not shown). We, therefore, implemented a reaction scheme assuming binding of the Dnmt3A-Dnmt3L complex to the DNA and two dissociation rate constants ($k_{-1}$ and $k_{-2}$) for release of Dnmt3L and Dnmt3A from the DNA. On the basis of the results obtained with both proteins in binary DNA binding experiments, we correlated the faster dissociation phase with Dnmt3L leaving the DNA, whereas the slower phase was assigned to dissociation of Dnmt3A.

This model fitted the data very accurately yielding $k_1 = 9.6 \times 10^5 \text{ s}^{-1} \text{ M}^{-1}$, $k_{-1} = 2.4 \times 10^{-3} \text{ s}^{-1}$, and $k_{-2} = 1.3 \times 10^{-4} \text{ s}^{-1}$ (for the error margins of these numbers see Supplemental...
Dnmt3A was immobilized. The overall binding constant (Dnmt3A-Dnmt3L complex was still present. As shown in Fig. Dnmt3L complex. The slow dissociation of this complex permits with CD-Dnmt3A and DNA (Fig. 7A). In the first phase, incubation with Dnmt3A led to the formation of the Dnmt3A-Dnmt3L complex. The slow dissociation of this complex permitted us to apply oligonucleotide to the surface, while the Dnmt3A-Dnmt3L complex was still present. As shown in Fig. 7A this led to a rapid dissociation of the Dnmt3A-Dnmt3L complex indicating that binding of DNA induced dissociation of the Dnmt3A-Dnmt3L complex. As expected, injection of buffer carried out as control did not have any effect on the kinetics of dissociation of Dnmt3A from Dnmt3L.

The conclusion that ternary complexes comprising Dnmt3A-Dnmt3L and DNA are not stable is also supported by successive binding of CD-Dnmt3A and CD-Dnmt3L to a sensor chip loaded with the CG30 oligonucleotide (Fig. 7B). In this experiment the immobilized DNA was incubated first with Dnmt3A. During the slow dissociation phase of Dnmt3A from the DNA, Dnmt3L was injected at a time when the Dnmt3A-DNA complexes were still present on the surface of the chip. Thereby, binding of Dnmt3L to DNA-Dnmt3A complexes could be studied. However, the comparison of the binding of Dnmt3L to the DNA-Dnmt3A surface with Dnmt3L binding to the DNA surface in the absence of Dnmt3A indicates no difference in the relative effects. This finding suggests that Dnmt3L does not interact with the Dnmt3A bound to DNA, again suggesting that ternary Dnmt3A-Dnmt3L-DNA complexes are not formed. In contrast to these results obtained after stepwise addition of Dnmt3A and Dnmt3L to the surface-bound DNA, clear differences were observed in the experiments where Dnmt3A and Dnmt3L were applied together to the DNA chip indicating that the Dnmt3A-Dnmt3L complex directly associates to the DNA (see above and Fig. 5C).

**DISCUSSION**

Interaction of an enzyme with a regulatory protein is a common principle in the regulation of enzyme activity. Examples include the regulation of protein and small molecule kinases, proteases, RNA and DNA polymerases, and G-proteins (48). The regulatory protein may affect the conformation of the enzyme, changing it into a more active or inactive state, or it might facilitate the binding of substrates and coenzymes. Both principles are exemplified in the case of the G-proteins (49). The GTPase-activating proteins interact with G-proteins in the GTP-bound state, stabilizing the active enzyme conformation and directly contributing to catalysis. In contrast, the guanine nucleotide exchange factors interact with G-proteins in the GDP-bound state. They induce opening of the active site and accelerate GDP to GTP exchange. In this work we investigate the Dnmt3L protein that has been shown to stimulate the activity of de novo DNA methyltransferases. Our results suggest that Dnmt3L accelerates the binding of the DNA substrate and AdoMet coenzyme to the methyltransferase, suggesting a similar role as guanine nucleotide exchange factors have for G-proteins.

In our experiments we observed a 15-fold stimulation of the activity of Dnmt3A and -3B by Dnmt3L. This level of stimulation is significantly higher than the stimulation factors previously reported from in vitro experiments, which are between 1.5- and 3-fold depending on the DNA substrate (43). The strong dependence of the stimulation on the relative concentrations of the MTases and Dnmt3L most likely explains this difference in results. We also noticed that lower levels of stimulation are observed with PCR fragments as substrates (data not shown) which were used by Suetake et al. to study the stimulation effect (43). The reason for this observation most likely is that methylation of PCR products is assayed at different ratios of enzyme to DNA. Another difference between our results and those of Suetake et al. is that they could not find DNA binding by Dnmt3L, which we have shown by two independent detection methods. The most likely reason for this discrepancy is that they only used gel retardation assays to investigate DNA binding, which is an assay that is not ideal for detection of weak and non-sequence-specific DNA binding.

We detected a comparable degree of stimulation of Dnmt3A and -3B by Dnmt3L, a result that supports a similar finding published recently by Suetake et al. (49). We show that stimulation of Dnmt3A and -3B by Dnmt3L depends on the interaction of the catalytic domains of the MTases and the C-terminal domain of Dnmt3L. This finding confirms and extends the observation of Suetake et al. who already demonstrated that the C-terminal domain of Dnmt3L is sufficient to obtain stimulation (43). Interestingly, so far all known effects of Dnmt3L knock-out in mice could be correlated to a loss of activation of the Dnmt3A enzyme (37-39) and the phenotype of Dnmt3L knock-out in germ cells (40). Also in cell culture experiments no stimulation of Dnmt3B could be found (41). Therefore, the biological role of the stimulation of Dnmt3B by Dnmt3L remains unclear in the light of these studies. One could speculate that a stimulation of Dnmt3B is only required in special cell types that so far were not investigated in the transgenic animals.
It had been the main focus of our study to understand the mechanism of activation of Dnmt3A by Dnmt3L in molecular details. To this end, we have studied the kinetics of DNA methylation by different MTases in the presence of Dnmt3L at different concentrations of DNA and coenzyme (AdoMet), using different DNA substrates and Dnmt3A variants. In addition, we have studied the kinetics of the protein-protein interactions of Dnmt3L with Dnmt3A and the interaction of both proteins with DNA in SPR experiments. Our results point toward a model in which Dnmt3A and Dnmt3L form a complex in solution. Thereby, the conformation of Dnmt3A changes into a more open form that accelerates DNA binding and increases AdoMet binding. In addition to the conformational change of Dnmt3A, the intrinsic DNA binding activity of Dnmt3L supports DNA binding by Dnmt3A-Dnmt3L complexes. After binding of DNA to the Dnmt3A-Dnmt3L complex, the affinity between Dnmt3A and Dnmt3L is reduced leading to the release of Dnmt3L from the Dnmt3A-DNA complex.

Each step in this model is based on direct experimental evidence, which will be briefly reviewed in the following section. We have observed that Dnmt3L improves DNA binding by Dnmt3A and it accelerates the rate of DNA binding by Dnmt3A ~20-fold. The ability of Dnmt3L to bind to DNA could contribute to this effect as suggested by the dependence of the stimulation on the length of the DNA substrate. Using short oligonucleotide substrates 12–16 bp in length we observed a lower level of stimulation than on substrates >20 bps. As the typical DNA footprint of a protein of the size of CD-Dnmt3A is expected around 12 bps, the short substrates most likely do not provide enough space for Dnmt3L and Dnmt3A to bind to the DNA at the same time. In addition, binding of Dnmt3L improves AdoMet binding of Dnmt3A, which could explain the basal level of activation observed with short oligonucleotide substrates. Our model predicts a complicated dependence of the rate enhancement on the conditions in the assay, because the overall effect depends on the influence of the rates of DNA and AdoMet binding on the catalytic rate. Conditions under which the catalytic step itself is slow will tend to reduce the stimulation by Dnmt3L, because the relative influence of the DNA and AdoMet binding steps on the overall rate decreases.

This was observed with a Dnmt3A variant carrying an amino acid exchange in the active site of the enzyme that was stimulated to a much lower degree than wild-type Dnmt3A. Similarly we observed a lower level of stimulation of methylation at altered target sites, like CA or CT sequences. This interpretation is supported by the finding that stimulation at CT sites is worse than at CA sites, which means the overall order of stimulation CG>CA>CT is identical to the rates of methyl group transfer observed with free Dnmt3A (30).

We show that the direct protein-protein interaction of Dnmt3A and Dnmt3L has a high affinity. Dnmt3A-Dnmt3L complexes form in solution and bind as a complex to the DNA. We have shown, in three independent sets of experiments, that the ternary Dnmt3A-Dnmt3L-DNA complex that is formed after DNA binding to Dnmt3A-Dnmt3L complexes is not stable. First of all, SPR experiments show that the addition of DNA leads to dissociation of Dnmt3A-Dnmt3L complexes. Second, the affinity of Dnmt3A for Dnmt3L (and vice versa) is reduced in the presence of DNA. Third, Dnmt3L only interacts with free Dnmt3A but not with Dnmt3A-DNAs complexes.

The slow DNA binding and release of Dnmt3A indicate that it exists in two conformations, one with the DNA binding cleft closed that allows tight interaction with DNA and catalysis, and another with the DNA binding cleft open that allows DNA exchange. In the free enzyme the closed conformation is preferred, leading to slow DNA binding. After DNA binding, the
interactions between Dnmt3A and the DNA stabilize the closed conformation, which traps the DNA and leads to a very low rate of DNA release from Dnmt3A. Our data suggest that Dnmt3L preferentially interacts with the open form of Dnmt3A. Therefore, it can stabilize this conformation and accelerate DNA binding. Because the open form is not stable after DNA binding, Dnmt3L tends to dissociate from Dnmt3A after DNA binding. The increased affinity of Dnmt3A-Dnmt3L complexes for AdoMet suggests the open conformation of Dnmt3A has a higher affinity for AdoMet as well, which is plausible because AdoMet suggests the open conformation of Dnmt3A has a higher affinity for AdoMet as well, which is plausible because AdoMet suggests the open conformation of Dnmt3A has a higher affinity for AdoMet as well, which is plausible because AdoMet.