De Novo Methylation of Nucleosomal DNA by the Mammalian Dnmt1 and Dnmt3A DNA Methyltransferases†

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ABSTRACT: In the cell, DNA is wrapped on histone octamers, which reduces its accessibility for DNA interacting enzymes. We investigated de novo methylation of nucleosomal DNA in vitro and show that the Dnmt3a and Dnmt1 DNA methyltransferases efficiently methylate nucleosomal DNA without dissociation of the histone octamer from the DNA. In contrast, the prokaryotic SssI DNA methyltransferase and the catalytic domain of Dnmt3a are strongly inhibited by nucleosomes. We also found that full-length Dnmt1 and Dnmt3a bind to nucleosomes much stronger than their isolated catalytic domains, demonstrating that the N-terminal parts of the MTases are required for the interaction with nucleosomes. Variations of the DNA sequence or the histone tails did not significantly influence the methylation activity of Dnmt3a. The observation that mammalian methyltransferases directly modify nucleosomal DNA provides an insight into the mechanisms by which histone tail and DNA methylation patterns can influence each other because the DNA methylation pattern can be established while histones remain associated to the DNA.

In mammals, DNA methylation occurs at CG sites that are modified to 70−80% in a cell type specific pattern (*1*−*3*); non-CG sites are modified at lower levels. DNA methylation is involved in many epigenetic processes such as regulation of gene expression, genomic imprinting, and X-chromosome inactivation and contributes to genomic stability. The mammalian DNA methyltransferase (MTase) Dnmt1 is involved in maintenance of the methylation pattern after replication and has a preference for methylation of hemimethylated target sites. However, the enzyme also shows a significant activity on unmethylated DNA (*4*, *5*), and it plays a role in de novo methylation of DNA (*6*, *7*). In addition, the Dnmt3a and Dnmt3b MTases are able to methylate DNA de novo (*8*, *9*), a process that mainly occurs during embryogenesis and development of germ cells. All three DNA MTases comprise a large N-terminal part that is implicated in protein targeting and enzyme regulation and a C-terminal catalytic domain (*10*). The C-terminal domains of Dnmt3a and Dnmt3b (*11*), but not of Dnmt1 (*5*), function as DNA MTases independent of the N-terminal part.

The fundamental unit of chromatin is the nucleosome, which consists of 147 bp of DNA wrapped around a histone octamer (*12*). The packaging of DNA in nucleosomes, however, poses serious problems for DNA interacting proteins. For example, many transcription factors show reduced affinity for their binding sites when these are located within nucleosomes (*13*). In addition, most restriction enzymes and nucleases are inhibited by nucleosomes (*14*, *15*). Prokaryotic methyltransferases have also been reported to methylate nucleosomal DNA inefficiently (*16*). Under physiological conditions, the reduced accessibility of the target sites on nucleosomal DNA is overcome by chromatin remodeling, which involves ATP-dependent movement and displacement of nucleosomes (*17*). To regulate access to chromatin, eukaryotes have evolved a series of epigenetic signals including histone tail acetylation, methylation, or phosphorylation and DNA methylation (*2*, *18*). The various epigenetic modifications appear to be interrelated. For example, de novo methylation of DNA is triggered by histone 3 at lysine 9 methylation (*19*, *20*), and erasure of CG methylation reduces the H3 K9 methylation (*21*−*23*). In addition, DNA methylation and histone deacetylation have synergistic effects on chromatin condensation (*2*).

The pattern of DNA methylation is maintained after DNA replication when the DNA is likely to be accessible for enzymatic modification. In contrast, de novo DNA methylation occurs within a chromatin context in the cell, which leads to the question if de novo methylation of DNA might depend on chromatin remodeling. This assumption is supported by the finding that chromatin remodeling factors are essential for DNA methylation in different model systems, although a direct influence of these factors on substrate accessibility of DNA MTases has not been demonstrated (*24*−*26*). However, it has been shown recently that the maintenance MTase Dnmt1 can directly methylate hemimethylated CG sites in the nucleosomal context (*27*), suggesting that de novo methylation also might occur directly on

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nucleosomes, which has not yet been investigated. Here, we study whether the DNA MTases Dnmt1 and Dnmt3a are capable of methylating nucleosomal DNA.

MATERIALS AND METHODS

Preparation and Analysis of Nucleosomes. To study methylation of nucleosomal DNA, nucleosomes were reconstituted using three different 147 bp DNA fragments produced by PCR. One DNA fragment was amplified from the mammary tumor virus 3′ long terminal repeat nucleosome A site (*28*), the second from the H19 CTCF binding site, and the third from the Air promoter (*27*). Mononucleosomes were reconstituted by salt dilution using recombinantly expressed *Xenopus laevis* histone octamers (*29*) and Cy5-labeled DNA (*28*). The yield of nucleosome formation was monitored in 4.5% acrylamide gels by fluorescence imaging of the Cy5-labeled DNA where nucleosomal DNA ran as a single band. The amounts of free and nucleosomal DNA were quantified using AIDA software (Raytest).

Nucleosomes containing histone H3 methylated at lysine 9 were prepared by native peptide ligation. Briefly, this involved the ligation of a synthetic methylated peptide (Albachem) to the globular domain of recombinant histone H3. A vector for the expression of truncated H3 containing an N-terminal cysteine at position 28 was generated by site-directed mutagenesis of the previously described histone H3 expression plasmid (*29*). The synthetic peptide consisted of histone H3 amino acids 1−27 containing trimethylated lysine 9 and a C-terminal thioester. Ligation was performed in 6 M GnCl and 0.2 M phosphate buffer, pH 7.3, containing 2% thiophenol for 16 h at 20 °C. The ligation product was separated from free peptide and globular H3 by ion-exchange chromatography using SOURCE 15 S (Pharmacia) resin in 7 M urea and 20 mM NaOAc, pH 5.2, using a gradient from 0 to 2 M NaCl. Fractions containing the ligation product were identified, pooled, and concentrated. The protein was refolded in the presence of H4, H2A, and H2B to form the histone octamer as described previously (*29*).

Methylation and Binding of Nucleosomes by Murine MTases. The MTases were purified as described (*5*, *9*, *11*). M.SssI was purchased from NEB. For the methylation reactions, 150 nM reconstituted nucleosomes which contained a 4:1 molar ratio of nucleosomes (120 nM) and free DNA (30 nM) were incubated with enzyme in 10 μL of methylation buffer (20 mM HEPES, pH 7.5, 1 mM EDTA, 25 μg/mL BSA) at 37 °C in the presence of 0.7 μM [*methyl*-^{3}H]AdoMet (2700 GBq/mmol; Amersham). Enzyme concentrations in the assays were 0.6 μM Dnmt3a and catalytic domain of Dnmt3a (CD-3a), 0.4 μM Dnmt1, and up to 5 μM catalytic domain of Dnmt1 (CD-D1). M.SssI was used in a final amount of 0.1 unit/μL. After defined times, 500 μM unlabeled AdoMet (Sigma) was added to stop the incorporation of radioactivity. To disrupt the interaction between MTase and nucleosomes, 300 mM NaCl was added to the reaction mix together with 3% (w/v) Ficoll (Sigma). The samples were incubated for 15 min at ambient temperature. The free DNA and nucleosomes were separated by agarose gel (0.8%) electrophoresis in 0.5× TBE. The bands containing the nucleosomes and free DNA were visualized by ethidium bromide staining and excised from the gel, and the radioactivity was analyzed by liquid scintillation counting.

To analyze binding of MTases to nucleosomes, 150 nM Cy5-labeled mononucleosomes were incubated with MTases for 10 min in methylation buffer. The reactions were loaded on a 3.5% or 4% acrylamide gel after addition of 3% (w/v) Ficoll. Electrophoresis was carried out in TBE buffer. The shifts were monitored by fluorescence imaging. In addition, DNA binding was detected by nitrocellulose filter binding performed using as described (*30*) in 50 mM HEPES, pH 7.5, and 1 mM EDTA.

Bisulfite DNA Sequencing. For bisulfite methylation analysis, nucleosomes were reconstituted as described above using a 193 bp DNA fragment that comprises the Nuc A site of the MMTV 3′LTR sequence and primer binding sites for retrieval of DNA after the bisulfite treatment. The methylation reaction was carried out as described using Dnmt3a MTase and unlabeled AdoMet (Sigma; final concentration 100 μM). The methylated nucleosomal DNA was retrieved from agarose gel by the gel extraction kit (Machery & Nagel). The DNA was treated with bisulfite reagent as described (*31*, *32*). The primers used to amplify the top and bottom strands after the bisulfite treatment were designed such that the central 147 bp occupied by the histone octamer were amplified. The PCR products from the converted top and the bottom strand of DNA were cloned using the TOPO-TA cloning kit (Invitrogen), and the clones were sequenced (SEQLAB).

RESULTS

Methylation and Binding of Nucleosomal DNA by MTases. To investigate the activity of methyltransferases on nucleosomal DNA, purified recombinant DNA MTases (Dnmt1 and Dnmt3a) (Figure 1 in Supporting Information) and reconstituted mononucleosomes were used. Nucleosomes were assembled onto a 147 bp DNA sequence that contains 10 CG sites using unmodified recombinant histones purified from *Escherichia coli* (*29*). The reconstitution of nucleosomes resulted in a mixture of nucleosomal and free DNA. DNA methylation reactions were performed in the presence of ^{3}H-labeled *S*-adenosyl-L-methionine (AdoMet) at 37 °C. The free DNA and nucleosomes were separated by agarose gel electrophoresis. The bands containing the nucleosomes and free DNA were excised from the gel, and the radioactivity incorporated into the DNA was analyzed (Figure 2 in Supporting Information). In this experimental setup, the methylation of nucleosomal and naked DNA takes place in competition, which allows a precise comparison of the substrate preferences of the MTases. Moreover, separation of the nucleosomal and free DNA after the methylation reaction on an agarose gel (see Materials and Methods) ensures a distinctly separate detection of the DNA methylation on nucleosomal and free DNA. The methylation of any disrupted nucleosome would eventually appear in the methylation activity on the free DNA, because disrupted nucleosomes cannot reconstitute back under these reaction conditions. However, under our experimental conditions disruption of nucleosomes was below 10% of the total amount of nucleosomal DNA (see, for example, Figure 2 in Supporting Information), similarly to that observed for other nucleosomal substrates by Okuwaki and Verrault (*27*).

Our results clearly show that both Dnmt1 and Dnmt3a methylate nucleosomal DNA without disruption of its

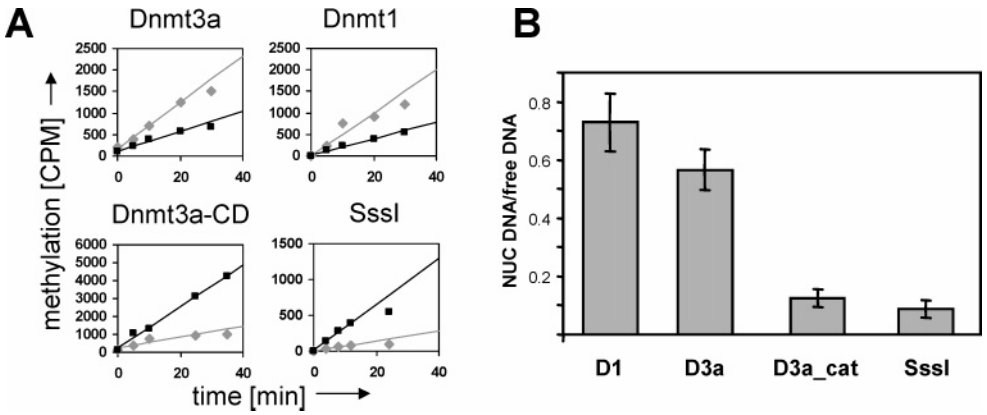

FIGURE 1: (A) Representative time courses of the methylation of nucleosomal (120 nM, gray diamonds) and free DNA (30 nM, black squares). (B) Relative activity of DNA MTases on nucleosomal vs free DNA, determined from four to six independent experiments after correction for the relative amounts of free and nucleosomal DNA in the reaction mix. Error bars represent the standard deviation between the individual experiments.

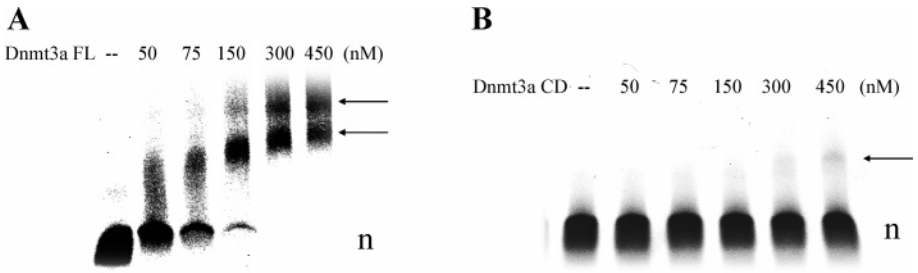

FIGURE 2: Binding of nucleosomes (150 nM) by increasing amounts of Dnmt3a (A) or CD-3a (B). "n" represents the free nucleosomes, and arrows indicate the shifted nucleosomes.

interaction with the histone octamer (Figure 1A). After correction for the relative amounts of nucleosomal and free DNA, only a slight reduction of enzymatic activity by nucleosomes was observed (Figure 1B). To check if this property was specific for mammalian MTases, we used the prokaryotic SssI DNA MTase, which has the same recognition sequence as the mammalian enzymes. In agreement with reports in the literature, we observed a strong inhibition of M.SssI by nucleosomal DNA (*16*, *27*). We further investigated the methylation activity of the catalytic domain of Dnmt3a (*11*) (CD-3a) on nucleosomes. To our surprise, CD-3a was also strongly inhibited by nucleosomal DNA, inferring a role of the N-terminal domain in the methylation of histone-bound DNA. Apparently, M.SssI and CD-3a behave like many nucleases and other DNA interacting enzymes, which show a pronounced inhibition by nucleosomes.

To study the interaction of DNA MTases with nucleosomes, an electrophoretic mobility shift assay (EMSA) was performed. Upon addition of Dnmt3a to nucleosomes, a clear band shift was observed, demonstrating that Dnmt3a interacts with nucleosomes (Figure 2). At higher concentrations of Dnmt3a, a second retarded band appeared, suggesting that at least two Dnmt3a molecules can bind to one nucleosome. In contrast, binding of CD-3a to nucleosomes was very weak and only detectable at higher protein concentrations (Figure 2). Therefore, binding of the full-length Dnmt3a to nucleosomal DNA has much higher affinity than binding of the isolated catalytic domain. These results support a role for the N-terminal region of Dnmt3a in nucleosome binding, in agreement to the observations in the methylation experiments. Similar effects were observed with Dnmt1 and CD-Dnmt1 (Figure 4 in Supporting Information), suggesting that the N-terminal part of Dnmt1 is also required for interaction with nucleosomes.

In the absence of clear shifts of the MTases with naked DNA in EMSA, we performed nitrocellulose filter binding studies of Dnmt3a, Dnmt1, CD-3a, and CD-Dnmt1 with the naked DNA, which were quantitatively analyzed. Under these conditions similar binding affinities of full-length MTases and their respective catalytic domains were observed (data not shown). Therefore, the N-terminal domains of the MTases do not assist in the interaction with the naked DNA. This finding supports the conclusion that the N-terminal parts of Dnmt3a and Dnmt1 interact with the nucleosomes.

In the cell, the PWWP domain of Dnmt3a has been shown to be important for the interaction of Dnmt3a with heterochromatin (*33*). In an attempt to investigate the type of interaction between the N-terminal domain of Dnmt3a and the nucleosome, we purified the PWWP domain as a recombinant protein and investigated its binding to the nucleosomes by EMSA. However, we could not detect any binding of the PWWP domain to the nucleosomes (Gowher and Xu, unpublished results), suggesting that in vivo other proteins might mediate the heterochromatic interaction of the PWWP domain.

Mapping the Sites of Methylation. The accessibility of the DNA bound to nucleosomes could influence the methylation of target cytosines depending on where they are positioned in the nucleosome. Consequently, we investigated the distribution of methylation on the nucleosomal DNA. Since Dnmt3a is a bona fide de novo MTase, all forthcoming experiments examining the de novo methylation of nucleosomes were done using only Dnmt3a. Following

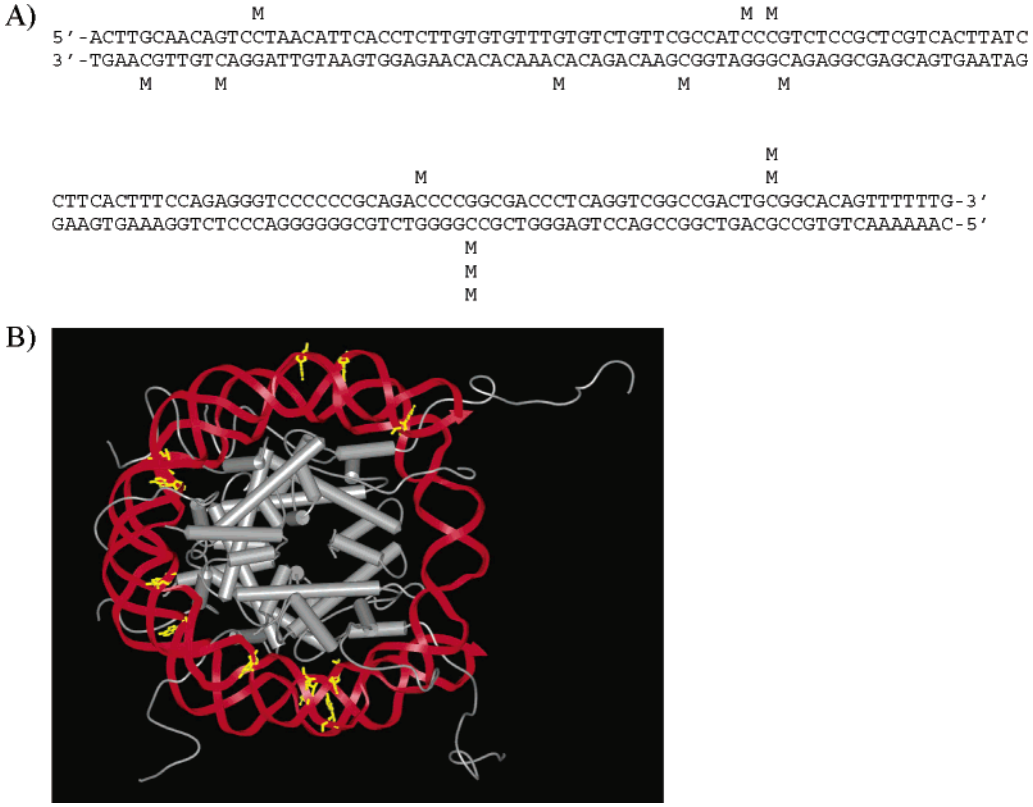

FIGURE 3: Results of the bisulfite methylation analysis of nucleosomal DNA by full-length Dnmt3a. (A) DNA sequence of the MMTV nucleosome. The nucleotides found to be methylated in the bisulfite analysis are indicated. (B) Superposition of the methylated cytosines on the structure of the nucleosome (PDB code: 1KX5). The DNA is shown in red, and the histone proteins are shown in gray. The methylated cytosines are shown as yellow stick models.

the methylation by Dnmt3a and isolation from agarose gels, the nucleosomal DNA was treated with bisulfite reagent, which deaminates unmethylated cytosines but does not react with methylated cytosines. The converted DNA was amplified by PCR, cloned, and sequenced (Figure 3 in Supporting Information). In 55 clones we detected 14 methylation events at CG and non-CG sites (Figure 3A). This finding is in agreement with the observation that Dnmt3a also methylates DNA at non-CG sites (*9*, *34*, *35*). Upon alignment of the methylated sites on the structure of the nucleosome, we found that the methylated sites were statistically distributed over the surface of the nucleosomal DNA (Figure 3B). It is notable that the methylation sites are not clustered toward the ends of the DNA, which are typically more accessible than the core region of the nucleosome (*36*).

The bisulfite sequencing results also revealed that CGG is a preferred site of methylation by Dnmt3a in the nucleosomal context, because 6 out of 8 methylation events at CGs occurred at CGG sites (probability of random occurrence after correction for the distribution of sites = 4.9%). In control experiments using the same DNA without assembly into nucleosomes, only 3 out of 11 methylation events at CG sites took place at CGG sites, which is in agreement with the published flanking site preference of Dnmt3a on naked DNA (*37*, *41*). The different flanking sequence preferences of Dnmt3a on nucleosomal DNA most likely are due to the conformational changes of the DNA in the nucleosomal context.

Effect of the Nucleosomal Components on the Catalytic Activity of Dnmt3a. To investigate if the histone tails influence the methylation activity of Dnmt3a, we analyzed the binding and activity of Dnmt3a on nucleosomes that were reconstituted from tail-less variants of individual histones. No significant difference in binding (data not shown) and methylation (Figure 4A) between all four individual mutant nucleosomes and the wild type was observed. We further detected no difference in the activity of Dnmt3a on H3 K9 methylated nucleosomes in comparison to the unmethylated ones. These results imply that none of the histone tails individually provides a major contribution to the binding of MTases to nucleosomes and H3 K9 methylation has no direct influence on the MTase activity.

Next, we wanted to study if Dnmt3a methylates different DNA sequences to different extents when bound to nucleosomes. On the basis of the results published by Okuwaki and Verreault (*27*), we investigated the de novo methylation of the H19 CTCF binding region and Air promoter after reconstituting them into nucleosomes. In comparison to MMTV (used in all previous experiments) and the Air nucleosomes, the H19 nucleosomes were methylated to a slightly higher degree (Figure 4B). The higher methylation was not because of less stability of the H19 nucleosome since we did not detect any accumulation of free DNA, similarly to that observed by Okuwaki and Verrault (*27*). The small preference of Dnmt3a for H19 nucleosomes could be due to the presence of some preferred sites in the H19 nucleosomes in comparison to the other two nucleosomes. Control experiments demonstrated that the free DNAs were modified to a similar extent (data not shown). We conclude that Dnmt3a is able to methylate nucleosomal DNA irrespective of the actual DNA sequence.

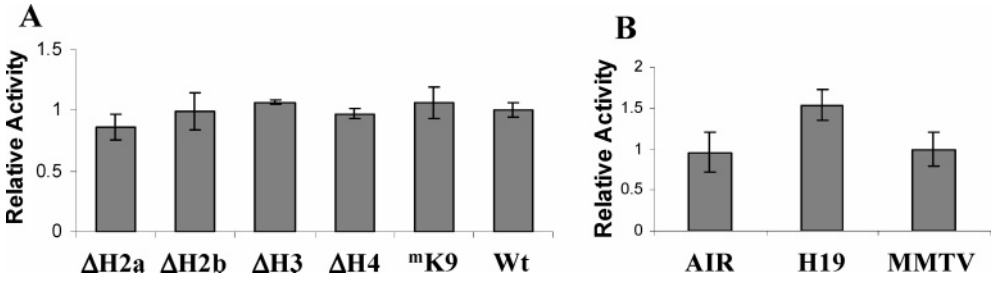

FIGURE 4: (A) Methylation of nucleosomes in which either the histone tails were individually removed (ΔH2A, ΔH2B, ΔH3, ΔH4) or H3 was methylated at lysine 9 (K9) by Dnmt3a. Data were normalized to the methylation activity observed with the wild-type MMTV nucleosomal DNA. (B) Methylation of nucleosomes reconstituted using Air promoter, H19 promoter, or MMTV DNA by Dnmt3a. Data were normalized to the methylation activity observed with the wild-type MMTV nucleosomal DNA.

DISCUSSION

To gain access to nucleosomal DNA, de novo MTases either need an intrinsic capability to methylate nucleosomal DNA or depend on chromatin remodeling factors. Here, we show that de novo methylation of DNA by Dnmt1 and Dnmt3a can occur directly on nucleosomal DNA. In contrast to the prokaryotic SssI MTase, the mammalian Dnmt3a and Dnmt1 MTases efficiently methylate nucleosomal DNA when compared to the methylation of free DNA. This mode of action provides a means by which information encoded in the histone tail modifications can influence the DNA methylation pattern, because DNA methylation can occur while the DNA is still connected to the histone proteins. In contrast, if methylation of nucleosomal DNA were inefficient, removal of histones would be required for DNA methylation, which would make it more difficult to directly link the two types of epigenetic modifications. In the simplest case, one could presume a direct regulation of DNA methyltransferases by histone modification. However, our observations indicate that individual histone tail deletions or K9 methylation of histone H3 does not significantly influence DNA methylation, although we cannot exclude the theoretical possibility that a synergistic interaction to several histone tails takes place. Consistent with this finding, the N-terminal parts of Dnmt1 and Dnmt3a lack known histone interaction motifs. This means that at least in the case of H3 K9 methylation a translation of the histone code into DNA methylation is likely to involve adaptor proteins that control the activity of the DNA MTases in response to the histone tail modification pattern or more complex patterns of histone modifications.

We find that the N-terminal regions of Dnmt3a and Dnmt1 are required for efficient methylation and binding of nucleosomal DNA. Since both N-termini contain at least one additional DNA binding site (*5*, *38*), we suppose these domains are organized such that they can interact efficiently with DNA within the context of the nucleosome. Thereby, a bidentate binding of the enzymes to the nucleosomal DNA (via their N-terminal domains and their catalytic domains) would increase the affinity of the enzyme for nucleosomal DNA and promote the action of the catalytic domain on nucleosomal DNA.

The catalytic mechanism of DNA MTases (*10*), which involves base flipping, fits the observation that DNA bound to nucleosomes can be efficiently modified. Since the enzyme is capable of rotating the target base out of the DNA double helix, it is conceivable that the DNA stays bound to histones during catalysis without causing a strong inhibition. Structural studies with several DNA MTases show that the catalytic domains of MTases approach their substrate DNA via the minor groove (for review, see ref *10*). Considering the even distribution of methylation on the surface of the nucleosomes that is not correlated to the accessibility of the minor groove found here and by Okuwaki and Verreault (*27*), the enzymes most likely trigger a transient dissociation of a short stretch of DNA from the nucleosomes that allows access to the target base. Interestingly, the uracil glycosylase, which flips out of its target base as well, similarly acts on nucleosomal DNA irrespective of the rotational positioning of the target site on the nucleosome (*39*), suggesting that a similar mechanism is operative in this enzyme as well.

It is interesting to compare the results presented here to a similar study published recently, where the maintenance methylation of certain hemimethylated CG sites by Dnmt1 was investigated, showing that Dnmt1 could methylate most target sites in a nucleosomal context (*27*). In agreement with our results, at most sites a moderate inhibition of Dnmt1 was observed, and methylation efficiency was not correlated to the exposure of the major or minor grooves on the nucleosome. However, it was shown that a hemimethylated CpG site placed in the Air promoter sequence was refractory to methylation by Dnmt1, although it was efficiently modified when placed in the H19 CTCF binding site (*27*). In our study, we found efficient de novo methylation of the Air promoter, the H19, and the MMTV sequence by Dnmt3a, demonstrating the ability of this enzyme to methylate nucleosomal DNA irrespective of differences in DNA sequence. Therefore, the Air promotor is not completely refractory to methylation. However, as we monitored global methylation of nucleosomal DNA, we cannot rule out differences in the accessibility of specific sites.

Several lines of evidence suggest that DNA methylation requires ATP-dependent chromatin remodeling. For example, in *Arabidopsis* mutations of DDM1 protein which is the member of SNF2 helicase subfamily cause 70% reduction of genomic methylation (*26*). A putative mammalian counterpart, Lsh, was described as a regulator of DNA methylation (*24*). In addition, there are reports that de novo MTases interact with chromatin remodeling factors (*40*). Our data demonstrate that de novo methylation can occur directly on nucleosomes in the absence of additional factors. Nonetheless, chromatin remodeling enzymes may further accelerate methylation within a nucleosomal context or assist in the methylation of more highly condensed chromatin structures.

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SUPPORTING INFORMATION AVAILABLE

Four figures showing purification of Dnmt1, Dnmt3a, and the catalytic domain of Dnmt3a, methylation of nucleosomal DNA by Dnmt1 and Dnmt3a, sequencing results obtained with bisulfite-converted DNA, and binding of nucleosomes by increasing amounts of Dnmt1. This material is available free of charge via the Internet at http://pubs.acs.org.

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