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Distinct DNA Methylation Activity of Dnmt3a and Dnmt3b towards Naked and Nucleosomal DNA

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In mammals, the resetting of DNA methylation patterns in early embryos and germ cells is crucial for development. *De novo* type DNA methyltransferases Dnmt3a and Dnmt3b are responsible for creating DNA methylation patterns during embryogenesis and in germ cells. Although their *in vitro* DNA methylation properties are similar, Dnmt3a and Dnmt3b methylate different genomic DNA regions *in vivo*. In the present study, we have examined the DNA methylation activity of Dnmt3a and Dnmt3b towards nucleosomes reconstituted from recombinant histones and DNAs, and compared it to that of the corresponding naked DNAs. Dnmt3a showed higher DNA methylation activity than Dnmt3b towards naked DNA and the naked part of nucleosomal DNA. On the other hand, Dnmt3a scarcely methylated the DNA within the nucleosome core region, while Dnmt3b significantly did, although the activity was low. We propose that the preferential DNA methylation activity of Dnmt3a towards the naked part of nucleosomal DNA and the significant methylation activity of Dnmt3b towards the nucleosome core region contribute to their distinct methylation of genomic DNA *in vivo*.

Key words: DNA methylation, DNA methyltransferase, Dnmt3a, Dnmt3b, nucleosome.

In vertebrates, the 5th positions of cytosine residues in CpG sequences in genomic DNA are often methylated (*1*, *2*). DNA methylation of the genome is essential for development (*3*) and plays crucial roles in a variety of biological processes, such as genomic imprinting (*4*) and carcinogenesis (*5*) *via* suppression of certain genes (*6*). In vertebrates, two types of DNA methyltransferase activities have been reported, *i.e.*, *de novo* and maintenance types. In mouse, *de novo* type DNA methylation creates tissue-specific methylation patterns at the implantation stage (*7*), and maintenance type methylation ensures clonal transmission of lineage-specific methylation patterns during replication. Dnmt1 is responsible for the latter activity.

Two DNA methyltransferases, Dnmt3a and Dnmt3b, are responsible for the creation of methylation patterns (*3*, *8*). Targeting of Dnmt3b gives a severer phenotype than that of Dnmt3a (*3*), and mutation of the *DNMT3B* gene, a human homologue of *Dnmt3b*, leads to hypomethylation in the satellite 2 and 3 regions of specific chromosomes (*9*), which is the cause of the ICF (immunodeficiency, centromeric region instability, and facial anomalies) syndrome (*3*, *10*, *11*). The results clearly indicate that Dnmt3a and Dnmt3b methylate distinct genomic regions *in vivo*. Although the specific DNA methylation activity of recombinant Dnmt3a is 1.2–1.5 fold higher than that of Dnmt3b when poly(dGdC)-poly(dGdC) is used as the substrate (*12*, *13*), both enzymes preferentially recognize dinucleotide sequence CpG. It was reported that Dnmt3a preferentially methylates CG flanked by pyrimidine (*14*), but, we could not find such a preference (*13*). Handa and Jeltsch reported that both Dnmt3a and Dnmt3b preferred to methylate RCGY with AT-rich flanks and disliked YCGR (*15*). This similar preference of Dnmt3a and Dnmt3b towards the target sequence, however, does not explain why distinct regions are methylated by Dnmt3a and Dnmt3b *in vivo*.

To explain why distinct DNA regions are methylated, the timing of expression of Dnmt3a and Dnmt3b should be considered. During early embryogenesis, Dnmt3b is highly expressed in totipotent embryonic cells, such as inner cell mass, epiblast, and embryonic ectoderm cells, while Dnmt3a is significantly and ubiquitously expressed in mesenchymal cells after E10.5 (*16*). This timing of expression of Dnmt3b coincides with the global methylation of genomic DNA at the implantation stage (*7*). Recently, we also reported that Dnmt3a2, a short form of Dnmt3a (*17*), and Dnmt3L, a necessary factor for DNA methylation in germ cells (*18–20*), are highly expressed in male germ cells in 14- to 18-day embryos (*21*), at which stage genome-wide DNA methylation occurs (*22*, *23*). As conditional targeting of the *Dnmt3a* gene in mouse germ cells erases the methylation imprint in germ cells (*24*), this stage-specific expression of Dnmt3a2 may contribute to the global methylation of genomic DNA in germ cells. Accordingly, such distinct timing of expression of DNA methyltransferases could be one reason why distinct DNA regions are methylated by these enzymes.

It should be noted that Dnmt3a is ubiquitously expressed in somatic cells without aberrant methylation (*16*). This clearly indicates that, other than the difference in timing of expression of Dnmt3a and Dnmt3b, these enzymes should methylate the distinct DNA regions by

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different mechanisms *in vivo*. DNA in the eukaryotic nucleus is packaged into a highly compact nucleoprotein complex, *i.e.*, chromatin (*25*, *26*), of which the elemental structure is nucleosomes. Interestingly, when the *Lsh* gene in mouse, the *ATRX* gene in man, or the *ddm1* gene in *Arabidopsis* is mutated, the DNA methylation level decreases (*27–31*). The products of these genes are components of ATP-dependent chromatin remodeling factors of the SWI2/SNF2 family. In addition, Dnmt3a and/or Dnmt3b interact directly with chromatin remodeling factor SNF2H, which is a member of the ISWI family (*32*, *33*), and with factors that modify core histones such as histone deacetylase, histone methyltransferase, and HP1, which specifically binds K9-methylated histone H3 (*32–37*). These modifications and binding proteins are the trigger for induction of the conversion of chromatin into inactive heterochromatin. Accordingly, the DNA methylation activity of Dnmt3a and Dnmt3b towards chromatin or its elemental structure, nucleosomes, should be considered to determine how specific regions of genomic DNA are methylated. Recently, DNA methylation activity towards reconstituted mononucleosomes was reported for DNMT1, a human homologue of maintenance-type DNA methyltransferase 1 (Dnmt1), and Dnmt3a (*38–40*). Okuwaki and Verreault reported that DNMT1 can methylate CpG even when it is packaged into nucleosomes, and the sequence specificity of DNMT1 becomes prominent when DNA is packaged into nucleosomes; and Robertson et al. reported that the activities of both DNMT1 and Dnmt3a decrease when the DNA is packaged into nucleosomes (*39*, *40*). On the contrary, Gowher et al. argued that Dnmt3a efficiently methylates nucleosomal DNA (*38*).

In the present study, we reconstituted mononucleosomes from recombinant histones H2A, H2B, H3, and H4 with DNAs differing in length and sequence, and then used them as substrates for determination of the DNA methylation activity of recombinant Dnmt3a and Dnmt3b. The DNA methylation activity of both Dnmt3a and Dnmt3b was severely inhibited on the formation of nucleosomes. Interestingly, the DNA methylation activity towards the naked DNA region of reconstituted nucleosomes was much higher for Dnmt3a than Dnmt3b, and that towards the DNA within the nucleosome core region was significant for Dnmt3b and negligible for Dnmt3a. We propose that the stage-specific high level expression of Dnmt3b contributes to the global methylation of the genome, even when it is packaged into nucleosomes; and the ubiquitous expression of Dnmt3a promptly methylates genomic DNA at the time when it becomes partially naked, for example, through the action of a chromatin-remodeling factor.

MATERIALS AND METHODS

Protein Expression and Purification—The cDNAs of human histones H2A, H2B, H3, and H4 were subcloned into an expression vector, pET22b (Merck), and then expressed in BL21-Codon Plus-R1L (Stratagene). The histone proteins were expressed as described elsewhere (*41*, *42*), and purified as described previously (*43*, *44*). Recombinant Dnmt3a and Dnmt3b were expressed in Sf9 cells, and then purified as described previously (*13*). The specific activities of the purified Dnmt3a and Dnmt3b are the highest hitherto reported (*13*).

Preparation of DNA Fragments for the Reconstitution of Nucleosomes—Mouse mammary tumor virus (MMTV)-A145 (145 bp) and MMTV-A242 (242 bp) (*45*), and the *Xenopus borealis* 5 S ribosomal RNA gene (5 S rDNA) fragments of 5S (RR) (155 bp) and 5S (RD) (220 bp), for which the sequence information was kindly provided by Dr. J. J. Hayes (*46*), were PCR-amplified with KOD DNA polymerase (Toyobo, Japan).

Naked DNA and nucleosomes electrophoresed in an agarose gel with 0.5× TBE buffer were stained with SYBR Green I (Invitrogen), and then the DNA was identified with an image analyzer, FluorImager 595 (Molecular Dynamics, CA), using program ImageQuant (Molecular Dynamics). The naked and nucleosomal DNA concentrations were spectrophotometrically determined (*44*).

Preparation of Nucleosomes from HeLa Cells—Nuclei were isolated from HeLa S3 cells as described elsewhere (*47*). Nuclei prepared from 2×10^6 cells were treated with 1 U of micrococcal nuclease (MNase) (Worthington, NJ) at 37°C for 15 min. Nucleosomes were passed through ionic exchange resin AG50-X2 (BioRad) (*48*), and then mononucleosomes were isolated by sucrose density gradient centrifugation (*49*). In brief, the nucleosomal fractions were layered on 5–20% (w/v) sucrose in 1 mM EDTA, 0.1 mM PMSF, 1 mM β-mercaptoethanol, and 10 mM Tris-HCl, pH 7.5, and then centrifuged at 230,000 × *g* in a Hitachi P40ST swing-type rotor at 4°C for 16 h. The fractions containing nucleosomes were concentrated with a Microcon YM-30 microconcentrator (Millipore). To prepare naked DNA, proteins in the nucleosomes were digested with proteinase K, extracted with phenol/chloroform, and then precipitated with ethanol.

Reconstitution of Nucleosomes—The reconstitution of the histone octamer and nucleosomes was performed as described elsewhere (*44*). The reconstituted nucleosomes were isolated from free DNA by centrifugation at 280,000 × *g* in a Hitachi P40ST swing-type rotor at 4°C for 3 h with a linear glycerol gradient of 5–20% (v/v) containing 1 mM EDTA, 0.1 mM PMSF, 1 mM β-mercaptoethanol, and 10 mM Tris-HCl, pH 7.5. The reconstitution of nucleosomes was confirmed by the protection of 145–147-bp DNA from MNase digestion (*50*). In brief, the reconstituted nucleosomes (25 ng DNA) were treated with or without MNase at 22°C for 10 min in a buffer comprising 10 mM KCl, 10% (v/v) glycerol, 1.5 mM $MgCl_2$, 2.7 μM $CaCl_2$, 0.5 mM EGTA, 1 mM DTT, 0.2 mM PMSF, 0.16 mg/ml bovine serum albumin, and 10 mM Hepes-KOH, pH 7.5. After the digestion, the 5′-ends of the DNA fragments were labeled with [γ-^{32}P]-ATP (Amersham Biosciences) and T4 polynucleotide kinase (Toyobo), then subjected to electrophoresis in a 5% polyacrylamide gel with 1× TBE buffer. The dried gels were subjected to autoradiography with an intensifying screen, X-Omat (Kodak, NY), at –80°C.

SDS–Polyacrylamide Gel Electrophoresis—To identify histones, and Dnmt3a and Dnmt3b, 18% and 7.5% polyacrylamide gels, respectively, were used, electrophoresis being performed as described elsewhere (*51*). The gels were stained with SYPRO Orange (Invitrogen) according to the manufacturer's instructions and the bands were visualized with FluorImager 595.

DNA Methylation Activity—DNA methylation activity was determined as described elsewhere (*13*). In brief, reaction mixtures containing 40 nM Dnmt3a, Dnmt3b or

16 U/ml SssI DNA methyltransferase (M.SssI) (New England Biolab, MA), the indicated amounts of naked DNA or nucleosomes, and 5.3 μM [^{3}H]-*S*-adenosyl-L-methionine (AdoMet) (15 Ci/mmol; Amersham Biosciences) in 25 μl of a reaction buffer comprising 2.7 M glycerol, 5 mM EDTA, 0.2 mM DTT, 25 mM NaCl, and 20 mM Tris-HCl, pH 7.4, were incubated at 37°C for 1 h. After the incubation, the radioactivity incorporated into DNA was determined with a liquid scintillation counter.

Determination of DNA Methylation Regions—To determine the DNA methylation sites in the 5S (RD) DNA, naked DNA and reconstituted nucleosomes were methylated with 35.2 μM [^{14}C]-AdoMet (57 mCi/mmol; Amersham Biosciences). After the incubation, the mixtures were treated with proteinase K, followed by phenol extraction and ethanol precipitation of the DNA. The DNAs thus prepared were digested with RsaI or HaeIII, and then electrophoresed in a 10% polyacrylamide gel with 1× TBE buffer. After electrophoresis, each gel was treated with EN^{3}HANCE (Amersham Biosciences). The gel was then exposed to an imaging plate (Fuji Photo Film, Japan), and the radioactivity was determined with a BAS2000 (Fuji Photo Film).

To determine the methylation levels of the nucleosome core region, naked and reconstituted nucleosomes of MMTV-A145 and 5S (RR) were methylated as above, and then digested with or without 3.75 U/ml MNase at 22°C for 10 min. An aliquot of the DNA was labeled with [γ-^{32}P]-ATP mixed with 2 μM cold ATP and T4 polynucleotide kinase to determine the recovery of DNA. The labeling efficiency was linear within the range of DNA concentrations used in the study. The [^{14}C]- and [^{32}P]-labeled DNAs were separately electrophoresed in a 5% polyacrylamide gel with 1× TBE buffer, and the radioactive bands were determined with a BAS2000 as above. The relative concentration of 5′-phosphorylated DNA was calculated from the specific radioactivity of [γ-^{32}P]-ATP/cold ATP, and was used to normalize the DNA methylation level.

Gel Ahift Assay—The conditions used for the binding of Dnmt3a or Dnmt3b (40 nM) to DNA or nucleosomes (30 ng) were identical to those for the determination of the DNA methylation activity, except for the use of cold AdoMet. The cold AdoMet used in this study was purified on a Sep-Pack C18 column (Waters, Japan) before use (*13*). After 1 h of incubation at 37°C, the reaction mixtures were loaded onto 0.7% agarose gels and electrophoresed in 0.5× TBE buffer. The DNA bands were stained with SYBR Green I.

Determination of Histones in the Nucleosomes, and Associated Dnmt3a and Dnmt3b after the DNA Methylation Reaction—Dnmt3a (0.36 μM) or Dnmt3b (1.1 μM) was incubated with nucleosomes containing 0.19 μM 5S (RD), which is the 220-bp fragment prepared from the *Xenopus borealis* 5 S ribosomal RNA gene, and 48 μM cold AdoMet on ice or at 37°C for 1 h. After the incubation, the reaction mixtures were loaded onto 0.7% SeaPrep agarose gels (Cambrex Bio Science, ME), and electrophoresed in 0.5× TBE buffer. The DNA bands were stained with ethidium bromide, and then each band was excised, melted, and TCA (20%, w/v)-precipitated. The proteins were electrophoresed in a SDS-polyacrylamide gel, and then determined with SYPRO Orange as described above.

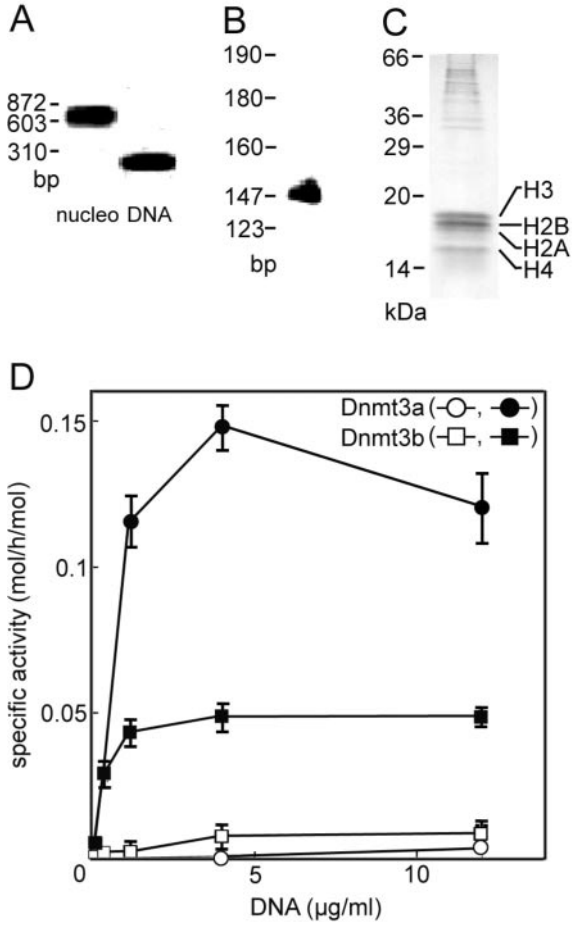

Fig. 1. **DNA methylation activity of Dnmt3a and Dnmt3b towards nucleosomes prepared from HeLa cell nuclei.** A: Nucleosomes prepared from HeLa cell nuclei (nucleo) and naked DNA prepared from the nucleosomes (DNA) were electrophoresed in a 0.7% agarose gel, and then the DNA was visualized with SYBR Green I. B: The size of the DNA packaged into HeLa nucleosomes was further examined by polyacrylamide gel electrophoresis. The DNA of around 147 bp was the major component of the nucleosomal DNA, indicating that the nucleosomes were mononucleosomes without linker DNA. C: Protein components of isolated nucleosomes were electrophoresed in an 18% SDS–polyacrylamide gel and then visualized with SYPRO Orange. The minor bands detected above 30 kDa were densitometrically determined to comprise about 15% of the total protein. D: The DNA methylation activities of Dnmt3a (circles) and Dnmt3b (squares) towards naked DNA (filled symbols) and nucleosomes (open symbols) are shown as specific activity in mol/h/mol of Dnmt3a or Dnmt3b, as means ± SD (n = 3, respectively).

RESULTS

DNA Methylation Activity of Dnmt3a and Dnmt3b towards Nucleosomes Prepared from HeLa Cells—The majority of genomic DNA *in vivo* is in the form of chromatin, of which the elemental structure is nucleosomes, a complex composed of DNA and a histone octamer. To mimic the *in vivo* state, it is important to examine the DNA methylation activity of Dnmt3a and Dnmt3b towards nucleosomes as a methyl acceptor. Firstly, we prepared nucleosomes from HeLa cell nuclei by means of MNase treatment and partially purified them (Fig. 1A). These

nucleosomes contained DNA of around 147 bp as the major DNA component (Fig. 1B), indicating that they were mononucleosomes lacking linker DNA. The nucleosomal fraction contained a histone octamer comprising histones H2A, H2B, H3, and H4, as major protein components. In addition, protein components of above 30 kDa in size and constituting about 15% of the total protein were identified in the preparation (Fig. 1C). The DNA methylation activity of Dnmt3a and Dnmt3b towards these nucleosomes was determined and compared with that towards deproteinized naked DNA (Fig. 1D). The DNA methylation activity of both Dnmt3a and Dnmt3b towards the nucleosomes was severely inhibited. The activity of Dnmt3b towards the nucleosomes, however, was significant, while that of Dnmt3a was almost below the detection level, *i.e.*, 0.3 ± 3.7 mmol/h/mol for Dnmt3a and 7.6 ± 3.2 mmol/h/mol for Dnmt3b at 4 μg/ml DNA. On the other hand, Dnmt3a showed more than two-fold higher activity than Dnmt3b towards naked DNA. The specific activity of Dnmt3a towards naked DNA, which was 0.15 ± 0.01 mol/h/mol, was three times higher than that of Dnmt3b, which was 0.05 ± 0.01 mol/h/mol, with 4 μg/ml DNA. The naked DNA prepared from HeLa nucleosomes seemed not to be a good substrate for the enzymes, since Dnmt3a and Dnmt3b showed about one order of magnitude higher specific activity towards poly(dGdC)-poly(dGdC) (*13*).

DNA Methylation Activity of Dnmt3a and Dnmt3b towards Nucleosomes Reconstituted from MMTV-LTR DNA Fragments—Native nucleosomes prepared from HeLa cell nuclei contained protein components other than core histones, and the histones may have undergone modifications such as acetylation, methylation, phosphorylation, and so on. In addition, since 80% of the CpG sequences are already methylated in somatic cells (*2*), the capacity of HeLa nuclear DNA for DNA methylation is limited. Such decorations or modifications might affect the DNA methylation activity of Dnmt3a and Dnmt3b. To avoid the effects of histone modifications, DNA methylation state, and/or other factors, we next reconstituted mononucleosomes from bacterially expressed histones, which are expected to have undergone no modification, and a DNA fragment of 145 bp prepared from mouse mammary tumor virus 3′-LTR (MMTV-A145), which is well known to form stable nucleosomes that completely lack a linker region (*45*). The purified nucleosomes were contaminated by free DNA, although the amount was very small (Fig. 2B). The DNA in nucleosomes was resistant to MNase digestion (Fig. 2C), indicating that the reconstituted nucleosomes were properly packaged.

The DNA methylation activity of Dnmt3a and Dnmt3b towards MMTV-A145 nucleosomes was determined (Fig. 2D). When MMTV-A145 nucleosomes that completely lack linker DNA were used as the substrate, the DNA methylation activity of both Dnmt3a and Dnmt3b was extremely low compared to that towards the naked MMTV-A145. Nevertheless, the activity towards the MMTV-A145 nucleosomes was significant even with Dnmt3a, *i.e.*, 0.08 ± 0.04 and 0.06 ± 0.02 mol/h/mol for Dnmt3a and Dnmt3b, respectively, with 2.50 μM CpG. The DNA methylation activity of Dnmt3a and Dnmt3b towards a higher concentration of MMTV-A145 nucleosomes, which was 0.16 ± 0.02 and 0.10 ± 0.01 mol/h/mol for Dnmt3a and Dnmt3b, respectively, with 7.5 μM CpG, did not reach the level of that towards the naked MMTV-A145. The finding that Dnmt3a showed very low DNA methylation activity towards the MMTV-A145 nucleosomes is quite distinct from the recent report by Gowher *et al.*, who demonstrated that Dnmt3a efficiently methylates nucleosomes reconstituted from 147-bp MMTV-DNA, which is identical to the MMTV-A145 used in the present study except for an extra two nucleotide sequence (*38*).

On the other hand, the maximum activity of Dnmt3a towards naked MMTV-A145, which was 1.09 ± 0.13 mol/h/mol, was more than 4 times higher than that of Dnmt3b, which was 0.25 ± 0.04 mol/h/mol, with 0.84 μM CpG. The titration profiles of the DNA methylation activities of Dnmt3a and Dnmt3b towards the naked and nucleosomal MMTV-A145 resembled those towards the naked DNA and nucleosomes prepared from HeLa nuclei, except for the remarkable difference that Dnmt3a apparently showed a significant level of DNA methylation activity towards the reconstituted nucleosomes, which was similar to that of Dnmt3b. Unlike Dnmt3a and Dnmt3b, the DNA methylation activity of M.SssI, a bacterial DNA methyltransferase, was not strongly inhibited on the formation of nucleosomes (Fig. 2D, lower panel).

As Dnmt3a preferred to methylate naked DNA, next we determined whether or not partially naked DNA specifically enhances the activity of Dnmt3a. For this, the length of MMTV-A145 was increased to 242 bp (MMTV-A242) for the reconstitution of nucleosomes (*45*). The expected position of the histone octamer is illustrated in Fig. 2A. However, the exact probability of this position is not known. Increasing of the DNA length to produce a linker region remarkably enhanced the DNA methylation activity of Dnmt3a, but did not greatly alter that of Dnmt3b, the levels being 0.32 ± 0.05 and 0.08 ± 0.02 mol/h/mol, respectively, with 1.50 μM CpG. Dnmt3a preferred to methylate the reconstituted nucleosomes with linker DNA (Fig. 2E). The activity of Dnmt3a towards naked MMTV-A242 was about 5 times higher than that of Dnmt3b as well. M.SssI activity towards the reconstituted nucleosomes, although for some reason inhibited by the addition of a linker region, was still significant (Fig. 2E, lower panel).

DNA Methylation Activity of Dnmt3a and Dnmt3b towards Nucleosomes Reconstituted from 5S rDNA—To further determine whether or not Dnmt3a and Dnmt3b can methylate nucleosomes that have no linker region, and, in addition, whether or not Dnmt3a preferentially methylates nucleosomes containing a linker region, a DNA fragment prepared from the *Xenopus borealis* ribosomal RNA gene (5 S rDNA), which is also known to form stable nucleosomes (*50*), was employed for the reconstitution of nucleosomes. The partial sequences of 5 S rDNA of two different lengths, 5S (RR) (155 bp) and 5S (RD) (220 bp) (*46*), were used for the reconstitution. The reported major positions of the histone octamer in the reconstituted nucleosomes are shown in Fig. 3A. The reconstituted nucleosomes again were contaminated by a very small amount of free DNA (Fig. 3B), and MNase digestion of the nucleosomes revealed a resistant DNA size of around 146 bp (Fig. 3C), indicating that they were properly reconstituted.

When the DNA methylation activity of Dnmt3a and Dnmt3b towards 5S (RR) nucleosomes was determined, their specific activity was again significantly lower,

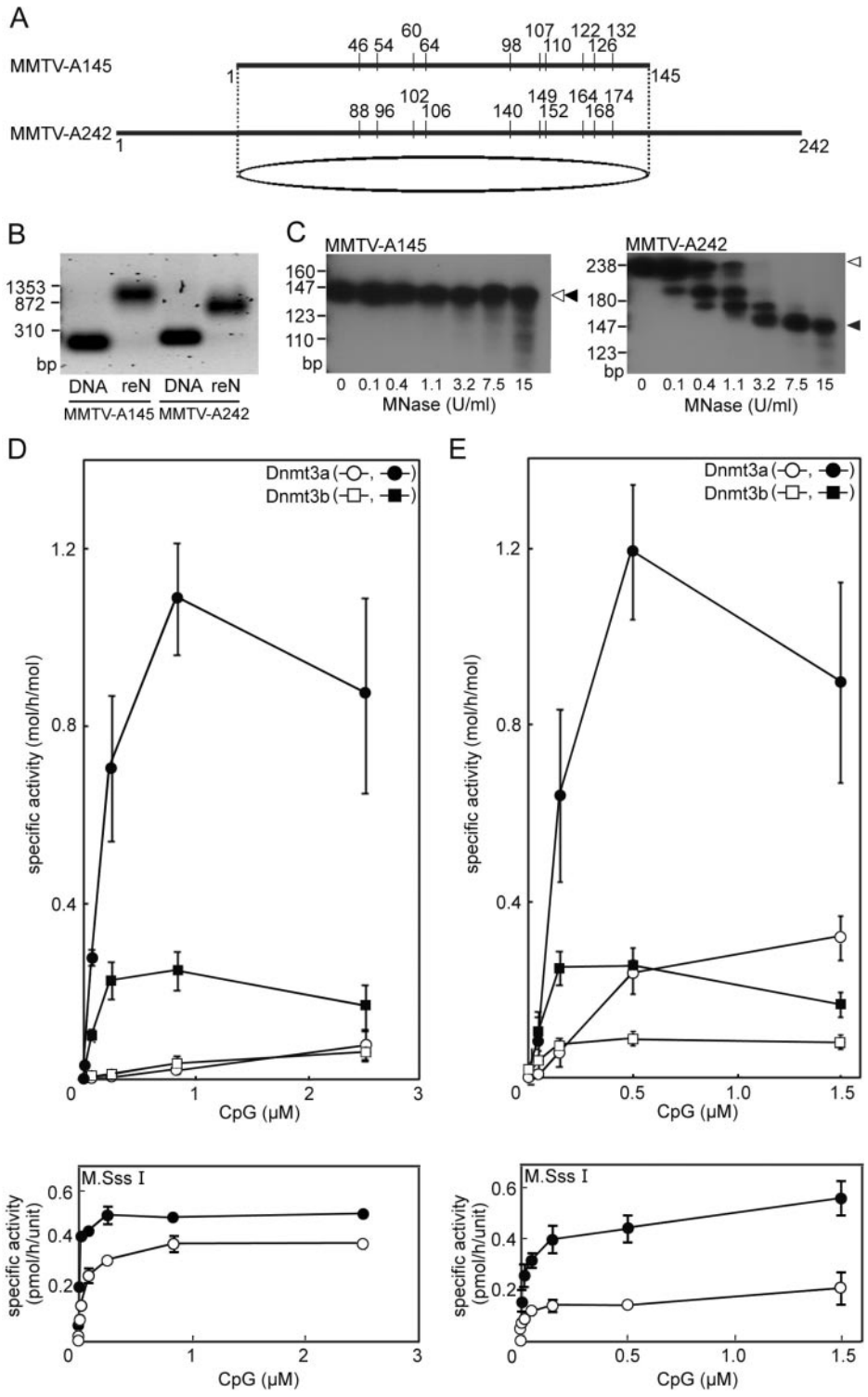

Fig. 2. **DNA methylation activity of Dnmt3a and Dnmt3b towards reconstituted nucleosomes with MMTV-LTR DNA.** A: The reported location of the histone octamer (ellipse), and the positions of cytosines in the CpG sequences with the nucleotide numbers in the nucleosomes reconstituted from MMTV-A145 and MMTV-A242 are indicated. B: MMTV-A145 and MMTV-A242 DNA (DNA), and reconstituted nucleosomes (reN) with MMTV-A145 and MMTV-A242 were electrophoresed in a 0.7% agarose gel and then visualized with SYBR Green I. A very small amount of free DNA was detected in the reconstituted nucleosomes. C: Reconstituted nucleosomes were treated with the indicated amounts (U/ml) of MNase, their 5′-ends were labeled with ^{32}P, and then they were electrophoresed in 5% polyacrylamide gels, the DNA fragments being visualized by autoradiography. The initial (open arrowheads) and MNase-resistant (filled arrowheads) DNA fragments are indicated. The reconstituted nucleosomes gave a nuclease-resistant band at around 146 bp. D and E: The DNA methylation activities of Dnmt3a (circles), Dnmt3b (squares), and M.SssI towards naked (filled symbols) and nucleosomal (open symbols) MMTV-A145 (D), and MMTV-A242 (E) are shown as specific activity in mol/h/mol for Dnmt3a and Dnmt3b, and pmol/h/unit for M.SssI, as means ± SD (n = 3, respectively). One mol double-stranded DNA with 1 CpG site was calculated to be 2 mol.

i.e., 0.19 ± 0.06 and 0.15 ± 0.01 mol/h/mol, respectively, with 2.35 μM CpG, than those towards the naked 5S (RR), *i.e.*, 1.05 ± 0.12 and 0.53 ± 0.09 mol/h/mol, respectively, with 0.78 μM CpG (Fig. 3D). Similar to the case of MMTV-A145 nucleosomes, both Dnmt3a and Dnmt3b apparently methylated the 5S (RR) nucleosomes containing very short linker DNAs. Note that the activity of Dnmt3a towards the naked 5S (RR) was about 2 times higher than that of Dnmt3b. On the contrary, the DNA methylation activity of M.SssI towards 5S (RR) nucleosomes was not severely inhibited compared to that towards the naked 5S (RR) (Fig. 3D, lower panel), which was also similar to the case of MMTV-A145.

When 5S (RD) nucleosomes containing long linker DNA were used for the DNA methylation activity assay, the specific activity of Dnmt3a, which was 0.86 ± 0.08 mol/h/mol

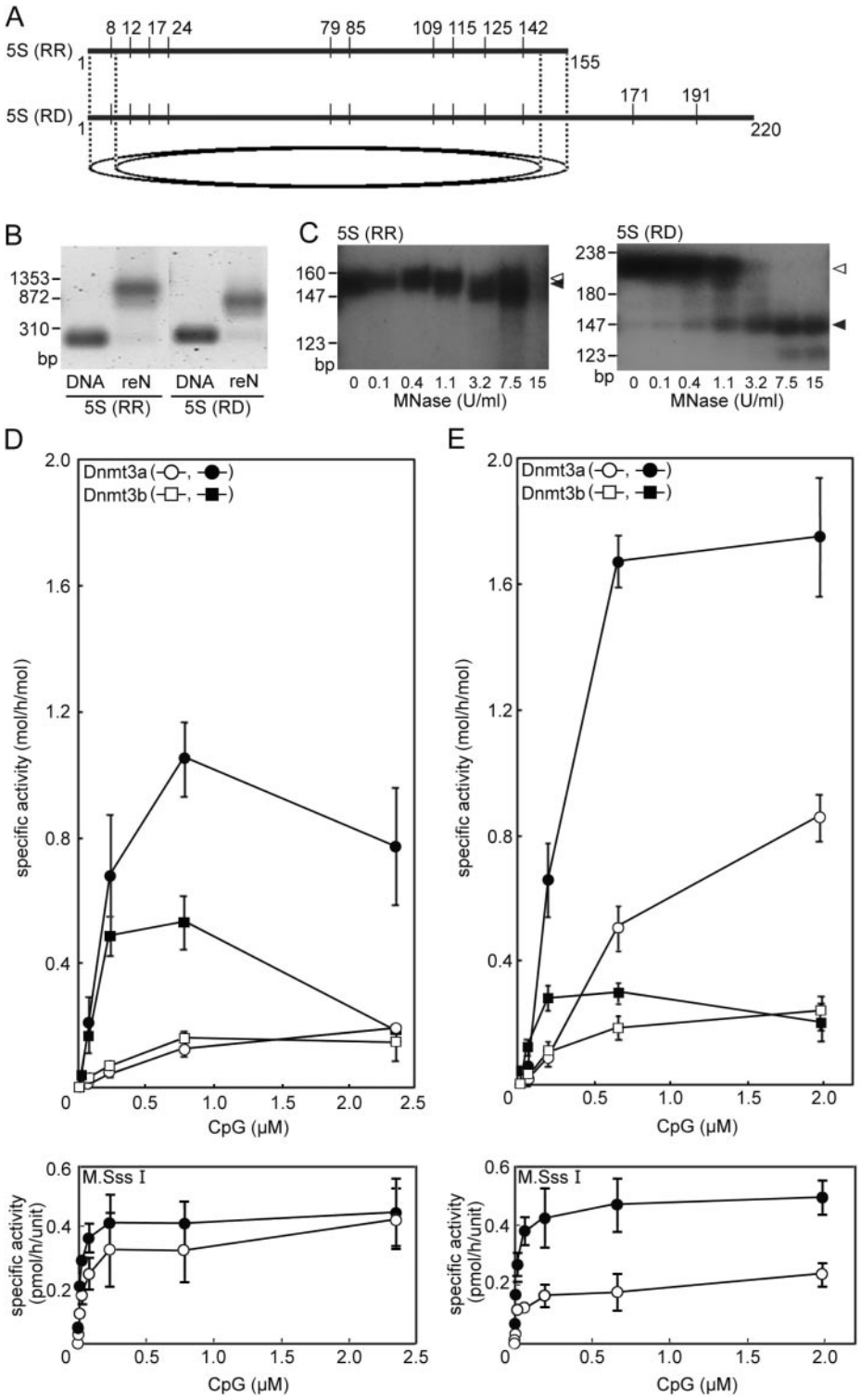

Fig. 3. **DNA methylation activity of Dnmt3a and Dnmt3b towards reconstituted nucleosomes with 5 S rDNA.** A: The reported locations of the histone octamers (ellipses), and the positions of cytosines in the CpG sequences with their nucleotide numbers in the nucleosomes reconstituted with the 5S (RR) and 5S (RD) DNA fragments are indicated. B: DNAs of 5S (RR) and 5S (RD) (DNA), and reconstituted nucleosomes (reN) with 5S (RR) and 5S (RD) were electrophoresed in a 0.7% agarose gel, and then the DNA was visualized as in Fig. 2B. A very small amount of free DNA was detected in the reconstituted nucleosomes. C: The sizes of MNase-resistant DNA fragments were determined for 5S (RR) (left) and 5S (RD) (right) nucleosomes, as in Fig. 2C. The initial (open arrowheads) and MNase-resistant (filled arrowheads) DNA fragments are indicated. The reconstituted nucleosomes were protected from MNase treatment. D and E: The DNA methylation activities of Dnmt3a (circles), Dnmt3b (squares), and M.SssI towards naked (filled symbols) and nucleosomal (open symbols) 5S (RR) (D) and 5S (RD) (E) are shown as specific activity in mol/h/mol for Dnmt3a and Dnmt3b, and pmol/h/unit for M.SssI, as means ± SD (n = 3, respectively). One mol double-stranded DNA with 1 CpG site was calculated to be 2 mol.

with 1.98 μM CpG, was about 4.5 times higher than that towards 5S (RR) nucleosomes, which was 0.19 ± 0.06 mol/h/mol with 2.35 μM CpG. On the other hand, the specific activity of Dnmt3b towards 5S (RD) nucleosomes, which was 0.24 ± 0.06 mol/h/mol with 1.98 μM CpG, was about 1.6-fold higher than that towards 5S (RR) nucleosomes, which was 0.15 ± 0.01 mol/h/mol with 2.35 μM CpG. Unlike Dnmt3b, Dnmt3a preferentially methylated the 5S (RD) reconstituted nucleosomes containing long linker DNA, that is, partially naked DNA. Interestingly, the addition of linker DNA again slightly inhibited the activity of M.SssI. When naked 5S (RD) was used as the substrate, the specific activity of Dnmt3a was more than 5 times higher than that of Dnmt3b. Therefore, as described

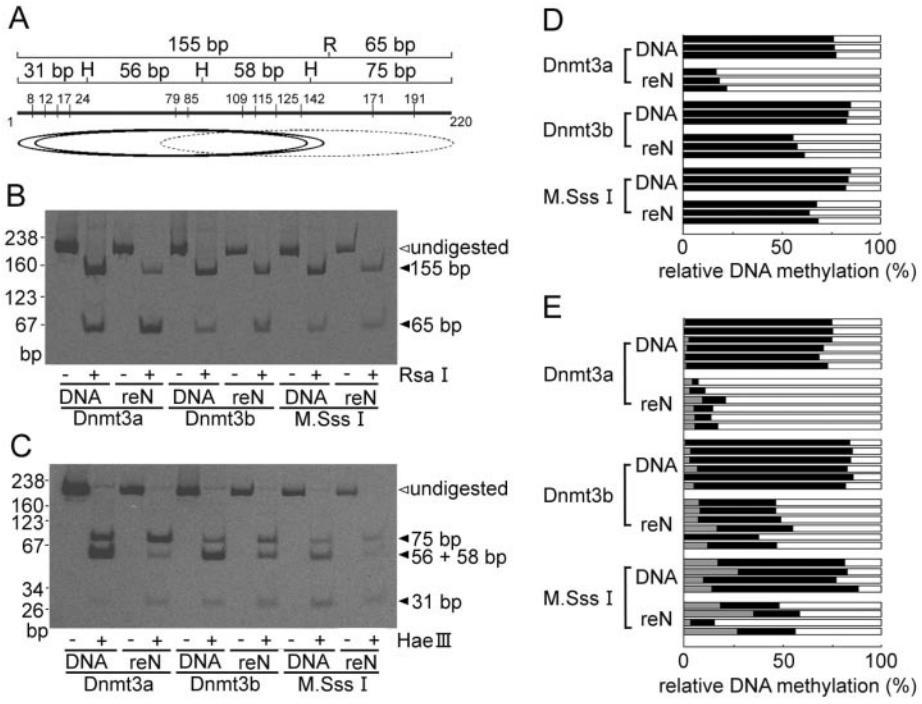

Fig. 4. **DNA methylation sites for Dnmt3a and Dnmt3b.** A: The reported locations of the histone octamers (ellipses), and the positions of cytosine residues in the CpG sequences of 5S (RD) are illustrated. For the histone octamer positioned at the 3′-end, the covering sequence is indicated by a dotted ellipse. The restriction sites for *Rsa*I (R) and *Hae*III (H), which were used for the dissection, are indicated. B and C: Naked 5S (RD) (DNA) or that in nucleosomes (reN) was methylated with Dnmt3a, Dnmt3b, M. SssI, and [^{14}C]-AdoMet. After the reaction, the 5S (RD) was digested with or without *Rsa*I (B) or *Hae*III (C). DNA was electrophoresed in a 10% polyacrylamide gel. The radioactive bands were visualized with an image analyzer. D and E: The relative radioactivity incorporated into a 155-bp band (black bars) and a 65-bp band (white bars) produced on the *Rsa*I digestion (D), and that into a 31-bp band (gray bars), 56- and 58-bp bands (black bars), and a 75-bp band (white bars) produced on the *Hae*III digestion (E) were determined. Each bar indicates the result of an independent experiment.

above, naked DNA is a better substrate for Dnmt3a than for Dnmt3b, irrespective of the difference in substrate DNA sequence.

Dnmt3a Prefers to Methylate DNA within the Linker Region—The results presented above indicate that Dnmt3a and Dnmt3b apparently could methylate DNA within the nucleosome core region, although weakly. In addition, Dnmt3a preferred to methylate the nucleosomes that contained linker portions. The question arose of whether or not this enhanced activity of Dnmt3a was due to methylation of the DNA within the nucleosome core region or only the CpGs in the linker sequence, that is, the naked DNA region.

We determined whether Dnmt3a methylated the added linker DNA or was induced to methylate the CpGs that were in the sequence within the nucleosome core region. For this, the methylation sites in 5S (RD) nucleosomes, in which 80% of core histones reside at the indicated positions in Figs. 3A and 4A (*52*), were analyzed. After DNA methylation, the naked and nucleosomal 5S (RD) were digested with RsaI, followed by polyacrylamide gel electrophoresis. *Rsa*I digestion generates a 5′ end 155-bp fragment containing 10 CpGs, at which the histone octamer is positioned with about 80% probability (*52*), and a 3′ end 65-bp fragment expected to exist mainly as a linker containing 2 CpGs (Fig. 4A). The radioactivity of each band was determined and typical results are shown in Fig. 4B. The relative radioactivity incorporated into a 155-bp band was low when the nucleosomes were methylated with Dnmt3a. On the other hand, Dnmt3b methylated the 155-bp fragment in 5S (RD) nucleosomes more efficiently. The relative radioactivity incorporated into the 155- (black bars) and 65-bp (white bars) fragments was determined in three independent experiments, and is shown in Fig. 4D. The 65-bp fragment in nucleosomes, which was expected to be in the linker region of nucleosomes, was efficiently methylated, especially by Dnmt3a. The results indicate that the enhancement of the DNA methylation activity of Dnmt3a on the addition of the linker DNA region was mainly due to the preferential methylation of the naked linker region of the nucleosomes.

Since not all the histone octamers in the 5S (RD) nucleosomes were positioned in the 155-bp region (*52*), some of them must be positioned elsewhere. Even though the histone octamer was positioned at the very 3′ end of the 5S (RD), the CpGs in the middle (cytosine residues at positions 79, 85, 109, 115, 125, and 142, see Fig. 4A) were never out of the nucleosome core region. To determine and compare the methylation levels of the CpGs in the 3′ major linker region and the middle part, another restriction enzyme, *Hae*III, was utilized. *Hae*III digestion generates a 31-bp fragment containing 4 CpGs from the 5′ end, which becomes exposed only when a histone octamer is positioned at the 3′ end (Fig. 4A, dotted lined ellipse); 56- and 58-bp fragments derived from the middle portion containing 2 and 4 CpGs, respectively, which never exist outside of the packaged position, and a 3′ end 75-bp fragment containing 2 CpGs, which mainly exists as a linker. Typical results are shown in Fig. 4C. The relative radioactivity incorporated into the 56- and 58-bp fragments was much lower when the nucleosomes were methylated with Dnmt3a rather than Dnmt3b or M.SssI. As was observed above, Dnmt3a quite efficiently methylated the 75-bp fragment in the nucleosomes. The relative radioactivity was determined in 6, 6, and 4 independent experiments for Dnmt3a, Dnmt3b, and M.SssI, respectively, and those for the 31-bp (gray bars), 56- and 58-bp (black bars), and 75-bp (white bars) fragments are shown (Fig. 4E). The

results support the conclusion drawn from RsaI digestion that the enhancement of the DNA methylation activity of Dnmt3a on the addition of the linker DNA region was mainly due not to the methylation of the nucleosome core region but to the preferential methylation of the naked linker region in the nucleosomes. Although Dnmt3b and even M.SssI tended to methylate the linker region in nucleosomes, the preferential methylation by Dnmt3a of the naked region in the nucleosomes was prominent.

The relative radioactivity incorporated into the 5′ end 31-bp fragment in the 5S (RD) nucleosomes due to the DNA methylation activity of Dnmt3a and Dnmt3b was even lower than the levels incorporated into the 56- and 58-bp fragments. This may not be due to the high probability of positioning of the histone octamer in this region, since the 31-bp fragment was scarcely methylated even in the naked 5S (RD) (see Fig. 4E). It is possible that the 31-bp fragment was partially lost during the treatment of the gels before exposure due to its small size. However, since the 31-bp fragment was significantly methylated in the case of M.SssI, loss of this fragment cannot solely be responsible for the poor methylation with Dnmt3a or Dnmt3b. It seems difficult for Dnmt3a and Dnmt3b to methylate the CpGs at the very end of the DNA molecule.

DNA Methylation of the Nucleosome Core Region by Dnmt3a and Dnmt3b—It should be noted that Dnmt3a quite inefficiently methylated the nucleosome core region (Fig. 4). However, it was also shown that Dnmt3a and Dnmt3b apparently methylated nucleosomes reconstituted from MMTV-A145 and 5S (RR), which possessed no and very short linker DNA, respectively, to similar levels (Figs. 2 and 3). Considering that each of the nucleosome preparations was contaminated by a small amount of free DNA, and Dnmt3a methylated the naked DNA region quite efficiently, it is possible that the apparent methylation activity observed for MMTV-A145 and 5S (RR) nucleosomes with Dnmt3a was that towards the free naked DNA. To determine whether or not Dnmt3a and Dnmt3b directly methylate the nucleosome core region, free DNA contaminating the nucleosome preparations was digested with MNase after the DNA methylation reaction. Then the radioactivity incorporated into the MNase-resistant DNA fraction was determined (Fig. 5).

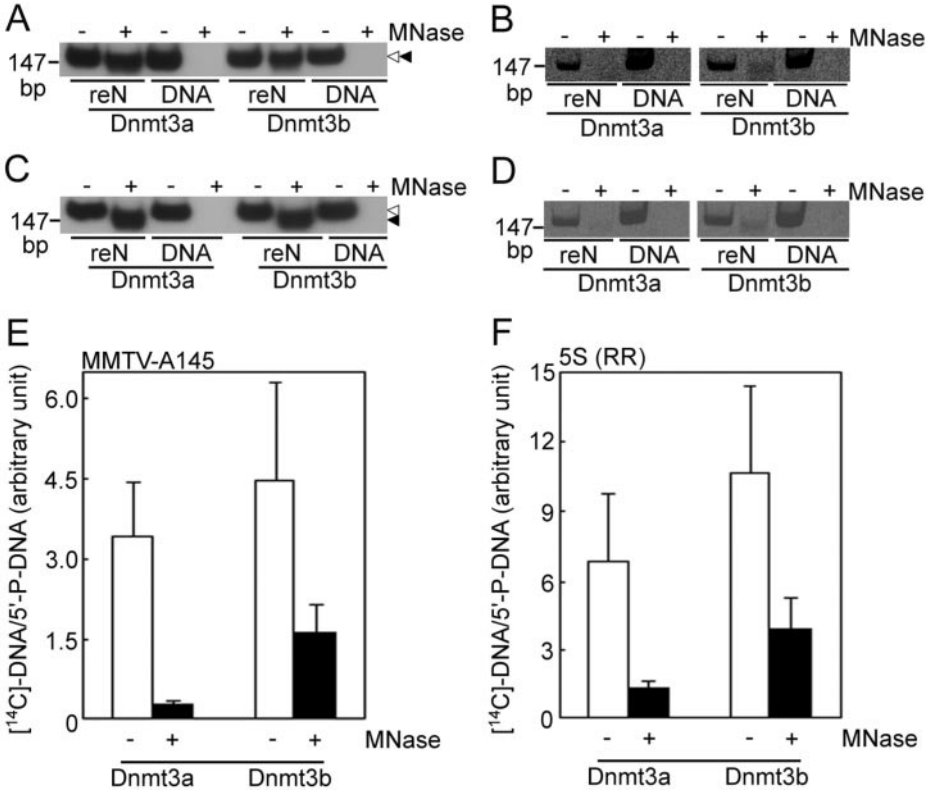

Fig. 5. **MNase digestion of nucleosomes methylated with Dnmt3a and Dnmt3b.** Naked DNA of, and nucleosomes reconstituted with MMTV-A145 (A and B) and 5S (RR) (C and D) were methylated as in Fig. 4 with Dnmt3a or Dnmt3b, and then digested with or without MNase. An aliquot of each sample before and after MNase digestion was phosphorylated with [γ-^{32}P]-ATP and T4 polynucleotide kinase, and then the recovery of the 5′-phosphorylated DNA after MNase digestion was determined (A and C). Remaining fractions were determined for the ^{14}C-labeled (methylated) DNA (B and D). The radio-labeled DNA methylated and phosphorylated samples were separately electrophoresed in 5% acrylamide gels and the radioactivity was determined with a BAS2000. The open and filled arrowheads in panels A and C indicate the DNA fragments before and after MNase digestion, respectively. The methylation level ([^{14}C]-DNA), normalized with respect to the 5′-phosphorylated-DNA (5′-P-DNA), before and remaining after the MNase digestion was determined in 6 independent experiments for MMTV-A145 (E) and 5S (RR) (F). 50–90% and 50–70% of the DNA was recovered after MNase digestion in each experiment for the nucleosomes reconstituted from MMTV-A145 and 5S (RR), respectively. White and black bars indicate the average specific activities in arbitrary units with standard deviations before and after the MNase digestion, respectively.

After DNA methylation of MMTV-A145, either naked or packaged into nucleosomes, the mixtures were treated with MNase. Under the conditions used, 50 to 90% of the DNA in nucleosomes was protected from the digestion in each treatment, while the naked DNA was completely digested (Fig. 5A). The radioactivity incorporated into the DNA in the nucleosome fraction methylated with Dnmt3a almost completely disappeared on MNase treatment (Fig. 5B). This indicates that the majority of methylated DNA was not nucleosomal but contaminating free DNA. On the other hand, a significant amount of the methylated DNA with Dnmt3b was protected (Fig. 5B). Similar to the case of MMTV-A145, 50–70% of the DNA in 5S (RR) nucleosomes was protected on MNase treatment, while the naked DNA was completely digested (Fig. 5C). Under the conditions used, again almost all the methylated DNA with Dnmt3a disappeared, while a significant amount of that with Dnmt3b remained at a position corresponding to around 146 bp, which is the size of the DNA wrapping histone octamer (Fig. 5D). The radioactivity of methylated DNA normalized with respect to that of the recovered 5′-phosphorylated DNA determined in 6 independent experiments, with three independently reconstituted nucleosome preparations for of MMTV-A145 and 5S (RR) and two different enzyme preparations of Dnmt3a and Dnmt3b, is summarized in panels E and F. The results clearly demonstrated that Dnmt3a mainly methylated not the nucleosome core region but the free DNA contaminating the nucleosomal fraction. On the other hand, Dnmt3b significantly methylated the nucleosome core region.

Both Dnmt3a and Dnmt3b Can Bind to Nucleosomes—The high and low DNA methylation activity of Dnmt3a towards nucleosomes with and without a linker DNA, respectively, and the significant methylation activity of Dnmt3b towards the nucleosome core region might depend on their affinity for the substrate nucleosomes. To investigate this possibility, we next examined the binding of Dnmt3a and Dnmt3b to nucleosomes with and without a linker DNA by means of a gel-shift assay. As a result of the assay (Fig. 6A), after the incubation of nucleosomes with an about 3- to 5-fold molar excess of Dnmt3a in the presence of AdoMet, all the nucleosomes reconstituted with MMTV-A145, MMTV-A242, 5S (RR), and 5S (RD) were completely shifted. Dnmt3a bound equally to the nucleosomes regardless of the presence or absence of linker DNA under the assay conditions used. On the other hand, not all the nucleosomes seemed to be occupied by Dnmt3b, especially those without a linker region, such as MMTV-A145 and 5S (RR) ones (Fig. 6B). These results suggest that the high DNA methylation activity of Dnmt3a towards the nucleosomes with linker DNA and the significant DNA methylation activity of Dnmt3b toward the nucleosome core region may not correlate with their affinity or binding capacity to the nucleosomes, at least as determined by gel-shift assay. The result suggests that the binding mode of the enzymes is important for their DNA methylation activity.

Dnmt3a and Dnmt3b Methylated the DNA Packaged into Nucleosomes without Changing the Constituents of the Nucleosomes—Dnmt3b significantly methylated the DNA within the nucleosome core region, although the activity was low. This may indicate that Dnmt3b can directly methylate the CpGs in the region enclosing the histone octamer. Since no free DNA was detected even after incubation of nucleosomes in the presence of AdoMet (see Fig. 6), and the methylated DNA was protected from MNase digestion (Fig. 5), nucleosomes were not likely to be destroyed by Dnmt3b during the DNA methylation.

To further examine whether Dnmt3b destroyed the nucleosome structure while methylating the nucleosome core region, the compositions of histones in nucleosomes before and after the DNA methylation reaction were determined. For this, nucleosomes reconstituted with 5S (RD) and ones bound to Dnmt3a or Dnmt3b were isolated by agarose gel electrophoresis after DNA methylation at 37°C or on ice for 1 h. Under the conditions used, almost

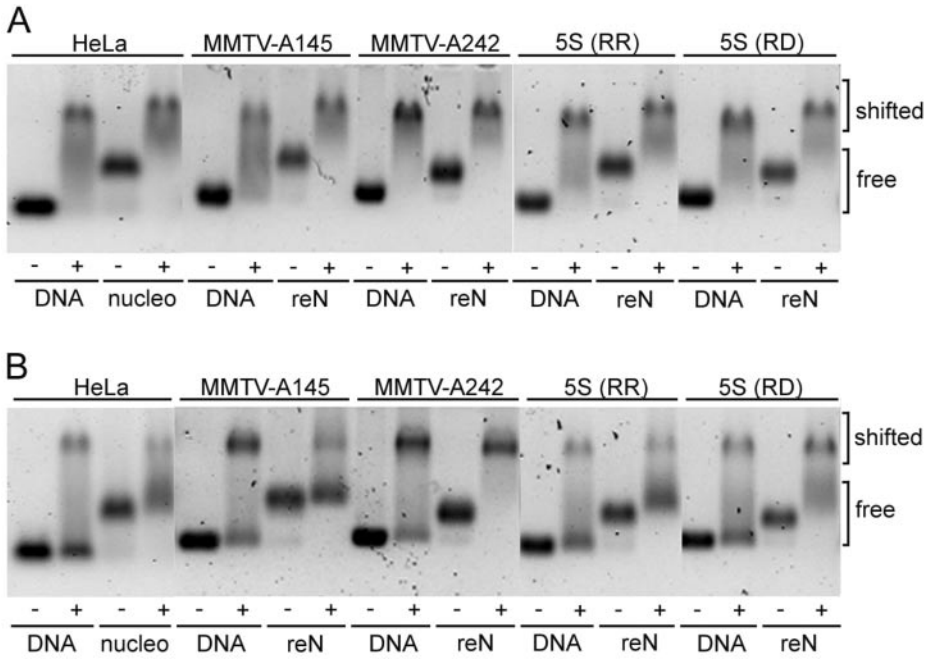

Fig. 6. **Binding of Dnmt3a and Dnmt3b to naked DNA and nucleosomes.** The nucleosomes prepared from HeLa cells (nucleo), reconstituted nucleosomes (reN), and naked DNAs (DNA) used in this study were incubated with or without Dnmt3a (A) or Dnmt3b (B) under identical buffer conditions for determination of the DNA methylation activity, and then electrophoresed in 0.7% agarose gels. DNA bands were visualized with SYBR Green I.

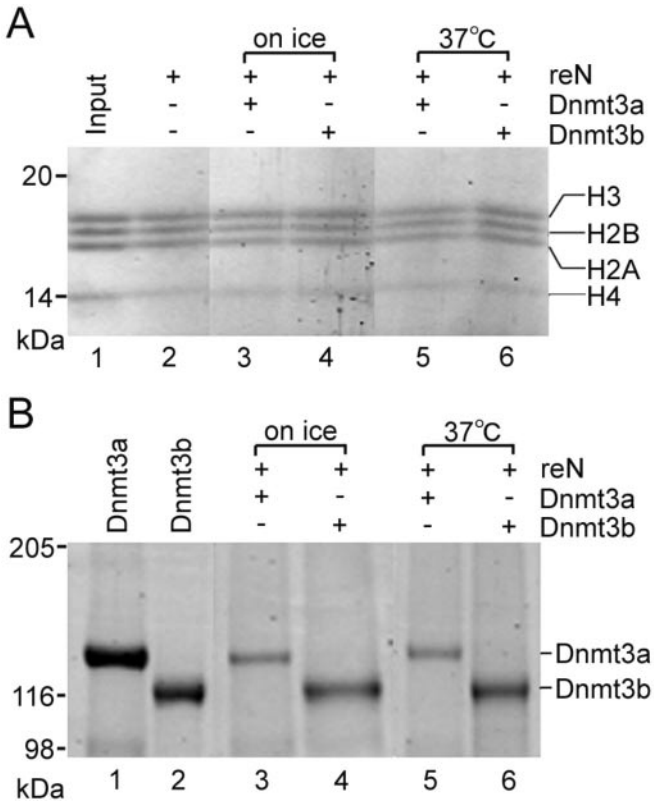

Fig. 7. **Dnmt3a and Dnmt3b did not change the histone composition of nucleosomes.** Nucleosomes reconstituted from 5S (RD) were incubated with Dnmt3a or Dnmt3b in the presence of AdoMet on ice or at 37°C for 1 h, and then electrophoresed in an agarose gel to separate free nucleosomes and those associated with Dnmt3a or Dnmt3b, and then the protein fractions were TCA-precipitated. The precipitated histones (A), and Dnmt3a and Dnmt3b (B) were analyzed in 18% and 7.5% SDS–polyacrylamide gels, respectively. An equivalent amount of extracted protein was loaded on each lane except for the "input," Dnmt3a, and Dnmt3b lanes, *i.e.*, a half the equivalent of the other lanes for "input" (A, lane 1), 0.25 pmol Dnmt3a (B, lane 1), and 0.25 pmol Dnmt3b (B, lane 2), being loaded, respectively. Lane 2 in panel A contains nucleosomal histones without incubation extracted from the agarose gel.

all nucleosomes were bound to Dnmt3a or Dnmt3b, and no DNA methylation activity was detected after 1 h of incubation on ice (data not shown). The compositions and ratios of histones H2A, H2B, H3, and H4 in the nucleosomes bound with or without Dnmt3a or Dnmt3b were determined (Fig. 7A). Even after the DNA methylation, the compositions and ratios of the histones in the nucleosomes were identical to those in the input nucleosomes. The recovery of total histones and the ratios of individual histones in nucleosomes incubated with or without Dnmt3a or Dnmt3b were densitometrically quantitated and are summarized in Table 1. Similar amounts of total histones and stoichiometric amounts of individual histones compared to those incubated on ice and without incubation were recovered in the nucleosomes after DNA methylation at 37°C. Under the conditions used, amounts of Dnmt3a and Dnmt3b corresponding to those of histones were recovered in the nucleosomal fractions (Fig. 7B and Table 1).

It has been reported that RNA polymerase II, nucleosome assembly factors, and chromatin remodeling factors destabilize nucleosomes, and thereby facilitate the loss of histones H2A and H2B (*53–55*). In the present study, neither Dnmt3a nor Dnmt3b affected the recovery or composition of the histones in nucleosomes. Thus, it can be concluded that Dnmt3b can directly methylate the CpGs within the nucleosome core region without destroying its structure.

DISCUSSION

Distinct Methylation of the Nucleosome Core Region by Dnmt3a and Dnmt3b—In the present study, we compared the activities of two *de novo* DNA methyltransferases, Dnmt3a and Dnmt3b, and found that Dnmt3a could scarcely methylate nucleosomes prepared from HeLa cell nuclei, but Dnmt3b significantly could. Utilizing reconstituted nucleosomes with unmodified histones and DNA of known sequence, we concluded that this was due to the poor and significant methylation activity of Dnmt3a and Dnmt3b, respectively, towards the DNA in the nucleosome core region. Furthermore, in comparison with Dnmt3b, Dnmt3a preferentially methylated naked DNA and the naked part of nucleosomal DNA. Dnmt3a and Dnmt3b

Table 1. **Recovery of histones, Dnmt3a and Dnmt3b in the nucleosomal fraction after the DNA methylation reaction.** Nucleosomes were incubated with Dnmt3a and Dnmt3b, and then analyzed by SDS polyacrylamide gel electrophoresis as in Fig. 7. Histones, Dnmt3a and Dnmt3b were stained with SYPRO Orange, and the stained bands were densitometrically quantitated.

Recovery	reN (or Dnmt3) input	reN[a] without incubation[b]	reN + Dnmt3a		reN + Dnmt3b	
			on ice	37°C	on ice	37°C
total histones	100	71 ± 9 (6)	58 ± 15 (3)	52 ± 14 (3)	51 ± 8 (3)	53 ± 21 (3)
ratios of histones[c]						
H2A	100	90 ± 14 (6)	91 ± 9 (3)	86 ± 15 (3)	86 ± 4 (3)	99 ± 5 (3)
H2B	100	81 ± 10 (6)	75 ± 4 (3)	72 ± 16 (3)	80 ± 2 (3)	80 ±12 (3)
H3	100	100	100	100	100	100
H4	100	97 ± 25 (6)	98 ± 13 (3)	84 ± 12 (3)	103 ± 20 (3)	96 ± 21 (3)
Dnmt3a or Dnmt3b[d]	100	—	63 (2)	55 (2)	63 (2)	58 (2)

The values indicated are averages (±SD). The numbers in parentheses indicate the numbers of independent experiments performed to calculate the percentages. [a]reN, reconstituted nucleosomes. [b]Nucleosomes without incubation were electrophoresed in an agarose gel, extracted, and then analyzed for recovery and composition. [c]The staining of each histone was calibrated with respect to that of the histone octamer in input. Densitometrically determined histones in each extract were normalized with respect to histone H3. [d]Dnmt3a and Dnmt3b were calibrated with known amounts of co-migrating Dnmt3a and Dnmt3b, respectively.

are known to interact with many protein components to modify chromatin *in vivo*, including histone deacetylase, histone methyltransferase, and HP1, which specifically binds to K9-methylated histone H3 (*32–37*). It is likely that these modifiers of core histones may differently affect the DNA methylation activity of Dnmt3a and Dnmt3b; nevertheless, the distinct methylation activity of Dnmt3a and Dnmt3b towards the linker and nucleosome core regions discovered in the present study may contribute to their methylation of distinct genomic regions *in vivo*.

In Vivo DNA Methylation by Dnmt3a Is Expected to Be Dependent on the Creation of a Naked Region in Genomic DNA—Although at a low level, Dnmt3a is expressed ubiquitously in most tissues and cell types (*16*, *56*). Therefore, Dnmt3a should be under stringent regulation. To prevent aberrant DNA methylation, the fact that Dnmt3a inefficiently methylates the nucleosome core region is quite important. In the present study, we also demonstrated that Dnmt3a is highly active towards the naked DNA region of nucleosomes. Under physiological conditions, however, such naked DNA regions seldom exist. Linker DNA is occupied by histone H1 and/or other DNA binding proteins, and thus Dnmt3a may not be able to methylate DNA freely. It has been reported that mutation of the genes encoding chromatin remodeling factors, such as *ddm1* in Arabidopsis, *ATRX* in man, and *Lsh* in mouse, induces hypomethylation in certain genomic regions (*27–31*). These SWI/SNF type chromatin remodeling factors are known to produce a short piece of naked DNA, and may provide Dnmt3a with sites to be methylated.

Similar to our results, it has been reported that the DNA methylation activity of Dnmt3a is severely inhibited when DNA is packaged into nucleosomes (*40*). On the contrary, Gowher et al. recently reported that Dnmt3a efficiently methylates nucleosomes with no linker DNA (*38*). In their study, almost identical DNA, which is two base pairs longer than MMTV-A145, was used for the reconstitution of nucleosomes, and, thus, the reason for the efficient methylation activity of Dnmt3a towards the nucleosomes is unknown. One possible explanation may be as follows. Gowher et al. used reconstituted nucleosomes contaminated by 20% of free DNA, and determined the methyl group incorporated into nucleosomes by separating them from free DNA by agarose gel electrophoresis after methylation. If the separation of nucleosomes from free DNA was insufficient, the free DNA that was preferentially methylated by Dnmt3a would have been present as a contaminant. In our assay system, we determined the DNA methylation of the nucleosome core region using nucleosomes separated from most of the free DNA by glycerol gradient centrifugation, and in some experiments a trace amount of free DNA remaining in the nucleosomal fraction was removed by MNase digestion.

Nucleosomes Are Not a Good Substrate Even for Dnmt3b—Even though Dnmt3b was able to methylate the DNA within the nucleosome core region, the activity was extremely low compared to that towards naked DNA (see Figs. 2D and 3D). Unlike *de novo* DNA methyltransferases, Dnmt1, which is a maintenance-type DNA methyltransferase and preferentially methylates hemimethylated DNA, can efficiently methylate the CpG in the nucleosome core region with some sequence specificity (*39*). As M.SssI, a bacterial DNA methyltransferase, methylates nucleosomes without a linker DNA quite efficiently (see Figs. 2 and 3), the nucleosome structure seems not to be an obstacle for M.SssI to methylate DNA. The inhibitory effect of nucleosome formation on the DNA methylation activity of Dnmt3a and Dnmt3b may be due to steric hindrance of access to CpG and/or direct interaction of the enzymes with histones (*57*). It may be important that nucleosomes are not good substrates for *de novo* DNA methyltransferases compared to naked DNA under physiological conditions so as to avoid aberrant DNA methylation.

In Vivo DNA Methylation by Dnmt3b Is Regulated through Stage- and Cell-Specific Timing of Expression—Dnmt3b could methylate CpGs even when they were within the nucleosome core region. This property of Dnmt3b is dangerous for cells. The expression of Dnmt3b, however, is restricted as to cell type and developmental stage (*16*). So far, Dnmt3b has been reported to be highly expressed only in embryonic stem cells and early stages of development around E4.5–7.5, at which stage global DNA methylation occurs (*16*, *58*). The expression levels of Dnmt3b are one order of magnitude higher than that of ubiquitously expressed Dnmt3a (*16*, *58*). This stage-specific high expression of Dnmt3b may contribute to the global methylation of the genome through direct methylation of the nucleosome core region. It has also been reported that some cancer cells express Dnmt3b (*59*). It may be important to strictly restrict the expression of Dnmt3b to certain cell types and developmental stages so as to prevent aberrant methylation through its *de novo* methylation activity.

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