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Distinct Enzymatic Properties of Recombinant Mouse DNA Methyltransferases Dnmt3a and Dnmt3b

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Recombinant mouse Dnmt3a and Dnmt3b were expressed in sf9 cells and purified to near homogeneity. The purified Dnmt3a and Dnmt3b gave specific activities of 1.8 ± 0.3 and 1.3 ± 0.1 mol/h/mol enzyme towards poly(dGdC)-poly(dGdC), respectively, which were the highest among those reported. Dnmt3a or Dnmt3b showed similar K_m values towards poly(dIdC)-poly(dIdC) and poly(dGdC)-poly(dGdC). The K_m values for *S*-adenosyl-L-methionine were not affected by the methyl-group acceptors, poly(dI-dC)-poly(dIdC) and poly(dG-dC)-poly(dGdC). The results indicate that the enzymes are *de novo*–type DNA methyltransferases. Dnmt3a and Dnmt3b activities were inhibited by Mn^{2+} and Ni^{2+} and showed broad pH optima around neutral pH. Both enzymes were susceptible to sodium ions, which inhibited their activity at around physiological ionic strength. However, Dnmt3a was fully active at physiological potassium concentration, but Dnmt3b was not. Using designed oligonucleotides for the analysis of cytosine methylation, we demonstrated that, in addition to CpG, Dnmt3a methylated CpA but not CpT and CpC, and that Dnmt3b methylated CpA and CpT but scarcely CpC. The relative activity of Dnmt3b towards nonCpG sequences was higher than that of Dnmt3a. These differences in enzymatic properties of Dnmt3a and Dnmt3b may contribute to the distinct functions of these enzymes *in vivo*.

Key words: DNA methyltranasferase, Dnmt3a, Dnmt3b, expression and purification.

Abbreviations: AdoMet, *S*-adenosyl-L-methionine; CBB, Coomassie Brilliant Blue R-250; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate.

In vertebrates, the 5th positions of cytosine residues in CpG sequences in genomic DNA are often methylated (*1*). Dynamic regulation of DNA methylation is known to contribute to physiological or pathological phenomena such as gene expression (*2–4*), genomic imprinting (*5*), X chromosome inactivation (*6*), and carcinogenesis (*7*). 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 activity creates gene-specific methylation patterns at the implantation stage of embryogenesis (*8*), and maintenance-type activity 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 at an early stage of embryogenesis (*9*, *10*) and have been shown to possess *de novo*-type DNA methylation activity *in vitro* (*11–14*). When either *Dnmt3a* or *Dnmt3b* is targeted, the residual *Dnmt3b* or *Dnmt3a* gene, respectively, partly compensates for the lack of the other gene (*10*), suggesting that Dnmt3a and Dnmt3b possess similar functional specificities. On the other hand, targeting of *Dnmt3b* gives a severer phenotype than that of *Dnmt3a*, and a mutation in *DNMT3b*, a human homologue of *Dnmt3b*, leads to hypomethylation of the satellite 2 and 3 regions of specific chromosomes, which is the cause of ICF (immunodeficiency, centromeric region instability, and facial anomalies) syndrome (*10*, *15–17*). These reports suggest that Dnmt3a and Dnmt3b possess distinct functions, and this may be explained at least partly by the difference in the timing of cell-type specific expression of Dnmt3a and Dnmt3b proteins during an early stage of the development. Dnmt3b is specifically expressed in totipotent embryonic cells, such as inner cell mass, epiblast and embryonic ectoderm cells, while Dnmt3a is significantly and ubiquitously expressed after E10.5 (*18*). However, stage- and cell-type-specific expression of the two enzymes may not fully explain the phenomena. For example, it is not known why Dnmt3b but not Dnmt3a is responsible for the methylation of the pericentromeric satellite repeats of specific chromosomes (*17*). It is thus important to compare the difference of *in vitro* enzymatic properties of Dnmt3a and Dnmt3b to understand the molecular mechanisms underlying their distinct functions *in vivo*. It has been reported that Dnmt3a and Dnmt3b possess similar kinetic parameters towards two substrates, DNA and *S*-adenosyl-L-methionine (AdoMet) (*12*). Both Dnmt3a and Dnmt3b have the ability to methylate nonCpG sequences (*11*, *12*). In addition to CpG, Dnmt3a favors CpA, while Dnmt3b favors CpA and CpT (*12*). However, partially purified enzymes with large GST proteins at their amino-terminus were used for these determinations. Recently, it was reported that the truncated catalytic domain of Dnmt3a methylates DNA in a distributive manner, which means at-random recognition of the methylation site, while that of Dnmt3b does so in

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processive manner, that is, Dnmt3b favors methylation near the methylated site (*19*).

To improve the quality of the results obtained with partially purified enzymes by Aoki *et al.* (*12*), and to look for differences between Dnmt3a and Dnmt3b in enzymatic properties *in vitro*, we prepared highly purified recombinant mouse Dnmt3a and Dnmt3b without GST protein utilizing a baculovirus system. Using the purified proteins, we confirmed that Dnmt3a and Dnmt3b exhibit similar *de novo* methylation activity with similar kinetic parameters to those reported previously (*12*), except for the specific activities and *Km* values for DNA. Removal of the GST protein improved the *Km* values for DNA substrate. Both Dnmt3a and Dnmt3b were inhibited by 120 mM NaCl. But, interestingly, Dnmt3a was fully active at KCl concentrations under 120 mM. It was also confirmed that, in addition to CpG, Dnmt3a methylated CpA but not CpT and CpC, and Dnmt3b methylated both CpA and CpT but scarcely CpC.

MATERIALS AND METHODS

Construction of Recombinant Baculoviruses—Recombinant His-tagged Dnmt3a and Dnmt3b were produced in sf9 cells using the Bac-to-Bac baculovirus expression system (Invitrogen, Tokyo). The ATG coding initiation methionines of mouse Dnmt3a and Dnmt3b (*9*) were directly ligated the *Bam*H1 linker without any spacer sequence and then subcloned into the *Bam*H1 site of the multi-cloning site of the $pF_{AST}B_{AC}HTb$ vector (Invitrogen, Tokyo). Recombinant baculoviruses were constructed according to the manufacturer's instructions, and the viruses were amplified by three rounds of infection to obtain high titer baculovirus stock. Sf9 cells were maintained in Grace's medium containing 10% (v/v) fetal bovine serum at 27°C.

Purification of His-Dnmt3—The recombinant baculovirus harboring Dnmt3a or Dnmt3b was infected into 5×10^8 sf9 cells at M.O.I. (multiplicity of infection) = 2. After the infection, the cells were incubated for 60 and 72 h for Dnmt3a and Dnmt3b, respectively, then harvested. All the procedures for the purification were performed at 4°C or on ice. The cells were washed twice each with Dulbecco's phosphate buffered saline (PBS) and buffer A [1 M sucrose, 3 mM $MgCl_2$, 0.3% (w/v) Triton X-100, 15 mM 2-mercaptoethanol, 0.1% (v/v) protease inhibitor cocktail (Nakalai Tesque, Kyoto), 20 mM Tris-HCl, pH 7.4]. The nuclear fraction was recovered by centrifugation at 2,400 $\times g$ for 10 min at 4°C. The washed nuclei were suspended in buffer A containing 1 M NaCl and incubated on ice for 20 min. The mixture was centrifuged at 400,000 $\times g$ for 10 min at 4°C, and the supernatant fraction was recovered. The extract was applied onto a 5-ml DEAE Sepharose CL-6B (Amersham Biosciences, Tokyo) column, and the unbound fraction was recovered. The fraction was filtered through a 0.45-μm MILEX-HA filter (Millipore, Tokyo), then loaded onto a 5-ml HiTrap Chelating Sepharose HP column (Amersham Biosciences, Tokyo), which was preloaded with Ni^{2+} and equilibrated with buffer B [1 M NaCl, 0.29 M sucrose, 3 mM $MgCl_2$, 0.1% (v/v) Nikkol (Nikkoh Chemicals, Tokyo), 15 mM 2-mercaptoethanol, 70 mM Tris-HCl, pH 7.6]. The column was washed with 20 column volumes of buffer B at a constant flow rate of 0.5 ml per min, and proteins were eluted with 50 ml of a linear gradient from 0 mM to 125 mM histidine in buffer B. The main fractions were pooled, the protease inhibitor cocktail and dithiothreitol (DTT) were added to final concentrations of 0.1% (v/v) and 1 mM, respectively, and the mixtures were frozen in liquid nitrogen, then stored at –80°C until use. The protein concentrations of the enzymes were monitored by SDS-polyacrylamide gel electrophoresis (PAGE). The protein bands were stained with Coomassie Brilliant Blue R250 (CBB) and determined with an image analyzer (MCID; Imaging Research, Canada), using bovine serum albumin as a standard.

Table 1. **Oligonucleotides used for the substrates of DNA methylation activities shown in Fig. 7.**

28-0:	5′-TGTTGTGGGTCAGTGCCTGATACTGTAC-3′
28c-0:	5′-GTACAGTATCAGGCACTGACCCACAACA-3′
28-3:	5′-TGTTGCGGGTCAGCGCCTGATACGGTAC-3′
28c-3:	5′-GTACCGTATCAGGCGCTGACCCGCAACA-3′

"28-0" and "28c-0" are the complementary sequences of 28mer oligonucleotides for the substrate that does not contain CpG. "28-3" and "28c-3" are the complementary sequences of 28mer oligonucleotides for the substrate that contain 3 pairs of CpG. The CpG sites were underlined.

Methyl-Group Acceptors for Dnmt3a and Dnmt3b Activities—Poly(dIdC)-poly(dIdC) (dIdC) and poly(dGdC)-poly(dGdC) (dGdC) (Amersham Biosciences, Tokyo) were used as methyl-group acceptors to determine total and *de novo* methylation activities, respectively, as described elsewhere (*12*, *20*). In addition, the synthesized oligonucleotides listed in Table 1 were used. The complementary oligonucleotides were annealed as described (*21*) and used for determining the methylation activity.

To analyze the DNA sequence specificity of Dnmt3a and Dnmt3b by the bisulfite method, the 141-bp fragment having *Bam*HI site at one end and *Xho*I site at the other was designed and subcloned into the pBSII multicloning sites. The nucleotide sequence of the fragment was 5′-ggatccgagtatggagttgtggtgggctcgccaggcagccgctacggcacctgcagctccggcggctgccagaattcactgccgcagctcaccgagcaccggacgaagcttccactgctccgtttggaatgggatagtgtggaagctcgag-3′, which contains 10 each of the dinucleotide sequences, CpA, CpC, CpG, and CpT, in the 85 bp between the primer sequences used for the amplification of bisulfite-treated DNA (indicated with underlines).

DNA Methylation Activity—The DNA methylation activity was determined as described elsewhere (*12*). In brief, 25 μl of the reaction buffer (2.7 M glycerol, 5 mM EDTA, 0.2 mM DTT, 25 mM NaCl, 20 mM Tris-HCl, pH 7.4) contained 50 ng (about 0.5 pmol) of the purified enzyme, 0.1 μg of dIdC or dGdC, 133 pmol (2.0 μCi and 5.3 μM) of [^{3}H]-*S*-adenosyl-L-methionine (AdoMet) (15Ci/mmol; Amersham Biosciences, Tokyo) unless otherwise stated. The methylation rates were linear for the first 90 min of incubation at 37°C under the conditions. After 1 h of incubation, the reaction was terminated with 1.5 mM non-radioactive AdoMet. The mixtures were incubated with 0.1 μg of proteinase K (Nakalai Tesque, Kyoto) at 50°C for 20 min, then the radioactivities were determined as described previously (*20*).

To determine the sequence specificity of Dnmt3a and Dnmt3b, the methylation reactions were performed basically as described above, except for using 0.1 μg of artifi-

Table 2. **Purification of Dnmt3a.** Sf9 cells (1×10^6 cells/ml in 500 ml of culture medium) were infected with the recombinant baculovirus at M.O.I = 2 and harvested after 60 h of incubation. The recovery of Dnmt3a was determined as CBB-stained bands in the SDS-polyacrylamide gel in an image analyzer, using bovine serum albumin as standard.

Purification	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Dnmt3a (mg/ml)	Total Dnmt3a (mg)	Purity (%)	recovery (%)
Cell extract	15	4.8	72.5	0.55	8.25	11.4	100*
DEAE	15	4.7	70.8	0.54	8.00	11.3	97
Chelate	3	0.24	0.72	0.23	0.69	95.2	8.4

*The recovery of Dnmt3a was normalized to the amount in the cell extract.

Table 3. **Purification of Dnmt3b.** Sf9 cells (1×10^6 cells/ml in 500 ml of culture medium) were infected with the recombinant baculovirus at M.O.I = 2 and harvested after 72 h of incubation. The recovery of Dnmt3b was determined as CBB-stained bands in the SDS-polyacrylamide gel in an image analyzer, using bovine serum albumin as standard.

Purification	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Dnmt3b (mg/ml)	Total Dnmt3b (mg)	Purity (%)	Recovery (%)
Cell extract	15	10.6	154.2	1.14	17.1	11.1	100*
DEAE	15	10.3	150.5	1.11	16.7	11.0	97
Chelate	3	0.41	1.22	0.40	1.19	97.4	7.0

*The recovery of Dnmt3a was normalized to the amount in the cell extract.

cially designed *Bam*HI–*Xho*I fragment as the methyl-group acceptor, and 2.5 nmol (100 μM) non-radioactive AdoMet purified with Sep-Pack Plus C18 (Waters, Tokyo) and 0.8 μg (5 pmol), 1 μg (10 pmol) or 5 μg (50 pmol) of purified Dnmt1, Dnmt3a or Dnmt3b were added to 25 μl of the reaction mixture. Purified recombinant Dnmt1 was generously provided by Daichika Hayata of our laboratory. After 1 h of incubation at 37°C, the mixtures were used for bisulfite modification.

Bisulfite Treatment of DNA—Bisulfite reaction was performed basically as described by Clark *et al.* (*22*), with a slight modification. Briefly, after the methylation reaction, 1 μg of herring sperm DNA was added to the reaction mixture as a carrier. The mixture was treated with neutralized phenol/chloroform, and then the DNA was precipitated with ethanol. The recovered DNA was treated with 0.3 M NaOH at 37°C for 30 min, and then incubated in 5.65 M $Na_2S_2O_5$ and 106 mM hydroquinone, pH 5. The modification reaction comprised three cycles of incubation at 95°C for 2 min and at 55°C for 1 h. The modified DNA was recovered with Wizard DNA Clean-Up System (Promega, Tokyo), treated with 0.2 M NaOH at room temperature for 10 min, and ethanol-precipitated in the presence of 3.75 M ammonium acetate, pH 7.0. The modified DNA thus prepared was PCR-amplified with the primers BS-f, 5′-gagtatggagttgtggtggg-3′ and BS-r, 5′-cttccacactatcccattcc-3′. The amplification reaction comprised 35 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 2 min. The amplified fragment was subcloned into the *Sma*I site of pBSII, and the sequences were determined by dideoxy methods (*23*).

RESULTS AND DISCUSSION

Expression and Purification of Dnmt3a and Dnmt3b—We previously, reported the partial purification of recombinant Dnmt3a and Dnmt3b with GST proteins fused at their amino-termini that were expressed in *Escherchia coli*, and determination of the kinetic parameters (*12*). In these preparations, however, a large protein of GST was added to the amino-terminus and some impurity still existed. To confirm the properties of the enzymes previously reported, we constructed the recombinant Dnmt3a and Dnmt3b in baculovirus without GST protein, expressed them in sf9 insect cells, and improved the purity of the final preparations.

The recombinant baculoviruses encoding Dnmt3a and Dnmt3b were infected into sf9 cells at M.O.I. = 2. Dnmt3a and Dnmt3b showed the highest expression levels at around 60 and 72 h after the infection, respectively (data not shown). The harvested cells were extracted and purified by DEAE Sepharose and Chelating Sepharose chromatographies as described in Materials and Methods. Typical purification steps are summarized in Tables 2 and 3. The contents of expressed Dnmt3a and Dnmt3b

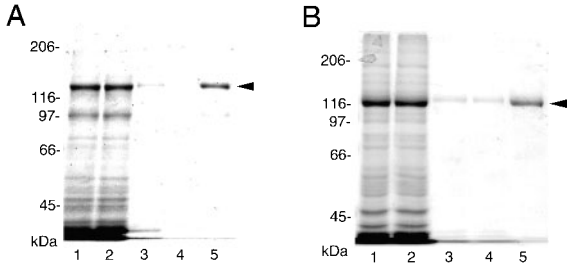

Fig. 1. **SDS-PAGE of Dnmt3a and Dnnmt3b.** A: An equivalent amount each of the fractions of solubilized sf9 cells expressing Dnmt3a (lane 1), flow-through from DEAE Sepharose CL6B (lane 2), flow-through from HiTrap Chelating Sepharose HP column (lane 3), and wash (lane 4), and twice the equivalent amount of the eluate (lane 5) were electrophoresed in SDS–7.5% acrylamide gel. The protein bands were stained with CBB. The purity of the purified Dnmt3a (lane 5) was more than 95% by densitometrical determination. B: Like that of Dnmt3a, the purification of Dnmt3b was monitored at each step by SDS-PAGE. Each lane corresponds to the same step as that in (A) with an equivalent amount each of the fractions for lanes 1–4, and twice the equivalent for lane 5. The arrowheads indicate the positions of Dnmt3a and Dnmt3b. The molecular weight markers are indicated at the left side of each gel.

Table 4. **Comparison of the specific activities of the purified Dnmt3a and Dnmt3b preparations.** The activities are shown in mol/h/mol enzyme.

Tag	Host	Dnmt3a	Dnmt3b	References
His	*E. coli*	0.07[1]	—	(*11*)
GST	*E. coli*	0.52 ± 0.09	0.43 ± 0.12	(*12*)
GST	mammalian cells	0.16[2]	—	(*13*)
His	baculovirus	0.026 ± 0.001[1]	—	(*14*)
His	baculovirus	1.8 ± 0.3	1.3 ± 0.1	present work

[1]Taken from the value of K_{cat} in the corresponding reference. [2]Calculated from the kinetic data shown in Fig. 5 of the reference.

were more than 10% of the total cell extracts, and the final yields of the purified proteins were about 7–9%. The monitoring of the samples at each step of the purification by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) is shown in Fig. 1. The purities of the final preparations of Dnmt3a and Dnmt3b were more than 95% as judged from the CBB staining. The specific activities of purified Dnmt3a and Dnmt3b, respectively, were 2.1 ± 0.1 and 1.5 ± 0.2 mol/h/mol enzyme towards dIdC, and 1.8 ± 0.3 and 1.3 ± 0.1 mol/h/mol enzyme towards dGdC. The activities indicate that both Dnmt3a and Dnmt3b are *de novo*-type DNA methyltransferases.

Several reports have appeared on the purification of recombinant Dnmt3a and/or Dnmt3b (*11–14*). The tags and expression systems used and their specific activities are summarized in Table 4. Although the conditions for the determination of activities differed, the Dnmt3a prepared in this work showed one of the highest specific activities. As for full-length Dnmt3b, only one example has hitherto been reported, which is by our group (Table 5) (*12*). Compared to that, the specific activities as well as the purities were improved in the present preparations.

Kinetic Parameters—We have determined the kinetic parameters K_m for DNA (dIdC and dGdC) and AdoMet, and the V_{max} values. The methyl-group acceptors dIdC and dGdC are usually utilized to determine total (maintenance plus *de novo*) and *de novo* methylation activities, respectively (*12*, *24*, *25*). Typical profiles of DNA methylation activities under different concentrations of dIdC, dGdC and AdoMet for Dnmt3a and Dnmt3b are shown in Figs. 2 and 3. K_m values for dIdC, dGdC and AdoMet determined from three independent experiments are summarized in Table 5 and 6. The K_m values of Dnmt3a towards dIdC and dGdC were similar, at 1.2 ± 0.1 and 1.6 ± 0.2 μM, respectively, as were those of Dnmt3b towards the same substrates, 0.4 ± 0.1 and 0.5 ± 0.1 μM, respectively (Table 5). The K_m values of each enzyme for AdoMet were also similar to each for both methyl-group acceptors (Table 6). The results thus indicate that Dnmt3a and Dnmt3b recognize dIdC and dGdC in a similar manner, and confirm our previous conclusion that both enzymes are "*de novo*–type" DNA methyltransferases (*12*).

The K_m^{DNA} values determined in the present study were two times lower than those determined for the

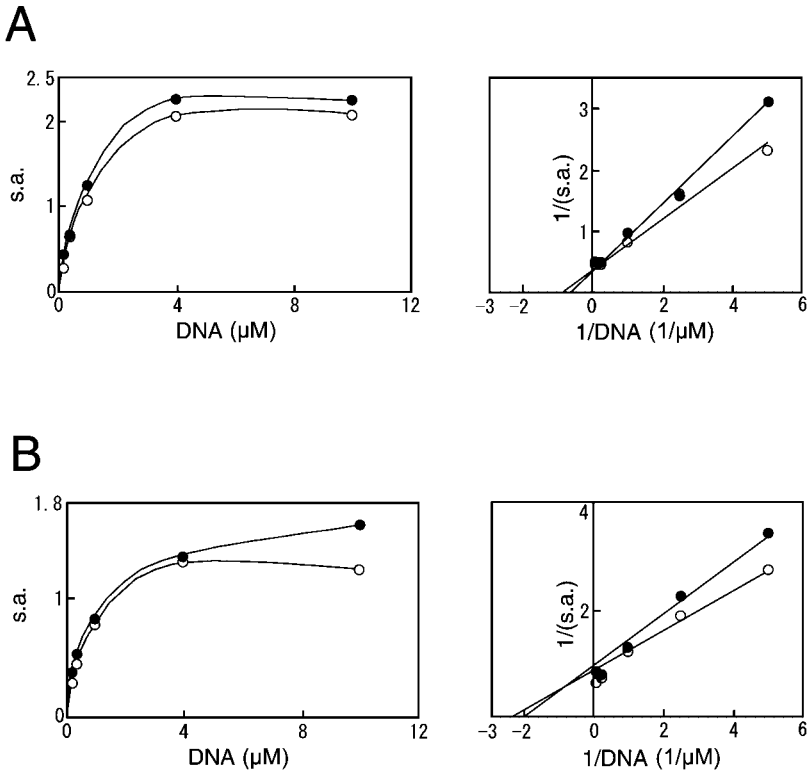

Fig. 2. **Effect of DNA concentration on the activity of Dnmt3a and Dnmt3b.** The DNA methylation activities [specific activity (s. a.) in mol/h/mol Dnmt3a or Dnmt3b] of Dnmt3a (A) and Dnmt3b (B) were titrated with dIdC (open circles) or dGdC (filled circles). One mole of double-stranded DNA with one CpI or one CpG site was calculated to be 2 mol. A typical example for each enzyme is demonstrated. Right panels show double reciprocal plots of representative titration curves.

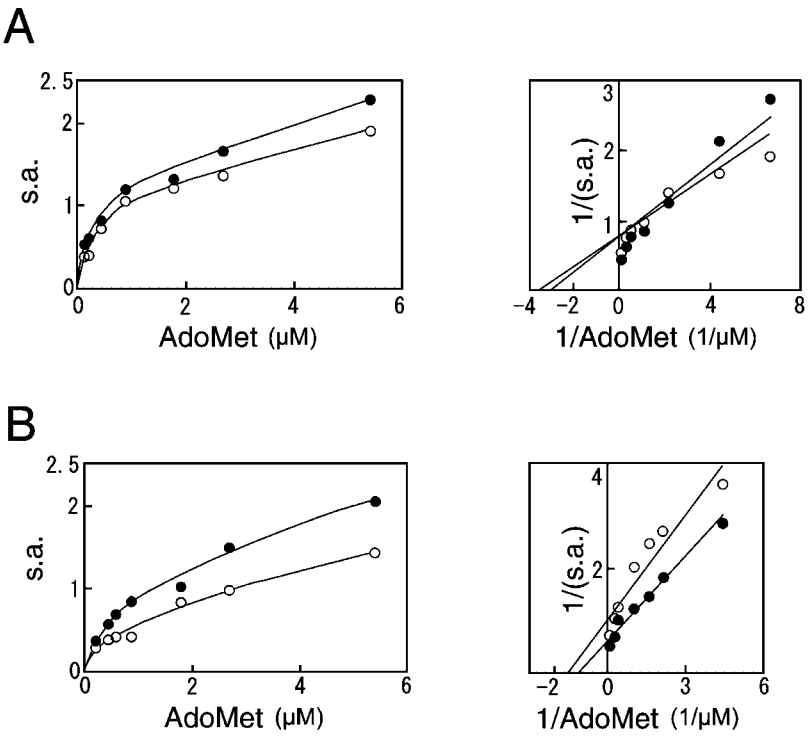

Fig. 3. **Effect of AdoMet concentration on the activity of Dnmt3a and Dnmt3b.** The DNA methylation activities [specific activity (s. a.) in mol/h/mol Dnmt3a or Dnmt3b] of Dnmt3a (A) and Dnmt3b (B) were titrated with AdoMet (left). As methyl acceptors, dIdC (open cicles) and dGdC (filled circles) were used. A typical example for each enzyme is demonstrated. Right panels show double reciprocal plots of the respective titration curves.

recombinants having a GST protein at the amino-terminus, namely, 2.7 ± 0.4 μM (towards dIdC) and 3.5 ± 1.2 μM (towards dGdC) for Dnmt3a, and 1.3 ± 0.4 μM (towards dIdC) and 1.0 ± 0.3 μM (towards dGdC) for Dnmt3b (*12*). The lower K_m^{DNA} values of both enzymes may reflect an increase in the binding affinity to the substrate DNA. On the other hand, the removal of GST protein did not significantly affect the K_m^{AdoMet} values of Dnmt3a and Dnmt3b. The binding motif of AdoMet is expected to be in the carboxyl-terminal half, which is the catalytic domain (*9*, *26*). As for the DNA binding, Dnmt3b and possibly Dnmt3a bind DNA via PWWP motif resides in their amino-terminal domain as well as the catalytic carboxyl-terminal domain (*27*). It is reasonable to estimate that the large GST protein added to the amino-terminus may negatively affect the DNA binding to the PWWP domain, which is close to the amino-terminus. If this is the case, it can be further estimated that the DNA binding of PWWP motif contributes positively to the kinetic parameters of the enzymes.

Effect of Salts, pH and Divalent Cations on the Activities—The activities of Dnmt3a and Dnmt3b were almost completely inhibited at NaCl concentrations above 120 mM (Fig. 4). On the other hand, interestingly, when KCl was chosen as an effector, the inhibition profiles of Dnmt3a and Dnmt3b differed. The activities of both enzymes were more resistant to KCl than NaCl salts, especially that of Dnmt3a. As the intracellular potassium ion concentration is high, it may be physiologically important that the enzymes are resistant to KCl concentration. At the physiological concentration of KCl, which is about 120 mM, Dnmt3a, but not Dnmt3b, was almost fully active. Contrary to the specific expression of Dnmt3b in pluripotent cells, Dnmt3a is ubiquitously expressed in somatic cells (18, unpublished observation), though in low level. Dnmt3a may play a role in changing the methylation status depending on various physiological stimuli, such as that for terminal differentiation. Since Dnmt3b is known to be responsible for the methylation of the pericentromeric satellite region (*10*, *16*, *17*), the enzyme should work *in vivo* too. To work as a *de novo* DNA methyltransferase under physiological salt compositions, some factors (mechanisms) may be present that support its activity *in vivo*.

Dnmt3a and Dnmt3b activity showed pH optimum of 7.0–7.8 and 6.5–7.8, respectively (Fig. 5). The pH dependencies of Dnmt3a and Dnmt3b were similar towards dIdC and dGdC. As for divalent cations, Ca^{2+} had no effect or rather an enhancing effect on the DNA methylation activity of both enzymes (Fig. 6). Mg^{2+} slightly inhibited the activities above 1 mM, and Mn^{2+} and Ni^{2+} strongly inhibited the activities. Mn^{2+} and Ni^{2+} may not

Table 5. **K_m values of Dnmt3a and Dnmt3b for DNA.**

	K_m^{DNA} (μM)		V_{max}^{DNA} (mol/h/mol Dnmt3)	
	dIdC	dGdC	dIdC	dGdC
Dnmt3a	1.2 ± 0.1[1]	1.6 ± 0.2	2.3 ± 0.1	2.0 ± 0.2
Dnmt3b	0.4 ± 0.1	0.5 ± 0.1	1.7 ± 0.0	1.4 ± 0.1

Three independent experiments similar to that shown in Fig. 2 were performed using different Dnmt3 preparations. [1]Values are mean ± standard deviation.

Table 6. **K_m values of Dnmt3a and Dnmt3b for AdoMet.**

	K_m^{AdoMet} (μM)		V_{max}^{AdoMet} (mol/h/mol Dnmt3)	
	dIdC	dGdC	dIdC	dGdC
Dnmt3a	0.3 ± 0.01[1]	0.3 ± 0.1	2.3 ± 0.0	2.0 ± 0.2
Dnmt3b	0.7 ± 0.2	0.7 ± 0.3	1.8 ± 0.3	1.3 ± 0.1

Three independent experiments similar to that shown in Fig. 3 were performed using different Dnmt3 preparations. [1]Values are mean ± standard deviation.

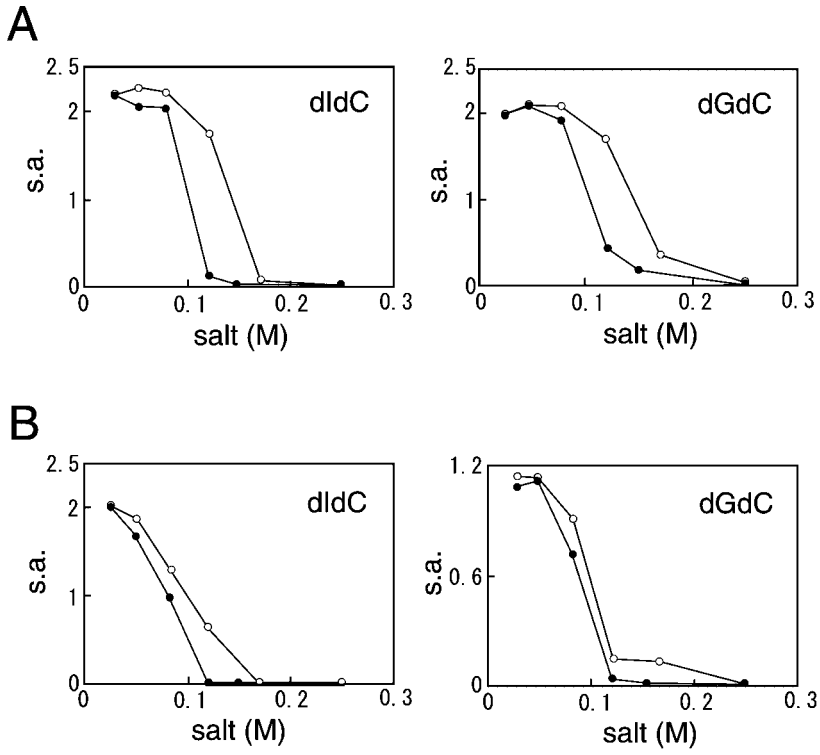

Fig. 4. **Effect of salt concentration on the activity of Dnmt3a and Dnmt3b.** The DNA methylation activities [specific activity (s. a.) in mol/h/mol Dnmt3a or Dnmt3b] of Dnmt3a (A) and Dnmt3b (B) were titrated with NaCl (filled circles) or KCl (open circles). As methyl acceptors, dIdC (left panels) and dGdC (right panels) were used.

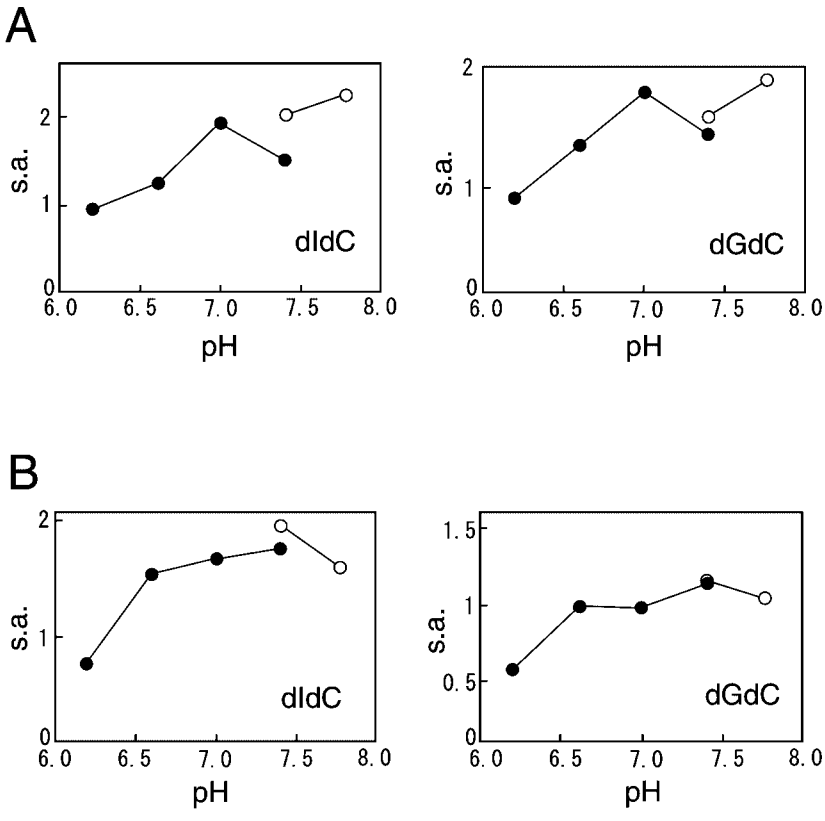

Fig. 5. **Effect of pH on the activity of Dnmt3a and Dnmt3b.** The DNA methylation activities [specific activity (s. a.) in mol/h/mol Dnmt3a or Dnmt3b] of Dnmt3a (A) and Dnmt3b (B) were determined at different pHs with 20 mM MOPS buffer (filled circles) or 20 mM Tris-HCl buffer (open circles) in the reaction mixture. As methyl acceptors, dIdC (left panels) and dGdC (right panels) were used.

exist in the order of mM in nuclei, and the effect of such the divalent cations is not important under physiological circumstances. However, it should be noted when determining the activities of His-tagged Dnmt3a and Dnmt3b, as the His-tagged enzymes are usually purified by use of a nickel-chelating matrix. The methylation activity of the enzymes should be determined in the presence of EDTA.

Sequence Specificity of DNA Methylation by Dnmt3a and Dnmt3b—We have reported that Dnmt3a and Dnmt3b methylates cytosine not only in the CpG

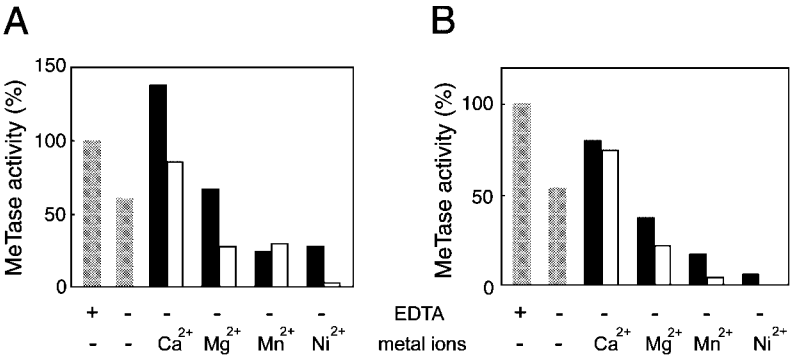

Fig. 6. **Effect of divalent cations on the activity of Dnmt3a and Dnmt3a.** The relative DNA methylation activities of Dnmt3b (A) and Dnmt3b (B) in the absence and presence of Ca^{2+}, Mg^{2+}, Mn^{2+}, or Ni^{2+} are normalized against that in the presence of 5 mM EDTA. Gray bars indicate the activities in the presence or absence of EDTA, and black and white bars indicate those activities in the presence of 1 or 5 mM cations, respectively. As methyl-group acceptor, dGdC was used.

sequence but also in the CpA and CpT sequences (*12*). Previously, we used the sequence of *myoD* gene as a methyl-group acceptor, and determined the methylation sites by the bisulfite method. The result, however, might be slightly biased, for the following reasons. Firstly, it was not possible to design primers at the pre-modified sequences that do not contain cytosine for the amplification of the modified sequences. The cytosine residues even in nonCpG sequences are in some cases methylated due to nonCpG methylating activities of the enzymes and might not be modified. Secondly, the *myoD* sequence contains unequal numbers of four dinucleotide sequences of CpN. For these, the determined sequence specificities of methylation acitivity of Dnmt3a and Dnmt3b might not be quantitative.

To improve these points, we designed a 141-bp DNA containing 10 each of CpA, CpC, CpG, and CpT in the 85-bp sequence between the PCR amplifying primer sequences, which omitted the cytosine residue in the upper strand. Using recombinant Dnmt3a or Dnmt3b and this designed DNA as substrate, we reexamined the sequence specificity of the activities of Dnmt3a and Dnmt3b. As shown in Table 7, it became clear that Dnmt3a and Dnmt3b preferentially recognize and methylate the CpG sequence. In addition, Dnmt3a methylated CpA but not CpT and CpC, while, Dnmt3b methylated CpT as well as CpA, and scarcely methylated CpC. As reported previously (*12*), Dnmt3b methylates nonCpG sequences more effectively than Dnmt3a.

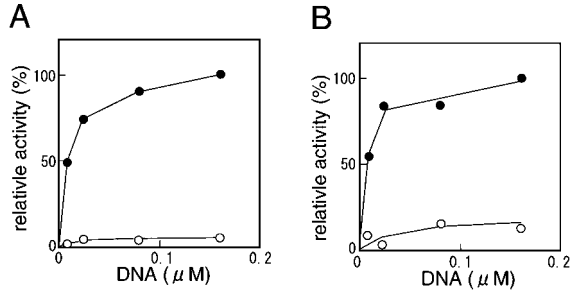

Fig. 7. **DNA methylation activities of Dnmt3a and Dnmt3b towards oligonucleotides containing or not containing the CpG sequence.** The relative DNA methylation activities, which were normalized against the activity determined at 0.16 μM oligonucleotide with three CpG, of Dnmt3a (A) and Dnmt3b (B) were titrated with oligonucleotide containing three CpG (filled circles) and no CpG (open circles). The concentration of each oligonucleotide is indicated not as CpG but as the whole molecule.

When the frequencies of nonCpG methylating activity of Dnmt3a and Dnmt3b were compared, the activity of Dnmt3b seemed to be higher than that of Dnmt3a (see Table 7). But this methylation was done with extremely high concentrations of the enzymes and might not occur under *in vitro* conditions where the rate of methylation activities is linear. To confirm whether the nonCpG methylation activity of Dnmt3b was higher than that of Dnmt3a, we next designed 28mer oligonucleotides containing or lacking CpG. One double-stranded oligonucleotide ("28-0") did not contain CpG but contained 7 CpA, 3 CpT and 3 CpC; and the other one ("28-3") contained 6 CpG, 4 CpA, 2 CpT and 4 CpC. As shown in Fig. 7, in the presence of excess amount of DNA, the sequence of which did not contain CpG and was different from that used for bisulfite analysis, both Dnmt3a and Dnmt3b showed significant activities. This result supports the conclusion that the nonCpG methylating activity detected by bisulfite analysis was not an artifact. Furthermore, Dnmt3b showed higher relative activity than Dnmt3a towards the oligonucleotides containing no CpG sequence. This also confirms that Dnmt3b possesses higher nonCpG methylating activity than Dnmt3a.

Lin *et al*. reported that when the –2 and +1 positions of CpG are pyrimidine (C or T), the cytocine in CpG is efficiently methylated (*13*). However, in the present study using the 141-bp DNA, we could not detect such a phenomenon. Recently, in primary effusion lymphoma (PEL) cells, nonCpG methylation on CC(A/T)GG has been reported, and this modification functions in repressing *B29* gene expression (*28*). The methylation sequence is at CA or CT, suggesting that Dnmt3b or/and Dnmt3a might be responsible for this type of unusual DNA methylation. Many tumor cells seem to express high levels of DNMT3B

Table 7. **Sequence specificity of the DNA methylation activities of recombinant Dnmt1, Dnmt3a and Dnmt3b.**

	CpG	CpA	CpT	CpC
Dnmt1	272 (100)[1]	0 (0)	7.8 (2.9)	0 (0)
Dnmt3a	263 (100)	10.5 (4.0)	0 (0)	0 (0)
Dnmt3b	195 (100)	13.9 (7.1)	26.8 (13.7)	6.0 (3.1)
No enzyme	0	3.0	0	0

The methylation of cytosine in the designed sequences of 85 bp was determined by the bisulfite method. Totals of 2496, 2457, 1716, and 1443 of CpN were analyzed after treatment with Dnmt1, Dnmt3a, Dnmt3b, and no enzyme, respectively, and the numbers of methylated dinucleotides were determined and normalized against 1,000 CpN. [1]The parentheses indicate the percentage of the methylation normalized against the methylation of CpG in each enzyme.

(*29*, *30*), a human homologue of mouse Dnmt3b, and suppression of DNMT3B in tumor cells induces apoptosis (*29*). NonCpG methylation activity of Dnmt3b may contribute to the oncogenesis.

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