J. Biochem. **140**, 553–559 (2006)
doi:10.1093/jb/mvj185

Stimulation Effect of Dnmt3L on the DNA Methylation Activity of Dnmt3a2

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Received July 11, 2006; accepted August 28, 2006

Quantification of DNA methyltransferases Dnmt3a and Dnmt3a2, and Dnmt3L in isolated male gonocytes in day 16.5 embryos confirmed that not Dnmt3a but Dnmt3a2 and Dnmt3L were the major Dnmt3s. The expression level of Dnmt3L constituted 5- to 10-fold molar excess compared to that of Dnmt3a2. The stimulation property of the DNA methylation activity of Dnmt3a2 with Dnmt3L towards substrate DNA in naked or nucleosomes was similar to that of Dnmt3a. However, the DNA methylation activity of not Dnmt3a but Dnmt3a2 was severely inhibited at the physiological salt concentration. Interestingly, the activity of Dnmt3a2 was significantly detected in the presence of Dnmt3L even at the physiological salt concentration. This indicates that Dnmt3a2 functions only in the presence of Dnmt3L in male gonocytes, and may explain why Dnmt3L is required specifically in mouse gonocytes for DNA methylation.

Key words: DNA methyltransferase, DNA methylation, Dnmt3a2, Dnmt3L, male germ cells.

Abbreviations: AdoMet, *S*-adenosyl-L-methionine; ES cells, embryonic stem cells; MNase, micrococcal endonuclease; PBS, Dulbecco's phosphate-buffered saline; PGC, primordial germ cells.

DNA methylation of the genome is essential for development (*1*) and plays crucial roles in a variety of biological processes, such as genomic imprinting (*2*) and carcinogenesis (*3*) *via* suppression of certain genes (*4*). In mammals, genome-wide DNA methylation occurs in implantation stage embryos and during gametogenesis (*5*). Two DNA methyltransferases, Dnmt3a and Dnmt3b, have been determined to create DNA methylation patterns (*1, 6*). It was shown that the *Dnmt3a* and *Dnmt3b* genes compensate for each other, and at the same time contribute to the methylation of distinct genomic regions on analyses of knockout mice and embryonic stem (ES) cells (*1, 7*). This partial compensation may well be explained by their stage-specific expression in mouse embryos, that is, Dnmt3b is highly expressed in totipotent embryonic cells, such as epiblast and embryonic ectoderm cells, in which genome-wide methylation occurs, and Dnmt3a is ubiquitously expressed in mesenchymal cells in embryos after day 10.5 (*1, 8*). However, the stage-specific expression of Dnmt3a and Dnmt3b may not fully explain their methylation of distinct genomic regions *in vivo*. When naked DNA is used as the substrate, Dnmt3a and Dnmt3b show similar sequence specificity and kinetic parameters for the DNA methylation activity *in vitro* (*9, 10*). However, when nucleosomes are used as the substrate, Dnmt3a preferentially methylates the naked part of nucleosomal DNA and Dnmt3b is able to methylate the nucleosome core region (*11*). The distinct DNA methylation activity of Dnmt3a and Dnmt3b towards nucleosomes as well as their distinct expression profiles during embryogenesis may contribute to their distinct DNA methylation properties *in vivo* (*11*).

In male germ cells in embryos on days 16–18, on which other global DNA methylation including imprinted genes occurs (*12–15*), Dnmt3a2, a product of the *Dnmt3a* gene, contributes to the DNA methylation (*16, 17*). Dnmt3a2 is an isoform lacking the amino-terminal 219 amino acid residues of Dnmt3a (*18*), and is reported to be highly expressed in ES cells (*19*) and male gonocytes in days 14–18 embryos (*17*). Interestingly, not Dnmt3a or Dnmt3b, but Dnmt3a2 and Dnmt3L are the major Dnmt3s in male gonocytes at these stages (*17*). Dnmt3L protein, which has no motifs responsible for the catalytic activity conserved by DNA methyltransferases, does not exhibit DNA methylation activity (*20–22*). Dnmt3L is expressed mainly in the chorion in days 7.5–8.5 embryos and in fetal male germ cells in days 14–18 embryos, and dramatically decreases after birth (*17, 21, 23–25*). Interestingly, Dnmt3L has been genetically proven to be a necessary factor for the DNA methylation during the differentiation of germ cells but not for that during embryogenesis (*21, 25, 26*), in spite of its expression during both stages. Homozygous *Dnmt3L* knockout male mice are sterile due to a defect in spermatogenesis accompanied by a loss of methylation in spermatogonial stem cells (*21, 25*), leading to the down regulation of gonad-specific genes (*27*). Dnmt3L directly interacts with Dnmt3a and Dnmt3b to enhance their DNA methylation activity *in vitro* (*22, 28*). Ectopically expressed Dnmt3L in culture cells efficiently enhances the DNA methylation activity of co-expressed Dnmt3a, Dnmt3a2, and Dnmt3b (*29*).

Since Dnmt3a2 is the major DNA methyltransferase in male germ cells at the stage of global DNA methylation, differences in the enzymatic properties and stimulatory

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effect of Dnmt3L between Dnmt3a2 and Dnmt3a or Dnmt3b should be considered to understand the absolute requirement of Dnmt3L for DNA methylation in germ cells. In the present study, we determined the expression levels of Dnmt3a2 and Dnmt3L in isolated gonocytes, and confirmed that Dnmt3a2 and Dnmt3L were highly expressed in the gonocytes, and the expression level of Dnmt3L was 5–10-fold that of Dnmt3a2. To discriminate the enzymatic properties of Dnmt3a2 and Dnmt3a or Dnmt3b, especially from the aspect of the effect of Dnmt3L, we have purified recombinant Dnmt3a2 and Dnmt3L. The profile of stimulation of DNA methylation activity with Dnmt3L and the activity towards reconstituted nucleosomes were almost identical to those in the case of Dnmt3a. However, the DNA methylation activity of not Dnmt3a but Dnmt3a2 was below the detection level at the physiological salt concentration. Intriguingly, the addition of Dnmt3L significantly enhanced the activity of Dnmt3a2 to a significant level. This strongly suggests that Dnmt3a2 can show DNA methylation activity only in the presence of Dnmt3L at the physiological salt concentration. Therefore, gonocytes, in which Dnmt3a2 is the major DNA methyltransferase, require Dnmt3L as a cofactor for *de novo* DNA methylation.

MATERIALS AND METHODS

Isolation of Primordial Germ Cells (PGC) and Western Blotting—PGC were isolated from transgenic mice carrying EGFP cDNA under the control of the Oct4 18-kb 5′ upstream genomic fragment as described (*30*). Briefly, encapsulated testes were dissected and incubated in Dulbecco's phosphate-buffered saline (PBS) containing 1 mM EDTA for 15 min at room temperature, added with PBS supplemented with collagenase (Sigma, Japan) and DNaseI (Invitrogen, Japan), and then incubated for 20 min at 37°C. Cell sorting was performed with a FACS Vantage flow cytometer equipped with CELL Quest software (Becton Dickinson, NJ). The total cell numbers were determined, and Western blotting was performed using specific antibodies recognizing Dnmt3a, Dnmt3a2 and Dnmt3L (*17*). The Dnmt3a, Dnmt3a2, and Dnmt3L bands were detected by means of alkaline phosphatase–coupled second antibodies, and quantified with an image analyzer, MCID (Imaging Research, Canada) using the recombinant Dnmt3a, Dnmt3a2, and Dnmt3L as standard as described elsewhere (*8*).

Expression and Purification of Recombinant Dnmt3a2 and Dnmt3L—The coding sequences of mouse Dnmt3a2 and Dnmt3L cDNAs were subcloned into the pF_{AST}-B_{AC}HTb vector (Invitrogen). The cDNAs of Dnmt3L and Dnmt3a2 were kindly provided by Drs. Kenichiro Hata (National Institute of Genetics, Japan) and En Li (Novartis, MA). The recombinant full-length Dnmt3a2 and Dnmt3L with a His-tag at the amino-terminus were purified basically as described (*10*). In brief, Sf9 cells were infected with the recombinant baculoviruses harboring Dnmt3a2 and Dnmt3L cDNAs, and then cultured for 72 and 48 h, respectively. All the purification steps were performed at 4°C or on ice. The harvested cells were washed twice with PBS, and then lysed in buffer A [1 M sucrose, 3 mM $MgCl_2$, 0.3% (w/v) Nikkol (Nikkoh Chemicals, Japan), 15 mM 2-mercaptoethanol, 0.1% (v/v) protease inhibitor cocktail (Nacali Tesque, Japan), and 20 mM Tris-HCl, pH 7.4]. The cell suspension was centrifuged at 2,400 × *g* for 20 min. The supernatant fraction was collected and subjected to purification. Dnmt3a2 was recovered on 5–25% saturation with ammonium sulfate, and the precipitate was dissolved in buffer A containing 1 M NaCl, and then purified on HiTrap Chelating Sepharose HP (GE Healthcare, Japan). As for Dnmt3L, the supernatant fraction was adjusted to a final concentration of 0.5 M NaCl with 5 M NaCl, and then the mixture was centrifuged at 100,000 × *g* for 10 min. The supernatant fraction was loaded onto HiTrap Chelating Sepharose HP. The column was washed with 10 bed volumes of buffer A containing 0.5 M NaCl and 20 mM imidazole, and then Dnmt3L was eluted with buffer A containing 0.5 M NaCl and 0.25 M imidazole. Dnmt3L thus eluted was dialyzed against 0.5 M NaCl, 15 mM 2-mercaptoethanol, 0.1% (v/v) protease inhibitor cocktail, and 20 mM Tris-HCl, pH 7.4. The protein concentration was determined with a BCA kit (Pierce Biotechnology, Il). The purified proteins were electrophoresed in a 12% polyacrylamide gel and then stained with Coomassie Brilliant Blue R-250.

Preparation of DNA Fragments and Reconstitution of Nucleosomes—DNA fragments of 5S ribosomal DNA, 5S (RR) (155 bp), 5S (RD) (220 bp), and MMTV-A145 (146 bp) were prepared as described (*11*). Briefly, DNA fragments were amplified by PCR using KOD polymerase (Toyobo, Japan) with the p5S (RD) plasmid harboring the *Xenopus borealis* 5S rRNA gene and the plasmid containing mouse mammary tumor virus LTR as templates.

Nucleosomes were reconstituted from the histone octamer and 5S (RR) DNA fragment (*11*). In brief, nucleosomes were reconstituted by the salt dialysis method, and the reconstituted product was purified by ultracentrifugation in a 5–20% (v/v) glycerol gradient. The nucleosomal DNA concentration was spectrophotometrically determined (*31*).

DNA Methylation Activity—DNA methylation activity was determined as described elsewhere (*10*). The reaction mixture (25 μl) comprising 40 nM Dnmt3a2 or Dnmt3a, 4 μg/ml of naked DNA or nucleosomes, 5.3 μM [^{3}H]-*S*-adenosyl-L-methionine (AdoMet) (15 Ci/mmol; GE Healthcare), and the indicated amount of Dnmt3L was incubated at 37°C for 1 h in the R buffer comprising 2.7 M glycerol, 5 mM EDTA, 0.2 mM DTT, 30 mM NaCl, and 20 mM Tris-HCl, pH 7.4. Indicated concentrations of KCl and $MgCl_2$ were added only when stated. As methyl group acceptors, 5S (RD), MMTV-A145, and poly(dGdC)·poly(dGdC) (dGdC) (GE Healthcare) were used. After the incubation, the radioactivity incorporated into DNA was determined with a liquid scintillation counter.

The methylation of the nucleosome core region was determined as described elsewhere (*11*). The 5S (RR) nucleosome was methylated with 35.2 μM [^{14}C]-AdoMet (57 mCi/mmol; GE Healthcare), and then digested with or without 3.75 U/ml micrococcal endonuclease (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 quantitated with a BAS2000 (Fuji Photo Film,

Table 1. **Expression levels of Dnmt3a, Dnmt3a2 and Dnmt3L in male gonocytes.** The expression levels of Dnmt3a, Dnmt3a2, and Dnmt3L were determined in two independent experiments by Western blotting using recombinant proteins as standards. The expression level of each protein is shown as fmol protein/10^5 cells.

	Dnmt3a2	Dnmt3L	Dnmt3a
exp. 1	110	520	nd[a]
exp. 2	68	650	nd
C2C12 cells[b]	nd	nd	10

[a]Expression level could not be detected (nd) due to the low expression level. [b]Expression level was calculated from the data reported by Watanabe *et al.* (*8*).

Japan). 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.

Binding of Dnmt3L with Dnmt3a2 and Dnmt3a—Direct binding of Dnmt3L with Dnmt3a2 or Dnmt3a was determined as described (*22*) with a slight modification. Dnmt3L (60 nM) and Dnmt3a2 and/or Dnmt3a (40 nM) in a reaction mixture comprising 5.3 μM cold AdoMet, with or without 4 μg/ml 5S (RR) DNA, in 1 ml of R buffer were incubated at 37°C for 1 h. After the incubation, the anti-Dnmt3L antibody was added, followed by incubation at 4°C overnight. To the reaction mixture, 15 μl of a 50% (v/v) slurry of Protein A–Sepharose CL-4B in R buffer was added, followed by further incubation at room temperature for 2 h. The matrix was washed four times with 100 μl of buffer R. The input, unbound, and matrix-bound fractions were loaded onto 12% SDS–polyacrylamide gels. The gels were stained with SYPRO Orange (Invitrogen) according to the manufacturer's instructions, and then the protein bands were determined with a FluorImager 595 (Molecular Dynamics, CA).

RESULTS AND DISCUSSION

Quantification of Dnmt3a2 and Dnmt3L in Male Gonocytes—We have reported that not Dnmt3a but the isoform Dnmt3a2 is highly expressed in male gonocytes in days 14–18 embryos, as well as Dnmt3L (*17*). This strongly suggests that the inhibition of DNA methylation on conditional knockout of the *Dnmt3a* gene in gonocytes at this stage reported by Kaneda *et al.* (*16*) was due to the deletion of Dnmt3a2. It has also been reported that Dnmt3L is a prerequisite factor for the DNA methylation in germ cells (*21, 25–27*), and enhances the DNA methylation activity through direct interaction with Dnmt3a or Dnmt3b (*22, 28*).

Our previous study clearly showed that Dnmt3a2 and Dnmt3L are specifically expressed in gonocytes in days 14–18 embryos (*17*). However, the experiments mainly involved the immuno-fluorescence technique, and semi-quantitative studies were only performed for whole testes, and thus were not quantitative. In the present study, we determined the expression levels in gonocytes using recombinant Dnmt3a, Dnmt3a2, and Dnmt3L as standards. The gonocytes were purified from the testes of days 16.5 embryos of transgenic mice expressing EGFP under the control of the *Oct4* promoter by FACS (*30*). Dnmt3a2 expressed in gonocytes in days 16.5 embryos amounted to 110 and 68 fmol/10^5 cells, and Dnmt3L to 520 and 650 fmol/10^5 cells, in two different preparations (Table 1). The expression of Dnmt3a was below the detection level in these cells. On the other hand, C2C12 myoblast cells express Dnmt3a, about 10 fmol/10^5 cells, but not Dnmt3a2 (*8*). It was confirmed that in gonocytes at this stage, not Dnmt3a but Dnmt3a2 was mainly expressed as well as Dnmt3L, and thus Dnmt3a2 and Dnmt3L were confirmed to be the major players in the DNA methylation. The molar ratio of expressed Dnmt3a2 and Dnmt3L was about 1:5–10.

Dnmt3a2 Bound to Dnmt3L with Similar Affinity to as for Dnmt3a—The mice with the *Dnmt3a* gene destroyed in germ-line specific manner show quite similar phenotypes to those with the *Dnmt3L* gene destroyed, *i.e.*, inhibition of methylation of the genomic regions in male germ cells and spermatogenesis (*16, 21, 25–27, 32*). It has been reported that Dnmt3L is expressed in early stage embryos. However, different from germ cells, *Dnmt3L* gene–knockout mice show apparently normal development (*21, 26*). In early stage embryos, in which global DNA methylation occurs, a high level of Dnmt3b and a low level of Dnmt3a are expressed (*8*). Dnmt3L and Dnmt3a2 are also highly expressed in embryos at this stage and/or embryonic stem cells (*18, 21, 23*).

It is curious that Dnmt3L is necessary for the DNA methylation in germ cells but not in early stage embryos. The reason for the absolute requirement of Dnmt3L during spermatogenesis should be the different properties of Dnmt3a2 and Dnmt3a or Dnmt3b, since the gonocytes at the stage of the global methylation almost exclusively express Dnmt3a2 (Table 1) (*17, 33*), while early stage embryos express Dnmt3b and Dnmt3a in addition to Dnmt3a2 (*1, 8*). We expected that the enzyme activity of Dnmt3a2 and Dnmt3a and/or stimulation property of Dnmt3L toward Dnmt3a2 and Dnmt3a are distinct. To understand the underlying mechanisms, Dnmt3a2 and Dnmt3L were expressed in Sf9 cells and purified to near homogeneity, as well as Dnmt3a and Dnmt3b (Fig. 1A).

We compared the binding affinity of Dnmt3a and Dnmt3a2 as to Dnmt3L. A similar amount of Dnmt3a or Dnmt3a2 was pulled down with Dnmt3L (Fig. 1B, left panel). When equal concentrations of Dnmt3a and Dnmt3a2 were incubated with Dnmt3L, almost equal amounts of Dnmt3a and Dnmt3a2 were pulled down with anti-Dnmt3L antibodies (Fig. 1B, right panel), indicating that the binding affinities of the two enzymes for Dnmt3L were similar. This agrees with the report that Dnmt3L binds to the C-teminal half of Dnmt3a, which is common to Dnmt3a2, and stimulates its activity (*28, 34*). This suggests that the binding affinity of Dnmt3a2 for Dnmt3L may not be a crucial factor to explain the absolute requirement of Dnmt3L for the DNA methylation only in gonocytes. It has been reported that Dnmt3L dissociates from Dnmt3a as soon as the complex binds to DNA (*28*). However, in the present study, it was shown that even when a DNA fragment was added to the binding reaction, the binding between Dnmt3L and Dnmt3a or Dnmt3a2 was not affected (Fig. 1B, right panel; compare lanes 9 and 12), indicating that Dnmt3L and Dnmt3a or Dnmt3a2 form a stable complex even in the presence of DNA.

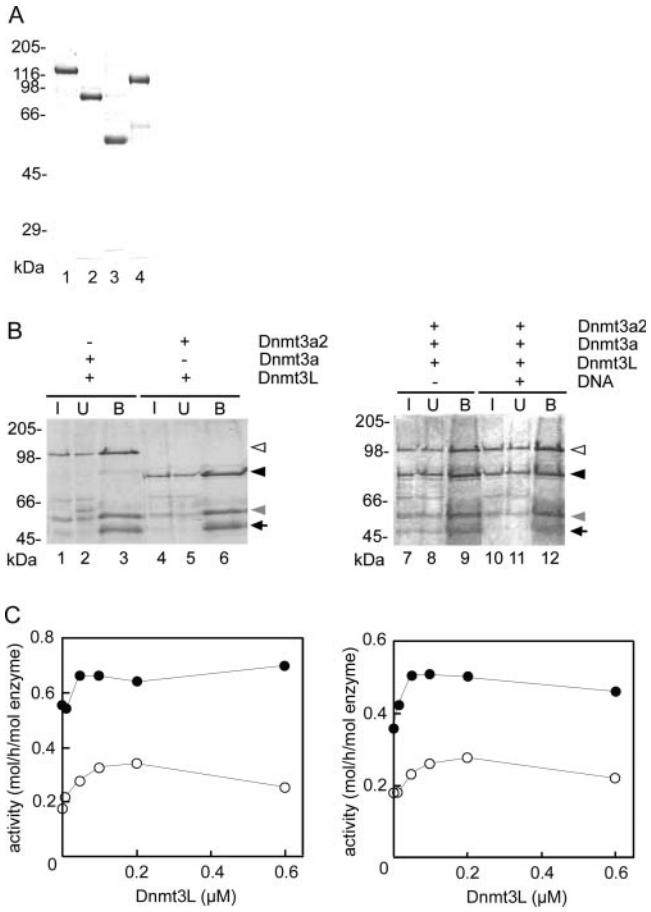

Fig. 1. **Interaction and stimulatory effect of Dnmt3L with Dnmt3a and Dnmt3a2.** A: SDS–polyacrylamide gel electrophoresis of recombinant Dnmt3a (lane 1), Dnmt3a2 (lane 2), Dnmt3L (lane 3), and Dnmt3b (lane 4) is shown. Protein bands were visualized by Coomassie Brilliant Blue R-250 staining. The purity of proteins was more than 95%. Molecular mass markers (kDa) are indicated at the left. B: Dnmt3L was incubated with Dnmt3a or Dnmt3a2 (left panel, lanes 1–6), or Dnmt3a and Dnmt3a2 (right panel, lanes 7–12), and then Dnmt3L was immunoprecipitated with anti-Dnmt3L antibodies. The 5S (RD) DNA fragment (4 μg/ml) was added to lanes 10–12. (+) and (–) indicate the presence and absence, respectively, of the factors indicated on the right side of the panels. Equivalent amounts of input (I) and unbound (U), and a ten times amount of bound (B) fractions were loaded onto a 12% SDS–polyacrylamide gel. The protein bands were stained with SYPRO Orange. The bands of Dnmt3a (open arrowheads), Dnmt3a2 (filled arrowheads), Dnmt3L (gray arrowheads), and IgG (arrows) are indicated. Molecular mass markers (kDa) are indicated at the left of each gel. C: The stimulation of Dnmt3a (filled circles, 40 nM) and Dnmt3a2 (open circles, 40 nM) activities with increasing amounts of Dnmt3L was determined using 5S (RD) (left panel) and MMTV-A145 (right panel) as methyl-group acceptors. The vertical axis represents the specific activity (mol/h/mol enzyme), and the horizontal axis represents the concentration of Dnmt3L in the reaction mixture.

Stimulation of Dnmt3a2 with Dnmt3L—We next determined the stimulation effect of Dnmt3L on the DNA methylation activity of Dnmt3a2 using a naked DNA fragment as a substrate. Similar to for Dnmt3a, the DNA methylation activity of Dnmt3a2 was stimulated in a dose-dependent manner, the maximum 2-fold stimulation being obtained with an about 5-molar excess of Dnmt3L. Apparently, Dnmt3a required less Dnmt3L for its maximal stimulation (Fig. 1C). Since the expression level of Dnmt3L in embrynic day 16.5 gonocytes was 5–10 times higher than that of Dnmt3a2 (see Table 1), the expression level of Dnmt3L was sufficient for the maximal stimulation of Dnmt3a2 in gonocytes. Due to the similar stimulation properties by Dnmt3L of Dnmt3a2 and Dnmt3a, again the results do not explain the necessity of Dnmt3L for DNA methylation in gonocytes.

DNA Methylation Activity of Dnmt3a2 toward Nucleosomal DNA—Recently, we showed that Dnmt3a hardly methylates the nucleosome core region, but that Dnmt3b significantly does, although its activity is low (*11*). Considering that Dnmt3b is highly expressed at the stage of implantation at which global methylation occurs (*8*), we proposed that the ability of Dnmt3b to methylate the nucleosome core region may contribute to genome-wide methylation (*11*). Similar to the expression level of Dnmt3b in implantation stage embryos, the expression level of Dnmt3a2 was quite high at the stage of global methylation in male germ cells. This led us to the idea that Dnmt3a2 might have the ability to methylate the nucleosome core region.

To examine this possibility, we determined the activity of Dnmt3a2 towards nucleosomes with a very short linker reconstituted from 155 bp DNA prepared from ribosomal DNA [5S (RR)] (Fig. 2A) (*11*), and compared it with those of Dnmt3a and Dnmt3b. The methylated DNA in the nucleosome core region was determined after MNase digestion to remove the contaminating naked DNA and short linker DNA exposed outside of the nucleosome core (*11*). As shown in Fig. 2B (upper panel), 50 to 90% of DNA in nucleosomes remained after MNase digestion, while naked DNA was completely digested. The radioactivity of methylated DNA normalized as to that of the recovered DNA is summarized in Fig. 2C. The DNA methylation activity of Dnmt3a2 towards the nucleosome core region was much lower than that of Dnmt3b and was similar to that of Dnmt3a.

It was reported that the DNA methylation activity of ectopically expressed human Dnmt3a2 is apparently stimulated by co-expressed human Dnmt3L in cultured cells (*29*). Thus we next examined whether or not Dnmt3L specifically stimulates the DNA methylation activity of Dnmt3a2 more efficiently than that of Dnmt3a towards the nucleosome core region. Images and the amounts of radioactivity of methylated DNA normalized as to that of the recovered DNA are shown in Fig. 2, D and E. The fold stimulation of the DNA methylation activity of Dnmt3a2 towards the nucleosome core region with Dnmt3L was similar to that of Dnmt3a. Considering the finding that Dnmt3a2 methylates the nucleosome core region poorly, similar to Dnmt3a, the DNA methylation activity towards nucleosomes cannot explain the requirement of Dnm3L in gonocytes for global methylation.

Effect of the Salt Concentration on the DNA Methylation Activity of Dnmt3a2 in the Presence or Absence of Dnmt3L—We have reported that the DNA methylation activity of Dnmt3a is susceptible to salt concentration (*10*), and is severely inhibited around the physiological ionic strength adjusted with NaCl. On the other hand, the activity is rather resistant to potassium ion, which is the major positive ion inside the cells (*10*). We expected

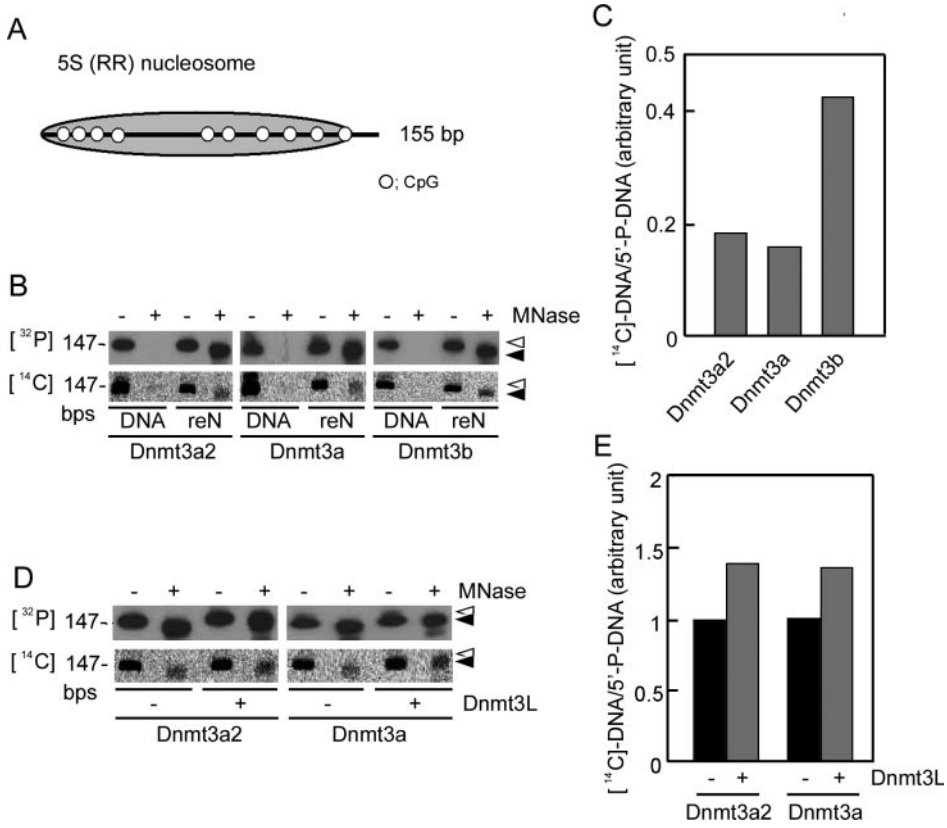

Fig. 2. **DNA methylation activity of Dnmt3a2 towards DNA in the nucleosome core region.** A: Schematic illustration of the nucleosome reconstituted with 5S (RR) DNA used in the experiments. The ellipse indicates the reported location of the histone octamer. The position of the CpG dinucleotide is indicated by open circles. B: Naked DNA (DNA) and nucleosomes (reN) were methylated with Dnmt3a2, Dnmt3a, or Dnmt3b, and then digested with (+) or without (–) MNase. An aliquot of each sample before and after MNase digestion was radio-labeled with [γ-^{32}P]-ATP and T4 polynucleotide kinase, and then the recovery of DNA after MNase digestion ([^{32}P]) was determined. The rest was used to quantitate the methylated DNA ([^{14}C]). The DNA was electrophoresed in a 5% polyacrylamide gel, and then the radioactivity was determined with a BAS2000. The open and filled arrowheads indicate the positions of DNA fragments before and after MNase digestion, respectively. C: The methylation activities of Dnmt3a2, Dnmt3a, and Dnmt3b towards nucleosomes normalized as to the recovered DNA calculated from panel B are shown as a histogram. The relative values as to those before MNase digestion are shown. D and E: A similar experiment was performed as in (C and D) in the presence (+) or absence (–) of a 2.5 molar excess of Dnmt3L using nucleosomes as the substrate for Dnmt3a and Dnmt3a2. The methylation activities of Dnmt3a2 and Dnmt3a in the presence of Dnmt3L were normalized as to those without Dnmt3L, respectively.

that susceptibility of the stimulation effect of Dnmt3L on the DNA methylation activity of Dnmt3a and Dnmt3a2 to salt concentration could be different. To this end we next examined the DNA methylation activity of Dnmt3a2 with 0–250 mM KCl and 30 mM NaCl, this NaCl concentration being inevitable due to the solution of purified Dnmt3a2 and Dnmt3L. The DNA methylation activity of Dnmt3a2 towards naked DNA (dGdC) determined in the presence of 30 mM NaCl was severely inhibited on the addition of 90 mM KCl, which is close to the physiological salt concentration and was almost below the detection level (Fig. 3B, open circles). On the other hand, Dnmt3a retained a significant level of activity at an identical salt concentration (Fig. 3A, open circles). Intriguingly, however, although its activity was low, significant activity was detected for Dnmt3a2 in the presence of a 2.5 molar excess of Dnmt3L (Fig. 3B, filled circles). Almost identical results were obtained when a different DNA sequence, 5S (RD), was used as the substrate (supplementary Fig. 1). It was clearly indicated that when the salt concentration was in the physiological range, Dnmt3a2 absolutely requires Dnmt3L for its DNA methylation activity. To confirm this result, the DNA methylation activity of Dnmt3a and Dnmt3a2 was determined in a reaction mixture containing fixed salt concentrations, 30 mM NaCl (Fig. 3, C and D), or 120 mM KCl and 30 mM NaCl (Fig. 3, E and F), using different enzyme preparations. It was reproducibly shown that with 120 mM KCl and 30 mM NaCl, Dnmt3a2 showed negligible DNA methylation activity, but showed significantly restored activity when Dnmt3L was present, although the activity was one order of magnitude lower than that of Dnmt3a (compare Fig. 3, E and F). The high expression level of Dnmt3a2 in gonocytes, which was about one order of magnitude higher than that expressed in somatic cells such as C2C12 cells (see Table 1), may compensate for this low activity of Dnmt3a2. On the other hand, Dnmt3a retained its activity even with 120 mM KCl and 30 mM NaCl. We propose that the absolute requirement of Dnmt3L for the DNA methylation in gonocytes is due mainly to this salt-sensitive DNA methylation activity of Dnmt3a2. Dnmt3L may somehow compensate for this Dnmt3a2 property. Since the affinity of Dnmt3a and Dnmt3a2 for Dnmt3L

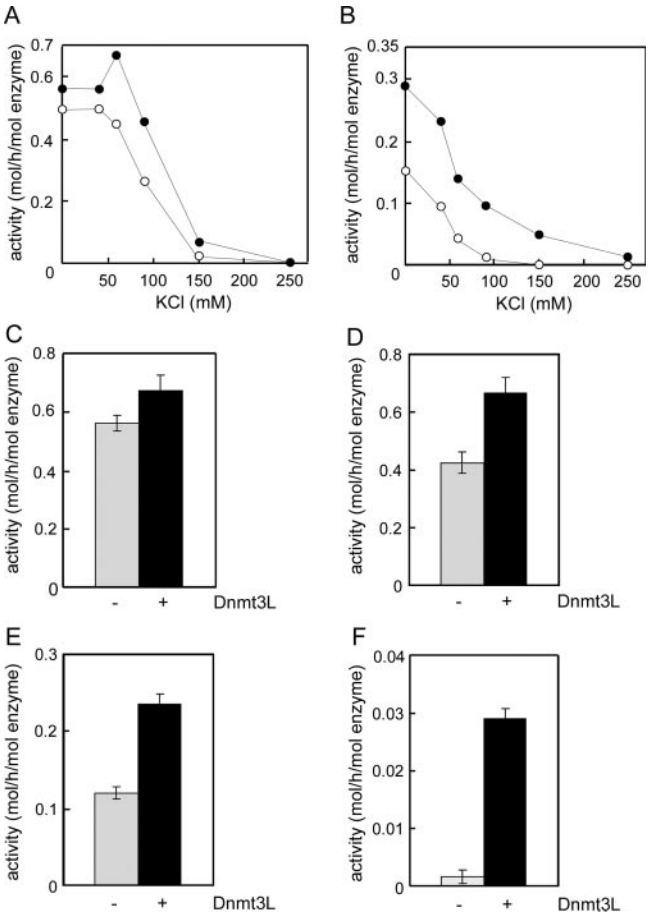

Fig. 3. **Effect of Dnmt3L on the activity of Dnmt3a2 and Dnmt3a with different salt concentrations.** The DNA methylation activities of Dnmt3a (A) and Dnmt3a2 (B) in the presence (filled circles) or absence (open circles) of Dnmt3L were titrated with increasing KCl concentrations. The reaction mixtures contained 30 mM NaCl and 0.5 mM $MgCl_2$ in addition to the indicated KCl concentration. dGdC was used as the methyl-group acceptor. The horizontal axes indicate the specific activity (mol/h/mol enzyme). The effect of Dnmt3L on the Dnmt3a (C and E) and Dnmt3a2 (D and F) methylation activity was determined with fixed salt concentrations. The buffer contained 30 mM NaCl (C and D), and 120 mM KCl and 0.5 mM $MgCl_2$ in addition to 30 mM NaCl (E and F). The activities were determined in the absence (gray bars) or presence (black bars) of a 2.5 molar excess of Dnmt3L. dGdC was used as the methyl-group acceptor. The activity (mol/h/mol enzyme) is shown as means ± SE (n = 3). The specific activity of Dnmt3a2 determined with 30 mM NaCl apparently differed from one purification lot to another, as shown in panels B and D in this figure. This may be due to the small difference in the NaCl concentration in the final preparation of Dnmt3a2, since its activity is quite sensitive to the salt concentration.

was not significantly affected by the salt concentration (supplementary Fig. 2), Dnmt3L may facilitate movement of the bound Dnmt3a2 to the target DNA as proposed by Gowher *et al.* (*28*).

Although the mice with the *Dnmt3a* gene destroyed in germ-line specific manner show a similar phenotype to that of *Dnmt3L* ones, the loss of DNA methylation in certain regions is different (*16, 25, 32*). The *IAP* locus is methylated in *Dnmt3a* gene knockout mice but demethylated in *Dnmt3L* knockout mice (*16, 25, 27*). On the contrary, the *Dlk1-Gtl2* imprinted cluster remains methylated in *Dnmt3L* knockout mice and demethylated in *Dnmt3a* knockout mice (*16, 25*). These results suggest that the stimulatory effect of Dnmt3L on Dnmt3a2 is not always the crucial step. DNA methyltransferases other than Dnmt3a2 may play a role in the *IAP* locus. It has been reported that Dnmt3b is expressed in male gonocytes in day 16 embryos, although at a very low level (*17*). Although no candidate factor is reported up to date, it might be possible that an unknown factor for the stimulation of DNA methylation of Dnmt3a2, other than Dnmt3L may contribute to the stimulation the *Dlk1-Gtl2* imprinted cluster. Or, undetectable level of Dnmt3a may contribute to the methylation.

Supplementary data are available at JB online.

This work was supported by Grants-in-Aid for Scientific Research (B), and for Young Scientists (IS) from the JSPS, and the National Project on Protein Structural and Functional Analyses.

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