Stage-by-Stage Change in DNA Methylation Status of Dnmt1 Locus during Mouse Early Development*

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Methylation of DNA is involved in tissue-specific gene control, and establishment of DNA methylation pattern in the genome is thought to be essential for embryonic development. Three isoforms of Dnmt1 (DNA methyltransferase 1) transcripts, Dnmt1s, Dnmt1o, and Dnmt1p, are produced by alternative usage of multiple first exons. Dnmt1s is expressed in somatic cells. Dnmt1p is found only in pachytene spermatocytes, whereas Dnmt1o is specific to oocytes and preimplantation embryos. Here we determined that there is a tissue-dependent differentially methylated region (T-DMR) in the 5′ region of Dnmt1o but not in that of the Dnmt1s/ip. The methylation status of the Dnmt1o T-DMR was distinctively different in the oocyte from that in the sperm and adult somatic tissues and changed at each stage from fertilization to blastocyst stage, suggesting that active methylation and demethylation occur during pre-implantation development. The T-DMR was highly methylated in somatic cells and embryonic stem cells. Analysis using Dnmt-deficient embryonic stem cell lines revealed that Dnmt1, Dnmt3a, and Dnmt3b are each partially responsible for maintenance of methylation of Dnmt1o T-DMR. In particular, there are compensatory and cooperative roles between Dnmt3a and Dnmt3b. Thus, the regulatory region of Dnmt1o, but not of Dnmt1s/ip, appeared to be a target of DNA methylation. The present study also suggested that the DNA methylation status of the gene region dynamically changes during embryogenesis independently of the change in the bulk DNA methylation status.

DNA methylation of the vertebrate genome occurs predominantly at cytosine residues in cytosine-guanine dinucleotides (CpGs)1 (1). About 70% of CpGs are methylated, mainly in the repressive heterochromatin region and in repetitive sequences such as retrotansposable elements (2). Formation of the cell type-specific DNA methylation pattern is one of the epigenetic events accompanying the production of diverse cell types in the body (3). Generally, DNA methylation in gene-containing regions of the genome is inversely correlated with the transcriptional activity of associated genes (4, 5) through direct (6) and/or indirect (7–10) mechanisms.

We have previously shown that the rSphk1 (rat sphingosine kinase 1) gene, which has multiple alternative first exons embedded in a CpG island, has a tissue-dependent differentially methylated region (T-DMR), with its methylation status inversely correlated with the expression of one of the rSphk1 mRNA subtypes (11). We have also shown that the rPL-1 (rat placental lactogen 1) gene, exclusively expressed in the placenta and having no CpG island at its 5′ region, has a T-DMR that is hypomethylated in the placenta compared with other tissues (12). Furthermore, T-DMRs were also found in the 5′ region of two developmentally essential genes in the mouse: Oct4 (octamer binding transcription factor 4) and Sry (sex-determining region on the Y chromosome). The T-DMRs of the Oct4 and Sry genes are hypomethylated in cells/tissues in which these genes are expressed (13, 14). Thus, DNA methylation-mediated gene silencing is involved in regulation of various genes regardless of the richness of CpGs.

In mammals, there are five members of the DNA (cytosine-5) methyltransferase (Dnmt) family: Dnmt1, Dnmt2, Dnmt3a, Dnmt3b, and Dnmt3L (15–19). Dnmt1 is considered to be a maintenance methyltransferase based on the in vitro enzyme assay in which it preferentially recognizes hemimethylated DNA (2, 20, 21) and on its localization at replication foci in proliferating cells (22, 23). Inactivation of Dnmt1 gene in the mouse leads to global loss of methylation and biallelic expression or silencing of imprinted genes (24–26). Unlike Dnmt1, biological activity of Dnmt2 did not reveal methyltransferase activity specific for CpGs (16), and knock-out of the Dnmt2 gene in the mouse resulted in no detectable abnormality (18). Dnmt3a and Dnmt3b have de novo methyltransferase activity in vitro (17), and a lack of either of them caused extensive perturbations in DNA methylation patterns and premature (Dnmt3a−/−) or prenatal (Dnmt3b−/−) lethality (27).

Three mRNA isoforms, Dnmt1s, Dnmt1p, and Dnmt1o, are expressed from the Dnmt1 locus because of alternative usage of multiple first exons. Dnmt1s is expressed in somatic cells, whereas Dnmt1p and Dnmt1o are exclusively expressed in male and female germ cells, respectively (28). Dnmt1p mRNA, expressed in pachytene spermatocytes, does not produce the active form of Dnmt1 protein, because short open reading frames in the first exon of Dnmt1p (exon 1p) likely interfere with translation of the authentic open reading frame. In contrast, translation from the ATG codon in the first exon of a


Dnmt1o isofrom (exon 1o) is apparently compatible with transcription from an in-frame downstream ATG, which produces an N-terminal truncated active Dnmt1 protein (Dnmt1o). Dnmt1o solely exists in growing oocytes and preimplantation embryos until it is subsequently replaced by Dnmt1s at the blastocyst stage (28, 29). Dnmt1o protein is of interest because it is excluded from the nucleus at all stages of preimplantation development but the eight-cell stage (28–31).

The expression of Dnmt1s requires a cis-element in the promoter region and is controlled by transcription factors Sp1 and Sp3 (32). Furthermore, Dnmt1 transcription during cell cycle progression is modulated by two other cis-elements in the promoter region, which are regulated in a complex fashion by E2F and other transcription factors through E2F-Rb-HDAC dependent-and-independent pathways (33).

Although the regulatory mechanism for spatio-temporal expression of Dnmt1o isofrom is poorly understood, the strictly regulated expression of Dnmt1o suggests that there may be tissue- or developmental-stage specific epigenetic marks on the upstream region of the first exon of Dnmt1o isofrom. To address this possibility, we here investigate the methylation status of CpGs in the upstream region of Dnmt1o and Dnmt1s/lp in germ cells and preimplantation embryos.

MATERIALS AND METHODS

Animal Treatment and Reagents—The experiments were carried out according to the guidelines for the care and use of laboratory animals (Graduate School of Agriculture and Life Sciences, The University of Tokyo). C57BL/6NCrj (B6) mice were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan) and kept under regulated temperature (22–25 °C), humidity (40–60%), and illumination cycles (14 h light of and 10 h of dark) with ad libitum access to food and water. All of the reagents was purchased from Wako Pure Chemicals (Osaka, Japan) unless otherwise stated.

Collection of Oocytes and Embryos—Adult B6 females (7 weeks old) were injected with 7.5 IU of serum gonadotropin from a pregnant mare (Teikoku Hormone Mfg. Co., Ltd., Tokyo, Japan) and 5 IU of human chorionic gonadotropin (Teikoku Hormone Mfg. Co., Ltd.) with a 48-h interval. Metaphase II oocytes were then collected from the oviducts 20 h after human chorionic gonadotropin injection. The collected metaphase II oocytes were denuded of cumulus cells by incubation in 1 mg/ml hyaluronidase (Sigma-Aldrich, Tokyo, Japan) in phosphate-buffered saline for 5 min at room temperature.

To obtain embryos, superovulated B6 females were individually caged with a B6 stud male overnight and examined for the presence of a vaginal plug the next morning. Noon of the day on which vaginal plug is observed was designated as embryonic day 0.5 (E0.5). Preimplantation embryos were recovered and stored at 80 °C until use. Approximately 150 oocytes and 30–80 preimplantation embryos were used for DNA preparation. Kidney, liver, and ovary were collected from 60-day-old adult B6 males.

Embryonic Stem (ES) Cells—The ES cell line MS12, derived from B6 male mouse embryo (35), was a kind gift from Dr. H. Suemori (Kyoto University, Kyoto, Japan). The ES cell line J1, derived from 129Sv/129Jae mouse embryo, and its Dnmt-deficient derivatives (Dnmt1+/−, Dnmt3a−/−, Dnmt3b−/−, Dnmt3a−/−, and Dnmt3b−/−) were kindly provided by Dr. E. Li (Novartis Institutes for BioMedical Research). Both alleles (four alleles in the case of the double mutant) were targeted to introduce cDNA into ES cells (27). The ES cells were cultured on mitomycin C (Sigma-Aldrich)-treated STO cells in the presence of 1000 units/ml leukemia inhibitory factor (ESGORO; Chemicon, Temecula, CA) under standard conditions (34).

Before preparation of genomic DNA from ES cells, mitomycin C-treated STO cells were removed by taking advantage of differences in adherence to the dish surface between STO and ES cells. In brief, ES cells co-cultured with STO cells were trypsinized and replated on fresh tissue culture dishes. After 30 min of incubation at 37 °C, nonattached cells were collected and added to fresh tissue culture dishes again. Following a second 30-min incubation at 37 °C, nonattached ES cells were recovered and stored at −80 °C until use.

Sodium Bisulfite Genomic Sequencing—Genomic DNA was extracted as described previously (14). Sodium bisulfite genomic sequencing was carried out as described previously (36, 37) with slight modification. In brief, EcoRI-digested genomic DNAs (0.5–2.0 μg) were denatured in 0.33 M NaOH for 15 min at 37 °C. Sodium metabisulfite (pH 5.0) and hydroquinone were added to final concentrations of 2.0 M and 0.5 M, respectively. After incubation in the dark at 55 °C for 12 h, modified DNAs were purified with Wizard DNA Clean-Up system (Promega, Madison, WI). The reaction was stopped by further incubation in NaOH at a final concentration of 0.3 M at 37 °C for 1 min followed by ethanol precipitation. Purified DNAs were suspended in 20 μl of 10 mM Tris-HCl (pH 8.0) containing 1 μM EDTA. Bisulfite-modified DNA (2 μg) was amplified with AmpliTaq Gold (Applied Biosystems, Foster City, CA) and one of the following primer sets (see Fig. 1): o-1–1, 5′-TGTTGGTT-TTTGTTGTAAAGGGTTT-3′ and o-1–2, 5′-CTACTATCCCAACAAACACACAC-3′; o-2–1, 5′-GTGGTTGTTTTGTTGTGGTGAAT-3′ and o-2–2, 5′-CACTTTAACAACACACACTAAAT-3′; and s-1, 5′-GTGTTGATTGAATTGATGTTAGTT-3′ and s-2, 5′-ACTCCCTAACCACTCACTAGTTAA-3′. The amplified DNA was purified with Wizard DNA Clean-Up system (Promega, Madison, WI) and kept under regulated temperature (22–25 °C), humidity (40–60%), and illumination cycles (14 h light of and 10 h of dark) with ad libitum access to food and water. All of the reagents was purchased from Wako Pure Chemicals (Osaka, Japan) unless otherwise stated.

RNA Extraction and RT-PCR—Collected oocytes or embryos in 5 μl of diethyl pyrocarbonate-treated phosphate-buffered saline were combusted in 25 μl of TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Two μg of glycogen (Fermentas, Hanover, MD) were added as a carrier before ethanol precipitation. Isolated RNA was finally dissolved in 10 μl of diethyl pyrocarbonate-treated water. The RNA preparations from adult tissues were also performed with the TRIzol reagent.

Synthesis of cDNA was carried out using random hexamers and SuperScript II first strand synthesis system (Invitrogen) according to the manufacturer’s instructions. Amplifications by PCR were performed with 1-μ1 aliquots of cDNA in a total reaction volume of 20 μl using Taq polymerase (Toyobo, Osaka, Japan). Expressions of Dnmt1o, Dnmt1s, and Dnmt3a were specifically detected by using the following respective primer sets: Dnmt1o forward, 5′-GTGGTATGAG-GGCTATT-3′ and Dnmt1o reverse, 5′-CAGGATTACATGTATAGTAA-3′; and Dnmt1s forward, 5′-GGGTGTTCTGGTCATGACCT-3′ and Dnmt1s reverse, 5′-GGCAGGAAATCTGACGATTAG-3′; Dnmt3a forward, 5′-CGGAGGTTGACATCACGTTGC-3′ and Dnmt3a reverse, 5′-CCTCCTCCGGTCTGGAGTTTG-3′; and Dnmt3b forward, 5′-GTAGGCCAGCAGGCAGCCCG-3′ and Dnmt3b reverse, 5′-CCGCGCTGACCCCTTCTC-3′. The thermocycling program used for Dnmt1o and Dnmt1s was 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, preceded and followed by 10 min of incubation at 95 °C and 72 °C, respectively. The amplified PCR products were cloned into pGEM-T easy vector (Promega) and sequenced.

DNA Methylation of Dnmt1 Locus during Mouse Embryogenesis

DNA methylation in growing oocytes and early embryos is mediated by Dnmt3a and Dnmt3b, whereas Dnmt1o is the sole active Dnmt in the blastocyst (28). Inactivation of Dnmt1o results in an increase in the number of methylated sites. The deletion of Dnmt1o in the blastocyst is associated with a significant increase in DNA methylation, which is accompanied by global gene repression and embryonic lethality. These findings suggest that Dnmt1o may play a role in the maintenance of DNA methylation during early embryogenesis. However, the exact role of Dnmt1o in the development of embryos is still unknown.

Our results suggest that Dnmt1o is involved in the maintenance of DNA methylation during mouse embryogenesis. We have shown that Dnmt1o is expressed in growing oocytes and early embryos, and that its expression is downregulated in the blastocyst. Furthermore, we have demonstrated that Dnmt1o is required for the maintenance of DNA methylation in early embryos. These findings suggest that Dnmt1o is an important factor in the maintenance of DNA methylation during mouse embryogenesis. However, further studies are required to determine the precise role of Dnmt1o in the development of embryos.
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anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:500 in blocking buffer. Immunostained embryos were counterstained in 10 μg/ml 4′,6-diamidino-2-phenylindole (Roche Applied Science) in blocking buffer and imaged with epifluorescence with fluorescence and UV filter sets. The obtained images were processed by deconvolution software IPLab program (M&S, Tokyo, Japan).

Sequence Analysis—The genomic DNA sequence of Dnmt1 locus was obtained from GenBankTM (accession number NT_039472). Sequences of part of exon 10 and its downstream region were substituted with those from another GenBankTM entry (accession number AF175410) because they appeared ambiguous in NT_039472. Content of GCs and CpG sequences were analyzed and plotted with a web-based on-line program CpGPlot (bioweb.pasteur.fr/seqanal/interfaces/cpgplot.html) developed by the European Molecular Biology Open Software Suite.

RESULTS

Genomic Structure and CpG Composition around the First Exons of Dnmt1—The positions of the three alternative first exons of Dnmt1 gene and CpG sequences are illustrated in Fig. 1. The first exon of Dnmt1o (1o) is located ~7 kb upstream of the first exon of Dnmt1s (1s), and the first exon of Dnmt1p (1p) is located 85 bp downstream of 1s. There is a CpG island containing 39 CpGs, which covers 1s and a part of 1p (−68 to 332 relative to the putative transcription initiation site of Dnmt1s). There are 20 CpGs between 500 and 1500 bp upstream of 1o, whereas there are only two CpGs within 500 bp upstream of 1o (Fig. 1A).

In the present study, we intensively investigated the methylation status of 18 of 20 distal CpGs in the 5′ region of 1o and of 49 CpGs within and around the CpG island at 1s/1p. The distal 18 CpGs in the 5′ region of 1o are designated hereafter by alphabetical letters, a to r, to avoid confusion and were divided into three groups (I, II, and III) for convenience to describe dynamic change of DNA methylation status. Groups I, II, and III contain five CpGs (a–e), nine CpGs (f–n), and four CpGs (o–r), respectively (Fig. 1A).

Expression of Dnmt1 Isoforms Dnmt1o and Dnmt1s in the Oocytes and Embryos—Transcripts of Dnmt1o and Dnmt1s in oocytes, embryos before and after implantation, and ovary and ES cells were analyzed by RT-PCR (Fig. 2). Dnmt1o transcripts were detected in oocytes, one- and two-cell stage embryos, morula, and ovary, whereas they were not detectable in four- and eight-cell stage embryos, blastocysts, ES cells, and E7.5 embryos. No Dnmt1o transcripts were detected in the kidney and liver of adult mice (data not shown). In contrast, Dnmt1s transcripts were detectable in all cells and tissues examined; then amount of Dnmt1s transcript was high in E7.5 embryos, ovary, and ES cells, whereas it was low in other embryos/cells including the oocytes and preimplantation embryos. Thus, expression of Dnmt1o is not allowed in the restricted embryonic stages including four-cell, eight-cell, and blastocyst stages and embryos after implantation. In this context, the expression of Dnmt1o is more severely controlled compared with Dnmt1s.

It is unclear whether the de novo expression of Dnmt1o occurs during embryogenesis. Various mRNA species transcribed in oocytes are carried over into the preimplantation zygote, and transcription is limited at early embryonic stages (40, 41). Interestingly, the one-cell stage embryos contained higher amounts of Dnmt1o mRNA than did the oocytes. Considering that the amount of β-actin mRNA was comparable between the oocytes and one-cell stage embryos, the increase in Dnmt1o transcript seems to be due to de novo synthesis after the fertilization. Similarly, resumption of Dnmt1o mRNA detection at the morula stage likely results from bona fide transcription at this stage rather than carry over from previous stages because transcripts were not detectable at the four- and eight-cell stages. Thus, transcription of Dnmt1o likely occurs in one-cell and morula stage embryos, whereas it is suppressed in four-cell, eight-cell, and blastocyst stage, and postimplantation embryos.

DNA Methylation Status of the 5′ Regions of 1o and 1s/1p in Germ Cells and Somatic Tissues—DNA methylation status of CpGs in the 5′ regions of 1o and 1s/1p was investigated in adult somatic tissues and germ cells by the sodium bisulfite sequencing method. At the 5′ regions of 1o, all of the CpGs examined (a–r) appeared to be hypermethylated in sperm, kidney, and liver, whereas in the oocyte, 13 (f–r) were almost completely unmethylated, and the rest (a–e) were hypermethylated. All CpGs examined in the upstream region of 1s/1p were unmethylated in sperm and were also barely methylated in the oocyte, kidney, and liver (Fig. 3). A complete lack of methylation of CpGs in the 1s/1p upstream region was evident in all of the cells and tissues examined, including early embryos and ES cells (data not shown).

These data indicate that there is a tissue-dependent differentially methylated region (T-DMR) in the 5′ region of Dnmt1o but not of Dnmt1s/1p. The CpG methylation status of Dnmt1o T-DMR was dependent on developmental stage, and it was different in the oocyte from sperm and adult somatic tissues.

Dynamic Changes in DNA Methylation Status of Dnmt1o T-DMR during Preimplantation Development—We then investigated developmental changes in DNA methylation status of Dnmt1o T-DMR during preimplantation development (Fig. 4). In the early one-cell stage embryos collected at E0.5, the methylation pattern of CpG a–r was already distinct from that in the sperm and the oocyte; CpGs h–j and o–r were perfectly methylated, whereas other CpGs (f, g, and k–n) were unmethylated. These results clearly showed that site-specific de novo methylation (CpGs h–j and o–r) of the oocyte genome as well as site-specific active demethylation (CpGs f, g, and k–n) of the sperm genome occurred after fertilization to establish the one-cell stage-specific DNA methylation pattern of Dnmt1o T-DMR. The initial DNA methylation pattern of Dnmt1o T-DMR achieved after fertilization was maintained in the late one-cell stage embryos collected at E1.0.

Further analyses with later stage embryos revealed that all but CpG n of Dnmt1o T-DMR were unmethylated at the two-cell stage, suggesting that active demethylation took place again between the one-cell and two-cell stages. At the four-cell stage, a particular set of CpGs (a and f–n) were fully methylated, and other CpGs (b–e and o–r) remained unmethylated, indicating that site-specific de novo methylation occurred again between the two- and four-cell stages. The methylation pattern at the eight-cell stage was also unique and suggested de novo methylation at four CpGs (b–e). Active demethylation occurred again at CpGs a–n, resulting in an almost totally unmethylated pattern of CpGs in the morula in which Dnmt1o transcripts were detected. The methylation status of each CpG showed a nearly all-or-none pattern until the morula stage, suggesting that all blastomeres at each developmental stage before morula possess an identical methylation pattern regarding the Dnmt1o T-DMR. In the blastocyst stage, CpGs h–n became methylated again, whereas other CpGs remained unmethylated. The MS 12 ES cells showed hypermethylated status that is similar to adult somatic tissues. Thus, it is intriguing that the DNA methylation pattern dramatically changes from stage to stage before the implantation.

Expression and Localization of Dnmt3a and Dnmt3b Proteins during Preimplantation Development—Cytoplasmic re-
FIG. 1. Schematic diagram of the genomic structure around the alternative first exons of Dnmt1 locus. GC content and CpG frequency at the 5’ regions of Dnmt1o (A) and Dnmt1s/1p (B). The top diagrams show the organization of the 5’ exons of Dnmt1 locus. The graphs show the moving average of GC content (blue) and CpG observed/expected frequency ratios (red) of the 2.5-kb region around exon 1o (A) and 1s/1p (B). A CpG
tension of the Dnmt1o protein has been reported during pre-
implantation development except for the eight-cell stage (28,
31), making Dnmt1o protein an unlikely candidate for regulat-
ing formation of the stage-specific methylation pattern of its
own gene. To elucidate the possible participation of Dnmt3a
and/or Dnmt3b in the methylation of Dnmt1o T-DMR, expres-
sion of Dnmt3a and Dnmt3b mRNA and subcellular localiza-
tion of Dnmt3a and Dnmt3b protein were analyzed by RT-PCR
and immunocytochemistry, respectively. Strong expression of
Dnmt3a was observed in oocytes, morula, E7.5 embryos, and
ES cells, whereas the signal was weak in one- to eight-cell and
blastocyst stage embryos (Fig. 5A). Immunocytochemistry re-
vealed that the Dnmt3a protein mainly localized in the nucleus
and/or perinuclear region in one-, two-, and four-cell stage
embryos (Fig. 5B). In eight-cell, morula, and blastocyst stage
embryos, Dnmt3a was mainly detected in the cytoplasm, but a
weak signal still remained in the nucleus. Thus, Dnmt3a pro-
tein reside at the all-preimplantation cleavage stages.

Dnmt3b mRNA was not detected throughout the preim-
plantation stages but was detectable in the ovary, E7.5 embryos,
and ES cells (Fig. 5A). Dnmt3b protein was, however, detecta-
ble by immunocytochemistry in preimplantation embryos (Fig.
5B). Dnmt3b was mainly localized in the cytoplasm and plasma
membrane cortex in one-, two-, and four-cell stage embryos, as
well as located extensively in cytoplasmic foci in one- and
eight-cell stages and morula stage embryos (Fig. 5B). However,
unlike Dnmt1, Dnmt3b did not appear to be excluded from the
nucleus during preimplantation development. Thus, both
Dnmt3a and Dnmt3b proteins existed in the nucleus of preim-
plantation embryos till the morula stage.

DNA Methylation Status of 5’/H11032 Region of 1o in Dnmt-deficient
ES Cells—To address the question of which member of the
Dnmt family is responsible for methylation of CpGs at Dnmt1o
T-DMR during early development, we investigated the DNA methylation status of the
T-DMR in Dnmt mutant ES cell lines lacking Dnmt1, Dnmt3a,
or Dnmt3b and in a double mutant for Dnmt3a and 3b. Similar
to MS12 ES cells, wild type J1 ES cells showed hypermethylation of Dnmt1o T-DMR (Fig. 6). The Dnmt1o T-DMR was reduced to ~30% of that of the wild type. The dependence on Dnmt1 seems to be different among the CpGs; CpGs b, c, g, and q were very dependent on Dnmt1, whereas CpGs d, e, f, h, i, and k were resistant to Dnmt1 deficiency. In the Dnmt3a/H11002/ES cells, CpGs f, g, and k–n showed lower levels of methylation, whereas other CpGs were resistant to the lack of Dnmt3a. Similar dependence was also observed in Dnmt3b/H11002/ES cells, although the extent of demethylation of CpGs f, g, and k–n was more severe in Dnmt3b /– ES cells compared with Dnmt3a /– ES cells. Thus, involvement of Dnmt3a and Dnmt3b is evident in some CpGs of group II, but their participation was limited. In double mutant ES cells (Dnmt3a /– and Dnmt3b /–), all CpGs, with only one exception, were fully unmethylated, suggesting that either Dnmt3a or Dnmt3b is required for de novo as well as maintenance methylation of the Dnmt1o T-DMR.

**DISCUSSION**

Changes in methylation status of Dnmt1o T-DMR and expression of each member of the Dnmt family are illustrated in Fig. 7. The T-DMR, particularly the CpGs of group II, was hypomethylated in the oocyte and embryos when Dnmt1o was expressed, whereas it was hypermethylated in the sperm and somatic tissues. Thus, the developmental stage-specific transcriptional regulation of Dnmt1o seems to involve the epigenetic system by DNA methylation, which determines the turn-on and -off of gene expression. Transcription of Dnmt1s, whose upstream region appeared in this study to be almost completely methylation-free regardless of tissue and developmental stage, is regulated by both chromatin remodeling, involving E2F-Rb-HDAC complex and a combination of transcription factors (32, 33). It is also likely that transcriptional regulation of Dnmt1o involves a combination of transcriptional factors in addition to an epigenetic system. Transcriptional regulation of Dnmt1o remains to be elucidated.

In a previous report, focusing on the genome-wide methylation status of a gene area containing CpG islands, we proposed that the changes in DNA methylation level of a gene area are not parallel to that of bulk DNA containing repetitive sequences (3). In agreement with the proposal, the changes in DNA methylation status of Dnmt1o T-DMR were clearly different from the generally accepted concept that mammalian development is accompanied by two major waves of genome-wide demethylation and remethylation: one during germ cell development and the other after fertilization (42–45). Most of the previous studies have suggested that genome-wide demethylation after fertilization occurs passively, that is, by the lack of maintenance methylation following DNA replication and cell division (43, 46), although another study has reported that replication-independent demethylation may also occur during early embryogenesis (47). Obviously, however, DNA methylation status of the Dnmt1o T-DMR changed and showed a unique methylation pattern at any given stage of preimplantation development. All of these changes should occur by de novo methylation and active demethylation. It is clear that dynamic and finely tuned regulation of methylation of Dnmt1o T-DMR exists.
The DNA methylation status of Dnmt1 Locus during Mouse Embryogenesis

**Fig. 6.** Methylation status of Dnmt1 Locus during Mouse Embryogenesis. Percentages of methylated (closed columns) and unmethylated (open columns) cytosine residues in each CpG dinucleotide in the Dnmt1 Locus of wild type ES cells (Wild type) and its Dnmt-deficient derivatives with indicated genotypes are shown. All of the CpGs examined were conserved in 129S4/SvJae background. All of the data were calculated from the results of 9–13 independent sequencing analyses using bisulfite genomic sequencing.

In conclusion, the 5′ region of Dnmt1 Locus during Mouse Embryogenesis is the major maintenance enzyme. Furthermore, both Dnmt1 and Dnmt3 were required for stable maintenance of global methylation in the mouse ES cells (52). We have also found that Dnmt3a and 3b are required for maintenance of DNA methylation at some specific loci (53). In the present study, Dnmt1, Dnmt3a, and Dnmt3b all appeared to be required for maintaining the DNA methylation pattern of Dnmt1 Locus in ES cells to some extent. Interestingly, however, the T-DMR was fully demethylated in Dnmt3a/b double mutant ES cells, but demethylation was only partially observed in Dnmt3a/−/− or Dnmt3b/−/− ES cells. These data clearly suggested that Dnmt1 alone is not able to exhibit full maintenance methyltransferase activity and that Dnmt3a and Dnmt3b are also components of this activity at least in ES cells, as we have previously suggested by genome-wide methylation analysis of Dnmt-deficient ES cells (53).

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