DNA methylation occurs at cytosine-phosphodiester-guanine sites (CpG sites), and its patterns are established and maintained during cell differentiation and cell division by DNA methyltransferases (DNMTs). In primordial germ cells (PGCs), the genome-wide methylation state inherited from parents is erased in a certain period, and new methylation patterns are established [1, 2]. The relationship between methylation and the regulation of imprinted genes in germ cells has recently attracted considerable attention [3–7]. In mammals, the methylation levels of maternal and paternal imprinted genes are different between methylation and the regulation of imprinted genes in germ cells. However, the methylation state of the promoter in mature germ cells is still unknown. Here, we investigated the methylation levels of these genes and the pluripotency marker gene Nanog in chicken sperm as compared with the Alb gene in the liver. CpG islands and/or promoter motifs such as TATA box, GC box and CAAT box were found within the putative promoter regions that we identified. By using the bisulfite reaction, CpG sites in the putative promoters were converted, and they were analyzed by sequencing. The putative promoters of Ddx4, Dnd1, Dazl and Nanog showed very low methylation levels in sperm, but they were highly methylated in the liver. Conversely, the Alb gene promoter was highly methylated in sperm and hypomethylated in the liver. However, no transcripts of Ddx4, Dnd1, Dazl and Nanog were detected in sperm or the liver. Also, no transcripts of Dnmt1 and Dnmt3a were detected in sperm. Our present results may indicate that these germ cell-specific genes and the pluripotency marker gene are ready to express any time after fertilization. Our findings showing that low methylation and selective DNA methylation of specific genes are present in chicken sperm contribute to our understanding of fertilization and embryogenesis of birds.

Key words: CpG site, DNA methylation, Gene expression, Germ cell-specific genes, Sperm
on this inactivity of transcription, in sperm, it is considered that there is an epigenetic mechanism that makes gene expression easier in the zygote. However, the methylation pattern of germ cell-specific genes in mature germ cells remains unknown. Thus, in the present study, we focused on the putative promoter region of germ cell-specific genes and the pluripotency marker gene Nanog. We first analyzed the methylation states of these genes in sperm.

**Materials and Methods**

**Chicken samples**

Chicken sperm and livers were collected from White Leghorn chickens. The semen was separated into 90% and 45% layered Percoll solutions (MP Biomedicals, Solon, OH, USA) by centrifuging for 15 min at 1000 g and 4 C. All procedures were performed under the control of the Guidelines for Animal Experiments of the Faculty of Medicine, Kyushu University, and in compliance with Law No. 105 and Notification No. 6 of the Government of Japan.

**Genomic DNA extraction and bisulfite reaction**

Genomic DNA was isolated using an AllPrep DNA/RNA Micro Kit (Qiagen, Valencia, CA, USA) following the manufacturer’s protocol. Unmethylated cytosine in the genomic DNAs isolated from each sample was converted to uracil using a MethylEasy Xceed Rapid DNA Bisulfite Modification Kit (Human Genetic Signatures Pty, Randwick, Australia) according to the manufacturer’s protocol.

**Genomic DNA subcloning and sequencing**

Genomic DNA sequences, CpG islands, and transcription factor binding sites were identified using the NCBI Entrez database (http://www.ncbi.nlm.nih.gov/Entrez/), MethPrimer (http://www.urogene.org/methprimer/), and TFBIND (http://tfbind.hgc.jp/), respectively. The target sequences were isolated from the genomic DNAs treated with the bisulfite reaction by PCR. The primer sets used for the PCR reaction are listed in Supplemental Table 1 (on-line only). The PCR reaction was performed in a total volume of 10 µl containing 40 ng genomic DNA, 1 µl of 10 × PCR buffer, 0.2 mM of dNTP, 0.25 units TaKaRa Taq HS (Takara Bio, Otsu, Japan) and 250 nM of each specific primer. The target sequences were ligated with T-Vector pMD20 (Takara Bio) using a DNA Ligation Kit, Mighty Mix (Takara Bio), and the construct was transformed into E. coli HST08 (Takara Bio) for sequencing.

**Total RNA extraction and reverse transcription**

Total RNA was isolated using an RNasy Mini Kit (Qiagen) according to the manufacturer’s protocol. RNA samples were treated with RNase-free DNase (Wako, Osaka, Japan). cDNA was synthesized with oligo (dT)15 and random primers using a GoTaq 2-Step RT-qPCR System (Promega, Madison, WI, USA).

**RT-PCR**

RT-PCR was performed in a 10-µl volume containing 45 ng cDNA, 1 µl of 10 × PCR buffer, 0.2 mM of dNTP, 0.25 units TaKaRa Taq HS (Takara Bio), and 250 nM of each specific primer (Supplemental Table 2: on-line only). All primer pairs were designed to span introns to prevent amplification of products from genomic DNA. PCR products were electrophoresed using 1.5% agarose gel and were detected by Midori Green DNA Stain (Nippon Genetics, Tokyo, Japan).

**Results**

**Searching for promoter motifs upstream of the first exon in the chicken**

Since the chicken genomic database still does not contain sufficient data to identify the promoter, we searched for promoter motifs such as TATA box, GC box, and CAAT box upstream of their first exon as compared with the mouse genomic database. The structures of chicken Ddx4, Dnd1, Dazl and Nanog were investigated. The Alb gene was used as a reference. Since each chicken gene was constructed by several exons, we focused on a region upstream of the first exon as a putative promoter. A CpG island was present near the first exon of Dnd1 and Dazl in the chicken. Chicken Ddx4, Nanog and Alb lack CpG islands within the investigated region. However, highly homologous sequences of TATA box, GC box, and CAAT box were located near the first exon of each chicken gene (Fig. 1). Consequently, the region approximately 500 bp upstream from the first exon may function as a promoter.

**Expression of DNA methyltransferases in sperm and the liver**

To further confirm whether the methylation level results on the expression levels of the Dnmt family, the transcripts of Dnmt1, Dnmt3a and Dnmt3b were analyzed in sperm and liver samples. As shown in Fig. 2, the transcripts of Dnmt1 and Dnmt3a were detected in liver samples, but not in sperm.

**Identification of methylated CpG sites in the putative promoters**

To identify the number of CpG sites in the putative promoters of Ddx4, Dnd1, Dazl, Nanog and Alb, we searched for the contiguous sequence of cytosine and guanine in the region 500 bp upstream from the first exon. The results are shown in Fig. 3A. The number of CpG sites was diverse among these five genes, and one CpG island existed in the 5′UTR of Dnd1 (–185 bp to –324 bp) and Dazl (–185 bp to –388 bp). Genomic DNA samples were prepared from sperm and liver samples to analyze their methylation states. After applying the bisulfite reaction to the target sequences, 12 clones were obtained from each gene. The methylation levels of CpG sites in all genes examined were very low in sperm samples, while Alb was highly methylated. Conversely, most CpG sites in Ddx4, Dnd1, Dazl and Nanog were highly methylated in liver samples, while the methylation level of Alb was low (Fig. 3B, Table 1).

**Expression of the target genes in sperm and the liver**

To further investigate the relationship between gene transcription and methylation in sperm and the liver, mRNA samples were subjected to RT-PCR. No transcripts of Ddx4, Dnd1, Dazl or Nanog were detected in either sperm or liver samples (Fig. 4). However, the transcript of liver Alb, which was hypomethylated in the putative promoter, was detected in liver samples.
Germ cell-specific genes such as Ddx4, Dnd1, and Dazl play critical roles in the proliferation and survival of germ cells. These proteins are all classified in the RNA binding protein family, and Dnd1 is associated with germ cell survival and the mitotic arrest of male PGCs [26, 27]. Dazl-defective mice are infertile and exhibit a decrease in germ cells [15]. In oligoasthenoteratozoospermic men, incorrect methylation in the Dazl promoter region is only slightly decreased in sperm cells. In contrast to the histone acetyltransferase and histone deacetylase [47–49], DNA methylation is a strict modification with a process that is regulated by the Dnmt family, including Dnmt1, Dnmt3a, Dnmt3b and Dnmt3L. Of the Dnmt family, Dnmt1 is conserved in all species, while Dnmt3a and Dnmt3b are involved in de novo methylation, and they work during early development and gametogenesis in mammals [54, 55]. De novo methylation occurs primarily in germ cells and pluripotent cells for the establishment of individual methylation patterns [56]. The above studies strongly suggest a relationship between methylation of the promoter and the expression of Dnmts in germ cells. In the present study, transcripts of Dnmt1 and Dnmt3a, but not Dnmt3b, were detected in liver samples but were not expressed in sperm. Conversely, it is very reasonable that the putative promoters of germ cell-specific genes are highly methylated in the liver, where they are not expressed. Interestingly, the methylation level of the Alb promoter was very low in liver samples, while the gene is constantly expressed, while epigenetic mechanisms [39–44]. At the TATA box, TFIIB and TFIIF, the basic transcription factors, promote gene transcription. One of the well-known transcriptional factors, Sp1, promotes gene transcription via its binding to the GC box in the promoter [45]. DNA methylation can potentially inhibit the binding of transcription factors and repress gene transcription [46]. The methylation patterns of some genes are different among organisms and between sexes. In mice, methylation in the promoter of the Brahma decreases during spermatogenesis but increases during oocyte maturation [20]. In the present study, we identified the highly homologous promoter motifs including TATA box. The presence of the highly homologous TATA box suggests the possibility of the combination of basic transcription factors, and the region works as a promoter.

In contrast to the histone acetylation involved in histone acetyltransferase and histone deacetylase [47–49], DNA methylation is a strict modification with a process that is regulated by the Dnmt family, including Dnmt1, Dnmt3a, Dnmt3b and Dnmt3L. Of the Dnmt family, Dnmt3L is lacking in chickens [50]. Dnmt1 maintains DNA methylation patterns from parental cells to daughter cells [51–53]. Dnmt3a and Dnmt3b are involved in de novo methylation, and they work during early development and gametogenesis in mammals [54, 55]. De novo methylation occurs primarily in germ cells and pluripotent cells for the establishment of individual methylation patterns [56]. The above studies strongly suggest a relationship between methylation of the promoter and the expression of Dnmts in germ cells. In the present study, transcripts of Dnmt1 and Dnmt3a, but not Dnmt3b, were detected in liver samples but were not expressed in sperm. The lack of gene expression of Dnmt in sperm indicates that the activity of the DNA methylation is low in sperm, while it is high in the liver. We also found that methylation levels in the putative promoters of germ cell-specific genes and Nanog were low in sperm, indicating that these genes may be easily expressed in sperm. However, no transcripts were detected in sperm. Conversely, it is very reasonable that the putative promoters of germ cell-specific genes are highly methylated in the liver, where they are not expressed.

Discussion

Germ cell-specific genes such as Ddx4, Dnd1, and Dazl play critical roles in the proliferation and survival of germ cells. These proteins are all classified in the RNA binding protein family, including Dnmt1, Dnmt3a, Dnmt3b and Dnmt3L. Of the Dnmt family, Dnmt1 is conserved in all species, while Dnmt3a and Dnmt3b are involved in de novo methylation, and they work during early development and gametogenesis in mammals [54, 55]. De novo methylation occurs primarily in germ cells and pluripotent cells for the establishment of individual methylation patterns [56]. The above studies strongly suggest a relationship between methylation of the promoter and the expression of Dnmts in germ cells. In the present study, transcripts of Dnmt1 and Dnmt3a, but not Dnmt3b, were detected in liver samples but were not expressed in sperm. The lack of gene expression of Dnmts in sperm indicates that the activity of the DNA methylation is low in sperm, while it is high in the liver. We also found that methylation levels in the putative promoters of germ cell-specific genes and Nanog were low in sperm, indicating that these genes may be easily expressed in sperm. However, no transcripts were detected in sperm. Conversely, it is very reasonable that the putative promoters of germ cell-specific genes are highly methylated in the liver, where they are not expressed.
Fig. 3. Methylation of CpG sites within the putative promoter region containing the region 500 bp upstream from the first exon. (A) Localization of CpG sites and a CpG island within the putative promoter region. Ddx4, Nanog and Alb lack CpG islands. Vertical line: CpG site. Shadow: CpG island. (B) Methylated CpG sites in the putative promoter regions of Ddx4, Dnd1, Dazl, Nanog and Alb in chicken sperm and liver samples. Left: sperm; right: liver. Filled circles and open circles represent the methylated and unmethylated CpG sites, respectively. Each line represents an examined clone. The localizations of the CpG sites in each gene shown in A (vertical line) and B (circle) represent the same positions.
Germ cells arise soon after fertilization [9, 23, 58]. Thus, since germ-cell-specific genes are hypomethylated in chicken sperm. These genes, the pluripotency marker gene and the albumin gene in chicken sperm and liver samples.

### Table 1. Methylation levels in the promoters of the germ cell-specific genes, the pluripotency marker gene and the albumin gene in chicken sperm and liver samples

<table>
<thead>
<tr>
<th>Genes</th>
<th>No. of CpG sites</th>
<th>Sperm</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ddx4</td>
<td>1</td>
<td>33</td>
<td>75</td>
</tr>
<tr>
<td>Dnd1</td>
<td>30</td>
<td>3</td>
<td>75</td>
</tr>
<tr>
<td>Dazl</td>
<td>28</td>
<td>3</td>
<td>88</td>
</tr>
<tr>
<td>Nanog</td>
<td>24</td>
<td>5</td>
<td>82</td>
</tr>
<tr>
<td>Alb</td>
<td>4</td>
<td>71</td>
<td>17</td>
</tr>
</tbody>
</table>

Fig. 4. Analyses of the expression of Ddx4, Dnd1, Dazl, Nanog and Alb in chicken sperm and liver samples. Sperm and liver mRNA samples were subjected to RT-PCR for expression of Ddx4, Dnd1, Dazl, Nanog and Alb. Gapdh was used as an internal control.

it was very high in sperm. Thus, genes are selectively methylated by unidentified mechanisms. Our present study supports a recent report demonstrating that the promoter CpG islands of germ cell-specific genes are hypermethylated in mouse somatic tissues [57].

One of the prospective reasons for this selectivity is that the genes having a hypomethylated putative promoter are necessary for the development of germ cells and embryos in early embryogenesis. Some studies have indicated the importance of the maternal factors stored in the egg for the zygotic gene activation in zygotes [21, 22]. Additionally, in the embryo of most vertebrates, the precursors of the paternal genome undergo meiosis. This is the most distinctive event for germ cells, and many genes are involved in this process. Elucidation of the relationship between methylation and expression of functional genes is important for clarification of the mechanism of reproduction. In conclusion, the present study provides novel evidence that the putative promoters of the germ cell-specific genes Ddx4, Dnd1 and Dazl and the pluripotency marker gene Nanog are hypomethylated in chicken sperm. These genes, which have critical roles in the proliferation and survival of germ cells and the pluripotency of embryonic cells, may be ready to be expressed soon after fertilization. Conversely, the promoter of the Alb gene, which is not essential for their proliferation and survival, is highly methylated in chicken sperm. Our findings showing that low methylation and selective DNA methylation of specific genes are present in chicken sperm contribute to our understanding of fertilization and embryogenesis of birds, but the molecular mechanism(s) remains to be clarified in the future.

**Conflict of interest:** The authors declare that no conflicts of interest exist that would prejudice the impartiality of this study.

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