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Analysis of Developmental Changes in Avian DNA Methylation Using a Novel Method for Quantifying Genome-wide DNA Methylation

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Individual differentiated somatic cells and undifferentiated stem cells have common genome, although their functions or morphological characters are very different. These differences are derived from difference of gene expression pattern. DNA methylation is generally key factor of suppression of gene and its level is globally change during mammalian early development. But, in birds, whether genome-wide changes in DNA methylation occur during embryonic development is still unknown. Here, we show that genome-wide DNA methylation to assess occurrence during early chick embryonic development. We found that the methylation status at stage 1 was approximately 57%, after which it gradually decreases, reaching a minimum at stage 10 (33%). After stage 10, DNA methylation gradually increased. These results should contribute to clarify the epigenetic mechanisms in birds.

Key words: chick embryo, DNA methylation, early development, isoschizomer, methylation-sensitive restriction enzyme

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Introduction

Methylation of CpG dinucleotides is a heritable epigenetic mechanism that is involved in a broad range of biological processes in vertebrates, plants, and fungi (Bird, 2002). DNA methylation is the most common modification of vertebrate genomes and is primarily associated with transcriptional repression (Chen and Li, 2004). In mammals, DNA methylation is coordinately regulated by three DNA methyltransferases, DNMT1, DNMT3A and DNMT3B (Ballestar and Wolffe, 2001), and it plays a crucial role in the regulation of gene expression, silencing of parasitic elements, genomic imprinting, and embryogenesis (Bird, 2002). The mouse genome is highly methylated, whereas this modification is virtually absent or very infrequent in some invertebrate species, including *Drosophila melanogaster* and *Caenorhabditis elegans*. Some invertebrates, however, have significant levels of methylcytosine (Regev *et al.,* 1998). For example, in the invertebrate chordate *Ciona intestinalis,* genes seem to be methylated and transposable elements non-methylated, which contrasts with the usual situation where methylation appears to silence repetitive sequences and transposable elements.

Global methylation levels differ not only between species but also between different stages of embryonic development. In the mouse, genome-wide demethylation occurs in the fertilized egg, just before zygotic transcription starts at the two-cell stage (Monk *et al.,* 1987; Kafri *et al.,* 1992; Mayer *et al.,* 2000; Oswald *et al.,* 2000). This hypomethylated state is gradually reversed following implantation. The function of these global changes in methylation status, however, is still unknown, but it may be related to reprogramming of the epigenetic marks before embryonic development. Alternatively, global demethylation may serve to derepress the genome on a global scale at the stage where the embryonic genome becomes transcriptionally active for the first time. In agreement with this hypothesis, Stancheva and Meehan (2000) reported that depletion of DNA methyltransferase leads to premature onset of embryonic gene transcription in the frog *Xenopus laevis*. Both hypotheses predict that developmental demethylation occurs globally in species with highly methylated genomes. In the zebrafish *Danio rerio,* however, genome-wide changes in DNA methylation are not observed during embryonic development

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(Macleod *et al.,* 1999).

In birds, the expression of some genes which is controlled by CpG methylation (Kransdorf *et al.,* 2006; Ramachandran *et al.,* 2007) changes in DNA methylation are known to occur during embryonic development. But, in birds, whether genome-wide changes in DNA methylation occur during embryonic development is still unknown. Here, we report that the genomic DNA methylation in the chick changes on a global scale during embryonic development.

Materials and Methods

Experimental Animals

All procedures described within were reviewed and approved by the Animal Care and Use Committee of Shinshu University, and were performed in accordance with the Guiding Principle for the Care and Use of Laboratory Animals.

Freshly laid fertilized eggs from White Leghorn chickens were used in the present studies. The developmental stages of the embryos were determined as described by Hamburger and Hamilton (1951).

Ruby Script Analysis

Chick genomic sequence data was obtained from the University of California Santa Cruz (UCSC) Genome Browser (http://genome.ucsc.edu/). Consider the following: Ruby script was used for the initial determination of the number of CpG dinucleotide and CCGG sites. To determine the number of CpG dinucleotides and CCGG sites around the proximal transcription start site, we randomly selected the following genes from the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/): *Collagen alpha-1* (M13496), *AANAT* (U46502), *BMP4* (X75915), *FGF8* (U41467), (*β-actin* (L08165), *RPLP0* (L28704), *PGK1* (L37101), *CDH1* (M22190), *POMC* (AB019555) and *EDNRB* (AF472616).

Isolation of Genomic DNA

Staged embryos were gently homogenized in homogenization buffer (10 mM Tris-HCl (pH 8.0), 25 mM ethlenediamine tetraacetic acid (pH 8.0), 100 mM NaCl, 0.5% sodium dodecylsulphate and 0.6 mg/m*l* proteinase K), and the homogenate was incubated for more than 16 h at 50℃. An equal volume of phenol/chloroform (1: 1) was added to the incubated samples. The samples were centrifuged for 10 min at 11,935× *g* after which the aqueous layer was transferred to a new tube, and ethanol precipitation was performed to collect the genomic DNA.

Restriction Enzyme Digestion

Digests with *Hpa*II and *Msp*I (New England BioLabs, Inc., Beverly, MA, USA) were carried out using 10 μg DNA and 100 U of each enzyme in NEBuffer 1 or 2 for 10 h at 37℃. Digestion of the DNA was verified by 1.0% agarose gel electrophoresis.

Quantitation of DNA Methylation

Methylated λ DNA was obtained by incubating λ DNA (Takara Bio, Inc, Shiga, Japan) with 3 U/μg *Sss*I methylase (New England BioLabs) and 160 μM of S-adenosylmethionine at 37℃ for 3 h. The methylated λ DNA was resistant to *Hpa*II, confirming completion of the methylation reaction. DNA digested with *Hpa*II and *Msp*I was subjected to phenol/chloroform extraction and ethanol precipitation. Purified DNA was incubated with 2 U/μg of Klenow fragment (FERMENTAS CANADA INC, Burlington, Canada) in the presence of 50 nM Cy3.5 -dCTP (GE Healthcare Amersham Biosciences Corp, Piscataway, CA, USA) and 1 μM dGTP (Invitrogen, Carlsbad, NJ, USA) at 37℃ for 2 h. For the removal of excess dye, the reaction mixture was purified twice using a Microcon ultrafiltration unit with a YM-30 membrane (Amicon, Inc., Beverly, MA, USA). Purified DNA was analyzed for the incorporation efficiency of Cy3.5-dCTP by measuring absorption at 260 nm and 581 nm.

Results and Discussion

Using Ruby script which was originally made, we first investigated the number of the CpG dinucleotides and CCGG sites in the chick genomic sequence. These sites should be recognized by the methylation-sensitive restriction enzymes *Hpa*II, whereas *Msp*I whick is isoschizomer of *Hpa*II and not the methylation-sensitive restriction enzymes. The frequency of the CpG dinucleotides was about 1% in the chick genomic sequence, which is slightly higher than the frequency of the CpG dinucleotides in the human genome sequence. Each chromosome had constant CpG dinucleotide and CCGG sites (Fig. 1A and B). The frequency of CCGG/CpG sites was approximately 6% in each chromosome.

We next determined the number of CpG dinucleotides and CCGG sites around the proximal transcription start site of randomly selected genes in the chick genome. We found that both CpG dinucleotides and CCGG sequences are concentrated around the transcription start site (Fig. 1 C and D). These results suggest that DNA methylation is a control mechanism used to control expression in the chick genome DNA and that it would be possible to use CpG dinucleotides within CCGG sites to evaluate DNA methylation in the chick genome.

Genomic DNA was isolated and analyzed using two methylation-sensitive restriction enzymes. Both *Hpa*II and *Msp*I cut the sequence CCGG, but if the CpG sequence in the middle is methylated, only *Msp*I can cut the sequence. Chick genomic DNA was found to be hypomethylated irrespective of the developmental stage, although there seemed to be a slight change in the level of methylation (Fig. 2).

To quantify the change in methylation, digested DNA was tagged with Cy3.5-dCTP and dGTP using Klenow fragment (Fig. 3A-C). The labeled, digested DNA absorbed at 581 nm, allowing quantitation of DNA digestion. To verify that this method was effective, we employed λ DNA. When the λ DNA was completely methylated *in vitro* with *Sss*I methylase, Cy-3.5 dCTP was incorporated with high efficiency (Fig. 4A). Next, we tested the genomic DNA isolated from chick embryos

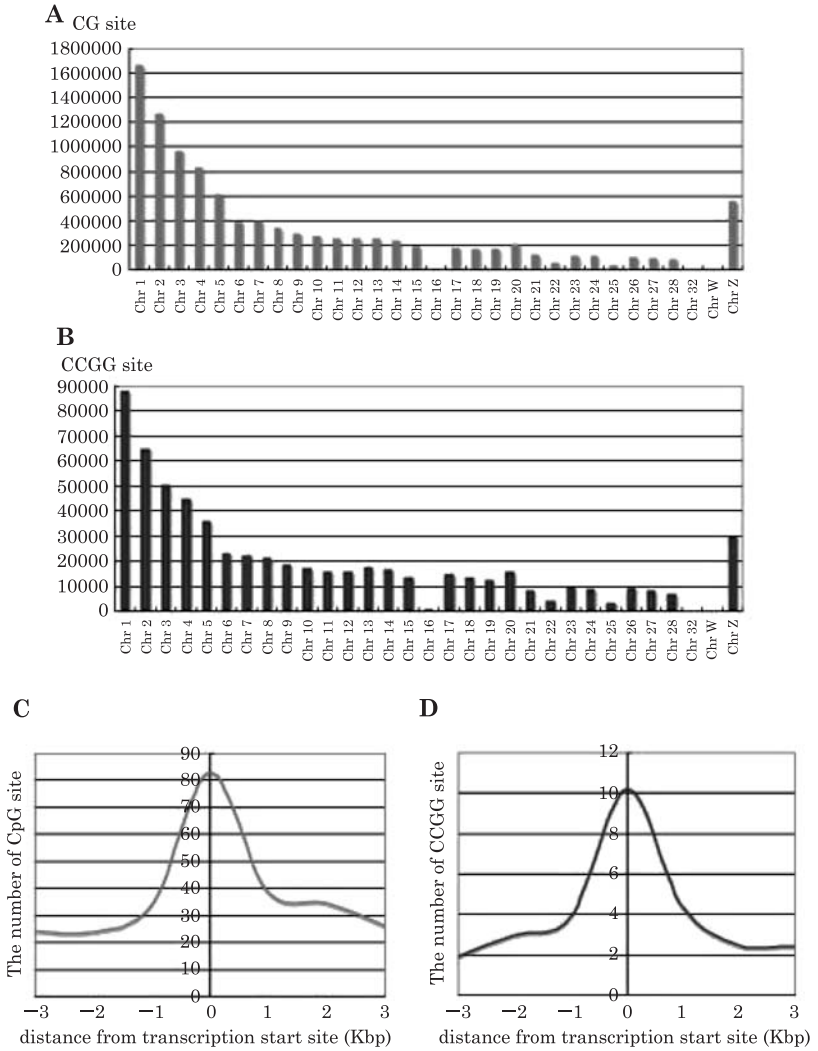

Fig. 1. **Analysis of chick genomic sequence by Ruby script.** A: The number of CpG site in each chromosome. B: The number of CCGG site in each chromosome. C: The number of CpG site around proximal transcription start site. D: The number of CCGG site around proximal transcription start site.

using this method. The level of DNA methylation was 57% to 64% between stages 1 and 7, and 33% to 40% between stages 10 and 17 (Fig. 4B). These results suggest that DNA methylation changes during embryonic development in birds.

We first established initial data on the frequency of CpG dinucleotides in the chick genome. We found that the chick genome has a higher frequency of CpG dinucleotides than the human genome, suggesting that, as in humans, DNA methylation controls gene expression in birds.

Moreover, we established a method for quantifying genome-wide DNA methylation. Using this method, we determined that global changes in genomic DNA methylation occur during chick development. Previous studies have examined genome-wide DNA methylation by electrophoresis (Mayer *et al.,* 2000; Stancheva and Meehan, 2000). At first, we also examined by electrophoresis (Fig. 2). But it was unclear that the dynamically change of genome-wide DNA methylation occurred in early development. DNA damage in the DNA extraction process could be caused the results. On the other hand, our method appears to be more sensitive, so that it may be able to detect small changes in methylation that would otherwise be missed.

Unexpectedly, the lowest level of DNA methylation was at stage 10 of chick development; we had expected the lowest level to be at stage 1, as the stage 1 was the most undifferentiated status after laid eggs and the avian blastoderm was equivalent to mammalian blastocyst (Naito *et al.,* 1990; Bird, 2002; Etches, 2006). This suggests that the change in DNA methylation in the chick differs from

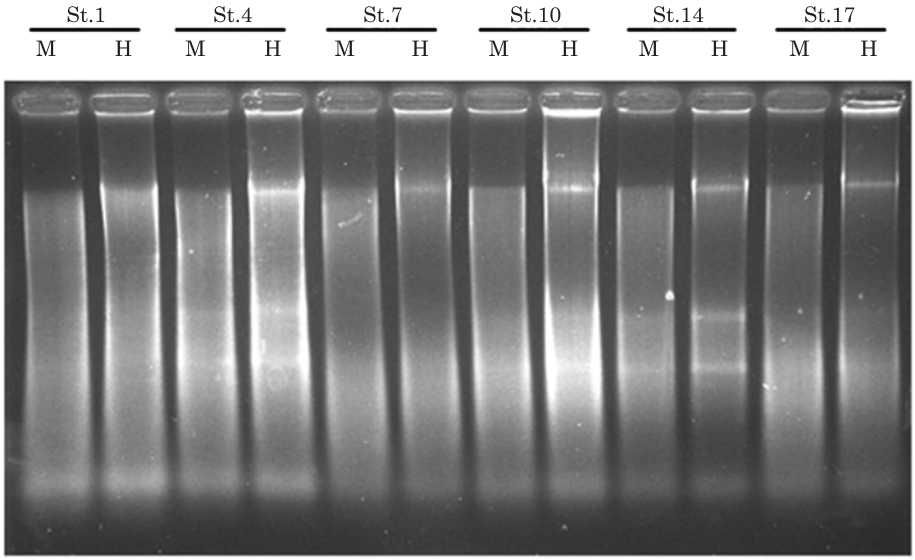

Fig. 2. **DNA mathyaltion during chick embryo development.** The genomic DNA was derived from stage1(St.1)-17(St.17). The methylation status of genomic DNA was tested by *Msp*I-digested DNA (M) and *Hpa*II-digested DNA (H). It was observed by ethidium bromide staining.

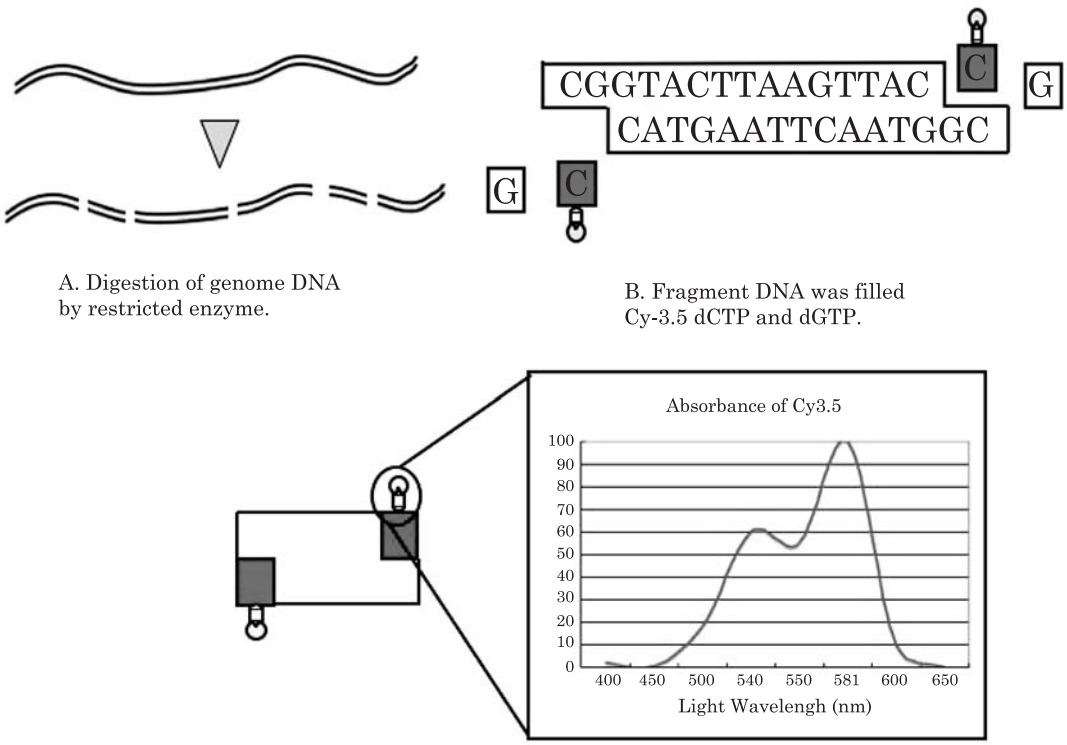

Fig. 3. **A schematic diagram of the novel method for quantification of genome wide DNA methylation.** A: Genomic DNA was digested by *Msp*I and *Hpa*II. B: Fragmental DNA which was digested restricted enzyme was filled Cy-3.5 dCTP and dGTP. C: Filled DNA had a specific absorbance (581 nm), as Cy-3.5 had a specific absorbance.

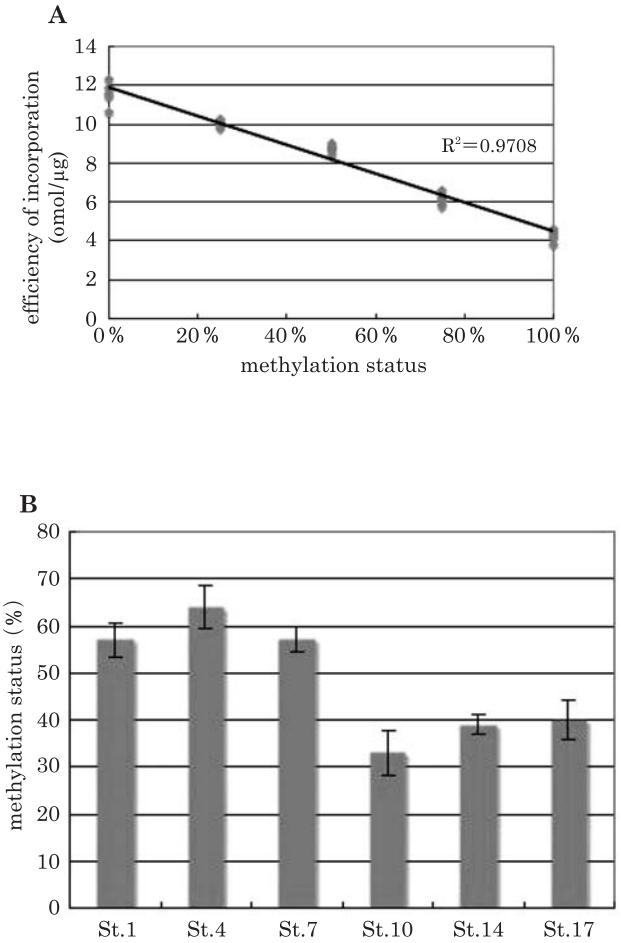

Fig. 4. **Quantification of genome wide DNA methylation in λ DNA and chick embryonic genome DNA.** A: λ DNA was completely methylated in vitro with *Sss*I methylase. The completely methylated DNA (100%) and unmethylated DNA (0%) were mixed by 1: 3 (25%), 1: 1 (50%) and 3: 1 (75%). The efficiency of Cy-3.5 dCTP incorporation was plotted by each methylation parcentage and there were made approximated curve. Date represent five independent experiments. B: Developmental change of DNA methylation in stage1 (St. 1)-stage17 (St. 17).

that in mammals. Further analysis by individual stage of chick development is needed to characterize the changes in more detail.

In conclusion, we developed a novel method for quantification of genome-wide DNA methylation that appears to be more precise than previous electrophoretic methods. Using this method, we show for the first time that the state of DNA methylation changes during embryonic development of the chick. In further studies, we will use this method, along with our previously established method for isolating circulating primordial germ cells (Yamamoto *et al.,* 2007), to investigate whether, as in mammals, DNA methylation changes during the differentiation of chick primordial germ cells into gonads.

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