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Biallelic expression of Z-linked genes in male chickens

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Abstract. In birds, females are heterogametic (ZW), while males are homogametic (ZZ). It has been proposed that there is no dosage compensation for the expression of Z-linked genes in birds. In order to examine if the genes are inactivated on one of the two Z chromosomes, we analyzed the allelic expression of the *B4GALT1* and *CHD-Z* genes on Z chromosomes in male chickens. One base substitution was detected among 15 chicken breeds and lines examined for each gene, and cross mating was made between the breeds or lines with polymorphism. cDNAs were synthesized from cultured cell colonies each derived from a single cell of an F1 male embryo. The allelic expression of the *B4GALT1* gene was examined by restriction fragment length polymorphism analysis of the PCR products digested with *Rsa*I, and that of the *CHD-Z* gene by the single nucleotide primer extension (SNuPE) method. Both of the genes displayed biallelic expression, suggesting that these Z-linked genes were not subject to inactivation in male chickens. Comparison between expression levels in males and females by real-time quantitative PCR suggested that expression was compensated for the *CHD-Z* gene but not for the *B4GALT1* gene.

Gene dosage compensation has evolved independently in many different animal groups using entirely distinct strategies, i.e., upregulation of the male X chromosome in *Drosophila* and downregulation of both X chromosomes in hermaphrodite *Caenorhabditis elegans*. In most mammalian species, males have a heterogametic XY chromosome pair, and gene dosage compensation is attained by inactivation of one X chromosome in females. In contrast, in birds, which have heterogametic females with a ZW chromosome constitution, no evidence of dosage compensation for Z-linked genes in males has been reported. It is widely accepted that dosage compensation does not occur in avian sex chromosomes (Baverstock et al., 1982), and differential expression of the Z-linked genes has been suggested to underlie the avian sex determination mechanism; it is possible that the double dosage of Z-linked genes may trigger sexual differentiation to the male phenotype (Smith et al., 1999). However, an imbalance between males and females in the dosage of a substantial number of genes might cause problems in development and other life processes. Thus, dosage compensation is thought to be an important mechanism related to the differential composition of sex chromosomes. Available evidence about the process of evolution of the Z and W chromosomes is in accordance with the idea that dosage compensation has been acquired evolutionarily in birds. In one extant avian lineage, the ratites (Palaeognathae), the Z and W chromo-

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somes are almost indistinguishable or only moderately differentiated. Morphological comparison between the sex chromosomes of ratites and carinates suggest that structural rearrangements such as deletions and inversions were the initial step of W chromosome differentiation from the ancestral homomorphic chromosome pair (Ogawa et al., 1998; Nishida-Umehara et al., 1999). In general, genetic degeneration of a sex chromosome, i. e., the Y chromosomes in flies and mammals, leads to the establishment of dosage compensation in X chromosomes (Marin et al., 2000). It is therefore speculated that the dosage compensation system was acquired during the evolutionary process of carinate species after the Z and W chromosomes became differentiated.

McQueen et al. (2001) compared the expression levels of some chicken Z-linked genes between the two sexes by using real-time quantitative PCR and thereby demonstrated that the majority of the Z-linked genes were compensated. In their study, at least six out of nine Z-linked genes showed equivalent levels of expression in males and females. This leads to the question, "What is the mechanism that underlies dosage compensation in birds?" If the birds use the same strategies as mammals for dosage compensation, one of the Z chromosomes of males would be inactivated to equalize the expression levels of Z-linked genes between males and females. However, several lines of evidence have implied that the dosage compensation in birds is not attributable to Z chromosome inactivation. The analysis of replication banding patterns of chromosomes by incorporation of 5-bromodeoxyuridine (BrdU) in three species of birds, including the chicken, revealed no evidence of asynchronous replication between euchromatic bands in the ZZ pair in males (Schmid et al., 1989). On the other hand, there have been only a few reports of molecular analyses of the expression of Z-linked genes in birds (Baverstock et al., 1982; McQueen et al., 2001). In order to determine if the genes are inactivated on one of the two Z chromosomes, we analyzed the allelic expression of two Z-linked genes, beta-1,4-galactosyl transferase *(B4GALT1)* and chromo-helicase-DNA-binding on the Z chromosome protein *(CHD-Z)*, in male chickens. The expression levels of these genes were compared between males and females by using real-time quantitative PCR.

Materials and methods

Processing of chicken samples

A total of 90 individuals from 15 different chicken breeds and lines, which were obtained from Hiroshima University and Nagoya University, were used to investigate DNA polymorphism of the Z-linked genes. These breeds and lines were Satsumadori, Barred Plymouth Rock, Oh-Shamo, Ko-Shamo, Nagoya, Don Tao, Rhode Island Red, Modern Game Bantam, Fayoumi, White Plymouth Rock, and five White Leghorn lines (DWR, Cornell-P, HA, HG and G). Peripheral blood samples were collected from adult chickens of each breed or line to look for DNA polymorphism. Cross matings were made between the breeds or lines that showed polymorphism in order to analyze the allelic expression of the *B4GALT1* and *CHD-Z* genes in the F1 hybrids.

Identification of DNA polymorphisms

Genomic DNAs were extracted from whole blood cells by proteinase K digestion and phenol-chloroform extraction. To identify DNA polymorphisms efficiently, PCR primers were designed to amplify the nucleotide sequences coding for the 3′-untranslated regions according to the sequences deposited in GenBank (*B4GALT1*, U19890; *CHD-Z*, AF004397). The following primers were used: *B4GALT1*, 5′-TTG CCA ACT GCA GCC GTG GTG CA-3′, 5′-CAC GCA CAG AGC TCT CCG AGG G-3′ and *CHD-Z*, 5′-CTG ACC TGT GCT TAT GTT TCA GGA-3′, 5′-CTG AAA AGC TTT CCA GGG CAC AG-3′. PCR was performed in a reaction volume of 50 µl containing 50 ng of genomic DNA. The PCR conditions were an initial denaturation at 96 °C for 2 min, followed by 30 cycles of incubation first at 94 °C for 30 s, then at an appropriate annealing temperature for 30 s, and then at 72 °C for 30 s, with a final extension at 72 °C for 5 min. The annealing temperature was 65 °C for *B4GALT1*, and 60 °C for *CHD-Z*. PCR products were purified using Microcon TM-100 (Millipore) and sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) and an ABI 377 DNA Sequencer.

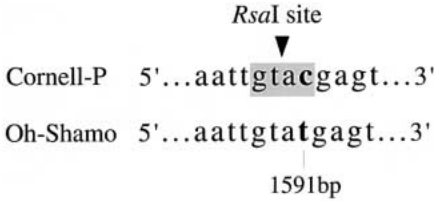

Fig. 1. The sequence difference at nucleotide position 1591 bp of the *B4GALT1* gene between the Cornell-P line and the Oh-Shamo breed (Table 1). This region is digested by *Rsa*I in the Cornell-P line but not in the Oh-Shamo breed.

Sexing and cell culture

Fertilized eggs of the F1 hybrids were incubated for 15 d in a humidified incubator at 38 °C with occasional rotation, and blood was collected from the embryos. DNA was extracted from whole blood cells of the embryos using Dr. GenTLE (TaKaRa) following the manufacturer's protocol. Sexing was performed as described by Fridolfsson and Ellegren (1999) with slight modification. PCR was performed in a reaction volume of 25 µl containing 25 ng of genomic DNA. The PCR conditions were an initial denaturation at 96 °C for 2 min, followed by 30 cycles of incubation at 94 °C for 30 s, annealing at 50 °C for 30 s, and incubation at 72 °C for 30 s, with a final extension at 72 °C for 5 min.

Fibroblast cells of the 15-day male embryos were cultured in Dulbecco's modified eagle medium (DMEM, Nissui) supplemented with 15% fetal bovine serum at 40 °C in 5% CO_2. Cultured cells were suspended in medium, and single cells were picked up using a capillary pipette under a stereoscopic microscope. Each cell was cultured in DMEM supplemented with 15% fetal bovine serum at 40 °C in 5% CO_2 using 96-well plates.

Preparation of RNA and RT-PCR

Total RNA was extracted from the colonies derived from single fibroblast cells using ISOGEN (Nippongene) following the manufacturer's protocol. RNA was treated with RNase-free DNase prior to cDNA synthesis by reverse transcription (RT). RT-PCR was carried out using a GeneAmp RNA PCR Kit (Perkin Elmer) according to the manufacturer's protocol. PCR primers and the conditions of the reaction were as described above.

Allelic expression analysis

Polymorphism was detected in the *B4GALT1* gene in the *Rsa*I restriction site (Fig. 1). To examine the allelic expression of the *B4GALT1* gene, the PCR products were digested with *Rsa*I at 37 °C overnight and separated by electrophoresis in a 2% agarose gel.

Allelic expression of the *CHD-Z* gene was examined using a single nucleotide primer extension (SNuPE) assay (Singer-Sam and Riggs, 1993; Yokomine et al., 2001) with the RT-RCR products as templates. SNuPE was performed in a 25 µl reaction volume containing 50 ng of purified RT-PCR products, 10 mM SNuPE primer: 5′-TGT GAA AAG AAG GCC TGA GTT G-3′, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 1 unit of Taq polymerase and 5 µCi of [a-^{32}P]dCTP or [a-^{32}P]dTTP as indicated in the text. The reaction was incubated at 95 °C for 2 min, 50 °C for 2 min, and 72 °C for 1 min and stopped by adding 10 µl of gel-loading buffer consisting of 10 mM EDTA in 98% formamide. DNA was denatured

at 95 °C for 5 min and electrophoresed on a 20 % denaturing polyacrylamide gel containing 7 M urea. DNA spots were visualized by autoradiography.

Real-time quantitative PCR

The cDNAs which were prepared from cell colonies derived from a single cell each from 11 male and 14 female 15-day embryos were examined. Sexing of embryos, RNA extraction from fibroblasts and cDNA synthesis were performed as described above. The following primers were used: *B4GALT1*, 5′-CGC TGC GCG TGG AGT T-3′, 5′-CTT GCA GTC CTT TGG AGC AAA-3′; *CHD-Z,* 5′-GCA GCT GGT TAT CCG GAC TAC-3′, 5′-CAT CAA TGC GTG CCT GAA AC-3′; and glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* (GenBank Acc. No. K01458), 5′-TGG TGC TAA GCG TGT TAT CAT CTC-3′, 5′-TGA CAA TTT TCA GGG ACT TGT CAT ATT-3′. PCR was performed using QuantiTect SYBR Green PCR Master Mix (QIAGEN), and the amplification was detected with the ABI PRISM 7700 Sequence Detection System. Relative quantitation was performed using the standard curve method (User Bulletin #2, October 2001, Applied Biosystems) and relative expression levels were calculated after correction according to expression of the *GAPDH* gene, which is linked with chicken chromosome 1.

The primers for the *CHD-Z* gene were designed to distinguish the *CHD-Z* sequence from the *CHD-W* sequence. Six base substitutions between *CHD-Z* and *CHD-W* genes were contained in the amplified region (GenBank Acc. No. AF181826). In order to confirm that the PCR products were derived from the *CHD-Z* gene, cloning of the PCR products was carried out using a TOPO TA Cloning Kit (Invitrogen) according to the manufacturer's protocol.

Statistical significance was assessed by 2-tailed *t* test for unrelated samples in which equal variance was not assumed. The *t* tests were conducted by comparing the expression of Z-linked genes based on the null hypothesis of no difference in the means or of a 2-fold difference between males and females (Baverstock et al., 1982; McQueen et al., 2001).

Results and discussion

The DNA polymorphisms identified in the 3′-untranslated region of the *B4GALT1* and *CHD-Z* genes are shown in Table 1. A single nucleotide substitution was detected in only one male of the Oh-Shamo breed (Japanese Large Game) for the *B4GALT1* gene. We crossed this male of the Oh-Shamo breed with females of the Cornell-P line, but the reciprocal cross was not made because of the absence of polymorphism in females of the Oh-Shamo breed. Another single nucleotide substitution was detected in the Satsumadori breed (fancy fowl originated from Kagoshima Prefecture, Japan) in the 3′-untranslated region of the *CHD-Z* gene. Reciprocal crosses were made between the Satsumadori breed and the Cornell-P line. However, the nucleotide substitution was heterozygous for A/G in the male Satsumadori, and therefore, two genotypes, A/A and A/G, were obtained in the F1 males by crossing of Satsumadori-breed males and Cornell-P-line females (Table 1). We used only heterozygous F1 embryos for the experiment after sequencing the PCR products amplified from the genomic DNAs of the embryos.

It is impossible to determine whether the biallelic expression in the F1 male embryos is caused by the random inactivation or the absence of inactivation when the RT-PCR products are derived from multiple cells. Thus RT-PCR was performed with mRNAs extracted from single-cell colonies to examine the allelic expression more precisely. Either paternal or maternal expression would be observed if the Z-linked gene was inactivated; in contrast, the Z-linked gene would display biallelic expression if inactivation did not occur.

Table 1. DNA polymorphism

Gene symbol	Line or breed (sex)	Polymorphism (position)[a]	Chromosome location
B4GALT1[b]	Oh-Shamo (♂)	C → T (1591)	Zq2.1
CHD-Z	Satsumadori (♀)	A → G (6372)	Zq1.6 → q2.1
	Satsumadori (♂)	A/A → A/G	

[a] The nucleotide position numbering is according to the sequences deposited GenBank: *B4GALT1*, U19890; *CHD-Z*, AF004397.
[b] Previous gene symbol was *GGTB2*.

The length of the RT-PCR product of the *B4GALT1* gene generated in this study was 385 bp, and this fragment of the Cornell-P line was cleaved into 241-bp and 144-bp fragments by *Rsa*I-digestion. The RT-PCR products of the Oh-Shamo breed were not digested with *Rsa*I because of the nucleotide substitution (Fig. 1). The presence of three DNA bands in the RT-PCR products amplified from the single-cell colonies of the 15-day F1 male embryos implied that the Z-linked *B4GALT1* gene was biallelically expressed in male chickens (Fig. 2). The biallelic expression of the *B4GALT1* gene was observed in all ten male embryos at 15 days. The same assay was also performed using RNA prepared from single-cell colonies from three and four samples of 5- and 10-day embryos, respectively, and all the samples showed the same results (data not shown). We next examined the allelic expression of the *CHD-Z* gene by the SNuPE assay. All cDNAs of the 15-day F1 male embryos obtained by reciprocal crosses showed two spots, suggesting that the *CHD-Z* gene was expressed biallelically (Fig. 3). These results suggest that neither of the two Z-linked genes is inactivated, at least in the fibroblasts from 5-, 10- and 15-day male embryos of chickens.

Kuroda et al. (2001) performed fluorescence in situ hybridization (FISH) analysis with nascent transcripts of five Z-linked genes to male chicken chromosomes, and detected biallelic expression of these genes. In agreement with their conclusions, our results obtained here confirmed the absence of Z chromosome inactivation in male chickens. However, it remains possible that some of the genes on the Z chromosome are subject to inactivation, because X inactivation does not operate on all genes on the mammalian X chromosome and several genes escape the inactivation. The genes on the X chromosome that escape dosage compensation are clustered in the pseudoautosomal region (PAR) where the X and Y chromosomes are recombined (Carrel et al., 1999). By analogy, it might be expected that avian genes located close to the PAR on the short arm of the Z chromosome would escape inactivation. However, the Z-linked genes examined in this study and the study of Kuroda et al. (2001) were not located in or close to the PAR. If dosage compensation occurs in birds, a different system from that in mammals seems to be operative in the avian genome.

We next compared the levels of expression of the Z-linked genes between the two sexes by real-time quantitative PCR. The *p* value was consistent with the null hypothesis of a male:female ratio equal to 1.0 for the *CHD-Z* gene, and the null

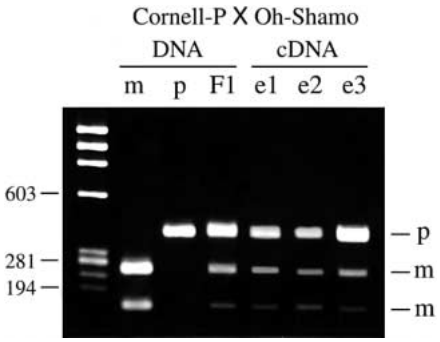

Fig. 2. Biallelic expression of the *B4GALT1* gene in male chickens. Genomic DNAs of maternal (m) and paternal (p) parents and the F1 male embryos (F1), and cDNAs of the15-day F1 male embryos (e1, e2 and e3), which were amplified from single-cell colonies. All PCR products were digested with *Rsa*I. Biallelic expression was seen in e1, e2 and e3. The molecular markers are a fX174/*Hae*III digest.

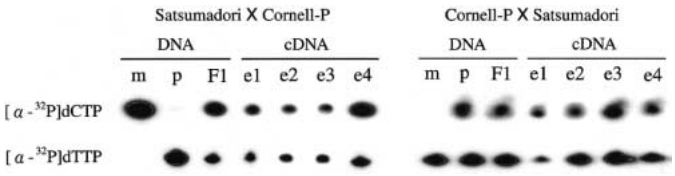

Fig. 3. Biallelic expression of the *CHD-Z* gene in male chickens demonstrated by SNuPE assay. Genomic DNAs of maternal (m) and paternal (p) parents and the F1 male embryos (F1), and cDNAs of 15-day F1 male embryos (e1, e2, e3 and e4), which were amplified from single-cell colonies. The parents clearly had different expression patterns. Biallelic expression was seen in e1, e2, e3 and e4.

Table 2. Expression levels and statistical analysis for Z-linked genes

Gene symbol	Sex[a]	N[b]	Level of expression[c]	Null hypothesis (1:1)[d]			Null hypothesis (2:1)[e]		
				t	d.f.	*p*	*t*	d.f.	*p*
B4GALT1	M	11	2.647 ± 1.098	3.81	13.88	0.00	0.25	21.59	0.81
	F	14	1.269 ± 0.547						
CHD-Z	M	10	0.834 ± 0.493	0.86	15.98	0.40	-2.04	21.93	0.05
	F	14	0.675 ± 0.372						

[a] M, males; F, females.
[b] Number of individuals.
[c] Relative expression was calculated after correction for expression of the *GAPDH* gene. Values are mean ± s.d.
[d] *t* tests were conducted on the null hypothesis of a 1:1 ratio of males:females.
[e] *t* tests were conducted on the null hypothesis of a 2:1 ratio of males:females.

hypothesis of a 2:1 ratio of males:females for the *B4GALT1* gene (Table 2). These results suggest that the expression was compensated for the *CHD-Z* gene, but not for the *B4GALT1* gene, at least in the fibroblasts from the 15-day embryos. McQueen et al. (2001) reported that six genes showed equivalent levels of expression between males and females in 3-day whole embryos, while for two other genes, including *GGTB2*, a synonym for *B4GALT1,* the expression levels were not determined, and for one gene the expression was not compensated. In agreement with their results, our findings indicated that the expression of the *B4GALT1* gene was not compensated in fibroblasts from 15-day embryos and that not all Z-linked genes could be compensated.

W chromosomes evolved independently via a lack of recombination after the differentiation of sex chromosomes in birds, and only four genes have been localized to the chicken W chromosome (Schmid et al., 2000). The *CHD-Z* and *CHD-W* genes are gametologues, and their homologies at the amino acid level are very high (Ellegren, 1996; Griffiths et al., 1996). It therefore seemed possible that the *CHD-Z* expression level appeared to be equal in males and females due to artifactual inclusion of the products of *CHD-W* expression. In this study we cloned six fragments from the RT-PCR products amplified with the primers of the *CHD-Z* gene and determined the nucleotide sequences of the clones. All of them represented sequences derived from the *CHD-Z* gene, suggesting that no products of the *CHD-W* gene were amplified under the present experimental conditions. Thus, our results support the possibility that the expression of the *CHD-Z* gene is compensated between the sexes and that the function of the *CHD-Z* gene is independent of that of the *CHD-W* gene. Further data on the expression levels of other gametologus genes on the Z and W chromosomes and comparison of their functions will be necessary to verify these possibilities.

As sex chromosomes have evolved independently in many different lineages, including birds, it is not surprising that there is a bird-specific gene dose-compensation mechanism. Many insights into the dosage compensation system have been derived from studies of *C. elegans*, *Drosophila* and mammals. However, little is known about how the compensatory system evolved when prototypes of sex chromosomes differentiated from an ancestral pair of autosomes, and about how the dosage compensation mechanisms gradually adapted to new demands encountered in the course of sex chromosome evolution. The chicken Z chromosome has highly conserved linkage homology with human chromosomes 5 and 9; on the other hand, human X-linked genes have been mapped to chicken autosomes, suggesting that the avian sex chromosomes have evolved indepen-

dently from mammalian X and Y chromosomes (Nanda et al., 1999, 2000). Further studies of dosage compensation in birds and comparison of the system in birds with that in mammals might shed some light on the evolution of the dosage compensation system in vertebrate sex chromosomes.

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