



Transcripts of the MHM region on the chicken Z chromosome accumulate as non-coding RNA in the nucleus of female cells adjacent to the *DMRT1* locus

Mika Teranishi¹, Yukiko Shimada¹, Tetsuya Hori¹, Osamu Nakabayashi^{1,2}, Tateki Kikuchi², Tracy Macleod³, Robert Pym³, Bruce Sheldon⁴, Irina Solovei⁵, Herbert Macgregor⁶ & Shigeki Mizuno^{1,7*}

¹Laboratory of Molecular Biology, Department of Molecular and Cell Biology, Graduate School of Agricultural Science, Tohoku University, 1-1 Tsutsumidori-Amamiyamachi, Aoba-ku, Sendai 981-8555 Japan; ²Department of Animal Models of Human Disease, National Institute of Neurosciences, NCNP, Kodaira, Tokyo 187-8502 Japan; ³Division of Animal Health and Production, University of Queensland, St Lucia, QLD, Australia; ⁴CSIRO, Division of Animal Production, Blacktown, NSW, Australia; ⁵Institute of Anthropology and Human Genetics, LMU, München, Germany; ⁶Department of Biology, University of Leicester, Leicester, LE1 7RH, UK; ⁷Present address: Department of Agricultural and Biological Chemistry, College of Bioresource Sciences, Nihon University, 1866 Kameino, Fujisawa 252-8510, Japan; Tel/Fax: (+81) 466 84 3943; E-mail: s-mizuno@brs.nihon-u.ac.jp

*Correspondence

Received 22 September 2000; received in revised form and accepted for publication by A. Sumner 19 December 2000

Key words: CpG methylation, *DMRT1* gene, *Gallus gallus domesticus*, MHM region, nuclear non-coding RNA, triploid chickens, W chromosome, Z chromosome

Abstract

The male hypermethylated (MHM) region, located near the middle of the short arm of the Z chromosome of chickens, consists of approximately 210 tandem repeats of a *Bam*HI 2.2-kb sequence unit. Cytosines of the CpG dinucleotides of this region are extensively methylated on the two Z chromosomes in the male but much less methylated on the single Z chromosome in the female. The state of methylation of the MHM region is established after fertilization by about the 1-day embryonic stage. The MHM region is transcribed only in the female from the particular strand into heterogeneous, high molecular-mass, non-coding RNA, which is accumulated at the site of transcription, adjacent to the *DMRT1* locus, in the nucleus. The transcriptional silence of the MHM region in the male is most likely caused by the CpG methylation, since treatment of the male embryonic fibroblasts with 5-azacytidine results in hypo-methylation and active transcription of this region. In ZZW triploid chickens, MHM regions are hypomethylated and transcribed on the two Z chromosomes, whereas MHM regions are hypermethylated and transcriptionally inactive on the three Z chromosomes in ZZZ triploid chickens, suggesting a possible role of the W chromosome on the state of the MHM region.

Introduction

The roles of the sex chromosomes; ZZ for males and ZW for females, in birds in sex determination

and sex differentiation are largely unknown. A gene on the W chromosome may play a positive role in triggering the pathway toward female sex development, like the *SRY/Sry* gene on the

mammalian Y chromosome which triggers the differentiation of testis (Sinclair *et al.* 1990, Gubbay *et al.* 1990), or the single dose of a gene on the Z chromosome may be important for triggering female development, on the assumption that a dosage compensation mechanism does not operate in birds (Nanda *et al.* 1999). The homologue of the *SRY/Sry* gene has not been identified in birds.

Recently, the *ASW/Wpkci* gene was found in the distal non-heterochromatic region on the short arm of the chicken W chromosome (O'Neill *et al.* 2000, Hori *et al.* 2000). *Wpkci* is actively transcribed during the early embryonic stage and encodes an altered form of PKCI (protein kinase C interacting protein). A gene encoding the chicken homologue of PKCI (*chPKCI*) was cloned and located near the centromere on the long arm of the Z chromosome (Hori *et al.* 2000). A model in which the role of *Wpkci* in female sex differentiation is implied has been proposed (O'Neill *et al.* 2000, Hori *et al.* 2000).

Several genes have been mapped to the chicken Z chromosome, and comparative mapping studies have shown that many of these are present on human chromosome 9 (Nanda *et al.* 1999, 2000). Among them, it is suggested that *DMRT1* (*doublesex* and *mab-3* related transcription factor 1) is involved in the differentiation of testis in humans, because XY individuals hemizygous for this gene region exhibit a high frequency of XY feminization (Raymond *et al.* 1998, 1999a). In chicken embryos, *DMRT1* is already expressed in the genital ridge at stage 25 (sexual differentiation takes place at stage 31). The level of expression of *DMRT1* is higher in the male (ZZ) than in the female (ZW) embryo, and this difference, perhaps reflecting gene dosage, may affect the determination of sex in birds (Raymond *et al.* 1999b). In post-hatched chickens, the expression of *DMRT1* is largely limited to the testis, and its expression becomes undetectable in the female in adult chickens (this study). Two other genes on the Z chromosome, *VLDL* and *ZOV3*, are related to the development of the ovum and ovary, respectively. The *VLDL* gene encodes a VLDL receptor which is responsible for the accumulation of vitellogenin and very low density lipoprotein (VLDL) in the ova (Barber *et al.* 1991). The *ZOV3* gene encodes an immunoglobulin super family glycoprotein expressed in the cell membrane

of ovarian granulosa cells and islets of cells in the ovarian theca externa layer, both of which are involved in the production of oestrogen (Kunita *et al.* 1997). Thus, sex-specific gonadal differentiation in birds may be triggered by the gene on the W chromosome but the expression of several genes on the Z chromosome is probably required for the process of gonadal differentiation and for the function of differentiated gonads.

In the present study we report on a 460-kb long region on the short arm of the chicken Z chromosome, where cytosines of CpG dinucleotides are highly methylated on the two Z chromosomes in males, but are much less methylated on the single Z chromosome in females. We call this region the MHM (male hypermethylated) region. The MHM status in chickens is established during early development, soon after fertilization, depending upon the absence or presence of the W chromosome. The MHM region and *DMRT1* gene lie close together on the Z chromosome and the female-specific, non-coding RNA transcribed from the MHM region accumulates adjacently to the *DMRT1* gene locus in the nucleus.

Materials and methods

cDNA clones

pCO10-6

Total RNA was prepared by the guanidinium thiocyanate–CsCl method (Sambrook *et al.* 1989) from diplotene-stage chicken oocytes (1–2.5 mm diameter). First strand cDNA was synthesized using random hexamers (Pharmacia) and SuperScript II RNaseH⁻ reverse transcriptase (Gibco BRL) in the presence of RNAGuard (Pharmacia). Second strand cDNA was synthesized with *E. coli* DNA polymerase I (Roche Molecular Biochemicals) in the presence of *E. coli* RNaseH (Takara Biomedicals) and *E. coli* DNA ligase (Takara Biomedicals). Double stranded cDNA was blunt-ended by the reaction with T4 DNA polymerase (Roche Molecular Biochemicals), ligated with *EcoRI*–*NotI*–*BamHI* adaptor and 5' ends were phosphorylated by the reaction with T4 polynucleotide kinase (Takara Biomedicals). Blunt-ended cDNA was elec-

trophoresed on a 1.2% agarose gel and 0.5–10 kb cDNA was recovered by the glass-powder method using EASYTRAP ver.2 (Takara Biomedicals). Size-selected cDNA was ligated with λ gt10 vector and a cDNA library was constructed by *in-vitro* packaging using GIGAPACK II Gold (Stratagene). The cDNA library was first probed with the genomic clone containing the mouse genomic sequence for 28S rRNA (a gift from Prof. R. Kominami, Niigata University) and negative clones were selected. These clones were then probed with a mixture of 32 P-labelled cDNA prepared from the mRNA of diplotene-stage oocytes. DNAs isolated from positive clones were subjected to Southern blot hybridization to genomic DNAs from male and female chickens and a clone showing a 2:1 (male to female) intensity ratio of hybridization was selected. The 0.5-kb cDNA insert of this clone was recloned into pBluescript SK+ to yield pCO10-6.

pCC1-11

Total RNA was prepared as above from cultured fibroblasts derived from an 8-day female chicken embryo. Double-stranded cDNA was synthesized as above but using the 5'-phosphorylated primer, 5'-ACAGATCCATT-3' (a sequence in the insert of pCO10-6) for the synthesis of the first strand cDNA. The double-stranded cDNA was ligated with λ ZAPII vector and a cDNA library was constructed as above. The library was screened with the insert of a genomic clone, pCOC10618 (described below), and a positive clone was isolated. The 3-kb cDNA insert of this positive clone was recloned into pBluescript SK+ to yield pCC1-11.

pcGAPDH

A pBluescript KS+ clone containing about 1 kb chicken *GAPDH* cDNA sequence (Dugaiczak *et al.* 1983).

Genomic clones

pCOC10618

A λ GEM12 genomic library of a male chicken was screened with the cDNA insert of pCO10-6. A positive clone was isolated and its ~16-kb insert was recloned into pBluescript SK+ to yield pCOC10618.

pSZ1S10

A pUC118 clone containing a part (about 10 kb) of *ZOV3*, a gene located on the short arm of the chicken Z chromosome, which encodes an immunoglobulin superfamily protein (Saitoh *et al.* 1993).

pEE0.6

pBluescript SK+ containing a 0.6-kb *EcoRI* fragment subcloned from p7AGW, a genomic clone containing the chicken W chromosome-specific non-repetitive sequence (Ogawa *et al.* 1997).

pBIREBP1300

pBluescript KS+ containing ~14 kb of the chicken *IREBP* (iron-responsive element-binding protein) gene sequence. *IREBP* is located close to the terminal heterochromatic region on the long arm of the Z chromosome (Saitoh *et al.* 1993).

DNA sequence analysis

Sequences of cDNA and genomic clones were determined using Thermo Sequenase fluorescent labelled primer cycle sequencing kit (Amersham) and a 373A DNA sequencer (PE Applied Biosystems), and analysed with DNASIS-Mac 3.0 (Hitachi Software Engineering).

Probes for hybridization

32 P-labelled probes

cDNA or genomic DNA inserts (sizes are shown in parentheses) of clones pCO10-6 (0.5 kb), pcGAPDH (1 kb), pCOC10618 (16 kb), pEE0.6 (0.6 kb) and pBIREBP (2-kb *PstI* fragment) were labelled by the random priming method (Feinberg & Vogelstein 1983) using [α - 32 P]dCTP (Amersham, 110 Tbq/mmol).

Biotinylated probes

One μ g DNA of pCOC10618 or *DMRT1* BAC clone 92F7 was labelled by nick translation with biotin-16-dUTP (Roche Molecular Biochemicals). The BAC clone 92F7 was selected from the female chicken BAC library with the 293-bp 3'-UTR sequence of *DMRT1* cDNA (nucleotide positions 1172–1464 of the chicken *DMRT1* cDNA sequence; accession number AF123456 in EMBL/

GenBank/DDBJ nucleotide sequence databases) as a probe. DNA of pSZ1S10 (0.5 μg) was sheared by sonication to 200–1000-bp fragments, heat-denatured, and labelled with biotin-14-dCTP using BIO PRIME DNA Labeling System (Gibco BRL).

Digoxigenin (DIG)-labelled probes

One μg DNA of pCOC10618 was labelled by nick translation with DIG-11-dUTP (Roche Molecular Biochemicals). pCO10-6 and pcGAPDH were linearized by digestion with an appropriate restriction enzyme within the multicloning site, and then DIG-labelled antisense or sense riboprobe was synthesized with DIG-11-UTP (Roche Molecular Biochemicals) and T7 or T3 RNA polymerase (Roche Molecular Biochemicals) according to Nakabayashi *et al.* (1998).

Preparation of DNA and RNA

High-molecular-weight DNA was prepared from the crude nuclei of blood cells according to Ogawa *et al.* (1997). When DNA was prepared from different tissues of chickens, frozen tissue was ground in a mortar in the presence of liquid nitrogen, suspended in PBS (10 mmol/L Na_2HPO_4 , 1.4 mmol/L KH_2PO_4 , 137 mmol/L NaCl, 2.7 mmol/L KCl, pH 7.4), crude nuclei prepared and DNA was extracted and purified as above. Sperm heads were prepared from the semen of a rooster by applying the method of Mizuno *et al.* (1973), suspended in 10 mmol/L Tris-HCl (pH 8.0), 10 mmol/L EDTA (pH 8.0) and DNA was extracted and purified as above. A 1-day (stage 6–7) chicken embryo was excised with a width of ~ 2 cm from surrounding extra-embryonic membrane, washed in PBS, and DNA was extracted as above.

Total RNA from different tissues of 40-day White Leghorn chickens was prepared by the guanidinium thiocyanate–CsCl method (Sambrook *et al.* 1989). Total RNA from cultured chicken embryonic fibroblasts was prepared by the acidic phenol extraction method according to the protocol of Sambrook *et al.* (1989). A part of the freshly excised liver (1 g) from a 1-day (post-hatched) female chicken was homogenized in 5 ml of HG buffer (50 mmol/L Tris-HCl (pH 7.5), 50 mmol/L KCl, 10 mmol/L MgCl_2 , 6 mmol/L 2-mercaptoethanol, 5 mmol/L dithio-

threitol, 10 U/ml of RNAGuard (Pharmacia)) in a Teflon homogenizer at about 600 rpm on ice. The homogenate was centrifuged at $12\,000 \times g$ for 5 min at 4 °C and the supernatant was saved as a cytoplasmic fraction. The pellet was suspended in HG buffer, centrifuged as above, and resuspended in HG buffer (a nuclear fraction). Total RNA was prepared from the cytoplasmic fraction or the nuclear fraction according to Brockdorff *et al.* (1992).

Poly(A)⁺ RNA was isolated from the total RNA preparation of the lung using an Oligotex-MAG mRNA Purification kit (Takara Biomedicals) and the fraction unbound to the Oligo(dT)30-magnetic beads was used as the poly(A)[−] RNA fraction.

Southern blot, Northern blot and slot-blot hybridization

A genomic DNA sample (15 μg) was digested with 180 U of *Hpa*II or *Msp*I at 37°C for 12 h in 10 mmol/L Tris-HCl (pH 7.5), 10 mmol/L MgCl_2 , 1 mmol/L dithiothreitol. The mixture was then adjusted to 60 mmol/L Tris-HCl (pH 7.5), 10 mmol/L MgCl_2 , 1 mmol/L dithiothreitol, and further digested with 75 U of *Bam*HI at 37°C for 10 h. The digested DNA was precipitated with ethanol, dissolved in TE (10 mmol/L Tris-HCl (pH 8.0), 1 mmol/L EDTA), and 10 μg of the digested DNA was electrophoresed on 1% agarose gel and subjected to Southern blot hybridization on a Hybond-N+ membrane (Amersham) with a ³²P-labelled probe in 0.5 mol/L sodium phosphate buffer (pH 7.2), 7% sodium dodecyl sulphate (SDS), 1 mmol/L EDTA at 63°C for 12 h, and the membrane was washed in $2 \times \text{SSC}$, 0.5% SDS at 65°C for 40 min.

A total RNA sample (20 μg) was subjected to 1% agarose gel electrophoresis under the denaturing conditions with formaldehyde and formamide according to the protocol of Sambrook *et al.* (1989), transferred to a Hybond-N+ membrane in 7.5 mmol/L NaOH and subjected to Northern blot hybridization with a DIG-labelled antisense or sense riboprobe or ³²P-labelled cDNA probe under the reaction conditions as above. Hybridization of the DIG-labelled probe was detected with 1:10 000-fold diluted alkaline phosphatase (AP)-conjugated anti-DIG antibody

(Roche Molecular Biochemicals). The total RNA, cytoplasmic RNA or nuclear RNA (30 µg each) was dissolved in 1 × SSC, 50% formamide, 7% formaldehyde and denatured by incubating at 68°C for 15 min, followed by rapid chilling. The denatured RNA solution was mixed with an equal volume of 20 × SSC, and aliquots containing 5, 10 and 15 µg RNA, respectively, were applied onto a Hybond-N+ membrane (Amersham) using a slot-blotter (MINIFOLD II; Schleicher & Schuell). The membrane was washed in 2 × SSC and subjected to hybridization with a ³²P-labelled cDNA probe as above.

Determination of the repetition frequency

Different amounts of the genomic DNA (0.15–5 µg) from male or female chickens or the purified DNA (0.06–0.5 ng) of pCOC10618 (1.93 × 10⁻⁵ pg/molecule), containing 8 copies of the *Bam*HI 2.2-kb repeating unit, were denatured and fixed onto a Hybond N+ membrane using a slot blotter as above. The blot was hybridized with ³²P-labelled cDNA clone, pCO10-6, as a probe under the same conditions as in the Southern blot hybridization. The intensity of hybridization was quantified with a FLA-2000 bioimage analyser (FUJIFILM) using an imaging plate. The reiteration frequency of the *Bam*HI 2.2-kb unit in the diploid genome (the diploid genome size = 2.54 pg (Mizuno *et al.* 1978)) was calculated according to the following equation: the number of diploid genomes: the number of plasmid molecules = intensity of hybridization to the genomic DNA/repetition frequency: intensity of hybridization to the plasmid/8. The actual numbers used for the calculation were: 2.46 × 10⁵: 3.24 × 10⁶ = 398/repetition frequency: 101/8, and the repetition frequency was determined as 415 times per diploid genome of the male.

Culture of chicken embryonic fibroblasts (CEFs) and treatment with 5-azacytidine

Fibroblasts derived from an 8-day (stage 34) female chicken embryo were cultured in DMEM (Dulbecco's modified Eagle's medium; SIGMA) supplemented with 8% fetal bovine serum (Gibco BRL), 2% chicken serum (SIGMA),

0.03% L-glutamine, 0.2% NaHCO₃, 50 U/ml penicillin-G, 50 µg/ml streptomycin, at 37°C under 5% CO₂/95% air. When the cells were treated with 5-azacytidine (5-AC), 5-AC was added to the above medium at the final concentration of 10 µmol/ml and the culture continued for about three cell generations.

Preparation of chromosomes

Mitotic chromosomes were prepared from CEFs after 4 h treatment with 20 ng/ml colcemid (Gibco BRL) according to Saitoh & Mizuno (1992), except that the hypotonic treatment of cells was carried out in the 4-fold diluted DMEM medium with Milli-Q water at room temperature for 20 min. Lampbrush chromosome spreads were prepared from chicken oocytes of about 1 mm diameter according to Solovei *et al.* (1993).

Fluorescence in-situ hybridization (FISH)

FISH to mitotic chromosome preparations was performed as described in Saitoh & Mizuno (1992) except that hybridization with a biotinylated or DIG-labelled probe was carried out in 50% formamide, 2 × SSC, 10% dextran sulphate and hybrids were detected by a series of reactions with: (1) fluorescein isothiocyanate (FITC)-labelled avidin DCS (Vector Labs), (2) goat biotinylated anti-avidin antibody (Vector Labs), and (3) FITC-labelled avidin DCS for the biotinylated probe; and with: (1) sheep anti-DIG antibody (Roche Molecular Biochemicals), and (2) rabbit rhodamine-labelled anti-sheep IgG (EY Labs) for the DIG-labelled probe. Chromosomes were counterstained with 0.2 µg/ml of 4,6-diamidino-2-phenylindole (DAPI) in 0.1 × PBS, 1.25% 1,4-diazabicyclo-(2.2.2) octane, 90% glycerol (pH 8.8), observed under DM-RB fluorescence microscope (Leica), and subjected to the image analysis with CytoVision (Applied Imaging). FISH to lampbrush chromosome preparations with the biotinylated or DIG-labelled DNA probe or riboprobe was carried out as described by Solovei *et al.* (1993, 1998) except that chromosome preparations were denatured in 70% formamide, 2 × SSC at 72°C for 3 min.

FISH to RNA transcripts and a gene locus in a nucleus was carried out according to Carter *et*

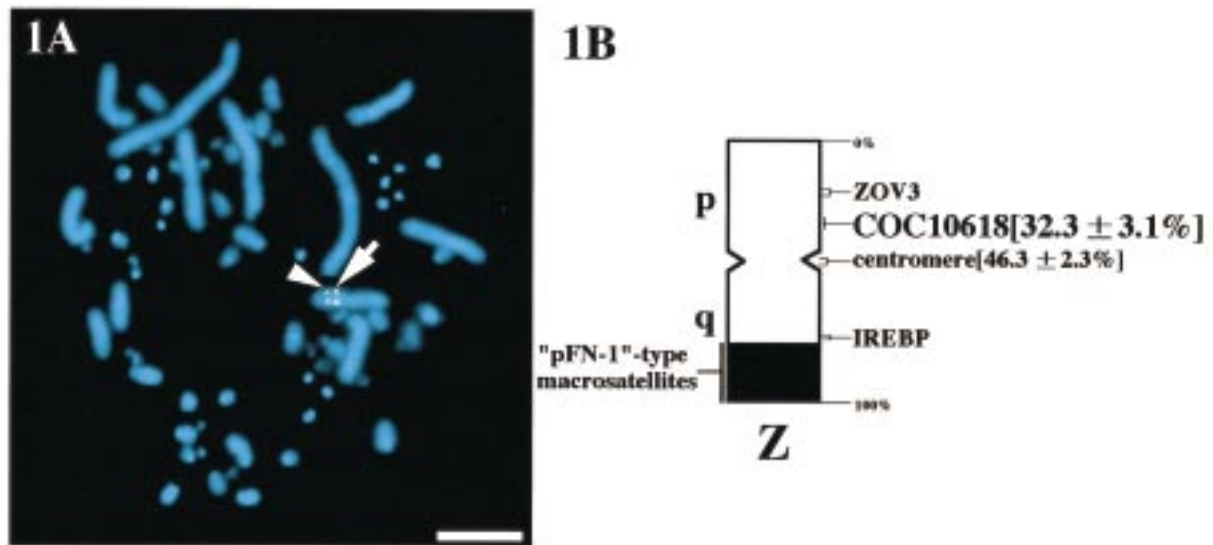


Figure 1. Location of the cloned sequence in pCOC10618 on the short arm of the chicken Z chromosome. (A) FISH to a female metaphase set with DIG-labelled pCOC10618 (arrow; detected with sheep anti-DIG antibody followed with rabbit rhodamine-labelled anti sheep IgG) and the biotinylated genomic probe pSZ1S10 for *ZOV3* (arrowhead; detected with FITC-conjugated avidin), a marker gene for the Carinatae Z chromosome. Chromosomes were counterstained with DAPI. Bar indicates 10 μm . (B) The COC10618 locus (designated the MHM region) on the chicken Z chromosome, determined by FISH to 80 metaphase sets.

al. (1991). Cells treated with 0.5% Triton X-100 and fixed with 4% paraformaldehyde were hybridized with DIG-labelled antisense riboprobe under non-denaturing conditions, washed, digested with RNase A, and hybrids were detected by the reaction with sheep anti-DIG antibody followed by rabbit FITC-labelled anti-sheep IgG. Cells were then fixed again with 4% paraformaldehyde. DNA was denatured in 0.07 N NaOH and hybridized with a biotinylated genomic probe. The DNA/DNA hybrids were detected with rhodamine-labelled avidin DCS (Vector Labs).

In-situ hybridization to tissue sections

Blocks of tissues were excised from the kidney of a 40-day-old male (2A + ZZ) or female (2A + ZW) chicken, or 94-week-old male or female intersex triploid chickens (3A + ZZZ or 3A + ZZW), fixed in PBS containing 4% paraformaldehyde at 4°C for 16 h, dehydrated and embedded in paraffin using a standard procedure. Three- μm -thick sections were cut and mounted on silane-coated glass slides. *In-situ* hybridization with a DIG-labelled

riboprobe and detection of hybrids with alkaline phosphatase-conjugated sheep anti-DIG antibody (Roche Molecular Biochemicals) followed by the colour-developing reaction were carried out according to Nakabayashi *et al.* (1998).

Results

Isolation of a Z chromosome-linked cDNA clone from chicken oocytes; chromosomal localization and transcription

A cDNA clone, pCO10-6, was obtained from the cDNA library of the lampbrush-stage chicken oocytes. This clone was suggested to be derived from a sequence on the Z chromosome because its cDNA insert hybridized to genomic DNAs from male and female chickens with an approximately 2:1 ratio of intensity (data not shown). A female chicken genomic library was screened with the insert of pCO10-6, and a clone, pCOC10618, containing about 16-kb genomic sequence, was obtained. FISH to metaphase chromosome sets from female chicken embryonic

fibroblasts (CEFs) with pCOC10618, together with the Z-linked genomic clone pSZ1S10 (for *ZOV3*; Saitoh *et al.* 1993), indicated that the sequence mapped to the centromere proximal side of the *ZOV3* locus on the short arm of the Z chromosome (Figure 1A), at the position of about 32% (of the entire chromosomal length) from the end of the short arm (Figure 1B).

When the insert of pCOC10618 was DIG-labelled and hybridized to the lampbrush ZW bivalent under different conditions to detect

hybridization to both DNA and RNA, RNA only or DNA only, it was shown that the sequence hybridized not only to DNA (Figure 2C) but also to RNA transcripts on a particular pair of loops at the expected region of the Z lampbrush chromosome (Figure 2A, B). The pattern of hybridization to the RNA transcripts implied that there might be several transcription units on the loop (Figure 2A, B). When similar experiments were performed with DIG-labelled strand-specific riboprobes, prepared by transcription of either strand of the

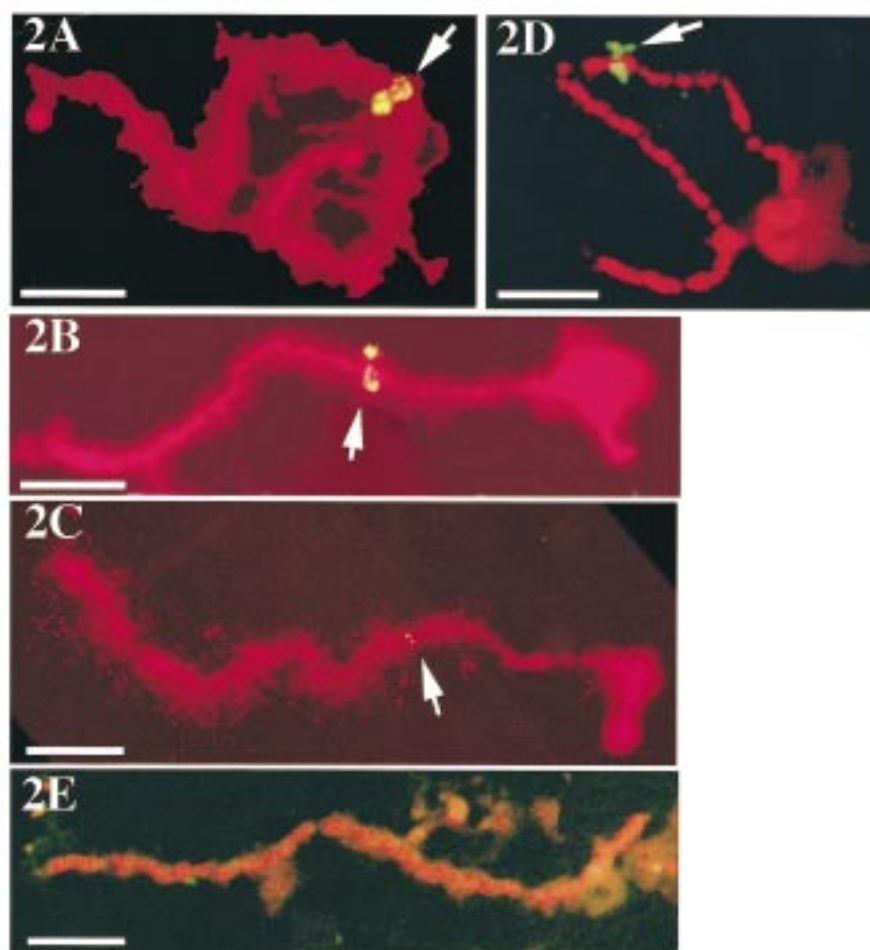


Figure 2. The MHM region is located and transcribed in a strand-specific manner on a particular pair of loops in the lampbrush Z chromosome from a chicken oocyte. FISH to the lampbrush ZW bivalent with the biotinylated pCOC10618 (A to C) or with the DIG-labelled antisense (D) or sense riboprobe (E), prepared from the MHM region cDNA clone, pCO10-6. Reactions were carried out under conditions allowing the probe to hybridize to both DNA and RNA (A), RNA (B, D, E) or DNA (C). Hybrids were detected with FITC-conjugated avidin for the biotinylated probes or with FITC-conjugated sheep anti-DIG Fab for the DIG-labelled probes (arrows). Chromosomes were counterstained with propidium iodide (PI). Bars indicate 10 μ m. Note that only the antisense riboprobe hybridized to RNA on a particular pair of loops (cf. D and E).

pCO10-6 cDNA clone, only one of the probes hybridized to the transcripts (cf. Figure 2D, E), indicating that a particular strand was used as a template for the transcription throughout the loop.

Male hypermethylation of the COC10618 sequence in the order Galliformes

When genomic DNA from blood cells of male or female chickens was digested with *Bam*H1 and subjected to Southern blot hybridization with ³²P-labelled pCOC10618, the probe containing a 16-kb genomic insert hybridized strongly to a 2.2-kb band in both males and females, which suggested that the majority of the cloned genomic sequence consisted of repeats of an approximately 2.2-kb-long unit (Figure 3A, lanes 3 and 6). The DNA preparations were then double-digested with *Bam*H1 and a restriction enzyme; *Hpa*II, *Hae*II, *Hha*I or *Bst*U1, all of which were unable to cleave recognition sequences when the cytosine of CpG in those sequences is methylated. Southern blot hybridization, as above, demonstrated that the DNA from the male was highly resistant to digestion with any of the methylation-sensitive restriction enzymes (Figure 3A, lanes 1, 7, 9 and 11), whereas the DNA from the female was digested more extensively (Figure 3A, lanes 4, 8, 10 and 12). The genomic region on the Z chromosome, which was hybridized with the pCOC10618 probe, was thus designated a male hypermethylated (MHM) region.

When digestion patterns of the MHM region with *Hpa*II (methylation-sensitive) versus *Msp*I (methylation-insensitive) were compared, bands produced with *Msp*I were not present in the *Hpa*II-digest of the DNA from the males (Figure 3A; cf. lanes 1 and 2), whereas all of the three major bands produced with *Msp*I were present in the *Hpa*II-digest of the DNA from the females (Figure 3A; cf. lanes 4 and 5), indicating that a substantial fraction of the MHM region in the female is unmethylated. The same results, as shown in Figure 3A, lanes 1 and 2 vs. lanes 4 and 5, were observed for DNA preparations from lung, liver and kidney of male and female chickens, and for the DNA preparations from testis vs. ovary (data not shown), indicating that hypermethylation in males and hypomethylation

in females of the MHM region are not tissue specific.

The contrasting hyper- and hypomethylation of the MHM region in males and females, detected with the pCOC10618 probe, was also demonstrated in different species (red jungle-fowl, Chukar partridge, common peafowl, Japanese quail and common turkey) belonging to the order Galliformes (Figure 3B). Hybridization of the pCOC10618 probe to genomic DNAs of species belonging to other orders was undetectable under the same conditions of stringency as applied in the experiments shown in Figure 3B (data not shown).

The sex-dependent hyper- or hypomethylation of the MHM region is established after fertilization

In order to examine when the states of hyper- or hypomethylation of the MHM region are established, DNA prepared from sperm cells, blood, or from male or female 1-day embryos were subjected to double-digestion with *Bam*H1 and *Hpa*II or *Msp*I, and Southern blot hybridization with ³²P-labelled pCOC10618 as a probe. The MHM region in sperm DNA was hypermethylated (Figure 4, lanes 1 vs. 2) as was the DNA from blood cells of the male (Figure 4, lanes 3 vs. 4). The MHM region in the DNA from 1-day male embryos was also hypermethylated to a similar extent to that in the sperm DNA or male blood cell DNA (cf. Figure 4, lane 7 and lanes 1, 3). On the other hand, the MHM region in the DNA from female 1-day embryos was much less methylated (Figure 4, lanes 9 vs. 10). The state of hypomethylation in the female 1-day embryo seems to be transient from the hypermethylated state as in the sperm DNA to the hypomethylated state as in the female blood cell DNA (cf. Figure 4, lanes 9 and lanes 1, 5).

Considering that one of the two Z chromosomes in the genome of the male embryo comes from the sperm and the other from the ovum, and the single Z chromosome in a genome of the female embryo comes from the sperm, and assuming that the MHM region in the DNA of the ovum is hypomethylated, it is conceivable that hypermethylation of the MHM region in the ovum-derived Z chromosome in the male and hypomethylation of the MHM region in the

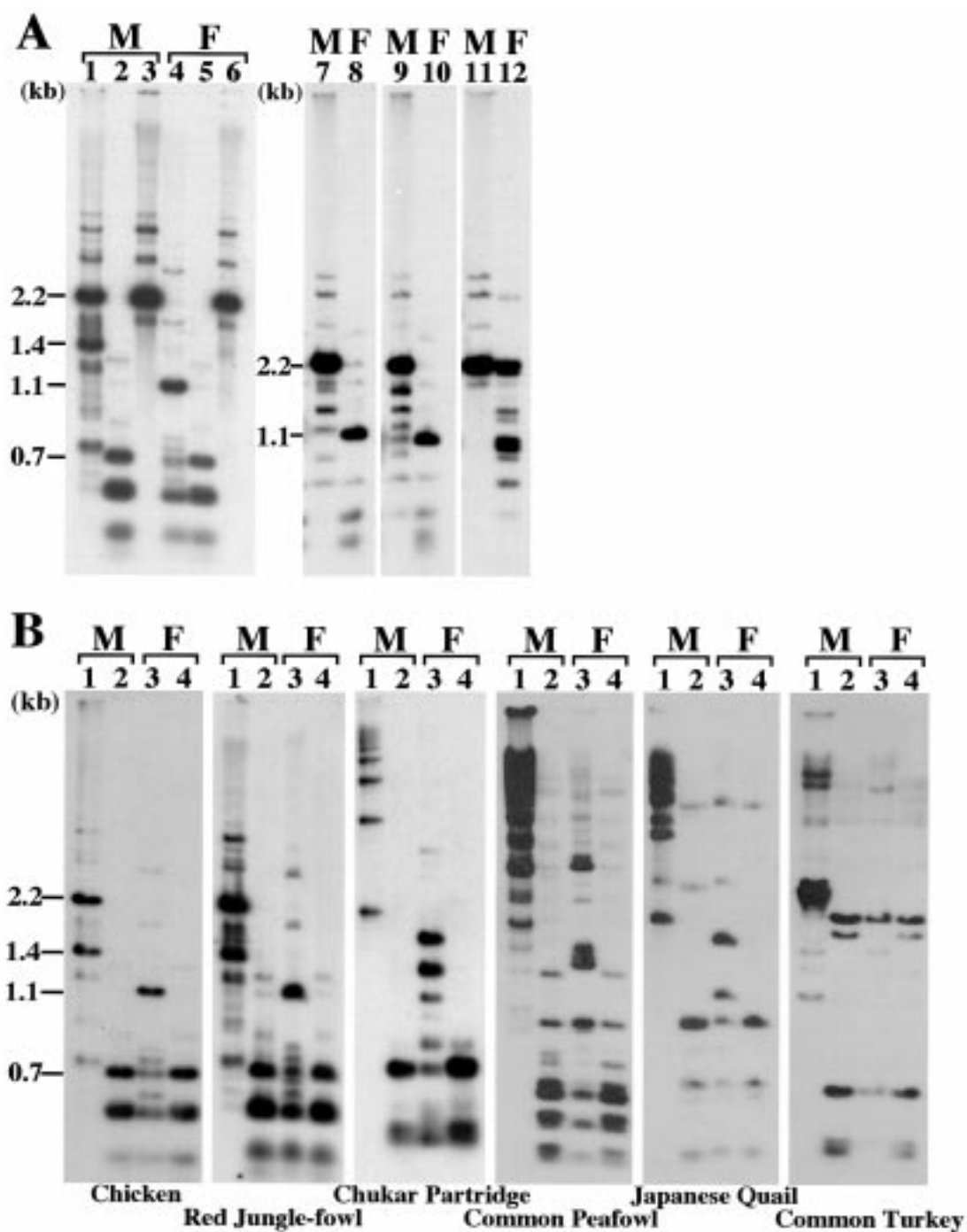


Figure 3. Hypermethylation of DNA in the MHM region in the male chicken and in male species belonging to the order Galliformes. (A) Southern blot hybridization with ^{32}P -labelled pCOC10618 to the genomic DNA prepared from blood cells of a male (M) or a female (F) chicken and digested with restriction enzyme(s) as indicated below. Lanes 1 and 4, *Bam*HI + *Hpa*II; lanes 2 and 5, *Bam*HI + *Msp*I; lanes 3 and 6, *Bam*HI; lanes 7 and 8, *Bam*HI + *Hae*II; lanes 9 and 10, *Bam*HI + *Hha*I; lanes 11 and 12, *Bam*HI + *Bst*UI. (B) Genomic DNAs prepared from male (M) or female (F) individuals of six different species, as indicated, were double-digested with *Bam*HI and *Hpa*II (lanes 1 and 3 in each panel) or *Bam*HI and *Msp*I (lanes 2 and 4 in each panel) and subjected to Southern blot hybridization with ^{32}P -labelled pCOC10618. Size markers in A and B are λ DNA digested with *Hind*III.

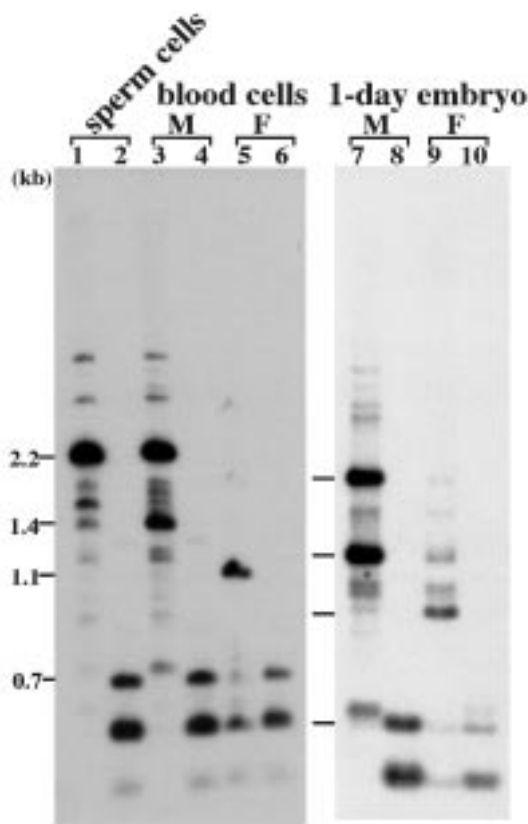


Figure 4. Hypermethylation in the male and hypomethylation in the female of the MHM region are established after fertilization and during early embryogenesis. Genomic DNAs prepared from sperm cells (lanes 1, 2), blood cells of a male (M) (lanes 3, 4) or a female (F) (lanes 5, 6) chicken and from male (lanes 7, 8) or female (lanes 9, 10) chicken embryos after 1-day incubation (stages 6 to 7), were double-digested with *Bam*HI and *Hpa*II (odd numbered lanes) or *Bam*HI and *Msp*I (even numbered lanes) and subjected to Southern blot hybridization with 32 P-labelled pCOC10618. Size markers are as in Figure 3.

sperm-derived Z chromosome in the female are established after fertilization and during early development up to about the 1-day embryo stage.

Methylation patterns of the MHM region in triploid chickens, suggesting a role of the W chromosome

The chicken line which produces a high frequency of triploid offspring (Thorne & Sheldon 1991, Thorne *et al.* 1997) was utilized to examine the state of methylation of the MHM region on the additional Z chromosome in ZZW intersex

females and ZZZ males. Triploids (3n autosomes+ZZZ or ZZW) were distinguished from diploids (2n autosomes+ZZ or ZW) by flow-cytometric comparison of nuclear DNA contents of blood cells according to Thorne *et al.* (1987), and ZZW intersex females were identified by the presence of W chromosome-specific *Xho*I-family repetitive sequence (Kodama *et al.* 1987). The ZW and ZZW constitutions of sex chromosomes were further distinguished by comparing the ratio of signal intensity values for the *IREBP* genomic sequence on the Z chromosome (Saitoh *et al.* 1993) and the EE0.6 genomic sequence on the W chromosome (Ogawa *et al.* 1997) in Southern blot hybridization (Figure 5A).

Southern blot hybridization of genomic DNA preparations, which were double-digested with *Bam*HI and *Hpa*II or *Msp*I, with the 32 P-labelled pCOC10618 probe revealed that patterns of hypermethylation of the MHM region were identical in diploid (ZZ) and triploid (ZZZ) male chickens (Figure 5B, lanes 1–4 versus lanes 5 to 8) and that patterns of hypomethylation of the MHM region were identical in diploid (ZW) female and triploid (ZZW) intersex female chickens (Figure 5B, lanes 9–12 vs. lanes 13–18). These results imply that hypo- or hypermethylation of the MHM region relates to the presence or absence of the W chromosome, and not the number of Z chromosome per genome.

Tandem repetition of the 2.2-kb non-coding sequence unit in the MHM region

Repetition frequency of the 2.2-kb *Bam*HI unit in the MHM region was quantified by comparing the levels of slot-blot hybridization to different amounts of genomic DNAs from male or female chickens and to different amounts of the genomic clone pCOC10618 (containing eight copies of the 2.2-kb *Bam*HI unit) with the 32 P-labelled pCO10-6 cDNA clone as a probe. It was calculated from these results (data not shown) that the 2.2-kb *Bam*HI unit was repeated about 208 times on the Z chromosome.

The entire cDNA insert (2911 bp) of pCC1-11 and a part (3867 bp) of the 16-kb insert of the genomic clone pCOC10618 from the MHM region were sequenced and deposited with DDBJ/EMBL/GenBank nucleotide sequence databases

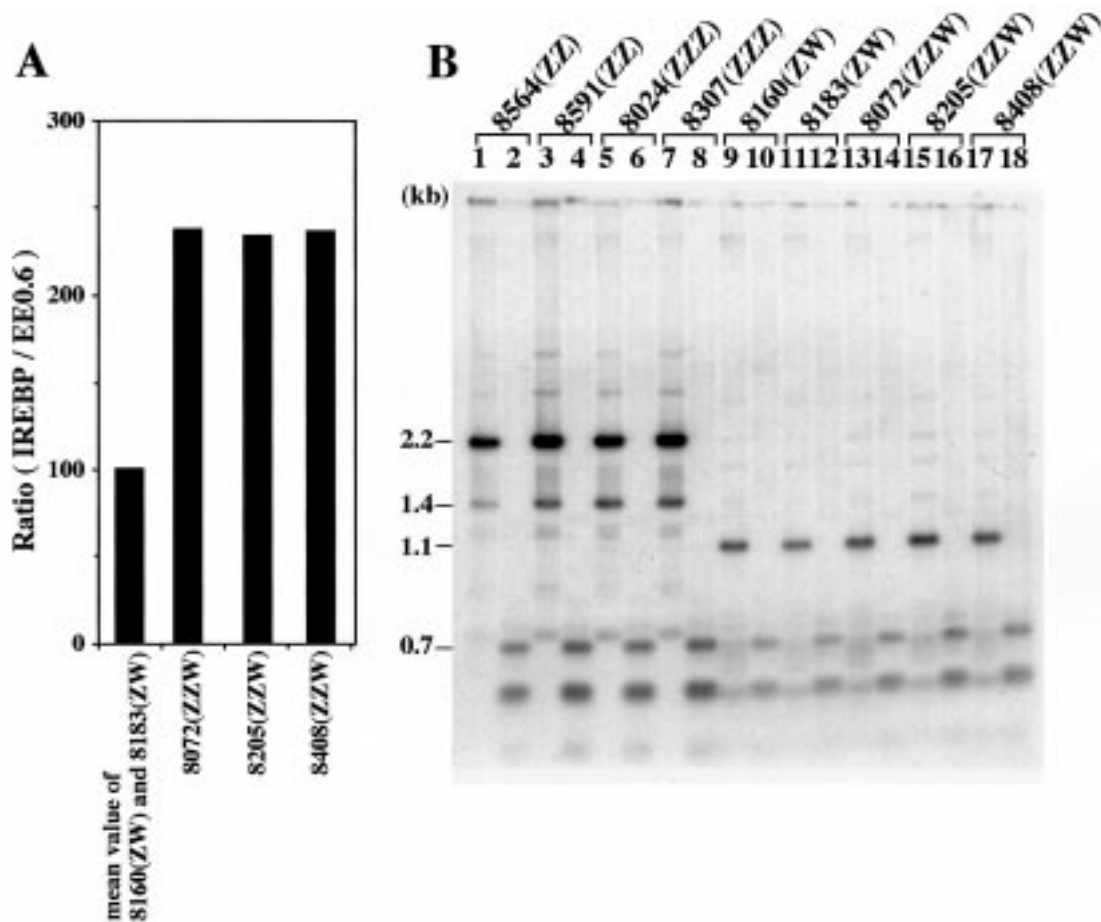


Figure 5. Comparison of the extent of methylation of the MHM region in diploid and triploid chickens. **(A)** The ZW and ZZW constitutions of sex chromosomes were distinguished by relative levels of Southern blot hybridization with the single-copy DNA probes; Z chromosome-specific *IREBP* and the W chromosome-specific *EE0.6*. **(B)** Genomic DNAs from individual diploid or triploid chickens, whose identification number and constitution of sex chromosomes are indicated, were double-digested with *Bam*HI and *Hpa*II (odd numbered lanes) or *Bam*HI and *Msp*I (even numbered lanes) and subjected to Southern blot hybridization with ³²P-labelled pCOC10618. Size markers are as in Figure 3.

under accession numbers AB046698 and AB046699, respectively. The homology plot analysis with these sequences revealed that the 2.2 kb unit was arranged tandemly (data not shown). The entire MHM region was calculated to be about 460 kb long, assuming that the entire region consisted of tandem repeats of the 2.2 kb unit (2.2×208).

When the determined genomic sequence of pCOC10618 and the sequence of the cDNA clone pCC1-11 were compared, these two sequences were nearly identical. Long open reading frames (ORFs) were not present in the cDNA sequence

in either strand or in any reading frame (the longest ORF was 426 bp). These results suggest strongly that the MHM region is transcribed but the transcripts have no protein-coding functions.

Female-specific transcription of the MHM region and repression of transcription in males by CpG methylation

Northern blot hybridization of total RNA preparations from various tissues of male and female chickens with the ³²P-labelled pCO10-6 cDNA

probe indicated that the MHM region is transcribed into heterogeneous and high molecular mass (around 9.5 kb) RNA in all the tissues examined only in the female (Figure 6A). Judging from these results and transcription of the MHM region

in the lampbrush-stage oocytes (Figure 2), it is likely that the MHM region is transcribed throughout female development.

When total RNA preparations from female and male CEFs were subjected to Northern blot

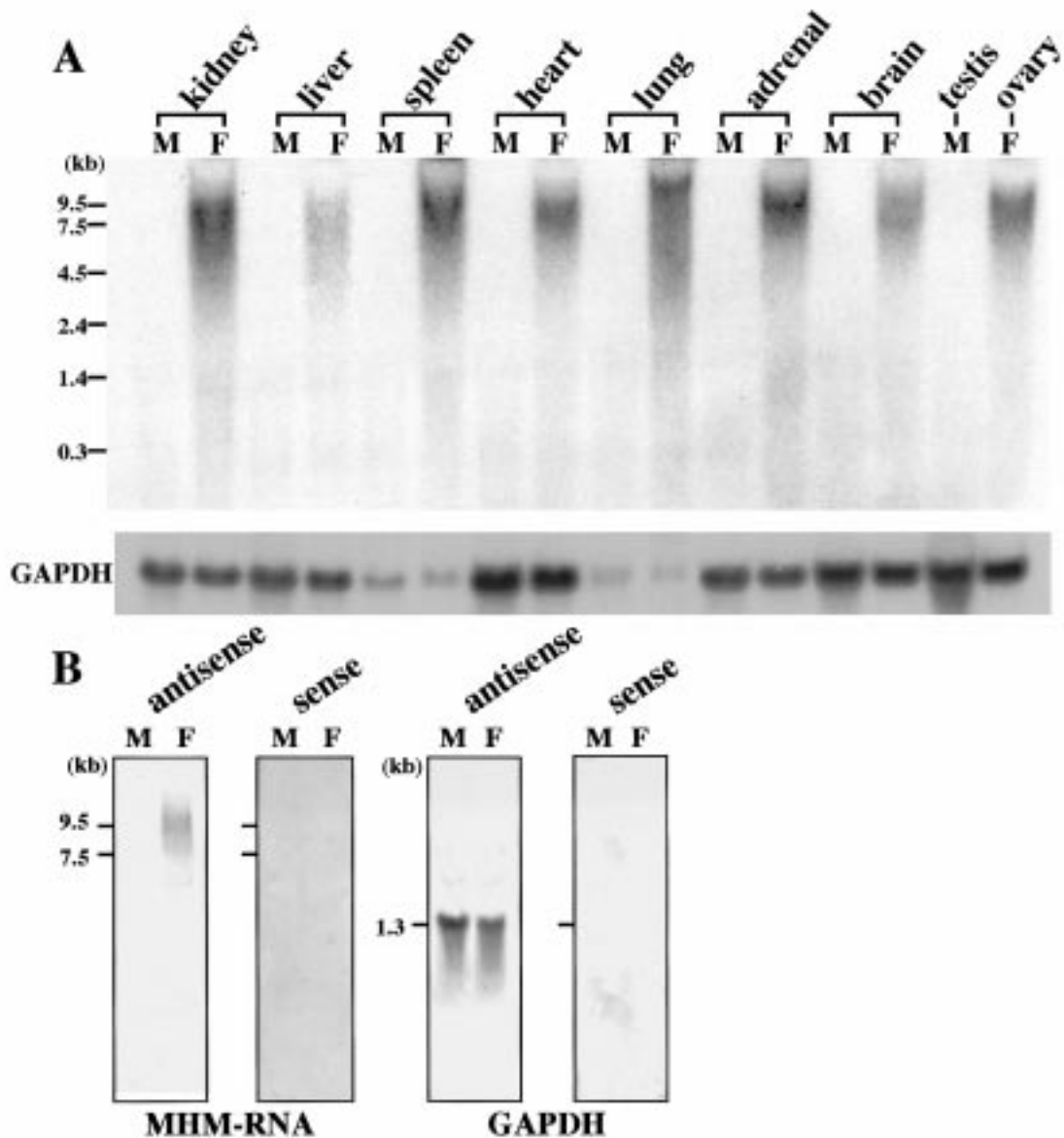


Figure 6. Transcription of high-molecular-mass heterogeneous RNA from the MHM region in female chickens is tissue non-specific but strand-specific. (A) Total RNA preparations from different tissues, as indicated, of a male (M) or a female (F) 40-day-old chicken were subjected to Northern blot hybridization with 32 P-labelled pCO10-6. The same blot was rehybridized with the 32 P-labelled pcGAPDH. Size markers are 0.24–9.5 kb RNA ladder (Gibco BRL). (B) Total RNA preparations from cultured fibroblasts derived from a male (M) or a female (F) chicken embryo were subjected to Northern blot hybridization with the DIG-labelled antisense or sense riboprobe prepared from pCO10-6 (the MHM region cDNA clone) or from pcGAPDH. Hybrids were detected by the reaction with AP-conjugated anti-DIG antibody.

hybridization with the DIG-labelled strand-specific riboprobes for the MHM-region transcripts or for the chicken glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) mRNA, only one of the probes, designated as antisense, hybridized to the high-molecular-mass heterogeneous RNA transcripts from the MHM region only in female-derived cells (Figure 6B). Only the antisense probe hybridized to about 1.3-kb *GAPDH* mRNA from both male and female-derived cells (Figure 6B). The same biotinylated antisense probe for the MHM region, but not the sense probe, also hybridized to a par-

ticular pair of loops on the lampbrush Z chromosome (cf. Figure 2D & E). These results indicate that only one of the strands in the MHM region serves as a template for transcription.

In order to examine whether hypermethylation is responsible for the absence of transcription of the MHM region in the male, cultured fibroblasts from male or female chicken embryos were treated with 5-azacytidine for about three cell generations, and the DNA and RNA extracted were subjected to Southern blot (Figure 7A) and Northern blot (Figure 7B) hybridization, respectively. After

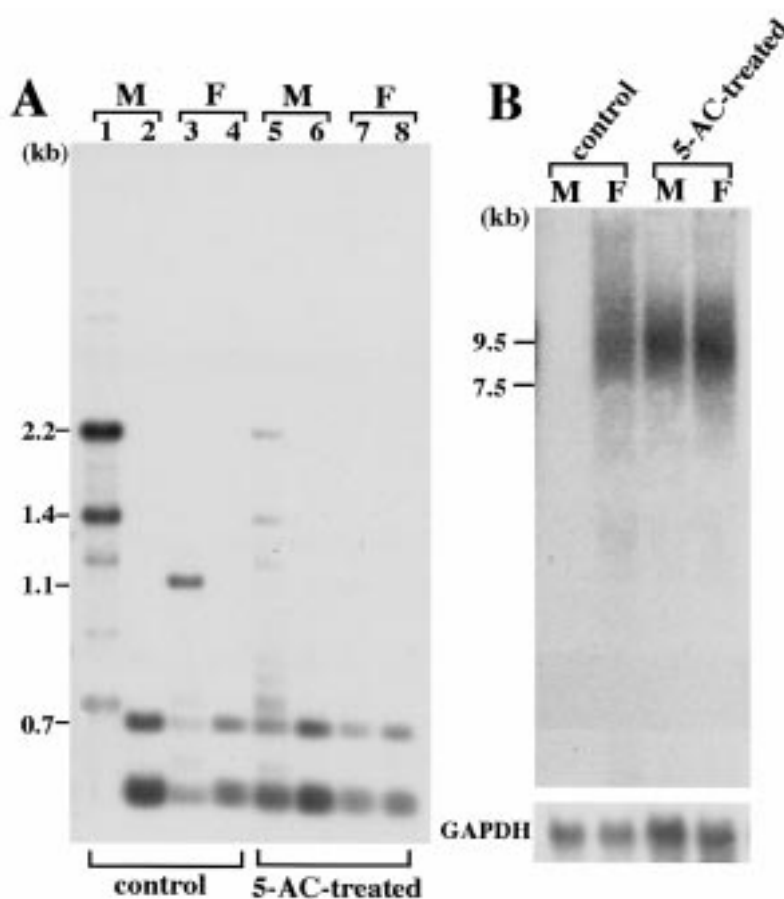


Figure 7. Demethylation and transcriptional activation of the MHM region in cultured fibroblasts derived from a male chicken embryo after treatment with 5-azacytidine. Fibroblasts derived from a male (M) or a female (F) chicken embryo were cultured in the absence (control) or presence of 5-azacytidine (5-AC-treated) for about three cell generations, and then DNA and total RNA were prepared. (A) DNA was double-digested with *Bam*HI and *Hpa*II (odd numbered lanes) or *Bam*HI and *Msp*I (even numbered lanes) and subjected to Southern blot hybridization with 32 P-labelled pCOC10618. (B) Total RNA was subjected to Northern blot hybridization with 32 P-labelled pCO10-6. The same blot was rehybridized with 32 P-labelled pcGAPDH. Note that transcripts from the MHM region were detected in the 5-AC-treated male embryonic fibroblasts.

the treatment with 5-azacytidine, the hypermethylated state of the MHM region in the male was largely lost and the digestion pattern with *Hpa*II became closer to that of *Msp*I (cf. Figure 7A, lanes 1 & 2 vs. 5 & 6). Although the MHM region in the female was hypomethylated originally, more extensive demethylation was effected by the treatment with 5-azacytidine as judged by the digestion patterns with *Hpa*II (cf. Figure 7A lanes 3 & 7). In accordance with the genomic demethylation, the MHM region in the male became transcriptionally active after the 5-azacytidine treatment and produced heterogeneous and high-molecular-mass RNA (Figure 7B, 3rd lane from left) which was similar to that produced from the MHM region in the 5-azacytidine-treated or untreated cells from the female (Figure 7B, 2nd and 4th lanes from left). These results strongly suggest that the absence

of transcription from the MHM region in the male is caused by hypermethylation of the DNA sequence in this region.

RNA transcripts from the MHM region accumulate in the nucleus and the majority of them are poly(A)⁻ RNA

Slot-blot hybridization to total RNA, cytoplasmic RNA or nuclear RNA, prepared from the female chicken liver, with ³²P-labelled MHM region cDNA probe or ³²P-labelled *GAPDH* cDNA probe, demonstrated that transcripts from the MHM region were localized in nuclei, whereas the majority of *GAPDH* transcripts were present in cytoplasm (Figure 8A).

When total RNA from the lung of the female chicken was fractionated into poly(A)⁺ and poly(A)⁻ RNA components, and these RNA pre-

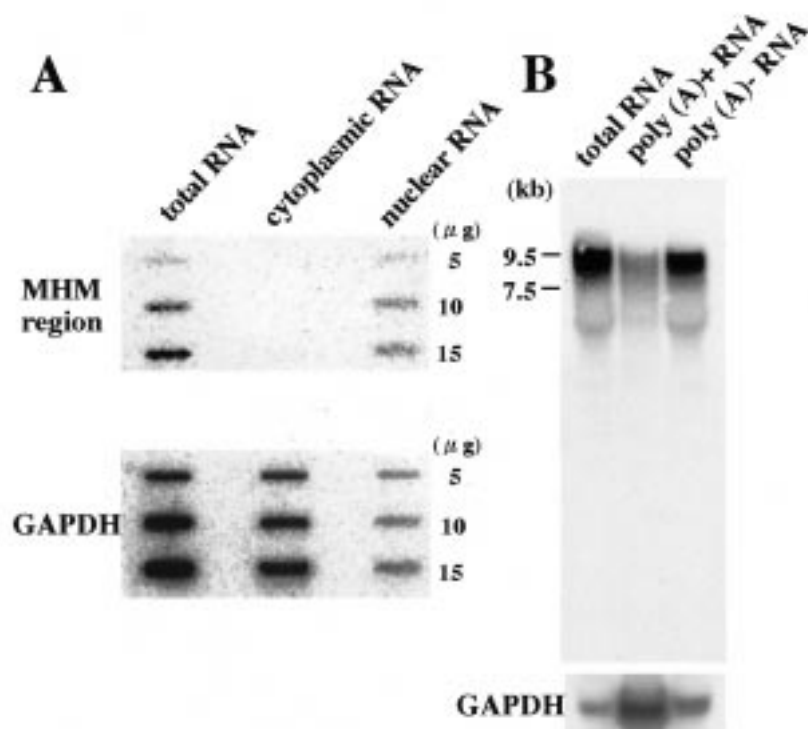


Figure 8. RNA transcripts from the MHM region are localized in nuclei and the majority of them are poly(A)⁻ RNA. (A) Total RNA, cytoplasmic RNA or nuclear RNA prepared from the female chicken liver was subjected to slot-blot hybridization with ³²P-labelled pCO10-6, followed by rehybridization with ³²P-labelled pcGAPDH. (B) Total RNA (20 μg/lane), poly(A)⁺ RNA (7.5 μg/lane) or poly(A)⁻ RNA (20 μg/lane) prepared from the female chicken lung was subjected to Northern blot hybridization with probes as in (A). The ratio of signal-intensity values; poly(A)⁺ RNA/total RNA, was 0.7 for transcripts from the MHM region, whereas the ratio was 7.2 for the *GAPDH* mRNA.

parations were subjected to Northern blot hybridization with ^{32}P -labelled probes as above, the ratio of signal intensity values for poly(A)⁺ RNA/total RNA was 7.2 for the *GAPDH* mRNA but was 0.7 for the MHM-region transcripts (Figure 8B). These results indicated that the majority of the MHM region transcripts were poly(A)⁻ RNA.

Accumulation of the MHM region transcripts in a nucleus was visualized by *in-situ* hybridization to paraffin sections of kidneys from male or female, diploid or triploid chickens, with DIG-labelled antisense or sense riboprobe. A single spot of hybridization was detected with the antisense probe in the diploid (ZW) nuclei (Figure 9A)

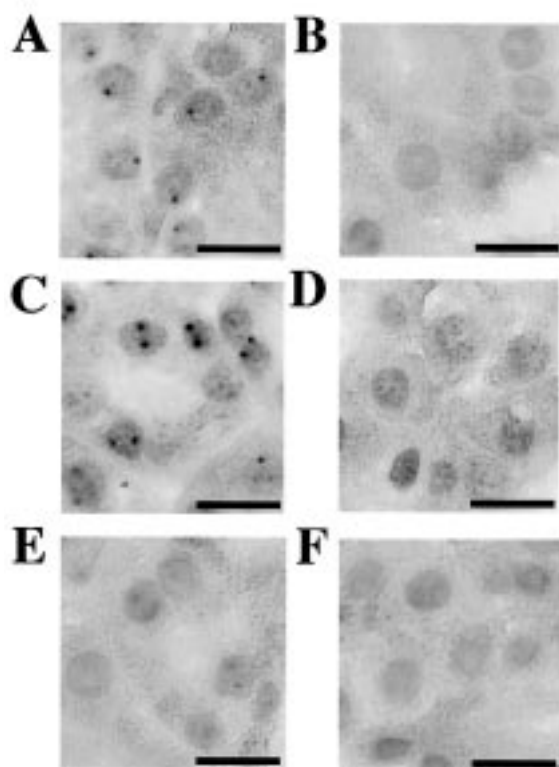


Figure 9. *In-situ* hybridization with riboprobes to paraffin sections of the chicken kidney, demonstrating that RNA transcripts from the MHM region accumulate as a single spot in the ZW diploid nucleus and double spots in the ZZW triploid nucleus. Sections were hybridized with DIG-labelled antisense (A, C, E, F) or sense (B, D) riboprobe prepared from the pCO10-6 cDNA clone and counterstained with haematoxylin. Genotypes of the nuclei are: ZW (A, B), ZZW (C, D), ZZ (E) and ZZZ (F). Bars indicate 10 μm .

and two spots of hybridization were detected in the triploid (ZZW) nuclei of the intersex female (Figure 9C). The antisense probe did not hybridize to the diploid (ZZ) and the triploid (ZZZ) nuclei of the male (Figure 9E, F). The sense riboprobe for the MHM region did not show any signal of hybridization in nuclei of the diploid female (ZW) or triploid intersex female (ZZW) chickens (Figure 9B, D). These results together with the results shown in Figure 5 indicate that hypomethylation and transcriptional activation of the MHM region are well correlated: the MHM region on the extra Z chromosome in the triploid (ZZZ) male is hypermethylated and transcriptionally inactive, whereas the MHM region on the extra Z chromosome in the triploid (ZZW) intersex female is hypomethylated and transcriptionally active. Moreover, the number of spots of hybridization in diploid (ZW) and triploid (ZZW) nuclei implied that the transcripts were accumulated at or in the vicinity of the MHM locus on the Z chromosome.

The MHM region is located very close to the DMRT1 locus on the Z chromosome

Recently, the gene locus for the chicken homologue of *DMRT1* was mapped by FISH to the middle of the short arm of the Z chromosome (Nanda *et al.* 1999). It has been suggested that the human *DMRT1* gene on chromosome 9 is involved in the differentiation of the male gonad because its mutation appears to cause XY sex reversal (Raymond *et al.* 1999a). We found by FISH that the MHM region and the *DMRT1* locus were located indistinguishably close both on the mitotic Z chromosome (Figure 10A) and the lampbrush Z chromosome (Figure 10B) of chickens.

Because transcription of the MHM region was female specific (Figure 6), the transcription of the nearby *DMRT1* gene was compared in males and females. Northern blot hybridization of the ^{32}P -labelled cDNA probe for the *DMRT1* transcripts to the total RNA preparations from 9–19-day embryonic gonads or from the testis or ovary of the post-hatched (2-, 40- or 200-day old) chickens, demonstrated that the level of transcription seemed to be dosage dependent, i.e. about 2 (male): 1 (female) in embryos as reported earlier (Raymonds *et al.* 1999b), but the expression

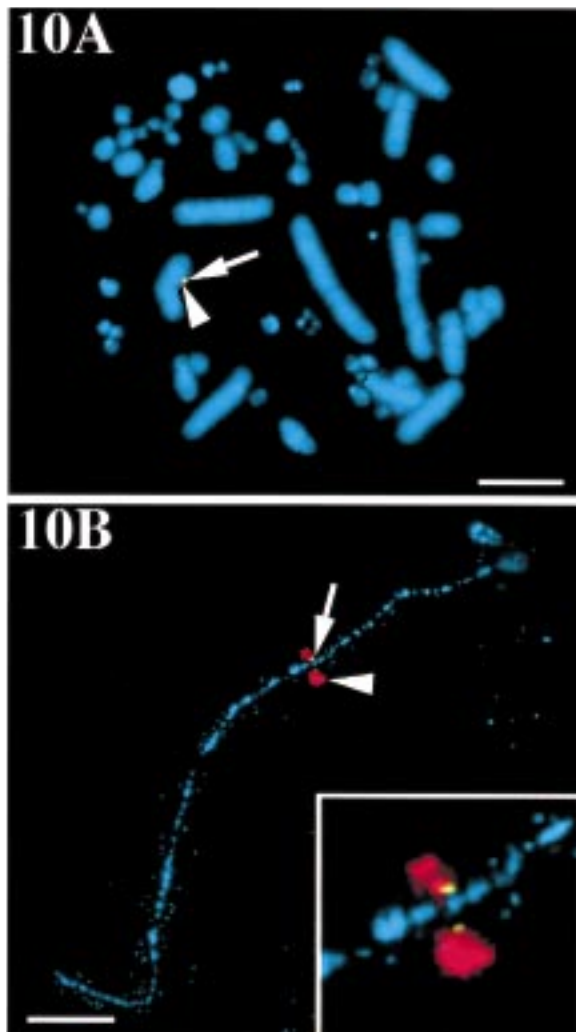


Figure 10. The MHM region and the *DMRT1* gene locus are colocalized by FISH on mitotic and lampbrush chromosomes of chickens. **(A)** FISH to a metaphase set prepared from a female-chicken embryonic fibroblast. The MHM region (rhodamine fluorescence; arrowhead) was hybridized with DIG-labelled pCOC10618, and the *DMRT1* gene locus (FITC fluorescence; arrow) was hybridized with the biotinylated BAC clone containing *DMRT1* gene. **(B)** FISH to a lampbrush ZW bivalent from a chicken oocyte under the conditions where probes hybridize to both DNA and RNA. The MHM region (arrowhead) and the *DMRT1* gene locus (arrow) were detected as in **(A)**. Bars indicate 10 μ m.

in the ovary became extremely low (in 2- or 40-day-old chickens) or undetectable (in 200-day-old chicken), in contrast to the continued expression in the testis, after hatching (data not shown). Thus, after hatching, the transcriptional

states of the adjacent loci (MHM region versus *DMRT1*) exhibit an inverse relationship, depending upon the sex of the chickens.

Transcripts of the MHM region accumulate in close contact with the DMRT1 locus in the nucleus

In nuclei of female chicken embryonic fibroblasts, the MHM region on the Z chromosome, as shown by FISH with the biotin-labelled pCOC10618 genomic probe, and transcripts from the MHM region, as shown by RNA FISH with the DIG-labelled antisense riboprobe, were shown to be almost colocalized (Figure 11A), which supported the notion (Figure 9) that the transcripts from the MHM region were accumulated at or very near to the site of transcription. Similar experiments demonstrated that transcripts from the MHM region accumulated adjacent to the *DMRT1* gene locus in the nucleus (Figure 11B), and the distance between them was indistinguishable from that between the site of accumulation of the MHM transcripts and the genomic MHM region itself (cf. Figure 11A and B).

Discussion

Unique features of the MHM region: comparison with genomic imprinting and X chromosome inactivation in mammals

Considering that the state of methylation of the MHM region on the Z chromosome is established after fertilization under the influence of the absence or presence of W chromosome, the MHM phenomenon is different from that of the genomic imprinting in mammals because the allele-specific differential CpG methylation, which is the probable basis of genomic imprinting, is erased in early stages of germ cell development but is re-established later in gametogenesis, and the essential patterns of CpG methylation related to genomic imprinting are maintained after fertilization (Constância *et al.* 1998).

The phenomenon of X chromosome inactivation in female mammals is also associated with CpG methylation. The ~35-kb X-inactivation centre (*Xic*) on the X chromosome contains an

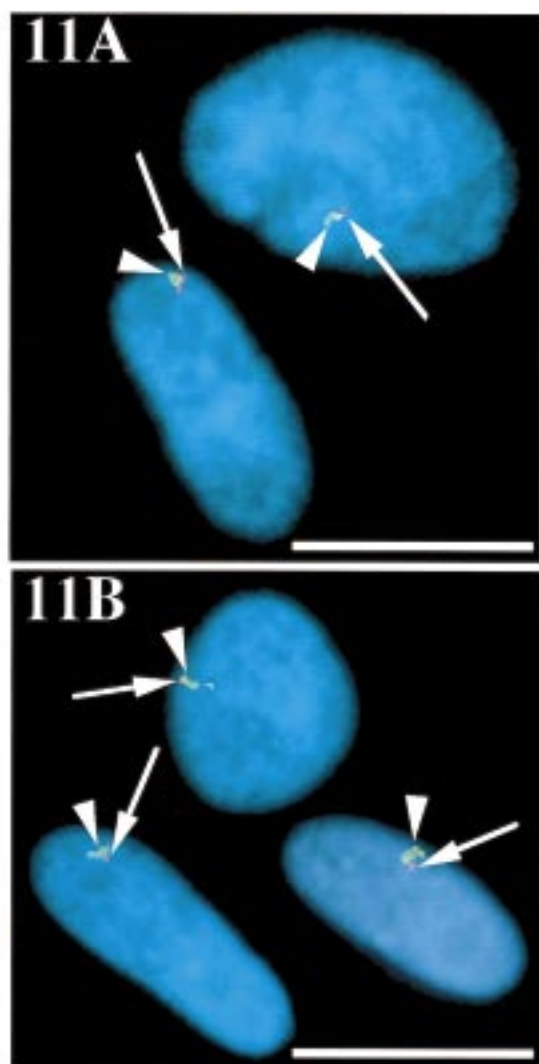


Figure 11. Transcripts from the MHM region accumulate at, or in close proximity to, the site of transcription and are located adjacent to the *DMRT1* gene locus in the nucleus of a female chicken embryonic fibroblast. **(A)** FISH to RNA transcripts from the MHM region (FITC fluorescence, green; arrowhead) with the DIG-labelled antisense riboprobe and to the MHM region DNA sequence (rhodamine fluorescence, red; arrow) with biotinylated pCOC10618. **(B)** FISH to RNA transcripts from the MHM region (FITC fluorescence; arrowhead) with the same probe as above and to the *DMRT1* gene sequence (rhodamine fluorescence; arrow) with the biotinylated BAC clone containing the chicken *DMRT1* gene. Nuclei were counterstained with DAPI. Bars indicate 10 μm .

Xist gene. The *Xist* gene on the inactive X chromosome is unmethylated, whereas the gene on the active X chromosome is CpG methylated. The unmethylated *Xist* gene is transcribed into *Xist*

RNA, which is heterogeneous in size and large (around 10 kb) nuclear RNA and does not contain ORFs larger than 300 nucleotides, like the MHM transcripts, but *Xist* RNA is spliced and polyadenylated. The transcription of *Xist* gene is activated and/or *Xist* RNA is stabilized by the factors expressed in male cells during embryogenesis and *Xist* RNA is spread in *cis* to coat the inactive X chromosome (Brown *et al.* 1991, Panning & Jaenisch 1998, Brockdorff 1998, Wutz & Jaenisch 2000).

MHM transcripts are also accumulated in the nucleus, but at, or very near to, the site of transcription. Hypermethylation of the MHM region takes place on both Z chromosomes in male cells during early development and the MHM regions on both Z chromosomes remain transcriptionally inactive. In the female, the MHM region is actively transcribed on the single Z chromosome. Thus, the situation is quite different from that in mammals where *Xist* RNA is produced from the inactive X chromosome; that is, one of the two X chromosomes in the female, whereas MHM RNA is produced from the single and largely active Z chromosome in the avian female.

The present results, demonstrating that the transcription of the MHM region in male CEFs became detectable after treatment with 5-azacytidine (Figure 7B) suggest strongly that the repression of transcription of the MHM region in male cells is caused by the hypermethylation of this region, although we could not completely deny a possibility that MHM transcripts in male cells are extremely unstable for some unknown reason and that they cannot be detected as a steady-state population. The sense riboprobe from the MHM region did not detect any hybridizable transcripts either in female or male cells (Figures 2E, 6B & 9). Thus, an antisense transcript like *Tsix* transcript in mammals, which causes either blocking of the function or repression of the expression of *Xist* gene at the X inactivation centre (Lee *et al.* 1999), does not seem to be involved in the MHM phenomenon.

Biological implication of the MHM phenomenon: a hypothesis

We have observed the MHM phenomenon in all the species that we have examined which belong

to the order Galliformes and in both diploid and triploid chickens. The results obtained with triploid chickens suggest that the MHM state is not simply determined by the number of Z chromosomes per genome but is more likely to be affected by the absence or presence of the W chromosome. The role of the W chromosome in this phenomenon is intriguing but, as yet, unknown. We propose the following hypothesis with regard to the biological significance of the MHM phenomenon.

An unknown gene (gene F) on the W chromosome is expressed early in embryogenesis in the female. Its translation product induces demethylation of the MHM region on the Z chromosome derived from the sperm and the MHM region becomes transcriptionally active, or the product of gene F induces transcription of the MHM region and consequently the region becomes hypomethylated. The non-coding high-molecular-mass RNA molecules (MHM RNA) transcribed from the MHM region accumulate at the site of transcription and remain near to that in the nucleus. The accumulation of MHM RNA may cause, to some extent, on the analogy of the *Xist* RNA, repression of the transcription of nearby gene(s), whose expression is disadvantageous to female differentiation. In the male, the MHM region is hypermethylated and transcriptionally inactive, and thus no such repressor RNA is formed and nearby gene(s) whose expression is advantageous to male differentiation is (are) actively transcribed.

The colocalization of the *DMRT1* gene with the MHM region, at the resolution of FISH, fits this hypothesis. The human *DMRT1* gene is mapped to 9p24.3. A gene whose mutations are responsible for a high incidence of XY feminization also maps to this locus, and the *DMRT1* gene has been suggested as a likely candidate (Raymond *et al.* 1999a). The deduced sequence of *DMRT1* contains a DNA-binding motif, called the DM domain, whose sequence is similar to those of the corresponding motifs in the products of *doublesex* (*dsx*; the sexual regulatory gene) of *Drosophila melanogaster* and *mab-3* (the male sexual regulatory gene) of *Caenorhabditis elegans* (Raymond *et al.* 1998). In human adults, the *DMRT1* gene is expressed only in the testis, but in early mouse embryos, its mRNA is detected

in the genital ridge of both male and female (Raymond *et al.* 1999b).

The chicken homologue of *DMRT1* is located on the Z chromosome. Its expression patterns are similar to those of mammalian *DMRT1* in that its mRNA is detected in the genital ridge of both male and female embryos but the expression level is approximately twice as high in the male as in the female (Raymond *et al.* 1999b, and this study). After hatching, the expression of *DMRT1* is detected mainly in the testis and to some extent in the ovary, but, later, the expression takes place almost exclusively in the testis (this study). If, as suggested in mammals, *DMRT1* is involved in the early differentiation of male gonads in birds, its about two-times higher level of expression in the embryonic gonads in the male may be important for its role. The expression of MHM-RNA in female embryonic gonads does not seem to be sufficient to repress the transcription of the *DMRT1* gene, probably due to the presence of excess amounts of positive transcriptional factors. On the other hand, if the continued expression of *DMRT1* in the male gonad and the nearly complete repression of the *DMRT1* gene in the female gonad after hatching are biologically important, then the local accumulation of MHM RNA and its possible interference in the transcriptional or post-transcriptional processes of the adjacent *DMRT1* gene may be regarded as one of the factors leading to the extreme and stable repression of the *DMRT1* gene in the female.

Acknowledgements

This work was supported by Grant-in-Aid for International Scientific Research (Joint Research) Grant, No.10044194, to S. Mizuno from the Ministry of Education, Science, Sports and Culture, Japan.

References

- Barber DL, Sanders EJ, Aebersold R, Schneider WJ (1991) The receptor for yolk lipoprotein deposition in the chicken oocyte. *J Biol Chem* **266**: 18761–18770.
- Brockdorff N (1998) The role of *Xist* in X-inactivation. *Curr Opin Genet Devel* **8**: 328–333.

- Brockdorff N, Ashworth A, Kay GF *et al.* (1992) The product of the mouse *Xist* gene is a 15 kb inactive X-specific transcript containing no conserved ORF and located in the nucleus. *Cell* **71**: 515–526.
- Brown CJ, Ballabio A, Rupert JL *et al.* (1991) A gene from the region of the human X inactivation centre is expressed exclusively from the inactive X chromosome. *Nature* **349**: 38–44.
- Carter KC, Taneja KL, Lawrence JB (1991) Discrete nuclear domains of poly(A) RNA and their relationship to the functional organization of the nucleus. *J Cell Biol* **115**: 1191–1202.
- Constância M, Pickard B, Kelsey G, Reik W (1998) Imprinting mechanisms. *Genome Res* **8**: 881–900.
- Dugaiczky A, Haron JA, Stone EM, Dennison OE, Rothblum KN, Schwartz RJ (1983) Cloning and sequencing of a deoxyribonucleic acid copy of glyceraldehyde-3-phosphate dehydrogenase messenger ribonucleic acid isolated from chicken muscle. *Biochemistry* **22**: 1605–1613.
- Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* **132**: 6–13.
- Gubbay J, Collignon J, Koopman P *et al.* (1990) A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes. *Nature* **346**: 245–250.
- Hori T, Asakawa S, Itoh Y, Shimizu N, Mizuno S (2000) *Wpkci*, encoding an altered form of *PKCI*, is conserved widely on the avian W chromosome and expressed in early female embryos: Implication of its role in the female sex determination. *Mol Biol Cell* **11**: 3645–3660.
- Kodama H, Saitoh H, Tone M, Kuhara S, Sakaki Y, Mizuno S (1987) Nucleotide sequences and unusual electrophoretic behavior of the W chromosome-specific repeating DNA units of the domestic fowl, *Gallus gallus domesticus*. *Chromosoma* **96**: 18–25.
- Kunita R, Nakabayashi O, Kikuchi T, Mizuno S (1997) Predominant expression of a Z-chromosome-linked immunoglobulin superfamily gene, *ZOV3*, in steroidogenic cells of ovarian follicles and in embryonic gonads of chickens. *Differentiation* **62**: 63–70.
- Lee JT, Davidow LS, Warshawsky D (1999) *Tsix*, a gene antisense to *Xist* at the X-inactivation centre. *Nature Genet* **21**: 400–404.
- Mizuno S, Whiteley HR, Whiteley AH (1973) The enrichment of egg-type RNA in cleavage stage embryos of the sand dollar *Dendraster excentricus*. *Differentiation* **1**: 339–348.
- Mizuno S, Tallman NA, Cox RF (1978) Estrogen withdrawal in chick oviduct. Characterization of RNA synthesized in isolated nuclei using a mercurated precursor. *Biochim Biophys Acta* **520**: 184–202.
- Nakabayashi O, Kikuchi H, Kikuchi T, Mizuno S (1998) Differential expression of genes for aromatase and estrogen receptor during the gonadal development in chicken embryos. *J Mol Endocrinol* **20**: 193–202.
- Nanda I, Shan Z, Schartl M *et al.* (1999) 300 million years of conserved synteny between chicken Z and human chromosome 9. *Nature Genet* **21**: 258–259.
- Nanda I, Zend-Ajusich E, Shan Z *et al.* (2000) Conserved synteny between the chicken Z sex chromosome and human chromosome 9 includes the male regulatory gene *DMRT1*: a comparative (re)view on avian sex determination. *Cytogenet Cell Genet* **89**: 67–78.
- Ogawa A, Solovei I, Hutchison N *et al.* (1997) Molecular characterization and cytological mapping of a non-repetitive DNA sequence region from the W chromosome of chicken and its use as a universal probe for sexing Carinatae birds. *Chromosome Res* **5**: 93–101.
- O'Neill M, Binder M, Smith C *et al.* (2000) *ASW*, a gene with conserved avian W-linkage and female specific expression in chick embryonic gonad. *Dev Genes Evol* **210**: 243–249.
- Panning B, Jaenisch R (1998) RNA and the epigenetic regulation of X chromosome inactivation. *Cell* **93**: 305–308.
- Raymond CS, Shamu CE, Shen MM *et al.* (1998) Evidence for evolutionary conservation of sex-determining genes. *Nature* **391**: 691–695.
- Raymond CS, Parker ED, Kettlewell JR *et al.* (1999a) A region of human chromosome 9p required for testis development contains two genes related to known sexual regulators. *Hum Mol Genet* **8**: 989–996.
- Raymond CS, Kettlewell JR, Hirsch B, Bardwell VJ, Zarkower D (1999b) Expression of *Dmrt1* in the genital ridge of mouse and chicken embryos suggests a role in vertebrate sexual development. *Dev Biol* **215**: 208–220.
- Saitoh Y, Mizuno S (1992) Distribution of *XhoI* and *EcoRI* family repetitive DNA sequences into separate domains in the chicken W chromosome. *Chromosoma* **101**: 474–477.
- Saitoh Y, Ogawa A, Hori T, Kunita R, Mizuno S (1993) Identification and localization of two genes on the chicken Z chromosome: implication of evolutionary conservation of the Z chromosome among avian species. *Chromosome Res* **1**: 239–251.
- Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning. A Laboratory Manual*, 2nd edn. Cold Spring Harbor laboratory Press. pp 7.10–7.11, 7.19–7.22, 7.43–7.45.
- Sinclair AH, Berta P, Palmer MS *et al.* (1990) A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. *Nature* **346**: 240–244.
- Solovei I, Gaginskaya E, Hutchison N, Macgregor H (1993) Avian sex chromosomes in the lampbrush form: ZW lampbrush bivalents from six species of bird. *Chromosome Res* **1**: 153–166.
- Solovei I, Ogawa A, Naito M, Mizuno S, Macgregor H (1998) Specific chromomeres on the chicken W lampbrush chromosome contain specific repetitive DNA sequence families. *Chromosome Res* **6**: 323–327.
- Thorne MH, Sheldon BL (1991) Cytological evidence of maternal meiotic errors in a line of chickens with a high incidence of triploidy. *Cytogenet Cell Genet* **57**: 206–210.
- Thorne MH, Collins RK, Sheldon BL (1987) Live haploid-diploid and other unusual mosaic chickens (*Gallus domesticus*). *Cytogenet Cell Genet* **45**: 21–25.
- Thorne MH, Nicholas FW, Moran C, Sheldon BL (1997) Genetic analysis of triploidy in a selected line of chickens. *J Hered* **88**: 495–498.
- Wutz A, Jaenisch R (2000) A shift from reversible to irreversible X inactivation is triggered during ES cell differentiation. *Mol Cell* **5**: 695–705.