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The long non-coding RNA, *MHM*, plays a role in chicken embryonic development, including gonadogenesis

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ABSTRACT

MHM is a chicken Z chromosome-linked locus that is methylated and transcriptionally silent in male cells, but is hypomethylated and transcribed into a long non-coding RNA in female cells. *MHM* has been implicated in both localised dosage compensation and sex determination in the chicken embryo, but direct evidence is lacking. We investigated the potential role of *MHM* in chicken embryonic development, using expression analysis and retroviral-mediated mis-expression. At embryonic stages, *MHM* is only expressed in females. Northern blotting showed that both sense and antisense strands of the *MHM* locus are transcribed, with the sense strand being more abundant. Whole mount *in situ* hybridization confirmed that the sense RNA is present in developing female embryos, notably in gonads, limbs, heart, branchial arch and brain. Within these cells, the *MHM* RNA is localized to the nucleus. The antisense transcript is lowly expressed and has a cytoplasmic localization in cells. Mis-expression of *MHM sense* and *antisense* sequences results in overgrowth of tissues in which transcripts are predominantly expressed. This includes altered asymmetric ovarian development in females. In males, *MHM* mis-expression impairs gonadal expression of the testis gene, *DMRT*1. Both *MHM* sense and antisense mis-expression cause brain abnormalities, while *MHM sense* causes an increase in male-biased embryo mortality. These results indicate that *MHM* has a role in chicken normal embryonic development, including gonadal sex differentiation.

Introduction

The chicken embryo has a ZZ male: ZW female sex chromosome system. The Z-linked gene, *DMRT*1, is required for testis development during embryonic development and is hypothesized to operate via gene dosage, with males (ZZ) having two doses and females (ZW) having one (reviewed in Chue and Smith, 2011). Z-linked *DMRT*1 is more highly expressed in testes *versus* ovaries, and knockdown of *DMRT*1 expression leads to feminization of male gonads (Smith et al., 2009). It is therefore postulated that *DMRT*1 is a key gene underlying sex determination in the avian system. The higher expression of *DMRT*1 in male tissues (ZZ) suggests that the gene escapes a dosage compensation mechanism. Indeed, birds lack a chromosome wide Z-inactivation system analogous to X chromosome inactivation in mammals (Baverstock et al., 1982; Kuroiwa et al., 2002; Ellegren, 2007; Itoh et al., 2007). On average, most Z-linked genes show mRNA expression levels that are greater than 1.5 fold higher in males (ZZ) compared to females (ZW), as assessed by microarray and individual gene expression studies (Kuroda et al., 2001; McQueen et al., 2001; Itoh et al., 2007; Zhang et al., 2010) reviewed in Arnold et al. (2008). However, there are regions across the Z chromosome that are compensated, or partially compensated (McQueen et al., 2001; McQueen and Clinton, 2009). These regions may harbor genes that are developmentally important and for which dosage inequality between the sexes cannot be tolerated.

A specific cluster of compensated genes has been reported on chicken Zp (Melamed and Arnold, 2007). Within this region is a gene called *MHM* (Male HyperMethylated). *MHM* is hypermethylated on the two Z sex chromosomes of male chickens and is transcriptionally silent. Treatment of male cells with the demethylating agent, 5-aza-cytidine, alters chromatin configuration and allows *MHM* expression in male cells (Itoh et al., 2011). In female cells (ZW), the *MHM* locus is hypomethylated on the single Z chromosome and is transcribed into a long non-coding

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RNA that coats the Z chromosome at the site of transcription (Teranishi et al., 2001; Itoh et al., 2011). The *MHM* sequence may mediate the partial dosage compensation seen on the chicken Z chromosome, either locally or more widely. In mammals, X inactivation is regulated by the X-linked gene, *Xist* (Leeb et al., 2009). While not structurally related to *Xist*, *MHM* shows similar features, encoding a high molecular weight non-coding RNA which coats one of the sex chromosomes in cis. It has therefore been speculated that *MHM* could specifically mediate dosage compensation of the Zp gene cluster in chicken, dubbed the "*MHM* valley", by modifying local chromatin organization (Arnold et al., 2008; Teranishi et al., 2001). However, whereas *Xist* is associated with X inactivation, *MHM* is associated with an active Z sex chromosome. Within this same region on the female Z sex chromosome, histone 4, lysine 16 is acetylated (Bisoni et al., 2005). In other dosage compensation systems, histone acetylation is associated with up-regulation of neighbouring genes (Bone et al., 1994; Smith et al., 2005). A causal relationship between MHM and H_4k_{16} acetylation is unclear. However, taken together, these observations have led to the hypothesis that *MHM* plays a role in dosage compensation by altering gene expression in females (ZW). It may do so by up-regulating a subset of genes on the female Z chromosome (those in the "MHM valley") to equalise dosage with that of males (ZZ), via a process that involves local female-specific hyperacetylation of histone H_4k_{16}. The *DMRT1* locus is adjacent to the MHM valley, and it has also been hypothesized that MHM RNA could function to repress *DMRT1* expression in female embryos, thereby contributing to sex determination in the chicken (Teranishi et al., 2001; Yang et al., 2010). To further explore the role of *MHM* in chicken embryonic development, we carried out detailed expression and mis-expression studies. We find that RNA transcription occurs from both the sense and antisense strands of the *MHM* locus and that these strands have different temporal and spatial expression patterns during embryonic development. Furthermore, mis-expression of these sequences causes embryonic abnormalities of the brain and gonads, consistent with a role in both dosage compensation and sex determination in the chicken embryo.

Methods

Cloning of the chicken 2.2 kb MHM repeat unit

The Chicken (*Gallus gallus*) *MHM* locus, first described by Teranishi et al. (2001), comprises a core *Bam*HI- 2.2 kb repeat that has at least 200 copies on the short arm of the Z sex chromosome. The transcript itself was estimated at 9 kb. Using this sequence data, we designed primers to amplify the 2.2 MHM sequence as described by Teranishi et al. (2001) (Genbank AB046698). A 2.2 kb PCR product obtained from chicken genomic DNA was cloned into pSLAX shuttle vector and its identity confirmed by automated sequencing. This sequence was then subcloned into the RCASBP(A) avian retroviral vector for mis-expression studies (Logan and Tabin, 1998).

Northern blot analysis

A 668 base pair *MHM* riboprobe, derived from the 2.2 kb sequence, was used for Northern blotting. Total RNA was isolated from several embryonic day 10.5 male and female gonad, limb and brain samples and approximately 10 μg of each were run on a 1.1% agarose/formaldehyde gel, together with RNA size standards (Promega Corp, Madison, WI, USA). RNA was transferred to a Hybond N membrane (Amersham Biosciences, UK) by capillary transfer and baked at 80 °C for two hours. Membranes were prehybridized with Ultrahyb (Ambion, Austin, Texas, USA) for one hour at 68 °C and then probed with 100 ng DIG-labelled probe overnight at 68 °C. *MHM sense* transcripts were detected with the 668 bp *MHM* antisense riboprobe, synthesized with SP6 RNA polymerase, while the *MHM-antisense* strand was detected on a separate membrane, using the sense probe (T7 RNA polymerase). The next day, membranes were washed to a stringency of 0.1 × standard saline citrate (SSC) and 0.1% sodium dodecyl sulfate (SDS) at 68 °C. Bound DIG-labelled riboprobe was detected using an anti-DIG antibody (Roche, Mannhein, Germany). Chemiluminescent signal generated by CDP-Star (Roche) substrate was detected by exposure of membranes to Kodak Hyperfilm (Amersham Biosciences, UK). Northern blots were performed twice. Chemiluminescent exposure time for the sense transcript was 1 min, while exposure for the antisense transcript was over 90 min.

Whole mount in situ hybridization

One hundred fertile chicken eggs were incubated at 37.8 °C and embryos were harvested throughout development. The whole mount *in situ* hybridization method (WISH) has been described in detail previously (Andrews et al., 1997; Smith et al., 1999). Embryos were sexed by PCR as described previously (Clinton et al., 2001), prior to manipulation of tissues for expression analysis. At least three embryos were used for each analysed time point. Briefly, tissues were dissected under RNase-free conditions and fixed overnight at 4 °C in 4% paraformaldehyde/PBS. Whole embryos (early stages) or isolated tissues were taken. Following fixation, tissues were dehydrated through a methanol series, and stored at −20 °C prior to rehydration and digestion with proteinase-K in PBS+0.1% Triton-X 100 (30 to 90 min, depending upon the size of tissues). Samples were then briefly re-fixed, and incubated overnight at 65 °C in prehybridisation buffer. Two different RNA probes were used in experiments that were repeated at least twice. In the first set of experiments, the same 668 bp fragment used for Northern blotting was used for WISH. The antisense probe (detecting *MHM-sense*) was synthesized using SP6 RNA polymerase and labeled with digoxygenin (DIG). The DIG "sense" probe (detecting MHM-antisense) was synthesized with T7 RNA polymerase. In the second experiment, the entire 2.2 kb repeat unit was used as a riboprobe. Probes were added to tissues overnight in hybridsation solution, washed at 1 × and 0.1 × strigency, and alkaline-phosphatase conjugated DIG antibody was added, following pre-blocking in TBTX buffer (pH 7.0) containing BSA and sheep serum. Tissues were then extensively washed in TBTX+0.1% BSA, and the chromagen was then added (NBT/BCIP in Tris-based buffer, pH 9.0). Color reactions were carried out at room temperature over 2–4 h, prior to imaging. For sectioning, tissues were placed back into chromogen solution and incubated at room temperature over two days, then cryoprotected by placing into 30% sucrose/PBS overnight at 4 °C. Following embedding in OCT compound and snap freezing on dry ice, tissue samples were cut on a cryostat at 10–18 μm and mounted onto slides with Aquamount. In some cases, tissues were treated with DAPI prior to mounting, allowing the visualization of RNA signals relative to the nucleus, followed by false coloring of the RNA signal against DAPI blue immunoflourescence.

Mis-expression of 2.2 kb MHM in ovo

To assess the effects on mis-expressing *MHM*, the RCAS avian viral vector was used (Fekete and Cepko, 1993; Logan and Tabin, 1998) (Smith et al., 2009). The 2.2 kb *Bam*HI MHM unit (Genbank AB046698) was subcloned from pSLAX shuttle plasmid into RCASBP/A proviral DNA and its sequence confirmed. The viral DNA was transfected into chicken fibroblastic DF1 cells and