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Female-specific hyperacetylation of histone H4 in the chicken Z chromosome

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Abstract

Birds undergo genetic sex determination using a ZW sex chromosome system. Although the avian mechanisms of neither sex determination nor dosage compensation are understood, a female-specific non-coding RNA (MHM) is expressed soon after fertilisation from the single Z chicken chromosome and is likely to have a role in one or both processes. We have now discovered a prominent female-specific modification to the Z chromatin in the region of the MHM locus. We find that chicken chromatin at Zp21, including the MHM locus, is strongly enriched for acetylation of histone H4 at lysine residue 16 in female but not male chromosomes. Interestingly, this specific histone modification is also enriched along the length of the up-regulated *Drosophila melanogaster* male X chromosome where it plays a vital role in the dosage compensation process.

Introduction

The chicken genome is organised into 39 chromosome pairs consisting of 28 pairs of small microchromosomes, 10 larger chromosome pairs and one pair of sex chromosomes. In birds, females are the heterogametic sex having non-homologous sex chromosomes denoted Z and W (the latter being a microchromosome). This chromosomal imbalance between ZW females and ZZ males is expected to necessitate sex chromosome dosage compensation as seen in the majority of species bearing sex chromosomes. Indeed, the expression levels of seven chicken Z chromosome genes have been shown by real-time PCR to be similar in males and females, suggesting the existence of an avian dosage compensation system (McQueen *et al.* 2001, Kuroiwa *et al.* 2002). This dosage compensation was detectable in embryos 4 days after fertilization (McQueen *et al.* 2001) and must, therefore, occur during early development.

Despite our knowledge of the ZW sex chromosome system, it is not clear whether avian sex is determined by a W-specific factor in the female or by a double dose of Z-linked elements in the male. The DMRT1 gene is located on the Z chromosome in chicken (Nanda *et al.* 2000), as well as in distantly related ratite birds (Shetty *et al.* 2002) and represents a strong sex-determining candidate gene (see Smith & Sinclair 2004 for review). DMRT1 is thought to have a role in sexual development in a variety of vertebrates and is strongly expressed in the male chick genital ridge prior to gonadal sex determination around day 5.5 (Raymond *et al.* 1999). Moreover, the DMRT1 gene is related to the likely sex-determining gene in Medaka fish (Matsuda *et al.* 2002) and has recently been assigned a sex chromosome location

in one of the unusual mammalian platypus sex chromosomes (Grutzner *et al.* 2004). In birds, the DMRT1 locus is cytogenetically indistinguishable from a locus known as MHM (for male hypermethylated), which is heavily methylated and silent in male *Galliformes* (Teranishi *et al.* 2001). The MHM locus consists of around 200 repeats of a 2.2-kb sequence and is transcribed into a heterogeneous non-coding RNA which accumulates in the female nucleus, locally to the site of transcription (Teranishi *et al.* 2001). Intriguingly, the female-specific expression (and male silencing) of MHM is established within the first day after fertilization (Teranishi *et al.* 2001). It has been suggested that this transcript has an epigenetic role in gene silencing of DMRT1 expression in female cells in order to suppress male gonadal development (Mizuno *et al.* 2002, Ellegren 2002). However, the evidence for DMRT1 as the primary sex-determining element remains inconclusive (Smith & Sinclair 2004) and neither is evidence available to support a silencing role for the MHM transcript.

The rox and Xist transcripts (from *Drosophila* and mouse, respectively) represent two well-studied examples of non-coding RNA transcripts that are expressed from the sex chromosomes and accumulate on their chromosome of expression in a sex-specific manner. Interestingly, both transcripts are integral components of the dosage compensation mechanism of that species. Dosage compensation requires stably inherited but ultimately reversible changes in chromosome-wide gene expression and is, therefore, effected via epigenetic mechanisms such as modified histones and DNA methylation (reviewed by Park & Kuroda 2001, Akhtar 2003, Jaenisch & Bird 2003). In female mammals, the accumulation of the Xist transcript is an initiating event in the silencing of one of the X chromosomes and is associated with extensive histone modification (and ultimately DNA methylation) of that chromosome as well as with the recruitment of polycomb proteins (Heard 2004). In *Drosophila melanogaster* the single male X chromosome doubles expression from the majority of genes (reviewed in Marin *et al.* 2000, Meller 2000, Park & Kuroda 2001). This upregulation also involves histone modifications, specifically acetylation of H4K16 along the entire male *Drosophila* X chromosome (Bone *et al.* 1994). This sex-specific H4K16 acetylation is now understood to play a pivotal role in the upregulation of gene expression in order to achieve dosage compensation in *Drosophila* (Smith *et al.* 2000). The MOF acetyltransferase, which is known to effect this modification, is targeted to the male *Drosophila* X chromosome in complex with four other MSL proteins but only in the presence of a non-coding RNA (rox-1 or rox-2) that is intimately associated with the MOF protein (Park *et al.* 2003).

The mechanism of the avian dosage compensation system is unknown. However, the possibility of male Z chromosome inactivation, analogous to the X inactivation seen in female mammals, is negated by observations of synchronous Z chromosome replication (Schmid *et al.* 1989) and biallelic expression from male Z chromosome genes (Kuroda *et al.* 2001, Kuroiwa *et al.* 2002). In the nematode worm *C. elegans*, gene expression is halved from both X chromosomes in the homogametic sex which, like the male X upregulation system of *Drosophila*, would result in biallelic expression of dosage-compensated sex chromosome genes. Either mechanism could feasibly be employed by birds.

In order to discover further epigenetic modifications of avian sex chromosomes, which might be associated with the dosage compensation mechanism, we have carried out an immunocytochemical screen. Using antibodies against a panel of histone modifications, we have discovered a marked sex-specific histone modification of the female avian Z chromosome.

Methods

Chicken fibroblast cultures

Adult fibroblasts were cultured from two separate hens. 5-mm^2 skin samples were incubated at 37°C in growth medium (MEM-alpha basic medium (GIBCO) supplemented with 10% foetal bovine serum and 1% chicken serum (GIBCO)) until fibroblast outgrowth was observed. Cells were then passaged by trypsinisation. Embryos were dissected from eggs at around 3 days of development and developmental stage was determined. Staged embryos (stages 17–19) were washed in cold Hanks salt solution and were mechanically disrupted by repeated pipetting in growth medium. Disrupted tissues were maintained at

37°C in culture medium until fibroblast outgrowth was observed. The sex of each embryo culture was determined using a 1-μl aliquot of cell suspension, removed after trypsinisation of monolayer culture, as template for sexing PCR reactions using established procedures (Clinton *et al*. 2001).

Immunocytochemistry

Fibroblast cultures were split 24 h prior to harvest for immunocytochemistry. Mitotic arrest was achieved during the last 2 h of culture by addition of KaryoMax Colcemid (GIBCO) to a final concentration of 0.1 μg/ml. Cells were harvested by trypsinisation and washed in culture medium followed by PBS before hypotonic treatment of approximately 10^5 cells/ml in prewarmed 75 mmol/L KCl at 37°C. 0.5 ml of swollen cells were gently spun onto individual glass slides at 500 rpm for 5 min in a Thermo Shandon Cytospin 4 cytofuge. Slides were briefly air dried and permeabilised at room temperature for 10 min in TKCM buffer (120 mmol/L KCl, 20 mmol/L NaCl, 10 mmol/L Tris-HCl pH 8.0, 0.5 mmol/L EDTA pH 8.0, 0.1 mmol/L Triton X-100). Slides were incubated with primary antibodies (described in Table 1) diluted in TKCM + 1% goat serum (or BSA fraction IV) in a humid box at room temperature for 2 h and were washed for 5 min each in TKCM and then KCM (120 mmol/L KCl, 20 mmol/L NACl, 10 mmol/L Tris-HCl pH 8.0, 0.5 mmol/L EDTA, pH 8.0). FITC-conjugated secondary antibody (anti-rabbit immunoglobulins from Sigma) was applied at room temperature for 30 min and slides were washed as before and fixed in 3% formaldehyde (biology grade SIGMA) in TKCM for 15 min at room temperature before mounting in Vectashield with DAPI. Slides were examined with an Olympus fluorescence microscope using filtre blocks for FITC and DAPI and were collected and visualised with a CCD camera and SoftWoRx deconvolution software.

Combined immunocytochemistry and FISH

Slides stained with the antibody against acetylated H4K16 were stored in the dark at 4°C then washed in distilled water for 5 min before RNase treatment, denaturation and hybridization overnight with biotin labelled DNA probes. BAC probes containing the ScII gene (BAC 76g06) and MHM sequences (BAC 004e19) were obtained by two-dimensional PCR screening of a chicken BAC library (Crooijmans *et al*. 2000), carried out by Richard Crooijman. BAC DNA was prepared using QIAGEN plasmid columns and following recommendations for low-copy plasmid purification. Biotin 16-dUTP (Boehringer) was incorporated by nick translation and labelled probes were concentrated by precipitation. 100 ng of probe were ethanol precipitated in the presence of 5 μg of sheared salmon sperm DNA as a carrier and probes were heat-denatured in hybridization buffer (50% formamide, 2 × SSC, 1% Tween, 10% dextran sulphate in distilled water). For the ScII probe only, 2 μg of sonicated chicken genomic DNA was pre-annealed with denatured probe for 15 min at 37°C to block repetitive sequences. Probes were detected with avidin-Texas red followed by biotinylated anti-avidin and an additional layer of avidin Texas red (all from Vector laboratories). Slides were mounted in antifadent containing DAPI and examined with a fluorescence microscope using filtre blocks

Table 1. Mitotic chromosome staining patterns for antibodies against specific histone modifications.

Modification detected	*Source and dilution*	*Male chromosome staining*	*Female chromosome staining*
H3K9 acetylation	UPSTATE 4 μg/ml	No significant enrichment	No significant enrichment
H3K9 methylation	Gift (P. Singh) 1:100	No significant enrichment	No significant enrichment
H4K5 acetylation	UPSTATE 1:500	Enriched on microchromosomes	Enriched on microchromosomes, Z hyperacetylation spots visible
H4K8 acetylation	UPSTATE 1:90	Enriched on microchromosomes	Enriched on microchromosomes, Z hyperacetylation spots visible
H4K12 acetylation	UPSTATE 1:90	No significant enrichment	Z hyperacetylation spots visible
H4K16 acetylation	Gift (M. Grunstein)/ SEROTEC 1:200	No significant enrichment	Strong hyperacetylation on Z

for FITC, Texas red and DAPI. Images were collected and visualised with a CCD camera and SoftWoRx deconvolution software.

Chromatin immunoprecipitation

Chromatin immunoprecipitation was carried out according to a modified protocol of Pattenden *et al*. 2002. Male and female samples were processed in parallel. Cultured chicken cells were cross-linked for 10 min at room temperature by addition of formaldehyde (final concentration 1%) to the growth medium. Cells were washed in cold PBS and harvested by scraping. Cells were rewashed in PBS and incubated on ice for 10 min in 900 µl lysis buffer (1% SDS, 10 mmol/L EDTA, 50 mmol/L Tris-HCl, pH 8.1, plus proteinase inhibitors) before sonication to obtain DNA fragments in the size range of 500–1000 bp. Sonicated chromatin was diluted 1:10 by the addition of IP buffer (1% Triton X-100, 2 mmol/L EDTA, 150 mmol/L NaCl, 20 mmol/L Tris-HCl, pH 8.1), and precleared by incubation with protein A-sepharose beads (Amersham) and 2 µg of sheared salmon sperm DNA. Chromatin was then incubated overnight at 4°C with antibody against acetylated H4K16 (SEROTEC) or as chromatin-only mock samples. Soluble chromatin was incubated at 4°C for 1 h with protein A-Sepharose beads that had been preblocked with tRNA and 2 µg of sheared salmon sperm DNA. Immunoprecipitates were washed in TSEI buffer (0.1% SDS, 1% TritonX-100, 2 mmol/L EDTA, pH 8.0, 20 mmol/L Tris-HCl, pH 8.1, 150 mmol/L NaCl), four times in TSEII buffer (0.1% SDS, 1% TritonX-100, 2 mmol/L EDTA pH 8.0, 20 mmol/L Tris-HCl pH 8.1, 500 mmol/L NaCl), once in Buffer III (0.25 mmol/L LiCl, 1% NP-40, 1% deoxycholate, 1 mmol/L EDTA pH 8.0, 10 mmol/L Tris-HCl pH 8.1) and finally three times in TE. Chromatin was then extracted by vortexing in 1% SDS, 0.1 mmol/L $NaHCO_3$ and cross-links were reversed at 65°C overnight. Finally the DNA was purified using the Qiaex II gel extraction/concentration kit (QIAGEN) according to the manufacturer's instructions. The primers used for PCR were as follows: for MHM 5′-gctgtcctggcgtttagctg-3′ and 5′-ccttgccttctgtggctc-3′ (expected product size of 280 bp); for DMRT1 5′-gtgatgtgtttgtagtgg-3′ and 5′-tacagctgcaactacagag-3′ (expected product 269 bp); for GAPDH 5′-gtccaagtggtggccatc-3′ 5′-caatctcagctctccaccttc-3′ (expected product 268 bp). PCR was carried out using Red Hot Taq polymerase under standard conditions.

Results

A single female chromosome shows a strong localised hyperacetylation signal that is absent on the male chromosomes

Immunocytochemistry (ICC) experiments were carried out on male and female chicken cells to look for sex-specific differences in the pattern of histone modification. In order to observe differences prior to sex determination, primary cultures of chicken embryo fibroblasts were obtained from staged day-3 embryos and were harvested within two or three passages in culture. At the second passage, a small aliquot of cells was removed for PCR whereby the sex of each culture was determined. Unfixed cells were spun onto slides for immunocytochemistry with a bank of antibodies raised against various histone modifications (antibodies shown in Table 1). Fibroblast cell lines from more than one female and male embryo were frequently tested in parallel. In all cases, the autosomal pattern was indistinguishable between males and females and, with some antibodies against acetylated histones, we saw signal enrichment in microchromosomes over macrochromosomes (Table 1). This confirms our previous findings that chicken microchromosomes are disproportionately gene rich (McQueen *et al*. 1996, McQueen *et al*. 1998). The observation of gene-rich microchromosomes is now corroborated by evidence from the chicken genome sequence (Hillier *et al*. 2004). However, we were surprised to note a striking enrichment of the antibody against acetylated H4K16 on a single metacentric chromosome in all female embryo cultures (Figure 1a–c). We observed the same single site of enrichment with our antibody on adult female chicken fibroblast cells grown in primary culture (Figure 1d) but not for the female established chicken cell line DT40 when treated in the same way. This pattern was not visible on any male chromosomes prepared under the same conditions (Figure 1e). The antibody against acetylated H4K16 is known to be

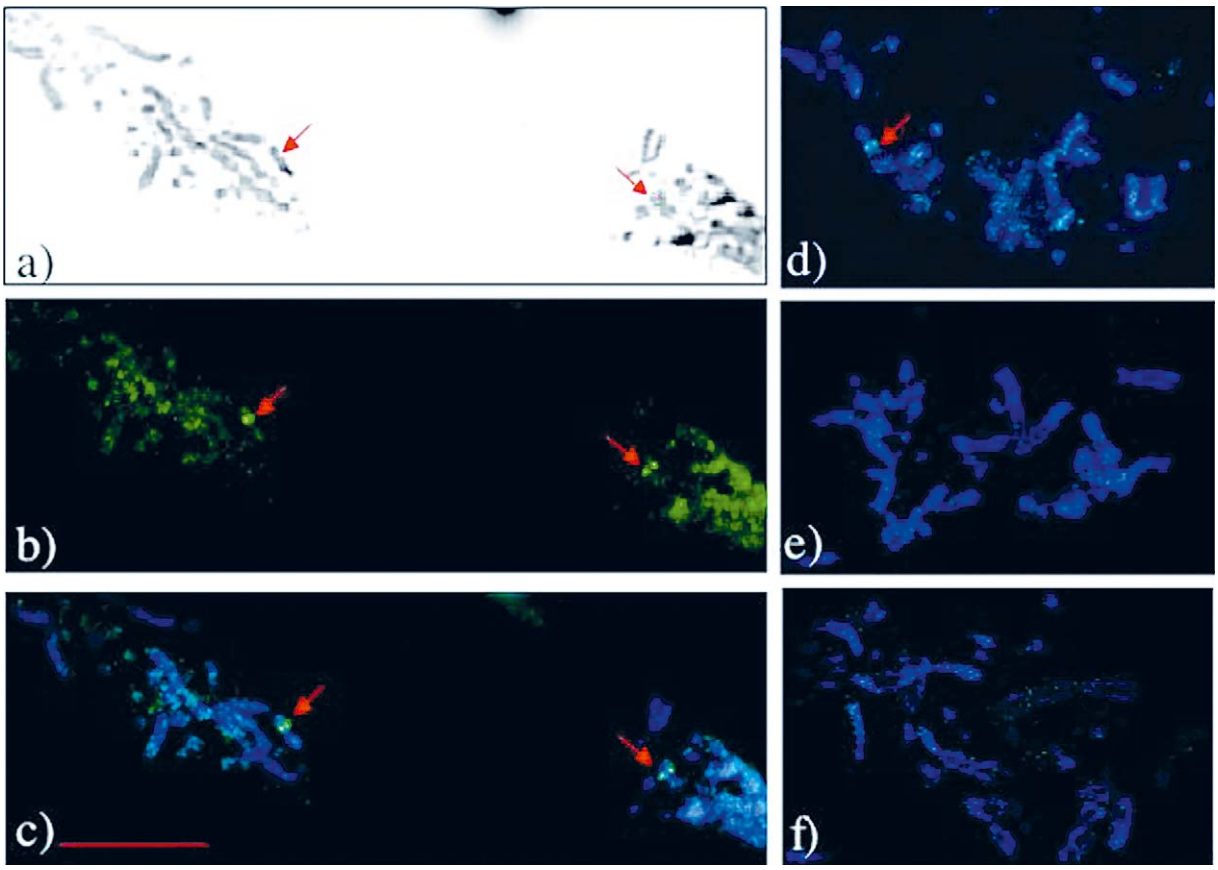

Figure 1. H4 hyperacetylation spots on a single female chicken chromosome. Chicken chromosomes stained with antibodies specific for histone modifications and showing signal enrichment at hyperacetylation spots (red arrows) on a single chromosome in female but not male cells. (**a**) Inverse DAPI staining of female embryo fibroblast chromosomes. (**b**) Female embryo fibroblast chromosomes stained with antibody specific for H4K16 acetylation which shows signal enrichment on a single chromosome. (**c**) Conjoined DAPI (blue) and H4K16 acetylation antibody staining (green) from (**a**) and (**b**). (**d**) Female adult fibroblast chromosomes stained with DAPI (blue) and H4K16 acetylation antibody (green). (**e** and **f**) Male embryo fibroblast chromosomes stained with DAPI (blue) and histone modification specific antibodies (green) showing no striking signal enrichment: (**e**) H4K16 acetylation antibody (green) and (**f**) H3K9 methylation antibody (green). Scale bar = 10 μm.

highly specific for that modified residue (Suka *et al.* 2001). On close examination, we could detect this female-specific enrichment, to a lesser degree, with other tested antibodies against acetylated H4 residues (H4K5AC, H4K8AC, H4K12Ac) (Table 1). No obvious enrichment or deficiency was seen for antibodies against acetylation or methylation of H3K9 in either males or females (Table 1, Figure 1f).

The female-specific hyperacetylation is located close to the MHM region on Zp

The size and morphology of the metacentric chromosome showing localised H4 hyperacetylation strongly suggested that it is the Z chromosome (see Figure 1a) and this was first investigated by reverse DAPI banding. The site of strong H4 hyperacetylation was seen to coincide with the prominent negative G band on the short arm of the Z chromosome recognised as Zp21 according to the standardised avian karyotype (Ladjali-Mohammedi *et al.* 1999). In order to confirm the Z location of the strong hyperacetylation signal, biotin-labelled probes for sequences on Zp and Zq were used in combined immunocytochemistry and DNA-FISH experiments. A BAC clone containing the ScII (Scaffold protein two) gene that maps to Zq1.5–1.6 (McQueen *et al.* 2001) clearly

demonstrated a Zp location for the H4 hyperacetylation signal on the female Z chromosome (Figure 2a, b). These results were particularly intriguing since the MHM region is also known to map to Zp21, proximal to the ZOV3 gene (Teranishi *et al.* 2001). We, therefore, repeated our DNA-FISH immunocytochemistry experiment using a biotin-labelled BAC containing part of the MHM region. Although the overlap of our hyperacetylation signal with the MHM region was not complete (Figure 2c), we found the two regions to be closely associated.

Female MHM chromatin is specifically enriched for H4K16 acetylation

In order to investigate whether chromatin in the MHM region was specifically affected by the histone modification described, we next carried out chromatin immunoprecipitation (ChIP) experiments. Anti-H4K16Ac antibody was used to precipitate sonicated chromatin preparations from one male and one female embryo, which were then assayed for specific sequences by PCR. Extracts from male and female cells were prepared and analysed in three independent experiments with additional repetition of the PCR for most experiments. We found the female- but not the male-precipitated chromatin to be enriched for MHM sequences while both showed modest enrichment for GAPDH (Figure 3a–c). The DMRT1 gene which is very closely located with MHM (Teranishi *et al.* 2001) but which is specifically expressed in embryonic genital ridge and male gonad (Raymond *et al.* 1999) showed no enrichment in precipitated chromatin from either male or female fibroblasts, as expected (Figure 3b).

Draft chicken genome sequence predicts a minimum distance of 23 kb between the DMRT1 gene and the MHM region

DMRT1 (accession number AF123456) and MHM (accession number AB046699) sequences were used to BLAST search the draft chicken genome sequence via the 'ensembl' browser (http://www.ensembl.org/Gallus_gallus/). Both sequences were found in supercontigs (whose length is guaranteed by read-pairing data but which contain gaps of estimated sizes) anchored to the Z chromosome. MHM repeats were found in contig 36572, around the 9.4 Mb position. The DMRT1 gene was found in the 336 kb contig 301 which has been assigned to the random portion of the Z chromosome due to the lack of physical mapping

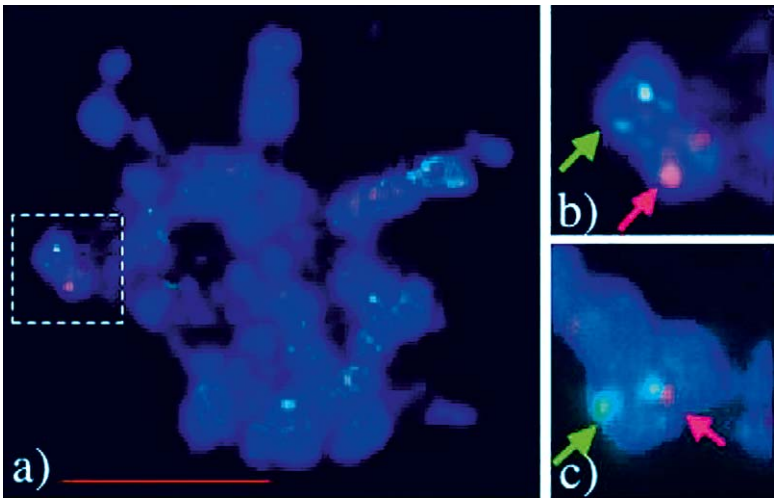

Figure 2. Combined immunocytochemistry and DNA-FISH maps the hyperacetylation spots to Zp close to the MHM locus. DNA-FISH with Z chromosome BAC probes (red arrows) hybridized to female chromosomes that are immunostained with antibody against acetylated H4K16 (green arrows). (**a**) ScII probe; (**b**) ScII probe showing enlargement of boxed area from (**a**); (**c**) MHM probe. Scale bar = 10 μm.

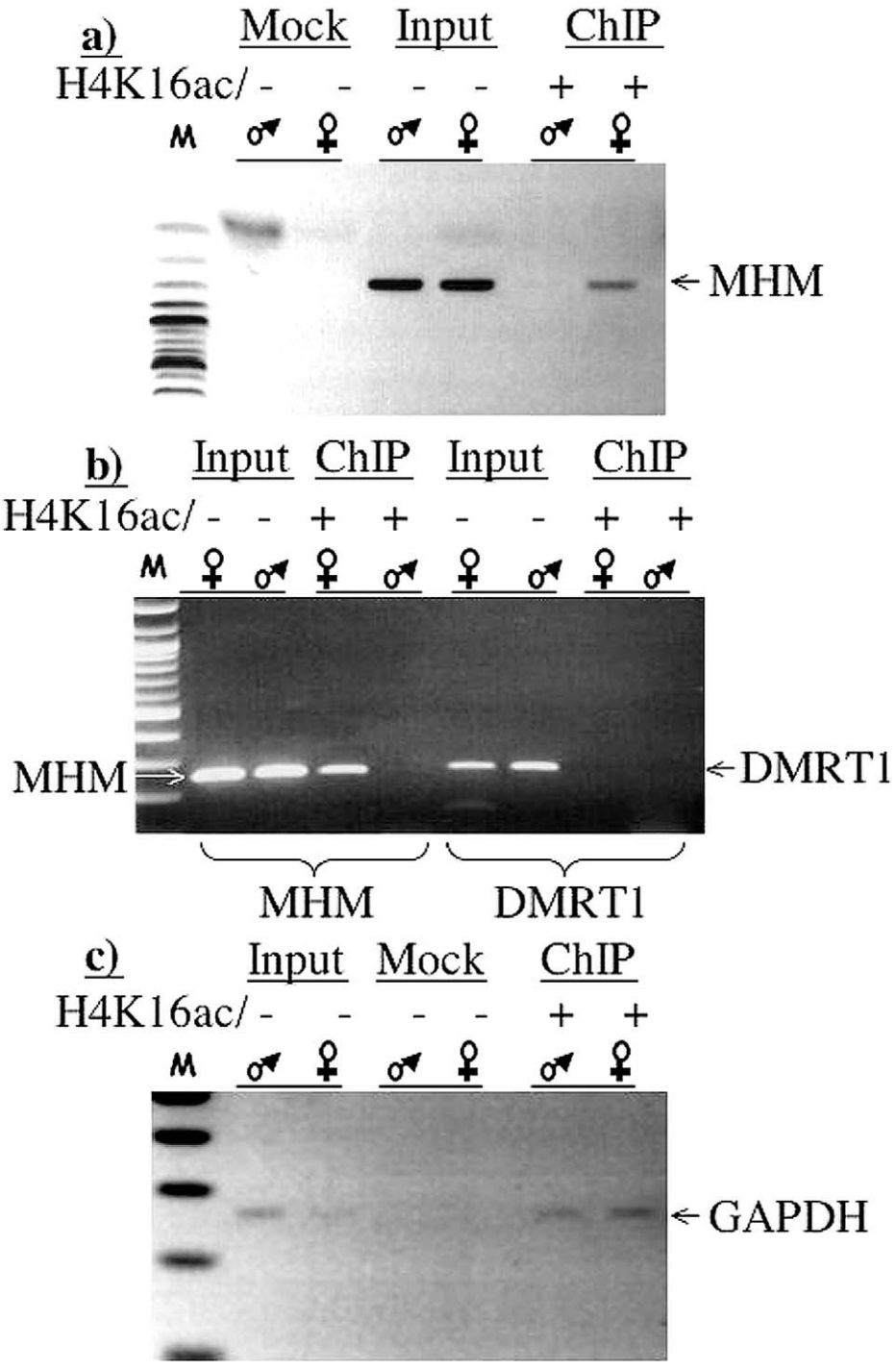

Figure 3. Chromatin immunoprecipitation showing female-specific enrichment of MHM in H4K16 acetylated chromatin. PCR amplification of male and female chicken embryo input chromatin and chromatin that has been precipitated in the absence (mock) or presence (ChIP) of H4K16ac antibody. M = marker. (**a**) Amplification with MHM primers demonstrates the female but not male MHM chromatin to specifically precipitate with H4K16ac antibody. (**b**) DMRT1 primers demonstrate lack of precipitation for DMRT1 chromatin while MHM chromatin shows female-specific precipitation in parallel. (**c**) GAPDH primers show non-sex-specific chromatin immunoprecipitation for this control sequence.

data. DMRT1 lies 23 kb from the end of contig 301 and no MHM-like sequence was found within this contig. Taken at face value, our results imply a minimum distance between MHM and DMRT1 of 23 kb, although it remains possible that the available sequence contains a local misassembly.

Discussion

We have identified a region of Z chromatin which is highly enriched with hyperacetylated histones in females but not in males. We have further mapped this area to a region close to the MHM locus by DNA FISH and have confirmed by chromatin immunoprecipitation that the MHM locus is specifically modified by hyperacetylation in female, but not male, chicken fibroblasts. Our findings represent the second described case of a sex-specific Z chromosome epigenetic modification in birds, the first being the male-specific DNA methylation of the MHM region. We further suggest that Zp histone hyperacetylation and expression of the MHM are related epigenetic events since both are female-specific and are detected during early embryonic development. Such hyperacetylation predicts chromatin hyperactivity. However, whether the chromatin hyperactivity is the cause or the consequence of female-specific MHM transcription remains unclear. Indeed, it has been noted that non-coding RNAs, although frequently associated with silencing, can alternatively be associated with local activation (Akhtar 2003).

The DMRT1 gene is cytogenetically coincident with the MHM locus, being found in the same loop of the lampbrush Z chromosome (Teranishi *et al.* 2001). DMRT1 maintains an opposite expression pattern to MHM in gonadal tissues, being specifically expressed in males (Raymond *et al.* 1999) but silent in somatic cells from both sexes. The lack of hyperacetylated chromatin from the DMRT1 locus in female chickens (Figure 3b) is therefore as expected for this gene. It is, however, interesting to consider the proximity of the silent DMRT1 locus in females to the hyperacetylated chromatin of the MHM domain which is estimated to cover at least 460 kb (Teranishi *et al.* 2001). If MHM and DMRT1 are closely located at the sequence level, it might be revealing to study the chromatin architecture of these two closely juxtaposed yet inversely expressed loci. If, as suggested by analysis of the first chicken genome sequence draft, these loci are more than 23 kb apart, MHM local accumulation might be expected to interact with other unspecified elements in this region, in addition to or instead of DMRT1. The precise relationship of the MHM and DMRT1 loci would be resolved by BAC-based sequencing of this area.

Since the sex-specific Z chromatin differences described in this paper along with MHM expression are not restricted to the gonad, like DMRT1 expression, but apply to embryonic and adult somatic cells, we suggest a role for both epigenetic modifications out-with sex determination. One possible role for these sex-specific epigenetic differences, which has previously been suggested for MHM (Ellegren 2002, Graves 2003), could be the process of dosage compensation. Indeed, dosage compensation is maintained in differentiated cells of mammals, *Drosophila* and *C. elegans* and was detected in the somatic cells of chicken embryos (McQueen *et al.* 2001).

Since biallelic Z chromosome gene expression argues against Z inactivation (Kuroda *et al.* 2001, Kuroiwa *et al.* 2002), the mechanism of avian dosage compensation is more likely to be Z gene up-regulation in females (akin to the mechanism employed by *Drosophila)*, or Z gene down-regulation in males (similar to the mechanism seen in *C. elegans)*. It is, therefore, provocative to note that enriched hyperacetylation of lysine 16, which we have demonstrated to be female specific on the chicken Z chromosome, is also a prominent and sex-specific feature of the *Drosophila* dosage compensation process. We have already noted that the *Drosophila* rox genes are analogous to the MHM locus in that their genes are located on the sex chromosome, their expression is limited to the heterogametic sex and they produce an untranslated RNA that accumulates locally on the sex chromosome *in cis*. It is tempting to speculate that avian MHM could function similarly to *Drosophila* rox by targeting chromatin modifying activity, such as acetyltransferase, to the Z. This might result in the sex-specific hyperacetylation observed and might also be a step in the upregulation of female Z chromosome genes for the purpose of dosage

compensation. Indeed, the existence of an avian version of the *Drosophila* dosage compensation machinery seems plausible given the recent discovery of critical components of this complex in other vertebrates (Neal *et al.* 2000, Sanjuan & Marin 2001). In line with this prediction, we have recently identified a putative mof homologue (clone 603116430F1) from the chicken EST database (Boardman *et al.* 2002) and shown by RT-PCR that it is expressed in fibroblasts from day-3 embryos (data not shown).

Unlike the extensively hyperacetylated *Drosophila* male X chromosome, the chicken Z hyperacetylation is not chromosome wide but concentrated specifically on part of the p arm. This suggests that this hyperacetylation *per se* cannot be the sole mechanism of up-regulation for all Z chromosome genes and that additional dosage compensation strategies must also be invoked. Nevertheless, parallels between the epigenetic features of the avian female Z chromosomes and of the *Drosophila* dosage compensation system are intriguing and further work comparing these systems will be most instructive.

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