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Avian sex determination: what, when and where?

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Abstract. Sex is determined genetically in all birds, but the underlying mechanism remains unknown. All species have a ZZ/ZW sex chromosome system characterised by female (ZW) heterogamety, but the chromosomes themselves can be heteromorphic (in most birds) or homomorphic (in the flightless ratites). Sex in birds might be determined by the dosage of a Z-linked gene (two in males, one in females) or by a dominant ovary-determining gene carried on the W sex chromosome, or both. Sex chromosome aneuploidy has not been conclusively documented in birds to differentiate between these possibilities. By definition, the sex chromosomes of birds must carry one or more sex-determining genes. In this review of avian sex determination, we ask what, when and where? What is the nature of the avian sex determinant? When should it be expressed in the developing embryo, and where is it expressed? The last two questions arise due to evidence suggesting that sex-determining genes in birds might be operating prior to overt sexual differentiation of the gonads into testes or ovaries, and in tissues other than the urogenital system.

Sex is determined in birds at the time of fertilisation through the inheritance of the sex chromosomes (ZZ male versus ZW female). While the mechanism of avian sex determination is still unknown, it must involve a sex-linked gene carried on either or both of the sex chromosomes. It has long been thought that, as in eutherian mammals, a sex-determining gene/s carried on the sex chromosome acts solely in the embryonic gonads, initiating testicular or ovarian differentiation. The developing gonads then synthesise and release sex steroid hormones to masculinise or feminise the gonadal ducts, external genitalia, brain and other tissues (Romanoff, 1960). In mammals, the key sex determinant is *SRY*, carried on the Y chromosome and transiently expressed in the embryonic male gonad to trigger a genetic cascade leading to testis formation. Testosterone released from the male gonad then masculinizes the tissues, which otherwise follow a female developmental program. Although *SRY* is absent in birds, it is generally thought that the principle is the same. That is, a sex determinant is first expressed in the embryonic gonads, triggering gonadal sex differentiation, followed by hormone production to induce secondary sexual differentiation (ducts, external genitalia and brain sex). In this review, we consider the evidence for this idea in birds. Firstly, what is the nature of the avian sex determinant? Does is depend on the presence/absence of a single gene, as in mammals? Secondly, when is it expressed? The two most promising sex-determining candidates in birds, the Z-linked *DMRT1* and W-linked *HINTW* genes, are expressed in the gonads well prior to sexual differentiation. Lastly, where is the sex determinant(s) expressed? There is evidence that candidate sex-determining genes may be expressed independently in other tissues in addition to the gonads, as occurs in marsupials (O et al., 1988) and probably also in eutherian mammals (Dewing et al., 2003).

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Chicken sex chromosomes and sex determination

The sex chromosomes in most birds are heteromorphic, characterised by a large Z chromosome and smaller heterochromatic W chromosome (Takagi and Sasaki, 1974; Solari,

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1993; Schmid et al., 2005). The exception is seen in the flightless ratites (emus, ostriches, etc.), in which the Z and W are essentially identical in size (homomorphic) (Takagi et al., 1972). The differentiated sex chromosomes of advanced, modern birds (carinates) have derived from a homomorphic autosomal chromosome pair that pre-dates their divergence from ratites (Fridolfsson et al., 1998; Ogawa et al., 1998). In the ratites, these chromosomes have differentiated little, but they are homologous to those of the chicken (Shetty et al., 1999) and they must carry sex determinants, as in all other birds. Our current understanding of avian sex determination largely derives from research on the chicken *(Gallus gallus domesticus)*. This species is widely used in developmental biology, it is of agricultural importance and its genome has now been sequenced (Hillier et al., 2004). As in other gallinaceous birds, the chicken has heteromorphic sex chromosomes. The large chicken Z sex chromosome has over 350 genes, while the smaller, heterochromatic W chromosome has very few, probably less than twenty (Mizuno et al., 2002; Stiglec et al., 2007). Recent studies indicate that the Z sex chromosome is strongly conserved across all birds, while the W is far less conserved and has undergone varying degrees of degradation (Shetty et al., 1999; Nanda and Schmid, 2002; Raudsepp et al., 2002; Berlin and Ellegren, 2006).

Sex could be determined by Z chromosome dosage (two for males, one for females), by a dominant W gene carried only in females, or by both mechanisms. The Z dosage hypothesis requires that the relevant gene is not subject to dosage compensation between the sexes. Indeed, the chicken Z chromosome does not appear to undergo widespread inactivation typical of the mammalian X chromosome (Kuroda et al., 2001; Ellegren, 2002). Although RT-PCR studies suggest dosage equalization between the sexes for several Z-linked genes (McQueen et al., 2001), recent microarray data indicate that dosage compensation in birds is weak in comparison to that seen in mammals (Itoh et al., 2007). Definitive sex chromosome aneuploidy (2A:ZZW or 2A:ZO) has not been reported in birds, and it may be lethal in embryo, at least in the case of ZO individuals (Graves, 2003). However, a line of triploid chickens with a 3A:ZZW genotype, reported by Thorne and Sheldon (1993), developed as intersexes. At hatching, these birds had a right testis and transient left ovotestis, and a female external phenotype. The ovarian component degenerated with age. This suggests that the W chromosome carries a female determinant, because some ovarian tissue could form despite the presence of two Z chromosomes (Lin et al., 1995). However, regression of this ovarian tissue in the ZZW triploid chickens implies that the putative W-linked female determinant is not dominant and that it can be 'overridden' by two Z chromosomes. A complication here, however, is the fact that these birds were complete triploids, so three copies of the entire genome may have influenced gonadal development. Recently, Arit et al. (2004) reported a female great reed warbler with an inferred 2A:ZZW genotype, based on the heterozygous inheritance of two Z-linked microsatellite markers. This also supports the notion that the W carries a female determinant, regardless of the number of Z chromosomes. However, the two Z alleles were apparently not passed on to the male offspring of this bird and its ZZW status was not confirmed by karyotyping. Both the Z dosage and dominant W hypotheses therefore remain viable.

Gonadal sex differentiation and conserved sex-determining genes

As in mammals, studies on chicken sex determination have focussed on the embryonic gonads, where sex-determining genes are logically thought to operate. The gonadal rudiments form on the medial surface of the embryonic (mesonephric) kidneys at approximately day 3.5 of development in the chicken embryo (developmental stage 20; Hamburger and Hamilton, 1951) (Fig. 1). At this stage, gonads are morphologically identical between the sexes (or 'bipotential') (Fig. 1A–C). Recent studies have shown that fibroblast growth factor signalling is important for the formation of the gonad during the indifferent stage (Yoshioka et al., 2005). Sexual differentiation is first detectable at the histological level at day 6.5 (developmental stage 30), when seminiferous cords begin to coalesce in the interior (medulla) of the male gonad, while the outer cortical layer starts to proliferate in females (Fig. 1B, C) (van Limbough, 1973; Ebensperger et al., 1988). Thus, morphological differentiation begins at the same time in the two sexes (in contrast to mammals, in which overt signs of sexual differentiation are first seen in males). Some researchers have reported sex differences in the size and distribution of primordial germ cells prior to day 6.5 (Zaccanti et al., 1990) but, since germ cells are not required for somatic differentiation of the gonads (McCarrey and Abbott, 1978, 1982), they will not be considered further here. Ovarian differentiation in the chicken embryo is characterised by asymmetry; although both gonads initially undergo cortical proliferation, this process is not maintained in the right gonad (Fig. 1A). As embryogenesis proceeds beyond day 6.5, cortical proliferation stops in the right gonad and, by day 8.5, the right cortex is reduced to a flattened epithelial monolayer. In contrast, the left gonad has a highly thickened cortex, populated by proliferating germ cells. Both female gonads have an underlying medulla, which is steroidogenic and characterised morphologically by extensive lacunae (fluid-filled cavities). In males, bilateral testes are characterised by well-developed seminiferous cords, containing supportive Sertoli cells and mitotically arrested germ cells.

Based on this morphology, sex-determining genes in the chicken embryo are expected to operate within the gonads just prior to the onset of sexual differentiation, which begins at day 6.5 (stage 30). Two genes expressed just prior to morphological differentiation are *SOX9* (SRY-like BOX, no. 9) and *CYP19A1* (aromatase*)* (Fig. 2). *SOX9* is expressed from stage 30 (day 6) only in male gonads, while *CYP19A1* is expressed from the same stage, but only in female gonads (Fig. 2) (Smith et al., 1997, 2005; Nakabayashi et al., 1998). *SOX9* is required for testis development in mammals, where

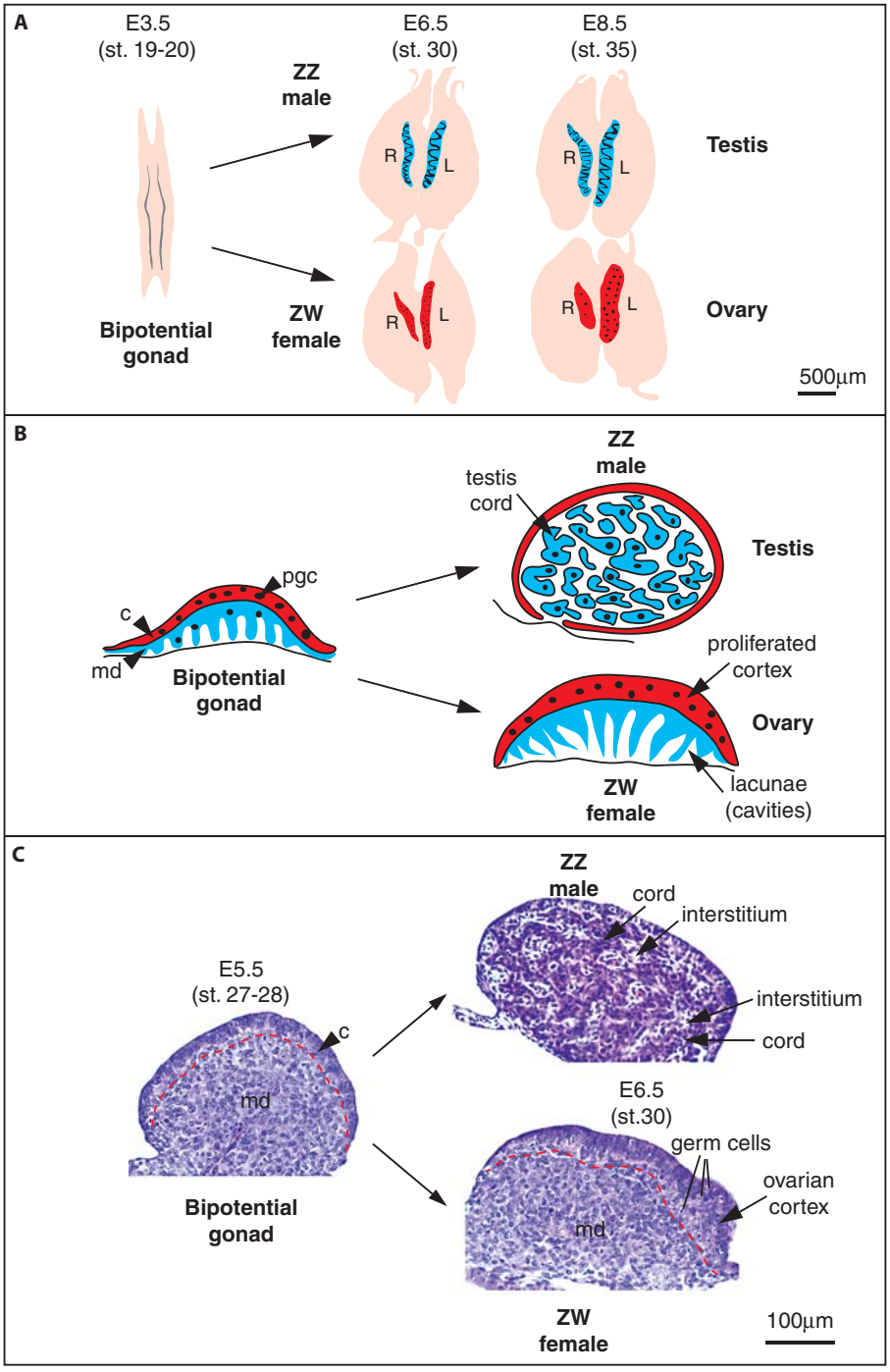
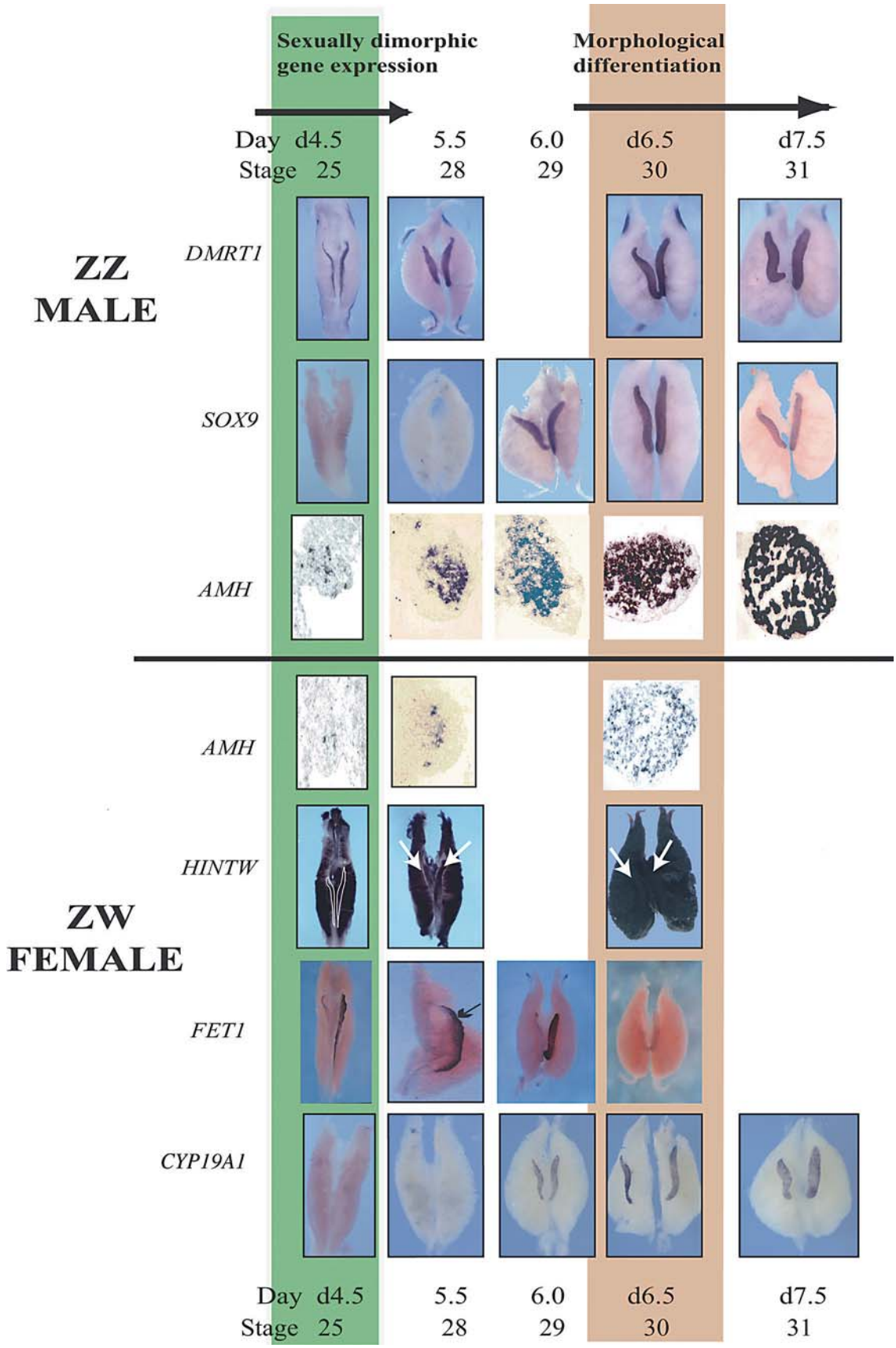


Fig. 1. Gonadal development and sexual differentiation in the chicken embryo. (**A**) Schematic of gonadal anatomy. At embryonic day 3.5 (stage 19–20), the gonads are undifferentiated or bipotential (shown in blue), on the medial surface of the mesonephric kidneys (pale brown). In ZZ males, bilateral testes develop, while, in ZW females, the left gonad becomes an ovary and the right regresses. (**B**) Schematic of gonadal histology. The bipotential gonad comprises an outer cortical layer (c), and underlying medulla (md). Primordial germ cells (pgc) are concentrated mainly in the cortex. Testis formation involves the condensation of medullary cords into seminiferous (testis) cords. The (left) ovary is characterised by cortical proliferation, while the medulla becomes reticulated, with numerous cavities (lacunae). (**C**) Gonadal histology in the chicken embryo. At embryonic day 5.5 (E5.5; stage 27–28) the gonads are histologically undifferentiated. The cortex (c) is distinct from the underlying medulla (md). In ZZ males, condensing cords are apparent by E6.5 (stage 30). The interstitium (site of Leydig cell development and testosterone synthesis) is present between the cords. In contrast, cortex proliferation, including germ cell proliferation, is apparent in ZW females.

it regulates Sertoli cell differentiation and seminiferous cord formation. It is also likely to perform the same role in birds (Kent et al., 1996). *CYP19A1* encodes the rate-limiting enzyme responsible for estrogen synthesis, which is required for ovarian differentiation in birds (reviewed in Smith and Sinclair, 2004). The embryonic female gonad in birds therefore synthesises estrogen from an early age (reviewed in Scheib, 1983). Estrogen receptor alpha (ERα) is expressed in both sexes, but expression declines in males as development proceeds (Andrews et al., 1997; Smith et al., 1997; Nakabayashi et al., 1998). ERα is primarily expressed in the gonadal cortex and may underlie the cortical hypertrophy typical of ovarian differentiation (Nakabayashi et al., 1998).

A number of other genes shown to play a role in mammalian gonadal sex differentiation have homologues in birds. These include *SF1* (Steroidogenic Factor-1, also called *Ad4BP*) and *DAX1* (Dosage sensitive sex reversal, *A*drenal hypoplasia congenita, X linked, no. 1). These genes encode


Sexually dimorphic gene expression
Morphological differentiation
Day d4.5 5.5 6.0 d6.5 d7.5
Stage 25 28 29 30 31
ZZ MALE
DMRT1
SOX9
AMH
AMH
HINTW
ZW FEMALE
FET1
CYP19A1
Day d4.5 5.5 6.0 d6.5 d7.5
Stage 25 28 29 30 31

orphan nuclear receptors. Other factors include signalling molecules such as *WNT4* (Wingless, Int-related, no. 4), and the *AMH* (Anti-Müllerian Hormone) gene (Smith et al., 1999a, b, 2000; Oreal et al., 2002). In the chicken embryo, these genes have expression profiles consistent with conserved roles in gonadal sex differentiation. However, none of the above genes are sex-linked, including *SOX9* and *CYP19A1*. While *SRY* represents the sex-determining trigger in mammals, this gene is absent in birds and an alternative genetic trigger(s) must exist.

What is the nature of the sex determinant(s)?

In mammals, the *SRY* gene encodes a transcription factor regulating gonadal sex differentiation. There is no reason to expect a priori that the avian sex determinant also encodes a transcription factor, although the only other master sex determinant identified in vertebrates, the *DMY* gene in some teleost fish species, also encodes a transcription factor (Matsuda et al., 2002; Nanda et al., 2002). The two most promising candidate avian sex determining genes are currently *DMRT1* (related to *DMY*), located on the Z sex chromosome, and *HINTW*, located on the W chromosome. *DMRT1* (Doublesex and Mab-3 Related Transcription factor, no. 1) encodes a novel nuclear transcription factor with a DNA-binding motif (the DM domain). *DMRT1* is unrelated to *SRY*. Deletions of human *DMRT1* are associated with XY male-to-female sex reversal (Raymond et al., 1999a) while mouse *Dmrt1* null mutants have (postnatal) testicular dysfunction, pointing to a role in male development (Raymond et al., 2000). *DMRT1* is conserved across vertebrates and even has homologues involved in sex determination in *Drosophila* and *Caenorhabditis elegans* (Raymond et al., 1998; Nanda et al., 1999). *DMRT1* is expressed specifically in the embryonic urogenital system of all vertebrates that have been examined, from fishes to birds and mammals, and it is more highly expressed in male gonads (Raymond et al., 1999b; Smith et al., 1999c; Shan et al., 2000; reviewed in Zarkower, 2001). Chicken *DMRT1* is Z-linked and is absent from the W sex chromosome. It is therefore present in two doses in males and in one dose in females. Furthermore, it is not dosage compensated (Nanda et al., 2000). In chicken embryos, it is expressed only in the gonads and Müllerian ducts, where expression is higher in males compared to females, from as early as day 3.5–4.5 (stage 20–25) (Fig. 2). Significantly, while many Z-linked genes in chicken map to both Z and W chromosomes of ratites, excluding them as universal avian sex determinants, *DMRT1* maps only to the Z chromosome of at least one ratite, the emu *(Dromaius novaehollandiae)* (Shetty et al., 2002). *DMRT1* is therefore a candidate avian sex determinant under the Z dosage hypothesis, with higher expression correlating with male (testicular) differentiation.

The dominant W hypothesis predicts an ovary determinant carried on the W sex chromosome that directs female development. In the absence of the W, male development would occur. One such candidate female gene is *HINTW* (HIstidine triad NucleoTide binding protein, W-linked). This gene was initially identified independently by two groups and was called *WPKCI,* W-linked Protein Kinase C inhibitor (Hori et al., 2000) and *ASW,* Avian Sex related, W-linked (O'Neill et al., 2000). Because it is now clear that this gene encodes a derived version of a histidine triad nucleotide binding protein (HINT), rather than a protein kinase inhibitor (PKCI), *HINTW* is the more appropriate and accepted nomenclature (see Ceplitis and Ellegren, 2004). HINT proteins form a branch of the HIT family of nucleotide hydrolase enzymes, they are conserved across animals and they specifically hydrolyse adenosine from lysine residues. However, *HINTW* is very unusual because it lacks the key catalytic motif (the histidine triad) that confers enzyme activity to all other bona fide HINT proteins, including a HINT homologue, *HINTZ*, which resides on the chicken Z sex chromosome. *HINTW* is reiterated over 40 times on the chicken W sex chromosome, it is conserved on the W in carinate birds, and phylogenetic studies indicate that this gene has undergone positive selection during evolution (Ceplitis and Ellegren, 2004; Backström et al., 2005). *HINTW* is widely expressed in female chicken embryos, including strong expression in the gonads (Fig. 2). The *HINTZ* gene, encoding a bona fide HINT enzyme, is expressed in the gonads of both sexes (Hori et al., 2000). Given its lack of a catalytic domain, it has been hypothesised that HINTW acts in a dominant negative fashion to block HINTZ function in the gonads, leading to female sexual differentiation (Pace and Brenner, 2003). This idea is supported by recent in vitro studies showing a direct interaction between recombinant HINTW and HINTZ, which inhibits the biochemical function of the latter (Moriyama et al., 2006). Taken together, these data point to a role for *HINTW* in avian sex determination. In the ratites, Southern blot analysis detects one band in both sexes; this indicates that the gene is not reiterated on the ratite W chromosome (Hori et al., 2000), and it is unclear at this stage whether a distinct *HINTW* gene exists in this group. If *HINTW* is absent in ratites, it negates this gene as a universal avian sex determinant. Alternatively, sex could be determined differently in ratites. This seems unlikely, given the common ancestry of ratite and carinate sex chromosomes.

Fig. 2. Timing of gene expression in embryonic chicken gonads, as assessed by whole mount and tissue section in situ hybridisation. The onset of morphological differentiation into testes or ovaries is shown (from day 6.5; stage 30). In ZZ males, *DMRT1* mRNA expression is detectable from day 3.5–4.5 (stages 20–25). In comparison, *SOX9* in males is first detectable at day 6.0 (stage 29). In ZW females, *HINTW* mRNA is expressed from days 3.5–4.5 (stages 20–25). *FET1* mRNA is also expressed from days 3.5–4.5, but asymmetrically expressed, with stronger expression in the left gonad. *FET1* expression is down-regulated in the gonads by day 6.5 (stage 30). In comparison, *CYP19A1* is first detectable at day 6.0 (stage 29). *AMH* is first detectable at stage 25 in both sexes, but appears higher in males, according to tissue section in situ hybridisation. By stage 28, this dimorphism in *AMH* is clear (left gonads only are shown). The onset of *AMH* expression precedes *SOX9* expression in males, and *CYP19A1* expression in females. The *AMH* expression is taken from Oreal et al. (1998) with permission.

The potential disruption of HINTZ by HINTW described above indicates that the Z and W sex chromosomes may directly interact to regulate avian sex determination. Another study also suggests direct interaction between these chromosomes, but in a different manner. Teranishi et al. (2001) identified a tandem repeat region on the chicken Z chromosome that is highly methylated in the two Z chromosomes of male cells (the male hypermethylated region, MHM), but far less so in females. Due to the lack of extensive methylation, the region is transcribed from the single Z of female cells, resulting in non-coding mRNA that accumulates at the site of transcription, near the *DMRT1* locus (within about 23 kb). This is reminiscent of *Xist*, the X-inactivation transcript in mammals. Interestingly, in triploid ZZW cells, the two Z chromosomes are hypomethylated and transcribed. In contrast, ZZZ triploid cells remain hypermethylated on the Z. This clearly points to the W chromosome as a potential regulator of methylation on the Z. The W may therefore encode a factor, such as a demethylating enzyme, that allows transcription of the non-coding RNA in females. This RNA then coats the Z and could silence or down-regulate potential male genes, such as *DMRT1* (Teranishi et al., 2001). Such potential interplay between the two sex chromosomes to control sex determination has not been reported in mammals or other vertebrates. The putative factor derived from the W chromosome remains to be identified. Interestingly, it has recently been reported that Z chromatin is hyperacetylated at histone 4 in female but not male chicken fibroblasts. This hyperacetylation is adjacent to the MHM locus and suggests a functional link, because hyperacetylation predicts chromatin hyperactivity (Bisoni et al., 2005). However, it is unclear at present whether female hyperacetylation is a cause or an effect of female transcription of the neighbouring MHM. Indeed, both processes could be related to dosage compensation and unrelated to sex determination.

When are sex-determining genes operating?

It is generally expected that the expression of sex-determining genes will begin in embryonic gonads just prior to overt gonadal sex differentiation (day 6.5, stage 30, in the chicken). However, current candidate genes do not meet this expectation. As shown in Fig. 2, the candidate male and female genes, *DMRT1* and *HINTW*, are both expressed in the gonads from at least as early as day 4.5, well prior to the onset of morphological differentiation and prior to the expression of the key genes, *SOX9* and *CYP19A1* (day 6.0–6.5). Therefore, these genes may be initiating sexual differentiation at the molecular level in a way that is not detectable morphologically until later. If so, they would be activating the *SOX9* and *CYP19A1* genes indirectly. This would make the avian system dissimilar to the mammalian system, in which SRY, for example, sets in motion *SOX9* expression and seminiferous cord formation over a short, tightly regulated developmental window. In birds, the sex determinants may be expressed very early, leading to further downstream gene expression well before histological differentiation becomes apparent. A potential intermediate gene in the chicken is the candidate aromatase regulator, *FOXL2*, which is expressed in the gonads female-specifically from day 5 (stage 28) (Loffler et al., 2003; Gorovoun et al., 2004; Hudson et al., 2005). *FOXL2* is autosomal but is likely to represent a link between the female determinant and *CYP19A1* (aromatase).

Another potential intermediate in the chicken sex-determining pathway may be Anti-Müllerian hormone (AMH). AMH is a hormone secreted by Sertoli cells, and it induces regression of the paired Müllerian ducts, which otherwise form oviducts (Fallopian tubes) in females. Accordingly, in the mammalian embryo, *AMH* is only expressed in males. In contrast, *AMH* is expressed in both male and female chicken embryos because the right Müllerian duct in females follows the male pattern and regresses. In mammals, *AMH* is expressed downstream in the male pathway. In the mouse, for example, *Amh* is expressed after *Sry* and *Sox9*, and indeed it has been shown that SOX9 acts together with WT1, SF1 and GATA4 to regulate the *Amh* gene (reviewed in Brennan and Capel, 2004). However, in the chicken embryo, *AMH* expression begins very early. According to tissue section and whole mount in situ hybridisation, low levels of *AMH* expression are first detectable in the gonads of both sexes stage 25–27 (stage day 4.5–5.0) (Oreal et al., 1998; Nishikimi et al., 2000). This is prior to the onset of histological differentiation and prior to the onset of *SOX9* expression in males (day 6.0; stage 29; Oreal et al., 1998). Recent evidence suggests that SF1 may be at least partly responsible to activating *AMH* gene expression in avian embryos (Takada et al., 2006). While *AMH* expression appears similar in both sexes at the very early stages, it becomes stronger in male chicken embryos by stage 28 (day 5.5–6) (Fig. 2). This again points to sexually dimorphic gene regulation prior to overt histological differentiation of the gonads. Because it is male-enriched at this early stage, *AMH* may respond to the higher levels of DMRT1 or another unknown male factor in ZZ embryos. Therefore, AMH may have a more 'upstream' position in the sex determining pathway of birds compared to mammals. However, a linear cascade linking *DMRT1, AMH* and genes such as *SOX9* has not been demonstrated in birds.

If sex is determined by gene dosage in birds, as suggested by the expression of *DMRT1*, then cumulative or threshold expression levels could be important. For example, although *DMRT1* is expressed sexually dimorphically from as early as day 3.5, it is possible that its function in sex determination requires a specific threshold level of expression to be achieved. *DMRT1* mRNA expression levels in male gonads appear to be highest at day 6 (stage 29) (Smith et al., 2003), which is just prior to morphological differentiation and coincides with the onset of *SOX9* expression. Similarly, *HINTW*, although expressed from day 2–3 in females, is most highly expressed in the gonads at day 5–6 (stages 27–29), just prior to aromatase activation (day 6; stage 30) (Hori et al., 2000). *DMRT1* and/or *HINTW* expression could regulate their own expression, leading to a threshold level re-

quired for triggering gonadal sex differentiation. In the case of *DMRT1*, alternative splicing may also play a role in defining its function. *DMRT1* is alternatively spliced in human testis (Cheng et al., 2006) and in the gonads of lower vertebrates (Sreenivasulu et al., 2002; Guo et al., 2005). Most recently this has also been shown for *DMRT1* transcripts in embryonic chicken gonads, with a male specific splice variant detected at stage 31, the time of gonadal sex differentiation (Zhao et al., 2007). It is noteworthy that differential splicing of the *Drosophila* homologue, *Doublesex,* is crucial to its sex-specific function. Therefore, different *DMRT1* isoforms may be produced at different embryonic stages in the avian embryo, with an isoform specific to days 5 and 6 triggering gonadal sex differentiation. This does imply, however, that another sex-specific factor controls sexually dimorphic alternative splicing of *DMRT1*, unless it is autoregulated.

Alternatively, *DMRT1* and *HINTW* may not be involved in avian sex determination. At present, there are few alternative sex-linked candidates. *FET1* (Female-Expressed Transcript, no. 1) is one potential W-linked candidate gene (Reed and Sinclair, 2002). This gene was isolated from a screen for novel sex-specific genes in chicken and it is expressed asymmetrically in female gonads from day 4.5 to day 6.5 (Fig. 2). While *FET1* does not appear to have a homologue on the chicken Z, its presence in other birds has not been definitely shown. Recent sequence analysis suggests that it encodes an avian retroviral element (C. Smith, unpublished), and there is no evidence that is it translated, which may undermine its possible involvement in sex determination. In addition, an ovary determinant in the chicken should be expressed in both left and right gonads, since *FOXL2* and *CYP19A1* are expressed in both gonads, while *FET1* is only expressed in the left gonad at the time of sexual differentiation (although it could activate genes in the contralateral gonad through indirect signalling). In a female-minus-male subtracted whole embryo cDNA macroarray, Yamada et al. (2004) isolated two novel W-linked sequences in chicken, 2d-2D9 and 2d-2F9. These sequences have no obvious Z-linked homologues. While 2d-2D9 has no obvious motifs, 2d-2F9 has homology to an ATPase superfamily. Both genes are expressed very early in the chicken embryo (from day 2, stages 12–13, before gonad formation) although their exact sites of expression are yet to be determined.

Where are sex-determining genes operating?

The widespread expression of the W-linked gene, *HINTW,* throughout the female chicken embryo, and early expression of the novel sequences 2d-2D9 and 2d-2F9 in whole embryos raise the question of where potential sex-determining genes are operating. In addition to being expressed in the gonads, sex-determining genes could be functioning directly in non-gonadal tissues in a cell autonomous fashion. This seems to apply to sexual differentiation of the avian brain. Scholz et al. (2006) reported sexually dimorphic gene expression in day 4 embryonic chicken brains, pre-dating gonadal sex differentiation and prior to aromatase-mediated estradiol synthesis in females. A number of genes were exclusively or more highly expressed in females, including *HINTW*, the Z-linked MHM non-coding RNA described above, and a novel W-linked sequence, *ABTW* (avian brain W-linked transcript). This suggests direct effects of sex-determining genes in tissues outside the gonads. The existence of gynandromorphic birds also supports this idea. Such birds are bilateral sex chimeras, with male features on one side of the body and female features on the other. In the case of a gynandromorphic zebra finch, the phenotype was female on the left side and male on the right. This included an ovary and female plumage on the left side of the body, with a testis and male plumage on the right side. *HINTW* was only expressed on the left side of the brain, which was feminised (Agate et al., 2003). Genotypically, the left side of the body, including the gonads, was ZW, while the right side was ZZ. It is highly unlikely that such a phenotype could arise from hormonal mechanisms alone, suggesting a direct genetic effect in individual cells. Agate et al. (2003) concluded that, at least in the brain, sexual differentiation is controlled by both the cell's genetic sex and its hormonal environment. This is consistent with the observation that gonadal steroid hormones alone cannot account for sexual dimorphisms seen in the neural song circuit of the brain, in plumage or sexual behaviour (Arnold, 1997; Gahr, 2003). Avian sex-determining genes might therefore directly influence sexual differentiation in tissues independently of the gonads.

Conclusions

Sex determination in birds involves genes acting in the gonads, but the exact nature of the sex determinant(s) and its onset of expression remain undefined. The *DMRT1* gene is currently the most promising candidate gene for avian sex determination under the Z chromosome dosage hypothesis. Meanwhile, the W-linked gene, *HINTW*, represents a very good candidate under the dominant W hypothesis. If either or both of these genes are involved in avian sex determination, their expression profiles in the chicken embryo suggest that they may act well before the onset of gonadal sex differentiation. The widespread expression of *HINTW* outside the urogenital system raises the possibility that avian sex determinants may act directly in tissues independently of the gonads. Under this scenario, sex in birds may be determined by direct genetic as well as hormonal mechanisms. Definitive proof of a role for the current candidates will involve gene over-expression or knockdown in ovo, using avian viral vectors (Chapman et al., 2005; Harpavat and Cepko, 2006). Recently, the isolation and genetic modification of chicken primordial germ cells for the production of transgenic poultry has been reported (van de Lavoir et al., 2006). This paves the way for the production of sex-reversed transgenic birds over-expressing candidate genes such as *DMRT1* and *HINTW*.

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