Expression of Dmrt1 in the Genital Ridge of Mouse and Chicken Embryos Suggests a Role in Vertebrate Sexual Development

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Sex-determining mechanisms are highly variable between phyla. Only one example has been found in which structurally and functionally related genes control sex determination in different phyla: the sexual regulators mab-3 of Caenorhabditis elegans and doublesex of Drosophila both encode proteins containing the DM domain, a novel DNA-binding motif. These two genes control similar aspects of sexual development, and the male isoform of DSX can substitute for MAB-3 in vivo, suggesting that the two proteins are functionally related. DM domain proteins may also play a role in sexual development of vertebrates. A human gene encoding a DM domain protein, DMRT1, is expressed only in the testis in adults and maps to distal 9p24.3, a short interval that is required for testis development. Earlier in development we find that murine Dmrt1 mRNA is expressed exclusively in the genital ridge of early XX and XY embryos. Thus Dmrt1 and Sry are the only regulatory genes known to be expressed exclusively in the mammalian genital ridge prior to sexual differentiation. Expression becomes XY-specific and restricted to the seminiferous tubules of the testis as gonadogenesis proceeds, and both Sertoli cells and germ cells express Dmrt1. Dmrt1 may also play a role in avian sexual development. In birds the heterogametic sex is female (ZW), and the homogametic sex is male (ZZ). Dmrt1 is Z-linked in the chicken. We find that chicken Dmrt1 is expressed in the genital ridge and Wolffian duct prior to sexual differentiation and is expressed at higher levels in ZZ than in ZW embryos. Based on sequence, map position, and expression patterns, we suggest that Dmrt1 is likely to play a role in vertebrate sexual development and therefore that DM domain genes may play a role in sexual development in a wide range of phyla.© 1999 Academic Press

Key Words: sex determination; DM domain; Dmrt1; mab-3; doublesex.

INTRODUCTION

Sexual differentiation is a nearly universal feature of animal development. Surprisingly, however, the genes directing this process are virtually unrelated between phyla, indicating that highly diverse molecular mechanisms have evolved to control sexual development (Cline and Meyer, 1996; Schafer and Goodfellow, 1996). Comparison of sex-determining genes between more closely related species also suggests that these genes may evolve particularly rapidly (de Bono and Hodgkin, 1996; Kuwabara, 1996; Marin and Baker, 1998; Whitfield et al., 1993). An apparent exception is the recent finding that the sexual regulator mab-3 (male abnormal 3) of Caenorhabditis elegans resembles the sexual regulator doublesex (dsx) of Drosophila (Raymond et al., 1998).

Several lines of evidence suggest that the similarity of mab-3 and dsx represents the evolutionary conservation of an ancient sex-determining gene. First, both genes encode proteins with a novel DNA-binding motif called the DM domain (for Doublesex and MAB-3) (Erdman and Burtis, 1993; Raymond et al., 1998). This domain contains conserved cysteines and histidines and chelates zinc, but it is distinct in sequence from other zinc-binding motifs (Erdman et al., 1996). Second, both genes act downstream in their respective sex-determining pathways (Baker and Ridge, 1980; Burtis and Baker, 1989; Shen and Hodgkin,
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Third, although dsx controls more aspects of sexual development than mab-3, both genes are required for the development of sex-specific sense organs and the transcriptional control of yolk protein genes (Bownes, 1994; Shen and Hodgkin, 1988; Yi and Zarkower, 1999). Fourth, the male-specific isoform of the Doublesex protein, DSX\(^ {\ast}\), can functionally substitute for MAB-3 in the peripheral nervous system of C. elegans (Raymond et al., 1998).

If a DM domain gene plays a role in mammalian sex determination, it would be expected to function in the early embryonic gonad. Male sexual development in mammals requires the formation and differentiation of the testis, which then produces the hormones Müllerian inhibiting substance (MIS; also called anti-Müllerian hormone, or AMH) and testosterone (Bogan and Page, 1994; Schafer and Goodfellow, 1996). MIS from Sertoli cells causes degeneration of the Müllerian ducts, and testosterone from Leydig cells promotes development of male sexual characteristics outside the gonad. The central role of the gonad in mammalian sex determination is illustrated by classical experiments. Surgical removal of the embryonic gonad prior to its sexual differentiation causes both XX and XY embryos to develop as females, demonstrating that the gonad is essential for male development (Jost, 1953). Conversely, transplantation of an XY gonad into an XX embryo results in male development, demonstrating that male gonad differentiation is sufficient to promote male development.

A number of genes with roles in sexual development of the mammalian gonad have been identified, and these can be roughly divided into three classes based on their order of action. First, there are genes required for the formation of the sexually indifferent gonad, such as SF1 (Luo et al., 1994) and WT1 (Kreidberg et al., 1993). Second, there are genes that determine whether the indifferent gonad will undergo a male or female mode of differentiation; these include the testis-determining factor on the Y chromosome, Sry (Sinclair et al., 1990), Sox9 (Wagner et al., 1994), and possibly Dax1 (Swain et al., 1998; Yu et al., 1998). Finally, a number of genes promote male or female sexual differentiation of the gonad once its sex has been determined, including SF1 and WT1 (Nachtigal et al., 1998) and Wnt-4 (Vainio et al., 1999). With the exception of Sry, all of these genes are expressed both in the gonad and elsewhere in the embryo and have other developmental roles that are distinct from sexual development.

Limited circumstantial evidence suggests that a DM domain gene may function in mammalian sexual development. We previously identified a DM domain-encoding cDNA clone from a human testis library and named the corresponding gene DMRT1 (doublesex- and mab-3-related transcription factor; formerly called DMT1) (Raymond et al., 1998, 1999). The map position of DMRT1 on chromosome 9 suggests that it may be required for human testis development (Raymond et al., 1998). XY individuals hemizygous for distal chromosome 9p exhibit a high frequency of XY feminization, ranging in severity from ambiguous genitalia to full XY sex reversal, suggesting that one or more genes in this region are needed in two copies for normal testis development (Bennett et al., 1993; Wilkie et al., 1993). Recent studies have narrowed the critical region for sex reversal to the most distal portion of 9p24.3, the region containing DMRT1 (Flejter et al., 1998; Guioli et al., 1998; Raymond et al., 1999; Veltia et al., 1997, 1998).

In birds, the chromosomal sex determination mechanism is distinct from that of mammals, with the female (ZW) being the heterogametic sex and the male (ZZ) the homogametic sex. Because avian sex determination is less extensively studied, it is unclear how many of the same factors control sex determination in mammals and birds. However, several differences are apparent. No sex-specifically expressed avian Sry equivalent has been identified and no sex-determining genes have been identified on the avian sex chromosomes. Sox9, which is autosomal, is expressed at higher levels in the male embryonic gonad of chickens, but Sox9 expression appears not to precede sexual differentiation (Kent et al., 1996; Moraes da Silva et al., 1996; Oreal et al., 1998; Smith et al., 1999).

Recently a DMRT1 homologue from chicken has been shown to be localized on the Z chromosome, which has extensive conserved synteny with human chromosome 9, including 9p24 (Nanda et al., 1999). ZZ embryos have a higher dose of the Dmrt1 gene than ZW embryos and thus potentially higher Dmrt1 expression. It has been proposed by Nanda et al. (1998) that higher expression of Dmrt1 in the avian embryo could lead to male development, while lower expression could lead to female development, much as we have suggested for human 9p deletions (Raymond et al., 1999).

Recent work suggests that nematodes and insects are more closely related than was previously believed (Agui-naldo et al., 1997); as a consequence molecular mechanisms identified in nematodes and insects are not necessarily expected to be found in more distantly related phyla such as vertebrates. Thus finding a sex-determining DM domain gene in a vertebrate would suggest that the involvement of such genes in sex determination predated the divergence of the ecdysozoa from the deuterostomia (Valentine et al., 1999). In addition, there are substantial differences in the biology of sex determination between vertebrates and invertebrates. In nematodes and insects, cells throughout the animal assess the sex chromosome composition and set the activity state of sex-determining regulatory pathways (reviewed by Cline and Meyer, 1996), whereas in mammals the primary events in sex determination appear to occur exclusively in the gonad (reviewed by Bogan and Page, 1994). For these reasons, involvement of DM domain genes in sexual development of vertebrates, nematodes, and arthropods, with their distant evolutionary relationships and extensive differences in sex-determining mechanisms, would imply a very widespread role for these genes among metazoan phyla.

Here we investigate the role of DMRT1 in vertebrates by characterizing the expression of homologues from mouse and chicken. We find that Dmrt1 mRNA is detectable in
the mouse embryo by E9.5 and is expressed exclusively in the genital ridges of both sexes from early stages of gonad formation. During gonad sexual differentiation Dmrt1 mRNA expression is maintained only in the XY gonad and becomes restricted to the developing seminiferous tubules of the testis. In chicken, Dmrt1 also is expressed in the early genital ridge, as well as Wolffian ducts. Expression is detectable by stage 19, prior to sexual differentiation, and Dmrt1 mRNA is more abundant in ZZ than ZZ gonads. Based on these results we suggest that in mammals and birds, as in nematodes and insects, DM domain containing genes are likely to play a role in sexual development.

MATERIALS AND METHODS

Genetic Nomenclature

We have used standard genetic nomenclature for Dmrt1 and, where possible, other genes. Specifically, italics are used to denote gene names and plain text for protein names (for example, Dmrt1 versus Dmrt1). The human gene name is entirely capitalized (DMRT1) and mouse and chicken genes have only the first letter capitalized (Dmrt1). To distinguish between mouse and chicken Dmrt1, we have specified in each case which gene is being discussed.

RT-PCR and RNA Blotting

Mouse tissues were dissected and total RNA isolated using the Trizol Reagent protocol (Life Technologies). cDNA was synthesized from 5 μg of total RNA by reverse transcription with Superscript II polymerase (Life Technologies) according to the manufacturer’s instructions, using random hexanucleotide primers (Life Technologies). Amplification was performed using 1 or 2 μl of cDNA reaction. An initial denaturation was performed for 1 min at 94°C, followed by 30 rounds of amplification at 94°C for 30 s, 55 to 65°C for 30 s, and 72°C for 30 to 60 s. All PCRs contained 10% DMSO. PCR fragments were analyzed on 1% agarose gels. Primers for amplification of Dmrt1 were the forward primer CR86 (CCGCCTCAAGGGCCACAAGCGC) from exon 1 and the reverse primer CR111 (CTCGAGGAGACTCAGACGAGG) from exon 3. Control RT-PCRs were performed using Hprt primers HprtF (CAGGACCTCTCGAAGTGGTAGT) and HprtR (GCATTAAAAGGAACTGGTGCAACG).

For RT-PCR from chicken embryos, cDNA synthesis was as described above. Dmrt1 amplification was for 40 cycles with an annealing temperature of 65°C in reactions containing 10% DMSO. PCR fragments were analyzed on 1% agarose gels. Primers for amplification of Dmrt1 cDNA were CD8RT1F (GAGGGAGGATGCTCTAGACGACGAGG) and CD8RT1REV (CTGGCCCAGGTATGAGGTGGGCGGGTAG). Amplification conditions were the same as described for other RT-PCRs except that annealing temperature was 60°C. The resulting 386-bp fragment, encoding the portion of Dmrt1 C-terminal to the DM domain, was cloned into the pCR2.1-TOPO vector (Invitrogen) to generate pcR4 (antisense orientation) and pcR5 (sense orientation). Probes for in situ hybridization were made by cutting the resulting plasmids with BamHI and transcribing with T7 RNA polymerase in the presence of digoxigenin-11-UTP, as described below.

In Situ Hybridization

For whole-mount in situ hybridizations, mouse embryos were harvested from pregnant CD1 females and fixed overnight in 4% paraformaldehyde at 4°C. For embryo staging, the morning of plug formation was designated day E0.5. After fixation, genital ridges or embryonic gonads were dissected from embryos, and whole-mount in situ hybridizations were performed as described (Henrique et al., 1995). Digoxigenin-11-UTP-labeled Dmrt1 antisense- and sense-strand RNA probes were synthesized using the Maxiscript in vitro transcription kit (Ambion). Hybridization was carried out overnight at 65°C and visualized with BM purple (Boehringer). Chickens in situ hybridization was performed using the same method. Chicken embryos were staged as described (Hamburger and Hamilton, 1951). Kit+/Kit++ male mice were purchased from The Jackson Laboratory.

For in situ hybridization to embryo sections, embryos were fixed in 4% paraformaldehyde overnight, dehydrated through an ethanol series, and embedded in paraffin. Paraffin blocks were cut into 5-μm sections, dewaxed in xylene, and rehydrated in phosphate-buffered saline. In situ hybridization with the DIG-labeled anti-sense RNA was carried out as described above. Sections were counterstained in 1% neutral red or eosin. For radioactive in situ hybridization, probes were labeled with [3H]UTP and hybridization
was performed as described (Coucouvanis and Martin, 1999). Sections were counterstained as indicated.

**Embryonic Germ Cell Purification**

Mouse germ cells were isolated by flow cytometry from pools of 10–12 gonads dissected from E13.5 Oct4:PEGFP transgenic male embryos as described (Anderson et al., 1999). Germ cell RNA was confirmed by amplification of endogenous Oct4 mRNA with primers Oct4F (GATGGCATATTGAGAGCTGGT) and Oct4R (CTGATGGCGGATGTGATGCTG). Contamination with somatic cells was tested by amplification of Steel cDNA with primers SteelFOR (GAGCTCCAGAAAACAGCTAAACGGGAGTCG) and SteelREV (CTGCCAATTACCCTCTCTTGAAATTCTCTCTCG).

**Sexing of Chicken Gonad DNA**

DNA was isolated from gonads/mesonephri by incubation at 55°C in TENS buffer (100 mM Tris, pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl) containing 100 μg/ml proteinase K, followed by phenol/chloroform and chloroform extraction and ethanol precipitation. DNA was resuspended in H2O at 100 ng/μl. To determine the chromosomal sex of the isolated DNA, we used primers spanning an intron of the CHD gene that differs in length by 17 bp between Z and W chromosome alleles (Griffiths et al., 1998). One microliter of DNA was amplified for 40 cycles with an annealing temperature of 58°C using primers CHDFORNEW (CAAGGATGATCCAGAATATCTTCTGC), and 2/5 of the reaction was separated by electrophoresis in 3% NuSieve GTG agarose.

**Fluorescence in Situ Hybridization (FISH) Mapping**

FISH was performed using standard protocols (Trask, 1997) and a Dmrt1 genomic lambda phage clone labeled by nick translation with digoxigenin-11-dUTP (Boehringer Mannheim) to probe metaphase chromosomes from 129/SvJ embryonic stem cells. Hybridization was detected with rhodamine-anti-digoxigenin. Signals were further amplified using rabbit anti-sheep and rhodamine anti-rabbit. Chromosomes 19 were identified by hybridization to a biotin-labeled chromosome 19-specific telomere probe ( Oncor) and detected with fluorescein-conjugated avidin. Chromosomes were counterstained with 4,6-diamidino-2-phenylindole. Twenty metaphase cells were examined using fluorescence microscopy and images were captured using Cytovision software (Applied Imaging). Comparison of mouse and human chromosome maps was based on data from the Mouse Genome Database (http://mgd.niai.affrc.go.jp/bin/query_homology).

**RESULTS**

**A Murine DMRT1 Homologue on Chromosome 19**

To investigate expression of DMRT1 in the embryo, we first used a degenerate PCR strategy to clone a murine homologue. Based on comparison of the DM domains of the DMRT1, MAB, and DSX proteins, we designed degenerate primers complementary to the most highly conserved sequences in the DM domain and amplified the Dmrt1 DM domain from a testis cDNA library (Materials and Methods). This DM domain cDNA fragment was then used as a probe to isolate a cDNA clone containing the full Dmrt1 coding sequence. The DM domains of mouse Dmrt1 and human DMRT1 are highly similar to those of MAB-3 and DSX, but outside the DM domain the only similarity among all of these proteins is the presence of a proline- and serine-rich region (Fig. 1A). The function of these proline- and serine-rich domains is unknown, but we speculate that they might mediate transcriptional regulation and/or protein–protein interaction. The predicted proteins encoded by Dmrt1 and DMRT1 are 98% identical in the DM domain and 82% identical over the entire coding region (Fig. 1B). Introns were identified by comparison of genomic and cDNA sequences, and all occur at the same positions as in DMRT1 (not shown).

We used FISH to map Dmrt1 and found that a Dmrt1 genomic DNA clone hybridizes to the central region of chromosome 19, at band C-2 or C-3 (Fig. 1C, and data not shown). Conserved synteny between mouse chromosome 19 and human distal chromosome 9p suggests that the mouse and human genes are at equivalent chromosomal locations. Based on sequence similarity, splice site position, and chromosomal position, we conclude that Dmrt1 is a murine homologue of DMRT1.

**Sex-Specific Expression of Dmrt1 mRNA in the Adult Gonad**

To test whether murine Dmrt1 mRNA is sex-specifically expressed in adult tissues, we used RT-PCR to analyze tissues from both sexes (Fig. 2). A Dmrt1 fragment was detected in testis cDNA, but not in ovary or in cDNA from five other tissues from both sexes (Fig. 2, top). As a positive control, we amplified the same cDNA samples with primers specific to Hprt and found that similar amounts of product were amplified from all of the tissues (Fig. 2, bottom). Thus murine Dmrt1 mRNA, like that of its human homologue DMRT1, is expressed in the adult testis but is not detectable in the ovary or in any other tissues tested.

**Early Expression of Murine Dmrt1 mRNA Is Restricted to the Genital Ridge**

The testis-specific adult expression of DMRT1 suggests a possible function in adult testis, but the key events in sex determination and gonad formation occur much earlier, during embryogenesis. We therefore examined embryonic expression of murine Dmrt1 mRNA using whole-mount in situ hybridization. In mouse the gonad primordium, the genital ridge, first becomes morphologically distinct at about day E10.5 in both XX and XY embryos. Sexual differentiation of the gonad becomes apparent at about E12 and continues for several days. Sex-determining genes therefore are expected to be expressed in the developing
gonad prior to E12, and genes involved in formation of the bipotential gonad should be expressed earlier, at or before E10.5.

During embryonic development, in situ hybridization detects Dmrt1 mRNA in the gonad, starting at the time of the initial appearance of the genital ridge and continuing through sexual differentiation of the gonad (Fig. 3). As a control, we also tested a sense RNA probe from the same template and detected no hybridization (data not shown).

At E10.5 and E11.5, as the genital ridges form from the surrounding intermediate mesoderm, Dmrt1 mRNA expression is detectable at similar levels in the genital ridges of both XX and XY embryos. At E12.5 and E13.5, as sex-specific structures are becoming apparent in the gonad, Dmrt1 mRNA is expressed in the developing sex cords of the testis as well as in a punctate pattern in the ovary. At E14.5 and E15.5, Dmrt1 mRNA expression declines in the ovary but is maintained in the testis.

We also assayed Dmrt1 expression by RT-PCR using RNA isolated from embryos and embryonic gonads (Fig. 4). Dmrt1 expression is detectable by RT-PCR at E9.5 and E10.5 in whole embryos and in mixed XX and XY gonads at E11.5, E12.5, and E13.5 (Fig. 4, left). Low expression at E9.5 and E10.5 is probably due to the use of whole embryos rather than dissected gonads at later stages. At E14.5, expression is slightly lower in ovaries relative to testes and

FIG. 1. Dmrt1 resembles DSX of Drosophila and MAB-3 of C. elegans and maps to chromosome 19. (A) Diagrams of the Dmrt1, DSX, and MAB-3 proteins. dsx is alternatively spliced to form mRNAs that encode the sex-specific proteins DSX^F and DSX^M. DM domains are indicated by filled boxes, and serine/proline-rich domains are indicated in gray. The female- and male-specific domains of the DSX proteins are indicated in red and blue, respectively. (B) Dmrt1 maps to chromosome 19. Mouse metaphase chromosomes hybridized with digoxigenin-labeled Dmrt1 genomic lambda phage probe (red) and chromosome 19-specific telomeric probe (green). Dmrt1 maps to the central region of chromosome 19 (arrows). (C) Sequence alignment of human DMRT1 and mouse and chicken Dmrt1. DM domain and proline/serine-rich region (P/S) are overlined. Chicken Dmrtl sequence is derived from a partial cDNA clone (accession number AF123456; Nanda et al., 1999) with three apparent sequence frameshifts corrected.

FIG. 2. Sex-specific expression of Dmrt1 in the adult gonad. (Top) RT-PCR of Dmrt1 from five tissues isolated from male (M) or female (F) adult mice, and from adult ovary and testes, using intron-spanning primers. (Bottom) Control RT-PCR of the same cDNA samples as in A, using intron-spanning Hprt primers. The Dmrt1-specific PCR primers used are complementary to sequences from the first and third exons of the Dmrt1 mRNA and amplify a fragment of 467 bp (Materials and Methods).
FIG. 3. Dmrt1 mRNA expression in embryonic genital ridge and gonads. (A) Whole-mount in situ hybridization. Embryonic day postcoitus is indicated. Upper left: E10.5 dissected embryo, including genital ridges and dorsal body wall. Dmrt1 mRNA expression (purple) is detected in genital ridge. Middle left: E11.5 genital ridges and mesonephri dissected from dorsal body wall. Lower left: E12.5 XY and XX gonads and mesonephri. At this and later stages expression of Dmrt1 mRNA is detected only in the gonad. Upper right: E13.5 XY and XX gonads and mesonephri. Middle right: E14.5 XY and XX gonads and mesonephri. Lower right: E15.5 XY and XX gonads and mesonephri. Scale bars, 0.5 mm. T, testis; O, ovary.
We performed several additional experiments to examine the cell types expressing Dmrt1. To confirm the primordial germ cell (PGC) expression, we purified PGCs from dissociated E13.5 gonads by flow cytometry and performed RT-PCR on the purified PGC RNA (Fig. 6A). PGCs were isolated from embryos of a transgenic mouse strain homozygous for a GFP reporter under the control of the germ cell-specific Oct4 promoter by selecting GFP-expressing cells from dissociated gonads (Anderson et al., 1999). For controls, we confirmed that the isolated cells are pure PGCs by showing that they do express the PGC-specific endogenous Oct4 mRNA and do not express the somatic cell-specific Steel mRNA. Dmrt1 mRNA is readily detectable in the purified PGCs.

To confirm expression of Dmrt1 in the Sertoli cell lineage, we performed two additional experiments. First, we isolated mRNA from adult Kit<sup>W</sup>/Kit<sup>W</sup> mutant testes and assayed Dmrt1 expression. Homozygous Kit<sup>W</sup> mutations cause failure of proliferation and migration of PGCs, resulting in testis cords containing mainly Sertoli cells (Buehr et al., 1993). In the mutant testes we found by Northern blot analysis (Fig. 6B) that expression of Dmrt1 is not reduced relative to wild type, indicating that Dmrt1 is expressed in somatic cells of the adult testis. Second, we performed in situ hybridization on wild-type and Kit<sup>W</sup>/Kit<sup>W</sup> adult testes. In wild-type testes (Figs. 6C and 6D), cells with Sertoli cell morphology located around the periphery of the seminiferous tubules express Dmrt1. Strong expression of Dmrt1 in Kit<sup>W</sup>/Kit<sup>W</sup> testis (Fig. 6E) confirms that at least some of the cells expressing Dmrt1 in the adult testis are Sertoli cells. In summary, both Sertoli cells and germ cells express Dmrt1, suggesting that the gene may play a role in the development or function of both cell types.

Expression of Dmrt1 in Chicken Embryos Suggests a Role in Sexual Development

The expression of Dmrt1 mRNA in the embryonic mouse gonad suggests a likely role for the gene in its development, sex determination, or sexual differentiation. Since birds have a distinct chromosomal sex determination mechanism (ZZ males; ZW females) and chicken Dmrt1 is Z-linked (Nanda et al., 1999), we also investigated the expression of Dmrt1 in the chicken embryo. We performed whole-mount in situ hybridization using a chicken Dmrt1 cDNA probe on embryos from stage 25, when the genital ridges are forming, to stage 31, when sexual differentiation is beginning (Figs. 7A–7C). Dmrt1 expression is detectable in the genital ridge at stage 25 (Fig. 7A). We also detected expression from stage 25 in the Wolffian ducts, the progenitors of male-specific internal reproductive structures (Fig. 7D; data not shown). About half of the embryos examined had stronger Dmrt1 expression in the genital ridge, and half had weaker expression, suggesting that expression might be higher in one sex than the other. These differences in expression were apparent at stage 25. We confirmed that the two sexes have different levels of Dmrt1 expression by...
using a PCR assay (Griffiths et al., 1998) to determine the chromosomal sex of strongly staining gonads and weakly staining gonads from stage 31 embryos (Fig. 7E). The stronger staining is in the ZZ gonads, and thus higher Dmrt1 expression in the bipotential gonad correlates with male development in birds.

In situ hybridization detects Dmrt1 at stage 25, while MIS, an early marker of testis differentiation, is reported to be expressed starting at stage 25–28 (Oreal et al., 1998; Smith et al., 1999). This suggests that Dmrt1 might be expressed prior to MIS and thus prior to sexual differentiation. To test this possibility, we assayed expression of Dmrt1 and MIS in RNA from dissected ZZ and ZW embryos using RT-PCR (Fig. 8). Dmrt1 expression is detectable by stage 19 but we did not detect significant MIS expression until stage 29, similar to previous reports (Oreal et al., 1998; Smith et al., 1999). In summary, chicken Dmrt1 is expressed in the genital ridge and its expression precedes sexual differentiation. Higher expression in ZZ than in ZW embryos suggests the possibility that Dmrt1 may be a dose-sensitive masculinizing gene in birds.

DISCUSSION

From data presented here and elsewhere, DM domain proteins appear likely to be involved in sexual regulation in at least three phyla. In nematodes and arthropods, the evidence is unambiguous: mab-3 is essential for male sexual differentiation of C. elegans, and dsx is essential for male and female sexual differentiation in Drosophila (Baker and Ridge, 1980; Shen and Hodgkin, 1988). The similar developmental roles (regulation of yolk protein transcription and sense organ differentiation), downstream action in sex-determining regulatory pathways, and functional interchangeability of mab-3 and dsx suggest that the sequence similarity of these two genes is likely to reflect the evolutionary conservation of sex-determining mechanisms between nematodes and insects.

We have presented several lines of evidence indicating that the DM domain gene Dmrt1 may have a role in vertebrate gonad formation and/or sex determination. First, Dmrt1 is related in sequence to known sex-determining genes from two other phyla. This evidence on its own is not compelling, as it is highly unlikely that all DM domain genes are involved in sex determination (Meng et al., 1999). More suggestively, however, murine Dmrt1 expression is found exclusively in the genital ridge as soon as that structure is morphologically distinct and later becomes restricted to the sex cords of the testis. Thus murine Dmrt1 mRNA is expressed at the appropriate time and place to play a role in directing sexual development and is not detectable anywhere else. The human DMRT1 gene is located in a short chromosomal interval (estimated at 250

FIG. 5. Dmrt1 mRNA expression is restricted to the embryonic gonad. (A) Nonradioactive in situ hybridization to section of E13.5 XY embryo, counterstained with neutral red. Expression (purple) is restricted to sex cords of differentiating testis. (B and C) Radioactive in situ hybridization to sections of E13.5 XY embryo. (B) Bright-field view of section stained with hematoxylin, with testis in center. (C) Dark-field view of same field, showing hybridization only to sex cords of testis. (D) Higher magnification view of section shown in A, hybridized with Dmrt1 probe. Nomarski optics. Expression is limited to the sex cords and is detected in virtually all cells, including pre-Sertoli and germ cells. (E) In situ hybridization of Sox9 probe (gift from H. Ingraham) to another section of the same embryonic testis, Nomarski optics. Expression is detected in cells with pre-Sertoli morphology located mainly around the periphery of the sex cord.
FIG. 6. Germ cell and Sertoli cell expression of Dmrt1. (A) RT-PCR of RNA from purified primordial germ cells (PGCs). cDNA was synthesized from purified PGC or non-PGC populations of cells isolated by flow cytometry from dissected E13.5 testes and amplified with primers specific to the PGC-specific gene Oct4 (left), the somatic cell-specific gene Steel (middle), or Dmrt1 (right). Reactions were done from mock cDNA syntheses (–RT) and compared to real syntheses (+RT). The isolated PGCs were collected based on expression of an Oct4:GFP transgene, and as expected they express Oct4, but not the somatic marker Steel. PGCs express Dmrt1 (far right lane). The non-PGC cell population expresses Steel (middle), confirming that the primers can efficiently amplify the Steel cDNA. (The somatic cell pools are always contaminated with low levels of PGCs, and thus this method cannot be used to test expression of Dmrt1 in somatic cells.)
which, when monosomic, is associated with male gonad dysgenesis and 46,XY sex reversal (Raymond et al., 1999).

Finally, in chickens, Dmrt1 is Z-linked and is expressed in the bipotential gonad at higher levels in ZZ than in ZW embryos. DM domain genes clearly are required for sex determination in nematodes and insects, which belong to ecdysozoan (molting) phyla and are relatively closely related. This suggests that other ecdysozoan animals are likely to have DM domain proteins with similar roles. If Dmrt1 plays a role in sex determination in the more distantly related vertebrates, it follows that similar genes are likely to be widely involved in sex determination in other bilaterian phyla (Aguinaldo et al., 1997; Valentine et al., 1999).

The gonad-specific expression of Dmrt1 in the mouse is particularly notable, as the only sex determination gene

FIG. 7. Expression of Dmrt1 in chicken genital ridge and Wolffian duct. (A) Stage 25, expression in genital ridge and Wolffian duct. The two middle embryos show higher Dmrt1 expression than the outer embryos. Wolffian duct expression is visible at left edge of second embryo from right. (B) Stage 28/29. Embryos show typical high and low expression of Dmrt1. Higher expressing embryos are presumed to be ZZ, but molecular sexing has been performed only on stage 31 gonads. (C) Stage 31. Expression is higher in ZZ gonad (right) than ZW gonad (left). (D) Stage 31, dorsal view, showing expression in Wolffian ducts. (E) Chromosomal sexing of stage 31 gonads. In situ hybridization to detect Dmrt1 was performed. DNA was then extracted from gonads with high or low expression and amplified as described under Materials and Methods. Amplification from one high- and one low-expressing gonad is shown. High-expressing gonads are from ZZ embryos (left lane) and low-expressing gonads are from ZW embryos (right lane).
known to be expressed exclusively in the gonad prior to sexual differentiation is Sry. Sry expression is restricted to XY embryos by virtue of its location on the Y chromosome, but it is unknown what restricts its expression to the genital ridge. An intriguing speculation is that Dmrt1 might activate Sry transcription in the genital ridge. The activation of Sry transcription by Dmrt1 would explain both the XY-specific phenotype of human 9p deletions and the gonad-specific expression of Sry. Consistent with such a possibility, we have detected Dmrt1 expression at E9.5, when the genital ridge begins to form, while Sry expression begins around E10.5 (Hacker et al., 1995).

Murine Dmrt1 mRNA initially is expressed at similar levels in the developing gonads of both XX and XY embryos. Dmrt1 might play a role in gonad development in both sexes, but there are two indications that mammalian Dmrt1 may be more important in testis development than in ovary development. First, Dmrt1 mRNA disappears during ovary differentiation. If Dmrt1 protein levels also drop in the ovary, any function of Dmrt1 in the later stages of gonad differentiation is likely to be testis-specific. In this regard Dmrt1 may resemble mab-3, which initially is expressed in both sexes, but becomes male-specific and is required only for aspects of male sexual development (Shen and Hodgkin, 1988; Raymond et al., 1998; W. Yi and D.Z., unpublished results). Second, while monosomy of the region of 9p containing DMRT1 causes XY sex reversal, 9p deletions have not been reported to affect XX gonadogenesis. Thus, if DMRT1 is the gene responsible for 9p sex reversal, it is likely that it is required in two copies only for testis development. In this respect DMRT1 in human is reminiscent of Dmrt1 in chicken, in which two copies of the gene and the consequent higher expression correlates with male development. There are other mechanisms by which Dmrt1 might function only in development of the XY gonad. For example, it might be translated or otherwise posttranscriptionally activated only in the XY gonad. Similarly, an XY-specific coregulator, such as Sry or Sox9, might be required for activity of Dmrt1, restricting its function to the developing testis. Finally, Dmrt1 activity might be blocked in the XX gonad by a female-specific gene.

Sex-specific expression of Dmrt1 in the bipotential chicken gonad makes this gene an especially good candidate to be involved in avian sex determination. The only other potential sex-determining gene identified in birds so far is Sox9. While murine Sox9 expression precedes the first signs of sexual dimorphism, it appears that avian Sox9 is not expressed prior to expression of MIS (Kent et al., 1996; Morais da Silva et al., 1996; Oreal et al., 1998; Smith et al., 1999). Thus in birds Sox9 appears unlikely to have a primary sex-determining role. Dmrt1 is expressed sex-specifically in the avian gonad prior to sex-specific MIS expression, and thus could potentially play a role in sex determination. Higher expression in the ZZ than in the ZW bipotential gonad makes Dmrt1 an obvious candidate to be a dose-sensitive sex-determining element on the Z chromosome. Indeed, Dmrt1 is the first Z-linked gene found to be expressed in the early gonad. Z chromosome inactivation appears not to occur in birds (Schmid et al., 1989), and there may be no Z chromosome dosage compensation mechanism (Baverstock et al., 1982). Our data indicate either that birds do not have Z chromosome dosage compensation or that Dmrt1 escapes such regulation.

DM domain genes are probably involved in a variety of developmental processes. From the data presented here, we suggest that among these processes is likely to be the regulation of vertebrate sexual development.

ACKNOWLEDGMENTS

We thank numerous colleagues for helpful discussions and other assistance, including Drs. Harry Orr, David Largaespada, Steven McLoon, Holly Ingraham, and Robert Anderson for valuable advice and materials, and Nduka Oemega for supplying chicken eggs. We especially thank Dr. Electra Coucouvanis for technical assistance and for critical reading of the manuscript. This work was supported by grants from the University of Minnesota Graduate School (D.Z. and V.J.B.), the STAGE program of the University of Minnesota (J.R.K.), the Minnesota Medical Foundation (D.Z.), and the NIH (D.Z., V.J.B.). C.S.R. was supported by an NIH predoctoral training grant.

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Received for publication June 28, 1999
Revised August 19, 1999
Accepted August 19, 1999