Male-biased expression of X-chromosomal DM domain-less Dmrt8 genes in the mouse

Anne-Marie Veith a,1, Jürgen Klattig b,1, Agnes Dettai a, Cornelia Schmidt a, Christoph Englert b, Jean-Nicolas Volff a,⁎

a Department of Physiological Chemistry I, Biozentrum, University of Würzburg, Am Hubland, D-97074 Würzburg, Germany
b Leibniz Institute for Age Research–Fritz Lipmann Institute, Jena, Germany

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Abstract

The vertebrate DMRT gene family encodes putative transcription factors related to the sexual regulators Doublesex (Drosophila melanogaster) and MAB-3 (Caenorhabditis elegans). They share a highly conserved DNA binding motif, the DM domain. In human and mouse seven DMRT genes (DMRT1–DMRT7) have been analyzed. DMRT8, a gene related to DMRT7, is located on the X chromosome in placental mammals. While DMRT8 is single copy in most mammals, three copies are present in mouse, rat, and rabbit. Despite the loss of the DM domain, DMRT8 genes have been maintained in the mammalian lineage, suggesting a DM domain-independent function. In adult mouse, two Dmrt8 genes are expressed exclusively in testis. Dmrt8.1 mRNA was detected in Sertoli cells by in situ hybridization. In embryos, Dmrt8.2 shows a dynamic expression restricted to male and female gonads and might therefore be involved in sexual development in the mouse.

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Introduction

Over the past few years the DMRT gene family (DMRT: DSX- and MAB-3-related transcription factor) has come to the fore because some of its members are involved in sex determination/differentiation in several metazoan phyla, representing the first known examples of conserved sexual regulatory genes [1,2]. Members of the DMRT gene family share a highly conserved protein motif, the DM domain. The DM domain is a small DNA binding motif that contains two distinct parts: first, an N-terminal DNA binding part containing six cysteines and two histidines at invariant positions, forming two intertwined zinc-finger-like DNA binding modules, and second, a C-terminal disordered tail, which acts as recognition helix for DNA binding. Unlike classical zinc finger modules, the DM domain binds into the minor groove of the DNA [3,4].

The most prominent member of the vertebrate DMRT gene family is DMRT1. In human, DMRT1, DMRT2, and DMRT3 are physically clustered on chromosome 9p24. Mutations and deletions in this region are associated with male-to-female sex reversal [5–7]. DMRT1 has been suggested to play a role during sexual development in a variety of vertebrate species, including fish [8–12], frogs [13], lizards and turtles [14,15], birds [16–18], and mammals [5,19–21]. In the teleost fish medaka (Oryzias latipes), two dmrt1 genes are present. In addition to dmrt1a, which is clustered together with dmrt2 and dmrt3 on linkage group 9 [10], an additional copy named dmrt1b or dmy is located in the sex-determining region on the Y chromosome, but not on the X chromosome. This copy shows attributes of a recently evolved master male sex-determining gene [22–25]. No orthologue of dmrt1b was found in more distantly related fish species, indicating that this gene is not a general master sex-determining gene in fish [26,27]. In birds, which have a chromosomal sex-determining system with ZZ males and ZW...
females, DMRT1 is located on the Z chromosome but not on the W. The expression of DMRT1 in the genital ridges prior to gonadal differentiation, with a higher expression in male embryos, is compatible with a role for DMRT1 as the male sex-determining gene in birds [16–18]. The observation that DMRT1 is Z-linked in birds and that the Y-chromosomal dmrt1by is the male-inducing factor in the fish medaka suggests that in some nonmammalian species a DMRT1 gene has played a role comparable to that of the mammalian male sex determination gene SRY (sex-determining region on the Y) during the evolution of sex chromosomes [18,24,25].

Recent analysis of other members of the DMRT gene family in mouse (Mus musculus), chicken (Gallus gallus), frog (Xenopus laevis), and fish (medaka, zebrafish Danio rerio) indicated that they show restricted and nonoverlapping expression patterns during embryogenesis. DMRT2 in mouse, chicken, and fish has an expression pattern consistent with a function during somitogenesis [29–32]. DMRT3 is expressed in a subset of dorsal interneurons in mouse, chicken, and medaka [31,33] and DMRT4 is expressed in the developing olfactory system in medaka as well as in the frog X. laevis [31,34]. Expression of Dmrt5 is detectable during embryogenesis in the brain of mouse [30]. In addition to dmrt1, some other DMRT genes also show gonadal expression in fish. Medaka dmrt2–4 share an expression in larval and adult gonads, but not during early gonadal development [31].

In mouse, three DMRT genes other than Dmrt1 (Dmrt3, Dmrt4, and Dmrt7) show an expression pattern consistent with a role in gonadal development [30]. Dmrt3 is expressed in interstitial cells of the testis. Dmrt4 is detected in gonads of both sexes and Dmrt7 is expressed in somatic and germ cells in the ovary, but solely in germ cells in testis [30]. Partial redundancy of these genes with Dmrt1 during gonadal development might explain the relative mild phenotype observed in Dmrt1-null mutant mice [20,30].

Another putative DMRT gene related to DMRT7 called DMRT8 (aka DMRTC1) is present on the X chromosome in human and mouse [35]. In contrast to all other known DMRT genes, the DM domain-encoding sequence of DMRT8 is not conserved in human and mouse. Based on comparison of human cDNA and genomic DMRT8 sequences, it has been doubted whether DMRT8 has the potential to encode functional DM domain-containing proteins in any species [30,35]. Here, we report the presence of several Dmrt8 genes on the X chromosome of mouse and analyze their expression and evolution in the mammalian lineage.

Results

**DMRT7 and DMRT8: two putative mammalian-specific DMRT genes**

DMRT7 and DMRT8 sequences from human and mouse [30,35] were used as initial queries in a BLAST analysis of public sequence databases (Table 1). Complete sequences for both DMRT7 and DMRT8 were identified in the genome drafts of chimpanzee, rhesus monkey, dog, cow, mouse, and rat. Partial DMRT7- and DMRT8-encoding sequences were detected for several other placental mammals (Table 1). In marsupials (opossum Monodelphis domestica) and monotremes (platypus Ornithorhynchus anatinus), only Dmrt7 could be identified. In nonmammalian species neither Dmrt7 nor Dmrt8 could be found despite using all available sequence resources, suggesting that these genes are mammalian-specific.

Most database information available for DMRT7- and DMRT8-encoding sequences corresponded to four distinct regions, which are conserved in DMRT7 and DMRT8 among species (regions I–IV, Table 1, Fig. 1a). Region I (75 aa) and region II (52 aa) include the DM domain (Figs. 1b and 1c). Regions III (35 aa) and IV (48 aa) are located downstream of the putative DM domain in the carboxy-terminus of DMRT7 and DMRT8. Region III and region IV are conserved (48–60% amino acid similarity) between DMRT7 and DMRT8, but not in other DMRT proteins, thus confirming a close relationship between DMRT7 and DMRT8 [2–35].

**DMRT8 is located on the X chromosome in mammals and triplicated in rabbit and rodents**

In all species for which information about chromosomal location is available (human, chimpanzee, mouse, rat, and dog), the DMRT8 genes are located on the X chromosome, but not on the Y chromosome, while DMRT7 is always located on autosomes (Table 1). This suggests that the common ancestor of all mammalian DMRT8 genes was X chromosomal. In contrast to the situation observed for most mammals, three Dmrt8 genes are present in rabbit, mouse, and rat. Phylogenetic analysis suggests that serial events of duplication occurred in the rabbit and rodent lineages after their divergence from other mammals, approximately 77 million years ago [36]. In rodents, triplication of Dmrt8 arose before the split between rat and mouse (Fig. 1d). The three Dmrt8 genes in mouse form an X-linked Dmrt8 gene cluster (Fig. 2a).

**Structural comparison and coding potential of DMRT7 and DMRT8**

Sequence comparison of DMRT7 genomic and cDNA sequences showed that the DM domain-encoding sequence is separated by a small intron (Figs. 1a and 2b, size of this intron is 83 bp in human and 74 bp in mouse). Analysis of region I and of region II (Figs. 1b and 1c) revealed that in DMRT8 a putative DM domain-encoding sequence is present and conserved between most investigated species (50–60% amino acid similarity). Similar to DMRT7, an intron separates the DM domain-encoding sequences of DMRT8 (81 bp in mouse Dmrt8.2). Thus, in DMRT7 and DMRT8, two separate exons encode the putative DNA-binding modules and disordered tail of the DM domain (Figs. 1b and 1c). The presence of an intron in the DM domain-encoding sequence is not unique for DMRT7 and DMRT8, since a similar situation has been also described for DMRT1 and medaka dmrt1by [10,12,22]. However, the position of this intron is not conserved in DMRT1 and DMRT7/8, suggesting an independent origin.
Based on their common sequence characteristics, we identified four orthologous groups of DMRT8 sequences (Fig. 1d). The first group consists of DMRT8 from primates (apes and other Old World monkeys). All amino acids at invariant positions of the DM domain are present, but in genomic DNA from the three apes (human, chimpanzee, and orangutan) the open reading frame of the potential DM domain of DMRT8 is disrupted by a stop codon (Fig. 1b). In the human DMRT8 cDNA sequence AJ291670 [35], the intronic sequence that separates the DM domain is retained in the cDNA. Consequently, the open reading frame is disrupted by a stop codon within the intronic sequence. Sequence comparison of human DMRT8 cDNA and genomic DNA of DMRT8 from all investigated primate species indicates that the donor splice site downstream of the zinc-finger module-encoding exon (region I) is mutated (GT to AT). A putative translation start site has been identified downstream of the DM domain-encoding region [35] (Fig. 1c). Therefore, the primate DMRT8 genes do not have the potential to encode a DM domain.

In contrast to the situation observed in primates, this splice site is intact in cat, dog, cow, and pig. In these species, one of the invariant histidines within the DM domain has changed either to glutamine or to glutamic acid. However, a second histidine is present next to this position (Fig. 1b). So far, this histidine is found only in Dmrt7 of opossum and in the investigated Dmrt8 sequences. Whether this histidine can

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DMRT7 and DMRT8 sequence sources: a http://www.ncbi.nlm.nih.gov/, b WGS (whole genome shotgun) or EST (expressed sequence tag) trace sequences (http://www.ncbi.nlm.nih.gov/BLAST/tracemb.shtml), nucleotide sequence positions of DMRT7 and DMRT8 genomic sequences in the genome drafts available at the UCSC genome browser (http://genome.uscs.edu/). Sequences encoding regions I, II, III, and IV are conserved in DMRT7 and DMRT8 (Fig. 1a). Regions I and II constitute the DM domain. Sequences encoding a truncated version of the DM domain are indicated with ∼; partial, incomplete WGS sequences for region I are in parentheses. Chr., chromosomal location.
compensate for the loss of the first one is unclear. Thus, the unique Dmr8 gene in cat, dog, cow, and pig might encode a protein with a DM-like domain, but whether it binds to DNA remains to be investigated.

The third group of sequences consists of the three Dmr8-encoding sequences from rabbit. As observed in some other placental mammals, one of the invariant histidines of the DM domain is substituted (Fig. 1b). Finally, three different Dmr8-encoding sequences are present in rodents. Dmr8.2 (mouse) and Dmr8.3 (mouse and rat) encode truncated versions of the zinc-finger module-containing part of the DM domain (Fig. 1b), while this sequence seems to be completely absent from mouse and rat Dmr8.1. In mouse and rat, a LINE element is inserted into the putative zinc-finger module-encoding exon of Dmr8.1, giving a hint about the degeneration of this module (Fig. 2b). The second part of the DM domain (region II) is present in all three rodent Dmr8 sequences (Fig. 1c).

Male-biased expression and alternative splicing of Dmr8.1 and Dmr8.2 in the mouse

Expression of Dmr8 genes in adult mice was analyzed by RT-PCR using several organs (Fig. 2c). In contrast to human DMRT8, which is expressed in various organs, including brain, lung, kidney, pancreas, and gonads of both sexes [35], expression of Dmr8.1 and Dmr8.2 was exclusively detected in testis of adult mice. For both Dmr8 genes, three different isoforms could be identified (Fig. 2c). All these isoforms are also expressed in mouse embryonic testis. Expression of Dmr8.3 could not be detected by RT-PCR in adult mice and embryos using several primer pairs (not shown). Whether Dmr8.3 is a pseudogene has to be further investigated.

Embryonic expression of Dmr8.1 and Dmr8.2 was assessed by quantitative real-time PCR (qRT-PCR) of cDNA from different tissues of single embryos at embryonic day (E) 13.5 with primers recognizing all Dmr8.1 and Dmr8.2 isoforms. Different tissues of single embryos at embryonic day (E) 13.5 by quantitative real-time PCR (qRT-PCR) of cDNA from Dmr8.3 embryos using several primer pairs (not shown). Whether Dmr8.3 also expressed in mouse embryonic testis. Expression of Dmr8.3 is a pseudogene has to be further investigated.

The spatial expression pattern of Dmr8.1 in adult mice was analyzed by in situ hybridization on testis sections. Dmr8.1 mRNA was detected only in Sertoli cells, a somatic key cell type in testis (Fig. 4b). The Sertoli cell-specific expression was confirmed by comparison to the expression pattern of Wt1 (Fig. 4c), a marker for Sertoli cells in adult mice [37]. We could not detect Dmr8.2 expression by in situ hybridization in adult testis, although expression was clearly detectable by RT-PCR. This might be due to a lower expression of Dmr8.2 compared to Dmr8.1.

The three Dmr8.1 and Dmr8.2 isoforms derived from different PCR products of testis (Fig. 2c) were cloned and analyzed. For Dmr8.1 and Dmr8.2, eight coding exons, and nine exons for Dmr7, are present (Fig. 2b). Comparison of Dmr8.1 genomic sequences from rat and mouse to other mammalian Dmr8 sequences indicates that the 3′ alternative terminal exon 7 of Dmr8.1 is apparently specific in mouse. In addition, exon 6 of Dmr7 is absent in all three Dmr8 genomic sequences (Fig. 2b, Dmr8.3 not shown). None of the Dmr8.1 isoforms encodes the first part of the DM domain because this exon is broken, most likely due to the insertion of a LINE element (Fig. 2b). Exon 2 (DM domain part II) is also absent in Dmr8.1b and Dmr8.1c (Fig. 2c). Here, exon 3 is elongated 5′ of the normal splice site (exon 3′) and contains a novel predicted translation start site. Dmr8.2c encodes a truncated version of the first part of the DM domain, while the intron that separates the two DM domain-encoding exons is present in Dmr8.2b, and similar to the situation in humans, a stop codon adjacent to the 5′ splice site disrupts the open reading frame (Fig. 2c). In summary, none of the investigated Dmr8 isoforms encodes the complete zinc-finger modules of the DM domain, suggesting that they probably do not have the DNA-binding capacity conferred by this domain.

**Discussion**

**Evolution of DMRT7 and DMRT8 in the mammalian lineage**

Based on currently available data from public databases, DMRT7 and DMRT8 are present in mammals, but not in nonmammalian vertebrate species, including chicken, frogs, and teleost fishes. Thus, these two DMRT genes might be mammalian-specific. The structural and sequence similarity of DMRT7 and DMRT8 is consistent with the hypothesis that DMRT8 originated by duplication of DMRT7 [35]. The presence of DMRT7 in monotremes, marsupials, and placental mammals suggests that DMRT7 was probably formed after the divergence of mammals from other vertebrates and before the divergence of placental mammals, one of the invariant histidines of the DM domain is substituted (Fig. 1b). Finally, three different Dmr8-encoding sequences are present in rodents. Dmr8.2 (mouse) and Dmr8.3 (mouse and rat) encode truncated versions of the zinc-finger module-containing part of the DM domain (Fig. 1b), while this sequence seems to be completely absent from mouse and rat Dmr8.1. In mouse and rat, a LINE element is inserted into the putative zinc-finger module-encoding exon of Dmr8.1, giving a hint about the degeneration of this module (Fig. 2b). The second part of the DM domain (region II) is present in all three rodent Dmr8 sequences (Fig. 1c).

**Fig. 1.** Organization and conserved domains in mammalian DMRT7 and DMRT8 proteins. (a) Schematic organization of DMRT7 and DMRT8 proteins. Regions I, II, III, and IV are conserved in DMRT7 and DMRT8. Region I and region II constitute the DM domain. The DM domain-encoding sequence is separated by an intron (arrowhead). (b, c) Sequence comparison of DMRT7 and DMRT8 putative DM domain translational products. The boundary between region I (DNA binding) and region II (disordered tail), which are encoded by different exons, is labeled with an arrowhead. In DMRT8 from human, chimp, and orangutan, the open reading frame is disrupted by a stop codon 5′ of the DM domain (indicated by a star). Only a partial DMRT7 sequence of region I is currently available for orangutan. (d) Phylogenetic analysis of DMRT7 and DMRT8. The tree, based on combined alignments from regions II, III, and IV (116 aa, bootstrap-neighbor-joining, 1000 pseudosamples), is unrooted. Sequences present only for regions III and IV were not included. Accession numbers are given in Table 1.
emergence of placental mammals approximately 210–310 million years ago [38]. Based on the current accessible data, the widespread distribution of DMRT8 in placental mammals suggests that this gene is at least 100 million years old. The duplication event that led to the formation of DMRT8 might have occurred either before the appearance of placental mammals or even within the placental mammalian lineage. Additional data, mainly from other marsupials and from

Fig. 2. Genomic organization and testis-specific expression of Dmrt8 genes in adult mouse. (a) X-chromosomal localization of Dmrt8.1, Dmrt8.2, and Dmrt8.3 in the mouse. (b) Genomic organization of Dmrt7, Dmrt8.1, and Dmrt8.2. Primers used are indicated as arrows. Dmrt8.2 encodes a truncated version of the DM domain. (c) RT-PCR-analysis of Dmrt8.1 and Dmrt8.2 isoforms in organs of adult mice and embryonic testis from E13.5. Tbp (TATA box-binding protein) was used as a control. As a negative control, water instead of cDNA was added to the PCR. The following primers were used: Dmrt8.1a, f1, r1; Dmrt8.1b, c, f3, r3; Dmrt8.2b, f1, r3; Dmrt8.2b, c, f4, r3 (r4 was used for sequencing the 3' end of Dmrt8.2a, b, c). Expression of Dmrt8.3 was not detectable (not shown). The putative translation start sites are indicated as arrows.
platypus, will be necessary to determine the evolutionary origin of \textit{DMRT8}.

Our data suggest that the common ancestor of all placental mammalian \textit{DMRT8} genes was located on the X chromosome. No homologue of \textit{DMRT8} was found on the Y chromosome, indicating that \textit{DMRT8} has been subsequently lost from the Y chromosome or was formed specifically on the X chromosome during sex chromosome evolution. Most investigated species have one copy of \textit{DMRT8}, while at least three copies are present in rabbit, mouse, and rat. Thus, \textit{Dmrt8} was duplicated several times in the rabbit, mouse, and rat lineage probably after their divergence from other mammals. The \textit{Dmrt8} copies might have been generated through two possible scenarios. Three \textit{Dmrt8} copies were already present in the common ancestor of mouse,
rat, and rabbit. Subsequently, these copies might have been homogenized through gene conversion independently in the rabbit and the mouse/rat lineages. Alternatively, the three Dmrt8 genes were formed by independent duplications after the separation of rabbit and rodents, approximately 55 million years ago.

During the evolution of DMRT8 several mutations occurred within the DM domain-encoding sequence. As a result, none of the analyzed DMRT8 genes encodes a typical DM domain. While Dmrt8 genes of cat, dog, cow, pig, and rabbit still encode a DM-like domain, a partial or complete loss of the DM domain has occurred in primates, mouse, and rat.

Do DMRT8 genes have DM domain-independent functions?

Despite the lack of the DM domain, DMRT8 genes have been maintained on the X chromosome in the placental mammalian lineage. Thus, DMRT8 proteins might exhibit a DM domain-independent function. Other functional domains located downstream of the DM domain might be present and important for DMRT8. In vertebrates, not much is known about the presence of other functional domains in DMRT proteins, as there is only little conservation outside the DM domain [2]. In Drosophila DSX, the carboxy-terminal-located domains are important for protein dimerization and recruitment of coregulatory factors [39,40]. Regions of high sequence similarity are present in the carboxy-terminus of DMRT7 and DMRT8 (Fig. 1a). It would be of special interest to investigate whether these regions correspond to functional domains involved in dimerization and recruitment of coregulatory factors. Dimerization seems to be important for DNA binding of the metazoan DM domain [3,40]. Therefore, DM domain-like or DM domain-less DMRT8 proteins could evolve as dominant-negative regulators as they might be able to form heterodimers with DMRT7 or other DMRT proteins and thus prevent DNA binding. Regulation of DNA binding mediated by such types of dominant-negative regulators has been discovered for some members of the basic helix–loop–helix, leucine-zipper, and homeodomain transcription factor families [41–43]. On the other hand, it is also possible that DMRT8 regulates the activity of other DMRT proteins indirectly by competition for coregulatory factors.

Male-biased expression of X chromosomal Dmrt8 genes in the mouse

In human and mouse, conflicting results have been reported about the presence of male-biased genes on the X chromosome [44,45]. On the one hand, the X chromosome appears to be enriched for male-biased genes, consistent with Rice’s hypothesis [46], which states that male-biased genes advantageous for male reproduction preferentially accumulate on the X chromosome. However, recent analysis of the genomic distribution of sex-biased genes in mouse revealed that the occurrence of X-linked male-biased genes depends on their temporal and spatial expression [45,47]. The X chromosome
accumulates male-biased genes expressed in male germ cells prior to MSCI (meiotic sex chromosome inactivation) and genes expressed in testicular somatic cells, but there is a lack of male-biased genes expressed in male germ cells during later stages of spermatogenesis [45,47,48]. During MSCI, which occurs in both sexes shortly after birth, sex chromosomes are transcriptionally inactive in germ cells, but not in somatic cells. Therefore, it has been suggested that male-biased genes expressed in male germ cells that underlie MSCI are eliminated rapidly from the sex chromosomes, and genes with important functions in these cells are located primarily on autosomes [45,47].

Dmrt8.1 and Dmrt8.2 in mouse show a male-biased expression in mature testis. Dmrt8.1 is expressed in the somatic Sertoli cells, suggesting that it might be involved in some Sertoli cell-mediated aspects of testicular function. Dmrt8.2 shows a dynamic expression during embryogenesis exclusively in male and female gonads, with a peak at E13.5 in males. Sry, the sex-determining gene in mouse, is expressed in males from E10.5 to E12.5 with a peak at E11.5 [49]. Thus, the time course of Dmrt8.2 expression suggests a potential role of Dmrt8.2 during male sex differentiation downstream of Sry. After birth, expression of Dmrt8.2 becomes restricted to testis, suggesting that Dmrt8.2 is expressed in somatic cells rather than in germ cells of the testis as the sex chromosomes have been silenced by MSCI. The expression patterns of Dmrt8 genes in mouse are consistent with the hypothesis of a preferential accumulation of male-biased genes on the X chromosome. From there, DM domain-less X-linked Dmrt8 genes in mouse might exhibit some functions advantageous in males.

Our observations support the involvement of several DMRT genes other than Dmr1 during sexual development in the mouse [30]. Dmr1 is expressed at similar levels in gonads of male and female mouse embryos from E11.5 to E13.5 [16,50]. At E14.5, Dmr1 expression becomes male-specific and in adults it is expressed in Sertoli cells and germ cells [16,17]. Dmr4 is detectable in gonads of both sexes from E11.5 on. In embryos at E15.5, Dmr3 is expressed mainly in interstitial cells. Dmrt7 expression is detected at higher levels in the developing ovary than in testis, suggesting a role for Dmrt7 during ovary differentiation in mouse [30]. One function of the Dmrt8 proteins in the mouse could be the direct or indirect regulation of Dmrt7 or other DMRT proteins, possibly as dominant negative regulators and thus to promote male sexual development.

Materials and methods

Sequence analysis and database screening

Sequence analysis and multiple sequence alignments were performed using BioEdit (sequence alignment editor, 1997–2004, Tom Hall, Isis Pharmaceuticals, http://www.mbio.ncsu/BioEdit) and implemented modules, like ClustalX [51]. Phylogenetic analysis was performed on single and combined amino acid alignments of regions I (75 aa), II (52 aa), III (35 aa), and IV (48 aa), with the neighbor-joining method [52]. 1000 pseudosamples as implemented in PAUP* (D.L. Swofford, Sinauer Associates, Sunderland, MA, USA, 1998). BLAST analysis was done using sequence databases accessible from the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST) and from the UCSC Genome Browser (http://genome.ucsc.edu/). DMRT8 exon/intron boundaries were identified by computer-assisted comparison between cDNA, putative proteins, and genomic DNA using sim4 [53] (http://bioinformatics.ube.ca/resources/tools) and Wise2 [54] (http://www.ebi.ac.uk/wise2).

Mice

Mice used in this study were from an MFl background. Embryos were collected from timed matings, with noon of the day on which the mating plug was observed designated as E0.5. Presence or absence of the Y chromosome was analyzed by PCR using Zfy-specific primers [55].

Analysis of Dmrt8 expression in embryonic and adult mice

For RT-PCR analysis several organs from adult male and female mice were dissected. Total RNA was isolated from up to 10 mg tissue using PegoGold Trifast (PepLabs). For qRT-PCR analysis, urogenital ridge regions from single E8.5 and E9.5 embryos, single pairs of urogenital ridges (mesonephros and gonad) from E10.5 and E11.5 embryos, single pairs of gonads from E12.5 to E15.5 embryos, single gonads of 1-day to 5-week-old mice, and various organs from single E13.5 embryos were prepared. Total RNA was isolated using the Absolutely RNA MicroPREP Kit (Stratagene). Reverse transcription and qRT-PCR analysis were performed as described by Barrionuevo and colleagues [56]. The relative expression in developing gonads was determined by comparison to a serial dilution of adult testis cDNA using the iCycler IQ optical system software version 3.1. Relative Dmrt8 expression levels determined by this method were normalized to the corresponding amounts of Tbp. The following primers were used for Tbp: Tbp-f (5′-GCCAAGGCTCTGAGCTA-3′), Tbp-r (5′-GGCCCTTCAGAGACTACTA-3′), 55°C for 25 cycles. For qRT-PCR the following primers were used: Dmrt8.1-f (5′-AAAGACGCTCTGTTAGA-GAG-3′), Dmrt8.1-r (5′-GTGGTTCTGCTGGTGTG-3′), Dmrt8.2-f (5′-TTTACAAGCATGCAAGAG-3′), Dmrt8.2-r (5′-TCACTGAGCATAGGCAGA-3′), 60°C for 40 cycles. Primers used for RT-PCR were, for Dmrt8.1a, Dmrt8.1-f1 (5′-ACTGAAAGAGGGCTCTG3′), Dmrt8.1-r1 (5′-CACGTGTAGTCCCTG-3′), 55°C for 35 cycles; for Dmrt8.2, Dmrt8.2-f4 (5′-AGCGGTCTTCGCGTTCCT-3′), Dmrt8.2-f3 (5′-GATGTTGGACCTGATCCAT-3′), 55°C for 25 cycles; for Dmrt8.2a, Dmrt8.2-f1 (5′-AGCGGTCTTCGCGTTCTG-3′), Dmrt8.2-f2 (5′-AGCGGTCTTCGCGTTCTG-3′), Dmrt8.2-r (5′-TCACTGAGCATAGGCAGA-3′), 55°C for 35 cycles; and for Dmrt8.2, Dmrt8.2-r4 (5′-ATGTTGGACCTGATCCCTG-3′), 55°C for 40 cycles.

Cloning and sequencing of DMRT8 PCR products

Dmrt8 RT-PCR products derived from testis cDNA were cloned into pBluescript II KS(+) (Stratagene) or into the pCRJ TOPO vector (Invitrogen Life Technologies). Sequencing reactions were performed using the CEQ DTCS dye terminator cycle sequencing kit and run on a CEQ 8000 DNA sequencing system (Beckman Coulter).

RNA in situ hybridization

In situ hybridizations were performed on paraffin sections of adult testis using digoxigenin-labeled (Roche) antisense and sense riboprobes [57]. For Dmrt8.1 a 610-bp cDNA fragment and for Dmrt8.2 a 609-bp cDNA fragment from testis, subcloned into pBluescript II KS(+), were used to generate riboprobes. A Wt1-specific probe was generated from a 1084-bp fragment containing the 3′ end of Wt1 mRNA.

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