Doublesex- and *Mab-3*-Related Transcription Factor-1 Repression of Aromatase Transcription, a Possible Mechanism Favoring the Male Pathway in Tilapia

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Doublesex- and *Mab-3*-related transcription factor-1 (Dmrt1) is an important transcription factor implicated in early testicular differentiation in vertebrates, but its target genes are largely unknown. In the Nile tilapia, estrogen is the natural inducer of ovarian differentiation. Our recent studies have shown that Forkhead-l2 up-regulated transcription of the *Cyp19a1a* gene (aromatase) in the gonads in a female-specific manner. However, the upstream factor(s) down-regulating *Cyp19a1a* expression during testicular differentiation remains unclear. In the present study, we used *in vitro* (promoter analysis) and *in vivo* (transgenesis and *in situ* hybridization) approaches to examine whether Dmrt1 inhibits *Cyp19a1a*'s transcriptional activity. The *in vitro* analysis using luciferase assays revealed that Dmrt1 repressed basal as well as Ad4BP/SF-1-activated *Cyp19a1a* transcription in HEK 293 cells. Luciferase assays with various deletions of Dmrt1 also showed that the *Doublesex* and *Mab-3* domain is essential for the repression. *In vitro*-translated Dmrt1 and the nuclear extract from tilapia testis could directly bind to the palindrome sequence ACATATGT in the *Cyp19a1a* promoter, as determined by EMSAs. Transgenic overexpression of Dmrt1 in XX fish resulted in decreased aromatase gene expression, reduced serum estradiol-17β levels, retardation of the ovarian cavity's development, varying degrees of follicular degeneration, and even a partial to complete sex reversal. Our results indicate that aromatase is one of the targets of Dmrt1. Dmrt1 suppresses the female pathway by repressing aromatase gene transcription and estrogen production in the gonads of tilapia and possibly other vertebrates. **(*Endocrinology* 151: 1331–1340, 2010)**

Among genes involved in sex determination and sexual differentiation, Dmrt1 (*Doublesex*- and *Mab-3*-related transcription factor-1) is the only one characterized to date containing a domain (the *Doublesex* and *Mab-3*, DM domain) that is conserved between phyla (1). Orthologs of Dmrt1 have been discovered in all the groups of vertebrates from fish to mammals (2–8). In the fish medaka, a duplicated copy of Dmrt1 (DMY/Dmrt1b) has been reported on the Y chromosome and DMY has been shown to have a role as the master male sex-determining gene, like its mammalian analog SRY (9). Dmrt1 orthologs have highly conserved roles in sexual differentiation from flies and worms to humans. *Dmrt1* expression is evident in male gonads during sexual differentiation and considerably up-regulated late in sex determination or early in testicular differentiation (4, 6, 7, 10–13). In non-mammalian vertebrates from fish to birds, Dmrt1 expression was found to be linked to the male phenotype (2, 8,

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

doi: 10.1210/en.2009-0999 Received August 24, 2009. Accepted December 4, 2009.
First Published Online January 7, 2010

Abbreviations: CMV, Cytomegalovirus; dah, day after hatching; DM, *Doublesex* and *Mab-3*; Dmrt1, *Doublesex*- and *Mab-3*-related transcription factor-1; E2, estradiol-17β; Fox12, Forkhead box 12; GFP, green fluorescent protein; H.E., hematoxylin-eosin; HEK 293, human embryonic kidney 293; hr, humanized recombinant.

10). Studies have shown Dmrt1 expression to be confined to Sertoli cell and germ cell compartments in the testis and Dmrt1 to be an important transcription factor implicated in early testicular differentiation in vertebrates (1, 3–5, 10–17). Even though Dmrt1 has a clear zinc-finger DNA-binding domain that can bind to the consensus sequence GATTTGATACATTGTTGC carrying the palindromic sequence ACATTGT, its target genes remain unknown (18).

Estrogens play a pivotal role in gonadal sexual differentiation in noneutherian vertebrates (17, 19–22). The P450 aromatase is the steroidogenic enzyme responsible for the synthesis of estrogens from androgens. Two aromatase genes, *Cyp19a1a* and *Cyp19a1b*, have been cloned and characterized in teleosts (23–28). The notion that estrogens and *Cyp19a1a* are involved in ovarian differentiation in fish has been well accepted. A recent paper went even further, placing estrogens and *Cyp19a1a* in a pivotal position to control not only ovarian, but also testicular differentiation, in both gonochoristic and hermaphrodite fish species (29). This working hypothesis states that up-regulated *Cyp19a1a* expression is needed for both triggering and maintaining ovarian differentiation and that the down-regulation of *Cyp19a1a* expression is the only step necessary for inducing testicular differentiation because either suppression of *Cyp19a1a* gene expression, inhibition of Cyp19a1a enzymatic activity, or blockage of estrogen receptivity is invariably associated with masculinization. Concerning the regulation of fish *Cyp19a1a* during gonadal differentiation, the transcription factor Forkhead box (Fox-l2) has been characterized as an ovarian-specific upstream regulator of the *Cyp19a1a* promoter that coactivates *Cyp19a1a* expression, along with some partner such as NR5A1(Ad4BP/SF-1) or cAMP. In contrast, upstream factors potentially down-regulating *Cyp19a1a* expression during testicular differentiation are still hypothetical (29).

In nonmammalian vertebrates, during normal development and sex reversal, levels of Dmrt1 and aromatase were found to be negatively correlated in the gonads. In normal males as well as sex-reversed genetic females, Dmrt1 expression was up-regulated, whereas aromatase expression was down-regulated. Conversely, in normal females as well as sex-reversed genetic males, the expression of aromatase was up-regulated and that of Dmrt1 was down-regulated, suggesting a negative link between them (8, 10, 17, 19) and leaving the possibility that Dmrt1 is the regulator of aromatase.

The Nile tilapia, a gonochoristic teleost fish with an XX/XY sex-determining system, provides an excellent model for studying gonadal sex differentiation because genetically all-female and all-male populations are available. Endogenous estradiol-17β (E2) was shown to be the natural inducer of ovarian differentiation in tilapia by our group (21, 30, 31). Recently the precise timing of the gonadal expression of 17 genes thought to be associated with gonadal sex differentiation in vertebrates was determined by real-time-PCR using gonads isolated from all-female and all-male tilapia before and after morphological sex differentiation. Consistent with our earlier reports, the transcripts of aromatase (*Cyp19a1a*) were detected only in XX gonads at 5 d after hatching (dah), with a marked elevation in expression thereafter. A close relationship between the expression of *Foxl2*, but not *nr5a1* (Ad4BP/SF-1), and that of *Cyp19a1a* in XX gonads suggests an important role for Foxl2 in the transcriptional regulation of *Cyp19a1a*. *Dmrt1* exhibited a male-specific expression in XY gonads from 6 dah onward, suggesting an important role for Dmrt1 in testicular differentiation (32). The expression profiles and localization of *DMRT1* during gonadal sex differentiation and hormone induced sex reversal were investigated in detail subsequently (33). Dmrt1 was detected in the gonial germ-cell-surrounding cells and the medullary-cell-mass cells adjacent to the germ-cell-surrounding cells of the XY gonad specifically before the appearance of any signs of morphological sex differentiation. The signals became localized in the Sertoli and epithelial cells comprising the efferent duct during gonadal differentiation. After the induction of XY sex reversal with estrogen, Dmrt1 decreased and then disappeared completely. In contrast, it was expressed in the germ-cell-surrounding cells during XX sex reversal with androgen. These results suggest that Dmrt1 is a superior testicular differentiation marker in tilapia (33).

Ad4BP/SF-1 was found to be involved in the regulation of *Cyp19a1a* gene expression in medaka and Nile tilapia (34, 35). Ad4BP/SF-1-binding sites were found in the promoter region of *Cyp19a1a* gene cloned from all fish species. Recently we have also shown that Foxl2 regulated the *Cyp19a1a* gene's transcription in gonads in a female-specific manner by binding to the promoter as well as interacting with Ad4BP/SF-1 (31). In this report, we present *in vitro* and *in vivo* data to demonstrate that Dmrt1 can suppress *Cyp19a1a* gene transcription in tilapia. Additionally, we found that the regulation of *Cyp19a1a*/*CYP19* gene expression by Dmrt1 in medaka and humans was similar to that in tilapia.

Materials and Methods

Animals

Nile tilapias, *Oreochromis niloticus*, were kept in recirculating freshwater tanks at 26 C before use. All-XX and all-XY progenies were obtained by crossing the pseudomale (XX male,

producing sperm after sex reversal) with the normal female (XX), and supermale (YY) with the normal female, respectively. In our experimental conditions, these all-XX and all-XY fish develop as all-female and all-male populations. Sex-reversed females (XY, treated with E2) and males (XX, treated with 17α-methyltestosterone) were obtained and maintained as described previously (30, 31). All experiments conformed to the Guide for Care and Use of Laboratory Animals and were approved by the Institutional Committee of Laboratory Animal Experimentation (National Institute for Basic Biology).

Plasmid constructs and deletion mutagenesis

Tilapia *Cyp19a1a* gene promoter (2.36 kb, blunt ligation into *Sma*I site), medaka *Cyp19a1a* promoter (2.0 kb, ligation into *Mlu*I/*Bgl*II sites) and human *CYP19* promoter (5.3 kb, ligation into *Nhe*I and *Bgl*II sites) fragments were generated by PCR and subcloned into the pGL3-basic vector (Promega Corp., Madison, WI). The promoters of medaka *Cyp11a1* (2.0 kb, ligation into *Mlu*I/*Xho*I sites) and *20β-HSD* (3.0 kb, ligation into *Mlu*I/*Xho*I sites) were also subcloned into the pGL3-basic vector and used as controls.

All transcription factors used in luciferase assays, including *Ad4BP/SF-1*, and *Dmrt1* from tilapia, medaka, and human were amplified and cloned into the pcDNA3.1 expression vector (pcDNA3.1/V5-His Topo TA expression kit; Invitrogen, Carlsbad, CA) using gene-specific open reading frame primers. 5′- and 3′-deletion mutations of *Dmrt1* expression constructs were made by designing gene-specific primers at the desired position. The mutant without a DM domain was generated by introducing a *Bam*H1 cutting site before and after the DM domain. All the mutants were cloned into the pcDNA 3.1 expression vector.

Plasmids used in transfection experiments were purified using a QIAfilter plasmid midi kit or QIAprep spin miniprep kit (QIAGEN Sciences, Germantown, MD), and the purity was verified by spectrophotometry and agarose gel electrophoresis. The constructs, mutations, and orientation of the insert were confirmed by direct sequencing.

Cell culture, transient transfections, and luciferase assays

Cell culture, transient transfections, and luciferase assays were performed as reported previously (31). Briefly, human embryonic kidney 293 (HEK 293) cells were transfected using Lipofectamine (Invitrogen) with the following plasmids: 1) 0.5 μg of normal or deletion constructs of the *Cyp19a1a* promoter cloned into the pGL3-basic luciferase reporter vector; 2) 0.05–0.5 μg of pcDNA3.1 expression plasmid (Invitrogen), containing the cDNAs encoding *tAd4BP/SF-1* and *tDmrt1*; and 3) pRL-TK (Promega), at 100 ng/well. *Renilla* luciferase from pRL-TK was used as an internal control for transfection efficiency. The transfection solution was made of 100 μl of Opti-MEM I reduced-serum medium containing precomplexed DNA and 2 μl of Lipofectamine reagent. Forty-eight hours after transfection, cells were washed in PBS and lysed in 100 μl of luciferase lysis buffer. Firefly luciferase and *Renilla* luciferase readings were obtained using the dual-luciferase reporter assay system (Promega) and LUMAT LB9507 luminometer (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). Relative luciferase activity was calculated by dividing the firefly luciferase activity with the *Renilla* luciferase activity. Results are presented as the mean ± SD of data from triplicate replicates. The data were analyzed using one-way ANOVA and the least significant difference on the GraphPad Prism 4 software (San Diego, CA).

VP16 activation cell-based assay

Coding sequences for mouse msDMRT1-VP16 (positive control), tDmrt1-VP16, tM3-VP16 (with only the DM domain), and tM6-VP16 (without the DM domain) proteins were inserted into pACT, a cytomegalovirus (CMV) promoter-driven expression vector, generating constructs fusing an SV40 nuclear localization signal and the VP16 strong transcriptional activation domain to the N terminus of the protein. tDmrt1 in pACT but without the VP16 sequences was used as a negative control. The luciferase-reporter plasmid containing four DMRT1-binding sites (18) was a gift from Dr. M. W. Murphy (group of David Zarkower, Department of Genetics, Cell Biology, and Development, University of Minnesota, Minneapolis, MN). Each VP16 fusion plasmid was cotransfected into HEK 293 cells with the appropriate reporter plasmid (normalization of transfection efficiency was done by using the *Renilla* luciferase from pRL-TK). Luciferase levels were compared with those of cells cotransfected with DMRT1 reporter plasmid and empty pACT (18).

EMSAs

EMSAs were basically performed as described previously (31). The TNT T7-coupled reticulocyte lysate system (Promega) was used to generate *in vitro*-translated tilapia recombinant Dmrt1 proteins. Nuclear extracts of tilapia ovary and testis were prepared as described previously (35). Oligonucleotide probes (sense 5′-TCGAATGCAT**CTACATATGTAAATA**TT-3′), which have a Dmrt1 consensus sequence and carry the palindromic sequence ACATATGT, were designed based on the sequence between −447/−424 bp of the Nile tilapia *Cyp19a1a* promoter (GenBank accession no. AB089924). The annealed oligonucleotides with overhanging *Xho*I ends were labeled with α-^{32}P deoxy-CTP by end fill-in from both ends using the Klenow fragment (Takara, Otsu, Shiga, Japan). The unincorporated α-^{32}P dCTP label was removed using a Sephadex-25 column. Protein-DNA binding reactions were performed using 3 μl of protein in a 20 μl volume of binding buffer, the radiolabeled probe (5000 cpm), and 1 μg of polydeoxyinosinic deoxycytidylic acid. Increasing amounts of *in vitro*-translated Dmrt1 protein and nuclear extraction (1, 3, and 6 μl for lanes 1 and 9, 2 and 10, and 3 and 11, respectively) to shift a constant amount of labeled double-stranded DNA to result in the intensified shift band. After 15 min of incubation on ice, 2 μl of loading dye were added, and the samples were fractionated on a 5% polyacrylamide gel at 130 V for 1.5 h. The gels were dried and exposed to a BAS-III imaging plate, and the hybridization signals were analyzed by a BAS 2000 Bio-Image analyzer (Fuji Film Co. Ltd., Tokyo, Japan). Competition experiments were performed in the presence of a 5- to 100-fold molar excess of unlabeled probes that were added 15 min before the labeled probe.

Overexpression of *Dmrt1* by transgenesis

Dmrt1 cDNA was subcloned into multiple cloning sites downstream of the CMV promoter in the pIRES-humanized recombinant (hr) green fluorescent protein (GFP)-1a vector (Stratagene, La Jolla, CA). *In vivo* transgenic overexpression of *Dmrt1* in XX fish *w*as carried out by injecting these GFP constructs into the fertilized eggs. Blood samples were collected from the caudal

veins of the 6-month-old transgenic fish as well as control fish. Serum E2 levels were measured using the estradiol enzyme immunoassay kit (Cayman Chemical Co., Ann Arbor, MI). Sample purification and assays were performed according to the manufacturer's instructions.

Histology and immunohistochemistry

The gonads of the injected fish were examined by monitoring the GFP signal and subjected to both histological and immunohistochemical analyses 3–6 months after the injection, using antibodies against tilapia aromatase. Gonads from normal XX control fish of the same age (hatched from the same batch) were used as controls. Gonads from both Dmrt1 transgenic fish and control fish were dissected and fixed in Bouin's solution at room temperature overnight. The tissues were then embedded in paraffin, and cross-sections were cut at 5 μm. Hematoxylin-eosin (H.E.) staining and immunostaining of aromatase were performed as described previously (30). Immunostained sections were counterstained with hematoxylin.

Results

Dmrt1 represses Ad4BP/SF-1 activated tilapia and medaka *Cyp19a1a* gene expression

Earlier we found that *Cyp19a1a* gene transcription was activated by Ad4BP/SF-1 alone and Foxl2 enhanced it further by luciferase assays using HEK 293 cells (31). In this study, we examined whether tilapia and medaka *Cyp19a1a* gene transcription is regulated by Dmrt1 in HEK 293 cells. Reduced luciferase activity was observed in the promoter assays when Dmrt1 was used alone, indicating it could suppress the basal transcription of *Cyp19a1a*. Furthermore, when cotransfected with Ad4BP/SF-1, Dmrt1 repressed Ad4BP/SF-1-activated *Cyp19a1a* expression in a dose-dependent (10–250 ng) manner (Fig. 1, A and B). When human *CYP19* was used in the luciferase assay, DMRT1 repressed Ad4BP/SF-1-activated *CYP19* expression also in a dose-dependent (5–100 ng) manner (supplemental Fig. S1, published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). In contrast, Dmrt1 activated medaka *Cyp11a1* but showed no effect on medaka *20β-HSD* expression (supplemental Figs. S2 and S3).

Regions of the Dmrt1 protein responsible for its repressive function

To determine which regions of the Dmrt1 protein are important for its suppressive effect, Dmrt1 mutants were constructed through sequential deletions of the wild-type protein from both the amino and carboxyl termini. In tilapia Dmrt1, the zinc finger DNA-binding domain (DM domain) is located in the region spanning amino acid residues 28–83. Cotransfection of Dmrt1 mutants with tilapia *Cyp19a1a* promoter-reporter constructs in HEK 293 cells and measurement of the luciferase activity revealed that the DM domain is essential for the repression of Ad4BP/SF-1-activated *Cyp19a1a* transcription. Mutants without the DM domain (M2 and M6) showed no repression. In contrast, deletions of the C terminus (M3, M4, and M5) resulted in a partial loss of repression, whereas deletion of the amino terminus (M1) had little effect on the repressor activity (Fig. 2).

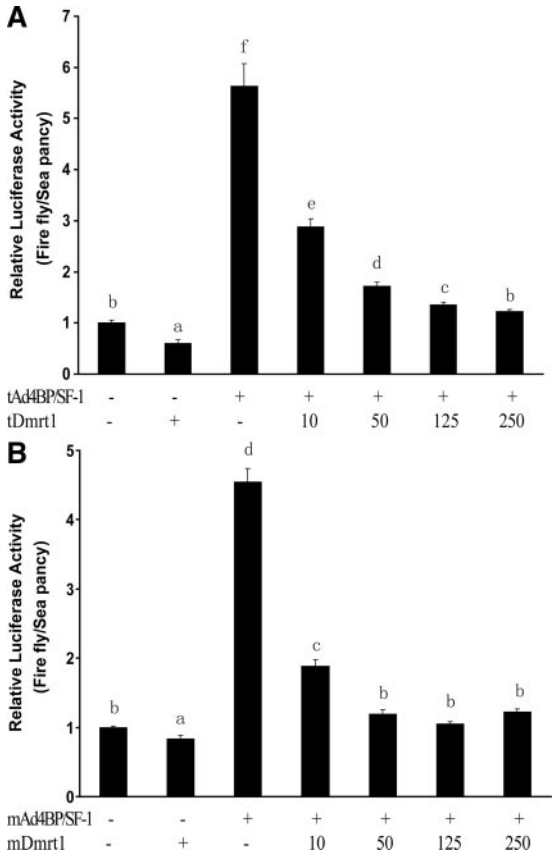

FIG. 1. Dmrt1 suppresses Ad4BP/SF-1-activated *Cyp19a1a* promoter activity. Dmrt1 and Ad4BP/SF-1 expression vectors (10–250 ng) were cotransfected into HEK 293 cells with the *Cyp19a1a* promoter construct (500 ng/well). The total amount of transfected plasmid, including the pRL-TK control vector (100 ng/well), was adjusted to 1.0 μg with empty vectors. Firefly and *Renilla* luciferase activities were measured 48 h after transfection. Relative luciferase activity was calculated by dividing the firefly luciferase activity with the *Renilla* luciferase activity. Results are the mean ± SD for triplicate transfections. +, 50 ng/well. A, Tilapia; B, medaka. *Bars bearing different letters* differ ($P < 0.01$) by one-way ANOVA.

Binding of tilapia Dmrt1 to the mouse DMRT1 response element consensus sequence and tilapia *Cyp19a1a* promoter

The ability of tilapia Dmrt1 to bind the recently published mouse DMRT1-binding sequence, GATTTGATACATTGTTGC, was tested by cotransfection of the luciferase reporter plasmid containing four DMRT1-binding sites and expression vectors encoding only VP16, msDMRT1-VP16, tDmrt1-VP16, or M3-VP16. Results showed a more efficient

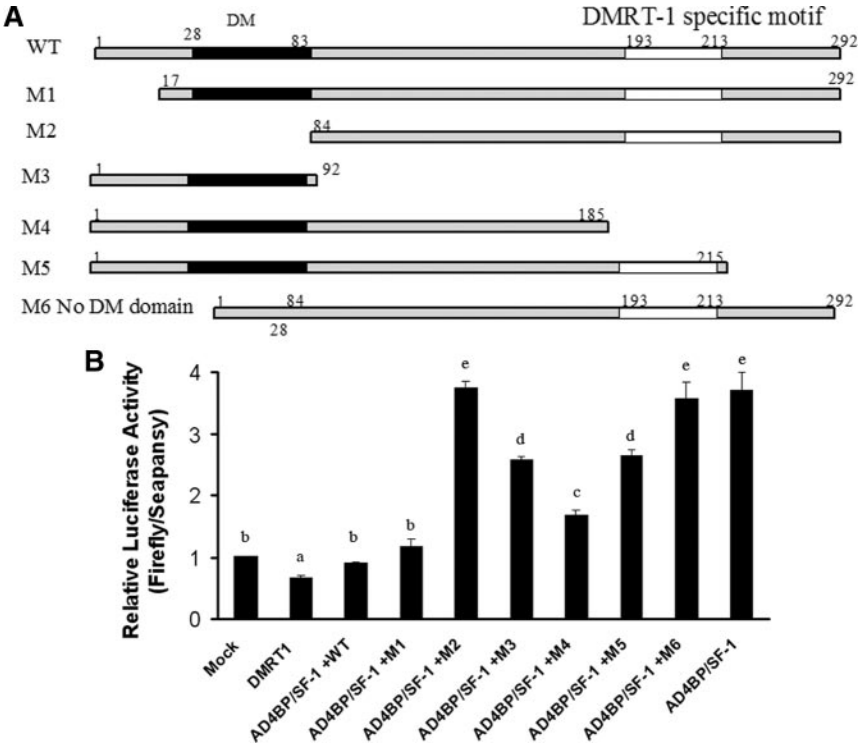

FIG. 2. Regions of the Dmrt1 protein responsible for its repressive function. A, Domain arrangement of Dmrt1 and construction of Dmrt1 mutants. The DNA-binding domain (DM domain) of Dmrt1 spans from the amino acid position 28 to 83 and carries an unusual zinc finger. Dmrt1 constructs with deletions of the amino terminus (M1) and DM domain (M2, M6) and sequential deletions of the carboxyl terminus (M3-5) were generated. B, The DM domain is essential for the repressive activity of Dmrt1 in HEK 293 cells. Deletion of the amino terminus had no effect, whereas sequential deletion of the carboxyl terminus resulted in a loss of repressor activity. Mutants without the DM domain (M2, M6) showed a complete loss of repressor activity. Mock indicates the pcDNA3.1 vector without any insert. *Bars bearing different letters* differ ($P < 0.01$) by one-way ANOVA.

binding of tDmrt1 (120-fold increase relative to the control) to the four DMRT1-binding sequences compared with msDMRT1 (4-fold), especially when M3-VP16 (180-fold) was used (Fig. 3A).

A very similar DMRT1-binding sequence, ATGCATC-**TACATATGTAAATA**TT, carrying the palindrome sequence **TACATATGTA**, was detected in the promoter region of the Nile tilapia *Cyp19a1a* gene, −447/−424 bp from the ATG start codon. EMSA was performed to test the ability of tDmrt1 to bind to this sequence. As shown in Fig. 3B, the *in vitro*-translated tDmrt1 protein with a c-Myc tag and nuclear extract of the testis bound to the radiolabeled oligonucleotides, resulting in the formation of a specific band of protein-DNA complex. This band was not seen when the nuclear extract from the ovary was used. Cold competitor (5–100 times) displaced the band in a dose-dependent manner. Addition of c-Myc monoclonal antibody removed part of the band, but no supershifted band could be seen (Fig. 3B).

Overexpression of *tDmrt1* in XX tilapia

To confirm the suppressive effect of Dmrt1 on aromatase gene transcription *in vivo* and on the gonad phenotype, *Dmrt1* open reading frame was subcloned into the multiple cloning sites downstream of the CMV promoter in the pIRES-hrGFP-1a vector. After injection into the fertilized egg, the transgene *Dmrt1* and the hrGFP sequence were transcribed as a single mRNA but were translated as two separate proteins. A mosaic GFP signal was observed in the gonads of the 3- to 6-month-old XX fish, indicating successful *Dmrt1* overexpression (Fig. 4, A and D). Many dark hollows could be observed on the large follicles with GFP signal (Fig. 4D). Histological analysis revealed that these ovaries, especially the vitellogenic follicles in the advanced stages, showed varying degrees of degeneration. The uneven shrinking of these follicles caused the formation of many deep hollows (Fig. 4E), which appeared as dark spots on the surface of the follicles under the stereomicroscope (Fig. 4D). Severe degeneration of follicles was observed in the regions with intense GFP expression, indicating high levels of the transgene in that particular area (Fig. 4, A, B, D, and E). Furthermore, the degenerating follicles and interstitial cells in these ovaries exhibited decreased levels of aromatase expression (Fig. 4E), compared with the control (Fig. 4F). In addition, some fish with *Dmrt1* overexpression exhibited a delay in the formation of the ovarian cavity, with an open ovarian cavity, even at 3 months after hatching (Fig. 4C). Occasionally special structures of unknown nature were found to have formed in some areas of the ovary (supplemental Fig. S4). These structures could not be seen in the normal developing ovaries. Whether these structures are indicative of the transition state of the gonad from ovary to testis is uncertain at this stage. A small proportion of the transgenic fish (less than 5%) exhibited complete sex reversal with the testis producing normal fertile sperm (Wang, D. S., and Y. Nagahama, unpublished data).

Impact of overexpression of Dmrt1 on serum E2 levels

E2 is the major estrogen found in tilapia serum. To investigate whether Dmrt1 influences E2 levels, we collected blood samples from the 6-month-old transgenic and control fish and measured E2 levels (Fig. 5). Overexpression of tDmrt1 in XX females resulted in decreased serum

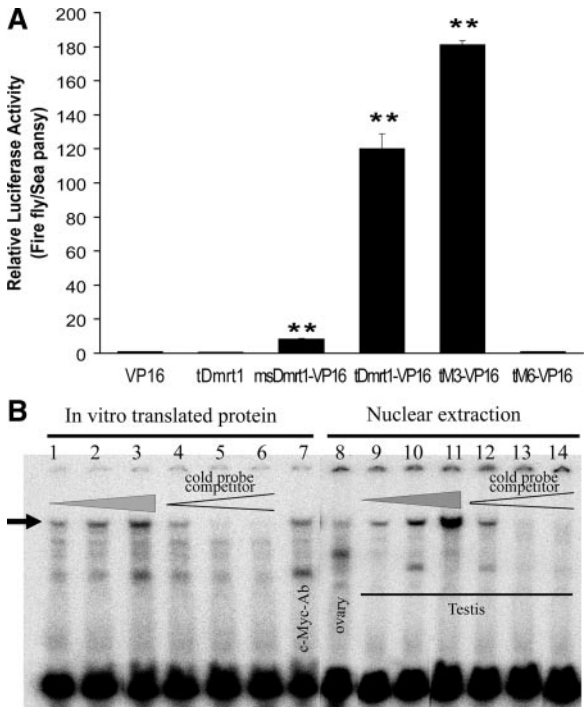

FIG. 3. DNA-binding specificity of tDmrt1. A, DNA recognition by tDmrt1 proteins in cultured cells. Transactivation by VP16 fusion proteins in HEK 293 cells transfected with expression vectors encoding the indicated VP16 only (negative control), tDmrt1 only (negative control), mouse msDmrt1-VP16 (positive control), tDmrt1-VP16, tM3-VP16 (with only the DM domain), and tM6-VP16 (without the DM domain) fusions and with the pGL3-promoter reporter plasmid containing four Dmrt1-binding consensus sequence upstream of the luciferase coding sequence. Values shown are averages for three experiments, with the *error bar* indicating the SEM. **, Significantly lower than the control with Student's *t* test, $P < 0.01$. B, EMSAs show binding of Dmrt1 protein to the tilapia *Cyp19a1a* promoter. A radiolabeled *Cyp19a1a* promoter probe was incubated with an *in vitro*-translated recombinant tDmrt1 with the c-Myc-tag and nuclear extracts from the tilapia testis and ovary. Excess unlabeled probe was used to demonstrate specific binding between Dmrt1 and the *Cyp19a1a* promoter. Addition of anti-c-Myc monoclonal antibody (c-Myc-Ab) resulted in reduced band intensity but failed to give a supershifted band. The positions of the Dmrt1-DNA probe complexes are shown with *arrows*.

E2 levels (6.9 ng/ml), which were about half the E2 levels in control females (14.7 ng/ml).

Discussion

The DM domain and the function of Dmrt1 in testicular differentiation are fairly well conserved in all classes of vertebrates examined to date. However, the molecular mechanism and targets of Dmrt1 remain unclear. Using a series of *in vitro* and *in vivo* approaches, we have shown in tilapia a well-established fish model that Dmrt1 is a natural repressor of aromatase, one of its targets.

Dmrt1 has long been speculated to be a repressor of female pathway genes. Furthermore, Ad4BP/SF-1 is well recognized as a stronger activator of *Cyp19/Cyp19a1a* transcription in vertebrates including tilapia (25–27, 34–39). In this study, Dmrt1 repressed basal as well as Ad4BP/SF-1-activated *Cyp19a1a* transcription in HEK 293 cells when tested with the tilapia and medaka *Cyp19a1a* promoters. Importantly, Dmrt1 also suppressed Ad4BP/SF-1-activated transcription of the human *CYP19* gene. Thus, Dmrt1 suppresses the female pathway by repressing aromatase gene transcription and the production of estrogen in the gonads in tilapia and possibly other vertebrates. Using the same *in vitro* transfection system, we also tested the effects of Dmrt1 on the transcription of two other steroidogenic enzyme genes of medaka, *Cyp11a1* (the gene that converts cholesterol to pregnenolone) and 20β-hydroxysteroid dehydrogenase (the gene that converts 17α-hydroxyprogesterone to 17α,20β-dihydroxy-4-pregnen-3-one, the maturation inducing hormone of most teleost fishes so far examined). In contrast to its repressive effect on *Cyp19a1a* expression, Dmrt1 activated medaka *Cyp11a1* transcription and had no effect on medaka *20β-HSD* expression. These results exclude the possibility of nonspecific effects of Dmrt1 on *CYP19/Cyp19a1a* promoters.

Luciferase assays with various deletions of Dmrt1 revealed that the DM domain was essential for the repression of Ad4BP/SF-1-activated *Cyp19a1a* transcription. As the zinc-finger-bearing DM domain is believed to be a DNA-binding domain, it could be assumed that Dmrt1 regulates aromatase gene transcription by binding directly to the promoter through the DM domain. However, the DM domain of human DMRT1 is also involved in its nuclear import via an interaction with importin (40), and therefore, loss of repression with the tDmrt1 mutant without DM domain could also be due to a failure of Dmrt1 to reach the nucleus. A recent publication demonstrated that mouse DMRT1 can bind to a consensus sequence, GATTTGATACATTGTTGC, which bears a palindrome sequence, ACATTGT (18). We tested the possibility that tilapia Dmrt1 binds to the same sequence using the 4XRE Dmrt1 luciferase reporter vectors and found it binds even more efficiently than mouse DMRT1. By using M3, a deletion mutant of Dmrt1 with only the DM domain, we showed that the binding occurs through the DM domain. These findings prompted us to analyze the tilapia *Cyp19a1a* gene's promoter sequence. Importantly, we found a mouse DMRT1 RE-like sequence −447/−424 bp from the ATG start codon. With EMSAs, we proved that this palindrome sequence shifted a clear band with *in vitro*-translated Dmrt1 protein and nuclear extract from the tilapia testis but not the ovary. The band was completely removed with cold probe competitor. Taken together, these results suggest that Dmrt1 represses aromatase gene expression by binding to the *Cyp19a1a* promoter

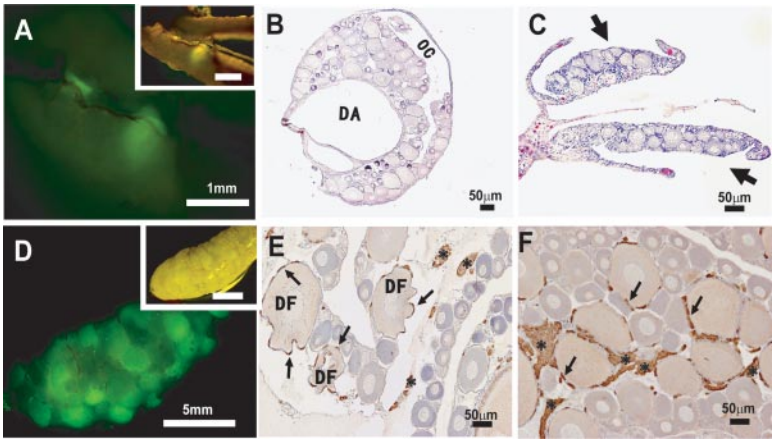

FIG. 4. Transgenic overexpression of *Dmrt1 in* the XX gonad (A–E). A, Ovary from a 5-month-old XX fish showing the mosaic expression of GFP in a dark field; *inset*, bright field. B, Cross-section of A through the region expressing GFP with H.E. staining to show the degenerated area (DA) and ovarian cavity (OC). C, Section of an ovary from a 3-month-old XX fish with H.E. staining showing the open ovarian cavity (*arrow*). Ovarian cavity should have been closed in 3-month-old normal female fish. D, Ovary from a 6-month-old XX fish showing the mosaic expression of signal in a dark field; *inset*, bright field. E, Cross-section from D with antiaromatase staining to show the decrease of aromatase expression in both interstitial cells (*asterisk*) and degenerating follicles (DF, *arrow*) in comparison with the control (F).

with its DM domain. However, at present, the possibility that Dmrt1 physically interacts with Ad4BP/SF-1 cannot be ruled out. Further detailed assessment of the functionality of the −447/−424 element will be required to determine whether Dmrt1 represses aromatase promoter activity by binding to DNA.

The suppressive effect of Dmrt1 on *Cyp19a1a* expression was further confirmed by transgenic experiments *in vivo*. Immunohistological analysis revealed that the transgenic overexpression of *Dmrt1* in XX tilapia down-regulated *Cyp19a1a* expression. Consistent with this, serum E2 levels in these fish were found to be reduced to only half of those in the control females, providing strong support to the claim that Dmrt1 represses aromatase gene transcription and reduces the production of E2. These results are consistent with those of our earlier studies showing that the androgen (17α-methyltestosterone)-induced sex reversal of XX tilapia fry was accompanied by a testicular structure with strong *Dmrt1* expression and down-regulation of the expression of several steroidogenic genes including *Cyp19a1a* (33, 41).

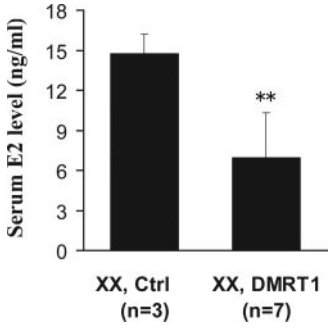

FIG. 5. Impact of transgenic overexpression of *Dmrt1* on tilapia serum E2 levels. Results are presented as the mean ± SD. Sample numbers are shown in the figure. **, Significantly lower than the control with Student's *t* test, $P < 0.01$.

Although exogenous estrogens have been shown to be important for the formation of the ovary in nonmammalian vertebrates, the exact role of endogenous estrogen is still unclear. One of the known functions of endogenous estrogen in fish is to induce the development of the ovarian cavity (30, 42). During normal development in tilapia, the ovarian cavity is formed around 2 months after hatching. However, some gonads with Dmrt1 overexpression had an open ovarian cavity, even at 3 months after hatching, indicating a delay in the formation of the cavity because of reduced E2 production. A similarly abnormal formation of the ovarian cavity was observed in transgenic XX tilapia with overexpression of dominant-negative mutants of Foxl2 (31). Some of these gonads failed to form an ovarian cavity, and yet some individuals had gonads with an open ovarian cavity, even at 5 months after hatching.

In the present study, overexpression of Dmrt1 in the XX gonad resulted in varying degrees of follicular degeneration and partial or complete sex reversal, a phenotype similar to that seen after overexpression of the Foxl2 dominant-negative mutant in XX tilapia (31). In nonmammalian vertebrates, estrogen-induced feminization of genetically male individuals caused an up-regulation of *Foxl2* expression and down-regulation of *Dmrt1* expression, whereas treatment with androgen or an aromatase inhibitor to masculinize genetic females caused a suppression of the expression of *Foxl2* and steroidogenic enzymes including aromatase, and up-regulation of *Dmrt1* expression, suggesting a direct link among Foxl2, Dmrt1, and estrogens (8, 10, 17, 19, 31, 41, 43–48). It is worth mentioning that before morphological sexual differentiation, *Foxl2* and *Dmrt1* were expressed in germ cell-surrounding cells in female and male gonads, respectively. Granulosa and Sertoli cells are counterparts of the female (ovary) and male (testis) gonads, respectively. Repressive effects of Foxl2 on the promoter activity of *Dmrt1* through the regulatory region also offered a potential mechanism for *Dmrt1* transcriptional silencing in granulosa cells of tilapia, as suggested in mice (46). The up-regulation of *Foxl2* expression in the XX gonad and XY sex-reversed gonad enhances aromatase gene expression, resulting in increased E2 levels that induce ovarian differentiation (34), whereas the up-regulation of *Dmrt1* expression in the XY gonad and XX sex-reversed gonad represses aro-

matase gene expression, resulting in low levels of E2 that favor testicular differentiation.

Experiments with Dmrt1 knockout mice indicated that females have normal ovaries and are fertile, whereas testicular differentiation in males is affected (49). The expression patterns of *Dmrt1* in developing gonads differ quite extensively between tilapia and mice, although the gene is highly expressed in the adult testis in both species. In tilapia, *Dmrt1* is expressed from as early as 6 dah, long before the morphological differentiation, which starts at 25 dah, and subsequently increases. In mice, whereas the dimorphic expression pattern of *Dmrt1* suggested a predominant role in male sexual development, its occurrence after the gonads begin their sex-specific programs argues against a role in the initial determination of gonadal sex. Another major difference in terms of sex determination and sexual differentiation between fish (tilapia) and mammals (mice) lies in steroids, which play a critical role in both processes in fish, but only in the latter process in eutherian mammals. In fish, such as tilapia and rainbow trout (21, 50), steroidogenic enzymes (including aromatase) are expressed in the XX gonad, but not the XY gonad, before the morphological differentiation starts, indicating delayed Leydig cell differentiation in the XY fish. In contrast, in eutherian mammals, steroidogenic enzymes are expressed once sex has been determined with an earlier expression in the XY gonad during sexual differentiation. Based on these differences, we speculate that if *Dmrt1* could be knocked out (now technically unfeasible) in tilapia, *Dmrt1* knockout XY fish should develop ovaries. This idea is supported by the present data from medaka. A duplicated copy of *Dmrt1*, *DMY/dmrt1b*, the master sex-determining gene in medaka, was also observed to be a strong suppressor of *Cyp19a1a*. The natural mutant, with a single insertion in exon 3 and the subsequent truncation of DMY, resulting in all XY female offspring (9), was found to lose part of the suppressive effect toward *Cyp19a1a* (supplemental Fig. S5). More recently we have shown that *DMY* knockdown using an RNA interference approach induces complete male-to-female sex reversal in XY medaka (Chakraborty, T., unpublished data). Finally, Dmrt1-knockout XY medaka (ENU mutants) were found to be sex reversed (Matsuda, M., unpublished data). Taken together, these data indicated that Dmrt1 is very important for both sex determination and sexual differentiation in tilapia but only important for testicular differentiation in mice.

Acknowledgments

We thank Dr. M. W. Murphy (Department of Genetics, Cell Biology, and Development, University of Minnesota, Minneapolis, MN) for providing the luciferase-reporter plasmid containing four DMRT1-binding sites.

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This work was supported by Grants 30770272, 30871905, and 30831160508 from the National Natural Science Foundation of China; Grant 2007AA10Z165 from the National High Technology Research and Development Program (863 program) of China; Grant 2009CB941200 from the National Basic Research Program of China; Grant IRT0859 from the Program for Changjiang Scholars and Innovative Research Team in University; and Grant CSTC, 2008BB1006 from the Natural Science Foundation Project of Chongqing, Chongqing Science and Technology Commission; and in part by a Grant-in-Aid for Scientific Research from the Solution-Oriented Research for Science, and Technology Research Project of the Japan Science and Technology Corp.

Disclosure Summary: The authors have nothing to disclose.

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