A W-linked DM-domain gene, DM-W, participates in primary ovary development in *Xenopus laevis*

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In the XX/XY sex-determining system, the Y-linked *SRY* genes of most mammals and the *DMY/Dmrt1bY* genes of the teleost fish medaka have been characterized as sex-determining genes that trigger formation of the testis. However, the molecular mechanism of the ZZ/ZW-type system in vertebrates, including the clawed frog *Xenopus laevis*, is unknown. Here, we isolated an *X. laevis* female genome-specific DM-domain gene, *DM-W*, and obtained molecular evidence of a W-chromosome in this species. The DNA-binding domain of DM-W showed a strikingly high identity (89%) with that of DMRT1, but it had no significant sequence similarity with the transactivation domain of DMRT1. In nonmammalian vertebrates, DMRT1 expression is connected to testis formation. We found *DMRT1* or *DM-W* to be expressed exclusively in the primordial gonads of both ZZ and ZW or ZW tadpoles, respectively. Although *DMRT1* showed continued expression after sex determination, *DM-W* was expressed transiently during sex determination. Interestingly, *DM-W* mRNA was more abundant than *DMRT1* mRNA in the primordial gonads of ZW tadpoles early in sex determination. To assess the role of DM-W, we produced transgenic tadpoles carrying a DM-W expression vector driven by ≈3 kb of the 5′-flanking sequence of *DM-W* or by the cytomegalovirus promoter. Importantly, some developing gonads of ZZ transgenic tadpoles showed ovarian cavities and primary oocytes with both drivers, suggesting that DM-W is crucial for primary ovary formation. Taken together, these results suggest that *DM-W* is a likely sex (ovary)-determining gene in *X. laevis*.

FISH | sex determination | transgenic | W-chromosome | ZZ/ZW

The sexual fate of metazoans is determined genetically or by environmental factors, such as temperature. In the former case, heterogametic sex chromosomes determine the male (XY♂) or female (ZW♀) fate in many species of vertebrates. In the XX/XY sex-determining system, the Y-linked *SRY* genes of most mammals and the *DMY/Dmrt1bY* gene of the teleost fish medaka have been characterized as sex-determining genes that initiate testis formation, leading to male sexual development (1–5). In contrast, the molecular mechanism for the ZZ/ZW sex-determining system remains unclear, because no sex-determining genes have been isolated.

The *Drosophila melanogaster doublesex* (*dsx*) and *Caenorhabditis elegans male abnormal* (*mab*)*-3* genes are known to control sexual development in these animals (6, 7). The two genes encode proteins containing a zinc finger-like DNA-binding motif called the DM domain. In vertebrates, the DM-domain gene *DMRT1* is implicated in sexual development. In the mouse, *DMRT1* is essential for postnatal testis differentiation (8, 9). In some other vertebrates, such as the chicken and turtle, DMRT1 expression is connected to testis formation in undifferentiated gonads (10–12). As mentioned above, the medaka fish gene *DMY/Dmrt1bY*, which is a coorthologue of *DMRT1*, causes testis formation as a sex-determining gene (3–5). In the chicken, which has the ZZ/ZW system, *DMRT1* is located on the Z chromosome, suggesting that gene dosage may induce male development (10, 11).

In some species of amphibians, sex determination is controlled genetically (13), even though the animals' sex chromosomes are morphologically indistinguishable from the autosomes. The South African clawed frog *Xenopus laevis* uses the ZZ/ZW system, which was demonstrated by backcrosses between sex-reversed and normal individuals (14), but its sex chromosomes have not yet been identified. Moreover, as with other animals that use the ZZ/ZW system, no sex-determining gene(s) has been identified. We recently showed that *X. laevis DMRT1* is expressed during embryogenesis and is then restricted to the primordial gonads. Furthermore, our *in vitro* experiments showed that the C-terminal region of DMRT1 is a transactivation domain (15). Here, we report the isolation of a W-linked paralogue of *DMRT1*, *DM-W*, in *X. laevis*. Although the DNA-binding domains of DM-W and DMRT1 shared high sequence identity (89%), their C-terminal regions had no significant sequence similarity. A comparative analysis of the *DM-W* and *DMRT1* mRNA expression patterns showed that *DM-W* was expressed predominantly in the primordial ZW gonads during early sex determination. These findings and phenotypic analyses of transgenic animals carrying DM-W expression vectors indicated that the W-linked gene, *DM-W*, is a probable sex (female)-determining gene in *X. laevis*.

Results

Isolation of *X. laevis* Female Genome-Specific Gene *DM-W*. To clarify the role of DM-domain genes in the sex-determining system of *X. laevis*, we previously isolated the *DMRT1* cDNA (15). Because medaka *DMY/Dmrt1bY* is located on the Y chromosome and chicken *DMRT1* is located on the Z chromosome (3, 4, 10, 11), we next examined whether *DMRT1* or its putative homologues were linked to the *X. laevis* sex chromosomes by performing Southern blot analyses of the genomic DNAs from adult females and males. Using the sequence encoding amino acids 292–336 of DMRT1, which does not include the DM domain, as a probe, we detected bands of ≈4.0 kb in both males and females. However, no specific bands in the female genome or doubly dense bands in the male genome were observed, suggesting that *DMRT1* was autosomal (Fig. 1*A*). Interestingly, the full-length cDNA probe, containing the DM-domain sequence, hybridized with an 8.0-kb DNA fragment only in samples from females, as well as with the 4.0-kb bands in both sexes (data not shown).

We cloned the 8.0-kb fragment by screening an *X. laevis* female genomic library with the full-length *DMRT1* cDNA. This

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The authors declare no conflict of interest.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AB259777 and AB365520).

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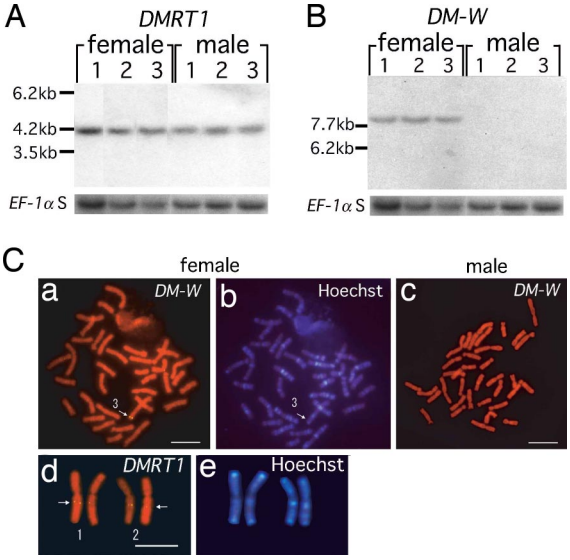

Fig. 1. *DM-W* is a female genome-specific DM-domain gene in *X. laevis*. (*A* and *B*) Southern blot analysis of *DMRT1* and *DM-W*. EcoRI-digested genomic DNA (20 μg) from *X. laevis* female and male liver was hybridized with the cDNA sequence corresponding to DMRT1 (amino acids 292–336) or DM-W (amino acids 124–194) as a probe. EF-1α (somatic) was used as a control. Numbers 1–3 correspond to the individual numbers. (*C*) Chromosomal localization of *DM-W* and *DMRT1*. Arrow indicates the fluorescence hybridization signal of *DM-W* (*a*) or *DMRT1* (*d*). FISH patterns of *DM-W* are shown on PI-stained metaphase spreads of female (*a*) and male (*c*) *X. laevis*. Hoechst-stained pattern of the same metaphase spread as *a* is shown in *b*. The *DM-W*-located W chromosome was identified as chromosome 3 (16). FISH pattern of *DMRT1* (*d*) and Hoechst-stained pattern (*e*) are shown on the same chromosomes, which corresponds to chromosomes 1 and 2 (16). (Scale bars, 10 μm.)

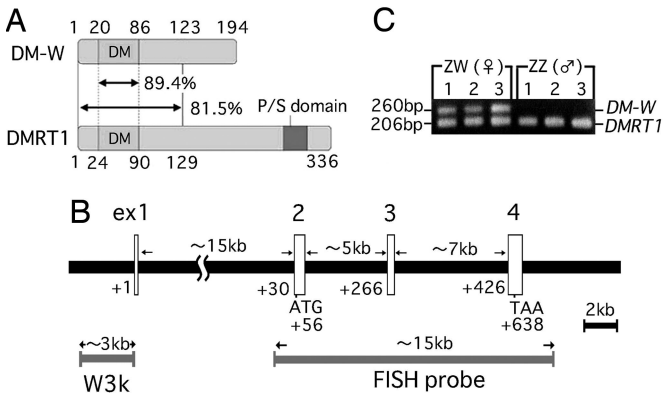

Fig. 2. Structures of the *DM-W* gene and its protein. (*A*) Schematic drawing of DM-W and DMRT1. DM-W and DMRT1 have high identity, except in their C-terminal regions. The P/S domain is proline- and serine-rich. DM, a zinc finger-like DNA-binding motif called the DM domain. (*B*) Genomic structure of the *DM-W* gene. *DM-W* consists of 4 exons, and the sequences corresponding to the initiation and stop codons lie within exons 2 and 4, respectively, as indicated. W3k, the ≈3 kb of the 5′-flanking region (see GenBank/EBI Data Bank accession number AB365520) used to construct the DM-W expression vector, pW3k-DM-W (see *Materials and Methods*); FISH probe, ≈15 kb of the genomic sequence, including exons 2–4, used for FISH analysis. (*C*) Genotyping ZW and ZZ individuals by PCR using genomic DNA isolated from liver. Sequence information of primers used is described in *Materials and Methods*. Numbers 1–3 correspond to the individual numbers in Fig. 1*A*.

screen revealed another DM domain-encoding sequence, corresponding to amino acids 75–129 of DMRT1 and with high identity to the DM domain of DMRT1. Next, we obtained the 5′- and 3′-flanking cDNA sequences of this partial DM domain by 5′- and 3′-RACE, and the full-length cDNA was amplified by PCR from the flanking sequences. As expected, a Southern blot probed with a part of this cDNA sequence that had little homology to *DMRT1* showed a single band of ≈8.0 kb in only the female samples (Fig. 1*B*). We named this female genome-specific DM gene *DM-W*, because it should be on the W chromosome. The nucleotide and deduced amino acid sequences of the *DM-W* cDNA were deposited in the GenBank/EBI Data Bank under accession number AB259777.

Identification of the W Chromosome in *X. laevis*, Which Has the ZZ/ZW Sex-Determining System. We next performed fluorescence *in situ* hybridization (FISH) for *DM-W* and *DMRT1* to determine their chromosomal locations (Fig. 1*C*). The Hoechst-stained bands obtained by the replication R-banding method, which correspond to G bands, made it possible to identify each chromosome. The hybridization signal of *DM-W* was observed on one chromosome in a female (Fig. 1*Ca*), and no signals were found on male metaphase spreads (Fig. 1*Cc*). We obtained the same results in two other females and another male (data not shown). These results indicated that the female-specific chromosome, where the *DM-W* was localized, is the W chromosome of *X. laevis*. This molecular evidence indicates the existence of a W chromosome in *X. laevis*, because the *X. laevis* sex chromosomes are morphologically indistinguishable from the autosomes (16). The W chromosome was identified as chromosome 3 (Fig. 1 *Ca* and *Cb*), following the nomenclature of *X. laevis* chromosomes in the report by Schmid and Steinlein (16). In contrast, the hybridization signals for *DMRT1* were found on two pairs of chromosomes, chromosomes 1 and 2 (Fig. 1*Cd* and *Ce*). The duplicate signals most likely reflect the duplication of a chromosomal pair during tetraploidization (16).

***DM-W* Is a Paralogue of *DMRT1*.** DM-W consisted of 194 aa residues, and its DM domain (amino acids 20–86) had a strikingly high identity (89%) with that of DMRT1 [Fig. 2*A* and supporting information (SI) Fig. 5]. In contrast, the DM domains of DMRT family members in individual vertebrates (medaka, zebrafish, and mouse) share ≈60–75% identity (17). In addition, the sequences flanking the DM domain of DM-W (amino acids 1–19 and 87–123) were conserved with the corresponding regions of DMRT1 (≈68% and ≈82% identity, respectively). Moreover, a phylogenetic analysis of vertebrate DMRT family members showed that DM-W belongs to the DMRT1 subgroup (SI Fig. 6). Because these observations suggest that *DM-W* may have evolved through a duplication of *DMRT1*, we concluded that *DM-W* is a paralogue of *DMRT1*. In contrast to the strong sequence similarity elsewhere, the C-terminal region (amino acids 124–194) of DM-W showed no significant sequence similarity to DMRT1. Therefore, the protein products of these two genes may function differently as transcription factors.

We next examined the gene structure of *DM-W* by using several genomic clones (see *Materials and Methods*). The structural analysis of the cDNA and genomic sequences indicated that the *DM-W* gene consisted of four exons, as shown in Fig. 2*B*. Exon 2 contained the ATG sequence (+56 to +58) for the initiation codon, and exon 4 contained the TAA sequence (+638 to +640) for the termination codon. Although we did not obtain the gene structure of *X. laevis DMRT1*, our recent analysis of the *DMRT1* gene in *X. tropicalis*, which is closely related to *X. laevis*, showed that it encodes a protein whose sequence shares ≈92% identity with *DMRT1* of *X. laevis* and consists of at least six exons (15). The N-terminal region (amino acids 1–123) of DM-W was encoded by exons 2 and 3 as is the corresponding region (amino acids 1–129) of *X. tropicalis* DMRT1. Moreover, the splice-junctional regions of exons 1–3 were conserved between *DM-W* and *X. tropicalis DMRT1*. In contrast, exon 4, which contained the coding sequence for the C-terminal region (amino acids

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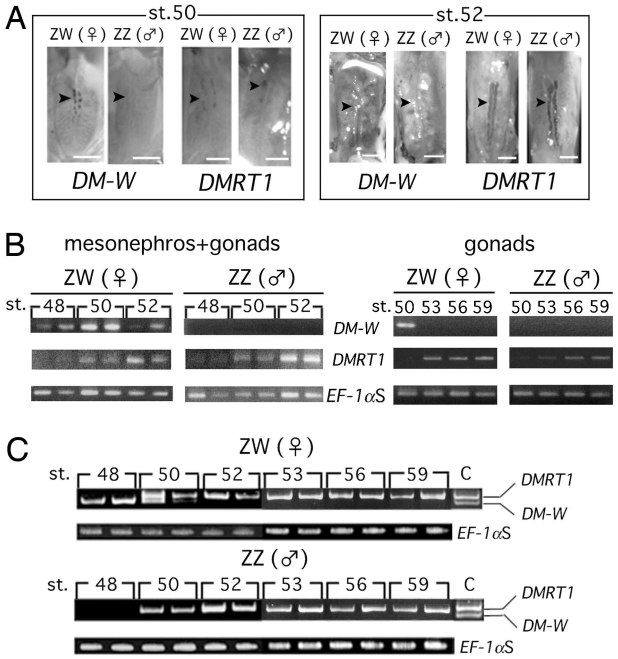

Fig. 3. Expression profiles of *DM-W* and *DMRT1* in the primordial gonads during the sex-determination period are shown. ZZ or ZW status was initially determined by PCR using genomic DNA from individuals, as described in *Materials and Methods*. (*A*) *In situ* hybridization of *DM-W* and *DMRT1* during sex determination (st. 50 and 52). The arrowhead indicates the primordial gonads. (Scale bars, 0.5 mm.) (*B*) RT-PCR of *DM-W* and *DMRT1* in primordial gonads at st. 48–59. Primordial gonads along with the mesonephros, from two ZZ and ZW tadpoles each at st. 48–52 were used (*Left*). The data from the same-stage ZZ and ZW individuals show similar expression levels. Because the primordial gonad without the mesonephros is too small to isolate sufficient RNA for RT-PCR at st. 48, RNA was isolated from five ZZ or ZW gonads at st. 50–59 (*Right*). (*C*) A comparison of mRNA levels using a competitive RT-PCR method for *DM-W* and *DMRT1* performed during and after sex determination. Total RNA was extracted from the gonad/mesonephros at st. 48–52 or from gonads at st. 53–59, respectively. RT-PCR was performed for each of two ZZ or ZW tadpoles at each stage, which confirmed that there were few individual differences. Lane C shows a control PCR in which the same amounts of pcDNA-FLAG-DM-W and pcDNA-FLAG-DMRT1 were used.

124–194) of DM-W, and had little homology with DMRT1, appears to be an evolutionarily unique feature of DM-W.

***DM-W* Shows Spatiotemporal Expression.** First, we developed a simple method for judging whether an individual is a genetic female (ZW) or male (ZZ) by PCR, by using specific primer pairs for the *DMRT1* and *DM-W* genes (Fig. 2*C* and SI Fig. 7). This method uses a small amount of genomic DNA and permits discrimination of the genetic sex even of embryos and tadpoles, and it is useful for analyzing whether a possible sex-related gene shows sex-dependent expression during development.

Next, we examined the distribution patterns of the *DM-W* and *DMRT1* transcripts by whole-mount *in situ* hybridization (WISH) in ZZ and ZW tadpoles at st. 50 and 52 during sex determination. At st. 50, *DMRT1* was expressed exclusively in the primordial gonads of both ZW and ZZ tadpoles, and *DM-W* showed almost the same pattern as *DMRT1* but in only the ZW tadpoles (Fig. 3*A Left*). This result was expected, because ZZ tadpoles do not carry the endogenous *DM-W* gene. Interestingly, *DM-W* transcripts were barely detectable at st. 52 in the ZW gonads, but the *DMRT1* transcript levels were maintained in the ZW and ZZ gonads (Fig. 3*A Right*).

We also examined the expression levels of these two genes by RT-PCR. We first had to include the mesonephros along with the gonads at the early stage of sex-determination (st. 48), because the primordial gonads are too small to dissect away from the mesonephros at this stage. The RT-PCR at st. 48–52 showed *DM-W* was highly expressed at st. 50, and *DMRT1* expression gradually increased in both ZZ and ZW individuals during this period (Fig. 3*B Left*). We next examined the expression levels in only gonads of the ZZ and ZW tadpoles at st. 50 and during early development into ovaries and testes (st. 53–59). *DM-W* was exclusively expressed in ZW primordial gonads only at sex determination (st. 50); *DMRT1* showed continued expression in both ZZ and ZW gonads during gonadal differentiation (Fig. 3*B Right*).

***DM-W* Shows Much Higher Expression than *DMRT1* in Primordial ZW Gonads at the Early Stage of Sex Determination.** Next, to compare in detail the expression levels of *DM-W* and *DMRT1* from the early stage of sex determination through its completion (st. 48–59) in ZW individuals, we performed comparative RT-PCR for the two mRNAs by using a protocol based on competitive PCR. A pair of primers was designed that recognized a common cDNA sequence in the two genes but would amplify different-sized cDNA fragments for each gene. Intriguingly, *DM-W* expression predominated early (st. 48), and *DMRT1* and *DM-W* showed similar levels of expression at st. 50 (Fig. 3*C*). This predominant expression of *DM-W* during early sex determination supports the idea that DM-W may play a role in (female) sex determination. Conversely, the *DMRT1* expression was much higher at st. 52. During the subsequent development of the gonads, *DM-W* expression was barely detectable, but *DMRT1* was continuously expressed in both the ZZ and ZW gonads. These results indicate that *DM-W* is specifically expressed in the heterogametic gonads during sex determination. This spatiotemporal expression pattern resembles that of the other known sex-determining genes, mammalian *SRY* (2) and medaka *DMY*/*Dmrt1bY* (3).

Exogenous *DM-W* Causes Developing Ovotestes in ZZ Tadpoles. To verify that DM-W plays a role in sex (ovary) determination or gonadal differentiation, we produced and analyzed transgenic tadpoles carrying an expression plasmid (pW3k-DM-W) that contained the *DM-W* cDNA with ≈3 kb of its 5′-flanking sequence (see Fig. 2*B*). The presence of the transgene and the ZW or ZZ status of the tadpoles were determined by genomic PCR (see *Materials and Methods*). We first examined the expression of the *DM-W* transgene in the primordial gonads of the ZZ transgenic tadpoles at st. 50 by RT-PCR. As shown in Fig. 4*A*, some transgenic ZZ gonads expressed more *DM-W* than normal ZW gonads, and others expressed less.

In the primary gonadal differentiation that occurs after sex determination in *X. laevis*, the germ cells migrate from the cortex to the medulla in genetically male gonads, but in genetic females they remain in the cortex of the gonads beginning to form ovarian cavities. At st. 56, the developing testes include primary spermatogonia but no meiotic cells; developing ovaries contain early meiotic oocytes, which are morphologically distinct from oogonia and spermatogonia (18–20). We confirmed these observations in normal, genetically female (ZW) or male (ZZ) tadpoles at st. 56 (Fig. 4*B Left*). We then analyzed DM-W's effect on the primary development of testes and ovaries in transgenic tadpoles at st. 56. The right gonad of each transgenic tadpole was sectioned, and the left one was used for RT-PCR. Intriguingly, three of the nine ZZ transgenic tadpoles (ZZ#1–3 in Fig. 4 *B* and *C* and/or SI Fig. 8*A*) developed ovotestes, which contained both ovarian cavities and testicular structures. Primary oocytes were observed near the ovarian cavities in these gonads (Fig. 4*B*). These three gonads expressed *DM-W* at almost the same level as normal ZW gonads. In contrast, developing testes of the two other ZZ transgenic tadpoles that we examined for *DM-W*

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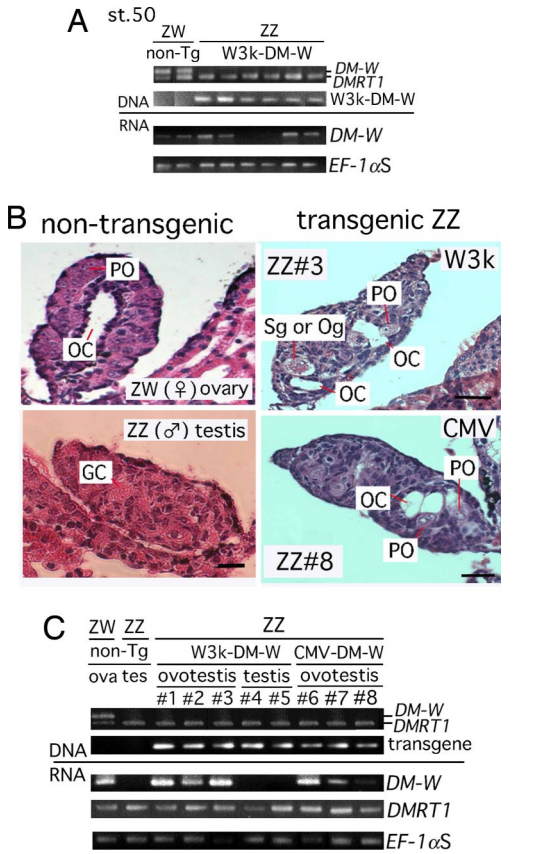

Fig. 4. Analysis of ZZ transgenic tadpoles carrying an expression vector for *DM-W*. (*A*) RT-PCR of *DM-W* of st. 50 tadpole gonads, including normal ZW gonads and transgenic gonads carrying pW3k-DM-W. Genomic PCRs for transgene insertion and to determine genetic sex are shown in the upper two panels. (*B*) Sections of normal developing ovary and testis (*Left*) and developing gonads with both testicular and ovarian structures (ovotestes) from transgenic ZZ tadpoles carrying pW3k-DM-W or pcDNA3-FLAG-DM-W (*Right*) at st. 56. OC, ovarian cavity; PO, primary oocyte; Sg, spermatogonium; Og, oogonium. Note that Sg and Og were morphologically indistinguishable. (Scale bars, 20 μm.) The ZZ transgenic tadpole, ZZ#3 or ZZ#8, carrying the DM-W expression vector pW3k-DM-W and pcDNA3-FLAG-DM-W, respectively, showed ovotestes. (*C*) RT-PCR of *DM-W* and *DMRT1* in normal gonads and transgenic gonads carrying pW3k-DM-W or pcDNA3-FLAG-DM-W, from st. 56 tadpoles. Genomic PCRs for transgene insertion and genetic sex are shown in the upper two panels. ZZ#1–ZZ#8 correspond to individual transgenic ZZ tadpoles described in the text, and ZZ#3 and ZZ#8 correspond to individual animals in *B*.

expression (ZZ#4 and ZZ#5 in Fig. 4*C* and SI Fig. 8*A*) expressed scarcely any *DM-W* (Fig. 4*C*). This result suggests that extremely low levels of exogenous *DM-W* expression in ZZ tadpoles could not induce the developing gonads to form ovarian structures. All 3 nontransgenic ZZ tadpoles and all 10 transgenic ZW tadpoles showed normal, genetically sex-appropriate, developing gonads (Table 1).

We also produced and analyzed transgenic tadpoles carrying another *DM-W* expression plasmid (pcDNA3-FLAG-DM-W) driven by a cytomegalovirus (CMV) promoter. At st. 56, three of the seven ZZ transgenic tadpoles (ZZ#6–8 in Fig. 4 *B* and *C* and/or SI Fig. 8*A*) showed ovotestes, and all five of the ZW transgenic tadpoles showed normal female gonads (Table 1). Exogenous *DM-W* was expressed in all three ZZ ovotestes (Fig. 4*C*).

We could not obtain enough RNA from only one (left) gonad of each transgenic tadpole, and then did not examine expressions of other genes, which could be involved in early sex differentiation. However, we barely examined expression of *P-450Arom*, the estrogen synthase (aromatase) gene only in the three gonads of the ZZ transgenic tadpoles (#2, #6, and #7) and normal gonads at st. 56 by RT-PCR. These ovotestes showed more than a few transcripts for *P-450Arom*, although we could not detect a band derived from the *P-450Arom* mRNA in normal ZZ testis (SI Fig. 8*B*).

Discussion

The existence of two sexes is nearly universal in vertebrates, but the mechanisms of sex determination are variable. In the XX/XY sex-determining system, *SRY* (in mammals) and *DMY/Dmrt1bY* (in medaka fish) have been isolated on the male-specific Y chromosome as a sex (male)-determining gene. However, no sex (female)-determining genes in the ZZ/ZW sex-determining system have been isolated so far. Here, we report a W-linked gene in *X. laevis* and a promising candidate for a (female) sex-determining gene in the ZZ/ZW system.

We characterized two DM-domain genes, *DMRT1* and its W-linked paralogue, *DM-W*, in *X. laevis*. The DNA-binding domain (amino acids 20–86) of DM-W had a strikingly high identity (89%) with that of DMRT1 (Fig. 2*A*). Recently, Murphy *et al.* reported that mouse DMRT1, -2, -3, -4, -5, and -7 can bind similar DNA sequences (21). They also showed that all six of these proteins bound the same DNA sequence in a gel mobility assay. We confirmed that DM-W and DMRT1 bound to this sequence as well (data not shown). The C-terminal region (amino acids 124–194) of DM-W showed no significant sequence similarity with the corresponding region (amino acids 130–336) of DMRT1 (Fig. 2*A*), which contains a transactivation domain (15). These findings suggest that the protein products of these two genes might function differently as transcription factors. It would be interesting to investigate whether DM-W functions as a competitor of DMRT1 in the ZW primordial gonads during sex determination.

The DNA sequence corresponding to the C-terminal region (amino acids 124–194) of DM-W is encoded by exon 4 (Fig. 2*B*), whereas the C-terminal region (amino acids 130–337) of *X. tropicalis* DMRT1 is encoded by exons 4–6 (15). The sequence of the *DM-W* exon 4 contained no significant homology among genomic and cDNA sequences derived from other species including *X. tropicalis*. If the exon–intron structure of the *X. tropicalis DMRT1* is the same as that of the *X. laevis DMRT1*, the unique exon 4 of *DM-W* might have emerged through or after a duplication of *DMRT1*, resulting in the functional difference between the two proteins.

Importantly, ectopic *DM-W* expression in some ZZ tadpoles induced the formation of ovarian cavities and primary oocytes in developing gonads (Fig. 4*B*). Analyses of the expression levels of the exogenous *DM-W* in primordial ZZ gonads at sex determination (st. 50; Fig. 4*A*) and early gonadal differentiation (st. 56; Fig. 4*C*) suggested that the level of transgene expression correlated with the gonadal phenotype. These findings indicated that DM-W participates in primary ovary formation. However, these *DM-W* expression vectors did not produce completely normal developing ovaries in ZZ tadpoles. It is possible that other W-linked factor(s) in ZW gonads are necessary for early development into ovaries or that some Z-linked factor(s) in ZZ gonads partially interferes with ovary formation through a gene-dosage effect. It would be interesting to produce transgenic ZW individuals carrying a DM-W knockdown vector to investigate the effect of *DM-W* down-regulation on sex determination and gonadal differentiation.

Our findings lead us to propose that ZZ/ZW sex determination in *X. laevis* by the female genome-specific gene *DM-W* and the presumptive testis-forming gene *DMRT1* may be regulated by competition for DNA-binding sites in ZW embryos. In this scenario, DM-W binds the target gene(s) of DMRT1 in ZW primordial gonads during sex determination, thus preventing

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Table 1. Genotyping and phenotyping of transgenic tadpoles carrying the DM-W expression vector driven by the 3-kb 5′-flanking sequence of *DM-W* or by the CMV promoter

Transgene	Genetic sex	Transgenesis	No. of tadpoles			
			Total no.	Developing testes	Developing ovaries	Ovotestes
pW3k-DM-W	ZZ	−	3	3	0	0
	ZZ	+	9	6	0	3
	ZW	+	10	0	10	0
pcDNA3-FLAG- DM-W	ZZ	−	4	4	0	0
	ZZ	+	7	4	0	3
	ZW	+	5	0	5	0

DMRT1 from interacting with its binding site and suppressing the formation of testes. This model would explain why *DM-W* was expressed at much higher levels than *DMRT1* in ZW gonads early (st. 48) in sex determination (Fig. 3*C*). To test this idea, it will be necessary to show that DMRT1 is a testis-forming factor. We also need to confirm that DM-W and DMRT1 are colocalized in specific cells in the ZW primordial gonad during sex determination and clarify for which gene(s) they may compete.

Beyond the sexual fate of individual animals, there are evolutionary implications of a sex-determining system mediated by DM-domain proteins. All metazoan species examined so far have multiple DM-domain genes, and at least one such gene may be required in each species for sexual determination and/or development (17), suggesting that the DM-domain genes and sexual reproduction may have coevolved in metazoans. Although Sry seems to have occupied the top position in the testis-formation cascade during mammalian evolution, some species of nonmammalian vertebrates may have developed a different regulatory system for DMRT1 expression or function, contributing to species diversity. In birds, *DMRT1* might have become Z-linked to exert its gene-dosage effect. In contrast, in *X. laevis*, *DM-W* might have emerged on the W chromosome, following the duplication of *DMRT1*, as the dominant-negative gene for *DMRT1*. Interestingly, we could not find a *DM-W* orthologue in the genome database of *X. tropicalis*, which is related to *X. laevis*. Because medaka *DMY/Dmrt1bY* is not the universal sex (testis)-determining gene in related species (22), the sex-determination mechanism of *X. laevis* may be different from that of *X. tropicalis*. It will be interesting to learn whether there is a *DM-W* orthologue in *X. borealis*, which is more closely related to *X. laevis*. In any case, we believe that the DM-domain genes, the sex-determining system, and species diversity could be closely related to one another.

Materials and Methods

Isolation of *DM-W*. *X. laevis* genomic libraries were constructed by using the ZAP Express and λFIX vectors (Stratagene) ligated, respectively, with ≈7–10 kb of EcoRI-digested or ≈8–16 kb of partially XhoI-digested genomic DNA fragments, which were derived from female liver. Genomic clones of the *DM-W* gene that included the third exon were isolated by plaque hybridization of the ZAP Express library by using a full-length *DMRT1* cDNA (15) probe. The DM-domain sequence of the third exon was used to obtain *DM-W* cDNAs by 5′ and 3′ rapid amplification of cDNA ends (RACE) by using a BD SMART PCR cDNA synthesis kit (Becton Dickenson Bioscience). The genomic clones for FISH or the DM-W expression vector were identified, respectively, by screening with the genomic library derived from the λFIX or ZAP Express vector, by using full-length *DM-W* cDNA or 80 bp of the 5′-untranslated flanking sequence of *DM-W*.

Southern Blot Analysis. EcoRI-digested genomic DNA from female or male liver was blotted and hybridized as described previously (23).

Cell Culture and Chromosome Preparation. Two males and three females were used for cell culture and chromosome preparations. After pithing, heart, lung, and kidney tissues were collected. The tissues were minced and cultured in DMEM (Invitrogen-GIBCO) supplemented with 15% FBS (Invitrogen-GIBCO), 1% insulin-transferrin-selenium-G supplement (Invitrogen-GIBCO), 100 μg/ml kanamycin, 1% antibiotic-antimycotic (PSA) (Invitrogen-GIBCO), and 2.5 μg/ml amphotericin B (Invitrogen-GIBCO). The fibroblast cell cultures were incubated at 26°C in a humidified 5% CO_2 atmosphere and maintained for 10–14 days in 65-mm plastic dishes (Iwaki). Primary cultured cells were harvested by using trypsin and then subcultured.

Replication R-banded chromosome preparations were made with the cultured cells at the third to fourth passage. BrdU (25 μg/ml) (Sigma-Aldrich) was added to the cell cultures at log phase, and the cell culturing was continued for 5 h, including 1 h of colcemid treatment (0.15 μg/ml), before harvesting. Chromosome slides were made according to a standard air-drying method. After being stained with Hoechst 33258 (1 μg/ml) for 5 min, the slides were heated to 65°C for 3 min on a hot plate and then exposed to UV light for an additional 5–6 min at 65°C (24).

FISH. FISH mapping was performed as previously described (24). A 15-kb fragment of the *DM-W* genomic DNA (see Fig. 2*B*) and a 1.6-kb fragment of the full-length *DMRT1* cDNA was labeled with biotin-16-dUTP (Roche Diagnostics) by using a nick translation kit (Roche Diagnostics) following a standard protocol. The labeled *DM-W* probe was ethanol-precipitated with 100× *X. laevis* genomic DNA that had been sonicated to suppress interspersed-type repetitive sequences. The chromosome slides hybridized with the *DM-W* probe were incubated with FITC-avidin (Vector Laboratories) and stained with 0.75 μg/ml propidium iodide (PI). The slides hybridized with the *DMRT1* cDNA were reacted with goat anti-biotin antibody (Vector Laboratories) and then stained with Alexa Fluor 488 rabbit anti-goat IgG (H+L) conjugate (Molecular Probes). The hybridization signals were observed under a Nikon fluorescence microscope with Nikon filter sets B-2A and UV-2A, and the FISH images were photographed with DYNA HG ASA 100 film (Kodak).

RT-PCR. Total RNA was isolated by using an RNeasy mini kit (Qiagen) from the primordial gonads and other tissues and was reverse transcribed with PowerScript (Clontech). PCR was carried out by using the resultant first-strand cDNA as a template and specific primer pairs for the target genes as follows: *DM-W*, 5′-CATTGCAAAGACAGCAAGCT-3′ and 5′-TCTGTGTTGCAGCATCAGCA-3′ following 5′-GAAGCTGGACTGCAGTAACT-3′ and 5′-AGACTACTAGACGAGGAGTG-3′; *DMRT1*, 5′- ATCACAGAAACCATCCAGCTG-3′ and 5′-TGGGTGGAGAAAGCACACTT-3′ following 5′-TACACAGACAACCAGCACAC-3′ and 5′-TGGGTGGAGAAAGCACACTT-3′. As controls, *EF-1αS* (somatic form of elongation factor-1 α) gene expression was examined by PCR with specific primers as follows: 5′-CCAGATTGGTGCTGGATATG-3′ and 5′-TTCTGAGCAGACTTTGTGAC-3′. Comparative RT-PCR, which was based on the competitive PCR method, was performed by using the common primers of *DM-W* and *DMRT1* cDNAs as follows: 5′-ATGCAAAACAATGAGGAACC-3′ and 5′-TATTCCYAGCTCCTCTTCCT-3′.

Determining ZW or ZZ Status in Individual Animals. Genomic DNA was isolated from the tail of a tadpole or liver of an adult frog by using a genomic DNA isolation kit (Promega) and was used to amplify the *DM-W* gene to determine the ZW type. The primers were 5′-CCACACCCAGCTCATGTAAAG-3′ and 5′-GGGCAGAGTCACATATACTG-3′. In the same reaction tube, the *DMRT1* gene was also amplified as a control by using primers 5′-AACAGGAGCCCAATTCTGAG-3′ and 5′-AACTGCTTGACCTCTAATGC-3′.

Plasmid Constructs. For the construction of the *DM-W* expression vector through its presumptive promoter, we first isolated a 3,155-bp 5′-flanking region (−3,155 to −1) of *DM-W* (GenBank/EBI Data Bank accession number

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AB365520) by screening the ZAP express genomic library, and the obtained fragments were ligated with the *DM-W* cDNA. The ligated fragment was inserted into the pIRESII-AcGFP vector (Clontech) to generate pW3k-DM-W. pcDNA3-FLAG-DM-W or pcDNA3-FLAG-DMRT1 was constructed by inserting the ORF of DM-W or DMRT1 in-frame downstream of the tag sequence of pcDNA3-FLAG vector (25).

Whole-Mount *in Situ* Hybridization. Whole-mount *in situ* hybridization was performed by using antisense and sense RNA probes for *DMRT1* (nucleotides 598–1,287) or *DM-W* (nucleotides 15–679), as described previously (26).

Production of Transgenic *Xenopus*. Transgenic tadpoles were produced by using restriction enzyme-mediated integration (REMI) on decondensed sperm nuclei followed by nuclear transplantation into unfertilized eggs (27). To detect the transgenes, PCR was performed by using the genomic DNA from the tail of the individual tadpole as the template and specific primer pairs: forward primer 5′-CATTGCAAAGACAAGCT-3′ and reverse primer 5′-TCTGTGTTGCAG-CATCAGCA-3′. The forward and reverse primers correspond to the sequences containing exons 3 and 4, respectively.

Histology. Gonads were fixed in Bouin's solution, dehydrated with methanol, embedded in paraffin wax, and cut into 7-μm sections. Hematoxylin/eosin staining was performed by using standard procedures.

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