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RESEARCH ARTICLE

Two DM Domain Genes, *DMY* and *DMRT1*, Involved in Testicular Differentiation and Development in the Medaka, *Oryzias latipes*

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The recent discovery of the *DMY* gene (DM domain gene on Y chromosome and one of the *DMRT1* family genes) as a key determinant of male development in the medaka (*Oryzias latipes*) has led to its designation as the prime candidate gene for sex-determination in this species. This study focused on the sites and pattern of expression of *DMY* and *DMRT1* genes during gonadal differentiation of medaka to further determine their roles in testis development. *DMY* mRNA and protein are expressed specifically in the somatic cells surrounding primordial germ cells (PGCs) in the early gonadal primordium, before morphological sex differences are seen. However, somatic cells surrounding PGCs never express *DMY* during the early migratory period. Expression of *DMY* persists in Sertoli cell lineage cells, from PGC-supporting cells to Sertoli cells, indicating that only *DMY*-positive cells enclose PGCs during mitotic arrest after hatching. *DMRT1* is expressed in spermatogonium-supporting cells after testicular differentiation (20–30 days after hatching), and its expression is much higher than that of *DMY* in mature testes. In XX sex-reversed testes, *DMRT1* is expressed in the Sertoli cell lineage, similar to the expression of *DMY* in XY testes. These results suggest strongly that *DMY* regulates PGC proliferation and differentiation sex-specifically during early gonadal differentiation of XY individuals and that *DMRT1* regulates spermatogonial differentiation. *Developmental Dynamics 231:518–526, 2004.* © 2004 Wiley-Liss, Inc.

Key words: *DMY*; *DMRT1*; sex determination; sex differentiation; primordial germ cell; Sertoli cell; medaka

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INTRODUCTION

In mammals, flies, and the worm *Caenorhabditis elegans,* the developmental cascade controlling sex determination is well understood. In most mammals, the male-inducing master gene, *SRY,* is located on the Y chromosome and, therefore, is absent in XX females. *SRY* seems to be specific to mammals (Swain and Lovell-Badge, 1999; Capel, 2000). In nonmammalian vertebrates, no master sex-determining gene has yet been identified. Recently, we reported an outstanding candidate for the first master sex-determining gene in a teleost fish, medaka (Matsuda et al., 2002). This gene, referred to as *DMY,* is located on the Y chromosome and is expressed exclusively in XY embryos. The *DMY* gene encodes a putative protein of 267 amino acids containing a DNA-binding domain called DM, which is also present in proteins (dsx in *Drosophila* and mab-3 in *C. elegans*) identified as transcriptional factors involved in sex determination in flies and nematodes (Shen and Hodgkin, 1988; Burtis and Baker, 1989; Yi et al., 2000).

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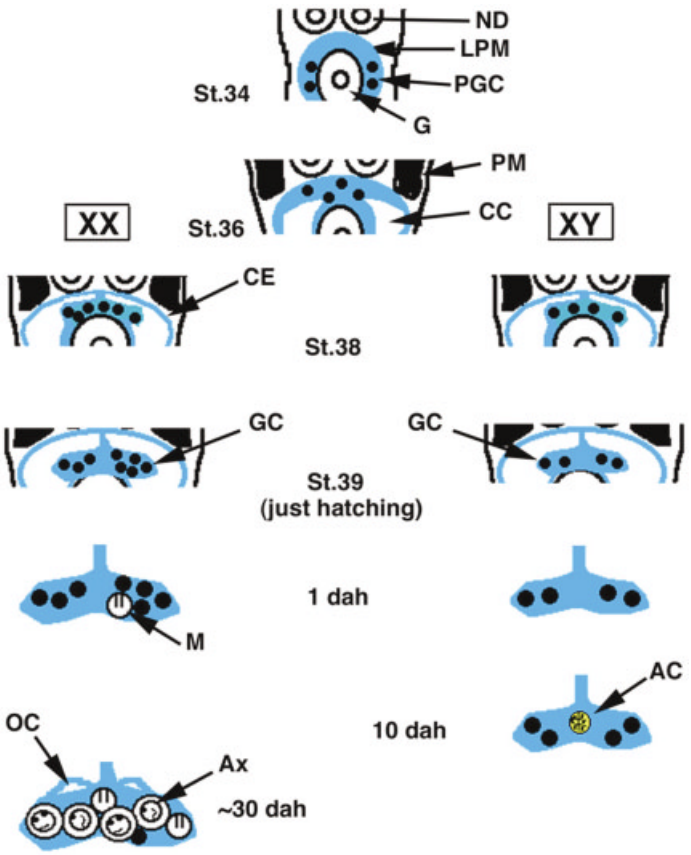

Fig. 1. Schematic representation of gonadal formation and sex differentiation in medaka. PM, paraxial mesoderm; LPM, lateral plate mesoderm; PGC, primordial germ cells; CE, coelomic epithelium; CC, coelomic cavity; ND, nephric duct; G, gut; M, meiotic cell; OC, ovarian cavity; AC, acinus structure; Ax, auxocyte. (Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.)

Our previous report indicates that *DMY* is essential for male medaka development, as spontaneous sex-reversed XY females produced a truncated DMY protein as a result of a single nucleotide insertion in exon 3. Furthermore, *DMY* mRNA was detected only in the somatic cells surrounding the germ cells of XY embryos at hatching. Of interest, *DMY* is a homolog of *DMRT1*, the putative transcription factor that is also involved in male development in other vertebrates including human, mouse, chicken, turtle, frog, and a teleost fish, tilapia (Raymond et al., 1998, 1999a,b; Smith et al., 1999; Kettlewell et al., 2000; Guan et al., 2000; Shibata et al., 2002). *DMRT1* appears to be involved in a specific type of XY sex reversal in humans (Veitia et al., 1997; Bennet et al., 1993; Flejter et al., 1998; Yi et al., 2000). However, it remains unclear how *DMY* controls male sex differentiation and whether *DMY* functions as a transcriptional factor.

Recent studies suggest that the medaka *DMY* gene was derived from the *DMRT1* gene through gene duplication (Nanda et al., 2002; Matsuda et al., 2003). In medaka, *DMRT1* is expressed predominantly in testis (Brunner et al., 2001). However, the relationship between *DMRT1* and *DMY* in male development remains unclear.

In this study, we investigated the characteristics of *DMY* and *DMRT1* expression during gonadal differentiation. Specifically, we focused on the following events: (1) the relationship between the timing of *DMY* expression and morphological sex differentiation; (2) the identity of *DMY* expressing cells; (3) the relationship between *DMRT1* expression and gonadal differentiation; and (4) *DMRT1* expression in XX testis. We show that the first appearance of morphological sex difference is the difference in germ cells (primordial germ cells, PGCs) number at stage 38 before hatching. We then show that *DMY* is specifically expressed in the somatic cells surrounding PGCs during early gonadal formation in XY embryos specifically, before morphological sex differences are apparent. Furthermore, for the first time, we demonstrate that DMY protein is localized in the nuclei of the somatic cells surrounding PGCs and Sertoli cells of XY individuals, indicating that DMY functions to direct development along the male pathway before and during gonadal sex differentiation. In contrast, *DMRT1* is expressed in Sertoli cells after testicular differentiation.

RESULTS

Morphological Gonadal Sex Differentiation

Figure 1 summarizes gonadal formation and sex differentiation in medaka. In brief, at stage 34, PGCs were migratory and localized in lateral plate mesoderm. At stage 36, lateral plate mesoderm differentiated into two layers, the somatic mesoderm and the splanchnic mesoderm, between which the body cavity was found. During this process, PGCs reached the gonadal region. By stage 38, formation of the gonadal anlage was completed and all PGCs were localized in it. This result is in accordance with a previous report (Hamaguchi, 1982). Previous reports have noted that the first appearance of morphological sex difference in medaka was the difference in the number of germ cells between the sexes (Satoh and Egami, 1972; Quirk and Hamilton, 1973; Hamaguchi, 1982). Although these reports indicated that a sex difference in germ cell number was detectable around the time of hatching, it was not known when the sex difference in germ cell number was first established. Changes in the number of germ cells during gonadal formation are shown in Figure 2. The first morphological sex difference was apparent as the difference in germ cells number between both sexes at stage 38 ($P < 0.05$). The

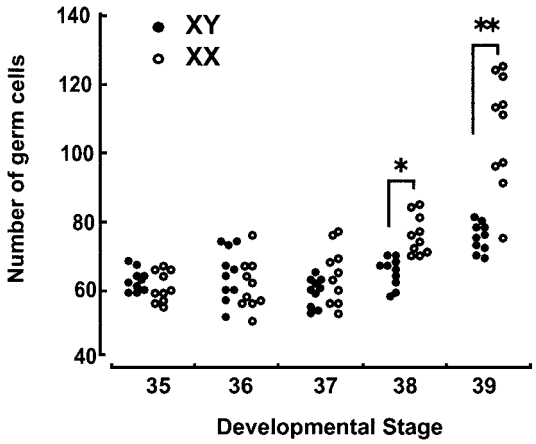

Fig. 2. Changes in germ cell number during gonadal differentiation. The number of germ cells during gonadal differentiation from stage 36 to stage 39 (just hatching) is shown. Each point represents the number of germ cells in each fry. Significant difference in germ cell number is observed between both sexes after stage 38. $*P < 0.05$; $**P < 0.01$.

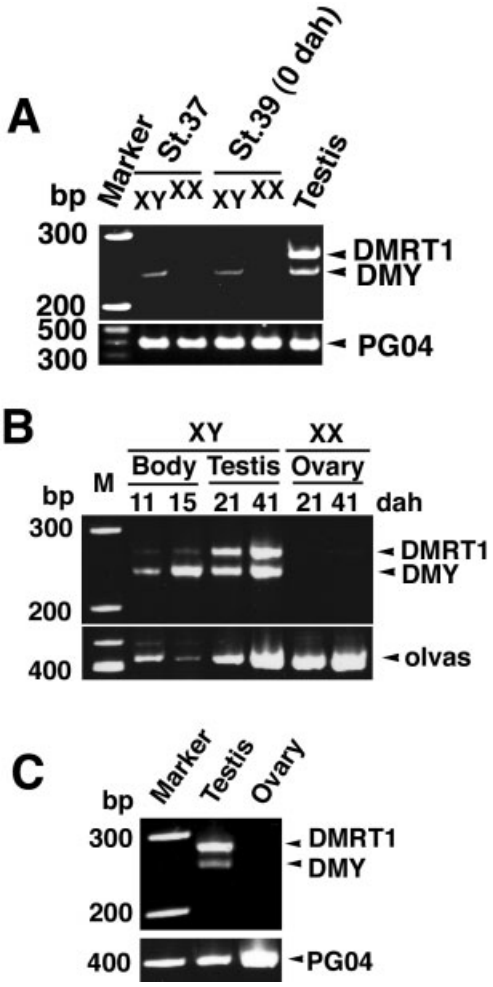

Fig. 3. Expression of *DMY* and *DMRT1* during gonadal differentiation and development. **A:** *DMY* and *DMRT1* expression around hatching, determined by reverse transcriptase-polymerase chain reaction (RT-PCR). *DMRT1* expression is undetectable around hatching, whereas expression of *DMRT1* and *DMY* is detectable in mature testes. **B,C:** Expression of *DMY* and *DMRT1* during testicular differentiation and development, determined by RT-PCR. St., stage; dah, days after hatching.

first appearance of meiotic cells was also seen in XX fry 1 days after hatching (dah). In XY fry, spermatogonial proliferation occurred after 20–30 dah.

The first morphological sex differences in somatic cells was regarded as the formation of the acinus (a globular structure that is the seminiferous tubule precursor), which occurred in XY gonads, and follicles in XX gonads after 10 dah, as previously reported (Shinomiya et al., 2001). The formation of an ovarian cavity as the key morphological sign for ovarian differentiation occurred after 30 dah (Fig. 1).

First Appearance of DMY During Gonadal Differentiation

Figure 3A shows the expression pattern of *DMY* and *DMRT1* mRNA in stage 37 and 39 (just hatching) embryos and in mature XY testes and XX ovaries, assessed by reverse transcriptase-polymerase chain reaction (RT-PCR). In mature XY testes, both *DMY* and *DMRT1* mRNA were detectable but the expression of *DMRT1* was relatively higher. Conversely, neither mRNA was detectable in XX ovaries. RT-PCR analysis also indicated that *DMY* was expressed specifically in XY individuals at stage 37 and 39 but not in XX individuals. At these stages, *DMRT1* mRNA was not detectable in either sex, suggesting that *DMY* is expressed before the first appearance of morphological sex difference (germ cell number in Fig. 2) and that *DMRT1* is not expressed around the time of hatching.

Although the in situ hybridization probe for *DMY* mRNA hybridizes to both *DMY* and *DMRT1* mRNAs because of their high similarity (93% at the nucleic acid level), RT-PCR showed that *DMRT1* is expressed at very low levels up to 15–20 dah, irrespective of genetic sex (see next section). Therefore, before this time, it is safe to assume that any hybridization signals identified with the *DMY* probe must derive from specific hybridization with *DMY* mRNA. In situ hybridization showed no detectable specific signals for DMY mRNA in any somatic cells during the migratory period of PGCs (Fig. 5A), although

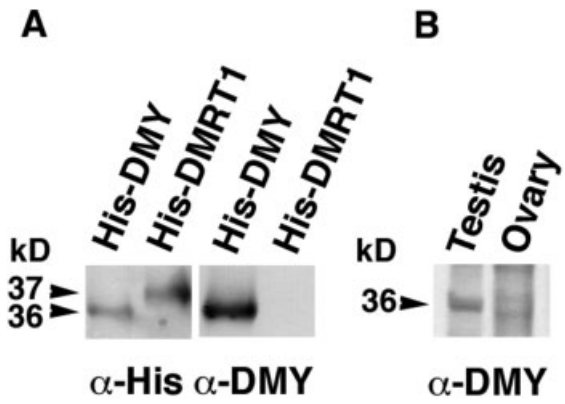

Fig. 4. Specificity of anti-DMY antibody. **A:** Recombinant His-tagged DMY and His-tagged DMRT1 protein were immunoblotted by anti-His antibody (α-His) and anti-DMY antibody (α-DMY). α-DMY recognizes DMY protein specifically and does not cross-react with DMRT1 protein. **B:** Immunoblots of α-DMY for testis (XY) and ovary (XX). α-DMY specifically recognizes a 37-kDa protein band in testis but not in ovary.

PGCs were already enclosed by somatic cells derived from lateral plate mesoderm during the migratory period and later become localized in the gonadal region (Hamaguchi, 1982). Specific signals for *DMY* mRNA became localized in the somatic cells surrounding PGCs at stage 36 (Fig. 5B), when PGCs were found in coelomic epithelium under the nephric duct (inset in Fig. 5B). After stage 36, *DMY* mRNA continued to be found only in PGC-supporting cells of XY embryos (Fig. 5B,C,E). As described above, *DMY* is one of the *DMRT1* homologs and is expected to function as a transcriptional factor.

To further define the expression pattern of DMY during gonadal sex differentiation, we produced a specific antibody against DMY. This antiserum (α-DMY) specifically recognized recombinant protein derived from His-tagged DMY and did not cross-react with recombinant DMRT1 protein. Further immunoblot analysis showed that DMY protein was present in XY testis (ca. 36-kDa protein) but not in XX ovary, suggesting that anti-DMY antibody specifically recognizes native DMY protein (Fig. 4). Immunohistochemical analysis using this antibody revealed that DMY protein was present primarily in the nuclei of PGC-supporting cells of XY fry during gonadal sex differentiation and testicular differentiation (Fig. 5D,E at stage 38; Fig. 6A,B at 5 dah).

First Appearance of DMRT1 During Gonadal Differentiation

During this period, *DMY* continued to be expressed in XY gonads. First-strand cDNA prepared from trunk body was applied to RT-PCR up to 20 dah. At that time, basal expression of *DMRT1* was detectable, depending upon the number of PCR cycles, irrespective of genetic sex. After 20 dah, *DMRT1* mRNA increased markedly in testes compared with the XX gonads, indicating that expression of *DMRT1* becomes elevated in testes after 20 dah. Similar expression of *DMY* and *DMRT1* was seen in testes at 40 dah (Fig. 3B). Thereafter, *DMRT1* mRNA continued to increase as compared with *DMY* mRNA; this increase was also found in mature testes (Fig. 3C).

DMY and DMRT1 Are Coexpressed in Sertoli Cells

In mature testes, *DMY* was localized in Sertoli cells during spermatogenesis and the epithelial cells of the intratesticular efferent duct, which is derived from Sertoli cells (Fig. 6D,F,G). Immunohistochemical analysis revealed that DMY protein was detected strongly in the nuclei and cytoplasm of A-type spermatogonium-supporting Sertoli cells (Fig. 6E,F). To clarify whether the localization of *DMY* and *DMRT1* is identical in testis, specific probes for *DMRT1* are required. However, a specific antibody for DMRT1 is not available, and the in situ hybridization probes for *DMY* and *DMRT1* hybridize with both mRNAs in mature testes (data not shown). To circumvent this problem, we used XX sex-reversed males having normal testes with spermatozoa. In XX sex-reversed testes, which never express *DMY*, *DMRT1* mRNA was detected specifically by in situ hybridization, localized in Sertoli cells and the epithelial cells of intratesticular efferent duct, similar to *DMY* in XY testes (Fig. 6H–J). This finding shows that *DMY* and *DMRT1* are coexpressed in Sertoli cells in mature testes of medaka.

DISCUSSION

DMY is a prime candidate for the master sex-determining gene in medaka and is required for male development (Matsuda et al., 2002). To further understand the roles and mechanisms of action of *DMY*, we focused on establishing detailed expression profiles for *DMY* and *DMRT1*, because the *DMY* gene appears to be derived from the duplication of the *DMRT1* gene (Nanda et al., 2002; Matsuda et al., 2003) and *DMRT1* is a candidate key factor for testicular differentiation in nonmammalian vertebrates (Koopman and Loffler, 2003). This article is the first report on the simultaneous expression profiles for *DMY* and *DMRT1*, and the results suggest that DMY functions as a protein directing male development.

Expression of DMY Before and During Testicular Differentiation

We previously reported the specific expression of *DMY* in somatic cells surrounding germ cells in gonads of XY fry at hatching (Matsuda et al., 2002). In this study, the first appearance of *DMY* was determined at both RNA and protein levels during early gonadal differentiation.

In mouse, *SRY*, a sex-determining gene on the Y chromosome, functions as a master switch to direct development along the male pathway (Koopman et al., 1991). *SRY* is expressed in XY genital ridges between approximately E10.5 and E13 with peak RNA expression at E11.5, al-

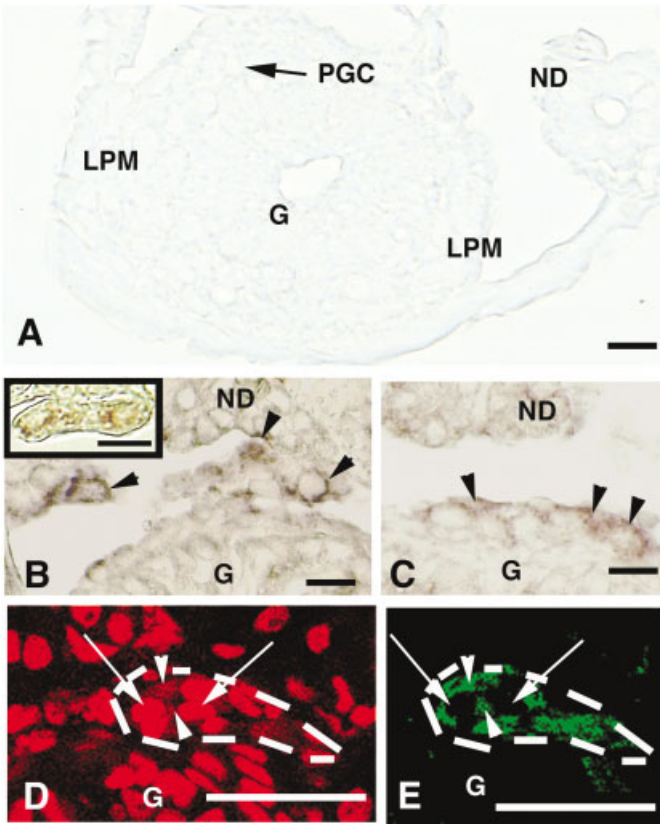

Fig. 5. *DMY* is expressed in Sertoli cell lineage, and its protein is localized in the nucleus before and during gonadal sex differentiation. **A:** Stage 34. Primordial germ cells (PGCs) are localized in lateral plate mesoderm (arrow). No signals for *DMY* are seen. **B:** Stage 36. PGCs become localized in coelomic epithelium under the nephric duct. Specific signals for *DMY* become localized in the somatic cells surrounding PGCs (arrowheads). Inset, PGCs stained with anti-vasa antibody at stage 36. **C:** Stage 39. *DMY* mRNA is localized in the somatic cells surrounding PGCs. D,E: XY fry at stage 38. **D:** Staining of nuclei (red) with TO-PRO-3. Arrows indicate the PGC. Arrowheads indicate PGC-supporting cells. The region enclosed by the dotted line indicates a gonadal anlage. **E:** Staining with α-DMY. Fluorescent signals (green) were localized specifically in the nucleus and cytoplasm of PGC-surrounding cells (arrowheads) in the gonadal region but not in germ cells (arrows). LPM, lateral plate mesoderm; G, gut; ND, nephric duct. Scale bars = 20 μm in A–C, 50 μm in D,E.

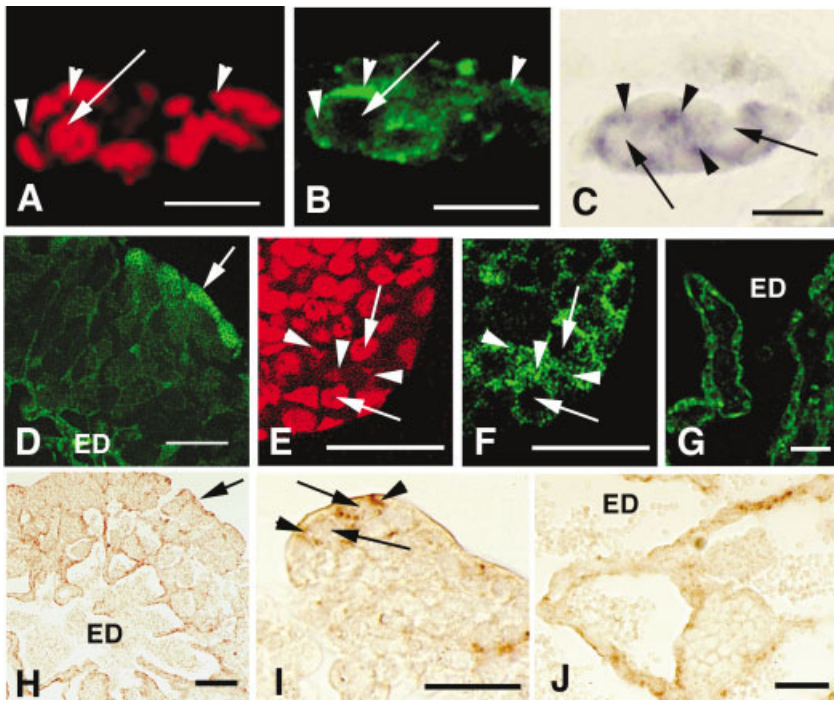

Fig. 6. DMY expression during testicular differentiation and localization of DMY protein in the nuclei of spermatogonium-supporting Sertoli cells. A–C: At 5 days after hatching (dah) gonad of XY fry. **A:** Staining with TO-PRO-3 (red). **B:** Staining with α-DMY (green). Immunohistochemical analysis shows that DMY is localized specifically in the nucleus and cytoplasm of the somatic cells surrounding germ cell. **C:** In situ hybridization for *DMY* mRNA. **D,F,G:** Mature XY testis. Staining with α-DMY (green). **E:** Staining with TO-PRO-3 (red). The nucleus and cytoplasm in A-type spermatogonia-supporting Sertoli cells (arrowheads) and the epithelial cells of intratesticular efferent duct (ED) are DMY-positive in XY testis. Arrows indicate A-type spermatogonium. **H–J:** XX sex-reversed testis. *DMRT1* mRNA is localized in the somatic cells, similar to the localization of *DMY* mRNA in XY testis. Arrows, spermatogonia. Arrowheads, germ cell supporting cells (Sertoli cells). Scale bars = 20 μm in A,B, 10 μm in C, 50 μm in D–J.

though little information is available on protein levels. Recent studies showed that *SRY* was expressed in the central region of genital ridges and then became localized nearer to the gonadal surface and cranial and caudal poles (Albrecht and Eicher, 2001; Bullejos and Koopman, 2001). A cell-tracing experiment using Sry-EGFP transgenic mice demonstrated that *SRY*-positive cells were closely associated with and sometimes partially surrounded each germ cell at E11. In medaka, on the other hand, *DMY* was not present in the somatic cells surrounding PGCs before PGCs reached to gonadal region, whereas PGCs are

already surrounded by the somatic cells derived from somatic mesoderm during PGC migration. *DMY* was detectable only in the somatic cells surrounding PGCs with the completion of the gonadal anlage. It is notable that both *SRY* and *DMY* are expressed by the somatic cells surrounding PGCs in the gonadal primordium specifically during gonadal formation, whereas the timing of the enclosure of PGCs by somatic cells is different between mouse and medaka. Although *SRY* and *DMY* are unrelated sex-determining genes, the timing of their expression during sex determination suggests that the encoded proteins may have similar actions on this process.

DMY Positive Cells Are Pre-Sertoli Cells

Previous reports suggested that *SRY* was expressed in pre-Sertoli cells (Burgoyne et al., 1988; Patek et al., 1991; Rossi et al., 1993; Swain et al., 1998; Salas-Cortes et al., 1999), although direct evidence was lacking. Recent detailed cell-tracing experiment using Sry-EGFP transgenic mice demonstrated that Sry was expressed in pre-Sertoli cells (Albrecht and Eicher, 2001). In medaka, PGCs are surrounded by the somatic cells derived from lateral plate mesoderm during PGC migration and contribute to gonadal formation (Hamaguchi, 1982; this study). At this time, *DMY* is expressed in the somatic cells surrounding PGCs and continues to be expressed only in the germ cell-supporting cell lineage of XY gonads during gonadal differentiation and development. Taken together, it is concluded that, in medaka, *DMY*-positive cells are pre-Sertoli cells and differentiate into Sertoli cells.

What Is the Function of DMY for Sex Determination and Differentiation?

In mouse, *SRY* is required for male differentiation by means of promotion of Sertoli cell differentiation (Swain and Lovell-Badge, 1999). Although the identity of its target genes remains unclear, Sry induces changes in proliferation of gonadal cells and the recruitment of peritubular and endothelial cells to the gonads (Capel, 2000). Although *DMY* is required for male development (Matsuda et al., 2002), its functions and mechanisms of action are unclear. It is well-known that the number of germ cells in many nonmammalian females is greater than in males around the time of morphological sex differentiation (Van Limborgh, 1975; Zust and Dixon, 1977; Nakamura et al., 1998). Thereafter, germ cells in females continue to proliferate and then enter into meiosis, in contrast to the male germ cells, which arrest in mitosis. In this study, we determined that the first appearance of the sex difference in germ cell number occurred at stage 38 before hatching in medaka (Fig. 2) and subsequently PGCs entered mitotic arrest in XY fry, whereas they go into meiotic arrest in XX fry (Satoh and Egami, 1972; Fig. 1). Later (at 10 dah), the formation of the acinus (a globular structure that is the seminiferous tubule precursor) occurred in XY gonads (Shinomiya et al., 2001; Fig. 1). These events are the first morphological signs of sex differentiation and testicular differentiation. They occur sequentially after the first indications of *DMY* expression but before *DMRT1* expression. These results suggest strongly that *DMY* is involved in PGC proliferation and testicular differentiation by means of Sertoli cells. Although in medaka the sex differences in germ cells, including the number and mitotic arrest, occur earlier than the sex differences in somatic cells, including testis cord formation, compared with mammals (Swain and Lovell-Badge, 1999; Capel, 2000), these observations suggest that Sertoli cells cause PGCs to enter mitotic arrest in both fish and mammals.

This study demonstrates for the first time that DMY protein is produced in the somatic cells surrounding PGCs and continues to be expressed during gonadal differentiation. *DMY* mRNA and protein were expressed with similar timing during gonadal differentiation. It is important to note that DMY is localized in the nuclei of the somatic cells surrounding PGC immediately after gonadal formation, strongly suggesting that DMY functions as a transcriptional regulator during early gonadal differentiation. Although binding of human DMRT1 to a DSX site was reported (Zhu et al., 2000), the DNA binding motif of DMRT1 homologs has not been identified in any vertebrate yet. We have demonstrated recently that tilapia and mouse DMRT1 have preferential DNA binding properties (Guan et al., unpublished observations). Taken together, these results indicate that one of the functions of DMY is to act as a transcriptional factor regulating the proliferation of PGCs in a sex-specific manner and testicular differentiation.

Expression of DMRT1 During Testicular Development

Unlike *DMY*, *DMRT1* expression first occurs at 20–30 dah. By this time, the formation of testis is completed (Shinomiya et al., 2001; Nanda et al., 2002). This timing of *DMRT1* appearance coincides well with the first occurrence of proliferation of A-type spermatogonia, suggesting the involvement of *DMRT1* in spermatogonial proliferation. However, this timing of *DMRT1* expression in medaka testis appears to be different from that reported in other species (Zarkower, 2001). These studies have shown that *DMRT1* expression is evident in male gonads during sex differentiation and considerably upregulated late in sex determination or during the early testis differentiation period (mouse; Raymond et al., 1999b; chicken, Smith et al., 2003; turtle, Kettlewell et al., 2000; trout, Marchand et al., 2000; tilapia, Kobayashi et al., unpublished observations). We also found that in tilapia, *DMRT1* transcripts were already present in Sertoli cells (or pre-Sertoli cells) of XY gonads before testicular differentiation, but not in XX gonads (Kobayashi et al., unpublished data). Thus, the timing of *DMRT1* expression in testes of these animals including tilapia appears to be different from that of medaka. Of interest, *DMY* and *DMRT1* have 93.0% identity at the levels of nucleotide bases. Besides medaka, there are no reports of other vertebrate species whose testes contain the sec-

ond form of *DMRT1*. Thus, medaka is unique to have two forms of *DMRT1* homolog genes in testes, although mice express several DM domain genes in the testis, including *DMRT1*, *DMRT3*, and *DMRT4* (Kim et al., 2003). This study also showed that DMY protein localized in both the nuclei and cytoplasm of A-type spermatogonia-supporting Sertoli cells, although *DMY* is regarded as a transcriptional factor. This finding may suggest the *DMY* activity in Sertoli cells during spermatogenesis. In XX sex-reversed medaka, complete spermatogenesis was observed and *DMRT1* was expressed in Sertoli cells, indicating that *DMY* is not required for spermatogenesis. Based on these findings, the present study suggests that, in medaka, *DMY* and *DMRT1* have different roles in male development: *DMY* is solely responsible for testicular differentiation, whereas *DMRT1* plays a major role for further testicular development including spermatogenesis.

In conclusion, although two closely related DM genes, *DMY* and *DMRT1*, are expressed in Sertoli cell lineage cells, their expression patterns are distinctly different. Our findings suggest that, in medaka, DMY functions in testicular differentiation, while DMRT1 might be an essential regulator of spermatogenesis. Therefore, it appears that DMY and DMRT1 have partitioned the functions performed by DMRT1 in other species, with DMY controlling testis differentiation and DMRT1 controlling spermatogenesis. We speculate that medaka may be early in the process of duplication and divergence of DMRT1 function, with DMY retaining the early functions of the ancestral DMRT1 gene and DMRT1 retaining the late functions. Interesting evolutionary questions are how *DMY* acquired its function as a sex-determining gene, and why *DMRT1* is expressed after testicular differentiation in medaka, unlike other non-mammalian vertebrates. Further studies will be necessary to clarify the transcriptional regulation and the biochemical characteristics of DMY and DMRT1 in medaka.

EXPERIMENTAL PROCEDURES

Strains

Most studies described used medaka (*Oryzias latipes*) from an inbred HdrR line. In this strain, the wild-type allele (R) of the r locus (a sex-linked pigment gene) is located Y-chromosome. Therefore, the female X^rX^r results in a white body color, and the male X^rY^R results in orange–red body color (Matsuda et al., 2002). Medaka from a HNI inbred line were used for detection of DMY protein since the antibody was generated by using DMY from this line (see below). Classification of developmental stage was according to the descriptions of Iwamatsu (1994) for medaka. XX sex-reversed males (white), which are naturally occurring (Nanda et al., 2003), were provided kindly from Dr. M. Sakaizumi (Niigata University).

RT-PCR for DMY and DMRT1

The data illustrated in Figure 3A were generated using the following protocol: first-strand cDNA was synthesized from 400 ng of total RNA in 20 μl using Omniscript (QIAGEN) with oligo-dT primers. PCR was carried out in a 25-μl reaction mixture containing 1 μl of the first-strand cDNA for *PG04* and *DMY/DMRT1* of testis and ovary, 2 μl for embryos. Using specific primer sets (PG04.1, PG04.2 for PG04 and PG17.41, PG17.42 for DMY/DMRT1), PCR was performed according to a previous report (Matsuda et al., 2002). For the data shown in Figure 3B,C, RT-PCR was performed by using an OneStep RT-PCR kit (Qiagen). For *olvas* (*vasa* homolog in *O. latipes*), 100 ng (RNA from trunk body) or 20 ng (RNA from gonads) of total RNA were used as template in 25-μl reactions. For DMY/DMRT1, 100 ng of total RNA was used as template in 25-μl reactions. PCR conditions were 30 min at 55°C; 15 min 95°C; 20 sec at 96°C, 30 sec at 55°C, 60 sec at 72°C for 35 cycles; and 5 min at 72°C. Specific primers for *olvas* (GenBank accession no. AB063484), a germ cell-specific marker gene (Shinomiya et al., 2000), were AGGCGCCAGGGACTCAGGAAATG and GCAGGATGGGCAGCAGGAACG.

Determination of Germ Cell Number During Gonadal Differentiation

Embryos and fry of HdrR inbred line were dissected to separate head and body. Dissected body portions were fixed in Bouin's fixative solution overnight and then embedded in paraffin. Each dissected head was used to determine genetic sex, according to a previous report (Shinomiya et al., 1999; see below). Cross-sections were cut serially at 5 μm thickness. All germ cells were counted in each fry. Ten individuals derived from the same parents were examined at each developmental stage for each sex. After cell counting, mean and SE were calculated for each sex at each stage and then differences between the sexes were evaluated statistically by paired *t*-test for each stage.

In Situ Hybridization

Gonads of fry 0–5 dah and greater than 10 dah were dissected with or without trunk body, respectively, and then fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4; 4% PFA) at 4°C overnight. For stage 25 to 39 embryos, whole embryos were fixed in 4% PFA, similarly. In situ hybridization was performed according to previous reports (Kobayashi et al., 2000; Matsuda et al., 2002).

Production of Antibody and Immunoblotting

To generate a specific antibody against DMY, an oligopeptide corresponding to a C-terminal amino acid sequence (PSSRPTP) predicted from *DMY* cDNA of the HNI strain (Matsuda et al., 2002) was synthesized, with the addition of a cysteine at the N-terminus to facilitate linkage to KLH as carrier protein. Female rabbits were immunized four times and then blood was collected. Serum was separated and then purified by affinity chromatography using the oligopeptide as antigen. To demonstrate that anti-DMY antibody specifically reacted with DMY protein, recombinant protein derived from His-tagged *DMY* and

DMRT1 expression vectors was extracted from *E. coli* lysate and purified using Ni+-agarose beads (Amersham–Pharmacia Co., Ltd.). Protein extracts were also prepared from testes and ovary according to a previous report (Kobayashi et al., 1996). Proteins (10 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12.5% SDS-polyacrylamide gel and then transferred to Immobilon membrane (Millipore) by semidry electroblotting (Kobayashi et al., 1996). Anti-DMY antibody and anti-His antibody (Qiagen) were used at dilutions of 1:250 and 1:1,000, respectively.

Immunohistochemistry

Fry and dissected testes from mature XY males were fixed in 4% paraformaldehyde in 0.1 M phosphate or cacodylate buffer (pH 7.4) overnight, dehydrated, and embedded in paraffin. Serial sections were cut at 5 μm thickness. Methodology used for immunohistochemistry was described in detail previously (Kobayashi et al., 1998, 2002). Anti-DMY and anti-vasa antibodies (Kobayashi et al., 2002) were used at 1:100 and 1:1,000, respectively. To detect DMY protein, sections were preincubated in 10 mM sodium citrate buffer (pH 6.0) for 5 min at 100°C before application of the primary antibody.

Distinction of Genetic Sex and RNA Extraction

The genetic sex of specimens was determined by PCR. For embryos, total RNA and genomic DNA were extracted from each embryo after homogenization with 350 μl of buffer RLT supplied with the RNeasy Mini Kit (Qiagen). The homogenized lysates were centrifuged and supernatants were used for RNA extraction by using the RNeasy Mini Kit with the RNase-Free DNase set protocol (Qiagen). Precipitated material was used for DNA extraction by using the DNeasy Tissue Kit (Qiagen) according to the manufacturer's protocol. For fry, the dissected head part was used for DNA extraction to examine genetic sex while the body part was used for RNA extraction or in situ hybridization analysis. RNA was extracted as above. Genomic PCR was performed using the primers for *DMRT1* and *DMY*: PG17.5, CCGGGTGCCCAAGTGCTCCCGCTG; PG17.6, GATCGTCCCTCCACAGAGAAGAGA. PCR conditions were 5 min 95°C, followed by 30 cycles of 20 sec at 96°C, 30 sec at 55°C, 30 sec at 72°C, followed by 5 min at 72°C.

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