Wild-Derived XY Sex-Reversal Mutants in the Medaka, *Oryzias latipes*

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ABSTRACT

The medaka, *Oryzias latipes*, has an XX/XY sex-determination mechanism. A Y-linked DM domain gene, *DMY*, has been isolated by positional cloning as a sex-determining gene in this species. Previously, we found 23 XY sex-reversed females from 11 localities by examining the genotypic sex of wild-caught medaka. Genetic analyses revealed that all these females had Y-linked gene mutations. Here, we aimed to clarify the cause of this sex reversal. To achieve this, we screened for mutations in the amino acid coding sequence of *DMY* and examined *DMY* expression at 0 days after hatching (dah) using densitometric semiquantitative RT-PCR. We found that the mutants could be classified into two groups. One contained mutations in the amino acid coding sequence of *DMY*, while the other had reduced *DMY* expression at 0 dah although the *DMY* coding sequence was normal. For the latter, histological analyses indicated that *Y<sup>Out</sup>*/*<sup>Out</sup>* (Y chromosome derived from an Oura XY female) individuals with the lowest *DMY* expression among the tested mutants were expected to develop into females at 0 dah. These results suggest that early testis development requires *DMY* expression above a threshold level. Mutants with reduced *DMY* expression may prove valuable for identifying *DMY* regulatory elements.

In vertebrates, the sex of an individual is established by the sex of the gonad and, in most cases, whether a gonad becomes a testis or an ovary is determined by the genome of that individual. In most mammals, the Y chromosome-specific gene *SRY*/Sry is the master male-determining gene (Gubbay et al. 1990; Sinclair et al. 1990; Koopman et al. 1991). The *SRY* gene encodes a testis-specific transcription factor that plays a key role in sexual differentiation and development in males (Lovell-Badge et al. 2002). Non-mammalian vertebrates also have a male heterogamic (XX–XY) sex-determination system, but no homolog of Sry could be found.

In the teleost medaka fish, *Oryzias latipes*, which has an XX–XY sex-determining system (Aida 1921), *DMY* (DM domain gene on the Y chromosome) has been found in the sex-determining region on the Y chromosome (Matsuda et al. 2002; NANDA et al. 2002). This gene is the first sex-determining gene to be found among non-mammalian vertebrates. *DMY* encodes a protein containing a DM domain, which is a DNA-binding motif found in *Drosophila melanogaster* and *Caenorhabditis elegans* (DSX and MAB-3, respectively) that is involved in sexual development (Raymond et al. 1998). The cDNA sequences of medaka *DMY* and *DMRT1* are highly similar and *DMY* appears to have originated through duplication of an autosomal segment containing the *DMRT1* region (Nanda et al. 2002; Kondo et al. 2004). *DMY* is specifically expressed in pre-Sertoli cells, somatic cells that surround primordial germ cells (PGCs), in the early gonadal primordium before any morphological sex differences are seen (Matsuda et al. 2002; Kobayashi et al. 2004). In the medaka, the first indication of morphological sex differences is a difference in the number of germ cells between the sexes at hatching (Satoh and Egami 1972; Quirk and Hamilton 1973; Hamaguchi 1982; Kobayashi et al. 2004). It is considered that one of the functions of *DMY* is to act as a factor that regulates the proliferation of PGCs via Sertoli cells in a sex-specific manner and controls testicular differentiation (Kobayashi et al. 2004).

In humans, mutations in the *SRY* gene result in XY sex reversal and pure gonadal dysgenesis (Jager et al. 1990). However, mutations in the *SRY* gene itself are considered to account for only 10–15% of 46,XY gonadal dysgenesis cases, and the majority of the remaining cases may have mutation(s) in *SRY* regulatory elements or other genes involved in the sex differentiation pathway (Cameron and Sinclair 1997). A number of genes have been identified as having roles in the sexual development pathway through analyses of human sexual anomaly cases and/or functional studies in mice (Koopman 2001). Sex-reversal mutants in medaka are also useful for revealing the molecular function of *DMY* and identifying...

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other genes involved in sex determination and differentiation. Analyses of such mutations may lead to further understanding of the molecular mechanisms of sex differentiation. In the present study, we identified two types of DMY mutants derived from wild populations of medaka. The first type is composed of loss-of-function mutants that contain mutations at the 3' region of the DM domain, suggesting that the 3' region of the DM domain is required for the normal function of DMY, male sex determination, and development. The second type is composed of reduced DMY expression mutants that have lower levels of DMY transcripts and contain a number of germ cells, including oocytes, at hatching. Taken together, these results suggest that early testis development requires DMY expression above a threshold level and support the hypothesis that DMY acts as a factor regulating PGC proliferation via Sertoli cells during early gonadal differentiation.

**MATERIALS AND METHODS**

**Fish and mating schemes:** In our previous study, we surveyed 2274 wild-caught fish from 40 localities throughout Japan and 750 fish from 69 wild stocks from Japan, Korea, China, and Taiwan and identified 25 XY females from 11 localities (Shinomiya et al. 2004). Genetic analyses revealed that the XY females from 8 localities produced all female XYm (Ym, Y chromosome derived from an XY female) progeny, while those from 3 localities yielded both male and female XYm progeny (Table 1), suggesting that all these wild XY females had Y-linked gene mutations. In this study, we established mutant strains from these XY female mutants. The XY females from northern populations were mated with XY males of an inbred strain Hdl-rR (Hyodo-Taguchi 1996) and the XY females from southern populations were mated with a congenic strain Hdl-rRInsY (Matsuda et al. 1998). The F1 progeny from each pair were grown and their genotypes were determined, since the DMY gene of the northern population, including the HNI inbred strain, contains 21 nucleotide deletions in intron 2 compared to the southern population, including the Hdl-rR strain (Shinomiya et al. 2004). When an XY female produced all female F1 XYm progeny, an F1 XYm female was mated with an F1 YYm male to produce F2 YYm females. Next, an F2 YYm female was mated with an F2 YYm male to establish mutant strains producing YYm females and YYm males in later generations. When an XY female produced male and female F1 XYm progeny, an F1 XYm female was mated with an F1 YYm male to produce F2 YYm females. Next, an F2 YYm male was mated with an F2 YYm female to establish mutant strains producing XYm males and females and YYm males in later generations. These mutant strains were used for RT–PCR and histological analyses.

**PCR and direct sequencing:** To screen for mutations in the amino acid coding sequence of DMY, exons 2–6 of DMY were PCR-amplified from caudal fin clip DNA using the following primer sets: exon 2: PG17ex2.1, 5'-GGG ATC TGC TGA CCC TCT TTT TCT GG-3' and PG17ex2.2, 5'-TTT CGG GTG AAC TCA CAT GGT TGT CG-3'; exon 3: PG17ex3.1, 5'-GCA ACA GAG AGT CGG ATT TAC GTG TCA-3' and PG17ex3.2, 5'-CTT TGG ACT TCA GGT TGA CAC ACC GCA TGA CCA TCT CGA-3'; exon 5: PG17ex5.1, 5'-CGC ATC TCA GGG GAT GAT GCC ACC-3' and PG17ex5.2, 5'-GGG AGC CAA AAA TGC GCC ACA TAA-3'; and exon 6: PG17ex6.1, 5'-GTC ATT AAC ACA ACG CAC AAC TTG-3' and PG17ex6.2, 5'-AAA AAC CAG ACC CGG GCA GAA GAC-3' (Figure 1A). The PCR products were sequenced directly in an ABI Prism 310 automated sequencer.

**RNA extraction and densitometric semi-quantitative RT–PCR:** Total RNA was extracted from embryos or fry at 9.5 days after fertilization in Kesen-numa G2, Shirene G2, Aizu-Wakamatsu G2, and Oura G3 generations using an RNeasy mini kit (QIAGEN) and subjected to RT–PCR using a OneStep RT–PCR kit (QIAGEN). Aliquots (20 ng) of the total RNA samples were used as templates in 25-μl reaction volumes. The PCR conditions were: 30 min at 55°C, 15 min at 95°C, cycles of 20 sec at 96°C, 30 sec at 55°C, and 60 sec at 72°C; and 5 min at 72°C. The number of cycles for each gene was adjusted to be within the linear range of amplification, specifically 36 cycles for DMY and 24 cycles for β-actin. The primers for DMY (DMYspe, 5'-TCC CGG AAC TGC TGA CCC TCT TTT TCT GG-3' and 5'-GGC ACT ACG CAC ACC GCA TGA CCA TCT CGA-3') amplified a 404-bp DNA fragment. The primers for β-actin (3b, 5'-CMG TCA GGA TCT TCA TSA GG-3' and 4, 5'-CAC ACC TGC TAC AAT GAG CTG A-3') amplified a 322-bp DNA fragment. Aliquots of 8 and 4 μl of the DMY and β-actin RT–PCR products, respectively, were electrophoresed in a 2% agarose gel in 1× TAE buffer and stained with ethidium bromide. The gels were visualized by UV transillumination and photographed with a Gelsdoc 2000 (Bio-Rad). The optical density of each band was quantified by densitometry using Quantity One Software (Bio-Rad) and represented as the ratio of each band to the 18S band. Two-ways ANOVA was used to test for statistically significant differences among XYm, XYInsY, and XY sex-reversal mutants, and the Games/Howell post hoc test for multiple comparisons was applied.

**Histological analysis in early gonadal development:** The fry of the Kesen-numa G2 and Oura G3 generations at 0 and 10 days after hatching (dah) were cut to separate the head and body. The body portions were fixed in Bouin’s fixative solution overnight and then embedded in paraffin. The heads were used to determine the genotype. Serial cross-sections (5 μm thick) of the body portions were cut and stained with hematoxylin and cosin. Germ cells were counted in each fry at 0 dah. After the cell counting, the mean number and SE were calculated for the Oura G3 generation and the differences between Yh/Yh and YhInsYh individuals were evaluated statistically by paired t-tests. Gonadal sex differentiation at 10 dah was examined for the presence or absence of diplotene oocytes.

**RESULTS**

**Mutations in the DMY amino acid coding sequence:** To identify mutations in the DMY amino acid coding sequence, we sequenced exons 2–6 of DMY (Figure 1A) in 23 XY sex-reversed females from 11 localities. Four types of mutations were identified in 15 XY females from 7 localities. One Awara (Matsuda et al. 2002), 2 Aomori, 1 Aizu-Bange, and 6 Kurobe XY females contained a C insertion in a polyC tracts specific to the northern population in exon 3 (Figure 1A, A and C), which caused a frameshift from residue 110 (designated N110insC) and resulted in premature termination at residue 139 (Figure 1B). An XY female from Suzu contained a C deletion in the same polyC tracts in exon 3 (Figure 1A, A and C), which caused a frameshift from residue 109
and resulted in premature termination at residue 203 (Figure 1B). An XY female from Aki contained a C deletion in another site in exon 3 (Figure 1, A and C), which caused a frameshift from residue 102 (designated S102fsdelC) and resulted in premature termination at residue 104 (Figure 1, B and C). Three XY females from Saigo had normal DMY exons 2–5, but PCR for exon 6 (using primers PG17ex6.1 and PG17ex6.2; Figure 1A) and 3′-RACE using RNA extracted from G2 YmYm (YmYm, Y chromosome derived from a Saigo XY female) fry at 0 dah did not produce any amplified bands (data not shown), suggesting that the DMY gene of these Saigo XY females may contain a large insertion or deletion in intron 5 and/or exon 6 and have no polyadenylation signal. The XY females from Awara, Aomori, Aizu-Bange, Kurobe, Suzu, Aki, and Saigo that contained mutations in the amino acid coding sequence produced all female XYm progeny in the G1 generation (Table 1), indicating their mutant DMY genes were nonfunctional.

**TABLE 1**

<table>
<thead>
<tr>
<th>Collection site</th>
<th>N</th>
<th>Phenotypic sex of XYm F1</th>
<th>Population</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aomori, Aomori Prefecture</td>
<td>2</td>
<td>All female</td>
<td>N</td>
</tr>
<tr>
<td>Aizu-wakamatsu, Fukushima Prefecture</td>
<td>2</td>
<td>Males and females</td>
<td>N</td>
</tr>
<tr>
<td>Aizu-Bange, Fukushima Prefecture</td>
<td>1</td>
<td>All female</td>
<td>N</td>
</tr>
<tr>
<td>Shirone, Niigata Prefecture</td>
<td>2</td>
<td>Males and females</td>
<td>N</td>
</tr>
<tr>
<td>Kurobe, Toyama Prefecture</td>
<td>6</td>
<td>All female</td>
<td>N</td>
</tr>
<tr>
<td>Suzu, Ishikawa Prefecture</td>
<td>1</td>
<td>All female</td>
<td>N</td>
</tr>
<tr>
<td>Awara, Fukui Prefecture</td>
<td>1</td>
<td>All female</td>
<td>N</td>
</tr>
<tr>
<td>Kesen-numa, Miyagi Prefecture</td>
<td>2</td>
<td>Males and females</td>
<td>S</td>
</tr>
<tr>
<td>Saigo, Shimane Prefecture</td>
<td>3</td>
<td>All female</td>
<td>S</td>
</tr>
<tr>
<td>Aki, Kochi Prefecture</td>
<td>1</td>
<td>All female</td>
<td>S</td>
</tr>
<tr>
<td>Oura, Kagoshima Prefecture</td>
<td>2</td>
<td>All female</td>
<td>S</td>
</tr>
</tbody>
</table>

From SHINOMIYA et al. (2004). N, number of XY females; Ym, Y chromosome derived from an XY female; N, northern Population; S, southern Population (Sakaizumi et al. 1989; Sakaizumi 1986).
Patterns of inheritance for sex reversal in Oura and Kesen-numa medaka: The XY females from Shirone, Aizu-Wakamatsu, Kesen-numa, and Oura produced sex-reversed XY\textsuperscript{m} female progeny in the G\textsubscript{1} generation, although the amino acid coding sequence of DMY was normal. To investigate the patterns of inheritance for sex reversal in more detail, we performed further genetic analyses for the Oura and Kesen-numa XY females.

For Oura, G\textsubscript{1} XY\textsuperscript{wOur} (Y\textsuperscript{Our}, Y chromosome derived from an Oura XY female) progeny produced from a mating between the XY female and an Hd-rR.Y\textsuperscript{HNI} male developed as all male (Figure 2A), suggesting that Y\textsuperscript{Our} had lost the male-determining function. Since Y\textsuperscript{HNI}Y\textsuperscript{Our} did not appear in the G\textsubscript{1} generation, another G\textsubscript{1} XY\textsuperscript{Our} female was mated with the Hd-rR.Y\textsuperscript{HNI} male. All 33 G\textsubscript{3} Y\textsuperscript{wOur}Y\textsuperscript{wOur} progeny produced from a mating between a G\textsubscript{2} XY\textsuperscript{Our} female and a G\textsubscript{2} Y\textsuperscript{HNI}Y\textsuperscript{Our} male developed as female (Figure 2A). This result confirmed that Y\textsuperscript{Our} has lost the male-determining function.

For Kesen-numa, G\textsubscript{1} XY\textsuperscript{wKsn} (Y\textsuperscript{Ksn}, Y chromosome derived from a Kesen-numa XY female) progeny produced from a mating between the XY female and an Hd-rR.Y\textsuperscript{HNI} male were either male or female, similar to the case for the Shirone XY female (Matsuda et al. 2002). Interestingly, we observed only 4 females among 18 XY\textsuperscript{wKsn} in the G\textsubscript{1} generation, while 22 XY\textsuperscript{wKsn} females were observed among 35 XY\textsuperscript{wKsn} in the G\textsubscript{2} generation (Figure 2B). Conversely, all 41 G\textsubscript{2} Y\textsuperscript{wKsn}Y\textsuperscript{wKsn} produced from a mating between a G\textsubscript{1} XY\textsuperscript{wKsn} female and a G\textsubscript{1} Y\textsuperscript{HNI}Y\textsuperscript{wKsn} male developed as male (Figure 2B), suggesting that two Y\textsuperscript{Ksn} chromosomes are sufficient for male determination and development. Y\textsuperscript{HNI}Y\textsuperscript{wKsn} and Y\textsuperscript{HNI}Y\textsuperscript{wOur}, which contained normal Y chromosomes from the Hd-rR.Y\textsuperscript{HNI} congenic strain, developed into normal males in adulthood.

Mutants with reduced DMY expression at the sex-determining period: The XY females from Shirone had reduced or faint DMY expression at 0 dah (Matsuda et al. 2002). To define the relationship between sex reversal and the DMY expression level, we detected DMY transcripts at 0 dah for Kesen-numa G\textsubscript{2}, Aizu-Wakamatsu G\textsubscript{2}, Shirone G\textsubscript{2}, and Oura G\textsubscript{4} generations that produced sex-reversed females, despite a normal amino acid coding sequence of DMY. To measure the DMY expression levels semiquantitatively, we performed densitometric RT–PCR. The mRNA levels of DMY were calibrated by those of β-actin (Figure 3A) and are shown as the ratios of DMY mRNA to β-actin mRNA in arbitrary units (Figure 3B). Statistical analyses using the Games/Howell post hoc test revealed that the DMY mRNA level was significantly lower in Y\textsuperscript{wKsn}Y\textsuperscript{wKsn} than in males of XY\textsuperscript{wKsn} and XY\textsuperscript{HNI}, although Y\textsuperscript{wKsn}Y\textsuperscript{wKsn} developed as all male in adulthood. The statistical analyses further revealed that the DMY mRNA levels were significantly lower in XY\textsuperscript{wKsn} and XY\textsuperscript{HNI}, Y chromosome derived from a Shirone XY female), and XY\textsuperscript{wKsn}, Y chromosome derived from an Aizu-Wakamatsu XY female), which developed as male or female in adulthood, as well as in Y\textsuperscript{wOur}Y\textsuperscript{wOur}, which developed as all female in adulthood, than in males of XY\textsuperscript{HNI}, XY\textsuperscript{HNI}, and Y\textsuperscript{wKsn}Y\textsuperscript{wKsn} (Figure 3B).

Early gonadal development of mutants with reduced DMY expression: We carried out histological observations for the Oura G\textsubscript{4} and Kesen-numa G\textsubscript{2} generations at 0 and 10 dah to reveal the early gonadal development of the mutants with reduced DMY expression. It is well known that the number of germ cells in many non-mammalian females is greater than that in males around the time of morphological sex differentiation (Van Nimbergh 1975; Zust and Dixon 1977; Nakamura et al. 1998). Thereafter, the germ cells in females continue to proliferate and then enter into meiosis, while the male germ cells arrest in mitosis. We counted the number of germ cells at 0 dah (Figure 4), which represents the time of the initial appearance of morphological
sex differences in medaka (Sato and Egami 1972; Quirk and Hamilton, 1973; Hamaguchi 1982). Around hatching, germ cells in XX individuals outnumber those in XY individuals (Hamaguchi 1982; Kobayashi et al., 2004) and subsequently germ cells enter mitotic arrest in XY fry, whereas they go into meiosis in XX fry after hatching (Sato and Egami 1972). Further, we examined the early gonadal development according to the presence or absence of diplotene oocytes at 10 dah (Table 2; Figure 5). In this period, the female germ cells are recognizable as oocytes from histological observations since the fry in putative male contain no germ cells in the meiotic prophase, while those in putative female contain germ cells both in mitosis and in the meiotic prophase (Sato and Egami 1972).

For Oura, $Y_{wOur}Y_{wOur}$, with the lowest $DMY$ expression, contained a number of germ cells, including oocytes, at 0 dah (Figure 4A), similar to normal XX females, and all eight $Y_{wOur}Y_{wOur}$ had diplotene oocytes at 10 dah (Table 2; Figure 5B). These results suggest that $Y_{wOur}Y_{wOur}$ develops as female in the same manner as normal XX females. $Y^{HN}Y_{wOur}$, with a normal Y chromosome from the HD-rRX$_{HNI}$ congenic strain, had a significantly lower germ-cell number at 0 dah than $Y_{wOur}Y_{wOur}$ (Figure 4A), and no oocytes were observed at either 0 or 10 dah (Table 2; Figure 5A). These results suggest that $Y^{HN}Y_{wOur}$ develops as male in the same manner as normal XY males.

For Kesen-numa, $Y_{wKsn}Y_{wKsn}$ and XY$_{wKsn}$, with reduced $DMY$ expression, had a number of germ cells, including oocytes, at 0 dah (Figure 4B). However, at 10 dah all eight XY$_{wKsn}$ had a small number of germ cells and no diplotene oocytes at 10 dah (Table 2; Figure 5D) and the remaining 2 XY$_{wKsn}$ had a small number of germ cells and no diplotene oocytes at 10 dah (Table 2; Figure 5C). These results are consistent with the observations that XY$_{wKsn}$ were either male or female in adulthood and that the number of females was greater than that of males in the $G_2$ generation. Some XY$_{wKsn}$ individuals would probably develop as females in the same manner as normal females. XY$_{HNI}$ and Y$_{wKsn}$, with a normal Y chromosome from the HD-rRX$_{HNI}$ congenic strain, contained no diplotene oocytes at either 0 or 10 dah (Table 2; Figure 5E), indicating that these individuals would develop as normal males.

**DISCUSSION**

**Characteristics of the $DMY$ mutations**: The results of this study indicate that all XY sex-reversal mutants
among wild populations of medaka are associated with defective DMY and can be classified into two types. One type contains mutations in the amino acid coding sequence of DMY (Awara, Aomori, Aizu-Bange, Kurobe, Suzu, Aki, and Saigo), while the other type has a normal coding sequence but reduced DMY expression at 0 dah (Shirone, Aizu-Wakamatsu, Kesennuma, and Oura).

In humans, mutations in SRY are associated with male-to-female sex reversal and a number of mutations have been identified within the SRY gene open reading frame (Shahid et al. 2004). Although nonsense mutations are scattered throughout the SRY gene, missense mutations tend to cluster in the central region of the gene, which encodes the high-mobility group (HMG) domain (Harley et al. 2003). In total, 43 of 53 missense mutations identified in the SRY gene open reading frame are located in the HMG domain (Shahid et al. 2004), strongly suggesting that the DNA-binding motif is essential in vivo. Conversely, only 10 mutations that lie outside the HMG domain have been detected. Among these, 7 are located in the 5’ region and 3 are located in the 3’ region of the HMG domain and all have different effects on the patient phenotype (Shahid et al. 2004). It is hypothesized that the region outside the HMG domain may be required to stabilize protein binding and/or to generate specificity by helping to discriminate between protein partners (Wilson and Koopman 2002).

### Table 2

Gonadal development at 10 dah in mutant lines

<table>
<thead>
<tr>
<th>Mutant line and generation</th>
<th>Genotype</th>
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<th>No. of fry</th>
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<tr>
<td>Oura G4</td>
<td>Y&lt;sub&gt;W&lt;/sub&gt;Our&lt;sub&gt;H&lt;/sub&gt;</td>
<td>8</td>
<td>8</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Y&lt;sub&gt;W&lt;/sub&gt;HNI</td>
<td>8</td>
<td>0</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Kesen-numa G&lt;sub&gt;2&lt;/sub&gt;</td>
<td>X&lt;sub&gt;W&lt;/sub&gt;HNI</td>
<td>8</td>
<td>0</td>
<td>8</td>
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</table>

DO, diplotene oocyte; Y<sub>W</sub>Our, Y chromosome derived from an Oura XY female; Y<sub>H</sub>NI, Y chromosome derived from an Hd-rR.Y<sub>H</sub>NI congenic strain male; Y<sub>Ksn</sub>, Y chromosome from a Kesen-numa XY female.

Figure 5.—Gonadal sex differentiation in the Kesen-numa and Oura mutant strains at 10 dah. The gonads of Y<sub>W</sub>Our<sub>H</sub> (B) and most XY<sub>Ksn</sub> (D) developed as female, while the gonads of Y<sub>H</sub>NI<sub>W</sub>Our (A), the remaining XY<sub>Ksn</sub> (C), Y<sub>T</sub>NY<sub>W</sub>Ksn (E), and Y<sub>W</sub>Ksn<sub>K</sub> (F) developed as male. Solid arrowheads indicate germ cells. Open arrowheads indicate diplotene stage oocytes. n, pronephric duct; g, gut. Bars, 10 μm.
The results of the present study demonstrate the occurrence of three types of DMY frameshift mutations. N110fsinsC was found in four populations (Awara, Aomori, Aizu-Bange, and Kurobe), while P109fsdelC was found in Suzu and S102fsdelC was found in Aki. The N110fsinsC and P109fsdelC frameshift mutations are present in the same polyC tract in exon 3, which is specific to the northern population. These mutations may have occurred independently in different populations, implying that this region is a mutational hot spot in the DMY gene, similar to the case for the human transcription factor hepatocyte nuclear factor (HNF)-1α, which contains a polyC tract in exon 4 (Kaisaki et al. 1997). The finding that all XYw containing each of N110fsinsC, P109fsdelC, and S102fsdelC in DMY developed as female in adulthood indicates that the mutated DMY genes do not have the male-determining function, although they contain the intact DM domain. Taken together, these results suggest that the 3’ region of the DM domain is indispensable for fulfilling the male-determining function of DMY.

**XY sex reversal is associated with reduced DMY expression**: In mice, XY sex reversal is associated with two types of mutation that appear to exert their effects by suppressing the Sry expression level. First, deletion of repeat sequences at some distance proximal to Sry on the mouse Y short arm results in reduced expression and sex reversal (Capel et al. 1993). Second, the sex reversal observed when crossing a *Mus musculus* strain *Poschiavinus* Y chromosome (YPOS) onto a *M. m. domesticus* background (in particular C57BL/6) (Eicher et al. 1982) has been attributed to a reduced Sry expression level from YPOS. Using a semiquantitative RT-PCR assay, Nagamine et al. (1999) showed that the levels of Sry expression from different mouse Y chromosomes are correlated with the degree of sex reversal they cause on an *M. m. domesticus* background. It appears that a critical threshold level must be achieved by a certain stage of genital ridge development for the supporting cell population to be pushed toward Sertoli cell differentiation.

In medaka, DMY expression first appears just before hatching and morphological sex differences are seen, whereas the expression of autosomal gene DMRT1 first occurs at 20–30 dah (Kobayashi et al. 2004). The present results demonstrate that the DMY expression levels at 0 dah in XYwKsn, XYwOu, XYwIcher, and YeOou YeOou, which produced sex-reversed females, are significantly reduced to less than half the levels in males of XYwKsn and XYwIcher. These results suggest that an underlying cause of XY sex reversal is a reduced level of DMY mRNA during a critical period of sex determination. It is interesting to note that YeOou YeOou developed as all male in adulthood, although their DMY mRNA level was significantly lower than that in XYwKsn and XYwIcher. Therefore, we consider that the certain threshold level of DMY mRNA required for male determination at 0 dah lies between the YeOou YeOou and XYwKsn mRNA levels.

**Lower levels of DMY transcripts lead the gonads to develop as phenotypic females**: In mice, Sry is expressed in pre-Sertoli cells (Albrecht and Eicher 2001) and required for male determination by means of promotion of Sertoli cell differentiation (Swain and Lovell-Badge 1999). In medaka, DMY is expressed in the pre-Sertoli cells (Matsuda et al. 2002; Kobayashi et al. 2004) and assumed to act as a factor that induces the development of pre-Sertoli cells into Sertoli cells in XY gonads, which is involved in the regulation of PGC proliferation as well as construction of the testicular tissue architecture (Matsuda 2005).

In the present study, we demonstrated reduced DMY expression at 0 dah in sex-reversed mutant strains. There are two possible causes for this reduced DMY expression: (1) the number of DMY-expressing cells is normal but the level of DMY transcription per cell is severely reduced or (2) the number of DMY-expressing cells is significantly reduced but the level of DMY transcription per cell is normal. Either of these possibilities could severely affect the development of pre-Sertoli cells into Sertoli cells. It is interesting to note that some XX recipient and XY donor chimeras of medaka develop into males that have only XX germ cells (Shinomiya et al. 2002). These results suggest that the XY somatic donor cells, the minor component in these chimeras, could induce sex reversal of the XX germ cells and the XX somatic cells. From these results, we infer that the latter possibility is unlikely to cause sex reversal, and it is reasonable to assume that the former possibility is the cause of the reduced DMY expression.

Our finding that YeOou YeOou develops as female in the same manner as normal XX females indicates that a low DMY mRNA level below the threshold level may lead to failure of DMY-positive cells to further differentiate into Sertoli cells. Since Sertoli cells are necessary for regulating PGC proliferation, lower levels of DMY transcripts lead to gonad development as phenotypic females. In contrast, the fact that YeOou YeOou develops as all male by 10 dah suggests that a DMY mRNA level above the threshold level at the sex-determining period is necessary for pre-Sertoli cells to undergo further differentiation. However, some YeKsn YeKsn individuals contain a number of germ cells, including oocytes, at 0 dah. This result implies that slightly reduced DMY expression may lead to a reduction in pre-Sertoli cell development into Sertoli cells. Moreover, some XYKsn develop as females and the remainder as males by 10 dah, although the DMY mRNA levels at 0 dah did not differ significantly among the six tested XYKsn individuals (data not shown). The increase in the sex-reversal ratio seen in Kesennuma G2 suggests the possibility that autosomal loci are related to the sex reversal in addition to a reduced DMY expression level, similar to the case for the tda genes in mice (Eicher et al. 1996).

The mutants with reduced DMY expression may contain regulatory mutations in the flanking region of
DMY: Future experiments will be focused on identifying the DMY expression control elements. These mutants may prove valuable for identifying DMY regulatory elements that are involved in transcriptional control.

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LITERATURE CITED


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