DMY gene induces male development in genetically female (XX) medaka fish

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Although the sex-determining gene *SRY/Sry* has been identified in mammals, homologues and genes that have a similar function have yet to be identified in nonmammalian vertebrates. Recently, *DMY* (the *DM*-domain gene on the Y chromosome) was cloned from the sex-determining region on the Y chromosome of the teleost fish medaka (*Oryzias latipes*). DMY has been shown to be required for the normal development of male individuals. In this study, we show that a 117-kb genomic DNA fragment that carries *DMY* is able to induce testis differentiation and subsequent male development in XX (genetically female) medaka. In addition, overexpression of *DMY* cDNA under the control of the CMV promoter also caused XX sex reversal. These results demonstrate that *DMY* is sufficient for male development in medaka and suggest that the functional difference between the X and Y chromosomes in medaka is a single gene. Our data indicate that *DMY* is an additional sex-determining gene in vertebrates.

sex differentiation | sex-determining gene | *Oryzias latipes* | sex reverse | transgenic

The sex of an individual is decided by the sex of the gonad, and in most cases the information required for a gonad to become either a testis or an ovary is encoded in the genome. The gene at the top of the sex-determination cascade for mammals is located on the Y chromosome and is called *SRY*. This gene was first identified during deletion analysis of the human Y chromosome (1). Furthermore, *Sry*, which is the mouse homologue of *SRY*, has been shown to be sufficient for the induction of male development in transgenic mice (2). In nonmammalian vertebrates, which also have a male heterogametic (XX-XY) sex-determining system, neither a homologue of *Sry* nor any other candidate genes recruited in sex determination was found until recently.

Recently, we identified a single candidate gene at the top of the sex determination cascade in the teleost medaka, *Oryzias latipes*, by positional cloning in the sex-determining region of the Y chromosome, and we named this gene *DMY* (3), although it has also been designated as *dmrt1bY* (4). This sex-determining region originated from a duplication of an autosomal region and it spans 258 kb, carrying only the *DMY* gene (4, 5). The expression of *DMY* is restricted exclusively to the somatic cells that surround the germ cells in the XY gonads, and the gene contains the highly conserved DM domain, which was originally described as a DNA-binding motif that is shared by *doublesex* (*dsx*) in *Drosophila melanogaster* and *mab-3* in *Caenorhabditis elegans* (6). The involvement of *DMY* in the process of sex determination was confirmed by the advent of two naturally occurring sex-reversed mutants, in which *DMY* was either truncated or expressed at reduced levels (3). Nevertheless, it remains to be seen whether *DMY* is sufficient for the development of an individual into a male.

In this study, we performed overexpression experiments using the *DMY* genomic region or *DMY* cDNA, which can induce testis development in genetic females (XX). We used the d-rR (7) and the Qurt (8) strains of medaka. In both strains, the genetic sex can be distinguished on the basis of body color. These experiments were designed to show that *DMY* is sufficient for male development and, consequently, that *DMY* is the sex-determining gene in medaka.

Results

Overexpression of *DMY* from the *DMY* Genomic Region. We subcloned the DMY^{HNI} genomic region, which encompasses ≈56 kb of the coding region, 60 kb of the upstream region, and 1.4 kb of the downstream region, from a bacterial artificial chromosome (BAC) genomic clone to the pCC1 vector (Fig. 1*A*). The vector containing DMY^{HNI} genomic DNA fragment (Fig. 1*A*) was injected into one-cell-stage medaka embryos of the d-rR strain. The transgene (DMY^{HNI}) and the host gene ($DMY^{d\text{-}rR}$) were distinguished either by restriction fragment length polymorphisms (RFPLs) of the genomic PCR products (Fig. 1*B*) or by differences in the nucleotide sequences of the respective RT-PCR products (Fig. 1*C*). The typical body coloration of the d-rR strain (white female and orange-red male) and the secondary sexual characteristics of the fins of medaka are shown in Fig. 2 *A* and *C*.

In the F_0 generation, we obtained 57 orange-red (XY) and 58 white (XX) adult-stage transgenic fish (Table 1). Of the 58 white fish, 13 (22.4%) showed male secondary sex characteristics (Table 1, Fig. 2). Genomic PCR-RFLP analyses revealed that none of these fish carried the $DMY^{d\text{-}rR}$ allele, which confirmed that they were XX. Eight of these 13 white males were found to be fertile, and 3 were sterile (i.e., we could not obtain fertilized eggs) (Table 2). Because some of the fish died before or during the mating experiments, we could not check their fertility (categorized as "not assessed" in Table 2).

To confirm the mRNA expression of the DMY^{HNI} transgene, we examined the integration and expression of the transgene in newly hatched transgenic fry by genomic PCR-RFLP, RT-PCR, and *in situ* hybridization with *DMY* as the probe. The genomes of 16 newly hatched transgenic fry were examined, and all were found by genomic PCR-RFLP to harbor the DMY^{HNI} transgene. Of these 16 fish, 5 were XX and 11 were XY. The levels of mRNA expression in the all XX and 27% of XY fry were examined by using RT-PCR (Table 3). All of the XX and XY transgenic embryos examined showed expression of the transgene

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The authors declare no conflict of interest.

Abbreviations: BAC, bacterial artificial chromosome; DAH, day(s) after hatching; RFLP, restriction fragment length polymorphism.

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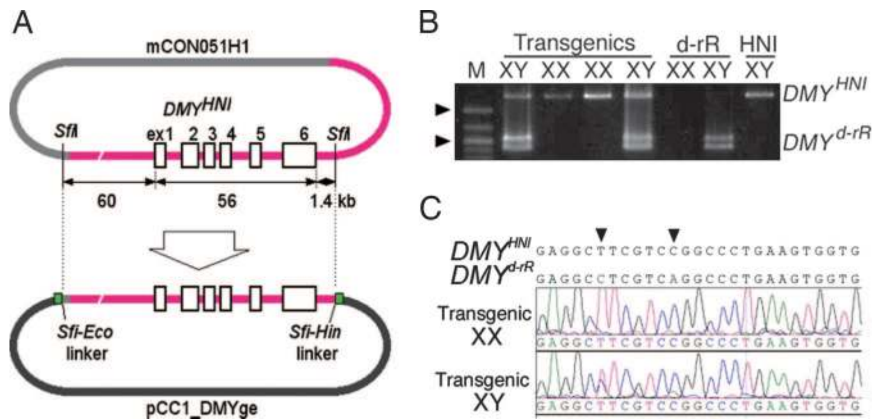

Fig. 1. Transgenic construct of the *DMY* genomic region. (*A*) Transgenic construct derived from the BAC clone mCON051H1, which contains DMY^{HNI} and two SfiI sites. The red coloration of the BAC clone represents the insert. This fragment was digested with SfiI and ligated into the pCC1 vector by using linkers. (*B*) Genomic PCR products digested with StuI. The $DMY^{d\text{-}rR}$ allele is digested, whereas the DMY^{HNI} allele is not digested. M represents the DNA marker. The arrowheads show the positions of the 1.5-kb and 1.0-kb DNA fragments. (*C*) Sequence chromatograms of the RT-PCR products of transgenic medaka. The DNA polymorphisms between DMY^{HNI} and $DMY^{d\text{-}rR}$ are shown by arrowheads. Transgenic XX individuals have only the DMY^{HNI} allele, whereas transgenic XY individuals have both the DMY^{HNI} and $DMY^{d\text{-}rR}$ alleles.

(DMY^{HNI}) in RT-PCR and direct sequencing experiments. The expression levels varied between individuals (data not shown). We examined an additional 21 newly hatched transgenic fry for DMY^{HNI} expression by *in situ* hybridization (Table 3 and Fig. 3). The sexes of these fry were assessed by genomic PCR-RFLP. Because the *in situ* hybridization method cannot distinguish the $DMY^{d\text{-}rR}$ (recipient) and DMY^{HNI} (transgene) alleles, we were unable to estimate transgene involvement in the signals obtained from the XY embryos. However, 26.7% of the XX transgenic embryos examined showed expression of the DMY^{HNI} transgene. The signals were found in somatic cells that surrounded the germ cells (pre-Sertoli cells), which resembles the pattern of endogenous expression of *DMY* in normal XY embryos.

To elucidate sex reversal during the overexpression of *DMY*, we analyzed histologically the gonads of the d-rR fish at 20 and 30 days after hatching (DAH). Genetic sexing was performed on the basis of body color. We analyzed 24 (12 XX and 12 XY) and 32 (15 XX and 17 XY) individuals at 20 and 30 DAH, respectively. All of the XY medaka were found to be normal. For the XX medaka, 8.3% (20 DAH) and 6.3% (30 DAH) displayed the acinus structure (a globular structure that is the seminiferous tubule precursor), whereas the remaining medaka had ovaries. However, we found that some of the XX fish had slowly developing ovaries, with either low numbers of germ cells or with incomplete follicles (see *Discussion*).

The first morphological sex difference manifested in the gonads is reflected in the number of germ cells (9). The number of germ cells in a *DMY* mutant identified from a wild population resembled that of the female (3). Therefore, we evaluated the effect of *DMY* on germ cell number in the transgenic F_0 generation. The genomic DNA fragment with DMY^{HNI} or Yamomoto's solution alone (control) was injected into one-cell-stage medaka embryos of the Qurt strain. The hatched fry were dissected into the head part, which was used for checking the genetic sex by genomic PCR-RFLP, and the body region, which was used for histological observation of the gonad (Fig. 4*A*). The numbers of germ cells and their stages in fry that overexpressed *DMY* were compared with those of the control fry (Fig. 4 *B–D*). The *DMY*-injected XX fry had fewer germ cells than the controls at both 0 DAH (Fig. 4*B*) and at 5 DAH (Fig. 4*C*). Interestingly, the *DMY*-injected XY fry also had fewer germ cells than the control at 0 DAH. These differences were confirmed as significant by the *t* test ($P < 0.05$). In *DMY*-injected XX fry at 5 DAH, the numbers of germ cells were reduced at various stages of germ cell development (Fig. 4*D*).

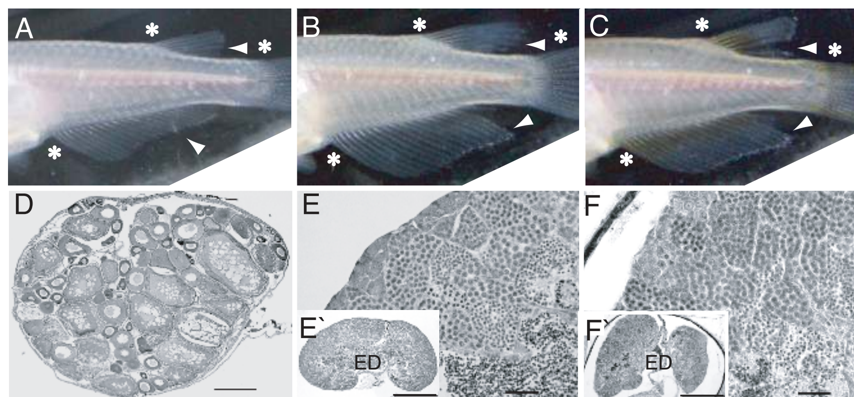

Fig. 2. Phenotypic analyses of *DMY* transgenic adult medaka. (*A–C*) The external secondary sexual characteristics and body coloration of the *DMY* transgenic fish in the adult stage are shown. The secondary sexual characteristics are manifested in the size and shape of dorsal and anal fins (white arrowhead). Both dorsal and anal fins of males are larger then those of females. The transgenic medaka include: a white (XX) female (*A*), a white (XX) male (*B*), and an orange-red (XY) male (*C*). Body coloration is clearly discernable at the basal parts of the fins (white asterisks). Gonadal histologies of a transgenic XX medaka, which has an ovary (*D*), an XX medaka, which has testes (*E* and *E*′), and an XY medaka (*F* and *F*′). ED, efferent duct. (Scale bars: *D*, *E*′, and *F*′, 200 μm; *E* and *F*, 50 μm.)

Table 1. Genotyping and phenotyping (secondary sexual characteristics) of transgenic fish using the *DMY* genomic region

		Phenotype	
Genotype	*n*	Female (%)	Male (%)
XY	57	0 (0)	57 (100)
XX	58	45 (77.6)	13 (22.4)

Overexpression of *DMY* Using the CMV Promoter. The overexpression of *DMY* using the *DMY* genomic region induced sex reversal. However, because the genomic region used was large (117 kb), it could not be ruled out that a region within this 117-kb DNA segment but outside the *DMY* open reading frame (ORF) was involved in the induction of sex reversal. Therefore, to investigate the ability of the *DMY* ORF to induce sex reversal, we constructed overexpression vectors in which CMV controlled the *DMY* or *DMRT1* cDNA. These constructs were injected into one-cell-stage medaka embryos of the Hd-rR strain, and the injected embryos were reared until the secondary sexual characteristics became apparent. Sex-reversed fish were obtained from the embryos that overexpressed *DMY*, whereas no sex-reversed fish were obtained from the embryos that overexpressed *DMRT1* (Table 4). The sex-reversed medaka were fertile (data not shown).

Discussion

A previous study has demonstrated that the *DMY* gene is necessary for normal male development (3). The present study shows that the overexpression of *DMY* by injecting the genomic region into XX individuals is sufficient to induce male development by reversing the sex of that individual. Furthermore, the overexpression of the *DMY* cDNA under the control of the CMV promoter also resulted in male development in XX individuals, which indicates that the protein encoded by the *DMY* ORF is sufficient for the induction of male development in XX medaka. Taken together with the previous data, our results indicate that *DMY* is the sex-determining gene in medaka. Thus, *DMY* is the first sex-determining gene to be identified in nonmammalian vertebrates.

Two lines of evidence suggest that *DMY* arose from a recent duplication event of the autosomal *DMRT1* genomic region. First, Y chromosome-linked *DMY* appears to have originated from a duplicate copy of autosomal *DMRT1* (4, 5, 10, 11), and then, in the Y chromosome, the duplicated *DMRT1* acquired a new function as a sex-determining gene, *DMY*. Second, *DMY* is also found in *Oryzias curvinotus*, which is most closely related to medaka (12), but is not found in other *Oryzias* species (*O. celebensis*, *O. mekongensis*) or in other fishes (guppy, tilapia, zebrafish, and fugu) (13). These results suggest that *DMY* is a recently evolved gene specific to some species of the genus *Oryzias*.

DMY represents a strong motive force for testis development in gonads. In transgenic experiments, F_0 embryos injected with DNA were typically mosaic. Likewise, F_0 embryos injected with the *DMY* genomic fragment were expected to have mosaic gonads. This situation is comparable to the XX recipient and XY donor chimeras of medaka, some of which developed as males despite having only a few XY donor cells and XX recipient cells in the gonad (14). It was found that some of the chimeras with only XX germ cells also developed into males. It is evident that relatively few XY somatic cells are sufficient to reverse the sex of the germ cells and somatic cells of the XX recipient gonad. These results suggest that even if *DMY* is present in only a minority of somatic cells, the gonad can ultimately develop into a testis. We believe that this is why we observed a low masculinization rate in the experiments in which gonad histology was examined at 20 and 30 DAH, whereas we observed a high masculinization rate for the secondary sex characteristics of the adult stage.

Table 2. Assessments of fertility of F_0 transgenic fish using the *DMY* genomic region

	n	Fertile	Sterile	Not assessed
XY male	57	53	1	3
XX female	45	35	0	10
XX male	13	8	3	2

Table 3. Transgene expression in embryos on the day of hatching (0 DAH)

			DMY expression		
Assay method	*n*	Genotype	−	+	Embryos expressing the *DMY*, %
RT-PCR	16	XY	0	3*	100
		XX	0	5	100
ISH	21	XY	0	6†	100‡
		XX	11	4	26.7

*Three individuals out of 11 were assayed.
†The transgene (DMY^{HNI}) and host gene ($DMY^{d\text{-}rR}$) could not be distinguished.

Some of the XX fish had slowly developing ovaries at 20 and 30 DAH. In this case, it appeared that the level of *DMY* expression in the XX gonad was low, and the gonad initially developed into an ovary. However, some of the somatic cells carried *DMY*, the expression of which should lead to testis development.

DMY plays a role in the development of germ cells in males. The number of germ cells in many nonmammalian females is greater than in males at around the time of morphological sex differentiation (15–17). The germ cells in the females continue to proliferate and subsequently enter meiosis, in contrast to the male germ cells, which arrest in mitosis (9, 18). Kobayashi *et al.* (2004) demonstrated that the first appearance of a sex difference in germ cell number occurs at stage 38, before the hatching of medaka. Furthermore, in the XY gonads of medaka, the formation of the acinous structure occurs ≈10 DAH (19). These events are the first morphological signs of sex differentiation, as well as of testicular differentiation. Nevertheless, *DMY* expression starts in the somatic cells surrounding the germ cells, which are found in the coelomic epithelium under the nephric duct at stage 34 (9). This phenomenon occurs before the emergence of any sex differences in terms of germ cell number. When *DMY* does not function (e.g., a *DMY* mutant), the germ cells in XY embryos proliferate and enter meiosis, just like the XX embryos. In the *DMY* overexpression experiments, the total number of germ cells at 0 DAH was significantly reduced in both the XX and XY fry. In the 5 DAH XX fry injected with *DMY*, not only was the total germ cell number reduced but the number of germ cells at various stages of development was also reduced. Because the total number of germ cells reflects the outcome of active mitosis, the reduced number of germ cells in the transgenic fry may be due to a signal or signals from the surrounding somatic cells that express *DMY*.

In mammals, the function of the X chromosome differs from that of the Y chromosome. The male-specific region of the Y chromosome contains 156 known transcriptional units, which include 78 protein-coding genes that collectively encode 27 distinct proteins, including 10 testis-specific genes and *SRY* (20). Chromosomally female transgenic mice that carry *Sry* develop into males that are sterile (2), which suggests that *Sry* is insuf-

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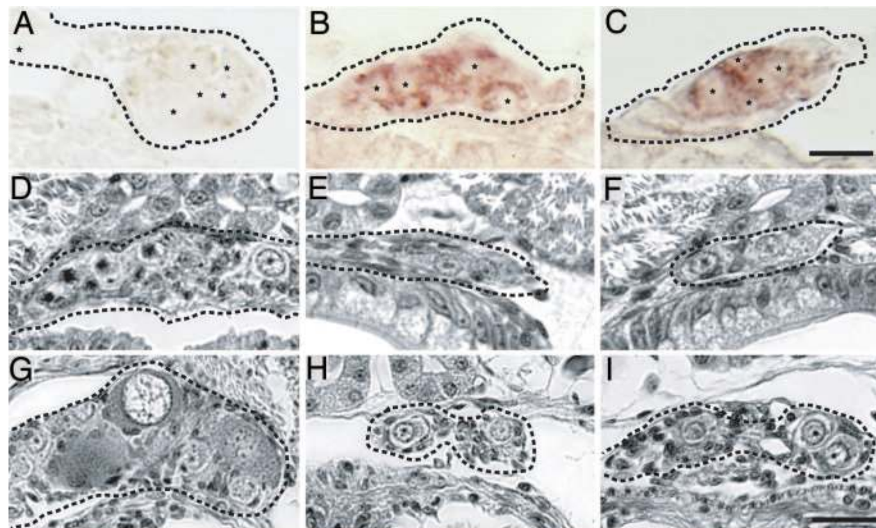

Fig. 3. Analyses of *DMY* transgenic medaka at different developmental stages. (*A–C*) *DMY* mRNA expression in the gonads of the fry is revealed by digoxigenin-labeled *in situ* hybridization of larval sections. (*A*) *DMY*-negative gonad of a transgenic XX fry. Strong signals for *DMY* are seen in the cells surrounding the germ cells in the XX fry (*B*) and XY fry (*C*). (*A–C*) Germ cells are denoted by asterisks. Gonad histologies at 2 DAH (*D–F*) and 30 DAH (*G–I*). (*E* and *H*) Transgenic XX medaka with testis-like gonads. (*F* and *I*) The transgenic XY individuals have gonads with typical testicular structure. The gonadal region is outlined by dotted lines. (Scale bars: 20 μm.)

ficient for complete male development. In contrast, the present study shows that *DMY* induces complete male development in XX medaka. This finding suggests that the functional difference between the X and Y chromosomes of medaka involves a single gene. Analyses of the genomic organization of the sex-determining regions and adjacent regions of the sex chromosomes of medaka have also suggested that *DMY* appears to be the only functional gene (5). Because most vertebrate species have chromosomal (genetic) sex-determining systems and no morphologically distinct sex chromosomes, as is the case with medaka, it is possible that the functional difference between the sex chromosomes is a single gene that determines the sex of the respective species.

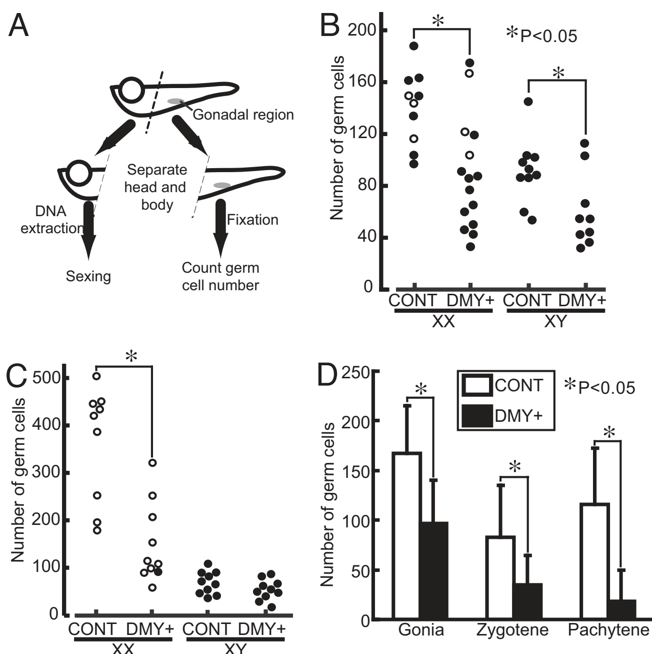

Fig. 4. Numbers of germ cells in transgenic and control medaka fry. (*A*) Schematic diagram depicting the procedure. Total numbers of germ cells at 0 DAH (*B*) and 5 DAH (*C*). Open circles represent individuals that have oocytes in the zygotene or pachytene stage, whereas filled circles represent individuals that lack oocytes in the zygotene or pachytene stage. (*D*) The numbers of germ cells at the different developmental stages of 5 DAH XX fry are shown. The control and *DMY* transgenic were compared within each stage. The results are presented as the mean ± SEM of each stage.

Materials and Methods

Strains. We used the three strains d-rR, Hd-rR, and Qurt. The d-rR and Hd-rR strains, in which the wild-type allele *R* of the *r* locus (a sex-linked pigment gene) is situated on the Y chromosome, was used to generate the transgenic fish with the *DMY* genomic region. In this case, the female X^rX^r had white body color, whereas the male X^rY^R had orange-red body color (7). Another strain, Qurt [*b*/*b*, *gu*/*gu*, *r*/*r*,X(*lf*)/Y(+)], in which the genetic sex of the XX and XY fish can be distinguished by the presence or absence of leucophore (8), was also used for the generation of transgenic fish.

Transgenic Constructs. The constructs for *DMY* transgenic fish were prepared by subcloning the BAC clone that was derived from the Y congenic strain Hd-rR.Y^{HNI} (3), which contains the sex-determining region from the Y chromosome of the HNI strain and has the genetic background of the Hd-rR strain. The BAC clone (mCON051H1) containing *DMY* was digested with SfiI, recognition sites for which were located ≈1.4 kb downstream of *DMY* exon 6 and the vector sequence (Fig. 1). A biased sinusoidal field gel electrophoresis system (GENOFIELD; Atto Co., Tokyo, Japan) was used to isolate the DNA fragment that contained *DMY*. The gel electrophoresis conditions were as follows: 1% low-melting-point agarose gel in 0.5× TBE (44.5 mM Tris/44.5 mM boric acid/1 mM EDTA, pH 8.0) at 20°C, dc 1.6 V/cm and ac 9.6 V/cm, and log-pulse time ramp from 0.01 to 0.2 Hz for 20 h. The DNA fragment was purified by using GELase (Epicentre Technologies, Madison, WI) and then ligated with the linker-ligated CopyControl pCC1 vector (Epicentre Technologies). This system combines the clone stability afforded by single-copy cloning with the advantages of the high yields of DNA obtained by "on-demand" induction of the clones to high copy number. The pCC1 vector was digested with HindIII and EcoRI and ligated with the Sfi-Hind linker (AGCTGAG) and

Table 4. CMV promoter-directed overexpression of *DMY* and *DMRT1*

cDNA	Embryos injected	Embryos hatched	Hatching rate (%)	XY male	XX female	XX male
DMY	195	86	44.1	20	-4	4
DMRT1	139	30	57.6	30	13	0

Sfi-Eco linker (AATGGG). TransforMax EPI300 electrocompetent cells (Epicentre Technologies) were used for transformation. Recombinant clones were grown in culture and induced by the addition of the CopyControl Induction Solution (Epicentre Technologies) to generate high plasmid copy numbers, according to the manufacturer's instructions. The clones were purified by using either a Large-Construct kit (Qiagen, Valencia, CA) or CsCl-ethidium bromide gradient ultracentrifugation, which was preceded by purification by using the QIAfilter Plasmid Maxi kit (Qiagen).

To construct CMV promoter-controlled overexpression vectors, the ORFs of *DMY* and *DMRT1* were amplified from the total RNA of whole embryos for *DMY* and of adult testes for *DMRT1*of the Hd-rR strain by using a OneStep RT-PCR kit (Qiagen) with the following primer sets: PG17.25 (CCC ACC AGA TCC TAT ACA AGT GAC) and PG17.89 (CAG CTT GTC GAC ATT TGG TTT CAC TGC TCA TGG A) for *DMY*, and PG17.25 and PG17.95 (CAG CTT GTC GAC ATT TGC TCT CGC TGC TCA TGG A) for *DMRT1*. Primer PG17.25 is located upstream of the ATG start site. Primers 17.89 and 17.95 are situated in the 3′ regions of *DMY* and *DMRT1*, respectively, and include the SalI restriction enzyme site but lack the stop codon. The RT-PCR products were cloned by using a Zero Blunt TOPO PCR Cloning kit (Invitrogen, Carlsbad, CA). The clones were sequenced to exclude sequence alterations, and the authentic clones were chosen. Selected clones were digested with EcoRI and SalI, purified by gel electrophoresis, and ligated to EcoRI and SalI-digested pIRES-hrGFP-1a vector (Stratagene, La Jolla, CA). In these constructs, the cDNAs were fused to three contiguous copies of the FLAG epitope tag, followed by the internal ribosome entry site, which was further linked to the hrGFP coding sequence. The clones were purified by using a QIAfilter Plasmid Maxi kit (Qiagen).

Microinjection. Fertilized eggs were collected within 15 min of spawning. The attaching filaments were scraped off the chorion on a piece of paper. Microinjection was carried out as described previously (21). For microinjection of the *DMY* genomic region, we used DNA at 50 ng/μl in Yamamoto's solution. For CMV promoter-directed overexpression, we used 45 ng/μl and 50 ng/μl of CMV-*DMY* and CMV-*DMRT1*, respectively, in Yamamoto's solution. The injected eggs were incubated at 26°C.

Sexing. The genetic sex of each specimen was determined by genomic PCR. For fry, the dissected head part was used for DNA extraction to assess the genetic sex while the body part was used for *in situ* hybridization analysis. Genomic PCR was performed by using the common primers for *DMRT1* and *DMY* (PG17.5, CCG GGT GCC CAA GTG CTC CCG CTG; and PG17.6, GAT CGT CCC TCC ACA GAG AAG AGA). PCR conditions were as follows: 5 min at 95°C, followed by 30 cycles of 20 sec at 96°C, 30 sec at 55°C, and 30 sec at 72°C, followed by 5 min at 72°C. The length of the *DMY* PCR product was different from that of *DMRT1*, which made it easier to judge the genetic sex by using 1% agarose gel electrophoresis.

Detection of the Transgene in the Genomes. Total RNA and genomic DNA samples were extracted from each hatched embryo after homogenization in a 1.5-ml tube with 350 μl of the RLT buffer supplied with an RNeasy Mini kit (Qiagen). The homogenized lysates were centrifuged, followed by RNA extraction protocol by using the RNeasy Mini kit and an RNase-Free DNase set (Qiagen). DNA was extracted from the pellet by using a DNeasy Tissue kit (Qiagen) according to the manufacturer's protocol.

Genomic PCR to detect the transgene was performed by using the *DMY*-specific primers PG17.19 (GAA CCA CAG CTT GAA GAC CCC GCT GA) and PG17.20 (GCA TCT GCT GGT ACT GCT GGT AGT TG). The PCR conditions were as follows: 5 min at 94°C, followed by 30 cycles of 15 sec at 96°C, 60 sec at 68°C, and a final step of 5 min at 72°C. To distinguish the $DMY^{d\text{-}rR}$ and DMY^{HNI} alleles, the PCR products were digested with StuI and electrophoresed in a 2% agarose gel.

RT-PCR was performed by using a OneStep RT-PCR kit (Qiagen). For the RT-PCR, the PG17.25 (CCC ACC AGA TCC TAT ACA AGT GAC) and PG17.48 (GGC TGG TAG AAG TTG TAG TAG GAG GTT T) primers were used, and 100 ng of total RNA was used as the template in a 25-μl volume. The PCR conditions were as follows: 30 min at 55°C, 15 min at 95°C, followed by 30 cycles of 20 sec at 96°C, 30 sec at 55°C, 60 sec at 72°C, and a final step of 5 min at 72°C. The RT-PCR products were directly sequenced.

Fertility Checking. To check the fertility of F_0 transgenic medaka, transgenic F_0 males were pair-mated with normal females or transgenic F_0 females.

Histological Analyses. For the histological analysis and counting of germ cell numbers, the embryos that were injected with the *DMY* genomic region were dissected into head and body segments. The body portions were fixed overnight in Bouin's fixative solution and then embedded in paraffin. Each dissected head was used to determine the genetic sex, according to the protocol listed in the *Sexing* section. Cross-sections were cut serially at 5-μm thickness, and after H&E staining, all of the germ cells were counted for each fry. After cell counting, the mean and standard error were calculated for each sex at each stage, and the differences between the sexes were evaluated statistically by using the paired *t* test for each stage.

***In Situ* Hybridization.** After the head part had been removed from the hatching fry, the body region with the gonad were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C overnight. *In situ* hybridization was performed as described previously (3, 9).

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Corrections

DEVELOPMENTAL BIOLOGY. For the article "*DMY* gene induces male development in genetically female (XX) medaka fish," by Masaru Matsuda, Ai Shinomiya, Masato Kinoshita, Aya Suzuki, Tohru Kobayashi, Bindhu Paul-Prasanth, En-lieng Lau, Satoshi Hamaguchi, Mitsuru Sakaizumi, and Yoshitaka Nagahama, which appeared in issue 10, March 6, 2007, of *Proc Natl Acad Sci USA* (104:3865–3870; first published February 28, 2007; 10.1073/pnas.0611707104), due to a printer's error, in Table 4, column 3, the number of *DMRT1* embryos hatched was given as "30"; it should instead be "80." Also, in Table 4, column 6, the number of *DMY* transgenic XX females was given as "−4"; it should instead be "4." The corrected table appears below.

Table 4. CMV promoter-directed overexpression of *DMY* and *DMRT1*

cDNA	Embryos injected	Embryos hatched	Hatching rate, %	XY male	XX female	XX male
DMY	195	86	44.1	20	4	4
DMRT1	139	80	57.6	30	13	0

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MEDICAL SCIENCES. For the article "The omega-3 fatty acid docosahexaenoate attenuates endothelial cyclooxygenase-2 induction through both NADP(H) oxidase and PKCε inhibition," by Marika Massaro, Aida Habib, Laura Lubrano, Serena Del Turco, Guido Lazzerini, Todd Bourcier, Babette B. Weksler, and Raffaele De Caterina, which appeared in issue 41, October 10, 2006, of Proc Natl *Acad Sci USA* (103:15184–15189; first published October 3, 2006; 10.1073/pnas.0510086103), the authors note that the following should be added as an affiliation for Marika Massaro: Department of Biological and Environmental Sciences and Technologies, University of Lecce, Ecotekne, 73100 Lecce, Italy. The corrected author and affiliation lines, and related footnotes, appear below.

Marika Massaro*[†‡], Aida Habib[‡§], Laura Lubrano[¶], Serena Del Turco[¶], Guido Lazzerini[¶], Todd Bourcier[‖], Babette B. Weksler, and Raffaele De Caterina[††‡‡]**

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MICROBIOLOGY. For the article "Specific polysaccharide conjugate vaccine-induced IgG antibodies prevent invasion of *Shigella* into Caco-2 cells and may be curative," by Yehuda Chowers, Joachim Kirschner, Nathan Keller, Iris Barshack, Simon Bar-Meir, Shai Ashkenazi, Rachel Schneerson, John Robbins, and Justen H. Passwell, which appeared in issue 7, February 13, 2007, of *Proc Natl Acad Sci USA* (104:2396–2401; first published February 7, 2007; 10.1073/pnas.0610833104), the authors note an error in the title. The title should read: "O-specific polysaccharide conjugate vaccine-induced antibodies prevent invasion of *Shigella* into Caco-2 cells and may be curative." The online version has been corrected. Additionally, on page 2396, in the first line of the Abstract, in the first line of the second paragraph of the main text, and in the Abbreviations footnote, "*O*-specific polysaccharide" should instead appear as "O-specific polysaccharide."

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MICROBIOLOGY. For the article "DgrA is a member of a new family of cyclic diguanosine monophosphate receptors and controls flagellar motor function in *Caulobacter crescentus*," by Matthias Christen, Beat Christen, Martin G. Allan, Marc Folcher, Paul Jenö, Stephan Grzesiek, and Urs Jenal, which appeared in issue 10, March 6, 2007, of *Proc Natl Acad Sci USA* (104:4112–4117; first published February 27, 2007; 10.1073/pnas.0607738104), on page 4112, column 1, the key term "c-di-Ghp" should instead appear as "c-di-GMP." The online version has been corrected.

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