A duplicated copy of *DMRT1* in the sex-determining region of the Y chromosome of the medaka, *Oryzias latipes*

Indrajit Nanda*†, Mariko Kondo†‡§, Ute Hornung‡, Shuichi Asakawa¶, Christoph Winkler‡, Atsushi Shimizu¶, Zhihong Shan‖, Thomas Haaf‖, Nobuyoshi Shimizu¶, Akihiro Shima§, Michael Schmid*, and Manfred Schartl‡**

*Institute for Human Genetics, and ‡Department of Physiological Chemistry I, Biocenter, University of Würzburg, D-97074 Würzburg, Germany; §School of Integrated Biosciences, University of Tokyo, Tokyo 113-0033, Japan; ¶Department of Molecular Biology, Keio University School of Medicine, Tokyo 160-8582, Japan; and ‖Max-Planck Institute for Molecular Genetics, D-14195 Berlin, Germany

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The genes that determine the development of the male or female sex are known in *Caenorhabditis elegans*, *Drosophila*, and most mammals. In many other organisms the existence of sex-determining factors has been shown by genetic evidence but the genes are unknown. We have found that in the fish medaka the Y chromosome-specific region spans only about 280 kb. It contains a duplicated copy of the autosomal *DMRT1* gene, named *DMRT1Y*. This is the only functional gene in this chromosome segment and maps precisely to the male sex-determining locus. The gene is expressed during male embryonic and larval development and in the Sertoli cells of the adult testes. These features make *DMRT1Y* a candidate for the medaka male sex-determining gene.

The overwhelming majority of multicellular animal species occur as both sexes, and in many cases the decision of whether an organism becomes a male or a female is determined by the genome. In most mammals, several flies, and the worm *Caenorhabditis elegans* the cascades of sex-determining genes are reasonably well understood. However, the master regulator encoded by the sex-determining locus (*SD*) on the Y chromosome of most mammalian species, *SRY*, is not functioning like that in some mammals (1). In nonmammalian species, which also have a XX/XY sex-determination system, *SRY* is not present at all. Neither the *sxl* gene of *Drosophila* nor *xol* of *C. elegans*, the genes at the top of the sex-determination cascade in these organisms, are functioning in the same way in more distantly related species (for review see ref. 2). This finding indicates that on the molecular level another dimension of diversity is added to the complex situation of multiple genetic systems for sex determination. In between worms and flies on the one side and mammals on the other side, there is a large gap in our knowledge about sex-determination genes.

Fishes are an attractive group of organisms for studying the evolution of sex determination because members of this class exemplify a broad range of various types of sex determination from hermaphroditism to gonochorism and from environmental to genetic sex determination (for review see refs. 3 and 4). The structure and expression of genes involved in sex determination and differentiation can be compared in species, which exhibit either similar or divergent sex-determination systems. Unfortunately, in both main fish models, the pufferfish (*Takifugu rubripes*) and the zebrafish (*Danio rerio*), no information exists on the mode of sex determination, the potential presence of sex chromosomes, and the process of sex differentiation.

The situation for a molecular analysis of sex determination is much more favorable in another fish model, the medaka (*Oryzias latipes*) (for review see ref. 5). Medaka has a XX/XY sex determination system like mammals. Male and female medakas are easily distinguished by a number of secondary sex characters (see ref. 6). A linkage map of the sex chromosomes with several molecular and phenotypic markers is available (7, 8). Especially useful is the *quart* strain, where the sex chromosomes express different alleles of the *lf* pigment marker. The presence of leucophores in the male and their absence in the female allow differentiation of both sexes as early as at 2–3 days of embryonic development (9).

From the genes known to be involved in sex determination/differentiation the *DMRT* genes are of special interest because of their widespread distribution. They form a family of genes, which share a highly conserved DNA-binding domain (10), the DM domain. *DMRT1* is a candidate downstream sex-determination gene in mammals and appears to be involved in a certain type of XY sex reversal in humans (11–13). It is conserved in a wide range of animals with diverse sex-determining mechanisms, including *C. elegans*, *Drosophila*, fish, reptiles, birds, and mammals (14–19).

We have investigated a potential role for *DMRT* genes in sex determination of medaka and found that a duplicated copy of *DMRT1* is present at the male *SD* and shows all features of a sex-determining gene.

Materials and Methods

Medaka Fish. All experimental animals were from inbred lines of Northern (Kaga, HNI) and Southern medakas (i-3, quart, HB32C, SOK) except for the Carbio strain, which is an outbred strain derived from the Southern population of medaka.

Cloning and Sequence Analysis. To obtain the Y-chromosomal *DMRT* gene the male-specific band from Southern blots of genomic DNA was excised and cloned. Briefly, the 3.3-kb male-specific *Eco*RI fragment from medaka (strain Carbio) was cloned by excising the 3- to 4-kb *Eco*RI fragments from a preparative 0.8% agarose gel. The DNA was ligated into λZapII *Eco*RI arms (Stratagene) and packaged *in vitro*. The resulting subgenomic library was screened with the 4-kb *Eco*RI fragment from medaka cosmid 73K2481, which contains exons 1–3 of the autosomal *DMRT1* gene.

Filters of arrayed medaka genomic cosmid libraries (nos. 73 and 74) were obtained from the Resource Center of the German Human Genome Project (Berlin) and screened with the human *DMRT1* cDNA under conditions of low stringency (hybridization: 35% formamide, 42°C; washing: 1× SSC/1% SDS, 63°C). Positive clones were initially characterized and arranged in groups by restriction fragment analysis and Southern blotting.

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Abbreviation: BAC, bacterial artificial chromosome.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AY129240 and AY129241).

†I.N. and M.K. contributed equally to this work.

**To whom reprint requests should be addressed. E-mail: phch1@biozentrum.uni-wuerzburg.de.

Restriction fragments cross-hybridizing with the human *DMRT1* probe were subcloned and sequenced.

For isolation of a medaka *DMRT1Y* cDNA, total RNA was isolated from 6-day-old embryos (strain Carbio). Reverse transcription was done with oligo(dT)$_{12-18}$ and Superscript II reverse transcriptase following the supplier's protocol (GIBCO/BRL). For PCR from 3 μl of the reverse transcription reaction, primers based on the genomic sequence of *DMRT1Y* (DMTYh: TCT GCT GAG CTC CCC GGG; DMTYi: GCC TCG CAG CTT CTC A) were used. The PCR was run for 35 cycles at an annealing temperature of 62°C. The sequence of *DMRT1Y* is deposited under GenBank accession no. AY129240.

Bacterial Artificial Chromosome (BAC) Isolation and Analysis. A genomic BAC library of the HNI strain with an average insert size of 160 kb was constructed (20). BAC clones containing the DM domain sequences were screened by colony hybridization under low stringency with the PCR fragment of the DM domain of medaka *DMRT4* (20). The clones containing *DMRT1Y* were selected by PCR screening with a specific primer set (DMTk: CAA CTT TGT CCA AAC TCT GA; DMTl: AAC TAA TTC ATC CCC ATT CC). The contig was extended by using PCR end fragments as probes, and BACs 168M02, 209O12, and 113N21 were shotgun-sequenced.

Sequences were analyzed by application of the NIX software tool (www.hgmp.mrc.ac.uk/NIX/). Putative exons and genes were compared at the nucleotide and amino acid levels to known genes by standard GCG programs and by BLAST and FASTA database searches. The relevant part of the contig is deposited under GenBank accession no. AY129241.

Southern Blot Analysis. DNA from individual fish was obtained from pooled organs as described. Five micrograms of genomic DNA was digested with restriction enzymes, separated on 0.8% agarose gels, and blotted onto nylon membranes (Hybond N+, Amersham Pharmacia). Membranes were hybridized either under conditions of moderate stringency [hybridization in 35% formamide, 0.1% Na-pyrophosphate, 50 mM Tris·Cl (pH 7.5), 5 × SSC, 1% SDS, 5 × Denhardt's, 100 μg/ml calf thymus DNA at 42°C, washing in 1 × SSC/1% SDS at 60°C] or high stringency (hybridization in the same buffer except that the formamide concentration was 50% and at the same temperature, washing in 0.1 × SSC/1% SDS at 68°C) with the following probes: *OlaDMRT1*, 4-kb *Eco*RI fragment from cosmid 73K2481; *OlaDMRT1Y,* 1.1-kb *Nhe*I/*Sst*I fragment from phage 2.1 of the subgenomic library; and *HsaDMRT1*, 1.5-kb *Eco*RI fragment from the human *DMRT1* cDNA.

Expression Analysis. Total RNA was extracted from pooled organs of several adult medaka fish or 10–40 pooled total embryos of defined stages (21) by using the TRIZOL reagent (GIBCO/BRL) according to the supplier's recommendation. After DNase treatment reverse transcription was done with 2 or 4 μg total RNA by using Superscript II reverse transcriptase (GIBCO/BRL) and random primers. cDNA from 10 ng (actin) to 200 ng (adult organs) or 600 ng (whole embryos) of total RNA was used for PCR with gene-specific primers: *Ola Actin*, MAct1(TTC AAC AGC CCT GCC ATG TA) and MAct 2 (GCA GCT CAT AGC TCT TCT CCA GGG AG) at an annealing temperature of 60°C for 25 cycles; *Ola DMRT1*, DMT1m (TCC GGC TCC ACA GCG GTC) and DMT1n(CAG ACA GAG GGT TGG GGG G) at an annealing temperature of 64°C for 35 cycles; *Ola DMRT1Y*, DMTYa (GGCCGGGTCCCCGGGTG) and DMTYc (CTG GTA CTG CTG GTA GTT GTG) at an annealing temperature of 64°C for 35 cycles.

RNA from sex-reversed embryos and adults was obtained after treating embryos from day 1 until hatching with 1 mg/ml 17β estradiol in the rearing medium essentially as described (22).

Whole-mount RNA *in situ* hybridization on adult testis was performed according to standard protocols (23). Samples were digested with proteinase K for 2 min before hybridization with a 488-nt *DMRT1Y* antisense riboprobe at 65°C. The riboprobe was generated from a partial cDNA that was obtained by reverse transcription–PCR using the DMTYa and DMTYc primers. Stained tissue samples were paraffin-embedded, sectioned, and counterstained with eosin.

Mapping. A sex-reversed XY female backcross panel was generated as reported (8). A total of 117 backcross progeny were analyzed for four STS markers (Yc-2, Casp6, SL1, Casp3B) that were previously isolated (7, 8, 24) and two phenotypic markers (lf: leucophore, y: male sex). *DMRT1Y* was mapped by PCR using primers DMTk and DMTn (TGA TGC AGC ATT TTG ACA CAT TTA). The products were electrophoresed on 6% acrylamide gels. Segregation of the markers was analyzed with Macintosh MAPMAKER version 2 (25).

Fluorescence *in Situ* Hybridization. BAC clones were labeled separately by standard nick translation using biotin-16-dUTP and digoxigenin-11-dUTP. For two-color hybridization equal amounts of labeled probes were mixed with hybridization solution at a final concentration of 10 μg/ml and used at 100 ng per slide. Before hybridizing with denatured medaka mitotic chromosomes the probe mixture was denatured and preannealed in the presence of excess genomic DNA. Hybridization sites for both probes were simultaneously detected by means of rhodamine-conjugated avidin (Vector Laboratories) and antidigoxigenin (monoclonal)-conjugated fluorescein (Sigma). Chromosomes were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Digitized images of the FITC and rhodamine signals were captured separately and displayed on DAPI-stained chromosomes by using EASY FISH 1.0 software (Applied Spectral Imaging, Mannheim, Germany).

Results

In our studies on sex-determining genes in the medaka we have recently found that the *DMRT1* homologue of medaka is located on an autosome in a gene cluster together with its paralogues, *DMRT 2* and *3* (26). cDNA probes from human and medaka *DMRT1* used on Southern blots of male and female DNA disclosed an additional fragment only in males (Fig. 1 *a–e*). The male-specific fragment was cloned from a partial genomic library and found to be a duplicated version of *DMRT1*, showing 93% identity on the nucleotide and 90% similarity on the amino acid level (Fig. 2). It has a nucleotide identity of 96% (92% amino acids) to the *DMY* gene isolated by Matsuda *et al.* (27). The sequence differences probably are caused by different strains of medaka that were used for analyses. PCR primers specific to the Y-chromosomal copy (designated *DMRT1Y*) were used for a linkage analysis (Fig. 1*f*). *DMRT1Y* was found in all 81 males but not in 57 females of the i-3 strain and in 226 tested females of seven other strains from the Northern and Southern medaka populations. For mapping we used the sex-reversed (XY female × XY male; ref. 8) backcross mapping panel, which spreads the map distances of Y-chromosomal markers in the vicinity of *SD* by a factor of 10 compared with the resolution obtained with a male backcross panel. No recombination between *SD* and *DMRT1Y* was detected (Fig. 1*g*), whereas all earlier described markers map with some distance left and right to *SD* (8, 24). Thus *DMRT1Y* and the male *SD* colocalize on the genetic map (linkage <0.24 cM, equivalent to approximately 125 kb according to refs. 7 and 28).

Sequence analysis of BACs from a genomic library of the HNI strain, cosmids from two independent genomic libraries of Northern (Kaga strain) and Southern (Carbio strain) medaka, and a full-length testis cDNA further confirmed that *DMRT1Y*

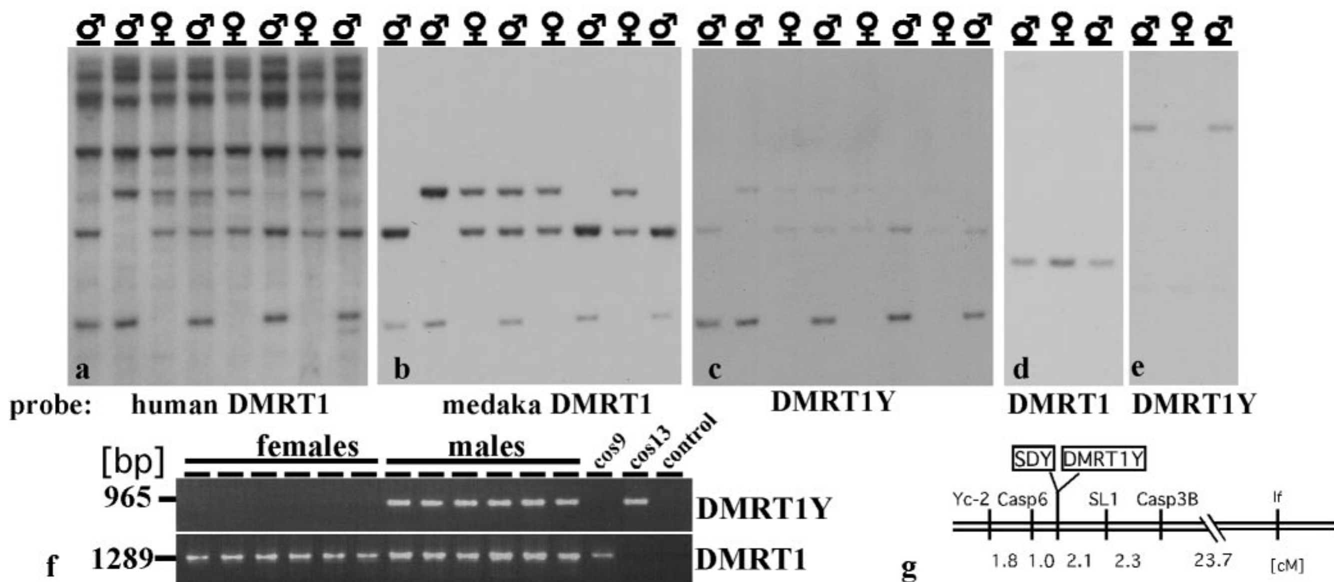

Fig. 1. Southern blot of *Eco*RI-digested male and female DNA with a human *DMRT1* cDNA (*a*), and rehybridized with medaka genomic *DMRT1* (*b*) and *DMRT1Y* (*c*) probes. *DMRT1* and *DMRT1Y* show some cross-hybridization to each other even under conditions of highest stringency because of the high sequence similarity. The hybridization conditions in *a* were moderate stringency and high stringency in *b* and *c*. (*d* and *e*) Hybridization under even further increased stringency conditions of *Pst*I-digested DNAs. (*f*) PCR from DNA of female and male medakas (strain i-3) with *DMRT1Y*- and *DMRT1*-specific primers. Cosmid 9 contains the autosomal *DMRT1* gene (26) and cosmid 13 contains *DMRT1Y*. (*g*) Genetic linkage map of the region flanking the male *SD*, based on meiosis in sex-reversed XY females. Numbers indicate genetic distances in centimorgans (cM). Markers *DMRT1Y* and *SDY* (locus determining the male sex phenotype) showed no recombination.

has all of the features of a functional gene and is not corrupted by mutation. BAC 15H17, which contains *DMRT1Y*, and adjacent BACs were used for fluorescence *in situ* hybridization analysis (Fig. 3) on male and female metaphases. BACs containing the marker sequences flanking the male-determining locus [SL1, Casp3B (data not shown), Casp6 (data not shown)] gave signals on both the X and Y chromosomes. Neighboring BACs that overlap with l5H17 hybridized to the Y and X chromosome as well. However, BAC 15H17 gave a strong hybridization signal only on the Y, but not the X. Weak signals were obtained with BAC 15H17 at the subtelomeric region of a chromosome pair that is equivalent to linkage group 9 of medaka. This is the location of the autosomal *DMRT* cluster.

Analysis of the contiguous sequence of three overlapping BACs (covering 380 kb, roughly equivalent to 0.74 cM; refs. 7 and 28) revealed the size and the borders of the duplicated region on the Y (Fig. 4). Downstream of the Y-specific region a gene is encoded that is the medaka orthologue of human *KIAA0032* (GenBank accession no. BAA04945). This gene (as well as the next one, *KIAA0914*, GenBank accession no. XP_003489) is also present on the X, thus defining the downstream limit of the Y-specific segment. At the upstream border of the Y-specific fragment another copy of *KIAA0032* is present. Thus, the size of the Y-specific fragment is about 260 kb. In the Y-specific region *DMRT1Y* is the only gene that is not corrupted by mutations. There are remnants of three other genes that are also found adjacent to *DMRT1* on the autosome and a fourth one from elsewhere in the genome, but they are all nonfunctional. From the BAC sequences no other functional gene is predicted by using 11 different gene or exon/intron prediction programs. However, a strikingly high number of transposons and other repetitive sequences were noticed, consistent with the expected genetic degeneration and recombinational isolation of the chromosomal region surrounding *SD* on the male-determining chromosome (29).

In adult fish *DMRT1Y* is expressed only in testes like the autosomal *DMRT1* (Fig. 5*a*). The transcript is localized in the Sertoli cells (Fig. 5*b*). During development *DMRT1Y* expression starts at the neurula stage and persists during embryogenesis and larval stages to adulthood (Fig. 5*c*). It is expressed only in male embryos. The expression of the autosomal *DMRT1* starts much later, around day 20.

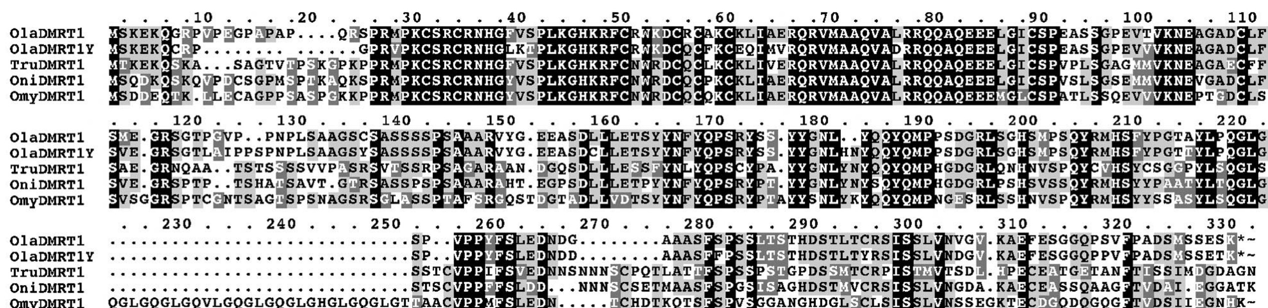

Fig. 2. Sequence comparison of the medaka *DMRT1* and *DMRT1Y* genes with the *DMRT1*s from other teleosts. Ola, *Oryzias latipes* (Medaka); Tru, *Takifugu rubripes*; Oni, *Oreochromis niloticus* (Tilapia); Omy, *Oncorhynchus mykiss* (rainbow trout).

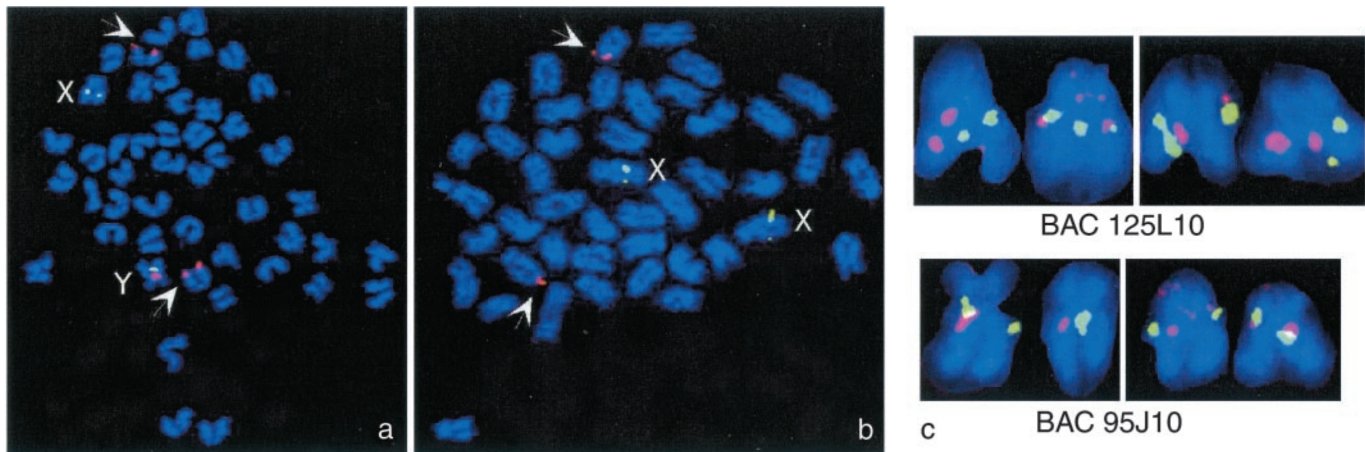

Fig. 3. Identification of the medaka Y chromosome: metaphases from male (*a*) and female (*b*) showing the hybridization signals of two BAC probes (15H17: *DMRT1Y*; 98C17: *SL1*). Note the presence of three hybridization spots for the BAC 15H17 in males as compared with the two spots in female (red signal). The additional fluorescence *in situ* hybridization signal in male is on the Y chromosome. The two relatively weak signals (arrows) in both male and female metaphase spreads represent the autosomal *DMRT1* locus (linkage group 9). The *SL1* marker containing BAC 98C17 detects both sex chromosomes (green signal). (*c*) Two highly enlarged XY chromosome pairs from two metaphases of male medaka showing hybridization to both sex chromosomes of BACs, which contain sequences flanking the Y-specific region on either side (95J10 and 125L10) (red signals). The *SL1* marker containing BAC98C17 (green signals) was used to identify the sex chromosomes.

To analyze whether *DMRT1Y* is an upstream sex-determining gene or a more downstream sex-differentiation gene we analyzed the expression of *DMRT1Y* in XY sex-reversed females. During the estrogen treatment and after hatching the expression of *DMRT1Y* was not affected. The transcript was even detected in the ovary of the adult XY females at levels comparable to testes (Fig. 5*d*).

Discussion

In medaka the pseudoautosomal region of the sex chromosomes is very large. In fact, sex chromosomal crossing-over occurs over the entire length of the chromosome, with the possible exception of the region immediately adjacent to *SD* on the Y chromosome where an extremely high density of markers could not be ordered because of lack of detectable recombination (7, 8, 24). Linkage group 1, which contains *SD*, is the largest one and is equivalent to one pair of homomorphic chromosomes of the largest group. All markers mapped on linkage group 1 (the sex chromosomes) are shared between the X and Y chromosomes, whereas the markers generated from the Y-specific fragment are present only on the Y chromosome (data not shown). This finding indicates that the Y-specific region may be very small. Together with the fact that *DMRT1Y* is highly similar to its ancestor *DMRT1*, which points to a recent rather than to an ancient duplication event, it appears that the sex chromosomes of medaka are at an early stage of evolution.

Gene hierarchy studies in the worm and the fly revealed that the corresponding *DMRT1* homologues *mab-3* and *DSX* are placed at the bottom of the sex-determination cascade. From an evolutionary point of view it appears that the genes at the top of the hierarchy, which rule the mechanism of sex determination, have become involved in this process only relatively recently. However, at least some of the downstream genes, like *DMRT1*, are conserved with respect to sequence and function (2, 30, 31). Based on findings that *DMRT1* is Z-linked in chicken it has been suggested that *DMRT1* in birds has been recruited as an upstream regulatory sex-determining factor (16, 32). In chicken *DMRT1* expression precedes expression of all other potential *SD* genes, is stronger in male than in female gonads, and is evident before the sex differentiation of the gonad anlage starts (18, 19).

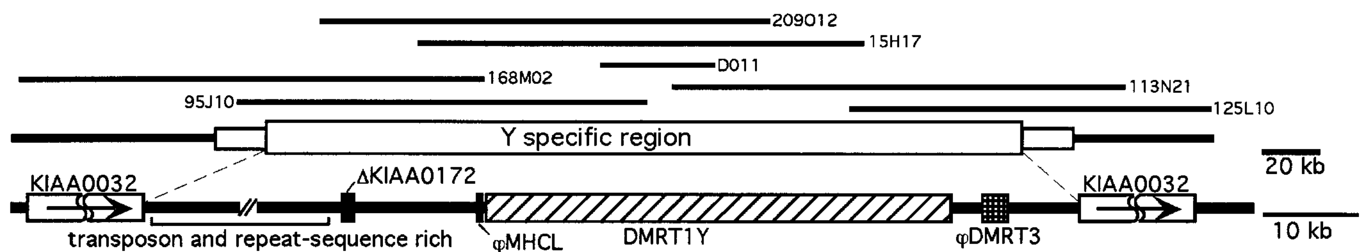

Fig. 4. Schematic representation of the *DMRT1Y*-containing region. Lines above show the analyzed BAC and cosmid clones. Genes and sequences with predicted homology to known genes are shown as boxes: striped, *DMRT1Y*; hatched, *φDMRT3*; light gray, *KIAA0032*; black, Δ*KIAA0172* and *φMHCL*. The region upstream of Δ*KIAA0172* contains only repetitive DNA and sequences with similarity to transposable elements of various organisms. Two genes upstream of *DMRT1*, a myosin heavy chain like gene (*MHCL*) and an ankyrin repeat containing gene (orthologous to human *KIAA0172*), are part of the duplicated fragment from linkage group 9 on the Y. The duplicated Y-chromosomal copy of *MHCL* is, however, destroyed by insertion of a poseidon element, a non-long terminal repeat retroposon (40), in Southern medaka and additionally a *TX-1*-related transposon in Northern medaka. The Y-chromosomal version of *KIAA0172* is corrupted by a deletion that takes out two exons. In addition, the 5′ part of the gene is missing, indicating the border of the duplicated fragment. In intron 4 of *DMRT1Y* an insertion has occurred. This insertion contains a duplicated copy of the putative medaka homologue of the human brain and testes antigen gene *MAP1* that is located on medaka linkage group 19 (M.K., H. Mitani, A.S., and M.S., unpublished work). The Y-chromosomal copy of *MAP1*, however, has a frameshift mutation that leads to a prematurely terminated protein. Downstream of the Y-chromosomal *DMRT1* a copy of *DMRT3* is found. But its coding sequence is lacking the ATG start codon and it has several frameshifts. *DMRT2*, which is the next gene following *DMRT3* on the autosomal cluster, is not found in the Y-specific region.

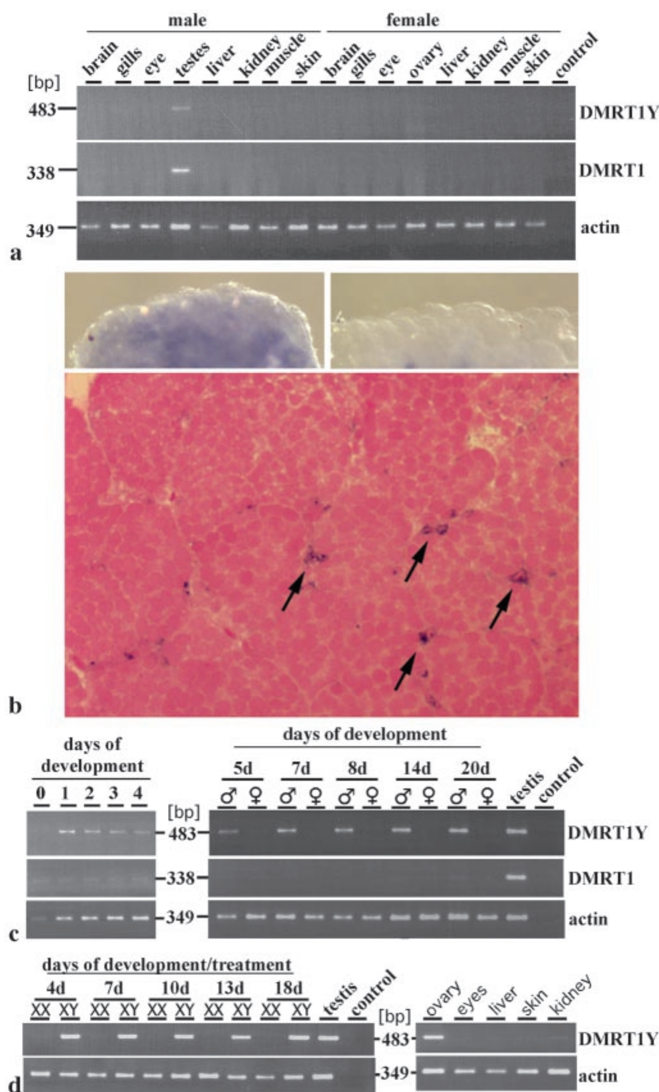

Fig. 5. Expression of *DMRT1Y* and *DMRT1*. (*a*) Reverse transcription–PCR with *DMRT1Y*-specific primers of total RNA from organs of adult male and female medaka. Actin expression was determined for calibration. (*b*) Whole-mount RNA *in situ* hybridization in adult testes with *DMRT1Y* antisense probe (*Upper Left*), *DMRT1* sense control (*Upper Right*), and section of testes showing staining in Sertoli cells (arrows, *Lower*). (*c*) Reverse transcription–PCR of medaka embryos and hatchlings with the same primers as in *a*. (*Left*) Analysis from Carbio strain samples. (*Right*) Analysis from Quart strain samples. (*d*) *DMRT1Y* expression in 17β estradiol-treated Quart embryos and sex-reversed adult XY females.

However, the exact map position of *DMRT1* on chicken Z in relation to the male *SD* locus remains to be determined. In the temperature-dependent sex determination of alligators and turtles, *DMRT1* exhibits the expected expression pattern for a sex-determining gene (19, 33). In rainbow trout it has been shown that *DMRT1* is expressed in the developing male gonad before morphological differentiation (15). In the mouse gonad specific expression of *DMRT1* is detected at embryonic day 9.5, whereas *Sry* expression begins around embryonic day 10.5 (18). Also in humans the simultaneous onset of *DMRT1* and *Sry* expression suggested a role of *DMRT1* in early events of sex determination (34).

What makes *DMRT1Y* in medaka a reasonable candidate for a sex-determination gene? The usual experimental tools to confirm a candidate gene cannot be applied here. In medaka, like in many other fish, sex can be experimentally reversed by steroid treatment or interfering with the activity of sex-differentiation genes. This means that transgenic expression of genes that act downstream in the sex-determination cascade or even sex-differentiation genes will lead to full sex reversal as well. Blocking *DMRT1Y* activity by antisense oligonucleotides cannot be used either, because the gonad is the last organ system to develop in medaka (35), and even morpholinos are not stable enough to be effective (data not shown). Matsuda *et al.* (27) found that a point mutation in the *DMY* (*DMRT1Y*) leads to XY male to female sex reversal. This finding shows convincingly that this gene is necessary for male sexual development. Also in humans the loss of *DMRT1* is connected to XY sex reversals (11–13, 36). *DMRT1* knockout mice revealed that the gene is essential for testis development (37). Thus, the data are not informative whether the gene in medaka has a different (more upstream) or a similar function (more downstream) like that in mammals. Of course, the linkage of *DMRT1Y* to the male *SD* (independently seen in this and another study, ref. 27) is pretty suggestive for its function as the primary sex-determining gene. As genetic mapping has only a certain resolution, a candidate gene may be located very close but not exactly at the locus encoding the phenotype in question. Thus further cumulative evidence should be helpful.

First, our finding of expression of *DMRT1Y* in XY sex-reversed females indicates that *DMRT1Y* is located upstream in the genetic hierarchy and is not one of the male sex-differentiation genes that have to be suppressed by sex-reverting hormone treatment. This experiment, however, does not rule out a difference in activity of *DMRT1Y* in sex-reversed animals, for instance if *DMRT1Y* is regulated posttranscriptionally. Second, the expression pattern of *DMRT1Y* is consistent with a sex-determination function. It is expressed early and exclusively in the male embryo. Most importantly, *DMRT1Y* in medaka is the only functional gene found in the Y chromosome-specific segment at the sex-determining region. The fact that androgen treatment during the sensitive period produces functional XX sex-reversed males that are fully fertile (6) excludes that spermatogenesis or other sex-differentiation genes are present on the Y, unlike the situation in mammals (38, 39). Hence, *DMRT1Y* is not involved in these processes but should have a function as a male sex-determination gene.

Experiments on the biochemical function of *DMRT1Y* are needed to understand how this gene functions in the male sex-determination process. The origin of *DMRT1Y* from an autosomal gene raises the question of what role gene duplication of sex determination and differentiation genes may play in the evolution of sex-determination systems. Such studies in suitable fish models might also help to contribute to our understanding of the function of *DMRTs* in mammals.

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1. Just, W., Rau, W., Vogel, W., Akhverdian, M., Fredga, K., Graves, J. A. & Lyapunova, E. (1995) *Nat. Genet.* **11,** 117–118.
2. Marin, I. & Baker, B. S. (1998) *Science* **281,** 1990–1994.
3. Baroiller, J. F., Guigen, Y. & Fostier, A. (1999) *Cell. Mol. Life Sci.* **55,** 910–931.

4. Devlin, R. H. & Nagahama, Y. (2002) *Aquaculture* **208,** 191–364.
5. Wittbrodt, J., Shima, A. & Schartl, M. (2002) *Nat. Rev. Genet.* **3,** 53–64.
6. Yamamoto, T. (1975) *Medaka (Killifish) Biology and Strains* (Keigaku, Tokyo).
7. Naruse, K., Fukamachi, S., Mitani, H., Kondo, M., Matsuoka, T., Kondo, S., Hanamura, N., Morita, Y., Hasegawa, K., Nishigaki, R., *et al*. (2000) *Genetics* **154,** 1773–1784.
8. Kondo, M., Nagao, E., Mitani, H. & Shima, A. (2001) *Genet. Res.* **78,** 23–30.
9. Wada, H., Shimada, A., Fukamachi, S., Naruse, K. & Shima, A. (1998) *Zool. Sci.* **15,** 123–126.
10. Zhu, L., Wilken, J., Phillips, N. B., Narendra, U., Chan, G., Stratton, S. M., Kent, S. B. & Weiss, M. A. (2000) *Genes Dev.* **14,** 1750–1764.
11. Raymond, C. S., Parker, E. D., Kettlewell, J. R., Brown, L. G., Page, D. C., Kusz, K., Jaruzelska, J., Reinberg, Y., Flejter, W. L., Bardwell, V. J., *et al*. (1999) *Hum. Mol. Genet.* **8,** 989–996.
12. Calvari, V., Bertini, V., De Grandi, A., Peverali, G., Zuffardi, O., Ferguson-Smith, M., Knudtzon, J., Camerino, G., Borsani, G. & Guioli, S. (2000) *Genomics* **65,** 203–212.
13. Ottolenghi, C. & McElreavey, K. (2000) *Mol. Genet. Metab.* **71,** 397–404.
14. Guan, G., Kobayashi, T. & Nagahama, Y. (2000) *Biochem. Biophys. Res. Commun.* **272,** 662–666.
15. Marchand, O., Govoroun, M., D'Cotta, H., McMeel, O., Lareyre, J., Bernot, A., Laudet, V. & Guiguen, Y. (2000) *Biochim. Biophys. Acta* **1493,** 180–187.
16. Nanda, I., Shan, Z., Schartl, M., Burt, D. W., Koehler, M., Nothwang, H., Grutzner, F., Paton, I. R., Windsor, D., Dunn, I., *et al*. (1999) *Nat. Genet.* **21,** 258–259.
17. Raymond, C. S., Shamu, C. E., Shen, M. M., Seifert, K. J., Hirsch, B., Hodgkin, J. & Zarkower, D. (1998) *Nature (London)* **391,** 691–695.
18. Raymond, C. S., Kettlewell, J. R., Hirsch, B., Bardwell, V. J. & Zarkower, D. (1999) *Dev. Biol.* **215,** 208–220.
19. Smith, C. A., McClive, P. J., Western, P. S., Reed, K. J. & Sinclair, A. H. (1999) *Nature (London)* **402,** 601–602.
20. Kondo, M., Froschauer, A., Kitano, A., Nanda, I., Hornung, U., Volff, J. N., Asakawa, S., Mitani, H., Naruse, K., Tanaka, M., *et al*. (2002) *Gene*, in press.
21. Iwamatsu, T. (1994) *Zool. Sci. (Tokyo)* **11,** 825–839.
22. Iwamatsu, T. (1999) *J. Exp. Zool.* **283,** 210–214.
23. Hauptmann, G. & Gerster, T. (1994) *Trends Genet.* **10,** 266.
24. Matsuda, M., Sotoyama, S., Hamaguchi, S. & Sakaizumi, M. (1999) *Genet. Res.* **73,** 225–231.
25. Lander, E. S., Green, P., Abrahamson, J., Barlow, A., Daly, M. J., Lincoln, S. E. & Newburg, L. (1987) *Genomics* **1,** 174–181.
26. Brunner, B., Hornung, U., Shan, Z., Nanda, I., Kondo, M., Zend-Ajusch, E., Haaf, T., Ropers, H. H., Shima, A., Schmid, M., *et al*. (2001) *Genomics* **77,** 8–17.
27. Matsuda, M., Nagahama, Y., Shinomiya, A., Sato, T., Matsuda, C., Kobayashi, T., Morrey, C. E., Shibata, N., Asakawa, S., Shimizu, N., *et al*. (2002) *Nature (London)* **417,** 559–563.
28. Ohtsuka, M., Makino, S., Yoda, K., Wada, H., Naruse, K., Mitani, H., Shima, A., Ozato, K., Kimura, M. & Inoko, H. (1999) *Genome Res.* **9,** 1277–1287.
29. Charlesworth, B. & Charlesworth, D. (2000) *Philos. Trans. R. Soc. London B* **355,** 1563–1572.
30. Wilkins, A. S. (1995) *BioEssays* **17,** 71–77.
31. Zarkower, D. (2001) *Nat. Rev. Genet.* **2,** 175–185.
32. Graves, J. A. & Shetty, S. (2001) *J. Exp. Zool.* **290,** 449–462.
33. Kettlewell, J. R., Raymond, C. S. & Zarkower, D. (2000) *Genesis* **26,** 174–178.
34. Moniot, B., Berta, P., Scherer, G., Sudbeck, P. & Poulat, F. (2000) *Mech. Dev.* **91,** 323–325.
35. Hamaguchi, S. (1992) *Fish Biol. J. Medaka* **4,** 11–17.
36. Ottolenghi, C., Veitia, R., Quintana-Murci, L., Torchard, D., Scapoli, L., Souleyreau-Therville, N., Beckmann, J., Fellous, M. & McElreavey, K. (2000) *Genomics* **64,** 170–178.
37. Raymond, C. S., Murphy, M. W., O'Sullivan, M. G., Bardwell, V. J. & Zarkower, D. (2000) *Genes Dev.* **14,** 2587–2595.
38. Lahn, B. T. & Page, D. C. (1997) *Science* **278,** 675–680.
39. Lahn, B. T., Pearson, N. M. & Jegalian, K. (2001) *Nat. Rev. Genet.* **2,** 207–216.
40. Volff, J. N., Hornung, U. & Schartl, M. (2001) *Mol. Genet. Genomics* **265,** 711–720.