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Zebrafish Androgen Receptor: Isolation, Molecular, and Biochemical Characterization[1]

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ABSTRACT

Androgens play an important role in male sexual differentiation and development. They exert their function by binding to and activating the androgen receptor (Ar), a member of the steroid hormone receptor superfamily. Here, we report on the isolation and characterization of zebrafish Ar. The complete transcript of zebrafish *ar* is 5.3 kb long encoding a putative polypeptide of 868 amino acids. Our experimental and bioinformatic analysis has found a single *ar* locus in zebrafish. Phylogenetic analysis using the ligand-binding domain showed that the zebrafish Ar clustered with its cyprinid orthologs to form a separate group, which was closer to the beta clade than to the alpha clade. Tissue-specific expression analysis revealed that the *ar* mRNA was expressed ubiquitously in all adult tissues tested, with sexually dimorphic expression in the gonad and muscle. While the *ar* transcript was maternally deposited into the embryo, signs of zygotic expression could be detected as early as 24 h after fertilization, and the expression level increased substantially afterwards. When analyzed during gonad development, the expression level of *ar* mRNA at 4 wk after fertilization was similar in both developing gonads but later became higher in the transforming testis, suggesting a potential role during male gonad differentiation. We also combined theoretical modeling with in vitro experiments to show that the zebrafish Ar is preferentially activated by 11-ketotestosterone.

11-ketotestosterone, androgen receptor, gonad differentiation, steroid hormones, testis

INTRODUCTION

Androgens direct male-specific aspects of development, physiology, reproduction, and behavior in vertebrates [1–3]. They exert their function by binding to and activating the androgen receptor (Ar), a member of the steroid hormone receptor superfamily [4]. Mutations in the *ar* gene [5–7] or in genes coding for androgen synthesizing enzymes [8, 9] result in abnormalities in male sexual differentiation and development. Structurally and functionally, all the nuclear receptors, including Ar, consist of three modular domains: N-terminal hypervariable transcriptional activation domain (TAD), a central highly conserved DNA-binding domain (DBD), and C-terminal ligand-binding domain (LBD) [3, 4, 10].

The *ar* genes appear to be specific to jawed vertebrates, as no such gene has been reported from *Agnatha,* and extensive attempts with arge combinations of degenerate *ar* primers failed to amplify a product from the lamprey [11]. The ancestral *ar* is thought to have been generated from an ancestral progesterone receptor gene as the most recent member of the nuclear receptor superfamily [11], presumably by the second whole genome duplication (2R) that happened during the evolution from the *Urochordates* to the last common ancestor of fishes and land vertebrates [12–14]. (Based on the analysis on hagfish Hox genes [15], we assume that the first genome duplication [1R] happened before the *Agnatha-Gnathostomata* split.)

Zebrafish (*Danio rerio, Cyprinidae*) serves as an important vertebrate model for studying development, genetics, and diseases [16–19]. Although most aspects of zebrafish biology have been studied extensively, our knowledge about the genetic regulation of zebrafish reproduction is limited. Histological studies have shown that juvenile zebrafish first develop an undifferentiated ovarylike structure, some of which later on transforms into a testis, whereas the rest matures into an ovary [20, 21]. Although the gonad differentiation is expected to be under genetic control, the final differentiation of the gonad in fish also depends on endocrine signals (i.e., estrogens and androgens [22, 23]). Like in many other teleost species, gonad differentiation in zebrafish can be affected by treating larvae/juveniles with sex steroids during early gonad development [24, 25]; however, the consequences of such hormonal effects at the molecular level have not been described in detail.

The major male-specific androgen in teleost fish is 11-ketotestosterone (KT). Several studies have shown increased levels of serum KT during natural sex change (ovary to testis) in several protogynous hermaphrodite fish species (e.g., *Epinephelus merra* [26] and *Thalassoma dupperrey* [27]). Moreover, exogenous treatment with KT induces female-to-male sex change [28]. In case of protogynous hermaphrodite honeycomb grouper, it has been suggested that KT might play a critical role during testicular differentiation [29]. On the other hand, the role of KT in gonad development and differentiation has not been studied in detail.

Although the *ar* gene has been isolated from a number of teleosts [30–34], including two cyprinids [35–37], the zebrafish ortholog has not yet been identified. In the present study, we cloned and characterized the zebrafish *ar* cDNA containing the

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full-length open reading frame together with its genomic locus. We also combined theoretical modeling with in vitro experiments to analyze the activation of the zebrafish Ar by various steroid hormones.

MATERIALS AND METHODS

Fish

Zebrafish individuals from the Toh and AB strains were raised, maintained, and crossed according to the standard protocol. Fish were reared in AHAB recirculation systems (Aquatic Habitats) at ambient temperature (26°C–28°C). The age of embryos is indicated as hours postfertilization (hpf), and the age of larvae as days postfertilization (dpf) or weeks postfertilization (wpf). All experimental procedures involving treatment or killing of live zebrafish were reviewed and approved by the Institutional Animal Care and Use Committee of Temasek Life Sciences Laboratory as well as the Agri-Food and Veterinary Authority of Singapore. They were performed according to the guidelines set by Responsible Use and Care of Laboratory Animals course.

RNA Isolation and cDNA Synthesis

To determine the expression of zebrafish *ar* in adult organs (gonad, brain, kidney, liver, skin, muscle, and eyes) in both sexes, RNA samples were collected from three adult male and three adult female individuals. RNA samples from different embryonic or larval stages (0 hpf, midblastula transition, 50% epiboly, 5 somite, 24 hpf, 48 hpf, 72 hpf, 7 dpf, and 14 dpf) were isolated from at least 15 individuals per stage. Gonadal samples from various stages (4–7 wpf) of differentiation were collected from four individuals transgenic to *vas::egfp* construct [38] per stage. Individuals were sorted into developing females and transforming males, as described previously [39]. Expression analyses were performed on pooled RNA samples.

Total RNA used in this study was extracted using Trizol-LS reagent (Life Technologies) according to the manufacturer's instructions. The precipitated RNA was dissolved in RNase-free water and immediately stored at −80°C. Concentration of total RNA was quantified by measuring the absorbance at the wavelength of 260 nm using Nanodrop (Nanodrop Technologies Inc.). First-strand cDNA was synthesized under standard conditions with the Superscript First-strand Synthesis System (Invitrogen) using oligo$(dT)_{15}$ primer (Roche).

Isolation, Cloning, and Sequencing of Zebrafish ar cDNAs

The cDNA of goldfish *ar* (AAM09278) was used to search for zebrafish expressed sequence tags (ESTs) in GenBank. Following clustering by the Sequencher software (v4.1.4), the zebrafish ESTs formed a 3.2-kb consensus sequence. To confirm its authenticity, RT-PCR was performed by primers AR_FL_F and _R binding to the end of the consensus sequence (see Supplementary Table 1, available at www.biolreprod.org, for detailed list of all primers used in the study) and using adult testis cDNA as a template. Extend Long Template PCR system (Roche) was used for PCR amplification. The cycling conditions were: initial denaturation step 95°C for 1 min, then 95°C for 30 sec, 58°C for 30 sec, 68°C for 3.5 min; 32 cycles.

A lambda-ZAP ovarian cDNA library was prepared from adult zebrafish and screened using digoxigenin-labeled, three-spined stickleback antisense *ar* RNA probe using the DIG RNA Labeling Kit (Roche) as previously described [40]. Several positive clones were obtained, and four were selected for further screening. Following three rounds of screening, three clones remaining were sequenced. All three clones were found to contain the same 3590-bp sequence spanning the 3′ end of the zebrafish *ar*. Following the assembly of the sequences obtained from RT-PCR and library screening we obtained a 5.3-kb zebrafish *ar* sequence spanning the entire coding region and the 3′ UTR. To determine the full-length mRNA of zebrafish *ar*, 5′- and 3′-RACE were performed using an RLM-RACE kit (Ambion) on total RNA isolated from adult zebrafish testis. The final products of the 5′- and 3′-RACE were cloned into pGEM-T Easy vector (Promega) for sequencing.

For transactivation assay, the full-length coding region of zebrafish *ar* was cloned into pCMVT_NT vector using VENT DNA polymerase (New England Biolabs) PCR system (97°C, 3 min × 1; [95°C, 30 sec; 60°C, 30 sec; 72°C, 3 min] × 35; 72°C, 10 min × 1).

Radiation Hybrid Mapping of the Zebrafish ar Locus

Radiation hybrid mapping with the LN54 radiation hybrid panel was performed as previously described [41, 42]. Polymerase chain reaction amplification was performed in duplicate for each of the 96-hybrid DNA templates using two sets of gene-specific primers. GoTaq Flexi DNA Polymerase (Promega) was used for PCR. Cycling conditions were as follows: initial denaturation step 95°C for 1 min, then 95°C for 30 sec, 58°C for 30 sec, 72°C for 1 min; 37 cycles. Polymerase chain reaction results were analyzed, and a map position was obtained using the mapping program found on the web page of the Dawid lab [43].

Southern Blot

Southern blot analyses were performed using 10 μg genomic DNA digested with *Ase*I, *Afl*II, and *Pst*I restriction enzymes at 37°C for 12 h. After electrophoretic separation, genomic DNA fragments were transferred to a Hybond N^+ nitrocellulose membrane (Amersham) under alkaline conditions [44]. Two PCR-generated, DIG-labeled DNA probes were synthesized using PCR DIG Probe Synthesis Kit (Roche). One probe was spanning over the 5′ UTR (1.8 kb), and other was from the 3′ UTR (1.7 kb). The hybridization was done according to the manufacturer's recommendation, and signals were visualized with CDP-Star detection reagent (Roche).

Phylogenetic Analysis of Teleost Ar Proteins

The available full-length amino acid sequence of 22 Ar proteins from 17 vertebrate species was retrieved from GenBank (see Supplementary Table 2, available online at www.biolreprod.org, for the full list of species and sequences). For those species with two apparent paralogs, the sequences of the two Ar proteins were compared by pairwise clustering using ClustalW (http://www.ebi.ac.uk/clustalw/; version 1.83; [45]) to ensure that they were not variants transcribed from the same locus. Besides the zebrafish Ar (EF42791), the protein sequences of the following four vertebrate androgen receptors were added: African clawed frog (GenBank ID: AAC97386), chicken (AB193190), mouse (AAA37234), and human (AAA51729). The sequences were then aligned by ClustalW [45]. Estimation of molecular phylogeny was carried out by the neighbor-joining method with Poisson correction model as implemented in MEGA (version 3.1) [46] using the Japanese eel progesterone receptor (Pr; GenBank: AB032075) as a root. Confidence in the phylogeny was assessed by bootstrap resampling of the data (1000).

Real-Time PCR Analysis

Real-time quantitative PCR was performed with the iCyler iQ Real-time Detection system using SYBR Green chemistry (Bio-Rad). The quantification of the transcripts was performed using a standard curve with 10-fold serial of dilution of testis cDNA. The 20-μl PCR mixture contained 10 μl of 2× iQ SYBR Green Supermix, 0.5 μl (10 mM) of each primer, and 1 μl cDNA. The following conditions were used: denaturation at 95°C for 3 min, 40 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 20 sec, followed by melt curve analysis (95°C for 2 min and decrease by 0.1°C at every 10 sec). Melt curve analysis was conducted to ensure that only a single PCR product was amplified. Samples were assayed in triplicate, and each experiment was repeated at least twice. Control reactions (without template) were run for each sample. To minimize variation due to differences in RNA loading, each sample was normalized to the expression level of the control gene, *beta actin* (*bactin*). As the expression of *bactin* varied over developmental time, to account for differences in its expression during early stages and adult liver and muscle, the level of expression within each group of samples was corrected by previously described formula [47–49]. The formula is as follows: individual value within a group/(mean value within a group/mean value of randomly selected control group). In early stages, 0 hpf was chosen as control group, whereas testis was selected as control for adult tissue analysis.

Statistical differences in relative mRNA expression between experimental groups were assessed by Student *t*-test. All experimental data are presented as mean ± SEM. Differences were considered statistically significant at $P < 0.05$.

Cell Culture and Activation Assay

The liver epithelial ZFL cell line (American Type Culture Collection) was used to determine the zebrafish Ar activity, as described previously [34]. At 90%–95% confluence, the cells were transfected with 0.6 μg DNA/well (270 ng zebrafish *ar*, 270 ng ARE-*slp*-LUC, and 60 ng pRL [Promega]). Transfections were performed using Lipofectamin 2000 (Invitrogen) according to the manufacturer's recommendations. At 24 h after transfection, the media was replaced with serum-free media containing steroids at various concentrations. Steroids used for the exposures were androstenediol (A; Steraloids), testosterone (T; Sigma), methyltestosterone (MT; Sigma), dihydrotestosterone (DHT; Sigma), KT (Steraloids), 11β-hydroxytestosterone (OHT; Steraloids), and 17β-estradiol (E2; Sigma). Ethanol, which was used as vehicle solvent for steroids, never exceeded 0.1% of the total medium volume. The exposure lasted

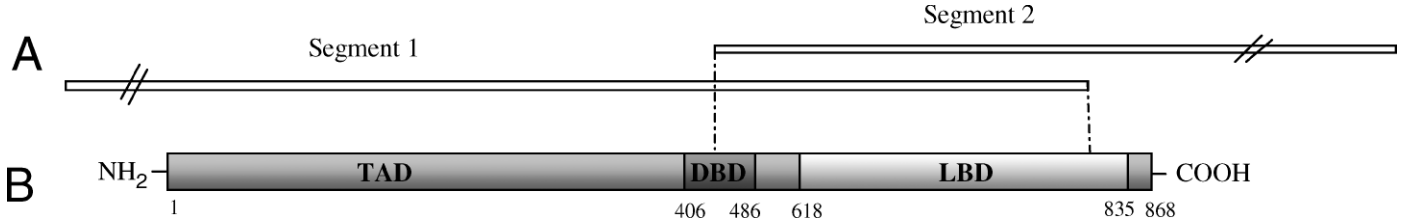

FIG. 1. The structure of the zebrafish *ar* mRNA and protein. **A**) The full-length mRNA of zebrafish *ar* was assembled from two overlapping cDNA clones. The sequence covering the 5′ end (segment 1) was obtained by RT-PCR with primers designed based on EST sequences, whereas the sequence covering the 3′ end (segment 2) was from a library screen. **B**) The zebrafish Ar protein shows conservation of the three major domains: transactivation domain (TAD), DNA-binding domain (DBD), and ligand binding domain (LBD).

for 40 h, whereafter the luciferase levels were measured in a TD 20/20 luminometer (Turner Designs) using a Dual Luciferase Assay Kit (Promega). All luciferase values received were normalized to their corresponding *Renilla* luciferase value.

Modeling the Binding Site

The interaction energies between the ligand and the Ar were computed using structures subjected to docking, relaxation, molecular dynamics (MD) simulation, and additional relaxation as previously described [50]. The crystal structure of the human Ar protein (pdb entry 1e3g) was used as a template for the modeling of the three-spined stickleback Ar and goldfish Ar, as previously described [50]. The goldfish Ar then was used as template for modeling the zebrafish Ar. The model was generated as a Cartesian Average of 10 models and energy minimized using the Amber99 forcefield. Monte-Carlo-simulated, annealing-based, flexible docking of the ligands into the Ar receptor was performed using automated docking as incorporated in molecular operating environment (MOE), with a maximum of 500 docking conformations evaluated for each system. The docked structures were evaluated based on the lowest S value (the objective function, based on evaluating the affinity ΔG scoring function; a combination of strain energy and mutual similarity score).

RESULTS

Isolation and Structural Analysis of Zebrafish ar *cDNA*

We used the goldfish *ar* cDNA (AAM09278) to acquire five zebrafish ESTs (BI1887100, AL924152, CD581002, CD604117, and CD597137) from GenBank. With these zebrafish ESTs, four more zebrafish ESTs (BI1979187, BI1979490, CD599579, and DT057202) and a partial cDNA sequence (AY510448) were retrieved from the GenBank. A hypothetical cDNA sequence of 1.5 kb in size was also obtained from the Ensemble database (v6; chromosome:Z-FISH6:9:39594802:39597140).

By clustering all of these, we ultimately generated a consensus sequence of more than 3.4 kb. RT-PCR amplification by primers designed to the end of this consensus sequence yielded a 3.2-kb product, which was subsequently cloned and sequenced.

By screening through a lambda-ZAP cDNA library generated from zebrafish ovarian tissue using the three-spined stickleback *ar* [34] as a probe, three clones were isolated. Sequencing confirmed that all three clones contained the same 3.3-kb insert. When clustered with the 3.4-kb clone described above, the two *ar* sequences overlapped and formed a consensus of 5.3 kb (Fig. 1), the ends of which were verified by 5′- and 3′-RACE using RNA isolated from adult testis as a template.

Sequence analysis of the full-length zebrafish *ar* cDNA revealed that there were two potential translation start sites (ATG) within the transcript. The sequences surrounding the first one (ACTATGG) were consistent with the Kozak rule [51] for efficient initiation of eukaryotic translation (PuNNATGPu). The 3′ UTR sequence was found to be rather long (more than 2.3 kb). Two potential polyadenylation signals (ATAAAA) were located at 456 bp and 535 bp downstream of the stop codon (TGA), respectively. Based on the above results, we concluded that the complete transcript of zebrafish *ar* is 5.3 kb long (Genbank ID: EF427915) encoding a putative polypeptide of 868 amino acids. Like other Ars, the putative amino acid sequence of the zebrafish Ar protein could be subdivided into three domains: TAD, DBD, and LBD (Fig. 1; see also Fig. S1 for the alignment of zebrafish Ar with its orthologs from goldfish and fathead minnow).

Bioinformatic and Experimental Analysis on Mapping and Characterization of the Zebrafish ar *Locus*

First, we used bioinformatic tools to determine the genomic location of the zebrafish *ar* locus. Neither the zebrafish genome assembly in the Ensembl database (Zv6) nor the NCBI MapView assembly based on Zv6 was able to assign the zebrafish *ar* locus to a specific position of a chromosome; rather, they indicated the putative presence of at least two loci. On the other hand, the VEGA (Vertebrate Genome Annotation) database (v25) of the Ensembl project, a central repository for frequently updated and manually annotated high-quality sequences showed a single locus for zebrafish *ar* on LG5 (18.64–18.75 Mb), which contained the full mRNA sequence and was in correct orientation.

To obtain experimental data, we tried to map the sequence onto the LN54 radiation hybrid mapping panel [41, 42]. The

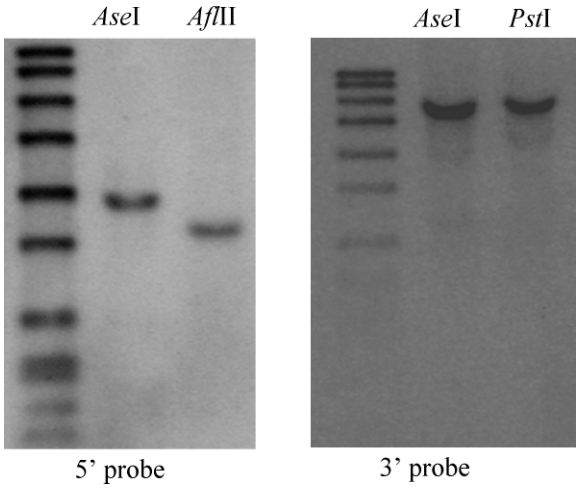

FIG. 2. Southern blot analysis of the *ar* gene in zebrafish genome. Genomic DNA digested with the indicated restriction enzymes was separated by agarose gel electrophoresis, blotted onto membrane, and hybridized with labeled *ar* fragments representing the 5′ UTR and 3′ UTR. The size standard (DIG Molecular-Weight Marker III; Roche) is shown on the left side of both panels.

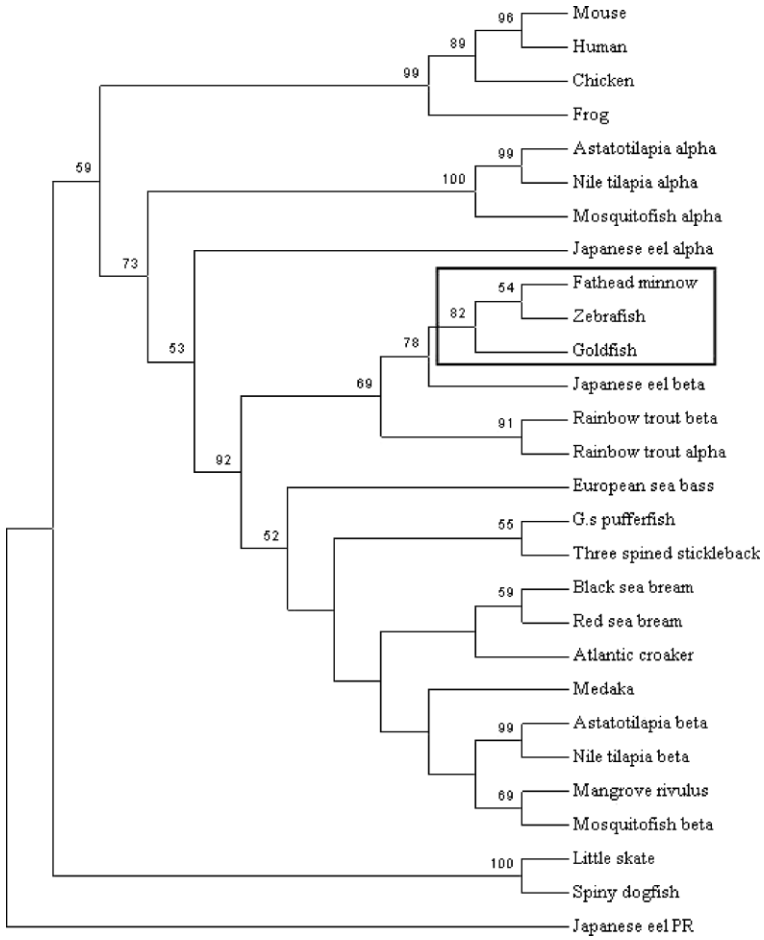

FIG. 3. Phylogenetic analysis of vertebrate Ar proteins based on the LBDs. All Ar sequences except the zebrafish were retrieved from GenBank (for accession numbers see *Materials and Methods* and Supplementary Table 2). Sequences were aligned by the CLUSTAL W software, whereas the phylogenetic tree was constructed with the neighbor-joining method with Poisson correction model as implemented in MEGA (version 3.1) and rooted by the progesterone receptor of the Japanese eel. Node labels above the branches show bootstrap values (>50% only).

results revealed two potential loci: one on LG5 (LOD score 8.5) and another on LG22 (LOD score 8.1). However, the software was unable to provide a precise position on either of the two LGs.

We then performed Southern blot analysis to find out whether a second *ar* gene exists in zebrafish genome that might be missing from the assembly. Single bands were detected using two pairs of probes binding to the 5′ and 3′ ends of the mRNA, respectively (Fig. 2). We have also looked for additional transcript(s) produced from a potential second *ar* locus: we performed RT-PCRs using different sets of primers binding to the LBD domain and producing overlapping products as well as 5′-RACE on the 3.5-kb-long partial *ar* cDNA isolated from the library. Neither of these attempts could detect a different type of zebrafish *ar* cDNA (data not shown).

At that point, the newest zebrafish genome assembly in the Ensembl database (zV7) became available, and we re-searched it with our full-length *ar* cDNA. The results have shown a

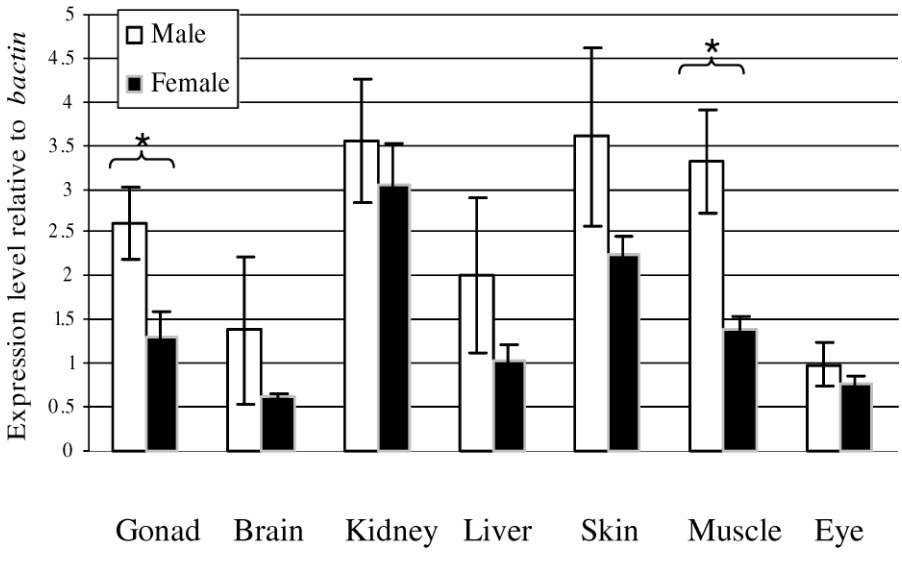

FIG. 4. The relative expression of zebrafish *ar* mRNA in adult organs. The level of expression was normalized to the expression of *bactin*. Values represent the means ± SEM (n = 3) of relative expression as determined by real-time PCR. Statistically significant differences in gene expression are denoted as follows: $*P < 0.05$ (Student *t*-test).

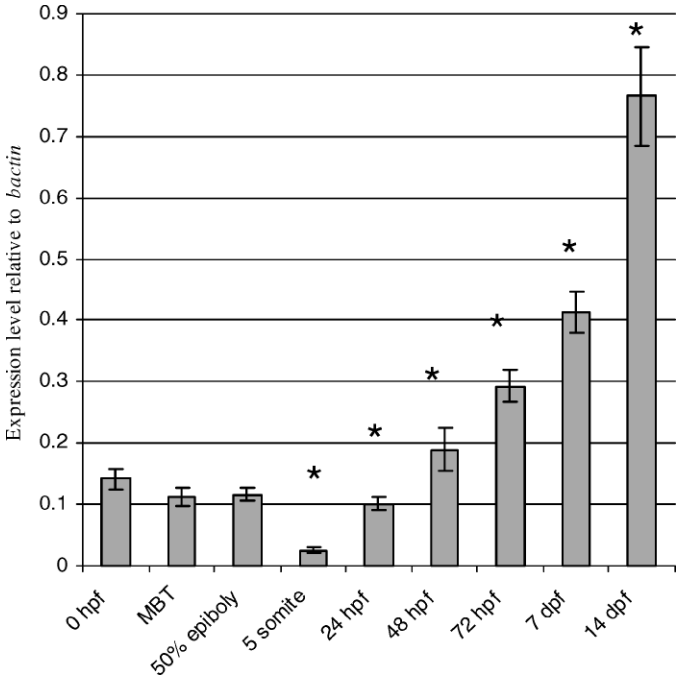

FIG. 5. The relative expression of zebrafish *ar* mRNA during early development. RNA samples were collected from whole embryos/larvae/juveniles at different stages of development. The level of expression was normalized to the expression of *bactin*. Values represent the means ± SEM (n = 15) of relative expression as determined by real-time PCR. Statistically significant differences in gene expression in comparison to the previous time point are denoted as follows: $*P < 0.05$ (Student *t*-test). MBT, midblastula transition.

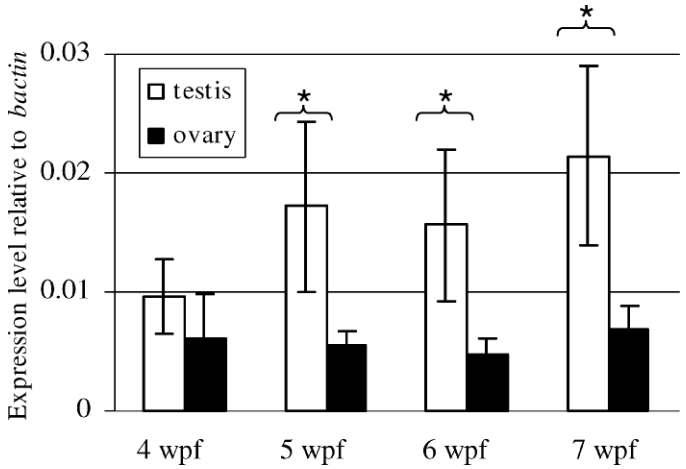

FIG. 6. The relative expression of *ar* mRNA during zebrafish gonad development and differentiation. RNA samples were isolated from developing male and female gonads collected from individuals transgenic to a reporter construct and sorted according to their enhanced green fluorescent protein level. The level of *ar* expression was normalized by the expression of *bactin*. Values represent the means ± SEM (n = 4) of relative expression as determined by real-time PCR. Statistically significant differences in gene expression are denoted as follows: $*P < 0.05$ (Student *t*-test).

single locus located at chromosome 5 (Chr:5) between 24 866 463 bp and 24 748 209 bp. Accordingly, the whole genomic locus is 118.24 kb long and contains 13 exons.

Although not all of the above results were able to narrow down the genomic location of zebrafish *ar* to a single position, the Southern hybridization, RT-PCRs, and BLAST results from the Zv7 assembly strongly indicate that there is only a single *ar* locus in the zebrafish genome, located on chromosome 5.

Sequence Homology and Phylogenetic Analysis of Vertebrate Ar

We compared the deduced amino acid sequence of the zebrafish Ar with those of other teleost Ar proteins. Comparison of three characteristic domains revealed that the TAD sequences were the most divergent among species: their sequence similarity ranged from 13% (tilapia Arα) to 78% (fathead minnow Ar; Supplementary Table 3, available online at www.biolreprod.org). In contrast, the DBD and LBD domains showed substantially higher levels of sequence similarity with those of other species: 77%–97% for DBD and 66%–96% for LBD. In the DBD of zebrafish Ar, the eight cysteine residues constituting two zinc finger motifs, the "P box" (GSCKV) and the "D box" (ASRND), were also conserved. Both of these motifs are known to be important for recognition of and binding to the hormone-responsive element of Ar target genes [52]. As expected, on the basis of taxonomic relationships, the whole zebrafish Ar protein showed the highest level of sequence similarity (84%) to the goldfish and fathead minnow Ars, whereas the lowest similarity (40%) was to tilapia Arα (Supplementary Table 3).

Phylogenetic analysis using putative amino acid sequences was carried out to determine the relative relationship of the zebrafish Ar to those with other vertebrate counterparts. Together, 22 fish Ar proteins (from 17 species; Supplementary Table 2) plus an additional four Ars from other vertebrates were retrieved from GenBank. For the five fish species with two Ar proteins (Supplementary Table 2), the two sequence pairs were compared by pairwise clustering, and all five pairs showed differences indicating the presence of two loci, as opposed to products of alternative transcription from the same locus. When the 27 Ar sequences (and the Japanese eel Pr used as a root) were analyzed by the neighbor-joining method, the results showed that zebrafish Ar clustered with the two other cyprinid orthologs into a separate subclade that is part of the Ar beta clade (Fig. 3).

The Expression Profile of ar *mRNA in Adult Zebrafish Tissues*

Real-time PCR was used to investigate the expression profile of zebrafish *ar* mRNA in seven different adult tissues from both sexes. The results showed that it was expressed ubiquitously in all tissues tested. In male gonad and muscle, the expression was significantly higher ($P = 0.04$ and $P = 0.02$, respectively) than the female counterpart (Fig. 4). Interestingly, we have not detected significant sexually dimorphic expression in the brain.

Expression of ar *mRNA During Zebrafish Development*

Using real-time PCR, we found that the *ar* transcript was maternally deposited into the embryo, and its level was maintained until 50% epiboly; then, it was drastically reduced by 5 somite stage ($P = 0.0002$; Fig. 5). Obvious signs of zygotic expression could be observed as early as 24 hpf ($P = 0.0001$). Expression levels increased substantially afterward during the course of development (Fig. 5).

Real-time PCR analysis of differentiating gonads during the 4–6 wpf period showed that the expression level of *ar* at 4 wpf

TABLE 1. Interaction energies (kcal/mol) between ligands and the zebrafish Ar vs. three-spined stickleback Ar.

	ΔE	
Hormone	Zebrafish Ar	Three-spined stickleback Ar[a]
KT	44.1	51.1
DHT	40.6	52.6
Testosterone	35.4	42.5
Androstenediol	43.4	NA
OHT	37.7	NA

[a] NA, Not available.

was similar in the developing testis and ovary, but at later stages the expression level increased significantly in the transforming testis in comparison with the ovary (5 wpf: $P = 0.01$; 6 wpf: $P = 0.0003$; 7 wpf: $P = 0.00421$; Fig. 6).

Computer Modeling of the LBD of the Zebrafish Ar Protein

Molecular modeling techniques were used to determine the ligand docking of the different compounds in the zebrafish Ar LBD. The energies of ligand-receptor interaction of the zebrafish Ar were compared to those of three-spined stickleback Ar that has previously been shown to be primarily activated by KT [34]. KT was the strongest activator of the zebrafish receptor, followed by DHT and T (Table 1). Although A and OHT do not activate the zebrafish Ar, their interaction energies were comparable to those of the activators, demonstrating that they localize to the ligand-binding pocket. Interaction energies were also determined for the KT-activated three-spined stickleback Ar, and in that species they were not found to be substantially different for KT and DHT and lower for T (Table 1).

Hormonal Treatments of Transiently Transfected ZFL Cells

ZFL cells transiently transfected with an expression vector containing zebrafish *ar* cDNA were exposed to KT, DHT, T, OHT, A, and E2 at different concentrations to evaluate their ability to activate the receptor. Of the tested steroids, KT was found to be the most potent activator of the zebrafish Ar, followed by MT, DHT, and T (Fig. 7). The fact that A was unable to activate the zebrafish Ar shows the importance of having a keto group in C3 position for an activator. Likewise, replacement of the 11-keto group, present in KT with a hydroxyl group, present in OHT, abolished receptor activation (Fig. 7).

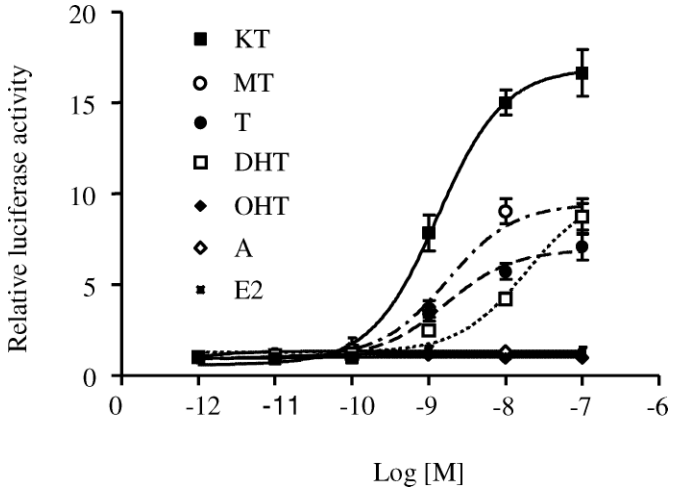

FIG. 7. Interaction of zebrafish Ar with a set of androgens and one natural estrogen. ZFL cells transfected with zebrafish *ar*, ARE-*slp*-luc, and the Renilla reporter vector were exposed to either DHT, KT, MT, A, OHT, T, or E2 as a control, respectively. All controls were transfected as the other cells but not treated with any inducer. The results are presented as mean values (n = 4) ± standard deviation. The presented results are from at least three independent measurements. Controls are arbitrarily set to one.

DISCUSSION

In the present study, we cloned the complete sequence of zebrafish *ar* cDNA and clarified its genomic location. Similarly to its orthologs, the zebrafish Ar protein contained all three domains characteristic of Ars and some other nuclear hormone receptors. We determined the expression pattern of zebrafish *ar* in adult tissues and during early development. The fact that the zebrafish Ar protein possessed *trans*-activating function when assayed by in vitro analysis proved that it is a functional receptor providing an important tool for various assays.

While all tetrapods have only a single *ar* locus, the number of loci described from fish varies between one and two. Of the 18 fish species from which an *ar* gene (or genes) has been deposited into GenBank, a single *ar* gene has been described from 13, and two genes from the remaining 5 (see Supplementary Table 2 for details). The ancestral genome of modern ray-finned fish likely contained two *ar* loci that were maintained as two active genes in some species and reduced to one in others during their evolution. Our experimental and bioinformatic analyses strongly indicate that there is only a single functional *ar* gene in zebrafish. Interestingly, the *Cyprinidae* family (to which zebrafish belong) also contains a number of "recent tetraploid" species (e.g., common carp and goldfish; for review, see Orban and Wu [53]) that are thought to have undergone yet an additional set of genome duplication (4R) in comparison to most other teleosts [54, 55]. In principle, these recent tetraploid species could have up to four *ar* loci. Therefore, it is surprising that from both cyprinid species analyzed so far (albeit not by genome sequencing or very extensive searches), namely, the "tetraploid" goldfish [36, 37] and the diploid fathead minnow [35], researchers have described only a single functional *ar* locus. Similarly, the whole *Salmonidae* family is known to be recent tetraploids [56], yet they only contain two types of Ar proteins. The possible explanation could be a loss of the second *ar* locus both from the cyprinids and salmonids before the 4R duplication and a repeated elimination of one *ar* paralog after the 4R duplication in the tetraploid cyprinids, indicating a possible selection pressure against having too many different *ar* loci in the genome.

The phylogenetic tree of vertebrate Ars contains four major branches: the first contains the *Elasmobranch* Ars (ray and shark); the second consists of those of the land vertebrates; the third, all the "single-locus Ars" together with the beta version from the "two-loci Ars," plus both Ars from rainbow trout; and the fourth, the alpha-type Ars from the two tilapias and mosquitofish. This is in good agreement with the trees produced by others [2, 35] prior to the isolation of zebrafish Ar, but it differs from that reported earlier by Olsson et al. [34]. The potential reason for the difference in the latter case could be the comparison between a rooted and unrooted tree. The fact that all single-locus Ar sequences (i.e., from species for which a second *ar* paralog has not been described) clustered together with the beta types of the two-loci Ars seems to indicate that the canonical form of teleost Ar is the beta type, whereas Ar alpha appears to be a duplicated copy generated later and lost subsequently from some of the fish species.

It would be very interesting to compare the functional characteristics of the receptor paralogs encoded by the two separate loci in the same teleost species with those from species with a single *ar* locus. Does the overlapping range of biochemical and physicochemical characteristics of the Ar paralogs reflect simple function sharing, or do they show wider steroid-binding specificities and/or new physical characteristics? Such results might provide clues to the puzzle as to why some fish species ended up with two different Ar proteins, whereas the majority appear to do well with a single one.

We used real-time PCR to quantitate the level of expression of *ar* transcript and at various developmental stages of embryo/larvae, as well as in differentiating gonads and in several adult tissues of both sexes. Our results from the embryos/larvae revealed that zebrafish *ar* was maternally deposited into the oocytes; the expression level then increased gradually from 50% epiboly onwards, indicating a potential role during late embryonic and early larval development. According to our knowledge, this is the first set of quantitative data about the temporal expression of *ar* in any teleost species during early development. It would be interesting to investigate the early function of Ar by creating temporary (Morpholino-based knockdown), permanent (mutant), or conditional loss-of-function situations.

Zebrafish were described previously as juvenile hermaphrodites [20]. Using the *vas::egfp* zebrafish transgenic line, we showed recently that a "juvenile ovary" developed in all individuals by approximately 3 wpf that can later either transform into a testis or develop further into a mature ovary [39]. The present study showed that the expression level of zebrafish *ar* mRNA was similar at the beginning of developing testis and ovary, but subsequently the expression increased significantly in the testis during the course of gonad development (Fig. 6). A similar observation regarding differential *ar* mRNA expression during gonad differentiation has been shown in the gonochoristic European sea bass [57] and the protandrous black porgy [58], albeit only with semiquantitative RT-PCR in the former case. Several previous observations from fish [59, 60] and mammals [61, 62] indicate that anti-müllerian hormone gene (*amh*) might play a potential role in testis development in protogynous teleosts through downregulation of the aromatase gene (*cyp19a1a*), a key gene for ovarian differentiation. A very recent study in fathead minnow revealed that the expression of *amh* was significantly downregulated while adult males were treated with an Ar antagonist, flutamide [63], whereas in medaka, flutamide exposure caused the formation of intersex gonad (ovo-testis) in males [64]. It would be worthwhile to investigate whether *ar* might be involved in "juvenile testis-to-ovary" transformation by directly or indirectly downregulating *amh*. Although our preliminary flutamide treatment experiment on 3-wpf larvae could not detect a significant effect on *amh* as well as on other steroidogenic genes (data not shown), additional treatments might be worthwhile to perform at later stages or with higher doses of the inhibitor to answer this question.

In adult zebrafish, *ar* mRNA was expressed ubiquitously. As expected, the testis showed a significantly higher level of *ar* expression than the ovary. For the first time in teleosts, we detected a substantially higher level of *ar* expression in male muscle compared with its female counterpart. Studies in mammals suggested the androgen involvement in the development of sexually dimorphic skeletal muscles [65]. Several clinical studies showed that testosterone administration resulted in upregulation of *ar* expression, which subsequently caused the increase of muscle protein synthesis, lean body mass, and muscle strength [66, 67].

In previous studies performed on a few fish species, semiquantitative PCR method was used to study the tissue-specific expression pattern of *ar* transcripts in teleosts. In European sea bass, a similar set of adult tissues were analyzed, and testis seemed to show the higher expression, followed by the ovary, brains, female liver, and male skin [57]. The beta form of *ar* showed restrictive tissue distribution in Japanese eel, whereas the expression of alpha was ubiquitous regardless of sexes [30]. In cases of rainbow trout, both forms of *ar* were expressed ubiquitously [33].

It has been shown previously that KT is the most active androgen in the three-spined stickleback [34]. In the present study, we show that this is also the case for the zebrafish. In the three-spined stickleback there was a discrepancy between ligand-binding affinity and receptor activation, as DHT bound stronger than KT [34]. However, when we determined the interaction energies for ligand-docking into the zebrafish Ar, we observed that KT bound with higher energy than DHT. As this was not in agreement with the ligand-binding data from three-spined stickleback, we also modeled the interaction between this receptor and KT, DHT, and T, respectively. In contrast to the three-spined stickleback Ar, which had comparable interaction energies for KT and DHT, in zebrafish Ar, KT had higher interaction energy than DHT. Comparison of the relative efficiency of zebrafish and three-spined stickleback Ar activation by different androgens shows that DHT is actually a weaker agonist in zebrafish compared with KT than it is in three-spined stickleback Ar [34]. Thus, it appears that the interaction energy correlates better with receptor activation than it does with the ligand-binding data. The data from ligand-dependent activation of the zebrafish Ar also show that the receptor is activated by androgens and that the keto groups at position C3 or at position C11 can not be replaced with hydroxyl groups without abolishing receptor activation.

NOTE ADDED IN PROOF

While this manuscript was in press, Bryant and colleagues [68] published biochemical data about a receptor capable of binding androstenedione (but not testosterone) from sea lamprey. It remains to be seen, whether this receptor is related to Ar or not.

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