There has been general acceptance that only one type of androgen receptor (AR) exists in an individual. This contrasts with other members of the nuclear receptor superfamily where multiple forms have been reported (e.g. estrogen receptor α/β, thyroid hormone receptor α/β, etc.). We have previously identified 11-ketotestosterone (a potent androgen in teleosts) as the spermatogenesis-inducing hormone of the Japanese eel and have cloned its receptor (eAR1) cDNA from eel testis. Here we report on the cloning of a cDNA encoding a second type of AR (eAR2) from the eel testis and the functional characterization of the encoded protein. This cDNA contains a complete open reading frame encoding 797 amino acid residues. The amino acid sequence of eAR2 shows high homology with other ARs, including eAR1, in the DNA-binding (98–88%) and ligand-binding (59–85%) domains, whereas the other domains show low homology (<35%).

In transient transfection assays of mammalian cells, the eAR2 protein displayed androgen-dependent activation of transcription from the androgen-responsive murine mammary tumor virus promoter. Tissue distribution of its mRNA was different from that of eAR1. We conclude that eAR2 is a novel AR in the eel, which we suggest should be named eel ARβ to distinguish it from eAR1 (eARα).

Since the initial cDNA cloning of nuclear steroid hormone receptors, the superfamily of nuclear receptors has rapidly grown. Nuclear receptors possess highly conserved DNA-binding domains and moderately conserved ligand-binding domains (LBDs)1 (1). It has been shown that members of this superfamily, such as estrogen receptor (ER), retinoic acid receptor, retinoid X receptor, and thyroid hormone receptor, have multiple subtypes and isoforms (1, 2). In the case of the ER, two subtypes (ERα and ERβ) exist, and ERβ has several isoforms designated ERβ1–5 (3) or ERβcx (4). Two isoforms of cDNA encoding rainbow trout AR (designated ARα and ARβ) (5) have been reported and are probably derived from salmonid tetraploidy. A novel subtype of the eel AR may exist because a cDNA encoding part of another AR was isolated during cloning of eel AR (eAR1) from testis (6).

In vertebrates, differentiation of the masculine phenotype is directed by secretion of steroid hormones, usually androgen (7). Recent studies in our laboratory, using a teleost, the Japanese eel (Anguilla japonica), have identified 11-ketotestosterone (11KT), a major androgen in some fish, as the spermatogenesis-inducing hormone (8–10). 11KT can induce the entire process of spermatogenesis from spermatogonial proliferation to sperm formation in both cultured testicular fragments (9) and cultured germ and somatic cells (10) from immature eels. 11KT activates Sertoli cells to stimulate production of activin B, which has been identified as the spermatogonial proliferator in the Japanese eel (11). However, the mechanisms underlying the regulation of eel spermatogenesis by 11KT are not fully understood. To understand the mechanism of androgen action and AR regulation of gene transcription, it is important to isolate and characterize subtypes of the AR.

Here, we describe the isolation and characterization of an eel cDNA containing an entire AR subtype coding region. The transactivation function of this AR was determined by expressing the cDNA in transiently transfected human kidney 293 cells. The AR mRNA levels in various tissues from eels were measured by reverse transcription-polymerase chain reaction (PCR).

**EXPERIMENTAL PROCEDURES**

**Animals and Treatment**—Cultivated Japanese eel males (150–200 g in body weight) were purchased from a commercial eel supplier. They were kept in recirculating freshwater tanks with a capacity of 500 liters at 20 °C. Fish were not fed throughout the experimental period.

**Isolation of cDNA Clones**—A cDNA fragment of an AR subtype (eAR2), whose sequence partially differed from eAR1, was isolated after a testis cDNA library from Japanese eels killed 1 and 3 days after human chorionic gonadotropin injection was screened using a PCR amplified product as a probe (6). Because the 3′ terminus of the clone was truncated, 3′-rapid amplification of cDNA ends (3′-RACE) was performed using a Marathon cDNA amplification kit (CLONTECH). The insert cDNA was nest-deleted at both ends using EcoRI/Mung bean nuclease. Sequencing was performed using the ABI PRISM Dye Terminator cycle sequencing kit (Applied Biosystems).

**Construction of Plasmid Vectors**—pcDNA-eAR2 was constructed by PCR amplification of the entire protein coding region (amino acids 1–798) of the eAR2, using primers that introduced an EcoRI site and in-frame Kozak sequence (12) at the 5′ end and an EcoRI site at the 3′ end. The EcoRI fragment was inserted at the EcoRI site of pcDNA3.1 (+) (Invitrogen), pcDNA-eARrev, which had the pcDNA-eAR2 inserted in the reverse direction, was also prepared as a negative control. pcDNA-eAR1 has been described elsewhere as pcDNA-eAR (6). An androgen-regulated reporter vector, named pGV2-MMTV, was also constructed as described previously (6).

**Transactivation Assays**—Human embryonic kidney 293 cells were seeded in 24-well plates at 5 × 104 cells/well in phenol-red free Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% charcoal-stripped fetal calf serum, and 50 μg/ml kanamycin. After 24 h, the cells were transfected with 250 ng of pGV2-MMTV, 250 ng of pRL-TK (for internal control to normalize for variation in transfection efficiency; contains the Renilla reniformis luciferase gene with the herpes simplex virus thymidine kinase promoter) and pcDNA-eAR2, pcDNA-eAR1, pcDNA-eARrev, or pcDNA-eAR. pcDNA-eAR is used as an internal control to normalize for variation in transfection efficiency; contains the Renilla reniformis luciferase gene with the herpes simplex virus thymidine kinase promoter.
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virus thymidine kinase promoter; Toyo Ink), and 50 ng of pcDNA-eAR2, pcDNA-eAR1, or pcDNA-eARrev, using Tfx™-20 reagent (Promega) according to the manufacturer's instructions. After 1 h of incubation, 1 ml of fresh medium containing 10% fetal calf serum plus steroid hormones was applied to the cells. After 48 h, the cells were collected, and the luciferase activities of the cells were measured by a chemilumines-

FIG. 1. Nucleotide sequence Japanese eel AR2 and the deduced amino acid sequence. The numbers on the right refer to the position of the nucleotides and the amino acids.

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cence assay using PicaGene Dual™ SeaPansy Assay System kit (Promega) according to the manufacturer’s instructions.

Reverse Transcription-PCR—Poly(A)1 RNA was prepared from gill, heart, head kidney, spleen, liver, muscle, testis, and brain of male eels and ovary of female eels by a method described previously (13). Random primed double strand cDNA was synthesized using a cDNA synthesis system (Amersham Pharmacia Biotech).

PCR was carried out in a 20-μl reaction mixture consisting of 50 mM KCl, 10 mM Tris-HCl, 1 mM MgCl2, 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 2.5 units of Taq polymerase (Perkin-Elmer), 1 mM sense primer (5’-TCCACAACTTCCTTGTCTG-3’, for eAR2), and 1 mM antisense primer (5’-GGGCTGGGTGGGAAGCTCAT-3’, for eAR2). Both sense and antisense primers for eAR1 and β-actin were the same as described previously (6, 14). 32 cycles of amplification for eAR2 and 28 cycles for eAR1 and β-actin were carried out under the following conditions: denaturation at 94 °C for 0.5 min, annealing at 62 °C for 0.5 min, and extension at 72 °C for 0.5 min. At completion of the PCR, fragments were separated on 1.5% agarose gels containing 0.5 mg/ml ethidium bromide.

Statistics—Data from transactivation experiments were analyzed by one-way analysis of variance. Significant differences between groups were identified by the Fisher’s protected least significant difference test. Differences were regarded as significant at p < 0.01.

RESULTS AND DISCUSSION

Sequence Homology with Other Steroid Hormone Receptors—
During cloning of eAR1 cDNA, one positive clone, whose sequence partially differed from that of eAR1 cDNA, was obtained. This insert is 1,718 base pairs in size, and the 3’-terminus of the cDNA was truncated. Thus, 3’-rapid amplification of cDNA ends (3’-RACE) was performed by using primer (corresponding to 1,594–1,622 base pairs) designed so that the PCR product overlapped with the 3’-terminus truncated clone at an eAR2 unique sequence. Based on the 3’-terminus truncated clone and the 3’-RACE product obtained, the nucleotide sequence and the deduced amino acid sequence of eAR2 were determined (Fig. 1). The sequence contains a long open reading frame encoding 797 amino acid residues (molecular mass, 94,688 Da). Comparison of the amino acid sequence of eAR2 with those of eel steroid hormone receptors and ARs of several species is shown in Fig. 2. The eAR2 gene sequence could be subdivided into 4–5 domains (A–F) as defined by Krust et al. (15). The putative DNA-binding domain (residues 436–522) and LBD (residues 548–797) show high homology with those of other ARs including eAR1 (DNA-binding domain, 98–88%; LBD, 59–85%). The other domains show low homology (>35%). Judging from these structures, eAR1 and eAR2 are not isoforms derived from translational initiation at two in-phase ATG codons, alternative splicing, or tetraploidy.

In tetraploid salmonids, two sets of genes for certain proteins have been found (16–18). Recently, two isoforms of AR (designated rtARα and rtARβ) were reported in rainbow trout (5).
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They are probably derived from salmonid tetraploidy, because homology of their full-length open reading frames is very high (85% in amino acid residues) compared with that of eAR1 and eAR2 (40% in amino acid residues). Notably, rtARβ did not activate transcription of an androgen-responsive gene. It has been reported that two types of gonadotropin α-subunit exist not only in salmonids but also in tetraploid common carp (19). Interestingly, when these subunits of common carp were expressed in insect cells by recombinant baculovirus, α1 associated with β subunit to form active gonadotropin, whereas α2 associated with β subunit but the resulting entity did not display gonadotropic activity (19). Thus, rtARs appear to be isoforms derived from tetraploidy as well as gonadotropin α of carp. Moreover, both rtARs are similar to eAR2 and different from eAR1 in structure. On the other hand, tetrapod ARs are similar to eAR1. Whether a subtype corresponding to eAR2 exists in tetrapods as well as other teleosts is an interesting question.

Although the N-terminal A/B domain displayed low homology overall, one conserved region is found among the ARs (residues 117–135 in the eAR2). It has been shown that the A/B domain contains transactivation function, which is dependent on cell and promoter types but less on ligand binding (20). Thus, it is interesting to determine whether the conserved regions in the A/B domain of ARs are involved in common functions, such as cell/promoter-specific transactivation.

In the DNA-binding domain, the position of cysteine residues, which constitute the two zinc finger motifs, and the P box (GSCKV), which is the important region for determination of the target gene (21), are also conserved in the eel AR2. Several amino acids in the LBD, which have been predicted as important in ligand binding based on crystallographic, genetic, or biochemical analysis (22), differ between hAR and eel ARs. For eAR2, these residues are Asn156 (eAR1, Asn16) (22), Leu626 (eAR1, Leu626), Tyr633 (eAR1, the same as hAR), Val666 (eAR1, Val670), Ser675 (eAR1, Thr679), Ser685 (eAR1, Asn616), Leu696 (eAR1, Leu617). Asn769 (eAR1, Cys820) (corresponding to Cys566 Gly743, Met749, Phe754, Lys777, Arg779, Met886, Val887, and Asp890 in the human AR) (22). These findings suggest that the affinity of...
eARs for various androgens may not differ greatly from that of the hAR. In addition, a leucine zipper motif, which was found in the C-terminal region of eAR1 LBD (residues 787–815) (6), is also conserved in the corresponding region of eAR2 (residues 736–764). The leucine zipper-like structure is conserved in many nuclear receptors, and is required for dimerization of receptors (23). Thus, this region in the eel AR could be involved in dimerization of ARs.

Androgen-dependent Transactivation Function of eAR2 Expressed in Mammalian Cells—Ligand specificity for the induction of androgen-regulated reporter activity was examined by incubation with 100 nM of various steroids (Fig. 3). Only androgens were effective in inducing luciferase activity. 11KT and the synthetic androgens, mibolerone (7α,17α-dimethyl-19-nortestosterone) and 17α-methyltestosterone, were most potent. Testosterone, 11β-hydroxytestosterone, and androstenedione were also effective, but their potencies were significantly lower than that of 11KT (p < 0.01). Other androgen-related steroids (11β-hydroxyandrostenedione, 11-ketoandrostenedione (adrenosterone), 11-ketoetiolanolone, and 5β-dihydrotestosterone) and other classes of steroids (cortisol, 17α,20β-dihydroxy-4-pregnen-3-one and estradiol-17β) were ineffective. When pcDNA-eARrev was transfected, none of the steroids induced luciferase activity (data not shown). These results show that the cloned eAR2 cDNA encodes a functional eel AR.

There were three minor differences in ligand affinity between eAR2 and eAR1, although the difference in homology of LBD between eAR1 and eAR2 (77%) is smaller than that between rat ERα and ERβ (53.5%) (2). First, synthetic androgens were significantly more effective than 5αDHT in inducing transcriptional activity of eAR2, a finding that differs from the pattern seen for eAR1 (Fig. 3B). Second, androstenedione induced weak transcriptional activity in eAR2, whereas eAR1 was not significantly stimulated by this steroid. Third, 17α,20β-dihydroxy-4-pregnen-3-one, which is the maturation-inducing steroid of many teleosts, did not induce any significant transcriptional activity of eAR2, whereas it induced weak transcriptional activity of eAR1. The ligand binding affinity of rat ERα and ERβ protein for physiological ligands is overall quite similar, certainly when only the order of competition is compared (24). It was also reported that the affinity for other estrogen-related substances (moxestrol, 17α-estradiol, etc.) differed between both ER subtypes (24). Similar differences between eel ARs were also observed in this study.

What is the native ligand of eARs? In the Japanese eel, 11KT (and not testosterone or 5αDHT) is the major androgen (quantitatively and physiologically) produced by testis during spermatogenesis (8, 9). 11KT stimulated luciferase activity through eAR2 and eAR1 (6) in a dose-dependent manner (Fig. 4). The minimum stimulatory dose of 11KT was 1 nM. Serum 11KT levels in human chorionic gonadotropin-treated and untreated Japanese eel males ranged from 0.8 to 26 nM (8). Physiological doses of 11KT are thus sufficient to activate luciferase activity via eel ARs. Thus, these results indicate that the major native ligand of ARs is 11KT.

Tissue Distribution of mRNA—The Levels of AR mRNA in various tissues from male Japanese eel were measured by reverse transcription-PCR. eAR2 mRNA was detected only in spleen, muscle, and testis, whereas eAR1 mRNA was observed in several other tissues as well (Fig. 5). Tissue distribution and/or the relative level of ERα and ERβ mRNA expression is quite different in rat (24). Differences in tissue distribution mRNA for eel ARs are predictable because their A/B domains, which contain a region of transactivation function strongly dependent on cell and promoter types but less on ligand binding, show low homology. Thus, difference in transactivation function of ARs on certain target genes may also exist in eel.

These results indicate that eAR2 is a novel AR subtype. Therefore, we suggest that this AR subtype be named eel ARβ to differentiate it from eAR1 (ARα) cloned previously (6). The biological significance of the existence of two different ARs is unclear and is being addressed in ongoing studies.

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