

# A novel progesterone receptor subtype in the Japanese eel, *Anguilla japonica*<sup>1</sup>

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**Abstract** A cDNA encoding a second type of a progesterone receptor (ePR2) was isolated from the same library as we had previously cloned a functional PR (ePR1) in eel testis. The amino acid sequence of the ePR2 shows low homology with ePR1 (34%), but both PRs showed progesterone-dependent transactivation in transfection experiment. Tissue distribution of ePR2 mRNA was clearly different from that of ePR1. Protein interaction between two PRs was demonstrated *in vitro* by a glutathione *S*-transferase pull-down assay. These results indicate that ePR2 is also a functional PR. This is the first isolation of two different functional PR molecules from a vertebrate. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

**Key words:** Nuclear receptor; Progesterone receptor  $\beta$ ; Progesterone; Spermiation; Sperm maturation; Japanese eel

## 1. Introduction

Progesterone receptor (PR) is a ligand-dependent transcriptional factor which belongs to a large family of nuclear receptors [1]. It has been shown that members of the nuclear receptor superfamily, such as retinoic acid receptor, retinoid X receptor and thyroid hormone receptor, have multiple subtypes and isoforms [1]. In the case of the steroid receptor subfamily, two or three subtypes have been reported for estrogen receptor (ER, ER $\alpha$ , ER $\beta$  and ER $\gamma$ ) in some mammals and teleost fishes [2,3,31] and androgen receptor (AR, AR $\alpha$  and AR $\beta$ ) in the Japanese eel, *Anguilla japonica* [4]. Although no PR subtypes have been reported, the PR has two isoforms (form A and form B). Chicken and human PR homologues originate from translational initiation at two in-phase ATG codons [5–7].

17 $\alpha$ ,20 $\beta$ -Dihydroxy-4-pregnen-3-one (17 $\alpha$ ,20 $\beta$ -DP) plays an important role in inducing oocyte maturation and spermiation in several teleost fishes including the Japanese eel [8].

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<sup>1</sup> The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with accession number AB028024.

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Although the mechanisms of the synthesis and action of this steroid have extensively been studied in the ovaries of several teleost fishes, the progesterone signaling system in gonads is not understood. In order to understand this problem, we have recently isolated and characterized a cDNA encoding a nuclear PR (ePR1) from a Japanese eel testis cDNA library. In transfection experiments using COS7 cells, the ePR1 showed progesterone-dependent activation of transcription. Thus, it was concluded that the ePR1 is a functional eel PR [9].

Here, we describe the isolation and characterization of another cDNA encoding a second type of ePR (ePR2) from the testis of the Japanese eel. Binding properties and the transactivation function of ePR2 were determined by expressing the cDNA in transiently transfected human embryonic kidney (HEK) 293 cells. The ePR mRNA levels in various tissues from eels were measured by reverse transcription-polymerase chain reaction (RT-PCR). The molecular interaction of both ePR proteins was also demonstrated by a glutathione *S*-transferase (GST) pull-down assay.

## 2. Materials and methods

### 2.1. Animals

Cultivated male Japanese eels (150–200 g body weight) were purchased from a commercial eel supplier (Marute Shouten, Hekinan City, Aichi Prefecture, Japan). They were kept in recirculating freshwater tanks with a capacity of 500 l at 20°C. Fish were not fed throughout the experimental period.

### 2.2. Isolation of cDNA clones

A fragment of ePR2 subtype, whose sequence was partially different from ePR1, 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 [10]. Since the 5'-terminus of the clone was truncated, 5'-rapid amplification of cDNA ends (5'-RACE) was performed using a Marathon cDNA Amplification kit (Clontech). The insert cDNA was nest-deleted at both ends using ExoIII/Mung bean nuclease. Sequencing was performed using the ABI PRISM Dye Terminator cycle sequencing kit (Applied Biosystems).

### 2.3. Construction of plasmid vectors

pcDNA-ePR2 was constructed by PCR amplification of the entire protein coding region (amino acids 1–689) of the ePR2, using primers which introduced an *EcoRV* site and in-frame Kozak sequence [11] at the 5'-end and a *XhoI* site at the 3'-end. The *EcoRV*–*XhoI* fragment was inserted at the corresponding site of pcDNA3.1(+) (Invitrogen). pcDNA-ePRrev, which had the pcDNA-ePR2 inserted in the reverse direction, was also prepared as a negative control. An ePR1 expression vector, named pcDNA-ePR1, was constructed after digestion an insert containing the entire protein coding sequence of the ePR1 from pSG5eDPR [9] with *EcoRI*, into the same site of pcDNA3.1(+). A

progesterone-regulated reporter vector, named MMTV-luc, was constructed as described previously [9]. Another progesterone-regulated reporter vector containing two copies of consensus steroid-responsive element, named SREX2-luc, was also constructed by ligating the SREX2 oligonucleotide (see below for sequence) into *MluI*-*BglII*-digested pGVP2 promoter vector which contains the *Photinus pyralis* luciferase gene (Toyo Ink). The SREX2 oligonucleotide was made after annealing 5'-CGCGTAGAACACAGTGTCTGATCCCGG-GACTAGAACACAGTGTCTA-3' and 5'-GATCTAGAACACTGTCTTAGTCCCGGGATCAGAACACTGTCTA-3'.

The GST expression vector, named pGST, was constructed by insertion of PCR amplification of the entire protein coding region (amino acids 1–240) of GST using pGEX-4T-2 (Pharmacia) as template, specific forward primer, which introduced an *EcoRV* site and in-frame Kozak sequence at the 5'-end, and reverse primer with a multiple cloning site of pGEX-4T-2, into pcDNA3.1(+) (Invitrogen) at the *EcoRV*-*NotI* site after digestion with *EcoRV* and *NotI*. The pGST-ePR1 and pGST-ePR2 were generated by in-frame ligation of the *NotI* fragment from PCR products of pcDNA-ePR1 and pcDNA-ePR2, respectively, using a primer which introduced a *NotI* site at the 5'-end and BGH reverse primer (Invitrogen) into the corresponding sites of pGST.

#### 2.4. Hormone-binding analysis

Hormone-binding analyses were carried out as described previously except for the use of HEK 293 cells and pcDNA3.1(+) vector [12]. In order to normalize for variation in transfection efficiency, pRL-TK, which contains the *Renilla reniformis* luciferase gene with the herpes simplex virus thymidine kinase promoter, was co-transfected with each expression vector for ePR.

#### 2.5. Transactivation assays

Transient transfection experiments were carried out as described previously [4].

#### 2.6. RT-PCR for PR mRNA

RT-PCR was carried out as described previously [4]. The sequences of the primers are as follows: 5'-TCATTGGCAGATAACGAAG-GACTGGC-3', for sense of ePR2; 5'-GCGACTTCAAGGAATTGTGTGTCTCA-3', for antisense of ePR2; 5'-CATACTCGGAG-TCTTTTGGGAGTTATGTG-3', for sense of ePR1; 5'-TCGC-AGTATTGGTCAGATAAGATG-3', for antisense of ePR1.

Both sense and antisense primers for eel  $\beta$ -actin were the same as described previously [13].

#### 2.7. GST pull-down assay

In the TNT-coupled RRL system (Promega) with T7 RNA polymerase, pcDNA-ePR1 or pcDNA-ePR2 was translated in the presence of [<sup>35</sup>S]methionine, and pGST-ePR1, pGST-ePR2 or pGST was translated in the absence of radiolabeled amino acids. 1  $\mu$ l of each ePR-containing lysate was mixed with 5  $\mu$ l lysate containing GST-ePR1, GST-ePR2 or GST protein. Samples had been incubated for 15 min on ice before 50  $\mu$ l glutathione-Sepharose (Pharmacia) diluted in phosphate-buffered saline (PBS) was added to each sample followed by 30 min of incubation on ice. The Sepharose beads were washed four times in PBS/0.1% Triton X-100, and bound proteins were eluted by incubation in SDS buffer for 2 min at 100°C. Hormone (17 $\alpha$ ,20 $\beta$ -DP; 10 nM) was included in all buffers, where applicable. Lysate of each ePR was loaded as input together with the eluted samples on a 12.5% SDS-PAGE. The gel was dried, exposed to an imaging plate and examined using an image analyzer BAS-2000 II (Fujix, Japan).

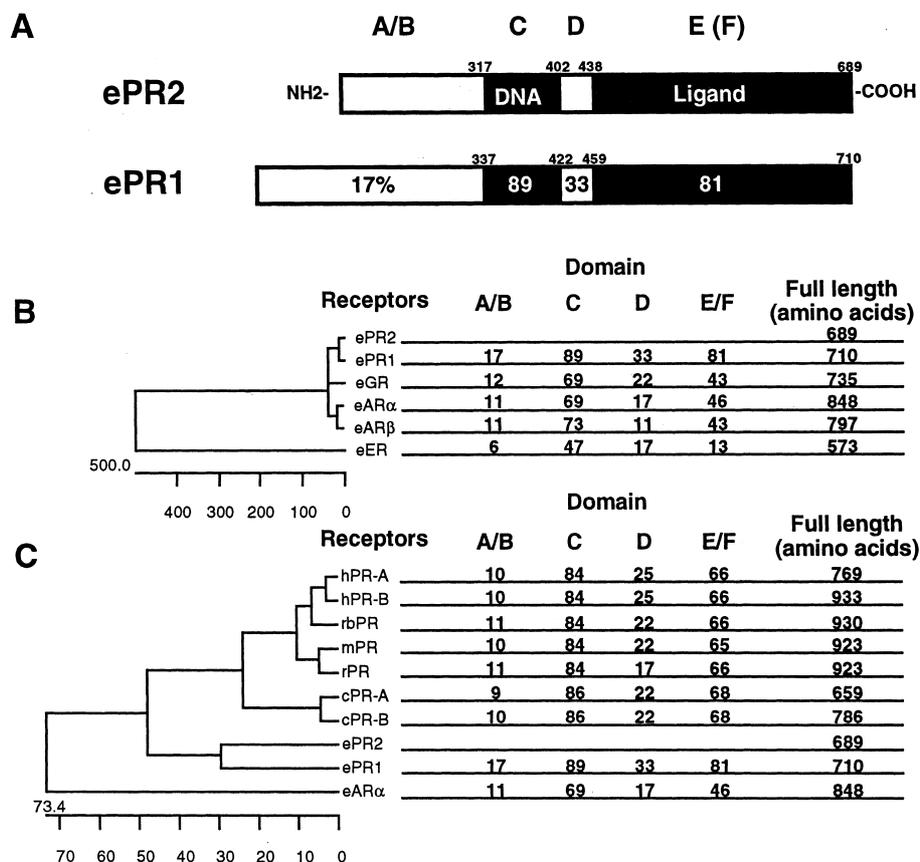


Fig. 1. Comparison of ePR2 protein with ePR1 (A), other steroid hormone receptors in eel (B) and PRs of several species (C). The functional A/B to E/F domains are schematically represented, with the numbers of amino acid residues indicated (A). Percentage homology of the domain relative to the Japanese eel PR2 is depicted. Clustal analysis of the full-length receptor sequences (B) and the E (ligand-binding) domains (C) using the MegAlign/DNASTAR software was used to develop alignments and phylogenetic trees. AR, androgen receptor; GR, glucocorticoid receptor; ER, estrogen receptor; e, eel; h, human; rb, rabbit; m, mouse; r, rat; c, chicken.

### 2.8. Statistics

Data were analyzed by one-way factorial analysis of variance and Scheffé's *F*-test as the multiple comparison test to determine significant difference between individuals;  $P \leq 0.01$  was considered significant.

## 3. Results

### 3.1. Sequence homology with other steroid hormone receptors

During cloning of eel AR $\alpha$  cDNA, one positive clone, whose sequence was partially different from ePR1, was obtained. This insert is 1538 bp in size, and the 5'-terminus of the cDNA was truncated. Thus, 5'-RACE was performed by using primer (corresponding to 180–209 bp of the clone) designed so that the PCR product overlapped with the 5'-terminus truncated clone at an ePR2-unique sequence. Based on the 5'-terminus truncated clone and the 5'-RACE product obtained, the nucleotide sequence and the deduced amino acid sequence of ePR2 were determined. The sequence contains a long open reading frame encoding 689 amino acid residues (molecular mass 76 312 Da). Comparison of the amino acid sequence of ePR2 with those of eel steroid hormone receptors and PRs of other species is shown in Fig. 1. The eel gene sequence could be subdivided into four to five domains (A–F) as defined by Krust et al. [14]. The putative DNA-binding domain (DBD; residues 317–402) and ligand-binding domain (LBD; residues 438–689) show high homology with those of other PRs including ePR1 (DBD: 84–89%, LBD: 65–81%). The other domains show low homology (< 33%).

### 3.2. Binding properties of ePRs expressed in HEK 293 cells

Whole cell extracts from pcDNA-ePRs transfected groups contained a protein which specifically bound 17 $\alpha$ ,20 $\beta$ -DP (Fig. 2A). Scatchard analysis using [<sup>3</sup>H]17 $\alpha$ ,20 $\beta$ -DP as a ligand revealed that ePR1 had a single class of high affinity binding sites for 17 $\alpha$ ,20 $\beta$ -DP, with a dissociation constant ( $K_d$ ) of 2.44 nM and a  $B_{max}$  of 1.25 pmol/relative luciferase unit (RLU) (Fig. 2B). Similar binding properties were observed for ePR2 with a  $K_d$  of 2.38 nM and a  $B_{max}$  of 1.13 pmol/RLU (Fig. 2C).

### 3.3. Progesterone-dependent transactivation function of ePR2 expressed in HEK 293 cells

The induction of progesterone-regulated reporter activity with 17 $\alpha$ ,20 $\beta$ -DP was shown in Fig. 3. ePR2 induced a three-fold response on both MMTV-luc and SREX2-luc when 100 nM 17 $\alpha$ ,20 $\beta$ -DP was added to the medium. ePR1 produced a larger fold induction of both reporters when examined under the same conditions. When pcDNA-ePRrev was transfected, no induction was found even with the hormone.

Ligand specificity for the induction of progesterone-regulated reporter activity was examined by incubation with 100 nM of various steroids (Fig. 3C). Four major progestogens in teleosts such as progesterone (P4), 17 $\alpha$ ,20 $\beta$ -DP, 20 $\beta$ -hydroxy-4-pregnen-3-one (20 $\beta$ -P) and 21-hydroxy-P4 (11-deoxycorticosterone; 11-DOC) [15] were effective in inducing luciferase activity. P4 and 17 $\alpha$ ,20 $\beta$ -DP were most potent. 20 $\beta$ -P and 11-DOC were also effective, but their potencies were significantly lower than that of 17 $\alpha$ ,20 $\beta$ -DP ( $P < 0.01$ ). Interestingly, 17 $\alpha$ ,21-dihydroxy-P4 (S) and 17 $\alpha$ ,20 $\beta$ ,21-trihydroxy-4-pregnen-3-one (20 $\beta$ -S) were ineffective in inducing luciferase activ-

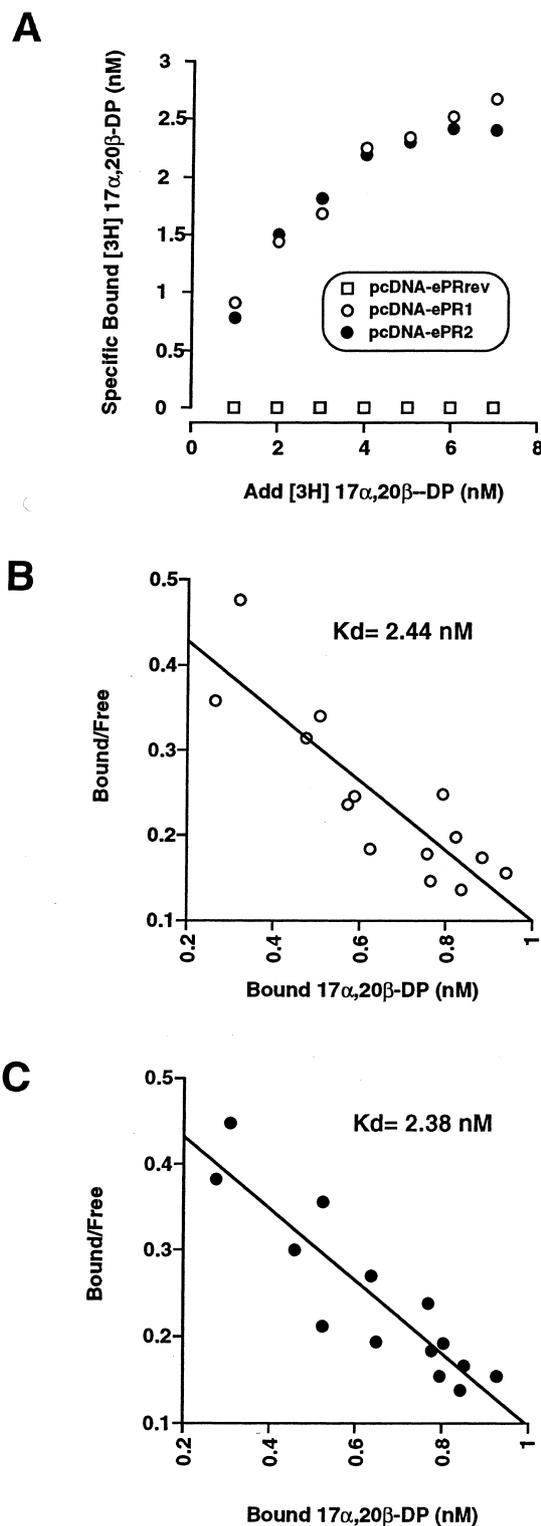


Fig. 2. Binding kinetics of 17 $\alpha$ ,20 $\beta$ -DP to ePRs. A: Saturation analysis of [<sup>3</sup>H]17 $\alpha$ ,20 $\beta$ -DP binding to ePRs expressed in HEK 293 cells. B, C: Scatchard plot of ePR1 (B) and ePR2 (C). The estimated dissociation constants ( $K_d$ ) for ePR1 and ePR2 are 2.44 nM and 2.38 nM, respectively.

ity in ePR2, although ePR1 was significantly stimulated by these steroids. Other progesterone-related steroids (17 $\alpha$ -hydroxy-P4 (17 $\alpha$ -P), 11 $\beta$ -hydroxy-P4, pregnenolone, and 17 $\alpha$ ,20 $\alpha$ -dihydroxy-4-pregnen-3-one (17 $\alpha$ ,20 $\alpha$ -DP)), and oth-

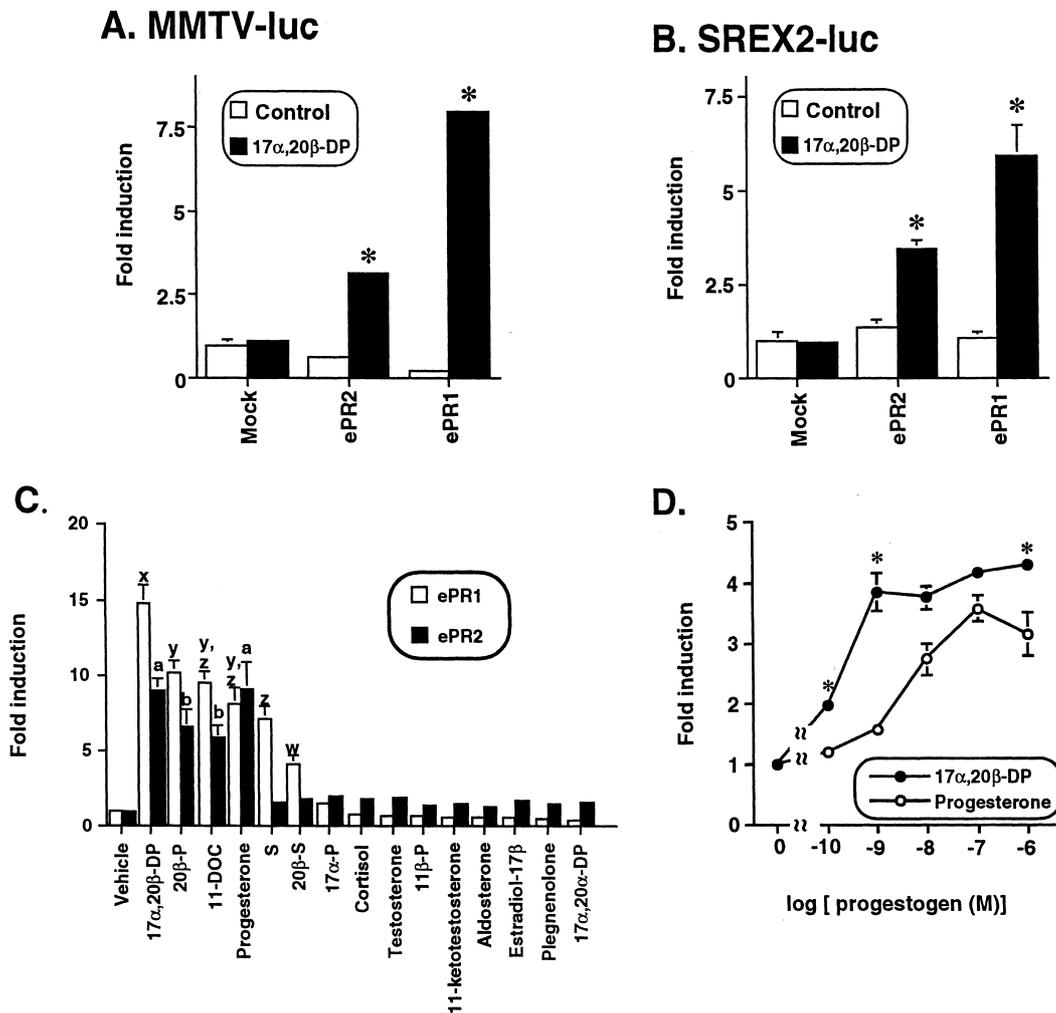


Fig. 3. Transcriptional activities of ePR2. A, B: 17 $\alpha$ ,20 $\beta$ -DP-dependent transcriptional activities of ePRs tested with the MMTV-luc (A) and SREX2-luc (B) reporter plasmids. HEK 293 cells were transiently transfected with an ePR expression vector together with the reporter vector. Cells were incubated with or without 100 nM of 17 $\alpha$ ,20 $\beta$ -DP for 48 h. Data are expressed as a ratio of each group: mock without 17 $\alpha$ ,20 $\beta$ -DP. \* $P$  < 0.001. C: Transcriptional activities for various steroids. Cells, transiently transfected with the MMTV-luc vector together with an ePR1 or ePR2 expression vector, were incubated with or without 100 nM of various steroids for 48 h. Data are expressed as a ratio of steroid: vehicle (ethanol). <sup>a,b,x,y,z,w</sup>Groups sharing the same letter codes were not significantly different at  $P$  < 0.01, and groups from a to b and from w to z were significantly different from vehicle group of ePR2 and ePR1, respectively, at  $P$  < 0.01. D: Dose-response profile of ePR2 activation by progestogens. Cells, transiently transfected with the MMTV-luc vector together with an ePR2 expression vector, were incubated with increasing concentrations of 17 $\alpha$ ,20 $\beta$ -DP or progesterone (0.01–100 nM) or with no ligand for 48 h. Asterisks show that the fold induction with 17 $\alpha$ ,20 $\beta$ -DP was significantly different from that of P4 at the same dose at  $P$  < 0.01. Each column represents the mean of triplicate determinations, and vertical bars represent the standard deviation. Lack of error bars on some columns is due to the errors being too small to show graphically.

er classes of steroids (cortisol, aldosterone, testosterone, 11-ketotestosterone, and estradiol-17 $\beta$ ) were ineffective.

Both 17 $\alpha$ ,20 $\beta$ -DP and P4 stimulated luciferase activity through ePR2 in a dose-dependent manner (Fig. 3D). The median effective doses of 17 $\alpha$ ,20 $\beta$ -DP and P4 were  $1.2 \times 10^{-10}$  nM and  $2.6 \times 10^{-9}$  nM, respectively. When doses less than 10 nM of steroids were used, the fold induction of reporter activity with 17 $\alpha$ ,20 $\beta$ -DP was significantly higher than that with P4 ( $P$  < 0.01).

#### 3.4. Tissue distribution of PR mRNA

To determine the relative distribution of ePR1 and ePR2 mRNA, poly(A)<sup>+</sup> RNA was isolated from various tissues from male and female eels and used for RT-PCR using primers specific for each ePR subtype. The relative distribution of both ePR subtypes was quite different, although this assay

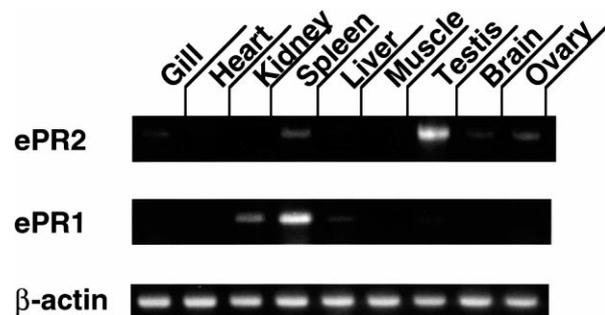


Fig. 4. Distribution of PRs mRNA in tissues of Japanese eel. Poly(A)<sup>+</sup> RNA was prepared from gill, heart, head kidney, spleen, liver, muscle, testis and brain of male eels and ovary of female eels.  $\beta$ -Actin was used as a positive control. mRNAs of both PR subtypes were detected by RT-PCR.

was only semiquantitative (Fig. 4). ePR2 mRNA was detected in gill, spleen, testis, brain and ovary, whereas ePR1 mRNA was observed in kidney, spleen, liver and testis.

### 3.5. GST pull-down assay

Both in vitro translated products of ePR1 and ePR2 were pulled down by GST-fused ePR1 or ePR2 proteins (Fig. 5). GST protein alone pulled neither ePR1 nor ePR2 down.

## 4. Discussion

The present study demonstrates the existence of a novel PR in the Japanese eel. The ePR2 has the putative DBD and LBD showing high homology with ePR1. The other domains show low homology. The size of ePR2 is shorter than ePR1 due to the shorter length of N-terminal A–B domain of ePR2. Judging from these structures, ePR1 and ePR2 are not isoforms derived from translational initiation at two in-phase ATG codons, alternative splicing, or tetraploidy. Phylogenetical analysis showed that ePR1 and ePR2 are distinct gene products and evolutionarily related but not recently duplicated. We have previously cloned and characterized two subtypes of ARs (AR $\alpha$  and AR $\beta$ ) from the eel testis cDNA library [9]. It is widely accepted that an ancestral steroid hormone receptor split four genes (AR, PR, glucocorticoid receptor (GR) and mineral corticoid receptor (MR)) by two waves of gene duplications in vertebrates [16,17]. However, it is likely that AR and PR diverged before the last gene duplication, because both AR and PR have two subtypes. It is important to determine whether two subtypes of GR and MR are also present in the Japanese eel.

In an earlier study, we showed that a bacterial recombinant ePR1-LBD (parts of the ePR1 molecule corresponding to the LBD of DPR, residues 459–710) bound 17 $\alpha$ ,20 $\beta$ -DP with a  $K_d$  of 24 nM [9]. In the present study, we synthesized the entire protein of ePR1 and ePR2 using HEK 293 cells. These two proteins showed similar biochemical properties and specifically bound 17 $\alpha$ ,20 $\beta$ -DP. However, their affinity for 17 $\alpha$ ,20 $\beta$ -DP was higher (approximately 10-fold) than that of the recombinant ePR1 protein.

A transactivation assay using a progesterone-regulated reporter vector containing the luciferase gene with the MMTV promoter into COS7 cells revealed that several progesterones including 17 $\alpha$ ,20 $\beta$ -DP were effective in inducing luciferase activity in ePR1, showing that ePR1 is a functional eel PR [9]. The present study also demonstrated the 17 $\alpha$ ,20 $\beta$ -DP-dependent transactivation function of ePR2 in HEK 293 cells. In the presence of 17 $\alpha$ ,20 $\beta$ -DP, both ePR1 and ePR2 activated the transcription of the luciferase gene under the control of MMTV or SREX2-SV40 promoter with a slightly lower luciferase activity in 293 cells transfected with ePR2. These results show that the cloned ePR2 cDNA also encodes a functional eel PR.

We have previously shown that the major native ligand of ePR1 is 17 $\alpha$ ,20 $\beta$ -DP [9]. This conclusion was based on: (1) 17 $\alpha$ ,20 $\beta$ -DP was the most effective steroid in inducing luciferase activity, (2) P4 is considered to be an intermediate in the synthesis of potent steroids in teleosts, and 17 $\alpha$ ,20 $\beta$ -DP and/or 20 $\beta$ -S are the major progesterones in teleosts, (3) 17 $\alpha$ ,20 $\beta$ -DP has been identified as the major oocyte maturation-inducing hormone (MIH) of Japanese eel, and (4) eel gonads produce a large amount of 17 $\alpha$ ,20 $\beta$ -DP in vitro, but

not 20 $\beta$ -P, 11-DOC, P4 or 20 $\beta$ -S, when the precursor is added to incubation medium [9]. The present study also showed that 17 $\alpha$ ,20 $\beta$ -DP was the most effective steroid in inducing luciferase activity in ePR2. P4 was also highly effective, but its median effective dose ( $2.6 \times 10^{-9}$  nM) was much higher than that of 17 $\alpha$ ,20 $\beta$ -DP ( $1.2 \times 10^{-10}$  nM). It is concluded from these results that like ePR1, the major native ligand of ePR2 is 17 $\alpha$ ,20 $\beta$ -DP.

Ligand specificity for the induction of progesterone-regulated reporter activity of ePR2 was very similar to that of ePR1. Steroids with high transcriptional activity in ePR1 and ePR2 have previously been shown to be highly effective in inducing final maturation of fish oocytes in vitro [14] with the exception that 20 $\beta$ -S, which has been identified as the MIH of spotted seatrout [18], was ineffective in inducing luciferase activity in ePR2, although some activity was found in ePR1. Pinter and Thomas [19] demonstrated the presence of the nuclear PR in the ovary of spotted seatrout. In their studies, 20 $\beta$ -S and 17 $\alpha$ ,20 $\beta$ -DP demonstrated very high affinity for this receptor. However, in the Japanese eel, the levels of 20 $\beta$ -S are very low in the eel ovary [20]. In the present study, the 20 $\alpha$  epimer of 17 $\alpha$ ,20 $\beta$ -DP, 17 $\alpha$ ,20 $\alpha$ -DP, did not induce transcriptional activity through ePRs. This steroid is found in testicular incubations or plasma of several teleosts [15], but has been shown to be less potent in inducing oocyte maturation. The precursors for 17 $\alpha$ ,20 $\beta$ -DP (17 $\alpha$ -P and pregnenolone) were also ineffective in inducing transcriptional activity of ePRs. Taken together, it is possible that ePRs play a role in the signaling system associated with progesterone-induced oocyte maturation in teleosts.

Affinity to C21 steroid of both ePRs was different from that of mammalian PR [21]. In mammal 11-DOC binds weakly to PR, but the relative binding affinity (RBA) of MR to that steroid (RBA = 75 ~ 100) is higher than that of PR (RBA = 25 ~ 50). S binds to MR, but not to PR in mammals. In the present study, 11-DOC induced transcriptional activity in both ePRs and S was effective in ePR1. These steroids have been shown to be produced in gonads of several teleosts and to induce final oocyte maturation (see [15]).

There are two minor differences in transactivity for ligands between ePR2 and ePR1. First, 20 $\beta$ -S and S did not show any significant transcriptional activity in ePR2, whereas they induced weak activity in ePR1. Second, when a concentration of 100 nM was used, P4 and 17 $\alpha$ ,20 $\beta$ -DP were equally effective in ePR2, whereas 17 $\alpha$ ,20 $\beta$ -DP was more effective than P4 in ePR1. The Scatchard analysis based on the present binding assay revealed that both proteins showed similar  $B_{max}$ . Since the variation in transfection efficiency was normalized in each experiment by using the same internal control vector, it is assumed that similar levels of both receptors were expressed after transient transfection in the reporter assay. We also reported previously that the transactivity for androstenedione and synthetic androgens differed in eel AR $\alpha$  and AR $\beta$  [4].

The physiological responses to estrogen are known to be mediated within specific tissues by at least two estrogen receptors, ER $\alpha$  and ER $\beta$  [22]. Studies of the receptors' tissue distribution and expression pattern in mammals indicate that ER $\alpha$  has a broad expression pattern, whereas ER $\beta$  has a more focused pattern with high levels in the ovary, prostate, epididymis, lung, and hypothalamus [23,24]. Similarly, tissue distribution of AR $\alpha$  and AR $\beta$  mRNA expression is quite different in eel. eAR $\alpha$  mRNA has a broad expression pattern,

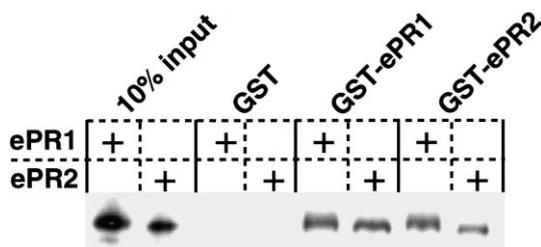


Fig. 5. GST pull-down assay. Both ePR1 and ePR2 proteins were labeled with [<sup>35</sup>S]methionine by translation in vitro using the TNT-coupled reticulocyte lysate system (Promega), shown in the first two lanes at the amount of 10% input. After incubation with GST, GST-ePR1 or GST-ePR2 proteins, samples were subsequently incubated with glutathione-Sepharose (Pharmacia), washed, eluted in SDS buffer, and separated on 12.5% SDS-PAGE.

whereas eAR $\beta$  mRNA was observed only in spleen, muscle, and testis [9]. In the present study, ePR2 mRNA was detected in gill, spleen, testis, brain, and ovary, whereas ePR1 mRNA was observed in kidney, spleen, liver, and testis. Differences in tissue distribution of ePR mRNA 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. In mammals, PRs were detected in uterus, ovary, vagina, testis, breast, brain, vascular endothelium, thymus, pancreatic islet, osteoblast-like cell, and lung [25]. In non-mammalian species, PRs were also detected in oviduct of chicken [5] and turtle [26], liver of turtle [27], testis of shark [28] and ovary of seatrout [20].

In mammals and chicken, several target genes of P4-PR have been reported (uteroglobin in rabbit uterus, osteonectin in mouse bone, and ovalbumin in chicken oviduct) [25]. No such studies have been performed in teleosts. It is necessary to identify target genes of piscine PRs to uncover their physiological roles. In this connection, it is of interest to note that although both ePR1 and ePR2 were expressed in spleen and testis in the Japanese eel, the relative expression of both PRs was different between two tissues. Recently, it has been reported that ER $\alpha$  and ER $\beta$  can heterodimerize with each other, suggesting their cross-talking possibilities [29,30]. Similarly, our results using a GST pull-down assay also show that ePR1 and ePR2 form heterodimers. It is thus possible that ePR1 and ePR2 may cooperate in regulating progesterone-responsive gene expression in cell types in which they are co-expressed. In this study, ePR1 and ePR2 mRNAs were co-expressed in spleen and testis. Further in situ hybridization studies will be necessary to determine whether both subtypes co-exist in the same cell.

In conclusion, a novel PR subtype, ePR2, was cloned and characterized. We suggest that this novel type of PR is named eel PR $\beta$  to differentiate it from ePR1 (PR $\alpha$ ) cloned previously [9]. Further studies are necessary to discern the full roles of each ePR in the progesterone signaling system.

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## References

- [1] Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schütz, G., Umezono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P. and Evans, R.M. (1995) *Cell* 83, 835–839.
- [2] Kuiper, G.G.J.M., Enmark, E., Pelto-Huikko, M., Nilsson, S. and Gustafsson, J.-A. (1996) *Proc. Natl. Acad. Sci. USA* 93, 5925–5930.
- [3] Chang, X.T., Kobayashi, T., Todo, T., Ikeuchi, T., Yoshiura, Y., Kajiuura-Kobayashi, H., Morry, C.E. and Nagahama, Y. (1999) *Zool. Sci.* 16, 653–658.
- [4] Ikeuchi, T., Todo, T., Kobayashi, T. and Nagahama, Y. (1999) *J. Biol. Chem.* 274, 25205–25209.
- [5] Schrader, W.T. and O'Malley, B.W. (1972) *J. Biol. Chem.* 247, 51–59.
- [6] Conneely, O.M., Maxwell, B.L., Toft, D.O., Schrader, W.T. and O'Malley, B.W. (1987) *Biochem. Biophys. Res. Commun.* 149, 493–501.
- [7] Kastner, P., Krust, A., Turcotte, B., Stropp, U., Tora, L., Gronemeyer, H. and Chambon, P. (1990) *EMBO J.* 9, 1603–1614.
- [8] Nagahama, Y. (1994) *Int. J. Dev. Biol.* 38, 217–229.
- [9] Todo, T., Ikeuchi, T., Kobayashi, T., Kajiuura-Kobayashi, H., Suzuki, K., Yoshikuni, M., Yamauchi, K. and Nagahama, Y. (2000) *FEBS Lett.* 465, 12–17.
- [10] Todo, T., Ikeuchi, T., Kobayashi, T. and Nagahama, Y. (1999) *Biochem. Biophys. Res. Commun.* 254, 378–383.
- [11] Kozak, M. (1986) *Cell* 44, 283–292.
- [12] Todo, T., Adachi, S. and Yamauchi, K. (1996) *Mol. Cell. Endocrinol.* 119, 37–45.
- [13] Nagae, M., Adachi, S. and Yamauchi, K. (1997) *Fish Physiol. Biochem.* 17, 179–186.
- [14] Krust, A., Green, S., Argos, P., Kumar, V., Walter, P., Bornert, J.M. and Chambon, P. (1986) *EMBO J.* 5, 891–897.
- [15] Kime, D.E. (1993) *Rev. Fish Biol. Fish.* 3, 160–180.
- [16] Escriva, H., Safi, R., Hanni, C., Langlois, M.-C., Saumitou-Laprade, P., Stehelin, D., Capron, A., Peirce, R. and Laudet, V. (1997) *Proc. Natl. Acad. Sci. USA* 94, 6803–6808.
- [17] Laudet, V. (1997) *J. Mol. Endocrinol.* 19, 207–226.
- [18] Thomas, P. and Trant, J.M. (1989) *Fish Physiol. Biochem.* 7, 185–191.
- [19] Pinter, J. and Thomas, P. (1995) *Biol. Reprod.* 52, 667–675.
- [20] Kazeto, Y. (1998) Dr. Thesis, Hokkaido University, Hakodate.
- [21] Ojasoo, T. and Raynaud, J.-P. (1978) *Cancer Res.* 38, 4186–4198.
- [22] Couse, J.F., Hewitt, S.C., Bunch, D.O., Sar, M., Walker, V.R., Davis, B.J. and Korach, K.S. (1999) *Science* 286, 2328–2331.
- [23] Kuiper, G.G.J.M., Carlsson, B., Grandien, K., Enmark, E., Häggblad, J., Nilsson, S. and Gustafsson, J.-Å. (1997) *Endocrinology* 138, 863–870.
- [24] Couse, J.E., Lindzey, J., Grandien, K., Gustafsson, J.-K. and Korach, K.S. (1997) *Endocrinology* 138, 4613–4621.
- [25] Graham, J.D. and Clarke, C.L. (1997) *Endocr. Rev.* 18, 502–519.
- [26] Reese, J.C. and Callard, I.P. (1989) *J. Steroid Biochem.* 32, 297–310.
- [27] Riley, D., Reese, J.C. and Callard, I.P. (1988) *Endocrinology* 123, 1195–1201.
- [28] Cuevas, M.E. and Callard, I.P. (1992) *Endocrinology* 130, 2173–2182.
- [29] Cowley, S.M., Hoare, S., Mosselman, S. and Parker, M.G. (1997) *J. Biol. Chem.* 272, 19858–19862.
- [30] Pettersson, K., Grandien, K., Kuiper, G.G. and Gustafsson, J.A. (1997) *Mol. Endocrinol.* 11, 1486–1496.
- [31] Hawkins, M.B., Thornton, J.W., Crews, D., Skipper, J.K., Dotte, A. and Thomas, P. (2000) *Proc. Natl. Acad. Sci. USA* 97, 10751–10756.