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Characterization of a testicular 17α,20β-dihydroxy-4-pregnen-3-one (a spermiation-inducing steroid in fish) receptor from a teleost, Japanese eel (*Anguilla japonica*)[1]

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Abstract A cDNA encoding a nuclear 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP, spermiation-inducing hormone in fish) receptor (DPR) was, for the first time, isolated from an eel testis cDNA library. The amino acid sequence of DPR shows high homology with those of human and chicken progesterone receptors. The affinity of the bacterial recombinant DPR ligand binding domain protein for 17α,20β-DP is higher than that of progesterone. In transfection experiments using COS7 cells, the DPR showed progestin-dependent activation of transcription. 17α,20β-DP was the most effective activator of transcription. These results indicate that the cDNA encodes a functional eel DPR, and show that 17α,20β-DP has a nuclear receptor-mediated action in eel testes.

Key words: Nuclear receptor; Progestin; Spermiation; Sperm maturation; Japanese eel

1. Introduction

Spermiation and sperm maturation occur during the final stage of spermatogenesis in most vertebrates, and are critical steps for successful fertilization. We have previously shown that a progestin, 17α,20β-dihydroxy-4-pregnen-3-one (17α, 20β-DP), plays an important role in inducing spermiation in several teleost fishes including the Japanese eel, *Anguilla japonica* [1–3]. Furthermore, we identified 17α,20α-dihydroxy-4-pregnen-3-one (17α,20α-DP) as the naturally occurring spermiation-inducing steroid in the testis of a frog, *Rana nigromaculata* [4]. However, the molecular mechanisms underlying the regulation of spermiation and sperm maturation by these steroids are not understood.

Steroid hormones generally act on their target cells through nuclear receptors. The nuclear receptors belong to a superfamily of ligand-dependent transcription factors which are characterized by a highly conserved DNA binding domain (DBD) and moderately conserved ligand binding domain (LBD) [5]. It is assumed that the action of 17α,20β-DP on eel testes is mediated through its nuclear receptor. A specific binding for 17α,20β-DP was detected by radioligand binding assay in a nuclear fraction from spotted seatrout ovaries and this nuclear receptor was considered to be a progesterone receptor (PR) [6]. However, there are some minor differences between fish nuclear 17α,20β-DP binding protein and mammalian PRs. For example, the affinity of fish PR for 11-deoxycorticosterone (11-DOC) is much higher than for mammalian PRs [6,7]. It is noteworthy that the affinities of mammalian mineralcorticoid receptors (MRs) for 11-DOC are higher than for mammalian PRs [7]. The isolation and characterization of the nuclear 17α,20β-DP receptor (DPR) molecule or its cDNA are necessary to compare the details of sequence and ligand specificity of DPR and PR.

In order to understand molecular mechanisms involved in 17α,20β-DP-induced spermiation and sperm maturation, we isolated and characterized a DPR cDNA, for the first time in any vertebrate, from the testis of the Japanese eel. The steroid binding properties of the DPR were determined using recombinant DPR-LBD protein expressed in *Escherichia coli*. The transactivation functions of the DPR were also examined by expressing the cDNA in transiently transfected mammalian COS7 cells. In addition, changes in DPR mRNA levels during human chorionic gonadotropin (HCG)-induced eel spermatogenesis were examined by Northern blotting.

2. Materials and methods

2.1. Isolation of cDNA clones

A conserved region in the DBD of steroid hormone receptors was amplified by polymerase chain reaction (PCR) as previously described [8]. A λZAPII cDNA library constructed from Japanese eel testes [9] was screened using the PCR product as probe. Subclones were prepared by in vivo excision. The insert cDNA was nest-deleted at both ends using ExoIII/Mung bean nuclease. Sequencing was performed using the ABI PRIZM Dye Terminator Cycle Sequencing kit (Applied Biosystems).

2.2. Construction of expression vectors

pQEeDPR-LBD was constructed by PCR amplification (with pBeSR2 as a template) of a fragment corresponding to amino acids 459–710 of the DPR (entire coding region of the LBD), using primers which incorporate a 5′ *Sph*I and a 3′ *Sal*I restriction site preceded by a stop codon, such that this fragment could be inserted into the corresponding sites of pQE-30 vector (Qiagen) which produces a fusion protein with six histidine residues in *E. coli*. pSG5eDPR was

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[1] The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with accession number AB032075.

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constructed by PCR amplification of the entire protein coding region (amino acids 1–710) of the DPR, using primers which introduce an *Eco*RI site and in-frame Kozak sequence [10] at the 5′ end and an *Eco*RI site at the 3′ end. The *Eco*RI fragment was inserted at the *Eco*RI site into pSG5 vector (Stratagene) [11]. All PCR reactions were carried out using Pfu DNA polymerase (Stratagene). A progestin-regulated reporter vector, named pGV2-MMTV, which contains the mouse mammary tumor virus-long terminal repeat (MMTV-LTR) and the *Photinus pyralis* luciferase gene, was constructed as previously described [8].

2.3. Hormone binding analysis

pQEeDPR-LBD was introduced into the *E. coli* M15 strain. A colony of the transformants was inoculated into 2×YT medium (16 g/l bacto-tryptone, 10 g/l bacto-yeast extract, 5 g/l NaCl), and grown at 37°C overnight. Fusion protein production was induced by treating with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h. Cells were pelleted by centrifugation at 4°C and resuspended in ice-cold phosphate-buffered saline (PBS) containing 1 mM phenylmethanesulfonyl fluoride and 5 μg/ml leupeptin. Cell density was checked by spectrophotometric measurement at OD_{600}, and cell suspension was diluted to 1 OD_{600} (approximately 1.5×10^8 cells/ml) with PBS.

100 μl of cell suspension (1.5×10^7 cells) was incubated with 0.2–20 nM [^{3}H]17α,20β-DP (generated from [^{3}H]17α-hydroxyprogesterone (17α-OHP; 1.8 TBq/mmol; New England Nuclear) as previously described [12]) or [^{3}H]progesterone ([^{3}H]P4; 36 TBq/mmol; New England Nuclear) in a total volume of 400 μl PBS. Parallel tubes containing the [^{3}H]17α,20β-DP or the [^{3}H]P4 plus 100-fold excess of radioinert 17α,20β-DP or P4 were also incubated for determination of non-specific binding. All samples were incubated in triplicate. After incubation for 4 h at 4°C with continuous rotation at 30 rpm, cells were pelleted by centrifugation at 4°C and washed three times with ice-cold PBS. Final cell pellets were resuspended in 100 μl PBS, and counted for radioactivity in Scintisol EX-H (Dojin). The specific binding data were analyzed by Scatchard's graphic method. To evaluate binding specificity, parallel samples were incubated with 10 nM [^{3}H]17α,20β-DP with and without a 100-fold excess of various radioinert steroids.

2.4. Transactivation assays

Transient transfection experiments were carried out as described previously except for using COS7 cells as host cell and pSG5eDPR or pSG5 as expression vector instead of HEK 293 cells and pcDNA3.1(+), respectively [8].

2.5. Northern blot analysis

Cultivated Japanese eel males were given a single intramuscular injection of HCG. Testes were sampled before injection of HCG and 1, 3, 6, 9, 12 and 15 days post injection. To obtain spermiating fish, males received weekly intramuscular injections of HCG for 10–14 weeks. Testes were collected from spermiating males. Total RNA and poly(A)$^+$ RNA were prepared from eel testes and 4 μg of the poly (A)$^+$ RNAs were analyzed by Northern blotting, using the methods previously described [13]. The same membrane was rehybridized with a Japanese eel β-actin cDNA probe [9]

3. Results

3.1. Isolation and sequencing of Japanese eel DPR cDNA

Using a PCR product as a probe, a cDNA library from eel testes was screened. From 3×10^5 phages, 25 positive clones were obtained after two rounds of screening. Based on partial sequence analysis of these clones, one clone named pBeSR2 was selected and fully sequenced. The pBeSR2 insert was 2841 bp in size, and contained a long open reading frame encoding 710 amino acid residues (molecular weight 78 815), together with a 306 nucleotide 5′ untranslated region and a 405 nucleotide 3′ untranslated region with an 18 residue poly(A) tail. The amino acid sequence of the cDNA showed the highest homology with those of PRs cloned in other species, suggesting that the cDNA encodes the DPR.

Comparison of the amino acid sequence from the DPR with those of human steroid hormone receptors [14–18], chicken PR [19,20] and eel estrogen receptor β (ERβ) [13] and androgen receptors (ARs) [8,21] is shown in Fig. 1. The eel gene sequence could be subdivided into 4–5 domains (A–F) as defined by Krust et al. [22]. The putative DBD (C domain, residues 337–421) and LBD (E domain, residues 459–710) showed high homology with those of human and chicken PRs (DBD: 85%, LBD: 65%). These domains also showed high homology with those of other receptors (human glucocorticoid receptor: GR; human MR; human and eel ARs), but their homologies were less than those in human and chicken PRs (DBD: 67–80%, LBD: 39–43%). The other domains showed little homology (3–12%).

3.2. Steroid binding abilities of the DPR-LBD expressed in E. coli

In order to examine the steroid binding properties of the

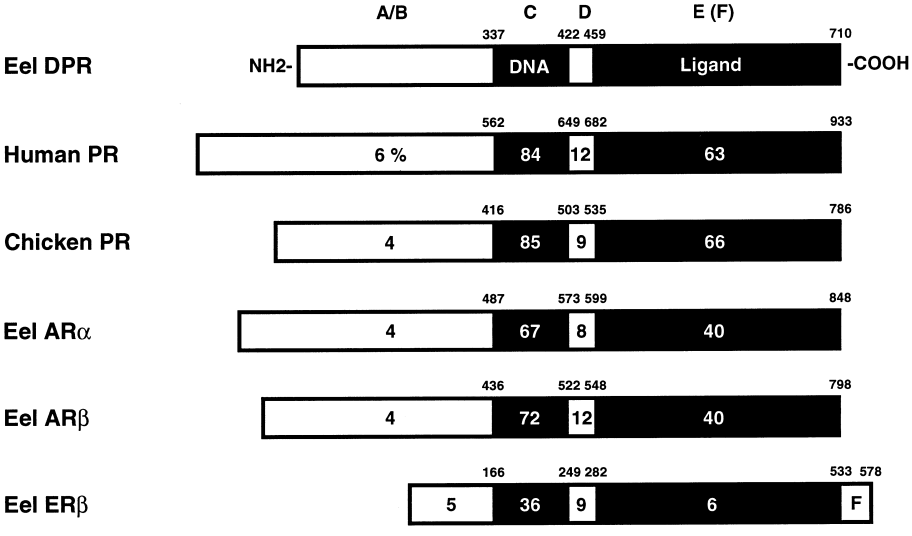

Fig. 1. Domain structure of the Japanese eel DPR, and homology with human steroid receptors, chicken PR and Japanese eel AR and ER. The numbers above each box refer to the position of amino acids in each domain. The figures within each box indicate the percentage homology of the domain relative to the Japanese eel DPR.

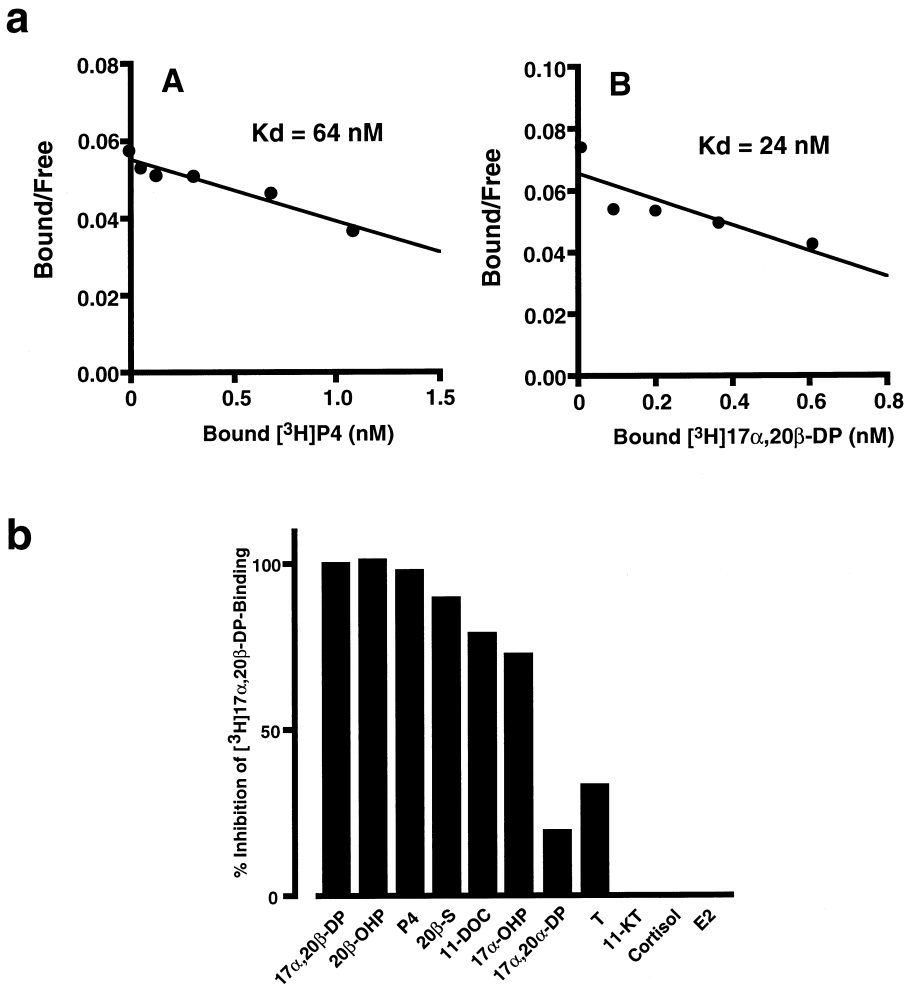

Fig. 2. (a) Scatchard analysis of [^{3}H]P4 and [^{3}H]17α,20β-DP binding to the DPR-LBD. Samples were incubated with a range of [^{3}H]P4 (A) or 17α,20β-DP (B) concentrations with or without a 200-fold excess of unlabeled each steroid. Specific binding data were analyzed by Scatchard's graphic method. Each point represents the mean of triplicate determinations. (b) Steroid binding specificity of the DPR-LBD. Samples were incubated with 10 nM [^{3}H]17α,20β-DP with and without 100-fold excess (1 μM) unlabeled competitor. Displacement was calculated as the percentage of maximum displacement as measured by the depression of binding in the presence of 1 μM 17α,20β-DP. Each column represents the mean of triplicate determinations.

DPR, the LBD of DPR (residues 459–710) was subcloned into a bacterial expression vector, and expressed as a fusion protein with six histidine residues in *E. coli*. After fractionation of the cell extracts into soluble and insoluble fractions, almost all the fusion protein was present in the insoluble fraction (data not shown). Thus, IPTG-induced 'intact' cells were used for subsequent binding analysis.

Scatchard analysis using [^{3}H]17α,20β-DP and [^{3}H]P4 as ligands showed a single class of high affinity binding sites for both 17α,20β-DP and P4 (Fig. 2a). However, the affinity of 17α,20β-DP was higher than that of P4 (equilibrium dissociation constant, K_d: 24 nM for 17α,20β-DP, 64 nM for P4). The specificity of progestin binding to the DPR was determined by competition of [^{3}H]17α,20β-DP binding with 100-fold excess unlabeled steroids (Fig. 2b). 17α,20β-DP, 20β-OHP and P4 were the most effective competitors, and other progestins, 17α,20β,21-trihydroxy-4-pregnen-3-one (20β-S) and 17α-OHP, and 11-DOC were also quite effective, although 17α,20α-DP was less effective. Testosterone (T) also competed for 17α,20β-DP binding sites, while 11-ketotestosterone (11-KT; a potent androgen in fish), cortisol and estradiol-17β (E2) had negligible effects.

3.3. Transactivation function of the DPR in mammalian cells

The DPR expression vector was cotransfected with a progestin-regulated reporter vector, pGV2-MMTV, containing the luciferase gene with the MMTV promoter, into COS7 cells. The ligand specificity for the induction of luciferase activity was examined by incubation with 100 nM of various steroids (Fig. 3). 17α,20β-DP was the most effective inducer of luciferase activity (approximately 7.5-fold compared with no hormone), and other progestins, 20β-OHP, P4, 20β-S and 17α-OHP, and 11-DOC were also effective. However, 17α,20α-DP and the other steroids, cortisol, T, 11-KT and E2, were ineffective.

3.4. Changes in testicular DPR mRNA levels during HCG-induced spermatogenesis

Northern blot analysis was conducted with testes from HCG-treated and untreated eels using the DPR cDNA frag-

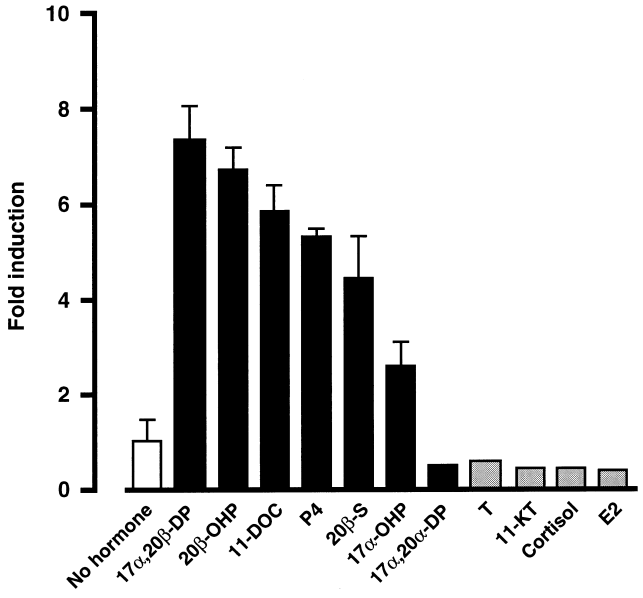

Fig. 3. Progestin-dependent transactivation function of Japanese eel DPR. COS7 cells were transiently transfected with the MMTV-luciferase vector together with pSG5eDPR. Cells were incubated with or without 100 nM of various steroids for 48 h. Data are expressed as a ratio of steroid:no hormone. Each column represents the mean of triplicate determinations, and vertical bars represent the S.E.M.

ment encoding the LBD as the probe (Fig. 4). Two mRNA bands corresponding to approximately 8.5 and 5 kb were detected in all testes. The levels of both mRNAs increased slightly until 6 days after single HCG injection, and then decreased 9 days after treatment. However, levels of both mRNAs increased slightly 12 and 15 days after treatment, and levels in testis mRNA obtained from spermiating fish treated with multiple HCG injections were higher than those from fish obtained at 15 days post injection.

4. Discussion

In the present study, a conserved region in the DBD of steroid hormone receptors was used to isolate a full-length cDNA encoding DPR from Japanese eel testis. The cloned DPR cDNA encodes a protein of 710 amino acids in length with a calculated molecular weight of 78 815. The size of DPR is shorter than PRs of chicken (787 amino acids) and human PRs (933 amino acids). The difference in size among DPR, chicken PR and human PR is due to the different lengths of N-terminal A/B domains. The DPR A/B domain has less homology with those of chicken and human PRs, although several conserved sequences have been found between chicken and human PRs [23]. It has been shown that the A/B domain contains transactivation function, named AF1, which is dependent on cell and promoter types but less on ligand binding [24]. Thus, the DPR AF1 may have functions different from those of chicken and human PRs.

Only two domains, DBD and LBD, are highly conserved between DPR and chicken and human PRs, similar to what has been found for eel ERβ [13], ARα [8] and ARβ [21]. The DBD is the most highly conserved region in DPR and other PRs (85%). This region is well conserved also in other steroid hormone receptors (67–80%). In the DBD, the positions of cysteine residues which constitute the two zinc finger motifs, and each P (GSCKV) and D box (AGRND) which are the important regions for determination of the target gene [25], are conserved in the DPR. The LBD of DPR exhibits less homology compared with other PRs. Recently, the crystal structure of P4-bound LBD of the human PR has been reported, and the amino acids bound to P4 have been identified [26]. All of those residues are conserved in the DPR, suggesting that the DPR binds progestins in a manner similar to that of the human PR.

We have also shown that the recombinant DPR protein binds progestins. We chose a bacterial expression system for the radioligand binding study, because it is easy to obtain a large amount of recombinants using the system. Since attempts to express full-length steroid receptors in bacteria were mostly unsuccessful [27,28], we decided to express only parts of the molecule corresponding to the LBD of DPR. The recombinant DPR-LBD bound both 17α,20β-DP and P4, but the affinity of DPR-LBD for 17α,20β-DP is higher than for P4. Furthermore, binding was specific for progestins, and other steroids, such as androgens, cortisol and E2, did not displace 17α,20β-DP from binding sites. These binding properties of the DPR-LBD are similar to those of a PR identified in the ovary of the spotted seatrout by radioligand binding assay [6], with a few minor differences: for seatrout PR, 20β-S is the second most effective competitor, and P4 is less effective than 20β-S and 11-DOC. These results clearly show that the characteristics of the DPR-LBD are consistent with known properties of steroid receptors, such as a limited capacity and specificity of ligand.

We have also demonstrated the progestin-dependent trans-

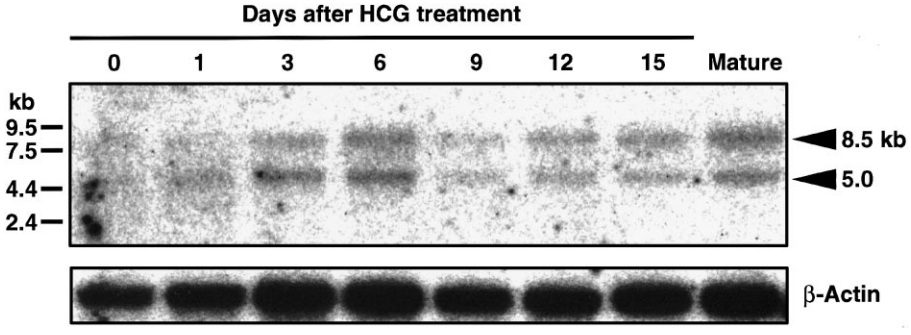

Fig. 4. Changes in testicular DPR mRNA levels during HCG-induced spermatogenesis. Cultivated male eels were given a single injection of HCG. Their testes were collected for RNA extraction before HCG injection and 1, 3, 6, 9, 12 and 15 days post injection. Mature testis was obtained after weekly injections of HCG for 10 weeks. 4 μg of poly(A)$^+$ mRNA was separated on a denaturing formaldehyde agarose (1%) gel, transferred to a nylon membrane and then hybridized with ^{32}P-labeled DPR cDNA.

activation function of the DPR. In the presence of 17α,20β-DP, the DPR activated the transcription of a luciferase gene under the control of MMTV-LTR which is well known as a progestin-regulated promoter [29]. Moreover, the regulation of transactivation by the DPR was progestin specific, and 17α,20β-DP was the most effective steroid. These results are in agreement with those of ligand binding assay using the DPR-LBD. It is noteworthy that unlike those of PRs, both binding activity and transactivation of DPR were highly stimulated by 11-DOC.

Together with the results from binding and transactivation studies, we conclude that the cloned cDNA encodes a functional DPR. The results also show that 17α,20β-DP is the most effective steroid in both binding activity and transactivation. In mammals and chicken, P4 is the major progestin, and P4 is considered to be a ligand for PRs [30]. However, in teleosts, 17α,20β-DP and/or 20β-S are the major progestins, and P4 is considered to be an intermediate in the synthesis of these steroids (see [1]). Both eel testis and ovary produce a large amount of 17α,20β-DP in vitro when the precursor is added to incubation medium [3,31,32], indicating that the eel gonads are capable of synthesizing 17α,20β-DP. Thin-layer chromatography analysis has shown that P4 is also produced by the ovary, but its levels were much lower than those of 17α,20β-DP [32], and P4 production by testis was not detected (Kobayashi et al., unpublished data). Other steroids which bind to and transactivate the eel DPR, 20β-OHP and 11-DOC, have not been identified as endogenous steroids, and 20β-S has been detected but its levels were very low in the eel ovary [32]. Taken together, these results confirm that 17α,20β-DP is the native ligand for the eel DPR.

Interestingly, 17α,20α-DP displayed neither binding nor transactivation function. The only structural difference between 17α,20β-DP and 17α,20α-DP is the position of a hydroxyl group at C20, suggesting that hydroxylation at C20 is essential for binding of ligand to the DPR. 17α,20α-DP has been found in mammals, and is thought to be an inactive progestin (see [33]). However, in a frog, *R. nigromaculata*, 17α,20α-DP has been identified as the spermiation-inducing steroid [4]. Therefore, it would be of considerable interest, from the standpoint of investigation of co-evolution of ligand and PR, to compare the molecular structures of PR-LBDs in various species.

Northern blot analysis showed that two DPR mRNAs with lengths approximately 8.5 and 5 kb were expressed in the eel testis. Multiple PR mRNAs were also detected in human (five mRNAs) [14] and chicken (three mRNAs) [19,20]. Both DPR mRNA levels in the eel testis fluctuated during HCG-induced spermatogenesis. DPR mRNA levels increased gradually until 6 days post injection, then decreased thereafter. The changes in DPR mRNA levels correlate with the progress of spermatogonial proliferation [34]. Involvement of progestins in early spermatogenesis has not been demonstrated in any vertebrate. In Japanese eel, 11-KT (a potent androgen in teleosts) has been identified as the spermatogenesis-inducing hormone (see [1]). It has also been shown that testicular AR (11-KT receptor) mRNA levels increased during HCG-induced eel spermatogenesis [8]. It is well known that the PR, AR, GR and MR act through a common hormone responsive element in their target genes [29]. Thus, the DPR may be involved in androgen-regulated eel spermatogenesis. DPR mRNA levels were relatively high in a spermiating eel. It has been shown that 17α,20β-DP plays an important role in final testicular maturation in Japanese eel [3]. However, the mechanisms underlying the regulation of testicular maturation by 17α,20β-DP are not fully understood. The cloned DPR cDNA will help to clarify the mechanism of 17α,20β-DP action in spermatogenesis and testicular maturation.

In conclusion, we have isolated a cDNA encoding a functional DPR. The recombinant protein expressed from the cDNA bound progestins, with limited capacity and progestin specificity. The cloned receptor is also capable of inducing transcription of a target gene only in response to progestins. In addition, strong evidence exists for 17α,20β-DP as the major native ligand for the DPR. The physiological actions of 17α,20β-DP mediated through a nuclear DPR are not understood in teleost fish, and the DPR cDNA should be a useful tool for study of the role of 17α,20β-DP in fish reproduction.

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