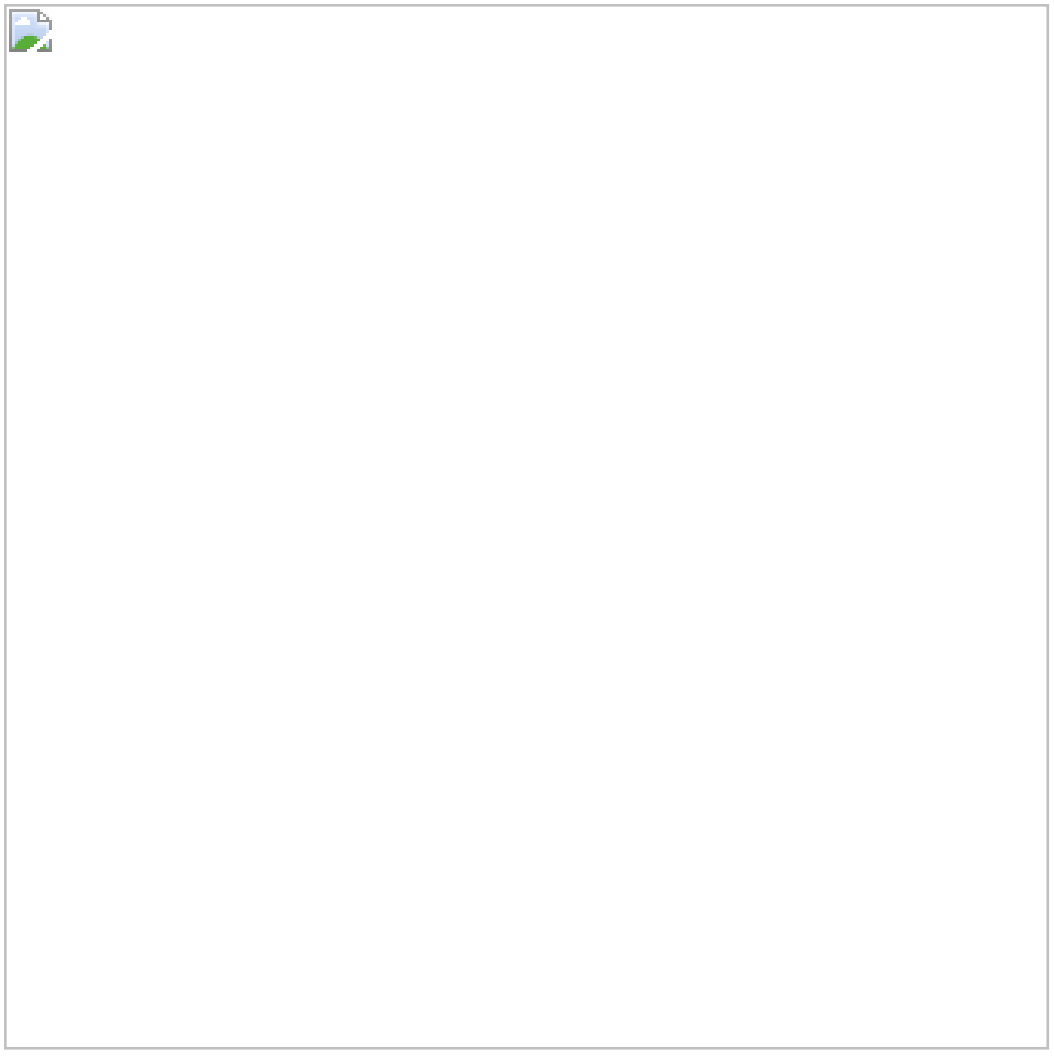

Hepatic Estrogen Receptors in the Japanese Eel, Anguilla japonica: Characterization and Changes in Binding Capacity during Artificially-induced Sexual Maturation

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Hepatic Estrogen Receptors in the Japanese Eel, *Anguilla japonica*: Characterization and Changes in Binding Capacity during Artificially-induced Sexual Maturation

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ABSTRACT—Estrogen receptors were identified in cytosolic and nuclear extracts of livers of female Japanese eel, *Anguilla japonica*. A single class of high affinity binding sites was found, with a Kd=0.97 nM for the cytosolic estrogen receptor (cER) and Kd=0.85 nM for the nuclear estrogen receptor (nER). Binding of both the cER and the nER was specific for estrogens (diethylstilbestrol: DES>estradiol-17β: E_2>>estriol: E_3>estrone: E_1). These binding characteristics of ERs were quite different from those of the serum estrogen-binding component; [^{3}H]E_2 binding to serum was not saturable, and was displaced by testosterone but not by DES, E_1 or E_3. The relationships between the levels of hepatic ERs, circulating E_2 and vitellogenin (VTG) during artificial maturation of cultivated female eels were examined, using eels injected weekly with chum salmon pituitary homogenate at a dose of 20 μg/g-body weight. Serum E_2 levels were constantly low during pre- to midvitellogenesis, and dramatically increased in the migratory nucleus stage. However, VTG levels gradually increased from early to midvitellogenesis, and were greatly elevated in the migratory nucleus stage. Hepatic cER levels slightly increased in early vitellogenesis, and then increased significantly from midvitellogenesis to the migratory nucleus stage. In contrast, nER levels did not change significantly, although nER levels in the migratory nucleus stage were higher than those at other stages. The changes in cER levels represent increased hepatic responsiveness to estrogenic stimuli during artificial maturation. Lack of change in nER levels may be a feature of artificial maturation compared to sexual maturation in nature.

INTRODUCTION

In teleosts, as in other oviparous vertebrates, estrogens are known to induce the production of vitellogenin (VTG), the precursor of the egg yolk proteins, in the liver (see [12]). It is assumed that estrogens act through a hepatic estrogen receptor (ER). Estrogens binding to ER is a prerequisite for hepatocyte stimulation. The resulting estrogen-ER complex acts on nuclear constituents and induces VTG gene transcription. Hepatic ERs have been found in many species of teleost (see [2, 8]). In addition, the ER is itself estrogen inducible, and it has been suggested that ER synthesis is involved in the induction of VTG synthesis [14].

Vitellogenesis has been correlated with elevated estradiol-17β (E_2) and VTG levels in the blood of various teleostean species (see [12]). However, changes in the concentration of ERs in the liver during the reproductive period have been studied in only one species of teleost, the spotted seatrout, *Cynoscion nebulosus* [23], and a few species of other oviparous vertebrates [15, 16, 18–20, 29]. These reports have shown that hepatic ER levels correlate well with circulating E_2 and VTG during vitellogenesis.

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Cultivated females of the Japanese eel (*Anguilla japonica*) have immature ovaries which do not enter vitellogenesis unless fish are treated with gonadotropic preparations such as salmon pituitary homogenate [5, 25, 27]. Therefore, the eel is a good model for studying the regulatory mechanisms of VTG synthesis, including the role of ERs in this process. In European eel, the hepatic ER has been characterized [10], and induction of ER synthesis by E_2 was also reported [11]. However, there is no information on changes in hepatic ER levels during the maturational process in eel.

In the present study, we have characterized the ERs in the liver of the Japanese eel. In addition, we have examined the relationships between the levels of hepatic ERs, circulating E_2 and VTG in females during artificially-induced sexual maturation.

MATERIALS AND METHODS

Fish and tissue handling

Female cultivated Japanese eels (369–956 g in body weight) were purchased from a commercial eel supplier. They were kept in recirculating seawater tanks with a capacity of 1,000 l at 20°C. Fish were not fed throughout the experimental period.

About 20 eels received weekly intramuscular injections of chum salmon pituitaries homogenized in eel Ringer [5] at a dose of 20 μg per g body weight. Sampling was performed at various stages of ovarian development, within 3 days of each injection. The fish were

anesthetized with 0.1% ethyl aminobenzoate. Blood was obtained from the caudal vasculature by cutting off the tail. Serum was separated by centrifugation at 2,000 g for 10 min at 4°C and stored at −30°C until use. Ovaries were dissected out, and follicles were isolated by dissection in ice-cold eel Ringer. Oocyte diameters were measured for 100 follicles per fish using a Profile projector (Nikon, Tokyo). The mean diameter of the largest group of 20 follicles was calculated to estimate the stage of oocyte development. The stages were classified as follows: pre-vitellogenesis (the oil droplet stage), 150–200 μm; early vitellogenesis, around 300 μm; midvitellogenesis, around 400–500 μm; late vitellogenesis, around 500–600 μm; migratory nucleus stage, above 700 μm [26].

Preparation of subcellular fractions from liver

All procedure were carried out at 0–4°C. After anesthesia and collection of blood, eels were laparotomized, and the livers were perfused by injection of ice-cold eel Ringer into the hepatic portal vein to remove blood completely. Livers were then removed, weighed, minced in ice-cold buffer A (0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$, 1 mM dithiothreitol (DTT), containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml each of leupeptin, pepstatin, chymostatin, antipain and benzamidine), and washed three times with buffer A. The minced liver was homogenized in 3 vol (w/v) of buffer A using a Polytron mechanical homogenizer for 30 sec, and then by five strokes with a motor-driven Teflon-glass homogenizer. The homogenate was passed through three layers of nylon screens (pore size: 200, 80, and 30 μm respectively) and then centrifuged at 1,800 g for 10 min. After lipid floating on top of the supernatant was removed, one tenth volume of glycerol was added to the supernatant. The supernatant was centrifuged at 100,000 g for 1 hr to obtain crude cytosol. Saturated ammonium sulfate solution was then slowly added to the supernatant under continuous stirring at 0°C to a maximum concentration of 33%. The suspension was left to settle for 1 hr and was then centrifuged at 10,000 g for 30 min. The supernatant was discarded and the pellet was dissolved in buffer B (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM DTT, 10% glycerol, containing 1 mM PMSF, 1 μg/ml each of leupeptin, pepstatin, chymostatin, antipain and benzamidine) to use as the cytosolic extract.

The remaining nuclear pellet was washed three times with 20 vol of buffer A, and resuspended in 1 vol/g liver of buffer C (buffer B containing 0.5 M KCl). The nuclear suspension was frozen in liquid nitrogen to lyse nuclei, allowed to thaw slowly at 0°C, and then homogenized with five gentle strokes of a glass-glass homogenizer. The nuclear lysate was incubated at 0°C for 1 hr with occasional mixing. The lysate was centrifuged at 100,000 g for 1 hr and the supernatant used as the nuclear extract. The cytosolic and nuclear extracts were frozen in liquid nitrogen and stored at −85°C until analysis.

Protein concentrations in the liver fractions were determined by the method of Bradford [1].

Preparation of serum for binding experiment

Vitellogenic eels were anesthetized and cut off the tail. Blood was collected directly into a chilled centrifugation tube, and mixed with protease inhibitors (1 mM PMSF, 1 μg/ml each of leupeptin, pepstatin, chymostatin, antipain and benzamidine). After collection, the blood was centrifuged at 2,000 g for 10 min at 4°C. The supernatant was collected and diluted with 1 vol of buffer B. The resulting solution was centrifuged at 100,000 g for 1 hr at 4°C. The supernatant was removed, treated with charcoal-dextran suspension (an equal volume of 0.5% Norit-A charcoal and 0.05% Dextran T-70 in buffer B) at 0–4°C for 5 min and centrifuged at 2,000 g for 10 min at 4°C. The supernatant was frozen in liquid nitrogen and stored at −85°C until use.

Measurement of [^{3}H]estradiol-17β binding

Samples of hepatic cytosol, nuclear extract or serum were usually incubated with 5 nM [2,4,6,7,-^{3}H]estradiol-17β ([^{3}H]E_2; 3.7 GBq/μmol; New England Nuclear, Boston, MA) in a total volume of 500 μl. Parallel tubes containing the [^{3}H]E_2 plus a 100-fold excess of radioinert diethylstilbestrol (DES) were also incubated for determination of nonspecific binding. All samples were incubated in duplicate or triplicate. After incubation for 6 hr at 4°C, the bound [^{3}H]E_2 was separated from free by treatment with charcoal-dextran suspension (an equal volume of 0.5% Norit-A charcoal and 0.05% Dextran T-70 in buffer B for cytosolic extract, or buffer C for nuclear extract) at 0–4°C for 5 min and centrifugation for 10 min at 1,800 g. Aliquots of the supernatant were counted for radioactivity in Aquasol II (New England Nuclear, Boston, MA). Specific binding was calculated by subtracting nonspecific (presence of DES) from total (absence of DES) binding. For Kd determinations, samples, obtained from a pool of 5 livers, were incubated with 0.2 to 10 nM [^{3}H]E_2 with and without a 100-fold molar excess of DES. The specific binding data were analyzed by Scatchard's graphic method [21]. To evaluate binding specificity, parallel samples were incubated with 5 nM [^{3}H]E_2 with and without a 100-fold excess of various radioinert steroids. In order to estimate ER concentrations at different stages, samples were incubated with increasing amounts of [^{3}H]E_2 with or without a 100-fold excess of DES, and the maximal binding capacity (Bmax) was calculated by the method of Scatchard [21], as described above.

Measurement of serum E_2 and VTG

Serum E_2 was assayed by radioimmunoassay according to the method described by Kagawa *et al.* [6].

The VTG levels in the serum were determined by single radial immunodiffusion (SRID) [9], and/or enzyme-linked immunosorbent assay (ELISA) [13], using a rabbit antiserum against eel yolk protein that specifically recognizes VTG. The minimal detectable serum concentration of VTG was 25 μg/ml in SRID, and 0.8 ng/ml in ELISA.

Statistical analysis

Data are presented as the mean ± SE. Statistical significance was assessed by analyses of variance (ANOVA), followed by Duncan's multiple range test.

RESULTS

Time course of [^{3}H]E_2 binding to cytosolic and nuclear extracts

Specific binding of [^{3}H]E_2 was measured in cytosolic and nuclear extracts from vitellogenic fish after various periods of incubation at 4°C. The association kinetics were similar in cytosolic and nuclear extracts (Fig. 1). Equilibrium was reached after 1 to 2 hr, and this binding was maintained for 12 hr. Since binding at 6 hr was slightly higher than at other periods, 6 hr was selected as the incubation time for subsequent binding experiments.

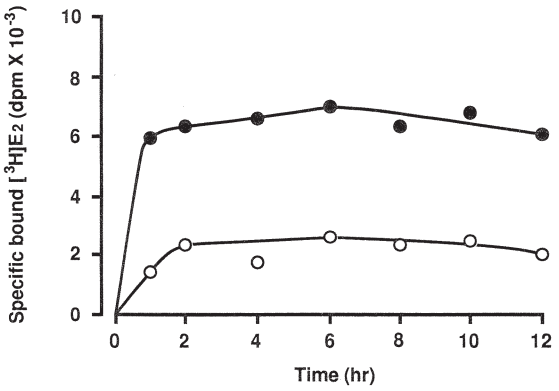

FIG. 1. Time course of $[^3H]E_2$ binding to hepatic cytosolic and nuclear extracts. Samples were incubated with 5 nM $[^3H]E_2$ in the absence (total binding) or presence (nonspecific binding) of 100-fold excess radioinert DES at 4°C for various periods. Specific binding was calculated as the difference between total and nonspecific binding. Specific binding in cytosolic and nuclear extracts are represented by open and closed circles, respectively. Each point represents the mean of triplicate determinations of a representative experiment from three experiments that were conducted on different liver pools.

Saturation analysis of cytosolic and nuclear estrogen binding

Using cytosolic extracts from a vitellogenic female, saturation binding assays and Scatchard plots showed a single class of high affinity binding sites with an equilibrium dissociation constant, Kd, of 0.97 nM and a maximal binding capacity (Bmax) of 78.4 fmol/mg protein (Fig. 2). In the nuclear extract, a single class of high affinity binding sites was found with a Kd of 0.85 nM and a Bmax of 37.3 fmol/mg protein (Fig. 3).

Serum samples were also subjected to saturation analysis. However, specific binding of $[^3H]E_2$ was not saturable in the concentration range of 0.2 to 20 nM, and binding could not be analyzed by Scatchard analysis (data not shown).

Steroid-binding specificity of serum and cytosolic and nuclear extracts

The specificity of estrogen binding to the liver fractions and serum was determined by competition of $[^3H]E_2$ binding with unlabeled steroids (Table 1). Binding in both cytosol and nuclear extracts was specific for estrogens. The synthetic estrogen DES was more potent than E_2 and the other natural estrogens, estrone and estriol, which were moderately effective competitors. Other steroid hormones did not compete with $[^3H]E_2$ for binding. This specificity profile was different from that of serum estrogen binding under the same assay conditions. In the serum, testosterone was an effec-

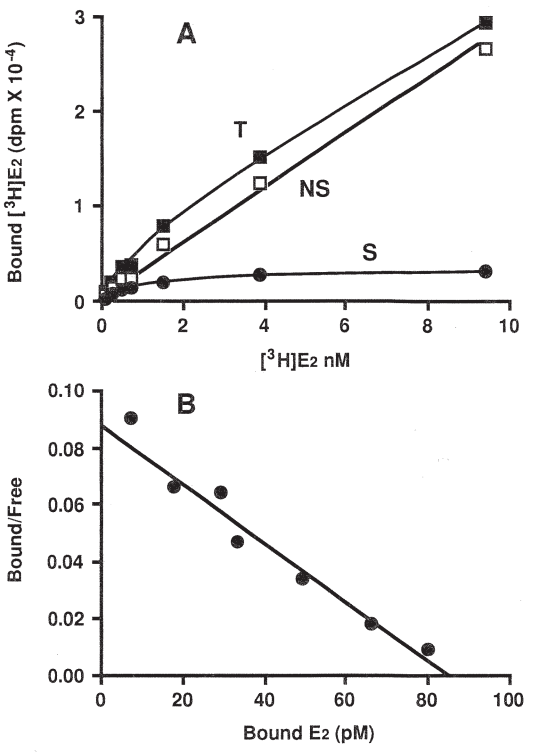

FIG. 2. Saturation analysis of $[^3H]E_2$ binding in a cytosolic extract. (A) The extract was incubated for 6 hr at 4°C with a range of $[^3H]E_2$ concentrations. T, total binding; NS, nonspecific binding; S, specific binding. (B) Scatchard plot of the specific binding in (A). Kd=0.97 nM, Bmax=78.4 fmol/mg protein. Each point represents the mean of triplicate determinations of a representative experiment from two experiments.

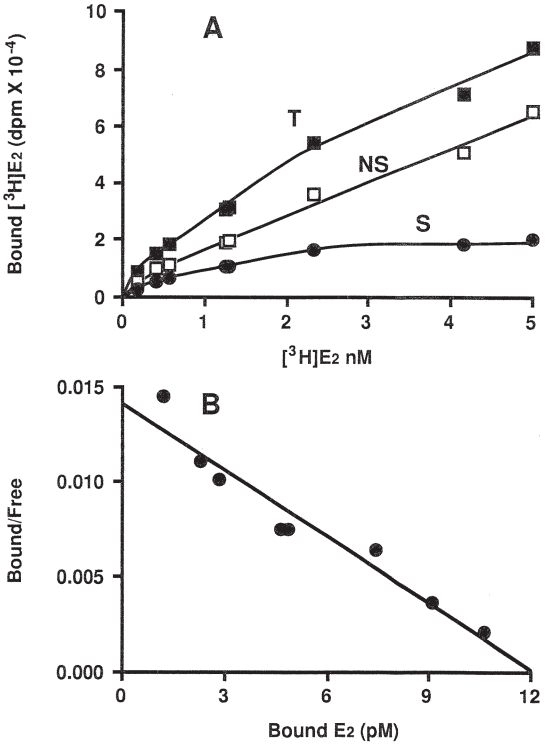

FIG. 3. Saturation analysis of $[^3H]E_2$ binding in a nuclear extract. (A) The extract was incubated for 6 hr at 4°C with a range of $[^3H]E_2$ concentrations. Calculation of specific binding and the symbols used are the same as in Figure 3. (B) Scatchard plot of the specific binding in (A). Kd=0.85 nM, Bmax=37.3 fmol/mg protein. Each point represents the mean of triplicate determinations of a representative experiment from two experiments.

TABLE 1. Steroid-binding specificity in liver cytosolic and nuclear extracts, and in serum

Competitor	% Displacement		
	Cytosol	Nuclear	Serum
Estradiol-17β	100	100	100
Diethylstilbestrol	136.5	115.4	8.5
Estrone	32.5	24.1	8.4
Estriol	90.8	41.8	14.6
Testosterone	8.0	0	70.9
11-Ketotestosterone	25.3	0	27.9
Progesterone	0	0	5.1
17α, 20β-Dihydroxy-4-pregnen-3-one	0	0	2.6
Cortisol	0	0	5.4

Note. Samples were incubated with 5 nM [^{3}H]E_2 with and without 100-fold excess radioinert competitor. Displacement was calculated as the percentage of maximum displacement as measured by the depression of binding in the presence of 500 nM E_2. Each point represents the mean of triplicate determinations of a representative experiment from two experiment.

tive competitor, while DES, estrone and estriol were poor competitors.

Changes in serum E_2 and VTG during artificial maturation

Changes in serum E_2 and VTG levels during artificial maturation of cultivated female are shown in Figure 4. In the present experiment, vitellogenesis started after 5–9 injections and was completed after 12–16 injections. Serum levels of E_2 were constantly low during pre- to mid-vitellogenesis (0.3–0.6 ng/ml), and dramatically increased in the migratory nucleus stage (5.18 ± 1.82 ng/ml, $p<0.01$). VTG could not be detected in initial controls (pre-vitellogenesis, <0.8 ng/ml), but increased from early (2.89 ± 1.36 mg/ml) to midvitellogenesis (4.65 ± 1.37 mg/ml). In the migratory nucleus stage, levels were much higher but varied among individuals (24.13 ± 11.398 mg/ml, $p<0.01$).

Changes in hepatic ERs during artificial maturation

Figure 5 shows the changes in hepatic ER levels during artificial maturation. Levels of cER increased slightly in early vitellogenesis, then increased significantly ($p<0.05$) in midvitellogenesis (105.72 ± 7.49 fmol/mg protein) and the migratory nucleus stage (93.58 ± 46.21 fmol/mg protein). In contrast, nER levels did not fluctuate significantly during gonadal development, but in the migratory nucleus stage, nER levels showed higher values (39.70 ± 3.10 fmol/mg protein) than those in other stages (pre: 33.38 ± 4.75, early: 21.71 ± 3.13, mid: 22.92 ± 6.95 fmol/mg protein). The affinities

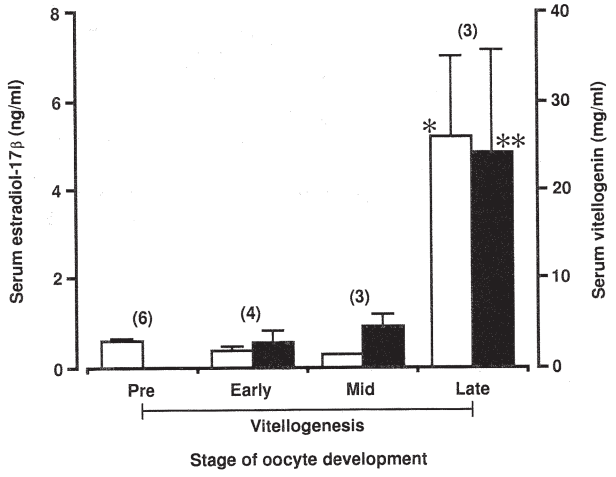

FIG. 4. Changes in serum E_2 and VTG levels during artificially-induced sexual maturation. Serum E_2 levels are represented by open columns, serum VTG levels are represented by closed columns. Vertical bars represent the SEM. Number of fish sampled is shown in parentheses. ND: non-detectable. *: Significantly different from other stages ($P<0.01$).

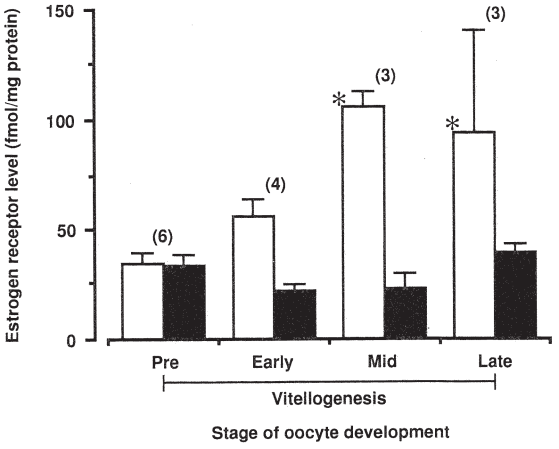

FIG. 5. Changes in hepatic cER and nER levels during artificially-induced sexual maturation. cER levels are represented by open columns, nER levels are represented by closed columns. Vertical bars represent the SEM. Number of fish sampled is shown in parentheses. *: Significantly different from other stages ($P<0.05$).

(Kd) of both ERs did not change significantly (0.4–0.9 nM) throughout sampling period (data not shown).

DISCUSSION

In the present study, the presence of ERs in hepatic cytosolic and nuclear extracts from Japanese eel was demonstrated. The ERs had a single class of high affinity estrogen-binding sites which were saturable. The binding affinities, Kd, of ERs (cytosol: 0.97 nM, nuclear: 0.85 nM) in Japanese eel liver were very similar to those reported in European eel [10] and other teleosts (see [8]). [^{3}H]E_2-binding to the eel ERs could be inhibited by estrogen only. Other steroids, such as androgens, progestogens, and cortisol, did not displace estradiol from the binding sites. Similar characteristics have been reported for ERs in the liver of salmonids (see [8]), spotted seatrout [22] and European eel [10]. These binding characteristics of the ERs in Japanese eel were consistent with the known properties of steroid receptors [2].

The serum of Japanese eel was also found to have estrogen-binding activity. However, the estrogen-binding characteristics of serum were quite different from those of receptors on the basis of its non-saturable binding of E_2, its lack of affinity for DES, estriol and estrone, and its relatively high affinity for testosterone. Recently, Chang *et al.* [3] have purified the sex steroid-binding protein (SSBP) from the plasma of Japanese eel. The molecular weight of the SSBP is 64 kDa, and the steroid-binding specificity of the SSBP is very similar to that of serum in the present study, i.e., high affinity for E_2, testosterone and androstenediol, low affinity for 11-ketotestosterone, and lack of affinity for other steroids. These results also indicate that the estrogen-binding component in liver fractions from Japanese eel found in the present study is the ER, and that contamination with SSBP is neglible.

In general, it has been considered that cER may be an artifact of homogenization. Free (unoccupied) ER is readily extracted from the nucleus into the cytosol with low ionic strength buffers. In contrast, receptors that are occupied by estrogen remain tightly bound to the chromatin under the same conditions, with extraction requiring 0.4–0.6 M salt. The enucleation [24], immunocytochemical [7], and recent molecular biological [28] experiments have clearly shown that free and occupied ERs are present in the nucleus. The characteristics of cER and nER in eel liver are quite similar to each other, strongly suggesting that they are the same molecule. In European eel, Messaouri *et al.* [11] have reported that 90% of cER disappeared quickly after E_2 injection, while nER increased. This result implies that cER tightly binds to the chromatin after binding E_2. Therefore, it appears that cERs are unoccupied, while nERs are occupied.

In the present study, hepatic cER levels gradually increased during vitellogenesis, while nER levels did not change significantly. The changes in cER levels seem to reflect the ability of hepatocytes to produce VTG, which correlates well with serum VTG levels. The higher levels of cERs in sexually mature females compared to those in immature females or males was also reported in brown trout [17] and spotted seatrout [22]. In *Xenopus*, after withdrawal of estrogen from estrogen stimulated males, the hepatic cER content remained elevated 8- to 12-fold above the unstimulated level for over 70 days [4]. The elevated cER levels are believed to be responsible for the faster and stronger vitellogenic response during a second estrogen stimulation. These observations in *Xenopus* argue for a role of cERs in conferring estrogen sensitivity to the liver. Therefore, the elevated levels of cER during artificial maturation might lead to increased estrogen sensitivity of hepatocytes.

It is difficult to explain the lack of change in nER levels during artificial maturation when serum VTG and cER levels increased. In spotted seatrout, changes in hepatic nER levels correlated well with serum VTG, E_2 and hepatic cER profiles during the reproductive cycle [23]. In masu salmon, *Oncorhynchus masou*, more drastic changes in hepatic nER levels were observed using a method similar to that of present study (Todo *et al.* unpublished data). The levels of nER started to increase rapidly from early vitellogenesis, and reached a maximum (the values were 30-fold above those of pre-vitellogenic fish) in midvitellogenesis. The elevated nER levels were maintained until late vitellogenesis, followed by a dramatic decrease during final maturation. The similar positive correlation between serum VTG, E_2 and hepatic nER levels during the reproductive period was also observed in other oviparous species [15, 16, 18, 19, 29]. Therefore, these changes seem to be a common phenomenon in natural reproductive cycles of oviparous vertebrates, and lack of changes in nER in the present study may be a feature of artificial induction of maturation in eel.

It has been shown that hepatic ER synthesis is auto-regulated by estrogen [8]. Messaouri *et al.* [11] reported that both cER and nER levels in the liver increased after E_2 treatment in silver females of European eel. Recently, we also found that ER mRNA was induced by E_2 in the liver of immature Japanese eel (unpublished data). Thus, in eel, hepatic ER synthesis is also up-regulated by E_2. However, in the migratory nucleus stage, both cER and nER levels did not increase despite dramatic increases in serum E_2 and VTG levels. Furthermore, lipid content in liver tissue increased abnormally during artficial maturation, expecially at the migratory nucleus stage when lipid droplets occupied large areas of the hepatocytes (unpublished data). In fact, the first supenatant of liver homogenate at the migratory nucleus stage contained large amounts of lipids, so that the yields of cytosol and nucleus were lesser than those at other stages. Thus, lipids might cause the loss of ERs during homogenization.

In summary, the presence of specific ERs in both hepatic cytosol and nucleus of Japanese eel was demonstrated. Both ERs have very similar properties. However, changes in the ERs during artifical maturation showed different patterns. Levels of cER gradually increased during vitellogenesis, while nER levels did not change significantly. In

artificial maturation, fish are treated with massive amounts of salmon pituitary homogenate which contains not only gonadotropin but also many unknown factors. These factors may be involved in VTG synthesis in the liver. In order to clarify the machanism of VTG synthesis in the eel, more intensive studies using more precise techniques, such as primary hepatocyte culture systems and a molecular approach, are required.

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