HOKKAIDO UNIVERSITY

Title	Studies on estrogen receptor system in the Japanese eel, Anguilla japonica
Author(s)	Todo, Takashi
Degree Grantor	北海道大学
Degree Name	博士(水産学)
Dissertation Number	甲第3724号
Issue Date	1995-12-25
DOI	https://doi.org/10.11501/3108191
Doc URL	https://hdl.handle.net/2115/51293
Type	doctoral thesis
File Information	000000292931.pdf

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主論文

Studies on estrogen receptor system in the Japanese eel, *Anguilla japonica*

Takashi Todo

1995

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Studies on estrogen receptor system in the Japanese eel, *Anguilla japonica*

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Contents

Acknowledgement ----------3

Chapter I. An introduction to studies on estrogen receptor system in the Japanese eel, *Anguilla japonica* ----------5

Chapter II. Characterization of hepatic estrogen receptors in the Japanese eel using radioreceptorassay ----------12

Chapter III. Molecular cloning and characterization of a Japanese eel estrogen receptor cDNA ----------35

Chapter IV. Expression of estrogen receptor mRNA in liver and testis of the Japanese eel ----------57

Chapter V. Summary and conclusions ----------72

References ----------79

Acknowledgments

I would like to take this opportunity to thank the following people whose help and co-operation have made this study possible:

My supervisor, Professor Kohei Yamauchi, Laboratory of Physiology, Department of Marine Biological Science, Faculty of Fisheries, Hokkaido University, for his guidance, support and encouragement throughout my graduate research.

Senior Lecturer Shinji Adachi, Laboratory of Physiology, Department of Marine Biological Science, Faculty of Fisheries, Hokkaido University, for his invaluable advice, instructive discussions and constant encouragement throughout this study.

Professor Akihiko Hara, Laboratory of Physiology, Department of Marine Biological Science, and Associate Professor Hiroshi Ueda, Toya Lake Station for Environment Biology, Faculty of Fisheries, Hokkaido University, for their encouragement, valuable discussions and critical reading the manuscript.

Professor Yoshitaka Nagahama, Laboratory of Reproductive Biology, National Institute for Basic Biology, for his encouragement and providing me a chance to complete this thesis.

Professor Graham Young, Department of Zoology, University of Otago, for his helpful discussion and critical reading of the manuscript.

Research Associate Takeshi Miura, Laboratory of Physiology, Department of Marine Biological Science, Faculty of Fisheries, Hokkaido University, for his encouragement, helpful advises and invaluable discussions.

Drs. Akihiro Takemura, Hideaki Yamada, Kiyoshi Soyano, Yasunori Koya, Koichiro Gen, Kazuhiko Mochida, Messrs. Komei Hotta, Hiroaki Chiba, Tsuyoshi Saga, Masaki Nagae, Kazuhiro Ura, Hideaki Kudo, Fumihiro Saeki, Mrs. Tomoko Ito, Miss Hiromi Okumura and all the colleagues who have been studied in Laboratory of Fresh-Water Fish-Culture, Faculty of Fisheries, Hokkaido University, during these years for their encouragement, assistance and stimulating discussions.

Chapter I.

An Introduction to the Studies on Estrogen Receptor System in the Japanese Eel, *Anguilla japonica*

The Japanese eel, *Anguilla japonica*, is a crucial important species in the aquaculture industry of Japan. However, the life history of the eel is complex, and little is known about its reproduction because sexually mature eels have never been found in the wild. Under the present condition, the supply of eel larvae for stocking farm is entirely dependent on capture in the wild. Therefore, it is important that a reliable supply of larvae is established. In this context, artificial control of gonadal development is very important for obtaining eel larvae.

Japanese eels start to enter sexual maturation when catadromous migration occur in autumn or early winter at the age of 5-12 years. At that time, eels have immature gonads which contain pre- or early vitellogenic oocytes in females and only spermatogonia in males. However, they do not undergo the further gonadal development under aquarium conditions. Therefore, hormone treatments have been demonstrated in order to promote the gonadal development (Yamauchi and Yamamoto, 1982).

Sexual maturity has been achieved by the treatments with salmon pituitary homogenate (SPH) for female and human chorionic gonadotropin (HCG) for male (Yamamoto *et al.*, 1972; 1974). Using these treatments, fertilized eggs and larvae have been obtained, although the larvae did not survive for more than 14 days (Yamamoto and

Yamauchi, 1974; Yamauchi *et al.*, 1976). However, the success rate of the SPH treatment is low, in contrast with males in which the induction of maturation is relatively easy (Yamauchi, 1990). For examples, the responsibility for the SPH is varied among individuals, and the majority do not undergo final maturation and ovulation (Yamauchi and Yamamoto, 1982; Ijili *et al.*, in press). Therefore, the basic research on the control mechanism of gonadal development is required, to establish a method for the artificial control of sexual maturation.

It is well established that in teleosts, as in other vertebrates, the pituitary-gonadal axis plays an important role in regulating gametogenesis. In most cases, gonadotropins (GTH) act through the biosynthesis of gonadal steroid hormones which in turn mediate various stages of gametogenesis including oocyte growth, oocyte maturation, spermatogenesis and spermiation (see Nagahama, 1987).

In all vertebrate species, estrogen, estradiol-17β (E_2) in most cases, is involved in many aspects of female reproduction. In teleosts, as in other oviparous vertebrates, a major effect of E_2 is the control of synthesis of vitellogenin (VTG), the precursor of yolk protein, in the liver (see Wallace, 1985). In the phase of oocyte growth termed vitellogenesis, E_2 is produced by the ovary under the influence of GTH, introduced into the circulatory system and stimulates hepatic synthesis

and secretion of VTG (summarized in Fig. 1). VTG is selectively incorporated into oocytes through a receptor-mediated endocytosis, and accumulated as yolk proteins which are going to be the important nutrition for embryo.

It is assumed that the action of E_2 is mediated by a specific receptor (estrogen receptor; ER) that is present in the nucleus of target cells. The ER is a transcriptional factor that directly binds to the specific target DNA sites and regulates the transactivation of target genes (Green and Chambon, 1988). The activation of the transcription of the VTG gene in hepatocytes of oviparous vertebrates, especially *Xenopus laevis*, has become an important model system for studies of estrogen action at the gene level (see Wahli, 1988). A number of studies in *Xenopus* clarified the mechanism of E_2 action on VTG gene expression as follows. After entering hepatocytes by diffusion, the E_2 binds to the ER in nucleus. The E_2-ER complex formed a homodimer binds tightly to the estrogen responsive elements (ERE) identified as palindrome sequence of AGGTCAnnnTGACCT and located upstream of VTG gene. This results in bending of DNA and activation and enhancement of transcription of VTG gene in association with other transcriptional factors (see Shapiro *et al.*, 1994). As described above, the ER is a

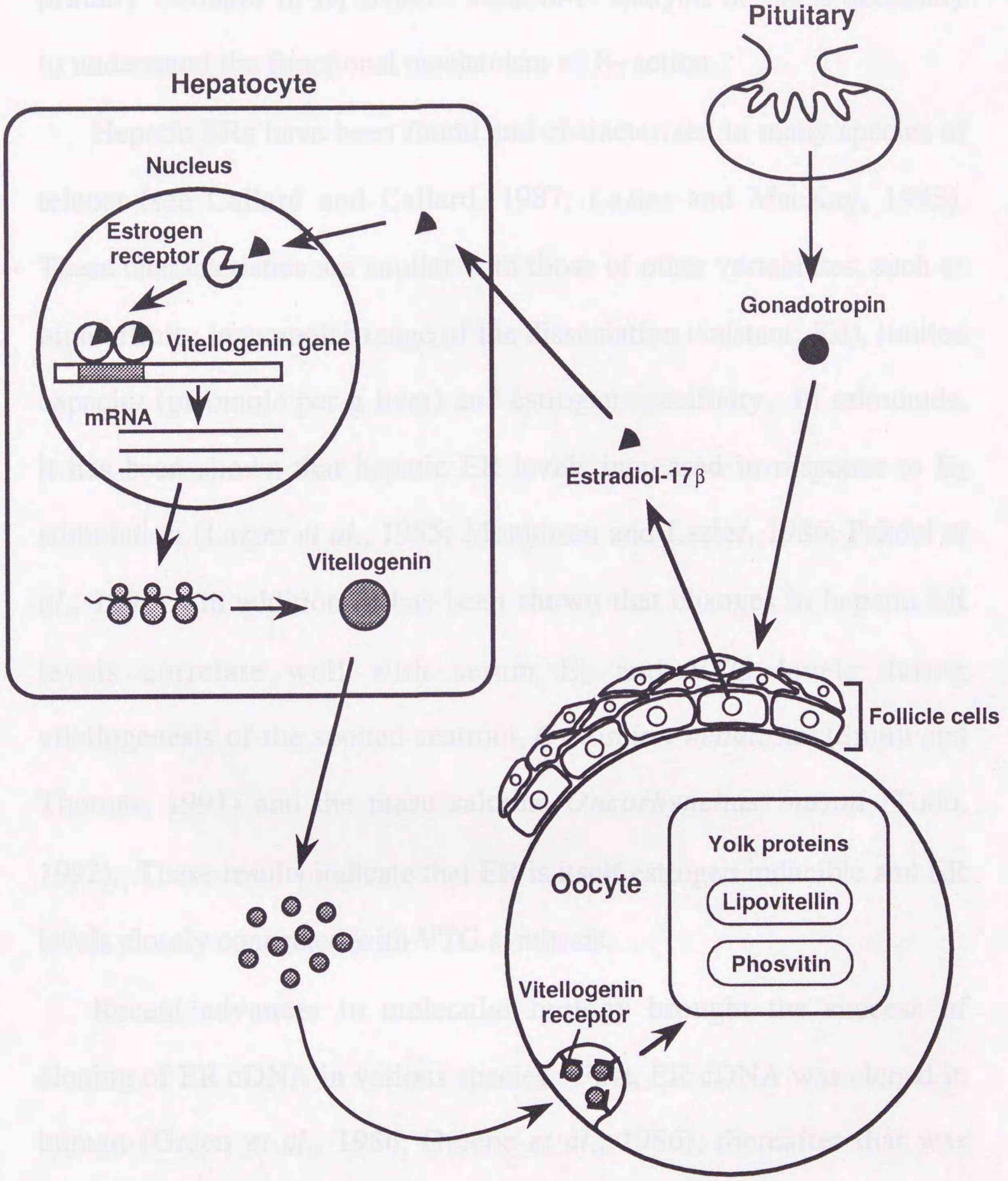


Fig. 1. Simplified schemaic representation of endocrine control of vitellogenesis

primary mediator of E_2 action. Therefore, analysis of ER is necessary to understand the functional mechanism of E_2 action.

Hepatic ERs have been found and characterized in many species of teleost (see Callard and Callard, 1987; Lazier and MacKay, 1993). These characteristics are similar with those of other vertebrates, such as high affinity (nanomolar range of the dissociation constant; Kd), limited capacity (picomole per g liver) and estrogen specificity. In salmonids, it has been shown that hepatic ER levels increased in response to E_2 stimulation (Lazier *et al.*, 1985; Mommsen and Lazier, 1986; Pakdel *et al.*, 1991). In addition, it has been shown that changes in hepatic ER levels correlate well with serum E_2 and VTG levels during vitellogenesis of the spotted seatrout, *Cynoscion nebulosus* (Smith and Thomas, 1991) and the masu salmon, *Oncorhynchus masou* (Todo, 1992). These results indicate that ER is itself estrogen inducible and ER levels closely connected with VTG synthesis.

Recent advances in molecular biology brought the success of cloning of ER cDNA in various species. First, ER cDNA was cloned in human (Green *et al.*, 1986; Greene *et al.*, 1986), thereafter that was cloned in other species utilizing the homology with human ER (other mammalian species: Koike *et al.*,1987; White *et al.*, 1987; chicken: Krust *et al.*, 1986; Maxwell *et al.*, 1987; *Xenopus laevis* : Weiler *et*

al., 1987; rainbow trout: Pakdel *et al*., 1990). On the basis of amino acid sequence homology, the ER has been divided into six domains designated A-F (Krust *et al*., 1986). The most conserved domain is C, which is basic and contains a number of cysteine residues able to form the zing fingers (Schwabe *et al*., 1990). The second most conserved region is E which is particularly hydrophobic. Functional studies indicate that C and E domains are required for DNA binding and ligand binding, respectively (Kumar *et al*., 1987). These two domains are highly conserved among various species from fish to mammals (C domain: 90-100%; E domain: 60-95%), indicating that the basic functions of ER have been conserved during evolution

In the present study, the ER system in the liver of the Japanese eel was investigated, in order to clarify the mechanism of E_2 action during the maturational process in eel. First, the hepatic ERs were characterized by radioreceptorassay, and changes in hepatic ER levels during artificial maturation were also examined. Next, a Japanese eel ER cDNA was isolated and characterized. Finally, the expression of eel ER mRNA in response to E_2 in the liver and changes in its levels during artificial maturation were examined by Northern blotting. In addition, ER mRNA expressed in testis was also investigated.

Chapter II.

Characterization of Hepatic Estrogen Receptors in the Japanese Eel using Radioreceptorassay

Introduction

Vitellogenesis has been correlated with elevated E_2 and VTG levels in the blood of various teleostean species (Mommsen and Walsh 1988). However, changes in the concentration of ERs in the liver during the reproductive period have been studied in only two species of teleosts, the spotted seatrout (Smith and Thomas 1991) and the masu salmon (Todo, 1992), and a few species of other oviparous vertebrates (Riley *et al.* 1987; Riley and Callard 1988a,b; Paolucci and Botte 1988; Yu and Ho 1989; Paolucci 1989). These reports have shown that hepatic ER levels correlate well with circulating E_2 and VTG during vitellogenesis.

In European eel, the hepatic ER has been characterized (Messaouri *et al.* 1991), and induction of ER synthesis by E_2 was also reported (Messaouri *et al.* 1992). However, there is no information on changes in hepatic ER levels during the maturational process in eel.

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

Material and Methods

Fish and tissue handling

Female cultivated Japanese eels (369-956g 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 SPH in eel Ringer (Ijiri *et al.* in press) 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 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.

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 (1976).

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 (Scatchard, 1949). 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, as described above.

Measurement of serum E_2

Serum E_2 was assayed by radioimmunoassay according to the method described by Kagawa *et al.* (1982).

Measurement of serum VTG

The VTG levels in the serum were determined by single radial immunodiffusion (SRID) (Mancini *et al.* 1965), and/or enzyme-linked immunosorbent assay (ELISA) (Okumura *et al.* 1995), 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 [^3H]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. 2). 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.

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. 3). 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. 4).

Serum samples were also subjected to saturation analysis. However, specific binding of [^{3}H]E_2 was not saturable in the concentration range

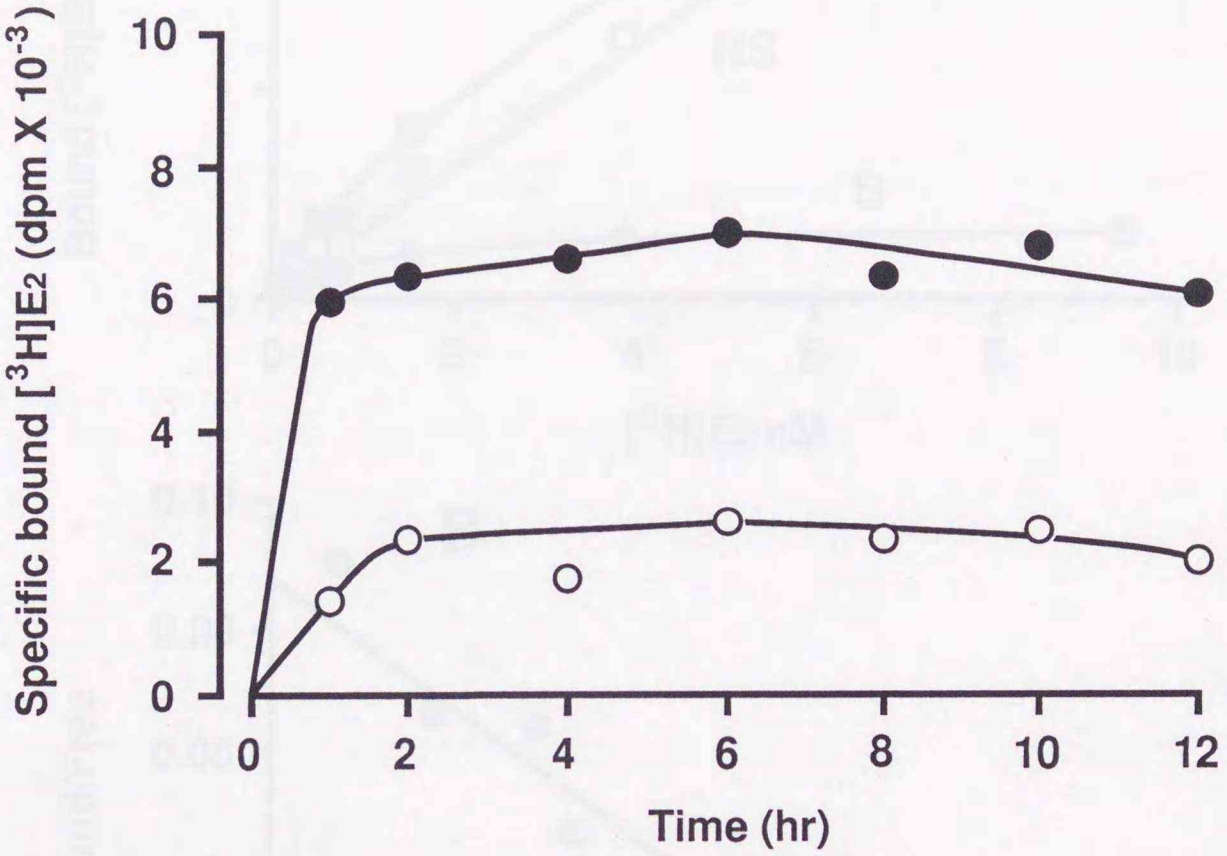


Fig. 2. Time course of [^{3}H]E2 binding to hepatic cytosolic and nuclear extracts. Samples were incubated with 5 nM [^{3}H]E2 in the absence (total binding) or presence (nonspecific binding) of 100-fold excess radioinert DES at 4°C for various periods. Specific binding was calculated as the difference between total and nonspecific binding. Specific binding in cytosolic and nuclear extracts are represented by open and closed circles, respectively.

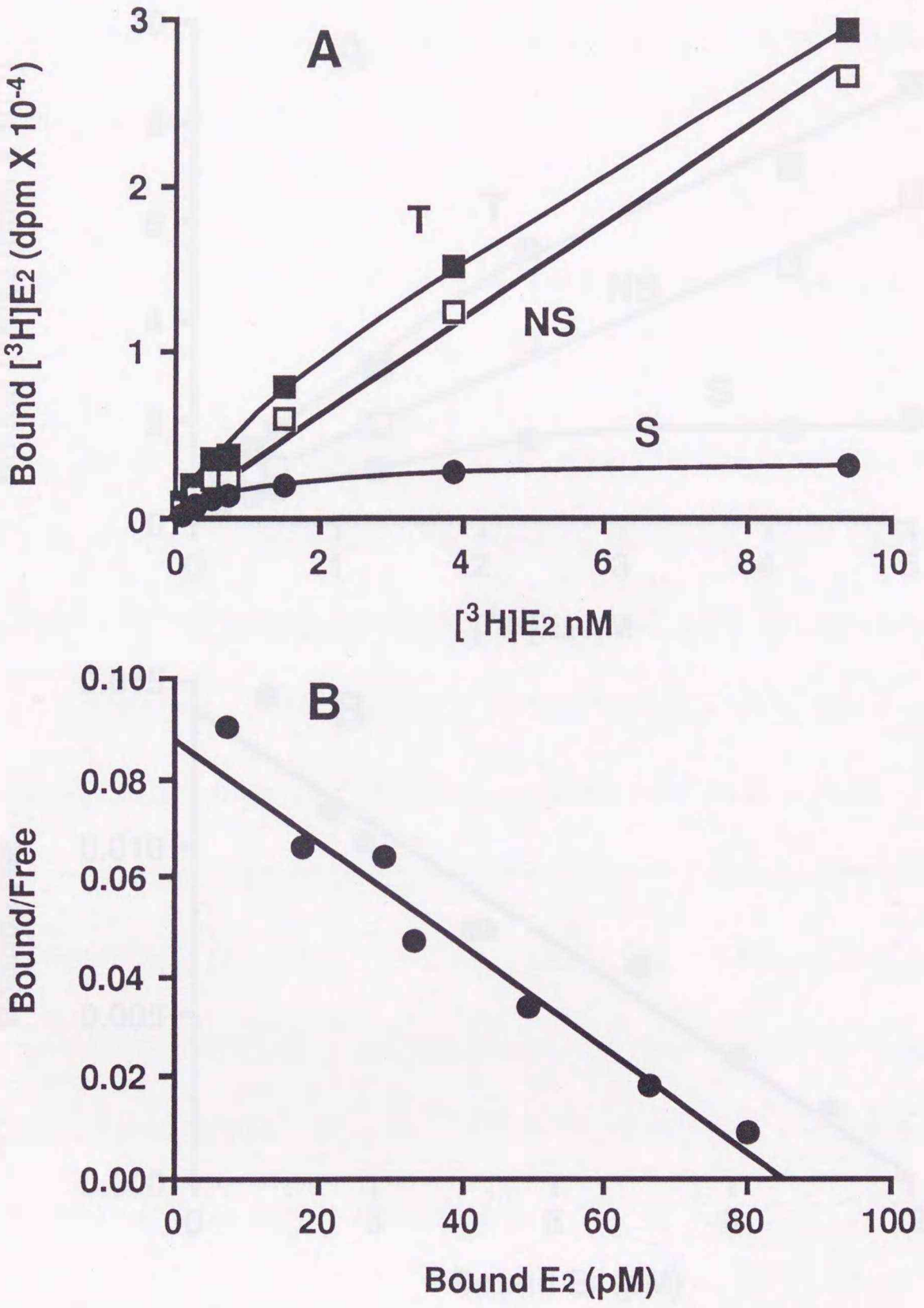


Fig. 3. Saturation analysis of [^{3}H]E2 binding in a cytosolic extract. (A) The extract was incubated for 6 hr at 4°C with a range of [^{3}H]E2 concentrations. T, total binding; NS, nonspecific binding; S, specific binding. (B) Scatchard plot of the specific binding in (A). Kd = 0.9 7 nM, Bmax = 78.4 fmol/mg protein.

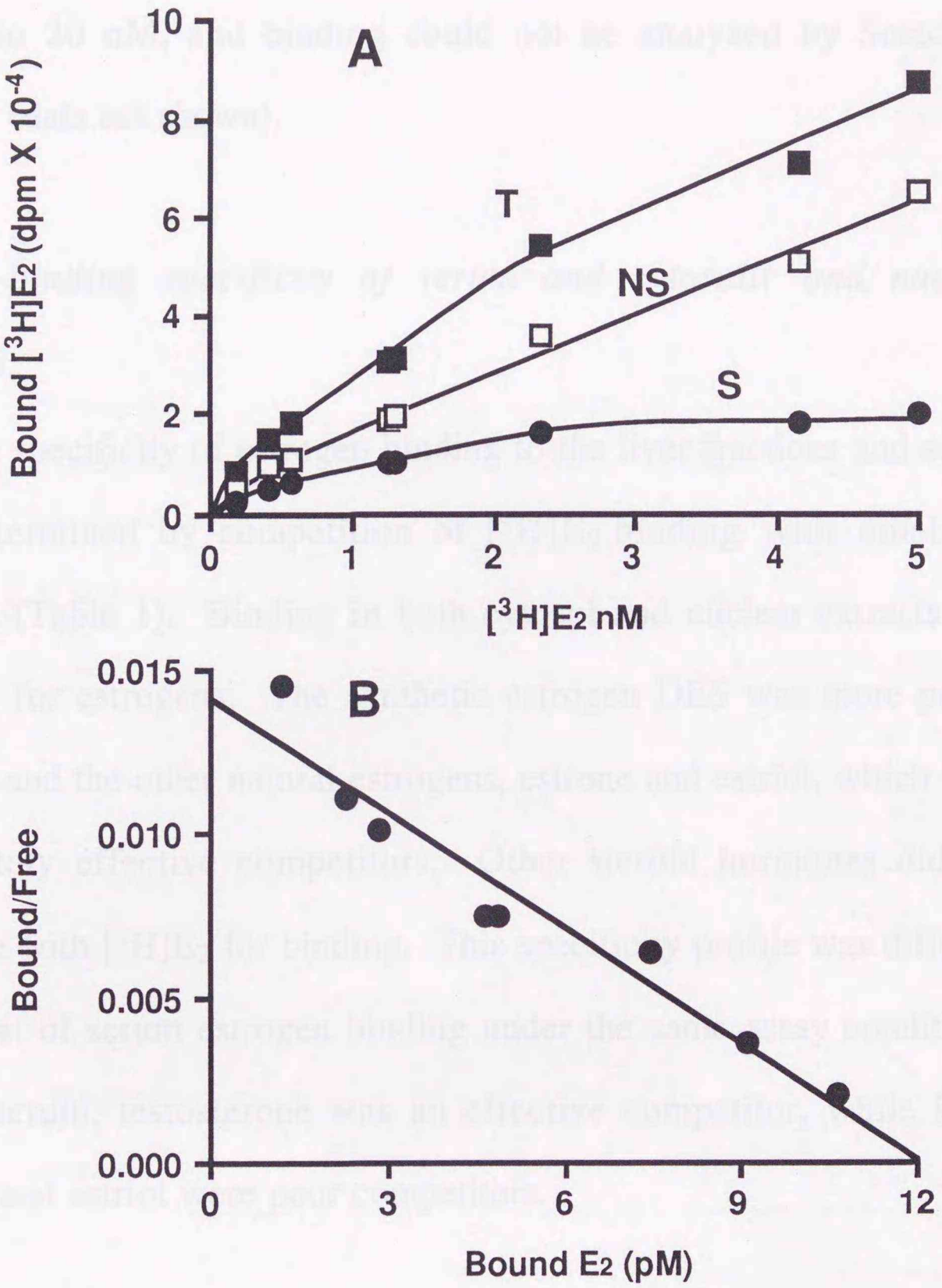


Fig. 4. Saturation analysis of [^{3}H]E2 binding in a nuclear extract. (A) The extract was incubated for 6 hr at 4°C with a range of [^{3}H]E2 concentrations. Calculation of specific binding and the symbols used are the same as in Fig. 3. (B) Scatchard plot of the specific binding in (A). Kd = 0.85 nM, Bmax = 37.3 fmol/mg protein.

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 effective 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 Fig. 5. In the present experiment, vitellogenesis started after 5-9 weeks and was completed after 12-16 weeks. Serum levels of E_2 were constantly low during pre- to

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]E2 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 E2 .

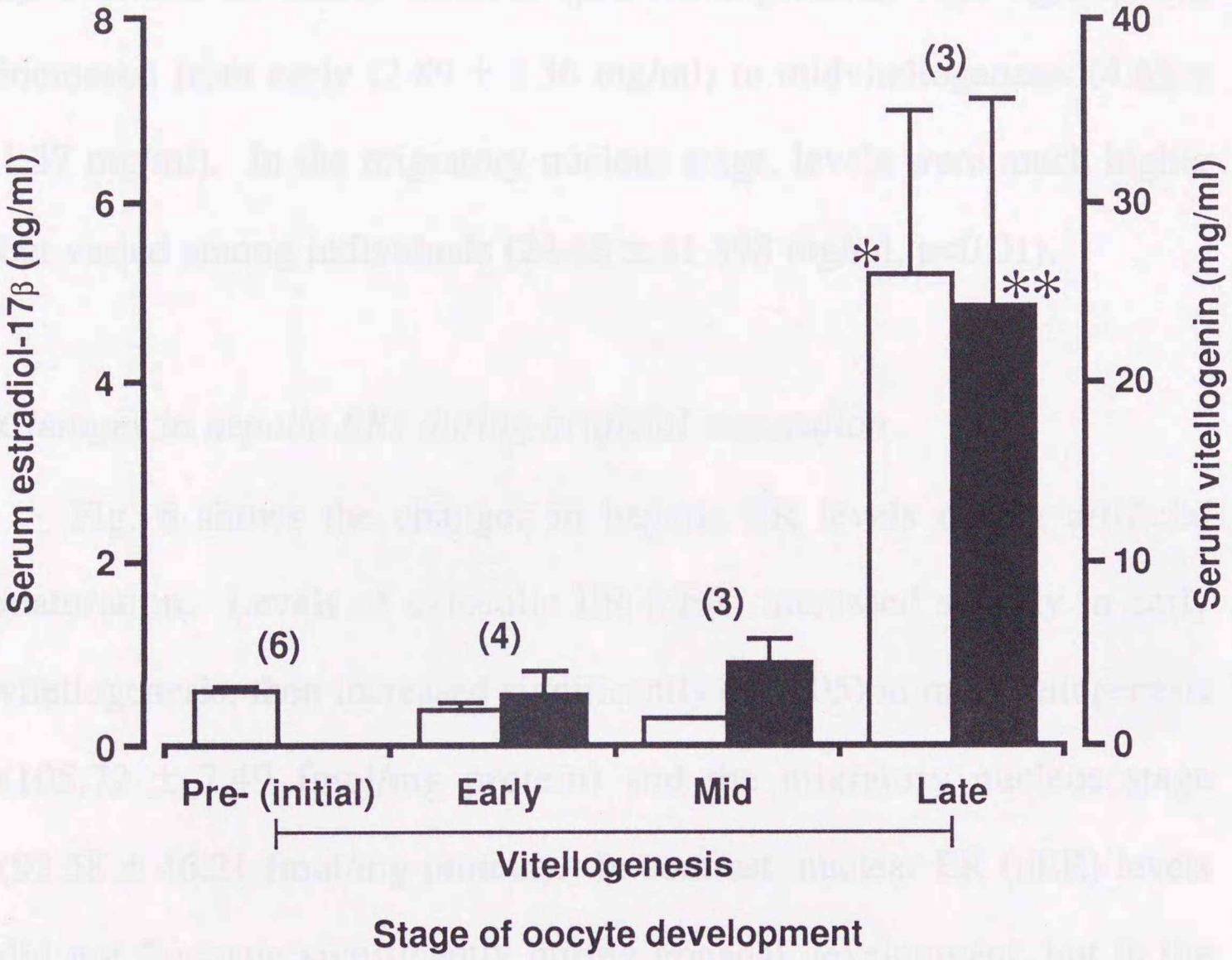


Fig. 5. Changes in serum E_2 and VTG levels during artificially-induced sexual maturation. Serum E_2 levels are represented by open columns, serum VTG levels are represented by closed columns. Vertical bars represent the SEM. Number of fish sampled is shown in parentheses. ND: non-detectable. *: Significantly different from other stages ($p<0.01$).

midvitellogenesis (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

Fig. 6 shows the changes in hepatic ER levels during artificial maturation. Levels of cytosolic ER (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, nuclear ER (nER) levels did not fluctuate significantly during gonadal development, but in the migratory nucleus stage, nER levels tended to be 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).

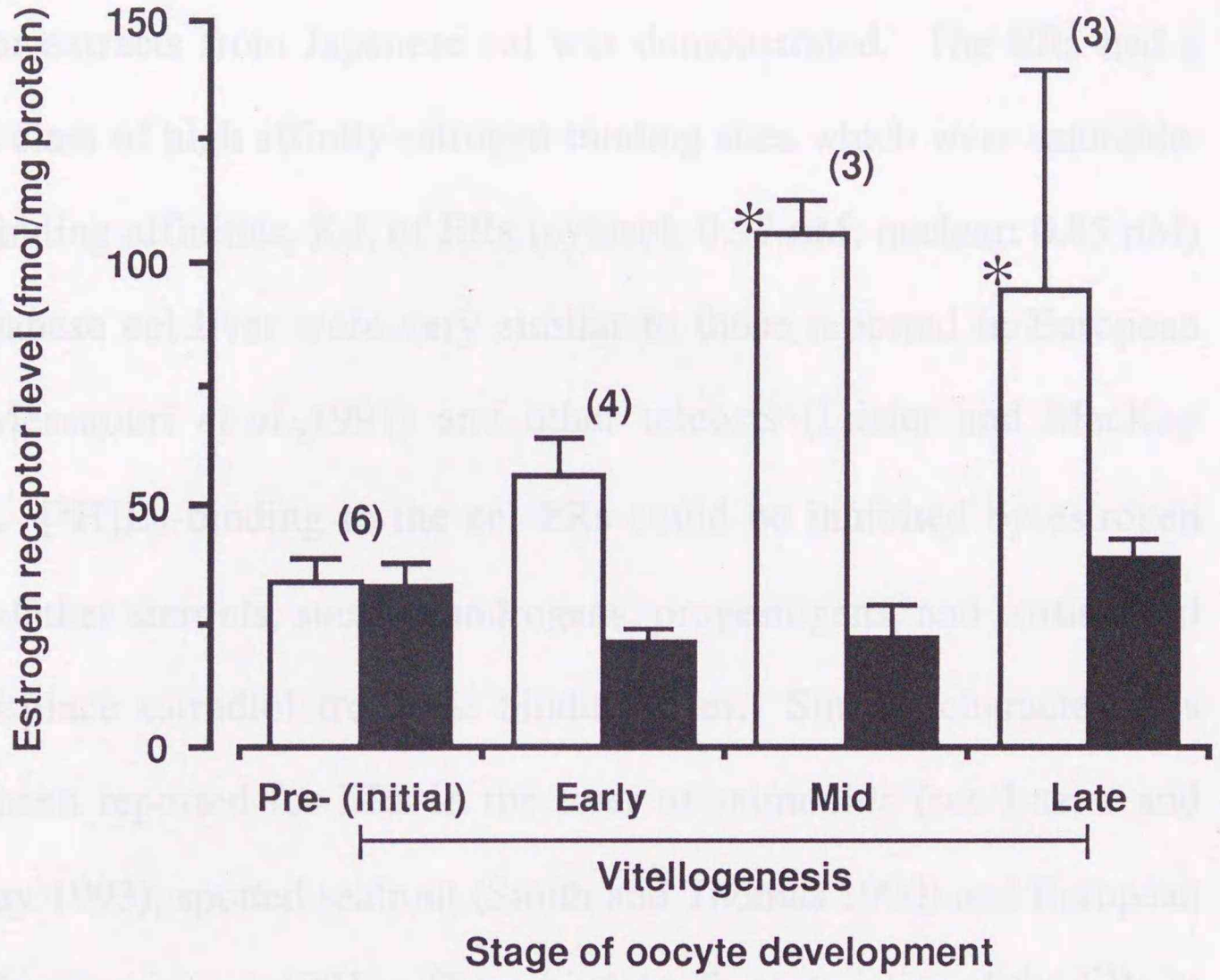


Fig. 6. Changes in hepatic cER and nER levels during artificially-induced sexual maturation. cER levels are represented by open columns, nER levels are represented by closed columns. Vertical bars represent the SEM. Number of fish sampled is shown in parentheses. *: Significantly different from other stages ($p<0.05$).

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 (Messaouri *et al.*,1991) and other teleosts (Lazier and MacKay 1993). [^{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 Lazier and Mackay 1993), spotted seatrout (Smith and Thomas 1990) and European eel (Messaouri *et al.*,1991). These binding characteristics of the ERs in Japanese eel were consistent with the known properties of steroid receptors (Callard and Callard 1987).

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.* (1994)

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 (Welshons *et al.* 1984), immunocytochemical (King and Greene 1984), and recent molecular biological (Ylikomi *et al.* 1992) 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.* (1992) has reported that 90% of cER disappeared quickly after E_2 injection, while nER increased. This result implies that cER is translocated to nuclei

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 (Pottinger 1986) and spotted seatrout (Smith and Thomas 1990). 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 (Hayward *et al.* 1980). 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 (Smith and Thomas 1991). 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). 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 (Riley and Callard 1988a,b; Paolucci and Botte 1988; Yu and Ho 1989; Paolucci 1989). 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 autoregulated by estrogen (Lazier and MacKay 1993). Messaouri *et al.* (1992) reported that both cER and nER levels in the liver increased after E_2 treatment in silver females of European eel. Recently, it was also found that ER mRNA was induced by E_2 in the liver of immature Japanese eel

(Chapter IV). 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 artificial maturation, especially at the migratory nucleus stage when lipid droplets occupied large areas of the hepatocytes (Okumura *et al.* unpublished). In fact, the first supernatant 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 artificial 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 SPH 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 mechanism 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.

Chapter III.

Molecular Cloning and Characterization of a Japanese Eel Estrogen Receptor cDNA

Introduction

In the previous chapter, the Japanese eel ER has been characterized. In addition, changes in hepatic ER levels during artificial maturation were also examined. However, it is difficult to completely explain the relationship among ER, E_2 and VTG levels, because these changes showed a complex pattern. In order to confirm the regulatory mechanism of VTG synthesis during artificial maturation, it is necessary to hold the ER as a molecule.

The ER belongs to the nuclear receptor superfamily that includes the receptors for steroid hormones, thyroid hormone, a number of vitamins and several whose ligands have yet to be identified (Evans, 1988; Beato, 1989). The ER cDNAs have been cloned in various species. In teleost, ER cDNA was cloned in only one species the rainbow trout, *Oncorhynchus mikyss* (Pakdel *et al.*, 1990). In the studies of trout ER, it has been shown that the structure and function of ER are conserved from mammal to teleost (see Le Dréan *et al.*, 1994).

In this chapter, a Japanese eel ER cDNA which contains a complete open reading frame has been isolated and characterized.

Materials and Methods

Fish and treatments

Silver (migrating) Japanese eel females were caught in the Hiranuma river in Aomori prefecture (Japan) during September 1992. They were transferred to Hakodate and raised in circulating artificial seawater tanks with a capacity of 1,000 l tanks at 20°C. To induce sexual maturation, fish received weekly injections of chum salmon pituitary homogenate (SPH) as described in Chapter II. Livers were collected from sexually mature (after 10 SPH injections) eels. The livers were quickly frozen in liquid nitrogen, and stored at -80°C until use.

Polymerase chain reaction (PCR)

Total RNA was prepared from the liver of a sexually mature silver female by the acid guanidium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987) using ISOGEN (Nippongene). Poly(A)$^+$ RNA was isolated by oligo(dT)-Latex beads (Oligotex-dT30, Takara). Random primed double-strand cDNA was synthesized using Amersham's cDNA synthesis system.

To isolate the ER homolog from the eel cDNA library, two PCR primers were produced with Applied Biosystems DNA Synthesizer model 391: 5' primer, 5'- G A C T A (C T) A T G T G (C T) C C (C T) G C G A C (C T) A A C C T G -3', and 3' primer, 5'- G T G A (C G) C G T C C A G C A T C T C C A G -3'. The 5' and 3' primers correspond to amino acid sequences which are highly conserved among species, DYMCPATNQ (DNA binding region) and LEMLDG(A)H (ligand binding region), respectively.

PCR was carried out in 100 µl reaction mixture consisting of 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 2.5 U Taq DNA polymerase (Perkin Elmer), 100 ng cDNA, and 1 µM of each primer. After overlaying the PCR mixture with mineral oil (100 µl), 35 cycles of amplification was carried out under the following conditions: denaturation at 94°C for 1 min, annealing at 50°C for 2 min and extension at 72°C for 5 min.

Isolation of ER cDNA

The PCR product was purified by 1% agarose gel electrophoresis followed by the NaI method using GENECLEAN II (BIO 101). The product was subcloned into a plasmid vector pUC18 (Pharmacia), designated peER, and sequenced using the Taq dye primer cycle

sequencing kit (Applied Biosystems). The PCR product had high homology to the conserved domain of ER. Thus, the product was used to isolate a cDNA clone containing the complete open reading frame.

A λgt10 library constructed from the liver of a sexually mature silver female was prepared using Amersham's cDNA cloning system. Phages were plated onto *Escherichia coli* C600 hfl, without amplification, with 20000-30000 phage per 15 cm Petri dish, and transferred onto nylon membrane (Hybond-N+, Amersham). Duplicate membranes were screened with the ^{32}P-labeled PCR product (2X10^6 cpm per membrane) overnight at 65°C in 20 ml of hybridization solution containing 6X SSC (1X SSC: 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 0.2% Ficoll, 0.2% polyvinilpyrrolidone (PVP), 0.1% sodium dodecyl sulfate (SDS), and 100 μg/ml calf thymus DNA. The membranes were washed twice at 65°C for 30 min with 6X SSC containing 0.1% SDS, followed by three 30 min washes at 65°C with 1X SSC containing 0.1% SDS. Five clones, positive after two rounds of screening, were isolated from 300,000 phages. The insert of one of them (λeER3), about 3 kbp in length, was selected, subcloned into pBluescript II SK M13+ (Strategene), designated peER3, and serial deletion mutants were produced with exonuclease III. Sequencing was performed using the Taq dye primer cycle sequencing kit. Sequence

analysis and comparison were carried out using DNASIS software (Hitachi).

Construction of transfection vector and its expression in COS7 cells

Eel ER expression plasmid, pSVLeER, was constructed as follows. The λeER3 insert fragment was blunt-ended by Klenow fragment, and ligated into the SmaI site of the expression vector pSVL (Pharmacia) which contains the SV40 enhancer promoter and splicing and polyadenylation signals. pSVL without insert was used as a control plasmid. Plasmid DNAs were transfected into COS7 cells (20 plates, 40-50% confluent cells/10 cm plate) by the DEAE-dextran method (Maruyama *et al.*, 1991). After 48 hr, the cells were washed twice with ice-cold phosphate-buffered saline, collected and suspended in binding buffer (20 mM Hepes, pH 7.4, 0.1 mM EDTA, 0.5 M KCl, 0.5 mM dithiothreitol, 1 mM phenylmethanesulphonyl fluoride, 5 μg/ml leupeptin and 20 % glycerol; 0.1 ml per plate). The suspension was frozen and thawed four times to lyse the cells, and then centrifuged at 105,000 X g for 1 hr at 4°C. The whole cell extract supernatant (WCE) was collected and protein concentration was determined by Bradford assay. The WCE was frozen in liquid nitrogen and stored at -80°C. Estradiol binding activity was measured by the dextran-coated charcoal

(DCC) method. Aliquots of WCE (50 µl) were incubated with [^{3}H]E_2 (3.7 GBq/µmol: New England Nuclear) with or without a 100-fold excess of unlabelled steroids. After incubation overnight at 4°C, an equal volume of DCC solution (0.5% Norit-A charcoal and 0.05% Dextran T-70 in 20 mM Hepes, pH 7.4) was added for 5 min on ice to remove free steroid. DCC was removed by centrifugation and aliquot of the supernatant was counted for radioactivity in Aquasol II (New England Nuclear). The dissociation constant (Kd) was determined by the method of Scatchard (1949).

Results

Isolation and the sequence of Japanese eel ER cDNA

Only one product, about 1 kbp in size, was obtained by PCR. This size was as expected from the sequences of ERs. The nucleotide sequence of the peER insert showed high homology with those of ERs (data not shown). Thus, the PCR product was confirmed as a partial ER clone. Using the PCR product, we screened the λgt10 library from mature female Japanese eel liver. The insert of one positive clone, λeER3, was subcloned into pBluescript plasmid, designated peER3 and sequenced.

The nucleotide sequence and the deduced amino acid sequence of peER3 insert are shown in Fig. 7. This clone contained a long open reading frame encoding 573 amino acid residues (molecular weight: 63,418), together with a 298 nucleotide 5'-untranslated region and a 1044 nucleotide 3'-untranslated region. This open reading frame was the longest and the DNA sequence surrounding the ATG codon was in agreement with the consensus sequence proposed by Kozak (1986) for translation initiation regions. At least two small open reading frames corresponding to 14 (ORF1), 47 (ORF2) and three (ORF3) amino acids were present in the 5'-untranslated region.

```
     ORF 1                                                                 ORF 2
A ATT CAA TGC TAA TTT CCA TCT CAC AAG ATA ATT CCA GTT GCA TAC TAT GAT ATA CGA CCA TAC TTA CGT AAC     73

TGG CGA TTT TAG TTT AGC TAT ACA TTT ATT TTG TAG TTT TAC TCG GCG CTA TCG CAC TGC GTC GGT AAA CGT GGA   148

CAC TTG CTT TTG CCG AAT AAG CGG CTA CAG GTA TCT GAT TTG AAG GAG CAG AAG GTT TTT CGT TAT CAG CTG AAA   223
                                                                              ORF 3
TAA GTA TTC GGG GCT GCG GCA AGA CAG CAG GGC TTC TCA GTG ACG AGG GCC TGT GGA TAA TGA GAT AGA TCC AAA   298

ATG GCT GGC TCT CCA GGC AAT GAG CTA CCT CTC CTG CAG CTT CAG GAG GTG GAC TCC AGC AAG GTT GGG GAA AGC   373
 M   A   G   S   P   G   N   E   L   P   L   L   Q   L   Q   E   V   D   S   S   K   V   G   E   S     25

GGG GGC TCT TCG GGG TTG CTG CCC ACC ATG TAT AAC GGT GCC CTG CCC GCG CTG AGC ATG GAA AGC CAT GCC GTC   448
 G   G   S   S   G   L   L   P   T   M   Y   N   G   A   L   P   A   L   S   M   E   S   H   A   V     50

TGC ATC CCG TCC CCC TAC ACG GAC AGC AGC CAC GAC TAC GCC GCC CTG ACC TTC TAC AGC CCC CCC ATC CTG AGC   523
 C   I   P   S   P   Y   T   D   S   S   H   D   Y   A   A   L   T   F   Y   S   P   P   I   L   S     75

CAC GGC GGC CCT GCC GTC CCC GAG AGC CCC GCC GCG CGC CAG TCC CTC AGC CCC TCC CTC TTC TGG CCG GCC CAC   598
 H   G   G   P   A   V   P   E   S   P   A   A   R   Q   S   L   S   P   S   L   F   W   P   A   H    100

GGC CAC CAC GGC CAC GTG TCC CCG CTG GCC CTG CAC TTC CAG CAG CCC CTG GTG TAC AGA GAG CCC GCC CAC TCC   673
 G   H   H   G   H   V   S   P   L   A   L   H   F   Q   Q   P   L   V   Y   R   E   P   A   H   S    125

CCG TGG GCG GAG CCC AAA CCG CTG GAG CAC GGC CAG GCC CAG ACC AGC AAG CTG GCG GGG AAG AGG ATG GCG GAG   748
 P   W   A   E   P   K   P   L   E   H   G   Q   A   Q   T   S   K   L   A   G   K   R   M   A   E    150

AGC GAG GAG GGG ACG AGC TCC GTC GGG GGC TGC TTC GCG GGG AAG GGA GAC ATG CAC TTC TGT GCC GTG TGC CAT   823
 S   E   E   G   T   S   S   V   G   G   C   F   A   G   K   G   D   M   H   F   C   A   V   C   H    175

GAC TAC GCC TCG GGG TAT CAC TAC GGG GTG TGG TCC TGC GAA GGC TGC AAG GCC TTC TTC AAG AGG AGC ATC CAA   898
 D   Y   A   S   G   Y   H   Y   G   V   W   S   C   E   G   C   K   A   F   F   K   R   S   I   Q    200

GGG CAC AAT GGC TAC ATC TGC CCC GCC ACC AAC CAG TGC ACC ATC GAC AAG AAC CGG CGC AAG AGC TGC CAG GCC   973
 G   H   N   G   Y   I   C   P   A   T   N   Q   C   T   I   D   K   N   R   R   K   S   C   Q   A    225

TGC CGA CTC CGC AAG TGC TAC GAA GTG GGC ATG ATG AAG TGT GGT GTG AGA CGG GAG CGT TGT ACC TAC CGG GGG  1048
 C   R   L   R   K   C   Y   E   V   G   M   M   K   C   G   V   R   R   E   R   C   T   Y   R   G    250

GCG CGA CAC CGC CGC ATG CCC CAC ATC CGC GAG TTG GCG GGC ACA GGG GGC GGG GCC AGG ACC CAG AGG CGG GGC  1123
 A   R   H   R   R   M   P   H   I   R   E   L   A   G   T   G   G   G   A   R   T   Q   R   R   G    275

GAG GGA GTC GTC CCT CAG ACG CAG GAG GCG CAG TCC TCG GCG CTC ACG CCG GAG CAG TTA ATC AAC CGC ATC ATC  1198
 E   G   V   V   P   Q   T   Q   E   A   Q   S   S   A   L   T   P   E   Q   L   I   N   R   I   I    300

GAG GCG GAG CCG CCG GAG ATC TAC CTC ATG AAG GAG CTG AAG AAG CCC TTC ACC GAG GAC AGC ATG ATG ATG TCA  1273
 E   A   E   P   P   E   I   Y   L   M   K   E   L   K   K   P   F   T   E   D   S   M   M   M   S    325

CTC ACC AAC CTG GCC GAC AAG GAG CTC GTC CTC ATG ATC AGC TGG GCC AAA AAG ATC CCT GGG TTT GTG GAG CTG  1348
 L   T   N   L   A   D   K   E   L   V   L   M   I   S   W   A   K   K   I   P   G   F   V   E   L    350

GAC CTG TCT GAC CAA GTA CAC CTG CTG GAG TGC TGT TGG CTG GAG GTG CTG ATG CTG GGC CTG ATG TGG AGG TCT  1423
 D   L   S   D   Q   V   H   L   L   E   C   C   W   L   E   V   L   M   L   G   L   M   W   R   S    375

GTG GAT CAC CCT GGG AAA CTC ATC TTT TCC CCA GAC CTC AAG CTC AAC AGG GAT GAG GGA AGT TGT GTG GAG GGG  1498
 V   D   H   P   G   K   L   I   F   S   P   D   L   K   L   N   R   D   E   G   S   C   V   E   G    400

ATC CTG GAA ATC TTC GAC ATG GTG CTG GCG GCA ACC TCC AGG TTT CGG GAG CTG AAG CTA CAG AGG GAG GAG TAT  1573
 I   L   E   I   F   D   M   V   L   A   A   T   S   R   F   R   E   L   K   L   Q   R   E   E   Y    425

GTG TGC CTC AAA GCC ATT ATC CTC CTC AAC CCC AAC CTG TGC ACA ACG TCT AGC GAG AAC CGC GAG GAG CTG GAG  1648
 V   C   L   K   A   I   I   L   L   N   P   N   L   C   T   T   S   S   E   N   R   E   E   L   E    450

AGC CGC AAC AAG CTG CTG CAC ATG CTG GAC TCC GTG ACC GAC GCG CTG GTG TGG ACC ATC GCC AAG AAG GGC CTG  1723
 S   R   N   K   L   L   H   M   L   D   S   V   T   D   A   L   V   W   T   I   A   K   K   G   L    475

ACC TTT CAG CAG CAG TCT GCC CGG CTG GCC CAC CTG CTG ATG CTG CTG GCA CAC ATC CGC CAC CTC AGT AAT AAG  1798
 T   F   Q   Q   Q   S   A   R   L   A   H   L   L   M   L   L   A   H   I   R   H   L   S   N   K    500

GGC ATG GAG CAC CTG TCC AAC ATG AAG AGG AAG AAC GTG GTG CCG CTC TAC GAC CTG CTG CTG GAG ATG CTG GAC  1873
 G   M   E   H   L   S   N   M   K   R   K   N   V   V   P   L   Y   D   L   L   L   E   M   L   D    525

GCC AAC ACC ATG CAC AGC AGC CGC ATG TCG GCG TCG TAC TCC TCC CAG CCG AGC CCG TGG TCC CAG GCC GCG CAG  1948
 A   N   T   M   H   S   S   R   M   S   A   S   Y   S   S   Q   P   S   P   W   S   Q   A   A   Q    550

AGC CAG CCT GGC CCG CCG CCA TCC TGC AGC GGG GAG TGC CCT TGT CCA CCC AAG GAA AGC AGT ACC ATC TAG CAT  2023
 S   Q   P   G   P   P   P   S   C   S   G   E   C   P   C   P   P   K   E   S   S   T   I   *          573

CCT GGA GTG GGG GAA CCT ACA GTT CTC TGT CAC TAG ATT GTC AGA GGG AAG TAT TTC ATA TTG TGG GAC ATT TAA  2098
CAT GAA TTT TAA GGA CAA ATA TAA TGT AGC TTT GTG AGC TAT GGA TTT CTG ACT GGG AAG GAT TAT TTA CAC ATG  2173
GTT TCT ACA GAC TCA TTT AAG GCC TCT ATG CAC CAT ACA ATA TTT TTT AAG TAT AAT ATC CTT TGT AGT ACA GTT  2248
CTT TCT GTG TGT GCT TGT TTT TTT TAG TGA AGT GTT TCC CTG CAA TTG CCT TCA GTT GAT CTT GCG CCA GTA TTC  2323
CTG GCA CTG GAT TGG TGG ATT TAG AGA AGT GTG AGA CTC TTC AGT AGT TCA GTC AGT CCC GTG CAT CTC CAT GAC  2398
AGA CAG GTC AGT TTC AGG CCA ACT TGG GTG CCA AAT CTG CGT CTC CAG GTG TGC ATA GTG AGG AGC CAT TAT GAG  2473
CAG TAG TCA CCT GAT GTT CTT TGC TGT TGA CAT GTA TGT CTG CTG ACC CCT TTG GGT TCA GAG ATA GAG AGA GAG  2548
AGA GAG GAA GGG TAG GGA TCC AGC TCT AGC TGA GGC ACA CCC ATC AGG AGA GGG GGA AAA AAA GAC AGG AGA CGG  2623
ACT GAA GGA CAG ACG GAG GAA GGG GTG TCA GAG CCG GGC CCT CGT CCG TGG GGA CGG GCC GGG AGC AGT GCA TTG  2698
TGG GAT TAC CTC GGC GGT GGG GCG CGC GGC CTC ATG CGG TCA GGC AGA GGC CCC AGC TGC GGA CCG GCG GAC TGA  2773
GGT GGG GGG TCT GTT GCC ATG GCG AGC TCC GTG CTG TGC TGG CGG GGC GGG GCG GCG GAG GAC AGG TAC CTG TAC  2848
GCC CCT CAC CCC AGG AGG CCG AGG TGA GTA ACC AAC CAC CTG TTT CCC CCA ATT TGG GCA AAC TTC CGC TCT TTA  2923
GGG AGC GTT TGT TTG AAG AGG CCG AGT GAT CTA GGG AGT GGC ATG AGT TTC TAT AGT CAG GAG GCA GAT CGG TTT  2998
ATC GAT TAA GGG GCG GTA AAG GTT TGT GAT ATC ACA AAA CCT CCG TTG TTC TTT TTA GTT TTT                  3061
```

Fig. 7. The nucleotide and deduced amino acid sequence of the Japanese eel ER cDNA. The numbers on the right refer to the position of the nucleotides and the amino acids. In the 5'-untranslated region, two putative short open reading frames (ORF1 and 2) and one very short open reading frame (ORF3) are underlined.

Sequence homology with other ERs

Comparison of the eel ER amino acid sequence with other ER sequences of human (Green *et al.*, 1986), chicken (Krust *et al.*, 1986), *Xenopus* (Weiler *et al.*, 1987) and rainbow trout (Pakdel *et al.*, 1990) is shown in Fig. 8. Using the nomenclature of Krust *et al.* (1986), the Japanese eel ER sequence could be subdivided into five to six domains. The putative DNA binding domain (C domain, residues 166-288) and ligand binding domain (E domain, residues 282-532) showed a high homology with those of other ERs, while other domains did not show significant homology (10-20%). Although the C domain was the most conserved region (77-82%) of the Japanese eel ER, its homology was less than that shown among other species (90-100%). However, the position of eight cysteine residues and other residues around them which constitute the two zinc finger motifs (Schwabe *et al.*, 1990) were conserved (Fig. 9a). In the E domain, three subdomains that showed sequence similarity could be distinguished (regions, α, β and γ in Fig. 9b), as shown in trout ER (Le Drén *et al.*, 1994). Fig. 10 shows the hydrophobicity index of eel, trout and human ERs. The profiles with hydrophilic C domain and a relatively hydrophobic E domains indicate that there is also good conservation of the protein structure.

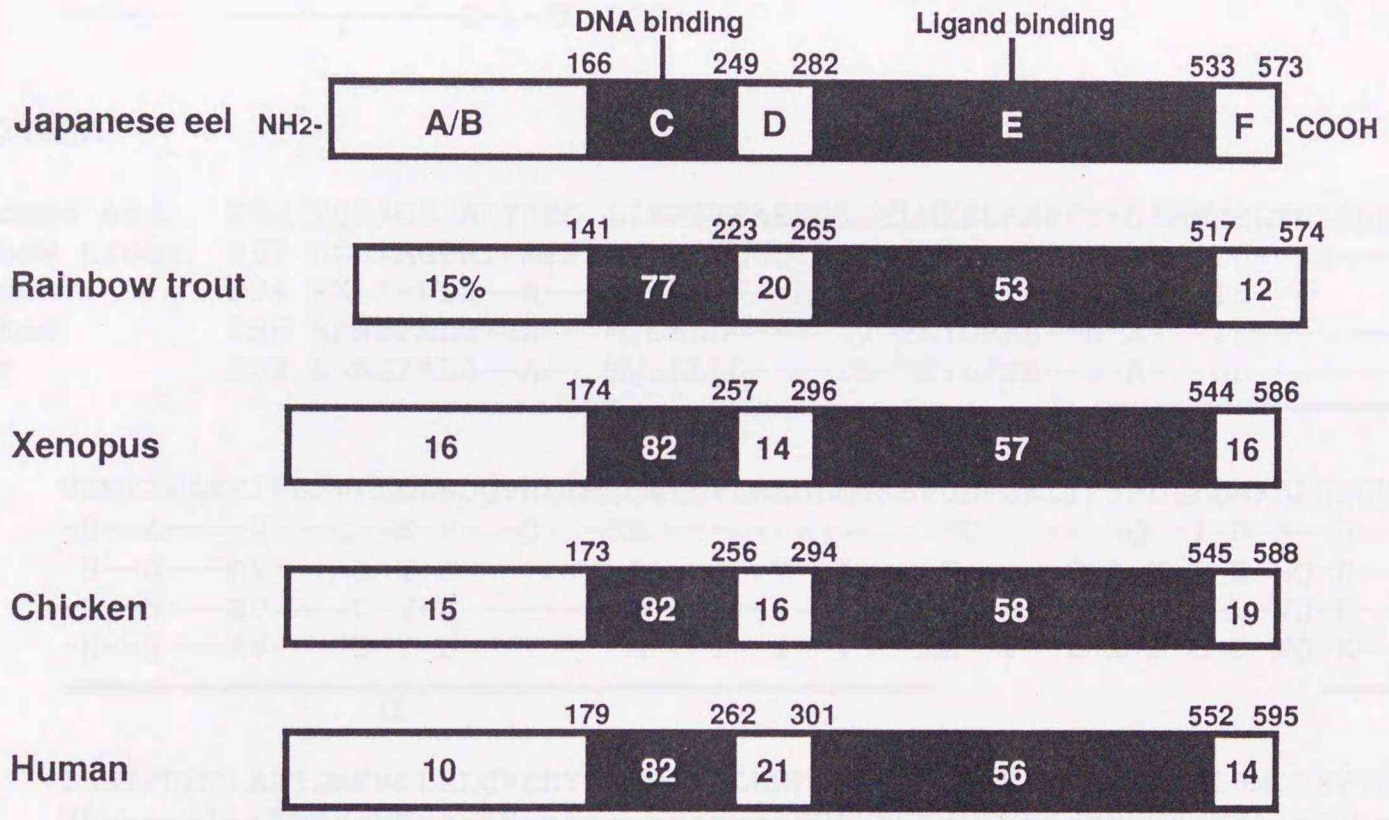


Fig. 8. Domain structure of the Japanese eel ER, and homology with rainbow trout, Xenopus, chicken and human ERs. The numbers above each box refer to the position of amino acids in each domain. The figures within boxes indicate the percentage homology of the domain relative to the Japanese eel ER.

a. C domain

```
                                        C I                                                                  C II
Japanese eel   166 GDMHFCAVCHDYASGYHYGVWSCEGCKAFFKRSIQGHNGYICPATNQCTIDKNR
Rainbow trout  141 NETRY----S-F---------------------------D-M--------M-R--
Xenopus        175 KETRY----S---------------------------D-M-------------
Chicken        175 KETRY----N---------------------------D-M-------------
Human          180 KETRY----N---------------------------D-M-------------

           RKSCQACRLRKCYEVGMMKCGVRRERCTY
           -----------------V-G-L-KD- GG
           -------------------G-I-KD-RGG
           -------------------G-I-KD-RGG
           -------------------G-I-KD-RGG
```

b. E domain

```
Japanese eel   282 TQEAQSSALTPEQ LINRIIEAEPPEIYLMKELKKPFTEDSMMMSLTNLADKEL
Rainbow trout  267 GGGWRGPRI-MPPEQVLFLLQGQT-ALCSRQKVAR-Y--VT--TL--SM-----
Xenopus        294 MKLSPVLS--A-- --SALM---A-IV-SEHDST--LS-A---TL---------
Chicken        296 KKNSPALS--A-- MVSALL-----IV-SEYDPNR--N-A---TL---------
Human          302 KKNSLALS--A-- MVSALLD----IL-SEYDPTR--S-A---GL---------

           VLMISWAKKIPGFVELDLSDQVHLLECCWLEVLMLGLMWRSVDHPGKLIFSPDLKLNRDEGSCVEG
           -H--A----V---Q--S-H---Q---SS-------------IHC------AQ--I-D-S--D----
           -H--N---RV----D-T-H--------A---I--V--I----E-----S-A-N-L-D-NQ-R----
           -H--N---RV----D-T-H--------A---I--I--V---ME-----L-A-N-L-D-NQ-K----
           -H--N---RV----D-T-H--------A---I--I--V---ME--V--L-A-N-L-D-NQ-K----
                                  α

           ILEIFDMVLAATSRFRELKLQREEYVCLKAIILLNPNLCTTSSENREELESRNKLLHMLDSVTDAL
           MA-----L--TV---GM---KP--F-----------GAFSFC-NSV-S-HNSSAVES---NI----
           LV-----LVTTAT---MMR-RG--FI---S-----SGVY-FL-STL-S--DTDLIHII--KII-T-
           MV-----L--TAA---MMN--G--F----S-----SGVY-FL-STLKS--ERDYIHRV--KI--T-
           MV-----L--TSS---MMN--G--F----S-----SGVY-FL-STLKS--EKDHIHRV--KI--T-
                                  β

           VWTIAKKGLTFQQQSARLAHLLMLLAHIRHLSNKGMEHLSNMKRKNVVPLYDLLLEMLDANTMHSS
           IHH-SHS-ASV---PR-Q-Q--L--S----M--------YSI-C--K------------GHRLQAP
           -HFM--S--SL---Q-------LI-S----M--------YS--C----------------HRI-TP
           IHLM--S--SL---HR---Q--L--S----M--------Y---C----------------HRL-AP
           IHLM--A---L---HQ---Q--L--S----M--------YS--C----------------HRL-AP
                                  γ
```

Fig. 9. Alignment of the amino acid sequences of the C and E domains of the Japanese eel, rainbow trout, Xenopus, chicken and human ERs. Sequences were positioned to yield maximum homology. Amino acids identical between Japanese eel ER and other ERs are shown by dashes (-). a: C domain. The cysteine residues involved in zinc coordination are indicated by black spots. b: E domain. Three conserved region (α,β and γ) are underlined.

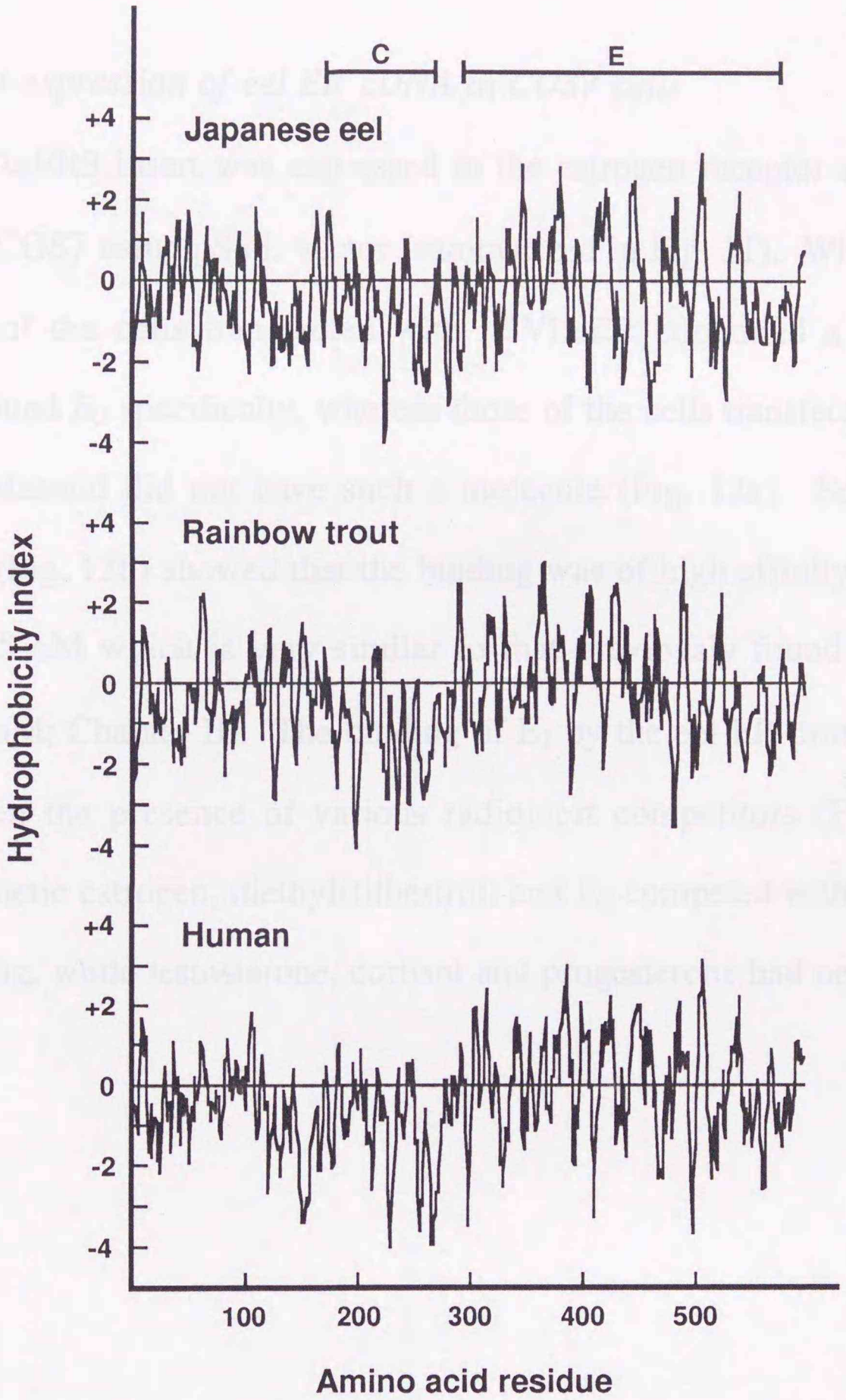


Fig. 10. Hydrophobic profiles of the Japanese eel, rainbow trout and human ER proteins. The profiles for three ERs were calculated using the algorithm of Kite and Doolittle. Positive and negative values on the ordinate indicate hydrophobic and hydrophilic degrees, respectively. The C and E domains are indicated at the top of the panel.

Transient expression of eel ER cDNA in COS7 cells

The λeER3 insert was expressed in the estrogen receptor negative cell line COS7 using pSVL vector (summarized in Fig. 11). Whole cell extracts of the cells transfected with pSVLeER contained a protein which bound E_2 specifically, whereas those of the cells transfected with control plasmid did not have such a molecule (Fig. 12a). Scatchard analysis (Fig. 12b) showed that the binding was of high affinity, with a Kd of 0.5 nM which is very similar to that previously found *in vivo* (0.8-0.9 nM; Chapter II). The binding of E_2 by the eel ER protein was assayed in the presence of various radioinert competitors (Fig. 13). The synthetic estrogen, diethylstilbestrol, and E_2 competed with $[^3H]E_2$ for binding, while testosterone, cortisol and progesterone had negligible effects.

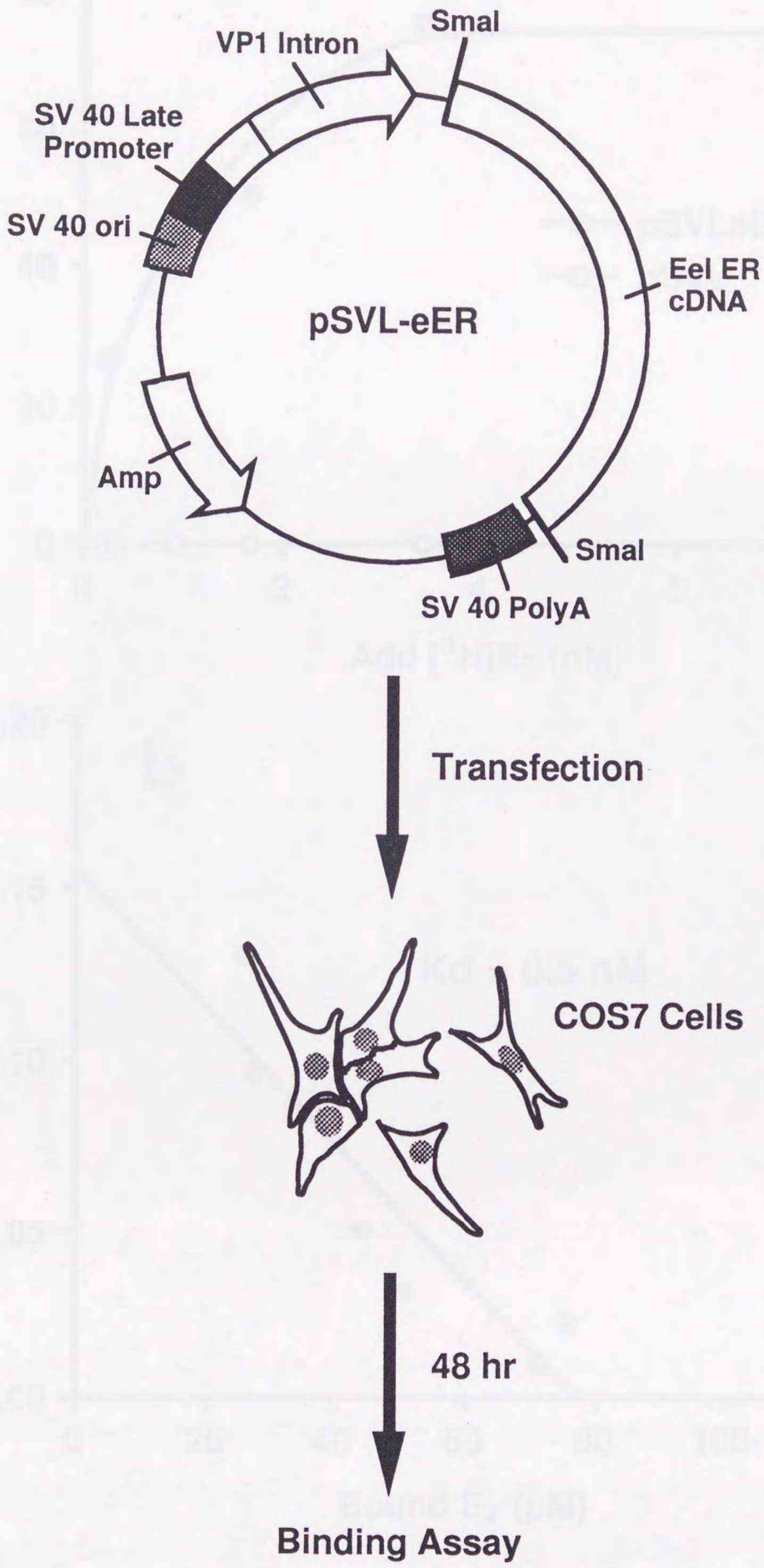


Fig. 11. Schematic representation of the Japanese eel ER fanctional assay

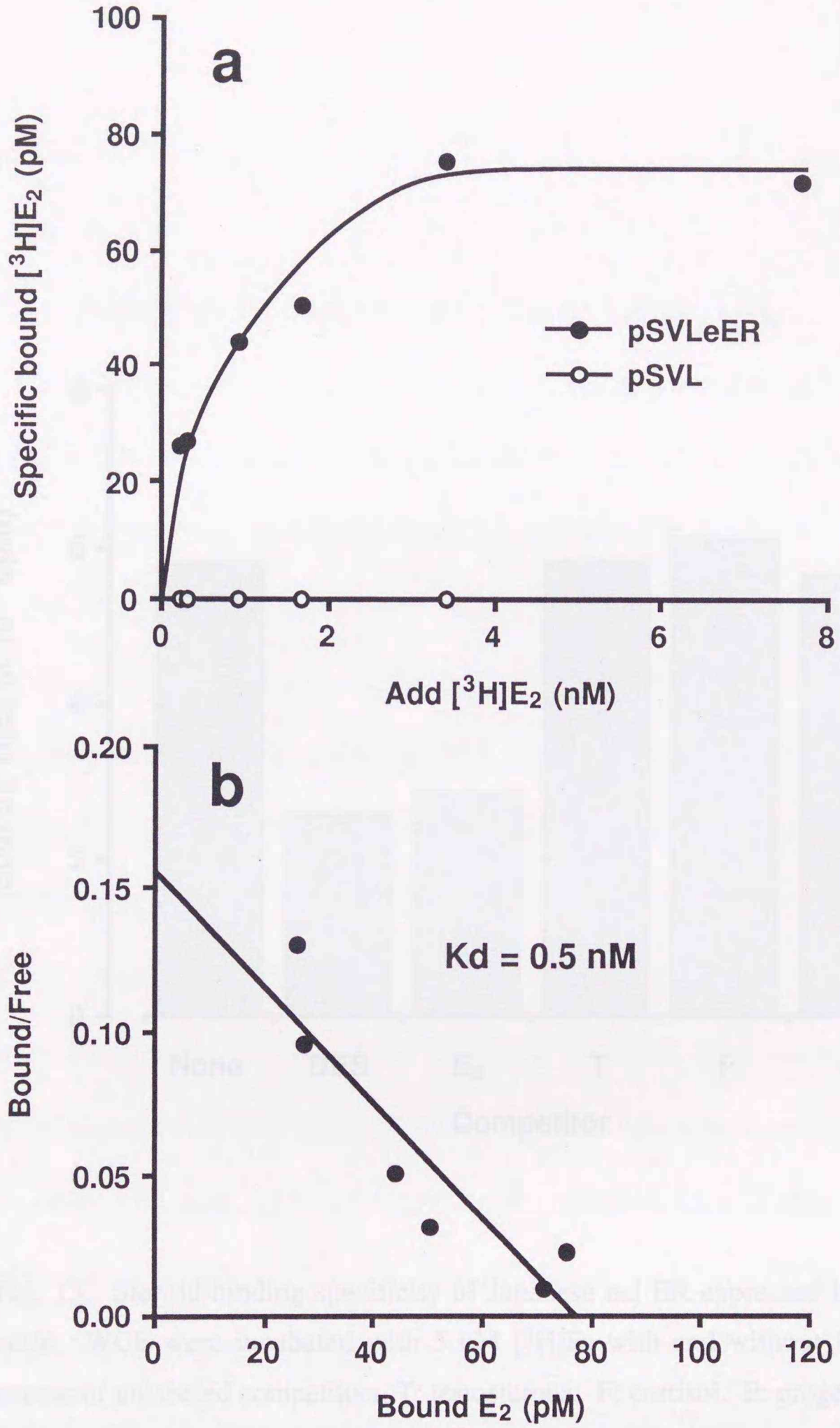


Fig. 12. Expression of Japanese eel ER cDNA in COS7 cells. a: Saturation analysis of [^{3}H]E2 binding. WCE of COS cells transfected with pSVLeER and those transfected with pSVL vector are represented by closed and open circles, respectively. b: Scatchard plot of eel ER expressed in COS7 cells. The estimated dissociation constant (Kd) is 0.5 nM. Each point represents the mean of triplicate determinations.

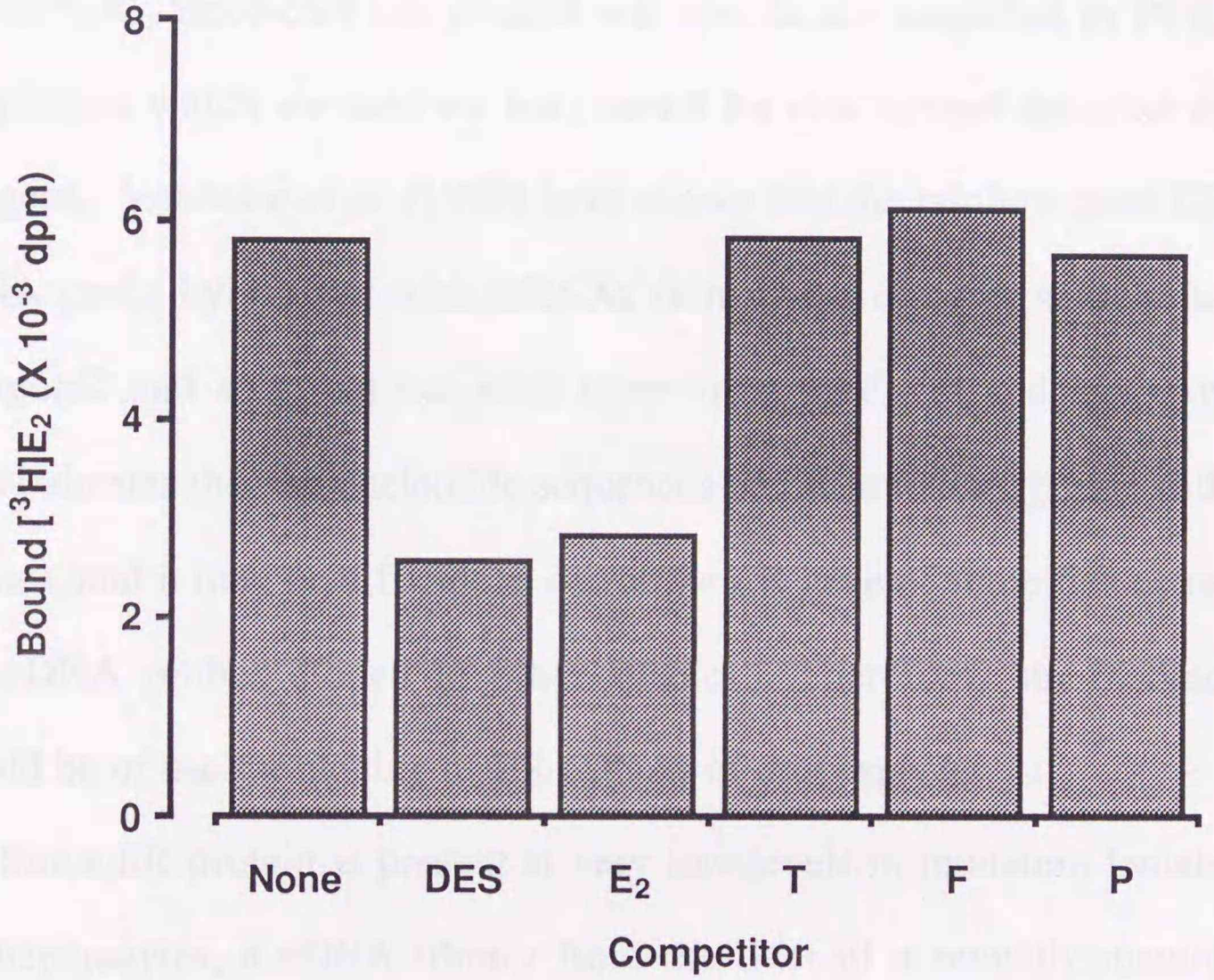


Fig. 13. Steroid-binding specificity of Japanese eel ER expressed in COS7 cells. WCE were incubated with 5 nM [^{3}H]E2 with and without 100-fold excess of unlabeled competitors. T: testosterone. F: cortisol. P: progesterone. Each bar represents the mean of triplicate determinations.

Discussion

In the present study, Japanese eel ER cDNA was isolated with the aid of PCR. Since only one product was specifically amplified by PCR, the primers which we used are very useful for cloning and detection of ER gene. Valotaire *et al.* (1993) have shown that the rainbow trout ER cDNA probe hybridized with mRNAs from livers of other salmonids, coregonid and sole, but not with those from pike, eel and lamprey. This indicates that the nucleotide sequences of ER in fish may vary with species, and it may be difficult to detect the ER gene of some fish using ER cDNA probes cloned in other species. Therefore, our method should be of use for cloning of ER cDNAs of other species.

Since ER protein is present at very low levels in immature female eel hepatocytes, a cDNA library from the liver of a sexually mature female, in which the ER concentration is about 4 to 5 times higher (chapter II), was used. Using the PCR product, a cDNA clone containing the complete open reading frame of the ER was isolated. This clone has at least two short open reading frames in the 5'-untranslated region. These short coding sequences upstream from the initiation codon were also found in ER cDNAs of other species (Green *et al.*, 1986; Krust *et al.*, 1986; White *et al.*, 1987; Weiler *et al.*, 1987;

Pakdel, *et al.*, 1990), but their function is still unknown. The comparison between the amino acid sequences of the Japanese eel ER and the ERs of four other species shows that the structure is not conserved in an homogenous way throughout the molecules and the two domains are highly conserved (C and E domains corresponding to DNA and ligand binding, respectively). The same conservation has been shown in the rainbow trout ER (Pakdel *et al.*, 1990). These results indicate that these two functional domains are mandatory necessary for estrogen action, and the fundamental functions of ER (namely, binding of estrogen and to target DNA sequences) have been conserved during evolution.

The C domain is the most highly conserved region in other species (92-100%) and in Japanese eel (80%). This region is well conserved also in the nuclear receptor family. In Japanese eel, several amino acid residues of the N and C terminal of this domain are different from those of other ERs. However, the distribution of eight cysteine and basic amino acid residues are almost the same as those of other ERs. It has been shown that these amino acid residues constitute the two zing finger motifs (Schwabe *et al.*, 1990), and these structures are necessary for binding to the target DNA sequences (Kumar *et al.*, 1987).

Therefore, the Japanese eel ER and human ER may interact with target DNA in a similar way.

The E (ligand binding) domains of eel and trout ERs exhibit less homology compared with other ERs. However, three conserved regions are found in all ERs. These regions may be involved in the major functions of the E domain, such as ligand binding, transactivation (Kumar *et al.*, 1987), receptor dimerization (Kumar and Chambon, 1988) and nuclear translocation (Ylikomi *et al.*, 1992). Several amino acids have been identified as important in estradiol-binding function in human and mouse ERs. These residues are conserved in the eel ER, such as Gly 395 and Lys 429 [corresponding to Gly 400 (Tora *et al.*, 1989b) and Lys 449 (Pakdel *et al.*, 1993) in the human ER], and Ile 494 and Gly 501 [corresponding to Ile 518 and Gly 525 in the mouse ER (Fawell *et al.*, 1990)]. Tyrosine residues in the E domain have been implicated in ligand binding (Migliaccio *et al.*, 1991; Koffman *et al.*, 1991). However, only one tyrosine residue, at position 517 in the eel ER, is conserved in all ERs. This tyrosine may be involved in ligand binding. Harlow *et al.* (1989) have identified Cys 530 in the human ER as the binding site of the aziridine group of an estrogen and antiestrogen analog. This residue is conserved in other ERs, but not in the eel ER (Arg 519). However, the adjacent residues (Lys 518, Lys 520 and Asn

521 corresponding to Lys 529, Lys 531 and Asn 532 in the human ER) are conserved. These amino acids were identified as residues involved in discrimination between estrogens and antiestrogens (Pakdel and Katzenellenbogen, 1992).

The other domains (A/B, D and F) in the eel ER do not show significant homology with other ERs. The functions of these domains are not fully understood. However, it has been shown that the A/B region has a transactivation function, although its activity is less than that of the E domain (Lees *et al.*, 1989). This transactivation function is hormone-independent, and is cell-type and promoter dependent (Tora *et al.*, 1989a; Bocquel *et al.*, 1989). Therefore, the reduced homology in the A/B domain between the eel and other ERs suggest that a species specific mechanism may exist in the transactivation function of ERs.

In addition to the sequence comparison, it has also shown that the λeER3 insert can synthesize a protein which binds with E_2 at the same high affinity as found for the eel ER *in vivo* (Chapter II). Furthermore, binding sites were specific to estrogens, and other steroids, such as testosterone, progesterone and cortisol, did not displace estradiol from the binding sites. These binding characteristics are consistent with known properties of steroid receptors (Callard and Callard, 1987).

Thus, these results confirm that the cDNA encodes the functional eel ER.

In conclusion, a cDNA clone encompassing the entire protein coding region of the Japanese eel ER has been isolated. It has also demonstrated that the cDNA encodes the functional receptor. The eel ER showed striking amino acid homology of the two important domains (DNA and ligand binding) with other ERs, while the other domains showed less homology. It remains to be determined whether the eel ER has transactivation functions similar to those of the mammalian ER. The cloned eel ER cDNA should be a useful tool for study of the mechanism of VTG synthesis at the molecular level.

Chapter IV.

Expression of Estrogen Receptor mRNA in Liver and Testis of the Japanese Eel

Introduction

It has been shown that VTG gene expression in hepatocyte is strictly dependent on the quantity of ER (Hayward *et al.*, 1982; Perlman *et al.*, 1984). In addition, it has been demonstrated that hepatic ER protein and mRNA are both induced by E_2 (Barton and Shapiro, 1988; Pakdel *et al.*, 1991; Flouriot, G. *et al.*, 1993). These results indicate that the increase in ER concentration resulted from E_2-induced expression of ER mRNA, increases cell sensitivity to E_2 and thus induces vitellogenesis.

In mammals, ER has been found in various tissues, although little is known about the biological functions of estrogens in those tissues (see Ciocca and Roig, 1995). In teleost, E_2 is involved in not only VTG synthesis but also other reproductive phenomena. Particularly, the feedback effect of E_2 on hypothalamus-pituitary axis has been well studied in many species (see Goos, 1987). Thus, in Japanese eel, ER may be present in various tissues, besides liver.

In this chapter, the expression of ER mRNA in the livers of E_2-treated eels and changes in its levels during artificial maturation were analyzed by Northern blotting using the eel ER cDNA as the probe. In addition, the tissue distribution of the ER mRNA was also examined.

Material and Methods

Fish and tissue handling

Immature cultivated Japanese eel (about 200 g in body weight) were purchased from a commercial eel supplier. They were kept in recirculating freshwater tanks with a capacity of 1,000 l at 20°C. Fish were not fed throughout the experimental period. Each 10 males and females received weekly intramuscular injections of E_2 (Sigma, 1 μg/g body weight) dissolved in propylene glycol. Various tissues were collected after 4 times of injection. For 20 males, a single injection of human chorionic gonadotropin (HCG) dissolved in eel Ringer was given intramuscularly at a dose of 5 IU per g body weight. Testes were sampled before injection of HCG and 3, 6 and 9 days postinjection.

Twenty females of the cultivated eels (369-956 g in body weight) received weekly injection of chum salmon pituitary homogenate as described in Chapter II. The livers were collected at various stages of ovarian development, within 3 days of each injection.

All collected tissues were quickly frozen in liquid nitrogen, and stored at -80°C until use.

Northern blot analysis

Total RNA and poly $(A)^+$ RNA were prepared from tissues as described in Chapter III. The poly $(A)^+$ RNAs (5 μg) were separated on 1 % agarose gel containing formaldehyde, transferred onto a nylon membrane (Hybond-N^+), and hybridized with 2 X 10^7 cpm of ^{32}P-labeled eel ER cDNA fragment (amplified by PCR as described in Chapter III) at 65°C overnight in 5X SSPE (1X SSPE: 0.18 M NaCl, 10 mM sodium phosphate and 1 mM EDTA, pH 7.7), 5X Denhardt's (1 X Denhardt's: 0.02% (W/V) each of Ficoll, PVP and bovine serum albumin), 0.5% SDS, and 100 μg/ml calf thymus DNA. Membrane was washed twice at room temperature for 10 min with 2X SSC containing 0.1% SDS, and then washed twice at 65°C for 15 min with 1X SSC containing 0.1% SDS, and analyzed by a BAS 2000 Bio-Image Analyzer (Fujifilm).

Results

Expression of ER mRNA in liver of E_2-stimulated and unstimulated Japanese eels

Northern blot analysis of livers from E_2-stimulated and unstimulated immature eels using the Japanese eel ER cDNA probe is shown in Fig. 14. In control male liver, no signals could be detected, while two mRNA bands corresponding to approximately 3.8 and 1.2 kb were detected in control female liver. Moreover, an additional mRNA of 5.6 kb was detected in both E_2-stimulated male and female livers, and its intensity was higher than those of other two bands.

Changes in ER mRNA levels in female liver during artificial maturation

Fig. 15 shows the changes in hepatic ER mRNA levels during artificial maturation of cultivated female Japanese eel. In pre-vitellogenesis (initial control), only two mRNA bands corresponding to 3.8 and 1.2 kb were detected. However, the 5.6 kb of mRNA was detected in early vitellogenesis. Moreover, levels of this 5.6 kb mRNA increased remarkably from midvitellogenesis to migratory nucleus stage, while levels of other two mRNA did not change.

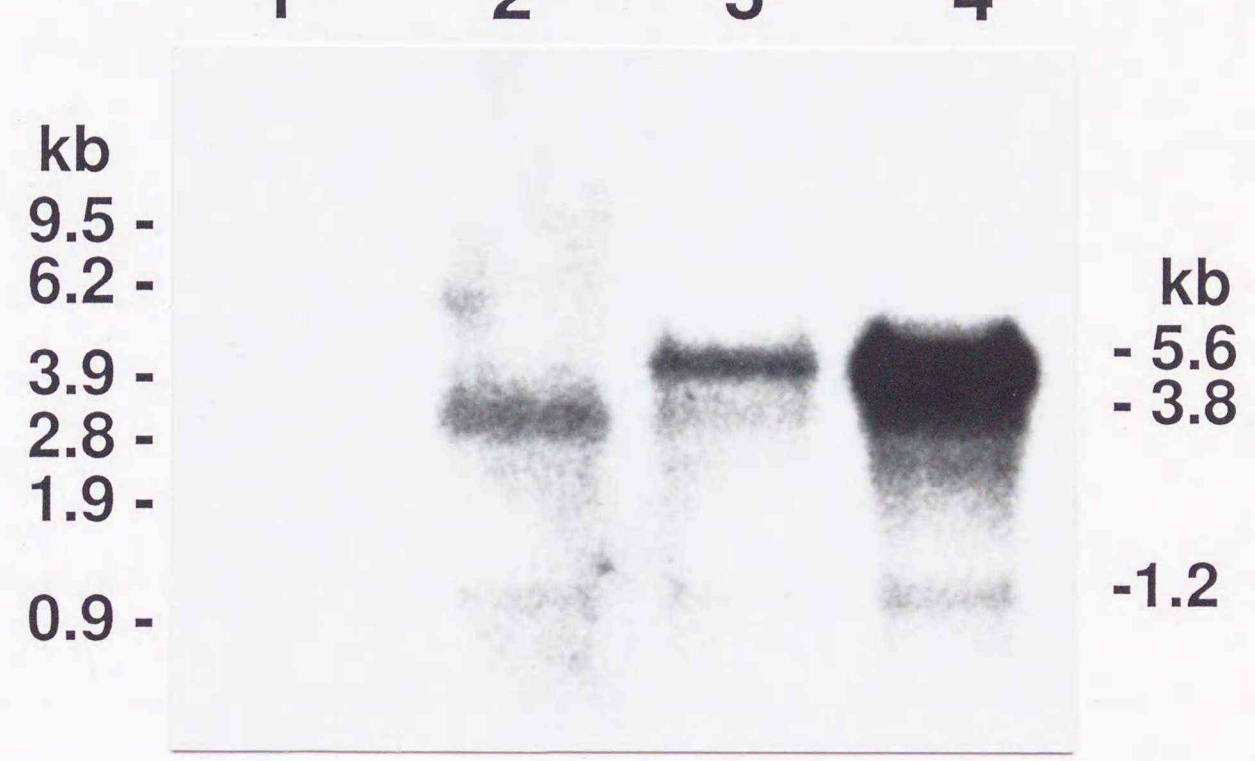


Fig. 14. Northern analysis of Japanese eel ER mRNA in the liver. Five μg of poly $(A)^+$ mRNA, isolated from E2-stimulated and unstimulated male and female livers, was separated on a denaturing formaldehyde agarose (1%) gel, transfered to a nylon membrane, and then hybridized with ^{32}P-labeled eel ER cDNA. Lane 1: untreated male. Lane 2: untrated female. Lane 3: E2-treated male. Lane 4: E2-treated female.

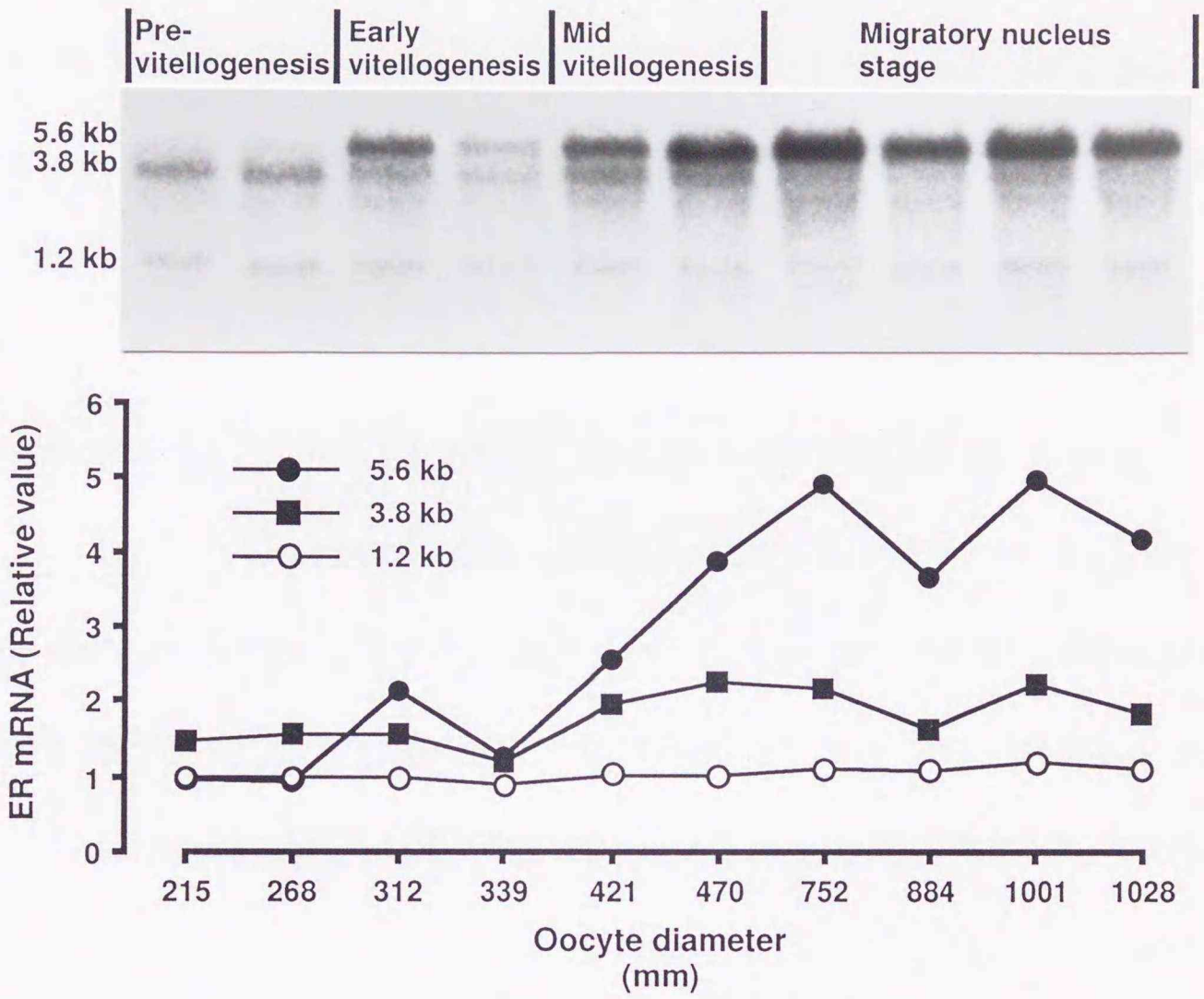


Fig. 15. Changes in hepatic ER mRNA levels during artificial maturation of female Japanese eel. Cultivated female eels were treated with SPH (20μg/g) every week. The livers were collected for RNA extraction at the appropriate time. The poly(A)+ RNAs were processed and analyzed as described in the legend of previous figure. Each ER mRNA levels were measured by scanning densitometory. Results were standarized by dividing by the 1.2 kb transcript of initial control.

Expression of ER mRNA in other tissues of E_2-stimulated eel

Expression of ER mRNA in various tissues from E_2-stimulated immature eels was analyzed by Northern blotting (Fig.16). A mRNA band corresponding to approximately 3.8 kb was only detected in testis. In other tissues, brain, heart, kidney, spleen and ovary, no signals were detected.

Changes in ER mRNA levels in testis after HCG treatment

Since it is possible that the ER mRNA in the testis of E_2-treated eel is induced abnormally by high dose of E_2, the mRNAs from untreated and HCG-treated testes were analyzed by Northern blotting using ER cDNA probe (Fig. 17). In untreated testis, the 3.8 kb mRNA were already detected. The level of this mRNA slightly increased 3 days after HCG injection, then gradually decreased between 6 and 9 days after treatment. By comparison, the level of 3.8 kb mRNA in E_2-treated testis was lower than that in untreated testis.

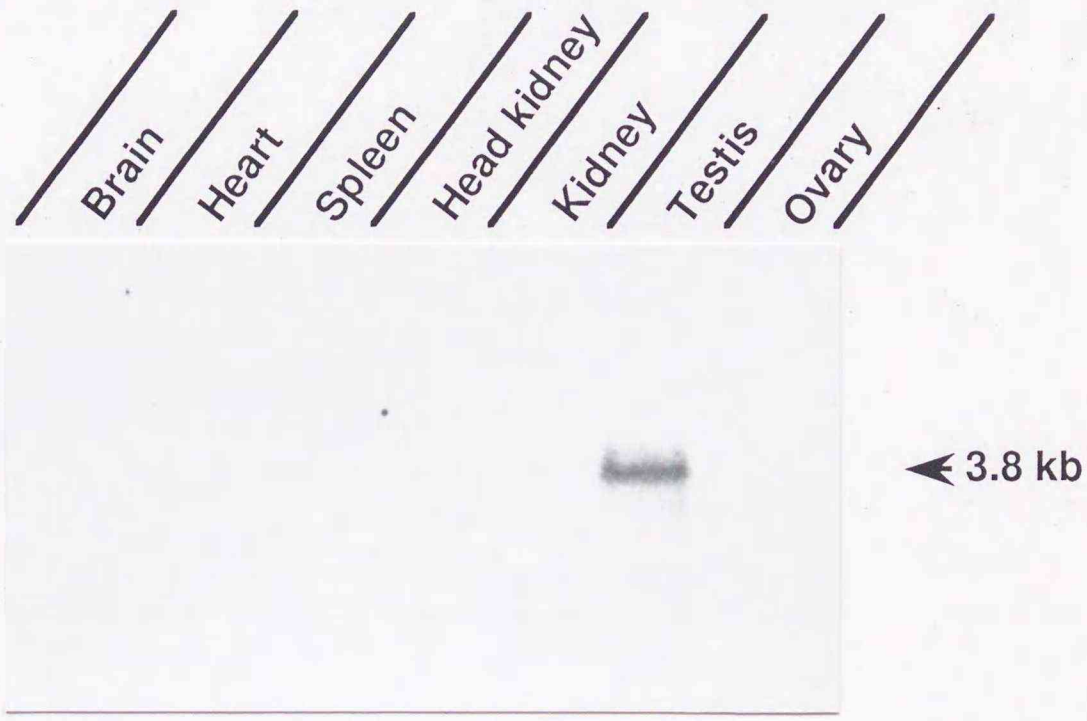


Fig. 16. Tissue distribution of ER mRNA. Immature cultivated eels were weekly received injection of E_2 at 1 μg/g body weight. The tissues were collected after the 4th injection. The poly(A)$^+$ RNAs were processed and analyzed as descrived in the legend of Fig. 14.

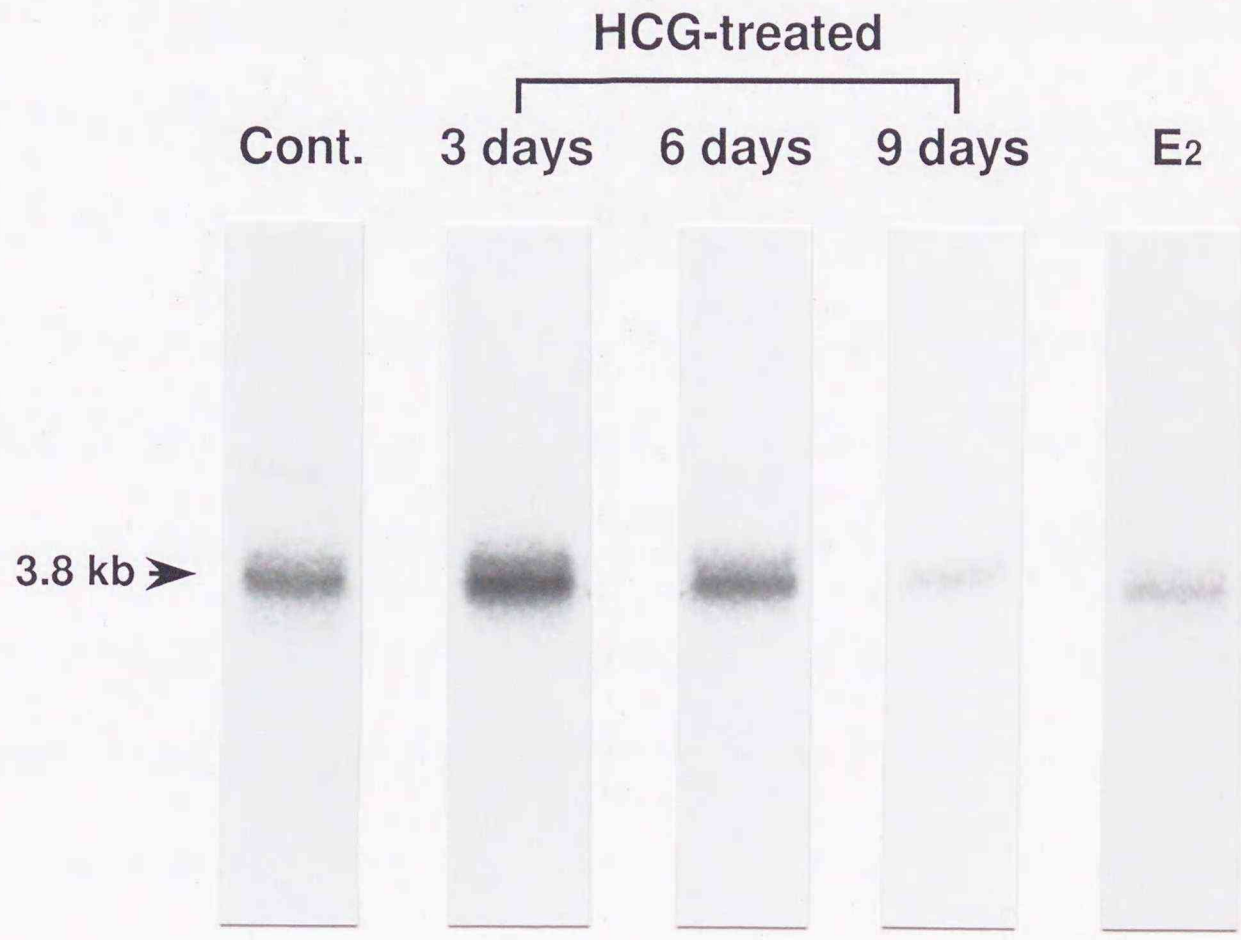


Fig. 17. Expression of ER mRNA in the testis of Japanese eel during HCG-induced spermatogenesis. Cultivate males were given a single injection of HCG (5 IU/g). The testes were collected for RNA extraction st the appropriate time. The poly(A)$^+$ RNAs were processed and analyzed as descrived in the legend of Fig. 14.

Discussion

Northern analysis showed that at least three ER mRNAs with lengths of approximately 5.6, 3.8 and 1.2 kb expressed in the Japanese eel liver. Multiple ER mRNAs were also detected in *Xenopus* (four mRNAs; Weiler *et el.* 1987) and trout (two mRNAs; Pakdel *et al.* 1989) liver. However, in both species, it is not known if all the ER mRNAs are functional, or if these mRNAs originate from the same gene. It is possible that both the 5.6 and 3.8 kb mRNAs encode the functional ER because their sizes are longer than that of the entire coding sequence of eel ER (1.7 kb).

Since the ER cDNA fragment encompassing the middle of the C domain to almost the end of the E domain was used as a probe, the 1.2 kb mRNA should also contain the coding sequence. However, the 1.2 kb transcript is shorter than the size of entire coding sequence. Similar short ER mRNAs were also found in mouse ovary (1.5 kb; Hillier *et al.*, 1989) and trout pituitary (1.4 kb; Pakdel *et al.*, 1990). In mouse ovary, the 1.5 kb mRNA contained only a part of E and the entire F domain (Hiller *et al.*, 1989). This specific ovarian messenger is considered to be a steroid-binding protein involved in estrogen action. Further hybridization studies, with various domain-specific ER probes,

will be necessary to characterize the function of the 1.2 kb messenger in Japanese eel liver.

It is possible that the three ER mRNAs in hepatocytes are transcribed from the same gene and that the size difference is due to alternative splicing. In mammals, the ER is encoded by single-copy gene (Koike *et al.*, 1987; White *et al.*, 1987; Ponglikitmongkol *et al.*, 1988). For the glucocorticoid, progesterone, thyroid hormone and retinoic acid receptors, different isoforms of mRNAs are generated through alternative splicing and/or are transcribed from distinct promoters from the same gene (Hollenberg *et al.*, 1985; Kastner *et al.*, 1990; Leid *et al.*, 1992; Lazar, 1993). However, two different genes encoding ERs were found in a trout genomic library (Le Roux *et al.*, 1993). Therefore, the possibility remains that the three mRNAs in eel liver are transcribed from different genes. Further analysis of multiple ER mRNAs in eel liver will help understand the mechanism of estrogen action and the evolution of the estrogen receptor system which are varied among species.

The 5.6 kb mRNA was induced by the treatment of salmon pituitary homogenate. Moreover, its levels gradually increased during vitellogenesis, while the other ER mRNAs did not change. The changes in 5.6 kb mRNA levels are consistent with the increase of hepatic cER

levels during artificial maturation (Chapter II). These results suggest that the increase of hepatic ER during artificial maturation is dependent on the amount of the 5.6 kb transcript . Moreover, in female liver, the amount of the 3.8 and 1.2 kb mRNAs were not altered by E_2 stimulation, while the 5.6 kb mRNA was induced by E_2. Thus, it is suggest that the 5.6 kb mRNA is predominantly involved in the action of estrogen (i.e., VTG synthesis) in Japanese eel liver.

The ER mRNA was also detected in the testis from E_2-stimulated fish. The size of this mRNA was 3.8 kb which is same as one of ER mRNAs expressed in the liver. Therefore, the ER mRNA in testis may be a functional. Moreover, the ER mRNA was also found in the testes of untreated and HCG-treated fish. Thus, the ER mRNA expresses usually in the testis of Japanese eel. The level of ER mRNA in untreated testes was higher than that in E_2-treated testis. This suggests that the ER mRNA levels in testis are down-regulated by E_2, in contrast to the result in liver. The levels of ER mRNA increased slightly 3 days after HCG treatment, then decreased on day 6 and 9. Miura *et al.* (1991) have shown that, on 3 days after a single HCG (5 IU/g body weight) injection, spermatogonia began proliferation and the late type B spermatogonia appeared. These results suggest that ER is involved in spermatogonial proliferation. ERs have been found in testes of

mammals (see Ciocca and Roig, 1995) and nonmammalian species except teleosts (see Callard and Callard, 1987). In mammals, ER is present in Sertoli, Leydig and germ cells (see Ciocca and Roig, 1995). However, the roles of estrogens on testicular function are not clarified yet. In contrast to other species, teleosts have no information on the participation of estrogens in spermatogenesis. It has been shown that the cultivated Japanese eel has many advantages to study about the control mechanism of spermatogenesis (Miura *et al.* 1994). Therefore, eels should be a good model for analysis of the roles of estrogens on testicular function.

In other tissues, the ER mRNA was not detected. It is well known that the brain is the target organ of estrogens (see Ciocca and Roig, 1995). In rainbow trout, the ER mRNA is expressed in the forebrain and pituitary (Salbert *et al.*, 1991). In also European eel, E_2 could be elevate gonadotropin releasing hormone levels in the brain and pituitary (Dufour *et al.*, 1985) and protein and mRNA levels of gonadotropin in the pituitary (Quérat *et al.*, 1991). Therefore, in Japanese eel, E_2 may be involved in the hypothalamus-pituitary axis, and ER may be present in the forebrain and pituitary. In order to clarify the existence and localization of ER mRNA in the brain, pituitary and other tissues of

Japanese eel, the intensive study using more sensitive technique, such as *in situ* hybridization, are required.

Chapter V.

Summary and Conclusions

Estrogen, estradiol-17β (E_2) in many cases, is known to be involved in many phenomena of female reproduction in vertebrates. In teleost, as in other oviparous species, the major effect of E_2 is the control of vitellogenin (VTG) synthesis in the liver. It is generally accept that the action of E_2 is mediated by a specific estrogen receptor (ER) that is present in the nucleus of target cells. In the present study, the ER system in the liver of the Japanese eel was investigated, in order to clarify the mechanism of E_2 action during the maturational process in female Japanese eel, *Anguilla japonica*.

First, characterization of the eel ERs was performed by radioreceptorassay. ERs were identified in cytosolic and nuclear extracts of livers of female eel. A single class of high affinity binding sites was found, with a Kd = 0.97 nM for the cytosolic ER (cER) and Kd = 0.85 nM for the nuclear ER (nER). Binding of both the cER and the nER was specific for estrogens (diethylstibestrol: DES > 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 fish

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.

A cDNA encoding the Japanese eel ER was isolated and sequenced. This cDNA contains a complete open reading frame encoding 573 amino acid residues, and the molecular weight of this protein is calculated to be 63,417. The amino acid sequence of the eel ER shows high homology of the DNA binding (80%) and ligand binding domains (55%) with those of other species. This indicates that the basic functions (DNA and ligand binding) of ER have been conserved during evolution. However, the other domains show greatly reduced homology

(10-20%). Thus, it is possible that a species specific mechanism exists in other functions (for example, transactivation function) of ER. When the cDNA was ligated to the pSVL vector and transfected into COS7 cells, a protein that had high affinity for E_2 and specifically bound estrogens was produced. These results confirm that the cDNA corresponds with a Japanese eel ER mRNA.

Northern analysis showed that three ER mRNAs with length of 5.6, 3.8 and 1.2 kb were expressed in eel liver. Their expression were E_2 inducible, with the 5.6 kb mRNA being strongly dependent on E_2 stimulation. The 5.6 kb mRNA was also induced by the treatment of salmon pituitary homogenate, moreover, its levels gradually increased during vitellogenesis, while the other ER mRNAs did not change. The changes in the 5.6 kb mRNA levels are consistent with those in cER levels. These results suggest that the 5.6 kb mRNA is predominantly involved in the action of estrogen (i.e., VTG synthesis) in eel liver.

The 3.8 kb of ER mRNA was also detected in the testis from E_2-treated fish, while any ER mRNAs were not found in other tissues. The ER mRNA were also found in the testes of untreated and HCG-treated fish. The level of ER mRNA in untreated testis was higher than that in E_2-treated testis. This suggests that the ER mRNA levels in testis are down-regulated by E_2, in contrast to the result in liver. The levels of

ER mRNA increased slightly 3 days after HCG treatment, then decreased on day 6 and 9. These result suggest that ER is involved in spermatogonial proliferation.

The conclusions of the endocrine control of vitellogenesis during artificial maturation of Japanese eel emerging from the present study are expressed diagrammatically in Fig. 18. The SPH acts on the ovary and stimulates the production of E_2. The E_2 enters hepatocyte by diffusion and binds to a ER in nucleus. The E_2-ER complex binds to the promoter region of VTG gene and activates transcription of VTG mRNA. The complex also recognizes the ER gene and induces or enhances expression of ER mRNAs, in particular the 5.6 kb mRNA. The increase of the 5.6 kb mRNA results in increase of ER protein levels, that is, responsiveness to E_2 and ability of VTG synthesis. However, there is a noticeable point that the massive amounts of SPH which contains not only GTHs but also many unknown factors, are used for artificial maturation. It has been demonstrated that pituitary factors are involved in VTG synthesis in several oviparous species (see Ho, 1987). In also European eel, it has been shown that the hypophysectomy reduces E_2-induced VTG production (Burzawa-Gerard and Vidal, 1991; Burzawa-Gerard and Delevallée-Fortier, 1992). Therefore, it is possible that the unknown factors in SPH are involved

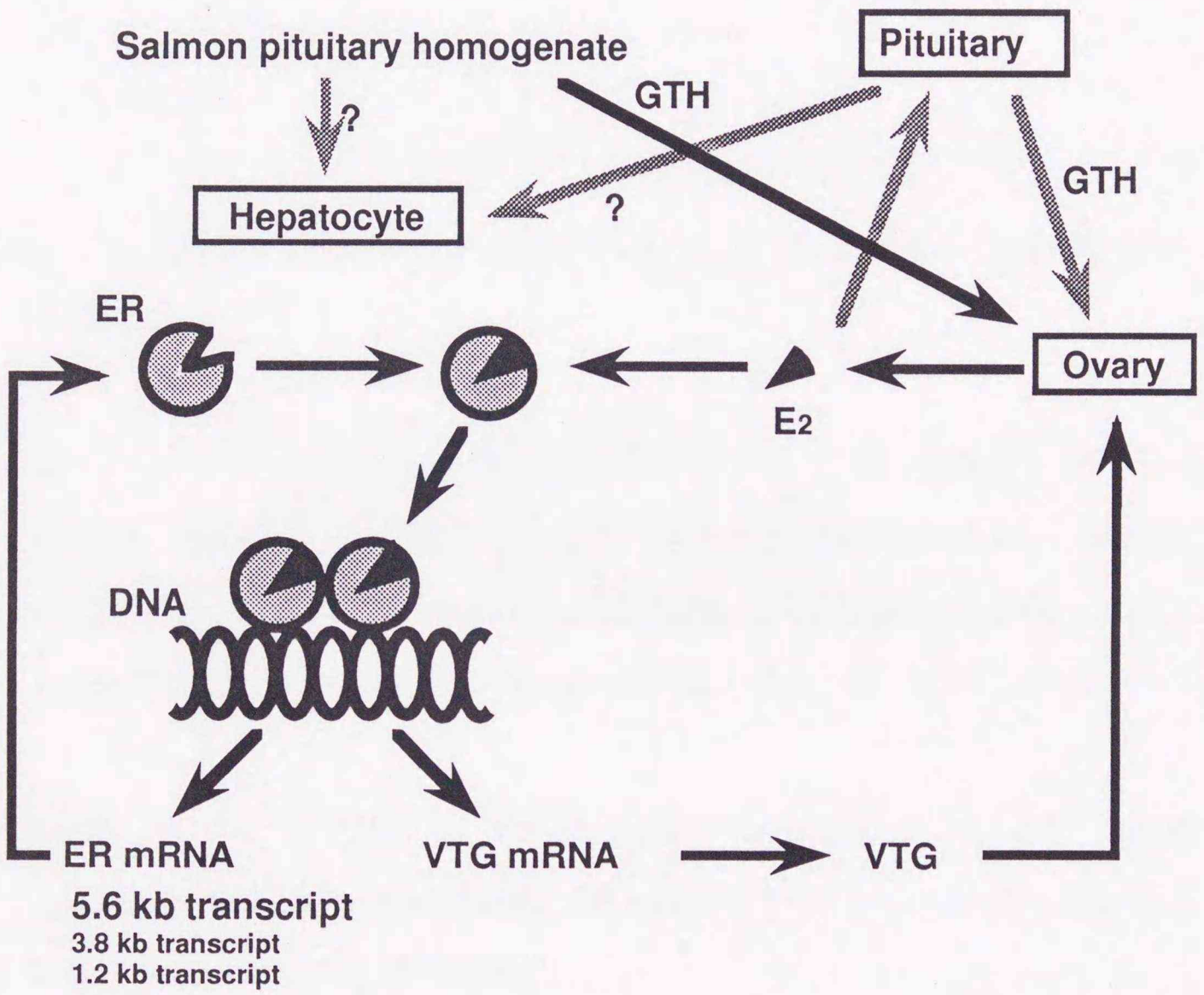


Fig. 18. Sheme summarizing the endocrine control of vitellogenesis during artificial maturation of Japanese eel

in VTG synthesis during artificial maturation. Identification of all the factors concerned in VTG synthesis is necessary for the establishment of the method of artificial maturation in the Japanese eel.

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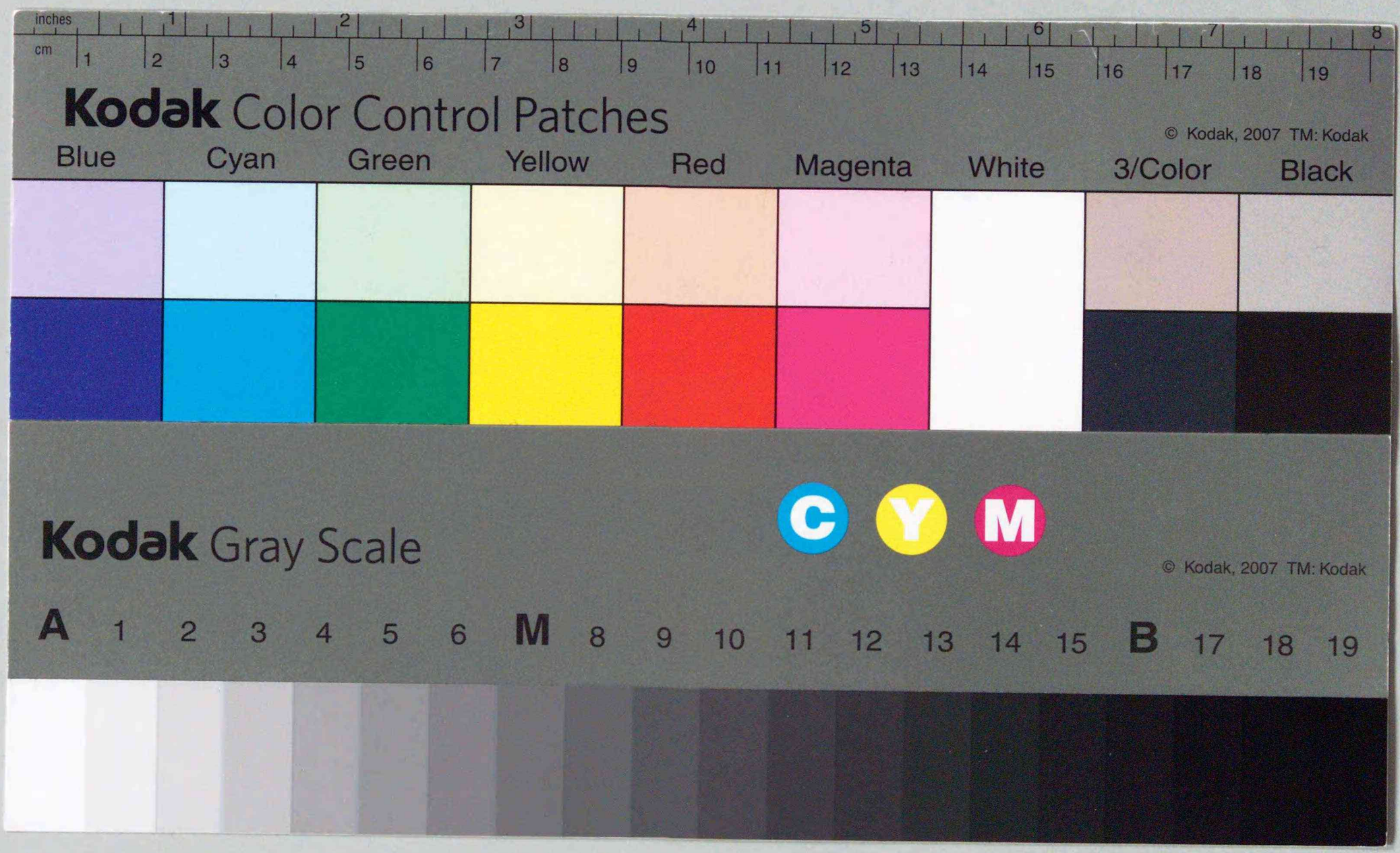
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