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主 論 文

Molecular biological studies on ovarian steroid hormone biosynthesis in Japanese eel, *Anguilla japonica*

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Abbreviation table

Technical term	Abbreviation
Aromatase	P450arom
17α-hydroxylase C_{17-20} lyase	P450c17
3β-hydroxysteroid dehydrogenase	3β-HSD
17β-hydroxysteroid dehydrogenase	17β-HSD
20β-hydroxysteroid dehydrogenase	20β-HSD
Carbonyl reductase	CR
Pregnenolone	Preg
17α-hydroxyprogesterone	17a-OHP
17α, 20β-dihydroxy-4-pregnen-3-one	17α, 20β-DHP
Dehydroepiandrosterone	DHA
Estradiol-17β	E2
Estrone	E1
Human kidney 293 cell	HK293
Thin layer chromatography	TLC
Polymerase chain reaction	PCR

Chapter I

An introduction to ovarian steroid hormone biosynthesis in Japanese eel, *Anguilla japonica*

The Japanese eel, *Anguilla japonica*, is a very important commercial species in the aquaculture industry of Japan. However, the life history of the eel is complex, and little is known about its reproduction as sexually maturing eels have never been caught from the wild. Eels captured from the wild have only immature ovaries or testes which are at pre- or early vitellogenic stage in females and at the spermatogonial proliferation stage in males. Furthermore, under culture conditions, they cannot undergo further gonadal development. Since the supply of elvers for stocking in eel farms is sustained entirely by supllies from the wild and since elver numbers have tended to decrease, the establishment of a reliable alternative supply of larvae has become an urgent and important problem. In order to solve this problem, artificial induction of gonadal maturation has been attempted by hormonal treatments.

Sexual maturity has been achieved by injections of chum salmon pituitary homogenates (SPH) in females (Yamamoto and Yamauchi, 1974; Yamauchi and Yamamoto, 1982) and human chorionic gonadotropin (HCG) in males (Yamamoto *et al.*, 1972). As a result of these treatments, fertilized eggs and larvae have been obtained successfully. However, the acquired larvae could not survive for more than two weeks, nor metamorphose into leptocephali (Yamauchi *et al.*, 1976). At present, techniques for artificial induction of maturation have become advanced and the success ratio for acquisition of larvae has increased. In addition, acquired larvae can now be fed and survive for longer than four weeks. Nevertheless, even at present, the success rate of SPH treatment in females is lower than that of HCG treatment in males, in which induction of maturation is relatively easy (Yamauchi, 1990). Furthermore, although it

is relatively easy to induce the process of vitellogenesis, most females cannot undergo final oocyte maturation and ovulation spontaneously (Yamauchi and Yamamoto, 1982). The fact that induction of maturation for females is more difficult than in males is caused by the complexity of oogenesis. Therefore, to establish an appropriate method for artificial induction of maturation of female Japanese eel, basic research on endocrine factors involved in gonadal development is required.

It is well-known that, in teleosts, oocyte growth and final oocyte maturation proceed under the control of gonadotropic hormone(s) secreted from the pituitary gland, and that their actions are mediated by steroid hormones secreted by follicular tissues enveloping the oocytes (Goetz, 1983; Nagahama, 1987). Therefore, studies on steroidogenesis in ovarian follicles are essential for understanding the mechanisms of the ovarian development.

Oocyte growth is mainly induced by C18 steroids (estrogens), especially estradiol-17β (E_2). During vitellogenesis, E_2 produced and secreted from ovarian follicles stimulates synthesis of vitellogenin (the precursor of yolk protein) in the liver. Vitellogenin, secreted into the bloodstream, is selectively incorporated into the oocytes resulting in oocyte growth (Wallace, 1985). In contrast, C21 steroids (progestogens), synthesized during maturational period, are associate with final oocyte maturation. A type of pregnene, 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-DHP) has been reported as the most effective inducer of final oocyte maturation *in vitro* in many species of teleosts (Goetz and Theofan, 1979; Nagahama *et al.*, 1983; Adachi *et al.*, 1988). Furthermore, 17α, 20β-DHP has been definitively identified as a natural maturation-inducing steroid (MIS) in

amago salmon *Oncorhynchus rhodurus* (Nagahama and Adachi, 1985) and Indian catfish *Clarias batrachus* (Haider and Rao, 1992).

In view of the importance of E2 and 17α, 20β-DHP on fish reproduction, changes in the plasma levels of these steroids, as well as the ability of the ovary to produce these steroids have been documented during the reproductive cycle. In many species of teleost, especially in salmonids, plasma E2 levels increase during vitellogenesis and decrease immediately prior to oocyte maturation (Kagawa *et al.*, 1983). In contrast, in amago salmon, 17α, 20β-DHP levels are very low during oocyte growth and strongly increase at the maturational phase (Young *et al.*, 1983). In addition to these changes in E2 and 17α, 20β-DHP levels, changes can be detected in the ability of ovarian follicles to produce these steroid hormones. Recently, changes in serum levels of steroid hormones and steroidogenic ability of ovarian follicles during artificial maturation of the Japanese eel were revealed (Ijiri *et al.*, 1995). Surprisingly, in artificially maturing female eel, serum E2 levels and the capacity to produce E2 in ovarian follicles were low during vitellogenesis and, unlike salmonids, rapidly increased at the maturational phase. Furthermore, 17α, 20β-DHP could not be detected in the serum throughout the period of artificial induction of maturation, whereas the ability to produce 17α, 20β-DHP by ovarian follicles at vitellogenic and maturational stages was enhanced following *in vivo* priming with SPH. These findings suggest that induction of steroidogenesis by SPH treatments is not normal. More detailed analysis on ovarian steroidogenesis is necessary to understand the regulation of steroid hormone synthesis.

Ovarian steroid hormones are synthesized from cholesterol via various intermediates through the action of specific several steroidogenic enzymes distributed in ovarian follicle layers. (Fig. 1). For example, P450c17 is a single enzyme which possesses two enzymatic activities, 17α-hydroxylase and C_{17-20} lyase activities, responsible for hydroxylation of the 17α-position of progestogens and cleavage of the C17-C20 bond of 17α-hydroxylated progestogens, respectively. Aromatase (P450arom) is essential for aromatization of aromatisable androgens (i. e., production of estrogens). Therefore, the particular steroidogenic enzymes expressed at any one time govern which steroids are produced. For this reason, in order to understand the control of steroid hormone production, the comprehensive knowledge on the mechanisms of gene expression of each steroidogenic enzyme is essential. Recent advances in molecular biological techniques have enabled the successful cloning of cDNAs encoding steroidogenic enzymes. cDNAs of main steroidogenic enzymes (Chung *et al.*, 1986; Lachance *et al.*, 1989; Chung *et al.*, 1987; Peltoketo *et al.*,1988) were cloned from human tissues, thereafter, steroidogenic enzymes from other vertebrate species, including fish were cloned, utilizing the amino acid homology with the human isoforms. Recently, in Japanese eel, the cDNA of one of the most important enzymes for production of estrogens, P450arom, was isolated and characterized (Ijiri *et al.*, unpublished). Northern blot analysis using the isolated cDNA revealed that the changes in mRNA levels of P450arom correlated well with both serum E_2 level and ovarian ability of E_2 production during oogenesis induced artificially by SPH treatments. However, the characterization of other steroidogenic enzymes is awaiting elucidation.

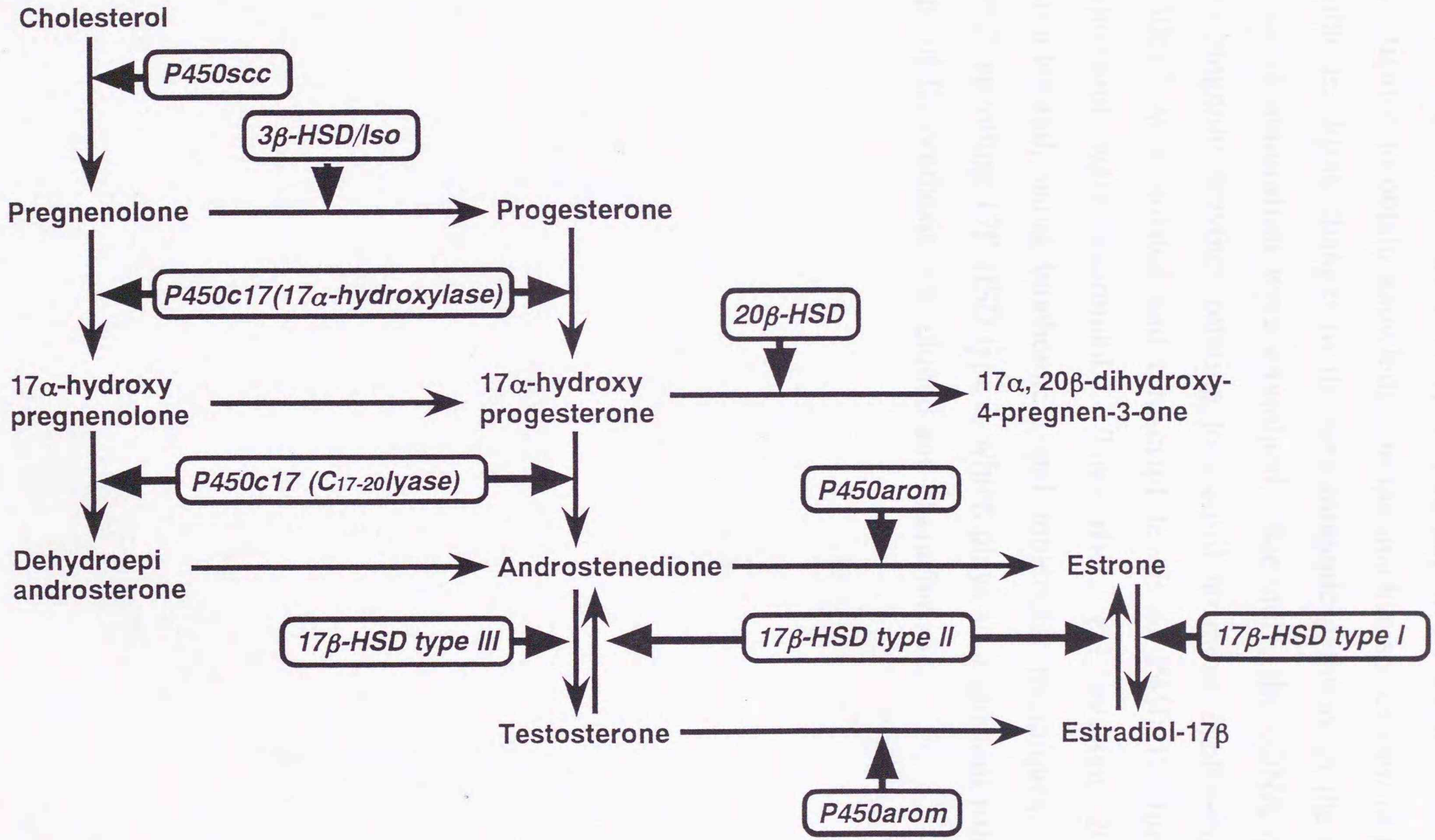


Fig. 1. General steroidogenic pathway

In the present study, ovarian steroidogenesis of the Japanese eel was investigated to obtain knowledge on the mechanism of steroid hormone synthesis. First, changes of the steroidogenic pathway in the ovary during artificial maturation were examined. Secondly, the cDNA of one of the steroidogenic enzymes relating to steroid hormone synthesis, eel ovarian P450c17, was isolated and transcript levels of P450c17 during artificial maturation were examined. Thereafter, eel ovarian 20β-HSD was characterized, using biochemical and molecular techniques. Finally, the cDNA encoding 17β-HSD type I, which plays an important role in the final steps of E_2 synthesis, was cloned and characterized.

Chapter II

Modulation of the steroidogenic pathway in Japanese eel ovarian follicles during artificial maturation

Introduction

In females of many teleost species, vitellogenesis and final oocyte maturation are controlled by two distinct steroid hormones, estradiol-17β (E_2) and 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-DHP), respectively (Nagahama, 1987). Previous studies on steroid synthesis have indicated that a change in steroidogenic pathway from production of estrogens, including E_2, or androgens, to progestins, including 17α, 20β-DHP, is observed during the transition from vitellogenesis to final oocyte maturation in many teleost species (Nagahama *et al.*, 1986; Schoonen *et al.*, 1989; Suzuki *et al.*, 1991). In some species, high concentrations of androgens in plasma and high potency in androgen production have been reported prior to the maturational phase (Kagawa *et al.*, 1983; Kanamori *et al.*, 1988; Suzuki *et al.*, 1989), yet the roles of androgens in female fish remain unknown .

Recently, changes in the capacity for the production of E_2 and 17α, 20β-DHP from exogenous steroid precursors of ovarian follicles during artificial maturation of the Japanese eel, have been elucidated (Ijiri *et al.*, 1995). Furthermore, 17α, 20β-DHP was identified as the major maturation-inducing steroid in this species (unpublished data), but the detailed steroidogenic pathway to produce these steroid hormones remains unclear.

This chapter was designed to elucidate the changes in steroidogenic pathway of ovarian follicles during artificial maturation in the Japanese eel.
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Materials and Methods

Chemicals

[4-^{14}C] Pregnenolone (50-60 mCi/mmol) was obtained from New England Nuclear (Boston, MA), and purified immediately prior to use by thin-layer chromatography (TLC) in a benzene-acetone (4:1, v/v) system. Authentic steroids were purchased from Sigma Chemical Company (St. Louis, MO) and Steraloids inc. (Wilton, NH). All the other reagents used were analytical grade.

Animals and tissue collection

Female Japanese eels at the silver stage were captured in the Hiranuma River, Aomori Prefecture.

After acclimation to artificial sea water, they were maintained without feeding in 1 m^3 tanks equipped with recirculating sea water at 20 °C and constant aeration. Eels received weekly intramuscular injections of acetone-dried salmon pituitary homogenate (20 mg/kg BW) suspended in eel ringer (NaCl 150 mM, KCl 3.0 mM, $MgCl_2$ 3.5 mM, $CaCl_2$ 5.0 mM, HEPES 10 mM, pH 7.4).

After decapitation or biopsies, ovarian tissues were removed from the fish and placed in ice-cold eel ringer.

Tissue preparation and incubation

After removal of ovarian tissues, follicles were separated from interstitial tissues with fine forceps. Follicles of the largest size-class were collected. Oocytes, measured with a Profile Projector (Nikon, Japan), early vitellogenic, mid-vitellogenic or migratory nucleus (post-

vitellogenic) stages and spanned 200-300 μm, 400-600 μm and 800-900 μm in diameter, respectively.

Ten intact follicles in each well were incubated in each well of a 24-well culture plate (Corning), containing 1 ml of eel ringer and [4-^{14}C] pregnenolone (about 1.0 x 10^5 cpm, 330 ng) dissolved in 10 μl propylene glycol, at 20 °C for 18 hours in a humidified incubator. Duplicate incubations were done for each experiment. At the end of incubation, final oocyte maturation was scored if the sign of germinal vesicle breakdown (GVBD) had occurred. Incubation media were collected and stored at -30 °C until analysis of steroids by thin-layer chromatography (TLC).

Extraction, separation, identification and quantification

Incubation media were extracted three times with about 2 ml of dichloromethane. The extracts were pooled and dried with anhydrous Na_2SO_4. After the addition of standard steroids (progesterone, androstenedione, 17α-OHP, testosterone, 17α, 20β-DHP, 20μg each), the extracts were applied to precoated silica gel TLC-plates (0.25 mm in thickness, 5 x 20 cm, E. Merk, Darmstadt, Germany) in a benzene-acetone (4:1, v/v) system. Radioactive spots on chromatograms were detected autoradiographically by an X-ray imaging analyzer (Fuji BAS 2000; Fuji film, Tokyo, Japan). These spots were removed from the plates separately with a scraper, and radioactive metabolites eluted from the silica gel with 1 ml of acetone after suspension in a small volume of water. Extraction was repeated four more times. After extraction, each radiolabeled metabolite was developed in several solvent systems by TLC using several authentic steroids as markers. When the metabolite had the same mobility as an

authentic steroid, attempts were made to identify it based on constant specific activities of crystals after repeated crystallization with the corresponding authentic steroid. Standard steroids were visualized under short-wave ultraviolet light (254 nm) or with ethanol-sulfuric acid (4:1, v/v). Radioactivities of crystals were measured in 5 ml of toluene containing 2,5-diphenyloxazole (DPO, 0.4% w/v) and 2,2'-p-phenylene-bis-(-5-phenylene oxazole) (POPOP, 0.01 w/v) with a scintillation counter (Beckman). Percentages of unused precursors and yields of identified metabolites were estimated as a ratio against of total radioactivities on the TLC plate using an X-ray imaging analyzer. All data were expressed as the mean $\pm$ SEM of two fish at the early vitellogenic stage, four fish at the mid-vitellogenic and three fish at the migratory nucleus stage.

Results

Oocyte maturation in vitro

GVBD could not be induced in ovarian follicles at the early and mid-vitellogenic stages by addition of pregnenolone to the incubation medium. However, when ovarian follicles at the migratory nucleus stage were incubated in the presence of precursor, GVBD occurred in all oocytes.

Identification of the metabolites

When precursor was provided for ovarian follicles at the mid-vitellogenic stage or migratory nucleus stage, one of the main radioactive products showed the same mobility as that of standard 17α-OHP. This metabolite was re-chromatographed in a system of dichloromethane-methanol (9:1, v/v) with authentic 17α-OHP and dehydroepiandrosterone (DHA) as markers. At the mid-vitellogenic stage, the recovered spot was separated into two metabolites, confirmed as 17α-OHP and DHA, respectively. At the migratory nucleus stage, this spot represented only one metabolite, which had identical mobility to that of 17α-OHP.

Some metabolites were dissolved in a small volume of organic solvent, for example dichloromethane, with the corresponding authentic steroids and the mixtures were recrystallized several times with various solvent systems. Some metabolites had constant specific radioactivities with the corresponding authentic steroids as shown in Table 1. Accordingly, androstenedione, DHA, 5α-androstanedione, progesterone, 17α-hydroxypregnenolone, 17α-OHP, and 17α, 20β-DHP were identified.

Table 1. Identification by recrystallization of the metabolites synthesized from [4- ^{14}C]pregnenolone by Japanese eel ovarian follicles

Metabolite	Specific activity of crystal (cpm/mg)	Solvent system
Androstenedione	590[a]	
	483	Dichloromethane - n - heptane
	483	Dichloromethane - n - hexane
	522	Dichloromethane - cyclohexane
Dehydroepiandrosterone	305[a]	
	243	Dichloromethane - n - hexane
	236	Dichloromethane - n - heptane
	238	Chloroform - cyclohexane
5α-androstanedione	545[a]	
	561	Dichloromethane - n - heptane
	488	Chloroform - n - hexane
	533	Acetone - water
Progesterone	600[a]	
	614	Dichloromethane - n - hexane
	586	Methanol - water
	571	Acetone - water
17α-hydroxypregnenolone	670[a]	
	680	Ethanol - water
	701	Acetone - water
	646	Methanol - water
17α-hydroxyprogesterone	925[a]	
	937	Dichloromethane - n - heptane
	924	Dichloromethane - n - hexane
	804	Chloroform - n - hexane
	802	Chloroform - cyclohexane
17α, 20β - dihydroxy - 4 - pregnen - 3 - one	556[a]	
	579	Dichloromethane - n - heptane
	522	Dichloromethane - n - hexane
	546	Chloroform - n - hexane
	582	Chloroform - cyclohexane

a ; Specific activity before recrystallization

Metabolism of steroids in ovarian follicles

Autoradiographs of steroid metabolites produced by ovarian follicles at different developmental stages and changes in the yields of main metabolites are shown in Fig. 2 and Fig. 3, respectively. Ovarian follicles at the early vitellogenic stage hardly metabolized any pregnenolone to the other steroid metabolites (Fig. 2 a), although there were some instances during which several metabolites, with mobilities identical to progesterone and DHA, were produced in small yields. In some cases, the production of unknown 1 was relatively high (Fig. 2 a).

At the mid-vitellogenic stage (Fig. 2 b,c), conversion of pregnenolone into other steroid metabolites increased greatly. At this stage, androstenedione and unknown 3 were produced as main metabolites in all cases, though 4-ene-C21 steroids, e.g. progesterone, 17α-OHP and 17α, 20β-DHP, were in some cases also detected. On one occasion, high production of 17α, 20β-DHP (about 10% yield) was observed (Fig. 2 c). When 4-ene-C21 steroids were not detected, the yields of C19 steroids (androstenedione and DHA) were notably higher than in those cases that did yield 4-ene-C21 steroids.

At the migratory nucleus stage (Fig. 2 d), the yields of androstenedione and unknown 3 decreased drastically and DHA was not detectable at all. In contrast, the yields of progesterone, 17α-OHP and 17α, 20β-DHP increased markedly and the total yield of these metabolites reached about 40% of all radioactive steroids. These metabolites were detected in all incubates. Metabolites with isopolarities to testosterone and 17α, 20β, 21-trihydroxy-4-pregnen-3-one were also observed. However, identification

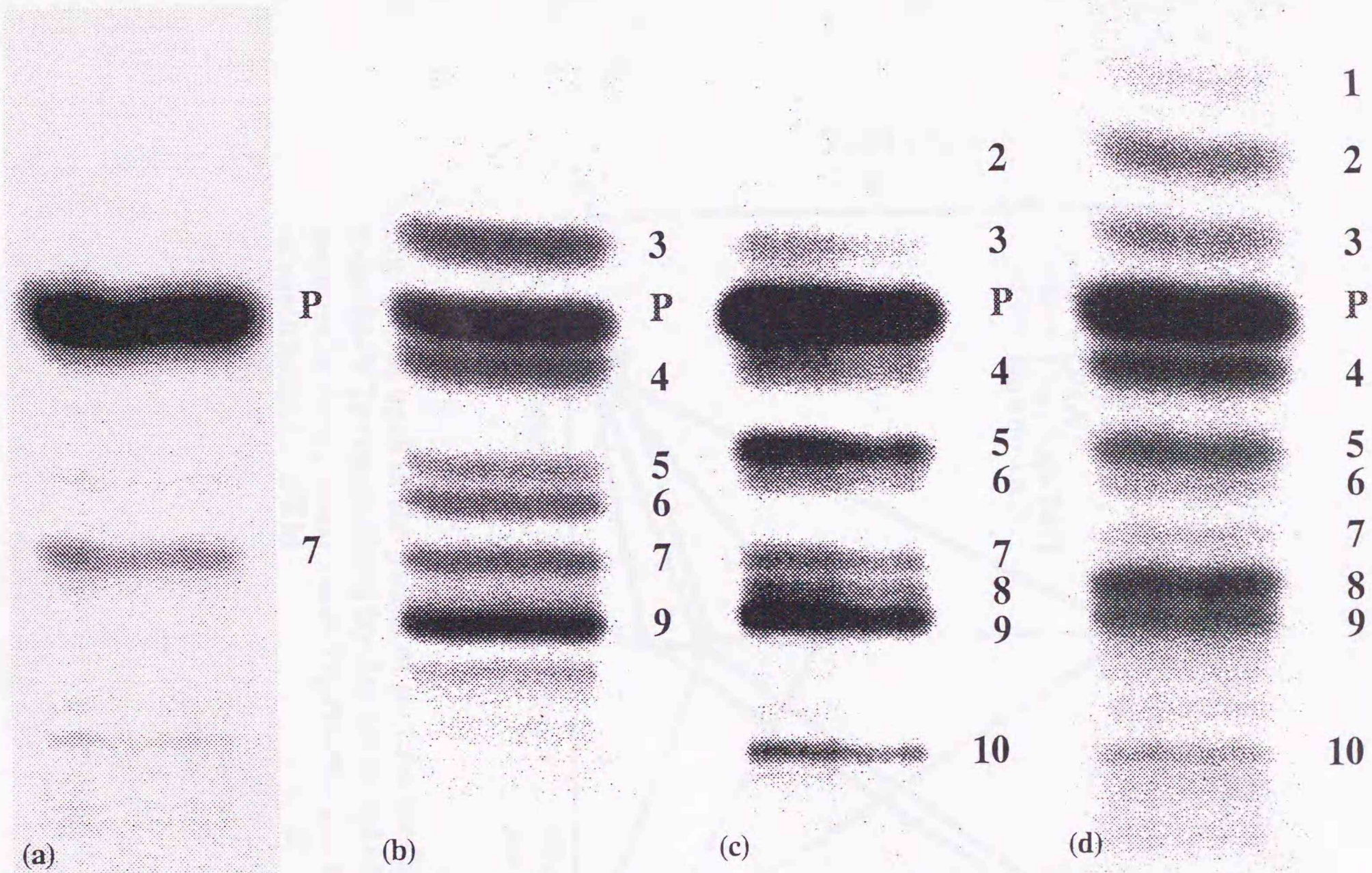


Fig. 2. Changes in autoradiograms of thin-layer chromatograms of the metabolites converted from [4-^{14}C]pregnenolone by the Japanese eel ovarian follicles at an early vitellogenic stage (a), a mid-vitellogenic stage (b, c) and a migratory nucleus stage (d). 1: 5α-Androstanedione, 2: Progesterone, 3: Androstenedione, 4: 17α-OHP and/or DHA, 5: 17α-hydroxypregnenolone, 6: unknown 1, 7: unknown 2, 8: 17α, 20β-DHP, 9: unknown 3, 10: unknown 4, P: Pregnenolone (precursor).

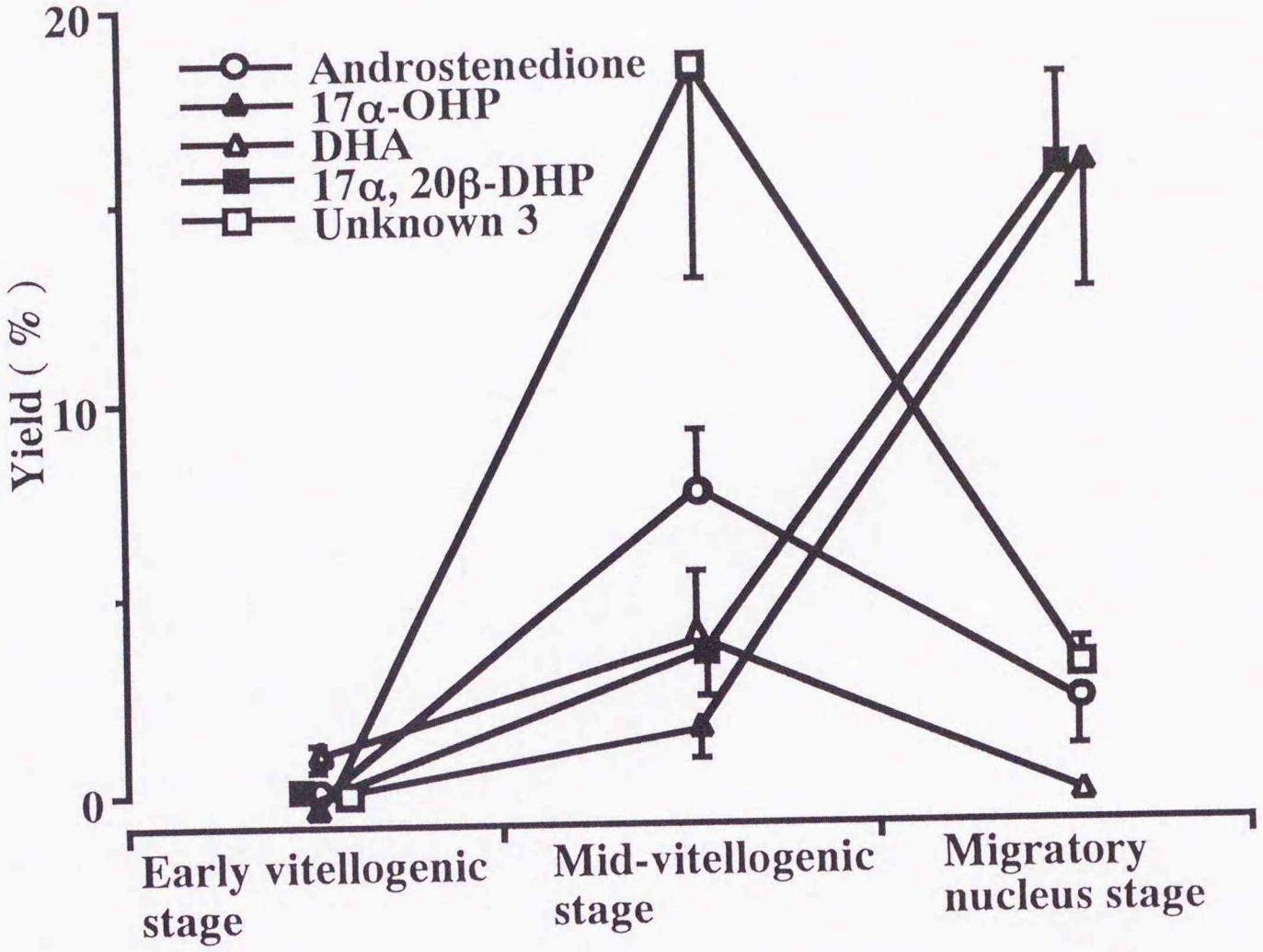


Fig. 3. Changes in the yields of metabolites synthesized from [4-^{14}C]pregnenolone by Japanese eel ovarian follicles *in vitro*. Each value represents the mean yield of metabolites ± SEM.

could not be confirmed by recrystallization because of the small amount of these products.

Discussion

In this chapter, changes in steroidogenic pathway during artificial induction of maturation in female Japanese eel were examined *in vitro*. Ovarian follicles in the early vitellogenic stage could hardly metabolize pregnenolone. In the European eel, *Anguilla anguilla*, pregnenolone was widely converted to other steroid metabolites by intact ovarian tissue or ovarian homogenates in the early vitellogenic stage (Colombo and Colombo-Belvedere, 1976; Querat *et al.*, 1986; Lambert *et al.*, 1991). The reason for the lack of conversion of pregnenolone in our experiments may be related to much smaller amounts of ovarian tissue used in this experiment than that in previous studies on the European eel.

In the mid-vitellogenic stage, pregnenolone was converted into several steroid metabolites. Androstenedione, DHA, progesterone, 17α-hydroxypregnenolone and unknown 3 were obtained as main metabolites. The precursor-product reactions indicate that activities of 3β-hydroxysteroid dehydrogenase / δ5-δ4 isomerase complex (3β-HSD), 17α-hydroxylase, $C_{17,20}$-lyase were induced or strongly enhanced. Therefore, the 4-ene pathway, reflecting conversion of pregnenolone into progesterone, progesterone into 17α-OHP and subsequently into androstenedione, appeared to be induced by SPH injections at this stage. Although 4-ene-C21 steroids could not be detected on one occasion, androstenedione and DHA were always present. This finding indicates that not only the 4-ene, but also the 5-ene pathway, reflecting pregnenolone conversion to 17α-hydroxypregnenolone, 17α-hydroxypregnenolone conversion to DHA, and DHA conversion into androstenedione, is used

Colombo and Colombo-Belvedere (1976) reported the predominance of the 5-ene over the 4-ene pathway in the European eel at the silver stage. This, together with the present findings, suggests that at first the 5-ene pathway may predominate rather than the 4-ene pathway, which takes over during the course of oogenesis, from the early to the mid-vitellogenic stage in the Japanese eel. This shift of the steroidogenic pathway may reflect enhanced 3β-HSD activity by SPH injections. Although unknown 3 was detected as one of the main metabolites, it could not be identified due to a lack of available standard steroids. Since the production of unknown 3 increased at the mid-vitellogenic stage and decreased at the migratory nucleus stage, this metabolite could be associated with a shift in the steroidogenic pathway, and should be investigated further. In one case, production of 17α, 20β-DHP (maturation-inducing steroid in this species) was very high at the mid-vitellogenic stage. 20β-HSD, which catalyzes the reduction 17α-OHP to 17α, 20β-DHP, was present in ovarian tissues during vitellogenesis in the European eel (Lambert *et al.*, 1991) and in the New Zealand longfinned eel (Lokman and Young, 1995). Enhanced 20β-HSD activity in ovarian follicles during vitellogenesis may be an unique feature in anguillids, although it has been reported that 20β-HSD could be significantly enhanced *in vitro* (Sakai *et al.*, 1987; Zhar W-X *et al.*, 1985; Young *et al.*, 1983; Suzuki *et al.*, 1988) and *in vivo* (Suzuki *et al.*, 1981; Schoonen *et al.*, 1989; Suzuki *et al.*, 1991) by gonadotropic treatment in many species of teleost fish. Alternatively activation of 20β-HSD at the mid-vitellogenic stage may not be a special feature of eels, but one of the effects of long-term gonadotropic treatment.

Ovarian follicles at the migratory nucleus stage, when incubated with precursor, produced large amounts of 17α, 20β-DHP, and this result confirmed that by Ijiri *et al.* (1995). Production of progesterone and 17α-OHP was very high, whereas that of androstenedione and DHA decreased markedly. These findings suggests that a pathway from pregnenolone to progesterone, 17α-OHP and, finally, 17α, 20β-DHP was induced by a decrease in $C_{17,20}$-lyase activity and an increase in 20β-HSD activity. In several teleost species whose maturation-inducing steroid was suggested to be 17α, 20β-DHP, this steroid has been detected in plasma during final gonadal maturational period (Idler *et al.*, 1959; Campell *et al.*, 1980; Nagahama *et al.*, 1991). In contrast, 17α, 20β-DHP was consistently undetectable in serum of the Japanese eel during artificial maturation (Yamauchi, 1990; Ijiri *et al.*, 1995). In this species, most females cannot spontaneously undergo oocyte maturation and ovulation, which may be one of the reasons for the absence of 17α, 20β-DHP in serum. Furthermore, when eels with follicles in the migratory nucleus stage received an injection of 17α-OHP to induce final maturation and ovulation, 17α, 20β-DHP was detected in the serum (unpublished data). Furthermore, 17α, 20β-DHP can detected in the serum of eels which could spontaneously undergo maturation and ovulation. Absence of serum 17α, 20β-DHP in mature eel and the production of 17α, 20β-DHP in response to exogenous substrate *in vivo* and *in vitro* suggest a lack of supply of endogenous steroid substrate, such as pregnenolone. The most effective inducer of GVBD *in vitro* in the Japanese eel was 17α, 20β-DHP, followed by 17α, 20β, 21-trihydroxy-4-pregnen-3-one and 17α-OHP (unpublished data). Since 17α-OHP was produced in considerable amounts at the prematurational stage, 17α-OHP

may have some roles in oocyte maturation. However, migratory nucleus stage ovarian follicles incubated with 17α-OHP rapidly produced large amounts of 17α, 20β-DHP (Ijiri *et al.*, 1995). Therefore, the maturation-inducing effect of 17α-OHP is attributable to the effect of 17α, 20β-DHP , synthesized from 17α-OHP. A trace amount of 17α, 20β, 21-trihydroxy-4-pregnen-3-one was ,nevertheless, observed in the present study. This steroid was reported as a major inducer of GVBD in Atlantic croaker (*Micropogonias undulatus*) (Trant and Thomas, 1989) and spotted seatrout (*Cynoscion nebulosus*) (Thomas and Trant, 1989). However, since this steroid was not detected in some cases, 17α, 20β, 21-trihydroxy-4-pregnen-3-one seemed to contribute little to oocyte maturation in the Japanese eel.

In conclusion, it is suggested that Japanese eel ovarian follicles undergo changes in steroidogenesis during vitellogenesis and final oocyte maturation. The shift in production of C19 steroids, androstenedione and DHA, to the formation of maturation-inducing steroid, 17α, 20β-DHP appears to occur during artificial maturation, especially during the transition from the mid-vitellogenic to migratory nucleus stage *in vitro*. These results indicate that this steroidogenic shift may occur by changes in activities of key enzymes involved in steroid metabolism, most notably, a decrease in $C_{17,20}$-lyase and an increase in 20β-HSD activities.

Chapter III.

Molecular cloning and characterization of Japanese eel ovarian P450c17 cDNA

Introduction

Cytochrome P45017α-hydroxylase/C_{17-20} lyase (P450c17) is essential for the production of sex steroids, androgens and estrogens, in gonadal tissues and for cortisol production in adrenal tissues of all vertebrates. P450c17 is a single enzyme that has both 17α-hydroxylase and C_{17-20} lyase activities (Nakajin *et al.*, 1984; Zuber *et al.*, 1986). For the production of sex steroids in gonads, enzymatic activities of both 17α-hydroxylase and C_{17-20} lyase are required. In contrast, only 17α-hydroxylase activity is required for the production of cortisol in adrenal tissues. Thus, P450c17 is one of the key enzymes involved in both steroidogenesis of both gonads and adrenal gland.

Recently, it was demonstrated by bioassay that SPH can greatly enhance the activities of several steroidogenic enzymes, such as 17α-hydroxylase and C_{17-20} lyase, in ovarian follicles (Ijiri *et al.*, 1995). Furthermore, in chapter II, there was a good evidence to suggest that a shift in the ovarian steroidogenic pathway from the production of androgens to the synthesis of 17α-hydroxylated progestins, 17α-OHP and 17α, 20β-DHP, occurs during the transition from the vitellogenic to the maturational stage *in vitro*. This finding suggests that C_{17-20} lyase activity decreases prior to the maturational phase, hence, P450c17 is one of the key enzymes involved in the prematurational steroidogenic shift in eel ovarian tissues.

In mammals, there are several reports on isolation of a cDNA encoding P450c17 and the regulation of its expression by gonadotropins (Voss and Fortune, 1993) or some growth factors (Sawetawan *et al.*, 1996). However, in fish, few reports have addressed the hormonal regulation of

P450c17 gene expression and only two studies on cloning of a cDNA encoding P450c17 have been performed, i.e., in rainbow trout (Sakai *et al.*, 1992) and spiny dogfish shark (Trant, 1995).

In this chapter, as the first step to investigate the mechanism regulating the steroidogenic shift in eel ovarian follicles, the cDNA encoding P450c17 was cloned from an eel ovarian cDNA library and eel P450c17 was characterized in a eucaryotic cDNA expression system using COS-7 cells. Furthermore, changes in the level of P450c17 mRNA were examined during ovarian development induced by hormonal treatment.

Materials and Methods

Animals

Silver female Japanese eels caught from the Hiranuma River, Aomori Prefecture, and cultivated eels, feminized by estradiol-17β administration (Tachiti and Nakagawa, 1992), were used. After acclimation to seawater, eels received weekly intramuscular injections of 20 (one silver eel, 6 times) or 40 μg/g BW (16 feminized eels, 0-9 times) acetone-dried salmon pituitary homogenate (SPH) suspended in eel ringer (NaCl 150 mM, KCl 3.0 mM, $MgCl_2$ 3.5 mM, $CaCl_2$ 5.0 mM, HEPES 10 mM, pH 7.4) to induce gonadal development. Two feminized eels with ovaries at the maturational stage were subsequently injected with 1 μg/g BW 17α, 20β-DHP to induce ovulation.

Ovarian tissues were removed from these fish, frozen in liquid nitrogen and kept at -80 °C, prior to preparation of poly(A)$^+$-RNA. Silver eel ovarian tissues in late vitellogenesis were used for construction of a cDNA library. For all other analyses, ovarian tissues from feminized eels were used.

Oocyte diameters were measured for 100 follicles per fish. The mean diameter of 20 follicles corresponding to the largest size class was calculated to estimate the stage of ovarian development. The stages were classified as follows: early vitellogenic stage, 200-400 μm; mid-vitellogenic stage, 400-600 μm; late vitellogenic stage, 600-800 μm; migratory nucleus stage, 800-1000 μm. These criteria were followed in following chapters.

Isolation of poly(A)$^+$-mRNA

Total RNA was extracted from ovarian tissues by guanidinium isothiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi 1987) using ISOGEN (Nippongene, Japan). Polyadenylated RNA was subsequently isolated from total RNA with Oligotex-dT-30 (Takara Shuzo, Japan). The cDNA was synthesized from oligo(dT)$^{12\text{-}18}$ primed mRNA using Superscript II Reverse Transcriptase (GIBCO BRL, U.S.A.).

Polymerase chain reaction (PCR) and isolation of P450c17 cDNA fragment

For amplification of a P450c17 cDNA fragment, upstream and downstream primers were designed from consensus sequences of P450c17 found in several vertebrate species. Upstream primer, 5'-GACATA(C)TTT(C)GGA(G)GCA(TCG)GG-3', and downstream primer, 5'-GCT(C)CCA(G)AAC(G)GGT(C)AAA(G)TA-3' were synthesized with an Applied Biosystems DNA synthesizer model 392 (Applied Biosystems, U.S.A.). PCR was carried out in a final volume of 50 μl containing 50 ng cDNA, 0.5 μM of each primer, 0.2 mM of each dNTP, 50 mM KCl, 10 mM Tris-HCl, 1.3 mM $MgCl_2$ and 2.5 U Taq DNA polymerase (Grainer, German). PCR was carried out for 30 cycles using an automatic thermal cycler (Gene Amp PCR System 2400, Perkin Elmer, U.S.A.) under the following conditions; denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min. After PCR, products were subcloned into the plasmid vector PCR™II, using a TA cloning kit (Invitrogen, U.S.A.). After subcloning, PCR products were sequenced with the Taq Dye Terminator Cycle Sequencing Kit (Perkin Elmer, U.S.A.) using a 373 DNA sequencer (Perkin Elmer, U.S.A.). Since the

amino acid sequence deduced from the PCR product had high homology to the corresponding regions of other P450c17 forms, the product was used as probe for screening of an eel ovarian cDNA library.

Library construction, screening and DNA sequencing

A λgt 10 cDNA library was constructed from oligo(dT)-primed ovarian mRNA using Amersham's cDNA cloning system. At least 100,000 clones were screened with a [^{32}P] dCTP-labeled eel P450c17 cDNA fragment obtained by PCR as described above, using a random-primed DNA labeling kit (New England Nuclear, U.S.A.) under the following conditions; 6 X SSC (1 X SSC: 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 0.2 % Ficoll and 0.2 % polyvinylpyrrolidone (this mixture is hereafter referred to as prehybrydization buffer), 0.1 % sodium dodecyl sulfate (SDS) and 0.1 % bovine serum albumin (BSA). After screening, four positive clones were isolated and digested with Eco RI to evaluate the length of the cDNA insert. The longest insert was about 2.2 kbp in length and subcloned into pUC19. For sequencing, the insert was digested with appropriate restriction enzymes and subcloned into the polylinker region of pUC19 or pBluescript II SK$^-$.

Expression of eel P450c17 in COS-7

To obtain the P450c17 cDNA containing the open reading frame only, an upstream primer, 5'-ATGGAGTGGATTTTTTGTTCG-3' and a downstream primer, 5'-GCGGAGAACCAAACTGATGGCTAA-3' were synthesized. PCR was carried out in the same solution as described above under the following thermal conditions: denaturation at 94 °C for 1 min,

annealing at 60 °C for 1 min and extension at 72 °C for 2 min. After PCR, the eel P450c17 product was obtained, sequenced for confirmation and subcloned into pSG5, one of the *in vitro* eucaryotic expression vectors for protein synthesis (Green *et al.*, 1988). For expression of P450c17, 1 x 10^5 COS-7 cells (Riken, Japan) were dispensed into the 6 wells of a tissue culture dish and transfected with 1.5 μg of the complex of eel P450c17 PCR product and pSG5 (eP450c17pSG5). Empty pSG5 were used as controls. Transfections were performed using the DOSPER Liposomal Transfection Reagent (Boehringer Mannheim, German). Transfected COS-7 cells were pre-cultured in Dulbecco's Modified Eagles medium (Nissui, Japan) containing 10 % fetal bovine serum at 37 °C for 36 hours. Cells were then incubated for 2, 8 or 16 hours in 2 ml of the same medium, in the presence of 0.1 μM of ^{14}C labeled pregnenolone or progesterone (New England Nuclear, U.S.A.) as precursors. Three replicate incubations were made for each experimental group. After incubation, media were collected and steroid metabolites were extracted three times with about 2 ml of dichloromethane. To evaluate enzymatic activities, thin-layer chromatography (TLC) analysis was conducted. The extracted steroid metabolites were loaded onto a TLC plate pre-coated with silica gel and separated in benzene-acetone (4;1, v/v). After separation, steroid metabolites were recovered and radioactivities quantified by liquid scintillation counting (Beckman, U.S.A.). The yield of each steroid metabolite was calculated as percentage total radioactivity.

Northern blot analysis

Poly(A)$^+$-RNA was isolated from ovarian tissues at pre-, early, mid- and late vitellogenic stages, the maturational stage and after ovulation (except for ovulated eggs). After denaturing at 70 °C for 10 min, 5 μg poly(A)$^+$-RNA and 2 μg RNA molecular weight ladder (GIBCO BRL, U.S.A.) were electrophoresed through a 1.1 % agarose gel containing 16 % formalin. The RNAs were transferred to a nylon membrane (Amersham, U.K.) and baked at 80 °C for 2 hours. The membrane was then prehybridized in prehybridization buffer containing 0.5 % SDS and 0.2 % BSA at 65 °C for 3 hours. After prehybridization, the membrane was hybridized overnight at 65 °C with a ^{32}P-labeled full-length eel P450c17 cDNA in prehybridization buffer containing 100 mg/ml salmon sperm DNA. The membrane was washed twice in 3 X SSC and twice in 1 X SSC, both containing 0.1 % SDS, at 65 °C for 30 min. The final washing was done in 0.1 X SSC containing 0.1 % SDS, at 65 °C for 20 min. After washing, the membrane was analyzed using a BAS 2000 Bio-Image analyzer (Fujix, Japan).

Results

Isolation and characterization of Japanese eel P450c17 cDNA

The screening of approximately 100,000 phage plaques using a cDNA fragment of eel P450c17 obtained by PCR resulted in isolation of four positively hybridizing clones. The longest clone was sequenced. The clone was 2228 bp in length and contained 55 bp of the 5'-untranslated region, a 1530 bp open reading frame starting from the third ATG and terminating at TAA, and 643 bp of 3'-untranslated region (Fig. 4).

The protein predicted from the open reading frame was 510 amino acid residues in length. Alignment of the amino acid residues of the eel P450c17 to those of rainbow trout (Sakai, *et al.,* 1992), spiny dogfish shark (Trant, 1995), chicken (Ono *et al.,* 1988), bovine (Zuber *et al.,* 1986(a)), rat (Fevolt, *et al.,* 1989) and human (Chung *et al.,* 1987) is shown in Fig. 5. The deduced amino acid sequence had high homology with the rainbow trout, shark and chicken forms (74 %, 61 % and 64 %, respectively) and between 45 % to 55 % with mammalian forms (bovine 55 %, rat 48 % and human 47 %). Moreover, the conserved P450c17 specific region, Ozols tridecapeptide region and the heme-binding region showed higher homology, viz. 87 %, 93 % and 85 %, respectively, than other areas when compared to those in rainbow trout.

gatgaatgacagtcaccgctgccggttgccttaagattgctaaacctgttggcg

ATG GAG TGG ATT TTT TGT TCG TTT CTA CTT TTC GTG GTC GCG CTC ACC GCG CTG CTA ATA AAA GCG
 M E W I F C S F L L F V V A L T A L L I K A
CGA ATC CAG AAG GCT CTG AAG GAC ACG CGA CCC CCA AGC CTT CCC TCG TTC CCC ATC ATC GGG AGT
 R I Q K A L K D T R P P S L P S F P I I G F
ATT CTC AGT TTG AGA AGC GAG CAC CCG CCG CAC GTG CTG TTT CAG GAG CTG CAG AAG AAG TAC GGT
 I L S L R S E H P P H V L F Q E L Q K K Y G
GAA ACC TAT TCG CTA AGG ATG GGC TCC CAC GAC GTG GTC ATC GTC AAC AGC CAC CAG CAT GCG AAG
 E T Y S L R M G S H D V V I V N S H Q H A K
GAA GTG CTC CTG AAA AGA GGC AAA ACC TTT GCA GGG AGA CCG AGA ACC GTC ACA ACA GAC ATT CTG
 E V L L K R G K T F A G R P R T V T T D I L
ACG AGG GAT GGG AAG GAT ATT GCA TTC GCA GAT TAC AGT GCC ACA TGG AGG TTC CAC AGA AAA ATG
 T R D G K D I A F A D Y S A T W R F H R K M
GTG CAT GGA GCT CTG TGT ATG TTC GGA GAG GGA TCA GCA TCC ATC GAA CAG ATA ATC TGC AGG GAG
 V H G A L C M F G E G S A S I E Q I I C R E
GCC AGC TCC ATG TGT CAG GCC CTA GTG GAG CTG CAG AGT ACT GCG GTT GAC CTG GCT CCA GAG CTG
 A S S M C Q A L V E L Q S T A V D L A P E L
ACC CGC GCT GTT ACC AAC GTG ATC TGC GCA CTC TGC TTC AGC TCC TCC TAC CAG CGC GGG GAC TGC
 T R A V T N V I C A L C F S S S Y Q R G D C
GAG TTT GAA GCC ATG CTG CAG TAC AGC CAG GGC ATA GTG GAC ACC GTG GCC AAG GAC AGC CTG GTG
 E F E A M L Q Y S Q G I V D T V A K D S L V
GAC ATC TTC CCC TGG CTG CAG ATA TTC CCC AAC AAA GAT CTA AAA ATC CTG AAG CAG TGT GTG ACT
 D I F P W L Q I F P N K D L K I L K Q C V T
GTC CGA GAC AGC CTG CTG CAG AAA AAG TAT GAC GAG CAC AAG GCG GAG TAC AGC GAT GAC ACG CAG
 V R D S L L Q K K Y D E H K A E Y S D D T Q
CGG GAC CTG CTG GAC GCG CTG ATC AGA GCC AAG CGC AGC GCG GAG AAC AAC AAC ACC AGC GCG ACG
 R D L L D A L I R A K R S A E N N N T S A T
CCG AGC CTG GGC CTG ACC GAC GAC CAC CTG CTG ATG ACC GTG GGG GAC ATC TTC GGG GCC GGC GTG
 P S L G L T D D H L L M T V G D I F G A G V
GAG ACC ACC ACC ACC GTG CTC AAA TGG GCC GTC GCC CTG CTC ATC CAC CAC CCC CAG GTG CAG AAG
 E T T T T V L K W A V A L L I H H P Q V Q K
AAG ATC CAG GAA GAG CTG GAC AGT AAA ATC GGG ATG AAC AGA CAC CCA AAA TTA AGC GAC AGA GGC
 K I Q E E L D S K I G M N R H P K L S D R G
AAC CTG CCG TAT CTT GAG GCC ACC ATC AGG GAG GTG CTG AGA ATC CGG CCG GTG TCG CCC CTC CTC
 N L P Y L E A T I R E V L R I R P V S P L L
ATA CCC CAC GTG GCC CTC GCT GAC TCC AGC ATC GGG GAG TAC ACA GTT GGA AAA GGG GCC AGA GTC
 I P H V A L A D S S I G E Y T V G K G A R V
ATC ATT AAT CTA TGG TCC CTG CAT CAT GAC GAG AAA GAG TGG AAG AAC CCT GAG ATC TTT GAT CCC
 I I N L W S L H H D E K E W K N P E I F D P
ACC CGC TTT CTG GAC GAG GAG GGC AGC AAC CTG TGC TGT CCC TCG GAG AGT TAC CTG CCG TTC GGG
 T R F L D E E G S N L C C P S E S Y L P F G
GCT GGA ATC AGG GTG TGT CTC GGG GAG TCC CTG GCG AAG ATG GAG CTG TTC CTC TTC CTC GCC TGG
 A G I R V C L G E S L A K M E L F L F L A W
ATC CTG CAG CGC TTC ACA TTG GAG GTT CCC CCA GGC CAG ACC CTG CCG GAC CTG GAG GGC AAA TTT
 I L Q R F T L E V P P G Q T L P D L E G K F
GGG GTG GTC CTG CAG CCG CAG AAG TAC AAA GTC TGC GCA CAG CTC AGG CCA TGC TGG GCG GAG AAC
 G V V L Q P Q K Y K V C A Q L R P C W A E N
CAA ACT GAT GGC TAA atccacaacaagcagggccaaagagtccaacccacttatgacatcacaataggttgggccataat
 Q T D G *

aaacccaatctgctgctgacatcacaatccactgcttacatcacaataagtcgggcaatgtaaaacccaacctccagctgacat
cccacagggcagggccaaagaacccaatcccctgcttacatcacaatgggttgggtcaaaaagacccaacctactgctaactat
cctgggcatgtaagtgggcagtcatgccactgggctacaataccagccattgtagcaataatatgtatatacatatatatcatt
tttgtcttctttggacagaacagggatgttgttcttccactaagaacatatccctgttggaatttttcacaaagtatgatgtaa
tttttaaaattctttttataatttcaaatgataaacagatgagagatggttttgcttcaggagaatagaagaaaagatctgtg
acattcagttcaatgccttttttatttatcttgcagttatttttaatttttaatataattaatttgtcattcaattgacacag
gagctgtgttcaaggatcactaatgaaacatactttagaaaagaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa

Fig. 4. Nucleotide and amino acid sequence of the cDNA encoding eel ovarian P450c17. The open reading frame, composed of 1530 bp, encodes 510 amino acid residues.

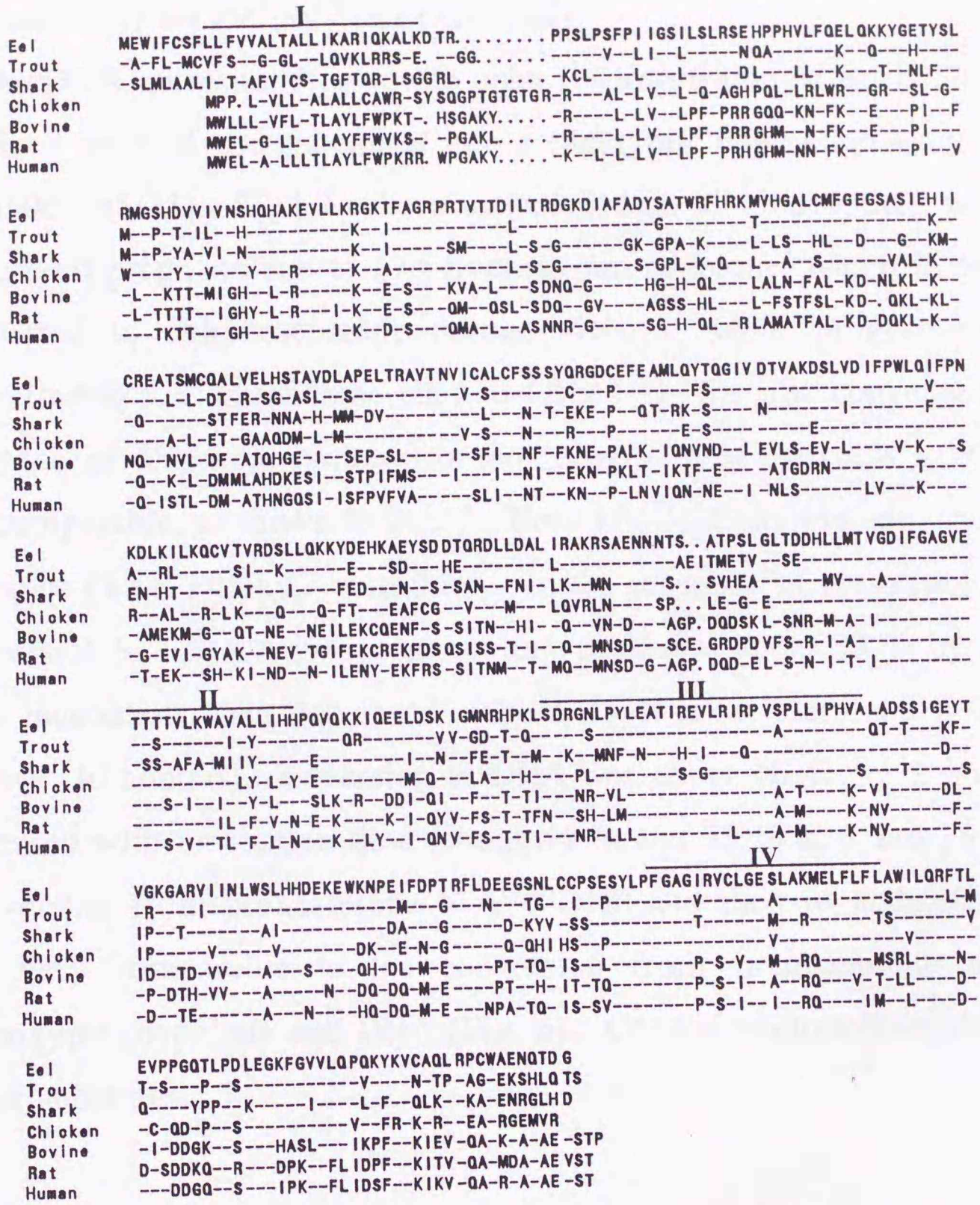


Fig. 5. Alignment of the deduced amino acid sequence of eel P450c17 with those of other animal species. Dashes indicate residues that are identical to the eel form. Dots indicate gaps introduced to facilitate alignment. I; the putative membrane spanning region, II; the conserved P450c17 specific region, III; the Ozols tridecapeptide region, IV; the heme-binding region

Expression of eel P450c17 in COS-7 cells

A cDNA corresponding to the open reading frame of eel P450c17 was obtained by PCR and subcloned into a eucaryotic expression vector, pSG5 (eP450c17pSG5). COS-7 cells transfected with eP450c17pSG5 were able to convert pregnenolone to 17α-hydroxypregnenolone, which in turn was converted to dehydroepiandrosterone (DHA), while progesterone was converted to androstenedione via 17α-OHP (Fig. 6). The conversion in the presence of δ5 (pregnenolone) or δ4 (progesterone)-steroids as substrate are comparable, as shown in Fig. 7. Both 17α-hydroxylase and C_{17-20} lyase activities of eel P450c17 were high. In the presence of pregnenolone, the conversion to 17α-hydroxypregnenolone peaked at about 23 % by 2 hours after incubation, then decreased (about 21 % at 8 hours and 2 % at 16 hours). In contrast, conversion to DHA was about 10 % by 2 hours and increased with incubation time (about 54 % and 85 % at 8 and 16 hours). Conversion from progesterone to 17α-OHP and then to androstenedione was very comparable to the conversion from pregnenolone to 17α-hydroxypregnenolone and DHA (Fig. 6). Control vectors did not convert either substrate.

Northern blot analysis of eel P450 c17 mRNA

Northern blot analysis revealed that only one transcript of 2.4 kb in length was present in eel ovarian tissues. P450c17 mRNA was not found before SPH treatment. However, the mRNA was detected after SPH injections, i.e., in early vitellogenic ovaries, and increased in mid-vitellogenic ovaries. Thereafter, the level of P450c17 mRNA did not change (Fig. 8).

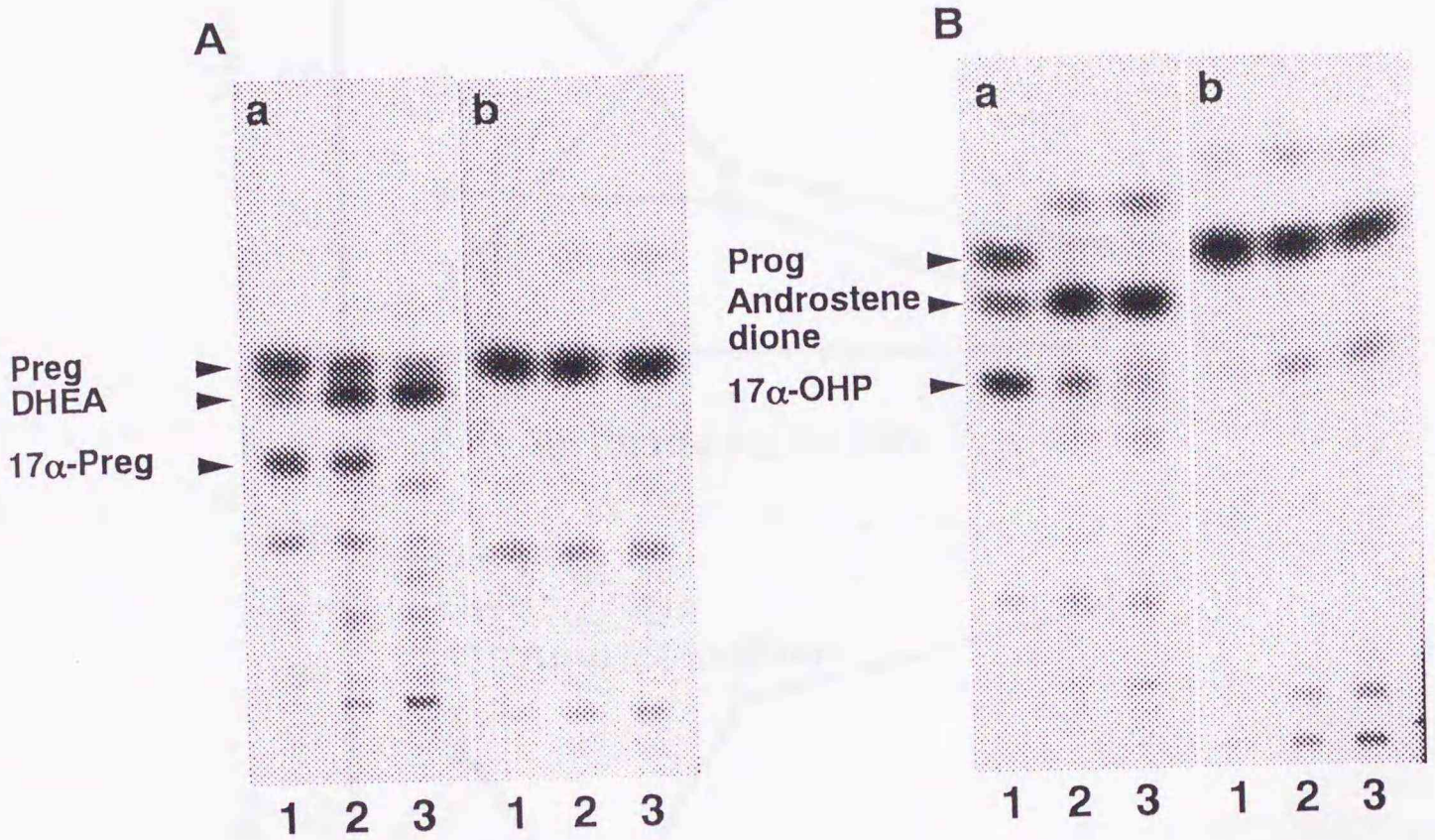


Fig. 6. Thin-layer chromatographs, illustrating the conversion of 0.1 μM ^{14}C-labeled pregnenolone (A) or progesterone (B) by COS-7 cells transfected with eP450c17pSG5 (a) or pSG5 (b).
Lane 1; incubation for 2 hr, lane 2; for 8 hr, lane 3; for 16hr.

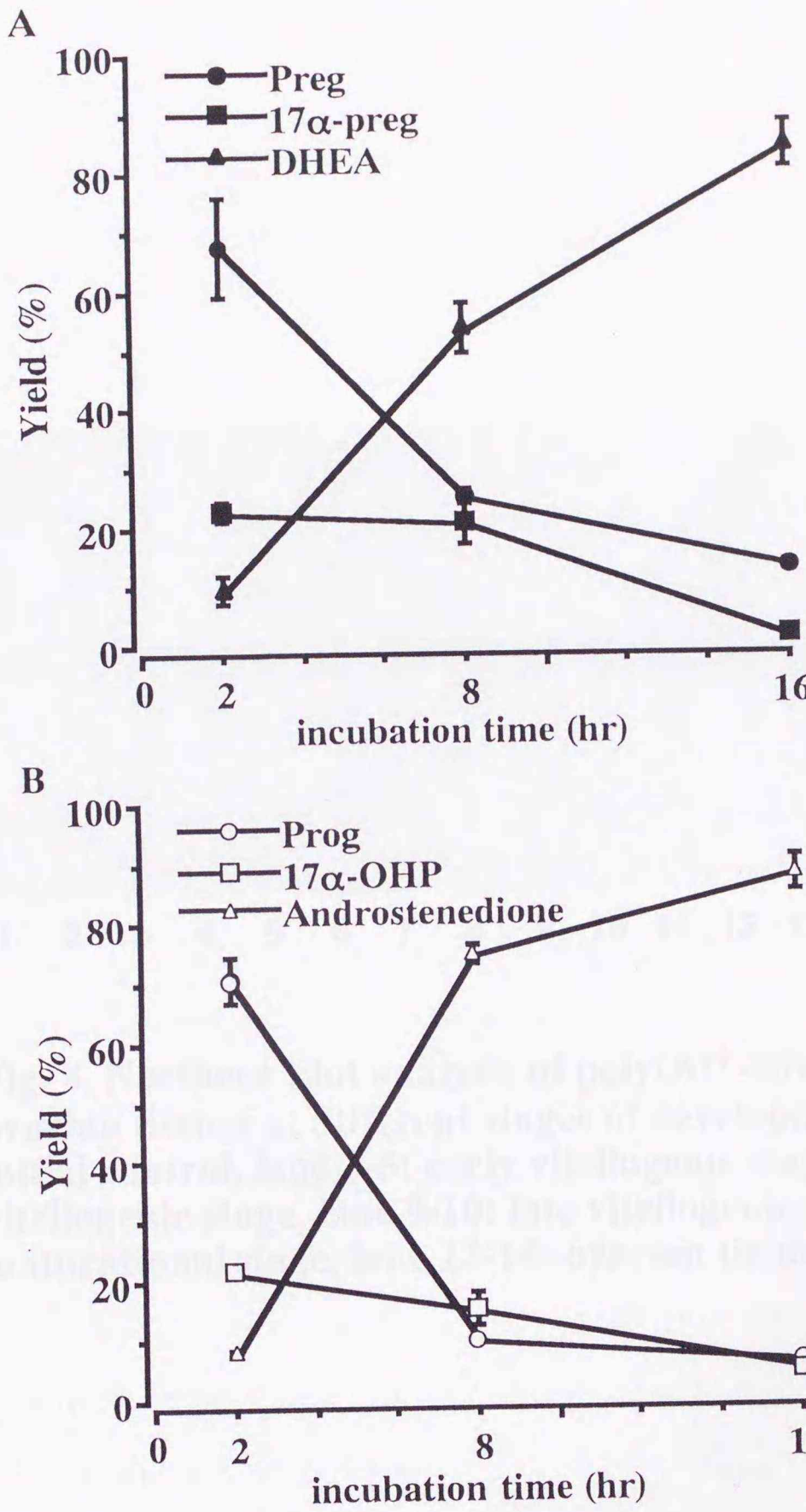


Fig. 7. Changes in yield of steroid metabolites produced from pregnenolone (Preg) or progesterone (Prog) by eel P450c17. ^{14}C-labeled pregnenolone (A) or progesterone (B) was added to COS-7 cells transfected with pSG5eP450c17. The bars represent the mean ± S.E.M. of three replicate measurements.

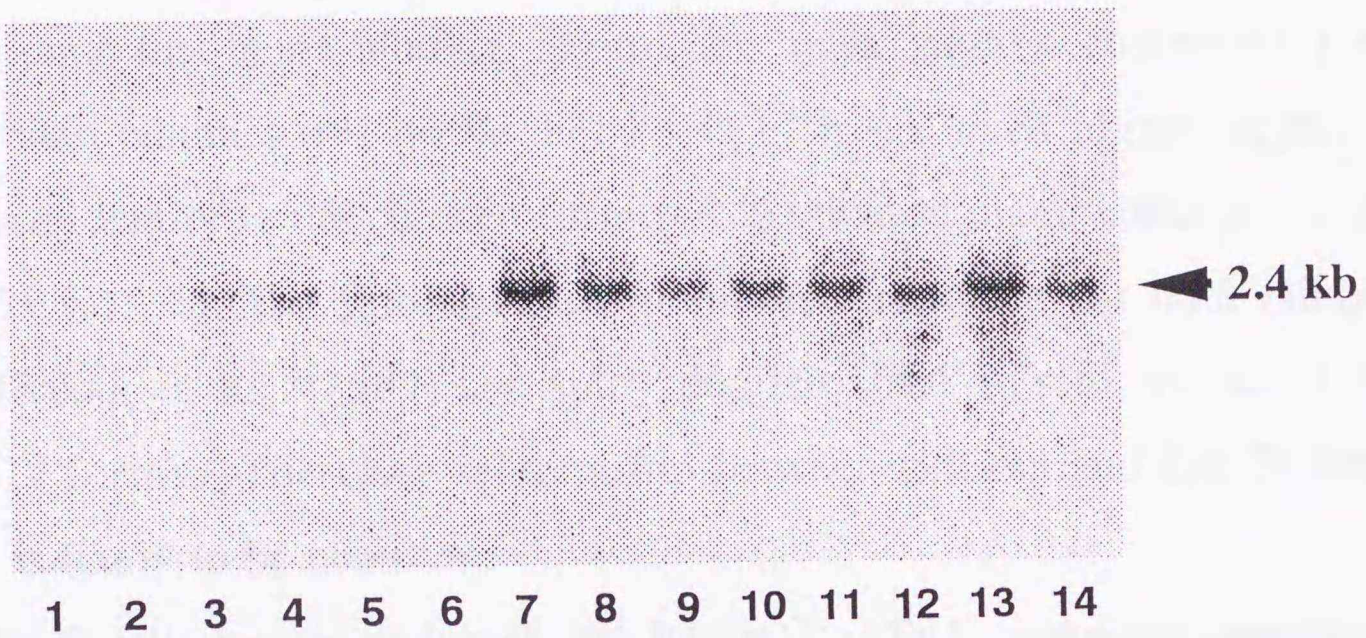


Fig. 8. Northern blot analysis of poly(A)$^+$-RNA (5μg) from ovarian tissues at different stages of development. Lane 1-2: initial control, lane 3-5: early vitellogenic stage, lane 6-8: mid-vitellogenic stage, lane 9-10: late vitellogenic stage, lane 11-12: maturational stage, lane 13-14: ovarian tissues after ovulation.

Discussion

In this chapter, a cDNA of approximately 2.2 kbp in length encoding eel P450c17 was isolated and the primary structure was characterized. The eel form of P450c17 has high homology to other animal forms, in particular, the amino acid residues in the conserved P450c17 specific region and the heme-binding region are quite highly conserved (viz., amino acid residues 301-460). Eel P450c17 has a much higher degree of sequence identity to the chicken form (64 %) than to mammalian forms (45 % to 55 %). Similar findings were previously reported for both rainbow trout (Sakai, *et. al.,* 1992) and spiny dogfish shark (Trant, *et. al.,* 1995) P450c17 forms. The close relationship between chicken and fish P450c17 forms is likely to be common.

COS-7 cells transfected with eel P450c17 cDNA converted exogenous pregnenolone to 17α-hydroxypregnenolone, and DHA, and exogenous progesterone to androstenedione via 17α-OHP. There was no difference in yields between conversion of pregnenolone to DHA via 17α-hydroxypregnenolone and that of progesterone to androstenedione via 17α-OHP in eel P450c17. To date, two types of P450c17 have been identified based on substrate specificities. One type possesses $C_{17\text{-}20}$ lyase activities for both δ4 (17α-OHP) and δ5 (17α-hydroxypregnenolone) steroid precursors, such as the rat isoform (eel form belongs to this type). The second type dose not have $C_{17\text{-}20}$ lyase activities for 17α-OHP, such as the human isoform. In human, $C_{17\text{-}20}$ lyase activity for 17α-OHP can be enhanced by electron-donating partners, such as cytochrome b5 (Katagiri, *et al.*, 1995). Furthermore, posttranslation of P450c17 by serine/threonine

phosphorylation selectively increase lyase activity, and dephosphorylation of this enzyme results in a decline of lyase activity (Miller *et al.*, 1997). Thus, the regulation of lyase activity by these factors governs whether or not androgens and estrogens are produced. There is no report on factors regulating lyase activity in fish, though such information may well be essential for elucidation of the mechanism of steroidogenic shift in fish gonads. In the European eel ovary at the silver stage, a predominance of the 5-ene over the 4-ene pathway was reported (Colombo and Colombo Belvedere, 1976) while in Japanese eel a steroidogenic shift from the 5-ene to 4-ene pathway was observed during oogenesis. However, eel P450c17 catalyzed the conversion of pregnenolone to 17α-hydroxypregnenolone and that of progesterone to 17α-OHP without much difference. For a 5-ene to 4-ene steroidogenic shift, both the period of expression of the 3β-hydroxysteroid dehydrogenase/ δ5-δ4 isomerase complex and its substrate specificity may be crucial rather than the characteristics of P450c17.

Human P450c17 can catalyze the conversion of progesterone to 16α-hydroxyprogesterone (Lorence *et al.*, 1990; Swart *et al.*, 1993). In this study, we could not demonstrate 16α-hydroxylase activity by eel P450c17. In P450c17s of trout (Sakai, *et. al.*, 1992), shark (Trant, *et. al.*, 1995) and other vertebrates, 16α-hydroxylase activity similarly could not be found . Thus, so far only the human form has 16α-hydroxylase activity. It deserves mentioning, however, rainbow trout head kidney has the ability to produces 16α-hydroxyprogesterone from progesterone (Andersson and Rafter, 1990). These findings suggest that a 16α-hydroxylase different from P450c17 exists in the adrenal gland of, at least some, fishes.

Northern blot analysis indicated that a single transcript was present and that it was 2.4 kb in length, which is the expected size based on other vertebrate forms of P450c17 reported to date. P450c17 transcripts were not found before SPH injections. After hormone treatment, the transcript was detected from eels at the early vitellogenic stage and the level of its mRNA increased in mid-vitellogenic ovaries, to remain constant thereafter. This finding indicates that expression of P450c17 can be induced by SPH treatment. In human testes, expression of P450 mRNA is controlled by gonadotropin and its second messenger, cAMP (Voutilainen and Miller, 1988). It is likely that in eel ovarian tissues gonadotropins, contained in SPH, mainly regulate expression of P450c17 also. Ijiri et al. (1995) reported that the serum testosterone level and the ability of follicles to produce testosterone from exogenous steroid substrates increased after SPH injections. These findings strongly support the notion that SPH is able to induce in enhancement both of 17α-hydroxylase and C_{17-20} lyase activities because these steroidogenic enzyme activities are essential for testosterone production. In chapter II, studies on the steroidogenic pathway *in vitro* in Japanese eel ovarian follicles revealed that the ovarian follicles produced androstenedione and 17α-hydroxylated progestogens, 17α-OHP and 17α, 20β–DHP, as among the main steroid metabolites at vitellogenic and maturational stages, respectively (unpublished data). Therefore, P450c17 contributes to production of androgens and 17α-hydroxylated progestogens at these respective developmental stages.

Chapter IV.

Characterization of ovarian 20β-HSD in the Japanese eel

Introduction

In teleost fish, final maturation of oocytes is induced by maturation-inducing steroid (MIS) produced in ovarian follicle layers under the control of gonadotropin (Nagahama, 1987). It has been reported that MIS typically is a progestogen, such as 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-DHP) in amago salmon, *Oncorhynchus rhodurus* (Nagahama and Adachi, 1985). Similarly, in many other species, including cyprinids and salmonids, 17α, 20β-DHP plays important roles in oocyte maturation and spermiation. Recently, in Japanese eel, 17α, 20β-DHP was also identified as MIS *in vitro* (unpublished data).

20β-hydroxysteroid dehydrogenase (20β-HSD) is known as the enzyme catalyzing the reduction of the 20-carbonyl group to 20β-hydroxylated products. This enzyme was first described in a procaryote, *Streptomyces hydrogenans* (Hübener *et al.*, 1958). In fish, 20β-HSD, the most important steroidogenic enzyme for 17α, 20β-DHP synthesis, utilizes 17α-hydroxyprogesterone (17α-OHP) as substrate for 17α, 20β-DHP synthesis. In many species of fish, changes in 20β-HSD activity during the reproductive cycle and up-regulation of 20β-HSD by gonadotropic treatment have been demonstrated (Suzuki *et al.*, 1981; Young *et al.*, 1983; Suzuki *et al.*, 1991). In 1995, it was revealed that 20β-HSD activity of intact ovarian follicles (accessed by the conversion 17α-OHP to 17α, 20β-DHP) was strongly enhanced by salmon pituitary homogenate treatment in Japanese eel also (Ijiri *et al.*, 1995). However, there is hardly any molecular information, including biochemical characteristics, primary structure of the enzyme, sequence of the cDNA encoding 20β-HSD, etc,

available from fish A decade ago, strong activity of 20β-HSD was demonstrated in the cytosolic fraction of neonatal pig testis, (Nakajin *et al.*, 1987) and this enzyme was purified (Nakajin *et al.*, 1988). More recently, a cDNA encoding pig testis 20β-HSD was isolated (Tanaka *et al.*, 1992). The deduced amino acid sequence from the cDNA showed high homology (85%) to human carbonyl reductase (CR). Furthermore, purified pig testicular 20β-HSD possessed wide substrate specificities for carbonyl compounds, like human carbonyl reductase, which suggests that cytosolic 20β-HSD in neonatal pig testis is equal to carbonyl reductase. In fish, ovarian 20β-HSD has also been suggested to correspond to CR (Ijiri *et al.*, 1995).

This chapter was designed to investigate characteristics of eel ovarian 20β-HSD and to address whether or not ovarian 20β-HSD is equal to CR.

Materials and methods

Animals and tissue collection

Glass eels captured in the wild were reared for two and a half years and feminized by estradiol-17β administration (Tachiki and Nakagawa, 1992). Forty feminized eels were used in this study. They were kept in recirculating seawater tanks with a capacity of 1 m^3 at 20 °C with constant aeration. Fish were not fed throughout the experimental period. Eels were anaesthetized with ethyl 4-aminobenzoate prior to treatment, which constituted received intramuscular injections with SPH (40 μg/g BW) suspended in eel ringer, once a week (0-11 times). After anaesthetization, ovarian tissues which had reached various developmental stages, were removed , frozen in liquid nitrogen and stored at -80 °C. Developmental stages were determined based on the criteria described in Chapter III.

As high 20β-HSD activity was anticipated in ovarian tissues in the migratory nucleus stage, investigation into the subcellular distribution of 20β-HSD were done using this tissue.

Subcellular preparations

Ovarian tissue was removed and homogenized in 4 vol (v/w) of 50 mM Tris-HCl buffer (pH 8.0) containing 0.2 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin and 1 mM EDTA (Buffer A) with a teflon homogenizer. The homogenate was centrifuged at 750 x g for 10 min. The pellet was discarded, while supernatant was centrifuged at 10,000 x g for 30 min and the resultant pellet, referred to as the mitochondrial preparation (mitochondria), was retained. Thereafter, the

supernatant fraction was centrifuged at 100,000 x g for 60 min and pellet and supernatant fractions were retained as microsomal (microsome) and cytosolic preparations (cytosol), respectively. The mitochondria and microsome were washed three and one time, respectively, resuspended in Buffer A, and recentrifuged at 10,000 x g for 30 min (mitochondria) or 100,000 x g for 60 min (microsome). All procedures were conducted at 0-4 °C.

Determination of 20β-HSD activity

After fractionation, each preparation (200-500 μg protein) was incubated with 1000 ng of a mixture of both [1, 2-^{3}H]17α-OHP (10^6 cpm) and non-labeled authentic 17α-OHP in 10 μl propyleneglycol and with a cofactors-generating system for 1 hour at 20 °C. This generating system was composed of 1 μmol β-nicotinamide adenine dinucleotide, 1 μmol β-nicotinamide adenine dinucleotide phosphate, 10 μmol glucose-6-phosphate, 2.5 IU glucose-6-phosphate dehydrogenase and 2 μmol $MgCl_2$ in 1 ml of 50 mM Tris-HCl buffer (pH 8.0) containing 1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin and 0.1 mM EDTA. After incubation, media were extracted twice with 3 ml of dichloromethane. The organic washes were pooled, dried with anhydrous sodium sulfate and evaporated at 45 °C under a stream of nitrogen gas. After addition of authentic 17α, 20β-DHP and 17α-OHP (20 μg each), extracts were subjected to TLC in a benzene-acetone (4:1, v/v) system. Radioactive spots on chromatograms were detected autoradiographically by an X-ray film (Amersham, UK). After separation of steroid metabolites by TLC, the area of corresponding to 17α, 20β-DHP was scraped off and eluted as described before (chapter II).

The resulting eluents were transferred into in scintillation vials, and ^{3}H-radioactivity of an aliquot was quantified in a toluene-DPO-POPOP system by a liquid scintillation counter. Specific activity of enzyme was expressed as pmol of the product formed per min per mg protein. Controls were nonspecific conversion of substrates accounted for by no protein blanks. Protein concentrations in each preparation were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Identification of 17α, 20β-DHP by recrystallization

The metabolite corresponding to 17α, 20β-DHP was separated by TLC, eluted, and, after addition of approximately 10 mg authentic 17α, 20β-DHP, the mixture was recrystallized in various solvent systems for four times. Based on constant specific activities of crystals after repeated crystallization, the identity of the metabolite was confirmed.

Isolation and sequence of cDNA encoding CR like 20β-HSD

For amplification of an eel CR cDNA fragment, one set of primers was designed from nucleotide sequences of several mammalian forms. An upstream primer, 5'-GAGGTGAATGT(ATGC)TC(ATGC)AG(CT)G G(ATGC)ATG-3', and a downstream primer, 5'-CCAGCCAGG(AG)CA (AG)CA(ATGC)GC(AG)TT-3' were synthesized. PCR was carried out with the set of primers for 30 cycles using an automatic thermal cycler (Gene Amp PCR System 2400, Perkin Elmer, U.S.A.). After PCR, products were subcloned into the plasmid vector PCR™II (Invitrogen, U.S.A.) and sequenced with the Taq Dye Terminator Cycle Sequencing kit (Perkin Elmer, U.S.A.). An isolated cDNA fragment showed high

homology to the corresponding regions of other CR forms. The product was used as probe for screening of a λgt 10 cDNA library constructed from eel ovarian tissues (see chapter III). After screening of 100,000 recombinant cDNA clones, one clone, which contained a complete open reading frame, was isolated and sequenced.

Expression of CR in COS-7

Eel CR cDNA, containing an open reading frame, was subcloned into pSG5. Subsequently COS-7 cells, transfected with the complex of eel CR cDNA and pSG5, were incubated with 0.1 μM ^{3}H-labeled 17α-OHP (New England Nuclear, U.S.A.) for 12 hours. After incubation, steroid metabolites in media were analyzed by thin-layer chromatography. The procedures for transfection of cDNA and analysis of steroid metabolites followed those described in Chapter III.

Northern blot analysis

Poly(A)$^{+}$-RNA was extracted from eel ovaries at various developmental stage. Northern blot analysis was carried out with 2 μg of poly(A)$^{+}$-RNA, using the cDNA that contained the open reading frame of eel CR. The resultant signals for CR were standardized over those of actins. The preparation of poly(A)$^{+}$-RNA and Northern analysis followed the procedures for described in Chapter III.

Results

Identification of 17α, 20β-DHP

In the presence of a cofactors-generating system and [1,2-^{3}H] 17α-OHP mitochondria and microsome synthesized one polar metabolite which was detected on TLC autoradiografically (Fig. 9). Since this metabolite had identical mobility to that of authentic 17α, 20β-DHP, specific activities following recrystallization were constant (Table 2), it was identified as 17α, 20β-DHP.

Subcellular distribution of 20β-HSD

Total activity of 20β-HSD was the highest among preparations of the Japanese eel ovarian tissues in the mitochondria, followed by the microsome and cytosol. The total activity of 20β-HSD was 61.1 % in the mitochondria (Table 3), while the microsome and cytosol were responsible for 27.9 % and 11.0 % of the total substrate conversion, respectively. Specific activity of 20β-HSD similarly was highest in the mitochondria, followed by the microsome and cytosol, reaching 2.3 and 89 fold those of the latter fractions, respectively.

Changes in 20β-HSD activity during ovarian development

Since specific activity of 20β-HSD was much higher in the mitochondria and microsome than that in the cytosol, activities in any these preparations were assessed to examine changes in specific activities of 20β-HSD during oocyte development (Fig. 10). Activities of 20β-HSD were present in the mitochondria (1.5-2.3 pmol/min/mg) and microsome (0.8-

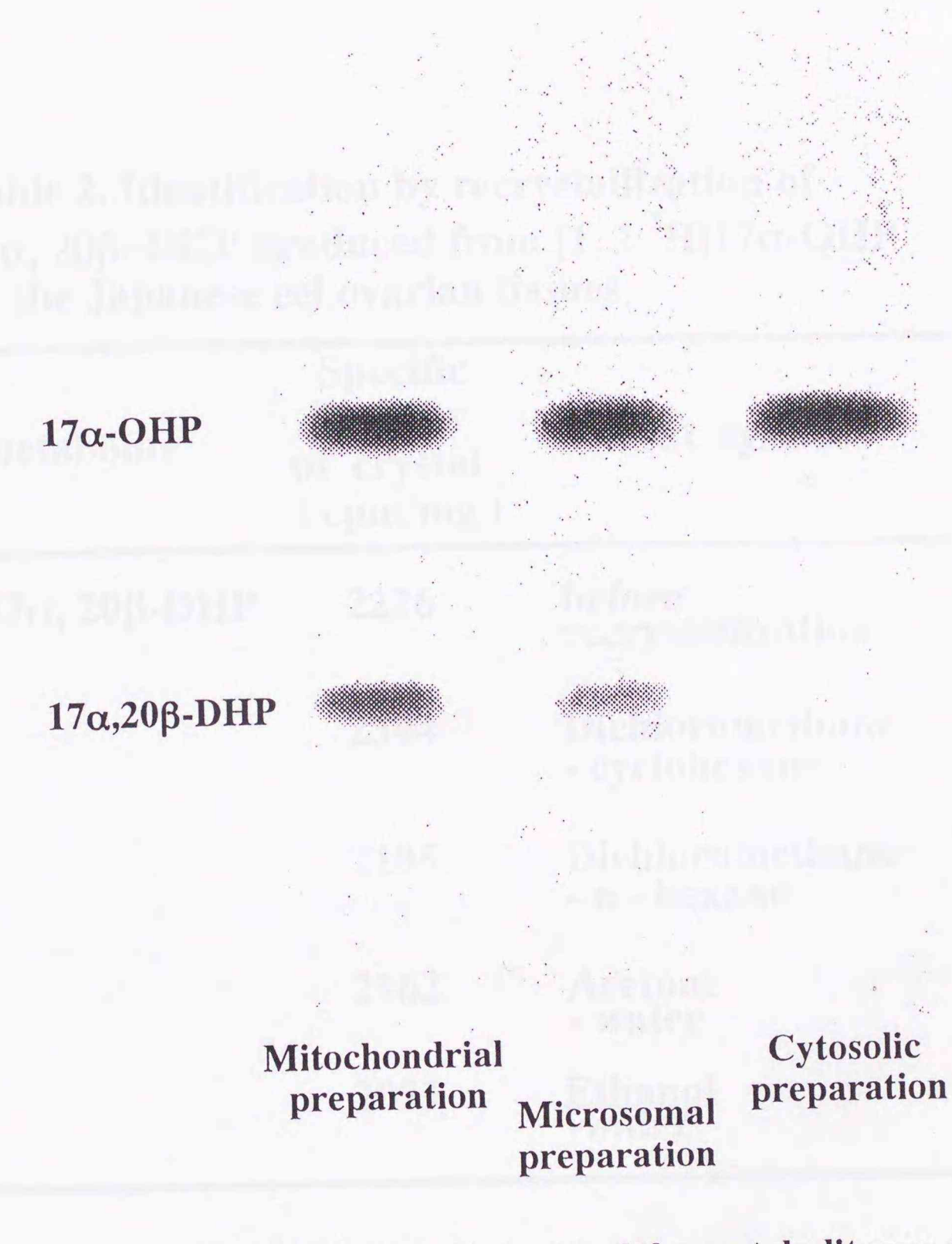


Fig. 9. Autoradiogram of the metabolite produced from [1, 2-^{3}H]17α-OHP by subcellular preparations of Japanese eel ovarian tissue

Table 2. Identification by recrystallization of 17α, 20β–DHP produced from [1, 2-^{3}H]17α-OHP by the Japanese eel ovarian tissues

metabolite	Specific activity of crystal (cpm/mg)	Solvent system
17α, 20β-DHP	2226	before recrystallization
	2344	Dichloromethane - cyclohexane
	2105	Dichloromethane - n - hexane
	2162	Acetone - water
	2031	Ethanol -water

Table 3. Subcellular distribution of 20β-HSD in Japanese eel ovarian tissues

Subcellular preparation	20β-HSD			
	specific activity (pmol/min/mg)	total protein (mg)	total activity (pmol/min)	(%)
Mitochondria	17.8	17.4	309.7	61.1
Microsome	7.8	18.2	142.0	27.9
Cytosol	0.2	279.7	55.9	11.0

Eel ovarian tissues (4.4g) were homogenized and separated by conventional differential centrifugationto obtain subcellular preparations

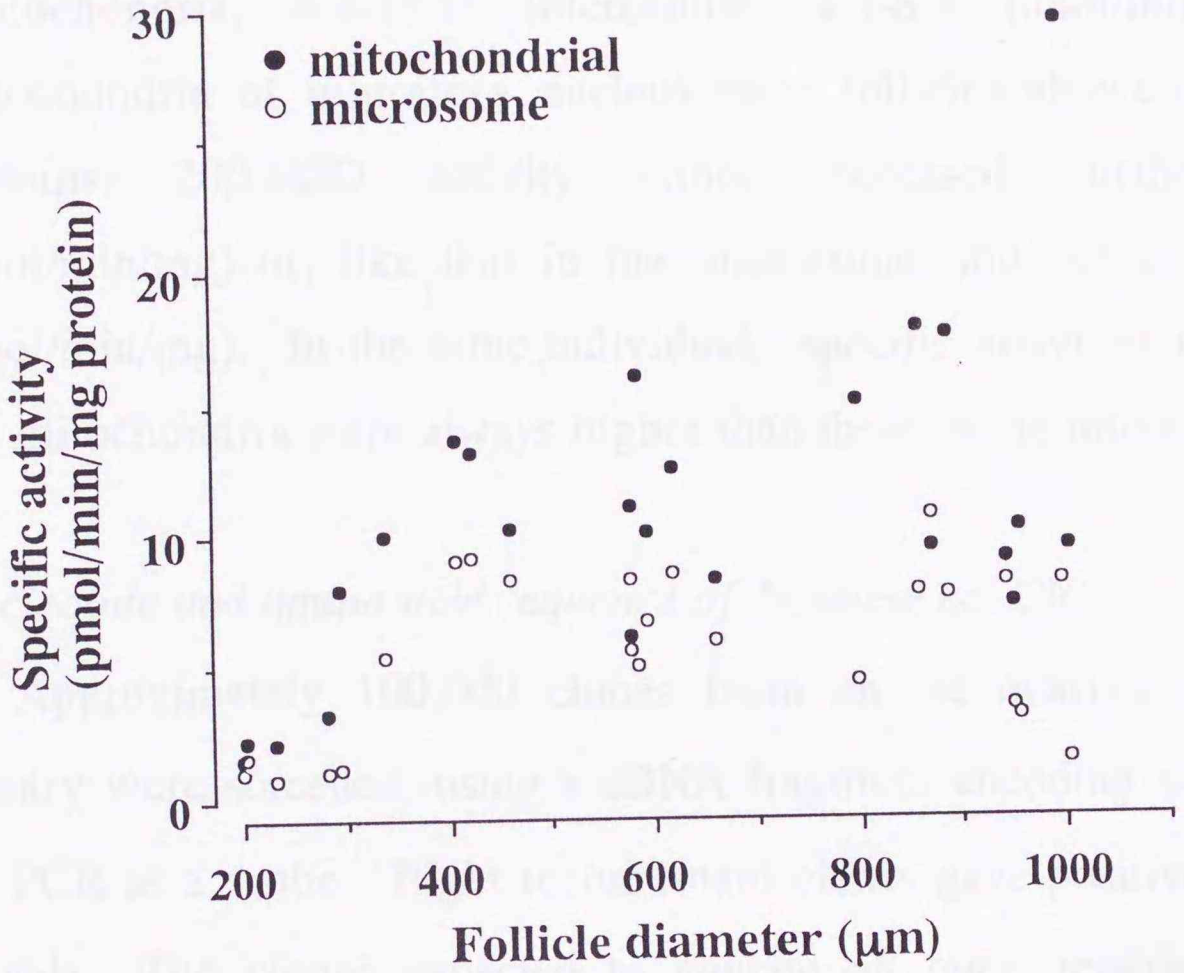


Fig. 10. Changes in specific activities of 20β-HSD in ovarian preparations of the Japanese eel

1.7 pmol/min/mg) at the pre-vitellogenic stage, prior to SPH treatment, and increased in both preparations at the early vitellogenic stage (mitochondria, 3.3-10.0: microsome, 1.1-5.4 pmol/min/mg), mid (mitochondria, 5.2-13.1: microsome, 4.5-9.2 pmol/min/mg) and late vitellogenic stages (mitochondria, 8.4-15.1: microsome, 4.5-8.5 pmol/min/mg). The mitochondria of migratory nucleus stage follicles showed either of two patterns: 20β-HSD activity either increased further (17.6-29.5 pmol/min/mg) or, like that in the microsome, did not change (7.3-10.3 pmol/min/mg). In the same individual, specific activities of 20β-HSD in the mitochondria were always higher than those in the microsome.

Nucleotide and amino acid sequence of Japanese eel CR

Approximately 100,000 clones from an eel ovarian λgt 10 cDNA library were screened, using a cDNA fragment encoding eel CR obtained by PCR as a probe. Eight recombinant clones gave positive hybridization signals The clones expected to contain an open reading frame were subcloned into pBluescript KS^- and sequenced. One clone included an open reading frame encoding 276 amino acid residues (Fig. 11), which showed high sequence identity to that of human (64%; Wermuth *et al.*, 1993), pig (56%; Tanaka *et al.*, 1992), two types of rat (gonadotropin-inducible and non-inducible CR; 63 and 52%; Wermuth *et al.*, 1995; Aoki *et al.*, 1997) and rabbit (59%; Gonzalez *et al.*, 1995) forms (Table 4). Hydropathicity profiles of eel and human, according to the method of Kyte and Doolittle (1982), is demonstrated in Fig. 12, and indicates good conservation of the secondary protein structure.

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                5'-tgacagtgtcgacgttgttgaactagttcctccagtttacagaaaaggatatctgcagat

ATG TCA ACA AAT AAA GTT GCA CTT GTC ACT GGT TCC AAC AAA GGC ATT GGC TTT
 M   S   T   N   K   V   A   L   V   T   G   S   N   K   G   I   G   F

GCA GTG GTG CGC GCA TTA TGT AAG GAG TTT CCC GGC GAC GTA TAC CTC AGT GCC
 A   V   V   R   A   L   C   K   E   F   P   G   D   V   Y   L   S   A

CGG GAT GTT GAC CGG GGT ACT GCG GCA GTA GAG AAC CTG AAA ACT GAA GGC TTA
 R   D   V   D   R   G   T   A   A   V   E   N   L   K   T   E   G   L

AAC CCA TTC TTT CAT CAG CTC GAT ATC ACC GAT CCG GCG AGC GTG CGC CAT GCC
 N   P   F   F   H   Q   L   D   I   T   D   P   A   S   V   R   H   A

CGA GAT TTC TTC AAG GAG AAA TAC GGC GGC CTT GAT GTA CTC GTT AAC AAC GCC
 R   D   F   F   K   E   K   Y   G   G   L   D   V   L   V   N   N   A

GGT ATT GCG TTC AAA GTT GCT GAT TCC ACA CCT TTT GGA ATC CAA GCT GAG GTC
 G   I   A   F   K   V   A   D   S   T   P   F   G   I   Q   A   E   V

ACT CTG AGA ACA AAC TTC CTT GCC ACC AGA GAT TTG TGC AAC GAG TTC CTC CCT
 T   L   R   T   N   F   L   A   T   R   D   L   C   N   E   F   L   P

ATC ATA AAA CCA GGA GGG AGA GTA GTT AAT GTT TCG AGT GGA ATG AGC TCC ATT
 I   I   K   P   G   G   R   V   V   N   V   S   S   G   M   S   S   I

GCT CTG AAG AGT TGC AGC TCA GAA CTC CAG GCA AGG TTC CGC AGC AAT GAC ATC
 A   L   K   S   C   S   S   E   L   Q   A   R   F   R   S   N   D   I

ACA GAG GAA GAG CTG GTG ATG CTG ATG GAG AAG TTT GTG CAG GAG GCC CAG AAA
 T   E   E   E   L   V   M   L   M   E   K   F   V   Q   E   A   Q   K

GGT GAA CAC ACC CAT AAA GGC TGG CCA AAC ACG GCC TAT GGG GTG TCC AAA ATT
 G   E   H   T   H   K   G   W   P   N   T   A   Y   G   V   S   K   I

GGC GTT ACG GTG CTG TCC AGG ATC CAG GCC CGC AGG CTT AGG GAG GAG AGG GCT
 G   V   T   V   L   S   R   I   Q   A   R   R   L   R   E   E   R   A

GGG GAC CAG ATC CTC CTT AAT GCC TGC TGC CCA GGC TGG GTG AGA ACC GAC ATG
 G   D   Q   I   L   L   N   A   C   C   P   G   W   V   R   T   D   M

GCA GGT CCC AAT GCC ACC AAA TCC CCC GAT GAG GGC GCT GTC ACC CCT GTC TAT
 A   G   P   N   A   T   K   S   P   D   E   G   A   V   T   P   V   Y

CTG GCC TTG CTG CCA GTT GGT GCG ACA GAG CCA CAG GGA CAG TTT GTG TCT GAG
 L   A   L   L   P   V   G   A   T   E   P   Q   G   Q   F   V   S   E

AAG CAG GTG CAG GTG TGG TGA  gcatatgaccttcacaaagtgaagtgataatctaatactgtt
 K   Q   V   Q   V   W   *

tattgcaatttaatgattggctcagtgacgtgatgtgtgtttttgtgtccaaatcaaaatattttcatt
aataaacaataaatgtatgctgacattatatgaaaaaaaaaaaaaaa
```

Fig. 11. Nucleotide and amino acid sequence of the cDNA encoding eel ovarian CR. The open reading frame is composed of 828 bp and encodes 276 amino acid residues.

Table 4. Homology of eel ovarian CR to the other homologous protein

Homologous protein	Homology (%)
Human CR	64
Pig 20β-HSD	56
Rat inducible CR	63
Rat noninducible CR	52
Rabbit CR	59

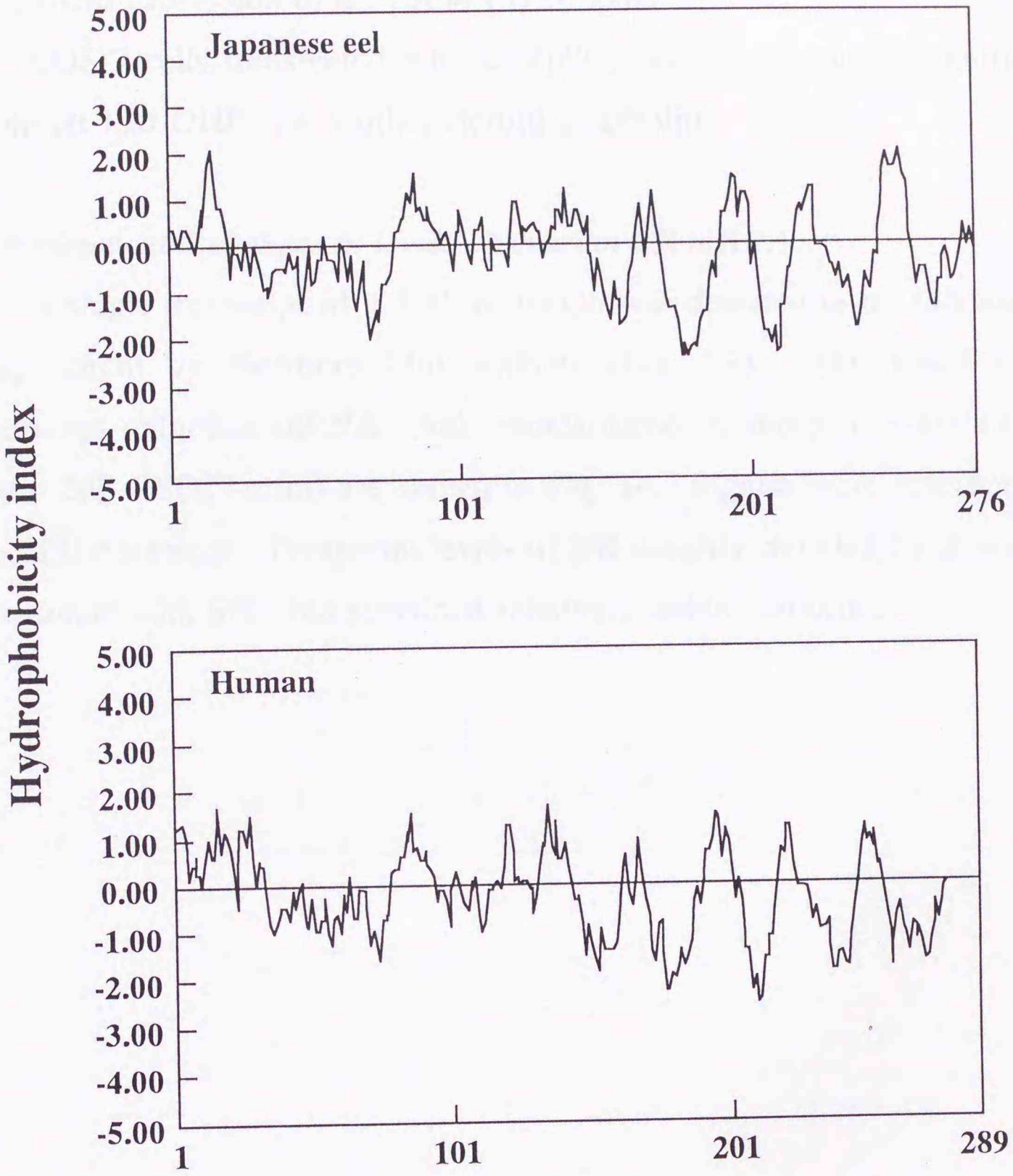


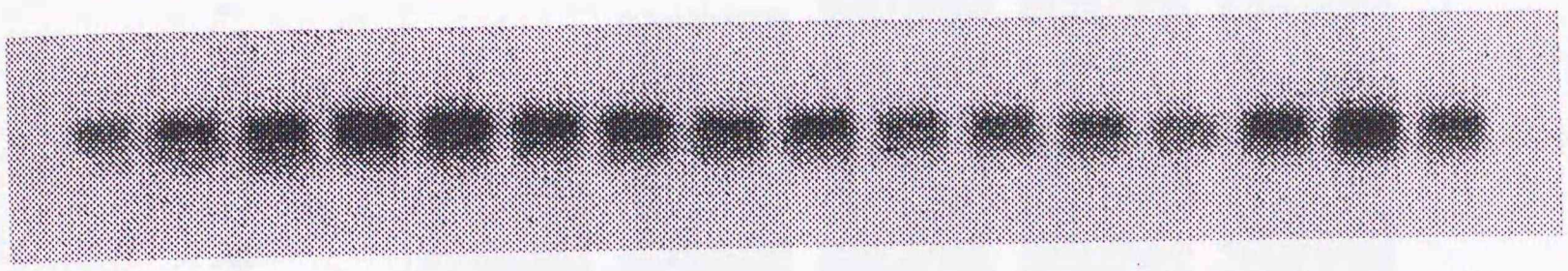
Fig. 12. Hydrophobicity profiles of Japanese eel and human CR proteins. The profiles for these CRs were calculated using the algorithm of Kite and Doolittle. Positive and negative values on the ordinate indicate hydrophobic and hydrophilic degrees, respectively.

Transient expression of eel CR in COS-7 cells

COS-7 cells transfected with eCRpSG5 were not able to significantly convert 17α-OHP to any other steroid metabolite.

Developmental changes in levels of ovarian CR mRNA

A single transcript of 1.3 kb in length was detected in all fish used for experiment by Northern blot analysis (Fig. 13). The mean relative carbonyl reductase mRNA levels standardized by those of β-actin (signal ratio 20β-HSD/β-actin) are shown in Fig. 14. Signals were detected prior to SPH treatment. Transcript levels of CR roughly doubled by 2 weeks of treatment with SPH, but remained relatively stable thereafter.



Fig, 13. Northern blot analysis of mRNA (2μg) from eel ovaries at various developmental stages. lane 1-3, previtellogenic ovaries; lane 4-6, early vitellogenic ovaries; lane 7-10, mid-vitellogenic ovaries; lane 10-13, late vitellogenic ovaries; lane 14-16, post-vitellogenic ovaries.

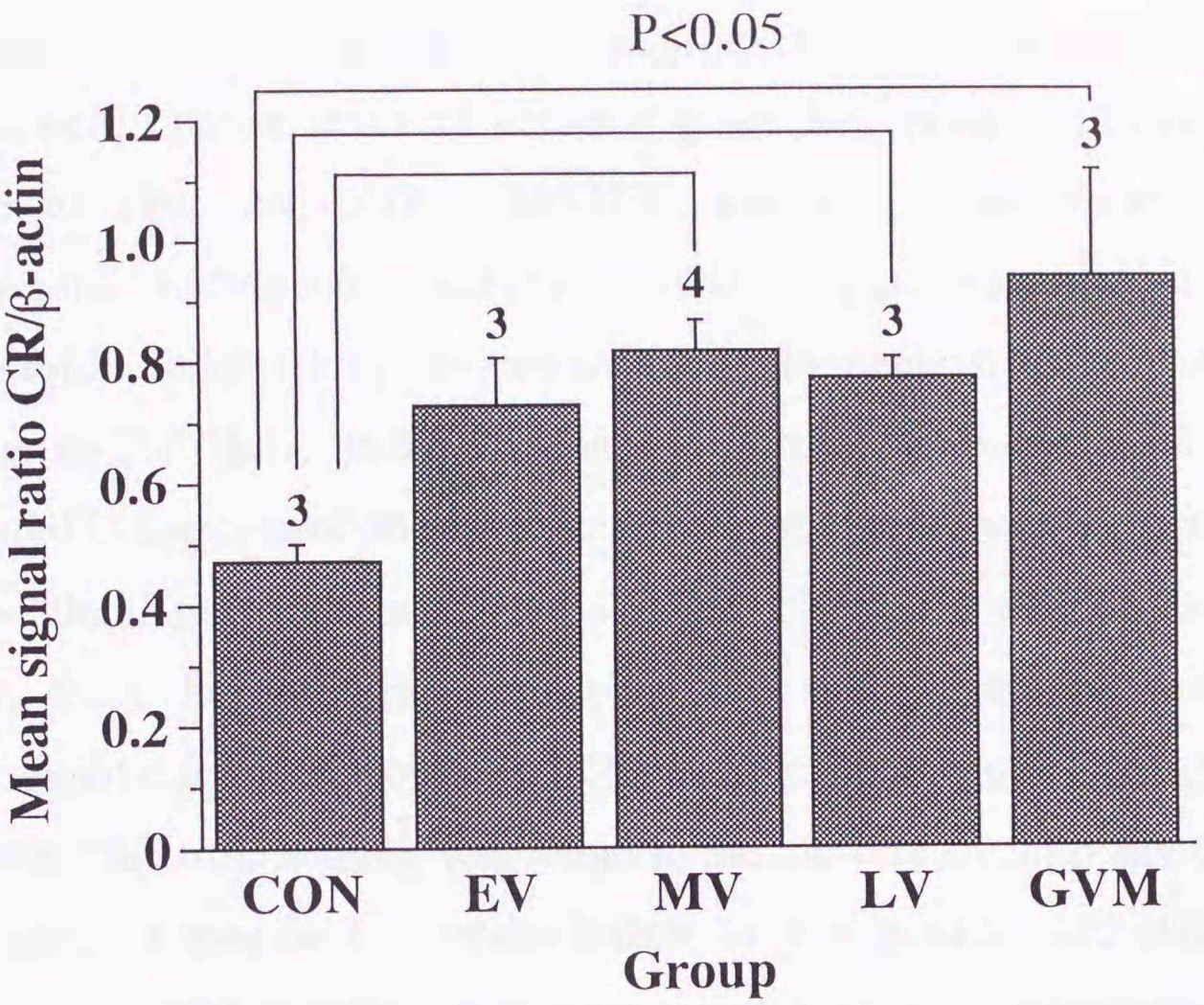


Fig. 14. Mean relative carbonyl reductase-like 20β-hydroxysteroid dehydrogenase mRNA levels referenced over those of β-actin (signal ratio 20β-HSD/β-actin) in Japanese eels artificially matured. CON: pre-treated control; EV: early vitellogenic; MV: mid vitellogenic; LV: late vitellogenic; GVM: mature, migratory nucleus stage. Numbers above bars represent sample sizes. Significantly different contrasts are indicated.

Discussion

When 17α-OHP was added to ovarian tissue preparations of the Japanese eel in the presence of cofactor generating system, 17α-OHP was reduced to 17α, 20β-DHP. 20β-HSD was distributed mainly in the mitochondria and specific activity of this enzyme was highest in the mitochondria followed by the microsome. In contrast, in neonatal pig testes, most 20β-HSD, which is equal to cytosolic carbonyl reductase, is distributed in the cytosol and specific activity of cytosol was highest among the subcellular preparations (Nakajin *et al.*, 1987). In ovarian tissues of teleosts, there has been no report on the subcellular distribution of steroidogenic enzymes. However, in testes of male amago salmon (Sakai *et al.*, 1989), 20β-HSD activity was found in the mixture of mitochondria and microsome. It may be a common feature of fish gonadal 20β-HSD to be distributed in mitochondrial and microsomal fractions. Accordingly, the distribution of 20β-HSD is different from that of mammalian carbonyl reductase (Wermuth *et al.*, 1981; Nakayama *et al.*, 1985).

Developmental changes in specific activity of 20β-HSD of Japanese eel ovary were assessed. At the pre-vitellogenic stage, low 20β-HSD activity was detected both in the mitochondria and microsome. At the mid- and late vitellogenic stages, the activities increased strikingly in both preparations. At the migratory nucleus stage, 20β-HSD activity did not change in the microsome, while it either increased further or did not change in the mitochondria. In the eel, Ijiri *et al.* (1995) reported that 20β-HSD activity (assessed by the conversion of 17α-OHP to 17α, 20β-DHP by 100 intact follicles) increased or decreased slightly in follicles at

the late vitellogenic stage. The present result is consistent with the previous report, and suggests that the change in 20β-HSD activity of follicles is largely dependent on mitochondrial 20β-HSD.

The cDNA encoding CR, thought to correspond to 20β-HSD, was isolated and characterized. The nucleotide and predicted amino acid sequence of eel CR showed high identity to other mammalian forms. Furthermore, the hydropathicity profile of eel CR was quite similar to that of the human form. These findings suggests that the structure of eel CR was highly conserved and that it may represent a cytosolic protein, like mammalian forms. This, together with the fact that 20β-HSD activity is mainly detected in mitochondria, suggests that the subcellular distribution of CR might be different from that of 20β-HSD in the eel ovary.

To investigate the changes in CR mRNA levels in the eel ovary during artificial maturation, Northern analysis was carried out. CR mRNA was detected before SPH treatment, the transcript levels of CR slightly increased at the early vitellogenic stage (induced gonadal development after 2 weeks of treatment with SPH). Thereafter, the level remained relatively stable. In the ovary of mouse and hamster, CR activity was enhanced by gonadotropins (Iwata *et al.*, 1993). Recently, two types of CR cDNA were identified (Aoki *et al.*, 1997). One is induced by GTH treatment, whereas the other is stably expressed. Moreover, the activity and content of rat ovarian CR were enhanced by estrogen-mediated gonadotropic effects, which caused transcription and translation rather than post-translational processing (Inazu *et al.*, 1992). Therefore, slight increased transcriptional levels after the beginning of SPH treatment may result from the effect of gonadotropins contained in SPH via E_2. The profile of the changes in

expression of the CR gene, however, did not clearly match changes in 20β-HSD activity of ovarian mitochondrial and microsomal fractions and of intact ovarian follicles reported previously by Ijiri *et al.*, 1995. This mismatch in developmental changes of CR and 20β-HSD, the differences in subcellular distribution of these enzymes and the absence of detectable 20β-HSD activity in COS-7 cells transfected with eel CR cDNA indicate that another 20β-HSD(s) may exist in the eel ovary.

In this chapter, the characteristics of 20β-HSD were examined and compared with CR in eel ovarian tissues. Although it is suggested that in fish CR is equal to functional 20β-HSD, this hypothesis dose not appear to be tenable in eel. In the fish ovary, studies on 20β-HSD are essential for understanding the mechanism of 17α, 20β-DHP synthesis, and hence, warrant the search for other type(s) of 20β-HSD involved in final oocyte maturation.

Chapter V

Molecular cloning and characterization of cDNAs encoding 17β-hydroxysteroid dehydrogenase type I from Japanese eel ovarian follicles

Introduction

Estrogens, especially estradiol-17β (E_2), plays important roles in female reproductive cycles of all female vertebrates. The 17β-hydroxysteroid dehydrogenases (17β-HSDs) are oxidoreductive steroidogenic enzymes that catalyze the conversion of 17-ketosteroids into 17β-hydroxylated steroids and vice versa. Hence, 17β-HSDs are responsible for the shifting ratios between estrone and E_2, androstenedione and testosterone, and dehydroepiandrosterone and 5-androstane-3β, 17β-diol. In mammals, cDNAs encoding five isoenzymes of 17β-HSD, known as types I-V, were cloned and characterized (Peltoketo *et al.*, 1988; Wu *et al.*, 1993; Geissler *et al.*, 1994; Adamski *et al.*, 1995; Labrie *et al.*, 1997). These isozymes are not only essential for sex steroid synthesis in gonads but also may regulate their levels in the target tissues because the catalytic properties and tissue distribution of these isozymes are different and they are present in various extragonadal tissues (Labrie *et al.*, 1997; Penning, 1997).

17β-HSD type I was at first purified from the cytosolic fraction of human placenta (Jarabak *et al.*, 1962) and the cDNA clone for this enzyme was isolated using a polyclonal antibody for this enzyme (Peltoketo *et al.*, 1988). This enzyme is also known as estrogenic 17β-HSD and plays a most important role for the biosynthesis of E_2 in the estrogenic tissues, including the ovary and placenta in human, because recombinant enzyme transiently expressed in mammalian cells catalyzed only reduction of estrone to E_2 (Poutanen *et al.*, 1993). Therefore, like aromatase, 17β-HSD type I is a crucial enzyme in the final step of E_2 production.

In teleost fish, knowledge on the mechanism of E2 synthesis is very important to understand and control reproduction because E2 synthesized and secreted from ovarian follicles is required for induction of vitellogenin synthesis in the liver, which, in turn, contributes to oocyte growth (Wallace, 1985). There are many reports on changes in serum E2 levels and the ability of ovarian follicles to produce E2 in many species of teleosts (Schreck and Hopwood, 1974; Whitehead *et al*,., 1983; Kanamori *et al*., 1988; Sakai *et al*., 1988). Especially in salmonids, it has been demonstrated that both these are high at the vitellogenic stage and that they decrease at the maturational stage. Ijiri *et al*., (1995) have shown that salmon pituitary homogenate injections induce the activities of several steroidogenic enzymes, including aromatase in ovarian follicles, which results in enhancement of E2 production from steroid intermediates. Furthermore, in this species, the serum E2 level increase at maturational stage, which is quite different from salmonids. Recently, cDNAs encoding aromatase were isolated and characterized from several fish (Tanaka *et al*., 1992b; Trant, 1994), including Japanese eel (Ijiri *et al*., unpublished data). However, there is no available information on 17β-HSD type I of fish. In this chapter, the cDNA encoding 17β-HSD type I was cloned from an eel ovarian cDNA library and the changes in the levels of its mRNA were examined during experimentally induced ovarian development.

Materials and methods

Animals and treatments

Silver female Japanese eels caught in Hiranuma River, Aomori Prefecture, and cultivated eels, either untreated males or fish feminized by estradiol-17β administration (Tachiki and Nakagawa, 1992), were used. Eels were not fed throughout the experimental period. Silver female eels and feminized eels received weekly intramuscular injections of 20 (6 times) or 40 μg/g BW (0-9 times) acetone-dried salmon pituitary homogenate (SPH), respectively, suspended in eel ringer. Ovarian tissues at various developmental stages and several tissues from fish at the maturational stage were collected. Male eels received a single injection of 1000 IU of human chorionic gonadotropin (HCG) dissolved in eel ringer. Testes from non-treated and HCG-treated (6 days post injection) fish were collected.

Tissues sampled were quickly frozen in liquid nitrogen and kept at -80 °C, before analyses.

Isolation and sequence analysis of cDNA

A λgt10 library was constructed from eel ovarian poly(A)$^+$-RNA as discribed in chapter III. A cDNA fragment of rat 17β-HSD type I (nucleotides 258-549) was obtained by PCR from E_2-primed rat ovarian tissues (Ghersevich *et al.*, 1994). 500,000 recombinant phage plaques were screened using a ^{32}P-labeled cDNA fragment of rat 17β-HSD I as probe under the following condition; 5 X SSC, 0.2 % polyvinylpyrrolidone, 0.2 % Ficoll 400, 0.2 % SDS, 0.2 % BSA, and 35 % formamide, at 42 °C for 18 hr. Six positive clones were identified and subcloned into a Bluescript

KS^+ vector. Its nucleotide sequences were determined with the Taq Dye Terminator Cycle Sequencing Kit (Perkin Elmer, U.S.A.) using an ABI 373 DNA sequencer (Perkin Elmer, U.S.A.).

Transient expression of 17β-HSD type I in human kidney 293 cells

The entire open reading frame of eel 17β-HSD type I was obtained by PCR using two oligonucleotides, 5'-ATGGAGCAGAGAGTG-3' and 5'-CTAGGGATCCTCTGG-3', as primers. The resulting PCR product was subcloned into the plasmid vector PCR™II, using a TA Cloning Kit (Invitrogen, U.S.A.) and sequenced for confirmation. Subsequently, the cDNA was inserted into an eucaryotic expression vector, pcDNA 3.1 (+), which contains the cytomegalovirus promoter (Funakoshi, Japan). Human kidney 293 cells (HSRRB; JCRB9068, Japan) were used for the expression experiment as it has been reported that COS cells possess endogenous 17β-HSD activity (Poutanen *et al.*, 1993). Cells were spread into wells of a 6-well plate at a density of 1×10^5 per well. Two days after plating, cells were transfected with the cDNA complex of eel 17β-HSD type I and pcDNA 3.1 (+), using the DOSPER Liposomal Transfection Reagent (Boehringer Mannheim, German) based on the manufacture's instructions. After 6 hours, medium was renewed and the transfected cells were incubated for 30 hours before measurement of 17β-HSD activity.

Measurement of 17β-HSD activity

After transfection of eel 17β-HSD type I in human kidney 293 cells, reductive and oxidative 17β-HSD activities were measured in triplicate. Cells were incubated with ^{14}C-labeled androstenedione (A), testosterone

(T), estrone (E_1) and E_2 (New England Nuclear, U.S.A.) at a concentration of 0.1 μM. After 12 hours of incubation, media were collected and steroid metabolites extracted with dichloromethane. The extracts were subjected to thin-layer chromatography (TLC) and co-applied with standard steroids (A, T, E_1 and E_2) in a benzene-acetone (4:1, v/v) system. Standard δ4 steroids (A and T) were visualized under ultraviolet (254 nm), while estrogens were detected by baking the TLC plate after spraying ethanol-sulfuric acid (4:1, v/v). Radioactive metabolites were detected and quantified using a BAS 2000 Bio-Image analyzer (Fujix, Japan). The activities of 17β-HSD are shown as the percentage yield of the products.

Northern analysis

Total RNA was extracted from eel tissues using ISOGEN (Nippongene, Japan), following the manufacturer's protocol. Polyadenylated RNA was subsequently isolated from total RNA with Oligotex-dT 30 (Takara shuzo, Japan). Poly(A)$^+$-RNA was isolated from brain, liver, spleen, head kidney, trunk kidney, heart, small intestine, testes (0 and 6 days after HCG injection) and ovarian tissues at pre-, early, mid-, late vitellogenic and maturational stages. Five μg of poly(A)$^+$-RNA from each sample was used for Northern blot. The procedure for Northern blot and the analysis of obtained results have been described previously (Chapter III).

Results

Nucleotide and amino acid sequence of Japanese eel 17β-HSD type I

After screening of 500,000 clones using rat 17β-HSD type I as probe, 6 positive clones were obtained. Two clones, expected to contain the open reading frame based on their length, were sequenced. Clone 1 and clone 2 were 1169 bp and 1459 bp in length and indeed contained the open reading frame. Coding regions of these clones consisted of 879 bp encoding 293 amino acid residues with 48 % and 51 % sequence identity to rat (Ghersevich *et al.*, 1994) and human (Peltoketo *et al.*, 1988) 17β-HSD type I. Moreover, five conserved regions that characterize the short-chain alcohol dehydrogenase (SCAD) family members, with homology of at least 62-83 % to mammalian forms, were identified (Fig. 15). Eel 17β-HSD type I is 35 and 51 amino acid shorter than the rat and human forms respectively. The coding regions of clones 1 and 2 showed 6 differences in nucleotide and 1 difference in amino acid residue, respectively. Furthermore, the 3'-untranslated region was identical between the stop codon (TAG) and the subsequent 45 nucleotides downstream, but thereafter exhibited dissimilar nucleotide sequences (Fig. 16). Six nucleotides (160-165) corresponding to Gly^{54} and Cys^{55} in rat and, Ala^{54} and Cys^{55} in human are lacking in the eel cDNAs (Fig. 15). Instead of these missing amino acid residues, the eel form possesses extra nucleotides encoding Arg^{196} and Arg^{197}. The active enzymatic site of members of the SCAD family, Tyr-X-X-X-Lys, is conserved between positions 154 and 158 (Ensor *et al.*, 1991; Obeid *et al.*, 1992). Moreover, four amino acid residues, Ser^{142}, Tyr^{155}, His^{221} and Glu^{272}, throughout to be involved in

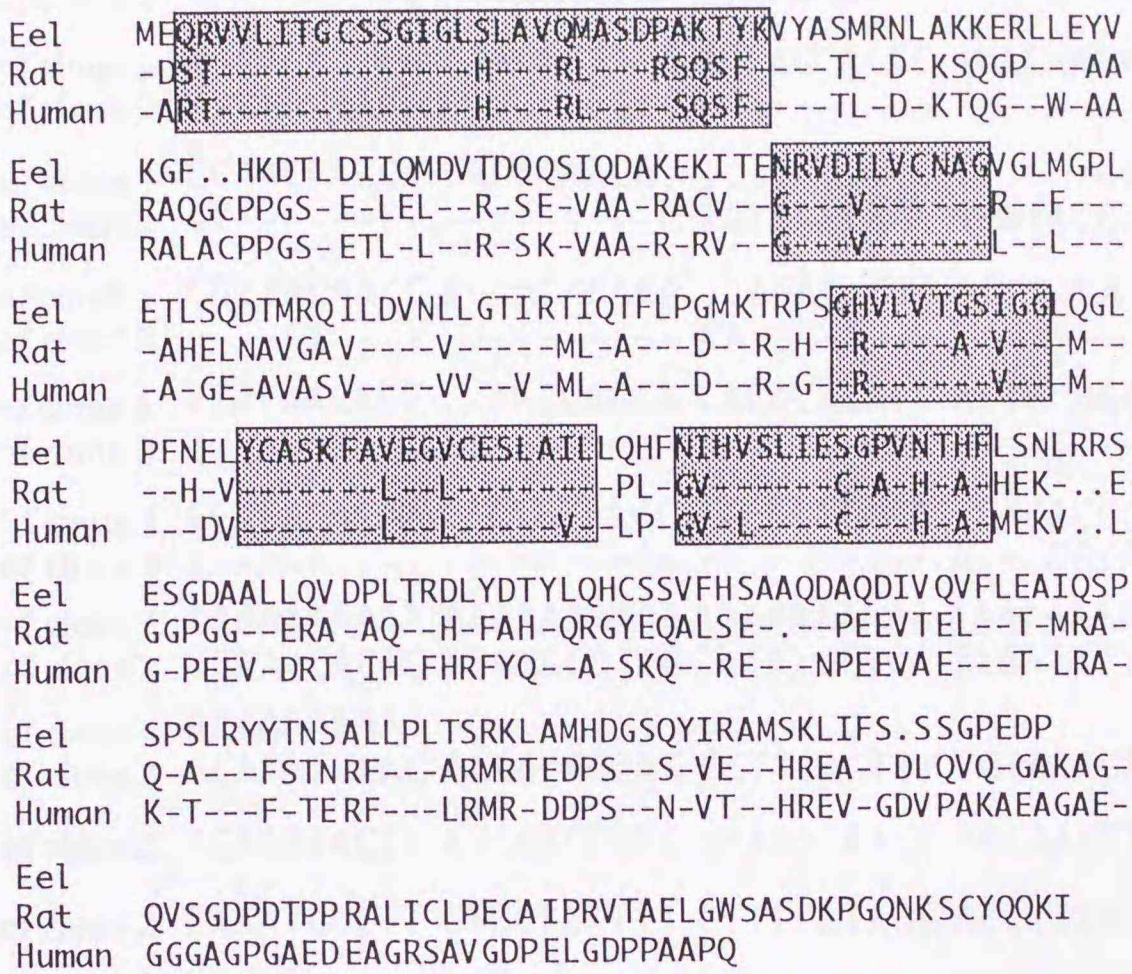


Fig 15. Alignment of the deduced amino acid sequence of eel 17β-HSD type I with that of rat and human. Dashes indicate amino acid residues that are identical to the eel form. Dots indicate gaps introduced to facilitate alignment.

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NCR of clone 1  TAGATACAGC CAGAACAAAC AACACTAAAA CACTATGACC
NCR of clone 2  ---------- ---------- ---------- ----------

NCR of clone 1  AGCACACA.. .......... .......CAA CACT..AACC
NCR of clone 2  --------TC ACTGTAACCA GCACACA--- ----GT----

NCR of clone 1  AGCACA..CA CAACACTATA ACCAGCACAC ACACAACACT
NCR of clone 2  ------TA-- -------G-- ---------- ----------

NCR of clone 1  GCAACCAGCA CACACAACA. .......... ......GCAA
NCR of clone 2  -T-------- ---------C TATAACCAGC ACATACA---

NCR of clone 1  CACTACAACC AGCACACACA ..ACACTGTA ACCAGCACA.
NCR of clone 2  ----GT---- ---------- CA-------- ---------T

NCR of clone 1  .CACAACACC ..AACCAGCA CACACACATC ACTGCAACCA
NCR of clone 2  A--------T GT-------- --------A- ----T-----

NCR of clone 1  GCACACACAA CACTGTAACC AGCACACACA .CACACCGTC
NCR of clone 2  ---------- ---------- ---------- A---TGTAAG

NCR of clone 1  AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA
NCR of clone 2  TGAAACACTG ATCCACCTTG CATATGCTCA TTAATATTTT

NCR of clone 1  AAAAAAAAA. .......... .......... ..........
NCR of clone 2  GCAAATGAAC AAGACAGGAG CCTTCAGTTT TGTAAAGAAG

NCR of clone 2  TGATTTACTA ATGAAGTGTC AAAAACATGG TGCAATTTTA

NCR of clone 2  CAAATGCCTT GTGCTGATTT CTTCCATAGT ACCTTTATCA

NCR of clone 2  TATCATGTAA ACCTGGCTTT GTTAGGGGTT GGGTATCAAT

NCR of clone 2  CACTGACAGC TATTTAACAA AGACATTATT AATAAATGAA

NCR of clone 2  AATTCAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA

NCR of clone 2  AAAAAAAAAA AAAAAAAA
```

Fig. 16. 3'-untranslated sequences of two cDNAs encoding eel 17β-HSD type I. Dashes indicate identical nucleic acids. Dots indicate gaps . Polyadenylation signal is underlined.

creating hydrogen bonds between E_2 and enzyme in the human 17β-HSD type I form (Labrie *et al.*, 1997), are completely conserved. However, sequence identity of C-terminal amino acid residues 192 to 293 positions is quite low, 30 % and 31 % compared to the rat and human isoforms, respectively.

Substrate specificity of eel recombinant 17β-HSD type I

A cDNA encoding eel 17β-HSD type I was obtained by PCR and subcloned into a eucaryotic expression vector, pcDNA 3.1(+). Human kidney 293 cells transfected with eel 17β-HSD type I showed exclusively estrogen reduction activity, catalyzing the conversion of E_1 to E_2, with the yield of E_2 of about 65 % in 12 hr, while other significant oxidative and reducing reactions were not detectable (Fig. 17, 18).

Developmental changes in levels of ovarian 17β-HSD type I

Northern blot analysis revealed that several transcripts between 1.8-3.2 kb in length were present and that transcriptional sizes of eel 17β-HSD in the ovarian tissues were different among individuals used for Northern blot analysis. 17β-HSD type I transcripts were not detected before SPH injections. However, signals were observed in ovaries from all SPH-treated eels, including maturing ovaries, and the intensity of the signals did not appear to change during ovarian development (Fig. 19).

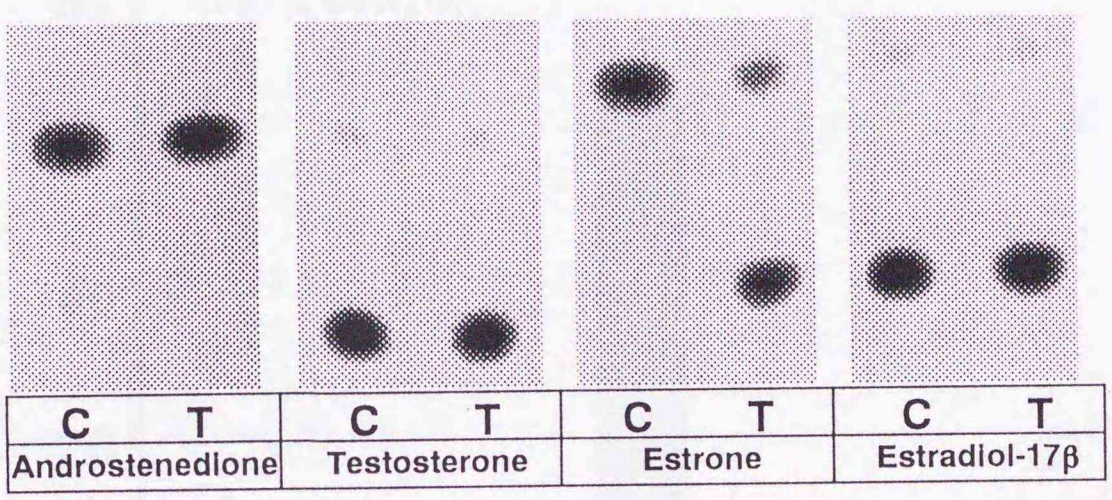


Fig. 17. Autoradiograph of thin-layer chromatography, illustrating the conversion of 0.1 μM ^{14}C-labeled substrate by human kidney 293 cells transfected with pcDNA 3.1 (C) or pcDNA3.1-17 β-HSD (T).

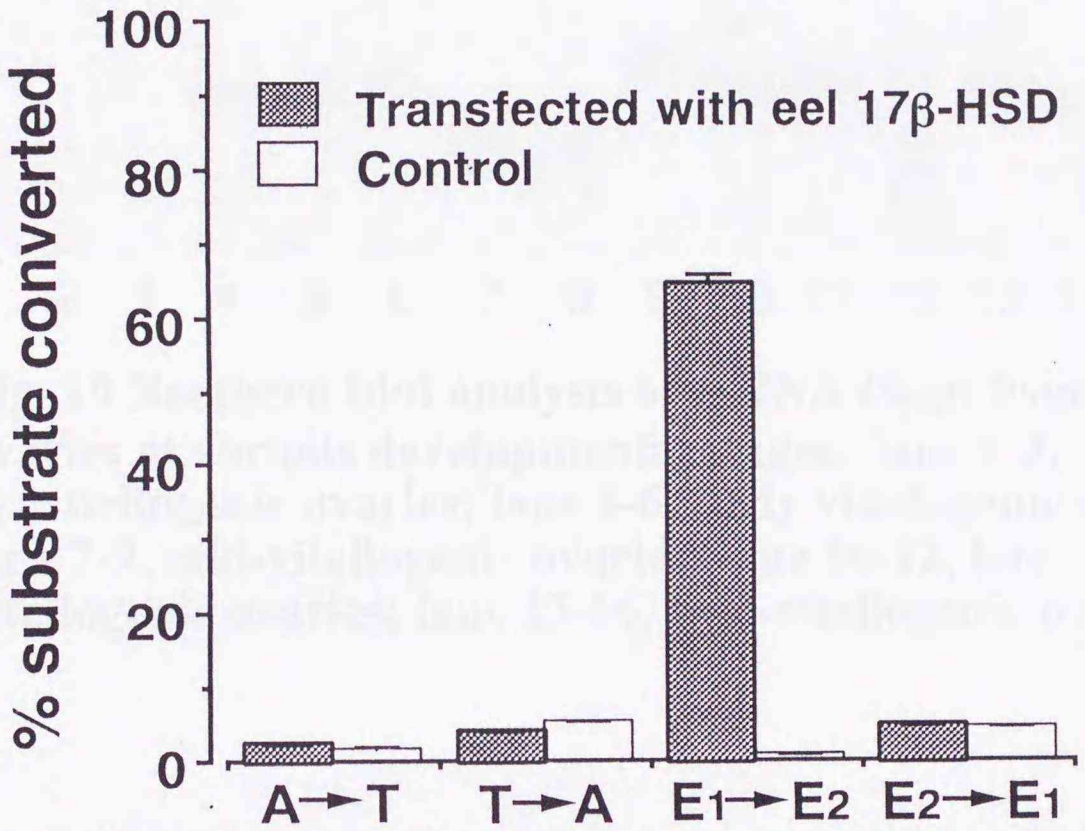


Fig. 18. Reducting and oxidative enzymatic activities of human kidney 293 cells transfected with control plasmid: ☐ or plasmid containing cDNA for eel 17β-HSD type I: ▩ . Bars represent the mean ±S.E. of three replicate measurements.

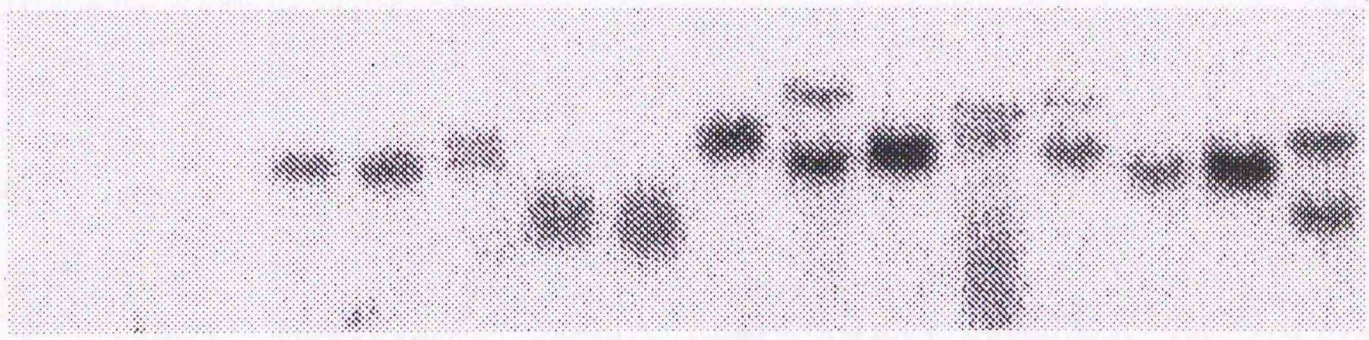


Fig. 19 Northern blot analysis of mRNA (5μg) from eel ovaries at various developmental stages. lane 1-3, previtellogenic ovaries; lane 4-6, early vitellogenic ovaries; lane 7-9, mid-vitellogenic ovaries; lane 10-12, late vitellogenic ovaries; lane 13-16, post-vitellogenic ovaries.

Tissue distribution of 17β-HSD type I mRNA

Northern blot analyses for 17β-HSD type I was carried out using testes and various tissues from a female eel to investigate tissue distribution of 17β-HSD type I transcripts. The result of Northern blot is shown in Fig. 20 and illustrates that mRNA for 17β-HSD type I was observed only in ovarian tissue.

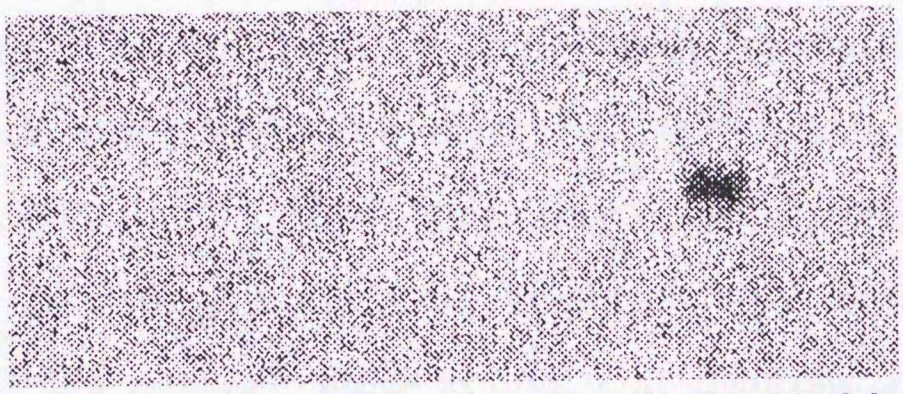


Fig. 15 Northern blot analysis of mRNA from several tissues of eel. lane 1, brain; lane 2; gill; lane 3, small intestine; lane 4, heart; lane 5, spleen; lane 6, liver; lane 7, head kidney; lane 8, posterior kidney; lane9, ovary (mid-vitellogenic); lane 10, testis (from untreated eel); lane 11, testis (from gonadotropin injected eel; 6 days p. i.).

Discussion

In this chapter, cDNAs encoding 17β-HSD type I of Japanese eel were cloned and sequenced. The deduced amino acid sequence of eel 17β-HSD type I shows high homology to rat and human forms and possesses five conserved regions of this steroidogenic enzyme. In man, it was demonstrated that 17β-HSD type I shows only catalytic activities for estrogens (Jarabak *et al.*, 1969; Poutanen *et al.*, 1993) and that four amino acid residues, Ser^{142}, Tyr^{155}, His^{221} and Glu^{272} were hypothesized to play important roles in estrogen substrate specificity by structural analysis (Labrie et al., 1997). In eel, these amino acid residues were completely conserved. Another report, using affinity labeling, suggested that a putative steroid-binding site of 17β-HSD type I is located between residues 205 and 224, with three histidine residues at the positions of 210, 213 and 221 thought to be important. However, in eel, this region shows low homology to the human form with only one His at position of 221 being conserved. Recently, mutagenesis studies indicated that, among the three His residues, only mutation of His^{221} to alanine resulted in reduction of catalytic activity. These facts suggest that His^{221} is especially important to catalyze the conversion of E_1 to E_2 of 17β-HSD type I and that the eel form is an enzyme involved in estrogen biosynthesis, like the human form.

The recombinant eel 17β-HSD type I synthesized in human 293 cells could catalyze only reduction of E_1 to E_2, like the human form. These enzymatic characteristics coincide with the results of structural analysis, which suggest that the main steroidogenic pathway for production of E_2 is via androstenedione and E_1 in eel ovarian follicles. Eel ovarian follicles at

the mid-vitellogenic stage produce androstenedione from pregnenolone as one of the main steroid metabolites *in vitro* (see Chapter II). Nevertheless, both serum E2 level and capacity of ovarian follicles to produce E2 *in vitro* are very low during vitellogenesis (Ijiri *et al.*, 1995). Furthermore, Ijiri *et al.*, recently, demonstrated that, in eel ovarian tissues, the transcription level of aromatase is low during vitellogenesis and that it increases at the maturational stage (unpublished data). These findings indicate that aromatase is the main limiting factor for synthesis of E2 in the eel ovary.

Northern blot analyses were performed to investigate the developmental changes in 17β-HSD type I mRNA and its tissue distribution. Before SPH treatment, the signal for 17β-HSD could not be observed in ovaries. After SPH injections, several transcripts of 17β-HSD type I, between 1.8-3.2 kb in length, were detected and the transcription level did not tend to change during ovarian development. This finding indicates that expression of 17β-HSD type I was enhanced by SPH, which contains many peptide hormones. In rat, it is suggested that transcription of 17β-HSD type I is mainly regulated by gonadotropins and E2. (Ghersevich *et al.*, 1994). Because SPH contains large amounts of gonadotropins, it is possible that gonadotropins enhance the level of 17β-HSD type I mRNA in the eel. The transcriptional sizes of 17β-HSD type I in the ovaries varied among individuals. Taken together with the observation that two isolated cDNAs had somewhat different nucleotide and amino acid sequences, it thus appears that several forms of 17β-HSD mRNAs may be present in the eel ovary, but the significance for these observations remains unclear. It has been reported that the mRNA of thyroid-stimulating hormone of European

eel (Berengere *et al.*, 1997) has multiple sizes. In fish, existence of multiple may not be rare.

Using RNase protection assay or Northern blot, 17β-HSD type I transcripts could be demonstrated in several tissues, including gonadal tissues, of human and rat (Rhéaume *et al*/., 1992; Akinola *et al.*, 1996), Other tissues, such as placenta, breast, endometrium, adipose tissues and skin, also transcribed the 17β-HSD type I gene and may thus contribute extragonadal estrogen and androgen synthesis. In eel, however, no significant expression of 17β-HSD type I was observed, except for the ovaries. No expression of 17β-HSD type I outside the ovary suggests that this enzyme is not involved in the steroid feedback system of the pituitary-gonadal axis. In serum from male eel 6 days after HCG injection, high levels of 17β-hydroxylated androgens, testosterone and 11-ketotestosterone, are observed (Miura *et al.*, 1991). Nevertheless, testes at 6 days after HCG injection did not possess 17β-HSD type I mRNA. It is plausible that other isoenzymes of 17β-HSD are expressed, similar to 17β-HSD type III in man, which may play a role in production of bioactive androgens by conversion of androstenedione to testosterone (Luu-The V *et al.*, 1996).

In man, the studies on multiple 17β-HSDs are most advanced and substrate specificities and distributions have been best demonstrated. It is strongly suggested that these isoenzymes of the 17β-HSD family are present to regulate intracellular concentrations of active estrogens and androgens. Recently, two type of estrogen receptor were identified in rat (Kuiper *et al.*, 1996). While in eel 'total estrogen recepter' mRNA was found not only in the liver but also in brain, coelomic wall and ovary (Nakamura *et*

al., unpublished data). Therefore, E2 may play some role in several target organs other than the liver in fish. Accordingly, the presence of multiple estrogen receptor subtypes may indicate the necessity for multiple forms of 17β-HSD and the resulting control for tissue specific function of estrogens and androgens in the target organs.

This is the first report in a non-mammalian vertebrate on the molecular characteristics of 17β-HSD. For the elucidation of the mechanisms on synthesis of sex steroids, characterization of other members of the 17β-HSD family and knowledge on the regulation of 17β-HSD isozymes are required

Chapter VI
Summary and Conclusions

Ovarian steroid hormones have important roles in reproduction in female vertebrates. In many species of fish, two steroid hormones, estradiol-17β (E_2) and 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-DHP), are identified as main inducers of vitellogenesis and final oocyte maturation, respectively. In general, steroid hormones are synthesized by consecutive catalytic reactions of steroidogenic enzymes distributed in ovarian follicle layers. In the present study, the steroidogenesis in the ovary of the Japanese eel was investigated, in order to clarify the mechanism of steroid hormone synthesis during oogenesis artificially induced in female Japanese eel, *Anguilla japonica*.

Changes in the eel ovarian steroidogenic pathway during artificial maturation were examined. A summary of the changes of the steroidogenic pathway in Japanese eel ovarian follicles is shown in Fig. 21. At the early vitellogenic stage (before hormonal treatment), the ovarian follicles could not converted from pregnenolone to the other steroid metabolites. Artificial induction of oogenesis by injections with SPH advanced ovarian follicles to the mid-vitellogenic stage, during which time they converted pregnenolone to two androgens, dehydroepiandrosterone and androstenedione. Thereafter, at the migratory nucleus stage, the yields of androgens decreased drastically. In contrast, the yields of 17α-hydroxylated 4-ene progestins, especially 17α-hydroxyprogesterone (17α-OHP) and 17α, 20β-DHP, increased markedly. These findings indicate that SPH can strongly enhance the expression of several steroidogenic enzymes to produce androgens and 17α-hydroxylated progestins, such as 3β-hydroxysteroid dehydrogenase, P450c17 (17α-hydroxylase and C_{17-20} lyase activities) and 20β-hydroxysteroid dehydrogenase (20β-HSD).

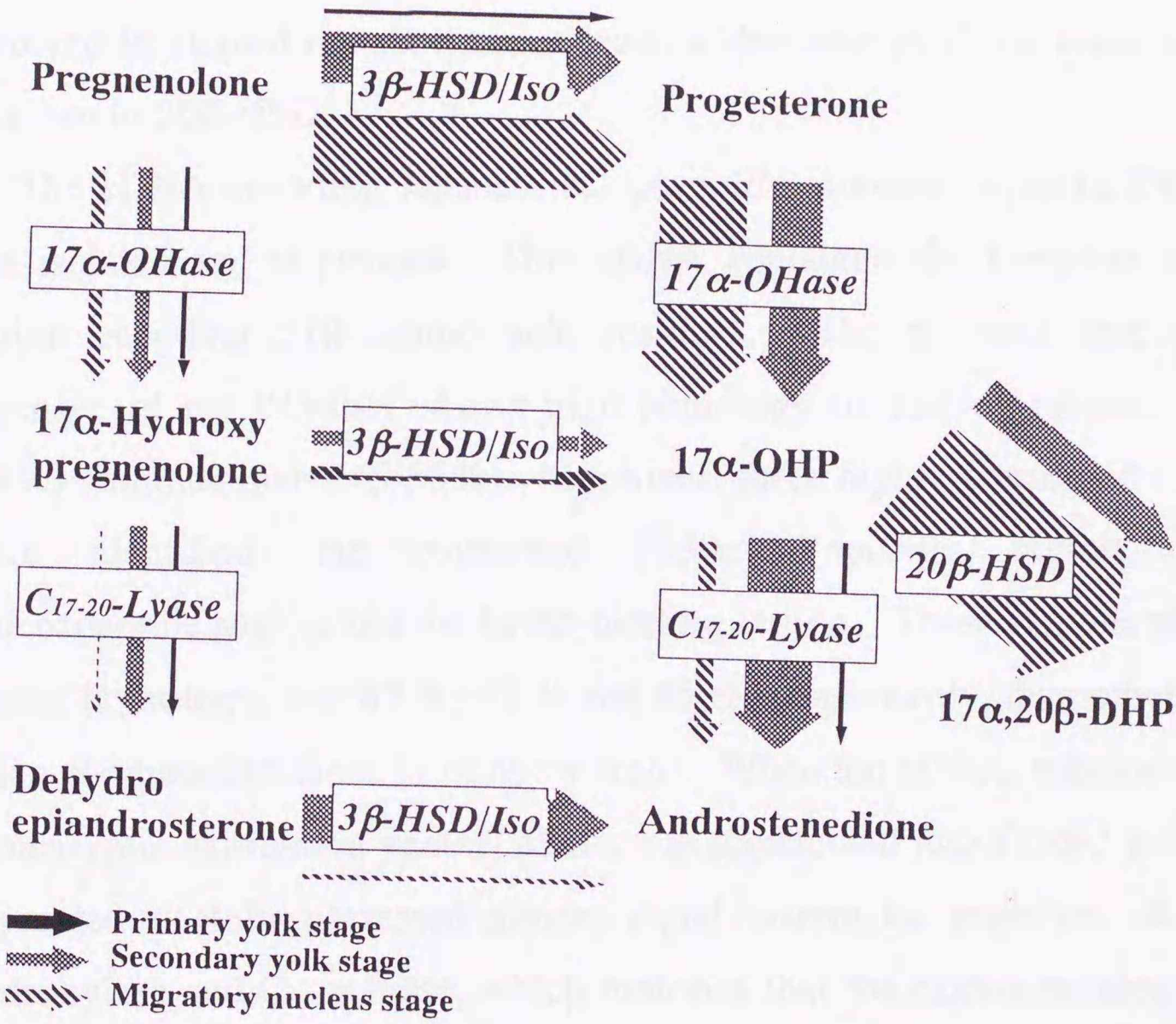


Fig. 21. Developmental changes in steroidogenic pathway in the ovarian follicles of Japanese eel.

Steroidogenesis induced by SPH is first directed forwards production of androgens but late shifts to production of maturation-inducing steroid, 17α, 20β-DHP, especially during the period from the mid-vitellogenic stage to the migratory nucleus stage *in vitro*. This result indicates that the steroidogenic shift may be caused by changes in activities of key enzymes involved in steroid metabolism, at least, a decrease in $C_{17,20}$-lyase and an increase in 20β-HSD.

The cDNA encoding Japanese eel (*Anguilla japonica*) ovarian P450c17 was isolated and sequenced. This cDNA contained the complete coding region encoding 510 amino acid residues. The deduced amino acid sequence of eel P450c17 shows high homology to that of rainbow trout (74%) and mammals (45-55%). Moreover, three highly conserved regions were identified, the conserved P450c17 specific region, Ozols tridecapeptide region and the heme-binding region. These regions showed higher homology, viz. 87 %, 93 % and 85 %, respectively, than other areas when compared to those in rainbow trout. When the cDNA, subcloned into a eucaryotic expression vector, pSG5, was transfected into COS-7 cells, the expressed protein possessed almost equal enzymatic activities of 17α-hydroxylase and $C_{17\text{-}20}$ lyase, which indicates that the cDNA isolated was a cDNA encoding eel ovarian P450c17. Northern blot analysis revealed that the mRNA of P450c17 was a single transcript which was 2.4 kb in length. The P450c17 gene was not transcribed before SPH injections. SPH treatment enhanced the transcription of the P450c17 gene, the transcript being detected in ovaries at the early vitellogenic stage. The mRNA level increased in mid-vitellogenic ovaries, and remained essentially unchanged thereafter. Further analysis of the ovarian steroidogenic pathway suggests

that the increase of 17α-hydroxylase activity and the decrease of $C_{17,20}$ lyase activity occurs during the transition from the vitellogenic to the maturational stage *in vitro*. Therefore, P450c17 appears to contribute to production of androgens and 17α-hydroxylated progestogens at these respective developmental stages.

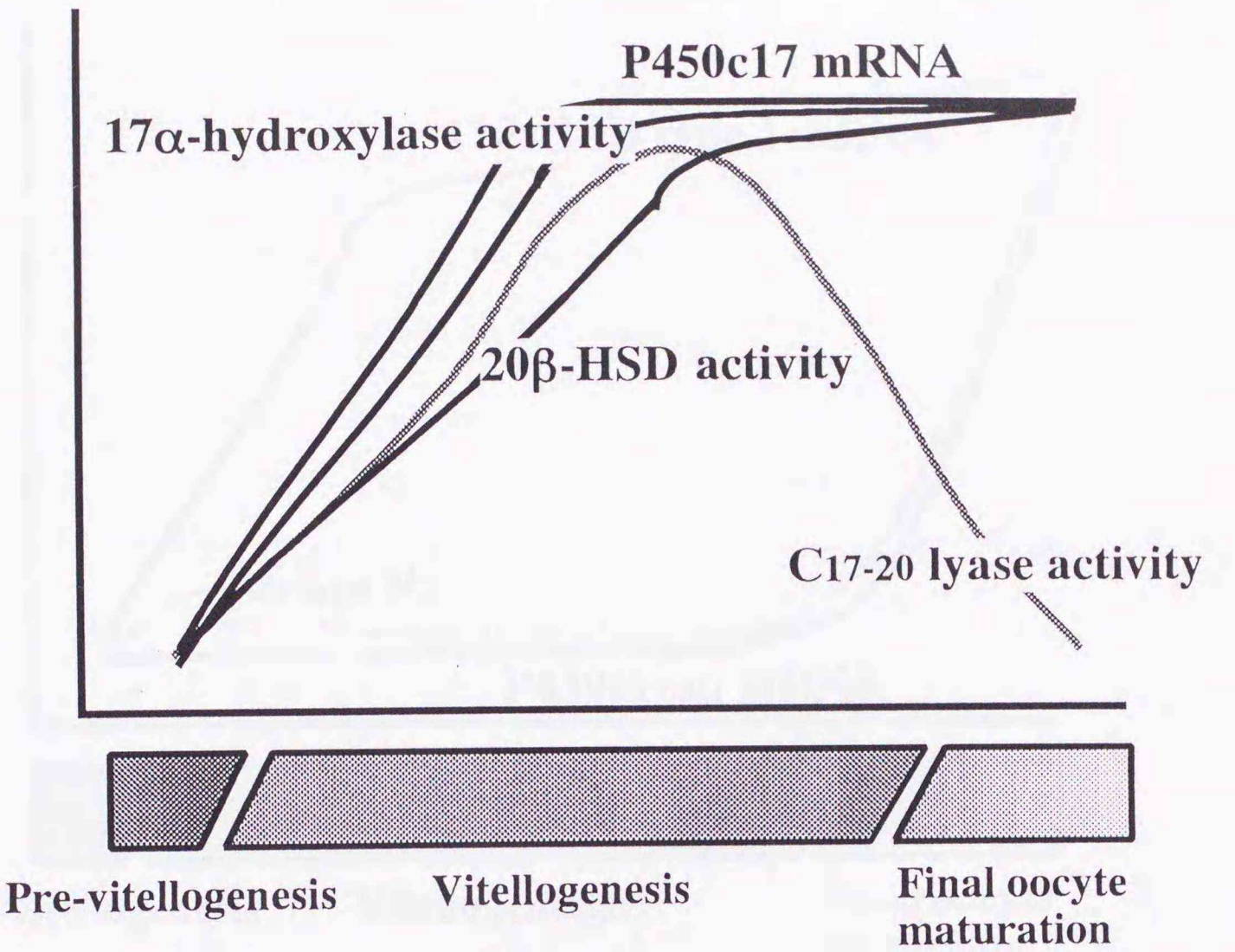
The second key-enzyme, 20β-hydroxysteroid dehydrogenase (20β-HSD), required for the production of 17α, 20β-DHP from 17α-OHP, was sequently characterized, both by biochemical and molecular biological methods. Thus, 20β-HSD activity was investigated in three subcellular fractions prepared from eel ovarian tissues by differential centrifugation methods. The mitochondrial fraction mainly possessed 20β-reducing activity, followed by the microsomal and cytosolic fractions. 20β-HSD activities of both mitochondria and microsome were detected at the pre-vitellogenic stage, before SPH treatment, and increased slightly at the early vitellogenic stage in response to hormone administration. Further increases in 20β-HSD activity were observed in the mid-vitellogenic stage (6-fold over that in the pre-vitellogenic stage), after which activity did not change significantly. To attempt further characterization of the enzyme that harbors 20β-oxidoreductive activity, the cDNA encoding carbonyl reductase (CR) was cloned and characterized. CR is a cytosolic protein and thought to be equal to 20β-HSD at least in mammals. The obtained eel CR cDNA showed high sequence identity to mammalian isoforms but, following transfection into COS-7 cells, did not show 20β-HSD activity. Moreover, hydropathicity profiles of eel and human forms were quite similar. These finding suggest that unlike 20β-HSD eel CR is a cytosolic protein. In order to clarify changes in the ovarian transcriptional levels of

CR, Northern blot analysis was carried out. Prior to SPH treatment, CR transcripts were detected, which roughly doubled by 2 weeks of treatment with SPH, but remained relatively stable thereafter. These changes in expression of the CR (putative 20β-HSD) gene did not clearly match changes in activities of 20β-HSD. This indicates that another 20β-HSD(s) may exist in the eel.

In order to investigate the control of E_2 synthesis in more detail, the cDNA encoding 17β-hydroxysteroid dehydrogenase type I (17β-HSD type I), which together with aromatase (P450arom) catalyzes the final steps of E_2 production, was isolated and characterized. The open reading frame of the cDNA obtained showed high homology to human and rat 17β-HSD type I cDNAs and the predicted amino acid sequence possessed five coserved regions like other isoforms. Human kidney 293 cells transfected with the complex of eel 17β-HSD type I cDNA and pcDNA3.1(+) vector could not convert androstenedione to testosterone but were effective in converting estrone (E_1) to E_2. This substrate specificity of eel 17β-HSD type I indicate that E_2 is mainly produced from androstenedione via E_1 in Japanese eel ovarian follicles. Changes in 17β-HSD type I transcript levels in ovary, as well as its tissue distribution were examined by Northern analysis. Signals of 17β-HSD type I transcripts were not observed in the ovaries from untreated eels. After SPH treatment, the transcripts were detected in the ovaries from all individuals at virtually unchanged levels throughout ovarian development. 17β-HSD type I transcripts were detected only in ovarian tissues. In human chorionic gonadotropin (HCG)-treated male eels, the serum levels of 17β-hydroxylated androgens and the capacity to produce 17β-hydroxylated androgens in the testes increase.

Nevertheless, 17β-HSD type I transcripts were not present in the testes. This result suggests like mammals that other type(s) of 17β-HSD are expressed in eel testes.

The summary diagram of the changes in the levels of enzymatic activities and mRNA of steroidogenic enzymes relates to the steroidogenic shift associated with the production of androgens to 17α, 20β-DHP, as shown in Fig. 22. These enzyme activities are quite low prior to SPH injections. After treatment with SPH, $C_{17\text{-}20}$ lyase activity increases at the vitellogenic stage, but decreases at the maturational stage. In contrast, 17α-hydroxylase activity slightly increases during vitellogenesis and continues to increase in the maturational stage. P450c17 gene transcripts, indicate of both $C_{17\text{-}20}$ lyase and 17α-hydroxylase activities, increase at the vitellogenic stage and remain stable thereafter. 20β-HSD activity is also observed before SPH treatment, and increases with advancing ovarian development. These steroidogenic enzyme changes induce the shift of ovarian steroidogenesis, from androgens to progestogens, in female Japanese eel. An improved diagrammatic representation on control of E_2 synthesis in the eel ovary and changes in the level of E_2 during artificial maturation is illustrated in Fig. 23. In general, two steroidogenic enzymes, 17β-HSD type I and P450arom, are responsible for the final steps in E_2 production in ovarian tissues. Eel 17β-HSD type I prefers E_1 over androstenedione as its substrate. Therefore, the main steroidogenic pathway to produce E_2 is from androstenedione to E_1, then to E_2. The transcription levels increase during vitellogenesis and then do not change, whereas P450arom mRNA hardly changes during vitellogenesis but increases at the maturational phase (Ijiri *et al.*, unpublished data). These



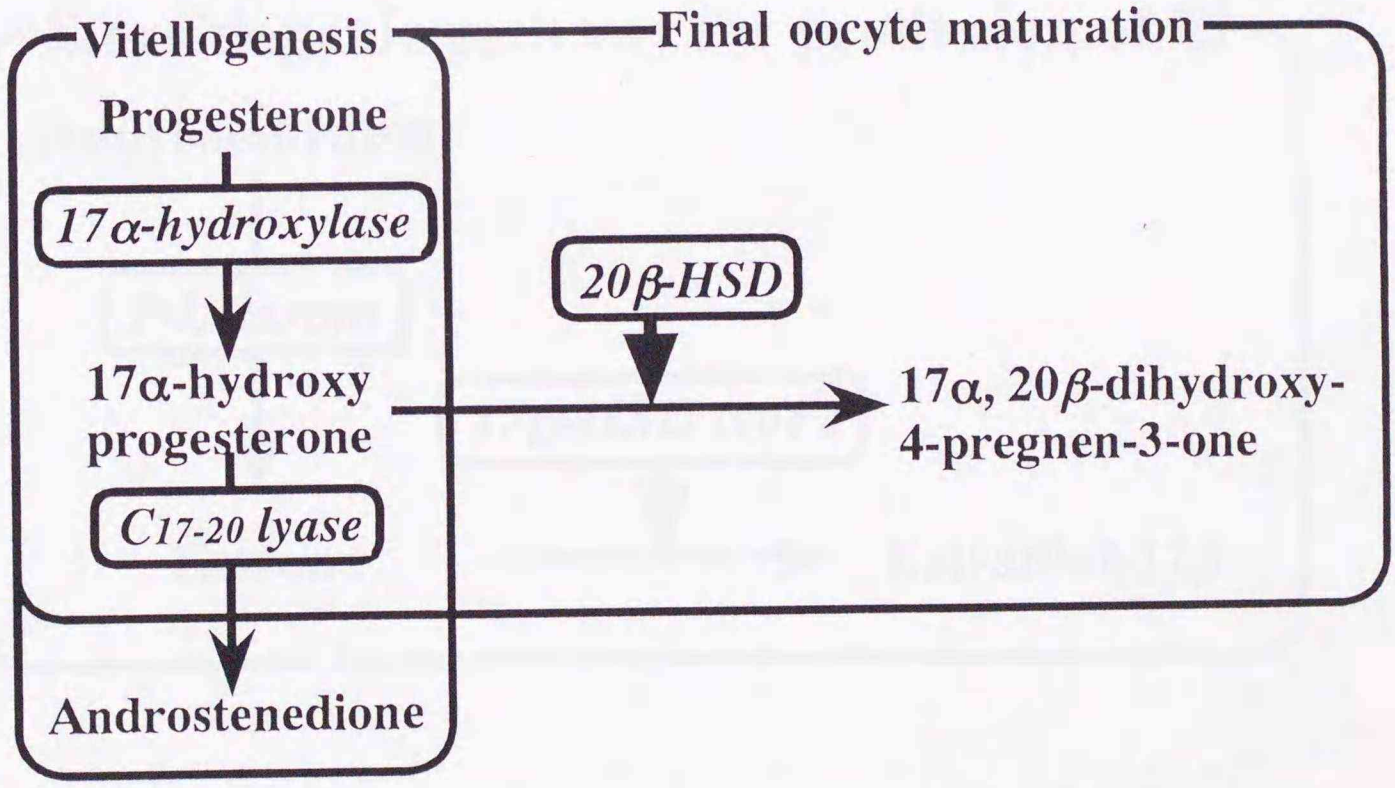
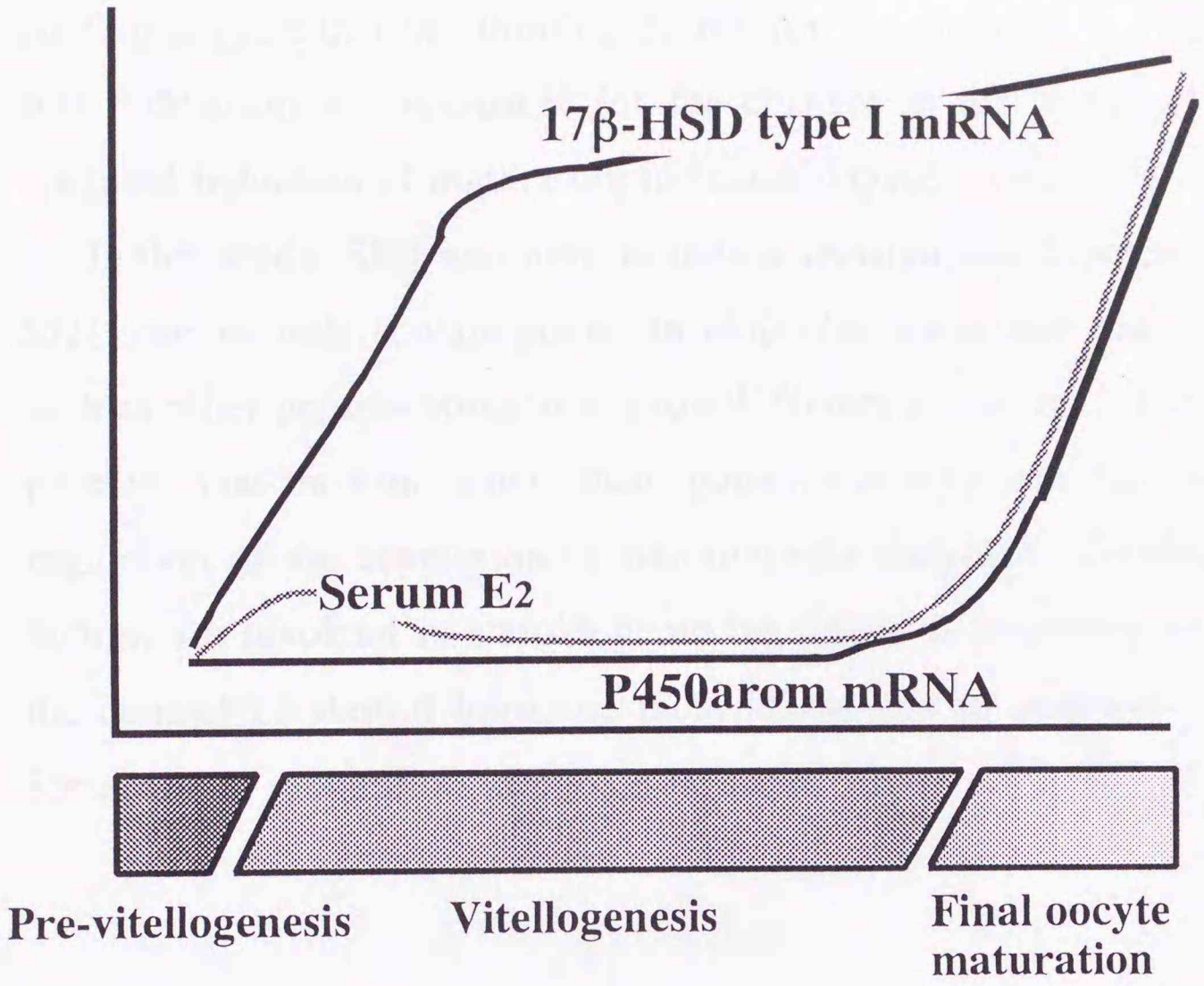


Fig. 22. Schematic illustration of the changes in the activities of 17α-hydroxylase , C17-20 lyase, and 20β-HSD and the transcript levels of P450c17.



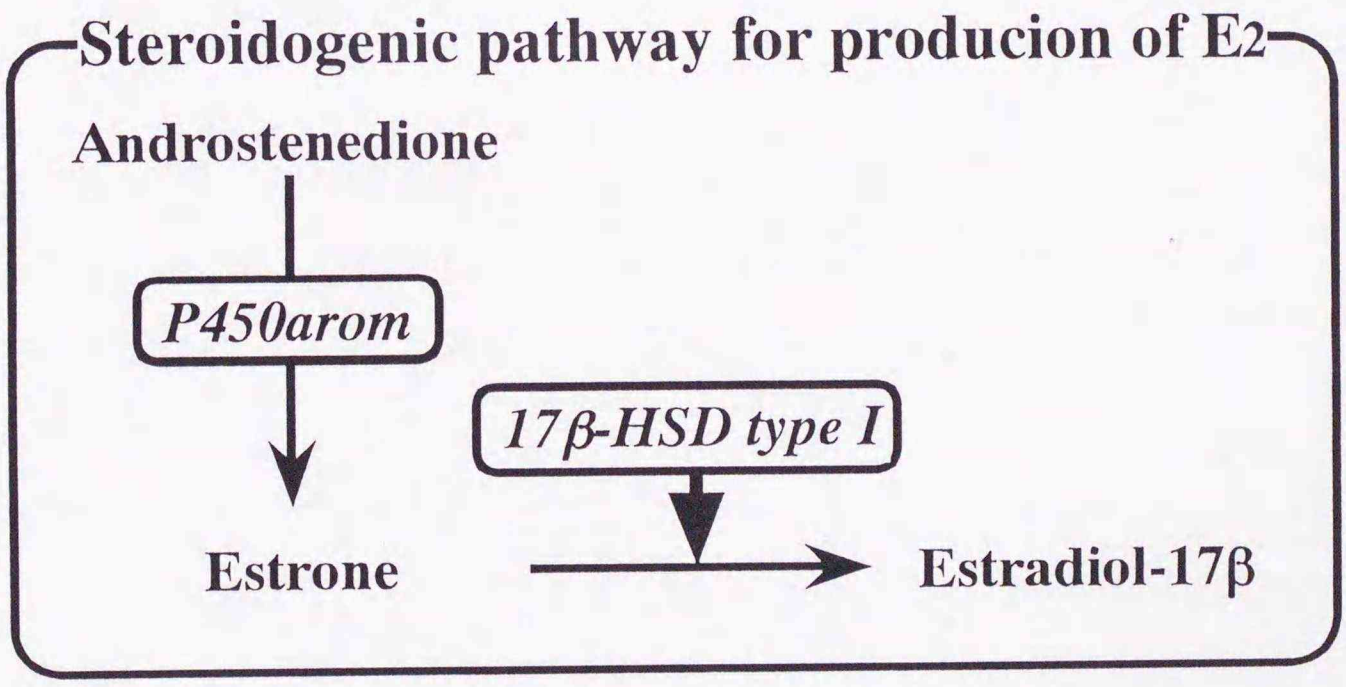


Fig. 23. Schematic illustration of the changes in serum E_2 levels, and the transcript levels of 17β-HSD type I and P450arom

finding suggest that the limiting factor for E_2 synthesis is P450arom and that P450arom is responsible for the changes in the levels of E_2 during artificial induction of maturation in female Japanese eel.

In this study, SPH was used to induce ovarian development. However, SPH dose not only contain gonadotropin(s) but also many unknown factors, such as other peptide hormones, growth factors and so on. Therefore, it is possible that factors other than gonadotropin(s) are involved in the regulation of the expression of steroidogenic enzymes. To identify which factors are involved in steroidogenic regulation is necessary to understand the control of steroid hormone biosynthesis and to improve the protocol for artificial maturation in female Japanese eel.

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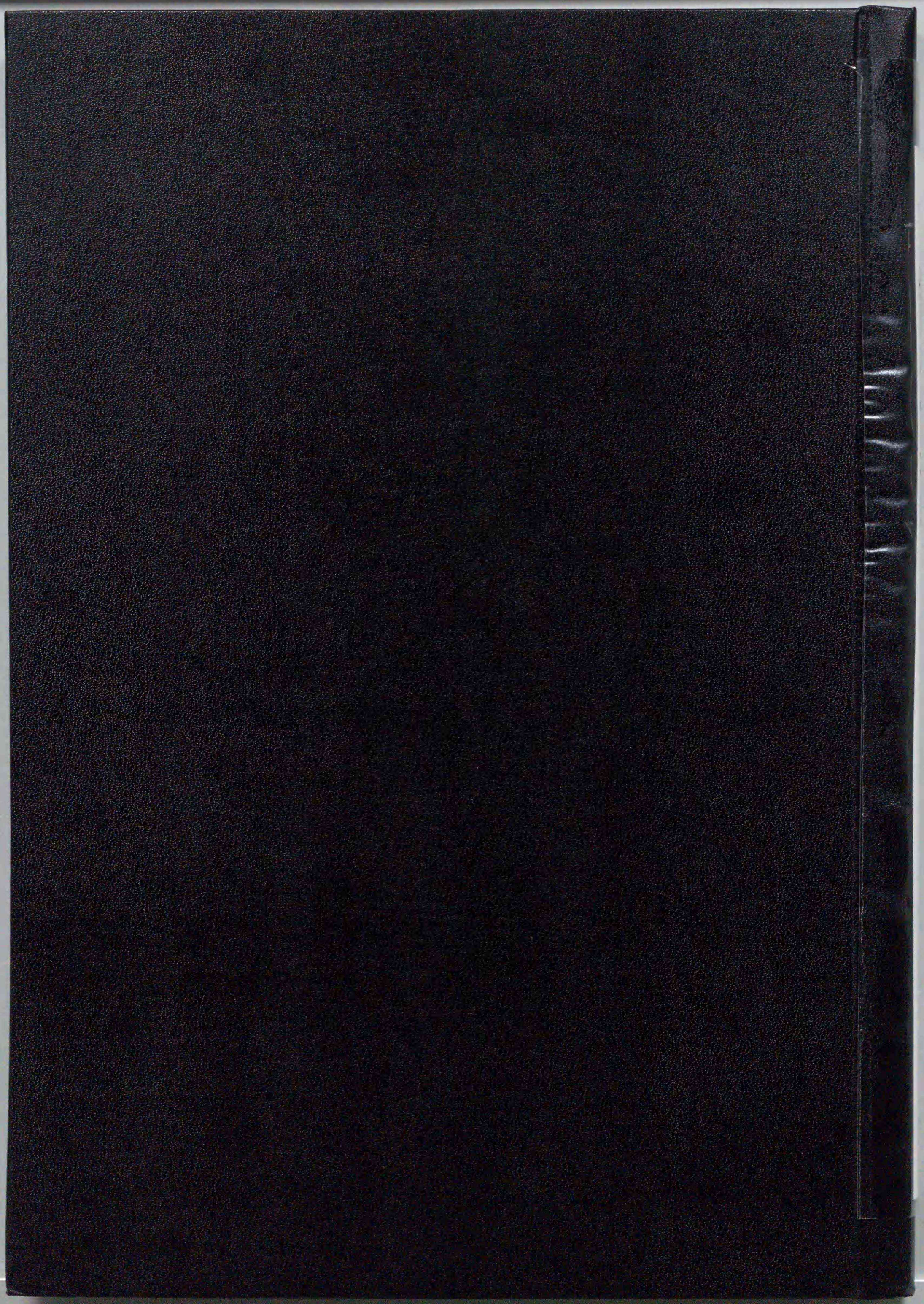
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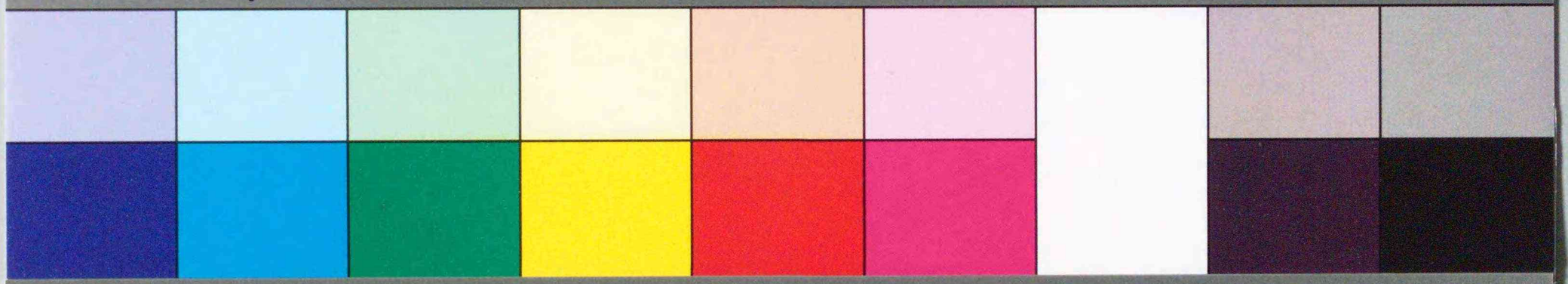
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