Ovarian Cytochrome P-450 17α-Hydroxylase/C17,20-Lyase of the Medaka (*Oryzias latipes*): Structure, Activity and Function

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Abstract

In nonmammlian vertebrates, ovarian follicle cells produce two different steroid hormones, estradiol-17β and maturation-inducing hormone (progestogens), in response to pituitary gonadotropins, which play important roles in two phases of oogenesis, vitellogenesis and oocyte maturation, respectively. Estradiol-17β promotes vitellogenesis in members of all nonmammalian vertebrates. On the other hand, a variety of progestogens have been shown to be effective in the initiation of meiotic maturation in fish and amphibian oocytes. A distinct shift from estradiol-17β to progestogens has been reported to occur in ovarian follicles immediately prior to oocyte maturation, and seems to be a prerequisite step for growing oocytes to enter the final stage of maturation. However, the regulatory mechanism of this steroidogenic shift is not clearly understood.

The medaka, *Oryzias latipes*, under a long photoperiod (14 hours light - 10 hours dark) at 26°C, spawns daily within 1 hr of the onset of light for a number of consecutive days. Under these conditions, the sequence of events leading to spawning such as vitellogenesis, oocyte maturation, and ovulation can be timed accurately. These features make medaka an ideal model in which to investigate the regulatory mechanism of the steroidogenic shift occurring in ovarian follicles prior to oocyte maturation.

The medaka ovarian follicle produces estradiol-17β during vitellogenesis and 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP, the most potent maturation-inducing hormone of medaka) during maturation. Although the cytochrome P-450 aromatase (P-450arom, the enzyme which converts testosterone to estradiol-17β) and 20β-hydroxysteroid dehydrogenase (20β-HSD, the enzyme which converts 17α-hydroxyprogesterone to 17α,20β-DP)

are known to be involved in the shift from estradiol-17β to 17α,20β-DP production in the medaka ovarian follicles, the key steroidogenic enzyme responsible for the shift remain unknown.

In this study, I first investigated changes in the steroidogenic pathway in medaka ovarian follicles during vitellogenesis and oocyte maturation by incubating the follicles *in vitro* with several radiolabeled steroid precursors, followed by thin layer chromatography (TLC) fractionation and recrystallizaion. When vitellogenic follicles collected at 18 hours before the expected time of spawning (vitellogenic follicles) were incubated with ^{3}H-labeled pregnenolone, the major metabolites were 17α-hydroxypregenenolone, 17α-hydroxyprogesterone, and androstenedione. Incubations of vitellogenic follicles with androstenedione produced testosterone and estradiol-17β. By contrast, when maturing follicles (postvitellogenic follicles undergoing maturation) collected at 10 hours before spawning were incubated with ^{3}H-labeled pregnenolone, the major metabolites were 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, and 17α,20β-DP; androstenedione was not detected. Neither vitellogenic and maturing follicles produced progesterone when they were incubated with ^{3}H-labeled pregnenolone, suggesting that in medaka ovarian follicles both estradiol-17β and 17α,20β-DP are synthesized by the ^{5}Δ-steroid pathway. Thus, there is a distinct shift in the steroidogenic pathway from estradiol-17β to 17α,20β-DP production in medaka ovarian follicles, and it is suggested that the decrease in C17,20-lyase activity is responsible for this shift.

In the next series of experiments, the effects of several reagents on C17,20-lyase activity in the medaka ovarian follicles were examined. The phosphodiesterase inhibitor IBMX, but not pregnant mare serum

gonadotropin (PMSG), enhanced androstenedione production in incubation of vitellogenic follicles with ^{14}C-labeled progesterone. Calcium ionophore A23187 and the phorbol ester TPA (a protein kinase C activator) blocked the stimulatory actions of IBMX on androstenedione production. These findings suggest that multiple signaling pathways may participate in the regulation of ovarian steroidogenesis, especially in the regulation of P-450c17 activity.

The preceding findings suggest that the rapid decrease in C17,20-lyase activity is responsible for the shift in the steroidogenic pathway from estradiol-17β to 17α,20β-DP production by medaka ovarian follicles prior to oocyte maturation. To further investigate the mechanism of this shift at the molecular levels, I have attempted to isolate cDNA clones encoding medaka *P*-450c17 from an ovarian cDNA library. Two types of *P*-450c17 cDNA (*P*-450c17L and *P*-450c17S: lacking 87 nucleotides comparing with *P*-450c17L in coding region) were isolated. The RNase protection assay and RT-PCR revealed that the existence of both transcripts. By comparing the sequences of these two cDNAs with that of genomic DNA, it was shown that nucleotides which were missing in *P*-450c17S was exon 4. *P*-450c17L products which were expressed in both mammalian COS 1 cells and *E. coli* showed both 17α-hydroxylase and C17,20-lyase activities, but P-450c17S product showed neither activity. These findings confirm that a single product of the cytochrome *P*-450c17 gene has both 17α-hydroxylase and C17,20-lyase activities, and further suggest that the differential regulation of 17α-hydroxylase and C17,20-lyase activities of P-450c17 is an important mechanism responsible for the shift in the steroidogenic pathway from estradiol-17β to 17α,20β-DP production by medaka ovarian follicles. Genomic Southern blot analysis revealed that medaka *P*-450c17 was a

single copy gene. Accordingly, two types of transcripts were synthesized by alternative splicing. Both transcripts were abundant in both ovary and testis. Northern blot analysis revealed two different sizes of transcripts, 2.4 and 6.4 kb, both of which are present in follicles during vitellogenesis and disappear during oocyte maturation. The amounts of these two transcripts begin increasing 29 hours before spawning and reach peaks around 23 hours. Amounts decrease to an almost undetectable level at 8 hours before spawning. Another small peak was observed at 47 hours. Thus, the amounts of *P*-450c17 transcripts present in medaka ovarian follicles correlate to a daily cycle of ovulation. The transcripts are present during vitellogenesis and begin to decrease prior to the production of maturation-inducing hormone (17 hours before spawning). These findings are consistent with the role of *P*-450c17 in the production of androgens and estradiol-17β during active vitellogenesis. However, the significance of two different sizes of *P*-450c17 transcripts remains unknown.

A possible involvement of cytochrome b_5 in the differential regulation of P-450c17 enzyme activity is also examined. Both NADPH:*P*-450 reductase and cytochrome b_5 transcripts were detected in medaka ovarian follicles. Expression of these mRNA levels was higher in follicles collected during vitellogenesis than those during oocyte maturation. NADPH:P-450 reductase purified from pig supported the metabolism of progesterone by medaka P-450c17, and pig cytochrome b_5 affect influenced the activities of P-450c17. These results are consistent with the notion that cytochrome b_5 play a role in the differential regulation of P-450c17 by medaka ovarian follicles.

I. Steroidogenesis in the Ovarian Follicles of the Medaka (*Oryzias latipes*) during Vitellogenesis and Oocyte Maturation

INTRODUCTION

In nonmammalian vertebrates, ovarian follicle cells produce two different steroid hormones, estradiol-17β and maturation-inducing hormone (progestogens), in response to pituitary gonadotropins, which play important roles in two phases of oogenesis, vitellogenesis and oocyte maturation, respectively. Estradiol-17β promotes vitellogenesis in members of all nonmammalian vertebrates (Ho, 1987). In each of these groups, the time of vitellogenin production corresponds to the period of elevated plasma estradiol-17β levels. Vitellogenin is the precursor molecule for egg yolk, which is of considerable importance as the source of metabolic energy for the developing embryo. In response to increased levels of plasma estradiol-17β, the liver synthesizes and secretes vitellogenin, which is carried in the bloodstream to the oocytes. The developing oocytes take up vitellogenin and convert it to egg yolk. On the other hand, a variety of progestogens have been shown to be effective in the initiation of meiotic maturation in fish and amphibian oocytes (Nagahama, 1987). Two progestogens, 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP) and 17α,20β,21-trihydroxy-4-pregnen-3-one, have been identified as the maturation-inducing hormone of several teleost fishes. These ovarian steroid hormones are known to be secreted from ovarian follicles in response to pituitary gonadotropins. A distinct shift from estradiol-17β to 17α,20β-DP has been reported to occur in ovarian follicles immediately prior to oocyte maturation (Nagahama, 1994), and seems to be a prerequisite step

for growing oocytes to enter the final stage of maturation. However, the regulatory mechanism of this steroidogenic shift is not clearly understood.

The medaka, *Oryzias latipes*, under a long photoperiod (14 hours light - 10 hours dark) at 26°C, spawns daily within 1 hour of the onset of light for a number of consecutive days. Under these conditions, the sequence of events leading to spawning such as vitellogenesis, oocyte maturation, and ovulation can be timed accurately (Iwamatsu, 1978). These features make medaka an ideal model in which to investigate the regulatory mechanism of the steroidogenic shift occurring in ovarian follicles prior to oocyte maturation. It has previously been shown that medaka ovarian follicles produce estradiol-17β during vitellogenesis and 17α,20β-DP during oocyte maturation (Fig. 1). During vitellogenesis, cytochrome P-450 aromatase (P-450arom) activity is high, but 20β-hydroxysteroid dehydrogenase (20β-HSD, the enzyme which converts 17α-hydroxyprogesterone to 17α,20β-DP) activity is non detectable, leading to the production of estradiol-17β. By contrast, during oocyte maturation, P-450arom activity is low, but 20β-HSD is high, leading to the production of 17α,20β-DP. 17α,20β-DP was identified as a major maturation-inducing hormone in medaka (Fukada *et al.*, 1994). Thus, there is a shift in steroidogenic enzyme activity (a decrease in P-450arom activity and an increase in 20β-HSD activity) and this shift is a mechanism regulating the shift from estradiol-17β to 17α,20β-DP production in medaka ovarian follicles. However, a shift in precursor production from testosterone to 17α-hydroxyprogesterone is also an important mechanism for the shift from estradiol-17β to 17α,20β-DP production. To determine a key enzyme(s) responsible for this shift in steroid precursor production, the precise information on the steroidogenic pathway during vitellogenesis and oocyte maturation is required.

In this study, the steroidogenic pathway in medaka ovarian follicles during vitellogenesis and oocyte maturation was determined. To this end, intact follicles or follicle cell layers, isolated at appropriate time before spawning, were incubated with radiolabeled steroid precursors, and metabolites were analyzed by TLC fractionation and recrystallization. Since the conversion of 17α-hydroxyprogesterone into androstenedione is expected to be a key step of the shift in medaka ovarian steroidogenesis from vitellogenesis to oocyte maturation, I focused on the production of androstenedione. I also tested the effects of several reagents on the production of androstenedione *in vitro*.

MATERIAL AND METHODS

Chemicals

[7-^{3}H(N)]-pregnenolone (370-925 GBq/mmol), [1,2-^{3}H]-17α-hydroxyprogesterone (1.48-2.22 TBq/mmol) and [4-^{14}C]-androstenedione (1.67-2.22 GBq/mmol) were obtained from NEN research products. These steroids were purified by TLC with appropriate solvent systems before use. Cold steroids, progesterone, androstenedione, 17α-hydroxyprogesterone, testosterone, and 17α,20β-DP were obtained from Sigma. Pregnant mare serum gonadotropin (PMSG) was purchased from Teikyo Zohki (Tokyo, Japan). The phosphodiesterase inhibitor 3-Isobutyl-1-methylxanthin (IBMX), calcium ionophore A23187, and the phorbol ester TPA (phorbol 12-myristate 13-acetate, a protein kinase C activator) were purchased from Sigma.

Ovarian follicle incubation

Medaka were obtained from a local fish farm (Yatomi, Aichi, Japan). Fish were killed by decapitation and ovaries were immediately removed and kept in cold modified Earl's medium 199 (Gibco BRL, containing 30 mg/liter streptomycin sulfate and penicillin G potassium. pH was adjusted with 25 mM Hepes-NaOH into 7.3, diluted into 90% of original concentration). Vitellogenic and maturing (follicles undergoing maturation) follicles were collected from several ovaries with the aid of fine forceps. In some experiments, follicle cell layers were obtained by squeezing out yolk from intact follicles. Isolated follicle cell layers or intact follicles were incubated in six well/flat bottom plastic cell wells containing 10 ml modified Earl's medium 199 with radiolabeled steroid precursors. In some experiments follicle cell layers or intact follicles were incubated in the presence of PMSG (100 units/ml) or other chemical reagents. Incubations were performed under humid conditions at 26°C.

Analysis of metabolites

Steroids were extracted from incubation media with 3 ml of dichloromethane five times and dried on warm sand bath under nitrogen gas. Dried extracted steroids were resuspended with a small volume of dichloromethane and applied on a TLC plate containing a fluorescent indicator (Merck). Mixture of progesterone, androstenedione, 17α-hydroxyprogesterone, testosterone, and 17α,20β-DP was applied on a TLC plate with samples as standards of mobility. Standard steroids were detected by UV absorption at 254 nm. The plates were usually developed in benzene:acetone (4:1; v/v) and in some experiments in other solvent systems.

When ^{3}H-labeled steroids were used as precursors, autoradiography was

carried out with ^{3}H-hyperfilms (Amersham). In case of ^{14}C-labeled precursors, results were analyzed with a BAS-2000 Bio-Imaging Analyzer (Fujifilm). Some of the bands were scraped and metabolites were extracted with dichloromethane. Extracted steroids were identified by recrystallization method. Recrystallization was performed in accord with Axelrod *et al.* (1965).

RESULTS

Incubations of vitellogenic and maturing follicles with [7-^{3}H(N)]-pregnenolone and [1,2-^{3}H]-17α-hydroxyprogesterone

Vitellogenic follicles (200/dish) collected at 18 hours before the expected time of spawning and maturing follicles (200/dish) (follicles undergoing maturation) collected at 10 hours were incubated with 5×10^{6} cpm of [7-^{3}H(N)]-pregnenolone for 3 hours and the steroid metabolites produced during incubations were separated by TLC-I, benzene:acetone (4:1). There was a marked difference in the pattern of radioactive steroid metabolites between vitellogenic and maturing follicles. The following steroid metabolites produced in vitellogenic follicle incubations were identified by their chromatographic mobilities in TLC: pregnenolone (precursor, metabolite 1), 17α-hydroxyprogesterone (metabolite 2), 17α-hydroxypregnenolone (metabolite 3), and androstenedione (metabolite 4) (Fig. 2A). The identities of the two metabolites as 17α-hydroxyprogesterone and androstenedione were confirmed by recrystallization to constant specific activity (Table 1). When maturing follicles were incubated with ^{3}H-labeled pregnenolone, two major metabolites (17α-hydroxypregnenolone and 17α-hydroxyprogesterone) and

two minor polar metabolites were found; however, no radioactivity comigated with androstenedione (Fig. 2B). Vitellogenic and maturing follicles were also incubated with 5 × 10^6 cpm of [1,2-^{3}H]-17α-hydroxyprogesterone for 3 hours. Autoradiographs of the steroid metabolites showed that 17α-hydroxyprogesterone was not extensively metabolized by either vitellogenic or postvitellogenic follicles. The yield of androstenedione was much higher in incubations of vitellogenic follicles than those of maturing follicles (Fig. 2C). Minor polar metabolites were found in incubations of maturing follicles (Fig. 2D). Vitellogenic and maturing intact follicles (follicle enclosed oocytes) were also incubated with 5 × 10^6 cpm of [1,2-^{3}H]-17α-hydroxyprogesterone for 3 hours. No significant difference between follicles and intact follicles was seen (Fig. 3). These observations indicate that the presence of oocytes did not affect the steroidogenic capacity of ovarian follicles.

Incubation of vitellogenic and maturing follicles with [4-^{14}C]-androstenedione

After chromatography of the vitellogenic follicle (190/dish) incubation with androstenedione gave several radioactive metabolites corresponding to carriers androstenedione (precursor, metabolite 1), testosterone (metabolite 2) and estradiol-17β (metabolite 3)(Fig. 4A). The testosterone area when rechromatographed in TLC-II, cyclohexane:ethanol (9:1), gave a major metabolite (metabolite 1) with the same mobility as the carrier standard testosterone (Fig. 5A). The estradiol-17β area when rechromatogaphed in TLC-II gave three metabolites: one of the metabolites was comigrated with estradiol-17β (Fig. 5B). Androstenedione was not extensively metabolized by maturing follicles with very few metabolites produced (Fig. 4B).

Incubation of vitellogenic follicles with [4-^{14}C]-17α-hydroxyprogesterone in the presence of PMSG

Medaka vitellogenic follicles collected at 22 hours before spawning do not undergo spontaneous maturation, but undergo maturation in response to 17α,20β-DP (M. Yoshikuni and Y. Nagahama, unpublished data). Intact follicles (200 /dish) were incubated in the presence or absence of PMSG for 2 and 12 hours. These follicles were further incubated with or without [4-^{14}C] 17α-hydroxyprogesterone for 6 hours. The pattern of steroidogenesis was similar to that seen in controls during the first 2 hours of incubation with PMSG (Figs. 6A and 6B). The major metabolites were androstenedione (metabolite 1) and a more polar metabolite. Incubation of intact follicles with PMSG for 18 hours resulted in marked changes in the pattern of 17α-hydroxyprogesterone metabolites; the pattern was similar to that seen in maturing follicles incubated with 17α-hydroxyprogesterone (Figs. 6C and 6D). Although there was no obvious decrease in androstenedione (metabolite 2) production, a polar metabolite corresponding to 17α,20β-DP (metabolite 3) was observed with several other polar metabolites (Fig. 6D).

Incubation of vitellogenic follicles with [4-^{14}C]-progesterone in the presence of IBMX alone and in combination with TPA and/or A23187

Effects of IBMX (a phosphodiesterase inhibitor) alone and in combination with TPA (a protein kinase C activator) and/or A23582 (a calcium ionophore) on progesterone metabolites were investigated using vitellogenic follicles collected at 18 hours before spawning. The follicles (100/dish) were incubated in media containing test compounds for 8 hours. After

incubation, 1.0×10^5 cpm of [4-^{14}C]-progesterone was added as a precursor. After further 3 hours of incubation, metabolites were extracted from incubation media and fractionated by two-dimensional TLC: benzene:acetone (4:1) in the first development system and cyclohexane:ethylacetate (1:1) in the second development system. IBMX (1 mM) stimulated androstenedione production (Fig. 7, column 2). This IBMX-stimulated androstenedione production was completely blocked by A23187 (5 μM) or TPA (0.5 μM) alone, or in combination with both compounds (Fig. 7, columns 3-6).

DISCUSSION

Data presented in this study indicate that there is a distinct shift in the steroidogenic pathway from estradiol-17β to 17α,20β-DP by medaka ovarian follicles. It was shown that both estradiol-17β and 17α,20β-DP are synthesized by the $^{5}\Delta$-steroid pathway (cholesterol → pregnenolone → 17α-hydroxypregnenolone → 17α-hydroxyprogesterone)(Fig. 8). Thus, in medaka ovarian follicles, the steroidogenic enzyme which serves as a branch point for estradiol-17β and 17α,20β-DP production is cytochrome P-450 17α-hydroxylase/C17,20-lyase (P-450c17). P-450c17 is a single cytochrome P-450 enzyme mediating both 17α-hydroxylase and C17,20-lyase activities (pig, Nakajin and Hall, 1981, Nakajin *et al.*, 1981, 1984; guinea pig, Tremblay *et al.*, 1994; bovine, Zuber *et al.*, 1987; rat, Namiki *et al.*, 1988; human, Yanase *et al.*, 1989; rainbow trout, Sakai *et al.*, 1992). Recently, I isolated a cDNA encoding P-450c17 from a medaka ovarian follicle cDNA library. The medaka P-450c17 expressed in non-steroidogenic COS 1 cells showed both 17α-hydroxylase and C17,20-lyase activities (see Chapter II, Kobayashi *et al.*, manuscript in preparation).

When medaka vitellogenic follicles were incubated with ^{3}H-labeled pregnenolone, the major metabolites were 17α-hydroxypregenenolone, 17α-hydroxyprogesterone, and androstenedione. By contrast, maturing follicles, under the same conditions, produce 17α-hydroxypregnenolone and 17α-hydroxyprogesterone. There is substantial reduction in the synthesis of androstenedione and, almost complete inhibition of testosterone and estradiol-17β. Subsequently, there is an increase in the synthesis of 17α,20β-DP. Furthermore, it was shown that the absence of androstenedione is not due to its rapid conversion to other metabolites.

These results suggest that there is a distinct decrease in C17,20-lyase activity of P-450c17 and this decrease is a critical mechanism that regulates the shift in the steroidogenic pathway. In mammals, inhibition of estradiol-17β production following the preovulatory LH surge is primarily the result of decrease in androgen production in theca and interstitial cells. In *in vitro* study with rat Grafian follicles in culture, inhibition of androgen secretion appeared to result from reduced 17α-hydroxylase and/or C17,20-lyase activity (Gore-Langton and Armstrong, 1988). In the tropical catfish, *Clarias macrocephalus*, human chorionic gonadotropin (hCG) treatment *in vitro* induces an switch in ovarian steroidogenesis from predominantly testosterone to 17α,20β-DP: a decrease in both 17α-hydroxylase and C17,20-lyase activities of P-450c17 and a rapid increase in 20β-hydroxysteroid dehydrogenase (20β-HSD) activity (Suzuki *et al.*, 1989). It was hypothesized that the shift is regulated by the inhibition of C17,20-lyase by gonadotropin (Scott and Baynes, 1982). However, our PMSG experiments using vitellogenic follicles collected at 22 hours before spawning demonstrated that gonadotropin is probably not directly responsible for the decrease in C17,20-lyase activity in medaka ovarian follicles. The fish described by Suzuki *et al.* (1989) were post-ovulatory. The differences in the effect of gonadotropin on ovarian steroidogenesis suggest that there may be major changes in steroidogenic activity over a very short time interval during the preovulatory phase. Further studies using larger numbers of fish at a more frequent sampling schedule are required to delineate more precisely the steroidogenic changes occurring during this period.

A very rapid steroidogenic pathway shift from androgen (11-ketotestosterone) to progestogen production has also been reported in several spermiating male fishes (Barry *et al.*, 1990). Thus, the differential

regulation of 17α-hydroxylase and C17,20-lyase activities of P-450c17 is important for the process of final gamete maturation in both female and male fish. Barry *et al.* (1990), using cultures of testicular fragments of spawning male common carp, *Cyprinus carpio*, demonstrated that gonadotropin was not probably directly responsible for controlling the shift in the steroidogenic pathway from androgen to progestogen production. On the basis of data from Inano *et al.* (1967), Baynes and Scott (1985) postulated that the steroidogenic shift in rainbow trout may be regulated by the inhibition of C17,20-lyase by 17α,20β-DP. In fact, Barry *et al.* (1989) tested this hypothesis in common carp and demonstrated that at least one of the two enzymes that convert testosterone to 11-ketotestosterone, 11β-hydroxylase and 11β-hydroxysteroid dehydrogenase, and probably C17,20-lyase, are inhibited in a dose-dependent manner by physiological levels of 17α,20β-DP. Certainly, this hypothesis merits further investigation using medaka ovarian follicles.

In this study, IBMX stimulated androstenedione production. It is of interest to note that A23187 and TPA suppressed IBMX-induced androstenedione production. These results suggest that calcium and C-kinase regulate C17,20-lyase activity in medaka ovarian follicles. In fact, the promoter regions of bovine and human *P*-450c17 contain not only cAMP responsive sequence (CRS) but also TPA responsive element (TRE). TPA was reported to suppress basal and cAMP stimulated transcription of *P*-450c17 through the TRE (Bakke *et al.*, 1992, Brentano *et al.*, 1990). Thus, it is possible that in medaka *P*-450c17 TRE also functions as a regulator of *P*-450c17 transcription in concert with CRS.

Another regulator of the enzymatic activities of P-450c17 is cytochrome b_5. It has been reported that in reconstituted systems, the C17,20-lyase

activity in human P-450c17 is virtually undetectable in the absence of cytochrome b_5. However, the addition of cytochrome b_5 enhances C17,20-lyase activity to a level much higher than 17α-hydroxylase activity (Katagiri *et al.*, 1995, Lee-Robichaud *et al.*, 1995). A similar enhancing effect of cytochrome b_5 on the C17,20-lyase activity is also seen in pig P-450c17 (Lee-Robichaud *et al.*, 1995). These results suggest that cytochrome b_5 play an important role in the differential regulation of the hydroxylase and lyase activities of P-450c17. Whether the level of cytochrome b_5 influences the C17,20-lyase activity in medaka ovarian follicles needs to be determined.

In summary, this study demonstrated that a distinct shift in the steroidogenic pathway from estradiol-17β to 17α,20β-DP production occurs in medaka ovarian follicles prior to oocyte maturation. The inhibition of C17,20-lyase activity may be directly responsible for controlling this shift. The inhibition of C17,20-lyase would lead not only to a decrease in estradiol-17β production, but also to an accumulation of 17α-hydroxyprogesterone, the immediate precursor of 17α,20β-DP, the maturation-inducing hormone of medaka. Fish ovarian follicles represent a useful model for future studies on the molecular mechanism of differential regulation of 17α-hydroxylase and C17,20-lyase activities of P-450c17.

II. Isolation, Characterization, and Expression of two cDNA Clones Encoding Medaka *P*-450c17

INTRODUCTION

In lower vertebrates, estradiol-17β and maturation-inducing hormone (progestogens) play very important roles in two different stages of oocyte development, vitellogenesis and maturation, respectively (Jalabert *et al.*, 1991; Nagahama, 1994). These two steroid hormones are synthesized in the same ovarian follicle cells, which means that production of each is regulated in a stage-specific manner.

In medaka, the change in enzymatic activities involved in ovarian steroidogenesis correlates with the daily spawning cycle. Coupled with the knowledge of the steroidogenic pathway in medaka, this predictability makes medaka an ideal animal to investigate molecular mechanism of ovarian steroidogenesis (Iwamatsu, 1987). A common steroidogenic pathway ($^{5}\Delta$ steroid-pathway) was shown to share for both estradiol-17β and maturation-inducing hormone production by medaka ovarian follicle cells (see Chapter I; also Kobayashi *et al.*, 1996). 17α-Hydroxyprogesterone is converted from pregnenolone via 17α-hydroxypregnenolone by 17α-hydroxylase and 3β-hydroxysteroid dehydrogenase activities. During vitellogenesis, C17,20-lyase further converts 17α-hydroxyprogesterone to androstenedione. Immediately prior to maturation, however, C17,20-lyase activity dramatically decreases and 20β-hydroxysteroid dehydrogenase activity increases to produce maturation-inducing hormone. The key enzyme in the choice of pathways leading to estradiol-17β and progestogens is the cytochrome *P*-450c17 (*P*-450c17). This enzyme is found in vertebrate

adrenals, testes, and ovaries. Interestingly, a single product of *P*-450c17 gene has both 17α-hydroxylase and C17,20-lyase activities in several species (pig; Nakajin and Hall, 1981, Nakajin *et al.*, 1981, 1984: bovine; Zuber *et al.*, 1986: guinea pig; Tremblay *et al.*, 1994: rat; Namiki *et al.*, 1981: human; Yanase *et al.*, 1989: rainbow trout; Sakai *et al.*, 1992). It has been suggested that cytochrome b_5 plays a role in regulating the ratio of 17α-hydroxylase and C17,20-lyase activities in various tissues. In human, *P*-450c17 product has low C17,20-lyase activity in the absence of cytochrome b_5 and cytochrome b_5 stimulates C17,20-lyase more than 17α-hydroxylase *in vitro* (Katagiri *et al.*, 1995). However, the mechanism by which a single polypeptide regulates these two distinct activities differentially through oogenesis remains to be elucidated.

To answer these questions as well as to provide a basis for studies on the role of P-450c17 in the steroidogenic shift occurring in medaka ovarian follicles immediately prior to oocyte maturation, I cloned *P*-450c17 cDNAs from medaka ovarian follicle cells. Changes in *P*-450c17 transcript levels have correlated with the changes in steroidogenic activities in medaka ovarian follicle cells. The presence of a novel type of *P*-450c17 transcript as well as the known type was detected. To verify transcripts as *P*-450c17, the enzymatic activities of both types of *P*-450c17 transcripts were determined using two kinds of heterologous expression systems, COS1 monkey kidney tumor cells and *Escherichia coli* (*E. coli*). Furthermore, a series of experiments were carried out to investigate whether cytochrome b_5 has an enhancing effect on 17α-hydroxylase and C17,20-lyase activities of medaka *P*-450c17 product. I also determined changes in the levels of cytochrome b_5 transcripts as well as those of NADPH:cytochrome *P*-450 reductase (NADPH:*P*-450 reductase) in medaka ovarian follicles during vitellogenesis

and oocyte maturation.

MATERIALS AND METHODS

cDNA library construction and screening

Total RNA from medaka ovarian follicle cells (isolated theca and granulosa cell layers) at 22 hours before the expected time of spawning was isolated using a Total RNA Isolation Kit (Invitrogen). Poly(A)$^+$ RNA was then purified from total RNA using OligotexTM-dT30 (Takara). A cDNA library was constructed from 50 ng of poly(A)$^+$ RNA using a cDNA Synthesis Kit (Pharmacia Biotech), λZAPII vector (Stratagene), and GigapackII Gold Packaging Extract (Stratagene). *P*-450c17 cDNA was screened from 8.9×10^4 plaques. Oligonucleotide primers 1 and 2 (Table 2) were synthesized and used as primers for PCR; primers were designed based on the sequence data of the medaka *P*-450c17 gene (M. Matsuyama, M. Tanaka, D. Kobayashi and Y. Nagahama, unpublished). PCR was performed in the presence of [α-^{32}P]-dCTP (Amersham) using AmpliTaq DNA polymerase (Perkin Elmer) with medaka *P*-450c17 genomic DNA fragment as a template, and PCR product was used as a probe for plaque hybridization.

Northern blot analysis of P-450c17

Total RNA was collected from medaka ovarian follicle cells at various times prior to spawning. Ten μg of total RNA was electrophoresed under denaturing conditions and immobilized on Hybond N$^+$ membranes (Amersham). Membranes were hybridized with the PCR probe described above. Hybridization signals were detected by the BAS-2000 Bio-Imaging Analyzer (Fujifilm).

RNA preparation for ribonuclease protection assay and RT-PCR

Intact follicles, collected at appropriate times before spawning, were immersed in ISOGEN (Nippongene) solution and shaken gently for 5 minutes to extract follicular cytosolic contents. Other tissues (testis, kidney, liver, brain, and spleen) were sonicated in ISOGEN solution. Total μg of total RNA was then extracted as described above.

Ribonuclease protection assay

A complementary RNA (cRNA) probe was designed to hybridize from positions 745 - 907 of the *P*-450c17 mRNA. This region contains 29 nucleotides of exon 4 and 11 nucleotides of vector sequence. The cRNA probe was synthesized by a RNA transcription kit (Stratagene) with T7 polymerase (Takara) in the presence of [α-^{32}P]CTP (Amersham T7/SP6 grade). The synthesized probe was purified by 6% polyacrylamide gel electrophoresis containing 8 M urea before hybridization (Ausubel et al.). Five μg of total RNA were hybridized with 1.4×10^4 cpm of cRNA probe at 55℃ and subsequently digested with RNase A (RNase protection kit, Boehringer Mannheim). Protected RNAs were eletrophoresed in a 6% polyacrylamide gel containing 8 M urea. Hybridization signals were detected with the BAS-2000 Bio-Imaging Analyzer (Fujifilm).

Southern blot analysis of RT-PCR products

Reverse transcription was performed using SuperscriptII (Gibco BRL) with primer 8 (Table 2). One μg total RNA extracted from ovarian follicle cells, testis, kidney, liver, brain, and spleen served as templates. Reverse transcription without RNA template was performed as a negative control. PCR was then carried out with 1/20th of the first strand DNA using primers

3 and 4 (Table 2).

The amplified DNA fragments were electrophoresed on 1.5% agarose gel and transferred to Hybond N^+ membrane (Amersham). Transferred DNAs were hybridized with an internal probe for *P*-450c17 to verify that the amplified products represented *P*-450c17 cDNA. The internal oligonucleotide probe (Table 2 - NO. 5) was labeled with digoxigenin(DIG)-11-ddUTP using the DIG Oligonucleotide 3'-End Labeling Kit (Boheringer Mannheim). Detection was performed using Anti-DIG, Fab fragment-alkaline phosphatase conjugate (Boheringer Mannheim) and CSPD^{TM} (Tropix).

Expression of medaka P-450c17 cDNA in COS1 cells and their enzymatic activities

Two types of medaka *P*-450c17 cDNA were ligated into the pSVL vector containing the SV40 late promoter. Transfection and incubation procedures were conducted according to Sakai *et al.* (1992). [4-^{14}C]-Progesterone was added as a precursor. Metabolized steroids were extracted, fractionated and identified by two dimensional thin layer chromatography (TLC, Merck), with benzene:acetone (4:1) (direction a) followed by cyclohexane:ethylacetate (1:1) (direction b). Cold progesterone, 17α-hydroxyprogesterone, androstenedione, testosterone, and 17α,20β-DP served as mobility markers detected by UV light. [1,2-^{3}H(N)]-17α-Hydroxyprogesterone and [7-^{3}H(N)]-pregnenolone were also used as precursors.

Expression of medaka P-450c17 cDNAs in E. coli and their enzymatic activities

The nucleotide sequence of both types of cDNAs surrounding the first methionine was modified from CGATGG to CCATGG using conventional PCR techniques; the modified cDNA sequences were confirmed by sequencing (Frohman et al., 1994). The resulting sequence is a recognition site of *Nco*I. *P*-450c17 cDNA containing *Nco*I and *Xho*I were digested and ligated into the *Nco*I and *Xho*I site of the pET15b expression vector (Novagen). *E. coli* BL21(DE3) were then transformed. Transformed E. coli was cultured in 40 ml of LB media containing 50 μg/ml ampicillin. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to 1 mM to induce the expression of *P*-450c17. After 3 hours of incubation with agitation at 37℃, bacteria were harvested, washed with 10 ml of MOPS buffer (50 mM MOPS (3-(N-Morpholino)propanesulfonic acid, 100 mM KCl, 1 mM EDTA and 1 mM dithiothreitol, pH 7.5), spun, and resuspended into 5 ml of MOPS buffer. [4-^{14}C]-Progesterone was added to the resuspeneded cells which were incubated at 28℃ with gentle shaking. Metabolized steroids were extracted after 24 hours of incubation, fractionated, and identified as described above.

Genomic Southern blot analysis

Genomic DNA of medaka was extracted from whole medaka (Hd-rR inbred strain) without viscera and gill (Fukada *et al.*, 1995). Genomic DNA was digested with *Dra*I, *Hind*III, *Pst*I, and *Pvu*II. The digested DNA fragments were fractionated by gel electrophoresis and transferred onto Hybond N^+ membranes. Membranes were then hybridized to a probe generated by PCR using primers 6 and 7 (Table-2), full length medaka *P*-

450c17 cDNA, and [α-^{32}P]-dCTP.

Reverse transcription and polymerase chain reaction to obtain PCR fragments of medaka cytochrome b_5 and NADPH:P-450 reductase

Total RNA was extracted from intact follicles collected at 32 hours before the expected time of spawning as described above. Reverse transcription and PCR were performed as described above with synthetic oligo dT primer for the first strand synthesis and primers 1-4 in Table 3 for PCR. Degenerate primer design was based on the reported cytochrome b_5 cDNA sequences of chicken, bovine, human, and rat or NADPH:*P*-450 reductase cDNA sequence of guinea pig, rabbit, and rat. NADPH:P-450 reductase amino acid sequence of brown trout was also available and this sequence was also used to determine the primer sites. These sequences were obtained from cytochrome P450 family in gene family database (Voltz and Fasman). Amplified cDNA fragments were fractionated by 1.5% agarose gel and recovered by GEANCLEANII (BIO 101), then ligated into the PCRII vector using a TA Cloning Kit (Invitorogen).

Northern blot hybridization of NADPH:P-450 reductase and cytochrome b_5

Total RNAs were collected from medaka ovarian follicle cells at 8, 14, 32, and 38 hours before the expected time of spawning. Twenty μg of total RNA were electrophoresed and immobilized on Hybond N^+ membrane (Amersham). These membranes were hybridized with PCR probes. PCR was carried out with PCR clones of medaka cytochrome b_5 or NADPH:*P*-450 reductase in the PCRII vector as templates, using AmpliTaq DNA Polymerase (Perkin Elmer) and primer sets shown in Table 3 (No. 5-8).

Expression of medaka P-450c17 cDNAs in E. coli and preparation of membrane fractions

The nucleotide sequence of both *P*-450c17S and *P*-450c17L around the first methionine was modified from CGATGG to CCATGG which results in a generation of a restriction sequence of *Nco*I. Six histidine residues, stop codon, and *Xho*I recognition sequence were added after the open reading frame (*P*-450c17S(His) and *P*-450c17L(His)), using a conventional PCR technique. Modified cDNA sequences were confirmed by sequencing. *P*-450c17 cDNAs which contain the *Nco*I and *Xho*I sites were digested with *Nco*I and *Xho*I and then ligated into the *Nco*I and *Xho*I sites of the pET15b expression vector (NOVAGENE) and transformed into E. coli BL21(DE3). The transformed E. coli were inoculated to 5 ml of LB media containing 50 μg/ml ampicillin. Overnight cultures of *E. coli* at 37℃ with a gentle shaking at 280 rpm were seeded into 500 ml of LB media. IPTG (2 mM) and 5-aminolevulinic acid (1 mM) were added to induce the expression of *P*-450c17 when A600 reached between 0.3 to 0.6. Membrane fractions were prepared according to Katagiri *et al.* (1995) with some modification as follows. After 3 hours of incubation at 37℃ with a gentle shaking at 280 rpm, bacteria were harvested and resuspended into 15 ml of TES buffer (0.1 M Tris-acetate, pH 7.8, 0.5 mM EDTA, 0.5 M sucrose). Lysozyme (200 mg) was added followed by 30 minutes of incubation on ice with continuous mixing. Sphroplasts were harvested and resuspended in 20 ml of ice-cold 0.1 M sodium phosphate, pH 7.2, containing 20% glycerol, 50 μM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (Wako), 1.0 μM pepstatin A (Peptide institute), and 5 μg/ml leupeptin (Peptide institute). After freezing and thawing, samples were homogenized by physcotrone followed by sonication. Unbroken cells and debris were pelleted at 3,000×

g for 10 minutes and supernatant were centrifuged at 225, 000×g for 30 minutes at 4℃. The resulting pellet was resuspended with 2 ml of 0.1 M sodium phosphate, pH 7.2, containing 20% glycerol using a tefron-glass homogenizer.

Western blot analysis

Fifteen amino acids at the C-terminal region of medaka P-450c17 without the last amino acid (CTPRPGCHSGLFPAN) were conjugated with hemocyanin, keyhole limpet (Calbiochem) and used as an antigen. Antiserum of rabbit (New Zealand white) was obtained and used for Western blotting.

E. coli itself or its membrane fractions were suspended in water and mixed with 2 × SDS (sodium dodecyl sulfate) buffer (Shmbrook et al., 1989) followed 5 minutes boil. These samples were electrophoresed in 12.5% polyacrylamide gel and transferred to a immobilone (Millipore). After a blocking step with 5% dried milk, the filter was hybridized first with an antiserum against C-terminal of medaka *P*-450c17 products (1:1000 dilution) and second with affinity isolated goat antibody anti-(rabbit IgG g and light chains) human Ig adsorbed alkaline phosphatase (Biosourse). Detection was carried out using nitro blue tetrazolium (Sigma) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma)

Measurement of enzymatic activities of P-450c17S(His) and P-450c17L(His) products in membrane fractions

17α-Hydroxylase and C17,20-lyase activities were analyzed as follows. Membrane fractions (50 μl, collected as described above) and 1 unit of pig liver NADPH:P-450 reductase were preincubated at 37 ℃ for 5 minutes in

the presence or absence of 200 pmol pig liver cytochrome b_5. Cold progesterone was mixed with 5×10^4 cpm of [4-^{14}C]-progesterone in reaction buffer (50 mM sodium phosphate, pH 7.2, 10 mM magnesium acetate, and 20% glycerol) and added up to 450 ml. Final concentration of progesterone was adjusted to 5 μM. After 5 minutes preincubation at 37℃, reactions were started by the addition of 50 ml of 10 mM NADPH. Aliquots (100 μl) were removed at 15, 30, 45, 60, and 120 min. Steroids were extracted with diethylether and evaporated on the warm sand bath under gentle nitrogen gas stream. Steroids were mixed with cold progesterone, 17α-hydroxyprogesterone and androstenedione and then separated by high performance thin layer chromatography (HPTLC, Silica Gel 60 F254, Merk) with chloroform:ethylacetate (4:1)(McCarthy *et al.*, 1988). Mixed cold steroids were detected by UV light and used as mobility markers. Data were analyzed by the BAS-2000 Bio-Imazing Analyzer (Fujifilm). NADPH:P-450 reductase and cytochrome b_5 of pig were kindly gifted from Dr. Harada (Division of Molecular Genetics, Fujita Health University, Aichi, Japan).

RESULTS

Cloning of two types of medaka P-450c17

A genomic fragment of the medaka *P*-450c17 gene was cloned previously (M. Matsuyama, M. Tanaka, D. Kobayashi and Y. Nagahama, unpublished). Northern blot hybridization using a region of this genomic fragment, showed the presence of two sizes of transcripts, 6.4 kb and 2.4 kb, in medaka ovarian follicle cells (Fig. 9A).

Since the steroidogenic capacity of medaka ovarian follicle cells fluctuates in a well-regulated daily cycle (Fukada *et al.*, 1994, , Saskai *et al.*, 1987, 1988), total RNA from various stages of ovarian follicle cells was used to determine if there is also a daily fluctuation of *P*-450c17 transcript levels. Northern blot analysis clearly demonstrated that the peak amount of both transcripts was observed at 23 and 47 hr before spawning (Fig. 9B). The amount of both transcripts decreases rapidly and becomes almost undetectable at 8 hours before spawning. The timing of the decrease is consistent with the onset of maturation-inducing hormone secretion from ovarian follicle cells, which was previously reported by Fukada *et al.* (1994) and Sakai *et al.* (1987, 1988).

Screening of cDNA library constructed from poly(A)$^+$ RNA collected at 22 hours before the expected time of spawning resulted in isolation of 13 positive clones. Two clones containing a 2.4 kb insert were selected for sequencing. These two cDNAs contained different size open reading frames (Fig. 10). One cDNA, which was expected to have a longer open reading frame (*P*-450c17L, 518 amino acid residues), was similar in sequence to *P*-450c17s of other species. The other cDNA was a novel type of cDNA (*P*-450c17S, 489 amino acids) lacking 87 nucleotides (boxed in Fig.

10) in the coding region. Comparing the cDNA sequences to the genomic sequence (M. Matsuyama, M. Tanaka, D. Kobayashi, Y. Nagahama, unpublished data), *P*-450c17L was found to consist of eight exons (Fig. 11). The introns were inserted at exactly the same positions as those of bovine, pig and human (Bhasker, 1989, Zhang, 1992, Picado-Leonard, 1987). The 87 nucleotides lacking *P*-450c17S corresponded to exon 4 and encoded 29 amino acids. PCR using primers 3 and 4 (Table 2) confirmed that two out of thirteen positive clones lacked exon 4 (Fig. 12). Medaka P-450c17L shows a high degree of homology with rainbow trout (76.8%), chicken (65.1%), and dogfish (61.5%) at the amino acid level (Sakai *et al.*, 1992, Ono *et al.*, 1988, Trant *et al.*, 1995); however, it showed less than 50% homology with those of mammalian species (Tremblay, 1994, Conley, 1992, Chung, 1987, Fevold, 1989, Youngblood, 1991, Fig. 13, Table 4). N-terminal hydrophobic region, conserved P-450c17 sequence, highly conserved regions in other species(Ono *et al.*, 1988), Ozols tridecapeptide region (Ozols, 1981), and heme binding region (Gotoh *et al.*, 1983) were also conserved in medaka P-450c17 (underlined in Fig. 10 a, b , c, and d, and Fig. 13 a, b , c, and d).

Two types of P-450c17 transcripts in medaka ovarian follicles

RNase protection assays and RT-PCR confirmed the presence of two types of transcripts. A cRNA probe was designed to hybridize 134 nucleotides of exon 5 region and 29 nucleotide of exon 4 region. This cRNA also contained 11 nucleotides of vector sequence. Therefore, fragments of 163 and 134 nucleotides were expected to be protected from RNase digestion for *P*-450c17L and *P*-450c17S transcripts, respectively. Protected fragments of appropriate sizes for *P*-450c17L and *P*-450c17S were detected in follicle cell RNA at 14, 21, 32, and 38 hours before spawning. No bands were detected

at 8 and 11 hours (Fig. 14). This indicates that *P*-450c17L and *P*-450c17S transcripts are present during vitellogenesis but absent during oocyte maturation.

RNase protection assays were also carried out using total RNA from several tissues (brain, kidney, liver and spleen in females; brain, kidney, liver, spleen and testis in males). There were no protected fragments except for testis and ovarian follicle cells (Fig. 15). RT-PCR with primers 3 and 4 (Table 2) also confirmed the presence of transcripts lacking exon 4. Total RNA was extracted from several different tissues. The amplified fragments were hybridized with oligonucleotide probe (NO. 5 in Table 2) whose sequence was found both in *P*-450c17S and *P*-450c17L (Fig. 10).

Medaka P-450c17 was a single copy gene

Exon 4 of medaka *P*-450c17 cDNA contained *Pst*I and *Pvu*II restriction enzyme sites. Genomic Southern blot analysis using *Pst*I or *Pvu*II digested genome cDNA generated only one positive band (Fig. 16, lanes 3 and 4). There was no difference between male and female genome. This clearly demonstrates that medaka *P*-450c17 is a single copy gene

17α-hydroxylase and C17,21-lyase activities of medaka P-450c17L product and no activities of P-450c17S product

Since it was reported that *E. coli* can express functional mammalian *P*-450c17s (Barnes *et al.*, 1991, Waterman, 1993, Imai *et al.*, 1993) and *E. coli* itself supports P-450c17 enzymatic activities (Jenkins et al., 1994), two types of medaka *P*-450c17 were expressed in *E. coli* to determine their enzymatic activities. After IPTG induction, *E. coli* was incubated in the presence of [4-^{14}C]-progesterone, and metabolized steroids were extracted

and identified by two dimensional TLC. The expression of *P*-450c17s was confirmed by Western blot analysis (Fig. 18). Two metabolized steroids extracted from *P*-450c17L-transfected *E. coli* comigrated with cold steroid markers 17α-hydroxyprogesterone and androstenedione (Fig. 19C). These steroids were not observed in control expression vectors without *P*-450c17 cDNA inserts (Fig. 19A). This indicates that *E. coli* which is transformed with the *P*-450c17L expression vector exhibits 17α-hydroxylase and C17,20-lyase activities. *E. coli* harboring *P*-450c17S cDNA showed neither 17α-hydroxyprogesterone nor androstenedione in its metabolites (Fig. 19B). Using 17α-hydroxyprogesterone as a substrate for C17,20-lyase activity, *E. coli* with *P*-450c17L cDNA produced androstenedione (Fig. 20C); however, *E. coli* with *P*-450c17S cDNA showed no C17,20-lyase activities (Fig. 20B).

When mammalian non-steroidogenic COS1 cells were transfected with expression vectors containing *P*-450c17L or *P*-450c17S cDNA, [4-^{14}C]-progesterone was converted to 17α-hydroxyprogesterone and androstenedione (Fig. 21). COS 1 cells, harboring *P*-450c17L also metabolized 17α–hydroxyprogesterone (Fig. 22) and pregnenolone (Fig. 23). In contrast, COS 1 cells which were transfected with expression vector containing *P*-450c17S showed neither 17α-hydroxylase and C17,20-lyase activity. These observations suggested that the product of *P*-450c17L cDNA has both 17α-hydroxylase and C17,20-lyase activities, but neither activity was found in the product of *P*-450c17S.

RT-PCR cloning of medaka cytochrome b_5 and NADPH:P-450 reductase

RT-PCR fragments of cytochrome b_5 and NADPH:*P*-450 reductase were obtained. Comparing the homology of these fragments with the cytochrome b5 and NADPH:P-450 reductase of pig, medaka cytochrome b_5

showed 59.0% homology to pig (Fig. 24) and NADPH:P-450 reductase showed 78.6% homology to pig (Fig. 25) at the amino acid level.

Expression level of cytochrome b_5 and NADPH:P-450 reductase during vitellogenic and maturation stages

Cytochrome b_5 and NADPH:*P*-450 reductase transcripts were detected in medaka ovarian follicle cells. The levels of transcripts were normalized with the levels at 8 hours before the expected time before spawning (Fig. 26). NADPH:*P*-450 reductase transcripts at 32 hours were 3.0 times higher than the levels at 8 hours. Cytochrome b_5 transcripts at 32 hours before the expected time of spawning were 1.6 times higher than the amount at 8 hours.

Effects of cytochrome b_5 on 17α-hydroxylase and C17,20-lyase activities of medaka P-450c17 in membrane fractions

The presence of *P*-450c17 products in membrane fractions was detected by Western blot analysis (Fig. 27). The conversion of progesterone to 17α-hydroxyprogesterone by *P*-450c17L products in membrane fractions was much faster in the presence of cytochrome b_5 (Fig. 28B) than its absence (Fig. 28A). Furthermore, androstenedione, though small in amount, was observed only in the presence of cytochrome b_5. Membrane fractions harboring *P*-450c17S products or mock did not convert progesterone (data not shown). In the absence of NADPH:P-450 reductase, membrane fractions containing *P*-450c17L products showed no conversion of progesterone (data not shown).

DISCUSSION

This study demonstrates, for the first time, the presence of two types of *P*-450c17 cDNA (*P*-450c17L and *P*-450c17S) which were obtained as 2.4 kb cDNA clones. The RNase protection assay and RT-PCR revealed that there were two types of transcripts in medaka ovarian follicle cells. By comparing the sequences of these two cDNAs with that of genomic DNA, it was shown that nucleotides which were missing in *P*-450c17S was exon 4. Exon 4 of medaka *P*-450c17L consists of 87 nucleotides and encodes 29 amino acids. Exon 4 of mammalian *P*-450c17 also consists of 87 nucleotides and encodes 29 amino acids (Bhasker *et al.*, 1989; Zhang *et al.*, 1992; Picado-Leonard *et al.*, 1987). Consequently, the structure of exon 4 is extremely conserved, which suggests that *P*-450c17S also occurs in mammalian species.

P-450c17L products which were expressed in both mammalian COS 1 cells and *E. coli* showed both 17α-hydroxylase and C17,20-lyase activities, but P-450c17S product showed neither activity. Amino acid positions 236L and 246R, both of which are present in exon 4, are highly conserved and thought to be involved in the substrate binding (Dong *et al.*, 1994). The possibility therefore exists that the lack of enzyme activity of *P*-450c17S products could be due to the change in the steroid binding site. The significance of the absence of exon 4 in terms of the steroid binding capacity and enzyme activity requires further investigation. It is also possible that the lack of the enzyme activity of P-450c17S is due to the absence of other factors in COS 1 cells and *E. coli* which are necessary for enzyme activity of the P-450c17S.

Genomic Southern blot analysis clearly showed that *P*-450c17 was a

single copy gene. The results of RNase protection assay, RT-PCR, and genomic Southern blot analysis revealed that these two transcripts were synthesized by alternative splicing. The exon/intron boundary sites were identical as those reported in other species (M. Matsuyama, M. Tanaka, D. Kobayashi, and Y. Nagahama, unpublished). Comparing the amino acid sequence to those of other species, *P*-450c17L showed a high degree of homology with chicken, rainbow trout, and dogfish, but low homology with mammals. This suggests that *P*-450c17 can be phylogenetically divided into two groups, mammalian and non-mammalian, based upon the degree of homology. Chicken *P*-450c17 is grouped with fish *P*-450c17 (Fig. 27). This situation appears to differ from that of cytochrome *P*-450 aromatase (*P*-450arom, *CYP*19), a member of the steroidogenic cytochrome P-450 family. Chicken *P*-450arom (McPhaul *et al.*, 1988) shows more similarity with mammalian *P*-450arom (Harada, 1988; Hickey *et al.*, 1990; Hinshelwood *et al.*, 1993; Terashima *et al.*, 1991) than with medaka (Tanaka *et al.*, 1995), rainbow trout (Tanaka *et al.*, 1992), and catfish (Trant, 1994)(Fig. 28). Thus, chicken *P*-450arom is grouped with the mammalian *P*-450arom. This difference in the phylogenetic relationship between *CYP*17 and *CYP*19 is of considerable interest in view of the molecular evolution of the *P*-450 multigene superfamily. To discuss this issue in more detail, it is necessary to gain more information regarding the sequences of these two genes from other vertebrate species such as reptiles and amphibians. It is also of important to obtain cDNA sequences of *CYP*21 (*P*-450c21) and *CYP*1A1 (*P*-4501A1), which belong to the same subfamily of *P*-450c17 (Degtyarenko *et al.*, 1993), of various species of vertebrates.

Northern blot analysis revealed two different sizes of transcripts, 2.4 kb and 6.4 kb. Although the significance of two different sizes of transcripts

remain unknown, the levels of transcripts exhibited a similar pattern of changes during vitellogenesis and maturation. The levels of these two transcripts begin increasing 29 hour before spawning and reach peaks around 23 hour. The decrease to an almost undetectable level at 8 hour before spawning (Fig. 9B). Another small peak was observed at 47 hour. Since medaka is a daily spawner, the amounts of *P*-450c17 transcripts present in ovarian follicle cells correlate to a daily cycle of ovulation. The transcripts are present during vitellogenesis and begin to decrease prior to the production of maturation-inducing hormone (17 hours before spawning). These findings indicate that *P*-450c17 is involved in both the decrease in estradiol-17β production and the increase in 17α,20β-DP production and further emphasize the importance for the shift in steroidogenesis in follicles immediately prior to oocyte maturation.

During vitellogenesis, 17α-hydroxylase and C17,20-lyase activities were found to be high in medaka ovarian follicle cells (see Chapter I). Presence of both activities results in the production of androstenedione from endogenous precursor pregnenolone via 17α-hydroxypregnenolone and 17α-hydroxyprogesterone. Androstenedione is further converted to estradiol-17β (indispensable steroid hormone for vitellogenesis) through testosterone. Prior to maturation, C17,20-lyase activity decreases, bringing about the disappearance of androstenedione. 17α-Hydroxyprogesterone is then used as a substrate for the production of maturation-inducing hormone, 17α,20β-dihydroxy-4-pregnen-3-one. These observations indicate that 17α-hydroxylase and C17,20-lyase activities of P-450c17 are differentially regulated during oogenesis in medaka. Elucidation of the molecular mechanisms regulating the decrease in only C17,20-lyase is critical to understand the shift in the steroidogenic pathway in medaka ovarian follicle

cells from vitellogenesis to oocyte maturation.

It has been reported that P-450c17 activities are supported by NADPH:P-450 reductase, and cytochrome b_5 affects two activities of P-450c17. In human, C17,20-lyase activity is low in the absence of cytochrome b_5 and is stimulated by the addition of cytochrome b_5 *in vitro* (Katagiri *et al.*, 1995; Lee-Robichaud *et al.*, 1995) in the reconstitute system with NADPH:P-450 reductase. This effect of cytochrome b_5 is also observed in pig; pig C17,20-lyase activity is more affected than 17α-hydroxylase activity by the addition of cytochrome b_5 (Lee-Robichaud *et al.*, 1995). These findings suggest that C17,20-lyase activity is more affected by the presence of cytochrome b_5 than 17α-hydroxylase activity and that cytochrome b_5 plays a role in the differential regulation of 17α-hydroxylase and C17,20-lyase activities. In the present study, both NADPH:*P*-450 reductase and cytochrome b_5 transcripts were detected in medaka ovarian follicles. Expression of these mRNA levels was higher in follicles during vitellogenesis than those during oocyte maturation. NADPH:P-450 reductase purified from pig supported 17α-hydroxylase and C17,20-lyase activities of medaka *P*-450c17L product expressed in *E. coli*. Moreover, pig cytochrome b_5 was found to influence the metabolism of progesterone by medaka P-450c17. These results suggest that cytochrome b_5 plays a role in the differential regulation of P-450c17 activities in medaka ovarian follicles.

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REFERENCES

Abe, K., Kimura, S., Kizawa, R., Anan, F. K., and Sugita, Y. (1985). Amino acid sequences of cytochrome b5 from human, porcine, and bovine erythrocytes and comparison with liver microsomal cytochrome b_5. *J. Biochem.*, **97**, 1659-68.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. Current Protocols in Molecular Biology, Mssachusetts General Hospital, Harvard Medical School.

Axelrod, L. R., Matthijssen C., Goldzieher, J. W., and Pulliam, J. E. (1965). Definitive identification of microquantities of radioactive steroids by recrystallization to constant specific activity. *Acta Endocrinol.* (Copenh), **99**, suppl. 7-77.

Bakke, M., and Lund, J. (1992). A novel 3',5'-cyclic adenosine monophosphate-responsive sequence in bovine *CYP17* gene is a target of negative regulation by protein kinase C. *Mol. Endocrinol.*, **6**, 1323-1331.

Barnes, H. J., Arlotto, M. P., and Waterman, M. R. (1991) Expression and enzymatic activity of recombinant cytochrome P450 17α-hydroxylase in *Escherichia coli. Proc. Natl. Acad. Sci. USA* 88, 5597-5601.

Bhasker, C. R., Adler, B. S., Dee, A., John, M. E., Kagimoto, M., Zuber M. X., Ahlgren, R., Wang, X., D., Simpson, E. R., and Waterman, M., R. (1989) Structural characterization of the bovine CYP17 (17α-hydroxylase) gene.*Arch. Biochem. Biophys.* **271**, 479-487.

Brentano, S. T., P-Leonard, J., Mellon, S. H., Moore, C. C. D., and Miller, W. L. (1990). Tissue-specific, cyclic adenosine 3',5'-monophosphate-induced, and phorbol ester-responded transcription from the human P450c17 promoter in

mouse cells. *Mol. Endcrinol.*, **4**, 1972-1979.

Conley, A. J., Graham-Lorence, S. E., Kagimoto, M., Lorence, M.,C., Murry, B. A., Oka, K., Sanders D., and Mason J. I. (1992) Nucleotide sequence of a cDNA encoding porcine testis 17α-hydroxylase cytochrome P-450. *Biochim. Biophys. Acta*, **1130**, 75-77.

Chung, B-C., P-leonard, J., Haniu, M., Bienkowski, M., Hall, P. F., Shively, J. E., Miller, W. L. (1987) Cytochrome P450c17 (steroid 17α-hydroxylase/17,20 lyase): cloning of human adrenal and testis cDNAs indicates the same gene is expressed in both tissues. *Proc. Natl. Acad. Sci. USA* **84**, 407-411.

Corbin, C. J., Graham-Lorence, S., McPhaul, M., Mason, J. I., Mendelson, C. R., and Simpson, E. R. (1988) Isolation of a full-length cDNA insert encoding human aromatase system cytochrome P-450 and its expression in nonsteroidogenic cells. Proc. *Natl. Acad. Sci. USA*, **85**, 8948-8952.

Degtyarenko, K. N., Archakov, A. I. (1993) Molecular evolution of P450 superfamily and P450-containing monooxygenase systems. *FEBS Lett.*, **332**, 1-8.

Dong, L., Lin-hua, Z., Eric, C., and Walter, L. M. (1994) Modeling and mutagenesis of the avtive site of human P450c17. *Mol. Endocrinol.*, 8, 392-402.

Fevold, H. R., Lorence, M. C., McCarthy, J. L., Trant, J. M., Kagimoto, M., Waterman, M. R., and Mason, J. I. (1989) Rat P450(17α) from testis: characterization of a full-length cDNA encoding a unique steroid hydroxylase capable of catalyzing both Δ4- and Δ5-steroid-17,20-lyase reactions. *Mol. Endcrinol.* **3**, 968-975.3

Frohman, M. A. (1994) in *The Polymerase Chain Reaction* (Mullis K. B., Ferré, F., Gibbs, R. A., eds) Brikhäuser, Boston.

Fukada, S., Tanaka, M., Iwaya, M., Nakajima, M., and Nagahama, Y. (1995) The Sox gene family and its expression during embryogenesis in teleost fish, medaka (Oryzias latipes). *Develop. Growth Differ.* **37**, 379-385.

Fukada, S., Tanaka, M., Matsuyana, M., Kobayashi, D., and Nagahama, Y. (1995) *Mol. Repod. Dev.*, submit.

Fukada, S., Sakai, N., Adachi, S., and Nagahama, Y. (1994) Steroidogenesis in the ovarian follicle of medaka (*Oryzias latipes*, a daily spawner) during oocyte maturation. *Develop. Growth & Differ.*, **36**, 81-88.

Gotoh, O., Tagashira, Y., Iizuka, T., and Fujii-Kuriyama, Y. (1983) Structural characteristics of cytochrome P-450. Possible location of the heme-binding cysteine in determined amino-acid sequences. *J. Biochem.* **93**, 807-817.

Haniu, M., Iyanagi, T., Miller, P., Lee, T. D., and. Shively, J. E. (1986) Complete amino acid sequence of NADPH-cytochrome P-450 reductase from porcine hepatic microsomes. *Biochemistry*, **25**, 7906-11.

Harada, N. (1988) Cloning of a complete cDNA encoding human aromatase: immunochemical identification and sequence analysis. *Biochem. Biophys. Res. Commun.* **156**, 725-732.

Hickey G. J., Krasnow, J. S., Beattie, W. G., and Richards, J. S. (1990) Aromatase cytochrome P450 in rat ovarian granulosa cells before and after luteinization: adenosine 3',5'-monophosphate-dependent and independent regulation. Cloning and sequencing of rat aromatase cDNA and 5' genomic DNA. *Mol. Endocrinol.* **14**, 3-12.

Hinshelwood, M. M., Corbin C. J., Tsang, P. C., and Simpson, E. R. (1993) Isolation and characterization of a complementary deoxyribonucleic acid insert encoding bovine aromatase cytochrome P450. *Endocrinol.* **133**, 1971-1977.

Ho, S. (1991). Vitellogenesis. *in* "Vertebrate Endocrinology: Fundamentals and Biomedical Implications" (Peter K. T. Pang and Martin P. Schreibman, eds.), Vol. 4, part A, Reproduction, pp. 91-114. Academic Press, San Diego.

Iwamatsu, T. (1978). Studies on oocyte maturation of the medaka (*Oryzias latipes*) V. On the structure of steroid that induce maturation *in vitro*. *J. Exp. Zool.*, **206**, 355-364.

Fukada, S., Sakai, N., Adachi, S., and Nagahama, Y. (1994). Steroidogenesis in the Ovarian follicle of medaka (*Oryzias latipes*, a daily spawner) during oocyte maturation. *Develop. Growth Differ.*, **36**, 81-88.

Imai, T., Globerman, H., Gertner, J. M., Kagawa, N., and Waterman, M. R. (1993) Expression and purification of functional human 17α-hydroxylase/17,20-lyase (P-450c17) in *Escherichia coli*. *J. Biol. Chem.* **268**, 19681-19689.

Inano, R., Nakano, H., Shikita, M. and Tamaoki, B. (1967). The influence of various factors upon testicular enzymes related to steroidogenesis. *Biochim. Biophys. Acta* **137**, 540-548.

Iwamatsu, T. (1978) Studies on oocyte maturation of medaka, *Oryzias latipes* VI. relationship between the circadian cycle of oocyte maturation and activity of the pituitary gland. *J. Exp. Zool.*, **206**, 355-364.

Jalabert, B., Fostier, A., Breton, B., and Weil, C. (1991) in *Vertebrate Endocrinology : Fundamentals and Biomedical Implications* (Pang, P. K. T., and Schreibman, M. P., eds) Vol. 4 part A pp. 23-61, Academic Press, New York.

Jenkins, C. M., Waterman, M. R. (1994) Flavodoxin and NADPH-flavodoxin reductase from *Escherichia coli* support bovine cytochrome P450c17 hydroxylase activities. *J. Biol. Chem.*, **269**. 27401-27408.

Katagiri, M., Kagawa, N., and Waterman, M. R. (1995) The role of

cytochrome b_5 in the biosybthesis of androgens by human P4510c17. *Arch. Biochem. Biophys.* **317**, 343-347human cyt b_5

Kobayashi, D., Tanaka, M., Fukada, S., and Nagahama, Y. (1996) Steroidogenesis in the ovarian follicles of medaka (Oryzias latipes) during vitellogenesis and maturation. (1996) *Zool. Sci.*, submit.

Lee-Robichaud P., Wright, J. N., Akhtar M. E., and Akhtar, M. (1995). Modulation of the activity of human 17α-hydroxylase-17,20-lyase (CYP17) by cytochrome b_5: endocrinological and mechanistic implications. *Biochem. J.*, **308**, 901-908.

McCarthy, J., L., and Waterman, M., R. (1988) Co-induction of 17α-hydroxylase and C-17,20-lyase activities in primary cultures of bovine adrenocortical cells in response to ACTH treatment. *J. Steroid Biochem.*, **29**, 307-312.

McPhaul, M. J., Noble, J. F., Simpson, E. R., Mendelson, C. R., and Wilson, J. D. (1988) The expression of a functional cDNA encoding the chicken cytochrome P-450arom (aromatase) that catalyzes the formation of estrogen from androgen. *J. Biol. Chem.* **263**, 16358-16363.

Nagahama, Y. (1987). Gonadotropin action on gametogenesis and steroidogenesis in teleost gonads. *Zool. Sci.*, **4**, 209-222.

Nagahama, Y., Matsushita, A., Iwamatsu, T., Sakai, N., and Fukada, S. (1991). A mechanism of the action of pregnant mare serum gonadotropin on aromatase activity in the ovarian follicle of the medaka, *Oryzias latipes*. *J. Exp. Zool.*, **259**, 53-58.

Nagahama, Y., Yoshikuni, M., Yamashita, and Tanaka, M. (1994). Regulation of oocyte maturation in fish. *In* "Fish Endocrinology" (Sherwood, N. M., Hew, C. L., eds.), Vol. XIII, Academic press, San Diego.

Nakajin, S., and Hall, P. F. (1981). Microsomal cytochrome P-450 from

neonatal pig testis. *J. Biol. Chem.*, **256**, 3871-3876.

Nakajin, S., Shively J. E., Yuan, P-M., and Hall, P. F. (1981). Microsomal cytochrome P-450 from neonatal pig testis: two enzymatic activities (17α-hydroxylase and C17,20-lyase) associated with one protein. *Biochemistry*, **20**, 4037-4042.

Nakajin, S., Shinoda, M., Haniu, M., Shively, J. E., and Hall, P. F. (1984). C21 steroid side chain cleavage enzyme from porcine adrenal. *J. Biol. Chem.*, **259**, 3971-3976.

Namiki, M., Kitamura, M., Buczki E., and Dufau M. L. (1988). Rat testis P-45017α cDNA: the deduced amino acid sequence, expression and secondary structural configration. *Biochem. Biophys. Res. Commun.*, **157**, 705-712.

Ono, H., Iwasaki, M., Sakamoto, N., and Zmizuno, S. (1988) cDNA cloning and sequence analysis of a chicken gene expressed during the gonadal development and homologous to mammalian cytochrome P-450c17. *Gene* **66**, 77-85.

Ozols, J., and Heinemann, F. S. (1981) *J. Biol. Chem.* **256,** 11405-11408

Abe, K., Kimura, S., Kizawa, R., Anan, F. K., and Sugita, Y. (1985). Amino acid sequences of cytochrome b_5 from human, porcine, and bovine erythrocytes and comparison with liver microsomal cytochrome b_5.

J Biochem, **97**, 1659-68

Picado-Leonard, J., and Miller, W. L. (1987) Cloning and sequence of the human gene for P450c17 (steroid 17α-hydroxylase/17,20 lyase): similarity with the gene for P-450c21. *DNA*, **6**, 439-448

Sakai, N., Iwamatsu, T., Yamauchi, K., and Nagahama, Y. (1987). Development of the steroidogenic capacity of medaka (*Oryzias latipes*) ovarian follicles during vitellogenesis and oocyte maturation. *Gen. Comp.*

Endcrinol., **66**, 333-342.

Sakai, N., Iwamatsu, T., Yamauchi, K.,Suzuki, N., and Nagahama Y. (1988). Influence of follicullar development on steroid production in the medaka (*Oryzias latipes*) ovarian follicle in response to exogenous substrates. *Gen. Comp. Endcrinol.*, **71**, 516-523.

Sakai, N., Tanaka, M., Miller, M. L., and Nagahama, Y. (1992). Rainbow trout cytochrome *P*-450c17 (17α-hydroxylase/17,20-lyase) cDNA cloning, enzymatic properties and temporal pattern of ovarian *P*-450c17 mRNA expression during oogenesis. *FEBS Lett.* , **301**, 60-64.

Shmbrook, J., Fritsch, E. F., and Maniatis, T. (1989) Detectin and analysis of proteins expressed from cloned genes. in "Molecular Cloning, 2 nd. ed.", Vol. 3, 18, Cold Spring Harbor Labotratory Press, New York.

Suzuki, K., Tan, E. S. P., and Tamaoki, B. (1989). Change of steroidogenic pathway in the ovary of a tropical catfish, *Clarias macrocephalus*, gunther, after hCG treatment. *Gen. Comp. Endocrinol.*, **76**, 223-229.

Tanaka, M., Fukada, S., Matsuyama, M., and Nagahama, Y. (1995) structure and promorter analysis of the cytochrome *P*-450 aromatase gene of the teleost fish, medaka (Oryzias latipes). *J. Biochem.* **117**,719-725.

Tanaka, M., Teleckey, T. M., Fukada, S., Adachi, S., Chen, S., and Nagahama, Y (1992) Cloning and sequence analysis of the cDNA encoding *P*-450 aromatase (*P*450arom) from a rainbow trout (*Oncorhynchus mykiss*) ovary; relationship between the amount of *P*450arom mRNA and the production of oestradiol-17β in the ovary. *J. Mol. Endocrinol.* **8**, 53-61

Terashima, M., Toda, K., Kawamoto, T., Kurabayashi, I., Ogawa, Y., Maeda, T., and Shizeta Y. (1991) Isolation of a full-length cDNA encoding mouse aromatase P450. *Arch. Biochem. Biophys.* **285**, 231-237.

Thompson, J. D., Higgins, D. G., and Gibon, T. J. (1994) CLUSTAL W:

improveing the sensitivity of progressive multiple sequence alignment. *Nuleic Acids Res.* 22, 4673-4680

Trant, J. M. (1994) Isolation and characterization of the cDNA encoding the channel catfish (*Ictalurus punctatus*) form of cytochrome *P*450arom. *Gen. Comp. Endocrinol.* **95**, 155-168.

Trant, J. M. (1995) Isolation and characterization of the cDNA encoding the spiny dogfish shark (Squalus acanthias) form of cytochrome P450c17. *J. Exp. Zool.* **272**, 25-33.

Tremblay, Y., Fleury, A., Beaudoin, C., Vallee, M., and Belanger, A. (1994). Molecular cloning and expression of guinea pig cytochrome P450c17 cDNA (steroid 17α-hydroxylase/17,20 lyase): tissue distribution, regulation, and substrate specificity of expressed enzyme. *DNA Cell Biol.*,**13**, 1199-1212

Waterman, M., R. (1993) Heterologous expression of cytochrome *P*-450 in *Escherichia coli. Biochem. Society Trans.* **21**, 1081-1085.

Yanase, T., Kagimoto, M., Suzuki, S., Hashiba, K., Simpson, E. R., and Waterman, M. R. (1989). Deletion of a phenylalanine in the N-terminal egion of human cytochrome P-450(17α) results in partial combined 17α-hydroxylase/17,20-lyase deficiency. *J. Biol. Chem.,* **264**, 18076-18082.

Youngblood, G.,L., Sartorius, C., Taylor, B.,A., and Payne, A. H. (1991) Isolation, characterization, and chromosomal mapping of mouseP450 17-a -hydroxylase/C17-20 lyase. *Genomics* ,10, 270-275.

Zhang, P., Nason, T. F., and Hall, P. F. (1992) Gene for 17α-hydroxylase/C(17-20) lyase P-450: complete nucleotide sequence of the porcine gene and 5' upstream sequence of the rat gene. *Biochem. Biophys. Acta* **1131**, 345-348.

Zuber, M. X., Simpson, E. R., and Waterman, M. R. (1986). Expression of bovine 17α-hydroxylase cytochrome P-450 cDNA in nonsteroidogenic (COS

1) cells. *Science*, **234**, 1258-1261.

TABLES AND FIGURES

Table-1 Specific Activities of Recrystallization

Steroid	Hours before spawning	Specifc activities of crystals (cpm/mg) 1st.	2nd.	3rd.	before crystallization
17α-hydroxyprogesterone	10	170	167	169	195
17α-hydroxyprogesterone	18	26.7	25.6	26.4	28.6
Androstenedione	18	15.4	14.8	14.6	17.1

Table 2 Oligonucleotides for primers and probe

NO.	sequence	position	direction
1	GTCACCAACGTCATCTGCTC	544-563	sense
2	CTGTAACCAGGGGAAGATGT	687-668	antisens
3	CGTCATCTGCTCGCTCTGTTTC	552-573	sense
4	CTGAACTCTGCGGCGGTGTTG	569-549	antisens
5	GTTTGAGGCCATGCTGCGCTAC	603-624	sense
6	GCAACAGAAGTACGGACAGACCT	183-204	sense
7	ACCCTCTCCAAACATGCACAGAG	429-407	antisens

Table 3 Oligonucleotide for PCR primers

NO.	sequence	target	direction
1	CT(GA)GA(GA)GAG(GA)T(GCT)CAGAAGCA(TC)AA	cyt b5	sense
2	TGG(CG)TGATCCC(ATG)GC(ATC)AT(AC)(GT)C	cyt b5	antisense
3	GG(GCA)GA(CT)CCCAC(CGT)GA(CT)AA(CT)GC	P-450red	sense
4	AA(GT)GGATT(CT)TT(AG)GCATC(GA)AA(GT)GG	P450red	antisense
5	ACATTAGAGGAGATAAGAGTACAC	cyt b5	sense
6	AGCCGGGATCAGCCAAAAGG	cyt b5	antisense
7	GGACCTCAAAACTCTTCAGGCG	P-450red	sense
8	CCCACGGATAACGCCCAGGAT	P-450red	antisense

Table-4 The homology of P-450c17 among 10 species at amino acid level.

	Trout	Dogfish	Chicken	Mouse	Rat	Guinia pig	Pig	Bovine	Human
Medaka	76.8	61.5	65.1	46.3	47.5	46.9	15.3	48.1	48.1
Trout		62.3	66.3	46.9	47.2	46.4	44.3	46.5	47.8
Dogfish			62.3	46.2	47.2	47.2	46.3	47.2	47.2
Chicken				46.5	48.2	46.4	47.1	48.1	48.2
Mouse					83.2	68.1	59.8	62.4	66.7
Rat						68.5	61.6	64.2	68.3
Guinia pig							61.8	64.0	70.5
Pig								77.6	65.9
Bovine									71.1

Fig. 1. Steroidogenesis pathway in ovarian follicle cells. The precise pathway and its shift mechanism are not known.

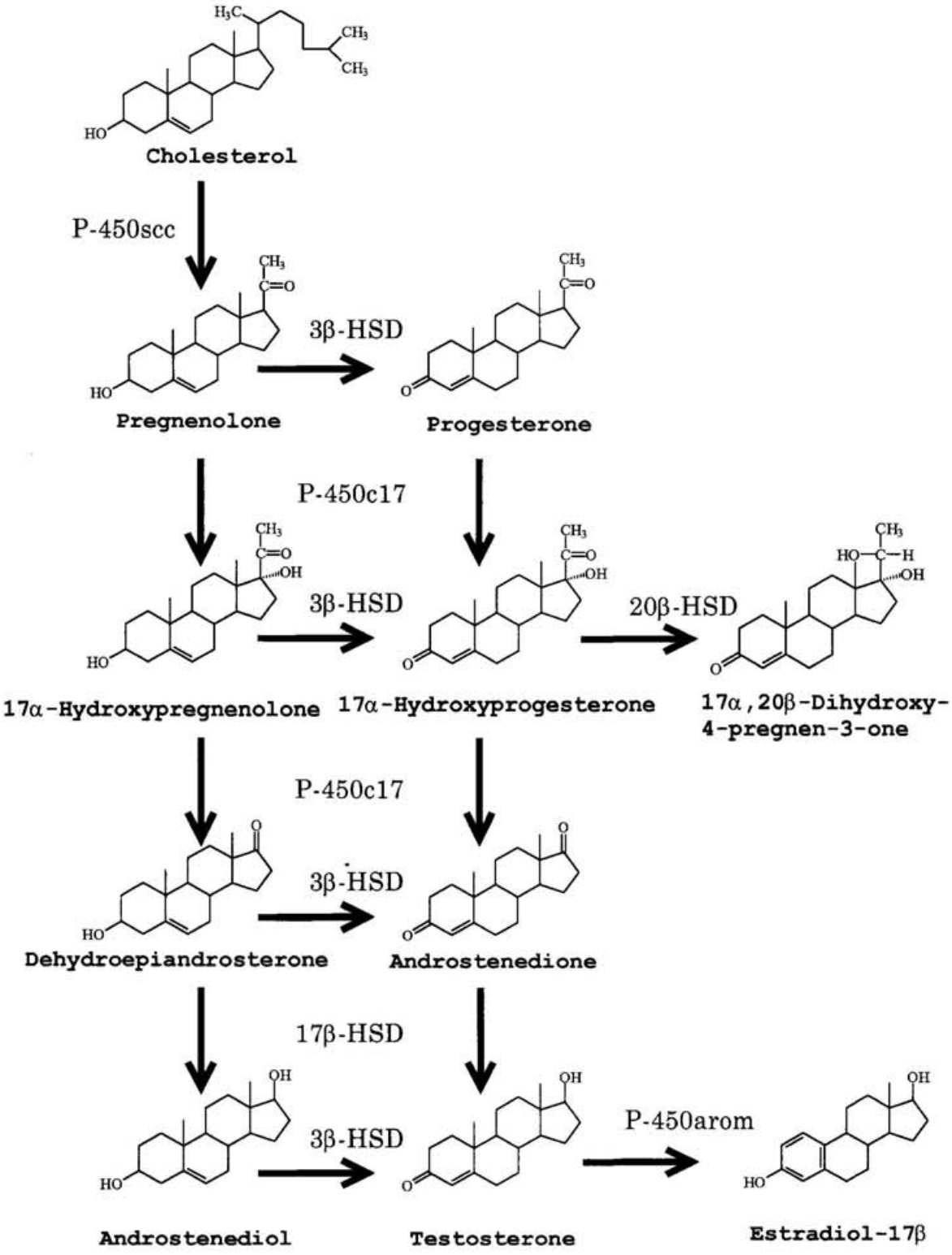
Cholesterol
P-450scc
Pregnenolone
3β-HSD
Progesterone
P-450c17
17α-Hydroxypregnenolone
3β-HSD
17α-Hydroxyprogesterone
20β-HSD
17α,20β-Dihydroxy-4-pregnen-3-one
P-450c17
Dehydroepiandrosterone
3β-HSD
Androstenedione
17β-HSD
Androstenediol
3β-HSD
Testosterone
P-450arom
Estradiol-17β

Fig. 2. Autoradiograms of steroid metabolites produced by medaka ovarian follicles incubated with [7-^{3}H(N)]-pregnenolone (A, B) or [1,2-^{3}H(N)]-17α-hydroxyprogesterone (C, D). Ovarian follicle cells were collected at 18 hours (A, C) or 10 hours (B, D) before spawning. 1, pregnenolone; 2, 17α-hydroxyprogesterone; 3, 17α-hydroxypregnenolone; 4, androstenedione.

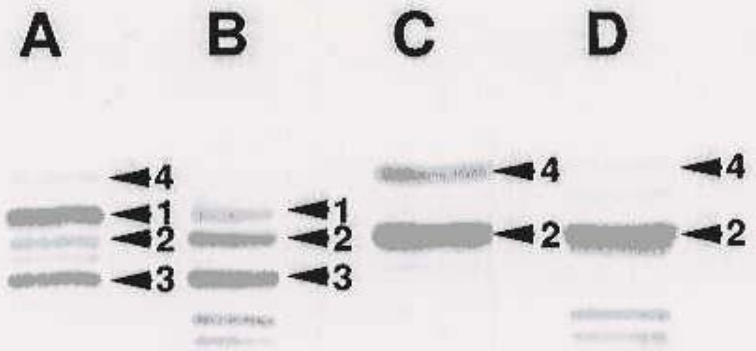
A
B
C
D
4
1
2
3
1
2
3
4
2
4
2

Fig. 3. Autoradiograms of steroid metabolites produced by medaka ovarian intact follicles incubated with [1,2-^{3}H(N)]-17α-hydroxyprogesterone. Ovarian intact follicle cells were collected at 18 hours (A) or 10 hours (B) before spawning. 1, 17α-hydroxyprogesterone; 2, androstenedione.

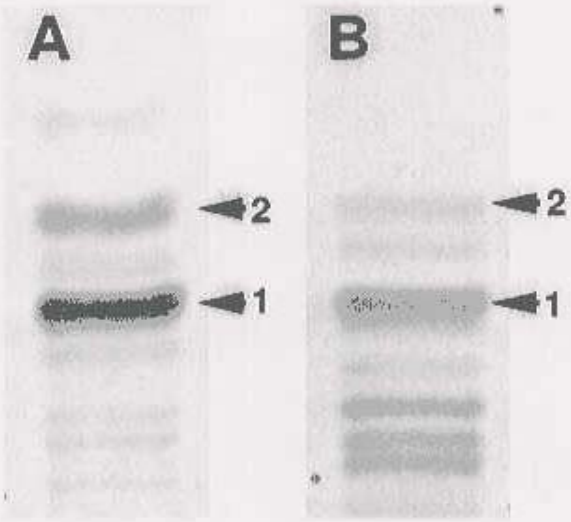
A
2
1
B
2
1

Fig. 4. Autoradiograms of steroid metabolites produced by medaka ovarian follicles incubated with [4-^{14}C] androstenedione. Ovarian follicle cells were collected at 18 hours (A) or 10 hours (B) before spawning. 1, androstenedione; 2, testosterone; 3, estradiol-17β.

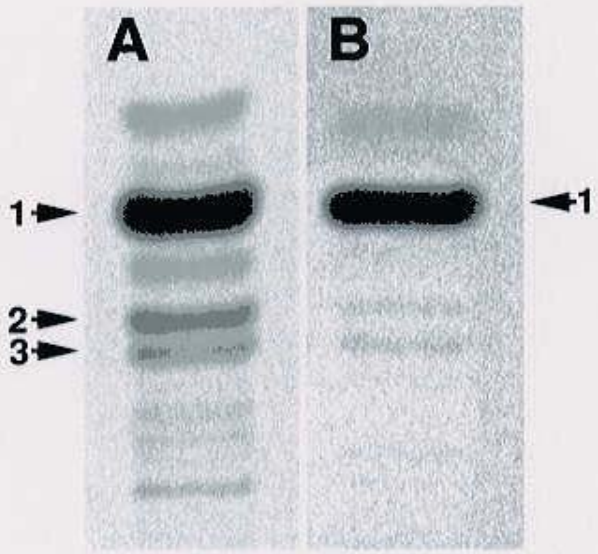
A
B
1
2
3
1

Fig. 5. Autoradiograms of steroid metabolites. The secondary TLC of testosterone and estradiol-17β, scraped from TLC plate in Fig. 2A. 1, testosterone; 2, estradiol-17β.

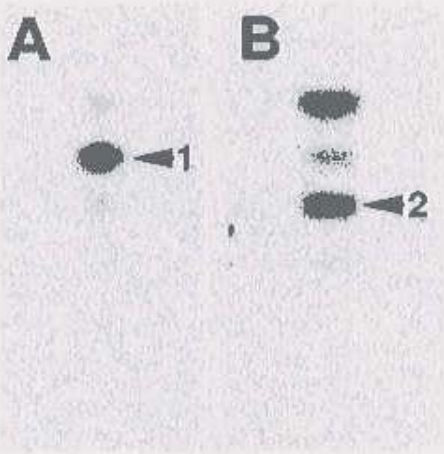
A
B
1
2

Fig. 6. Autoradiograms of steroid metabolites produced by medaka follicle enclosed oocytes incubated with [4-^{14}C]-17α-hydroxyprogesterone in the presence (B, D) or absence (A, C) of PMSG. [4-^{14}C]-17α-hydroxyprogesterone was added at 2 hours (A, B) or 12 hours (C, D) after the incubation started. Six hours after the addition of steroid, metabolites were extracted from media and applied to TLC fractionation. 1, 17α-hydroxyprogesterone; 2, androstenedione; 3, 17α,20β-DP.

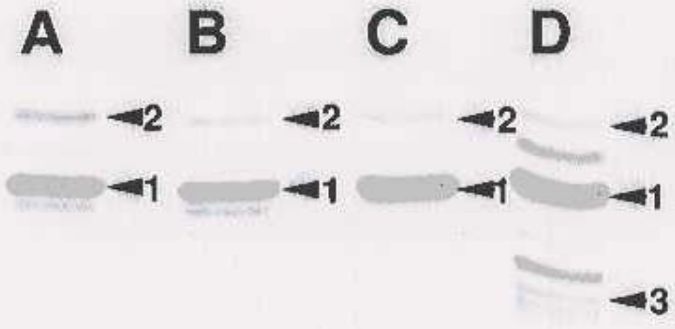
A
B
C
D
2
2
2
2
1
1
1
1
3

Fig. 7. Effects of IBMX, TPA, and A23187 on progesterone metabolites by vitellogenic follicles collected at 18 hours before spawning. Data are shown as a percentage of recovered radioactivity. 1, control; 2, IBMX (1 mM); 3, IBMX (1 mM) + A23187 (5 μM); 4, IBMX (1 mM) + TPA (0.5 μM); 5, IBMX (1 mM) + A23187 (5 μM) + TPA (0.1 μM); 6, IBMX (1 mM) + A23187 (5 μM) + TPA (0.5 μM).

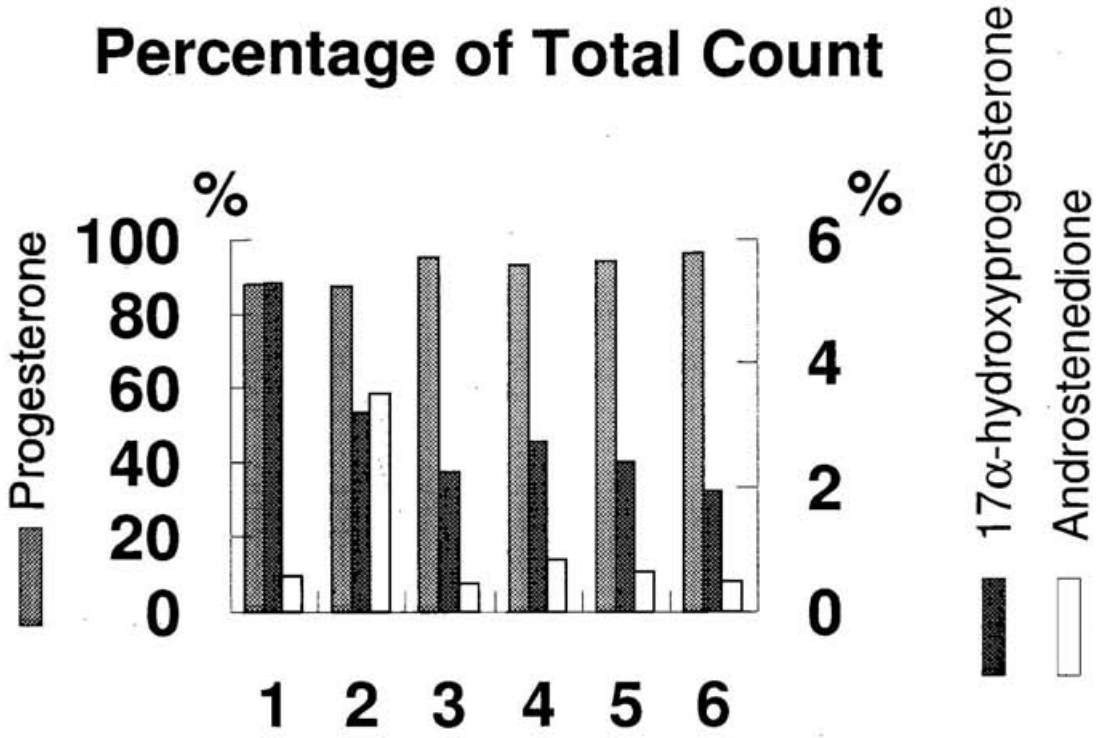
Percentage of Total Count
Progesterone
%
100
80
60
40
20
0
%
6
4
2
0
1 2 3 4 5 6
17α-hydroxyprogesterone
Androstenedione

Fig. 8. Steroidogenesis pathways of estradiol-17β and 17α,20β-dihydroxy-4-pregnen-3-one biosynthesis by medaka ovarian follicles during vitellogenesis and oocyte maturation.

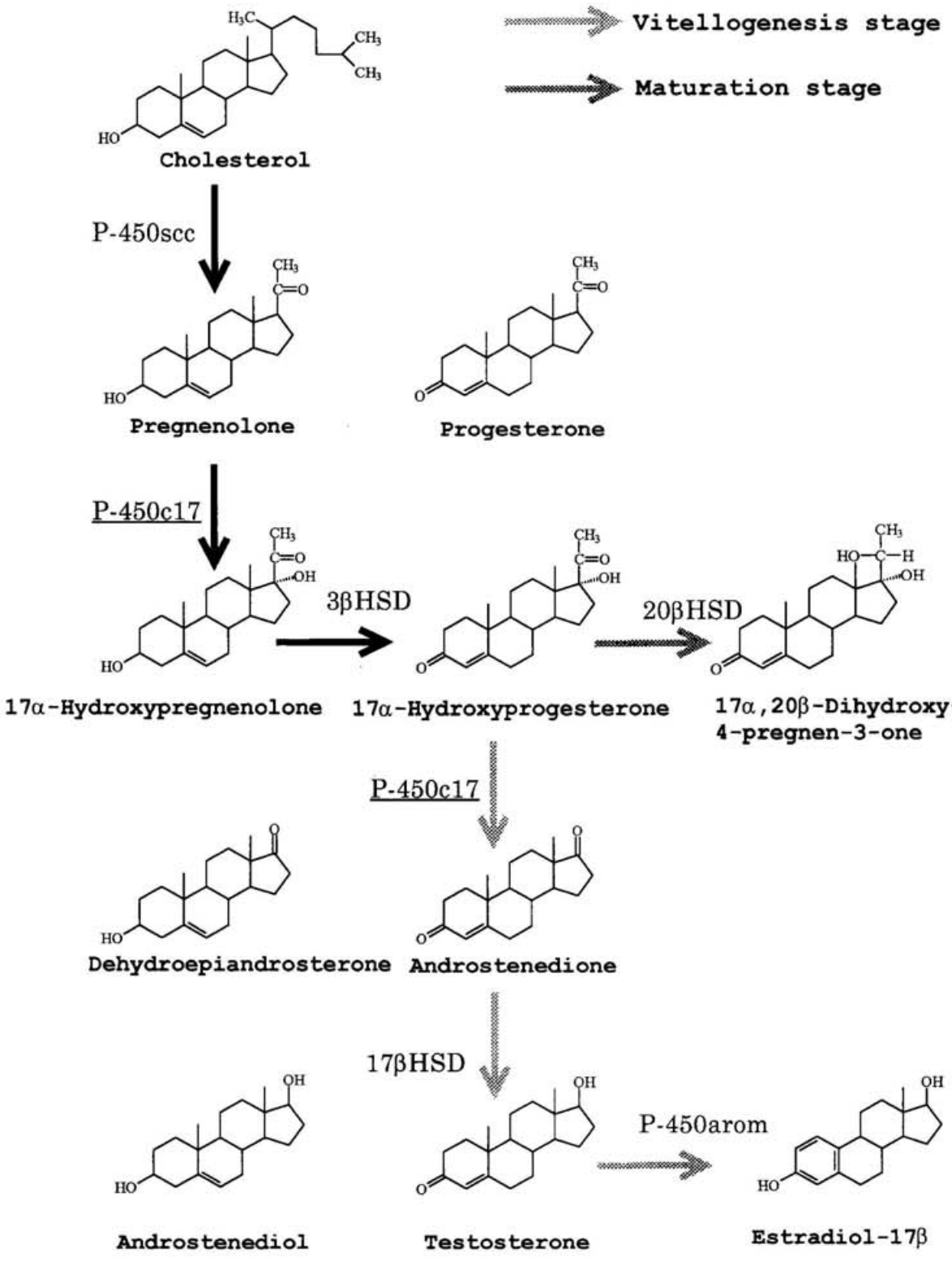
Vitellogenesis stage
Maturation stage
Cholesterol
P-450scc
Pregnenolone
Progesterone
P-450c17
3βHSD
20βHSD
17α-Hydroxypregnenolone
17α-Hydroxyprogesterone
17α,20β-Dihydroxy-4-pregnen-3-one
P-450c17
Dehydroepiandrosterone
Androstenedione
17βHSD
P-450arom
Androstenediol
Testosterone
Estradiol-17β

Fig. 9. Northern bolt analysis. A: 2.4 kb and 6.4 kb transcripts are detected in medaka ovarian follicle cells at 26 hours before the expected time of spawning. B: Time course change in the amount of *P*-450c17 transcripts in medaka ovarian follicle cells. Total RNA was extracted every 3 hours, blotted onto nylon membranes and hybridized with a PCR probe using genome fragment as a template. The relative amount of transcripts were normalized with the amount of 2.4 kb transcripts at 23 hours before spawning.

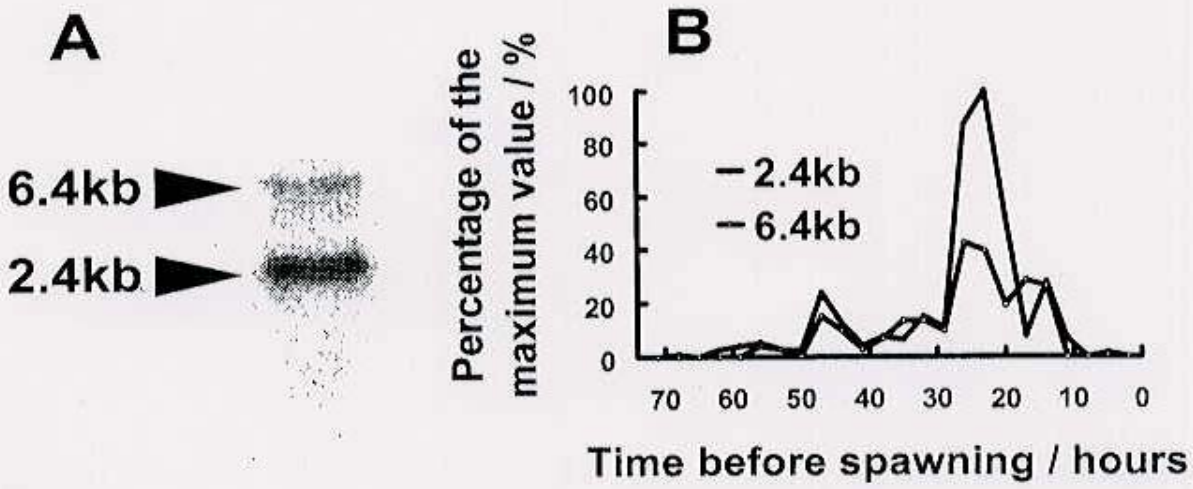
A
6.4kb
2.4kb
B
Percentage of the maximum value / %
100
80
60
40
20
0
2.4kb
6.4kb
70
60
50
40
30
20
10
0
Time before spawning / hours

Fig. 10. The nucleotide and deduced amino acid sequence of medaka *P*-450c17 cDNA. Exon 4, which is removed in *P*-450c17S is boxed. a: N-terminal hydrophobic sequence, b: conserved *P*-450c17 sequence (Ono *et al.*, 1988), c: Ozols tridecapeptide region (Ozols *et al.*, 1981), d: conserved heme binding region (Gotoh *et. al.*, 1983).

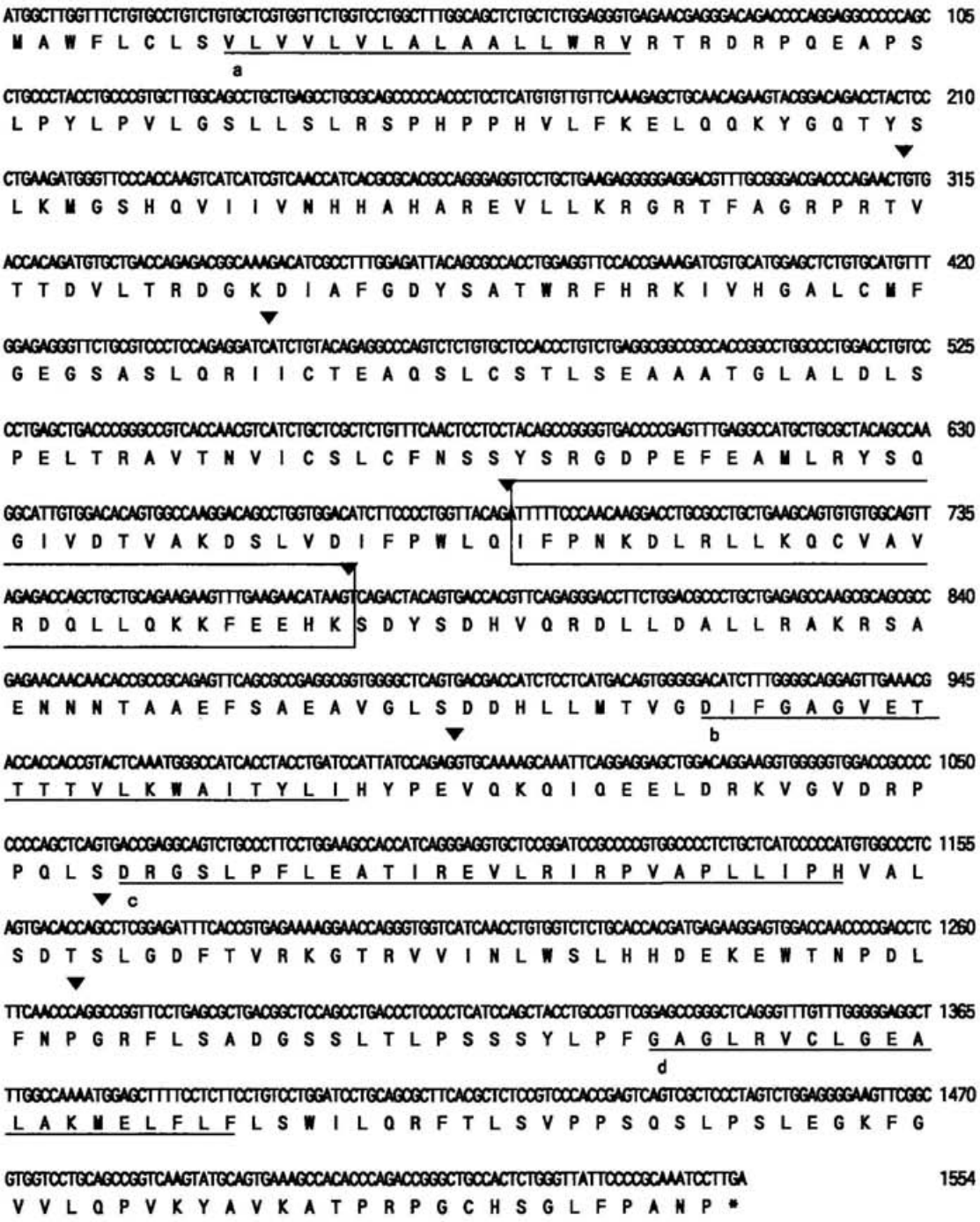
ATGGCTTGGTTTCTGTGCCTGTCTGTGCTCGTGGTTCTGGTCCTGGCTTTGGCAGCTCTGCTCTGGAGGGTGAGAACGAGGGACAGACCCCAGGAGGCCCCCAGC 105
M A W F L C L S V L V V L V L A L A A L L W R V R T R D R P Q E A P S
a
CTGCCCTACCTGCCCGTGCTTGGCAGCCTGCTGAGCCTGCGCAGCCCCCACCCTCCTCATGTGTTGTTCAAAGAGCTGCAACAGAAGTACGGACAGACCTACTCC 210
L P Y L P V L G S L L S L R S P H P P H V L F K E L Q Q K Y G Q T Y S
CTGAAGATGGGTTCCCACCAAGTCATCATCGTCAACCATCACGCGCACGCCAGGGAGGTCCTGCTGAAGAGGGGGAGGACGTTTGCGGGACGACCCAGAACTGTG 315
L K M G S H Q V I I V N H H A H A R E V L L K R G R T F A G R P R T V
ACCACAGATGTGCTGACCAGAGACGGCAAAGACATCGCCTTTGGAGATTACAGCGCCACCTGGAGGTTCCACCGAAAGATCGTGCATGGAGCTCTGTGCATGTTT 420
T T D V L T R D G K D I A F G D Y S A T W R F H R K I V H G A L C M F
GGAGAGGGTTCTGCGTCCCTCCAGAGGATCATCTGTACAGAGGCCCAGTCTCTGTGCTCCACCCTGTCTGAGGCGGCCGCCACCGGCCTGGCCCTGGACCTGTCC 525
G E G S A S L Q R I I C T E A Q S L C S T L S E A A A T G L A L D L S
CCTGAGCTGACCCGGGCCGTCACCAACGTCATCTGCTCGCTCTGTTTCAACTCCTCCTACAGCCGGGGTGACCCCGAGTTTGAGGCCATGCTGCGCTACAGCCAA 630
P E L T R A V T N V I C S L C F N S S Y S R G D P E F E A M L R Y S Q
GGCATTGTGGACACAGTGGCCAAGGACAGCCTGGTGGACATCTTCCCCTGGTTACAGATTTTTCCCAACAAGGACCTGCGCCTGCTGAAGCAGTGTGTGGCAGTT 735
G I V D T V A K D S L V D I F P W L Q I F P N K D L R L L K Q C V A V
AGAGACCAGCTGCTGCAGAAGAAGTTTGAAGAACATAAGTCAGACTACAGTGACCACGTTCAGAGGGACCTTCTGGACGCCCTGCTGAGAGCCAAGCGCAGCGCC 840
R D Q L L Q K K F E E H K S D Y S D H V Q R D L L D A L L R A K R S A
GAGAACAACAACACCGCCGCAGAGTTCAGCGCCGAGGCGGTGGGGCTCAGTGACGACCATCTCCTCATGACAGTGGGGGACATCTTTGGGGCAGGAGTTGAAACG 945
E N N N T A A E F S A E A V G L S D D H L L M T V G D I F G A G V E T
b
ACCACCACCGTACTCAAATGGGCCATCACCTACCTGATCCATTATCCAGAGGTGCAAAAGCAAATTCAGGAGGAGCTGGACAGGAAGGTGGGGGTGGACCGCCCC 1050
T T T V L K W A I T Y L I H Y P E V Q K Q I Q E E L D R K V G V D R P
CCCCAGCTCAGTGACCGAGGCAGTCTGCCCTTCCTGGAAGCCACCATCAGGGAGGTGCTCCGGATCCGCCCCGTGGCCCCTCTGCTCATCCCCCATGTGGCCCTC 1155
P Q L S D R G S L P F L E A T I R E V L R I R P V A P L L I P H V A L
c
AGTGACACCAGCCTCGGAGATTTCACCGTGAGAAAAGGAACCAGGGTGGTCATCAACCTGTGGTCTCTGCACCACGATGAGAAGGAGTGGACCAACCCCGACCTC 1260
S D T S L G D F T V R K G T R V V I N L W S L H H D E K E W T N P D L
TTCAACCCAGGCCGGTTCCTGAGCGCTGACGGCTCCAGCCTGACCCTCCCCTCATCCAGCTACCTGCCGTTCGGAGCCGGGCTCAGGGTTTGTTTGGGGGAGGCT 1365
F N P G R F L S A D G S S L T L P S S S Y L P F G A G L R V C L G E A
d
TTGGCCAAAATGGAGCTTTTCCTCTTCCTGTCCTGGATCCTGCAGCGCTTCACGCTCTCCGTCCCACCGAGTCAGTCGCTCCCTAGTCTGGAGGGGAAGTTCGGC 1470
L A K M E L F L F L S W I L Q R F T L S V P P S Q S L P S L E G K F G
GTGGTCCTGCAGCCGGTCAAGTATGCAGTGAAAGCCACACCCAGACCGGGCTGCCACTCTGGGTTATTCCCCGCAAATCCTTGA 1554
V V L Q P V K Y A V K A T P R P G C H S G L F P A N P *

Fig. 11. cDNA and genome structure of medaka *P*-450c17. Exon IV which is spliced out in *P*-450c17S is shadowed.

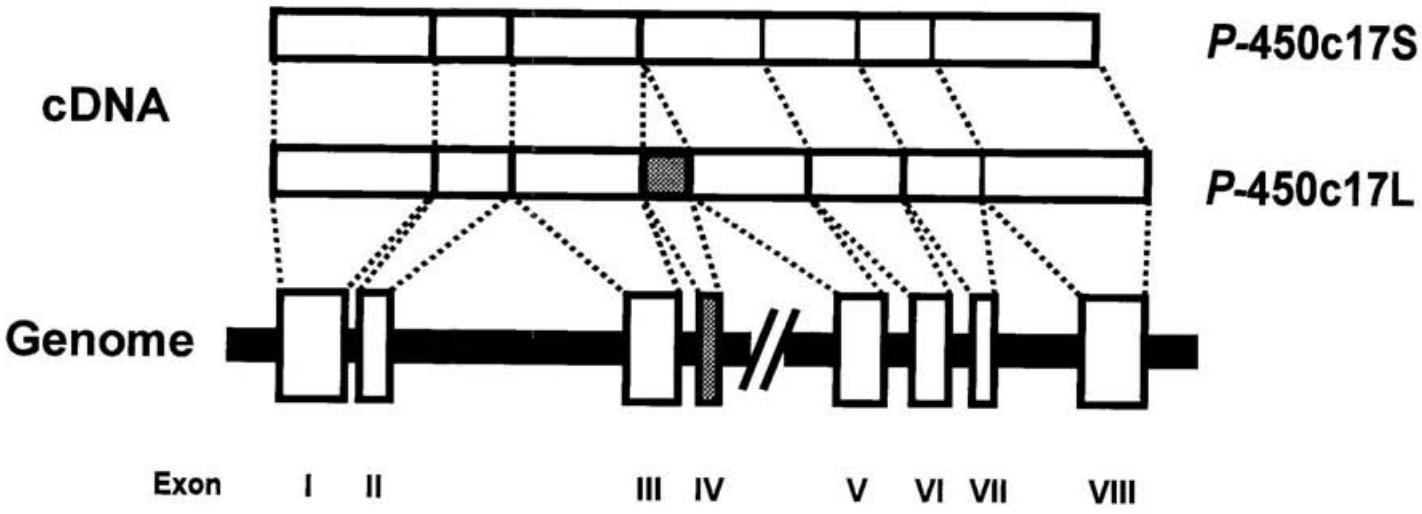
cDNA
P-450c17S
P-450c17L
Genome
Exon
I
II
III
IV
V
VI
VII
VIII

Fig. 12. Two out of 13 positive clones lacked exon 4. PCR was carried out using primers 6, 7 (Table 2). PCR fragments were electrophoresed on 1.5 % agarose gel and detected by ethidiumbromide staining and UV illumination. A, PCR amplified fragment containing exon 4.; B, PCR amplified fragment without exon 4.

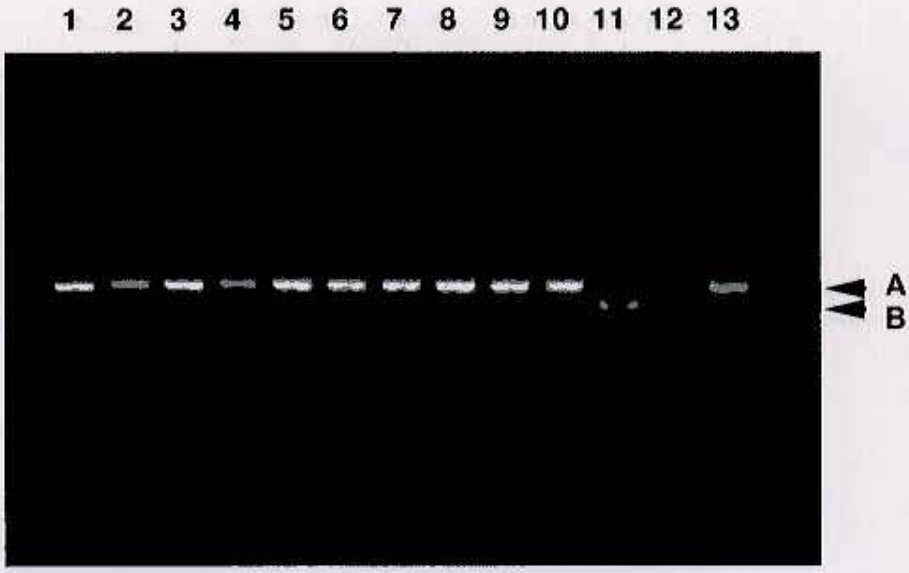
1 2 3 4 5 6 7 8 9 10 11 12 13
A
B

Fig. 13. Comparison of deduced amino acid sequences of medaka, rainbow trout, dogfish, chicken, mouse, rat guineapig, pig, bovine, and human *P*-45017. Alignment was made using GENETYX (Software development). Identical amino acids among the 10 species are indicated by *. a: N-terminal hydrophobic sequence, b: conserved *P*-450c17 sequence (Ono *et al.*, 1988), c: Ozols tridecapeptide region (Ozols *et al.*, 1981), d: conserved heme binding region (Gotoh *et. al.*, 1983).

```
                                a                                 *   *  ***  *
Medaka            1 :  MAWFLCLSVLVVLVLALAALLWRVRTRDRPQEAPSLPYLPVLGSLLSLR
Rainbow trout     1 : MAWFLCMCVFSVVGLGLLLLQVKLRRSLETRGGPPSLPVFPLIGSLLSLR
Dogfish           1 :  MSLMLAALILTVAFVICSLTGFTQRKLSGGRLPKCLPSFPLIGSLLSLR
Chicken           1 :  MPPLAVLLLALALLCAWRLSYSQGPTGTGTGRPRSLPALPLVGSLLQLA
Mouse             1 :       MWELVGLLLLILAYFFWPKSKTPNAKFPRSLPFLPLVGSLPFLP
Rat               1 :       MWELVGLLLLILAYFFWVKSKTPGAKLPRSLPSLPLVGSLPFLP
Guineapig         1 :       MWELVTLLGLILAYLFWPRQGSSGTKYPKSLPSLPVVGSLPFLP
Pig               1 :       MWVLLVFFLLTLTYLFWPKTKGSGAKYPRSLPVLPVVGSLPFLP
Bovine            1 :       MWLLLAVFLLTLAYLFWPKTKHSGAKYPRSLPSLPLVGSLPFLP
Human             1 :       MWELVALLLLTLAYLFWPKRRCPGAKYPKSLLSLPLVGSLPFLP

                        *       *   *                          *** * *  *
Medaka           50 : SPHPPHVLFKELQQKYGQTYSLKMGSHQVIIVNHHAHAREVLLKRGRTFA
Rainbow trout    51 : SNQAPHVLFQKLQQKYGHTYSLMMGPHTVILVNHHQHAKEVLLKKGKIFA
Dogfish          50 : SDLPPHLLFQKLQKTYGNLFSLMMGPHYAVVINNHQHAKEVLLKKGKIFA
Chicken          50 : GHPQLHLRLWRLQGRYGSLYGLWMGSHYVVVVNSYQHAREVLLKKGKAFA
Mouse            45 : RRGHMHANFFKLQEKYGPIYSLRLGTTTAVIVGHYQLAREVLVKKGKEFS
Rat              45 : RRGHMHVNFFKLQEKYGPIYSLRLGTTTTVIIGHYQLAREVLIKKGKEFS
Guineapig        45 : KSGHMHVNFFKLQKKYGPIYSFRLGSTTTVVIGHHQLARELLIKKGKEFS
Pig              45 : RRGHQHMNFFKLQDKYGPIFSFRLGSKTTVVIGDHQLAKEVLLKKGKEFS
Bovine           45 : RRGQQHKNFFKLQEKYGPIYSFRLGSKTTVMIGHHQLAREVLLKKGKEFS
Human            45 : RHGHMHNNFFKLQKKYGPIYSVRMGTKTTVIVGHHQLAKEVLIKKGKDFS

                     ***   *         *  **      *   **        *
Medaka          100 : GRPRTVTTDVLTRDGKDIAFGDYSATWRFHRKIVHGALCMFGEGSASLQR
Rainbow trout   101 : GRPRTVTTDLLTRDGKDIAFADYGATWRFHRKTVHGALCMFGEGSASIEK
Dogfish         100 : GRPSMVTTDLLSRGGKDIAFGKYGPAWKFHRKLVLSALHLFGDGSAGIEK
Chicken         100 : GRPRTVTTDLLSRGGKDIAFASYGPLWKFQRKLVHAALSMFGEGSVALEK
Mouse            95 : GRPQMVTLGLLSDQGKGVAFADSSSSWQLHRKLVFSTFSLF-RDDQKLEK
Rat              95 : GRPQMVTQSLLSDQGKGVAFADAGSSWHLHRKLVFSTFSLF-KDGQKLEK
Guineapig        95 : GRPLTTTVALLSDNGKGIAFADSSATWQLHRRLVLSSFSLFRDGEQKLEN
Pig              95 : GRPRVMTLDILSDNQKGIAFADHGTSWQLHRKLALSTFSLFKGGNLKLEN
Bovine           95 : GRPKVATLDILSDNQKGIAFADHGAHWQLHRKLALNAFALFKDGNLKLEK
Human            95 : GRPQMATLDIASNNRKGIAFADSGAHWQLHRRLAMATFALFKDGDQKLEK
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                          *  *    *              *          **      ***     *
Medaka          150 : IICTEAQSLCSTLSEAAATGLALDLSPELTRAVTNVICSLCFNSSYSRGD
Raibow trout    151 : IICREALSLC--DTLRESGSASLDLSPELTRAVTNVVCSLCFSSSYCRGD
Dogfish         150 : MICQEATSMC--STFERLNNAAHDMMPDVTRAVTNVICLLCFNSTYEKED
Chicken         150 : IICREAASLC--ETLGAAQDMALDMAPELTRAVTNVVCSLCFNSSYRRGD
Mouse           145 : MICQEANSLC--DLILTYDGESRDLSTLIFKSVINIICTICFNISFENKD
Rat             145 : LICQEAKSLC--DMMLAHDKESIDLSTPIFMSVTNIICAICFNISYEKND
Guineapig       145 : IICQELSALC--DFLATCDGQVKDLSSSIFMTVVNIICMICFSVSYKEGD
Pig             145 : IINQEIKVLC--DFLATRNGESIDLAQPLSLAMTNIVSFICFNFSFKKGD
Bovine          145 : IINQEANVLC--DFLATQHGEAIDLSEPLSLAVTNIISFICFNFSFKNED
Human           145 : IICQEISTLC--DMLATHNGQSIDISFPVFVAVTNVISLICFNTSYKNGD

                                  *          * *  *    **            *
Medaka          200 : PEFEAMLRYSQGIVDTVAKDSLVDIFPWLQIFPNKDLRLLKQCVAVRDQL
Rainbow trout   201 : PEFEAMLQFSQGIVDTVAKDSLVDIFPWLQVFPNADLRLLKQCVSIRDKL
Dogfish         200 : PEFQTMRKYSQGIVNTVAKDSLIDIFPWLQFFPNENLHTLKQCIATRDSI
Chicken         200 : PEFEAMLEYSQGIVDTVAKESLVDIFPWLQIFPNRDLALLKRCLKVRDQL
Mouse           195 : PILTTIQTFTEGIVDVLGHSDLVDIFPWLKIFPNKNLEMIKEHTKIREKT
Rat             195 : PKLTAIKTFTEGIVDATGDRNLVDIFPWLTIFPNKGLEVIKGYAKVRNEV
Guineapig       195 : MELVTIRRFTTGFVNSLSDDNLVDIFPWLKIFPNKTLEMIRKYTEIRGAM
Pig             195 : PALQAIVNFNDGILDAVGKEILYDMFPGIRILPSQTLENMKQCVRMRNEL
Bovine          195 : PALKAIQNVNDGILEVLSKEVLLDIFPVLKIFPSKAMEKMKGCVQTRNEL
Human           195 : PELNVIQNYNEGIIDNLSKDSLVDLVPWLKIFPNKTLEKLKSHVKIRNDL

                          *                                   *
Medaka          250 : LQKKFEEHKSDYSDHVQRDLLDALLRAKRSAENNNTAAEFSAEAVGLSDD
Rainbow trout   251 : LQKKYEEHKSDYSDHEQRDLLDALLRAKRSAENNNTA-EITMETVGLSED
Dogfish         250 : LQKKFEDHKANYSSDSANDLFNILLKAKMNAENNNSS-----VHEAGLTDD
Mouse           245 : LVEMFEKCKEKFNSESLSSLTDILIQAKMNAENNNTGE--GQDPSVFSDK
Chicken         250 : LQQKFTEHKEAFCGDTVRDLMDALLQVRLNAE-NNSP---LEPGLELTDD
Rat             245 : LTGIFEKCREKFDSQSISSLTDILIQAKMNSDNNNSCE--GRDPDVFSDR
Guineapig       245 : LSKILKECKEKFRSDSVSNLIDLLIQAK-VNENNNNSSL-DQDSNLFSDK
Pig             245 : L-REILENRKENYSRNSITNLLDIMIQAKTNAESNTGG-PDHNLKLLSDR
Bovine          245 : L-NEILEKCQENFSSDSITNLLHILIQAK-VNADNNNAGPDQDSKLLSNR
Human           245 : L-NKILENYKEKFRSDSITNMLDTLMQAKMNSD-NGNAGPDQDSELLSDN
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                          *   * b****** **      *    * * *          *   *
Medaka           300 : HLLMTVGDIFGAGVETTTTVLKWAITYLIHYPEVQKQIQEELDRKVGVDR
Rainbow trout    301 : HLLMTVGDIFGAGVETTSTVLKWAIAYLIHHPQVQQRIQEELDSVVGGDR
Dogfish          300 : HMVMTVADIFGAGVETSSTAFAWMIIYLIHHPEVQKKIQEEIDSNIGFER
Chicken          300 : HLLMTVGDIFGAGVETTTTVLKWAVLYLLHYPEVQKKIQEEMDQKIGLAR
Mouse            295 : HILVTVGDIFGAGIETTSSVLNWILAFLVHNPEVKRKIQKEIDQYVGFSR
Rat              295 : HILATVGDIFGAGIETTTTVLKWILAFLVHNPEVKKKIQKEIDQYVGFSR
Guineapig        295 : HILTTLGDIFGAGVETSSSVVLWVIAFLLHNPQVKKKIQEEIDHNVGFSR
Pig              295 : HMLATVADIFGAGVETSASVVKWIVAFLLHYPLLRKKIQDAIDQNIGFNR
Bovine           295 : HMLATIGDIFGAGVETTTSVIKWIVAYLLHHPSLKKRIQDDIDQIIGFNR
Human            295 : HILTTIGDIFGAGVETTTSVVKWTLAFLLHNPQVKKKLYEEIDQNVGFSR

                          *  c*       * *** * **  ** * ****    * * *     *
Medaka           350 : PPQLSDRGSLPFLEATIREVLRIRPVAPLLIPHVALSDTSLGDFTVRKGT
Rainbow trout    351 : TPQLSDRGSLPYLEATIREVLRIRPVAPLLIPHVAQTDTSIGKFTVRKGA
Dogfish          350 : TPKMSDKGNMNFLNATIHEILRIQPVSPLLIPHVALADSSIGDYTIPKGT
Chicken          350 : HPHLSDRPLLPYLEATISEGLRIRPVSPLLIPHVSLADTSIGEYSIPKGA
Mouse            345 : TPSFNDRTHLLMLEATIREVLRIRPVAPLLIPHKANIDSSIGEFAIPKDT
Rat              345 : TPTFNDRSHLLMLEATIREVLRIRPVAPMLIPHKANVDSSIGEFTVPKDT
Guineapig        345 : TPTFSDRNHLLMLEATIREVLRIRPVAPILIPHKANTDSSIGEFAIDKDT
Pig              345 : APSISDRNQLVLLEATIREVLRFRPVSPTLIPHRAIIDSSIGEFTIDKDT
Bovine           345 : TPTISDRNRLVLLEATIREVLRIRPVAPTLIPHKAVIDSSIGDLTIDKGT
Human            345 : TPTISDRNRLLLLEATIREVLRLRPVAPMLIPHKANVDSSIGEFAVDKGT

                             ***  ** * **  *     ****              ********d*
Medaka           400 : RVVINLWSLHHDEKEWTNPDLFNPGRFLSADGSSLNLPSSSYLPFGAGLR
Rainbow trout    401 : RIIINLWSLHHDEKEWKNPEMFDPGRFLNEEGTGLCIPSPSYLPFGAGVR
Dogfish          400 : RVIINLWAIHHDEKEWKNPDAFDPGRFLDEDGKYVCSSSESYLPFGAGTR
Chicken          400 : RVVINLWSVHHDEKEWDKPEEFNPGRFLDEQGQHIHSPSPSYLPFGAGIR
Mouse            395 : HVIINLWALHHDKNEWDQPDRFMPERFLDPTGSHLITPTPSYLPFGAGPR
Rat              395 : HVVVNLWALHHDENEWDQPDQFMPERFLDPTGSHLITPTQSYLPFGAGPR
Guineapig        395 : NVLVNLWALHHNEQEWDRPDQFMPERFLDPTGSQIIVPSSSYLPFGAGPR
Pig              395 : DVVVNLWALHHNEKEWLRPDLFMPERFLDPTGTQLISPSLSYLPFGAGPR
Bovine           395 : DVVVNLWALHHSEKEWQHPDLFMPERFLDPTGTQLISPSLSYLPFGAGPR
Human            395 : EVIINLWALHHNEKEWHQPDQFMPERFLNPAGTQLISPSVSYLPFGAGPR
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                          * ** **  * *      ***            *   *    *
Medaka           450 : VCLGEALAKMELFLFLSWILQRFTLSVPPSQSLPSLEGKFGVVLQPVKYA
Rainbow trout    451 : VCLGEALAKMEIFLFLSWILQRLTMTVSPGQPLPSLEGKFGVVLQPVKYK
Dogfish          450 : VCLGEMLARMELFLFTSWILQRFTVQVPPGYPPPDKEGKFGIVLQPLKFK
Chicken          450 : VCLGEVLAKMELFLFLAWVLQRFTLECPQDQPLPSLEGKFGVVLQVQKFR
Mouse            445 : SCIGEALARQELFIFMALLLQRFDFDVSDDKQLPCLVGDPKVVFLIDPFK
Rat              445 : SCIGEALARQELFVFTALLLQRFDLDVSDDKQLPRLEGDPKVVFLIDPFK
Guineapig        445 : SCVGEALARQEIFLITAWLLQKFDLEVPEGGQLPSLEGIPKIVFLIDPFK
Pig              445 : SCVGEMLARQELFLFTAGLLQRFDLELPDDGQLPCLVGNPSLVLQIDPFK
Bovine           445 : SCVGEMLARQELFLFMSRLLQRFNLEIPDDGKLPSLEGHASLVLQIKPFK
Human            445 : SCIGEILARQELFLIMAWLLQRFDLEVPDDGQLPSLEGIPKVVFLIDSFK

                          *
Medaka           500 : VKATPRPG-CHSGLFPANPC
Rainbow trout    501 : VNATPRAGWEKSHLQTSC
Dogfish          500 : VQLKLRKAWENRGLHD
Chicken          500 : VKARLREAWRGEMVR
Mouse            495 : VKITVRQAWKDAQVEVST
Rat              495 : VKITVRQAWMDAQAEVST
Guineapig        495 : VKITVRPAWKEAQAEGSA
Pig              495 : VKIKERQAWKEAHTEGSTSC
Bovine           495 : VKIEVRQAWKEAQAEGSTP
Human            495 : VKIKVRQAWREAQAEGST
```

Fig. 14. RNase protection analysis in medaka ovarian follicles. Lane 1: size marker (DNA), 2: probe, 3-8: 8, 11,14, 21, 32, 38 hours before spawning. Five micrograms of total RNA from ovarian follicles at indicated times before spawning were hybridized with 173 base cRNA probe indicated by arrowhead in lane 2. Two length of protected fragments, 163 base and 134, were detected during vitellogenic stage. 134 base band indicates the presence of transcript lacking exon 4 region.

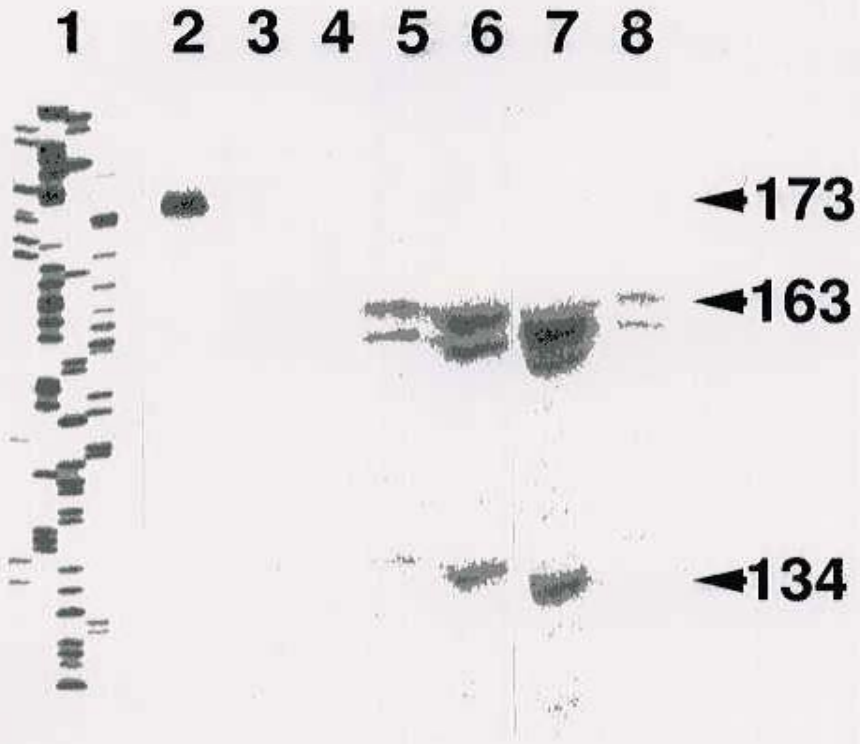

1 2 3 4 5 6 7 8
173
163
134

Fig. 15. RNase protection analysis in medaka tissues. Lane 1: size marker (DNA), 2: probe, 3-7: male, 3: testis, 4: kidney, 5: liver, 6: brain, 7: spleen. 8-12: female 8: ovary, 9: kidney, 10: liver 11: brain, 12 spleen. Five μg of total RNA from each tissue were hybridized with 173 base cRNA probe indicated by arrowhead in lane 2. Two length of protected fragments, 163 base and 134, were detected only in testis and ovary.


1
2
3
4
5
6
7
8
9
10
11
12
174
163
134

Fig. 16. Southern blot analysis of RT-PCR products. The template DNAs used for reactions are as follows. 1: without template DNA, 2: *P*-450c17L, 3: *P*-450c17S, The first strand DNAs were synthesized from total RNAs. 4-8: ovarian follicle cells, kidney, liver, brain, and spleen, of female, respectively, 9-13: testis, kidney, liver, brain, and spleen, of male, respectively. Reverse transcription was performed using SuperscriptII with primer 8 (Table 2). PCR was carried out with primers 3 and 4 (Table 2). PCR fragments were electrophoresed on 1.5% agarose and transferred to Hybond N^+ membrane (Amersham). Transferred DNAs were hybridized with an internal oligonucleotide probe for *P*-450c17 (Table 2 - NO.5)


1 2 3 4 5 6 7 8 9 10 11 12 13
A
B

Fig. 17. Genomic Southern blot analysis. Genomic DNAs were extracted from inbred strain Hd-rR and digested with the following restriction enzymes. PCR amplified DNA with primers 6 and 7 (Table-1) was used for the probe. 1: *Dra* I, 2: *Hind* III, 3: *Pst* I, 4: *Pvu* II. A: female, B: male. The digested DNA fragments were fractionated by gel electrophoresis and transferred onto Hybond N^+ membranes. Membranes were then hybridized to a probe generated by PCR using primers 6 and 7 (Table-2).

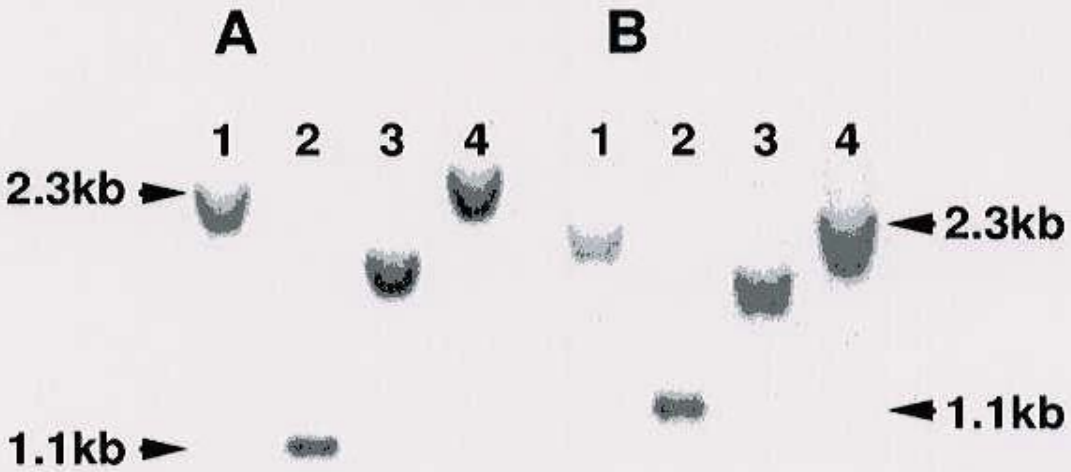

A
B
1
2
3
4
1
2
3
4
2.3kb
1.1kb
2.3kb
1.1kb

Fig. 18. Western blot analysis of *P*-450c17 products expressed in *E. coli*. 1, *P*-450c17L + IPTG; 2, *P*-450c17L - IPTG; 3, *P*-450c17S + IPTG; 4, *P*-450c17S, - IPTG; 5, Pre-stain molecular weight marker (Bio-rad), numbers are the size of standard proteins (kDa). A; *P*-4501c17L product, B; *P*-450c17S product.

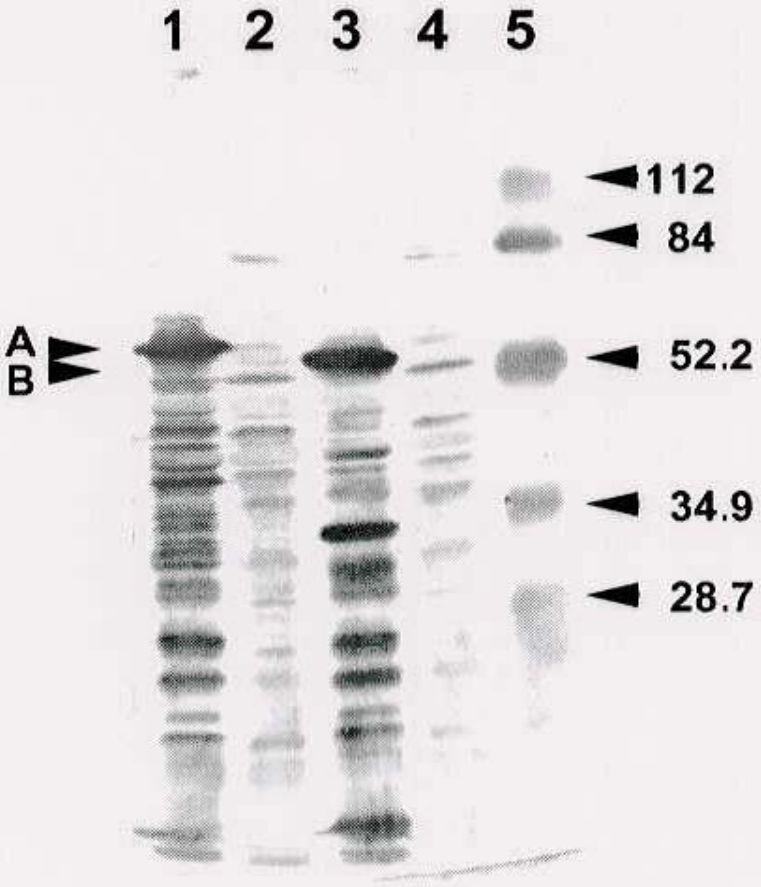

1
2
3
4
5
112
84
A
B
52.2
34.9
28.7

Fig. 19. Activities of *P*-450c17S and *P*-450c17L expressed in *E. coli*. [4-^{14}C]-Progesterone was added as a precursor. Steroids were fractionated by the two dimensional TLC system developed with benzene-acetone (4:1, direction a) and cyclohexane:ethylacetate (1:1, direction b). A: mock, B: *P*-450c17S, C: *P*-450c17L. Progesterone is indicated as spot 1. 17α-Hydroxyprogesterone (spot 2) and androstenedione (spot 3) were detected.

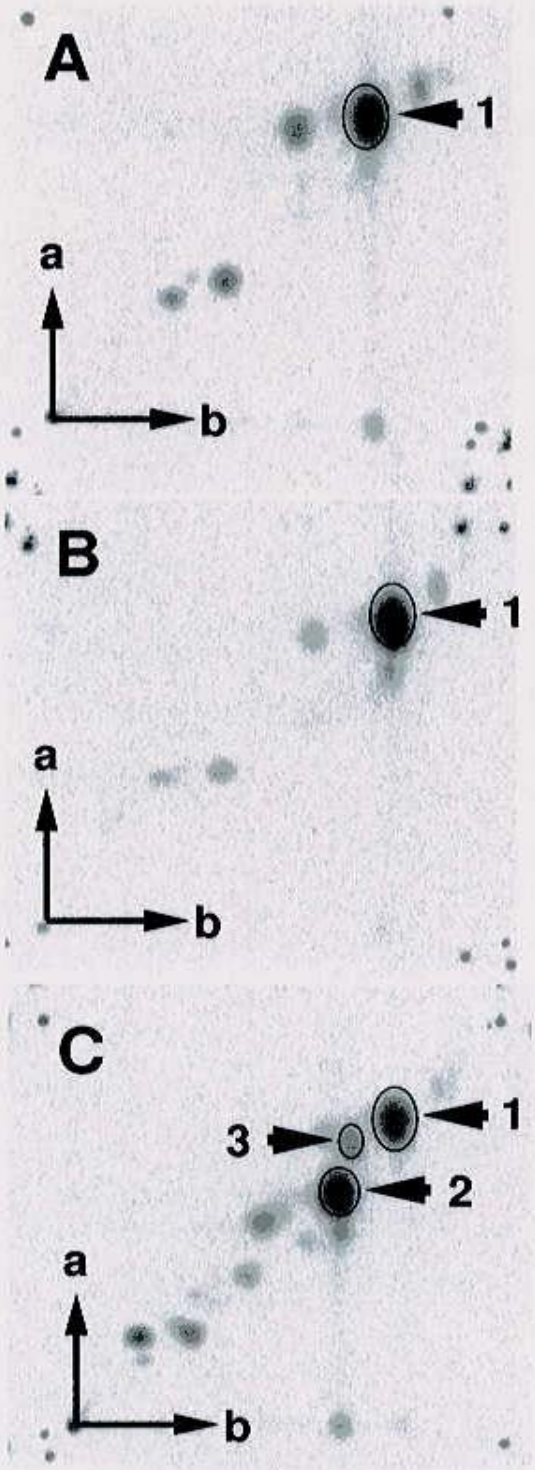

A
1
a
b
B
1
a
b
C
3
1
2
a
b

Fig. 20. Activities of *P*-450c17S and *P*-450c17L expressed in *E. coli*. [1,2-^{3}H(N)]-17α-Hydroxyprogesterone was added as a precursor. Steroids were fractionated by the two dimensional TLC system developed with benzene-acetone (4:1, direction a) and cyclohexane:ethylacetate (1:1, direction b). A: mock, B: *P*-450c17S, C: *P*-450c17L. 17α-Hydroxyprogesterone is indicated as spot 1. Androstenedione spot 2) were detected.

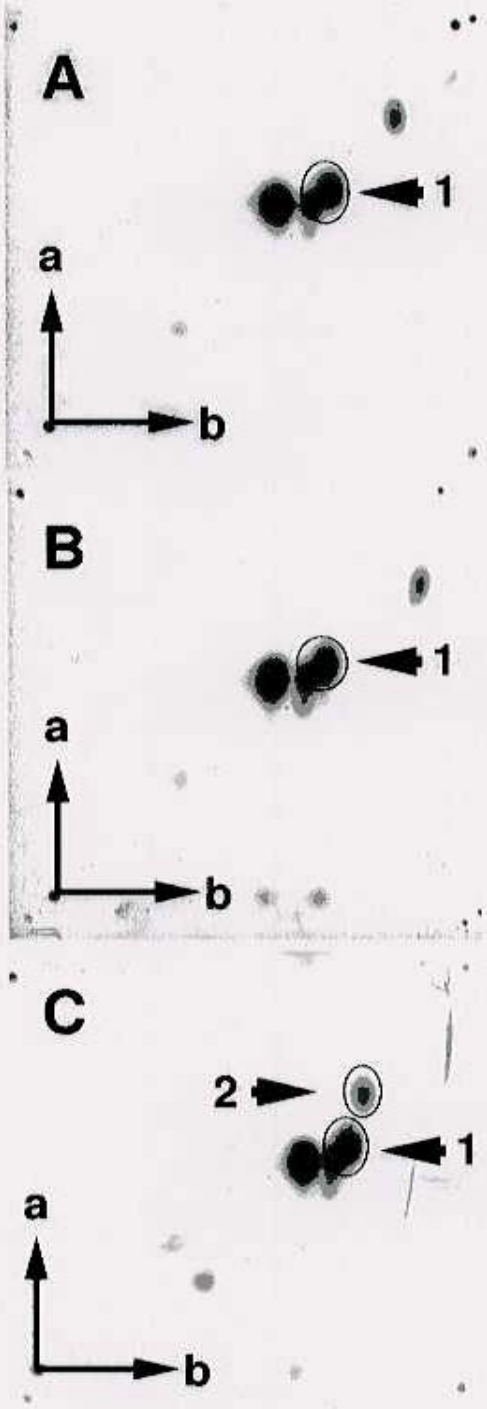

A
1
a
b
B
1
a
b
C
2
1
a
b

Fig. 21. Activities of *P*-450c17S and *P*-450c17L expressed in COS 1 cells. [4-^{14}C]-Progesterone was added as a precursor. Steroids were fractionated by two dimensional TLC system developed with benzene-acetone (4:1, direction a) and cyclohexane:ethylacetate (1:1, direction b). A: mock, B: *P*-450c17S, C: *P*-450c17L. Progesterone is indicated as spot 1. 17α-Hydroxyprogesterone (spot 2) and androstenedione (spot 3) were detected

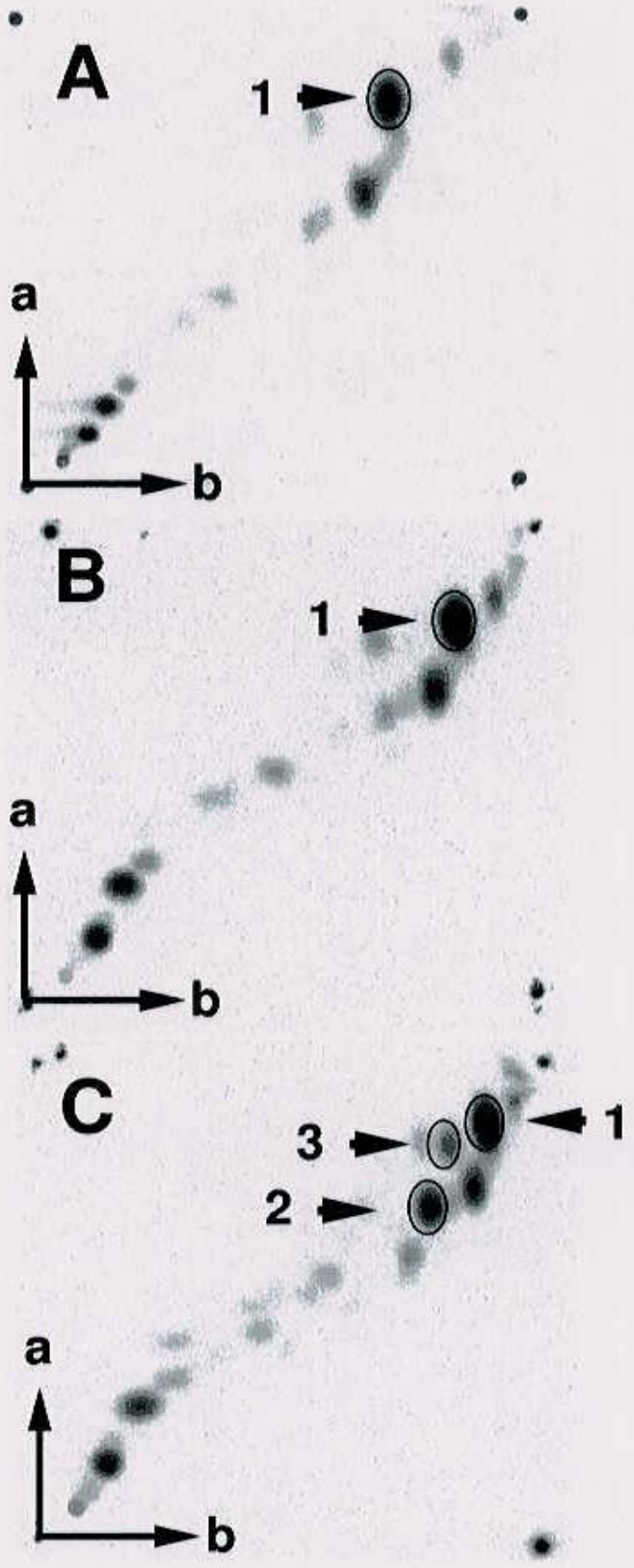

A
1
a
b
B
1
a
b
C
3
1
2
a
b

Fig. 22. Activities of *P*-450c17S and *P*-450c17L expressed in COS 1 cells. [1,2-^{3}H(N)]-17α-Hydroxyprogesterone was added as a precursor. Steroids were fractionated by the two dimensional TLC system developed with benzene-acetone (4:1, direction a) and cyclohexane:ethylacetate (1:1, direction b). A: mock, B: *P*-450c17S, C: *P*-450c17L. 17α-hydroxyprogesterone are indicated as spot 1. Androstenedione (spot 2) were detected.

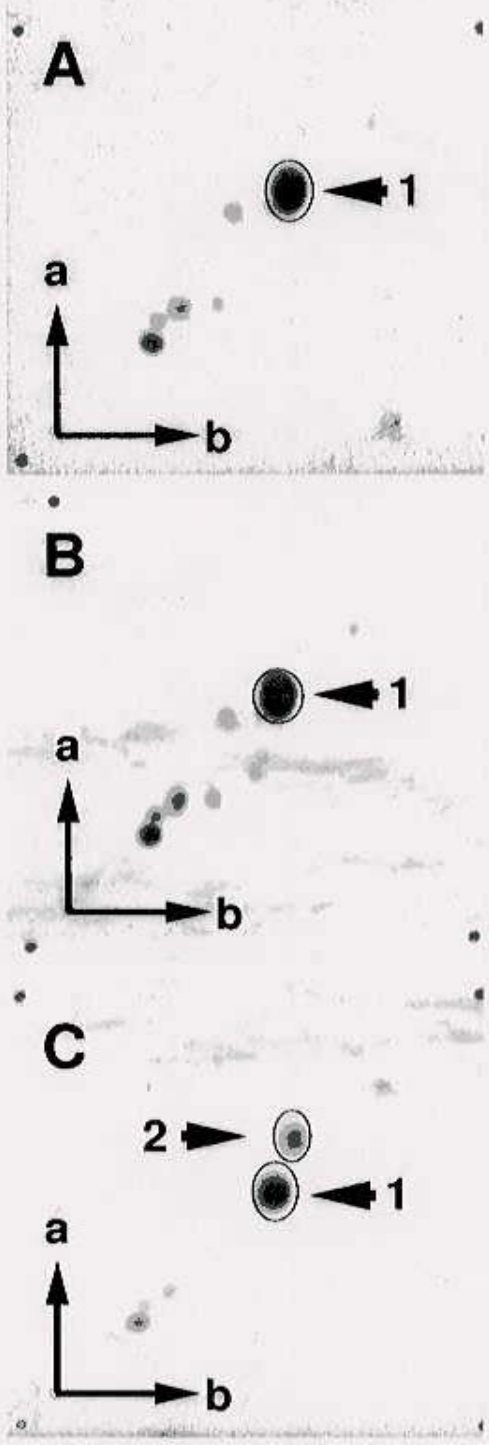

A
1
a
b
B
1
a
b
C
2
1
a
b

Fig. 23. Activities of *P*-450c17S and *P*-450c17L expressed in COS 1 cells. [7-^{3}H(N)]-Pregnenolone was added as a precursor. Steroids were fractionated by the two dimensional TLC system developed with benzene-acetone (4:1, direction a) and cyclohexane:ethylacetate (1:1, direction b). A: mock, B: *P*-450c17S, C: *P*-450c17L. Pregnenolone is indicated as spot 1. 17α-Hydroxypregnenolone (spot 2) and dehydroepiandrosterone (spot 3) were detected.

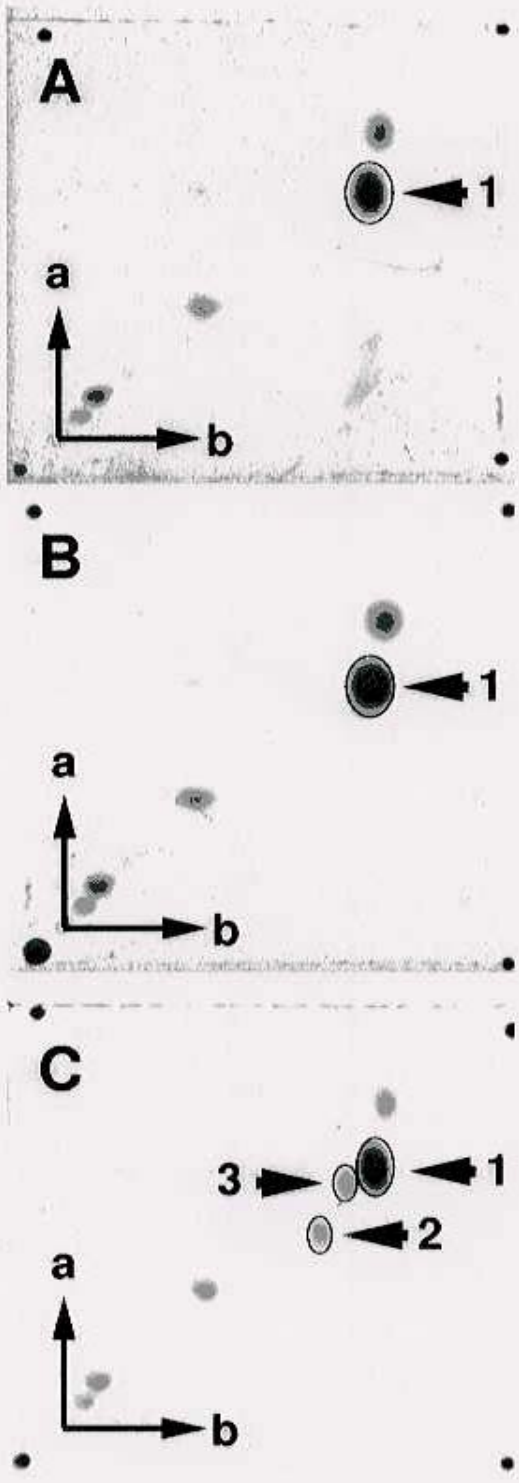

A
1
a
b
B
1
a
b
C
3
1
2
a
b

Fig. 24. Comparison of deduced amino acid sequences of medaka cytochrome b_5 PCR fragment with that of pig cytochrome b_5 (Abe *et al.*, 1985). Alignment was made using GENETYX (Software development). Identical amino acids are indicated by ˙

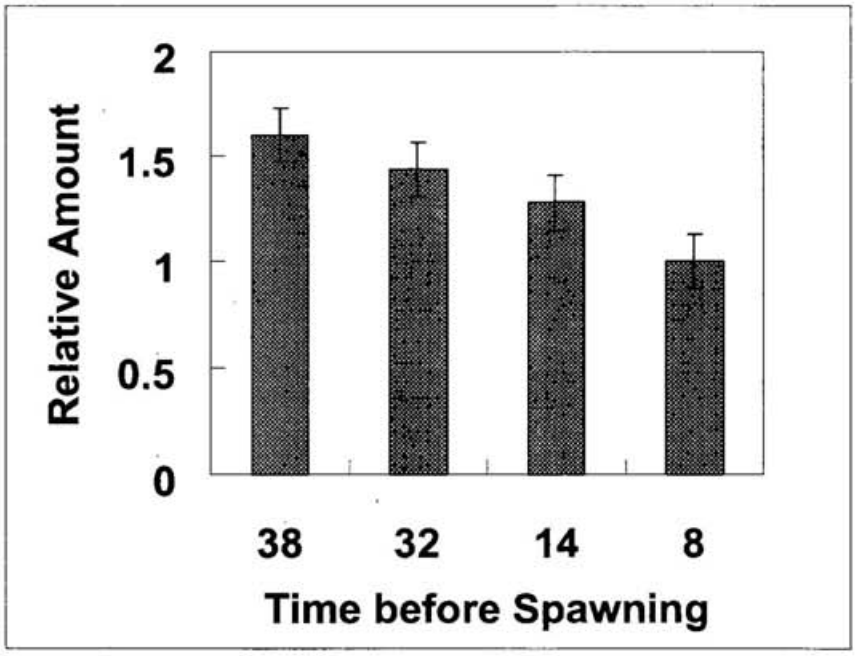
```
Pig     1 AEQSDKAVKYYTLEEIQKHNNSKSTWLILHHKVYDLTKFLEEHPGGEEVLREQAGGDATE
                  *****. **  ..***..*.****...*.********** **.*.****
Medaka  1           TLEEIRVHNMINDTWLVIHDKVYDISSFVEEHPGGEEVLLEQGGADATE

       61 NFEDVGHSTDARELSKTFIIGELHPDDRSKIAKPSETLITTVESNSSWWTNWVIPAISAL
          .******* ****. . . ***** .**.*  *  *   .. . .** ** *.***
       50 SFEDVGHSLDAREMLQQYYIGELHLADRKKEKKNVE---ASSSQESSSWTFWLIPA

      121 VVSLMYHFYTSEN
```

Fig. 25. Comparison of deduced amino acid sequences of medaka NADPH:P-450 reductase PCR fragment with that of pig NADPH:P-450 reductase (Haniu *et al.*, 1986). Alignment was made using GENETYX (Software development). Identical amino acids are indicated by *.

```
Pig       1 GDSNVDTGTTTSEMVAEEVSLFSATDMVLFSLIVGLLTYWFIFRKKKDEVPEFSKIETTT
         61 SSVKDSSFVEKMKKTGRNIIVFYGSQTGTAEEFANRLSKDAHRYGMRGMAADPEEYDLSD

Pig     121 LSSLPEIENALAVFCMATYGEGDPTDNAQDFYDWLQE-ADVDLTGVKYAVFGLGNKTYEH
                                 ********************  .* **.*..*.**.*********
Medaka    1                      GDPTDNAQDFYDWLQENDDEDLSGLNYTVFALGNKTYEH

Pig     180 FNAMGKYVDKRLEQLGAQRIFDLGLGDDDGNLEEDFITWREQFWPAVCEHFGVEATGEES
             .*************.***.************.******..******************...*
Medaka   40 YNAMGKYVDKRLEELGAKRIFDLGLGDDDSNLEEDFVSWREQFWPAVCEHFGVEASADEL

Pig     240 SIRQYELVVHTDMDTAVVYTGEMGRLKSYENQKPPFDAKNPFLAVVTTNRKLNQGTERHL
             ******* .*.*..  .  *.***.*****.* **********
Medaka  100 SIRQYELKLHNDVNMNKVFTGEIGRLKSFEVQKPPFDAKNP

         300 MHLELDISDSKIRYESGDHVAVYPANDSALVNQLGEILGTDLDIVMSLNNLDEESNKRHP
         360 FPCPTTYRTALTYYLDITNPPRTNVLYELAQYASEPSEQEQLRKMASSSGEGKELYLSWV
         420 VEARRHILAILQDYPSLRPPIDHLCERLPRLQARYYSIASSSKVHPNSVHICAVVVEYET
         480 KSGRVNKGVATSWLRAKEPAGENGRRALVPMFVRKSQFRLPFKATTPVIMVGPGTGVAPF
         540 IGFIQERAWLQEQGKEVGETLLYYGCRRSDEDYLYREELAQFHAKGALTRLSVAFSREQP
         600 QKVYVQHLLKRDKEHLWKLIHDGGAHIYICGDARNMARDVQNTFCDIVAEQGPMEHAQAV
         660 DYVKKLMTKGRYSLDVWS
```

Fig. 26. The relative amount of cytochrome b_5 (A) and NADPH:*P*-450 reductase (B) transcripts detected by Northern bolt analysis during vitellogenesis (32, 38 hours) and oocyte maturation (8, 14 hours). Twenty micrograms of total RNA were extracted at 8, 14, 32, and 38 hours before the expected time of spawning, blotted onto nylon membranes and hybridized with PCR probe using RT-PCR fragment as a template. All values were normalized to the value at 8 hours before spawning.

A


Relative Amount
2
1.5
1
0.5
0
38
32
14
8
Time before Spawning

B

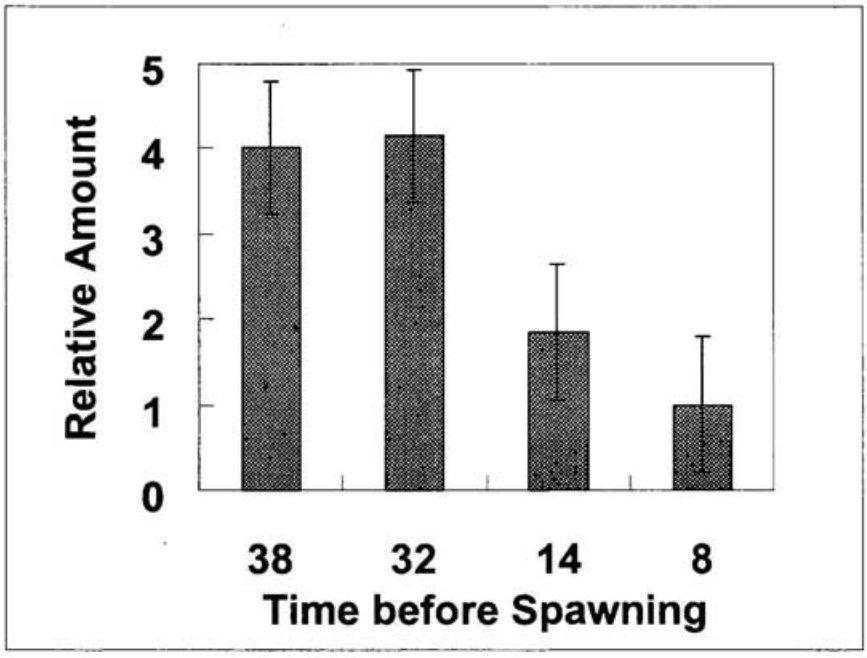

Relative Amount
5
4
3
2
1
0
38
32
14
8
Time before Spawning

Fig. 27. Western blot analysis of *P*-450c17 products in membrane fractions. 1, *P*-450c17S + IPTG; 2, *P*-450c17L + IPTG; 3, Pre-stain molecular weight marker (Bio-rad), numbers are the size of standard proteins (kDa). A; *P*-4501c17L product, B; *P*-450c17S product..

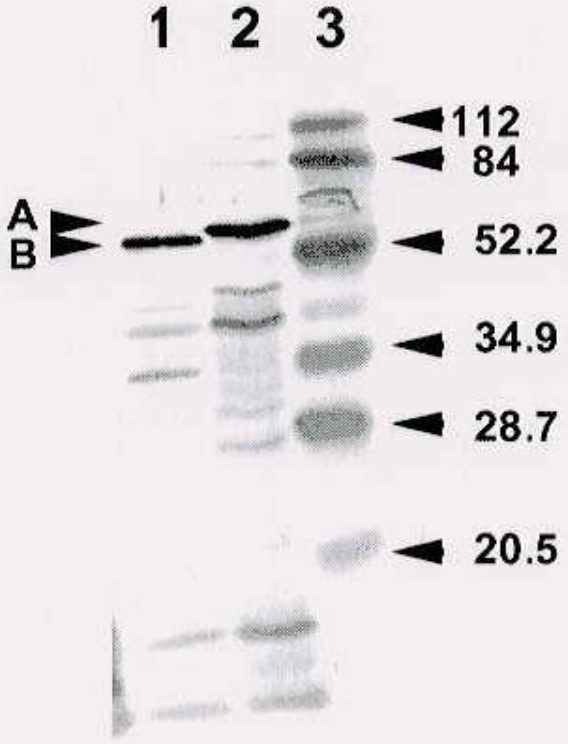
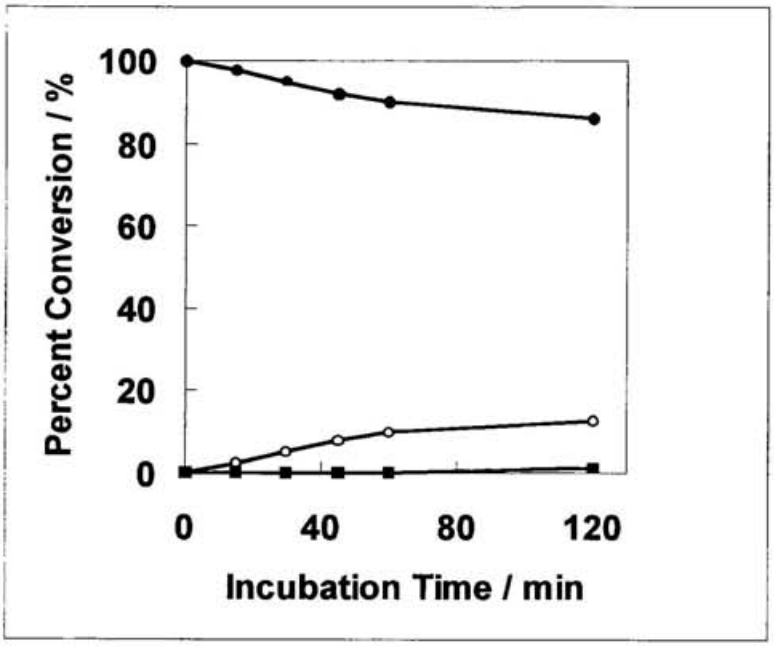

1
2
3
112
84
A
B
52.2
34.9
28.7
20.5

Fig. 28. Effects of cytochrome b_5 on profiles of metabolites produced by medaka *P*-450c17 products in membrane fractions. [4-^{14}C]-Progesterone was added as a precursor (●). The rate conversion of metabolites was calculated from the amount of radioactivity associated with each metabolite on TLC, using BAS-2000 Bio-Imazing Analyzer (Fujifilm). A: in the absence of cytochrome b_5. B: in the presence of cytochrome b_5. 17α-hydroxyprogesterone (○), androstenedione (■).

A


Percent Conversion / %
100
80
60
40
20
0
0
40
80
120
Incubation Time / min

B

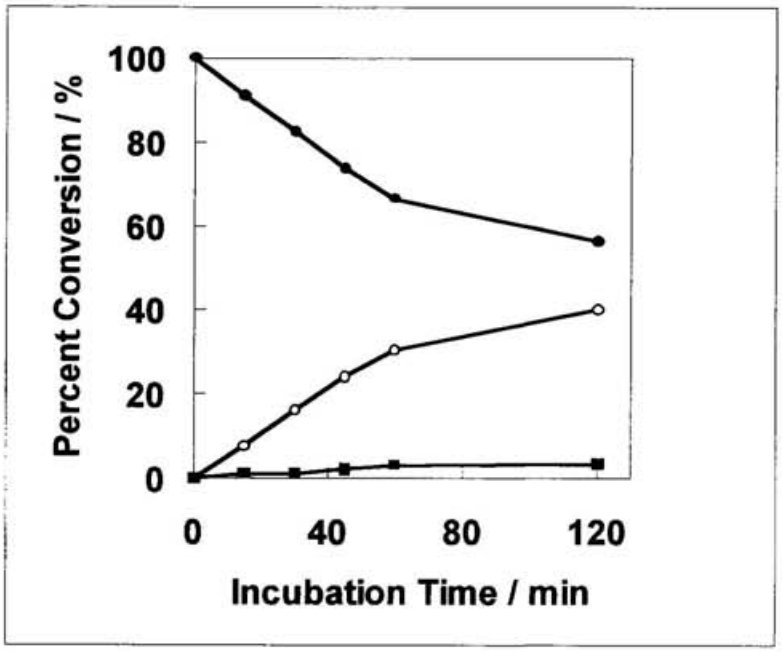

Percent Conversion / %
100
80
60
40
20
0
0
40
80
120
Incubation Time / min

Fig. 29. The relative amount of *P*-450c17S and *P*-450c17L transcripts detected by RNase protection assay. Five micrograms of total RNA from ovarian follicles and testis at indicated times before spawning were hybridized with 173 base cRNA probe. The relative amount of radioactivity between 163 base (*P*-450c17L) and 134 base (*P*-450c17S) fragments, were calculated, using BAS-200 Bio-Imazing Analyzer (Fujifilm).

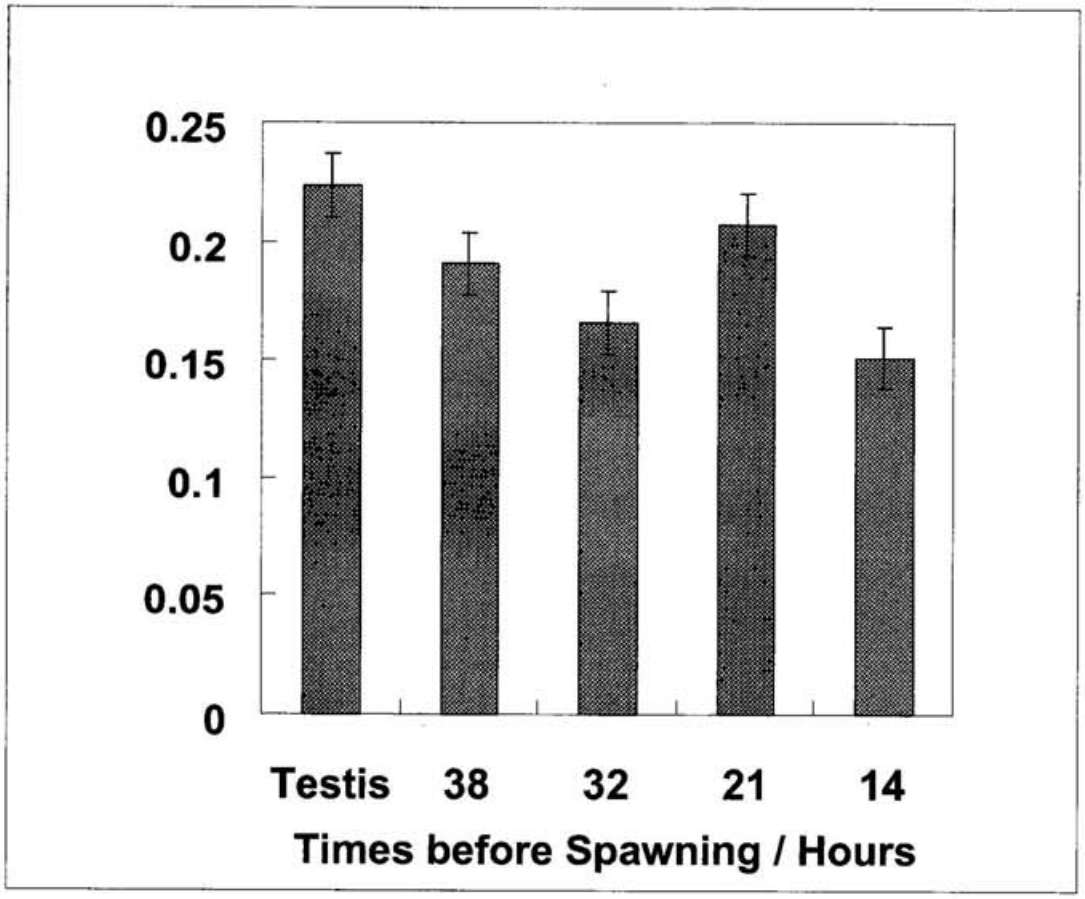

0.25
0.2
0.15
0.1
0.05
0
Testis
38
32
21
14
Times before Spawning / Hours

Fig. 30. Phylogenetic tree of vertebrate P-450c17. Phylogenetic relations were analyzed using CLUSTAL W (Thompson *et al.*, 1994). P-450c17 was divided into two groups, mammals and non-mammals.

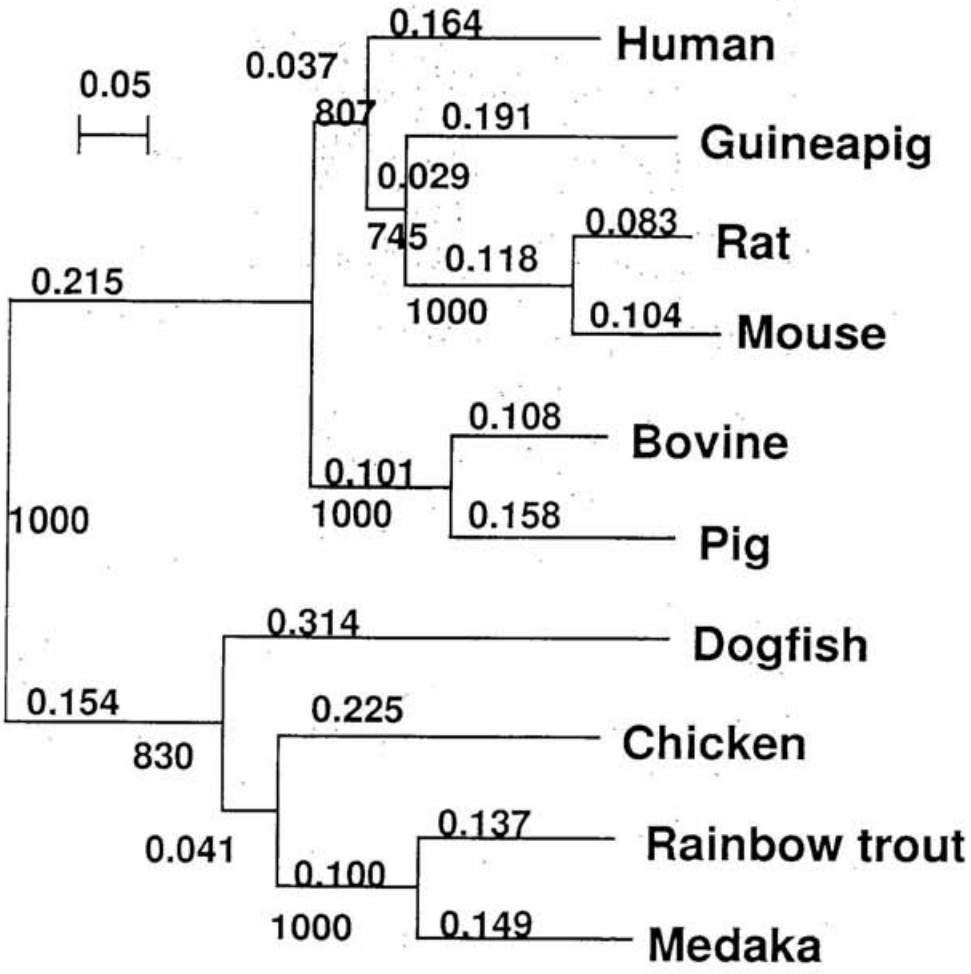
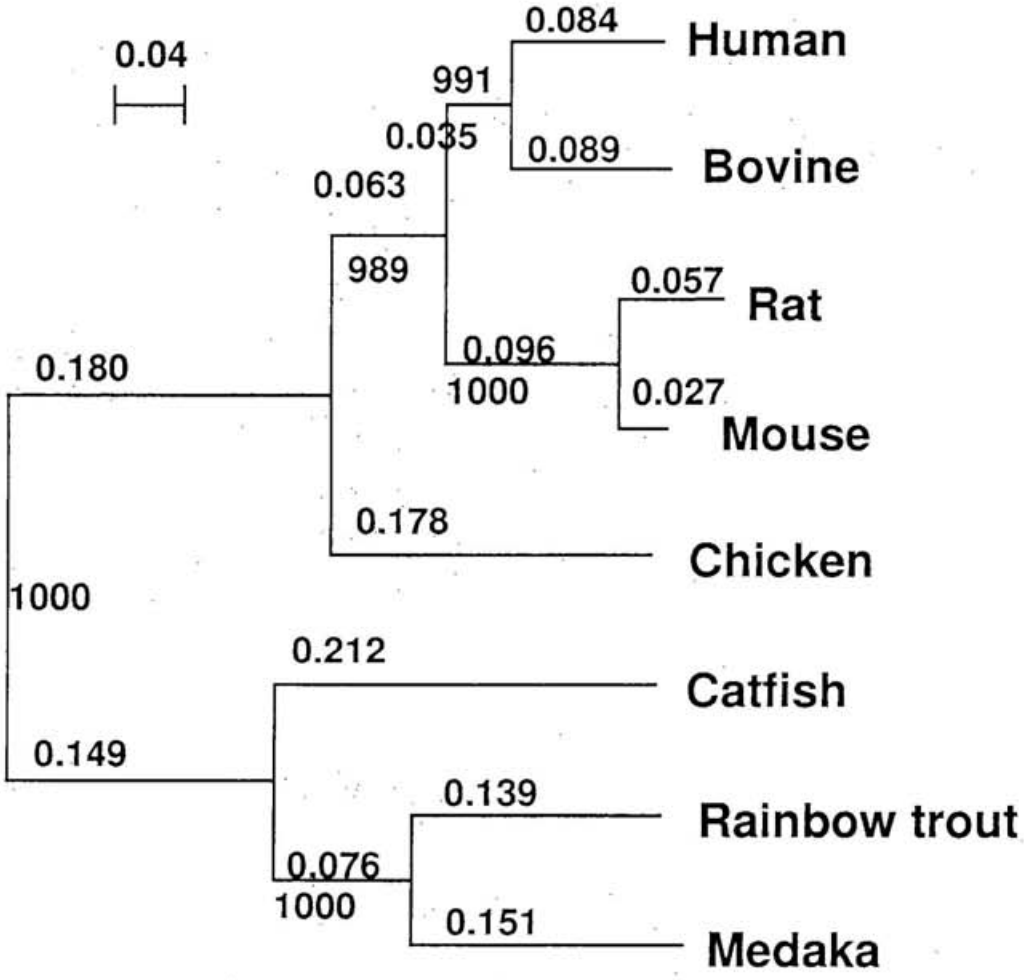

0.05
0.164
Human
0.037
807
0.191
Guineapig
0.029
745
0.083
Rat
0.118
0.215
1000
0.104
Mouse
0.108
Bovine
0.101
1000
1000
0.158
Pig
0.314
Dogfish
0.154
0.225
830
Chicken
0.137
Rainbow trout
0.041
0.100
1000
0.149
Medaka

Fig. 31. Phylogenetic tree of vertebrate P-450arom. Phylogenetic relations were analyzed using CLUSTAL W (Thompson *et al.*, 1994).


0.04
0.084
Human
991
0.035
0.089
Bovine
0.063
989
0.057
Rat
0.096
0.180
1000
0.027
Mouse
0.178
Chicken
1000
0.212
Catfish
0.149
0.139
Rainbow trout
0.076
1000
0.151
Medaka