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Isolation and expression of rainbow trout (*Oncorhynchus mykiss*) ovarian cDNA encoding 3β-hydroxysteroid dehydrogenase/Δ^{5-4}-isomerase

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Résumé

Un net changement de la stéroïdogenèse, passant de la production de testostérone à celle de 17α-hydroxyprogestérone, apparait dans les cellules ovariennes de la thèque des salmonidés, juste avant la maturation ovocytaire. Ce changement est un prérequis pour la production de 17α,20β-dihydroxy-4-pregnene-3-one (stéroïde inducteur de la maturation chez les salmonidés), par les cellules de la granulosa, durant la maturation ovocytaire. Le cytochrome P-450 portant l'activité 17α-hydroxylase/17,20-lyase (P-$450_{17\alpha}$) et la 3β-hydroxystéroïde déshydrogénase/Δ^{5-4}-isomérase (3β-HSD) sont les deux enzymes stéroïdogènes majeures impliquées dans la synthèse de la 17α-hydroxyprogestérone et de la testostérone. En utilisant des sondes ADNc de mammifère, nous avons isolé et caractérisé un ADNc complet codant pour ces deux enzymes, à partir d'une librairie d'ADNc de cellules ovariennes thèquales de truite arc-en-ciel (*Oncorhynchus mykiss*). Le clonage d'un ADNc, de 2,4 kilobase, codant pour le P-$450_{17\alpha}$, et l'expression transitoire de ce clone dans des cellules non stéroïdogènes de tumeur de rein, COS-1, de singe, ont été récemment rapportés (Sakai *et al.* 1992). Nous avons isolé un ADNc de 1,4 kilobase qui s'hybride avec l'ADNc mammalien de la 3β-HSD. L'expression de cet ADNc, dans les cellules COS-1, conduit à la production d'une enzyme capable de convertir la déhydroépiandrostérone en androsténédione. Une discussion est faite sur l'évolution des activités enzymatiques et de l'expression de la P-$450_{17\alpha}$ et de la 3β-HSD, dans l'ovaire de truite arc-en-ciel, en rapport avec la modification de la stéroïdogenèse apparaissant dans les enveloppes du follicule ovarien.

Abstract

A distinct shift in steroidogenesis from testosterone to 17α-hydroxyprogesterone occurs in the salmonid ovarian thecal cell layers immediately prior to oocyte maturation, and is a prerequisite for the production of 17α,20β-dihydroxy-4-pregnen-3-one (maturation-inducing hormone of salmonid fishes) by granulosa cells during oocyte maturation. 17α-Hydroxylase/17,20-lyase cytochrome P-450 (P-$450_{17\alpha}$) and 3β-hydroxysteroid dehydrogenase/Δ^{5-4}-isomerase (3β-HSD) are the two major steroidogenic enzymes involved in the production of 17α-hydroxyprogesterone and testosterone. Using mammalian cDNA probes, we isolated and characterized full-length cDNAs encoding these two enzymes from a rainbow trout (*Oncorhynchus mykiss*) ovarian thecal cell cDNA library. The cloning of 2.4-kilobase cDNA encoding P-$450_{17\alpha}$ and transient expres-

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sion of this clone in nonsteroidogenic monkey kidney tumor COS-1 cells have recently been reported (Sakai *et al.* 1992). We have isolated a 1.4-kilobase cDNA which is hybridized to the mammalian 3β-HSD cDNAs. Expression of this cDNA in COS-1 cells led to the production of an enzyme which is capable of converting dehydroepiandrosterone to androstenedione. In this study, enzymatic activities and expression of rainbow trout ovarian P-450$_{17\alpha}$ and 3β-HSD are discussed in relation of the steroidogenic shift occurring in the ovarian follicle layers.

Introduction

Oocyte growth and meiotic maturation in teleosts, like those in other vertebrates, are regulated by gonadotropins (Nagahama *et al.* 1993). However, these actions of gonadotropins are considered to be mediated predominantly by ovarian steroid hormones. In salmonid fishes, two ovarian steroid hormones have been implicated in the regulation of these two important processes of oogenesis: estradiol-17β for oocyte growth and 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP) for oocyte maturation (Nagahama and Adachi 1985; Nagahama 1987a). It is now established that ovarian granulosa cells are the site of production of these two steroidal mediators, but production by the ovarian follicle depends on the provision of precursor steroids by the thecal cells (two-cell type model) (Nagahama 1987b) (Fig. 1). During oocyte growth, the thecal cell layers synthesize testosterone, which is converted into estradiol-17β by aromatase activity in the granulosa cell layers. During oocyte maturation, however, the thecal cell layers synthesize 17α-hydroxyprogesterone, which is converted into 17α,20β-DP by 20β-hydroxysteroid dehydrogenase in the granulosa cell layers. Thus, a distinct shift in the salmonid steroidogenesis from testosterone to 17α-hydroxyprogesterone occurs only in the thecal cell layers immediately prior to oocyte maturation, and is a prerequisite for the growing oocytes to enter the final stage of maturation (Kanamori *et al.* 1988; Nagahama 1987b).

Ovarian steroidogenesis is regulated through changes in the relative activity of individual steroidogenic enzymes. 17α-Hydroxylase/17,20-lyase cytochrome P-450 (P-450$_{17\alpha}$) and 3β-hydroxysteroid dehydrogenase/Δ^{5-4}-isomerase (3β-HSD) are the two major steroidogenic enzymes involved in the production of 17α-hydroxyprogesterone and

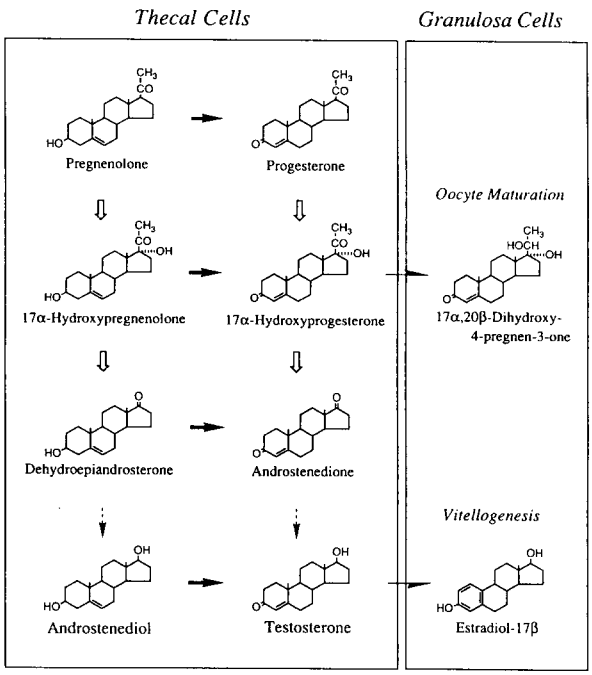

Fig. 1. Two-cell type model and possible pathways for ovarian steroidogenesis of rainbow trout: P-450$_{17\alpha}$ (17α-hydroxylase/17,20-lyase), ⇨: 3β-hydroxysteroid dehydrogenase/Δ^{5-4}-isomerase (3β-HSD), ➡: 17β-hydroxysteroid dehydrogenase (17β-HSD, ⇢: 20β-hydroxysteroid dehydrogenase (20β-HSD), →: P-450$_{\text{Aromatase}}$→.

testosterone. Thus, it is most likely that the steroidogenic shift in the salmonid thecal cell layers occurring prior to oocyte maturation is regulated by changes in the relative activity of these two enzymes. As a first step to address this question at the molecular level, we have utilized the techniques of molecular genetics to first isolate and characterize the cDNAs specific for salmonid ovarian P-450$_{17\alpha}$ and 3β-HSD. We have previously reported the trout P-450$_{17\alpha}$ cDNA cloning and enzymatic properties in nonsteroidogenic monkey kidney tumour COS-1 cells (Sakai *et al.* 1992). However, the clon-

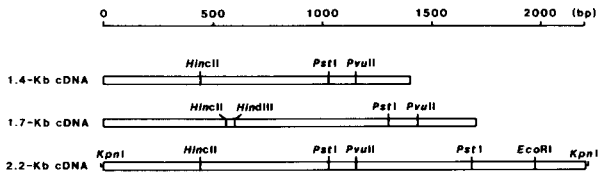

Fig. 2. Restriction map of the 1.4-kilobase cDNA insert, 1.7-kilobase cDNA insert and 2.2-kilobase cDNA insert. Restriction sites of *Kpn*I shown in 2.2-kilobase cDNA insert are in adaptor ligated into 2.2-kilobase cDNA insert, and were used for the preparation of *Kpn*I-*Hin*cII fragment and *Eco*RI-*Kpn*I fragment.

ing of cDNA encoding 3β-HSD has not been reported for nonmammalian vertebrates. This article describes the isolation of rainbow trout (*Oncorhynchus mykiss*) 3β-HSD cDNA and expression of the cDNA in COS-1 cell. These results, together with our earlier findings on the molecular cloning of rainbow trout P-450$_{17\alpha}$ cDNA, are discussed in relation to the steroidogenic shift occurring in the ovarian follicle layer prior to oocyte maturation.

Materials and methods

Mammalian 3β-HSD cDNA probes were kindly provided by Dr. J.I. Mason (U.S.A.) (rat testis 3β-HSD, Lorence *et al.* 1991; rat liver 3β-HSD, Naville *et al.* 1991; human placental 3β-HSD, Lorence *et al.* 1990). Three cDNA fragments containing the majority of the open reading frame encoding 3β-HSD were prepared: 1.1-kilobase cDNA fragment from rat testis 3β-HSD cDNA by digestion of *Eco*RI and *Hin*dIII, 1.1-kilobase cDNA fragment from rat liver 3β-HSD by digestion of *Eco*RI and *Hin*dIII, and 1.0-kilobase cDNA fragment from human 3β-HSD by digestion of *Eco*RI and *Pvu*II. Each cDNA fragment was labeled by Multiprime DNA labeling systems (Amersham) and used to screen the ovarian thecal cell cDNA library of rainbow trout under the same conditions as described by Sakai *et al.* (1992). cDNA inserts were isolated from positively hybridizing clones, and digested with *Eco*RI or *Kpn*I, then cloned into pBluescript KS(−). DNA sequencing was preformed using a 373A DNA sequencer with Dye primer thermal circular sequencing systems (Applied Biosystems Co.).

Expression of the cDNA in nonsteroidogenic monkey kidney tumour COS-1 cells was performed according to Tanaka *et al.* (1992). Metabolites from ^{14}C-labeled dehydroepiandrosterone by the cDNA transfected COS-1 cells were separated by thin layer chromatography and identified by recrystallization (Sakai *et al.* 1989). Northern blot analysis was carried out as described previously (Sakai *et al.* 1992).

Results

Approximately 1.0×10^5 plaques from the ovarian thecal cell cDNA library of rainbow trout were screened with each of the mammalian 3β-HSD cDNA probes. Sixty-four positive clones from rat testis 3β-HSD, 49 positive clones from rat liver 3β-HSD, and 71 positive clones from human placental 3β-HSD were identified. Twelve positive clones were randomly selected from 184 positive clones, the cDNA inserts were identified by southern blot analysis using rat testis 3β-HSD cDNA probe. Three different cDNA inserts in length (1.4-kilobase, 1.7-kilobase, and 2.2-kilobase) were found. Restriction maps of three cDNA inserts are different as shown in Fig. 2. The cDNA inserts were ligated into pBluescript KS(−) and partially sequenced from each end. The nucleotide sequence of 300 base from 5′-end in three cDNA inserts were almost the same, but that from 3′-end in three cDNA inserts were different (data not shown). Poly A was not found in the 1.7-kilobase cDNA insert. By northern blot analysis using 5′-end cDNA fragment (*Kpn*I-*Hin*cII fragment) and 3′-end cDNA fragment (*Eco*RI-*Kpn*I fragment) of the 2.2-kilobase cDNA insert, two different transcripts were found in poly $(A)^+$ RNA of trout post-ovulatory follicles. Therefore, it appears that the 2.2-kilobase cDNA insert is an artifact of two different cDNA connected when the cDNA library was constructed. The second cDNA in 2.2-kilobase cDNA did not bear any resemblance to 3β-HSD in partial sequence analysis (data not shown). The 1.4-kilobase cDNA insert, designated 3Bth2-10, was selected for further characterization. The complete nucleotide sequence of 3Bth2-10 and its deduced amino acid sequence will be described elsewhere.

Table 1. Recrystallization of the radioactive metabolite with the authentic androstenedione for identification

Specific activity before crystallization (cpm/mg)		101
Specific activity of crystal (cpm/mg)	1st	97
	2nd	102
	3rd	98

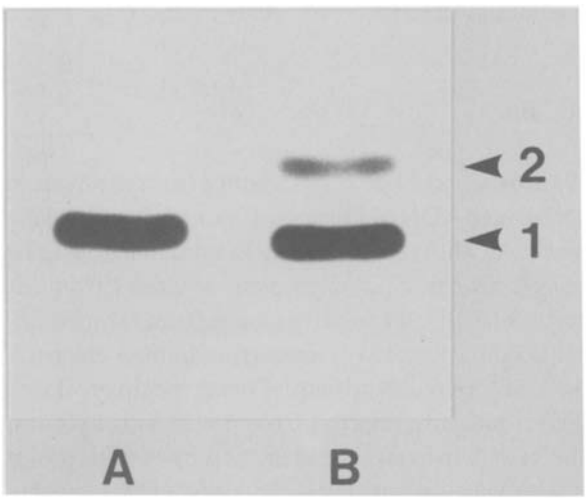

Fig. 3. Thin layer chromatographs showing 3β-hydroxysteroid dehydrogenase (3β-HSD) activity of COS-1 cells transfected with pSVL only (A) and the rainbow trout 3β-HSD expression vector (B). ^{14}C-Labeled dehydroepiandrosterone (1.0×10^5 cpm) was added to a COS-1 cell culture, and metabolites were extracted and separated on TLC. The bands corresponding to dehydroepiandrosterone (1) and androstenedione (2) are indicated.

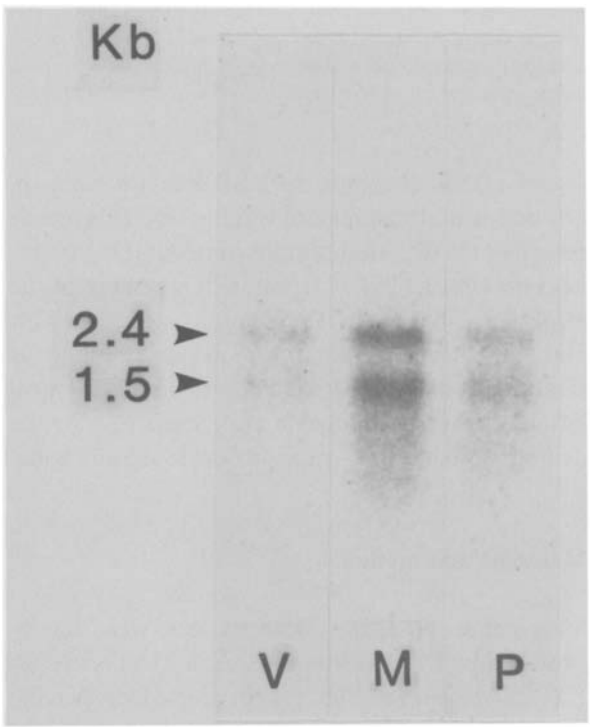

Fig. 4. Northern hybridization of rainbow trout P-450$_{17\alpha}$ (2.4-kilobase) and 3β-HSD (1.5-kilobase) to poly (A)$^+$RNA (2 μg) from three stages of ovarian follicles (V, vitellogenic follicles; M, follicles at the migrated nucleus stage; P, post-ovulatory follicles).

To determine whether the 3Bth2-10 cDNA clone encodes a protein exhibiting 3β-hydroxysteroid dehydrogenase/Δ^{5-4}-isomerase activity, the insert ligated into pSVL was used to transfect COS-1 cells. When ^{14}C-labeled dehydroepiandrosterone was used as substrate and the products were analyzed by thin layer chromatography, the major product identified as androstenedione by recrystallization (Table 1) was found in the COS-1 cell cultures with the recombinant trout 3β-HSD expression vector, but not in the cultures with pSVL only (Fig. 3).

Northern blot analysis of ovarian follicular poly (A)$^+$RNA at three stages of oogenesis with the trout 3β-HSD cDNA probe revealed the presence of a 1.5-kilobase mRNA species. To compare changes in the 2.4-kilobase transcript of P-450$_{17\alpha}$, rainbow trout P-450$_{17\alpha}$ cDNA also hybridized (Fig. 4). We confirmed the mRNA specific for rainbow trout 3β-HSD to be a single species in another experiment. Signals of the 1.5-kilobase transcripts were barely detected during vitellogenesis, markedly increased during oocyte maturation, and decreased after ovulation.

Discussion

Using the corresponding mammalian 3β-HSD cDNA probes, we have isolated and expressed, for

the first time in any nonmammalian vertebrates, cDNA specific for rainbow trout 3β-HSD. In our laboratory, three more cDNAs encoding rainbow trout ovarian steroidogenic enzymes, P-450$_{17\alpha}$, aromatase cytochrome P-450 (P-450$_{Arom}$) (Tanaka *et al.* 1992) and cholesterol side-chain cleavage cytochrome P-450 (P-450$_{SCC}$) (Takahashi *et al.* 1993) have also been characterized. Comparison of the nucleotide sequences of these three P-450 enzymes of rainbow trout with those of mammals indicates that the fish enzymes are approximately 55% homologous to those of mammals. It is of interest to know whether a similar sequence homology also exists between fish HSDs and mammalian HSDs. We are currently determining the complete nucleotide sequence of the rainbow trout 3β-HSD cDNA clone. Our observations demonstrate that mammalian cDNA probes can be used to clone gonadal and adrenal steroidogenic enzymes of various nonmammalian species.

We have used a eukaryotic expression vector system to transfect COS-1 monkey kidney tumor cells and thereby characterized the rainbow trout P-450$_{17\alpha}$ (Sakai *et al.* 1992) and 3β-HSD and their associated activities in cells that normally do not exhibit these steroidogenic functions. We also expressed the rainbow trout P-450$_{Arom}$ (Tanaka *et al.* 1992) and P-450$_{SCC}$ (Takahashi *et al.* 1993). Thus, these studies have also shown that the mammalian COS-1 cell system is useful for enzymatic studies of various fish steroidogenic enzymes.

Biosynthesis of testosterone requires both 17α-hydroxylase and 17,20-lyase activities, whereas that of 17α-hydroxyprogesterone required only 17α-hydroxylase activity. It has long been thought from many physiological studies that 17α-hydroxylase and 17,20-lyase are distinct enzymes. However, purification of mammalian testis and adrenal P-450$_{17\alpha}$ showed that a single protein mediated both activities (Nakajin and Hall 1981; Kominami *et al.* 1982; Nakajin *et al.* 1983). This was confirmed by recent findings that expression of mammalian P-450$_{17\alpha}$ cDNAs in COS-1 cells (Zuber *et al.* 1986; Namiki *et al.* 1988; Fovold *et al.* 1989; Lin *et al.* 1991), bacteria (Barnes *et al.* 1991) and yeast (Sakaki *et al.* 1989) led to production of a steroid hydroxylase which had both 17α-hydroxylase and 17,20-lyase activities. Thus, one important question is whether the fish 17α-hydroxylase and 17,20-lyase activities are catalyzed by the same protein. Molecular cloning and characterization of rainbow trout P-450$_{17\alpha}$ cDNA shows that the 17α-hydroxylase and 17,20-lyase activities reside within the same rainbow trout P-450$_{17\alpha}$ polypeptide chain (Sakai *et al.* 1992). Expression of trout P-450$_{17\alpha}$ in COS-1 cells indicates that the trout P-450$_{17\alpha}$ has more 17,20-lyase activity catalyzing 17α-hydroxypregnenolone to dehydroepiandrosterone than that catalyzing 17α-hydroxyprogrestrone to androstenedione. This characteristic may be important to explain for the synthesis of 17α-hydroxyprogesterone in trout ovarian thecal cells. Further studies on the substrate specificity of trout P-450$_{17\alpha}$ will be necessary using trout ovarian follicle homogenates.

The enzyme, 3β-HSD is a key enzyme which regulates the formation of Δ^4-3-ketosteroids from Δ^5-3β-hydroxysteroids, *i.e.*, the synthesis of the potent steroid hormones progesterone, 17α-hydroxyprogesterone, and androstenedione from their much less hormonally active precursors, pregnenolone, 17α-hydroxypregnenolone, and dehydroepiandrosterone, respectively. It has not yet been determined whether the synthesis of testosterone during oocyte growth and 17α-hydroxyprogesterone during oocyte maturation by the salmonid ovarian thecal cell layers involves either the Δ^4-steroid or the Δ^5-steroid pathway. This question is important in relation to the difference in substrate preference observed in rainbow trout P-450$_{17\alpha}$ (Sakai *et al.* 1992). It is possible that 3β-HSD may play a critical role in determining the steroidogenic pathway in the salmonid thecal cell layers. In this study, we demonstrated that the rainbow trout 3β-HSD cDNA was capable of converting dehydroepiandrosterone to androstenedione. We are currently determining whether the recombinant rainbow trout 3β-HSD can also catalyze the conversion of other Δ^5-steroids pregnenolone and 17α-hydroxypregnenolone to their corresponding Δ^4-steroids, progesterone and 17α-hydroxyprogesterone, respectively.

Isolation of cDNA clones specific for rainbow trout P-450$_{17\alpha}$ and 3β-HSD has made it possible to

determine changes in mRNA levels during follicular development and maturation in rainbow trout. The levels of P-450$_{17\alpha}$ and 3β-HSD were barely detectable during vitellogenesis, dramatically increased during oocyte maturation, and decreased markedly after ovulation. The dramatic increases in ovarian P-450$_{17\alpha}$ and 3β-HSD mRNA levels may be directly associated with the increased production of 17α-hydroxyprogesterone and 17α,20β-DP during oocyte maturation. The hormonal factor(s) responsible for the increased expression of P-450$_{17\alpha}$ and 3β-HSD during oocyte maturation remained to be determined. It is also interesting to determine whether other forms of P-450$_{17\alpha}$ and 3β-HSD mRNAs are expressed in the ovaries during vitellogenesis, a stage when testosterone and estradiol-17β are the major steroid hormones produced by ovaries.

The isolation and characterization of rainbow trout P-450$_{17\alpha}$ and 3β-HSD cDNAs will greatly facilitate further investigations of the molecular basis of the steroidogenic shift in the salmonid thecal cell layers. Resolution of the molecular events regulating this shift may provide new insight into the intracellular events regulating follicular development and maturation. Furthermore, the availability of cDNA clones specific for rainbow trout ovarian steroidogenic enzymes also provides the necessary probes for identification of steroidogenic enzyme genes of other fish species and for subsequent investigations on the molecular evolution of vertebrate steroidogenic enzymes.

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