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Characterization of Ovarian Cytochrome P450$_{C17}$ (17 α-hydroxylase/17,20-lyase) in *Rana dybowski*

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북방산 개구리 난소의 Cytochrome P450$_{C17}$ 유전자 특성

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ABSTRACT : 17 α-hydroxylase/17,20-lyase(P450$_{C17}$) is the key enzyme mediating the conversion of progesterone to 17 α-hydroxyprogesterone, ultimately to androstenedione during steroidogenesis. *R. dybowskii*'s ovarian P450$_{C17}$ cDNA was cloned to understand the regulatory mechanism of ovarian steroidogenic pathway at the molecular level in amphibian. A 2.5kb cDNA clone encoding a single open-reading frame with a 519 deduced amino acid was isolated with the screening of ovarian cDNA library. This sequence contained the three highly conserved domains as seen in P450$_{C17}$ of other species. The comparison of amino acid sequence of *Rana* P450$_{C17}$ with other animal's P450$_{C17}$ showed relatively high identity with 76% in *Xenopus*, 63% in chicken, 60% in rainbow trout, and 45% in human. Phylogenic analysis also indicated that *Rana* P450$_{C17}$ gene was evolutionary well conserved among vertebrate. Northern analysis indicated that the two different sizes of P450$_{C17}$ transcripts with approximately 2.5 and 3.6kb were detected in ovary tissue, but not in other tissues. The expression vector of *Rana* P450$_{C17}$ clearly showed the 17 α-hydroxylase activity converting the exogenous progesterone into 17 α-hydroxyprogesterone in the nonsteroidogenic COS-1 cells. Therefore, *Rana* P450$_{C17}$ cDNA is very useful to investigate the molecular mechanism of the ovarian steroidogenesis in amphibian.

Key words : P450$_{c17}$, Steroidogenesis, Ovary, Amphibian.

요 약 : 스테로이드 합성효소 중 17 α-hydroxylase/17,20-lyase(P450$_{C17}$)는 progesterone을 17 α-hydroxyprogesterone을 거쳐 androstenedione으로 변환을 담당하는 효소이다. 양서류 난소에서 스테로이드 합성의 분자적 조절과정의 연구에 사용할 목적으로 북방산 개구리(*Rana dybowskii*) 난소에서 P450$_{C17}$ cDNA를 클로닝 하였다. 북방산 개구리 난포세포의 cDNA library 검색을 통해 분리된 약 2.5kb의 cDNA는 529개의 아미노산을 가진 단일 번역틀을 가지고 있었다. 개구리 P450$_{C17}$의 아미노산 서열은 *Xenopus*와는 76%, 닭과는 63%, 그리고 사람과는 약 45%의 동일성을 보여 주였고, 동시에 진화적으로 척추동물에서 매우 잘 보존된 아미노산 서열을 가지고 있었다. 노던 분석에서 개구리의 P450$_{C17}$ 전사체는 난소에서만 2.5kb와 3.6kb 크기의 두 종류가 발견되었다. 그리고 개구리 *Rana* P450$_{C17}$ cDNA는 비스테로이드 합성 세포인 COS-1세포에서 분명한 17 α-hydroxylase/17,20-lyase 활성을 주었다. 따라서 클로닝된 개구리 P450$_{C17}$ 유전자는 양서류의 난소에서 스테로이드 합성의 분자적 기작을 연구하는데 매우 유용할 것으로 사료된다.

INTRODUCTION

In amphibian, it has been well established that the oocyte growth and maturation are regulated by the steroids produced by ovarian follicles under the control of gonadotropins(Schuetz, 1985). Many studies indicate that the estradiol(E_2) produced by follicle cells stimulate the synthesis of yolk protein in liver of female frogs(Wallace and Bergink, 1974; Schuetz, 1985) and the follicular progesterone(P_4) is also very important to stimulate the meiotic resumption before ovulation in *Xenopus* and Rana(Masui and Clarke, 1979; Maller, 1985; Kwon *et al.*, 1989). Thus, investigators have believed that two ovarian steroids are physiologically important to ovarian function in the frogs. In *Xenopus*, the steroidogenic shift in follicle was in first reported by Fortune (1983) that the medium-sized follicles mainly produced E_2, but the large follicles only secreted testosterone(T) and P_4. Steroidogenesis in *Rana* has been studied that E_2 was mainly produced by the medium-sized vitellogenic follicles, T by the intermediate-

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sized follicles, and P_4 by the fully grown follicles(Kwon *et al.*, 1991). During the development of follicles in *Rana* ovary, it appeared that the steroidogenic shift is closely related to the changes of specific steroidogenic enzyme activity(Kwon *et al.*, 1993, Kwon & Ahn, 1994 Ahn *et al.*, 1996).

Steroid 17α-hydroxylase converts pregnenolone to 17α-hydroxypregnenolone and converts progesterone to 17α-hydroxyprogesterone. These hydroxylated steroids may then be converted to dehydroepiandrosterone and androstenedione. These two enzyme activities are carried out by a single enzyme called cytochrome $P450_{C17}$ in all species revealed the nucleotide sequence of its cDNA until now(Hinshellwood *et al.*, 1993). In amphibian, the change of steroidogenesis enzyme activity during the development of ovarian follicleswas reported(Kwon *et al.*, 1993; Kwon & Ahn, 1994) and the steroidogenic shift in the cultured ovarian follicle at breeding season was also studied(Ahn *et al.*, 1993; 1996). It is thus strongly suggested that the increase of P_4 concentration at breeding season, which is a pivotal role in the induction of meiotic maturation, be closely related to the decrease of $P450_{C17}$ gene expression in frogs. To completely elucidate the steroidogenic shift during amphibian follicle development, it needs to examine the expression of gene encoding steroidogenic enzymes.

Thus, afull-length of cDNA encoding frog $P450_{C17}$ from theovarian cDNA library of *Rana dybowskii* has been cloned and the result has been reported in this paper.

MATERIALS AND METHODS

1. Construction of *Rana dybowskii* Ovarian Follicle Cell Layer cDNA Library

All other molecular biology techniques except the indicated method used in this experiment were performed as described previously(Maniatis *et al.*, 1989). The adult female frogs, *Rana dybowskii*, during hibernation were collected under a rock of mountain stream in area of Kwangju, Korea and kept at 4℃ before use. After injection of frog pituitary homogenate(0.05 arbitrary unit, FPH) to stimulate steroidogenesis(Kwon *et al.*, 1989), the follicles of different stage were obtained from the ovaries. The follicular cell layers were prepared by squeezing out the yolk part of follicles and rinsed three times with phosphate-buffered saline (PBS, pH 7.4). Total and poly(A)$^+$RNA were isolated by guanidium thiocyanate-CsCl gradient ultracentrifugation and oligo d(T) chromatography method, respectively. About 5mg of poly(A)+ RNA was converted into a double-stranded cDNA with cDNA synthesis kit(Pharmacia) and ligated with *EcoRI-NotI* linker. Linker ligated-cDNA was inserted into lambda ZAP Ⅱ vector(Stratagene) and added into phage lysate according to supplier's protocols. The average number of cDNA insert efficiency ligated with lambda phage DNA was $\sim 1\times10^6$/ug and the average size of cDNA insertwas ~1.8kb. The constructed cDNA library was amplified and used for the screening of frog $P450_{C17}$ cDNA.

2. Probe Preparation

From the conserved domain of the deduced amino acid of $P450_{C17}$ cDNA known in other species, two sets of degenerated oligomers were designed for polymerase-chain reaction(PCR). Primer set 1 is 5'-GACATMTTTGGGGCHG GNGTGGAGACC-3' and 5'-SCCNGCCCCAAAVGGCARGTARCT-3'. Primer set 2 is 5'-GGIGGIGGNVTNGARAC-3' and 5'-IATIAGIRKNGMNACNGG-3'. PCR reaction with *Rana dybowskii* follicular cell cDNA library was done with thermal cycler and AmpliTaq polymerase (Perkin-Elmer). Primer set 1 and 2 was produced about 420bp and 220bp DNA fragments, respectively, as expected. In the primer set 2, *EcoRI* site was tagged and used for the cloning of 220 bp of PCR product into pBluescript vector(Stratagene). The sequencing of 220bp of PCR product indicated that it encoded the highly homologue sequence with $P450_{C17}$ of other species(data is not shown). The PCR cloned 220bp fragment was labeled with ^{32}P by random primed method(Feinberg and Vogestein, 1983) and used with probe for isolating frog $P450_{C17}$ cDNA.

3. Screening, DNA Sequencing and Analysis

Approximately 2.0×10^5 colonies of *Rana dybowskii* follicular cell cDNA library were plated and transferred to nylon membrane(Hybond-N, Amersham). Plaque hybridization and the conversion of positive phage clone to plasmid vector were done according to manufacture's protocol(Stratagene).

The cDNA sequence was commercially determined with an Applied Biosystems DNA sequencer(Prism 877 model). All nucleotide sequence analysis, including primer design, finding of open-reading frame, the sequence comparison and phylogenic analysis using ClustalW algorithm, was performed with MacVector 8.0 program(Accelyrs).

4. Northern Blot Analysis

Total RNAs were isolated from various tissues of the adult frogs captured in spring by guanidium thiocyanate-phenol-chloroform extraction method(Chomczynski and Sacchi, 1987). Thirty mg of total RNAs were separated on 1.5% formaldehyde denatured agarose gel and transferred to nylon membrane. A full size of frog $P450_{C17}$ cDNA was used as a probe. Northern filters were hybridized with probe, and then the washed filters were exposed to X-ray film(Kodak) for 2 days.

5. Enzymatic Activity of *Rana* $P450_{C17}$

A 1.9 kb of *Rana* $P450_{C17}$ cDNA, including a complete open-reading frame and a poly(A)$^+$ additional site was inserted into *Hind*Ⅲ and *Pst*I site of pCDM8 expression vector(GIBCO-BRL). The resulted recombinant expression vector was purified by CsCl-ultracentifugation. COS-1 cells were cultured onto a 35 mm dish with DMEM(GIBCO-BRL) plus 10% fetal calf serum. Transfection of *Rana* $P450_{C17}$ expression vector into COS-1 cell was carried out by using Lipofectamine(GIBCO-BRL) according to manufacture's protocol. After 48hr culture, 1 μmCi of ^{14}C labeled-progesterone(98Ci/mol, Amersham) was added. After incubation for 7 and 14hr, the steroids containing into media were extracted. The steroid metabolites were analyzed with thin-layer chromatography(TLC) as described previously(Sakai *et al.*, 1992).

RESULTS AND DISCUSSION

To isolate the cDNA encoding $P450_{C17}$ in frog, the cDNA library from poly(A)$^+$ RNA isolated in the ovarian follicle cell layers of hibernated frogs, *Rana dybowskii*, was constructed. The hibernated frog's ovary substantially contained a very small amount of total RNA and poly(A)$^+$ RNA(data is not shown). It seems to be reflected on a lower cellular activity during hibernation. So, FPH was injected into the female frogs to stimulate the steroidogenesis and then placed at room temperature for a certain period. The sufficient poly(A)$^+$ RNA from the follicle cell layer of ovaries was isolated, pooled, and constructed cDNA library as described in Materials and Methods. It suggests that ovarian function and steroidogenesis may be directly controlled by gonadotropin at even the hibernation period in frog.

Ten positive clones were identified by screening approximately 2×10^5 plaques using a PCR cloned partial cDNA fragment of $P450_{C17}$ as a probe(Details see Materials and Methods). The longest clone with approximately 2.5 kb insertwas chosen and sequenced. The complete nucleotide sequence of frog $P450_{C17}$, as shown in Fig. 1, contains a single open reading frame of 1557 bp encoding 519 amino acid, a relatively long 5'-untranslated region of about 800 bp and 183 nucleotides of 3'-untranslated region. A putative poly(A)$^+$ addition signal(ATTAAA) is found 163 nucleotides downstream from the stop codon and 14 nucleotides upstream from the poly(A) track. The sequence of *Rana* $P450_{C17}$ cDNA has been deposited with the GenBank data library under Accession number AF042278.

The deduced amino acid sequence of the *Rana* $P450_{C17}$ was compared with that of *Xenopus*(AAG42003), chicken(P12394), trout(P30437), human(P05093), and rat(P11715). The result of comparison with ClustalW algorithm is shown in Fig. 2. The identity of amino acid sequence is greatest between frog and *Xenopus* $P450_{C17}$(76%), closely followed by chicken $P450_{C17}$ (625%). There is a significant drop in homology when comparing frog enzyme to human and rat $P450_{C17}$(45 and 44%, respectively). As expected, the two highly conserved sequences

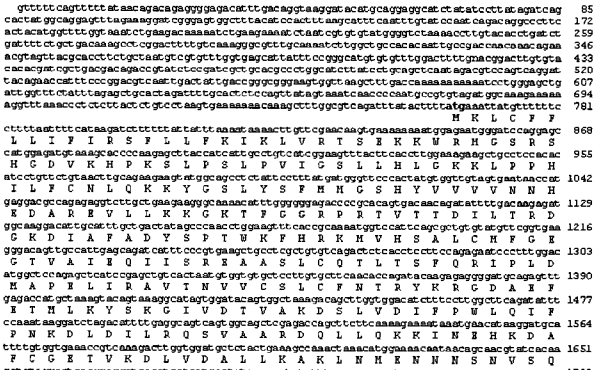

Fig. 1. The nucleotide sequence and deduced amino acid sequence of *Rana* $P450_{C17}$. The amino acid sequence deduced from an open reading frame is shown below the nucleotide sequence. Numbers in left indicate the position of nucleotide. The putative poly(A)$^+$ addition signal, ATTAAA, is underlined. The sequence has been deposited in the GenBank as accession number AF042278.

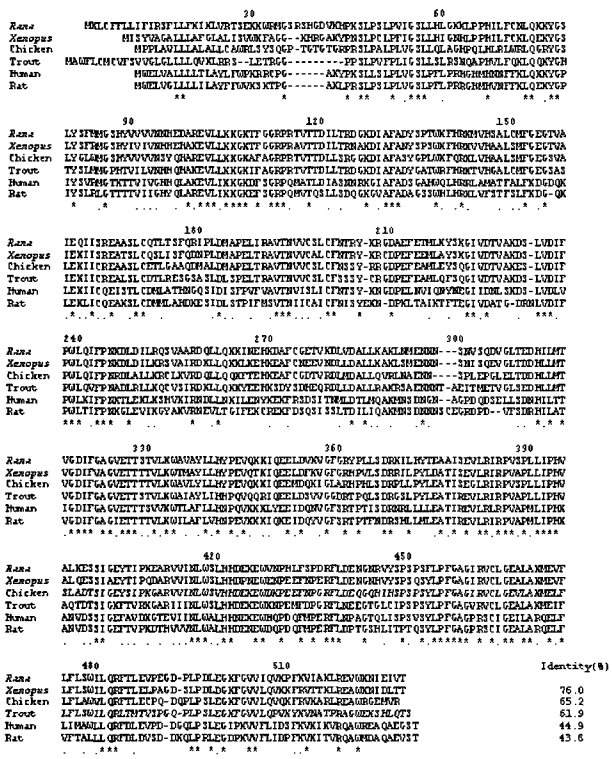

Fig. 2. The comparison of the deduced amino acid sequence of *Rana* P450$_{C17}$ with other vertebrate P450$_{C17}$. The aligned sequences of P450$_{C17}$ are *Xenopus*(AAG42003), chicken(P12394), trout(P30437), human(P05093), and rat (P11715). The sequence alignment was performed with ClustalW algorithm. The three conserved domain identified in P450$_{C17}$ gene family, the conserved P450$_{C17}$ sequence, the conserved Ozol's tridecapeptide region, and the conserved heme biding domain are located at amino acids 325-348, amino acid 374-401 and amino acid 464-483, respectively. These parts are highlighted with box. The gap indicates sites where the comparable amino acids are not present. The identical sequence is marked with asterisk and the homologous sequence is marked with dot below the amino acid sequence. The percentage of amino acid identity between frog and other species P450$_{C17}$ is noted in the end of sequence alignment.

of different P450$_{C17}$ gene families, the putative heme binding domain(Gotoh *et al.*, 1983) and Ozols tridecapeptide sequence (Ozols *et al.*, 1985) show high degree homology within P450$_{C17}$ from these six species. Also the highly homologous P450$_{C17}$ sequence noted by Ono *et al.*(1988) is present in *Rana* P450$_{C17}$. This comparison suggested that the cloned *Rana* P450$_{C17}$ cDNA is well conserved the feature of P450$_{C17}$ enzyme among vertebrate.

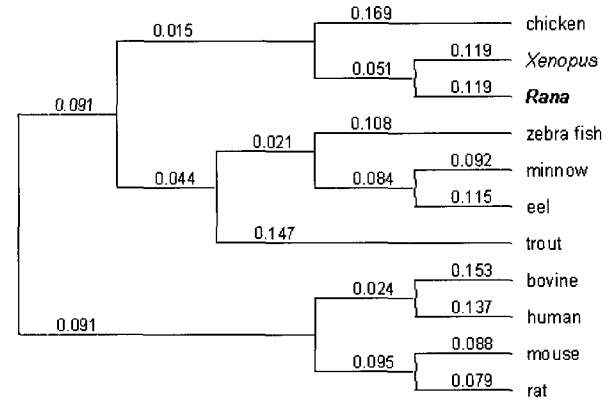

Fig. 3. Phylogenic relationship among vertebrate P450$_{C17}$. The tree was constructed by MacVector program(ver. 8.0), using the neighbor-joining method. GenBank accession numbers of P450$_{C17}$ enzymes are followed; bovine, P05185; chicken, P12394; eel, AAR88432; human, P05093; minnow, CAC38768; mouse, P27786 rat, P11715; trout, P30437; zebra fish, XP092837; *Xenopus*, AAG42003. The number marked in phylogram indicates the distance between nodes shown in phylogenetic units. A value 0.1 corresponds to a difference of 10% between two species.

For a detailed analysis of the relationship between vertebrate P450$_{C17}$ enzymes, a phylogenic analysis was performed. The amino acid sequences of 11 well-characterized vertebrate P450$_{C17}$ were aligned using the neighbor-joining method(Fig. 3). The result was generally consistent with the previous analysis(Sakai *et al.*, 1992; Arlt *et al.*, 2002). The frog P450$_{C17}$ is evolved from fish P450$_{C17}$ and diverged according to the divergence of frog species.

Northern analysis showed that the total RNA of ovary tissue only hybridized with the frog P450$_{C17}$ cDNA probe, but other tissues such as liver, brain and kidney did not transcribe the mRNA of frog P450$_{C17}$(Fig. 4). Two mRNAs of P450$_{C17}$ with 2.5 and 3.6kb were detected in the ovary tissues. The smaller size, 2.5 kb of mRNA, may be a final transcript for the translation of frog P450$_{C17}$ and 3.6kb mRNA may be a primary transcript of P450$_{C17}$. Alternatively, it is possible that two forms of frog P450$_{C17}$ gene transcripts may be produced by an alternative splicing or the use of two polyadenylation signal, as well established other steroidogenic enzyme genes such as aromatase in human (Dooby *et al.*, 1990) and rat(Dooby *et al.*, 1991; Leophart *et al.*, 1990). And it seems to be reflected on a different transcriptional regulation in frog P450$_{C17}$ gene expression unlike other species.

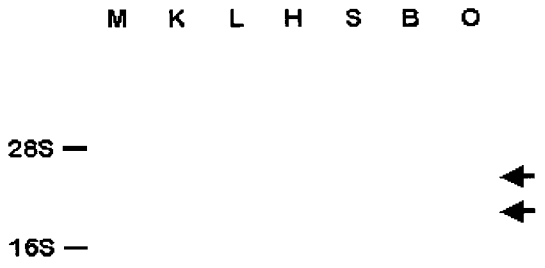

Fig. 4. Northern blot analysis of *Rana* $P450_{C17}$ gene expression. Total RNAs were isolated from muscle, kidney, liver, heart, stomach, brain, and ovary of the adult frog captured at spring and probed as described in the Materials and Methods. Numbers in the right panel indicate the position of ribosomal RNAs. Arrows mark the position of 2.5kb and 3.6kb transcripts. M: muscle, K: kidney, L: liver, H: heart, S: stomach, B: brain, O: ovary.

After insertion of the full-length *Rana* $P450_{C17}$ cDNA into pCDM8 expression vector, this plasmid was transfected into COS-1 cells. The activity of the expressed $P450_{C17}$ enzyme was examined by adding the exogenous progesterone. As shown in Fig. 5, the exogenous progesterone was converted to 17α-hydroxyprogesterone at 7hr-culture group and its amount is clearly increased in a 14hr-culture group. In addition, the androstenedione band was appeared with very weak intensity in a 14hr-culture extract(Data is not shown). The same result was confirmed by

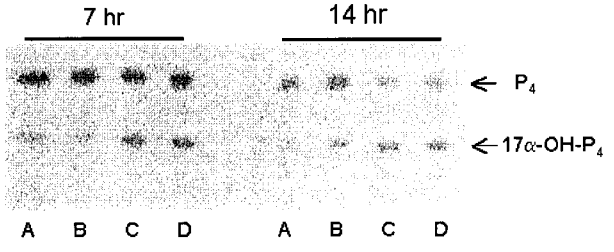

Fig. 5. 17 α-hydroxylase and 17,20-lyase activity of the cloned *Rana* $P450_{C17}$ in COS-1 cells. The expression vector was transfected into COS-1 cell and incubated with ^{14}C-progesterone. After culture for 7 and 14hr, steroids were extracted from media. The steroid metabolites were separated onto thin-layer chromatography and exposed onto BAS reader(Fuji). A: COS-1 cell only, no transfection; B: pCDM8 vector only, no $P450_{C17}$ cDNA insert; C and D: *Rana* $P450_{C17}$ expression vector. The arrows in left panel indicate the position of P_4 and 17 α -hydroxyprogesterone.

the steroid radioimmunoassay(Data is not shown). Thus, it means that the cloned cDNA has not only 17α-hydroxylase but also 17,20-lyase enzyme activity. The same enzymatic activity of $P450_{C17}$ expressed in COS-1 cells has already been reported in other species, human(Lin *et al.*, 1991), rat(Fevold *et al.*, 1989), and trout(Sakai *et al.*, 1992). It thus indicates that the cloned cDNA encodes the frog $P450_{C17}$ enzyme.

Therefore, the cDNA encoding frog $P450_{C17}$ in *Rana dybowskii* has been successfully identified and characterized. It is very valuable to study the mechanism and regulation of amphibian steroid synthesis related to amphibian reproduction cycle at molecular level. This work will ultimately be elucidated the mechanism of steroidogenic shift occurred during follicle growth and the control mechanism of the plasma progesterone level involved in the meiotic resumption of oocyte maturation in amphibian.

Ovarian progesterone is the natural trigger of amphibian oocyte maturation, known to the resumption of meiosis arrested at diplotene stage(Masui & Clarke, 1979). It has been well established that progesterone induced the activation of maturation promoting factor(MPF) via membrane-bound progesterone receptor, but not nuclear progesterone receptor(Maller, 1985). However, the recent evidence has strongly indicated that androgens, rather progesterone, are the dominant physiological mediators of oocyte maturation in *Xenopus*(Lutz *et al.*, 2001). Progesterone is rapidly converted to androstenedione in the isolated oocyte, indicating the involvement of $P450_{C17}$ as the primary enzyme mediating this process. It has suggested that $P450_{C17}$ might be expressed in the oocyte themselves, but rather than in follicular cells. Therefore, it needs to examine the expression of $P450_{C17}$ in *Rana* oocyte because it has been well established that the oocyte maturation of *Rana* species is also mediated by progesterone.

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