Volume 301, number 1, 60–64 FEBS 10916 April 1992

Rainbow trout cytochrome P-450$_{c17}$ (17α-hydroxylase/17,20-lyase)

cDNA cloning, enzymatic properties and temporal pattern of ovarian P-450$_{c17}$ mRNA expression during oogenesis

Noriyoshi Sakai[a], Minoru Tanaka[a], Shinji Adachi[a], Walter L. Miller[b] and Yoshitaka Nagahama[a]

[a]*Laboratory of Reproductive Biology, National Institute for Basic Biology, Okazaki 444, Japan* and [b]*Department of Pediatrics and the Metabolic Research Unit, University of California, San Francisco, CA 94143, USA*

Received 23 January 1992

A cDNA clone encoding cytochrome P-450$_{c17}$ (17α-hydroxylase/17,20-lyase) was isolated from a rainbow trout ovarian follicle cDNA library. The cDNA contained an open reading frame of 1,542 nucleotides encoding a protein of 514 amino acid residues. The amino acid sequence of trout P-450$_{c17}$ shows a much greater homology with chicken P-450$_{c17}$ than with that of human, bovine and rat. The trout P-450$_{c17}$ expressed in non-steroidogenic mammalian COS-1 cells showed both 17α-hydroxylase and 17,20-lyase activities. The cDNA only hybridized to a single species of mRNA (2.4 kb) isolated from rainbow trout ovaries; the 2.4 kb transcripts were abundant in trout ovaries during the later stages of oogenesis.

Rainbow trout; Cytochrome P-450$_{c17}$; cDNA cloning; Expression in COS-1 cell

1. INTRODUCTION

In vertebrates, the actions of gonadotropins on regulating germ cell development and maturation are considered to be mediated predominantly by gonadal steroid hormones. In salmonid fishes, two biologically important steroidal mediators of oocyte growth (estradiol-17β) and maturation (17α,20β-dihydroxy-4-pregnen-3-one; 17,20-DP) have been identified. 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 cell (two-cell type model) [1–4]. A dramatic switch in the salmonid steroidogenesis from testosterone to 17α-hydroxyprogesterone occurs only in the thecal cell layers immediately prior to oocyte maturation [5]. The formation of testosterone requires the activities of both 17α-hydroxylase and 17,20-lyase, whereas 17α-hydroxyprogesterone requires only 17α-hydroxylase. Recent studies of mammalian cytochrome P-450$_{c17}$ have shown that P-450$_{c17}$ is a single enzyme mediating both 17α-hydroxylase and 17,20-lyase activities in the synthesis of steroid hormones [6–11]. Therefore, it is of great interest to investigate the molecular mechanisms responsible for the differential regulation of these two enzymatic activities of P-450$_{c17}$ in thecal cell layers immediately prior to oocyte maturation.

To initiate these studies in salmonid fish, we cloned a full-length cDNA encoding cytochrome P-450$_{c17}$ from a rainbow trout (*Oncorhynchus mykiss*) ovarian thecal cell layer cDNA library. The enzymatic activities catalyzed by rainbow trout P-450$_{c17}$ were determined by expressing the cDNA in transiently transfected non-steroidogenic COS-1 cells. The expression of P-450$_{c17}$ mRNA was also examined in trout ovarian follicles at different stages in oogenesis.

Correspondence address: Y. Nagahama, Laboratory of Reproductive Biology, National Institute for Basic Biology, Okazaki 444, Japan. Fax: (81) (564) 53-7400

2. MATERIALS AND METHODS

2.1. *Cloning and sequencing*

Three-year-old rainbow trout were obtained from Samegai Trout Hatchery in 1990. Thecal cell layers were isolated from ovarian follicles at the mid-vitellogenic stage (oocyte diameter, 3.54 mm) with fine watchmaker's forceps. A cDNA library from ovarian thecal cell layer poly(A)$^+$ R.N.A was constructed as described previously [12]. A human cytochrome P-450$_{c17}$ cDNA [13] was digested at bases 828 and 1,458 with *Nsp*I and *Pvu*II to yield a 630 bp fragment which was subcloned into M13mp19. The human cDNA was labeled as described previously [12] and used to screen the ovarian thecal cell cDNA library of rainbow trout under the following conditions: 5× SSC (0.75 M NaCl/0.075 M sodium citrate), 35% (v/v) formamide, 200 μg/ml of denatured herring sperm DNA, 5× Denhardt's solution (0.1% polyvinylpyrrolidone/0.1% ficoll type 400/0.1% bovine serum albumin fraction V), 0.2% SDS, 10% dextran sulfate at 42°C. The cDNA inserts from positively hybridizing clones, were isolated and digested with *Kpn*I. The *Kpn*I fragments were rendered blunt-ended with Klenow polymerase, then cloned into the *Sma*I site of pBluescript KS(−). DNA sequencing was performed by using ExoIII/Mung bean nuclease deletion system (Takara Co.) [14] and a 373A DNA sequencer with Dye

Published by Elsevier Science Publishers B.V.

primer or Dye Deoxy Terminator thermal circular sequencing systems (Applied Biosystems Co.).

2.2. *COS-1 cell expression*

The rainbow trout $P\text{-}450_{17}$ expression vector was constructed by ligating the 2,081 bp cDNA fragment which was cut from the *Kpn*I fragment by digestion with *Sac*I into pSVL, an expression vector using the SV40 late promoter (Pharmacia LKB). 15 μg of the recombinant plasmid were transfected to 3×10^5 COS-1 cells plated onto a 60 mm tissue culture dish in 3 ml culture medium according to Tanaka et al. [12]. Thin layer chromatography (TLC) analysis of catalytic properties of trout $P\text{-}450_{17}$ was carried out using ^{14}C-labelled pregnenolone or progesterone as substrate as described previously [15].

2.3. *Northern blot analysis*

Northern blot analysis was carried out as described previously [12]. The 2,081 bp cDNA fragment as a probe was labelled by Multiprime DNA labelling systems (Amersham), and hybridization was performed under the following conditions: 5× SSC, 50% formamide, 200 μg/ml of denatured herring sperm DNA, 5× Denhardt's solution, 0.2% SDS at 42°C.

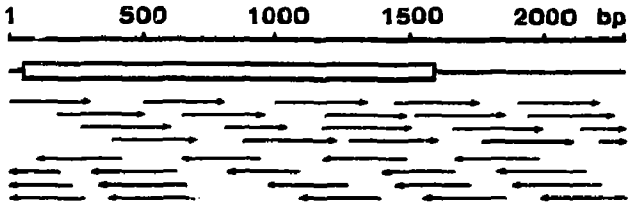

Fig. 1. Sequencing strategy for λth3-7. Location of the open reading frame is shown by an open box. Each arrow indicates the direction and extent of sequencing.

```
GTGACACTCTGATAACGACGCCAGCCCCCTCTCGGCCCAACATACAGCATGGCGTGGTTTTTTGTGCATGTGTGTATTCTCTGTGGTCGGG   89
                                                M  A  W  F  L  C  M  C  V  F  S  V  V  G    14

CTGGGTCTACTGCTCTTGCAGGTGAAACTTCGAAGGTCCCTGGAGACCAGAGGGGGCCCTCCGAGCCTGCCCGTGTTCCCACTCATCGGG  179
 L  G  L  L  L  L  Q  V  K  L  R  R  S  L  E  T  R  G  G  P  P  S  L  P  V  F  P  L  I  G   44

AGCCTGTTGAGCCTGCGAAGCAATCAGGCACCGCATGTCCTCTTCCAGAAGCTGCAGCAGAAATATGGACATACCTATTCTCTAATGATG  269
 S  L  L  S  L  R  S  N  Q  A  P  H  V  L  F  Q  K  L  Q  Q  K  Y  G  H  T  Y  S  L  M  M   74

GGCCCACACACAGTCATCCTTGTCAACCACCACCAGCACGCCAAAGAGGTCCTCCTTAAAAAGGGGAAAATCTTTGCAGGAAGACCCAGA  359
 G  P  H  T  V  I  L  V  N  H  H  Q  H  A  K  E  V  L  L  K  K  G  K  I  F  A  G  R  P  R   104

ACAGTAACCACAGACCTGTTGACGAGGGACGGTAAAGATATTGCCTTTGCAGACTACGGTGCCACTTGGAGATTTCATCGTAAAACAGTG  449
 T  V  T  T  D  L  L  T  R  D  G  K  D  I  A  F  A  D  Y  G  A  T  W  R  F  H  R  K  T  V   134

CATGGAGCCTTGTGTATGTTTGGTGAGGGCTCTGCCTCCATCGAGAAGATTATCTGTAGGGAAGCTCTCTCCCTGTGTGACACTCTGCGA  539
 H  G  A  L  C  M  F  G  E  G  S  A  S  I  E  K  I  I  C  R  E  A  L  S  L  C  D  T  L  R   164

GAGTCAGGAAGTGCATCGTTGGACTTGTCCCCAGAGCTGACAAGAGCTGTCACCAACGTGGTCTGCTCTCTCTGCTTCAGCTCCTCCTAT  629
 E  S  G  S  A  S  L  D  L  S  P  E  L  T  R  A  V  T  N  V  V  C  S  L  C  F  S  S  S  Y   194

TGCCGCGGCGACCCCGAGTTTGAGGCCATGCTGCAGTTCAGCCAGGGCATCGTGGATACGGTCGCTAAGGACAGTCTGGTGGACATCTTC  719
 C  R  G  D  P  E  F  E  A  M  L  Q  F  S  Q  G  I  V  D  T  V  A  K  D  S  L  V  D  I  F   224

CCATGGCTACAGGTCTTCCCCAACGCAGACCTGCGTCTCCTAAAGCAGTGTGTGTCAATCAGAGACAAGCTGCTTCAGAAAAAATACGAG  809
 P  W  L  Q  V  F  P  N  A  D  L  R  L  L  K  Q  C  V  S  I  R  D  K  L  L  Q  K  K  Y  E   254

GAACACAAGTCGGACTACAGCGATCATGAACAGAGAGACCTCCTGGACGCCCTGCTAAGGGCCAAACGCAGTGCTGAGAACAACAACACT  899
 E  H  K  S  D  Y  S  D  H  E  Q  R  D  L  L  D  A  L  L  R  A  K  R  S  A  E  N  N  N  T   284

GCCGAGATCACCATGGAGACCGTGGGCCTTAGTGAAGACCACCTGCTCATGACCGTGGGTGACATATTTGGAGCCGGAGTGGAAACCACG  989
 A  E  I  T  M  E  T  V  G  L  S  E  D  H  L  L  M  T  V  G  D  I  F  G  A  G  V  E  T  T   314

TCAACAGTCCTAAAATGGGCAATTGCCTACCTCATCCACCATCCACAGGTACAACAGCGAATTCAGGAGGAACTGGACAGTGTGGTGGGG 1079
 S  T  V  L  R  W  A  I  A  Y  L  I  H  H  P  Q  V  Q  Q  R  I  Q  E  E  L  D  S  V  V  G   344

GGAGACAGAACCCCCCAGCTCAGTGACAGGGGGAGCCTGCCCTACCTGGAGGCCACCATTAGAGAGGTGCTACGCATCCGGCCTGTTGCC 1169
 G  D  R  T  P  Q  L  S  D  R  G  S  L  P  Y  L  E  A  T  I  R  E  V  L  R  I  R  P  V  A   374

CCTCTACTCATCCCCCATGTAGCCCAAACAGACACCAGTATTGGCAAGTTCACTGTTAGGAAAGGTGCTCGCATCATCATCAACTTGTGG 1259
 P  L  L  I  P  H  V  A  Q  T  D  T  S  I  G  K  F  T  V  R  K  G  A  R  I  I  I  N  L  W   404

TCCCTGCATCACGATGAGAAGGAGTGGAAGAACCCAGAGATGTTTGACCCTGGTCGCTTCCTGAACGAGGAGGGGACAGGTCTGTGCATC 1349
 S  L  H  H  D  E  K  E  W  K  N  P  E  M  F  D  P  G  R  F  L  N  E  E  G  T  G  L  C  I   434

CCCTCGCCCAGCTACTTGCCGTTTGGGGCAGGGGTGAGAGTGTGTCTGGGGGAGGCGCTGGCTAAGATGGAGATCTTCCTCTTCCTCTCC 1439
 P  S  P  S  Y  L  P  F  G  A  G  V  R  V  C  L  G  E  A  L  A  K  M  E  I  F  L  F  L  S   464

TGGATCCTTCAGCGTTTAACTATGACCGTGTCCCCGGGCCAGCCACTGCCCAGCCTGGAAGGAAAGTTTGGGGTAGTCCTCCAGCCGGTC 1529
 W  I  L  Q  R  L  T  M  T  V  S  P  G  Q  P  L  P  S  L  E  G  K  F  G  V  V  L  Q  P  V   494

AAATACAAGGTCAATGCCACACCAAGAGCAGGCTGGGAGAAGAGCCATCTCCAGACCTCTTAGACCCCCCCCCCCCTCCACCCTTAACGA 1619
 K  Y  K  V  N  A  T  P  R  A  G  W  E  K  S  H  L  Q  T  S  *                              514

ATGACATGCATGGGTGTTAATGTTTAAACACTAATAGTGGTGCATTTGAATTGACCTTGGAATCTCATAGCTCCAGTTGGTCAAGTTGTG 1709

AAGATGATATAAATGCGTTCTAGTGCTTTTGAGGTTGGTACAACTCTACAATGAATTAGATTTTTAGCTAGAGCGCTAAGCAAGATAGTG 1799

TTGCTAATAATAAGTTGGTTAACTAGCTGACAATGTGTCAAACCATAGACTGTATTGGTCAAAGTAGCTTCTTTATCCACATAGCTAAAG 1889

GCATTGTTTTACCATCTTTTGGTCGCAAAGGTGACAGGTCATTGATGAAACGTCAGAACTCGATGATACTCAGGTATCATCATTCTGGCC 1979

TGAGTTTTTCACTTGGAACTTGGAGGGCATTGACTTGGAGGGCATTGTACTGGCTATTTTGGAGTCGGGAACTCGTATTTCCACATTTCA 2069

GAGAGCTCTCGAATGCACCATAGAACATGGAAACAGGCGTGAACTAAGGTTGTGATACATTACGCAGAGACAGCCCTTGTAATGACTGCT 2159

ATTTTTCACTGTATTGATGTTGAACAATGCTGTATATTTTTTTGCCTTAATGAGCTGTACTAATGTATTCCGTTACTACAGTTTTACATA 2249

ATACTGTCTTTCAGTGATGAATAAAAAATCATATGGCAAAAAAAAAAAAAA                                          2300
```

Fig. 2. Nucleotide and deduced amino acid sequences of rainbow trout ovarian $P\text{-}450_{17}$. Amino acid sequence deduced from an open reading frame is shown below the nucleotide sequence. The 'AATAAA' polyadenylation signal is underlined.

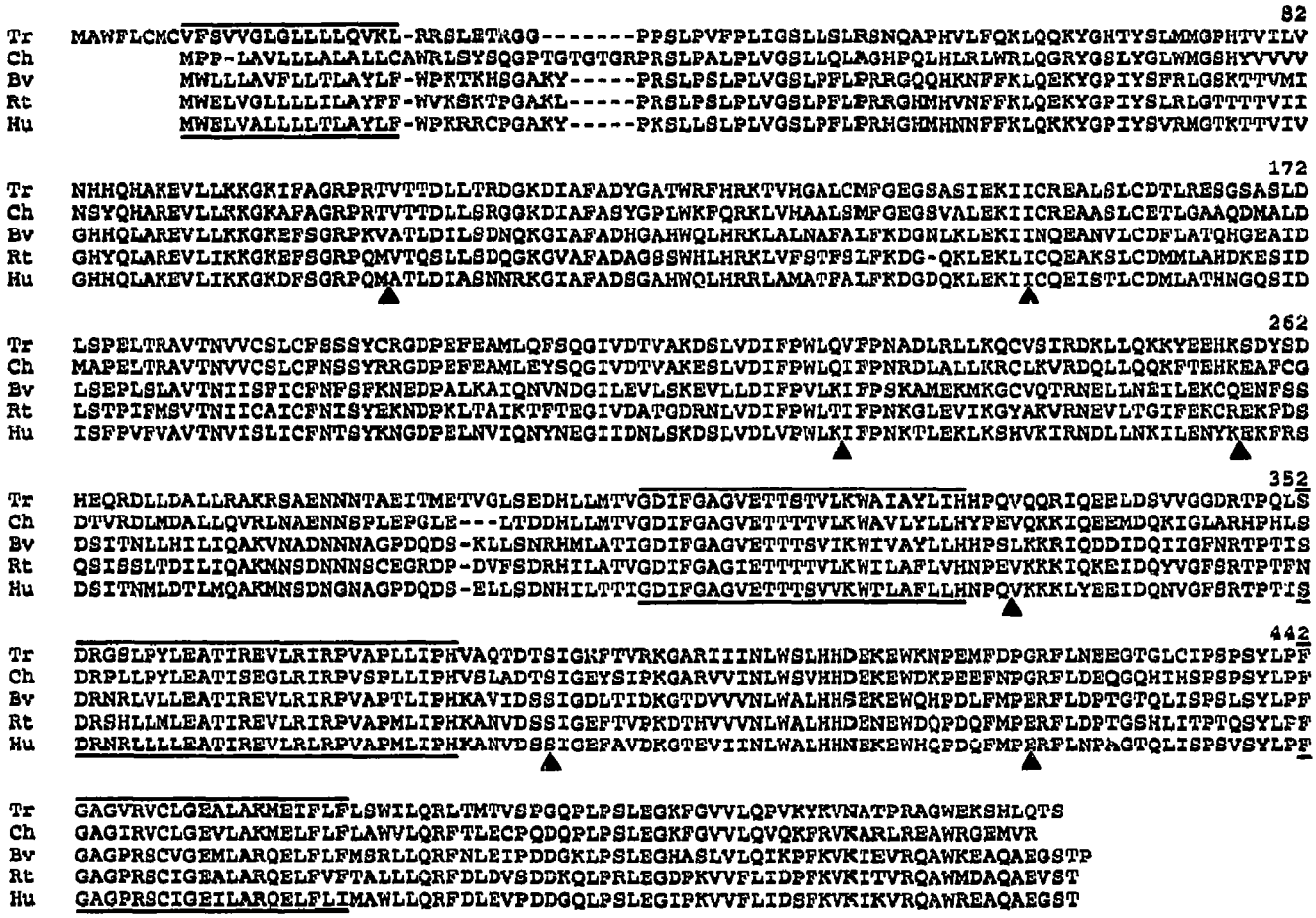

Fig. 3. Comparison of deduced amino acid sequences of rainbow trout (Tr), chicken (Ch) [18], bovine (Bv) [17], rat (Rt) [19] and human (Hu) [13] *P*-450$_{c17}$. The N-terminal hydrophobic sequence, the conserved *P*-450$_{c17}$ sequence (amino acids 311–335) identified by Ono et al. [15], the conserved (amino acids 361–383) Ozols tridecapeptide region [25], and the conserved (amino acids 449–470) heme binding region [26] are highlighted by both overlining and underlining. Triangles indicate the location of the introns reported in the human *P*-450$_{c17}$ gene [20].

3. RESULTS AND DISCUSSION

Screening of approximately 2.2 × 10^5 plaques from a rainbow trout ovarian thecal cell library yielded 30 positive clones. The longest of these, λth3–7, was chosen and sequenced by the strategy shown in Fig. 1. The nucleotide sequence of rainbow trout *P*-450$_{c17}$ contains an open reading frame of 1,542 nucleotides, starting from the first ATG codon and terminating at a TAG stop codon, which is predicted to encode 514 amino acid residues (Fig. 2). A second in a frame ATG codon is 18 bp downstream from the first. Although the CAGCATGG sequence surrounding the first ATG codon is similar to the consensus sequence proposed by Kozak [16], the ATG codon that initiates translation has not been proven. A typical poly-adenylation signal, AATAAA, was found 13 bp upstream from the poly(A) track.

Comparison of the amino acid sequence of trout *P*-450$_{c17}$ with that of human [13], bovine [17], chicken [18], and rat [19] *P*-450$_{c17}$ is shown in Fig. 3. We found considerable amino acid identity between trout and chicken *P*-450$_{c17}$ (64%, 318 residues identical in 500 residues), whereas 48% (243/505), 46% (233/505) and 47% (239/504) identity was found when trout *P*-450$_{c17}$ was compared with human, bovine and rat *P*-450$_{c17}$, respectively. Residues 152–228 of trout *P*-450$_{c17}$ which correspond to the amino acid sequence encoded by exon III of the human *P*-450$_{c17}$ [20], show an extremely high homology with chicken (79%, 61/77), although much lesser homology was found between trout and human (45%, 35/77), bovine (39%, 30/77), rat (47%, 36/77). When the amino acid sequence of exon III in human *P*-450$_{c17}$ was compared with that of other mammalian *P*-450$_{c17}$, the least homology (38%, 29/77) was found in exons from I to VIII. Residues 106–151 corresponding to exon II of the human *P*-450$_{c17}$ are also more conservative between trout and chicken (72%, 33/46) than between trout and human (39%, 18/46), bovine (46%, 21/46), rat (44%, 20/45).

It has been reported that the *P*-450$_{c17}$ and *P*-450$_{c21}$ genes are evolutionarily more closely related to each

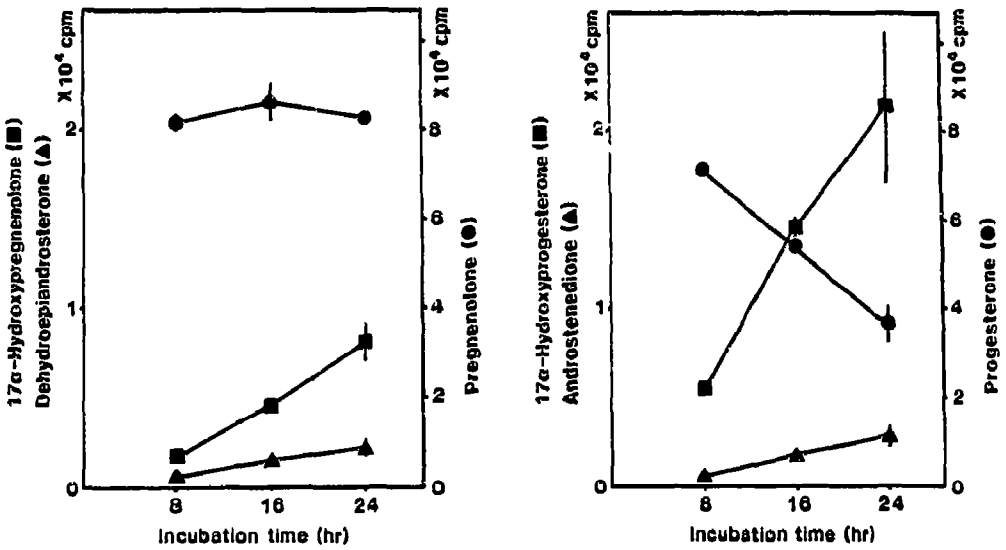

Fig. 4. Activities of 17α-hydroxylase and 17,20-lyase of trout $P\text{-}450_{c17}$ in COS-1 cells. ^{14}C-Labelled pregnenolone (1.4×10^5 cpm) or progesterone (1.3×10^5 cpm) were added to COS-1 cells, and metabolites from each substrate were separated by TLC and identified by recrystallization. The vertical lines represent the means ± S.E.M. of duplicate measures.

other [20]. When residues 237–265 of trout $P\text{-}450_{c17}$, which correspond to exon IV of the human $P\text{-}450_{c17}$, were used to search the Swiss Plot data base, the computer detected the amino acid sequence of exon VI of human $P\text{-}450_{c17}$ (41%, 12/29) [21,22] as well as the exon IV of human $P\text{-}450_{c17}$ (48%, 14/29) [20]. In bovine, exon IV of $P\text{-}450_{c17}$ (41%, 12/29) [23,24] rather than the exon IV of $P\text{-}450_{c17}$ (31%, 9/29) is similar to the region of trout $P\text{-}450_{c17}$.

With introduction of the trout $P\text{-}450_{c17}$ cDNA expression vector into COS-1 cells, exogenous pregnenolone was converted to 17α-hydroxypregnenolone and dehydroepiandrosterone, and exogenous progesterone was converted to 17α-hydroxyprogesterone and androstenedione (Fig. 4). The trout $P\text{-}450_{c17}$ favors conversion of progesterone to 17α-hydroxyprogesterone over the conversion of pregnenolone to 17α-hydroxypregnenolone. However, conversion of pregnenolone to dehydroepiandrosterone and that of progesterone to androstenedione are almost the same. These results indicate that the trout $P\text{-}450_{c17}$ has more 17,20- lyase activity catalyzing 17α-hydroxypregnenolone to dehydroepiandrosterone than that catalyzing 17α-hydroxyprogesterone to androstenedione. Activities of $P\text{-}450_{c17}$ expressed in COS-1 cells or yeast have been reported in bovine [9,10], human [11] and rat [19]. The 17,20-lyase activity of bovine and human $P\text{-}450_{c17}$ is greater with 17α-hydroxypregnenolone than with 17α- hydroxyprogesterone, whereas rat $P\text{-}450_{c17}$ has more 17,20-lyase activity catalyzing 17α-hydroxyprogesterone to androstenedione than that catalyzing 17α-hydroxypregnenolone to dehydroepiandrosterone. It seems that each $P\text{-}450_{c17}$ has a slightly different activity in terms of substrate specificity. Thus, it is interesting to determine whether different forms of $P\text{-}450_{c17}$ express in the trout thecal cell layers during vitellogenesis and oocyte maturation.

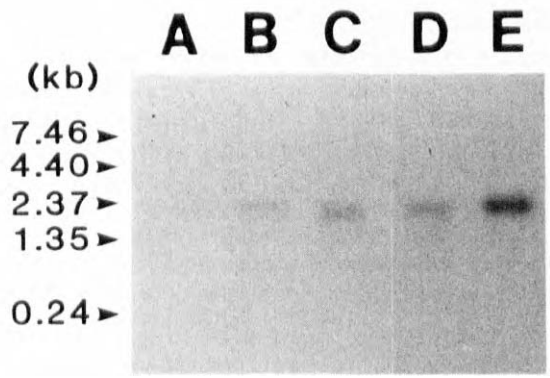

Fig. 5. Northern hybridization of poly(A)$^+$ RNA (2 μg) from various stages of ovarian follicles and isolated thecal cells. A, 1.81 mm diameter of follicle; B, 2.84 mm diameter of follicle; C, 4.68 mm diameter of follicle (immature); D, post-ovulated follicle; E, ovarian thecal cells isolated from 3.54 mm diameter of follicle.

By Northern hybridization analysis using a 2,081 bp λth3–7 cDNA fragment which had excluded 270 bp of 3′-region as a probe, we found the mRNA specific for rainbow trout $P\text{-}450_{c17}$ to be a single species, approximately 2.4 kb in length. The 2.4 kb transcripts were not found in ovaries during the early vitellogenic stage, barely detected during the mid-vitellogenic stage, and abundant during the post-vitellogenic stage and after ovulation (Fig. 5). These results are consistent with the increasing production of steroid hormones by salmonid ovarian follicles towards the end of the reproductive cycle [5].

REFERENCES

[1] Short, R.V. (1962) J. Endocrinol. 24, 59–63.
[2] Nagahama, Y. and Adachi, S. (1985) Dev. Biol. 109, 428–435.
[3] Voutilainen, R., Tapanainen, J., Chung, B., Matteson, K.J. and Miller, W.L. (1986) J. Clin. Endocrinol. Metab. 63, 202–207.
[4] Nagahama, Y. (1987) Dev. Growth Different. 29, 1–12.
[5] Kanamori, A., Adachi, S. and Nagahama, Y. (1988) Gen. Comp. Endocrinol. 72, 13–24.
[6] Nakajin, S. and Hall, P.F. (1981) J. Biol. Chem. 256, 3871–3876.
[7] Kominami, S., Shinzawa, K. and Takemori, S. (1982) Biochem. Biophys. Res. Commun. 109, 916–921.
[8] Nakajin, S., Shinoda, M. and Hall, P.F. (1984) Biochem. Biophys. Res. Commun. 111, 512–517.
[9] Zuber, M.X., Simpson, E.R. and Waterman, M.R. (1986) Science 234, 1258–1261.
[10] Sakaki, T., Shibata, M., Yabusaki, Y., Murakami, H. and Ohakwa, H. (1989) DNA 8, 409–418.
[11] Lin, D., Harikrishna, J.A., Moore, C.C.D., Jones, K.L. and Miller, W.L. (1991) J. Biol. Chem. 266, 15992–15998.
[12] Tanaka, M., Telecky, T.M., Fukada, S., Adachi, S., Chen and Nagahama, Y. (1991) J. Mol. Endocrinol., in press.
[13] Chung, B., Picard-Leonard, J., Haniu, M., Bienkowski, M., Hall, P.F., Shively, J.E. and Millar, W.L. (1987) Proc. Natl. Acad. Sci. USA 84, 407–411.
[14] Henikoff, S. (1984) Gene 28, 351–359.
[15] Sakai, N., Ueda, H., Suzuki, N. and Nagahama, Y. (1989) Biomed. Res. 10, 131–138.
[16] Kozak, M. (1986) Cell 44, 283–292.
[17] Zuber, M.X., John, M.E., Okamura, T., Simpson, E.R. and Waterman, M.R. (1986) J. Biol. Chem. 261, 2475–2482.
[18] Ono, H., Iwasaki, M., Sakamoto, N. and Mizuno, S. (1988) Gene 66, 77–85.
[19] Fevold, H.R., Lorence, M.C., McCarthy, J.L., Trant, J.M., Kagimoto, M., Waterman, M.R. and Mason, J.I. (1989) Mol. Endocrinol. 3, 968–975.
[20] Picado-Leonard, J. and Miller, M.L. (1987) DNA 6, 439–448.
[21] Higashi, Y., Yoshioka, H., Yamane, M., Gotoh, O. and Fujii-Kuriyama, Y. (1986) Proc. Natl. Acad. Sci. USA 83, 2841–2845.
[22] White, P.C., New, M.I. and Dupont, B. (1986) Proc. Natl. Acad. Sci. USA 83, 5111–5115.
[23] Chung, B., Natteson, K.J. and Miller, W.L. (1986) Proc. Natl. Acad. Sci. USA 83, 4243–4247.
[24] Yoshioka, H., Morohashi, K., Sogawa, K., Yamane, M., Kominami, S., Takemori, S., Okada, Y., Omura, T. and Fujii-Kuriyama, Y. (1986) J. Biol. Chem. 261, 4106–4109.
[25] Ozols, J. and Heinemann, F.S. (1981) J. Biol. Chem. 256, 11405–11408.
[26] Gotoh, O., Tagashira, Y., Iizuka, T. and Fujii-Kuriyama, Y. (1983) J. Biochem. 93, 807–817.