Volume 319, number 1,2, 45–48 FEBS 12195 March 1993

Rainbow trout ovarian cholesterol side-chain cleavage cytochrome P450 (P450scc)

cDNA cloning and mRNA expression during oogenesis

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

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

Received 30 November 1992

A cDNA clone encoding cholesterol side-chain cleavage cytochrome P450 (P450scc) was isolated from a rainbow trout ovarian follicle cDNA library. The cDNA contains an open reading frame of 1,542 nucleotides encoding a protein of 514 amino acids. The predicted amino acid sequence of trout P450scc shows 48% homology with that of human, and 46% homology with that of rat, bovine and pig. P450scc activity was confirmed by transfected COS-1 monkey kidney tumour cells with an expression vector for trout P450scc cDNA and subsequent detection of conversion from 25-hydroxycholesterol to pregnenolone by radioimmunoassay. The cDNA only hybridized to a single 1.8 kb RNA transcript. The transcript was not found in early vitellogenic follicles, barely detected in postvitellogenic follicles, and abundant in postovulatory follicles.

Rainbow trout; Cytochrome P450scc; Expression in COS-1 cells; Steroidogenesis

1. INTRODUCTION

In vertebrates, oocyte growth and maturation are regulated by pituitary gonadotropins and ovarian steroid hormones. In salmonid fishes, two steroidal mediators of oocyte growth and maturation are 17β-estradiol and 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP), respectively [1]. Two major somatic cell types in the ovary, the thecal cells and granulosa cells, participate in the synthesis of these two steroids [2,3]. During oocyte growth, the ovarian follicle layer predominantly produces 17β-estradiol. Immediately prior to or during final oocyte maturation, there is a drastic increase in 17α,20β-DP production by the follicle layer in response to the ovulatory surge of plasma gonadotropin [1].

One of the important steroidogenic enzymes involved in the synthesis of these two steroids is cholesterol side-chain cleavage cytohrome P450 (P450scc). This enzyme catalyzes the conversion of cholesterol to pregnenolone, which is the initial and rate-limiting reaction in the synthesis of steroid hormones. However, the role of P450scc in differential production of 17β-estradiol and 17α,20β-DP during oocyte growth and maturation has not been determined.

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

As a first step to analyze this question in rainbow trout (*Oncorhynchus mykiss*), we have isolated and cloned a full-length cDNA encoding P450scc from a rainbow trout ovarian thecal cell layer cDNA library. Comparisons have been made between the deduced amino acid sequences of rainbow trout and four species of mammals. Identification of rainbow trout P450scc was based on expression of the cDNA in transfected nonsteroidogenic COS-1 monkey kidney tumour cells. We also analyzed the expression of P450scc transcripts from rainbow trout ovarian follicles during oocyte growth and final maturation by Northern hybridization.

2. MATERIALS AND METHODS

2.1. *Cloning and sequencing*

Three-year-old rainbow trout were obtained from Samegai Trout Hatchery (Shiga Prefecture, Japan). 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 rainbow trout ovarian thecal cell layer poly(A)$^+$ RNA was constructed as described previously [4]. A human P450scc cDNA [5] was digested at nucleotide position 1,158 and 1,442 with *Pvu*II and *Sau*3AI to yield a 285 bp fragment which encompasses the highly conserved steroid-binding and heme-binding sites. This 284 bp fragment was subcloned into M13mp19 and labelled with [α-^{32}P]dCTP as a probe. About 2.4×10^5 plaques were blotted onto Hybond N$^+$ membranes, were prehybridized for 1 h in 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% polyvinylpyrolidone/0.1% Ficoll type 400/0.1% bovine serum albumin fraction V), 0.2% SDS, 10% dextran sulfate at 42°C, and then hybridized with 5×10^5 cpm of the probe at 42°C overnight. The inserts from positive plaques were isolated and digested with *Eco*RI. The resultant 1.8 kb DNA insert was subcloned into *Eco*RI site of pBluescript KS(+). Deletion clones were obtained by using *Exo*III/Mung bean nuclease deletion system and the single-stranded DNA were prepared using M13K07 an helper phage [6]. DNA sequencing was carried out by dideoxy chain-termination method using an M13 sequencing kit (Takara Shuzo Co.), a 7-DEAZA sequencing kit (Takara Shuzo Co.) or a *Bca* BEST Dideoxy Sequencing kit (Takara Shuzo Co.).

2.2. *COS-1 cell expression*

A 1,580 bp cDNA containing the entire codon region (nucleotide position 30–1609:P) and a 1,433 bp cDNA lacking the 5′ region encoding the putative extension (mitochondrial leader) peptide (nucleotide position 177–1609:M) were amplified by Polymerase Chain Reaction using synthetic primers with *Xho*I and *Sac*I sites. The amplified DNA fragments were cloned into an expression vector, pSVL (Pharmacia LKB) at *Xho*I and *Sac*I sites. The two recombinant plasmids, pSVL/P and pSVL/M, and pSVL (no insert) were transfected to 2.2×10^5 COS-1 cells as previously described [4]. On the next day, 25-hydroxycholesterol (Sigma) was added to the medium to 2 nmol/ml. The cells were cultured for 48 or 120 h. Pregnenolone was extracted from the culture medium with diethylether. P450scc activity was deduced by measuring levels of pregnenolone by radioimmunoassay as described previously [7]. The anti-pregnenolone-3-Succ-BSA serum (Teikoku Zoki Co.) crossreacts with pregnenolone, progesterone, and deoxycorticosterone at 100%, 15.6% and 0.1%, respectively. This antiserum crossreacts less than 0.1% with most of ovarian steroids and corticosteroids tested, such as 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, 17β-estradiol, and cortisol.

2.3. *Northern blot analysis*

Northern blot analysis was carried out as described previously [4]. The 1,789 bp cDNA fragment was labelled with [α-^{32}P]dCTP using the Random Primer Plus Extention Labelling System (NEN) as a probe, and hybridized in 5× SSC, 5× Denhardt's solution, 0.2% SDS, 200 μg/ml denatured herring sperm DNA with 5×10^5 cpm of the probe at 45°C overnight.

3. RESULTS AND DISCUSSION

To isolate the cDNA encoding P450scc, we used a cDNA library that was constructed from rainbow trout ovarian thecal cell poly(A)$^+$ RNA. Four positive clones were found by screening approximately 2.4×10^5 plaques using a human 284 bp *Pvu*II/*Sau*3AI fragment as a probe. The longest of these clones, 1.8 kb-long insert, was chosen and sequenced by the strategy shown in Fig. 1. The nucleotide sequence of rainbow trout P450scc 1.8 kb-long insert contains an 1,542 bp open reading frame starting from the first ATG codon and terminating at a TGA stop codon. The predicted protein contains 514 amino acids (Fig. 2). There are direct repeats (4 times, core sequence: CAGTGATATCATGAAGCCTCTACACAGCAGA) starting from 3 bp upstream from the

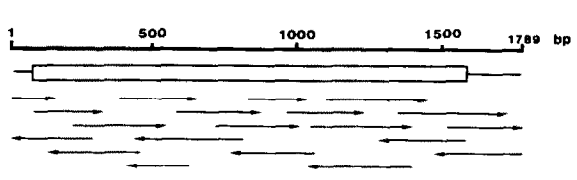

Fig. 1. Sequencing strategy for the 1.8 kb insert. Location of the open reading frame is shown by an open box. Each arrow indicates the direction and extent of sequencing.

```
AAAAGAAACAGACAGAAGAAGAGTTGGAAGAAGTGAGGTAGGATAGAGACGCTGCTATGA     60
                                                          M

TGGTGAGCTGGAGTGTGTGTCGCAGTTCCCTGGCTCTGCCAGCATGTGGACTACCCAGTG    120
M  V  S  W  S  V  C  R  S  S  L  A  L  P  A  C  G  L  P  S

CCCGCCACAACTCCAGTATGCCGGTGGTGCGCCAGGCTCTGTCCCCAGACAACAGCAGTA    180
A  R  H  N  S  S  M  P  V  V  R  Q  A  L  S  P  D  N  S  S

CGGTCCAGAACTTCAGTGAGATCCCAGGTCTCTGGAGAAACGGACTCGCCAACCTCTACA    240
T  V  Q  N  F  S  E  I  P  G  L  W  R  N  G  L  A  N  L  Y

GTTTCTGGAAACTAGACGGATTCAGGAACATCCACAGAGTCATGGTGCACAACTTCAACA    300
S  F  W  K  L  D  G  F  R  N  I  H  R  V  M  V  H  N  F  N

CCTTCGGTCCAATATACAGGGAGAAGATAGGCTACTATGATAGTGTAAACATTATAAAGC    360
T  F  G  P  I  Y  R  E  K  I  G  Y  Y  D  S  V  N  I  I  K

CGGAGATGCCAGCCATCTTGTTCAAGGCAGAAGGACACTACCCCAAGAGGTTAACGGTGG    420
P  E  M  P  A  I  L  F  K  A  E  G  H  Y  P  K  R  L  T  V

AGGCATGGACCTCATACAGAGACTACAGGAACAGGAAATATGGAGTCCTGCTCAAGAATG    480
E  A  W  T  S  Y  R  D  Y  R  N  R  K  Y  G  V  L  L  K  N

GGGAGGACTGGCGGTCCAACAGGGTGATTCTGAATAGAGAGGTGATCTCTCCCAAGGTGT    540
G  E  D  W  R  S  N  R  V  I  L  N  R  E  V  I  S  P  K  V

TGGGGAACTTTGTTCCTCTGTTGGATGAGGTTGGGCAGGACTTTGTGGCCCGAGTACATA    600
L  G  N  F  V  P  L  L  D  E  V  G  Q  D  F  V  A  R  V  H

AGAAGATAGAAAGGAGTGGACAGGACAAATGGACCACCGATCTTTCTCAAGAACTCTTCA    660
K  K  I  E  R  S  G  Q  D  K  W  T  T  D  L  S  Q  E  L  F

AATACGCTCTGGAATCGGTGGGTTCAGTTCTGTATGGGGAACGTCTGGGCCTGATGTTGG    720
K  Y  A  L  E  S  V  G  S  V  L  Y  G  E  R  L  G  L  M  L

ACTACATCAACCCTGAGGCCCAACACTTCATTGACTGCATCTCTCTGATGTTCAAGACTA    780
D  Y  I  N  P  E  A  Q  H  F  I  D  C  I  S  L  M  F  K  T

CCTCTCCCATGCTGTACATCCCCCCGGCCATGCTGAGGAGGGTAGGAGCCAAGATCTGGA    840
T  S  P  M  L  Y  I  P  P  A  M  L  R  R  V  G  A  K  I  W

GACATCACGTAGAGGCCTGGGATGGCATCTTCAACCAGGCGGACCGCTGCATCCAGAACA    900
R  D  H  V  E  A  W  D  G  I  F  N  Q  A  D  R  C  I  Q  N

TCTACAGGACGATGCGTCAGGACACTAACACCCACGGGAAGTATCCAGGAGTCCTGGCCA    960
I  Y  R  T  M  R  Q  D  T  N  T  H  G  K  Y  P  G  V  L  A

GCCTTCTGATGTTAGACAAGCTGTCTATAGAGGATATCAAGGCCAGCGTCACTGAACTGA   1020
S  L  L  M  L  D  K  L  S  I  E  D  I  K  A  S  V  T  E  L

TGGCTGGAGGGGTAGACACGACATCTATCACCCTGCTGTGGACTCTATATGAGCTTGCCA   1080
M  A  G  G  V  D  T  T  S  I  T  L  L  W  T  L  Y  E  L  A

GACACCCTGACCTCCAGGAAGAGTTGAGGGCTGAGGTGGCTGTAGCCAGACAGTCTACCC   1140
R  H  P  D  L  Q  E  E  L  R  A  E  V  A  V  A  R  Q  S  T

AGGGAGACATGCTACAGATGCTGAAGATGATACCGCTGGTCAAAGGAGCGCTGAAGGAAA   1200
Q  G  D  M  L  Q  M  L  K  M  I  P  L  V  K  G  A  L  K  E

CGCTGAGGCTTCATCCAGTTGCAGTCAGTTTACAGAGATACATTACAGAGGAAATCGTCA   1260
T  L  R  L  H  P  V  A  V  S  L  Q  R  Y  I  T  E  E  I  V

TTCAGAACTATCACATACCTTGTGGGACTCTGGTCCAGTTGGGTCTCTATGCGATGGGTA   1320
I  Q  N  Y  H  I  P  C  G  T  L  V  Q  L  G  L  Y  A  M  G

GAGACCCAGATGTGTTCCCCAGACCTGAGAAGTACCTCCCGTCCCGCTGGCTGCGGACAG   1380
R  D  P  D  V  F  P  R  P  E  K  Y  L  P  S  R  W  L  R  T

AGAACCAGTACTTCAGGAGCTTGGGCTTCGGATTTGGACCCAGACAGTGTCTTGGACGGC   1440
E  N  Q  Y  F  R  S  L  G  F  G  F  G  P  R  Q  C  L  G  R

GCATAGCTGAGACGGAGATGCAGCTCTTCCTTATACATATGCTGGAGAACTTCAGAGTAG   1500
R  I  A  E  T  E  M  Q  L  F  L  I  H  M  L  E  N  F  R  V

ATAAACAGCGTCAGGTGGAGGTGCACAGTACCTTCGAGTTGATCTTGTTGCCAGAGAAAC   1560
D  K  Q  R  Q  V  E  V  H  S  T  F  E  L  I  L  L  P  E  K

CCATTCTTCTGACCCTGAAGCCTCTAAAGAGCGGCCAGTGATATCATGAAGCCTCTACAC   1620
P  I  L  L  T  L  K  P  L  K  S  G  Q  *

AGCAGACAGTGATATCATGAAGCCTCTACACAGCAGACAGTGACATCATGAAGCCTCTAC   1680

ACAGCAGACAGTGACATCATGAAGCCTCTACTCTGTAAGGTCAAAGCGTAAGAAAAATTC   1740

TAACATTGTTTTAATAAAACAATAATATAACTTGAAAAAAAAAAAAAAA              1789
```

Fig. 2. Nucleotide and deduced amino acid sequence of rainbow trout P450scc. Amino acid sequence deduced from an open reading frame is shown below the nucleotide sequence. The AATAAA polyadenylation signal is underlined.

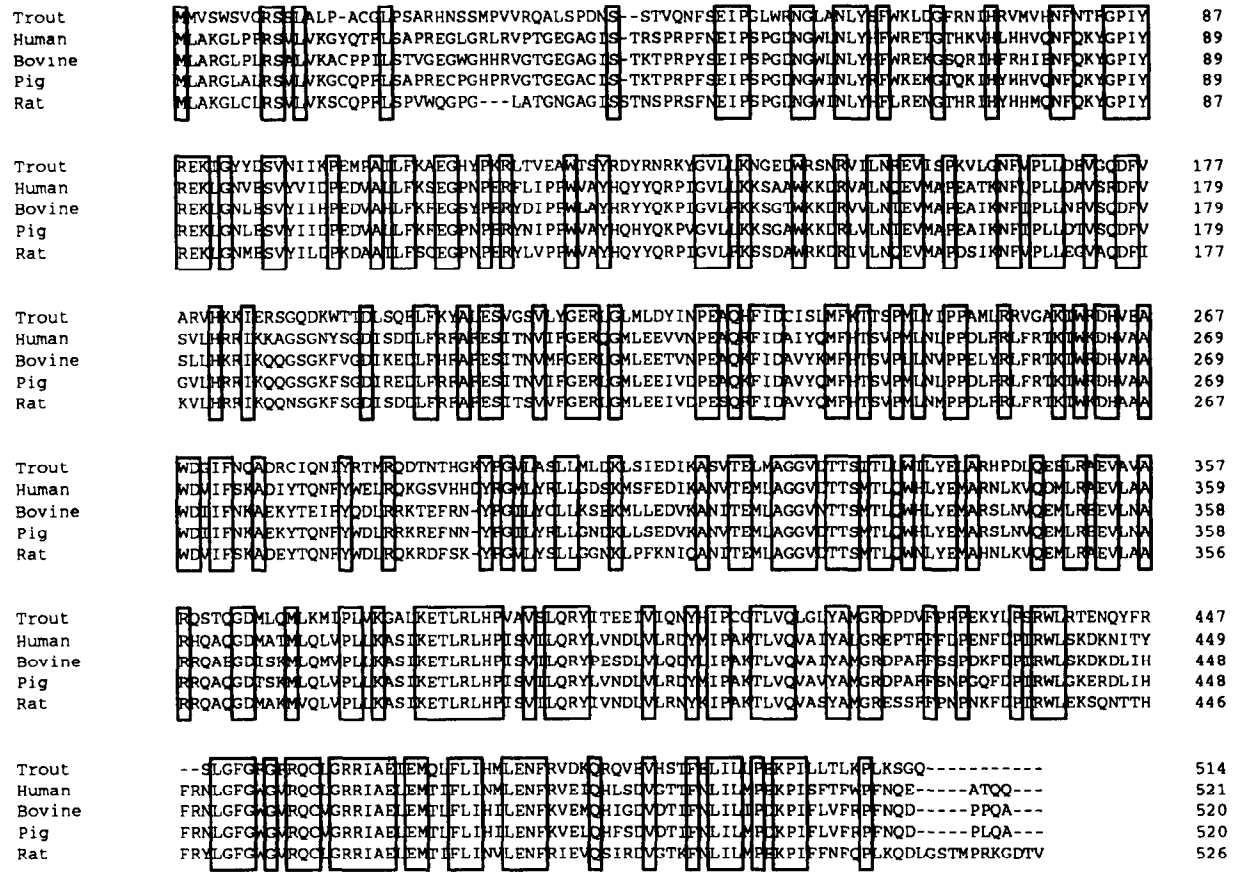

Fig. 3. Comparison of deduced amino acid sequence of rainbow trout (Trout), human, bovine, pig and rat P450scc. Alignment was made using Gene Works (IntelliGenetics, Inc.). Identical amino acids between five species are indicated as a box.

stop codon. A polyadenylation signal, AATAAA, was found 16 bp upstream from the poly(A)$^+$ track.

This is the first cDNA cloning of P450scc in a non-mammalian vertebrate. Therefore, we compared the deduced amino acid sequence of rainbow trout P450scc with that known from four species of mammals (human, rat, bovine and pig) (Fig. 3). An overall homology of 48% was found between the amino acid sequence of rainbow trout and that of human P450scc [5]. The rat [8], bovine [9] and pig [10] P450scc amino acid sequences share 46% homology with that of rainbow trout. A similar degree of amino acid sequence homology between rainbow trout and mammals was found in cytochrome P450c17 (17α-hydroxylase/17,20-lyase cytochrome, 46–48%) and in cytochrome P450arom (aromatase, 52%) [4,11]. The regions of greatest similarity between rainbow trout and mammalian P450scc were in the heme-binding region [12] at residues 451–471 (71%, 15 identical/21 amino acids), and in the steroid-binding region [13] at residues 373–395 (70%, 16 identical/23 amino acids).

Since the deduced amino acid sequence had a low homology compared with that of four mammalian cytochrome P450scc sequences, a recombinant expression vector containing either pSVL/P or pSVL/M was introduced into COS-1 cells to confirm that the cloned sequence encodes a polypeptide having the ability to convert 25-hydroxycholesterol to pregnenolone. The 1,580 bp sequence contains the entire open reading frame of rainbow trout P450scc cDNA, while the 1,433 bp cDNA lacks 5′ region containing N-terminal 39 amino acids. COS-1 cells transfected with the pSVL/P produced significantly more pregnenolone (23 ng/plate) than did pSVL/M (2.6 ng/plate), pSVL (no insert, 3.0 ng/plate) or no vector (2.6 ng/plate) (Table I). This re-

Table I

Activities of rainbow trout P450scc in COS-1 cells

	Pregnenolone (ng/plate)	
	48 h	120 h
pSVL	2.8	3.0
pSVL/P	12.9	23.2
pSVL/M	2.5	3.1

The recombinant plasmids, pSVL/P, pSVL/M, pSVL (no insert) were transfected to 2.2×10^5 COS-1 cells. After 24 h, 2 nmol/ml of 25-hydroxycholesterol was added and 48 or 120 h later, pregnenolone was extracted. P450scc activity was deduced by measuring levels of pregnenolone by radioimmunoassay.

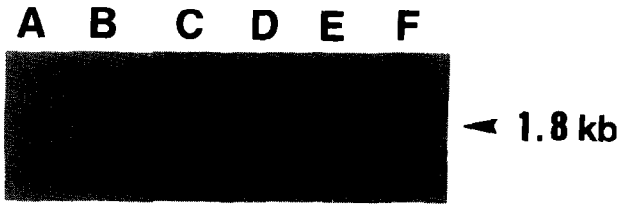

Fig. 4. Northern hybridization of poly(A)$^+$ RNA (2 μg) from various stages of follicles. (A) 1.8 mm diameter follicle; (B) 2.4 mm diameter follicle; (C) 2.9 mm diameter follicle; (D) 4.7 mm diameter follicle (immature); (E) 4.8 mm diameter follicle (immature); (F) postovulatory follicle.

sult clearly indicates that the 1,580 bp insert codes for rainbow trout P450scc.

pSVL/M did not significantly convert exogenous 25-hydroxycholesterol into pregnenolone in COS-1 cells (Table I), indicating that N-terminal 39 amino acids play an important part in expression of activity of trout P450scc. It is likely that the N-terminal region functions as a mitochondrial targeting peptide, although no significant homology could not be found between the trout N-terminal 39 amino acid sequence and the corresponding sequences of four mammalian P450scc enzymes (Fig. 3).

Northern hybridization analysis showed a single 1.8 kb long transcript. The 1.8 kb transcript was not found in early vitellogenic follicles, barely detected in post-vitellogenic follicles and abundant in postovulatory follicles (Fig. 4). The increased amounts of P450scc transcript during final oocyte maturation may be responsible for the rapid increase in the follicular production of maturation-inducing hormone of this species, 17α,20β-DP. The factors involved in increasing the abundance of P450scc transcript remain to be determined, although cAMP and several nuclear proteins have been implicated in mammalian systems [14–19].

Acknowledgements: We gratefully acknowledge Dr. S. Fushiki, Mr. T. Kobayashi and the Director and staff of the Samegai Trout Hatchery for providing the fish. COS-1 cells were provided by the Japanese Cancer Research Bank. This work was supported by Grant-in-Aid (0120210) to Y.N. for scientific research from the Ministry of Education, Science, and Culture of Japan.

REFERENCES

[1] Nagahama, Y. (1987) Zool. Sci. 4, 209–222.
[2] Kagawa, H., Young, G., Adachi, S. and Nagahama, Y. (1992) Gen. Comp. Endocrinol. 47, 440–448.
[3] Young, G., Adachi, S. and Nagahama, Y. (1986) Dev. Biol. 118, 1–8.
[4] Tanaka, M., Telecky, T.M., Fukada, S., Adachi, S., Chen, S. and Nagahama, Y. (1992) J. Mol. Endocrinol. 8, 53–61.
[5] Chung, B., Matteson, K.J., Voutilainen, R., Mohandas, T.K. and Miller, W.L. (1986) Proc. Natl. Acad. Sci. USA 83, 8962–8966
[6] Henikoff, S. (1984) Gene 28, 351–359.
[7] Abraham, G.E., Buster, J.E., Kyle, F.W., Corrales, P.C. and Teller, R.C. (1973) J. Clin. Endocrinol. Metab. 37, 40–45.
[8] Oonk, B.R., Parker, K.L., Gibson, F.L. and Richards, J.S. (1990) J. Biol. Chem. 265, 22392–22401.
[9] Morohashi, K., Fujii-Kuriyama, Y., Okada, Y., Sogawa, K., Hirose, T. and Inayama, S. (1984) Proc. Natl. Acad. Sci. USA 81, 4647–4651.
[10] Mulheron, G.W., Stone, R.T., Miller, W.L. and Wise, T. (1989) Nucleic Acids Res. 17, 1773.
[11] Sakai, N., Tanaka, M., Adachi, S., Miller, W.L. and Nagahama, Y. (1992) FEBS Lett. 301, 60–64.
[12] Gotoh, O., Tagashira, Y., Iizuka, T. and Fujii-Kuriyama, Y. (1983) J. Biochem. 93, 807–817.
[13] Picado-Leonard, J. and Miller, W.L. (1988) Mol. Endocrinol. 2, 1145–1150.
[14] Moore, C.C.D., Brentano, S.T. and Miller, W.L. (1990) Mol. Cell. Biol. 10, 6013–6023.
[15] Inoue, H., Watanabe, N., Higashi, Y. and Fujii-Kuriyama, Y. (1991) Eur. J. Biochem. 195, 563–569.
[16] Rice, D.A., Mouw, A.R., Bogerd, A.M. and Parker, K.L. (1991) Mol. Endocrinol. 5, 1552–1561.
[17] Honda, S.I., Morohashi, K.I. and Omura, T. (1991) J. Biochem. 108, 1042–1049.
[18] Lala, D.S., Rice, D.A. and Parker, K.L. (1992) Mol. Endocrinol. 6, 1244–1258.
[19] Moore, C.C.D., Hum, D.W. and Miller, W.L. (1992) Mol. Endocrinol. 6, 2045–2058