Bull. Inst. Zool., Academia Sinica, Monograph **16**: 37-59 (1991)

STUDIES ON PISCINE GONADOTROPIN—STRUCTURE, FUNCTION, MOLECULAR CLONING, EXPRESSION AND GENE STRUCTURE

TUNG-BIN LO, FORE-LIEN HUANG, CHEN-SHENG LIU, YEA-SHA CHANG, GEEN-DONG CHANG and CHANG-JEN HUANG

Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan 10765, Republic of China
and
Graduate Institute of Biochemical Sciences, National Taiwan University, Taipei, Taiwan 10765, Republic of China

T.-B. Lo, F.-L. Huang, C.-S. Liu, Y.-S. Chang, G.-D. Chang and C.-J. Huang (1991) Studies on piscine gonadotropins—Structure, function, molecular cloning, expression and gene structure. *Bull. Inst. Zool., Academia Sinica, Monograph* **16**: 37-59. Five piscine gonadotropins were isolated and purified from pituitaries of five species of fish (carp, silver carp, bighead carp, grass carp and pike eel). They are all composed of two non-identical (α and β), non-covalently linked subunits. Each subunit is a glycoprotein and the complete amino acid sequences were determined either by the conventional chemical method or by deduction from the nucleotide sequence of the corresponding cDNA. The amino acid sequences of α-subunit of fresh water fishes are almost identical, but show only 75% and 70% homology to marine fish and human lutropin, respectively. In β-subunit, the similar situation are observed, *i.e.* almost identical between fresh water fishes, and only 80% and 46% homology to marine fish and human lutropin, respectively.

The biological activity as tested both in tests of rat and carp for steroidogenesis showed distinct species specificity between mammalian lutropins and piscine gonadotropins, and β-subunit was shown as the hormone specific subunit as tested by reassociated hybrid molecules. The immunological cross-reactivities of piscine and mammalian gonatropins were also corresponded to the above-mentioned species specificity. The cDNA library was constructed from corresponding mRNA and screened either by synthetic oligonucleotide or known piscine cDNA. The nucleotide sequence of each cDNA was determined by the dideoxy nucleotide chain termination method.

The expression of carp gonadotropin subunit (α) was proceeded in insect cell line (Sf21-AE) by using a transfer plasmid (pAV6) as vector. One expressed subunit ($r\alpha_1$) was immunologically identical with native subunit, and the biological activity was also almost the same potency as tested by steroidogenesis after association with native β subunit. However, another recombinant α_2 was not hormonally active, and seemed to be a competitive inhibitor of α_1 subunit.

For determination of carp gonadotropin α subunit genes, the PCR amplified products were prepared from genomic DNA (obtained from carp erythrocytes) as template and synthetic oligonucleotides as primers. The subunit gene was shown as multigene family and was composed of four exons and three introns. The size was rather small (1.2 kb). For β subunit genes, genomic DNA was directly partially digested and screened with cDNA encoding β subunit. It was also smaller in size, and composed of three exons and two introns.

Key words: Piscine gonadotropins, Amino acid sequence, Hormonal function, Molecular cloning, Gene structure, Gene expression.

The chemical structure, hormonal function and immunological properties of mammalian gonadotropins (GTHs) have been well characterized, however not much work has been undertaken on piscine GTHs. The comparative studies on piscine pituitary hormones have been proceeded in our laboratory for more than ten years since 1980. This paper is the summarization of a part of this series of work on GTHs, including chemical structure, hormonal activity, immunological pro perty, molecular cloning of five species of fish, carp (*Cyprinus carpio*), silver carp (*Hypophthalmichthys molitrix*), bighead carp (*Hypophthalmichthys nobilis*), grass carp (*Ctenopharyngodon idellus*), and pike eel (*Muraenesox cinereus*), and expression and gene structure of carp GTH.

ISOLATION AND CHEMICAL CHARACTERIZATION

Piscine pituitary glands of both sexes, either freshly collected or preserved in acetone at −20°C, were homogenized and extracted with 6% ammonium acetate (pH 5.1) in 40% ethanol at 4°C, and crude glycoprotein fraction was recovered by alcohol precipitation (Huang *et al.*, 1981). After dialysis against 5 mM ammonium bicarbonate and lyophilization, GTH was purified by DEAE-cellulose chromatography and followed by gel filtration (Chang *et al.*,

Comparison of the amino acid sequence of α subunit

```
                                10                  20                  30
cGTH1  Y P R N D M N N F G C E E C K L K E N N I F S K P G A P V Y
cGTH2  - - - - Y - - - - - - - - - - - - - - - - - - - - - - - -
scGTH  - - - - - I T - - - - - - - - - - - - - - - - - - - - - - -
bcGTH  - - - - - I T - - - - - - - - - - - - - - - - - - - - - - -
gcGTH  - - - - - - T - - - - - - - - - - - - - - - - - - - - - - -
peGTH  - - N - E I S R G - - D - - R - - D - K F - - - - S - - I F
 eGTH  - - N - E - A R G - - D - - R - Q - - K - - - - - - S - - I F
sGTHI  - Q N S - - T - V - - - - - - - - - - K V - - N - - - - - - -
sGTHII - - N S - K T - M - - - - - T - - P - T - - X X - N X X I M
  hLH                 V Q D - P - - T - Q - - P F - - Q - - - - I L

                                40                  50                  60
cGTH1  Q C M G C C F S R A Y P T P L R S K K T M L V P K N I T S E
cGTH2  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
scGTH  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
bcGTH  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
gcGTH  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
peGTH  - - V - - - - - - - - - - - - - - - - - - - - - - D - - - -
 eGTH  - - V - - - - - - - - - - - - - - - - - - - - - - - - - - -
sGTHI  - - T - - - - - - - - - - - - Q - - - A - - - - - - - - - -
sGTHII - - T - - - - - - - - - - - - - - - Q - - - - - - - - - - -
  hLH  - - - - - - - - - - - - - - - - - - - - - - Q - - V - - - -

                                70                  80                  90
cGTH1  A T C C V A K E V K R V L V N D V K L V X N H T D C H C S T
cGTH2  - - - - - - - - F - Q - - - - - I - - - X - - - - - - - - -
scGTH  - - - - - - - - - - - - - - - - - - - - X - - - - - - - - -
bcGTH  - - - - - - - - - - - - - - - - - - - - X - - - - - - - - -
gcGTH  - - - - - - - - - - - - - - - - - - - - X - - - - - - - - -
peGTH  - - - - - - R - - T K X - X D N M - - X E - - - - - - - - -
 eGTH  - - - - - - R - - T - X - X D N M - - X E - - - - - - - - -
sGTHI  - - - - - - - - G E - - V - X - N I K L T - - - E - W - N -
sGTHII - - - - - - - - G E - - T T K - G F P - T - - - E - - - - -
  hLH  S - - - - - - - S Y N - - T - M G G F K - E - - - A - - - - -

                96
cGTH1  C Y Y H K S
cGTH2  - - - - - -
scGTH  - - - - - -
bcGTH  - - - - - -
gcGTH  - - - - - -
peGTH  - - - - - -
 eGTH  - - - - - F
sGTHI  - - H - - -
sGTHII - - - - - -
  hLH  - - - - - -
```

Fig. 1. Comparison of the amino acid sequence of α subunit. c, carp (Chang *et al.*, 1988b); sc, silver carp (Chang *et al.*, 1990); bc, bighead carp (Huang *et al.*, 1990); gc, grass carp (Unpublished data); pe, pike eel (Liu *et al.*, 1989); e, eel (Querat *et al.*, 1990a); s, salmon (Swanson *et al.*, 1989); hLH, human LH (Sairam, 1983); —, residue identical to that of cGTH; X, gap insertion for half cystine alignment.

Comparison of the amino acid sequence of ß subunit

```
                                10                  20                  30
 cGTH          S Y L P P C E P V N E T V A V E K E G C P K C L V L Q
scGTH          - F - - - - - - - - - - - - - - - - - - - - - - - F -
bcGTH          - F - - - - - - - - - - - - - - - - - - - - - - - F -
gcGTH          - F - - - - - - - - - - - - - - - - - - - - - - - F -
peGTH          - V - Q - - Q - I - - - I S - - - D - - - - - - - F -
 eGTH          - V - L - - - - I - - - I S - - - D - - - - - - - F -
sGTHII         - L M Q - - Q - I - E - - S L - - - - - - - T - - - I Q
sGTH I G T E C R Y G - R L N - M - I I - - R - D - H G S I T I X
hFSH             N S - - L T - I - I - L - - - - - - - T - - - I N
 hLH   S R E P L R - W - H - I - A I L - - - - - - - - - V - I T V N

                                40                  50                  60
 cGTH  T T I C S G H C L T K E P V Y K S P F S T V Y Q H V C T Y R
scGTH  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
bcGTH  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
gcGTH  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
peGTH  - S - - - - - - I - - D - S - - - - L - - - - - R - - - - -
 eGTH  - S - - - - - - I - - D - S - - - - L - - - - - R - - - - -
sGTHII T P - - - - - - V - - - - - F - - - - - - - - - - - - - - -
sGTHI  - - X - A - L - E - T D L N - Q - T W L P R S - G - - N F K
 hFSH  - - W - A - Y - Y - R D L - - - N - A R P K I - K T - - F K
 hLH   - - - - A - Y - P - M R M L L Q A V L P P - P - P - - - - -

                                70                  80                  90
 cGTH  D V R Y E T V R L P D C P P G V D P H I T Y P V A L S C D C
scGTH  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
bcGTH  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
gcGTH  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
peGTH  - - - - - - - - - - R - R - - - - - - V - F - - - - - - - -
 eGTH  - - - - - - - - - - - - R - - - - - - V - F - - - - - - - -
sGTHII - - - - - - I - - - - - - - W - - - - V - - - - - - - - - -
sGTHI  E W S - - K - Y - E G - - S - - E - F F X I - - - K - - - -
 hFSH  E L V - - - - - V - D - A H H A - S L Y - - - - - T Q - H -
 hLH   - - - F - S I - - - G - - R - - - - V V S F - - - - - - - R -

                              100                 110                 118     122
 cGTH  S L C T M D T S D C T I E S L Q P D F C M S Q R E D F L
scGTH  - - - - - - - - - - - - - - - - - - - Y - - - - - - - - P
bcGTH  - - - - - - - - - - - - - - - - - - - - - - - - - - - - P
gcGTH  - - - - - - - - - - - - - - - - - - - - - - - - - - - - P
peGTH  N - - - - - - - - - - A - Q - - R - - - - - - - - A P
 eGTH  N - - - - - - - - - - A - Q - - R - - - - - - - - A S L P A
sGTHII - - - N - - - - - - - - - - - - - - - - I T - - V L T D G D M W
 sGTHI I K - K T - N T - - D R I - M A T P S - I V N P L E M
 hFSH  G K - D S - S T - - - V R G - G - S Y - S F G E M K E
 hLH   G P - R R S - - - - G G P K D H - L T - D E N S K G
```

Fig. 2. Comparison of the amino acid sequence of β subunit. c, carp (Chang *et al.*, 1988b); sc, silver carp (Chang *et al.*, 1990); bc, bighead carp (Huang *et al.*, 1990); gc, grass carp (Unpublished data); pe, pike eel (Liu *et al.*, 1989); e, eel (Querat *et al.*, 1990b); s, salmon (Swanson *et al.*, 1989); hFSH, human FSH (Shome *et al.*, 1988); hLH, human LH (Shome and Parlow, 1973); —, residue identical to that of cGTH; X, gap insertion for half cystine alignment.

1988a). For further purification of GTH or dissociation of subunits, HPLC method was employed (Chang *et al.*, 1988b). The complete amino acid sequence of each subunit was determined either by conventional chemical method (Edman degradation) or by deduction from the nucleotide sequence of the corresponding cDNA. The complete amino acid sequences of five piscine GTH-α and -β subunits are listed with those of some other known GT Hsin Figs. 1 and 2, respectively. The

Table 1
Homology analysis of amino acid sequences

α subunit			homology (%)
cGTH	vs	scGTH	98
scGTH	vs	bcGTH	100
scGTH	vs	gcGTH	99
scGTH	vs	peGTH	74
peGTH	vs	eGTH	91
cGTH	vs	sGTH(I)	75
cGTH	vs	sGTH(II)	73
sGTH(I)	vs	sGTH(II)	72
peGTH	vs	sGTH(II)	67
scGTH	vs	hLH	70
scGTH	vs	bLH	72
hLH	vs	bLH	74
β subunit			homology (%)
cGTH	vs	scGTH	97
scGTH	vs	bcGTH	99
scGTH	vs	gcGTH	99
cGTH	vs	peGTH	80
peGTH	vs	eGTH	94
scGTH	vs	sGTH(I)	34
scGTH	vs	sGTH(II)	75
peGTH	vs	sGTH(II)	71
sGTH(I)	vs	sGTH(II)	31
peGTH	vs	hLH	45
peGTH	vs	bLH	38
scGTH	vs	hLH	46
scGTH	vs	bLH	41
scGTH	vs	hFSH	41
scGTH	vs	bFSH	41
hLH	vs	bLH	66
hLH	vs	hFSH	36
hFSH	vs	bFSH	91

homology analysis of amino acid sequences of α and β subunits are shown in Table 1. The α subunits of four fresh water fishes (carp, silver carp, bighead carp and grass carp, all Family Cyprinidae) are almost identical (98-100%), and show about 75% and 70% homology to marine fishes (pike eel and salmon) and human (hLH), respectively. As shown in Fig. 1, the variable regions are rather concentrated on two regions, 1-10 and 68-79 (less than 30% homology), and the homology of other regions, 11-67 and 80-96, is very high, 80% and 88%, respectively. Moreover, about 64% of the differences in amino acid residues at various position can be explained by a single base mutation in the codon. The heterogeneity at the N-terminus of the α subunit is also observed in bcGTHα (Huang *et al.*, 1990). It is obvious that the α subunit is quite conserved during the evolution from fish to higher vertebrate. The β subunits of four fresh water fishes are again almost identical (97-99%). About 80% homology is kept between fresh and marine fish, however not more than 50% homology exists between piscine and mammalian hormone. In contrast with α subunit, the variable regions are rather scattered all over the polypeptide chain of β subunit, however 12 half-cystine residues occupy identical positions. It implies that the hormone specific β subunit evolved from a common ancestral molecule.

HORMONAL ACTIVITY AND IMMUNOLOGICAL PROPERTIES

The isolated GTH preparation was first assayed by steroidogenesis in rat Leydig cells as usually employed for mammalian LH (Moyle and Ramachandran, 1973), however it showed extremely low potency (Huang *et al.*, 1981). When carp testes as well as carp ovarian follicles instead of rat Leydig cells were used, it showed very good response, while mammalian LH was not active in this system (Huang *et al.*, 1981; Chang *et al.*, 1988a). The quite obvious species specificities were observed. They were also characterized *in vivo* system either by the stimulation of ovulation in loach or by P^{32}-phosphate uptake in chick testes, and of course the RIA method was routinely used for convenience (Huang *et al.*, 1981; Chang *et al.*, 1988a). The typical relative potency of GTHs as assayed by steroidogensis in carp

testes system is shown in Table 2. Four fresh water piscine GTHs show practically identical potency, but marine fish, peGTH, shows rather lower potency and ovine LH shows almost no response in this system. However in rat Leydig cell assay, the results were completely reversed, all piscine GTHs showed no response.

Table 2
The relative biological activity of various gonadotropins in stimulation of androgen production by carp testis *in vitro*

Gonadotropin	Relative potency (%)
Carp GTH	100
Silver carp GTH	108
Bighead carp GTH	102
Grass carp GTH	94
Pike eel GTH	56
Ovine LH	0

The immunological properties also support the species specificity as shown in Table 3. Fresh water piscine GTHs were immunologically almost identical, and with marine fish, peGTH, the displacement curve is not parallel, they did cross react but not identical. It means their epitopes are not identical but some of them must be the same, as the polyclonal antibodies were used in this experiment. Mammalian hormone, oLH, shows no cross reactivity to any piscine GTH. Both immunological property and hormonal activity are very well coincided with their structural homology.

MOLECULAR CLONING

In recent years, the determination of nucleotide sequence of a

Table 3
Immunological cross-reactivities of piscine and mammalian gonadotropins

	Cross-reactivity (%)			
	Antiserum			
Antigen	Anti-cGTH	Anti-scGTH	Anti-peGTH	Anti-oLH
cGTH	100	136	np*	0
scGTH	89	100	np	0
bcGTH	81	125	np	0
peGTH	np	np	100	0
oLH	0	0	0	100

*: not parallel
c: carp; sc: silver carp; bc: bighead carp; pe: pike eel; o: ovine

gene or cDNA has been used for confirmation or prediction of the amino acid sequence of the corresponding protein. For example, as described before, the amino acid sequences of many piscine GTHs were established by this method. Furthermore also for the study of biosynthesis of GTH, the molecular cloning of GTH was proceeded in our laboratory. For the molecular cloning, the corresponding cDNAs were prepared by mRNA approach. Pitutary polyadenylated mRNA was prepared from freshly collected pituitaries by guanidinium/CsCl method (Ulrich *et al.*, 1977)

```
     GATCACATCTCGCAGGAAGTCAAGAACAAAGCAATC ATG TTT TGG ACA AGA TAT GCT  57
                                          Met Phe Trp Thr Arg Tyr Ala
                                          -23         -20
GGA GCA AGT ATA TTA TTG TTT TTT ATG CTT ATT CGT CTT GGA CAA CTG TAT 108
Gly Ala Ser Ile Leu Leu Phe Phe Met Leu Ile Arg Leu Gly Gln Leu Tyr
                        -10                                 -1   1
CCA AGA AAT GAT ATG AAT AAC TTT GGA TGT GAG GAG TGC AAA CTC AAG GAG 159
Pro Arg Asn Asp Met Asn Asn Phe Gly Cys Glu Glu Cys Lys Leu Lys Glu
                                  10
AAC AAC ATT TTC TCA AAA CCT GGA GCT CCT GTC TAT CAG TGT ATG GGA TGC 210
Asn Asn Ile Phe Ser Lys Pro Gly Ala Pro Val Tyr Gln Cys Met Gly Cys
     20                                     30
TGT TTT TCT AGG GCT TAC CCC ACG CCC CTG AGG TCC AAG AAA ACC ATG CTT 261
Cys Phe Ser Arg Ala Tyr Pro Thr Pro Leu Arg Ser Lys Lys Thr Met Leu
                 40                                  50
GTT CCA AAA AAT ATC ACA TCA GAA GCT ACA TGC TGT GTA GCC AAA GAA GTT 312
Val Pro Lys Asn Ile Thr Ser Glu Ala Thr Cys Cys Val Ala Lys Glu Val
                             60
AAG CGG GTG CTT GTC AAT GAT GTC AAA CTA GTG AAC CAC ACA GAC TGC CAC 363
Lys Arg Val Leu Val Asn Asp Val Lys Leu Val Asn His Thr Asp Cys His
 70                                      80
TGC AGC ACC TGC TAC TAT CAT AAG TCT TAA ACAAACAACAACAAAAACATTGTGACA 420
Cys Ser Thr Cys Tyr Tyr His Lys Ser ***
             90                  95
TTCAAGATTTGCTTGTGTTGGCCTAAAACTAGTTATATTCCCATCTACCTGTCATTTTACGCCACTT 487

ATTAGTTTTGTTAGCTGTGCATATTTACTTTTCTACCATGCAAATTATTCCCTAAATTGTCAATGCT 554

GGGTGTTTTTTCCACAACTTCTTTTCCAAAAGGTTGCCATCATTATTGCAACCAACATTTTAAGAGC 621

AAATAATGTGATATTTTATCATTAATAAAATGCAAGAAAACATCATCACTGCACTCTGTTTGTTATT 688

AGTTTAAGCCCTCTTTTGCTGGTAATTGCCCATTTATTTTGTCATCTTACTTATGTAAGATTGTTCA 755

GATCCATATATTAAATTAAAAATATGATTGCTTGTG-Poly A                          824
```

Fig. 3. The nucleotide sequence of cDNA encoding cGTH-α and its deduced amino acid sequence.

followed by oligo-dT cellulose column chromatography. Double stranded cDNA, synthesized by the method of Gubler and Hoffman (1983), was ligated with *Eco*RI site of pUC9. The *E. coli* strain JM101 was used

```
TTTTAACAGCCTGCTGAGCA ATG GGG ACA CCT GTC AAG ATT TTA GTT GTT CGA  53
                     Met Gly Thr Pro Val Lys Ile Leu Val Val Arg
                     -27                         -20
AAC CAC ATC CTA TTC TCT GTA GTT GTC CTA CTA GCT GTT GCT CAA AGC 101
Asn His Ile Leu Phe Ser Val Val Val Leu Leu Ala Val Ala Gln Ser
                         -10                                 -1
TCT TAT CTT CCA CCC TGT GAG CCA GTT AAT GAG ACT GTA GCT GTG GAA 149
Ser Tyr Leu Pro Pro Cys Glu Pro Val Asn Glu Thr Val Ala Val Glu
  1                              10
AAG GAG GGC TGT CCA AAA TGT CTG GTG TTA CAG ACC ACC ATC TGC AGC 197
Lys Glu Gly Cys Pro Lys Cys Leu Val Leu Gln Thr Thr Ile Cys Ser
             20                                  30
GGT CAC TGC CTG ACA AAG GAG CCT GTA TAC AAG AGC CCA TTT TCC ACT 245
Gly His Cys Leu Thr Lys Glu Pro Val Tyr Lys Ser Pro Phe Ser Thr
                         40
GTC TAC CAA CAC GTG TGC ACT TAC CGG GAT GTG CGT TAC GAG ACC GTC 293
Val Tyr Gln His Val Cys Thr Tyr Arg Asp Val Arg Tyr Glu Thr Val
     50                                  60
CGC TTG CCA GAT TGT CCT CCA GGG GTG GAC CCC CAT ATC ACC TAC CCG 341
Arg Leu Pro Asp Cys Pro Pro Gly Val Asp Pro His Ile Thr Tyr Pro
                    70                                      80
GTG GCT CTC AGC TGC GAC TGC AGC CTG TGC ACC ATG GAC ACA TCC GAC 389
Val Ala Leu Ser Cys Asp Cys Ser Leu Cys Thr Met Asp Thr Ser Asp
                              90
TGT ACG ATT GAA AGC CTG CAG CCT GAC TTT TGC ATG TCT CAG AGA GAG 437
Cys Thr Ile Glu Ser Leu Gln Pro Asp Phe Cys Met Ser Gln Arg Glu
            100                                 110
GAT TTT CTT GTA TAC TAG CCCCTTTTGGTCCCAAAACTACTATCCTGTGTTTAGCAC 494
Asp Phe Leu Val Tyr ***
                117
ATCAAACCAAAGTGTACACAAACTGATAAACTCTCACTCTAAATCAGATAAATGTTGTGTAGA 557

TGTATATCAATAAAAAGTGCATACTTCTTAAATTCATTTTCTACGCTAAAGCCAAACACATTT 620

TATTCTAGTAATGTAAATGGGCAGTTGGCGTGGATATTGAGCTGAAGCATATAGTTGTAAGTG 683

ATCATTAGAAAATCCAGGGCCAATGACCAGAAAGAAAAACAACAAGAGTTTAATTGATACAAA 746

AGTACAAATGCCAAATGAAAATCAGTTGTACTCTGTATACGGAGAAGCTTGTTAATTAAATTG 809

CAAAACTTTGATCATGGTATTCATCCATCACCATGATGAATTTAAATAACACTGTGCCACTTT 872

TTGAAATATGAAATAAAACACAAAAT-Poly A                                 909
```

Fig. 4. The nucleotide sequence of cDNA encoding cGTH-β and its deduced amino acid sequence.

as host. Thus constructed cDNA library was then screened either by the corresponding synthetic oligonucleotide or known cDNA as probe. Bacteria containing recombinant plasmids were first grown on nitrocellulose filters, lyzed with NaOH, and baked for colony hybridization by the method of Grunstein and Hogness (1975). For further confirmation, the plasmid DNAs of positive clones were extracted and digested with *Eco*RI followed by *in situ* hybridization to agarose gel with labelled probes (Kidd *et al.*, 1983). For nucleotide sequence analysis, the cDNAs encoding GTH-α and GTH-β were cleaved with *Pst*I and *Ava*II, respectively. After separation by electrophoresis in agarose and electroelution, the resulted DNA fragment were subcloned into pUC9. The nucleotide sequences were determined by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977). The typical results of the nucleotide sequences and the deduced amino acid sequences of cDNAs encoding cGTH-α and cGTH-β are shown in Figs. 3 and 4, respectively. It also becomes clear that all subunits are synthesized as prohormone with different size of signal peptide at N-terminus as shown in Figs. 3 and 4, and in β subunit, C-terminal two amino acid residues in prohormone are removed during post-translational processing as shown in Fig. 4. By this method, the complete amino acid sequences of cGTH-α, -β (Chang *et al.*, 1988b), scGTH-α, -β (Chang *et al.*, 1990), bcGTH-α, -β (Huang *et al.*, 1990) and gcGTH-α, -β (unpublished data) were determined as shown in Figs. 1 and 2.

EXPRESSION OF TWO FORMS OF cGTH-α SUBUNIT

In the study of carp cDNA, from 1,286 clones, we obtained six positive clones for α, and five clones for β subunit. All clones for β were almost the same size and showed identical base sequences in the region of reading frame, however among six α clones, four clones showed the same sequence as the subunit we isolated from pituitary gland while another two clones showed slightly different sequences. They differ in seven amino acid residues, three in signal peptide region and four in mature protein region as shown in Fig. 5a. They were designated as α_1 and α_2, and α_2 is not yet isolated from pituitary extract (Chang *et al.*, 1988b). Because of

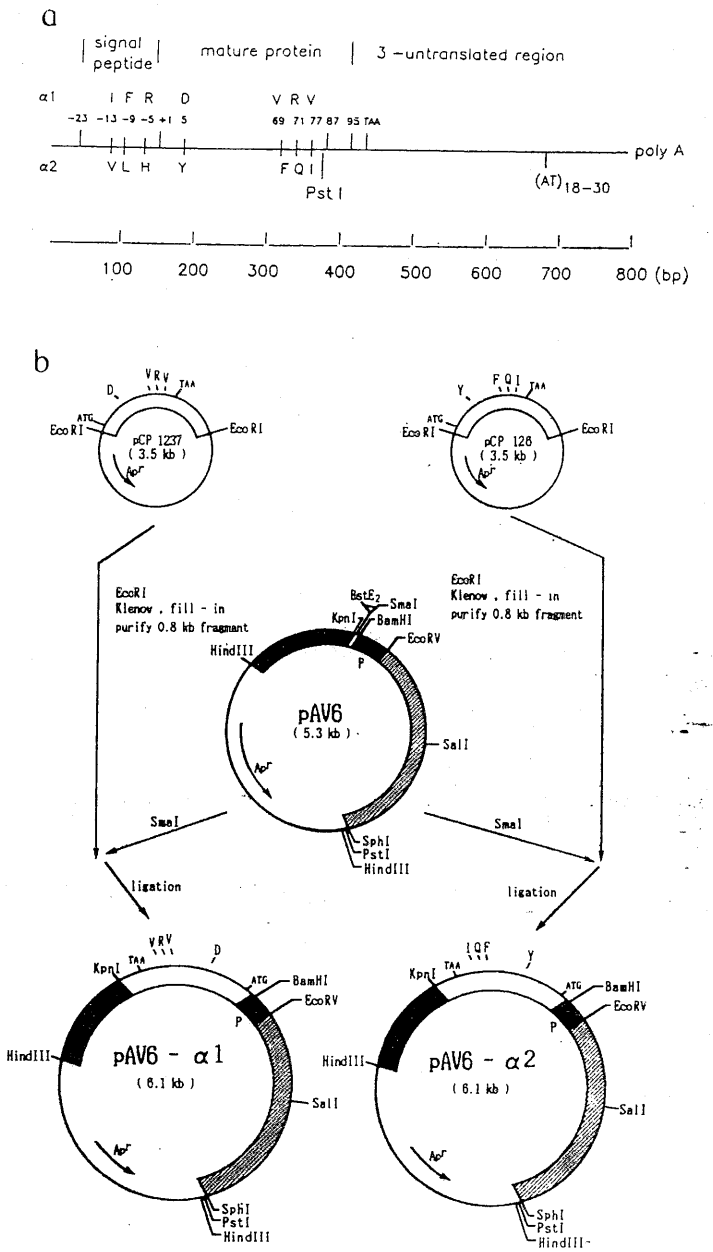


Fig. 5. (5a) Structural differences between α_1 and α_2 cDNAs. Both α_1 and α_2 cDNAs of cGTH have an internal *Pst*I site located at codon 86. Unusual A·T repeat sequences with varied length found only in the α_2 cDNA are indicated as $(AT)_{18-30}$. Amino acid differences are shown in single letter code.

(5b) Construction of transfer vectors. P, promoter of the polyhedrin gene. The upstream and downstream regions of the polyhedrin gene are shown by light and dark shading, respectively. ATG, start codon; TAA, stop codon; Ap^r, ampicillin-resistance gene.

the curiosity to know the properties of α_2 subunit as well as like to prepare various subunits with differently glycosylated forms, the expression study was proceeded by using a transfer plasmid (pAV6) as vector and Sf21-AE insect cells as host. In the vector pAV6, a part of polyhedrin gene was deleted and multiple cloning site (MCS) was inserted as shown in Fig. 5b. The filled region contains 0.6 kb of the downstream with polyadenylylation signal. The insert encoding the α_1 or α_2 subunit was cleaved by *Eco*RI and the 0.8 kb filled-in *Eco*RI fragment was isolated by agarose gel electrophoresis. This frament was ligated to the *Sma*I site of pAV6. Thus prepared transfer plasmids, pAV6-α_1 and pAV6-α_2, had upstream and downstream regions of the polyhedrin gene that allowed for homologous recombination with the same regions in the genome of the wild-type baculovirus. The expression of both cDNAs was controlled by the promoter from the polyhedrin gene. Each transfer plasmid DNA was cotransfected with the wild-type AcNPV viral DNA into insect cells, Sf21-AE (Summers and Smith, 1987). After transfection, pure recombinant viruses, vAV6-α_1 and vAV6-α_2 were screened by dot blot hybridization

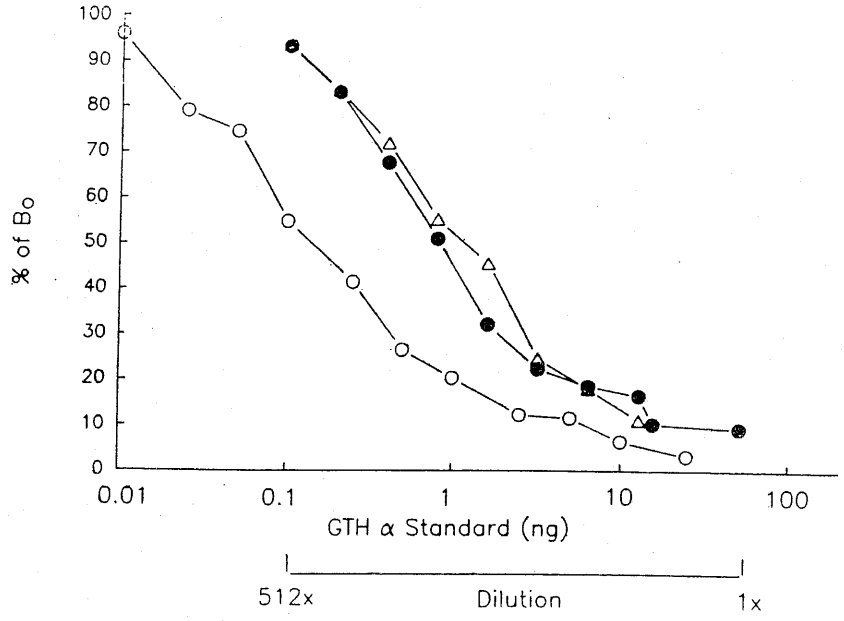


Fig. 6. Displacement of I^{123}-labelled native cGTH-α subunit from anti-cGTH-α by 0.01--100 ng of unlabelled native α (○) or various dilution of recombinant α_1 (●) or α_2 (△).

(Pen *et al.*, 1989), using either α_1 or α_2 cDNA as probe. For expression, monolayers of Sf21-AE cells were infected with recombinant viruses. The kinetics of synthesis and secretion, and molecular weights

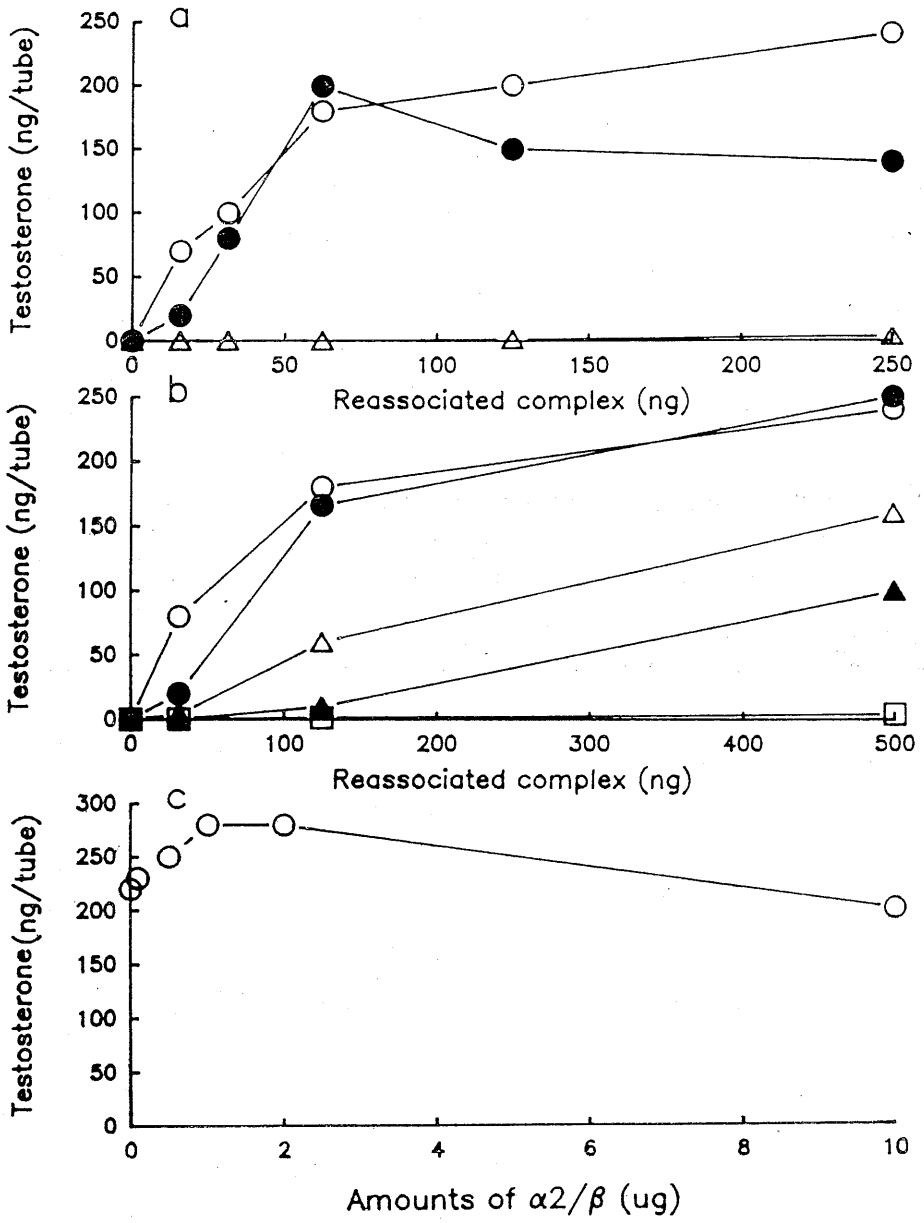


Fig. 7. Biological activity of GTH assay by steroidogenesis in carp testes. (a) Dose-response curve of native $\alpha+\beta$ subunit (○), r$\alpha_1+\beta$ (●), and r$\alpha_2+\beta$ (△). (b) Competition of rα_2 subunit with rα_1 subunit for association with native β subunit: native $\alpha+\beta$ (○), r$\alpha_1+\beta$ (●), r$\alpha_1+\beta$+20%rα_2 (△), r$\alpha_1+\beta$+50%rα_2 (▲), and r$\alpha_1+\beta$+100%rα_2 (□). (c) Addition of various amounts of preformed rα_2/β complex to 1 μg of rα_1/β complex.

of these two α_1 and α_2 subunits were almost the same, and immunologically also identical as shown in Fig. 6 (Huang *et al.*, 1991a). The hormonal activity was tested by the steroidogenesis in carp testes assay after associated with the native β subunit of cGTH isolated from pituitary gland, and the results are shown in Fig. 7. As shown in Fig. 7a, recombinant α_1 subunit ($r\alpha_1$) shows almost the same potency as native α, however $r\alpha_2$ is ineffective. Fig. 7b shows the effect of $r\alpha_2$ on association of $r\alpha_1$ and native β. The increased amount of $r\alpha_2$ (20%, 50% and 100% of $r\alpha_1$) was added in the system which contained equal amount of $r\alpha_1$ and native β. It is obvious that the presence of 100% $r\alpha_2$ completely devoid the association of $r\alpha_1$ and β. However the Fig. 7c shows the preformed α_1/β was not affected by the preformed α_2/β even at 10 fold higher concentration (Huang *et al.*, 1991a). The existence and property of α_2 subunit is very fascinating. It is a competitive inhibitor of α_1 to associate with β, thus it may serve as an regulator for controlling GTH level in the biological system, or it may be just one degenerating molecular species during the process of evolution. They differ only in four amino acid residues at position 5, 69, 71 and 77. The position 71 has been indicated to be an essential residue (Sairam, 1983). It is noticed that all known active α subunits are residued either as Arg or Lys at this position as shown in Fig. 1, however in α_2 it is Gln. It is an interesting observation for further studies of structure-function relationship of the α subunit.

GENE STRUCTURES OF cGTH α AND β SUBUNITS

For determination of gene structure, two different approaches were employed. The first one is the polymerase chain reaction (PCR) method using genomic DNA as template and synthetic oligonucleotides as primers for investigation of the cGTHα gene. Four set of primers used are shown in Fig. 8. All PCR amplified DNA fragments were purified from agarose gel electrophoresis and were ligated into *Sma*I site of plasmid vector pUC18, then subjected to DNA sequence analysis by dideoxy chain termination method. All the PCR amplified products of each set of primers contain two types of DNA sequences; one corresponds to cGTHα_1 cDNA, while the other matches with the

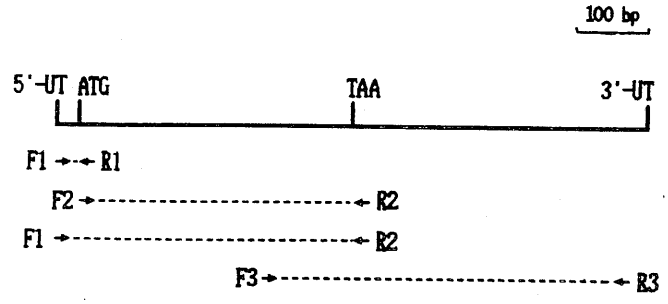
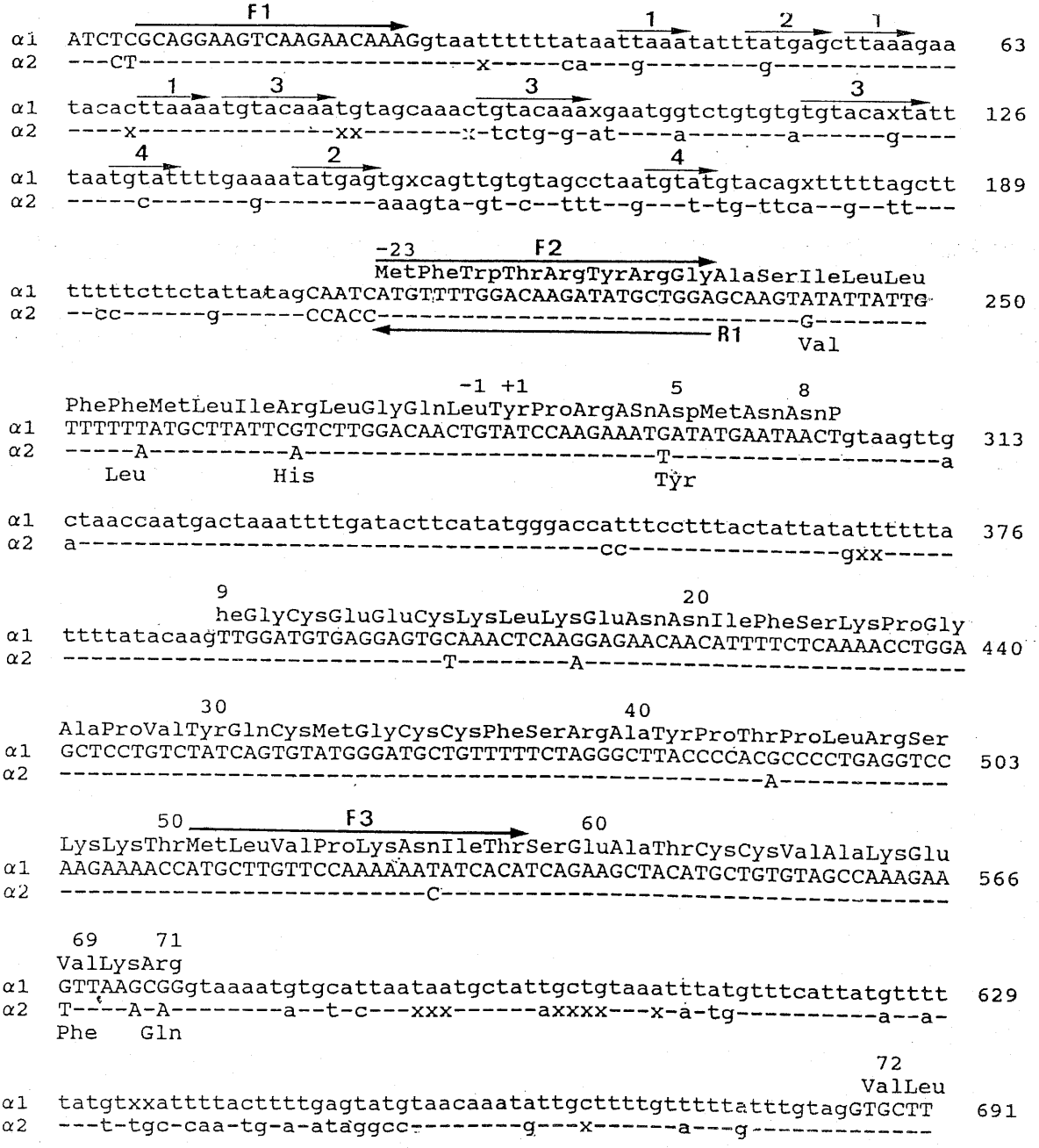


Fig. 8. The locations of primers and the strategy of using PCR to align the sequence of cGTHα gene. The sequences of the synthetic forward (F) and reversed (R) primers are indicated in Fig. 9. 5′-UT, 5′-untranslated region; 3′-UT, 3′-untranslated region; ATG, start codon; TAA, stop codon.

cGTHα_2 cDNA. They were aligned by using their overlapping regions, and shown in Fig. 9. Both cGTHα_1 and cGTHα_2 genes comprise four exons and three introns. The intron I is located in the 5′-untranslated regions, and is 177 bp in length for both genes with 74% homology in sequence. The other two introns, II and III, are located in the coding region and are 80 bp and 108 bp in cGTHα_1 gene, and 80 bp and 101 bp in cGTHα_2 gene. The sequence homology was 94% for intron II and 65% for intron III. An unusal AT-repeat is only present in the 3′-untranslated region of cGTHα_2 gene. A third cGTHα gene, α_2-1 (Fig. 10) with only 460 bp in length, was detected in PCR amplified products by using primers F3 and R3 (Fig. 8). Nucleotide sequence analysis of this fragment indicates that it is identical to part of the cGTHα_2 gene except intron III is missing. Whether it is a pseudogene or not is unknown. The above results indicate that multiple α genes are present in carp, while only single α gene has been found in mammals. The comparison of carp, rat, bovine, human, rhesus and mouse GTHα gene structures are shown in Fig. 10. The basic organization of all α genes are the same, consisting of four exons and three introns, and the splicing sites of α genes are very similar. The size of exons are almost the same, but the size of introns of cGTHα are much smaller than those of mammalian α genes as shown in Fig. 10 (Huang *et al.*, 1992).

For the determination of the β subunit gene, the second approach, the carp genomic DNA library was

directly used for screening of cGTHβ gene. The carp genomic DNA was partially digested with *Sau*3A1, and 15-20 kb DNA fragments were isolated by agarose gel electrophoresis, and ligated into *Bam*HI site of phage vector, EMBL 3, then after *in vitro* packaging, transfected into *E. coli* KW251 strain. Constructed genomic library was then screened



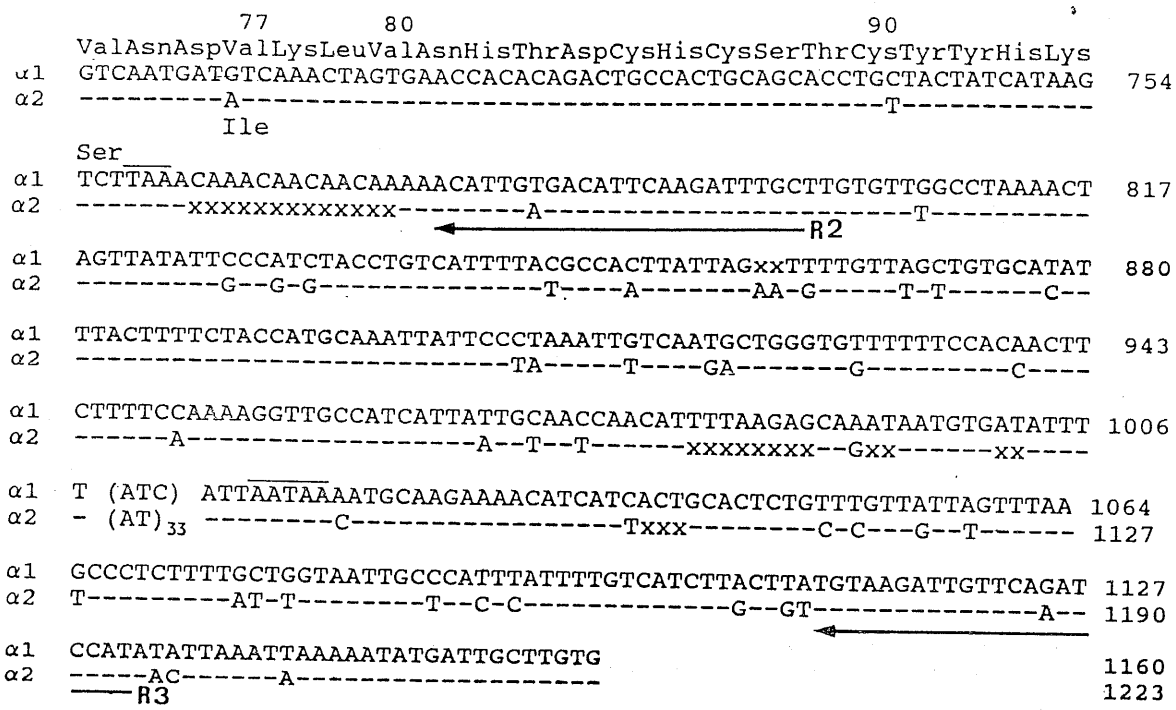


Fig. 9. The nucleotide sequences of cGTHα_1 and cGTHα_2 genes. —, in cGTHα_2 gene indicates nucleotides identical to those of cGTHα_1 gene; X, denotes deletions used for maximal alignment of sequence. Exons are shown by capitals and introns by small letters. The unusual AT-repeat sequences in cGTHα_2 gene are represented as $(AT)_{33}$ with repeat number denoted by the subscript. Several direct repeats are shown and numbered 1 to 5. Locations and sequences of PCR primers are also shown. TAA, stop codon; AATAA, polyadenylation signal.

with cDNA of cGTHβ as probe. The screened β gene was further subjected to restriction enzyme mapping, Southern hybridization, then subcloning and finally sequenced. The base sequence of cGTHβ gene is shown in Fig. 11. The size is also about 1.2 kb, and composed of three exons and two introns. The schematic drawing for comparison with some other GTHβ genes are shown in Fig. 12. They are not like α genes, almost the same in size, and consist of three exons and two introns. Mature protein regions are located in exons 2 and 3 (Chang *et al.*, 1992).

CONCLUDING REMARKS

It took us almost ten years to establish the most basic knowledges of piscine GTHs including chemical structure, hormone action, gene structure and expression. Although

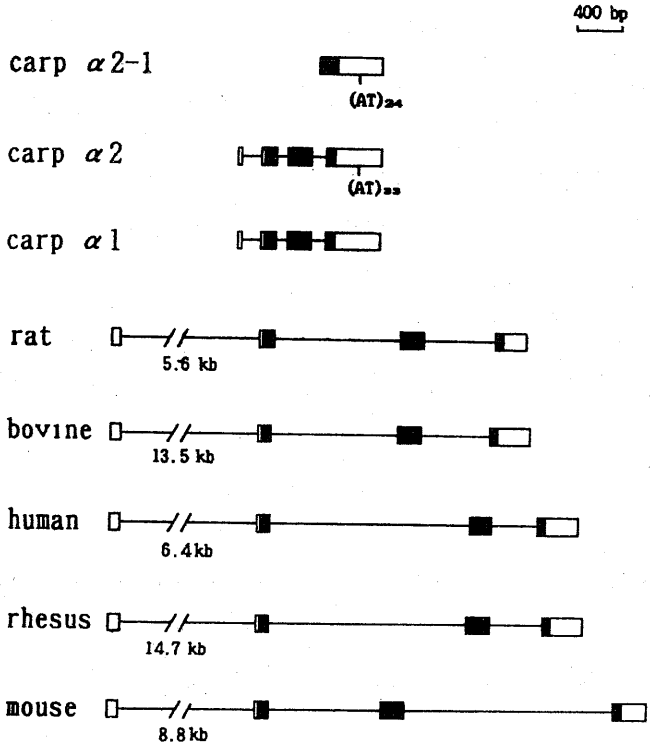


Fig. 10. Comparison of α gene structures of carp, rat (Burnside *et al.*, 1988), bovine (Goodwin *et al.*, 1983), human (Boothby *et al.*, 1981), rhesus (Golos *et al.*, 1991) and mouse (Gordon *et al.*, 1988). Boxes and lines represent exons and introns, respectively. Coding regions are shown as solid and noncoding regions as open boxes.

it is not complete, however based on those descriptive informations obtained, several comments should be mentioned. All mammalian pituitary gonadotropic hormones have been known to exist in two distinct molecular species, lutropin (LH) and follitropin (FSH), however we found only one type of GTH during our isolation procedures as described earlier. These GTHs isolated seem rather similar to LH as compared the sequence homology of the hormone specific β subunit, *i.e.* homology of scGTH-β to hLH-β and hFSH-β is 46% and 41%, respectively (Table 1). Recently, two types of GTH, GTHI and GTHII, have been isolated from chum salmon (Suzuki *et al.*, 1988a; Suzuki *et al.*, 1988b; Ito *et al.*, 1988) and coho salmon (Swanson *et al.*, 1989, 1991), and GTHI and GTHII are structurally homologous to mammalian FSH and LH, respectively. However only one GTH (type II) has been found in chinook salmon (Trinh *et al.*, 1986). Chum salmon GTHI-β and GTHII-β

```
gattagataatctactgtatataatatatgtgcactgaacgaacagcaggtgtcttttat -153
ctgttcattctgacctgatgaatttggagagttgttacgtgagaatggaaacacataatc - 93
ctcttgagatcagtgtgattaatttaaggtcagattttagtttaattgttggtttcatgg - 33
cactatataaatctacccagatgccgtgtgacTGTACACATAGAGCCACTTTTAACAGCC  28
        -27
          M  G  T  P  V  K
TGCTGAGCAATGGGGACACCTGTCAAgtaagtctaataaataattaatgcagtaatattg  88
ttcacagttttttttctttttctttttgcttctttgctgtttcaatggggcttcacgtta 148
                                        -20
                                       I  L  V  V  R  N  H  I  L
aatggaattgctctccttctttttccatctacagGATTTTAGTTGTTCGAAACCACATCCT 208
      -10                          -1  1
  F  S  V  V  V  L  L  A  V  A  Q  S  S  Y  L  T  T  C  E  P
ATTCTCTGTAGTTGTCCTACTAGCTGTTGCTCAAAGCTCTTATCTTCCACCCTGTGAGCC 268
    10                             20
  V  N  E  T  V  A  V  E  K  E  G  C  P  K  C  L  V  L  Q  T
AGTTAATGAGACTGTAGCTGTGGAAAAGGAGGGCTGTCCAAAATGTCTGGTGTTACAGAC 328
    30
  T  I  C  S  G  H  C  L  T  K
CACCATCTGCAGCGGTCACTGCCTGACAAAGgtgcttactttcttgtcagtgtctttttt 388
ttgtttgtttttacctgttcatttgcatgggaacatttttcagcaacattaatttgaat  448
tcagattaatatacaagcatcattatttccagtggtgatgacagtgtattatcttacatt 508
        40                             50
      E  P  V  Y  K  S  P  F  S  T  V  Y  Q  H  V  C  T  Y
tctagGAGCCTGTATACAAGAGCCCATTTTCCACTGTCTACCAACACGTGTGCACTTACC 568
        60                             70
R  D  V  R  Y  E  T  V  R  L  P  D  C  P  P  G  V  D  P  H
GGGATGTGCGTTACGAGACCGTCCGCTTGCCAGATTGTCCTCCAGGGGTGGACCCCCATA 628
        80                             90
I  T  Y  P  V  A  L  S  C  D  C  S  L  C  T  M  D  H  S  D
TCACCTACCCGGTGGCTCTCAGCTGCGACTGCAGCCTGTGCACCATGGACACGTCCGACT 688
       100                            110
C  T  I  E  S  L  Q  P  D  F  C  M  S  Q  R  E  D  F  L  V
GTACGATTGAAAGCCTGCAGCCTGACTTTTGCATGTCTCAGAGAGAGGATTTCCTTGTAT 748
117
Y  .
ACTAGCCCCTTTTGGTCCCAAAACTACTATCCTGTGTTTAGCACATCAAACCAAAGTGTA 808
CACAAACTGATAAATTCTCACTCTAAATCAGATAAATGTTGTGTAGATGTATATCAATAA 868
AAAGTGCATACTTCTTAAATTCATTTTCTACGCTAAAGCCAAACACATTTTATTCTAGTA 928
ATGTAAATGGGCAGTTGGCGTGGATATTGAGCTGAAGACATATAGTTGTAAGTGATCATT 988
AGAAAATCCAGGGCCAATGACCAGAAAGAAAAACAACAAGAGTTTAATTGATACAAAAGT 1048
ACAAATGCCAAATGAAAATCAGTTGTACTCTGTATACGGAGAAGCTTGTTAATTAAATTG 1108
CAAAACTTTGATCATGGTATTCATCCATCACCATGATGAATTTAAATAACACTGTGCCAC 1168
TTTTTGAAATATGAAATAAAACACAAAATAAATAAAATTAAAACAAATGaattaatgcat 1228
tccgcaggcaaaagttattgttctcctttgaagatcactcagaggagaacatgcattctg 1288
```

Fig. 11. The nucleotide sequence of cGTH-β gene. Exons are shown by capital and introns by small letters.

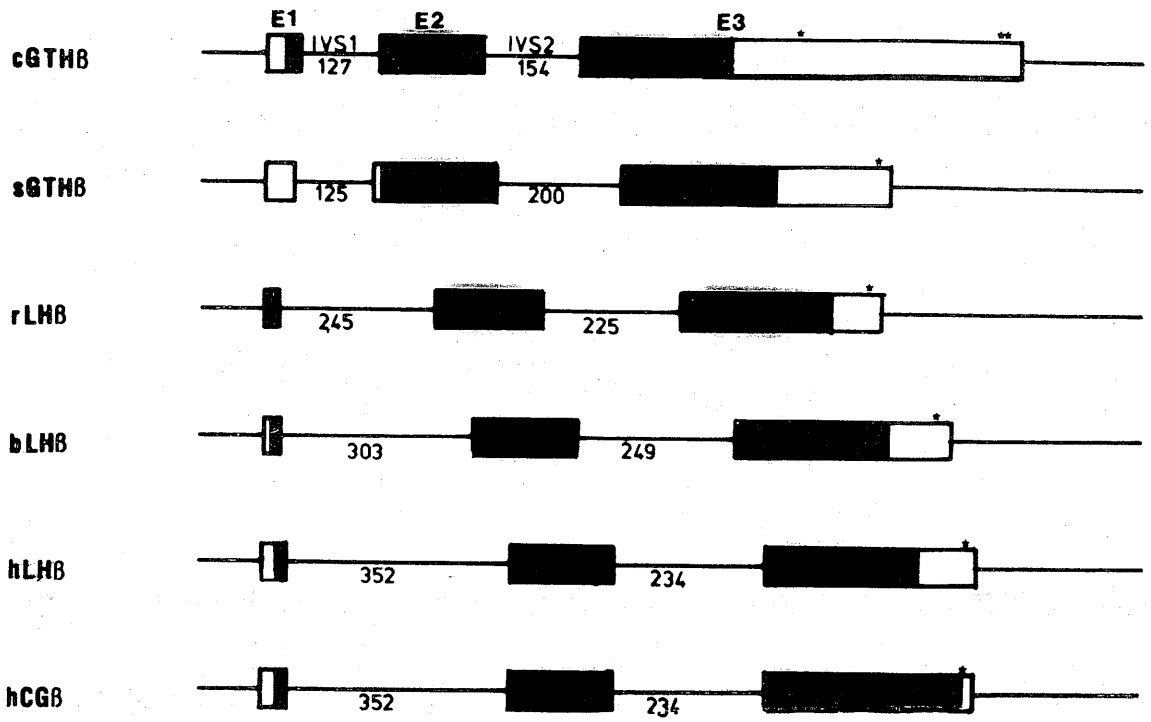


Fig. 12. Comparison of cGTH-β gene with s: salmon (Xiong and Hew, 1991), r: rat (Jameson *et al.*, 1984), b: bovine (Virgin *et al.*, 1985), h: human LH-β and CG-β (Talmadge *et al.*, 1984) genes. Boxes and lines represent exons (E) and intron (IVS: intervening sequences), respectively. Coding regions are shown as solid and noncoding regions as open boxes. The asterisk (*) indicates the polyadenylation site of the gene.

have only about 31% amino acid sequence identity to each other, and both α subunits are unexpectedly not identical but show only 72% sequence homology (Itoh *et al.*, 1990). However both GTHI and GTHII could not be differentiated by *in vitro* steroidogenesis assay, both were almost equally active in these assayes. Based on the limited observation obtained, Swanson *et al.* (1991) suggest relationships of GTHI with gonadal growth and GTHII with final maturation of the gonads similar to those of FSH and LH in mammals. Apparently more direct and further verifications are necessary. Based on structural homology, GTHs we isolated are apparently belong to the type of GTHII isolated from salmon. As mentioned before, we did not find other type of GTH, however we are not sure whether type I GTH exists in Cyprinidae fish or not. Further study is in progress.

The discovery of hormonally ineffective α_2 subunit in carp cDNA library is very interesting. Again

we do not know whether it is really expressed or exists in pitutitary extract or not. The more extensive search of this fraction in pituitary extract is now in progress. Three other fresh water fish cDNA libraries were constructed under the same way as in carp, however we could not screened any clone equivalent to ineffective α_2 of carp. It is interesting to know whether it is happened only in carp or not. Moreover the structure-function relationship between α_1 (active) and α_2 (inactive) subunits, including both protein and oligosaccharide moieties, and the role of oligosaccharide moiety during receptor binding and signal transduction, remain subjects for our further investigations.

REFERENCES

Boothby, M., R.W. Ruddon, C. Anderson, D. McWilliams and I. Boime (1981) A single gonadotropin α subunit in normal tissue and tumor-derived cell lines. *J. Biol. Chem.* **259**: 5121-5127.

Burnside, J., R.R. Buckland and W.W. Chin (1988) Isolation and characterization of the gene encoding the α-subunit of the rat pituitary glycoprotein hormones. *Gene* **70**: 67-74.

Chang, Y.S., F.L. Huang, C.T. Chen and T.B. Lo (1988a) Isolation and properties of the pituitary gonadotropin from silver carp (*Hypophthalmichthys molitrix*). *Int. J. Peptide Protein Res.* **31**: 150-156.

Chang, Y.S., C.J. Huang, F.L. Huang and T.B. Lo (1988b) Primary structure of carp gonadotropin subunits deduced from cDNA nucleotide sequences. *Int. J. Peptide Protein Res.* **32**: 556-564.

Chang, Y.S., C.J. Huang, F.L. Huang, C.S. Liu and T.B. Lo (1990) Purification, characterization and molecular cloning of gonadotropin subunits of silver carp (*Hypophthalmichthis molitrix*). *Gen. Comp. Endocrinol.* **78**: 23-33.

Chang, Y.S., F.L. Huang and T.B. Lo (1992) Isolation and sequence analysis of carp gonadotropin β subunit genes. *Mol. Marine Biol. Biotech.* (in press).

Golos, T.G., M. Durning and J.M. Fisher (1991) Molecular cloning of the rhesus glycoprotein hormone α-subuit gene. *DNA and Cell Biol.* **10**: 367-380.

Goodwin, R.G., C.L. Moncman, F.M. Rottman and J.H. Nilson (1983) Characterization and nucleotide sequence of the gene for the common α subunit of the bovine pituitary glycoprotein hormones. *Nucleic Acids Res.* **11**: 1910-1920.

Gordon, D.F., W.M. Wood and E.C. Ridgway (1988) Organization and nucleotide sequence of the mouse α-subunit gene of the pituitary glycoprotein hormones. *DNA* **7**: 679-690.

Grunstein, M. and D. Hogness (1975) Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene. *Proc. Natl. Acad. Sci. USA* **72**: 3961-3965.

Gubler, U. and B. J. Hoffman (1983) A simple and very efficient method for generating cDNA library. *Gene* **25**: 263-269.

Huang, C.J., F.L. Huang, G.D. Chang, Y.S. Chang, C.F. Lo, M.J. Fraser and T.B. Lo (1991a) Expression of two forms of carp gonadotropin α subunits in insect cells by recombinant baculovirus. *Proc. Natl. Acad. Sci. USA* **88**: 7486-7490.

Huang, C.J., F.L. Huang, Y.C. Wang, Y.S. Chang and T.B. Lo (1992) Organization and nucleotide sequence of carp gonadotropin α subunit genes. *Biochim. Biophys. Acta* **1129**: 239-242.

Huang, F.L, C.J. Huang, S.H. Lin, T.B. Lo and H. Papkoff (1981) Isolation and characterization of gonadotropin isohormones from the pituitary gland of pike eel (*Muraenesox cinereus*). *Int. J. Peptide Protein Res.* **18**: 69-78.

Huang, F.L., Y.S. Chang, L.C. Yu and T.B. Lo (1990) Purification, characterization and molecular cloning of bighead carp (*Hypophthalmichthys nobilis*) gonadotropin and its subunits. *J. Chinese Biochem. Soc.* **19**: 10-24.

Itoh, H., K. Suzuki and H. Kawauchi (1988) The complete amino acid sequences of β subunits of two distinct chum salmon GTHs. *Gen. Comp. Endocrinol.* **71**: 438-451.

Itoh, H., K. Suzuki and H. Kawauchi (1990) The complete amina acid sequences of α subunits of chum salmon gonadotropins. *Gen. Comp. Endocrinol* **78**: 56-65.

Jameson, L., W.W. Chin, A.N. Hollenberg, A.S. Chang and J.F. Habener (1984) The gene encoding the β-subunit of rat luteinizing hormone. *J. Biol. Chem.* **259**: 15474-15480.

Kidd, V.J., R.B. Wallace, K. Hakura and S.L.C. Woo (1983) α_1-antitrypsin deficiency detection by direct analysis of the mutation in the gene. *Nature* (*London*) **304**: 230-234.

Liu, C.S., F.L. Huang, Y.S. Chang and T.B. Lo (1989) Pike eel (*Muraenesox cinereus*) gonadotropin: amino acid sequences of both α and β subunits. *Eur. J. Biochem.* **186**: 105-114.

Moyle, W.R. and J. Ramachandran (1973) Effect of LH on steroidogenesis and cyclic AMP accumulation in rat Leydig cell preparations and mouse Leydig cells. *Endocrinology* **93**: 127-134.

Pen, J., G.W. Welling and S. Welling-Wester (1989) An efficient procedure for the isolation of recombinant baculovirus. *Nucleic Acids Res.* **17**: 451.

Querat, B., M. Jutisz, Y.A. Fontaine and R. Counis (1990a) Cloning and sequence analysis of the cDNA for the pituitary glycoprotein hormone α-subunit of the European eel. *Mol. Cell. Endocrinol.* **71**: 253-259.

Querat, B., M. Moumni, M. Jutisz, Y.A. Fontaine and R. Counis (1990b) Molecular cloning and sequence analysis of the cDNA for the putative β subunit of the type-II gonadotropin from the European eel. *J. Mol. Endocrinol.* **4**: 257-264.

Sairam, M.R. (1983) *Gonadotropic Hormones: relationship between structure and function with emphasis on antagonists. In*: "Hormonal proteins and peptides" (Li, C.H. ed.) Vol. 11, Academic Press, New York. pp. 1-79.

Sanger, F., S. Nicklen and A.R. Coulson (1977) DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**: 5463-5467.

Shome, B. and A.F. Parlow (1973) The primary structure of the hormone-specific, β-subunit of human pituitary luteinizing hormone (hLH). *J. Clin. Endocrinol. Metab.* **36**: 618-621.

Shome, B., A.F. Parlow, W.K. Liu, H.S. Nahm, T. Wen and D.N. Ward (1988) A reevaluation of the amino acid sequence of human follitropin β-subunit. *J. Protein Chem.* **7**: 325-339.

Summers, M.D. and G.E. Smith (1987) A manual of methods for baculovirus vectors and insect cell culture procedures. *Texas Agric. Exp. Stn.* (*Bull.*) No. 1555.

Suzuki, K., H. Kawauchi and Y. Nagahama (1988a) Isolation and characterization of two distinct gonadotropins from chum salmon pituitary glands. *Gen. Comp. Endocrinol* **71**: 292-301.

Suzuki, K., H. Kawauchi and Y. Nagahama (1988b) Isolation and characterization of subunits of two distinct gonadotropins from chum salmon pituitary glands. *Gen. Comp. Endocrinol.* **71**: 302-306.

Swanson, P., M. Bernard, M. Nozaki, K. Suzuki, H. Kawauchi and W.W. Dickhoff (1989) Gonadotropins I and II in juvenile coho salmon. *Fish Physiol. and Biochem.* **7**: 169-176.

Swanson, P., K. Suzuki, H. Kawauchi and W.W. Dickhoff (1991) Isolation and characterization of two coho salmon gonadotropins, GTH I and GTH II. *Biology of Reproduction* **44**: 29-38.

Talmadge, K., N.C. Vamvakopoulos and J.C. Fiddes (1984) Evolution of the subunits of human chorionic gonadotropin and luteinizing hormone. *Nature (London)* **307**: 37-49.

Trinh, K.Y., N.C. Wang, C.L. Hew and L.W. Crim (1986) Molecular cloning and sequencing of salmon gonadotropin β subunit. *Eur. J. Biochem.* **159**: 619-624.

Ulrich, A., J. Shine, J. Chirgwin, R. Pitet, E. Tischer, W.J. Ratter and H.M. Goodman (1977) Rat insurin genes: construction of plasmids containing the coding segments. *Science* **196**: 1313-1319.

Virgin, J.B., B.J. Silver, A.R. Thomason and J.M. Nilson (1985) The gene for the β subunit of bovine luteining hormone encodes a gonadotropin mRNA with an unusually short 5′-untranslated region *J. Biol. Chem.* **260**: 7072-7077.

Xiong, F. and C.L. Hew (1991) Chinook salmon (*Oncorhynchus tschawytscha*) gonadotropin II β subunit gene encodes multiple messenger ribonucleic acids. Int. Symposium on Reproductive Biology in Aquaculture. April 22-27, Taipei, Taiwan. pp. 79.