Molecular cloning of the cDNAs for pituitary glycoprotein hormone α subunits of two species of duck and their gene regulation

Ya-Lun Hsieh, Abhijit Chatterjee, Jung-Tsun Chien and **John Yuh-Lin Yu**

Endocrinology Laboratory, Institute of Zoology, Academia Sinica, Taipei, Taiwan 115, Republic of China

(Requests for offprints should be addressed to John Yuh-Lin Yu, Endocrinology Laboratory, Institute of Zoology, Academia Sinica, Taipei, Taiwan 115, Republic of China; Email: johnyu@ccvax.sinica.edu.tw)

ABSTRACT

The cDNAs encoding pituitary glycoprotein hormone α subunits (PGHαs) of two species of duck (Muscovy duck, *Cairina moschata* and Pekin duck, *Anas platyrhynchos domesticus*) were cloned and sequenced to better understand the phylogenic diversity and evolution of PGHα molecules in vertebrates. Oligonucleotide primers were designed and used for reverse transcription PCR (RT-PCR) amplification of PGHα cDNA fragments from total cellular RNA of pituitary glands. The remaining sequences were completed by rapid amplification of the cDNA ends. The nucleotide sequence of isolated PGHα cDNA of both ducks are identical, including 81 bp of 5′ untranslated region (UTR), 360 bp of coding region, and 272 bp of 3′-UTR followed by a 13 bp poly(A)$^+$ tract. The total number of amino acids deduced from the cDNA of the duck PGHα is 120 with a signal peptide of 24 amino acids and a mature protein of 96 amino acids. PGHαs of the ducks (order Anseriformes) share 96% homology of amino acid sequence in signal peptide, and 100% homology in mature proteins with chicken, quail and turkey (order Galliformes). Our data thus demonstrate identical inter-order homology of PGHα mature protein in birds. Ten cysteine residues, presumably forming five disulfide bonds within the α subunit, and four proline residues, presumably responsible for folding of the molecule, are conserved in the α subunit of ducks. Northern blot analysis revealed that PGHα mRNA is expressed only in the pituitary. In order to study factors regulating the gene expression of PGHα mRNA, duck pituitary fragments were incubated with GnRH, TRH, testosterone, or triiodothyronine (T_3). GnRH and TRH increased, while testosterone and T_3 decreased, PGHα mRNA levels. This is the first report in birds of TRH up-regulation and down-regulation by testosterone and T_3 under *in vitro* conditions. The present study demonstrates both ducks have the same cDNA nucleotide and deduced amino acid sequences in the PGHα subunit, exhibiting identical inter-genus homology within the family of Anatidae. The findings from mRNA expression work suggest that hypothalamic GnRH and TRH up-regulate, while testosterone and T_3 down-regulate, PGHα gene expression in ducks.

Journal of Molecular Endocrinology (2001) **27,** 339–347

INTRODUCTION

The pituitary glycoprotein hormones (luteinizing hormone, LH; follicle-stimulating hormone, FSH; and thyroid-stimulating hormone, TSH) are members of the glycoprotein hormone family. Each hormone is a heterodimeric molecule, consisting of α and β subunits. The α subunit is common to all glycoprotein hormones and the main role is to confer biological action through the signal transduction pathway, whereas the β subunit is specific to each hormone and determines the hormonal activity and species specificity (Pierce & Parsons 1981, Quérat 1994). The α and β subunits are initially synthesized as separate proteins from different genes and are associated in cytoplasm by non-covalent bonding to form biologically active dimer molecules (Naylor *et al*. 1983, Kourides *et al*. 1984, Gharib *et al*. 1990). The cDNA and amino acid sequences of pituitary glycoprotein hormone α

0952–5041/01/027–339

subunits (PGHαs) have been studied much more extensively in mammals and teleosts than in birds. There are only three species of birds for which the cDNA and amino acid sequences of PGHα have been investigated: turkey (Foster & Foster 1991), chicken (Foster *et al*. 1992) and quail (Ando & Ishii 1994), which all belong to the order Galliformes. For better understanding of the phylogenic diversity and evolution of PGHα molecules in birds, we have cloned cDNAs for PGHαs from pituitaries of two species of bird of the order Anseriformes, Muscovy duck (*Cairina moschata*) and Pekin duck (*Anas platyrhynchos domesticus*). Their nucleotides and deduced amino acid sequences are compared with those of other birds and selected species of other vertebrate classes. It has been demonstrated that hypothalamic gonadotropin-releasing hormone (GnRH) and thyrotropin-releasing hormone (TRH) stimulate, while gonadal steroid hormones and thyroid hormones inhibit PGHα gene expression. To our knowledge, no or little information in this regard has been available in birds. We thus also investigated the hormonal influence on the expression of PGHα mRNA in the ducks under *in vitro* condition.

MATERIALS AND METHODS

Oligonucleotide design

Oligonucleotide primers for amplification of duck PGHα cDNAs were designed based on the conserved region of chicken, turkey and quail PGHαs (Foster & Foster 1991, Foster *et al*. 1992, Ando & Ishii 1994). The sense primer and antisense primers used for cloning duck PGHαs are 5′-ATG GATTGCTACAGGAAGTAT-3′ and 5′-TAGG ATTTATGGTAGTAGCAG-3′ respectively. The two primers used for amplification of the 5′ and 3′ ends of the duck PGHα cDNAs are 5′-TACACAG CACGTGGCTTCTGATGTAAT-3′ and 5′-GGA GCACCCATTTACCAGTGCACTGGG-3′ respectively; they were designed from duck PGHα subclone sequence. To estimate the internal standard in reverse transcription PCR (RT-PCR) analysis, we also included the β-actin primers in the same PCR procedure. The sense primer and antisense primer of β-actin are 5′-ACGTCGCACT GGATTTCGAG-3′ and 5′-TGTCAGCAATGC CAGGGTAC-3′ respectively. The β-actin primers were designed based on a chicken β-actin sequence from the GenBank database (Accession No. #L08165).

RNA extraction and RT-PCR

Six adult male Muscovy ducks and six Pekin ducks were purchased from a commercial breeder (Nankang, Taipei, Taiwan) and used as a source for this experiment. The ducks were killed and pituitary glands were placed into liquid nitrogen immediately. The pituitary total cellular RNA was extracted with the total RNA miniprep system kit (Viogene, Sunnyvale, CA, USA). The quality of RNA was measured at $A_{260\,nm}/A_{280\,nm}$ (Kontron Spectrophotometer, UVIKON 810). Only RNAs with A_{260} nm/A_{280} nm ratios of 1·6–2·0 were used for RT-PCR. Reverse transcription was performed with Moloney-murine leukemia virus reverse transcriptase (MMLV-RT) (Stratagene, CA, USA), according to the procedure supplied by the manufacturer. Oligo(dT) primer (100 ng) and total cellular RNA (500 ng) from pituitary glands were heated to 65 °C for 5 min. Then, 10 units of MMLV-RT were added to each reaction tube, which was incubated for 15 min at 37 °C. The PCR reactions were performed under hot-start conditions (94 °C, 2 min) with pfuTurbo DNA polymerase (Stratagene) for 30 cycles of 94 °C, 52 °C and 72 °C (1 min each), and then 10 min at 72 °C before holding at 4 °C.

After production of a 360-base duck PGHα cDNA fragment, the remaining duck PGHα sequence was obtained with the Marathon cDNA amplification kit (Clontech, Palo Alto, CA, USA). PCR reactions were performed under hot-start conditions for 30 s at 94 °C, 5 cycles of 5 s at 94 °C and 3 min at 72 °C, and 30 cycles of 5 s at 94 °C and 3 min at 68 °C.

Ligation and transformation

The purified PCR products were inserted into the pPCR-Script Amp sk(+) cloning vector with T_4 ligase (Stratagene). The transformation procedure was according to the manufacturer's direction with XL10-Gold kan ultracompetent cells (Stratagene). Six white colonies were selected and subjected to the alkaline lysis miniprep procedure (Davis *et al*. 1986). The plasmids were then analyzed by restriction enzyme digestion for the duck PGHα cDNA fragment.

Sequence analysis of PCR products and plasmids

The PCR products and the plasmid inserts were sequenced in both directions using the ABI DNA Sequencer (Applied Biosystems, Foster City, CA, USA). PCR products were sequenced using 100 ng of PCR product as template and 32 pg of either sense or antisense primer. The plasmid inserts were cut with SacI and KpnI restriction enzymes (Takara Biomedicals, Shiga, Japan) and sequenced

using 200 ng as template and with 23 ng of either T_3 or T_7 primer (Stratagene). For both PCR products and plasmid inserts, the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer, Foster City, CA, USA) was used with 5 units of AmpliTaq DNA polymerase in the sequencing of all products. The thermocycler program was composed of 25 cycles of 10 s at 96 °C, 5 s at 50 °C, and 4 min at 60 °C.

Pituitary tissue culture

Pituitary tissue cultures were done based on the procedures described by Foster & Foster (1991) and Gregory & Porter (1997) with minor modifications. Pituitaries were removed from ducks, immediately washed in 1 × Hanks buffer (Life Technologies, Grand Island, NY, USA) twice and placed in 35 mm culture dishes. Extraneous tissue was removed and pituitaries were sliced into six to eight pieces and placed in sterile phenol red free Dulbecco's Modified Eagles Medium (DMEM) plus antibiotic antimycotic (Life Technologies, Grand Island, NY, USA). The pituitary cultures were divided into five groups (four separate pituitary incubations in each group) and cultured for 24 h (42 °C; 95% O_2, 5% CO_2) in a defined serum-free medium (to avoid the stimulating effects of hormones and growth factors present in fetal calf serum): mammalian GnRH (10^{-8} M), TRH (10^{-8} M), testosterone (10^{-8} M), T_3 (10^{-8} M), and controls (without hormones) according to the procedures described by Foster & Foster (1991) and Gregory & Porter (1997). Following treatment, tissues were collected and snap-frozen in liquid nitrogen for isolation of RNA and Northern blot analysis.

Northern blot analysis for regulation of PGHα mRNA expression and tissue specificity of PGHα of the ducks

Samples from pituitary tissue culture treatments and brain cortex, liver and thyroid without hormone incubations for tissue specificity studies were used for Northern analysis. Total cellular RNAs of 10 μg of each tissue were used for Northern blot analysis, separated by electrophoresis on a 1·5% agarose–formaldehyde gel, and stained with ethidium bromide. RNAs were then transferred to a nylon filter membrane by capillary action and cross-linked in a u.v. cross-linker (Spectroline, XL-1000). The membrane was hybridized to digoxigenin (DIG)-labeled 360-base antisense riboprobe for duck PGHα in DIG EASY Hyb (Boehringer Mannheim, Germany) at 50 °C overnight. The membrane was then washed with 2 × SSC–0·5% SDS at 22 °C and 0·1 × SSC–0·1% SDS at 60 °C for 30 min. The membrane was treated with blocking reagent to prevent nonspecific attraction of the antibody, and was then incubated with a dilution of anti-DIG-alkaline phosphatase. The membrane carrying the hybridized probe and bound antibody conjugate was reacted with a chemiluminescent substrate and exposed to X-ray film for 1 h. The probe was then removed from the membrane after incubation with 0·1% SDS at 95 °C for 15 min. This step was repeated twice to ensure complete removal of the probe. The membrane was then re-hybridized with a chicken β-actin cDNA probe for normalization of the loading condition.

Statistical analysis

The data obtained from Northern blot analysis of the regulation of PGHα mRNA expression were subjected to one-way ANOVA. Differences between control (without hormone), GnRH, TRH, testosterone or T_3-treated were tested using the Scheffé test (Scheffé 1953). Differences were considered significant at $P<0·05$.

RESULTS

Cloning and amino acid sequence analysis of PGHα for Muscovy duck and Pekin duck

A PCR subclone product containing 360 bp was obtained and appeared as a single band in 1·5% agarose gel; this subclone was identified to encode the duck PGHα subunit precursor molecule from its nucleotide sequence. To determine the remaining 3′ and 5′ portions of duck PGHα cDNA sequence, 5′ and 3′ rapid amplification of cDNA ends (RACE) were performed, and the entire reading frame of PGHα was sequenced along with 81 bp of the 5′ untranslated region and 272 bp of the 3′ untranslated region followed by a 13 bp poly(A)$^+$ tract (Fig. 1). The open reading frame, 360 bp in length, encoded a putative signal peptide of 24 amino acids and a mature protein of 96 amino acids. The nucleotide sequences and amino acid sequences of PGHαs in Muscovy duck and Pekin duck are identical. Ten cysteine residues of duck PGHα are conserved at positions 11, 14, 25, 32, 36, 63, 64, 86, 88 and 91 and two presumptive asparagine-linked glycosylation sites (positions 57 and 82) are also conserved in ducks (Fig. 2).

Northern blot analysis for regulation of PGHα mRNA expression and tissue specificity of PGHα of the ducks

Northern blot analysis showed that the size of the duck PGHα mRNA transcript was approximately

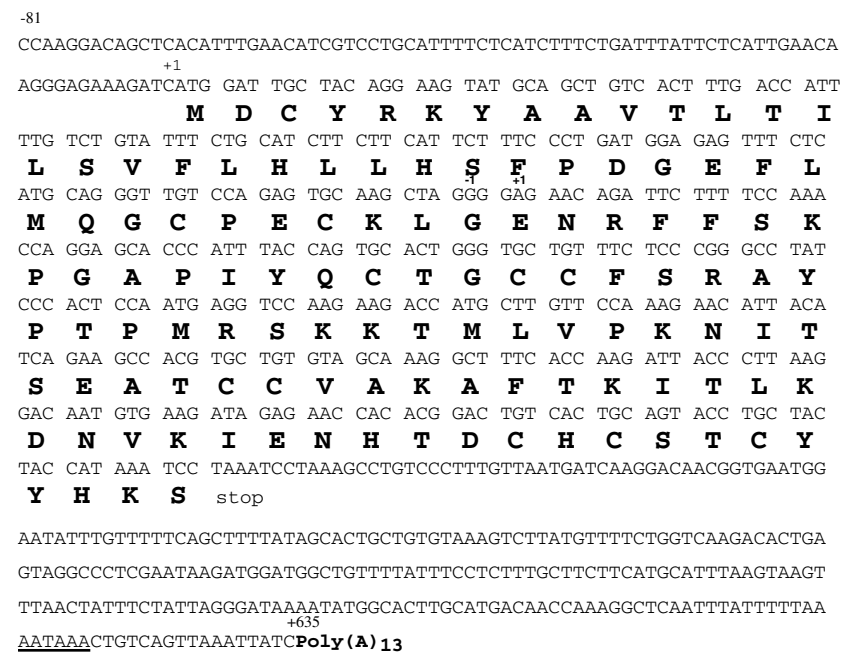

FIGURE 1 The nucleotide sequence of duck PGHα cDNA includes 81 bp of 5′ untranslated region, 360 bp of coding region, and 272 bp 3′ untranslated region followed by a 13 bp poly(A)$^+$ tract. The predicted open reading frame contains 120 amino acids as shown under the nucleotide sequence. The putative polyadenylation signal (AATAAA) is underlined.

800 bp (Fig. 3). To study PGHα mRNA expression of the ducks, pituitary fragments were cultured and treated for 24 h with GnRH, TRH, testosterone, and T_3. As shown in Fig. 3, PGHα mRNA levels of the GnRH-treated group and the TRH-treated group are 94% and 43% higher than those of the control group respectively, (P<0·05; n=4). However, PGHα mRNA levels of both the testosterone- and T_3-treated groups are 42% and 50% lower than those of the controls respectively (P<0·05; n=4).

To determine whether or not the expression of duck TSHβ cDNA was specific for pituitary gland, total cellular RNAs isolated from pituitary gland, brain cortex, liver, and testis were also compared by Northern blot analysis. As indicated in Fig. 3, the approximately 800 bp bands appeared in pituitaries, but not in other tissues.

DISCUSSION

In the present study, we have isolated full-length cDNA clones encoding the PGHα from two species of duck. Several lines of evidence indicate that the cDNAs isolated in the present study are those of duck PGHαs. First, the nucleotide and amino acid sequences of the duck PGHαs share high homology with other avian PGHαs. Secondly, the number and positions of ten cysteine residues and four proline residues (presumably responsible for folding of the molecule) are conserved in the sequence alignments in all the vertebrate species. Thirdly, the analysis of various tissues showed the expression of PGHα mRNA only occurs in the pituitary. And finally, GnRH and TRH increased, while testosterone and T_3 decreased, PGHα mRNA levels as analyzed by Northern blot analysis of the pituitary cultures.

The nucleotide and amino acid sequences of PGHαs of Muscovy duck and Pekin duck are identical. Although the mature proteins of PGHαs are identical among the birds so far studied (order Anseriformes: Muscovy duck and Pekin duck; order Galliformes: chicken, quail and turkey), their signal peptides are slightly different from each other (Fig. 2 and Table 1). The amino acid sequence of the signal peptides are, however, identical among the species within the same order (Fig. 2 and Table 1). As indicated in Table 1, the identities in PGHα signal peptides are always lower than the identities in PGHα mature proteins when comparisons are made between the ducks and other vertebrates. The identities of PGHα signal peptides between ducks and teleosts are found to be particularly low. A phylogenic tree of PGHα

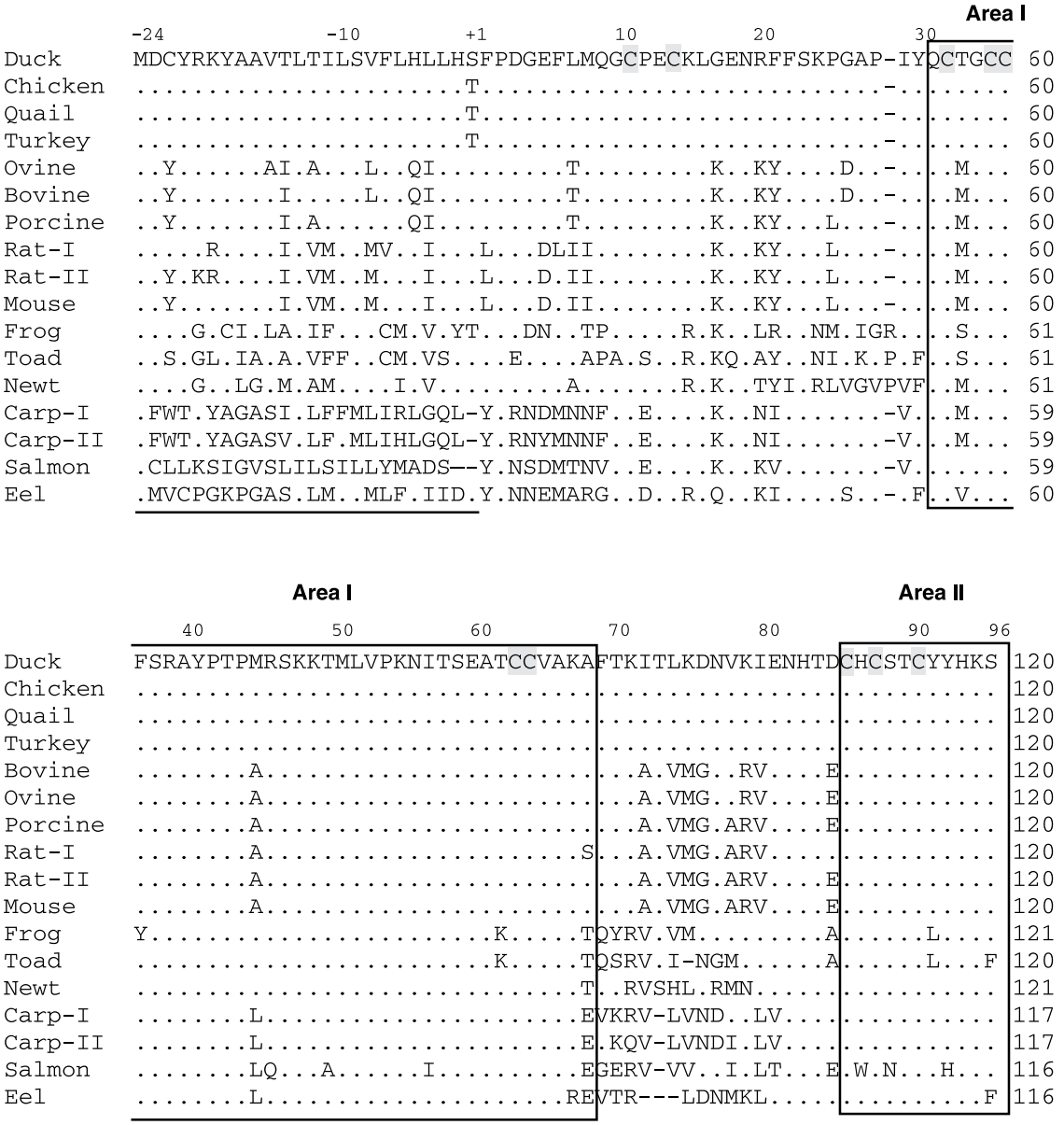

FIGURE 2 Comparisons of amino acid sequence of PGHα between duck and other vertebrates, including chicken (Foster *et al*. 1992), turkey (Foster & Foster 1991), quail (Ando and Ishii 1994), bovine (Nilson *et al*. 1983*a*), ovine (Bello *et al*. 1989), porcine (Hirai *et al*. 1989); rat-I and -II (Godine *et al*. 1982), mouse (Chin *et al*. 1981), bullfrog, toad, newt (Arai *et al*. 1998), carp-I and -II (Chang *et al*. 1988), salmon (Sekine *et al*. 1989) and eel (Quérat *et al*. 1990). Amino acids identical to those of the duck α-subunit are each indicated by a dot (.). Hyphens (-) have been inserted to obtain maximum homology. Amino acids −24 to −1 (underlined) represent the signal peptide sequence. The regions blocked off (Area I and Area II) indicate high levels of identity between species. Ten conserved cysteine residues are designated as 'C's with shading.

subunits based on the mature protein sequence from selected vertebrate species was constructed (Fig. 4). The present study demonstrates that both cDNA nucleotide and amino acid sequence of the PGHα subunit are identical in the two species of duck, thus exhibiting identical inter-genus homology within the family of Anatidae. The identical amino acid sequences of PGHα mature proteins among all five

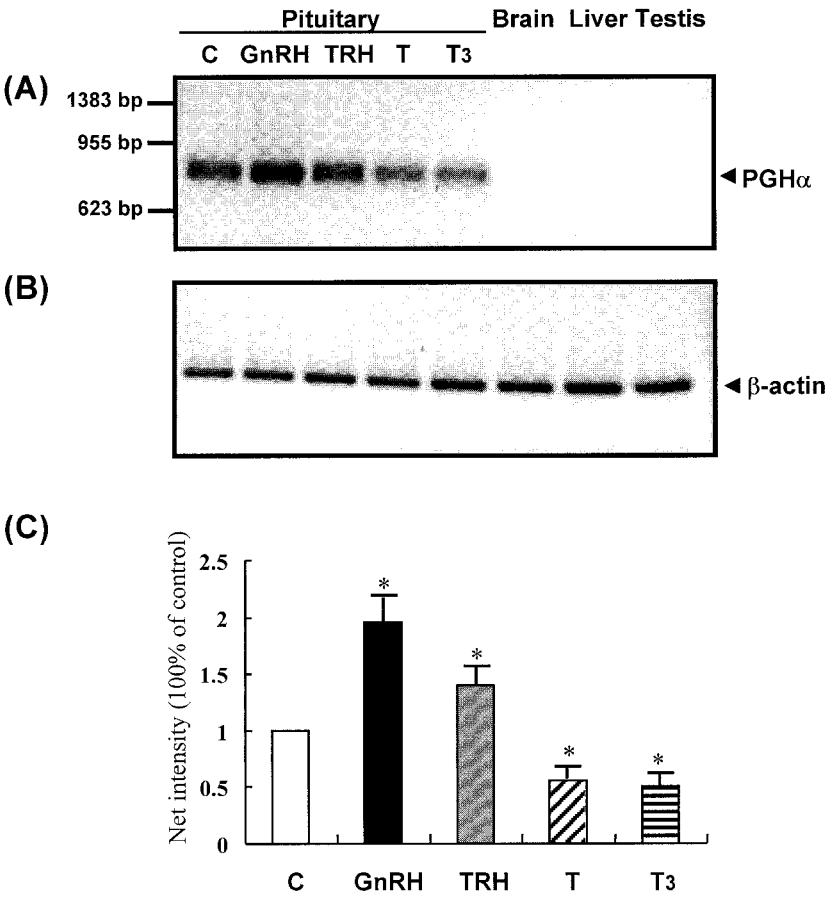

FIGURE 3 Regulation of PGHα mRNA expression and tissue-specific expression in duck pituitary cultures. Pituitary cultures were treated for 24 h with GnRH (10^{-8} M), TRH (10^{-8} M), testosterone (T; 10^{-8} M), T_3 (10^{-8} M), or without hormone (control, C). Total pituitary RNA prepared from each pituitary treatment, as well as from duck brain, liver and testis was analyzed by Northern blotting. (A) The expression of duck PGHα mRNA in pituitary culture treated with different hormones. The three small bars represent the location of 623 bp, 955 bp and 1383 bp of RNA marker. (B) The blot reprobed with chicken β-actin for normalization of RNA loading. (C) The PGHα mRNA intensities in different treatments as shown in (A) were analyzed by Kodak Digital Science ID image analysis software, Ver 3.0. Values represent the means ± S.D. of four separate experiments. *P<0·05.

avian species so far studied in the two orders of birds revealed that identical inter-order homology of PGHα subunits exists in birds. By contrast, such a pattern of identical inter-order homology has not been found in other vertebrate classes, where over 20 species each in mammals and teleosts have been investigated. It may be inferred that the PGHα subunits are more conserved in birds than in mammals and in teleosts. In addition, the lengths of PGHα mature protein of avian and mammalians are 96 amino acids, while amphibians are 96–97, and teleosts are 93–95 (Table 1). It appears that the lengths of PGHα mature protein are highly conserved among species of the same vertebrate class.

In mature PGHα subunits, there are two distinct areas that are highly conserved among the species from different vertebrate classes (Fig. 2). In Area I (amino acid residues between 31 and 68), there is 87–95% identity between birds and mammals, 92–97% identity between birds and amphibians, and 90–95% identity between birds and teleosts. The amino acid residues between 34 and 49 in Area I have been reported to play important roles in

TABLE 1. Comparison of the size and percentage identity of pituitary glycoprotein hormone common α subunit between ducks and other selected vertebrates. Muscovy duck and Pekin duck have identical α subunits

	cDNA of PGHα mature protein		**PGHα signal peptide**		**PGHα mature protein**	
	Length (bp)	Identity (%)	Length (aa)	Identity (%)	Length (aa)	Identity (%)
Species						
Muscovy duck	288	—	24	—	96	—
Pekin duck	288	—	24	—	96	—
Chicken	288	96	24	96	96	100
Turkey	288	97	24	96	96	100
Quail	288	96	24	96	96	100
Bovine	288	76	24	79	96	85
Porcine	288	76	24	79	96	84
Ovine	288	77	24	71	96	85
Rat-I	288	73	24	71	96	80
Rat-II	288	73	24	67	96	81
Mouse	288	75	24	75	96	81
Frog	291	72	24	50	97	75
Toad	288	70	24	46	96	67
Newt	291	76	24	67	97	73
Carp-I	285	68	23	13	95	73
Carp-II	285	67	23	17	95	72
Salmon	285	61	22	5	95	67
Eel	279	66	24	25	93	74

The references for PGHαs of other vertebrates used for comparisons are indicated in Fig. 2.
aa, amino acids.

dimer formation (Bielinska & Boime 1992, Xia *et al*. 1994, Grossmann *et al*. 1996), receptor binding and hormone action (Leinung *et al*. 1991, Yoo *et al*. 1993, Xia *et al*. 1994, Zeng *et al*. 1995). In Area II (amino acid residues between 85 and 96), there is 100% identity between birds and mammals, 82–91% identity between birds and amphibians, and 75–100% identity between birds and teleosts. The sequences 92–96 in Area II have also been indicated to be responsible for receptor binding and signaling, and play roles in bioactivity of hormone action (Chen *et al*. 1992, Grossmann *et al*. 1995). Ten cysteine residues of duck PGHαs are conserved at positions 11, 14, 25, 32, 36, 63, 64, 86, 88 and 91, presumably forming five disulfide bonds within the α subunit; two presumptive asparagine-linked glycosylation sites (positions 57 and 82) are also conserved in ducks.

Although the mode and mechanisms of hormonal regulation in PGHα expression have been extensively investigated in mammals (Nilson *et al*. 1983*b*, Chin *et al*. 1985, Shupnik *et al*. 1986, Dalkin *et al*. 1991, Winters *et al*. 1992, Holdstock & Burrin 1994), little is known in birds. It has been demonstrated in mammalian species that GnRH and TRH enhance the formation of PGHα in pituitary cells (Shupnik *et al*. 1986, Holdstock & Burrin 1994); while estradiol, testosterone, and thyroid hormones suppress PGHα mRNA expression in pituitary under both *in vitro* and *in vivo* conditions (Nilson *et al*. 1983*b*, Chin *et al*. 1985, Winters *et al*. 1992). TRH is present in the avian hypothalamus, and the chicken pituitary TRH receptor has been cloned and characterized (Thompson *et al*. 1981, Sun *et al*. 1998, Geris *et al*. 1999). In birds, TRH stimulates the release of TSH from the adenohypophysis of chicken *in vitro* (Breneman & Rathkamp 1973, Scanes 1974). We report here, for the first time in avian species, that PGHα mRNA levels of the ducks were increased by TRH, while decreased by testosterone and T_3, under *in vitro* conditions. Our observations that GnRH enhanced duck PGHα mRNA levels are in agreement with the reports by Foster *et al*. (1992) and Foster & Foster (1991) that GnRH increased PGHα mRNA levels in chicken and turkey pituitary cultures under *in vitro* conditions. In the present study, cultures of the duck pituitary tissues were performed for investigating the regulation of PGHα mRNA expression; the cultured duck pituitaries thus consisted of both thyrotropes and gonadotropes. Since the PGHα gene is present in both cells, GnRH and testosterone presumably act on gonadotropes, while TRH and T_3 act on thyrotropes, for PGHα mRNA expression. Studies using isolated pituitary gonadotropes or thyrotropes will provide a clear answer.

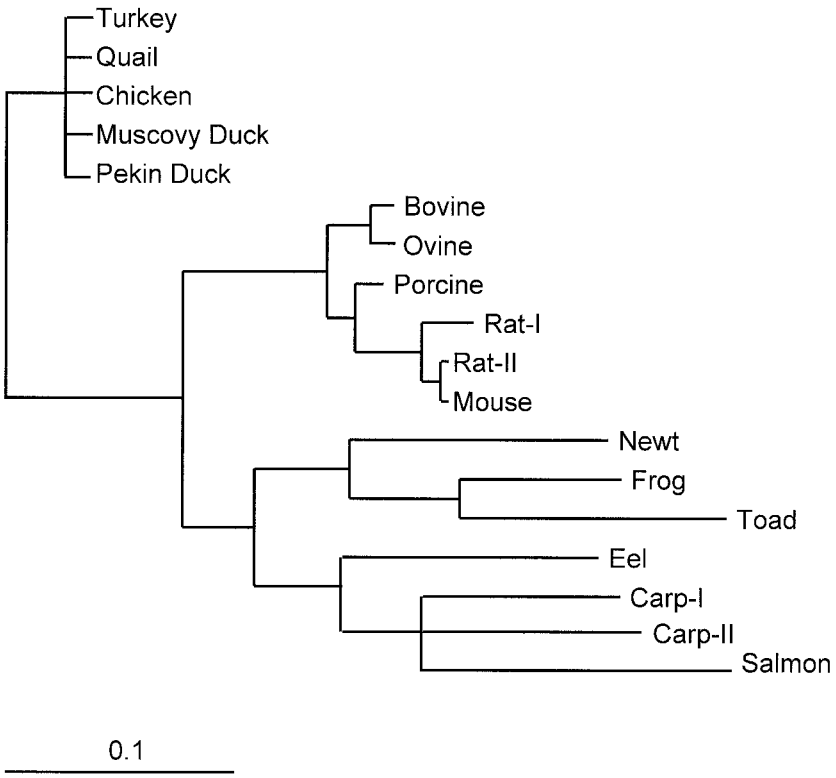

FIGURE 4 A phylogenic tree of amino acid sequences of selected vertebrate PGHα subunits. The tree was constructed with the ClustalW program by pair-group cluster method (EMBL-European Bioinformatics Institute) and Tree View (Rod Page, University of Glasgow, UK) based on available data in birds and amphibians, and selected species of mammals and teleosts as described in Fig. 2. No data are yet available for reptilian PGHα. The value indicated is the number of substitutions per site.

Our results suggest that hypothalamic GnRH and TRH up-regulate, while gonadal steroids and thyroid hormones down-regulate the PGHα mRNA expression in the ducks. The findings from the present study, together with others, thus suggest that the mode of regulation of PGHα gene expression in the hypothalamo–pituitary–target organs (gonad and thyroid) axis has been conserved in birds and mammals.

ACKNOWLEDGEMENTS

This study was supported by a grant from Academia Sinica, Taipei, Taiwan, ROC.

REFERENCES

Ando H & Ishii S 1994 Molecular cloning of complementary deoxyribonucleic acid for the pituitary glycoprotein hormone α-subunit and luteinizing hormone β-subunit precursor molecule of Japanese quail (*Coturnix coturnix japonica*). *General and Comparative Endocrinology* **93** 357–368.

Arai Y, Kubokawa K & Ishii S 1998 Cloning of cDNA for the pituitary glycoprotein hormone alpha subunit precursor molecules in three amphibian species, *Bufo japonicus*, *Rana catesbeiana*, and *Cynops pyrrhogaster*. *General and Comparative Endocrinology* **121** 46–53.

Bello PA, Mountford PS, Brandon MR & Adams TE 1989 Cloning and DNA sequence analysis of the cDNA for the common alpha-subunit of the ovine pituitary glycoprotein hormones. *Nucleic Acids Research* 17 **24** 10494.

Bielinska M & Boime I 1992 Site-directed mutagenesis defines a domain in the gonadotropin alpha-subunit required for assembly with the chorionic gonadotropin beta-subunit. *Molecular Endocrinology* **6** 261–271.

Breneman WR & Rathkamp W 1973 Release of thyroid stimulating hormone from chick anterior pituitary glands by thyrotropin releasing hormone (TRH). *Biochemical and Biophysical Research Communications* **52** 189–194.

Chang YS, Hung CJ, Huang FL, Liu CS & Lo TB 1988 Primary structures of carp gonadotropin subunits deduced from cDNA nucleotide sequences. *International Journal of Peptide Protein Research* **32** 556–564.

Chen F, Wang Y & Puett D 1992 The carboxy-terminal region of the glycoprotein hormone alpha-subunit: contributions to receptor binding and signaling in human chorionic gonadotropin. *Molecular Endocrinology* **6** 914–919.

Chin WW, Kronenberg HM, Dee PC, Maloof F & Habener JF 1981 Nucleotide sequence of the mRNA encoding the pre-α-subunit of mouse thyrotropin. *PNAS* **80** 4649 –4653.

Chin WW, Shupnik MA, Ross DS, Habener JF & Chester Ridgway E 1985 Regulation of the α and thyrotropin β-subunit messenger ribonucleic acids by thyroid hormones. *Endocrinology* **116** 873–878.

Dalkin AC, Paul SJ, Haisenleder DJ, Ortolano GA, Yasin M & Marshall JC 1991 Gonadal steroid effect similar regulation of gonadotropin subunit mRNA in both male and female rats. *Journal of Endocrinology* **132** 39–45.

Davis LG, Dibner MD & Battey JF 1986 *Basic Methods in Molecular Biology*. New York: Elsevier.

Foster DN & Foster LK 1991 Cloning and sequence analysis of common α-subunit complementary deoxyribonucleic acid of turkey pituitary glycoprotein hormones. *Poultry Science* **70** 2516–2523.

Foster DN, Galehouse D, Giordano T, Min B, Lamb IC, Porter DA, Intehar KJ & Bacon WL 1992 Nucleotide sequence of the cDNA encoding the common α subunit of the chicken pituitary glycoprotein hormone. *Journal of Molecular Endocrinology* **8** 21–27.

Geris KL, Handt ED, Kühn ER & Darras VM 1999 Thyrotropin-releasing hormone concentrations in different regions of the chicken brain and pituitary: an ontogenetic study. *Brain Research* **818** 260–266.

Gharib SD, Wierman ME, Shupnik MA & Chin WW 1990 Molecular biology of the pituitary gonadotropins. *Endocrine Reviews* **11** 177–199.

Godine JE, Chin WW & Habener JF 1982 α-Subunit of rat pituitary glycoprotein hormones. Primary structure of the precursor determined from the nucleotide sequence of cloned cDNA. *Journal of Biological Chemistry* **257** 8368–8371.

Gregory CC & Porter TE 1997 Cloning and sequence analysis of a cDNA for β subunit of chicken thyroid-stimulating hormone. *General and Comparative Endocrinology* **107** 182–190.

Grossmann M, Szkudlinski MW, Zeng H, Kraiem Z, Ji I, Kraiem Z, Tropea JE, Ji TH & Weintraub BD 1995 Role of the carboxy-terminal residues of the alpha-subunit in the expression and bioactivity of human thyroid-stimulating hormone. *Molecular Endocrinology* **9** 948–958.

Grossmann M, Szkudlinski MW, Dias JA, Xia H, Wong R, Puett D & Weintraub BD 1996 Site-directed mutagenesis of amino acids 33–44 of the common alpha-subunit reveals different structural requirements for heterodimer expression among the glycoprotein hormones and suggests that cyclic adenosine 3′,5′-monophosphate production and growth promotion are potentially dissociable functions of human thyrotropin. *Molecular Endocrinology* **10** 769–779.

Hirai T, Takikawa H & Kato Y 1989 Molecular cloning of cDNA for precursors of porcine pituitary glycoprotein hormone common a-subunit and of thyroid stimulating hormone β-subunit. *Molecular and Cellular Endocrinology* **63** 209–217.

Holdstock JG & Burrin JM 1994 Regulation of glycoprotein hormone free α-subunit secretion and intracellular α-subunit content in primary pituitary cells. *Endocrinology* **134** 685–694.

Kourides IA, Barker PE, Gurr JA, Pravtcheva DD & Ruddle FH 1984 Assignments of the genes for the α and β subunits of thyrotropin to different mouse chromosomes. *PNAS* **81** 517–519.

Leinung MC, Reed DK, McCormick DJ, Ryan RJ & Morris JC 1991 Further characterization of the receptor region of the thyroid-stimulating hormone alpha subunit: a comprehensive synthetic peptide study of the alpha-subunit 26–46 sequence. *PNAS* **88** 9707–9711.

Naylor SL, Chin WW, Goodman HM, Lalley PA, Grzechik KH & Sakaguchi AY 1983 Chromosome assignment of genes encoding the α and β subunits of glycoprotein hormones in man and mouse. *Somatic Cell Genetics* **9** 757–770.

Nilson JH, Thomason AR, Cserbak MT, Moncman CL & Woychik RP 1983*a* Nucleotide sequence of a cDNA for the common α subunit of the bovine pituitary glycoprotein hormones. Conservation of nucleotides in the 3′-untranslated region of bovine and human pre-α-subunit mRNAs. *Journal of Biological Chemistry* **258** 4679–4682.

Nilson JH, Nejedlik MT, Virgin JB, Crowder ME & Nett TM 1983*b* Expression of α subunit and luteinizing hormone β gene in the ovine anterior pituitary. *Journal of Biological Chemistry* **258** 12087–12090.

Pierce JG & Parsons TF 1981 Glycoprotein hormones: structure and function. *Annual Review of Biochemistry* **50** 465–495.

Quérat B 1994 Molecular evolution of the glycoprotein hormones in vertebrates. In *Perspective in Comparative Endocrinology*, pp 27–35. Eds KG Davey, RE Peter & SS Tobe. Toronto: National Research Council of Canada.

Quérat B, Jutisz M, Fontaine YA & Counis R 1990 Cloning and sequence analysis of the cDNA for the pituitary glycoprotein hormone alpha-subunit of the European eel. *Molecular and Cellular Endocrinology* **71** 253–259.

Scanes CG 1974 Some *in vitro* effects of synthetic thyrotropin releasing factor on the secretion of thyroid stimulating hormone from the anterior pituitary gland of the domestic fowl. *Neuroendocrinology* **15** 1–9.

Scheffé H 1953 A method for judging all contrasts in the analysis of variances. *Biometrika* **48** 87–104.

Sekine S, Saito A, Itoh H, Kawauchi H & Itoh S 1989 Molecular cloning and sequence analysis of salmon gonadotropin cDNAs. *PNAS* **86** 8645–8649.

Shupnik MA, Greenspan SL & Chester Ridgway E 1986 Transcriptional regulation of thyrotropin subunit genes by thyrotropin-releasing hormone and dopamine in pituitary cell culture. *Journal of Biological Chemistry* **261** 12675–12679.

Sun YM, Millar RP, Ho H, Gershengorn MC & Illing N 1998 Cloning and characterization of the chicken thyrotropin-releasing hormone receptor. *Endocrinology* **139** 3390–3398.

Thompson DF, Taylor R & Burt DR 1981 TRH receptor binding in avian pituitary and brain. *General and Comparative Endocrinology* **44** 77–81.

Winters SJ, Ishizaka K, Kitahara S, Troen P & Attardi B 1992 Effects of testosterone on gonadotropin subunit messenger ribonucleic acids in the presence or absence of gonadotropin-releasing hormone. *Endocrinology* **130** 726–734.

Xia D, Henry LJ, Gerard RD & Deisenhofer J 1994 Crystal structure of the receptor-binding domain of adenovirus type 5 fiber protein at 1·7 A resolution. *Structure* **2** 1259–1270.

Yoo J, Zeng H, Ji I, Murdoch WJ & Ji TH 1993 COOH-terminal amino acids of the alpha subunit play common and different roles in human choriogonadotropin and follitropin. *Journal of Biological Chemistry* **268** 13034–13042.

Zeng H, Ji I & Ji TH 1995 Lys 91 and His 90 of the alpha-subunit are crucial for receptor binding and hormone action of follicle-stimulating hormone (FSH) and play hormone-specific roles in FSH and human chorionic gonadotropin. *Endocrinology* **136** 2948–2953.

RECEIVED 25 July 2001
ACCEPTED 30 July 2001