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Cloning of the Genes for the Pituitary Glycoprotein Hormone α and Follicle-Stimulating Hormone β Subunits in the Japanese Crested Ibis, *Nipponia nippon*

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ABSTRACT—We have isolated a part of the gene for the pituitary glycoprotein hormone common α subunit (PGHα) and the whole gene for the follicle-stimulating hormone β subunit (FSHβ) in the Japanese crested ibis (*Nipponia nippon*), a critically endangered bird species in East Asia. The nucleotide sequence of a part of the PGHα gene (5026 bp) contained three exons holding the whole coding and 3' untranslated regions, but lacked a 5' untranslated region. Its exon-intron structure was similar to that in mammals, but different from that in teleosts in the location of the second intron. For the FSHβ gene, the nucleotide sequence of 7633 bp was assembled from two phage clones. The exon-intron structure of three exons and two introns was similar to that observed in mammals and teleosts. In the putative promoter region of the ibis FSHβ gene, a progesterone responsive element (PRE)-like sequence and two AP-1 responsive element-like sequences reported in the ovine FSHβ gene were not conserved in complete form. The increased number of ATTTA motifs in the putative 3' untranslated region in comparison with those in Japanese quail and chicken FSHβ cDNA suggested that more rapid degradation of FSHβ mRNA occurs in this species. Deduced amino acid sequences of the ibis PGHα and FSHβ showed high similarities with those of the corresponding subunits of other avian species. This is the first report on the genomic sequences of the PGHα and FSHβ in an avian species.

Key words: gonadotropin, PGHα, FSHβ, gene, the Japanese crested ibis

INTRODUCTION

Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are the main members of gonadotropins and they also belong to the pituitary glycoprotein hormone family which also includes thyroid-stimulating hormone (TSH). All these hormones are heterodimeric molecules composed of a common α subunit and a hormone-specific β subunit. The α and β subunits are encoded by different genes and synthesized as separate peptides. So far, the genes encoding the gonadotropin subunits have been characterized in mammals and teleosts. As for gonadotropin subunits in avian species, cDNA sequences have been reported in Japanese quail (Ando and Ishii, 1994; Kikuchi *et al*., 1998), chicken (Noce *et al*., 1989; Foster *et al*., 1992; Shen and Yu, 2002), turkey (Foster and Foster, 1991; You *et al*., 1995) and two species of duck (Hsieh et al., 2001), and amino acid sequences have been chemically determined in ostrich (Koide *et al*., 1996). However, genomic information such as exon-intron structure and regulatory sequences in the 5' flanking region has not been available in avian species. Accordingly, cloning of avian gonadotropin subunit genes will provide us important information on the evolution of gonadotropin genes and their regulation.

The Japanese crested ibis *Nipponia nippon* (order Ciconiiformes, family Threskiornithidae) is a critically endangered species in East Asia. This species was formerly widespread throughout Japan, Korea, China and an adjacent part in southeastern Russia. At present, the only wild population of approximately 170 individuals survives in Yang Xian, Shaanxi Province, China. In addition, captive populations are kept in China and Japan. Since 1980s, Ishii and his collaborators (Ishii, 1999; Wingfield *et al*., 2000) have developed endocrinological methods for the artificial breeding of

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endangered birds. They have shown that hormone therapy using gonadotropins from closely related species in reproductively quiescent female Japanese quails resulted in gonadal maturation, egg-laying and production of reproductively active offspring (Wakabayashi *et al.*, 1992; Ishii, 1999). Thus, homologous gonadotropin administration is considered to be effective in stimulating reproductive activity in the ibis. However, chemical isolation of gonadotropins from the ibis or related species for the hormone therapy program is almost impossible. Rather, it seems practical to clone the genes for the gonadotropin subunits of the ibis and to generate large quantities of gonadotropins using the recombinant technique.

Thus, cloning of the genes for the gonadotropin subunits in the Japanese crested ibis is invaluable because it provides not only the first information on the genomic structure of these subunits in avian species, but also the molecular information which is necessary for recombinant gonadotropins. Here, we report the isolation and characterization of a nucleotide sequence of a part of the gene for the pituitary glycoprotein hormone common α subunit (PGHα) and the sequence of the whole gene for the follicle-stimulating hormone β subunit (FSHβ) in the Japanese crested ibis.

MATERIALS AND METHODS

Isolation of the ibis PGHα gene

When the last male Japanese crested ibis of the Japanese origin (named Midori) died in 1995, most of his organs were preserved in liquid nitrogen (Ishii, 1999). Genomic DNA was extracted from approximately 15 mg of the preserved kidney using a GenomicPrep Cells and Tissue DNA Isolation Kit (Amersham Biosciences, NJ). The extracted DNA was digested with *Eco*RI and separated in agarose gel electrophoresis. DNA fragments of 4–10 kb long were recovered from the gel and ligated with the Lambda ZAPII vector (Stratagene, CA). An original library of 2.5×10^5 clones was generated and amplified once to 1.3×10^{11} clones. Approximately 2.5×10^5 clones in the amplified library were screened by plaque hybridization. To prepare a hybridization probe, the PGHα cDNA fragment of Japanese quail (Ando and Ishii, 1994) was randomly labeled with [α-^{32}P]dCTP (Amersham Biosciences, UK) and used as a probe. Prehybridization was performed at 42°C for more than 2 hr with denatured salmon sperm DNA (0.2 mg/ml) in a hybridization buffer containing 6×SSC (1×SSC: 150 mM NaCl, 15 mM sodium citrate, pH7.0), Denhardt's solution (0.02% Ficoll, 0.02% bovine serum albumin, and 0.02% polyvinylpyrrolidone), and 0.1% SDS. Hybridization was carried out at 55°C overnight in the hybridization buffer containing the labeled probe. Membranes were washed once with 1×SSC containing 0.1% SDS at 60°C for 20 min and hybridization signals on the membranes were analyzed with a BAS-2000II Bio-Imaging Analyzer (Fuji Photo Film, Japan). After third round of screening, ten positive clones were isolated and the insert fragments were subcloned into the pBluescript phagemid vector. After identification of these clones by restriction enzyme digestion, a clone (pIA1) was selected for sequence analysis. Nucleotide sequence was determined with a Thermo Sequenase Cycle Sequencing Kit (USB Corporation, OH) with a DNA sequencer Model 4000L (LI-COR, NE).

Isolation of the ibis FSHβ gene

In order to obtain a nucleotide sequence of a part of the FSHβ gene, PCR was carried out with genomic DNA as a template. Primers used were as follows: FSH-F, 5'-TG(T/C)TCIGGITA(C/T)TG(C/T)T(A/T)(A/C)ACIA(A/G)(A/G)G-3' and FSH-R, 5'-CA(A/G)TCIGT(A/G)CT(A/G)TCI(C/G)T(A/G)TC(A/G)CAI(G/T)TI(C/T)C(A/G)CA(A/G)TG(A/G)C-3', for the sense and antisense, respectively. They were originally used for amplifying a part of the FSHβ cDNA in Japanese quail (Kikuchi et al., 1998). The PCR was performed with 30 cycles of 1 min at 95°C, 1 min at 50°C, 3 min at 72°C using a Premix Ex Taq (Takara Shuzo, Japan). The PCR products were subjected to agarose gel electrophoresis and then cloned into pCR2.1 plasmid vector (Invitrogen, CA). After sequencing, a clone (pIF-PCR) was selected for further study.

Another genomic library was constructed in the Lambda EMBL3 phage vector (Stratagene, CA) with genomic DNA partially digested with *Bam*HI. An original library of 5.0×10^4 clones was amplified once to 8.0×10^9 clones, and 1.0×10^5 clones were screened by plaque hybridization. The pIFPCR insert was randomly labeled with [α-^{32}P]dCTP and used as a probe. Prehybridization was performed as described above, and hybridization was carried out at 60°C overnight. Membranes were washed once with 1×SSC containing 0.1% SDS at 60°C for 20 min and once with 0.1×SSC containing 0.1% SDS at 60°C for 20 min. After third round of screening, one positive clone (IF1) was isolated. The insert fragment was digested with *Eco*RI and subcloned into pBluescriptII phagemid vector (Stratagene, CA) for sequencing.

Because IF1 was found to cover only a 5' part of the FSHβ gene, screening was repeated to obtain the remaining 3' part. The pIFPCR insert was digested with *Bam*HI and then a fragment of approximately 180 bp was recovered to exclude a part overlapping with IF1. This fragment was randomly labeled with [α-^{32}P]dCTP and used as a probe. Since the genomic library did not give any positive signal, we constructed other genomic library with the Lambda EMBL3 vector and *Bam*HI-digested genomic DNA. The resultant library was 1.6×10^5 clones and was screened without amplification. Hybridization was carried out at 50°C overnight, and membranes were washed once with 3×SSC containing 0.1% SDS at 50°C for 20 min. Consequently, three positive clones were isolated. Based on identification by restriction enzyme digestion, a clone (IF4) was selected and a region of this insert was subcloned into the pBluescriptII phagemid vector. Nucleotide sequence was determined with a BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit and an ABI PRISM 377 DNA Sequencing System (Applied Biosystems, CA).

Analysis of the second intron in the ibis FSHβ gene in two individuals

Nucleotide sequences in the second intron of the FSHβ gene were determined in two individuals. One was Midori of Japanese origin, and the other was Long-Long which had been lent from China to Japan and died in Japan in 1994. The genomic DNA of Long-Long was extracted from the cryo-preserved kidney (Ishii, 1999). Genomic DNA extraction, PCR using the primers FSH-F and FSH-R, cloning and sequencing were performed as described above. After sequencing, the PCR products were digested with *Eco*RI and subjected to electrophoresis.

RESULTS

Isolation and nucleotide sequence of the ibis PGHα gene

Ten positive clones were isolated from the genomic library and nucleotide sequence of a clone (pIA1) was determined (Fig. 1). The determined sequence of 5026 bp was compared with PGHα cDNA sequences of turkey (Foster and Foster, 1991), chicken (Foster *et al.*, 1992), Japanese quail (Ando and Ishii, 1994) and two species of duck (Hsieh *et al.*, 2001). Comparison results showed remarkable

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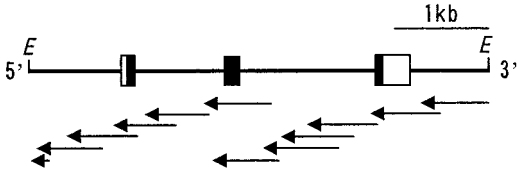


Fig. 1. Structure and sequencing strategy of a part of the gene for the PGHα in the Japanese crested ibis. Solid and open boxes indicate the coding and the untranslated region of exons, respectively. Lines represent introns and 3' flanking region. Arrows indicate the direction and extent of sequencing. Restriction sites for *Eco*RI are indicated with *E*.

sequence similarities which enabled us to infer the exon-intron structure (Fig. 2). We could recognize three putative exons containing the whole coding region and 3' untranslated region within this sequence, but we were unable to find a sequence corresponding to most of 5' untranslated region. Exon-intron junctions were determined according to the GT-AG rule (Breathnach and Chambon, 1981). Then, amino acid sequence was deduced and aligned with those of PGHα of turkey (Foster and Foster, 1991), chicken (Foster *et al*., 1992), Japanese quail (Ando and Ishii, 1994), ostrich (Koide *et al*., 1996), and two species of duck (Hsieh *et al*., 2001) (Fig. 3). Three amino acid residues in the putative ibis PGHα precursor were different from those in each of the other birds over a signal peptide of 24 residues, while 96 residues in a mature protein were completely identical to those of the other birds except for ostrich (ostrich mature protein differed in three amino acid residues from those of the other birds). Ten cysteine residues forming disulfide bonds and two putative N-linked glycosylation sites were conserved among all these birds. These findings assured that the acquired nucleotide sequence encoded the ibis PGHα precursor molecule.

All the PGHα genes previously reported in mammals and teleosts consist of four exons and three introns (Fiddes and Goodman, 1981; Goodwin *et al*., 1983; Burnside *et al*., 1988; Gordon *et al*., 1988; Kato *et al*., 1990; Golos *et al*., 1991; Huang *et al*., 1992; Suzuki *et al*., 1995). The exon-intron structure in the ibis PGHα gene was compared with that of carp (Huang *et al*., 1992), mouse (Gordon *et al*., 1988), bovine (Goodwin *et al*., 1983) and human (Fiddes and Goodman, 1981) (Fig. 4). The result indicated that the clone we obtained lacked a 5' part corresponding to the first exon of other vertebrates. By aligning amino acid sequences, locations of the introns in the coding region were compared. The location of the first intron in the part of the ibis PGHα gene was identical with that of the corresponding intron (the second intron) in mammals, but different from that in teleosts with an amino acid residue (Fig. 5), while the location of the second intron (the third in mammals and teleosts) was conserved through the ibis, mammals and teleosts.

Isolation and nucleotide sequence of the ibis FSHβ gene

Strategy to obtain the ibis FSHβ gene is illustrated in Fig. 6. The PCR gave a single band of approximately 800 bp and the determined 780 bp sequence was compared with FSHβ cDNA sequences of Japanese quail (Kikuchi *et al*., 1998) and chicken (Shen and Yu, 2002). This sequence was found to contain an intron of 582 bp flanked by partial exons of 23 and 175 bp of the putative FSHβ gene. A clone (IF1), which was isolated from the genomic library, contained an insert of approximately 10 kb, of which the nucleotide sequence of 4059 bp around the 3' part was determined. Comparison of the sequence with the FSHβ cDNA sequences of the two bird species revealed that IF1 contained a 5' part of the FSHβ gene, but lacked its 3' part. Then, three clones were isolated by repeated screening of the other genomic library. A partial nucleotide sequence of a clone (IF4), 3580 bp in size, was compared with the FSHβ cDNA sequences of the two bird species. IF4 was shown to contain a 3' part of the FSHβ gene which was missing in IF1.

By collecting the results from the two phage clones (IF1 and IF4) and the PCR fragment (pIFPCR), a sequence of 7633 bp was acquired (Fig. 7). Exon-intron structure was inferred by sequence similarities with the FSHβ cDNA in Japanese quail and chicken and exon-intron junctions were determined based on the GT-AG rule (Breathnach and Chambon, 1981). Particularly, we inferred the putative transcriptional start site and the polyadenylation site referring to the 5' and 3' end of the chicken FSHβ cDNA determined by rapid amplification of cDNA end (RACE) method. Finally, the assembled nucleotide sequence was found to contain 2820 bp of the 5' flanking region followed by three exons of 34, 160 and 2549 bp, separated by two introns of 458 and 582 bp. An amino acid sequence was deduced and aligned with those of Japanese quail (Kikuchi *et al*., 1998), chicken (Shen and Yu, 2002) and ostrich (Koide *et al*., 1996) (Fig. 8). The ibis amino acid sequence showed remarkable similarities to those of FSHβ of Japanese quail (94.7%) and chicken (95.4%). High similarity (93.4%) was also observed with the ostrich FSHβ except for a deletion of five residues in the C-terminus. Twelve cysteine residues and two putative N-linked glycosylation sites were conserved through these four bird species. These results enabled us to conclude that the nucleotide sequence we obtained encoded the FSHβ gene of the Japanese crested ibis.

The ibis FSHβ gene showed a structure with three exons and two introns. The similar exon-intron structure has been previously reported in mammals and teleosts (Jameson *et al*., 1988; Kim *et al*., 1988; Gharib *et al*., 1989; Hirai *et al*., 1990; Guzman *et al*., 1991; Kumar *et al*., 1995; Sohn *et al*., 1998; Rosenfeld *et al*., 2001). Locations of the first intron in the 5' untranslated region and the second intron between amino acid residue 33 and 34 of mature protein in the ibis FSHβ gene were also similar to locations reported for mammalian and teleostean counterparts. Comparison of the nucleotide sequence in the 5' flanking region between the ibis and non-avian species (teleosts and mammals)

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                      -20                                             -10
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Leu His Ala Phe Pro Asp Gly Glu Phe Leu Met Gln
             +1                                +9
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Pro Gly Ala Pro Ile Tyr Gln Cys Thr Gly Cys Cys Phe Ser Arg Ala Tyr Pro Thr Pro Met Arg
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TCC AAG AAG ACC ATG CTC GTT CCA AAG AAC ATT ACA TCA GAA GCA ACG TGC TGC GTA GCA AAG GCT
Ser Lys Lys Thr Met Leu Val Pro Lys Asn Ile Thr Ser Glu Ala Thr Cys Cys Val Ala Lys Ala
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Fig. 2. Nucleotide sequence of the part of the PGHα gene in the Japanese crested ibis. Exons are shown in capital letters, and introns and flanking regions are shown in lowercase letters. The polyadenylation signal, AATAAA, is underlined. Deduced amino acid residues are represented with three letter codes below the nucleotide sequence and numbered sequentially from the N-terminus. The nucleotide sequence data in this figure is available in the EMBL/GenBank/DDBJ Data Bank with Accession No. AB089503.

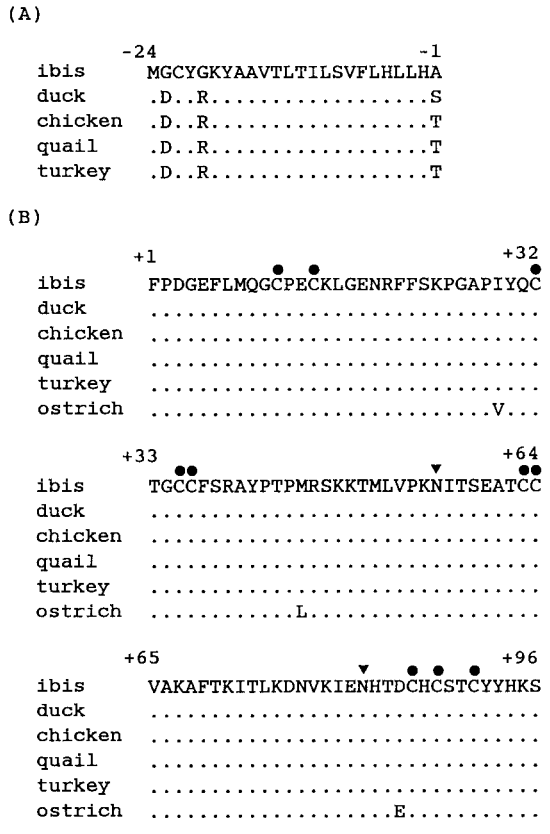


Fig. 3. Alignment of amino acid sequences of signal peptides (A) and mature proteins (B) of the PGHα precursor molecules of the Japanese crested ibis, duck (Hsieh *et al*., 2001), chicken (Foster *et al*., 1992), Japanese quail (Ando and Ishii, 1994), turkey (Foster and Foster, 1991) and ostrich (Koide *et al*., 1996). Residues identical to those in the Japanese crested ibis are indicated with dots. Ten conserved cysteine residues and two putative N-linked glycosylation sites are denoted by ● and ▼, respectively.

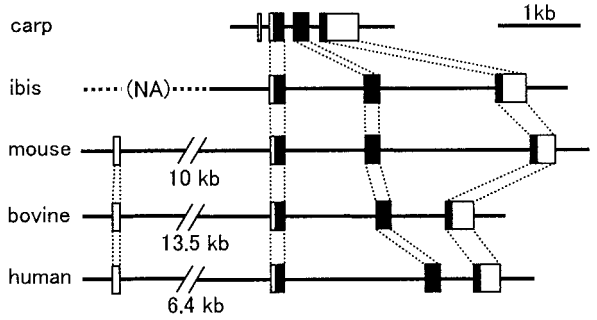


Fig. 4. Comparison of the exon-intron structure of the part of the PGHα gene in the Japanese crested ibis with those of PGHα gene of carp (Huang *et al*., 1992), mouse (Gordon *et al*., 1988), bovine (Goodwin *et al*., 1983) and human (Fiddes and Goodman, 1981). Solid and open boxes represent the coding and the untranslated regions of exons, respectively. Solid lines indicate introns and flanking regions. Thin broken lines between boxes link corresponding exons. A thick broken line in the Japanese crested ibis represents part whose nucleotide sequence is not available at present.

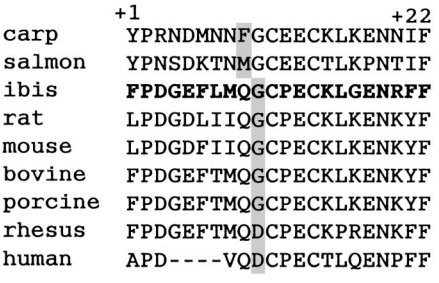


Fig. 5. Alignment of a part of amino acid sequences of PGHα for comparison of location of the second intron. Locations of the intron are shaded. Numbers at the top represent positions of amino acid residues in the PGHα in the Japanese crested ibis. References are as follows; carp (Huang *et al*., 1992), salmon (Suzuki *et al*., 1995), rat (Burnside *et al*., 1988), mouse (Gordon *et al*., 1988), bovine (Goodwin *et al*., 1983), porcine (Kato *et al*., 1990), rhesus (Golos *et al*., 1991), and human (Fiddes and Goodman, 1981).

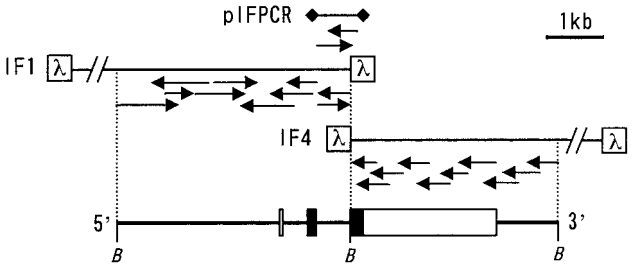


Fig. 6. Structure and sequencing strategy of the FSHβ gene in the Japanese crested ibis. Insert fragments of pIFPCR, IF1 and IF4 are shown with arrows which indicate the direction and extent of sequencing. At the bottom, the structure of ibis FSHβ gene is shown with exons indicated as boxes. Solid and open boxes represent coding and untranslated regions, respectively. Restriction sites for *Bam*HI are indicated with *B*.

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                                                                          Met Lys Thr Val
                                                                          -20
AAT TGT TAT GTG CTG TTA TTT TGC TGG AAA GCA ATT TGT TGC AAT AGC TGT CAG CTT ACC AAC ATT
Asn Cys Tyr Val Leu Leu Phe Cys Trp Lys Ala Ile Cys Cys Asn Ser Cys Gln Leu Thr Asn Ile
                         -10                                          +1
ACC ATA GCA GTG GAA AGA GAA GAA TGT GAA TTC TGT ATT ACA GTG AAT GCC ACG TGG TGC TCA GGA
Thr Ile Ala Val Glu Arg Glu Glu Cys Glu Phe Cys Ile Thr Val Asn Ala Thr Trp Cys Ser Gly
            +10                                  +20
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Tyr Cys Phe Thr Arg
    +30
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                                                                Asp Pro Val Tyr Lys Tyr Pro
                                                                +34                      +40
CCA GTA TCA TCT GTT CAG CAA ACA TGT ACT TTC AAA GAG GTT GTG TAT GAA ACA GTG AAG ATC CCA
Pro Val Ser Ser Val Gln Gln Thr Cys Thr Phe Lys Glu Val Val Tyr Glu Thr Val Lys Ile Pro
                                  +50                                  +60
GGC TGT GGT GAC CAT CCC GAA TCT TTT TAT TCG TAC CCA GTA GCT ACT GAG TGC CAT TGT GAG ACC
Gly Cys Gly Asp His Pro Glu Ser Phe Tyr Ser Tyr Pro Val Ala Thr Glu Cys His Cys Glu Thr
                              +70                                  +80
TGC GAC ACT GAC AGC ACT GAC TGC ACC GTG CGA GGG CTG GGG CCA TCC TAC TGT TCT TTC AGT CAG
Cys Asp Thr Asp Ser Thr Asp Cys Thr Val Arg Gly Leu Gly Pro Ser Tyr Cys Ser Phe Ser Gln
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Asn Gly Ser Asn Gln Stop
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Fig. 7. Nucleotide sequence of the FSHβ gene and its flanking region in the Japanese crested ibis. Exons are shown in capital letters, and introns and flanking regions are shown in lowercase letters. The two potential TATAAA sequences and the five potential polyadenylation signals, AATAAA, are underlined. The bent arrow indicates the putative transcriptional start site deduced from 5' end of chicken FSHβ cDNA (Shen and Yu, 2002). Eight ATTTA motifs in the putative 3' flanking region are boxed. Deduced amino acid residues are represented with three letter codes below the nucleotide sequence and numbered sequentially from the N-terminus. The nucleotide sequence data in this figure is available in the EMBL/GenBank/DDBJ Data Bank with Accession No. AB089502.

revealed that the ibis sequence could be well aligned with the sequences in mammals within the promoter region (Fig. 9).

Analysis of the second intron of the ibis FSHβ gene in two individuals

Results of sequencing the PCR fragments of Midori and Long-Long showed that two types of sequences were present in the second intron of the FSHβ gene (Fig. 10). The difference between the two types was the 32 bp sequence in the 5' portion. Ten and five clones were sequenced for Midori and Long-Long, respectively. Only the shorter type was observed in Midori, whereas both the shorter (in two clones) and longer (in three clones) types were found in Long-Long. In addition to the length difference, three nucleotide substitutions were observed. A substitution, which was located in the most 3' position, changed the sequence "GAATTC" (the recognition site of *Eco*RI) in the shorter type into "CAATTC" in the longer type. Utilizing this sequence difference, we could differentiate the two types by *Eco*RI digestion of the PCR products. Namely, the PCR product containing only the shorter type (approximately 780 bp) was divided into two fragments of 550 and 230 bp, while the PCR product of the longer type (approximately 810 bp), was not cleaved. The digestion results showed that only two fragments of 550 and 230 bp were observed for Midori and three fragment of 810, 550 and 230 bp for Long-Long, indicating that Midori had only the shorter type and Long-Long had both types.

DISCUSSION

In the present study, we have isolated and characterized a nucleotide sequence of a part of the PGHα gene and

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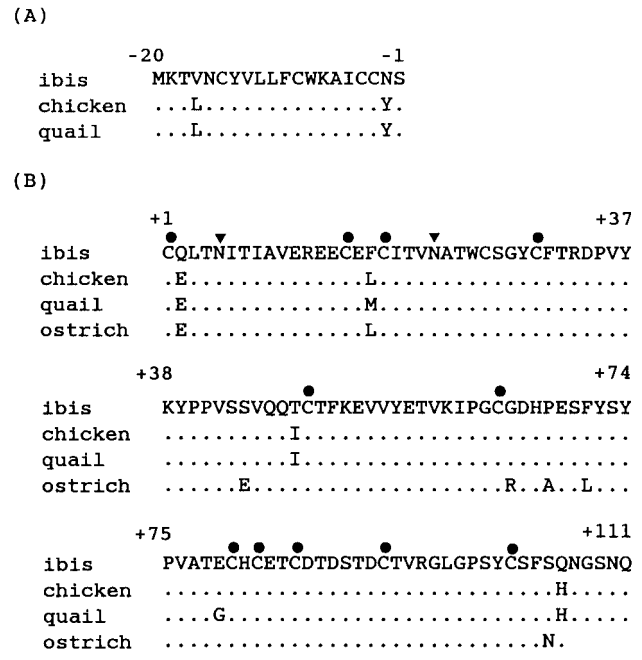


Fig. 8. Alignment of the amino acid sequences of signal peptides (A) and mature proteins (B) of FSHβ precursor molecules of the Japanese crested ibis, chicken (Shen and Yu, 2002), Japanese quail (Kikuchi *et al*., 1998), and ostrich (Koide *et al*., 1996). Residues identical to those in the Japanese crested ibis are indicated with dots. Twelve conserved cysteines and two putative N-linked glycosylation sites are denoted by ● and ▼, respectively.

the sequence of the entire FSHβ gene in the Japanese crested ibis. This is the first isolation and characterization of gonadotorpin subunit genes in avian species. The exon-intron structure of the ibis PGHα and FSHβ genes was basically similar to that of the corresponding gene in mammals and teleosts. This result suggests that the same exon-intron structure exists generally through all avian species. However, there was a minor difference among animal groups. It was the location in the second intron (corresponding to the first intron in the partial ibis gene) in the PGHα gene. This intron was shifted with an amino acid residue in teleosts compared to the ibis and mammals (Fig. 5). It is difficult to conclude from this fact that the teleostean type is more primitive than avian and mammalian type, since Arai *et al*. (1998) reported that most teleosts are considered to be genetically highly specialized group. Arai *et al*. (1998) aligned amino acid sequences of PGHα precursors in various vertebrates and revealed that only Australian lungfish has an insertion of an amino acid residue (alanine) around a position corresponding to the second exon-intron boundary. At present, the genomic structure of lungfish PGHα gene has not been clarified, it may provide some information to explain the location difference of this intron.

In the ibis FSHβ gene, we deduced the location of the putative transcriptional start site and the polyadenylation site referring to the 5' and 3' end of the chicken FSHβ cDNA (Shen and Yu, 2002). However, two TATAAA sequences in the putative promoter region and five potential polyadenylation signals in the putative 3' untranslated region were observed (Fig. 7). The proximal TATAAA sequence, which was 27 bp apart from the putative transcriptional start site, was conserved through the ibis and mammals, whereas the distal sequence was unique to the ibis and did not exist in mammals (Fig. 9). Then, the proximal sequence is considered to be more possible than the distal one, but the other possibility that the distal sequence is utilized cannot be excluded. Further studies are required to obtain a definite conclusion of the transcriptional start site and the polyadenylation site of the ibis FSHβ gene.

Kikuchi *et al*. (1988) and Shen and Yu (2002) have reported that five ATTTA motifs were found in the 3' untranslated region of the FSHβ cDNA in Japanese quail and chicken, respectively. This motif has been characterised as a signal for rapid degradation of mRNA coding for cytokine or proto-oncogenes (Shaw and Kamen, 1986; Chen *et al*., 1995). We found eight ATTTA motifs in the putative 3' untranslated region of the ibis FSHβ gene. Akashi *et al*. (1994) have demonstrated that the number of this motif in the 3' untranslated region correlates with instability of mRNA using the chimeric rabbit β-globin gene. It is possible that the increased number of this motif in the ibis FSHβ gene induces more rapid degradation of the mRNA than the other birds.

The nucleotide sequence in the 5' flanking region of the ibis FSHβ gene showed no appreciable similarity to those in teleosts and mammals apart from those in the promoter region in mammals (Fig. 9). In the promoter region of the ovine FSHβ gene, a progesterone responsive element (PRE)-like sequence and two AP-1 responsive element-like sequences have been demonstrated to be responsible for progesterone and gonadotropin-releasing hormone (GnRH) regulation of the gene expression, respectively (Webster *et al*., 1995; Huang *et al*., 2001). In the bovine and porcine FSHβ genes, these sequences were completely identical, but several substitutions occurred in each of the sequences in the rat and human FSHβ genes. Function of these sequences in rat and human has not been clear. The corresponding sequences in the ibis had several substitutions from the mammalian sequences and the consensus sequence. These results suggest a possibility that the ibis and other vertebrates (teleosts and mammals) apply different regulation mechanisms to the FSHβ gene expression. Further analysis on the 5' flanking region will reveal the mechanism of ibis FSHβ gene expression.

Among avian species we compared, the amino acid sequences of the PGHα and FSHβ showed remarkable similarities. According to the taxonomy, extant birds are classified into two lineages, the Palaeognathae and the Neognathae. The Palaeognathae includes ostrich, and the Neognathae consists of two clades; the first clade contains two orders of Galliformes (chicken, Japanese quail and turkey) and Anseriformes (duck), and the second clade contains the rest orders (van Tuinen *et al*., 2000). Our study has

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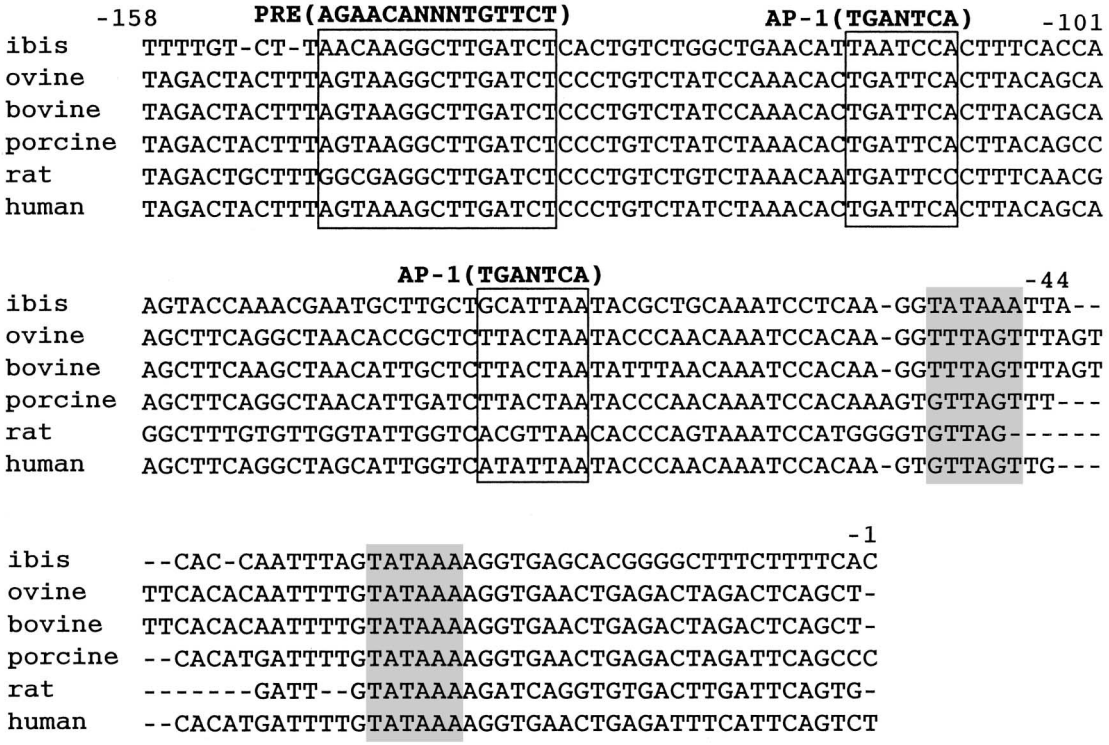


Fig. 9. Comparison of nucleotide sequences of the promoter region of FSHβ genes of the Japanese crested ibis, ovine (Guzman *et al*., 1991), bovine (Kim *et al*., 1988), porcine (Hirai *et al*., 1990), rat (Gharib *et al*., 1989) and human (Jameson *et al*., 1988). The nucleotide sequence is numbered from the putative transcriptional start site in the ibis FSHβ gene. A progesterone responsive element (PRE)-like sequence (Webster *et al*., 1995) and two AP-1 responsive element-like sequences (Huang *et al*., 2001) reported in ovine FSHβ gene are boxed with solid line with consensus sequence attached in parenthesis. Parts in which TATAAA sequences are observed in the ibis are shaded.



Fig. 10. Nucleotide sequences of the second intron of the FSHβ gene in the Japanese crested ibis, (L) the longer type and (S) the shorter type. Dashes indicate a gap inserted to maximize identity. Three nucleotide mismatches are marked with a reverse triangle. Part recognized by *Eco*RI in the short type is boxed.

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provided the first information on the amino acid sequences of the PGHα and FSHβ in a bird from the second clade of the Neognathae. High similarities of the PGHα and FSHβ among ibis, chicken, Japanese quail and ostrich indicate that FSH is generally highly conserved over wide range of avian species.

Lastly, we found two (the shorter and longer) types of the sequence in the second intron of the FSHβ gene in the Japanese crested ibis. The shorter type was observed in both of two individuals we used, Midori of the Japanese origin and Long-Long of the Chinese origin, whereas the longer type was found only in Long-Long. These results suggested that geographic divergence occur in this species. At present, we are extending the sequence analysis of this intron to other individuals of both the Japanese and Chinese origins. Sequence analysis of the mitochondrial DNA of this species is also proceeding.

ACKNOWLEDGMENTS

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