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Cloning of the Gene for the Thyrotropin β Subunit in the Japanese Crested Ibis, *Nipponia nippon*

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ABSTRACT—We isolated a putative gene for the thyrotropin β subunit (TSHβ) from two types of genomic libraries of the Japanese crested ibis, *Nipponia nippon*. Exon-intron structure was deduced by comparing the determined sequence with those of TSHβ cDNA of other birds. The deduced amino acid sequence shows extensive similarities to those of the other birds, which assures our assumption that the acquired nucleotide sequence represents the TSHβ gene. The assembled genomic fragment is 4192 bp in size and consists of 1937 bp of putative 5' flanking region followed by exon-intron structure with three exons and two introns, similar to those observed in rat, human and goldfish counterparts. Locations of introns are also similar to those in mammals and goldfish. Comparison of the 5' flanking region of the ibis TSHβ gene with those of mammals reveals that several regulatory sequences, such as negative thyroid hormone responsive element (nTRE), Pit-1 responsive element, and AP-1 responsive element, which were characterized in mammalian TSHβ genes, are also found in the promoter region. This is the first report on the exon-intron structure and 5' flanking region of the TSHβ gene in an avian species.

Key words: thyrotropin (TSH), cloning, gene, Japanese crested ibis (*Nipponia nippon*)

INTRODUCTION

In most vertebrates, thyrotropin (thyroid-stimulating hormone, TSH) is synthesized and secreted by thyrotropes of the adenohypophysis. It regulates the growth and metabolism of an organism by stimulating thyroid hormone production in the thyroid gland. Furthermore, in birds, it has been reported that this hormone controls molting and may influence reproduction that is principally regulated by gonadotropins, i.e. follicle-stimulating hormone (FSH) and luteinizing hormone (LH). The TSH, FSH and LH are members of the pituitary glycoprotein hormone family and all of them are heterodimeric molecules consisting of two subunits, α and β. Within a single species, the α subunit is common between each of these hormones whereas the β subunit is unique. The sequences for the TSHβ gene have been determined in rat (Carr *et al*., 1987), mouse (Gordon *et al*., 1988), human (Wondisford *et al*., 1988; Guidon *et al*., 1988), and goldfish (Sohn *et al*., 1999). As for avian species, cDNA for the TSHβ has been characterized in the Japanese quail (Kato *et al*., 1997), chicken (Gregory and Porter, 1997), and the Muscovy duck (Hsieh *et al*., 2000). No information has thus far been available regarding its genomic sequence.

The Japanese crested ibis *Nipponia nippon* (order Ciconiiformes, family Threskiornithidae) is a severely endangered bird species in East Asia. This species had once been widely distributed over Japan, Korea, China and an adjacent part in eastern Siberia. However, wild populations of this species started to disappear in all of these areas in the 20th century, and at present, only a single population of approximately 170 individuals survives at Yang Xian, Shaanxi Province, China. Under such circumstances, it is urgently necessary to place this endangered species under careful protection and also to obtain genetic information for this species on the hormone molecules which are related to reproduction. In Japan, wild populations had become extinct and only an old female bird captured in 1968 still survives. This female is the only living individual of non-Chinese origin in the world. Fortunately living cells of several individuals of Japanese origin and frozen tissues of one of the Japanese origin have been preserved for scientific study (Ishii, 1999), we have constructed genomic libraries from the tissue sample and have tried to clone the genes encoding molecules of the members of the pituitary glycoprotein hormone family.

Here, we report the isolation and characterization of the

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gene for Japanese crested ibis TSHβ. We expected to obtain information not only on structure of the molecule but also on transcriptional regulation of the gene by analyzing the genomic sequence in the 5' flanking region. The results represent the first information on the genomic structure of the TSHβ gene not only in avian species, but also in non-mammalian tetrapods.

MATERIAL AND METHODS

Material

A male *Nipponia nippon* individual of Japanese origin (named "Midori") died in 1995, and most of his organs were preserved in liquid nitrogen (Ishii, 1999). We used part of the frozen kidney as a source of genomic DNA.

Genomic DNA extraction

Genomic DNA was extracted from approximately 15 mg of the frozen kidney with a GenomicPrep Cells and Tissue DNA Isolation Kit (Amersham Pharmacia Biotech, NJ).

PCR amplification (1)

In order to obtain a partial sequence of the ibis TSHβ gene, PCR was performed with genomic DNA as a template. The following oligonucleotides were designed as sense and antisense primers, referring to nucleotide sequences conserved through TSHβ cDNA of other vertebrates: TSH-F1 (5'-TGCCTI(AG)CCAT(CT)AA(CT)-ACIAC(CT)(AG)T(CT)TG-3') and TSH-R1 (5'-G(CT)C(AT)GT(AG)-TTACAI(TG)T(TG)(CT)CACA-3'). 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 Taq (Ex Taq version) (Takara Shuzo, Japan). The amplified product gave a single band by electrophoresis on an agarose gel. The DNA was extracted from the band and cloned into a pCR2.1 plasmid vector (Invitrogen, CA). The clone (*pITPCR*) was found to contain an insert with the expected size and was then selected for sequence analysis. The nucleotide sequence was determined with a Thermo Sequenase Cycle Sequencing Kit (USB Corporation, OH) with a DNA sequencer Model 4000L (LI-COR, NE).

Construction and screening of the genomic library (1)

A genomic library was constructed using the Lambda EMBL3 phage vector (Stratagene, CA) with genomic DNA partially digested with *Bam*HI. A primary library of 5.0×10^4 plaques was amplified once to obtain 8.0×10^9 plaques from which 1.0×10^5 plaques were screened by plaque hybridization. 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 60°C overnight in the hybridization buffer containing a DNA insert of *pITPCR* randomly labeled with [α-^{32}P]dCTP (Amersham Pharmacia Biotech, UK) as a probe. After hybridization, 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. Hybridization signals on the membranes were analyzed with a BAS-2000II Bio-Imaging Analyzer (Fuji Photo Film, Japan). After third round of screening, three positive clones were isolated. DNA was purified from these clones, digested with *Bam*HI and/or *Eco*RI and analyzed by electrophoresis. These clones contained an insert of the same size, and one (*IT3*) was selected, subcloned into a pBluescriptII phagemid vector (Stratagene, CA) for sequencing.

PCR amplification (2)

Another PCR was performed to obtain the 5' flanking region of *IT3*, which harbors the 5' untranslated exon of the ibis TSHβ gene. PCR primer, TSH-1 (5'-GAATTCAGCTGACAAGAGGT-3', for sense) was designed from nucleotide sequences of the 5' untranslated region of TSHβ cDNA in the Japanese quail, chicken, and the Muscovy duck, and TSH-2 (5'-TCAGGCCAAAGAGGAGAGAC-3', for antisense) was generated from the nucleotide sequence of *IT3*. PCR was performed with 30 cycles of 20 sec at 94°C and 15 min at 68°C with a One Shot LA PCR Mix (Takara Shuzo, Japan). The product gave a single band in an agarose gel electrophoresis. The DNA was extracted and cloned into a pCR2.1 vector (Invitrogen). The clone (*pITPCR2*) was found to contain the insert of the expected size and selected for sequence analysis.

Construction and screening of the genomic library (2)

Genomic DNA was partially digested with *Sau*3AI and fragment size examined on an agarose gel. DNA fragments of the appropriate size were dephosphorylated with bacterial alkaline phosphatase and ligated to a Lambda EMBL3 phage vector (Stratagene), and a library of 3.5×10^4 plaques was generated. To prepare a hybridization probe, PCR was performed with the primers TSH-1 and TSH-2 using *pITPCR2* as a template. To get a 5' fragment which does not overlap with *IT3*, the PCR product was digested with *Bam*HI, separated on an agarose gel and DNA fragment of approximately 220 bp was purified from the gel. This fragment was randomly labeled with [α-^{32}P]dCTP and used as a probe. Prehybridization was carried out as described above and hybridization was performed at 50°C overnight in the hybridization buffer containing the labeled probe. Membranes were washed once with 3× SSC, 0.1% SDS at 55°C for 20 min, and hybridization signals were analyzed as described above. After the third round of screening, one positive clone was isolated and named *IT7*. DNA was purified and digested with *Sal*I and *Bgl*II, and a DNA fragment of approximately 3.5 kb was subcloned into a pBluescriptII phagemid vector (Stratagene). The nucleotide sequence was determined using a BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, CA) and an ABI PRISM 377 DNA Sequencing System (Applied Biosystems, CA).

RESULTS

Isolation of the putative TSHβ gene in the Japanese crested ibis

The strategy used to obtain the nucleotide sequence of the putative ibis TSHβ gene is illustrated in Fig. 1. The first

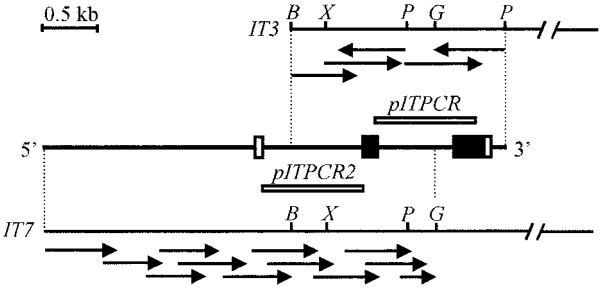

Fig. 1. Structure and sequencing strategy of the putative TSHβ gene in the Japanese crested ibis. Top and bottom lines indicate the insert of *IT3* (10kb) and *IT7* (17kb), respectively. Arrows indicate the direction and extent of sequencing. Open bars represent the cloned PCR products. The center line (in bold) shows the structure of the putative ibis TSHβ gene with exons indicated as boxes. Solid and open boxes indicate coding and untranslated regions, respectively. Restriction enzymes, *Bam*HI, *Xba*I, *Pst*I and *Bgl*II are indicated with *B, X, P,* and *G,* respectively.

```
gatctgtcctctgcctatattcttacaggaaacgttgaggacactttaaacctggacgtgggggtcttcctgtcagaacccatgcgtaca
aatacagctcccattgcctaaagctgtgcttgtaaatcactgtgaaatctttagagaaaagaatcagctgtccctacttcagctatgcat
gcaagggtcacatgcaatgttcttgctttaggaatgccatcttttattcttatttgggcactttctgtcctctccaggatttagtttta
tgaattgtttccttttccttcaggatcgttgtctcctttgaaatactcttaagattttctgtgattaaagttttaaaaaaccaaacccca
tacacatatatgtagtgtatacgtgtgtatataatatgtgtgtgtgtgcgtatacacattatctacatatgggtgtatgtatgtgtatac
tcatatagcctgtatgtttaactgcacttaaaatatgtcagaaaaatatagagttaaataatgtattggagtagtagcaaaataacagcg
ttatacttctgttgctaagtgtggatggacagatgttactgagagtccatttaatgctctgctcatgtccttaagccagaaagagttgta
tttaattcctgaaaagttatatggtagaaattaatccataattaaagactccatgaaagcatttggctagtcttaatagaacaacaactc
tatcatgttatccaaaaaacccctttggggaattagccagggacacagagctgtgtgcagcagcccaaaaccacaggaaaggaggcaatat
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tttatcgtatttcttatggctatgtaaatcaagaacgtggttgacctgtgtaagacatacatcagctcagagtcccaatggccgttgggt
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tggccgagtgcgaaggctggccggccgctgatgaagcagtagaagacagtgggtttgtgctgaatcagtgcaagacgtgcatgctgactc
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accacactgcagagcaattgctttgatttgagatttgctactttctgtgtacagcactgcatctctcagttgtgcctgctgggtaacgag
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atagccttcctgtcatgatgttgctgtcctaggtagcaattaagttaatcaagagtaacttttttaaagcactggttagtttaaaagcct
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tttgtggttcagtatgaattttcaatagatgactttgcgataagaaagcagcaattcagatgcaatta[tataaa]cactaagatcagccag

                                                    ↱
gaattagcctggagag[tataaa]gcacacacagagcttgtgctgggtcACTGCCTCTCCCAGCTCAAGGCAGAGGCAGGTTCCTGTAACAG
GGAAGAATTCAGCTGACAAGAGgttggtctttccagacttgcattgttgcagctacagggtaaaccaggctctgtaatgaacggctttttg
cgatgggaactgacagctggaagagactagagggtgatgtccatatgtttgtagacactgtcatttagagtttgtggtttagaggctata
gagtagaccctggaattgtctgggagttaatcaaagctttgttctggggatcccccagcaagtctgaggaatgaggtcagcacaacagac
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cccatttcatcacgcctctaagccaactggaaagtaaaaaatacagtggtatgcctgatatgcacagctgtttctttttctctcttgaac
attttataagcagctttctttagaaaattatttttaaaccagtgagattattttagcatctctggttaattctagagctcatgttctctc
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aagtctgagattggaaagaatttgtcaggctgtagctaaaaagttaagattacgtctagtctgtgactatcataactgttcttgtcaaat
tccgagaaaagggctggaaggtacacctaattttacttagggtttgctttgtttgtttgttttttcccccatctagC ATG AGT CCC
                                                                                  Met Ser Pro
                                                                                  -20
TTC TTT GTG ATG TCT CTC CTC TTT GGC CTG ACT TTC GGT CAA ACA GCA TCA CTT TGT GCT CCT TCT
Phe Phe Val Met Ser Leu Leu Phe Gly Leu Thr Phe Gly Gln Thr Ala Ser Leu Cys Ala Pro Ser
                          -10                                   -1  +1
GAG TAT ATA ATC CAT GTG GAG AAA AGG GAA TGT GCC TAC TGC CTG GCC ATC AAC ACC ACC ATC TGC
Glu Tyr Ile Ile His Val Glu Lys Arg Glu Cys Ala Tyr Cys Leu Ala Ile Asn Thr Thr Ile Cys
               +10                                      +20
GCT GGA TTT TGC ATG ACT CGG gtacaaggcattactctgttttaggcagttgtttctataatggccatcactgcaaaacaagt
Ala Gly Phe Cys Met Thr Arg
       +30             +34
gcctgttctcattggaaaatggtgtgtaaatgatccagttctaatactgtttgtcacagaaaaatcacactgtcatgttttcaagttagc
tttttgcgaaggtatggatgagaaatgccaagcctccggaactacggagtttataacaccagtggcacagcagagaactgactgctgctg
tcactgcagggcctctgtagtcagtttgtggggtcaggatggaacaggtgagaagcagattatatttagccactcttatttaatgtaaac
gacttgggtaacttctactcccactactgttacgacagctagctgtggagaatattttgcacataggcatggcaaacactatgcttgagt
ttcgctgttcacacagcatttgaagttccaccttggtcagtaggtagccaacacagattacaaagcactgttagcactccgagccaagcg
gattagttaaaaccatacaagcaaggtgaacctatctactggggcaaagcgccccaatcacaggcagattcaaaatcacagatctgaaca
gaagcgcactgaaaatatcagagccaatcaaagtgtaaagaggtttgctttctgcacgtttgagacgatgtcaaaagaagtcttctgacg
gtccggtatgcttccgtgaaacaacccatttctttttttcttttgtag GAC AGC AAT GGC AAG AAG CTG CTA CTC AAA
                                                  Asp Ser Asn Gly Lys Lys Leu Leu Leu Lys
                                                  +35                 +40
AGT GCT CTG TCG CAG AAC GTG TGC ACG TAC AAA GAG ATG TTG TAT CAA ACA GCA CTG ATT CCG GGG
Ser Ala Leu Ser Gln Asn Val Cys Thr Tyr Lys Glu Met Leu Tyr Gln Thr Ala Leu Ile Pro Gly
                    +50                                     +60
TGT CCT CAT CAC ACC ATC CCT TAC TAT TCC TAC CCT GTG GCT GTG AGC TGC AAG TGT GGT AAA TGT
Cys Pro His His Thr Ile Pro Tyr Tyr Ser Tyr Pro Val Ala Val Ser Cys Lys Cys Gly Lys Cys
           +70                                     +80
AAC ACT GAT TAC AGT GAC TGT GTT CAT GAG AAG GTT AGG ACA AAC TAT TGC ACT AAA CCA CAG AAG
Asn Thr Asp Tyr Ser Asp Cys Val His Glu Lys Val Arg Thr Asn Tyr Cys Thr Lys Pro Gln Lys
    +90                                     +100                                    +110
CTC TGC AAC ATG TAA GCTTCCAACAGAATGTGGCTGAAATGTACCTCTCTGCTGAACTAAACATA[AATAAA]AGTGTATTTCATAA
Leu Cys Asn Met stop
          +114
CAGGTTTGCaaattggtgtgcacaaagttttattctaaagatgtatcaccagttacttcaattaactgggtattctgtgacacgtgcaaa
agcagaatcgtaggactgcag
```

Fig. 2. Nucleotide sequence of the TSHβ 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 TATAA sequences and the polyadenylation signal, AATAAA, are underlined. The bent arrow indicates the putative transcriptional start site deduced from 5' end of TSHβ cDNA sequence in the Muscovy duck (Hsieh *et al.*, 2000). Deduced amino acid residues are represented with three letter codes below the nucleotide sequence and numbered sequentially from the amino terminus. The nucleotide sequence data in this figure is available in the EMBL/GenBank/DDBJ Data Bank with Accession No. AB089501.

PCR, performed with a primer set designed from conserved nucleotide sequences in TSHβ of other vertebrates, produced a single band of approximately 950 bp. DNA from this band was cloned and the nucleotide sequence of a clone, named *pITPCR* , was determined. This 957 bp sequence was compared with those of TSHβ cDNA from the Japanese quail (Kato *et al*., 1997), chicken (Gregory and Porter, 1997) and the Muscovy duck (Hsieh *et al*., 2000). We concluded that this sequence contains a portion of the putative TSHβ gene of the Japanese crested ibis. The screening of the genomic library constructed with *Bam*HI-digested genomic DNA with *pITPCR* as a probe gave three positive clones. Further characterization of these clones with *Bam*HI and/or *Eco*RI digestion indicated that all of them represent identical clones with an insert of approximately 10 kb. One of them, named *IT3*, was selected for sequence analysis. A nucleotide sequence of 1985 bp was determined and compared with TSHβ cDNA sequences of the Japanese quail, chicken and the Muscovy duck. The result showed that *IT3* contains a 3' portion of the putative ibis TSHβ gene, corresponding to the whole coding exons, but lacks its 5' portion including the 5' untranslated region.

In an attempt to obtain the missing sequence from *IT3*, we performed another PCR using a primer designed from nucleotide sequences in the 5' untranslated region of TSHβ cDNA of other birds (TSH-1) and a primer generated from the sequence of *IT3* (TSH-2). A PCR product of approximately 900 bp, named *pITPCR2*, was cloned and sequenced. Comparing the subsequently determined 923 bp sequence with that of *IT3* revealed that the product contains a 5' portion of *IT3* (700 bp) and newly determined 5' portion (223 bp) of the putative ibis TSHβ gene. One positive clone was obtained by screening of the library constructed with genomic DNA partially digested with *Sau*3AI. The insert of this clone, named *IT7*, is approximately 17 kb. A region of this insert, 3625 bp in size, was compared with those of both *IT3* and 5' untranslated region of TSHβ cDNA of other birds. It was shown that the sequence overlaps with 1418 bp of 5' portion of *IT3* and contains 2207 bp of the newly determined 5' portion of the putative ibis TSHβ gene containing a 5' untranslated exon.

By combining the results obtained from two phage

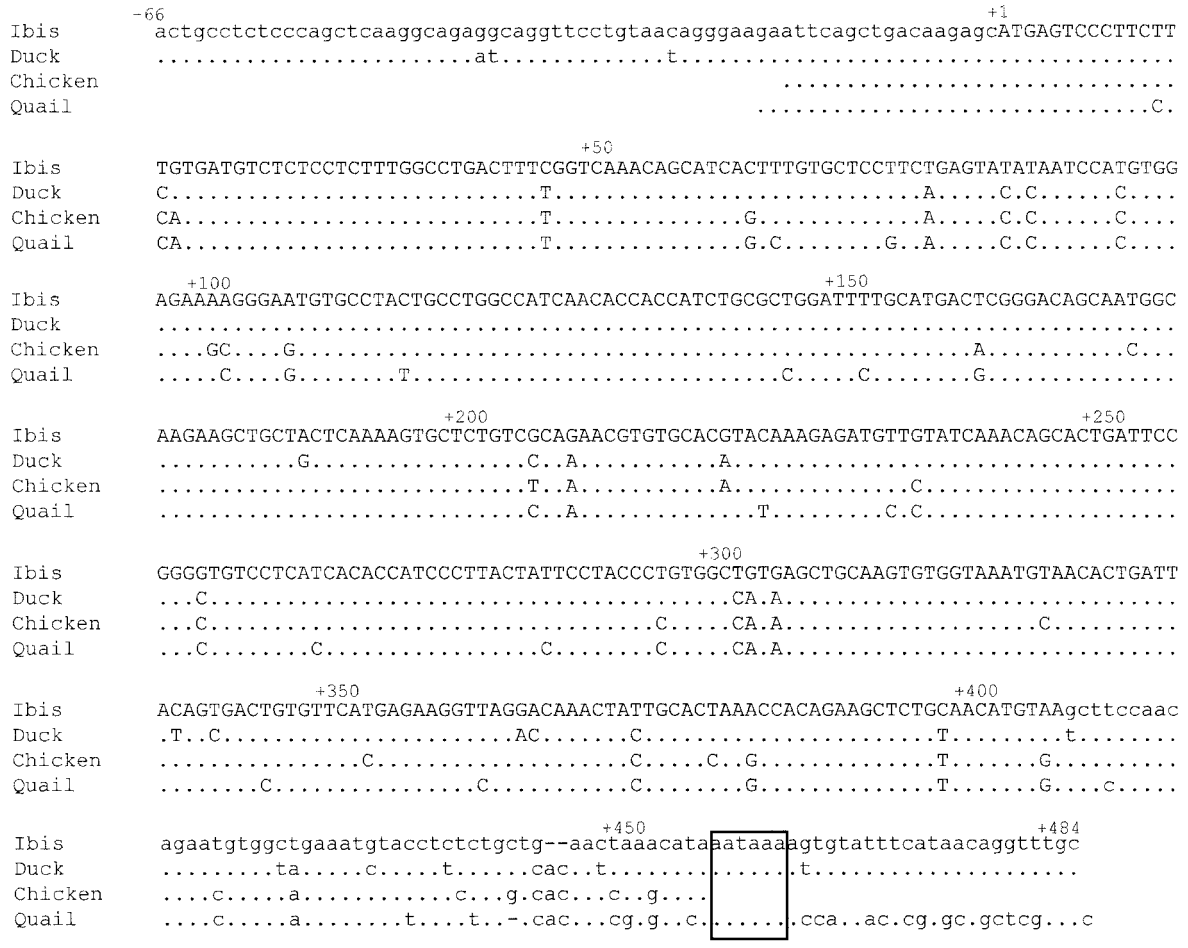

Fig. 3. Comparison of the exon nucleotide sequence of the TSHβ gene of the Japanese crested ibis and the sequences of TSHβ cDNA in the Japanese quail (Kato *et al*., 1997), chicken (Gregory and Porter, 1997), and the Muscovy duck (Hsieh *et al*., 2000). Protein coding regions are shown with capital letters, while 5' and 3' untranslated sequences are shown in lowercase letters. Dots indicate matched nucleotides with ibis TSHβ and hyphens represent spaces inserted to obtain the maximal homology. The polyadenylation signal, AATAAA, is boxed.

clones (*IT3* and *IT7*), a nucleotide sequence of 4192 bp was assembled (Fig. 2). The exon-intron structure was deduced by comparison of the nucleotide sequence with those of the TSHβ cDNA of the Japanese quail, chicken, and the Muscovy duck. The exon-intron junctions were determined based on the GT-AG rule on the splicing-donor site (Breathnach and Chambon, 1981). We inferred the transcriptional start site and the polyadenylation site by comparing the sequence with that of the Muscovy duck TSHβ cDNA (Hsieh *et al.*, 2000). Taken together, it was concluded that the nucleotide sequence contains 1937 bp of a 5' flanking region followed by three exons of 65 bp, 163 bp and 322 bp, separated by two introns of 864 bp and 739 bp. The nucleotide sequence data is available in the EMBL/GenBank/DDBJ Data Bank with Accession No. AB089501.

Comparison of ibis and other avian TSHβ at the nucleotide and amino acid sequence level

The nucleotide sequences of the three exons in the putative ibis TSHβ gene were aligned with those of TSHβ cDNA of other three birds (Fig. 3). The extent of similarities of the ibis sequence with those of the Japanese quail, chicken, and the Muscovy duck are 88%, 92%, and 94%, respectively. An amino acid sequence of 134 residues was deduced from the nucleotide sequence of the putative coding region. It consists of a signal peptide with 20 residues and a mature protein with 114 residues. The amino acid sequence was aligned with those of the three birds (Fig. 4). Similarities of ibis TSHβ with those of the Japanese quail, chicken, and the Muscovy duck are 96.3%, 96.3%, and 97.8%, respectively.

```
          -20                        +1            +15
Ibis      mspffvmsllfgltfgqtasLCAPSEYIIHVEKRE
Duck      ..........................T.......
Chicken   .....m..............V......T.......
Quail     ....lm..............V......T.......

          +16                                      +50
Ibis      CAYCLAINTTICAGFCMTRDSNGKKLLLKSALSQN
Duck      ....................................
Chicken   ....................................
Quail     ....................................

          +51                                      +85
Ibis      VCTYKEMLYQTALIPGCPHHTIPYYSYPVAVSCKC
Duck      ............................I....
Chicken   .......F......................I....
Quail     ............................I....

          +86                            +114
Ibis      GKCNTDYSDCVHEKVRTNYCTKPQKLCNM
Duck      ..............P.............
Chicken   ............................
Quail     ............................
```

Fig. 4. Comparison of the deduced amino acid sequence of avian TSHβ. Amino acid residues of a putative signal peptide and a mature protein are indicated by lowercase letters and capital letters, respectively, and the residues identical to those in the Japanese crested ibis TSHβ are indicated with dots. Amino acid sequence data are cited from Kato *et al.* (1997) for the Japanese quail, Gregory and Porter (1997) for the chicken, and Hsieh *et al.* (2000) for the Muscovy duck.

Comparison of ibis, goldfish and mammalian TSHβ genes

Comparison of the structure of the ibis TSHβ gene with those of rat (Carr *et al.*, 1987), mouse (Gordon *et al.*, 1988), human (Wondisford *et al.*, 1988; Guidon *et al.*, 1988) and goldfish (Sohn *et al.*, 1999) shows that the ibis TSHβ gene has similar exon-intron organization to those of other vertebrates, except for mouse TSHβ gene which has two additional untranslated exons (Fig. 5). Intron 1 of the ibis TSHβ gene is much shorter than that in rat and human but almost same as goldfish. The locations of introns are identical to those of rat, human and goldfish counterparts, namely, intron 1 (intron 1, 2, and 3 for mouse) is located in the 5' untranslated region and intron 2 (intron 4 for mouse) is located in the three amino acids downstream from the fifth cysteine residue in the coding region (Fig. 2 and Fig. 6).

Comparing the nucleotide sequence of the 5' flanking region with those of rat, mouse, human and goldfish TSHβ gene reveals that two TATAA sequences and several regulatory sequences reported in mammalian TSHβ genes, such

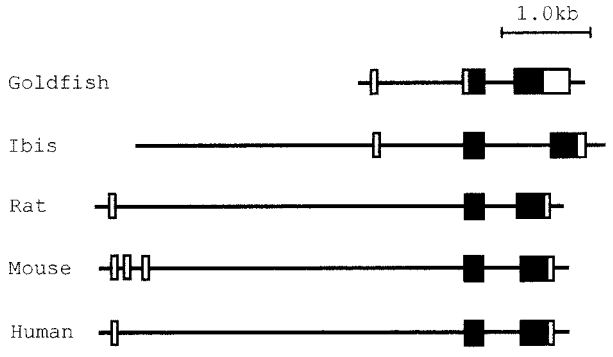

Fig. 5. Comparison of the TSHβ exon-intron structure of ibis, goldfish and mammals. Solid and open boxes represent the coding and the untranslated regions of exons, respectively. Thin lines indicate introns and flanking regions. References: goldfish, Sohn *et al.* (1999); rat, Carr *et al.* (1987); mouse, Gordon *et al.* (1988); human, Wondisford *et al.* (1988) and Guidon *et al.* (1988).

```
              +27          ▽      +40
Goldfish      CMGFCFSR DSNVKE
Ibis          CAGFCMTR DSNGKK
Mouse         CAGYCMTR DINGKL
Rat           CAGYCMTR DINGKL
Human         CAGYCMTR DINGKL
```

Fig. 6. A diagram showing the conserved location of the intron 2 in TSHβ genes (intron 4 in the mouse TSHβ gene). The inverted triangle indicates the position of the intron. Numbers at the top represent positions of amino acid residues in the ibis TSHβ molecule. Sequences are aligned with respect to the conserved cysteine residues (shaded). References; Sohn *et al.* (1999) for the goldfish, Carr *et al.* (1987) for the rat, Gordon *et al.* (1988) for the mouse, and Wondisford *et al.* (1988) and Guidon *et al.* (1988) for the human.

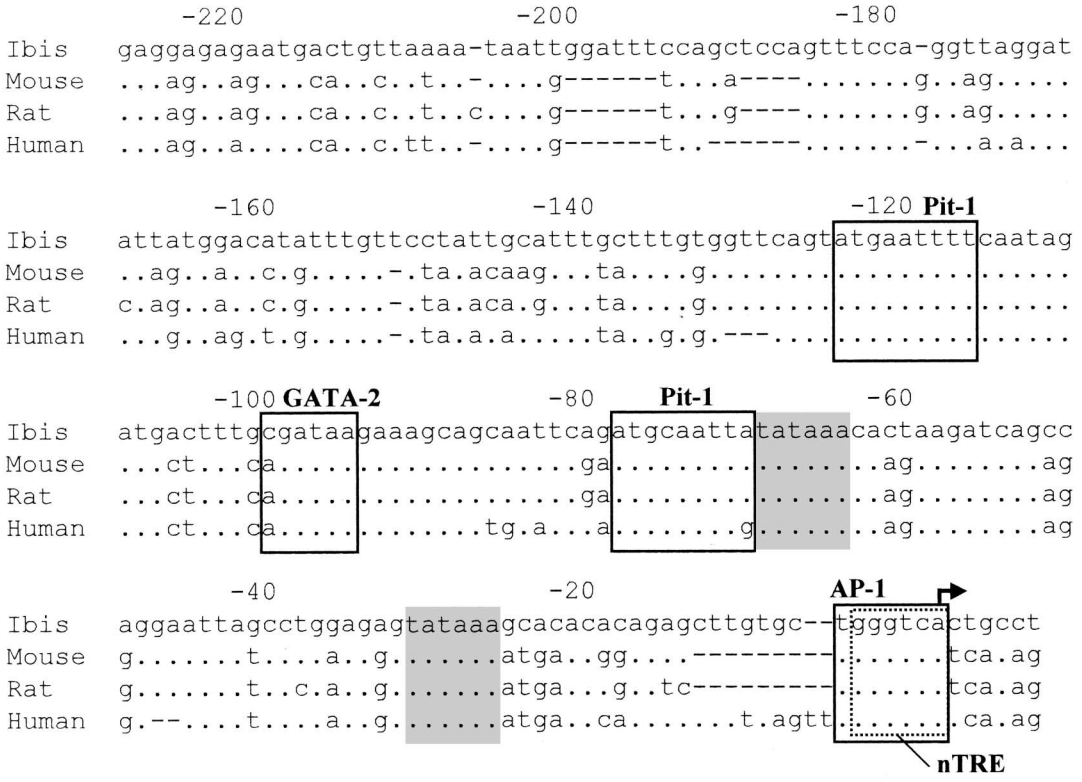

Fig. 7. Comparison of nucleotide sequences of the 5' flanking region of TSHβ genes of the Japanese crested ibis, mouse (Gordon *et al.*, 1988), rat (Carr *et al.*, 1987) and human (Guidon *et al.*, 1988). The nucleotide sequence is numbered from the putative transcriptional start site in ibis TSHβ gene (indicated by a bent arrow). Two TATAA sequences are shaded. Two Pit-1 responsive elements (Steinfelder *et al.*, 1992; Lin *et al.*, 1994), an AP-1 responsive element (Wondisford *et al.*, 1993) and a GATA-2 responsive element (Gordon *et al.*, 1997) are boxed with solid lines, while a negative thyroid hormone responsive element (nTRE) (Cohen *et al.*, 1995) is boxed with a dotted line.

as negative thyroid hormone responsive element (Cohen *et al.*, 1995), Pit-1 responsive element (Steinfelder *et al.*, 1992; Lin *et al.*, 1994), and AP-1 responsive element (Wondisford *et al.*, 1993), are found within the promoter region (Fig. 7). However, in the ibis TSHβ gene, a nucleotide is substituted from consensus sequence of GATA-2 responsive element reported in mouse (Gordon *et al.*, 1997).

DISCUSSION

In the present study, we clarified the entire exon-intron structure and 5' flanking region of the TSHβ gene for the first time in any avian species. The exon-intron structure was deduced by comparing the determined ibis sequence with TSHβ cDNA sequence ever characterized in avian species; the Japanese quail (Kato *et al.*, 1997), chicken (Gregory and Porter, 1997) and the Muscovy duck (Hsieh *et al.*, 2000). Although exact positions of the transcriptional start site and the polyadenylation site remain to be determined, they were inferred by comparison of the gene sequence with the TSHβ cDNA sequence of the Muscovy duck. These assignments appear to be appropriate considering their distance from the TATAA sequence and the polyadenylation signal (AATAAA), respectively. Comparison of the nucleotide sequence of exons of the ibis TSHβ gene with TSHβ cDNA sequences of other birds reveals similarities as high as 88–94% (Fig. 3), and at the amino acid level, similarities are higher than 96% (Fig. 4). These results strongly support our assumption that the nucleotide sequence we obtained encodes the gene for the TSHβ in the Japanese crested ibis.

Comparison of the exon-intron structure of ibis TSHβ gene with those of rat (Carr *et al.*, 1987), mouse (Gordon *et al.*, 1988), human (Wondisford *et al.*, 1988; Guidon *et al.*, 1988) and goldfish (Sohn *et al.*, 1999) shows that ibis TSHβ gene has a similar exon-intron structure to rat, human and goldfish which have three exons and two introns (Fig. 5). An exception is the mouse TSHβ gene which has five exons and four introns. The difference in gene structure of the mouse TSHβ from the others is caused by an addition of two 5' untranslated exons. Among the three 5' untranslated exons in mouse, exon 1 is ascertained to be homologous to exon 1 in rat and human. In contrast, exon 2 and 3 in mouse are thought to appear by nucleotide substitutions which generate additional splicing-donor sites within the region (Gordon *et al.*, 1988; Guidon *et al.*, 1988). Therefore, apart from the difference of the number of 5' untranslated exons, the basic structure of the mouse TSHβ gene is considered to be the same to those of other vertebrate species including ibis. Locations of introns are also conserved among these vertebrates; intron 1 (intron 1, 2, and 3 for mouse) is located in

the 5' untranslated region, whereas intron 2 (intron 4 for mouse) is located in the three amino acids downstream from the fifth cysteine residue in the coding region (Fig. 6). The organization of three exons and two introns and the location of intron 2 conserved among the TSHβ genes are also characteristics observed in all the genes for the β subunits of other glycoprotein hormones characterized so far in mammals and teleosts (Chang *et al*., 1992; Sohn *et al*., 1998; Sohn *et al*., 1999). These data suggest that the exon-intron structure common to most of the genes for the glycoprotein hormone β subunits is also conserved in TSHβ gene in the Japanese crested ibis, and may also be the case in other avian species. This conserved exon-intron structure supports the idea that the genes for the β subunits evolved from a common ancestral gene (Ishii, 1991). In addition, it may reflect importance of the structure of these subunit molecules to maintain functions of thyrotropin and gonadotropins since changes in exon-intron structure may change structure of proteins.

By comparing the 5' flanking region of ibis TSHβ gene with those of mammals, several regulatory sequences characterized in mammalian TSHβ genes were also found in the promoter region (Fig. 7). First, two TATAA sequences are noted both in ibis and mammals. In rat and mouse TSHβ genes, the presence of two transcriptional start sites suggests that both TATAA sequences are utilized, although transcription from the downstream site dominates that from the upstream site (Carr *et al*., 1987; Gordon *et al*., 1988). In human TSHβ gene, transcription from only the downstream site has been detected (Wondisford *et al*., 1988; Guidon *et al*., 1988). In ibis TSHβ gene, the marked similarity with the 5' end of Muscovy duck TSHβ cDNA (Hsieh *et al*., 2000) suggest that, at least, transcription from the downstream site is likely to occur, although transcription from the upstream site is also possible. Secondly, a negative thyroid hormone responsive element (nTRE) is present in this region. It is positioned around the transcriptional start site. Cohen *et al.* (1995) reported that this element mediates thyroid hormone inhibition of the TSHβ gene in human and mouse. In avian species, Hsieh *et al.* (2000) reported that the TSHβ mRNA level was decreased by the action of triiodothyronine (T3) and thyroxine (T4) in primary pituitary cells of the Muscovy duck. Taken together, it is possible that this nTRE mediates inhibition of gene expression by thyroid hormone in ibis TSHβ gene. Lastly, two Pit-1 responsive elements and an AP-1 responsive element, which stimulate TSHβ gene expression in mammals, are conserved in ibis TSHβ gene. The pituitary-specific transcription factor, Pit-1 has been shown to mediate both hypothalamic thyrotropin-releasing hormone (TRH) and cAMP stimulation of the TSHβ gene in mammals. In this regard, two Pit-1 responsive elements have been reported in human (Steinfelder *et al*., 1992) and mouse (Lin *et al*., 1994). In the ibis, it is possible that Pit-1 binds to these two responsive elements and stimulates the TSHβ gene expression. However, it has been suggested in the mouse TSHβ gene that Pit-1 stimulation through the upstream element needs interaction with GATA-2 which binds to its responsive element (Haugen *et al*., 1996; Gordon *et al*., 1997). Nucleotide sequence of the GATA-2 responsive element in ibis TSHβ gene has a substitution when compared with the mammalian consensus sequence. Therefore, whether the upstream Pit-1 responsive element and the GATA-2 responsive element are functional in ibis TSHβ gene remains to be investigated. On the other hand, the AP-1 element overlaps with the nTRE and Wondisford *et al.* (1993) suggested that TRH may decrease the magnitude of thyroid hormone inhibition on the TSHβ gene through an interaction between a transcription factor, AP-1 (*c-jun/c-fos*), and thyroid hormone receptor at this overlapping element. Arrangement of these elements in ibis TSHβ gene is identical to that in mammals. Thus, such interaction between AP-1 and thyroid hormone receptor is possible in ibis TSHβ gene expression. In avian species, Hsieh *et al.* (2000) reported that TSHβ mRNA level was increased by TRH in primary pituitary cells in the Muscovy duck. Therefore, it is possible that TRH stimulates TSHβ gene expression through these Pit-1 and AP-1 responsive elements in the Japanese crested ibis. Taken altogether, these data suggest that both birds and mammals may adopt to a certain extent a common system for the regulation of the TSHβ gene expression. However, possibility of the presence of avian- or ibis-specific regulatory system cannot be neglected. To elucidate regulation system of the TSHβ gene expression in avian species or in the Japanese crested ibis, further studies will be needed.

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