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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
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 1999, and most of his organs were preserved in liquid nitrogen (Ishi, 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'-TGCCTTAGCAATGCGTGTGG-3') and TSH-R1 (5'-GCTCTACTTACCTTGCTTT-3') and TSH-R1 (5'-GCTCTACTTACCTTGCTTT-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 on agarose gel electrophoresis. The DNA was extracted from the band and cloned into a pCR2.1 vector (Invitrogen, CA). The clone (pITPCR1) was found to contain an insert with the expected size and was then selected for sequence analysis. The nucleotide sequence was determined using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, CA).

Construction and screening of the genomic library (2)

Genomic DNA was partially digested with Sau3AI 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 3.5×10^6 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 TIT3, the PCR product was digested with BamHI, separated on an agarose gel and DNA fragments of approximately 220 bp was purified from the gel. This fragment was randomly labeled with [α-32P]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 3x 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 SalI and BglII, 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.](image-url)
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 BamHI-digested genomic DNA with pITPCR as a probe gave three positive clones. Further characterization of these clones with BamHI and/or EcoRI 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 Sau3AI. 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

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Ibis  -66                      +1
Duck   ....................................................
Chicken ...................................................
Quail  ....................................................

Ibis  +50                      +150
Duck   C...................................................
Chicken ...................................................
Quail  ....................................................

Ibis  +200                     +250
Duck   ...................................................
Chicken ...................................................
Quail  ....................................................

Ibis  +300                     +350
Duck   ...................................................
Chicken ...................................................
Quail  ....................................................

Ibis  +450                     +500
Duck   ...................................................
Chicken ...................................................
Quail  ....................................................
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**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.

### 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 untranscribed 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

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**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.

**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).

**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.
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).

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

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 (Steinfeld 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.
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 characterized 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 hormones characterized so far in mammals 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 (Steinfeld 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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