Cloning of a Partial cDNA for Japanese Quail Thyroid-Stimulating Hormone β and Effects of Methimazole on the Thyroid and Reproductive Axes

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ABSTRACT The purposes of this study were to develop a probe for the detection of thyroid-stimulating hormone (TSH) β subunit mRNA, to validate the usefulness of that probe in measuring TSH, and to use it to investigate the effects of thyroid suppression on TSH and the reproductive axis in Japanese quail. The objectives of experiment 1 were to isolate and characterize a partial cDNA for quail TSH and validate a riboprobe transcribed from this cDNA. This riboprobe was then used to assess changes in TSH β mRNA levels in Japanese quail. We isolated a cDNA of 168 bp with 94% identity to the corresponding sequence in chicken TSH β. The transcribed riboprobe was shown to be pituitary gland specific, and differences in TSH β mRNA levels were detectable with 2.5 µg of total RNA in Northern blot analysis. In experiment 2, our objective was to determine if thyroid inhibition would result in a detectable change in TSH β mRNA and alterations in the pituitary luteinizing hormone (LH) or indices of gonadal function. We used adult, reproductively active, male Japanese quail on a long-day photoperiod. Treatment with a goitrogen, methimazole (MMI), increased (P < 0.05) thyroid gland and liver weights and decreased (P < 0.05) serum thyroxine (T4) concentrations compared to control birds. We detected increased TSH β mRNA in the pituitaries of MMI-treated birds compared to controls. There was no effect of MMI treatment on the reproductive variables measured, including LH β mRNA levels, serum androgen and estradiol concentrations, gonad weight, or cloacal gland area. Therefore, it appears that thyroid axis inhibition and the consequent increase in TSH β mRNA did not have direct effects on reproductive axis function in male Japanese quail.

(Key words: Japanese quail, thyroid, methimazole, reproduction, thyroid-stimulating hormone β)

INTRODUCTION Thyroid-stimulating hormone (TSH) is a glycoprotein composed of an α- and β-subunits and is produced by thyrotrophs in the anterior pituitary gland. The α-subunit of TSH is common to the other pituitary glycoproteins, luteinizing hormone and follicle-stimulating hormone. The β-subunit is unique to TSH and confers its biological specificity. The production and release of TSH is stimulated by thyroid-releasing hormone (TRH) and inhibited through a negative feedback loop by thyroid hormones, thyroxine (T4) and triiodothyronine (T3), (McCann and Ojeda, 1996).

It has been demonstrated previously that goitrogens and exogenous thyroid hormones will affect the thyroid axis in avian species (Almeida and Thomas, 1980; Iqbal et al., 1987; Siopes, 1997; Wilson and McNabb, 1997). Methimazole (MMI) administration causes significant decreases in plasma T4 and T3 in chick embryos and growing chicks (Iqbal et al., 1987). Thyroid gland hypertrophy is evident in quail after MMI treatment, indicating increased stimulation by TSH (Wilson and McNabb, 1997). Gregory et al. (1998) showed that embryonic chick TSH β mRNA levels are affected by exogenous T3.

Study of the thyroid function in birds has been limited in some aspects because of the lack of tools for measuring avian TSH. The cloning of the TSH β gene has been accomplished in species of several vertebrate classes, including mammals (Gurr et al., 1983; Croyle and Maurer, 1984; Maurer et al., 1984), amphibians (Buckbinder and Brown, 1993), fish (Ito et al., 1993; Salmon et al., 1993), and birds (Gregory and Porter, 1997; Kato et al., 1997a,b). However, direct stimulation of TSH by TRH and negative feedback effects of thyroid hormones on TSH have been difficult to study in avian species in vivo because a TSH assay has not yet been developed. Localization of thyrotrophs producing TSH in the avian pituitary has been shown using heterologous TSH antibodies (drakes, Sharp et al.,

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Abbreviation Key: LH = luteinizing hormone; MMI = methimazole; TRH = thyroid-releasing hormone; TSH = thyroid-stimulating hormone; T4 = thyroxine; T3 = triiodothyronine.
The thyroid axis is important for the development and maturation of the reproductive axis, as well as regulation of the seasonality of the reproductive axis in some species. There is evidence of interactions at all levels of these axes—the hypothalamus, pituitary, and target glands—although the details of these interactions are not well understood (for review, see Jannini et al., 1995). Kirby et al. (1996) have shown that transient hypothyroidism during reproductive development in the male chick results in permanent alteration of adult reproductive characteristics. Other studies in birds support the hypothesis that the thyroid axis plays a permissive role in reproduction in seasonally breeding species (Follett and Nicholls, 1985; Wilson and Reinert, 1996; Bentley et al., 1997).

The objective of experiment 1 was to clone a partial cDNA for Japanese quail TSHβ and to use it as a template for transcribing a riboprobe. This riboprobe was then used for Northern blot analyses to localize and identify changes in steady-state TSHβ mRNA levels. The riboprobe was validated by determining tissue specificity and sensitivity of detection. The objective of experiment 2 was to determine if treatment with the thyroid inhibitor, MMI, would change steady-state levels of TSHβ mRNA. In addition, we also assayed plasma androgen levels and LHβ mRNA to determine if a change in the thyroid axis of reproductively active males would affect the reproductive axis.

**MATERIALS AND METHODS**

**Cloning of a Partial cDNA for Quail TSHβ**

Japanese quail (Coturnix coturnix japonica, University of Maryland quail colony) were housed in a controlled environment under 15 L: 9 D with food (Purina Game Bird Layena) and water available ad libitum. Quail pituitaries (n = 6) were collected from three males and three females that ranged in age from 3 to 72 wk of age. Pituitaries were snap frozen, and a sample of liver was collected for a negative control. Total RNA was extracted from pituitaries using the acid guanidine-phenol-chloroform method (Chomczynski and Sacchi, 1987) or TriReagent. Total RNA concentration was determined by optical density reading at 260 nm and electrophoresis in a 1% formaldehyde agarose gel. RNA was reverse transcribed using Superscript II reverse transcriptase and two different primers. An oligo (dT)12 primer was used to create a cDNA library. A degenerate primer β3 designed for isolation of cDNA for chicken TSHβ mRNA by Gregory and Porter (1997) was used to specifically reverse transcribe TSHβ mRNA. Total liver RNA served as a negative control. Three degenerate primers (β1, β2, and β3) designed for isolation of cTSHβ by Gregory and Porter (1997) were used to amplify portions of the TSHβ gene (Sambrook et al., 1989). The cDNA produced from the two RT reactions were combined with these primers and Taq DNA polymerase in two sequential reactions. Hot start PCR (30 cycles) with primers β1 and β3 resulted in amplification of a cDNA sequence of 280 bp. The subsequent PCR with a nested primer β2 for primer β3 produced a cDNA of 225 bp. The reverse transcribed product from liver RNA provided a negative control, and a partial cDNA sequence for chicken TSHβ was a positive control. PCR samples ligated into pGEM-T Easy vector were used to transform Max Efficiency DH5α bacteria. Plasmids isolated from three clones contained an insert of appropriate size (200 bp). These clones were individually sequenced in both directions by the DNA sequencing facility, UMCP using M13(-21) forward and M13 reverse sequencing primers.

**Validation of the TSHβ Riboprobe**

Isolated plasmid was linearized with BamHI, and the antisense riboprobe was transcribed using T7 polymerase and DIG RNA labeling mix. The sense riboprobe was transcribed by SP6 polymerase after linearizing the plasmid with NcoI. Three Northern blot analyses were run for riboprobe validation to determine tissue specificity and detection sensitivity. Total brain, heart, liver, muscle, ovary, pituitary, and testis RNA was extracted (TriReagent) from adult male Japanese quail.

Total RNA (5 μg) was electrophoresed, transferred to a nitran membrane following electrophoresis, cross-linked, hybridized with antisense riboprobe (100 ng/mL) overnight at 42°C, and then subjected to stringent washes (two washes at 0.1 × SSC/0.1% SDS; 15 min each at 66°C). DIG with anti-DIG-alkaline phosphatase conjugate and CDP-Star was used instead of a radioactive label.

For the third Northern, pituitary RNA was isolated from 11 adult male quail. Dilutions of RNA (1, 2.5, 5, 7.5, 10, 12.5, and 15 μg) were electrophoresed, transferred to a nitran membrane, hybridized with antisense riboprobe, and visualized as described above.

**MMI Treatment**

Adult reproductive male Japanese quail were housed individually beginning 1 wk before and throughout the experiment. They were provided feed (Purina Game Bird Layena7) and water ad libitum. The light cycle was 15 L:9 D, but the time of lights on was 1 h later from their...
previous light cycle. Birds were randomly assigned to the treatment groups: control (200 µL of 0.9% saline once a day) and MMI (10 mg of MMI in 200 µL of saline) n = 8 per group. Saline and MMI solutions were made in one large batch, divided into weekly aliquots, and stored at −20°C. Treatments were administered by oral intubation with soft tubing attached to a 1-mL tuberculin syringe, once a day at 1230 h. Birds were treated for 4 wk. Blood samples and body weights were taken weekly. Cloacal gland length and width were recorded at the beginning and end of the treatment period. Cloacal gland area was estimated by multiplying cloacal gland length by cloacal gland width.

Observations of molting were also noted. Three control birds and two MMI birds molted during the treatment period. Molting was most likely due to the 1-h shift in the onset of daylight or the transfer of the birds to a new room, rather than a result of treatment. Thyroid gland, heart, liver, and gonad weights were recorded at the end of the experiment; pituitaries were collected and snap frozen.

**Northern Blot Analysis for TSHβ and LHβ mRNA**

Northern blots were used to assess differences in steady-state TSHβ and LHβ mRNA levels in the pituitary due to treatments. We used the turkey LHβ riboprobe, isolated and developed by Doug Foster’s lab (University of Minnesota; You et al., 1995), and previously validated for use in quail (Ottinger and Foster, unpublished data). From our work in experiment 1, we found that the DIG-labeled riboprobes proved difficult to quantify. Therefore, the TSHβ riboprobe and the LHβ riboprobe were radiolabeled with 32P-CTP. Pituitaries were pooled (two per pool) for a total of n = 4 pooled samples for each treatment.

Samples were extracted, and 2 µg of total RNA for each sample was subjected to electrophoresis, transferred to a nylon membrane, and hybridized with the LHβ riboprobe at 42°C overnight. After hybridization with the LHβ riboprobe, the membrane was washed twice at 50°C in 2× SSC with 0.1% SDS and four times at 58 and 65°C in 0.2× SSC with 0.1% SDS. Film was exposed to the membrane with intensifying screens at −80°C for 3 d. Riboprobe was stripped from the membrane by boiling in 0.2× SSC for 40 min, and hybridization was repeated with TSHβ riboprobe following the same methods used for LHβ riboprobe. Film was exposed to the membrane for 3 wk. TSHβ, LHβ, and 18S bands were quantified on an Opeco image analysis system. Variability in RNA loading, as demonstrated by 18S ribosomal RNA band intensity, was an artifact of protein contamination inherent in extracting small tissue samples (two pituitaries) with Tri-Reagent.

All samples were run within one assay for each hormone measured. Serum T4 and T3 were measured in double antibody RIA according to methods developed by McNabb and Hughes (1983). Primary antibodies* were produced in rabbits; 125I-T4 and 125I-T3 were purchased. The carrier immunoglobulin was a rabbit protein. John McMurtry (USDA, Beltsville, MD) kindly provided the secondary antibody, a sheep anti-rabbit antibody. Assay volumes for T4 and T3 were 12.5 µL and 25 µL, respectively. The sensitivity for the T4 RIA was 1.25 ng/mL and for the T3 RIA was 0.125 ng/mL. Precision for the T4 and T3 assays, as CV, were 3.1 and 2.6% for T4 and T3, respectively (McNichols and McNabb, 1988).

The steroid RIA were single antibody assays for serum androgen and estradiol. Serum was double-extracted using seven volumes of diethyl ether. The ether extract was then dried and reconstituted in a standard buffer diluent supplied with the assay. The antibody used in the androgen RIA had 100% cross-reactivity with testosterone and 44% with 5α-dihydrotestosterone. The antibody used in the estradiol RIA had 100% cross-reactivity with estradiol and 1.3% with estrone. Assay volumes for androgens and estradiol were 50 and 100 µL, respectively. The intraassay coefficient of variation for androgen was 4.23%. For estradiol, the intraassay coefficient of variation was 1.29%. The sensitivity for both steroid RIA was 0.01 ng/mL.

**Statistics**

Statistical analyses were conducted for all variables by one-way analysis of variance using the PROC MIXED procedure of SAS software. Variances were partitioned to correct for heterogeneous variances when appropriate. The best-fit model was determined by Schwartz’s Bayesian criterion value. Means were compared by Fisher’s protected least significant difference. Weekly measurements of serum T4 concentrations and BW were analyzed by repeated measures using PROC MIXED procedure of SAS software.

**RESULTS**

In experiment 1, we were successful in isolating and characterizing a partial cDNA of quail TSHβ (Figure 1). The three isolated clones had identical sequences. The quail cDNA sequence between the primers (168 bp) was 94% identical to the corresponding cDNA sequence in chicken, with a difference of 10 nucleotides. This portion of quail TSHβ is only 50% identical to quail LHβ. The derived amino acid sequence for this portion of quail TSHβ was compared to the corresponding sequence in other species (Figure 2). Within this partial amino acid sequence, there is 98% identity of quail to chicken, 68% to bovine, 57% to frog, and 68% to rat.

The transcribed riboprobe was validated for use in quail by determining tissue specificity and sensitivity by Northern blot analysis. Tissue specificity was indicated

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FIGURE 1. Comparison of 168-nucleotide partial quail cDNA (Quail; GenBank Accession #AF541922) sequence with previously isolated quail cDNA (Quail2; Kato et al., 1997a,b) and chicken cDNA sequences for thyroid-stimulating hormone β (Gregory and Porter, 1997). There is a difference of two nucleotides between the two quail sequences. The quail and chicken sequences share 94% identity with 10 nucleotide substitutions.

FIGURE 2. Comparison of partial thyroid-stimulating hormone β amino acid sequences of quail, chicken (Gregory and Porter, 1997), bovine (Maurer et al., 1984), frog (Buckbinder and Brown, 1993), and rat (Croyle and Maurer, 1984). Within this sequence there is 98% identity of quail to chicken, 68% to bovine, 57% to frog and 68% to rat.

by antisense probe binding, or lack thereof, to various tissues, including quail brain, heart, liver, muscle, ovary, pituitary, and testes (Figure 3). When probed with the quail TSHβ riboprobe, signal for TSHβ mRNA was only detected in the pituitary gland RNA. The signal detected in other tissues was nonspecific binding of the TSHβ riboprobe to ribosomal RNA. Binding of the riboprobe to only ribosomal RNA also occurred in the brain and muscle tissue (not included in Figure 3). The sense probe showed no evidence of specific binding (data not shown). In the total pituitary RNA titration Northern blot analysis, riboprobe sensitivity was indicated by the differences that were detected in TSHβ mRNA concentrations when as little as 2.5 µg of total RNA was loaded using the DIG-labeled riboprobe (data not shown).

The results of experiment 2 suggest that MMI suppressed thyroid gland function (decreased plasma T4 by the final week of the study; P < 0.05) resulting in a negative feedback effect that increased pituitary TSH release (thyroid gland hypertrophy; P < 0.05), see Table 1. The hyperzeugmatophagy of the thyroid gland in the MMI-treated birds suggested an increase in TSH, which was confirmed by the TSHβ mRNA measurements. The results from the Northern blot for TSHβ provided evidence that MMI treatment increased TSHβ mRNA (Figure 4). Note the increased band darkness in the Northern blot for MMI treatment for TSHβ mRNA compared to the control group. Ethidium bromide staining of 18S RNA indicated that roughly equivalent amounts of RNA were loaded for both groups. T3 concentrations (Table 2) and body weights were not affected by MMI treatment.

Alteration of the thyroid system did not affect the reproductive variables we measured (Tables 1 and 2). Measurements of the reproductive tract (gonad weight and cloacal gland area) were not significantly different between the two groups (Table 1). There were no consistent effects of MMI treatment on LHβ mRNA levels (figure not shown) or on gonadal androgens or estradiol (Table 2).

FIGURE 3. Tissue specificity Northern blot hybridized with thyroid-stimulating hormone β(TSHβ) antisense riboprobe. The TSHβ riboprobe specifically bound to pituitary TSHβ mRNA at approximately 600 bases. The small arrow indicates nonspecific binding of the riboprobe to ribosomal RNA. The large arrow indicates specific binding of the riboprobe to TSHβ mRNA.

TABLE 1. Means ± SEM (n = 8) for each of the selected variables after 4 wk of methimazole treatment

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Methimazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gonad (g)</td>
<td>3.03 ± 0.34</td>
<td>3.06 ± 0.34</td>
</tr>
<tr>
<td>Thyroid (mg)</td>
<td>10.3 ± 1.1</td>
<td>58.4 ± 14.7*</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>1.76 ± 0.10</td>
<td>2.19 ± 0.10*</td>
</tr>
<tr>
<td>Heart (g)</td>
<td>1.28 ± 0.07</td>
<td>1.25 ± 0.07</td>
</tr>
<tr>
<td>Cloacal area (mm²)</td>
<td>156 ± 16</td>
<td>150 ± 16</td>
</tr>
</tbody>
</table>

*Significant differences (P < 0.05) are denoted within each variable.

DISCUSSION

A quail TSHβ riboprobe was developed and validated for measuring changes in quail TSHβ mRNA in different
physiological states. We found a difference of only one amino acid between quail and chicken within the isolated partial cDNA. The one different amino acid appeared to be a highly variable site in all TSHβ sequences presented (Figure 2). Additionally, the amino acids at this site for quail (leucine) and chicken (phenylalanine) TSHβ have nonpolar side chains. Therefore, it is unlikely that this amino acid substitution resulted in a functional change. Based on this limited analysis, quail TSHβ appeared to be most related to that of the chicken and least related to that of the frog.

After initiation of this project, another group (Kato et al., 1997a) reported the isolation of a quail TSHβ cDNA (Figure 1). Our isolated partial cDNA sequence differs from this previously isolated cDNA sequence for quail TSHβ by two nucleotides. However, both sequences yielded the same amino acid sequence (Figure 2). The difference in nucleotide sequence may be due to different isolation methods. Because of the small amount of difference between the two sequences and the identical derived amino acid sequence, it was difficult to determine which was the true cDNA sequence. It should be noted that our sequence was from three isolated clones, and the Kato et al. (1997a) sequence is from one isolated clone.

The transcribed riboprobe was validated for use in the quail by determining tissue specificity and sensitivity. The riboprobe was found to be pituitary specific. Differences in TSHβ mRNA concentrations can be detected when as little as 2.5 µg of total RNA is loaded using the DIG-labeled riboprobe. For tissue specificity and RNA titration Northern blot, the signal was observed at approximately 600 bases, which is similar to observations with the chicken TSHβ riboprobe (Gregory and Porter, 1997). Radiolabeled riboprobes were used in the MMI experiment because their greater sensitivity was useful to analyze TSHβ and LHβ mRNA levels in the smaller amounts of total RNA isolated in that experiment.

In experiment 2, we looked at the effects of a thyroid inhibitor on steady-state levels of TSHβ mRNA and tested if a change in the thyroid axis would affect the reproductive axis of adult male quail. The results of experiment 2 indicated that MMI affected the thyroid axis by inhibiting T4 production and resulted in decreased circulating hormone. This decrease would decrease the negative feedback effects of the thyroid hormones on the pituitary gland. Consequently, an increase in TSH would explain the increase in thyroid gland weight. Consistent with this interpretation, we found greater levels of TSHβ mRNA using the TSHβ riboprobe developed in this study. The lack of consistent change in LHβ in response to MMI and in contrast to the increase in TSHβ mRNA supported the specificity of the TSHβ riboprobe. Moreover, crossreaction with the LHβ mRNA was unlikely because of low homology (50% identical) and because TSHβ and LHβ riboprobes showed differing results. These results are consistent with the findings of Iqbal et al. (1987), which also showed that plasma thyroid hormone concentrations decreased with MMI treatment. Therefore, our results extend the information from this earlier study in that MMI treatment resulted in decreased plasma thyroid hormone levels.

It has been shown in mammals that a decrease in T4 with normal T3 concentrations results in an increase in TSH (Larsen et al., 1981). Moreover, local 5′-deiodination of T4 to T3 provides additional ligand for receptors within the pituitary. Studies in chickens indicate that avian deiodination is very similar to that of mammals (Rudas and Bartha, 1993; Rudas et al., 1993, 1994). The concurrent
increase in TSHβ mRNA levels and decrease in T4 in the absence of a significant change in T3 concentrations in the MMI-treated birds in this study supported the hypothesis that the feedback signals and responses in birds are similar to those observed in mammals (Haynes, 1990).

Alteration of the thyroid system did not affect the reproductive variables we measured. Because gonadal weight (Ottinger and Bakst, 1981) and cloacal gland area (Siopes and Wilson, 1975) are dependent on circulating levels of testosterone, the absence of thyroid system effects parallels no effect on plasma androgen levels. Studies by Follett and Nicholls (1985) have shown that the timing of thyroid system change may be a key factor in the ability to show seasonal regression of the reproductive system. Further studies report that thyroidectomy, during the long days of reproductive activity, does not affect reproductive activity (Follett and Nicholls, 1985).

It is possible that the impact of the thyroid system on the reproductive axis requires sustained exposure to thyroid system alterations. A decrease in T4 was observed that reproductive changes might have been observed if there was a time delay in the response to the treatment. Further, it could be predicted that subsequent impact on the reproductive axis would require additional time. Therefore, the consequences of thyroid system alterations on the reproductive axis would be delayed. As such, it is possible that reproductive changes might have been observed if the experiment was over a longer period of time.

We were successful in developing and validating a riboprobe for Japanese quail TSHβ. We were also able to alter the thyroid axis with MMI and detect these changes in steady-state levels of TSHβ mRNA with this riboprobe. The changes in the thyroid axis did not result in detectable changes in the reproductive variables we measured.

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