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Thyroid-Stimulating Hormone Regulation and Transcription in Hypothyroidism

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1. Introduction

Thyroid hormones play essential roles in mammalian life, especially in neurodevelopment (Porterfield & Hendrich, 1993). This fact is clearly shown in patients with neurological deficits from endemic cretinism, who reside in iodine-deficient areas (DeLong et al., 1985). In addition to neurological impairments, thyroid dysfunctions, such as hyperthyroidism and hypothyroidism, lead to a wide variety of clinical manifestations. Hypothyroidism is defined as deficient thyroid hormone action. It is caused most often by decreased thyroid hormone production, although in rare cases it is caused by reduced tissue responsiveness to or consumptive degradation of the hormone (Huang et al., 2000; Refetoff, Weiss, & Usala, 1993). There are two types of deficient thyroid hormone production: primary (thyroidal) hypothyroidism and central hypothyroidism. The former is commonly caused by iodine deficiency (DeLong et al., 1985) or chronic autoimmune thyroiditis, known as Hashimoto's thyroiditis (Dayan & Daniels, 1996). In iodine-sufficient areas, Hashimoto's thyroiditis is a major cause of primary hypothyroidism. The loss of functional follicles caused by intrathyroidal lymphocytic infiltration is attributable to impaired thyroid hormone production. Central hypothyroidism is due to reduced thyroid stimulation by thyroid-stimulating hormone (TSH) resulting from pituitary disease (secondary hypothyroidism) or hypothalamic disease (tertiary hypothyroidism) (Lania et al., 2008). Pituitary macroadenomas or radiotherapy of brain tumours and pituitary adenomas are frequently associated with insufficient TSH production in adults (Rose, 2001). In some cases of central hypothyroidism, abnormally glycosylated TSH with a reduced bioactivity is secreted (Faglia et al., 1979; Taylor & Weintraub, 1989). Because thyroid hormones negatively regulate pituitary TSH synthesis, decreased serum thyroid hormone concentrations lead to the stimulation of TSH production. Therefore, in primary hypothyroidism, serum TSH levels are increased, even at the stage of subclinical hypothyroidism (Fatourehchi, 2009). By contrast, an increase in serum TSH is generally not observed in central hypothyroidism because of the impaired hypothalamic-pituitary-thyroid axis. Based on these observations, serum TSH serves as a useful indicator for the presence and the type of hypothyroidism. Therefore, measurements of TSH are quite useful in clinical practice (Ladenson et al., 2000).

2. Regulation of thyroid-stimulating hormone synthesis

TSH is produced in pituitary thyrotrophs and activates thyroid follicular cells by binding to the TSH receptor (Magner, 1990). This hormone promotes thyroid cell proliferation and thyroid hormone synthesis by inducing expression of thyroglobulin, thyroid peroxidase, sodium iodide symporter and type I iodothyronine deiodinase (D1) (Tang et al., 1995). TSH consists of an α and a β subunit (Shupnik et al., 1989). The α subunit is common to both TSH and gonadotropins. The β subunit is a prerequisite for the bioactivity of TSH. Both subunits are glycosylated posttranslationally, which is controlled by the thyrotropin-releasing hormone (TRH) and is essential for exerting sufficient hormonal bioactivity (Menezes-Ferreira et al., 1986; Taylor & Weintraub, 1989). In fact, abnormally glycosylated TSH with reduced bioactivity is found in some patients with central hypothyroidism (Faglia et al., 1979; Petersen et al., 1978).

TSH synthesis is largely dependent on serum thyroid hormone levels. Patients with primary hyperthyroidism or primary hypothyroidism consistently demonstrate suppressed or increased serum TSH levels, respectively (Ladenson et al., 2000). Clinically, serum TSH concentrations serve as a sensitive indicator of thyroid dysfunction since patients with abnormal thyroid function have altered serum TSH levels, even at a subclinical stage (Fatourech, 2009). Thus, the measurement of serum TSH is routine for a thyroid evaluation in daily clinical practice. In addition to TSH production, the metabolic clearance rate of TSH is also influenced by serum thyroid hormone levels, i.e., increased in hyperthyroidism and decreased in hypothyroidism (Ridgway et al., 1974).

In contrast to the negative regulation of TSH production by thyroid hormones, the production is positively regulated by TRH. Mice devoid of the TRH gene exhibit hypothyroidism accompanied by low circulating TSH levels and reduced numbers of TSH-immunopositive cells in their pituitary glands (Yamada et al., 1997). In TRH and thyroid hormone receptor (TR) β -subunit double knockout mice, basal serum TSH levels are low, and hypothyroidism fails to increase serum TSH concentrations (Nikrodhanond et al., 2006). These studies have demonstrated the pivotal role of TRH in the regulation of TSH production.

Recent studies have demonstrated the presence of TSH receptors in the hypothalamus and pituitary folliculo-stellate cells (Crisanti et al., 2001; Prummel et al., 2000), suggesting short and ultra-short loop feedback regulation of TSH secretion (Prummel et al., 2004). However, their significance remains unknown.

3. Thyrotropin-releasing hormone as a positive regulator of thyroid-stimulating hormone production

TRH is synthesised in neurons located in the parvocellular part of the hypothalamic paraventricular nucleus (PVN) (Fekete & Lechan, 2007). These neurons project their axons to the median eminence where TRH is released into the portal vein, and the hormone subsequently reaches the anterior pituitary. The binding of TRH to its receptor activates phospholipase C and is followed by calcium mobilisation and protein kinase C (PKC) activation (Carr et al., 1991). However, recent studies suggest that cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) rather than the calcium

and PKC signalling pathways may play a central role in TRH-stimulated TSH synthesis (Hashimoto et al., 2000). In addition, the transcription factor Pit-1, which is pivotal in the differentiation of thyrotrophs, lactotrophs and somatotrophs (Andersen & Rosenfeld, 1994), induces a synergistic increase in TSH β subunit gene transcription in the presence of CREB-binding protein (Hashimoto et al., 2000).

TRH synthesis is negatively regulated by thyroid hormones. In the brain, up to 80% of the triiodothyronine (T3) bound to nuclear TRs is locally generated through the conversion of thyroxine (T4) to T3 by the type 2 deiodinase (D2) (Crantz et al., 1982). Therefore, rather than T3, the amount of T4 in circulation is pivotal for the maintenance of adequate T3 levels in the brain. However, D2 is expressed in the tanycytes lining the third ventricle and in astrocytes, but not in neurons (Guadano-Ferraz et al., 1997; Tu et al., 1997). The T3 produced by tanycytes is the primary source of T3 in TRH neurons. In these neurons, the cellular uptake of T3 is mediated by monocarboxylate transporter 8 (MCT8) (Heuer & Visser, 2009). T3 binds to the TRs, of which there are three isoforms (TR α 1, β 1 and β 2) in the brain (Cheng, 2005). Notably, TR β 2 is central in the suppression of TRH production since mice devoid of the TR β 2 gene exhibit increased TRH gene expression in the PVN (Abel et al., 2001). There are consensus sequences for thyroid hormone response element (TRE) half-sites (AGGTCA) in the TRH gene, which may be involved in the negative regulation of TRH gene expression by T3 (Wilber & Xu, 1998). The promotor region of the gene also contains a cAMP response element (CRE) that overlaps with a TRE (Wilber & Xu, 1998), which suggests that there is cross talk between the thyroid hormone and cAMP-dependent signalling pathways.

4. Triiodothyronine is a negative regulator of thyroid-stimulating hormone production

Transcription of the TSH α and β subunit genes is negatively regulated by T3 (Magner, 1990; Shupnik et al., 1989). Mice lacking the TR β gene have been shown to exhibit inappropriate secretion of TSH (Abel et al., 1999; Weiss et al., 1997). By contrast, TSH β subunit expression was not altered in mice devoid of the TR α gene (Wikstrom et al., 1998). Thus, a series of studies suggest a pivotal role for the TR β isoforms, especially TR β 2, in T3-mediated TSH suppression. There is a TRE half-site-like sequence (GGGTCA) in the β subunit gene, and previous studies have suggested that it might act as a negative TRE in thyrotrophs (Carr et al., 1989). However, later studies demonstrated that this putative negative TRE was not required in TSH suppression (Matsushita et al., 2007). Instead, the transcription factor GATA2 interacts with Pit-1 and TR to play an essential role in both the T3-induced suppression and the TRH-induced potentiation of TSH expression (Matsushita et al., 2007; Nakano et al., 2004).

Approximately 50 to 60 % of T3 bound to TRs is locally produced by D2-mediated T4 to T3 conversion in the rat pituitary gland (Silva & Larsen, 1978). Human pituitary tissues also contain D2 (Itagaki et al., 1990). D2 inhibition by iopanoic acid results in an increase in rat serum TSH levels (Obregon et al., 1980), which indicates that D2-mediated T4 deiodination is the primary source of T3 in the pituitary. D2 activity is increased in hypothyroidism and is decreased in hyperthyroidism (Bianco et al., 2002). Thus, the intrapituitary T3 levels are carefully maintained by the fine-tuning of D2 activity.

5. Neuronal control of thyroid-stimulating hormone secretion (Fig. 1)

TSH secretion is indirectly controlled by neuronal afferents innervating hypothalamic TRH neurons. Adrenergic input from the C1-3 brainstem stimulates TRH synthesis in response to cold exposure (Arancibia et al., 1996; Arancibia et al., 1989). Catecholamine binding to $\alpha 1$ adrenergic receptors leads to CREB phosphorylation and subsequent activation of the TRH promoter (Thonberg et al., 2002). Adrenergic neurons that are in contact with the TRH neurons also contain cocaine- and amphetamine-regulated transcript (CART) and neuropeptide Y (NPY) (Wittmann et al., 2002, 2004). Previous studies have demonstrated that CART stimulates TRH synthesis, whereas NPY inhibits it (Fekete et al., 2000, 2001).

III, the third ventricle; AGRP, agouti-related protein; ARC, hypothalamic arcuate nucleus; C1-3, C1-3 adrenergic area of the brainstem; CART, cocaine- and amphetamine-regulated transcript; D2, type 2 iodothyronine deiodinase; DMN, hypothalamic dorsomedial nucleus; α -MSH, α -melanocyte stimulating hormone; ME, median eminence; NPY, neuropeptide Y; PVN, hypothalamic paraventricular nucleus; TRH, thyrotropin-releasing hormone; TSH, thyroid stimulating hormone.

Fig. 1. Regulatory mechanism of the hypothalamic-pituitary-thyroid axis.

Peptidergic input from the arcuate nucleus may mediate a fasting-induced decrease in TRH production (Lechan & Fekete, 2006). Leptin administration during fasting prevents the inhibition of TRH synthesis, suggesting its involvement in this process (Legradi et al., 1997). The arcuate nucleus also sends axon terminals containing NPY and agouti-related protein (AGRP) or α -melanocyte stimulating hormone (α -MSH) and CART to the TRH neurons and thus negatively or positively regulates TRH gene expression (Elias et al., 1998; Hahn et al.,

1998; Mizuno et al., 1998). Also, α -MSH-containing neurons innervate the hypothalamic dorsomedial nucleus (DMN), and the DMN subsequently projects to the TRH neurons in the PVN (Mihaly et al., 2001). Thus, there are two pathways to the TRH neurons: the direct arcuate-PVN and the indirect arcuate-DMN-PVN.

6. Drugs affecting thyroid-stimulating hormone secretion

Glucocorticoids can lower serum TSH concentrations through TRH suppression in the PVN (Alkemade et al., 2005; Wilber & Utiger, 1969). TRH neurons possess glucocorticoid receptors, and a response element to the hormone has been identified in the TRH gene (Cintra et al., 1990).

Dopamine can reduce TSH production and secretion through its binding to dopamine D2 receptors in the pituitary gland (Shupnik et al., 1986). Interestingly, it stimulates TRH secretion in the rat (Lewis et al., 1987), but this effect cannot override its inhibitory effect on the pituitary gland.

Somatostatin suppresses TSH secretion from the pituitary gland (Lamberts et al., 1989), and its analogues are therefore used for the treatment of TSH-producing adenomas (Beck-Peccoz et al., 1989).

In addition to drugs, several cytokines, such as interleukin-1 β (IL-1 β), IL-6 and tumour necrosis factor- α (TNF α), have been shown to inhibit TSH secretion (Bartalena et al., 1994; Pang et al., 1989). These cytokines can stimulate the D2 activity in pituitary cells (Baur et al., 2000), suggesting increased T4 to T3 conversion as one possible mechanism for the suppressed TSH production in cytokine-treated animals.

7. Quantitative analysis of thyroid-stimulating hormone transcription in hypothyroidism (Sugimoto et al., 2007)

TSH (thyrotropin) is the primary regulatory peptide for the synthesis and secretion of thyroid hormones, including T3 and T4. TRH secretion from the hypothalamus stimulates the release of TSH from the anterior lobe of the pituitary gland. TSH is then secreted into the blood to stimulate the release of T4, which is produced by the thyroid gland, and T3, which is produced by both the thyroid gland and by conversion of T4 in peripheral tissues. T3 has stronger biological effects than T4. This TRH-TSH-thyroid hormone (T3, T4) secretion relationship is called the hypothalamic-pituitary-thyroid axis (HPT axis), which operates on both short- and long-feedback mechanisms (O'Shea & Williams, 2002). The plasma levels of T3 and T4 are maintained by this mechanism.

Thyroid hormones are essential for maintaining many physiological functions, including metabolism, growth, and development. In hypothyroidism, TSH and TRH levels are elevated, owing to the lack of a suppressive action of the T3. Hypothyroidism in rats, induced by propylthiouracil (PTU) administration, is associated with high TSH mRNA expression, which is measured semiquantitatively by northern blotting or *in situ* hybridisation; however, these results varied widely from 3 to 22 times the level seen in control rats (Carr & Chin, 1988; Franklyn et al., 1987; Samuels et al., 1989; Shupnik & Ridgway, 1987; Steel et al., 1990; Taylor et al., 1990).

The LightCycler® system (Roche Applied Science, Switzerland) was developed for the quantitative analysis of gene expression by real-time polymerase chain reaction (PCR). It combines a thermocycler and a microvolume fluorimeter (Lyon, 2001) with the fluorescence-based assay requiring less manipulation than a basic PCR assay (Contini et al., 2005). Therefore, the LightCycler® is a highly sensitive quantitative method for the detection of RNA expression (Emrich et al., 2002; Schuster et al., 2004; Tan et al., 2004).

Posttranscriptional control of mRNA steady-state levels can occur at many steps after the synthesis of the initial heterogeneous nuclear RNA (hnRNA) transcript. hnRNA is therefore the primary transcript produced by RNA polymerase, which includes both the exonic and the intronic regions of the DNA. Transcriptional control of the mRNA occurs at the levels of hnRNA stability, splicing, polyadenylation, capping, methylation, editing, the nuclear-cytoplasmic transport of mature mRNA, and mRNA stability. Mature mRNA synthesised in the nucleus translocates into the cytoplasm, where it is stabilised, translated into protein, or degraded (Kren & Steer, 1996). The half-life of mRNA is comparatively longer than that of hnRNA, and changes in mRNA levels in the cell do not necessarily reflect the transcriptional level. It takes at least 0.5 h, and sometimes up to 2 h, for mRNA to accumulate to detectable levels after the start of transcription for most genes (Kren & Steer, 1996). A more reliable measure for evaluating the rate of transcription is hnRNA because of its short half-life of 15–30 min (Darnell, 1983), which makes it a valuable quantitative indicator of transcriptional activation.

In 2002, Johnson et al. (Johnson et al., 2002) reported a quantitative real-time reverse transcription (RT)-PCR analysis of prostaglandin endoperoxide H synthase hnRNA. Since then, several studies have successfully detected hnRNA expression using real-time PCR systems (Danzi et al., 2005; Ginsberg et al., 2006; Johnson et al., 2003; Kuroda et al., 2005; Li et al., 2005). However, only semiquantitative analyses of hnRNA have been reported for TSH β gene transcription (Franklyn et al., 1987; Samuels et al., 1989; Shupnik & Ridgway, 1987; Taylor et al., 1990). In this study, we performed a quantitative analysis of TSH β gene expression by real-time RT-PCR using the LightCycler® system. We present here the first quantitative demonstration of increased mRNA and hnRNA expression of TSH β under a chronic condition of hypothyroidism in rats. This method for the detection of quantitative hnRNA is illustrated in Fig. 2.

7.1 Materials and methods

7.1.1 Animals and the induction of hypothyroidism

Adult male Wistar rats (Nippon Clea Inc., Shizuoka, Japan) weighing 280 g were allowed free access to food and water and were maintained on a 12 h light/12 h dark cycle (lights on 07:00–19:00 h). The rats were divided into two groups of four animals each: a hypothyroidism group and a control group. The rats were allowed free access to either 0.05% methimazole (MMI) in water (hypothyroid group) or water alone (controls). Fourteen days later, all rats were decapitated between 08:00 and 10:00 h, and blood was collected from their trunks to avoid contamination with the pituitary portal blood, which contains high TSH levels. The sample was collected into a tube containing ice-cold ethylenediaminetetraacetic acid (EDTA) and centrifuged at 1008 $\times$ g for 30 min. Serum T3 and T4 levels were measured to assess the degree of hypothyroidism induced by MMI administration.

All animal experiments were conducted in accordance with the international standards for animal welfare from the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Animal Experiments Guidelines of the Institute for Animal Experimentation, Tohoku University Graduate School of Medicine.

Real-time PCR was performed using the cDNA as a template, which was reverse transcribed from total RNA. The intron-specific primer pair was used to amplify hnRNA for primary transcript quantification. Figure modified from Strachan, T & Read, AP. (2004). DNA structure and gene expression, In: *Human Molecular Genetics 3rd*, pp15, Garland Science, ISBN 0-8153-4184-9, New York, USA.

Fig. 2. Our method for the detection of quantitative hnRNA.

7.1.2 Assay of serum T3 and T4 by EIA

Serum samples taken from the trunk blood were analysed for T3 and T4 levels by an Enzyme Immunoassay (EIA) kit (IMX Dynapac, Abbott Japan, Tokyo, Japan). The sensitivities of the assay were <15 ng/dl for T3 and 1.0 ug/dl for T4.

7.1.3 RNA isolation and reverse transcription for real-time PCR

Following decapitation, the rat brains were removed within 1 min. The pituitaries were dissected out, snap frozen in liquid nitrogen, homogenised and treated with a combination of Trizol and chloroform (Invitrogen, San Diego, CA) to extract total RNA (Guevremont et al., 2006). The RNA from the rat pituitary was purified using DNase I to remove genomic DNA, and the RNA concentration was determined by absorbance readings at 260 nm on a UV spectrophotometre (Bio-Rad, Hercules, CA). A total of 2 µg of RNA was reverse transcribed using the Omniscript Reverse Transcription (RT) kit (Qiagen, Hilden, Germany) with random hexamer primers. Refined cDNA was synthesised in a total volume of 20 ul, treated with RNase H and stored at -20°C until use.

The integrity of the RNA used for the cDNA preparations was tested by PCR amplification (using a thermal cycler) of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene (primer sequences are listed in **Table 1**). After electrophoresis on 2% agarose gels, staining with ethidium bromide and visualisation by a UV light, the sizes of the PCR products were verified by comparing them against molecular weight markers.

	Forward primer 5' – 3'	Reverse primer 5' – 3'	Product size
TSHβ mRNA	ggcaaactgtttcttcccaa	gttggttttgacagcctcgt	210 bp
TSHβ hnRNA	gaccagtgatccagtcggtt	cgggctgtagaaaccaggta	447 bp
GAPDH mRNA	tgaacgggaagctcactgg	tccaccacctgttgctgta	307 bp

Primers were designed using the computer programme Primer 3 Software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). All the produced sequences were checked for homology by the NCBI database BLASTn.
Table 1. Primer sequences for quantitative real-time RT-PCR.

7.1.4 Primer design and quantitative analysis by real-time PCR

TSHβ primers were designed to amplify two specific regions in the TSHβ RNA. To amplify mRNA, the exon-specific primer pair, which was designed to target sequences from exon 3, was used (**Table 1**). The intron-specific primer pair, which was designed to target sequences found in intron 1, was used to amplify hnRNA (**Table 1**). The amplified mRNA and hnRNA products were 210 and 447 bp, respectively. All RT-PCR assays were normalised against rat GAPDH using commercial PCR primers (Nihon Gene Research Laboratories) that amplify a 307 bp product (**Table 1**).

Quantification of the TSH β mRNA and hnRNA was performed by real-time PCR using the LightCycler $^{\circledR}$ system (Roche Diagnostics, Japan) with SYBR green detection of amplification products. PCR for all genes was performed in a final volume of 20 μ l using the LC FastStart DNA Master SYBR Green I (Roche Molecular Biochemicals) with 2 μ l of each primer at 0.5 M, 3 mM MgCl $_2$ and 2 μ l of extracted cDNA, but without dimethyl sulfoxide (DMSO).

Table 2 shows the LightCycler $^{\circledR}$ PCR amplification programmes. A single fluorescence reading for each sample was taken at the annealing step. Quantitative results were determined from the crossing point (CP), which marked the cycle when the fluorescence of a given sample significantly exceeded the baseline signal, and are expressed as a fractional cycle number. CP values plotted against known concentrations of TSH β mRNA and hnRNA were used to obtain a standard curve. The TSH β mRNA and hnRNA counts for

Gene		Programme for Real-time LightCycler			Melting curve		
		Denaturation	Annealing	Elongation			
TSH β mRNA (cycles 45)	Temperature ($^{\circ}$ C)	95	62	72	95	62	96
	Incubation time (sec)	10	10	20	0	15	0
	Temperature transition rate ($^{\circ}$ C/sec)	20	20	20	20	20	0.1
TSH β hnRNA (cycles 45)	Temperature ($^{\circ}$ C)	95	62	72	95	62	96
	Incubation time (sec)	10	10	20	0	15	0
	Temperature transition rate ($^{\circ}$ C/sec)	20	20	20	20	20	0.1
GAPDH mRNA (cycles 45)	Temperature ($^{\circ}$ C)	95	62	72	95	62	96
	Incubation time (sec)	10	10	20	0	15	0
	Temperature transition rate ($^{\circ}$ C/sec)	20	20	20	20	20	0.1

Table 2. LightCycler PCR amplification conditions.

a given rat sample were calculated by interpolation from this standard curve (Software LightCycler® 3.5). The melting point of each amplified product was calculated to check the PCR specificity (Fukushima et al., 2003), and transcription of each cDNA was quantified (Fig. 3).

The horizontal and vertical axes show the PCR cycle number and the degree of fluorescence, respectively. In the hypothyroid rat group, the PCR amplification lines curve upward at lower PCR cycle numbers than those of the control group, indicating that the initial hnRNA content was higher in the hypothyroid rat group than in the control group.

Figure Inset: The unknown relative concentration of hnRNA can be calculated from the sample concentration (the horizontal axis; log X) and the cycle number (the vertical axis).

Fig. 3. Original graph from the LightCycler® 3.5 system for the quantification of TSHβ hnRNA.

7.1.5 Statistical analysis

All data are expressed as means ± S.D. T3 and T4 measurements and real-time PCR data were analysed for statistical significance using an unpaired *Student's t-test*. A *p*-value less than 0.01 was considered statistically significant difference.

7.2 Results

7.2.1 Serum T3 and T4 levels

The serum T3 and T4 levels for the hypothyroid rats and control rats are listed in **Table 3**. Oral administration of 0.05% MMI ad libitum was sufficient to induce hypothyroidism in rats. MMI administration resulted in a complete suppression of T3 levels.

	T3	T4
Hypothyroidism	n.d.*	1610 ± 1100*
Control	48.7 ± 2.1	6730 ± 210

Values are means ± SD. *p < 0.01 compared with the control group. Not detected (n.d.).

Table 3. Serum T3 and T4 levels (ng/dl).

7.2.2 Quantitative analysis of TSHβ mRNA and hnRNA by real-time PCR

TSH gene regulation at the transcriptional and posttranscriptional levels was evaluated by real-time RT-PCR to assess mRNA and hnRNA (mRNA precursor) expression. The total RNA extracts from pituitaries isolated from weight-matched rats were treated with DNase I to remove contaminating genomic DNA prior to RT-PCR. The TSHβ-specific primers were subsequently checked for their ability to amplify during PCR reactions in the thermal cycler using total RNA with or without DNase I treatment. Although a PCR product was created from the total RNA template in the absence of DNase I (**Fig. 4**), the presence of DNase I inhibited the reaction, and no product was produced (data not shown).

The TSHβ mRNA and hnRNA levels measured from real-time RT-PCR analysis were estimated relative to the values of GAPDH (the internal standard) and expressed as percentages of the control (**Fig. 5**). The expression levels of both TSHβ mRNA and hnRNA in hypothyroidism were approximately fourfold higher than the respective levels in control rats. The difference in CP between TSHβ mRNA and hnRNA was approximately 3–5 (**Fig. 3**), indicating that hnRNA expression was 8- to 32-fold lower than mRNA expression in chronically hypothyroid rats.

7.3 Discussion

The aim of this study was to detect hnRNA expression by quantitative real-time PCR analysis, and, in particular, the up-regulation of TSHβ gene transcription under the condition of hypothyroidism. Previous reports have shown a range of levels for the up-regulation of TSHβ mRNA (**Table 4**). Although these results were obtained under different experimental conditions, such as the length of PTU administration and the method of mRNA detection, our results confirmed a significant increase in TSHβ mRNA expression in hypothyroid rats.

Both bands of the TSHβ mRNA and the hnRNA bands in the hypothyroid rats group looked were thicker than that those in the control group. The far left lanes show ladder is a DNA marker ladder. After checking the size of the PCR amplicons length by this ladder, we can forward proceed to the next step for a quantitative real-time PCR. Hypothyroid rats group: lanes 1-4. Control group: lanes 5-7.

Fig. 4. Detection of the RT-PCR amplicons for TSHβ mRNA and hnRNA by thermal cyclor

TSHβ mRNA and hnRNA levels in hypothyroid rats were approximately fourfold higher than those of the control rats. Control values were normalised to 100%. Data are expressed as means ± S.D. * $p < 0.01$ compared with the control group.

Fig. 5. Quantitative analysis of TSHβ mRNA and hnRNA levels in control and hypothyroid rats using a LightCycler® PCR system.

Most previous reports have used PTU to induce hypothyroidism (Franklyn et al., 1987; Samuels et al., 1989; Shupnik & Ridgway, 1987; Taylor et al., 1990). However, there is ample evidence that MMI should be used first as an antithyroid drug for Graves’ disease (Ginsberg et al., 2006). In addition, some studies have shown that MMI is more effective than PTU at equivalent doses by reducing thyroid hormone levels more rapidly and achieving euthyroidism more quickly (Ginsberg et al., 2006). In this study, free access to a 0.05% MMI solution in drinking water for 2 weeks induced hypothyroidism in rats (**Table 3**). Because of its longer half-life, MMI can also be used as a single daily agent and is therefore more likely to be associated with patient compliance (Cooper, 1984, 1986). Most importantly, MMI may have a more favourable safety profile than PTU (Ginsberg et al., 2006).

Reference	Duration of PTU administration	TSH β mRNA	Method
(Taylor et al., 1990)	2 weeks	4-fold up-regulation	<i>in situ</i> hybridisation
	4 weeks	16-fold up-regulation	
(Steel et al., 1990)		22-fold up-regulation	<i>in situ</i> hybridisation
(Samuels et al., 1989)	6 weeks	3-fold up-regulation	Northern blot analysis
(Carr & Chin, 1988)		6- to 8-fold up-regulation	Northern blot analysis
(Shupnik & Ridgway, 1987)	6 weeks	10-fold up-regulation	Northern blot analysis
(Franklyn et al., 1987)	3 weeks	10-fold up-regulation	Northern blot analysis
	10 weeks	20-fold up-regulation	

PTU administration; oral administration of 0.05% propylthiouracil in the drinking water

Table 4. Previous reports of semi-quantitative analysis of TSH β mRNA expression in rat hypothyroidism.

In this study, we accurately measured the increased rate of TSH gene transcription in hypothyroid rats by detecting mRNA and hnRNA expression levels over time using the LightCycler® system. Our main recommendations for success with this system are as follows: (1) the extracted total RNA should be treated with DNase to ensure exclusion of genomic DNA and (2) degenerate oligonucleotide random primers should be used instead of the degenerate oligonucleotide dT primers that are more commonly used for mRNA detection.

The poly-A tail of a mature mRNA is immediately appended by poly-A polymerase at the final step of RNA processing. Oligonucleotide dT primers attach to the poly-A tail of the

mRNA for cDNA synthesis until the RT reaction stops. Therefore, only the exonic regions of the DNA are reflected by RT-PCR when use of Oligonucleotide dT primers. However, in our experiments, we used a random hexamer primer, which attaches to DNA complementarily and randomly. When a hexamer primer attaches to any intronic region of the hnRNA, the RT reaction accurately makes cDNA, reflecting primary transcription. Using this cDNA containing intronic sequences as a template, we successfully amplified primary TSH β transcripts with a TSH β -specific intron primer.

The only difficulty in our method was dissecting tissues of the same size and location from the animals. In this study, we easily removed pituitary glands of the same size because the pituitary gland differentiates from a dual embryonic origin, unlike other nearby tissues (Kelberman et al., 2009), which allows the pituitary gland to be easily separated. However, in the case of continuous tissues, such as the hypothalamus, it may be difficult to dissect an area from the same location within the brain. If the dissected area does not contain the target neurons, the internal gene of the area should show a relative increase.

The increased level of TSH β mRNA was nearly equal to that of TSH β hnRNA in hypothyroid rats. Since the transcriptional rate is often masked by the abundance of pre-existing mature mRNA, quantitative detection of hnRNA is important for the precise examination of transcriptional changes during an acute response. Therefore, the use of a quantitative real-time PCR method is beneficial for the analysis of genes with low expression levels, such as those genes that are undetectable when using an *in situ* hybridisation method.

In this report, we demonstrated for the first time that optimal conditions for real-time PCR enable the detection of TSH β hnRNA expression levels in hypothyroid rats. In addition to its critical role in homeostasis, thyroid hormone can also regulate gene transcription in relation to various aspects of brain function (Refetoff et al., 1993), including synapse formation (Nicholson & Altman, 1972). Thus, our results are of particular importance for neuroendocrinological studies.

Finally, we determined the exact ratio for the up-regulation of TSH β mRNA and hnRNA levels in hypothyroid rats using quantitative real-time PCR. Using this method, we can perform similar investigations on the transcription rates of other genes.

8. Conclusion

We have summarised TSH regulation (e.g., TSH secretion and synthesis), the HPT axis as a positive/negative regulator of TSH, the neuronal control of TSH, and drugs affecting TSH in hypothyroidism. We also described a method for the quantitative analysis of TSH β hnRNA and mRNA expression using real-time PCR in induced hypothyroidism. Both the TSH β mRNA and hnRNA expression levels in hypothyroidism induced by methimazole administration were increased approximately fourfold over the respective levels in control rats. Further, the level of TSH β hnRNA expression was 8- to 32-fold lower than that of TSH β mRNA in chronically hypothyroid rats. Using this method, we can perform similar investigations of the transcription rates for other genes.

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10. References

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Hypothyroidism is the most common thyroid disorder and it is significantly more frequent than presented - millions of people suffer from this disease without knowing it. People with this condition will have symptoms associated with slow metabolism. Estimates of subclinical hypothyroidism range between 3 to 8 %, increasing with age, whereas it more likely affects women than men. About 10% of women may have some degree of thyroid hormone deficiency. Hypothyroidism may affect lipid metabolism, neurological diseases or other clinical conditions. The book includes studies on advancements in diagnosis, regulation and replacement therapy, thyroid ultrasonography and radioiodine therapy for hypothyroidism. "Hypothyroidism - Influences and Treatments" contains many important specifications, results of scientific studies and innovations for endocrine practice.

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