THE HUMAN prepro THYROTROPIN-RELEASING HORMONE (TRH) GENE; CLONING, CHARACTERIZATION, HORMONAL REGULATION, AND GENE LOCALIZATION*

JOHN F. WILBER, and (*by invitation*) MASANOBU YAMADA, UN J. KIM, PEI FENG, N. ERIC CARNELL

BALTIMORE

Our laboratory has demonstrated recently that thyrotropin (TSH) secretion is regulated in part by thyroid hormone inhibition of TSH-releasing hormone (TRH) synthesis and secretion, both *in vitro* and *in vivo*. Minute amounts of triiodothyronine (T_3) can suppress TSH secretion when administered intracerebraventricularly, whereas identical quantities of T_3 administered by a peripheral route do not lower circulating TSH concentrations (1). In addition, TRH secretion is augmented *in vitro* from experimentally hypothyroid rats and secretion is below normal from thyrotoxic rat hypothalami, and T_3 (10^{-9}–10^{-11} M) introduced into incubational media can prevent ouabain-activated TRH secretion *in vitro* (2). Most recently, we have demonstrated that there is augmented TRH messenger RNA in experimentally hypothyroid rats and reduced messenger RNA in the hypothalamic paraventricular nucleus in thyrotoxic rats, whereas no changes were seen in either group when *whole* hypothalamic RNA was hybridized (1). On the basis of these considerations, we wanted to determine whether thyroid hormones were involved also in the inhibitory regulation of the *human* TRH gene. Two X 10^6 plaques of a human lung fibroblast Lambda Fix genomic library were screened with a ^{32}P-cDNA *rat* preproTRH (ppTRH) gene probe, which included the last three TRH coding sequences of the third exon. Under conditions of moderate stringency, an initial 15 kb DNA fragment was identified, which included the complete ppTRH genomic sequence of 3.3 kb, containing three exons separated by two introns of 1,050 and 650 base pairs respectively Figure 1 (3). The inferred cDNA sequence was confirmed by PCR amplification of hypothalamic cDNA derived from freshly prepared human hypothalamic RNA. Human hypothalamic ppTRH cDNA contained 6 identical repeats encoding TRH in the third exon, with 7 intervening cryptic peptides which can be processed from the prohormonal peptide to subserve functions potentially as neuromodulators and/or neurotransmitters. The rat ppTRH gene, in contrast, contains 5 sequences encoding TRH. Of interest is that the third exon

* From The Division of Endocrinology and Metabolism University of Maryland Medical School, Baltimore, MD. 21201

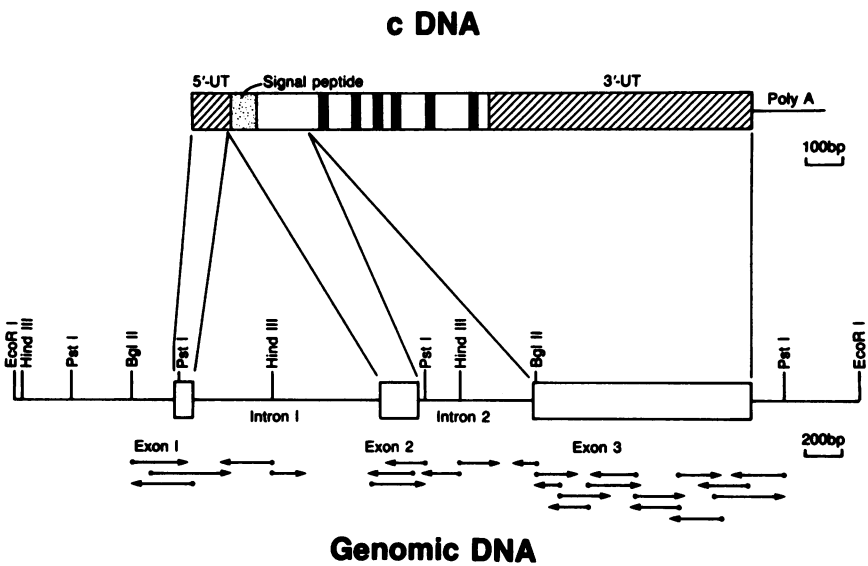

FIG. 1. Structure of Human preproTRH cDNA and Gene and the Sequencing Strategy. The top line shows a schematic diagram of human preproTRH cDNA. Solid boxes represent TRH coding sequences. Representation of the human preproTRH gene is schematized below. Exons are represented by open boxes. Vertical connecting lines indicate relationships between structural regions of the cDNA and the genomic DNA. The horizontal arrows beneath the genomic DNA indicate the direction and extent of sequencing.

open reading frame of 700 base pairs of the human gene, shares 52% homology at the DNA level with the chromogranin A gene, a common endocrine-associated gene present in the hypothalamus, pituitary, parathyroid, and adrenal medulla. Homology of the human ppTRH gene with the rat ppTRH gene is 73.3% and 59.5% at the nucleic acid and amino acid levels respectively (3).

The 5′ flanking region of the human ppTRH gene contains four features of particular interest (3). First, there is a TATA box at −25 to −30 bp from the start site of transcription. Second, there is a potential CREB binding site at −53 to −60 bp. Third, two GC rich inverted repeats further upstream likely represent SP-1 binding sites. Fourth and most importantly, 2 octameric sequences were identified at −158 and −165 bp and at −183 and −190 bp that could serve as thyroid hormone recognition elements (TREs). The sequence between −183 and −190 interestingly is identical to that in the human ppTSH β-chain gene, which is regulated negatively by L-T_3 (1).

To investigate whether or not L-T_3 regulates the human ppTRH gene, chimeric plasmid constructs were generated with a SV-40 virus cassette

containing the luciferase gene as a reporter element. The TRH flanking region inserted into the multicloning site represented base pairs −900 to +25 of the genomic sequence. Transfection of this construct into pituitary GH_3 cells was accomplished by the calcium phosphate method (4). No significant inhibitory effects of L-T_3 (10^{-11}M–10^{-9} M) upon the pSV40-enhancerless-luciferase control construct were observed (Fig. 2). In striking contrast, L-T_3 over an identical concentration range, progressively inhibited light generation in plasmids containing the human TRH 5′ flanking region minus the SV40 promoter. Significant inhibition was seen at all concentrations of L-T_3 (10^{-9} M T_3, −24%, $P > 0.02$; 10^{-10} M T_3, −45%, $P > 0.01$; 10^{-9} M T_3 −71%, $P > 0.001$) (Fig. 3). These studies

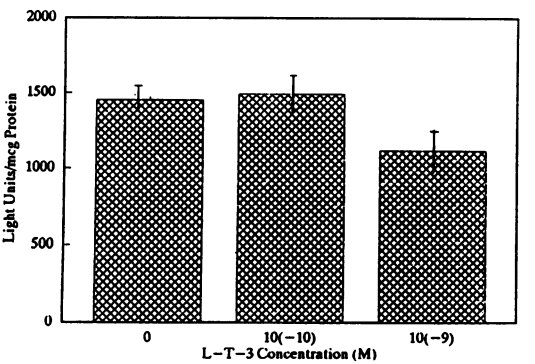

FIG. 2. The effect of L-T_3 (10^{-10} M, 10^{-9} M) *in vitro* upon the activity of the chimeric construct, pSV40-Enhancerless-*luciferase* (LUC). There were no significant inhibition of *luciferase* activity by L-T_3. The apparent 18% reduction in *luciferase* activity at 10^{-9} M T_3 was not statistically significant ($P > 0.1$). "Enhancerless" indicates that SV40 promoter was utilized in lieu of the human preproTRH promoter in the chimeric plasmid constructs.

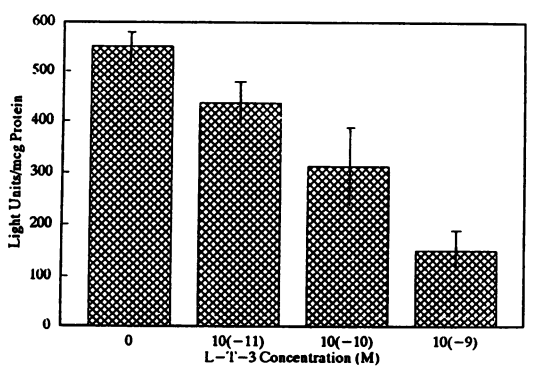

FIG. 3. The effect of L-T_3 *in vitro* upon the activity of the transfected chimeric construct, "pSVO-TRH-LUC" (containing the human ppTRH 5′ flanking sequence −900 to +54 bp) in cultured GH_3 cells. Progressive and significant inhibition was seen at all L-T_3 concentrations (10^{-11} M, −24%, $P < 0.02$; 10^{-10} M, −45%, $P < 0.01$; 10^{-9} M, −71%, $P < 0.001$).

indicate that L-T_3 *in vitro* does indeed inhibit the expression of the human pTRH 5′ flanking sequence ligated to the reporter gene *luciferase*, and they support to the concept that thyroid hormones can regulate not only TRH action at the level of pituitary but also inhibit ppTRH gene expression. This effect, we hypothesize, is expressed at the −183 to −190 octameric sequence (GCCAGTCG) and/or at the downstream homologous octimer at −158 to −166 pp (GGTCCCAC). Studies are currently in progress to corroborate what DNA sequences are involved specifically in the observed inhibitory effects of T_3 using transient transfection studies and Avidin-Biotin DNA binding assays with TRE-containing synthetic oligonucleotides (4).

Because genomic Southern analyses of the human ppTRH gene, after digestion of human white cell DNA with 4 separate restriction endonucleases, have demonstrated a single copy gene, we have now investigated the precise chromosomal localization of this gene to help to elucidate the molecular basis of human genetic disorders interrupting TRH synthesis. Localization of the human gene was accomplished with hamster-human somatic cell hybrids, using cell lines obtained from Bios, Inc. (New Haven CT) (5). The human chromosomal content of hybrid cells was determined by marker enzyme analyses and karyotyping. Filters for Southern analyses and appropriate conditions for digestion with endonucleases, agarose electrophoresis, and hybridization have been described previously (6). Hybridization was performed with the full human ^{32}P cDNA sequences as a probe. In Figure 4, a single hybridization band to the far left is observed with human WBC DNA as expected. No hybridization signals were identified, in contrast, with Chinese hamster DNA, the far left columns of the two right-hand sections, indicating that all hybridization signals on these transfers represented human, and not hamster DNA. Hybridization was seen in cell lines 3, 4, 13, 20–22, and 29 (Fig. 4). These cell lines indicated complete concordance between chromosome 3 and the TRH genome, allowing definitive assignment of the ppTRH gene to this chromosome. It is of note that the gene for cERBA-β protein, which serves as one of the variants of L-T_3 intracellular binding proteins, is located also on chromosome 3 (7). It will be of interest to ascertain if the two genes are closely linked since L-T_3 bound to CErbA-β can inhibit TRH gene expression. The assignment of the human ppTRH gene to chromosome 3 should also facilitate genetic analyses of hereditary disorders of ppTRH gene mutations by restriction-fragment length polymorphisms (RFLPs) and mutant gene sequencing. Since TRH is encoded by six identical repeats, mutational causes would likely involve mechanisms such as frame shift, non-sense, or promoter mutations, in lieu of point mutations in the sequences encoding TRH *per se*.

TRH has been discovered to be resident in a number of loci not only

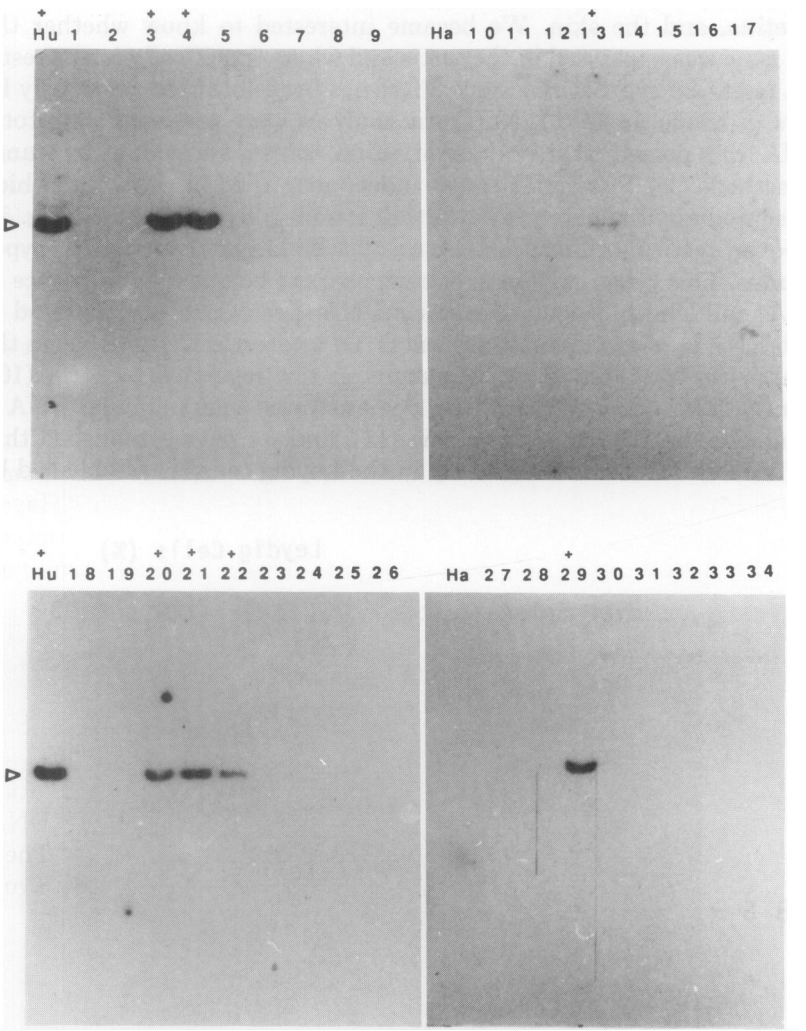

FIG. 4. Genomic Southern transfers of human-Chinese hamster hybrid cell DNA after EcoRI digestion. ^{32}P-labeled human preproTRH cDNA was used as a probe. A single band of 4.8 kb was observed with total human WBC DNA lanes (labeled "Hu") which was identified also in cell lines 3, 4, 13, 20–22, and 29. No hybridization signals were seen in any of the other cell lines examined. Note that no band was seen with Chinese hamster (CHO) DNA, labeled "Ha." Thus, the hybridization shown represent only human DNA in these hamster-human cell lines.

outside the hypothalamus but outside of the central nervous system (8). These loci include the gastrointestinal tract, the islets of Langerhans, the retina, and the skin. We became interested to know whether the TRH gene was expressed in the testis and where specifically in the testis TRH might be synthesized since TRH has been localized previously by others in whole testis (9). Northern analyses were prepared from total mRNA from pooled rat hypothalami, testis, spleen, and kidney by standard methods (1). TRH mRNA was indeed identified in rat testis, which represented approximately 13% of that found in hypothalamus (Fig. 5). Moreover, testicular TRH mRNA was 0.4 kb larger than that in hypothalamus. This larger mRNA species appears to be due to a difference in poly A^+ tail length, since the larger mRNA species can be converted to 1.6 kb RNA by digestion with RNase H. Of interest is that mRNA in the testis, in contrast in the hypothalamus, is not regulated by L-T_3 (10). Recently, Lee and co-workers have demonstrated that testicular RNA is regulated negatively by testosterone (11), and we have established that TRH mRNA is located exclusively in the Leydig cells, as established by

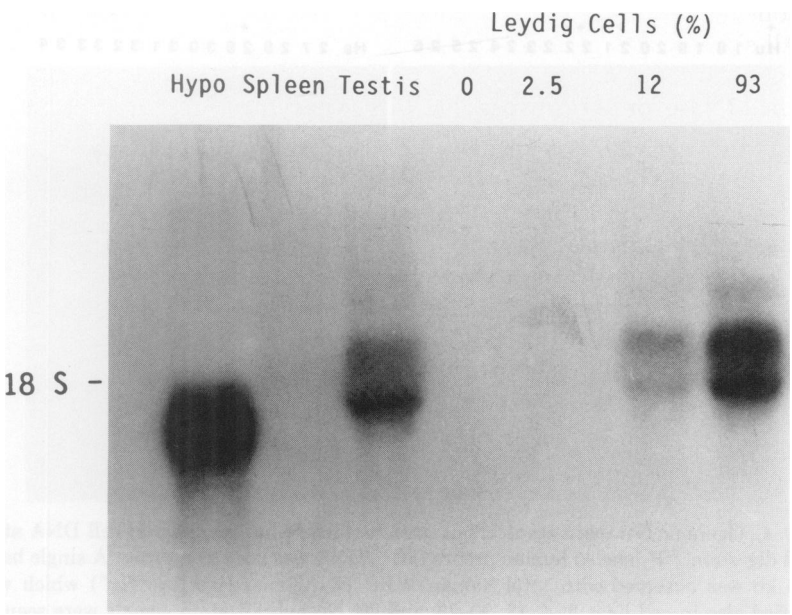

FIG. 5. Northern analyses of total RNA polyA$^+$ RNA from rat testis and Leydig cell-enriched fractions after cell elutriation. Note the strong, single band from hypothalamic mRNA at 1.6 kb. There was no hybridization with splenic RNA. Testis and Leydig cell fractions exhibited identical, dual hybridization signals of 1.8 kb and 2.0 kb. This polymorphism is attributable to variable poly A tail length because the double bands can be transformed to single bands at 1.6 kb by digestion with RNase H.

cell elutriation. This observation is in accord with the demonstration, by immunocytochemistry, that TRH is located also in the Leydig cell exclusively, and TRH concentrations increase during development (Fig. 6). We postulate that *testicular* TRH plays a paracrine or autocrine role in the regulation of testosterone synthesis and/or secretion (2).

Until now, TRH gene transcription outside of the central nervous system has been identified only in the testis, placenta, and the pancreas (8). Since the heart has the capacity to biosynthesize and secret other neuropeptides, notably atrial natriuretic peptide (ANP), we wondered whether TRH, which is known to stimulate blood pressure when administered to man in pharmacological doses, might also be formed in the heart. To investigate this question, both whole rat heart and individually dissected cardiac chambers were used to generate messenger RNA for Northern analyses, using a ^{32}P-labeled TRH antisence cRNA probe provided by Drs. Lee and Goodman. Cardiac TRH identity with synthetic TRH was confirmed by passing whole heart extracts through Sephadex G25 columns and incubation that with the enzyme Pyroglutamate Aminopeptidase. TRH RNA was identified in all cardiac chambers. Concentrations in the atria were 10.1 higher than those found in ventricles (Fig. 6). All signals were 1.6 kb, identical to the size of hypothalamic ppTRH RNA. The presence of ppmRNA in the rat myocardium was confirmed by the RNase protection assays. TRH concentrations, in contrast to

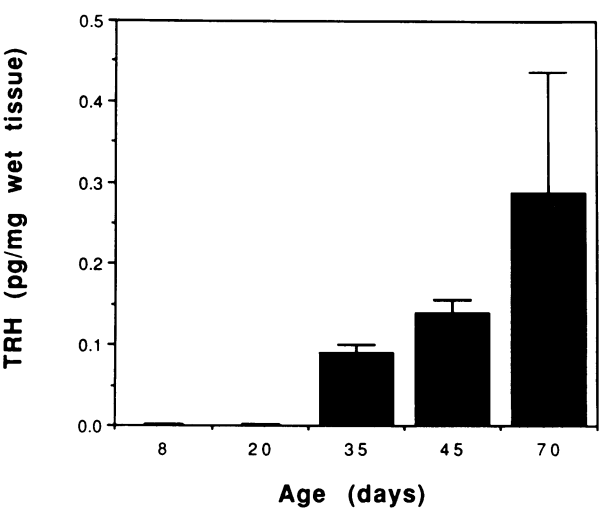

FIG. 6. TRH concentrations in whole rat testis between ages 8 and 70 days. Note progressive increments in TRH vs. time. The concentrations at days 45 and 70 were not statistically different ($P > 0.5$) from day 35. Days 35–70 were higher significantly from days 8 and 20 ($P < 0.01$).

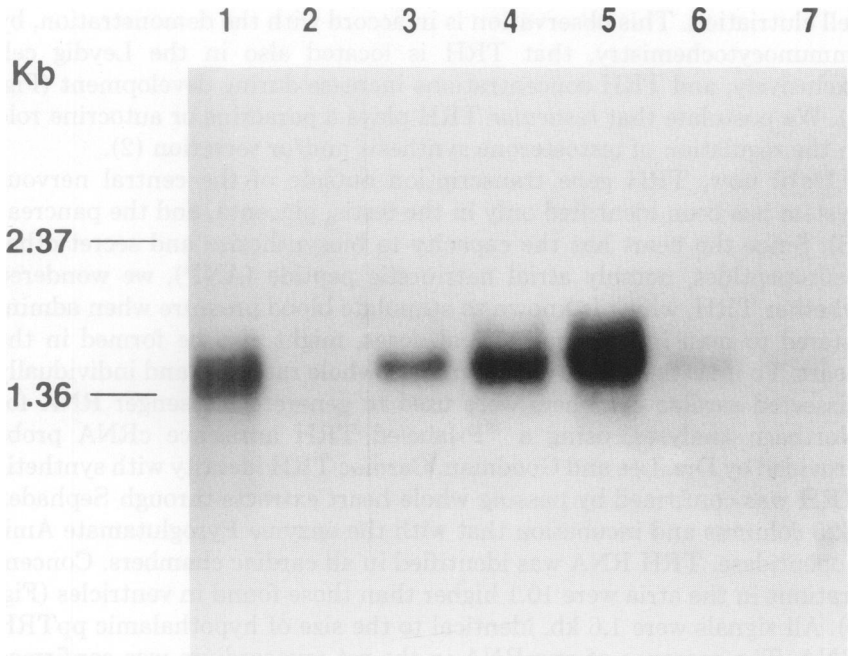

FIG. 7. TRH mRNA Densities in Whole Rat Heart and Individual Cardiac Chambers (1 = Rat hypothalamus, 2 = liver, 3 = whole heart, 4 = left atrium, 5 = right atrium, 6 = left ventricle, and 7 = right ventricle). Thirty mcg of total RNA/lane was loaded for Northern transfers. Mean atrial TRH mRNA densities were 10× higher than ventricular hybridization signals.

TRH mRNA, exhibited two-fold higher concentrations in the ventricles, suggesting that there must be post-transcriptional and/or post-translational regulation of preproTRH mRNA to account for the dissociation of TRH and TRH mRNA activities. These data raise the intriguing possibility that TRH may play an autocrine or paracrine role also in the heart, possibly by influencing the contractile state of the myocardium. Studies are currently in process to examine the role of TRH messenger RNA and TRH in the human heart in health and disease.

REFERENCES

1. Yamada M, Wilber JF. Reciprocal regulation of preprothyrotropin-releasing hormone (TRH) mRNA in the rat anterior hypothalamus by thyroid hormone: Dissociation from TRH concentrations during hypothyroidism. *Neuropeptides* 1990; 15: 49.
2. Iriuchijima T, Rogers D, Wilber JF. TSH secretory regulation: New evidence that triiodothyronine (T_3) and thyroxine (T_4) can inhibit TRH secretion both in vivo and in vitro. In: Medeiros-Neto G, Gaitan E, eds. *Frontiers in Thyroidology* New York: Plenum Medical Book Co; 1986; 1: 233.

3. Yamada M, Radovick S, Wondisford FE, et al. Cloning and structure of human genomic DNA and hypothalamic cDNA encoding human prepro thyrotropin-releasing hormone. *Mol Endocrinol* 1990; 4: 551.
4. Yamada M, Radovick S, Wondisford FE, et al. The human preproTRH (ppTRH) gene: Inhibition of expression in GH_3 cells of plasmid chimeric luciferase constructs by L-triiodothyronine ($L\text{-}T_3$). *Proceedings of The 10th Annual International Thyroid Congress,* The Hague, The Netherlands, 1991.
5. Yamada M, Wondisford FE, Radovick S, et al. Assignment of human prepro thyrotropin-releasing hormone (TRH) gene to chromosome 3. *Somat Cell Mol Genet* 1991; 17: 97.
6. Carlock LR, Smith D, Wasmuth JJ. *Somat Cell Mol Genet* 1986; 12: 163.
7. Weinberger C, Thompson CC, Ong ES, Lebo R, Groul DJ, Evans RM. *Nature* 1986; 324: 641.
8. Wilber JF, Yamada M. Thyrotropin-releasing hormone: Current concepts. In: Greer MA, ed. *The Thyroid Gland.* New York: Raven Press, Ltd.; 1990: 127.
9. Pekary AE, Bhasin S, Smith V, et al. Thyroid hormone modulation of thyrotropin-releasing hormone (TRH) and TRH-Gly levels in the male rat reproductive system. *J Endocr* 1987; 114: 271.
10. Feng P, Kim U, Yamada M, et al. Rat preproTRH gene expression: Selective alterations by thyroid status in the paraventricular nucleus (PVN) but not in testis, a new extra-CNS locus for ppTRH gene expression. *Proceedings of The 10th Annual International Thyroid Congress,* The Hague, The Netherlands, Feb. 1991.
11. Yang IM, Krieg RJ, Lee SL. Hormonal regulation of thyrotropin releasing hormone (TRH) gene expression in rat testes. *Thyroid* 1991; 1: S-81.