toxicity or activation for lymphokine production, we hypothesize that these cells might be activated at sites of antigen-specific immune responses through the interaction of IL-2 with IL-2Rβ. The role of IL-2Rβ on resting T cells is less clear. It is possible that it may serve principally or solely to provide a substrate for formation of high-affinity IL-2 receptors when IL-2Rα is expressed. Thus, the relatively large amount of IL-2Rβ present on LGLs may allow IL-2–induced recruitment of bystander LGLs at the sites of immune responses, while the smaller amount on resting T cells may allow less nonspecific recruitment but rapid expression of high-affinity IL-2 receptors and response after antigen-specific induction of IL-2Rα.

Note added in proof: Since this manuscript was submitted, two other groups have also reported the expression of IL-2Rβ on LGLs (*24*).

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20. The 40-fold greater affinity of the high-affinity IL-2 receptor for IL-2 than for anti-Tac [see (*12*)] necessitates use of considerable excess anti-Tac to prevent binding of IL-2. Our conditions ensured <2% high-affinity receptor occupancy by IL-2. The ratio of anti-Tac to IL-2 used by others is often difficult to ascertain but generally far less than used in this study. Concentrations of anti-Tac sufficient to saturate receptors and to block proliferation of activated T cells (a frequently used control) may not block other IL-2R–dependent responses if there are differences in the requirements for receptor occupancy or the IL-2 concentrations used.
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Thyroid Hormone Regulates TRH Biosynthesis in the Paraventricular Nucleus of the Rat Hypothalamus

THOMAS P. SEGERSON, JOHN KAUER, HUBERT C. WOLFE, HAMID MOBTAKER, PING WU, IVOR M. D. JACKSON, RONALD M. LECHAN*

Thyroid hormone is important in the regulation of synthesis and secretion of thyroid-stimulating hormone (TSH) in the anterior pituitary, but its role in the control of hypothalamic thyrotropin-releasing hormone (TRH) is controversial. To determine whether thyroid hormone regulates the function of TRH in the hypothalamic tuberoinfundibular system, a study was made of the effect of hypothyroidism on thyrotropin-releasing hormone messenger RNA (proTRH mRNA) and TRH prohormone in the rat paraventricular nucleus. Extracts of rat hypothalamic paraventricular nucleus were examined by quantitative Northern blot analysis, and coronal sections of rat brain were examined by in situ hybridization histochemistry and immunocytochemistry. A nearly twofold increase in proTRH mRNA was observed in hypothyroid animals; this increase could be obliterated by levothyroxine treatment, suggesting an inverse relation between circulating thyroid hormone and proTRH mRNA. In situ hybridization showed that this response occurred exclusively in medial parvocellular neurons of the paraventricular nucleus. A simultaneous increase in proTRH mRNA and immunoreactive TRH prohormone in this region suggests that hypothyroidism induces both transcription and translation of the TRH prohormone in the paraventricular nucleus.

ALTHOUGH IT IS CLEARLY ESTABLISHED that thyroid hormone exerts negative feedback control on thyroid-stimulating hormone (TSH) secretion by anterior pituitary thyrotropes (*1*), its effect on hypothalamic thyrotropin-releasing hormone (TRH), the principal regulator of TSH secretion, has been a subject of controversy (*2*). Measurements of TRH content in the hypothalamus in altered states of thyroid function have been conflicting, possibly because there are many TRH neurons that are not part of the tuberoinfundibular system (*3, 4*) (and hence might be regulated differently by thyroid hormone), and because content alone may be separately affected by rates of synthesis, transport, secretion, and degradation. The recent isolation of a complementary DNA (cDNA) that encodes the TRH prohormone (proTRH) (*5*) and the development of antisera that interact specifically with proTRH (*6*) have provided the tools to determine whether thyroid hormone regulates the function of the TRH tuberoinfundibular system.

Hypothyroidism was induced in male Sprague-Dawley rats by injecting them intraperitoneally with propylthiouracil (1.0 mg per 100 g of body weight) and putting 0.02% methimazole in their drinking water. Control animals received either an initial intraperitoneal injection of normal saline alone or were treated as the hypothyroid animals but given a daily intraperitoneal injection of levothyroxine (3 μg per 100 g of body weight). Animals were decapitated after 21 days of treatment and their blood was assayed for TSH by reagents supplied by the NIADDK. The brains were snap-frozen in hexanes containing Dry Ice, and a 1.5-mm coronal section containing the entire paraventricular nucleus was cut with

T. P. Segerson and R. M. Lechan, Department of Medicine, Endocrine Division, New England Medical Center Hospitals, Boston, MA 02111.
J. Kauer, Department of Neurosurgery, New England Medical Center Hospitals, Boston, MA 02111.
H. C. Wolfe and H. Mobtaker, Department of Pathology, New England Medical Center Hospitals, Boston, MA 02111.
P. Wu and I. M. D. Jackson, Department of Medicine, Division of Endocrinology, Brown University, Rhode Island Hospital, Providence, RI 02902.

*To whom correspondence should be addressed.

razor blades on a template. With a 14-gauge stainless steel needle, the paraventricular nucleus was punched from the tissue section and extracted for RNA. Equal amounts of RNA were subjected to denaturing gel electrophoresis, blotted onto a nylon membrane and probed for TRH prohormone mRNA with an antisense [^{32}P]guanosine triphosphate–labeled transcript generated from a 1241-bp Eco RI–Pst I fragment of proTRH cDNA cloned antisense in pSP65 (Promega-Biotech) (*5*). Normalization of RNA samples was confirmed by reprobing blots with a 28*S* ribosomal RNA (rRNA) probe (*7*). The autoradiographs were densitometrically analyzed by computer (*8*) and the concentrations of proTRH mRNA estimated by comparison to a standard curve generated by hybridization to known concentrations of sense transcript of the proTRH cDNA fragment (*9*).

To determine whether the effect of thyroid hormone is exclusively on the paraventricular nucleus, we also prepared brains from hypothyroid and control groups for in situ hybridization histochemistry by intracardiac perfusion with 4% paraformaldehyde. Autoradiographs were prepared from brain sections (20 μm) through the paraventricular nucleus that were probed in situ for proTRH mRNA with a [^{35}S]uridine triphosphate–labeled antisense probe, coated with photographic emulsion, and developed after exposure. Density of silver grains accumulating over neurons was evaluated under darkfield microscopy, digitized, and analyzed by computer imaging. Vibratome tissue sections (50 μm) were also prepared for immunocytochemistry by the avidin-biotin-peroxidase (ABC) technique (*10*) with the use of antiserum that recognizes the TRH prohormone (*6*).

After 21 days of thioamide treatment, proTRH mRNA in the paraventricular nucleus nearly doubled in concentration (Fig. 1, A and B). Estimated proTRH mRNA levels increased from 469 attomoles ± 85 SEM in normal saline controls to 914 attomoles ± 126 SEM in the hypothyroid animals (T_4 μg/dl: hypothyroid <1.0, versus normal saline control 3.5 ± 0.16 SEM; TSH ng/ml: hypothyroid 35.9 ± 1.31 SEM versus normal saline control 2.23 ± 0.24 SEM, $P < 0.01$; Student's t test). Thioamide-treated animals treated with levothyroxine showed normal levels of thyrotropin when compared to normal saline-treated controls (2.65 ± 1.5 SEM) and no increment in the levels of proTRH mRNA (Fig. 1C).

By in situ hybridization histochemistry, hypothyroid rat brains showed a selective increase in the accumulation of silver grains over paraventricular nuclear perikarya in the medial parvocellular division but not the anterior parvocellular division or other hybridizing neurons in the hypothalamus (Fig. 2, A and B). When plotted histographically, the majority of medial parvocellular neurons in control animals had density ratios of 0 to 2 (median range 0.5 to 1.0) in contrast to hypothyroid animals that showed a wide range of density ratios from 0.5 to 19 (median range 5.0 to 5.5) (Fig. 3), indicating that some neurons may increase their content of proTRH mRNA severalfold.

A marked increase in the intensity of staining of immunoreactive proTRH also occurred in the medial parvocellular division of the paraventricular nucleus that filled the cytoplasm and processes of neuronal peri-

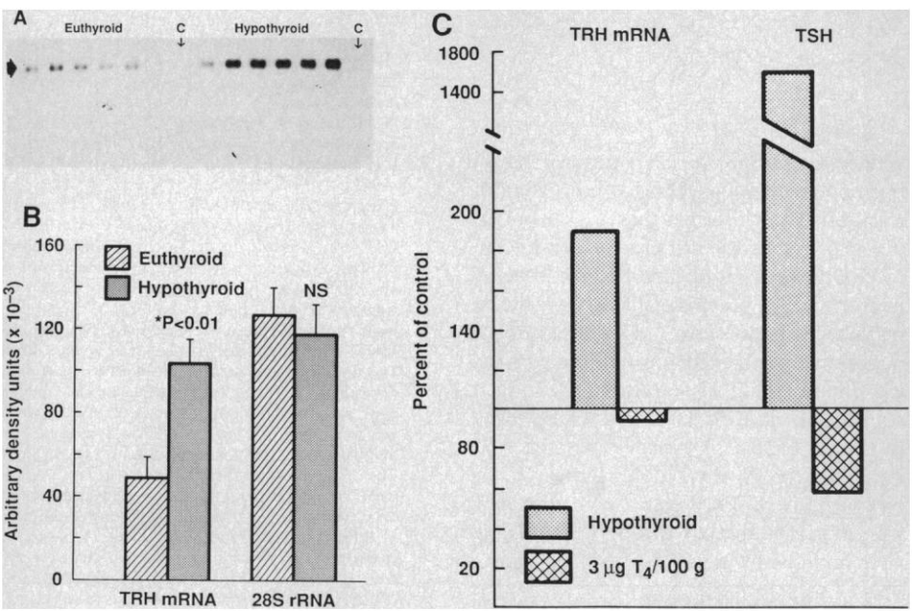

Fig. 1. (**A**) Northern blot of proTRH mRNA from euthyroid (normal saline controls) and hypothyroid (thioamide-treated) animals. Each lane (six in each group) represents a sample of RNA extracted from the paraventricular nucleus of two animals (*17*). Lane C represents RNA extracted from a micropunch of cerebral cortex. A hybridizing band of approximately 1.6 kb (arrowhead), representing proTRH mRNA, is present only in PVN extracts. (**B**). Comparison of density units of proTRH mRNA from euthyroid and hypothyroid animals determined by computer image analysis of Northern blot in (A). A significant increase in proTRH mRNA is visible in the hypothyroid group (Student's t test). Linearity within the density range was established by hybridization of standards of sense transcript in parallel with the PVN RNA Northern blots (*9*). Normalization of RNA samples was confirmed by the absence of a significant difference in rRNA in the two groups of extracts. Blots were reprobed with 10^6 cpm/ml of a ^{32}P-labeled RNA probe for rat 28*S* rRNA (*7*) after elution of hybridized probe in 2 liters of 50 m*M* EDTA (*p*H 8.0) at 100°C for 15 minutes. (**C**) TRH mRNA and TSH in hypothyroid and levothyroxine-treated animals as a percentage of TRH mRNA and TSH in normal, saline-treated, euthyroid control animals. The concentration of proTRH mRNA and serum TSH in thioamide-treated animals replaced with levothyroxine was not significantly different from that of the euthyroid control animals.

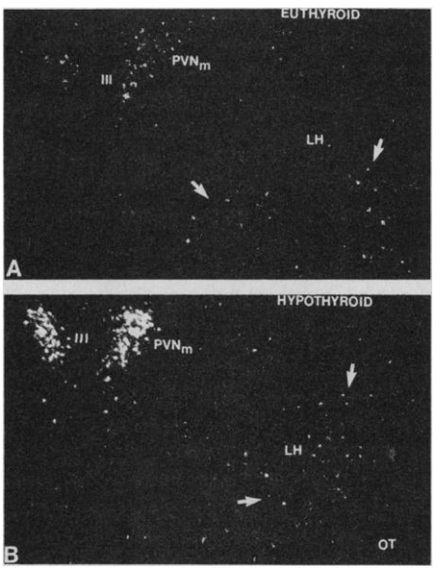

Fig. 2. In situ hybridization histochemistry (darkfield illumination) of the hypothalamus (20-μm coronal section) at the level of the medial parvocellular division of the paraventricular nucleus using a probe for proTRH mRNA in (**A**) euthyroid (normal saline controls) and (**B**) hypothyroid animals. A marked increase in hybridization is seen in medial parvocellular neurons of the paraventricular nucleus neurons (PVN_m) but not lateral hypothalamic neurons (arrows) in hypothyroid animals (LH, lateral hypothalamus; OT, optic tract; III, third ventricle). Original magnification, ×79. Hybridization was performed as previously described (*5*, *15*) in buffer containing 5×10^5 cpm of [^{35}S]uridine triphosphate–labeled antisense probe, transcribed from pSP65 containing the proTRH cDNA insert, linearized with Hind III. Slides were coated with NTB-2 photographic emulsion (Eastman Kodak, Rochester, New York) and the autoradiograms developed after 6 days.

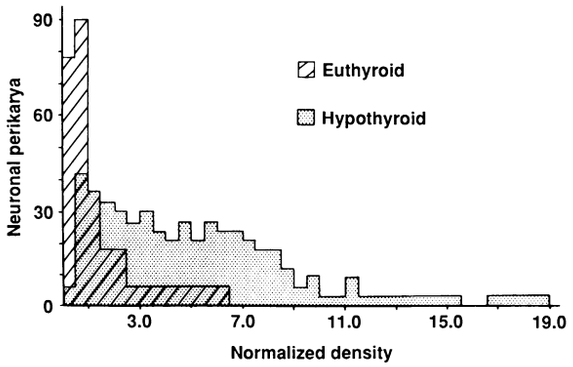

Fig. 3. Computer-generated histogram of PVN cell densities normalized to mean lateral hypothalmic cell density after in situ hybridization with a [^{35}S]uridine triphosphate–labeled single-stranded antisense probe for proTRH mRNA. Hypothyroid animals show a marked shift to the right indicating a relative increase in hybridization density. Slides were examined under dark-field microscopy with an Eyecom TV camera, the image digitized by a Deanza image processor to a 512 × 512 pixel array and analyzed using a Vax 11/780 computer. A median smooth transformation defined an outline around clusters of silver grains (cells) and touching clusters were separated by removing background points. An isodensity contour-following program delineated boundaries and integrated density values over each cluster (*16*).

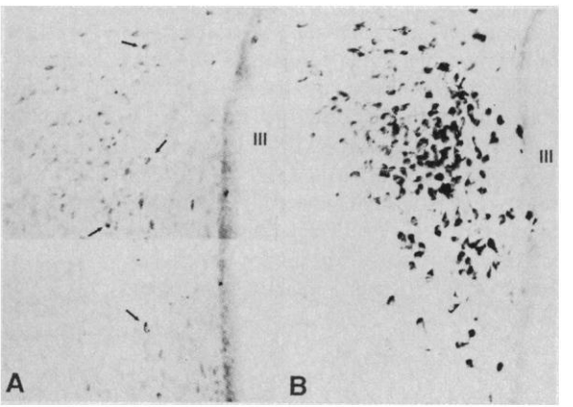

Fig. 4. Immunoreactive proTRH in the paraventricular nucleus of (**A**) euthyroid and (**B**) hypothyroid animals. A marked increase in the intensity and distribution of perikaryal staining is seen in (B). Arrows in (A) denote perinuclear staining in control animals (III, third ventricle). Sections were incubated for 24 to 48 hours at 4°C with proTRH antiserum (No. 351) diluted 1:750 in tris-buffered saline containing 0.3% Triton X-100. The reaction product was developed with 3,3′-diaminobenzidine tetrahydrochloride activated with 0.01% H_2O_2. Original magnification, ×200.

karya, in contrast to the more characteristic perinuclear pattern of staining in control animals (Fig. 4, A and B) (*6*).

These studies demonstrate that hypothyroidism results in a significant increase in proTRH mRNA exclusively in the paraventricular nucleus, the thyrotropic area of the hypothalamus (*4*), and that this response can be inhibited by levothyroxine. An inverse relation between circulating thyroid hormone and proTRH mRNA concentrations in the paraventricular nucleus is thereby established. Because immunoreactive proTRH increases simultaneously with proTRH mRNA in these cells, it is likely that hypothyroidism induces both transcription and translation of the TRH prohormone in the paraventricular nucleus. We propose, therefore, that a negative feedback effect on the thyroid hormone occurs centrally to regulate hypophysiotropic TRH secretion in addition to the well-documented effect on TSH secretion from anterior pituitary thyrotropes (*2*).

The selective effect of hypothyroidism on medial parvocellular neurons of the hypothalamus indicates that either cellular factors specific to these paraventricular neurons or afferent input to these neurons determine this response. It is unknown whether paraventricular nucleus neurons possess thyroid hormone receptors (*11*). However, anatomical studies have shown that TRH-containing neurons of the paraventricular nucleus receive direct synaptic input from adrenergic neurons (*12*), preferentially to the medial parvocellular division (*13*). Because epinephrine and norepinephrine have convincing effects on TRH secretion, resulting in stimulation of both TSH and TRH release in the rat (*14*), it is possible that the feedback control of thyroid hormone on the biosynthesis of TRH may be mediated, at least in part, indirectly through central catecholamine pathways.

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9. Transcripts of an Eco RI–Pst I 1241-bp fragment of the TRH cDNA, cloned in native orientation in pSP64, were quantified by ultraviolet optical density. Samples of 1, 5, 10, 50, and 100 pg of RNA transcript were subjected to electrophoresis, blotted, and probed in parallel with the PVN extractions. Sense transcript (250 pg) was added to an extract of a micropunch from the cerebral cortex and a portion of this sample was treated identically to estimate the recovery and loss during extraction. Density of hybridizing bands (1.2 kb) of sense transcript were analyzed to establish linearity in the density range and to correct the estimated concentration of TRH mRNA in the PVN extracts for extraction loss.
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17. Tissue punches were homogenized in 6*M* urea, 3*M* LiCl, and 10 m*M* vanadyl ribonucleoside complex, incubated overnight at 0°C, and centrifuged at 16,000*g* at 4°C for 30 minutes. The pellets were resuspended in 10 m*M* tris-HCl (*p*H 7.5) containing 5 m*M* EDTA and 0.1% sodium dodecyl sulfate (SDS) and extracted with phenol and chloroform, and the RNA was precipitated with 0.1*M* sodium chloride and ethanol. Aliquots from each sample were subjected to electrophoresis on a 1% agarose gel containing 1.0 μg of ethidium bromide per milliliter and photographed under ultraviolet light. The 28*S* ribosomal bands on the negative image were analyzed on a Quik-Scan Jr. (Helena Laboratories) densitometer. Standards of known dilutions of extracts from cerebral cortex were subjected to electrophoresis simultaneously to determine proportional concentrations of RNA extracts. Equal amounts of RNA from each sample were subjected to electrophoresis in a 1% agarose gel containing 2.2*M* formaldehyde. The blot was hybridized according to the manufacturer's protocol for 48 hours at 68°C in buffer containing 10^7 cpm/ml of the ^{32}P-labeled antisense RNA probe. After washing in 0.1× SSC containing 0.1% SDS at 72°C, the membrane was exposed to Kodak XAR5 film at −70°C and developed after 4 to 48 hours in a Kodak X-omat processor. Density of hybridizing bands on autoradiographs was determined by computer analysis.

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