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DETECTION OF THYROTROPIN-RELEASING HORMONE (TRH) mRNA BY THE REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION IN THE HUMAN NORMAL AND TUMORAL ANTERIOR PITUITARY

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To investigate the presence of TRH mRNA in the human anterior pituitary tissue, total RNA from human normal and tumoral anterior pituitary, hypothalamus (positive control) and muscle tissues (negative control) was reverse transcribed (RT) to the first strand of cDNA. RT products were then amplified by polymerase chain reaction (PCR) using a set of three exon-specific primers (two external 5' and 3' primers and one internal 3' primer) for a target sequence of the TRH gene including an intronic sequence of about 650 base pairs (bp). Southern analysis of the RT-PCR products specifically hybridizing with a 45-mer TRH probe showed two bands of the predicted sizes (399 and 351 bp) far more intense in hypothalamus than in normal and tumoral anterior pituitary tissue. The 399 and 351 bp RT-PCR products contained the BglII enzyme restriction site included in the TRH cDNA sequences spanned by the primers and the two respective digested fragments which were, as predicted, 337 and 289 bp long, hybridized with the TRH probe. Based on these results, we can conclude that the RT-PCR products generated from RNA tissue were the target TRH sequences in the human normal and tumoral anterior pituitary tissue as well as in the hypothalamus. Our data imply TRH gene expression in the human anterior pituitary.

Originally isolated from the mammalian hypothalamus (1,2), Thyrotropin-Releasing Hormone (TRH) has been found ubiquitously distributed in the central nervous system and other tissues (3,4). TRH synthesis has been demonstrated in few extrahypothalamic tissues (5,6). TRH has been also observed in the anterior pituitary and several arguments

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favor its endogenous biosynthesis. In the rat, TRH and proTRH peptides have been demonstrated in long-term primary cultures of anterior pituitary tissue (7,8). In man, several reports from our laboratory have shown that TRH was present in the normal and tumoral anterior pituitary and was released in vitro in large amounts by these tissues (9,10). The hypothesis of an anterior pituitary synthesis of TRH needed to be substantiated by the demonstration of TRH gene expression in this tissue.

The human TRH gene and cDNA have been sequenced recently (11). We used a reverse transcription-polymerase chain reaction (RT-PCR) protocol (12) to investigate the presence of TRH mRNA in human anterior pituitary tissues.

MATERIALS AND METHODS

Tissue collection

Three human GH-secreting and two nonsecreting pituitary adenomas were collected in the operating room. Normal anterior pituitary tissue was obtained at autopsy from three subjects 4 to 8 h after death. Human hypothalamus and muscle used as positive and negative control tissues, respectively, were obtained in the same conditions. Each tissue was dissected with clean instruments to eliminate cross-tissue contamination. All samples were rapidly frozen in liquid nitrogen.

Oligonucleotide Primers

Three 21-mer primers were synthesized (Eurogentec) according to the published hTRH gene sequence (11). The 5' primer (5'-CTC-TTC-CTC-CGG-GAA-AAC-ATC-3') is located in the second exon. The outermost 3' primer (5'-CTG-GCG-TTT-TTC-AGG-CAT-CAG-3') and the internal 3' primer (5'-CTC-TTC-TTC-CCA-GCT-CCT-TTG-3') were located in the third exon. The predicted size of the two fragments amplified by RT-PCR primed with either the two external primers or the 5' and the internal 3' primers are 399 and 351 bp in length respectively. They contain a complementary sequence for a 45-mer probe (Eurogentec) for the hTRH cDNA sequence encoding the [+79aa/+93aa] region of the human proTRH (11). The predicted RT-PCR amplified fragments contain the BglII restriction endonuclease site included in the hTRH cDNA sequences spanned by the primers. The anticipated size of the RT-PCR products digested with BglII enzyme were of 337 and 62 bp for the 399 bp fragment, and 289 and 62 bp for the 351 bp fragment. The complementary sequence for the hTRH probe was conserved in the 337 and 289 bp digested products. Genomic DNA includes one intronic region of about 650 bp between the 5' and the internal 3' primers used for the RT-PCR. As positive control of RT-PCR techniques carried out with human anterior pituitary RNA as template, a parallel amplification of hGH RT RNA was performed with a set of three 21-mer primers for the hGH gene by the RT-PCR methods described below. The three primers were expected to amplify two cDNA sequences of 529 and 509 bp in length. They spanned three large intronic regions of the hGH gene (13).

RT-PCR amplification of total RNA

Total RNA was extracted from the tissues by the method of Chirgwin et al. (14). - Following the protocol of the Superscript Preamplification System for first strand cDNA synthesis (BRL), 5 μg of total RNA were primed with oligo$(dT)_{12\text{-}18}$, reverse transcribed with Moloney Murine leukemia virus RNase H^- enzyme at 42°C and treated with E. coli RNase H (BRL) before the PCR reaction. As negative control, water was substituted for the total RNA samples (blank RT).

PCR was carried out according to instructions provided with the GeneAmp DNA Amplification Reagent Kit (Perkin Elmer/Cetus) with some modifications (12,15). Two microliters of RT reaction from each sample was incubated with 0.2 mM of each dNTP,

a set of 5' and outermost 3' primers (0.5 μM) and 2.5 units of Taq polymerase (Perkin-Elmer/Cetus). The polymerase amplification was carried out using a Hybaid Thermal Reactor (Seralabo) for two sets of 20 PCR cycles. Initially, a first cycle of amplification (94°C, 1.5 min; 56°C, 1.5 min; 72°C, 1.5 min) was followed by 19 cycles (94°C, 0.5 min; 56°C, 1.5 min; 72°C, 1.5 min), after which the internal 3' primer (2 μM) was added to each sample for an additional 20 cycles and a final extension for 10 min at 72°C. The samples were then rapidly cooled to 2°C and kept on ice or frozen until analyzed.

Controls were run in parrallel with the test tissues. To reduce the risk of contamination tissue, RT RNA of normal and tumoral anterior pituitary, muscle, blank RT and hypothalamus RT RNA were respectively treated in this order. Further negative control of polymerase chain reaction was performed by substituting water for reverse transcript samples (blank PCR).

Restriction enzyme analysis

Twenty microliters of each RT-PCR sample were ethanol precipitated, redissolved in 1x reaction buffer and divided in two aliquots. One of them was treated with BglII endonuclease.

Southern blot analysis

Ten microliters of the RT-PCR samples and 10 μl of the products of the restriction enzyme analysis were electrophoresed on 2 % agarose gels in Tris borate/EDTA (TBE). Gels were stained with ethidium bromide, examined on 312-nm UV light and prepared for capillary transfer onto nylon Hybond-N membrane (Amersham)(16). The Southern blots were prehybridized for 4-6 h at 42°C in 5x SSPE, 5x Denhardt's solution, 100 μg/ml denatured salmon sperm DNA, 0.5% sodium dodecyl sulfate (SDS) and 50% deionized formamide. Hybridization was performed for 18 h at 42°C in the prehybridization solution with addition of 10% dextran sulfate and 10 ng/ml of the 3'end-labeled oligonucleotide hTRH probe (17). The blots were washed under high stringency conditions and exposed to X-AR5 film Kodak at -70°C with Dupont-Cronex Lightning plus intensifying screens.

RESULTS

PCR amplification of the RT RNA of human normal anterior pituitary tissue with the three hGH primers generated very large amounts of two products displaying the anticipated sizes of 529 and 509 bp (Fig. 1). For TRH, two bands corresponding to the predicted sizes of 399 and 351 bp (see material and methods) were readily observed after ethidium bromide staining and UV light when hypothalamic, normal anterior pituitary, GH-secreting and nonsecreting adenoma RT RNA was amplified with the set of three primers specific for hTRH cDNA. Heavy bands corresponding to amplification of genomic DNA were not observed. Amplification products were absent when muscle RNA was used in the RT-PCR with the two sets of primers.

To ascertain that the bands seen in UV light after ethidium bromide staining were amplified fragments of hTRH cDNA, Southern blot analysis was performed with a ^{32}P-labeled hTRH probe that was complementary to a part of the sequences to be amplified. Both the 399 and 351 bp RT-PCR fragments obtained with human normal and tumoral anterior pituitary and hypothalamic RNA hybridized under highly stringent conditions with the probe (Fig. 2). The two bands were less intense in the normal and tumoral anterior

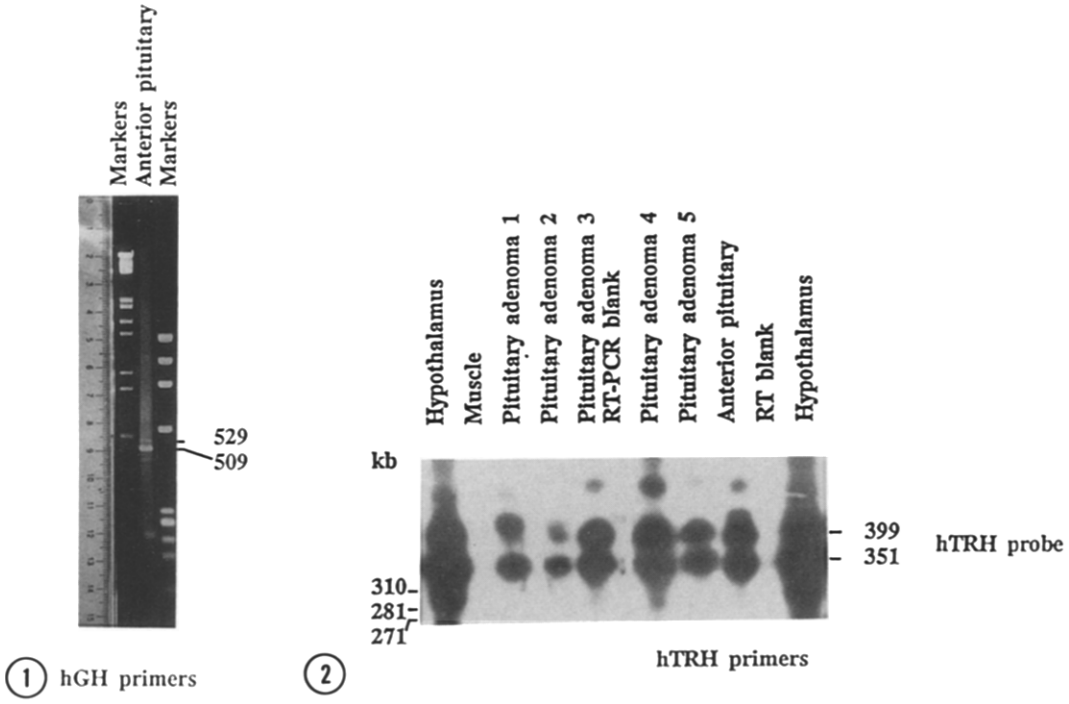

Figure 1 . Positive control of RT-PCR protocol performed with total RNA from human normal anterior pituitary tissue. After electrophoresis on 2 % agarose gel of RT-PCR products generated with the hGH primers, two intense bands with predicted sizes of 529 and 509 bp were detected by ethidium bromide staining in human normal anterior pituitary RNA. As result of the RT-PCR protocol we followed (see material and methods), the heavier 529 bp band was more intense than the 509 bp band.

Figure 2 . Evidence of mRNA TRH in human normal and tumoral anterior pituitary tissue. Southern blots of RT-PCR products from total RNA of human tissues were hybridized in 50% formamide with the 45 mer hTRH probe at 42°C for 18 h and exposed for 8 h. The two bands with predicted sizes of 399 and 351 bp were observed in both hypothalamus and tumoral or normal anterior pituitary. No signal was detected in muscle and blank even though the blot was exposed for 72 h. For these cases, the best resolutions for the hypothalamus and GH-secreting adenoma 3 were obtained after 1 and 2 h of exposure, respectively.

pituitary tissue than in the hypothalamus. No more than ethidium bromide staining and UV light did Southern blot analysis detected any amplification of genomic DNA or any positivity in the muscle and blank sample reactions.

The restriction analysis of the RT-PCR products generated with the hTRH primers showed that the BglII endonuclease digestion of the 399 bp RT-PCR product yielded the two expected fragments of 62 and 337 bp, and that of the 351 bp product two fragments of 62 and 289 bp. In Southern blot analysis, the 337 and 289 bp digested fragments of both hypothalamic and anterior pituitary RT-PCR products strongly hybridized with the hTRH probe (Fig. 3).

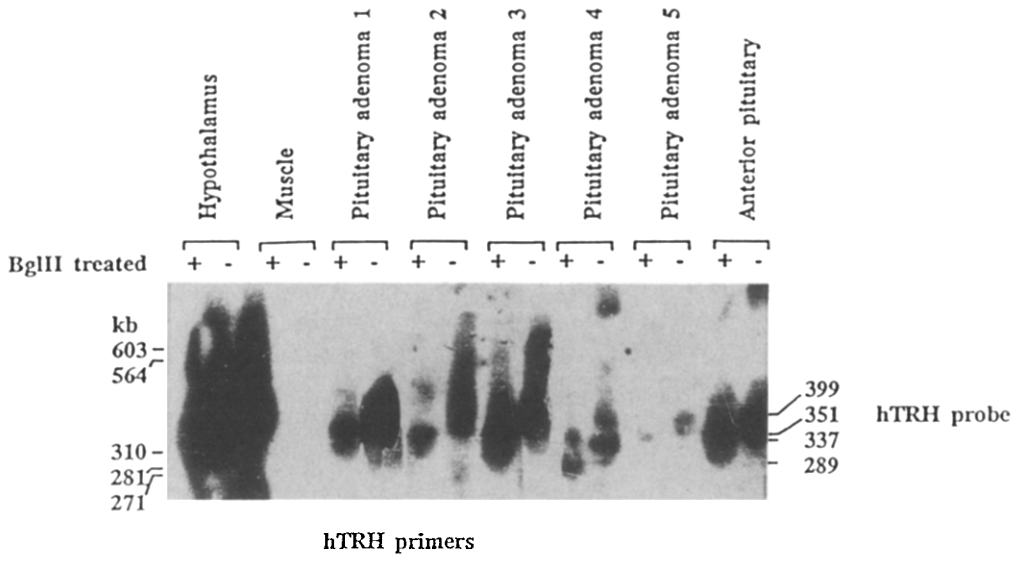

Figure 3 . Restriction analysis of RT-PCR products generated by hTRH primers. After BglII digestion, the anticipated 337 and 289 bp fragments produced respectively by the 399 bp and 289 bp amplification products still hybridized with hTRH probe. As in Fig. 2, blots in hypothalamus and the GH-secreting tumor 3 were overexposed to allow an easy detection of signal in other anterior pituitary tissue.

DISCUSSION

The present results demonstrate for the first time the presence of TRH mRNA in human anterior pituitary tissue. This demonstration is based on the identity of the RT-PCR products which were generated from the human hypothalamus, a well-established site of TRH synthesis, and the human normal and tumoral anterior pituitary, using specific hTRH primers. In these RT RNA preparations, the amplification products and the BglII restriction fragments had the predicted sizes, and the complementary sequence for a hTRH probe was conserved. The sizes of the amplification products ruled out they could have resulted from amplification of genomic DNA. The possibility that our results might be accounted for by cross-tissue contamination seemed highly unlikely since no RT-PCR products were detected when the hTRH or the hGH primers were used with muscle RT RNA and since RT blanks and PCR blanks remained always negative. Furthermore, the possibility of sample contamination in the RT-PCR protocol were reduced by always adding RNA of normal and tumoral anterior pituitary tissue first, of hypothalamus last and negative controls between them. Identical results were obtained in three repetitions of these experiments. Under these conditions, we showed that amplified sequences of the hTRH cDNA could be generated from human normal and tumoral anterior pituitary RNA.

Resorting to a RT-PCR amplification protocol specifically designed to detect a small number of copies of mRNA (12) was based upon the anticipated low abundance of

hTRH mRNA in the anterior pituitary tissue. In a previous unpublished study, we could detect a hTRH hybridization signal in human normal and tumoral anterior pituitary by RNA dot blot technique but we failed to demonstrate the presence of hTRH mRNA in these tissues by northern analysis.

Our study is in keeping with an increasing number of reports showing that several neuropeptides classically associated with the hypothalamus are also produced by the anterior lobe of the pituitary gland (18,19). Our data strongly support the hypothesis that TRH is endogenously synthesized by the anterior pituitary and could be therefore involved in local regulatory mechanisms.

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