Genomic Organization and Promoter Function of the Human Thyrotropin-releasing Hormone Receptor Gene*

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We isolated and characterized the gene for the human thyrotropin-releasing hormone receptor. The gene spanned more than 30 kilobases and contained three exons and two introns. Intron 1 exists in the 5'-untranslated region, and intron 2 is more than 25 kilobases in length which interrupts the coding region before the beginning of the putative sixth transmembrane domain. Exon 3 encodes the rest of the coding region and the entire 3'-untranslated region. The 3'-flanking region contains four potential polyadenylation signals, and 3'-rapid amplification of cDNA ends studies showed that only a signal at 2076 base pairs downstream of the stop codon was functional.In the anterior pituitary, Primer extension and anchor-polymerase chain reaction studies indicated a transcriptional start site at 344 base pairs upstream of the translational start site. The promoter region does not contain either a TATA box or a CAAT box in the appropriate location. Transient transfection study revealed significant activity of the promoter in GH4C1 cells, and the region between –338 and –933 bp from the transcriptional start site worked as a negative regulator. Knowledge of the genomic organization and the promoter region of thyrotropin-releasing hormone (TRH) receptor gene will allow further studies of possible disorders of the TRH receptor, as well as facilitate elucidation of transcriptional control of the human TRH receptor gene.

Thyrotropin-releasing hormone (TRH) is a major stimulator of the synthesis and secretion of thyrotropin (TSH) and prolactin in the anterior pituitary (1, 2). In addition, it is known to be distributed in various tissues, including the central nervous system, gastrointestinal tract, and reproductive systems, and may play a role as a neurotransmitter or neuromodulator (3). It has been reported that TRH receptor (TRHR) and its mRNA are also distributed in these tissues suggesting that the effects of TRH are mediated through its specific receptor (4–9). In the anterior pituitary, the majority of the effects of TRH are known to be mediated by activation of the inositol phospholipid-calcium protein kinase C transduction pathway (10–12).

Following cloning of the mouse TRHR cDNA by Straub et al. (16), we and others have cloned a human pituitary TRHR cDNA and its partial genomic DNA (13–17). The deduced amino acid sequence from the cDNA revealed that TRHR is a member of the family of G protein-coupled receptors containing putative seven transmembrane domains. The human TRHR has a high degree of homology with those of the rat and mouse (18–21). However, the regions corresponding to the carboxyl terminus differ completely between these species; the mouse TRHR is shortest, 393 amino acids in length, while the human and rat TRHRs contain an additional 5 and 19 amino acids, respectively. Using these cDNAs as probes, TRHR mRNA levels in the anterior pituitary and GH cells were shown to be regulated transcriptionally or post-transcriptionally by thyroid hormones, estrogen, dexamethasone, and epidermal growth factor (EGF) (22–27). The presence of response elements for these factors in the promoter region of the TRHR gene is therefore anticipated. However, the complete genomic organization and the promoter region of the TRHR gene have not yet been determined in any species. As a step toward elucidation of possible genetic disorders of the TRHR and transcriptional regulation of the TRHR gene, we cloned and characterized the human TRHR gene and investigated its promoter activity.

MATERIALS AND METHODS

Isolation of Human TRH Receptor Genomic Clones—A human genomic DNA library derived from placental DNA in EMBL3 SP6/T7 (Clontech) was used in this study. Approximately 1 × 10^6 recombinants were screened using a ^32P-labeled rat TRHR cDNA (rTRHR-2) encoding the region between the first and fifth transmembrane domains of the rat TRHR that we recently cloned (21). Filter hybridization was performed at 37 °C using the previously described method (28). Filters were washed twice at room temperature for 15 min in 2 × SSC and twice at 50 °C for 15 min in 2 × SSC and 1% SDS. Two genomic clones were isolated and characterized by restriction endonuclease mapping. The restriction digests were subjected to electrophoresis, transferred onto a nylon membrane, and hybridized with the rTRHR1 probe. All hybridized genomic fragments were subcloned into pGEM4Z or pGEM3Z for further restriction analysis and sequenced by the dideoxy chain-termination method using Sequenase 2.0 and sequence-specific oligonucleotides. To obtain the 3' portion of the human TRHR gene, another screening was performed under the same conditions with the rat TRHR 2 cDNA that encodes the region between the sixth transmembrane domain and carboxyl terminus of the rat TRHR.

Preparation of Total RNA and Poly(A)^+ RNA—Poly(A)^+ RNAs and total RNA were prepared from GH-secreting adenomas and normal pituitaries obtained from operations or autopsies at Gunma University as described previously (17). Informed consent was obtained from each family, and this study was approved by the ethical committee on human research of Gunma University.

Reverse Transcription-Polymerase Chain Reaction—PCR was used to determine the sizes of introns and exon-intron boundaries. Two μg of poly(A)^+ RNA from a GH-secreting adenoma were subjected to reverse transcription using Moloney murine leukemia virus reverse transcriptase and purified according to the manufacturer's instructions (GIBCO BRL). The resulting cDNA was used as a template for amplification by PCR using primer pairs specific for the human TRHR coding region.
transcription for 2 h at 37 °C. PCR amplification of cDNA and 100 ng of human genomic DNA extracted from leukocytes was performed essentially as described previously (28). Forty cycles of amplification were carried out using a touchdown program (94 °C for 1 min, 60 °C for 2 min, 72 °C for 2 min), followed by a 15-min final extension at 72 °C. Primers were designed to span the exon 1/exon 2 boundary (E1, 5'-attaggcaatactgctgtgctg-3'; E2, 5'-gatttaaagccacactcctagctg-3'), as shown in Fig. 2A. Amplification products were gel purified, subcloned into the pGEM4Z plasmid, and sequenced.

Southern Blot Hybridization Analyses of the Human TRHR Gene—Approximately 10 ng of human genomic DNA extracted from human leukocytes digested with BstEII and combinations of BstEII with BamHI, EcoRI, Sall, or XhoI were separated on a 0.8% agarose gel and then transferred onto a nylon membrane. The membrane was hybridized with 32P-labeled probe A or B and washed at 68 °C in 0.1 × SSC, 0.1% SDS as described previously (Fig. 2B) (28). Probe A, a 0.8-kb fragment, corresponded to the region between the adapter (5'-GCCGCTTGCAATGGGATCC-3') and the EII site on the intron 2/exon 3 boundary (5'-GCCGCTTGCAATGGGATCC-3'). These two probes were end-labeled with [γ-32P]ATP, hybridized to 40 µg of total RNA extracted from the human pituitary GH-secreting tumor, and extended using avian myeloblastosis virus reverse transcriptase. Forty µg of total RNA from the human liver was used as a negative control. Norther blot hybridization analysis revealed that human TRHR mRNA was not present in the liver (data not shown). The primer extended products were separated on an 8% urea, 6% polyacrylamide gel. The gel was then dried and exposed to Kodak XAR-5 film. The sizes of the resulting labeled primer-extended products were inferred from their co-migration with a sequencing ladder, which was obtained using the same primer with an exon 1-containing clone.

Identification of the Transcription Start Site by Anchor-PCR—To confirm the results obtained by primer extension and due to the difficulty of obtaining a normal intact pituitary, anchor-PCR was performed using 0.28 µg of poly(A)-RNA and 5 µg of total RNA obtained from an anterior pituitary. First-strand cDNA was extended with random di- gonucleotides using avian myeloblastosis virus reverse transcriptase at 42 °C. The extended products were purified from excess primer using GENO-BIND (Clontech). Ligation of cDNA to the anchor oligonucleotide (5'-aggccattagacctgccaacctgga-3') was carried out at room temperature overnight with 5 units T4 RNA ligase in T4 RNA ligase buffer (50 mm Tris-HCl (pH 8.0), 10 mm MgCl2, 1 mm hexamine cobalt chloride, 20 µM ATP, 25% polyethylene glycol 8000). After ligation was terminated, the first PCR amplification was carried out using 1 µl of ligated cDNAs and 25 pmol of an internal oligonucleotide (AP1 (5'-agccctttccaactaatgacaaacc-3') or AP2 (5'-agccctttccaactaatgacaaacc-3')) and a complementary anchor oligonucleotide (5'-aggccattagacctgccaacctgga-3') in a similar fashion. The PCR products were analyzed electrophoretically using 1.5% agarose gels and visualized by ethidium bromide staining. PCR amplification products were gel purified, subcloned into pGEM4Z, and sequenced.

Analysis of 3’-Untranslated Region by 3’-Rapid Amplification of cDNA Ends (3’-RACE)—To detect functional polyadenylation signals in the 3’-untranslated region of the human TRHR gene, 3’-RACE was performed as described previously (28). Briefly, the first strand was synthesized with 5 µg of total RNA from the anterior pituitary and an oligo(dT)17 primer and a downstream internal primer (PA316, 5'-tggccctaatgagaacatacacg-3'; PA317, 5'-tggccctaatgagaacatacacg-3'), as shown in Fig. 4B. The PCR products were analyzed by electrophoresis, subcloned, and sequenced.

RESULTS

Isolation and Characterization of the Human TRHR Gene—Comparison of the genomic sequence with that of the cDNA established the organization of the human TRHR gene as three exons and two introns (Fig. 1). As shown in Fig. 2A, a polymerase chain reaction product generated from the cDNA prepared from a human pituitary GH-secreting adenoma had the exact size and nucleotide sequence predicted for a transcript of the cDNA. The reaction was initiated by the addition of 200 µl of 0.2 µM dNTPs and light emission was measured for 10 s using a luminometer. Luc activity was expressed as arbitrary light units/µg of cellular protein. Transcriptional activity corrected for the efficiency of transfection by measuring β-galactosidase (pSIV-Gal, Promega) activity.

Phenotypically using 1% agarose gels and subjected to Southern blot analysis with a 32P-labeled genomic fragment containing the 3’-untranslated region as a probe. Positive fragments were subcloned and sequenced as described above.

Cell Culture and Transfection—GH4C1, CV-1, and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 µg/ml) (Life Technologies, Inc.), and amphotericin B (0.25 µg/ml) (Sigma). Cells were plated 24 h before transfection into 60-mm tissue culture dishes at subconfluent density. Transient transfection was performed by the calcium phosphate precipitation method using Cellfectin (Pharmacia Biotech Inc.) with 3 µg of reporter construct. Glycerol shock was performed 16 h after transfection for 2 min with 15% glycerol in phosphate-buffered saline. The cells were then harvested after an additional 48 h.

Plasmid Construction—pA3Luc is a promoterless luciferase expression vector containing a polynucleotide linker sequence upstream of the HindIII-SacII fragment derived from pSV5a5 and was propagated in pGEM3 (a gift from Dr. W. M. Wood, University of Colorado Health Sciences Center). TKLuc contains the herpes simplex virus TK promoter linked to pA3Luc. The human TRHR gene PstI-HindII fragment (containing 933 bp of the promoter region, 256 bp of the exon 1, and 136 bp of the first intron), the HindII fragment from –337 to +256 and 136 bp of the first intron, and the PstI fragment from –337 to –228 were subcloned into pA3Luc and were named pPH1.4Luc, pPH0.8Luc, and pPH0.7Luc, respectively. These constructs were transfected into GH4C1, HeLa, and CV-1 cells as described above.

Luciferase Assay—To determine luciferase (Luc) activity, cell monolayers were rinsed twice with phosphate-buffered saline and then lysed with 400 µl of 25 mm glycylglycine, pH 7.8, containing 15 mm MgSO4, 4 mm EGTA, 1 mm dithiothreitol, and 1% (w/v) Triton X-100. Cells were scraped from the dishes and centrifuged at 12,000 × g for 5 min at 4 °C. Assays for Luc activity were performed using 100-µl aliquots of cell lysate and 350 µl of 25 mm glycylglycine, pH 7.8, containing 15 mm MgSO4, 4 mm EGTA, 1 mm dithiothreitol, and 2 mm ATP. The reaction was initiated by the addition of 200 µl of 0.2 µm d-luciferin, and light emission was measured for 10 s using a luminometer. Luc activity was expressed as arbitrary light units/µg of cellular protein. Transcriptional activity corrected for the efficiency of transfection by measuring β-galactosidase (pSIV-Gal, Promega) activity.

Statistical Analysis—Statistical analysis was performed by the Dunnet’s multiple range test.

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digested with BstEII and XhoI, only the BstEII fragment hybridized with probe A was reduced in size, indicating that intron 2 was at least 25 kb in length.

Analysis of the 5' Flanking Sequence of the Human TRHR Gene—Primer extension with 40 μg of total RNA from the human pituitary GH-secreting adenoma and the PE-1 primer gave a signal at 344 bp from the translational initiation site, whereas no significant signals were found in liver total RNA or yeast transfer RNA. No extension was identified using primer PE-2 or PE-3, suggesting that there are no other start sites in more upstream regions (data not shown).

To confirm the results of the primer extension studies, we performed anchor-PCR of the human TRHR transcript in intact anterior pituitary (Fig. 4B). Sequence analysis revealed that all eight anchor-PCR clones had residue 344 as the transcriptional start site. On the basis of these observations, the transcriptional start site of the human TRHR gene was assigned to the adenosine residue 344 bp upstream of the translational initiation site.

Inspection of the sequence of the TRHR promoter region indicated that there is no typical TATA box, CAAT box, or GC-rich sequence in close proximity to the transcriptional start site. However, several possible regulatory elements were identified including complete sequence matches for PEA-3 (at −427, AGGAAG, underline indicates nucleotides match to the consensus sequence), GATA motif (at −2219 and −2346, AGATAT; at −286, CTATCA), two 6/7 match Pit-1 binding sites (at −451, TTTCAT; at −384, TTTCAT), a 6/7 match AP-1 site (at −149, TGTCCTA), two 5/6 match half sites of thyroid hormone response element (TRE) (at −355 and −252, AGCTCA), and a 12/15 match palindromic site of glucocorticoid response element (at −246, GGTTGACTAGTTCT).

Analysis of the 3' Flanking Region of the Human TRHR Gene—Sequence analysis revealed four potential polyadenylation signals (AATAAA) in the 3' untranslated region of the human TRHR gene (Fig. 3). To determine which is functional in the anterior pituitary, three sets of 3'-RACE experiments were performed using the primers shown in Fig. 5. A single band was
amplified using the primers RA315 and 316 and was subcloned into pGEM4Z. No amplification was observed with primers NTR1 and RA307 or with RA307 and RA308. Sequence analysis indicated only a single functional polyadenylation signal 2076 bp from the stop codon in the anterior pituitary, and thymidine residue 26 bp downstream of the signal was the polyadenylation site. In addition, there are eight copies of the ATTTA pentamer motif, which may affect mRNA stability, in the 3'-untranslated region of the human TRHR gene.

Promoter Activity of the Human TRHR Gene—To determine whether the putative promoter region is functional, a 1.4-kb fragment containing approximately 900 bp of the promoter region was subcloned into a luciferase reporter plasmid. As illustrated in Fig. 6A, significant expression was observed only in GH4C1 cells transfected with either pPH1.4Luc or pH0.8Luc (data not shown) constructs. Luciferase activity of pH0.8Luc in GH4C1 cells was approximately 15,000 arbitrary light units/10 s/100 μg of protein; when the expression of pPH1.4Luc in GH4C1 cells was set as 100%, the activities of pPH0.8Luc and pPP0.7Luc were 474.9 ± 31.5 and 9.47 ± 5.5%, respectively (Fig. 6B).

DISCUSSION

We previously reported the existence of an intron in the coding region of the human TRHR gene based on its partial genomic sequence (17). The partial genomic sequence of the mouse TRHR has also recently been reported (13). However, the complete structure, including transcriptional start site, number of introns, and functional polyadenylation signals etc., has not been reported. In the present study, we established the complete structure of the human TRHR gene, which contains three exons and two introns and spans a region of more than 30 kb. The first exon contains only the 5'-untranslated region, the second exon begins from 88 bp upstream of the translational initiation site, and the second intron started just before the beginning of the sixth transmembrane domain and is more than 25 kb in length. The third exon contains the rest of the coding sequence and the entire 3'-untranslated region. The organization of the human TRHR gene differed from that of the mouse TRHR gene (16). Although the mouse TRHR gene possesses an intron at an identical location in the 5'-untranslated region, it does not have an intron in the transmembrane region, but instead, it has one intron in the region close to the carboxyl terminus and another in the 3'-untranslated region. These insertions or deletion of the intron may be the result of evolution of the TRHR gene. Further cloning of TRHR genes in other species may be helpful for understanding evolutionary changes of the TRHR gene.

Primer extension and anchor-PCR studies revealed a single transcriptional start site at adenine nucleotide 344 bp upstream of the translational start site. This start site is characteristic of other mammalian genes with consensus sequences PyCAPy, where the deoxyadenosine residue is the start of transcription (29, 30). Inspection of the sequence of the promoter region of the human TRHR gene revealed that consensus TATA and CAAT boxes are not located at the appropriate FIG. 3. Nucleotide sequence of the human TRHR gene. Exons are shown in upper case letters. Introns are described in lower case letters. Proposed transcriptional initiation site is shown with an asterisk and is numbered +1. Several potential cis-acting sequences (PEA-3, Pit-1, thyroid hormone response element, glucocorticoid response element, AP1, and GATA motif) and dinucleotide TG repeats are double-underlined. Predicted transmembrane domain and ATTATA pentamer motif in the 3'-untranslated region are underlined. Classical polyadenylation signals (AATAAA) are represented in bold letters.
The absence of typical TATA and CAAT sequences are characteristic of other G protein-coupled receptors such as those for β-adrenergic, α1b-adrenergic, luteinizing hormone, TSH, and gonadotropin-releasing hormone (GnRH) (31–39). Therefore, these genes may be regulated by common mechanisms.

There are several d(TG)n dinucleotide repeats present in the 5′-region of the human TRHR gene, similar to the mouse TRHR gene (13). These repeats are often found in areas that serve as regulatory elements for gene expression and are thought to be potential Z-DNA-forming sequences. It has been suggested that Z-DNA, which appears at critical control regions of genes, might be involved in regulation of gene expression particularly since a similar dinucleotide repeat has been shown to exert a negative effect on transcription of the rat prolactin gene (40). Functional studies are needed to determine whether the d(TG)n repeat plays a role in regulating transcription of the human TRHR gene.

We identified several possible cis-acting regulatory sequences in the promoter region. Of special interest was the identification of two Pit-1 binding sites. Pit-1, an anterior pituitary-specific transcription factor, has been reported to be involved in the regulation of anterior pituitary hormones, for example, in the activation of theGH, TSH, and prolactin genes (41–43). Because TRHR mRNA was also identified in TSH, prolactin, and GH-producing tumors, Pit-1 may be involved in the regulation of anterior pituitary TRHRs through activation of the TRHR gene.

GH4C1 cells used in the transfection study were derived from rat pituitary tumor cells and are known to express a number of TRHRs, and CV-1 and HeLa cells were derived from embryonic monkey kidney and human chorionic carcinoma cells, respectively. Therefore, GH4C1 cells might be expected to express the TRHR gene. As expected, the PH1.4 fragment including the 933 bp putative promoter region was transcriptionally active only in GH4C1 indicating that this fragment may be sufficient for tissue-specific expression. Furthermore,
Characterization of Gene for Human TRH Receptor

Fig. 6. Analysis of the human TRHR promoter. A, constructs containing TRHR gene upstream fragments were subcloned in front of the luciferase reporter gene and transiently transfected into GH4C1, HeLa, and CV-1 cells. The data are expressed as relative luciferase activity (arbitrary light units of the TRHR promoter/that of the TK promoter). Values represent means ± S.D. of triplicate determinations in the data in GH4C1 cells set to 100%. At least three independent experiments were performed. B, the physical maps of the luciferase fusion constructs are shown. The first exon is indicated by a box. GH4C1 cells were transiently transfected with the indicated plasmids, and luciferase activity was measured. The value of pH1.4Luc was set as 100%, and other values are presented as means ± S.D. of triplicate determinations. The control used was the promoterless luciferase plasmid, the typical activity of which measured ~500 arbitrary light units/10^6 g of protein.

deletion of the upstream fragments PH0.7 (–933 to approximately −388) from pH1.4 (–933 to approximately +256) led to a significant increase in the promoter activity, suggesting that this region works as a negative regulatory element such as a repressor in the human TRHR gene.

In conclusion, the genomic structure and the promoter region of the human TRHR gene were characterized. The characterization of the human TRHR gene and its promoter should facilitate further study of the genetic diseases involving this gene and the mechanisms involved in the transcriptional regulation of its expression.

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