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MOLECULAR CLONING OF A FUNCTIONAL HUMAN THYROTROPIN-RELEASING HORMONE RECEPTOR

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Summary: A cDNA encoding the human thyrotropin-releasing hormone receptor (hTRH-R) was isolated from a human brain cDNA library. Screening of 1.2 million clones resulted in 2 candidates. The largest clone contained TRH-R homologous sequences starting in the third transmembrane domain and included a long 3′ untranslated sequence. The smaller clone contained a potential start of the open reading frame, but was interrupted by an intron in the sixth transmembrane domain. The two clones had 497 bp of overlapping identical sequences and it was possible to assemble a complete cDNA thus restoring the assumed coding sequence. Electrophysiological studies of frog oocytes injected with *in vitro* transcribed mRNA showed TRH-specific inward currents, demonstrating that the reconstituted cDNA encoded a functional receptor. The predicted amino acid sequence of the hTRH-R protein showed high homology with the rat and mouse TRH-Rs with the exception of their C-terminal region. The human TRH-R gene seems to contain two introns.

Thyrotropin-releasing hormone (TRH), a small neuropeptide ($pGluHisProNH_2$) is widely distributed throughout the central and peripheral nervous system as well as in extraneural tissues. The peptide is synthesized in the hypothalamus and transported by the portal vascular system to the anterior pituitary where it acts on thyrotropic and lactotropic cells to promote secretion of TSH and prolactin, respectively (1). Functional aspects of the TRH receptor, TRH-R, have been studied in detail using rat and mouse anterior pituitary tumour cell lines. TRH binding to the receptor excerts bifunctional effects mediated through different stimulatory G-proteins: activation of a phosphoinositol-4,5-bisphosphate-specific phospholipase C via $G\alpha_q$ and/or $G\alpha_{11}$ (2) and adenylyl cyclase via $G\alpha_s$ or a homologous protein (3-5). Ligand binding and oocyte expression studies suggested that there might be two types of TRH-Rs in the rat GH4C1 pituitary cells (6,7).

Recently, cDNAs encoding the TRH-R were isolated from rat and mouse tumour cell lines. The mouse TRH-R cDNA was isolated by expression cloning using mRNA from the mouse pituitary thyrotropic TtT tumour cells (8). The 3.8 kb cDNA encoded a protein of 393 amino acids. The rat TRH-R cDNA, isolated by PCR-cloning using mRNA from rat GH4C1 cells (9) had a size of 3.5 kb and encoded a protein of 412 amino acids. Both receptors showed a high degree of homology except for sequences in their C-terminal region. In a parallel work, a rat TRHR cDNA

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was isolated using mRNA from GH3 cells (10). A third rat TRH-R cDNA was isolated from an anterior pituitary cDNA library (15).

In the present work we describe the cloning of a human TRH-R cDNA isolated from a central nervous system (CNS) library. Two candidates (clone I and II) containing highly homologous sequences to the mouse and rat TRH-Rs were identified after screening 1.2 million clones. Curiously, none of these contained an open reading frame which was entirely colinear with the mouse and rat TRH-R sequences. Both isolated cDNAs seemed to have been synthesized from partially spliced mRNAs. We were, however, able to assemble from the two clones a complete colinear cDNA with an intact coding sequence based on species homology considerations. The deduced human TRH-R amino acid sequence showed 95% identity with the rat and mouse sequences, except for its C-terminal region where the TRH-Rs from all three species differed considerably. The reconstituted human cDNA was in vitro transcribed, injected into frog oocytes and the following electrophysiological studies showed characteristic membrane potential changes where a biphasic inward current was elicited dose-dependently reaching a maximum at 10^{-6} M. This demonstrated that mRNA made from the reconstituted cDNA encodes a functional receptor.

MATERIALS AND METHODS

Preparation of a TRH-R-specific probe using the polymerase chain reaction (PCR). Three degenerate PCR primers were constructed; one forward primer (A) and two in the reverse direction (B,C) using sequence information from two mouse TRH-R cDNA regions which exhibited a high homology to other G-protein coupled receptors. The sequence of the forward primer was taken from the first transmembrane domain while the two reverse primer sequences were located to the sixth transmembrane domain:

A) 5′- GCGCATGGATCC ATT GTI GGI AAT ATT ATG GT-3′
A A
B) Outer reverse primer: 5′- GGCCGCGGATCCTAG IGT ICT GTA IGG CAT CCA-3′
G
C) Inner reverse primer: 5′-GCGCTGGATCC G CAT CCA IAA IAG IGCAAAC,
G A G

where I represents inosine. The TRH-R-specific probe was generated by two rounds of PCR using as template mRNA which was isolated from rat anterior pituitary tumour cells (GH4C1 cells). 5 µg of total mRNA was reverse transcribed using MMLV-H$^-$-reverse transcriptase (superscript, Gibco-BRL) to make the first DNA strand according to the manufacturers protocol. One quarter of this reaction mixture was amplified by PCR using the primers A and B. 2% of this reaction mixture was reamplified in a second round of PCR now using primers A and C. PCR conditions were as following: denaturing at 95°C, 1 min; annealing at 45°C, 2 min and extension at 72°C, 2 min.

Screening of a human CNS cDNA library. The PCR products were purified by GeneClean (BIO 101, Inc.), cleaved with the restriction enzyme *Bam*HI and cloned into Bluescript SKII$^+$ (Stratagene). Inserts were sequenced by the dideoxy chain termination method (16). One identified rTRH-R insert of 700 bp was excised from the vector and the purified fragment was then labelled with ^{32}P in order to be used as a probe to screen a fetal human (female) cDNA library made in lambda ZAPII. Hybridization was carried out for 16 hr at 42°C in 25% formamide/6xSSC/ 10% dextran sulfate/0.1% SDS/5x Denhardt's solution (12). No salmon sperm DNA was used. Filters were washed twice for 5 min at room temperature and twice for 30 min at 42°C in 2x SSC/ 0.1% SDS. The positive clones which were found with the TRH-R-specific probe ("hTRHR I-V") under stringent conditions were picked, rescued into Bluescript SK$^-$ and the plasmid mapped with restriction endonucleases. Two clones that gave the strongest hybridization signals were subcloned and verified by sequence determination. Each insert was sequenced in both directions.

Expression of hTRH-R in *Xenopus* oocytes. *Xenopus laevis* frogs were obtained from dr. Horst Kähler (Institut für Entwiklungsbiologie, Kollaustr. 113b, D-2000 Hamburg 61, Germany). Stage VI oocytes were manually defolliculated using fine watchmaker forceps, and then rolled in poly-L-lysine coated Petri dishes to remove all remnants of follicle cells. The *in vitro* synthesized mRNA was dissolved in H2O at a concentration of 0.1 mg/ml and pressure injected (50 nl) into the oocytes. Injected oocytes were incubated at 19 oC for 48 hr in Barth's medium. Traditional two electrode voltage clamping using a Biologic (Claix, France) amplifier, was employed to examine the oocytes for expression of functional hTRH-receptors (7).

RESULTS AND DISCUSSION

When this work was initiated the only TRH-R sequence information available was the mouse sequence (8). In order to obtain a probe to screen a human CNS cDNA library we performed PCR on reverse transcribed mRNA from rat pituitary tumour cells. Degenerate primers were synthesized using sequence information from conserved regions in the first and sixth transmembrane domain of the cloned mouse TRH-R. One forward primer sequence from the first transmembrane domain and two reverse primers from the latter region were designed. Two successive rounds of PCR with the two pairs of primers gave a product of the expected size of 700 bp. Sequence determination confirmed the nature of the DNA product as a rTRH-R specific fragment (9).

The cloned PCR product was further used to probe a human CNS cDNA library. A total number of 1.2 million clones was screened at low stringency and resulted in 5 candidates, two of which (hTRHR-I and II) gave strong signals also at high stringency. Restriction endonuclease analysis of the rescued plasmids showed that all five had a unique restriction map (see Fig. 1). Two clones, hTRHR-I and II, were selected for further analysis and both clones were found to contain sequences with strong homology to the mouse and rat TRH-R sequences. Curiously, none of them represented a full length cDNA. The largest clone I (hTRHR-I), with a 4 kb insert contained a TRH-R sequence starting in the third transmembrane domain and included a long 3′ untranslated sequence (Fig. 1).In clone II with a 2.5 kb insert, we identified the potential

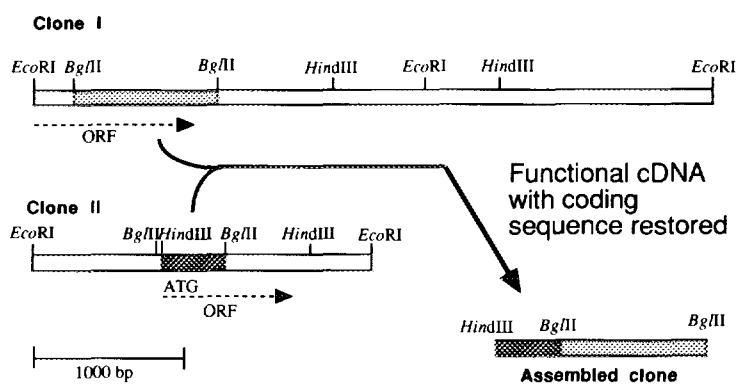

Figure 1. Assembly of the complete cDNA for the human TRH receptor from two positive clones. A *Hin*dIII subclone of clone II was digested with *Bgl*II and *Bam*HI (in the polylinker) and a *Bgl*II subfragment of clone I was inserted. One resulting clone with insert of correct orientation was sequenced and found to contain a full TRH-R coding sequence. The fragment from clone I is indicated as dotted area and that from clone II as cross-hatched area. ORF designates "open reading frame".

translation startcodon which was missing in clone I. In addition we found a 789 bp sequence downstream of the ATG that was highly homologous to the first part of the rat and mouse TRH-R sequences (8,9,10). After this position, which corresponded to the sixth transmembrane domain, the sequence abruptly diverged relative to the mouse and rat sequence containing a site with strong homology to the splice site consensus sequence (hTRH-R: CAG/GTAAGC compared to the 5'-splice site consensus sequence MAG/GTAAGT (17) where M=A or C). Maps of the two clones are shown in Fig.1. As is the case for the dopamine2 receptor (13) the intron in the sixth transmembrane region could give rise to isoforms of the TRH-R by alternative splicing. This region has been implicated in G-protein coupling and might mediate activation of different effector molecules. Analysis of ^{3}H-TRH binding has indicated the presence of different sites with high and low K_Ds (6), and recently de la Pena *et al.* (11) reported two isoforms of rTRH-R generated by alternative splicing (11).

The two clones contained 497 bp identical overlapping sequences. It was possible to assemble a complete cDNA where the assumed full coding sequence was restored as illustrated in Fig. 1. The DNA sequence of this reconstituted cDNA is shown in Fig. 2. The deduced amino acid sequence predicts a protein of 398 amino acids with a molecular mass of 45 kDa. The functionality of this putative hTRH-receptor was examined by microinjection of mRNA transcribed from the cDNA template, into *Xenopus* oocytes. Water injected or non injected control oocytes never responded to TRH stimulation. However, addition of TRH to mRNA injected oocytes resulted always in a characteristic electrophysiological response consisting of a biphasic inward current at a holding potential of -70 mV (Fig. 3a). The reversal potential of this current was close to -30 mV (Fig. 3b) and the magnitude of the current response saturated at a TRH concentration of approximately 10^{-6} M (Fig. 3c). Both the wave form and the reversal potential of the TRH-induced current strongly suggests that the response was due to an activation of the endogenous inositol trisphosphate second messenger-mediated pathway of the oocytes. We therefore concluded that the cDNA encoded a fully functional hTRH-receptor.

An alignment of the hTRH-R with the mouse and rat TRH-R amino acid sequences is shown in Fig. 4. The overall homology is very high. Differences between the species are often conservative in order to maintain the function. Changes seem to be clustered between long stretches of identity suggesting the presence of regions with less functional importance. Compared to the mouse and rat sequences the human receptor shows 94.6% and 95.7% identity, respectively, in the first 392 amino acids. After amino acid position 392 the three sequences diverge; the mouse sequence contains one additional amino acid, the rat sequence 20 amino acids and the human receptor has a six amino acids extension. The functional significance of this difference, if any, remains to be elucidated.

The rTRH-R gene has been characterized by de la Pena et al. (11). They reported the occurrence of two reverse transcribed cDNAs which code for functional TRH-R when expressed in oocytes.These transcripts are probably generated by alternative splicing of a retained intron (54bp) of a gene represented only once in the rat genome. The two isoforms show indistinguishable functional properties when expressed in *Xenopus laevis* oocytes. Their results demonstrate a perfect colinearity between genomic DNA and cDNA in the two isoforms except for the retained intron. From genomic mapping of the TRH-R in mouse (15) it was found that the 20 amino acids extension in the C-terminal of the rat TRH-R (9) is also present in the mouse gene, but separated by a 233 bp long intron. The human sequence with a unique six amino acid

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ATG GAA AAC GAG ACA GTC AGT GAA CTG AAC CAA ACA CAG CTT CAG CCA CGA GCA GTG GTG  60
Met glu asn glu thr val ser glu leu asn gln thr gln leu gln pro arg ala val val  20

GCC TTA GAA TAC CAG GTG GTC ACC ATC TTA CTT GTA CTC ATT ATT TGT GGC CTG GGT ATT 120
ala leu glu tyr gln val val thr ile leu leu val leu ile ile cys gly leu gly ile  40

GTA GGC AAC ATC ATG GTA GTC CTG GTT GTC ATG AGA ACC AAG CAC ATG AGG ACC CCC ACA 180
val gly asn ile met val val leu val val met arg thr lys his met arg thr pro thr  60

AAC TGC TAC CTG GTG AGC CTG GCA GTA GCT GAT CTC ATG GTC TTG GTG GCC GCA GGC CTC 240
asn cys tyr leu val ser leu ala val ala asp leu met val leu val ala ala gly leu  80

CCC AAC ATA ACA GAC AGT ATC TAC GGT TCC TGG GTC TAT GGC TAT GTT GGA TGC CTC TGC 300
pro asn ile thr asp ser ile tyr gly ser trp val tyr gly tyr val gly cys leu cys 100

ATT ACT TAC CTC CAG TAT TTG GGA ATT AAT GCA TCC TCT TGT TCA ATA ACA GCC TTT ACC 360
ile thr tyr leu gln tyr leu gly ile asn ala ser ser cys ser ile thr ala phe thr 120

ATT GAG AGG TAC ATA GCA ATC TGT CAC CCC ATC AAA GCC CAG TTT CTC TGC ACA TTT TCC 420
ile glu arg tyr ile ala ile cys his pro ile lys ala gln phe leu cys thr phe ser 140

AGA GCC AAA AAG ATT ATC ATC TTT GTC TGG GCT TTC ACA TCT CTT TAC TGT ATG CTC TGG 480
arg ala lys lys ile ile ile phe val trp ala phe thr ser leu tyr cys met leu trp 160

TTC TTC TTG CTG GAT CTC AAT ATT AGC ACC TAC AAA GAT GCT ATT GTG ATA TCC TGT GGC 540
phe phe leu leu asp leu asn ile ser thr tyr lys asp ala ile val ile ser cys gly 180

TAC AAG ATC TCC AGG AAT TAC TAC TCA CCT ATT TAC CTA ATG GAC TTT GGT GTC TTT TAT 600
tyr lys ile ser arg asn tyr tyr ser pro ile tyr leu met asp phe gly val phe tyr 200

GTT GTG CCA ATG ATC CTG GCT ACC GTC CTC TAT GGA TTC ATA GCT AGA ATC CTT TTC TTA 660
val val pro met ile leu ala thr val leu tyr gly phe ile ala arg ile leu phe leu 220

AAT CCC ATT CCT TCA GAT CCT AAA GAA AAC TCT AAG ACA TGG AAA AAT GAT TCA ACC CAT 720
asn pro ile pro ser asp pro lys glu asn ser lys thr trp lys asn asp ser thr his 240

CAG AAC ACA AAT CTG AAT GTA AAT ACC TCT AAT AGA TGT TTC AAC AGC ACA GTA TCT TCA 780
gln asn thr asn leu asn val asn thr ser asn arg cys phe asn ser thr val ser ser 260

AGG AAG CAG GTC ACC AAG ATG CTG GCA GTG GTT GTA ATT CTG TTT GCC CTT TTA TGG ATG 840
arg lys gln val thr lys met leu ala val val val ile leu phe ala leu leu trp met 280

CCC TAC AGG ACT CTA GTG GTT GTC AAC TCA TTT CTC TCC AGT CCT TTC CAA GAA AAT TGG 900
pro tyr arg thr leu val val val asn ser phe leu ser ser pro phe gln glu asn trp 300

TTT TTG CTC TTT TGC AGA ATT TGC ATT TAT CTC AAC AGT GCC ATC AAC CCG GTG ATT TAC 960
phe leu leu phe cys arg ile cys ile tyr leu asn ser ala ile asn pro val ile tyr 320

AAT CTC ATG TCC CAG AAA TTC CGT GCA GCC TTC AGA AAG CTC TGC AAC TGC AAG CAG AAG 1020
asn leu met ser gln lys phe arg ala ala phe arg lys leu cys asn cys lys gln lys 340

CCA ACA GAG AAA CCT GCT AAC TAC AGT GTG GCC CTA AAT TAC AGC GTC ATC AAG GAG TCA 1080
pro thr glu lys pro ala asn tyr ser val ala leu asn tyr ser val ile lys glu ser 360

GAC CAT TTC AGC ACA GAG CTT GAT GAT ATC ACT GTC ACT GAC ACT TAC CTG TCT GCC ACA 1140
asp his phe ser thr glu leu asp asp ile thr val thr asp thr tyr leu ser ala thr 380

AAA GTG TCT TTT GAT GAC ACC TGC TTG GCT TCT GAG GTA TCC TTT AGC CAA AGT TGA
lys val ser phe asp asp thr cys leu ala ser glu val ser phe ser gln ser OPA
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Figure 2. Nucleotide and deduced amino acid sequences of the assembled human TRH receptor cDNA.

extension also has a splice site consensus sequence at the point of divergence. From our findings we suggest that the human TRH-R gene is interrupted by at least two introns, one starting in position 790 and one in position 1177 of the cDNA (Fig.1).

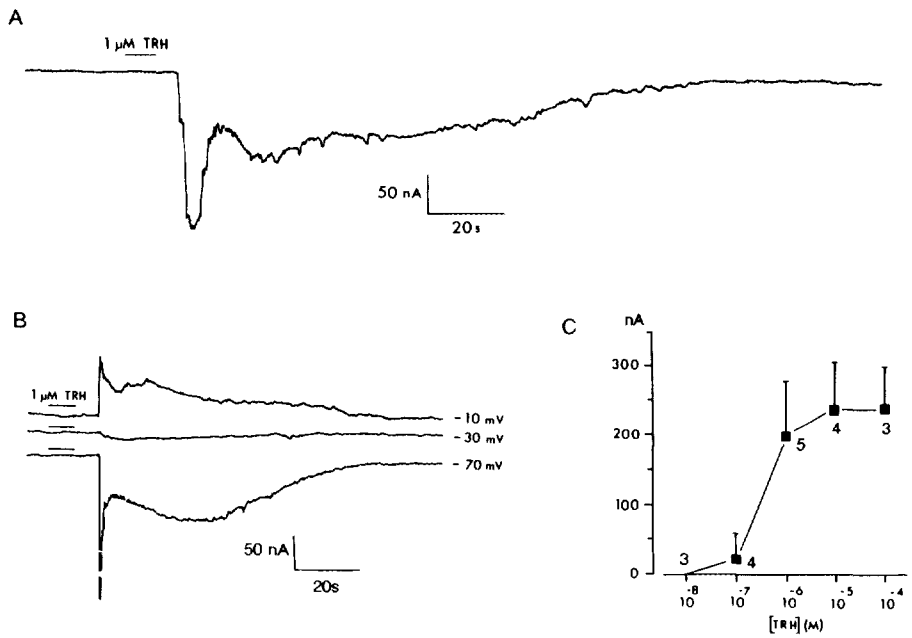

Figure 3. Expression of hTRH-R mRNA in *Xenopus* oocytes. Panel A: a typical TRH induced biphasic inward current elicited 48 hr after mRNA injection and at a holding potential of -70 mV. Water injected or non-injected controls showed no response to TRH stimulation. Panel B: The reversal potential of the TRH specific currents was close to -30 mV. Panel C: the initial transient inward current component at -70 mV reached maximum at approximately 10^{-6} TRH. The number of oocytes examined at each concentration is given in the figure. Mean ± SD is shown.

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                                    ••••••••••TMS1•••••••••          ••••TMS2•
mouse   MENDTVSEMNQTELQPQAAVALEYQVVTILLVVIICGLGIVGNIMVVLVVMRTKHMRTPTNCYLVSLAVA
rat     ---E----L-----P--V--------------V----------------------A-----------
human   ---E----L---Q---R-V-------------L-----------------------------------

        ••••••••••••                ••••••••••TMS3•••••••••
mouse   DLMVLVAAGLPNITDSIYGSWVYGYVGCLCITYLQYLGINASSCSITAFTIERYIAICHPIKAQFLCTFS
rat     ---------------------------------------------------------------------
human   ---------------------------------------------------------------------

            ••••••••••TMS4••••••••••                          •••••••••TMS5••••
mouse   RAKKIIIFVWAFTSIYCMLWFFLLDLNISTYKNAVVVSCGYKISRNYYSPIYLMDFGVFYVVPMILATVL
rat     ----------------------------------D-I-I----------------------M--------
human   --------------L----------------D-I-I---------------------------------

        •••••                                                  •••••••••TMS6••
mouse   YGFIARILFLNPIPSDPKENSKMWKNDSIHQNKNLNLNATNRCFNSTVSSRKQVTKMLAVVVILFALLWM
rat     ----------------------T-----T---M-----T-------------------------------
human   ----------------------T-----T---T---V-TS-----------------------------

        ••••••••          •••••••••TMS7•••••••••
mouse   PYRTLVVVNSFLSSPFQENWFLLFCRICIYLNSAINPVIYNLMSQKFRAAFRKLCNCKQKPTEKAANYSV
rat     ---------------------------------------------------------------------
human   ---------------------------------------------------------------------P-----

mouse   ALNYSVIKESDRFSTELEDITVTDTYVSTTKVSFDDTCLASEN
rat     ------------------D-----------------------KNGPSSCTYGYSLTAKQEKI
human   -----------H-----D--------L-A-------------VSFSQS
```

Figure 4. Comparison of the amino acid sequences from the mouse, rat and human TRH receptor proteins. Identities are indicated by solid lines and differences between species are marked with the respective one letter code. The putative transmembrane domains (TM1-7) are indicated above the sequence by a dotted line.

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