Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 9514–9518, December 1990
Biochemistry

Expression cloning of a cDNA encoding the mouse pituitary thyrotropin-releasing hormone receptor

(hypothalamic releasing factor/guanine nucleotide-binding regulatory protein-coupled receptor/signal transduction)

RICHARD E. STRAUB*†, GEORGES C. FRECH‡, ROLF H. JOHO‡, AND MARVIN C. GERSHENGORN*§

*Division of Endocrinology and Metabolism, Department of Medicine, and Department of Cell Biology & Anatomy, Cornell University Medical College, New York, NY 10021; and ‡Department of Physiology and Molecular Biophysics, Baylor College of Medicine, Houston, TX 77030

Communicated by B. L. Horecker, August 1, 1990 (received for review June 18, 1990)

ABSTRACT **Thyrotropin-releasing hormone (TRH) is an important extracellular regulatory molecule that functions as a releasing factor in the anterior pituitary gland and as a neurotransmitter/neuromodulator in the central and peripheral nervous systems. Binding sites for TRH are present in these tissues, but the TRH receptor (TRH-R) has not been purified from any source. Using *Xenopus laevis* oocytes in an expression cloning strategy, we have isolated a cDNA clone that encodes the mouse pituitary TRH-R. This conclusion is based on the following evidence. Injection of sense RNA transcribed *in vitro* from this cDNA into *Xenopus* oocytes leads to expression of cell-surface receptors that bind TRH and the competitive antagonist chlordiazepoxide with appropriate affinities and that elicit electrophysiological responses to TRH with the appropriate concentration dependency. Antisense RNA inhibits the TRH response in *Xenopus* oocytes injected with RNA isolated from normal rat anterior pituitary glands. Finally, transfection of COS-1 cells with this cDNA leads to expression of receptors that bind TRH and chlordiazepoxide with appropriate affinities and that transduce TRH stimulation of inositol phosphate formation. The 3.8-kilobase mouse TRH-R cDNA encodes a protein of 393 amino acids that shows similarities to other guanine nucleotide-binding regulatory protein-coupled receptors.**

Thyrotropin-releasing hormone (TRH), which was initially isolated from the hypothalamus and thought to function solely as a releasing factor for the anterior pituitary gland, has been shown to be distributed widely throughout the central and peripheral nervous systems as well as in extraneural tissues (1, 2). TRH receptors (TRH-R) are present in the pituitary (3) and brain (4). Binding sites for TRH, which probably represent receptors that serve as transmembrane signaling proteins, have also been found in other tissues. Pharmacological evidence suggests that the pituitary and brain receptors are similar but that they exhibit different isoelectric points (5). The question of whether there are TRH-R subtypes, as is the case for other guanine nucleotide-binding regulatory protein (G protein)-coupled receptors (6), remains open.

Transmembrane signaling by the pituitary TRH-R has been studied intensively, and it has been shown that TRH acts via the inositol phospholipid–calcium–protein kinase C transduction pathway (7). Indirect evidence (8, 9) is consistent with the hypothesis that a G protein couples the TRH-R to a phospholipase C that hydrolyzes phosphatidylinositol 4,5-bisphosphate. Therefore, it has been assumed that the TRH-R is a member of the large family of G protein-coupled receptors. However, attempts to purify the TRH-R protein by conventional biochemical techniques have not been successful (10, 11). To study in detail the molecular mechanisms of TRH-R signaling, the regulation of TRH-R number and mRNA levels under different physiological conditions, and whether different subtypes exist for the TRH-R, we isolated a cDNA encoding the mouse pituitary TRH-R. In this report we describe the isolation and sequence¶ of this cDNA and present ligand binding and response characteristics of the receptor expressed in *Xenopus* oocytes and in transfected mammalian cells that show that it encodes the pituitary TRH-R.

MATERIALS AND METHODS

Expression Cloning of the Mouse TRH-R cDNA. A directional, size-selected cDNA library was constructed by using our previously reported method (12). Poly(A)$^+$ RNA was isolated from a mouse pituitary thyrotropic TtT tumor (13) and fractionated on a sucrose gradient. The fraction that gave the highest specific TRH-R mRNA activity when injected into stage 5 or 6 *Xenopus laevis* oocytes (14, 15) was used for preparative cDNA synthesis. To find an enzyme that would generate the highest yield of long cDNAs, we compared seven lots of murine leukemia virus reverse transcriptase from four manufacturers using as test templates RNA fractions from the sucrose gradient adjacent to the one with the highest specific TRH-R mRNA activity. cDNAs of between 3 and 5 kilobases (kb) were purified by agarose gel electrophoresis and electroelution. The size-selected cDNAs were ligated directionally into λZAP (Stratagene) such that transcription using T7 RNA polymerase would yield sense RNA. A library of 1.2×10^6 clones was constructed that was >95% recombinant. The primary library was immediately divided and amplified individually in 60 sublibraries of approximately 20,000 clones each. Phage λ DNA was prepared by the cetylmethylammonium bromide DNA precipitation method (16). The DNA was digested with *Not* I, proteinase K-treated, extracted, and precipitated. *In vitro* transcription of sense RNA was carried out by a modification of the procedure used in cloning the serotonin type 1c receptor (17). Transcripts from 20 pools of 20,000 clones each were injected individually into *Xenopus* oocytes (18). Two or 3 days later the oocytes were placed under voltage clamp, and 1 μM TRH was administered in the bath. One pool gave a clear TRH-evoked current response in the oocyte, while the other 19 pools were negative. The positive pool of 20,000 clones was reduced by division to pools of 2000, 200, 30, 10, and finally a single "positive" clone was isolated that contained a 3.8-kb insert.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: TRH, thyrotropin-releasing hormone; TRH-R, TRH receptor; G protein, guanine nucleotide-binding regulatory protein.

†Present address: Molecular Genetics Laboratory, College of Physicians and Surgeons at Columbia University, Box 58, 722 West 168th St., New York, NY 10032.

§To whom reprint requests should be addressed at: Room A328, Cornell University Medical College, 1300 York Ave., New York, NY 10021.

¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. M37490).

DNA Sequencing. The plasmid Bluescript (pBSmTRHR) was excision-rescued from the TRH-R λZAP clone by coinfection with helper phage R-408. Restriction fragments of the cDNA were subcloned into M13mp19 and sequenced by the dideoxy chain-termination method of Sanger *et al.* (19).

Expression and Pharmacological Characterization in Oocytes. *Xenopus* oocytes were injected with 5 ng of sense RNA transcripts and were assayed for specific binding of TRH and electrophysiological responses to TRH. Binding to intact oocytes was performed as described (20) with 2 nM [^{3}H]methylTRH as the labeled ligand and unlabeled TRH and chlordiazepoxide as competitive inhibitors. In separate experiments, the electrophysiological response to TRH was measured.

Antisense Inhibition of the Oocyte TRH Response. For use in inhibition-of-expression experiments, a 1.8-kb *Bgl* II/*Hin*dIII fragment was subcloned into the *Bam*HI site of pBluescript. After linearization with *Sac* II, antisense RNA was transcribed by using T7 RNA polymerase. Antisense RNA (5 ng) was mixed with total RNA (300 ng) isolated from normal rat anterior pituitary glands and kept on ice for 1 hr prior to injection into oocytes (21). Responses to TRH and carbachol were assayed 48–72 hr after injection.

Expression in COS-1 Cells. The entire TRH-R cDNA contained in a *Not* I/*Hin*dIII fragment was cloned directionally into the eukaryotic expression vector pCDM8 (22) (pCDM8m-TRHR), and COS-1 cells were transfected by using the DEAE dextran method (23). After 48–72 hr, the cells were assayed for [^{3}H]methylTRH binding and TRH-stimulated inositol phosphate formation (24).

RESULTS AND DISCUSSION

Expression Cloning. Poly(A)$^+$ RNA isolated from a mouse pituitary thyrotropic (TtT) tumor was size-fractionated, and the fractions were assayed for mRNA activity for the TRH-R in *Xenopus* oocytes. A typical response evoked by a maximally effective dose of TRH (1 μM) in voltage-clamped oocytes after injection of 20 ng of size-fractionated TtT poly(A)$^+$ RNA is illustrated in Fig. 1, left trace. A directional cDNA library of 1.2 × 10^6 clones was constructed in λZAP by using response-selected RNA. RNA transcribed *in vitro* from a single clone isolated by serial division of this library conferred responsiveness to TRH (Fig. 1, center trace). Responses can be obtained by injection of as little as 1 pg of RNA transcripts. RNA transcribed from any other individual clone isolated from the same library did not confer TRH responsiveness (e.g., Fig. 1, right trace).

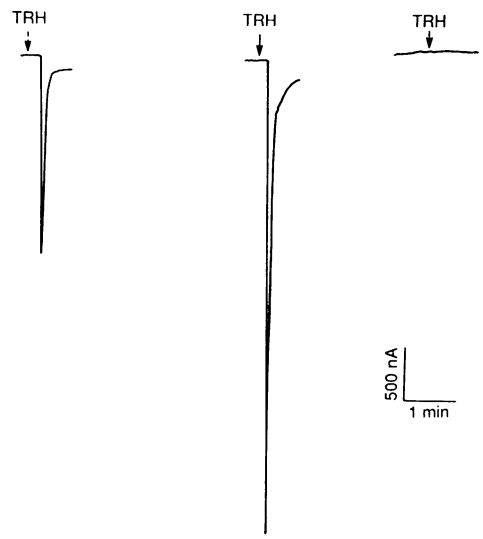

FIG. 1. Electrophysiological responses to TRH in *Xenopus* oocytes injected 48 hr previously with 20 ng of sucrose gradient fractionated TtT poly(A)$^+$ RNA (left trace), 2 ng of RNA transcribed *in vitro* from the TRH-R cDNA (pBSmTRHR) (center trace), or 20 ng of RNA transcribed from a different clone from the same cDNA library (right trace).

Expression and Pharmacological Characterization in Oocytes. Analysis of the ligand binding and dose–response characteristics of the receptor expressed in oocytes after injection of RNA transcribed *in vitro* from pBSmTRHR demonstrated that this cDNA encodes a functional TRH-R. Inhibition of the binding of [^{3}H]methylTRH, an analog with 10-fold higher affinity than TRH for the pituitary TRH-R (25), by TRH and by chlordiazepoxide, a competitive antagonist of TRH binding in the pituitary (26), is illustrated in Fig. 2 *Upper*. The apparent inhibition constants (K_i) for TRH and chlordiazepoxide were calculated to be approximately 30 nM and 3 μM, respectively, values close to those obtained for TRH receptors in GH$_3$ pituitary cells (26).

The concentration dependency of the TRH-induced oocyte electrophysiological response is shown in Fig. 2 *Lower*. As little as 1 nM TRH evoked a response, 10 nM TRH evoked half-maximal responses and 100 nM TRH evoked the maximal response. This dose dependency is the same as that

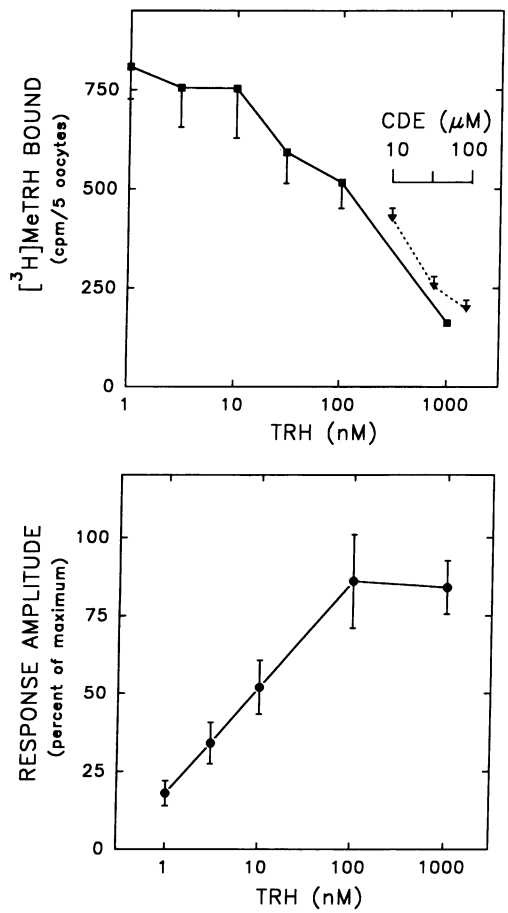

FIG. 2. Expression of TRH-Rs in *Xenopus* oocytes injected with RNA transcribed *in vitro* from the plasmid containing the TRH-R cDNA (pBSmTRHR). (*Upper*) TRH (■) and chlordiazepoxide (CDE) (▼) competition for the binding of [^{3}H]methylTRH to oocytes expressing TRH-Rs 3 days after injection of 5 ng of *in vitro* transcribed RNA. Results are presented as means ± SEM of duplicate determinations in two experiments. In *Upper*, only half of the error bars are shown; the error for 1000 nM TRH (not shown) is ± 10. (*Lower*) Concentration–response relationship of oocytes 3 days after injection of 5 ng of *in vitro* transcribed RNA. Results are presented from two experiments with $n \geq 6$.

observed in GH_3 cells when rapid effects of TRH, such as generation of inositol 1,4,5-trisphosphate or elevation of cytoplasmic calcium, were measured (9, 27).

Antisense RNA Inhibition of the Oocyte TRH Response. To ascertain if the cloned mouse TRH-R sequence is similar to endogenous rat pituitary TRH-R mRNA, we determined whether antisense RNA transcribed *in vitro* from a portion of the TRH-R cDNA in pBluescript (pBSmTRHR1.8) would inhibit TRH-R expression in oocytes injected with RNA isolated from normal rat anterior pituitary glands. Injection of RNA isolated from normal rat pituitaries led to acquisition of responses to TRH and carbachol, a muscarinic agonist (Fig. 3 *Upper*, left trace); there were no intrinsic responses to TRH or carbachol in uninjected collagenase-treated oocytes. In Fig. 3 *Upper*, right trace, are responses from an oocyte injected with RNA from normal rat pituitaries that had been incubated with antisense TRH-R RNA. The TRH response in the oocyte was abolished, but the carbachol response was unaffected. As shown in the compilation of data in Fig. 3 *Lower*, when antisense RNA was allowed to hybridize to rat pituitary RNA prior to injection, the response to TRH was inhibited by 87%, whereas the response to carbachol was not significantly inhibited.

Expression in COS-1 Cells. To examine both ligand binding and response characteristics of the cloned receptor in a mammalian system, we transfected COS-1 cells transiently with the TRH-R cDNA subcloned into the eukaryotic expression vector pCDM8 that contains the cytomegalovirus promoter (22) (pCDM8mTRHR). No specific binding or TRH-stimulated inositol phosphate formation was detectable in untransfected COS-1 cells or cells transfected with pCDM8 alone (data not shown). [^{3}H]methylTRH binding and displacement by TRH and chlordiazepoxide were measured (Fig. 4 *Upper*). The K_i values for TRH (10 nM) and for chlordiazepoxide (20 μM) agree with those found in both oocytes injected with RNA transcribed *in vitro* from pBSmTRHR (Fig. 2 *Upper*) and in GH_3 cells (26). TRH-stimulated inositol phosphate formation is shown in Fig. 4 *Lower*. TRH exhibited an EC_{50} of approximately 10 nM, which is similar to the EC_{50} found in oocytes injected with RNA transcribed *in vitro* from pBSmTRHR (Fig. 2 *Lower*) and in GH_3 cells (9, 27).

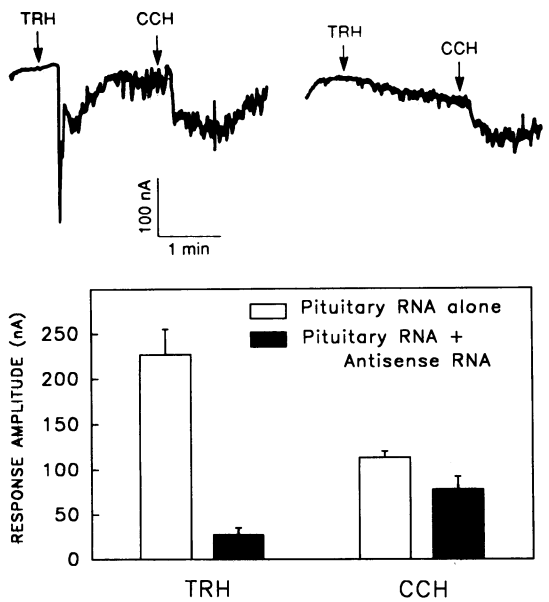

FIG. 3. Effect of antisense TRH-R RNA on the acquired TRH and carbachol responses in *Xenopus* oocytes. Antisense RNA (5 ng) transcribed *in vitro* from pBluescript containing a portion of the TRH-R cDNA (pBSmTRHR1.8) was incubated for 1 hr with 300 ng of RNA isolated from normal rat pituitary glands. The mixture was injected into oocytes, and electrophysiological responses to 1 μM TRH were assayed 3 days later. (*Upper*) Representative responses in the absence (left trace) or presence (right trace) of antisense TRH-R RNA. (*Lower*) Antisense inhibition of the TRH response but not the carbachol response. Results are presented from two similar experiments with $n \geq 6$.

Primary Structure of the TRH-R. The nucleotide sequence and complete deduced amino acid sequence of the mouse TRH-R are shown in Fig. 5. The λZAP clone isolated contained a cDNA insert of approximately 3.8 kb, and we sequenced both strands of the 5'-most 1752 bp. The open reading frame of 1179 bp from nucleotides 259 to 1437 encodes a protein of 393 amino acids with a predicted molecular weight of 44.5 kDa. Hydropathy analysis using the Kyte and Doolittle (28) and Eisenberg (29) algorithms predicts seven transmembrane domains, in agreement with the topology proposed for other G protein-coupled receptors (6). Two potential N-linked glycosylation sites with the Asn-Xaa-(Thr or Ser) consensus sequence are present in the N terminus at positions 3 and 10. Two cysteines homologous to those in the β_2 receptor that

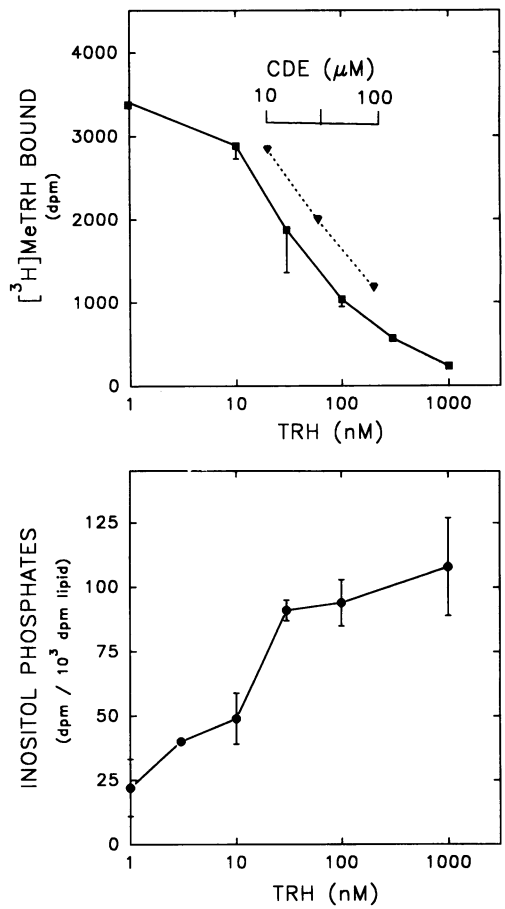

FIG. 4. Expression of TRH-Rs in COS-1 cells transfected with pCDM8mTRHR. COS-1 cells were transfected with the TRH-R cDNA in the mammalian expression vector pCDM8 (pCDM8mTRHR) and assayed for binding and responses 48 hr later. (*Upper*) TRH (■) and chlordiazepoxide (CDE) (▼) competition for the binding of [^{3}H]methylTRH. COS-1 cells transfected with pCDM8mTRHR were assayed for the binding of [^{3}H]methylTRH in the presence of increasing amounts of either unlabeled TRH or chlordiazepoxide. Results are presented as means ± range of data (only half of range shown) from duplicate determinations in one of two similar experiments. (*Lower*) Stimulation of inositol phosphate formation by TRH. COS-1 cells transfected with pCDM8mTRHR were labeled with *myo*-[^{3}H]inositol for 48 hr, washed, and tested for dose-dependent stimulation of inositol phosphate production by TRH. Results are presented as means ± SD of triplicate determinations from one of two similar experiments.

```
1                                          GAATTCCGGAGGGTTTAGAGGAACTGCCGCTCTGAAGACTGAGCCTCTGCTAAGTGATCTTCCTGCCAGACAGACTGGACAAGATTTCTTCTGCAG
97   GATTGGAAACTTGGACCTATTAGCACTTCATCTACCAGAGAAACAGGCAGCGTGACAGAGTGAAGAGGGGAAAGAACTACTGCAAAAACAAACAGACAGAAAGGTGAAGGCTGGAAAGA
216  TGTTTTAGAGTCCCCGTGTCAGAGAAGCTTCAAGCCACTGAAG ATG GAG AAT GAT ACT GTC AGT GAA ATG AAC CAA ACC GAG CTC CAG CCA CAA GCA GCT
1                                                Met Glu Asn Asp Thr Val Ser Glu Met Asn Gln Thr Glu Leu Gln Pro Gln Ala Ala

316  GTG GCC CTC GAG TAC CAG GTG GTT ACC ATC TTA CTT GTG GTC ATT ATT TGT GGA CTG GGC ATT GTG GGC AAC ATC ATG GTA GTC CTG GTG
20   Val Ala Leu Glu Tyr Gln Val Val Thr Ile Leu Leu Val Val Ile Ile Cys Gly Leu Gly Ile Val Gly Asn Ile Met Val Val Leu Val

406  GTC ATG AGA ACA AAG CAC ATG AGA ACC CCT ACA AAC TGT TAC CTG GTA AGT CTG GCT GTG GCA GAT CTC ATG GTT CTG GTG GCT GCA GGA
50   Val Met Arg Thr Lys His Met Arg Thr Pro Thr Asn Cys Tyr Leu Val Ser Leu Ala Val Ala Asp Leu Met Val Leu Val Ala Ala Gly

496  CTC CCC AAC ATA ACC GAC AGT ATC TAT GGT TCC TGG GTC TAT GGC TAT GTT GGC TGC CTC TGC ATT ACA TAT CTC CAG TAC CTA GGC ATT
80   Leu Pro Asn Ile Thr Asp Ser Ile Tyr Gly Ser Trp Val Tyr Gly Tyr Val Gly Cys Leu Cys Ile Thr Tyr Leu Gln Tyr Leu Gly Ile

586  AAT GCA TCT TCA TGT TCA ATA ACG GCC TTT ACC ATT GAA AGG TAC ATA GCA ATC TGT CAC CCC ATC AAA GCC CAG TTT CTC TGC ACG TTT
110  Asn Ala Ser Ser Cys Ser Ile Thr Ala Phe Thr Ile Glu Arg Tyr Ile Ala Ile Cys His Pro Ile Lys Ala Gln Phe Leu Cys Thr Phe

676  TCC AGA GCC AAA AAA ATC ATC ATC TTT GTC TGG GCC TTC ACA TCC ATT TAC TGT ATG CTC TGG TTC TTC CTG CTG GAT CTC AAC ATC AGC
140  Ser Arg Ala Lys Lys Ile Ile Ile Phe Val Trp Ala Phe Thr Ser Ile Tyr Cys Met Leu Trp Phe Phe Leu Leu Asp Leu Asn Ile Ser

766  ACC TAC AAA AAC GCT GTT GTG GTT TCC TGT GGC TAC AAG ATC TCC AGG AAC TAC TAC TCA CCT ATT TAC CTA ATG GAC TTT GGT GTC TTT
170  Thr Tyr Lys Asn Ala Val Val Val Ser Cys Gly Tyr Lys Ile Ser Arg Asn Tyr Tyr Ser Pro Ile Tyr Leu Met Asp Phe Gly Val Phe

856  TAT GTT GTG CCA ATG ATC CTG GCC ACT GTG CTT TAT GGA TTT ATA GCT AGA ATC CTC TTC TTA AAC CCC ATT CCT TCA GAC CCT AAA GAA
200  Tyr Val Val Pro Met Ile Leu Ala Thr Val Leu Tyr Gly Phe Ile Ala Arg Ile Leu Phe Leu Asn Pro Ile Pro Ser Asp Pro Lys Glu

946  AAC TCT AAG ATG TGG AAA AAT GAC TCA ATC CAT CAG AAC AAG AAT TTG AAT TTA AAT GCC ACC AAC AGA TGC TTC AAC AGC ACT GTA TCT
230  Asn Ser Lys Met Trp Lys Asn Asp Ser Ile His Gln Asn Lys Asn Leu Asn Leu Asn Ala Thr Asn Arg Cys Phe Asn Ser Thr Val Ser

1036 TCC AGG AAG CAG GTC ACC AAG ATG CTC GCA GTG GTT GTA ATT CTG TTT GCC CTT TTA TGG ATG CCC TAC AGG ACT CTT GTG GTT GTC AAC
260  Ser Arg Lys Gln Val Thr Lys Met Leu Ala Val Val Val Ile Leu Phe Ala Leu Leu Trp Met Pro Tyr Arg Thr Leu Val Val Val Asn

1126 TCA TTT CTC TCC AGC CCT TTC CAG GAA AAT TGG TTC TTG CTC TTT TGC AGA ATT TGC ATT TAT CTC AAC AGT GCC ATC AAC CCA GTG ATT
290  Ser Phe Leu Ser Ser Pro Phe Gln Glu Asn Trp Phe Leu Leu Phe Cys Arg Ile Cys Ile Tyr Leu Asn Ser Ala Ile Asn Pro Val Ile

1216 TAC AAC CTC ATG TCT CAG AAA TTT CGT GCA GCC TTC AGG AAG CTC TGC AAT TGC AAG CAG AAG CCC ACA GAA AAA GCT GCT AAC TAC AGT
320  Tyr Asn Leu Met Ser Gln Lys Phe Arg Ala Ala Phe Arg Lys Leu Cys Asn Cys Lys Gln Lys Pro Thr Glu Lys Ala Ala Asn Tyr Ser

1306 GTG GCC CTA AAT TAC AGT GTC ATC AAG GAG TCA GAC CGC TTC AGC ACA GAG CTA GAA GAT ATC ACC GTC ACC GAT ACG TAT GTG TCA ACC
350  Val Ala Leu Asn Tyr Ser Val Ile Lys Glu Ser Asp Arg Phe Ser Thr Glu Leu Glu Asp Ile Thr Val Thr Asp Thr Tyr Val Ser Thr

1396 ACA AAA GTG TCC TTT GAT GAC ACC TGC TTG GCC TCT GAG AAC TAA AGAAGGCCATAAAGAACCCTATAATGTTCTTCATGTATATTTATTTCATAATTATGATC
380  Thr Lys Val Ser Phe Asp Asp Thr Cys Leu Ala Ser Glu Asn ***

1500 TCAAATAATTCAGCAACAGATGTCTTCAGGCAAGTTACATACTGGGAAGCAGTTCACTATTAAGATTTTCAGCAACTTACGGACTACATCAAGTGACAAGTTTAACCTATGGTGTGCTT
1619 AATTCAAAACTCAAATAATCTTCTTTGACAAGAGTGATCATGGATAAAAATGGACTCAGTTCTTGTGCATATAGCTTTTCTTTGACTGCCAAACAAGAGCGAATATGAACTTCTTCAAG
1738 AACATCTTGAACTGG ----- 3'
```

FIG. 5. Nucleotide and deduced amino acid sequences of the mouse TRH-R cDNA.

have been proposed to engage in a disulfide bond (30) are present in extracellular loops 1 (Cys-98) and 2 (Cys-179). These residues also may form a disulfide bond in the TRH-R because it has been shown that TRH binding is markedly reduced when disulfide bonds are disrupted (31). A cysteine present in the C terminus close to transmembrane domain M-VII (Cys-335) is homologous to the cysteine that may be acylated by palmitic acid in the β_2 receptor (32). Fifteen of the last 52 amino acids are either serine or threonine, potential sites for regulatory phosphorylation (6). Candidates for phosphorylation by protein kinase C (33) include three residues in intracellular loop 3 (Thr-250, Ser-259, and Ser-260) and four in the C terminus (Ser-324, Thr-342, Ser-360, and Thr-379). The third intracellular loop has been shown to be important in

```
                                                                                  M-I                                  M-II                                        M-III
          *                   *       *        *                  ------------------**--*-----      *  *----**---*------------        *      * ------------*---------
M TRH-R   ME                  NDTVSEM NQTELQ   PQAAVALEYQVVT      ILLVVIICGLG       IVGNIMVVLVVMRTKHMRTPT  NCYLVSLAVADLMVLVAAGLPNITDSIYGS  WVYGYVG CLCITYLQYLGINASSCSITAFTI
R SPR     MDNVLPMDSDLFP       NIST    NTSESNQFVQPTWQI                 VLWAAAYTVIVVTSVV  GMVVVIWIILAHKRMRTVT  NYFLVNLAFAEACMAAFNTVVNFTYAVHNV  WYYGLFY CKFHNFFPIAALFASIYSMTAVAF
O RHOD    MVESTTLV            NQTWWY  NPTVDIH  PHWAKFDPIPDA       VYYSVGIFIGVVGIIGILGNGVVIYLFSKTKSLQ  TPANMFIINLAMSDLSFSAINGFPLKTISAFMKK WIFGKVA CGLYGLLGGIFGFMSINTMAMISI
R 5HT2    MEILCED             NISLSSIPNSLMQLGDG PRLYHNDFN 43 EKNWSALLTTVVI  ILTI AGNILVIMAVSLEKKLQNAT  NYFLMSLAIADMLLGFLVMPVSMLTILYGYR WPLPSKL CAIWIYLDVLFSTASIMHLCAISL
T BAR     MGDGWLPPDCGPH       NRSGGGGA TAA     PTGSRQVSA 3 QWEAGMSLLMALVVLL  IVAGNVLVIAAIGRTQRLQTLT  NLFITSLACADLVMGLLVVPFGATLVVRGT  WLWGSFL CEDWTSLDVLCVTASIETLCVIAI
D RDC8    MSTMGSW                                             VYITVELAIVLA      IL GNVLVCWAVWLNSNLQNVT  NYFVVSLAAADIAVGVLAIPFAITISTGFCAACHNCLFFACFVLVLTQSSIF  SLLA  IAI
R D2      MDPLNLSWYDDDLERQNWSRPF  NGSEGKADR PHYNYY           AMILTLLIFI        IVFGNVLVCMAVSREKALQTTT  NYLIVSLAVADLLVATLVMPWVVYLEVVGE  WKFSRIH CDIFVTLDVMMCTASILNLCAISI
R M5      MEGESY              NESTV   NGT      PVNHQALERHGLWE     VITIAVVTAVVSLMTIV GNVLVMISF KVNS QLKTVNNYYLLSLACADLIIGIFSMNLYTTYILMGR  WVLGSLA CDLWLALDYVASNASVMNLLVISF

                                        M-IV                                              M-V
          ***    *              --*---*----------*----------       *                 -----*---*-------*----
M TRH-R   ERYIAICHPIKAQFLCTFSRAKKIIIFV WAFTSIYCM LWFFLLDLNISTYKNAVVVSCGYKISRNYYSPIYL     MDFGVFYVVPMILATVLYGFIARILFLNPIPSDPKENSKMWKNDSIHQNKNLNLNATNRCFNSTVSSRKQVTK
R SPR     DRYMAIIHPLQPRLSATATK    VVIFVIWVLALLLAF PQGYYS    TTETMPSRVVCMIEWPEHPMRTYEKAYHICVTVLIYFL  PLLVIAYAYTVVGITLWASEIPGDSSD                       RYHEQVSAKRKVVKM
O RHOD    DRYNVIGRPMAASKKMSHRRAFLMIIFV WMWSIVWSVGPVFNWGAYVPEGILTS    CSFDYLSTDPSTRSFILCMY   FCGFML PIIIIAFCYFNIVMSVSNHEKEMAAMAK                 RLNAKELRKAQAGASAEMKLAK
R 5HT2    DRYVAIQNPIHHSRFNSRTKAFLKIIAV WTISVGISM PIPVFGL  QDDSKVFKEGSCLLADDN          FVLIGSFVAFFI PLTIMVITYFLTIKSLQKEATLCVSDLS 24 KLFQRSIHREPGSYAGRKTMQSISNEQKACKV
T BAR     DRYLAITSPFRYQSLMT RARAKVIICTVWAISALVSFLPIMMHWW RDEDPQALK  CYQDPGCCDFVTNR AYAIASSIIS FYI PLLIMIFVYLRVYREAKEQIRKIDRCEG 15 LPQHQPILGNGRASKRKTSRVAMAMREHKALK
D RDC8    DRYIAIRIPLRYNGLVTGTRAK GIIAVCWVLSFAIGLTPMLGWNNCSQPKEGRNYSQGCGEGQVACLFEDVVPMNYMVYYNFFAFVLVPLLLMLGVYLRIFLAARRQLKQMESQP                         LPGERARSTLQKEVHAAKS
R D2      DRYTAVAMPMLYNTRYSSKRRVTVMIAIVWVLSFTISC PLLFGL      NNTDQNECIIAN          PAFVVYSSIVSFYV PFIVTLLVYIKIYIVLRKRRKRVNTKR  86 EIQTMPNGKTRTSLKTMSRRKLSQQKEKKATQ
R M5      DRYFSITRPLTYRAKRTPKRAG IMIGLAWLVSFILWA PAILCWQYLV GKRTVPPDECQIQFLSEPTIT      FGTAIAAFYI PVSVMTILYCRIYRETEKRTKDLADLQ 186 KDPSTKGPDPNLSHQMTKRKRMVLVKERKAAQ

              M-VI                            M-VII
          --------*---*-*-------          ---*---------------**--*          *    *          *
M TRH-R   MLAVVVILFALLWMPYRTLVVVNSFLSSPF          QENWFLLFCRICIYLNSAINPVIY    NLMSQKFRAAFRKL     CNCKQKPTEKAANYSVALNYSVIKESDRFSTELEDITVTDTYVSTTKVSFDDTCLASEN
R SPR     MIVVVCT FAICWLPFHVFFLLPYINPDLYLKKFIQQVYLASMWLAMS         STMYNPIIYCCLNDRFRLGFKHAFR         CCPFISAGDYEGLEMKSTRYLQTQSSV 30 SLDLTSMGSSRSMSKTMTESSSFYSNMLA
O RHOD    ISMVIITQFMLSWSPYAIIDAARRAQELE           MEMLSSPVLFAKASAIH  NPIVYSVSHPK     FREAIQTTFPWLLTCCQFDEKECEDANDAEEEVVASERGGE 60 PPQGYPPQGAPPQVEAPQGAPPQGVDNQAYQA
R 5HT2    LGIVFFL FVVMWCPFFITNIMAVICKESCNENVIGALLNVFVWIGYLSSAV      NPLVYTLFNKTYRSAFSRY IQ         CQYKENRKPLQLILVNTIPALAYKSSQ 16 DDCSMVTLGKQQSEENCTDNIETVNEKVSCV
T BAR     TLGIIMGVFTLCWLPFFLVNIVNVFNRDLVP          DWLFVFFNWLGYANSAFNPIIYCRSPD     FRKAFKRLL          CFPRKADRRLHAGGQPAPLPGGFISTL 70 IVKLFEDATCTCPHTHKLKMKWRFKQHQA
D RDC8    LAIIVGL FALCWLPLHIINCFTFFCPECSHAP        LWLMYLTIVLSHTNSVVNPFIYAYRIRE    FRQTFRKIIRSHVLRRREPFKAGGTSARALAAHGSDGEQI 51 LSHELKGACPESPGLEGPLAQDGAGV
R D2      MLAIVLGVFIICWLPFFITHILNIHCDCNIPP         VLYSAFTWLGYVNSAV  NPIIYTTFNIE     FRKAFMKILH         C
R M5      TLSAILLAFIITWTPYNIMVLVSTFCDKCVPVTLWH     LGYWLCYVNSTI      NPICYALCN     RTFRKTFKLLLL       CRWKKKKVEEKLYWQGNSKLP
```

FIG. 6. Alignment of the deduced amino acid sequence of the mouse TRH-R with the rat substance P receptor (R SPR) (43), octopus rhodopsin (O RHOD) (44), rat serotonin type 2 receptor (R 5HT2) (45), turkey β_1 receptor (T BAR) (46), putative dog thyroid receptor RDC8 (D RDC8) (47), rat dopamine D_2 receptor (R D2) (48), and rat muscarinic M5 receptor (R M5) (49). Sequences were chosen for alignment based on searches of the Genpept (GenBank) and Swiss-Prot (EMBL) data bases using the program FASTA (42). An initial alignment was produced by using the program GENALIGN (50) and adjusted manually to align the conserved residues indicated by asterisks. Putative membrane spanning domains for the TRH-R were assigned based on hydrophobicity analysis (28, 29). Numbers present in the sequences indicate the number of residues not shown within each gap.

coupling to G proteins (6). In the TRH-R, amino acids 227–235 of this region can be predicted (34) to form an amphipathic helix, a secondary structure that may be necessary for G-protein activation (35).

Prolines, which in bacteriorhodopsin impart bends to the transmembrane helices (36), are present in transmembrane domains M-V, M-VI, and M-VII, but there is no proline in M-IV. While most receptors in this family have prolines in all four final transmembrane domains, some receptors, including the thyrotropin receptor (37), luteinizing hormone–chorionic gonadotropin (LH–CG) receptor (38), and *mas*-encoded protein (39) do not. M-III does not contain aspartic acid which has been shown to be necessary for agonist binding to the β receptor (40) and is present in all adrenergic, muscarinic, serotonin, and dopamine receptors but is absent in the visual opsins, *mas*-encoded proteins, and receptors for thyrotropin, LH–CG, substance K, and substance P. Likewise, the TRH-R does not contain the serines in M-V that have been proposed to interact with the hydroxyl groups of ligands that contain a catechol moiety (41).

Sequence Identity with Other Receptors. When the mouse TRH-R amino acid sequence was used to search (42) the Genpept (GenBank) and Swiss-Prot (EMBL) data bases, the results indicated significant sequence identity with only some of those receptors proposed to couple to G proteins. The alignment of the TRH-R with these members of the family of G protein-coupled receptors is shown in Fig. 6. When the transmembrane domains only are compared, the percent identity is as follows: rat substance P receptor (43), 21.5%; octopus rhodopsin (44), 24.1%; rat serotonin type 2 receptor (45), 29.4%; turkey β_1 receptor (46), 28.9%; putative dog thyroid receptor RDC8 (47), 28%; rat dopamine D_2 receptor (48), 32.5%; rat muscarinic M5 receptor (49), 28.8%; and human α_2 receptor (51), 29.5%. Analysis of the significance of these sequence identities with the Pearson program RDF2 (42) confirmed that these receptors share common ancestry and also that the mouse TRH-R is much less related to other receptors such as the dog thyrotropin receptor (37), pig LH–CG receptor (38), rat *mas*-encoded protein (39), yeast A factor (52), *Dictyostelium* cAMP receptor (53), and pHS1-2 (54).

In conclusion, we have cloned the mouse pituitary TRH-R. This isolation of a cDNA encoding the TRH-R, which may be the initial cloning of a cDNA for any hypothalamic releasing factor receptor, will permit analysis of the structure, function, and regulation of the receptor to proceed at the molecular level.

This work was supported by National Institutes of Health Grant BRSG S07 RR05396 (to M.C.G.) and by an Advanced Technology Program Grant from the State of Texas (to R.H.J.). We thank C. S. Narayanan for helping with the construction of pBSmTRHR1.8 and W. Pan and F. C. Bancroft of Mount Sinai School of Medicine for helping with pCDM8mTRHR.

1. Lechan, R. M. & Segerson, T. P. (1989) *Ann. N.Y. Acad. Sci.* **553**, 29–59.
2. Hokfelt, T., Tsuruo, Y., Ulfhake, B., Cullheim, S., Arvidsson, U., Foster, G. A., Schultzberg, M., Schalling, M., Arborelius, L., Freedman, J., Post, C. & Visser, T. (1989) *Ann. N.Y. Acad. Sci.* **553**, 76–105.
3. Hinkle, P. M. (1989) *Ann. N.Y. Acad. Sci.* **553**, 176–187.
4. Sharif, N. A. (1989) *Ann. N.Y. Acad. Sci.* **553**, 147–175.
5. Johnson, W. A., Nathanson, N. M. & Horita, A. (1989) *Ann. N.Y. Acad. Sci.* **553**, 137–146.
6. Raymond, J. R., Hnatowich, M., Caron, M. G. & Lefkowitz, R. J. (1990) in *ADP-Ribosylating Toxins and G Proteins*, eds. Moss, J. & Vaughan, M. (Am. Soc. Microbiol., Washington, D.C.), pp. 163–188.
7. Gershengorn, M. C. (1989) *Ann. N.Y. Acad. Sci.* **553**, 191–196.
8. Hinkle, P. M. & Phillips, W. J. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 6183–6187.
9. Straub, R. E. & Gershengorn, M. C. (1986) *J. Biol. Chem.* **261**, 2712–2717.
10. Johnson, W. A., Nathanson, N. M. & Horita, A. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 4227–4231.
11. Phillips, W. J. & Hinkle, P. M. (1989) *Mol. Pharmacol.* **35**, 533–540.
12. Frech, G. C. & Joho, R. H. (1989) *Gene Anal. Techn.* **6**, 33–38.
13. Gershengorn, M. C. (1983) in *Molecular Basis of Thyroid Hormone Action*, eds. Samuels, H. H. & Oppenheimer, J. H. (Academic, New York), pp. 388–412.
14. Oron, Y., Straub, R. E., Traktman, P. & Gershengorn, M. C. (1987) *Science* **238**, 1406–1408.
15. Oron, Y., Gillo, B., Straub, R. E. & Gershengorn, M. C. (1987) *Mol. Endocrinol.* **1**, 918–925.
16. Del Sal, G., Manfioletti, G. & Schneider, C. (1989) *Biotechniques* **7**, 514–520.
17. Julius, D., MacDermott, A. B., Axel, R. & Jessell, T. M. (1988) *Science* **241**, 558–564.
18. Straub, R. E., Oron, Y. & Gershengorn, M. C. (1989) in *Methods in Neurosciences, Vol. 1: Genetic Probes*, ed. Conn, P. M. (Academic, Orlando, FL), pp. 46–61.
19. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
20. Straub, R. E., Oron, Y., Gillo, B., Thomson, R. & Gershengorn, M. C. (1989) *Mol. Endocrinol.* **3**, 9070–9140.
21. Melton, D. A. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 144–148.
22. Seed, B. (1987) *Nature (London)* **329**, 840–842.
23. Cullen, B. R. (1987) *Methods Enzymol.* **152**, 684–704.
24. Imai, A. & Gershengorn, M. C. (1987) *Methods Enzymol.* **141**, 100–101.
25. Taylor, R. L. & Sharif, N. A. (1981) *Neuroendocrinology* **32**, 301–316.
26. Gershengorn, M. C. & Paul, M. E. (1986) *Endocrinology* **119**, 833–839.
27. Albert, P. R. & Tashjian, A. H., Jr. (1984) *J. Biol. Chem.* **259**, 5827–5832.
28. Kyte, J. & Doolittle, R. F. (1982) *J. Mol. Biol.* **157**, 105–132.
29. Rees, D. C., DeAntonio, L. & Eisenberg, D. (1989) *Science* **245**, 510–513.
30. Dohlman, H. G., Caron, M. G., Deblasi, A., Frielle, T. & Lefkowitz, R. J. (1990) *Biochemistry* **29**, 2335–2342.
31. Sharif, N. A. & Burt, D. R. (1984) *J. Neurochem.* **42**, 209–214.
32. O'Dowd, B. F., Hnatowich, M., Caron, M. G., Lefkowitz, R. J. & Bouvier, M. (1989) *J. Biol. Chem.* **264**, 7564–7569.
33. Kikkawa, U., Kishimoto, A. & Nishizuka, Y. (1989) *Annu. Rev. Biochem.* **58**, 31–44.
34. Schiffer, M. & Edmundson, A. B. (1967) *Biophys. J.* **7**, 121–135.
35. Strader, C. D., Dixon, R. A. F., Cheung, A. H., Candelore, M. R., Blake, A. D. & Sigal, I. S. (1987) *J. Biol. Chem.* **262**, 16439–16443.
36. Popot, J.-L., Engelman, D. M., Gurel, O. & Zaccaï, G. (1989) *J. Mol. Biol.* **210**, 829–847.
37. Parmentier, M., Libert, F., Maenhaut, C., Lefort, A., Gérard, C., Perret, J., Van Sande, J., Dumont, J. E. & Vassart, G. (1989) *Science* **246**, 1620–1622.
38. McFarland, K. C., Sprengel, R., Phillips, H. S., Kohler, M., Rosemblit, N., Nikolics, K., Segaloff, D. L. & Seegurg, P. H. (1989) *Science* **245**, 494–499.
39. Young, D., O'Neill, K., Jessell, T. & Wigler, M. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 5339–5342.
40. Strader, C. D., Sigal, I. S., Candelore, M. R., Rands, E., Hill, W. S. & Dixon, R. A. (1988) *J. Biol. Chem.* **263**, 10267–10271.
41. Strader, C. D., Candelore, M. R., Hill, W. S., Sigal, I. S. & Dixon, R. A. F. (1989) *J. Biol. Chem.* **264**, 13572–13578.
42. Pearson, W. R. (1990) *Methods Enzymol.* **183**, 63–98.
43. Hershey, A. D. & Krause, J. E. (1990) *Science* **247**, 958–962.
44. Ovchinnikov, Y. A., Abdulaev, N. G., Zolatarev, A. S., Artamonov, I. D., Bespalov, I. A., Dergachev, A. E. & Tsuda, M. (1988) *FEBS Lett.* **232**, 69–72.
45. Julius, D., Huang, K. N., Livelli, T. J., Axel, R. & Jessell, T. M. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 928–932.
46. Yarden, Y., Rodriguez, H., Wong, S. K.-F., Brandt, D. R., May, D. C., Burnier, J., Harkins, R. N., Chen, E. Y., Ramachandran, J., Ullrich, A. & Ross, E. M. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 6795–6799.
47. Libert, F., Parmentier, M., Lefort, A., Dinsart, C., Van Sande, J., Maenhaut, C., Simons, M.-J., Dumont, J. E. & Vassart, G. (1989) *Science* **244**, 569–572.
48. Bunzow, J. R., Van Tol, H. H. M., Grandy, D. K., Albert, P., Salon, J., Christie, M., Machida, C. A., Neve, K. A. & Civelli, O. (1988) *Nature (London)* **336**, 783–787.
49. Liao, C.-F., Themmen, A. P. N., Joho, R., Barberis, C., Birnbaumer, M. & Birnbaumer, L. (1989) *J. Biol. Chem.* **264**, 7328–7337.
50. Martinez, H. M. (1988) *Nucleic Acids Res.* **16**, 1683–1691.
51. Kobilka, B. K., Matsui, H., Kobilka, T. S., Yang-Feng, T. L., Francke, U., Caron, M. G., Lefkowitz, R. J. & Regan, J. W. (1987) *Science* **238**, 650–656.
52. Hagen, D. C., McCaffrey, G. & Sprague, G. F., Jr. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 1418–1422.
53. Klein, P. S., Sun, T. J., Saxe, C. L., Kimmel, A. R., Johnson, R. L. & Devroetes, P. N. (1988) *Science* **241**, 1467–1472.
54. Rapiejko, P. J., George, S. T. & Malbon, C. C. (1988) *Nucleic Acids Res.* **16**, 8721.