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27. We thank J. Oberlindacher for preparation of the manuscript and A. Krensky and M. Davis for thoughtful reviews. Supported by the Howard Hughes Medical Institute and by NIH grants CA 39612 and HL 33942 to G.R.C., by American Cancer Society grant IM 457 and an American Heart Association Established Investigator award to E.L., and by an NIH Clinical Investigator Award to D.B.D.
26 July 1989; accepted 13 November 1989

Molecular Cloning of the Thyrotropin Receptor

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The pituitary hormone thyrotropin, or thyroid-stimulating hormone (TSH), is the main physiological agent that regulates the thyroid gland. The thyrotropin receptor (TSHR) was cloned by selective amplification with the polymerase chain reaction of DNA segments presenting sequence similarity with genes for G protein–coupled receptors. Out of 11 new putative receptor clones obtained from genomic DNA, one had sequence characteristics different from all the others. Although this clone did not hybridize to thyroid transcripts, screening of a dog thyroid complementary DNA (cDNA) library at moderate stringency identified a cDNA encoding a 4.9-kilobase thyroid-specific transcript. The polypeptide encoded by this thyroid-specific transcript consisted of a 398–amino acid residue amino-terminal segment, constituting a putative extracellular domain, connected to a 346-residue carboxyl-terminal domain that contained seven putative transmembrane segments. Expression of the cDNA conferred TSH responsiveness to *Xenopus* oocytes and Y1 cells and a TSH binding phenotype to COS cells. The TSHR and the receptor for luteinizing hormone–choriogonadotropin constitute a subfamily of G protein–coupled receptors with distinct sequence characteristics.

THYROTROPIN (TSH) STIMULATES the function and the proliferation of thyrocytes and induces the expression of differentiation (*1*). Most of its effects are mediated by adenosine 3′,5′-monophosphate (cAMP) (*1*). Like the other pituitary and placental glycoprotein hormones [follicle-stimulating hormone (FSH), luteinizing hormone (LH), and choriogonadotropin (CG)], TSH is a heterodimer. All these hormones have identical α subunits; the β subunits, although possessing sequence similarity, are specific for each of the hormones (*2*). The activated TSH, FSH, and LH-CG receptors stimulate adenylyl cyclase in their respective target cells through the G protein G_s (*3*). In man, the TSH receptor (TSHR) can be the target of autoimmune reactions that lead to hyper- or hypostimulation of the thyroid gland by autoantibodies in Grave's disease and in idiopathic myxoedema, respectively (*4*).

With the use of the polymerase chain reaction (PCR) (*5*), we have taken advantage of the sequence similarity displayed by all known G protein–coupled receptors to amplify and clone new members of this gene family (*6*). The method involves the use of degenerate oligonucleotide primers corresponding to conserved regions in transmembrane segments of the known receptors. Previously, when applied to cDNA from thyroid tissue with primers corresponding to transmembrane segments III and VI, the method did not result in the cloning of TSHR. However, it led to the cloning of four new members of the G protein–coupled receptor family (*6*).

As most G protein–coupled receptor genes do not contain introns in their coding sequence, we have used a similar strategy with new sets of degenerate primers and

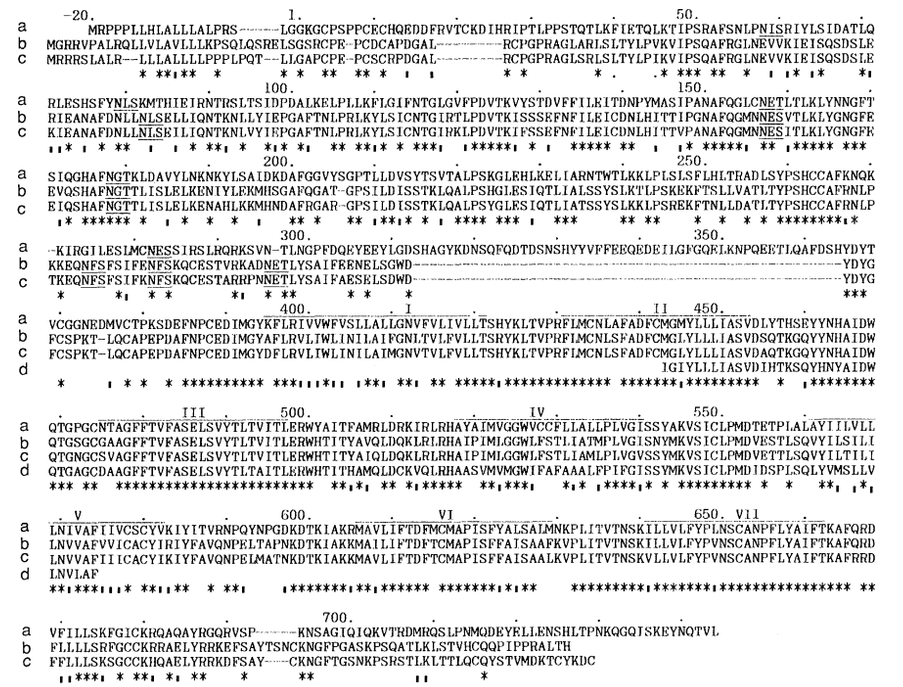

Fig. 1. Primary structure of the dog TSH receptor (a), as deduced from the nucleic acid sequence of the clone dTSHR (*18*). The sequence was aligned (*19*) with full-length rat (b) and pig (c) LH-CG receptor sequences (*16, 17*) and with the HGMP09 partial sequence (d). Numbering is given from the predicted first residue of the mature polypeptide (*12*). Every tenth residue is marked with a dot. Identical residues and conservative replacements in TSHR and the LH-CG receptors are indicated by an asterisk and a vertical bar, respectively. Potential sites for N-linked glycosylation are underlined. Putative transmembrane segments are overlined and numbered I through VII. Lambda phages containing dTSHR inserts were subcloned in M13 and sequenced on both strands (Applied Biosystems, Model 370A sequencer) by a combination of forced cloning and exonuclease III deletions (*20*).

with human genomic DNA as starting material. Eleven clones displaying sequence similarity with G protein–coupled receptor genes were obtained (*7*). One of these clones, HGMP09, which was amplified with primers corresponding to transmembrane segments II and VII, had sequence characteristics that suggested that it belonged to a distinct subfamily of receptors (Fig. 1); in particular, it does not contain the canonical AspArgTyr (DRY) tripeptide close to transmembrane segment III (*8*) and lacks the Asp residue implicated in the binding of charged amines in adrenergic (Asp^{113}), muscarinic, dopaminergic, and serotonergic receptors (*9*).

The clone HGMP09 was used as a probe both in Northern (RNA) blotting experiments with thyroid and nonthyroid tissues, and in screening of a dog thyroid cDNA library. HGMP09 did not hybridize to thyroid mRNA but identified a prominent 2.6-kb transcript present in the ovary and, to a lesser extent, in the testis (Fig. 2A). However, under moderate conditions of stringency it hybridized to one out of 50,000 thyroid cDNA clones, suggesting cross-hybridization with a relatively abundant putative receptor of the thyroid. We thus hypothesized that HGMP09 encoded a fragment of a receptor, distinct from TSHR, but with sequence characteristics that would be expected from a closely related receptor, such as the LH or FSH receptors. A full-length cross-hybridizing clone (dTSHR) was isolated and used as a probe in Northern blots of ten different dog tissues. It hybridized to a 4.9-kb transcript present only in the thyroid gland and in cultured thyrocytes (Fig. 2B). The signal was stronger in cultured thyrocytes that had been exposed for several days to forskolin (which stimulates adenylyl cyclase) than in thyroid tissue. This is a characteristic that would be expected for TSHR, the expression of which is stimulated by cAMP agonists in cultured cells (*10*). A 4417-bp cDNA clone was sequenced completely (*11*). It contains an open reading frame of 764 amino acids beginning with a 20-residue signal peptide, as predicted by von Heijne's algorithm (*12*) (Fig. 1). Comparison to other G protein–coupled receptors and hydropathy profile analysis (*7*) suggested that the protein consisted of a 398-residue NH_2-terminal structure constituting a large putative extracellular domain, followed by 346 amino acids, comprising seven putative transmembrane segments.

The encoded polypeptide was identified as TSHR by expression of the cDNA in a variety of systems. Microinjection of recombinant mRNA in adrenocortical Y1 cells and in *Xenopus* oocytes conferred a TSH-responsive phenotype to both systems. Y1 cells responded to TSH by a characteristic morphological change that is triggered in these cells by an increase in the cytoplasmic concentration of cAMP (Fig. 3) (*13, 14*). *Xenopus* oocytes showed a dose-dependent increase in cAMP (Fig. 4A) that was specific for stimulation by TSH and corresponded to the expected sensitivity of the dog receptor to bovine TSH (half-maximal effect around 0.3 n*M*) (*15*). Transient expression of the receptor cDNA was obtained in COS7 cells (Fig. 4B). Specific binding of ^{125}I-labeled TSH to membranes was observed only in transfected cells. The displacement curves of the label by TSH presented characteristics very similar to that obtained with membranes from dog thyrocytes (half-maximal displacement at 0.4 n*M* and 0.16 n*M* for COS cells and thyrocytes, respectively) (Fig. 4B).

Comparison of TSHR with the LH-CG receptor (*16, 17*) reveals common characteristics that make them members of a subfamily of G protein–coupled receptors. They both display a long NH_2-terminal extension containing multiple sites for N-linked glycosylation (five sites in TSHR). TSHR has an extra 52-residue insert close to the junction between the putative extracellular domain and the first transmembrane segment (Fig. 1). The overall sequence similarity between the extracellular domains of the TSH and LH-CG receptors is 45% (Fig. 1). The similarity between a segment of soybean lectin and the rat LH-CG receptor (*16*) is not conserved in TSHR. The COOH-terminal half of TSHR, containing the transmembrane segments, is 70% similar to both the pig and rat LH-CG receptors (Fig. 1). The homology is particularly strong in the transmembrane segments themselves, where stretches of up to 24 consecutive identical residues are observed (transmembrane region III). Also, the COOH-terminal region of the third putative intracellular loop,

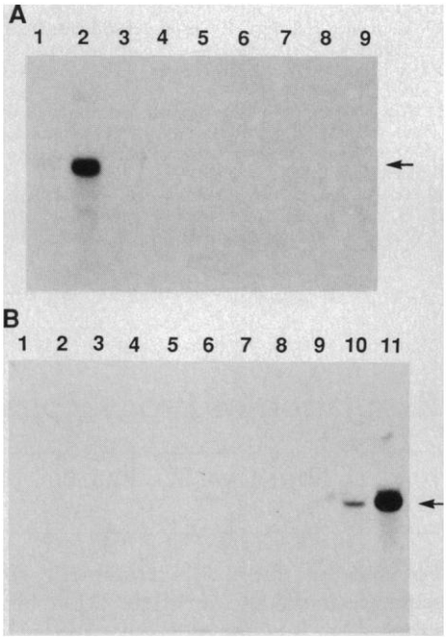

Fig. 2. Tissue distribution of transcripts corresponding to clone HGMP09 (**A**) and the TSH receptor clone (**B**). (A) A Northern blot of poly(A)$^+$ RNA (10 μg per lane) prepared from dog tissues was hybridized with ^{32}P-labeled HGMP09 (*21*). The position of a 2.6-kb transcript [size estimated from DNA size markers (BRL)] is marked with an arrow. Tissues examined were testis, ovary, brain, lung, liver, kidney, spleen, stomach, and thyroid (lanes 1 to 9, respectively). (B) A Northern blot of poly(A)$^+$ RNA (10 μg per lane) prepared from dog tissues was hybridized with ^{32}P-labeled dTSHR (*21*). Tissues examined were testis, ovary, brain, lung, heart, liver, kidney, spleen, stomach, thyroid, and dog thyrocytes that had been cultured for 3 days in the presence of $10^{-5}M$ forskolin (*22*) (lanes 1 to 11, respectively). The position of a 4.9-kb transcript is marked with an arrow.

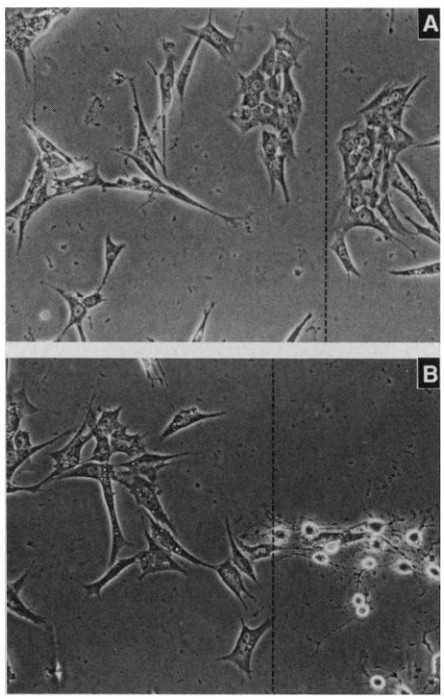

Fig. 3. TSH-induced morphological changes in Y1 cells that had been microinjected with TSHR receptor mRNA. Y1 cells were microinjected with recombinant TSHR mRNA (0.1 pl at a concentration of 0.25 μg/μl) (right of dotted line) or water (left of dotted line) as previously described (*14*) and incubated in control medium (**A**) or with TSH (0.1 n*M*) (**B**). The phosphodiesterase inhibitors RO 201724 and isobutylmethylxanthine ($10^{-6}M$ each) were present in all incubations.

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which is particularly short in TSH and LH-CG receptors and which has been implicated in the interaction with the α subunit of G_s ($G_s\alpha$) (*8, 9*), is identical in both receptor types. This pattern of similarities gives support to the view that the extracellular domain functions in ligand recognition (*4*), whereas the membrane-inserted domain is responsible for the activation of $G_s\alpha$ (*16, 17*). The partial clone HGMP09 also appears 70% identical to corresponding regions of TSHR and the LH-CG receptors (Fig. 1). The fact that HGMP09 obviously belongs to the same G protein–coupled receptor subfamily, together with the tissue distribution of its transcript (Fig. 2A), makes HGMP09 a possible candidate for the FSH receptor.

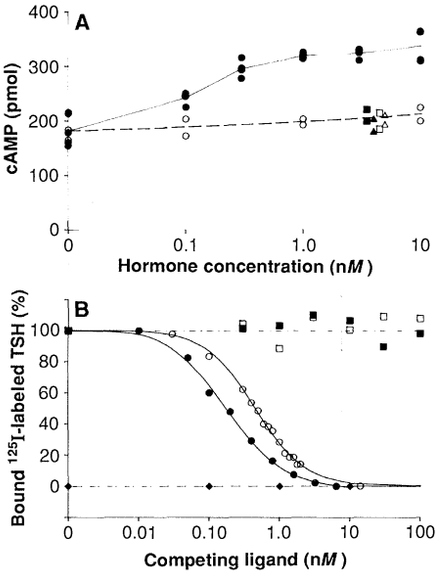

Fig. 4. (**A**) TSH-induced cAMP accumulation in *Xenopus* oocytes that had been microinjected with TSH receptor mRNA. *Xenopus* oocytes were handled as described (*23*) and injected with water (open symbols) or recombinant TSHR mRNA (*14*) (50 nl at a concentration of 0.1 μg/μl) (filled symbols). After 3 days in control medium, batches of 35 oocytes were incubated for 90 min in medium supplemented with various concentrations of TSH (circles), LH (squares), or FSH (triangles). cAMP was determined as described (*15*). RO 201724 and isobutylmethylxanthine ($10^{-6}M$ each) were present in all incubations. Results were concordant in three independent experiments. Incubation of control oocytes with $10^{-4}M$ forskolin resulted in a doubling of the cAMP concentration (M. Parmentier *et al.*, unpublished data). (**B**) Displacement of ^{125}I-labeled TSH from TSH receptors expressed in COS7 cells. COS7 cells were transfected with TSHR cDNA subcloned in pSVL (*24*). After 72 hours, cells were harvested and a membrane fraction was prepared (*25*). Membranes were similarly prepared from wild-type COS7 cells and from dog thyrocytes in primary culture (*22*). Binding of ^{125}I-labeled TSH (Trak, Henning Gmbh, Berlin, FRG) was performed at 0°C for 120 min in the presence of various concentrations of competitors (TSH; Armour, Chicago, IL. FSH and LH; UCB Bioproducts, Brussels, Belgium). Bound radioactivity was separated by centrifugation and measured. Results are expressed as a percentage of the ^{125}I-labeled TSH bound by transfected cells in the absence of competitor (3000 cpm) after correcting for nonspecific binding (radioactivity bound in the presence of 100 n*M* unlabeled TSH, 800 cpm). Open and filled circles, displacement by unlabeled TSH from COS7 cells and thyrocyte membranes, respectively. Open and filled squares, displacement for COS7 cells by LH and FSH, respectively. Diamonds, displacement by unlabeled TSH from untransfected COS7 cells. Similar displacement curves were obtained in three independent experiments.

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26. M. Parmentier and F. Libert contributed equally to the present work. We thank C. Massart for help with *Xenopus* oocytes, cAMP determinations, and artwork, and T. Velu, A. Donda, and S. Swillens for discussions. Supported by grants from the Ministère de la Politique Scientifique, the Fonds de la Recherche Scientifique Médicale, NIH (R01-DK21732), Solvay S.A., and the Association Recherche Biomédicale et Diagnostic. C.M. is a fellow from the Institut pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture (IRSIA). F.L. is Aspirant of the Fonds National de la Recherche Scientifique.

17 October 1989; accepted 14 November 1989

Regulation of Proenkephalin by Fos and Jun

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Fos and Jun form a heterodimeric complex that associates with the nucleotide sequence motif known as the AP-1 binding site. Although this complex has been proposed to function as a transcriptional regulator in neurons, no specific target gene has yet been identified. Proenkephalin mRNA increased in the hippocampus during seizure just after an increase in c-*fos* and c-*jun* expression was detected. Fos-Jun complexes bound specifically to a regulatory sequence in the 5′ control region of the proenkephalin gene. Furthermore, c-*fos* and c-*jun* stimulated transcription from this control region synergistically in transactivation assays. These data suggest that the proenkephalin gene may be a physiological target for Fos and Jun in the hippocampus and indicate that these proto-oncogene transcription factors may play a role in neuronal responses to stimulation.

THE PROTO-ONCOGENES, c-*fos* AND c-*jun*, are members of the class of inducible genes termed cellular immediate-early genes. Their protein products, Fos and Jun, have been proposed to function as transcriptional regulators that couple extracellular signals to alterations in cellular

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