Isolation of TSH and LH/CG Receptor cDNAs from Human Thyroid: Regulation by Tissue Specific Splicing

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A TSH receptor (TSH-R) cDNA has been isolated from a human thyroid λGT11 library. Unexpectedly, several cDNAs encoding the human LH/CG receptor (LH/CG-R), previously thought to be expressed solely in gonadal cells, were also isolated from the thyroid library. The receptors are structurally related, consisting of a signal sequence, a large extracellular amino terminal domain, seven membrane spanning domains, and a short carboxyl-terminal portion. The TSH-R is encoded by a single 4.2 kilobase mRNA specific to the thyroid. Introns were not present in any hTSH-R cDNAs examined, however, sequencing of several LH/CG-R cDNAs and RNase protection experiments demonstrated that the majority of hLH/CG-R mRNA in the thyroid is incompletely spliced. Consequently, tissue-specific splicing may be an important step in the regulation of the glycoprotein hormone receptor family. (Molecular Endocrinology 4: 1264–1276, 1990)

INTRODUCTION

The heterodimeric glycoprotein hormones, human CG (hCG), LH, TSH, and FSH are the largest, most complex mammalian hormones known, with mol wts from 28,000–38,000 (1). They share a common α-subunit, which is required for hormone binding, and a divergent β-subunit, which is also required for binding and determines hormone specificity.

LH and hCG, and FSH each bind to specific receptors on a variety of gonadal cells while TSH binds to a receptor on the thyroid follicular cell, resulting in the activation of adenylate cyclase in each case (2–4). The isolation of cDNAs encoding the rat and porcine LH/hCG receptors was reported recently (5, 6). While this manuscript was in preparation, isolation of the canine (7) and human (8, 9) TSH-R cDNAs were also reported.

Binding of TSH to its receptor on the thyroid follicular cell results in a rapid activation of adenylate cyclase which has been shown to be responsible for a diverse array of events in the cell, including an increase in thyroglobulin expression and secretion, an increase in iodide uptake, an increase in the processing of thyroglobulin into thyroid hormone, and entry of the cell from G_0 into the cell cycle (10).

The TSH receptor is of interest not only because of its central role in controlling thyroid cell metabolism. TSH also plays a key role in the growth and development of the thyroid gland. For example, in the absence of TSH the thyroid gland does not develop properly (11, 12). The thyroid gland of the hyt/hyt mouse strain is also rudimentary (13) and evidence suggests that this mutant strain may have a defective TSH receptor.

Finally, autoantibodies directed against the TSH receptor are directly responsible for the pathogenesis and

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hyperthyroidism of Graves' disease, a common autoimmune disorder (14). The cloning of the TSH receptor is therefore a critical step for furthering our understanding of both normal and abnormal thyroid function.

RESULTS

Cloning of the hLH/CG and TSH Receptor (hLH/CG-R, hTSH-R) cDNAs

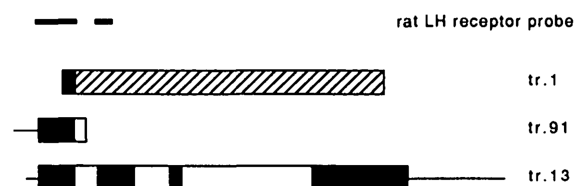
The strategy used for cloning the TSH receptor was based on the prediction that the structure of the receptor would be highly conserved relative to the other glycoprotein hormone receptors. The rat LH/CG receptor was previously purified from pseudopregnant rat ovary (15), and a full-length cDNA obtained (5). A human thyroid λGT11 cDNA library was screened at low stringency using a 622 base pair probe corresponding to the amino terminus of the rat LH/CG receptor. Three strongly hybridizing clones were isolated and sequenced. All three clones contained introns (Fig. 1) and one clone, tr. 13, contained an apparently full length coding sequence of 684 amino acids. The rat LH/CG cDNA probe was found to span portions of the first two exons. Clone tr. 1 was found to contain a consensus splice donor sequence at the first exon/intron boundary followed by intron sequences not found at the corresponding positions in clones tr. 91 and tr. 13. Additionally, introns 1 and 2 in clone tr. 13 did not contain consensus splice signals (Fig. 2A) suggesting partial aberrant splicing of these introns. The third intron in tr. 13 contains consensus splice signals and may represent the normal full-length intron at this position.

The amino acid sequence encoded by tr. 13 is almost identical in structure to the rat and porcine LH/CG receptors reported previously, with the exception of a small deletion corresponding to amino acids 288–301 of the rat LH/CG-R. The significance of this deletion is not yet understood, but may be the result of aberrant splicing, seen elsewhere in this clone. The human LH/CG-R amino acid sequence is greater than 90% identical to the rat receptor and >94% identical to the porcine receptor. Clones tr. 1, tr.91, and tr.13 appear therefore to encode incompletely processed versions of the human LH/CG receptor. Southern hybridization analysis (not shown) using the tr. 91 insert as a probe demonstrated the presence of a single LH/CG-R sequence in the human genome. In addition to the three strongly hybridizing clones, seven weakly hybridizing clones were purified, subcloned and mapped by restriction endonuclease digestion. Each of the seven cDNAs was unique and therefore we felt it was necessary to develop an alternative strategy for cloning the TSH receptor.

Degenerate oligonucleotides (24,000- and 32,000-fold degenerate, see *Materials and Methods*) for use in polymerase chain reaction (PCR) were designed using amino acid sequences from the third and sixth transmembrane regions which were identical in both the rat and human LH/CG-R. First strand cDNA was prepared using total RNA from bovine thyroid, a human Graves' disease thyroidectomy sample, human testis, and human ovary. When PCR was performed on these templates using the oligonucleotides based on the LH/CG receptor sequences, amplified products of the appropriate size [431 nucleotides (nt)] were obtained from bovine and human thyroid only. These bands were subcloned and sequenced. The amino acid sequence of the bovine and human fragments were 84% identical, and the human fragment clearly encoded a receptor highly related to but different from the hLH/CG-R, making both PCR sequences likely candidates for the TSH-R.

The human PCR fragment was then used to rescreen the thyroid library at high stringency. Screening 10^6 recombinant phage, twelve clones were isolated, 9 of which were approximately 4.2 kb in length. Three

A. Clones isolated by low stringency hybridization



B. Clones isolated by polymerase chain reaction

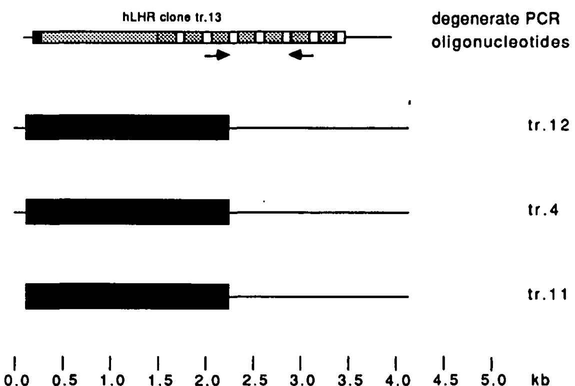


Fig. 1. Structure of hTSH-R and hLH/CG-R cDNA Clones
Solid bars indicate protein coding sequences, *open* and *hashed bars* indicate intron sequences, and *lines* indicate 5'- and 3'-untranslated sequence. Identical coding sequences among each set of clones are aligned vertically, with the scale in kilobases shown below. A, Human LH/CG-R clones showing intron/exon structure and the approximate location of the homologous rat LH/CG-R sequences used for low stringency screening. B, Human TSH-R clones showing the approximate location, relative to the assembled hLH/CG-R coding sequence, of the degenerate oligonucleotides used for PCR amplification of a TSH-R receptor fragment. The hLH/CG-R coding sequence is not drawn to scale. Clones were subsequently isolated by screening the thyroid library at high stringency with the TSH-R fragment.

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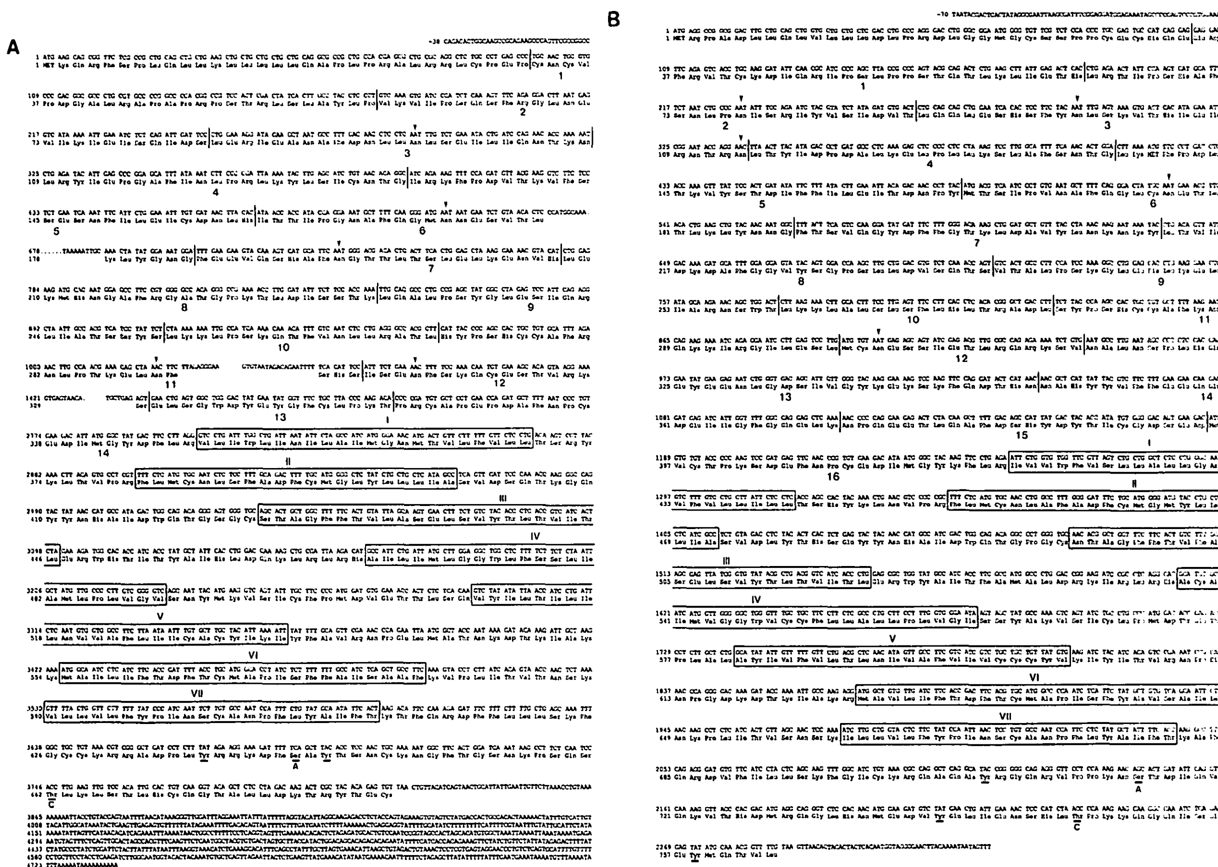

Fig. 2. Sequence of hLH/CG-R and hTSH-R cDNAs
Intron sequences (not shown) are represented by *dots*. *Vertical lines* represent approximate location of leucine rich glycoprotein (LRG) repeats and each repeat is *numbered underneath* the amino acid sequence. Membrane spanning domains are *boxed* and putative sites of N-linked glycosylation are shown with *arrows*. Potential tyrosine kinase phosphorylation sites in the COOH-terminal tail are underlined and consensus sites for cAMP-dependent protein kinase or protein kinase C phosphorylation are *underlined* and marked with an A or C, respectively. A, Sequence of the hLH/CG-R. B, Sequence of the hTSH-R.

clones were subcloned and restriction mapped (Fig. 1) and two clones (tr. 12 and tr. 4) were used to determine the complete sequence of the cDNA (Fig. 2B). While one of the hTSH-R sequences reported recently (9), isolated using amino acid sequence provided by us, yielded a predicted amino acid sequence nearly identical to that presented here, the other sequence reported had 16 amino acid differences, and two deletions present in the amino terminus (8).

Structure of the Glycoprotein Hormone Receptors

The nucleotide and predicted amino acid sequences of the hLH/CG-R and TSH-R are shown in Fig. 2, A and B. Only a portion of the sequence of the three introns found in the LH/CG-R clone tr. 13 is presented here. The predicted amino acid sequences of the hLH/CG-R, human TSH-R (hTSH-R), and rat FSH-R (rFSH-R, Ref. 67) were aligned using genalign (Intelligenetics) with the final adjustments made by hand (Fig. 3). The receptors are very similar structurally beginning with putative signal sequences at the amino terminus of each. The signal sequences are followed by a large extracellular amino terminal domain of approximately 323 and 395 amino acids (aa) for the hLH/CG-R and hTSH-R, respectively. The amino terminus is made up of a reiterated leucine-rich sequence (LxxLPxxLLxxLxxLxx-LDLSxNx) found in a number of serum and membrane glycoproteins known collectively as the leucine-rich glycoproteins (LRGs). Examples include α2-glycoprotein, a human serum protein (16), platelet membrane glycoprotein 1b, known to bind von Willebrand factor (17), and chaoptin, a drosophila membrane glycoprotein involved in photoreceptor cell morphogenesis (18). In each glycoprotein hormone receptor, this domain contains several potential N-linked glycosylation sites (Figs. 2 and 3). The rFSH receptor contains the fewest (three) while the hTSH and hLH/CG receptors contain five and six putative sites respectively. Five of the six sites are conserved in the LH/CG receptor sequence from rat, pig, and man, and several sites are also conserved among the different receptor species, suggesting a possible role of glycosylation in ligand binding. The difference in length between the hTSH-R and the hLH/CG-R and rFSH-R is due primarily to the presence in the hTSH-R sequence of an additional two LRG repeats (50 amino acids).

The spacing of amino acids in the extracellular amino

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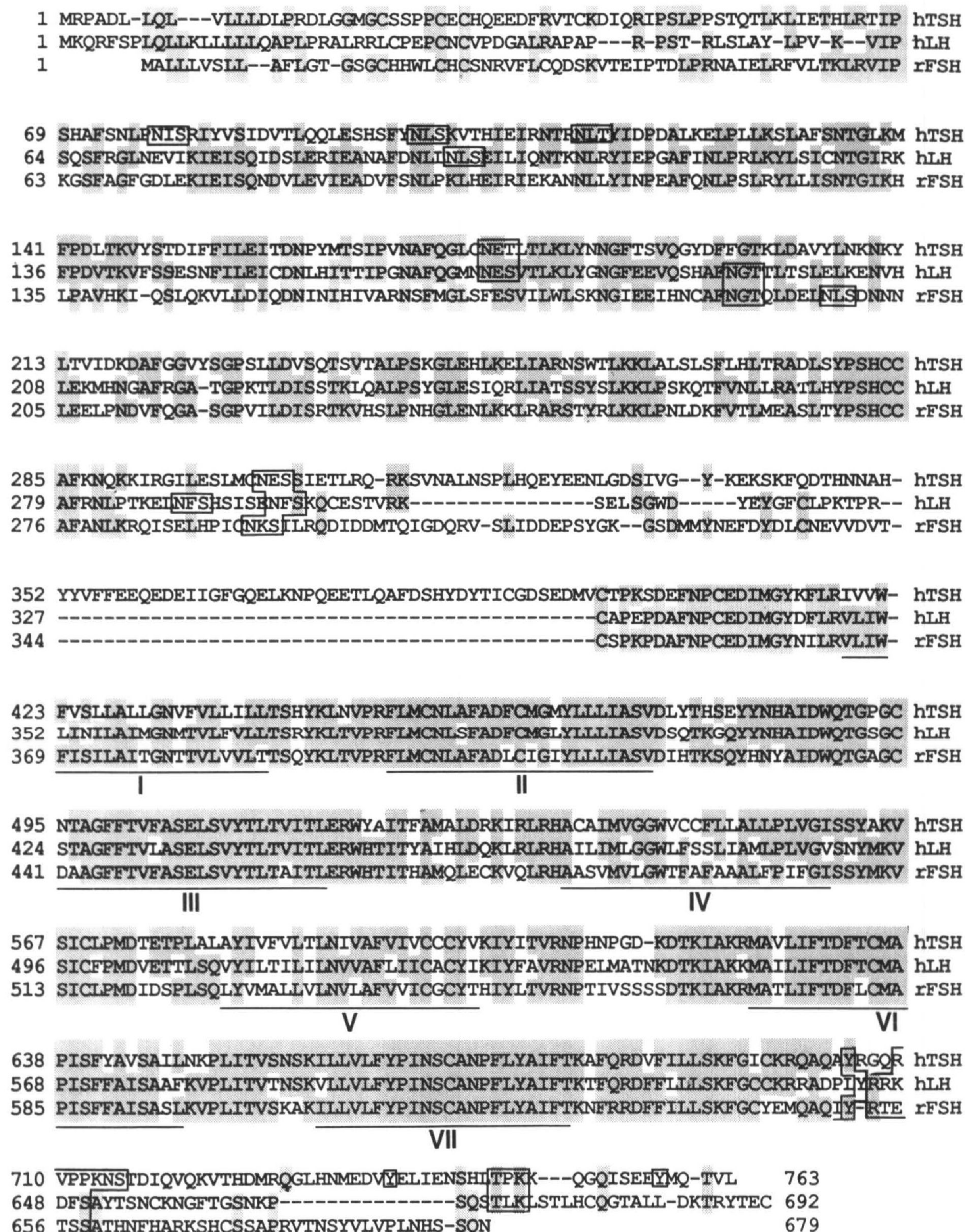

Fig. 3. Sequence Comparison of the hLH/CG-R, hTSH-R, and rat FSH-R

The human LH/CG receptor, hTSH-R, and rat FSH receptor amino acid sequences were aligned using the genalign program (Intelligenetics). Additional alignment was performed manually. Identical sequences are highlighted, and putative N-linked glycosylation sites, and phosphorylation sites are *boxed*.

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terminal domain is highly conserved in all three receptors through the first 11 LRG repeats. There then appears a hinge region consisting of a variable length repeat (LRG 12), two additional repeats unique to the hTSH receptor (LRG 14 and 15), and a ubiquitous highly conserved repeat (>70% amino acid identity) just prior to the first transmembrane segment, labeled 16 in the hTSH-R and 14 in the hLH/CG-R sequences. The amino acid sequences of the hTSH-R and hLH/CG-R are approximately 38% identical in LRG domains 1–12, and approximately 72% identical across the seven transmembrane domains. Little amino acid conservation is seen after the first 20 amino acids COOH-terminal to the last transmembrane domain. However, a potential phosphorylation site for cAMP-dependent protein kinase (19), protein kinase C (20) and several sites for tyrosine kinases (21) are found in the putative COOH-terminal intracellular domain of each receptor. The positioning of the cAMP-dependent protein kinase site and protein kinase C site is conserved among receptor species as is one of the tyrosine kinase phosphorylation sites (Fig. 3).

Tissue Distribution of the TSH-R and LH/CG-R mRNAs

Expression of hLH/CG-R and hTSH-R mRNAs was examined using four techniques: polymerase chain reaction (Fig. 4), Northern hybridization (Fig. 5), *in situ* hybridization (Fig. 6), and RNase protection (see Fig. 8). Polymerase chain reaction (PCR) was used as a highly sensitive method for detecting hLH/CG-R and hTSH-R mRNA expression. First-strand cDNA was prepared using 5 μg total cellular RNA from a variety of human tissues (Fig. 4A). PCR was performed with this material using oligonucleotides designed to prime the amplification of a 140 nt product from the 5′-end of the mRNA (see *Materials and Methods*). The amplified product was found, as expected, in reactions containing RNA from human testis and ovary, and also in a Graves' disease thyroidectomy sample (Fig. 4A). No hLH/CG-R mRNA expression was seen in the HeLa or IM9 lymphocyte cell lines, nor in several other human tissues tested including melanoma, pancreas (not shown) and placenta (not shown). Since the thyroid library was constructed using RNA from the thyroid of a single individual, the possibility existed that the LH/CG mRNA was aberrantly expressed in the thyroid of this individual, or perhaps only in Graves' disease. To test this hypothesis, RNA and first strand cDNA was prepared from three different Graves' disease thyroidectomy samples, and a cold nodule, a thyroid growth unable to capture iodide with the same efficiency as the surrounding thyroid tissue (hence, the appearance of a cold spot

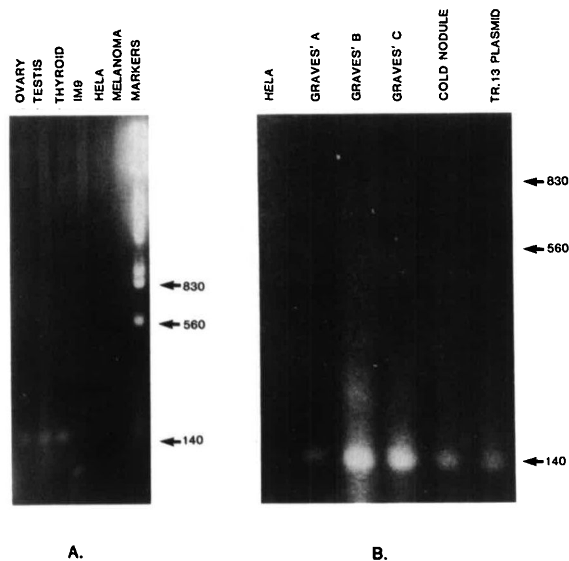

Fig. 4. PCR Analysis of hLH/CG-R Expression

PCR was performed as described (*Materials and Methods*) on first strand cDNA from the tissues shown using oligonucleotides capable of amplifying a portion of the 5′-end of the hLH/CG-R sequence. PCR products were electrophoresed on a 2% agarose TBE gel and stained with ethidium bromide. Size markers shown in A only, and are λ-phage DNA treated with *Eco*RI and *Hind*III. A, Expression of hLH/CG-R mRNA in a variety of human tissues (ovary, testis, thyroid, melanoma) and cell lines (IM-9 lymphocytes, hela cervical carcinoma). B, Expression of hLH/CG-R mRNA in a variety of human thyroid samples. As a positive control, PCR was performed on 100 ng pBS(−) plasmid containing the full-length hLH/CG-R cDNA insert.

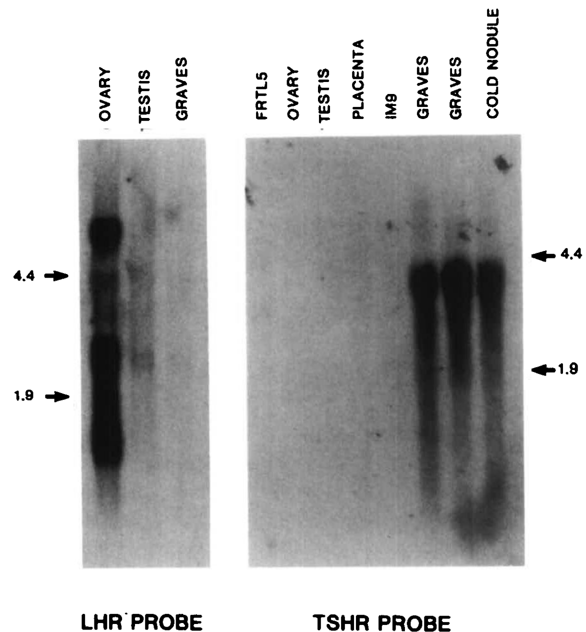

Fig. 5. Northern Analysis of LH/CG-R and TSH-R Expression

Autoradiographs of Northern blot probed with either a hLH/CG-R probe (*left panel*) or hTSH-R probe (*right panel*). Each lane contains 20 μg total RNA. Samples are pseudopregnant mouse ovary, human testis and human Graves thyroidectomy (*left panel*), and FRTL5 rat thyroid cell line, pseudopregnant mouse ovary, human testis, human placenta, human IM-9 lymphocyte cell line, Graves disease thyroidectomy samples, and a human thyroid cold nodule (*right panel*). Size markers are the human 18S and 28S ribosomal RNAs as visualized using UV shadowing. The autoradiographs resulted from a 3-day (LH/CG-R probe) or 6-day (TSH-R probe) exposure.

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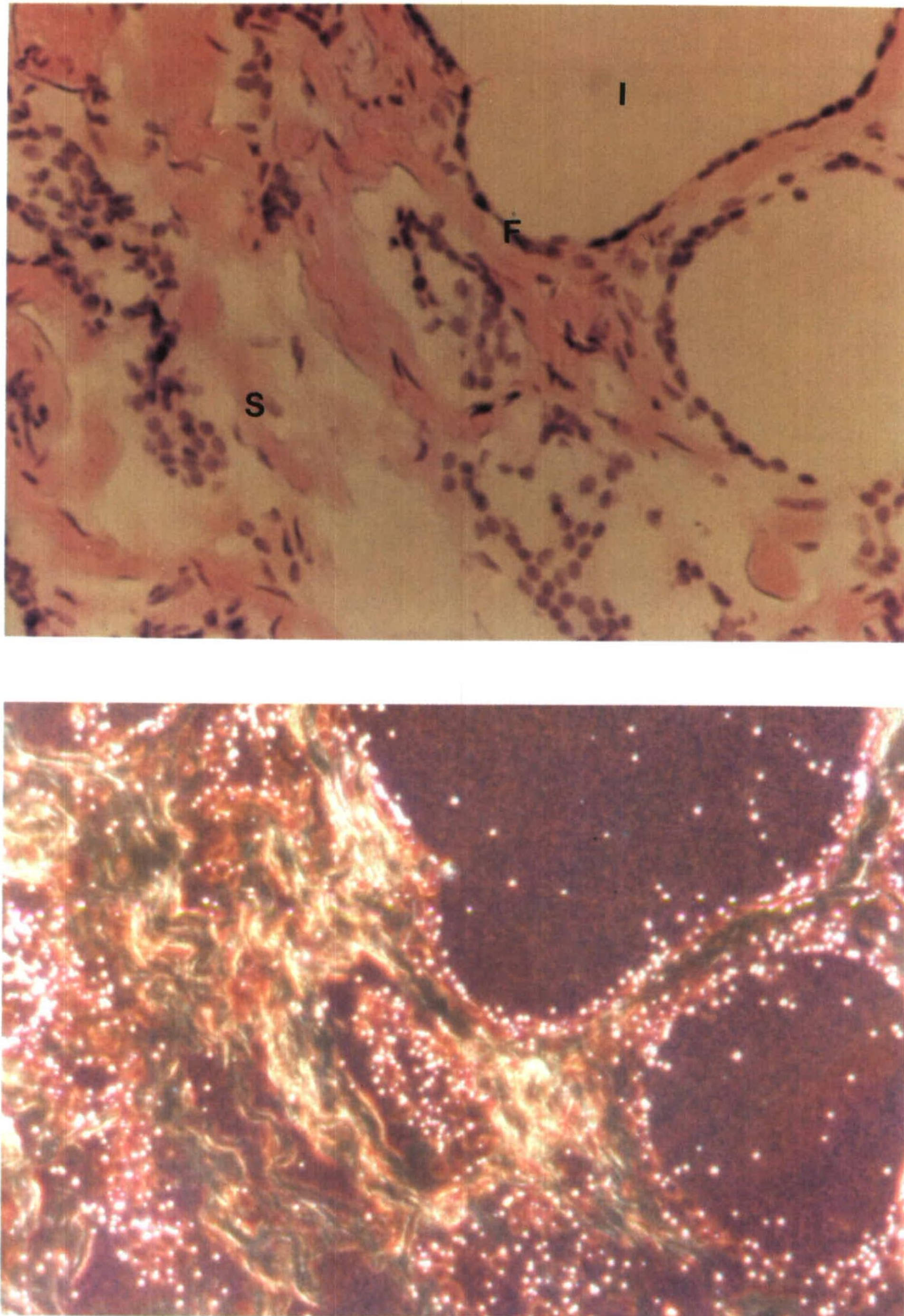

Fig. 6. *In Situ* Hybridization with an Anti-Sense ^{35}S-RNA Probe Complementary to the Human TSH receptor

A, Lightfield micrograph of an hematoxylin and eosin stained section of human thyroid at 75× magnification showing follicle cells (F), stromal cells (S), and the colloid intrafollicular zones (I). B, Darkfield micrograph (same field and magnification) showing autoradiographic signal (bright spots) overlying the thyroid follicular cells. The long exposure times (4 weeks) necessary for the visualization of the signal resulted in moderate levels of uniform nonspecific staining in intrafollicular zones and over stromal cells.

on a ^{125}I thyroid scan). PCR clearly demonstrated the presence of LH/CG-R mRNA in all four thyroid samples (Fig. 4B), but not in the control HeLa cell sample. Preliminary PCR results suggest that hTSH-R mRNA expression may also occur in the testis, at very low levels, however, since no hTSH-R mRNA could be detected by Northern analysis (see below, and Fig. 5, *right panel*).

Messenger RNA was examined by Northern hybridization analysis using a 600 nt probe corresponding to the amino terminal fragment of the hLH/CG-R or a 431 nt fragment, encoding transmembrane domains 3–6, of

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the hTSH-R. Probes were radioactively labeled to similar specific activities using the random-priming method (22). Three major [6.7, 2.6, and 1.2 kilobase (kb)] and three minor (4.5, 3.0, and 2.0 kb) LH/CG-R mRNA species are expressed in pseudopregnant mouse ovary, while two species (4.5 and 2.6 kb) are seen in human testis and thyroid (Fig. 5, *left panel*). Pseudopregnant mice were used as a source of ovarian tissue due to the extremely low amounts of mRNA seen in adult ovarian tissue (5). The different length LH/CG-R mRNAs, which may result from alternative splicing or varible length 3′-noncoding sequence (6, 23), vary in abundance from species to species and in testicular *vs.* ovarian tissue. While the two species of mRNA in testis and thyroid appear to migrate identically, we do not yet know whether they are the same and whether they are processed or unprocessed mRNA species. This question was examined in more detail by RNase protection (see below).

In contrast to LH/CG-R mRNA expression, found in both gonadal tissues and thyroid, TSH-R mRNA expression, by Northern analysis, appears restricted to the thyroid (Fig. 5, *right panel*). In the three human thyroid samples examined, the TSH-R is encoded by a single 4.2 kb mRNA species. In the clonal FRTL5 rat thyroid cell line, two TSH-R mRNA species of 3.0 and 4.4 kb are seen. No mRNA expression was detected by Northern analysis in human ovary, testis, or placenta, nor was any expression detected in the human IM9 lymphocyte line, previously shown to express high affinity TSH binding sites (24). The low level of mRNA expression seen in the FRTL5 cell line may be due to suboptimal hybridization with a human receptor probe, or may represent a decrease in receptor mRNA expression in this line.

In situ hybridization histochemistry revealed specific hybridization of anti-sense hTSH-R probe to the thyroid follicular cells, known to respond to TSH, while stromal cells (areas staining green) showed no specific staining (Fig. 6). Hybridization of similar sections with the sense probe revealed only non-specific hybridization.

Functional Expression of the TSH-R Protein

To demonstrate that clone tr. 12 encoded a full-length TSH receptor cDNA, we chose to examine its ability to couple TSH binding to activation of adenylate cyclase. A retrovirus expression vector, pLJ (25), containing the entire tr. 12 cDNA sequence was transfected into human 293 cells, and intracellular cAMP concentrations were measured 60 h later using a ^{3}H-cAMP displacement assay, after treatment with hCG, hFSH, or hTSH. As shown in Fig. 7, 100 ng/ml hFSH or hCG have little effect, while the same amount of hTSH elevated intracellular cAMP over 6-fold. Half-maximal intracellular concentrations of cAMP were obtained with approximately 60 picomolar hTSH. In several experiments, a 15-fold elevation of intracellular cAMP was induced by application of 100 ng/ml hTSH (not shown). Transfection of the retrovirus vector alone, with no hTSH-R insert, produced no elevation of intracellular cAMP over background in cells treated with 100 ng/ml TSH. Expression of the human LH/CG receptor was attempted using identical methods, however, no elevation of cAMP was seen after treatment with any of the glycoprotein hormones. This could result from any number of problems, including, for example, the deletion found in clone tr.13, or perhaps inefficient removal of the LH/CG-R introns in the nongonadal 293 cell line.

Tissue-Specific Regulation of hLH/CG-R mRNA Splicing

The presence of introns in all 3 hLH/CG-R clones isolated suggested that the LH/CG-R mRNA was not efficiently processed in the thyroid. To examine this question in more detail, we constructed an RNase protection probe by inserting a fragment of the hLH/CG-R cDNA into the PBS (−) vector (Stratagene, La Jolla, CA). The fragment spanned 323 nucleotides of exon 1 and 62 nucleotides of intron 1 (Fig. 8A). ^{32}P-labeled antisense RNA corresponding to this region was hybridized to 20 μg total RNA from human testis, human thyroid, and the FRTL5 cell line. After hybridization at 45C and RNAse digestion, the probe protected nearly equivalent amounts of an approximately 380 nucleotide fragment from all three samples, although the FRTL5 band appeared to be slightly smaller in size (Fig. 8B). However, significant amounts of spliced LH/CG-R mRNA, represented by a 323 nucleotide protected fragment, were seen in the lane representing human testis but not in human or rat thyroid. Under more stringent conditions (55 C hybridization), the rat LH/CG-R mRNA species was no longer protected while the same bands were protected in the two human samples (Fig. 8C). The smaller amount of protected RNA in thyroid at 55 C may have been due to experimental error since equivalent amounts of the 380 nucleotide band were seen in testis and thyroid in other hybridizations performed at 65 C. Additional bands seen at 267 and 210 nt may be due to splicing within exon 1. Receptor isoforms resulting from alternative splicing have been reported for the rat LH/CG-R mRNA (6, 23). Undegraded probe RNA ran significantly higher than the 380 nt band due to the presence of vector sequences.

DISCUSSION

The glycoprotein hormone receptors are structurally homologous polypeptides which represent a unique subgroup of the G protein-linked receptor family. It has been noted previously for both the TSH receptor (26) and the LH/CG receptor (27) that a high affinity hormone binding domain could be released from cells, most likely by proteolysis. Affinity crosslinking experiments using ^{125}I-labeled TSH initially suggested the TSH receptor was composed of two disulfide-bonded sub-

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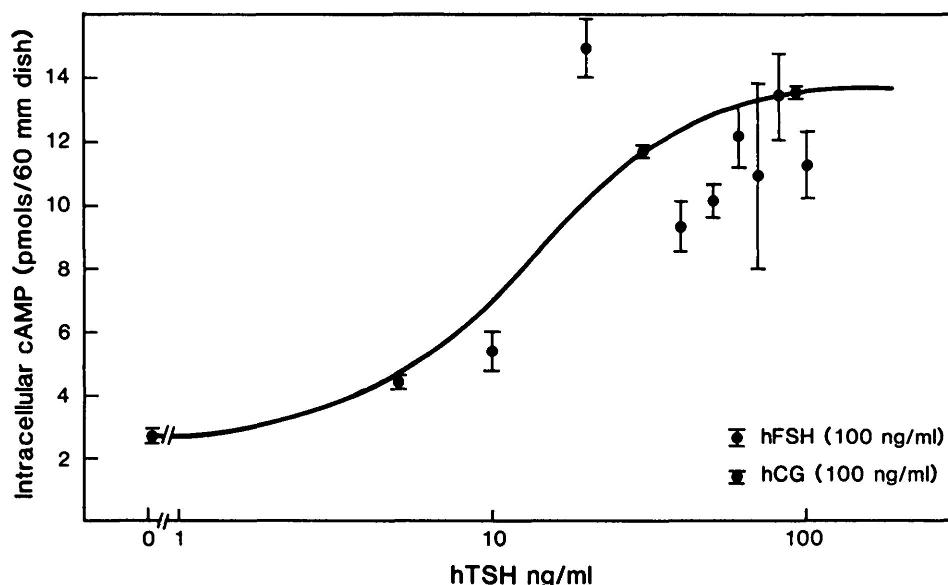

Fig. 7. Functional Expression of the TSH Receptor Protein
Intracellular cAMP (*ordinate*) was measured as a function of hormone concentration (*abcissa*) after transient transfection of an hTSH-R expression plasmid into the human 293 cell line. Hormones were obtained from the National Pituitary Program and were tested at a range of concentrations (hTSH) or at 100 ng/ml only (hFSH and hCG). *Points* indicate the mean and *bars* the range of the data.

units, a 25kd transmembrane subunit and a 45 kilodalton (KDa) extracellular hormone binding subunit (28). A similar size hormone binding component has been reported by others using different methodologies (29–31). However, more recent experiments on the TSH receptor protein present on rat FRTL5 cells have demonstrated the presence of larger precursors from which both subunits may derive (32). The existence of a protease-releasable hormone binding domain, plus the very large size of the glycoprotein hormones contradicted a simple seven membrane spanning structure for the glycoprotein hormone receptors. The G protein-linked receptors characterized thus far bind small ligands, such as the 15 Å long retinal molecule in the case of rhodopsin (33), in a pocket formed by three or more of the membrane-spanning segments (34, 35). In contrast, TSH has a Stokes radius of 33 Å (1) and thus would have a predicted diameter four times that of retinal. The paradox of how the glycoprotein hormone receptors could be related to the other G protein-linked receptors, and still bind a 28–38kDa ligand via a protease-releasable binding domain has now been partially resolved with the elucidation of the structure of these receptors.

Two models of TSH-R structure are presented in Fig. 9. The large extracellular LRG domain is likely to encode a high affinity hormone binding site (5, 26). In both models the β subunit is shown in contact with the receptor, particularly the LRG domain where the most sequence divergence is found among the glycoprotein hormone receptors, to emphasize its role in determining hormone binding specificity. It is predicted, however, based on studies of glycoprotein hormone binding, that multiple contacts are made between both hormone subunits and receptor (5, 31). Additionally, the carbohydrate moities on both TSH subunits are predicted to be involved in functional activity of hormone. Naturally occurring forms of TSH with a lower carbohydrate content (36) or chemically deglycosylated forms of hFSH and hCG (37, 38) do not disrupt high affinity binding of hormone to receptor, but do attenuate the ability of hormone to activate adenylate cyclase.

In the first model high affinity binding of glycoprotein hormone to the LRG domain indirectly induces a conformational change in the seven transmembrane domains without binding deep in a pocket formed by multiple transmembrane segments (Fig. 9A). In this model we predict that the conformation of the membrane-spanning segments may be very similar to that of other G protein-linked receptors.

Another model (Fig. 9B) would have glycoprotein hormone interacting directly with both the LRG domain and the membrane spanning domains. In this model the LRG domain is more closely associated with the cell membrane. The periodic distribution of hydrophobic residues is predicted to allow the LRGs to form amphipathic structures, which in the case of chaoptin is proposed to mediate the protein's tight association with the plasma membrane in the absence of a discernable transmembrane domain (18). In the second model, multiple contacts between hormone and both receptor domains would require a significantly larger ligand binding pocket than envisioned for other G protein-linked receptors.

Many hormones possess the capacity to regulate their cognate receptors (39). However, despite some

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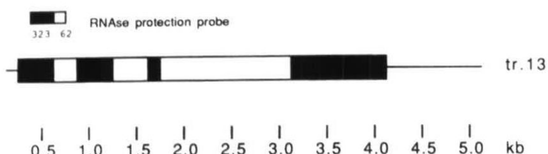

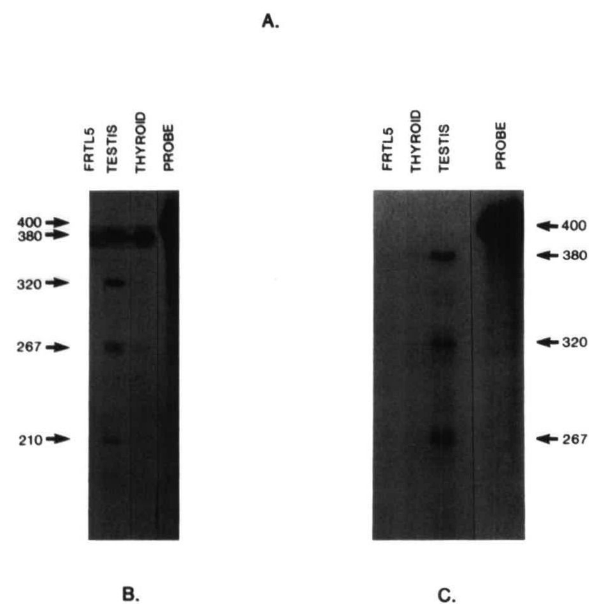

Fig. 8. RNase Protection Analysis of LH/CG Receptor mRNA

Radiolabeled antisense RNA spanning a portion of the hLH/CG-R sequence was hybridized with equal amounts (20 μg) of total RNA from the cell lines and tissues shown. Protected products were electrophoresed on a 6% acrylamide sequencing gel. A, Location of the antisense probe showing introns (*open boxes*), and exons (*lines* and *solid boxes*). Hybridizations were performed at 45C (B) and 55C (C). Samples are, *from left to right*, the rat FRTL5 thyroid cell line, human Graves' disease thyroid, human testis, and full-length anti-sense probe. Size markers were obtained from a DNA sequencing ladder electrophoresed on the same gel.

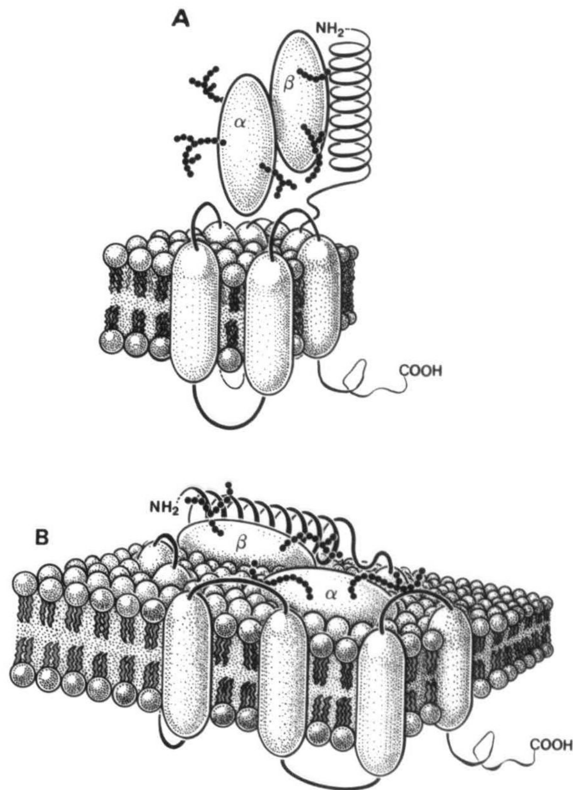

Fig. 9. Models of TSH Receptor Structure

Both models predict a specific interaction between the β-subunit of TSH and the amino terminal domain of the TSH-R, represented here in an a-helical configuration. One model (A) predicts no direct interaction between hormone and the 7 membrane spanning domains while in the second model (B) a larger pocket is formed by receptor, allowing direct conformational changes in the seven transmembrane domains as a result of hormone binding.

conflicting reports (40, 41) many experiments suggest that the binding of TSH or stimulatory Graves' autoantibodies to the TSH-R does not down-regulate receptor number or responsiveness (42–44). It is of interest to note that the TSH-R does not have a canonical consensus sequence for phosphorylation by cAMP-dependent protein kinase (Arg/Lys,Arg/Lys,X_{1-2}Ser/Thr) in contrast to the LH/CG-R and FSH-R (Fig. 3). LH/CG receptor down-regulation has been clearly demonstrated (45, 46).

The TSH receptor sequence will also allow a more detailed understanding of the etiology of Graves' disease. It is tempting to speculate that TSH and Graves immunoglobulin G (IgG) binding both take place in the highly divergent region of the amino terminal extracellular domain (LRG repeats 11–14 in the TSH-R). Synthetic peptides may be used to precisely locate the antigenic epitopes on the receptor. Two major classes of anti-receptor antibodies are seen in Graves' patients, which are distinguished by their behavior as either agonists or antagonists (14, 47). Both groups compete with TSH for binding and appear to recognize a limited number of epitopes (48, 49). Definition of the antibody binding sites on the receptor will allow a more detailed understanding of the mechanism of autoimmunity in this disease, a more detailed understanding of the hormone binding site and also the potential development of diagnostics and therapeutics for the treatment of the disease.

Two separate lines of evidence suggest hLH/CG receptor mRNA is expressed in the thyroid but is not completely processed. All hLH/CG receptor clones isolated from the thyroid library were found to contain intron sequences and RNase protection indicated that the majority LH/CG mRNA in adult thyroid remains incompletely spliced, while approximately 50% of the same mRNA in the testes is spliced. Presumably, appropriate splicing of this mRNA in gonadal tissue leads to expression of the *bona fide* LH/CG receptor. We cannot yet rule out the possibility that there may be additional LH/CG-R genes expressed in gonadal tissue with significant sequence variation from that presented here.

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The glycoprotein hormone receptors may be an example of a receptor family in which tissue distribution of the receptors is regulated, at least in part, by tissue-specific splicing. We are currently looking to determine whether minor amounts of TSH receptor mRNA is expressed in testis or ovary and whether the FSH receptor mRNA is expressed in thyroid. In addition to tissue-specific splicing, it is apparent that LH/CG-R mRNA is not efficiently spliced in normal testis (Fig. 8), and thus the amount of normal LH/CG receptor protein expressed in gonadal tissues may also be regulated by splicing. Given the structural similarities between the TSH and LH/CG receptor mRNAs, one would predict that there are tissue-specific factors recognizing small sequence differences between these mRNAs which result in the tissue-specific splicing.

Human CG is able to stimulate thyroid cells both *in vitro* and *in vivo*, and this had been attributed to an interaction between hCG and the TSH receptor (50–52). However our observations show that LH/CG-R mRNA is present in the thyroid and raise the possibility of expression of functional LH/CG receptor in the thyroid. There is increasing evidence to show that human chorionic gonadotropin plays a role in the regulation of thyroid function during early pregnancy. In the first 10 weeks of pregnancy, when levels of hCG are as much as eight times the value seen in postpartum women, levels of free T_3 and free T_4 are elevated by 30–35% (53) but TSH values fall within the normal range. While levels of hCG decrease as pregnancy progresses, there is a small but significant rise in TSH (54–56). Additionally, the precipitous drop in hCG levels which occurs after first trimester termination of pregnancy is associated with a doubling of TSH, suggesting that hCG had been subserving the role as thyroid stimulator during early pregnancy (57). If the LH/CG receptor is more efficiently spliced during pregnancy it would represent a mechanism for hCG-induced thyroid stimulation, and an intriguing example of hormonal regulation of mRNA splicing.

MATERIALS AND METHODS

Construction of a Human Thyroid cDNA Library

Poly(A)$^+$ RNA was isolated from a single Graves' disease thyroidectomy sample using a guanidinium thiocyanate procedure (58), followed by oligo (dT) cellulose chromatography. Complimentary DNA was synthesized using oligo (dT) primers (59) and species greater than 2 kb were selected by Sephadex G50 chromatography and inserted into the λGT 11 vector (60).

Isolation of LH/CG-R and TSH-R cDNA Clones

Five × 10^6 clones were screened with a 622 nt rat LH/CG receptor probe (5) radioactively labeled using the random primer method (22). Hybridization was performed in a solution containing 1 M NaCl, 50 mM Tris, pH 7.4, 10% Denhardts solution, 0.1% NaPyrophosphate, 0.2% sodium dodecyl sulfate (SDS), 10% dextran sulfate, 30% deionized formamide, 100 μg/ml salmon sperm DNA, 10^6 cpm/ml ^{32}P-labeled probe (SA > 5 × 10^8 cpm/μg) for 12 h at 42 C. Filters were washed in 2 × SSC, 0.1% SDS for 2 × 15 min at approximately 22 C, 2 × 30 min at approximately 42 C, 1 × 45 min at approximately 55 C, and 1 × 25 min at approximately 58 C.

Degenerate oligonucleotides for PCR (61) were designed to include all possible sequences encoding transmembrane domains 3 and 6 of the rat and hLH/CG-R (amino acids 498–506 and 633–641 of the hLH/CG-R), with the addition of an *Eco*R1 and *Hind*III restriction site respectively: ACAGAATTCGG[ACGT]TT[TC]TT[TC]AC[ACGT]GT[ACGT]TT[TC]GC[ACGT][TA][CG][ACGT]GA;ACAAGCTT[AG]AA[ACGT][CG][AT][AGT]AT[ACGT]GG[ACGT]GCCAT[AG]CA[ACGT]GT[AG]AA. First strand cDNA was prepared by reverse transcription of 5 μg total thyroid RNA from a second Graves' thyroidectomy sample. Thirty cycles of PCR amplification was performed with 1 min of denaturation at 94 C, 1 min of annealing at 50 C, and 2 min of extention at 72 C in 0.1 ml containing 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM each dNTP, 10 mM Tris, pH 8.3, and 2.5 Units *T. aquaticus* DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT). One tenth of the reaction was reamplified for 30 cycles after the addition of fresh nucleotides and polymerase. The amplified product was subcloned into PBS (−) (Stratagene) and sequenced using the dideoxy method (62). The fragment was subsequently used for rescreening 10^6 thyroid cDNA clones at high stringency (same as above except for 50% formamide in the hybridization and a final filter wash at 68 C in 0.1 × SSC, 0.1% SDS).

DNA Sequencing

The hLH/CG and hTSH receptor cDNAs were subcloned into the PBS(−) vector (Stratagene) and sequenced using the dideoxy method (62). The hLH/CG receptor vector was digested 5′ of the insert with *Sph*I and *Bam*HI and then treated with exonuclease III for varying lengths of time. The resulting set of overlapping clones were then sequenced to assemble the complete cDNA sequence. The 5′-terminal 2500 nucleotide portion of the TSH receptor cDNA was restriction mapped and a number of small fragments were subcloned into PBS(−) and sequenced on both strands. Nucleic acid sequence analysis was performed using software from Intelligenetics.

Northern Analysis

Total RNA (20 μg) from each of the tissues shown was electrophoresed on a 1% agarose, 2.2 M formaldehyde gel, transferred to Nytran filters (Schleicher & Schluell, Keene, NH) and hybridized at high stringency, as described above, with ^{32}P-labeled probes.

In Situ Hybridization

Human thyroid tissue was obtained from the Tumor Bank, Department of Pathology, New England Medical Center Hospital (Boston, MA), from total thyroidectomy samples removed from euthyroid patients with medullary thyroid carcinoma. Eight micromolar cryostat sections of normal appearing thyroid follicles were prepared on gelatin-coated slides for *in situ* hybridization. A 1-kb fragment of cDNA encoding the human TSH-R was used as a template for *in vitro* transcription. The specific activity of ^{35}S-labeled sense and anti-sense transcripts was approximately 10^8 cpm/μg probe. Tissue sections were pretreated with detergent and protease and incubated in hybridization buffer for 16 h at 42 C with 3 × 10^6 cpm probe as described previously (63). After ribonuclease treatment, sections were washed extensively at 37 C, dehydrated, and immersed in Kodak NTB-2 photographic emulsion. After exposure times of 1–4 weeks slides were developed, fixed, counter-stained with haematoxylin and eosin; and examined by light and darkfield microscopy.

Rnase Protection

Ribonuclease protection was performed essentially as described (64). A 385 nucleotide fragment of the human LH/CG-

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R from clone tr. 91 was subcloned into the pBS-vector (Stratagene). Anti-sense RNA was transcribed in the presence of 50 μCi [a-^{32}P]CTP using T7 RNA polymerase. Total RNA from each of the tissues shown was denatured at 85 C for 5 min., hybridized with 5 × 10^5 cpm of probe in 0.4 M NaCl, 1 mM EDTA, 40 mM PIPES (Piperazine-*N*,*N*-bis[2-ethanesulfonic acid]; 1,4-Piperazinediethane sulfonic acid) pH 6.4, 80% deionized formamide for 12 h at 45–65 C. The hybridization products were then digested with 40 μg/ml ribonuclease A and 2 μg/ml ribonuclease T1 for 1 h at 30 C. The protected products were treated with proteinase K, phenol extracted, precipitated, and analyzed by acrylamide gel electrophoresis as described (64).

Transient Expression Analysis

The entire 4.2 kb clone tr. 12 was inserted into the *Bam*HI site of the retroviral vector pLJ (25). Ten micrograms of the expression plasmid or the pLJ plasmid with no insert were then transfected onto separate 60-mm dishes of exponentially growing human 293 cells using a modification of the $CaPo_4$ method (65). The plates were rinsed twice with Dulbecco's modified Eagle's medium plus 10% calf serum after 18 h. After 60 h the plates were rinsed twice with Dulbecco's modified Eagle's medium containing 1 mg/ml BSA and 0.5 mm IBMX. The cells were then incubated for 30 min at 37 C in the same medium containing the various concentrations of hTSH, hFSH, or hCG shown. The cells were then rinsed twice with Hanks buffered salts at 4 C and lysed with 1 ml 60% ethanol. The cells were scraped and pelleted and the supernatants were lyophilized. The resulting pellets were resuspended in water and the cAMP in each sample was quantified using an assay method (Amersham, Arlington Heights, IL) which measures the ability of cAMP in the sample to displace [8-^{3}H] cAMP from a high affinity cAMP binding protein (66). Duplicate plates were analyzed for each data point. *Bars* indicate the range of values obtained and points indicate the mean. All hormones, kindly provided by the National Pituitary Program, NIDDK, were the purest grades available. The hTSH had a bioactivity of 15.0 I.U./mg.

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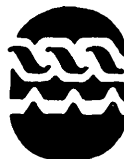

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