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Cloning, chromosomal assignment, and regulation of the rat thyrotropin receptor: Expression of the gene is regulated by thyrotropin, agents that increase cAMP levels, and thyroid autoantibodies

(FRTL-5 cell line/chorionic gonadotropin/lutropin/guanine nucleotide-binding protein)

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ABSTRACT **A rat thyrotropin (thyroid-stimulating hormone, TSH) receptor cDNA was isolated that encoded a protein of 764 amino acids, M_r 86,528. Transfection of the cDNA caused COS-7 cells to develop a TSH-sensitive adenylate cyclase response and the ability to bind ^{125}I-labeled TSH; both activities were similar to those of rat FRTL-5 thyroid cells and not duplicated by lutropin. The gene represented by the cDNA was assigned to mouse chromosome 12 and human chromosome 14. Northern analyses identified two species of mRNA, 5.6 and 3.3 kilobases, in FRTL-5 thyroid cells; the transcripts appeared to differ only in the extent of their 3′ noncoding sequences. There were minimal amounts of the two mRNAs in rat ovary, and neither was detected in RNA preparations from rat testis, liver, lung, brain, spleen, and FRT thyroid cells, which do not have a functional TSH receptor. TSH decreased both mRNA species 3- to 4-fold within 8 hr in FRTL-5 thyroid cells; down-regulation was dependent on TSH concentration and duplicated by forskolin, cholera toxin, or 8-bromo-cAMP but not by a phorbol ester. Down-regulation was also duplicated by thyroid-stimulating autoantibodies, which increased cAMP levels, but not by thyrotropin binding-inhibiting autoantibodies, which actually increased TSH receptor mRNA levels.**

The rat FRTL-5 thyroid cell has become a widely used model of a normal, functioning endocrine cell; its growth and function depend on thyrotropin (thyroid-stimulating hormone, TSH) (1–3). It is further used to measure and study the action of autoantibodies in patients with autoimmune thyroid disease (1–3). Defining the structure of the rat TSH receptor and its role in the growth and function of FRTL-5 cells has thus become critically important to a multiplicity of research and clinical programs. In the present work we have cloned the TSH receptor from rat FRTL-5 thyroid cells.§ Its amino acid sequence is ≈90% identical with the dog (4) and human (5–7) TSH receptor sequences recently reported.

In this report we additionally describe the mouse and human chromosomal localization of the gene. More important, we show that expression of the gene is down-regulated by TSH, thyroid-stimulating autoantibodies (TSAbs), or any agent with a cAMP signal; in contrast, autoantibodies that interact with the receptor and inhibit TSH binding (TBIAbs) actually increase gene expression. Since data are provided showing that the interaction of both types of antibodies with thyroid cells is dependent on the presence of TSH receptor mRNA, this report establishes that the antibodies interact with different epitopes of the receptor and these epitopes have different physiological roles.

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MATERIALS AND METHODS

Cell Culture. The rat FRTL-5 thyroid cells (ATCC CRL 8305) are a continuous line of functioning cells derived from normal Fischer rats (1, 2). FRT thyroid cells are also a line derived from normal Fischer rats but have no functional TSH receptor (8). Both were maintained in culture as described (1, 2, 8).

Probe Preparation Using the Polymerase Chain Reaction. Cloning was based on the presumed (9) structural relationship between the rat TSH receptor and the rat receptor for lutropin (luteinizing hormone, LH)/chorionic gonadotropin (CG) (10). cDNA templates were synthesized using 5 μg of rat testis poly(A)$^+$ RNA (Clontech), murine reverse transcriptase (Pharmacia), and Pharmacia's protocol. The 286-base-pair (bp) cDNA fragment was amplified (11) with *Thermus aquaticus* DNA polymerase and 25 pmol of each of the primers identified in Fig. 1 (wavy lines). Each cycle was 1 min at 94°C for denaturation, 2 min at 55°C for hybridization, and 3 min at 72°C for extension. Rescreening used a 177-bp probe representing the 5′ end of clone 16B1 (Fig. 1 legend), synthesized using 10 ng of pGEM-7Z plasmid (Promega) containing the 16B1 insert and appropriate primers.

Isolation of Rat FRTL-5 TSH Receptor cDNA by Plaque Hybridization, Subcloning, and DNA Sequencing. A FRTL-5 cell cDNA library constructed in λgt11 was screened by plaque hybridization (12, 13). The initial screening used low-stringency conditions (55°C); subsequent screenings used a temperature of 65°C. cDNA fragments were prepared, subcloned, and sequenced as described (12–14). DNA and nucleotide sequence alignments were performed using PC-GENE software (IntelliGenetics).

RNA Isolation and Northern Analysis. Poly(A)$^+$ RNAs from FRTL-5 thyroid cells were prepared and Northern analyses using Nytran membranes (Schleicher & Schuell) were performed as described (12, 13). The probes used were the purified inserts from either clone 16B1 or 4A2 and a β-actin cDNA (kindly provided by B. Paterson, National Cancer Institute). Filters were washed as described (13), with final

Abbreviations: TSH, thyrotropin; LH, lutropin; CG, chorionic gonadotropin; TSAb, thyroid-stimulating antibody; TBIAb, antibody that inhibits TSH binding.

§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M34842).

washings in 1× SSPE (0.18 M NaCl/10 mM phosphate, pH 7.4/0.5 mM EDTA) plus 0.1% SDS at 65°C.

Transfection Studies. Transfection experiments with COS-7 cells (ATCC CRL 1651) used an electroporation technique (Bio-Rad). The expression vector was constructed by subcloning *Eco*RI T8AFB (Fig. 1) cDNA inserts into the *Eco*RI site of simian virus 40-promoter-driven pSG5. Cells (10^7 per ml), washed and resuspended in 0.8 ml of sucrose/phosphate electroporation buffer, were incubated with plasmid DNA (80 μg in 10 μl of water) for 10 min in an ice-water bath before being pulsed (330 V, 25 μF). Cells were plated in 10-cm dishes (4×10^6 cells per dish); TSH-stimulated cAMP production or TSH binding was measured after culture for 40 hr; cell viability was ≈50% after electroporation.

Assays of TSH Binding and cAMP Levels. Highly purified bovine TSH (NIDDK-bTSH-I-1, 30 units/mg) was radioiodinated and binding was measured essentially as described (15) with the exception that the washing and incubation buffer

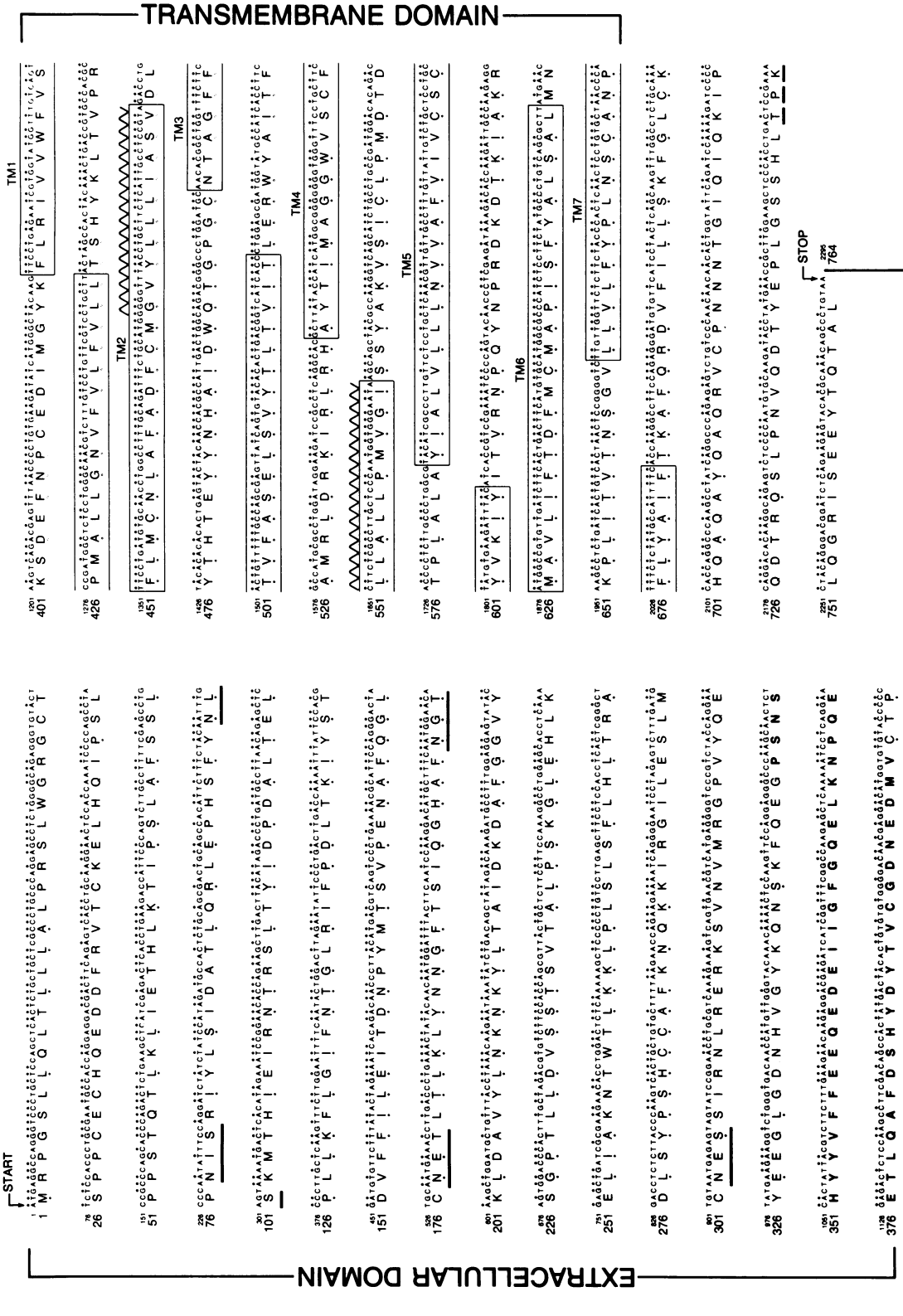

FIG. 1. (*Legend appears at the bottom of the opposite page.*)

was modified Hanks' balanced salt solution (NaCl replaced by 222 mM sucrose) containing 0.5% bovine serum albumin and 20 mM Hepes at pH 7.4. Incubation mixtures contained 4×10^6 cpm of ^{125}I-labeled TSH (120 μCi/μg; 1 μCi = 37 kBq) and unlabeled TSH or LH as noted. Specific binding was calculated by subtracting values obtained in the presence of 0.1 μM unlabeled TSH. cAMP levels were assayed as described (2). Cell pellets were in each case solubilized with 1 M NaOH for protein determinations. Protein was measured using Bio-Rad reagents and a bovine serum albumin standard.

Chromosomal Mapping. Mapping of the human chromosome used a human fibroblast line (GM2658) with the karyotype 46,XX,t(2;6)(q11,q15) and human–Chinese hamster somatic cell hybrids (12). Chromosomal location of the mouse gene was determined by Southern blotting of restriction enzyme-digested DNAs purified from hamster–mouse somatic cell hybrids (13). In both cases the full-length cDNA, T8AFB, was used as the probe.

Materials. Purified bovine TSH and LH (USDA-bLH-B5, 2.1 units/mg) were obtained from the hormone-distribution program of the National Institute of Diabetes and Digestive and Kidney Diseases. Other materials were from the sources previously stated (12, 13).

RESULTS

Cloning. From 8×10^5 plaques screened at low stringency with the rat testis LH/CG receptor probe, 20 rat FRTL-5 thyroid cell clones were obtained. Eighteen, with insert sizes 1.4 to 4.2 kilobases (kb), contained a transmembrane domain exhibiting 70% amino acid sequence identity with the comparable region of the rat LH/CG receptor (10). Compared to the LH/CG receptor, the two longest clones, 4.2 and 2.4 kb in length (4A2 and 16B1, respectively), had an incomplete 5′ end (Fig. 1 legend). After rescreening with a 0.18-kb probe identical to the 5′ end of clone 16B1 (*Materials and Methods*), a 2.8-kb cDNA clone (T8AFB) was obtained with characteristics compatible with its encoding the full-length TSH receptor and only a small portion of the 3′ noncoding region defined by clones 4A2 and 16B1 (Fig. 1).

The nucleotide sequence of T8AFB, 2834 bp long, contains an open reading frame encoding a protein, M_r 86,528, with 764 amino acids (Fig. 1). The first in-frame ATG is followed by codons specifying a hydrophobic sequence (Fig. 1, first 21–23 residues) defined as a signal peptide in the LH/CG receptor (10). There is a long hydrophilic region with five potential N-linked glycosylation sites (Fig. 1, underlined) followed by a region with seven hydrophobic, membrane-spanning domains (boxed and numbered) and a cytoplasmic region containing one potential protein kinase C phosphorylation site (broken underline). The TAA stop codon is followed by a polyadenylylation signal at nucleotides 2686–2691 (Fig. 1, underlined). The peptide present in the TSH receptor but not the LH/CG receptor is noted (Fig. 1, bold).

The homology between the entire coding regions defined by the rat TSH and LH/CG receptors is 64% and 48% for nucleotides and amino acids, respectively. The homology in the transmembrane region is, in contrast, 69% and 70%, respectively (Fig. 1). The overall amino acid homology with the human and dog TSH receptors (4–7) is 86% and 89%, respectively.

Expression in COS-7 Transfectants. When T8AFB in the correct orientation was transfected into COS-7 cells, the encoded protein caused the cells to become sensitive to TSH in cAMP assays. The relative increase in total cAMP induced by 0.1 nM TSH in the transfected cells was comparable to increases measured in the F1 subclone of FRTL-5 thyroid cells in the same experiment, 5-fold vs. 10-fold above basal, respectively (>20 pmol/mg of protein vs. <5 pmol/mg of protein). By comparison, LH did not significantly increase cAMP above basal levels when tested at a 10-fold higher (1 nM) concentration, and COS-7 cells transfected identically with constructs containing the cDNA insert in the opposite orientation did not have a TSH-increased adenylate cyclase activity.

The development of a TSH-sensitive adenylate cyclase response in the transfected COS-7 cells was accompanied by the appearance of specific binding of TSH. Binding of ^{125}I-labeled TSH to COS-7 cells transfected with the insert in the correct orientation, but not in the opposite orientation, exhibited a curvilinear isotherm similar to that of FRTL-5 thyroid cells (15) and was inhibited 50%, 75%, and >90%, respectively, by 0.3, 3, and 30 nM unlabeled TSH but not by 10 nM LH. The K_d values for the high- and low-affinity binding sites were estimated to be 1.3×10^{-10} M and 5.1×10^{-8} M, respectively; these compare favorably with values for rat FRTL-5 cells (15), 5.9×10^{-10} M and 1.7×10^{-8} M.

Chromosomal Assignment. The gene for the mouse TSH receptor, *Tshr*, was assigned to chromosome 12 by Southern blot analysis of DNAs of interspecies backcross mice. The restriction enzyme *Sca* I identified a polymorphism in the parental strains of this cross: NFS/N (8.4 kb) and *Mus musculus* (Skive) (7.1 kb). Inheritance of the NFS/N fragment in the backcross (NFS × *musculus*) × *musculus* showed that *Tshr* is linked to *Igh*, with 12 recombinants in 72 mice (16.7 ± 4.4 centimorgans). Linkage relationships among genes on mouse chromosome 12 are conserved on human chromosomes 2 and 14. The human TSH receptor gene, *TSHR*, was mapped to chromosome 14. Interestingly, the thyroid peroxidase gene also maps to mouse chromosome 12 but is on human chromosome 2 (13).

Gene Expression. Northern analyses of poly(A)$^+$ RNA preparations from rat FRTL-5 thyroid cells identified two mRNA species, 5.6 and 3.3 kb in size (Fig. 2, lane 1). The same two mRNAs were barely detected in rat ovary (lane 4) and were not detected in rat testis, brain, liver, lung, or spleen (lanes 3 and 5–8, respectively). A probe derived from the midportion of the extracellular domain of the T8AFB clone (Fig. 1) hybridized with both species of transcripts, 5.6 and 3.3 kb. In contrast, a 0.7-kb cDNA probe derived from the 3′-terminal, nontranslated portion of clone 4A2 (Fig. 1) hybridized only with the 5.6-kb transcript. These results indicate that the 5.6-kb mRNA transcript is larger primarily because it contains a longer 3′ noncoding region.

Receptor Regulation by TSH and Autoantibodies to the TSH Receptor. Poly(A)$^+$ RNA from FRTL-5 rat thyroid cells maintained in the absence of TSH for 7 days (Fig. 2, lane 1) had significantly higher levels of both the 5.6- and 3.3-kb transcripts than did cells maintained in the presence of TSH (lane 2), suggesting that TSH down-regulated expression of

FIG. 1 (*on opposite page*). Nucleotide and deduced amino acid sequence of the TSH receptor derived from clones 4A2, 16B1, and T8AFB, which represent nucleotides 1045 to 5214, 551 to 2920, and −54 to 2780, respectively. Dots denote residues that are the same as in the rat LH/CG receptor as determined by the computer-derived best-fit comparisons of the two sequences. Five potential glycosylation sites (extracellular domain, underlined) and one potential phosphorylation site (broken underline) are noted. Numbered boxes (TM1–TM7) denote the seven hydrophobic regions of the transmembrane domain. The wavy lines above two of these boxes show the approximate endpoints of the LH/CG receptor probe used to screen the FRTL-5 library. Solid underlining in the nontranslated 3′ region indicates polyadenylylation signals. Residues presented in bold type represent the peptide region unique to the TSH receptor.

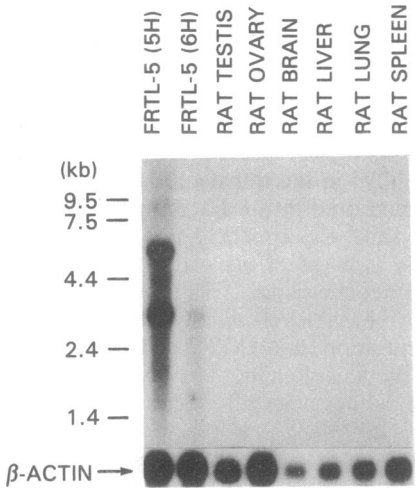

FIG. 2. Northern analysis of poly(A)$^+$ RNA (5 μg per lane) isolated from rat FRTL-5 thyroid cells maintained in the absence (5H) or presence (6H) of TSH compared with preparations from rat testis, ovary, brain, spleen, liver, and lung (Clontech). Hybridization was with the insert from the 4A2 clone (Fig. 1) and with β-actin cDNA.

this gene. Down-regulation was rapid, 3- to 4-fold within 8 hr of TSH challenge (Fig. 3*A*); was dependent on TSH concentration (Fig. 3*B*); and could be duplicated by cholera toxin, forskolin, or 8-bromo-cAMP but not by phorbol 12-myristate 13-acetate (Fig. 3*B*). Measured at the same time and under the same conditions as in Fig. 3*B*, TSH binding to cells decreased 62 ± 10% whether TSH, cholera toxin, forskolin, or 8-bromo-cAMP was the agent.

Patients with autoimmune thyroid disease have circulating autoantibodies that increase cAMP levels (TSAbs) and that inhibit TSH binding (TBIAbs) (1–3). When IgG preparations

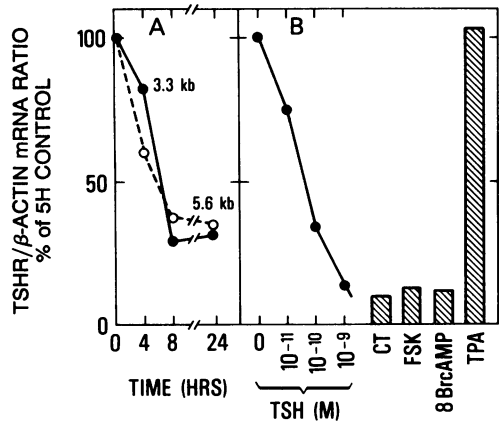

FIG. 3. Expression of TSH receptor mRNA in FRTL-5 cells at various times after exposure to 0.1 nM TSH (*A*) or 24 hr after exposure to various concentrations of TSH or to cholera toxin (CT, 10 ng/ml), forskolin (FSK, 10 μM), 8-bromo-cAMP (8 BrcAMP, 1 mM), or phorbol 12-myristate 13-acetate ("12 *O*-tetradecanoylphorbol 13-acetate," TPA, 20 nM) (*B*). Cells were maintained 6–7 days with no TSH in the medium. Poly(A)$^+$ RNA was prepared from cells at the times noted after TSH challenge (*A*) or 24 hr after challenge (*B*). Equal amounts of poly(A)$^+$ RNA (5 μg per lane) were subjected to sequential Northern analysis using the 16B1 (*A*) or T8AFB (*B*) cDNA inserts and a β-actin probe. After autoradiography, quantitation in arbitrary units was made by densitometry (LKB laser densitometer). After the densitometry value in the cells with no TSH (5H) at zero time was set as the reference value for both the TSH receptor (TSHR) and β-actin probes, the ratios were calculated. Data are presented as percentages of control ratio values from cells maintained in the absence of TSH (5H). In *B*, the sum of the 5.6- and 3.3-kb mRNAs is plotted.

Table 1. Ability of IgG preparations from patients with autoimmune thyroid disease to down-regulate TSH receptor mRNA in FRTL-5 cells

Addition to medium	TBIAb index	TSAb activity	TSH receptor/β-actin mRNA ratio, % of 5H control
Normal IgG	0	100	116
TSH (0.1 nM)	—	—	15
Graves IgG			
Patient 1	84*	1065*	22
Patient 2	80*	107	92
Patient 3	79*	122	103
Primary hypothyroidism IgG			
Patient 1	88*	86	260*
Patient 2	95*	77	189*

IgG preparations were prepared using protein G (Genex). Patients with active, untreated autoimmune Graves disease (hyperthyroidism) or primary hypothyroidism were diagnosed as described (1–3, 16). TSAb activity (ability to increase cAMP levels) was measured using FRTL-5 thyroid cells and the indicated IgG at 1 mg/ml (2); values are expressed as the percent increase over 5H control cells exposed to normal human IgG. TBIAb activity (ability to inhibit TSH binding) was measured in a radioreceptor assay (17); the TBIAb index measures the ability of patient IgG to inhibit relative to normal IgG (17). The effect of IgG (1 mg/ml) on TSH receptor mRNA levels was measured after 24 hr as described in the legend to Fig. 3.

*Statistically significant ($P < 0.01$).

from patients with Graves disease were tested, those which increased cAMP levels like TSH also down-regulated TSH receptor mRNA levels (Table 1, Graves IgG no. 1, representative of six tested). IgG preparations from patients with primary hypothyroidism, which have potent TBIAb but no TSAb activity, increased TSH receptor mRNA levels (Table 1, representative of six tested). Reactivity of both types of antibodies with FRTL-5 cells is associated with the presence of the TSH receptor in the cell (Fig. 4). Thus, TSAbs and TBIAbs, as well as TSH, reacted with FRTL-5 rat thyroid cells (Fig. 4*A*; refs. 1–3 and 16) but not with FRT rat thyroid cells (Fig. 4*A*). FRT, a continuously growing line that, like FRTL-5, is derived from Fischer rats and has an apparently normal adenylate cyclase complex sensitive to cholera toxin and forskolin (1–3, 16), did not express TSH mRNA (Fig. 4*B*). Interestingly, IgG from Graves patients having as potent

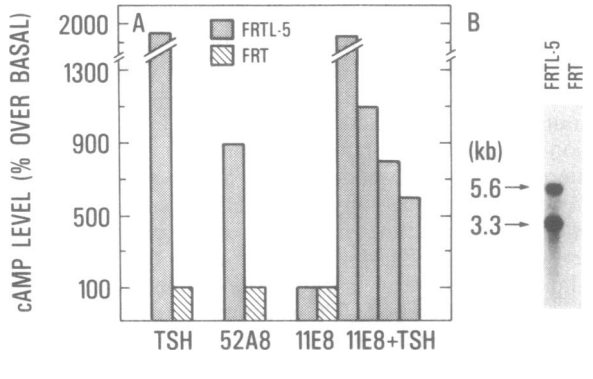

FIG. 4. (*A*) Effect of monoclonal TSH receptor antibodies 52A8 and 11E8 (16) on cAMP levels in rat FRTL-5 and FRT thyroid cells. The ability to increase cAMP was measured as described (2), using purified IgG at 50 μg/ml; the ability of 11E8 to inhibit TSH-increased cAMP levels was measured with antibody at 30, 70, and 150 μg/ml. (*B*) Expression of TSH receptor mRNA in FRTL-5 and FRT cells. Northern analyses were performed using poly(A)$^+$ RNA (5 μg per lane) as in Fig. 2; hybridization of the FRT cell RNA with the β-actin cDNA was performed to ensure that the lack of reaction with the TSH receptor probe was not due to mRNA degradation.

a TBIAb activity as IgG from patients with primary hypothyroidism, but a weaker TSAb activity, did not increase TSH receptor mRNA levels (Table 1).

DISCUSSION

The submission of this report followed the presentation of the amino acid sequence of the dog and human TSH receptors (4–7). Nevertheless, since FRTL-5 rat thyroid cells are now used by many laboratories in multiple fields, definition of the rat TSH receptor as well as the mechanisms by which it regulates the growth and function of FRTL-5 cells has importance beyond its cloning. This is illustrated in the following observations.

Agonist desensitization of a receptor coupled to a cAMP signal, the β-adrenergic receptor for example, can result from sequestration, phosphorylation, and/or from down-regulation of its mRNA (18). The present report shows that any hormonal agonist, or TSH or a Graves TSAb, that evokes a cAMP signal can down-regulate TSH receptor mRNA; this is associated with decreased TSH binding. This result explains a report (19) showing that a TSAb-positive Graves IgG could desensitize the TSH receptor in FRTL-5 cells; the same IgG used in that report is used here (Graves IgG no. 1). Since desensitization in the FRTL-5 and other thyroid systems has been associated with mechanisms other than the cAMP signal (20), these results predict that, as in the β-adrenergic system, multiple mechanisms of desensitization will exist. Unlike the β-adrenergic receptor (18), however, desensitization by phosphorylation will be a kinase C-mediated activity.

We have observed that TBIAbs from autoimmune hypothyroid patients—i.e., patients with no TSAb activity—increase TSH receptor mRNA levels with no effect on cAMP levels. The different actions of the TBIAbs and TSAbs on TSH receptor gene expression indicate that the antibodies recognize different epitopes of the TSH receptor, consistent with conclusions from mixing studies using monoclonal antibodies to the TSH receptor (16). Since the data in Fig. 4 establish that both types of antibodies interact with the receptor, they additionally show that these different epitopes have different bioactivities.

In the β-adrenergic system, the interaction of an antagonist with the receptor inhibits the action of the agonist to increase cAMP and decrease receptor mRNA (21) by acting at a receptor level. The inability of some IgGs from patients with active Graves disease (Table 1, Graves IgG nos. 2 and 3) to increase cAMP and decrease TSH receptor mRNA may be the pathophysiologic analog of this antagonist–agonist phenomenon. The mechanism may, however, be distinct since TSAbs and TBIAbs do not interact with the same receptor epitope, have different actions on receptor mRNA levels, and can, in some cases, stimulate the Ca^{2+}/arachidonate signal system (16). The possibility that this does not represent antagonism at a receptor level but rather antagonism at a signal level and via separate guanine nucleotide-binding proteins could be investigated in mixing experiments using monoclonal and patient TBIAbs and TSAbs. Rapid down- and up-regulation of the TSH receptor mRNA via different guanine nucleotide-binding proteins, in a cell where both growth and function depend on TSH, may be a means of differentially regulating these two processes.

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