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Gene Usage and Regulation of Gsα Gene Expression in Thyroid Cells

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The TSH receptor is a G-protein-coupled seven transmembrane segment receptor. The interaction between TSH and its receptor mediates signal transduction by activating adenylyl cyclase through Gsα. There are four forms of Gsα (two short [45 kDa] and two large [52 kDa]), arising from alternative splicing of exon 3 of the Gsα gene. Gsα-1 and -2 contain exon 3, whereas exon 3 is spliced out in Gsα-3 and -4. The inclusion of a serine residue at the 3' splice junction of exon 3 distinguishes Gsα-2 and -4 from Gsα-1 and -3. The expression of different Gsα forms appears to be tissue-specific. In this study, we have examined the Gsα splice variants in 26 human thyroid tumor specimens and rat thyroid tissues as well as a rat FRTL-5 cell line. Furthermore, we have studied the regulation of the Gsα gene expression by TSH and cAMP in FRTL-5 cells. We found that Gsα-1 and -4 mRNA were present in both human and rat thyroid cells, although Gsα-4 was more abundant in human thyroid cells as compared to rat thyroid and FRTL-5 cells. The Gsα mRNA can be easily amplified by RT-PCR regardless of tumor type and stage, suggesting that Gsα gene expression in thyroid tumors may not be markedly affected by dedifferentiation of thyroid cells.

Both TSH and 8-bromo-cAMP, a cAMP analog, can stimulate the Gsα gene expression in FRTL-5 cells with maximal effect by 6 h and 1 h, respectively. The addition of cycloheximide to the culture of FRTL-5 cells abolished the effect of bTSH, but not that of 8-bromo-cAMP, on the expression of the Gsα gene. Cellular cAMP measurements showed that bTSH-stimulated cAMP production was significantly reduced to the basal level after addition of cycloheximide. These results suggest that regulation of the Gsα gene expression by TSH is mediated by a cAMP-dependent process and requires new protein synthesis.

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Introduction

The heterotrimeric guanine nucleotide binding proteins (G proteins), composed of α, β, and γ subunits, act as molecular switches between numerous cell surface receptors and various effecters. These effecters include adenylyl cyclase, phospholipase A2, phospholipase C, phosphodiesterase, and calcium and potassium channels. Agonist-activated receptors catalyze the exchange of guanosine diphosphate (GDP), bound to the α subunit, for guanosine triphosphate (GTP), resulting in the subsequent dissociation of the α-GTP complex from the βγ heterodimer. The α-GTP complex and the free βγ dimer then interact with various effecters to generate regulatory molecules or second messengers. Termination of the signal occurs when the intrinsic GTPase activity of the α subunit hydrolyses GTP to GDP, thus creating the inactive α-GDP complex, which reassociates with the βγ dimer (Gilman, 1987; Simon et al., 1991).

The TSH receptor is a member of the G-protein-coupled seven transmembrane segment receptors (Nagayama and Rapoport, 1992; Vassart and Dumont, 1992). TSH binding to its receptor activates adenylyl cyclase and phospholipase C through Gsα and $Gq/_{11}$, respectively (Allgier et al., 1994), to mediate differentiated thyroid cell function and growth (Nagayama and Rapoport, 1992; Vassart and Dumont, 1992).

There are two species of Gsα proteins, which migrate on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with apparent molecular weights of 45 kDa (Gsα-S) and 52 kDa (Gsα-L) (Sternweis et al., 1981; Gilman, 1987). Human cDNAs that encode four different forms of Gsα were characterized (Bray et al., 1986). These forms arise from alternative splicing of exon 3 and the inclusion or exclusion of a serine codon at the 3' splice junction of exon 3 of the Gsα gene (Bray et al., 1986; Robishaw et al., 1986; Kozasa et al., 1988). Gsα-1 and Gsα-3 are identical, except that the latter lacks the 45 nucleotides

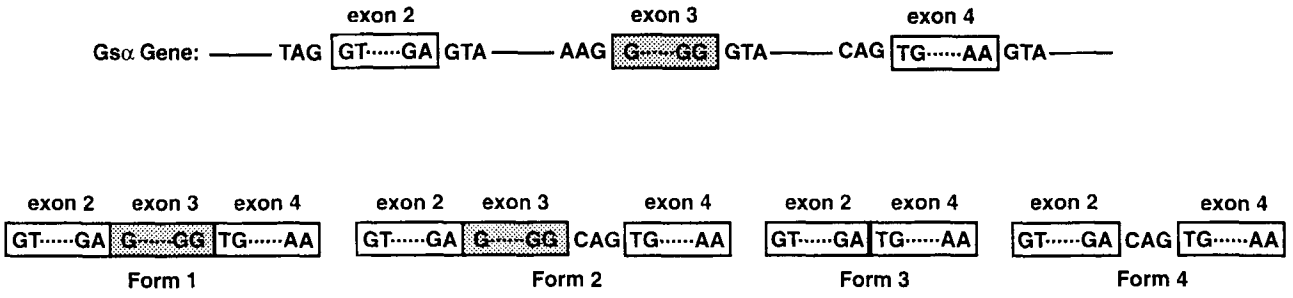

Fig. 1. Schematic representation for the origin of four different Gsα mRNAs by alternative splicing. The Gsα gene is shown in the upper panel. Exons 2 and 4 are shown by open boxes; exon 3 is shown by a shaded box. Nucleotide sequences of exon-intron boundaries are shown. Four Gsα mRNAs are indicated by Form 1, 2, 3, and 4.

encoded by exon 3. Gsα-2 and Gsα-4 carry three additional nucleotides (CAG) encoding a serine residue (Fig. 1). All four forms are present in the human and rat brain (Bray et al., 1986; Granneman and Bannon, 1991), whereas only Gsα-1 and Gsα-4 exist in bovine adrenal (Robishaw et al., 1986) and human liver tissues (Mattera et al., 1986), suggesting that splice site usage is tissue specific.

We now report on the investigation of the splice site usage of the Gsα gene in human and rat thyroid tissues as well as a FRTL-5 cell line. The regulation of Gsα gene expression by TSH was also studied in FRTL-5 cell line.

Results

Two cDNA fragments (295 and 245 bp) were amplified by PCR from all 26 thyroid tumor specimens (Fig. 2). The two fragments were of the expected size of the cDNA fragments comprising Gsα exons 2 through 4 (Gsα-L, 295 bp), and one in which exon 3 was spliced out (Gsα-S, 245 bp). It appears that the Gsα pre-mRNA was either equally spliced into the large (Gsα-L) and the small forms (Gsα-S) of the transcripts or with a slight predominance of Gsα-L in human thyroid tissues (Fig. 2). The Gsα mRNA can be easily amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) regardless of tumor type and stage, suggesting that Gsα gene expression in thyroid tumors may not be as profoundly affected by dedifferentiation of thyroid cells, as those genes encoding thyroglobulin, thyroperoxidase, or TSH receptor, which were reported to be poorly transcribed with increasing tumor dedifferentiation (Brabant et al., 1991; Shi et al., 1993).

In contrast to human thyroid tissue, we found FRTL-5 cells to exhibit predominantly the 295-bp band (representing Gsα-L) and could not see the 245-bp Gsα-S band on ethidium bromide stained gel, whether or not the cells were stimulated with bTSH (Fig. 3A). Southern blot analyses of Gsα RT-PCR product, however, have detected very small amounts of the 245 bp Gsα-S (Fig. 3B), indicating that the splicing efficiency was significantly lower in FRTL-5 cells than in human thyroid cells. In order to find out whether the presence of predominant Gsα-L was characteristic of FRTL-5 cells or of rat tissues in general, we amplified Gsα

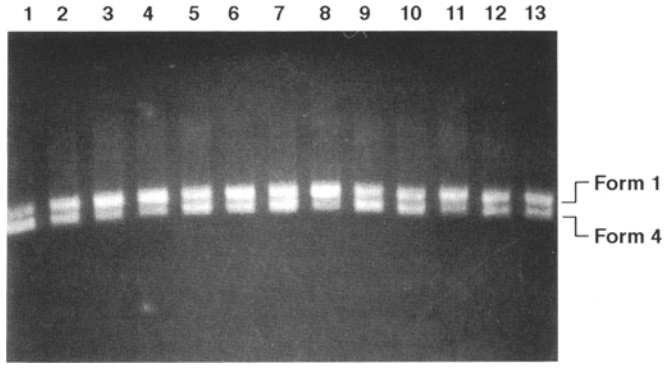

Fig. 2. Analysis of Gsα splicing variants from thyroid tumor mRNA by RT-PCR. DNA fragments were generated by PCR from single-stranded cDNA (synthesized from RNA by reverse transcriptase) using primers covering the region for exon 2-4 of Gsα gene, and electrophoresed on 2% agarose gel. Shown are representative of 13 thyroid tumor specimens. Lanes 10–12, multinodular goiters; Lane 7–9, anaplastic carcinomas; remaining samples are from papillary carcinoma.

cDNA fragments from rat thyroid and liver. Both tissues exhibited both large and small forms of Gsα, but Gsα-L was much more abundant, particularly in the thyroid (Fig. 3).

We next sequenced both the large and small cDNA fragments of Gsα mRNA after subcloning them into a TA cloning vector to determine the splicing patterns in the thyroid. We found that the large fragment contains exon 3 (Gsα-1 isoform), whereas the small fragment lacks the exon 3 but has three additional nucleotides encoding a serine residue at the exon 2/exon 4 junction (Gsα-4 isoform) (data not shown). The rat thyroid and FRTL-5 cells exhibited Gsα pre-mRNA splicing patterns identical to those seen in its human counterpart, which utilize form-1 and form-4.

Last, we investigated whether TSH regulates Gsα gene expression in FRTL-5 cells. To this end, the cells were cultured with or without $10^{-8}M$ bTSH for periods up to 72 h, and Gsα gene expression was analyzed by Northern hybridization. As shown in Fig. 4, Gsα transcripts start to increase by 4 h with maximal stimulation by 6 h. It then declined slightly but remained steady by 24 h, and declined further by 72 h. The permeable cAMP analog, 8-bromo-

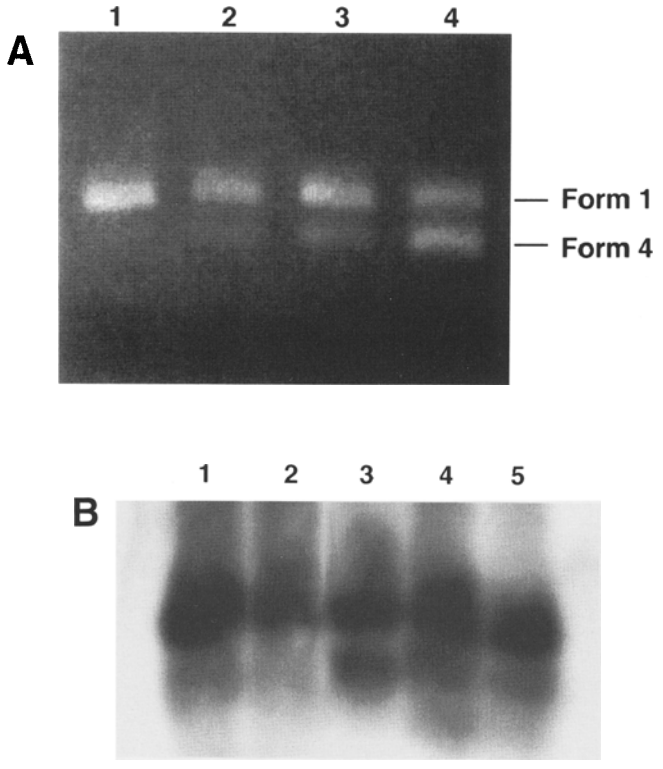

Fig. 3. Analysis of Gsα splicing variants from human and rat thyroid, rat liver, and FRTL-5 cell line mRNAs by RT-PCR. DNA fragments were generated by PCR from single-stranded cDNA (synthesized from RNA by reverse transcriptase) using primers covering the region for exon 2–4 of Gsα gene. **(A)** 5 μL of PCR products were electrophoresed on 2% agarose gel and stained with ethidium bromide. Lane 1, FRTL-5 cell line; lane 2, rat thyroid; lane 3, rat liver; lane 4, human thyroid. **(B)** 2 μL of PCR products were electrophoresed on 1.5% agarose gel (5 μL loaded in lane 1), transferred to the nylon membrane and hybridized with a Gsα probe. Lanes 1 and 2, FRTL-5 cell line; lane 3, human thyroid; lane 4, rat thyroid; lane 5, rat liver.

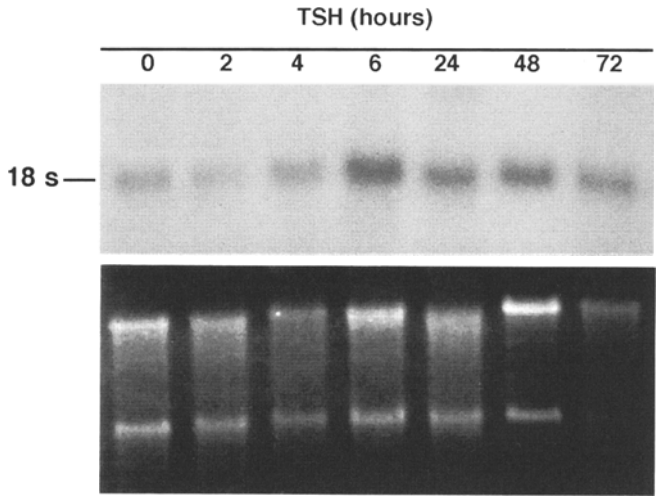

Fig. 4. Effects of bTSH on the expression of Gsα mRNA in FRTL-5 cells. FRTL-5 cells were cultured in the absence or presence of bTSH ($10^{-8}M$) for 2–72 h. Total RNA was prepared from the cells at the end of the incubation and Gsα gene expression was analyzed by Northern blot hybridization as described in Materials and Methods. The figure shown in the upper panel is a representative of three separate Northern blot experiments from FRTL-5 cells probed with the Gsα cDNA fragments. The position of 18S ribosomal RNA is indicated. Lower panel: ethidium bromide staining of the RNA loaded for Northern blot to monitor the actual RNA loading.

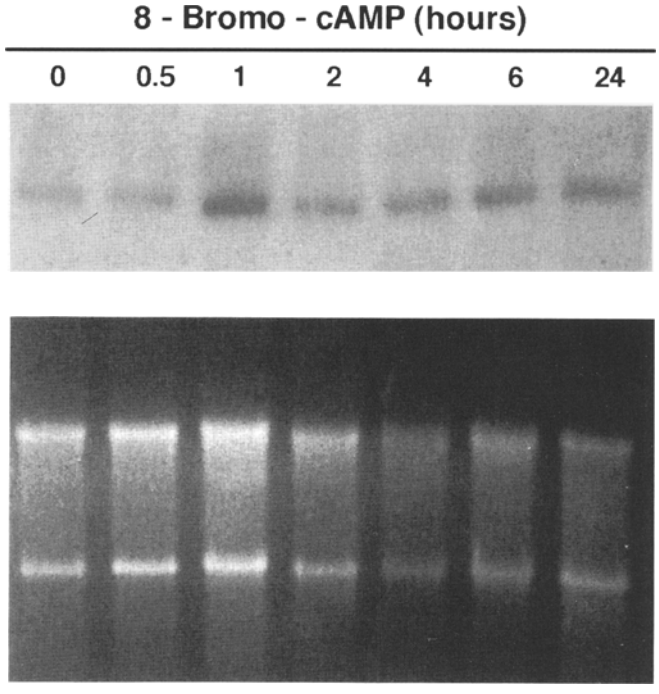

Fig. 5. Effects of 8-Bromoadenosine 3', 5'-cyclic monophosphate on the expression of Gsα mRNA in FRTL-5 cells. FRTL-5 cells were cultured in the presence or absence of 1 m*M* 8-bromo-cAMP for 30 min–24 h. Total RNA was prepared from the cells at the end of the incubation and Gsα gene expression was analyzed by Northern blot hybridization. The figure shown in the upper panel is a representative of three separate Northern blot experiments from FRTL-5 cells probed with the Gsα cDNA fragments. The position of 18S ribosomal RNA is indicated. Lower panel: ethidium bromide staining of the RNA loaded for Northern blot to monitor the actual RNA loading.

cAMP, can also stimulate Gsα gene transcription, but faster than TSH. As shown in Fig. 5, Gsα transcripts reached maximal stimulation in 1 h and then declined but remained steady by 24 h. These patterns of Gsα mRNA induction are similar to those described in several tissues exposed to G-protein coupled receptor agonists and cAMP analog (Longabaugh et al., 1989; Saunier et al., 1990; Dib et al., 1994).

To investigate whether new protein synthesis is required to induce Gsα gene expression by TSH and cAMP, FRTL-5 cells were cultured with cycloheximide for 6 h in the presence or absence of bTSH ($10^{-8}M$) or 8-bromo-cAMP (1 m*M*). As shown in Fig. 6, the steady state of Gsα transcripts is slightly decreased by cycloheximide in the absence of bTSH. Addition of bTSH in the presence of cycloheximide did not increase Gsα transcripts. It is thus apparent that new protein synthesis is necessary for maintaining the basal level of Gsα transcripts, and particularly for TSH-mediated Gsα gene expression. In contrast, cycloheximide has no effect on 8-bromo-cAMP mediated Gsα expression

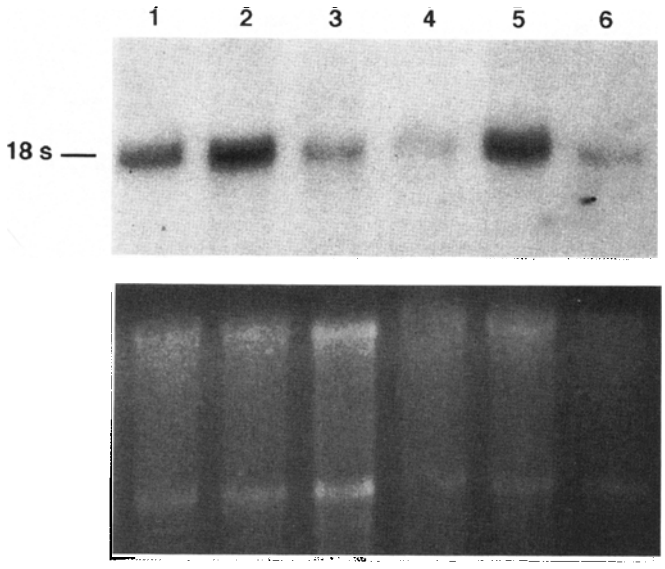

Fig. 6. Effects of cycloheximide on the expression of Gsa mRNA in FRTL-5 cells. FRTL-5 cells were cultured with cycloheximide (5 μg/mL), bTSH (10^{-8} *M*), 8-bromo-cAMP (1 m*M*), cycloheximide plus bTSH, or cycloheximide plus 8-bromo-cAMP, respectively, for 6 h. Total RNA was prepared from the cells at the end of the incubation and Gsa gene expression was analyzed by Northern blot hybridization. The figure shown in the upper panel is a representative of three separate Northern blot experiments from FRTL-5 cells probed with the Gsa cDNA fragments. The position of 18S ribosomal RNA is indicated. Lane 1, FRTL-5 cells + bTSH; lane 2, FRTL-5 cells + 8-bromo-cAMP; lane 3, FRTL-5 cells + cycloheximide + bTSH; lane 4, FRTL-5 cells + cycloheximide; lane 5, FRTL-5 cells + cycloheximide + 8-bromo-cAMP; lane 6, FRTL-5 cells without treatment. Lower panel: ethidium bromide staining of the RNA loaded for Northern blot to monitor the actual RNA loading.

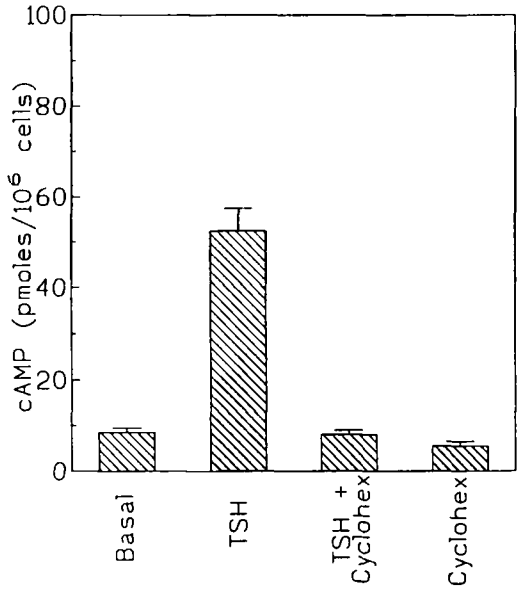

Fig. 7. Effects of cycloheximide on the cellular cAMP production in FRTL-5 cells. FRTL-5 cells were cultured with cycloheximide (5 μg/mL), bTSH (10^{-8} *M*), and cycloheximide plus bTSH, respectively, for 6 h in the presense of 1 m*M* isobutyl methylxanthine. Cellular cAMP was measured by RIA. Two replicates are used for each data point and data are presented as mean + SD of three experiments.

(Fig. 6) and, therefore, new protein synthesis is not required for cAMP-mediated Gsα expression.

In order to find out whether the inhibition of TSH-induced Gsα expression by cycloheximide results from reduced cAMP production or is through a cAMP independent pathway, we measured cellular cAMP levels after treatment of the cells with bTSH, bTSH plus cycloheximide, or cycloheximide alone, respectively. As shown in Fig. 7, cycloheximide reduces basal level of cAMP by 20% and abolishes the effect of TSH-stimulated cAMP production. The extent of cAMP reduction parallels with that of inhibition of TSH-stimulated Gsα transcription by cycloheximide (Fig. 7), indicating that Gsα gene transcription is mediated by cAMP.

Discussion

We have examined Gsα pre-mRNA splicing patterns in various tissue specimens using PCR and DNA sequence analysis. In human thyroid both form-1 (Gsα-L) and form-4 (Gsα-S) mRNAs are equally or near equally present. Although these two forms also exist in Sprague-Dawley rat thyroid and liver as well as FRTL-5 cells, form-1 is predominant, particularly in FRTL-5 cells.

Early studies suggested that Gsα-L may have greater ability to support hormone-stimulated adenylyl cyclase activity (Sternweiss et al., 1981, Robishaw et al., 1986). Later studies indicated that the ability for both variants to induce adenylyl cyclase activity was equivalent (Graziano et al., 1987; O'Donnell et al., 1991), although subtle differences cannot be excluded. The 15 amino acid residues present in exon 3 of Gsα-1 and Gsα-2 constitute a relatively hydrophilic, negatively charged region placed on the surface of the Gsα protein (Bray et al., 1986; Kozasa et al., 1988). Moreover, serine-82, present in Gsα-1 and Gsα-2, but not in Gsα-3 and Gsα-4, is a potential phosphorylation site for cAMP-dependent protein kinase A, whereas serine-87 in Gsα-2 and serine-72 in Gsα-4 are potential phosphorylation sites for protein kinase C, suggesting that the alternative use of these splice sites may confer Gsα proteins with differential regulatory properties (Bray et al., 1986; Kozasa et al., 1988). To what extent these phosphorylation sites are utilized and the roles of phosphorylation in regulating Gsα isoform function is unclear.

Although almost equal amounts of Gsα-L and -S mRNA are present in human thyroid cells (Fig. 2), careful inspection of the figures in the paper by Allgeier et al. (1994) show a predominance of Gsα-S protein (Gsα-4 isoform) in human thyroid membranes, yet Gsα-L (Gsα-1 isoform) appears to have greater TSH-induced photolabeling by [α-^{32}P]GTP azidoanilide. These findings imply a greater efficiency of translation of Gsα-S in human thyroid cells,

whereas Gsα-L seemingly have greater ability to mediate TSH-stimulated adenylate cyclase activity.

A variety of rat tissues, including thyroid and liver in this study, have been shown to express two Gsα isoforms, although Gsα-L protein or mRNA is more abundant (Granneman et al., 1990; Granneman and Bannon, 1991; Zeitler et al., 1993). It is likely that the efficiency of the splicing machinery is variable in different rat tissues and is much lower as compared to that of human thyroid. Given that the TSH-induced activation of adenylyl cyclase through Gsα is comparable between FRTL-5 cells (Gsα-L predominant) and human thyroid cells (both Gsα-L and -S are equally present), it is tempting to suggest that some functional redundancy may exist among Gsα isoforms.

In the present study, we have demonstrated that TSH can stimulate the Gsα gene expression in FRTL-5 cells with maximal effect by 6 h. The up-regulation of the Gsα gene was also observed with 8-bromo-cAMP, a cAMP analog, with maximal stimulation by 1 h. The difference in time-course of Gsα transcription by bTSH and 8-bromo-cAMP is reminiscent of those induced in astroglial cells by isoproterenol on the one hand, and forskolin and 8-bromo- cAMP on the other (Dib et al., 1994). The intermediacy of cellular regulatory steps between the activation of the receptors by agonists and transcription of Gsα as well as the resistance of 8-bromo-cAMP to degradation by cAMP phosphodiesterase likely accounts for these differences.

The addition of cycloheximide to the cultures of FRTL-5 cells significantly inhibits TSH, but not 8-bromo-cAMP, mediated Gsα expression. It suggests that new protein synthesis is required at the level of TSH receptor and/or adenylyl cyclase for TSH-induced Gsα expression. Previous studies have shown that Gsα protein and mRNA were up-regulated by both TSH and forskolin in porcine thyroid cells. These increases were counteracted by the protein kinase C activator, TPA (tetradecanoylphobol acetate) (Dib et al., 1994), and required new protein synthesis (Saunier et al., 1990; Dib et al., 1994). Our results are consistent with those obtained in porcine thyroid cells. Two classes of cis-acting elements were characterized within the promoter region of the cAMP-regulated genes, the cAMP-responsive elements, and the activator protein 2 (AP-2) binding site. These sequences have the properties of enhancers (Roesler et al., 1988). Although the Gsα gene is regulated by a cAMP-dependent pathway, neither classical cAMP-responsive elements nor AP-2 sequence were found in the promoter region of the Gsα gene (Kozasa et al., 1988). Given that the initiation of Gsα gene transcription is fast and does not require new protein synthesis, we conjecture that nonclassic cAMP response elements and AP-2 sequence may be involved in the transcriptional activation of the Gsα gene.

In summary, we have shown that Gsα variants 1 and 4 are near equally expressed in human thyroid tissue (Bray et a:L, 1986). Gsα gene expression appears to be retained in multinodular goiter tissues as well as carcinoma samples of different degrees of dedifferentiation, suggesting that Gsα gene expression in thyroid tumors may not be as markedly affected by dedifferentiation of thyroid cell function as those genes for thyroglobulin, thyroperoxidase, or TSH receptor (Farid et al., 1994). By contrast to human thyroid, rat thyroid and liver as well as FRTL-5 cells exhibit predominantly the Gsα-1, the larger form. We have further shown that TSH stimulates Gsα gene expression through a cAMP dependent pathway in FRTL-5 cells, and that this induction requires new protein synthesis.

Materials and Methods

All human thyroid tissues were from tumor specimens obtained at surgery, and were immediately frozen in liquid nitrogen and stored at −70°C until processed. Twenty-six thyroid tumor specimens were included in the study: 3 nodules from patients with multinodular goiter, 15 papillary carcinomas, 4 follicular carcinomas, and 4 anaplastic carcinomas. Rat thyroid and liver tissues were obtained from Sprague-Dawley rats. The FRTL-5 cell line (Ambesi-Impiombato et al., 1980), obtained from ATCC (Rockville, MD), was maintained in Ham's F12 medium supplemented with 5% calf serum, penicillin (100 U/mL), streptomycin (100 μg/mL), fungizone (25 μg/mL), and six hormones: insulin (10 μg/mL), hydrocortisone (10 n*M*), transferrin (5 μg/mL), glycyl-L-histidyl-L-lysine acetate (10 ng/mL), somatostatin (10 ng/mL), and bovine TSH (10 mI.U./mL) (all Sigma, St. Louis, MO) at 37°C in humidified atmosphere containing 5% CO_2.

RNA Extraction and RT-PCR Procedure

Total RNA was extracted by the guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). RT-PCR procedure was performed as described previously (Zou et al., 1994). Briefly, 5 yg of total RNA was reverse transcribed into cDNA in 15 μL vol, using Pharmacia's (Piscataway, NJ) first-strand cDNA synthesis kit. The cDNA was then amplified by PCR using two primers (5'-CAG GAGCCAGAATGACAA-3'and 5'-TTCA ATCGCCTCTTTCTTCAG-3'), which are flanking exon 2 and 4 of the Gsα gene. Samples were denatured at 94°C for 4 min and submitted to 25 cycles of amplification as follows: 40s denaturation at 94°C, 40s annealing at 42°C, and 40s extension at 72°C. 5 μL of each PCR product were run on 2% agarose gel containing ethidium bromide, visualized with UV light, and photographed.

Northern Blot Hybridization

Ten micrograms of total RNA were fractionated on 1% agarose gel containing 2.2*M* formaldehyde and blotted onto nylon membranes (Hybond-N, Amersham, Arlington Heights, IL) by capillary transfer. The accuracy of RNA

loading was monitored by ethidium bromide staining of the ribosomal RNA (Zou et al., 1993). The Gsα cDNA probes were obtained by PCR amplification of the two fragments comprising exon 2 and 4 with or without exon 3. They were labeled with [α-^{32}P]dCTP to a specific activity of 10^9cpm/μg, using Pharmacia's random primer labeling kit. Hybridization was performed at 42°C for 18 h in 6X SSPE, 10 m*M* EDTA, 5X Denhardt's solution, 0.5% SDS, 100 μg/mL denatured salmon testis DNA, and 50% formamide. The membranes were then washed twice in 2X SSPE at 65°C and exposed to Kodak XAR-5 film at −70°C with intensifying screens.

Southern Blot Hybridization

Southern blot analysis was performed by running 2–5 μL of PCR products on 1.5% agarose gel, and blotted onto nylon membranes (Hybond-N, Amersham) by capillary transfer. The filter was then hybridized with Gsa probe as described in Northern Blot Hybridization.

DNA Sequencing Analysis

DNA sequencing was performed by the dideoxy chain termination method after cloning the PCR products into TA cloning vector (Invitrogen, San Diego, CA).

Regulation of the Gsa Gene Expression by TSH and cAMP

FRTL-5 cells were cultured in 75-cm^2 culture flasks in Ham's F12 medium containing 5% calf serum and the six hormones at 37°C in a humidified incubator containing 5% CO_2. When the cells reached 70–80% confluence, the medium was replaced; the cells were washed twice with phosphate buffered saline (PBS) and maintained in 5H medium (without TSH) for 5 d. The cells were then cultured in the absence or presence of bTSH (10^{-8}*M*), 8-bromo-adenosine 3', 5'-cyclic monophosphate (1 m*M*, Sigma, St. Louis, MO), cycloheximide (5 μg/mL, Sigma) for different periods of time as indicated in the text. Total RNA was prepared from the cells after treatment and Gsα gene expression was analyzed by Northern hybridization.

Cellular cAMP Measurements

Cells were cultured in 24-well plates in Ham's F12 medium containing 5% calf serum and the six hormones at 37°C in a humidified incubator containing 5% CO_2. When the cells reached 70–80% confluence, the medium was replaced; the cells were washed twice with phosphate buffered saline (PBS) and maintained in 5H medium (without TSH) for 5 d. bTSH was added to the medium at a final concentration of 10^{-8}*M* together with 1 m*M* isobutyl methylxanthine in the presence or absence of 5 μg/mL cycloheximide. After 6 h incubation at 37°C, cellular cAMP was extracted with 500 μL absolute ethanol and measured by radioimmunoassay using Du Pont-New England Nuclear's (Boston, MA) cAMP assay kit.

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