GH mRNA Levels are Elevated by Forskolin but not GH Releasing Hormone in GHRH Receptor-Expressing MtT/S Somatotroph Cell Line

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Running title: Elevated cAMP in MtT/S somatotrophs

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Summary

The MtT/S somatotroph cell line should be a growth hormone-releasing hormone (GHRH)-responsive model system for the study of physiological control of growth hormone (GH) transcription because GH secretion from these cells is stimulated by GHRH. To examine the GH transcriptional activity of these cells, endogenous GH mRNA levels were measured using a ribonuclease protection assay following treatment under a variety of hormonal conditions. While omission of serum led to reduction of GH mRNA to 22% of control levels by 2 days and to 8% by 5 days ($p < 0.05$ for both), GH mRNA levels were maintained at control values in serum-free medium containing 5 nM dexamethasone and 30 pM triiodothyronine (TDM). However, the addition of 10 nM GHRH under any treatment condition did not significantly alter GH mRNA levels. Characterization of the MtT/S cells showed that GHRH-receptor (GHRH-R) mRNA was detectable by reverse transcription-polymerase chain reaction (RT-PCR) amplification. Measurement of extracellular cAMP showed that the MtT/S cells have basal levels of 20 nmol/10^6 cells/hour in both serum-containing and serum-free media, and that GHRH had no effect on cAMP levels, suggesting constitutive activation. To rule out the possibility of autocrine stimulation by GHRH produced endogenously, GHRH mRNA was not detectable in MtT/S cells using RT-PCR amplification. The stimulatory G-protein subunit, mutations of which are known to activate adenylate cyclase constitutively in acromegaly, was sequenced but found not to differ from normal pituitary in the regions most commonly mutated. Finally, treatment with 10 μM forskolin, to directly activate adenylate cyclase, increased GH mRNA to 140% of controls in TDM, and to 163% in serum-free medium after 2 days, and to 166% in TDM-treated cells and 174% in serum-free culture after 5 days (all $p < 0.05$). Taken together, these data indicate that although MtT/S cells express the GHRH-R, GHRH cannot stimulate adenylate cyclase to increase GH transcription due to constitutive elevation of cAMP levels, by a means that may be similar to that in cases of acromegaly not caused by oncogenic *gsp* mutations.

Introduction

Growth hormone-releasing hormone (GHRH) is an important physiological stimulator of the anterior pituitary somatotroph cell type, causing increases in both growth hormone (GH) synthesis and secretion (Fukata et al., 1985; Theill and Karin, 1993; Torchia et al., 1998). As synthesis of GH is regulated at the transcriptional level, GHRH increases the GH transcription rate by 200-300% in primary pituitary cultures. This increase is evident in as little as 10 minutes, indicating that the initial events in stimulation occur rapidly (Barinaga et al., 1985; Barinaga et al., 1983). Following 24 hours of GHRH treatment, steady-state levels of GH mRNA are increased by over 200%, and these increases are maintained for at least 4 days, suggesting that the effects of GHRH are not transient (Gick et al., 1984).

The effects of GHRH are mediated by a specific seven transmembrane G-protein coupled receptor which is expressed primarily in the anterior pituitary (Mayo, 1992). Ligand binding to the GHRH receptor leads to activation of adenylate cyclase (Bilezikjian and Vale, 1983). The resulting increase in intracellular cAMP levels activates protein kinase A (PKA) (Bilezikjian et al., 1987), which is required for cAMP-dependent stimulation of GH transcription (Shepard et al., 1994). Once PKA is activated, it translocates to the nucleus where it phosphorylates a number of targets (Hagiwara et al., 1993). However, the mechanism by which PKA stimulates GH transcription remains to be elucidated.

Delineating the mechanisms by which GHRH regulates GH mRNA expression has been hindered by the lack of GHRH responsive cell line models. Although primary pituitary cultures are derived from normal tissue and are GHRH responsive, they contain multiple hormone-secreting cell types (Billestrup et al., 1986; Hoeffler and Frawley, 1987; Hoeffler et al., 1987; Tashjian, 1979). The heterologous composition of these cultures makes molecular and biochemical techniques difficult to interpret. Several immortal cell lines derived from pituitary tumors produce GH at high levels (Bancroft, 1981). These cell lines typically also produce detectable prolactin (PRL) making them a model of the somatomammotroph cell, which is defined by the production of both GH and PRL. However, such cell lines do not typically express the GHRH receptor or

respond to GHRH at the level of GH secretion, or synthesis of mRNA or protein (Zeytin et al., 1984).

The rat MtT/S cell line was cloned by limiting dilution from an estrogen induced pituitary tumor (Inoue et al., 1990). The cell line expresses GH at high levels without detectable PRL under normal conditions. GHRH treatment results in elevated MtT/S cell GH secretion and GH protein synthesis (Inoue et al., 1990). Further, MtT/S cells are unique in the production of GHRH-R mRNA (Miller and Mayo, 1997b). Thus, the MtT/S cell line may be a useful model to study control of GH transcription by GHRH, especially because transcription from the endogenous GH gene can be studied.

Materials and Methods

Preparation of media and hormone stocks

MtT/S cells were routinely cultured in serum-containing medium as previously described (Inoue et al., 1990). Complete Medium (CM) was composed of DMEM/F12 (1:1) medium containing 3151 mg/L D-glucose, 365 mg/L L-glutamine, and 2.438 g/L sodium bicarbonate supplemented with 10% donor horse serum, 2% certified fetal bovine serum, 50 units/ml penicillin G sodium, 50 µg/ml streptomycin sulfate, and 100 µg/ml kanamycin sulfate (Life Technologies, Grand Island, NY). Serum-free medium (SFM) was CM without either horse or bovine serum, while T3+Dex medium (TDM) was SFM containing 30 pM T3 and 5 nM Dex, values chosen for correlation with previous studies of GHRH treated primary pituitary cultures (Bilezikjian and Vale, 1983). Growth hormone-releasing hormone (GHRH 1-29 NH_2) was kindly provided by Peptide Research Laboratories (Tulane University Medical Center, New Orleans, LA). Lyophilized GHRH was dissolved in 0.01 M acetic acid to prepare a 1 mg/ml (0.294 mM) primary stock, while forskolin (Alexis, San Diego, CA) and dexamethasone (Sigma, St. Louis, MO) were each dissolved in 100% ethanol, and triiodothyronine (T3, Sigma) stock was prepared in 1 N sterile sodium hydroxide. All were diluted to working concentrations with DMEM/F12 medium.

Maintenance of MtT/S cell cultures

MtT/S rat somatotroph cells were obtained live from the Riken Cell Bank (Tsukuba Science City, Japan). Following expansion of cultures, cells were slow-frozen in CM supplemented with 5% DMSO and placed under liquid nitrogen for long term storage. MtT/S cells have a loosely adherent phenotype and were removed from culture flasks by gentle pipetting. Cells were routinely plated at 2 million cells per 75 cm^2 tissue culture flask (Corning) in 10 ml CM and incubated at 37° C in 5% CO_2 and 100% humidity. Cells were passaged every seven days at a density of 6-8 million cells/flask. All experiments were performed on cells at passages 3-10 after thawing from liquid nitrogen.

Treatment of MtT/S cells with hormones and pharmacological agents

Following incubation for 7 days with CM in 75 cm^2 flasks, cells were dissociated by gentle pipetting. Viable cell number was determined using a hemacytometer and trypan blue (Life Technologies) exclusion. Cells were centrifuged for 3 minutes at 500 x g to remove medium and resuspended in fresh CM at a concentration of 100,000 cells/ml. 100,000 cells were placed in each well of a 6-well tissue culture plate (Falcon), and CM was added to 5 ml final volume.

At the beginning of plateau phase (9 days of culture, 1 million cells/well), medium was removed from wells by pipetting. Cells were washed 3 times with 5 ml SFM taking care not to dislodge loosely attached cell clusters. Wash media was discarded without centrifuging to reclaim detached cells. 5 ml treatment media with or without the indicated hormone or pharmacological agent then was added to each well. Each treatment was performed on triplicate wells. Cultures were incubated with treatment media for the indicated amount of time at 37° C in 5% CO_2, and 100% humidity.

Isolation of total RNA

Cells were removed from wells by gentle pipetting, and the media was removed following centrifugation at 500 x g for 3 minutes. Total RNA was isolated from cell pellets using a single

step phenol/chloroform extraction in the presence of 14 M guanidine salts and urea (Ultraspec reagent, Biotecx, Houston, TX). After RNA was ethanol precipitated from the aqueous phase and resuspended in sterile ultrapure water, spectrophotometric absorbance at 260 nm was used to determine yield of total RNA. Each well routinely yielded between 45-55 µg total RNA.

Measurement of GH mRNA expression

A region of the GH gene was amplified by polymerase chain reaction (PCR) from rat anterior pituitary cDNA. Amplified product size was confirmed by agarose gel electrophoresis, then cloned into pCR 2.0 plasmid vector (InVitrogen, Carlsbad, CA) containing T7 and SP6 RNA polymerase promoters. The cloned GH template was sequenced to confirm insert orientation, then linearized with Xmn I restriction enzyme (Life Technologies), predicted to produce a 325 nucleotide probe that would be reduced to a 245 nt protected fragment after RNase digestion. A mouse -actin probe was generated from commercially-supplied plasmid (Ambion, Austin, TX). Antisense RNA probes for chemiluminescent detection were generated by *in vitro* transcription reactions using SP6 RNA polymerase to incorporate biotinylated CTP (BIOTINscript Kit, Ambion). Probe synthesis reactions were denatured and electrophoresed on an 8 M urea, 5% polyacrylamide gel (Life Technologies) so that full length transcripts could be visualized by UV shadowing on a fluorescent TLC plate (Ambion). Probes were eluted from the excised gel fragment by 12 hour incubation at 37° C in buffer containing 0.5 M ammonium acetate, 1 mM EDTA, 0.2% SDS, then phenol-chloroform extracted, ethanol precipitated, and resuspended in sterile ultrapure water. Probe yield was determined by spectrophotometric absorbance at 260 nm. The specific activity of the GH probe was reduced by adding 10 fold excess of unlabeled *in vitro* transcribed antisense GH RNA.

For analysis of GH mRNA expression, 2.5 µg MtT/S cell RNA, 5 ng GH probe, 25 µg yeast carrier RNA, and Pellet-Paint precipitant (Novagen, Madison, WI) were co-precipitated. After hybridization to target RNA, single stranded RNA was digested, RNase was inactivated and protected fragments were analyzed according to the manufacturer's directions (RPA II kit, Ambion). Protected probes were separated by electrophoresis on an 8 M urea, 5 % polyacrylamide

gel for 45 minutes at 200 volts in 1X TBE buffer. Protected fragments were electrotransfered from the gel to a positively charged nylon membrane (Ambion) for 2 hours at 300 mA in 0.5 X TBE buffer. RNA was covalently bound to the membrane by exposure to 120 mjoules 254 nM UV light (Stratalinker 2400, Stratagene, La Jolla, CA). Chemiluminescent detection of protected biotinylated fragments was performed using streptavidin-conjugated alkaline phosphatase and CDP-Star substrate according to manufacturer's instructions (Ambion). Membranes were exposed to BioMax Light Film (Kodak, Rochester, NY) for 1, 2, 5, 7, 10, and 15 minutes.

After processing (GBX, Kodak), all films were exposed to constant intensity transillumination (FotoDyne, Madison, WI) so that images could be digitally captured using a CCD video camera (Hamamatsu C2400) and a Macintosh Quadra 950 computer (Apple Computer). Bands were identified and the integrated optical density of each band was compared using Gel Pro Analyzer 2.0.1 software (Media Cybernetics, Silver Spring, MD). Multiple film exposures of each gel were compared to ensure that film was in the linear response range. Identical samples were loaded in triplicate on each gel to allow comparison between gels. Probes for -actin were also included and quantified in each sample in order to provide for a normalization standard.

Measurement of extracellular cAMP levels

Cells were seeded in 6-well plates as described above for determination of GH mRNA. At the beginning of plateau phase, medium was removed from wells by gentle pipetting. Cells were washed 3 times with 10 ml SFM taking care not to dislodge loosely attached cell clusters. This washing procedure was also followed prior to 24 hour pretreatment in 5 ml SFM. For determination of extracellular cAMP levels, cells were incubated for 3 hours in 3 ml SFM with or without 10 nM GHRH. A 1 ml aliquot of medium was collected from each well and centrifuged at 500 x g for 5 minutes to remove any loose cells. A 500 μl aliquot of the medium was removed without disturbing the cell pellet and stored frozen at -80° C until cAMP levels were measured. Immediately following collection of media, cells were collected and cell numbers determined on a hemacytometer. Treatments were performed on triplicate wells and each treatment was repeated in

two independent experiments. Radioimmunoassay (RIA) for cAMP was performed to determine cAMP levels in the media as previously described (Culler et al., 1984).

Assay of GHRH-receptor mRNA

Determination of the presence of GHRH-R mRNA in total RNA from MtT/S cultures was performed by RT-PCR assays as previously described (Miller and Mayo, 1997a; Miller and Mayo, 1997b).

Analysis of GHRH mRNA expression

RNA was isolated from MtT/S cells grown in CM. First strand cDNA was synthesized from 5 µg MtT/S cell total RNA using the Superscript Preamplification System (Life Technologies) according to the manufacturer's directions. Multiple dilutions of MtT/S cDNA were amplified by PCR using rat GHRH specific oligonucleotide primers (sense primer, CATGCAGACGCCATCTTCAC; antisense primer, TTTGTTCCTGGTTCCTCTCC). Thermal profile used for 32 cycles of amplification consisted of denaturation at 94° C for 1 minute and annealing/extension at 69° C for 2.5 minutes. PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining and UV transillumination.

Analysis of $G_s\alpha$ nucleotide sequence

MtT/S cDNA was amplified by PCR using rat G_s specific oligonucleotide primers (sense primer, CCTCGGCAACAGTAAGACC; antisense primer, GAATTAAATTTGGGCGTTCC) designed by computer (Oligo 5.0, National Biosystems). The thermal profile used for 30 cycles of amplification consisted of denaturation at 94° C for 1 minute and annealing/extension at 68° C for 1 minute. PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining and UV transillumination. PCR products purified by matrix binding (Life Technologies) were sequenced using internal gene specific oligonucleotide primers (sense primer, CCTGCTACGAGCGCTCCAAC; antisense primer, CAGAGCCTCCTGCAGACGGT), and fluorescent dye-labeled dideoxy terminators with FS AmpliTaq DNA polymerase (Applied Biosystems, Inc.). Sequencing reactions were resolved using a 373A automated nucleotide

sequencing instrument (Applied Biosystems, Inc.). Electropherograms were evaluated using Factura 1.2.Or6 and Sequence Navigator 1.0.1 software (Applied Biosystems, Inc.).

Statistical Analysis

Statistical analysis of data was performed using SuperANOVA software (Abacus Concepts, Berkeley, CA). As warranted by ANOVA test results, Student-Newman-Keuls *post hoc* tests were performed, and were considered significant for p   0.05.

Results

Measurement of GH mRNA by chemiluminescent-ribonuclease protection assay (c-RPA)

The use of the c-RPA required testing for accuracy and linearity of measurement. Antisense GH probe was hybridized with yeast RNA followed by incubation without or with RNase. No protected fragments of the probe remained after RNase treatment, indicating that hybridization was specific and digestion was complete (Figure 1, left lane). GH antisense probe was hybridized with MtT/S target RNA, then incubated with RNase. As expected, RNase-treated GH probe migrated farther than untreated probe indicating that the vector sequence is removed by RNase (Figure 1, center and right lanes). As a further test of the assay, protected GH mRNA signal was compared from samples of 2.5 µg and 5 µg of total RNA from untreated MtT/S cells. The signal (121 ± 2.2 intensity units; n=3) of the 5 µg samples was 2.4 fold that of the signal (50 ± 2.5 units; n=3) from the 2.5 µg samples, indicating that GH probe is in excess of the amount of GH target mRNA present in 2.5 µg MtT/S total RNA. In all samples, the amount of   -actin mRNA also determined by RPA was determined for normalization.

Effects of serum, serum-free, and serum-free supplemented with T3 and Dex media on GH mRNA expression

Different types of media supplements were tested for their effect on levels of GH mRNA in the MtT/S cell cultures. Following 2 and 5 day treatment of MtT/S cells with CM, GH mRNA

levels measured by c-RPA were increased significantly to 127%±15 and 128%±10, respectively, (p < 0.05 for both) from levels measured immediately before treatment (Figure 2). However, following 2 and 5 day treatments of MtT/S cells in SFM, GH mRNA expression was decreased significantly to 22%±2 and 8%±1 (p < 0.05 for both) of levels immediately before treatment, respectively (Figure. 2). Following 2 and 5 day treatment of MtT/S cells in TDM medium, to replace hormones likely to be important for GH expression, GH mRNA levels were not significantly different from levels prior to treatment (Figure 2).

Effects of GHRH on GH mRNA expression

In order to analyze the effects of GHRH on GH mRNA levels in MtT/S cells, cells were treated with 10 nM GHRH for 2 or 5 days in all three types of media. After either 2 or 5 days of treatment, GH mRNA levels measured by c-RPA were not significantly different from GH expression under any of the conditions (Figure 3A and B). In order to ensure the potency of the GHRH preparation, medium prepared from aliquots of the GHRH stock was tested for stimulation of GH secretion from rat primary cultures. Measurements of GH secretion from these primary cultures corroborated the calculated concentration of GHRH (data not shown). Treatments were also performed where GHRH was replenished every 24 hours during the 2 to 5 day period, but these results were identical to those in Figure 3 (data not shown).

Detection of GHRH-R mRNA in MtT/S cells

The presence of GHRH-R in these cultures was confirmed by RT-PCR assay of total RNA from both untreated and GHRH-treated MtT/S cells after either 2 or 12 h of culture. In these samples, a band with size of 489 bp was predominant after amplification (Figure 4, top panel). This size agrees with previous analysis of the GHRH-R mRNA (Miller and Mayo, 1997a); two isoforms of the GHRH-R mRNA in MtT/S cells can be detected under other conditions (Miller and Mayo, in preparation). Each sample was also amplified for ribosomal protein L19 (RPL19) mRNA as a positive control (Fig. 4, bottom panel), yielding a product of the predicted size of 196 bp.

Effects of GHRH on extracellular cyclic AMP level

Because there was no effect of GHRH upon GH mRNA levels, it was important to assay another aspect of cellular activation in response to GHRH treatment. Extracellular cAMP levels were measured in MtT/S cells following two treatments: immediate serum withdrawal by transfer into SFM at time 0, or after 24 hour pretreatment in SFM. GHRH treatment did not significantly increase extracellular cAMP levels under either treatment condition (Figure 5). Furthermore, pretreatment with SFM for 24 hours did not reduce extracellular cAMP levels compared to levels measured immediately following serum withdrawal under either GHRH-stimulated or unstimulated conditions (Figure 5). Measured cAMP levels at shorter times (0.25, 1, 3 and 6 hours) with or without GHRH treatment were also elevated (data not shown).

Analysis of endogenous GHRH mRNA expression

In order to determine whether the MtT/S cells were producing endogenous GHRH that resulted in constitutive autocrine stimulation, amplification of GHRH from MtT/S cell cDNA by RT-PCR was performed. These assays did not result in any detectable products (Figure 6). As a positive control, amplification of plasmid DNA containing the GHRH coding sequence with GHRH specific primers displayed a single product of the predicted size (Fig. 6). The sensitivity of the PCR assay was approximated by dividing the number of plasmids in the control by the number of MtT/S cells used in the assay. According to this calculation, the assay will detect as few as 26 GHRH cDNA molecules per MtT/S cell. Amplification of the MtT/S cDNA for GH, known to exist in these cells, displayed a single product of the predicted size (data not shown). Negative control amplifications displayed no detectable products.

Analysis of the G-protein stimulatory alpha subunit nucleotide sequence

Sequence analysis of the G-protein stimulatory alpha subunit (G_s) cDNA was performed to determine if the elevated cAMP levels in MtT/S cells were due to mutations of the G_s protein, as is the case in nearly 50% of group 2 acromegalics (Spada et al., 1993). RT-PCR was

performed to produce a nearly full-length product from the G_s mRNA in both normal rat pituitary and MtT/s cells. Direct sequencing of the region of interest, containing codons 201 and 227 (Figure 7), was then performed with internal primers and fluorescent dye terminators. The sequences of both normal pituitary and the MtT/S G_s were identical to the published sequence (Figure 7), encoding Arg at codon 201 and Gln at codon 227 (Landis et al., 1989).

Effects of direct stimulation of adenylate cyclase on GH mRNA expression

To test whether GH mRNA levels in MtT/S cells could be altered by direct stimulation of adenylate cyclase, treatment with forskolin was evaluated. Following 2 days of treatment in CM, 10 μM forskolin did not significantly increase GH mRNA expression relative to the control (Figure 8A). However, following 2 day treatment with 10 μM forskolin, GH mRNA expression was significantly increased *vs*. control conditions (p < 0.05) in cells treated with SFM (163%±21) or TDM (140%±10; Fig. 8A). Following 5 day treatment with 10 μM forskolin, GH mRNA expression was increased *vs*. control levels in all treatments (Figure 8B), either CM (140%±18), SFM (174%±15), or TDM (166%±31). All of these measured increases were statistically significant *vs*. controls (p < 0.05).

Discussion

MtT/S cells are a pure somatotrophic cell line expressing high levels of GH (Inoue et al., 1990). In order to investigate the potential use of MtT/S cells for the analysis of GH transcriptional regulation, GH mRNA levels in response to a variety of stimulatory conditions were examined. Under unstimulated growth conditions, MtT/S cells maintain a high level of GH mRNA transcripts. Results in serum-free conditions suggest that such high level GH mRNA expression requires some factor(s) present in serum, and it has been shown that added T3 and Dex are capable of maintaining GH mRNA expression at control levels. The ability of T3 and/or Dex to maintain MtT/S GH mRNA expression is consistent with earlier reports that rat GH transcription requires T3 and/or Dex (Dobner et al., 1981; Evans et al., 1982; Spindler et al., 1982). Therefore, culture

of MtT/S cells with T3 and Dex allows subsequent stimulatory GHRH treatments to be performed under defined conditions similar to those performed on rat primary pituitary cultures (Gick et al., 1984).

MtT/S cells are a unique model of the somatotroph because of the reported release of GH into the medium after GHRH treatment (Inoue et al., 1990). While the initial release studies were performed with GHRH(1-44), and the present study used GHRH(1-29), these two forms of GHRH do not differ in terms of biological activity (Frohman and Jansson, 1986; Spiess et al., 1982). However, under a variety of culture conditions, it was found that addition of GHRH to the medium had no effect upon cellular GH mRNA level, suggesting that there is a defect in GHRH-receptor signaling to the GH transcriptional apparatus. Detection of GHRH-R mRNA by RT-PCR suggests that the GHRH-R is present in MtT/S cells and is consistent with the report of increased GH secretion following treatment with GHRH (Inoue et al., 1990). Importantly, GH release was measured at 1 hour after GHRH treatment (Inoue et al., 1990) rather than the longer times of the present study. Because GHRH-induced stimulation of GH release requires increases in intracellular calcium, while stimulation of GH transcription requires increases in intracellular cAMP (Barinaga et al., 1985), it is possible that signaling to the GH transcriptional apparatus is defective while signaling to the release pathway is intact.

Further investigation of GH transcriptional activation pathways in MtT/S cells was performed by measuring cAMP levels under a variety of culture conditions. Measurement of extracellular cAMP was performed, because this value parallels intracellular cAMP levels (Bilezikjian and Vale, 1983). Cellular treatment conditions immediately following serum withdrawal were deliberately similar to those used for measurement of MtT/S cell GH secretion in response to GHRH (Inoue et al., 1990). Under these conditions, GHRH does not stimulate MtT/S cell extracellular cAMP levels, suggesting that the lack of GH mRNA responsiveness is due to a defect in signaling from the GHRH-R to adenylate cyclase. In primary pituitary cultures treated under similar control conditions, cAMP secreted is less than 10 pmoles per million cells per hour (Bilezikjian and Vale, 1983). The cAMP secreted by MtT/S cells is more than 1000 fold greater

than that reported for primary pituitary cells, suggesting that the alteration in MtT/S cell GHRH signaling pathway constitutively activates adenylate cyclase. As further evidence for this notion, the high-level of MtT/S cAMP secretion is not dependent on serum factors, because 24 hour pretreatment with serum-free medium does not decrease cAMP levels. These data suggest that MtT/S cells may be unresponsive to GHRH at the transcriptional level due to constitutive activation of adenylate cyclase.

The likely causes of elevated cAMP levels in MtT/S cells were systematically investigated. Endogenous synthesis and secretion of GHRH has been associated with many GH-producing pituitary tumors (Joubert et al., 1989; Levy and Lightman, 1992; Wakabayashi et al., 1992). Because autocrine action of GHRH could produce high levels of cAMP under control conditions, endogenous GHRH expression was evaluated in MtT/S cells. The results of the highly sensitive RT-PCR assay suggest that GHRH is not synthesized by MtT/S cells. Thus, some other mechanism must cause the observed elevations in cAMP levels.

A number of GH-producing tumors which are not responsive to GHRH have greatly elevated cAMP, classified as group 2 adenomas (Spada et al., 1993). Nearly 50% of the members of group 2 have a constitutively active heterotrimeric G-protein which stimulates adenylate cyclase (Vallar et al., 1987). This constitutive activation of adenylate cyclase is caused by point mutations at two codons, Arg201 and Gln227, in G_s which block hydrolysis of GTP (Landis et al., 1989). Therefore, the nucleotide sequence encoding the G_s from MtT/S cells was determined. Despite the frequent occurrence of G_s mutations that result in *gsp* oncogenic transformation of somatotrophs into GH-secreting microadenomas, the MtT/S cells showed no sequence differences from either normal pituitary cDNAs or from the published sequence. Thus, MtT/S cells apparently belong to the group 2 class of tumors with elevated cAMP due to unknown etiology (Spada et al., 1993).

Treatment of MtT/S cells with forskolin was used to directly activate adenylate cyclase. Although cAMP was not measured after forskolin treatment, the increase in GH mRNA suggests that adenylate cyclase activation occurred. The result that forskolin does stimulate GH mRNA levels in MtT/S cells demonstrates that despite elevated cAMP levels in the untreated MtT/S cells,

GH mRNA expression has not reached maximal levels. Furthermore, the results suggest that the GH transcriptional machinery is intact in MtT/S cells, and is capable of responding to increases in cAMP levels. As a direct stimulator of adenylate cyclase (Seamon and Daly, 1986), forskolin has been shown to activate GH transcription by approximately 4 fold in primary pituitary cultures (Barinaga et al., 1985). Thus, the slight increases in forskolin-induced MtT/S cell GH mRNA levels are much less than those expected for primary cultures. However, these small increases are consistent with the finding that forskolin can stimulate cAMP levels in group 2 GH-producing tumors with basal cAMP elevation (Vallar et al., 1987).

In summary, MtT/S cells show elevated basal cAMP levels that can be increased by direct stimulation of adenylate cyclase activity but apparently not *via* the GHRH-R. It is likely that this condition is caused by some alteration in the cellular machinery that transduces signals from the GHRH-R to adenylate cyclase. Thus, the lack of change in endogenous GH transcription in these cells after GHRH treatment is apparently due to constitutive activation of adenylate cyclase by an undetermined mechanism, although this was not tested directly using pharmacological inhibitors. These cells should provide a model of group 2 acromegaly with elevated cAMP **not** caused by *gsp* mutations, constituting over 50% of this group (Landis et al., 1989; Spada et al., 1993; Vallar et al., 1987). Elucidation of the mutational cause of elevated cAMP in the MtT/S cells could be tested to see if analogous human mutations exist in acromegaly. Finally, with the clarification of the source of the elevated cAMP in MtT/S, it will be possible to use these cells as a culture model for the dissection of the physiological regulation of the endogenous GH transcriptional apparatus. By studying transcriptional regulation of the entire GH gene in a pituitary-derived cell, it is likely that somatotroph-specific aspects of the complex regulatory control system (Xu et al., 1998) could be elucidated.

Acknowledgements

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Figure Legends

Figure 1. Chemiluminescent-RPA for GH mRNA. Trials of antisense GH probe specificity showed that the probe intensity was robust without RNase treatment, was completely removed by RNase treatment when no GH mRNA was present, and was protected at the predicted size when total RNA from MtT/S cells was added.

Figure 2. Effects of serum containing, serum-free, and serum-free supplemented with 30 pM T3 and 5 nM Dex media on GH mRNA expression. MtT/S cells were incubated with the above media for 2 or 5 days and GH mRNA expression was measured using a chemiluminescent ribonuclease protection assay (c-RPA). Results are the mean of 6 samples from two independent experiments and are expressed as % of expression at the beginning of treatment. Error bars represent standard error of the mean (SEM). Values which are significantly different from the values at the beginning of treatment are designated by *, $p < 0.05$.

Figure 3. Effects of GHRH on GH mRNA expression. MtT/S cells were incubated for 2 (A) or 5 (B) days in various control media with or without 10 nM GHRH. GH mRNA expression was measured using c-RPA. Results are the mean of 6 treatments from two independent experiments and are expressed as % of control without GHRH. Error bars denote SEM. No values are statistically different from controls ($p < 0.05$, considered significant).

Figure 4. Expression of GHRH-receptor mRNA in MtT/S cells. Total RNA extracted from MtT/S cells was reverse transcribed and then amplified to detect either GHRH-R (upper panel) or ribosomal protein L19 (RPL19; lower panel) in cells grown in CM (left two lanes) or in TDM (right two lanes), either untreated (lanes marked with C) or treated with 10 nM GHRH (lanes marked with T). Product sizes of 489 bp for GHRH-R and 196 bp for RPL19 indicated on the left of each panel were determined from molecular weight markers (not shown).

Figure 5. Effects of GHRH and serum factors on cAMP levels. Cells were treated following growth in CM or following 24 hours pretreatment in serum-free media. Cells were then incubated with or without 10 nM GHRH in the absence of serum for 3 hours. Secreted cAMP was measured by RIA. Results are the mean of 3 treatments and are expressed as nMol cAMP secreted per 1×10^6 cells per hour. Similar results were obtained in two independent experiments. Error bars denote SEM.

Figure 6. Expression of GHRH mRNA in MtT/S cells. RT-PCR was used to amplify GHRH cDNA from a defined number of MtT/S cells. A negative control was performed in the absence of cDNA template. Positive controls consisting of dilutions of a plasmid containing the GHRH coding sequence amplified the expected size product (115 bp). Sensitivity of the RT-PCR was approximated by dividing the copy number of GHRH plasmid by the number of cells used in the RT-PCR assay.

Figure 7. Nucleotide sequence analysis of G_s in MtT/S cells. A) is the published sequence of the wild-type G_s in the rat from codons 200 to 230, with the amino acid translation below the codons; B) is the nucleotide sequence for either oncogenic G_s proteins, with amino acid changes shown below; C) is the sequence determined from rat pituitary; and D) is the sequence determined from MtT/S cells.

Figure 8. Effects of direct adenylate cyclase stimulation on GH mRNA expression. MtT/S cells were incubated for 2 (A) or 5 (B) days in various control media with or without 10 μM forskolin. GH mRNA expression was measured using c-RPA. Results are the mean of 6 treatments from two independent experiments and are expressed as % of control without forskolin. Error bars denote SEM. Values which are significantly different from the controls are designated by *, $p < 0.05$.
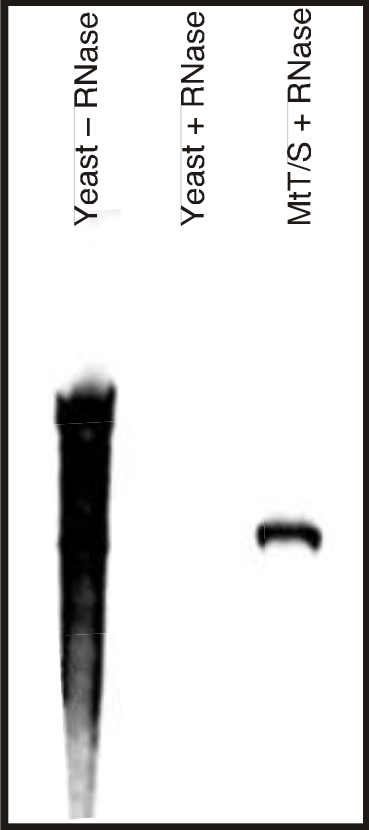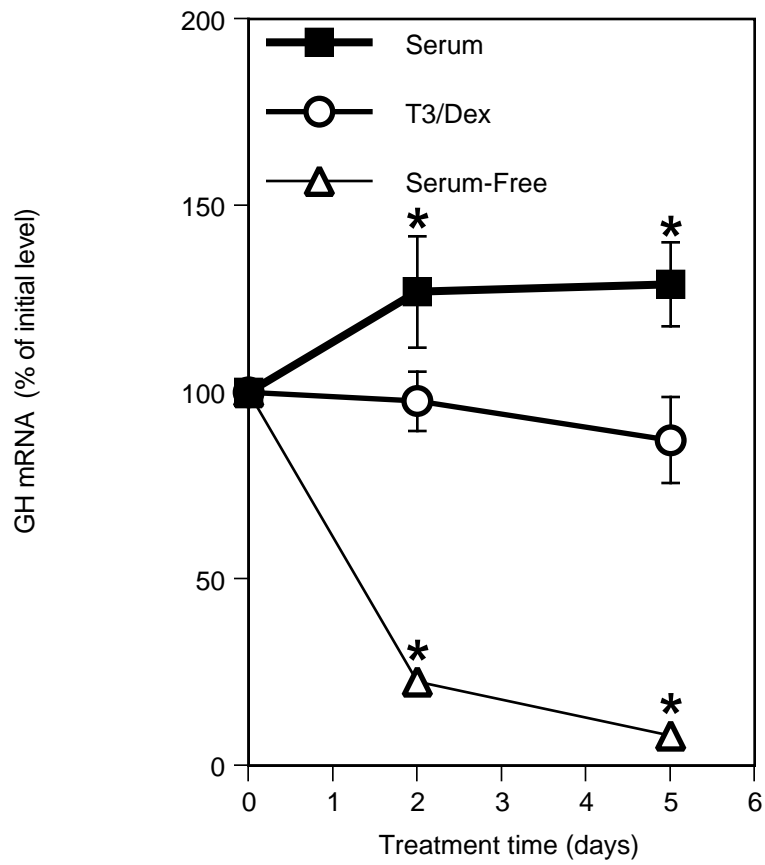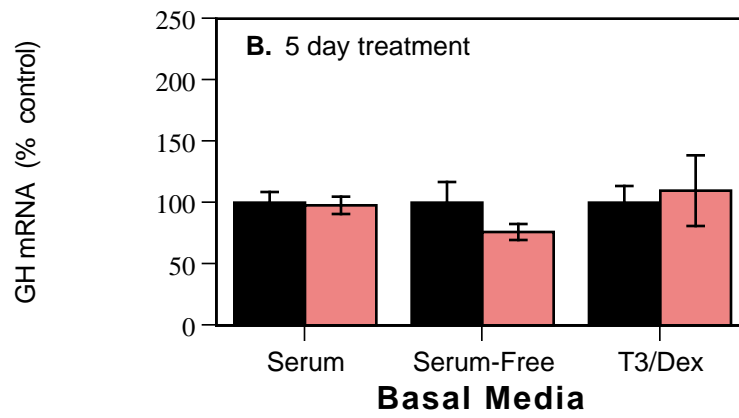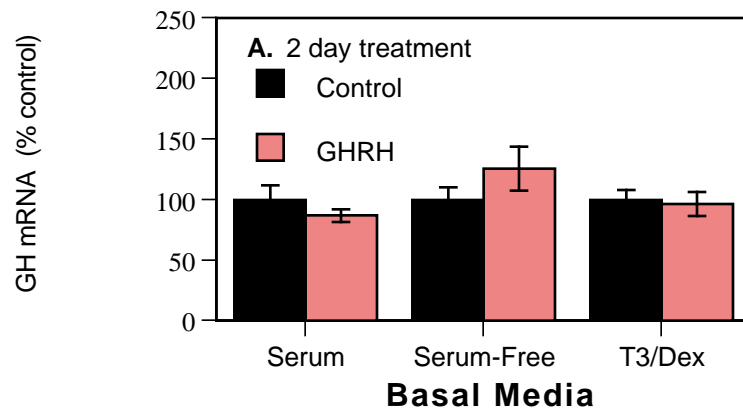
Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

GHRH
(115 bp)

300bp

200bp

100bp

A)
```
                  201                                                                                              227
  GAC CTG CTT CGC TGC CGC GTC CTG ACC TCT GGA ATC TTT GAG ACC AAG TTC CAG GTG GAC AAA GTC AAC TTC CAC ATG TTC GAT GTG GGC GGC CAG CGC
   D   L   L   R   C   R   V   L   T   S   G   I   F   E   T   K   F   Q   V   D   K   V   N   F   H   M   F   D   V   G   G   Q   R

                  CAC
B) ... ... ...   TGC ... ... ... ... ... ... ... ... ... ... ... ... ...                                          CGG ...
                  C or H                                                                                           R

C)  GAC CTG CTT CGC TGC CGC GTC CTG ACC TCT GGA ATC TTT GAG ACC AAG TTC CAG GTG GAC AAA GTC AAC TTC CAC ATG TTC GAT GTG GGC GGC CAG CGC

D)  GAC CTG CTT CGC TGC CGC GTC CTG ACC TCT GGA ATC TTT GAG ACC AAG TTC CAG GTG GAC AAA GTC AAC TTC CAC ATG TTC GAT GTG GGC GGC CAG CGC
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Figure 7

Figure 8