

# Glucocorticoids Regulate Pituitary Growth Hormone-Releasing Hormone Receptor Messenger Ribonucleic Acid Expression\*

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## ABSTRACT

Glucocorticoids regulate GH synthesis and secretion by influencing both hypothalamic and pituitary function. With respect to GH-releasing hormone (GHRH), an important GH secretagogue, glucocorticoids are reported not only to suppress hypothalamic GHRH expression but also to augment pituitary responsiveness to GHRH. To investigate further this latter observation, we have determined the effects of this steroid on expression of the GHRH receptor (GHRH-R) gene in the rat pituitary *in vivo* and in pituitary cells *in vitro*. Adult male rats were adrenalectomized or sham operated and treated with sc implants of cholesterol or corticosterone. Adrenalectomized animals showed substantially reduced pituitary GHRH-R mRNA levels, when compared with untreated sham-operated animals. Conversely, administration of corticosterone increased pituitary GHRH-R mRNA levels in intact, as well as adrenalectomized rats. We also analyzed

the effects of the synthetic glucocorticoid, dexamethasone, on GHRH-R mRNA expression in cultured rat anterior pituitary cells. GHRH-R mRNA was significantly increased by dexamethasone, with a maximal response observed in the presence of 100 nM hormone. This dose of dexamethasone substantially elevated GHRH-R mRNA after 6 h, 12 h, and 24 h of treatment. Dexamethasone did not increase GHRH-R mRNA in the presence of the transcriptional inhibitor actinomycin D, indicating that the predominant effect of the hormone is to increase transcription of the GHRH-R gene. These data demonstrate that GHRH-R mRNA levels are directly stimulated by glucocorticoids, both in the presence and absence of hypothalamic influences, providing a probable explanation for the ability of this steroid to alter pituitary responsiveness to GHRH. (*Endocrinology* 138: 2458–2465, 1997)

**G**H SYNTHESIS and secretion is regulated predominantly by the opposing actions of the stimulatory hypothalamic peptide GH-releasing hormone (GHRH) and the inhibitory peptide SS (1, 2). These hormones exert their actions on the somatotrope cells of the anterior pituitary by interacting with specific membrane receptors. Recently, cDNAs for the GHRH receptor (GHRH-R) were cloned and characterized from several mammalian species (3–7). These receptors are members of a growing subfamily of G protein-coupled receptors whose ligands are structurally related peptide hormones (8–10). The interaction of GHRH with its receptor activates adenylate cyclase through a G protein-coupled pathway, increasing the levels of the second messenger cAMP (3, 9, 10). The critical role of the GHRH-R in stimulating GH release is apparent in the *little* mouse, a dwarf phenotype resulting from a point mutation in the amino-terminal domain of the protein (4, 9, 10) that leads to loss of receptor function. More recently, human GH deficiency has been associated with a nonsense mutation in the amino-terminal domain of the GHRH-R (11).

Corticosteroids are important physiological regulators of GH synthesis and secretion (12). In general, glucocorticoids exert a suppressive effect on the GH axis in mammals (13),

causing growth retardation in laboratory rats (14, 15) and decreased growth rates in chronically treated children (12). In addition, patients with Cushing's syndrome, which clinically presents as hypercortisolemia, have almost lost the ability to secrete GH (12). Glucocorticoids seem to exert these growth-suppressive effects by acting at multiple levels of the GH pathway (1, 12). Previously, it was demonstrated that GHRH-binding sites were down-regulated in dispersed pituitary cells from adrenalectomized rats, and hormone replacement with the synthetic glucocorticoid, dexamethasone, was able to restore GHRH binding (16, 17). Significantly, these changes in GHRH binding were not caused by altered binding affinity, suggesting that differences in receptor number were responsible for the corticosteroid effects. To characterize further the mechanisms of corticosteroid action on the GH axis, we analyzed the effects of glucocorticoids on the expression of the rat GHRH-R gene in the intact pituitary and in enzymatically dispersed pituitary cells, using RT, followed by the PCR, to measure GHRH-R mRNA levels. We demonstrate that glucocorticoids increase GHRH-R mRNA levels both *in vivo* and *in vitro*. We also demonstrate that this induction requires new RNA synthesis, indicating that glucocorticoids act to regulate gene transcription, rather than mRNA stability.

## Materials and Methods

### Reagents

Powdered DMEM, FBS, and HBSS were obtained from GIBCO/BRL (Grand Island, NY). Corticosterone was purchased from ICN Biomedicals (Costa Mesa, CA). RQ1 deoxyribonuclease (DNase) I and avian

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myeloblastosis virus-RT were from Promega (Madison, WI). Random primers were from Pharmacia (Piscataway, NJ) and Taq polymerase was from Perkin Elmer (Foster City, CA). Radionucleotides were purchased from Amersham Life Sciences (Arlington Heights, IL). All other reagents, including trypsin (pancreatic, type II), DNase I (pancreatic, type IV), soybean trypsin inhibitor, BSA, cholesterol, dexamethasone, dimethyl-sulfoxide (DMSO), and actinomycin D were purchased from Sigma (St. Louis, MO).

### Animals

Adult male Sprague-Dawley rats (Charles River, Portage, MI; and Harlan, Indianapolis, IN), weighing 200–250 g, were housed in facilities approved by the American Association for Accreditation of Laboratory Animal Care, under a light:dark cycle of 14:10, with water and food provided *ad libitum*. The experimental protocols used in these studies were approved by the Northwestern University Institutional Animal Care and Use Committee.

### *In vivo effects of corticosterone*

Male rats were bilaterally adrenalectomized or sham operated under light anesthesia between 0900 and 1200 h on day 1 of the experiment. Immediately after surgery, and continuing for the duration of the experiment, the adrenalectomized rats were maintained on 0.9% saline in their drinking water. The animals received either 2 cholesterol pellets (average pellet weight of 340 mg) or 2 corticosterone pellets (average pellet weight of 375 mg), made according to previously described methods (18) and implanted sc under light metofane (Methoxyflurane, Pitman-Moore, Mundelein, IL) anesthesia at 0900 h on day 6 of the experiment. Animals were killed by decapitation between 1030 and 1230 h on day 9 of the experiment. Pituitaries were removed, frozen on dry ice, and stored at  $-80^{\circ}\text{C}$  until RNA was extracted. Trunk blood was collected on ice and allowed to clot, and serum was stored at  $-20^{\circ}\text{C}$  for subsequent hormone measurement by RIA. Adrenal gland atrophy (14) and reduced body weight (14, 15) have previously been reported as indicators of high circulating levels of corticosterone. Body weight was therefore monitored for each animal throughout the course of the experiment, and the adrenal glands were removed from the sham-operated animals and dissected, and the paired wet weight was measured.

### *In vitro effects of dexamethasone*

Anterior pituitary glands were obtained from 30–40 male rats at 0900 h. Primary anterior pituitary cultures were prepared, as previously described (19), with some modifications. Briefly, the anterior lobes were separated from the neurointermediate and posterior lobes and placed into ice-cold, sterile HEPES buffer (25 mM HEPES, pH 7.4; 137 mM NaCl; 5 mM KCl; 0.7 mM  $\text{Na}_2\text{HPO}_4$ ; 10 mM glucose) containing 100 U/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin, and 0.25  $\mu\text{g}/\text{ml}$  amphotericin B. The anterior pituitaries were washed in HEPES buffer four times, then minced with sterile scalpel blades on a siliconized glass slide into 1- to 2-mm<sup>3</sup> pieces. The pituitary fragments were pooled and washed twice with HEPES buffer. The cells were dispersed two times, at 30 C for 60 min each time, in a Bellco flask containing 25 ml enzymatic trypsin solution (HBSS containing: 4 mM  $\text{NaHCO}_3$ , 10 mM HEPES, pH 7.4; 0.1% wt/vol trypsin; 0.2% wt/vol glucose; 0.4% wt/vol BSA; 10  $\mu\text{g}/\text{ml}$  DNase I type IV). The cells were collected by low-speed centrifugation (270  $\times$  g) and dissociated by trituration in DMEM containing 10  $\mu\text{g}/\text{ml}$  DNase I type IV and 25  $\mu\text{g}/\text{ml}$  soybean trypsin inhibitor. Three to five million cells were plated into 12-well plates containing DMEM with 5% (vol/vol) charcoal-stripped FBS (20), 100 U/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin, and 0.25  $\mu\text{g}/\text{ml}$  amphotericin B, then incubated at 37 C. After 40 h, the cells were treated with ethanol vehicle or the synthetic glucocorticoid, dexamethasone, (1 nM to 1  $\mu\text{M}$ ) in fresh medium and harvested at various time points. In experiments involving the RNA polymerase inhibitor, actinomycin D (21), the cells were pretreated with 5  $\mu\text{g}/\text{ml}$  (4  $\mu\text{M}$ ) actinomycin D or DMSO vehicle for 30 min, before treatment with dexamethasone. The actinomycin D treatment was continued for the duration of the experiment.

### RNA isolation

RNA was extracted from individual pituitaries by homogenization in guanidinium thiocyanate and purified by ultracentrifugation through a cesium chloride density gradient (22). RNA was isolated from primary anterior pituitary cultures by extraction of acidic guanidinium thiocyanate lysates with phenol and chloroform (23), followed by RQ1 DNase I digestion of the RNA.

### RT-PCR

Oligonucleotide primer pairs of 22 nucleotides (RPRJA; 5'-CATCTCCTAGGTCCAAACCAGC-3' and RPRJC; 5'-GAAGTTCAGGGTCATGCCATA-3'; 50–54% G+C content) and 21 nucleotides (RPL19A, 5'-CTGAAGTCAAAGGGAATGTG-3'; and RPL19B, 5'-GGACAGAGTCTTGATGATCTC-3'; 42–47% G+C content) were synthesized, based on the sequences of rat GHRH-R (3) and ribosomal protein L19 (24), respectively. Primers were designed such that the expected products would be 489 bp for the GHRH-R and 196 bp for ribosomal protein L19 (RPL19). We first established optimal conditions for this assay by determining the range of the assay with regard to the number of amplification cycles and RNA input, as previously described (25). This revealed the appropriate range of the assay to be 22 to 35 amplification cycles for both the GHRH-R and RPL19 primers (data not shown), and 28 cycles were utilized in all subsequent experiments.

Total RNA from intact pituitaries (2.5  $\mu\text{g}$ ) or primary pituitary cell cultures (0.5  $\mu\text{g}$ ) were reverse transcribed at 42 C in a 20- $\mu\text{l}$  reaction using random hexamer primers and avian myeloblastosis virus-RT. Five microliters of each reverse transcription reaction was amplified in a 25- $\mu\text{l}$  reaction in the presence of either the GHRH-R or the RPL19 primers (125 ng each),  $\alpha^{32}\text{P}$ -dCTP (0.5  $\mu\text{Ci}$  at 3000 Ci/mmol), and Taq DNA polymerase (0.625 units) in 10 mM Tris (pH 8.3), 50 mM KCl, 2.2 mM  $\text{MgCl}_2$ , 0.01% gelatin and overlaid with mineral oil. Amplification was carried out for 28 cycles, using an annealing temperature of 65 C (primers RPRJA and RPRJC) or 55 C (primers RPL19A and RPL19B), on a Perkin Elmer thermocycler. The samples were chloroform extracted and 10  $\mu\text{l}$  of each sample were size separated by electrophoresis on a 5% nondenaturing polyacrylamide gel. After autoradiography, the gel was analyzed on a Molecular Dynamics Phosphorimager using ImageQuant version 3 software (Molecular Dynamics, Sunnyvale, CA). The intensity of the GHRH-R signal was normalized to the RPL19 control and plotted as arbitrary phosphorimage units.

### RIA

Serum corticosterone was measured using a double-antibody kit from ICN Biomedicals employing [<sup>125</sup>I] corticosterone-3-carboxymethyl-oxime as the tracer. The intraassay variation was 2%.

### Statistical analysis

Unless otherwise stated, data shown are mean plus SEM. For the *in vivo* experiments, n refers to the number of individual pituitaries for each treatment group. For the primary pituitary cells, n refers to the number of cell culture wells for each treatment group, and each experiment was repeated at least twice. ANOVA was performed using CRISP statistical software (CRUNCH Software, San Francisco, CA). *Post hoc* comparisons of the effects of dexamethasone were performed using the Newman-Keuls test. *P* less than 0.05 was considered statistically significant.

## Results

### *Corticosterone modulation of GHRH-R mRNA in vivo*

It previously has been demonstrated that binding of GHRH to pituitary homogenates or dispersed cells is decreased by adrenalectomy and restored by the addition of dexamethasone (16, 17). Using a similar paradigm, GHRH-R mRNA levels in the pituitaries of sham-operated and adrenalectomized rats were analyzed by RT-PCR, and the effect of exogenous corticosteroid was measured (Fig. 1). Bilateral adrenalectomy decreased pituitary GHRH-R mRNA expres-

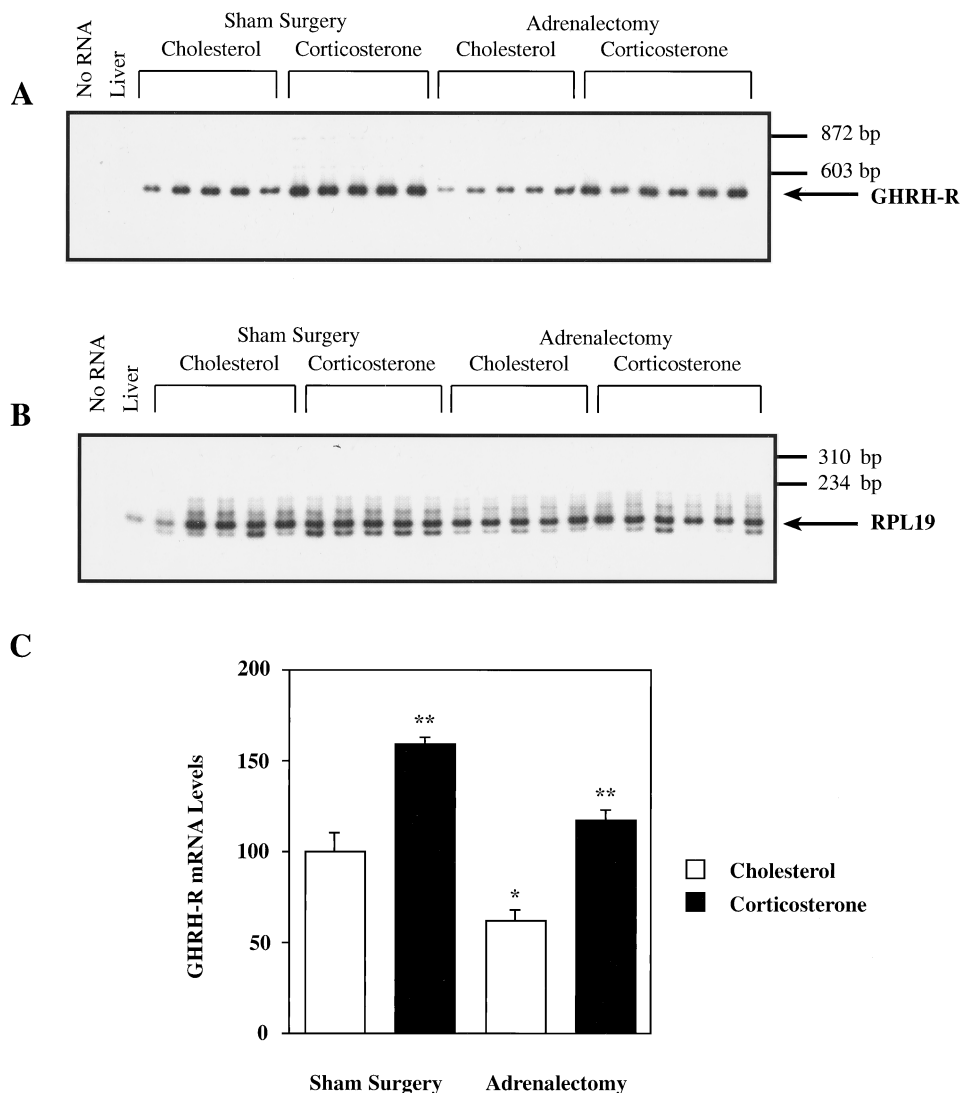


FIG. 1. Adrenalectomy decreases and corticosterone increases GHRH-R mRNA expression *in vivo*. Total RNA (2.5  $\mu$ g) isolated from individual pituitaries of sham-operated or bilaterally adrenalectomized rats treated with cholesterol or corticosterone for 3 days was analyzed by RT-PCR for amounts of GHRH-R (panel A) and RPL19 (panel B) mRNAs. Autoradiograms of the dried 5% polyacrylamide gels on which the PCR products were separated by electrophoresis are shown. The first and second lanes in panels A and B represent a control without RNA and a control with liver RNA, respectively. The position of the expected 489-bp GHRH-R and 196-bp RPL19 PCR products, as well as the positions of the size markers, are indicated. The GHRH-R and RPL19 products were quantitated by phosphorimage analysis, and the level of GHRH-R mRNA relative to that of RPL19 was plotted as arbitrary phosphorimage units (panel C). \*,  $P < 0.0004$ , relative to cholesterol treated sham; \*\*,  $P < 0.0001$ , relative to cholesterol-treated animals;  $n = 5-6$ .

TABLE 1. Physiological effects of corticosterone treatment

Treatment	Initial weight	Final weight	Adrenal weight	Corticosterone	N
Sham + Chol	218.4 g $\pm$ 3.9	235.2 g $\pm$ 4.4	56.2 mg $\pm$ 0.7	4.44 $\mu$ g/dl $\pm$ 0.8	5
Sham + Cort	216.2 g $\pm$ 2.9	193.0 g $\pm$ 4.6 <sup>a</sup>	42.0 mg $\pm$ 2.1 <sup>c</sup>	66.37 $\mu$ g/dl $\pm$ 19.6 <sup>d</sup>	5
Adx + Chol	223.3 g $\pm$ 4.4	251.7 g $\pm$ 6.5	N/A	<1.0 $\mu$ g/dl	6
Adx + Cort	222.2 g $\pm$ 3.5	223.3 g $\pm$ 2.8 <sup>b</sup>	N/A	50.85 $\mu$ g/dl $\pm$ 1.43 <sup>d</sup>	6

Body weight, adrenal weight and circulating corticosterone were monitored as markers of the effects of surgery and hormone treatment. Changes in posttreatment body weight were statistically significant: <sup>a</sup>  $P < 0.0001$  relative to cholesterol treated shams; <sup>b</sup>  $P < 0.0001$  relative to surgery matched cholesterol treated animals. The weights before steroid treatment were not statistically different. Wet weights of the paired adrenal glands were significantly decreased: <sup>c</sup>  $P < 0.0002$  relative to cholesterol treated sham. Serum corticosterone levels were significantly elevated: <sup>d</sup>  $P < 0.0001$  relative to surgery matched cholesterol treated animals.

sion to approximately 60% of the level observed in cholesterol-treated intact animals ( $P < 0.0004$ ). This effect was reversed by the addition of exogenous corticosterone, resulting in a 1.9-fold increase in GHRH-R mRNA levels relative to cholesterol-treated, adrenalectomized rats ( $P < 0.0001$ ). In addition, treatment of intact animals with corticosterone resulted in a 1.6-fold increase in GHRH-R mRNA levels relative to cholesterol-treated, intact animals ( $P < 0.0001$ ).

The physiological effects of surgery and corticosteroid treatment were monitored throughout this *in vivo* study (Table 1). There was no significant difference in the weight of the animals before treatment, regardless of surgery status. However, the effects of surgery and treatment on the final body weight were both statistically significant ( $P < 0.0001$ ). Animals treated with cholesterol, regardless of surgery status, gained weight (average percent BW gain of  $+7.69 \pm 0.72\%$  and  $+12.64 \pm 1.08\%$  for sham and adrenalectomy, respec-

tively), whereas there was a loss in body weight or lack of weight gain in animals receiving corticosterone ( $-12.14 \pm 1.31\%$  and  $+0.56 \pm 1.03\%$  for sham and adrenalectomy, respectively). The level of circulating corticosterone, as measured by RIA ( $66.37 \mu\text{g}/\text{dl} \pm 19.6$  for intact rats and  $50.85 \mu\text{g}/\text{dl} \pm 1.43$  for adrenalectomized rats), was statistically elevated ( $P < 0.0001$ ) when compared to cholesterol treated animals. Additionally, the paired adrenal weight of sham-operated rats that received corticosterone was decreased to 75% of that in cholesterol-treated animals ( $P < 0.0002$ ).

#### Dexamethasone modulation of GHRH-R mRNA *in vitro*

To determine if the glucocorticoid-induced changes observed *in vivo* could be elicited in the absence of any hypothalamic input, the effects of dexamethasone on GHRH-R mRNA expression were monitored in a primary pituitary cell culture system. Rat anterior pituitaries were enzymatically dispersed and grown in culture for 40 h before hormone treatment. Cultured cells were treated with fresh media or fresh media containing dexamethasone. The expression of GHRH-R and RPL19 mRNAs, after 12 h of treatment with various doses of dexamethasone, was monitored by RT-PCR (Fig. 2). GHRH-R mRNA levels were significantly elevated by all doses of dexamethasone ( $P < 0.0004$ ), with a maximal increase of approximately 5-fold in the presence of 100 nM dexamethasone.

Using the maximally effective dose of 100 nM dexamethasone; GHRH-R mRNA, and RPL19 mRNA levels were monitored by RT-PCR after various times of treatment (Fig. 3). Although there was no significant change in GHRH-R mRNA levels after 3 h of treatment with 100 nM dexamethasone, there was a significant increase in GHRH-R mRNA after 6 h (6.1-fold), 12 h (5.2-fold) and 24 h (4.1-fold) dexamethasone treatment, as compared with the time-matched controls ( $P < 0.0001$ ). *Post hoc* comparisons of the dexamethasone-treated groups indicated that the effect of hormone treatment on GHRH-R mRNA levels was increased with time ( $P < 0.05$ ). Parallel studies, not shown here, indicated that GH mRNA was elevated after 12 h treatment with 1  $\mu\text{M}$  dexamethasone and 24 h exposure to 100 nM dexamethasone ( $P < 0.05$ ).

#### Glucocorticoid-induced changes in GHRH-R mRNA require new RNA synthesis

To determine whether the increased level of GHRH-R mRNA in response to dexamethasone treatment of dispersed anterior pituitary cells was caused by increased gene transcription or increased mRNA stability, the effects of dexamethasone were monitored in the presence and absence of the RNA polymerase inhibitor actinomycin D. Primary anterior pituitary cells were dispersed, as described previously, and allowed to stabilize for 40 h before treatment. Cultured cells were exposed to fresh media or fresh media containing actinomycin D for 30 min before treatment with dexamethasone. The actinomycin D treatment was continued through the 12 h of dexamethasone treatment. Total RNA was isolated from the harvested cells, as described previously, and the expression of GHRH-R and RPL19 mRNAs was monitored by RT-PCR (Fig. 4). A 4.4-fold increase in GHRH-R

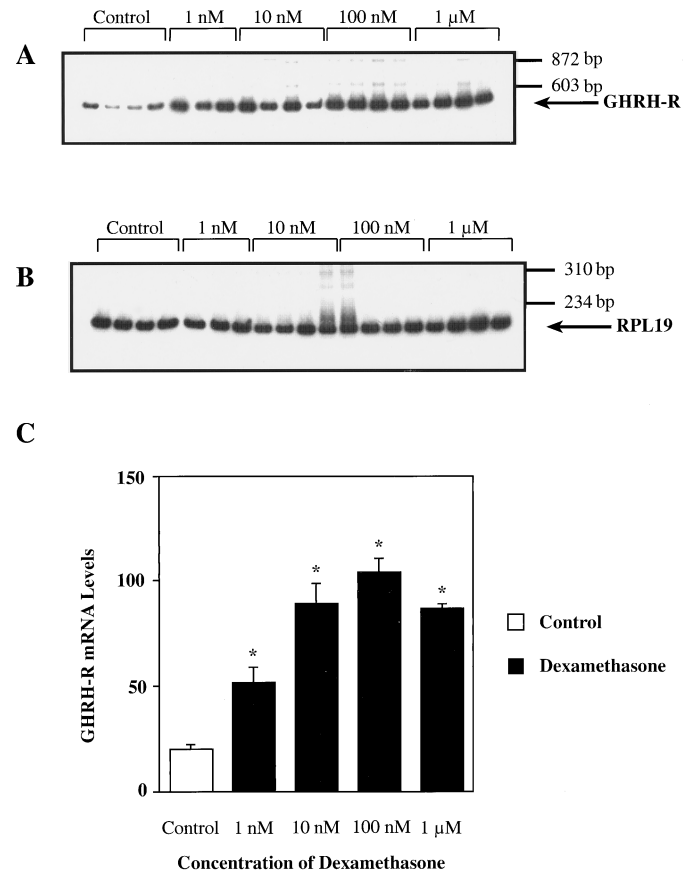


FIG. 2. Dose-dependent increases in GHRH-R mRNA expression in primary pituitary cell cultures, in response to dexamethasone. Total RNA ( $0.5 \mu\text{g}$ ) isolated from enzymatically dispersed anterior pituitary cells, treated with vehicle (ethanol), 1 nM, 10 nM, 100 nM, or 1  $\mu\text{M}$  dexamethasone for 12 h, was analyzed by RT-PCR for GHRH-R (panel A) and RPL19 (panel B) mRNAs. The position of the expected 489-bp GHRH-R and 196-bp RPL19 PCR products, as well as the positions of the size markers, are indicated. The GHRH-R and RPL19 products were quantitated by phosphorimage analysis, and the level of GHRH-R mRNA relative to that of RPL19 was plotted as arbitrary phosphorimage units (panel C). Similar results were observed in an independent experiment. \*,  $P < 0.0004$ , relative to vehicle-treated control;  $n = 4$ .

mRNA expression in response to glucocorticoid treatment was observed in the absence of actinomycin D treatment ( $P < 0.0001$ ), consistent with the inductions observed in Figs. 2 and 3. In contrast, treatment with actinomycin D completely abolished the ability of dexamethasone to increase GHRH-R mRNA in pituitary cells ( $P < 0.0001$ ).

#### Discussion

The studies presented here have characterized further the mechanism by which corticosteroids influence the GH axis. The effect of glucocorticoids on GHRH-R mRNA expression was monitored *in vivo*, as well as in cultured anterior pituitary cells. Adrenalectomy decreases GHRH-R mRNA expression, and corticosterone treatment increases GHRH-R mRNA levels, in the pituitary gland of adrenalectomized and intact male rats. Similarly, dexamethasone elevates GHRH-R mRNA expression in anterior pituitary cells grown in primary culture. Treatment with actinomycin D prevents the

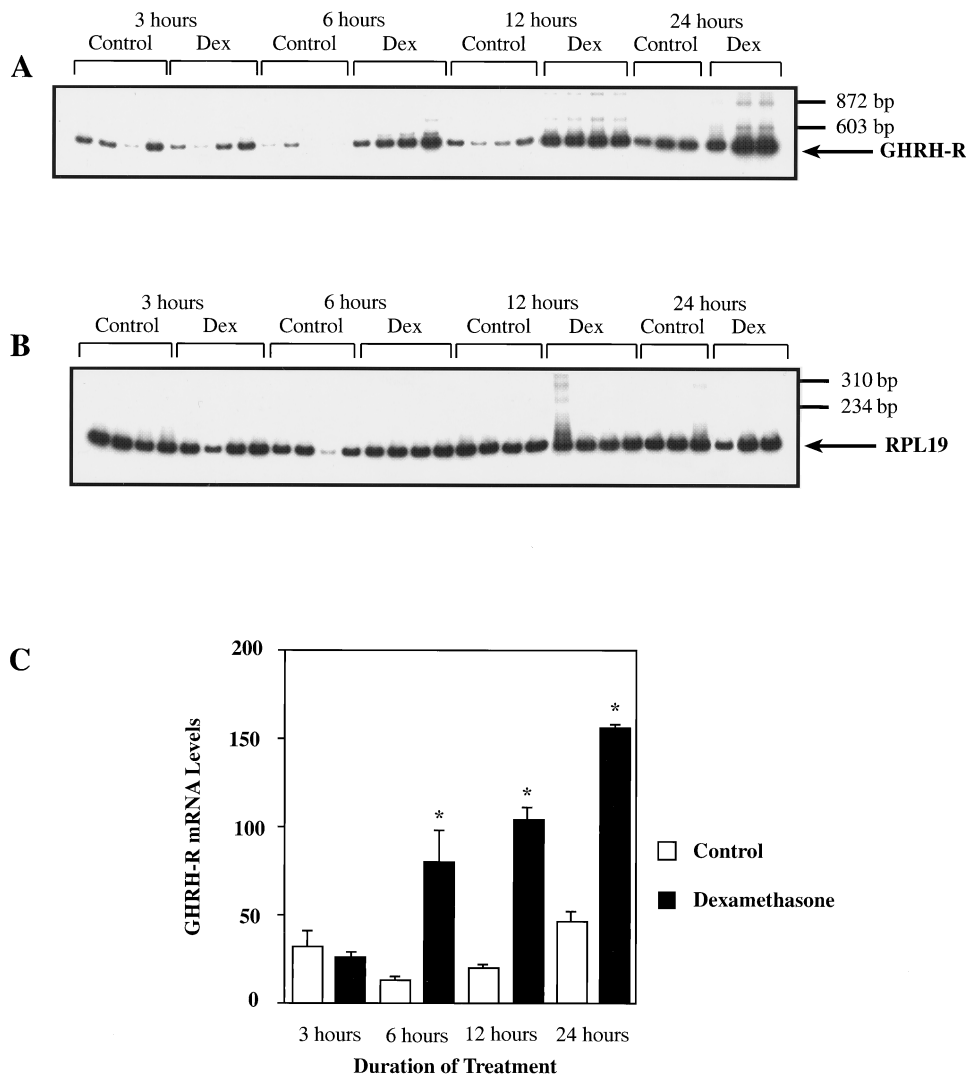


FIG. 3. Time-dependent increases in GHRH-R mRNA expression in cultured anterior pituitary cells, in response to dexamethasone. Total RNA (0.5  $\mu$ g) isolated from enzymatically dispersed anterior pituitary cells treated with vehicle (ethanol) or 100 nM dexamethasone for 3, 6, 12, or 24 h, were analyzed by RT-PCR for GHRH-R (panel A) and RPL19 (panel B) mRNAs. The position of the expected 489-bp GHRH-R and 196-bp RPL19 PCR products, as well as the positions of the size markers, are indicated. The GHRH-R and RPL19 products were quantitated by phosphorimager analysis, and the level of GHRH-R mRNA relative to that of RPL19 was plotted as arbitrary phosphorimager units (panel C). Similar results were observed in an independent experiment. \*,  $P < 0.0001$ , relative to time-matched, vehicle-treated controls;  $n = 4$ .

dexamethasone-induced increase in GHRH-R mRNA, suggesting that glucocorticoids exert their effects predominantly by stimulating transcription of the GHRH-R gene. These results extend previous observations that GHRH-binding sites in pituitary homogenates and dispersed cells are decreased in response to adrenalectomy and subsequently elevated after dexamethasone treatment (16, 17) and therefore define a likely mechanism by which glucocorticoids increase the responsiveness of the somatotrope cell to GHRH.

Previous studies have demonstrated that glucocorticoids have diverse effects at multiple levels of the GH axis. These adrenal hormones influence downstream effectors, such as IGF-I (26), regulate GH feedback mechanisms by altering the expression of the GH receptor gene (27, 28), and modify both hypothalamic and pituitary function. At the level of the pituitary, glucocorticoids can stimulate directly GH synthesis and secretion in the absence of any hypothalamic input (29). Implanting the pituitary gland under the kidney capsule removes the regulatory effects of GHRH and SS on GH expression. Under these conditions, adrenalectomy decreases pituitary GH mRNA levels, whereas subsequent treatment with dexamethasone restores GH synthesis (29).

GH release (30) and GH mRNA accumulation (31) are both stimulated by glucocorticoids in cultured pituitary cells. Glucocorticoid regulation of the GH gene occurs primarily through a direct interaction of the glucocorticoid receptor with a response element within the first intron of the GH gene (32).

Glucocorticoids influence hypothalamic control over GH expression by maintaining a balance between the positive and negative influences of GHRH and SS. Previous studies have suggested that chronic exposure to glucocorticoids augments somatostatinergic tone at the level of the hypothalamus (1, 12). Expression of SS mRNA is increased in the arcuate nucleus after 48 and 72 h of dexamethasone treatment (27); however, prolonged exposure to the hormone decreases SS mRNA levels in the periventricular and paraventricular regions of the hypothalamus (28, 33). Total hypothalamic SS content is increased in response to glucocorticoid treatment during the same period (33, 34). Conversely, GHRH mRNA expression in the arcuate nucleus of the hypothalamus is decreased after prolonged treatment with dexamethasone (27, 28), and decreases in total hypothalamic

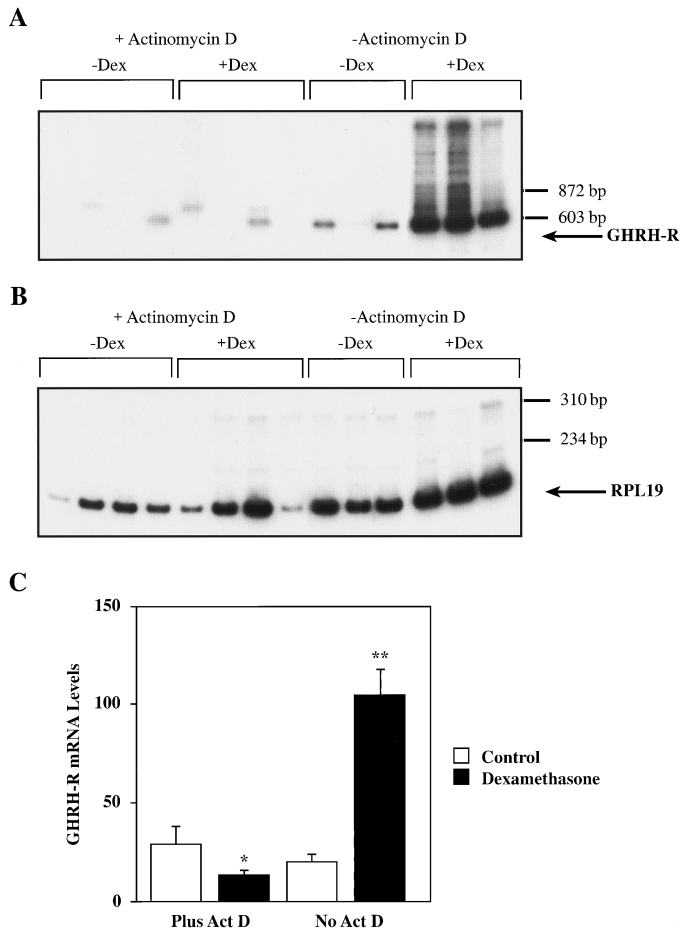


FIG. 4. Dexamethasone does not increase GHRH-R mRNA in the presence of the transcriptional inhibitor, actinomycin D. Total RNA (0.5  $\mu$ g), isolated from enzymatically dispersed anterior pituitary cells treated with vehicle (DMSO) or 4  $\mu$ M actinomycin D for 30 min before treatment with dexamethasone (Dex) for 12 h, were analyzed by RT-PCR for GHRH-R (panel A) and RPL19 (panel B) mRNAs. The position of the expected 489-bp GHRH-R and 196-bp RPL19 products, as well as the positions of the size markers, are indicated. The GHRH-R and RPL19 products were quantified by phosphorimage analysis, and the level of GHRH-R mRNA relative to that of RPL19 was plotted as arbitrary phosphorimage units (panel C). \*,  $P < 0.0001$ , relative to no actinomycin D, plus dexamethasone treatment; \*\*,  $P < 0.0001$ , relative to no actinomycin D, no dexamethasone treatment;  $n = 3-4$ .

GHRH content are detected after 3 days exposure to the hormone (34).

The glucocorticoid-induced changes in GHRH and SS expression at the level of the hypothalamus are similar to alterations in pituitary expression of the GHRH and SS receptors in response to glucocorticoid treatment. Some *in vivo* studies have suggested that glucocorticoids act by suppressing GHRH-stimulated GH release (34); however, the duration of glucocorticoid treatment has been shown to have profound effects on the GHRH response (35). Significantly, other *in vivo* studies have demonstrated that treatment with glucocorticoids in the presence of SS inhibitors (36) or antibodies specific for SS (37) enhances the response of GH to GHRH. In primary rat pituitary cultures, glucocorticoids augment both basal and GHRH-induced GH release (30, 34)

and reduce the ability of SS to inhibit GH release in response to GHRH (30). Importantly, glucocorticoids have been shown to exert differential effects on GHRH (16, 17) and SS binding (38). The observed changes in GHRH and SS binding are not caused by altered receptor affinity, suggesting that differences in receptor numbers are responsible for the corticosteroid effects. In support of these observations, mRNAs for SS receptor (SSTR) subtypes SSTR1, SSTR2, and SSTR3 are all decreased in pituitary cell cultures after 24 h exposure to glucocorticoids (39). Our observation that GHRH-R mRNA levels are induced after 6 h of glucocorticoid treatment of primary pituitary cells and remain elevated up to 24 h after initiation of treatment further supports the hypothesis that glucocorticoids differentially regulate SS and GHRH, as well as their corresponding receptors.

Corticosteroids exert a broad spectrum of physiological effects in the animal; therefore, multiple components could be involved in eliciting the increase in GHRH-R mRNA levels that is observed *in vivo*. The high level of circulating corticosterone, the lack of weight gain, and the substantial decrease in adrenal gland weight in the animals that received hormone implants suggest that the paradigm used for analyzing GHRH-R mRNA regulation by corticosteroids mimics the chronic exposure previously demonstrated to inhibit both linear growth (12-15) and GH release (34). Although it is possible that the increases in pituitary expression of GHRH-R mRNA are caused by the catabolic effects of corticosterone, glucocorticoids remain capable of enhancing GHRH-R gene expression in a primary pituitary cell culture system. It recently has been demonstrated that glucocorticoids directly inhibit GHRH neurons in the arcuate nucleus of the hypothalamus, whereas the inhibitory effects of this hormone on the SS neurons of the periventricular nucleus are mediated by GH (28). It is therefore possible that the changes in GHRH-R expression, in response to corticosterone that we have observed *in vivo*, are contributed to by GH and/or glucocorticoid-mediated changes in GHRH or SS expression in the hypothalamus. In support of this observation, it has recently been demonstrated that passive immunization with GHRH antiserum and treatment with exogenous GH both cause decreased GHRH-R mRNA expression *in vivo* (40). However, our finding that dexamethasone increases GHRH-R mRNA expression in cultured anterior pituitary cells suggests that glucocorticoids can act directly at the level of the pituitary to exert its effect.

Thyroid hormone also has profound effects on the GH axis (41, 42), and in many ways, these parallel the effects of glucocorticoids on the hypothalamic peptides SS and GHRH and their pituitary receptors. Thyroid ablation decreases SS mRNA, while simultaneously increasing GHRH mRNA. Similarly, GHRH-R mRNA expression is decreased in response to thyroidectomy (41) and antithyroid treatment (42); however, SSTR1 seems to be unaffected by thyroid hormone manipulations. In addition, thyroid hormone can directly stimulate GH gene transcription (31). Both glucocorticoid and thyroid hormones, therefore, act to increase somatostatinergic tone at the level of the hypothalamus and increase pituitary responsiveness to GHRH secretion by altering GHRH-R levels. This change in sensitivity could help to explain the observation that GH is expressed at an earlier

developmental time, in fetal rats that have been treated with dexamethasone, than in control animals (43).

Glucocorticoids are well-characterized activators of transcription, exerting their effects through specific cellular receptors that interact directly with hormone response elements located in the regulatory regions of responsive genes (44). Steroid hormones, including glucocorticoids, also may contribute to changes in mRNA stability (45). In pituitary cells, glucocorticoids have been shown both to increase transcription of the GH gene (31) and to enhance the stability of the GH mRNA (46). It was therefore important to determine whether the effects of glucocorticoids on GHRH-R mRNA accumulation were caused by increased gene transcription or by alterations in GHRH-R mRNA stability. We have attempted to assess directly the effects of glucocorticoids on GHRH-R gene transcription by performing nuclear run-on experiments to measure gene transcription rates, but this assay lacks sufficient sensitivity, presumably because of the extremely low levels of GHRH-R expression and the heterogeneous population of cells present in primary pituitary cultures. We therefore investigated the ability of an inhibitor of transcription to block the glucocorticoid induction of GHRH-R mRNA *in vitro*. Actinomycin D is a well-characterized inhibitor of gene transcription that acts by intercalating into DNA, thus preventing the progression of RNA polymerase (18). Actinomycin D also can affect protein synthesis by interfering with the association of mRNAs with the ribosome (47). Although there are clearly limitations to using this potent metabolic inhibitor, short duration treatments with actinomycin D can provide important insights into transcriptional *vs.* posttranscriptional regulatory mechanisms. For example, actinomycin D has been used previously in pituitary primary cell cultures to assess hormonal modulation of the gonadotropin subunit mRNAs (48). We found that actinomycin D completely blocked the dexamethasone-induced increase in GHRH-R mRNA levels we had previously observed in cultured pituitary cells, consistent with the hypothesis that glucocorticoids act directly on pituitary cells to stimulate transcription of the GHRH-R gene.

Our studies have clearly defined a mechanism by which glucocorticoids can modify pituitary responsiveness to GHRH released from the hypothalamus, and thus, increase GH synthesis and secretion *in vivo*. Given the positive actions of glucocorticoids on both GHRH-R and GH within the pituitary somatotrope cell, the overall growth suppressive effects of this steroid are indicative of its complex actions at multiple levels of the GH axis. During the preparation of this manuscript, others reported similar effects of glucocorticoids on GHRH-R mRNA levels *in vivo* (49) or *in vitro* (50). Lam and co-workers (49) used RNA blot analysis to demonstrate that pituitary GHRH-R mRNA levels were increased in dexamethasone-treated intact rats, whereas Tamaki and co-workers (50) used a dispersed pituitary cell system to demonstrate increased GHRH-R mRNA levels in response to dexamethasone. Although these recent studies used animal and cell culture models somewhat different than those described here, in general, these findings agree with both our *in vivo* and pituitary cell culture experiments demonstrating that glucocorticoids are important positive regulators of GHRH-R mRNA expression. Our observation that actino-

mycin D can inhibit the ability of dexamethasone to increase GHRH-R mRNA expression further suggests that this is predominantly a transcriptional response and that glucocorticoid receptors are likely to directly interact with the promoter of the GHRH-R gene to stimulate its activity.

The GHRH-R gene has been characterized recently from several species (51–54), and glucocorticoid response elements have been identified in the 5' flanking region of the human GHRH-R gene (53). This observation suggests that glucocorticoids might modulate GHRH-R mRNA expression in the human pituitary gland as they do in the rat, and indeed, the human gene seems to be glucocorticoid-responsive (53). The presence of glucocorticoid response elements in the GHRH-R gene promoter is consistent with a direct interaction of the glucocorticoid receptor with this gene to regulate its transcription. Glucocorticoid receptors have been shown to colocalize in the rat pituitary, predominantly with the corticotropin and GH secreting cells (54) and, therefore, are appropriately localized to mediate transcriptional responses in somatotrope cells. Our studies demonstrating that glucocorticoids stimulate expression of the GHRH-R gene both *in vivo* and *in vitro* provide a likely explanation for the ability of this steroid to enhance pituitary responsiveness to GHRH. To understand the mechanism of glucocorticoid action in this system in more detail will clearly require the identification and characterization of the DNA elements within the GHRH-R gene that mediate glucocorticoid responsiveness.

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