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Homologous Upregulation of Gonadotropin-Releasing Hormone Receptor mRNA Occurs through Transcriptional Activation Rather than Modulation of mRNA Stability

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In a previous study, we showed that even continuous application of gonadotropin-releasing hormone (GnRH) could increase the steady-state levels of GnRH receptor (GnRH-R) mRNA if treated for a relatively short period (6 h). Therefore, in the present study we examined whether GnRH-induced increment of GnRH-R mRNA is owing to stabilization of the preexisting GnRH-R mRNA or new synthesis of GnRH-R mRNA or both. Initially, to examine the effect on new RNA synthesis, the transcription inhibitor, actinomycin D (2 μ*M*), was added to primary cultured rat anterior pituitary cells. In the presence of transcription inhibitor, GnRH-induced augmentation of GnRH-R mRNA levels was completely abolished. This result indicates that homologous upregulation of GnRH-R mRNA expression occurs at least through new RNA synthesis of GnRH-R gene. We further assessed the effects of GnRH on the turnover rate of GnRH-R mRNA using actinomycin D (2 μ*M*). The basal half-life of GnRH-R mRNA was estimated to be approx 21 h. The application of GnRH tended to slightly suppress the basal turnover rate of GnRH; however, there was no statistically significant difference, compared with the group treated with actinomycin D alone. Collectively, our results suggest that the homologous upregulation of GnRH-R mRNA may occur through transcriptional activation of GnRH-R gene rather than enhancement of GnRH-R mRNA stability, although we did not examine the transcription rate of GnRH-R gene.

Key Words: Gonadotropin-releasing hormone receptor; gonadotropin-releasing hormone; anterior pituitary; transcription; stability; rat.

Introduction

Gonadotropin-releasing hormone (GnRH) plays a pivotal role in regulating the reproductive functions. GnRH is released into the hypothalamo-hypophyseal portal vein and binds to its GnRH receptors (GnRH-Rs) on the pituitary gonadotropes to regulate the synthesis and secretion of gonadotropins *(1,2)*. The pituitary GnRH-R mediates the effects of GnRH on the secretion and biosynthesis of gonadotropins, and appears to be associated with the responsiveness of gonadotropes to GnRH *(3–5)*. Thus, the regulation of GnRH-R expression in the pituitary gland is a crucial point in the process of reproduction.

GnRH has been known to regulate its own receptor, and many studies have examined the effects of mode, duration, and doses of GnRH treatment on GnRH-R expression. Especially, cloning of GnRH-R cDNA in several species allowed the investigation of the molecular basis underlying the homologous regulation of GnRH-R gene expression. It is known that low concentrations of GnRH or pulsatile administration of GnRH upregulates the expression of GnRH-R mRNA, whereas high doses of GnRH or continuous GnRH administration downregulates the amount of GnRH-R mRNA expression in the pituitary gland *(6–9)*. However, in a previous study *(10)*, we clearly demonstrated that even continuous application of GnRH is able to upregulate the expression of pituitary GnRH-R mRNA, if treated for a relatively short period (6 h). Therefore, it appears that any modes of GnRH administration for a relatively short period (6 h) upregulate the amount of GnRH-R mRNA in the pituitary gonadotropes. In this event, the alterations in the steady-state levels of a particular mRNA may be owing to the change in the turnover rate of mRNA, or altered tran-

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scription rate of the gene or both. However, the mechanism by which GnRH upregulates the expression of GnRH-R mRNA has not been clearly determined. Therefore, the present study attempted to determine whether GnRH-induced upregulation of GnRH-R mRNA expression occurs through suppression of the turnover rate of GnRH-R mRNA or transcriptional activation of GnRH-R gene. In the present study, pituitary GnRH-R mRNA levels were determined by competitive reverse transcription-polymerase chain reaction (RT-PCR) employed in our previous work *(10)*.

Results

Homologous Upregulation of GnRH-R mRNA May Be Mediated by New RNA Synthesis

For the construction of the standard curve, 100 fg of mutant GnRH-R cRNA was coamplified with different amounts of native GnRH-R cRNA (10, 25, 50, 100, 250, 500, 1000, and 2500 fg). The plot for ratios of native:mutant signals vs amounts of native cRNA revealed a linear relationship (Fig. 1). The regression coefficient (*r*) of the standard curve was above 0.950. GnRH-R mRNA in pituitary total RNA (0.5 μg) competed with 100 fg of mutant GnRH-R cRNA. GnRH-R mRNA levels were calculated based on the standard curve.

To examine whether upregulation of GnRH-R mRNA by GnRH requires new RNA synthesis, pituitary cells were exposed to GnRH in the presence or absence of transcription inhibitor, actinomycin D (2 μ*M*). Actinomycin D alone exerted no significant effect on the basal levels of GnRH-R mRNA (Fig. 2A). Continuous application of GnRH (0.2 n*M*) for 6 h increased pituitary GnRH-R mRNA levels. However, when GnRH was added to pituitary cells together with actinomycin D, the stimulatory effect of GnRH on GnRH-R mRNA levels was completely abolished. On the contrary, luteinizing hormone (LH) release was still significantly increased by the administration of GnRH, in spite of simultaneous application of actinomycin D, although GnRH-induced augmentation of LH release was reduced (Fig. 2B).

Effects of New Protein Synthesis Blockade on GnRH-Induced Increase in GnRH-R mRNA and LH Release

To determine whether homologous upregulation of GnRH-R mRNA requires new protein synthesis, pituitary cells were exposed to GnRH for 6 h in the presence of the protein synthesis inhibitor cycloheximide (CHX) (1 μg/mL). In spite of the presence of CHX, GnRH upregulated pituitary GnRH-R mRNA levels, and any significant alteration was not observed in the stimulatory action of GnRH on GnRH-R mRNA, compared with the group treated with GnRH alone (Fig. 3A). In contrast to GnRH-R mRNA expression, GnRH-induced augmentation of LH release was significantly reduced in the presence of CHX, although GnRH still stimulated LH release significantly (Fig. 3B).

GnRH Does Not Enhance Stability of GnRH-R mRNA

To explore the effect of GnRH on the basal turnover rate of preexisting GnRH-R mRNA, pituitary cells were exposed to actinomycin D (2 μ*M*) in the presence or absence of GnRH (0.2 n*M*) for 6, 12, and 24 h. The decay curves of GnRH-R mRNA in the presence of actinomycin D were obtained from the linear regression analysis. The half-life of GnRH-R mRNA in the absence of GnRH was estimated to be approx 21 h (Fig. 4). When GnRH was added to the cultures, the turnover rate of GnRH-R mRNA tended to be slightly suppressed, but not significantly. In addition, we further delineated the effect of GnRH on the stability of GnRH-R mRNA in cultured pituitary cells. To increase preexisting GnRH-R mRNA levels, all groups were pretreated with GnRH (0.2 n*M*) for 6 h. Thereafter, GnRH-containing medium was washed out, and the cultures were further exposed to actinomycin D (2 μ*M*) in the presence or absence of GnRH (0.2 n*M*) for an additional 6, 12, and 24 h. The decay curves of GnRH-R mRNA were obtained from the linear regression analysis. The administration of GnRH also tended to increase slightly the stability of GnRH-R mRNA, but there was no statistical significance (Fig. 5).

Discussion

In a previous study *(10)*, we demonstrated that continuous GnRH application for a relatively short period (6 h) increased the amount of pituitary GnRH-R mRNA. However, the mechanism by which GnRH upregulates the expression of GnRH-R mRNA has not been determined. Recently, Lin and Conn *(11)* demonstrated that GnRH administration stimulated the promoter activity of GnRH-R gene using the transient transfection system in which GGH3 cells were transfected with GnRH-R-Luc vector (vector containing luciferase reporter gene and promoter fragment of mouse GnRH-R gene). However, there is increasing evidence that hormonal regulation for the stability of preexisting mRNA can affect the amount of mRNA expression of a certain gene, along with its regulation of transcriptional activation. Indeed, Joyeux et al. *(12)* found that in breast cancer cells a progestin-induced rise in the amount of fatty acid synthetase (FAS) mRNA expression was owing to stabilization of the preexisting FAS mRNA as well as transcriptional activation of FAS gene. In addition, Chedrese et al. *(13)* showed that in the αT3 cells GnRH augmented the expression of glycoprotein α-subunit mRNA by both increasing the stability of the mRNA and stimulating the transcriptional activity of the gene. Moreover, in a previous study *(14)*, we demonstrated that progesterone together with estradiol increased the steady-state levels of LH β-subunit mRNA in primary cultured rat pituitary cells by enhancing the stability of LH β-subunit mRNA. Collectively, these studies indicate that the stabil-

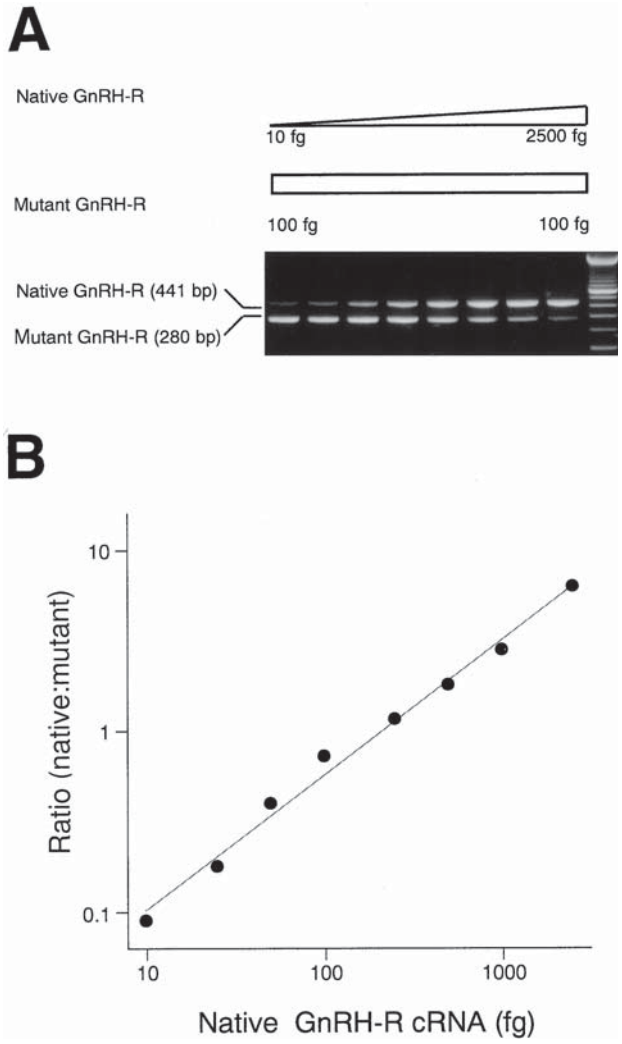


Fig. 1. Standard curve of competitive RT-PCR for quantitation of GnRH-R mRNA levels. A constant amount of mutant GnRH-R cRNA was coamplified with various concentrations of native GnRH-R cRNA. **(A)** PCR product was separated on a 2.0% agarose gel. **(B)** Plot of ratios of native:mutant GnRH-R signals against different amounts of native GnRH-R cRNA revealed a linear relationship. Mutant GnRH-R cRNA (100 fg) was coamplified with 10, 25, 50, 100, 250, 500, 1000, and 2500 fg of native GnRH-R cRNA.

ity of preexisting mRNA is also an important determinant in the regulation of the steady-state levels of certain mRNA. Therefore, the present study examined whether GnRH-induced augmentation of the amount of GnRH-R mRNA is owing to a suppressive effect of GnRH on turnover rate of the preexisting GnRH-R mRNA, activation of GnRH-R gene transcription, or both.

The present study revealed that the application of GnRH tended to increase slightly the basal half-life of GnRH-R mRNA, but the increase was statistically insignificant. The basal half-life of GnRH-R mRNA was estimated to be approx 21 h. Although information about the basal turnover

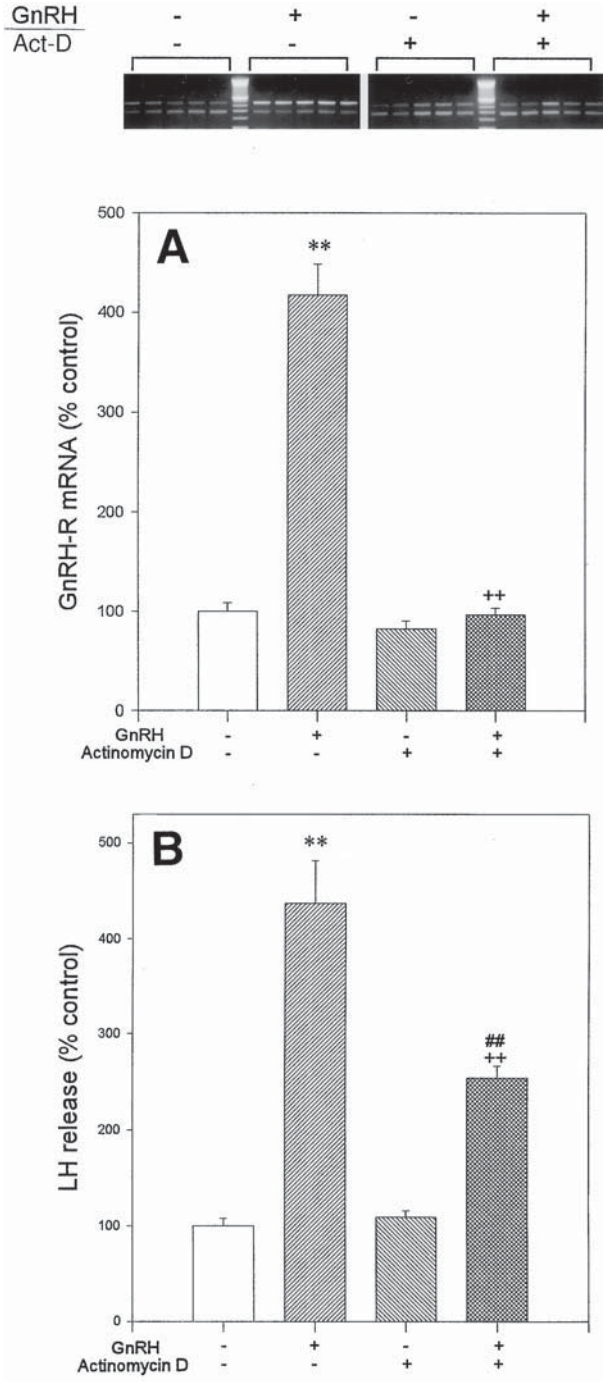
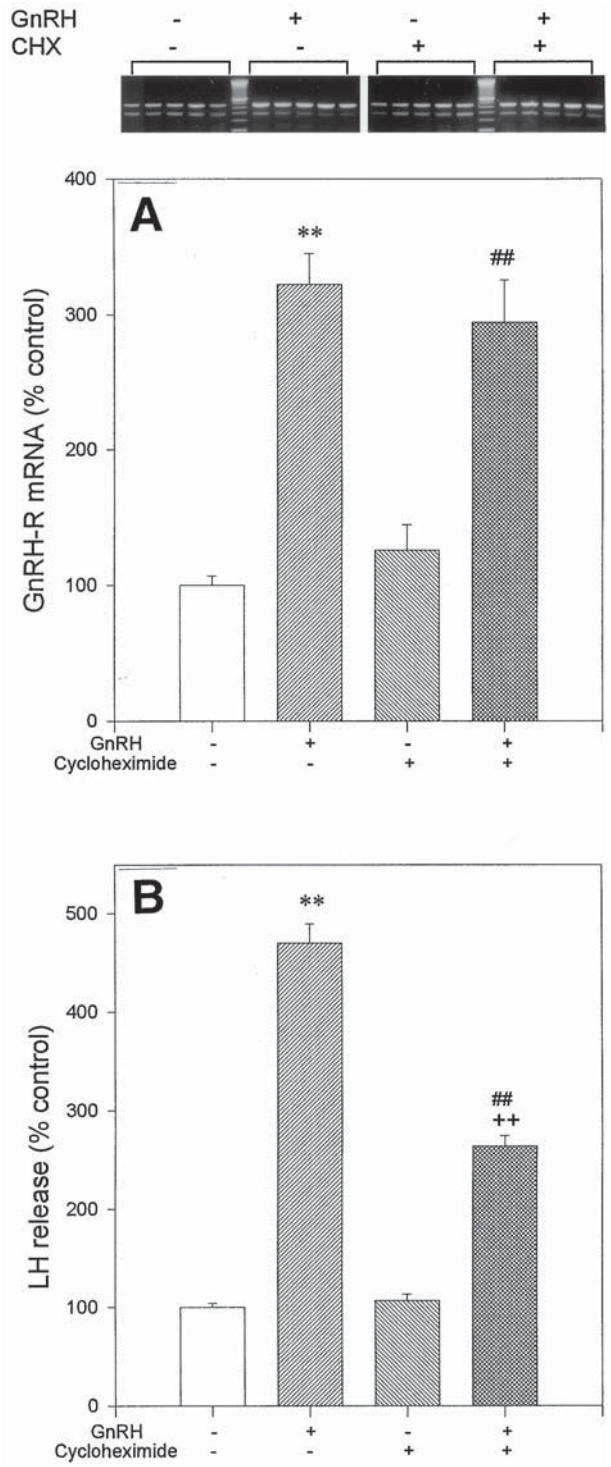


Fig. 2. Effects of transcription blockade on homologous upregulation of GnRH-R mRNA expression **(A)** and GnRH-stimulated LH release **(B)**. Pituitary cells were continuously exposed to GnRH (0.2 n*M*) for 6 h in the absence or presence of transcription blocker, actinomycin D (Act-D) (2 μ*M*). The values of GnRH-R mRNA are expressed as a percentage of the value in control (medium alone) after calculation based on the standard curve. Each accumulated LH release for 6 h is expressed as a percentage of that from control (medium alone). PCR product was separated on a 2.0% agarose gel **(top)**. Each bar represents the mean ± SEM ($n = 14 – 16$ from four independent experiments). $^{**}p < 0.01$ (vs control); $^{\#\#}p < 0.01$ (vs Act-D alone); $^{++}p < 0.01$ (vs GnRH alone).



Sig. 3. Effects of protein synthesis blockade on homologous upregulation of GnRH-R mRNA **(A)** and GnRH-stimulated LH release **(B)**. Pituitary cells were continuously exposed to GnRH (0.2 n*M*) for 6 h in the absence or presence of protein synthesis blocker, CHX (1 μg/mL). The values of GnRH-R mRNA are expressed as a percentage of the value in control (medium alone). Each accumulated LH release for 6 h is expressed as a percentage of that from control (medium alone). PCR product was separated on a 2.0% agarose gel **(top)**. Each bar represents the mean ± SEM (n = 11 to 12 from three independent experiments). $^{**}p < 0.01$ (vs control); $^{\#\#}p < 0.01$ (vs CHX alone); $^{++}p < 0.01$ (vs GnRH alone).

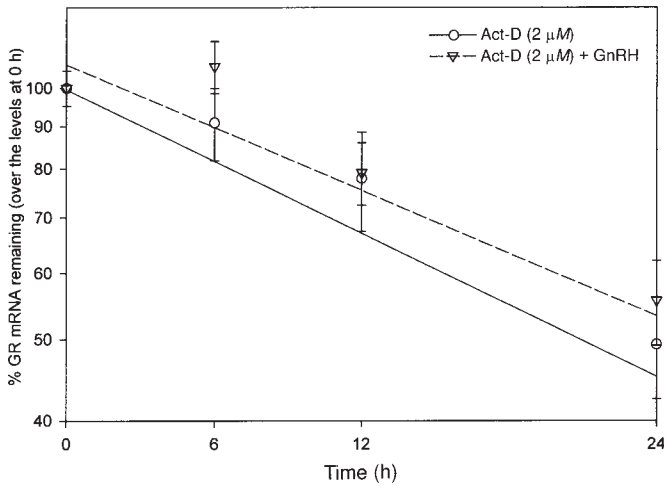


Fig. 4. Effect of GnRH on the basal turnover rates of GnRH-R mRNA. After preculture for 72 h, pituitary cells were exposed to actinomycin D (Act-D) (2 μ*M*) with or without GnRH (0.2 n*M*) for the indicated times. The levels of GnRH-R mRNA are presented on a semilog scale as a percentage of the levels in untreated cells at 0 h. Each bar represents the mean ± SEM (from three independent experiments).

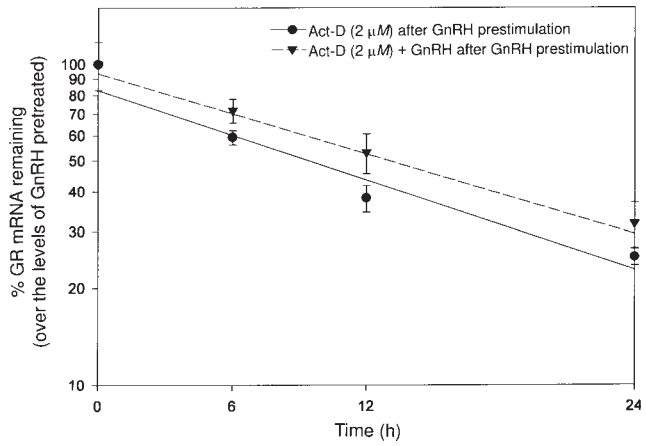


Fig. 5. Effect of GnRH on the turnover rates of GnRH-R mRNA in GnRH-prestimulated cells. After preculture for 72 h, pituitary cells were pretreated with GnRH (0.2 n*M*) for 6 h, and then thoroughly washed out of the medium. Pituitary cells were additionally exposed to actinomycin D (Act-D) (2 μ*M*) with or without GnRH (0.2 n*M*) for the indicated times. The levels of GnRH-R mRNA are presented on a semilog scale as a percentage of the levels in GnRH-pretreated cells for 6 h. Each bar represents the mean ± SEM (from two independent experiments).

rate or the regulation of GnRH-R mRNA stability is very rare, Li et al. *(15)* investigated the basal half-life of GnRH-R mRNA in GT1-7 neurons. They reported that the basal half-life of GnRH-R mRNA is approx 27 h. However, the concentration of actinomycin D (10 μg/mL, approx 8 μ*M*) in their study was four times higher than the dose of actinomycin D (2 μ*M*) in the present study. It is known that there are several methods to determine the basal half-life of cer-

tain mRNAs *(16)*. To delineate the half-life of preexisting mRNA using transcription blockers is the most convenient of several methods in primary cell cultures. However, the use of actinomycin D as transcription inhibitor has some drawbacks. One is that the half-life of certain mRNAs is inversely related to adapted doses of actinomycin D *(17,18)*. Furthermore, Harrold et al. *(16)* pointed out that in order to assess the exact half-life of certain mRNAs, more than two methods must be used simultaneously. Therefore, the half-life of certain mRNAs should be carefully interpreted when actinomycin D is used. Because the main purpose of our study was to delineate the involvement of any change in mRNA stability in the homologous upregulation of GnRH-R mRNA rather than to calculate the exact half-life of GnRH-R mRNA, the use of actinomycin D seemed to be acceptable.

Our results showed that the basal half-life of GnRH-R mRNA was estimated to be approx 21 h and 9 h in GnRH-unstimulated cells and GnRH-prestimulated cells, respectively. However, this does not mean that GnRH destabilizes GnRH-R mRNA. These results indicate that the basal turnover rate of GnRH-R mRNA is accelerated in GnRH-prestimulated cells, compared with GnRH-unstimulated cells. We are not able to explain this acceleration in GnRH-prestimulated cells. Information on the regulation of activity of certain cytoplasmic ribonucleases has not been available until now. Nevertheless, this acceleration of decay rate of GnRH-R mRNA would be secondary to a massive increase in GnRH-R mRNA. If this is the case, it is possible to hypothesize that the excessive augmentation of GnRH-R mRNA levels may accelerate the basal turnover rate of mRNA, to restore the extraordinarily increased GnRH-R mRNA levels to the basal levels required physiologically. However, additional studies are required to understand how GnRH-prestimulation accelerates the basal turnover rate of GnRH-R mRNA.

Actinomycin D is known to prevent mRNA transcripts from properly attaching to the ribosome as well as to block nearly all new RNA synthesis *(16)*. Thus, there is a possibility that actinomycin D might potentially inhibit the synthesis of labile proteins, which may be involved in regulating the steady-state levels of certain mRNA. Indeed, the blockade of new protein synthesis was found to prevent significantly the stimulatory effects of *n*-butyrate on the expression of plasminogen activator inhibitor type 1 mRNA in the Hep G2 cells *(19)*. Furthermore, administration of the protein synthesis inhibitor, CHX, clearly impaired the augmentation of β-casein mRNA levels, which was induced by a combined application of insulin, prolactin, and glucocorticoids in the COMMA D mouse mammary epithelial cell line *(20)*. These studies suggested that inhibition of protein synthesis might disturb the expression of certain mRNAs. Therefore, the possibility cannot be ruled out that the abolishment of homologous upregulation of GnRH-R mRNA expression by actinomycin D may be owing to this potential inhibitory effect of actinomycin D on protein synthesis. Thus, we further delineated whether inhibition of the protein synthesis changes the stimulatory effects of GnRH on GnRH-R mRNA expression. However, homologous upregulation of GnRH-R mRNA expression was not significantly changed by a blockade of new protein synthesis, suggesting that a potential inhibitory effect of actinomycin D on other protein biosynthesis can be ruled out.

In the present study, it was observed that GnRH-induced augmentation of LH release was reduced by the application of CHX or actinomycin D. However, GnRH-induced LH release was still significantly enhanced in spite of blockade of protein synthesis or transcription. It has been demonstrated that the administration of GnRH stimulated the synthesis of both α and LHβ polypeptide *(21–23)*, as well as LH release *(21,24)*. However, the synthesis of LH subunit peptides is not coupled directly to LH release *(25,26)*, as revealed in the present study. In addition, LH release from pituitary cells exposed to GnRH for 6 h as used in the present study reflects release of not only preexisting (stored) LH but also newly synthesized LH *(25)*. Therefore, it is likely that the partial inhibition of GnRH-stimulated LH release by CHX is owing to the fact that the preexisting LH release is not prevented. In addition, the GnRH-stimulated neosynthesis of LH subunit peptides appears to result from the augmentation of subunit mRNA levels *(14,22,23,27,28)* via transcriptional activation *(29–31)* and/or enhancement of mRNA stability *(13)*. Thus, it seems that a reduction in the response of LH release to GnRH by actinomycin D is secondary to the inhibition of transcription of LH subunit genes. However, the potential inhibitory effect of actinomycin D on other protein synthesis cannot be overlooked.

In summary, the present study showed that the blockade of new transcription completely abolished the homologous upregulation of GnRH-R mRNA expression and that the administration of GnRH did not elicit significant suppression of the turnover rate of GnRH-R mRNA. It is therefore concluded that the homologous upregulation of GnRH-R mRNA expression may occur through transcriptional activation of GnRH-R gene rather than enhancing the stability of preexisting GnRH-R mRNA.

Materials and Methods

Preparation of Anterior Pituitary Cell Cultures

Pituitary glands from female Sprague-Dawley rats (150–200 g; Yuhan Research Center, Korea) at random stages of the estrous cycle were used for the preparation of cell cultures, because there was no significant difference in the GnRH-induced increase in GnRH-R mRNA levels in primary cultures between the pituitary glands from estrous rats and those from random cycling rats. Anterior pituitary cells were prepared by enzymatic dispersion, with modifi-

cations to previously described methods *(32)*. Following decapitation, the anterior pituitary glands were removed and washed in Spinner's minimal essential medium (S-MEM) (Gibco-BRL, Gaithersburg, MD) containing 0.3% bovine serum albumin (BSA) (fraction V) (Sigma, St. Louis, MO) and 10 m*M* HEPES (Sigma). Anterior lobes were cut into several pieces in S-MEM/BSA and then enzymatically digested with 20 mL of S-MEM/BSA containing 0.25% trypsin (1:250) (Difco, Detroit, MI) and DNase (10 μg/gland) (Sigma) for 1 h at 4°C and a further 30 min at 37°C.

Dispersion was facilitated by repeated aspiration and expulsion of the tissue fragments with a fire-polished Pasteur pipet. Dispersed cells were then centrifuged at 400*g* for 10 min. The pellet was resuspended in 20 mL of S-MEM/BSA containing trypsin inhibitor (5 μg/mL) (Sigma) and filtered through lens paper to remove residual tissue fragments. The cell suspension was briefly centrifuged, and the cell pellet was resuspended in α-minimal essential medium (α-MEM) (Gibco-BRL), containing 2.5% fetal bovine serum (Gibco-BRL), 10% horse serum (Gibco-BRL), and antibiotics (100 U of penicillin and 100 mg of streptomycin/mL) (Sigma). All sera were dextran-charcoal stripped for the removal of residual steroids. The cells were more than 95% viable, as measured by trypan blue exclusion. Aliquots of these cells (1×10^6 cells/mL) were incubated in multiwell culture plates (Falcon) in 5% CO_2/air at 37°C. Following preincubation, the cells were washed twice with Dulbecco's phosphate-buffered saline (Sigma) to remove serum and nonadherent cells, and thereafter they were further incubated in serum-free medium (α-MEM) containing 0.3% BSA for experiments.

Total RNA Extraction

Total cytoplasmic RNA from the pituitary was extracted by the acid guanidinium thiocyanate-phenol-chloroform method *(33)*. Briefly, 600 μL of denaturing solution containing 4 *M* guanidinium thiocyanate, 25 m*M* sodium citrate (pH 7.0), 0.5% *N*-lauroyl sarcosine, and 0.1 *M* 2-mercaptoethanol was added to each well and collected in a microcentrifuge tube. Sixty microliters of 2 *M* sodium acetate (pH 4.0), 600 μL of water-saturated phenol, and 120 μL of chloroform:isoamyl alcohol mixture (49:1) were added. After cooling on ice for 15 min, samples were centrifuged (10,000*g*) at 4°C for 20 min and precipitated with ethanol. After washing with 75% ethanol, the RNA pellet was dried under a vacuum and dissolved in 20 μL of sterilized distilled water. RNA content was then quantified at an absorbance of A_{260}.

Competitive RT-PCR

Competitive RT-PCR to determine GnRH-R mRNA levels was performed as previously described *(10,34)*. GnRH-R primers were synthesized based on the sequence of the rat GnRH-R cDNA *(35)*. The upstream primer was 5'-CTTGAAGCCCGTCCTTGGAGAAAT-3' and the downstream primer is 5'GCGATCCAGGCTAATCAC CACCAT-3'. Primers were designed such that the predicted sizes of PCR products were 441 and 280 bp for native and mutant GnRH-R, respectively. After linearization of plasmids containing native and mutant GnRH-R cDNA, native and mutant GnRH-R cRNAs were synthesized by T7 RNA polymerase using an in vitro transcription system kit from Promega (Madison, WI). Concentrations of native and mutant GnRH-R cRNAs were measured with an ultraviolet (UV) spectrophotometer at A_{260}.

Briefly, native cRNA templates or GnRH-R mRNA in pituitary total RNA and a fixed amount of mutant cRNA template were coreverse transcribed by 200 U of RNaseH$^-$ Moloney murine leukemia virus RT (Gibco-BRL). Subsequently, the PCR reaction mixture containing 50 pmol of up- and downstream primers and 2.5 U of *Taq* DNA polymerase (Promega) was added. When native and mutant cDNAs are coamplified in the same tube, the sequence homology between them may cause hybrids of native and mutant DNA at the annealing temperature *(36)*. Therefore, PCR amplification was carried out with a two-step procedure to reduce hybrids (step 1: denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min, and primer extension at 72°C for 2 min; step 2: denaturation at 85°C for 1 min, primer annealing at 60°C for 1 min, and primer extension at 72°C for 2 min). To determine GnRH-R mRNA levels, 33 cycles of step 1 and 6 cycles of step 2 were used. Ten-microliter aliquots of PCR products were electrophoresed on a 2.0% agarose gel in TAE buffer, stained with ethidium bromide, and photographed under UV illumination with Polaroid 665-type negative and positive films (Polaroid, Cambridge, MA). Negative film was used for densitometric scanning of native and mutant signals.

Radioimmunoassay of LH

LH was measured by a double-antibody radioimmunoassay using reagents kindly provided by the National Pituitary Agency/NIDDK. Tracer (rLH-I-9) was iodinated by chloramine T. Radiolabeled tracer (^{125}I-rLH) was separated by gel permeation chromatography with Sephadex G-50. The antisera (rLH-S-10) and the reference preparations (rLH-RP-2) were dissolved in 0.01 *M* phosphate buffer containing 0.1% (w/v) BSA. The antisera were diluted from the stock solutions (1:18.75), so that the final tube dilution was 1:135,000. To measure LH concentration, radiolabeled tracer (10,000–15,000 cpm/100 μL), antisera (100 μL; 1:45,000), and unknown samples (100 μL) were incubated for 3 h at 37°C. The antigen–antibody complex was precipitated by adding 0.1 mL of EDTA, 0.1 mL of normal rabbit serum (2%), 0.1 mL of antirabbit IgG (Sigma), and 0.6 mL of polyethylene glycol (mol wt 8000). After centrifugation at 3000*g* for 20 min, the supernatant was discarded and the radioactivity present in the precipitate was counted in a gamma counter (Packard, Meriden, CT).

Assay sensitivity was 0.1 ng/mL. The intra- and interassay coefficients of variation were 6.8 and 9.2%, respectively.

Data Analysis

GnRH-R signals on negative film were measured with a densitometric scanner (Hoefer, San Francisco, CA). The amounts of GnRH-R mRNA were calculated from the native:mutant ratio using a standard curve. Data was statistically evaluated using one-way analysis of variance, followed by Fisher's least significant difference test for a post-hoc comparison. The slope of the decay curves in the presence of actinomycin D was obtained from the linear regression analysis and compared using the student's *t*-test. All values are given as the means ± SEM. Statistical significance was set at $p < 0.05$.

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