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Expression and Regulation of Gonadotropin-Releasing Hormone (GnRH) and Its Receptor mRNA Transcripts During the Mouse Ovarian Development

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The present study examines the expression and regulation of gonadotropin-releasing hormone (GnRH) and its receptor (GnRH-R) mRNA levels during mouse ovarian development. A fully processed, mature GnRH mRNA together with intron-containing primary transcripts was expressed in the immature mouse ovary as determined by Northern blot analysis and reverse transcription-polymerase chain reaction (RT-PCR). The size of ovarian GnRH mRNA was similar to that of hypothalamus, but its amount was much lower than that in the hypothalamus. Quantitative RT-PCR procedure also revealed the expression of GnRH-R mRNA in the ovary, but the estimated amount was a thousand-fold lower than that in the pituitary gland. We also examined the regulation of ovarian GnRH and GnRH-R mRNA levels during the follicular development induced by pregnant mare's serum gonadotropin (PMSG) and/or human chorionic gonadotropin (hCG). Ovarian luteinizing hormone receptor (LH-R) mRNA was abruptly increased at 48 h after the PMSG administration and rapidly decreased to the basal level thereafter. Ovarian GnRH mRNA level was slightly decreased at 48 h after the PMSG administration, and then returned to the basal value. GnRH-R mRNA level began to increase at 24 h after the PMSG treatment, decreased below the uninduced basal level at 48 h, and gradually increased thereafter. HCG administration did not alter ovarian GnRH mRNA level, while it blocked the PMSG-induced increase in GnRH mRNA level. Taken together, the present study demonstrates that the expression of GnRH and GnRH-R mRNA are regulated by gonadotropin during follicular development, suggesting possible intragonadal paracrine roles of GnRH and GnRH-R in the mouse ovarian development.

Gonadotropin-releasing hormone (GnRH), which is a decapeptide released from hypothalamic neurons, regulates the synthesis and release of gonadotropin from the anterior pituitary by binding to its specific GnRH receptor (GnRH-R; Stojilkovic et al., 1994). In addition to this neuroendocrine function, extrapituitary action of GnRH has been implicated in the regulation of reproductive events, such as steroid synthesis (Clayton, 1989; Peng et al., 1994), oocyte maturation (Hillensjo and LeMaire, 1980; Yoshimura et al., 1991), ovulation (Ekholm et al., 1981), follicular atretic processes (Billig et al., 1994), and growth of placenta (Wolfahrt et al., 2001).

GnRH-R is a G protein-associated, membrane-bound glycoprotein with a molecular mass of 50-60 kDa, and is characterized by the absence of carboxy-terminal cytoplasmic domain involved in the desensitization and internalization (Clayton, 1989; Stojilkovic et al., 1994). In accordance with the suggested extrapituitary action of GnRH, GnRH-R transcripts are expressed in the ovarian cells (Peng et al., 1991; Minaretzis et al., 1995; Whitelaw et al., 1995). Bauer-Dantoin and Jameson (1995) reported the localization and quantitative changes of GnRH-R mRNA transcripts in the rat ovary during the estrus cycle. Recently, Park et al. (2001) demonstrated the negative regulation of GnRH-R mRNA expression by FSH through a cAMP-protein kinase A pathway in the rat granulosa cells. Although previous results indicate the local functions of GnRH in the ovary, its precise mechanism of regulation is not completely resolved. In fact, whether mature GnRH mRNA is expressed in the mouse ovary remains still unclear. In this study, we demonstrate functional GnRH and GnRH-R transcripts in the mouse ovary. Moreover,

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we examined the regulation of GnRH and GnRH-R mRNA levels in an immature mouse ovary in which follicular development is induced by pregnant mare's serum gonadotropin (PMSG) and/or human chorionic gonadotropin (hCG) administration.

Material and Methods

Animals

Immature ICR mice (21 d old) were obtained from the Animal Breeding Center at Seoul National University and maintained under 14 h light and 10 h dark photocycle (light on at 06:00) with water and food supplied *ad libitum*. To induce follicle development, immature mice were intraperitoneally injected with 5 I.U. pregnant mare's serum gonadotropin (PMSG). HCG (5 I.U.) was administered to the PMSG-primed mice at 48 h after PMSG injection.

Total RNA preparation

Total RNAs were isolated by the acid guanidium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). The tissues were homogenized with a tissue homogenizer in 600 µl of solution D (4 M guanidium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% N-lauryl sarcosine, and 0.1 M 2-mercaptoethanol) on ice. Subsequently, 0.1 volume of 2 M sodium acetate (pH 4.0), one volume of water-saturated phenol, and 0.2 volume of chloroform-isoamyl alcohol (49:1) were added to the homogenate. After thorough mixing by vigorous vortexing, the mixture was incubated on ice for 10 min. Total RNA was then fractionated by centrifugation at 12,000 rpm for 30 min at 4℃ and precipitated from supernatant in the presence of one volume of isopropanol. The pellet was washed twice with 75% ethanol, and dissolved in distilled water. Unless otherwise mentioned, diethyl pyrocarbonate-treated water was used in all experiments. Total RNA concentration was assessed by absorbance at 260 nm.

Northern blot analysis

Total RNAs (10 µg) were isolated from various tissues of immature mice, denatured in the presence of 50% formamide, 2.2 M formaldehyde, 20 mM MOPS (3-[N-morpholino] propansulfonic acid), 4 mM sodium acetate, and 0.5 mM EDTA at 65℃ for 10 min. After electrophoresis on a 1.2% agarose gel containing 2.2 M formaldehyde, RNA was transferred onto nylon membrane (Nytran, 0.45 µm pore size; Schleicher and Schuell) by capillary action under 10x SSPE (1x SSPE: 0.18 M NaCl, 10 mM Na_2HPO_4, pH 7.7, and 1 mM EDTA). RNA transfer and loading efficiency were estimated by staining the extra membrane in 0.1% methylene blue. RNA intactness was estimated by comparing the band intensity between the 28 S and 18 S ribosomal RNAs. Membrane was washed with 6x SSPE for 5 min, air-dried and RNA was permanently attached to the membrane by UV illumination for 1 min. Hybridization was performed at 42℃ overnight in a heat-sealable polyethylene bag containing 40 ml of hybridization buffer (5x SSPE, pH 7.4, 50% SDS, 0.2 mg/ml heat-denaturated salmon sperm DNA, and 50% formamide) and hybridization probes. ^{32}P-labeled antisense RNA transcripts (5×10^8 cpm/µg RNA) of mouse GnRH and GnRH-R were used as hybridization probes. The membranes were then exposed to X-ray film at -70℃ for 1 wk. To control the loading efficiency, the membranes were rehybridized with [^{32}P]-labeled cDNA for the 18 S ribosomal RNA.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA (1 µg) isolated from the various tissues were denatured in the presence of 100 pmol random hexanucleotide in a final volume of 10.75 µl at 75℃ for 10 min (Shim et al., 1997). After brief centrifugation at 4℃, 9.25 µl of master mix [200 U RNase H-MMLV reverse transcriptase, 4 µl dNTP mix (2.5 mM each), 0.25 µl RNasin (26 U/µl), and 4 µl of 5 x RT buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, and 10 mM dithiothreitol)] were added, and further incubated at 37℃ for 1 h. Temperature was raised to 95℃ for 5 min to terminate the reaction. The PCR amplification was carried out with 2 µl of RT reaction mixture in 40 µl of PCR reaction solution containing 4 µl of 10x PCR buffer (supplied by Perkin Elmer Cetus), 3.2 µl dNTP mix (2.5 mM each), and 1 U Ampli-Taq polymerase (Perkin Elmer Cetus). In each reaction, 10 pmol of PCR primers were added. The sample was then overlaid with 40 µl of mineral oil and subjected to amplification on a PCR cycler (Pharmacia LKB, Gene ATAQ controller). The PCR was performed under following parameters; 94℃ for 1 min, 60℃ for 1 min, and 72℃ for 1.5 min for 32 cycles. The final extension was performed at 72℃ for 10 min, and second amplification step (85℃ for 1 min, 60℃ for 1 min, and 72℃ for 1.5 min for 5 cycles) was included to minimize native/mutant heteroduplex formation (Shim et al., 1997). Six µl of PCR products were analyzed on a 3% agarose gel. Gels were then stained with ethidium bromide and photographed under UV illumination. For the evaluation of luteinizing hormone receptor (LH-R) mRNA level in the mouse ovary, co-amplification of RT-PCR with β-actin as an internal control was performed as described previously (Shim et al., 1996). The PCR primers used in this study were designed on the bases of cDNA sequences for the mouse GnRH, GnRH-R, and rat LH-R (Mason et al., 1986; Reinhart et al., 1992; Tsai-Morris et al., 1991). Southern blot hybridization was performed according to the method of Sambrook et al. (1989). Briefly, the PCR-amplified cDNAs on the stained gel were blotted onto the Nytran membrane. The membrane was then washed with 6x SSPE,

air-dried, and DNA was permanently attached by UV illumination. Hybridization was performed as described in Northern blot analysis.

Competitive RT-PCR

The strategy for construction of the truncated mutant cDNA and synthesis of mutant cRNA transcripts were the same as previous described (Shim et al., 1997). First, we determined the concentrations of GnRH and GnRH-R transcripts present in the defined RNA mass that was isolated from hypothalamus, pituitary, and ovary. Then, mutant cRNAs (serially diluted from 10 fg to 10 pg) were externally added into total RNA samples (1 μg) before RT reaction and PCR amplification was performed in the presence of specific primers (Fig. 3A). The PCR reaction produced two amplified products for native and mutant transcripts: for GnRH, 396 and 297 bp; for GnRH-R, 441 and 280 bp, respectively. After electrophoresis on a 2% agarose gel, we calculated the relative densitometric ratios of the native to mutant PCR products. Estimated levels of GnRH transcripts present in one μg of total RNA were 0.2 pg in immature mouse ovary, and 2 pg in the hypothalamus, respectively. In pituitary gland, GnRH mRNA was not detectable by RT-PCR and only the mutant bands were visible on agarose gel. In contrast, GnRH-R mRNA that was expressed in all tissues tested was detectable at the range of 1 fg to 10 pg mutant concentrations. An RT-PCR amplification was performed with serially diluted total RNAs (0.125-5 μg) isolated from immature ovaries in the presence of 2 pg GnRH and 30 fg GnRH-R mutant cRNAs. Relative densities between native and mutant bands were calculated and expressed as a function of RNA input.

Data analysis

Signals for GnRH and GnRH-R mRNA on photographs obtained by competitive RT-PCR were analyzed by densitometric scanning (Hoefer Scientific Instruments). The data (mean ± SEM) were evaluated using one-way analysis of variance (ANOVA) followed by Fisher's least difference test for a post-hoc comparison. Statistical significance was set at $P < 0.05$.

Results

Expression of GnRH mRNA transcripts in the mouse ovary

To determine whether GnRH gene is expressed in the mouse ovary, GnRH and GnRH-R mRNA levels were examined by Northern blot analysis and RT-PCR. Total RNAs were isolated from various tissues, including hypothalamus, pituitary, and ovary of sexually immature female mice and subjected to Northern blot hybridization with ^{32}P-labeled antisense cRNA probes specific to the mouse GnRH and GnRH-R mRNAs. Northern blot analysis revealed the discrete signals for GnRH mRNA transcript (0.7 kb in size) in the ovary and hypothalamus, but not in the pituitary gland, and other tissues examined (Fig. 1A). To further analyze the GnRH transcripts by RT-PCR, several sets of primers were designed as shown in Fig. 2A. We were able to detect the primary GnRH transcripts as well as the fully processed, mature transcript using various combinations of these primers. Mature GnRH mRNA without intron sequences was detected only in the hypothalamus and ovary (Fig. 1B, Fig. 2B). Intermediate transcript containing the first intron sequence was seen in most of the tissues tested, such as hypothalamus, pituitary, brain cortex, skeletal muscle, and ovary (Fig.

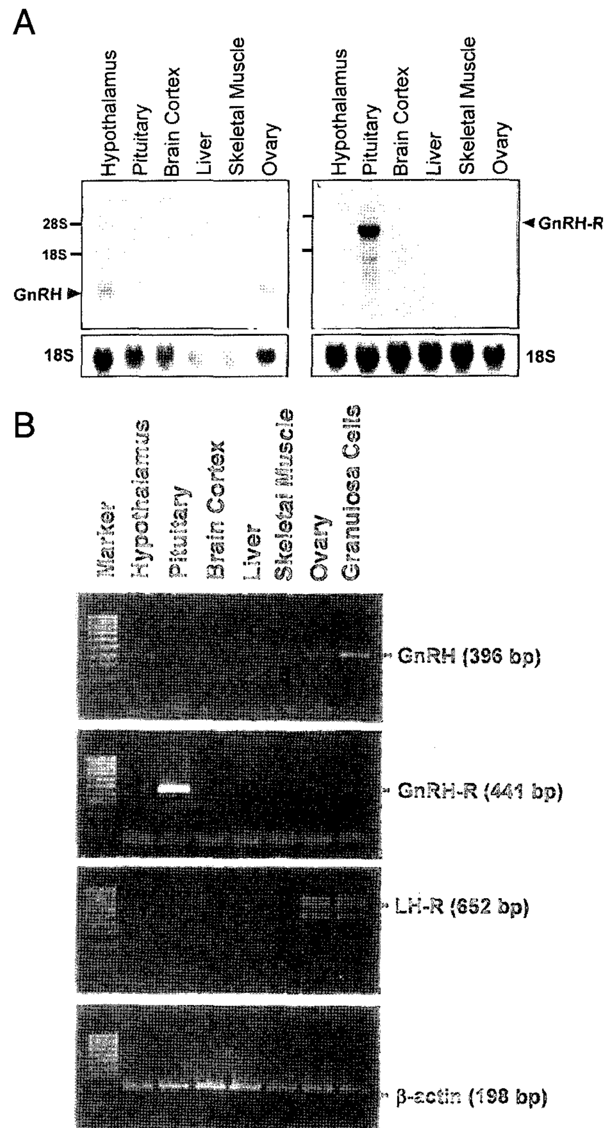
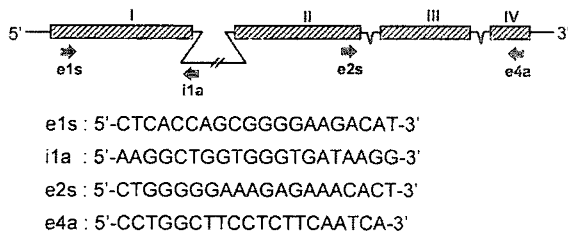
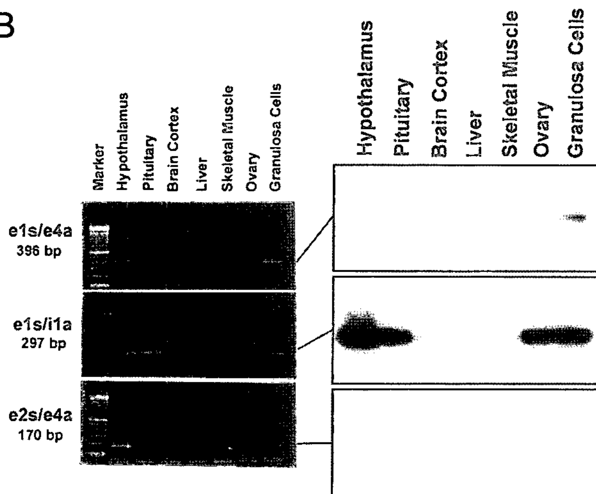


Fig. 1. A, Northern blot analysis of GnRH and GnRH-R mRNA transcripts in the mouse ovary. B, RT-PCR analysis for GnRH, GnRH-R, and LH-R mRNAs. The β-actin was used as a positive control and an 100 bp ladder marker was used as a size standard. The sizes of amplified products are indicated in parentheses.





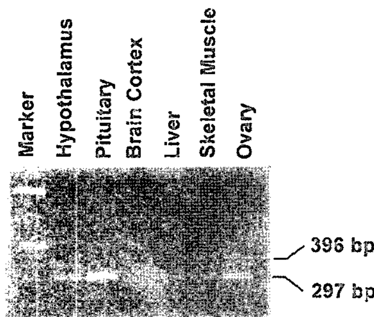


Fig. 2. Characterization of ovarian GnRH transcripts using an RT-PCR. A, The structure of mouse GnRH transcripts. Shaded box represents the exons, and solid line represents the 5'- or 3'-untranslated region and introns. Locations of RT-PCR primers are indicated by arrows, and nucleotide sequences of each primer used in the RT-PCR amplification are listed below. B, The results of RT-PCR amplification (left panels) and subsequent Southern hybridization (right panels) of GnRH transcripts. Specific primer sets used in the PCR amplification and the expected sizes of PCR products are indicated at the left side of each panel. C, Relative ratio of mature GnRH transcripts to intron-containing primary transcripts in the various tissues. RT-PCR was performed in the presence of three primers, e1s, i1a, and e4a in one tube, producing two PCR amplimers (396 bp and 297 bp products for mature transcript and primary transcript, respectively). An 100 bp ladder maker was used as a size standard.

2B). LH-R mRNA was expressed only in the ovary and granulosa cells as shown in Fig. 1B. Our PCR primers for LH-R were designed to detect a 652 bp PCR product, but two additional bands were also observed. This result might be due to the presence of incomplete, possibly inactive forms of LH-R transcripts in the ovary as suggested by others (Aatsinki et al., 1992; Bacich et al., 1994).

Expression of GnRH-R mRNA transcripts in the mouse ovary

Northern blot analysis detected two GnRH-R mRNA transcripts (about 1.5 and 4 kb in size) in the pituitary gland, but not in the ovary as shown in Fig. 1A. Although we failed to detect discrete signals for GnRH-R mRNA in the ovary by Northern blot analysis, RT-PCR procedure clearly demonstrated the PCR product (441 bp) of GnRH-R mRNA in the granulosa cells isolated from the immature mouse ovary (Fig. 1B). Quantification of GnRH-R mRNA concentration using a competitive RT-PCR revealed that the ovary GnRH-R mRNA level was about a thousand-fold lower than that seen in the pituitary (Fig. 3A).

Regulation of GnRH and GnRH-R mRNA expression during mouse ovarian development

To precisely analyze the regulation of GnRH and GnRH-R mRNA expression during the mouse folliculogenesis, we adopted a competitive RT-PCR procedure. Fig. 3A shows that 0.1 pg, 10 pg, and 30 fg of GnRH-R mRNA per 1 µg total RNA were present in the mouse hypothalamus, pituitary, and ovary, respectively. Fig. 3B and C represent standard curves for quantification of GnRH and GnRH-R mRNAs in the mouse ovary. An immature female mouse model was used for this study. PMSG was intraperitoneally injected into an immature female mouse to induce follicular development. At the indicated time points after the hormone treatment, ovaries were dissected and the expression levels of GnRH and GnRH-R mRNA were analyzed by competitive RT-PCR (Fig. 4A and Fig. 4B). We also tested if PMSG administration properly induced follicular development and subsequent ovulation either by counting the ovulated eggs in the morning of day 3 after hormone treatment (data not shown; Hogan et al., 1994), or by examining the expression levels of LH-R mRNA (Fig. 4C). LH-R mRNA level was increased 24 h after the PMSG treatment, peaked at 48 h and rapidly decreased to the basal level thereafter (Fig. 4). In contrast, injection of saline (vehicle injection control) failed to induce LH-R mRNA (data not shown). Treatment with hCG resulted in a decrease in PMSG-induced LH-R mRNA level. Although the decreased level was sustained by 24 h after hCG treatment, it exceeded the control level by several fold at 48 h. As shown in Fig. 4A, the PMSG treatment did not exert any notable effect on the GnRH mRNA level for up to 44 h after the treatment. GnRH mRNA level, however, decreased by two-fold at 48 h, which was recovered to the untreated level at 96 h. hCG treatment at 48 h after the PMSG administration blocked the PMSG-induced decrease in GnRH mRNA level. The decrease in the mature mRNA transcript (395 bp) relative to the first

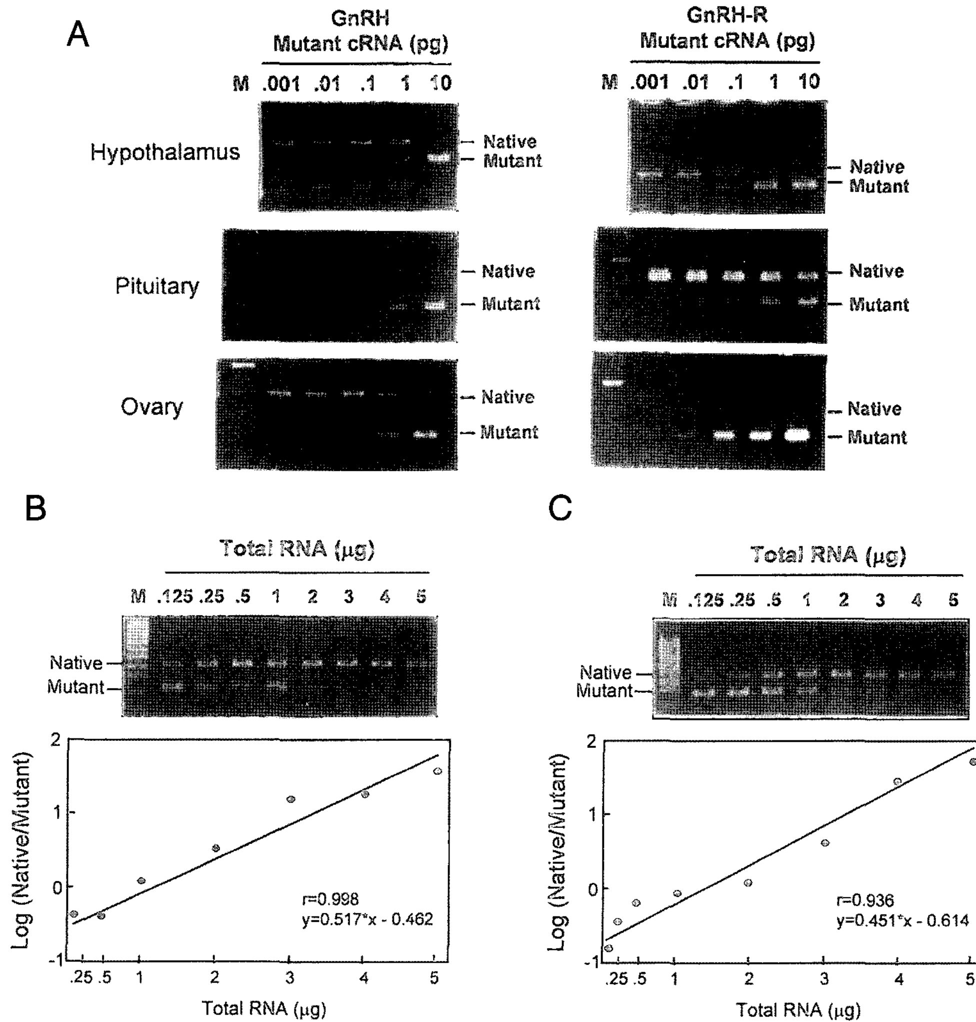


Fig. 3. A, Quantitative analysis of GnRH and GnRH-R mRNA levels in the mouse hypothalamus, pituitary, and ovary using a competitive RT-PCR procedure. B and C, The evaluation of competitive RT-PCR procedure by constructing the standard curves for GnRH (B) and GnRH-R (C) as a function of mRNA input.

intron-containing primary transcript (297 bp) shown in Fig. 5 appeared to be responsible for the PMSG-induced decrease in GnRH mRNA level. As shown in Fig. 4B, GnRH-R mRNA level was increased at early time points by 44 h after the PMSG administration, and then rapidly decreased to the uninduced level and thereafter exceeded the uninduced level by several fold at 96 h, while the hCG treatment completely suppressed this increase to the undetectable level.

Discussion

In the present study, the presence of GnRH and GnRH-R transcripts in the mouse ovary were evidently demonstrated by Northern blot and competitive RT-PCR. The presence of GnRH transcript containing the first intron sequence in most of the tissues tested is consistent with the previous report (Kakar and Jennes, 1995). Furthermore, Goubau et al. (1992) reported that the major GnRH transcript from rat granulosa cells retained the intronic sequences, while the minor GnRH transcript was a genuine GnRH transcript as found in the hypothalamus. The major GnRH transcripts detected by Goubau et al. (1992) consisted of exons 2, 3, and 4, introns B and C plus an unidentified sequence, presumably a short sequence of the first intron. Recent report by Seong et al. (1999) demonstrated that the GnRH primary transcripts are more prevalent than mature GnRH mRNA in non-GnRH producing tissues, such as ovarian cells. They claimed that the extremely low level of mature GnRH mRNA, as compared with GnRH the primary transcripts, is most likely due to the intrinsic weakness of the intron A splice sites. Our results, together with others, suggest that the primary GnRH transcripts are present in the mouse ovary/ granulosa cells and that they may retain intron A, which was not spliced because of the intrinsic weakness of its splice site.

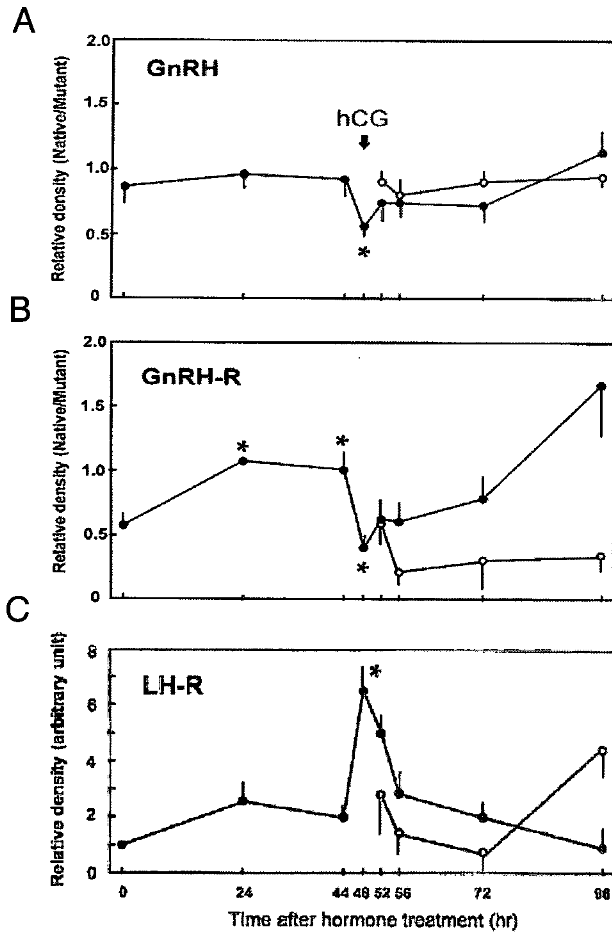


Fig. 4. Changes in the GnRH (A), GnRH-R (B), and LH-R (C) mRNA levels during the follicular development induced by PMSG/hCG administration in the immature mouse ovary. Animals were intraperitoneally injected with PMSG (5 I.U.), and sacrificed at indicated time points (closed circle). HCG (5 I.U.) was administered to PMSG-primed mice at 48 h after PMSG injection (open circle). Total RNAs were isolated and competitive RT-PCR was performed in the presence of mutant GnRH and GnRH-R cRNAs as described in Fig. 3. Data (mean ± SEM of 7-9 independent determinations) are presented as a relative density between native and mutant signals. For LH-R mRNA levels, co-amplification of LH-R with β-actin as an internal control was performed, and signals for LH-R were compensated with β-actin signals. Results are presented as a mean ± SEM of five independent experiments. The RNA level at 0 h sets 1. * $P < 0.05$.

There are two GnRH-R transcripts (about 1.5 and 4 kb in size) in the mouse pituitary gland, but not in the ovary as shown in Fig. 1A. This result is consistent with the previous report showing that alpha T3 cells and mouse pituitary gland expressed two GnRH-R transcripts (1.6 and 3.5 kb; Reinhart et al., 1992). In contrast, the rat pituitary gland and ovary expressed a single 4.6 kb transcript for GnRH-R (Reinhart et al., 1992). Although we failed to detect discrete signals for GnRH-R mRNA in the mouse ovary by Northern blot analysis, RT-PCR analysis clearly demonstrated the GnRH-R mRNA in the granulosa cells isolated from the immature mouse ovary (Fig. 2B). Quantification of GnRH-R mRNA levels using competitive RT-PCR revealed that the mouse ovary contained about a

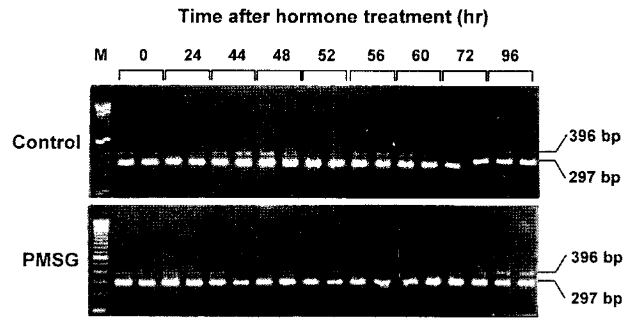


Fig.5. Time-dependent changes of mature GnRH mRNA level relative to the primary transcript after PMSG treatment. Total RNAs were isolated from the ovaries of mice injected with saline (the control group) or PMSG at indicated time points, and RT-PCR was performed as described in Fig. 2B to detect mature GnRH transcript (396 bp) as well as primary transcript (297 bp) in a single reaction. The results are from independent RNA samples of duplicate experiments.

thousand-fold lower amount of GnRH-R mRNA than the pituitary did (Fig. 3A). This result partly explained why we could not detect GnRH-R mRNA by Northern hybridization. In the human ovary, GnRH-R mRNA level is about 200-fold lower than that in the pituitary (Minaretzis et al., 1995). Taken together, these results clearly demonstrate that GnRH and GnRH-R mRNA are expressed in the mouse ovary. Moreover, this is the first time to report the presence of fully processed, mature GnRH transcript in the mouse ovary, which is same as that found in the hypothalamus. Although several reports previously suggested the presence of GnRH transcript in rat by *in situ* hybridization, RT-PCR, and Northern blot analysis (Oikawa et al., 1990; Clayton et al., 1992; Goubau et al., 1992; Peng et al., 1994), it was a primary GnRH transcript different in size from that in the hypothalamus, containing intron sequences. Therefore, it has long been hypothesized that the GnRH transcripts found in extrahypothalamic regions, including the ovary and testis, may differ from hypothalamic one in the structure as well as in the biological functions (Goubau et al., 1992; Peng et al., 1994; Kakar and Jennes, 1995). The failure to detect mature GnRH mRNA in the earlier studies is likely due to the use of adult ovaries as the RNA source rather than immature ovaries. In fact, we also failed to detect mature GnRH mRNA in the ovaries from adult mice by Northern hybridization (data not shown).

The possible roles of GnRH and GnRH-R mRNA expression in ovarian development were examined using an immature female mouse model where PMSG injected into an immature female mice could induce follicular development. As shown in Fig. 4C, LH-R mRNA level peaked at 48 h after the PMSG injection, which could be decreased by hCG treatment. Although the decreased LH-R mRNA level was sustained by 24 h after the hCG treatment, it exceeded the control level by several fold at 48 h. Our results were highly consistent with the previous report showing gonadotropin-induced changes of LH-R mRNA expression in the rat

ovary (Nakamura et al., 1991; Camp et al., 1991; Peng et al., 1991), indicating that our hormone treatment strategy properly induced follicle development in the immature female mice. The disappearance of mature GnRH mRNA transcript relative to the first intron-containing primary transcript (Fig. 5) may be responsible for the PMSG-induced decrease in GnRH mRNA level (Fig. 4A). However, it is uncertain whether it is the result of transcriptional turn-off or altered processing of the primary transcripts. Definitive roles of ovarian GnRH and GnRH-R expression on follicle development still remain controversial, probably due to pleiotrophic actions by GnRH as suggested previously. Recently, Bauer-Dantoin and Jameson (1995) found significant changes in GnRH-R mRNA expression only in two types of follicles, corpora lutea and atretic follicles during the 4-d estrus cycle of the rats, suggesting involvement of GnRH in the atretic process in vivo. Our results, in part, support this hypothesis. It is plausible to assume that a rapid decrease in both GnRH and GnRH-R mRNA levels at 48 h after the PMSG treatment may be correlated with the onset of ovulatory follicles and removal of atretic follicle from the ovary at this time. In fact, GnRH-R mRNA was decreased in the preovulatory follicles that normally express the gonadotropin receptors (Camp et al., 1991). It is worth mentioning that LH-R mRNA was at the highest level when either GnRH or GnRH-R mRNA was decreased to the lowest level (Fig. 4C). Whether GnRH and GnRH-R expression are actively involved in the atretic process remains to be determined.

In conclusion, our study suggests that the expression of GnRH and GnRH-R mRNAs in the mouse ovary is regulated by gonadotropin during follicular development, indicating intragonadal paracrine roles of GnRH and GnRH-R in the mouse ovarian development.

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