

Quantitative Gonadotropin-Releasing Hormone Gene Expression and Immunohistochemical Localization in Human Endometrium Throughout the Menstrual Cycle¹

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ABSTRACT

GnRH is one of the paracrine/autocrine regulators of hCG secretion produced by the human trophoblast during pregnancy. We hypothesized that GnRH may play a role in the embryonic/endometrial dialogue during early implantation. To examine this hypothesis, we assessed GnRH and GnRH-receptor mRNA and protein expression in human endometrium throughout the menstrual cycle of premenopausal fertile patients. Quantitation of the mRNA was performed by reverse transcription (RT)-competitive polymerase chain reaction (PCR) in the presence of a competitive cDNA fragment. RT-PCR revealed that unfractionated endometrium and isolated endometrial stromal and epithelial cells express GnRH and GnRH-receptor mRNA throughout all phases of the menstrual cycle. Quantitative PCR showed a dynamic pattern in the GnRH mRNA expression throughout the cycle, with a significant increase ($p < 0.05$) in the secretory phase as compared to the proliferative phase. Furthermore, quantitative competitive PCR of isolated glandular and stromal cells showed higher mRNA levels ($p < 0.05$) in the luteal phase in both compartments. GnRH immunostaining was localized in all major compartments, with the most intense staining during the luteal phase. On the basis of these data, we suggest that during reproductive life, endometrial GnRH may play a paracrine/autocrine role in the early stages of implantation by modulating embryonic trophoblastic secretion of hCG.

INTRODUCTION

Almost thirty years have passed since the isolation, determination of structure, and synthesis of GnRH, the hypothalamic hormone controlling the secretion of both FSH and LH from the anterior pituitary [1, 2]. GnRH binds to specific high-affinity pituitary receptors to trigger the release of the gonadotropins; low levels of GnRH are trophic to the receptor and stimulatory to the pituitary-gonadal axis [3].

An increasing body of evidence indicates that, in addition to the central action, a variety of human tissues express extrahypothalamic GnRH that is immunologically, biologically, and chemically identical to the hypothalamic hormone [4–6]. Recent studies have demonstrated the expression of mRNA for the human GnRH-receptor in several

extrapituitary organs such as the placenta, myometrium, breast, prostate, ovary, and testis [7–14]. In addition to this, there are indications that the presence of low-affinity/high-capacity binding sites for GnRH are present both in normal human endometrium and in endometrial carcinomas [15–19].

Since the synthesis of GnRH by Schally [1] in the early 1970s, interest in the clinical application of GnRH agonist (GnRH-a), has grown. The role of GnRH-a in reproductive medicine as well as in the treatment of prostatic cancer, precocious puberty, endometriosis, and uterine fibroids has been fully demonstrated [20–23]. GnRH when administered in a pulsatile fashion binds to the pituitary receptors and activates the gonadotrophs to both synthesize and release LH and FSH. Paradoxically, long-term administration of GnRH-a has an inhibitory effect on the pituitary gonadotropic secretion, causing a reversible medical gonadectomy [7]. GnRH-a has been successfully and routinely used for pituitary desensitization before ovarian stimulation with gonadotropins in in vitro fertilization (IVF) programs [20]. Different protocols have been designed, among them the “long protocol,” in which the GnRH-a administration is started in the midluteal phase of the cycle preceding the gonadotropins and is continued until ovulation induction by hCG administration; this has become the most widely employed regimen and has improved clinical outcome, producing greater numbers of preovulatory follicles and embryos as well as increased pregnancy rates over other regimens [20].

The inadvertent exposure of human pregnancy during the early stages of embryonic implantation to GnRH-a has been reported in IVF patients, and the clinical experience in those cases has suggested not only that undesired effects are unlikely but that the analogue might enhance implantation [24–36]. Moreover, it has been reported that patients with long history of infertility and unsuccessful treatment by different modalities have conceived repeatedly under the administration of GnRH-a [26, 37], with the suggestion that these conceptions may have been favored by the analogue.

It is well-established that the human placenta produces and secretes GnRH and that this immunoreactive GnRH is present in both cytotrophoblast and syncytiotrophoblast [38–40]. The placenta produces the highest concentrations of GnRH in the early gestation, before the 15th week, which correlates temporally with hCG production. For this reason, placental GnRH has been implicated as one of the primary regulators of the synthesis and secretion of hCG [41]. It has also been demonstrated that GnRH receptor mRNA is expressed in both cytotrophoblast and syncytiotrophoblast, and that the mRNA levels exhibit changes paralleling the time course of hCG secretion during pregnancy,

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providing a paracrine/autocrine regulatory mechanism of hCG secretion by human placental GnRH during the first trimester of pregnancy [42]. We hypothesize that the local expression of GnRH, as well as its receptor, may play an autocrine/paracrine role in the human endometrium during early embryonic implantation.

We chose to use competitive, quantitative polymerase chain reaction (PCR) to examine the amount of GnRH-mRNA in the endometrial samples throughout the menstrual cycle because of its high accuracy and because of its ability to detect and quantitate mRNA in small amounts of tissue [43, 44]. In the study reported here, we determined that GnRH is expressed in a dynamic pattern at both the mRNA and the protein level in the normal human endometrium throughout the entire menstrual cycle of fertile women. In addition to this, the GnRH receptor is present at the mRNA level in all phases of the menstrual cycle and in both endometrial compartments. These results provide evidence that GnRH, either directly or as a mediator, may play a role in the early embryo-maternal dialogue.

MATERIAL AND METHODS

Tissue Collection

Endometrial samples were obtained from 35 fertile premenopausal women aged 17-43 yr, undergoing laparoscopic surgery for various benign gynecological reasons. Patients with endometriosis and pelvic inflammatory disease were excluded.

Written consent from the patients and approval by the Institutional Committee on the use of human subjects in research at Stanford University were obtained before collection of tissue samples for this study.

Endometrial samples were taken using a Novak (Novak Inc., Palo Alto, CA) curette in the operating room immediately before surgical procedures. One part of the tissue was fixed with 4% paraformaldehyde; the rest was washed in 0.9% sodium chloride solution in order to remove contaminating blood and was directly processed for RNA extraction.

Tissue samples were classified according to time of collection and histological dating according to the method of Noyes et al. [45] into six different groups: early proliferative (n = 5), midproliferative (n = 6), late proliferative (n = 5), early secretory (n = 6), midsecretory (n = 7), and late secretory phase (n = 6).

Tissue Processing

Fixed tissue was embedded in paraffin, sectioned, and mounted. Twelve serial sections (6 μ m) from each sample were then prepared for immunohistochemistry, and the first and last sections were stained with hematoxylin-eosin and examined with a Nikon microphot-FXA microscope (Nikon Instruments, Garden City, NY).

Immunohistochemical Analysis

To determine the presence of GnRH in human endometrium, a double-antibody technique was employed according to the avidin-biotin alkaline phosphatase technique.

Sections were deparaffinized with xylene and quickly rehydrated through graded alcohols. Excess liquid was removed, and sections were washed with PBS (pH 7.4). In order to block unspecific binding, sections were preincubated in humidity chambers for 30 min at room temperature in PBS containing 2% normal goat serum (Sigma

Chemical Co., St. Louis, MO). After further washing in PBS (pH 7.4) with 0.05% Tween-20 (PBS-T; Sigma Chemical Co.; three times for 5 min each), sections were incubated for 90 min at 37°C with rabbit anti-[Lys⁸]GnRH as first antibody at a dilution of 1/100 each section. After a PBS-T washing, sections were incubated with a secondary antibody, biotinylated anti-rabbit IgG (dilution 1/800; Sigma Chemical Co.), in humidity chambers for 90 min at room temperature.

Immunohistochemical controls were incubated with PBS containing 2% goat serum without primary antibody. To amplify the signal, sections were washed with PBS-T, and then the avidin-biotin alkaline phosphatase-staining method (Vector Lab., Inc., Burlingame, CA) was used. Endogenous alkaline phosphatase activity was inhibited by the addition of levamisole to the buffer used to prepare the substrate solution. Finally, slides were counterstained with 25% hematoxylin, cleared, coverslipped, and examined by a Nikon DX-DB2 camera and a Nikon microphot-FXA microscope under \times 100-400 magnification. A red precipitate indicated positive staining by the primary antibody.

Isolation of Human Endometrial Stromal and Epithelial Cells

Endometrial tissue samples from the proliferative (n = 7) and luteal phase (n = 9) were washed with Dulbecco's Modified Eagle's medium (DMEM; Cellgro, Palo Alto, CA) to remove excess blood. Then the tissue samples were minced into tiny pieces (approximately 1 mm³) and digested overnight at room temperature with DMEM containing 0.1% collagenase type IA and 0.02% deoxyribonuclease type I (Worthington Biochemical Corporation, Freehold, NJ).

After tissue digestion, the stromal and epithelial cells were isolated as follows: the cell solution was allowed to settle for 5-10 min, and then the supernatant (stromal-rich fraction) was filtered to a new tube with cell strainer (70- μ m nylon; Falcon Plastics, Los Angeles, CA). The settled pellet (epithelial-rich fraction) was rinsed with DMEM. The process was repeated two more times.

In order to purify the settled epithelial pellet from residual stromal cells and undigested clumps, the epithelial cells were placed in a 75-cm² Falcon flask with DMEM containing 2% charcoal-Dextran-treated fetal bovine serum (Gemini, The Woodlands, TX) and incubated at 37°C in a 95% air:5% CO₂ atmosphere. The residual stromal cells attached to the plastic within 20-30 min. The medium containing the unattached epithelial glands was transferred to a new tube. This procedure was repeated twice, and then cell viability was ascertained using trypan blue dye.

In order to confirm the identity of the isolated cells, immunohistochemical localization of cytokeratin and vimentin as markers for epithelial and stromal cells was carried out. The isolated stromal and epithelial cells prepared by this method contained less than 0.1% of endometrial epithelial or vascular cells and less than 5% of endometrial epithelial or stromal cells, respectively [46].

RNA Extraction

The extraction of RNA from the tissue sample was carried out as described previously [47] with the RNA-STAT-60 reagent (Tel-Test "B" Inc., Friendswood, TX). Briefly, tissue samples were washed three times in PBS (Gibco BRL, Grand Island, NY) to remove blood contamination.

TABLE 1. Primers used for PCR.

cDNA	Ref #	Size of amplified fragment	Position of primers on cDNA	3'/5'-end*	Sequence of oligonucleotide
β -Actin	48	838 bp	294–325 1100–1131	5' 3'	5' → ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG CG → 3' 5' → CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC → 3'
GnRH	49	399 bp	47–67 445–426 445–426 +213–198	5' 3' 3'C	5' → AAA ACT CCT AGC TGG CCT TAT → 3' 5' → GGA ATA TGT GCA ACT TGG TG → 3' 5' → GGA ATA TGT GCA ACT TGG TGA GCG TTG GGT TTC TGC → 3'
GnRH-receptor	50	524 bp 231 bp	300–319 823–801 477–496 706–687	5'o 3'o 5'i 3'i	5' → GAT TGT CAT GCC ACT GGA TG → 3' 5' → TTA GAG TCT TCA GCC GTG CTC → 3' 5' → CAA CAG CAA AGT CGG ACA GT → 3' 5' → AGC ATG AAA AGA GGG ATG ATG → 3'

* 3'C, primer used to construct competitive cDNA for GnRH; 5'o/3'o/5'i/3'i: outer and inner primers for the nested PCR of GnRH-receptor.

One hundred milligrams of tissue was homogenized in 1 ml of RNA-STAT-60 reagent. Total RNA was separated from DNA and proteins by adding chloroform and was precipitated using isopropanol. The precipitate was washed two times in 75% ethanol, air-dried, and re-diluted in diethylpyrocarbonate (DEPC)-treated dH₂O. Amount and purity of extracted RNA was quantitated by spectrophotometry in a GenQuant RNA/DNA calculator (Pharmacia Biotech Ltd., Cambridge, UK).

Primers for Reverse Transcription (RT) and PCR

Sequences of cDNA-clones for the mRNAs to be detected in human endometrial samples (β -actin [48], GnRH [49], GnRH-receptor [50]) were obtained from the GenBank Database of the National Center for Biotechnology Information (NCBI) of the National Institutes of Health (NIH, Internet address: <http://www.2.ncbi.nlm.nih.gov/cgi-bin/genbank>). One corresponding set of primers for GnRH and GnRH receptor were found with the help of the program OLIGO 5.0 Primer Analysis Software (National Bioscience, Plymouth, MN) and synthesized by the "protein, amino acid and nucleic acid-(PAN) facility," Beckman-Center, Stanford University, Stanford, CA. To ensure that the detected product resulted from amplification of cDNA rather than contaminating genomic DNA, primers were designed to cross intron/exon boundaries. The human β -actin primers that were used to amplify an external standard were obtained from Clontech Laboratories Inc., Palo Alto, CA. β -Actin mRNA expression was employed as an external positive control, being detected in all the samples studied, thus assuming the integrity of the RNA and the RT-PCR process. The primer sequences, locations on the cDNA, and sizes of the amplified fragments are listed in Table 1.

RT

For each mRNA to be detected, 19 μ l RT-mastermix was prepared (5 mM MgCl₂ solution, single-strength PCR-buffer II, 2.5 μ l DEPC-treated dH₂O, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 1 mM dTTP, 2.5 μ M oligo(dT)₁₆, 20 IU RNase inhibitor [all Perkin-Elmer, Foster City, CA], 100 IU murine leukemia virus reverse transcriptase [Gibco BRL], and 1 μ g total RNA diluted in 1 μ l DEPC-treated H₂O) and filled into a 0.5-ml thin-wall PCR tube (Applied Scientific, South San Francisco, CA). RT-MasterMix in PCR-tubes was covered with 50 μ l of light white mineral oil (Sigma) and kept on ice until the RT. RT was carried out in the DNA Thermal Cycler 480 (Perkin-Elmer) using a program with the following parameters: 42°C, 15 min; 99°C, 5 min; 4°C, infinity. After the reaction was com-

pleted, samples were stored at -20°C until the PCR. As negative control, 1 μ l DEPC-treated H₂O without RNA sample was subjected to the same RT reaction.

Construction of the Competitive- and Target-cDNA Fragments for GnRH

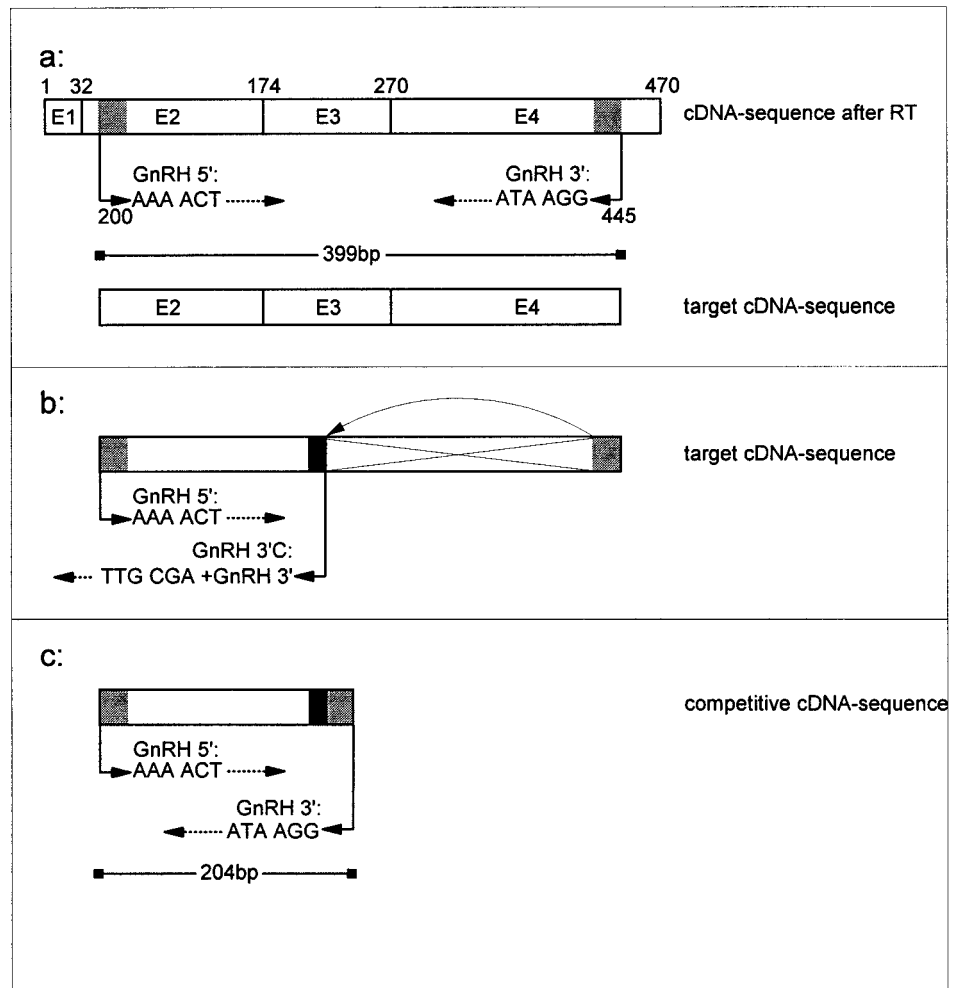
A 399-base pair (bp) fragment of native GnRH-cDNA (the target) was obtained by PCR amplification of reverse-transcribed total RNA from a luteal-phase endometrial biopsy (Fig. 1a) with the regular 3' and 5' primers (Table 1). The PCR product was visualized by agarose gel electrophoresis stained with ethidium bromide (ETB), and the cDNA was extracted from the gel, purified with an agarose gel extraction kit (Boehringer Mannheim, Germany), and quantitated by spectrophotometry (Pharmacia Biotech Ltd., Cambridge, UK). Independent sequence analysis was performed to confirm the identity of the expected sequence and amplified cDNA.

After sequence confirmation, 1 ng of target cDNA was amplified using a modification of a method previously described [43, 44] to construct a competitive cDNA fragment: a floating primer with a sequence complementary to the cDNA between the 3' and 5' primer binding sites was designed by attaching this sequence to the reversed complementary sequence of the binding site of the original 3'-GnRH primer (Fig. 1b). After one round of PCR with the regular 5'-primer and the 3'-floating primer, the PCR-product was visualized by agarose gel electrophoresis stained with ETB, the cDNA was extracted from the gel and purified, and the concentration was determined as described above. This step resulted in a cDNA fragment of 208 bp with a 195-bp deletion compared to the target cDNA and with the 3'-end and 5'-end primer binding sites on its ends (Fig. 1c). Again, sequence analysis was performed to confirm the identity of the expected sequence and amplified cDNA for this competitive cDNA fragment.

Standard-Curve and Competitive PCR for GnRH

The standard curve for GnRH was constructed by a co-amplification of a constant amount of competitive cDNA (30 amol) with declining amounts of target cDNA obtained by serial dilution. The amounts of target and competitive cDNA added to each PCR are shown in Figure 2. Five microliters of the cDNA mix was added to 95 μ l PCR-mastermix containing 1.9 mM MgCl₂ solution, single-strength PCR buffer II, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM dTTP, 2.5 U polymerase-gold (all Perkin-Elmer), 4 μ l 3'+5' primer-mix (5 μ M each) for GnRH, and 65 μ l DEPC-treated H₂O. The reaction mix was cov-

FIG. 1. **a)** Construction of the target cDNA by amplification of a 399-bp fragment from cDNA obtained after RT of 1 μ g total RNA extracted from human luteal endometrium. Primers used for this step were 3' and 5' GnRH (Table 1). **b)** Creation of an artificial deletion in the target cDNA from **a**. By amplification of the target cDNA with the 5' GnRH primer and the 3'C GnRH floating primer (Table 1), a 195-bp deletion was obtained. **c)** Sequence of the competitive cDNA for GnRH. The cDNA sequence is identical with the target cDNA except for the 195-bp deletion created in **b**. The sequences of the primer binding sites for GnRH 3' and GnRH 5' are identical for the target cDNA and native cDNA, suggesting that in a coamplification of either of these cDNAs with the competitive cDNA, the cDNAs will truly compete for the primers and for the polymerase and will be amplified with the same affinity.



ered with 50 μ l light white mineral oil, put in the DNA Thermal Cycler 480, and heated to 99°C for 9 min to denature all proteins and to activate the polymerase gold. After completion of 30 cycles of 94°C for 45 sec, 56°C for 45 sec, and 72°C for 60 sec, the reaction was terminated at 72°C for 5 min and cooled down to 4°C. PCR products were stored at -20°C until 2% agarose gel electrophoresis was carried out in the presence of ETB. After completion of electrophoresis, the agarose gel was analyzed on the GelDoc 1000 system (Bio-Rad Laboratories, Hercules, CA). DNA size calculation and UV densitometry were carried out by using the Molecular Analyst Software (Bio-Rad Laboratories).

The logarithmically transformed ratios of target cDNA to competitive cDNA were plotted against the log amount of initially added target cDNA in each PCR to obtain the standard curve shown in Figure 2. This standard curve was highly reproducible and linear over two orders of magnitude. The values obtained from the regression line of the standard curve ($y = b + mx$) allowed us to calculate the amount of cDNA transcripts in an unknown sample: 30 amol of competitive cDNA was added to each unknown sample before PCR. The ratio of the densities of the competitive cDNA band (208 bp) and of the cDNA band obtained from the sample (399 bp) was logarithmically transformed and compared to the values obtained from the standard curve ($x = (y-m)/b$). Quantitative competitive PCR was carried out on at least two aliquots from the RT cDNA

of each patient, and the results did not differ more than $\pm 5\%$.

PCR for β -Actin

Five-microliter aliquots from each RT product were mixed with 95 μ l of the PCR-mastermix described above with 4 μ l 3'+5' primer-mix (5 μ M each) for β -actin. Program parameters were 30 cycles of 94°C for 45 sec, 54°C for 45 sec, and 72°C for 60 sec. For this PCR, no competitor was added.

Nested PCR for the GnRH Receptor

RT products were split for the first round of PCR. Two microliters of RT product was added to 48 μ l of PCR-1-mix (2 mM MgCl₂ solution, 10-strength PCR buffer II, 33.5 μ l DEPC-treated dH₂O, 0.24 mM of each dNTP, 2.5 U polymerase gold [all Perkin-Elmer], and 2.4 μ l outer 3'+5' primer-mix [5 μ M each, Table 1]). After all components were mixed in a 0.5-ml thin-wall PCR cup, the reaction mix was covered with 50 μ l light white mineral oil. After completion of 30 cycles of 94°C for 45 sec, 54°C for 45 sec, and 72°C for 60 sec, the reaction was terminated at 72°C for 5 min and cooled down to 4°C. First-round PCR products were stored at -20°C until the second PCR. For the second PCR, 5 μ l of first-round PCR products was added to 95 μ l PCR2 reaction mix (1.9 mM MgCl₂ solution, 10-strength PCR buffer II, 65.0 μ l DEPC-treated H₂O

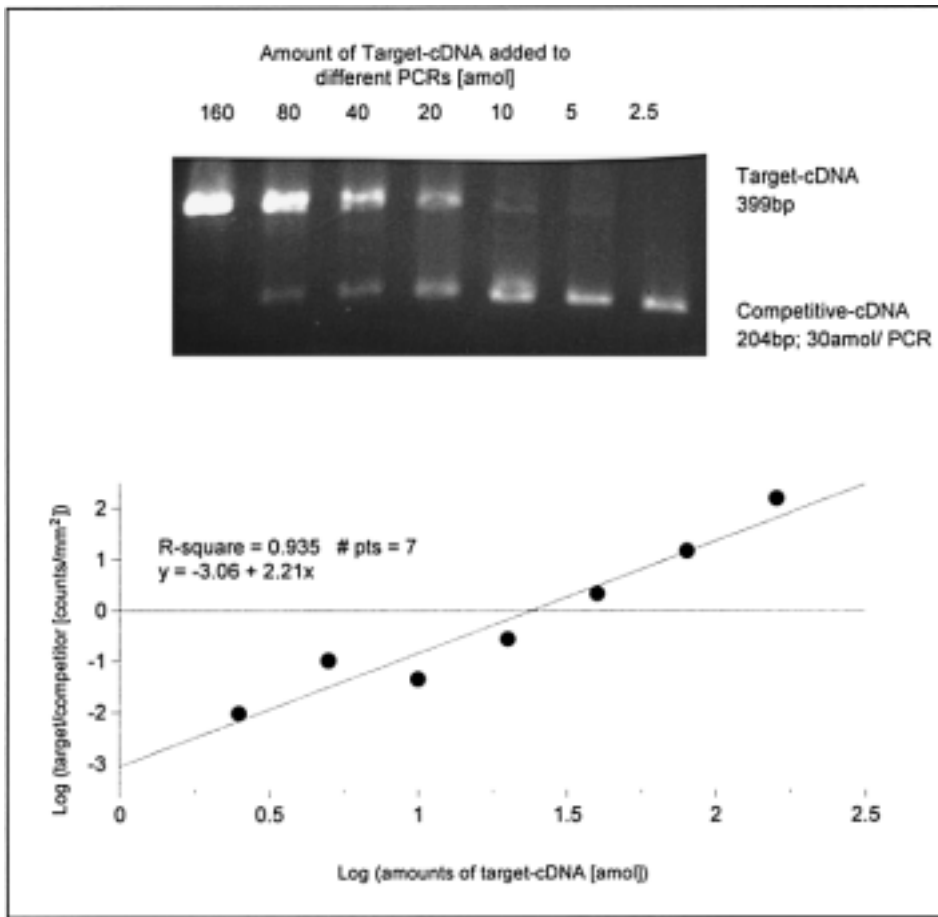


FIG. 2. The upper panel shows a 2% agarose gel stained with ETB. Declining amounts of target cDNA were coamplified with 30 amol competitive cDNA. The lower panel shows the standard curve obtained from this gel. The log ratio of target to competitive product density was plotted against the log amount of target initially added to the PCR.

[dist.], 0.2 mM of each dNTP, 2.5 U polymerase gold [all Perkin-Elmer], and 4 μ l inner 3'+5' primer mix [5 μ M each, Table 1]), in a thin-wall PCR tube, and covered with 50 μ l mineral oil. Program parameters were identical with those of the first round of PCR. After the second round of PCR was completed, samples were stored at -20°C until agarose gel electrophoresis was carried out.

Data Analysis

Statistical analysis was performed by ANOVA and independent sample *t*-test. The analysis was carried out using the Statistical Package for Social Science (SPSS Inc., Chicago, IL) with a *p* value < 0.05 considered statistically significant.

RESULTS

RT-PCR Analysis

RT-PCR was employed to increase the sensitivity of detection, and a sequence of 399 bp of GnRH mRNA was amplified in all the endometrial samples analyzed from fertile women in both the follicular and luteal phases of the menstrual cycle.

The localization of the expected fragment (524 bp) for the GnRH receptor mRNA was also examined in the endometrial samples from the same patients, and amplified signal was detected using one round of PCR (40 cycles) in 30% of the samples studied. For this reason, the sensitivity was further increased by performing two rounds of nested PCR (30 cycles in the first round and 35 in the second) in order to amplify a signal. Electrophoresis of RT-PCR frag-

ments of each sample produced a 231-bp size band corresponding to the fragment produced by the specific primer used in the second round PCR. By this method we were able to detect GnRH receptor in all the samples studied throughout the different phases of the menstrual cycle (Fig. 3).

β -Actin mRNA expression was ascertained in all the samples studied, thus confirming the integrity of the RNA and the RT-PCR process.

To examine the quantitative amount of GnRH mRNA in the endometrial cells from individual subjects throughout the menstrual cycle, we coamplified the patient sample for 40 cycles in the presence of defined amounts of internal standard cDNA for GnRH. Figure 2 shows the standard curve obtained for GnRH mRNA by plotting the logarithmically transformed ratios of the densities of target cDNA to competitive cDNA against the log amount of initially

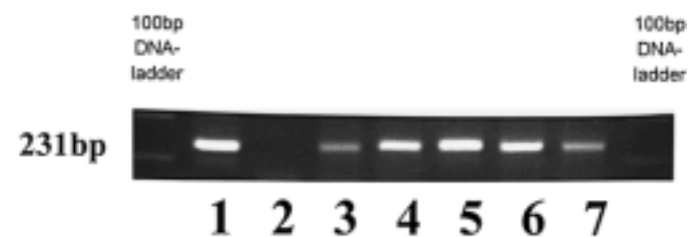


FIG. 3. GnRH receptor mRNA expression in the different phases of the menstrual cycle: 1) Positive control (2nd trimester human placenta); 2) Negative control; 3) midproliferative endometrium; 4) late proliferative endometrium; 5) early luteal endometrium; 6) midluteal endometrium; 7) late luteal endometrium.

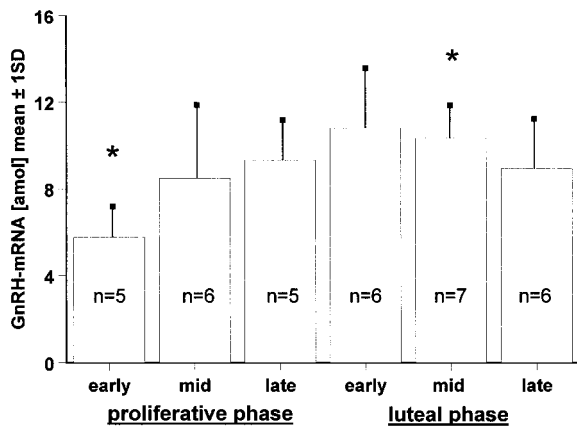


FIG. 4. Quantitative competitive PCR of GnRH mRNA from endometrial samples studied throughout the menstrual cycle. mRNA levels showed a progressive increase from the early proliferative phase to the midluteal phase. * $p < 0.05$ between these two phases.

added target cDNA in each PCR. The quantitative PCR of the different endometrial samples studied throughout the menstrual cycle showed a progressive increase in the mRNA levels from the early proliferative phase to the midluteal phase, which was statistically significant ($p < 0.05$) between these two phases (Fig. 4).

After the separation of the glandular epithelium from stromal cells, the presence of mRNA for GnRH and receptor in both endometrial compartments independently was demonstrated. Quantitative PCR of isolated glandular and stromal cells showed higher GnRH mRNA levels ($p < 0.05$) in the luteal phase than in the follicular phase in both compartments; however, the increase in mRNA levels was more pronounced in epithelial than in stromal cells (Fig. 5).

Immunoreactive GnRH was present in all major endometrial compartments (luminal epithelium, glands, and stroma cells) throughout the entire menstrual cycle according to an avidin-biotin alkaline phosphatase technique. No staining was seen in cells of hematopoietic origin or vascular smooth muscle. The most intense immunoreactivity was noted during the luteal phase in all compartments as compared to the follicular phase (Fig. 6).

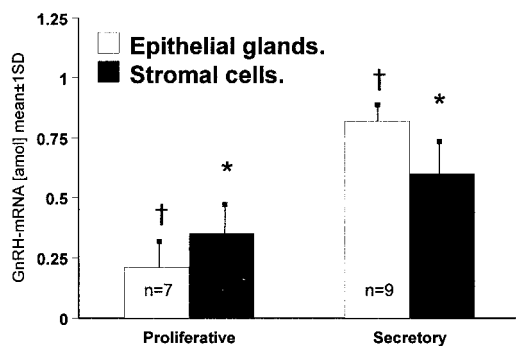


FIG. 5. Quantitative, competitive PCR of isolated glandular and stromal cells. mRNA levels showed an increase in the luteal phase in both endometrial compartments; however, these increases were more evident in the epithelium than in the stroma. * and †, Bars with the same symbols showed significant differences at $p < 0.05$ and $p < 0.01$, respectively. Values are means \pm SD.

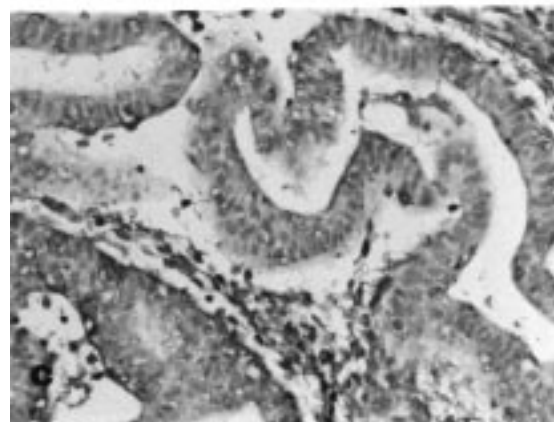
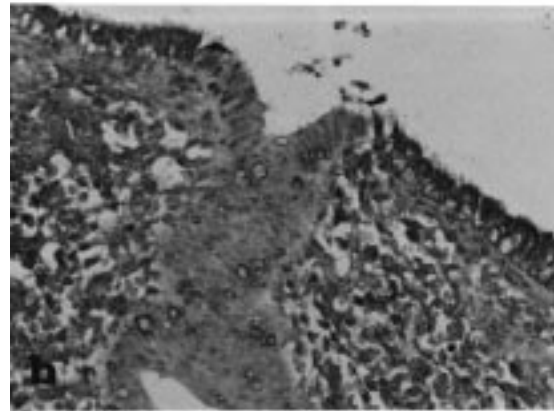
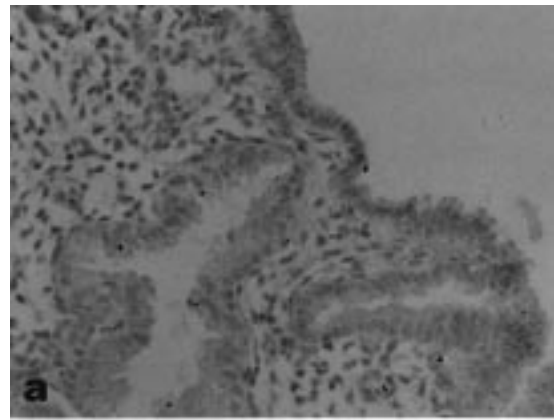


FIG. 6. GnRH immunoreactivity in sections of human endometrium. a) Negative control; b) and c) midluteal phase endometrium. GnRH immunoreactivity was highly expressed in the luminal epithelium (b) as well as in the stroma cells (c). $\times 200$.

DISCUSSION

We have shown for the first time that both GnRH and its receptor are expressed at the mRNA level in vivo by the human endometrium throughout the entire menstrual cycle of fertile patients. Isolated endometrial stromal and epithelial cells expressed both GnRH and its receptor, with greater GnRH mRNA expression in stromal cells compared to epithelial cells during proliferative phase. However, increased epithelial expression of GnRH mRNA occurred during the luteal phase. This is consistent with the immunohistochemical data, which showed affinity-purified antibody to GnRH bound to the endometrial stroma of each section of every patient studied both in the follicular and luteal phases of

the menstrual cycle, with the most intense reactivity in the luteal phase. We also localized the GnRH staining to both the glandular and luminal epithelium, with increased signal in the luteal phase.

Human implantation is a complex series of steps that under normal circumstances begins even before the blastocyst reaches the uterine cavity and attaches to the endometrial epithelium after the loss of zona pellucida. In order to complete this series of events and to accomplish successful implantation and placentation, the embryo and the uterine endometrium must be synchronized during this limited period of uterine receptivity known as the "window of implantation" [46].

This enigmatic process is the result of an embryonic-maternal dialogue, in which the embryo and the endometrium induce changes in each other to promote receptivity. Growth factors and cytokines are secreted by the developing blastocyst to enhance uterine receptivity, directly by influencing the endometrial epithelial cells to undergo cellular changes such as down-regulation of cell polarity, or indirectly by stimulation of ovarian steroidogenesis through hCG, until placental progesterone production is sufficient to maintain the continuing pregnancy [51].

After adhesion of the embryonic pole of the human blastocyst to the endometrial epithelial surface, trophoblastic differentiation into cytotrophoblast and syncytiotrophoblast develops as the trophoblast invades the stromal endometrium. Human CG secretion rises in a logarithmic fashion from 6 days after conception until the wk 10-11 after the last menstrual period, at which time the level peaks. This is followed by a decline to a nadir reached about wk 18 and remaining constant until term [52]. One of the primary functions of hCG during the early steps of pregnancy is to stimulate progesterone production by the corpus luteum as well as to enhance uterine receptivity, since progesterone has immunosuppressive properties that potentially prevent maternal rejection [52].

The role of GnRH in controlling placental hCG production and secretion has been fully demonstrated both in vitro [38-42, 53] and in vivo [54, 55], especially in first-trimester placenta. The stimulatory effect appears to be receptor-dependent, since GnRH antagonist (GnRH-ant) blocks both the GnRH- and GnRH-a-induced effects. Furthermore, GnRH-ant reduces the amplitude of spontaneous placental hCG pulses, suggesting a direct blockage of endogenous placental GnRH [56]. Recently, an antibody against GnRH has been described in the maternal circulation of pregnant women with previous miscarriages and low levels of hCG [55], reinforcing the important role of GnRH in human implantation and placentation.

In previous studies, GnRH receptor mRNA was reported to be present in 23% of the nonmalignant endometrial samples studied and in 77% of endometrial cancers [57]. In our study, we detected the mRNA of GnRH receptor in 30% of the endometrial samples by performing one round of PCR; however, by nested PCR we were able to detect the receptor in all the endometrial tissue specimens studied both in the follicular and in the luteal phase. This indicates that although the GnRH receptor is expressed at low levels, it is present in the human endometrium of fertile women throughout the entire menstrual cycle.

Seshagiri et al. [58] showed that immunoreactive GnRH and CG were produced in vitro by cultured rhesus monkey embryos during the entire peri-attachment period, from morula to attached blastocyst stage, and that the GnRH secretion commenced before that of hCG. Moreover, embryos

that failed to hatch and attach secreted lower amounts of GnRH into the medium in contrast to those that did reach later stages of development; at the same time, CG was very low or absent in the medium of these embryos.

No data are available from human embryos during the peri-attachment period, and future studies should investigate the presence of GnRH and receptor during human embryonic development. Nevertheless, after embryo-endometrial adhesion, as the embryo invades the uterine endometrial stroma, trophoblastic differentiation into cyto- and syncytiotrophoblast layers occurs, and both are known to produce GnRH as well as to express the GnRH receptor in the first-trimester placenta [38-41].

Encouraged by the evidence that large doses of GnRH or GnRH-a were able to induce abortion or delay implantation in rats and baboon monkeys [7, 59], one of the first clinical GnRH-a experiments in humans was to assess a possible postcoital and postimplantation contraceptive effect. The surprising outcomes of those studies was that high doses of super-reactive GnRH-a not only failed to block implantation or induce abortion in humans [60] but rather seemed to enhance implantation [24-36]. The exact mechanism by which GnRH-a favors pregnancy during the peri-implantation period has remained unknown until present.

The presence of GnRH and receptor with a dynamic pattern in human endometrium both in the epithelium and stroma provides evidence that this hormone may play a substantial role as a molecular autocrine/paracrine regulator in embryo/endometrial interactions during early implantation.

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