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Placental Progonadotropin-Releasing Hormone (Pro-GnRH) in the Rhesus Monkey

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Gonadotropin-releasing hormone (GnRH) has been shown to play a role in the regulation of human chorionic gonadotropin (hCG) secretion by the human placenta. Molecular studies have demonstrated that human placental trophoblast cells synthesize a progonadotropin-releasing hormone (pro-GnRH) identical to its human hypothalamic counterpart. However, far less is known about nonhuman primates. To determine whether pro-GnRH exists in the rhesus placenta, pro-GnRH mRNA was cloned, sequenced, and shown to be 97.6% homologous to its human placental counterpart. A single base difference (base 1167) in the domain encoding GnRH results in the same amino acid, arginine, in position 8, whereas four base differences (bases 1200, 1253, 1268, 1292) in the domain encoding GnRH-associated peptide (GAP) result in four different amino acids in positions 19, 37, 42, and 50. The absence of a basic amino acid in position 50 suggests the rhesus sequence may be cleaved to yield GAP peptides different from the human placenta. Thus, these data justify the use of mammalian GnRH in studies of rhesus placental function, but indicate the need to investigate the roles unique GAP peptides may play in placental/uterine function.

Key Words: Rhesus monkey; placenta; GnRH; GAP; base sequence.

Introduction

The role hypothalamic gonadotropin-releasing hormone (GnRH) plays in the regulation of secretion of the pituitary gonadotropins follicle-stimulating hormone and luteinizing hormone is well-established. However, much less is known about the role of placental GnRH in the regulation of secretion of the placental gonadotropin, human chorionic gonadotropin (hCG), which is required for implantation and the early maintenance of pregnancy *(1–8)*. Following the first demonstration of a single high-affinity binding site for GnRH in pituitary membranes *(9)*, putative receptor sites were identified in a number of extrahypothalamic sites, including the placenta *(10)*. Given the short half-life of GnRH in serum (<5 min), Khodr and Siler-Khodr *(11)* postulated that there was a local source of GnRH within the placenta that would act in an autocrine or paracrine manner to regulate the secretion of hCG. Subsequently, GnRH immunostaining of the cytotrophoblast and syncytiotrophoblast cells of the human placenta was shown *(11–14)*. Duello et al. *(15)* used primers based on the sequence of hypothalamic progonadotropin-releasing hormone (pro-GnRH) mRNA *(16)* to demonstrate pro-GnRH mRNA in first trimester, second trimester, and term human placental samples, and showed its sequence to be identical to its hypothalamic counterpart. Concurrently, Kelly et al. *(17)* confirmed the presence of pro-GnRH mRNA using a solution hybridization assay and also demonstrated placental pro-GnRH mRNA levels to be approx 0.1–1% of the level of hCGβ mRNA, consistent with GnRHs suggested paracrine role in hCG regulation.

Far less is known about the role of placental GnRH in nonhuman primates. Seshagiri et al. *(18)* studied the production and timing of GnRH secretion in peri-implantation embryos of rhesus monkeys. Secretion of GnRH was at low levels during the prehatching blastocyst stage, but increased over time as did concentrations of chorionic gonadotropin (CG). Prehatching blastocysts, attached blastocysts, and monolayer cultures of placental cells were all shown to immunostain for GnRH. To further validate the use of rhesus monkey placenta as a model for the study of human placental hormone secretion, a gene amplification/sequence analysis study has been undertaken to examine the presence of pro-GnRH mRNA in the rhesus monkey placenta and to compare its sequence to its human placental counterpart.

Results

RT-PCR methodologies were used to amplify pro-GnRH mRNA from human (8-wk gestation) and rhesus monkey placental tissues (36 or 73 d gestation) under conditions

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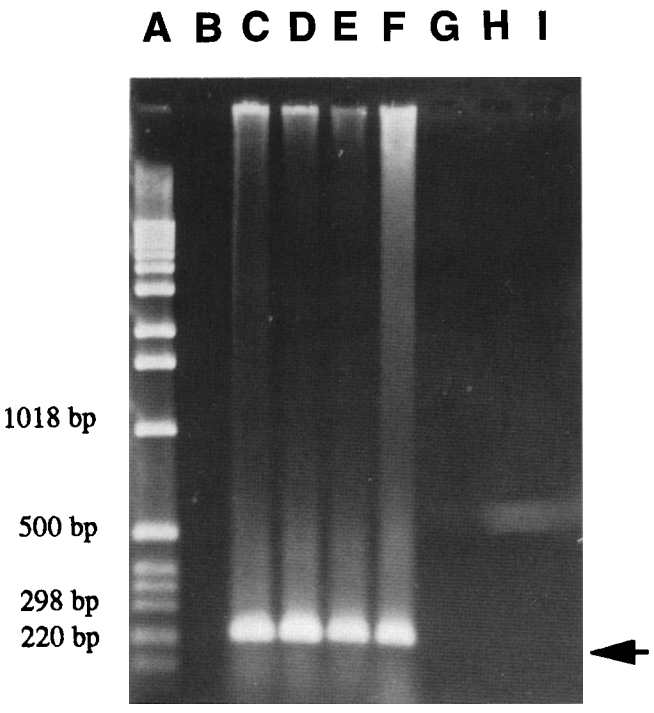

Fig. 1. RT-PCR amplification of pro-GnRH mRNA from 8-wk human (lane C), 36 d rhesus monkey (lanes D and E), and 73 d rhesus monkey placentas (lane F). Controls for sample shown in lane E; upper primer only (lane G); lower primer only (lane H); cDNA omitted (lane I).

identical to those previously used to amplify pro-GnRH mRNA from human placental tissues *(15)*. The primer combination based on the sequence of human hypothalamic pro-GnRH *(16)* predicted a transcript of 207 bps. A single band of ~200 bps was amplified from both human (lane C) and rhesus monkey (lanes D–F) placental RNA (Fig. 1). No bands resulted when amplifications were carried out using only the upper primer (lane G) or only the lower primer (lane H) indicating that none of the bands were a result of self priming. In addition, no bands resulted when the cDNA was omitted (lane I), indicating that there was no RNA contamination. These controls were performed on all placental samples, however, only those for the 36 d gestation rhesus placental sample (lane E) are shown.

Following cloning, DNA from positive clones was analyzed by restriction digestion and three clones were identified that were expressing a transcript of ~200 bps. Sequence analysis revealed these transcripts to be 97.6% homologous to human placental pro-GnRH mRNA. A comparison of the human pro-GnRH sequence and the rhesus monkey placental sequence is shown in Fig. 2. (The asterisks indicate the location of the termination site, which was immediately 3' to the lower primer.) Five base differences were noted, one within the first 30 nucleotides encoding GnRH proper (bases 1144-1173; Fig. 2, GnRH, solid line) and four within the sequence encoding GnRH-associated peptide (GAP) (bases 1183-1350; Fig. 2, GAP, dashed line). The single base difference corresponding to base 1167 of the human sequence resulted in the encoding of the same amino acid, arginine, in position 8. Thus, there is no difference in the amino acid sequence of GnRH (amino acids 1 to 10) between the human and rhesus monkey placentas. Of the four additional base differences in the GAP sequence, one (base 1253) resulted in the substitution of a basic amino acid for a different basic amino acid—histidine for arginine in position 37—and one (base 1292) in the deletion of a basic amino acid—glutamine for arginine in position 50. The remaining two base differences corresponding to bases 1200 and 1268 resulted in methionine residues, rather than isoleucine and threonine, in positions 19 and 42, respectively.

Discussion

Khodr and Siler-Khodr *(11)* first postulated that there was a 'hypothalamus' within the placenta that produced GnRH and that GnRH acted in a paracrine fashion to regulate secretion of hCG. They demonstrated GnRH immunostaining of the cytotrophoblast and syncytiotrophoblast cells of the human placenta *(11)* as well as GnRH stimulation of hCG secretion *(1)*. However, it was not possible on the basis of immunostaining alone to determine whether the GnRH was maternal, fetal, or placental in origin. Thus, molecular approaches were taken to address this issue. Petraglia et al. *(14)* demonstrated *in situ* labeling of cytotrophoblast cells from term placentas using antisense riboprobes to pro-GnRH mRNA and immunostained the same cells using an antiserum to [Lys^8]GnRH. In contrast, Duello et al. *(15)* showed labeling of both the cytotrophoblast and syncytiotrophoblast cells of first trimester placental samples using an *in situ* hybridization approach in conjunction with laser scanning confocal microscopy. Labeling of the syncytiotrophoblast may have represented contribution of pro-GnRH mRNA to the syncytiotrophoblast at the time of fusion of the cytotrophoblast cells or *de novo* synthesis by the syncytiotrophoblast cells. Both trophoblast layers were also shown to immunostain for pro-GnRH using an antisera to the peptide that bridges the proteolytic cleavage site of pro-GnRH (amino acids 6–12) *(19)*. In addition, Kelly et al. *(17)* demonstrated the presence of both antisense and sense pro-GnRH mRNA using a solution hybridization assay and showed placental pro-GnRH mRNA levels to be approx 0.1–1% of the level of hCGβ mRNA, consistent with GnRHs suggested paracrine role in hCG regulation.

The only study of the role of GnRH in rhesus monkey placenta was conducted by Seshagiri et al. *(18)* who demonstrated GnRH secretion by rhesus monkey embryos and trophoblast cells. GnRH secretion by embryos was initially low during the prehatching blastocyst stage (0.32 pg/mL ± 0.05 SEM), but increased over time (≥13 d) to the point of hatched blastocyst attachment and proliferation of trophoblast (1.30 pg/mL ± 0.23 SEM). GnRH immunostaining of

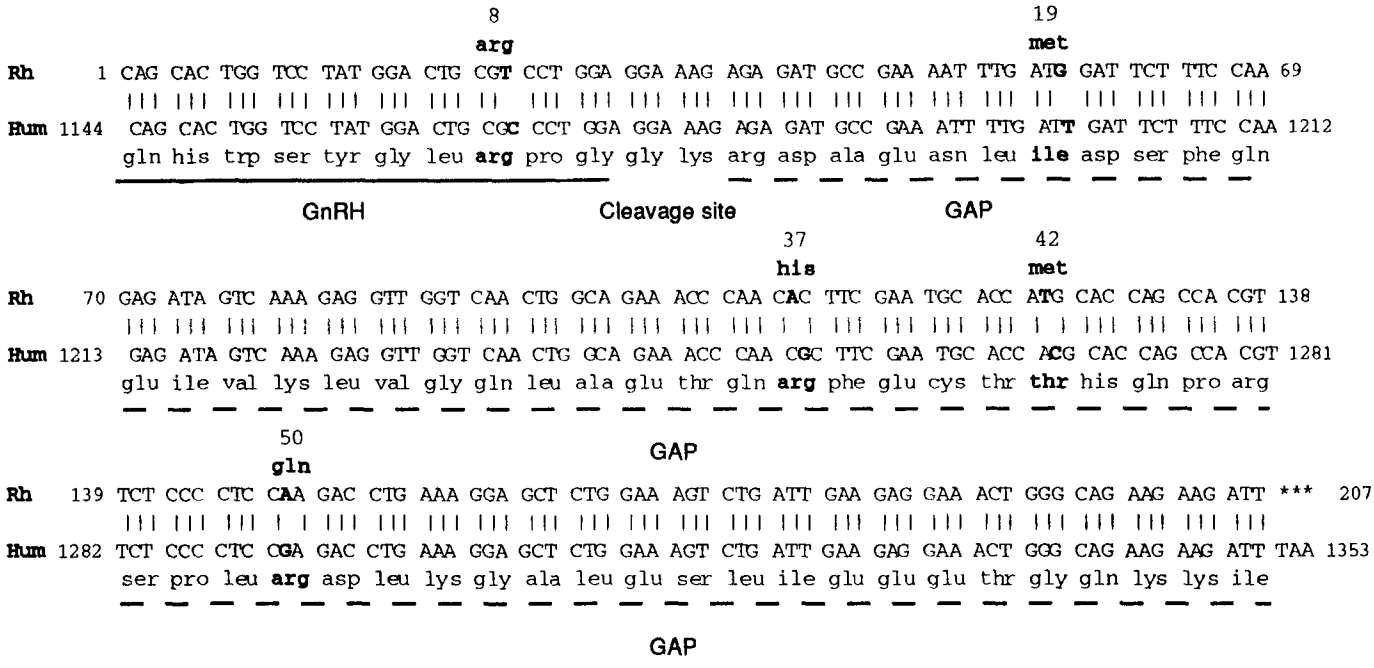

Fig. 2. Comparison of the base (capital letters) and amino acid sequences (small letters) of rhesus monkey (Rh) and human (Hum) placental pro-GnRH mRNA. Base #, row ends; amino acid #, above rows; GnRH, solid line; GAP, hatched line; asterisks, position of termination codon.

embryos and placental derived trophoblast was also demonstrated using an antiserum to mammalian GnRH. In the study reported here, we set out to determine if there is a source of GnRH within the rhesus monkey placenta and, if so, to assess its similarity to human placental pro-GnRH mRNA. As hypothesized, sequence analysis of rhesus monkey placental pro-GnRH mRNA demonstrated a high degree of homology (97.6%) between rhesus monkey and human placental pro-GnRH mRNA. As in mammalian hypothalami, rhesus monkey placental pro-GnRH mRNA encodes placental GnRH, a gly-lys cleavage site, and placental GAP. The structure of rhesus monkey placental GnRH was identical to that of its human hypothalamic/placental counterpart, possibly reflecting a pivotal role in reproductive function in the placenta. However, it is not clear that regulation of CG is GnRHs only role or even its major role, since an identical pro-GnRH transcript is present in the bovine placenta, which lacks a CG *(20)*. Thus, it is of particular interest to determine if there are additional endpoints for GnRH action that have to date eluded us.

The sequence of rhesus monkey placental GAP was also shown to be highly conserved. Whereas the sequence of rhesus monkey GAP differed by only four amino acids from human GAP, the absence of a basic amino acid (cleavage site) at position 50 suggests a relevant GAP peptide in the rhesus placenta may be represented by amino acid residues 38–66, rather than 38–49 and 51–66 as in the human GAP molecule. Surprisingly, the role of GAP peptides in placental function have not been studied in primates, though one could envision many possibilities. They may act in an autocrine fashion to regulate secretion of placental hormones or growth factors by the same placental cell or in a paracrine fashion to affect the secretion of adjacent cells. Given that a prolactin-inhibitory peptide was identified within the hypothalamic GAP molecule *(21)*, a similar peptide may act within the placenta to regulate the production of prolactin-like transcripts. Likewise, they may regulate secretion of tubal/uterine factors required to nourish the embryo or permit implantation. Although it is possible that GAP peptides play no role in placental function or reproduction, as a rule evolution does not appear to select for waste.

In conclusion, the demonstration that rhesus monkey placental pro-GnRH mRNA encodes a GnRH molecule identical to human hypothalamic/placental GnRH supports the use of synthetic mammalian GnRH in studies of regulation of rhesus monkey CG. It also further establishes the legitimacy of a rhesus monkey placental model to further our understanding of human placental function. Finally, whereas the role of GAP peptides in pituitary function are unclear, their role in placental function is a totally unexplored field that warrants investigation.

Materials and Methods

Total RNA Isolation

Human placental tissue (8-wk gestation) was collected from local sources after elective termination with the approval of the University of Wisconsin Human Subjects Committee. Two rhesus monkey *(Macaca mulatta)* placentas were harvested at 36 d gestation and one at 73 d gesta-

tion by Dr. Thaddeus Golos. Total cellular RNA from all tissues was prepared by the guanidium isothiocyante/ cycle method *(22)*. Concentrations were determined spectrophotometrically.

Reverse Transcription-Polymerase Chain Reaction Analysis

Reverse transcription-polymerase chain reaction (RT-PCR) analysis was used to amplify pro-GnRH mRNA from the three rhesus placental samples. Five μg total RNA was reverse transcribed in a final volume of 20 μL. The reaction mixture included 1.0 × RT buffer (50 m*M* Tris-HCl, pH 8.3; 75 m*M* KCl; 3 m*M* $MgCl_2$), 0.1 mg/mL BSA, 1.0 m*M* DTT, 30 pmol oligo dT12-18 (Clontech Laboratories, Inc., Palo Alto, CA), 0.2 m*M* dNTP, 0.5 U/μL RNAsin (Promega, Madison, WI), and 50 U SUPERSCRIPT™II RNase H-reverse transcriptase (Life Technologies, Gaithersburg, MD). The reaction mixture was heated to 70°C for 10 min, incubated 2 h at 42°C, heated to 95°C for 10 min, and quenched on ice.

For PCR amplification, 5 μL of RT was amplified in a final volume of 50 μL. The reaction mixture included 1× reaction buffer (10 m*M* Tris-HCl, pH 9.0 at 25°C, 50 m*M* KCl, 0.1% Triton X-100), 1.5 m*M* $MgCl_2$, 0.2 m*M* dNTP, 2.0 U Taq polymerase (Promega), and the following primers (Operon Technologies Inc., Alameda, CA) based on the human hypothalamic sequence *(16)*:

5' primer-5' CAGCACTGGTCCTATGGACTG
(bases 1144 to 1164)

3' primer-5' AATCTTCTTCTGCCCAGTTTCC 3'
(bases 1329 to 1350)

Amplification conditions were 35 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min, and elongation at 72°C for 2 min, followed by final extension at 72°C for 7 min. The PCR products were run on 1% agarose gels, stained with ethidium bromide, and visualized using a UV illuminator.

Cloning

The RT-PCR reaction mixture from the amplification of pro-GnRH mRNA from a 36-d gestation placental sample was used for ligation (1/1) into the pCR™II vector using the Original TA Cloning Kit (Invitrogen, San Diego, CA) transformed with TOP10F' OneShot™ Competent cells. Following ligation and transformation, the transformed cells were spread on LB agar plate and incubated at 37°C for at least 18 h. They were then held at 4°C for 2–3 h to allow proper color development of white colonies corresponding to interruption of the lacZ gene. The plasmid DNA was recovered using the alkaline lysis miniprep protocol *(23)*, a portion of which was analyzed by restriction digestion with EcoRI restriction enzyme (Promega) to identify clones with transcripts of the predicted size. The remaining plasmid DNA was prepared for sequencing using the Qiagen Plasmid Mini kit (Qiagen, Chatsworth, CA).

Sequence Analyses

An ABI Automated DNA Sequencer using dideoxy NTP chemistry was used to determine the sequence of the pro-GnRH transcripts (Dr. Gerald Bergtrom, Department of Biological Sciences, University of Wisconsin-Milwaukee, Milwaukee, WI). The software of the Genetics Computer Group (Madison, WI) was used to compare the human and rhesus monkey placental pro-GnRH gene sequences.

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