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Nucleotide Sequence Analyses Predict that Human Pituitary and Human Placental Gonadotropin-Releasing Hormone Receptors Have Identical Primary Structures

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Gonadotropin-releasing hormone (GnRH) interacts with a putative receptor in human placenta to cause the dose-dependent release of human chorionic gonadotropin (hCG) in a manner analogous to hypothalamic GnRH stimulation of luteinizing hormone (LH), and follicle-stimulating hormone (FSH) by the pituitary gland. However, GnRH agonists bind a placental binding site at a lower affinity than they bind the pituitary GnRH receptor, suggesting the two sites differ. To address this issue, human placental GnRH receptor mRNA from an 8-wk placental sample was amplified using primers based on the sequence of the human pituitary receptor, cloned, and sequenced. The sequence of the placental transcript was found to be identical to its pituitary counterpart. Thus, as with the pituitary receptor, this placental receptor transcript appears to encode a single polypeptide chain with seven intramembranous domains and two glycosylation sites. However, it is possible these receptors differ in their posttranslational processing or there is more than one placental GnRH receptor.

In addition, subsequent gene amplification studies, modified to increase the sensitivity of the method, revealed a band of the predicted size (~1051 bp) in a 6-wk and two 8-wk placental samples, but not in 5-wk and 7-wk samples. The failure to amplify GnRH receptor mRNA at these gestational ages did not appear to be an issue of cell health or sample degradation, since GnRH, αhCG, and βhCG mRNA were amplified from each of these tissues. Instead these data suggest GnRH receptor mRNA may have a short half-life or the mRNA may be translated as rapidly as it is transcribed, with the result that it has not accumulated in the cell.

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Introduction

Gonadotropin-releasing hormone (GnRH) is known to stimulate the secretion of human chorionic gonadotropin (hCG), serum concentrations of which increase throughout the first trimester of pregnancy, peaking at 8–10 wk, and then decline throughout the second trimester to basal levels *(1–7)*. Human chorionic gonadotropin (HCG) in turn stimulates the secretion of placental progesterone, which is required for the early establishment and maintenance of pregnancy. This cascade is analogous to hypothalamic GnRH stimulating the secretion of pituitary luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which in turn stimulate the secretion of ovarian progesterone.

The cDNA sequences of hypothalamic and placental GnRH are known to be identical *(8–10)*. However, it has been suspected that the structures of the receptors differ based on the finding that GnRH agonists bind the pituitary receptor with a 100-fold greater affinity than they bind the placental site *(7)*. The structure of human pituitary GnRH receptor mRNA was elucidated by Kakar et al. *(11)* and Chi et al. *(12)*. It was shown to have a 987-bp open reading frame sequence that encodes a single polypeptide chain and appears to be a member of the G-protein-coupled receptor superfamily. However, the placental GnRH receptor(s) has not been identified or characterized. Thus, the goal of the studies described herein was to use primers based on the sequence of the human pituitary GnRH receptor to amplify, clone, and sequence transcripts of interest and to determine their homology to the human pituitary GnRH receptor sequence. The data generated would contribute to our understanding of the similarities and differences between the hypothalamic/pituitary and placental GnRH axes, which will in turn contribute to our overall understanding of hCG regulation.

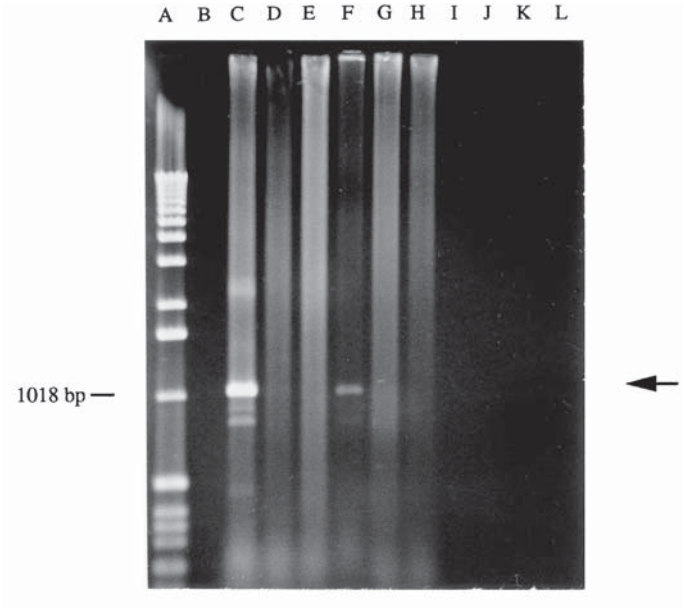

Fig. 1. RT-PCR amplification of an 8-wk placental RNA using primers designed for amplication of the human pituitary GnRH receptor. DNA marker—lane A. Note the ~1051 bp predicted band amplified from human pituitary mRNA (lane C) and from 8-wk human placental mRNA (lane F). A control lacking RNA is shown in lane I. For each sample, use of only the sense primer did not result in an amplified band (lanes D, G, and J), nor did amplification with only the antisense primer (lane E, H, and K).

Results

GnRH Receptor mRNA Amplification, Cloning, and Sequencing

GnRH has been shown to act through a putative receptor to regulate the secretion of hCG, maternal serum concentrations of which increase exponentially for the first 6 wk of gestation, peak at weeks 8–10, and then decrease throughout the second trimester to basal levels *(1–7)*. The elevated levels of hCG in early pregnancy are consistent with its role in maternal recognition of pregnancy, i.e., binding of hCG to the LH receptor in the ovary to perpetuate the secretion of progesterone and thus perpetuate the pregnancy. Early first-trimester placental samples (5–8 wk) were therefore selected for gene amplification studies using primers based on the sequence of the human pituitary GnRH receptor *(8)*. After 35 cycles of amplification (1 × 35), only a single 8-wk placental sample showed a band of the predicted size of the GnRH receptor transcript (~1051 bp) (Fig. 1, lane F). Figure 1 shows a prominent band of ~1051 bp which resulted from amplification of reverse-transcribed human pituitary mRNA (lane C). When the amplification was carried out on the human pituitary sample with only the sense primer (lane D) or only the antisense primer (lane E), no bands resulted, ruling out the possibility that a single primer could act as both the sense and antisense primer to amplify an unknown transcript. A distinct band resulting from the amplification of RNA from the 8-wk human placental tissue (lane F) was shown to be the same size as the band amplified from human pituitary (compare lanes C and F). Again, when amplification was carried out on human placental sample using only the sense primer (lane G) or the antisense primer (lane H), no bands resulted. Since an additional method control, when the reverse-transcribed GnRH receptor mRNA (RT #1) was omitted from the reaction mixture, no bands resulted, ruling out the possibility of amplification of a contaminant (lanes I–K).

Because amplification of the 8-wk placental sample satisfied all method controls, the amplified reaction mixture (RT-PCR #1) was used for ligation into a vector for subsequent cloning of GnRH receptor mRNA. Note that the amplification reaction was carried out at low stringency (54°C annealing temperature) to increase the probability of amplifying any transcripts similar to GnRH receptor mRNA. Following completion of the standard reverse transcriptase polymerase chain reaction (RT-PCR) reaction (RT-PCR #1) (1 × 35), 5 μL of the amplified reaction was transferred to a second tube with fresh reagents and subjected to an additional 35 cycles of amplification (2 × 35) (modified protocol A). These modifications served to increase the amount of GnRH receptor transcript available for ligation, which in turn resulted in more positive clones. Restriction digestion of the DNA from the cloned 8-wk placental sample revealed that 5 of 60 clones contained a single transcript of ~1051 bp.

Once the clones were sequenced, the GelMerge Program of the Genetics Computer Group (GCG) was used to compile the partial sequences from each individual clone into a full-length transcript. The GelAssemble Software was then used to compile the transcripts from the five individual clones into a consensus sequence. Sequence analyses revealed transcripts with a 987-bp sequence with 100% homology to human pituitary GnRH receptor mRNA (Fig. 2). The sequence appears to encode a single polypeptide sequence with seven probable transmembrane domains (Fig. 2, underlined) and two potential sites of N-linked glycosylation at the amino acids 18 and 102 (Fig. 2, bold print) *(11)*.

Experiments to Address False Negatives owing to Low GnRH Receptor mRNA Levels

Given the role of GnRH in the regulation of hCG, it seemed highly improbable that first-trimester human placenta would only be producing GnRH receptors in the 8th wk of gestation. To test for the possibility of false negatives owing to low levels of GnRH receptor mRNA, modifications to the RT-PCR procedure were employed to increase the amount of transcript available for amplification (modified protocol B). First, a standard RT reaction (RT #1) was carried out. A fourfold concentration of this reaction (RT #1) was used as the template in the PCR reaction (RT-PCR #2), which was carried out for 35 cycles (1 × 35). Five microliters of RT-PCR #2 were then transferred to a second tube with fresh reagents and subjected to

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    ATG GCA AAC AGT GCC TCT CCT GAA CAG AAT CAA AAT CAC TGT TCA GCC ATC AAC  54
 1  Met Ala Asn Ser Ala Ser Pro Glu Gln Asn Gln Asn His Cys Ser Ala Ile Asn

    AAC AGC ATC CCA CTG ATG CAG GGC AAC CTC CCC ACT CTG ACC TTG TCT GGA AAG 108
19  Asn Ser Ile Pro Leu Met Gln Gly Asn Leu Pro Thr Leu Thr Leu Ser Gly Lys

    ATC CGA GTG ACG GTT ACT TTC TTC CTT TTT CTG CTC TCT GCG ACC TTT AAT GCT 162
37  Ile Arg Val Thr Val Thr Phe Phe Leu Phe Leu Leu Ser Ala Thr Phe Asn Ala

    TCT TTC TTG TTG AAA CTT CAG AAG TGG ACA CAG AAG AAA GAG AAA GGG AAA AAG 216
55  Ser Phe Leu Leu Lys Leu Gln Lys Trp Thr Gln Lys Lys Glu Lys Gly Lys Lys

    CTC TCA AGA ATG AAG CTG CTC TTA AAA CAT CTG ACC TTA GCC AAC CTG TTG GAG 270
73  Leu Ser Arg Met Lys Leu Leu Leu Lys His Leu Thr Leu Ala Asn Leu Leu Glu

    ACT CTG ATT GTC ATG CCA CTG GAT GGG ATG TGG AAC ATT ACA GTC CAA TGG TAT 324
91  Thr Leu Ile Val Met Pro Leu Asp Gly Met Trp Asn Ile Thr Val Gln Trp Tyr

    GCT GGA GAG TTA CTC TGC AAA GTT CTC AGT TAT CTA AAG CTT TTC TCC ATG TAT 378
109 Ala Gly Glu Leu Leu Cys Lys Val Leu Ser Tyr Leu Lys Leu Phe Ser Met Tyr

    GCC CCA GCC TTC ATG ATG GTG GTG ATC AGC CTG GAC CGC TCC CTG GCT ATC ACG 432
127 Ala Pro Ala Phe Met Met Val Val Ile Ser Leu Asp Arg Ser Leu Ala Ile Thr

    AGG CCC CTA GCT TTG AAA AGC AAC AGC AAA GTC GGA CAG TCC ATG GTT GGC CTG 486
145 Arg Pro Leu Ala Leu Lys Ser Asn Ser Lys Val Gly Gln Ser Met Val Gly Leu

    GCC TGG ATC CTC AGT AGT GTC TTT GCA GGA CCA CAG TTA TAC ATC TTC AGG ATG 540
163 Ala Trp Ile Leu Ser Ser Val Phe Ala Gly Pro Gln Leu Tyr Ile Phe Arg Met

    ATT CAT CTA GCA GAC AGC TCT GGA CAG ACA AAA GTT TTC TCT CAA TGT GTA ACA 594
181 Ile His Leu Ala Asp Ser Ser Gly Gln Thr Lys Val Phe Ser Gln Cys Val Thr

    CAC TGC AGT TTT TCA CAA TGG TGG CAT CAA GCA TTT TAT AAC TTT TTC ACC TTC 648
199 His Cys Ser Phe Ser Gln Trp Trp His Gln Ala Phe Tyr Asn Phe Phe Thr Phe

    AGC TGC CTC TTC ATC ATC CCT CTT TTC ATC ATG CTG ATC TGC AAT GCA AAA ATC 702
217 Ser Cys Leu Phe Ile Ile Pro Leu Phe Ile Met Leu Ile Cys Asn Ala Lys Ile

    ATC TTC ACC CTG ACA CGG GTC CTT CAT CAG GAC CCC CAC GAA CTA CAA CTG AAT 756
235 Ile Phe Thr Leu Thr Arg Val Leu His Gln Asp Pro His Glu Leu Gln Leu Asn

    CAG TCC AAG AAC AAT ATA CCA AGA GCA CGG CTG AAG ACT CTA AAA ATG ACG GTT 810
253 Gln Ser Lys Asn Asn Ile Pro Arg Ala Arg Leu Lys Thr Leu Lys Met Thr Val

    GCA TTT GCC ACT TCA TTT ACT GTC TGC TGG ACT CCC TAC TAT GTC CTA GGA ATT 864
271 Ala Phe Ala Thr Ser Phe Thr Val Cys Trp Thr Pro Tyr Tyr Val Leu Gly Ile

    TGG TAT TGG TTT GAT CCT GAA ATG TTA AAC AGG TTG TCA GAC CCA GTA AAT CAC 918
289 Trp Tyr Trp Phe Asp Pro Glu Met Leu Asn Arg Leu Ser Asp Pro Val Asn His

    TTC TTC TTT CTC TTT GCC TTT TTA AAC CCA TGC TTT GAT CCA CTT ATC TAT GGA 972
307 Phe Phe Phe Leu Phe Ala Phe Leu Asn Pro Cys Phe Asp Pro Leu Ile Tyr Gly

    TAT TTT TCT CTG TGA  987
325 Tyr Phe Ser Leu *
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Fig. 2. The placental GnRH receptor cDNA sequence. The nucleotide sequence is represented in the upper line and is numbered on the right side, whereas the predicted amino acid sequence is the represented in the lower line and is numbered on the left side of the figure. The underlined segments indicate the seven probable transmembrane domains, the potential N-linked glycosylation sites (amino acids 18 and 102) are in bold print, and the stop codon is denoted by an asterisk (*).

an additional 35 cycles of amplification (2 × 35). This approach served to increase the number of GnRH receptor transcripts available for cloning by both increasing the amount of template to be amplified and doubling the number of cycles.

Previously the 8-wk placental sample shown in Fig. 3, lane H, was the only one to show a transcript of the predicted 1051 bp using a standard protocol. Using modified protocol B, bands of ~1051 bp are evident in the 6-wk human placenta (Fig. 3, lane D) as well as in two additional 8-wk placentas (lanes F and G). However, the 5-wk (lane C) and 7-wk (lane E) placental samples still did not demonstrate a band of the predicted size under these conditions. Reverse-transcribed GnRH, αhCG, and β-hCG mRNAs were all amplified in all placental samples studied (data not shown). Thus, it is improbable that the absence of a 1051-bp band was owing to a problem of the general health of the tissue or sample degradation.

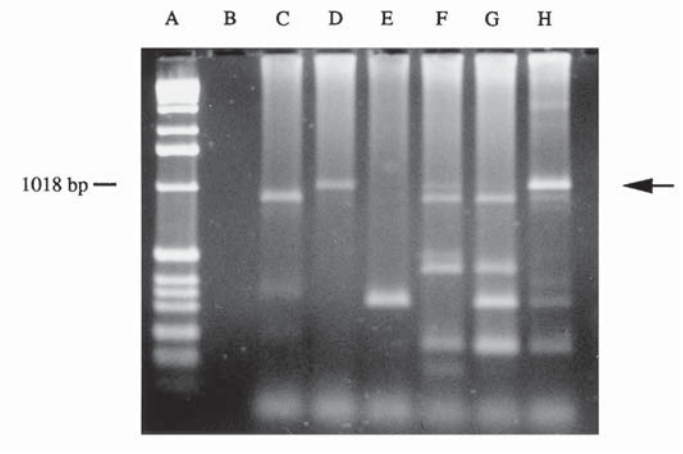

Fig. 3. RT-PCR amplification of first-trimester placental samples using primers designed for amplification of the human pituitary receptor. DNA marker—lane A. Note the presence of the ~1051 predicted band amplied from a 6-wk placenta (lane D) and three 8-wk placentas (lanes F, G, and H). In contrast, the 5-wk (lane C) and 7-wk (lane E) placentas failed to amplify the band in question.

Discussion

In this study, we have shown the sequence of a placental GnRH receptor cDNA to be the same as the pituitary GnRH receptor cDNA reported by Kakar et al. *(11)* and Chi et al. *(12)*. The pituitary receptor has been described as a 987 bp sequence that encodes a single polypeptide chain of 328 amino acids, is believed to be a member of the G-protein-coupled superfamily, and possesses seven probable hydrophobic transmembrane domains with an extracellular amino-terminus and an intracellular carboxy-terminus. The receptor also appears to have two potential N-linked glycosylation sites present at amino acids 18 and 102, several probable phosphorylation sites in the cytoplasmic domains at serine and threonine residues, and four extracellular cysteines, which may be involved in disulfide bonds acting to stabilize the structure of the functional protein. Although the cDNA sequence of the pituitary and placental receptors are identical, the placental receptor certainly may exhibit differences in its posttranslational processing. Given that the sequence of the placental GnRH receptor identified here and the pituitary receptor are identical, the placental receptor can be expected to retain many of the same properties. In these studies, the amplification of a placental GnRH receptor mRNA was carried out at low stringency (54°C annealing temperature) to increase the probability of amplifying "GnRH receptor-like" transcripts. Sixty clones were sampled to derive the data reported. Nonetheless, the possibility remains that more extensive sampling might reveal another form of placental GnRH receptor mRNA present in small amounts or the use of first-trimester placental libraries which were not available at the time these studies were initiated

These findings are surprising in light of binding data, which suggested the receptors may differ. Currie et al. *(13)* first demonstrated that native GnRH binds both the pituitary and placental receptors with the same affinity ($K^A = 6.2 \times 10^7 M^{-1}$). However, GnRH agonists bind the pituitary receptor at a 100-fold greater affinity than they bind the placental receptor. This suggested the pituitary and placental receptors differ. Three hypotheses have been put forth as possible explanations for this difference. First, because GnRH agonists contain d-amino acids substituted to render them resistant to degradation, Currie et al. *(13)* suggested the placenta may possess large amounts of a protease that acts on sites within the agonists not protected by d-amino acid substitutions. Thus, the agonist is degraded into fragments that bind the receptor with an affinity similar to the native peptide. However, Bramley et al. *(14)* studied the inactivation of various GnRH tracers following incubation with either rat pituitary or human placental membranes. It was shown that although different isoforms were degraded at different rates in these tissues, differences in the ability of the isoforms to bind to placental vs pituitary sites were not related to differences in degradation of the tracers, but rather to differences in their specificity for the receptor. Second, it was hypothesized that GnRH or GnRH agonists bind to a placental site, which was in fact a GnRH-degrading enzyme. Menzies and Bramley *(15)* demonstrated that this was not the case and reported that GnRH binding appeared to be associated primarily with the placental plasma membrane. Third, Currie et al. *(13)* suggested placental GnRH differed structurally from hypothalamic GnRH, Thus, it was not the ligand intended for the placental GnRH receptor. Duello et al. *(10)* demonstrated a placental GnRH identical to hypothalamic GnRH. However, sequencing was carried out directly from an RT-PCR sample, rather than from a cloned product. Thus, the possibility of other forms of GnRH being present in lower quantities remained a possibility. To check for other forms of placental GnRH, we cloned and sequenced placental GnRH, but found no other forms (unpublished data). An additional fourth possibility is that the respective transcripts differ in their posttranslational processing yielding proteins that differ in their phosphorylation or glycosylations states. Last, but not least, the findings presented here may appear inconsistent with the findings of Currie et al. *(7)*, because the placentas studied were of different gestational ages. We studied human placental samples collected during the first trimester when serum hCG levels are elevated because of our interest in early pregnancy events. Currie and colleagues studied placentas collected at term when serum hCG levels have been low for approx 20 wk. Thus, it is possible different GnRH receptors are important at different times during gestation, or that the term placenta binding site identified is not a receptor *per se* or not a functional receptor.

The data presented offer possibilities concerning why the sequence of the placental GnRH receptor has eluded us. The technical modifications required to amplify placental GnRH receptor mRNA suggest *(1)* it is present in small

amounts or (2) if transcription equals translation, it will not accumulate giving the false impression that GnRH is not playing an active role at that "snapshot" in time. First, the fourfold increase of RT template in the PCR reaction (RT-PCR #2) and the addition of an another 35 cycles of amplification (2 × 35) acted to increase the amount of GnRH receptor transcript in the final amplified product. Thus, it was possible to amplify the ~1051 bp predicted band in placental samples that failed to produce a band using standard RT-PCR protocols. Second, even after all modifications were employed, the 5- and 7-wk placental samples still did not show a band of the predicted size, suggesting the levels of GnRH receptor mRNA were below the level of detection by the methods used or the trophoblast cells were actively transcribing the receptor mRNA, but it was translated as fast as it was expressed, with the result that there was no accumulation and therefore no detection. In addition, the possibility exists that the small fraction of the placenta sampled may simply not be active producers of GnRH. Thus, a thorough sampling would be necessary to compare trophoblast overlying the chorionic villi, in the trophoblastic columns that anchor the placenta to the endometrium, in the trophoblastic shell that surrounds the implanted blastocyst, infiltrating the decidua in the basal plate, within the spiral arteries at the placental site, and underlying the chorioamnion that surrounds the entire endometrial cavity in late gestation *(16)*. Under any circumstances, it would be premature to suggest an entire placenta is not involved in GnRH production on the basis of an absent band or an unlabeled collection of cells. Additional studies with more extensive sampling and more sensitive approaches will be required to understand fully the expression patterns of this receptor.

Then why do maternal concentrations of hCG decline across gestation? It does not appear to be a deficiency of GnRH, since molecular studies have shown the concentration of GnRH mRNA to remain constant across gestation *(9,10)* and immunocytochemical experiments have demonstrated the translated peptide in the second and third trimesters *(17,18)*. Lin et al. *(19)* reported that GnRH receptor mRNA levels paralleled the decline of maternal serum hCG concentrations on the basis of an *in situ* hybridization study using probes based on the sequence of the human pituitary receptor *(8)*. However, it is difficult to assess these results, since it is not clear whether the sampling was sufficient and nonspecific labeling does not appear to have been subtracted from total labeling. Results of our study would suggest the level of GnRH receptor mRNA differs between placental samples of the same gestational age. Further studies will be required to determine whether this pattern continues through the second and third trimesters.

In the midst of all of the unknowns, a possible explanation for the declining hCG levels involves a consideration of hormone dynamics along with placental cytoarchitecture. First, we have shown GnRH is both transcribed (*in situ* hybridization) and translated (immunocytochemistry) by the cytotrophoblast cell population *(10)*. Second, cytotrophoblast cells are known to fuse with the syncytiotrophoblast cell layer (thus, its multinucleated, syncytial appearance). Third, it is known the cytotrophoblast cell layer thins across gestation with few cells evident at term *(20)*. Thus, as the cytotrophoblast layer thins, there are fewer cytotrophoblasts in direct apposition to the syncytiotrophoblast. Therefore, though the placenta is producing GnRH, there are a decreasing number of cytotrophoblast cells in apposition to the syncytiotrophoblast. Therefore, less GnRH is delivered to the syncytiotrophoblast to supply sufficient GnRH stimulation to keep serum hCG levels elevated. This would be true under both an autocrine and paracrine model of secretion.

In conclusion, the demonstration of a human placental GnRH receptor mRNA that is identical to its human pituitary counterpart is consistent with the high degree of conservation evident in reproductive systems. GnRH in the hypothalamus or its anlage is believed to be 500 million years old, whereas placentation is believed to be 85–100 million years old *(21)*. Thus, it appears the placenta has "borrowed" a very highly conserved process from the hypothalamus and pituitary, which ensures that ovulation will occur, and has used it to ensure that the now fertilized egg will implant and successfully proceed to term. Given the significant body of data documenting the effect of native GnRH on hCG secretion, there is reason to believe the transcript identified in this article may well encode a physiologically significant receptor through which GnRH mediates the secretion of hCG. The possibility that additional placental GnRH receptors might exist is a very interesting topic worthy of additional study, the existence of which need not be in conflict with the findings presented here.

Materials and Methods

Collection of Tissue / RNA Isolation

The University of Wisconsin Human Subjects Committee Human approved collection of human pituitary tissue from autopsies as well as human placental tissues (5–8 wk of gestation) from local sources following elective termination. Total cellular RNA from the tissue was prepared by the guanidium isothiocyanate/cycle method *(22)*. Concentrations were determined spectrophotometrically.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analyses

RT-PCR analyses were used to amplify GnRH receptor from the human placental samples. Five micrograms of total RNA were reverse-transcribed in a final volume of 20 μL. The reaction mixture included 1.0 x 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 Corporation, 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 for 2 h at 42°C, heated to 95°C for 10 min, and quenched on ice (RT #1).

Primers for human placental GnRH receptor based on the human pituitary GnRH receptor mRNA sequence *(11, 12)* follow:

5' sense 5' ATGGCAAACAGTGCCTCTC 3'
(bases 56–74)
3' antisense 5' TCCCAGATGGAGAGATTC 3'
(bases 1087–1104)

For the initial amplifications, 5 μL of RT were amplified in a final volume of 50 μL. The reaction mixture included 1X 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 Corp.), and the primers shown above (Operon Technologies Inc., Alameda, CA). Amplification conditions for each transcript were 35 cycles (1 × 35) 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 (RT-PCR #1). This procedure was followed for the data shown in Fig. 1.

In subsequent experiments, two modifications were employed. In modified protocol A, 5 μL of RT-PCR #1 were transferred to a second tube with a fresh reagents and subjected to an additional 35 cycles of amplification (2 × 35). This procedure was used to produce the amplified reaction mixture used for cloning. In modified protocol B, RT #1 was as stated above. However, a fourfold concentration of RT #1 was aliquoted into a fresh PCR reaction mixture (identical concentrations) to produce RT-PCR #2. This sample was then used as starting material for a second round of 35 cycles of amplification (2 × 35) as detailed in modified protocol A. These procedures were followed to generate the data shown in Fig. 3.

The PCR products were electrophoresed on 1% agarose gels, stained with ethidium bromide, and visualized using a UV illuminator.

Cloning

An aliquot of RT-PCR #1 amplified 2 x 35 cycles from an 8-wk placental sample was used for ligation (1:1) into the pCR™2.1 vector using the Original TA Cloning Kit (Invitrogen Corp., San Diego, CA) transformed with TOP10F' OneShot™ Competent cells. Following ligation and transformation, the transformed cells were plated on LB agar plates and incubated at 37°C for a minimum of 18 h. They were then held at 4°C for 2–3 h to allow proper color development of white colonies, which correspond to interruption of the *lac*Z gene. A portion of the plasmid DNA was recovered using the alkaline lysis miniprep protocol *(23)*, which was analyzed by restriction digestion with *Eco*R1 restriction enzyme (Promega Corporation,) to identify 5 of 60 clones with transcripts of the predicted size. The remaining plasmid DNA was prepared for sequencing using the Qiagen Plasmid Mini kit (Qiagen, Inc., Chatsworth, CA).

Sequence Analyses

An ABI Automated DNA Sequencer using dideoxy-NTP chemistry was used to determine the forward and reverse sequences of five GnRH receptor clones (Protein/DNA Technology Center, Rockefeller University, New York, NY). The initial sequencing used the following universal primers:

M13 Forward (-47)
5' CGC CAG GGT TTT CCC AGT CAC GAC 3'
M13 Reverse
5' CAG GAA ACA GCT ATG AC 3'

Additional sequencing was accomplished using primers (Operon Technologies Inc., Alameda, CA) based on internal domains of the human pituitary GnRH receptor mRNA *(11,12)*.

5' sense 5' CTA GCT TTG AAA AGC AAC AG 3'
(bases 494–513)
3' antisense 5' CAT TGC AGA TCA GCA TGA T 3'
(bases 731–749)
5' sense 5' GCT CTC AAG AAT GAA GCT GC 3'
(bases 271–290)
3' antisense 5' GAC ATA GTA GGG AGT CCA GC 3'
(bases 891–910)

The GelMerge and GelAssemble Programs of the Wisconsin Sequence Analysis Package Software (Version 9.2) of the Genetics Computer Group (Madison, WI) was used to compile and assemble the individual sequences into a full-length transcript. The Bestfit Program was then used to compare the putative human placental GnRH receptor mRNA sequences to the human pituitary GnRH receptor mRNA sequence.

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