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Up-Regulation of the Human Prolactin Receptor in the Endometrium

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In humans, uterine endometrial stromal cells differentiate (decidualize) into decidual cells that express prolactin (PRL). Decidual PRL expression continues throughout pregnancy, thus decidual cells lining fetal membranes of term placenta synthesize and secrete PRL. To examine the hypothesis that PRL may play an autocrine role in the decidual cells, we examined the expression of the PRL receptor (PRL-R) during in vitro decidualization of stromal cells and in term decidua. In endometrial stromal cells decidualized by treatment with 1 μ*M* medroxyprogesterone and 10 n*M* estradiol for 3, 6, and 9 d, respectively, a 12.7 kb PRL-R transcript increased 3–3.5-fold, 16.5–17-fold, and 23.5–24-fold, respectively, compared with untreated controls, in duplicate experiments. Progesterone-dependent PRL-R and PRL expression were stimulated by 1 μ*M* prostaglandin E_2. Term decidua expressed the long form of the PRL-R and five major PRL-R transcripts (12.7, 9.7, 7.0, 3.6, and 2.8 kb). In contrast, human liver expressed two major transcripts (12.7 and 9.7 kb) while HepG2 cells expressed a single 7.0-kb-sized transcript. These studies demonstrate that PRL-R expression is stimulated upon progesterone-induced PRL gene expression in endometrial stromal cells supporting the hypothesis that PRL may have an autocrine effect in the endometrium and decidua.

Key Words: Decidualization; endometrium; prolactin; receptor; human.

Introduction

In humans, decidualized (differentiated) stromal cells of the uterine endometrium and decidual cells lining fetal membranes of the placenta, synthesize and release large amounts of prolactin (PRL) (Andersen, 1990; Handwerger et al., 1991). Although the function(s) of PRL in these tissues is poorly defined, the identification of PRL receptor (PRL-R) mRNA, specific PRL binding sites, and PRL-R protein in human decidua, placenta, and fetal membranes supports a putative autocrine/paracrine action of PRL (McWey et al., 1982; Maaskant et al., 1996). The initial step in the biological action of PRL involves its interaction with a specific transmembrane receptor, which belongs to the cytokine receptor family whose members share similar extracellular structural domains (Kelly et al., 1992). A single gene encodes the PRL-R in humans, yet six major protein species of the PRL-R have been identified in decidua and placenta by Western blot analysis using a rat monoclonal antibody against the extracellular domain of the PRL-R (Kelly et al., 1992; Maaskant et al., 1996). Apart from a recent study of human chorio-decidual cells, in which presence of mutiple PRL-R transcripts was detected (Maaskant et al., 1996), there are few studies of the PRL-R in normal human tissues. Human hepatoma cells (HepG2) and breast cancer cells have been used to clone and characterize the human PRL-R (Boutin et al., 1989; Clevenger et al., 1995). We determined the species of PRL-R in human term decidual cells compared with liver, and present evidence that differential transcription initiation occurs in the 5' region of the PRL-R gene. We also examined whether the PRL-R is expressed in human uterine endometrial stromal cells and if PRL-R mRNA expression is regulated during stromal cell decidualization. We and others have developed an in vitro model to study decidualization in human endometrial stromal cells, in which PRL expression can be used as a marker of decidualization (Irwin et al., 1989; Brar et al., 1995). In this model, progesterone plays a critical role in stromal cell decidualization, as in vivo, whereas several growth factors and other agents modulate progesterone-dependent decidualization (Fay and Grudzinskas, 1991; Irwin et al., 1991; Tabibzadeh, 1991). We also determined if PRL-R mRNA levels are regulated by prostaglandin E_2 (PGE_2), which enhances progesterone-dependent decidualization and PRL expression in human endometrial stromal cells (Frank et al., 1993).

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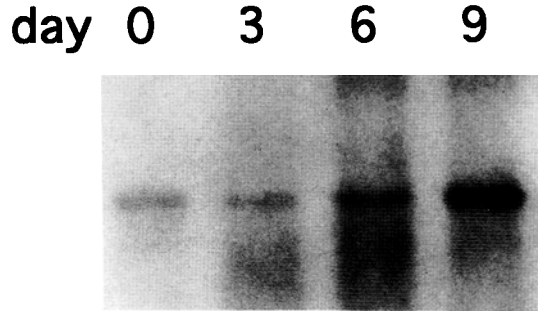

Fig. 1. Northern blot analysis of cultured human endometrial stromal cells (10 μg total RNA). Levels of PRL-R mRNA (12.7-kb-sized transcript) during decidualization of human endometrial stromal cells were progressively increased upon treatment of cells with cultured 1 μ*M* MPA and 10 n*M* E_2 for 3, 6, and 9 d. Following electrophoresis and transfer to nylon membranes, the samples were hybridized with ^{32}P-labeled PRL-R cDNA probe (H1/H2 clone) and quantified by Phosphorimage analysis.

Results

Expression of PRL-R mRNA is Increased During Decidualization of Endometrial Stromal Cells

There is a progressive increase in PRL synthesis and release by stromal cells treated continuously with medroxyprogesterone acetate (MPA) and estradiol (E_2) beginning at approx 3–6 d of treatment (Brar et al., 1995). To determine if PRL-R mRNA levels are also induced by MPA and E_2, Northern blot analysis was performed using total RNA from cells treated with MPA and E_2 for 3, 6, and 9 d. A single 12.7-kb-sized transcript was detected by Northern blot analysis of human endometrial stromal cells before (day 0) and following treatment (Fig. 1). PRL-R mRNA levels were increased signficantly ($p < 0.01$) with MPA and E_2 treatment for 3–6 d. Upon exposure of stromal cells to MPA and E_2 for 3, 6, and 9 d, PRL-R mRNA levels increased 3–3.5-fold, 16.5–17-fold, and 23.5–24-fold, respectively, compared with untreated controls, in duplicate experiments.

PGE_2 Stimulates PRL-R mRNA Levels in Endometrial Stromal Cells

Progesterone-dependent PRL release is significantly ($p < 0.01$) enhanced upon coincubation of endometrial stromal cells with 1 μ*M* PGE_2, MPA, and E_2 (Frank et al., 1993). We examined whether PRL-R mRNA levels, in addition to PRL, were also altered with PGE_2 treatment by ribonuclease protection assays using an RNA probe complementary to the 5' region of the PRL-R (*see* Fig. 4B, probe 1, later in article). PRL-R mRNA levels in endometrial stromal cells treated with PGE_2 were approx 12-fold greater than cells cultured in the absence of PGE_2 (Fig. 2, lane 1). In addition to an increase in the doublet of protected bands (arrow PRL-R), smaller sized transcripts were also detected in stromal cells treated with PGE_2 in combination with MPA and E_2 (lane 2).

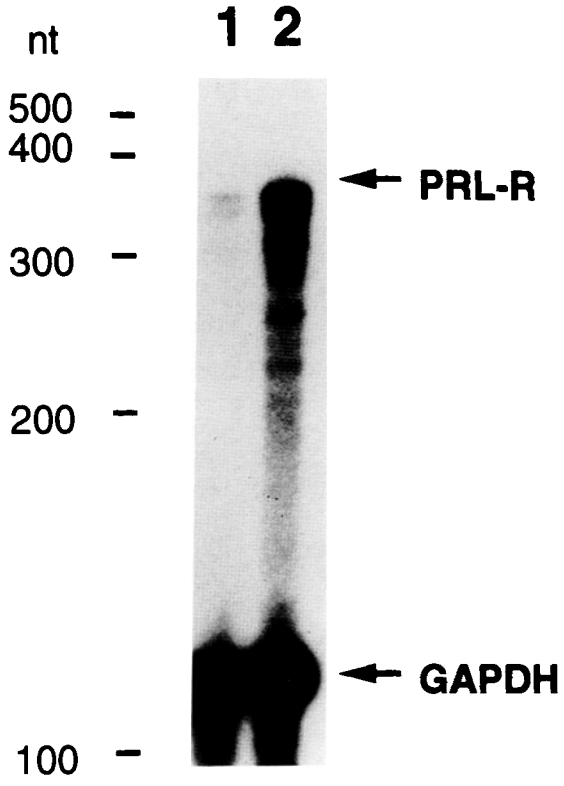

Fig. 2. Ribonuclease protection assays of endometrial stromal cells was performed using probe 1 (Fig. 4B) which hybridizes to the 5' region of human PRL-R. Five micrograms of total RNA was analyzed from stromal cells treated with MPA and E_2 for 24 d; (lane 1) or with 1 μ*M* PGE_2 in combination with MPA and E_2 (lane 2). The simultaneous detection of GAPDH transcripts was used to normalize the intensity of the signal for PRL-R for the same sample upon analysis of the data by Phosphorimage screens. Transcription of a doublet of protected fragments present in control cells was increased in PGE_2-treated cells (PRL-R arrow).

Multiple PRL-R Transcripts Expressed in Decidua

We next examined whether decidual cells of pregnancy also express a single-sized PRL-R transcript, seen in endometrial stromal cells during the menstrual cycle (Fig. 1). Expression of PRL-R mRNA species in human decidua was also compared with transcripts in liver and HepG2 cells, which has not previously been reported. Northern blot analysis of total RNA from term decidua using a human PRL-R cDNA probe showed that at least five major PRL-R transcripts are expressed. The sizes of the PRL-R transcripts are 12.7, 9.7, 7.0, 3.6, and 2.8 kb (Fig. 3, lane 2). In contrast, human liver expressed PRL-R transcripts of 12.7 and 7.0 kb whereas HepG2 cells the major PRL-R transcript expressed was 7.0-kb size (Fig. 1, lanes 1 and 3, respectively).

To determine if the multiple PRL-R transcripts in decidua and liver represent alternatively transcription or splicing in the 5' or 3' regions of the PRL-R domains, ribonuclease protection assays were performed. RNA probes extending to different 5' regions of the PRL-R were used in the assay (Fig. 4, probes 1–3). Transcription of multiple-sized products occurred, which were similar in decidua and

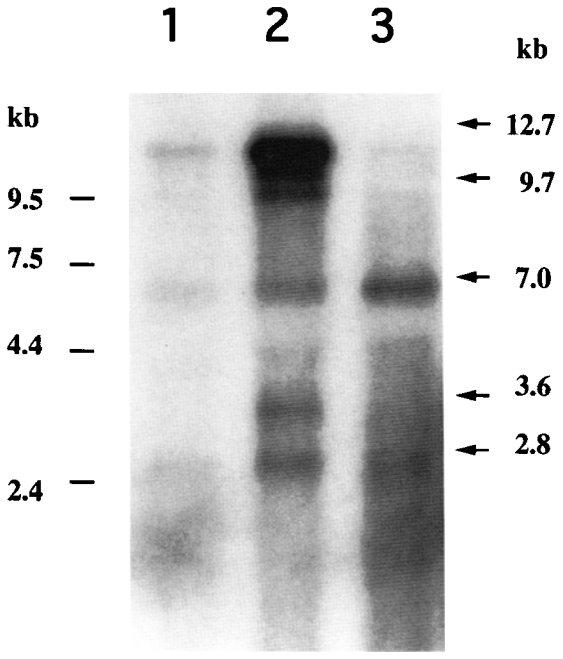

Fig. 3. Northern blot analysis of PRL-R mRNA from human liver, decidua, and HepG2 cells. Lane 1: human liver (10 μg total RNA); lane 2: human decidual tissue (10 μg total RNA); lane 3: Hep G2 cells (5 μg of poly A^+ RNA). The membrane was hybridized with ^{32}P labeled PRL-R cDNA (H1/H2 clone, 2556 bp). On the left, the position of molecular weight markers is indicated, and on the right arrows indicate the sizes of the PRL-R transcripts detected.

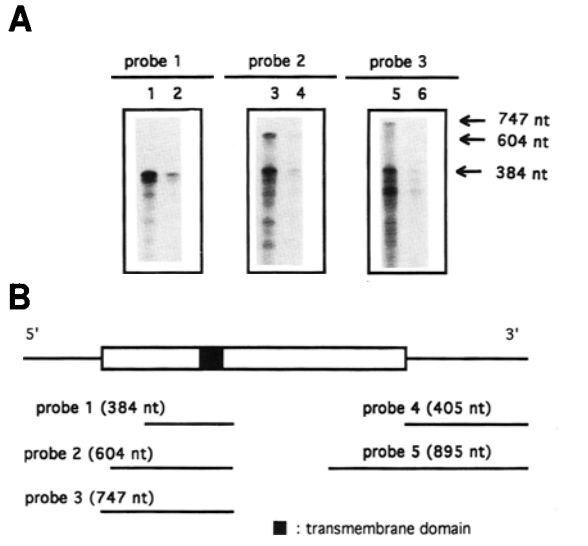

Fig. 4. Transcription of PRL-R mRNA in human decidua and liver by ribonuclease protection analysis: Fig. 4A, lanes 1, 3, and 5 shows human decidua (5 μg total RNA); lanes 2, 4, and 6 shows human liver (10 μg total RNA). Probes 1–3 in the 5' region of human PRL-R mRNA were used in the assays as indicated schematically in Fig. 4B. The sizes of the probes are indicated in parenthesis.

liver, with a prominent doublet at approx 384 nt with probe 1 (Fig. 4A). When probes encoding the 3' region of PRL-R (Fig. 4B, probes 4 and 5) were used in similar experiments, single-sized protected fragments were detected in RNA from decidua and liver (data not shown).

RT-PCR Analysis of PRL-R in Decidua

Since multiple-sized PRL-R transcripts are expressed in decidua and liver, we used RT-PCR with Southern blot analysis to determine whether one or more of the transcripts represent deletions in the extracellular or intracellular domains of the PRL-R previously characterized in HepG2 cells and breast cancer (T-47D) cells (Boutin et al., 1989). A single PCR product of the predicted size was detected using primers for each of the domains in HepG2 cells, decidual cells, and liver (Fig. 5). Sequence analysis of the PCR products from decidual cells revealed that the nucleotide sequence of this PRL-R mRNA in decidua was identical to regions of the PRL-R identified in HepG2 cells and breast cancer (T-47D) cells (Boutin et al., 1989).

Discussion

The present article demonstrates that in human endometrial stromal cells, expression of PRL-R mRNA increases in parallel with progesterone-dependent induction of the PRL gene and decidualization. In addition, PGE_2, which enhances the ability of progesterone to induce expression of the PRL gene (Frank et al., 1993), also stimulated expression of the PRL-R in endometrial stromal cells. Moreover, human decidual cells at term, which express high levels of PRL, also express the PRL-R gene. These findings suggest that both the PRL-R and PRL are present in decidualized uterine endometrium prior to blastocyst implantation and probably through pregnancy to term in decidual cells. Whereas the cultured decidual cell preparations used in our studies may represent a heterogenous population of cells including large granular lymphocytes that may also express PRL-R mRNA, the decidual cells represent the majority (approx 90–95%) of the population.

We found multiple (at least five major species) PRL-R transcripts are expressed in human decidual cells. Three mRNA species (10.5, 3.5, and 2.8 kb) identified by Northern blot analysis have been reported in human choriodecidua and placental trophoblast cells (Maaskant et al., 1996). We identified two additional species of the PRL-R (12.7 and 7.0 kb) in human decidua using a modification of the standard Northern blot analysis procedure optimized to increase sensitivity (Beckers et al., 1994). Our studies suggest that differential splicing or alternative transcription start sites in the 5' region of the PRL-R gene could result in multiple PRL-R transcripts in human decidual cells and liver. Multiple transcripts in other species that all have a single PRL-R gene, arise either as a result of alternative splicing of the PRL-R gene in rodents and sheep or because of differential splicing in the 5' and 3' untranslated region of the PRL-R gene in rabbits (Shirota et al., 1990; Dusanter-Fourt et al., 1991; Anthony

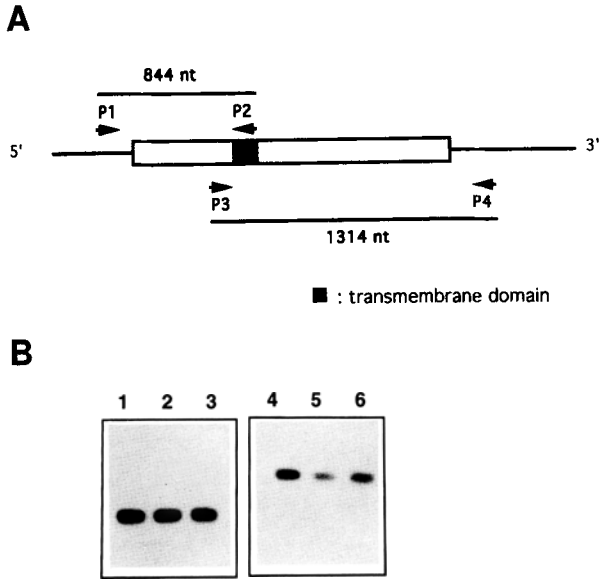

Fig. 5. Schematic representation of the human PRL-R. Oligonucleotide sense and anti-sense primer pairs (P1-4) used as primers for RT-PCR and the predicted sizes of the PCR products are indicated **(A)**. Additional antisense primers (not shown) selected within in the PCR products were used as probes for each Southern blot analysis **(B)**. Lanes 1–3 shows the 844 bp product obtained using primers P1 and P2 and DNA from HepG2 (lane 1); decidua (lane 2); human liver (lane 3); lanes 4–5 show the 1314 bp product obtained using primers P3 and P4 and DNA from HepG2 (lane 4); decidua (lane 5); and human liver (lane 6).

et al., 1995). It is likely that similar mechanisms that give rise to PRL-R isoforms in these animals or the presence of incomplete transcripts, result in expression of some of the additional PRL-R transcripts in human decidua. The functional significance of multiple sized PRL-R transcripts and also several protein species (Maaskant et al., 1996) in human decidual cells as in other species and tissues, remains to be determined. We detected high expression of a single 12.7-kb PRL-R transcript in endometrial stromal cells whereas the decidua expressed at least five major species including a 12.7-kb PRL-R transcript. The presence of multiple PRL-R species in decidual cells may be indicative of a more diverse role of PRL in the decidua during pregnancy compared with that in the endometrium during the menstrual cycle.

In humans, the sequence of the PRL-R has been deduced from complementary DNA clones in HepG2 hepatoma cells, T-47D breast cancer cells, fetal chorionic larvae, and lymphoid cells (Boutin et al., 1989; Pellegrini et al., 1992). Two naturally occurring iso-forms (a long-form 82 kDa and a short-form 42 kDa) of the PRL-R that differ in their cytoplasmic domains, have been cloned in several organs from different species (Boutin et al., 1988; Shirota et al., 1990). The structure of the PRL-R, characterized in the relatively few human cells to date (Boutin et al., 1989; Pellegrini et al., 1992), is similar to the long form of the PRL-R identified in the rat liver and rabbit mammary gland (Edery et al., 1989; Kelly et al., 1989). Our studies indicate that the long form of the PRL-R is present in human decidua and liver, the sequence of which is homologous to the form previously cloned from HepG2 cells (Boutin et al., 1989). We also demonstrated that the PRL-R mRNA species expressed in the human liver (12.7 and 9.7 kb) are larger than in Hep G2 cells, which express a single 7.0-kb-sized transcript that has not previously been reported. In contrast to the human liver, in the rat liver, the short form of the PRL-R predominates, and the sizes of PRL-R RNA transcripts are much smaller ranging in size from 5.5–1.8 kb (Shirota et al., 1990).

The expression of intermediate sized (60–62 kDa) isoforms of the PRL-R have been reported in Nb2 lymphoma and human breast carcinoma, which arise as a result of deletions in the cytoplasmic domain of the long form of the PRL-R (Ali et al., 1991; Clevenger et al., 1995). Our PCR analysis studies do not indicate the presence of the intermediate form of the PRL-R in the decidua or liver arising as a result of a deletion of the cytoplasmic region of the long form of the PRL-R, as reported in human breast cancer cells or Nb2 cells (O'Neal et al., 1991; Clevenger et al., 1995). In contrast to rat tissues that express both the long and short isoforms of the PRL-R, there is no evidence that normal human tissues express this short isoform of the PRL-R (Kelly et al., 1991; Nagano and Kelly, 1994). The presence of novel isoforms of the human PRL-R that may arise either upon translation of some of the transcripts identified in the decidua or because of small deletions, was not investigated and remains to be determined.

The levels of PRL-R mRNA in human endometrial stromal cells are stimulated by progesterone upon induction of the PRL gene supporting the hypothesis that PRL may up-regulate the expression of its receptor in decidualized human endometrial cells. An autocrine role of PRL is suggested by the finding that elevated PRL-R levels occur in PRL-secreting tumors from patients with markedly increased serum PRL levels (Ciccarelli et al., 1995). Circulating concentrations of PRL have also been demonstrated to regulate the number of PRL binding sites in the rat hypothalamus (Muccioli and Di Carlo, 1994). However, PRL has also been reported to down-regulate its receptor expression in rat and mouse peripheral lymphocytes and Nb2 cells (O'Neal et al., 1991; Di Carlo et al., 1995). These differences in the regulation of PRL-R expression may be indicative of tissue-specific functions of the PRL gene that is known to have diverse effects in a range of tissues. The effect of progesterone on PRL-R may also be tissue-specific. Whereas we found that progesterone stimulated PRL-R expression in human endometrial stromal cells, in the mouse mammary gland progesterone inhibits PRL-R mRNA levels (Nishikawa et al., 1994). Therefore, the different effects of PRL and progesterone on the PRL-R must be interpreted as a reflection of the role(s) of PRL in a specific tissue.

In conclusion, our studies demonstrate an increase in the expression of the PRL-R during progesterone-dependent decidualization of the human endometrium that is characterized by the induction of PRL expression. Increased translation of PRL-R mRNA may not be required to increase the number of active PRL-R proteins and an increase in PRL-R mRNA levels during decidualization may not directly represent a higher number of active PRL-R protein species. Moreover, coexistence of PRL and the PRL-R in the same tissue does not necessarily mean that the locally produced PRL acts on the local PRL-R. However, parallel increases in PRL and its receptor support the possibilty that PRL could have an autocrine function in the human endometrium and decidua.

Materials and Methods

Tissue and Cell Preparation

Decidual tissue was obtained at term and decidual cells were obtained after dissection away from amniotic and chorionic membranes as previously described (Markoff et al., 1983). Decidual and Hep G2 cells (American Type Culture Collection, Rockville, MD) used were cultured on plastic in Dulbeccois Modified Medium (DMEM) containing 4500 mg/l glucose (Gibco-BRL Life Technologies Inc., Grand Island, NY) with 10% fetal bovine serum (Hyclone Laboratories Inc., Logan, UT) and 25 U/mL penicillin G, 25 μg/mL streptomycin, and 2.5 μg/mL amphotericin B. Uterine endometrial tissue was obtained from women with normal menstrual cycles at the time of elective tubal ligation. Informed consent was obtained from patients and the study was approved by the Institutional Review Boards of Children's Hospital Medical Center and the University of Cincinnati. Proliferative or secretory phase endometrium was removed by suction biopsy and stromal cell cultures were prepared as previously described (Brar et al., 1995). Enriched cultures of stromal cells were obtained using a method of selective attachment to plastic and brief trypsinization of subcultures. Stromal cell cultures prepared by this method were verified to contain less than 0.1% of nonstromal cells using antisera against vimentin and cytokeratin as immunohistochemical markers (Irwin et al., 1989). Stromal cells were cultured in DMEM/ 2% FBS, 25 U/mL penicillin G, 25 μg/mL streptomycin, and 2.5 μg/mL amphotericin B.

In Vitro Decidualization of Human Endometrial Stromal Cells

Decidualization of endometrial stromal cells was induced by incubating subconfluent cells in media containing 1 μ*M* MPA (Sigma Chemical Co., St. Louis, MO) and 10 n*M* E_2 (Sigma) as previously described (Brar et al., 1995). These steroids were dissolved in ethanol and added to the media before use. The final concentration of ethanol in the media never exceeded 0.09% (v/v). Phase contrast microscopy was used to verify morphological changes associated with differentiation in vitro in response to MPA and E_2 (Irwin et al., 1989; 1991). In some experiments, cells were treated with 1 μ*M* PGE_2 (Sigma) in combination with MPA and E_2. PRL secretion used as a marker of decidualization was measured in conditioned media by standard radioimmunoassay (Golander et al., 1979).

Northern Blot RNA Analysis

Total RNA from cells and tissue was isolated by the guanidium thiocyanate-phenol-chloroform extraction method of Chomczynski and Sacci (1987) using Tri-reagent (Molecular Research Center Inc., Cincinnati, OH). Ten micrograms of total RNA was fractionated by electrophoresis through 1% agarose-6% formaldehyde gels in HEPES buffer. Following fixation of the RNA with mild alkali treatment, the RNA was transferred to a positively charged nylon membrane (Nytran, Schleider and Schuell, Keen, NH) as previously described (Beckers et al., 1994). Blot hybridization was performed at 65°C with cDNA of human PRL-R; (H1/H2 clone, 2556 bp, gift from Dr. P. Kelly, Paris, France). The probes (25 ng) were labeled with [^{32}P]dCTP by random priming (Prime-It labeling kit, Stratagene Cloning Systems, La Jolla, CA), which generated probes with specific activities of 1–3 × 10^9 dpm/μg. Hybridization signals were quantified by exposure of blots to Phosphorimager screens followed by analysis of the bands using ImageQuant 3.2 software (Molecular Dynamics, Sunnyvale, CA). The values obtained are expressed as relative arbitary units. Ethidium bromide staining of the gel prior to transfer of the RNA to a blot showed that equal amounts of total RNA were loaded in each lane.

Analysis of RNA by Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Sequencing

Total RNA (200 ng) was first reverse transcribed using M-MLV reverse transcriptase (Gibco-BRL) and oligo $(dT)_{16}$ primers. The reverse transcribed RNA was then amplified by PCR for 35 cycles using specific primers encoding different regions of human PRL-R: Oligonucleotide sequences used for PCR of human PRL-R (Boutin et al., 1989), shown schematically in Fig. 5A, were as follows: sense primer (P1) 5'-TGAGGATGCTTTCCACATGAACC located at -72 to -49 nucleotides and antisense primer (P2) 5'-AAGCCACTGCCCAGACAATAATC located at 772 to 749 nucleotides gave a 844-bp product; sense primer (P3) 5'-AGTGCATGGAGTCCAGCGAC located at 646 to 665 nucleotides and antisense primer (P4) 5'-GTCTGACTACATTCTTGAGCATTTC located at 1959 to 1937 nucleotides gave a 1314-bp product. For each sample, primers for GAPDH (Brar et al., 1996) were added to the same reaction tube as those used to amplify the PRL-R. The denaturing temperature was 94°C for 1 min, annealing temperature was 65°C for 1 min, and extending temperature was

72°C for 2 min. The PCR products obtained were electrophoresed on a 1% agarose gel, stained with ethidium bromide, then transferred to a nylon membrane (Nytran). Southern hybridization was performed with ^{32}P-end labeled oligonucleotide sequences that were specific for a region each of the predicted PCR products (Fig. 1A). Signals on autoradiographs were quantified by Phosphorimage analysis (Molecular Dynamics). In addition, the PCR products obtained were subcloned into the pCRII plasmid using the TA cloning kit (Invitrogen, Corporation, San Diego, CA) and sequenced using a DNA sequencing kit (Sequenase version 2.0, United States Biochemical, Cleveland, OH) as directed by the manufacturers. All samples were analyzed in at least three experiments.

Ribonuclease Protection Assay

PRL-R mRNA were quantitated in a solution hybridization ribonuclease protection assay using a kit from Ambion Inc., Austin, TX. Antisense transcripts to be used as probes (Fig. 1B) were synthesized from linearized plasmid DNA using ^{32}P-UTP. The residual DNA template was removed by incubating for 30 min at 37°C with 1 U RNase-free DNase I (Promega). Radiolabeled transcripts were purified by polyacrylamide gel electrophoresis. Two to 10 µg of total RNA was hybridized with 1×10^5 cpm of labeled probes in buffer containing 80% formamide at 45°C for 16 h. Residual transcripts were digested with RNase A and RNase T1 at 37°C for 30 min. The protected transcripts were ethanol-precipitated and resolved by electrophoresis through a 5% polyacrylamide/8*M* urea DNA sequencing gel. Dried gels were exposed to Phosphorimager screens and the bands analyzed using ImageQuant 3.2 Software (Molecular Dynamics). The size of the protected fragments was determined by reference to a RNA marker transcribed with ^{32}P-UTP (Ambion Inc.). The intensity of the PRL-R transcripts was normalized to the intensity of protected GAPDH (~150 nt) fragments in the same sample, and results are expressed as corrected Phosphorimager units. All ribonuclease protection assays shown were performed in duplicate.

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