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Expression of Prolactin Gene in Mouse Placenta During Late Pregnancy: Detection of mRNA and Its Translation Product

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Abstract. To examine the existence of PRL messenger ribonucleic acid (mRNA) in the mouse placenta during late pregnancy, reverse transcriptase-polymerase chain reaction (RT-PCR) and Southern blot analysis were carried out followed by nucleotide sequence analysis of cDNA. Total RNA extracted from each tissue was reverse-transcribed, followed by PCR with two oligonucleotide primers specific for a part of mouse PRL (mPRL) cDNA. An amplified RT-PCR product of predicted size was detected in all samples from the placenta of days 16 and 18 pregnant mice. This product was specifically hybridized with a probe overlapping an entire sequence of mPRL cDNA in Southern blot analysis. Nucleotide sequence analysis also provided evidence that the amplified cDNA had a nucleotide sequence completely identical to the mPRL cDNA sequence reported previously. Furthermore, mPRL with a slightly bigger molecular weight than that of pituitary PRL was detected in the placenta of days 12, 14, 16 and 18 pregnancy by immunoblot analysis. These results suggest that PRL mRNA and its translation product are synthesized in mouse placenta during late pregnancy.

Key words: Mouse PRL, Placenta, RT-PCR, Southern blot, Immunoblot

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PRL exhibits a wide variety of activities in vertebrates, including stimulation of growth and differentiation of mammary epithelium, lactation, osmoregulation and parental behavior in teleosts, amphibian development, broodiness in hens and the production of cropsac 'milk' in pigeons [1–4]. Moreover in addition to these activities, the involvement of PRL in the immune system is also focused on PRL-stimulated lymphocyte proliferation [5]. Although PRL is secreted mainly from the PRL cells of the anterior pituitary gland into the blood, PRL protein and/or its mRNA have been found in a variety of tissues including the mammary gland in the rat [6–10] and mouse [11], milk in various species [12, 13], rat brain [14–20], the human immune system [21–24], and human placenta [25–32]. These reports suggest that the existence of PRL in nonpituitary tissues is due not only to receptor binding form but also to its synthesis and secretion from these tissues presumably for autocrine or paracrine stimulation. However, the definitive role of PRL in these tissues has not yet been clarified.

Among these tissues, we focused on the placenta to determine whether PRL is synthesized here in mice as it is in humans. Although it is well known that the placenta in rodents synthesizes various kinds of placental lactogens and PRL-like proteins [33, 34], there is no report on the existence of PRL in the rodent placenta. In the present study, we attempted to examine the presence of PRL mRNA in mouse placental tissue by means of RT-PCR and Southern blot analysis followed by nucleotide

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sequence analysis. Immunoblot analysis was also carried out to detect PRL protein in the placenta during late pregnancy.

Materials and Methods

Animals

The management of mice and experimental procedures in this study were performed according to the guidelines for animal experiments of this university. ICR strain mice were housed under controlled temperature conditions (22 ± 2 °C) in an artificially-illuminated room (12 h light and 12 h dark). Food and tap water were available *ad libitum*. At 70–90 days of age, females were mated with males and the day a vaginal plug was present was designated day 0 of pregnancy. Mice were killed by cervical dislocation on days 12, 14, 16 and 18 of pregnancy. The pituitary glands and placenta were removed and used immediately for experiments, or stored at −80 °C until further analysis.

RNA extraction and the detection of PRL mRNA

Total RNA was isolated from 100 mg of placental tissue or pooled pituitary glands by the acid guanidinium-phenol-chloroform method [35]. RNA concentration was estimated at 260 nm with a spectrophotometer. Expression of mouse PRL (mPRL) mRNA in the tissues was determined by the reverse transcriptase-polymerase chain reaction (RT-PCR). The cDNA for mPRL was synthesized in 20 μl buffer containing 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, dNTP at 1 mM each, 1 U/ml RNase inhibitor, 2.5 U/ml RAV-2 reverse transcriptase (Takara, Kyoto, Japan), 0.75 μM antisense primer for mPRL cDNA and 1 μg total RNA. The reaction was performed at 42 °C for 30 min and terminated by heating for 5 min at 95 °C. The sample was then stored at 4 °C until the following PCR reaction. The target cDNA obtained by reverse transcriptase reaction was amplified by PCR for 40 cycles (1 cycle: 94 °C-1 min, 55 °C-2 min, 72 °C-2 min) in a Thermal Cycler (Funakoshi, Tokyo, Japan) in buffer containing synthesized cDNA solution, 2 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 U Taq DNA polymerase (Takara, Kyoto, Japan), and 0.75 μM sense primer for mPRL (total vol. = 100 μl). The mPRL primers used in this study corresponded to the mPRL cDNA sequences reported by Harigaya *et al.* [36] shown in Fig. 1. The predicted size of the PCR product was 654 bp between a and c primers and 418 bp between b and d primers. β-actin primers, according to the mouse sequence [37], corresponding to amino acids 35-41 (5'-GTGGGCCGCTCTAGGCACCA-3') for the sense strand and to 116-108 (5'-CGGTTGGCCTTAG-GGTTCAGGGGGG-3') for the antisense strand, were used for the RT-PCR control reaction.

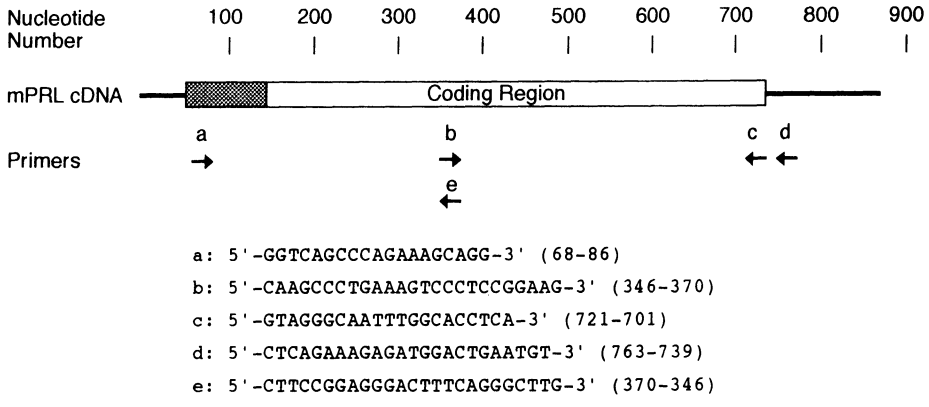

Fig. 1. mPRL cDNA and primers used for PCR reaction. Primers and their locations corresponding to the nucleotide sequence of mPRL cDNA are indicated by arrows in the 5'-3' direction. Primer sequences and nucleotide number in parentheses are described under the scheme. Primers a, b, c and d were used for the PCR reaction and primers b and e were used for nucleotide sequence analysis.

DNA blot hybridization analysis

DNA blot hybridization analysis was performed following the procedure of Southern [38]. The PCR products were run on 2% agarose gel electrophoresis. The gel was then blotted onto an Immobilon-S membrane (Millipore, Bedford, MA, USA) and the detection of mPRL cDNA was performed with a Polar Plex Chemiluminescent blotting kit (Millipore) according to the supplier's recommendation, with an entire sequence of mPRL cDNA [36] as a probe labeled with biotin and a random primer labeling kit (Millipore).

Nucleotide sequence analysis

PCR-amplified cDNAs with mPRL primers were ligated to pCRII vector and introduced into INVαF'-competent cells with a TA cloning kit (Invitrogen, San Diego, CA, U.S.A.). Transformed cells were cultured for 24 h at 37 °C in ampicillin- and Xgal-containing medium, and white colonies, which contained cDNA-inserted pCRII plasmid were selected. Cloned pCRII plasmids were amplified and then subjected to nucleotide sequence analysis. Nucleotide sequence of the cDNA was determined by the dye terminator cycle-sequencing method with a 373A DNA Sequencer from Applied Biosystems (Foster City, CA, U.S.A.) following the manufacturer's instructions.

Immunoblot analysis

Immunoblot analysis was performed according to the method of Towbin *et al.* [39]. Pituitary glands and placenta were homogenized in a Teflon-glass homogenizer. The homogenate was centrifuged at 10,000 × g for 10 min, and the supernatant was dissolved in sample buffer [62.5 mM Tris-HCl (pH 6.8)/5 mM EDTA/10% (v/v) glycerol/2% (w/v) sodium dodecyl sulphate (SDS)/10% (v/v) 2-mercaptoethanol (2-ME) / 0.01% (w/v) bromophenol blue] and heated at 95 °C for 5 min. Samples were applied to SDS-polyacrylamide gel electrophoresis [40]. Proteins were then transferred electrophoretically to Immobilon-P membrane (Millipore). The membrane was treated with rabbit antiserum raised against recombinant mPRL [41, 42] as a primary antibody. Antigen-antibody complexes were detected by a biotinylated secondary antibody and a streptavidin-biotin-horseradish peroxidase complex with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, U.S.A.). The band specific for mPRL was stained with diaminobenzidine as a chromogen.

Results

A single amplified product (245 bp) obtained by PCR analysis with β-actin primers was seen in agarose gel electrophoresis of all samples used in the present study (data not shown). The amplified fragment of RT-PCR corresponding in length to the predicted size between two mPRL primers b and d in Fig. 1 (418 bp) was obtained from all samples of pituitary glands and placenta from days 16 and 18 of pregnancy (Fig. 2). To clarify the accuracy of these products, Southern blot analysis was performed after agarose gel electrophoresis. These PCR products were hybridized with a probe containing an entire sequence of mPRL cDNA (Fig. 2).

Furthermore, RT-PCR was performed again with different PRL primers (a and c in Fig. 1) to obtain longer cDNAs (654 bp) from placenta of days 16

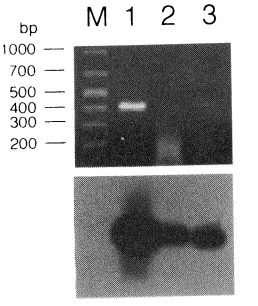

Fig. 2. Agarose gel electrophoretic patterns of RT-PCR products (upper panel) and following Southern blot analysis with mouse cDNA probe (lower panel). Primers used in this PCR experiment were b and d in Fig. 1. The predicted size of amplified products was 418 bp. The lanes contain PCR products obtained from the pituitary gland of day 18 of pregnancy (lane 1), placenta from day 16 (lane 2) and day 18 (lane 3) of pregnancy and a size standard DNA marker (lane M).

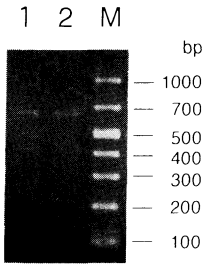

Fig. 3. Agarose gel electrophoretic pattern of RT-PCR products for plasmid construction and nucleotide sequence analysis. Primers used in this PCR experiment were a and c in Fig. 1. The predicted size of amplified products was 654 bp. The lanes contain PCR products obtained from the placenta of day 16 (lane 1) and day 18 (lane 2) of pregnancy and a size standard DNA marker (lane M).

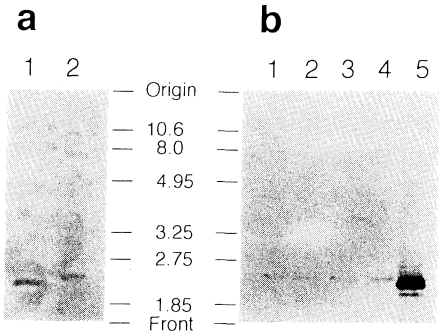

Fig. 4. Immunoblot analysis of placental PRL. Samples were subjected to 12.5% SDS-PAGE and immunoblot analysis, followed by reaction with rabbit mPRL antiserum. The lanes contain extracts from pituitary gland (lane 1) and placenta (lane 2) of day 18 of pregnancy in panel a, and extracts from placenta of day 12 (lane 1), day 14 (lane 2), day 16 (lane 3), day 18 (lane 4) of pregnancy and pituitary gland from adult male mouse (lane 5) in panel b. Samples were prepared in concentration of proteins for adequate staining intensity in panel a, and a quarter volume of final extract from each tissue in panel b. Phosphorylase B (10.6 K), bovine serum albumin (8.0 K), ovalbumin (4.95 K), carbonic anhydrase (3.25 K), soybean trypsin inhibitor (2.75 K) and lysozyme (1.85 K) were used as molecular weight standards.

and 18 pregnancy for nucleotide sequence analysis (Fig. 3). These cDNAs were ligated in pCRII vector plasmids to perform the nucleotide sequence analysis in order to reveal whether cDNAs had a sequence identical to that of mPRL cDNA. One colony from each sample at days 16 and 18 of pregnancy was used for sequence analysis which was determined in both directions of cDNA with a primer derived Sp6 or T7 promoter sequence contained in the vector plasmid, or b or e PRL primer as shown in Fig. 1. The result showed that the PCR product with PRL primers had a nucleotide sequence exactly identical to that of the mPRL cDNA reported previously [36].

Immunoblot analysis also revealed the existence of mPRL which confirmed the synthesis of PRL in placenta at day 18 of pregnancy (Fig. 4a). However, the molecular size of the positive band from the placenta was slightly bigger than that from the pituitary gland. In addition, the existence of PRL was also observed in placenta from days 12, 14 and 16 of pregnancy, although the amounts of PRL were very small in all placentas (Fig. 4b).

Discussion

The placenta is a major source of hormones during pregnancy and secretes members of the GH/PRL family such as placental lactogen (PL) and its variants in many species [43]. Moreover in humans, it is reported that a PRL identical to pituitary PRL is synthesized in placenta [25–32]. However, it has not yet been reported in rodents whether PRL is synthesized and secreted in the placenta during pregnancy. In rats and mice, many kinds of placental lactogens (e.g., PLI, PLII, PLIv), PRL-like proteins (PLP) and PRL-related peptides (PRP) have been reported [34, 44, 45]. Some of these substances can bind to PRL receptors and are therefore PRL-like agents, but others do not bind or show any sign of PRL-like activity. The question of the role of these materials in the mothers and/or fetuses during pregnancy remains unanswered [34].

As there was no report on the presence or absence of PRL in the placenta of mice, we searched for PRL mRNA and PRL protein in the mouse placenta during pregnancy. By means of RT-PCR, amplified fragments that coincided with the expected molecular size of the PCR product were obtained. This RT-PCR product was confirmed as

PRL cDNA by Southern blot analysis followed by nucleotide sequence analysis. Immunoblot analysis also revealed the existence of PRL protein in the placenta from day 12 to day 18 of pregnancy, although the molecular size of the band positive for mPRL was slightly larger than that of pituitary PRL. The nucleotide sequence analysis provides evidence that the placental PRL sequence is identical to the pituitary PRL sequence in the amplified region. Although the nucleotide sequence of RT-PCR cDNA determined in the present study is not comparable to full-length mPRL cDNA, it covers almost the entire coding region of mature protein and a part of the signal peptide. The meaning of this size difference is not known. It is possible that posttranslational processing of PRL in the placenta is different from that in the pituitary. The complete nucleotide sequence analysis of placental PRL may be necessary to clarify this PRL size difference.

PRL is thus present in the placenta during the latter half of pregnancy in mice. However, its function remains ambiguous, as is true for placental lactogens and related proteins. Since extrapituitary PRL may affect the tissue producing it through an autocrine and/or paracrine pathway [46, 47], placental PRL may be an local mediator affecting cell growth or expression of other placental proteins. The possibility of placental PRL entering the blood circulation of the mother and/or the fetus also is real.

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