Cellular localization and evolution of prolactin receptor mRNA in ovine endometrium during pregnancy

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Abstract In this study, we have investigated the expression of the prolactin receptor gene in ovine endometrium during oestrus cycle and pregnancy. Using reverse transcription-PCR analysis, we provided evidence that the prolactin receptor gene is specifically transcribed in this tissue. As shown by Northern blot analysis, the level of the prolactin receptor transcripts increased dramatically during late pregnancy. In situ hybridization experiments revealed that prolactin receptor mRNA was specifically expressed in the glandular compartment and confirmed the dramatic increase of its expression that occurs at the end of pregnancy. Taken together, these findings are consistent with a putative role of prolactin and/or related molecules in the regulation of the proliferation of the glandular compartment and/or in the control of the secretory activity of the endometrium.

Key words: Prolactin receptor; Endometrium; Ovine species; In situ hybridization

1. Introduction

Prolactin (PRL) is a pleitropic hormone involved in a large number of physiological actions that can be divided into the following categories: water and electrolyte balance, growth and development, endocrinology and metabolism, brain and behavior, reproduction and immunoregulation and protection [1]. The effects of PRL are mediated through an interaction with a specific high affinity cell surface receptor. The presence of prolactin receptor (PRL-R) has been detected in numerous organs including uterus. Indeed, PRL-R gene expression has been demonstrated in human endometrium [2,3] as well as in rat [4,5], rabbit [6], pig [7] and bovine uterus [8]. In the uterine tissues, PRL and its related hormones are believed to be involved in the establishment and maintenance of an appropriate environment for embryonic and fetal development. The effects of PRL on uterine endometrium have been particularly studied in pig [7] and rabbit [9]. In this latter species, PRL has been involved in hypertrophy and glandular differentiation [10] and in the alteration of uterine secretions [11,12]. More recently, inactivation of the mouse PRL-R gene provided evidence of the critical role of PRL in the implantation and embryonic development [13]. However, the precise role of PRL on the regulation of the uterine environment remains to be determined.

Although the presence of the PRL-R in ovine uterus has been previously described [6], the target tissues of PRL and/or placental lactogen have not been determined. To further understand the putative role of PRL and/or placental lactogen on the uterus during pregnancy, we report experiments demonstrating the transcription of the PRL-R gene in the ovine endometrium during oestrus cycle and pregnancy. Moreover, the cellular localization of PRL-R mRNA in the endometrium has been undertaken by in situ hybridization.

2. Material and methods

2.1. Animals

Procedures relating to the care and use of animals were approved by the French Ministry of Agriculture according to the French regulations for animal experimentation (guideline 19/04/1988). Primiparous ewes of the Préalpes du Sud breed were used. Oestrus cycles were synchronized as previously described [14]. At various days of pregnancy and lactation, animals were killed and samples of the uterus were collected and treated for total RNA extraction and in situ hybridization.

2.2. Preparation of the hybridization probes

The 324 bp fragment beginning at the *Afl*II restriction site and ending at a *Xba*I restriction site of the ovine PRL-R cDNA, kindly provided by Dr Bignon [15], was randomly labelled using a random primed DNA labelling kit (Boehringer Mannheim, Meylan, France). For in situ hybridization, recombinant Bluescript SK(−) transcription vector (Stratagene, Ozyme, Montigny-le-Bretonneux, France) containing the full length ovine PRL-R cDNA was linearized. Antisense and sense cRNA probes were transcribed using respectively T3 or T7 RNA polymerases (Gibco BRL-Life Technologies, Eragny, France) and [α^{35}S]UTP (> 1000 Ci/mmol) was used as labelled ribonucleotide triphosphate. Full length ovine PRL-R cRNA sense probe was also synthesized and used as a control for in situ hybridization.

2.3. RNA isolation and blotting

Total RNA was prepared from each ovine endometrium by the guanidium isothiocyanate method described by Chomczynski and Sacchi [16] and modified by Puissant and Houdebine [17]. 20 μg Of the formamide-formaldehyde denaturated total RNA was fractionated on a 1.5% agarose gel, transferred to a Zeta-probe membrane (Bio-Rad, Ivry sur Seine, France), hybridized at 65°C in a buffer containing 0.5 M sodium dihydrogenophosphate pH 7.2, 7% SDS, 1 mM EDTA and 0.5% non-fat dry milk and washed at 65°C in 4×SSC (1×SSC: 150 mM sodium chloride, 15 mM sodium citrate, pH 7) and 0.5% SDS.

2.4. Oligonucleotides

PRL-R primers were kindly provided by Dr C. Bignon. Upstream primer (5′-AGCCTTCTGAATGGACAGTCACCTCCTGAAAAACC-3′) and downstream primer (5′-TCCTTCACTGGGAAGTCATTAGGTATCTGG-3′) correspond to the nucleotide positions 58–92 and 672–701 of the ovine PRL-R cDNA sequence respectively [15]. The expected size of the PRL-R amplified fragment was 644 bp.

2.5. Reverse transcription (RT)

RT was performed on 2 μg of total mRNA with Superscript II (GIBCO BRL, Life Technologies SARL, Cergy Pontoise, France) reverse transcriptase using random hexamers in a 20 μl reaction, 1 h at 42°C. A negative control which contained the first strand synthesis reagents but no RNA sample was performed.

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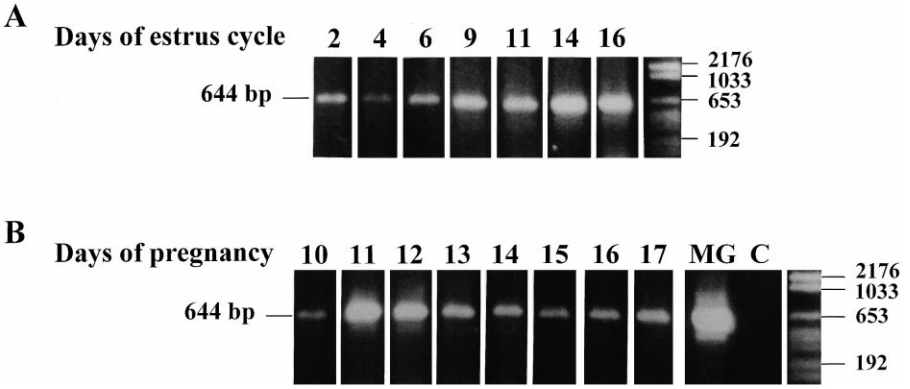

Fig. 1. Amplification of PRL-R mRNA by PCR in ovine endometrium during the oestrus cycle (A) and early pregnancy (B). Size of the amplified DNA fragment and those of the molecular size standard are given on the left and right respectively.

2.6. PCR and analysis of amplification products

One-fifth of the first strand synthesis reaction were amplified for 30 cycles in 25 mM $MgCl_2$, 200 μM dNTPs, 1×PCR Buffer (Promega, Lyon, France), 50 pM each primer and 5 U Taq Polymerase (Promega, Lyon, France). After the initial denaturation step (94°C for 10 min), the amplification conditions were as follows: denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min on a Perkin Elmer Cetus gene Amp PCR system 480 (Perkin Elmer Cetus, Saint Quentin en Yvelines, France). After amplification, the samples were separated on a 1% agarose gel stained with ethidium bromide, transferred on a Zeta-probe membrane (Bio-Rad, Ivry sur Seine, France) and hybridized with an appropriate probe as subsequently described.

2.7. In situ hybridization

Uterine tissues from ewes at various stages of the oestrus cycle, pregnancy and lactation were dissected and prepared for in situ hybridization as previously described for mammary tissue [14]. Antisense and sense probes were produced by using previously described ovine PRL-R cDNA in plasmid Bluescript (Stratagene, Montigny-le-Bretonneux, France). Cryostat sections were processed for in situ hybridization as previously described [14].

3. Results

3.1. RT-PCR analysis of PRL-R mRNA in ovine endometrium during oestrus cycle and pregnancy

Fig. 1 represents the ethidium bromide staining of the electrophoresed PRL-R PCR products. A single band identical in size to that amplified in the control RNA (644 bp) was obtained in endometrial samples of the ewes during the oestrus cycle and at the beginning of pregnancy. No amplification was observed when RNA was replaced by distilled water.

The same experiments were carried out with RNA extracted from ovine endometrium and placenta during late pregnancy. As depicted in Fig. 2, a DNA fragment of 644 bp was amplified in ovine endometrium and placental tissues. Subsequent Southern blot analysis of the same gel revealed a single band of 644 bp hybridizing with the ovine 324 bp PRL-R probe, confirming that the ovine PRL-R gene is expressed in these tissues. No amplification was observed in RNA samples from amniotic membranes.

3.2. Northern blot analysis of the uterine PRL-R mRNA

In order to appreciate the levels of PRL-R mRNA in ovine endometrium during pregnancy and lactation, Northern blot analysis was performed. Ovine mammary gland and endometrium PRL-R transcripts both yielded hybridizing bands of similar sizes of 10.2 and 4.5 kb. These sizes are consistent with those previously described in ovine mammary gland [14]. The PRL-R mRNA level in ovine endometrium at day 14 of pregnancy was too weak to allow detection by Northern blot experiments. This level dramatically increased during the second half of pregnancy (Fig. 3). During lactation, no PRL-R transcripts were detected. In the placenta, PRL-R mRNA

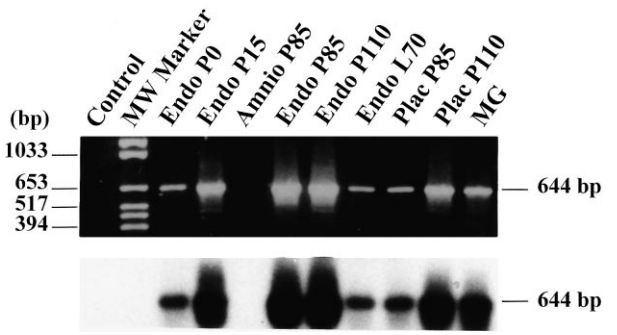

Fig. 2. Amplification of PRL-R mRNA by PCR in ovine endometrium (Endo) at day 0 (day of insemination)(P0), 15 (P15), 85 (P85) and 110 (P110) of pregnancy and day 70 (L70) of lactation: in amniotic membranes at day 85 of pregnancy (Amnio P85) and in placenta (Plac) at day 85 and 110 of pregnancy. Mammary gland (MG) was used as positive control. Lane C corresponds to a negative control which represents amplification in the absence of RNA. Sizes of the amplified DNA fragment and those of the molecular size standard are given on the left and right respectively.

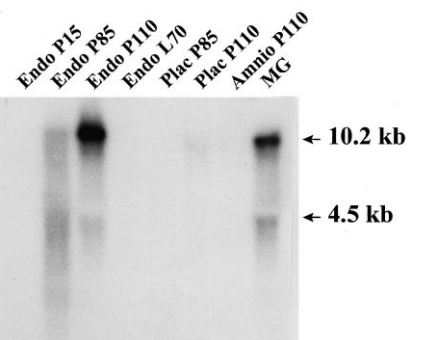

Fig. 3. Northern blot analysis of ovine PRL-R transcripts in ovine endometrium at day 15 (P15), 85 (P85), 110 (P110) of pregnancy and day 70 (L70) of lactation, in placenta at day 85 (P85) and 110 (P110) of pregnancy and in the amniotic membrane. MG: mammary gland. The size of the transcripts is indicated on the right margin.

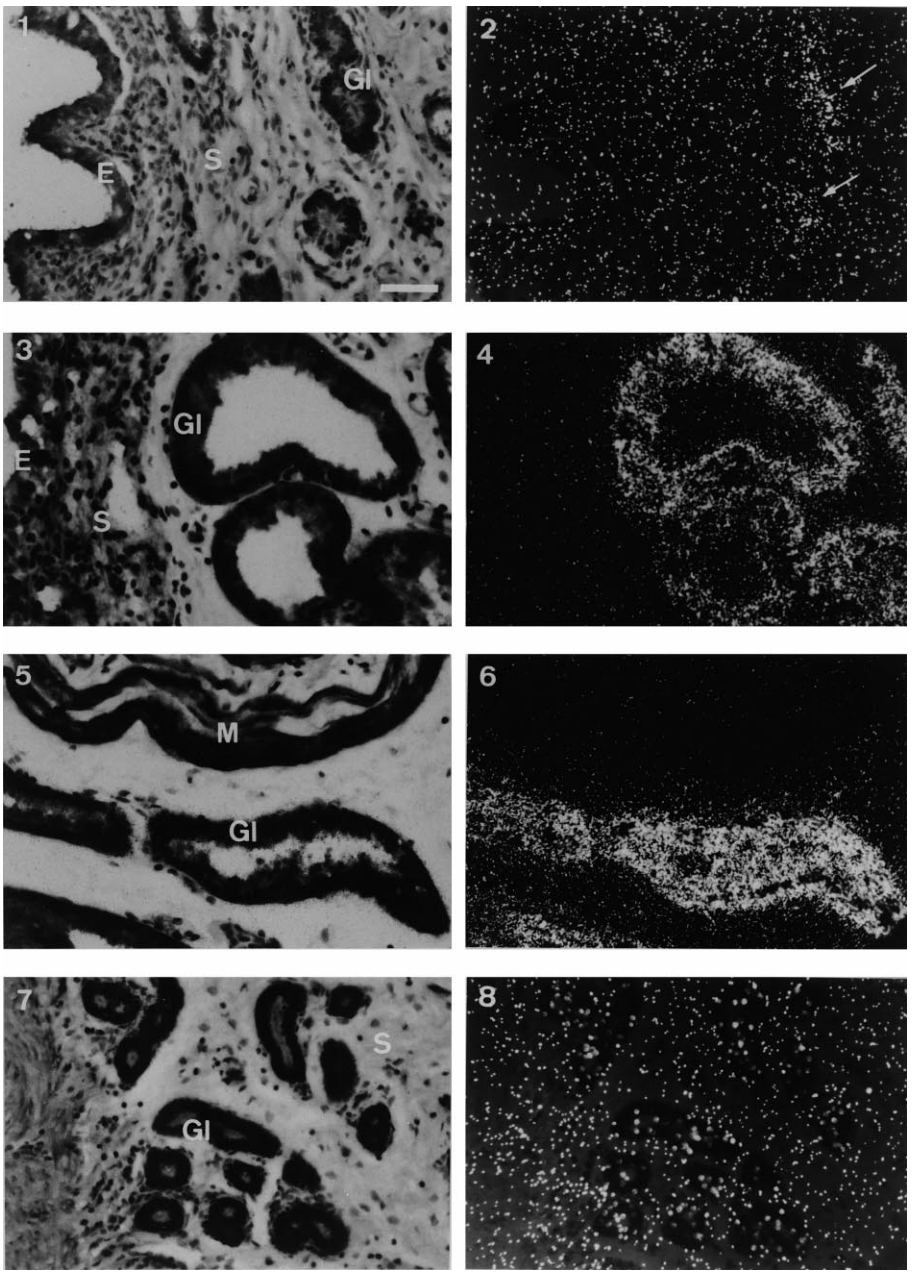

Fig. 4. Cellular localization of PRL-R mRNA in ovine endometrium at day 15 (panels 1, 2) and 90 (panels 3–6) of pregnancy. A section of the endometrium at day 15 of pregnancy (panels 7,8) was hybridized with the sense probe and used as a control of specificity. Shown are the bright-field (right column) and the epi-illuminated (left column) photomicrographs. Bar = 50 μm.

was barely detectable at day 110 of pregnancy. On the contrary, PRL-R transcripts were undetectable in the amniotic membrane.

3.3. Localization by in situ hybridization of PRL-R mRNA in ovine endometrium

To precise the cellular localization of PRL-R mRNA synthesis, in situ hybridization was performed (Fig. 4). As described in Section 2, a ^{35}S-labelled ovine PRL-R cRNA antisense probe was used to detect specific PRL-R mRNA and a ^{35}S-labelled ovine PRL-R cRNA sense probe was used as a negative control. The ovine placental cotyledonary compartment, the luminal epithelium, the stroma and the myometrium exhibited no signal above that observed with the sense probe (Fig. 4, panels 7, 8). In endometrium at day 15 of pregnancy, a weak hybridization signal was observed within the cells of the glandular epithelium and more precisely within the deepest glands (Fig. 4 panels 1, 2). This signal was dramatically up-regulated during late pregnancy as illustrated by the in situ hybridization experiments performed on the uterus at day 90 of pregnancy (Fig. 4 panels 3–6). This increase is associated with a dramatic hypertrophy and enlargement of the epithelial endometrial glands.

4. Discussion

This study clearly demonstrates that PRL-R mRNA is expressed in ovine endometrium during oestrus cycle and preg-

nancy. Data obtained by Northern blotting show that expression of the PRL-R gene is up-regulated during late pregnancy. The endometrial PRL-R mRNA pattern was identical to that previously described in the mammary gland [14]: a major transcript of 10.2 kb and a second one of 4.5 kb. Bovine and ovine PRL-R amino acid sequences are closely related (91.6% of identity) [8,15]. Both ovine and bovine PRL-R are characterized by the presence of two types of mRNA encoding a long and a short form of PRL-R. The alternative splicing that leads to the generation of the two forms of PRL-R seems to be the same between bovine and ovine [15]. However, the pattern of the PRL-R transcripts in the endometrium differs between bovine and ovine species. Indeed, Scott and colleagues [8] have described two transcripts in the bovine endometrium, a major one of 3.8 kb and a second one of less abundance of about 4.4 kb. The transcript of 10.2 kb present in ovine endometrium has not been detected in the bovine species, thus suggesting that bovine and ovine transcripts might result from different alternative splicing processes.

This study represents the first demonstration of the localization of the PRL-R mRNA in the endometrium of ruminant. Indeed, PRL-R gene is strongly expressed in the epithelial cells of the endometrial glands. Although those glands derive from the luminal epithelium, no hybridization signal indicating the presence of PRL-R mRNA in this region was detected. This suggests that PRL-R gene expression is specific to one type of uterine cells. Results of in situ hybridization revealed no obvious signal in the caruncular endometrium (non-glandular areas) which constitute the maternal part of the cotyledon. In the same way, no hybridization of the PRL-R antisense probe was observed in the placenta (data not shown). However, the presence of PRL-R mRNA in ovine placenta was established by RT-PCR at days 85 and 110 of pregnancy. This divergence may be explained by the difference in the sensitivity of the two techniques. Indeed the presence of the RT-PCR amplified fragment in the placenta may be the result of a very weak expression of the PRL-R gene not detectable by in situ hybridization. Nevertheless, this fragment may also reflect a possible contamination of the placental tissue by RNA extracted from endometrial glands during the dissection. The data in this report, concerning the expression and the localization of PRL-R mRNA in ovine endometrium are in agreement with those previously described in rabbit [12]. In cow, it has been also suggested that the PRL-R gene was expressed at the level of intercaruncular endometrium [8,18]. On the contrary, in species characterized by decidualization of the maternal stroma like human and rodents, PRL-R gene expression observed in both the stromal and glandular compartments is mainly associated with the decidual cells [3,19,20]. Taken together, these results suggest that the role of PRL in the endometrium might be different in nature and time course between species where decidualization occurs or not.

The expression of the PRL gene is essentially restricted to the glandular cells of the pituitary. However, PRL is also synthesized by the decidualized stromal cells of the endometrium in human [21,22]. In ruminants, neither decidualization of the maternal stroma nor endometrial secretion of PRL have been described until now. However, ovine as well as human placenta secretes a polypeptidic hormone named placental lactogen identified by its ability to bind the PRL-R [23] and care structural and functional similarity to the pituitary PRL [24]. Consequently, these lactogen molecules may be involved in the development and maintenance of a favorable environment for embryonic and fetal growth in the ewe, as it has been suggested in mice by the inactivation of the PRL-R gene [13].

The precise role of PRL in ovine endometrium during oestrus cycle and pregnancy has not yet been clarified. However, several effects have been previously reported in other species. Indeed, in rabbit, PRL promotes a thickening of the uterine endometrium by stimulating proliferation of glandular epithelial cells, an increase of the glandular differentiation and may also alter the cellular composition of the endometrium [10,25]. In pig, PRL might influence the ionic content of the uterine environment and the secretory activity of the endometrium [11]. The effect of PRL on the secretory activity of the endometrium can not be dissociated from that of oestrogens and progesterone. Indeed, it has been established that the treatment of immature rabbits by oestrogens and progesterone induced an increase of the level of mRNA encoding uteroglobine. This increase was higher when animals were additionally treated with PRL [12]. Thus, the PRL treatment might be involved in the enhancement of the uterine sensitivity to progesterone through an increase in progesterone receptor content [10,26]. A servomechanism explaining this process has been described by Chilton and colleagues [9]: acting through its receptor, PRL induces an increase in the concentration of the progesterone receptor. In return, progesterone acting through its own receptor promotes the expression of the PRL-R. By this way, PRL and progesterone may act synergistically to regulate the expression of progesterone-sensitive genes like uteroglobine. In the ewe, such a phenomenon has not been described yet. However, various elements indicate that PRL might influence the proliferation and secretory activity of the endometrial glands. The proliferation of the ovine glandular epithelial cells of the endometrium and the hypertrophy of these glands during pregnancy have been previously described by Wimsatt [27] and might be linked to the increase in PRL-R mRNA level which occurs concomitantly. Moreover, ovine endometrium is characterized by an intense secretory activity during pregnancy and it has been shown that progesterone may influence the expression of two glycoproteins called 'uterine milk proteins' [28–31].

In conclusion, the present study clearly demonstrates that the PRL-R gene is specifically expressed in the ovine endometrium and more precisely in the endometrial glands. The level of PRL-R mRNA strongly increases during late pregnancy. PRL has multiple functions throughout the body including growth and differentiation and immune response [1] and it remains difficult to assign a precise role for uterine PRL. It will therefore be of great interest to further investigate a possible role for PRL and/or related hormones acting through the PRL-R in the establishment of an adequate uterine environment allowing the maintenance of pregnancy.

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