Expression of mRNA encoding the prostaglandin F_{2α} receptor in bovine corpora lutea throughout the oestrous cycle and pregnancy

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The abundance of mRNA encoding the PGF_{2α} receptor in bovine corpora lutea at different phases of the oestrous cycle and pregnancy was examined in relation to the number of [³H]PGF_{2α} binding sites. Corpora lutea were removed from cyclic (early: 3–5 days after ovulation; mid-cycle: 8–12 days after ovulation; late: 15–18 days after ovulation; and regressed: 20–21 days after ovulation) and pregnant (early: fetal size 9–13 cm (2–3 months); mid-cycle: fetal size 42–43 cm (5–6 months); and late: fetal size 78–80 cm (8 months)) cows and subjected to total RNA preparation, *in situ* hybridization and membrane preparation for [³H]PGF_{2α} binding assay. Northern blot analysis demonstrated that expression of PGF_{2α} receptor mRNA progressively increased from the early phase to the late phase of the oestrous cycle, and was markedly reduced at the regressed phase; while constant amounts of mRNA were observed in early and middle pregnant corpora lutea, and there was a significant reduction at late pregnancy. Specific high affinity [³H]PGF_{2α} binding sites with K_d values of 18.3–31.1 nmol⁻¹ were observed in the luteal membrane during the oestrous cycle and pregnancy; this is consistent with the expression of PGF_{2α} receptor mRNA. The number of receptors decreased at the regressed phase and in early pregnancy. These results strongly suggest that PGF_{2α} is involved in not only luteolysis but also luteal functions in both pregnant and nonpregnant cows.

Introduction

Since prostaglandin F_{2α} (PGF_{2α}) was first identified as one of the prostanoids to cause contraction of smooth muscle (Änggård and Bergström, 1963; Änggård and Samuelsson, 1963; Perkins 1975), a variety of its biological actions have been reported in diverse tissues (Moncada *et al.*, 1985; Coleman *et al.*, 1989). The corpus luteum is a transient endocrine organ formed from the ruptured follicle after ovulation and its primary function is considered to be the production of progesterone, which prepares the uterine endometrium for implantation and maintains early pregnancy. If pregnancy does not occur, the corpus luteum must regress to allow the next cycle of follicle development to begin. Among the biological actions of PGF_{2α}, the best known physiological role is its involvement in luteolysis, especially in the initiation of luteal regression in cows, through its binding to a specific receptor on the luteal membrane (Horton and Poyser, 1976; Auletta and Flint, 1988; Steele and Leung, 1993). Previous studies show that an increased concentration of PGF_{2α} in utero-ovarian venous blood is observed in the late oestrous cycle, while PGF_{2α} secretion is markedly reduced at early pregnancy (Gleeson *et al.*, 1974; Moeliono *et al.*, 1977). Administration of PGF_{2α} to

cows causes luteal regression following a precipitous decline in serum progesterone concentration as early as 4–6 days after ovulation (Gleeson, 1974; Hansel and Convey, 1983), demonstrating that the synthesis and secretion of PGF_{2α} in ruminants, including cows, are regulated strictly during the oestrous cycle and pregnancy. Specific [³H]PGF_{2α} binding sites have been reported in corpora lutea of cows (Kimball and Lauderdale, 1974; Powell *et al.*, 1975; Rao, 1975). The concentration of high affinity PGF_{2α} binding sites has been quantified on bovine corpora lutea (Rao *et al.*, 1979) and luteal cells (Chegini *et al.*, 1991) taken at different stages of the oestrous cycle, and high binding activity of [³H]PGF_{2α} was observed at the late oestrous phase. Despite rapid luteolysis following PGF_{2α} administration *in vivo* and the presence of binding sites for PGF_{2α} in bovine corpus luteum, direct effects of PGF_{2α} on corpus luteum function *in vitro* are modest.

In previous studies, we and others have isolated functional cDNA clones for bovine (Sakamoto *et al.*, 1994), mouse (Sugimoto *et al.*, 1994) and human (Abramovitz *et al.*, 1994) PGF_{2α} receptor with seven transmembrane domains characteristic of G-protein-coupled receptors. We reported that the PGF_{2α} receptor is coupled to phosphoinositide metabolism via G_q in cDNA transfected Chinese hamster ovary cells (Ito *et al.*, 1994). We also demonstrated, by RNA blot analysis, that the PGF_{2α} receptor mRNA is highly expressed and accumulated in

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large luteal cells of the bovine corpus luteum at an unspecified stage of the oestrous cycle (Sakamoto *et al.*, 1994). In initial attempts to resolve the disparity in $\text{PGF}_{2\alpha}$ actions *in vivo* and *in vitro*, and to characterize the transcriptional regulation of the gene encoding the bovine $\text{PGF}_{2\alpha}$ receptor in the corpus luteum, we performed northern blot analysis and *in situ* hybridization in relation to binding activities for [^3H] $\text{PGF}_{2\alpha}$ to the crude luteal membrane during the oestrous cycle and pregnancy.

Materials and Methods

Dissociation of corpora lutea

Ovaries with corpora lutea from Holstein–Friesian cows were collected at a local abattoir within 30 min after slaughter. The luteal phase was classified as early (3–5 days after ovulation), middle (8–12 days after ovulation), late (15–18 days after ovulation) or regressed (20–21 days after ovulation) stages by macroscopic observation of the ovary as described by Okuda *et al.* (1988). The gestational ages were determined from fetal crown–rump length (Kristoffersen, 1960) and classified as: early: 2–3 months (9–13 cm); middle: 5–6 months (42–43 cm); and late: 8 months (78–80 cm). After determination of the stages at the abattoir, the corpora lutea were separated immediately from the remaining ovarian tissue, frozen rapidly in liquid nitrogen and stored at -80°C until processed.

Northern blot analysis

Total RNAs were prepared from bovine corpora lutea by the acid guanidinium thiocyanate method described by Chomczynski and Sacchi (1987), and poly(A) $^+$ RNAs were purified using the mRNA purification kit (Pharmacia LKB Biotechnology, Piscataway). Two micrograms of poly(A) $^+$ RNAs were subjected to electrophoresis on 1.0% (w/v) agarose–formaldehyde gel and transferred on to a Nytran nylon membrane filter purchased from Schleicher & Schuell (Keene). The clone SN463 contains the 230 bp insert DNA corresponding to transmembrane domains IV–VI of the bovine $\text{PGF}_{2\alpha}$ receptor (Sakamoto *et al.*, 1994). The 230 bp DNA, amplified by polymerase chain reaction (PCR), was labelled by ^{32}P by the Megaprime DNA labelling system (Amersham Corp., Arlington Heights) and used as a probe DNA. Hybridization was performed under standard hybridization conditions: 50% (v/v) formamide; 0.2% (w/v) SDS; $5 \times$ Denhardt's solution; $5 \times$ standard saline phosphate (SSPE); and 100 μg sonicated salmon sperm DNA ml^{-1} . Hybridization was carried out at 40°C and the membrane was washed with $1 \times$ SSPE and 0.5% (w/v) SDS at 42°C .

Hybridization in situ

For the preparation of the anti-sense riboprobe, BC2211 DNA carrying the full-length cDNA for bovine $\text{PGF}_{2\alpha}$ receptor (Sakamoto *et al.*, 1994) was digested with EcoRV and used as template for *in vitro* transcription in the presence of digoxigenin-11-UTP (Boehringer-Mannheim, Mannheim). The

paraformaldehyde-fixed tissue sections of ovaries including corpora lutea were hybridized with the digoxigenin-labelled riboprobe in the following solution at 45°C : 50% (v/v) formamide; 4% (w/v) dextran sulfate; 250 μg salmon sperm DNA ml^{-1} ; 250 μg yeast RNA ml^{-1} ; $1 \times$ Denhardt's solution; 0.2% (w/v) SDS; 0.75 mol NaCl l^{-1} ; 25 mmol piperazine- $\text{N,N}'$ -bis (2-ethanesulfonic acid) l^{-1} . Hybridized sections were then incubated with RNaseA for 30 min at 37°C . The hybridization material was reacted with alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer-Mannheim, Mannheim) and signals were detected by incubation with medium containing nitroblue tetrazolium, 5-bromo-4-chloro-3-indolylphosphate and levamisole.

Binding of [^3H] $\text{PGF}_{2\alpha}$ to luteal membrane fraction

Corpora lutea at each stage were homogenized in four volumes of the homogenizing buffer containing 0.15 mol NaCl l^{-1} , 1 mmol EDTA l^{-1} , and 10 mmol KPi l^{-1} , pH 7.4, at 4°C with a Polytron homogenizer. The homogenate was filtered through two layers of cheesecloth and the filtrate was centrifuged at 1000 g for 10 min. The supernatant was centrifuged at 26 000 g for 20 min and the homogenized material sedimenting between 1000 and 26 000 g was resuspended in buffer A containing 0.1 mol NaCl l^{-1} , 1 mmol EGTA l^{-1} and 10 mmol KPi l^{-1} , pH 6.0, and used as the membrane fraction for binding assay. The [^3H] $\text{PGF}_{2\alpha}$ (168 Ci mmol^{-1}) was purchased from Du Pont-New England Nuclear. In preliminary experiments, specific bindings of 5 nmol [^3H] $\text{PGF}_{2\alpha} \text{l}^{-1}$ to the 1000–26 000 g pellet obtained with and without 0.3 mol sucrose l^{-1} in the homogenizing buffer were not different (data not shown). Binding assay of [^3H] $\text{PGF}_{2\alpha}$ to the membrane was carried out at 23°C essentially as described by Negishi *et al.* (1987). The affinity and concentration of the $\text{PGF}_{2\alpha}$ receptor in corpora lutea at each stage were determined from Scatchard plots after an incubation of 200 μg of the membrane with various concentrations of [^3H] $\text{PGF}_{2\alpha}$ for 1 h in the presence or absence of a 1000-fold excess of unlabelled $\text{PGF}_{2\alpha}$.

Results

Northern blot analysis

In a previous report (Sakamoto *et al.*, 1994), we described the complete nucleotide sequence and the deduced primary structure of bovine $\text{PGF}_{2\alpha}$ receptor. Clone SN463, screened by the standard PCR using degenerated primers, carries the 230 bp insert DNA comprising transmembrane domains IV–VI of the $\text{PGF}_{2\alpha}$ receptor. Because this cDNA fragment represents the unique sequence for bovine $\text{PGF}_{2\alpha}$ receptor, the SN463 230 bp DNA fragment was commonly used as probe DNA for northern blot hybridization.

SN463 230 bp probe gave two RNA bands, one major 5.0 kb and another minor 2.0 kb RNA (Fig. 1a). Since both hybridizing signals remained intense even after washing twice in $1 \times$ SSPE at 50°C , these two bands represent specific mRNA species which may encode bovine $\text{PGF}_{2\alpha}$ receptor protein. Over the course of the oestrous cycle, the 5.0 kb mRNA was increasingly expressed and accumulated, with a peak in the late

Fig. 1. Northern blot analysis of poly(A)⁺ RNA isolated from bovine corpora lutea. The poly(A)⁺ RNAs used (2 µg of each) were isolated from bovine corpora lutea obtained from different phases of the oestrous cycle (lanes 1–4) and pregnancy (lanes 5–7): lane 1: early oestrus; lane 2: mid-oestrus; lane 3: late oestrus; lane 4: regressed oestrus; lane 5: early pregnancy; lane 6: mid-pregnancy; and lane 7: late pregnancy. The same Nytran membrane filter was hybridized sequentially with a ³²P-labelled SN463 230 bp probe (a) and G3PDH probe (b). The positions of 18S and 28S rRNAs are indicated on the right.

cyclic phase (approximately three times the intensity of that in the early cyclic phase) and then a marked reduction of the major RNA band at the regressed phase (day 21). In total, three independent RNA preparations were examined; one showed a fivefold increase of intensity in the late cyclic phase compared with the early cyclic phase, and another gave only a 1.5-fold increase (data not shown). Although the extent of expression of 5.0 kb mRNA was dependent on the preparation lot, the tendency of expression during the oestrous cycle was the same. Compared with the 5.0 kb mRNA, the diversity of the 2.0 kb mRNA was small, and no significant alteration coupled with the cyclic phase was detected. This finding suggests that these two RNAs have independent regulatory mechanisms for transcription. Virtually no 2.0 kb mRNA band was detected in the corpora lutea obtained from pregnant cows. However, a 5.0 kb mRNA band of comparable intensity to that in the late oestrous phase was observed in the early and middle pregnant corpora lutea. There was a significant reduction of this mRNA signal in the late pregnant corpus luteum. A different preparation gave far fewer hybridized signals in the corpus luteum

obtained at mid- and late pregnancy than at early pregnancy (data not shown). The same filters were rehybridized with an internal control, G3PDH DNA, and a similar intensity of signal was detected among these RNA samples (Fig. 1b).

Hybridization in situ

Cellular distribution of mRNA encoding PGF_{2α} receptor was examined by *in situ* hybridization (Fig. 2). The hybridization signal was strictly localized in the region of corpus luteum obtained from the mid-oestrous phase (Fig. 2b), but no signal, or only a faint signal, was detected in the early oestrous phase (Fig. 2a). The signal taken in the late oestrous phase was as intense as that of the mid-oestrous phase (data not shown). Because no hybridizing signal was detected by the incubation with the sense RNA probe (Fig. 2b'), this signal represents the sense mRNA encoding receptor protein.

More detailed information was obtained by further characterizing *in situ* hybridization using bovine corpora lutea obtained from each phase of the oestrous cycle and pregnancy, at higher magnification (Fig. 2c–i). As described by Sakamoto *et al.* (1994), many large luteal cells in the corpus luteum were detected with the anti-sense riboprobe prepared from BC2211 cDNA. The localization of the hybridization signal to large luteal cells and a lack of the signal in smaller cells in the corpus luteum is illustrated in Fig. 2j. The number of receptors expressed in luteal cells was represented as the intensity of hybridizing signals on the cell. Far fewer signals were detected in the regressed phase (Fig. 2f) than in the late cyclic phase. Consistent with the RNA blot analysis, fewer signals were observed in the early luteal phase (Fig. 2c) or in late pregnancy (Fig. 2i).

Binding of [³H]PGF_{2α} to luteal membrane fraction

The binding of [³H]PGF_{2α} to the membrane derived from the corpus luteum at an early phase of the oestrous cycle is shown in Fig. 3. Specific binding was more than 80%, except for the maximum concentration (52 nmol l⁻¹) used here, and saturable. The Scatchard plot analysis showed an apparently single high-affinity binding site. Although the binding properties varied between preparations of corpora lutea at the same stage, such a single high-affinity binding site was observed with a preparation at all stages of the oestrous cycle and pregnancy. The capacity of [³H]PGF_{2α} binding increased as the oestrous cycle progressed, together with the K_d values (Fig. 4). This was consistent with the northern blot analysis. Table 1 summarizes B_{max} and K_d values of high-affinity [³H]PGF_{2α} binding sites on the corpora lutea at each stage of the oestrous cycle and pregnancy. The capacity (B_{max}) at the early, middle, and late phases of the oestrous cycle are 1.17 ± 0.16, 1.38 ± 0.17, and 1.58 ± 0.28 pmol mg⁻¹ protein, respectively, and the affinity (K_d), 18.3 ± 2.12, 19.1 ± 2.65, and 22.4 ± 1.26 nmol l⁻¹. The binding capacity was significantly reduced at the regressed phase, with a B_{max} value of 0.17 ± 0.05 pmol mg⁻¹ protein and a K_d value of 31.1 ± 2.99 nmol l⁻¹.

A high affinity binding site for [³H]PGF_{2α} was also observed on the membrane fraction of corpora lutea at different stages of

Fig. 2. *In situ* hybridization of bovine ovaries with a digoxigenin-labelled riboprobe for $\text{PGF}_{2\alpha}$ receptor. Bright-field photomicrographs show the *in situ* hybridization of bovine ovaries with digoxigenin-labelled sense (b') and anti-sense (a, b, c-j) RNA probes synthesized from a cDNA clone for $\text{PGF}_{2\alpha}$ receptor *in vitro*. A highly specific hybridization signal is seen in the corpus luteum (CL) obtained at the mid-oestrous phase (b), while it is absent in the early oestrous phase (a). The sense riboprobe fails to express a hybridization signal in the corpus luteum obtained at the mid-oestrous phase (b'). (j) Hybridization-positive large luteal cells in the corpus luteum obtained at the mid-oestrous phase. Note that much smaller cells interspersed in these large cells remain negative for mRNA encoding the $\text{PGF}_{2\alpha}$ receptor (small arrows in j). Panels c-i show hybridization-negative or hybridization-positive luteal cells in the corpus luteum, prepared from ovaries at different luteal phases as follows: cyclic (c, early; d, middle; e, late; f, regressed) and pregnant (g, early; h, middle; i, late). Scale bars represent 2 mm (a and b), 100 μm (b'-j).

Fig. 3. Binding of [³H]PGF_{2α} to the corpus luteal membrane at the early stage of the bovine oestrous cycle. The luteal membrane was incubated for 60 min at 23°C with increasing concentrations of [³H]PGF_{2α} in the presence (●) or absence (○) of a 1000-fold excess of unlabelled PGF_{2α} (inset). Specific [³H]PGF_{2α} binding (□) was obtained by subtraction of nonspecific binding from total binding at the indicated concentrations of [³H]PGF_{2α}. Each point represents the mean ± SEM of triplicate determinations.

Fig. 4. Scatchard plots of [³H]PGF_{2α} binding to the luteal membrane at different phases of the bovine oestrous cycle. Scatchard plot analyses were carried out by subtraction of nonspecific binding from total binding at the indicated concentrations of [³H]PGF_{2α}; each point represents the mean ± SEM. Membranes were prepared from corpora lutea at early (□), middle (Δ), late (○), and regressed (●) stages.

pregnancy. The binding capacity was higher at mid-pregnancy (1.17 ± 0.03 pmol mg⁻¹ protein) than at early pregnancy (0.57 ± 0.05), but K_d values were similar. In the late phase of pregnancy, one of three preparations of the membrane showed a high-affinity binding site for [³H]PGF_{2α} ($B_{max} = 1.91$ pmol mg⁻¹ protein, $K_d = 22.8$ nmol l⁻¹), but the other two preparations did not exhibit high-affinity binding sites by Scatchard plot analysis.

Table 1. The number and affinity of high-affinity [³H]PGF_{2α} binding to corpora luteal membranes at different stages of the oestrous cycle and pregnancy in cows

Stage	Number of samples	B_{max} (pmol mg ⁻¹ protein)	K_d (nmol l ⁻¹)
Oestrus			
Early	5	1.17 ± 0.16	18.3 ± 2.12
Mid-	3	1.38 ± 0.17	19.1 ± 2.65
Late	3	1.58 ± 0.28	22.4 ± 1.26
Regressed	3	0.17 ± 0.05	31.1 ± 2.99
Pregnancy			
Early	3	0.57 ± 0.05	28.8 ± 9.14
Mid-	3	1.17 ± 0.03	23.7 ± 2.95
Late	3 ^a	1.91	22.8

^aTwo of three membrane preparations from corpora lutea at late pregnancy exhibited no high-affinity binding activity to [³H]PGF_{2α}.

Discussion

The present study demonstrates the abundance of mRNA encoding the PGF_{2α} receptor in the bovine corpus luteum in relation to [³H]PGF_{2α} binding capacity of the luteal membrane throughout the oestrous cycle and pregnancy. In the cyclic corpora lutea, specific hybridization signals to the large 5.0 kb mRNA showed a progressive increase from the early phase, reached the highest value in the late cyclic phase, just before the corpora lutea began to regress. Our binding analysis showed the gradual increase in binding capacity of [³H]PGF_{2α} to the crude luteal membrane fraction over the course of the oestrous cycle. This is consistent with a previous report by Rao *et al.* (1979). When the process of luteolysis was completed in the regressed phase (day 21), both the amount of mRNA encoding the PGF_{2α} receptor and the binding activity of PGF_{2α} on the membrane were markedly reduced. Therefore, the increase in mRNA expression of PGF_{2α} receptor in the late oestrous phase may be responsible for the accumulation of receptor protein on the plasma membrane that initiates luteal regression. We detected high affinity [³H]PGF_{2α} binding sites, with a K_d value of 16.9 nmol l⁻¹, on the luteal membrane taken from the corpora lutea at the beginning of the early oestrous stage (data not shown), suggesting that expression of mRNA encoding PGF_{2α} receptor may start together with the formation and growth of the corpus luteum. Our results contrast with a previous report that, even with the existence of a considerable number of PGF_{2α} receptors in the middle of the oestrous cycle, the affinity ($K_d = 3458$ nmol l⁻¹) of the bovine PGF_{2α} receptor in day 13 corpora lutea was more than 200 times lower than that ($K_d = 17$ nmol l⁻¹) in day 20 corpora lutea (Rao *et al.*, 1979). The reason for the discrepancy remains unknown. However, the present results agree with the reports that exogenous PGF_{2α} administered *in vivo* in cows can initiate luteal regression as early as 4–6 days after ovulation (Gleeson, 1974; Hansel and Convey, 1983). We have shown that expression of high-affinity [³H]PGF_{2α} binding sites may be high enough to produce luteolysis in the bovine corpus luteum at the early stage, and so the present study supports the notion that the synthesis and release of PGF_{2α} is strictly regulated

during the oestrous cycle and pregnancy (Gleeson *et al.*, 1974; Moeliono *et al.*, 1977).

Although [^3H]PGF $_{2\alpha}$ binding activity and amount of PGF $_{2\alpha}$ receptor mRNA in cyclic corpora lutea altered progressively through the cycle, less correlation was observed between them in pregnant corpora lutea. While the amount of receptor mRNA varied little in early and middle pregnancy, much higher binding activity was detected on the corpora lutea of mid-pregnancy than on those of early pregnancy. The corpus luteum produces progesterone and maintains pregnancy until the placenta replaces the function of the corpus luteum. The time of the luteoplacental shift (Auletta and Flint, 1988) in cows is much later than in primates (more than 200 days into the 280 day gestation), and so the corpus luteum remains viable even in late pregnancy. The expression of abundant receptors for PGF $_{2\alpha}$ during the oestrous cycle and pregnancy implies the involvement of PGF $_{2\alpha}$ in luteal functions besides luteolysis.

As is clearly indicated by *in situ* hybridization, mRNA encoding the PGF $_{2\alpha}$ receptor was expressed precisely on corpora lutea obtained from the mid-oestrous phase, but only a faint signal was detected in the early oestrous phase. Receptor mRNA was located mostly in large luteal cells but not or much less in small luteal and nonluteal cells. This is consistent with our previous results (Sakamoto *et al.*, 1994) and also the location of [^3H]PGF $_{2\alpha}$ receptor binding sites and receptors observed by quantitative light microscope autoradiography by Chegini *et al.* (1991). The cellular localization of receptor mRNA and, consequently, of receptor protein, does not change in the ovary.

We demonstrated that 5.0 kb mRNA produces a functional receptor protein and gives typical electrophysiological responses to PGF $_{2\alpha}$ in *Xenopus* oocytes (Sakamoto *et al.*, 1994). Besides the 5.0 kb major RNA band, northern blot analysis sometimes gave a fainter 2.0 kb RNA band. The content of this 2.0 kb mRNA was critically dependent on the preparation lot. So far, we have no indication of whether it encodes a functional receptor protein. Our data from northern blot analysis suggest that the cycle-dependent fluctuation of mRNA amount is quite different between these two RNA populations. The amount of 2.0 kb mRNA, unlike that of the 5.0 kb mRNA appeared to be constant during the oestrous cycle, even in the regressed phase, suggesting that it has a different regulatory mechanism of transcription. By genomic analysis, we established that the bovine haploid genome carries a single copy gene for the PGF $_{2\alpha}$ receptor (T. Ezashi, K. Sakamoto, K. Miwa, E. Okuda-Ashitaka, S. Ito and O. Hayaishi, unpublished). Therefore, at least two different promoters must conduct initiation of these two transcripts, one constitutively promoting smaller RNA synthesis, and the other activating transcription for larger RNA in an oestrous cycle-dependent manner. Specific trans-acting factors may be involved in the accumulation of 5.0 kb mRNA in the late-phase luteal cells. However, the difference in mRNA stability must be considered. An experiment by the 3'RACE-PCR method revealed the existence of two potential poly(A) tails, implying that the difference in mRNA stability is promoted by the external 3' untranslated region (K. Sakamoto, T. Ezashi, K. Miwa, E. Okuda-Ashitaka, S. Ito and O. Hayaishi, unpublished). Determination of the exact transcription units of these RNAs is likely to be of prime importance in explaining the diversity in the amount of mRNA during the oestrous

cycle. Identification of the transcription initiation sites and characterization of promoter activity of the PGF $_{2\alpha}$ receptor gene are in progress in our laboratory.

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