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Acute Expression of the PRL Receptor Gene after Ovariectomy in Midpregnant Mouse Mammary Gland

YASUSI MIZOGUCHI, JAE YOUNG KIM, TETSUO SASAKI*, TARO HAMA*, MASATO SASAKI*, JUMPEI ENAMI* AND SENKITI SAKAI

Department of Animal Breeding, Faculty of Agriculture, University of Tokyo, Tokyo 113, and
** Research Laboratory, Zenyayu Kogyo Co., Ltd., Tokyo 178, Japan*

Abstract. In order to examine the time-dependent expression of the PRL receptor (PRL-R) gene at lactogenesis, the level of PRL-R mRNA was determined following ovariectomy in pregnant mouse mammary gland. Following reverse transcription, the quantity of mRNA was measured by the competitive polymerase chain reaction. The casein (a 22000 molecular weight component) mRNA level was measured as a marker for milk synthesis. Following ovariectomy, the onset of abortion occurred mostly at 22–23 h and the level of casein mRNA began to increase at 12 h. The long and short forms of PRL-R mRNAs were detected in a molar ratio of 1:0.2 on day 12 of pregnancy. Eight h after ovariectomy, the long form of PRL-R mRNA began to increase, showing a bell-shaped profile with the highest peak at 16 h. The short form of PRL-R mRNA was at low levels and remained constant. The levels of the long form of PRL-R mRNA decreased similarly in the presence and absence of foster pups from 24 to 48 h. Conversely, casein mRNA were maintained at high levels by supplying foster pups. The level of the long form of PRL-R mRNA reached a maximum prior to abortion. The present experiments demonstrated that the acute expression of the PRL-R gene occurred in the mammary gland at lactogenesis.

Key words: Casein mRNA, Lactogenesis, Mammary gland, PRL receptor mRNA

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PRL stimulates mammary gland functions by firstly binding to its receptor (PRL-R) present on the plasma membrane of the cell. It is well-known that placental lactogen binds to mammary PRL-R [1, 2] and it is capable of initiating the synthesis of milk *in vitro* [3]. Although placental lactogen is at high levels during the second half of pregnancy [4–6], the synthesis of milk is completely suppressed. The active synthesis of milk occurs following parturition. The binding of PRL to PRL-R is minimal throughout pregnancy but begins to increase before or after parturition [7–9]. At present, the time-dependent change in the number of PRL-Rs especially before parturition remains unknown, since it is difficult to predict precisely the onset-time of parturition *in vivo*. The changes in mammary gland functions are examined by means of the ovariectomy-initiated lactation system as a model of lactogenesis [10–14]. It is difficult to determine the actual number of PRL-Rs by the PRL-binding assay during mid- to late pregnancy, since most PRL-Rs are occupied with placental lactogen at high levels [12], but the determination of the PRL-R mRNA level itself is slightly affected by the presence of placental lactogen. In order to determine the expression of the PRL-R gene, the time-dependent change in the level of PRL-R mRNA was examined following ovariectomy in midpregnant mouse mammary gland. It is well-known that the synthesis of casein is regulated by

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Correspondence to: Dr. Senkiti SAKAI, Department of Animal Breeding, Faculty of Agriculture, University of Tokyo, Yayoi 1–1–1, Bunkyo-ku, Tokyo 113, Japan

PRL [15, 16]. γ-Casein mRNA encodes a mouse milk casein component with a molecular weight of 22,000 [17] and its level is used as a marker for milk synthesis.

The long and short forms of PRL-R cDNA have been previously isolated and sequenced [18–22]. In the mouse ovary, one long and three short forms of PRL-R mRNAs are isolated [23]. The long form of PRL-R (PRL-R_L) which is involved only in milk protein synthesis [22] has an extra and unique amino acid sequence in the intracellular region. The nucleic acid sequence of mouse γ-casein cDNA has also been reported [17]. In the present experiments, chimeric DNA (competitor DNA) capable of annealing with the sense and antisense primers for the candidate cDNA was constructed. Mammary gland mRNA was reverse-transcribed to cDNA, and quantitatively determined by using competitor DNA in the polymerase chain reaction (competitive RT-PCR) [24, 25].

Materials and Methods

Materials

Phagemids pBluescript II SK(+) was obtained from Stratagene (La Jolla, CA). Taq DNA Polymerase and SUPERSCRIPT™ II RNase H⁻ Reverse Transcriptase were from GIBCO BRL (Grand Island, NY). An mRNA Separator kit was from Clonteck (Palo Alta, CA). Random heximer primers were from Toyobo (Osaka, Japan). Restriction endonucleases were from Takara (Kyoto, Japan). The 100 bp ladder marker was from Pharmacia (Upsala, Sweden). All synthetic primers used in PCR were manufactured by Toa Synthesis Co. (Tokyo, Japan). Steroid hormones were from Sigma (St Louis, MO).

Animals

ICR mice were supplied by Clea (Tokyo, Japan). The day on which a vaginal plug was found was designated as day zero of pregnancy. Bilateral ovaries were removed under pentobarbitone sodium anesthesia at 1000–1130 h on day 12 of pregnancy. The completion of ovariectomy was taken as time zero of ovariectomy. The onset of abortion occurred mostly at 22–23 h after ovariectomy. Adrenalectomy was performed following ovariectomy. Cortisol (1 mg) or progesterone (1 mg) was dissolved in 0.2 m*l* olive oil and administered at 0 h of ovariectomy. For foster-nursing, the pups were obtained one day after normal delivery. Ten pups, separated for 6 h from their mother, were supplied to each mouse at 24 h of ovariectomy.

Extraction of RNA and synthesis of cDNA

Whole mammary glands were collected immediately after sacrifice. Total RNA was extracted from fresh mammary glands according to the procedures described by Chomczynski & Sacchi [26]. According to the SUPERSCRIPT II instructions, cDNA was transcribed from 1.25 μg of total RNA, 200 units of reverse transcriptase, 200 nmol of each dNTP and 100 pmol of random heximer primers. The reaction was performed at 37 °C for 90 min in a total volume of 20 μ*l*. The enzyme was inactivated by heating at 70 °C for 15 min and the cDNA was stored at −50 °C until use.

In order to determine the mRNA concentration-dependency of reverse-transcription (RT), the poly(A) RNA rich fraction was prepared from mammary gland on day 0 of lactation with an mRNA Separator kit. Poly(A) RNA which ranged between 12.5 ng and 125 ng was added to RNA of the 0 h-ovariectomized and pregnant control (the reference RNA standard). The reverse-transcription reaction was performed as described above.

Construction of competitive DNA for target cDNAs

The procedures for competitive PCR were originally described by Gilliland *et al.* [24]. The long form of mouse PRL-R (PRL-R_L) cDNA (1827 bp, DDBJ database: accession No. D10214) was inserted into pBluescript II SK(+). The *Pst* I/*Bam* HI (327 bp) and *Eae* I/*Hind* III (562 bp) fragments of PRL-R cDNA clone and the *Mbo* I/*Eae* I fragment (728 bp) of pBluescript II SK(+) were ligated. The chimeric DNA was inserted into pBluescript II SK (+). Competitor DNA (1638 bp) was prepared by digesting plasmid DNA with *Pst* I and *Xho* I.

Mouse γ-casein competitor DNA was constructed in pBluescript II SK(+) by replacing the *Nhe* I/*Kpn* I fragment of mouse γ-casein cDNA clone (DDBJ database: accession No. D10215) with the *Nhe* I/*Kpn* I fragment of mouse PRL-R cDNA.

Competitive PCR

The amount of competitor DNA for PRL-R cDNAs varied between 0.1 and 12.5 atto(a)-mol/reaction. The sense primer hybridized with the region (285–309 nucleotide (nt)) of both the long and short forms of PRL-R (PRL-$R_{L\&S}$) cDNA. One antisense primer hybridized the same region (765–789nt) of PRL-$R_{L\&S}$ cDNA, and the other antisense primer was specific to the unique sequence (846–870nt) of PRL-R_L cDNA. The structure of competitor DNA used for the determination of PRL-R_L and PRL-$R_{L\&S}$ mRNAs is shown in Fig. 1. γ-Casein competitor DNA varied from 2 to 240 amol. A set of the primers was as follows:

PRL-$R_{L\&S}$ 5′-CATCACAGTAAATGCCACGAACGAA-3′ (sense; 285–309nt, Tm; 70) 5′-AAAGATGCAGGTCATCATGCTATAA-3′ (antisense; 765–789nt, Tm; 66)

PRL-R_L 5′-CATCACAGTAAATGCCACGAACGAA-3′ (sense; 285–309nt, Tm; 70) 5′-GGCACTCAGCAGTTCTTCAGACTTG-3′ (antisense; 846–870nt, Tm; 72)

γ-casein 5′-GCTGGACAATAGCGTGTTCTTCC-3′ (sense; 114–136nt, Tm; 70) 5′-GGGAAGGTGTAATCCTTACTGGG-3′ (antisense; 505–527nt, Tm; 70).

Twenty μl of the PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$) contained 1 unit of Taq DNA polymerase, 10 pmol of each primer, various amounts of competitor DNA and 4 nmol of each dNTP. One μl of cDNA was added to the reaction mixture above, and the reaction was cycled 30 times. Each cycle consisted of 94 °C-1 min for denaturing, 58 °C-1 min for annealing and 74 °C-1 min for extension. The last reaction was carried out for 2 min at 74 °C and then terminated at 4 °C. The optimum concentration of cDNA was obtained by serial dilution with water. The PCR products (5 μl) were separated through a 1.5% agarose gel. The gel was stained with ethidium bromide (0.3 μg/ml) and photographed on a Polaroid 665 positive/negative film. The negative film was scanned in a scanning densitometer. All RNA samples were assayed for DNA contamination by doing PCR without the reverse transcription step.

The reference RNA standard was constructed by pooling RNA of 10 pregnant mice ovariectomized at 0 h. An aliquot of the standard RNA was applied for each competitive RT-PCR assay.

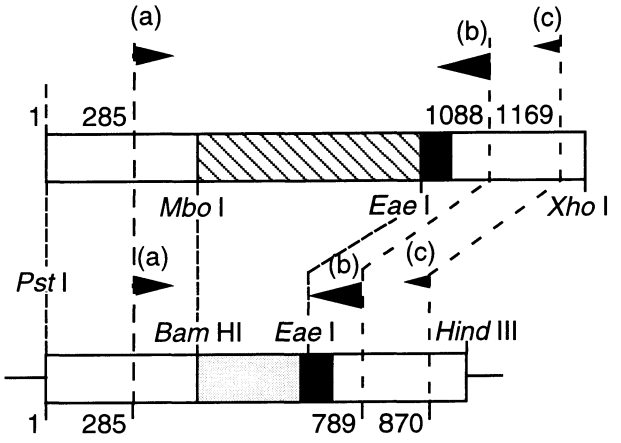

Fig. 1. Schematic structure of competitor DNA used for the determination of PRL-R mRNA. Competitor DNA (upper) was constructed by replacing the *Bam* HI/*Eae* I fragment (dotted) of mouse PRL-R_L cDNA clone (lower) with the *Mbo* I/*Eae* I fragment (hatched) of the pBluescript II SK(+). The positions hybridized with primers are shown by arrows and the transmembrane region by the black column. PRL-$R_{L\&S}$ and PRL-R_L sense primer (a); PRL-$R_{L\&S}$ antisense primer (b); PRL-R_L antisense primer (c).

Data analysis

Data are expressed as the mean ± SEM. Statistical analysis was made by Student's *t*-test, linear regression analysis or analysis of variance (ANOVA). Differences were considered to be significant when probability (*P*) was less than 0.05.

Results

Measurement of PRL-R mRNA by competitive RT-PCR

Figure 2a shows the representative results for the PCR products obtained with the antisense primer specific to PRL-R_L. In each lane, two bands were clearly recognized. The upper bands (885 bp) were derived from competitor DNA while the lower bands (586 bp) were from PRL-R_L cDNA. The amplification of PRL-R_L cDNA was competitively inhibited depending on the increase in competitor DNA, since the density of PRL-R_L

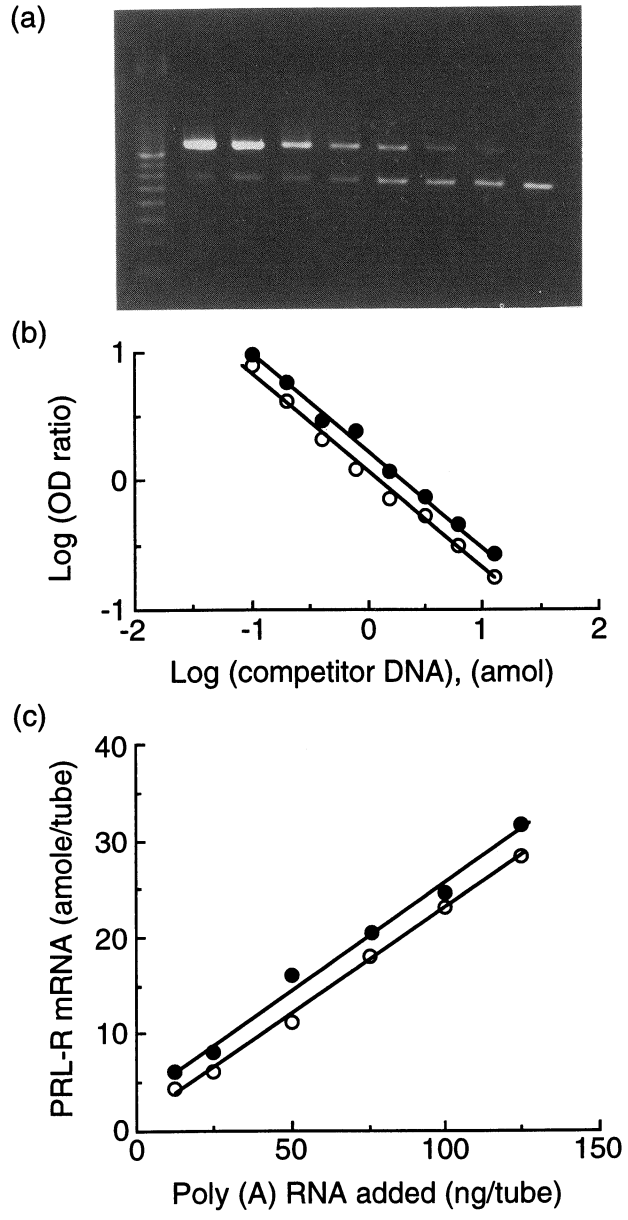

Fig. 2. Construction of competitive PCR. a: PCR was carried out with a fixed amount of cDNA (1 μl of RT reaction mixture) in the presence of various amounts of competitor DNA (0.1 to 12.5 amol). The PCR products (right 8 lanes) and the 100 bp ladder marker (left). b: The OD ratio of the two bands plotted against the amount of competitor DNA. The OD of the 586 bp product was corrected with a factor 885/586. c: Poly(A) RNA, isolated from mammary glands on day 0 of lactation, was increased from 12.5 ng to 125 ng. RNA, adjusted to 1.25 μg with the reference RNA standard, and was reverse-transcribed to cDNA. Results are expressed as the corresponding quantity of competitor DNA added to PCR. (○); the long form, (●); the long and short forms.

cDNA decreased gradually. Due to the difference in the length of PCR products, the optical density (OD) of the 586 bp product was corrected with a factor of 885/586. The relationship between the corrected OD ratio of the two bands and the amount of competitor DNA was logarithmically linear ($r=-0.99$) (Fig. 2b). The ratio "0" on the vertical axis indicated that the quantity of PRL-R_L cDNA was equal to that of competitor DNA. The quantity of PRL-R_L mRNA was determined by extrapolation from that of competitor DNA on the horizontal axis. Under the present assay conditions, DNA amplification in PCR was reproducible with small intra- and interassay coefficients of variation (less than 10%). In the PRL-$R_{L\&S}$ competitive PCR, two bands were identified: one band of 804 bp was derived from competitor DNA and the other band of 505 bp was from PRL-$R_{L\&S}$ cDNAs. The regression line was parallel to that in the PRL-R_L PCR but shifted to the right due to the presence of PRL-R_S cDNAs. Reverse-transcription was performed by adding specific amounts of poly(A) RNA (Fig. 2c). The PCR products for PRL-R_L and PRL-$R_{L\&S}$ increased in a linear manner ($r>0.98$) with the increasing amounts of poly(A) RNA. The regression lines were parallel to each other. Poly(A) RNA was quantitatively converted to cDNA. PRL-R_S mRNA was determined by subtracting PRL-R_L mRNA from PRL-$R_{L\&S}$ mRNA.

In the mouse γ-casein competitive RT-PCR, two bands were identified: one band of 609 bp was derived from competitor DNA and the other band of 414 bp was from γ-casein cDNA. The results were essentially similar to those in Fig. 2 (data not shown).

Levels of PRL-R_L and PRL-R_S mRNAs in ovariectomized and ovariectomized-adrenalectomized mouse mammary glands

Table 1 shows the levels of PRL-R_L and PRL-R_S mRNAs in ovariectomized and ovariectomized-adrenalectomized mouse mammary glands. At 20 h of ovariectomy, the level of PRL-R_L mRNA was 3.5-fold higher than that of the 0 h control ($P<0.01$). The level of PRL-R_S mRNA was low and only insignificantly changed. The administration of progesterone inhibited an increase in the level of PRL-R_L mRNA ($P<0.01$), while PRL-R_S mRNA was at the same level as that of the vehicle-injected

Table 1. Levels of PRL-R_L and PRL-R_S mRNAs in mammary glands

	PRL-R_L (relative mRNA level)[a]	PRL-R_S
pregnancy (12 d)[b]	1.0 ± 0.4	0.2 ± 0.2
ovariectomy[c]		
+ oil	3.5 ± 0.6	0.5 ± 0.2
+ progesterone	1.6 ± 0.2	0.5 ± 0.2
ovari-adrenalectomy[c]		
+ oil	1.5 ± 0.1	0.2 ± 0.1
+ cortisol	2.3 ± 0.2	0.3 ± 0.2
pregnancy (18 d)[d]	1.8 ± 0.6	0.4 ± 0.1
lactation (0 d)[d]	4.7 ± 0.4	0.3 ± 0.7

[a] The PRL-R_L mRNA level in the reference RNA standard was taken as 1. Mean ± SEM (n=4). [b] Mice were assayed at 0 h of ovariectomy. [c] Mice given 1 mg of progesterone or cortisol at 0 h of operation were assayed 20 h later. [d] Intact mice.

control. Adrenalectomy inhibited an ovariectomy-induced increase in the PRL-R_L mRNA level ($P<0.05$), but the levels of PRL-R_S mRNA were unaffected by adrenalectomy and/or cortisol therapy. As shown in the same table, the level of PRL-R_L mRNA increased by 2.6 times the day before and after parturition ($P<0.05$). No changes in mammary PRL-R_S mRNA levels were found following parturition as compared with those on day 18 of pregnancy.

Time-dependent changes in PRL-R_L mRNA and γ-casein mRNA

PRL-R_L and γ-casein mRNAs in the mammary gland were measured over a 40 h period (Fig. 3). The onset of abortion occurred mostly at 22–23 h after ovariectomy. PRL-R_L mRNA began to increase at 12 h ($P<0.05$), reached a maximum at 16 h and then decreased linearly with time. The highest levels were 4.1 times higher than those of the 0 h-ovariectomized mice ($P<0.01$). At 40 h, levels were still higher than those at 0 h ($P<0.05$). Levels of PRL-R_S mRNA were low and remained constant. γ-Casein mRNA remained at low levels until 8 h but increased sharply between 8 and 16 h and maintained high levels until 32 h. γ-Casein mRNA levels at 16 h were 4.8 times greater than those at 0 h. From 32 to 40 h, it decreased by 60% ($P<0.01$). At the earlier stages of ovariectomy, levels of PRL-R_L mRNA changed in parallel with those of γ-casein mRNA. The levels of PRL-R_L mRNA decreased faster than those of γ-casein mRNA (ANOVA, $P<0.05$).

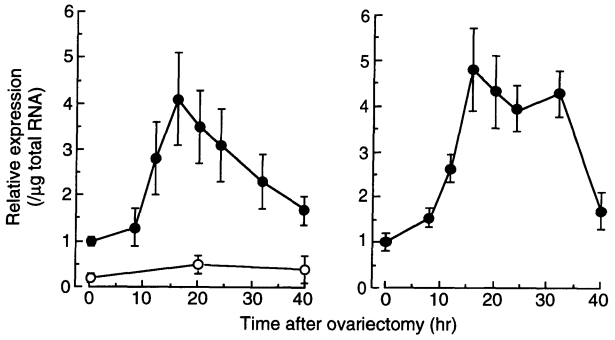

Fig. 3. Time-dependent changes in PRL-R mRNA and γ-casein mRNA following ovariectomy. PRL-R_L (●) and PRL-R_S (○) mRNAs are shown on the left and γ-casein mRNA is shown on the right. Data were compared with PRL-R_L or γ-casein mRNAs in the reference RNA standard. Samples were collected at 0, 8, 12, 16, 20, 24, 32 and 40 h after operation. Data for PRL-R_S obtained at 0, 20 and 40 h are shown. Each vertical bar indicates the SEM for four mice.

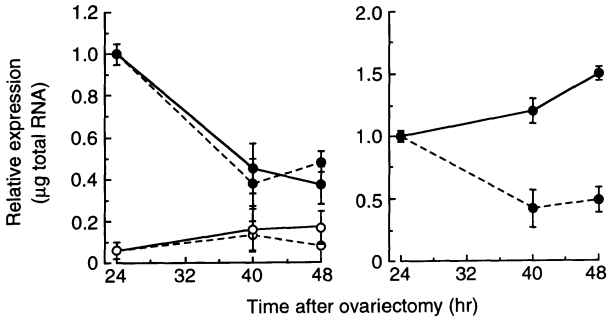

Fig. 4. Effects of foster pups on levels of PRL-R mRNA and γ-casein mRNA. Ten pups (solid lines) and no pups (dotted lines) were supplied 24 h after ovariectomy. PRL-R_L (●) and PRL-R_S (○) mRNAs are shown on the left. γ-Casein mRNA is shown on the right. Levels of PRL-R_L and γ-casein mRNAs in 24 h-ovariectomized mice were taken as 1. Each vertical bar indicates the SEM for four mice. The SEMs for PRL-R_S mRNA are not shown.

Effect of foster-nursing on levels of PRL-R_L and γ-casein mRNAs

In order for ovariectomized mice to continue milk synthesis, the pups were supplied to mice at 24 h after ovariectomy. Levels of PRL-R_L and γ-casein mRNAs were determined at 40 and 48 h

after ovariectomy and were compared with those obtained at 24 h after ovariectomy (Fig. 4). Results showed that the levels of PRL-R_L mRNA decreased similarly with or without foster pups. At 40 and 48 h, differences between the two groups were insignificant. In mice with and without foster pups, levels of PRL-R_S mRNA were unchanged. In mice without foster pups, γ-casein mRNA decreased significantly ($P<0.05$), but, with foster pups, it remained at high levels and at 48 h, it was elevated further to 1.4 times more than that at 40 h ($P<0.05$). Differences between the two groups were significant at 40 h ($P<0.05$) and 48 h ($P<0.01$).

Discussion

It is well-established that the mammary gland initiates the synthesis of milk following ovariectomy in pregnant mice and rats. The present data indicate that both PRL-R_L and PRL-R_S mRNAs existed in the mammary gland, but the level of PRL-R_L mRNA was always higher than that of PRL-R_S mRNA over a 48 h period after ovariectomy. The level of PRL-R_L mRNA was elevated clearly high after ovariectomy. The PRL-R_S mRNA was at low levels throughout the experiments. It has been reported that PRL-R_L mRNA is dominant in the mammary glands of lactating mice [27], rabbits [28] and rats [29], but the dominant PRL-R mRNA species is for PRL-R_S mRNA in the mouse [19] and rat liver [30]. Although three PRL-R_S mRNA species are reported in the mouse ovary [23], 5'- and 3'-primers used here are able to anneal with these PRL-R_S cDNAs and to be used for amplification in PCR. It is concluded that by ovariectomy, PRL-R_L mRNA is selectively expressed in the mouse mammary gland at midpregnancy.

At midpregnancy and following ovariectomy, the amount of PRL binding increases in a time-dependent manner and reaches a maximum 32 h after operation [14]. The presence of high levels of placental lactogen, particularly before abortion in the present system, makes it difficult to accurately determine the number of PRL-Rs due to the masking of PRL-R with the hormone [12]. Furthermore it is difficult to predict precisely the onset time of parturition *in vivo*. The present experiments clearly showed that the level of PRL-R_L mRNA was low at midpregnancy but began to rise sharply after 8 h of ovariectomy. The PRL-R_L mRNA acutely expressed prior to the onset of abortion. Concomitantly, the level of casein mRNA was sharply increased. The expression of the casein gene occurred in parallel with that of the transcription of PRL-R_L. It has been shown that PRL-R_L is involved only in milk protein synthesis [22]. This suggests that PRL-R_L mRNA is rapidly translated to PRL-R_L in the mammary gland. Administration of progesterone inhibits both increases in PRL binding and lactose synthesis in the ovariectomized midpregnant mouse mammary gland [10]. It is probable that the increase in the number of PRL-R_Ls at lactogenesis is associated with the expression of the PRL-R_L mRNA. The serum PRL concentration does not change after ovariectomy in midpregnant mouse [11]. Placental lactogen is capable of initiating milk synthesis [3] by binding to mammary PRL-R [1, 2]. Placental lactogen is at high levels during the second half of pregnancy [4–6]. The level of PRL-R_L mRNA began to increase about 10 h before abortion. Based on the present findings, it is speculated that as a result of the increased levels of PRL-R_L mRNA in the mammary gland, placental lactogen initiates the synthesis of casein mRNA by binding to PRL-R.

The level of casein mRNA remained high for a longer period than that of PRL-R_L mRNA. Hereafter, casein mRNA dropped to low levels, probably due to the accumulation of milk. This is supported by the fact the high casein mRNA level is sustained by supplying foster pups. But the level of PRL-R_L mRNA decreased regardless of the presence or absence of foster pups, suggesting that the degree of the PRL-R gene expression differed according to the stage of differentiation. By *in situ* hybridization in early lactating rats, most PRL-R_L mRNAs are localized in cuboidal alveolar cells with relatively narrow lumens and the expression is very weak in flattened alveolar epithelium with large lumens filled with milk [31]. PRL-R is a membrane-integrated protein, while casein is transported into the alveolar lumen after translation. It is speculated that, after translation, PRL-R_L is retained for a relatively long period by the mammary cells.

Kuhn [13] proposed that the trigger for lactogenesis was the withdrawal of progesterone in the circulation. Circulating progesterone is at high levels during the second half of pregnancy but begins

to decrease at the end of pregnancy [32]. Progesterone is unable to increase the amount of PRL binding to mammary cell *in vitro* [33] and suppresses the expression of the PRL-R gene at the level of transcription [34], while glucocorticoid increases the amount of PRL binding to mammary cells *in vivo* [35] and *in vitro* [33]. This study indicated that cortisol stimulated the expression of PRL-R genes. The glucocorticoid receptor level is high during the second half of pregnancy [36]. As progesterone is able to bind to mammary glucocorticoid receptors [37], it is expected that most glucocorticoid receptors are occupied with progesterone during the second half of pregnancy. After ovariectomy, progesterone in plasma decreased from 50 to 9 ng/m*l* in the first 2 h and its levels were lowered to below to 1 ng/m*l* at 16 h, and corticosterone in plasma increased from 78 to 600 ng/m*l* during the first 2 h and its concentrations were at levels higher than 700 ng/m*l* until 8 h (unpublished data). It is probable that both the high level of glucocorticoid and the low level of progesterone are essential for the expression of the PRL-R gene in the mammary gland.

It is important to make clear the hormonal regulation of lactogenesis at the level of the gene expression. The present experiments reveal the time-dependent change in the PRL-R gene expression following ovariectomy in the midpregnant mouse mammary gland. The expression of the PRL-R gene is initiated about 10 h earlier than the onset of abortion. Most transcripts of the PRL-R gene are processed for the synthesis of PRL-R_L. The expression of the PRL-R gene is a contributing factor in the increase in the number of PRL-Rs. The synthesis of casein mRNA is initiated prior to abortion. Since PRL regulates the synthesis of casein by binding particularly to PRL-R_L, it is concluded that the initiation of milk synthesis is regulated at the stage of PRL-R gene expression.

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