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Hormone-Dependent Expression of γ -Casein mRNA in Mouse Mammary Epithelial Cells Cultured on Floating Collagen Gels

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ABSTRACT—Mammary epithelial cells isolated from pregnant mice were plated on a collagen gel matrix and cultured in a serum-free medium supplemented with combinations of insulin, dexamethasone and prolactin (PRL). After the cells formed a monolayer, the collagen gel was detached from the culture dish and allowed to float in the medium. Quantification of γ -casein mRNA by a competitive PCR method revealed that the cells on the floating gel accumulated considerably larger amounts of γ -casein mRNA than the cells on the gel remained attached to the dish. Under these floating collagen gel culture conditions, addition of both dexamethasone and PRL to the insulin-supplemented basal medium maximally stimulated the accumulation of γ -casein mRNA. These observations strongly suggest that the status of the extracellular matrix as well as hormones controls the differentiation of mouse mammary epithelial cells at the mRNA level.

INTRODUCTION

Coordinated actions of multiple hormones control the synthesis and secretion of milk proteins by the lactating mammary gland *in vivo* (Nandi, 1959). Organ culture studies using mammary explants from mid-pregnant mice demonstrated that a combination of insulin, glucocorticoids and prolactin (PRL) is essential for induction of milk protein synthesis *in vitro* (Enami and Nandi, 1977; Topper and Freeman, 1980; Prop, 1981). Further studies using monolayer cultures of isolated mammary epithelial cells established that these hormones act directly on the epithelial cells. The level of milk protein synthesis under the monolayer culture conditions, however, was extremely low as compared with the lactating mammary epithelial cells *in vivo* (Enami, 1977). To observe an augmented production of milk proteins in culture, various extracellular matrices were used as culture substrata. It was then revealed that floating collagen gel matrix supports enhanced and continued production of milk proteins by mammary epithelial cells (Emerman *et al.*, 1977; Emerman and Pitelka, 1977; Enami *et al.*, 1979; Shannon and Pitelka, 1981). Later, augmentation of milk protein synthesis was also observed with reconstituted basement membranes derived from the Engelbreth-Holm-Swarm (EHS) tumor (Li *et al.*, 1987; Blum *et al.*, 1987). Thus, the importance of the source and the status of extracellular matrix has been suggested.

To address a question whether or not the extracellular matrices and hormones control the milk protein synthesis at the mRNA level, we first developed a competitive PCR method

for measurement of γ -casein mRNA. We then measured the γ -casein mRNA contents in mammary epithelial cells cultured under various hormonal and extracellular matrix conditions. The results obtained indicated that not only hormones but also the status of the collagen gel strongly affects the expression of milk protein mRNA.

MATERIALS AND METHODS

Primary culture of mammary epithelial cells

Epithelial cells were isolated from 13- to 16-day-pregnant BALB/c mouse mammary glands after digestion with collagenase and actinase as described previously (Enami *et al.*, 1979, 1987). The epithelial cells were suspended in Dulbecco's modified Eagle's medium (DME) supplemented with 10% fetal bovine serum (FBS; GIBCO/BRL), penicillin (100 U/ml) and streptomycin (100 μ g/ml), and plated on a bed of collagen gel (Cellmatrix I-A; Nitta Gelatine, Osaka) (Enami *et al.*, 1987; Enami and Tsukada, 1993) at a density of 2.5×10^6 cells/cm² in a 16-mm well (Corning Cell Wells). Prior to hormone treatment, mammary epithelial cells were adapted in a basal serum-free medium [a mixture of equal volume of DME and Ham's F12 containing 10 mM Hepes (pH 7.4), 1 μ g/ml of insulin (28.6 I.U./mg; bovine pancreatic; Sigma), 2.5 mg/ml of bovine serum albumin (BSA; Nitta Gelatine, Osaka) and antibiotics] for 2 or 3 days.

Floating collagen gel culture and hormone treatment

After the epithelial cells attached and spread on the collagen gel, the gel was detached from the plastic surface by carefully trimming the edge around the culture well with a sterile needle. Dexamethasone (Sigma) and PRL (ovine; Sigma) were added to the basal medium at final concentrations of 1 μ g/ml and 5 μ g/ml, respectively. Medium change was done every other day.

RNA isolation and reverse transcription

Total RNA was isolated from the cells on collagen gels by the

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acid guanidinium-phenol-chloroform method of Chomczynski and Sacchi (1987) with the following modifications. A bed of collagen gel with mammary epithelial cells growing on it was placed on several sheets of filter paper to remove the culture medium, transferred into 0.9 ml of solution D and then quickly homogenized in a polytron homogenizer. One hundred and thirty μ l of 2 M sodium acetate (pH 4.0), 1.3 ml of water-saturated phenol and 0.4 ml of chloroform were then added and mixed well. The mixture was left on ice for 15 min. After centrifugation at $20,000 \times g$ at 4°C for 20 min, RNA in the aqueous phase was precipitated with 2-propanol, redissolved in 0.5 ml of solution D and reprecipitated with 2-propanol. The RNA pellet was rinsed twice with 80% ethanol and finally dried. The RNA content was determined by measuring the O.D.₂₆₀ of the water-dissolved samples. First strand cDNA was synthesized in a volume of 20 μ l containing up to 1.5 μ g of total RNA, 1 pmol of random hexamer primer (Takara Shuzo, Kyoto), 200 units of SuperScript™ II reverse transcriptase (GIBCO/BRL) and a buffer system supplied by the manufacturer. After incubation at 37°C for 90 min the reaction mixture was heated at 95°C for 5 min. The first strand cDNA was stored at -80°C until use.

Quantification of mouse γ -casein mRNA by competitive PCR

Quantification of mouse γ -casein mRNA was done by applying the competitive PCR method of Gilliland *et al.* (1990). A chimeric DNA (Fig. 1) was constructed in pBluescript II (Stratagene) by replacing the *NheI/KpnI* fragment of mouse γ -casein cDNA (Sasaki *et al.*, 1993) with the *NheI/KpnI* fragment of mouse PRL receptor cDNA (Sasaki *et al.*, 1996). The competitor DNA fragment was made by cutting the above newly constructed vector with *XbaI* and *SspI*. Competitive PCR was performed for 35 cycles (94°C , 1 min; 60°C , 1 min; 72°C , 1 min) followed by a final extension of 9 min at 72°C . The reaction mixture contained cDNA (0.1–1.0 μ l of the above reverse transcription product), various amounts of the competitor, 2.5 pmol each of sense and antisense primers, 2.5 nmol each of dNTPs and 0.25 unit of *TaKaRa Taq* DNA polymerase (Takara Shuzo, Kyoto) in a final volume of 12.5 μ l. After amplification a 10 μ l portion was electrophoresed on a 1% agarose gel. The gel was stained with ethidium bromide and photographed on a Polaroid 665 positive/negative film. The density of each band on the negative film was quantified by scanning in a densitometer (Shimadzu, CS-9000). The ratio of the densities of the γ -casein and the competitor bands (γ -casein/competitor) was multiplied by 1.47 (molecular weight correction factor) and plotted against the reciprocal of the amount of added competitor expressed in moles. The amount of double-strand γ -casein cDNA was determined from the amount of competitor corresponding to the ratio "1" on the vertical axis. This value was multiplied by 2 to give the amount of single-strand γ -casein cDNA initially present in the reaction mixture. The amount of single-strand γ -casein cDNA thus obtained was directly used to express the relative amount of γ -casein mRNA.

RESULTS

Quantification of mouse γ -casein mRNA by competitive PCR

To measure γ -casein mRNA content in cultured mammary epithelial cells, we first developed a sensitive quantification method using competitive PCR. The competitor DNA, which serves as an internal standard, was constructed so that γ -casein cDNA and competitor DNA differ in size and also differ in sequences except for the primer regions (Fig. 1). An electrophoretic analysis of the PCR products showed that the presence of the competitor (609 bp) in the same reaction mixture inhibited the amplification of γ -casein cDNA (414 bp) competitively (Fig. 2, insert photograph). After PCR using two different concentrations of γ -casein mRNA the ratios of

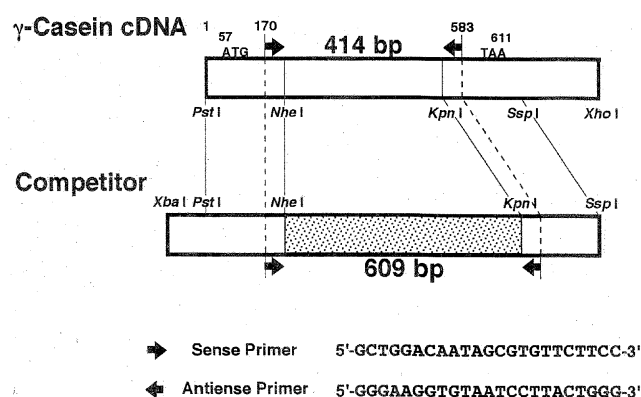


Fig. 1. Schematic representation of mouse γ -casein cDNA and a competitor DNA for competitive PCR. The numbers on top indicate the nucleotide positions in the cDNA. "414 bp" and "609 bp" indicate the sizes of the PCR products. Stippled box shows the sequence derived from mouse PRL receptor cDNA. Sense and antisense primer sequences are shown at the bottom.

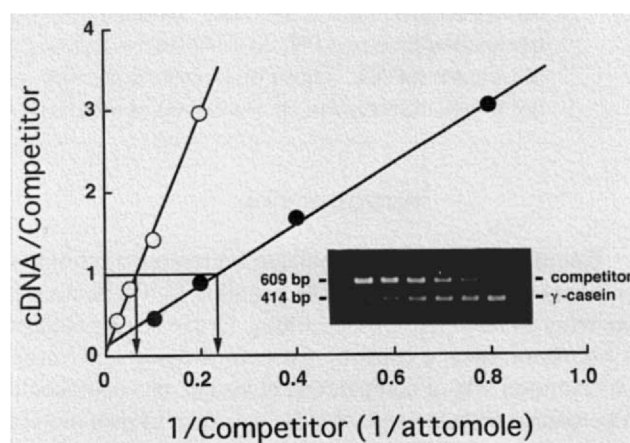


Fig. 2. Quantification of γ -casein cDNA by competitive PCR. Reverse transcription was done in a volume of 20 μ l using 0.375 μ g (●) or 1.5 μ g (○) of total RNA obtained from mammary epithelial cells cultured on floating collagen gels in the presence of dexamethasone and PRL in the insulin-supplemented basal serum-free medium. A portion (0.1 μ l) of the first strand cDNA was mixed with various amounts of the competitor and served as a template for competitive PCR. After competitive PCR the ratio of the densities of the ethidium bromide-stained γ -casein and competitor bands (γ -casein/competitor), shown in the insert photograph, was corrected for the difference in molecular weight and plotted against the reciprocal of the amount of added competitor. The lines were drawn from linear regression analyses of the data. The amount of γ -casein cDNA in the sample was obtained after doubling the reciprocal of the value pointed by the arrows.

the densities of electrophoretic bands (γ -casein/competitor) were corrected for the difference in molecular weight and plotted against the reciprocal of the amount of competitor added (Fig. 2). The results showed that the 4-fold difference in the amount of γ -casein mRNA in the original sample was correctly reflected in the final PCR products. Thus, the linearity of the quantification method was established.

Effect of collagen gel status on expression of γ -casein mRNA

Using the competitive PCR method we examined whether or not the status of the collagen gel affects the expression of γ -casein mRNA in mammary epithelial cells. Mouse mammary epithelial cells were plated on a bed of collagen gel at a high density (2.5×10^6 cells/cm²) and cultured for 3 days in the basal serum-free medium containing insulin alone. Dexamethasone and PRL were then added and the culture was continued for one week. The total RNA content/well remained unchanged as long as the collagen gel remained attached to the culture well surface. When the collagen gel was detached from the culture surface and allowed to float in the medium, however, the total RNA content/well decreased to approximately a half of the initial level in 4 days (Fig. 3A). By contrast, γ -casein mRNA content in cells cultured for 4 days on floating collagen gels increased to a level 140-fold higher than that in cells on attached gels. This difference further increased to 330-fold after 7 days in culture. When the flotation of the collagen gel was delayed, a delayed but dramatic increase in γ -casein mRNA content was seen (Fig. 3B).

Concerning the difference between the attached and the floating collagen gels we have consistently observed the changes in gel size. As soon as the collagen gel was detached from the culture surface, the gel started to contract and within a few days the diameter of the floating gel became 1/3 of that of the attached gel (data not shown). This observation is consistent with previous observations (Emerman *et al.*, 1977; Emerman and Pitelka, 1977; Enami *et al.*, 1979; Shannon and Pitelka, 1981).

Effect of combinations of hormones on expression of γ -casein mRNA

We next examined the effects of combinations of hormones on total RNA and γ -casein mRNA contents in

mammary epithelial cells cultured on floating collagen gels. The total RNA contents gradually decreased when the cells were cultured in the absence of hormones. This decrease could not be prevented by supplementation of either dexamethasone or PRL alone (Fig. 4A). However, supplementation of both PRL and dexamethasone prevented the decrease and slightly increased the total RNA content. Thus, cooperative action of PRL and dexamethasone on maintenance of total RNA content was shown. With regard to the changes in γ -casein mRNA content, the effect of hormones were more clearly discernible. A 2,800-fold increase in γ -casein mRNA content was attained in 4 days in the presence of both dexamethasone and PRL (Fig. 4B). Cultivation in the basal medium or in the presence of dexamethasone alone was without effect. PRL, however, significantly increased the expression of γ -casein mRNA, although the level was 10-fold lower than that attained by the combination of dexamethasone and PRL. These results suggest that PRL is essential for induction of γ -casein mRNA in mammary epithelial cells of the pregnant mouse and that the γ -casein mRNA level is increased by dexamethasone.

Effect of PRL on expression of γ -casein mRNA

Based on the above observation suggesting the essential role of PRL in the turnover of RNAs, we examined the effects of different doses of PRL on total RNA and γ -casein mRNA contents in mammary epithelial cells cultured on floating collagen gels. Mouse mammary epithelial cells were cultured on floating collagen gels in the presence of 1 μ g/ml of dexamethasone and different doses of PRL for 4 days. PRL stimulated the increase in total RNA content in a dose-dependent manner (Fig. 5A). A 2-fold increase in total RNA content was attained with 1 μ g/ml of PRL. PRL also stimulated the increase in γ -casein mRNA content in a dose-dependent manner. A 3,400-fold increase in γ -casein mRNA content was

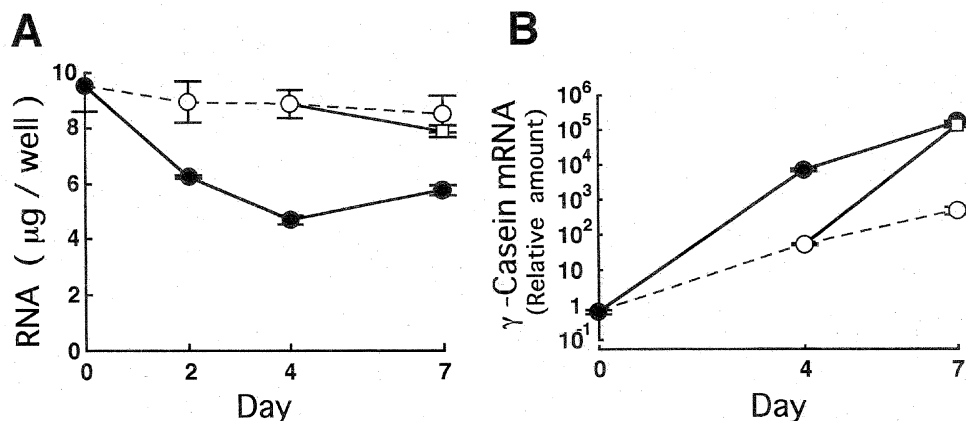


Fig. 3. Effect of collagen gel matrix status on total RNA content (A) and γ -casein mRNA content (B) in cultured mammary epithelial cells. After serum-free cultivation of mammary epithelial cells (2.5×10^6 /cm²) for 3 days on collagen gels, dexamethasone (1 μ g/ml) and PRL (5 μ g/ml) were added (Day 0). Some of the collagen gels were detached from the culture surface on days 0 and 4. Total RNA content/well was determined by measuring O.D.₂₆₀. γ -Casein mRNA content/well was measured by the competitive PCR method and expressed as the amount relative to 0.1 attomol of γ -casein cDNA. Solid lines, floating (detached) collagen gel; broken line, attached collagen gel. The data represent the mean $\pm$ SEM from triplicate cultures.

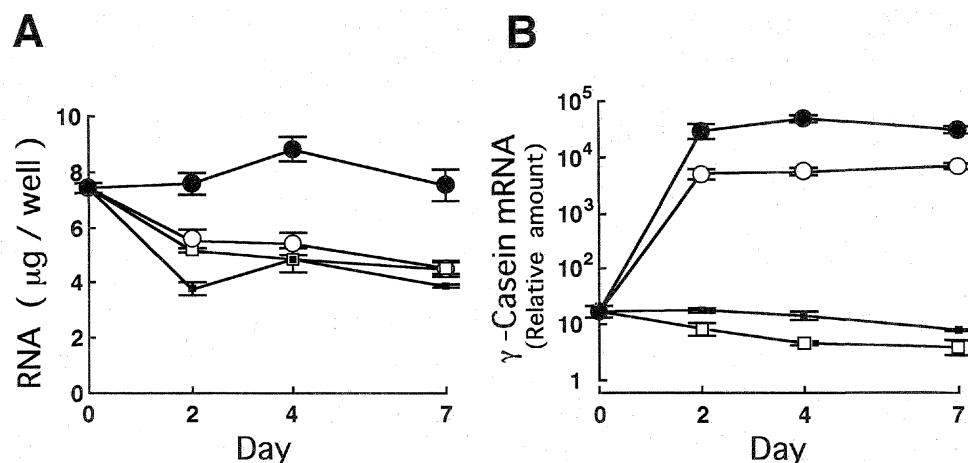


Fig. 4. Effects of hormones on total RNA content (A) and γ -casein mRNA content (B) in mammary epithelial cells cultured on floating collagen gels. After cultivation of mammary epithelial cells on floating collagen gels for 3 days in the basal serum-free medium supplemented with insulin alone, various combinations of hormones were added (Day 0) and the culture was continued for 7 days. γ -Casein mRNA content/well was expressed as the amount relative to 0.1 attomol of γ -casein cDNA.

□, insulin alone; ■, insulin + dexamethasone (1 μ g/ml); ○, insulin + PRL (5 μ g/ml); ●, insulin + dexamethasone + PRL. The data represent the mean $\pm$ SEM from triplicate cultures.

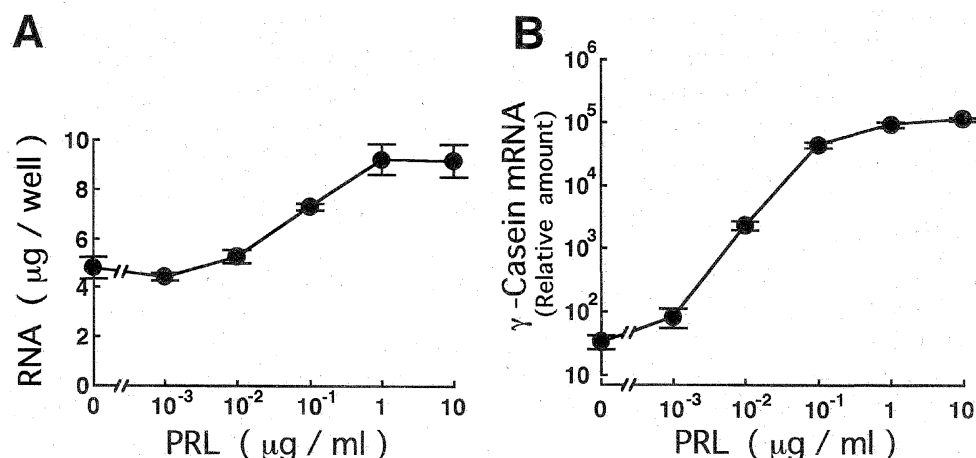


Fig. 5. Effects of different doses of PRL on total RNA content (A) and γ -casein mRNA content (B) in mouse mammary epithelial cells cultured on floating collagen gels. Mammary epithelial cells were plated on collagen gels and cultured for 2 days in DME supplemented with 10% FBS. The cells were then cultured for 2 days in the basal serum-free medium supplemented with insulin alone. On day 4, the collagen gel was floated in the medium, and dexamethasone (1 μ g/ml) and different doses of PRL were supplemented to the basal medium. γ -Casein mRNA content was expressed as the amount relative to 0.1 attomol of γ -casein cDNA. The data represent the mean $\pm$ SEM from triplicate cultures.

observed with the same dose of PRL (Fig. 5B). The large difference in the fold-increases in total RNA and γ -casein mRNA contents suggest that PRL specifically induce γ -casein mRNA among other RNA species.

DISCUSSION

We have recently isolated a cDNA encoding mouse γ -casein (Sasaki *et al.*, 1993). Based on its nucleotide sequence we developed a competitive PCR method for quantification of mouse γ -casein mRNA. The application of the competitive PCR method enabled us to measure a wide range of γ -casein

mRNA content in mammary epithelial cells cultured under various conditions.

It has been reported that mouse mammary epithelial cells differentiate morphologically and biochemically to synthesize and secrete milk proteins when grown *in vitro* on floating collagen gels (Emerman *et al.*, 1977; Emerman and Pitelka, 1977; Enami *et al.*, 1979; Shannon and Pitelka, 1981). As for the mechanisms by which floating collagen gel supports differentiation of mammary epithelial cells, two factors, (1) cell shape change and (2) exposure of the basal cell surface to the culture medium, have been implicated to play important roles. The collagen gel begins to contract as soon as the gel

is released from the culture dish and floated in the medium. This contraction coincides with the change in cell shape from squamous to cuboidal/columnar. Additionally, flotation of the collagen gel renders the basal cell surface exposed to the culture medium. Thus, nutrients and various factors in the medium may become more easily accessible to the basal surface. Our present study demonstrated that cultivation of mammary epithelial cells on floating collagen gels, but not on attached collagen gels, induces an increased expression of γ -casein mRNA in response to dexamethasone and PRL. These results suggest that the status of the collagen gel determines the inducibility of milk-specific genes in mammary epithelial cells. Cell shape change and exposure of the basal surface to the medium as mentioned above may somehow be related to this process. The clarification of the mechanisms of extracellular matrix-dependent expression of milk-specific genes awaits further experimentation at the mRNA level.

Recently, it has been reported that the binding of PRL to its receptor activates the JAK2 tyrosine kinase (Dusanter-Fourt *et al.*, 1994; Rui *et al.*, 1994). JAK2 in turn activates the novel transcription factor, Stat5/MGF, by tyrosine phosphorylation (Wakao *et al.*, 1994). The activated Stat5 eventually binds to the specific DNA sequence and stimulates transcription of milk protein genes (Gouilleux *et al.*, 1994). We are currently studying whether or not the activation of the JAK2-Stat5 signal transduction pathway by PRL is also under the control of the status of the culture substratum.

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