Hormonal Modulation of the Casein Gene Expression in a Mammogenesis-Lactogenesis Culture Model of the Whole Mammary Gland of the Mouse*

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Whole mammary glands from immature virgin female BALB/c mice, primed in vivo with estradiol-17β and progesterone, were incubated in a serum-free medium, containing combinations of different hormones. The initial incubation for 6 days (Step I) in medium with the mammogenic hormones (insulin, prolactin, estradiol, progesterone, and growth hormone) stimulated the development of pregnancy-like lobuloalveolar structures. The lobuloalveolar morphogenesis of the gland in vitro also resulted in a pronounced increase in epithelial cell number. Hybridization assays using a cDNA probe to 15 S casein mRNA (cDNA,csn) showed that casein mRNA (mRNA,csn) is virtually nondetectable in the ductal glands from unprimed and primed animals. Concentration of the mRNA,csn was 0.00067%, with 147 molecules/cell in the lobuloalveolar glands obtained after the 6-day Step I incubation in the corticosteroid-free mammogenic medium. This low level of mRNA,csn in the gland was unaltered after additional 3 days of culture in prolactin- or progesterone-deficient mammogenic hormone medium. After the lobuloalveolar glands were transferred to the Step II culture medium containing the lactogenic hormone combination (insulin, prolactin, and cortisol) an increase in mRNA,csn accumulation was measurable by the cDNA,csn probe. This stimulation of mRNA,csn concentration in total RNA of the gland was progressive. Between 0 to 9 days in the lactogenic medium, mRNA,csn level rose from 0.00067% to 0.99%, a 154-fold increase. During the same period, the number of epithelial cells per g of tissue remained essentially unaltered, while the cellular accumulation of the mRNA,csn increased 255-fold, from 147 to 37,524 molecules/cell. Thus, the results of the present two-step, mammogenesis-lactogenesis culture model show that the presence of cortisol in a medium containing prolactin is essential for the stimulation of casein gene expression in murine mammary gland.

Cortisol and prolactin are required for the induction of casein in murine mammary glands (1-3). Despite several studies, the specific role(s) of the steroid and the polypeptide hormones in regulation of casein and its mRNA (4-6) is still uncertain. Direct assessment of hormonal modulation of the casein mRNA (mRNA,csn) is now feasible because mRNA,csn can be translated in cell-free protein synthesis systems (7, 8), and cDNA probes to purified casein mRNA have been prepared (9, 10). Although glucocorticoid is essential for the maintenance of translational activity of the mRNA,csn in the postpartum mammary gland (11), intensity of suckling, a stimulus known to regulate the pituitary prolactin release (12), may significantly influence the level of the mRNA,csn. Glucocorticoid is also required for the maintenance as well as replenishment of the prolactin receptor on mammary cells of lactating mice (13). Moreover, casein (14) and its mRNA (8) appear in the mammary gland in vivo as early as midpregnancy, indicating that the pregnancy mammary cells are under active stimulation of the lactogenic hormones. Consequently, the responses of mammary cells measured in fragments of pregnancy mammary tissue in conventional short term "organ culture" (15) may not exclusively reflect action(s) of the hormones present in the medium. Thus, the complexities of the systemic endocrine environment of the animal limit the scope of reliable studies on the mechanisms of multiple hormone regulation of expression of the casein gene and mammary cell differentiation in vivo.

The immature mammary parenchyma in an isolated whole gland sequentially mimics pregnancy-like lobuloalveolar growth (mammogenesis) and differentiation (lactogenesis) in medium containing appropriate hormone combinations (16). The glands in medium with a controlled combination of the mammogenic hormones fail to elicit casein and translatable mRNA,csn, whereas abundant casein and its mRNA are produced during subsequent cultivation in presence of the lactogenic hormones. Therefore, the two-step culture of the whole mammary gland provides a suitable model system for measuring the initial molecular responses of the mammary cells evoked by the lactogenic hormones in a chemically defined medium. In this report, we present the results of studies on hormonal mediation of mRNA,csn production in the mammary cells and the quantitation of mRNA,csn molecules during the two-step, 15-day culture of the whole mammary gland.

EXPERIMENTAL PROCEDURES

Organ Culture—The detailed procedures for organ culture of the whole mammary gland have been previously described (16, 17). As a prerequisite for the whole gland culture, 3- to 4-week-old BALB/c female mice were primed for 9 days by daily subcutaneous injections of 1 μg of estradiol-17β and 1 mg of progesterone/animal in 0.9% saline. The entire second thoracic mammary gland was then excised under sterile conditions and incubated in Waymouth's medium (18). In the present experiments, the glands were incubated initially for 6

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days with 5 μg/ml each of the hormones insulin, prolactin, growth hormone, plus estradiol-17β (0.001 μg/ml), and progesterone (1 μg/ml) for 9 days. Incubation was then continued in medium containing the lactogenic hormones, insulin, prolactin, and cortisol, 5 μg/ml each for 9 days. Incubation was done at 37°C in a 5% O₂, 5% CO₂ atmosphere.

**Cell Dissociation**—The number of cells per g of tissue was determined by dissociating the mammary tissue with a mixture of collagenase, hyaluronidase, and DNase, collecting the epithelial cells by centrifugation, and counting the number of cells in a haemocytometer, as previously described (19). This procedure yields mostly epithelial cells and the viability of the cells was 85 to 90% as determined by the trypan blue exclusion test.

**Casein mRNA Purification and cDNA Synthesis**—Total RNA of the lactating mammary glands of postpartum BALB/c mice was isolated by the phenolchloroform method (20), and the 15 S mouse casein mRNA purified essentially as described (10). Briefly, total RNA was dissolved in 0.5 M KCl, 10 mM Tris-HCl, pH 7.6, and heat-denatured at 70°C for 10 min prior to fractionation on an oligo(dT) cellulose column (21). The poly(A) containing RNA obtained after three successive oligo(dT) cellulose chromatography was dissolved in 1 mM NaEDTA, pH 5.0, heat-denatured, and centrifuged at 130,000 × g for 16 h at 2°C on a 10 to 30% linear sucrose gradient in 0.1 M NaCl, 1 mM Na2EDTA, 10 mM NaOAc, pH 5.0. At each step of purification, the total and casein mRNA activity of the RNA samples was determined by translational activity in a cell-free protein-synthesizing system derived from wheat germ ribosomes (22). Casein was determined by specific immunoprecipitation with the rabbit antibody to mouse casein (23) and 93.4% of the translational product directed by the RNA in 15 S region of the sucrose gradient was immunoprecipitable. RNA in this fraction migrates as a doublet on denaturing 2.5% agarose gels (10).

DNA complementary to purified mRNA was synthesized as previously described (10). The cDNA mRNA was sedimented in an 8 to 18% alkaline sucrose gradient and cDNA molecules larger than 1000 nucleotides were pooled, precipitated overnight with ethanol in the presence of 40 μg/ml of Escherichia coli DNA as carrier, dissolved in distilled water, and stored in aliquots at -20°C.

**Quantitation of Casein mRNA Molecules per Cell**—The number of casein mRNA molecules per cell (obtained by enzymatic dissociation of the gland) was estimated as described by McKnight et al. (25), assuming a molecular weight of 550,000 for the 15 S casein mRNA doublet and that 1 A₂₆₀ = 40 μg of RNA.

**RESULTS**

Table I outlines the developmental sequences of mammosgeneis and lactogenesis in the two-step culture model of the whole mammary gland used in the present study. As illustrated earlier (16), the ductal parenchyma of the whole mammary gland after 6-day incubation in the Step I mammogenic medium with insulin, prolactin, growth hormone, estrogen, and progesterone developed into pregnancy-like lobuloalveolar structures. The lobuloalveolar morphogenesis also resulted in a 10-fold increase of epithelial cell number (Table II). The increased epithelial cell number reached at the end of Step I remained essentially unaltered throughout the 9-day period of Step II culture in medium containing the lactogenic hormones insulin, prolactin, and cortisol.

**Kinetics of mRNA. Accumulation Measured by the cDNA mRNA Probe**—RNAs from unprimed and estrogen-progesterone-primed virgin mammary glands in vivo failed to hybridize to the cDNA mRNA to a significant level even at Rot values up to 10,000 mol. s⁻¹ liter⁻¹ (Fig. 1). Although RNA from the lobuloalveolar glands after 6 days of incubation in the Step I mammogenic medium hybridized to the cDNA mRNA, the Rot values were relatively high. mRNA mRNA comprised 0.00067% of the total RNA from these glands at the end of Step I.

**TABLE I**

| Culture stage | No. cells/g tissue | Per cent casein mRNA | No. casein mRNA molecules/cell | Increase in molecules/cell over IPrlF
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<tr>
<td>Step I</td>
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<td></td>
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<tr>
<td>Priming Glands</td>
<td>0.54 ± 0.13</td>
<td></td>
<td>3.42</td>
<td>147</td>
</tr>
<tr>
<td>6-day IPrlF</td>
<td>5.32 ± 0.31</td>
<td>0.0067</td>
<td>3.32</td>
<td>147</td>
</tr>
<tr>
<td>1-day IPrlF</td>
<td>5.55 ± 0.50</td>
<td>0.029</td>
<td>3.28</td>
<td>147</td>
</tr>
<tr>
<td>3-day IPrlF</td>
<td>6.09 ± 0.71</td>
<td>0.068</td>
<td>3.12</td>
<td>147</td>
</tr>
<tr>
<td>6-day IPrlF</td>
<td>5.43 ± 0.62</td>
<td>0.09</td>
<td>3.12</td>
<td>147</td>
</tr>
<tr>
<td>9-day IPrlF</td>
<td>4.53 ± 0.75</td>
<td>0.09</td>
<td>3.12</td>
<td>147</td>
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**FIG. 1.** Hybridization of cDNA mRNA to total RNA from glands at different stages of organ culture. [³H]cDNA mRNA (500 cpm/reaction) was hybridized under conditions of RNA excess for varying time periods at 68°C. Total RNAs were extracted from mammary glands at the following culture points: ▲ — ▲, glands freshly isolated from virgin 3-week-old BALB/c mice (unprimed); □ — □, glands from 3-week-old virgin BALB/c mice after 9 days of priming with a mixture of estrogen and progesterone; Δ — Δ, glands grown for 6 days in the mammogenic medium; △ Δ, glands from 6-day IPrlF; ○ — ○, IPrlF + 1-day IPrlF; □ — □, 6-day IPrlF + 3-day IPrlF; ○ — ○, 6-day IPrlF + 6-day IPrlF; and ■ — ■, 6-day IPrlF + 9-day IPrlF. I, insulin; Prl prolactin; E, estradiol-17β; P, progesterone; GH, growth hormone; F, cortisol.

**TABLE II**

| Casein mRNA Molecules per Cell | No. cells/g tissue | Per cent casein mRNA | No. casein mRNA molecules/cell | Increase in molecules/cell over IPrlF
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<td>147</td>
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a The values reported are a mean of five determinations.

**Twelve-seventy primed glands (weight 0.5 g) were pooled for each determination.**

1 Insulin, Prl prolactin; E, estradiol-17β; P, progesterone; GH, growth hormone; F, cortisol.

2 Twelve to sixteen glands (weight 0.2-0.3 g) from either IPrlF or IPrlF were pooled for each determination.

2 The values reported are a mean of five determinations.

2 Twenty-seven primed glands (weight 0.5 g) were pooled for each determination.

2 Twelve to sixteen glands (weight 0.2-0.3 g) from either IPrlF or IPrlF were pooled for each determination.
RNA from the glands incubated in the Step II medium with the lactogenic hormone combination (insulin, prolactin, and cortisol) hybridized to the cDNA\textsubscript{con} at progressively reduced R\textsubscript{0.1/2} values (Fig. 1, Table II). After 9 days in Step II culture, the mRNA\textsubscript{con} level in the glands increased markedly to 0.09% of the total RNA. This reflects a 134-fold increase of the mRNA\textsubscript{con} between 0 to 9 days culture of the gland in the lactogenic medium (Table II). Translation assays in the wheat germ ribosome system also showed a progressive increase of casein mRNA activity during the 9-day Step II culture period (data not shown).

**Quantification of Casein mRNA Molecules per Cell—Quantitation of mRNA\textsubscript{con} molecules in the ductal mammary glands of unprimed and primed animals was not feasible, because RNA from these glands failed to hybridize to the cDNA\textsubscript{con} to a significant level (Fig. 1). Estimates of mRNA\textsubscript{con} per cell in the lobuloalveolar glands were based on the number of epithelial cells obtained after enzymatic dissociation of the glands. Data in Table II show that the lobuloalveolar gland, after 6 days in Step I culture contained 147 molecules/cell. Transfer of the glands to Step II culture in the medium containing insulin, prolactin, and cortisol stimulated a sharp rise of the number of mRNA\textsubscript{con} molecules per cell within 24 h. Between 0 and 9 days in the same medium, the number rose from 147 to 37,524 molecules/cell, resulting into a 255-fold increase (Table II).

**Omission of Prolactin or Progesterone during Step I Culture—**In order to assess the role of prolactin present in the mammogenic medium, the ovarian steroid-primed glands were initially incubated for 6 days in the standard mammogenic medium. Subsequently, the cultivation was continued for another 3 days in a medium containing prolactin-free mammogenic hormones. Hybridization of the RNA from these glands to the cDNA\textsubscript{con} probe showed that 3-day culture in the prolactin-deficient medium did not cause any significant change in the R\textsubscript{0.1/2} value, compared to the RNA from the glands after 6-day incubation in the prolactin containing mammogenic medium (Table III). The glands after 3 days in prolactin-deficient mammogenic medium were then incubated for an additional 3 days in the lactogenic medium. RNA from these glands hybridized to the cDNA\textsubscript{con} probe at a R\textsubscript{0.1/2} similar to that of glands initially incubated for 6 days in the standard mammogenic medium, followed by 3 days with the lactogenic hormones in Step II. This suggests that 3-day incubation in the prolactin-free medium does not alter the potential of the glands to respond to the lactogenic hormones in the usual manner.

The mammogenic medium contained progesterone, the ovarian steroid which has been reported to be antagonistic to lactogenesis, and mRNA\textsubscript{con} accumulation in the mammary gland (26–28). Accordingly, the level of mRNA\textsubscript{con} in the glands cultivated in progesterone-free mammogenic medium was also analyzed. After the usual 6 days in Step I mammogenic medium, the glands were incubated for another 3 days in a progesterone-free mammogenic medium. Hybridization of the cDNA\textsubscript{con} to RNA from these glands (Table III) showed little difference of the R\textsubscript{0.1/2} values compared to RNA from glands after 6 days of incubation in the complete mammogenic medium.

**DISCUSSION**

In the two-step culture model of the whole mammary gland, the immature parenchyma mimics pregnancy-like alveolar morphogenesis and differentiation in a chemically defined medium, supplemented with combinations of appropriate hormones. The substantial increase of epithelial cell number, after 6-day lobuloalveolar growth in the mammogenic medium is consistent with our earlier findings of increased RNA, protein, and DNA synthesis in the gland in presence of a similar growth-promoting hormone mixture (29). The increase of epithelial cell number in the gland in culture appears to mimic the pattern of increase of mammary alveolar cell DNA during pregnancy (30). The maximal lobuloalveolar development of the mammary gland is accomplished after 5 to 6 days of incubation in vitro (31, 32). The high epithelial cell number attained during this period was maintained during the 9-day culture period in the lactogenic medium containing prolactin and cortisol. This characteristic of the gland in vitro is also in agreement with the fact that cortisol and prolactin do not increase mammary cell DNA in postpartum animals (33).

Results of molecular hybridization showed that between 0 and 6 days of culture with the mammogenic hormones, the concentration of mRNA\textsubscript{con} in the lobuloalveolar gland was 0.00067% with 147 molecules/cell. The low level of mRNA\textsubscript{con} remained unaltered after additional 3 days of incubation in the prolactin- or progesterone-deficient mammogenic medium, although the possibility exists that residual prolactin or progesterone carried over from preceding incubation in the complete mammogenic medium may influence these results. Nonetheless, the corticosteroid-free mammogenic medium seems to maintain a basal level of 147 molecules/cell even though the gland contains the alveolar secretory structures. The tubular gland cells of chick oviduct in culture under uninuced condition contain a basal level of 40 and 500 molecules of ovalbumin and conalbumin mRNA, respectively (34).

The basal level of the mRNA\textsubscript{con} in the pregnancy-like alveolar glands obtained in the corticosteroid-free culture medium, is in contrast with the presence of an appreciable amount of the mRNA\textsubscript{con} (8) and casein (14) in pregnancy mammary gland in vivo. It should be noted that the levels of circulating cortisol and prolactin are elevated during late pregnancy (35, 36). mRNA\textsubscript{con} and casein can also be obtained in the gland during the alveolar morphogenesis in culture when the present mammogenic hormone combination is supplemented with a corticosteroid (aldosterone), or in medium with insulin, prolactin, and aldosterone (7, 16). This moderate expression of the differentiated function of the mammary cells in the corticosteroid-containing mammogenic medium is apparently due to the limited glucocorticoid activity of aldosterone. The maximal response of the cells, however, requires subsequent incubation of the gland in medium containing prolactin and hydrocortisone. The corticosteroid-free mammogenic hormone combination used in the present study constitutes a controlled hormonal environment which sup-

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**TABLE III**

<table>
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<tr>
<th>Culture conditions</th>
<th>mRNA\textsubscript{con} Levels in glands cultivated in different hormonal combinations</th>
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<tbody>
<tr>
<td>A. Complete mammogenic medium</td>
<td>( R_{0.1/2} )</td>
</tr>
<tr>
<td>6-day IPrlEPGH*</td>
<td>525</td>
</tr>
<tr>
<td>6-day IPrlEPGH + 3-day IEPGH</td>
<td>469</td>
</tr>
<tr>
<td>6-day IPrlEPGH + 3-day IEPGH + 3-day IPrlF*</td>
<td>105</td>
</tr>
<tr>
<td>C. Progesterone-free</td>
<td>6-day IPrlEPGH + 3-day IPrlE</td>
</tr>
</tbody>
</table>

\* I, insulin; Prl, prolactin; E, estradiol-17β; F, progesterone; GH, growth hormone; F, cortisol.

The \( R_{0.1/2} \) value of glands grown for 6 days in standard mammogenic medium followed by 3 days in lactogenic medium, i.e. 6-day IPrlEPGH + 3-day IPrlF is 12, giving a per cent mRNA\textsubscript{con} of 0.029. **
ports pregnancy-like lobuloalveolar morphogenesis without expression of the differentiated function in the gland in culture.

During the Step II culture, the mammary gland in medium containing prolactin and cortisol showed measurable amounts of the mRNA\textsubscript{mRNA} within 24 h. The concentration of mRNA\textsubscript{mRNA} in total RNA progressively increased from 0.00067% to 0.09%, between 0 to 9 days of incubation in presence of the lactogenic hormones. Moreover, cellular accumulation of the mRNA\textsubscript{mRNA} during the same period rose from 147 molecules to 37,524 molecules/cell, reflecting a 255-fold increase.

The number of epithelial cells during the same culture period, however, remained essentially similar. Therefore, it is conceivable that this increase of cellular accumulation of the mRNA\textsubscript{mRNA} rising from a basal level may involve a de novo initiation, followed by a sustained transcription of the casein gene in the Step II culture medium containing cortisol and prolactin. As noted earlier, absence of prolactin in the corticosteroid-free mammogenic medium does not alter the basal level of the mRNA\textsubscript{mRNA}, in the mammary cells during culture Step I. In the medium with prolactin, however, cortisol can raise the cellular concentration of the mRNA\textsubscript{mRNA} 255-fold over the basal level. Thus, it is reasonable to conclude that presence of the glucocorticoid is obligatory to increased accumulation of the mRNA\textsubscript{mRNA} in the murine mammary gland in vitro. Studies (10) in vitro have shown that adrenalectomy of the lactating mouse causes a 80% reduction of mRNA\textsubscript{mRNA} level in the mammary gland and a single injection of hydrocortisone can replenish this loss over 4-fold, within 48 h. This glucocorticoid activation of the mRNA\textsubscript{mRNA} accumulation is concomitant with a 2-fold stimulation of specific transcription, measured by hybridization of the cDNA\textsubscript{cDNA} to mercury-labeled RNA (H\textsubscript{r}RNA) synthesized in vitro in isolated mammary cell nuclei. This indicates that the glucocorticoid is involved in the modulation of transcription of the casein gene in the lactating mammary gland in vivo. Since these studies were done in nonhypophysectomized animals, the action of glucocorticoid may be synergistic with the endogenous prolactin. The two-step culture model described in this report should permit assessment of this possibility.

Extracts of murine mammary tissue fragments obtained from pregnant animals respond to prolactin and cortisol (37) in culture and produce casein during short term incubation in insulin-containing medium (4). In recent studies (38, 39), mammary gland fragments from pregnant rats were initially incubated for 48 h with insulin and cortisol. The incubation was then continued for another 24 to 48 h in fresh medium containing insulin and prolactin. After the second incubation, a marked increase of mRNA\textsubscript{mRNA} accumulation in the explants was measurable by a specific cDNA probe. Based on this observation it has been concluded that prolactin is the inducer of the casein gene and presence of cortisol is not necessary for evoking this response of the mammary cells (38). However, the pregnancy mammary tissue used in these studies was already exposed to the glucocorticoid in vivo, and was again preincubated for 48 h with cortisol. Thus, response of the explants to prolactin during the short term culture in the corticosteroid-free medium is likely to be synergistic with the residual glucocorticoid retained in the tissue from preceding 48-h preincubation in medium containing insulin and cortisol. The failure of prolactin to promote cellular accumulation of the mRNA\textsubscript{mRNA} in the corticosteroid-free mammogenic medium observed in the present study is consistent with the above possibility.

The inherent shortcomings, such as exposure of the pregnancy mammary cells to the corticosteroid in vivo, presence of mRNA\textsubscript{mRNA} in the pregnancy mammary explants, and the short term duration of the culture, limit reliable interpretation of the results of studies with pregnancy mammary tissue organ culture.” On the other hand, the results presented in this report clearly demonstrate that, in the two-step culture of the whole gland, the mammary parenchyma develops into a pregnancy-like lobuloalveolar gland in a corticosteroid-free controlled hormone environment in vitro. Virtually no mRNA\textsubscript{mRNA} accumulates in the gland at this stage even though prolactin is present in the mammogenic medium. These glands during subsequent culture in the medium containing prolactin and cortisol accumulate abundant mRNA\textsubscript{mRNA}. Therefore, in future studies on the expression of the casein gene in the whole mammary gland organ culture model, the responses observed can be related to the hormonal environment in vitro. Such results will be interpretable with reliability.

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