Absolute requirement of glucocorticoid for expression of the casein gene in the presence of prolactin

(BALB/c mouse/whole mammary gland *in vitro*/casein mRNA/cDNA hybridization/cortisol retention)

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ABSTRACT   Second thoracic mammary glands of immature BALB/c female mice were stimulated to pregnancy-like lobuloalveolar (LA) development after 6 days of incubation in a corticosteroid-free step I culture medium containing insulin, prolactin, estradiol, progesterone, and growth hormone. A low basal level (0.0009%) of casein mRNA (mRNA$_{csn}$) sequences was detectable in the LA glands by a specific cDNA probe. Subsequent incubation of the LA glands for 3 days in medium containing insulin and prolactin or insulin and cortisol failed to elicit mRNA$_{csn}$ above the basal level, indicating that neither prolactin nor cortisol alone can support casein gene expression. However, an increase in mRNA$_{csn}$ levels was observed when the 3-day incubation with insulin and cortisol or insulin and prolactin was followed by 3 days of culture in presence of insulin, prolactin, and cortisol. When a 3-day incubation with insulin and prolactin was followed by 3 days in insulin and cortisol medium, mRNA$_{csn}$ levels in the gland remained similar to the basal level. However, a 20-fold increase in the mRNA$_{csn}$ levels ensued when the LA glands were sequentially incubated for 3 days in insulin and cortisol and then for another 3 days in insulin and prolactin medium. After a preincubation in insulin and cortisol medium, the LA glands retained residual cortisol during subsequent incubation in insulin and prolactin medium, and the mRNA$_{csn}$ levels in these glands were related to the level of residual cortisol present. When mRNA$_{csn}$ and the residual cortisol level reached a minimum, addition of fresh cortisol to the medium caused a 20-fold increase in the mRNA$_{csn}$ levels. This indicates that cortisol is a limiting factor in insulin and prolactin medium and its presence is absolutely required for casein gene expression.

The requirement that prolactin and cortisol be present for lactogenesis was delineated over 2 decades ago (1, 2). Since then, numerous studies have shown that, during lactation, a marked increase of mammary cell RNA and protein, including casein, is dependent upon stimulation by prolactin and cortisol (3, 4). We have shown that cortisol is required for the maintenance of casein-synthesizing polysomes, poly(A)$^+$RNA synthesis, and casein mRNA (mRNA$_{csn}$) accumulation in the lactating mammary gland of the mouse (5–7). Moreover, a regulatory action of the glucocorticoid on transcription of the casein gene in the mammary gland *in vivo* has been demonstrated (7). However, complexities in the animal limit elucidation of the discrete role of polypeptide and steroid hormones which regulate specific expression of the casein gene in breast cells.

Fragments of mammary tissue from pregnant mice were demonstrated to be capable of synthesizing casein in a culture medium containing the lactogenic hormones plus insulin (8). This provided an *in vitro* model for studying molecular responses of the mammary cells to prolactin and glucocorticoid

action in a chemically defined medium containing insulin, which is needed for viability of the mammary parenchyma *in vitro* (9). Subsequently, it was observed that prolactin stimulates casein synthesis as well as transcriptional activity in the explants that were preincubated with cortisol (10, 11). It was then concluded that prolactin acts as an inducer for casein gene expression. With a similar experimental protocol, recently it has been observed that the endogenous mRNA$_{csn}$, normally present in pregnant rat mammary tissue, is decreased after an initial incubation of the explants with cortisol (12, 13). Subsequent incubation with prolactin in a cortisol-free medium results into a pronounced increase of mRNA$_{csn}$ transcription in the explants (13). Based on these findings it has been concluded that prolactin is "the" hormone required for expression of the casein gene, and cortisol is not necessary for evoking this response in the mammary cells (12, 13). This conclusion assumes that the explants, preincubated with cortisol, remain completely free of the steroid hormone during the subsequent short-term culture with prolactin alone. However, mammary tissue is known to retain steroid hormones, including cortisol, for a prolonged period of time (14, 15). The concentration of the residual glucocorticoid has been reported to be sufficient to support casein synthesis, measured by [^{32}P]orthophosphate uptake, in the explants in presence of prolactin alone (15). This raises the important question of whether responses of the mammary cells to prolactin measured (during last 15 years) in explants preexposed to cortisol reflect an action of the polypeptide hormone alone or a synergistic action of prolactin and the residual cortisol retained by the tissue.

Earlier we have shown that, in a two-step culture model of the whole mammary organ, the immature parenchyma develops pregnancy-like lobuloalveolar (LA) structures in a step I corticosteroid-free medium containing insulin, prolactin, growth hormone, and the ovarian steroids (16). The LA glands containing the secretory structures elicit only a basal level of mRNA$_{csn}$. In the step II culture medium containing insulin, prolactin, and cortisol, abundant mRNA$_{csn}$ accumulates in the LA glands within 24 hr, and the concentration of the specific sequences increases 255-fold during the 9-day incubation (16). This increase of mRNA$_{csn}$ is accompanied by accumulation of casein in the gland (17). The possibility of a carryover of residual cortisol from step I to step II medium is virtually absent in this unique *in vitro* model, because the step I mammogenic medium does not contain any adrenal glucocorticoid. In the present study, this two-step culture model of the whole mammary organ was used to assess the action of the two principal lactogenic

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Abbreviations: LA glands, lobuloalveolar mammary glands obtained after 6-day incubation in mammogenic medium; mRNA$_{csn}$, casein mRNA; R$_0$t, mol of ribonucleotide per liter × time (sec); R$_0$t$_{1/2}$, R$_0$t necessary for 50% hybridization.

hormones, prolactin and cortisol, on expression of the casein gene in a controlled hormonal environment. The results clearly show that expression of the casein gene is not inducible by either prolactin or the adrenal glucocorticoid alone.

MATERIALS AND METHODS

Organ Culture. Immature 3- to 4-week-old BALB/c female mice obtained through the National Cancer Institute (Bethesda, MD) were used. The procedures for whole mammary gland organ culture have been described (16, 18, 19). In the present study the entire thoracic glands of the estrogen/progesterone primed mice were first incubated for 6 days in a step I mammogenic medium containing insulin, prolactin, estradiol, progesterone, and growth hormone for pregnancy-like LA growth. The LA glands were then incubated in medium with different combinations of insulin, prolactin, and cortisol for various time intervals. Incubations were done at 37°C in a 95% O_2/5% CO_2 atmosphere. Medium was changed on alternate days during the initial step I culture and then every 24 hr subsequently.

Assessment of [^{3}H]Cortisol Retention by the Mammary Gland. After step I culture in cortisol-free medium, the LA glands were first incubated for 48 hr in insulin and cortisol (each at 5 μg/ml). During the last 24 hr the medium contained 5 μCi of [^{3}H]cortisol (New England Nuclear, 93 Ci/mmol; 1 Ci = 3.7 $\times$ 10^{10} becquerels) per ml. Subsequently, the glands were transferred to a medium containing insulin plus prolactin. At different time intervals glands were collected, rinsed with saline, and blot dried. The level of [^{3}H]cortisol in the homogenate of these glands was determined by thin-layer chromatography as described (20), except that the LH-20 chromatographic step was omitted.

Quantitation of mRNA$_{csn}$ Level. The level of casein mRNA in the glands was measured by a specific [^{3}H]cDNA probe to purified 15S mouse casein mRNA (7). Total RNA from the glands was extracted by the phenol/chloroform method and RNA excess hybridization followed by S1 nuclease digestion was done as described (7, 16).

RESULTS

Responses of the LA Glands in Medium with Prolactin or Cortisol Alone. Consistent with our earlier findings (16), the LA glands contained an extremely low level of mRNA$_{csn}$ at the end of a 6-day incubation in a cortisol-free mammogenic medium (Table 1). We have also shown that this low basal level of mRNA$_{csn}$ in the LA glands is not influenced by the absence of

Table 1. Level of mRNA$_{csn}$ in LA glands incubated with different hormone combinations

Culture*	$R_0t_{1/2}$[†]	% mRNA$_{csn}$
IPrlEPGH, 6 days	417	0.0009
IF, 3 days	832	0.0005
IF, 3 days → IPrlF, 3 days	5.02	0.076
IPrl, 3 days	ND[‡]	ND
IPrl, 3 days → IPrlF, 3 days	10	0.038

* I, insulin (5 μg/ml); Prl, prolactin (5 μg/ml); E, estradiol-17β (1 ng/ml); P, progesterone (1 μg/ml); GH, growth hormone (5 μg/ml); F, cortisol (5 μg/ml). The details of the hormones used have been described elsewhere (16, 19). Bovine growth hormone was a gift from the National Pituitary Agency (National Institute of Arthritis, Metabolism and Digestive Diseases). All glands were incubated for first 6 days in IPrlEPGH medium. The concentration of each hormone used during subsequent incubation was the same as described above.
[†] $R_0t_{1/2}$ of purified mRNA$_{csn}$ is 0.0038 mol sec/liter.
[‡] ND, not detectable.

prolactin or progesterone from the medium (16). This indicates that the casein gene remains in an uninduced condition in the LA glands. Thus, this whole mammary gland culture model is suitable to assess the role of prolactin and cortisol on the expression of casein gene in a controlled hormonal environment.

It is evident from the results shown in Table 1 that the LA glands fail to accumulate mRNA$_{csn}$ sequences above the basal level even after 3 days of incubation in insulin/cortisol or insulin/prolactin medium. This indicates that neither prolactin nor cortisol alone is capable of stimulating mRNA$_{csn}$ accumulation in the glands. However, when preincubation of the LA glands in the medium with cortisol or prolactin was followed by incubation with both prolactin and cortisol, a marked increase in mRNA$_{csn}$ levels was evident in the glands. These results reveal that the mammary epithelium remains highly responsive to the lactogenic hormone combination even after a 3-day preincubation in absence of cortisol or prolactin, and the glands require the stimulation of both hormones for expression of the casein gene.

Effect of Preincubation of the LA Glands with Cortisol or Prolactin. We then examined whether the mammary glands are capable of accumulating mRNA$_{csn}$ if the preincubation in insulin/prolactin medium is followed by an incubation with insulin/cortisol or, conversely, if preincubation with insulin/cortisol is followed by incubation in insulin/prolactin medium. When the LA glands were incubated with insulin/cortisol after a preincubation in insulin/prolactin medium, the mRNA$_{csn}$ sequences in the glands became measurable by the cDNA probe (Table 2). However, the level remained close to the basal condition (0.0009%) seen in the LA glands at the end of step I culture. It is possible that some residual prolactin retained by the LA glands from the preincubation medium may have acted synergistically with cortisol, added in the medium subsequently, and this may account for the slight stimulation. Mammary tissue may retain residual prolactin at least for the initial 4 hr at a level sufficient to stimulate RNA synthesis in the explants *in vitro* (21). The mRNA$_{csn}$ levels may have remained low (0.0012%) at the end of the 3-day culture period due to degradation of the residual peptide hormone. Because the turnover rate of the mRNA$_{csn}$ in the mammary tissue *in vitro* is fairly low (13), it is likely that the mRNA$_{csn}$ sequences accumulated during the initial hours of interaction between cortisol and residual prolactin may not be lost completely at the end of the 3-day culture period.

On the other hand, an 18-fold increase in mRNA$_{csn}$ over the basal level occurred when the LA glands were incubated with insulin/prolactin for 3 days after preincubation in the insulin/cortisol medium. This pronounced increase cannot be ascribed solely to prolactin action because the results presented

Table 2. Effect of preincubation of LA glands with cortisol or prolactin on accumulation of mRNA$_{csn}$ sequences after subsequent incubation in medium containing prolactin or cortisol

Culture*	$R_0t_{1/2}$	% mRNA$_{csn}$
IPrl, 3 days	ND[†]	ND
IPrl, 3 days → IF, 3 days	316.0	0.0012
IF, 3 days	832.0	0.0005
IF, 3 days → IPrl, 3 days	23.4	0.016

* All the glands were first incubated for 6 days in the mammogenic medium for LA development. Subsequently, these glands were incubated with different hormone combinations as indicated. The concentration of each hormone was the same as described in Table 1.
[†] ND, not detectable.

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in the preceding paragraphs show that prolactin alone cannot stimulate accumulation of the mRNA$_{csn}$ sequences in the LA glands. Alternatively, it is possible that prolactin acts synergistically with the residual cortisol retained by the LA glands from the insulin/cortisol preincubation medium. It has been shown that the mammary gland can take up glucocorticoid (22, 23) and retain it for a prolonged period (15). Thus, the present results, showing a marked increase of mRNA$_{csn}$ levels in glands incubated with prolactin alone after a preincubation with cortisol, may reflect an action of prolactin in combination with the residual cortisol. Accordingly, studies were done to assess the ability of the LA glands to retain residual cortisol from the preincubation medium under the present experimental conditions.

Cortisol Retention and mRNA$_{csn}$ Accumulation in the LA Glands. At 24 hr after removal of cortisol from the medium, a substantial amount of the steroid hormone was retained by the LA glands (Fig. 1). Subsequently, the residual cortisol declined, and 6 days after cortisol withdrawal the steroid level reached 2 ng/g of tissue. During the first 48 hr in insulin/prolactin medium, the mRNA$_{csn}$ levels increased 25-fold (0.0006% to 0.016%) and thereafter it declined to 0.0027% by the day 6. However, addition of fresh cortisol to the medium at this point, causes a 20-fold increase in mRNA$_{csn}$ levels in the glands in the presence of prolactin.

Although the level of cortisol decreased >90% within the first 24 hr after its removal (Fig. 1), during the 6-day culture in insulin/prolactin medium the glands retained a measurable level
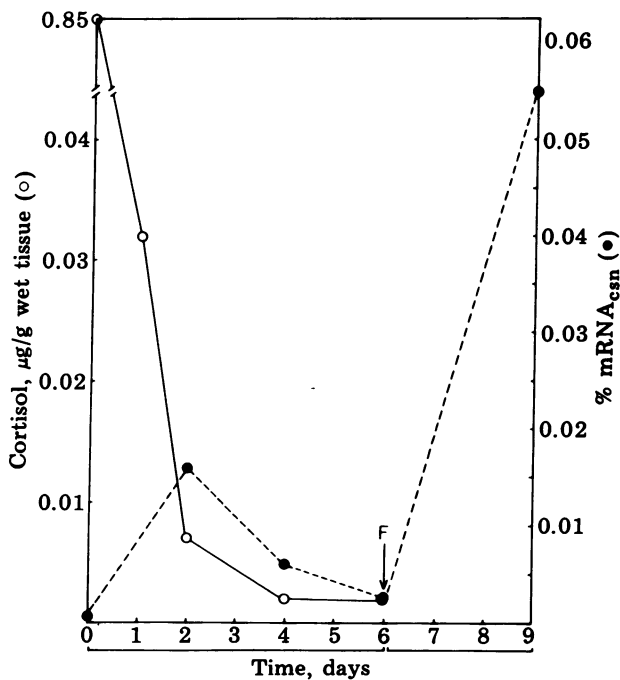
Table 3. Level of cortisol retained by LA glands incubated with insulin/prolactin after 48-hr preincubation with insulin/cortisol

| | Cortisol | |
Days with IPrl*	nmol/g tissue	Molecules/g tissue × 10⁻¹⁴
0	2.0	12.0
1	0.09	0.54
2	0.02	0.12
4	0.006	0.036
6	0.006	0.036

* Glands were incubated for six days with IPrlEPGH and then for 2 days with IF (see Table 1 for abbreviations and concentrations). During the last 24 hr of incubation with IF, [³H]cortisol was added to the medium, and the amount of cortisol retained by the glands was determined as described in Fig. 1.

of cortisol (Table 3). Recently, it has been shown that addition of cortisol as low as 3 μM (3 nmol/ml of medium) to insulin/prolactin medium can cause a 2-fold increase in casein synthesis in pregnancy mammary explants (24). Accordingly, the amount of cortisol retained by the glands, at least during the first 48 hr after its removal, appears to be enough to exert its action along with prolactin in the medium. Thus, the increase in mRNA$_{csn}$ levels 48 hr after cortisol withdrawal may be a result of the interaction between prolactin and the residual cortisol during the initial period of culture. Under a similar incubation protocol, casein synthesis also has been shown to increase during the initial incubation period in insulin/prolactin medium after a 48-hr preincubation of the pregnancy mammary explants in medium with insulin and cortisol (15). Because the minimum level of cortisol required for its action with prolactin and the half-life of the mRNA$_{csn}$ under the conditions described in Fig. 1 are not known, at present it is not possible to determine whether the decrease in mRNA$_{csn}$ levels after 48 hr in insulin/prolactin medium is a result of transcriptional block or due to degradation of the accumulated mRNA$_{csn}$ sequences. Nevertheless, the results clearly demonstrate that cortisol is a limiting factor for expression of the casein gene in insulin/prolactin medium and its subsequent replenishment can cause an enormous increase in the mRNA$_{csn}$ levels, indicating an absolute requirement for the glucocorticoid for the functional differentiation of the mammary gland.

DISCUSSION

Studies in animals have revealed that, although cortisol therapy can promote expression of the casein gene (5–7), suckling, a stimulus known to maintain an elevated level of circulating prolactin (25), also influences mRNA$_{csn}$ concentration in the mammary gland *in vivo* (5). This shows that complexities of the endocrine environment in the animal make the *in vivo* model inadequate for further elucidation of the role of adrenal glucocorticoid and pituitary polypeptide hormones regulating expression of a specific gene in the mammary cells. The short-term *in vitro* model, derived from fragments of mammary gland of pregnant animals, also has serious limitations. The pregnancy mammary parenchyma, rich in glucocorticoid receptor (26), is exposed to an increased level of circulating glucocorticoid in the pregnant animal (27). The glands also contain abundant casein and its mRNA (28, 29). Consequently, preincubation in a medium with insulin and cortisol is required to reduce the endogenous mRNA$_{csn}$ in the explants (12, 13). This exposure to the steroid hormone is likely to enrich explants in cortisol because the mammary tissue is known to retain cortisol for a prolonged period (15). Therefore, a marked stimulation

FIG. 1. Relationship between the amount of cortisol retained and mRNA$_{csn}$ accumulated by the LA glands in insulin/prolactin medium after a 48-hr preincubation with insulin/cortisol. During the last 24 hr of the 48 hr preincubation period [³H]cortisol (5 μCi/ml) was added. At the end of the preincubation (time 0) the glands were transferred to the medium containing insulin and prolactin. The medium was changed every 24 hr and, at different time intervals, the glands were collected, rinsed with cold saline, and blot dried. Tissue (0.1 g) was homogenized in 1 ml of deionized water and extracted with ethyl acetate at room temperature. The organic phase was dried under N$_2$, dissolved in 0.1 ml of chloroform/methanol, 1:1 (vol/vol) containing 5 mg of unlabeled cortisol per ml, and chromatographed on thin-layer chromatographic plates according to Carson *et al.* (20). Arrow indicates the time when fresh cortisol (5 μg/ml) was added to the medium.

of casein gene expression during subsequent short-term incubation of the explants with prolactin may not reflect a stimulatory action of the polypeptide hormone alone (12, 13). Instead, it is more likely that the results reflect a synergistic action of prolactin and the residual cortisol. Results of the present and other recent studies (15) seem to confirm this possibility. Thus, it is difficult to delineate the discrete role of the polypeptide and steroid hormones for the casein gene expression by using an *in vitro* model derived from the fragments of pregnancy mammary tissue.

The two-step culture model of the whole mammary organ seems to offer uniquely suitable conditions because pregnancy-like LA morphogenesis of the parenchyma is accomplished in a corticosteroid-free medium *in vitro*. The mammary cells in the LA glands remain in an uninduced state, eliciting only a basal level of $mRNA_{csn}$. This then eliminates preincubation of the glands with cortisol, a step required to reduce the endogenous $mRNA_{csn}$ in explants from pregnancy mammary tissue. Moreover, the lack of requirement for preincubation with cortisol also avoids the complex problem of residual cortisol carryover into the step II medium. The present results demonstrate that LA glands not preincubated with cortisol fail to elicit $mRNA_{csn}$ in medium with cortisol or prolactin, indicating that neither the steroid nor the polypeptide hormone alone is capable of stimulating the casein gene in the LA whole mammary organ. However, the glands remain competent and accumulate abundant $mRNA_{csn}$ sequences when exposed to both cortisol and prolactin during subsequent incubation. Thus, future studies using this two-step culture model of the whole mammary organ should provide elucidation of the complex interactions between the steroid and the polypeptide hormones regulating mammary cell differentiation. Until then, assigning specific regulatory function to either of the two lactogenic hormones at the transcriptional or posttranscriptional level of control of the casein gene will remain conjecture.

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