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Glucocorticoid modulation of casein gene transcription in mouse mammary gland*

(hydrocortisone/isolated nuclei/synthesis of Hg-containing RNA/cDNA hybridization)

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ABSTRACT **The influence of cortisol and prolactin on casein gene expression in the mammary gland of lactating BALB/c mice was measured by using a specific cDNA probe to 15S casein mRNA ($cDNA_{csn}$). Casein mRNA ($mRNA_{csn}$) level in the mammary gland was decreased by 85% 5 days after adrenal ablation, but then was increased 4.4-fold 12 hr after a single injection of hydrocortisone-21-acetate. An 80% decrease in serum prolactin level, induced by the prolactin inhibitor 2-bromo-α-ergocryptin (CB-154), did not alter the level of $mRNA_{csn}$ in the gland. Specific transcription of the casein gene in nuclei isolated from lactating mammary glands was measured by $cDNA_{csn}$ hybridization to the *in vitro* synthesized Hg-CTP-containing RNA (Hg-RNA), which was purified by SH-agarose chromatography. The level of the $mRNA_{csn}$ in Hg-RNA synthesized in the isolated nuclei was 0.09% and this was decreased 85% by α-amanitin, indicating that the $mRNA_{csn}$ sequences in the Hg-RNA were the products of RNA polymerase II-directed DNA-dependent RNA synthesis. Transcription of the $mRNA_{csn}$ in isolated nuclei was decreased by 70% 5 days after adrenalectomy and a single injection of the glucocorticoid then increased the transcription level 2-fold at 6 hr. Essentially no alteration of the level of transcription was detectable in mammary nuclei isolated from lactating mice with 80% decreased serum prolactin level, induced by CB-154 treatment. The results thus demonstrate a glucocorticoid involvement on the modulation of casein gene expression at the transcriptional level of control.**

Since the establishment that cortisol and prolactin are the two principal hormones required for lactogenesis (2, 3) (differentiation) as well as production of casein (4) in murine mammary gland, numerous studies have attempted to ascertain the role(s) of the adrenal steroid and the pituitary polypeptide hormones in regulation of the milk protein, casein (5–8), and its mRNA ($mRNA_{csn}$) (9, 10). But understanding of the discrete role of the steroid and the polypeptide hormone during multiple hormone regulation of mammary cell differentiation as yet has remained obscure, principally because the available technology used in previous studies provided only indirect information. Our recent findings (11) that mammary cell nuclei *in vitro* support abundant synthesis of mercury-labeled 15S RNA (Hg-RNA), which can be isolated by SH-agarose chromatography (12), have improved the prospects for measuring hormonal modulation of expression of the casein gene, because the $mRNA_{csn}$ in the Hg-RNA transcripts can be measured by hybridization to specific cDNA. The usefulness of SH-agarose chromatography of Hg-RNA synthesized *in vitro* for monitoring of specific gene transcription has been demonstrated (13, 14). This report presents the results on the influence of the lactogenic hormones on transcription of the casein gene in postpartum mammary gland of the mouse.

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MATERIALS AND METHODS

Animals and Treatments. Two groups of 5-day postpartum BALB/c mice, each mouse nursing 5–7 pups, were bilaterally adrenalectomized under pentabarbitol anesthesia. Five days later mice in one group were killed. The second group of 5-day adrenalectomized mice were given a single subcutaneous injection of 250 μg of hydrocortisone-21-acetate in 0.1 ml of 0.9% saline. Batches of these animals were killed at different times after the injection. In order to obtain lactating animals with reduced serum prolactin level, unoperated mice in another group were given daily injections of 100 μg of the prolactin inhibitor (15), 2-bromo-α-ergocryptene (CB-154), for 3 days starting on the 8th day of lactation. These animals were killed on the 10th day of lactation, 6 hr after the last injection of the ergot alkaloid. Ten-day lactating mice were used as nontreated controls. All the animals were allowed to nurse their pups throughout the experimental period. Immediately after the animals were killed mammary glands were removed, frozen in liquid nitrogen, and stored at −80°C.

***In Vitro* RNA Synthesis in Isolated Nuclei and SH-Agarose Chromatography.** Isolation of the nuclei and *in vitro* RNA synthesis in the presence of a mercury-labeled nucleotide (Hg-CTP) were as described (11). Essentially, the same number of nuclei (200 μg of DNA per ml of reaction mixture) was used in each 1-ml assay mixture. At the end of a 60-min incubation at 25°C, RNA was extracted by the phenol/chloroform method (11) in the presence of wheat germ high molecular weight RNA as carrier (150-fold excess over endogenous nuclear RNA). After 10 min of heat denaturation at 75°C, the Hg-RNA was isolated by SH-agarose column chromatography as described (11, 12) and precipitated with ethanol in the presence of *Escherichia coli* tRNA. For quantitation, Hg-RNA synthesized *in vitro* was labeled with [α-^{32}P]UTP (New England Nuclear) of low specific activity (50 cpm/pmol).

Casein mRNA Purification, Synthesis of $cDNA_{csn}$, and Molecular Hybridization. Phenol/chloroform-extracted (16) mammary gland RNA from 8- to 11-day lactating mice was heat denatured and subjected to two successive oligo(dT)-cellulose chromatographic purifications according to standard

Abbreviations: Hg-CTP, 5-mercuricytidine triphosphate; Hg-RNA, RNA containing Hg-CTP; $mRNA_{csn}$, 15S casein mRNA; $cDNA_{csn}$, complementary DNA to $mRNA_{csn}$; CB-154, 2-bromo-α-ergocryptene; R_0t, moles of ribonucleotide per liter × time (sec); $R_0t_{1/2}$, R_0t necessary for 50% hybridization.

* Preliminary report was presented at the 18th Annual Meeting of the American Society for Cell Biology (1).

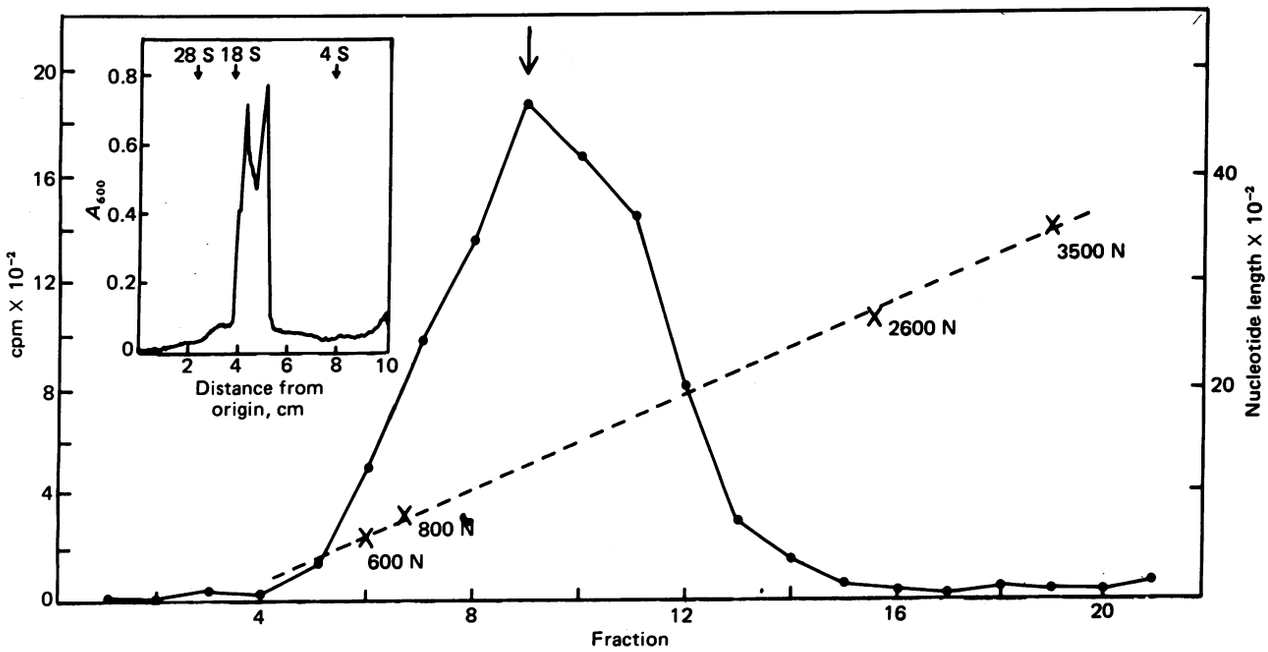

FIG. 1. Alkaline sucrose gradient centrifugation of [^{3}H]cDNA$_{csn}$. cDNA, made against mRNA$_{csn}$, was centrifuged in an 8–18% alkaline sucrose gradient in 0.1 M NaOH/0.9 M NaCl/5 mM EDTA at 5°C for 24 hr at 38,000 rpm in a Spinco SW 41 rotor, and an aliquot of each gradient fraction was assayed for radioactivity. X, Position of the ^{3}H-labeled viral marker DNAs centrifuged on a parallel gradient. The arrow shows the peak fraction of the synthesized cDNA, which has a nucleotide (N) length of about 1250. (*Inset*) Agarose gel electrophoresis of purified casein mRNA. After purification of RNA, 10 μg of RNA from the 15S region of the sucrose gradient was electrophoresed on a 2.5% agarose gel in 0.025 M citric acid, pH 3.5/6 M urea for 4 hr at 3.5 mA per gel tube. Stained (1% methylene blue) gels were scanned at 600 nm. The marker RNAs (arrows) were electrophoresed in parallel gels.

procedures (17). The poly(A) RNA, eluted from the second column with H_2O, was heat denatured (70°C, 1 min), cooled rapidly, and centrifuged in 10–30% linear sucrose gradients at 130,000 × *g* for 16 hr at 2°C as described (18). Most of the RNA sedimented slower than 18 S. At each step of purification, the activity of total and casein mRNA was measured in a wheat germ ribosome translational system (19, 20). Casein in the reaction product was determined by specific immunoprecipitation (20) with antibody to mouse casein (21). In agreement with an earlier report (20), 95% of the translational activity of the RNA in the 15S sucrose gradient fraction was that of casein mRNA. The 15S mRNA (mRNA$_{csn}$) resolved as a doublet after agarose gel electrophoresis (Fig. 1 *Inset*).

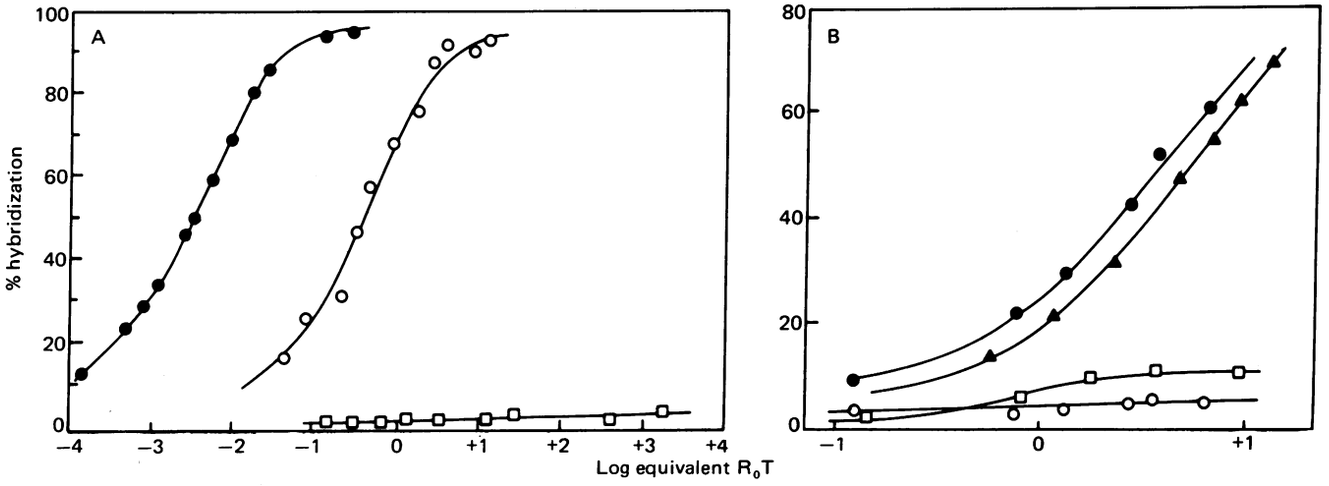

FIG. 2. RNA excess hybridization of the cDNA$_{csn}$ probe. Test RNAs were hybridized to [^{3}H]cDNA$_{csn}$ (500 cpm per reaction) probe at 68°C for varying time periods as described (23). After digestion with S1 nuclease (Miles) at 1000 units per ml for 2 hr at 37°C in 0.1 M sodium acetate, pH 4.5/0.2 M NaCl/1.2 mM $ZnCl_2$, cDNA·RNA hybrids were precipitated with trichloroacetic acid on Millipore filters and assayed in a Beckman LS-350 liquid scintillation counter. The 100% hybridization values were determined for each time point and the S1 nuclease-resistant background was subtracted from each value. Products of 33 *in vitro* assays were pooled for each determination. (*A*) cDNA$_{csn}$ hybridized to its own template (●), to total mammary RNA (○), and to total liver RNA of lactating mice (□). (*B*) Hybridization of cDNA$_{csn}$ to Hg-RNA synthesized in isolated lactating mammary cell nuclei (●), to Hg-RNA transcribed in isolated liver cell nuclei of lactating mice (□), and to total lactating mammary cell nuclear RNA *in vivo* (▲). ○, Control assay for checking nonspecific binding of non-Hg-RNA to the SH-agarose column. Equivalent amounts (DNA value) of nuclei of lactating mammary cells were incubated in the RNA synthesis reaction mixture without ribonucleoside triphosphates; the reaction mixture was extracted by phenol/chloroform and chromatographed on SH-agarose column as described (11). The bound fraction was ethanol precipitated, dissolved in same volume of water as the Hg-RNA from lactating mammary cell nuclei, and hybridized. R_0t, initial concentration of RNA (moles per liter) × time (sec).

Table 1. Influence of hydrocortisone on accumulation of the $mRNA_{csn}$ sequences in postpartum mammary gland

Animals	% $mRNA_{csn}$	Fold increase over 5-day adrenalectomized
10-day lactating	1.56	—
5 days after adrenalectomy	0.25	—
Hr after hydrocortisone injection		
1	0.25	—
3	0.25	—
6	0.74	3.0
12	1.10	4.4
24	0.50	2.0

For each determination, mammary glands from 4–6 animals were pooled, total RNA was extracted (16), and $mRNA_{csn}$ sequences were measured by RNA excess–$cDNA_{csn}$ hybridization as in Fig. 2.

The $mRNA_{csn}$ was used as the template for $cDNA_{csn}$ synthesis by using avian myeloblastosis virus reverse transcriptase according to the standard procedure (22). After the initial Sephadex G-50 chromatography and alkali treatment, the [^{3}H]-$cDNA_{csn}$ was characterized on an alkaline sucrose gradient (22). A peak of radioactivity around 1250 nucleotides was obtained. Based on its sedimentation value, $mRNA_{csn}$ is estimated to have a complexity of about 4.5×10^5, and this corresponds to 1400 nucleotides. Accordingly, the majority of the cDNA synthesized represents essentially a complete copy of its template, and only molecules of 1000 nucleotides or more were pooled for further use (Fig. 1). Hybridization of the $cDNA_{csn}$ to different RNA samples was carried out in 0.01 M Hepes, pH 7/0.6 M NaCl/0.002 M Na_2EDTA as described (23).

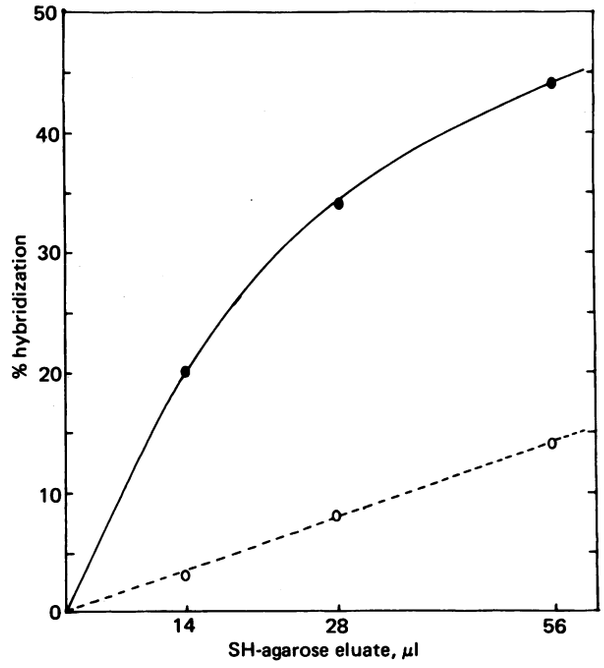

FIG. 3. Hybridization of $cDNA_{csn}$ to the Hg-RNA synthesized in isolated nuclei of lactating mammary gland in absence (●) or presence (○) of 0.05 mM α-amanitin (Calbiochem). Identical amounts of nuclei were incubated in the RNA synthesis assay mixture, extracted by phenol/chloroform, and chromatographed on an SH-agarose column. Samples of Hg-RNA were dissolved in identical volumes of water and aliquots of same volume were used for hybridization. Increasing volumes were hybridized to a constant amount of $cDNA_{csn}$ (500 cpm) for 24 hr at 68°C and S1 nuclease-resistant, trichloroacetic acid-precipitable material was assayed on Millipore filters. Mammary tissue pooled from 6–8 animals was used for nuclear isolation. Reaction products of nine *in vitro* assays were pooled for each determination.

Table 2. Effect of CB-154 treatment on serum prolactin and $mRNA_{csn}$ level in 10-day lactating mice

Animals	Serum prolactin, ng/ml	% $mRNA_{csn}$
10-day lactating	583.5 ± 81.7	1.56
10-day lactating + CB-154	108.0 ± 23.0	1.16

Total mammary RNA from 4–6 animals in each group was extracted by phenol/chloroform (16) and the level of $mRNA_{csn}$ was assessed by RNA excess–$cDNA_{csn}$ hybridization as described in Fig. 2. Sera from three animals in each determination were assayed by duplicate radioimmunoassay as described (25).

RESULTS

Specificity of $cDNA_{csn}$ Probe. Fig. 2*A* shows the results of RNA excess hybridization to the $cDNA_{csn}$ probe. The purified $mRNA_{csn}$ hybridized to the $cDNA_{csn}$ probe 114 times faster than did total mammary RNA, reaching a 90% level of hybridization with a single transition ($R_0t_{1/2}$ 3.5×10^{-3} mol·s·liter^{-1}). Mouse liver RNA failed to show any significant hybridization to the $cDNA_{csn}$ even at R_0t values greater than 1000 mol·s·liter^{-1}, demonstrating the specificity of the $cDNA_{csn}$.

Glucocorticoid Action on Casein Gene Expression. Table 1 shows data on the level of the $mRNA_{csn}$ in the lactating mammary gland, measured by molecular hybridization of the $cDNA_{csn}$ probe to mammary gland RNA. Five days after ad-

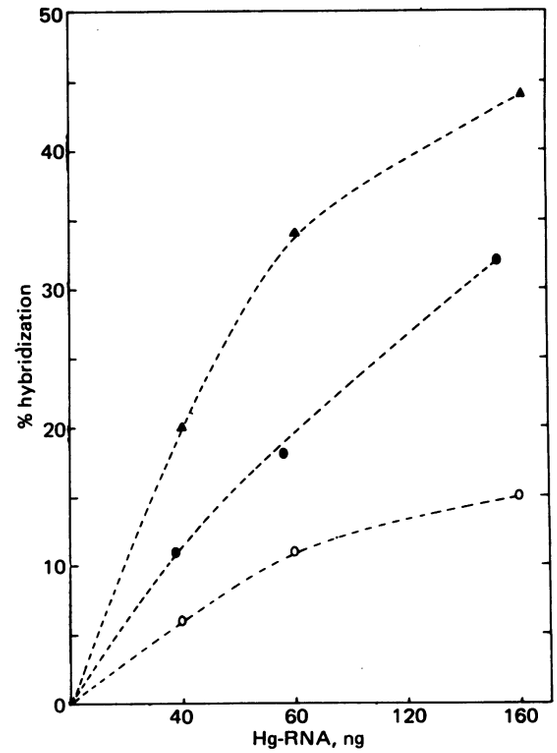

FIG. 4. Effect of adrenalectomy and cortisol treatment on $mRNA_{csn}$ transcription *in vitro*. Hg-RNA was synthesized in isolated mammary nuclei, processed, and hybridized to $cDNA_{csn}$ as described in Fig. 3. ▲, 10-day unoperated lactating mice; ○, 5 days after adrenalectomy; ●, 5 days after adrenalectomy and 6 hr after one hydrocortisone injection. For isolation of nuclei, mammary tissue from 6–8 animals was used and nine *in vitro* assay products were pooled for each determination.

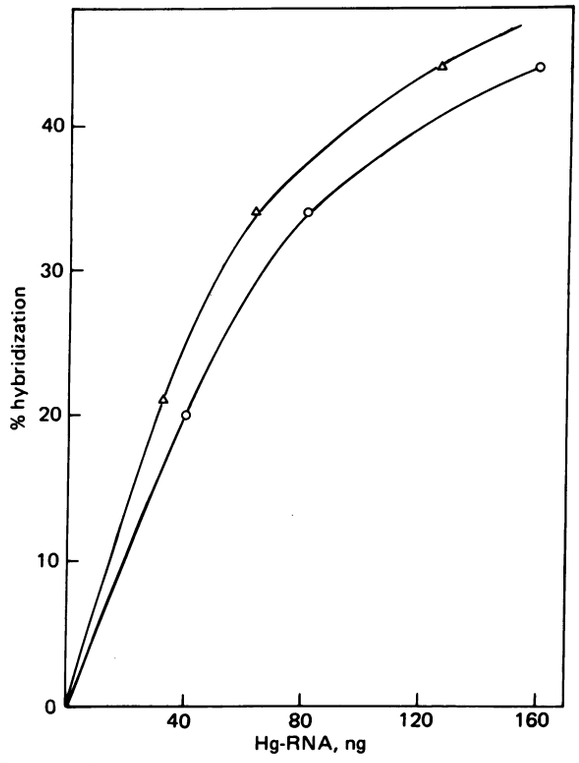

FIG. 5. Effect of decreased serum prolactin level on $mRNA_{csn}$ transcription in isolated mammary cell nuclei. Hg-RNA synthesized in isolated nuclei was hybridized to $cDNA_{csn}$ as described in Fig. 4. ○, 10-day lactating mice; △, 10-day lactating mice treated with CB-154. Hybridization conditions were as described for Figs. 3 and 4. Mammary tissue from 6–8 animals was used in each group for isolation of nuclei and nine *in vitro* assay products were pooled for each determination.

renalectomy, the level of $mRNA_{csn}$ in the mammary gland was decreased by 85%. Twelve hours after a single injection, hydrocortisone stimulated a 4.4-fold increase of $mRNA_{csn}$ in the gland above the level of adrenalectomized mice. This rise was followed by a decline at 24 hr. The increase in the level of $mRNA_{csn}$ at 6 and 12 hr after glucocorticoid injection was, however, preceded by a 3-hr lag period.

As expected, the data in Fig. 2*B* show that the Hg-RNAs transcribed by the isolated nuclei from mammary cells of 10-day lactating mice contained a high level of $mRNA_{csn}$ sequences. Estimates based on $R_0t_{1/2}$ values and the standard equation (24) showed that 0.09% of the Hg-transcripts were sequences of $mRNA_{csn}$. It was also estimated that 0.07% of the total nuclear RNA of the lactating mammary cells *in vivo* was $mRNA_{csn}$ sequences. Hybridization of the Hg-RNA transcribed in liver nuclei to the $cDNA_{csn}$ probe failed to exceed the 10% level, even at R_0t values up to 10 mol·s·liter^{-1}. Assessment of the level of possible endogenous non-Hg-RNA nonspecifically binding to the SH-agarose column consistently showed none. Furthermore, the Hg-RNA synthesized in the isolated nuclei in the presence of α-amanitin showed an 85% decreased hybridization to the $cDNA_{csn}$ (Fig. 3).

Data in Fig. 4 show that transcription of $mRNA_{csn}$ in the isolated nuclei of mammary cells of 5-day adrenalectomized postpartum mice was only $\frac{1}{3}$ that of the controls, but was restored to $\frac{2}{3}$ of the control if the mice were given a single injection of hydrocortisone 6 hr before the animals were killed.

Decreased Serum Prolactin Level and Casein Gene Expression. It is evident from the data in Table 2 that three daily injections of CB-154 for 3 days caused an 80% decrease in the serum prolactin level, whereas the $mRNA_{csn}$ level in the mammary glands of these postpartum animals remained virtually unaltered. Furthermore, isolated mammary nuclei from animals with drastically decreased serum prolactin levels were capable of *in vitro* $mRNA_{csn}$ synthesis at a level as high as that of nuclei from 10-day lactating mammary glands of untreated mice (Fig. 5).

DISCUSSION

Hybridizations of the $cDNA_{csn}$ to Hg-RNA synthesized in isolated nuclei of lactating mammary cells show a substantial level (0.09%) of $mRNA_{csn}$ in the *in vitro* transcripts. Failure of the isolated nuclei of liver cells to synthesize any significant level of $mRNA_{csn}$ sequences under similar conditions indicates that tissue specificity of casein mRNA transcription is maintained in the *in vitro* reaction mixture. As reported earlier (14, 26) heat denaturation of the RNA samples done in the present study should have prevented contamination of the Hg-RNA by aggregating endogenous casein mRNA. Excess (150 fold) carrier wheat germ RNA added to the reaction mixture should dilute the endogenous casein mRNA, again reducing possible aggregation between endogenous mRNA and Hg-RNA. Furthermore, consistent with an earlier observation in oviduct nuclei (27), the present results also showed that the level of $mRNA_{csn}$ sequences in the Hg-RNA transcribed in the presence of α-amanitin was decreased over 85%, demonstrating that $mRNA_{csn}$ present in the Hg-RNA is the product of RNA polymerase II-directed DNA-dependent RNA synthesis in the isolated nuclei. Thus, the casein mRNA sequences in the Hg-RNA, hybridizing to the $cDNA_{csn}$, almost entirely represent the sequences transcribed *in vitro*.

An 85% decrease in the level of the $mRNA_{csn}$ after adrenal ablation and a 4.4-fold increase after cortisol treatment are consistent with our earlier findings on the level of casein mRNA measured by a translational assay (28). The initial 3-hr lag period before a detectable manifestation of the action of the glucocorticoid is interesting because a similar pattern also has been observed during different steroid hormone-induced specific gene expression in other target organs (29, 30).

The $\frac{2}{3}$ decrease in transcription of the $mRNA_{csn}$, caused by adrenalectomy, and the 100% increase within 6 hr after the single injection of hydrocortisone show that the action of the glucocorticoid in regulation of the casein mRNA is mediated at the transcriptional level of control. Therefore, the present results on measuring of specific transcription strongly indicate that the corticosteroid exerts a marked modulatory influence on expression of the casein gene. This observation is consistent with our earlier reports on glucocorticoid-induced increase of precursor incorporation into lactating mammary cell RNA, including the 15S RNA (31, 32). Addition of prolactin in medium containing hydrocortisone stimulates synthesis of rapidly labeled RNA and accumulation of casein mRNA sequences in mammary explants of pregnant mouse and rat, respectively (33, 34). The postulation, based on these observations, that prolactin is the inducer of the casein gene appears to be a conjecture, because maximal response to prolactin action was measured in the presence of hydrocortisone and specific transcription of the casein gene was not measured in either of these studies.

Because adrenalectomized lactating animals maintain a high serum prolactin level (35), and prolactin-dependent lobuloalveolar secretory structures are sustained after adrenalectomy (28), the action of the glucocorticoid may be synergistic with endogenous prolactin. However, virtually no alteration of the level of transcription of the casein gene in the mammary nuclei of lactating mice with 80% decreased serum prolactin suggests that change of serum prolactin level does not influence the level

of casein gene transcription. Because a residual level of serum prolactin was present in the CB-154-treated animals, the synergistic action of endogenous prolactin remains a possibility. Nevertheless, results of the direct measure of specific transcription in isolated mammary nuclei clearly indicate a modulatory action of the glucocorticoid on expression of the casein gene. Elucidation of the precise mechanism(s) of glucocorticoid action at the transcriptional level can be obtained under conditions of controlled hormonal environment in a serum-free organ culture of the whole mammary gland (36).

Presence of a specific glucocorticoid nuclear receptor complex in lactating mammary cells has been documented (37, 38). Therefore, glucocorticoid-induced specific transcriptional responses of the casein gene in the mammary cells appear consistent with the concept (39) of receptor-mediated steroid hormone regulation of specific gene expression in target organs.

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