Vol. 98, No. 2, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 30, 1981 Pages 380-387

PROLACTIN-INDUCED ACCUMULATION OF CASEIN mRNA IN MOUSE MAMMARY EXPLANTS: A SELECTIVE ROLE OF GLUCOCORTICOID

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Received November 17, 1980

SUMMARY: The role of glucocorticoid in the prolactin-induced accumulation of casein mRNA in mammary explants from midpregnant mice has been studied after an initial 4-day incubation to allow the level of messenger to decline to undetectable levels. Subsequent culture for 3 days: 1) with insulin and glucocorticoid did not result in detectable accumulation of messenger; 2) with insulin and prolactin resulted in a very small accumulation; 3) with insulin, glucocorticoid and prolactin elicited a 20-fold greater accumulation of casein mRNA than the system with only insulin and prolactin. Therefore, although glucocorticoids are not an absolute requirement for casein gene expression in mouse mammary tissue, they are necessary for massive accumulation of casein mRNA induced by prolactin. It appears that this dependence is not a result of either mRNA stabilization or alteration in prolactin receptors. By contrast, stimulation of total epithelial RNA synthesis by prolactin does not have this glucocorticoid dependency.

INTRODUCTION

It has been shown that glucocorticoids are essential for the formation of phosphorylated casein (1) and casein peptide (2) by mouse mammary explants. However, the methods used provided no information about glucocorticoid involvement in the formation of casein mRNA ($mRNA_{csn}$)[4].

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[4]Abbreviations: $mRNA_{csn}$, casein mRNA; F, cortisol; P, prolactin; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; AMV, avian myeloblastosis virus; I, insulin; $cDNA_{csn}$, DNA complementary to purified $mRNA_{csn}$.

0006-291X/81/020380-08$01.00/0

Development of specific cDNA probes for $mRNA_{csn}$ has enabled investigators to study effects of F and P on the expression of the casein gene. Recently, it was reported (3) that F, in addition to P, is essential for the accumulation of $mRNA_{csn}$, by cultured mammary glands from virgin mice. This report shows that, although F is required for the accumulation of appreciable amounts of $mRNA_{csn}$, the mammary epithelial cells in explants from midpregnant mice can respond to P in this regard to a very limited extent in the absence of glucocorticoid. Furthermore, it is shown than, unlike $mRNA_{csn}$, prolactin-induced formation of other types of RNA is independent of F, and some mechanistic information about F in relation to mammary RNA metabolism is provided.

MATERIALS AND METHODS

Ovine prolactin (NIH-P-S-13) was kindly provided by the Hormone Distribution Program, NIAMDD, and crystalline porcine insulin was a gift from Eli Lilly Company. Cortisol was obtained from Calbiochem; Hepes, from Sigma Chemical Company; and *E. coli* DNA, from P. L. Biochemicals. AMV reverse transcriptase was generously supplied by Dr. J. W. Beard, Life Sciences Research Laboratories, St. Petersburg, Florida. [5-^{3}H]dCTP (15-30 Ci/mmol), [8-^{3}H]dGTP (5-20 Ci/mmol) and [methyl-^{3}H]TTP were obtained from Amersham Corp., while [5-^{3}H]uridine (30 Ci/mmol) and [8-^{3}H]dATP (10-20 Ci/mmol) were from New England Nuclear Corp. Medium 199 was a product of Grand Island Biological Company. Ribonuclease-free deoxyribonuclease, type I (≥2000 units/mg), was from Millipore Corp. and nitrocellulose filters (Metricel membrane filter, 0.45 μm) were from Gelman Instrument Company. S_1 nuclease (≥400 units/μg), yeast RNA and yeast transfer RNA were purchased from Boehringer Mannheim. Polypropylene cryotubes were from Vangard International, Inc. (Neptune, New Jersey). Phenol was freshly distilled prior to use for RNA isolation.

The abdominal glands from C3H/HeN mice, 10-12 days into their first pregnancy, were removed under sterile conditions and cultured at 37° under air in Medium 199 with Hanks' salts and 20 mM Hepes, pH 7.6 (4). The concentrations of hormones used in the culture medium were I (5 μg/ml), F (1 μg/ml) and P (5 μg/ml). The medium initially contained either I or IF for the specified time period and then was changed to one containing either I, IF, IP or IFP. The medium was changed daily.

[^{3}H]DNA, complementary to purified 15S mRNA (5), was synthesized as previously described (6) using AMV reverse transcriptase; the concentration of each deoxynucleoside triphosphate was 600 μM and KCL was omitted. The $cDNA_{csn}$ was purified on alkaline sucrose gradients (5-20%, linear) according to the method of Stavnezer *et al.* (7). The resulting cDNA had a specific activity of 1.3×10^8 dpm/μg and was between 1,100 and 1,200 nucleotides long.

Total RNA was isolated from cultured explants by homogenization in a detergent buffer, extraction with phenol-chloroform and precipitation with ethanol, as previously described (5). The samples were redissolved in 80 µl of phosphate-buffered saline containing 10 mM $MgCl_2$, and 20 µl of a deoxyribonuclease solution (100 µg/ml) was added. After a 1 h incubation at 37°, the reaction mixture was re-extracted with phenol-choloroform and precipitated with ethanol. The recovery of RNA was not affected by the deoxyribonuclease.

RNA excess hybridization was carried out in polypropylene cryotubes. The hybridization mixture contained RNA (about 300 µg/ml) isolated from mammary explants subjected to various hormonal treatments, yeast transfer RNA (200 µg/ml), 0.6 M NaCl, 30 mM Hepes (pH 7.0), 1 mM EDTA and about 1,500 cpm of $cDNA_{csn}$. The reaction mixture was covered with paraffin oil and heated to 100° for 3 min in order to denature the nucleic acids. The reaction mixture was then incubated at 66° to R_0t values; no degradation of cDNA or mRNA was detected during this incubation.

At appropriate intervals, 20-µl aliquots were removed, diluted in 1.1 ml of nuclease buffer (0.1 M NaOAc, pH 4.5, 0.2 M NaCl and 1.2 mM $ZnCl_2$) and stored at -20°. At the end of the hybridization, 0.5 ml aliquots of each sample were diluted to 1 ml with nuclease buffer containing 10 µg of denatured *E. coli* DNA with and without 3,500 units of S_1 nuclease. After 40 min at 37°, 100 µg of yeast RNA and 1 ml of cold 20% trichloro-acetic acid were added, and the samples were filtered through nitrocellulose membranes. The filters were heated to 100° for 15 min before they were counted in a toluene-based scintillation cocktail. The lower limit of sensitivity under these conditions was a $mRNA_{csn}$ concentration of 0.0005%.

Total RNA synthesis was determined in mammary gland explants from midpregnant mice after the explants were initially cultured with I or IF for 96 h followed by a 12-h incubation with IP or IFP, respectively. During the final 4 h, the explants were labeled with [^{3}H]uridine (0.5 µCi/ml) and the amount of RNA synthesized was determined by the method of Green and Topper (8). Since F inhibits RNA synthesis in fat cells, giving rise to lower baseline values in an IF system (8), the results are expressed as percent stimulation by P over baseline values in either the I or IF system, respectively. These values reflect RNA synthesis in epithelial cells, since cleared fat pads do not respond to P in regard to RNA synthesis (8).

RESULTS AND DISCUSSION

Fig. 1 shows the hybridization curves of casein cDNA with total RNA isolated from mammary glands of virgin, pregnant and 7-8 day lactating mice. The purified 15S doublet mRNA exhibits 75-80% hybridization to the $cDNA_{csn}$ probe with a single transition; the $R_0t_{\frac{1}{2}}$ value is 5.0×10^{-3} mol·s/L, which is similar to other reported values (6,9). The $R_0t_{\frac{1}{2}}$ for RNA isolated from lactating glands is 3.5×10^{-1} mol·s/L and that from mammary glands of pregnant mice is 1.1×10^{0} mol·s/L, demonstrating that 1.4% and 0.45% of the total RNA, respectively, is composed of

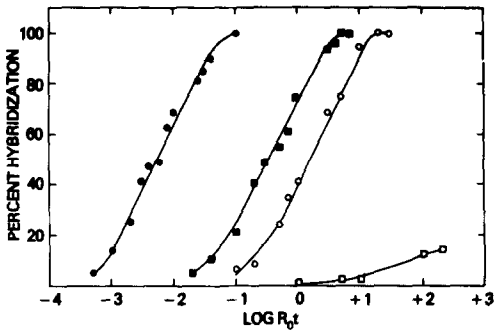

Fig. 1. Hybridization of mouse mammary gland cDNA$_{csn}$ with purified mRNA$_{csn}$ (●) or total RNA isolated from mammary gland of lactating (■), pregnant (○) or virgin mice (□). cDNA was synthesized from 15S doublet casein mRNA obtained from lactating mammary glands. Maximum hybridization was achieved between 75 and 80%; however, the values were normalized to 100% for the purpose of comparison.

mRNA$_{csn}$ sequences. RNA from virgin glands does not hybridize more than 10-15% even when hybridization is carried out to $R_o t$ values as high as 10^3.

Data in Table 1 indicate the relative contributions of F and P to mRNA$_{csn}$ accumulation. It can be seen that the endogenous level of the messenger falls progressively with time in the presence of I alone, and is no longer detectable after 96 h. Addition of F to the I_{96} system (line 7) elicits no detectable accumulation of mRNA$_{csn}$. By contrast, addition of P does evoke a small, progressive rise in the level of the messenger (lines 5 and 8). This reflects an influence of P in the absence of residual, endogenous glucocorticoid, since the latter is absent after only 48 h. Thus, mRNA$_{csn}$ is no higher in the $I_{24} \rightarrow IP_{24}$ system than in the $I_{24} \rightarrow IF_{24}$ system (line 2), whereas addition of both F and P (line 3) evokes a large increment. Furthermore, Ganguly *et al.* (3) have shown that even when the mouse mammary gland is charged with F by incubation in medium containing 5 µg of F/ml, enough of this steroid is lost during a four-day incubation in an F-free medium such that mRNA$_{csn}$ levels are not affected.

However, addition of both F and P to the I_{96} system (line 9) causes

TABLE I

Hormonal regulation of casein mRNA accumulation in mammary gland explants from midpregnant mice

	First incubation	Second incubation	$R_o t_{\frac{1}{2}}$ (mol·s/L)	Casein mRNA (percent)
1.	I_{24}	-	9.0×10^1	0.0055
2.	I_{24}	I_{24}, IF_{24} or IP_{24}	3.5×10^2	0.0014
3.	I_{24}	IFP_{24}	1.0×10^1	0.050
4.	I_{96}	-	n.d.[a]	-
5.	I_{96}	IP_{24}	6.0×10^2	0.0008
6.	I_{96}	IFP_{24}	3.0×10^2	0.0017
7.	I_{96}	I_{72} or IF_{72}	n.d.	-
8.	I_{96}	IP_{72}	2.5×10^2	0.0020
9.	I_{96}	IFP_{72}	1.1×10^1	0.045

Mammary gland explants from midpregnant mice were cultured in Medium 199 with I for the indicated hours (subscript) before the medium was changed to I, IF, IP or IFP. $R_o t_{\frac{1}{2}}$ values obtained from several experiments ranged between 9% and 15% of the average values given below. $R_o t_{\frac{1}{2}}$ value for the pure casein mRNA was 5.0×10^{-3} mol·s/L and this was used to calculate the percent of casein mRNA in the total RNA extracted from the explants.

[a]Not detectable ($R_o t_{\frac{1}{2}} >> 10^3$).

a 20-fold greater increase in the level of $mRNA_{csn}$ after 72 h than addition of P alone (line 8). Furthermore, the rate of accumulation during day 2 and day 3 after the addition of both F and P (lines 6 and 9) is 35-fold greater than that following addition of P alone (lines 5 and 8).

It should be noted that the lower levels of $mRNA_{csn}$ in the IF and IP systems, compared to those in the IFP systems, are not a reflection of differences in non-casein RNA, since the total RNA isolated from each experimental group does not differ significantly and averages 0.66 ±

0.03 μg/mg explant. Since a previous report (10) has shown that neither DNA synthesis in mammary gland explants from midpregnant mice nor the mitotic indices of the mammary epithelial cells differ among the IF, IP and IFP systems, and since the DNA content in both an I and an IFP system are the same (11), it can be assumed that the observed differences in $mRNA_{csn}$ represent different concentrations per epithelial cell. The possibility that the RNA extracted from the IP system might contain an inhibitor of hybridization was entertained, but it was rejected after this RNA failed to inhibit hybridization when mixed with RNA isolated from an IFP system (data not shown).

The following conclusions can be reached from the data in Table I: 1) F cannot evoke detectable $mRNA_{csn}$ in the absence of P; 2) P can evoke a small accumulation in the absence of F, but 3) F and P together can cause a relatively massive build-up of the messenger. These results differ qualitatively from those of Ganguly *et al.* (3), who reported that in the absence of F, P is unable to "turn on" detectably the casein gene in mammary tissue from virgin mice. The disparity may reflect a difference between the tissue from virgin and pregnant mice. Also, the present results differ quantitatively from those of Guyette *et al.* (12) who reported a much smaller dependence on F of P-induced $mRNA_{csn}$ in rat mammary tissue. This has been discussed by Ganguly *et al.* (3). Rabbit mammary tissue is independent of F in this regard (13).

A point to be emphasized in this report is that, although P can effect a small accumulation of $mRNA_{csn}$ in mammary explants from pregnant mice in the absence of glucocorticoid, under these conditions the tissue is largely unresponsive to P in this regard. A fuller understanding of this unresponsiveness has been sought. It is clear from Table I, line 2, that F does not simply stabilize $mRNA_{csn}$ formed under the influence of P; the level in the $I_{24} \rightarrow IF_{24}$ system has declined to the same level as that in the $I_{24} \rightarrow I_{24}$ system. It appears, then, that the ability of

TABLE II

Effect of cortisol on the prolactin-induced enhancement of both total epithelial RNA synthesis and accumulation of casein mRNA in mammary gland explants from midpregnant mice

Culture conditions	Stimulation of total epithelial RNA synthesis		$R_o t_{\frac{1}{2}}$	Casein mRNA
	cpm/mg[a]	percent	(mol·s/L)	(percent)
1. $I_{96} \rightarrow IP_{12}$	87/262	+33	n.d.[b]	-
2. $IF_{96} \rightarrow IFP_{12}$	70/201	+35	6.6×10^{1}	0.0075

Mammary gland explants from midpregnant mice were cultured in Medium 199 with I or IF for 96 h before the medium was changed to IP or IFP, respectively, for an additional 12 h. During the final 4 h, the explants were labeled with [^{3}H]uridine (0.5 μCi/ml); baseline synthesis was determined in an I_{108} and an IF_{108} system, respectively. $R_o t_{\frac{1}{2}}$ value for the pure casein mRNA was 5.0×10^{-3} mol·s/L and this was used to calculate the percent of casein mRNA in the total RNA extracted from the explants.

[a] Prolactin-induced stimulation/baseline synthesis.

[b] Not detectable ($R_o t_{\frac{1}{2}} >> 10^3$).

P to affect only a very small accumulation of the messenger in the absence of glucocorticoid reflects diminished P-induced transcription. A critical question is whether similar, reduced P-responsiveness is manifested in terms of other types of RNA in glucocorticoid-free conditions. Data in Table II indicate that this is not the case. P stimulates total epithelial RNA synthesis as much in the absence of F as in its presence.

Previous studies (14,15) have shown that mouse mammary cells lose a large fraction of their P receptors *in vitro* and *in vivo* in the absence of glucocorticoid. Nevertheless, the P receptor-deficient cells respond to P as well as the normal mammary epithelial cells in regard to total RNA synthesis (Table II). The results suggest that the partial P-unresponsiveness of the cells in the I → IP system may be ascribable to a defect distal to the P receptor. Therefore, the great potentiation that F exerts on P-induced accumulation of $mRNA_{csn}$ is not a result of either mRNA stabilization or alteration in P receptors.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. Tadashi Yamamoto for performing the methylmercury gel electrophoresis and Dr. Takami Oka for providing rabbit antimouse casein serum. The authors are also grateful to Dr. S. K. Arya, Dr. S. Muthukrishnan and Dr. G. Aulach for many helpful discussions.

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