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AN ESSENTIAL ROLE FOR GLUCOCORTICOID IN CASEIN GENE EXPRESSION IN RAT MAMMARY EXPLANTS

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SUMMARY: It is demonstrated that the accumulation of 42 K casein mRNA in mammary tissue from adrenalectomized, virgin rats is almost 20x higher in the presence of exogenous hydrocortisone than in its absence. Accumulation of 25 K casein mRNA in this tissue is totally dependent on the steroid. The results indictate a much greater dependency on hydrocortisone than was appreciated previously, and also show that this dependency does not reflect a loss of cell viability in the absence of the steroid.

It has been shown that glucocorticoid is essential for casein gene expression in explanted mouse mammary tissue (1,2). By contrast, it was reported (3) that glucocorticoid is not essential, although it is potentiative for casein gene expression in cultured rat mammary tissue. We have considered two possibilities to acccount for the reported difference between the mouse and rat tissues. First, it might represent another authentic species difference in mammary tissue (4). Second, the incomplete dependence of the rat tissue might only be apparent in that it might reflect the presence of some residual, endogenous glucocorticoid in the isolated rat tissue (5). In an effort to distinguish between these two possibilities, mammary tissue from virgin rats which had been deprived of the adrenal source of glucocorticoid for a prolonged period was studied.

MATERIALS AND METHODS

Mammary Gland Explant Culture: Virgin, intact or adrenalectomized (10 weeks old) Sprague-Dawley rats (Taconic Farms, Germantown, NY) were used. Explants were prepared from the mammary glands of adrenalectomized rats 18 days after ablation. All

ABBREVIATIONS
I, insulin; F, hydrocortisone; P, prolactin; $mRNA_{csn}$, casein mRNA; Adrex, adrenalectomized

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explants were prepared from the fourth abdominal mammary glands and cultured in Medium 199 as described previously (5). The hormones, insulin, cortisol and prolactin were added to the media at the concentration of 1 µg/ml, each.

RNA Extraction From Mammary Gland Explants: RNA was extracted from approximately 50 mg tissue by homogenization in 1.5 ml of 5 M guanidine thiocyanate buffer, pH 7.0, containing 1% Sarkosyl, 0.5 M β-mercaptoethanol, and 25 mM potassium acetate (6), and purified by centrifugation through a 1.3 ml cushion of 5.7 M CsCl containing 0.1 potassium EDTA, pH 7.5 (7). The RNA pellet was dissolved in sterile water, and routinely stored at -20°C in 0.2 M potassium acetate, pH 5.0, and 79% ethanol. The RNA concentration of the extracts were determined by measuring the absorbance at 260 nm using $A_1\ 1\%_{cm} = 200$.

Assay of mRNA: The construction and characterization of recombinant pBR322 plasmids containing cDNA inserts to mRNAs for rat 42K casein and 25K casein has been described (8). Clone p-42K 303, corresponding to 42K casein, and clone p-25K 530, corresponding to 25K casein were selected from a lactating rat mammary gland cDNA library by hybrid selection, cell-free translation of complementary RNAs and immunological identification of the translational products (9). The cDNA insert of p-42K 303 is 1080 base pairs long and has the same restriction enzyme map as that of the alpha-casein clone of Richards et al., (10). The cDNA insert of p-25K 530 is 1050 base pairs long and has a restriction enzyme map similar to that of the beta-casein clone of Richards et al., (10). Plasmid DNA was prepared from p-42K 303 and P-25K 530 clones as described (9) and purified free of RNA and linear DNA by centrifugation three times in a CsCl gradient (d-1.5732) containing ethidium bromide (150 µg/ml) in VTi 50 rotor (Beckman) at 48,000 rpm for 12 h.

Papers in the aminobenzyloxymethylcellulose form (Schleicher and Schuell, Inc., Keene, NH) were activated to the diazobenzyloxymethyl (DBM) form by soaking in a solution containing 4.6 mM $NaNO_2$ and 1.3 M HCl for at least 30 min., then washed in several changes of 0.2 M sodium citrate, pH 4.0, and blotted dry. RNA (1 or 2 ug) in 10 ul of 0.2M sodium acetate, pH 4.0, was spotted onto the surface of DBM-paper disks (11 mm. diameter) which were dried overnight at room temperature. RNA covalently linked to the DBM paper circles was washed for at least 4 h at 42°C with pre-hybridization solution, consisting of 50% formamide, 5 x SSC, 5 x Denhardt's solution, 50 nM sodium phosphate, pH 6.5, 1% glycine and sonicated denatured salmon sperm DNA (500 µg/ml) as previously described (11). Each filter was then hybridized for 18 h in a polypropylene vial PPC-4 (Isolab, Inc., Akron, Ohio) containing 50 ul of the pre-hybridization solution and heat denatured ^{32}P-labelled plasmid probe (2 to 4 x 10^6 cpm/ml). The amount of plasmid probe added to each vial was 12-18 ng. After completion of the hybridization the papers were washed in STE-SDS buffer (0.15 M NaCl 0.01 M Tris-HCl, pH 7.4, 1 mM EDTA and 0.5% SDS), briefly 3 times at room temperature, once at 50°C for 15 min and twice with a 3 times dilution of STE-SDS buffer at 50°C for 15 min. The radioactivity remaining on the paper disks was counted in a toluene based scintillant. The concentrations of the mRNAs extracted from explant tissue were usually calculated as a percentage relative to the concentration of mRNA present in RNA extracted from mammary tissue of lactating rats.

Electrophoresis and Autoradiography: RNA (8 µg per lane) was electrophoresed in 0.8% agarose gel containing formaldehyde (12), transferred to DBM paper by southern technique (13) using 1 M sodium acetate, pH 4, and hybridized with ^{32}P-labelled plasmid DNA as previously described (14). The ^{32}P-labelled plasmid-mRNA hybrid was detected by autoradiography (18 h) with the use of Kodak XR paper and intensifying screens at -70°C.

RESULTS

DBM Paper Disk Hybridization Assay. To determine whether hybridization of RNA with casein cDNA plasmids could be used to quantitate casein specific mRNA, different amounts of RNA were spotted onto DBM-paper circles. Hybridization to

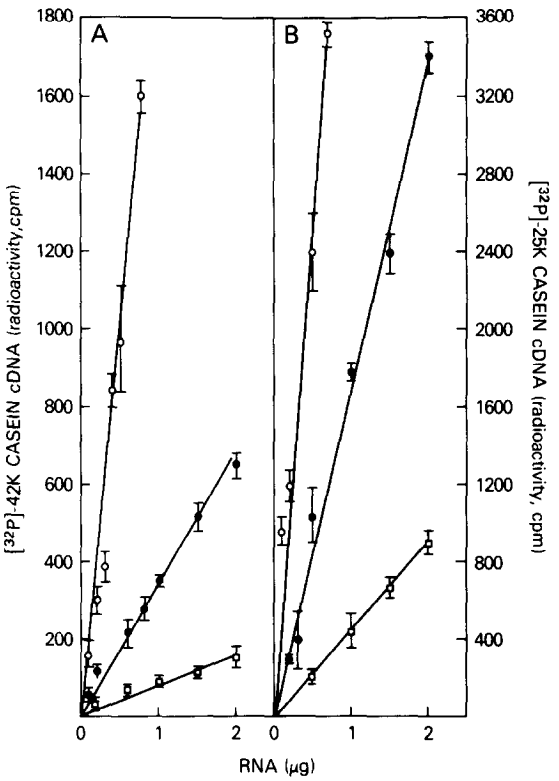

Figure 1. Quantitation of casein mRNA coupled to DBM-paper. The RNA extracted from lactating tissue (o) or mid-pregnant mammary explants cultured in IFP (•) or I (□) for 4 days was analyzed for (A) 42 K casein mRNA or (B) 25 K casein mRNA by the DBM paper disk hybridization assay as described in Materials and Methods. The RNA extracted from the mammary tissue was made to 2 ug per filter with yeast RNA. The error bars indicate the range of duplicate hybridizations. Culture medium contained I, F and P at concentrations of 1 ug/ml, each.

increasing amounts of RNA (0.1 to 2.0 ug) from lactating mammary tissue, or mid-pregnant mammary tissue explants previously cultured for 3 days on medium containing insulin, or insulin, hydrocortisone and prolactin, increased linearly with increasing RNA coupled to the paper (Figure 1). Hybridization to increasing amounts of RNA from rat liver, heart or kidney, or from yeast remained at background levels over the entire range of RNA concentrations that were used (data not shown). The limit of sensitivity of the assay, based on purified 42K or 25K casein mRNA, was 20-50 pg. Experiments with uniformly ^{32}P-labelled total mammary gland RNA indicated that greater than 90% of the RNA applied to DBM-paper remained covalently bound to the paper after washing. These experiments showed that hybridization between RNA coupled to DBM paper and casein cDNA plasmids 303 or 530 could be used to quantitate 42 K casein mRNA or 25 K casein mRNA, respectively, in a fashion similar to that which

had been used previously for other tissue mRNAs (11,15,16). The recent observation (3) that this assay is non-linear at different concentrations of casein mRNA was due to the use of total RNA at concentrations greater than 2 ug; this has been confirmed in the present study (data not shown).

On the basis of these experiments, 1 or 2 ug of total RNA extracted from cultured explants was applied to DBM-paper. For each hybridization, a set of standards was included; these were filters which contained 0.1 to 0.5 ug of RNA extracted from lactating mammary tissues. Filters containing RNA from rat liver, kidney, or yeast were also included in each DBM paper hybridization assay to correct for background counts.

Levels of Casein mRNA in Tissue From Intact or Adrenalectomized Virgin Rats. Mammary tissue isolated from virgin rats which had been adrenalectomized 18 days previously would be expected to contain less glucocorticoid than tissue from intact virgin animals. On this basis, if glucocorticoid plays a critical role in the induction of rat casein mRNAs, the tissue from the adrenalectomized animals should exhibit a greater dependence on exogenous hydrocortisone than tissue from intact animals. Consequences of such glucocorticoid depletion on hormone-dependent accumulation of casein mRNAs are presented in Figures 2, 3 and 4. It can be seen that the t_o values are much lower in tissue from adrenalectomized than in that from intact virgin rats. This suggests a dependence of casein mRNA levels *in vivo* on glucocorticoid. Figure 2 shows that the level of 42 K casein mRNA induced by IFP in tissue from intact, virgin rats is twice that present in the tissue at time zero. However, the IP-level is lower than the initial level. Furthermore, the IFP/IP ratio is about 9. Studies with tissue from adrenalectomized, virgin rats shows that a small induction above the initial level does occur in the IP system. However, this tissue is highly dependent on exogenous hydrocortisone since the IFP/IP ratio, calculated after subtraction of the t_o value, is about 17.

Similar studies on the 25 K casein mRNA are described in Figure 3. After culture of tissue from the intact virgin the IFP/IP ratio was about 24, denoting a very high dependence on exogenous glucocorticoid. Tissue from the adrenalectomized animals was very responsive to IFP, but was completely unresponsive in the absence of exoge-

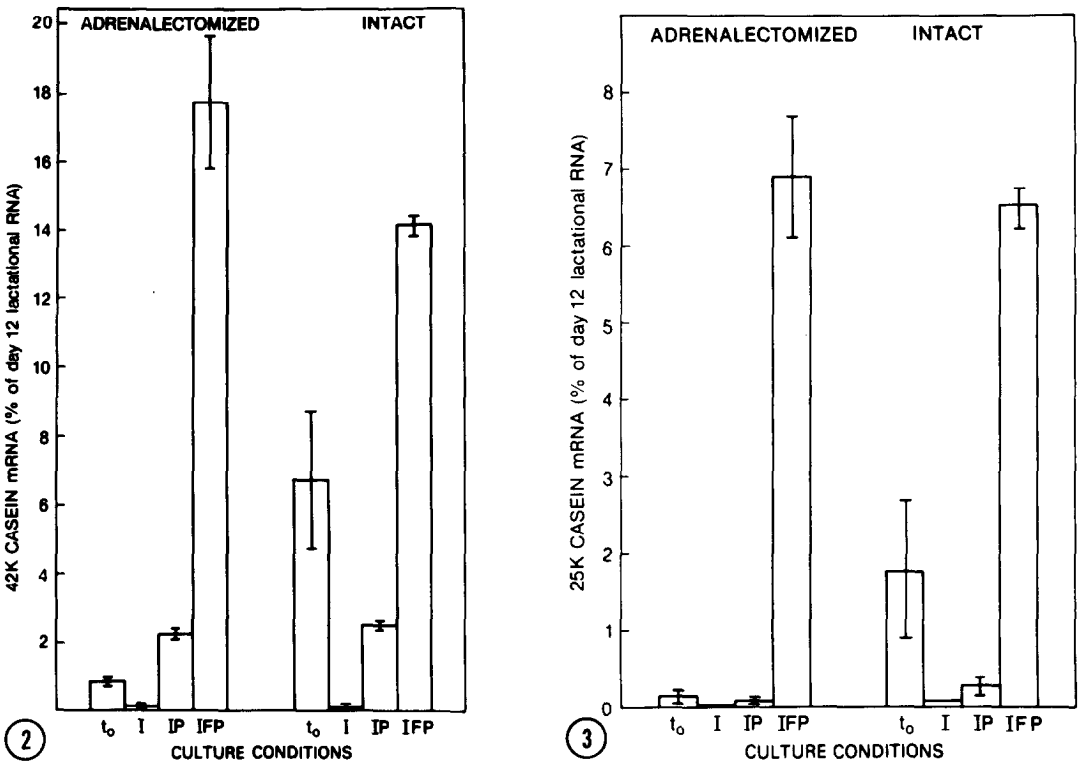

Figure 2. Effect of hormones on 42 K casein mRNA accumulation in mammary explants of adrenalectomized or intact virgin rats. Explants were cultured for 4 days in media containing I, IP or IFP. Each hormone was used at a concentration of 1 ug/ml. Time zero cultures are shown as t_o. Extraction of total RNA and quantitation of mRNA are described in Materials and Methods. The amount of RNA extracted per mg wet weight tissue in these experiments is given in Table 1, and 1-2 ug of the RNA was covalently linked to DBM-paper for hybridization assay. The values of 42 K casein mRNA are expressed as percentages of the levels in the day 12 lactational RNA preparations which were included in each hybridization assay. Each value represents mean $\pm$ S.E.M. for 3 groups of rats, with 3 animals in each group. Ten percent of 12 day lactational RNA represents 840 cpm (above a background of 30 cpm) of ^{32}P-labelled cDNA hybridized to 2 ug RNA bound to filter. Counts obtained for 1 ug RNA per filter were half those obtained for 2 ug RNA per filter.

Figure 3. Effect of hormones on the accumulation of 25 K casein mRNA in mammary explants of (A) adrenalectomized or (B) intact virgin rats. Experimental details were the same as described in the legend of Figure 2. Five percent of 12 day lactational RNA represents 860 cpm (above background of 30 cpm) of ^{32}P-labelled cDNA hybridized to 2 ug RNA bound to filter. Counts obtained for 1 ug RNA per filter were half those obtained for 2 ug RNA.

nous glucocorticoid. The results shown in Figs. 2 and 3 are in agreement with the autoradiography pattern of the electrophoresed and hybridized total RNA shown in Fig. 4.

It was concluded earlier (3) that rat mammary tissue loses viability when cultured in the absence of hydrocortisone since it was observed that IP-explants have less total extractable RNA than IFP-explants. By contrast, the data in Table

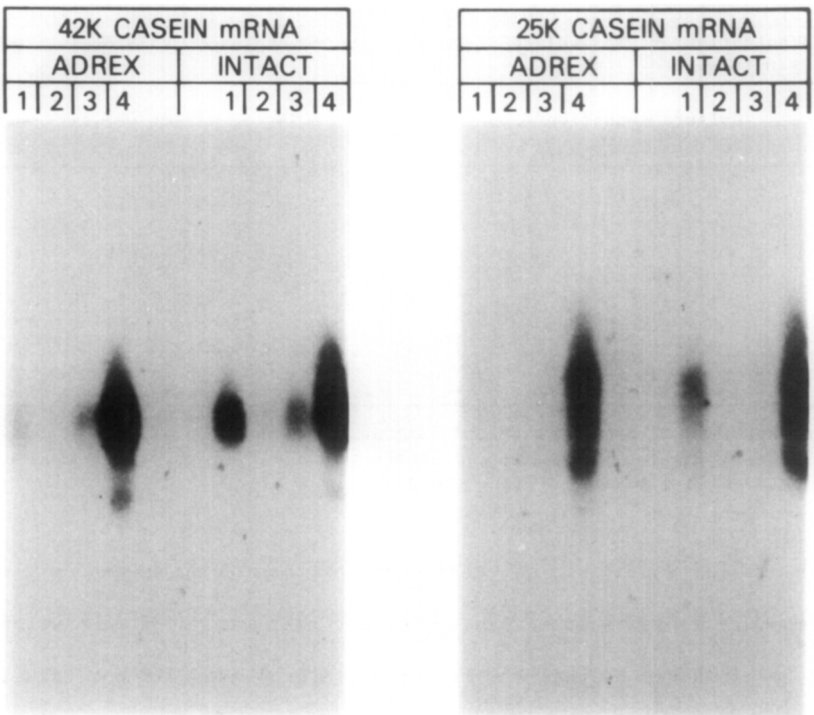

Figure 4. Autoradiogram of 42 K and 25 K casein mRNA from adrenalectomized and intact virgin rat mammary tissue. The RNA was isolated, electrophoresed, transferred to DBM paper hybridized with ^{32}P-labelled plasmid DNA and detected by autoradiography as described in the Methods section. The lanes contained 8 ug of RNA, from fresh mammary tissue (lane 1), or from tissue cultured for 4 days with I (lane 2), IP (lane 3) or IFP (lane 4). The RNA in each lane contains equal amounts of the pooled RNA derived from each of the 3 experimental groups of animals shown in Figs. 2 and 3.

1 demonstrate that the IP-tissue contains at least as much total extractable RNA as the IFP-tissue.

DISCUSSION

The results demonstrate that cultured mammary tissue from virgin rats which had been deprived of glucocorticoid by adrenalectomy 18 days previously is highly dependent on exogenous glucocorticoid in terms of the accumulation of casein mRNA. Although a small increment in the accumulation of the 42 K casein mRNA does occur in the absence of exogenous hydrocortisone, almost 20 times greater accumulation occurs in the presence of the steroid. In this instance hydrocortisone had a massive potentiative effect. However, accumulation of the 25 K casein mRNA appears to be totally dependent on hydrocortisone.

TABLE 1

Concentration of RNA isolated from the mammary explants of adrenalectomized virgin rats.

Culture conditions	RNA; μg per mg wet tissue
t_o	0.46 ± 0.025
I	1.03 ± 0.049
IP	0.91 ± 0.064
IFP	0.83 ± 0.060

The experimental details were the same as those described in the legends to Figs. 2 and 3. Each value represents the mean ± S.E.M. for 3 groups of rats, with 3 animals in each group.

A much smaller dependence of casein gene expression on exogenous glucocorticoid in mammary explants from intact pregnant rats was reported previously (3). This might be explained by the fact that the protocol used did not take into account fully the observation that isolated mammary tissue from pregnant rats retains about 30% of the hydrocortisone to which it had been exposed 6 days previously (5). This explanation might also apply to the protocol (3) in which tissue from pregnant rats, adrenalectomized only 2 days before sacrifice, was used. In both cases, it is likely that the tissue had retained some endogenous glucocorticoid during culture.

It appears that casein gene expression in isolated rat mammary tissue is highly, and perhaps entirely, dependent on glucocorticoid. This dependency does not reflect a loss of cell viability in the absence of the steroid.

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