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CONDITIONS REQUIRED FOR PREDOMINANT SYNTHESIS OF mRNA AND ASSESSMENT OF CHROMATIN-FREE RNA POLYMERASES IN ISOLATED MOUSE MAMMARY CELL NUCLEI

by

Ranjan Ganguly

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For the Degree of Doctor of Philosophy

School of Life Sciences

Under the Supervision of Professor Mihir R. Banerjee

Lincoln, Nebraska

August, 1978

TITLE

Conditions Required for Predominant Synthesis of mRNA and Assessment of Chromatin-free RNA Polymerases in Isolated Mouse Mammary Cell Nuclei

BY

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A. GENERAL INTRODUCTION

It was postulated by Karlson (1963) that the hormones exert their physiological effect by modulating gene expression in the target organs. Increased cellular RNA synthesis after estradiol injection is possibly the first evidence in support of this concept (Mueller et al., 1958). Later, Clever and Karlson (1960) showed that in Chironomous, a dipteran, chromosomal puffs could be induced by the hormone, ecdysone. This event is known to be an indication of gene expression at the chromosomal level. Estrogen administration also showed an increased rate of RNA synthesis in rat uterus and chick oviduct (Mueller, 1965; Gorski et al., 1965; Hamilton, 1968; O'Malley et al., 1968, 1969; Williams-Ashman and Reddi, 1971; Jensen and DeSombre, 1972). Hormones are also found to induce synthesis of specific proteins in several eukaryotic organisms. It has been shown that steroid hormones can induce the synthesis of tissue-specific proteins, viz., ovalbumin and avidin in chick oviduct (Oka and Schimke, 1969; Comstock et al., 1972; O'Malley and Means, 1974); uteroglobin in rabbit uterus (Arthur and Daniel, 1972; Bullock and Willen, 1974); and vitellogenin in laying hen liver (Schejeide et al., 1963; Greengard et al., 1964, 1965). Multiple hormonal regulation of the synthesis of the male rat liver-specific protein, α_{2u}-globulin, also has been studied extensively (Roy and Neuhaus, 1967; Roy et al., 1975; Kurtz and Feigelson, 1977). These reports confirm the earlier concept that the hormones regulate at the level of gene expression (Karlson, 1963).

The mammary gland, which is known to synthesize casein and several milk proteins, is dependent on steroid and polypeptide hormones for its structural and functional differentiation (Peterson, 1944; Folley and Malpress, 1948; Lyons et al., 1958; Nandi, 1959; Cowie, 1961; Denamur,

1971; Anderson, 1974; Banerjee, 1976). Interest in recent years centers around the hormonal regulation of casein synthesis. Earlier studies indicated that the synthesis of this major milk protein in mouse mammary gland was prolactin and cortisol dependent (Banerjee et al., 1971a; Denamur, 1971; Banerjee, 1976; Banerjee et al., 1977). Recent studies further demonstrated the requirement of cortisol for the induction of casein mRNA in mouse mammary gland (Terry et al., 1977a,b; Banerjee et al., 1977). These studies have thus generated an interest in understanding the exact modes of prolactin and cortisol action on the expression of the casein gene.

The present investigation has been undertaken to understand hormonal regulation of casein mRNA synthesis in mouse mammary gland. For this purpose, an in vitro RNA-synthesizing system is required which will transcribe mRNA in isolated mammary cell nuclei for a prolonged period of time. Further, a tool is needed with which RNA synthesis in vitro can be separated free of endogenous nuclear RNAs and finally, a molecular probe is essential to monitor casein mRNA in the in vitro transcripts.

1. The Mammary Gland of the Mouse

Extensive work has been done over the past two decades to understand the morphological development of the mouse mammary gland during post-natal life (Nandi, 1959; Nandi and Bern, 1961; Ichinose and Nandi, 1964; Singh et al., 1970; Mehta and Banerjee, 1975). The mammary glands of prepuberal females exhibit a conspicuous branching of the ducts with terminal end-buds. During post-puberal life extensive ramification of the ducts becomes evident, the end-buds become infrequent and a few

alveoli are visible. With the onset of pregnancy, morphogenesis of the parenchyma is characterized by the development of alveoli and these aggregate into lobule-like structures. This process is known as mammogenesis. At the later half of pregnancy the mammary fat pad becomes filled with the lobuloalveolar structures and the ductal system becomes inconspicuous. The lobuloalveolar structures constitute the secretory units which actively produce milk in post-partum females.

Earlier studies on the endocrine regulation of the mammary gland of the mouse revealed that in ovariectomized, adrenalectomized and hypophysectomized (triply operated) pre-puberal mice, ductal branching could be induced by a combination of estradiol, deoxicorticosterone acetate and growth hormone (Nandi and Bern, 1961). A limited alveolar development in these triply operated mice was inducible by injections of a combination of estradiol-17β, progesterone and growth hormone. To achieve pregnancy-like lobuloalveolar development, prolonged treatment with the ovarian steroids, along with deoxicorticosterone acetate plus prolactin (or growth hormone) was required.

Although studies in vivo showed that growth of the mammary parenchyma was endocrine dependent, systemic complexities in the animal limited the scope in understanding the discrete role of each hormone in mammogenesis and accordingly, an organ culture system was needed. While earlier attempts to develop an appropriate culture model met with limited success (Prop, 1961; Rivera, 1964; Gadkari et al., 1968; Elias, 1957, 1962), Ichinose and Nandi (1964, 1966) first demonstrated that pregnancy-like alveolar growth in virgin Balb/C mouse mammary glands could be induced in culture medium supplemented with ovarian steroids and/or

aldosterone + insulin, prolactin and growth hormone. However, daily injections of the virgins with estradiol-17β and progesterone were required before cultivation of the glands. Subsequently, it was also found that the extent of priming was variable in different strains of mice (Singh et al., 1970). Mehta and Banerjee (1975) observed that insulin alone or in combination with steroids or the pituitary peptide hormones failed to produce alveolar development. Insulin, prolactin and aldosterone (or ovarian steroids) were found to be the minimal hormonal combination required to induce full lobuloalveolar growth in culture (Ichinose and Nandi, 1966; Singh et al., 1970; Mehta and Banerjee, 1975; Wood et al., 1975).

a. Molecular events associated with alveolar morphogenesis

1. DNA synthesis: Boivin et al. (1948) and Mirsky and Ris (1949) first demonstrated that deoxyribonucleic acid (DNA) content remained constant per somatic cell of multicellular organisms and this provided a molecular parameter to estimate biological growth. Using this parameter, it was observed earlier that DNA content per unit weight of dry fat tissue of Swiss Webster mouse mammary gland increased continuously from virgin to late pregnancy (Brookreson and Turner, 1959; Wada and Turner, 1959). In subsequent studies, efforts were made to estimate the rate of DNA synthesis, rather than its content by monitoring ^{3}H-TdR uptake in the mammary tissue. Bresciani (1968) reported that in 5 to 6 month-old virgins, rate of mammary DNA synthesis was greater in the terminal end-buds. With the onset of pregnancy, the rate of DNA synthesis was found to increase and this was accompanied by a higher mitotic index (Banerjee, 1962; Banerjee and Walker, 1967; Traurig, 1967a,b). These results thus demonstrate that there is a direct relationship between morphological

growth and increase in DNA content and its rate of synthesis.

Banerjee and Rogers (1971) have shown that while DNA synthesis in the mammary epithelium of young virgin mice was highly sensitive to the ovarian hormones, the rate of ^{3}H-TdR incorporation in the stromal cells remained non-responsive. Interestingly, DNA synthesis in the epithelial cells of the end buds showed a positive responsiveness both to estradiol and progesterone, but the ductal epithelium responded only to progesterone (Besciani, 1968).

Ovarian steroids also have been found to modulate the cell cycle in mouse mammary gland (Banerjee and Walker, 1967; Besciani, 1965). It was observed that daily injections of estradiol and progesterone decreased the length of mammary epithelial cell S-phase (time of DNA synthesis), from 20 to 8.8 hours (Bresciani, 1965). Banerjee and Walker (1967) have shown that in outgrowths of pregnant mammary tissue obtained after mammary fat pad transplantation into a virgin host, the duration of S-phase increased to 21.5 hours, which is otherwise only 8 to 9 hours in the pregnant animals. The ovarian steroids are also known to modulate the DNA polymerase activity in the mammary cells (D. N. Banerjee et al., 1971; Banerjee et al., 1973).

DNA synthesis in the explants of mid-pregnant mouse mammary gland has been reported to be higher in the insulin-containing medium as compared to the no hormone medium after 24 hours of cultivation (Stockdale and Topper, 1966). This increased rate of DNA synthesis, however, was found to drop by 50% at the 48th hour in media containing insulin alone. Such a reduction was not observed when the medium was supplemented with insulin + prolactin or insulin + prolactin + cortisol. These results have

been interpreted to mean that insulin initiates DNA synthesis in mammary cells. However, the validity of this postulation is questionable, since estradiol is reported to be inhibitory and progesterone ineffective for DNA synthesis (Turkington et al., 1967a; Turkington and Hilf, 1968). On the contrary, the ovarian steroids are known to be stimulatory for mammary growth in vivo (Bresciani, 1965, 1968; Banerjee and Walker, 1967; Banerjee and Rogers, 1971).

During alveolar growth of the ductal parenchyma in estrogen and progesterone-primed virgin mouse mammary gland, DNA synthesis was found to reach a peak at the second and fourth day of culture in presence of insulin + prolactin + aldosterone (or estradiol + progesterone) (Banerjee et al., 1973; Mehta and Banerjee, 1975). The first peak was believed to be the DNA synthesis initiated in vivo during priming (Mukherjee et al., 1973). The second peak represented the cells which initiated DNA synthesis in the culture medium. The reports by Mehta and Banerjee (1975) indicate that estradiol and progesterone together with insulin + prolactin are stimulatory rather than inhibitory to DNA synthesis as observed in the mid-pregnant explant cultures (Turkington et al., 1967a; Turkington and Hilf, 1968). These authors also observed that the ovarian steroids are replaceable by aldosterone in the culture for increased DNA synthesis as well as increased DNA polymerase activity (Mehta and Banerjee, 1975). The molecular responses of the whole mammary gland to the hormones in culture thus appear to mimic the characteristics of the in vivo condition.

2. RNA synthesis: It has been observed that daily injections of estradiol and progesterone enhanced the rate of ^{3}H-uridine uptake of DNA

and protein synthesis (Banerjee and Rogers, 1971). Density gradient centrifugation and gel electrophoresis of the mammary RNA showed that little rapidly labeled RNA was present in the virgin mammary gland (Banerjee and Banerjee, 1973; Banerjee et al., 1973). Daily injections of the ovarian steroids for 9 days, however, induced the synthesis of different molecular species of RNA, including HnRNA (Banerjee et al., 1973).

The rate of ^{3}H-uridine uptake into 15 day pregnant mammary glands has been reported to be six times greater when compared to that in virgins (Banerjee et al., 1971a). This increased rate of RNA synthesis was reduced only by 20 to 30% after adrenalectomy. It is generally believed that HnRNA synthesis reflects the metabolic activation of the tissue. In mammary glands of mice, HnRNA was first detectable at the onset of pregnancy (Banerjee and Banerjee, 1973).

It has been discussed earlier that in insulin + prolactin + aldosterone containing medium, estradiol + progesterone-primed virgin mouse mammary glands undergo lobuloalveolar morphogenesis (Mehta and Banerjee, 1975). RNA synthesis during alveolar development in the whole mammary glands in organ culture reached maximum level on the third day of culture and this was followed by a rise in DNA synthesis. Substitution of aldosterone by the ovarian steroids showed a similar result. It has been noted also that synthesis of 4S, 18S and 28S RNA was maximum in the presence of all three hormones (insulin + prolactin + aldosterone), as compared to insulin alone, insulin + steroids or insulin + prolactin. However, RNA synthesis was greater in the latter combination of hormones (Mehta and Banerjee, 1975). The authors interpreted these results to

indicate that a combination of the polypeptide and steroid hormones favor the synthesis of RNA in the mammary glands.

3. Protein synthesis: Information on hormonal regulation of protein synthesis during mammary lobuloalveolar morphogenesis in vivo is rather limited. Most studies were done with the explants of mammary glands grown in culture media. Cole and Hopkins (1962) reported that estrogen and progesterone in presence of prolactin were able to stimulate glutamine-aspartic-transaminase activity in the mammary glands of hypophysectomized and ovariectomized mice in vivo. In mid-pregnant mouse mammary explants, increased activity of glucose-6-phosphate dehydrogenase (G6PD), malic enzymes, UDP-glucose phosphopyrolase, ATP citrate lyase, lactate dehydrogenase was observed in the medium containing insulin, prolactin and cortisol (Jones and Forsyth, 1969). However, the stimulation of these enzymes was not seen in the prolactin-deficient medium. In contrast, increased synthesis of histones, non-milk proteins, RNA and DNA polymerases and G6PD was observed in the mid-pregnant mouse mammary explants in the medium containing insulin alone (Turkington, 1969, 1972; Turkington and Ward, 1969a,b). These observations, thus, indicate that both prolactin and insulin are required for increased protein synthesis. To elucidate the mechanism of actions of these hormones, mammary explants were cultivated in presence of RNA and protein synthesis inhibitors (Leader and Barry, 1969; Rivera and Cummins, 1971). It was observed that under these conditions, a rise in G6PD and 6-phosphoglucose dehydrogenase activity was abolished. These observations thus indicate that the initial RNA synthesis is needed for the hormone-induced increased activity of these enzymes.

During the lobuloalveolar morphogenesis of virgin mouse mammary glands in culture, total protein synthesis was found to be dependent upon the presence of specific hormones (Mehta and Banerjee, 1975). Addition of insulin to the culture medium increased the total protein synthesis by about three fold as compared to the medium containing no hormone. No significant increase was observed when prolactin was added with insulin. However, protein synthesis increased by another 1.6 fold in the mammary gland cultured in medium containing insulin + prolactin + aldosterone. The adrenocorticosteroid was found to be replaceable by the ovarian steroids (Mehta and Banerjee, 1975). This indicates that prolactin and aldosterone or ovarian steroids may act synergistically to increase protein synthesis. The results on macromolecular synthesis during alveolar morphogenesis of the parenchyma in culture thus appear to be consistent with the similar endocrine regulated events in vivo.

b. Functional differentiation

It is evident from the discussion in the preceding section that morphogenetic development of the mammary glands is completed during pregnancy and the hormones required for this process are well delineated. After parturition, the mammary glands undergo functional differentiation and this is characterized by the production of milk which contains casein and α-lactalbumin, specific milk proteins. In addition, hormonal regulation of milk protein synthesis as well as several other associated metabolic events take place during lactation and these are briefly discussed in the following section.

1. DNA synthesis: Banerjee et al. (1971b) have reported that in lactating mouse mammary glands the DNA content is about 30% higher than the expected diploid value. Similarly, a higher DNA content was observed in rabbit (Sod-Moriah and Schmidt, 1968) and rat (Simpson and Schmidt, 1969). Moreover, during the initial days of lactation a higher ^{3}H-TdR labeling index in the lactating mouse mammary gland was not accompanied by an increased mitotic index (Banerjee et al., 1971b). This DNA synthesis is associated with the GC-rich fraction of the DNA. While these authors (Banerjee et al., 1971b) suggested that this event was an example of gene amplification, no functional significance of this unscheduled DNA synthesis is known. Recently Cumins and Stockdale (1975) were unable to detect such phenomenon in the explants of mammary tissue of pregnant mice in culture medium containing insulin + prolactin + cortisol. However, prolactin induced preferential increase in GC-rich DNA synthesis was observed in pseudopregnant rabbit (see Banerjee, 1976).

During lactogenesis in the mid-pregnant mammary explants in culture medium, DNA synthesis and its relation to milk protein synthesis has been also investigated. The mid-pregnant mammary explants were found to reach a peak of DNA synthesis at 24 hours in a medium containing insulin (Stockdale et al., 1966; Turkington, 1968). Although addition of prolactin and cortisol did not alter the rate of DNA synthesis, the casein peak appeared at 48 hours in the medium containing all three hormones. When the explants were cultured in the presence of these hormones along with inhibitors which cause mitotic arrest or DNA synthesis inhibition at S-phase, synthesis of casein and α-lactalbumin was also blocked (Stockdale and Topper, 1966; Turkington and Topper, 1967; Mayne and Berry, 1970).

On the basis of these observations, the authors postulated that a final round of DNA synthesis or "critical mitosis" was essential for the functional differentiation of the mammary cells (Topper, 1968; Topper and Vonderhaar, 1974). However, Owens et al. (1973) showed that if the explants were exposed to the inhibitors, such as ara-C or FUdR which blocks DNA synthesis at the G_1 - S phase, the mammary cells could still produce casein. These authors believe that no "critical mitosis" or DNA synthesis in culture is required for the functional differentiation of the mid-pregnant mammary explants (Topper, 1968; Turkington, 1972). Mukherjee et al. (1973) have reported that mid-pregnant mammary explants represent cells initiated to the replicative process in vivo and insulin in culture permits completion of this process in vitro.

2. RNA and milk protein synthesis: It has been observed in several species that the RNA/DNA ratio increased at the onset of lactogenesis (Baldwin, 1966; Yanai and Nagasawa, 1971), and this increase was due to the stimulation of RNA synthesis from the beginning of lactation (Denamur, 1974). In C3H and Balb/C mice, ^{3}H-UR uptake in 5-day lactating mammary glands was ten fold higher than that in the virgins (Banerjee et al., 1971a). Sirakov and Rychlik (1968) have observed in strain H mice that ^{3}H-UR uptake was four times greater at 12 to 16 days of lactation when compared to 18 days of pregnancy. It has been reported also that adrenalectomy during lactation in mice caused a significant reduction in the rate of RNA synthesis as measured by ^{3}H-UR uptake (Banerjee et al., 1971a). Exogenous cortisol treatment, however, induced RNA synthesis in these adrenalectomized mice. This suggests that during the lactational

period cortisol alone or in synergism with other hormones regulates the rate of RNA synthesis in vivo.

An autoradiographic determination of ^{3}H-UR uptake in the lactating mammary cells showed a preferential increase of nucleolar RNA synthesis (Banerjee and Banerjee, 1971). A similar phenomenon has been also observed in the lactating mouse (Denamur, 1974). This suggests an increased rRNA synthesis in the lactating mouse. In this connection it should be mentioned that increased RER (rough endoplasmic reticulum) in lactating mouse mammary gland was totally abolished after adrenalectomy as judged by electron microscopy (Banerjee and Banerjee, 1971). This suggests that, adrenocorticosteroids not only induce rRNA synthesis in the adrenalectomized lactating mice but may also have some function in maintaining the RER system in the cell.

Casein constitutes nearly 80% of the proteins in milk and hormones needed for the induction and maintenance of this major milk protein is fairly well understood (Banerjee, 1976). Banerjee et al. (1971a) have shown that general protein synthesis in 10-day lactating C3H mice, as measured by ^{3}H-leucine uptake, decreased by about 50 and 70%, 1 and 3 days after adrenalectomy, respectively. When these mice were injected with cortisol, protein synthesis increased as function of time and 3 hours after the injection the increase was more than 100%. Determination of casein synthesis by Ca^{++}-rennin precipitable radioactivity, revealed that the inhibitory effect of adrenalectomy was more apparent on this milk protein and 3 hours after cortisol injection a 250% increase of casein synthesis was observed (Banerjee et al., 1971a). Although these studies indicate the positive role of cortisol on casein synthesis in the

mouse mammary gland in vivo, it is not possible to conclude whether the glucocorticoid acts independent of the endogenous prolactin.

Juergens et al. (1965) first demonstrated that casein synthesis could be induced in the explants of mid-pregnant mouse mammary glands when cultivated in a culture medium containing insulin, prolactin and cortisol. Lockwood et al. (1966) showed that, there was very little difference in the synthesis of α-lactalbumin and β-lactoglobulin in explants cultured in media containing insulin alone or with cortisol or prolactin. However, a two-fold increase of the milk protein synthesis was observed in medium containing all three hormones. After 48 hours of cultivation in a similar medium, a five-fold increase in casein synthesis was observed (Lockwood et al., 1966). Turkington (1972) reported that casein and α-lactalbumin synthesis could be induced in mammary explants cultured in medium containing insulin + cortisol or cortisol + prolactin or prolactin + insulin. However, maximum synthesis was seen only when all three hormones were present in the culture medium (Turkington, 1972). These results, therefore, indicate that both cortisol and prolactin are required for the synthesis of milk proteins in mammary explants. In this connection it is also interesting to note that in the explants of the mid-pregnant mammary gland, cortisol induced formation of the RER system which is needed for the storage of the secretory product of a cell (Mills and Topper, 1970).

Oka (1974) reported that spermidine mimics the lactogenic properties of cortisol in mouse mammary explants. The author also observed that cortisol or prolactin can induce increased spermine or spermidine synthesis when present along with insulin. Thus, it has been postulated that

the effect of cortisol actually is on the synthesis of these polyamines (Oka, 1974).

In recent years, most studies have centered around the hormonal regulation of casein synthesis at the molecular level. To pursue these studies a sensitive radioimmunoassay was used by several investigators (Feldman, 1974; Terry et al., 1975a, Enami and Nandi, 1977; Emerman et al., 1977). Using this technique it was observed that quantity of casein was similar in the late pregnant and lactating mouse mammary gland (Terry et al., 1975a). On the other hand, casein was undetectable in the estrogen + progesterone-primed virgin mouse mammary gland. Cultivation of these treated glands for 6 days in lobuloalveolar growth promoting medium (insulin + prolactin + growth hormone + ovarian steroids) failed to produce any detectable level of casein (Terry et al., 1977a). However, after lobuloalveolar growth, cultivation of the whole mammary gland in medium containing insulin + prolactin + cortisol (lactogenic medium) produced a very high level of casein (Terry et al., 1977a). Recently, Enami and Nandi (1977) have shown that virgin mouse mammary explants (induced to lobuloalveolar structures by 5-week treatment with ovarian steroid pellet) synthesize higher levels of casein in insulin + prolactin + cortisol supplemented medium on the 4th day in culture as compared to the medium containing insulin + prolactin or insulin + cortisol. The authors also showed by SDS-polyacrylamide gel electrophoresis, that all three components of casein were synthesized in this whole mammary gland organ culture. Similar effect of all three hormones was seen in dissociated mammary cells grown in a floating collagen gel culture system (Emerman et al., 1977). The same study also revealed that the mammary cells failed to show

hormone-induced casein synthesis when cultured in a dispersed condition in multi-well culture dishes which did not provide any contact with collagen gel like substances. These observations reiterate that both prolactin and cortisol are required for the synthesis of casein in the mammary gland. Furthermore, studies of Emerman et al. (1977) also indicated that the metabolic function of the mammary cells was dependent on their physical condition.

It is evident from the discussion in the preceding paragraph that intact mammary glands from virgin mice can be induced to undergo an in vivo like functional differentiation and secondly, the hormonal regulation of casein synthesis can be monitored by a sensitive radioimmunoassay. Using anti-casein serum it was possible to detect casein synthesized by mammary RNA in a cell-free translational system and this provided a tool to measure the level of casein mRNA, rather than the protein itself, in mammary glands (Gaye et al., 1973; Terry et al., 1975b; Rosen et al., 1975). Using these techniques it was demonstrated that adrenalectomy of lactating mice virtually abolished the casein mRNA (Terry et al., 1977b; Banerjee et al., 1977). A normal level of casein mRNA, however, could be maintained when the adrenalectomized animals were injected with cortisol. Casein mRNA in the mammary polysomes was found to be similarly influenced by the hormones. Terry et al. (1977a) have also shown that whole mammary gland contained a very high level of casein mRNA when cultured in a medium containing insulin + prolactin + cortisol after it had been induced to lobuloalveolar stage in the growth promoting medium. It is noteworthy to mention that casein mRNA in the mammary glands cultured for 6 days in the growth promoting medium was found to be very low. These