A SEARCH FOR PREFERENTIAL SYNTHESIS OF SPECIFIC NUCLEAR DNA'S DURING MAMMARY GLAND DEVELOPMENT

E.P. Cummins and Frank E. Stockdale
Departments of Medicine and Biology
Stanford University School of Medicine
Stanford, California 94305

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SUMMARY: The hormone-dependent differentiation of mammary gland epithelium of mature mice in vitro requires the synthesis of DNA. Only the daughter cells are capable of milk protein synthesis upon exposure to hormones. This coupling of cell proliferation to initiation of specific protein synthesis has suggested that gene amplification could be involved in the regulation of milk protein synthesis during lactation. It has been reported that DNA with a different buoyant density appears in the mammary gland at lactation in the mouse. The experiments reported here demonstrate that there is no change in the buoyant density of DNA in mammary epithelial cells either at lactation or during hormone-dependent differentiation of mammary tissue in tissue culture.

Augmented rates of milk protein synthesis have been demonstrated to be preceded by and dependent upon DNA synthesis in mammary tissue growing in vitro (1, 2). In this system it appears that cell division of mammary epithelium from nulligravida mature mice is responsible for the production of daughter cells capable of specific protein synthesis in response to prolactin and insulin. The possibility that gene amplification could be a factor in the apparent coupling between DNA synthesis and cell differentiation in this and other hormonally responsive systems is apparent and has been suggested. Banerjee and Wagner (3) have reported that as much as 1/3 of newly synthesized DNA in the lactating mammary gland of mice has a buoyant density distinctly greater than the main band or satellite DNA. This additional band is not seen in DNA from mammary gland of virgin or mid-pregnant mice. Prolactin locally applied to the mammary gland of the rabbit has also been reported to result in an increase in the specific activity of DNA on the more dense side of the main band DNA extracted from the gland after an injection of tritiated thymidine (4). A 1.8-fold increase in DNA capable of hybridization with r-RNA (an increase from 0.025% to 0.046% of the total DNA) has been demonstrated in human liver cells exposed in vitro to triiodothyronine (5). Because of the importance of the finding of Banerjee and Wagner to understanding the control of milk protein synthesis demonstrated in vitro, we attempted to confirm these studies and to extend them to mammary gland tissue undergoing hormone-dependent differentiation in vitro.

MATERIALS AND METHODS:

Animals and Cultures: Females of strain C3H and Balb/C were used in all studies. The animals were injected with 100 µCi of tritiated thymidine (specific activity 20 Ci/mM) one hour prior to sacrifice.

Extraction of DNA: Mammary gland tissue was removed and placed in cold homogenizing solution (Tris, 0.1 M pH 8; 0.1 N NaCl; 0.005 M EDTA). The tissue was minced and homogenized in teflon glass motor-driven homogenizer. The homogenate was made 1% with sodium lauryl sarcosine and shaken for five minutes. This solution was centrifuged for 10 minutes at 600 x g at 4° C, and the supernatant was made 1 M with sodium perchlorate. The solution was extracted three times with chloroform:isoamyl alcohol (24:1), and the aqueous phase was made 1 M with sodium acetate. Nucleic acids were collected on a glass rod after the addition of ethanol. This material was then dissolved in 1 x SSC and treated for two hours with ribonuclease (Worthington, 50 µgm/ml) followed by two hours with pre-digested pronase (Worthington, 50 µg/ml). This solution was then re-extracted three times with chloroform:isoamyl alcohol, the DNA precipitated with ethanol and finally dissolved in 1 x SSC.

Density Gradient Centrifugation: Aliquots of DNA extracts were added to recrystallized CsCl to give a refractive index of 1.4720. Neutral CsCl gradients were formed in a SW-40 rotor by centrifuging samples at 20° C at 39,000 RPM for 48 hours. The tubes were punctured from the bottom, and optical density tracings were automatically recorded at 2,540 Å on a Chromatronix Spectrometer. Fractions were collected and precipitated with 10% TCA on glass fiber filters. The filters were counted in a counting solution composed of toluene, PPO and POPOP (6).

Tissue Culture Methods: Tissue from mammary gland of mature non-pregnant and mid-pregnant mice was cut into explants and incubated in organ culture in Medium 199 as previously described (7, 8).

RESULTS: DNA was extracted from mammary gland tissue of non-pregnant (6-8 week old) female mice, 15-day pregnant females and 6-day lactating C3H mice. The DNA was centrifuged to equilibrium on cesium chloride, and the optical density tracing was obtained (Figure 1). As can be seen, the optical density profiles of the DNA for each of these animals were exactly the same. The same experiments were performed on Balb/C females on Day 1, 3, 5, 6 of lactation, and in each case the patterns were identical to those obtained from C3H females. Some animals were also injected intraperitoneally with tritiated thymidine one hour prior to sacrifice and the DNA extracted from their mammary glands. The radioactivity patterns obtained by fractionating the gradients were identical to the optical density tracings shown in Figure 1.

In an effort to induce different patterns of DNA synthesis in virgin or pregnant mice, C3H females were injected with 5 mg of ovine prolactin for three consecutive days. On the fourth day they were injected intraperitoneally with tritiated thymidine, and one hour later the DNA was extracted from their mammary glands. In each case the optical density tracing and the radioactive profile were identical to the saline-injected control animal DNA (Figure 2).

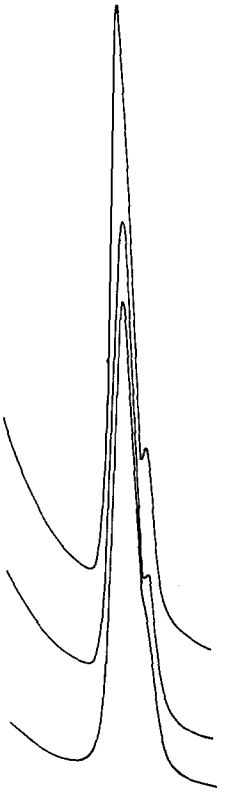

FIGURE 1. Optical density profiles of neutral cesium chloride gradients of DNA isolated from the mammary gland of C3H mice. (top) Six-day lactating mammary gland; (middle) 12-day pregnant mammary gland; (bottom) non-pregnant virgin (3 months of age) mammary gland. The top gradient contained 1.4 OD_{260}; the middle, 1.0 OD_{260}; and the bottom, 0.7 OD_{260} of DNA.

Using mammary gland tissue from mature non-pregnant or pregnant animals, organ culture experiments were performed to determine if the hormones required for initiation of milk protein synthesis changed the banding pattern of the DNA formed in vitro. It has been shown previously that the peak rate of DNA synthesis in response to hormones in vitro is reached at 24 hours of incubation for explants from pregnant animals and at 48 hours for explants from non-pregnant animals. Tritiated thymidine was added to such cultures from the 20-24th hour for explants from pregnant animals and from the 44-48th hour for tissues from non-pregnant animals. In some experiments the tissue was exposed for the entire culture period to tritiated thymidine. Tissues were incubated in the presence of insulin or prolactin alone; insulin plus prolactin; or insulin,

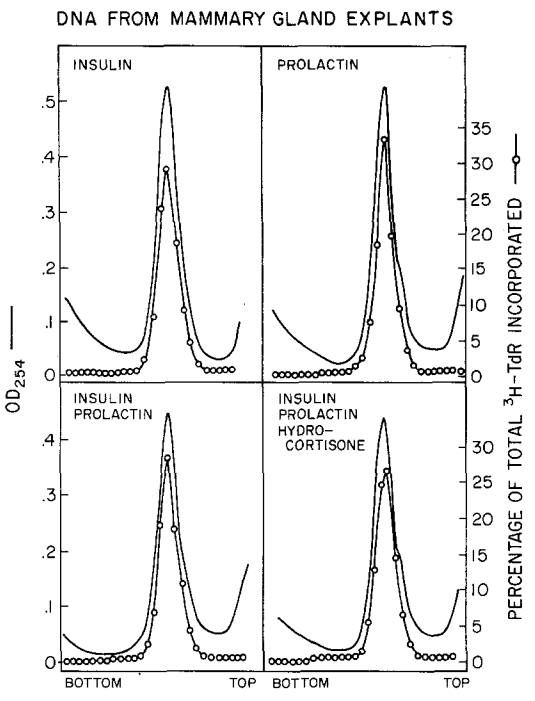

FIGURE 2. DNA synthesis in mammary gland explants of mid-pregnant mice. Explants were incubated for 48 hours in the presence of insulin; prolactin; insulin + prolactin; or insulin, prolactin and hydrocortisone. The DNA was extracted from these explants as indicated in Materials and Methods. The DNA was then centrifuged to equilibrium in cesium chloride, and fractions were collected from the bottom of the tube. Optical density was measured at 254, and tritiated thymidine incorporated during the last four hours of incubation (1 μC/ml) was determined by scintillation counting of each fraction.

prolactin, and hydrocortisone. Only the combination of insulin, hydrocortisone and prolactin results in increased milk protein synthesis. DNA extracted from either explants from mature non-pregnant females (not shown) or explants from mid-pregnant females, sedimented with identical patterns (Figure 3). Though the specific activity of the DNA is changed by hormonal addition in these culture experiments, there was not a selective increase in specific activity on the heavy side of the main band of the DNA nor did there emerge a new band of radioactive DNA.

DISCUSSION: Differentiation is frequently coupled with DNA synthesis and cell proliferation (1,9,10,11,12,13). In erythrogenesis there appears to be a pre-programmed series of cell cycles culminating in the fully differentiated red blood cell. In the chick oviduct and the mammary epithelium of mature

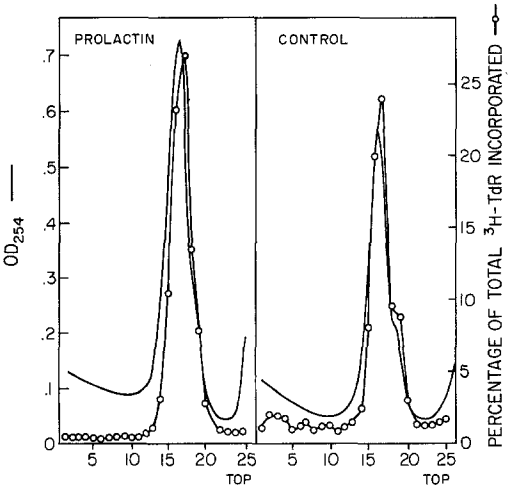

FIGURE 3. DNA synthesis in prolactin-injected animals. C3H animals were injected as indicated in Materials and Methods with prolactin (2 mg/day for 3 days), and the animals were then injected four hours prior to sacrifice with 100 μC of tritiated thymidine, and the DNA was extracted from their mammary glands and centrifuged to equilibrium in cesium chloride. Fractions were obtained from the bottom of the tube, and continuous optical density tracings were obtained. The incorporation of tritiated thymidine was determined in each fraction by scintillation counting.

female mice, hormonally induced cell proliferation also precedes specific protein synthesis. These observations can be interpreted in terms of a gene amplification model of cell differentiation. The work of Souleil and Panijel (12) on the appearance of DNA with a different buoyant density during antigen stimulated transformation of lymphocytes and the demonstration by Koch and Cruceanu (5) that liver cells contain additional copies of r-RNA following exposure to triiodothyronine are consistent with such a model.

In the most thoroughly studied example of gene amplification during cell differentiation, the amphibian oocyte, the amount of selective DNA synthesis can constitute as much as a third of the germinal vesicle DNA (14). The amplified r-RNA appears as a heavy band separate from main band DNA when DNA is extracted from X. laevis germinal vesicles and is centrifuged to equilibrium in cesium chloride. The studies reported here demonstrate that there is neither a change in the optical density pattern in cesium chloride gradients nor the pattern of tritiated thymidine incorporation during the growth of the mammary gland epithelium in pregnancy or lactation. Nor does one see a change in the buoyant density of DNA in a setting where hormone-

dependent differentiation is accompanied by DNA synthesis in mammary gland epithelium in vitro. These observations suggest that if amplification of specific DNA's occurs during development of the mammary gland, it either has the same density as main band DNA or it constitutes such a small amount of the total DNA (5) that it is not apparent in the gradient. To determine definitively whether selective DNA amplification does occur in the mammary gland system, one must await the isolation of m-RNA specific for milk proteins and its use in a quantitative study by RNA-DNA hybridization. There is no apparent reason for the discrepancy between our observation and those of Banerjee and Wagner (3).

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