# GENE AMPLIFICATION IN MAMMARY GLAND AT DIFFERENTIATION.

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SUMMARY: Mouse mammary gland DNA was analyzed by isopycnic density gradient centrifugation in CsCl. The buoyant density of the pre-existing and newly made DNA of the mammary glands of virgin, pregnant and weaned mice showed the characteristic major and minor-(satellite) components of mouse DNA. However, most of the radioactive DNA of early lactating gland equilibriated as slightly heavier (0.004 g/cm$^3$) than the major band of mouse DNA and this heavily labeled peak in 6 day lactating gland was associated with a shoulder on the optical density curve. The results indicate a periodic amplification of the mammary cell genome at differentiation. Similarity of the buoyant density of the heavy DNA peak with that of the G+C rich mouse DNA also suggests involvement of the ribosomal genes.

In post-partum females, the lobulo-alveolar structure of the mammary gland is the site of a series of complex metabolic events resulting in the secretion of a unique product, the milk. Earlier we reported that the functional differentiation of the breast tissue in mice is marked by sharply increased RNA synthesis prior to the onset of milk protein synthesis and the initial low DNA synthesis in the post-partum mammary gland suddenly rises to a peak on the 6th day followed by a rapid decline (1, 2, 3, 4). This abrupt rise of DNA synthesis in the early lactating gland, however, is not accompanied by a corresponding alteration of the mitotic activity. This non-mitotic DNA synthesis results in a 30% rise of nuclear DNA content in the lactating mammary cells. Similar increased nuclear DNA in the lactating cells has also been observed by Feulgen microspectrophotometric and chemical analysis of rabbit and rat mammary glands (5, 6). Since the lobulo-alveolar mouse mammary tissue is characterized by a diploid karyotype (7), the presence of the excess nuclear DNA in the lactating tissue thus suggests the occurence of a periodic amplification of a portion of the cellular genome at differentiation. This

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Abbreviations: SLS, sodium lauryl sarcosine; SSC, standard saline citrate.

report presents the results of the isopycnic banding (equilibrium centrifugation) patterns of mouse mammary gland DNA in CsCl density gradient.

MATERIALS AND METHODS:

Animals and isolation of DNA: Six week old nuliparous BALB/c mice were bred in our laboratory and mammary gland DNA was labeled in vivo by a single intraperitoneal injection of 100µCi $^{3}$H-thymidine (6.7Ci/mM, New England Nuclear Corp., Boston, Mass.) and 1 hour later mice were killed by cervical dislocation. Mammary glands were excised, freed of lymph nodes, frozen and stored at -20°C.

DNA was prepared according to a modification of Marmur's method (8). The minced mammary tissue was homogenized in a tris-HCl buffer (0.1M tris, 0.1 NaCl, 0.005 M EDTA; pH 8) and after adding 0.1 volume of 10% SLS, the homogenate was shaken for 5 min. The solution was then made 1M $NaClO_4$, 1 volume Sevag's solution (chloroform and isoamyl alcohol, 10:1 [v/v]) was added. The mixture was shaken for 45 min at room temperature and then centrifuged at 10,000 rpm for 10 min. The aqueous layer was removed and the extraction was repeated 3-6 times. After adding 0.1 vol 4M NaOAc to the solution, the nucleic acid was precipitated by cold ethanol (-20°C), removed by wrapping on a glass rod and dissolved overnight in 0.01 X SSC at 4°C. 0.1 vol 10 X SSC was then added and the solution was incubated at 37°C with RNase (20µg/ml) for 2 hours and then with pronase (50µg/ml, predigested) for another 2 hours. The solution was re-extracted 2-3 times as described above, DNA was precipitated by cold ethanol, collected, dissolved in 0.01 X SSC and stored at 4°C with a drop of $CHCl_3$.

Isopycnic banding in CsCl: Preparative, isopycnic centrifugation of mammary gland DNA in CsCl was done as previously described (9). Gradients for 40 rotor were prepared in a volume of 4.5ml by adding 4.3750 g optical grade CsCl (Harshaw Chemical Co.) to 3.4ml 0.01M tris buffer (pH 8.2). Refractive index was read in an Abbe-3L refractometer (Bausch and Lomb) at 25°C, densities were determined from standard tables and the initial density

range of the solution was 1.710-1.715g/cm$^3$. The tubes were centrifuged in a Spinco Model L-2 at 33,000 rpm for 60-65 hours at 25°C. Drop fractions were collected from the bottom and the optical density of each fraction was measured in a Beckman DB-GT recording spectrophotometer. Radioactivity of the DNA in the fractions was determined after $CCl_3COOH$ precipitation by the filter paper method (10) and the samples were counted in a Nuclear Chicago scintillation spectrometer (Unilux II).

RESULTS:

Figure 1 illustrates a typical optical density profile after isopycnic banding of the mammary gland DNA in a CsCl gradient. E. coli DNA used as a density marker was clearly resolved and the buoyant densities of the DNA samples were consistent with densities of E. coli (1.713) and the mouse (1.702) DNA as previously reported (11, 12). In the mammary tissue of virgin mice $^3$H-dT radioactivity was congruent with the optical density pattern of the DNA after centrifugation to equilibrium (Fig. 2). In the mid-pregnant animal both the major and the minor satellite components of mouse DNA (13, 14) were detectable in the optical density as well as in the corresponding radioactivity distribution of the fractions of the mammary gland DNA.

Buoyant density and radioactivity patterns of the DNA from lactating mammary gland are shown in Figs. 4a-4d. In the lactating gland the radioactivity distribution of the DNA was markedly different from that of the virgin and the pregnant mammary gland. When DNA from 5 day lactating tissue was centrifuged to equilibrium a slightly visible shoulder was present on the ascending arm of the optical density curve and the radioactive DNA resolved into distinct peaks, one was associated with the usual major DNA component and the second, heavily labeled peak was coincident with the shoulder in the heavier region of the gradient (Fig. 4a). In addition to the low radioactivity corresponding to the main DNA band a heavily labeled peak associated with a slightly heavier buoyant density region was also evident in the gradients

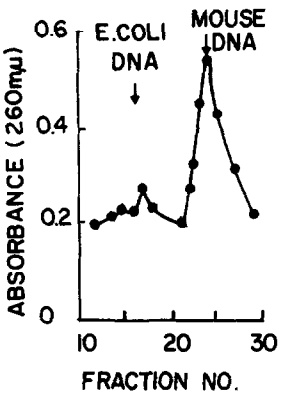



Fig. 1.

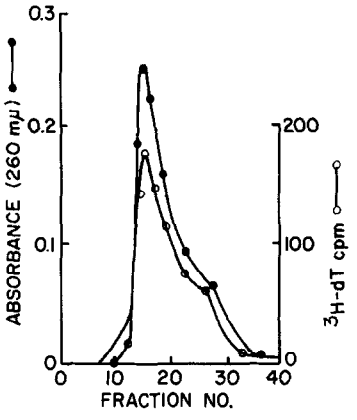



Fig. 2.

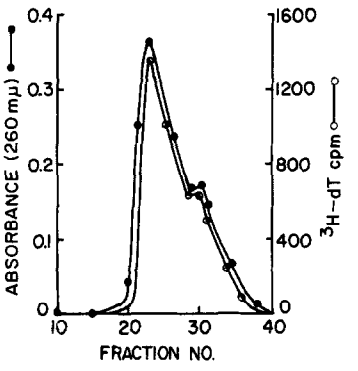



Fig. 3.

Figure 1. A mixture of ultraviolet absorption profiles, unlabeled E. coli DNA (Sigma) and mouse DNA (6 day lactating mammary gland) banded in CsCl. E. coli was used as a density marker. 50μg E. coli and 238μg mouse DNA.

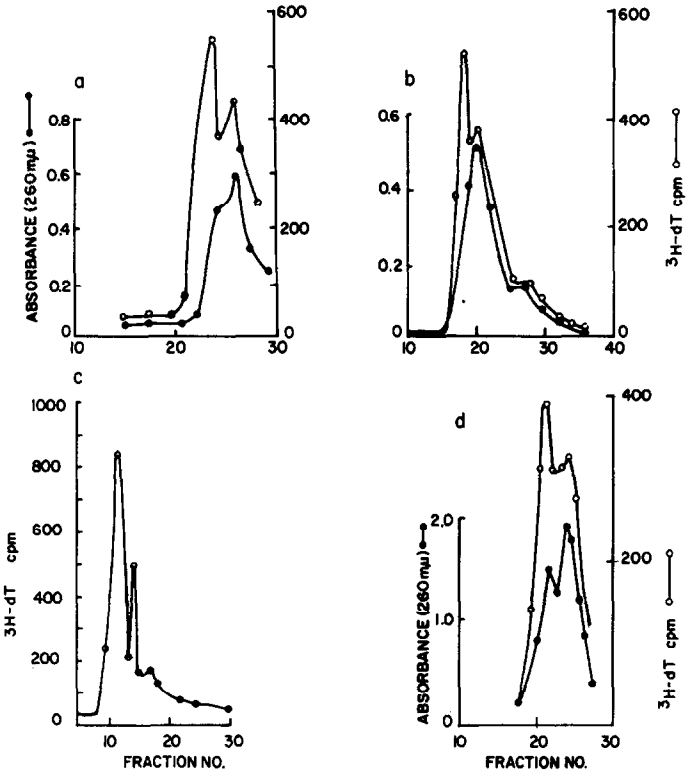
Figure 2. Buoyant density patterns of labeled DNA isolated from mammary glands of adult virgin mice. 57 μg DNA.

Figure 3. Buoyant density patterns of labeled DNA isolated from mammary glands from 12 day pregnant mice. 75 μg DNA.

containing 6 day lactating mammary gland DNA (Figs. 4b, 4c). When a gradient with an excess amount of 6 day lactating gland DNA was centrifuged to equilibrium a peak on the optical density curve, corresponding to the heavily labeled peak was detectable in the heavier buoyant density region of the gradient (Fig. 4d). Both the optical density and the radioactivity profiles of the DNA from involuted mammary gland failed to show an additional peak of higher buoyant density and the banding patterns of the DNA from this tissue were characteristically similar to that of the virgin mammary gland DNA (Fig. 5).

DISCUSSION:

The buoyant density of the mammary gland DNA from virgin, pregnant and weaned mice are in agreement with previous results on CsCl density gradient analysis of mouse DNA (9, 14). The increased $^3$H-dT incorporation




Figures 4a to 4d. Ultraviolet absorption and labeling patterns of DNA isolated from lactating mammary glands after centrifugation to equilibrium in CsCl. a) 5 day lactating, 362 μg DNA; b) 6 day lactating, gradient contained nuclear preparations (15), 142 μg; c) 6 day lactating, only radioactive profile, 153 μg; d) 6 day lactating gradient, 795 μg DNA.

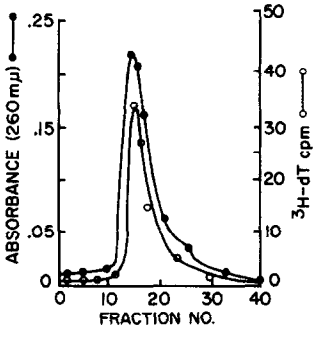



Figure 5. Buoyant density patterns of labeled DNA isolated from involuted mammary glands, 14 days after weaning, 80 μg DNA.

into DNA of mid-pregnant mammary tissue is consistent with the highly proliferative nature of this tissue.

The isopycnic banding patterns in CsCl of the newly made DNA in the lactating mammary cells on the other hand is markedly different and reveal some interesting phenomenon. The radioactivity in the lactating mammary cell DNA equilibriated as three distinct buoyant density fractions. Two of these were associated with the usual major and minor-(satellite) components of mouse DNA (14), the other most heavily labeled third peak corresponded to a slightly heavier buoyant density region. Since bulk of the radioactivity was present in this third heavier fraction of the newly made DNA the results indicate that a substantial portion of the lactating mammary cells active in DNA replication are preferentially synthesizing a type of DNA which has a buoyant density slightly higher than the average buoyant density of mouse DNA. The above interpretation is also supported by our earlier observations that most of the early lactating cells active in DNA synthesis do not proceed to mitosis and the $^{3}$H-dT specific activity is significantly lower than that of the mammary gland DNA of pregnant mice, although the labeling index in the two tissues is virtually identical (4). The reduced level of radioactivity associated with the major and the lighter satellite bands of the DNA may reflect the usual pre-mitotic DNA synthesis. A low level of 0.1-0.2% mitosis is evident in the mammary tissue throughout the period of lactation (4). The present results thus provide further evidence concerning the occurence of amplification of a portion of the cellular genome during functional differentiation of the mouse mammary gland and the fact that the extra DNA is not present in the involuted gland also suggest that the amplification may be periodic. The occurence of periodic "gene amplification" as a means of increasing the number of gene copies to support the high rate of RNA and protein synthesis in differentiated tissues has been postulated (16). It may be of interest to mention that the rapid rate of milk protein synthesis in the lactating mammary cells is accompanied by a selective rise of nucleolar RNA synthesis and membrane-bound ribosomes (1, 2).

Our preliminary estimates indicate that the density difference between the heavier and the usual major DNA band of lactating mammary cells is approximately 0.004 g/cm$^3$. In mouse, the G+C rich DNA has been indicated to have a similar buoyant density difference with the main DNA band (17) and this suggests that the amplified portion of the lactating cell DNA may represent the G+C rich sequences of the genome, hence possibly the ribosomal cistrons. However, more detailed studies such as analytical density gradient, molecular hybridization and base ratio analysis of the DNA will be needed for further elucidation of the molecular nature of the amplified portion of the genome. The possible influence of hormones on regulation of gene amplification as a mechanism of control on differentiation in endocrine target organs has been discussed (18) and knowledge about the role of the amplified DNA in regulation of milk protein synthesis in the mammary cells will be of much interest.

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