[CANCER RESEARCH 37, 3644–3647, October 1977]

# Regulation of Mammary Tumor Virus Production by Prolactin in BALB/cfC3H Mouse Normal Mammary Epithelial Cells *in Vitro*[1]

**Jason Yang,[2] J. Enami, and S. Nandi**

*Cancer Research Laboratory, University of California, Berkeley, California 94720*

## SUMMARY

Hormonal regulation of mammary tumor virus (MTV) production, has been analyzed with normal mammary epithelial cells from chronically infected BALB/cfC3H mice. The effect of prolactin in terms of increased MTV production was not reproducibly observed in cells cultured in tissue culture dishes, whereas the cells grown on floating collagen gels consistently responded to prolactin in a dose-dependent manner. Of the three media tested, Dulbecco's modified Eagle's medium was found to be the best in terms of responsiveness to prolactin and in maximal MTV production. Specificity studies with other pituitary and placental hormones in place of prolactin have shown that both growth hormones and human placental lactogen can replace prolactin, whereas follicle-stimulating hormone, luteinizing hormone, and thyrotrophin were ineffective. Contrary to the mammary tumor systems, where it has been shown that insulin and glucocorticoid can maximally stimulate MTV production, these hormones alone elicited only a small response in the absence of prolactin in normal mammary epithelial cells. Although prolactin alone had very little effect by itself, its presence was necessary (permissive effect) in order for the glucocorticoids to be able to maximally stimulate MTV production in normal cells.

## INTRODUCTION

Hormonal regulation of mouse mammary tumor virus expression *in vitro* has been studied, in the past, only in the mammary tumor epithelial cells both in primary monolayer cultures (4, 10, 12, 24) and cell lines (8, 18, 19). Similar studies with normal mammary epithelial cells from MTV[3] infected mice are lacking, however. We have recently analyzed the correlation between casein and MTV production with BALB/cfC3H mouse normal mammary epithelial cells grown in tissue culture dishes and on floating collagen gels.[4] The effect of prolactin in terms of increased MTV production was not reproducibly observed with the tissue culture dishes, but with the use of floating collagen gel, the normal mammary epithelial cells consistently responded to prolactin. This is in contrast to the mammary tumor epithelial cells, in which prolactin has been shown to have no effect (3, 12). In this paper, we extend our finding and present a detailed analysis of hormonal regulation of MTV expression in chronically infected normal mammary epithelial cells. The effect of prolactin in 3 different media, prolactin dose-response studies, and specificity studies with other pituitary and placental hormones in place of prolactin have been done. To our knowledge our report is the 1st detailed analysis of hormonal regulation of MTV production in normal mammary epithelial cells *in vitro*.

## MATERIALS AND METHODS

Mammary glands were obtained from either 10- to 13-day midpregnant BALB/cfC3H mice or 3- to 5-month-old virgin BALB/cfC3H mice. In order to stimulate the mammary gland of virgin BALB/cfC3H mice, each mouse was implanted s.c. for 4 to 5 weeks with a pellet containing 0.08 mg of 17$\beta$-estradiol, 20 mg of deoxycorticosterone, and 5 mg of cholesterol. This hormonal treatment results in lobuloalveolar development in virgin mouse comparable to the level seen in the mammary gland of midpregnant mice (6). Cell dissociation of normal mammary gland (7) and preparation of collagen solution and collagen gels (5, 13) have been previously described. The dissociated cells were plated on collagen gels in 35-mm Falcon plastic dishes at a seeding density of approximately $10^6$ cells/sq cm in DME with 10% fetal calf serum, 100 units of penicillin per ml, and 100 $\mu$g of streptomycin per ml. After 48 hr, various combinations of hormones in serum-free test medium (with antibiotics) were added, and the collagen gels were detached from the dishes.

MTV production was monitored by duplicate determinations (which differed by not more than 10%) of reverse transcriptase activity from approximately 6 ml of tissue culture fluid pooled from a 24-hr incubation period of triplicate 35-mm dishes for each hormone combination. The supernatant reverse transcriptase activity is a specific indicator of MTV production, as reported previously (4), by showing an association of the viral activity with physical, morphological, and immunological properties of MTV. Details of the assay have been recently described (4, 23). Assays were performed under conditions in which the incorporation of [$^3$H]dGTP was directly proportional to the concentration of

[1] This investigation was supported by Grants CA05388 and CA09041, awarded by the National Cancer Institute, Department of Health, Education and Welfare.

[2] To whom requests for reprints should be addressed.

[3] The abbreviatons used are: MTV, mammary tumor virus; DME, Dulbecco's modified Eagle's medium; TSH, thyrotrophin; FSH, follicle-stimulating hormone; LH, luteinizing hormone.

[4] J. Enami, J. Yang, and S. Nandi. Production of Casein and Mammary Tumor Virus in Mouse Mammary Epithelial Cells Grown on Collagen Gels, submitted for publication to *Proceedings of the National Academy of Sciences of the United States of America*.

Received March 21, 1977; accepted July 14, 1977.

MTV in the reaction mixture and to incubation time. A standard preparation of MTV, with a known incorporation of [$^3$H]dGTP, was assayed in parallel in order to compare relative MTV production among experiments. The presence of type C virus was monitored by replacing 10 mM $MgCl_2$ with 0.5 mM $MnCl_2$, and no evidence for significant type C virus production was noted.

Ovine prolactin (NIH-PS11), human growth hormone (NIH-HS201), ovine growth hormone (NIH-S11), bovine growth hormone (NIH-B17), and ovine TSH (NIH-S5) were gifts to Dr. H. Bern, University of California, Berkeley, Calif., from NIH. Human placental lactogen (catalogue 100712, 10t 3334) was supplied by Dr. Y. J. Topper, NIH, Bethesda, Md. Ovine FSH and LH were obtained from Dr. H. Papkoff, University of California, San Francisco, Calif. Powdered media (DME, Medium 199, and Waymouth MB 752/1) were obtained from Grand Island Biological Co., Grand Island, N. Y.

## RESULTS

MTV production was monitored in pelleted BALB/cfC3H mouse normal mammary epithelial cells cultured in tissue culture dishes and on floating collagen gels with 3 different media (DME, Medium 199, and Waymouth), with 3 hormone combinations: (*a*) insulin alone; (*b*) insulin and hydrocortisone; and (*c*) insulin, hydrocortisone, and prolactin. As shown in Chart 1, cells cultured on floating collagen gels produced more MTV than cells in tissue culture dishes regardless of the media used. The effect of prolactin in terms of increased MTV production was not reproducibly observed with the tissue culture dishes, but with the use of floating collagen gel, the normal mammary epithelial cells consistently responded to prolactin. However, the response to prolactin varied, depending on the media used in floating collagen gels. In general, the best response to prolactin was observed with DME and the least with Waymouth's medium. Despite this variation, addition of prolactin to insulin- and hydrocortisone-containing media consistently increased MTV production. As previously shown[4] and also in Chart 4, prolactin alone had very little effect. However, its presence seemed to allow maximal stimulation of MTV production by hydrocortisone (permissive effect).

Similar studies, in normal mammary epithelial cells from midpregnant BALB/cfC3H mice, were also performed in order to demonstrate that the mammary epithelial cells from both pelleted and midpregnant mice respond similarly to various hormone combinations in terms of MTV production, and therefore these cells can be used interchangeably. Chart 2 shows the time course of MTV production in normal mammary epithelial cells from midpregnant BALB/cfC3H mice on floating collagen gels with 3 different media, with 3 hormone combinations. Qualitatively similar results had been obtained in terms of responsiveness to prolactin in 3 different media.

These studies indicated that (*a*) mammary gland cells from midpregnant and pelleted mice behaved similarly, (*b*) cells cultured on floating collagen gels responded more consistently to prolactin and produced more MTV than the same cells cultured in tissue culture dishes regardless of the media, and (*c*) DME was the best medium in terms of prolactin responsiveness and maximal MTV production. Therefore, subsequent studies were performed on floating collagen gels with DME. Dose response of prolactin with

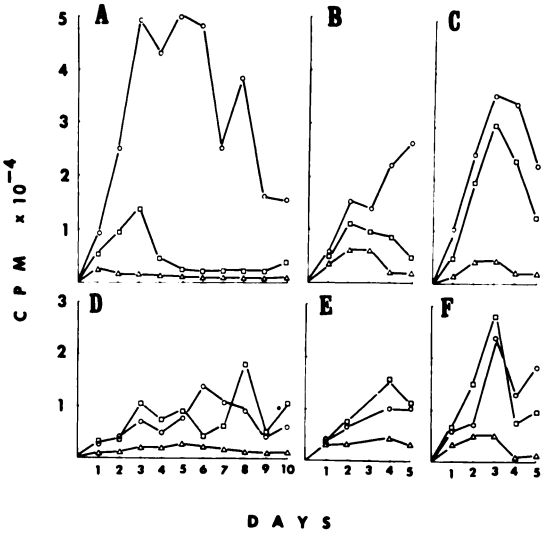


Chart 1. Time course of MTV production in mammary epithelial cells from pelleted BALB/cfC3H mice. Cells were cultured on floating collagen gels (*A* to *C*) and in tissue culture dishes (*D* to *F*) in the presence of 10 μg of insulin per ml (△), 10 μg of insulin per ml and 5 μg of hydrocortisone per ml (□), and 10 μg of insulin per ml, 5 μg of hydrocortisone per ml, and 10 μg of prolactin per ml (○). *A* and *D* had DME; *B* and *E* had Medium 199, and *C* and *F* had Waymouth's medium.

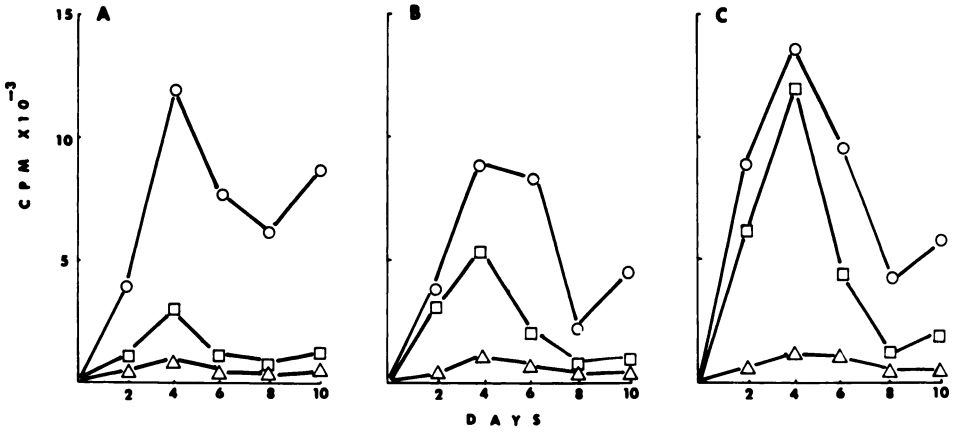


Chart 2. Time course of MTV production in mammary epithelial cells from midpregnant BALB/cfC3H mice. Cells were cultured on floating collagen gels in the presence of 10 μg of insulin per ml (△), 10 μg of insulin per ml and 5 μg of hydrocortisone per ml (□), and 10 μg of insulin per ml, 5 μg of hydrocortisone per ml, and 10 μg of prolactin per ml (○). *A* had DME; *B* had Medium 199; and *C* had Waymouth's medium.

these conditions is shown in Chart 3. When prolactin was added to insulin- and hydrocortisone-containing media, MTV production increased in a dose-dependent manner, with maximal production at doses around 5 to 10 μg/ml. Chart 4 shows the result of specificity studies in which prolactin was replaced by various pituitary and placental hormones. Among the pituitary hormones tested, both the growth hormones and human placental lactogen were able to mimic the prolactin effect. When both prolactin and growth hormone were added to insulin- and hydrocortisone-containing media, the stimulatory effect did not exceed that seen in cultures treated with prolactin, insulin, and hydrocortisone (data not shown). Among the growth hormones tested, bovine growth hormone gave the least response, although MTV production was substantially greater than that seen in insulin- and hydrocortisone-containing media. FSH, LH, and TSH were ineffective in replacing prolactin.

## DISCUSSION

We have analyzed the hormonal regulation of MTV production in normal mammary epithelial cells from both midpregnant and pelleted BALB/cfC3H mice. Cells from both sources responded similarly to the effect of prolactin in 3 different media with the floating collagen gels. Cells cultured on floating collagen gels consistently responded to prolactin and produced more MTV compared with the same cells cultured in tissue culture dishes. The central role of prolactin in normal mammary epithelial cells in terms of MTV production has been established, since addition of prolactin to insulin- and hydrocortisone-containing medium significantly increased MTV production. Although addition of hydrocortisone to medium containing insulin alone increased MTV production, a maximal stimulatory response is not fully expressed until prolactin is added. This is in contrast to the mammary tumor system, where insulin and glucocorticoid can maximally stimulate MTV production (4, 12). In fact, it has been reported that prolactin has no effect on BALB/cfC3H mouse mammary tumor cells in monolayer cultures (3, 12).

The nature of the stimulatory effect of prolactin in normal cells but not in the tumor cells is only speculative at this time. Since prolactin is believed to interact with the membrane receptors and alterations in membrane properties are believed to take place with transformation, it is quite possible that the receptors are available and functional only in the normal cells but not in the tumor cells. Consistent findings of prolactin effect in floating collagen gels compared with monolayers could be attributed to the exposure of a basal side more rich in prolactin receptors, which is not accessible on a tissue culture dish. In addition, since it is known that cell density is one of the factors controlling MTV production in mammary tumor cells in monolayers (4, 10, 23, 24), the shrinking of gels with time may contribute to changes in cell density which in turn may affect MTV production.

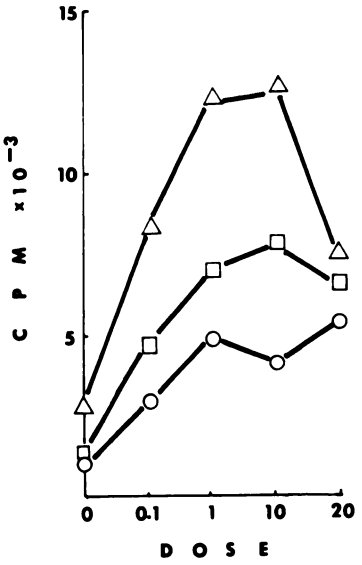


Chart 3. Dose response (μg/ml) of prolactin. MTV production was monitored in culture media collected on Days 2 (○), 5 (△), and 6 (□). All cultures contained 10 μg of insulin and 5 μg of hydrocortisone per ml.

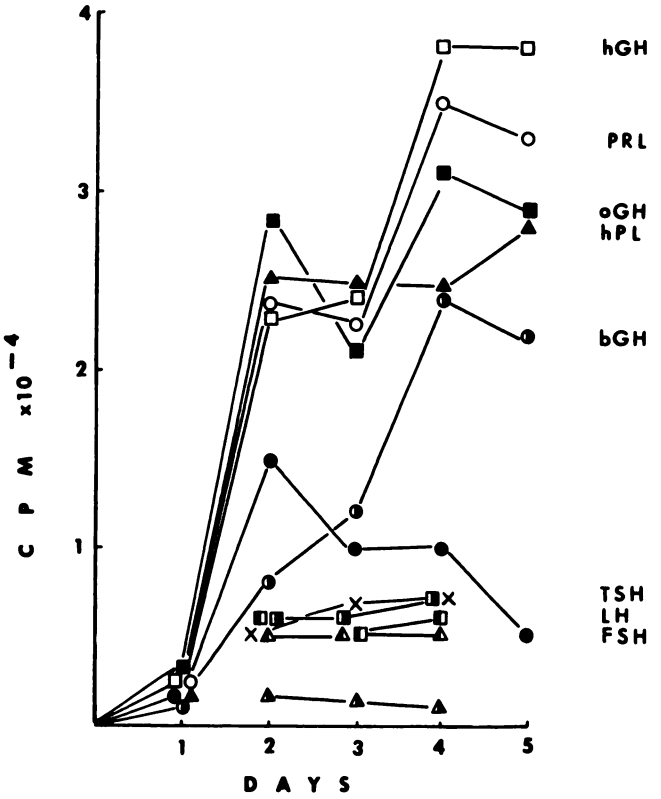


Chart 4. Time course of MTV production in the presence of various pituitary and placental hormones. Prolactin (10 μg/ml) (○) was replaced by 10-μg/ml amounts of various pituitary hormones as follows: human growth hormone (□), ovine growth hormone (■), bovine growth hormone (◑), human placental lactogen (▲), ovine TSH (◧), ovine FSH (△), and ovine LH (◨). All cultures also contained 10 μg of insulin per ml and 5 μg of hydrocortisone per ml. Also shown are cultures treated with 10 μg of insulin per ml (△), 10 μg of insulin and 5 μg of hydrocortisone per ml (●), and 10 μg of insulin per ml and 10 μg of prolactin per ml (×). Cumulative MTV production for 5 days indicate the following relative production as compared with prolactin (100%): human growth hormone (107%), ovine growth hormone (96%), bovine growth hormone (58%), human placental lactogen (88%), ovine TSH (21%), ovine FSH (19%), and ovine LH (22%). Insulin alone had 5%, insulin plus hydrocortisone had 36%, and insulin plus prolactin had 17% compared with cultures treated with all 3 hormones.

The reason for the difference in the magnitude of prolactin effect with different media is not known. The best response to prolactin was observed with DME and the least with Waymouth's medium. This may be partly due to the fact that MTV production was relatively high, even in the absence of prolactin using Waymouth's medium, whereas the production was low without the addition of prolactin in DME. It has been suggested that the biochemical action of prolactin involves the modulation of the action of other hormones, rather than acting directly by itself, often by controlling precursor pools (9). It is possible that 1 of the actions of prolactin in cells grown in DME may be to produce materials or its precursors necessary for enhanced MTV production. The same material may already be present in Waymouth's medium, thus explaining the high level of MTV production even without the addition of prolactin.

Dose-response studies of prolactin and specificity studies involving replacement of prolactin by other pituitary hormones have been performed with normal mammary epithelial cells from midpregnant BALB/cfC3H on floating collagen gel with DME. In the presence of insulin and hydrocortisone, cells responded to prolactin in a dose-dependent manner in terms of increased MTV production. This effect of prolactin can be mimicked by replacement of prolactin with growth hormones (human, ovine, and bovine) or human placental lactogen, but not with FSH, LH, or TSH. Human growth hormones and human placental lactogen are known to possess properties of prolactin in terms of biological activities and structural similarities (22). However, the possibility of prolactin contaminant in other growth hormone preparations used cannot be ruled out.

Since prolactin is an important factor in mouse mammary carcinogenesis (15) and is also important in the development and differentiation of the normal mammary gland (1, 14), it is of interest that prolactin is an absolute requirement for maximal production of MTV in normal cells but not in tumor cells. Although increased MTV expression *per se* is not responsible for noduligenesis nor tumorigenesis, there seems to be some correlation between the mammary tumor incidence in different strains and the MTV expression in milk (16, 17, 21) and in their tissues (16, 20). In addition, the concentration of MTV antigen determined by immunodiffusion assay (2) or radioimmunoassay (11) indicate that mice with a higher level of MTV expression have a greater tendency to develop mammary tumors.

## ACKNOWLEDGMENTS

We thank Dr. P. Bowman for critically reading the manuscript and John Underhill for photographic assistance.

## REFERENCES

1. Banerjee, M. R. Responses of Mammary Cells to Hormones. Intern. Rev. Cytol., *47:* 1–97, 1976.
2. Blair, P. B. Detection by Immunodiffusion of Mouse Mammary Tumor Virus in Milk Samples and Correlation with Tumor Development. Cancer Res., *29:* 745–748, 1969.
3. Cardiff, R. D., Young, L. J. T., and Ashley, R. L. Hormone Synergism in the *in vitro* Production of the Mouse Mammary Tumor Virus. J. Toxicol. Environ. Health *1* (Suppl.)*:* 117–129, 1976.
4. Dickson, C., Haslam, S., and Nandi, S. Conditions for Optimal MTV Synthesis *in vitro* and the Effect of Steroid Hormones on Virus Production. Virology, *62:* 242–252, 1974.
5. Elsdale, T., and Bard, J. Collagen Substrata for Studies on Cell Behavior. J. Cell Biol., *54:* 626–637, 1972.
6. Enami, J. Hormonal Control of Milk Protein Synthesis in Mouse Mammary Epithelial Cells *in vitro*. Ph.D. Dissertation, University of California, Berkeley, Calif., 1977.
7. Enami, J., Nandi, S., and Haslam, S. Production of Three-dimensional Structures—"Domes"—from Dissociated Mammary Epithelial Cells. *In Vitro*, *8:* 405, 1973.
8. Fine, D. L., Plowman, J. K., Kelly, S. P., Arthur, L. O., and Hillman, E. A. Enhanced Production of Mouse Mammary Tumor Virus in Dexamethasone-treated 5-iododeoxyuridine-stimulated Mammary Tumor Cell Culture. J. Natl. Cancer Inst., *52:* 1881–1886, 1974.
9. Horrobin, D. E. Prolactin: Physiology and Clinical Significance, pp. 13–21. Montreal: MTP Medical and Technical Publishing Co., 1973.
10. Kimball, P. C., Boehm-Truitt, M., Schochetman, G., and Schlom, J. Characterization of Mouse Mammary Tumor Viruses from Primary Tumor Cell Cultures. I. Immunologic and Structural Studies. J. Natl. Cancer Inst., *56:* 111–117, 1976.
11. LoGerfo, P., Silverstein, G., and Charney, J. Radioimmunoassay for Mouse Mammary Tumor Virus-associated Antigen. Surgery, *76:* 16–22, 1974.
12. McGrath, C. M. Replication of Mammary Tumor Virus in Tumor Cell Cultures: Dependence on Hormone-induced Cellular Organization. J. Natl. Cancer Inst., *47:* 455–467, 1971.
13. Michalopoulos, G., and Pitot, H. C. Primary Culture on Parenchymal Liver Cells on Collagen Membranes. Exptl. Cell Res., *94:* 70–78, 1975.
14. Nandi, S., and Bern, H. A. The Hormones Responsible for Lactogenesis BALB/cCrgl Mice. Gen. Comp. Endocrinol., *1:* 195–210, 1961.
15. Nandi, S., and McGrath, C. M. Mammary Neoplasia in Mice. Advan. Cancer Res., *17:* 353–414, 1973.
16. Noon, M. C., Wolford, R. G., and Parks, W. P. Expression of Mouse Mammary Tumor Viral Polypeptides in Milks and Tissues. J. Immunol., *115:* 653–658, 1975.
17. Parks, W. P., Howk, R. S., Scolnick, E. M., Oroszland, S., and Gilden, R. V. Immunochemical Characterization of Two Major Polypeptides from Mouse Mammary Tumor Virus. J. Virol., *13:* 1200–1210, 1974.
18. Parks, W. P., Ransom, J. C., Young, H. A., and Scolnick, E. M. Mammary Tumor Virus Induction by Glucocorticoids. Characterization of Specific Transcriptional Regulation. J. Biol. Chem., *250:* 3330–3336, 1975.
19. Ringold, G., Lasfargues, E. Y., Bishop, J. M., and Varmus, H. E. Production of Mouse Mammary Tumor Virus by Cultured Cells in the Absence and Presence of Hormones: Assay by Molecular Hybridization. Virology, *65:* 135–147, 1975.
20. Varmus, H. E., Quintrell, N., Mederiros, E., Bishop, J. M., Nowinski, R. C., and Sarkar, N. H. Transcription of Mouse Mammary Tumor Virus Genes in Tissues from High and Low Tumor Incidence Mouse Strains. J. Mol. Biol., *79:* 663–679, 1973.
21. Verstraeten, A. A., van Nie, R., Kwa, H. G., and Hageman, P. C. Quantitative Estimation of Mouse Mammary Tumor Virus Antigens by Radioimmunoassay. Intern. J. Cancer, *15:* 270–281, 1975.
22. Wallis, M. The Molecular Evolution of Pituitary Hormones. Biol. Rev., *50:* 35–98, 1975.
23. Yang, J., and Nandi, S. Cyclic AMP Regulation of MTV Production. J. Virol., *21:* 815–819, 1977.
24. Young, L. J. T., Cardiff, R. D., and Ashley, R. L. Long-term Primary Culture of Mouse Mammary Tumor Cells: Production of Virus. J. Natl. Cancer Inst., *54:* 1215–1221, 1975.

# Regulation of Mammary Tumor Virus Production by Prolactin in BALB/cfC3H Mouse Normal Mammary Epithelial Cells *in Vitro*

Jason Yang, J. Enami and S. Nandi

*Cancer Res* 1977;37:3644-3647.