AD_____

GRANT NO: DAMD17-94-J-4452

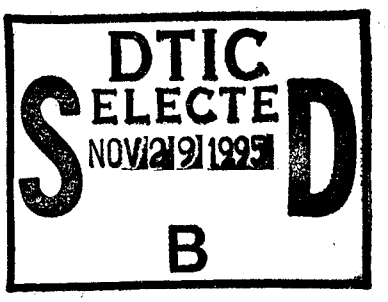
TITLE: Role of Mammary Prolactin in Carcinogenesis

PRINCIPAL INVESTIGATOR(S): Nira Ben-Jonathan

CONTRACTING ORGANIZATION: University of Cincinnati
Cincinnati, Ohio 45267-0553

REPORT DATE: October 1995

TYPE OF REPORT: Annual



PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1995	Annual 15 Sep 94 - 14 Sep 95

4. TITLE AND SUBTITLE
Role of Mammary Prolactin in Carcinogenesis

5. FUNDING NUMBERS
DAMD17-94-J-4452

6. AUTHOR(S)
Dr. Nira Ben-Jonathan

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Cincinnati
Cincinnati, Ohio 45267-0553

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

Prolactin (PRL) is a mitogenic hormone that shares characteristics with growth factors. Recent reports that rat mammary tissue expresses PRL mRNA led us to hypothesize that PRL acts as an autocrine/paracrine growth factor in the mammary gland, and may be a determinant in mammary carcinogenesis. To examine this, mammary tumors were induced in rats by injecting the carcinogen nitrosomethylurea (NMU). *In vitro* studies utilized an NMU-derived mammary tumor cell line. The mammary tumors and the cell line express mRNA for both PRL and PRL receptor, as assessed by reverse transcriptase (RT)-PCR. Immunoreactive PRL was detected in the NMU-induced tumors. NMU cells were cultured with PRL antiserum. The PRL antiserum inhibited cell proliferation 40-50% as compared to normal rabbit serum (NRS) or GH antiserum. In summary, we have shown that NMU-induced mammary tumors express mRNA for PRL and PRL receptor. The addition of PRL antiserum to cultured NMU cells inhibited their growth. We propose that PRL may be acting as a local growth factor that stimulates proliferation of mammary tumors.

14. SUBJECT TERMS
rat mammary tumors, prolactin, prolactin receptor, cell proliferation, carcinogen
breast cancer

15. NUMBER OF PAGES
11

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

GENERAL INSTRUCTIONS FOR COMPLETING SF 298

The Report Documentation Page (RDP) is used in announcing and cataloging reports. It is important that this information be consistent with the rest of the report, particularly the cover and title page. Instructions for filling in each block of the form follow. It is important to ***stay within the lines*** to meet ***optical scanning requirements***.

Block 1. Agency Use Only *(Leave blank)*.

Block 2. Report Date. Full publication date including day, month, and year, if available (e.g. 1 Jan 88). Must cite at least the year.

Block 3. Type of Report and Dates Covered. State whether report is interim, final, etc. If applicable, enter inclusive report dates (e.g. 10 Jun 87 - 30 Jun 88).

Block 4. Title and Subtitle. A title is taken from the part of the report that provides the most meaningful and complete information. When a report is prepared in more than one volume, repeat the primary title, add volume number, and include subtitle for the specific volume. On classified documents enter the title classification in parentheses.

Block 5. Funding Numbers. To include contract and grant numbers; may include program element number(s), project number(s), task number(s), and work unit number(s). Use the following labels:

C	- Contract	PR	- Project
G	- Grant	TA	- Task
PE	- Program Element	WU	- Work Unit Accession No.

Block 6. Author(s). Name(s) of person(s) responsible for writing the report, performing the research, or credited with the content of the report. If editor or compiler, this should follow the name(s).

Block 7. Performing Organization Name(s) and Address(es). Self-explanatory.

Block 8. Performing Organization Report Number. Enter the unique alphanumeric report number(s) assigned by the organization performing the report.

Block 9. Sponsoring/Monitoring Agency Name(s) and Address(es). Self-explanatory.

Block 10. Sponsoring/Monitoring Agency Report Number. *(If known)*

Block 11. Supplementary Notes. Enter information not included elsewhere such as: Prepared in cooperation with...; Trans. of...; To be published in.... When a report is revised, include a statement whether the new report supersedes or supplements the older report.

Block 12a. Distribution/Availability Statement. Denotes public availability or limitations. Cite any availability to the public. Enter additional limitations or special markings in all capitals (e.g. NOFORN, REL, ITAR).

- **DOD** - See DoDD 5230.24, "Distribution Statements on Technical Documents."
- **DOE** - See authorities.
- **NASA** - See Handbook NHB 2200.2.
- **NTIS** - Leave blank.

Block 12b. Distribution Code.

- **DOD** - Leave blank.
- **DOE** - Enter DOE distribution categories from the Standard Distribution for Unclassified Scientific and Technical Reports.
- **NASA** - Leave blank.
- **NTIS** - Leave blank.

Block 13. Abstract. Include a brief *(Maximum 200 words)* factual summary of the most significant information contained in the report.

Block 14. Subject Terms. Keywords or phrases identifying major subjects in the report.

Block 15. Number of Pages. Enter the total number of pages.

Block 16. Price Code. Enter appropriate price code *(NTIS only)*.

Blocks 17. - 19. Security Classifications. Self-explanatory. Enter U.S. Security Classification in accordance with U.S. Security Regulations (i.e., UNCLASSIFIED). If form contains classified information, stamp classification on the top and bottom of the page.

Block 20. Limitation of Abstract. This block must be completed to assign a limitation to the abstract. Enter either UL (unlimited) or SAR (same as report). An entry in this block is necessary if the abstract is to be limited. If blank, the abstract is assumed to be unlimited.

*U.S.GPO:1993-0-358-779

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

NBJ Where copyrighted material is quoted, permission has been obtained to use such material.

NBJ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

NBJ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

NBJ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

NBJ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

NBJ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

NBJ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

NBJ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories. ...

Nika Ben-Jonath 10/1/95

PI - Signature Date

Table of Contents:

INTRODUCTION:

Prolactin (PRL) is a pituitary polypeptide hormone whose classic target is the mammary gland. The mitogenic effects of PRL in the mammary gland support a role for the hormone in mammary carcinogenesis. However, circulating PRL levels correlate poorly with the incidence and severity of breast cancer, and treatments that suppress pituitary PRL release have not improved outcome (1). Consequently, PRL has been largely disregarded as a significant determinant in breast cancer. The recent demonstration that mammary tissue produces PRL (2-5) raises the possibility that PRL acts as a locally produced growth factor, exerting paracrine or autocrine effects, independent of its circulating levels.

Growth factors are emerging as important mediators of carcinogenesis and have been implicated in malignant transformation, tumor cell proliferation and tumor progression. PRL shares several characteristics with growth factors, including multiple extrapituitary sites of synthesis, wide distribution of receptors, homology of the PRL receptor to those of the cytokine/hematopoietic growth factor family, similarities in signal transduction pathways, and mitogenic and morphogenic actions. Indeed, PRL has been shown to act as an autocrine growth factor in two cell types, the somatomammotroph (GH_3) cell line (6) and lymphocytes (7).

In this study, we examined the hypothesis that PRL is produced by mammary tumors and acts as a local mitogen involved in tumorigenesis. For an *in vivo* model, we used rat mammary tumors induced by the carcinogen nitrosomethylurea (NMU; 8). As an *in vitro* model, we used an NMU-derived rat mammary tumor cell line. The objectives were to: a) demonstrate local expression of both PRL and PRL receptor, and b) determine whether local PRL affects proliferation of mammary tumor cells.

BODY:

Materials and Methods

Animals

Forty-five day old female Fischer 344 rats were given two i.v. injections, one week apart, of NMU at 5 mg/100g BW. Mammary tumors developed within 80-100 days. Rats with large tumors (2-3 cm in diameter) were either sacrificed or were ovariectomized (OVEX) and sacrificed 10 days later. Tumors were removed and stored at -70C until use. Mammary tissue from lactating rats (1-2 days postpartum) served as controls. All experiments were approved by the University of Cincinnati Institutional Animal Care and Use Committee.

RNA isolation and RT-PCR

Tissues were homogenized in guanidinium thiocyanate buffer and total RNA was isolated by phenol/chloroform extraction. Reverse transcription of 1μg RNA was performed using Superscript reverse transcriptase primed with oligo-dT and then subjected to PCR, using the following primers. For rPRL, the 5' primer, located in the 3rd exon, had the sequence 5'-GATCGTGAGTTTATTGCCAAGGCC-3' (nucleotides 268 to 291) and the 3' primer, located in the 5th exon, was 5'CTTGCAGGGATGGGAGTTGTGACC-3' (nucleotides 578 to 601), with a

predicted product size of 334bp. For the rPRL receptor, 2 sets of primers were used to detect the long or short isoform. The upstream primer for both, 5'-ATGGATACTGGAGTAGATGGAGCC -3', was derived from a sequence in the region common to the two receptors (nucleotides 620 to 643). Utilizing downstream primers specific to the long (5' -CCAGAGTCACTGTCGGGATC TAAG-3', (nucleotides 1011 to 1034), or short (5'-GAGGCTCCTATTTGAGTCTGCAGC-3', (nucleotides 935 to 958) forms, yielded product sizes of 415 and 339bp, respectively. After denaturation at 95C for 5min, the reaction was carried as follows: 94C for 30s, 65C for 15s, and 72C for 45s. The PCR products were visualized on ethidium bromide-stained agarose gels. For Southern analysis, PCR products were transferred to nylon membranes and hybridized to cDNA probes, random primer labelled with ^{32}P-dCTP. Each experiment was repeated 3 times.

PRL Immunoreactivity

Tissues were homogenized in 1N acetic acid, centrifuged at 10,000xg for 10 min and the supernatants dialyzed overnight and lyophilized. Samples were reconstituted in RIA assay buffer and analyzed in serial 2-fold dilutions. Rat PRL RIA was performed using reagents obtained from the NIDDK hormone distribution program, with an assay sensitivity of 50 pg/tube. Intra- and inter-assay coefficients of variation were 7% and 9%, respectively.

Cell culture and cell proliferation assay

NMU cells were obtained from ATTC and cultured in DMEM containing 10% fetal bovine serum (FBS). Before an experiment, cells were incubated for 24h in DMEM/1% FBS, detached by trypsinization and plated at a density of 2 to 16 X10^3 cells/well. After confirming cell attachment, the cells were incubated with dialyzed antisera to rPRL (NIDDK, IC4) or rGH (NIDDK, IC1), or with dialyzed NRS at a dilution of 1:300. After 48h, relative cell density was determined by the MTT assay. In this assay, cell number is proportional to optical density at 570 nm. All data are presented as mean ± SEM; significant differences were calculated by two-way analysis of variance with Duncan's multiple range test. Each experiment was performed 3 times.

Results

Detection of PRL in mammary tissue

PRL transcript was detected in the lactating mammary gland, NMU-induced mammary tumors, and the NMU cell line (Fig. 1, upper panel). Products of the expected size were seen, and confirmed by Southern hybridization to a rPRL cDNA probe (Fig. 1, lower panel). The mammary and tumor PRL PCR products were sequenced and found identical to pituitary PRL. Interestingly, an additional band of approximately 210bp was seen on the Southern blot in the tumor, but not in the lactating mammary tissue. OVEX resulted in a marked decrease (>50%) in the size of the tumors studied. However, the effect of OVEX on the expression of PRL was neither large nor consistent (Fig. 1). Immunoreactive PRL was detected in the mammary tumors (Fig. 2). Homogenates of tumor tissues contained an average of 0.3 ± 0.3 ng/mg protein. The dilution curve of mammary tumor PRL was parallel to the standard curve.

Expression of the PRL receptor by mammary tissue

All mammary tissues expressed mRNA for the PRL receptor, including the long and short isoforms (Fig. 3, upper panel). The PCR products were of the expected sizes, and hybridized

strongly to a cDNA probe common to the two isoforms (Fig. 3, lower panel). A predominance of the long form was seen in the lactating mammary tissue, whereas equal amounts of the two isoforms were observed in the tumors. The short form could not be detected in the NMU cell line (Fig. 3); although a PCR product of greater than expected size was seen, it did not hybridize on the Southern analysis and was considered an artifact. OVEX had no consistent effect on the expression of the PRL receptor, nor on the relative amounts of the two isoforms in the tumors. An additional, smaller PCR product was seen in the reaction for the long form of the receptor in the tumor, which also hybridized to the PRL receptor probe; this product was not seen in the lactating mammary tissue.

Suppression of NMU cell proliferation by PRL antisera

The addition of rPRL antiserum to NMU cells markedly inhibited their proliferation, compared to cells incubated with either NRS or rGH antiserum (Fig. 4). This inhibitory effect was dependent on cell density, resulting in 65-70% suppression at the low initial cell density and 35-40% at the higher density. This inhibition was statistically significant at all initial cell densities ($P<0.05$). It is of interest that such marked inhibitory effect was not seen with two other PRL antisera, i.e., IC5 from NIDDK or an antiserum produced by this laboratory (data not shown). Exogenous rPRL did not reverse the effect of the PRL antiserum, and caused only a modest stimulatory effect (20-25%) on NMU cell growth in the absence of PRL antisera (data not shown).

Discussion

Expression of PRL mRNA in rat mammary tissues was recently demonstrated by Northern analysis (2,5), RT-PCR (3,5), and *in situ* hybridization (2). We have extended these findings to both NMU-induced mammary tumors and a cell line derived from such tumors. The demonstration of detectable levels of immunoreactive PRL further indicate that the message is translated into protein. This is supported by the finding of PRL mRNA in the polysomal fraction of ovine mammary tissue (5). The concentration of immunoreactive PRL in the tumor (0.3 ng/mg protein), while significantly lower than that of pituitary PRL, is similar in magnitude to that reported in the brain, and could reach significant high concentration in the vicinity of responsive cells. It cannot be excluded that the PRL present in mammary tissues is derived, in part, from non-mammary cells, e.g. lymphocytes. However, the localization of PRL mRNA to the mammary epithelium by *in situ* hybridization (2), together with our finding of PRL expression by the NMU cell line, argue against lymphocytes being the major source of mammary PRL.

The rat mammary tissue expresses both short and long forms of the PRL receptor (Fig. 3). Since the difference between the two isoforms lies in the intracellular domain of the receptor, they could be linked to different signal transduction pathways. Predominant expression of the long form of the receptor by normal mammary gland has been previously reported (9). Our results indicate that the NMU-induced tumors express approximately equal amounts of the two receptors. The physiological significance of an altered ratio of the two receptors in the tumor is yet unknown. Similarly, the presence of a PRL receptor variant in the tumor, as suggested by the additional hybridizing band in the PCR reaction for the long form of the receptor, may be important. Indeed, a mutation of the PRL receptor, due to a large deletion in the intracellular domain, appears to confer PRL-dependent growth on the NB_2 lymphoma cell line (10).

Ovariectomy is known to result in a marked decrease in the size of the majority of NMU-induced tumors (8), an effect also seen in the present studies. However, both PRL and PRL receptor transcripts remained easily detectable after OVEX, suggesting that estrogen is not required for their basal expression in the rat mammary gland. Two laboratories have examined the effect of OVEX on PRL receptors in NMU-induced mammary tumors using ligand binding analysis (11, 12). Both found a modest (25-30%) decrease in binding in the OVEX tumors. Additional, more quantitative, experiments would be necessary to resolve this issue in the NMU tumors.

To examine whether local PRL acts as a mitogen in the mammary tumor, we added PRL antiserum to NMU cells cultured under serum-free conditions. The marked inhibition of cell proliferation suggests that endogenous PRL contributes to the maintenance of basal cell growth of this mammary tumor cell line. An autocrine or paracrine effect of PRL on cell proliferation has previously been demonstrated in pituitary cells (6) and lymphocytes (7). The addition of exogenous PRL did not reverse the inhibitory effect of the antibody in the NMU cell line. This was also seen in the GH_3 cells, and was apparently due to the presence of different PRL isoforms in the reference preparation with opposing effects on cell proliferation (6).

We are puzzled by the finding that the IC4 antiserum, but not two other PRL antisera, inhibits proliferation of NMU cells. Cell growth was inhibited by the IC4 antiserum after extensive dialysis and by the IgG fraction following ammonium sulfate precipitation. It is of interest that variable effects of different PRL antisera on cell proliferation were also reported for lymphocytes (7) and GH_3 cells (6). Together, these findings suggest that only certain PRL isoforms might act as mitogens. Given the large number of PRL isoforms and their diverse biological effects, it is possible that the NMU cells are producing a specific PRL isoform which is recognized by IC4 but not the other antisera.

CONCLUSIONS:

We have demonstrated that rat mammary tissues, including mammary tumors, express PRL messenger RNA and contain immunoreactive PRL. Mammary tumors, but not lactating mammary tissue, appear to express a smaller PRL variant. In addition, these tissues express PRL receptor mRNA, including both the long and short isoforms. The NMU-induced tumors also may express PRL receptor variants, smaller than the expected size. The addition of PRL antiserum to cultures of an NMU cell line markedly inhibited cell proliferation. This effect was not seen with either NRS or GH antiserum.

Whereas the expression of PRL by mammary tissue is now well established, previous reports have not addressed the possible function(s) of local PRL. Preliminary evidence is provided here that locally produced PRL may act as a growth factor in the mammary gland. These autocrine/paracrine actions of PRL in mammary tissue could bear important implications to the field of breast cancer. The presence of a locally produced hormone, acting as a growth factor in the mammary gland, raises the possibility that disturbances in its regulation, such as overexpression or mutation of PRL or its receptor, promote malignancy or enhance its progression. These effects are now well documented for other growth factors in the breast, including EGF, TGF-α, and c-erbB-2.

REFERENCES:

1. Fernandes PA 1992 Prolactin physiology in the etiology of breast cancer. Sem Rep Endocrinol 10:258-265
2. Steinmetz RW, Grant AL, Malven PV 1993 Transcription of prolactin gene in milk secretory cells of the rat mammary gland. J Endocr 136:271-276
3. Kurtz A, Bristol LA, Toth BE, Lazar-wesley E, Takacs L, Kacsoh B 1993 Mammary epithelial cells of lactating rat express prolactin messenger ribonucleic acid. Biol Reprod 48:1095-1103
4. Fields K, Kulig E, Lloyd RV 1993 Detection of prolactin messenger RNA in mammary and other normal and neoplastic tissues by polymerase chain reaction. Lab Invest 68:354-360
5. Le Provost F, Leroux C, Martin P, Gaye P, Djiane J 1994 Prolactin gene expression in ovine and caprine mammary gland. Neuroendocrinology 60:305-31
6. Krown KA, Wang Y-F, Ho TWC, Kelly PA, Walker AM 1992 Prolactin isoform 2 as an autocrine growth factor for GH3 cells. Endocrinology 131:595-602
7. Hartmann DP, Holoday JW, Bernton EW 1989 Inhibition of lymphocyte proliferation by antibodies to prolactin. FASEB J 3:2194-2202
8. Manni A, Rainieri J, Arafah BM, Finegan HM, Pearson OH 1982 Role of estrogen and prolactin in the growth and receptor levels of N-Nitrosomethylurea-induced rat mammary tumors. Cancer Res 42:3492-3495
9. Ouhtit A, Morel G, Kelly PA 1993 Visualization of gene expression of short and long forms of prolactin receptor in rat reproductive tissues. Biol Reprod 49:528-536
10. Ali S, Pellegrini I, Kelly PA 1991 A prolactin-dependent immune cell line (Nb2) expresses a mutant form of prolactin receptor. J Biol Chem 266:20110-20117
11. Arafah BM, Gullino PM, Manni A, Pearson OH 1980 Effect of ovariectomy on hormone receptors and growth of N-nitrosomethylurea-induced mammary tumors in the rat. Cancer Res 40:4628-4630
12. Turcot-Lemay L, Kelly PA 1980 Characterization of estradiol, progesterone, and prolactin receptors in nitrosomethylurea-induced mammary tumors and effect of antiestrogen treatment on the development and growth of these tumors. Cancer Res 40:3232-3240

APPENDIX:

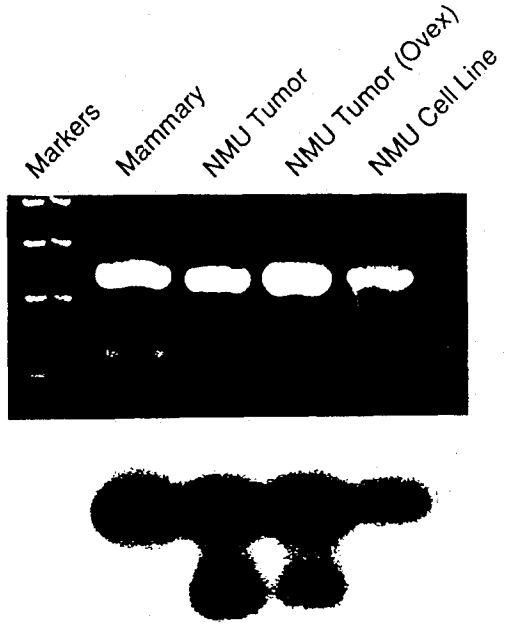


Figure 1. PRL expression in mammary tissues as detected by RT-PCR. Upper panel: PCR amplification of PRL mRNA in lactating mammary tissue, NMU-induced mammary tumors and the NMU cell line. Lower panel: Southern blot analysis of the PCR products. The PCR products were transferred to nylon membranes and hybridized to the full length, ^{32}P-labelled rPRL cDNA probe. All samples demonstrated detectable levels of PRL transcript.

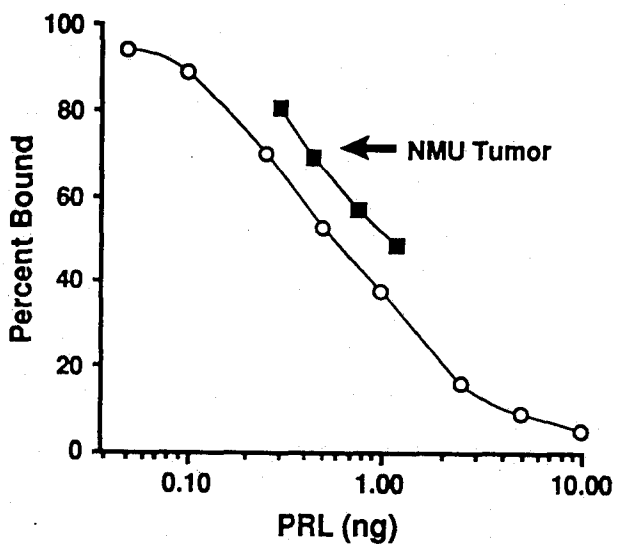


Figure 2. Immunoreactive PRL in the NMU-induced mammary tumor. An NMU-induced mammary tumor was extracted with 1N acetic acid, dialyzed and lyophyllized. Serial 2-fold dilutions were assayed for PRL. Displacements of tumor extract were parallel to the standard curve. This is a representative example of one of three extracted tumors.

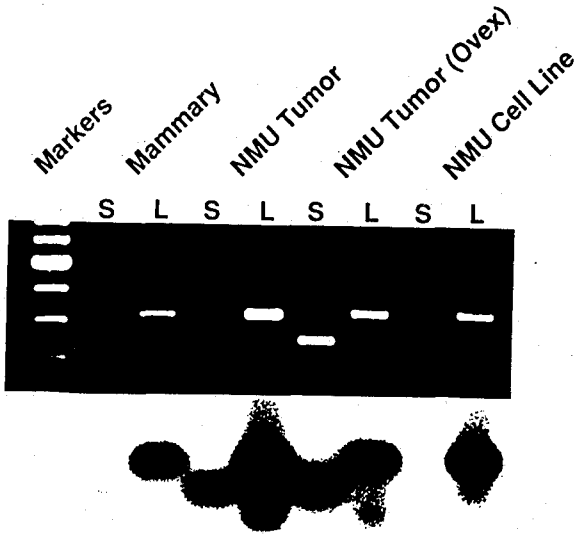


Figure 3. PRL receptor expression in mammary tissues determined by RT-PCR. Upper panel: PCR amplification of PRL receptor mRNA in lactating mammary tissue, NMU-induced mammary tumors and NMU cell lines. S and L denote long and short receptor isoforms, respectively. Lower Panel: Southern blot analysis of the PCR products. Following transfer to membrane, the PCR products were hybridized to a segment of the rPRL receptor which is common to the two isoforms, allowing comparison of the relative band intensities. All samples contain both PRL receptor isoforms, with the exception of the NMU cell line, which lacks the short isoform.

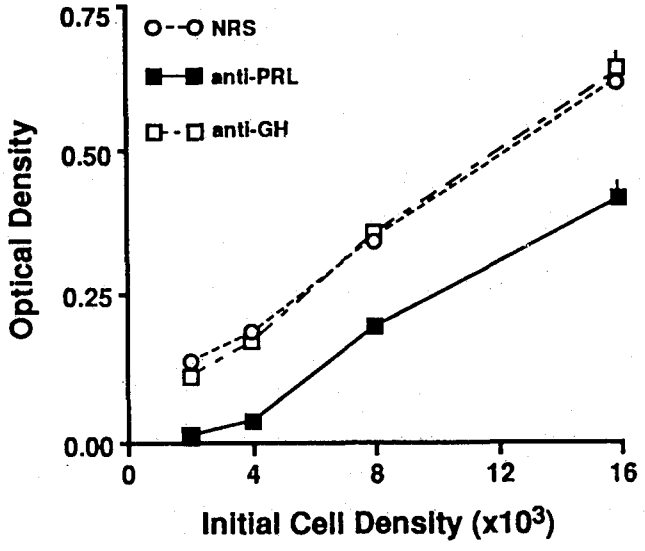


Figure 4. Inhibition of NMU cell proliferation by 48h incubation with PRL antiserum (anti-PRL) and lack of effect of anti-GH and normal rabbit serum (NRS), each at a dilution of 1:300. Optical density (570 nm), as determined by the MTT assay, is proportional to cell number. The inhibitory effect of PRL antiserum was more pronounced at low cell density.