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We propose to generate a novel inducible bitransgenic system to examine the effect of the expression of a target oncogene, the polyoma middle T antigen, on the onset of mammary epithelium hyperplasia. Using this inducible system, the specific expression of a transgene can be initiated by the administration of an external compound. To establish such a regulatable system, we have introduced the regulator and the inducible target separately into mouse embryos to generate transgenic mouse lines. To target the expression of the regulator in mammary glands, we have placed the regulator under the control of the MMTV-LTR. Transgenic lines of the regulator and the target, polyoma middle T antigen have been generated. These lines are currently being crossed to generate bitransgenic lines and the expression of the target oncogene will be induced by administration of progesterone antagonists. The regulated expression of polyoma middle T antigen in the mammary gland of bitransgenic mice will allow the investigation of the potential protective effects of avarian steroid hormones on the development of mammary tumors in response to specific oncogene expression. The success of this approach may have far-reaching effects on the future understanding of the mechanisms of oncogenesis in mammary epithelium.

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Sophia T. Lai 10/28/96

PI - Signature Date

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INTRODUCTION

Significance

Breast cancer continues to be a prevailing disease among women in the United States. Though numerous models for breast cancer have been developed, the etiology of the disease remains obscure. The transgenic mice technology has offered a means of targeting oncogenes to the mammary glands to help better understand the role these oncogenes play in the development of breast cancer. However, the constitutive expression of oncogene does not allow the assessment of the critical time points in which the mammary glands are most susceptible to oncogenesis. Thus, it is most imperative that a regulatable system be established to turn genes on and off, rendering it feasible to define the role of a candidate oncogene plays in mammary gland oncogenesis.

A novel inducible bitransgenic mice system is being generated to meet this goal. It is made up of two lines of mice. The first line of mice, the regulator mice, targeted to the mammary gland via the MMTV promoter, carries a chimeric transcription factor which is activated by an exogenous ligand. This chimeric regulator consists of three functional domains, an activation domain from HSV-VP16, a Gal4 DNA binding domain and a modified progesterone receptor ligand binding domain which responds to anti-progesterone, such as RU486, but not to progestins or other endogenous ligands. The second line of mice, the target mice, carries an oncogene under the control of four yeast transcription factor Gal4 binding sites (referred to as 17X4) where the regulator can bind to. Two minimal promoters have been chosen for the target genes namely, the E1B TATA and the TK promoter. When these transgenic lines are crossed to create the bitransgenic mice, the regulator is activated upon the administration of RU486 which then binds to the Gal4 recognition sequences upstream of the target oncogene. The expression of an oncogene in the bitransgenic mice can be induced at specific windows of development and the effects of this oncogene on mammary gland oncogenesis can be assessed. With this system, one can also study the interaction between hormones and the target oncogene during mammary gland development.

To test our regulatable system, we are using the polyoma middle T antigen (PyT) as our target oncogene in our breast cancer model. PyT is a potent oncogene which has been known to cause multi-focal tumors in the mammary glands of female mice [1]. This target will be regulated by our transactivator, which will be targeted for expression in the mammary glands. We will also use the int-2 target mice generated by the laboratory of Dr. Phil Leder. Our justification lies on the fact that the int-2 target mice are inducible [2]

BODY

Our proposed statement of work (*SOW*) for the first two years are as follows:

First year: Generation of a regulatable system for preferential gene targeting to the mammary gland.

A. Construction of a regulator which is specifically expressed in mammary tissues.
B. Construction of regulatable reporters using polyoma virus middle T antigen.
C. Functional studies of the regulator and the regulatable target gene.

Second year: Start generating bitransgenic mouse lines containing both regulator and target oncogene and examine the effects of regulatable expression of the PyT and int-2 oncogene.

A. Investigation of the effects of the expression of the regulator gene in transgenic mice.
B. Investigation of the expression of the target gene alone in transgenic mice.
C. Establish the bitransgenic transactivator system in mice.

Summary of previous work

Our previous work indicated that among three MMTV-KCR-GLVP lines tested for the expression of the regulator in the mammary gland, only one of the lines, 7134, showed expression as detected by RT-PCR. Three additional new lines have not been tested at that time.

The low expression of the regulator might be contributed by the presence of 275bp of pBR322 plasmid sequence on our microinjected fragment. To circumvent this problem, we have generated two improved regulators devoid of this plasmid sequence. These are the MMTV-KCR-HAGLVP1bGHpA (MBGH) and MMTV-KCR-HAGLVP1rBGHpA (BHMM) . In addition, by placing the VP16 activation domain at the C-terminal portion of our regulator and extending the length of the ligand binding domain of progesterone receptor, we generated a more potent regulator which showed a stronger induction in CAT activity in transfections and a higher affinity to RU486. Based on this finding, we generated another regulator line (MVPC) which would enable us to use lower levels of RU486 in our studies and thereby minimizing the undesirable pleiotropic effects.

In our last report, we also mentioned that we have obtained two 17X4-TK-PyT target lines and were in the process of breeding them. We also indicated that we would be using the int-2 target mice provided by Dr. Phil Leder to further validate the functionality of our system since these target mice have been proven to work previously [2].

(Work from Oct. 1995 to Oct. 1996)
Analysis of regulator expression of the old MMTV-KCR-GLVP lines

To enhance the sensitivity for the detection of the expression of the regulator, we have designed new primers to analyze the expression of the remaining untested MMTV-KCR-GLVP lines by RT-PCR. The sequence of the primers are as follows:

p1: 5′ TCGGCAAATATCGCATGC 3′
p2: 5′ CATGTCCAGATCGAAATCGTCTTG 3′
p3: 5′ CGTCTCCGCTCGTCACTTATCC 3′

The experimental scheme of the RT-PCR methodology used to analyze regulator expression is denoted in Figure 1. A reverse primer (**p1**) was used to prime the 1st strand cDNA synthesis from mRNA transcribed from the regulator transgene and two primers (**p2** and **p3**) flanking an intron were used in the subsequent PCR to amplify specific regulator cDNA. The primers used for PCR, **p2** and **p3**, have higher T_m values than **p1**. The use of two primers flanking the intron would allow for the discrimination between contaminating DNA present in total RNA and the reverse-transcribed cDNA. The contaminating genomic product is larger than the cDNA product (**350bp**). Total RNA isolated from 10-day old lactating mouse mammary gland was used for the analysis.

Figure 2 denotes the visualization of the RT-PCR amplified products. All the tested regulator lines express the regulator as summarized in Table 1. Since the male founder 9391 failed to mate and hence could not pass the regulator transgene to his offspring, we could not analyze the regulator expression from this line. The failure to detect the expression of the regulator in any of the MMTV-KCR-GLVP lines by Northern blots prompted us to initiate other means to obtain higher regulator expression.

Transactivation potential of the new regulators

To minimize the cost of generating transgenic mice, the functionality of the 3 improved regulator constructs were first assayed in transient transfections. These regulators are diagrammed in Figure 3. bGHpA denotes the polyadenylation signal derived from the bovine growth hormone gene and rBGpA denotes the polyadenylation signal from the rabbit betaglobin gene. HA denotes the hemagglutin epitope recognized by the 12CA5 monoclonal antibody. This tag will facilitatethe detection of expressed regulator protein using the 12CA5 antibody.

Transient transfection assays were carried out in CV1 cells to determine the transactivation potential of the various MMTV-regulators on the induction of the target reporter activity. Figure 4 shows that significant levels of the CAT reporter activity are seen only in the presence of RU486. Thus, all the tested regulators are functional and can induce reporter activity upon RU486 treatment. (+) denotes the addition of RU486 while (-) denotes the addition of vehicle (i.e. 80% alcohol). MBGH, BHMM and MVPC represent the MMTV-regulators.

Analyses of improved transgenic regulator lines

Having verified the functionality of these regulator constructs, we used them to generate improved regulator lines. These lines are MMTV-KCR-HAGLVP1bGHpA (MBGH), MMTV-KCR-HAGLVP1rBGHpA (BHMM) and MMTV-KCR-GLVPC'rBGpA (MVPC). The characterization of these lines are summarized in Table 2. We obtained 4 MBGH, 3 BHMM and 6 MVPC founder lines. Genotypic analyses by PCR and Southerns showed that all but two lines passed the transgene to their progeny in a normal Mendelian fashion. The founder MBGH 6219 was a multiple integrant while the MVPC 6920 founder line was a mosaic. So far none of the segregated lines derived from the MBGH 6219 multiple integrant expressed the regulator at a level detected via Northerns**. By Northern analysis, 3 lines have been shown to express the regulator. The MBGH 6229 line is a high expressor while both the BHMM 7386 and MVPC 6921 are low expressors. Thus, we have obtained 3 regulator lines that express the regulator at higher levels than the previous MMTV-KCR-GLVP lines. We believe that these 3 regulator lines have a better potential to induce the expression of our target genes than any of our previous MMTV-KCR-GLVP lines.

Tissue distribution of regulator expression

The expression profile of the regulator in different tissues of both male and female MBGH 6229 regulator line was assessed by a Ribonuclease protection assay (RPA) . The results of the RPA analyses are summarized in Table 3. In the female, regulator expression is seen highly in the mammary gland and moderately in the lung. Total RNA was isolated from a female mouse sacrificed at day 10 lactation. In the male, low levels of the regulator were detected in the seminal vesicles, the ampullary gland, the prostate and the lung. An 18S rRNA riboprobe was used to quantitate loading. These results show that the targeting of the regulator to the mammary gland is fairly specific and resembles the normal pattern of MMTV-directed expression. Analyses of the other two regulator lines are underway.

Consequence of regulator expression in transgenic mice

While we have not analyzed the effects of regulator expression at the molecular level (i.e. analyze any perturbations in the expression of mammary specific genes due to regulator expression) in transgenic mice, we have not observed any unusual gross anomalies and detrimental effects on the well-being of these mice. Both the male and female regulator mice are fertile and have not shown any palpable tumors for a period of up to 1 year.

It is also highly imperative that the regulator expression does not perturb the normal development of these transgenic mice. With sufficient female mice, we will begin to analyze the effects of the regulator expression especially on mammary development by examining mammary gland specific markers (i.e. keratin-specific markers and milk proteins).

Generation of 17X4-PyT target mice

We generated two 17X4-TK-PyT target lines (5988 and 5989) but no 17X4-E1bTATA-PyT lines. The 5988 line is a high-copy founder while the 5989 is a low-copy founder. The failure to obtain the TATA lines could be a consequence that the TATA-PyT transgene preferentially serves as a better enhancer trap than the TK-PyT transgene. Thus, ectopic expression of this gene might result in embryonic lethality [3]. In transient transfection studies, target genes driven by the Gal4 binding sites and TK promoter tend to have high basal activity; however, we believe that this may not be the case in transgenic mice due to site of integration and chromosomal effects. Instead, the presence of GC boxes in the TK promoter will enable the Sp1 protein to bind to and perhaps co-operate with the activated regulator in bringing about a higher level of induction when RU486 is administered.

With the availability of these two targets to work with (i.e. the PyT and the int-2 mice) and in the interest of minimizing the cost of our experiments, we have decided not to generate the activated h-ras target mice. We justify our choice by the following reasons. At least in the mammary gland, the PyT oncogene is much more potent than the activated h-ras oncogene in generating tumors. Moreover, the tumors generated by PyT are rapid and multi-focal implying little subsequent mutations needed for tumorigenesis. Above all, the tumorigenic process of PyT is clearly not a stochastic event much akin to that of activated h-ras.

Tissue distribution of 17X4-PyT target expression in target mice

To ensure that the target line is silent, we sought to analyze the tissue-profile of target gene expression in both male and female target mice by the more sensitive RT-PCR method. In the initial breeding stages, we had fewer females and so we begun to analyze the expression of the male target mice first. The results summarized in Table 4 are derived from male target mice. In the 5988 line, no expression of the target is seen in the tested tissues. In the 5989 line, we observe low levels of target expression in the brain. Depending on the threshold levels of PyT needed to induce any phenotypic change in the brain, the consequence of PyT expression here may not be significant. This fact is perhaps substantiated by the observation that no target mice has succumbed to any palpable tumors for up to a year.

The tissue-profile analysis of target gene expression in the female PyT target mice is currently underway. Based on the finding that the female target mice are fertile, we are relatively confident that little or no expression of the target is seen in these mice. No RT-PCR analyses were performed on the int-2 target mice as the target line has been reported to be silent in the absence of the transactivator gene [2].

Generation of bitransgenic mice

The int-2 target mice was chosen as one of our other targets because this line of mice has been shown to be transactivated by the MMTV-Gal4 transactivator mice [2]. Moreover, our regulated system

allows us to time the expression of the int-2 target gene which has not been done before. This is important because the phenotype observed is usually a consequence of the time point when the causative gene is first expressed [4-6]. Thus, expressing the int-2 gene at different time points may give different phenotypes. Besides, the int-2 target could potentially serve as a slow model for mammary tumorigenesis, allowing the dissection of the role of this gene.

We crossed the 6229 MBGH regulator line with the int-2 target line to generate bitransgenic mice. Since the int-2 mice are homozygotes, we would expect to get bitransgenic and int-2 target mice only. We would also only expect the bitransgenic mice given RU486 to develop hyperplasia ultimately culminating in tumors. Likewise, we have also crossed our 6229 MBGH line to both the 17X4-PyT target lines (5988 and 5989 lines).

Induction of hGH reporter

Our unpublished results indicated that our liver-specific regulator could induce the expression of human growth hormone in serum when RU486 was administered to bitransgenic mice (these bitransgenic mice are the result of a cross between the liver-specific regulator and the growth hormone target). To ensure the regulatory system works in mammary gland, we mated our MBGH regulator line to a hGH target line to generate mice bitransgenic for both the regulator and the hGH target.

RNA isolated from mammary biopsies collected from a lactating female bitransgenic mice before and following administration of a dose of 500ug/Kg body-weight RU486 were subjected to RPA. Our preliminary results showed a significant induction of the growth hormone message when RU486 was added. In the absence of RU486, no hGH expression was seen. We also assayed the expression of the regulator and observed no significant change in the presence or absence of RU486.

ONGOING EXPERIMENTS

Induction of int-2 and PyT targets

The preliminary result on the hGH induction experiment suggests to us that the regulator line is functioning in the mammary gland. This in turn prompted us to examine the induction of the int-2 and PyT expression. We are currently analyzing the induction of int-2 expression in bitransgenic mice following administration of RU486. We will also analyze the bitransgenic mice at the lactating time point whereby the regulator expression is maximal. With sufficient mice, we will begin a dose-response study of the effects of the levels of RU486 on target gene induction. We will also determine that the induction of the target gene during mammary gland development.

Tissue -profile of PyT expression in female target mice

We have obtained tissues from both the TK-PyT 5988 and 5989 female mice and are in the process of analyzing the expression of the regulator in different tissues of female target mice.

Tissue-profile of regulator expression

With sufficient BHMM 7386 and MVPC 6921 female lactating mice, we will begin our analyses on the tissue-profile of regulator expression.

Molecular consequences of regulator expression

It is important that the regulator expression does not perturb the normal development of the transgenic mice. We will analyze via Northerns if the regulator expression perturbs a number of mammary specific markers like mammary-epithelial keratin markers and milk proteins.

CONCLUSIONS

We have generated three regulator lines that express the regulator at significant high levels to be detected by Northerns. Each of these three lines are being crossed to both the *int-2* and PyT target lines to generate bitransgenic mice, allowing the examination of the functionality of our system.

Our most promising line is the high expressor MBGH 6229 line. In lactating females, the regulator is expressed highly in the mammary gland and to a much lower level in the lung. This enunciates the rather specific expression characteristic of transgenes driven by the MMTV promoter. Therefore, we focus our efforts on mating this line to the available targets to test our system. The ability to induce the target gene only in the presence of RU486 is vital to our success in generating an inducible model for breast cancer. This inducible model will have far-reaching effects in our understanding of the initiation and progression of mammary tumorigenesis by PyT.

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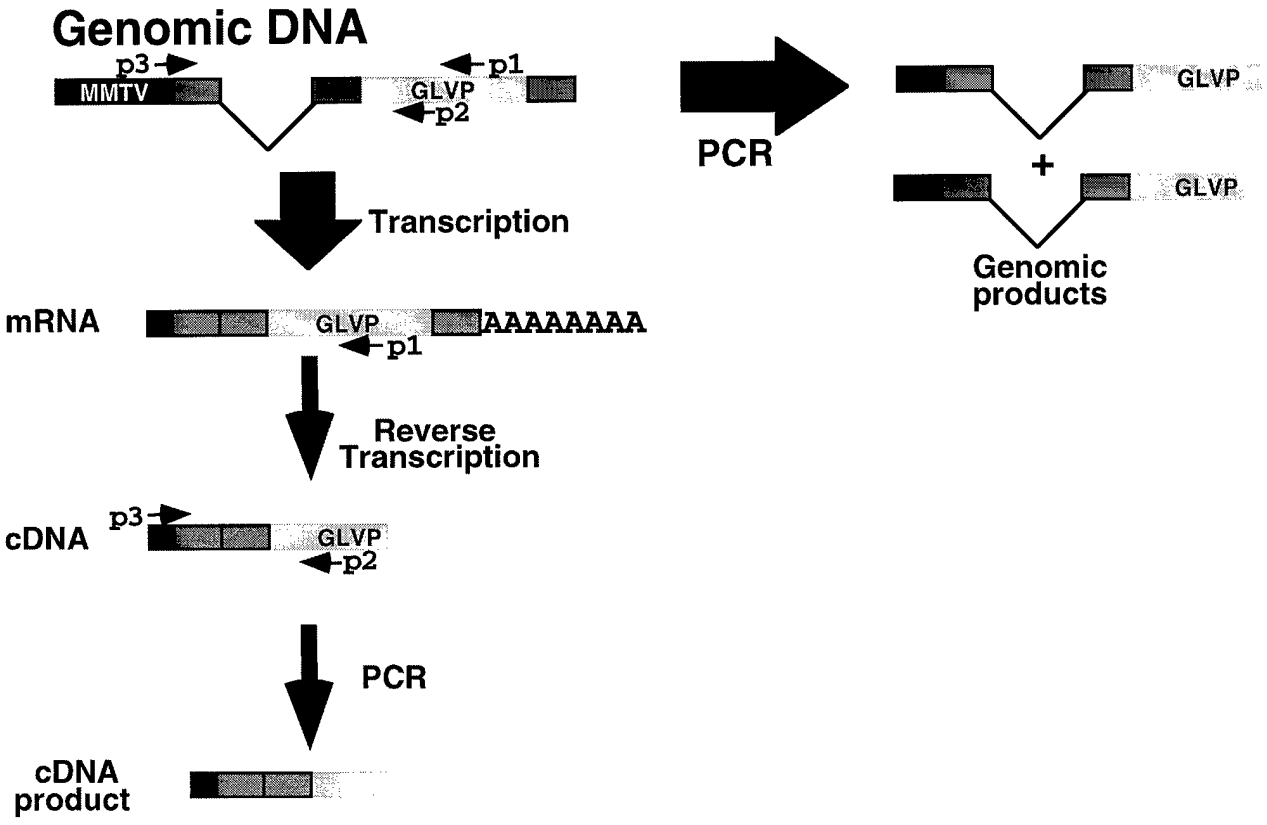
APPENDIX

Figure 1-4
Table 1-4


Genomic DNA
p3
MMTV
GLVP
p1
p2
PCR
GLVP
+
GLVP
Genomic products
Transcription
mRNA
GLVP
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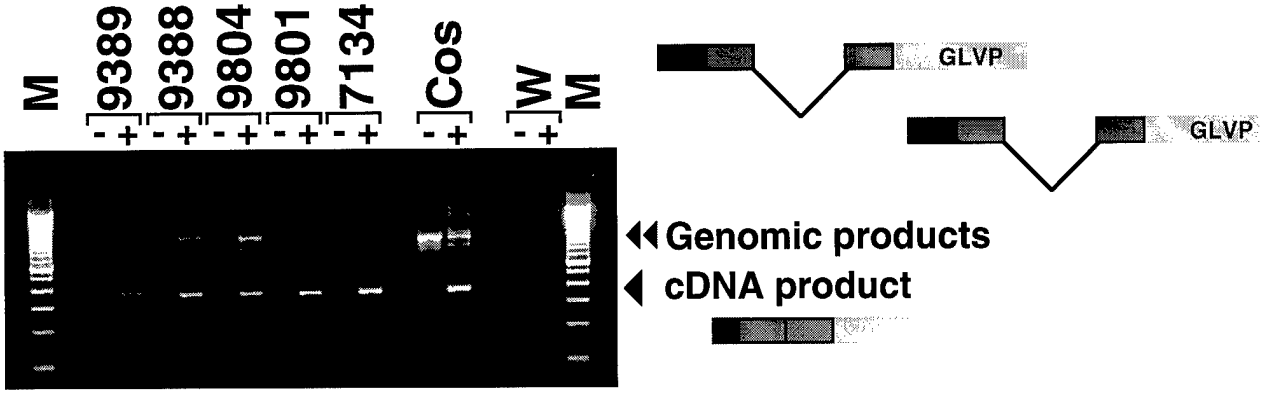
Reverse Transcription
cDNA
p3
GLVP
p2
PCR
cDNA product

Figure 1


M
9389
9388
9804
9801
7134
Cos
W
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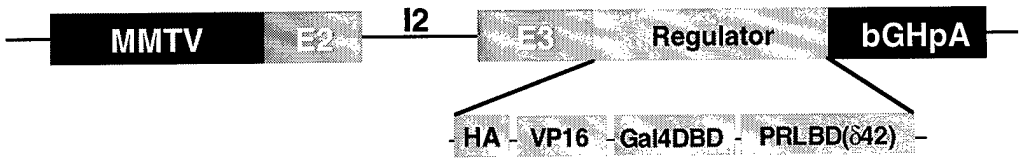
- + - + - + - + - + - + - +
GLVP
GLVP
Genomic products
cDNA product

Figure 2

MBGH



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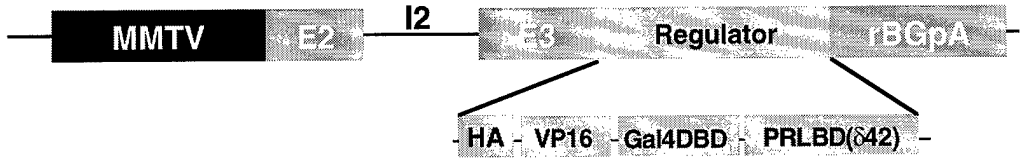

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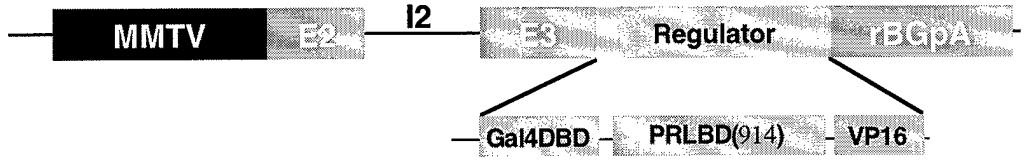

Figure 3

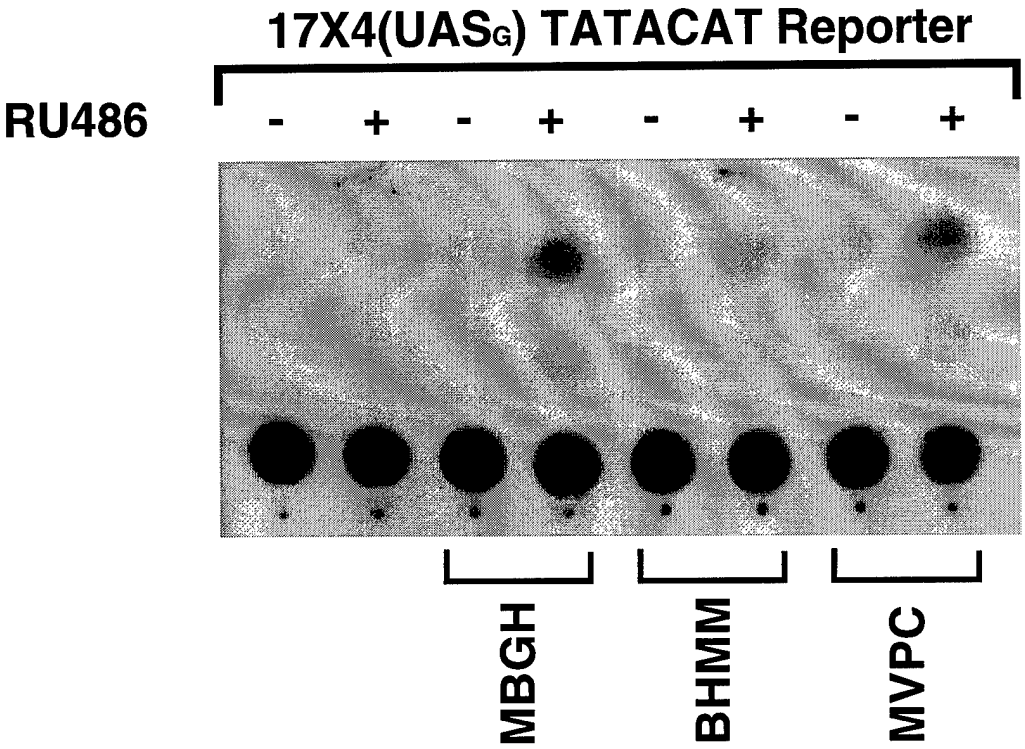

Figure 4

Founder #	Mammary Gland Exp.
7134	Yes
9388	Yes
9389	Yes
9391	Not tested
9801	Yes
9804	Yes

Table 1

	Founder #	Transgene passage	Mammary Gland Exp.	Cross to int-2
MBGH	6217	Normal	No	No
	6219	Multiple integrant	No**	No
	6226	Normal	No	No
	6229	Normal	High	Yes
BHMM	6949	Normal	No	No
	6955	Normal	No	No
	7386	Normal	Low	Yes
MVPC	6917	Normal	No	No
	6920	Mosaic	Not tested	No
	6921	Normal	Low	Yes
	6925	Normal	No	No
	6927	Normal	No	No
	6928	Normal	No	No

Table 2

	Organ	Expression
MBGH Female	Mammary Gland	High
	Lung	Med
	Salivary Gland	Low
	Spleen	Low
	Brain	No
	Liver	No
	Heart	No
	Kidney	No
MBGH Male	Lung	Low
	Seminal Vesicle	Low
	Ampullary Gland	Low
	Anterior Prostate	Low
	Ventral Prostate	Low
	Dorsal/Lateral Prostate	Low
	Vas Deferens	Low
	Preputial Gland	No
	Urethra	No
	Brain	No
	Spleen	No
	Bladder	No
	Heart	No
	Testis	No

Table 3

	Tissue	Expression
Male 5989	Lung	No
	Testis	No
	Urinary Tract (urethra/prostate)	No
	Brain	Yes
	Salivary gland	No
Male 5988	Lung	No
	Testis	No
	Urinary Tract (urethra/prostate)	No
	Spleen	No
	Liver	No
	Brain	No
	Heart	No

Table 4