

# Conditional Gene Expression in Secretory Tissues and Skin of Transgenic Mice Using the MMTV-LTR and the Tetracycline Responsive System

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**Abstract** Molecular mechanisms of development and disease can be studied in transgenic animals. Controlling the spatial and temporal expression patterns of transgenes, however, is a prerequisite for the elucidation of gene function in the whole organism. Previously we reported that mice carrying a *tetR*/VP16 hybrid gene (tTA), under the control of the human cytomegalovirus immediate early 1 (HCMV-IE1) gene promoter, can be used to temporally activate the expression of transgenes under the control of a promoter containing *tetop* sequences. We now show that the MMTV-LTR can be used to target expression of tTA to the epithelial cells of secretory organs and skin in transgenic mice. Notably, nearly uniform expression of a *tetop*-lacZ transgene was found in seminal vesicle, salivary gland, and Leydig cells of mice carrying also the MMTV-tTA transgene. More heterogeneous patterns of gene expression were observed in mammary epithelial cells and basal cells of the epidermis. Different MMTV-tTA lines had comparable tissue expression patterns. Transcriptional activation mediated by tTA was up to several hundredfold, and it was abrogated after the administration of tetracycline. The MMTV-tTA mice established in this work will be useful for experiments examining the roles of biological factors at defined developmental stages in the epithelial cells of salivary gland, seminal vesicle, mammary gland, and skin and the Leydig cells of testes. In addition, in combination with the CRE/lox recombination system, these mice will be useful to achieve gene deletions at defined time points in these organs.

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Dominant gain of function experiments in transgenic animals has been useful to elucidate the role of mammalian gene products in development and oncogenesis [Jaenisch, 1988]. Although it is possible to direct the expression of transgenes to defined tissues by employing specific genetic regulatory elements, conditional control of gene expression has been a challenge. For example, the promoter of the whey acidic protein (WAP) gene can target gene expression to

mammary alveolar cells [Pittius et al., 1988], but its temporal activation during puberty and pregnancy cannot be controlled by experimental conditions in vivo [Burdon et al., 1991; McKnight et al., 1992; Paleyanda et al., 1994]. The inability to directly control the temporal expression of transgenes has important consequences for transgenic studies designed to elucidate the role of proteins involved in development. The phenotype observed will frequently reflect the stage of development at which the transgene encoding the regulatory protein was first activated. For example, if expression of the transgene-encoded protein causes lethality at a defined stage of development, it will not be possible to study its effect on later developmental stages. This is particularly important in cases in which a protein can play different roles which depend on the stage of development and the cell type. For

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example, depending on the time of expression during pregnancy, TGF- $\beta$  can interfere with either ductal or alveolar development [Jhappan et al., 1993; Kordon et al., 1995; Pierce et al., 1993].

The development of regulatory circuits based on the tetracycline resistance operon *tet* from *E. coli* transposon Tn10 opened a new approach to controlling gene expression in eukaryotic cells [Gossen and Bujard, 1992; Furth et al., 1994]. In this system, a transactivator protein (tTA) composed of the *tet* repressor and the activating domain of viral protein VP16 of herpes simplex virus activates transcription from a minimal promoter fused to seven *tet* operator sequences from Tn10 (*tetop*). When an HCMVIE1 enhancer/promoter is used to direct tTA expression in transgenic mice, *tetop*-controlled target gene expression is activated in several different cell types, although the strongest activation is observed in muscle cells [Furth et al., 1994]. Transcriptional activation can be repressed by administration of tetracycline to the animal.

Many studies of the transforming potential of oncogenes and growth factors in transgenic mice have been focused on secretory tissues, such as the mammary gland, salivary gland, and male reproductive organs [Sinn et al., 1987; Muller et al., 1988, 1990; Stewart et al., 1984; Andres et al., 1987; Stoecklin et al., 1993; Schönenberger et al., 1988, 1990; Groner, 1992; Guy et al., 1992]. Conditional control over the expression of oncogenes and/or growth factors in these tissues would provide a new tool to study oncogenesis in these organs. For instance, oncogene and growth factor expression can be targeted to cells at specific stages of differentiation, the duration of oncoprotein or growth factor can be directly controlled, and expression of oncogenes or growth factors during embryogenesis can be avoided if desired. To establish a conditional gene expression system in the epithelial cells of different secretory tissues, we generated a hybrid gene composed of the MMTV-LTR and the coding region of the tTA transactivator protein (MMTV-tTA). The function of the MMTV-tTA hybrid gene was evaluated in both tissue culture cells and several different tissues of transgenic mice. Mice carrying this MMTV-tTA transgene were bred with mice transgenic for a *tetop*- $\beta$ -galactosidase gene (*tet $\beta$ <sub>n</sub>*) or *tetop*-luciferase gene (*tetL*). The expression pattern in double transgenic MMTV-tTA/*tet $\beta$ <sub>n</sub>* mice was examined to

determine the cell type and number of expressing cells in the absence and presence of tetracycline. The double transgenic MMTV-tTA/*tetL* mice were used to quantitate the degree of activation in different tissues in the absence and presence of tetracycline.

## MATERIALS AND METHODS

### Expression Vectors

The MMTV-LTR-tTA expression vector (MMTV-tTA) is composed of MMTV-LTR sequences from -1100 to -1 [Hennighausen et al., 1994] linked to the tTA gene flanked at the 3' end by the rabbit  $\beta$ -globin intron and a polyadenylation (polyA) signal. It was constructed by substituting the MMTV-LTR for the HCMVIE1 enhancer/promoter in pUHG15-1 [Furth et al., 1994b]. The nonreplicating nuclear localized  $\beta$ -galactosidase *tetop* expression vector (*tet $\beta$ <sub>n</sub>*) has been described previously [Furth et al., 1994]. The replicating nuclear localized  $\beta$ -galactosidase *tetop* expression vector (BPV- $\beta$ <sub>n</sub>) is composed of the bovine papillomavirus type 1 (BPV-1) genome linearized at the BamHI site, the *tetop* promoter, the n $\beta$ G gene flanked at the 3' end by the MPI intron, and poly(A) signal [a] and pML2d sequences. It was constructed by substituting a fragment containing the *tetop* promoter and the n $\beta$ G gene flanked at the 3' end by the MPI intron and poly(A) signal from the nonreplicating *tet $\beta$ <sub>n</sub>* vector for a fragment containing the MT-1 promoter linked to the EC-SOD gene flanked at the 3' end by rabbit  $\beta$ -globin sequences in pS30.

### Transfections and Generation of Stable Cell Lines Expressing the MMTV-tTA Gene

NIH3T3 cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, antibiotics, and glutamine. To generate clones of NIH3T3 cells which stably expressed tTA under the control of the MMTV-LTR, electrotransformations were performed on  $10^6$  cells, using 5  $\mu$ g of MMTV-tTA, 5  $\mu$ g of an MMTV-neomycin construct, and 10  $\mu$ g of salmon sperm DNA as carrier. Standard procedures for generating clones of stable transfectants were followed [Sambrook et al., 1989]. Five cell lines derived from stable clones were then used in transient transfection assays. For transient assays in cells which stably expressed tTA from the MMTV-LTR, electrotransformations were performed on  $10^6$  cells, using 10  $\mu$ g of *tet $\beta$ <sub>n</sub>* and

20  $\mu\text{g}$  of salmon sperm DNA as carrier. In situ staining for  $\beta$ -galactosidase activity in the transfected cells was performed 48 h after transfection. For transient assays in NIH3T3 cells which did not carry the MMTV-tTA gene, cells were transfected with 20  $\mu\text{g}$  of MMTV-tTA, 20  $\mu\text{g}$  of either  $\text{tet}\beta_n$  or BPV- $\beta_n$ , and 10  $\mu\text{g}$  of salmon sperm DNA as carrier. Cells were harvested 48 h after the transfection, and 10  $\mu\text{g}$  of cellular protein extract was analyzed for  $\beta$ -galactosidase activity. Cells treated with tetracycline were exposed to the drug (1  $\mu\text{g}/\text{ml}$ ) for 24 h prior to transfection. Transfection efficiency was unaffected by the presence of tetracycline [Li and Furth, unpublished data]. Both transient and stable transfectants which received tetracycline were exposed to the drug for the entire experiment. The tetracycline containing media was changed every 24 h.

#### Analysis of $\beta$ -Galactosidase Activity in Tissue Culture Cells

For analysis of  $\beta$ -galactosidase activity in cell lines with stable expression of tTA, in situ staining for nuclear localized  $\beta$ -galactosidase activity was performed on 10 cm plates of cells transiently transfected with  $\text{tet}\beta_n$ . Before staining, the cells were fixed with 0.5% glutaraldehyde in  $1\times$  PBS for 15 min. They were stained at  $30^\circ\text{C}$  in a solution containing 0.05% 4-chloro-5-bromo-3-indolyl- $\beta$ -D-galactopyranoside (X-gal), 3 mM  $\text{MgCl}_2$ , 3 mM  $\text{K}_3\text{Fe}(\text{CN})_6$ , and 3 mM  $\text{K}_4\text{Fe}(\text{CN})_6 \cdot 6\text{H}_2\text{O}$  for 24 h. After staining was complete, the cells were examined under the microscope for the appearance of blue nuclei. For analysis of  $\beta$ -galactosidase activity in cells transiently transfected with  $\text{tet}\beta_n$  and MMTV-tTA or BPV- $\beta_n$  and MMTV-tTA, cellular extracts were prepared by freeze-thawing. The protein concentration of the cellular extracts was determined using the Bradford assay (Pierce). Ten micrograms of cellular extract were analyzed for  $\beta$ -galactosidase activity after incubation at  $30^\circ\text{C}$  in 4 mg/ml ONPG, 100 mM  $\text{NaPO}_4$ , pH 7.0, 10 mM KCl, 1 mM  $\text{Mg}_2\text{SO}_4$ , and 50 mM  $\beta$ -mercaptoethanol until a yellow color developed. Sample O.D.s were determined at 410 nm.

#### Northern Blot Analysis of RNA Expression From the MMTV-tTA Gene in Stably Transfected NIH3T3 Cells

Expression levels of tTA RNA were analyzed in two of the three stable cell lines which demon-

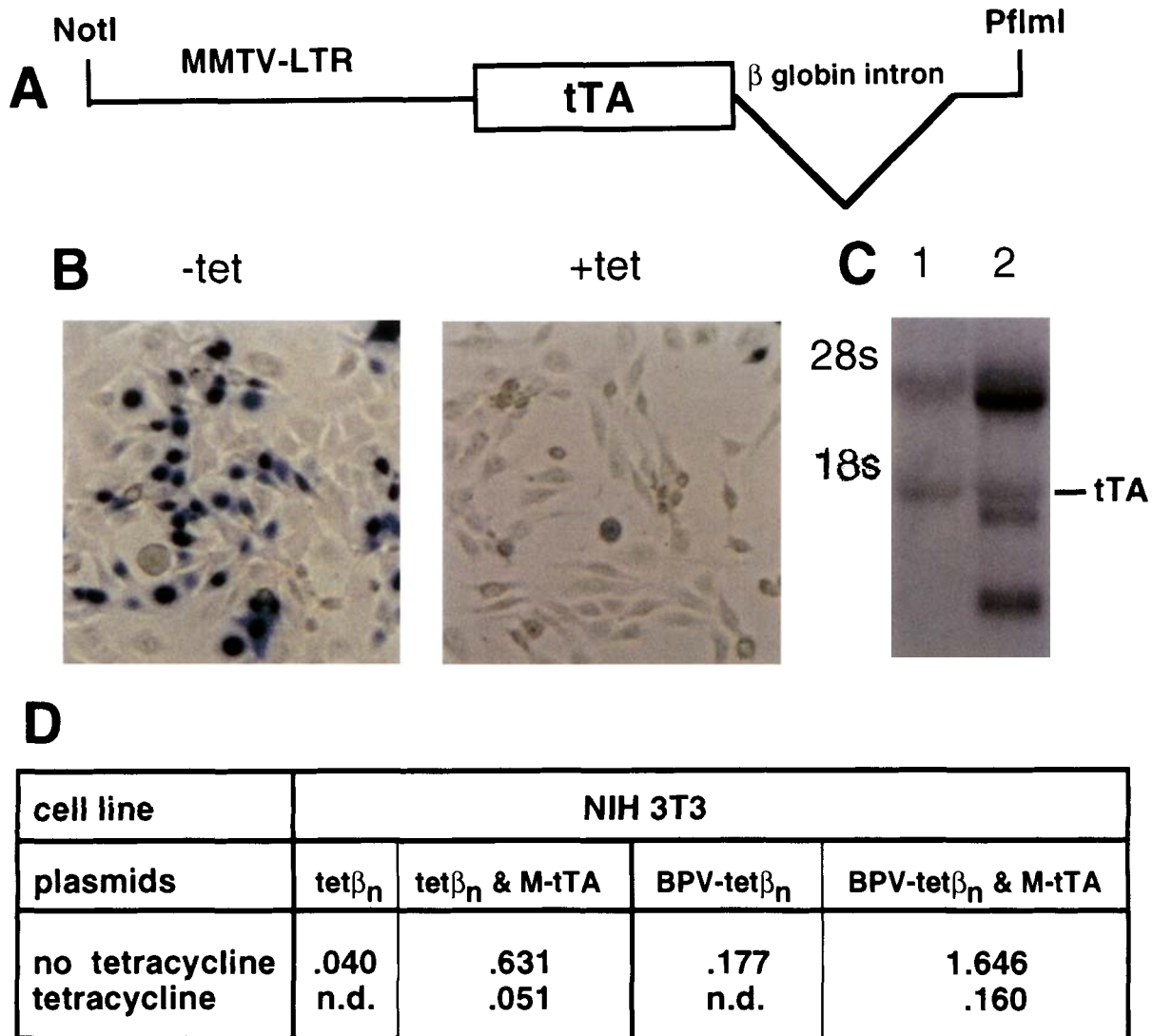
strated in situ staining for  $\beta$ -galactosidase activity after transient transfection with  $\text{tet}\beta_n$ . Total RNA was isolated from the individual stable cell lines and the parent NIH3T3 cell line by a modification of a method first described by [Chomczynski and Sacchi, 1987]. The RNA was quantitated on a spectrophotometer, and 10  $\mu\text{g}$  of each sample was fractionated on a formaldehyde agarose gel, transferred to a nylon membrane, and fixed on the membrane by UV irradiation. The membrane was hybridized overnight to a random primer labelled probe composed of the tTA coding sequences.

#### Generation of the Transgenes and Transgenic Mice

The MMTV-tTA transgene (Fig. 1A) was separated from the vector with XhoI and PflmI and isolated as a 3.5 kb fragment from an agarose gel using electroelution. The DNA fragments were injected into fertilized eggs at a concentration of approximately 5 ng/ $\mu\text{l}$ . Transgenic mice were generated according to standard procedures, and founder mice were analyzed using the polymerase chain reaction (PCR) and Southern hybridization. The tTA transgene was identified using primers corresponding to the MMTV-LTR from -50 to -33 (5' CTG ATC TGA GCT CTG AGT G 3') and *tetR* sequences (5' GCA AAA GTG AGT ATG GGT CC 3'). The resulting PCR product was 280 bp in size. Transgenic mice carrying the tetop-luciferase and tetop-n $\beta$ gal transgenes have been described [Furth et al., 1994]. The reporter genes were identified with primers corresponding to the HCMV promoter and the luciferase gene (5' GCA ATT GTT CCA GGA ACC AGG GCG 3') or the nuclear localization signal of the  $\beta$ -galactosidase gene (5' CGG GAT CCC CCA TGC TCC CC 3'). The PCR product for the luciferase gene had a size of 320 bp, and that for the  $\beta$ -galactosidase transgene was 269 bp in length.

#### Administration of Tetracycline

Slow release tetracycline pellets (Innovative Research of America, Toledo, OH) were implanted subcutaneously in the shoulder region using a trochar according to the manufacturer's directions. These pellets released 0.7 mg tetracycline hydrochloride per day. Pellets were kept in place for 3-15 days before levels of transgene expression were measured.



**Fig. 1.** Structure and function of the MMTV-tTA hybrid gene. The MMTV-tTA hybrid gene (A) contains 1.1 kb of the MMTV-LTR, the tTA coding region, and the  $\beta$  globin intron and poly(A) site. B: NIH3T3 cells, which have stably integrated a plasmid containing the MMTV-tTA gene, were transiently transfected with a plasmid containing the tet $\beta_n$  reporter gene. Cells were grown for 48 h in the absence (-tet) and presence (+tet) of tetracycline and were subsequently stained for  $\beta$ -galactosidase

activity. C: Northern blot analysis of RNA from cell lines carrying a silent (lane 1) or a transcribed (lane 2) MMTV-tTA transgene. The positions of the 18s and 28s RNA and the tTA mRNA are indicated. D: Analysis of tTA activity in NIH3T3 cells stably transfected with the MMTV-tTA hybrid gene. The nonreplicating tet $\beta_n$  and the replicating BPV-tet $\beta_n$  plasmids were transiently transfected.

#### Analysis of $\beta$ -Galactosidase Activity in Transgenic Mice

To analyze  $\beta$ -galactosidase activity, mice were killed using cervical dislocation and the individual tissues or organs were removed.  $\beta$ -galactosidase activity was assayed in whole tissue samples and in frozen sections. To analyze activity in whole tissue samples, 5 mm cubes of selected tissues were fixed in 2% paraformaldehyde and 0.02% glutaraldehyde in PBS for 1 h

and then rinsed twice in PBS. Staining for  $\beta$ -galactosidase activity was done at 30°C in a solution containing 0.1% 4-chloro-5-bromo-3-indolyl- $\beta$ -D-galactopyranoside (X-gal), 2 mM MgCl<sub>2</sub>, 5 mM EGTA, 0.02% (w/v) Nonidet P-40, 5 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, and 5 mM K<sub>4</sub>Fe(CN)<sub>6</sub> · 6H<sub>2</sub>O for 24–28 h. After staining was complete, the specimens were embedded in paraffin, and the sections were cut at 10  $\mu$ m, counterstained with nuclear fast red, and examined for the presence

of blue-colored nuclei. To analyze activity on frozen sections, tissue samples were embedded in O.C.T. (Tissue-Tek; Miles Inc., Elkhart, IN), sectioned on a cryostat at 10  $\mu\text{m}$ , placed on gelatin-coated slides, and air-dried. Slides are fixed for 2 min in 0.5% glutaraldehyde in 1 $\times$  PBS and then stained at 30°C in a solution containing 0.1% 4-chloro-5-bromo-3-indolyl- $\beta$ -D-galactopyranoside (X-gal), 100 mM sodium phosphate, pH 7.3, 1.3 mM  $\text{MgCl}_2$ , 3 mM  $\text{K}_3\text{Fe}(\text{CN})_6$ , and 3 mM  $\text{K}_4\text{Fe}(\text{CN})_6 \cdot 6\text{H}_2\text{O}$  for 24–48 h. After staining was complete, the sections were counterstained with nuclear fast red and examined for the presence of blue nuclei.

#### Analysis of Luciferase Activity in Transgenic Mice

To analyze luciferase activity, mice were normally killed using cervical dislocation, and tissues samples were removed. In one case, a mammary gland biopsy was performed. In three cases, biopsies of earskin were performed. Prior to the biopsy the mouse was anesthetized using avetin, .7 ml intraperitoneally. After the biopsy was completed, a tetracycline pellet was placed into the mouse. Seven days later, the mouse was killed as described above. Tissues to be analyzed were homogenized by using a Polytron in lysis buffer containing 25 mM glycyl-glycine, 15 mM  $\text{Mg}_2\text{SO}_4$ , 2 mM EDTA, 1 mM dithiothreitol, and 1% Triton X-100. The homogenate was centrifuged for 5 min at 12,000 rpm, and 100  $\mu\text{l}$  of the supernatant was used in the luciferase assay (25 mM glycyl-glycine, 15 mM  $\text{Mg}_2\text{SO}_4$ , 5 mM ATP). Luciferase activity was measured using a Berthold Lumat (Berthold, Göttingen, Germany) after the injection of 100  $\mu\text{l}$  of a 0.05 mM luciferin solution. The protein concentration of the homogenate was determined using the Bradford assay. Luciferase activities were calculated as relative light units (rlu) per milligram of total cellular protein.

## RESULTS

### Function and RNA Analysis of the MMTV-tTA Hybrid Gene in NIH3T3 Cells

The expression of the MMTV-tTA hybrid gene (Fig. 1A) was analyzed in stably transfected NIH3T3 cells. Five individual neomycin-resistant colonies were selected for analysis. Each individual cloned cell line was transiently transfected with  $\text{tet}\beta_n$  in the absence and presence of tetracycline. In situ  $\beta$ -galactosidase activity was measured 48 h after transfection. Three of the

five cell lines revealed  $\beta$ -galactosidase activity in the absence of tetracycline, and this activity was greatly reduced in the presence of tetracycline. An example of the  $\beta$ -galactosidase activity in the absence and presence of tetracycline in one of the cloned cell lines (tTA5) is shown in Figure 1B. RNA from two of the three functional cell lines (tTA1 and tTA5) was analyzed on Northern blots for the presence of tTA mRNA. A band corresponding to the predicted size of the mature mRNA encoded by the MMTV-tTA gene was detected in 10  $\mu\text{g}$  of total RNA in one of the cell lines (Fig. 1C). The 1.4 kb band corresponds in size to the mature mRNA encoded by MMTV-tTA hybrid gene. The 0.6 kb fragment most likely represents an aberrantly spliced RNA, and the approximately 4 kb fragment probably results from read-through into plasmid sequences. Although a second cell line subjected to Northern blot analysis expressed functional tTA protein demonstrated by in situ  $\beta$ -galactosidase staining, the mRNA expression level was below the level of detection using 10  $\mu\text{g}$  of total RNA (data not shown).

Functional activity of MMTV-tTA was also evaluated after transient cotransfection with either a nonreplicating ( $\text{tet}\beta_n$ ) or a replicating (BPV- $\beta_n$ ) vector into NIH3T3 cells in the absence of presence of tetracycline. Both  $\text{tet}\beta_n$  and BPV- $\beta_n$  were transfected individually into NIH3T3 cells to evaluate expression levels from each vector in the absence of tTA protein. Expression from both  $\text{tet}\beta_n$  and BPV- $\beta_n$  was activated by MMTV-tTA in the absence of tetracycline, and this activity was suppressed to basal levels in the presence of tetracycline (Fig. 1D). Higher basal expression levels were seen with the replicating vector, suggesting that gene copy number is one factor influencing the degree of basal expression found from the *tetop* promoter.

### Generation of Transgenic Mice Expressing the MMTV-tTA Transgene

Six lines of transgenic mice that carried MMTV-tTA transgenes were analyzed in detail. The activity of the MMTV-tTA transgenes in each of these lines was analyzed in several tissues in double transgenic mice which carried an MMTV-tTA transgene and either the  $\text{tet}\beta_n$  or the  $\text{tetL}$  transgene. Mice carrying both MMTV-tTA and  $\text{tet}\beta_n$  transgenes were used to analyze gene activation and repression on a single cell level. Mice carrying both the MMTV-tTA and  $\text{tetL}$  transgenes were used to quantitate the

degree of gene activation and repression in whole tissues. From the six lines analyzed, five activated both the  $tet\beta_n$  and the  $tetL$  transgenes.

### The MMTV-tTA Transgene Targets $\beta$ -Galactosidase Expression to the Epithelial Cells of Secretory Tissues and Skin

Analysis of double transgenic mice which carried the MMTV-tTA and  $tet\beta_n$  transgenes revealed strong nuclear staining in epithelial cells of the salivary gland, seminal vesicle, mammary gland, and skin and the Leydig cells in testes (Fig. 2). Similar patterns of expression were observed in the five expressing MMTV-tTA lines. Heterogeneous patterns of expression were observed in all tissues, but the degree of heterogeneity varied markedly between tissues. A large proportion of epithelial cells in salivary gland and seminal vesicle revealed  $\beta$ -galactosidase activity, whereas the number of epithelial cells in the mammary gland and skin staining blue was more variable. In the testis, the majority, if not all, of the Leydig cells demonstrated blue nuclei. After 7 days of tetracycline exposure, nearly all sections revealed no blue nuclei. Single blue nuclei were found in only a few sections (data not shown).

### MMTV-tTA Transgene Activation in Secretory Tissues and Skin Was Abrogated by the Administration of Tetracycline

Individual double transgenic mice carrying the MMTV-tTA and  $tetL$  transgenes were used to quantitate the degree of activation in the absence of tetracycline and repression by tetracycline in salivary gland, seminal vesicle, mammary gland, and skin (Table I). MMTV-tTA-mediated activation of the  $tetL$  transgene was abrogated after the administration of tetracycline. A mammary gland biopsy was performed in one mouse and skin biopsies were performed in three mice, so that tissue from the same mouse could be analyzed in the absence and presence of tetracycline. In the presence of tetracycline, luciferase expression was reduced to basal levels in salivary gland, seminal vesicle, and mammary gland. In skin, luciferase expression was reduced to basal levels in one mouse, and in another two mice it was reduced to 1.6% and 4.4% of the levels measured in the absence of tetracycline.

### The MMTV-tTA Transgene Is Activated in a Broad Range of Tissues

The ability of MMTV-tTA to activate expression from the tetop promoter was analyzed in additional tissues from individual mice (Table II). While the highest levels of activation were seen in secretory tissues and skin, lower levels of expression were found in a broad range of tissues. Tissues with the highest luciferase activities were those which demonstrated the most homogeneous  $\beta$ -galactosidase staining.

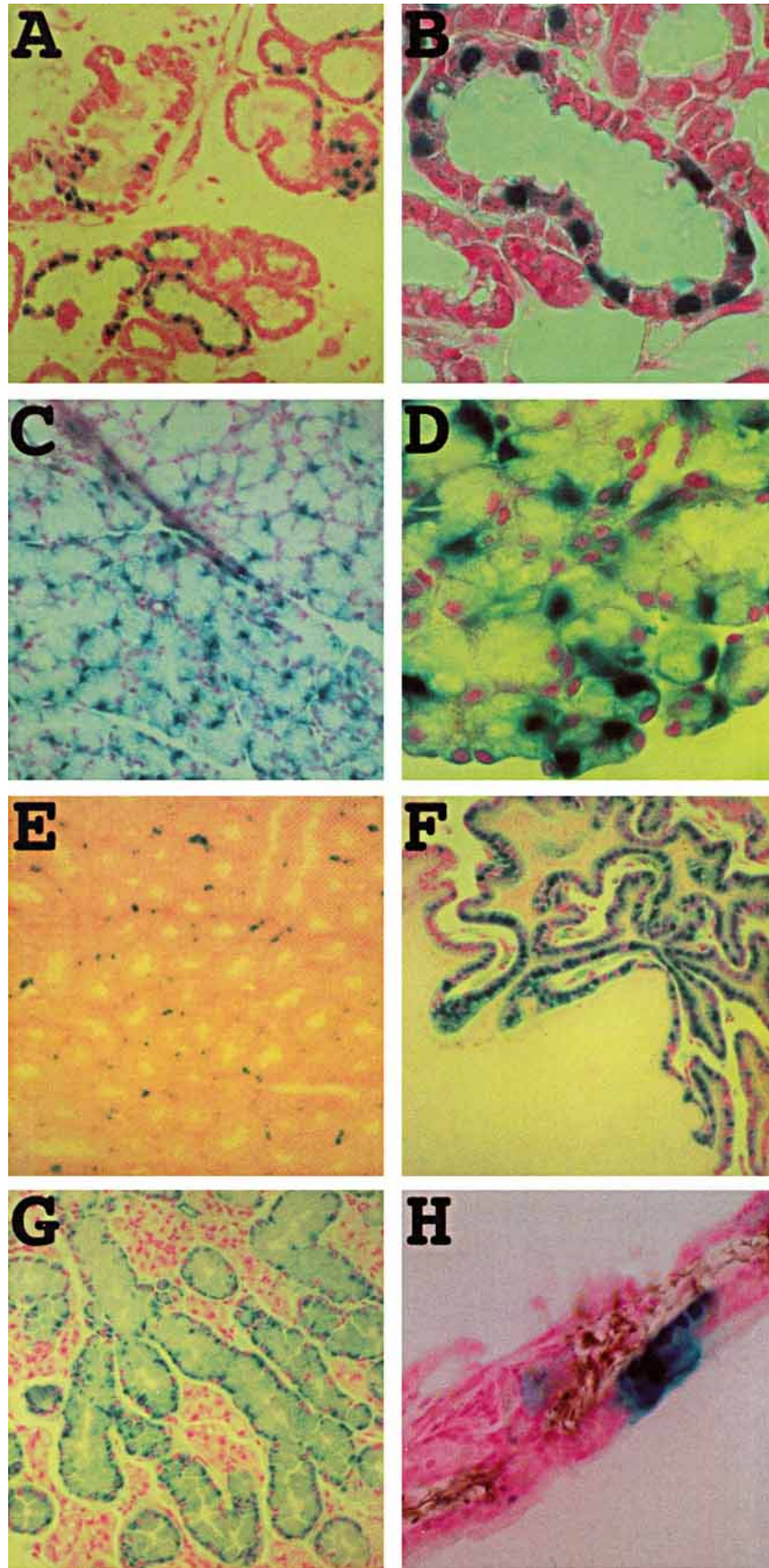
## DISCUSSION

### Conditional Gene Expression as a Tool to Study Development and Tumorigenesis in Transgenic Mice

The establishment of links between the expression pattern of individual genes and the development and pathophysiology of specific tissues remains a challenge in biology. Experimental approaches include dominant gain of function experiments, in which a gene is either ectopically expressed or overexpressed, and the controlled deletion of genes from the germline of mice. Conditional gene expression systems which permit temporal control over the expression pattern of an individual gene enable experimental approaches which can be used to focus on specific developmental time points, evaluate effects of the duration of expression, and explore the reversibility of phenotypes linked to specific gene expression.

Targeting transgene expression to many specific cell types has been achieved. Regulatory elements from genes expressed in specialized cell types have been isolated and will convey the same cell specificity as the endogenous gene when used in a transgene. For example, promoter sequences from mammary specific genes can target gene expression in transgenic mice to mammary tissue [Hennighausen, 1992]. The *tet* responsive system can be combined with tissue-specific promoters to conditionally target expression to selected organs and tissues.

In this study, the MMTV-LTR was selected to target gene expression to secretory tissues. Similar to previous reports, lower levels of gene expression were detectable in a number of tissues. The presence of significant levels of gene expression in the basal cells of the epidermis was not predicted. In the absence of the MMTV-tTA transgene the  $tetL$  and  $tet\beta_n$  reporter genes are virtually silent [Furth et al., 1994]. In tissues



**Fig. 2.** Analysis of nuclear  $\beta$ -galactosidase activity in tissue sections from double transgenic mice, carrying both the MMTV-tTA and tet $\beta_n$  transgenes. Nuclei were counterstained with nuclear fast red, and the blue nuclei indicate expressing cells. **A:** Mammary tissue from a female at day 10 of lactation.  $\times 200$ . **B:**

As **A** but at  $\times 630$  magnification. **C:** Salivary gland from a 6-week-old virgin.  $\times 200$ . **D:** As **C** but at a  $\times 630$  magnification. **E:** Testicular tissue of an 8-week-old male. The blue cells correspond to the Leydig cells. **F:** Seminal vesicle from the same male. **G:** Salivary gland from the same male. **H:** Ear skin.

**TABLE I. Luciferase Activities in Salivary and Mammary Tissue, Seminal Vesicle, and Skin in the Absence and Presence of Tetracycline in Individual Double Transgenic Mice Containing the MMTV-tTA and tetL Transgenes\***

MMTV-tTA line	Tissue	-tet	+tet	Days of tet
MTA1-02	Salivary gland	26,305		
MTA1-05	Salivary gland	3,139		
MTA1-10	Salivary gland	165,000		
MTA3-12	Salivary gland	320,000		
MTA1-13	Salivary gland	15,784		
MTA1-07	Salivary gland		220	11
MTA1-08	Salivary gland		562	11
MTA1-11	Salivary gland		141	3
MTA3-12	Seminal vesicle	1,994,000		
MTA3-15	Seminal vesicle		388	70
MTA1-4	Mammary gland	1,082,000		
MTA1-3	Mammary gland		296	11
MTA1-20	Mammary gland		626	11
MTA3-01	Mammary gland	2,230		
MTA3-11	Mammary gland	2,274	225	7
MTA3-10	Mammary gland	2,015		
MTA3-14	Mammary gland		238	14
MTA1-4	Skin	2,296,000		
MTA3-12	Skin	364,000		
MTA1-7	Skin	1,548,234	25,407	11
MTA1-11	Skin	812,860	779	3
MTA3-14	Skin	51,930	2,316	11

\*Data is presented as relative light units (rlu) per milligram of total cellular protein.

which were targeted by the MMTV-tTA transgene, gene expression was activated up to several hundredfold. This activation was abrogated in several secretory tissues after at least a 3 day exposure of the animal to therapeutic levels of tetracycline. However, complete abrogation of

gene expression in some tissues—for example, skin—may require higher levels of tetracycline administration or the use of different tetracycline analogues. While tetracycline is broadly distributed in tissues, the pharmacokinetics of tetracycline delivery for the *tet* responsive system in individual tissues may vary. This may be relevant for studies related to embryonic development where rapid gene induction and repression may be required. The degree of repression and induction required for individual studies will also influence the utility of the *tet* responsive system to address specific experimental questions.

Other systems which permit conditional control of transgene expression are currently being explored. Another approach to controlling gene activity uses the *tetR* to inhibit gene transcription. In this case *tetR* molecules bind to *tetO* sequences located at the transcriptional start site and block gene transcription in the absence of tetracycline. In the presence of tetracycline, binding of the *tetR* to the *tetO* is greatly reduced, and transcription is activated [Gatz et al., 1991]. O'Malley and colleagues have described a C-terminal deletion mutant of the progesterone receptor which fails to bind progesterone but can bind its antagonist RU486 and can be used to activate genes containing progesterone response elements [Wang et al., 1994]. With RU486 as an agonist, a chimeric protein consisting of the steroid binding domain, the DNA binding domain of GAL4, and the VP16 activation domain can activate in cell lines a minimal promoter linked to GAL4 binding sites. Establishment of the utility of this system in transgenic animals is still pending. Another system being explored is based on the ecdysone receptor from *Drosophila*.

#### Mosaic Expression of Transgenes

A highly mosaic activity pattern of the  $tet\beta_n$  reporter gene has been observed in muscle of mice carrying an HCMVIE1-tTA transgene and a G4- $tet\beta_n$  transgene [Furth et al., 1994]. In contrast, the staining pattern in salivary gland and seminal vesicle in double transgenic mice carrying the MMTV-tTA transgene and a G4- $tet\beta_n$  transgene is far more homogenous. Up to 80% of the cells in these tissues demonstrated  $\beta$ -galactosidase activity. This suggests that an important determinant of the degree of mosaicism is the expression pattern of the transcriptional regulatory region used to activate the tTA



**TABLE II. Luciferase Activity in Tissues From Individual Double Transgenic Mice Containing the MMTV-tTA and tetL Transgenes\***

Tissue	LU5	MTA1-4	Fold induction	MTA3-12	Fold induction
Testes	360	n.d.	—	227,000	600
Epididymis	250	n.d.	—	286,000	1,100
Ovary	70	35,000	500	n.d.	—
Thymus	220	16,000	70	116,000	500
Tongue	590	n.d.	—	34,000	60
Spleen	130	16,000	120	7,200	50
Kidney	50	15,000	300	5,300	100
Heart	20	9300	460	10,000	500
Liver	20	900	50	11,000	600
Thigh muscle	350	4500	13	1,400	4

\*Data is presented as relative light units (rlu) per milligram of total cellular protein. Luciferase activity in single transgenic mice containing only the tetL transgene is listed as reference and the fold induction is calculated. n.d. = not determined.

gene. At the same time, markedly heterogeneous  $\beta$ -galactosidase staining was found in mammary tissue of double transgenic lactating mice. This indicates that the degree of heterogeneity from an individual promoter can vary from between tissues. Extensive mosaic transgene expression has also been observed in mammary tissue of mice carrying other hybrid genes under the control of the MMTV-LTR [Hennighausen et al., 1994; Stoecklin et al., 1993; Muller et al., 1988] and the WAP gene promoter [Li et al., 1994]. Finally, some variation in the degree of heterogeneity in individual tissues between animals from a single line was observed in these experiments, although the overall pattern of gene expression remained the same.

Mosaic transgene expression in the *tet* responsive system can be controlled for if the number of expressing cells can be identified. This may be accomplished by immunohistochemistry, by *in situ* techniques, by coexpressing a  $\beta$ -galactosidase reporter gene after cointegration, or by including the  $\beta$ -galactosidase sequences in the transgene using an internal ribosome entry site. Alternatively, identification of expressing cells could be performed by the introduction of a specific tag sequence (e.g., the flu tag) into the target gene followed by immunohistochemistry. Tissues and animals which demonstrate the most homogeneous staining patterns generally would be the most appropriate choices for further studies. In some tissues, biopsies can be performed to follow the targeted transgene expression pattern and evaluate the temporal course of genetic and histological changes which result from conditional expression of the transgene. If the targeted transgene is an oncogene or a secreted protein, it is possible that clear biological effects

will be observed even when expression is not completely homogeneous.

Homogenous transgene expression may be achieved by choosing other promoter elements to control tTA expression. For example, in contrast to the MMTV-LTR, the promoter/upstream region of the mouse WAP gene generally conveys a homogenous expression in mammary tissue [G. Robinson and L. Hennighausen, submitted]. The introduction of sequences which can mediate more reliable transgene activity, such as MAR elements, could provide more homogeneous expression of tTA [McKnight et al., 1992]. Another route of achieving reproducible expression pattern may be the introduction of the tTA into an endogenous gene via homologous recombination in ES cells.

#### The Use of MMTV-tTA Transgenic Mice

The availability of transgenic mice expressing the MMTV-tTA transgene has implications for several research areas. Since tTA is expressed at high levels in salivary gland, seminal vesicle, mammary gland, and skin, these mice can be used to study the role of developmental genes and oncogenes in the context of different cell types. For example, we have generated double transgenic mice carrying an MMTV-tTA transgene and a transgene consisting of the *tet* promoter and the coding region for the SV40 T antigen. Expression of the tetop-SV40T transgene was dependent on the presence of tTA and was detected with antibodies against the T-antigen in secretory tissues [P.A. Furth, S. Efrat, and L. Hennighausen, unpublished]. Another new and potentially powerful research tool comes from the combination of the *tet* responsive with the CRE/lox recombination system. This is cur-

rently being developed by several groups. Regulating the temporal activity of the CRE gene will permit gene deletions from the genome at specific time points. This is particularly important for genes whose deletion by traditional homologous recombination in ES cells results in embryonic lethality. Temporal deletions will permit the generation of mice in which the loss of gene function can be studied at different time points.

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#### REFERENCES

- Andres A-C, Schönenberger C-A, Groner B, Hennighausen L, LeMeur M, Gerlinger P (1987): Ha-ras oncogene expression directed by a milk protein gene promoter: Tissue specificity, hormonal regulation, and tumor induction in transgenic mice. *Proc Natl Acad Sci USA* 84:1299–1303.
- Burdon T, Sankaran L, Wall RJ, Spencer M, Hennighausen L (1991): Expression of a whey acidic protein transgene during mammary development: Evidence for different mechanisms of regulation during pregnancy and lactation. *J Biol Chem* 266:6909–6914.
- Chomczynski P, Sacchi N (1987): Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159.
- Furth PA, St. Onge L, Boger H, Gruss P, Gossen M, Kistnes A, Bujard H, Hennighausen L (1994): Temporal control of gene expression in transgenic mice by a tetracycline responsive promoter. *Proc Natl Acad Sci USA* 91:9302–9306.
- Gatz C, Kaiser A, Wendenburg R (1991): Regulation of a modified CaMV 35S promoter by the Tn 10–encoded Tet repressor in transgenic tobacco. *Mol Gen Genet* 227:229–237.
- Gossen M, Bujard H (1992): Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci USA* 89:5547–5551.
- Groner B (1992): Oncogene expression in mammary epithelial cells. *J Cell Biochem* 49:128–136.
- Guy CT, Cardiff RD, Muller WJ (1992): Induction of mammary tumors by expression of polyomavirus middle T oncogene: A transgenic mouse model for metastatic disease. *Mol Cell Biol* 12:954–961.
- Hennighausen L (1992): The prospects for domesticating milk protein genes. *J Cell Biochem* 49:325–332.
- Hennighausen L, McKnight RA, Burdon T, Baik M, Wall RJ, Smith GH (1994): Whey acidic protein extrinsically expressed from the mouse mammary tumor virus long terminal repeat results in hyperplasia of the coagulation gland epithelium and impaired mammary development. *Cell Growth Differ* 5:607–613.
- Jaenisch R (1988): Transgenic animals. *Science* 240:1468–1474.
- Jhappan C, Geiser AG, Kordon EC, Bagheri D, Hennighausen L, Roberts AB, Smith GH, Merlino G (1993): Targeting expression of a transforming growth factor  $\beta$  1 transgene to the pregnant mammary gland inhibits alveolar development and lactation. *EMBO J* 12:1835–1845.
- Kordon E, McKnight RA, Jhappan C, Hennighausen L, Merlino G, Smith GH (1995): Ectopic TGF $\beta$ 1 expression in the secretory mammary epithelium induces early senescence of the epithelial stem cell population. *Dev Biol* 167:47–61.
- Li B, Greenberg N, Stephens LC, Meyn R, Medina D, Rosen JM (1994): Preferential overexpression of a 172 Arg-Leu mutant p53 in the mammary gland of transgenic mice results in altered lobuloalveolar development. *Cell Growth Differ* 5:711–721.
- McKnight RA, Shamay A, Sankaran L, Wall RJ, Hennighausen L (1992): Matrix attachment regions impart position independent regulation of a tissue specific gene in transgenic mice. *Proc Natl Acad Sci USA* 87:6943–6947.
- Muller WJ, Sinn E, Pattengale PK, Wallace R, Leder P (1988): Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. *Cell* 54:105–115.
- Muller WJ, Lee FS, Dickson C, Peters G, Pattengale P, Leder P (1990): The int-2 gene product acts as an epithelial growth factor in transgenic mice. *EMBO J* 9:907–913.
- Paleyanda RK, Zhang D-W, Hennighausen L, McKnight RA, Lubon H (1994): Regulation of human protein C gene expression by the mouse WAP promoter. *Transgenic Research* 3:335–343.
- Pierce DF Jr, Johnson MD, Matsui Y, Robinson SD, Gold LI, Purchio AE, Daniel CW, Hogen BLM, Moses HL (1993): Inhibition of mammary duct development but not alveolar outgrowth during pregnancy in transgenic mice expressing active TGF- $\beta$ 1. *Genes Dev* 7:2308–2317.
- Pittius CW, Hennighausen L, Lee E, Westphal H, Nicols E, Vitale J, Gordon K (1988): A milk protein gene promoter directs the expression of human tissue plasminogen activator cDNA to the mammary gland in transgenic mice. *Proc Natl Acad Sci USA* 85:5874–5878.
- Sambrook S, Fritsch EF, Maniatis T (1989): “Molecular Cloning: A Laboratory Manual,” 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Schönenberger C-A, Andres A-C, Groner B, van der Valk N, LeMeur M, Gerlinger P (1988): Targeted c-myc gene expression in mammary glands of transgenic mice induces mammary tumors with constitutive milk protein gene transcription. *EMBO J* 7:169–175.
- Schönenberger C-A, Zuk A, Groner B, Jones W, Andres A-C (1990): Induction of the endogenous whey acidic protein (WAP) gene and a WAP-myc hybrid gene in primary murine mammary organoids. *Dev Biol* 327–337.
- Sinn E, Muller W, Pattengale P, Tepler I, Wallace R, Leder P (1987): Coexpression of MMTV/v-Ha-ras and MMTV/c-myc genes in transgenic mice: Synergistic action of oncogenes in vivo. *Cell* 49:465–475.
- Stewart TA, Pattengale PK, Leder P (1984): Spontaneous mammary adenocarcinoma in transgenic mice that carry and express MTV/myc fusion genes. *Cell* 38:627–637.
- Stoecklin E, Botteri F, Groner B (1993): An activated allele of the c-erbB-2 oncogene impairs kidney and lung function and causes early death of transgenic mice. *J Cell Biol* 122:199–208.
- Wang Y, O’Malley BW Jr, Tsai SY, O’Malley BW (1994): A regulatory system for use in gene transfer. *Proc Natl Acad Sci USA* 91:8180–8184.