Time-Sensitive Reversal of Hyperplasia in Transgenic Mice Expressing SV40 T Antigen

Dagmar Ewald, Minglin Li, Shimon Efrat, Gert Auer, Robert J. Wall, Priscilla A. Furth,* Lothar Hennighausen*

The role of viral oncoprotein expression in the maintenance of cellular transformation was examined as a function of time through controlled expression of simian virus 40 T antigen (TAg). Expression of TAg in the submandibular gland of transgenic mice from the time of birth induced cellular transformation and extensive ductal hyperplasia by 4 months of age. The hyperplasia was reversed when TAg expression was silenced for 3 weeks. When TAg expression was silenced after 7 months, however, the hyperplasia persisted even though TAg was absent. Although the polyploidy of ductal cells could be reversed at 4 months of age, cells at 7 months of age remained polyploid even in the absence of TAg. These results support a model of time-dependent multistep tumorigenesis, in which virally transformed cells eventually lose their dependence on the viral oncoprotein for maintenance of the transformed state.

Tumorigenesis is a multistep process that is thought to involve the successive activation of oncogenes and the inactivation of tumor suppressor genes [reviewed in (*1*)]. This hypothesis is strongly supported by experiments with transgenic mice expressing oncogenes (*2*–*6*) under the control of various promoters. Such mice develop solitary tumors in a stochastic fashion, which indicates that additional genetic or cellular events are required for the development of irreversible cellular transformation. However, the length of time required for oncogene-induced phenotypic changes to become irreversible is unknown because it has not been possible to inactivate the transforming transgenes. We used the tetracycline response system (*7*–*9*) to examine the role of a viral oncogene in maintaining the transformed phenotype as a function of time. In this system, the oncogene is transcribed in the absence of tetracycline and is silent in the presence of tetracycline.

The simian virus 40 (SV40) T antigen (TAg) binds to and inactivates two tumor suppressor proteins, p53 and the retinoblastoma protein pRb (*10*–*12*). Transgenic mice expressing TAg show cellular changes that generally increase with the duration of TAg expression and result in hyperplasias (*13*–*16*) and tumor formation (*14*, *15*, *17*, *18*) in the targeted tissues. To determine whether the TAg-induced cellular changes were reversible once the oncoprotein stimulus was removed, we generated transgenic mice in which TAg could be conditionally expressed in the salivary gland. Mice carrying the tetracycline-responsive transactivator (*tTA*) gene under the control of the mouse mammary tumor virus long-terminal repeat (MMTA-LTR) (*MTA* transgene) (Fig. 1A) (*9*) were bred to mice carrying the SV40 TAg coding sequence linked to a *tet*-op promoter (*T* transgene) (Fig. 1A) (*19*) to generate *MTA-T* double transgenic offspring. Transgene activity was detected in the striated ducts of the submandibular gland (Fig. 1B). The tTA-mediated TAg expression was detected in striated ducts by immunocytochemistry (Fig. 1B) and by immunoblot analysis (*20*) (Fig. 1C). No TAg was detected in mice carrying only the *T* transgene (Fig. 1C).

The temporal progression of TAg-induced morphological changes in the submandibular gland, and their potential reversibility, were investigated over a 7-month period. Differences between the glands in nontransgenic and MTA-T mice were first observed when the mice were 2 weeks old. At that age, normal submandibular tissue consists largely of serous acini with little ductal development (Fig. 2A). In the MTA-T mice, scattered foci of transformed cells were detectable (Fig. 2D). By 4 months, the glands of MTA-T mice were distinctly abnormal. The density of striated ducts was increased and the ducts were composed of numerous small cells with a high nuclear:cytoplasmic volume ratio (Fig. 2E). In contrast, the striated ductal cells in nontransgenic mice and in mice carrying either transgene alone displayed an intensely

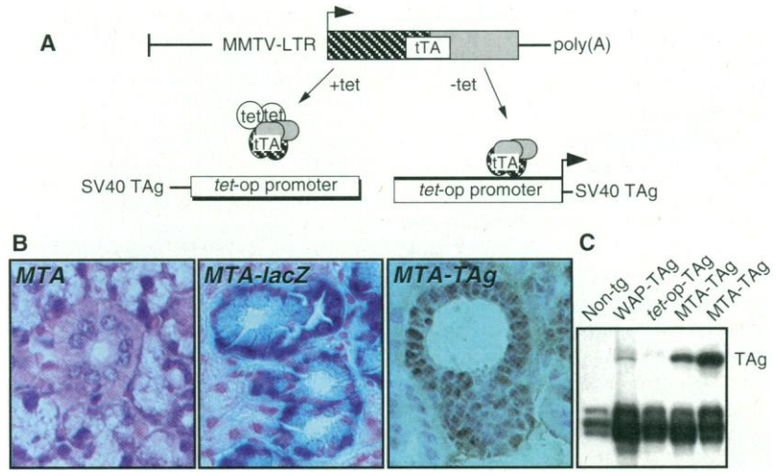

Fig. 1. The tetracycline-responsive transactivator tTA under the control of an MMTV-LTR induces transcription of a *tet*-op–SV40 TAg gene in striated ductal cells of the salivary gland of transgenic mice. (**A**) Structure of the *tTA* (*9*) and the *tet-op-TAg* transgene (*19*); poly(A), polyadenylation signal. The tTA binds to the *tet*-op sequences in the absence (−tet) but not in the presence (+tet) of tetracycline. (**B**) Left, striated ductal cells from the submandibular gland of a 4-month-old transgenic female carrying the *MMTV-tTA* gene. Center, striated ductal cells from a double transgenic female carrying *MMTV-tTA* and a marker *tet-op-lacZ* transgene (*9*), showing that expression of nuclear β-galactosidase is confined to the striated ductal cells. Right, immunostaining with TAg-specific antibodies of striated ducts from a double transgenic female carrying *MMTV-tTA* and *tet-op-TAg* genes, showing expression of TAg in the nuclei. (**C**) Immunoblot analysis of protein from salivary tissue of a nontransgenic female (non-tg), from mammary tissue of a mouse in which TAg expression had been targeted to that tissue (WAP-TAg) (15), from salivary tissue of a *tet*-op-TAg transgenic mouse (*tet*-op-TAg), and from salivary tissue of two independent double transgenic MTA-T mice.

D. Ewald and L. Hennighausen, Laboratory of Biochemistry and Metabolism, National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, USA.
M. Li and P. A. Furth, Division of Infectious Diseases, Department of Medicine, University of Maryland Medical School and the Institute of Human Virology, Baltimore, MD 21201, USA.
S. Efrat, Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, NY 10461, USA.
G. Auer, Department of Oncology and Pathology, Karolinska Institute, S-17176 Stockholm, Sweden.
R. J. Wall, Agricultural Research Service, United States Department of Agriculture, Beltsville, MD 20705, USA.
*To whom correspondence should be addressed at furth@ncifcrf.gov (for P. A. Furth) or hennighausen@nih.gov (for L. Hennighausen).

eosinophilic cytoplasm with a low nuclear: cytoplasmic volume ratio (Fig. 2B). The number of striated ducts and the hyperproliferative changes increased with age. When the MTA-T mice were 7 months old, the number of serous cells had declined and the glandular structure was completely disrupted with extensive ductal hyperplasia (Fig. 2F). The hyperproliferative changes remained confined to the striated ductal epithelium.

We studied the reversibility of these hyperplastic changes after silencing TAg expression by administering tetracycline to these mice at 4 or 7 months of age (*21*). In the absence of tetracycline, hyperplastic ducts composed of transformed cells filled the submandibular glands of 4-month-old females (Fig. 2E). TAg was detected in the majority of ductal cells (Fig. 3E). After a 3-week treatment with tetracycline, the gland changed dramatically in appearance. Although the density of ductal structures remained high, transformed ductal cells were no longer detectable (Fig. 2C). This cellular change correlated with the loss of TAg expression (Fig. 3F). Administration of tetracycline to 7-month-old mice yielded different results. Large areas of transformed ductal cells persisted (Fig. 2H and Table 1) despite the complete loss of TAg expression (*22*). The clonality and temporal appearance of hyperplastic ducts were evaluated in 4 month-old-mice that had been on tetracycline for 3 weeks and were then taken off the drug. Within 3 weeks after reactivation of the *TAg* transgene, multiple hyperplastic foci were detected (*23*).

Submandibular tissue from transgenic mice with an active or silenced *TAg* transgene was evaluated for the presence of polyploidy (*24*). Epithelial cells in TAg-induced hyperplastic ducts (Fig. 3A) of 4-month-old mice were polyploid (Fig. 3C). Hyperplasia and polyploidy of individual cells correlated with the presence of TAg (Fig. 3E). Upon reversal of hyperplasia (Fig. 3B), the cells had an average DNA content of 2N (*25*) (Fig. 3D). In 4-month-old mice, few selected foci remained hyperplastic (Fig. 3B) in the absence of TAg staining (Fig. 3F). When the same experiment was performed with 7-month-old mice, relatively few ducts showed reversal of hyperplasia, and the cells that did not revert remained polyploid despite the absence of TAg (*25*).

Our findings support the notion that

Table 1. Characteristics of submandibular glands in 4- and 7-month-old transgenic mice expressing or not expressing TAg. No interaction between age and tetracycline treatment was detected ($P = 0.652$). On average, 4-month-old mice had more total ducts per field than did 7-month-old mice (119 ± 3 versus 98 ± 4, $P < 0.01$). Tetracycline (tet) treatment had no influence on the total number of ducts (102 ± 4 versus 112 ± 3, $P = 0.016$). Numbers of ducts are means ± SD. There was an interaction between age and tetracycline treatment for hyperplasia ($P < 0.01$). Tetracycline significantly reduced the hyperplasia in 4-month-old mice, but the reduction was less dramatic in 7-month-old mice.

Treatment	Mice (*n*)	Age (months)	TAg expression	Total ducts (*n*)	Hyperplastic ducts (*n*)	Ploidy of ductal cells
−tet	4	4	Yes	127 ± 4†	110 ± 4†	Polyploid
+tet	4	4	No	112 ± 5†‡	17 ± 3‡	2N
−tet	4	7	Yes	103 ± 4‡	96 ± 5†	Polyploid
+tet	2	7	No	93 ± 5‡	71 ± 12§	Polyploid
+tet/−tet*	2	4	Yes	106 ± 6	41 ± 7	ND

*The tetracycline pellet was removed after 3 weeks and submandibular tissue was analyzed after an additional 3 weeks during which TAg was expressed. Polyploidy was not determined (ND). †‡§The means within a column with different footnote symbols are statistically different ($P < 0.01$).

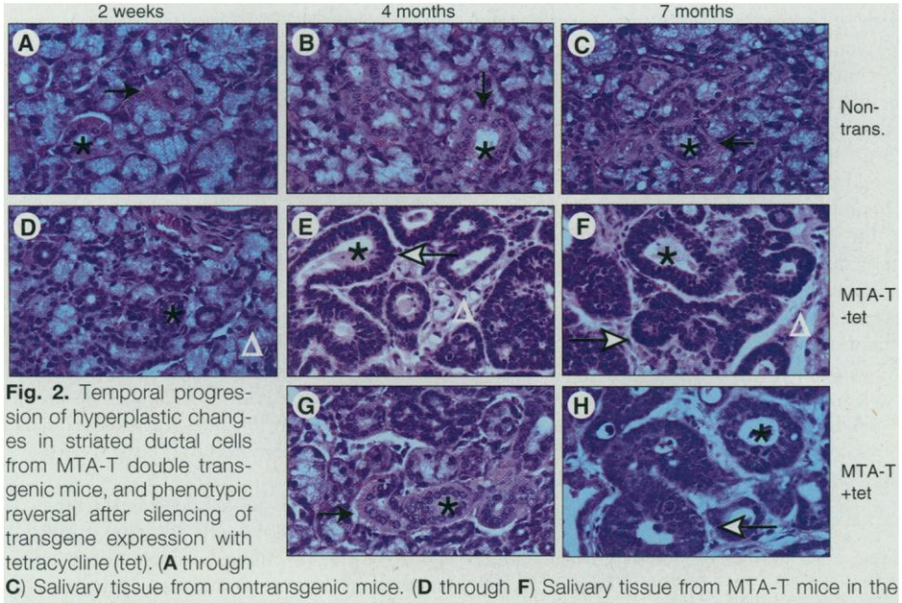

Fig. 2. Temporal progression of hyperplastic changes in striated ductal cells from MTA-T double transgenic mice, and phenotypic reversal after silencing of transgene expression with tetracycline (tet). (**A** through **C**) Salivary tissue from nontransgenic mice. (**D** through **F**) Salivary tissue from MTA-T mice in the absence of tetracycline (–tet). (**G** and **H**) Salivary tissue from MTA-T mice that had been exposed to tetracycline for 3 weeks (+tet). Tissue was stained with hematoxylin and eosin (H&E) and the magnification was ×630. Four MTA-T mice were analyzed at 2 weeks of age. Twelve 4-month-old MTA-T mice were analyzed without tetracycline treatment and nine were analyzed after tetracycline treatment. Eight 7-month-old MTA-T mice were analyzed without tetracycline treatment and seven were analyzed after tetracycline treatment. Asterisks indicate ductal luminae, triangles indicate serous acini, black arrows indicate normal striated ductal cells, and white arrows indicate hyperplastic ducts.

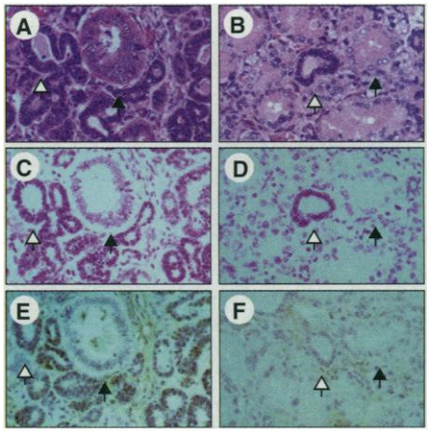

Fig. 3. Correlation of cellular ploidy with TAg expression in the submandibular gland of 4-month-old transgenic mice in which TAg had been continuously active since birth. (**A**) H&E staining, (**C**) Feulgen staining, and (**E**) immunocytochemical staining for TAg. The black arrow points to a nontransformed duct (A) with 2N ploidy (C) and no TAg expression (E). The white arrow points to a polyploid hyperplastic duct that expresses TAg. Serial sections were analyzed to follow a particular ductal structure. (**B**) H&E staining, (**D**) Feulgen staining, and (**F**) immunocytochemical staining for TAg of 4-month-old transgenic mice in which TAg expression had been silenced 3 weeks before analysis. The white arrow points to a rare remaining hyperplastic duct (B) which is polyploid (D) but does not express TAg (F). The black arrow points to a phenotypically reverted duct.

cumulative changes occur in TAg-expressing mice between 4 and 7 months of age that prohibit reversal of cellular transformation even in the absence of the primary transforming stimulus. The nature of the these changes remains to be identified.

REFERENCES AND NOTES

1. R. A. Weinberg, *Ann. N.Y. Acad. Sci.* **758**, 331 (1995).
2. E. Sinn *et al.*, *Cell* **49**, 465 (1987).
3. W. J. Muller, E. Sinn, P. K. Pattengale, R. Wallace, P. Leder, *ibid.* **54**, 105 (1988).
4. T. A. Stewart, P. K. Pattengale, P. Leder, *ibid.* **38**, 627 (1984).
5. L. A. Donehower *et al.*, *Genes Dev.* **9**, 882 (1995).
6. A. Elson, C. Deng, J. Campos-Torres, L. A. Donehower, P. Leder, *Oncogene* **11**, 181 (1995).
7. M. Gossen and H. Bujard, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5547 (1992).
8. P. A. Furth *et al.*, *ibid.* **91**, 9302 (1994).
9. L. Hennighausen, R. Wall, U. Tillmann, M. Li, P. A. Furth, *J. Cell. Biochem.* **59**, 463 (1995).
10. J. A. DeCaprio *et al.*, *Cell* **54**, 275 (1988).
11. D. P. Lane and L. V. Crawford, *Nature* **278**, 261 (1979).
12. D. I. H. Linzer and A. J. Levine, *Cell* **17**, 43 (1979).
13. J. M. Adams and S. Cory, *Science* **254**, 1161 (1991).
14. N. M. Greenberg *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 3439 (1995).
15. M. Li, J. Hu, K. Heermeier, L. Hennighausen, P. A. Furth, *Cell Growth Differ.* **7**, 3 (1996).
16. J. C. Pascall, M. A. Surani, S. C. Barton, T. J. Vaughan, K. D. Brown, *J. Mol. Endocrinol.* **12**, 313 (1994).
17. H. Symonds *et al.*, *Cell* **78**, 703 (1994).
18. Y. J. Tzeng, E. Guhl, M. Graessmann, A. Graessmann, *Oncogene* **8**, 1965 (1993).
19. S. Efrat, D. Fusco-DeMane, H. Lemberg, O. Al Emran, X. Wang, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 3576 (1995).
20. Salivary gland tissue was homogenized in 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, phenylmethylsulfonyl fluoride (10 mg/ml), aprotinin (30 μl/ml) (Sigma), and 100 mM sodium orthovanadate in phosphate buffered saline. Proteins (20 μg) were separated in an 8% SDS–polyacrylamide gel and transferred onto a NOVEX polyvinylidene diflouride membrane by means of a NOVEX Western Transfer Apparatus. After transfer and blocking with buffer [5% nonfat milk, 10 mM tris-HCl (pH 7.5), 100 mM NaCl, and 0.1% Tween-20] for 1 hour at room temperature, the membrane was exposed to a 1:400 dilution of mouse monoclonal antibody Pab 101 (Santa Cruz Biotechnology, Santa Cruz, CA) followed by exposure to a 1:4000 dilution of peroxidase-conjugated rabbit polyclonal antibody to mouse immunoglobulin G (Jackson Immunochemical, West Grove, PA) for 1 hour at room temperature. The proteins were visualized with the ECL protein immunoblotting protocol (Amersham).
21. Slow-release tetracycline pellets (Innovative Research of America, Toledo, OH) were implanted subcutaneously in the shoulder region with the use of a trochar according to the manufacturer's directions. These pellets released 0.7 mg of tetracycline hydrochloride per day over a 21-day period.
22. P. A. Furth, M. Li, L. Hennighausen, unpublished data.
23. P. A. Furth and L. Hennighausen, unpublished data. Quantative analyses on two mice revealed that approximately 40% of the ducts were hyperplastic.
24. The degree of ploidy was determined on Feulgen-stained paraffin sections.
25. L. Hennighausen and G. Auer, unpublished data.

26. We thank G. Robinson for critical reading of the manuscript and K. Heermeier, X. Liu, K.-U. Wagner, and J. Hu for technical advice. Supported in part by a Pangborn Award from the University of Maryland Medical School (to P.A.F.).

13 March 1996; accepted 25 June 1996

Correction of the Mutation Responsible for Sickle Cell Anemia by an RNA-DNA Oligonucleotide

Allyson Cole-Strauss,* Kyonggeun Yoon,* Yufei Xiang, Bruce C. Byrne, Michael C. Rice, Jeff Gryn, William K. Holloman, Eric B. Kmiec†

A chimeric oligonucleotide composed of DNA and modified RNA residues was used to direct correction of the mutation in the hemoglobin β^S allele. After introduction of the chimeric molecule into lymphoblastoid cells homozygous for the β^S mutation, there was a detectable level of gene conversion of the mutant allele to the normal sequence. The efficient and specific conversion directed by chimeric molecules may hold promise as a therapeutic method for the treatment of genetic diseases.

Sickle cell anemia is the classic prototype of a hereditary hemoglobinopathy resulting from a point mutation in the β-globin gene (*1*, *2*). The clinical condition results from homozygosity of the sickle cell allele β^S, in which an A-to-T mutation within the sixth codon of the β-globin coding region changes the normal glutamic acid residue to a valine. The severity and prevalence of this disease have made sickle cell anemia a candidate for gene therapy. Over the past several years a variety of strategies for genetic modification have been devised. These have included transduction methods based on retrovirus and adeno-associated virus vectors to deliver the normal β- or γ-globin gene to hematopoietic progenitor cells (*3*). Nevertheless, the attendant random integration of retrovirus vectors and genomic rearrangements accompanying integration of adeno-associated viruses are undesirable consequences of gene transfer mediated by viral vectors. Correction of the β^S allele to β^A through a gene conversion mechanism would provide a means for gene therapy that circumvents this problem.

In a recent study from this laboratory, a procedure was reported for correcting the mutated form of an extrachromosomal gene present on a plasmid by use of a chimeric oligonucleotide composed of DNA and RNA residues (*4*). The design of this molecule was prompted by the discovery that RNA-DNA hybrids were highly active in homologous pairing reactions in vitro and that hairpin caps at the ends of hybrid molecules were no impediment to pairing (*5*). These observations suggested a strategy for targeted correction in which a short, double-stranded oligonucleotide vector is activated for recombination by incorporating RNA residues and protected from exonucleolytic degradation by capping both ends. The 2′-O-methyl modification of ribose of the RNA added protection against cleavage by ribonuclease (RNase) H activities.

To correct the β^S mutation, we designed the chimeric oligonucleotide (SC1) as a single molecule (with two sequences that were inverted and complementary) capable of folding back on itself to form a duplex structure (Fig. 1). The molecule was composed of DNA residues with two intervening blocks of 10 2′-O-methyl RNA residues flanking a short stretch of five DNA residues. When the molecule was folded into the duplex conformation, the sequence of one strand comprised all DNA residues whereas the other strand contained the RNA-DNA blocks. In this case, the internal sequence is complementary to the β^S-globin sequence over a stretch of 25 residues that span the site of the β^S mutation, with the exception of a single base (T). The five DNA residues flanked by RNA residues were centered about the mutant T residue in the β^S coding sequence. A control chimeric oligonucleotide (SC2) was designed in the same manner with the exception of one base (A; designated in bold and with an asterisk in Fig. 1).

The chimeric molecule was introduced into lymphoblastoid cells (B cells) homozygous for the β^S allele by means of a commercial liposome formulation, and the cells were assayed for correction after 6 hours (*6*, *7*). Correction of the single base mutation was assessed by taking advantage of restriction fragment length polymorphisms (RFLPs) resulting from the β^S mutation (*2*): the A-to-T transversion in the β^S allele results in the loss of a Bsu 36I restriction

A. Cole-Strauss, K. Yoon, Y. Xiang, M. C. Rice, E. B. Kmiec, Department of Pharmacology, Kimmel Cancer Center, Thomas Jefferson University, 233 South 10th Street, Philadelphia, PA 19107, USA.
B. C. Byrne and J. Gryn, Division of Hematology-Oncology, Department of Medicine, Cooper Hospital University Medical Center, Camden, NJ 08103, USA.
W. K. Holloman, Department of Microbiology, Cornell University School of Medicine, 1300 York Avenue, New York, NY, USA.

*These authors contributed equally to this work.
†To whom correspondence should be addressed.