Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 3576–3580, April 1995
Biochemistry

Conditional transformation of a pancreatic β-cell line derived from transgenic mice expressing a tetracycline-regulated oncogene

(diabetes/inducible oncogene/insulinoma/simian virus 40 large tumor antigen)

SHIMON EFRAT*, DAVID FUSCO-DEMANE, HADAS LEMBERG, OBAIDULLAH AL EMRAN, AND XIAORONG WANG

Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, NY 10461

Communicated by Stanley G. Nathanson, Albert Einstein College of Medicine of Yeshiva University, Bronx, NY, January 10, 1995

ABSTRACT **Conditional oncogene expression in transgenic mice is of interest for studying the oncoprotein requirements during tumorigenesis and for deriving cell lines that can be induced to undergo growth arrest and enhance their differentiated functions. We utilized the bacterial tetracycline (Tet)-resistance operon regulatory system (*tet*) from Tn*10* of *Escherichia coli* to control simian virus 40 (SV40) large tumor (T) antigen (TAg) gene expression and to generate conditionally transformed pancreatic β cells in transgenic mice. A fusion protein containing the *tet* repressor (*tet*R) and the activating domain of the herpes simplex virus protein VP16, which converts the repressor into a transcription activator, was produced in β cells of transgenic mice under control of the insulin promoter. In a separate lineage of transgenic mice, the TAg gene was introduced under control of a tandem array of *tet* operator sequences and a minimal promoter, which by itself is not sufficient for gene expression. Mice from the two lineages were then crossed to generate double-transgenic mice. Expression of the *tet*R fusion protein in β cells activated TAg transcription, resulting in the development of β-cell tumors. Tumors arising in the absence of Tet were cultured to derive a stable β-cell line. Cell incubation in the presence of Tet led to inhibition of proliferation, as shown by decreased BrdUrd and [^{3}H]thymidine incorporation. The Tet derivative anhydrotetracycline showed a 100-fold stronger inhibition compared with Tet. When administered *in vivo*, Tet efficiently inhibited β-cell proliferation. These findings indicate that transformed β cells selected for growth during a tumorigenesis process *in vivo* maintain a dependence on the continuous presence of the TAg oncoprotein for their proliferation. This system provides an approach for generation of β-cell lines for cell therapy of diabetes as well as conditionally transformed cell lines from other cell types of interest.**

Conditional regulation of oncogene expression in transgenic mice may provide important insights into their role in tumorigenesis. The ability to turn the oncogene expression on or off at various stages during tumor progression may allow the assessment of the oncoprotein requirement at each stage. In addition, such a system will permit the generation of conditionally transformed cell lines. Targeted oncogenesis in transgenic mice has proved to be a valuable approach for generation of cell lines from rare cell types. However, the transformed cells are often dedifferentiated with respect to highly specialized functions, when compared to their nonproliferating counterparts. The ability to induce growth arrest in the cells following the establishment of the cell line may provide fully differentiated cells for functional studies.

The simian virus 40 (SV40) gene encoding the large tumor (T) antigen (TAg) has been widely used for cell transformation in transgenic mice (1). Unlike most other oncogene products, TAg alone can transform cells in the absence of other oncoproteins. This is due to its multiple effects on cell replication, including inactivation of the growth-suppressing proteins p53 and Rb (retinoblastoma protein), stimulation of gene transcription, and DNA unwinding (2). The use of temperature-sensitive TAg mutants allowed the establishment of conditional cell lines from a number of cell types (3). However, this approach does not allow the manipulation of TAg gene expression *in vivo*. The bacterial *lac* repressor/operator system has been employed to regulate TAg gene expression when introduced into cultured cells (4). However, the use of this approach *in vivo* is limited by the difficulty to control gene expression by the administration of lactose analogs such as isopropyl β-D-thiogalactoside. Another approach, relying on DNA recombination using the Cre recombinase–*lox* (locus of crossing over) strategy to delete an intervening DNA sequence that inhibits TAg gene expression, was successfully applied to activate TAg gene expression in lens cells in transgenic mice (5). However, the recombination event is irreversible and does not allow repeated on–off switching of the gene.

A different bacterial control system has been described that is based on the tetracycline (Tet)–resistance operon (*tet* from Tn*10* of *Escherichia coli*; ref. 6). The *tet* repressor protein called *tet*R, inhibits expression of bacterial Tet-resistance genes by binding to cognate operator sequences in their regulatory regions. The repressor is inactivated in the presence of Tet, thereby allowing gene transcription and resistance to the antibiotic effects of Tet. Labow *et al.* (7) have demonstrated that the *E. coli lac* repressor can be converted into a transcription activator by fusion with the transcription activation domain of the herpes simplex virus (HSV) VP16 protein. Similarly, the fusion of the *tet*R to VP16 by Gossen and Bujard (6) has generated a hybrid transactivator that specifically stimulates transcription of promoters that include *tet* operator sequences. In the presence of Tet, the binding of the transactivator to the promoter is impaired, resulting in shutoff of a reporter gene expression in cultured cells (6) and in transgenic mice (8). Employing *tet*R as part of an activator allows tighter regulation of the target gene, compared with its use to inhibit gene expression.

TAg gene expression in transgenic mouse pancreatic β cells under control of the rat insulin promoter (RIP) results in general islet hyperplasia (9). A small number of the islets progress to develop into tumors, indicating that other events, in addition to TAg activity, are required for tumor formation (9). β tumor cell (βTC) lines can be derived from the insulinomas that maintain many characteristics of normal β cells (10,

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: Tet, tetracycline antibiotic; *tet*, tetracycline-resistance operon from Tn10 of *Escherichia coli*; ATet, anhydrotetracycline; βTC, beta tumor cells; HSV, herpes simplex virus; RIP, rat insulin promoter; TAg, large tumor (T) antigen; SV40, simian virus 40; *tet*R, *tet* repressor; tTA, Tet-controlled transcription activator.

*To whom reprint requests should be addressed.

11). We utilized the *tet* system to regulate TAg gene expression in β cells in transgenic mice. The *tet*R-VP16 fusion protein, denoted Tet-controlled transcription activator (tTA), was produced in β cells of transgenic mice under control of RIP. In a separate lineage of transgenic mice, the TAg gene was introduced under control of a tandem array of *tet* operator sequences and a minimal promoter. In the double-transgenic mice, tTA expression in β cells activated transcription of the TAg gene as judged by the development of β cell tumors. Cells from a βTC line derived from a tumor undergo growth arrest in the presence of Tet both in culture and *in vivo*. This suggests that β cells transformed by TAg require the oncoprotein activities for their continuous proliferation.

MATERIALS AND METHODS

Plasmid Constructs. To construct the tet-TAg plasmid, the *Xho* I–*Xba* I fragment from pUHD-10-3 (6), containing a tandem array of seven copies of the *tet* operator sequence and a cytomegalovirus minimal promoter, was placed in front of the TAg gene in pRIP-TAg (9) by deleting the *Aat* II–*Xba* I fragment containing the RIP from pRIP-TAg and converting the *Aat* II and *Xho* I sites into blunt ends. To generate pRIP-tTA, the *Eco*RI–*Bam*HI tTA fragment from pUHD-15-1 (6) was made blunt-ended with Klenow fragment and ligated into the *Sma* I site of pMLSIS.CAT (12) after removal of the *Pst* I–*Sma* I fragment of the gene encoding chloramphenicol acelyltransferase (CAT). This placed the tTA gene downstream of a hybrid intron element and upstream of the SV40 late polyadenylylation site. The combined 1630-bp fragment was inserted between the *Xba* I and *Sal* I sites of pRIP-TAg downstream of the RIP.

Transgenic Mice. Linearized plasmid DNA was microinjected into one-cell C3HeB/FeJ mouse embryos. Transgenic mice were generated and bred according to established procedures (13).

Cell Culture. Tumors were excised from the pancreas, and a β-cell line, denoted βTC-tet, was established and propagated as described (10). All media supplies were from GIBCO. Cells were grown in Dulbecco's modified Eagle's medium containing 25 mM glucose and supplemented with 15% (vol/vol) horse serum, 2.5% (vol/vol) fetal bovine serum, 100 units of penicillin per ml, and 100 μg of streptomycin per ml. Tet (United States Biochemical) and anhydrotetracycline (ATet) (Lederle) were included at the indicated concentrations.

Cell Implantation. Cells were trypsinized, washed in phosphate-buffered saline (PBS), and resuspended in PBS at 5×10^6 cells per ml. Recipient C3H mice were injected (i.p.) with 10^6 cells each and were maintained on regular drinking water or on water containing 1 mg of Tet per ml and 2.5% sucrose. Each group included four or five mice. They were monitored weekly for blood glucose with Glucometer strips. To generate diabetic mice, 12 C3H male mice were injected i.p. with one dose of 200 mg of streptozotocin (Sigma) per kg of body weight, followed 7 days later by three doses of 50 mg/kg on 3 consecutive days, which caused hyperglycemia within 6–9 additional days. Eight mice were then injected i.p. with 2×10^6 βTC-tet cells, while four mice were kept as diabetic controls. Mice were monitored weekly for blood glucose levels. When euglycemia was obtained in the cell-implanted group, four of the mice in this group were implanted subcutaneously with a slow-release Tet pellet (Innovative Research of America) designed to release 3.3 mg per day and were followed by weekly blood glucose checks. Glucose levels below 40 mg/dl were determined with a Beckman glucose analyzer.

Immunohistochemistry. Cells were plated in 16-well slides (Nunc) for the indicated period in the absence or presence of Tet or ATet. For BrdUrd incorporation assay, cells were pulsed for 60 min with 10 μM BrdUrd (Sigma) and stained with an anti-BrdUrd monoclonal antibody (Becton Dickinson) according to the manufacturer's recommendations. The bound antibody was visualized with biotinated anti-mouse IgG and horseradish peroxidase-conjugated avidin (ABC Kit, Vector Laboratories) and a diaminobenzidine (DAB) substrate. Cells in separate wells were stained with a rabbit anti-TAg serum as described (14). The bound antibody was visualized with a horseradish peroxidase-conjugated goat anti-rabbit antibody and DAB. Mice with βTC-tet tumors were injected i.p. with 100 μg of BrdUrd per g of body weight. One hour later they were sacrificed, and the tumors were removed, fixed with 4% buffered formaldehyde, processed for paraffin embedding, and sectioned. Tumor sections were stained with anti-TAg and anti-BrdUrd antibodies as described above.

Thymidine Incorporation Assay. Cells (2×10^4) were seeded into 96-well plates. Following the indicated incubation, they were pulsed with 1 μCi (37 kBq) of [*methyl*-^{3}H]thymidine (Amersham, 78 Ci/mmol) for 6 hr. The cells were then lysed in water with a cell harvester, and the DNA was retained on a glass fiber filter (Whittaker). The filters were dried, and the radioactivity incorporated into DNA was quantitated with a scintillation counter. Each condition was assayed in quadruplicate.

RESULTS

The tTA gene was placed under control of the RIP (Fig. 1), and the construct was used to generate transgenic mice in which tTA would be constitutively expressed specifically in β cells. In a separate lineage of transgenic mice, the TAg gene was introduced under control of a minimal promoter and a tandem array of *tet* operator sequences (Fig. 1). This promoter does not allow expression of the TAg gene by itself. Therefore, as expected, these transgenic mice did not develop tumors. The two lines of mice were crossed to generate double-transgenic mice. In these mice the *tet*R part of the tTA protein is expected to bind to the target *tet* operator sequences in β cells and allow the VP16 part of the molecule to activate transcription of the TAg gene. This resulted in the development of multiple β-cell tumors by 5–6 months of age. No tumors were detected in other organs, demonstrating the need for the tTA-induced expression of TAg for the β-cell-specific tumor development.

Cells from the tumors were cultured to derive a stable cell line, denoted βTC-tet. When incubated in the presence of Tet at 1 μg/ml, the cells undergo growth arrest, as demonstrated by the difference in cell number and size of the colonies in *A* and *B* of Fig. 2. This effect is reversible. Removal of Tet after a 5-week incubation resulted in resumed cell replication (not shown). The effect of Tet on DNA replication in the βTC-tet cells was analyzed by visualizing BrdUrd incorporation with an anti-BrdUrd monoclonal antibody. In the absence of Tet, many replicating cells incorporated BrdUrd during a 1-hr pulse (Fig. 2*C*). After a 3-day incubation with Tet at 1 μg/ml, only a small

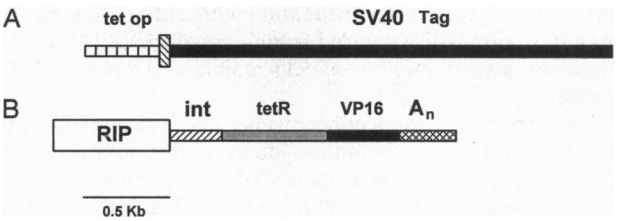

FIG. 1. Gene constructs used in the conditional transformation strategy. (*A*) tet-TAg construct. The SV40 TAg gene was placed under control of a tandem array of *tet* operator sequences and a minimal promoter (hatched box). (*B*) RIP-tTA construct. A fusion gene encoding the *tet*R protein and the activating domain of the herpes simplex virus VP16 protein was placed under control of the RIP, downstream of an intron element (int) and upstream of a polyadenylylation signal (A_n).

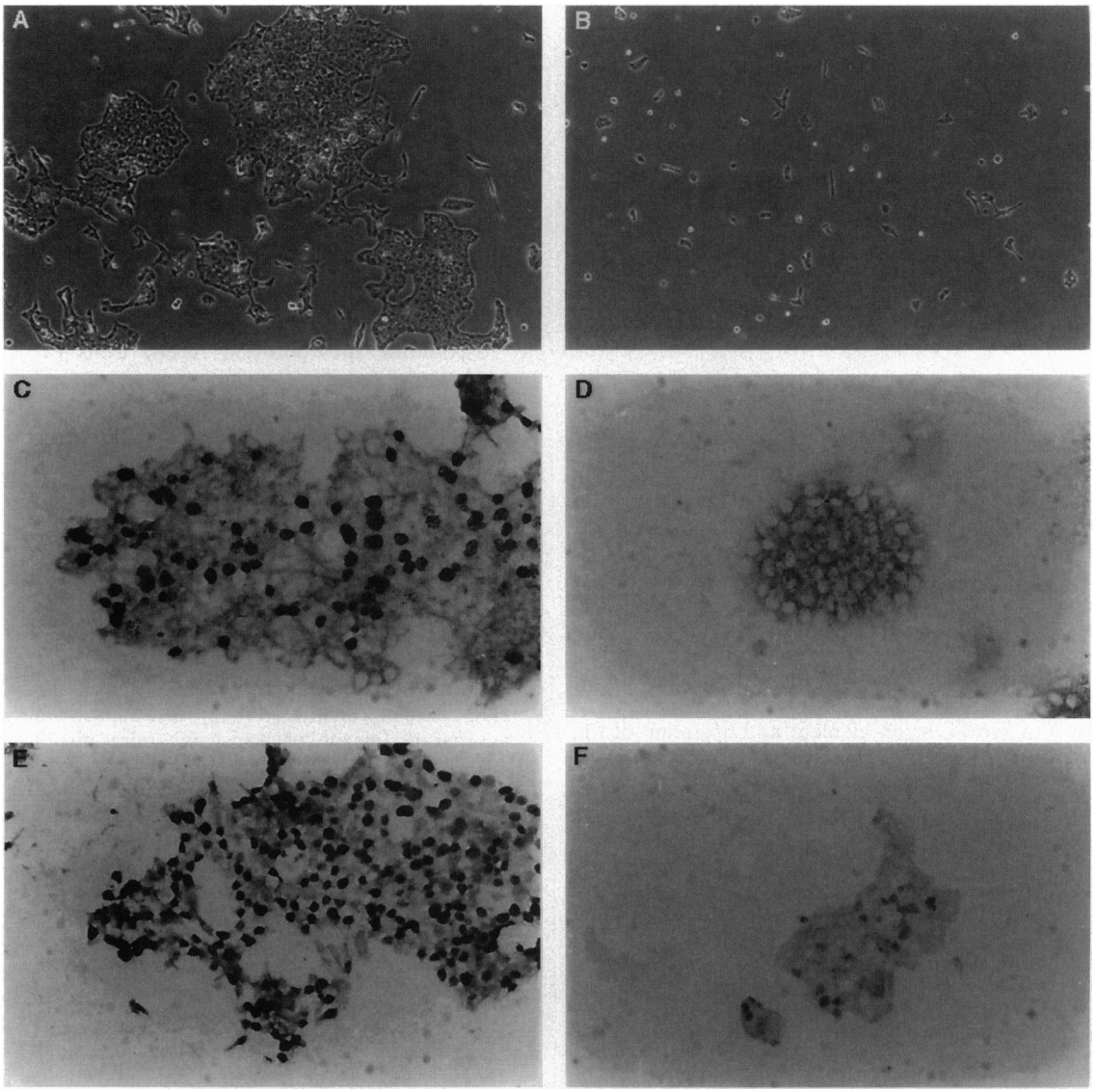

FIG. 2. Effect of Tet on βTC-tet cell growth and TAg gene expression. Equal numbers of βTC-tet cells were seeded into two series of wells. They were grown for 3 weeks in the absence (*A*) or presence (*B*) of Tet at 1 μg/ml and photographed in a phase-contrast microscope. Similar cells were incubated in 16-well slides for 7 days in the absence (*C* and *E*) or presence (*D* and *F*) of Tet at 1 μg/ml. Cells were pulsed for 1 hr with BrdUrd (*C* and *D*) and stained with an anti-BrdUrd monoclonal antibody. Cells in separate wells were stained with an anti-TAg antiserum (*E* and *F*). The bound antibodies were visualized with horseradish peroxidase-conjugated second antibodies. The cells shown are representative of three independent experiments. (×200.)

number of cells incorporated BrdUrd (data not shown). After a 7-day incubation, no cells were observed to incorporate BrdUrd (Fig. 2*D*). The effect of Tet on TAg gene expression was analyzed by immunohistochemistry with an anti-TAg antiserum. After a 7-day incubation with Tet at 1 μg/ml, TAg immunostaining disappeared from most of the cells (Fig. 2*F*). However, a small number of cells maintained detectable TAg staining.

The effect of various concentrations of Tet on cell growth was analyzed by [^{3}H]thymidine incorporation into replicating DNA (Fig. 3). A complete shutoff of replication was achieved in the presence of Tet at 100 ng/ml. The Tet derivative ATet has been shown to bind *tet*R 35 times more strongly than it binds Tet (15). As shown in Fig. 3, incubation of βTC-tet cells in the presence of ATet at 1 ng/ml resulted in complete growth arrest.

Cells from another β-cell line, βTC3 (10), which was derived from mice expressing a RIP-controlled TAg gene that is not responsive to the Tet regulation, were used as controls in these experiments. Incubation of βTC3 cells in the presence of either Tet or ATet at 1 μg/ml did not affect their growth rate, BrdUrd and [^{3}H]thymidine incorporation, and TAg staining (not shown).

To test the ability of Tet to regulate cell growth *in vivo*, syngeneic C3H mice were injected with 10^6 βTC-tet cells i.p. βTC cell lines are tumorigenic and form benign tumors at the site of injection (10). Tumor development leads to hypoglycemia and can be detected by monitoring blood glucose levels. Mice in one group were maintained on drinking water containing Tet. None of them developed tumors within 14 weeks, as judged by blood glucose measurements and a careful autopsy. No abnormalities were observed as a result of the prolonged Tet treatment. Mice maintained in the absence of Tet developed hypoglycemia and tumors within 8–13 weeks. When hypoglycemia was detected, one subgroup received Tet-containing drinking water for 7 days, while another subgroup continued to drink regular water. The mice were then pulsed with BrdUrd and sacrificed, and the tumors were removed and processed for immunohistochemical analyses. Tumors from mice that were not treated with Tet contained

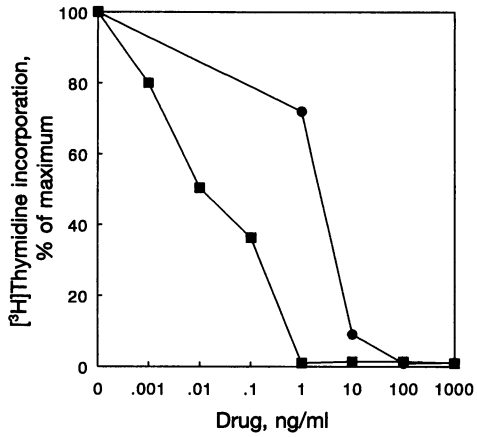

FIG. 3. Growth arrest of βTC-tet cells after incubation with Tet and ATet. Cells (2×10^4) in quadruplicate wells were incubated for 7 days in the presence of the indicated concentration of Tet (●) or ATet (■). They were then pulsed with [^{3}H]thymidine for 6 hr, followed by quantitation of the radioactivity incorporated into DNA. Values represent the percentage of cpm in the absence of drugs, averaging 4×10^4 cpm per well.

numerous cells that stained for BrdUrd and TAg (Fig. 4 *A* and *C*). In contrast, tumors from mice treated for 7 days with Tet showed no BrdUrd and TAg staining (Fig. 4 *B* and *D*). These results demonstrate that Tet effectively inhibits TAg gene expression and β-cell proliferation *in vivo*.

The βTC-tet cells demonstrate correct responsiveness to glucose in the physiological concentration range (data not shown). To evaluate their capacity to maintain glucose homeostasis *in vivo*, βTC-tet cells were implanted into diabetic recipients (Fig. 5). The cell implantation led to correction of hyperglycemia within 2 weeks, demonstrating the ability of βTC-tet cells to function as normal β cells *in vivo*. As observed in the past with other βTC lines, the implanted cells continued to proliferate in mice not treated with Tet, which resulted in hypoglycemia and premature death. In contrast, in mice implanted with slow-release Tet pellets, blood glucose levels were stabilized in the normal range. The normal blood glucose levels were maintained for as long as the mice were followed—4 months after Tet implantation. These results indicate that the cells undergo growth arrest as a result of Tet-induced inhibition of TAg gene expression but remain viable and capable of normal glucose sensing and insulin production and secretion.

DISCUSSION

These results demonstrate the ability to regulate oncogene expression and cell proliferation by controlling the binding of the *tet*R to its cognate operator sequence in the presence of Tet or its derivative ATet. The concentration of Tet required for complete inhibition of cell replication is <0.1 μg/ml. ATet, which binds the *tet*R protein with a higher affinity, can achieve this effect at a concentration lower by 2 orders of magnitude. At the same time, ATet has a very weak antibiotic activity, since its binding to the bacterial ribosome is greatly reduced, compared with Tet (15). These properties render ATet a more attractive ligand for prolonged treatments *in vivo*.

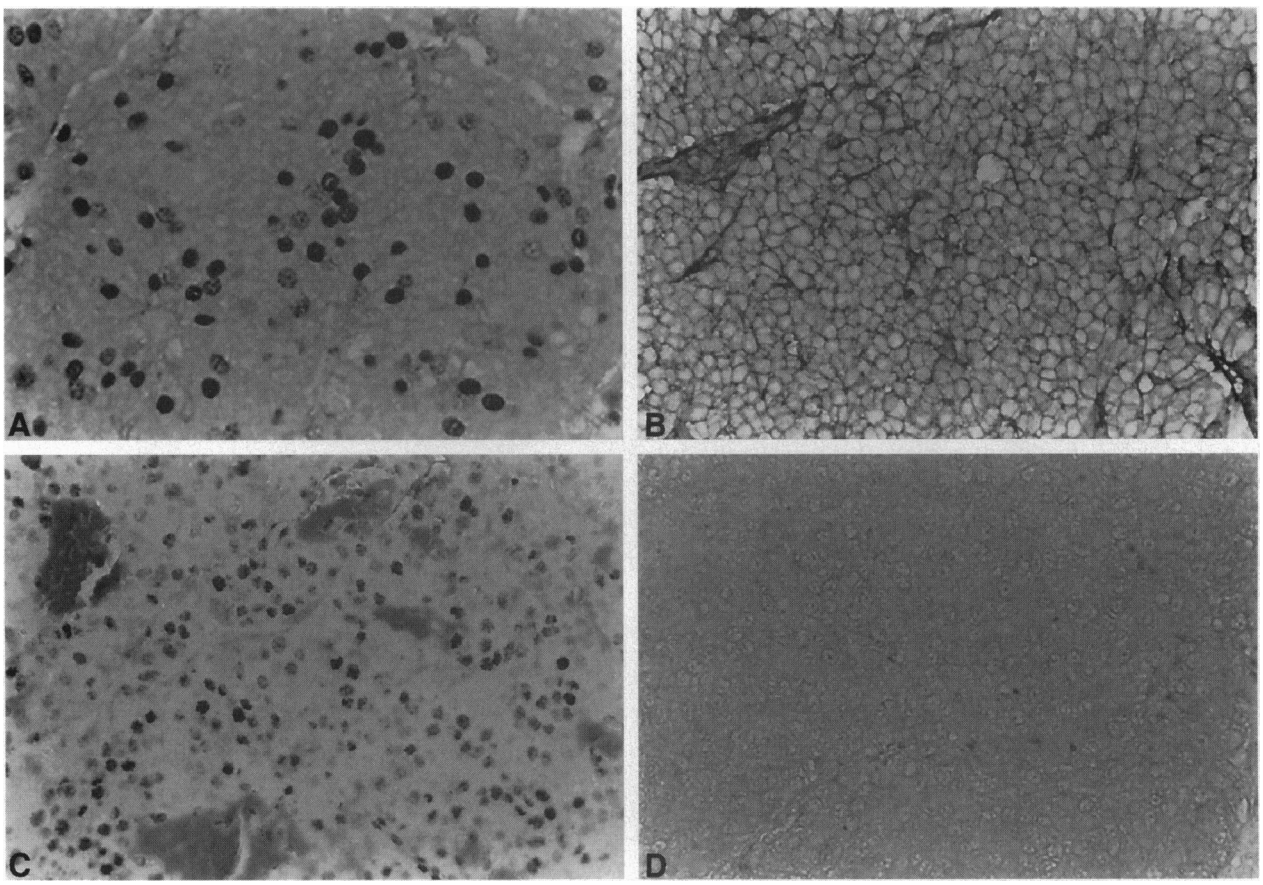

FIG. 4. Effect of Tet on TAg gene expression and βTC-tet cell proliferation *in vivo*. Mice with βTC-tet tumors received regular drinking water (*A* and *C*) or water containing Tet (*B* and *D*) for 7 days. They were then pulsed with BrdUrd, and the tumors were removed, sectioned, and stained with anti-BrdUrd (*A* and *B*) and anti-TAg (*C* and *D*) antisera. The bound antibodies were visualized with horseradish peroxidase-conjugated second antibodies. (×230.)

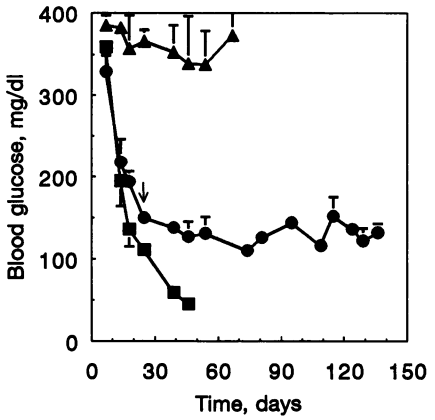

FIG. 5. βTC-tet cells maintain normal blood glucose levels in diabetic recipients. Mice made diabetic by treatment with streptozotocin were implanted i.p. with 2×10^6 cells (●, ■) or received no cell implant (▲). The time of cell implantation is shown as day 0. Blood glucose levels were measured weekly. When blood glucose was corrected, mice in one group (●) were implanted with slow-release Tet pellets (day 25, arrow). Blood glucose levels in this group remained stable, while in the group that was not treated with Tet (■), blood glucose continued to decrease as a result of uncontrolled proliferation of the insulin-secreting cells. Hypoglycemia in this group resulted in death of one tumor-bearing mouse at 32 days and two mice at 50 days. Values are means ± SEM ($n = 4$). The difference between the two groups injected with cells ± Tet at the 39- and 46-day time points is significant by the t test ($P < 0.01$).

A 7-day incubation of βTC-tet cells in the presence of Tet at 1 μg/ml did not eliminate completely the TAg protein from all of the cells, as judged by immunohistochemical analysis. In contrast, after a 7-day Tet treatment *in vivo*, no TAg was detected (Fig. 4). This may represent prolonged stability of TAg protein or mRNA in culture or leakiness of the regulatory system. However, it should be pointed out that the transforming activity of TAg requires threshold levels of the oncoprotein (16) that are sufficient for stoichiometric interactions, such as titration of tumor suppressor gene products. As demonstrated by the BrdUrd and [^{3}H]thymidine incorporation assays, the Tet treatment likely down-regulated TAg levels below this functional threshold.

These results reveal the dependence of the transformed β cells on continuous expression of the TAg oncoprotein for their proliferation. The development of β-cell tumors in these mice is a rare event, occurring in 1–2% of the islets. This has suggested the involvement of additional genetic changes in the cells. However, our results indicate that these changes most likely do not include mutations in genes that regulate the cell cycle, since the cells continue to require the activities of TAg to remain in cycle.

The ability to control cell proliferation *in vivo* by administration of Tet in the drinking water or with slow-release pellets and the fact that inhibition of gene expression by Tet is reversible upon drug removal provide an experimental system for studying the role of the TAg oncoprotein at various stages of tumorigenesis. In addition, the tet-TAg mice will allow the derivation of conditionally transformed cell lines from other cell types by targeting the expression of the tTA fusion protein with appropriate cell-specific promoters. Similarly, the mice expressing the tTA protein in β cells can be used to obtain reversible expression of other genes of interest in these cells by crossing them with mice expressing such genes under control of the *tet* operator minimal promoter.

The βTC-tet cell line will allow studies on the effect of cell proliferation on the expression of differentiated functions in β cells. The results obtained with cells transplanted into diabetic mice demonstrate that insulin secretion from the growth-arrested βTC-tet cells remains correctly regulated, which enables them to maintain blood glucose levels in the physiological range. To determine the effect of cell proliferation on glucose-induced insulin synthesis and secretion in these cells, cells propagated in culture and induced to undergo growth-arrest in the presence of Tet will be studied in comparison with actively proliferating cells cultured in the absence of Tet. The strategy described here will contribute to the development of β-cell lines for cell therapy of diabetes and to generation of conditionally transformed cell lines from other cell types with therapeutic potential.

Note Added in Proof. In the experiment of Fig. 5, the Tet pellets were removed from the mice at the end of the 4-month period of treatment. This resulted in the development of hypoglycemia and tumors within an additional 1 month. This finding indicates that the βTC-tet cells maintain a proliferative capacity during a prolonged period of growth arrest, which allows renewed cell replication once TAg expression is restored.

We thank Dinah Carroll for paraffin sections, Dr. H. Bujard for the pUHD-10-3 and pUHD-15-1 plasmids, and Dr. G. A. Ellestad for ATet. This work was funded by a program project grant from the Juvenile Diabetes Foundation International (to S.E.).

1. Hanahan, D. (1988) *Annu. Rev. Genet.* **22,** 479–519.
2. Fanning, E. & Knippers, R. (1992) *Annu. Rev. Biochem.* **61,** 55–85.
3. Jat, P. S., Noble, M. D., Ataliotis, P., Tanaka, Y., Yannoutsos, N., Larsen, L. & Kioussis, D. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 5096–5100.
4. Deuschle, U., Hipskind, R. A. & Bujard, H. (1990) *Science* **248,** 480–483.
5. Lakso, M., Sauer, B., Mosinger, B., Lee, E. J., Manning, R. W., Yu, S.-H., Mulder, K. L. & Westphal, H. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 6232–6236.
6. Gossen, M. & Bujard, H. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 5547–5551.
7. Labow, M. A., Baim, S. B., Shenk, T. & Levine, A. J. (1990) *Mol. Cell. Biol.* **10,** 3343–3356.
8. Furth, P. A., St. Onge, L., Böger, H., Gruss, P., Gossen, M., Kistner, A., Bujard, H. & Henninghausen, L. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 9302–9306.
9. Hanahan, D. (1985) *Nature (London)* **315,** 115–122.
10. Efrat, S., Linde, S., Kofod, H., Spector, D., Delannoy, M., Grant, S., Hanahan, D. & Baekkeskov, S. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 9037–9041.
11. D'Ambra, R., Surana, M., Efrat, S., Starr, R. G. & Fleischer, N. (1990) *Endocrinology* **126,** 2815–2822.
12. Huang, M. T. F. & Gorman, C. M. (1990) *Nucleic Acids Res.* **18,** 937–947.
13. Hogan, B., Costantini, F. & Lacy, E. (1986) *Manipulating the Mouse Embryo* (Cold Spring Harbor Lab. Press, Plainview, NY).
14. Efrat, S. & Hanahan, D. (1987) *Mol. Cell. Biol.* **7,** 192–198.
15. Degenkolb, J., Takahashi, M., Ellestad, G. A. & Hillen, W. (1991) *Antimicrob. Agents Chemother.* **35,** 1591–1595.
16. Efrat, S. & Hanahan, D. (1989) in *Transforming Proteins of DNA Tumor Viruses*, eds. Knippers, R. & Levine, A. J. (Springer, Berlin), pp. 89–95.