Generation of a tightly regulated all-*cis* β cell-specific tetracycline-inducible vector

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Ability to induce protein expression at will in a cell is a powerful strategy used by scientists to better understand the function of a protein of interest. Various inducible systems have been designed in eukaryotic cells to achieve this goal. Most of them rely on two distinct vectors, one encoding a protein that can regulate transcription by binding a compound X, and one hosting the cDNA encoding the protein of interest placed downstream of promoter sequences that can bind the protein regulated by compound X (e.g., tetracycline, ecdysone). The commercially available systems are not designed to allow cell- or tissue-specific regulated expression. Additionally, although these systems can be used to generate stable clones that can be induced to express a given protein, extensive screening is often required to eliminate the clones that display poor induction or high basal levels. In the present report, we aimed to design a pancreatic β cell-specific tetracycline-inducible system. Since the classical two-vector based tetracycline-inducible system proved to be unsatisfactory in our hands, a single vector was eventually designed that allowed tight β cell-specific tetracycline induction in unselected cell populations.

INTRODUCTION

Time-controlled expression in cell lines or transgenic animals is often crucial for fully understanding the functionality of a protein of interest. Various inducible systems have therefore been developed to achieve this goal. Most commercially available inducible systems use two different plasmids. The first encodes a protein that can differentially regulate gene transcription upon binding to a given inducer molecule. The second allows subcloning of the cDNA encoding the protein of interest downstream of promoter sequences that can recruit the protein regulated by the inducer molecule. For example, in a system developed by the laboratory of Ronald Evans (1) and commercialized by Stratagene, the first plasmid encodes the ecdysone receptor (EcR). In mammalian cells, EcR heterodimerizes with the retinoid-X-receptor (RXR), allowing the complex to bind to ecdysone-responsive elements (EREs). Binding of ecdysone (or its analog ponasterone A) turns the EcR-RXR complex into a transcription activator. The second plasmid bears EREs upstream of a multiple cloning site allowing subcloning of the cDNA of interest. Cells expressing both plasmids will therefore produce the protein of interest when incubated with ecdysone.

The most widely used inducible system relies on the use of tetracycline as an inducer molecule (2). Commercialized by Clontech, this system takes advantage of one plasmid encoding reverse tetracycline-responsive transactivator (rtTA), which is a reversed version of a fusion protein between the tetracycline repressor of the *Tn10Tc* operon of *Escherichia coli* fused to the C-terminal portion of the herpes simplex virus protein VP16. The rtTA protein, in contrast to the nonreversed tTA form, allows gene transcription when bound to tetracycline (or the analog doxycycline). The other plasmid provided in the system bears multiple tetracycline-responsive elements (TREs) coupled to a minimal promoter and located upstream of multiple restriction sites, allowing subcloning of the cDNA of interest (3). Cell lines and mouse strains expressing the rtTA protein are available. Using such lines and strains, researchers can speed up the development of inducible systems for their protein of interest.

Commercial systems allowing tissue- or cell type-specific regulated expression of a protein of interest are not, to our knowledge, yet available. In the context of our current research, we wished to express at will, and specifically in pancreatic β cells, an anti-apoptotic protein that corresponds to

the N-terminal fragment of the RasGAP protein (called fragment N) (4,5). We have shown earlier that the constitutive expression of fragment N potentially protects β cells against various detrimental conditions and stress stimuli (6). Fragment N and the pathways it regulates could therefore be the target for the development of anti-diabetic drugs. However, cell and mouse systems, in which fragment N could be induced at will, represent better models to assess the therapeutic potential of fragment N. We have therefore assessed various tetracycline-based inducible systems for their ability to provide the tightest and most efficient induction of fragment N. We then modified the best system to ensure β cell-specific expression.

MATERIALS AND METHODS

Materials

pTRE-tight vector was from Clontech (Mountain View, CA, USA). Tetracycline was from Sigma (St. Louis, MO, USA). Lipofectamine 2000 was obtained from Invitrogen (Carlsbad, CA, USA). The anti-hemagglutinin (HA) antibody and the anti-green fluorescent protein (GFP) antibody were purchased from Covance (Princeton, NJ, USA) and BD Biosciences (San Jose, CA, USA), respectively. Hoechst 33342 was purchased from Invitrogen. Triton X-100 was from Fluka (Sigma-Aldrich, St. Louis, MO, USA). Sodium pyruvate was from Sigma. β -mercaptoethanol was from Gibco (Invitrogen). Sodium dodecyl sulfate (SDS) was from Sigma. Bromophenol blue was from Acros Organics (Thermo Fisher Scientific, Waltham, MA, USA). The secondary antibody used in Western blot analysis was an IRDYE 800-conjugated, affinity purified, goat anti-mouse immunoglobulin G (IgG) (H&L) from Rockland Immunochemicals (Gilbertsville, PA, USA). The secondary antibody used in immunocytochemistry experiments was a Cy3-conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA).

Plasmids

HA-N(D157A).TREtight encodes a caspase-resistant form of fragment N (4) under the control of TREs consisting

Figure 1. Construction of pTRE-tight-mod. The identical repeats (sequence indicated at the top of the figure) separating the tetracycline-responsive elements (TREs) in pTRE-tight were deleted and replaced with different random intervening sequences flanking the TREs. Insertion of these sequences was performed by using the indicated annealed oligonucleotides that were subcloned in the different constructs using the indicated restriction enzymes.

of seven identical 19-bp sequences (tetO 19-mers) connected by seven identical 17-bp-long intermediate sequences. This plasmid was created by subcloning the klenowed *ClaI/BamHI* insert containing the HA-tagged fragment N in plasmid RIP-HA-N(D157A).xf3 into pTRE-tight (Becton Dickinson; BD Biosciences) opened with *SmaI*. RIP-HA-N(D157A).xf3 was made by subcloning a blunted *BamHI/Sall* insert of HA-N(D157A).bs into plasmid RIP-vMos.xf3 (a kind gift from Dr. Bernard Thorens, Lausanne University, Lausanne Switzerland) opened with *XbaI/HindIII* and blunt-ended. Plasmid HA-N(D157A).bs was created by subcloning a blunt *BamHI/XhoI* HA-N(D157A).dn3 (4) insert into pBluescriptII SK+ (Stratagene, La Jolla, CA, USA) opened with *EcoRV*. HA-N(D157A).dn3 bears the cDNA coding for the uncleavable form of fragment N under the control of a cytomegalovirus (CMV) promoter (4).

HA-N(D157A).TRETightmod is similar to HA-N(D157A).TRETight except that its 17-bp-long intermediate sequences are random and different

from one another. HA-N(D157A). TREtightmod was created by subcloning a blunt *ClaI/BamHI* insert from RIP-HA-N(D157A).xf3 into pTRE-tight-mod opened with *SmaI*. The latter plasmid was made as described in Figure 1 by sequential insertion of the indicated annealed oligonucleotides into progressively longer constructs (pTRE-tightΔ6 to pTRE-tight-mod).

Plasmid tTRKRAB-TREtight is a construct bringing the CMV promoter into the vicinity of TREs. It was built by subcloning a blunt 1634-bp *EcoRI* fragment from pLVUTHsip53-tTRKRAB (7) into pTRE-tight opened with *Clal* and blunt-ended.

HA-N(D157A).RIP-tTRKRB-TREtight bears the cDNA of the uncleavable form of fragment N under the control of a TRE-regulated rat insulin promoter (RIP). It was made by ligating the 2126-bp fragment resulting from the *Bam*HI, *Sca*I, and *Apa*I-mediated digestion of RIP-HA-N(D157A).xf3 together with the annealed sense 5'-ACTCAATGACACAGTGGATTGAGCGGCCGGC-3' and antisense

5'-GGCCGCCGGCCGCTCAATCCA
CTGTGTCATTGAGT-3' oligonucleotides into tTRKRAB-TREtight opened with *Bam*HI and *Not*I.

HA-N(D157A).tTRKRAB-TREtight is a RIP-less version of HA-N(D157A).RIP-tTRKRAB-TREtight. It was made by self-ligating the 4.3-kb fragment of HA-N(D157A).RIP-tTRKRAB-TREtight digested with *Bam*HI and *Cl*al. This plasmid bears a minimal CMV promoter downstream of the TREs.

The pEGFP-C1 plasmid was obtained from Clontech and encodes an enhanced GFP (EGFP) under the control of the CMV promoter.

Cell Culture

HEK 293 tet-on, COS, and HEK 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) plus Glutamax from Gibco (Invitrogen) supplemented with 10% decompemented fetal calf serum (FCS) at 37°C and 5% CO₂. Min6 cells were grown in DMEM supplemented with 15% decompemented FCS, 1 mM sodium pyruvate, and 0.1 mM β -mercaptoethanol at 37°C and 5% CO₂. β TC3 cells were grown in RPMI (Roswell Park Memorial Institute)-1640 medium (Gibco) plus Glutamax with 10% decompemented FCS and 1 mM sodium pyruvate at 37°C and 5% CO₂.

Cell Transfection

Cells were transfected using Lipofectamine 2000 as described previously (8).

Western Blot Analysis

After the indicated treatments described in the figure legends, the cells were lysed on ice in mono Q-C buffer (4) supplemented with one tablet of EDTA-free protease inhibitor (Roche, Basel, Switzerland) per 50 mL. Protein concentration was measured by a Bradford assay using bovine serum albumin (BSA) as a standard. Lysates were mixed with sample buffer [62.4 mM Tris-HCl, pH 6.8, 10% glycerol, 5% (v/v) β -mercaptoethanol, 2% (w/v) SDS, 0.01% (w/v) bromophenol blue] before loading on SDS polyacrylamide gel electrophoresis (SDS-PAGE) gels. Western blot analysis was performed as described previously (9).

Figure 2. Tetracycline-responsive vectors for gene induction. The chosen gene whose expression is to be regulated by tetracycline is the un-cleavable RasGAP-derived fragment N [(N(D157A))]; this fragment could be replaced by any other cDNA of interest. (A) The pTRE-tight vector from Clontech contains seven tetracycline-responsive elements (TREs) derived from the bacterial tetracycline operator sequence (dark blue elements in the scheme) separated by seven identical 17-bp-long sequences (light blue elements). In the tet-on system, an additional vector encodes the reverse tetracycline-responsive transactivator (rtTA) that binds TREs when interacting with tetracycline and drives the expression of genes downstream of the TREs. (B) The pTRE-tight-mod vector is essentially similar to pTRE-tight except that the intervening sequences between the TREs are composed of random sequences that are all different from one another (multicolor elements in the scheme). (C) Krüppel-associated box (KRAB) is a transcriptional repressor. The fusion protein between KRAB and the tetracycline repressor (tetR) of *Escherichia coli* binds TREs in the absence of tetracycline and represses neighboring genes. In the construct depicted here, the sequences downstream of the TREs correspond to the cDNAs of N(D157A) and the tetR-KRAB fusion protein separated by an encephalomyocarditis virus internal ribosome entry site (EMCV-IRES). As soon as tetR/KRAB is expressed, it blocks N(D157A) expression as well as its own. In the presence of tetracycline, tetR/KRAB can no longer interact with the TREs, and efficient expression of N(D157A) ensues. (D) Same mode of action as in panel C, except that the promoter controlling the expression of the sequence of interest is the rat insulin promoter. This ensures specific expression of the cDNAs present in the construct insulin-producing cells.

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Figure 3. Tetracycline-regulated expression of fragment N. The different expression systems described in Figure 2 were used in non-insulin-expressing cells and in the Min6 insulinoma cell line. (A) HEK 293 cells constitutively expressing the reverse tetracycline-responsive transactivator (rtTA) protein (293 tet-on) were transfected with the HA-N(D157A).TREtight and HA-N(D157A).TREtightmod plasmids. The cells were treated or not with 1 μ M tetracycline for 48 h. Fragment N expression was assessed by Western blot analysis using an anti-hemagglutinin (HA) antibody. (B) HEK 293T cells were transfected with the HA-N(D157A).tTR-KRAB plasmid. The cells were then treated as in panel A. (C) HEK 293T, COS, Min6, and β TC3 cells were transfected (T) or not (NT) with the HA-N(D157A).RIP-tTR-KRAB plasmid. The cells were then treated as in panel A. (D) Min6 cells were transfected with plasmid HA-N(D157A).RIP-tTR-KRAB together with a green fluorescent protein (GFP)-expressing vector. The cells were then incubated or not for 48 h with 1 μ M tetracycline. Finally, the cells were stained with an anti-HA antibody (see Materials and Methods section for details). The nuclei were labeled with Hoechst 33342. Quantitation of the cells co-expressing GFP and HA-N is shown on the bar graph (mean $\pm$ SD 50–150 cells were counted in each of three independent experiments).

Immunocytochemistry

Cells on coverslips were washed in phosphate-buffered saline (PBS; 116 mM NaCl, 10.4 mM Na_2HPO_4 , 3.2 mM KH_2PO_4 , pH 7.25), fixed with 2% paraformaldehyde obtained from Acros Organics for 15 min at room temperature, and stained with 10 μ M Hoechst 33342. Following

an additional washing with PBS, the cells were permeabilized with 0.2% (v/v) Triton X-100 in PBS for 15 min. The cells were then washed in DMEM containing 10% FCS and incubated for 1 h at room temperature with a 1/100 dilution in DMEM, 10% FCS of the primary antibody against the HA tag. After a washing step in PBS, the cells were incubated in the same medium,

with a 1/500 dilution of the secondary antibody. Finally, six washes over a 16-h period were performed in PBS before mounting the coverslips in mounting medium for fluorescence (Vectashield; Vector Laboratories, Burlingame, CA, USA). Images were acquired using a Zeiss AxioVis C1 microscope (Carl Zeiss, Jena, Germany).

Pulse-Chase Experiments

HEK293T cells were co-transfected with plasmids encoding fragment N [HA-N(D157A).dn3] and GFP (pEGFP-C1). The pulse-chase experiments were performed as described previously (10).

RESULTS AND DISCUSSION

Various tetracycline-inducible systems (Figure 2) were used to evaluate time-controlled expression of fragment N. First we tested the tet-on system commercialized by Clontech. It is based on the ability of the rtTA to bind TREs in the presence of tetracycline (or the related compound doxycycline) and to drive the expression of downstream cDNAs (11) (Figure 2A). The pTRE-tight vector can be used to subclone the cDNA of interest downstream of TREs, while the expression of rtTA is driven by another plasmid. There are however, commercially available cell lines that stably express the rtTA-encoding plasmid, such as the HEK293 tet-on cells. These cells transfected with a fragment N-encoding pTRE-tight plasmid only displayed weak induction of fragment N expression in presence of tetracycline (Figure 3A, first two lanes). There is evidence that repetition of the same intervening sequences between the TREs can negatively affect the induction induced by tetracycline in pTRE-tight and related vectors (12). We therefore replaced each intervening sequence with a different random sequence and created the vector called pTRE-tightmod (Figures 1 and 2B). However, this did not generate satisfactory results as a constitutive nontetracycline-regulated expression was obtained when this vector was used in HEK293 tet-on cells (Figure 3A, last two lanes).

Recently, a new lentiviral-based induction system was reported that combines the ability of the tetracycline receptor (tetR) to interact with TREs

in the presence of tetracycline and the capacity of the Krüppel-associated box (KRAB) domain found in some vertebrate transcription regulators to repress gene expression. A fusion protein between tetR and KRAB binds TREs and efficiently represses the expression of downstream genes. In the presence of tetracycline, the fusion protein no longer interacts with the TREs, allowing transcription of the gene of interest to resume (7). This system requires a slight initial leakage in order to produce a small amount of the tetR/KRAB fusion protein to block further expression. We isolated the elements that encode the tetR/KRAB fusion protein in the lentiviral vector and subcloned them in pTRE-tight to create a simpler vector that could be used in transfection experiments. The sequence encoding fragment N was then placed upstream of the tetR/KRAB cDNA and was separated from the latter by an internal ribosomal entry site (IRES) (Figure 2C). Expression of fragment N in HEK293T cells expressing this plasmid [HA-N(D157A).tTR-KRAB]

was strongly induced by tetracycline (Figure 3B). A weak expression of fragment N was detected in the absence of tetracycline (Figure 3B, first lane) as could be expected from the fact that initial leakage allowing expression of the repressor will also lead to fragment N expression (see Figure 2, C and D). To determine if β cell-specific expression of fragment N could be obtained with the above system, a rat insulin promoter was incorporated in the plasmid used in Figure 3B (Figure 2D). This new construct, called HA-N(D157A).RIP-tTR-KRAB, could no longer lead to the expression of fragment N in HEK 293T or COS cells in the presence of tetracycline (Figure 3C, upper blot). In contrast, in the Min6 and β TC3 insulin-producing cell lines, this construct allowed strong tetracycline-dependent fragment N expression (Figure 3C, lower blot). As expected, the inherent leakage of the system induced some expression of fragment N in the noninduced state, but this induction remained very low (Figure 3C, lower blot,

second and fifth lanes). To determine if there was a homogenous tetracycline-mediated induction of fragment N in the transfected cell population, the expression of fragment N was assessed at the cell level by immunocytochemistry. Min6 cells were co-transfected with HA-N(D157A).RIP-tTR-KRAB and a GFP-encoding plasmid and then induced or not with tetracycline. Figure 3D shows that tetracycline induced strong induction of fragment N in the vast majority (~95%) of the transfected cells (i.e., GFP-positive cells). Less than ~5% GFP-positive cells were found to be weakly positive for HA-N in the absence of tetracycline. Our system allows, therefore, a tightly regulated and homogenous expression of a sequence of interest within a cell population.

The results presented in Figure 3, C and D, demonstrate that a single plasmid can bear all the elements that will permit tetracycline-regulated expression of a cDNA of interest in β cells. To determine the kinetics of gene expression and repression on β cells expressing such

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a plasmid, Min6 cells transfected with HA-N(D157A).RIP-tTR-KRAB were incubated with tetracycline for various periods of times and then cultured in the absence of the drug. To account for plasmid dilution due to cell division, the expression of fragment N was normalized against GFP levels. Figure 4, A and B, show that it takes about 48 h for tetracycline to optimally induce the expression of fragment N. A period of ~100 h is required to reach basal levels of fragment N expression after removal of the drug. Fragment N has a longer half-life than EGFP (10.5 versus 5.7 h) (Figure 4C). Normalization of fragment N levels against those of GFP might therefore overestimate the actual fragment N expression in situations where both proteins are not synthesized, such as when plasmids are diluted out upon cell division. Consequently, the

Figure 4. Reversibility of fragment N expression using the RIP-tTR-KRAB system. (A) Min6 Cells were transfected or not with plasmid HA-N(D157A).RIP-tTR-KRAB together with a green fluorescent protein (GFP)-expressing vector (NT, not transfected). The cells were then left untreated or stimulated with tetracycline as indicated under the blot. Fragment N and GFP expression was assessed by Western blot analysis using anti-hemagglutinin (HA) and anti-GFP antibodies. The lower expression of fragment N in cells lysed 168 h after transfection compared with those lysed 48 h after transfection (compare lanes 5 and 7) is most likely due to dilution of the plasmids as cells are dividing. (B) Cells were transfected as in panel A. The following day, the cells were treated or not with 1 µg/mL tetracycline and lysed after the indicated time periods (upper blot). Alternatively, following a 48-h tetracycline treatment, the cells were washed and cultured in control culture medium for the indicated number of hours. The cells were analyzed for HA-N and GFP expression as described in panel A. Variations in fragment N expression after induction by tetracycline, followed by its removal are depicted graphically under the blots. The chart represents the fold increase in fragment N expression over the levels found in nontreated cells. The results are normalized against GFP expression to account for plasmid dilution as cells divide in culture (pooled data from four independent experiments). (C) Cells were transfected with HA-N(D157A).dn3 and a GFP-expressing plasmid. Forty hours later, the cells were pulsed with ³⁵S-methionine for 15 min and chased for the indicated time periods (see Material and Methods section). HA-tagged fragment N and GFP were then isolated by immunoprecipitation and visualized by autoradiography following migration on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. Results from two independent experiments are shown as the ln of the band intensities of the proteins on the gel as a function of time. The half-life of the proteins was determined as: -ln 2 divided by the slope of the linear regression line.

levels of fragment N in the right part of the curve presented in Figure 4B should be considered as an upper limit. The decrease in fragment N expression upon tetracycline withdrawal is therefore likely to be more pronounced than pictured in Figure 4B. Altogether, the results presented in Figure 4 indicate that the RIP-tTR-KRAB vector allows a tight and reversible tetracycline-inducible expression of a gene of interest specifically in β cells.

Tetracycline-based systems have been used in research for time-controlled induction of gene expression both in vitro (11) and in vivo (2). They were designed initially as two-vector systems. The first vector encodes the transcription regulator that binds tetracycline and the second vector bears the cDNA of interest. Transfection of the two plasmids in cell cultures results in a

heterogeneous cell population that, upon selection for stable integrations, will consist of clones displaying different inducibility patterns. Depending on the site of integration and consequently the impact of neighboring regulatory sequences (11), some clones will show high background levels of expression, others will not be able to respond to tetracycline, and some will be appropriately induced by the drug. Screening for the latter type might turn out to be time-consuming. In a recent study performed in our laboratory, we established clones derived from the rtTA-expressing HEK 293 Clontech cell line that can be induced to express the IB1 protein upon addition of tetracycline (10). However, the screening process took months, since we had to test hundreds of individual stably-transfected clones before we could isolated two that gave satisfactory results (10). In this context, a self-regulated single vector system would obviously have been advantageous.

To circumvent some of the problems linked to the presence of neighboring sequences that could alter the proper regulation of tetracycline-inducible systems, single lentiviral vectors bearing both the tetracycline binding protein and the protein of interest under the control of the same promoter elements have been designed. These systems are autoregulated because the tetracycline-binding protein controls its own expression (7,13). They appear to generate a more homogenous induction profile within individual clones after their introduction in a cell population (13). We have used here elements of one such autoregulated system (7), together with the insulin promoter, to create a single vector allowing tetracycline-inducible pancreatic β cell-specific expression of a cDNA of interest. By substituting different tissue-specific promoters for the insulin promoter in our construct, this system can be potentially adapted for any cell type-specific expression. This, however, remains to be experimentally assessed.

Single-vector tetracycline-based systems containing both the sequences encoding rtTA and the tetracycline response elements have been described earlier (14,15). Bäckman and collaborators have combined in a single vector the *Renilla* luciferase cDNA under the control of various versions of TREs placed downstream of, and in a

reverse orientation to, the rtTA reverse transactivator under the control of a constitutive promoter. An induction in relative luciferase activity of up to ~50-fold could be recorded using the best construct (14). However, it is difficult from such enzymatic assay to quantitatively determine the level of luciferase induction at the protein level. Lottmann and collaborators have designed a transgene containing the rtTA reverse transcriptase under the control of the mouse insulin promoter followed by a PDX-1 antisense sequence, whose expression was controlled by TREs. Expression of this vector in an insulinoma cell line resulted in a strong tetracycline-mediated induction of the antisense construct. However, the construct was not tested in non-insulin-producing cells, and therefore the specificity of the tetracycline-mediated expression was not demonstrated (15).

Nevertheless, these two studies indicate that combining in a same vector the rtTA cDNA, and a sequence of interest under the control of TREs can lead to efficient tetracycline-mediated induction of the RNA of interest. However, these one-vector systems do not ensure that the regulator (i.e., the rtTA molecule) and the regulated sequence (e.g., the cDNA of interest) are co-regulated because they are still under the control of different promoters. In contrast, the system we describe here provided a tight co-regulation between the regulator (i.e., the tetR-KRAB fusion protein) and the sequence of interest, because both of these elements are controlled by the same TRE-containing promoter (Figure 2, C and D). Moreover, even in the vicinity of strong enhancer sequences, our construct would ensure that the expression of the sequence of interest in the noninduced situation is repressed to very low levels, because of the concomitant expression of the tetR-KRAB repressor. This might not be the case with the other systems. Our system, therefore, provides a tight tetracycline-mediated regulation of a sequence of interest, but as indicated above, it requires a slight initial expression leakage of both the regulator and the sequence of interest. This might make it incompatible with sequences of interest that encode highly toxic molecules.

The vast majority of earlier strategies to generate mice with tissue-specific

tetracycline-regulated expression of a given construct [sense and antisense cDNAs, small interfering RNA (siRNA)] required first the generation of two lines of transgenic mice corresponding to the two vectors needed and secondly the crossing of these two lines with each other. This is time-consuming, and the efficacy of the construct can only be appreciated in the offspring resulting from the interbreeding of the two transgenic mice. The vector that we have described here avoids these problems and may therefore be particularly useful for the rapid generation of mice in which a protein of interest can be induced by tetracycline in specific tissues.

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COMPETING INTEREST STATEMENT

The authors declare no competing interests.

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