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Conditional cell ablation by stringent tetracycline-dependent regulation of *barnase* in mammalian cells

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ABSTRACT

Conditional expression of suicide genes *in vivo* has a wide range of applications in biological research and requires a minimal basal promoter activity in the uninduced state. To reduce basal activity of tetracycline (tc)-inducible target promoters we combined synthetic *tet* operators in varying numbers with a core promoter derived from the plant viral 35S promoter. An optimized promoter, P_{TF}, was found to exert a stringent regulation of luciferase in combination with tTA and rtTA in different mammalian cell lines. We linked P_{TF} to the *barnase* gene, coding for a highly active RNase from *Bacillus amyloliquefaciens*. Stable cell clones expressing *barnase* under control of tTA exerted cell death only after tc withdrawal, correlating with a 10-fold induction of *barnase* mRNA expression. Directing tTA expression through a neuron-specific enolase promoter (P_{NSE}) leads to *barnase* expression and cell death in neuronal cells after tc withdrawal. Taken together, our data demonstrate that a stringent control of *barnase* expression in the uninduced state improves cell ablation studies, as high frequencies of transgene propagation in both cell lines and in transgenic mice are observed.

INTRODUCTION

For many applications in gene therapy and medical research, the expression of a suicide gene at defined time points is of great advantage. In particular, the cell-specific induction of toxin genes represents a promising approach for the functional analysis of distinct cell types *in situ*. In many human diseases, loss of distinct cell types leads to detrimental diseases, for instance in the central nervous system (CNS). Until now, conditonal cell ablation has been achieved either by introduction of the herpes simplex thymidine kinase (HSV-*tk*) cassette for functional analysis of astrocytes in transgenic mice (1,2) or by tetracycline (tc)-dependent expression of diphteria toxin A chain (DTA) in cell lines (3) and transgenic mice (4). These approaches faced several limitations: the HSV-*tk* gene is only applicable to ablation of proliferating cells within the CNS. Tetracycline controlled expression of DTA led to very low yields of recombinant cell clones of transgenic mice, as the original target promotor (5) revealed leakiness in the uninduced state.

The tc-dependent expression system is based on a chimeric transcriptional transactivator tTA, which is a fusion between the bacterial Tet repressor (TetR) and the constitutive activation domain of herpes simplex protein 16 (VP16). Binding of tTA to a target promoter consisting of multiple operators upstream of a core promoter leads to activation of transcription, which can be abrogated by addition of tc or the commonly used analog doxycycline (dox), which interferes with DNA binding of tTA. In addition, a TetR mutant that binds DNA only in the presence of tc has been exploited for the construction of rtTA, yielding a tc-inducible promoter (6).

In previous reports it has been demonstrated that the efficiency of the tc system is dependent on the cell line tested and is even more complex in transgenic mice where residual activity, even in the absence of the transactivator, depends on the tissue (7,8). The difference in expression levels between induced and uninduced transgenic tissue is 100 in optimal cases and varies in different tissues. Residual activity might be due to transcription factor binding to the CMV core promoter (7). The problem of leakiness is even more pronounced when rtTA is used as a transcriptional activator.

Three different approaches have been undertaken to reduce leakiness of the tc-dependent expression systems. Most recently, Urlinger *et al.* (9) isolated new rtTA alleles yielding lower activation in the uninduced state. Forster *et al.* (10) and Imhof *et al.* (11) used rtTA to drive tc-inducible expression of a suitable target promoter which was at the same time repressed by tTA in the absence of tc. This sophisticated arrangement reduced background activity by a factor of six, but has the disadvantage of requiring the simultaneous expression of two trans factors. Hoffmann *et al.* (12) used a modified mouse mammary tumor virus promoter (ΔMTV) in which the steroid-responsive elements were replaced by tetO sequences. However, as access of tTA depends critically on chromatin (13), induction levels are on average 10-fold lower than in the CMV based target promoter.

In this work we constructed a new target promoter with reduced leakiness, by exchanging the viral cytomegalovirus core promoter by a plant viral promoter and by avoiding identical spacer sequences between the multimerized operators. Promoter constructs were tested for basal activity either with tTA or with rtTA in different mammalian cell lines. As our major goal is to ablate neuronal cell types within the mouse

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CNS, we included in our analyses cell lines representing the rodent nervous system. To assess the usefulness of the optimised promoter in cell ablation experiments, we combined it with the lethal RNase gene from *Bacillus amyloliquefaciens* (*barnase*; 14), thus demonstrating a stringent cell ablation strategy for mammalian cells and tissues.

MATERIALS AND METHODS

Plasmids and cloning

The promoters P_{TAX} and P_{TF} were from plasmids pBHTAX and pBHTF (15). For cloning, the promoter sequences were amplified by PCR using primers with *Hin*dIII linkers. As control plasmids for tc-dependent luciferase expression, plasmids pXTC7C53Luc (P_{XTC}-Luc; 5) and pMtetO$_5$Luc (P_{MtetO5}-Luc; 12) were used. The plasmids pTAX-Luc and pTF-Luc were obtained by digesting pMtetO$_5$Luc with *Hin*dIII, and ligation of the *Hin*dIII promoter fragments into the respective vector sites. For construction of an expression vector containing the tc-inducible cDNA for Barnase, P_{TF} was fused to the Barnase coding region (16). Addition of a mammalian polyadenylation signal was achieved by cloning it into the expression vector SBC-1 (17) using *Cla*I and *Hin*dIII. As a regulator plasmid, we used pUHD15-1 (5) or pUHD17-1 (6) encoding the tc-dependent transactivator (tTA or rtTA, respectively) under control of a human cytomegalovirus (hCMV) promotor (P_{CMV}–tTA or P_{CMV}–rtTA). For cell-specific expression of tTA, the hCMV promotor was removed by cleaving with *Xho*I, filled-in with Klenow DNA Polymerase and digested with *Eco*RI. A 1.8 kb neuronal-specific enolase (NSE) promotor-fragment was excised from pCRToPo-cloning vector (Invitrogen, Heidelberg, Germany) with *Eco*RI and *Pvu*II and ligated into unique restriction sites of pUHD15 to construct the neuronal regulator plasmid P_{NSE}–tTA.

The plasmids pRSVLacZII and pEGFP-N3 (Clontech, Heidelberg, Germany), containing β-galactosidase cDNA (lacZ) or GFP under control of constitutive promotors, respectively, were used as reporter plasmids.

Cell culture and transfections

HeLa (ATCC CCL 2), CHO*dhfr*⁻ (ATCC CRL 9096) hamster ovary fibroblasts, C6 rat glioma cells (ATCC CCL 107), 293T human embryonal kidney cells (provided by Dr Jan Cornelis, DKFZ, Heidelberg, Germany), PC12 rat adrenal pheochromocytoma (ATTC CRL 1721), mouse motoneuron-like cell line NSC-19 (18), and Neuro-2A neuroblastoma cells (ATCC CCL 153) were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% (v/v) foetal calf serum (FCS; Biochrom KG, Berlin, Germany); CHO*dhfr*⁻ cells were cultured in the presence of Prolin (5 mM) and HT supplement (0.1 mM hypoxanthin, 0.01 mM thymidine). The cell line CHOAA8 (Clontech, Heidelberg, Germany), stably expressing the tc-dependent regulator tTA (plasmid pUHD15-1neo), was cultured in α-MEM supplemented with 10% FCS and 100 µg/ml G418 (Gibco BRL). Neuronal cell lines and C6 cells were transfected with Lipofectamine (Promega, Madison, WI), CHO*dhfr*⁻ and 293T cells by using the calcium phosphate method. The transfection efficiencies were usually >20%, as determined by control transfections with pEGFP. Depending on the cell type, $1–3.5 \times 10^5$ cells were seeded into 30 mm plates and constructs were added 24 h later in the absence or presence of dox (Sigma, Steinheim, Germany). The dishes with dox were preincubated with 1.0 µg/ml 24 h before transfection. Transient cotransfections were performed with ratios of 5:1 regulator plasmid to target plasmid (*luciferase* or *barnase*) and cells were analysed 48–72 h after transfection. Luciferase constructs were stably integrated by electroporating 10 µg *Pvu*I linearised plasmid in a Bio-Rad electroporator with 200 V and 480 µF. For stable transfection of *barnase* constructs, cotransfections were done in 1:10 and 1:20 ratios of pBSΔpac (CHOAA8) or pEGFP-neo (Neuro-2A) to P_{TF}–*barnase*. Three days after transfection, transfectants were selected either with 3.5 µg/ml puromycin or 400 µg/ml G418 (Luciferase plasmids and pEGFP-neo).

Luciferase and β-galactosidase assays

Forty-eight hours after transfection, cells were lysed by the addition of 150 µl lysis buffer (supplied with the Dual-Light kit). Activities of β-galactosidase and luciferase were measured by the Dual-Light Chemieluminescent Reporter Gene Assay (Tropix, Bedford, MS) using a Lumat LB 9507 (EG&G Berthold, Bad Wildbad, Germany). Enzyme activities were adjusted to protein content of cell lysates, which were determinated by the BCA method (Pierce, Rockford, IL).

Quantification of GFP-positive cells

GFP, P_{TF}–*barnase* and P_{CMV}–tTA (or P_{NSE}–tTA) cotransfected cells, subsequently cultured in the absence or presence of dox, were analysed 48 and 72 h after transfection by UV. GFP-positive cells were counted in similar sections of a number of microscopic fields. Similarly, transfected cells were rinsed from the plates with 1 ml of phosphate buffered saline (PBS) containing 0.05 mM EDTA. Numbers of GFP-positive cells were determined in a fluorescence activating cell sorter (FACS; Beckton-Dickinson) using an argon ion laser at 488 nm excitation wavelength.

Isolation of RNA and RT–PCR

Total RNAs from cultured cells were isolated by the NucleoSpin RNA II preparation method (Macherey-Nagel, Düren, Germany) with an included DNase I digest. Usually, 0.5–1 µg of total RNA in 20 µl reaction volume was subjected to reverse transcription (RT) using superscript reverse transcriptase (Life Technologies GmbH, Eggenstein, Germany). After RT, 2 µl of a 1:5 dilution was used for PCR amplification in a total volume of 20 µl, containing MasterMix-Taq (Qiagen, Hilden, Germany).

For the bacterial RNase *barnase* the primers were bar_s (5′-AGGTCTGATAATGGTCCGT-3′) and bar_{as} (5′-AAGGGAA-CCTGGCAGACGTC-3′); for ribosomal protein L7 mRNA, $L7_s$ (5′-AGATGTACCGCACTGAGATTC-3′) and $L7_{as}$ (5′-ACTTACCAAGAGACCGAGCAA-3′).

Primers were obtained from TIB MolBiol (Berlin, Germany). PCR conditions for *barnase*, 4 min initial denaturation at 94°C, 20–25 cycles with 1 min denaturation at 92°C, 1 min annealing at 60°C, 45 s elongation at 72°C and finally 10 min extension at 72°C; for *L7* amplification, 4 min initial denaturation at 94°C, 18–22 cycles with 1 min denaturation at 94°C, 45 s annealing at 62°C, 45 s elongation at 72°C, 10 min extention at 72°C. The resulting amplication products (*barnase*, 210 bp;

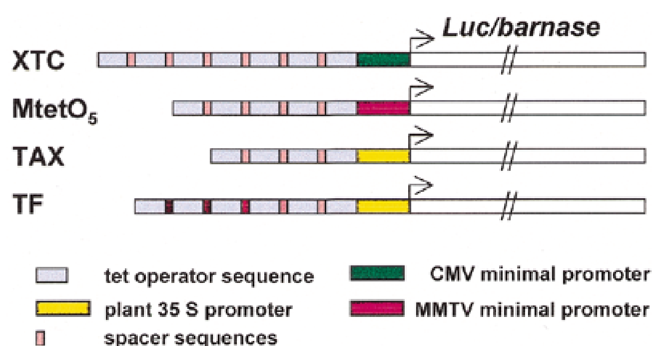

Figure 1. Target promoter constructions (P_{XTC}, P_{MtetO5}, P_{TAX}, P_{TF}) linked to *luciferase* or *barnase* used for analyses. Transcription start sites are indicated by arrows. Note that in P_{TF}, the spacer sequences between *tet* operators differ from each other.

L7, 352 bp) were separated by electrophoresis on 2% agarose gels and visualised by ethidium bromide staining.

Dot blot analysis

PCR products were diluted by 20× standard saline citrat buffer (SSC), boiled and transferred via Slot blot (Minifold^{R}II, Schleicher & Schuell, Dassel, Germany) to nylon membranes (Pall Biodyne B, Pall, Dreieich, Germany). The DNA were fixed by baking. The blots were hybridised with [α-^{32}P]dATP random labelled PCR amplifications of *barnase* and *L7*. After exposition (Bio-Imager, Bio-Rad, Göttingen, Germany), the signals were quantified by Bio-Rad Multi-Analyst™ version 1.1.

Documentation

Photodocumentation was done on a Zeiss Axiophot fluorescence microscope with phase contrast and UV microscopy. Images were processed by Adobe Photoshop version 6.0.

RESULTS AND DISCUSSION

Optimisation of a tc-dependent target promoter

In order to reduce the background activity of the target promoter P_{XTC} (5), two new promoters (both of them containing multiple *tet* operator sites upstream of a minimal promoter) were constructed and fused to *luciferase* as a reporter gene. The sequences of both domains of the target promoter P_{XTC} (5) (the minimal promoter as well as the region encoding the operators) were altered (Fig. 1). The minimal promoter, which is in the original $P_{hCMV*-1}$ the region –53 to +75 of the hCMV, was exchanged against the region –45 to +1 of the plant viral cauliflower mosaic virus 35S promoter (15,19). In P_{Tax}, the 'enhancer region' consists of four operators and three identical spacer regions, which are different from those in $P_{hCMV*-1}$. In P_{TF} the 'enhancer region' was exchanged against a fragment encoding six *tet* operators. The difference to P_{Tax} is that the sequences between the operators are not multimerized, but each spacer encodes a different sequence. The rationale for this alteration is that weak binding sites between operator sites might lead to background activity due to synergistic effects. These promoters were tested by transient transfection assays with tTA and rtTA in different cell lines and in stable cell clones of CHO cells (Fig. 2). As control promoters, we used P_{XTC}, the original target promoter described by Gossen and Bujard, and a mouse mammary tumor virus (MMTV) LTR derived promoter (P_{MtetO5}), developed as tc-dependent promoter with reduced basal activity (12).

In all cell lines tested, P_{TF} and P_{MtetO5} revealed the lowest basal Luc activities in the uninduced state, which is at least 4–10-fold depending on the cell line. P_{Tax} yielded higher background activities than P_{TF}, indicating that avoiding the multimerisation of identical spacer sequences indeed helps to reduce background activity. In the induced state, P_{TF} reaches at least 60% of the expression levels of P_{XTC} in cell lines HeLa, CHO, PC12 and C6. In all these cell lines, P_{TF} activity was higher than in pMtetO$_5$, this effect being most pronounced in HeLa cells.

In stable cell clones of CHO cells containing P_{CMV}–tTA and a single copy integration of respective promoters, the reduction in basal activity is 12-fold with P_{TF} whereas induction is only reduced by 25% compared to P_{XTC}. Thus, with this simple approach, basal activity of the target promoter was drastically reduced whereas induced activities were only reduced by 50%. This resulted in an increase of the activation of gene expression upon withdrawal of tc from 16- to 140-fold. Also, in combination with rtTA, these promoters yielded lower background activities in different cell lines, which was between 8- and 12-fold.

Analysis of conditional *barnase* expression

The optimised tc-dependent target promoter P_{TF} was used for conditional suicide gene expression. P_{TF} was fused to the *barnase* gene to give P_{TF}–*barnase*. The *barnase* gene contains the coding region for a *B.amyloliquefaciens* RNase, a small extracellular RNase protein of 110 amino acid residues, which is *in vivo* inhibited by barstar (14). The *barnase* gene has been used in transgenic plants for cell-ablation studies (20).

The constructs P_{TF}–*barnase*, P_{CMV}–*tTA* (ratio 1:5) and a GFP (pEGFP) vector were cotransfected and numbers of surviving cells were determined by counting GFP positive cells (Fig. 3). Usually, 48–72 h after transfection, cell numbers were reduced by 80% after tc withdrawal in all cell lines used. The observed residual cell survival might be due to the lack of simultaneous presence of all three constructs in one single cell.

Analysis of conditional gene expression in neuronal cells

To assess the regulated expression of *barnase* in neuronal cells, we generated stable Neuro2A cell clones comprising a low copy number of P_{TF}–*barnase* integrates. We received *barnase* positive Neuro2A clones (70% of all G418 resistant cell clones) that survived the selection procedure and exerted a conditional *barnase* expression after transient transfection with P_{CMV}–tTA, P_{CMV}–rtTA, P_{NSE}–tTA (Fig. 4), which led to cell reduction of at least 70% after 48 h. As a comparable induction rate would be achieved with a number of weak, cell-specific promoters, demonstrated by the neuronal-specific enolase (P_{NSE}) promoter, we argue that even a low induction of the *barnase* gene *in vivo* is sufficient to ablate specific cell types in transgenic mice.

To analyse the kinetics of conditional *barnase* expression, we generated individual CHO-AA8 (contains a stable integrate of P_{CMV}–tTA) cell clones containing stable integrates of P_{TF}-*barnase*. Out of 12 CHO cell clones positive for *barnase*,

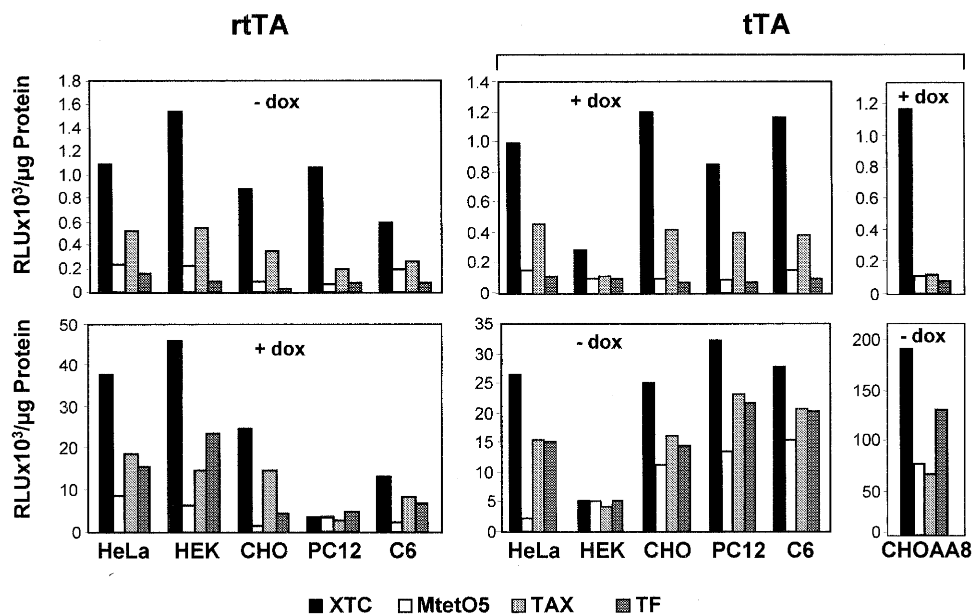

Figure 2. Comparative transactivation measurements of promoter–*luciferase* constructs in cell lines HeLa, HEK293, CHO, PC12 and C6 cells with either rtTA or tTA, with (+) dox (1 µg/ml) or without (–) dox. Mean values of five independent experiments 48 h after transient transfection of 100 ng target promoter construct and 500 ng P_{CMV}-rtTA or P_{CMV}-tTA. The mean values of promoter activity from six different CHOAA8 (P_{CMV}-tTA) cell clones comprising low copy integrations of promoter–*luciferase* constructs in the uninduced state and 48 h after dox withdrawal are shown to the right.

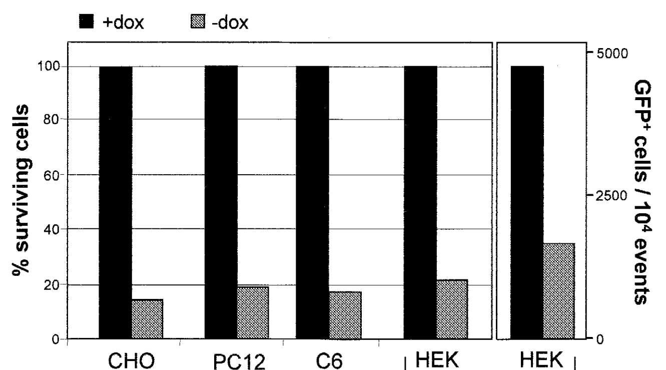

Figure 3. Tetracycline-induced cell ablation by *barnase* in cell lines CHO, PC12, C6 and HEK with the P_{TF}–*barnase* construct. Cells were cotransfected with P_{TF}–*barnase* (100 ng), P_{CMV}–tTA (500 ng) and with 100 ng pEGFP with (+) or without (–) dox; 48 h after transfection, cell numbers were determined as mean values by counting GFP⁺ cells in 10 independent fields of vision under the UV microscope (magnification 100×). Right diagram, for comparison, the number of HEK cells transfected as above were determined by FACS analysis.

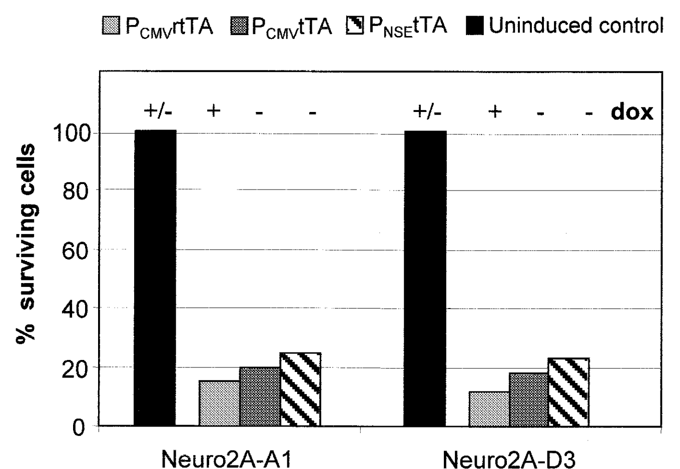

Figure 4. Tetracycline-dependent ablation of two stable *barnase* containing Neuro2A cell clones by P_{CMV}–rtTA, P_{CMV}–tTA and P_{NSE}–tTA. Neuro2A clones A1 and D3 were transfected with 500 ng of regulator construct. 48 h after transfection either in the presence (+dox) or in the absence (–dox) of inducer, cell numbers were determined by counting 10 independent fields of vision. Cells transfected with the regulator constructs in the uninduced state (+ for tTA; – for rtTA) were taken as 100% control by definition.

seven cell cones survived for a longer time period and *barnase* expression was tc-inducible. From this calculation, >60% of all cell clones obtained revealed conditional cell ablation by the *barnase* gene. A representative induction experiment with a double-stable CHO cell clone is shown in Figure 5. The mRNA levels of *barnase* were determined by semiquantitative RT–PCR (Fig. 5A). Induction of *barnase* is maximal 7 days after tc withdrawal and drops to lower levels later on (Fig. 5C). At this time point, cell morphology appeared normal whereas 9 days after tc withdrawal, a clear-cut change in cell morphology was detectable (Fig. 5B and C). and no cell showed normal morphology compared to the uninduced state, demonstrating a complete cell ablation. The reason for the delayed kinetics of Barnase accumulation or toxicity compared to maximal levels of mRNA induction is unknown. However, a similar kinetic of luciferase accumulation has been observed with this promoter in transgenic plants (19). Similar ablation results were obtained with irradiated CHO cells. These cells were also completely ablated 11 days after tc withdrawal demonstrating that even

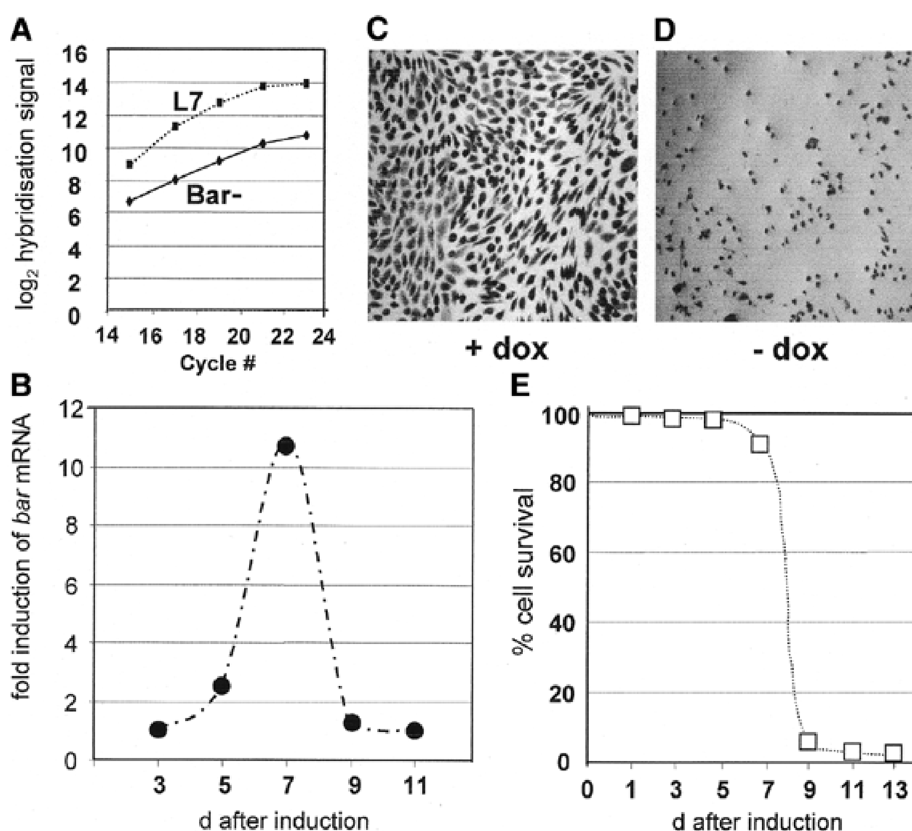

Figure 5. Tetracycline-dependent cell ablation in a CHO cell clone double transgenic for P_{CMV}–tTA and P_{TF}–*barnase*. (**A**) Increment of *barnase* and *L7* RT–PCR products from RNA of *barnase* expressing CHO cells with total cycle number, quantified as Bio-Imager units. Values are given as binary logarithms. From these data, 18 cycles were chosen for subsequent experiments to ensure maximal sensitivity and linearity of the PCR reaction. (**B**) Time-dependent induction of *barnase* mRNA levels, determined by RT–PCR. Mean values of three independent samples are given as fold-induction of *barnase* (*bar*) mRNA at respective days (d) after dox withdrawal. (**C** and **D**) Morphology of a CHO cell clone with stable integrates of P_{TF}–*barnase* and P_{CMV}–tTA cultured either in the presence (+dox; C), or after withdrawal of dox (–dox; D). Cells were stained with crystal violet. Whereas cells in the presence of dox (C) show normal fibroblast morphology, only some remaining dead cells or cell debris can be seen 11 days after dox withdrawal in (D). Scale bar in (D), 5 µm. (**E**) Time-dependent cell survival of CHO cells at respective days after tc withdrawal. Note that the drop in cell number is delayed towards maximal *barnase* mRNA induction by 2 days.

under non-proliferative conditions, Barnase can act as suicide gene (data not shown).

We also injected the P_{TF}–*barnase* construct in pronuclei to generate transgenic mice. So far, similar to the observations in cell lines, we obtained high frequencies of *barnase* positive offsprings: from 10 born mice, 2 founder mice aroused, which were viable and had a normal phenotype. These results imply that conditional *barnase* expression with P_{TF} resulted in higher frequencies of viable littermates as observed with a P_{XTC}–DTA construct, for which an ~1% rate of viable offsprings (3 per 317 born offsprings; 4) was obtained.

In conclusion, we have presented a conditional suicide system based on a modified tc-dependent expression system and combined it with a prokaryotic RNase. We found that the low basal activity of P_{TF} allows a high percentage of transgenic propagation in cell lines and mice. In combination with *barnase*, even low induction levels of the target promoter, usually achieved by tTA expression under cell-specific promoters, are sufficient to exert conditional cell ablation. Thus, our novel cell ablation strategy might have an impact on the molecular analysis of pathological processes in transgenic animals, in particular in the CNS.

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