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RESEARCH ARTICLE

A Tetracycline Controlled Activation/Repression System with Increased Potential for Gene Transfer into Mammalian Cells

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Abstract

Background Tight control of gene activity has been achieved in cells and transgenic organisms using the Tet regulatory systems. Unregulated basal transcription can, however, be observed whenever integration of target genes driven by promoters responsive to tetracycline controlled transcriptional activators (tTA, rtTA) does not occur at suitable chromosomal sites. Moreover, in viral vectors containing both the tTA coding sequence and the regulated target gene, proximity of the enhancer element driving tTA/rtTA expression to the responsive unit will lead to elevated background levels. Similarly when tTA/rtTA responsive transcription units are in a non-integrated state as eg., during transient expression, intrinsic residual transcription persists in their 'off' state, which can differ in intensity among different cell types.

Methods To efficiently repress such background activities we generated tetracycline controlled transcriptional silencers (tTS) that bind promoters responsive for rtTA in absence of the effector doxycycline (Dox). Addition of Dox prevents binding of tTS thus relieving repression, promotes binding of rtTA and thereby switches the promoter from an actively repressed to an activated state.

Results Of several tTS – fusions between a modified Tet repressor and transcriptional silencing domains – tTS^{Kid} was found to be most effective in reducing the activity of two target promoters. Ten to 200 fold repression is seen in transient expression whereas in stably transfected HeLa cells the regulatory range of the rtTA system was increased by three orders of magnitude.

Conclusions The new system appears particularly suited for the transfer of toxic genes into appropriate chromosomal sites as well as for tight regulation of genes carried by viral or episomal vectors.

Keywords gene expression; regulation; tetracycline

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Introduction

The tetracycline controlled expression systems [1,2] are widely used to regulate gene expression and were shown to function in cell lines (for review see [3]) plants [4], yeast [5], Drosophila [6,7], mice [8–11] and rats

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[12,13]. In one of the systems, transcription factor tTA, a fusion between a bacterial Tet repressor (TetR) and a transcriptional activating domain activates a minimal promoter that is fused downstream of an array of *tet* operator (*tetO*) sequences, the cognate binding sites of TetR and thus of tTA. Tetracyclines, and particularly doxycycline (Dox), prevent binding of tTA to $P_{hCMV}{}^{*}{}_{-1}$ [1], a tTA responsive promoter and abolish transcription. A complementary system based on a *tetR* mutant functions in opposite fashion [2]: rtTA (reverse tetracycline controlled transactivator) requires doxycycline to bind to *tetO* and thus transcription will only be activated in the presence of the effector.

The Tet control systems allow to regulate gene activities over a range of up to five orders of magnitude in mammalian cells [1] and in transgenic mice [8]. Two features are responsible for this wide regulatory window: First, when fully activated, the tTA/rtTA responsive promoter $P_{hCMV}{}^{*}{}_{-1}$ is one of the strongest RNA polymerase II promoters described so far [14]; second, when properly embedded into chromatin, this promoter shows no measurable intrinsic activity [1,8]. Tight regulation can therefore be achieved whenever a tTA/rtTA responsive transcription unit is integrated into a chromosomal locus where no nearby enhancer can activate the minimal promoter contained in $P_{hCMV}{}^{*}{}_{-1}$. This is best supported by the finding that even the gene encoding diphteria toxin A can be stably maintained and controlled in mice via Dox [15]. By contrast, in transient expression, ie., when tTA/rtTA responsive transcription units are not integrated in the chromosome, an intrinsic basal activity is observed which can reach considerable levels depending on the cell type and the experimental conditions [1,3,16]. The main reasons for this background activity are the absence of chromatin repression and the high copy number of the template in the cell. Regulation factors measured in transient expression experiments are therefore smaller than those found in carefully selected stable cell lines. While for many studies a certain background is acceptable and a regulation factor between 50 and around 1000 fold is sufficient, it will be prohibitory for others, in particular when the product of the target gene is not well tolerated by the cell. Thus, even low levels of expression in the transient state can prevent the establishment of a cell line despite the fact that upon integration into an appropriate locus the gene of interest would be controlled tightly enough to warrant stable experimental conditions. Moreover, when tTA/rtTA controlled target genes are transferred together with the expression unit for the transactivator eg., in a single viral vector, nearby enhancers that affect the tTA/rtTA responsive promoter are unavoidable.

While these limitations also hold true for other regulatory systems that are based on the principle of promoter activation, the Tet systems offer a rather simple solution to this problem. By appropriately combining transcriptional activators with transcriptional silencers, both susceptible to regulation by tetracyclines, a promoter like $P_{hCMV}{}^{*}{}_{-1}$ can be actively repressed as well as activated in a Dox dependent manner.

Here, we report the generation of tetracycline controlled transcriptional silencers (tTS) which are fusions between a TetR variant and domains known to function as repressors of transcription of which a first version has been described by Deuschle *et al.* [17] in an attempt to control the cytomegalovirus IE promoter by repression. Among the domains examined, KRAB derived from the human kidney protein Kid-1 [18] turned out to be the most suitable one in our context. The resulting TetR-fusion, tTS^{Kid}, can be coexpressed with rtTA without forming heterodimers due to modification of the dimerization surface of the TetR moiety. In the absence of Dox, tTS^{Kid} binds to the operators of $P_{hCMV}{}^{*}{}_{-1}$ and represses its residual activity, whereas in the presence of Dox rtTA occupies the *tetO* sites and activates the promoter. We show that this combined tTS/rtTA system has an expanded regulatory range due to a strong reduction of the basal activity of tTA/rtTA responsive promoters in the state of transient expression as well as in cell lines where such promoters are susceptible to activation from nearby enhancers. The system should permit the study of a variety of genes which previously appeared difficult to control and may prove particularly useful for vectors which remain in an episomal state.

Materials and methods

Cloning of tTS

To facilitate cloning of different silencing domains a basic cloning vector was generated. For this purpose a double stranded oligonucleotide ($^{5'}$AAAAAAGAAGAGAAAGCTAGCATCGATTAACTAAGTAAGGATCC) was ligated into pUHD141-1/X [19] previously cut with SmaI. The resulting plasmid (pUHS 1-1) encodes TetR[B] followed by the SV40 Tag NLS, a small multiple cloning site comprising NheI and ClaI restriction sites followed by 3 TAA stop codons in all reading frames and a BamHI site. PCR fragments encoding the silencing domain of erbA (aa 389–632), eve (aa 140–246) and Kid-1 (aa 12–74) were inserted into this vector as NheI-ClaI fragments giving rise to pUHS 2-1 encoding tTS^{erb}, pUHS 3-1 encoding tTS^{eve} and pUHS 4-1 encoding tTS^{Kid} respectively.

To prevent heterodimerisation between TetR moities derived from rtTA and tTS a chimeric TetR consisting of the DNA binding domain of TetR [B] (aa 1–44) and the rest of the protein core from TetR[E] (aa 45–211) was generated. The sequence encoding the N-terminal 44 amino acids was obtained by PCR on pUHD 14-1 [1] (upstream primer: $^{5'}$GACACCGGGACCGATCCAGCC downstream primer: $^{5'}$GCTTATTTTTCACGTGCCAATACAATGTAGGCTGCTC) and the amplified material was digested with XbaI and PmlI. pWH610 encoding TetR[E] served as template to amplify the sequences encoding aa 45–211 (upstream primer: $^{5'}$GTATTGGCACGTGCGCAACAAGC downstream primer: $^{5'}$CGATGCTAGCTTTCTCT-

TCTTTTTTGGTTTATTACCATCCTCAATGGGTGTATGC) and this PCR product was digested with PmlI and NheI. Both fragments were simultaneously ligated into plasmid pUHS 1-1 which was previously cut with XbaI and NheI giving rise to plasmid pUHS 5-1. The Kid-1 derived silencing domain was cloned into this plasmid as a NheI-PvuII fragment obtained from pUHS 4-1 resulting in plasmid pUHS 6-1.

Transfection and quantitation of reporter gene activity

Introduction of DNA into cells via calcium phosphate transfection and determination of luciferase activity from whole cell extracts was done as described previously [1]. Transfection using Lipofectamine (Gibco) was done according to the recommendations of the manufacturer.

Western blotting

Whole cell extracts were prepared as described in [1]. To detect the different silencers a polyclonal rabbit serum (laboratory stock) directed against the TetR moiety was used.

Generation of triple stable cell lines

HRL9, a HR5 derived cell line that carries rtTA and the Tet-regulated luciferase encoded by plasmid pUHC13-3 stably integrated into the genome was transfected with pUHS6-1, encoding tTS[B|E]^{Kid} and pSV2pac [20] a plasmid that encodes the puromycin resistance gene driven by the SV40 promoter. Clones were selected in the presence of 2 μg/ml puromycin and subsequently tested for expression of functional silencer by determining luciferase activity in the presence (10 ng/ml and 1 μg/ml) and absence of doxycycline.

Results

Generation and characterization of tetracycline controlled transcriptional silencers (tTS)

The basal background activity of $P_{hCMV}{}^{*}{}_{-1}$ was determined in five different cell lines after transfection of these cells with plasmid pUHC13-3 [1] carrying the luciferase gene under $P_{hCMV}{}^{*}{}_{-1}$ control. As shown in Figure 1, luciferase activity varies significantly among the different cell lines and when compared to the activity in HeLa cells, it is increased 20 to 70 fold in NIH 3T3, CHO, HEK293 and COS-1 cells, respectively. In these cells, we have examined the function of 3 tTS constructs that were generated by fusing at the DNA level TetR with silencing domains of ErbA [21], EVE [22] and Kid-1 [18] (Figure 2A). Coding sequences of the three fusion proteins are preceeded by the promoter of the immediate early genes of CMV (P_{hCMV}) and followed by the SV40 poly A site. Expression of all silencers was verified by transiently transfecting plasmids encoding tTS^{erb}, tTS^{eve} and tTS^{Kid} into HeLa cells. Analysis of total cellular extracts by Western blotting (Figure 2B) using a polyclonal serum directed against TetR reveals three proteins of the expected molecular weight tTS^{erb} : 52kD, tTS^{eve} : 35 kD and tTS^{Kid} : 32 kD. A control transfection with a TetR encoding plasmid yielded a species with a molecular weight of 23 kD as expected for the monomer of the Tet repressor.

Stoichiometric amounts of pUHC13-3 and plasmids encoding either one of the three silencer constructs were cotransfected (together with a β-galactosidase expressing vector as transfection standard) into the respective cell lines which were grown in the absence or presence of Dox, respectively. As a control, pUHC13-3 was also cotransfected with a homologous plasmid encoding TetR

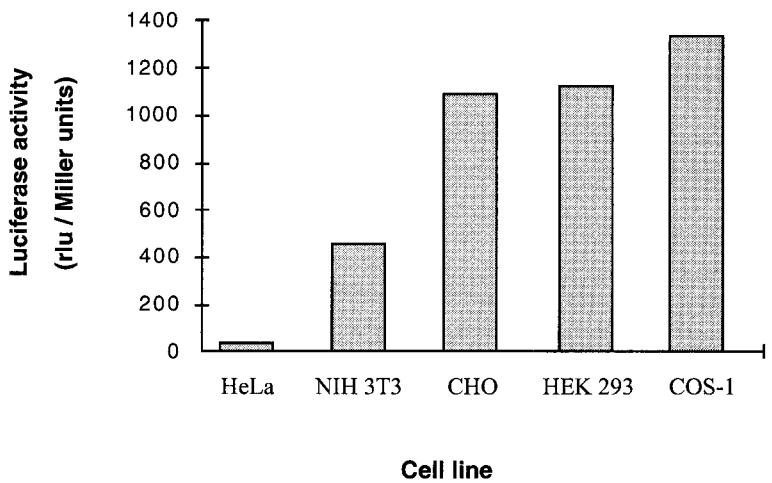

Figure 1. Basal activity of $P_{hCMV}{}^{*}{}_{-1}$ in different cell lines. Cells were seeded into 35 mm dishes and transfected the next day at 50% confluency via the calcium phosphate method with a DNA mix containing the Tet-regulated luciferase reporter gene and a constitutively expressed *lacZ* gene. 24 h after transfection cells were harvested and luciferase activity was determined in whole cell extracts as described [1]. Values given correspond to the mean of two independent experiments. To normalise for differences in transfection efficiency, luciferase activity was standardized to the specific activity of β-galactosidase

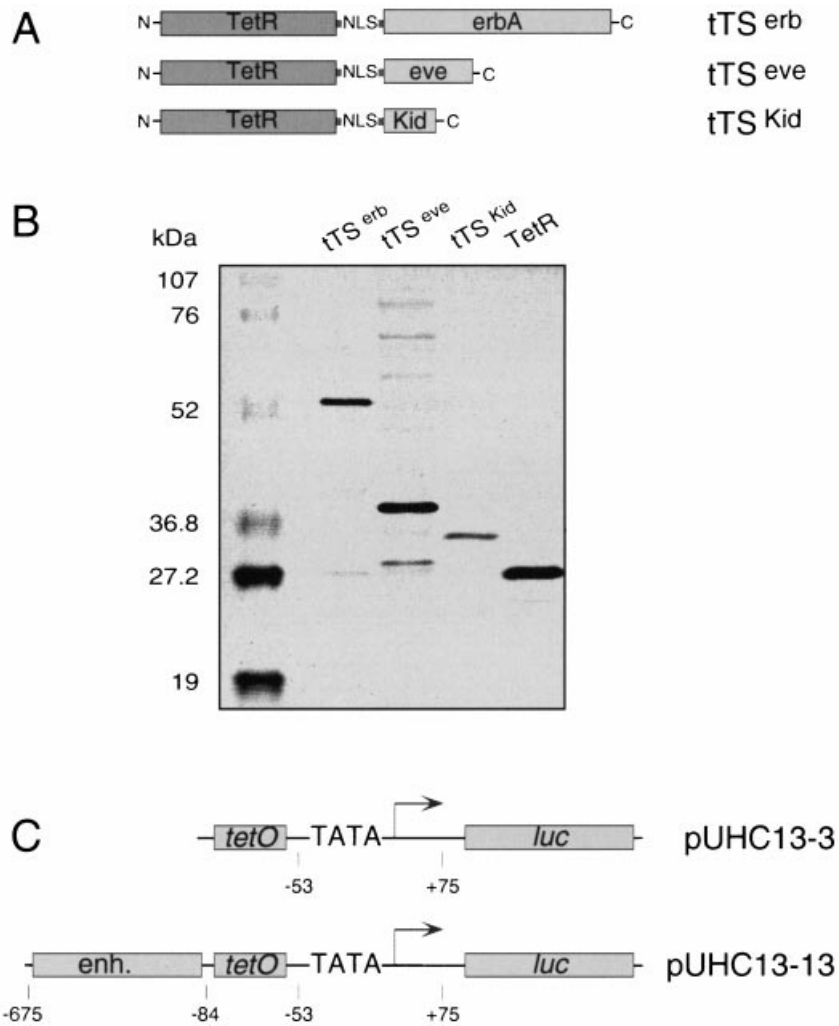

Figure 2. Tetracycline controlled silencers and target promoters. (A) Representation of silencer constructs. The fusion proteins consist of TetR, the nuclear localization sequence (NLS) of SV40 Tag [26] and the silencing domains erbA, eve or Kid, respectively. (B) Identification of silencers produced in HeLa cells. HeLa cells grown to 50% confluency were transiently tranfected with plasmids: pUHS 2-1, pUHS 3-1, pUHS 4-1 encoding tTS^{erb}, tTS^{eve}, and tTS^{Kid} and the cloning vector pUHS 1-1 encoding TetR. After 30 h, cell extracts were prepared and electrophoretically separated on 12% PAA gels. Western blot analysis using a polyclonal α-TetR antibody reveals proteins of a molecular weight of: lane 2: 52 kD, lane 3: 35 kD, lane 4: 32 kD and lane 5: 23 kD respectively which corresponds to the expected molecular weight of tTS^{erb}, tTS^{eve}, tTS^{Kid} and TetR. Lane 1 contains the molecular weight standard. (C) Representation of $P_{hCMV}{}^{*}{}_{-1}$ and the $P_{tetO\text{-}13}$ promoter enhancer construct of pUHC13-13. enh. denotes the enhancer region of P_{hCMV}, *tetO*, an array of 7 *tet* operator sequences [1] and luc, the luciferase gene. Outline is not drawn to scale

alone. Under these conditions the three silencer constructs reduce luciferase activity in the various cell lines to a similar extent as shown in Figure 3. Whereas in HeLa cells that show the lowest basal activity (Figure 1), repression was about ten fold, it is about 20, 30 and 50 fold in NIH 3T3, C 243 and HEK293 cells, respectively.

Beside suppressing the residual activity of a minimal promoter in the transient state before integration into the genome occurs, tTS ideally should be effective in counteracting the stimulatory effect of nearby enhancers on integrated promoters. To mimic the influence of enhancers on $P_{hCMV}{}^{*}{}_{-1}$, we generated $P_{tetO\text{-}13}$ which contains upstream of the 7 *tetO* sequences the complete CMV enhancer region spanning position −84 to −675 [23] (Figure 2C). In transient transfection assays as described above, luciferase activity conferred by pUHC13-13 carrying $P_{tetO\text{-}13}$ was reduced 180-fold by tTS^{Kid}, five-fold by tTS^{erb} and two-fold by tTS^{eve} in HeLa cells.

Thus, while all three silencers appear about equally efficient in reducing luciferase activity resulting from a minimal promoter in transient transfections, tTS^{Kid} was most effective when repression of the enhancer containing construct was examined. From these data, we conclude that tTS^{Kid} is the most versatile silencer generated. Therefore, we used only tTS^{Kid} in the remaining part of the study.

Combining tTS^{Kid} with the rtTA-dependent activating system

tTS^{Kid} binds to a target promoter in the absence of the effector and thus can be combined with the rtTA activating system where activation of gene transcription

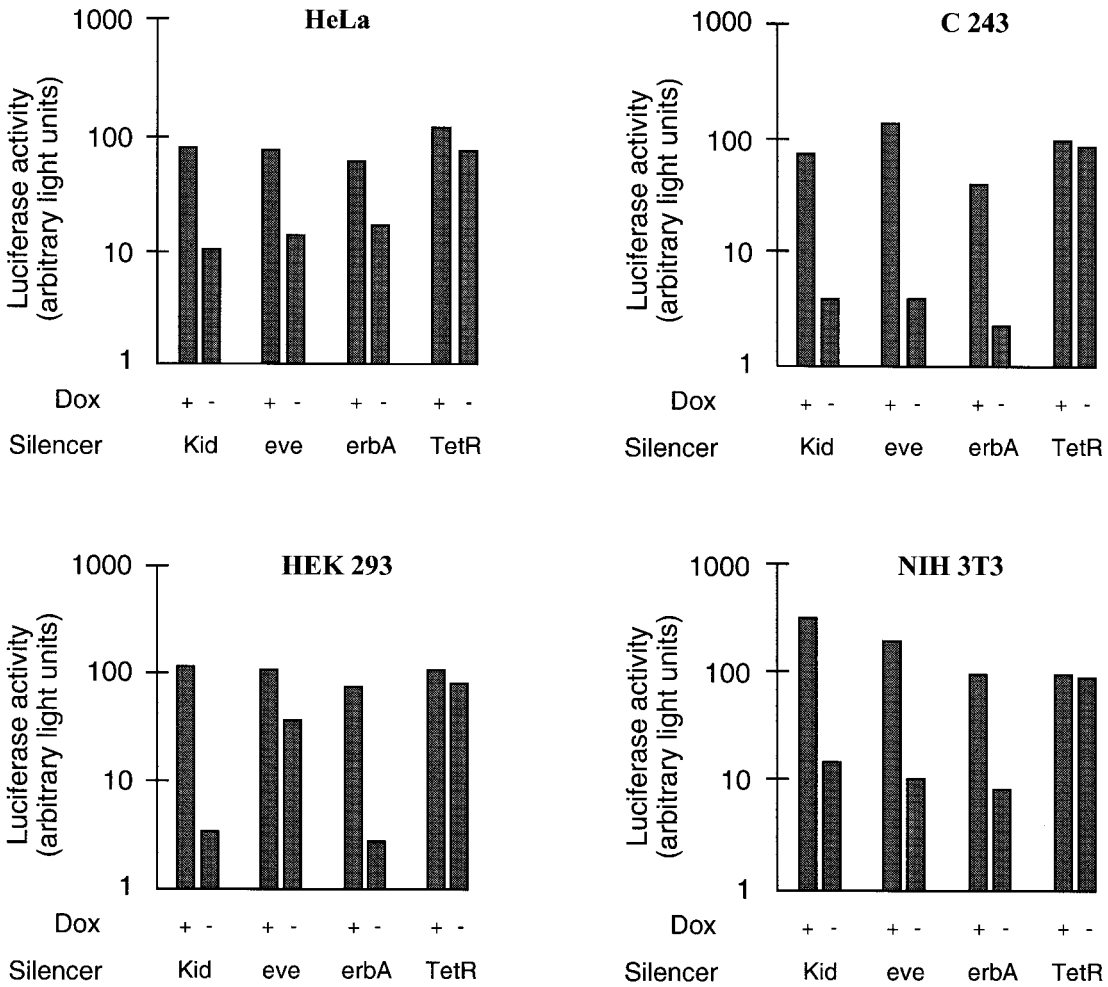

Figure 3. Luciferase gene expression is repressed after cotransfection with a silencer. Different cell lines were transfected via the calcium phosphate method with the regulated luciferase reporter gene (encoded by pUHC13-3) and a silencer construct or the TetR encoding plasmid as a control as indicated. The same transfection mix was split between cells that were grown in medium containing 1 μg/ml (+) or not containing (−) doxycycline. Cells were harvested after 36 h and luciferase activity was measured. Data shown correspond to the mean of two independent experiments. To determine the effect of a given silencer in the different cell lines luciferase activity obtained in the cotransfection with the TetR-encoding plasmid was arbitrarily set as 100

will occur only in the presence of doxycycline. Because both regulatory proteins bind to *tetO* operator sequences as dimers, coexpression of tTS^{Kid} and rtTA in the same cell will not only lead to the formation of tTS^{Kid} and rtTA homodimers, but also to tTS^{Kid}/rtTA heterodimers [24] that will diminish the number of active molecules for either system (activating or repressing) and most likely compromize the overall efficiency of the system. Recent studies [24] have shown that heterodimer formation does not occur between the natural Tet repressors of the B and the E class. As both rtTA and tTS^{Kid} are based on the repressor of the B class, we altered the dimerization specificity of tTS^{Kid} by replacing the relevant portion of the repressor moiety by the corresponding sequence of $TetR^{E}$ resulting into $tTS^{Kid}B/E$ (encoded by pUHS 6-1).

Previously we have shown that tTA binds *tetO* DNA at doxycycline concentrations below 10 ng/ml while rtTA associates with these sequences only above 100 ng/ml [1,2]. From this we reasoned that combination of $tTS^{Kid}B/E$ and rtTA in the same cell will lead to activated transcription at doxycycline concentrations above 100 ng/ml while residual expression of a target gene (e.g. luciferase reporter gene) should be actively repressed at effector concentrations below 10 ng/ml. At intermediate effector concentrations, neither rtTA nor $tTS^{Kid}B/E$ should bind to the target promoter and reporter gene activity detected should reflect the unregulated background expression level. To examine this model, HeLa cell line HR5 [2] that carries the rtTA gene stably integrated was transiently transfected with $tTS^{Kid}B/E$ and the luciferase reporter unit (pUHC13-3). Cells were incubated for 24 h at different concentrations of doxycycline before they were harvested and luciferase activity was determined in cell extracts. Figure 4 shows that indeed luciferase expression is activated by rtTA at concentrations above 100 ng/ml. At effector concentrations between 10 and 100 ng/ml neither rtTA nor $tTS^{Kid}B/E$ bind and thus luciferase activity reflects the level of basal expression. Reporter expression is reduced to background levels below 3 ng/ml of doxycycline when

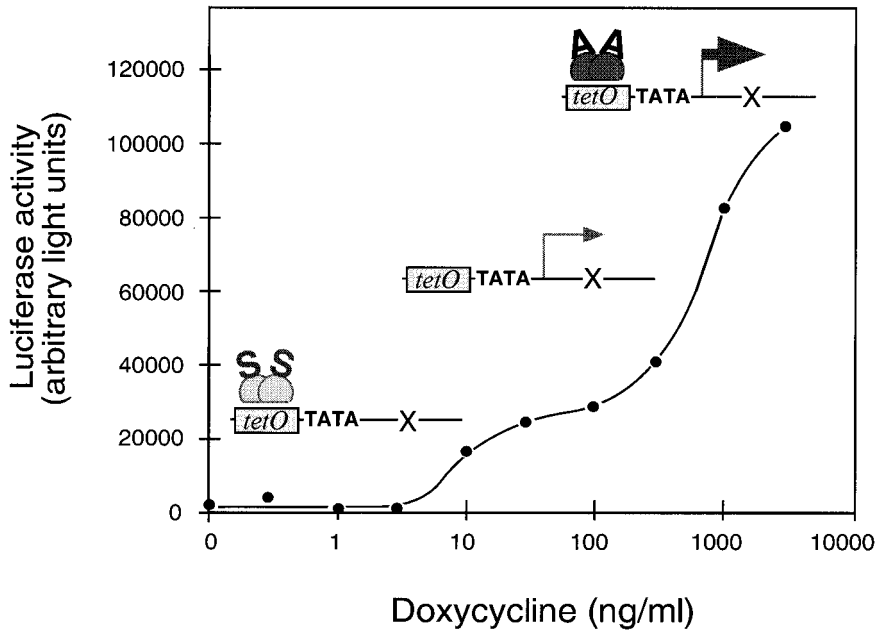

Figure 4. Dose-response curve demonstrating repression of basal luciferase expression by tTS^{Kid}. HR5 cells [2] which constitutively express rtTA were transiently transfected with pUHC13-3 and pUHS 6-1. The same transfection mix was split between cells grown in the presence of different concentrations of doxycycline. Cells were harvested 24 h later and luciferase activity, normalised to the constitutively expressed β-galactosidase control was determined. Values given for luciferase activity correspond to the mean of two independent experiments. The inserts indicate schematically the three different states of activity of the tTS/rtTA system at the respective Dox concentration

tTS^{Kid}B/E binds the target promoter. These results confirm our model with regard to doxycycline-controlled binding of tTS^{Kid}B/E and rtTA to the *tetO* sequences of $P_{hCMV}{}^{*}{}_{-1}$ (Figure 4).

When the combined activating/repressing system was transferred in those cell lines which had previously been shown to exhibit the highest basal activity, namely HEK293, CHO and COS-1, an efficient reduction of the unregulated background activity was observed in transient expression experiments (Figure 5). Transfection of cells with DNA encoding either rtTA or both rtTA and tTS^{Kid}B/E showed not only that, in absence of doxycycline, the silencer was able to reduce background activities by a factor of 10–50 but also that maximal expression of luciferase is achieved upon induction, i.e. in presence of 1 μg/ml doxycycline (Figure 5). Thus, the presence of tTS^{Kid}B/E does not impair the activation potential of the reverse transactivator.

The regulation potential of the combined tTS/rtTA system

To quantify the effect of tTS^{Kid} on the regulatory range of the rtTA system, we generated HeLa cell lines that contain the luciferase reporter unit stably integrated and which produce constitutively rtTA as well as tTS^{Kid}B/E. The parental cell line, HRL9, expresses the rtTA-gene under control of P_{hCMV} while the luciferase gene controlled by $P_{hCMV}{}^{*}{}_{-1}$ is located in a "high background locus" resulting in significant luciferase expression in the absence of doxycycline. Therefore, upon addition of doxycycline, luciferase activity can be induced only around 27 fold (Table 1). HRL9 cells were cotransfected with tTS^{Kid}B/E DNA (pUHS 6-1) and a plasmid carrying the puromycin resistance gene as a selectable marker. Cells resistant to puromycin were examined for luciferase activity. As shown in Table 1, in five clones (analyzed from a total

Table 1. Regulation of luciferase by the combined tTS^{Kid}B/E rtTA system in stably transfected HeLa cells. The HeLa cell line HRL9 which constitutively expresses the reverse transactivator and in addition contains the regulated luciferase gene stably integrated was transfected with pUHS6-1 and pSV2pac conferring puromycin resistance. Luciferase activity was determined from puromycin resistant clones that were grown in the presence (1 μg/ml and 10 ng/ml) and absence of doxycycline until they had reached 60% confluency at most

	Luciferase activity [rlu/μg of protein]			
clone	+ Dox [1 μg/ml]	+ Dox [10 ng/ml]	−Dox	factor of regulation
HRL9	50 711 ± 2 628	2 824 ± 45	1 897 ± 25	2.7×10^1
HRL/tTS^{Kid}-1	60 653 ± 3 215	1 607 ± 84	⩽2	$\geqslant 3 \times 10^4$
HRL/tTS^{Kid}-2	48 880 ± 1 852	849 ± 64	24 ± 2	2×10^3
HRL/tTS^{Kid}-3	110 229 ± 1 989	3 645 ± 258	⩽2	$\geqslant 5 \times 10^4$
HRL/tTS^{Kid}-5	35 933 ± 1 828	1 202 ± 75	⩽2	$\geqslant 1.5 \times 10^4$
HRL/tTS^{Kid}-7	105 041 ± 16 287	4 087 ± 1 696	⩽2	$\geqslant 5 \times 10^4$

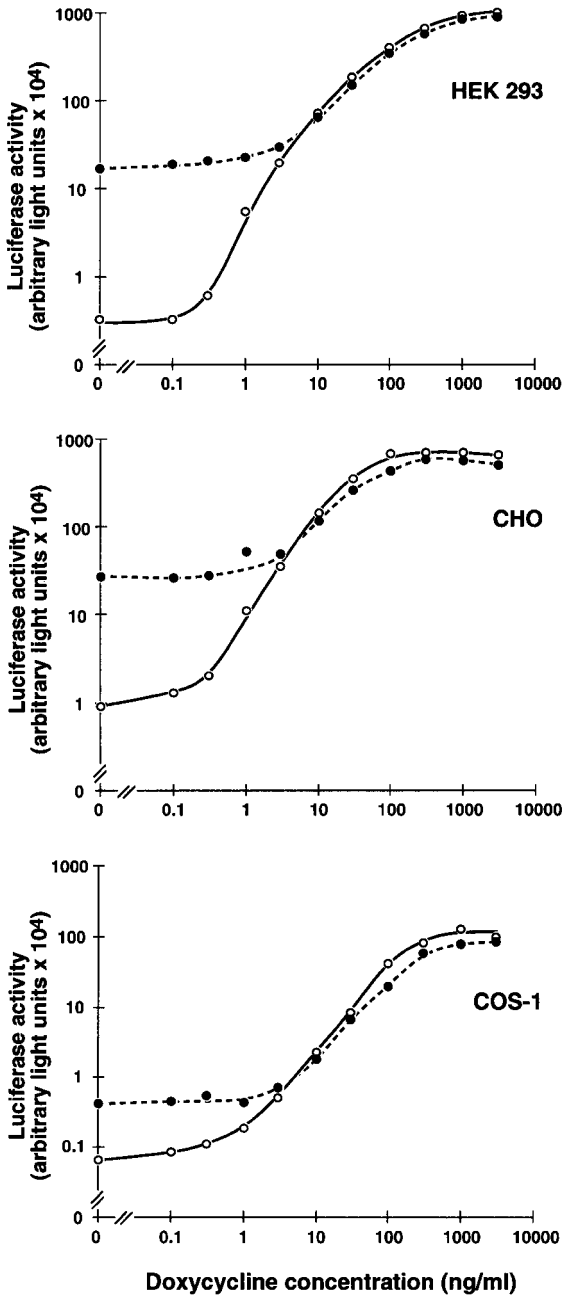

Figure 5. Combining the activating system with the repressing system in different cell lines. Luciferase activity is measured at different concentrations of doxycycline after transient transfection of cells with Lipofectamine and a DNA mix containing vectors encoding rtTA and the regulated luciferase (filled circles) or rtTA, the regulated luciferase and tTS^{Kid} (open circles). A plasmid encoding the constitutively expressed β-galactosidase gene was included for standardization in all experiments. Each point represents the mean luciferase activity of two independently transfected dishes

of seven selected), luciferase activity is approximately the same as in the parental HRL9 line at 1 μg/ml doxycycline. At 10 ng/ml of doxycycline, it is similar to the unregulated-basal level as obseved in HRL9 cells (variation of luciferase activity by a factor of two-three between the parental cells and the five triple stable lines is likely due to clonal variation). In the absence of doxycycline, however, luciferase activity is reduced to instrumental background in four out of five triple stable clones increasing the respective regulation factors by two to three orders of magnitude when compared to the HRL9 parent cell line. With these cell lines, induction kinetics of the rtTA and the combined rtTA/ tTS^{Kid}B/E system were compared in order to examine whether this parameter would be affected by the presence of the silencer. Thus, luciferase activity in HLR9 and HRL/tTS^{Kid}-7 cultures (Table 1) was monitored during a 40-hour-period following the addition of Dox to the medium. The time course of induction depicted in Figure 6 shows that in both cell lines maximal luciferase activity is seen after eight to 12 hours demonstrating that the rapid kinetics of activation of the rtTA system is not affected by the presence of the silencer tTS^{Kid}B/E.

Discussion

To maintain specificity, RNA polymerase II promoters like the artificial tTA/rtTA responsive promoters which are solely controlled by highly defined activators, require shielding from outside signals which may stimulate the minimal promoter element placed under tTA/rtTA control. This can rather readily be achieved at the cellular as well as at the organismal level whenever selection or screening procedures allow the identification of an appropriate integration event, ie., when the rtTA/ tTA responsive transcription unit has integrated in a "silent" chromosomal locus. In our experience, around five to 15% of insertions depending on the cell line occur at loci where no basal activity eg., of luciferase can be detected but activation of the promoter to high levels can nevertheless be induced through addition or withdrawal of Dox. The tetracycline controlled transcriptional silencers described here now allow to actively shield the minimal promoter contained in $P_{hCMV}{}^{*}{}_{-1}$ from outside stimulatory influence. In the HRL9 HeLa cell line, the tTA/rtTA expression unit is obviously not inserted in a "silent" but rather in a "high background" locus and due to this background activity there is only a modest 27 fold upregulation of luciferase by rtTA (although the absolute expression level reached is high). This background activity was reduced by tTS^{Kid}B/E to an extent that luciferase is not detectable anymore in four out of five clones resulting in a regulation factor which is two to three orders of magnitude higher. Importantly, the presence of the silencer does neither interfere with the absolute level of expression after induction by Dox nor with the kinetics of induction. Therefore, in cell lines which coexpress rtTA and tTS^{Kid}B/E the number of loci that permit tight regulation of a target gene should be considerably larger and the increased probability to hit such loci will facilitate the generation of cell lines in which a gene of interest is well regulated via Dox.

The situation is qualitatively different in transient expression experiments. Here, the transcription units are not embedded in a chromatin structure comparable to

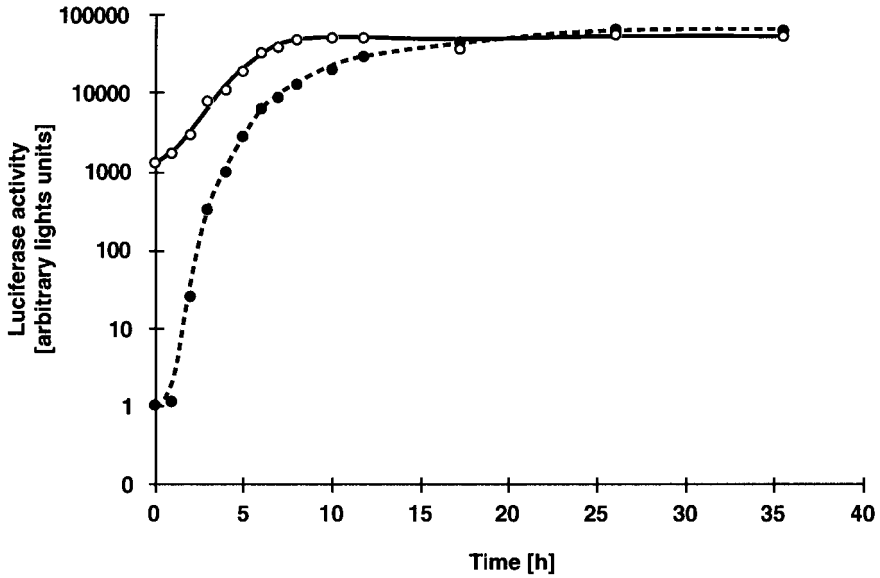

Figure 6. Kinetics of luciferase induction in HRL9 and HRL/tTS^{Kid}-7 cells after activation with doxycycline. HRL9 (open circles) and HRL/tTS^{Kid}-7 (filled circles) cells were plated at a density of 5×10^4 cells per 35 mm dish. After attachment of the cells the medium of all cultures was renewed (time=0) and replaced by medium containing doxycycline (1 μg/ml). Each point represents the mean of the normalized luciferase activity from 2 independently grown dishes of cells harvested at the times indicated

the integrated state rendering the transcription unit susceptible to unspecific transcription events. The intensity of such transcription can even vary among different cell types, possibly reflecting varying compositions of transcription factors. In addition, multiple copies of the template are present in the cell. Together these parameters prevent low background activities and high regulation factors when compared to properly selected stable cellular clones or mouse lines. Nevertheless, as shown herein, presence of silencers in the transient state of expression significantly reduces background activities particularly in cell types that have shown high residual expression such as HEK293 and CHO cells. The ten to 50 fold reduction demonstrated is probably not the maximum that may be achieved after optimization of the experimental conditions which would eg., reconcile the intracellular silencer to template ratio. Nevertheless, reduction of the residual activity and the concommitant increase of the regulatory window will expand the applicability of Tet regulation in experiments that are bound to be carried out at the level of transient expression. In this regard, cell lines that stably produce both rtTA and tTS^{Kid}B/E will be most useful.

More importantly, the background reduction in the transient state of expression will greatly facilitate the generation of cell lines where target genes are tightly controlled via doxycycline. This will be of particular advantage for genes whose products are not well tolerated by cells.

Finally, the detailed structural information on Tet repressors and the possibility of modifying these interesting allosteric proteins by genetic means in *E. coli* have allowed the generation of quite intriguing TetR variants. Here, we have made use of an altered dimerization surface for tTS^{Kid}B/E which prevents the formation of rtTA: tTS^{Kid} heterodimers and instead allows the production of two populations of TetR-based homodimeric proteins in the same cell. Together with respecified DNA recognition properties [24], the Tet regulatory system will increasingly permit adaptation to a variety of specific experimental needs. This may include its application *in vivo* as first results with Tet regulated transplanted cells support a low immunogenicity of TetR [25].

Conclusions

A major requirement for gene therapy is to keep transgene expression in target cells under "outside" control. The Tet systems offer tight control over the level and timing of transgene expression. However, depending on the specific experimental conditions like the cellular background chosen or when using single vectors to deliver the gene for the regulatory protein (tTA/rtTA) together with the target gene, enhancer sequences driving expression of the transactivator may increase the basal expression level of the target gene and thus interfere with tight regulation. In order to shield the Tet-controlled expression cassette from outside effects, we have generated tTS, a tetracycline controlled transcriptional silencer that we combined with the rtTA-dependent activating system. In this new system target gene activity is highly induced via rtTA in the presence of doxycycline. In the absence of Dox, tTS will actively repress the transgene thus abolishing background expression. This combined activating/repressing system seems particularly useful for the expression of potentially toxic genes or when using episomal vectors.

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References

1. Gossen M, Bujard H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci U S A* 1992; **89**: 5547–5551.
2. Gossen M, Freundlieb S, Bender G, Müller G, Hillen W, Bujard H. Transcriptional activation by tetracyclines in mammalian cells. *Science* 1995; **268**: 1766–1769.
3. Freundlieb S, Baron U, Bonin AL, Gossen M, Bujard H. Use of tetracycline-controlled gene expression systems to study mammalian cell cycle. *Methods Enzymol* 1997; **283**: 159–173.
4. Weinmann P, Gossen M, Hillen W, Bujard H, Gatz C. A chimeric transactivator allows tetracycline-responsive gene expression in whole plants. *Plant J* 1994; **5**: 559–569.
5. Gari E, Piedrafita L, Aldea M, Herrero E. A set of vectors with a tetracycline-regulatable promoter system for modulated gene expression in Saccharomyces cerevisiae. *Yeast* 1997; **13**: 837–848.
6. Girard F, Bello B, Laemmli UK, Gehring WJ. *In vivo* analysis of scaffold-associated regions in Drosophila: a synthetic high-affinity SAR binding protein suppresses position effect variegation. *Embo J* 1998; **17**: 2079–2085.
7. Bieschke ET, Wheeler JC, Tower J. Doxycycline-induced transgene expression during Drosophila development and aging. *Mol Gen Genet* 1998; **258**: 571–579.
8. Kistner A, Gossen M, Zimmermann F, *et al*. Doxycycline-mediated quantitative and tissue-specific control of gene expression in transgenic mice. *Proc Natl Acad Sci U S A* 1996; **93**: 10933–10938.
9. Efrat S, Fusco DeMane D, Lemberg H, al Emran O, Wang X. Conditional transformation of a pancreatic beta-cell line derived from transgenic mice expressing a tetracycline-regulated oncogene. *Proc Natl Acad Sci U S A* 1995; **92**: 3576–3580.
10. Mayford M, Bach ME, Huang YY, Wang L, Hawkins RD, Kandel ER. Control of memory formation through regulated expression of a CaMKII transgene. *Science* 1996; **274**: 1678–1683.
11. Ewald D, Li M, Efrat S, *et al*. Time-sensitive reversal of hyperplasia in transgenic mice expressing SV40 T antigen. *Science* 1996; **273**: 1384–1386.
12. Fishman GI, Kaplan ML, Buttrick PM. Tetracycline-regulated cardiac gene expression *in vivo*. *J Clin Invest* 1994; **93**: 1864–1868.
13. Harding TC, Geddes BJ, Murphy D, Knight D, Uney JB. Switching transgene expression in the brain using an adenoviral tetracycline-regulatable system. *Nat Biotechnol* 1998; **16**: 553–555.
14. Yin DX, Zhu L, Schimke RT. Tetracycline-controlled gene expression system achieves high-level and quantitative control of gene expression. *Anal Biochem* 1996; **235**: 195–201.
15. Lee P, Morley G, Huang Q, *et al*. Conditional lineage ablation to model human diseases. *Proc Natl Acad Sci U S A* 1998; **95**: 11371–11376.
16. Gossen M, Bonin AL, Bujard H. Control of gene activity in higher eukaryotic cells by prokaryotic regulatory elements. *Trends Biochem Sci* 1993; **18**: 471–475.
17. Deuschle U, Meyer WK, Thiesen HJ. Tetracycline-reversible silencing of eukaryotic promoters. *Mol Cell Biol* 1995; **15**: 1907–1914.
18. Witzgall R, O'Leary E, Leaf A, Onaldi D, Bonventre JV. The Kruppel-associated box-A (KRAB-A) domain of zinc finger proteins mediates transcriptional repression. *Proc Natl Acad Sci U S A* 1994; **91**: 4514–4518.
19. Baron U, Gossen M, Bujard H. Tetracycline-controlled transcription in eukaryotes: novel transactivators with graded transactivation potential. *Nucleic Acids Res* 1997; **25**: 2723–2729.
20. Vara JA, Portela A, Ortin J, Jimenez A. Expression in mammalian cells of a gene from Streptomyces alboniger conferring puromycin resistance. *Nucleic Acids Res* 1986; **14**: 4617–4624.
21. Martin B, Renkawitz R, Muller M. Two silencing sub-domains of v-erbA synergize with each other, but not with RXR. *Nucleic Acids Res* 1994; **22**: 4899–4905.
22. Han K, Manley JL. Transcriptional repression by the Drosophila even-skipped protein: definition of a minimal repression domain. *Genes Dev* 1993; **7**: 491–503.
23. Boshart M, Weber F, Jahn G, Dorsch-Hasler K, Fleckenstein B, Schaffner W. A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. *Cell* 1985; **41**: 521–530.
24. Baron U, Schnappinger D, Helbl V, Gossen M, Hillen W, Bujard H. Generation of conditional mutants in higher eukaryotes by switching between the expression of two genes. *Proc Natl Acad Sci U S A* 1999 (In Press).
25. Bohl D, Naffakh N, Heard JM. Long-term control of erythropoietin secretion by doxycycline in mice transplanted with engineered primary myoblasts. *Nat Med* 1997; **3**: 299–305.
26. Kalderon D, Roberts BL, Richardson WD, Smith AE. A short amino acid sequence able to specify nuclear location. *Cell* 1984; **39**: 499–509.

