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Tetracycline-inducible expression systems with reduced basal activity in mammalian cells

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ABSTRACT

We describe a modification of the tetracycline-inducible eukaryotic gene expression system with decreased basal levels of expression in HeLa cells. It employs the tetracycline-inducible transactivator and a tetracycline-regulated repressor fusion acting on the same promoter. To avoid heterodimerization or competition for the same DNA site, each was provided with different DNA recognition and/or protein dimerization specificities. We achieved active silencing in the uninduced state resulting in ~6-fold reduced levels of basal transcription and several hundred-fold activation of gene expression upon addition of tetracycline.

Regulatable gene expression methods for mammalian cells allowing tight and specific regulation are rare. Using the regulatory elements of the Tn*10*-encoded tetracycline (tc) resistance operon from *Escherichia coli* (for review see 1), an inducible system of over five orders of magnitude was developed for HeLa cells. TetR is converted into a transcriptional activator by fusion with the VP16 transcriptional activation domain (tTA, 2). Subsequently, a reversed TetR–VP16 transactivator (rtTA) was developed which efficiently binds *tetO* only in the presence of tc (3). Tc-regulated gene expression has been successfully employed in mammalian cell cultures (4,5) and in transgenic mice (6,7). Some applications are hampered by the residual basal activity in the uninduced state (6–8).

We present two approaches in which activation by rtTA is combined with repression in the uninduced state. For this purpose the transactivator and repressor are expressed in the same cell, but are in opposite ways controlled by the inducer. Transcriptional repression is mediated by a chimeric protein consisting of the N-terminus of the KRAB repressor domain of the mammalian Kox1 protein fused to TetR. This transrepressor can bind to *tetO* in the absence of tc to silence transcription of the gene of interest (9). In the presence of tc the transrepressor dissociates from *tetO* and binding of rtTA (3) takes place, resulting in activation of transcription (Fig. 1A). Since TetR forms dimers *in vivo*, heterodimerization could obscure these phenotypes. This heterodimerization does not seem to have detrimental effects in a similar system developed for regulated gene expression in yeast (10). Nevertheless, we made use of the modular architecture of TetR (11) and combined the class B DNA binding domain (amino acids 1–50) and the class E dimerization region (amino acids 51–211) in the TetR–KRAB fusion [TetR(B/E)–KRAB].

While in the former strategy rtTA and TetR(B/E)–KRAB bind to seven *tetO* elements upstream of the TATA box (Fig. 1A) in the reporter construct pUHC13-3 (2), we also constructed rtTA(4C), which has a new DNA binding specificity (12) and only binds efficiently to the *tetO*-4C variant. Seven of these *tetO*-4C elements are located upstream of the TATA box in the construct pUHC13-3(4C+). TetR(B/E)–KRAB binds to wild type *tetO* located 2 bp downstream of the TATA box in this new reporter construct (Fig. 1B). Thus, transactivator and transrepressor bind exclusively to different sites.

The regulator constructs encoded TetR(B/E)–KRAB and rtTA, each expressed under the control of the human cytomegalovirus IE promoter/enhancer. Furthermore, we used the synthetic, bicistronic constructs pCMV–KRAB–rtTA or pCMV–KRAB–rtTA(4C) which were designed to simultaneously express TetR(B/E)–KRAB and rtTA joined by an internal ribosome entry site (IRES) under the control of a single hCMV promoter/enhancer.

To investigate tc-dependent regulation of the combined activator/repressor strategy, we performed transient co-transfection experiments in HeLa cells using the luciferase encoding reporter plasmids pUHC13-3 or pUHC13-3(4C+) together with the various regulator constructs.

Luciferase expression from pUHC13-3 (Fig. 2A) is driven by the transactivator rtTA (pUHD17-1neo, 3) in the presence of inducer, as evidenced by the 170-fold increase when only rtTA was transfected. The transrepressor TetR(B/E)–KRAB alone led to an ~4-fold decrease of the basal expression level, which is alleviated in the presence of tc. Expression without tc was repressed ~6-fold when rtTA and TetR(B/E)–KRAB were co-transfected. Addition of tc led to an ~900-fold increase of luciferase expression, reaching a level similar to the one obtained with rtTA alone. Supplying both transregulators in the bicistronic construct pCMV–KRAB–rtTA led equally to an ~6-fold repression without tc, but the activation was only 360-fold (Fig. 2A).

This regulatory system also proved to be efficient in the Jurkat T cell line, where we obtained ~30-fold activation with the

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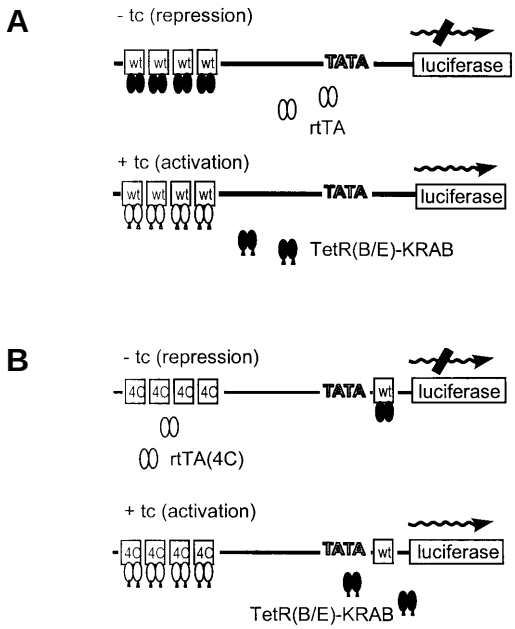

Figure 1. Combination of the tc-dependent repressor TetR(B/E)–KRAB with the activator rtTA. (**A**) In the reporter construct pUHC13-3 TetR(B/E)–KRAB (black) binds to wild type *tet* operators located upstream of the TATA box only in the absence of tetracycline thus silencing basal transcription of luciferase. By tc-induced binding of rtTA (white) to *tetO* transcription of the luciferase gene is activated. (**B**) Reporter construct pUHC13-3(4C+) with different binding sites for transrepressor and transactivator. In the absence of tc, TetR(B/E)–KRAB (black) binds to a single wild type *tetO* 2 bp downstream of the TATA box resulting in repression of the luciferase gene transcription. Upon addition of inducer, the transrepressor dissociates off the operator and rtTA(4C) (white) binds to *tetO*-4C sequences upstream of the TATA box thus activating gene expression. pUHC13-3(4C+) was constructed by exchanging *tetO* for *tetO-4C* (12) and by inserting a synthetic DNA fragment coding for the TATA box and an additional *tetO* sequence into *Stu*I/*Sac*I digested pUHC13-3(4C). pCMV–TetR(B/E)–KRAB was constructed by exchanging *tetR* (class B) for the PCR amplified tetR(B/E) chimera via *Eco*RI and *Sal*I sites in vector pCMV–TetR–KRAB (9). For the bicistronic constructs a polioviral IRES sequence was amplified from plasmid pVBC3 (H. J. Hauser, GBF Braunschweig, Germany) introducing a *Sma*I restriction site at the 3′ end, and inserted into *Bam*HI linearized pCMV–TetR(B/E)–KRAB. The transactivator rtTA was excised from pUHC17-1neo (3) with *Eco*RI and *Bam*HI and the blunt ended fragment was ligated to the newly constructed *Sma*I site. This gave rise to pCMV–KRAB–rtTA. The plasmid pCMV–KRAB–rtTA(4C) was constructed analogously except that an rtTA of 4C-binding specificity was used.

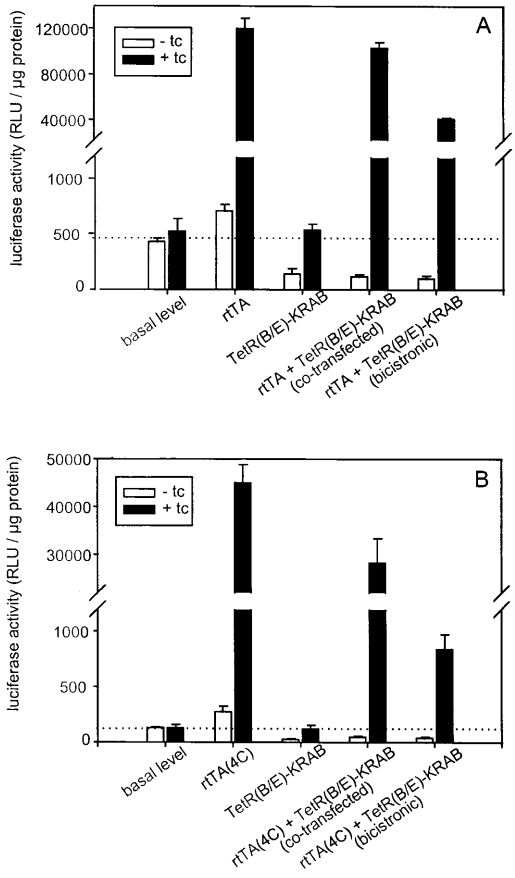

Figure 2. (**A**) Tetracycline-induced silencing and activation of the luciferase reporter gene by TetR(B/E)–KRAB and rtTA bound upstream of the TATA box. (**B**) Silencing and activation of the luciferase gene by TetR(B/E)–KRAB and rtTA(4C) bound to separate *tetO* sequences (wild type and 4C). HeLa cells were transiently transfected with the respective reporter plasmids pUHC13-3 or pUHC13-3(4C+) and the various regulator constructs as indicated. Basal level was detected by transfection of the reporter constructs alone. Transfections were performed with 2 µg of DNA and lipofectamine (Gibco Life Technologies, Basel) according to the manufacturer's instructions. For induction 10 µg/ml anhydrotetracycline were added (+tc, black bars). Cells were harvested after 36 h and cell lysates were prepared. Luciferase activity of HeLa cell lysates was determined with 100 mM potassium phosphate pH 7.8, 15 mM $MgSO_4$, 5 mM ATP and 0.5 mM D-luciferin (Boehringer, Mannheim) and is shown in relative light units (RLUs) per microgram of protein. Protein concentrations were measured with the BioRad protein assay kit. RLUs are controlled for β-galactosidase activity as a measure of transfection efficiency. Data shown are a representative experiment performed in duplicate.

construct pCMV–KRAB–rtTA and 70–100-fold activation by cotransfecting the monocistronic regulators. Repression ranged from 2- to 6-fold with both systems (data not shown).

Transfection experiments in HeLa cells further were performed with the respective mono- and bicistronic constructs together with the reporter plasmid pUHC13-3(4C+). The transactivator used in these experiments was rtTA(4C). The results are shown in Figure 2B. Basal transcription was repressed ~5-fold by the transrepressor in the absence of tc, demonstrating that TetR(B/E)–KRAB can also exert the silencing effect when bound downstream of the TATA box. The transactivator rtTA(4C) mediated a 170-fold activation of luciferase expression in the presence of tc, which is identical to the activation by rtTA with wild type DNA binding specificity (Fig. 2A). Repression in the absence of tc was 6-fold upon co-transfection of rtTA(4C) and TetR(B/E)–KRAB. After addition of inducer, an ~600-fold activated expression of luciferase was measured. When both transregulators were provided by the bicistronic construct pCMV–KRAB–rtTA(4C), repression in the absence of tc was indistinguishable from repression by the monocistronic TetR(B/E)–KRAB construct. However, activation by IRES-expressed rtTA(4C) was only 21-fold.

This study demonstrates that the tc-dependent activation of gene expression by rtTA can be efficiently combined with a repressor strategy, both in HeLa and T cell lines, similar to the activator/repressor dual system developed for tight tc-dependent regulation in budding yeast (10). The level of basal activity can be ~6-fold repressed by TetR(B/E)–KRAB without significant reduction of the luciferase expression level in the induced state. The

combined activator/repressor system shows maximal efficiency when both transregulators are co-expressed as monocistronic constructs. Decline in transcriptional activation in the bicistronic constructs might result from low efficiency of IRES-dependent translation (13,14) of the transactivators rtTA or rtTA(4C). Thus, the independent genes strategy is currently advisable.

In summary, tc-induced expression in mammalian cells can be regulated more tightly when combined with active silencing in the uninduced state. The described repressor/activator strategy might be useful for the expression and analysis of toxic gene products, as basal levels of transcription are decreased in comparison to systems using only activator constructs.

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