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Tetracycline-controlled transcription in eukaryotes: novel transactivators with graded transactivation potential

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ABSTRACT

Several tetracycline-controlled transactivators (tTA) were generated which differ in their activation potential by >3 orders of magnitude. The transactivators are fusions between the Tet repressor and minimal transcriptional activation domains derived from Herpes simplex virus protein 16 (VP16). By reducing the VP16 moiety of the previously described tTA to 12 amino acids, potential targets for interactions with various cellular transcription factors were eliminated, as were potential epitopes which may elicit a cellular immune response. When compared with the originally described tTA, these new transactivators are tolerated at higher intracellular concentrations. This will facilitate establishment of tet regulatory systems under a variety of conditions, but particularly when cell type-restricted tetracycline-controlled gene expression is to be achieved in transgenic organisms via homologous recombination.

INTRODUCTION

The tetracycline-controlled transcription activation system described previously (1) was shown to function as an efficient genetic switch in a variety of eukaryotic cells, including mammalian (2), plant (3) and yeast (Garí,E., personal communication) cells. It also allows effective control of gene activities at the level of the organism, as shown in plants (3), mice (4) and *Drosophila* (Gehring,W., personal communication). One of the key components of the tet system is the tetracycline-controlled transactivator (tTA), a fusion protein between the repressor of the (Tn*10*) tetracycline resistance operon of *Escherichia coli* and a C-terminal portion of VP16 that contains domains capable of activating transcription (5). In the absence of the effector tetracycline (Tc), tTA will activate transcription from a suitably engineered minimal promoter by binding to an array of *tet* operator (*tetO*) sequences positioned upstream. In the presence of Tc, tTA is prevented from binding to its target and thus transcription is abolished. Using a TetR mutant, a transactivator with a reverse phenotype (rtTA) was generated which, when compared with tTA, functions in the opposite fashion: it requires Tc derivatives, like doxycycline or anhydrotetracycline, for binding to its operator and thus activates transcription only in the presence but not in the absence of the tet effector. Transcriptional regulation via rtTA has been shown in mammalian cells (6) and in mice (4). Despite their widespread application, the present tet regulatory systems may still be developed further to fulfil specific experimental requirements. Here, we focus on modification of the activation domains of tTA and rtTA which, when overexpressed, can be deleterious to cell metabolism.

The intracellular concentration of transcriptional activators appears well controlled, as expected for regulatory proteins. Overexpression of transcription factors results in 'squelching' (7), which is seen as a consequence of titrating components of the transcriptional machinery from their respective intracellular pools. For VP16, as one of the most potent transactivators, it has been demonstrated that its overexpression, e.g. as a fusion protein with GAL4, is not tolerated by cells (8,9). Considering that VP16 interacts with a variety of essential components of the transcriptional machinery, including the adaptor/co-activator protein ADA2 in *Saccharomyces cerevisiae* (10) and its human homolog (11), with TFIIB (12), TFIID (13), TFIIH (14) and dTAFII40 (15), this is not surprising.

Accordingly, Gilbert and co-workers (16) found a correlation between squelching and growth arrest, which indicates that toxicity through squelching is a quantitative problem where the intracellular concentration and the strength of the activation domains are crucial parameters.

We attribute the fact that tTA and rtTA were nevertheless shown to function well in numerous systems to the exceptional specificity of the Tet repressor/operator interaction (17). This specificity warrants a high occupancy of *tetO* sequences by the transactivator at low intracellular concentrations of tTA/rtTA. Random integration of tTA/rtTA expression units into chromosomes allows screening for integration sites where the synthesis of tTA/rtTA is sufficiently high to yield good activation but low enough to prevent the deleterious effects of squelching. For example, we estimate the concentration of tTA in our HeLa X1 cell line to be ~4000 molecules/cell (18), hardly sufficient to seriously affect pools of basal transcription factors but nevertheless capable of activating a chromosomally integrated tTA responsive promoter >10^5-fold.

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This cell line, like numerous others, as well as several tTA- and rtTA-producing mouse lines, is perfectly stable in our laboratory and over several years has demonstrated that the intracellular concentration of the transactivator lies within a 'physiological' window.

There are, however, experimental strategies where screening or selection for an appropriate intracellular concentration of the transactivator is not possible. For example, to achieve cell type-specific regulation of a gene in transgenic organisms it appears attractive to place, via homologous recombination, a tTA/rtTA gene under the control of the promoter which directs expression of the gene of interest. Given the proper design of the vector used for recombination, the integration event will, at the same time, inactivate the target gene; its coding sequence controlled by a tTA/rtTA responsive promoter can be provided independently. While such an experimental 'knock in/knock out' strategy would allow for cell type-specific expression of tTA/rtTA and thus for an equally specific Tc-controlled regulation of the gene of interest, the effective intracellular concentration of the transactivator will be primarily a function of the transcriptional activity of a particular locus, a parameter which appears unpredictable and impossible to control. One way to overcome these limitations would be to adapt the activation potential of the transactivator to the expression level of a specific locus.

Here we describe a panel of novel Tc-controlled transactivators which contain VP16-derived minimal activation domains and which possess a graded transactivation potential spanning >3 orders of magnitude. These transactivators are tolerated in cells at higher concentrations and, therefore, appear suitable for experimental approaches as described above.

MATERIALS AND METHODS

Oligonucleotides encoding minimal activation domains

The minimal activation domains of this study were derived from VP16 and comprise positions 436–447 according to Seipel *et al.* (19). Synthetic oligonucleotides encoding this domain and variations thereof were designated [F], [GF], [FG], [GG] and [Y] respectively, whereby the letters designate the amino acids at position 442 (triplets underlined). Sequences of the coding strands are given. The oligonucleotides encode one or two minimal domains as indicated by the letters in brackets.

Oligo [F], 5′-CCGGCCGACGCCCTGGACGACTTCGACCTGGACATGCTG-3′;
Oligo [GF], 5′-CCGGCCGACGCCCTGGACGACGGCGACCTGGACATGCTGC-CTGCTGATGCTCTCGATGATTTCGATCTCGATATGCTCC-3′;
Oligo [FG], 5′-CCGGCCGACGCCCTGGACGACTTCGACCTGGACATGCTGCC-TGCTGATGCTCTCGATGATGGCGATCTCGATATGCTCC-3′;
Oligo [GG], 5′-CCGGCCGACGCCCTGGACGACGGCGACCTGGACATGCTGCC-TGCTGATGCTCTCGATGATGGCGATCTCGATATGCTCC-3′;
Oligo [Y], 5′-CCGGCCGACGCCCTGGACGACTACGACCTGGACATCCTC-3′.

The protruding 5′-ends of the double-stranded oligonucleotides are compatible with the cleavage site of restriction endonuclease *Xma*I.

Plasmids

The ColE1-based plasmid pUHD141-1 (20) contains the TetR coding sequence which is optimized at the 5′-end for efficient initiation of translation (21). Transcription of the *tetR* gene is controlled by the human cytomegalovirus IE promoter (22). To allow insertion of DNA in-frame with the 3′-end of the *tetR* open reading frame via *Xma*I cleavage, pUHD141-1 was linearized with *Afl*II, whose site overlaps the *tetR* stop codon. Protruding DNA 5′-ends were removed by mung bean nuclease and the synthetic oligonucleotide 5′-CCCGGGTAACTAAGTAA-3′ was ligated into the vector using standard cloning procedures. The resulting plasmid, pUHD141-1/X, containing a *Xma*I cleavage site at the very 3′-end of the *tetR* gene, was verified by sequence analysis.

Cell culture and transient transfections

HeLa X1/6 cells containing chromosomally integrated copies of the luciferase reporter construct pUHC13-3 (18) and HeLa (wt) cells were maintained at 37°C and 5% CO_2 in Earl's modified Eagles medium (E-MEM; Gibco) supplemented with 10% fetal calf serum. Transfections by calcium phosphate co-precipitation were performed according to standard protocols with the following modifications. HeLa X1/6 cells were grown in 35 mm dishes to 50–60% confluency. One hour prior to transfection, the culture medium was renewed and the cells were incubated at 37°C and 6% CO_2. The calcium phosphate/DNA precipitate contains 1.5 µg plasmid DNA [consisting of 0.5 µg transactivator construct, 0.4 µg *lacZ* expression vector (pUHD16-1), included for normalization of transfection efficiency, and 0.6 µg pUC18 as non-specific carrier DNA]. The precipitate (100 µl/dish) was added to X1/6 cells which were then further incubated at 37°C and 6% CO_2 for 30 h. Transfection efficiency, as determined by *in situ* β-galactosidase staining of parallel cultures, was between 60 and 80%.

Luciferase assay

Dishes (35 mm) containing transfected X1/6 cells were washed with 3 ml phosphate-buffered saline (PBS) and lysed in 125 µl lysis buffer containing 25 mM Tris–phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM diaminocyclohexane tetraacetic acid, 10% glycerol and 1% Triton X-100 for 10 min at room temperature. The lysates were scraped off the dishes and centrifuged for 10 s in an Eppendorf centrifuge. Luciferase activity in these extracts was measured as described (1) and luciferase values were normalized to β-galactosidase activity by performing standard liquid *O*-nitrophenyl β-galactopyranoside assays (23).

DNA retardation assay

HeLa cells were grown in 10 cm dishes to 50–60% confluency and transfected via the calcium phosphate procedure with 20 µg plasmid DNA encoding the various tTAs. Thirty hours post-transfection total cell extracts were prepared as follows. Cells (~2×10^6) were washed with PBS, centrifuged, resuspended in 250 µl buffer containing 10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride and incubated for 20 min at 0°C before they were quickly frozen and thawed. NaCl was added to a final concentration of 250 mM and, after incubation for 20 min at 0°C, the samples were centrifuged in a Beckman TL-100 ultracentrifuge at 435 000 *g* and 0°C for 10 min. Aliquots of the extracts (10 µl) were mixed with 10 µl binding buffer (20 mM $MgCl_2$, 20 mM Tris, pH 7.5, 10% glycerol, 2 mg/ml herring sperm DNA and 1 mg/ml bovine serum albumin) and 2 fmol ^{32}P-labeled *tetO* DNA isolated from

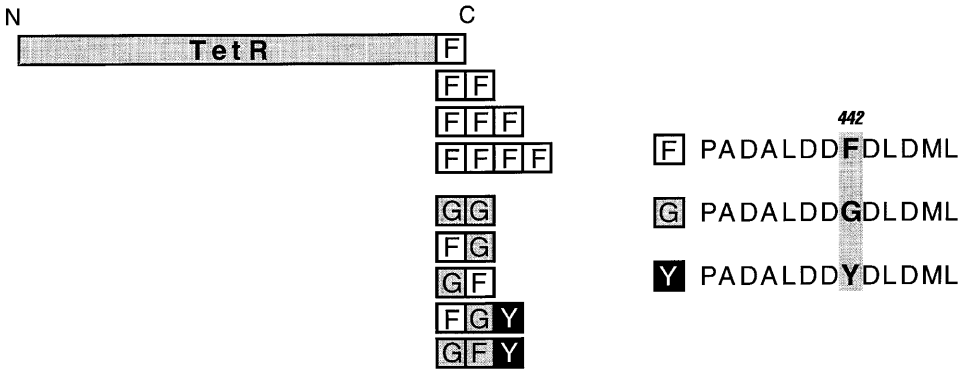

Figure 1. Fusions between TetR and minimal acidic activation domains. TetR was fused to minimal activation domains derived from VP16. The amino acid sequence of the domains is outlined at right. [F] denotes the wild-type sequence between position 436 and 447 of VP16 which contains a phenylalanine at positon 442. In the mutated minimal domains [G] or [Y], Phe^{442} is substituted by glycine and tyrosine respectively. Various combinations of the minimal domains were fused to TetR, resulting in the panel of fusion proteins outlined at left.

pUHC13-3 (1) as a 42 bp *Taq*I fragment after filling in the protruding ends with T4 DNA polymerase in the presence of [α-^{32}P]dCTP. After 25 min, the reaction mixture was loaded onto a 5% polyacrylamide/0.13% bisacrylamide gel containing 5% glycerol. Electrophoresis was carried out in 45 mM Tris base, 45 mM boric acid and 1 mM EDTA at 7 V/cm.

Generation of stably transfected cell lines

HeLa X1/6 cells were grown in 35 mm dishes and transfected with 2 µg linearized plasmid DNA as described above. The transfection mixture contained plasmid pHMR272 (24), carrying the *hyg* gene, and plasmid pUHD15-1, pUHD19-1 or pUHD26-1 (containing the Kozak sequence upstream of the tTA gene). The molar ratio between the plasmid in question and the selection marker was 40:1. After 24 h, cells were transferred to 10 cm dishes and maintained in medium containing 300 µg/ml hygromycin. Resistant clones were isolated, expanded separately and analyzed for luciferase activity (1). To further investigate tTA-dependent activation of the luciferase gene in those clones, cells were seeded at a density of 10 000 cells/35 mm dish and grown in the presence or absence of Tc (1 µg/ml). After 5 days, cell extracts were prepared as described above and luciferase activity was measured. The protein content of the lysates was determined according to Bradford (25).

Generation of cell pools stably expressing various transactivators and quantitation of relative intracellular tTA concentrations

Plasmids pUHD15-1, pUHD19-1, pUHD20-1 and pUHD26-1 were modified by inserting a selectable marker gene. In each case an expression cassette containing the *neo* gene was inserted into the *Xho*I site located upstream of P_{hCMV} (1). The resulting plasmids were designated pUHD15-1*neo*, pUHD19-1*neo*, pUHD20-1*neo* and pUHD26-1*neo* respectively.

HeLa cells were grown in 10 cm dishes to 50% confluency and transfected with 20 µg linearized plasmid DNA as described above. After 24 h, cells were transferred to 14.5 cm dishes and maintained in medium containing 500 µg/ml G418. Resistant clones were then pooled, seeded into 14.5 cm dishes and grown under selective pressure until they reached confluency. Extracts from cell pools were prepared and DNA retardation assays were carried out as described above. Total protein content of the extracts was determined according to Bradford (25). Protein–DNA complexes were detected and quantified by a phosphoimager. In all HeLa cell extracts a protein with some affinity to DNA was observed. This protein, marked with an asterisk in Figure 3, was used as an internal marker for quantitation of the various transactivators.

RESULTS

Fusions between TetR and minimal activation domains derived from VP16

VP16 contains two distinct transcriptional activation domains characterized by bulky, hydrophobic amino acids positioned in a highly negatively charged environment (26). Each domain was shown to activate transcription when fused to a heterologous DNA binding domain such as that of GAL4 (19). An oligonucleotide [F] encoding the acidic domain delineated by positions 436–447 was synthesized (Fig. 1) and inserted into plasmid pUHD141-1/X in-frame with the 3′-end of the *tetR* gene. Due to multiple integrations, sequences were generated which encode transactivators containing one, two, three or four activation modules. They were designated TetR–F, TetR–FF, TetR–FFF, TetR–FFFF respectively. To reduce possible structural constraints induced by the repeat units, the individual domains were joined by a proline, which also connects the first domain to TetR. Each transactivator construct was verified by sequence analysis.

Tc-dependent binding of the novel TetR fusion proteins to *tetO* sequences

Binding of the new TetR chimeras to *tetO* was examined by DNA retardation experiments. The various proteins were produced by transient expression of plasmids pUHD141-1/X and pUHD18-1 to pUHD21-1 (Table 1) in HeLa cells. Thirty hours after transfection, extracts were prepared and incubated with radiolabeled *tetO* DNA. Electrophoretic separation of the protein–DNA complexes shows that the new fusion proteins bind *tetO* DNA with an efficiency comparable with that of TetR (Fig. 2A) and form complexes which migrate according to the molecular weight of the respective fusion proteins. Presence of Tc in the

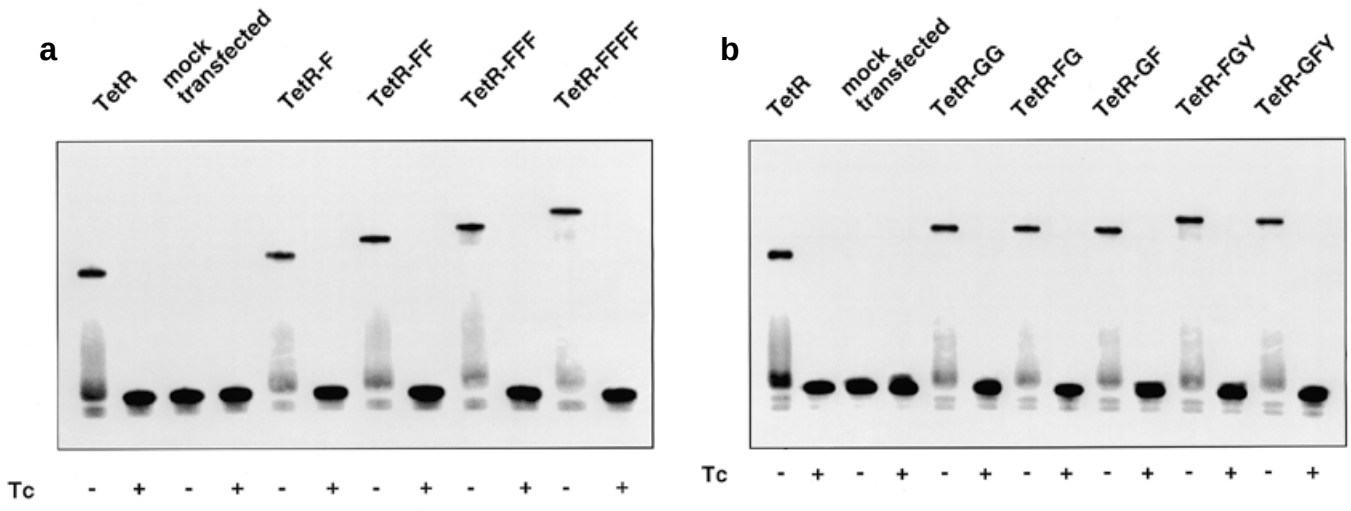

Figure 2. Characterization of the various TetR fusions by DNA retardation assays. HeLa cells grown in 10 cm dishes to 40% confluency were transiently transfected with plasmid DNA encoding either TetR or one of the fusion proteins shown in Figure 1. Cell extracts prepared after 36 h were combined with radiolabeled *tetO* DNA in the presence or absence of Tc. Protein–DNA complexes were separated electrophoretically and detected using a phosphorimager. (**a**) Mobility shift of TetR–[F] fusions; (**b**) fusions between TetR and [F], [G] and [Y] domains. Mock-transfected cells contained vector DNA without a tTA encoding insert.

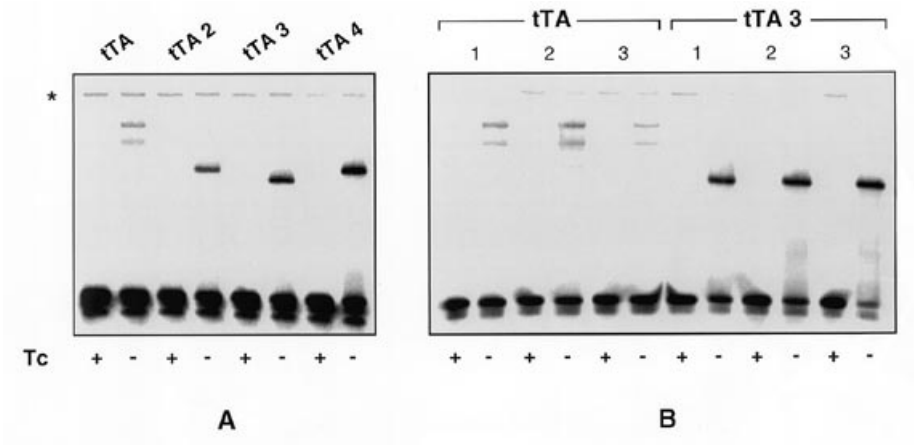

Figure 3. Comparison of intracellular concentrations of transactivators. Protein extracts prepared from cells stably expressing various transactivators were subjected to electrophoretic mobility shift assays with radioactively labeled *tetO* DNA. Protein and DNA were mixed in the presence or absence of Tc (±Tc) before comparable amounts were applied to the polyacrylamide gel. (**A**) Extracts from pools of HeLa cells stably transfected with DNA encoding tTA, tTA2, tTA3 and tTA4 respectively under the control of P_{hCMV}. (**B**) Analysis of individual clones producing tTA or tTA3. tTA: lane 1, extract from X1/5 cells (1); lane 2, extract of the X1/6-tTA cell line in Table 2; lane 3, extract of a clone picked from the tTA-transfected HeLa cell pool described in (A). tTA3: lanes 1 and 2, extracts of the tTA3-producing cell lines in Table 2; lane 3, extract of a clone picked from the tTA3-producing HeLa cell pool described in (A). *A marker used for quantitation of the signals.

binding assay prevents complex formation. Furthermore, this analysis suggests that the new fusion proteins are stable, as no degradation products are detectable.

Activation potential of the new TetR–[F] fusions

To assess the activation potential of the new TetR fusions, HeLa X1/6 cells were transiently transfected with plasmids encoding the respective proteins. Luciferase activity in this cell line is barely detectable but can be greatly increased by transient expression of a tTA-encoding gene; this activity is abolished by Tc (Table 1). Induction of the luciferase gene is entirely dependent on the activation domain fused to TetR, as TetR alone has no effect (Table 1). When the different TetR–[F] fusions were examined in this assay, a gradual increase in luciferase activity is observed whereby TetR–FF reaches ~40%, TetR–FFF almost 100% and TetR–FFFF ~230% of the activity conferred by tTA. Interestingly, TetR–F containing a single minimal domain does not activate under these conditions.

TetR fusions containing mutated minimal domains

Mutational analysis of the acidic activation domains of VP16 has revealed that the phenylalanine at position 442 is crucial for function (26). When replaced by aromatic amino acids like Tyr or Trp or by hydrophobic amino acids such as Leu, Ile or Ala the activation potential of truncated VP16 was reduced ~3- and 10-fold respectively. All other substitutions caused an even larger loss of activity.

Table 1. Transcription activation by fusions between TetR and various minimal domains

TetR fusion	Arbitrary luciferase activity		Relative activation (%)	Designation of transactivator	Plasmids encoding TetR and TetR fusions
	+Tc	–Tc			
TetR–VP16	20	265 410	100	tTA	pUHD15-1
TetR	26	32	0		pUHD141-1/X
TetR–F	21	21	02		pUHD18-1
TetR–FF	27	102 828	39	tTA3	pUHD19-1
TetR–FFF	33	259 556	98	tTA2	pUHD20-1
TetR–FFFF	33	607 264	230	tTA1	pUHD21-1
TetR–GG	28	30	0		pUHD22-1
TetR–FG	24	88	0.03	tTA7	pUHD23-1
TetR–GF	28	1 500	0.6	tTA6	pUHD24-1
TetR–FGY	16	12 080	4.6	tTA5	pUHD25-1
TetR–GFY	25	37 217	14	tTA4	pUHD26-1

HeLa cell line X1/6, which contains the luciferase gene under transcriptional control of the chromosomally integrated tTA-dependent promoter $P_{hCMV*-1}$ (1), was grown in 35 mm dishes to 50% confluency. Cells were transiently transfected with DNA encoding either TetR, tTA or one of the new fusion proteins. Cultures were incubated in the presence or absence of Tc (1 µg/ml) for 30 h before luciferase activity was measured and standardized to β-galactosidase activity (introduced by co-transfection with pUHD16-1). The measurements of two independent transfection experiments are shown and related to the activity of tTA (100%).

Table 2. Analysis of HeLa X1/6 cell clones stably expressing tTA, tTA3 or tTA4

Cell line	Luciferase activity (RLU/µg protein)		Regulation factor
	+Tc	–Tc	
X1/6-tTA (clone 1)	4 (±0.2)	1 062 283 (±44221)	~2.5×10^5
X1/6-tTA3 (clone 1)	1 (±0.3)	228 363 (±15608)	~2.2×10^5
X1/6-tTA3 (clone 3)	3 (±0.1)	462 184 (±21585)	~1.5×10^5
X1/6-tTA4 (clone 7)	2 (±0.2)	89 010 (±3220)	~4.4×10^4
X1/6	1 (±0.2)	1 (±0.4)	

HeLa cell line X1/6 was transfected with pHMR272 and plasmids encoding the respective transactivators. Clones resistant to hygromycin were analyzed for Tc-dependent luciferase activity. Cells of four clones selected for efficient regulation were seeded at a density of 10 000 cells/ 35 mm dish and grown in the presence or absence of Tc (1 µg/ml) for 5 days. Values given are arithmetic means of five independent cultures.

To broaden the range of activation potential of fusions between TetR- and VP16-derived minimal domains, we have varied the sequence of the latter by replacing Phe with Gly or Tyr. Several TetR fusions containing various combinations of mutated (G, Y) and wild-type (F) domains were generated (Fig. 1) and examined for their *tetO* binding as well as for their ability to activate transcription from $P_{hCMV*-1}$ in X1/6 cells. When produced in HeLa cells, all fusion proteins appear to efficiently bind *tetO*, as evidenced by DNA retardation experiments (Fig. 2B). When their activation potential was analyzed (Table 1), no activity was found for TetR–GG. However, by combining a [G] with an [F] domain, low but distinct activation is produced, amounting to ~0.03 (TetR–FG) and 0.6% (TetR–GF) of the activation potential of tTA respectively. Higher levels of activation are conferred by the combinations FGY and GFY, which correspond to 4.6 and 14% of the tTA activity. Together with the [F] domain-containing TetR fusions described above, these combinations establish a panel of Tc-controlled transactivators which covers a range of activation strength of >3 orders of magnitude. For simplicity, the TetR fusions capable of activating transcription are designated tTA1–tTA7, as indicated in Table 1.

Control of luciferase activity in HeLa X1/6 cells constitutively producing tTA3 and tTA4

To characterize the properties of some of the novel transactivators in stably transfected cells, the genes encoding tTA3 or tTA4 controlled by P_{hCMV} were transferred into HeLa X1/6 cells. Co-transfection with pUHD19-1 or pUHD26-1 (Table 1) and pHMR272, which conveys hygromycin resistance (24), leads to the isolation of resistant clones, which were examined for luciferase activity in the presence and absence of Tc. In the resistant clones luciferase activity in the presence of Tc is indistinguishable from the activity of non-transfected X1/6 cells (Table 2), whereas in the absence of the effector it can be stimulated >10^4-fold. These data confirm the functionality of the two new transactivators tTA3 and tTA4 under stable cellular conditions. They both allow tight regulation of transcription via a tTA/rtTA-responsive promoter. It should be emphasized that in the clones tested the level of activation conveyed by TetR–FF and TetR–GFY cannot be compared with the data obtained in transient transfections (Table 1). In the latter experiments the same amount of transactivator-encoding DNA was introduced

into the cells, resulting in comparable intracellular concentrations of the tTA proteins. Therefore, the different levels of activation reflect the properties of the respective TetR fusions. In contrast, in stably transfected cells the genes encoding the transactivators are randomly integrated into the genome. Their expression is both copy number and locus dependent and, consequently, their intracellular concentration will differ from clone to clone. These concentration differences, rather than the properties of the respective transactivators, are thus reflected in the different levels of activation.

Intracellular concentrations of transactivators

To examine whether transactivators with minimal domains are tolerated at higher intracellular concentrations than tTA, HeLa cells were transfected in parallel with plasmids encoding tTA, tTA2, tTA3 or tTA4. The corresponding plasmids (Table 1) were equipped with a *neo* resistance marker (see Materials and Methods) to ensure that clones resistant to G418 would also express the transactivator gene. Selection for G418 resistance led to pools of 300–500 colonies. Such pools were grown up and protein extracts were analyzed for transactivator protein by electrophoretic mobility shift experiments with radioactively labeled *tetO* DNA. As shown in Figure 3A, all transactivators consisting of TetR and minimal activation domains are present in the cell at higher concentrations than the TetR–VP16 fusion protein tTA. Interestingly, tTA2, which has the same activation potential as tTA (Table 1), is nevertheless tolerated at a 3-fold higher concentration. Among the new transactivators, however, the intracellular concentration increases inversely with the respective activation potential. Thus, tTA3 and tTA4 concentrations are 5- and 9-fold higher respectively than that of tTA. When individual clones producing either tTA or tTA3 were analyzed for relative abundance of the transactivators, again by DNA retardation assay, the same picture emerged: the intracellular concentration of tTA3 was again about five times higher than that of tTA (Fig. 3B). Extracts from HeLa cells expressing tTA show a second protein–DNA complex in the DNA retardation assay, which appears to be a degradation product of tTA (Fig. 3). This product is also found to a variable extent in other cell lines. From the mobility of this complex it can be estimated that ~40 amino acids have been cleaved off, most likely from the C-terminus, since a deletion of this size from the N-terminus would abolish the operator binding capacity of the transactivator. Therefore, this degradation product has most likely lost the second (C-terminal) activation domain of the VP16 moiety. It is not clear whether such a truncated protein will still act as a transactivator.

DISCUSSION

The transcriptional transactivators described herein are fusions between the Tet repressor and minimal activation domains derived from a 12 amino acid 'acidic activation domain' of VP16. Combination of several of these minimal domains using wild-type as well as mutated sequences yielded a panel of transactivators (tTA1–tTA7, Table 1) which differ in their activation potential by >3 orders of magnitude, whereby tTA1 exceeds the activation strength of the previously described tTA 2.3-fold. The new transactivators activate the previously described tTA responsive promoter $P_{hCMV*-1}$ (1), despite the fact that a number of sites known to interact with cellular transcription factors were eliminated. Thus, when compared with VP16, tTA1–tTA7 have lost sites which contact Oct-1 (27) and the host cell factor HCF (28), both required for formation of the C1 complex comprising Oct-1, HCF, VP16 and DNA (27). Similarly, deletion of the second C-terminal acidic activation domain of VP16, known to contact TAFII40 (15) and ADA2 (10), is expected to further reduce the interaction of the new transactivators with those factors. Therefore, we assume that these tTA proteins have gained specificity while their capacity for squelching is reduced. This assumption is supported by the finding that tTA2 is tolerated in HeLa cells at 3-fold higher concentrations than the original TetR–VP16 fusion (tTA), although both transactivators possess the same activation potential (Table 1). It thus appears that elements of VP16 were removed which limit expression to a lower level. When the intracellular concentrations of tTA2, tTA3 and tTA4 are compared, an inverse correlation with the respective activation potential is revealed. It thus appears feasible to use the panel of transactivators described here for the adjustment of transactivating capacities to expression signals of different strength.

Fusion of acidic domains to DNA binding proteins as described here increases the negative charge of the molecule and thus may affect its affinity for DNA. However, DNA retardation experiments shown herein demonstrate that all TetR fusions bind to *tetO* sequences with comparable efficiency, although minor differences between the various binding constants would not be revealed by this assay. This is in contrast to findings by others (Schaffner,W., personal communication) and indicates a low susceptibility of the TetR/DNA interaction to C-terminally located negative charges.

Fusing a single [F] domain to TetR yielded a protein that does not activate transcription. However, fusing a second minimal activation domain to TetR–F, resulting in TetR–FF (tTA3), generates a transactivator which reaches ~40% of the activity of tTA. Adding further [F] domains to tTA3 increases the activation capacity ~2.5-fold per domain, as seen for tTA2 and tTA1. There may be several reasons why TetR–F is not effective in transactivation. Either the single activation domain, which is only 12 amino acids in length, is burried within the TetR protein and has to be placed at a distance from TetR to make physical contact with the transcription initiation apparatus at the promoter or, alternatively, this negatively charged domain may become neutralized by the positively charged amino acid residues at the C-terminus of TetR. Finally, it may be possible that a single acidic activation domain alone is not sufficient to stimulate transcription and full activation requires two modular activation domains, as the data presented herein suggest.

Comparing TetR–F with TetR–GF (tTA6) indicates that adding a [G] domain, which by itself is transcriptionally inactive, since TetR–GG is ineffective, suffices to generate a functional transactivator, tTA6. The transactivator with the inverse order of the two minimal domains, TetR–FG (TA7), is less active than tTA6, indicating that steric factors contribute to a functional arrangement of activating domains. Since tTA7 nevertheless has a measurable activity we must conclude that the negative charges of the [G] domain contribute to transcriptional activity as well. tTA6 and tTA7 are both very weak transactivators. By simply exchanging the glycine for a phenylalanine the activation potential of the resulting transactivator (tTA3) is increased ~60-fold (tTA6) or even >1000-fold (tTA7). From this we conclude that in our system at least two minimal activation modules acting synergistically are required for efficient stimulation of transcription. The activation

properties of tTA5 and tTA4 may again be explained by steric and synergistic effects exerted by the combination of the respective minimal domains, whereby addition of the [Y] domain to both tTA6 and tTA7 increases the activation potential 20-fold.

The panel of Tc-controlled transactivators described here offers a number of advantages. First, it allows adaptation of the capacity of a transactivator to the strength of a given promoter. This opens up new possibilities for achieving cell type-restricted Tc-controlled regulation in transgenic organisms by placing a tTA coding sequence under control of a cellular promoter via homologous recombination. Since neither the strength of the targeted promoter nor the intracellular tTA concentration originating from such a locus can be readily predicted, choice of transactivators differing in strength will give an additional degree of freedom to finding the appropriate promoter/transactivator combination. Second, due to their increased specificity and their reduced squelching capacity, the new tTAs should facilitate the generation of cell lines and transgenic animals constitutively producing tTA in proper amounts. Third, by reducing the size of the activation domain of the original tTA, numerous sequence motifs potentially capable of eliciting a cellular immune response were eliminated. Therefore, the transactivators characterized here may be preferred whenever interference with the cellular immune response is expected, although such a response has not been observed for tTA/rtTA in the mouse model so far (29). Finally, the small size of the new transactivators may be of advantage when integration into vector systems with limited capacity for foreign sequences is considered.

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