TetR hybrid transcription factors report cell signaling and are inhibited by doxycycline

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BioTechniques 39:529-536 (October 2005)
doi 10.2144/000112002

We developed a bigenic reporter system composed of a hybrid transcription factor that combines the regulatory and activation domains of either Elk-1 or cyclic AMP-responsive element binding protein (CREB) with the DNA binding, dimerization, and regulatory domains from a synthetic variant of the bacterial Tet repressor (TetR). The novel hybrid transcription factor TetR-Elk-1 was regulated by MAPK ERK kinase 1 (MEK-1) overexpression, and TetR-CREB was regulated by protein kinase A (PKA) overexpression or elevation of cyclic AMP levels. These hybrid transcription factors could be useful reporters of cell signaling pathways because, unlike previous GAL4 hybrid reporters, TetR hybrid transcription factors are inhibited by the administration of doxycycline. We validated this system in cell culture transfection experiments utilizing luciferase assays to monitor reporter gene expression and Western blot analysis to monitor transcription factor expression and phosphorylation levels. This system may be useful in creating temporally restricted windows of response to cell signaling and may be of value in the advancement of methods used to study signal transduction.

INTRODUCTION

Hybrid transcription factor reporter systems take advantage of the modular nature of transcription factors and have been used extensively to study transcriptional regulation. For example, the first hybrid transcription factor was composed of the GAL4 activation domain and the LexA DNA binding domain and demonstrated the independence of the DNA binding domain and the activation domain by activating genes with LexA operators (1). Another hybrid composed of the DNA binding domain of GAL4 and a region of herpes virus protein 16 (VP16) yielded an unusually active transcriptional activator that has proven useful for expressing very high levels of a desired gene (2). GAL4 hybrid transcription factors have been utilized extensively to study protein-protein interactions (3). They have also been used with great success to study the activation of regulated transcription factors by combining portions of the transcription

factor of interest with GAL4. For example, GAL4-Elk-1 hybrid transcription factors were applied in the original identification of the Elk-1 activation domain (4), and LexA-Elk-1 hybrid transcription factors were employed to identify critical regulatory sites within the activation domain (5). GAL4-cyclic AMP (cAMP)-responsive element binding protein (CREB) hybrid transcription factors were used to demonstrate that CREB activation could drive gene expression in response to calcium and cAMP signaling, each of which is important in the cellular response to electrical signals (6), and GAL4-CREB was also exploited to map the kinase-inducible and constitutively active domains of CREB (7). GAL4-CREB hybrids were then used to investigate the relationship of CREB and two important interacting proteins, cAMP-responsive element modulator (CREM) (8) and CREB binding protein (CBP) (9). GAL4-CREB has also been applied in *Aplysia* sensory neurons to demonstrate gene

activation in response to drugs that produce long-term facilitation (10). In transgenic mice, GAL4 hybrids have been adapted to produce constitutive (11) or regulated (12) expression of a second transgene mediated by the upstream activation sequence (UAS). Although reporter systems regulated by transcription factor binding sites have been used in transgenic mouse studies (13), bigenic reporter systems utilizing hybrid transcription factors that respond to cell signaling events have not yet been used successfully in transgenic mice.

Like GAL4 or LexA hybrid transcription factors, the tetracycline-sensitive transactivator (tTA) takes advantage of the modularity of transcription activators, as tTA consists of the bacterial Tet repressor (TetR) fused to the activation domain of VP16 (14). tTA binds to the tetracycline-responsive operator (*tetO*) DNA sequences to initiate transcription but can be inactivated by the administration of tetracycline or derivatives such as

doxycycline (14). This ability to control gene expression has the important advantage of temporal control of expression and can even be utilized to titrate expression levels (14,15). The tTA system has been adapted for use in transgenic mice (16) and *Drosophila* (17) and has proven useful in the study of the brain (18), mammary gland (19), lung (20), and other tissues (21). Synthetic tTA has also been optimized for mammalian codon usage (22). Although TetR has been combined with mammalian transcriptional activation domains to lower toxicity (23) or compare efficiency (24), TetR has not previously been combined with a regulated transcription factor to drive expression selectively in response to regulatory cell signaling events. We therefore hypothesized that replacing the DNA binding components used in traditional hybrid reporter approaches with the doxycycline-regulated DNA binding properties of TetR would improve the hybrid reporter strategy to produce a temporally controllable reporter system with regulated activation domains from transcription factors of interest. Because TetR

hybrids contain a regulated activation domain, they would not be an appropriate replacement for previous tetracycline-regulated transactivators such as tTA in cases where dual regulation is not desired. However, TetR hybrids represent an improvement on previous hybrid reporter systems because TetR hybrids replicate the function of previous hybrid reporter systems with the additional benefit of Tet regulation, allowing a greater variety of potential applications and additional control experiments in existing strategies.

We created TetR-Elk-1 and TetR-CREB hybrid transcription factors, consisting of the regulatory and transcriptional activation domains of Elk-1 (amino acids 307–428) or CREB (amino acids 1–261) and the DNA binding, dimerization, and regulatory domains (amino acids 1–211) of the tetracycline transactivator tTA2s (22), a synthetic variant of TetR. To determine if these hybrid transcription factors could act as reporters of cell signaling, we tested the ability of TetR-Elk-1 to regulate expression of a *tetO*-driven reporter gene in response to overexpression of the upstream kinase MAPK

ERK kinase 1 (MEK-1), a kinase that activates extracellular signal-regulated kinase (ERK), which then activates Elk-1 (4,5). We also tested the ability of TetR-CREB to regulate expression in response to overexpression of protein kinase A (PKA), which can activate CREB directly by phosphorylation (8). We tested the ability of TetR-CREB to regulate expression in response to the adenylyl cyclase agonist forskolin (Sigma-Aldrich, St. Louis, MO, USA) and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich), which artificially elevate cAMP levels within the cell. Additionally, we tested the ability of doxycycline to inhibit reporter gene expression mediated by TetR hybrids. We antici-

pated that if TetR hybrid transcription factors could respond to signaling activity, they would have a distinct advantage over GAL4 and LexA hybrid transcription factors (2,4,5,25) because the binding of TetR to *tetO* can be inhibited by tetracycline analogs for additional regulatory control over gene expression. These TetR hybrid transcription factors may be useful for applications such as transgenic animal studies or selective screening, where controlling unwanted expression is an important constraint in the detection of cell signaling events.

MATERIALS AND METHODS

Construction of Plasmids

The plasmid pUHT61-1 (22), containing synthetic tTA2s optimized for mammalian codon usage, was a kind gift of Hermann Bujard (ZMBH, Heidelberg, Germany). The region of tTA2s encoding three F activation domains (15) was removed by digestion with *Xma*I and *Eag*I (New England Biolabs, Beverly, MA, USA), leaving the pUHT61-1 backbone containing the synthetic TetR regulatory, dimerization, and DNA binding domain of tTA2s (amino acids 1–211). The region encoding the activation domain of the human CREB (amino acids 1–261) was amplified by PCR from the pNexδ-WT plasmid (10), a kind gift of Bong-Kiun Kaang (Seoul National University, Seoul, Korea), using the primers EBCREBfor (5'-TCTCGGCCGTACG AATGACCATGGAATCTGGGCCG-3') and XACREBrev (5'-TCCCCC GGGACCGGTAGGAAGTGCTGG GGAGGATGCC-3'). The resulting PCR product contained restriction endonuclease recognition sequences used for subcloning. PCR product was digested with *Xma*I and *Eag*I, and ligated into the pUHT61-1 lacking F activation domains to produce the plasmid MKp18:TetR-CREB. MKp18:TetR-CREB was digested sequentially with *Bsi*WI and *Age*I to remove the region encoding CREB(1–261). The remaining vector containing the synthetic TetR regulatory, dimerization, and DNA binding domain was used in ligation reactions to

Figure 1. The TetR hybrid transcription factor reporter system responds to cell signaling events and can be inhibited by doxycycline. (A) The bigenic system includes a reporter gene (green) regulated by a tetracycline-responsive operator (*tetO*) promoter and a hybrid transcription factor consisting of the regulatory, dimerization, and DNA binding domains of Tet repressor (TetR; gray) fused to the regulated activation domain from a transcription factor of interest (blue), which is driven by a constitutively active cytomegalovirus promoter (CMV). In the basal state, the hybrid transcription factor is bound to the *tetO* promoter but is inactive. (B) Phosphorylation of the regulated activation domain by upstream kinase activity (gold) during cell signaling leads to the expression of the reporter gene. (C) Doxycycline (red) inhibits interaction between TetR domains and the *tetO* promoter so that the hybrid transcription factor does not mediate transcription of the reporter gene, regardless of its phosphorylation state.

produce three plasmids: MKp50:TetR(-), MKp51:TetR-CREB(SA119), and MKp37:TetR-Elk-1. MKp50:TetR(-) was produced by ligation without insertion. The DNA fragment encoding mutant CREB(SA119) used to create MKp51:TetR-CREB(SA) was amplified by PCR from pNex δ -SA119 (10), a kind gift of Bong-Kiun Kaang, using EBCREBfor and XACREBrev described above. The DNA fragment encoding amino acids 307–428 of Elk-1 used to produce MKp51:TetR-Elk-1 was amplified by PCR from pFA2-Elk-1 (GAL4-Elk-1) (Stratagene, La Jolla, CA, USA) using primers MKelkF (5'-CCCCGTACGCTCCCGAGCCGAG-3') and MKelkR (5'-TCCCACCGG TTCATGGCTTCTGGGG-3'), then digested sequentially with *Bsi*WI and *Age*I. All subcloning was confirmed by restriction digest followed by sequencing of ligation sites and PCR-amplified insertion sequences. Plasmids pFA2-CREB (GAL4-CREB), pCRE-Luc, pFC-MEK1, pFC-PKA, and pFR-Luc (UAS-Luc) were obtained from Stratagene. Plasmid pRG-TK, which constitutively expressed *Renilla* luciferase to control for transfection efficiency, was obtained from Promega (Madison, WI, USA). Plasmid pBI-L (*tetO*-Luc) was obtained from BD Biosciences Clontech (Mountain View, CA, USA).

Cell Culture and Transfections

Human embryonic kidney (HEK) 293 cells (ATCC, Manassas, VA, USA) were maintained in minimum essential media with Earle's salts (Invitrogen, Carlsbad, CA, USA) supplemented with 10% horse serum (ATCC) and 1% penicillin/streptomycin (Invitrogen). Preceding transfection, cells at 70% confluence were incubated in serum-free media supplemented with 1% penicillin/streptomycin for 5 h. FuGENE[®] 6 Transfection Reagent (Roche Applied Science, Indianapolis, IN, USA) was then used to transfect the cells according to the manufacturer's protocol, with 1 μ L FuGENE 6/100 μ L of serum-free media for each transfection mixture. For the dual luciferase assay, each experiment was performed in triplicate on 12-well plates. Each well was transfected with 10 ng pRG-TK, 50 ng of the reporter gene plasmids *tetO*-Luc or pFR-Luc (UAS-Luc) reporter plasmid, 25 ng of the upstream activator plasmids pFC-MEK-1 or pFC-PKA, and 25 ng of the hybrid transcription factor plasmids MKp37:TetR-Elk-1, MKp18:TetR-CREB, MKp50:TetR(-), MKp51:TetR-CREB(SA119), pFA2-Elk1 (GAL4-Elk-1), or pFA2-CREB (GAL4-CREB). Doxycycline was added at a concentration of 1 ng/ μ L, which did not interfere with the viability, morphology, or transfection efficiency (data not shown). The adenylyl cyclase agonist forskolin and the phosphodies-

terase inhibitor IBMX were dissolved in dimethyl sulfoxide (DMSO), diluted with culture media, and added to cell culture 16 h after transfection to a final concentration of 10 μ M. KCl was also added to a final concentration of 50 mM. The vehicle consisted of DMSO at a final concentration of 0.04% by volume. Cell culture and transfections for Western blot analysis experiments were carried out in a similar manner except that 60-mm² culture dishes were used for cell culture and all treatments were scaled up proportionate to the surface area.

Luciferase Assay

Twenty-four hours following transfection, luciferase activity was assayed using the Dual-Luciferase[®] Reporter Assay System (Promega). Briefly, cells were washed once with 500 μ L warm phosphate-buffered saline (PBS) before lysing in 200 μ L Passive Lysis Buffer and flash-freezing in dry ice/ethanol. To measure firefly luciferase activity, 15 μ L of lysate were added to 100 μ L of the substrate solution, and luminescence was measured for 10 s using a Monolight[™] 2010 luminometer (BD Biosciences, San Jose, CA, USA). To measure *Renilla* luciferase activity, 100 μ L Stop and Glo[®] reagent (Promega) were injected, and luminescence was measured for 10 s. Firefly luciferase activity was corrected for transfection efficiency by dividing by *Renilla* luciferase activity and then normalized to obtain relative light units (RLU) by dividing by the basal expression value, defined as TetR hybrid-mediated expression in the absence of stimulation. Each set of experiments was transfected, treated, and assayed in parallel.

Western Blot Analysis

Cells were harvested after 24 h with 500 μ L Western Lysis Buffer [100 mM dithiothreitol (DTT), 50 mM Tris-HCl, 2% sodium dodecyl sulfate (SDS), pH 6.8] per 60 mm² tissue culture dish. Samples were boiled 10 min, centrifuged at 8000 $\times$ g, and the supernatant was transferred to new tubes. The RC DC[™] Protein Assay (Bio-Rad Laboratories, Hercules,

Figure 2. Regulation of *tetO* reporter gene expression by MEK-1 overexpression or doxycycline treatment. Firefly luciferase expression mediated by (A) Tet repressor (TetR(-)), (B) TetR-Elk-1, or (C) in the absence of transactivators was measured in response to doxycycline (dox) and MAPK ERK kinase 1 (MEK-1) overexpression (MEK). Values are normalized to TetR-Elk-1-mediated expression in the absence of doxycycline or MEK-1. Error bars indicate SEM of triplicate experiments. RLU, relative light unit.

CA, USA) was used to determine the concentration of protein within each experimental group. Twenty micrograms of protein were loaded per well on a 4% stacking, 10% separating polyacrylamide gel. Electrophoretic transfer was then performed overnight onto a Protran[®] pure nitrocellulose membrane (Schleicher & Schuell BioScience, Keene, NH, USA) at 398 mA. Membranes were blocked for 1 h in 5% NFDM buffer [5% nonfat dry milk, 0.1% Tween[®] 20 (Bio-Rad Laboratories), and Tris-buffered saline (TBS), pH 7.6], washed 3 times, and then incubated 1:500 with phospho-Elk-1 or Elk-1 primary antibodies (New England Biolabs) overnight with agitation in 5% bovine serum albumin (BSA; Sigma-Aldrich), 0.1% Tween 20 and TBS, pH 7.6. Membranes were then washed 3 times and incubated overnight in a 1:1000 dilution of polyclonal rabbit secondary antibody (Chemicon, Temecula, CA, USA) in 5% NFDM, 0.1% Tween 20, TBS, pH 7.6. For chemiluminescent detection, the ECL Plus[™] Western Blotting Detection System (Amersham Biosciences, Piscataway, NJ, USA) was used according to the manufacturer's instructions and detected on BioMax[®] film (Kodak, Rochester, NY, USA).

RESULTS

TetR-Elk-1 Is Activated by MEK-1 and Inhibited by Doxycycline

To validate the TetR hybrid reporter strategy (Figure 1), experiments were conducted in HEK 293 cells using luciferase assays to determine if TetR-Elk-1 hybrid transcription factor could be activated by upstream cell signaling events and inhibited by tetracycline analogs. Cells transfected with TetR(-), which consisted of the synthetic TetR domains but no activation domain, showed basal levels of expression, regardless of treatment (Figure 2A). However, cells transfected with TetR-Elk-1 demonstrated approximately 50-fold greater expression in response to MEK-1 cotransfection (Figure 2B). This increased expression was inhibited by doxycycline. Cells that were not transfected with any transcription factor

(Figure 2C) demonstrated that the reporter plasmid alone was sufficient to produce the baseline levels seen in TetR(-) or TetR-Elk-1 groups.

Western blot analysis was used to determine how kinase cotransfection and/or doxycycline treatment affected hybrid transcription factor expression and phosphorylation levels (Figure 3). Membranes were incubated with either Elk-1 or phospho-Elk-1 antibodies followed by polyclonal rabbit secondary antibody and were developed using chemiluminescence. The Elk-1 Western blot analysis, which detected both phosphorylated and unphosphorylated protein, indicated that the TetR-Elk-1 hybrid protein was expressed at high levels in all TetR-Elk-1 experimental groups. The addition of MEK-1 resulted in electrophoretic mobility shifting, as has been

previously described following the phosphorylation of multiple sites on the Elk-1 activation domain (4,26,27). This band shifting precluded the use of densitometry; however, the Elk-1 Western blot analysis is consistent with the prediction that MEK-1 and doxycycline would not dramatically affect TetR-Elk-1 protein levels. The TetR(-) experimental groups as well as the experimental groups lacking transactivator did not have detectable signal, demonstrating the specificity of the antibodies and an absence of detectable endogenous Elk-1 in HEK 293 cells.

The phospho-Elk-1 Western blot demonstrates high levels of phosphorylated TetR-Elk-1 in response to cotransfection of MEK-1 and TetR-Elk-1. Background levels of phosphorylated TetR-Elk-1 in groups not cotransfected with MEK-1 were nearly undetectable.

Figure 3. Western blot analysis of Elk-1 and phospho-Elk-1 following MEK-1 overexpression or doxycycline treatment. Elk-1 directed or phospho-S383 Elk-1 directed antibodies were used to detect protein 24 h after transfection in the presence and absence of MAPK ERK kinase 1 (MEK-1) overexpression (MEK) and doxycycline treatment (dox). Band shifting occurred as a result of Tet repressor (TetR)-Elk-1 phosphorylation following MEK-1 overexpression.

Figure 4. Regulation of *tetO* reporter gene expression by PKA overexpression or doxycycline treatment. Firefly luciferase expression mediated by (A) Tet repressor (TetR)-cyclic AMP (cAMP)-responsive element binding protein (CREB)(SA), (B) TetR-CREB, or (C) in the absence of transactivators was measured in response to doxycycline (dox) and protein kinase A (PKA) overexpression. Values are normalized to TetR-CREB-mediated expression in the absence of doxycycline or PKA. Error bars indicate SEM of triplicate experiments. RLU, relative light unit.

Phospho-Elk-1 Western blot analysis was also consistent with the prediction that doxycycline would not interfere with phosphorylation of TetR-Elk-1 by MEK-1. Together with the results from the reporter expression experiments, these Western blot observations support the prediction that doxycycline inhibits TetR-Elk-1 by inhibiting the interaction of TetR and the *tetO* DNA element rather than interfering with activation of the Elk-1 domains. No bands were present on the phospho-Elk-1 Western blot of either the TetR(-) transfected controls or the experimental groups not transfected with transcription factors, indicating the specificity of the antibody and the absence of detectable levels of endogenous phospho-Elk-1.

TetR-CREB Is Activated by PKA or cAMP and Inhibited by Doxycycline

We also tested the dual regulation of the analogous transactivator TetR-CREB (Figure 4). PKA greatly

increases the amount of luciferase reporter activity to levels approximately 75-fold above baseline, whereas the administration of doxycycline returns reporter expression to baseline levels. Because CREB activation by PKA is mediated by phosphorylation of serine at amino acid 119 (28), we also tested TetR-CREB(SA), which has a serine-to-arginine point mutation at amino acid 119 and cannot be activated (10). This mutant TetR-CREB(SA) did not induce transcription levels significantly over background levels (Figure 4A). To determine if TetR-CREB could respond to endogenous signaling events in the absence of doxycycline, we cotransfected TetR-CREB and *tetO*-luc into HEK 293 cells and then treated cells with forskolin/IBMX/KCl to elevate cAMP levels (Figure 5). In the presence of elevated cAMP levels, reporter gene expression increased approximately 4.6-fold ($P < 0.01$). Expression of the *tetO*-Luc reporter gene was not induced by forskolin/IBMX/KCl in the presence of doxycycline (Figure 5).

In the Absence of Doxycycline, TetR Hybrids Perform Comparably with Previous Systems

We compared both TetR-Elk-1 and TetR-CREB directly with previously established reporter systems. The results indicate that TetR-Elk-1 activity induced by MEK-1 cotransfection

was greater than the induced activity of GAL4-Elk-1 (Figure 6A). We also compared TetR-CREB response to PKA cotransfection with that of GAL4-CREB and CRE-luc, a construct containing cAMP response elements upstream of a luciferase reporter. In response to PKA cotransfection, GAL4-CREB mediated approximately 25% higher levels of activity than TetR-CREB, although both produced higher levels of expression than the CRE-luc *cis*-reporter when cotransfected with PKA (Figure 6B). Batch effects accounted for variability in the normalized assay values (Figure 2B and Figure 6A), but error bars demonstrate the low variability within experimental batches.

DISCUSSION

The results demonstrate the dual regulation of the doxycycline-sensitive hybrid reporter systems TetR-Elk-1 (Figure 2) and TetR-CREB (Figure 4). To test the TetR-Elk-1 response to cell signaling events, we overexpressed MEK-1, which does not directly phosphorylate Elk-1 but rather activates ERK, which in turn activates Elk-1 (4,5). In response to overexpression of MEK-1, TetR-Elk-1 activated expression of a reporter gene placed downstream of a tetracycline-responsive element, and this expression

Figure 5. Regulation of TetR-CREB-mediated reporter gene expression by drugs that stimulate endogenous signaling. The adenylyl cyclase agonist forskolin (10 μ M) and the phosphodiesterase inhibitor IBMX (10 μ M) were delivered along with KCl (50 mM) to trigger downstream endogenous cell signaling events in the absence of doxycycline. Forskolin/3-isobutyl-1-methylxanthine (IBMX)/KCl treatment had no significant effect on reporter gene expression in the presence of doxycycline. Values are normalized to Tet repressor (TetR)-cyclic AMP (cAMP)-responsive element binding protein (CREB)-mediated expression in the absence of drugs. Error bars indicate SEM of triplicate experiments. Control groups (-) were transfected with the hybrid transcription factors and reporter constructs but were treated with vehicle [dimethyl sulfoxide (DMSO) in culture media, final concentration 0.04%]. RLU, relative light unit.

Figure 6. TetR hybrid transcription factors compared with previous signal-responsive reporter systems. (A) Tet repressor (TetR)-Elk-1-mediated and GAL4-Elk-1-mediated expression of a reporter gene was measured following regulation by MAPK ERK kinase 1 (MEK-1) overexpression (MEK). Values are normalized to TetR-Elk-1-mediated expression in the absence of MEK-1. (B) TetR-cyclic AMP (cAMP)-responsive element binding protein (CREB), GAL4-CREB, and CRE-mediated expression of a reporter gene was measured following regulation by protein kinase A (PKA) overexpression. Values are normalized to TetR-CREB-mediated expression in the absence of PKA. Control groups (-) were transfected with the hybrid transcription factors and reporter constructs but without the kinase. RLU, relative light unit.

could be inhibited by doxycycline (Figure 2). Likewise, TetR-CREB-mediated reporter gene expression increased in response to PKA overexpression (Figure 4) or forskolin/IBMX/KCL treatment (Figure 5), which stimulates endogenous pathways to activate CREB. Gene expression mediated by TetR-CREB was both doxycycline sensitive and serine 119-dependent (Figure 4). By comparing the TetR hybrids directly with the equivalent GAL4 hybrids, we demonstrated that, in the absence of doxycycline, the TetR-based hybrids perform comparably with previous methods (Figure 6).

The regulation of expression by two independent conditions, one controlled by cell signaling events and one controlled by the investigator, has important implications for the experimental design of future research in cell signaling. The TetR hybrid system might be useful in screening strategies, such as screening a library of potential signaling molecules and selecting clones in which survival genes are turned on by TetR hybrid transcription factors. The dose-dependency of the Tet system (15) could be particularly useful in optimizing conditions for such a strategy. Temporal control of the TetR hybrid transcription factor could prove useful in transgenic animal studies where one often wants to examine cell signaling during a specific time window during development or following an experimental challenge. For example, when hippocampi and amygdalae from CRE-lacZ reporter mice were examined following contextual learning, CREB-mediated reporter expression in control groups accounted for approximately 50% of the induced level of reporter gene expression (29). The TetR hybrid reporter system may provide a mechanism to dramatically lower this background by temporally restricting the opportunity for reporter gene expression using doxycycline. The TetR hybrid reporter system produced slightly higher background expression than the traditional system (Figure 6), attributable to the *tetO*-luc reporter plasmid (Figure 2C). If necessary, the *tetO*-luc reporter plasmid could be altered in future experiments to reduce background expression in the absence

of cell signaling. The ratio of induction was still greater for TetR-Elk-1 compared with GAL4-Elk-1 (Figure 6A), although not for TetR-CREB compared with GAL4-CREB (Figure 6B).

TetR hybrid reporters represent an improvement on previous hybrid reporters because of their potential future applications. The TetR hybrid transcription factor could be expressed using tissue-specific promoters (18–21) to combine spatial and temporal specificity. The TetR hybrid reporter system could also be adapted in transgenic mouse strategies by breeding with an array of existing reporter mouse strains, such as those expressing green fluorescent protein (GFP) (30) or luciferase (16). These properties could be of potential use in the development of in vivo imaging of whole animals (30) or synaptic plasticity (31). In addition to reporter genes, effector genes could be expressed to produce desired phenotypes in a manner responsive to endogenous cell signaling to create temporally restricted and activity-dependant manipulation of cellular processes. Thus, the combination of doxycycline-regulated TetR with kinase-regulated transcriptional activation to produce a TetR hybrid reporter system provides a feasible strategy for the future study of cell signaling.

ACKNOWLEDGMENTS

The authors thank Marcelo Wood, Klaus Kaestner, Julie A. Blendy, Lewis Chodosh, and Debu Chakravarti for helpful discussions and comments on the manuscript. We thank Bong-Kiun Kaang and Hermann Bujard for generously supplying plasmids. We also thank Carolina Isiegas for technical training and assistance and Richard Shultz for use of equipment. This research was supported by grant no. R01MH060244 from the National Institute of Mental Health (to T.A.) and The David and Lucile Packard Foundation Fellowship in Science and Engineering (to T.A.).

COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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Received 2 February 2005; accepted 8 March 2005.

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