Cytotechnology (2011) 63:211–216
DOI 10.1007/s10616-011-9335-z

BRIEF REPORT

Development of a tightly-regulated tetracycline-dependent transcriptional activator and repressor co-expression system for the strong induction of transgene expression

Hiroshi Hosoda · Takahisa Miyao · Shusaku Uchida · Shinsuke Sakai · Satoshi Kida

Received: 17 September 2010 / Accepted: 5 January 2011 / Published online: 20 February 2011

Abstract The teteracycline (Tc)-dependent and -inducible transcriptional activator (rtTA) system has been used to express regulated transgene expression in vitro and in vivo. However, previous reports have demonstrated that, even in the absence of Tc, the rtTA binds weakly to the tetracycline response element (TRE), leading to a low level of background activity. In order to reduce the leaky gene expression induced by rtTA, we previously established a tightly regulated system (A-IRES-R system) that makes use of both the rtTA (A) and a Tc-dependent repressor (TetR-Kruppel-associated box; KRAB) (R). In addition, others have described a transactivator rtTA2-M2 (M2) that displays higher sensitivity to Dox than rtTA. In this study, to further develop the A-IRES-R system, we generated a derivative Tc system (M2-IRES-R system) that co-expresses both rtTA-M2 and TetR-KRAB from a single vector. We show that compared to the A-IRES-R system, the M2-IRES-R system leads to a greater level of induced TRE-mediated transcription in the presence of doxycycline (Dox) and yet displays a similar level of basal TRE-mediated transcription in the absence of Dox. Furthermore, the M2-IRES-R system also displays less leaky gene expression in the absence of Dox compared to rtTA-M2 and rtTA systems. Taken together, our results suggest that the M2-IRES-R system enables to tightly regulate and highly induce the expression of transgene compared to other systems.

Keywords rtTA-M2 · Tight regulation · Transgenic technique · Basal transcription

H. Hosoda, T. Miyao and S. Kida contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s10616-011-9335-z) contains supplementary material, which is available to authorized users.

H. Hosoda · T. Miyao · S. Kida (✉)
Department of Bioscience, Faculty of Applied Bioscience, Tokyo University of Agriculture, 1-1-1 Sakuragaoka, Setagaya-ku, Tokyo 156-8502, Japan
e-mail: kida@nodai.ac.jp

S. Uchida · S. Sakai · S. Kida
Department of Agricultural Chemistry, Faculty of Applied Bioscience, Tokyo University of Agriculture, 1-1-1 Sakuragaoka, Setagaya-ku, Tokyo 156-8502, Japan

Introduction

Genetic techniques have proven powerful for the study of the molecular bases of biological phenomena and for the development of gene therapies. However, there are serious limitations to these methods. For example, conventional gene targeting technology may lead to severe developmental deficits or the development of compensatory mechanisms. To overcome these problems, methods should be developed that temporally and spatially regulate the mutation. For the regulation of somatic mutagenesis, a

transgenic system is necessary, in which the expression of target proteins, such as Cre recombinase and mutated proteins, is tightly regulated by exogenous compounds.

Bujard et al. have developed regulatable gene expression systems for use in mammals that are based on Tc-responsive transcription factors (Gossen and Bujard 1992; Gossen et al. 1995). Indeed, two types of engineered Tc-responsive transcription factors have been generated through modification of the repressor of the Tn*10* tetracycline (Tc) operon of *Escherichia coli* (TetR), which binds to its operator sequence (tetracycline response element; TRE) in the absence of Tc. The transcription activation domain, VP16, of herpes simplex virus was fused with TetR (TetR-VP16; tTA) or a mutant of TetR (rTetR) that binds to TRE only in the presence of Tc (rTetR-VP16; rtTA). The resulting fusion activators, tTA and rtTA, activate TRE-mediated transcription in the absence or presence of Tc, respectively (Gossen and Bujard 1992; Gossen et al. 1995). These activators have been used to regulate TRE-dependent transgene expression in transgenic mice (Mayford et al. 1996; Chen et al. 1998; Mansuy et al. 1998; Tremblay et al. 1998; Yamamoto et al. 2000).

rtTA is thought to be more useful than tTA because the administration of Tc, rather than its removal, induces the de novo synthesis of target proteins. However, the expression of rtTA has been shown to result in leaky Tc-independent expression because rtTA binds weakly to the TRE, even in the absence of Tc (Kistner et al. 1996; Forster et al. 1999; Freundlieb et al. 1999; Baron and Bujard 2000; Wang et al. 2000; Uchida et al. 2006). Severe problems may result when this rtTA system is used to regulate the expression of toxic proteins or proteins whose low level of expression is enough to affect the function of endogenous proteins. Therefore, in order to reduce this leaky expression, a system was developed in which the Tc-dependent activator (rtTA) and Tc-dependent transcription repressor (TetR-KRAB) are co-expressed from a single vector using an internal ribosome entry site (IRES; Bornkamm et al. 2005; Saqr et al. 2006; Uchida et al. 2006). In this system, only TetR-KRAB binds to the TRE in the absence of Tc and represses leaky rtTA-dependent transcription. This system has been used successfully for the regulation of transgene expression in the brain (Uchida et al. 2006).

Other tetracycline-inducible transcription activators have been developed that show an increase in sensitivity to doxycycline (Dox), a derivative of Tc (Urlinger et al. 2000). For example, rtTA-M2 is active at a Dox concentration that is tenfold lower than that necessary when using rtTA, and displays transgene expression in the absence of Dox that is less leaky than that observed with rtTA.

In this report, to further develop transgenic systems that enable tightly regulated transgene expression, we generated a derivative Tc-based system (M2-IRES-R) that co-expresses rtTA-M2 and TetR-KRAB from a single expression vector. We compared the regulation of transgene expression by the M2-IRES-R system with that observed with the rtTA-M2 and A-IRES-R systems. We show that the M2-IRES-R system drives a greater level of induced transgene expression in the presence of Dox and that it nevertheless displays a basal level of TRE-mediated transcription in the absence of Dox that is comparable to that observed with the A-IRES-R system.

Materials and methods

Plasmid constructions

A TRE-reporter construct, pTRE-Luc, and expression plasmids encoding rtTA or tTA (prtTA or ptTA, respectively) were purchased from Clontech (Mountain View, NJ). The β-galactosidase expression plasmids, pCH110 and pCMVβ, were purchased from Amersham (Piscataway, NJ, USA) or Clontech, respectively. pA-IRES-R was constructed as previously described (Uchida et al. 2006). prtTA-M2 expressing a tTA mutant containing five amino acid substitutions relative to wild-type tTA was constructed as previously described (Urlinger et al. 2000). Furthermore, *Kpn*I and *Asc*I, and *Eco*RI, *Mlu*I and *Cla*I restriction enzyme sites were introduced into the 5′- and 3′-ends of the rtTA-M2 coding region, respectively, by PCR based site-directed mutagenesis. To generate prtTA-M2, the *Kpn*I-*Eco*RI fragment of rtTA-M2 was subcloned into the corresponding restriction sites in pcDNA3. To generate pM2-IRES-R, the *Cla*I fragment encoding rtTA-M2 from prtTA-M2 was inserted into the corresponding restriction sites in pA-IRES-R.

The doxycycline solution

Doxycycline (Dox)-hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in water to a final concentration of 2 mg/ml and sterilized using a 0.22-μm filter. Aliquots of the Dox stock solution were stored at −20 °C.

Cell culture and transient transfection

NIH3T3 and COS-1 cells were maintained in DMEM supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C in 5% CO_2 as previously described (Hosoda et al. 2004). PC12 cells were maintained in DMEM supplemented with 10% FBS, 5% horse serum, penicillin (100 U/ml), and streptomycin (100 μg/ml).

Reporter assay

For the reporter assay, NIH3T3, COS-1 and PC12 cells grown in 24-well plates were transiently transfected with plasmids using Lipofectamin-plus and the Lipofectamin 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Fifteen hours following transfection, Dox (10–1,000 ng/ml) was added to the cell culture medium. pCH110 (100 ng) or pCMVβ (100 ng) was used as a control for normalization of transfection efficiency. Forty-eight hours following transfection, we measured the activities of luciferase and β-galactosidase as previously described (Hosoda et al. 2004). Luciferase activity was normalized to β-galactosidase activity, and the relative luciferase activity was then calculated as a ratio relative to that observed in control cells transfected with pTRE-Luc and pcDNA3 in the absence of Dox. All reporter assays were performed in triplicate in three independent experiments. Each value represents the mean ± SD. Statistical analyses were performed using the unpaired Student's t test. A p value <0.05 was considered to be statistically significant.

Results and discussion

We first confirmed the previous observations that compared to rtTA, rtTA-M2 display reduced basal activity in the absence of Dox and increased sensitivity to Dox. NIH3T3 cells were transiently transfected with a TRE-reporter plasmid, together with an expression plasmid encoding rtTA (prtTA) or rtTA-M2 (prtTA-M2). In the absence of Dox, cells expressing rtTA displayed significantly greater basal transcription from the TRE-dependent promoter compared to control cells transfected with pcDNA3, indicating that expression of rtTA led to leaky transcription (***Online Resource). Consistent with the results of a previous report (Deuschle et al. 1995), this leaky expression was not observed following expression of rtTA-M2 (Online Resource). In the presence of Dox, cells expressing rtTA-M2 displayed significantly greater induced transcription from the TRE-dependent promoter compared

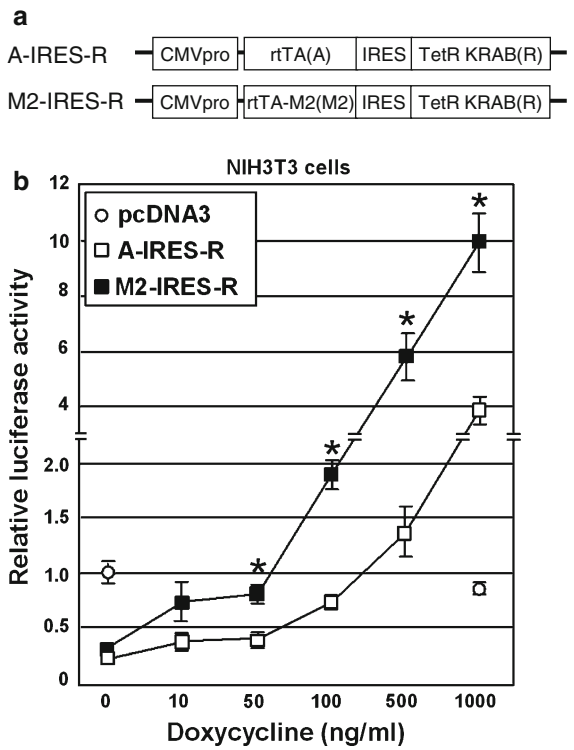

Fig. 1 Effect of M2-IRES-R and A-IRES-R on basal and Dox-inducible TRE-mediated transcription. **a** Structures of expression units. *CMVpro* cytomegalovirus promoter. **b** NIH3T3 cells were transiently co-transfected with the pTRE-Luc reporter plasmid (10 ng), together with pM2-IRES-R (100 ng) or pA-IRES-R (100 ng). pCH110 (100 ng) was used as a control for transfection efficiency. Empty vector (pcDNA3) was used to standardize the total amount of transfected DNA (210 ng). Dox (10–1,000 ng/ml) was added to the cell culture medium. An asterisk indicates $p < 0.05$ compared with A-IRES-R in the presence of each dose of Dox (Student's t test)

to cells expressing rtTA. Importantly, rtTA-M2 but not rtTA displayed activation of TRE-mediate transcription even following the application of a low dose of Dox (100 ng/ml). These observations are consistent with previous findings and indicate that compared to rtTA, rtTA-M2 displays a lower level of leaky transcription and a greater level of inducible transcription in response to DOX.

To reduce leaky transcription activation by rtTA, we previously developed a bicistronic expression system (A-IRES-R) that allows co-expression of both a Dox-dependent activator (rtTA; A) and repressor (TetR-KRAB; R) form a single vector incorporating an IRES. We next examined the function of rtTA-M2 in this bicistronic expression system. To test this, NIH3T3 cells were transiently transfected with pA-IRES-R or pM2-IRES-R. In the absence of Dox, co-expression of rtTA-M2 or rtTA with TetR-KRAB lead to a decrease in the basal activity of the TRE-dependent promoter compared to that observed in control cells transfected with pcDNA3 (Fig. 1), indicating that both systems exhibit a lower level of leaky transcription. Moreover, in the presence of more than 50 ng/ml Dox, the M2-IRES-R system showed significantly greater levels of induced transcription compared to the A-IRES-R system (Fig. 1). These results indicate that the M2-IRES-R system displays a greater level of inducible expression compared to the A-IRES-R system, while nonetheless displaying a comparable level of basal transcription from the TRE-dependent promoter. Therefore, these results suggest that the M2-IRES-R system may be useful to induce high-level expression of a transgene whose leaky expression should be prevented owing to toxicity of the gene product, such oncoproteins or factors that trigger apoptosis.

Previous reports have shown that rtTA-M2 itself displays a lower level of leaky TRE-mediated transcription than rtTA (Welman et al. 2006). We next compared the levels of basal and induced TRE-mediated transcription from the M2-IRES-R and rtTA-M2 systems, the latter of which only expresses rtTA-M2 and not the TetR-KRAB repressor, in the absence and presence of Dox in NIH3T3 cells. In the

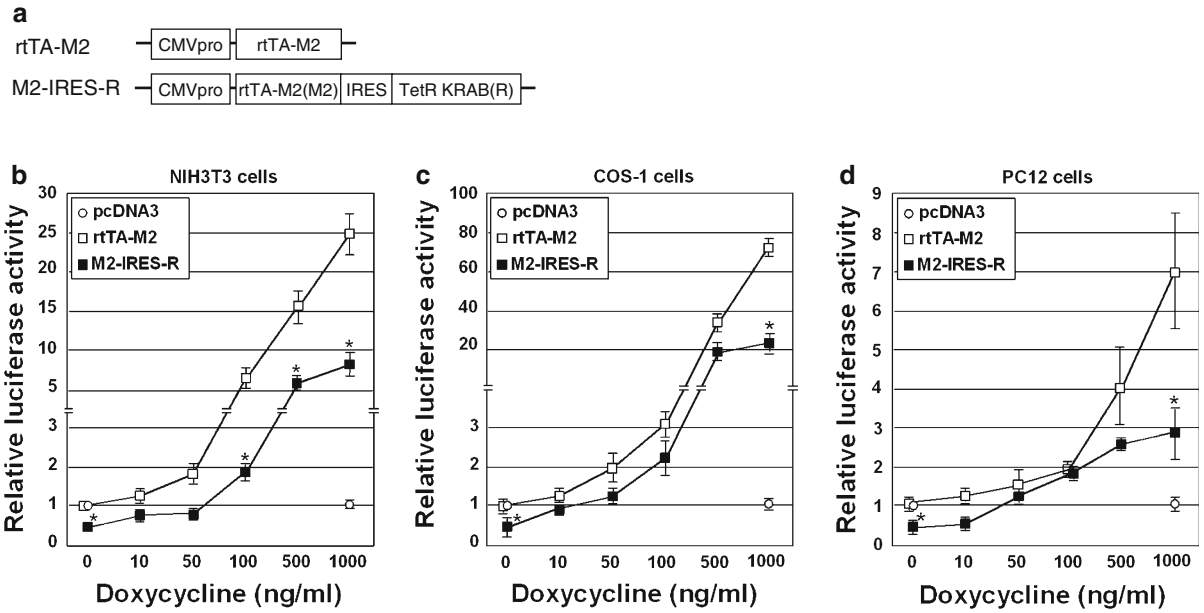

Fig. 2 Effect of M2-IRES-R and rtTA-M2 on basal and Dox-inducible TRE-mediated transcription. **a** Structures of expression units. CMVpro; cytomegalovirus promoter. **b** NIH3T3, **c** COS-1 and **d** PC12 cells were transiently co-transfected with the pTRE-Luc reporter plasmid (10 ng), together with pM2-IRES-R (100 ng) or prtTA-M2 (100 ng). pCH110 (100 ng, NIH3T3 cells) or pCMVβ (100 ng, COS-1 and PC12 cells) were used as controls for transfection efficiency. Empty vector (pcDNA3) was used to standardize the total amount of transfected DNA (210 ng). Dox (10–1,000 ng/ml) was added to the cell culture medium. An asterisk indicates $p < 0.05$ compared with M2-IRES-R in the presence of each dose of Dox (Student's t test)

absence of Dox, the M2-IRES-R system displayed a lower level of basal transcription from the TRE-dependent promoter compared to the rtTA-M2 system (Fig. 2a). In contrast, in the presence of more than 100 ng/ml of Dox, the rtTA-M2 system displayed significantly greater level of induced TRE-mediated transcription than the M2-IRES-R system (Fig. 2a). These results indicated that M2-IRES-R system gave rise to a lower level of basal transcription that was observed with the rtTA-M2 system, although the level of Dox-induced expression observed with the M2-IRES-R system is lower than that observed with the rtTA-M2 system.

We finally examined whether the M2-IRES-R system displays tight regulation of TRE-mediated transcription in other types of cell, specifically Cos-1 cells, a monkey kidney cell line, and PC-12 cells, a rat neuronal cell line. As was observed in NIH3T3 cells, the M2-IRES-R system gave rise to lower levels of basal and induced TRE-mediated transcription than the rtTA-M2 system in both the absence and presence of Dox, respectively, in COS-1 cells and PC12 cells (Fig. 2b, c). Taken together, these results suggest that the M2-IRES-R system functions in various types of cells including neuron-like cells.

In this report, we improved the A-IRES-R system by incorporating the Dox-dependent transcription activator, rtTA-M2. The resulting M2-IRES-R system displayed a greater level of induction of TRE-mediated transcription by Dox that was observed with the A-IRES-R system and also gave rise to a lower level of basal transcription that was observed with the rtTA and rtTA-M2 systems, although the level of Dox-induced expression observed with the M2-IRES-R system is lower than that observed with the rtTA-M2 system (Fig. 1). These results indicate that the M2-IRES-R system is better suited for the inducible expression of toxic proteins, such as those that could induce embryonic lethality or developmental defects, compared to other systems, whereas the rtTA-M2 system should be used when greater expression of the gene of interest is required.

Acknowledgment This work was supported by a Grant-in Aid for High Technology Research and Priority Areas—Molecular Brain Science—from the ministry of Education, Science and Culture, Japan and the Research Grant for Nervous and Mental Disorders from the Ministry of Health, Labor and Welfare, Japan.

References

Baron U, Bujard H (2000) Tet repressor-based system for regulated gene expression in eukaryotic cells: principles and advances. Methods Enzymol 327:401–421

Bornkamm GW, Berens C, Kuklil-Roos C, Bechet JM, Laux G, Bachl J, Korndoefer M, Schlee M, Holzel M, Malamoussi A, Chapman RD, Nimmerjahn F, Mautner J, Hillen W, Bujard H, Feuillard J (2005) Stringent doxycycline-dependent control of gene activities using an episomal one-vector system. Nucleic Acids Res 33:e137

Chen J, Kelz MB, Zeng G, Sakai N, Steffen C, Shockett PE, Picciotto MR, Duman RS, Nestler EJ (1998) Transgenic animals with inducible, targeted gene expression in brain. Mol Pharmacol 54:495–503

Forster K, Helbl V, Lederer T, Urlinger S, Wittenburg N, Hillen W (1999) Tetracycline-inducible expression systems with reduced basal activity in mammalian cells. Nucleic Acids Res 27:708–710

Freundlieb S, Schirra-Muller C, Bujard H (1999) A tetracycline controlled activation/repression system with increased potential for gene transfer into mammalian cells. J Gene Med 1:4–12

Gossen M, Bujard H (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc Natl Acad Sci USA 89:5547–5551

Gossen M, Freundlieb S, Bender G, Muller G, Hillen W, Bujard H (1995) Transcriptional activation by tetracyclines in mammalian cells. Science 268:1766–1769

Hosoda H, Motohashi J, Kato H, Masushige S, Kida S (2004) A BMAL1 mutant with arginine 91 substituted with alanine acts as a dominant negative inhibitor. Gene 338:235–241

Kistner A, Gossen M, Zimmermann F, Jerecic J, Ullmer C, Lubbert H, Bujard H (1996) Doxycycline-mediated quantitative and tissue-specific control of gene expression in transgenic mice. Proc Natl Acad Sci USA 93:10933–10938

Mansuy IM, Winder DG, Moallem TM, Osman M, Mayford M, Hawkins RD, Kandel ER (1998) Inducible and reversible gene expression with the rtTA system for the study of memory. Neuron 21:257–265

Mayford M, Bach ME, Huang YY, Wang L, Hawkins RD, Kandel ER (1996) Control of memory formation through regulated expression of a CaMKII transgene. Science 274:1678–1683

Saqr HE, Omran O, Dasgupta S, Yu RK, Oblinger JL, Yates AJ (2006) Endogenous GD3 ganglioside induces apoptosis in U-1242 MG glioma cells. J Neurochem 96:1301–1314

Tremblay P, Meiner Z, Galou M, Heinrich C, Petromilli C, Lisse T, Cayetano J, Torchia M, Mobley W, Bujard H, DeArmond SJ, Prusiner SB (1998) Doxycycline control of prion protein transgene expression modulates prion disease in mice. Proc Natl Acad Sci USA 95:12580–12585

Uchida S, Sakai S, Furuichi T, Hosoda H, Toyota K, Ishii T, Kitamoto A, Sekine M, Koike K, Masushige S Murphy G, Silva AJ and Kida S (2006) Tight regulation of transgene expression by tetracycline-dependent activator and repressor in brain. Genes Brain Behav 5:96–106

Urlinger S, Baron U, Thellmann M, Hasan MT, Bujard H, Hillen W (2000) Exploring the sequence space for tetracycline-dependent transcriptional activators: novel

mutations yield expanded range and sensitivity. Proc Natl Acad Sci USA 97:7963–7968

Wang Z, Zheng T, Zhu Z, Homer RJ, Riese RJ, Chapman HA Jr, Shapiro SD, Elias JA (2000) Interferon gamma induction of pulmonary emphysema in the adult murine lung. J Exp Med 192:1587–1600

Welman A, Barraclough J, Dive C (2006a) Generation of cells expressing improved doxycycline-regulated reverse transcriptional transactivator rtTA2S-M2. Nat Protoc 1:803–811

Welman A, Barraclough J, Dive C (2006b) Generation of cells expressing improved doxycycline-regulated reverse transcriptional transactivator rtTA2S-M2. Nat Protoc 1:803–811

Yamamoto A, Lucas JJ, Hen R (2000) Reversal of neuropathology and motor dysfunction in a conditional model of Huntington's disease. Cell 101:57–66