Attenuation of leakiness in doxycycline-inducible expression via incorporation of 3′ AU-rich mRNA destabilizing elements

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BioTechniques 45:155-162 (August 2008)
doi 10.2144/000112896

Tetracycline-regulated expression systems have been widely used for inducible protein expression in cultured mammalian cells. With these systems, however, leakiness in expression of the target gene in the absence of the inducing agent is a frequent problem. Here we describe a novel approach to overcome this problem that involves the incorporation of AU-rich mRNA destabilizing elements (AREs) into the 3′ untranslated regions of the tetracycline-inducible constructs. Using the inducible expression of sphingosine kinase 1 and 2 in HEK293 cells as model systems, we found this ARE approach to be remarkably successful in ablating expression of these proteins in the absence of doxycycline through decreasing stability of their mRNAs. We show that this undemanding and flexible process results in a substantial decrease in the leakiness of the tetracycline-inducible expression system while maintaining a high level of target protein expression following induction.

INTRODUCTION

Inducible regulation of transgene expression in mammalian cells is a valuable tool in the study of gene function. Control of both the timing and level of protein expression not only minimizes potentially detrimental consequences of high, long-term overexpression on cell signaling and viability (1), but also increases the likelihood of observing physiologically relevant cellular effects.

The most commonly used inducible protein expression systems are those regulated by tetracycline (Tet) and its derivatives. Various Tet-regulated expression systems have been developed, including repression systems using the Tet-repressor (TetR) protein to block target gene transcription in the absence of Tet (2), or transactivation systems using TetR-mammalian transcription factor fusion derivatives to either switch transcription of a target gene on or off in response to Tet (Tet-On and Tet-Off systems) (3,4). While possessing many advantages over other current systems, basal leakiness still remains the main problem reported with these Tet controlled systems (5,6). The high basal expression levels in Tet systems are most probably due to the site of chromosomal integration, which has been described to be an important factor in tight regulation of the Tet promoter (7). False promoters or cryptic initiation signals may also contribute to a leaky expression of the Tet system under noninduced conditions (8).

Various approaches have been developed to tighten the control of gene expression of the Tet-regulated systems. Most involve the reduction of gene dosage either by low–copy number episomal vector systems (9,10) or by single-copy chromosomal integration through the use of retroviral vectors (11–13). Others have tried to decrease basal activity of the promoter by means of point mutations. However, results were generally disappointing as these modified systems do not sustain a high level of gene expression (14,15). Other strategies, which have met with some success, have involved the use of more efficient repressors and the use of combinations of different repressors to reduce basal levels while maintaining good induction ratios (16–19). Here, we report an alternative novel, simple, and broadly applicable method to overcome basal leakiness of inducible expression systems. We show, using Tet-inducible expression of two distinct sphingosine kinases as model systems, that incorporation of AU-rich mRNA destabilizing elements (AREs) in the 3′ untranslated region (UTR) of inducible constructs results in a significant decrease in the leakiness of the Tet-inducible expression system while maintaining high levels of inducibility.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), HEPES buffer solution, penicillin, and streptomycin were purchased from CSL Biosciences (Parkville, Australia) and tetracycline-free fetal bovine serum (FBS) was obtained from BD Biosciences (Palo Alto, CA, USA). Protease inhibitors (Complete) were purchased from Roche Diagnostics GmbH (Mannheim, Germany); doxycycline from Sigma Aldrich (St. Louis, MO, USA); Flp-In T-Rex HEK293 cells, pOG44 recombinase, and Benchmark pre-stained protein standards from Invitrogen (Carlsbad, CA, USA); nitrocellulose membranes from Schleicher and Schuell (Keene, NH, USA); D-*erythro*-Sphingosine from Biomol Research Laboratories, Inc. (Plymouth, PA, USA); and [γ^{32}P]ATP and [α^{32}P]dATP from Perkin Elmer (Melbourne, Australia).

Construction of Expression Plasmids

The human sphingosine kinase 1 and 2 (SK1 and SK2) cDNAs containing *C*-terminal FLAG epitope tags (20,21) were cloned into pcDNA5/FRT/TO (Invitrogen) by digestion with *Kpn*I and *Not*I. Subsequently, AREs (underlined) were incorporated into the 3′ UTRs of these plasmids using the oligonucleotides 5′-GGCCGC<u>ATTTATTTATTTATTTATTTA</u>GGTACCTGCAG<u>TTTATTTATTTATTTATTTA</u>AGCTTC-3′ and 5′-TCGAGAAGCT<u>TAAATAAATAAATAAATAAA</u>CTGCAGGTACC<u>TAA</u>

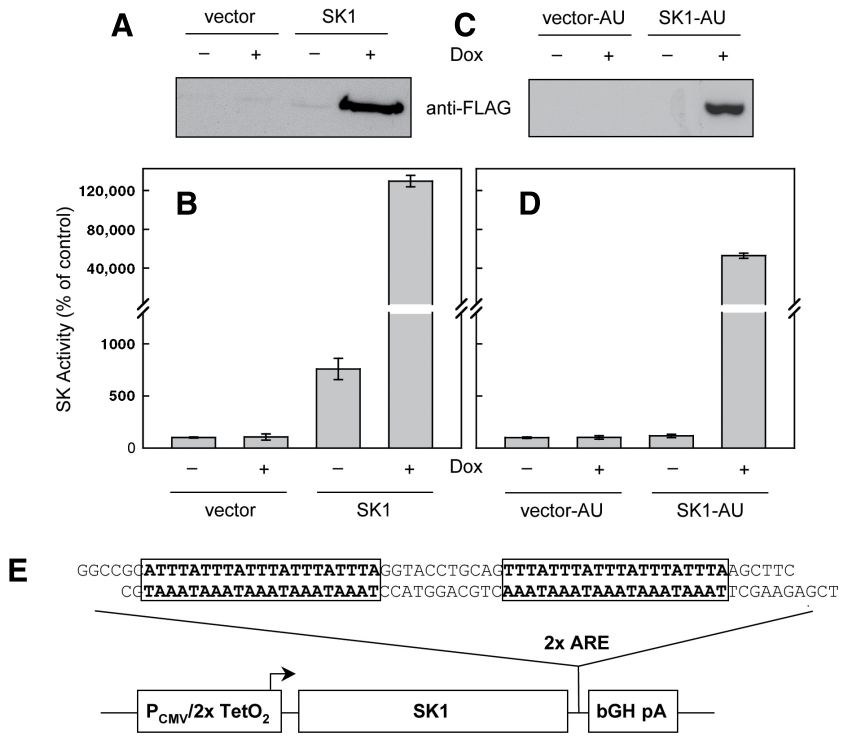

Figure 1. Leakiness in SK1 expression in the doxycycline-inducible system is strongly attenuated by incorporation of AU-rich mRNA destabilizing elements (AREs) into the 3′ untranslated region (UTR). Flp-In T-Rex HEK293 cells containing inducible FLAG epitope–tagged SK1 without or with AREs in the 3′ UTR (SK1 and SK1-AU, respectively), and corresponding vector control cells (vector and vector-AU, respectively) were cultured for 24 h either in the absence or the presence of 1 µg/mL doxycycline. The expression of SK1 was then examined in cell lysates by immunoblot analysis with (A, C) anti-FLAG antibodies and (B, D) sphingosine kinase (SK) activity assays. Data represent the mean ± SEM from more than five experiments. (E) Schematic representation of ARE incorporation into the 3′ UTR of the Tet-inducible Flp-In T-Rex vector system. A previously identified, optimized ARE (boxed sequence) (28) was incorporated, in duplicate, into the 3′ UTR of the Flp-In T-Rex expression vector as described in the Construction of Expression Plasmids section. Boxed segments within the schematic represent important regions within the expression vector; P_{CMV}/2× $TetO_2$, the Tet-inducible promoter; SK1, the coding region of the SK1 cDNA; and bGH pA, the polyadenylation sequence of the bovine growth hormone cDNA.

ATAAATAAATAAATAAATGC-3′ (Geneworks, Adelaide, Australia). These oligonucleotides were heated at 95°C for 5 min, and annealed by cooling to room temperature. These AREs were then ligated into pcDNA5/FRT/TO, pcDNA5/FRT/TO-SK1, and pcDNA5/FRT/TO-SK2 following digestion with *Not*I and *Xho*I, leaving the multiple cloning site largely intact. Constructs were sequenced to verify incorporation of the desired AREs.

Cell Culture and Generation of Stably Transfected Inducible HEK293 Cell Lines

Flp-In T-Rex HEK293 cells were cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, 0.2% (w/v) sodium bicarbonate, 1.2 mg/mL penicillin, and 1.6 mg/mL streptomycin. The cells were co-transfected with the pOG44 vector encoding the Flp recombinase, and pcDNA5/FRT/TO-, pcDNA5/FRT/TO-AU-, pcDNA5/FRT/TO-SK1-, pcDNA5/FRT/TO-SK1-AU-, pcDNA5/FRT/TO-SK2-, or pcDNA5/FRT/TO-SK2-AU-inducible mammalian expression constructs in 9:1 ratio using the Lipofectamine 2000 reagent (Invitrogen) as described by the manufacturer. Two days after transfection the cells were passaged and after the cells had attached, the growth medium was replaced with a selective medium containing 150 µg/mL Hygromycin B and 15 µg/mL Blasticidin (Invitrogen). The selective medium was changed every 3 to 4 days until the desired number of cells was grown. Experiments were performed with pools of Hygromycin B–resistant cells, which by the nature of the Flp-In system are isogenic (22).

To induce SK1, cells were exposed to various concentrations of doxycycline, harvested 24 h later, and lysed by sonication (2 W for 30 s at 4°C) in extraction buffer containing 50 mM Tris/HCl (pH 7.4), 10% glycerol, 0.05% Triton X-100, 150 mM NaCl, 1 mM dithiothreitol, 2 mM Na_3VO_4, 10 mM NaF, 1 mM EDTA, and Complete protease inhibitors. Cells ectopically expressing SK2 were treated and harvested in the same manner, but using extraction buffer lacking Triton X-100, since this detergent has been reported to inhibit the activity of SK2 (23). Protein concentrations in cell homogenates were determined with Coomassie Brilliant Blue reagent (Bio-Rad Laboratories, Hercules, CA, USA) with BSA as standard.

Western Blotting

Cell lysates were subjected to SDS-PAGE and the proteins transferred to nitrocellulose membranes. SK1 and SK2 were detected via their FLAG-epitope tags with the monoclonal M2 anti-FLAG antibody (Sigma Aldrich).

Sphingosine Kinase Activity Assays

Sphingosine kinase activity was determined using D-*erythro*-sphingosine and [γ^{32}P]ATP as substrates as described previously (21). SK1 assays were performed using sphingosine solublized with Triton X-100, while assays for SK2 were performed with sphingosine solublized with BSA. One unit (U) of activity is defined as 1 pmol of sphingosine 1-phosphate formed per min per mg of protein.

Northern Blotting

Flp-In T-Rex HEK293 cells were left uninduced or induced with either 1 ng/mL or 10 ng/mL doxycycline for SK1 and SK1-AU cells, respectively. This difference in doxycycline concentration was used in an attempt

to generate similar cellular levels of each mRNA. After 24 h of induction the cells were treated with actinomycin D (10 µg/mL) to inhibit any further transcription. Total RNA was then extracted at various time intervals using TRIzol reagent (Invitrogen) and analyzed by Northern blotting. Briefly, 20 µg of total RNA was separated on a 1% agarose gel, blotted onto Nylon membranes (Hybond-N; GE Healthcare BioSciences, Piscataway, NJ, USA), and then hybridized with [α^{32}P]dATP-labeled cDNA probes for SK1 and normalized with actin. The membranes were analyzed by phosphorimaging.

RESULTS AND DISCUSSION

Sphingosine kinases are important signaling enzymes that catalyze the phosphorylation of the lipid sphingosine to generate the bio-active phospholipid sphingosine 1-phosphate (24). Almost all previous studies that have examined the role of the sphingosine kinases through overexpression of these enzymes have used expression systems driven by strong, constitutive promoters. With these systems, both known human sphingosine kinases, SK1 and SK2, express very efficiently in mammalian cells; in the case of SK1, the cellular sphingosine kinase activity is up to 1000-fold higher than endogenous levels (25).

To more thoroughly study the cellular roles of SK1 we wished to generate an inducible system for expression of this enzyme. To do this we used the Flp-In T-Rex system, which employs the TetR protein to block target gene transcription in the absence of Tet, so that SK1 cDNA is introduced in a silent state and transcription is activated via addition of the Tet derivative doxycycline. Following establishment of an isogenic HEK293 cell line containing inducible FLAG-tagged SK1 we analyzed its effectiveness in maintaining tight, inducible expression of this enzyme. Immunoblot analysis with anti-FLAG antibodies demonstrated strong induction in response to doxycycline, but also suggested the possibility of some leakiness in SK1 expression in the absence of the inducer (Figure 1A). This leakiness in expression of SK1 was better seen by performing more sensitive sphingosine kinase activity assays, which showed that in the absence of doxycycline there was approximately eightfold higher sphingosine kinase activity in lysates from cells containing the inducible SK1 construct, compared with empty vector control cells (Figure 1B). Since the physiological regulation of endogenous SK1 activity involves changes in activity in the order of two- to fivefold (20,26) the observed leakiness resulting from the Tet-regulated system was considered likely to be problematic for functional analysis. Therefore, we searched for ways to ablate the leakiness in SK1 expression in this system.

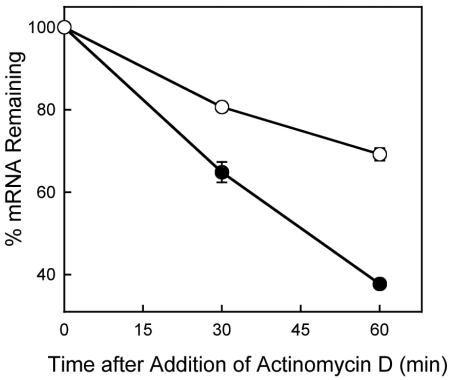

Figure 2. Degradation of SK1 mRNA is enhanced by insertion of AU-rich mRNA destabilizing elements (AREs) in the 3′ untranslated region (UTR). Flp-In T-Rex HEK293 cells containing inducible SK1 without (○) or with (●) AREs in the 3′ UTR were cultured for 24 h in the presence of 1 µg/mL doxycycline. The cells were then treated with 10 µg/mL actinomycin D to inhibit further transcription, harvested at the indicated times, and total RNA isolated for Northern blot analysis. The results show that the half-life of SK1 and SK1-AU mRNA were 90 min and 47 min, respectively. Data represent the mean ± SEM from two experiments.

While a variety of approaches have been used to overcome this leakiness (9,11,16), most are either labor-intensive, not readily available, or achieve only limited success. Since protein expression can be regulated through mRNA stability (27), we hypothesized that incorporating instability in the mRNA may provide an alternate approach for reducing basal leakiness in these systems through decreasing the half-life of mRNA transcribed in the absence of the inducing agent. Thus, we took the novel approach of incorporating AREs into the 3′ UTR of the inducible SK1 construct. To generate the construct, a previously identified, optimized ARE (28) was incorporated, in duplicate, into the Flp-In T-Rex expression vector in the 3′ UTR of the SK1 construct (Figure 1E). A HEK293 cell line was then generated containing this SK1-AU-inducible expression construct.

To examine the effect of AREs in reducing leakiness in SK1 expression in the inducible system we analyzed both SK1 protein expression as well as the sphingosine kinase activity in the SK1-AU HEK293 cells both before and after induction by doxycycline. The presence of the ARE significantly reduced ectopic expression of SK1 protein in the absence of doxycycline, since sphingosine kinase activity in lysates of SK1-AU HEK293 cells grown in the absence of doxycycline were comparable to the control cells (Figure 1D). Notably, since SK1 has high intrinsic catalytic activity that is not dependent on posttranslational modifications (25), any ectopic SK1 expression directly correlates with an increase in cellular sphingosine kinase activity. Thus, this firmly indicated that the AREs successfully ablated leakiness in SK1 expression. Doxycycline-induced SK1 protein expression in these cells was slightly decreased compared with that observed for cells containing constructs lacking AREs, but could still be efficiently induced to levels approximately 600-fold higher than that for endogenous SK1 (Figure 1, C and D).

To confirm that the AREs lead to attenuation of SK1 expression in the absence of doxycycline by mRNA destabilization, Northern blot analysis was performed using total RNA extracted from Flp-In T-Rex HEK293 cell lines containing inducible SK1 constructs with or without the AREs. Following induction of SK1 expression with doxycycline for 24 h, further transcription was then inhibited by the addition of actinomycin D. RNA was then harvested at various time points over a 60 min period and the stability of the SK1 mRNA determined by assessing the levels of residual SK1 mRNA. The results indicate that the rate of SK1 mRNA disappearance was twofold greater in cells containing

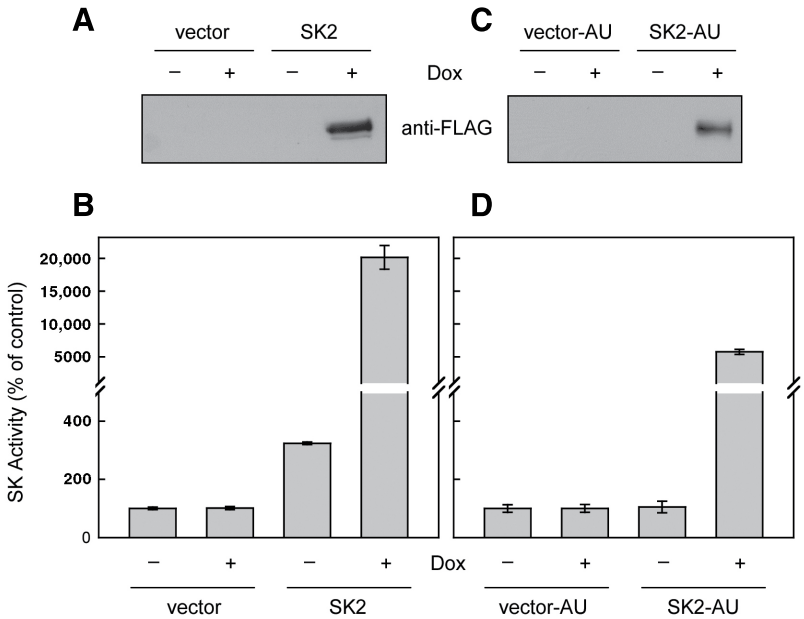

Figure 3. AU-rich mRNA destabilizing elements (AREs) also strongly attenuate leakiness in SK2 expression in the doxycycline-inducible system. Flp-In T-Rex HEK293 cells containing inducible FLAG epitope–tagged SK2 without or with AREs in the 3′ UTR (SK2 and SK2-AU, respectively) and corresponding vector control cells (vector and vector-AU, respectively) were cultured for 24 h either in the absence or presence of 1 µg/mL doxycycline. The expression of SK2 was then examined in cell lysates by immunoblot analysis with (A, C) anti-FLAG antibodies and (B, D) sphingosine kinase (SK) activity assays. Data represent the mean ± SEM from more than five experiments.

SK1-AU compared with that observed for the cells containing the SK1 construct lacking the AREs (Figure 2). This strongly suggests that incorporation of the AREs into the 3′ UTR promoted mRNA degradation and is likely to be the mechanism whereby attenuation of SK1 expression in the absence of doxycycline is achieved.

Having established the effectiveness of the incorporation of AREs in reducing leakiness of SK1 expression in the Tet-inducible system, we next examined whether this approach may be more broadly applicable to reducing doxycycline-independent expression of other proteins. Therefore, we examined the effect of incorporation of AREs in reducing leakiness of Tet-inducible SK2 expression. While SK2 shows some sequence similarity to SK1, it originates from a different gene and is substantially larger than SK1 due to the presence of two additional polypeptide regions at its *N*-terminus and within the middle of its sequence that are quite distinct from SK1 (23). Thus, these distinct differences between the two sphingosine kinases allowed the use of SK2 to examine the broader applicability of ARE incorporation in reducing doxycycline-independent expression in the Tet-inducible system.

HEK293 cell lines were generated containing Flp-In T-REx Tet-inducible SK2 expression vectors with and without the incorporation of AREs within the 3′ UTR. These cell lines were then analyzed for the expression of SK2 prior to and following addition of doxycycline. In the absence of doxycycline, SK2 activity was elevated in these cells approximately threefold over that seen in empty vector control cells (Figure 3B), even though expression of SK2 protein could not be detected via immunoblot analysis for its FLAG epitope (Figure 3A). Like the situation with SK1, however, this doxycycline-independent expression of SK2 was abolished by the presence of AREs (Figure 3D). Again, doxycycline still strongly induced SK2 protein expression in these SK2-AU cells (Figure 3, C and D), albeit at somewhat lower levels than that observed for the inducible constructs lacking AREs. These results suggest that incorporation of AREs into the 3′ UTR of expression constructs may provide a broadly applicable approach to reduce the leakiness of Tet-inducible expression systems.

In conclusion, using the inducible expression of both SK1 and SK2 in HEK293 cells as model systems, we found that incorporation of AREs into the 3′ UTR of inducible expression constructs was remarkably successful in ablating leakiness in protein expression in the absence of inducing agent. Importantly, the presence of AREs had only a moderate effect on the levels of protein expression upon induction by doxycycline. One of the major advantages of this approach over previously published approaches is its ease of use, since the AREs can be incorporated into any expression construct via a single digestion/ligation or PCR step. This approach also has a high degree of flexibility. In the current study we incorporated two AREs into the 3′ UTR of the SK1 and SK2 expression constructs, which efficiently overcame the low level of leakiness in expression of these proteins in the absence of doxycycline. Additional AREs could, however, be incorporated to further increase mRNA destablization (28)

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and more strongly reduce leakiness for other constructs where higher doxycycline-independent expression may exist. Reducing the half-life of the mRNA can have the additional potential benefit of shortening the response time. Where rapid up- or down-regulation of gene expression is advantageous, the short mRNA half-life results in a more rapid approach to steady state following induction or repression of transcription in response to addition or removal of the inducer doxycycline. Furthermore, while we used this approach to a TetR-based repression system, because it relies on mRNA destablization, this approach would also be applicable for reducing leakiness in Tet transactivation-based systems, and indeed, any other inducible expression system.

ACKNOWLEDGEMENTS

This work was supported by a Fay Fuller Fellowship and a Senior Research Fellowship from the National Health and Medical Research Council of Australia (to S.M.P.).

COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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Received 18 March 2008; accepted 29 April 2008.

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