METHOD

A versatile ribosomal protein promoter-based reporter system for selective assessment of RNA stability and post-transcriptional control

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ABSTRACT

Assessment of post-transcriptional control relies on use of transcriptional inhibitors and is masked by copious and cryptic transcriptional induction. We screened several cellular promoters that are constitutively active yet noninducible to external stimuli. The ribosomal protein RPS30 promoter was chosen; its TATA signal and sp1 site location were optimized. The modified promoter (RPS30M) is selective to post-transcriptional effects of AU-rich elements (ARE) in the 3'UTR, while it is not transcriptionally responsive to a wide variety of agents including pro-inflammatory cytokines and RNA-binding proteins. Specific *cis*-acting elements can be appended to RPS30M by a cloning-free approach to allow coupled transcriptional/post-transcriptional assessment, as demonstrated with NF- κ B and β -catenin/wnt signaling experiments. Moreover, efficient tetracycline-regulated RPS30M was created for quantitative assessment of the half-lives of mRNAs containing AREs. The described approach provides enhanced versatility and suitability for selective post-transcriptional assessment with or without transcriptional induction.

Keywords: post-transcriptional control; RNA stability; AU-rich elements; reporter systems

INTRODUCTION

Post-transcriptional regulation of gene expression, which has gained further attention in recent years, is essential for normal functioning of eukaryotic cells, and aberrations in this regulation can contribute to certain disease processes (Fan et al. 2005; Khabar 2005). Unlike reporter assays for transcriptional analysis that are widely used, there is a need for selective and versatile post-transcriptional regulation assessment systems.

The 3'untranslated region (3'UTR) of eukaryotic mRNA is a major target for transacting factors, such as RNA-binding proteins or miRNAs that affect the stability or the translation of specific mRNAs (Glisovic et al. 2008; Hobert 2008; Bartel 2009; Zhang and Su 2009). The 3'UTRs often contain *cis*-acting elements that mediate post-transcriptional control of gene expression including RNA stability and

translation. Among these sequences are the AU-rich elements (AREs) that are found in more than 10% of the human or mouse genome (Halees et al. 2008).

ARE-mediated pathways are responsive to inflammatory stimuli like endotoxin, pro-inflammatory cytokines, and other stress inducers. The signals are transduced to the ARE mainly through signaling cascades, such as the p38 MAPK pathway that phosphorylates ARE-binding proteins like tristetraprolin (Wang et al. 1999; Winzen et al. 1999; Lasa et al. 2000; Chrestensen et al. 2004; Hitti et al. 2006). The same stimuli are also strong inducers of a wide range of promoters including the most commonly used exogenous expression viral promoters CMV and SV40 (Lee et al. 2004; Ramanathan et al. 2005). The inducibility of these promoters may mask true post-transcriptional assessment in reporter assays.

In order to study selective post-transcriptional effects on gene expression, we developed an EGFP reporter expression system that relies on a constitutively active cellular promoter that is noninducible by external stimuli. This is different from the behavior of viral promoters such as CMV and SV40 virus that harbor binding sites for transcription factors that are activated by different signaling

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pathways. This system also allows the use of convenient fluorescence-based reporter assay rather than methods that are based on the inhibition of transcription and mRNA detection. Inhibition of transcription by drugs like actinomycin D can cause significant nonspecific effects, such as toxicity, especially for mRNAs with long half-lives, and enhancement of mRNA stability in certain conditions (Rajagopalan and Malter 1996; Blaxall and Port 2000). In this paper, we used ribosomal protein RPS30 promoter that was further modified for increased strength. Furthermore, transcriptional responsive elements can be added to RPS30M by a simple cloning-free strategy to allow coupled transcriptional and post-transcriptional effects. Moreover, the RPS30M can be rendered TetO-regulated allowing transcriptional block and mRNA half-life determination. We propose a versatile and enhanced system for the analysis of post-transcriptional and coupled transcriptional/post-transcriptional regulation that is suitable for both in-depth analysis and large-scale screening efforts.

RESULTS AND DISCUSSION

Screening for noninducible and strong cellular promoters

Several cellular promoters were assessed for their expression strength and responsiveness to the pro-inflammatory cytokine TNF- α . First, we selected several candidate cellular promoters from microarray experiments and bioinformatics (Al-Zoghaibi et al. 2007) that have strong expression signals or EST counts, respectively. We have found in microarray screening experiments that the selected cellular gene promoters, particularly those of ribosomal protein genes, namely, *RPL0*, *RPL1*, *RPL3*, *RPS23*, and *RPS30*, are abundantly expressed in different cell types, their expression does not change with different stimuli, and they do not contain inducible transcriptional elements, such as those of NF- κ B sites (data not shown). The CMV and SV40 minimal promoters, commonly used viral promoters in reporter gene assays, were used as controls. The elongation factor α (EEF1A1) promoter was used as a control cellular promoter. The promoters were amplified from genomic DNA and cloned upstream of EGFP in a mammalian expression vector (Fig. 1A).

The EGFP reporter fluorescence, generated from HEK293 cells that were transfected with each of the promoter constructs, was assessed. As expected the CMV promoter gave the highest expression (Fig. 1B, inset with different scale) when compared with cellular promoters—about 10-fold ($P < 0.001$). The minimal SV40 promoter, commonly used in reporter constructs was weakly expressed. The cellular promoters derived from EEF1A with its first intron or with the eIF4g intron gave higher expression than the ribosomal protein genes (*RPL0*, *RPL1*, and *RPL3*)—these promoters also contain their first introns. Since introns are known to

enhance transgenic expression by increasing mRNA accumulation (Nott et al. 2003), we tested this effect on RPS23 promoter-linked expression. The intron of RPS23 (RPS23I) enhanced the expression of RPS23 promoter-linked reporter activity by eightfold ($P < 0.001$) (Fig. 1B). The RPS30 that also contains its first intron caused higher expression than RPS23. Both RPS23 and RPS30 promoters had the highest expression level of all cellular promoters tested here.

The CMV was strongly responsive to TNF- α induction (2.2-fold) (Fig. 1B, inset). The SV40 minimal promoter was increased by 2.3-fold due to TNF- α treatment. Unlike CMV and SV40, all the ribosomal protein promoters including RPS30 were not responsive to TNF- α . The inducibility was also tested at the mRNA level by real-time (RT) PCR; in contrast to CMV, the RPS30 promoter was neither responsive to TNF- α nor IL1- α (Fig. 1C). Because of its enhanced expression and lack of cytokine stimulation, we focused on the RPS30 promoter for further evaluation and suitability in post-transcriptional assessment assays.

Modification of the RPS30 promoter and response to stimuli

Post-transcriptional gene regulation can lead to a substantial reduction in mRNA or protein levels. Therefore, the need of a promoter with a sustained high level of detectable expression is crucial. A modification was made to the RPS30 promoter to make it stronger. In the modified RPS30 (RPS30M), the TATA-like box was replaced with the canonical TATA signal and the promoter was truncated to minimize potential transcriptional induction while bringing the distant sp1 site toward the transcriptional start site for higher constitutive expression (Fig. 2A). The modified RPS30M was clearly stronger (approximately ninefold) than the wild-type RPS30 (Fig. 2B). Although intratest transfection efficiency variation is always low (<6% CV), normalization by cotransfection with red fluorescent protein (RFP) was performed and yielded similar results (data not shown). Likewise, RPS23 with the modification in a similar manner as with RPS30 was superior to the wild-type RPS23 (Fig. 2B).

We tested the responsiveness of the RPS30M and CMV promoters to a variety of external stimuli that triggers different signaling cascades like dsRNA (an RNA viral intermediate analog), H₂O₂ (oxidative stress), lithium (β -catenin pathway), calyculin (phosphatase inhibitor), CoCl₂ (hypoxia), PMA (protein kinase C inducer), ionomycin (calcium signaling), and the p38 mitogen-activated protein kinase activator, anisomycin (Fig. 2C). Unlike CMV, the RPS30M promoter was not responsive to all inducers except the combination of PMA and ionomycin, where the response was weak and statistically nonsignificant ($P = 0.174$) when compared with the CMV promoter.

Further, we performed experiments to test the ability of the RPS30M system to specifically detect post-transcriptional

FIGURE 1. Construction and performance of cellular promoter vectors. (A) Schematic representation of the promoter constructs and their forms. The vector constructs comprise different promoters (dashed boxes), EGFP reporter coding region, and the stable BGH 3'UTR. pCMV contains the CMV promoter with intron A; pSV40 contains the SV40 promoter; pEF1 contains the elongation factor α promoter with or without the eIF4G 5'UTR; pRPL contains the ribosomal protein gene promoters of RPL0, RPL1, and RPL30; and pRPS contains the ribosomal protein gene promoters of RPS23 or RPS30 with or without their first introns (I). (B) Analysis of promoter strength and inducibility. HEK293 cells in 96-well microplates were transfected with the indicated promoter constructs (100 ng DNA) for 18 h and then treated with or without TNF- α (10 ng/mL) for an additional 18 h. Reporter activity as fluorescence was assessed from images captured by a BD automated Bioimager and quantitated by ProXcell as described in Materials and Methods. Readings are mean $\pm$ SEM of fluorescence intensities from quadruplicate wells. CMV promoter data are shown independently because it has a higher scale. (C) The response of CMV and the RPS30 promoters at mRNA levels. HEK293 cells were transfected with CMV and RPS30 constructs overnight and treated with TNF- α or IL-1 α for 4 h. Total RNA was extracted at the indicated time points and subjected to RT-PCR using a primer pair/TaqMan probe specific to EGFP mRNA. The TaqMan primer/probe spans an intron and exon junction sequence for efficient control of DNA contamination. Data are mean $\pm$ SEM of three independent experiments. * $P < 0.05$. ** $P < 0.01$.

regulation due to pro-inflammatory cytokines. First, we evaluated the reporter response to the presence of the mRNA destabilization elements, ARE, and found that they have a better suppressive response on the reporter activity from the RPS30M-linked construct when compared with the reporter activity from the CMV promoter construct (Fig. 2D). The EGFP reporter under the control of the RPS30M promoter and linked to the stable BGH 3'UTR that lacks ARE, was not responsive in the HEK293 cell line to either interleukin-1 (IL-1 α), okadaic acid (OA), or a combination of both agents (Fig. 2E). In contrast, when the same reporter is fused to the ARE-containing IL-8 3'UTR and TNF- α 3'UTR, a strong and significant selective response to OA or OA in combination with IL-1 α could be detected (Fig. 2E). OA is an inhibitor of protein

phosphatase 1 and 2A and can increase ARE-containing-mRNA stability, possibly by the inhibition of TTP dephosphorylation leading to TTP phosphorylation and loss of its mRNA-binding action (Pshenichkin and Wise 1995; Benjamin et al. 2004; Hitti et al. 2006).

The experiments above indicate that the RPS30M system is suitable for the study of selective post-transcriptional regulation in response to a wide range of stimuli including pro-inflammatory inducers.

The assessment of post-transcriptional regulation using the RPS30 system

The RPS30M system was tested using another cell line, Huh-7. This well-differentiated hepatocellular cell line

FIGURE 2. Modification and performance of the RPS23 and RPS30 promoters. (A) Schematic diagram of the RPS30 promoter and its modifications in RPS30M. Numbers show the nucleotide position in relation to the transcriptional start site (TSS). Vertical lines indicate positions of sp1 sites. E: exon. Vertical arrow is translational start. (Lower panel) The sequences of the modified promoter, underlined are sp1 sites and modified TATA signal. (B) Effects of RPS modifications on reporter expression in HEK293. Cells were transfected with 100 ng of the reporters for 18 h; images were captured by an automated BD Bioimager and quantitated by ProXcell. (C) Response of CMV and RPS30M promoters to several external stimuli and signaling inducers. HEK293 cells in 96-well microplates were transfected with CMV and RPS30M constructs that harbor the stable BGH 3'UTR and treated with the indicated inducers overnight. (D) Response of RPS promoter-linked reporter to ARE-3'UTR. HEK293 cells were transfected with 150 ng of EGFP reporter vectors that are under the transcriptional control of the ribosomal protein RPS30M or CMV promoter. The EGFP reporter contains either control stable 3'UTR or 3'UTR, which contain 200 bases of IL-8 or TNF- α 3'UTR that contain ARE. (E) HEK293 cells were transfected with vectors as indicated for 18 h and then treated for 6 h with 2 ng/mL of IL-1 α , 100 nM okadaic acid (OA), or a combination. Reporter activity as fluorescence was assessed from high resolution images captured by a BD Bioimager and quantitated by ProXcell as described in Materials and Methods. All data above are mean $\pm$ SEM (quadruplicate) of one of three experiments. * P < 0.02, ** P < 0.01, *** P < 0.001.

responds to a variety of pro-inflammatory cytokines and has been customarily used for hepatitis and cytokine research (Bevan and Raynes 1991; Preiss et al. 2008; Ait-Goughoulte et al. 2009). An additional advantage of Huh-7 is its excellent transfection efficiency ($\sim$ 70%). In the case of the BGH-3'UTR that lacks the ARE, no or minimal induction by the cytokines, OA, or combination was observed at the mRNA level (Fig. 3A, left panel). With the reporter constructs that are fused with IL-8 3'UTR or TNF 3'UTR, treatment with IL-1 α or TNF- α led to an increase (from two- to threefold) in the reporter mRNA levels and no further increase was observed with addition of OA (Fig. 3A, middle, right panels).

Despite the increased effects, due to cytokine treatment, on reporter mRNA levels from the ARE-3'UTR constructs (Fig. 3B, middle, right panels), treatment with the cytokines had minimal effects on the protein expression (Fig. 3B, middle, right panels). It has been shown that the ARE can mediate blockage of the ARE-mRNA translation, and release from blockage can be mediated by certain inducers (Zhang et al. 2002; Vasudevan et al. 2007). Indeed, treatment with OA and particularly in combination with IL-1 α or TNF- α led to a considerable up-regulation of protein expression (from 4.0- to 5.5-fold) (Fig. 3B, middle, right panels) suggesting that combined OA and cytokine treatment releases a translation block.

FIGURE 3. The effect of pro-inflammatory cytokines on the RPS30M-driven post-transcriptional reporter system. HuH-7 cells were transfected with vectors containing the RPS30M promoter-linked EGFP constructs fused with the control non-ARE BGH 3'UTR, IL8 3'UTR, or TNF- α 3'UTR. Cells were treated with 2 ng/mL of IL1 α , 2 ng/mL of TNF- α , 100 nM okadaic acid (OA) or a combination as indicated. Fluorescence due to the activity of the EGFP protein (A) was quantified by imaging (after 6 h) and the mRNA levels (B) were quantified by real-time QPCR after 4 h. Mean $\pm$ SEM of three independent experiments. * P < 0.02, ** P < 0.01, *** P < 0.001.

The RPS30M reporter system with a fluorescence end point allows the assessment of the integrated outcome of mRNA stability and translation, and thus, it is more relevant in drug screening approaches. Using the EGFP-based assay and the *Taqman* RT-PCR permits analysis of mRNA stabilization and the regulation of translation in a single reporter assay.

RPS30M-based post-transcriptional assessment of RNA-binding protein activity

Because RNA-binding proteins can affect a number of transcriptional factors as evidenced from microarray studies (Lal et al. 2004; Lopez de Silanes et al. 2005; Lai et al. 2006; Winzen et al. 2007; Stoecklin et al. 2008), selective post-transcriptional assessment of their effect on reporter constructs is usually performed using transcriptional inhibitors. Thus, the RPS30M-based model is a practical alternative to study selective post-transcriptional effects of RNA-binding proteins without secondary transcriptional effects.

HuH-7 cells were cotransfected with RPS30M-EGFP reporter vector and either empty pcDNA3.1 or pcDNA3.1 expressing the ARE-binding and stabilizing protein HuR and the dominant negative form of TTP, C124R, which stabilizes ARE-mRNA (Lai et al. 2000; Franks and Lykke-Andersen 2007; Al-Ahmadi et al. 2009). Recently, we

showed that C124R TTP has a stabilizing effect on mRNAs with ARE-containing HuR 3'UTR (Al-Ahmadi et al. 2009). We also observed a nonspecific effect of C124R TTP and HuR on the CMV promoter. There was increased activity from the reporter gene fused with the control 3'UTR (no ARE) driven by the CMV promoter due to overexpression of C124R TTP and HuR, fivefold and 2.5-fold, respectively (Fig. 4A). In contrast, neither HuR nor C124R TTP has nonspecific effects on RPS30M promoter activity (Fig. 4A). Increased expression of EGFP, due to the effect of the RNA-binding proteins, was observed only when the reporter was linked to IL-8 and TNF 3'UTRs (Fig. 4B,C).

To further confirm the specificity of the effect of C124R TTP on ARE itself, it was tested against the 60 nucleotides (nt) of ARE derived from TNF- α 3'UTR and a mutated form of this region. The effect of the TTP zinc finger mutant was observed only with the wild-type but not the mutant, ARE (Fig. 4D).

Transcriptionally coupled assessment of post-transcriptional regulation

A useful variation of the RPS30M promoter-linked reporter system is the ability to append any inducible *cis*-acting transcriptional response element (TRE) to study single or coupled transcriptional and post-transcriptional effects employing a simplified and versatile cloning-free approach

FIGURE 4. The effect of RNA-binding proteins on the RPS30M-driven reporter system on post-transcriptional regulation. Huh-7 cells in 96-well microplates were cotransfected with 50 ng reporter vector with either empty pcDNA3.1 vector, or pcDNA3 expressing HuR, or a dominant negative RNA stabilizing mutant form of TTP (C124R). The reporter constructs are fused with (A) RPS30M or CMV-driven EGFP with the control BGH 3'UTR that lacks AREs; (B) RPS30M-driven reporter fused with IL8 3'UTR; or (C) RPS30M-driven reporter fused with TNF- α 3'UTR; or (D) RPS30M-driven fused with TNF- α 60 nt ARE, or its mutant form. *** $P < 0.001$.

(Fig. 5A; al-Haj et al. 2009). We constructed linear reporters that have Tcf/lef sequence elements that respond to the β -catenin/wnt pathway inducer, lithium chloride (Hedgepeth et al. 1997). Lithium inhibits glycogen synthase kinase 3 β resulting in the cytoplasmic accumulation of β -catenin and movement into the nucleus to bind Tcf/lef transcription factors that activate Tcf/lef TRE-containing target genes (Pap and Cooper 1998). The β -catenin/wnt pathway also regulates the stability of a number of mRNAs like PITX2, c-Jun, cyclin D1, and cyclin D2, in an ARE-dependent manner (Briata et al. 2003; Gherzi et al. 2006).

HEK-293 cells were transfected with Tcf/lef reporter constructs and then treated with different concentrations of lithium. At the transcriptional level, a dose response ($r = 0.98$, $P < 0.001$) was observed when the Tcf/lef TRE was linked to a truncated (RPS30M -68) and to a full-length RPS30M promoter (Fig. 5A,B). In the absence of Tcf/lef TRE, the RPS30M promoter was not induced by lithium, except at the high concentration of 30 mM, where a slight but statistically nonsignificant induction was seen (Fig. 5B). A RPS30M-driven construct with TNF 3'UTR was responsive to lithium, indicating selective post-transcriptional action of the β -catenin induced pathway (Fig. 5C, left side). To analyze coupled transcriptional and post-transcription regulation in response to the same stimuli, we compared

the effect of lithium on constructs with or without the Tcf/lef TRE fused to the RPS30M promoter in the presence or absence of TNF- α 3'UTR (Fig. 5C, right side). Interestingly, transcriptional induction alone (Tcf/lef TRE-RPS30M in the absence of TNF 3'UTR with 20 mM lithium) increased reporter activity 2.5-fold (Fig. 5B) whereas post-transcriptional induction alone increased reporter activity by fivefold (Fig. 5C). The coupled transcriptional and post-transcriptional effect (Tcf/lef TRE-RPS30M with TNF- α 3'UTR) leads to more than 10-fold induction of the EGFP reporter (Fig. 5C). These results suggest that the coupled effect is at least additive and can be synergistic.

The RPS30M transcriptional/post-transcriptional coupled system was also used to analyze response to IL-1 α . This cytokine activates the NF- κ B signaling pathway to induce transcription of many inflammation related genes, as well as the p38 MAPK signaling pathway, which stabilizes and enhances the translation of ARE containing mRNAs. The effect of IL-1 α treatment on reporter constructs in HEK293 cells in the presence or absence of NF- κ B TRE sites

with or without IL-8 3'UTR was analyzed. Similar to the β -catenin/wnt pathway, enhanced coupled transcriptional/post-transcriptional effects were observed. The presence of NF- κ B TRE increased the reporter levels due to IL-1 α treatment by 1.7-fold; and when the IL8 3'UTR is present, the coupled transcriptional/post-transcriptional effect increased to more than 2.5-fold (Fig. 5D). Parallel experiments were performed using truncated forms of RPS30M (-68 RPS30M and -168 RPS30M) fused or not fused to NF- κ B TRE and in the presence or absence of TNF- α 3'UTR. As expected the coupled effect was observed particularly with the -68 RPS30M (Fig. 5E). These results show that the RPS30M system is versatile and can be used to study transcriptional, post-transcriptional, and coupled processes.

TetO-regulated RPS30M promoter for assessment of post-transcriptional effects

RPS30M promoter-driven expression can become regulated by fusing the promoter to TetO elements, which bind a transactivator that is inactivated by doxycycline. We used the cloning-free approach (Fig. 5A) to generate TetO -24 RPS30 and TetO -68 RPS30 promoter fusion constructs. We first evaluated the performance and degree of leakiness

FIGURE 5. Coupled transcriptional and post-transcriptional regulation. (A) Schematic diagram showing the generation of PCR products employing the cloning-free method (al-Haj et al. 2009) in which the forward primer contains the transcriptional response element (TRE) of interest at the 5'-end and the 3'-end part is a sequence target specific location of the promoter. Nucleotide number shown is in relation to the TATA signal. The 3'UTR is with or without ARE. (B) Lithium dose-dependent induction of reporter activity due to the RPS30M promoter that was generated by PCR using a forward primer that has two copies of Tcf/lef and a partial RPS30M promoter (–68; *left* columns) or the full promoter (*middle* columns). A construct without Tcf/lef was used as a control (*right* columns). (C) Coupled transcriptional and post-transcriptional effects due to Tcf/lef TRE and TNF- α 3'UTR. Cells were transfected with the reporter constructs with TNF 3'UTR in the absence (*left* side) or the presence of Tcf/lef TRE in the RPS30M promoter of the constructs (*right* side); cells were treated with lithium for 18 h. The control is 100% of reporter activity with no Li treatment. (D) Coupled transcriptional and post-transcriptional effects due to NF- κ B signaling. Cells were transfected with the reporter construct with IL-8 3'UTR (ARE, *right* side) or without (–ARE, *left* side) in the absence or presence of NF- κ B TRE sites in the RPS30M promoter of the constructs. Reporter activity in the absence of IL-1 α (10 ng/mL) was taken as control of 100%. (E) Cells were transfected with the reporter construct containing the TNF- α 3'UTR (ARE) in the absence or the presence of two copies of NF- κ B constructs in –68 RPS30M and –168 RPS30M. Reporter activity in the absence of IL-1 α (10 ng/mL) was taken as a control of 100%. Data above are mean $\pm$ SEM (triplicate readings) of quantitated captured images from one experiment of at least three independent experiments. *P*-values: **P* < 0.05, ***P* < 0.01, ****P* < 0.001. ms: Marginally significant.

of TetO-mediated shutoff. The activity of the EGFP reporter from the TetO –24 RPS30 and TetO –68 RPS30 constructs was reduced by 71% and 84% when the cells were treated with doxycycline before transfection (Fig. 6A). We selected the TetO –68 RPS30 for the doxycycline chase experiment where cells were first transfected overnight to allow the reporter expression. At 24-h post-doxycycline treatment, a 60%–80% reduction of reporter activity was detected in the case of ARE 3'UTRs that represent unstable mRNAs (Fig. 6C) compared with the control stable 3'UTR-linked reporter, which was reduced by $\sim$ 30% (the half-life of the expressed EGFP >20 h). These conditions allowed optimal differential results between the constructs with stable mRNA and unstable mRNA (Fig. 6C). The use of the

PCR-facilitated inclusion of TetO without cloning indicates the versatility of the RPS30M-driven plasmid with any ARE or 3'UTR of interest.

If mRNA half-life determinations are required, then the TetO-RPS30M can be used for this purpose, instead of using transcriptional inhibitors, such as actinomycin D (AcD) that can perturb the normal biology of RNA stability and cause nonspecific toxicity (Harrold et al. 1991; Rajagopalan and Malter 1996; Blaxall and Port 2000). Indeed, the TetO-linked system has been well developed and used extensively for mRNA half-life determinations (Xu et al. 1998; Loflin et al. 1999; Chang et al. 2004; Tchen et al. 2004; Chen et al. 2007, 2008, 2009; Winzen et al. 2007). Thus, we have carried out mRNA half-life determinations showing the utility of the

FIGURE 6. TetO-regulated RPS30M-linked EGFP for specific assessment of post-transcriptional regulation and half-life determinations. (A,B) TetO-conversion of RPS30M into TetO-regulated constructs using a cloning-free approach (Fig. 5A). HeLa-Tet off cells in 96-well microplate were transfected overnight with purified PCR products that contain three copies of TetO linked to the -68 RPS30M1 region. Cells were treated with 100 ng/mL of doxycycline for 30 h. Reporter activity as fluorescence was assessed from processed images captured by a BD Bioimager and quantitated by ProXcell. Data are mean $\pm$ SEM (quadruplicate) of one experiment of two. * $P < 0.01$, *** $P < 0.001$, ms: marginally significant. (C) Half-life measurements using doxycycline (1 μ g/mL) or actinomycin D (10 μ g/mL) that shut off transcription from TetO-linked RPS30M reporters with or without AU-rich elements. Decay curves of two constructs are shown using one phase decay model. Data are mean $\pm$ SEM (triplicate) of one representative experiment of two performed. (D) An overall scheme of the transcriptional/post-transcriptional assessment using the RPS30M reporter system. ARE: AU-rich elements; TRE: transcriptional response elements; Dox: doxycycline; and AcD: actinomycin D.

the cloning-free construction of TetO-RPS30M and response to doxycycline-mediated shut-off transcription (Fig. 6B). The half-life of the reporter mRNA linked to TNF 3'UTR was 0.55 h, which is significantly shorter than the reporter mRNA with the control 3'UTR (>5 h) and correlated with the protein levels modulated by doxycycline. However, we always detect basal levels of unstable transcripts of $\sim 20\%$ from 6 h and even after 8 h of doxycycline treatment suggesting potential leakiness in transcription. This limitation may be circumvented with usage of more than three copies of TetO—though this has not been evaluated in this system. We used AcD to block transcription and performed experiments using doxycycline and an AcD chase in parallel, in which comparable results were observed (Fig. 6B). Thus, the leakiness is not always detectable with the Northern blotting method, which has been previously used with the Tet system, compared

with the highly sensitive quantitative PCR (QPCR) that has a much lower threshold of detecting a mRNA copy number. Thus, in the case of sensitive QPCR, it is advisable to use the mRNA decay curve fit and mRNA half-life determination that consider plateaus, such as the one-phase decay model (Khabar et al. 2003; Al-Ahmadi et al. 2009). Figure 6D shows an overall scheme of the RPS30M system versatility.

The use of a fluorescence-based reporter system employing a modified ribosomal protein gene promoter (RPS30M), which is constitutively active and not subject to promoter stimulation, allows its use as a simpler approach compared with RNA-based assessment that requires RNA extraction, use of transcriptional inhibitors, and RNA quantification. The RPS30M system with a fluorescence end point, or other detection reporters, such as luciferase, allows the assessment of the integrated outcome of mRNA stability and translation, and thus, it is more relevant in

drug screening approaches. Different in-depth analyses can also be performed with the RPS30M promoter-driven system. Using the EGFP based assay and the *Taqman* RT-PCR permits analysis of mRNA stabilization and the regulation of translation of a single reporter assay. The mRNA half-life determination is simplified with a strategy that utilizes the simple cloning-free approach of the TetO-mediated transcriptional block. Individual or coupled transcriptional/post-transcriptional effects due to a single transcriptional factor response element can also be performed with the cloning-free approach.

MATERIALS AND METHODS

Cloning of the ribosomal protein and other cellular promoters

The EGFP-Gwiz plasmid, which is under the control of CMV/Intron A, was obtained from Genlantis. The plasmid backbone was used to construct different promoters in place of the CMV promoter/Intron A cassette, which was removed by using inverse PCR with forward primer TCTTTTCTGCAGTCACCGTC and reverse primer CACTATGATATCTGTGCGGTATTTACAC that contains an *EcoRV* site (underlined). Ribosomal protein promoters were amplified from the genomic DNA of the HEK293 cell line, whereas SV40 and EF promoters were cloned from SV40 pGL3 (Promega, Inc.) and pORF (Invitrogen) plasmids, respectively. The promoters with or without their introns were amplified from genomic DNA using PCR with primers specific to the flanking region of each promoter/intron sequence of the following genes: *PRPL0*, *PRPL1*, *RPL3*, *RPS23*, and *RPS30*. The gene structures of the ribosomal promoters were obtained from the Ribosomal Protein Database (<http://ribosome.med.miyazaki-u.ac.jp>). The forward and reverse primers, depending on the orientation of the promoters in the genomic DNA, included the restriction sites, *EcoRV* and *Sall* sites. PCR was carried out with the proofreading *Pfu* polymerase (Stratagene). Amplified DNA fragments were resolved in 1.2%–1.5% agarose gel and the desired amplicons were excised, purified, and cloned into the promoterless Gwiz-EGFP backbone. All the promoters including SV40 and pEF promoters were cloned into the same vector backbone.

Modification of the RPS23 and RPS30 promoters

A 5'-truncated RPS23M promoter (480 bases) with first exon and intron (501 bases) was constructed by custom gene synthesis (GeneArt, Inc.). A 5' addition of 68 bases of Sp1 site-containing sequences of RPS23 (+806 to +872 from TSS) was included and nine bases of RPS23 exon 2 were added for splicing. The ATG site in exon 1 of RPS23 was mutated to CTG to force translation initiation of the linked EGFP reporter. The RPS30M promoter construct was synthesized by GeneArt and contains a 5'-truncated RPS30 promoter (418 bases), first exon (45 bases), first intron (268 bases), and exon 2 (eight bases) for splicing. The EGFP coding region was fused with exon 2 of the RPS. The putative TATA box was improved from TACAAATA to TATAAATA. BamHI site CACTGAG was eliminated by mutating to CACCTTGAG since this site is used for 3'UTR cloning. The RPS30M promoter now contains three sp1 sites.

Cloning of AU-rich regions and ARE

The AU-rich 237-bp region (972–1209 nt: NM_000584) that belongs to the 1250-bp IL-8 3'UTR or 250 bases from TNF- α 3'UTR (1200–1450 bp, NM_00059) were amplified by RT-PCR using specific forward and reverse primers with additional 5' sequences that contain BamHI and XbaI, respectively. The forward primer is 5'-CAGCAGGATCCGATGTTGTGAGGACATGTG-3' and the reverse primer is 5'-CGACCTCTAGAACCC TGATTGAAATTTAT-3' for IL-8 3' UTR. For TNF- α 3'UTR, the forward primer is 5'-CAGCAGGATCCAGAATGCTGCAGGACTTGAG-3' and the reverse primer is 5'-CGACCTCTAGACTATGTTCAGCTCCGTTT-3'. The PCR products were cut by BamHI and XbaI sequentially and followed by phenol extraction and ethanol precipitation. Short DNA duplexes were made by annealing two synthetic complementary oligonucleotides of 70 bases that correspond to TNF- α ARE or a mutant form with BamHI and XbaI overhangs. The TNF- α oligonucleotide ARE sequence is GATCCTTGATTTATTTATTTATTTATTTATTTATTTATTTATTTACAGATGAATGTATTTATTTGGGAGAT and their mutant forms (ATT to CCG). The purified DNA products were ligated into the RPS30M or CMV promoter expression vectors with bovine growth hormone 3'UTR that has BamHI and XbaI sites. Recombinant colonies were verified by PCR and sequencing.

Cell lines, compounds, and RNA-binding protein plasmids

The HEK293 cell line was obtained from American Type Culture Collection (ATCC) and cultured in DMEM medium (Invitrogen) supplemented with 10% FBS and antibiotics. The Tet-off HeLa cell line was obtained from Clontech and was maintained in DMEM with selection drug G418 (Sigma). The Huh-7 cell line was propagated in DMEM medium with 10% FBS and antibiotics. The following are the stimuli and compounds that were used in this study: Recombinant human IL-1 α (R&D Systems, Inc.), TNF- α (R&D systems), okadaic acid (Calbiochem), poly I:C (Calbiochem), calyculin (Sigma-Aldrich), doxycycline (Sigma), cobalt chloride (Sigma), PMA (Sigma), ionomycin (Sigma), lithium (Sigma), anisomycin (Santa Cruz Biotech), hydrogen peroxide (Sigma), actinomycin D (Sigma), and doxycycline (Sigma). The RNA-binding protein expression plasmids, HuR and mutant C124R TTP, were kindly provided by Dr. Christopher Moroni (University of Basel) and Dr. Perry Blackshear (NIH), respectively.

Reporter transfection and reporter activity assessment

Cells in 96-well clear bottom black microplates were transfected with 50–100 ng of the reporter vectors using lipofectamine 2000 reagent (Invitrogen). All transfections were performed in several replicates as indicated in the text. The variance in GFP fluorescence among replicate microwells was <6%; thus, with this minimum variance, experiments do not warrant transfection normalization (Al-Zoghaibi et al. 2007). Automated laser-focus image capturing was performed using the high-throughput BD Pathway 435 imager (BD Biosciences). Image processing, segmentation, and fluorescence quantification were facilitated by the ProXcell program and previously described (al-Haj et al. 2009). Data are presented as mean values $\pm$ standard error (SEM) of

total fluorescence intensity in each well with replicate readings ranging from three to four as indicated in the text. Student's *t*-test was used when comparing two data groups, while analysis of variance (ANOVA) was performed for each data set having three or more data groups.

RNA, reverse transcription, and RT-PCR

For total RNA preparations, transfections were performed in 10-cm plates with 4 μ g vector DNA using lipofectamine 2000. After 24 h, the cells were split into six-well plates to ensure equal proportions of transfected cells in each plate. Total RNA was extracted with TRI Reagent (Sigma). Reverse transcription was performed using Superscript II and Oligo dT primer (Invitrogen). RT-PCR was performed using a custom-made Taqman primer and probe set (Applied Biosystems). The primers span the RPS30M intron to control DNA contamination. The 6-carboxyfluorescein (6FAM)-labeled TaqMan probe that targets the CMV exon 1-EGFP (exon 2) junction sequence was used. The probe design allowed further control of DNA contamination. The control RPL0 mRNA probe is labeled with a 5' reporter VIC dye (Applied Biosystems). The specificity for the cDNA of the Taqman primer was tested on a negative control containing plasmid DNA. The endogenous control RPLP0 was used for normalization. RT-PCR was performed in multiplex in the Chroma 4 DNA Engine cycler (BioRad). Cell-culture experiments for the mRNA half-life determination were performed with the HeLa Tet-off cell line that was treated with doxycycline (1 μ g/mL) to shut off transcription.

Transcriptional coupling and TetO conversion of RPS30M promoter

The RPS30M promoter-linked EGFP expression construct was turned transcriptionally inducible by generating an expression active PCR product directly from the vector using two primers flanking the full expression cassette. The forward primer has 17–18 bases of complementary sequence to the RPS30M plasmid template (full RPS30M promoter, –68, or –168 from the TATA box) and 5'-end sequences that contain transcriptional response elements. The TREs used were three copies of the Tcf/lef binding site TTAAAGATCAAAG, NF- κ B site AGGGGACTTTCCTGA, or TetO CATCCCTATCAGTGATAGAC. The reverse primer is complementary to a downstream region of the poly(A) site. PCRs were carried out using the following reagents and conditions: 2.5 U HotStart Taq (Qiagen) and 0.2 U Pfx polymerase (Invitrogen) mix, 2 μ L (100–200 ng) of the vector template, 1X PCR buffer, 0.2 mM dNTPs, and 0.2 μ M primers, with the following cycle conditions: 95°C for 12 min; 31 cycles of: 94°C, 1 min; 51°C, 1 min; 72°C, 4 min; and final extension at 72°C for 7 min. The PCR products were purified using PCR purification columns (Qiagen) to eliminate the primers, small PCR products, buffer, and enzymes. The PCR products were finally eluted in sterile water. The PCR products were run on a 1.2% agarose gel and visualized by ethidium bromide under UV light to verify size and quality.

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