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A translation control reporter system (TCRS) for the analysis of translationally controlled processes in the vertebrate cell

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ABSTRACT

Regulation of translation is critical for the accurate expression of a broad variety of genes that function in cell cycle progression and cell differentiation, as well as in the adaptation to cellular stress. The aetiologies of a number of human diseases, including cancer, have been linked to mutations in genes that control mRNA translation, or in *cis*-regulatory mRNA-sequences. Therefore, research on translational control and its therapeutic appliance has become most important. However, to date only a limited number of therapeutic drugs are known to affect translational control. Here we describe a novel, straightforward approach for the detection of cellular translational activity. We developed a Translational Control Reporter System (TCRS), which utilizes the *cis*-regulatory upstream open reading frame (uORF) from the *c/ebpα* locus to direct the translation of a dual reporter gene into two unique reporter peptides. The peptides contain a pre-pro-trypsin (PPT) signal for secretion into the medium and distinct immunogenic epitopes for detection and quantification purposes. TCRS-peptide expression levels reflect changes of translation initiation induced by serum growth factors, drugs or translation factor mutants. TCRS can be tailored to various research settings and the system may accomplish a broad application to uncover links between translational control and drugs.

INTRODUCTION

Extra-cellular stimuli, i.e. hormones, growth factors or nutrients, may induce global changes in protein synthesis. The translation of subgroups of mRNAs, however, may be modified differentially. These mRNAs are characterized by specific translation *cis*-regulatory elements that allow extracellular stimuli to selectively induce or suppress the expression of a subset of proteins. Translation *cis*-regulatory elements are typically found in transcripts of key regulatory proteins, e.g. growth factors, cell cycle regulators and regulators of apoptosis.

In the past decade translational control has been implicated in various human diseases, highlighting the significance of this regulatory mechanism (1,2). Some human diseases are genetically linked to mutations in components of the translational signalling network, such as Wolcott-Rallison Syndrome (WRS) (3) and Leucoencephalopathy with vanishing white matter (VWM) (4), in which mutations affecting the regulation of translation initiation factor (eIF) 2 have been identified. Alternatively, mutations in *cis*-regulatory elements or translational silencing of target mRNAs may contribute to disease development, e.g. mutations affecting upstream open reading frames (uORFs) in the thrombopoietin (TPO) gene causes hereditary thrombocythaemia (5), and translational silencing of C/EBPα expression is found in chronic myeloid leukaemia (CML) (6). In addition, deregulated translation as a result of disturbed PI3-kinase–PKB/Akt signalling contributes to tumourigenesis, e.g. in cancer-prone syndromes Cowden, Chermite-Dudos and Bannayan-Zonana with mutations in the tumour suppressor PTEN (7). Finally, some drugs that are believed to act by interfering with translational-control-signalling, have been applied in the treatment of cancer, or are in clinical trails, underscoring the therapeutic relevance of translation control (1,8,9). Albeit the increasing significance of this approach, the list of translationally active drugs is short. Therefore, there is an urgent need for research tools that can be used specifically for the identification and analysis of translational control processes in the cell.

Initiation of translation is the rate-limiting and the most regulated step during protein synthesis (10). Small uORFs have been identified as key *cis*-regulatory mRNA elements of translation initiation in a number of regulatory genes,

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including C/EBP (11), SCL/Tal1 (12), ATF-4 (13,14), TPO (5), CLN3 (15) and BACE-1 (16,17). Several studies have shown that uORFs function by monitoring the activity of eIFs (18,19). Thereby they may regulate translation of the main coding frame *per se* (20), or translation from alternative downstream initiation sites by ribosome scanning and re-initiation (Figure 1a). In the latter case, at moderate translational activity the suboptimal context of the uORF initiation codon allows part of the ribosomes to read through the uORF and to initiate translation at the proximal AUG-codon, generating a long isoform. Poor efficiency of re-initiation at the distal site further inhibits the expression of the small isoform. In contrast, at increased translational activity ribosomes may recognize and translate the uORF, resume scanning and efficiently re-initiate translation at the distant downstream AUG-codon, generating a small isoform. Efficiency of re-initiation at the downstream site depends on reloading with the ternary eIF2/GTP/Met-tRNA$_i^{Met}$ complex needed for the re-initiation. In addition, eIF4E as part of the eIF4F-complex has been shown to stimulate the efficiency of uORF translation *per se* and the eIF4F-complex is required for efficient scanning and re-initiation following uORF translation (11,19). Intriguingly, in this way different proteins with different functions can be expressed from a single mRNA transcript, which may determine cell-fate as in the case of C/EBPα and β, and SCL/ Tal1 (11,12).

In order to facilitate the study of mechanisms in translational control and to exploit translation control in drug

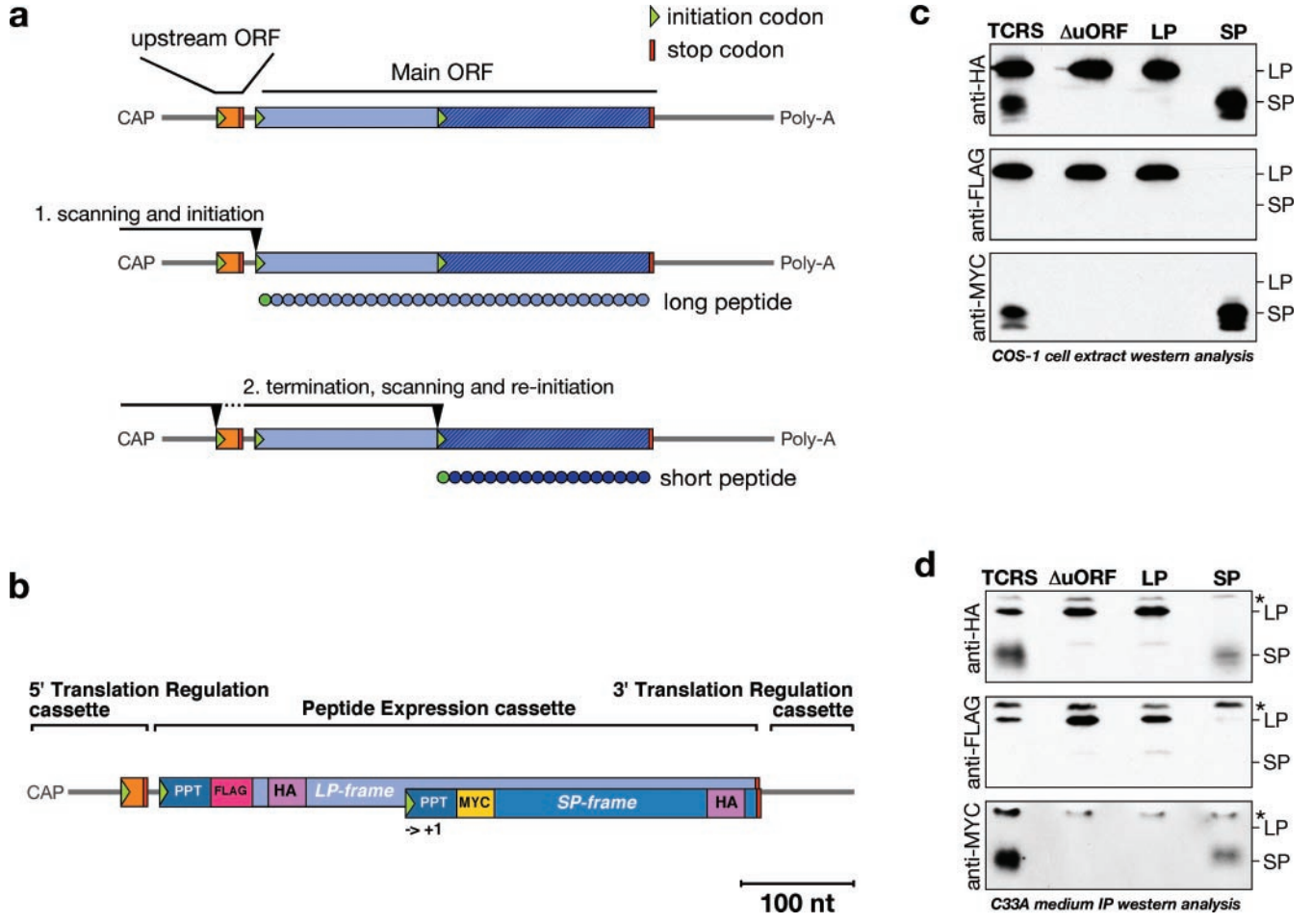


Figure 1. Design and function of the TCRS. (**a**) General scheme of a functional uORF directing translation from two alternative downstream initiation codons. The suboptimal context of the uORF initiation codon allows ribosomes to read through the uORF and to initiate translation at the first AUG of the main ORF, generating a long peptide (LP). In contrast, ribosomes that have translated the uORF resume scanning and may re-initiate translation at the downstream site, generating a Short Peptide (SP). The efficiency of uORF translation, ribosome re-scanning and translation re-initiation at the distal initiation site depends on eIF-levels and/ or -activities (**b**) Schematic representation of the TCRS. TCRS contains three cassettes, a 5′ translation regulation cassette with the uORF as the *cis*-regulatory element, a peptide expression cassette, and a 3′ translation regulation cassette. An LP can be expressed from the peptide expression cassette, containing N-terminal PPT sequences for secretion, FLAG and HA immuno-epitopes for detection and immobilization purposes, respectively, and eight cysteine residues for radioactive labelling. Alternatively, an SP can be expressed in a different reading frame (+1), also containing N-terminal PPT sequences, MYC and HA immuno-epitopes and eight cysteine residues. (**c**) Transient expression of TCRS- and the control constructs with deleted (Δ) uORF, LP-expression and SP-expression followed by immuno-detection in COS-1 cells. Both LP and SP could be detected using anti-HA antibodies. LP and SP could be distinctively detected using anti-FLAG antibodies and anti-MYC antibodies, respectively. (**d**) Secretion of TCRS-peptides into the culture medium. LP and SP are secreted into conditioned medium of TCRS-C33A cell lines. LP and SP were isolated from the medium by immuno-precipitation using anti-HA antibodies against the common HA-epitope and individually detected by their unique FLAG- and MYC-epitopes, respectively. Asterisk indicates the cross-reacting light chain of the anti-HA antibody employed for the immuno-precipitation.

discovery, we have developed an uORF-based Translational Control Reporter System (TCRS). We show that TCRS rapidly and efficiently reports changes in the translational activity of cells exposed to altered growth conditions, translationally active drugs or mutant translation factors.

MATERIALS AND METHODS

Plasmid construction

The TCRS encoding sequences were prepared by annealing of synthetic oligonucleotides and PCR, and cloned in pGEM 7Zf(+) (for sequence see Supplementary Figure 1). The final TCRS constructs were cloned as BamHI–EcoRI fragments into the expression vectors pSG5 and pcDNA3. Construction of the human eIF2α-pBabe^{puro} and human eIF4E-pBabe^{puro} constructs are described in Calkhoven *et al*. (11).

Cell culture, transfection and retroviral infection

COS-1, C33A, HEK293A and Phoenix E cells (G.P. Nolan, Stanford University School of Medicine, Stanford, CA; ATCC, SD 3444) were grown in DMEM supplemented with 10% fetal calf serum (FCS) in a humidified atmosphere with 5% CO_2 at 37°C. COS-1 cells were transiently transfected using 5 μg of pSG5 based expression vectors using a DEAE-Dextran method (21) and harvested 24 h after transfection. C33A and HEK293A cells were transfected with 5 μg of pcDNA3-based expression vectors using the calcium phosphate DNA precipitation method (22). Pools of cells stably expressing TCRS were selected for G418-resistance (C33A 0.8 mg/ml; HEK293A 0.7 mg/ml). Upon selection, cells were maintained in medium supplemented with G418 (0.3 mg/ml). Ecotropic retroviral packaging Phoenix E cells were transiently transfected with empty pBabe^{puro}, eIF2α-pBabe^{puro} or eIF4E-pBabe^{puro} constructs using the calcium phosphate DNA precipitation method, and infectious virus was harvested after 48 h. C33A cells (5×10^5) harbouring TCRS were infected as described previously (11), selected for puromycin resistance (2 μg/ml; Sigma) and pooled. Stable TCRS-C33A and TCRS-HEK293A cells were treated with 1 μM rapamycin (Calbiochem), 5 mM 2-aminopurine (2-AP) (Sigma) or 400 nM Thapsigargin (Calbiochem).

Immune-precipitation

A total of 10 ml culture medium was harvested in siliconized falcon tubes and immune-precipitated over night with 7 μg HA11 antibody (MMS-101R, Covance) followed by 2 h incubation with Protein-G Sepharose-beads (Amersham Biosciences), rinsed in 5× IP buffer [100 mM NaCl, 50 mM Tris (pH 8) and 0.003% NP40]. The protein was eluted from the beads with SDS-loading buffer and subsequent boiling. The entire immune precipitate was used for western blot analysis, as described below.

Western blot analysis

Cells were harvested in siliconized tubes. Sample preparation included rapid lysis in 0.5 M NaOH, neutralization with 0.5 M HCl, addition of SDS-loading buffer, sonication and boiling. 15% SDS–polyacrylamide gels were used for protein separation, which were then electro-blotted on a PVDF membrane (Immobilon-P, Millipore). Western blot analysis was performed followed by luminescent detection according to the manufacturer's instruction (Amersham Life Technologies, ECL system). For quantification purposes, western blot analysis was performed and fluorescent dye-conjugated second antibody (Alexa-Flour 680 goat anti-mouse antibody) was employed and made visible with the LI-COR Odyssey Infrared Imaging System according to the manufacturer's instruction (LI-COR Biosciences). Quantification was directly performed on the blot using the LI-COR Odyssey Analysis program. Antisera were used at the following concentrations: 1:200 eIF2α (C-20) (Santa Cruz Biotechnology), 1:200 4E-BP1 (R113) (Santa Cruz Biotechnology), 1:450 mouse anti-FLAG M2 (F 3165) (Sigma), 1:500 anti-eIF4E (610269) (BD Transduction Laboratories), 1:1000 mouse anti-HA11 (MMS-101R) (Covance), 1:1000 anti-Myc (9E10) (Santa Cruz Biotechnology), 1:1000 phospho-eIF2α (Ser51) (Cell Signaling Technology), 1:2000 anti-goat IgG horseradish peroxidase (HRP) (sc2020) (Santa Cruz Biotechnology), 1:3000 PKR (M515) (Santa Cruz Biotechnology), 1:5000 ECL anti-mouse IgG HRP (NA931V), 1:5000 ECL anti-rabbit IgG HRP (NA934V), for Odyssey analysis: 1:2500 goat anti-mouse (Alexa-Flour 680).

Enzyme-linked immunosorbent assay (ELISA)

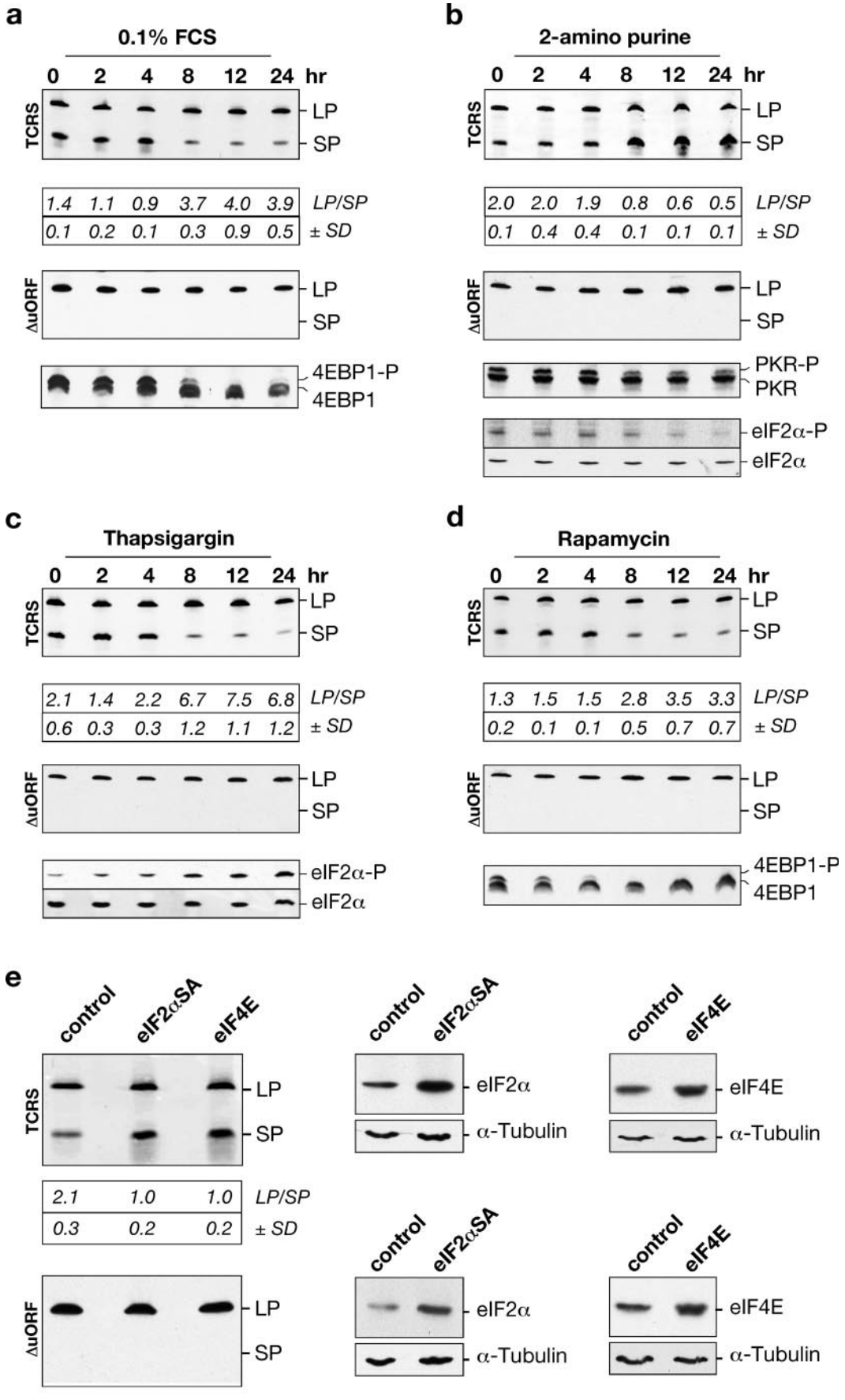
The plates with 96-wells were coated over night using rabbit anti-HA (1:250) (Novus Biologicals) in a carbonate based coating buffer (pH 9.6), blocked for 1 h with 10% FCS in phosphate-buffered saline (PBS), washed with 3× PBS/ 0.05% Tween and incubated with cell lysates for 2 h. For this purpose 1×10^7 cells were harvested in siliconized tubes, re-suspended in 400 μl IP buffer, ruptured by sonication and diluted 1:4 to 1:256 in PBS. Upon incubation, the plates were washed three times with PBS/ 0.05% TWEEN and incubated with mouse anti-FLAG M2 (F 3165) (1:300) (Sigma) or mouse anti-Myc (9E10) (1:200) (Santa Cruz Biotechnology) for 1 h, washed again three times and incubated for 1 h with HRP-linked rabbit F(ab′)2 anti-mouse IgG (1:3000). For detection purposes, the plates were rinsed with PBS/ 0.05% Tween and the substrate solution (BDopt EIA) added according to manufacturer's (BD Biosciences) instructions. The reaction was stopped with 2.5 N H_2SO_4 and analysed at 450–570 nm.

RESULTS

Design, construction and functioning of the TCRS

TCRS consists of three successive parts (Figure 1b, for complete sequence see Supplementary Figure 1). The 5′-translation-regulatory-cassette contains the uORF of C/EBPα, which serves as the *cis*-regulatory element that controls the site of translation initiation. The peptide expression-cassette codes for two peptides, one Long Peptide (LP) expressed from an initiation site proximal to the uORF, and a Short Peptide (SP) initiated from a site more distal and in a different reading frame. Finally, the 3′-cassette possesses a polyadenylation (polyA) signal required for efficient translation.

The peptides contain a common HA-epitope for the immobilization to antibody-coated surfaces, and for detection


a
0.1% FCS
0 2 4 8 12 24 hr
TCRS
LP
SP
1.4 1.1 0.9 3.7 4.0 3.9 LP/SP
0.1 0.2 0.1 0.3 0.9 0.5 ± SD
ΔuORF
LP
SP
4EBP1-P
4EBP1
b
2-amino purine
0 2 4 8 12 24 hr
TCRS
LP
SP
2.0 2.0 1.9 0.8 0.6 0.5 LP/SP
0.1 0.4 0.4 0.1 0.1 0.1 ± SD
ΔuORF
LP
SP
PKR-P
PKR
eIF2α-P
eIF2α
c
Thapsigargin
0 2 4 8 12 24 hr
TCRS
LP
SP
2.1 1.4 2.2 6.7 7.5 6.8 LP/SP
0.6 0.3 0.3 1.2 1.1 1.2 ± SD
ΔuORF
LP
SP
eIF2α-P
eIF2α
d
Rapamycin
0 2 4 8 12 24 hr
TCRS
LP
SP
1.3 1.5 1.5 2.8 3.5 3.3 LP/SP
0.2 0.1 0.1 0.5 0.7 0.7 ± SD
ΔuORF
LP
SP
4EBP1-P
4EBP1
e
control
eIF2αSA
eIF4E
TCRS
LP
SP
2.1 1.0 1.0 LP/SP
0.3 0.2 0.2 ± SD
ΔuORF
LP
SP
control
eIF2αSA
eIF2α
α-Tubulin
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α-Tubulin
control
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purposes. The specific epitopes, FLAG and MYC in LP and SP, respectively, serve for the detection and discrimination of the expressed peptides. In addition, both peptides have eight cysteine residues for S^{35}-radioactive labelling, to allow analysis by radioactive-based assays. Both LP and SP start with N-terminal pre-pro-trypsin (PPT) sequences for targeting to the endoplasmic reticulum (ER) and secretion. In this way peptide expression levels may be identified directly in the culture medium in a convenient way.

To examine the function of TCRS in cell culture, the TCRS-sequence was cloned into the expression vectors pSG5 and pcDNA3 and transfected into different cell lines. The expression of four constructs was analysed by SDS–PAGE and western-blotting: the complete TCRS with a functional uORF and full coding TCRS-sequence as depicted in Figure 1b, ΔTCRS in which the uORF has been rendered non-functional by a point mutation in the uORF initiation codon, and control constructs, entirely lacking the 5′ regulation cassette and solely coding for either LP or SP. All peptides that contain the HA-epitope could be detected in COS-1 cell extracts by immuno-blotting using anti-HA antibody (Figure 1c). Staining of the same western blot with anti-FLAG antibody reveals only the LP, whereas immuno-staining with anti-MYC antibody selectively identifies the SP. In addition, both peptides can be detected through their individual immuno-tags after HA-immuno-precipitation from conditioned medium of stably TCRS-expressing C33A cells (Figure 1d) or HEK293A cells (data not shown). Importantly, elimination of the uORF initiation site in ΔTCRS results in expression of the LP only, and abrogates expression of the SP (Figure 1c and d). Thus similar to the translational regulation of C/EBPα, β (11) and SCL/Tal1 (12), translation of the SP is exclusively dependent on an intact uORF in the 5′ translational-regulatory-cassette. Hence, the C/EBPα-mRNA *cis*-regulatory uORF sequence functions as an independent translation regulatory unit and can as such be employed in the TCRS.

TCRS detects changes in regulated translation initiation induced by serum growth factors or specific drugs

Translation initiation from ordinary initiation AUG-codons as well as re-initiation at alternative initiation codons following uORF translation is critically controlled by the initiation factors eIF2 and eIF4E (11,18). Binding of eIF4E to the mRNA-cap is a rate-limiting step in the assembly of the eIF4F-complex. In addition, the eIF4F-complex is essential for efficient ribosome scanning and re-initiation subsequent to uORF translation by unknown mechanisms (19). eIF2 delivers the initiation methionyl-tRNA required for initiation and re-initiation (23).

An important rationale to design TCRS was to establish an assay system for the identification of translationally active compounds. To examine the capacity of TCRS to perform this task, TCRS-expressing C33A and HEK293A cell lines were exposed to conditions known to affect translational control and analysed by quantification of immuno-blotted TCRS-peptides using fluorescent dye-conjugated secondary antibodies (see Materials and Methods). Figure 2a shows that serum starvation, which causes suppression of both eIF2 and eIF4E function and results in the attenuation of translation initiation, could be followed in the TCRS expression profile as a 3-fold increase in the LP/SP ratio in the TCRS-HEK293A cell line.

Attenuation of protein synthesis at the ER by phosphorylation of eIF2α is a cellular stress response conserved from yeast to vertebrates (24). Inhibitory binding of the tumour promoting drug thapsigargin to ER calcium pumps (SERCAs) causes an ER stress response with rapid activation of ER-bound eIF2α-kinase PERK, successive phosphorylation of eIF2α and inhibition of translation initiation (13,25). Figure 1b shows that inhibition of eIF2 function by 400 nM thapsigargin resulted in a 3-fold increase of LP/SP ratio after 8 h of treatment.

Conversely, inhibition of eIF2α-kinases by the potent ATP-binding site directed inhibitor 2-AP prevents phosphorylation of the eIF2α subunit, resulting in activation of eIF2 function (26) and an increase in translation initiation. Figure 2c shows that activation of eIF2 function by 5 mM 2-AP was tracked by a gradual 2.5- to 4-fold decrease in the LP/SP ratio after 8 through 24 h upon treatment in TCRS-HEK293A cells.

Regulation of eIF4E levels due to inhibitory binding to hypo-phosphorylated 4E-binding proteins (4E-BPs) is an additional major and conserved mechanism to control initiation of translation under various cellular conditions (27). The mammalian target of rapamycin (mTOR) stimulates eIF4E-mediated initiation of translation by maintaining 4E-BPs in a hyper-phosphorylated and inactive state (28). Rapamycin, the specific inhibitor of mTOR, and its derivatives have anti-proliferative activities on tumour cells, and their application in cancer chemotherapy is currently investigated in several studies and clinical trials (9,29,30). Figure 2d shows that 1 μM rapamycin reduces the phosphorylation of 4E-BP1

Figure 2. TCRS monitors adjustments in translation initiation induced by drugs, serum or eIF activity. (**a**) Serum deprivation (0.1% FCS) causes a general attenuation of translation initiation with hypo-phosphorylation of 4E-BP1 (lower panel), and results in an increase in LP/SP from the TCRS construct (TCRS, upper panel). Expression of SP was not detected from a related construct lacking the *cis*-regulatory uORF (ΔuORF, middle panel). (**b**) 2-AP (5 mM) inhibits eIF2α-kinases including PKR, resulting in reduced PKR auto-phosphorylation (lower panel) and hypo-phosphorylation and activation of eIF2α function (lower panels). The increase in translation resulted in a decrease in LP/SP ratio (TCRS, upper panel). In a related construct devoid of the *cis*-regulatory uORF, SP-expression was not detectable (ΔuORF, middle panel). (**c**) Thapsigargin (400 nM) causes repression of translation (re-)initiation through hyper-phosphorylation of eIF2α (lower panel), resulting in an increase in LP/SP ratio (TCRS, upper panel). Expression of SP was not detected from a mutant construct deficient in the *cis*-regulatory uORF (ΔuORF, middle panel). (**d**) Rapamycin (1 μM) inhibits mTOR signalling and attenuates translation through the hypo-phosphorylation of 4E-BP1 (lower panel) and subsequent sequestering of eIF4E, which results in an increase in LP/SP ratio (TCRS, upper panel). From a mutant construct lacking the *cis*-regulatory uORF, SP-expression was not detected (ΔuORF, middle panel). (**e**) Expression of the dominant positive mutant eIF2αSA or over-expression of eIF4E increases translation (re-) initiation and results in enhanced expression of SP relative to LP (TCRS, upper left panel). Expression of SP was not detected from a related construct lacking the *cis*-regulatory uORF (ΔuORF, lower left panel). Control blots show over-expression of eIF2αSA (upper middle for TCRS blot, lower middle for ΔuORF blot) or eIF4E (upper right for TCRS blot, lower right for ΔuORF blot). Experiments were performed in HEK293A (a–d) and C33A (e) cells. Ratios of LP versus SP were measured by quantification of immuno-blotted TCRS-peptides using fluorescent dye-conjugated secondary anti-HA antibodies. Shown are the mean values of three experiments with standard deviations (±SD); differences between the ratios are significant ($P < 0.05$).

and that the inhibitory effect on translation initiation can be monitored by an increase in LP/SP ratio in TCRS-HEK293A cells, up to 2.5-fold. Removing the functional translation *cis*-regulatory uORF in TCRS abolishes the expression of SP in all abovementioned cases (Figure 2a–e, panels labelled with ΔuORF).

Taking the data together, changes in translational activity in vertebrate cells induced by drug-treatment are thoroughly reported by the TCRS.

Mutant eIF activities are detected by TCRS

Recently mutations in components of the translational machinery, or aberrant expression of translation factors have been associated with the aetiology of human diseases (1). To examine the performance of TCRS in detecting translational changes caused by aberrant eIF activities, we ectopically overexpressed eIF4E or a dominant positive mutant of the α-subunit of eIF2 (eIF2α SA) in C33A-TCRS cells by retroviral transfer of eIF-pBabe^{puro} constructs followed by selection for puromycin resistance. TCRS expression was analysed by quantification of immuno-blotted TCRS-peptides using fluorescent dye-conjugated secondary antibodies (see Materials and Methods). Enhanced translation initiation as a consequence of over-expression of eIF4E or a dominant positive mutant of the α-subunit of eIF2 (eIF2αSA) (31), could be monitored by a 2-fold decrease in LP/SP ratios (Figure 2e). Thus translation of the downstream SP-frame is enhanced when translation re-initiation is improved, which emphasizes the functionality of the system.

Performance of TCRS in an ELISA setting

To test the suitability of TCRS in an ELISA setting, extracts from C33A cell lines that contain TCRS or the control constructs, with deleted (Δ) uORF, expressing LP only and expressing SP only, were applied on HA-coated ELISA dishes. The HA-immobilized LP and SP peptides could be distinctively detected using anti-FLAG and anti-MYC antibodies, respectively (Figure 3a). Attenuation of translation initiation in TCRS-expressing HEK293A cells following treatment with 400 nM thapsigargin for 12 h could be detected as a 2-fold increase in LP/SP ratio measured as a decrease in MYC signal relative to FLAG signal (Figure 3b). Thus in concordance with western blot analysis, a shift in the LP/SP ratio can be followed using TCRS in an ELISA system.

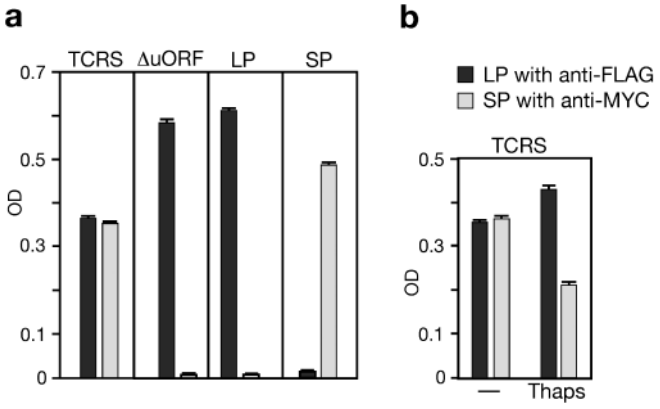

Figure 3. Application of TCRS in an ELISA system. (**a**) Extracts from C33A cell lines were applied containing TCRS or the control constructs, with deleted (Δ) uORF, expressing LP only, and expressing SP only. LP- and SP-expression levels were detected through their specific FLAG (black bars) and MYC (grey bars) immuno-tags following immobilization on HA-coated ELISA dishes. (**b**) Thapsigargin-induced down-regulation of translation initiation in HEK293A cells could be measured in an ELISA setting as an increase in LP/SP ratio through their specific FLAG (black bars) and MYC (grey bars) immuno-tags, respectively. The bars represent mean values of duplicate experiments with standard deviations.

DISCUSSION

Translation control has now been recognized as a crucial mechanism to regulate the expression of major regulatory genes involved in cellular functioning, cell cycle progression and differentiation. Hence it is not surprising that deregulation of translation is involved in the aetiology of human diseases. Although the importance of translational control has been firmly established, the knowledge about the function of *cis*-regulatory mRNA elements in conjunction with cellular signalling and its significance for novel therapeutic concepts is still limited. Nevertheless, some drugs are known to exert their effects by interfering with the translation machinery. Particularly, the drug rapamycin has attracted attention since it is highly active against a broad range of tumour cells (9,29).

Here we have presented an efficient method to identify and analyse changes in translational control in the vertebrate cell through a TCRS. This system is advantageous in terms of rapid and easy application and its potential adaptation for high-throughput screening. In this study the TCRS method is validated by the detection of changing translational conditions in the cell, induced by various stimuli: serum growth factors, drugs and mutant translation initiation factors (eIFs). We chose the C/EBPα-uORF as the element that controls translation of the dual reporter gene in TCRS. Previously we demonstrated that this *cis*-regulatory element may convert eIF activity into a translational outcome in its natural setting, and it functions as an independent *cis*-regulatory sequence when separated from its natural sequence context (11). In addition, the strict conservation of the C/EBPα-uORF sequence reflects its pivotal role in *c/ebpa* gene regulation. Moreover, uORFs with similar functions were identified in other genes.

Although the uORF mediated translational response is not understood in all detail, the eIF related effects on TCRS translation can be explained based on several studies on yeast (18) and vertebrate genes (11,12,19,32). Our study implies that probably any alteration in cellular signalling pathways that affects translation (re-) initiation efficiency through the delivery of Met-tRNA$_i^{Met}$ by the eIF2-GTP-Met-tRNA$_i^{Met}$ ternary complex, and/ or through cap-binding and scanning functions of eIF4E, are rapidly detected by TCRS with a maximum effect of the readout after 8 h of treatment.

In accordance with the uORF function to monitor cellular eIF activity and to regulate the alternative use of downstream initiation codons, enhanced eIF activity results in a relative increase in translation from the SP initiation site in the TCRS transcript, reflected in a lower LP/SP ratio. Conversely, low eIF activity results in a relative decrease in SP levels,

reflected in higher LP/SP ratios. TCRS was designed to exclusively identify changes in regulated translation: the TCRS readout reports differential translational activity through the expression levels of the two reporter peptides, LP and SP. Because both reporter peptides are synthesized from a single transcript it ensures segregation of changes in translation control from changes due to other regulatory events, such as transcription-rates or mRNA-stability.

TCRS has a modular design enabling the distinct elements to be exchanged independently from each other. Foremost, different (potential) regulatory elements can be cloned into both the 5′- and 3′-translation regulation cassettes for examination. In addition, a construct lacking a functional PPT leader sequence in LP and SP would allow discrimination between cytoplasmic-resident translation and translation-coupled secretion through the ER. The application of TCRS in ELISA techniques and further development into high-throughput protocols is tenable by its design with one immunogenic epitope in common for immobilization and a distinct second epitope for detection. With the adaptation of TCRS in transgenic animal models, *in vivo* studies in translation control could be performed.

Our work demonstrates that the TCRS is a propitious method to analyse changes in the translational control status in the vertebrate cell, and it may be used for the quantification of a translational response following extra-cellular stimulation. TCRS may become a highly valuable tool in the development of novel therapeutics to treat human diseases associated with aberrant translation.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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