

Use of mRNA- and protein-destabilizing elements to develop a highly responsive reporter system

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ABSTRACT

Reporter assays are widely used in applications that require measurement of changes in gene expression over time (e.g. drug screening). With standard reporter vectors, the measurable effect of a treatment or compound (altered reporter activity) is substantially diluted and delayed, compared with its true effect (altered transcriptional activity). This problem is caused by the relatively long half-lives of both the reporter protein and its mRNA. As a result, the activities of compounds, ligands or treatments that have a relatively minor effect, or a substantial but transient effect, often remain undetected. To circumvent this problem, we introduced modular protein- and mRNA-destabilizing elements into a range of commonly used reporters. Our data show that both elements are required for maximal responses to both increases and decreases in transcriptional activity. The double-destabilized reporter vectors showed markedly improved performance in drug screening, kinetic assays and dose-response titrations.

INTRODUCTION

Reporter proteins are characterized as having an easily measured activity (e.g. luminescence or fluorescence) that serves as a read-out in a variety of biological assays. In this paper the term 'reporter assay' relates to the use of reporter gene vectors, which link a regulatory element of interest to the coding sequence of a reporter protein. Within cells containing the reporter vector, any change in reporter levels (or activity) is

then indicative of changes in the activity of the gene regulatory element. In many applications, the regulatory element is the promoter/enhancer region from a gene of interest, such that reporter activity mimics transcriptional activity. Similarly, the linking of specific transcription factor binding sites to a reporter allows measurement of the activation state of a signal transduction pathway of interest. Alternatively, reporter genes linked to relevant *cis*-acting regulatory elements can be used as indicators of general cellular conditions, such as cell growth, hypoxia or cytotoxicity. For a review of the reporter gene technology, see (1).

Due to their simplicity and versatility, reporter assays have evolved to become an important research tool in the study of signal transduction pathways and gene expression. For many such applications, reporter assays are the most efficient and economical option and as such, they are widely used in cell-based models for drug discovery programs.

Standard reporter genes encode products with very slow clearance rates that cause a substantial delay and dilution of the measurable response to treatments that alter their expression. For example, chloramphenicol acetyl transferase (CAT), beta galactosidase (β gal) and enhanced green fluorescent protein (EGFP) have protein half-lives of ~50 (2), 30 (3) and 24 h (4,5), respectively. Therefore, even a rapid and complete block in transcription can only be detected at the level of reporter activity after a protracted period of time. Less apparent is the fact that long half-lives also diminish the ability to measure responses to treatments that increase transcription. This is because long half-life proteins are accumulated over a very long period (days), such that any increase in production that occurs during a few to several hours of treatment has proportionally little impact on the very high steady-state levels already present. Extending the period of treatment could introduce unwanted secondary effects, such as cytotoxicity.

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

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Furthermore, long incubation periods would provide little, if any, assistance to the detection of transient responses or responses to compounds that do not maintain their activity for long periods in cell culture.

To address this problem of discordance between transcriptional changes and the subsequent effects on reporter levels, protein-destabilizing elements (DE) have been used to reduce the half-life of various reporter proteins. Li *et al.* (6) described the use of a PEST sequence to destabilize the EGFP thereby reducing its half-life to ~2 h. Similarly, Leclerc *et al.* (7) used a PEST sequence to reduce the protein half-life of firefly luciferase from 3.68 to 0.84 h. Other methods for destabilizing proteins utilize the N-end rule (8) and ubiquitin fusion degradation pathways (9). Destabilizing the reporter protein, however, only partly addresses the problem, since reporter clearance rates are also dependent on the half-life of the reporter mRNA. As long as the pre-existing reporter mRNA remains intact, it continues to produce new reporter protein via translation.

One measure of the true delay between altered transcription and altered reporter protein levels would be the time taken for reporter protein levels (or activity) to reduce by 50% following a block in transcription. We term this the 'effective half-life' and describe a novel system for its measurement. The simplicity of this system makes it an attractive alternative to actinomycin D-chase studies for measuring mRNA stability effects. Using this and other systems, we analysed the benefits of including both mRNA- and protein-DE in a variety of reporter genes. These 'double-destabilized' (dd) reporter constructs provide substantial improvements in the responsiveness of commonly used reporter genes and are a vastly more effective tool for identifying or measuring changes in transcription or

upstream signalling pathways in a variety of applications, including high throughput drug screening (HTS).

METHODS

DNA constructs

The reporter constructs used here are summarized in Figure 1. All constructs were created within a pGL3-Basic (Promega) plasmid backbone, between the multiple cloning site and the XbaI site, which follows the stop codon and marks the beginning of the SV40 3'-untranslated region (3'-UTR) and poly(A) signal. RNA DE generated by PCR or hybridization of synthetic DNA oligonucleotides were cloned into the XbaI site to create a longer, chimeric 3'-UTR, consisting of the RNA DE [N4 (10), *myc* (11) or *fos* (12)] followed by the original SV40 3'-UTR.

Protein DE included the P1 and P2 PEST sequences, as contained in pd1EGFP-N1 and pTRE-d2EGFP (Clontech), respectively, and were positioned in frame with the reporter coding region at its C-terminus; i.e. connected to the last amino acid of the reporter via an engineered HindIII site. The ubiquitin sequence was generated by PCR of human genomic DNA and inserted upstream of and in frame with the reporter. Non-cleavable ubiquitin (Ub) fusion constructs were generated by changing the Gly residue at position 76 to Val using the QuickChange site-directed mutagenesis kit (Stratagene) (9). Cleavable ubiquitin (cUb) constructs included additional codons immediately following the ubiquitin to provide an optimized 'N-end rule' degron; i.e. to provide a reporter protein with an N-terminal Arg, and Lys at positions 3 and 15 following cleavage of the ubiquitin (13,14).

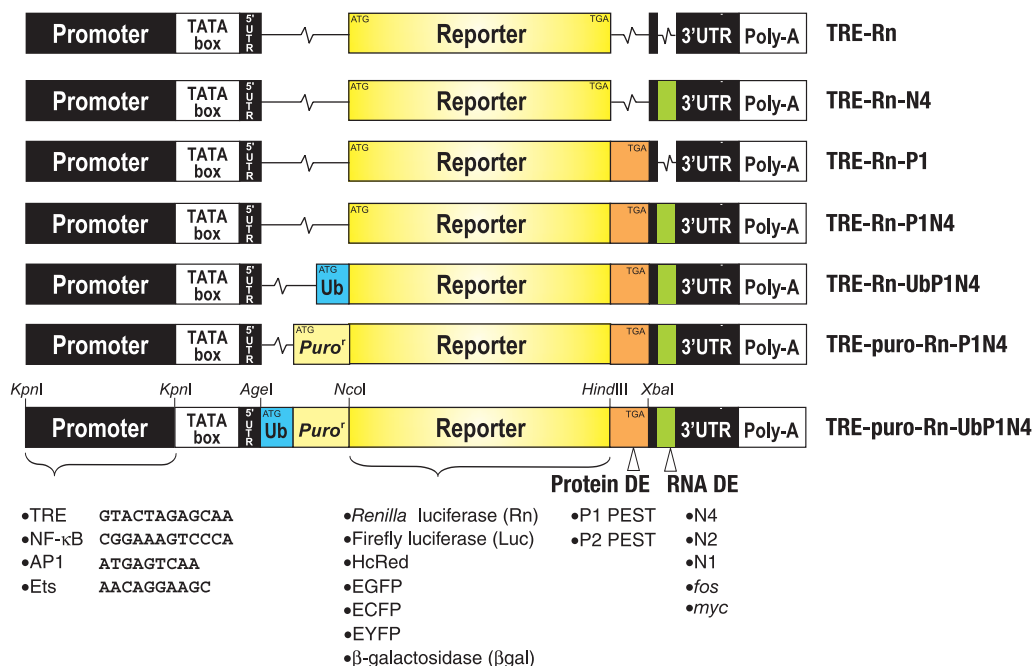


Figure 1. Schematic representation of the reporter constructs used in this study. Destabilized reporter constructs contained either the P1 or P2 PEST protein DE, fused to the 3' end of the reporter coding region; an RNA DE, consisting of 1–4 copies of the nonamer (N1, N2 or N4) or derived from the *c-myc* or *c-fos* 3'-UTR, inserted in the 3'-UTR; or both a protein DE and an RNA DE. To enable further destabilization, a cleavable ubiquitin (cUb) or mutant, non-cleavable ubiquitin (Ub), moiety was fused to the 5' end of the reporter-coding region. To assist in the selection of stable cell lines, some constructs contained the puromycin resistance gene (*Puro'*) fused in frame to the reporter gene. Examples of construct names are shown on the right side.

The tetracycline responsive element (TRE) promoter, including TATA box and 5'-UTR, were derived from pTRE-d2EGFP (Clontech) and cloned upstream of the Kozak-ATG translation start signal (15). In some constructs, the TRE enhancer elements were removed by digestion with KpnI and replaced with synthetic DNA oligonucleotides containing tandem copies of the binding sites for NF κ B (16,17) (8 copies), AP1 (18,19) (6 copies) or Ets (16,17) (6 copies).

To create vectors expressing different reporter activities, the coding regions of these reporters were generated by PCR of commercially available vectors and cloned into the appropriate sites of the above mentioned vectors (see Figure 1) using restriction enzyme sites engineered within the PCR primers. For example, reverse primers omitting the stop codon and including a HindIII site were used to create reporter-PEST fusion constructs, whereas alternative primers including the stop codon followed by an XbaI site allowed construction of reporters without the PEST sequence. Ligation of the same PCR products into vectors with and without an RNA DE provided constructs differing only in the presence or absence of that element.

Finally, puro-reporter fusions were generated by PCR of the puromycin resistance gene from pBabe-puro (20) and ligation immediately upstream of and in frame with the reporter coding region. The forward primer introduced an optimized Kozak sequence (15) to enhance translation and the reverse primer included a flexible glycine linker (21) to assist correct protein folding.

Cell culture and transfections

Tet-off HeLa (Clontech, USA), Tet-off CHO (Clontech, USA), MDA468 (ATCC, USA) and SKBR3 (ATCC, USA) cell lines were maintained in RPMI supplemented with 10% heat-inactivated fetal bovine serum (FBS; CSL, Australia), 100 U/ml penicillin and 100 μ g/ml streptomycin (Invitrogen, USA) at 37°C in a humidified atmosphere containing 5% CO₂.

Cells pre-grown to 70% confluency in 75 cm² flasks were transfected with a total of 5–6 μ g plasmid DNA using LipofectamineTM 2000 (Invitrogen, USA) according to the manufacturer's instructions. Six hours post-transfection, the cells were sub-cultured as required into 60 mm culture dishes or 96-well tissue culture plates. Transfected cells were incubated for 24 h post transfection (~70% confluence) before treatment with activators or inhibitors. To create stably expressing cells, the transfected cells were subjected to puromycin selection in media containing 1 μ g/ml puromycin (Sigma, USA) for at least 4 weeks.

Activators and inhibitors

Unless otherwise stated, the following final concentrations were used: 2 μ g/ml doxycycline (Dox; Clontech, USA), 20 ng/ml phorbol-12-myristate-13-acetate (PMA; Calbiochem-Novabiochem, USA), 2 nM epidermal growth factor (EGF; Sigma, USA), and 5 μ M each of AG555, AG825, AG1478, SB203580, bisindolylmaleimide I (BDM) and Ro-32-0432 (Calbiochem-Novabiochem, USA). Appropriate ethanol or dimethyl sulfoxide solutions prepared in culture media were used as carrier controls.

Luciferase assays

Stably or transiently transfected cells were sub-cultured onto black, clear-bottomed 96-well plates (Corning, USA). After treatment, cells were harvested by aspiration of the media followed by the addition of 25 μ l of Passive Lysis Buffer (Promega, USA) per well, supplemented with one freeze-thaw cycle. Luciferase assays were performed *in situ* using the Dual-Luciferase[®] Reporter Assay System (Promega) according to the manufacturer's instructions and measured on a Wallac VictorTM 1420 Multilabel luminometer (Perkin Elmer, USA).

Flow cytometry

Cells expressing fluorescent protein were harvested with trypsin and kept on ice in RPMI containing 50% FBS prior to the measurement of fluorescence on a Coulter Epic XL flow cytometer (Coulter, USA). Data were analysed using the FlowJo software package (Tree Star, USA) and values presented as the fluorescence of the percentile corresponding to the median of positive (fluorescent) cells at $t = 0$ h.

RESULTS

Construct design and nomenclature

Figure 1 shows a schematic representation of the reporter genes used in this study. The various different promoters, reporters and DE used are indicated at the bottom. Throughout this paper, construct names are designated as (promoter name)-(reporter name)-(DE) and example names, using the TRE promoter and *Renilla* luciferase (*Rn*) reporter are indicated to the right in Figure 1. Additional constructs contained promoters comprising the consensus binding sites for NF κ B, AP1 or Ets as indicated. Other reporters such as firefly luciferase (Luc) and various fluorescent proteins (HcRed, EGFP, EYFP and ECFP) were also used. Protein DE included P1, P2, cUb and Ub. RNA DE included 1, 2 or 4 copies of the nonamer TTATTTATT (10) (N1, N2 and N4, respectively) and AU-rich elements (ARE) from the *c-fos* (12) (*fos*) and *c-myc* (11) (*myc*) 3'-UTR (data not shown). Double-destabilized constructs are defined here as those including an RNA DE and at least one protein DE.

Improved responses to transcriptional changes

The TRE promoter can be repressed in Tet-off cells (Clontech) by the addition of tetracycline or Dox to the transfected cells. We used this system to determine the effect of various mRNA- and protein-DE on the effective half-life of various reporter constructs. The standard-*Rn* (TRE-*Rn*; Figure 2A) and standard Luc (TRE-Luc; Figure 2B) constructs showed effective half-lives of 14–17 h. Both mRNA and protein contributed to this delayed response since including either a potent PEST protein degradation signal (P1) or 4 copies of the RNA destabilizing nonamer (N4) reduced the effective half-life to 6–12 h. However, when both elements were included (P1N4), the effective half-lives were further reduced to ~3 h. This rapid rate of decay resulted in levels approaching zero at 12 h, when the standard reporters still displayed >50% of the initial activity ($t = 0$ h).

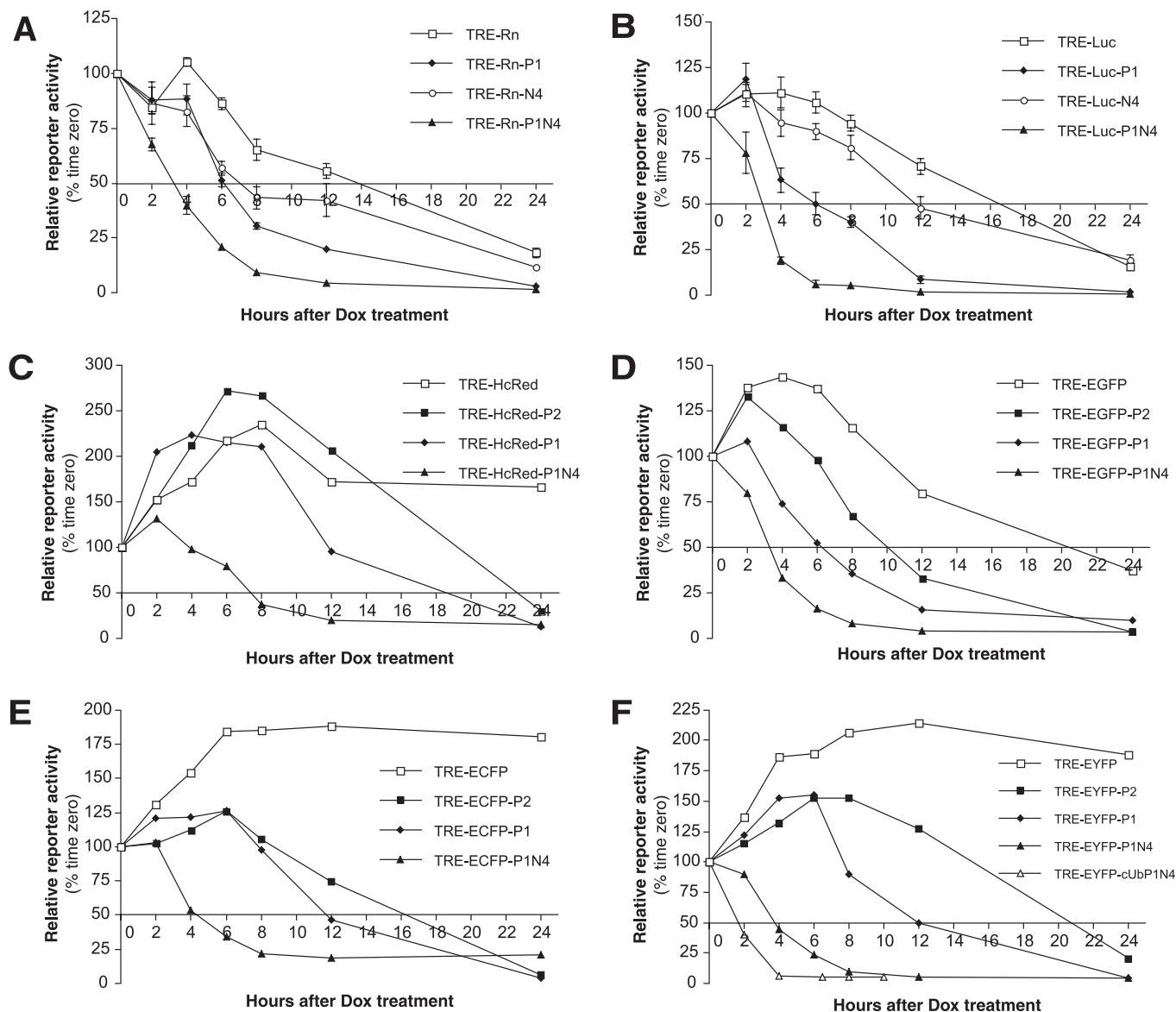


Figure 2. The effects of protein- and RNA-DE on effective reporter half-life. Tet-off HeLa cells were transiently transfected with TRE-driven reporter plasmids encoding *Rn* (A); Luc (B); HcRed (C); EGFP (D); ECFP (E); or EYFP (F). Reporter activity was measured following the inhibition of transcription by Dox treatment and expressed as a percentage of $t=0$ h values. Results are expressed as means \pm SEM ($n=4$) of a single experiment (A and B) or are representative of at least 3 similar experiments (C–F); and show standard reporter constructs (open square) as well as those containing: the P1 protein DE (closed diamond), P2 protein DE (closed square), N4 RNA DE (open circle) or both P1 protein DE and N4 RNA DE (closed triangle). In the case of EYFP (F), an additional construct that further contained an N-terminal cUb was included. The results show a substantial reduction in effective half-life when both protein- and RNA-DE are included in the reporter construct.

The combined effects of RNA DE (N4) and protein DE (P1) were also evident when applied to constructs for HcRed (Figure 2C), EGFP (Figure 2D), ECFP (Figure 2E) and EYFP (Figure 2F). Reporter constructs containing the P2 PEST sequence, equivalent to that of d2EGFP (Clontech), are included for comparison in Figure 2C–F. Also included in Figure 2F is a construct (TRE-EYFP-cUbP1N4) containing an ‘N-end rule degron’ in addition to the P1 and N4 DE. This was achieved by altering the N-terminal amino acid from Met to Arg, through the generation of a cUb reporter chimera. The further improvements in responsiveness seen with this construct demonstrate the potential benefits of including two separate protein DE, together with an RNA DE.

Taken together, these results demonstrate that the modular protein- and mRNA-DE work well in combination for a wide range of commonly used reporter systems. To confirm the effectiveness of this destabilization strategy in other cell types, we performed a similar comparison of standard- versus dd-*Rn* constructs in Tet-off Chinese hamster ovary cells and Tet-off Jurkat T cells (data not shown). The results showed comparable or enhanced destabilization compared to those achieved in HeLa cells. The strategy was also shown to be effective in MDA468 and SKBR3 cells (see Figure 5E and F and Figure 5G and H, respectively). Collectively, these data demonstrate the applicability of the reporter destabilization strategy to different cell types.

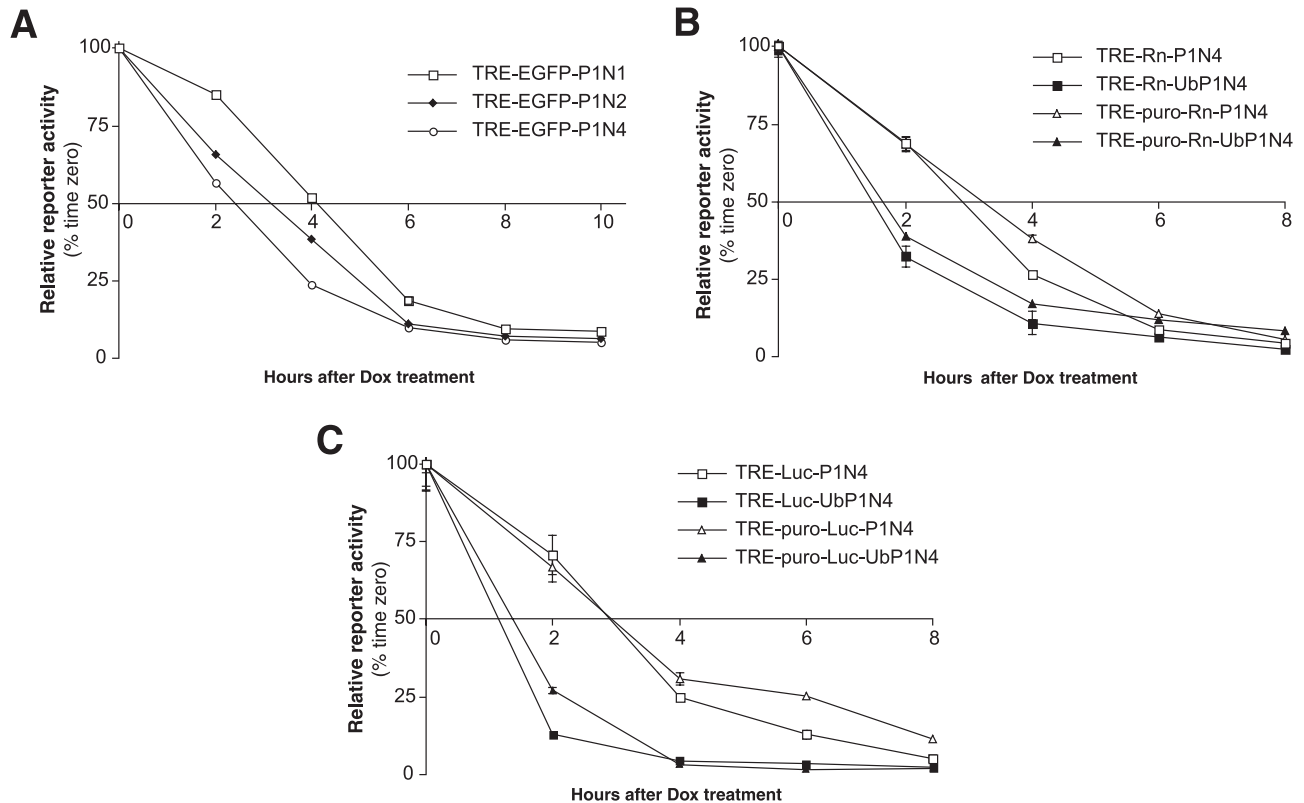


Figure 3. Effect of additional fusion domains. Dox time-course experiments were performed in Tet-off HeLa cells as described in Figure 2. (A) TRE-EGFP reporter constructs containing the P1 protein DE plus an RNA DE consisting of either 1 (open square), 2 (closed diamond) or 4 (open circle) copies of the nonamer, showing a progressive reduction in effective reporter half-life with increasing copy number. (B and C) The dd-*Rn* (B) and dd-Luc (C) constructs (open square) were fused in frame to either a puromycin-resistance gene (puro; open triangle) or a non-cleavable ubiquitin (Ub) N-terminal moiety (closed square) or both (closed triangle). The Ub moiety caused a marked reduction in effective half-life in all cases. Results are representative of at least three similar experiments (A) or are expressed as mean \pm SEM ($n = 3$) of a single experiment (B and C).

Optimizing the mRNA- and protein-fusion domains

Having developed an improved assay for quantifying the effect of mRNA- or protein-DE, we used this system to determine the optimal fusion domains and degradation signals for use in transcriptional reporter assays. The nonamer UUAU-UUAUU has been shown to be the minimal unit required for effective mRNA destabilization and Zubiaga *et al.* showed that two nonamers have a stronger effect than one (10). Our data confirm these findings and demonstrate further destabilization with 4 nonamers (Figure 3A). Increasing the nonamer copy number to 6 had little, if any, additional effect (data not shown).

Two well known and potent mRNA-degradation signals are the AREs present within the 3'-UTRs of *c-myc* (11) and *c-fos* (12) mRNAs. Four copies of the nonamer provided a level of mRNA destabilization equivalent to that provided by the *c-myc* or *c-fos* AREs (data not shown). However, we felt that utilizing the nonamers was preferable to using the much larger and more complex *c-myc* or *c-fos* ARE since the smaller size of the nonamer minimizes the chance of introducing undesirable RNA response elements.

With respect to protein destabilization, we demonstrated additive effects when two different DE were included in a single EYFP (Figure 2F) construct. Such a strategy also increases the responsiveness of *Rn* (Figure 3B) and Luc (Figure 3C) constructs, with effective half-lives of just 1.5 h

being achieved through the combination of P1 and Ub protein DE (in conjunction with the N4 RNA DE).

The data shown in Figures 2 and 3 are derived from transient transfection assays. However, many applications of reporter assays require the generation of cell lines stably expressing the reporter construct. Such studies typically utilize a separate antibiotic resistance gene, often located on a separate co-transfected plasmid. In order to negate the need for a separate resistance gene or plasmid, we developed constructs encoding fusion proteins of the puromycin-resistance gene and the destabilized versions of *Rn* (Figure 3B), Luc (Figure 3C) or a variety of fluorescent proteins (data not shown). All of these constructs encoded functional reporter activity, as shown here, and puromycin resistance (see legend Figure 4). The puromycin resistance domain showed some evidence of a weak stabilizing effect but did not substantially impair the rapid response (Figure 3B and C).

Improved responses in stably transfected cells

To determine the effect of our destabilization strategy in stably transfected cells, we co-transfected TRE-puro-*Rn*-P1N4 and TRE-Luc into Tet-off HeLa cells and selected them in puromycin. The reciprocal cells, i.e. co-transfected with TRE-puro-Luc-P1N4 and TRE-*Rn* were prepared and tested in parallel experiments. Pools of puromycin-resistant

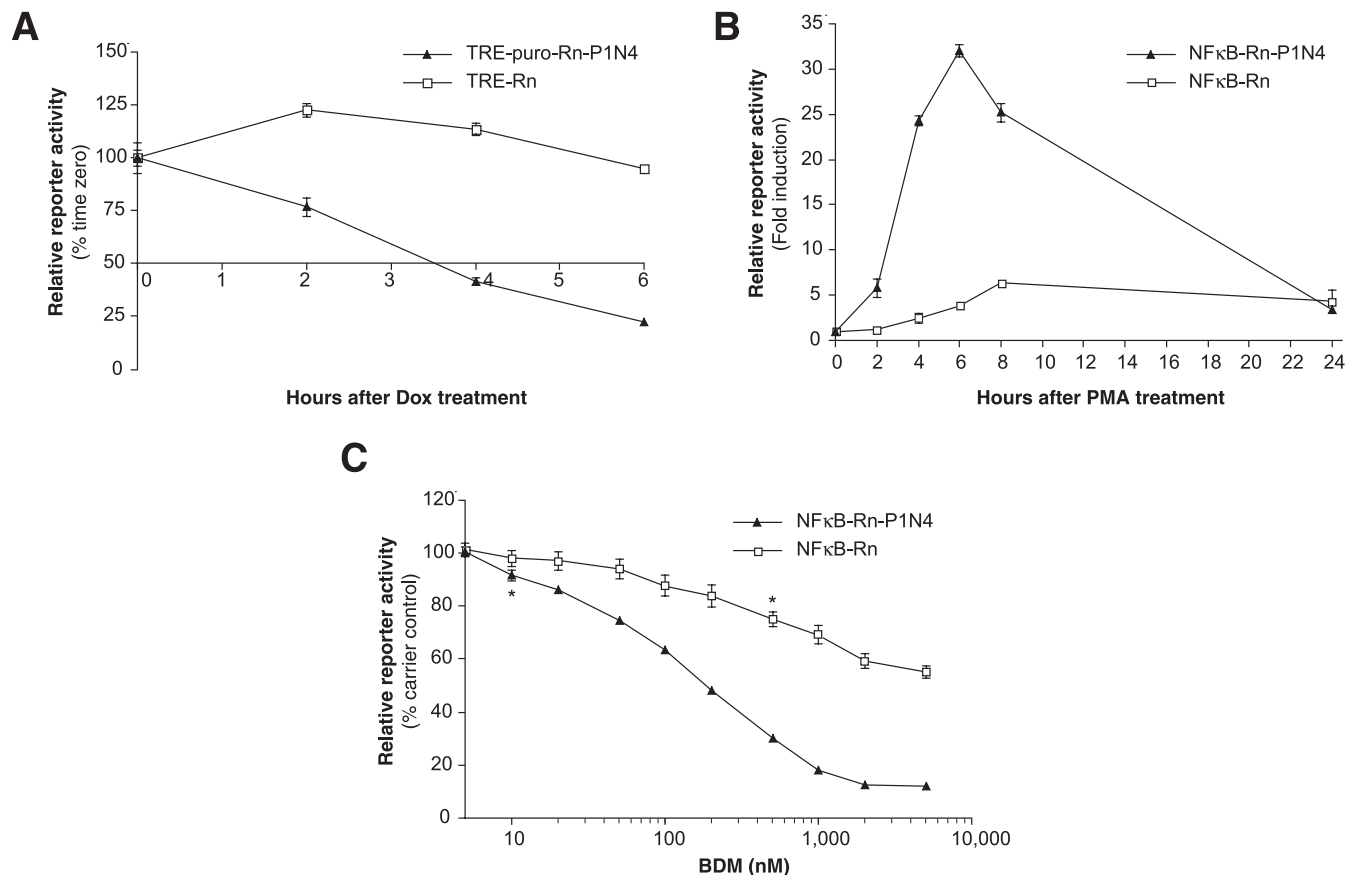


Figure 4. Testing the effectiveness of dd-reporter constructs in stably transfected cells. Experiments performed in 96-well plates using pools of puromycin-selected Tet-off HeLa cells. (A) Time course after addition of Dox to cells stably transfected with TRE-*Rn* or TRE-puro-*Rn*-P1N4. Activities are expressed as the percentage of time zero; mean \pm SEM ($n = 6$). The dd-construct (closed triangle) showed a rapid decline in reporter activity, whereas the standard construct (open square) showed little response. (B) Cell lines stably expressing NFκB-driven standard- (open square) or dd- (closed triangle) *Rn* constructs (puromycin-resistance provided by co-transfected TRE-puro-Luc-P1N4) were treated with PMA for the indicated times. Fold induction of *Rn* activities (+PMA/−PMA) are expressed as mean \pm SEM ($n = 3$) and show a substantially stronger and faster response with the dd-construct compared to its standard counterpart. (C) Concentration–response curves for BDM, a known PKC inhibitor. Cells stably expressing NFκB-driven standard- (open square) or dd- (closed triangle) *Rn* constructs were treated with PMA for 3 h before addition of BDM at the concentrations indicated for a further 3 h. *Rn* activities are expressed as the percentage of carrier control (0 nM BDM) values and represent the mean \pm SEM of combined data from four separate experiments ($n = 19$). Asterisk indicates the minimum detectable dose ($P < 0.001$; compared to 0 nM using Student's *t*-test).

cells were seeded onto 96-well plates and analysed in a dual-luciferase assay following Dox treatment. Figure 4A shows that the improved responsiveness (faster decay) of dd-*Rn* constructs, previously seen in transient transfections, was also evident in these stable transfections. Similar results were obtained for the Luc constructs (data not shown).

To establish a model of inducible reporter expression, we linked standard- and dd-luciferase constructs to a promoter consisting of eight NFκB elements in tandem repeats (see Figure 1). NFκB activity can be stimulated in cell culture by the addition of PMA (22). A separate pool of stably-expressing cells was created for each of the four NFκB-driven constructs (standard- and dd-; *Rn* and Luc) by co-transfecting each with a puromycin-resistance plasmid (see legend, Figure 4) and selection in puromycin. Compared with its standard counterpart, the dd-*Rn* construct provided both a faster response to PMA and a substantially higher maximal induction (32-fold versus 7-fold; Figure 4B). Similar results were obtained in a comparison of the two Luc constructs

(data not shown). These data show that the combined use of mRNA- and protein-DE provides a marked improvement in responses to increased as well as decreased transcription.

The induction of NFκB transcriptional activity following addition of PMA is mediated primarily via protein kinase C (PKC) (22). Therefore, we used the cells stably expressing NFκB-driven *Rn* to compare the responsiveness of standard-versus dd-reporter constructs to BDM, a known PKC inhibitor (23). Figure 4C shows the combined results from four separate dose–response studies. Compared to the standard-*Rn* construct, the dd-*Rn* construct showed a more pronounced response and a lower variance at all doses. The much steeper dose–response curve provided by the dd-construct is likely to provide a more accurate measure of parameters such as the dose required for 50% of maximal activity (EC50). Moreover, the minimal detectable dose of BDM with a $P < 0.001$ was 50-fold lower with the dd-reporter compared with the standard reporter. This increased sensitivity would provide a marked improvement in the ability to detect weakly active compounds.

Improved sensitivity in drug screening assays

To confirm the anticipated benefits of dd-reporter constructs in drug screening applications, we tested their ability to detect known PKC inhibitors [BDM (23) and Ro-32-0432 (24)] and epidermal growth factor receptor (EGFR) inhibitors [AG1478 (25)] and distinguish them from inhibitors of other protein kinases. Figure 5 shows the results of 4 separate experiments, including (as indicated in the Figure legend) both transient and stable transfections as well as co-transfections and separate transfections. Separate transfections enable the comparison of standard versus dd-versions of the same reporter type (e.g. *Rn*), albeit in separate cell lines, whereas co-transfections (standard Luc and dd-*Rn*) enable the comparison of two different reporters within the same cells in the same (dual-luciferase) assay, thus controlling for any sample to sample variations. The validity of the latter strategy is supported by our data showing that equivalent versions of *Rn* and Luc have similar response rates (e.g. compare Figure 2A and B).

Figure 5A shows that with the dd-construct both BDM and Ro-32-0432 can be readily identified as potent PKC inhibitors by virtue of their ability to block induction of NF κ B by PMA. While the standard reporter (Figure 5B) also revealed these active compounds, the degree of differentiation between compounds with and without an anti-PKC effect was considerably less than that seen with the dd-construct.

Figure 5C and D depict a similar assay, except using cells stably transfected with reporter constructs driven by a synthetic promoter consisting of six tandem repeats of the binding site for AP1 (see Figure 1), another PKC-activated transcription factor (18). Although the level of induction by PMA was considerably less than that seen with the NF κ B-driven constructs, the dd-construct still enabled identification of the active PKC inhibitors (Figure 5C). In contrast, the standard construct showed no detectable induction by PMA and as such, the PKC inhibitors could not be distinguished from the other compounds when using this construct (Figure 5D).

We next used a synthetic promoter consisting of six tandem repeats of the Ets binding site (16,17) in combination with EGF-treatment to identify EGFR inhibitors. Two different transiently transfected cell lines were tested; MDA468 cells (Figure 5E and F), which express very high levels of EGFR (26) and SKBR3 cells (Figure 5G and H), which express moderate levels of EGFR (but very high levels of its co-receptor, ErbB2) (26). Interestingly, it was SKBR3 cells that showed higher induction of Ets by EGF. The only biologically active EGFR inhibitor was AG1478 and this was clearly evident when using the dd-reporter construct on either MDA468 (Figure 5E) or SKBR3 (Figure 5G) cells. With the standard reporter construct, the effect of AG1478 was undetectable when using MDA468 (Figure 5F), but was evident when using the more responsive SKBR3 cells (Figure 5H). Note that AG555 and AG825 are also EGFR inhibitors but were developed in cell-free assays and later shown not to be active in cellular assays (27,28).

Taken together, the results shown in Figures 4C and 5 and other data presented here, indicate that the dd-reporter system provides increased sensitivity to transcriptional changes in all cases. In achieving this increased responsiveness, there is a reduction in the absolute levels of reporter protein present within a transfected cell. For example, we have observed a

5- to 10-fold reduction in dd-*Rn* luciferase activity compared with the standard *Rn* in co-transfection studies (data not shown). However, this was not problematic in the present study, as we continued to achieve typically >100-fold background levels, even in our miniaturized (96-well) assays. Notwithstanding this, ongoing optimization of coding sequences has enhanced absolute expression levels of dd-*Rn* by 16-fold (data not shown) without compromising the speed of the response.

DISCUSSION

The versatility and cost-effectiveness of reporter assays have led to their widespread application in numerous fields of biological and pharmaceutical research. Many of these applications require measurement of changes in gene expression over time or in response to a specific treatment. It is for these applications that there is a particular need for reporter genes with improved responsiveness to transcriptional changes.

Our data show that the speed and magnitude of response to a transcriptional change are determined by the half-lives of both the reporter protein and its mRNA. The incorporation of protein DE alone certainly improves responsiveness. However, as long as the reporter mRNA remains intact, it continues to produce new protein. In effect, much of the reporter activity measured in current drug screening assays actually derives from transcription that took place before drug addition.

We applied a reporter dd strategy, which combined the use of both RNA- and protein-DE for the first time. We show that these elements work cooperatively to provide improved measurable responses to both increases and decreases in transcription. A major implication of these data is that drugs or treatments shown in standard reporter assays to have little or no detectable effect (and thus disregarded), may often elicit an actual change in transcription of 10-fold or more. This is well illustrated by our Dox/TRE experiments, which showed no detectable effect of up to 8 h Dox treatment when using any of the standard reporter constructs, despite the rapid and potent effect of Dox in this system, as evident with our dd-reporter constructs. Furthermore, the dd-*Rn* vector was able to detect the anti-PKC effect of BDM at a 50-fold lower concentration than with a standard-*Rn* vector. The dd-reporter vectors also provided a much greater magnitude of response which, coupled with faster reporter clearance rates, provided for steeper concentration–response curves, thus facilitating greater accuracy in EC₅₀ determinations. Finally, these attributes also provided more accurate kinetic assays.

One of the strengths of our destabilization strategy is the ability to improve the performance of reporter assays without the need for additional reagents or equipment. Importantly, the DE described here are modular and proved effective with all reporters and cell lines tested. Therefore, the same DE can be incorporated into each of the reporter genes used within a multi-reporter system, including those intended for use as an internal control gene, which should be as similar in sequence as possible to the test reporter gene. Furthermore, the components of our destabilization strategy have also been shown to be effective in plants (29–32) and yeast (8,9,33,34).

We also describe a novel system for measuring the effect of *cis*-acting mRNA stability elements. The same system could

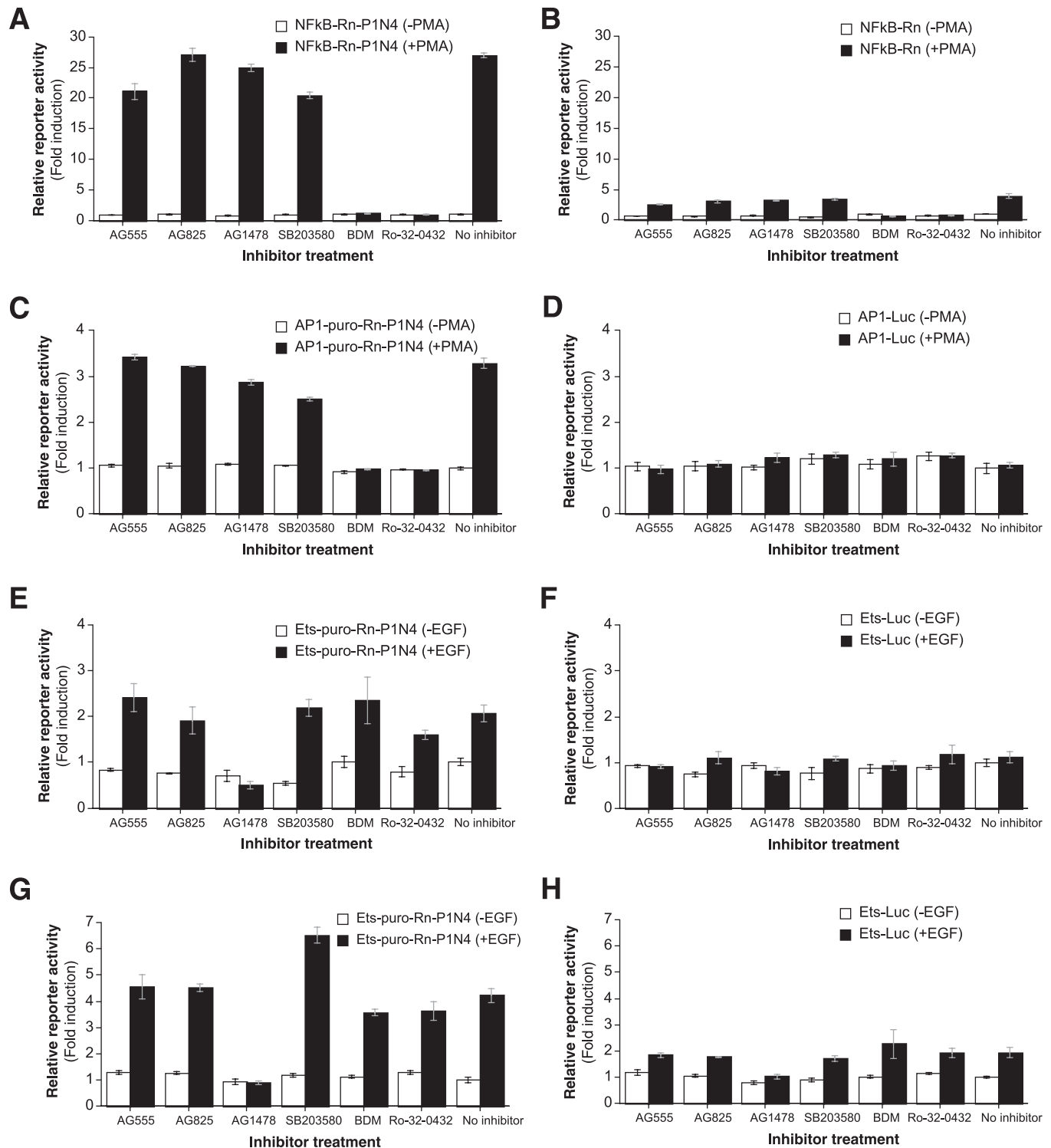


Figure 5. Comparing the effectiveness of standard- versus dd- reporter constructs in drug screening assays. (A and B) HeLa cell lines stably transfected with NFκB-driven dd- (A) or standard- (B) *Rn* constructs were seeded into 96-well plates and treated with 5 μM of the indicated kinase inhibitors or carrier control for 1 h before addition of PMA or carrier control for an additional 5 h. Reporter activities are normalized against mean carrier control values and presented as mean ± SEM ($n = 3$). The results demonstrate the greater efficiency of the dd-construct in detecting the two PKC inhibitors, BDM and Ro-32-0432. (C and D) As above, except using HeLa cells stably co-transfected with dd-puro-*Rn* and standard Luc constructs, each driven by the AP1 promoter. The AP1 promoter was less sensitive to PMA induction than the NFκB promoter, such that inhibition by BDM and Ro-32-0432 could only be detected with the dd-construct (C). (E and F) As above, except using transiently transfected MDA468 cells and the EGF-responsive Ets promoter. EGF replaced the PMA treatment. An effect specific to the known EGF inhibitor, AG1478, could be clearly identified with the dd-construct (E), but not with the standard construct (F). (G and H) An identical study to (E and F), except performed in SKBR3 cells, which showed a higher degree of EGF-responsiveness. Inhibition of this pathway AG1478 was evident with both constructs although the dd-reporter (G) showed an improved differentiation between this inhibitor and the others.

be used to study *trans*-acting factors such as RNA-binding proteins or to screen for drugs that influence these *cis*- or *trans*-acting factors. The study of mRNA stability typically requires highly time consuming and inaccurate assay procedures. Xu *et al.* (35) and Loffin *et al.* (36) summarize these problems and describe an improved system utilizing the TRE promoter. We have further simplified that system by using short half-life reporter proteins to allow measurement of reporter activity rather than quantifying the reporter mRNA directly. The new simplified system provides an attractive alternative to standard methods, particularly in higher throughput assays.

Reporter assays are not only used to measure the transcriptional activity of a specific gene but also to measure the activity of a single transcription factor or even an upstream pathway. For example, we used a synthetic promoter consisting of NF κ B-binding sites to identify and evaluate the efficacy of PKC inhibitors and Ets-binding sites to evaluate EGFR inhibitors. In this context, promoters from cell-cycle regulated genes or toxic-response genes can be used to monitor cell growth or toxicity, respectively. Furthermore, reporter assays are not limited to cell-based assays but can also be used in whole live animals. For example, a photon detection system is used to monitor luciferase activity in tumour xenografts (37).

In the context of drug screening, the dd system offers several advantages, including; (i) the detection of compounds or treatments that have only a minor to moderate effect on transcription (<10-fold); (ii) the detection of compounds having a substantial but transient effect (including chemically unstable compounds); (iii) the ability to utilize much shorter incubation periods, thereby significantly improving the economics of reporter assays in HTS applications; (iv) the ability to utilize cell line/promoter combinations that produce only relatively small transcriptional changes; (v) the use of the puro-reporter fusions described here will assist in the construction of stable cell lines for reporter analysis and remove the requirement for a separate promoter to drive the selectable marker gene, thus preventing potential errors resulting from promoter cross-talk. Finally, the lower variance and stronger effects obtained using our system should result in more drug leads and fewer false positives.

In summary, we present a strategy for improving the performance of reporter assays by including modular RNA- and protein-DE into the reporter gene. We have validated the system using a variety of different reporters and cell lines in both transient and stable transfection assays. Our data includes transcriptional induction and suppression as well as drug screening, kinetic assays and dose-response studies. In all cases, the dd-reporter vectors, which we have named 'RapidReporter', outperformed standard reporter genes and those incorporating only a protein DE. We propose that this novel system will provide considerable benefits to the pharmaceutical industry and any investigator seeking to detect transcriptional changes over time or after a specific treatment.

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REFERENCES

- Naylor,L.H. (1999) Reporter gene technology: the future looks bright. *Biochem. Pharmacol.*, **58**, 749–757.
- Thompson,J.F., Hayes,L.S. and Lloyd,D.B. (1991) Modulation of firefly luciferase stability and impact on studies of gene regulation. *Gene*, **103**, 171–177.
- Gonda,D.K., Bachmair,A., Wunning,I., Tobias,J.W., Lane,W.S. and Varshavsky,A. (1989) Universality and structure of the N-end rule. *J. Biol. Chem.*, **264**, 16700–16712.
- Chalfie,M., Tu,Y., Euskirchen,G., Ward,W.W. and Prasher,D.C. (1994) Green fluorescent protein as a marker for gene expression. *Science*, **263**, 802–805.
- Corish,P. and Tyler-Smith,C. (1999) Attenuation of green fluorescent protein half-life in mammalian cells. *Protein Eng.*, **12**, 1035–1040.
- Li,X., Zhao,X., Fang,Y., Jiang,X., Duong,T., Fan,C., Huang,C.C. and Kain,S.R. (1998) Generation of destabilized green fluorescent protein as a transcription reporter. *J. Biol. Chem.*, **273**, 34970–34975.
- Leclerc,G.M., Boockfor,F.R., Faught,W.J. and Frawley,L.S. (2000) Development of a destabilized firefly luciferase enzyme for measurement of gene expression. *Biotechniques*, **29**, 590–591, 594–596, 598 passim.
- Bachmair,A., Finley,D. and Varshavsky,A. (1986) *In vivo* half-life of a protein is a function of its amino-terminal residue. *Science*, **234**, 179–186.
- Johnson,E.S., Ma,P.C., Ota,I.M. and Varshavsky,A. (1995) A proteolytic pathway that recognizes ubiquitin as a degradation signal. *J. Biol. Chem.*, **270**, 17442–17456.
- Zubiaga,A.M., Belasco,J.G. and Greenberg,M.E. (1995) The nonamer UUAUUUAUU is the key AU-rich sequence motif that mediates mRNA degradation. *Mol. Cell. Biol.*, **15**, 2219–2230.
- Yeilding,N.M., Rehman,M.T. and Lee,W.M. (1996) Identification of sequences in *c-myc* mRNA that regulate its steady-state levels. *Mol. Cell. Biol.*, **16**, 3511–3522.
- Shyu,A.B., Greenberg,M.E. and Belasco,J.G. (1989) The *c-fos* transcript is targeted for rapid decay by two distinct mRNA degradation pathways. *Genes Dev.*, **3**, 60–72.
- Suzuki,T. and Varshavsky,A. (1999) Degradation signals in the lysine-asparagine sequence space. *EMBO J.*, **18**, 6017–6026.
- Dantuma,N.P., Lindsten,K., Glas,R., Jellne,M. and Masucci,M.G. (2000) Short-lived green fluorescent proteins for quantifying ubiquitin/proteasome-dependent proteolysis in living cells. *Nat. Biotechnol.*, **18**, 538–543.
- Kozak,M. (2000) Do the 5' untranslated domains of human cDNAs challenge the rules for initiation of translation (or is it vice versa)? *Genomics*, **70**, 396–406.
- Kuprash,D.V., Osipovich,O.A., Pokholok,D.K., Alimzhanov,M.B., Biragyn,A., Turetskaya,R.L. and Nedospasov,S.A. (1996) Functional analysis of the lymphotoxin-beta promoter. Sequence requirements for PMA activation. *J. Immunol.*, **156**, 2465–2472.
- Voon,D.C., Subrata,L.S., Karimi,M., Ulgiati,D. and Abraham,L.J. (2004) TNF and phorbol esters induce lymphotoxin-beta expression through distinct pathways involving Ets and NF-kappaB family members. *J. Immunol.*, **172**, 4332–4341.
- Lee,W., Mitchell,P. and Tjian,R. (1987) Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. *Cell*, **49**, 741–752.
- Sun,Y., Wenger,L., Brinckerhoff,C.E., Misra,R.R. and Cheung,H.S. (2002) Basic calcium phosphate crystals induce matrix metalloproteinase-1 through the Ras/mitogen-activated protein kinase/c-Fos/AP-1/metalloproteinase 1 pathway. Involvement of transcription factor binding sites AP-1 and PEA-3. *J. Biol. Chem.*, **277**, 1544–1552.
- Morgenstern,J.P. and Land,H. (1990) Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection

- markers and a complementary helper-free packaging cell line. *Nucleic Acids Res.*, **18**, 3587–3596.
21. Brinkmann, U., Gallo, M., Brinkmann, E., Kunwar, S. and Pastan, I. (1993) A recombinant immunotoxin that is active on prostate cancer cells and that is composed of the Fv region of monoclonal antibody PR1 and a truncated form of *Pseudomonas* exotoxin. *Proc. Natl Acad. Sci. USA*, **90**, 547–551.
 22. Baldwin, A.J. (1996) The NF-kappa B and I kappa B proteins: new discoveries and insights. *Ann. Rev. Immunol.*, **14**, 649–683.
 23. Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E., Loriolle, F. *et al.* (1991) The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. *J. Biol. Chem.*, **266**, 15771–15781.
 24. Wilkinson, S.E., Parker, P.J. and Nixon, J.S. (1993) Isoenzyme specificity of bisindolylmaleimides, selective inhibitors of protein kinase C. *Biochem. J.*, **294**, 335–337.
 25. Levitzki, A. and Gazit, A. (1995) Tyrosine kinase inhibition: an approach to drug development. *Science*, **267**, 1782–1788.
 26. Kraus, M.H., Popescu, N.C., Amsbaugh, S.C. and King, C.R. (1987) Overexpression of the EGF receptor-related proto-oncogene erbB-2 in human mammary tumor cell lines by different molecular mechanisms. *EMBO J.*, **6**, 605–610.
 27. Oshero, N., Gazit, A., Gilon, C. and Levitzki, A. (1993) Selective inhibition of the epidermal growth factor and HER2/neu receptors by tyrophostins. *J. Biol. Chem.*, **268**, 11134–11142.
 28. Kleinberger-Doron, N., Shelah, N., Capone, R., Gazit, A. and Levitzki, A. (1998) Inhibition of Cdk2 activation by selected tyrophostins causes cell cycle arrest at late G1 and S phase. *Exp. Cell Res.*, **241**, 340–351.
 29. Ohme-Takagi, M., Taylor, C.B., Newman, T.C. and Green, P.J. (1993) The effect of sequences with high AU content on mRNA stability in tobacco. *Proc. Natl Acad. Sci. USA*, **90**, 11811–11815.
 30. Lagrange, T., Hakimi, M.A., Pontier, D., Courtois, F., Alcaraz, J.P., Grunwald, D., Lam, E. and Lerbs-Mache, S. (2003) Transcription factor IIB (TFIIB)-related protein (pBrp), a plant-specific member of the TFIIB-related protein family. *Mol. Cell. Biol.*, **23**, 3274–3286.
 31. Bachmair, A., Becker, F. and Schell, J. (1993) Use of a reporter transgene to generate arabidopsis mutants in ubiquitin-dependent protein degradation. *Proc. Natl Acad. Sci. USA*, **90**, 418–421.
 32. Worley, C.K., Ling, R. and Callis, J. (1998) Engineering *in vivo* instability of firefly luciferase and *Escherichia coli* beta-glucuronidase in higher plants using recognition elements from the ubiquitin pathway. *Plant Mol. Biol.*, **37**, 337–347.
 33. Vasudevan, S. and Peltz, S.W. (2001) Regulated ARE-mediated mRNA decay in *Saccharomyces cerevisiae*. *Mol. Cell*, **7**, 1191–1200.
 34. Hoyt, M.A., Zhang, M. and Coffino, P. (2003) Ubiquitin-independent mechanisms of mouse ornithine decarboxylase degradation are conserved between mammalian and fungal cells. *J. Biol. Chem.*, **278**, 12135–12143.
 35. Xu, N., Loflin, P., Chen, C.Y. and Shyu, A.B. (1998) A broader role for AU-rich element-mediated mRNA turnover revealed by a new transcriptional pulse strategy. *Nucleic Acids Res.*, **26**, 558–565.
 36. Loflin, P.T., Chen, C.Y., Xu, N. and Shyu, A.B. (1999) Transcriptional pulsing approaches for analysis of mRNA turnover in mammalian cells. *Methods*, **17**, 11–20.
 37. Contag, C.H., Jenkins, D., Contag, P.R. and Negrin, R.S. (2000) Use of reporter genes for optical measurements of neoplastic disease *in vivo*. *Neoplasia*, **2**, 41–52.