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A Dual Luciferase System for Analysis of Post-Transcriptional Regulation of Gene Expression in *Leishmania*

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Abstract

Gene expression in kinetoplastid parasites is regulated via post-transcriptional mechanisms that modulate mRNA turnover, translation rate, and/or post-translational protein stability. To facilitate the analysis of post-transcriptional regulation, a dual luciferase system was developed in which firefly and *Renilla* luciferase reporters genetically fused to compatible drug resistance genes are integrated in place of one allele of the gene of interest and of an internal control gene, respectively, in a manner that preserves the cognate pre-mRNA processing signals. The sensitivity and reproducibility of the assay coupled with the ability to rapidly assemble reporter integration constructs render the dual luciferase system suitable for analysis of multiple candidates derived from global expression analysis platforms. To demonstrate the utility of the system, regulation of three genes in response to purine starvation was examined in *Leishmania donovani* promastigotes. This dual luciferase system should be directly applicable to the analysis of post-transcriptional regulation in other kinetoplastids.

Keywords

Leishmania; Firefly luciferase; *Renilla* luciferase; gene regulation; translational regulation; nutrient stress response

Transcription in kinetoplastid parasites is polycistronic, resulting in the production of long multigene pre-mRNAs that require coupled trans-splicing and polyadenylation reactions for processing into mature single-gene mRNAs (1). Consequently, regulation of gene expression in these organisms occurs primarily post-transcriptionally through mechanisms that control mRNA abundance, translation rate, and post-translational protein stability (2). Numerous studies have shown that control of mRNA and translational levels is mediated predominantly by elements encoded within the 5' and 3' untranslated regions (UTRs) of mRNAs (3–5), though regulatory elements have been also been found in coding sequences

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(CDS) (6,7). The application of systems-level approaches (e.g., RNA-seq and whole proteome profiling) to the study of global gene regulation in these parasites is becoming more common (8–14). While these approaches typically yield a multiplicity of candidates with altered mRNA or protein abundance in response to a particular growth condition or developmental program (9,10), understanding the contributions of translational and post-translational mechanisms to the regulation of individual candidates usually requires additional downstream analysis.

A variety of heterologous reporter systems (e.g., chloramphenicol acetyltransferase, β -galactosidase, β -glucuronidase, firefly (**Fluc**) and *Renilla* (**Rluc**) luciferases) have been employed to examine translational regulation and to identify *cis*-regulatory elements controlling mRNA abundance and translation in kinetoplastids (4,15–17). The studies presented herein describe the development of a Fluc/Rluc dual luciferase reporter system that allows post-transcriptional regulation to be readily assessed for multiple candidates derived from systems-level gene expression studies of kinetoplastid parasites. A key feature of this system is the utilization of versions of the *Fluc* and *Rluc* genes fused in-frame with each of five differing drug resistance genes (referred to as **Luc-DRG** fusions; see Supplementary Materials and Methods). This enables direct selection for replacement of the CDS from one allele of the gene of interest with a luciferase reporter in a manner that preserves the cognate pre-mRNA processing signals; hence, the contribution of the 5' and 3' UTRs to regulation should be reflected in luciferase expression from the *Luc-DRG* allele. Integration of the reporter gene should maintain physiological levels of reporter message, and circumvents potential reproducibility issues due to cell-to-cell variation in copy number and non-physiological expression in episome-based reporter systems. In our preferred configuration of this system, *Rluc* fused to a puromycin resistance gene (**Rluc-PAC**) is integrated in place of one allele of a control gene, the expression of which does not change under the conditions of the experiment (Fig1A). The resultant cell line serves as the recipient for subsequent transfections of *Fluc*-blastidicin resistance gene fusions (**Fluc-BSD**) targeting various genes of interest. The power of a dual luciferase system lies in the ability to sequentially measure Fluc and Rluc reporter luminescence from the same aliquot of a cell extract using a luminometer. In our system, the presence of an *Rluc* reporter integrated at a control locus in the same cell allows normalization of Fluc luminescence, adjusting for experimental variations such as cell number, pipetting errors, and efficiency of cell lysis.

The availability of a variety of *Luc-DRG* fusions provides the flexibility to find a compatible pair of *Fluc* and *Rluc* reporters for integration into cell lines that may already express one or more drug resistance markers. To enhance the efficiency with which the system can be implemented, the luciferase-drug resistance gene fusions were incorporated into donor vectors compatible with a previously described method from our laboratory for rapidly generating gene targeting constructs via multi-fragment ligation (18)(Fig1B). In this method, all of the targeting vector components (5' and 3' targeting sequences, a luciferase-drug resistance gene fusion, and a minimal plasmid backbone) are digested with SfiI, gel purified, and combined for directional ligation to form the completed targeting vector (Fig. 1C). Luciferase reporter targeting vectors can be assembled in three to four days and several

constructs can be processed in parallel, greatly facilitating the analysis of multiple candidates.

As a first step in validating the system, it was important to examine the sensitivity and linear range of detection for integrated *Fluc* and *Rluc* reporter constructs. An *L. donovani* promastigote line in which *Fluc-BSD* and *Rluc-PAC* reporters had replaced one allele each of the *LdNT2* and UMP synthase (*UMPS*) genes, respectively, was grown to mid logarithmic phase and 5-fold serial dilutions of the culture were independently processed for analysis via a dual luciferase assay. Both *Fluc* and *Rluc* luminescence could be reliably detected from lysates corresponding to as few as 2000 cells and the luminescence signal increased linearly with increasing cell number over the 625-fold range of the assay (Fig. 2A). In practice, the sensitivity of detection will depend on the level of expression conferred by the UTRs flanking the reporter as a consequence of the site of integration. Importantly, the *Fluc/Rluc* ratio was essentially equivalent at all cell numbers assayed, highlighting the robustness of normalization using *Rluc* as an internal control (Fig. 2B).

The primary motivation for developing the dual luciferase reporter system was to facilitate the assessment of post-transcriptional regulation in candidates derived from our RNA-seq and proteomic analyses of the adaptive response of *L. donovani* promastigotes to purine starvation (8). An important step in implementing this system was to identify and validate a gene unaffected by purine starvation to serve as a control locus for *Rluc-BSD* reporter integration. The *UMPS* gene (also known as *OMPDC-OPRT*) (19), which catalyzes the final two steps of *de novo* pyrimidine biosynthesis, was chosen to be the internal control because our previously published studies demonstrated that *UMPS* mRNA and protein abundance do not change following 24 or 48 h purine starvation (8,20). To validate *UMPS* as an internal control for the studies presented here, the effect of 48 h purine starvation on *UMPS* mRNA levels was examined via real-time quantitative reverse transcription PCR (qRT-PCR), using a second gene as a normalizer that was shown to be unaffected by purine starvation (8) (Table 1; Supplementary Materials and Methods). This confirmed that *UMPS* mRNA was essentially unchanged by purine starvation and, by extension, validates the use of *UMPS* mRNA as an internal normalizer for qRT-PCR analysis of gene regulation in response to purine scarcity (Table 1). Second, it was important to demonstrate that the *Fluc-BSD* and *Rluc-PAC* reporters did not themselves encode elements that conferred responsiveness to purine starvation at either the mRNA or protein level. To evaluate this possibility, the *Fluc-BSD* and *Rluc-PAC* reporters were independently integrated into the *UMPS* locus in separate cell lines and the expression of reporter mRNA and protein in response to 48 h purine starvation was monitored via qRT-PCR and western blot analysis, respectively (Table 1 and Fig. 2C). The mRNA abundance of *Fluc-BSD* and *Rluc-PAC* integrated at the *UMPS* locus did not change in comparison to the *UMPS* mRNA expressed from the remaining wild type *UMPS* allele (Table 1), intimating that neither reporter encodes *cis*-elements that affect mRNA abundance in response to purine stress, and that both reporters accurately reflect expression from the *UMPS* locus. Similarly, western blot analysis revealed that abundance of *Fluc-BSD* and *Rluc-PAC* protein was not altered by purine starvation (Figs 2C and 2D). Taken together, these data validate *Fluc-BSD*, *Rluc-PAC*, and the *UMPS* locus as

components of a dual luciferase system for studying the translational response to purine stress.

The regulation of several candidates identified through comprehensive global proteomics and RNA-seq analyses of purine starved *L. donovani* promastigotes has been assessed following 6 or 24 h of purine deprivation using the dual luciferase system (8). To further demonstrate the usefulness of the dual luciferase system, the regulation of three examples following 48 h purine starvation is presented here. These examples include two purine nucleobase transporters, LdNT3 and LdNT4, and a hypothetical protein of unknown function, LinJ.31.2490 (*HYP31* in Table 1). Independent cell lines were generated in which the *Fluc-BSD* reporter was integrated in place of one allele of each candidate gene using a cell line with *Rluc-PAC* inserted at the *UMPS* locus as the recipient. Following purine starvation for 48 h, luciferase activity was determined via dual luciferase assay and changes in mRNA abundance for both the endogenous allele and the *Fluc-BSD* replacement allele were assessed by qRT-PCR. Overall, the calculated fold-changes in *Fluc* luciferase activity showed low variability between the two biological replicates (Table 1), emphasizing the value of normalization with the internal *Rluc-PAC* control. Comparing the change in *Fluc* luciferase activity to the change in the corresponding *Fluc-BSD* mRNA at a given locus allows the relative contributions of mRNA abundance and translational mechanisms to the regulation of the respective gene to be determined. In the case of *Fluc-BSD* integrated at the *LdNT3* locus, luciferase activity increases 23-fold in response to purine starvation, while *Fluc-BSD* mRNA levels increase only 4-fold, intimating that the *LdNT3* 5' and/or 3' UTRs mediate a ~5-fold increase in translation as well as an increase in mRNA abundance (Table 1). In contrast, the increase in both luciferase activity and *Fluc-BSD* mRNA levels in response to purine deprivation is similar for *Fluc-BSD* integrated at the LinJ.31.2490 locus (*HYP31* in Table 1), hence the increase in luciferase activity in this case likely results solely from the change in mRNA abundance. The lack of a significant change in luciferase activity or mRNA abundance for *Fluc-BSD* integrated at the *LdNT4* locus (Table 1) is consistent with our published data (8,20), and indicates that the system reliably reflects the absence of a response to a given stimulus. Hence, the dual luciferase system demonstrates a broad dynamic range that will enable the detection of both small (< 4-fold) and large (>23-fold) regulatory responses.

In summary, the dual luciferase system presented here was shown to be sensitive and reproducible, with a broad dynamic range. The system provides a means to distinguish between regulation at the level of mRNA abundance and translation and, because the *Luc-DRG* reporter integrates in place of the coding sequence, makes it possible to determine if the UTRs of a candidate gene are sufficient to confer regulation. Once *Rluc* expressing control cell lines are generated, the system is rapid to implement. The simplicity and robustness of the system and relative ease of targeting construct generation combine to make the dual luciferase system a valuable tool for discerning mechanisms of post-transcriptional regulation for multiple candidates resulting from global expression analysis platforms (8). The dissection of UTRs to identify regulatory elements may prove difficult using the dual luciferase system in its current configuration. Because the point of crossover during recombination is unpredictable, the intended alterations to the UTRs (i.e., point mutations or

deletions) may not be included during integration of the luciferase reporter in place of the gene of interest. Modifications of the system designed to circumvent this limitation are currently being evaluated. Although tested in *L. donovani*, the general approach and components of this dual luciferase system should be directly applicable to a variety of kinetoplastid parasites.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Fluc	firefly luciferase
Fluc-BSD	firefly luciferase fusion to blasticidin S deaminase
Rluc	<i>Renilla</i> luciferase
Rluc-PAC	<i>Renilla</i> luciferase fusion to puromycin acetyltransferase
UMPS	uridine monophosphate synthase
UTR	untranslated region.

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Highlights

A dual luciferase system was created for analysis of gene expression in *Leishmania*.

A firefly luciferase gene is integrated in place of one allele of a gene of interest.

A *Renilla* luciferase gene is also integrated at a control locus for normalization.

The dual luciferase system is sensitive and reproducible, with a broad dynamic range.

The dual luciferase reporter system can be applied to multiple kinetoplastid species.

A.

B.

C.

Fig. 1. Configuration and key components of the dual luciferase system

(A) *Rluc*-puromycin resistance gene (*Rluc*-PAC) and *Fluc*-blasticidin resistance gene (*Fluc*-BSD) fusions flanked by appropriate targeting sequences (TS) are sequentially transfected to replace the CDS of one allele of either a control gene or a gene of interest, respectively, via homologous recombination. The cognate spliced-leader acceptor site (SLAS; represented by black ovals) and polyadenylation site (represented by the “A” adjacent to the SLAS) are preserved and drive expression of the drug resistance gene, obviating the need for exogenous pre-mRNA processing signals that could disrupt regulation of the reporter. (B)

The *Fluc* and *Rluc* genes were fused to genes encoding resistance to blasticidin, hygromycin, neomycin, phleomycin, and puromycin and inserted into pCRm to generate donor vectors. All of the luciferase-drug resistance gene (*Luc-DRG*) fusions retain robust luciferase activity and the ability to confer drug resistance upon chromosomal integration (data not shown). The *Luc-DRG* fusions are flanked by SfiI restriction sites designed to present non-identical 3'-overhangs upon SfiI digestion (designated SfiI-B and SfiI-C) that fit into our modular targeting vector construction strategy (18). (C) The targeting fragment, consisting of a *Luc-DRG* reporter flanked by the appropriate 5' and 3' TSs, can be released from the targeting vector by digestion with either PacI or PmeI. GenBank accession numbers for the pCRm-Luc-DRG donor vectors, as well as construction details for the *Luc-DRG* fusions, donor vectors, and targeting vectors are provided in the Supplementary Materials and Methods.

Fig. 2. Validation of the dual luciferase system

(A and B) An *L. donovani* promastigote line (*LdNT2/ldnt2::Fluc-BSD*, *UMPS/umps::Rluc-PAC*) was grown to mid-exponential phase. Starting at 6.25×10^7 cells/ mL, four 5-fold serial dilutions were made, in triplicate, and 1 mL of each was pelleted, lysed in 1 mL passive lysis buffer, and 20 μ L of the lysate was assayed with a Dual-Luciferase® Reporter Assay System (Promega) as described in (8). Cell number equivalents correspond to the number of cells present in 20 μ L of each dilution. The data represent the mean of the triplicates for each dilution. (A) Luminescence is linear with increasing cell number for both Fluc ($r^2=0.9999$) and Rluc ($r^2=1.000$). (B) The Fluc/Rluc ratio is independent of the number of cells assayed; data plotted are the same as shown in panel A. Parallel cultures of two *L. donovani* promastigote lines, *UMPS/umps::Fluc-BSD* (C) and *UMPS/umps::Rluc-PAC* (D), were cultured in the presence (+) or absence (-) of purine for 48 h and subjected to western

blot analysis using antibodies against Fluc, Rluc, UMPS, or the loading control arginase (ARG) as indicated (see Supplementary Materials and Methods for experimental details; parasite propagation and purine starvation conditions are described in (8)). The expression of arginase protein is unaffected by purine starvation (20).

Table 1

Changes in mRNA and luciferase activity following 48 h of purine starvation.

Allele	Fold-Change qRT-PCR	Fold-Change Luciferase
<i>UMPS</i>	1.12 ± 0.05	-
<i>umps::Fluc-BSD</i>	1.06 ± 0.01	-
<i>umps::Rluc-BSD</i>	1.29 ± 0.02	-
<i>HYP31</i>	3.59 ± 1.30	-
<i>hyp31::Fluc-BSD</i>	2.92 ± 0.12	3.82 ± 0.35
<i>LdNT3</i>	5.56 ± 1.39	-
<i>ldnt3::Fluc-BSD</i>	4.15 ± 0.05	22.54 ± 0.47
<i>LdNT4</i>	0.81 ± 0.15	-
<i>ldnt4::Fluc-BSD</i>	0.89 ± 0.09	1.32 ± 0.07

Wild type *L. donovani* promastigotes or cell lines with an *Fluc-BSD* or *Rluc-PAC* reporter inserted in place of one allele of the indicated gene were incubated in the presence or absence of exogenous purine for 48 h and the change in luciferase activity and/or mRNA abundance between starved and non-starved cultures was measured via dual luciferase assay or qRT-PCR as described (8). RNA isolation for qRT-PCR analysis and luciferase assays were performed in parallel on aliquots from the same culture. For the cell lines with integrated luciferase reporters, qRT-PCR was performed using primers specific for *Fluc*, *Rluc*, or the indicated wild type gene, and the fold-changes in response to purine starvation were determined via the 2^{-CT} method using *UMPS* mRNA as the normalizer as described by Martin and colleagues (8); the mean and standard deviation of two biological replicates are presented. The change in *UMPS* mRNA abundance from wild type cells was determined similarly, except that the fold change in response to purine starvation was determined by normalizing to threonyl-tRNA synthetase (XM_003864679) mRNA, which is unchanged following 24 h purine starvation according to RNA-seq analysis, and has been shown by proteomic analysis to be unaffected at the protein level after 24 or 48 h purine starvation (8). Cell lines with *Fluc-BSD* insertions in the indicated genes also contained *Rluc-PAC* inserted at the *UMPS* locus as a normalization control. Dual luciferase assays were performed to derive Fluc and Rluc relative light unit (RLU) values from the same cell extract. To calculate the fold-change in Fluc reporter expression due to purine starvation, the Fluc RLU value was divided by the Rluc RLU value to give a normalized value, then the normalized value from purine starved condition was divided by the normalized value from the non-starved condition to give the fold-change. The standard deviation is provided for the mean of two biological replicates. HYP31 (LinJ.31.2490, or LDBPK_312490) is a hypothetical protein of unknown function encoded on chromosome 31, which was shown via RNA-seq analysis to be upregulated following 24 h purine starvation (8). Primers and conditions for qRT-PCR, as well as details on the construction and integration of the luciferase targeting vectors are provided in (8); primers for *HYP31* targeting vector generation are included in the Supplementary Materials and Methods.