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Integrating Ribosomal Promoter Vectors that Offer a Choice of Constitutive Expression Profiles in *Leishmania donovani*

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Abstract

We have designed a novel series of integrating ribosomal RNA promoter vectors with five incrementally different constitutive expression profiles, covering a 250-fold range. Differential expression was achieved by placing different combinations of synthetic or leishmanial DNA sequences upstream and downstream of the transgene coding sequence in order to modulate pre-mRNA processing efficiency and mRNA stability, respectively. All of the vectors have extensive multiple cloning sites, and versions are available for producing N- or C- terminal GFP fusions at each of the possible relative expression levels. In addition, the modular configuration of the vectors allows drug resistance cassettes and other components to be readily exchanged. *In toto*, these vectors should be useful additions to the toolkit available for molecular and genetic studies of *Leishmania donovani*.

Abstract

Graphical Abstract

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Summary: Novel integrating ribosomal promoter vectors with incrementally different constitutive expression profiles are available for *Leishmania donovani*. Fluorescence intensity distributions from promastigotes expressing GFP from the six vectors are shown.

Keywords

Leishmania donovani; expression vector; ribosomal RNA promoter; green fluorescent protein

Integration of expression vectors into the ribosomal RNA (rRNA) locus provides uniform transgene expression that is stable in the absence of drug selection, thus avoiding the primary deficiencies of episomal vectors in *Leishmania* [1-3]. Current configurations of vectors for integrating into the rRNA array in *Leishmania* species express at very high levels, which is a consequence of strong RNA polymerase I transcription combined with pre-mRNA processing signals and mRNA stability elements from abundantly expressed endogenous genes [1-3]. While this is desirable for recombinant protein production [3] or monitoring parasite infectivity in macrophages [2] and live animals [4,5], many experimental paradigms (e.g., protein localization studies, complementation of gene knockouts) would benefit from lower expression levels. The overall goal of this work was to generate a collection of integrating rRNA promoter vectors (pRP vectors) with a range of constitutive transgene expression options.

The general configurations of the pRP vectors and the rRNA array are depicted in Fig. 1. The pRP vectors contain a 550 bp fragment encoding the *L. donovani* rRNA promoter and ~250 bp of adjacent downstream sequence [6], which confers robust RNA polymerase I transcription and provides a targeting sequence for integration into the rRNA array via homologous recombination. To provide a second region of homology for recombination into the rRNA array, a 649 bp DNA fragment corresponding to the region between the end of the *L. donovani* 28S rRNA gene and the beginning of the 64 bp repeats found upstream of the

rRNA promoter was included (as this DNA sequence was previously proposed to function in transcription termination [7], it will be referred to as a “terminator”). For integration, the pRP vector can be linearized by cutting at one of the three unique restriction sites between the terminator and the rRNA promoter (Fig. 1A and 1B). The extensive multiple cloning site (MCS) includes a pair of *Sfi*I restriction sites to allow efficient directional cloning [8,9] (Supplementary Fig. S1). The neomycin drug resistance cassette (NEO) is flanked by *Sbf*I and *Asc*I restriction sites that are compatible with the drug cassette “donor vectors” from our previously described modular system for rapid assembly of gene targeting vectors [9], allowing ready exchange of drug resistance markers. Similarly, the 5′- and 3′-untranslated regions (UTRs) are flanked by unique restriction sites to facilitate their substitution. As described below, the 5′- and 3′-UTRs differ between vectors (Table 1). To allow N- and C-terminal tagging of transgenes, versions of each pRP vector were generated that include an enhanced GFP gene encoded with a leishmanial codon bias (Table S1 and Supplementary Materials and Methods).

In kinetoplasts, genes are expressed as long multi-gene transcripts that require signals within the 5′- and 3′-UTRs of each gene for processing into mature single-gene mRNAs. The efficiency of pre-mRNA processing (i.e., *trans*-splicing and polyadenylation) and regulatory elements in the UTRs play pivotal roles in establishing steady state mRNA abundance and overall expression levels [10]. To achieve the goal of producing vectors with a range of expression profiles, pRP vectors were created that encoded various combinations of 5′- and 3′-UTRs chosen for their potential to enhance or diminish expression (Table 1). For vectors capable of low-level expression, a truncated version of the modified *TbPARP* 5′-UTR and *trans*-splicing signal described by Siegel and colleagues [11] was chosen for its potential to attenuate the robust RNA polymerase I expression driven from the rRNA promoter (see Fig. S2 for sequence). The wild type *T. brucei PARP* gene 5′-UTR was shown to function poorly in *L. major* when driven by weaker RNA polymerase II transcription [12]. The two nucleotides preceding the splice acceptor site of the *TbPARP* 5′-UTR were mutated to the consensus nucleotides preceding leishmanial splice acceptor sites (referred to as *LdPARP* 5′-UTR) [13], which was anticipated to improve *trans*-splicing efficiency and provide an intermediate expression profile (see Fig. S2 for details). The *Crithidia fasciculata* phosphoglycerate kinase B intergenic region (*PGKB* 5′-UTR) drives high-level expression in the pNUS episomal vectors (P. Yates, unpublished observations; [14]) and was therefore included to enable high expression from the pRP vector series. Two different 3′-UTRs were utilized: 1) The *L. major* α tubulin gene (*LmTub*) intergenic region, which was expected to give rise to stable mRNAs; and 2) *LmSIDER1270*, which is a DNA fragment encoding both a polyadenylation signal and a member of the *L. major* *SIDER2* retroposon family shown by Bringaud and colleagues [15] to act as an mRNA instability element. These 3′-UTRs were combined with each of the above 5′-UTRs to give six different pRP vectors (Table 1). To enable quantitative comparison of the expression levels, the firefly luciferase (*Fluc*) reporter gene was inserted into the *Sfi*I sites of each version of pRP.

The *Fluc*-encoding pRP vectors were each linearized and transfected into a previously generated *L. donovani* promastigote line expressing *Renilla* luciferase (Rluc) from the *LdNT2* locus that serves as an internal control for normalizing *Fluc* expression between

transfectant lines as monitored via dual luciferase assays [16]. Bulk (non-clonal) transfectant cultures were maintained as promastigotes or converted to axenic amastigotes prior to analysis; bulk cultures, rather than clones, were analyzed because it was expected that this would best represent the average performance of a given vector, and account for variation between clones due to integration position within the rRNA array or differences in the number of integrated vectors (addressed below).

The relative Fluc expression from logarithmic phase cultures of the various pRP vector transfectant cultures was determined via dual luciferase assays and, in general, the performance of individual vectors reflected the predicted properties of the 5'- and 3'-UTRs chosen to modulate their expression (Table 1). Changing the two nucleotides upstream of the *TbPARP* 5'-UTR to the leishmanial consensus (*LdPARP* 5'-UTR) increased Fluc expression by about five- to six-fold in promastigotes and three to six-fold in axenic amastigotes, presumably by increasing the efficiency of *trans*-splicing (Table 1; compare pRP-L_B to pRP-M and pRP-VL to pRP-L_A). In both lifecycle stages, the *PGKB* 5'-UTR increased expression of Fluc 40- to 60-fold over *TbPARP* (Table 1; compare pRP-L_B to pRP-VH and pRP-VL to pRP-H), emphasizing the weakness of the *TbPARP* 5'-UTR as a *trans*-splicing signal in *Leishmania* [12]. Varying the 3'-UTR allowed further modulation of expression in both promastigotes and amastigotes, with *LmSIDER1270* reducing Fluc expression three- to six-fold compared to the *LmTub* intergenic region, a degree of diminution consistent with the five-fold reduction described by Bringaud and colleagues [15] (Table 1; compare pRP-VL to pRP-L_A, pRP-L_B to pRP-M, and pRP-H to pRP-VH). Two combinations of 5'- and 3'-UTRs resulted in essentially identical expression, giving rise to the low expressing vectors, pRP-L_A and pRP-L_B. Overall, the strategy of modulating constitutive transgene expression by employing combinations of 5'- and 3'-UTRs with differing properties was effective in generating a series of vectors with a wide range of defined relative expression levels. While it was expected that the different expression profiles among the vectors reflected differences in steady-state mRNA abundance mediated by the UTRs, it should be noted that relative mRNA abundances were not directly examined.

Previous studies in both *Leishmania* and *T. brucei* demonstrated that expression of constructs integrated into the rRNA locus showed clone-to-clone variability, presumably relating to the position of integration within the rRNA array [2,17]. To test for variation in pRP vector expression, normalized Fluc activity was compared between ten independently-derived pRP-M-*Fluc* clones (Fig. 2). Fluc activity was similar for clones 1 through 7, while the activity for clones 8 through 10 was three to four-fold lower than the others. This suggested that most rRNA loci are similarly permissive, resulting in essentially equivalent expression from the pRP vectors. An alternative possibility, which was not tested, is that differential expression represents differences in the number of vectors integrated into the rRNA array. The potential integration of pRP vectors into less permissive rRNA loci or at different copy numbers will introduce some degree of cell-to-cell variability in bulk transfectants, but expands the potential range of expression between individual clones in a manner that will facilitate the identification of clones with ideal expression profiles for a

given experiment. It may be possible to circumvent this clonal variability by marking a single rRNA locus for vector integration as has been done in *T. brucei* [17].

One limitation of the pRP vectors is that they are specific for *L. donovani* or closely related species (*L. infantum* and *L. chagasi*) due to the surprisingly divergent nature of the rRNA promoter and intergenic regions among *Leishmania* species [7], which renders integration into the rRNA array of other *Leishmania* species via homologous recombination unlikely (as was the case in *L. major* and *L. mexicana*; P. Yates, data not shown). Other vectors that utilize transcription from the rRNA promoter [1,3] have a broad host range because they were designed to integrate into the highly conserved *SSU* rRNA gene. Replacement of the rRNA promoter and terminator sequences in the pRP vectors with *SSU* rRNA sequences would enable their use in other *Leishmania* species. In this regard, it is expected that the strategy of modulating expression via combinations of 5'- and 3'-UTR sequences will be generally applicable, though the relative expression profiles from the various combinations of UTRs may vary between species.

The configuration of the pRP vectors reflects the original intent to develop vectors that could either integrate into the *L. donovani* rRNA array or be utilized as stable low copy episomes. Boucher and colleagues [6] described a 550 bp fragment encoding the *L. donovani* rRNA promoter and ~250 bp of adjacent downstream sequence (containing a putative replication origin and associated segregation functions) that facilitated low copy number episomal replication and stability in the absence of drug selection. This 550 bp fragment was included in the pRP vectors but failed to allow low copy episomal replication in this context. Instead, transfection of circular pRP vectors variably and unpredictably resulted in either high copy number episomal replication or, as has been seen for ribosomal promoter vectors in *T. cruzi* [18], integration into the rRNA array (P. Yates – data not shown). Hence, optimal and consistent performance requires linearization of the vectors prior to transfection.

In summary, the novel pRP vector series described herein exhibits a number of useful features, including: 1) stable and uniform expression due to integration into the rRNA array; 2) the choice of vectors with five different relative expression profiles; 3) an extensive multiple cloning site that includes a pair of *Sfi*I restriction sites for highly efficient directional transgene insertion; 4) vectors for generating N- and C-terminal fusions to a *GFP* gene encoded with a leishmanial codon bias; and 5) a modular design that simplifies the exchange of drug resistance cassettes and mRNA regulatory elements. Bulk cultures of pRP transfectants, in which the majority of cells will express the encoded transgene at a similar level, will suffice for most studies. Alternatively, clonal cell lines with uniform expression can be established and screened for the most suitable expression profile. The pRP vectors, as well as donor vectors for exchanging drug resistance markers [9], can be obtained directly from the authors upon request.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

UTR	untranslated region
rRNA	ribosomal RNA
Fluc	firefly luciferase

Research highlights

- Six ribosomal RNA promoter (pRP) vectors were made for integration into the rRRNA array.
- The pRP vectors provide five incrementally different expression profiles covering a 250-fold range.
- Different expression profiles were achieved by varying 5'- and 3'-UTR sequences.
- pRP vectors are available for making N- and C-terminal transgene fusions to GFP.
- The pRP vectors are specific for *Leishmania donovani* and closely related species.

Fig. 1.

(A) Schematic of a generic pRP vector. Individual components are described in the text; Term = putative RNA polymerase I terminator sequence; rRNA pro = *Leishmania donovani* rRNA promoter; MCS = multiple cloning site. The black arrow indicates the direction of RNA polymerase I transcription from the rRNA promoter. White arrows indicate the orientation of protein coding genes. The thickened line flanking the ampicillin resistance gene (AMP^R) denotes the *E. coli* plasmid backbone. The restriction sites listed are unique in the vector and allow the individual components to be easily exchanged for those with alternative properties. (B) Integration of a linearized pRP vector into the rRNA array. A portion of the rRNA array is depicted: white box with black arrow = rRNA promoter; rRNA = the rRNA genes; vertical lines represent the 64 bp repeats; GOI = gene of interest; 5' = 5'-UTR; 3' = 3'-UTR. Recombination into the rRNA array (indicated by the crossed lines) occurs via homology between the rRNA promoter and the terminator. See Supplementary Materials and Methods for vector construction details and Table S1 for GenBank accession numbers.

Fig. 2.

Clonal variation in expression from pRP vectors. Normalized luciferase values were determined for ten independently derived *L. donovani* promastigote clones transfected with pRP-M-*Fluc*, as described in Table 1 and the Supplementary Materials and Methods. The Fluc/Rluc ratio represents the mean and standard deviation from triplicate luciferase measurements of each culture.

Table 1

Relative firefly luciferase (Fluc) expression from various pRP vectors in *L. donovani* promastigotes and amastigotes. pRP vectors with the indicated combinations of 5'- and 3'-UTRs and expressing Fluc were stably transfected into a cell line containing a *Renilla* luciferase (*Rluc*) gene integrated into the *LdNT2* locus (16), which served as an internal normalization control. The promastigote and amastigote cultures were derived from the same non-clonal transfectant cultures, and luciferase assays were performed in triplicate on parallel cultures in mid-logarithmic growth to avoid variation in expression due to differences in culture growth phase. Fluc/Rluc ratios represent the mean and standard deviation from two independent experiments for each pRP transfectant culture. Relative expression values were derived by dividing the Fluc/Rluc ratio for each vector by that of the lowest expressing vector (pRP-VL). The vectors were given the following qualitative designations to reflect their relative expression levels: VL = very low; L = low; M = medium; H = high; VH = very high. For additional experimental details, see the Supplementary Materials and Methods.

Vector	5'-UTR	3'-UTR	Promastigote		Amastigote	
			Fluc/Rluc	Relative	Fluc/Rluc	Relative
pRP-VL	<i>TbFARP</i>	<i>LmSIDER1270</i>	0.22 ± 0.03	1.00	1.69 ± 0.07	1.00
pRP-L _A	<i>TbFARP</i>	<i>LmTub</i>	1.29 ± 0.09	5.86	9.30 ± 0.40	5.50
pRP-L _B	<i>LdFARP</i>	<i>LmSIDER1270</i>	1.34 ± 0.04	6.07	9.93 ± 0.06	5.88
pRP-M	<i>LdFARP</i>	<i>LmTub</i>	6.86 ± 0.76	31.16	33.00 ± 6.61	19.53
pRP-H	<i>PGKB</i>	<i>LmSIDER1270</i>	10.94 ± 0.91	49.73	107.74 ± 0.35	63.75
pRP-VH	<i>PGKB</i>	<i>LmTub</i>	54.51 ± 4.64	247.77	359.96 ± 2.18	212.99