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Short communication

Tests of heterologous promoters and intergenic regions in *Leishmania major*

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Trypanosoma brucei and *Leishmania major* now have many powerful genetic tools available, and offer different advantages for the study of gene expression. For example, functional genetic rescue is simplest in *L. major* and inducible gene expression is best established for *T. brucei* [1–5]. Although these two genera are evolutionarily distantly related [6,7], they share many basic features of gene expression and cell biology including an abundance of GPI-anchored surface molecules, RNA editing, polycistronic transcription, and *trans*-splicing [8–10]. For many studies, it would be convenient to test gene function directly in both species, without the need for transfer into species-specific vectors. Optimism that this would be possible has been prompted by reports showing that several signals involved in trypanosomatid gene expression (such as *trans*-splice acceptor sites) functioned in heterologous species, and that *Leishmania* splice acceptor requirements may be relatively relaxed [11–14]. To assess the generality of this, we tested the ability of RNA polymerase I promoters and RNA processing sequences present within the intergenic regions of *T. brucei* and *Crithidia fasciculata* to direct mRNA and protein synthesis in *L. major*.

A variety of constructs were tested, each containing the following elements: test promoter, intergenic region (containing a splice acceptor), reporter gene, and in most cases, a second intergenic region (also containing a splice acceptor).

Abbreviations: UTR, untranslated region.

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These were transfected into *L. major* promastigotes (Tables 1 and 2) or *T. brucei* procyclic cells (not shown), and expression of reporter genes was assayed after overnight cultivation [15–17]. All vectors additionally carried a phage T7 RNA polymerase promoter, allowing transfection into *L. major* expressing T7 RNA polymerase [18] to serve as a control for active promoter-driven transcription. Previously, we showed that the T7 promoter/RNA polymerase showed elevated expression in transient transfections [18], an effect evident in Tables 1 and 2.

In the first set of experiments involving transfection of *Leishmania* and expression of an *E. coli* β-galactosidase reporter gene, we compared the activity of the *T. brucei PARP* (Procyclic acidic repetitive protein or procyclin) promoter and splice acceptor with that of a *Leishmania* splice acceptor (Lm *DST*). The *DST* transcripts are located immediately 3′ of the *L. major DHFR-TS* gene [18–20]. The BGAOS construct bearing the Lm *DST* splice acceptor showed good activity in wild-type *Leishmania*, but insertion of the *T. brucei PARP* promoter resulted in little stimulation of β-galactosidase activity (Table 1, constructs BGAOS versus pXβGAL-PARP-P). If the Lm *DST* splice acceptor was replaced by the *PARP* splice acceptor, β-galactosidase expression was almost completely abolished (less 4-fold over background), even when transcription was driven by T7 polymerase (Table 1, construct PARP.P5′βGAL). These results suggested that neither the *PARP* promoter nor the *PARP* intergenic region (splice acceptor and 5′-UTR) functioned well in *L. major*.

The plasmid BGAOS yielded no detectable β-galactosidase in transient transfections of *T. brucei*. However, constructs bearing the *PARP* promoter showed comparable β-galactosidase expression, with both the *PARP* splice acceptor/5′-UTR (PARP.P5′GAL) or the *L. major*-derived *DST* intergenic region (pXβGAL PARP-P; not shown). This indicates that the *DST* intergenic region present in these vectors was compatible with high expression in trypanosomes.

Table 1
Transfection of *PARP* promoter constructs into *Leishmania major*

Constructs[a]	Promoter	5′ flanking splice acceptor	Transfected cell line[b]			
			Wild-type		+T7 RNA polymerase	
			Relative βGal[c]	% BGAOS	Relative βGal[c]	% BGAOS
673-73-βGAL	–	–	0.0091	0.7	0.031	0.1
PARP.P5′βGAL	*PARP*	Tb *PARP*	0.024	1.8	0.12	0.5
BGAOS	–	Lm *DST*	1.3	100	27	100
pXβGAL PARP-P	*PARP*	Lm *DST*	1.4	101	20	81

[a] Test plasmids contained a *lacZ* reporter gene, usually preceded by a splice acceptor (*L. major DST* [19,33] or *T. brucei PARP*), which in turn was preceded by the *T. brucei PARP* promoter as indicated. 673-73-BGAL contained the *lacZ* gene inserted in a pSP64 vector containing a modified polylinker sequence, and lacks any trypanosomatid sequences. PARP.P5′βGAL was created by inserting the *PARP* promoter and splice acceptor into the *Sma*I site located just 5′ of the *lacZ* gene of pXβGAL; a T7 promoter is located 3 kb upstream of *lacZ* present in pXβGAL. pXβGAL PARP-P was created by inserting the *Kpn*I-*Sma*I *PARP* promoter fragment of pHD1 [16] into the *Kpn*I-*Nsi*I sites of pX-βGAL [33]. BGAOS was derived from pX63NEO-βGAL [15] by insertion of a 56 bp sequence containing the T7 promoter [18] into the unique *Afl*II site, located 300 bp upstream of the DST splice acceptor joined to *lacZ*.

[b] Promastigotes of *L. major* were transfected with 10 μg of the indicated circular plasmid DNA plus 10 μg of pX63NEO-GUS [15]. Two clonal lines were transfected: the wild-type CC-1 line [34], or CC-1 stably transfected with the plasmid pX63HYG-T7nRP ('+T7 RNA polymerase'), which expresses a modified T7 RNA polymerase containing the SV40 nuclear localization signal [18]. Reporter enzyme assays were performed 15–20 h after transfection as described [15,17].

[c] Relative βGAL activity was expressed as the ratio of the β-galactosidase (experimental plasmid) to β-glucuronidase (control plasmid; pX63-GUS) activity, obtained following co-transfection with 10 μg of each plasmid. The average of two determinations is shown; the standard deviation for the relative βGal values was less than ± 10%.

Table 2
Activity of heterologous promoters and splice acceptors in transient transfections of *Leishmania major*[a]

Construct[b]	Promoter	5′ flanking splice acceptor	Reporter	3′ flanking splice acceptor	Transfected cell line			
					Luciferase		β-galactosidase	
					Wild-type	+T7 RNA polymerase	Wild-type	+T7 RNA polymerase
None	–	–	–	–	7 ± 3	9 ± 1	4.5	8.5
BGAOS	–	Lm *DST*	gal	–	–[c]	–	1450	76 700
PHD 183	Cf rRNA	Cf *PGK-C*	gal	Tb actin	–	–	5.5	1990
pHD 16	*PARP*	Tb aldolase	LUC	Tb aldolase	14 ± 7	840 ± 110	–	–
pHD 50	Tb rRNA	Tb actin	LUC	Tb actin	18	76	–	–
pHD 51	*PARP*	Cf *PGK-C*	LUC	Tb actin	8 ± 6	150 ± 25	–	–
pHD 54	–	Tb actin	LUC	Tb actin	7 ± 4	23 ± 4	–	–
PALT-LUC[d]	–	Le tubulin	LUC	–	110 ± 9	129 ± 9[d]	–	–
PT-ST7X.LUC	–	Lm *DHFR-TS*	LUC	Lm *DST*	120 ± 56	5400 ± 1200	–	–
BG7S.LUC	–	Lm *DST*	LUC	–	178	10 300	–	–

[a] *Leishmania* cell lines, transfection protocols, and reporter enzyme assays were described in the legend to Table 1, except that co-transfection with the pX63-βGUS was not performed. '+T7 RNA polymerase' refers to transfections in *L. major* expressing T7 RNA polymerase. Luciferase activity (LUC) is expressed in light units, β-galactosidase activity (β-gal) is expressed in fluorescent units. Cf, *Crithidia fasciculata*; Lm, *Leishmania major*; Le, *Leishmania enrietti*; and Tb, *Trypanosoma brucei*.

[b] *T. brucei* expression vectors pHD 16, 50, 54, 183 and 51 have been described [22,23,25]. pALT-LUC was obtained from D. Wirth (Harvard School of Public Health, Boston) and contains flanking sequences from *L. enrietti* (personal communication). BG7S.LUC is similar to BGAOS (Table 1), but has the luciferase coding region from pGEM-LUC replacing the *lacZ* gene. pT-ST7X.LUC contains the 3 kb fragment of pX containing the *DHFR-TS* flanking regions, with the *NEO* gene replaced by the luciferase coding region of pGEM-LUC and the T7 promoter of BGAOS inserted upstream. This assembly is carried in the vector pT (unpublished data).

[c] '–' means not performed.

[d] This construct lacks a T7 promoter.

The activities of a broader selection of test constructs in transient transfections of *L. major* are shown in Table 2. Luciferase activity was surprisingly low, even when driven by *Leishmania* sequences (pALT-LUC, pT-ST7X.LUC, or BG7S.LUC; Table 2). However, Gay et al. [21] reported more luciferase activity with constructs bearing gp63 intergenic regions driving the luciferase gene in *L. chagasi*, although the activity was much less than seen in transient transfections of *T. brucei* [22].

Overall, the highest activities were obtained from the *Leishmania* vectors BG7S.LUC, pT-ST7X.LUC, pALT-LUC and βGAOS, in which the reporter genes were flanked by the various 5′- and 3′-flanking regions from the *L. major DHFR-TS* gene (Lm DHFR-TS and Lm DST splice acceptors; Table 2) or that of *L. enrietti* tubulin. The absence of a 3′ flanking intergenic region did not affect activity, although the presence of a fortuitous RNA processing site located in the bacterial vector backbone may fulfill this requirement [18]. The use of T7 RNA polymerase to increase expression allowed a more sensitive determination of the effect of heterologous intergenic regions on reporter gene activity. Only two heterologous flanking regions showed detectable activity in *L. major*: *C. fasciculata* phosphoglycerate kinase C (*PGK-C*) locus (pHD183 and pHD51), and the *T. brucei* aldolase (*ALD*) locus (pHD16). However, the heterologous flanking regions were much less active than those of *Leishmania* (1.4–2.7 and 8–15% of that obtained with constructs BGAOS and pT-ST7X.LUC, respectively).

Comparing activities in the absence of T7 polymerase, we found that none of the heterologous RNA polymerase I promoters has any detectable function in *L. major* (Table 2; pHD183, pHD16, pHD50, pHD51). In *T. brucei*, homologous polymerase I promoters give up to 10-fold less activity than the T7 phage polymerase promoter, when polymerase is not limiting [23].

These results indicate that it will not be possible to use RNA polymerase I promoters from one trypanosomatid species to drive high-level expression in another. Similarly, the putative *Crithidia* RNA polymerase I promoter tested here does not function in *T. brucei* [24,25], the *T. cruzi* rRNA promoter does not show detectable activity in a wide variety of other trypanosomatids [26], and the *L. amazonensis* rRNA promoter was inactive in *T. cruzi*, *Crithidia* or *Endotrypanumi* [27]. Even within *Leishmania*, variable activity was observed following transfection of RNA polymerase I promoter constructs into strains from different species groups [21,27]. RNA polymerase I promoters are highly species-specific [28,29], and given the evolutionary distance separating trypanosomes and *Leishmania* [6,7], the lack of heterologous promoter activity is not surprising.

We observed that different splice acceptor sites yielded very different levels of gene expression in *L. major*. This was somewhat surprising in view of the somewhat relaxed requirements for *trans*-splicing in *Leishmania*, commonly believed to consist of little more than a polypyrimidine tract followed by an AG [12,13]. The results in Table 2 as well as other data suggest that *trans*-splice site acceptor requirements may be more complex than previously appreciated [30].

Our studies provide useful data that could permit the design of 'universal' trypanosomatid vectors in the future. For example, to obtain an expression vector that would function efficiently in transient assays in both *L. major* and *T. brucei*, one strategy would be to use the *DST* upstream region from *L. major* (the 5′ splice acceptor contained in the pX-series vectors), in combination with a bacteriophage promoter and RNA polymerase. To obtain permanent transformation of *T. brucei*, an additional requirement would be inclusion of a region of DNA suitable for targeting into the *T. brucei* genome by homologous recombination, or alternatively, sequences conferring episomal replication [31,32].

These data suggest that caution should be exercised when interpreting certain negative results arising from tests involving intergenic regions in heterologous species. This is particularly relevant to those considering the use of *T. brucei* genomic libraries for functional rescue studies within *Leishmania*; in this case, a negative result may arise because a gene is absent, or because of heterologous expression failure. For these purposes, construction of a *T. brucei* cDNA or genomic DNA library in a *Leishmania* expression vector may be a better alternative.

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