# Identification of promoters in trypanosomes

## Identificación de promotores en tripanosomas

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**ABSTRACT**

The trypanosomes diverged early in eukaryotic evolution, reflected in the existence of many unusual biological features such as polycistronic transcription and antigenic variation. The mechanisms by which genes are expressed has been an area of intensive study over the last two decades and some of the peculiar aspects of gene expression are briefly discussed. Promoters for RNA polymerase II have eluded detection in these organisms and in their absence, an *in vitro* system which would permit the dissection of transcription cannot be established. Here, I concentrate on the search for promoters with particular reference to an approach recently used to isolate them from *Trypanosoma brucei*.

**Key words**: Trypanosomes. Transcription. Post-transcriptional control. Promoters.

**RESUMEN**

Los tripanosomátidos divergieron tempranamente en la evolución eucariótica, por lo cual presentan características biológicas inusuales, como la transcripción policistrónica o la variacion antigénica. Los mecanismos de expresión génica en estos organismos han sido estudiados intensamente durante las dos últimas décadas y algunos aspectos peculiares de la expresión génica se discuten brevemente. Los promotores tipo RNA polimerasa II no han sido descubiertos en los tripanosomátidos, de modo que no ha podido ser establecido un sistema *in vitro* que pueda permitir la disección de la transcripción. Este artículo se concentra en la búsqueda de promotores haciendo particular referencia a las técnicas que fueron recientemente utilizadas para aislar secuencias promotoras en *Trypanosoma brucei*.

**Palabras clave**: Tripanosomas. Transcripción. Control post-transcripcional. Promotores.

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## INTRODUCTION

The trypanosomes are flagellated protozoans which are responsible for a variety of diseases. In Africa, *Trypanosoma brucei* causes both acute and chronic forms of human sleeping sickness (*T. brucei rhodiense* and *T. brucei gambiense*, respectively), and diseases of domestic livestock (*T. brucei*). In Central and South America, *T. cruzi* infects many species of mammals and causes Chagas disease in humans. The control of gene expression in trypanosomes differs in many aspects from that of other eukaryotes (reviewed in 1,2). While promoters which recruit RNA polymerases (RNAP) I and generally resemble those of other organisms, the polycistronic nature of transcriptional units, coupled to rapid *trans*-splicing, has hindered the identification of class II promoters. With the exception of a few cases, mRNAs are processed from large, polycistronic precursors with no apparent control of transcription at the level of individual genes. Thus, the eukaryotic paradigm of a gene consisting of an open reading frame preceded by a specific promoter containing a minimal core promoter and upstream elements which bind factors, the interaction of which determine its pattern of expression, does not generally apply to trypanosomes. Dissection of the processes whereby the RNAP II transcription complex is recruited to, and assembled on, DNA, and transcription is initiated and controlled, requires the development of *in vitro* transcription. This cannot be established in the absence of functional promoters. Here, I briefly review some unusual features of gene expression in trypanosomes and discuss some techniques used to map transcriptional start sites, concentrating on an approach designed to circumvent some of the problems associated with previous attempts to identify promoters.

## TRANSCRIPTION

There are about 100 copies of the rRNA transcription unit in *Trypanosoma brucei*, present in tandem arrays on several chromosomes (3). The mature rRNAs are derived from a large precursor, the promoter for which is located 1.2 kb upstream of the first rRNA gene (4). This promoter shares structural features with those of other eukaryotes, with a core region of approximately 70 bp directing a basal level of transcription which is stimulated by an upstream control element centered at position -200 (5). An enhancer element, present in other organisms, has not been found. In the spacer region upstream of each promoter in the array is a transcriptionally silent region which implies the existence of a terminator, as yet uncharacterised. In other eukaryotes, RNAP I exclusively transcribes rRNA. In trypanosomes, however, a number of observations have indicated that this polymerase may also synthesise other RNAs.

The major surface protein of the bloodstream stage is the variant surface glycoprotein (VSG) which is transcribed at the end of a 40 - 60 kb polycistronic transcription unit termed the expression site (ES; reviewed in 2, 6). There are up to 20 of these in the genome, located at telomeres, and consisting of a promoter, several (8 - 10) expression site associated genes (ESAGs), a series of 76 bp repeats and the VSG gene. Only one ES is active at a time so that a single VSG is exclusively expressed in the cell. The remaining sites are repressed by a mechanism which remains to be identified. The polymerase which transcribes this locus is pharmacologically distinct from RNAP II and shares the same resistance to á-amanitin and sensitivity to sarkosyl as RNAP I (7-9). These are features also exhibited by the polymerase which transcribes the Procyclic Acid Repetitive Protein (PARP; 8-10) and the metacyclic VSG (mVSG; 11) loci, the major surface coat proteins of the procyclic and metacyclic stages in the insect, respectively. This polymerase (or polymerases) also differs from RNAP I in some of its other characteristics such as cation requirement (12), which has led to a long-running controversy over its identity. Since the spliced leader RNA (SL RNA) has a 5′cap structure, this dispenses with the need for the transcribing polymerase to incorporate a cap and permits polymerases other than RNAP II to transcribe mRNA precursors. It is therefore possible that due to its efficiency, RNAP I has been recruited to transcribe the genes encoding the major surface coat proteins since they need to be highly expressed. Indeed, structural features are shared between the rRNA, PARP and VSG promoters (5,13,14) i.e. a core element containing two boxes, the importance of their relative spacing and the presence of an upstream control element. There is also a functional relationship, as evidenced by the activity of hybrid promoters containing elements of the PARP, VSG and rRNA promoters (5, 14), further strengthening the argument that they are transcribed by the same, although possibly modified, polymerase.

To date, all mRNAs known to be transcribed by RNAP II are expressed polycistronically in large precursor RNAs, implying that there is no control of transcription at the level of initiation for individual genes. In addition, no significant variation in the rate of transcription across RNAP II transcribed polycistronic units has ever been demonstrated in nuclear run-on experiments. When a reporter gene cassette was integrated into three different genes transcribed by RNAP II, it yielded similar activities in each locus, supporting this observation (15). Thus, it appears that expression of some, if not all, RNAP II transcribed genes is controlled exclusively by post-transcriptional mechanisms. Analysis of the large subunit of RNAP II of *T. brucei* (16,17) has shown that the C-terminal domain lacks the repetitive heptamer structure characteristic of this subunit in other organisms. The functional significance of this difference is not clear. As the 5′cap of

the mRNA is provided by the SL RNA, the transcriptional complex may also lack the associated capping activity found in other eukaryotes. Finally, none of the elements such as initiators, TATA boxes or enhancers which are typically present in the promoters have been found in trypanosomes. Such observations clearly indicate that many aspects of RNAP II transcription in these parasites are significantly different from the eukaryotic paradigm.

A number of tRNA and small RNA genes such as those involved in splicing have been characterised (18-20) and found to be transcribed by RNAP III, as expected. They are often found in close proximity and the intragenic promoter elements (A and B boxes) of the tRNA genes have been shown to also function as extragenic regulatory elements of the linked small RNAs (20).

## PROCESSING OF POLYCISTRONIC TRANSCRIPTS

Polycistronic precursor RNAs are processed by *trans*-splicing and polyadenylation to yield mature mRNAs (reviewed in 6,21), as illustrated in Figure 1. During *trans*-splicing, a small SL RNA is added to the 5′end of what will become the mRNA. In *T. brucei,* the SL RNA, which is 35 nt in length with a 4 nt cap structure, is synthesised as part of a 140 nt RNA which is then processed to yield the mature SL RNA. The precursor is transcribed from a 1.35 kb unit, of which there are about 200 copies in the genome (22,23). Each unit is transcribed individually by a polymerase, the identity of which

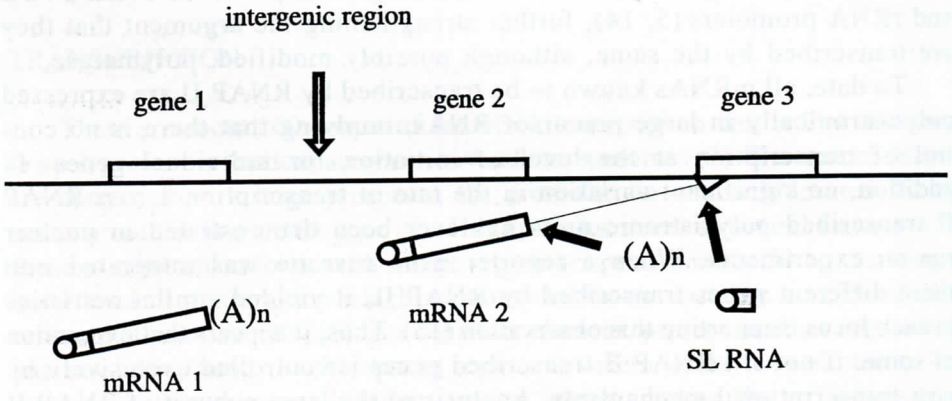


Fig. 1.—Processing of polycistronic transcripts. Three genes and an intergenic region containing the signals for splicing and polyadenylation are shown. Following transcription, the RNA is *trans*-spliced, cleaved and polyadenylated. The SL RNA (hatched box) includes a 5′cap (open circle). Open boxes represent coding sequences and $(A)_n$, polyA tails. Based on Graham (53).

is still not clear. Early work suggested that it was RNAP III (12), however more recent experiments have indicated that it may be RNAP II (24). Genes are generally arranged in clusters with related genes often in tandem arrays (6). The intergenic regions typically contain polypyrimidine-rich stretches which, together with the dinucleotide AG, make up the splice acceptor site (SAS) to which the SL RNA is joined. Trypanosomes do not use the eukaryotic consensus signal for polyadenylation, AAUAAA, but rather the site of cleavage and polyadenylation is located a fixed distance upstream (100 - 150 nt in *T. brucei*; 25) of the SAS of the downstream pre-mRNA. Thus, *trans*-splicing and polyadenylation are linked. This was demonstrated by altering the position of the SAS, resulting in a similar shift in the position of the upstream site of polyadenylation. In contrast, elimination of the wild type site of polyadenylation did not affect the site of downstream SL RNA addition and polyadenylation continued to occur upstream from this at a similar distance as in the wild type situation (25,26). In reality, polyadenylation usually occurs at several points within a small region rather than at a single site.

## POST-TRANSCRIPTIONAL CONTROL

Post-transcriptional control may be exerted at a number of levels such as RNA processing and maturation, mRNA transport or turnover, and translation. The use of alternative splicing and polyadenylation sites may play a role in regulation of gene expression. The phosphoglycerate kinase (PGK) locus contains 3 related genes (PGK A, PGK B and PGK C; 27) which are transcribed at approximately the same rate in both bloodstream and procyclic stages but are highly developmentally regulated. The level of PGK A transcripts is very low in both bloodstream and procyclic forms, probably as a result of its very inefficient SAS (28). In contrast, PGK B mRNA is up-regulated in the insect stage while PGK C is highly expressed in the bloodstream stage, effects which are mediated by their 3' UTRs and demonstrable by 3' UTR swap experiments (29). Studies have demonstrated that the 3' UTR of VSG stabilises reporter gene transcripts in bloodstream parasites but significantly reduces their relative level in procyclic stages as a result of decreased efficiency of mRNA maturation (30). VSG transcripts disappear from the cell quickly on differentiation from the bloodstream to the insect form. This regulation may be attributable to effects on RNA turnover since VSG mRNA has a turnover rate of 4.5 hours in bloodstream forms which decreases rapidly to 1.5 hours following the application of conditions which force the parasites to begin to differentiate (31). In Leishmania, deletion of part of the 5' UTR of the *hsp83* gene causes a loss in the temperature-dependant regulation of a reporter

gene (32). These and other studies have convincingly demonstrated the major role that 3' UTRs can play in gene expression in trypanosomes.

## APPROACHES TO IDENTIFY PROMOTERS IN TRYPANOSOMES

As a result of *trans*-splicing, techniques such as primer extension and RNA protection usually reveal only the site of SL RNA addition. It is possible, however, to stabilise large RNA precursors before processing by subjecting cells to treatment with sinefungin or low levels of UV irradiation (33). This approach was taken by Pays and coworkers in an attempt to localise a putative transcriptional start site upstream of the actin locus (34). An alternative method to map promoters is by measuring the susceptibility of a transcribed region to UV irradiation (35). Essentially, the more distant a sequence is from its promoter, the more sensitive its transcription is to UV because thymine dimers are formed in the DNA as a function of DNA length and polymerases cannot transcribe through them. Thus, the further away a sequence is from its promoter, the higher the probability of the formation of dimers between the promoter and sequence of interest and therefore, the more sensitive is its transcription. This technique allowed Lee to map putative promoters in the intergenic regions of the *hsp*70 gene cluster (36). When the putative actin and *hsp70* promoters were assayed in transient assays, their activities were reported to be low, approximately 1% of that of RNAP I promoters, or undetectable (34,36,37).

The development of autonomously replicating plasmids (38) and artificial mini-chromosomes (TACs) in *T. brucei* (39,40) was anticipated to be of use in the design of promoter traps. In one study (41), random genomic fragments were cloned upstream of a hygromycin resistance gene (*hyg*) in an episomal construct, introduced into parasites and hygromycin resistant transformants selected. The only promoter recovered was the PARP promoter (41) which was proposed to play a role in plasmid DNA replication. Transfection studies in *Leishmania enriettii* (42) have shown that non-directed transcription occurs from plasmid sequences, even in the absence of parasite-derived DNA, a process which might obscure specific transcription from promoters. This phenomenon can also occur in *T. brucei*, is probably attributable to transcription by RNAP II (43) and in the absence of effective transcriptional terminators, cannot be avoided. The formation of large, multimeric episomes seems to favour this effect and expression of reporter genes located on such molecules can easily reach 1% or more of the level obtained from a single rRNA promoter driving a single copy of a reporter gene (43). When analysing sequences for their ability to specifically direct transcription in a transcriptionally active background, it is difficult to differentiate between genuine promoter activity and merely enhancement of *trans*-splicing of transcripts which initiate

further upstream. It is therefore essential that the transcriptional silence of an environment must be established before promoter analysis. Thus, there is a potential difficulty with all approaches which rely on extrachromosomal constructs. This may not affect the identification or analysis of highly active sequences but as RNAP II promoters might be anticipated to have activities of just 1-10 % of class I promoters (based on run-on data, integration of reporter genes and activities of the putative RNAP II promoters; discussed above), such approaches may be problematic.

## DESIGN OF A CONSTRUCT FOR PROMOTER TRAPPING AND ANALYSIS

We chose to search for, and subsequently assay, putative promoter fragments of *T. brucei* in a genomic context, based on the following considerations. Potential problems associated with the assessment of fragments integrated into the genome include the possibility of transcriptional read-through from upstream sequences into the reporter gene, as discussed above. This may be avoided by directing the promoter trap to a transcriptionally silent region of the genome such as the rRNA intergenic locus (4,44). Constructs integrated here will be in the nucleolus. Experiments in yeast have established that the RNAP II transcription complex is not excluded and is capable of recognising and initiating transcription from class II promoters integrated in the rRNA array (45). However, to ensure that nuclear compartmentalisation did not affect the behaviour of putative promoters, constructs were also designed which integrate into the silent region upstream of the SL RNA promoter. Analysis of a number of different species of trypanosomes and Leishmania has demonstrated that the sequences which direct transcription of the SL RNA precursor are generally located within 100 bp upstream of the transcriptional start site and that termination signals are contained within about 100 bp downstream of the site of transcriptional termination (e.g. 24,46). We therefore reasoned that there may exist a region of approximately 900 bp in the SL RNA repeat unit of *T. brucei* which is transcriptionally silent. This region had not previously been investigated but our results indicate that it is possible to integrate sequences here and that there is no detectable transcription originating from upstream (see below). By targeting constructs to both these loci, it is possible to assess activities in two different chromosomal and nuclear locations, minimising positional effects.

Since class II promoters were anticipated to be considerably less active than those of RNAP I, a reporter gene rather than a selectable marker was employed for the detection of promoter activity. The use of selection would probably favour transformants which expressed high levels of the selectable marker and transcription of its gene in these cells would presumably be under

the control of an RNAP I promoter. Also, in the abscnce of *bone fide* RNAP II promoters, it was somewhat difficult to determine the drug concentration which kills wild type cells while permitting transformants with a low level of expression of the selectable marker to survive. Finally, a reporter gene permits quantification, allowing the categorisation of transformants based on their activity in an assay. To this end, firefly luciferase reporter gene (*luc*) was favoured as the assay is sensitive, quick and simple. The hygromycin resistance gene (*hyg*) was used for selection of transformants, under the control of the PARP promoter. If tandem copies of the construct are integrated, the reporter gene of the downstream copy may be transcribed by the polymerase initiating from the upstream promoter driving the drug resistance gene. To prevent this possibility, a promoter which directs a polymerase sensitive to a transcriptional terminator may be employed. Such a terminator, specific for the polymerase which transcribes the PARP locus, has recently been described (47). It does not, however, completely abolish transcription, so the *luc* gene was placed in the opposite direction to that of the *hyg* gene. This prevents transcriptional read-through in the event of the integration of tandem repeats, regardless of their orientation. The basic construct, pHD526 (Fig. 2), was designed to integrate into the rRNA spacer region, integration occuring exclusively through homologous recombination in *T. brucei* (48). The plasmid consists of the *hyg* gene with 5′ and 3′ UTRs, driven by the PARP promoter. Upstream of this, such that they are in a head to head orientation, is the *luc* gene, flanked by the actin 5′ and 3′ UTRs. The 3′ actin UTR is juxtaposed to a second actin 5′ region as this promotes correct polyadenylation and supports a high level of expression (49), increasing sensitivity. A unique *Bgl*II site was introduced between the *hyg* and *luc* genes to permit the cloning of sequences to be assessed for their ability to drive expression of the reporter gene. Close to the *Bgl*II restriction site is a *Pst*I site used to determine the orientation of inserts isolated from transformants in a library screen. By replacing the rRNA targeting region for an SL RNA sequence, pHD572 was generated, permitting analysis of inserts of interest in this second location.

## THE CONSTRUCTS FUNCTION AS PREDICTED

To test this approach, constructs were transfected into procyclic trypanosomes and stable cell lines established following limiting dilution (50). In constructs lacking promoters to drive the *luc* gene (pHD526 and pHD572; Fig. 3), the level of luciferase activity was indistinguishable from instrumental background (270±79 RLU). To demonstrate that promoters function in this context, the rRNA promoter (51) was cloned into both pHD526 and pHD572. This gave an average of 273 000 RLU/2x$10^5$ cells (pHD546) when integrated in the

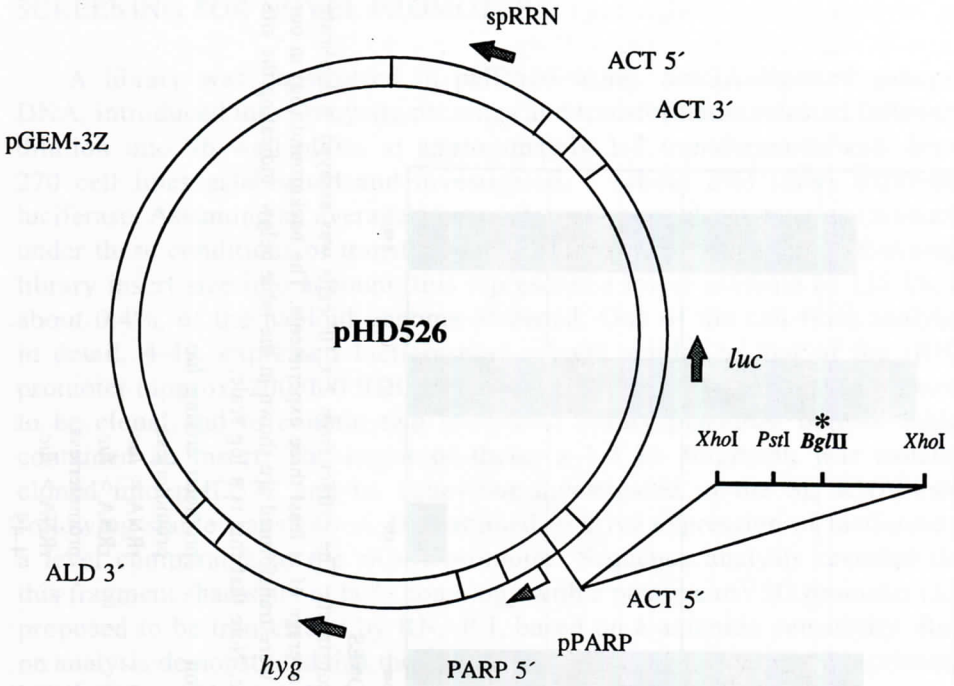


Fig. 2.—Map of pHD526. The sequences necessary for propagation in *E. coli* are from pGEM-3Z, as shown. The orientation of the two open reading frames, *luc* and *hyg*, are indicated by arrows, as is the direction of the intergenic rRNA spacer (spRRN) when the construct is integrated. This region was replaced with a targeting sequence for the SL RNA locus to generate pHD572. Actin 5′ and 3′ UTRs (ACT) flank *luc*, and the position of the PARP 5′ UTR and aldolase 3′ UTR (ALD) are shown. The site of transcription initiation within the PARP promoter (pPARP) is shown by an arrow. The region encompassing the *Bgl*II (*) site into which fragments are cloned is enlarged and the relative position of the *Pst*II site indicated (see text).

rRNA spacer and 465 000 RLU/2x$10^5$ cells (pHD576) when integrated in the SL RNA array. In the opposite orientation, (pHD545 and pHD575) the activity was at background levels, indistinguishable from the promoterless constructs. Thus, the rRNA promoter gave activities that were three orders of magnitude higher than background. If one arbitrarily considers twice background as the limit of significant activity (here, about 500 RLU/2x$10^5$ cells), activities could be detected that are just over 0.1% of an RNAP I promoter, 10 - 100-fold less than might be expected from a class II promoter. Of crucial importance, integration of DNA *per se* does not result in expression of *luc* (pHD545 and pHD575), indicating that a library constructed using these plasmids could be used in a screen for novel promoters.

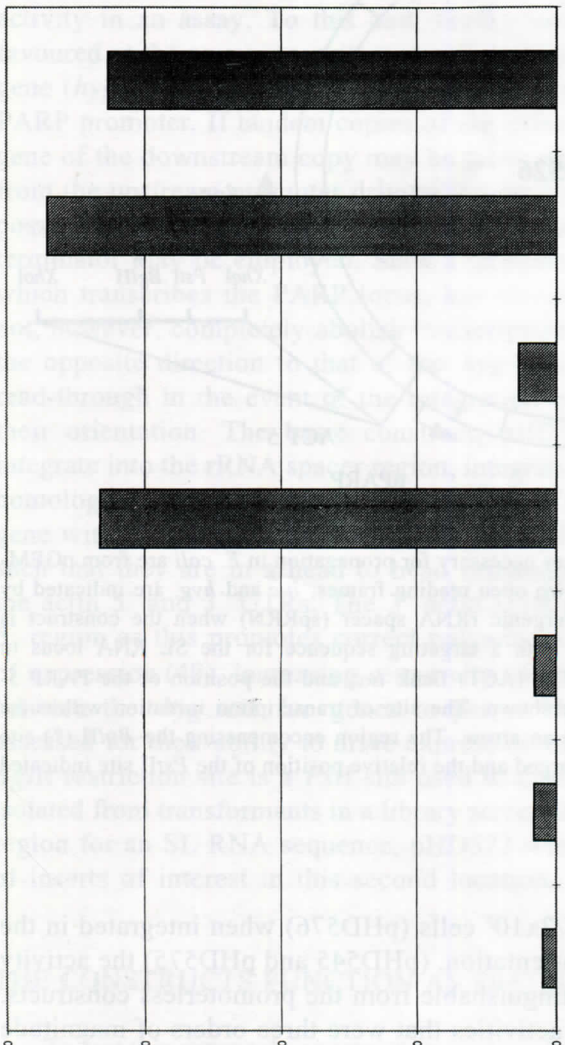


Fig. 3.—Luciferase activities of clones stably transformed with plasmid constructs. The extract from 2 x $10^5$ cells was assayed in duplicate and is expressed as relative light units (RLU). The results shown are the average of at least three experiments. The background (bg) in our hands was approximately 250 RLU. The constructs with which the clones were transformed are shown in the table below, indicating the site of integration and the identity and orientation of the promoter driving *luc*. Note that this is a log scale.

| CONSTRUCT | Site of integration | promoter driving *luc* |
|---|---|---|
| pHD526 | rRNA spacer | promoterless |
| pHD545 | rRNA spacer | rRNA < *luc* |
| pHD546 | rRNA spacer | rRNA > *luc* |
| pHD572 | SL RNA spacer | promoterless |
| pHD576 | SL RNA spacer | rRNA > *luc* |
| 4-49 | SL RNA spacer | 4-49 |

## SCREENING FOR NOVEL PROMOTERS

A library was constructed in pHD526 using *Sau*3A-digested genomic DNA, introduced into procyclic parasites and transformants selected following dilution into 96 well plates at approximately 1-2 transformants/well. From 270 cell lines established and investigated, 5 (about 2%) stably expressed luciferase. Assuming an average of one integration event per genome (favoured under these conditions of transformation), and taking orientation and average library insert size into account, this represents a lower estimate of 135 kb, or about 0.4%, of the haploid genome screened. One of the cell lines analysed in detail, 4-49, expressed luciferase at a level similar to that of the rRNA promoter (approx. 200 000 RLU/2x$10^5$ cells; Fig. 3). This cell line was shown to be clonal and to contain two integrated library plasmids, both of which contained an insert. The larger of these, a 1.3 kb fragment, was isolated, cloned into pHD572 and its behaviour investigated in the SL RNA locus following stable transfection. It continued to drive expression of luciferase at a level comparable to the rRNA promoter. Sequence analysis revealed that this fragment shares about 60% homology with a putative mVSG promoter (52), proposed to be transcribed by RNAP I, based on á-amanitin sensitivity. Run-on analysis demonstrated that the polymerase recruited by this putative promoter isolated from clone 4-49 has a similar sensitivity to á-amanitin.

## SUMMARY

Trypanosomal gene expression exhibits many peculiarities such as polycistronic transcription, *trans*-splicing and the prominence of post-transcriptional control. One area of particular interest is the mechanism by which the RNAP II initiates transcription. To date, no sequences typical of class II promoters in other eukaryotes have been described, and no transcriptional start sites or promoters used by this polymerase have been unequivocally demonstrated, leading to the suggestion that they may not exist. Approaches to detect such promoters based on techniques successful in other organisms have failed in trypanosomes due primarily to the combination of polycistronic transcription and rapid *trans*-splicing. The use of extrachromosomal constructs is compromised by non-specific transcription which makes it difficult to distinguish between transcription initiation and an enhanced level of *trans*-splicing, and effectively reduces the sensitivity of promoter traps. The constructs described here were designed to assess promoter activities in a transcriptionally silent, genomic environment. In addition to their use in promoter dissection and comparison, these constructs can be used to trap novel promoters, as demonstrated.

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