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## Temperature shift activates bloodstream *VSG* expression site promoters in *Trypanosoma brucei*

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### Abstract

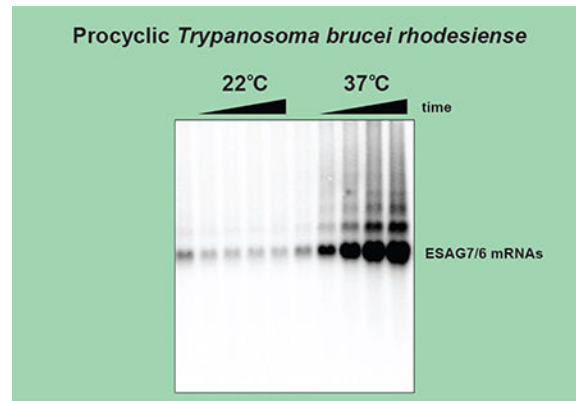
*Trypanosoma brucei* relies on two types of variant surface glycoprotein (*VSG*) expression sites (ESs) for RNA polymerase I (Pol I) transcription of *VSG* pre-mRNA. Trypanosomes developing into infectious metacyclic cells in the tsetse vector use metacyclic *VSG* ESs (MESs) and proliferating parasites in the mammalian host deploy bloodstream *VSG* ESs (BESs). Unlike the monocistronic MESs, BESs are polycistronic and their highly conserved promoters differ considerably from the MES promoters. The significance of the divergent sequences of MES and BES promoters remains to be determined. We used a reporter system to specifically test the effect of temperature on the activity of MES and BES promoters in procyclic trypanosomes and our results demonstrate that transcription from the MES promoter is largely insensitive to changes in temperature. In contrast, the BES promoter drives rapid activation of transcription upon a change of temperature from 28°C to 37°C. Additionally, endogenous BESs respond similarly to the elevation of temperature and initiate increased production of BES pre-mRNA and mRNA. Our results indicate that the sequence of the BES promoter is a specificity signal which triggers BES activation *in vivo* upon entry into the mammalian host.

### Graphical abstract

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## Keywords

*Trypanosoma brucei*; transcription; VSG; expression site; promoter

*Trypanosoma brucei rhodesiense* is one of the two subspecies of the protozoan parasite *T. brucei* that cause human African trypanosomiasis. All members of the *T. brucei* group use a monolayer of variant surface glycoproteins (VSGs) on their exterior to outmaneuver the mammalian immune system by switching the expressed VSG [1,2]. Only a single *VSG* gene is highly active at any given time point from a large repertoire of *VSG* sequences in the *T. brucei* genome [3]. Although definitive explanation for the molecular mechanism driving monoallelic *VSG* expression is still elusive, the localization of the active *VSG* gene in an extranucleolar focus called expression site body (ESB) [4] is a defining feature of the active gene. RNA polymerase I (Pol I) transcribes the expressed *VSG* gene from a chromosome locus termed *VSG* expression site (ES), positioned adjacent to a telomere [1,2]. Two types of *VSG* ESs exist in the *T. brucei* genome. In the tsetse fly vector salivary glands trypanosomes developing to metacyclic cells use exclusively metacyclic *VSG* ESs (MESs) for the *de novo* synthesis of a VSG coat on the parasite surface, a critical aspect of their development leading to acquisition of infectivity. A ubiquitous characteristic of MESs is that they are short (3 to 6 kb) and monocistronic, i.e. there are no additional coding sequences between the MES promoter and the telomere except the *VSG* gene [5]. The “minimalistic” nature of MESs likely lowers the metabolic costs for synthesizing the VSG coat in trypanosomes that are in the process of acquiring transmission competence accompanied by a transition to quiescence [6]. In contrast, dividing bloodstream form trypanosomes in the mammalian host use bloodstream ESs (BESs) to transcribe the expressed *VSG* in any single cell. BESs are up to 60 kb long and polycistronic, containing multiple expression site associated genes (*ESAGs*) and a long run of 70 bp repeats in addition to the single telomeric *VSG* gene [1,2]. Even though recent evidence supports recombinatorial transfer of metacyclic *VSGs* to the active BES [7], on rare occasions, a MES can be active for *VSG* expression in dividing bloodstream *T. brucei* [8].

Although both metacyclic and bloodstream *VSG* ESs are transcribed by the multifunctional *T. brucei* RNA polymerase I (Pol I), the BES and MES promoter sequences differ considerably (Fig. 1). The highly conserved BES promoter present in all of the ~15 BESs is

shorter than 70 bp [9] and binds a multi-subunit protein complex named Class I Transcription Factor A (CITFA), which also recognizes the longer procyclin and ribosomal RNA Pol I promoters [10]. The MES promoters are also short and exhibit a higher degree of heterogeneity [3,5,11], compared to the BES promoters. Despite their different sequences, MES and BES promoters are nevertheless recognized with similar high affinity by the transcription factor CITFA [12]. This factor was shown to be indispensable for transcription of *rRNA*, procyclin and BES *VSG* genes in *T. brucei* [10,13–15] and was demonstrated to be present not only in the nucleolus but also in the *VSG*ESB [14–16]. In addition, promoter occupancy of the active BES by CITFA is higher than that of silent BESs, likely reflecting a critical role for CITFA in BES activation [16].

The reason for the divergent sequences of MES and BES promoters has so far remained unclear. Possible roles could include acting as a specificity signal for MES activation during metacyclic development, or alternatively, for MES inactivation and/or BES activation during the *in situ* *VSG* switch upon entry into the mammalian host. MES activation occurs in *T. brucei* cells that are attached to the tsetse salivary gland epithelium, is induced by an unknown mechanism and can be accomplished *in vitro* in the absence of fly tissues by forced expression of the RNA-binding protein genes *RBP6* (accession number Tb927.3.2930) [6,11] or *RBP10* (accession number Tb927.8.2780) [17]. Metacyclic VSGs are rapidly replaced with VSGs expressed from BESs in the initial stages of mammalian infection [18], suggesting that ES switching occurs very early in this process. The precise triggers that initiate the MES to BES *VSG* switching have remained unknown.

To gain insight into the process of switching from MESs to BESs upon entry into the mammalian host, we decided to study the effect of temperature on the activity of MES and BES promoters in *T. b. rhodesiense* parasites. We chose this particular subspecies since its procyclic form exhibits the most robust growth *in vitro* under our laboratory conditions when compared to other procyclic *T. brucei* strains, even at suboptimal temperatures. In order to minimize other variables, we chose to abstract our experiment from the influence of genome positioning and epigenetic factors that affect *VSG* expression at telomeres [1,2]. For this reason, we targeted our reporter constructs for integration in the actively Pol II-transcribed  $\beta/\alpha$ -*tubulin* locus. The reporter cassettes consisted of the MES (*VSG397*) or the BES (*VSG2*) promoter, immediately followed by the paraflagellar rod protein 2 (*PFR2*) intergenic region containing the *PFR2* *trans*-splice site, the blasticidin-S deaminase (BSD) coding sequence and a large portion of the  $\beta/\alpha$ -*tubulin* intergenic segment, which serves both as a target for chromosomal integration and provides the pre-mRNA processing signals necessary for proper polyadenylation of the BSD transcript (Figs. 1 and S1). The two constructs were transfected separately into procyclic *T. b. rhodesiense* cells, because we wanted to avoid competition with the active BES *VSG* promoter in bloodstream-form cells. After selection of stable transformants with blasticidin at 28°C, equal volumes of cell cultures of the same density in the absence of blasticidin were grown overnight at 28°C in duplicate, and transferred to 22°C or 37°C. Equal volume aliquots of the cultures were used to prepare RNA samples at the zero time point (28°C) of the experiment and every hour after placing the cultures at 22°C or 37°C for 4 hours. Northern blotting was the method chosen for monitoring the levels of the BSD reporter mRNA and several other transcripts in the corresponding total RNA samples (Fig. 2). The BSD mRNA from the MES promoter

construct showed limited change in steady-state levels at either 22°C or 37°C for the tested time period (Figs. 2A and S2). In contrast, the BSD mRNA had lower initial levels in the cells containing the BES promoter reporter compared to the MES construct and these levels remained low during the incubation for 4 hours at 22°C (Figs. 2A and S2). However, the temperature shift to 37°C resulted in rapid increase of BSD mRNA, noticeable even after only 1-hour incubation at the elevated temperature (Figs. 2A and S2). Because all elements (except the promoters) of our experimental design were identical between the two cell lines, including the 5'- and 3'-UTRs and lack of selecting drug pressure (blasticidin was removed from the cultures), we can exclude the possibility that the observed increase in BSD mRNA levels was the result of a change in pre-mRNA processing, mRNA export to the cytoplasm, mRNA translation or mRNA degradation. Our conclusion is that the observed elevation of BSD mRNA levels at 37°C in the cells containing the BES promoter reporter cassette was solely the result of increased transcription from the BES promoter.

One of the mRNAs we chose to monitor codes for an ortholog of the mammalian heat shock factor binding protein 1 (*HSBP1*; accession number Tb927.9.13070) (Fig. 2B). The sequence of the protein in all available trypanosomatid species shows remarkable similarity to the human polypeptide and the structure of the *T. brucei* protein can be modeled on the available crystal structure of the mammalian HSBP1 [19] (data not shown). This protein has been shown to modulate the activity of heat shock factor 1 (HSF1) [20], a transcription factor that does not appear to be present in trypanosomes. Nevertheless, since this is a known transcription factor regulator and it has highly elevated levels (both protein and mRNA) in metacyclic trypanosomes in comparison to procyclics [6], we decided to test if its inducible expression in *T. brucei* Lister 427 (29–13) cells will trigger rapid activation of metacyclic VSG expression. Over the course of 24 hours of inducible *HSBP1* expression we could not detect MES activation (data not shown). This observation does not exclude the possibility that HSBP1 in trypanosomes could be involved in regulating transcription by RNA polymerases I, II or III.

Our result demonstrating an increase in transcription from a BES promoter of the reporter mRNA at 37°C prompted us to test whether endogenous BESs responded to this temperature shift. *ESAG7* and *ESAG6* genes encode the two subunits of the *T. brucei* transferrin receptor heterodimer and are always positioned at the very beginning of the BES Pol I transcription unit that generates the *VSG* mRNA [21]. We used a probe that simultaneously detects both *ESAG7* and *ESAG6* mRNAs to visualize their abundance. There was a small decrease in the already low abundance of *ESAG7/6* mRNAs in both cell lines incubated at 22°C (Figs. 2B and S2). The temperature shift to 37°C caused a rapid and very obvious increase in the steady-state levels of endogenous *ESAG7/6* mRNAs (Figs. 2B and S2). We additionally observed discrete longer RNA species, likely representing partially processed pre-mRNA molecules containing 2, 3 or more protein coding sequences as well as a smear from the major mRNA band to the gel wells. The putative *ESAG7-ESAG6* di-cistronic RNA has been observed previously [21], however its intensity in our northern blot combined with the presence of a smear and a higher-order polycistronic RNA species are likely indicators of inefficient pre-mRNA processing. We were able to exclude the possibility that increased stability of *ESAG7/6* mRNAs is solely responsible for their rapid accumulation at 37°C, because a promoterless construct with identical UTRs (Fig. S3) integrated in endogenous

BES exhibited almost identical behavior (Fig. S4), confirming that endogenous BES promoters are similarly activated at a temperature that is characteristic for a mammalian host. The increased activity of the BES promoters at higher temperature was also observed in two other *T. brucei* strains, TREU 927 and Lister 427 (Fig. S5).

Our results agree with previous data showing that BES promoters are generally silent in procyclic cells under normal conditions, regardless of their genome context [22]. A partial derepression in procyclics of BES promoters when placed in the rDNA spacer [22,23] or in artificial proximity to a procyclin promoter [24] is likely the result of subjecting the promoter to a high local concentration of CITFA and Pol I complexes. On plasmid episomes the BES promoter has been reported to be equally active in bloodstream and procyclic form trypanosomes, however the authors acknowledged that the transcriptional activity was low [25,26]. Additionally, numerous previous studies assessing BES promoter activity in nuclear run-on assays, while still informative, should not be regarded as an accurate readout of true levels of transcription, considering the rapid loss of activity by cooling of the cells and nuclei during the procedure for isolating trypanosome nuclei [27].

In conclusion, we demonstrate that the different sequences of MES and BES promoters determine their different behavior during the shift in temperature from the tsetse vector to the mammalian host. MES promoters are active at all temperatures tested (22°C, 28°C and 37°C) and this could explain the reason for the occasional isolation of bloodstream trypanosome clones expressing *VSG* from a MES [8]. In contrast, BES promoters, particularly in their natural genomic context, are highly active only at mammalian body temperature and this likely contributes to the lack of activation of BESs during the development of infectious metacyclics in the tsetse salivary glands. The specific temperature-sensitive sequence determinants of the BES promoter and the identity of the biomolecule functioning as a temperature sensor and triggering BES promoter activation are currently unknown.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## ACKNOWLEDGMENTS

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## Abbreviations:

<b>VSG</b>	variant surface glycoprotein
<b>ES</b>	expression site
<b>BES</b>	bloodstream expression site
<b>MES</b>	metacyclic expression site

<b>CITFA</b>	class I transcription factor A
<b>Pol I</b>	RNA polymerase I
<b>ESAG</b>	expression site associated gene.

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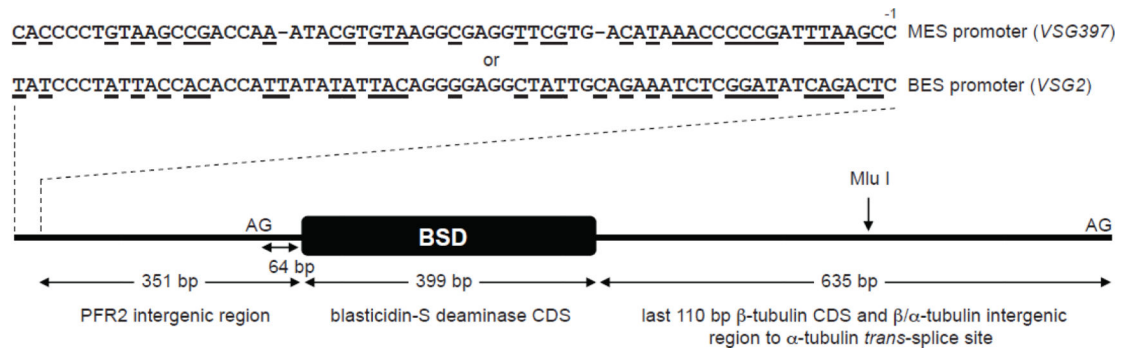
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**Highlights**

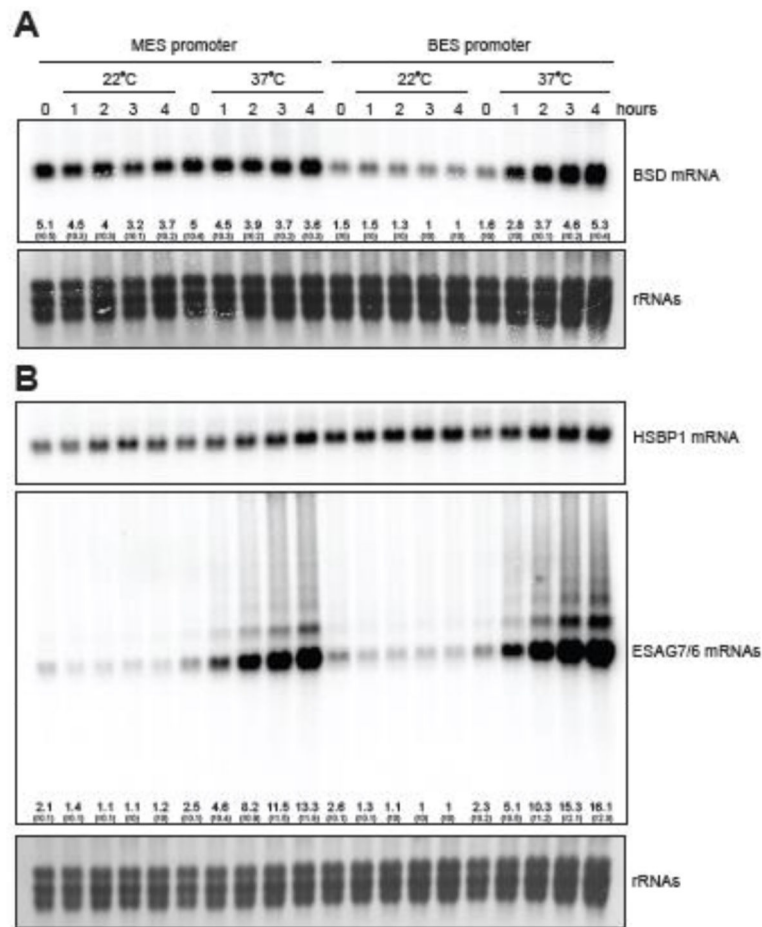
- Metacyclic *VSG* expression site (ES) promoters are insensitive to changes in temperature
- Bloodstream *VSG* ES (BES) promoters are repressed at ambient temperature
- BES promoters are activated at mammalian body temperature





**Fig. 1.**

A diagram of the ES promoter reporter constructs. The difference between the two cassettes is only the identity of the promoter, MES (from the *T. brucei* Lister 427 VSG397ES) or BES (from the *T. brucei* Lister 427 VSG2ES). Different nucleotides between the two promoters are underlined and the position immediately upstream of the Pol I transcription start site is marked with  $-1$ . The backbone of the constructs is pBluescript II KS(+). AGs indicate the *trans*-splice sites upstream and downstream of the BSD coding sequence. The Mlu I restriction site present in the  $\beta/\alpha$ -tubulin intergenic region was used to linearize the plasmids prior to transfection.

**Fig. 2.**

Increase in temperature activates BES promoters. *T. b. rhodesiense* strain YTat 1.1, carrying either the MES or BES promoter reporter construct was grown at 28°C in Cunningham's medium supplemented with 10% heat inactivated FBS and 2 mM L Gln. The two cultures (grown in parallel) were diluted in duplicate to  $1 \times 10^6$  cells/mL and incubated overnight at 28°C. Time point 0 hours represents the cultures at 28°C. One flask of each culture was placed at 22°C and the other at 37°C. Total RNA was prepared with Trizol reagent (Invitrogen) and Northern blotting was performed using a standard protocol. The probes used, (A) BSD, (B) HSBP1 and ESAG7/6, are indicated on the right (probe details are available upon request). The probe against ESAG7 and ESAG6 recognizes a region with high degree of identity between the two genes. Numbers in the lanes indicate the levels of the detected mRNAs from three independent experiments ( $\pm$  SEM) normalized relative to the HSBP1 mRNA amounts in the samples. The lowest steady-state level is set to 1. The large rRNAs were visualized by staining with methylene blue.