***Trypanosoma brucei* variant surface glycoprotein regulation involves coupled activation/inactivation and chromatin remodeling of expression sites**

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***Trypanosoma brucei* is an extracellular protozoan parasite that cycles between mammalian hosts and the tsetse vector. In bloodstream-form trypanosomes, only one variant surface glycoprotein gene (VSG) expression site (ES) is active at any time. Transcriptional switching between ESs results in antigenic variation. No VSG is transcribed in the insect procyclic stage. We have used bacteriophage T7 RNA polymerase (T7RNAP) to study the transcriptional accessibility of ES chromatin *in vivo*. We show that T7RNAP-mediated transcription from chromosomally integrated T7 promoters is repressed along the entire length of the ES in the procyclic form, but not in the bloodstream form, suggesting that the accessible chromatin of inactive bloodstream-form ESs is remodeled upon differentiation to yield a structure that is no longer permissive for T7RNAP-mediated transcription. In the bloodstream form, replacing the active ES promoter with a T7 promoter, which is incapable of sustaining high-level transcription of the entire ES, prompts an ES switch. These data suggest two distinct mechanisms for ES regulation: a chromatin-mediated developmental silencing of the ES in the procyclic form and a rapid coupled mechanism for ES activation and inactivation in the bloodstream form.**
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Introduction

Transcriptional control of the variant surface glycoprotein gene (VSG) expression sites (ESs) in the protozoan parasite *Trypanosoma brucei* represents a complex regulation of a multigene family in an early branched eukaryote. Hybridization analyses have suggested the presence of ~20 ESs, which share a high degree of sequence homology (Cully *et al.*, 1985; Zomerdijk *et al.*, 1990; Navarro and Cross, 1996). In the bloodstream form of *T. brucei*, however, only one ES is highly transcribed at any time. By switching VSGs, the parasite undergoes antigenic variation and eludes the host immune response (Borst *et al.*, 1993; Vanhamme and Pays, 1995; Cross, 1996). VSGs can be

switched by transposing a non-transcribed basic copy VSG into an already active ES, displacing the resident VSG, or by activating another ES with concomitant inactivation of the previously active one, also known as *in situ* ES activation. The ES is developmentally down-regulated in the procyclic form, the insect midgut stage of the parasite, where another glycoprotein, the procyclic acidic repetitive protein (PARP; also called procyclin), replaces VSG on the membrane (Hehl and Roditi, 1994).

The mechanisms that allow only one ES to be active in the bloodstream form and all ESs to be down-regulated in the procyclic form are unknown. Transcription from the ES promoter, which is located 40–60 kb upstream of the telomeric VSG (Pays *et al.*, 1981; De Lange and Borst, 1982; Johnson *et al.*, 1987; Kooter *et al.*, 1987), is driven by an α -amanitin-resistant polymerase, inferred to be Pol I (Rudenko *et al.*, 1991; Zomerdijk *et al.*, 1991). The promoter and surrounding sequences of characterized ESs are 90% identical, and *in situ* ES activation can occur without DNA rearrangements or sequence changes at the promoter (Pays *et al.*, 1990; Zomerdijk *et al.*, 1990; Gottesdiener *et al.*, 1991; Navarro and Cross, 1996). In addition, the 5' end of all ES promoters is flanked by a 20–40 kb region of irregular 50 bp repeats, whose function is unknown. In a previous study, we determined the transcriptional activities of the ES promoter in the different stages of the parasite life cycle *in vivo* (Navarro and Cross, 1998). Transcription of a reporter gene, inserted 1 kb downstream of the ES promoter, differed up to 1000-fold between an active and an inactive ES in the bloodstream form. Upon differentiation to the procyclic form, the activity of previously active and inactive ES promoters were identical, 100-fold below the level of an active bloodstream-form ES. These data suggested the presence of two distinct mechanisms controlling ES regulation in bloodstream and procyclic forms.

Recently, transcriptional studies have focused on the role of chromatin structure as a means to regulate the accessibility of DNA templates (reviewed by Grunstein, 1998; Pirrotta, 1998). Several studies performed in different systems have used a heterologous RNA polymerase to measure chromatin accessibility from different templates (Jenuwein *et al.*, 1993, 1997; McCall and Bender, 1996). Transcription of a reporter gene, inserted into different loci and driven by a heterologous polymerase, might distinguish between different transcriptional regulatory mechanisms. Thus, mechanisms that rely on protein–protein interactions within an endogenous transcriptional complex should not affect transcription driven by a heterologous RNA polymerase, while mechanisms involving more general chromatin changes may affect the ability of a small heterologous RNA polymerase to access its promoter or to elongate from it. Indeed, differential effects on T7 RNA polymerase (T7RNAP) transcription *in vivo*

have been described for elements that are thought to operate through different transcriptional activation mechanisms (Chen *et al.*, 1987). In addition, T7RNAP-mediated transcription is stimulated by the presence of matrix-attachment regions upstream of a T7 promoter in chromatin templates *in vitro* (Jenuwein *et al.*, 1997). Together, these studies suggest that T7RNAP-mediated transcription of chromatin provides a sensitive probe to distinguish between different transcriptional regulatory mechanisms.

In an attempt to understand the molecular mechanisms underlying ES regulation, we have investigated the role of chromatin at the level of transcriptional accessibility to T7RNAP. *Trypanosoma brucei* is amenable to this approach *in vivo*, because T7RNAP-mediated transcription of chromatin is processive and transcripts are capped *in trans*, allowing efficient protein expression from transcripts generated by the heterologous polymerases (Wirtz *et al.*, 1994, 1998). The T7 promoter was inserted into different chromosomal locations, including different locations within an ES, in trypanosomes engineered to express T7RNAP.

Results

T7RNAP-mediated transcription is repressed only in the ES of the procyclic form

This study was performed using a trypanosome clone (19B) tagged with a neomycin phosphotransferase gene (*NEO*) 1 kb downstream of the active 121 ES promoter,

which allowed us to monitor the activity of the wild-type ES (Navarro and Cross, 1996). To study chromatin accessibility within a given ES *in vivo*, we established stable expression of T7RNAP in this clone. Constitutive nuclear expression of a T7RNAP was achieved by inserting a promoterless cassette containing the T7RNAP gene with SV40 nuclear localization sequences into the tubulin locus (Wirtz *et al.*, 1994). Among several clones obtained, 19BT1 expressed the highest levels of a functional T7RNAP, as determined by transient transfection with a plasmid bearing a luciferase reporter gene (*LUC*) driven by the T7 promoter (data not shown).

In order to target different chromosomal regions, we designed a cassette containing a bleomycin resistance (*BLE*) and *LUC* gene, flanked by appropriate polyadenylation and splicing sequences, driven by the T7 promoter, located downstream of the desired targeting sequence. Since homologous recombination occurs efficiently in *T. brucei*, the whole construct was inserted in the target locus by a single crossover event (Figure 1). After insertion of the cassette, reporter activity reveals the level of T7RNAP-mediated transcription in the targeted chromosomal context. Stable transformants were obtained in the bloodstream form and single clonal lines were differentiated to the procyclic form *in vitro* (Overath *et al.*, 1986), allowing analysis of individual clones in both developmental stages.

Initially, we integrated the cassette into three non-ES loci, to obtain baselines for T7RNAP-mediated transcrip-

Fig. 1. Insertion of the T7 promoter-driven reporter cassette into different chromosomal contexts. **(A)** Three loci, including rDNA, one allele of *RNP1* and the *VSG* 118 basic copy, were used as controls for the transcription of a *LUC*–*BLE* cassette by T7RNAP. Solid bars indicate the targeting sequences used for each locus. Relevant restriction sites, which linearized the targeting sequences, are indicated in the native locus. **(B)** Schematic diagram of the 121 and 221 ESs in *T. brucei* clone 19BT1. ES-associated genes (*ESAGs*) are indicated by boxes between the promoter (represented as a flag) and the telomeric *VSG*. The 50 bp repeats are represented by a hatched box upstream of the promoter. The *NEO* cassette is 1 kb downstream from the transcription initiation site of the active 121 ES (Navarro and Cross, 1996). Arrows indicate the three different insertions into the inactive 221 ES; upstream of the ES promoter, *ESAG3* and the *VSG* Ψ . 18S, 18S ribosomal RNA; *RNP1*, large subunit of RNA polymerase I; UTR, untranslated region; *LUC*, luciferase gene; *BLE*, bleomycin gene.

tion at different chromosomal loci (Figure 1). These regions were the non-transcribed ribosomal DNA (rDNA) spacer sequence, located between two Pol I transcription units, a region upstream of the Pol II-transcribed gene for the large subunit of RNA polymerase I (*RNP1*), and a region upstream from the non-transcribed basic copy of *VSG* 118, located at an internal chromosomal position. To select for insertion of the cassette into potentially repressed loci, all stable transformants were selected with a low concentration of phleomycin (1 µg/ml). Several independent transformant clones were obtained for all three loci. In the bloodstream form, the transcriptional activity of T7RNAP, as measured by luciferase activity, was similarly high in the rDNA spacer sequences, the *RNP1* locus and in the basic copy of *VSG* 118 (Figure 2).

To study the transcriptional accessibility of an inactive ES in the bloodstream form, we inserted the cassette upstream of the 221 ES promoter, which is inactive in clone 19BT1, which expresses *VSG* 121. Three independent stable transformants were selected with a low concentration of phleomycin and maintained for several weeks in the absence of any drug selection. The luciferase activity of these bloodstream clones indicated a transcriptional activity of the T7RNAP similar to the clones where the cassette was inserted into the three control loci (Figure 2), demonstrating that the regions around the inactive 221 ES promoter are accessible to the heterologous polymerase.

To study chromatin accessibility at these different chromosomal loci in the procyclic form, all bloodstream-form clones were simultaneously differentiated to the procyclic form and maintained under these conditions for 2 weeks. As measured by luciferase activity, efficient T7RNAP-mediated transcription was observed in *RNP1* and *VSG* 118 basic-copy loci and in the rDNA spacer, where it was somewhat elevated (Figure 2). In contrast, T7RNAP-mediated transcription from a T7 promoter inserted upstream of the 221 ES promoter was dramatically

repressed. We obtained additional clones in which the T7 promoter-driven cassette with upstream ES promoter targeting sequences had integrated into two uncharacterized inactive ESs, as determined by chromosomal and Southern hybridization (data not shown). T7 reporters inserted upstream of these other inactive ES promoter regions gave similarly high activities in the bloodstream form, and were also silenced upon differentiation, suggesting that all ESs are down-regulated by a T7RNAP-sensitive transcriptional repression in the procyclic form. The luciferase activity in these clones was 100-fold lower in the procyclic than in the bloodstream form, and a similar quantitative difference has been observed in the activity of the endogenous ES promoter in the bloodstream versus the procyclic form (Navarro and Cross, 1998). Northern analysis was too insensitive to detect a T7-driven *LUC* mRNA from the procyclic-form ES, but confirmed constant levels of T7RNAP mRNA in both developmental states (data not shown). Incorrect processing and concomitant degradation of *LUC* mRNA are highly unlikely, for several reasons, to be responsible for the observed down-regulation of luciferase activity derived from the ES in procyclic forms. The *LUC-BLE* cassette, including the untranslated regions containing splicing and polyadenylation signals, was identical in every construct. Insertion of the constructs by a single crossover event positioned a 3 kb DNA fragment of neutral pGEM sequences at the 3' end of the cassette, rather than trypanosome DNA. We also found no differences in the size of the *LUC* mRNAs arising from different chromosomal locations, both in the bloodstream and the procyclic forms, when luciferase mRNA was detectable. These observations strongly suggest that incorrect RNA processing cannot account for the down-regulation of luciferase activity derived from the ES in the procyclic form, and support the interpretation that chromatin-mediated silencing prevents T7RNAP from processively transcribing the procyclic-form ES.

Fig. 2. Luciferase expression from a T7 promoter inserted into different chromosomal contexts. The *LUC-BLE* cassette driven by the T7 promoter was inserted into different chromosomal loci, which are indicated below the bars. Values are means of three different measurements in the bloodstream form, or of three individual differentiation experiments in the procyclic form, from at least two independent clones. Error bars indicate standard deviations from the mean. RLU are represented on a logarithmic scale. For comparison, the two horizontal bars show the range of luciferase activities from the same *LUC-BLE* cassette driven by an active ES promoter in the bloodstream or procyclic form (Navarro and Cross, 1998). Background RLUs from wild-type cell lysates were maximally 0.03. The enzymatic activity of a purified luciferase protein (Boehringer) added to wild-type cell extracts was 3-fold higher in the procyclic than in the bloodstream form. To compensate for this difference, assays were performed with 3×10^5 cells in bloodstream or 10^5 in procyclic forms.

Fig. 3. ES promoter replacement strategy. Schematic diagram of the active 121 ES tagged with a *NEO* cassette. The active ES promoter, together with the *NEO* cassette, was replaced by a T7 promoter-driven *LUC-BLE* cassette or, as a control, by an ES promoter-driven *LUC-BLE* cassette, by homologous recombination. Solid black bars indicate the targeting sequences, which included sequences between the *SpeI* and *MscI* restriction sites upstream of the 121 ES promoter and 300 bp of the pGEM vector located at the 3' end of the *NEO* cassette. Recombinant clones obtained by this strategy were tested for their sensitivity to G418 before confirming proper integration by Southern and chromosomal blot analysis. *NEO*, neomycin; *LUC*, luciferase; *BLE*, bleomycin; UTR, untranslated region.

The repressed chromatin domain spans the entire ES

To define the extent of the repressed chromatin domain, we analyzed transcriptional accessibility from two other locations downstream of the 221 ES promoter. We inserted the T7 promoter cassette into a VSG pseudogene (*VSG Ψ*), which is located upstream of the telomeric VSG 221, and into *ESAG3*, which is located in a more central position within the ES (Figure 1). Several independent clones were obtained for each construct and luciferase activities were analyzed. In the bloodstream form, the level of T7RNAP-mediated transcription from these two regions within the ES was similar to that obtained when the T7 promoter was inserted upstream of the ES promoter (Figure 2). When these clones were differentiated to the procyclic form, the reporter activity was drastically down-regulated (Figure 2). Only the *VSG* 221 and the telomeric sequences remain downstream of the T7 promoter inserted into the *VSG Ψ*, and the T7 promoter is flanked by the 50 bp repeats region when inserted upstream of the ES promoter (Figure 1). Thus, T7RNAP-mediated transcription accessibility suggests that the repressed chromatin domain encompasses the entire ES, extending some 50 kb from the 50 bp repeats to the *VSG*.

Replacement of the ES promoter with the T7 promoter leads to a switch

Since transcription from a T7 promoter appeared to be as efficient as from an ES promoter in bloodstream forms, we decided to attempt to replace the active ES promoter with a T7 promoter in clone 19BT1, containing a *NEO* cassette 1 kb downstream of the active 121 ES promoter (Figure 3). As a control, we replaced the *NEO* cassette with the 121 ES promoter sequences and the *LUC-BLE* cassette. Four independent clones were obtained with a T7 replacement construct, at a frequency similar to that seen with constructs targeting other loci. Southern and pulsed-field gel electrophoresis analysis confirmed the proper replacement of the ES promoter and *NEO* cassette

Fig. 4. Replacement of an active ES promoter by a T7 promoter results in an *in situ* ES switch. (A) Western blot analysis of clones in which the ES promoter was replaced by an ES promoter and the *LUC-BLE* reporter cassette (ESR1), clones in which the T7 promoter-reporter cassette was inserted into the *VSG Ψ* (T7Ψ3), and clones in which the ES promoter was replaced by the T7 promoter-reporter cassette (T7R1, T7R4, T7R5 and T7R7). The 221 and 121 VSGs were detected using polyclonal rabbit antisera depleted of cross-reacting antibodies (121Ab, 221Ab). (B) Northern blot analysis (4 µg of total RNA) of different clones, including a control with the ES promoter replacement (ESR1), the T7 promoter replacement clones T7R1, 4, 5, 7 and a 221 wild-type clone (221wt). The blot was hybridized with the different probes indicated on the right. *LUC*, luciferase; *TUB*, tubulin.

with the T7 promoter-driven *LUC-BLE* cassette (data not shown). These clones all showed high luciferase activities, in the range of 200–400 relative light units (RLU). However, as measured both by Western and Northern blot analysis, these clones no longer expressed *VSG* 121, while all control clones stably expressed *VSG* 121 (Figure 4). Indirect immunofluorescence analysis indicated that, in one of these clones (T7R4), >99% of the cells expressed *VSG* 221. Thus, deletion of the formerly active 121 ES

promoter by the T7 replacement construct led to the stable activation of the 221 ES in this clone. The other three recombinant clones switched transcription to other ESs, different from the 221 ES, indicating that disruption of the active 121 ES promoter led to non-directed, random switching events.

The *in situ* ES switching frequency in *T.brucei* strain 427 is generally very low, $\sim 10^{-6}$ (Horn and Cross, 1997b). The inactivation frequency of the specific *NEO*-tagged 121 ES clone used in the present study was between 2.3×10^{-6} and 8×10^{-7} (Navarro and Cross, 1998), and the frequency with which specific stable transformants were obtained was approximately one in 10^5 of the cells that survived electroporation. The coincidence of both events, which would be necessary for a spontaneous switch to occur in the few transformed cells, is therefore highly unlikely. We therefore conclude that the observed switching events must have occurred almost instantaneously upon deletion of the active ES promoter, in each of the four recombinant clones. Otherwise, we would have observed a delay in outgrowth of resistant clonal cell lines, and/or a mixed population of cells expressing different VSGs. Thus, these data indicate that replacement of the ES promoter by the T7 promoter, which drives transcription of the marker gene at a high level but fails to reach the 50 kb remote VSG, is accompanied by a rapid *in situ* switching event.

Discussion

Many transitions in chromatin structure are thought to occur during development, and such global alterations can contribute to establishing and maintaining states of differential transcriptional activity (Dillon and Grosveld, 1994; Vermaak and Wolffe, 1998). In the present study, we have investigated chromatin accessibility in *T.brucei*, using T7RNAP-mediated transcription from a T7 promoter as a reporter *in vivo*. We found that heterologous transcription was repressed in the procyclic ES, in contrast to other loci, suggesting a specific role for chromatin remodeling in the developmental down-regulation of the ES.

The use of a heterologous promoter excluded the possibility of inherent transcriptional regulation, as has been observed with endogenous promoters. Previous studies using an endogenous Pol I promoter suggested that a chromatin-mediated silencing mechanism contributed to the regulation of ES transcription in the bloodstream form (Horn and Cross, 1995). A ribosomal RNA (rRNA) promoter inserted into an inactive ES was repressed. However, this repression was not specific for the ES locus, since repression was also observed in the non-telomeric basic copy of *VSG118* (Horn and Cross, 1997a). Upon differentiation to the procyclic form, the rRNA promoter inserted into the basic copy of the *VSG118* was fully active, while in the ES it regained only 10% of its maximum activity (Horn and Cross, 1997a). Thus, the rRNA promoter was actually repressed in the ES upon differentiation, relative to the basic-copy *VSG118* locus. These results agree with our observed silencing of the ES-associated T7 promoter in the procyclic form.

Transcriptional silencing of T7RNAP was not restricted to the ES promoter region, but spanned the entire ES. The repressed chromatin domain presumably extended between

two stretches of DNA repeats, the 50 bp direct repeats at the 5' end of the ES promoter and the telomeric repeats downstream of the VSG. Such repeats might insulate the ES, establishing the boundaries of the repressed chromatin domain, as has been described in higher eukaryotes (Dunaway *et al.*, 1997; Robinett *et al.*, 1997).

The molecular mechanisms responsible for this developmental chromatin remodeling are unknown. Initiation and elongation by T7RNAP *in vitro* are inhibited by higher eukaryote nucleosomes on reconstituted templates (Wolffe and Drew, 1989; O'Neill *et al.*, 1992) or in cell nuclei (Jenuwein *et al.*, 1993). In *Drosophila* embryos, however, conditions that increase histone acetylation increase T7RNAP-mediated transcription (McCall and Bender, 1996). T7RNAP transcriptional repression by nucleosomes *in vitro* seems to require the presence of histone N-terminal domains (Chirinos *et al.*, 1998). These N-terminal tails are thought to direct the association or dissociation of histones with the DNA (Luger *et al.*, 1997; Wade *et al.*, 1997). Thus, N-terminal histone modification might represent a possible molecular mechanism responsible for the developmental silencing of the ES in the procyclic form. Unfortunately, nothing is known about histone modification in *T.brucei*.

A striking difference between the bloodstream and procyclic-form ESs, at the level of DNA modification, is that the 50-bp and telomeric repeats of the bloodstream form contain an abundant nucleotide modification, β -D-glucosyl-hydroxymethyluracil, or base J (van Leeuwen *et al.*, 1997). This DNA modification may lead to developmental differences in the coating of the entire ES domain by non-histone proteins, from the 50 bp repeats region to the telomeric end.

The transcriptional mechanisms underlying ES *in situ* activation/inactivation in bloodstream forms are not understood. A rRNA promoter replacing the ES promoter has been reported to be switched efficiently on and off, suggesting that the ES switching is promoter independent (Rudenko *et al.*, 1995). However, there is evidence from other lower eukaryotes that only a subset of the rRNA gene copies is active, suggesting that a rRNA promoter can be in either an active or an inactive state (Dammann *et al.*, 1993, 1995). Thus, it seems possible that epigenetic transcriptional switching events represent an intrinsic feature of Pol I promoters, which might explain why the switchable polycistronic ES is transcribed by Pol I instead of Pol II.

T7RNAP transcription of an inactive ES in the bloodstream form was not repressed. We cannot rule out that a subtle Pol I-sensitive and T7RNAP-insensitive chromatin modification down-regulates inactive ESs in the bloodstream form. The mechanism ensuring transcriptional inactivity of the bloodstream-form ESs could operate via a silencing mechanism akin to the hop-and-skip model proposed for the polycomb group (PcG) (reviewed by Pirrotta, 1998). The precise means by which PcG complexes silence transcription has not been elucidated, but empirically, it does not prevent access by T7RNAP to its promoter (McCall and Bender, 1996).

We and others found no significant differences in chromatin structure between active and inactive ESs in the bloodstream form, either at the promoter region (Navarro and Cross, 1998), or at the telomeric VSG region (Greaves

and Borst, 1987), as measured by micrococcal-nuclease sensitivity assays. Conversely, chromatin sensitivity assays using DNase I in isolated nuclei have shown an increased accessibility of the active telomeric VSG sequences compared with their chromosome-internal non-expressed basic copies (Pays *et al.*, 1981; Pays and Nolan, 1998). Likewise, the active *vsg* was preferentially digested by single-strand-specific endonucleases (Greaves and Borst, 1987). Nevertheless, a tagged ES promoter region did not show preferential DNase I cleavage in active versus inactive transcriptional states (M. Navarro and G.A.M. Cross, unpublished results). Thus, higher nuclease accessibility of the active ES in bloodstream form may be restricted to the telomeric end, rather than at the promoter region. In addition, previous studies identified a prominent nuclease-hypersensitive site common to active and inactive ES promoters, suggesting that inactive ES promoters, in the bloodstream form, contain factors that are poised and ready to recruit the Pol I holoenzyme when the ES is activated (Navarro and Cross, 1998). Our current results, showing rapid switching after ES promoter disruption, support the idea that, in bloodstream forms, inactive ESs comprise potentially active, rather than actively repressed, chromatin domains.

We were able to provoke antigenic switches by disrupting the active ES promoter with the T7 promoter, reproducing a real *in situ* ES activation. This suggests that ES activation depends upon inactivation of the formerly active ES, indicating that activation and inactivation are coupled processes. Disruption of other aspects of ES organization also increased switching rates (Davies *et al.*, 1997). These observations suggest that disabling transcription of the active ES permits cells to switch to an alternative ES, as has been suggested elsewhere (Cross *et al.*, 1998). The ability of an inactive ES to be rapidly activated suggests that the epigenetic regulation of the ES in the bloodstream form might be mediated by a nuclear compartmentalization phenomenon, as previously suggested (Shea *et al.*, 1987). The existence of a high level of DNA structural organization in the interphase nucleus of *T. brucei* has been hypothesized to play a role in the control of gene expression (Chung *et al.*, 1990). We favor a model in which the active ES is located in a unique transcriptional environment within the nucleus. While inactive ESs inefficiently compete with the active one for this location, disrupting Pol I transcription of the active ES might lead to a vacancy in this compartment, allowing occupancy by another ES. In sum, our results suggest the existence of two independent transcriptional control mechanisms for ES regulation in *T. brucei*: a chromatin remodeling of the developmentally down-regulated ES in the procyclic form, and a nucleation site recruitment-dependent activation of a transcriptionally accessible ES in the bloodstream form, which allows rapid antigenic switching.

Materials and methods

Trypanosomes and differentiation conditions

Trypanosoma brucei clone 221a [Molteno Institute Trypanozoon antigenic type 1.2 (MITat 1.2) clone 221a] was derived from strain 427 (Johnson and Cross, 1979). The *NEO* cassette was inserted into the inactive 121 ES in the 221a clone and the 121 ES was subsequently

selected for the active state (Navarro and Cross, 1996). Trypanosomes were routinely cultured in HMI-9 at 37°C (Hirumi and Hirumi, 1989). The homogeneity of trypanosome populations (based on counts of ~500 parasites) was verified by indirect immunofluorescence using specific antibodies against VSG 121 and VSG 221, together with DAPI as a nuclear stain. Cultured bloodstream cells were differentiated to procyclic forms by transferring 2×10^7 cells into 5 ml of depleted trypanosome medium (DTM) with 15% fetal bovine serum and 3 mM citric acid cycle intermediates at 27°C (Overath *et al.*, 1986), without feeder cells. Procyclic cultures were established for 2 weeks prior to analysis.

Plasmid constructs and transformation conditions

To obtain a clone constitutively expressing T7RNAP, the plasmid pHD328 (Wirtz *et al.*, 1994) was inserted in the 121 ES *NEO*-tagged cell line described previously (Navarro and Cross, 1996) and stable transformant clones selected with 2 µg/ml of hygromycin. The reporter cassette containing the *LUC* gene flanked by *T. brucei* actin splice acceptor and a truncated version of the aldolase polyadenylation sequence that does not confer developmental regulation (Hotz *et al.*, 1995), together with the *BLE* gene flanked by *T. brucei* actin untranslated regions, located downstream of the *SpeI*-*SstI* 121 ES promoter restriction fragment (pMig20) was described previously as the control construct (Navarro and Cross, 1998). The *LUC*-*BLE* cassette driven by a T7 promoter, which targets the rDNA locus (pLew82), was described elsewhere (Wirtz *et al.*, 1998). To construct *RPNI*-targeted reporter pLew113, pLew112 was first generated by blunt-end cloning of a *NotI*-*Asp718I* fragment of pLew107, a close relative of pLew29 (Wirtz *et al.*, 1999) into the *PvuII* site of pLew19 (Wirtz *et al.*, 1998). pLew113 was constructed from pLew112 and pLew82 (Wirtz *et al.*, 1998) by exchange of the *PvuI*-*PacI* fragments. pLew118 was constructed from pMig20 (Navarro and Cross, 1998) through cloning intermediates, pLew93, pLew97 and pLew109. pLew93 was made by replacement of the *PmaCI*-*BglIII* fragment of pMig20 with a similarly digested PCR product generated using oligonucleotide primers EW33 (5'-GAGGCGTTCTTT-TCCCACGAG-3') and EW35 (5'-GAAGATCTACCGGTGCAGTCTG-ATATCTGAGATTTC-3'), with pMig20 as template. This effectively changed the ES promoter start site CA dinucleotide to GC and inserted *AgeI* and *BglIII* sites immediately downstream, at the same time deleting the downstream region to the *BglIII* site of pMig20. pLew97 was made from pLew93 by inserting an annealed, kinased oligonucleotide pair, consisting of EW12 and EW13 (Wirtz *et al.*, 1998), into the *AgeI* site of pLew93. A 168 bp *PvuII*-*EcoRV* fragment of Bluescript SK+ (Stratagene), containing the T7 promoter and part of the multiple cloning site, was inserted into the *BglIII* site of pLew97 after filling the 5' overhangs with Klenow, to give pLew109. *PmaCI*-digested pLew109 was partially digested with *BssHI*, and the 6.7 kb partial product was ligated to a *PmaCI*-*BssHI*-digested PCR product generated using primers EW33 and EW46 (5'-TTGGCGCGCTTAAGCCGATTAGCAGTCTG-3'), with pLew109 as template, yielding pLew118. A DNA fragment obtained by digestion of VSG Ψ plasmid (Horn and Cross, 1997a) with *AlwNI* and *MluI* (filled by Klenow) containing the VSG Ψ (1.1010 pseudogene) was ligated to pLew118 digested with *AlwNI* and *AlfII* (filled by Klenow) (restriction site located upstream of the T7 promoter in pLew118), which generated pMig39. The 221ES *ESAG3* targeting sequences were generated by PCR using the oligonucleotide 5'-GGGCC-GCAACTAGTGCCTAGATGGGTTCTGAAATTTT-3' priming upstream of *ESAG3*, subcloned previously (Horn and Cross, 1997a), and 5'-AATCCGAGGCTTAAGTGCGGCCATCTTGGCCATGC-3'. This DNA fragment was digested with *SpeI* and *AlfII* and ligated to pLew118 digested with the same enzymes, to generate pMig44. The region upstream of the 221 ES promoter was obtained by PCR, using pRK8(+) 221 ES promoter genomic clone as a template (Zomerdijsk *et al.*, 1991) and the oligonucleotide 5'-TAGAGCGTAATAATGATATTTATAT-3', priming upstream of the *SpeI* site, located upstream of the 221 ES promoter, and the oligonucleotide 5'-CACCCTGCAGGCCATCTTGG-CCCACCTCTCTCTCTCCGAGAG-3' digested with *SpeI* and *SfiI* (located upstream of the T7 promoter in pMig44) and ligated to pMig44 digested with the same, to generate pMig63. The targeting sequences for the insertion of the T7 promoter into the VSG 118 basic copy were obtained by digestion of the plasmid 5' of VSG 118 (Horn and Cross, 1997a), digested with *SpeI* and *MscI* and ligated to pMig44 digested with *SpeI* and *SfiI* (T4 trimmed), to generate pMig65. The 5' targeting sequences used for the replacement of the ES promoter were generated by PCR using the oligonucleotide 5'-GGCCGCTCTAGAAGTGTGG-GAA-3' and the oligonucleotide 5'-CCGGCGGCCATCTTGGCCCAA-CGTTAATGCAACTCCTC-3', priming at ~169 bp of the initiation site, using the 121 ES promoter clone pMig20 as a template. This DNA

fragment was digested with *SpeI* and *SfiI*, and ligated to pMig44 digested with the same enzymes to generate pMig57. As a 3' targeting sequence, we used 300 bp of the vector pGEM, located at the 3' end of the *NEO* cassette. The presence of the pGEM fragment provided a unique targeting sequence, which, together with the upstream region of the 121 ES promoter, produced the chosen replacement. All constructs were sequenced at The Rockefeller University Shared Technology Resource Center, from which synthetic oligonucleotides were also obtained. Plasmid DNA for transfection was prepared using anion exchange columns (Qiagen Inc). Trypanosomes were grown in HMI-9 at 37°C and electroporated in Cytomix (van den Hoff *et al.*, 1992) with 10 µg of linearized plasmid DNA in each cuvette. After electroporation, surviving cells were counted and distributed in a 24-well plate (1–4×10⁴/well). The next day, one volume of medium containing 2 µg/ml phleomycin (Cayla, Toulouse, France) was added to the wells. Clonal cell lines were derived directly from drug-resistant positive wells (5–10% of the wells were positive). Cultures were subsequently grown in the presence of 2 µg/ml of hygromycin, to assure the expression of T7RNAP, but in the absence of phleomycin.

RNA analysis and probes

RNA was isolated using RNastat60 (Tel-Test Inc), and electrophoresed using 4 µg RNA/lane in a formaldehyde-agarose gel, then transferred to Hybond membrane and cross-linked to the membrane in a UV Stratalinker 1800 (Stratagene). All the restriction fragments used as probes were described previously (Navarro and Cross, 1996), except for the luciferase probe, which was a *HindIII*–*BamHI* restriction fragment obtained by digestion of the reporter plasmid. Probes were generated using the Prime-It II labeling kit (Stratagene) with a Prep-A-Gene (Bio-Rad) gel-purified DNA. All hybridization and washing of filters was at 65°C in a hybridization oven (Hybaid). Post-hybridization washes were twice in 2× SSC and twice for 30 min at 65°C in 0.2× SSC and 0.1% SDS. RNA size was estimated according to the migration of an RNA ladder (Gibco-BRL). After hybridization, the probes were stripped as recommended by the manufacturer (Amersham). Western blotting was performed as described previously (Horn and Cross, 1995).

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