

Analysis of *Trypanosoma brucei* *vsg* expression site switching in vitro

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Abstract

Trypanosoma brucei can undergo antigenic variation by switching between distinct telomeric variant surface glycoprotein gene (*vsg*) expression sites (ESs) or by replacing the active *vsg*. DNA rearrangements have often been associated with ES switching, but it is unclear if such rearrangements are necessary or whether ES inactivation always accompanies ES activation. To explore these issues, we derived ten independent clones, from the same parent, that had undergone a similar *vsg* activation event. This was achieved in the absence of an immune response, in vitro, using cells with selectable markers integrated into an ES. Nine of the ten clones had undergone ES switching. Such heritable changes in transcription state occurred at a frequency of approximately 6×10^{-7} . Comparison of switched and un-switched clones highlighted the dynamic nature of *T. brucei* telomeres, but changes in telomere length were not specifically associated with ES switching. Mapping within and beyond the ESs revealed no detectable DNA rearrangements, indicating that rearrangements are not necessary for ES activation/inactivation. Examination of individual cells indicated that ES activation consistently accompanied inactivation of the previously active ES. In some cases, however, we found cells that appeared to have efficiently established the switched state but which subsequently, at a frequency of approximately 2×10^{-3} , generated cells expressing both pre- and post-switch *vsgs*. These results show that ES activation/inactivation is usually a coupled process but that cells can inherit a propensity to uncouple these events. © 1997 Elsevier Science B.V.

Keywords: Antigenic variation; Expression site; Telomere; *Trypanosoma brucei*; Variant surface glycoprotein

Abbreviations: ES, expression site; TRF, telomere restriction fragment; Vsg (*vsg*), variant surface glycoprotein (gene).

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1. Introduction

In the blood and tissue spaces of the mammalian host, *Trypanosoma brucei* escapes elimination by the host immune response by a process of antigenic variation (reviewed in [1,2]). Antigenic

variation involves the mutually exclusive and sequential expression of individual variant surface glycoprotein (Vsg) genes (*vsg*) derived from a repertoire of approximately 1000 *vsgs* and pseudo-*vsgs* [3]. The expressed *vsg* is invariably located at the telomere-proximal end of a polycistronic transcription unit known as an expression site (ES). A number of ES-associated genes are also present in these polycistronic transcription units and the ES promoter is located approximately 50 kb upstream of the *vsg*. Antigenic variation is spontaneous and can occur by a number of mechanisms, but most commonly involves replacement of the active *vsg*, by gene conversion at the active ES, or by switching transcription from one ES to another (reviewed in [1,2]). The *vsgs* at silent telomeric ESs are more likely to become expressed and can be activated by either of these mechanisms [4].

We [5] and others [6] have recently shown that position effects repress transcription at a silent *T. brucei* ES. We wish to discover how ES domains faithfully and heritably maintain their transcriptional status over many generations, yet infrequently allow alterations of state. DNA rearrangements can alter gene expression in a number of organisms [7] and rearrangements within an ES, or upstream of the promoter region, have been found after ES switching, (reviewed in [8]), but it is unclear if any of these changes are necessary for ES switching. We wanted to determine, using more stringent experimental protocols than hitherto possible, whether any DNA rearrangements are necessary for ES switching, or whether ES switching can be attributed solely to epigenetic phenomena. We also wanted to determine if ES inactivation invariably accompanies ES activation. There are estimated to be approximately 20 bloodstream-form ESs in the *T. brucei* genome [9–12]. Usually, only one ES is active at a time in individual bloodstream-form cells and ES activity is controlled at or near transcription initiation [10], with notable exceptions. In a switch associated with a large insertion upstream of the silenced *vsg*, the region upstream of the insertion continued to be transcribed [13]. Furthermore, stable expression of two *vsgs* from different ESs in individual cells has been reported in

T. equiperdum [14], suggesting that ESs can switch on and off independently.

In *T. brucei* clone 118a, a single copy of *vsg221* is present at a transcriptionally repressed ES. This *vsg* can be activated via duplication or ES switching [15]. Activation of the *vsg221* ES occurred with no detectable rearrangement up to 55 kb upstream of *vsg221* [15] and inactivation occurred without a single point mutation within 3.4 kb spanning the ES promoter [10]. The active ES in clone 118a can also undergo inactivation and subsequent reactivation [16]. To capture and study multiple examples of activation of the *vsg221* ES, we inserted genes encoding resistance to phleomycin and G418 into the silent *vsg221* ES in 118a cells [5]. Acquisition of drug-resistance can consequently be exploited to capture clones in which the *vsg221* ES has become transcriptionally active, or the marker cassette has been duplicated to a transcriptionally active locus. Activation events can be captured immediately while minimizing sub-culturing or other procedures that could allow unrelated DNA rearrangements to accumulate. Also, because drug selection, as opposed to immune selection, does not require inactivation of the previously active *vsg* for survival, our approach allows the isolation of cells that activate a second ES without inactivating the previously active ES.

In this study, we show that detectable DNA rearrangements are not necessary for ES switching and that ES activation usually accompanies inactivation of the previously active ES. Our switched clones, however, inherited different phenotypes. Some of these clones inherited a propensity to uncouple ES activation and inactivation leading to the generation of cells expressing two *vsgs*.

2. Materials and methods

2.1. Cells

Cells were maintained and cloned as previously described [5]. *T. brucei* Mitat 1.5 clone 118a was derived from stablate 049 and Mitat 1.2 clone 221a was derived in culture from stablate 052 [17,18]. G418 was purchased from Gibco and phleomycin from Cayla.

2.2. DNA analysis

Genomic DNA was isolated and Southern blotting was carried out as previously described [5]. Agarose blocks containing intact *T. brucei* chromosomes were prepared as described for mammalian cells [19]. Restriction enzyme digestion of DNA in agarose was carried out according to the manufacturer (New England Biolabs). DNA embedded in agarose was size-fractionated using a Rotating Agarose Gel Electrophoresis apparatus (Stratagene). Gels were prepared from SeaKem LE agarose (FMC) in 0.5 × TBE and the electrophoresis temperature was kept constant at 12°C. *Hansenula wingei*, *Saccharomyces cerevisiae*

and *Schizosaccharomyces pombe* chromosome markers were from Bio-Rad and low-range pulsed field gel electrophoresis markers were from New England Biolabs. Large DNA fragments were visualized and transferred to membrane as described [19]. All post-hybridization washes were at 65°C in 0.2 × SSC and 0.2% SDS.

2.3. Protein analysis

Western blotting and immunofluorescence analysis were carried out as previously described [5]. For detection of Neo or Ble, protein from 3 × 10⁵ cells was loaded per lane. For detection of Vsg, protein from 1.5 × 10⁵ cells was loaded per lane. Vsg, Neo or Ble proteins were separated in 10, 12.5 and 17.5% polyacrylamide gels, respectively. The antiserum to Vsg221 was depleted of antibody to the cross-reacting determinant [20].

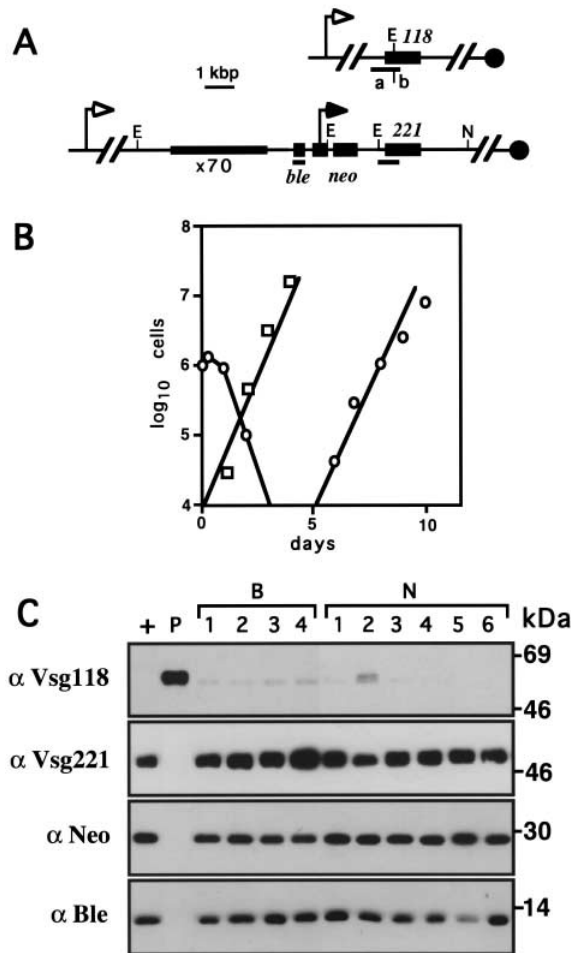


Fig. 1. Selection of switched cells in vitro. (A) Physical maps of the *vsg118* and *vsg221* ES loci in 118⁺/bRn cells, which express *vsg118* [5]. The endogenous ES promoters and the inserted rRNA promoter are indicated as open and filled arrowheads respectively. The telomere is indicated downstream of each *vsg*. ×70 represents imperfect '70 bp' repeats, and some relevant restriction sites are shown. Abbreviations used for restriction enzymes are E, *Eco*RI; N, *Nar*I. Probes used for Southern analysis are indicated as bars below the maps; *vsg118* probe a contains 730 bp directly upstream of the *Eco*RI site; *vsg118* probe b contains 200 bp directly downstream of the *Eco*RI site; the *ble* probe contains the entire coding region from pUT-56 [26] and the *vsg221* probe is a 770-bp fragment containing the first 520 bp of the coding region. (B) Selection of drug-resistant cells from 118⁺/bRn cultures. 10⁶ 118⁺/bRn cells were transferred to medium containing 1 μg ml⁻¹ phleomycin (O). As a control, approximately 10⁴ cells were grown in medium without phleomycin (□). Cell density was determined in both cultures after 6 h and every day for ten days. (C) Western analysis of ten independent drug-resistant clones. 221⁺/bRn cells act as a positive control for cells that have activated the linked *ble*, *neo* and *vsg221* genes. This strain was generated by inserting selectable markers directly into an active *vsg221* ES [5]. Protein was isolated from 221⁺/bRn cells (lane +), 118⁺/bRn parental cells (lane P), four independent clones derived by phleomycin selection (selection for activation of *ble*, lanes B1–B4) and six independent clones derived by G418 selection (selection for activation of *neo*, lanes N1–N6). Proteins were detected using the primary antibodies indicated to the left of each panel.

3. Results

3.1. Selection of cells in which *vsg221* was activated

We have previously reported insertion of a cassette (pbRn), containing an rRNA promoter flanked by selectable markers encoding resistance to phleomycin (*ble*) and G418 (*neo*), just upstream of *vsg221* in cells expressing *vsg118* (118^+ /bRn cells, see Fig. 1A). Transcription from the inserted rRNA promoter is repressed at this locus and the upstream ES promoter is inactive, so the recombinant cells were resistant to only low levels of G418 and remained completely sensitive to phleomycin [5]. 118^+ /bRn cells continue to express Vsg118 and do not express detectable Vsg221 (see Fig. 1C) because transcription from the inserted but repressed rRNA promoter is approximately 80-fold lower at this locus relative to an active ES [5]. Because *ble*, *neo* and *vsg221* were closely linked in 118^+ /bRn cells, these markers were expected to be activated concomitantly, whether by duplicative or non-duplicative mechanisms (Fig. 1A). The design of these experiments allowed us to examine the activation and inactivation of two well characterized ESs [10,11,21–24] in a panel of independent clones.

Indirect immunofluorescence analysis indicated that more than 99% of 118^+ /bRn cells were expressing Vsg118 and that cells expressing Vsg221 were undetectable (<0.1%). This showed that *vsg221* remained stably repressed in 118^+ /bRn cells [5]. In order to see if *ble* and *neo* could be expressed at increased levels in 118^+ /bRn cells, they were grown in drug concentrations above their previously determined IC_{50} values [5]. Using phleomycin ($IC_{50} < 0.2 \mu\text{g ml}^{-1}$) at $1 \mu\text{g ml}^{-1}$, we expected to select for cells in which the upstream ES promoter was activated, or in which the cassette was duplicated and/or translocated downstream of a fully active promoter. Using G418 ($IC_{50} = 13 \mu\text{g ml}^{-1}$) at $50 \mu\text{g ml}^{-1}$, we expected to select for the same events and, possibly, for the independent activation of the inserted rRNA promoter (Fig. 1A). This type of event could conceivably lead to antigenic variation because an rRNA promoter can replace the ES

promoter at the active ES [6]. *T. brucei* cells appear to produce a growth inhibitor [25]. To ensure that this inhibitor did not prevent the growth of any drug-resistant cells, drugs were applied to cultures at a density of $10^5 \text{ cells ml}^{-1}$ in 10 ml cultures. In this way we obtained phleomycin-resistant cells, which grew at a similar rate to the parental cells (Fig. 1B). We did not obtain any G418 resistant cells in this particular experiment (see below). To estimate the frequency at which phleomycin-resistant cells arose, 24 independent sub-clones were derived from 118^+ /bRn cells. 2×10^5 cells of each clone were transferred to 2 ml cultures containing $1 \mu\text{g ml}^{-1}$ of phleomycin. Three of the 24 sub-clones generated resistant cells, giving an estimate of *ble* activation frequency of 6.25×10^{-7} . Phleomycin kills cells by breaking DNA [26], so we were concerned that this might affect the *vsg* switching mechanism or stimulate a switch. We therefore used G418 to select additional switched clones. 10^6 cells from ten of the 118^+ /bRn sub-clones that had not generated phleomycin-resistant cells were placed in $50 \mu\text{g ml}^{-1}$ G418 in 10 ml cultures. Six of the ten sub-clones generated G418-resistant cells, giving an estimate of *neo* activation frequency of 6×10^{-7} . All ten independent drug-resistant cultures (B1–B4 and N1–N6) exhibited high-level resistance to both drugs, indicating that selection in G418 did not lead to independent activation of the rRNA promoter (see Fig. 1A) and that intermediate increases in drug-resistance probably did not occur. The ten resistant cultures were immediately cloned, to ensure that all resistant cultures were derived from a single activation event, and expanded in the absence of drug selection so that we could see how stable the resistant phenotype was. DNA and protein samples were prepared from each clone as soon as possible, to minimize any subsequent phenotypic or genotypic changes. Western blotting (Fig. 1C) with antibodies against Vsg118 (the antiserum to Vsg118 was not depleted of antibody to the cross reacting determinant [20], so some cross reaction with the faster migrating Vsg221 is visible in this panel), Vsg221, Neo and Ble, indicated that every drug-resistant clone was expressing Vsg221, in place of Vsg118 (residual Vsg118 was detectable in clone N2), as well as

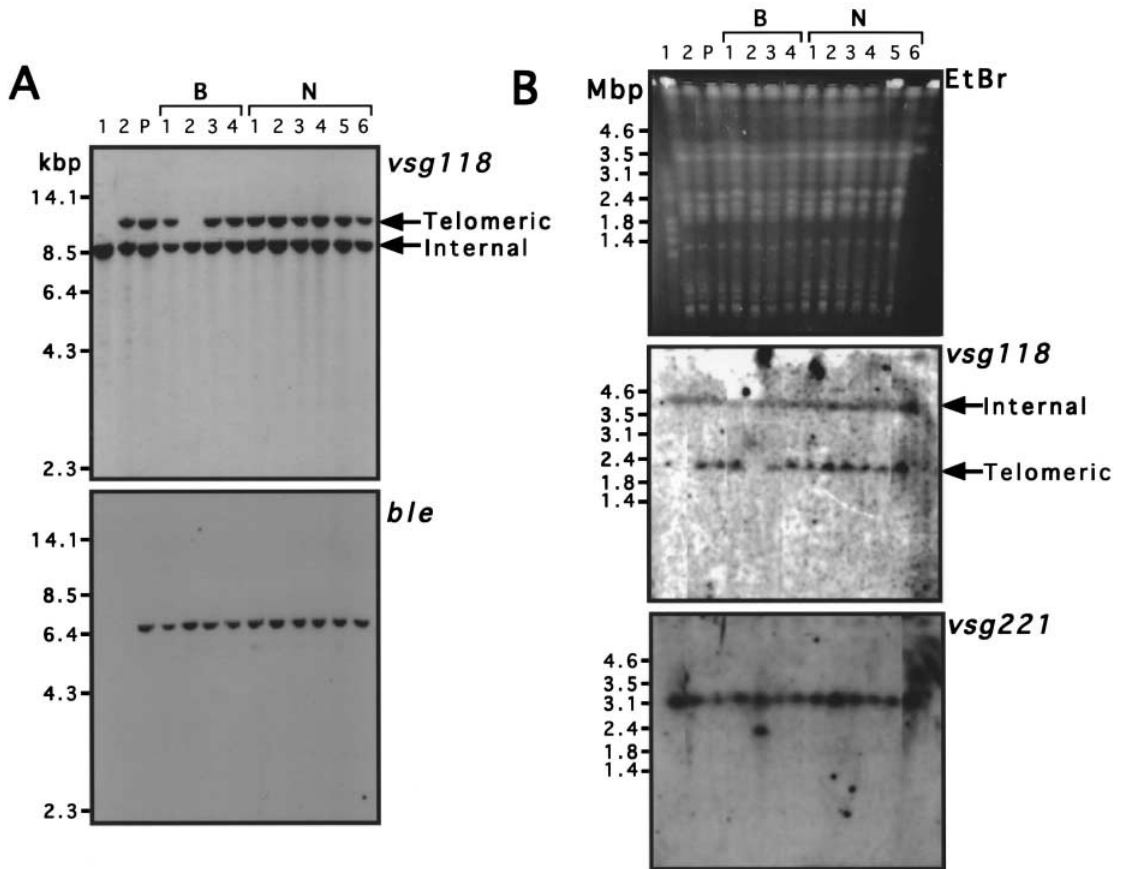


Fig. 2. Chromosomal analysis. (A) Genomic DNA (3 μ g) from wt221a (lane 1), wt118a (lane 2), 118⁺/bRn parental cells (lane P), and the ten *vsg221*-expressing (switched) clones (B1–B4 and N1–N6) was digested with *Eco*RI and the resulting blot was sequentially hybridized with the *vsg118* (probe a) and *ble* probes (see Fig. 1A). The chromosome-internal and telomeric copies of *vsg118* are indicated. (B) Chromosomal DNA from the same strains listed in (A) was separated by Rotating Agarose Gel Electrophoresis (see Section 2) in a 0.8% gel. The rotation angle was set at 106°. Pulse field conditions were a linear ramp, 100–300 s for 10 h at 120 V, followed by a linear ramp, 1000–2500 s for 80 h at 50 V. *H. wingei* and *S. pombe* size markers can be seen in the extreme left and right un-numbered lanes respectively. Molecular size markers are indicated in megabase pairs (Mbp). The top panel shows the ethidium bromide stained gel. A blot of this gel was hybridized with *vsg118* (probe a, middle panel) and *vsg221* (lower panel).

high levels of Neo and Ble. This indicated that the switched phenotype was stable in the absence of drug selection, in most cases.

3.2. *vsg* Switching occurred via *vsg* duplication or expression site switching

A Southern blot hybridized with a *vsg118* probe indicated that nine of the ten clones generated from 118⁺/bRn cells retained the telomeric copy of *vsg118* found in the parental cells (Fig. 2A).

These results suggested that *vsg221* was activated in most of the clones by a non-duplicative mechanism. The telomeric copy of *vsg118* has been replaced by another *vsg* in wt221a cells [4] (for a description of the lineage of the clones used in these experiments see Materials and methods and [17,18]). Similarly, disappearance of the telomeric copy of *vsg118* in one of the switched clones (B2) suggested that *vsg221* may have been duplicated, replacing *vsg118* in this clone. Rehybridizing this blot with a *ble* probe showed that each clone was

indistinguishable from the parent in this digest. Any alterations in the length of the block of '70 bp' repeats at the activated *vsg221* locus [27] would have been detected in this fragment (see Fig. 1A). It has previously been shown that alterations of the 70-bp repeat region are not necessary for ES switching to occur [28] and consistent with these results, we detected no rearrangements in this region following ES switching.

To determine whether large-scale changes had occurred in chromosome size, and to determine which mechanisms were involved in *vsg* switching, we separated chromosomal DNA by pulsed field gel electrophoresis (Fig. 2B). A chromosome-internal copy of *vsg118* was present in all of the clones on a chromosome of approximately 4 Mbp [29]. In 118a (lane 2) and 118⁺/bRn parental cells (lane P), the expressed copy of *vsg118* is on a chromosome of approximately 2 Mbp. The chromosome blot hybridized with a *vsg118* probe confirmed the absence of the telomeric copy of *vsg118* in wt221a cells (lane 1) and one of the switched clones (B2). Hybridization of the same blot with a *vsg221* probe indicated that *vsg221* was duplicated, replacing *vsg118* in clone B2. In all the other clones, the single copy of *vsg221* was on a chromosome of approximately 3 Mbp, indicating that the ES containing *vsg221* was activated via ES switching in nine out of the ten switched clones.

3.3. Telomere length measurements following expression site switching

Rearrangements at the telomere have been reported to be specifically associated with ES switching [30]. We measured the telomere restriction fragment (TRF) length, at the activated (*vsg221*) and inactivated (*vsg118*) ES, in 118⁺/bRn cells and in all nine clones that had undergone ES switching. Two other *vsg* cDNA clones (*vsgbR2* [4] and *vsgB* [12]), which have copies at silent telomeric loci in 118⁺/bRn cells, allowed us to also measure the TRF length at two non-switched loci. *EcoRI* digestion allowed us to determine TRF length at all four telomeres on the same blot (Fig. 3). From initial TRF length

data, using clones that had undergone ES switching, it was unclear if TRF length changes were associated with cloning or switching. We therefore derived nine sub-clones from 118⁺/bRn parental cells (independent from the 24 118⁺/bRn sub-clones described in Section 3.1) for comparison (Fig. 3). The panel of sub-clones indicated that the silent telomeres (*vsg221*, *vsgbR2*, *vsgB*) remained relatively stable. Phosphorimager analysis of the distribution of TRF lengths at the active telomere (*vsg118*) in the parental 118⁺/bRn population indicated that a heterogeneous distribution had accumulated during clonal growth (Fig. 3). This suggested that cloning involves selection of cells with different TRF lengths rather than inducing shortening. Re-cloning after activation of the *vsg221* ES can also account for the different *vsg221* TRF lengths seen in the switched clones. We saw no increases in TRF length, in any of the clones, beyond what would be expected during normal mitotic growth [31,32].

The chromosome-internal *vsg118* can be seen at approximately 3 kbp and we assume that the *vsgbR2* and *vsgB* bands that do not display length heterogeneity also represent non-telomeric fragments.

3.4. No rearrangements were detectable within or upstream of regulated expression sites

To determine if rearrangements at more telomere-distal loci were associated with ES switching, we mapped upstream of the regulated ESs in all of our switched clones, using *PmeI* and *RsrII* restriction enzymes (Fig. 4A). We used a *ble* probe to detect *vsg221*-associated fragments, because there is an *RsrII* site directly downstream of *ble*. The other bands representing ESs appear at slightly different sizes because they are telomeric fragments, and this indicates that we can detect relatively small size differences in these gels (compare Figs. 3 and 4B). Most of the *RsrII* digests were incomplete in these experiments, as indicated by the bands at more than 200 kb. Wt118a cells have previously been reported to have a duplicated ES promoter linked to *vsg118* [11,24]. We confirmed that

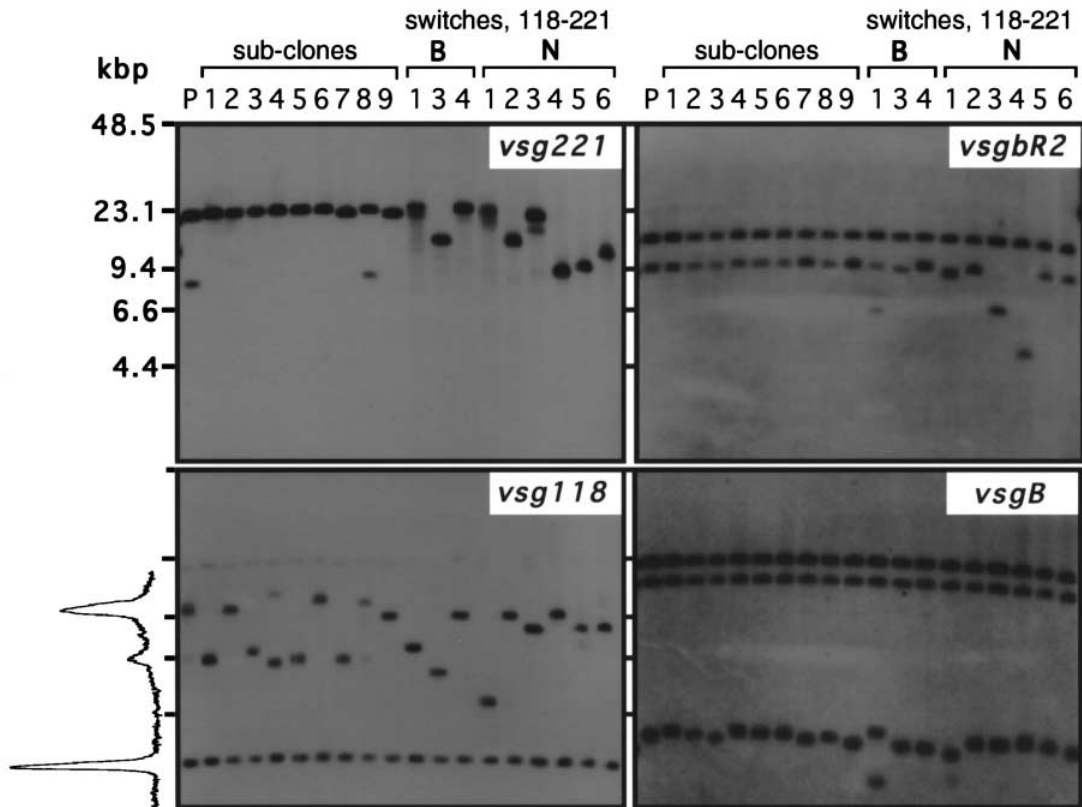


Fig. 3. Determination of telomere restriction fragment length. Chromosomes from 118^+ /bRn parental cells (lane P), 9 118^+ /bRn sub-clones and all nine clones in which ES switching had occurred (B1, B3, B4 and N1-N6) were digested with *EcoRI* (see Fig. 1A) and separated by Rotating Agarose Gel Electrophoresis in a 1% gel. The rotation angle was set at 120° and a linear ramp, 1–2 s pulses, was applied for 17 h at 150 V. The resulting blot was sequentially hybridized with the probes indicated in the upper right hand corner of each panel. The *vsg118*, probe b (see Fig. 1A) was used with this blot. The *vsgbR2* [4] and *vsgB* [12] probes contained 330 and 340 bp from the 5'-end of their respective cDNA clones (obtained by reverse-transcriptase polymerase chain reaction (RT-PCR) using cells derived from other *vsg* switching experiments [12], K.P. Davies, unpublished data). A phosphorimager scan of the 118^+ /bRn parental cell DNA hybridized with the *vsg118* probe is shown at the left hand side of this track.

118^+ /bRn cells retain this duplicated promoter (data not shown), but none of our nine clones that had undergone ES switching exhibited the previously reported [11] loss of 15 kb at the inactivated locus (Fig. 4B, *PmeI* digests). No other rearrangements were detected up to 80 kb (*vsg118*, inactivated) or 180 kb (*vsg221*, activated) upstream of the *vsg*. We also checked for rearrangement in the sub-telomeric sequence just downstream of *vsg221*. We found no rearrangement down to the *NarI* site (Fig. 1A and data not shown) suggesting that the conserved sequence 3' of the *vsg* [33] was unchanged.

3.5. Expression of two *vsgs* by a sub-population of switched cultures

We detected residual Vsg118 in one of the clones that had undergone ES switching (Fig. 1C, clone N2). To determine if Vsg118 was being expressed independently of Vsg221, we carried out immunofluorescence analysis. The majority of these cells appeared to express exclusively Vsg221, while approximately 10% expressed both Vsg118 and Vsg221, and less than 1% expressed exclusively Vsg118 (Fig. 5). These cells had been sub-cloned following the switch and were derived by

selection in G418, indicating that a single G418-resistant cell could generate this heterogeneity. The accumulation of cells expressing exclusively Vsg118 is possible because each resistant popula-

tion was sub-cloned and expanded in the absence of drug (see Section 3.1). To determine the reproducibility of this phenomenon and to determine if it is dependent upon selection in G418, we generated further independent switches in each drug. Because we knew the switching frequency of $118^+/\text{bRn}$ cells was approximately 6×10^{-7} , rather than sub-cloning parental cells, we placed 100 cells ($< 0.1\%$ expressing Vsg221 as determined by immunofluorescence) in 20 10 ml flasks and added phleomycin ($1 \mu\text{g ml}^{-1}$) to ten and G418 (50 mg ml^{-1}) to the other ten when the density reached approximately $10^5 \text{ cells ml}^{-1}$ ($10^6 \text{ cells flask}^{-1}$). We generated new switches so that we could examine cells as soon as possible after the switch (approximately 1 week or 20 generations, when 10^6 drug-resistant cells were available: drug selection was maintained throughout this period).

Immunofluorescence analysis indicated cells expressing both Vsg118 and Vsg221 in one of three phleomycin and one of three G418-selected cultures, constituting approximately 1% of cells in each culture. The appearance of cells expressing two VsGs under phleomycin selection indicates that the ES promoter, rather than just the rRNA promoter, is active in the *vsg221* ES (see Fig. 1A). Cultures in which we did not detect cells expressing both VsGs were grown for a further ten generations, but cells expressing both VsGs remained absent or undetectable. It is possible that the 'founder' cell for these heterogeneous cultures expressed Vsg221 alone or both Vsg118 and Vsg221. Because cells expressing both VsGs were more abundant after longer periods of growth following the switch (1% after 20 generations and 10% after approximately 100 generations), we favor the hypothesis that the 'founder' cell expressed Vsg221 alone. The accumulation of cells expressing both VsGs is then easily explained by a modified version of the Galton–Watson model (see [34], equation 1), which was designed to measure mutation frequencies based on the accumulation of cells bearing a particular mutation. This equation is also suitable for measuring a phenotype switch not involving mutation, as many of the considerations are the same for both situations. The model predicts that the population of cells expressing two VsGs should increase in a linear fashion with the

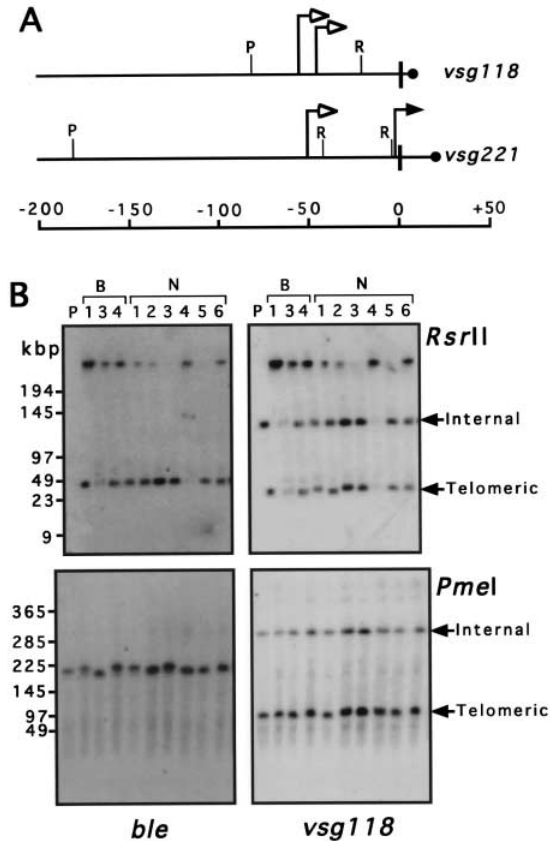


Fig. 4. Mapping upstream of regulated expression sites. (A) Physical maps spanning upstream of telomeric ESs containing *vsg118* and *vsg221* as determined in $118^+/\text{bRn}$ parental cells. ES and rRNA promoters are indicated as open and filled arrowheads respectively. Relevant restriction sites are shown and the abbreviations used for restriction enzymes are P, *PmeI* and R, *RsrII*. The scale indicates positions in kbp relative to each *vsg* start codon. (B) Chromosomal DNA from $118^+/\text{bRn}$ parental cells (lane P) and all 9 clones in which ES switching had occurred (B1, B3, B4 and N1–N6) were digested with *RsrII* or *PmeI* and separated by Rotating Agarose Gel Electrophoresis in 1% gels. The rotation angle was set at 120° . Pulse field conditions for *RsrII* digested DNA were a linear ramp, 2–15 s for 18 h at 150 V; and for *PmeI* digested DNA was a linear ramp, 10–30 s for 20 h at 150 V. Both blots were sequentially hybridized with the probes indicated below the panels. The *vsg118*, probe a (see Fig. 1A) was used with these blots.

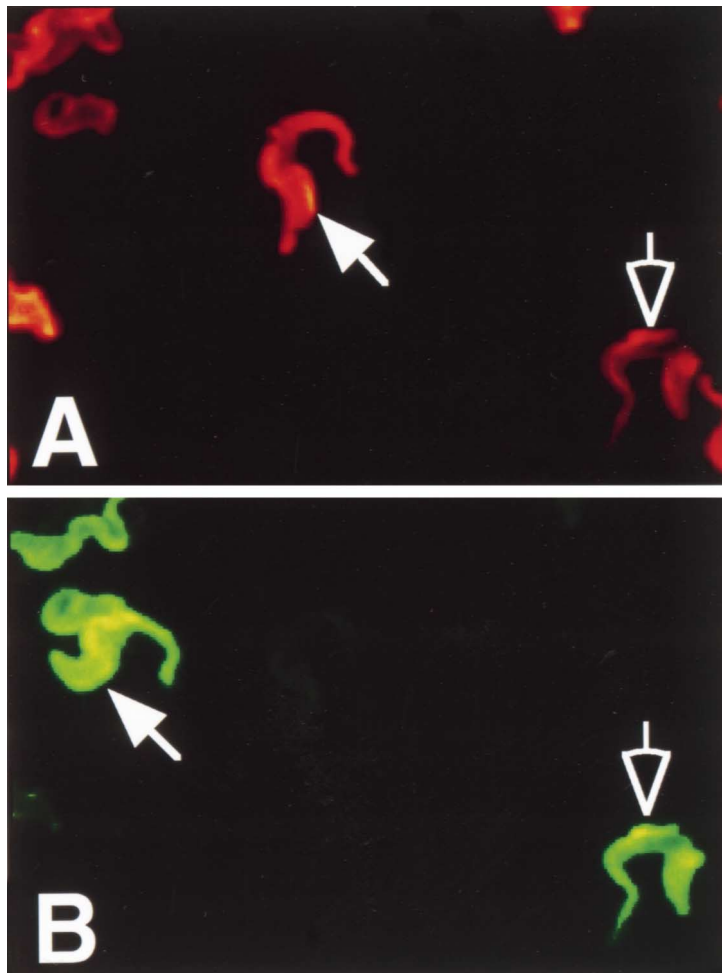


Fig. 5. Expression of two *vsgs* by a sub-population of switched cultures. Fixed cells from the switched clone N2 were incubated with rat antiserum to Vsg221 and rabbit antiserum to Vsg118. Secondary antibodies were rhodamine-conjugated goat anti-rat and FITC-conjugated goat anti-rabbit. Pictures of the same field indicate cells expressing either Vsg221 (panel A) or Vsg118 (panel B, closed arrowheads) and a cell expressing both Vsgs (open arrowheads). A field containing an untypically high proportion of double-expressing cells and a Vsg118 expressing cell was chosen for illustration.

number of generations, which is consistent with our observations. We used this model to estimate the switch frequency (Vsg221 expression to Vsg221/Vsg118 double-expression). Assuming that cells do not switch back to expressing only Vsg221, and that the growth rate of cells expressing one or two Vsgs is not significantly different (see [35]), we derived a switch frequency of approximately 2×10^{-3} .

4. Discussion

Several DNA rearrangements have been found in association with ES switching (reviewed in [8]). To determine if ES switching requires DNA rearrangements or is controlled by an epigenetic mechanism, we generated a panel of clones in which the same ES switch has occurred. A search for rearrangements within and beyond the regu-

lated ESs, as well as an examination of changes in telomere length, indicated that antigenic variation can occur without any of the previously reported or any novel rearrangements. We also found that ES activation usually involves inactivation of the previously active ES, but that cells expressing two *vsgs* subsequently accumulate in some cultures.

We wanted to capture a panel of independent clones, all derived from the same clone, immediately following a similar switching event (*vsg118* to *vsg221* expression). A selectable marker cassette inserted into a repressed *vsg221* locus in 118⁺/bRn cells was exploited to generate clones in which selectable marker expression was activated concomitantly with *vsg221*. This was achieved in vitro and allowed accurate measurements of switching frequency. Antigenic variation in *T. brucei*, whether by recombinatorial mechanisms or otherwise, has been estimated to occur at a frequency ranging from 2.2×10^{-7} to 2.6×10^{-6} in vitro [36]. These estimates were generated using a clone with the same active ES as that in 118⁺/bRn cells. In our experiments, a specific switching event occurred at a frequency of 6×10^{-7} , and we obtained a similar frequency using selection for *neo* or *ble* expression. From our panel of ten switched clones, nine occurred via ES switching. We detected no examples of the independent activation of the inserted rRNA promoter, suggesting that the ES is regulated as a single unit and may represent a chromatin domain, see [37]. Also, if repressed domains are assembled from the telomere, as in *Saccharomyces cerevisiae*, this result may indicate continuous spreading of a repressed chromatin structure along the ES (see [38]).

Changes in telomere length have been reported to be associated with ES switching. ES inactivation appeared to correlate with a reduction in telomere length while activation appeared to correlate with an increase in telomere length [30]. Change in telomere length is probably due to addition and deletion of T₂AG₃ repeats [39,40]. In other organisms, telomeres appear to interact with each other and with the nuclear envelope [41]. An attractive model for ES switching is that the ESs interact physically during the switch process. A model in which rearrangements between the two telomeres leads to telomere end exchange is ap-

pealing. This does not appear to be the case, however, as we only see telomere loss and not a single example of significant gain in telomere length. Most if not all telomeres grow in *T. brucei*, with the active telomere growing faster and undergoing progressive length reductions leading to telomere length heterogeneity within a clone [31,32]. Our panel of switched clones was derived by sub-cloning 118⁺/bRn cells, from which switches were selected (switching may also be considered as sub-cloning) and subsequently sub-cloned again before generating DNA samples. Sub-cloning, and not ES switching, at points when *vsg118* and *vsg221* were active, respectively, can explain the TRF length heterogeneity found in the switched clones. A cloning method involving heat-shock was reported to induce telomere shortening [31]. Our data show that changes in telomere length may be accounted for by the dynamic nature of telomeres rather than necessarily being associated with ES switching or heat shock.

An ES with promoter repeats exhibited rearrangement associated with inactivation [11,24] and activation [12]. The rearrangement involved loss of a tandemly repeated promoter via intra-chromosomal homologous recombination [11,42]. This rearrangement, however, is not necessary for regulation of transcription as these promoters can exist in an active or silent ES with single or tandem copies [11,12,24]. Similar rearrangements also occur independently of ES switching [12]. We detected no rearrangement in the promoter region of the same ES following inactivation of this ES in nine independent examples. Also, no rearrangements were detected in related ESs following ES switching [43]. Activation of an ES has been associated with another rearrangement upstream of the promoter, within 50 bp repeat sequences [24]. The resolution of our gels allowed detection of insertions or deletions of greater than 5 kb in this region, and we detected no such rearrangements.

The relationship between some rearrangements and *vsg* switching, in cases in which a small number of samples were examined, may be incidental. Other rearrangements, in the promoter region [11,12,24,42], do appear to be associated with some ES switching events, however. These

rearrangements may be sufficient but not obligatory for switching. Alternatively, these rearrangements may occur at a relatively high frequency and subsequently influence the switching frequency of a particular ES. If, for example, telomere length influences the frequency of ES switching, it is possible that telomere growth and breakage could re-program the likely order of ES switching.

Transient [44] and stable [45] transfection in bloodstream and procyclic-form *T. brucei* indicates the presence of factors necessary for transcription initiation at multiple ES promoters in both life-cycle stages. Despite the presence of multiple ESs, *vsg* expression is usually mutually exclusive in bloodstream-form cells. The discovery of trypanosomes in which two *vsg* ESs were stably transcribed [13,14] suggested that ESs can switch on and off independently and that it was the immune response that usually eliminates cells expressing both pre- and post-switch Vsgs. When selecting for *vsg* switching by drug selection rather than by immune selection, survival is not dependent upon inactivation of the previously expressed *vsg*. In our experiments, ES inactivation accompanied ES activation in nine independent examples in the absence of immune selection. These results suggest that the activation/inactivation process is usually coupled and occurs as a coordinated event or series of events. An examination of individual cells, however, did reveal the presence of cells expressing both Vsgs in some of our switched cultures. Our results suggest that a mutually exclusive switch is efficiently established and relatively efficiently maintained, but that repression at the inactivated locus is lost at a frequency of 2×10^{-3} following some switch events. This predisposition toward generating cells expressing two Vsgs is a heritable phenotype, which is maintained after sub-cloning, as indicated by the switched clone N2. In addition, some of these cells switch back to exclusive expression of the previously active Vsg, at what appears to be a similarly high frequency. Taken together, these observations show that ES activation/inactivation is clearly a coupled event but that the machinery maintaining all but one ES in a repressed state can be compromised in a heritable fashion (possibly during the

establishment step), which allows the activation/inactivation event to become uncoupled.

Isogenic cells can exist in mitotically stable yet phenotypically distinct epigenetic states (reviewed in [46]). Our results suggest that an epigenetic mechanism, and not DNA alteration, controls ES transcription in bloodstream-form *T. brucei*. This hypothesis is supported by several lines of evidence. ES activation occurs in conjunction with ES inactivation and no detectable DNA rearrangements are necessary for ES switching (this paper). Inactivation and reactivation of metacyclic [47] and bloodstream-form *vsgs* [48], during the life cycle, is thought to occur in the absence of DNA rearrangements. Based on these results, we cannot rule out that small undetected DNA rearrangements may be specifically associated with ES switching. If spontaneous DNA mutations were responsible for switching, however, each phenotype would be expected to be extremely stable. Mitotically metastable phenotypes have been demonstrated in early bloodstream-form infections, when metacyclic *vsgs* (*vsgs* activated in the insect salivary gland, see[49]) are expressed [50] and, as demonstrated in this paper, in some clones expressing *vsgs* from bloodstream-form ESs. These *vsg* phenotypes switch at a significantly faster rate (2.7×10^{-2} and 2×10^{-3} , respectively) than would be expected by spontaneous mutation. Repression is position-dependent and promoter-independent [5,6], suggesting that it is mediated by an altered chromatin structure (see [46]). *vsg* ES switching appears to occur in a stochastic manner, as does activation of a particular ES from the metacyclic [49,51] and bloodstream-form [52] sub-set during the life cycle. We therefore suggest that an epigenetic mechanism controls *vsg* activation and switching in metacyclic and bloodstream-form cells.

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