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Manipulation of the *vsg* co-transposed region increases expression-site switching in *Trypanosoma brucei*

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Abstract

Disruption of a region of DNA in *Trypanosoma brucei* immediately upstream of the expressed telomere-proximal variant surface glycoprotein gene (*vsg*), known as the co-transposed region (CTR), can cause a dramatic increase in the rate at which the active expression site (ES) is switched off and a new ES is switched on. Deletion of most of the CTR in two ESs caused a greater than 100-fold increase in the rate of ES switching, to about 1.3×10^{-4} per generation. A more dramatic effect was observed when the entire CTR and the 5′ coding region of the expressed *vsg*221 were deleted. In this case a new ES was activated within a few cell divisions. This switch also occurred in cell lines where a second *vsg* had been inserted into the ES, prior to CTR deletion. These cell lines, which stably co-expressed the inserted and endogenous Vsgs, in equal amounts, did not differ from the wild type in growth rate or switching frequency, suggesting that simultaneous expression of two Vsgs has no intrinsic effect. CTR deletion did not disturb the inserted *vsg*117. We tentatively conclude that it was not the disruption of the *vsg*221 in itself that destabilized the ES. All of the observed switches occurred without additional detectable DNA rearrangements in the switched ES. Deletion of the 70-bp repeats and/or a *vsg* pseudogene upstream of the CTR did not affect ES stability. Several speculative interpretations of these observation are offered, the most intriguing of which is that the CTR plays some role in modulating chromatin conformation at an ES. © 1997 Elsevier Science B.V.

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

Abbreviations: CRD, cross-reacting determinant; CTR, co-transposed region; ES, expression site; *esag*, ES-associated gene; GPI, glycosylphosphatidylinositol; *hyg*, hygromycin phosphotransferase gene; KO, knock-out; *luc*, luciferase gene; MITat, Molteno Institute Trypanozoon antigen type; *neo*, neomycin phosphotransferase gene; NKO, non-knockout; Parp, procyclic acidic repetitive protein; PAS, polyadenylation site; PFGE, pulsed-field gel electrophoresis; SAS, splice acceptor site; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; Sw, switched variant; Vsg, variant surface glycoprotein; *vsg*, Vsg gene; WT, wild-type.

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1. Introduction

The bloodstream form of *Trypanosoma brucei* survives the host immune response by sequentially changing its surface coat, which is composed of approximately 10^7 molecules of a single molecular species of variant surface glycoprotein (Vsg) [1]. The mechanisms regulating *vsg* expression are probably among the most complex employed by pathogenic microorganisms, and are likely to operate at several levels, including transcription initiation, elongation, RNA maturation, stability, and translation efficiency [2]. Although *T. brucei* contains hundreds of *vsgs* [3], only one is normally expressed at a time [2,4–7]. The expressed *vsg* is always the most telomere-proximal gene in a polycistronic transcription unit known as an expression site (ES). Hybridization and mapping data suggest that there may be as many as 20 ESs in *T. brucei* [8,9]. ES promoters sequenced so far show more than 90% sequence identity [10–12]. The 5′ end of the ES promoter is flanked by a region of imperfect direct repeats of 50 bp, which covers at least 10 kbp in the 221 ES [9].

One of the central puzzles of antigenic variation is what mechanism ensures the apparently normal singularity of ES transcription? There are several mechanisms by which *T. brucei* achieves *vsg* gene switching (antigenic variation). They include ES activation/inactivation in situ, reciprocal recombination, duplicative transposition of a silent non-telomeric *vsg* into an active telomeric ES, and duplicative transposition of large telomeric domains [7]. The mechanisms controling ES activation and inactivation in situ remain obscure, but appear to be very stringent. It seems exceptional for two ES to be simultaneously active. *T. brucei* cells displaying two Vsgs (but not necessarily transcribing both genes simultaneously) can be found as a minor population in rats infected with metacyclic forms from the Tsetse, during the first infection cycle, when there is rapid switching from metacyclic to bloodstream-form *vsgs* [13]. These double-expressing cells do not multiply for long, probably because metacyclic *vsgs* form a subclass of telomeric *vsgs* that are transcribed monocistronically and are not linked to ES-associated genes (*esags*) [14–18]. There has been one report of *T. equiperdum* apparently simultaneously expressing two *vsgs* from two ESs, in a situation that was stable for about 30 generations in vitro but could not be propagated beyond a few generations in mice [19]. As we show here and explore in more detail elsewhere [20], there appears to be no intrinsic disadvantage to the simultaneous expression of expressing multiple Vsgs on the trypanosome surface.

Microorganisms often achieve a low rate of activation or inactivation of gene expression by DNA rearrangements [21]. However, current evidence suggests that this is not the mechanism used for ES switching in *T. brucei*. Although DNA rearrangements may occur during ES activation and inactivation [10,22–24], switches can also occur without detectable DNA rearrangements over a region extending from the telomeric *vsg* to more than 150 kbp upstream of the ES promoter [25,26].

Recent reports [27,28] have highlighted the possibility that an effect similar to telomere-position-effect silencing in yeast [29,30] may operate in *T. brucei*. However, the type of telomere silencing seen in *T. brucei* would have to extend much further than the 5-kb limit of silencing occurring at yeast telomeres since the ES promoter is 55–60 kb upstream of the telomere. The way in which telomeric silencing might allow a single ES to be active has not been addressed. Also, as has recently been shown by replacing the core of the ES promoter with a *T. brucei* rRNA promoter [28], the ES promoter per se is not unique, but its chromosomal context may be of special importance.

A novel base, β-D-glucosylhydroxymethyluracil [31], initially detected because of its interference with restriction enzyme-mediated cleavage, occurs within and around silent telomeric *vsgs* [32,33]. Although the absence of this modification from a telomeric *vsg* correlated with activation of the corresponding ES, most of the modification probably occurs within the telomeric TTAGGG repeats [34]. It is unclear to what extent the modification correlates with ES activity, and whether its presence is a cause or consequence of ES silencing.

Once a new ES is activated, the old ES must shut down: a mixed Vsg coat would presumably result in the killing of the parasite by antibodies elicited by the first Vsg. The rate at which inactivation of

one ES and the (presumably simultaneous) activation of another occurs is somewhat controversial but in laboratory-adapted bloodstream forms it has been reported to be around 10^{-7} per generation [35]. The other *vsg*-expressing life-cycle stage is the metacyclic form. Metacyclic *vsgs* are found in a subset of telomeric ESs, which have a very different architecture from bloodstream-form ESs and switch off at a rate of 10^{-2} per generation [36]. Much higher switch frequencies (7×10^{-3}) have been reported for bloodstream forms after cycling through the Tsetse [37], but this may reflect switches from metacyclic *vsgs*.

Both chromosome-internal and telomeric *vsgs* are flanked by a co-transposed region (CTR), which consists of 1–2 kbp of DNA between the *vsg* and the so called barren region, which consists of 2–10 kbp or more of imperfect 70-bp repeats [3,38–41]. The 5′ and 3′ boundaries of gene conversion, during duplicative transposition of a silent chromosome-internal *vsg*, are generally found within the 70 bp repeats and either in the *vsg* 3′ coding or untranslated region, which contains several short, highly conserved, sequences. All of the known CTR sequences (there are about ten entries in GenBank) are distinct and the function of this region is unknown. We have found that the deletion of the CTR from two ESs resulted in greater than 100-fold increases in ES switching frequency. An even more dramatic effect was observed when the entire CTR was deleted, along with the start codon of the expressed *vsg*221. In this case, a new ES was activated within a few cell divisions. This is the first report that manipulation of the trypanosome genome can increase ES switching. The implications for antigenic variation in trypanosomes and the potential to destabilize an expression site by genetic manipulation, are discussed.

2. Materials and methods

2.1. Trypanosomes and culture conditions

Bloodstream-form *T. brucei* strain 427 was used in all these studies. The variant antigen types have been previously described, being derived either from the Molteno Institute Trypanozoon antigen types (MITat) 1.5 clone 118a, referred to as WT118, or MITat 1.2 clone 221a, referred to here as WT221 [1,42,43]. Cultures were maintained in medium HMI-9 at 37°C [44].

2.2. Plasmid construction and genetic manipulation

DNA integration and cloning of bloodstream forms were performed essentially as previously described [45]. All targeting plasmids were digested with appropriate restriction enzymes, to release linear targeting fragments from plasmid DNA, prior to transfection by electroporation. Cells to be transfected were confirmed to be homogeneously (based on counts of approximately 200 parasites) expressing the correct Vsg by immunofluorescence with the appropriate antibody. Cloned drug-resistant recombinant cell lines were derived by culture in 2.5 μg ml^{-1} hygromycin or G418, added 16 h after transfection [46]. Until the clones were used for switching experiments, they were always cultured in the presence of hygromycin or G418. The correct integrations of all constructs were confirmed by restriction mapping and pulsed-field gel electrophoresis (PFGE) of chromosomal DNA. Genetic maps of the ES recombinants are illustrated in Fig. 1. The plasmid pBEAHA5′(NKO118) was constructed to target the *vsg*118 ES in WT118 cells. This plasmid contains the Tn5 hygromycin phosphotransferase gene (*hyg*), flanked by the splice acceptor site (SAS) and polyadenylation site (PAS) from a *T. brucei* aldolase gene (GenBank locus TBFBPAA; nucleotides 2150–2345 and 3473–4179 for SAS and PAS respectively). The *hyg* cassette was flanked upstream by a 5′ 118 ES-targeting sequence from *esag*1 (nucleotides 366–1445 in GenBank locus TRBESAGA; amplified from pGE117a.10 [8]) and downstream by a 3′ ES-targeting sequence (from position 1965 in GenBank locus TBGP05 to position 1051 in GenBank locus TBMITAT15; amplified from pc118-29I [39]) consisting of the first 892 bp of the *vsg*118 coding sequence and 333 bp upstream of the translation initiation codon, which includes the *vsg*118 SAS. The resulting clones were called 118CTKO (118 CTR knock-out).

Several plasmids were designed to target the *vsg*221 ES (Fig. 1). In one set of clones, the entire

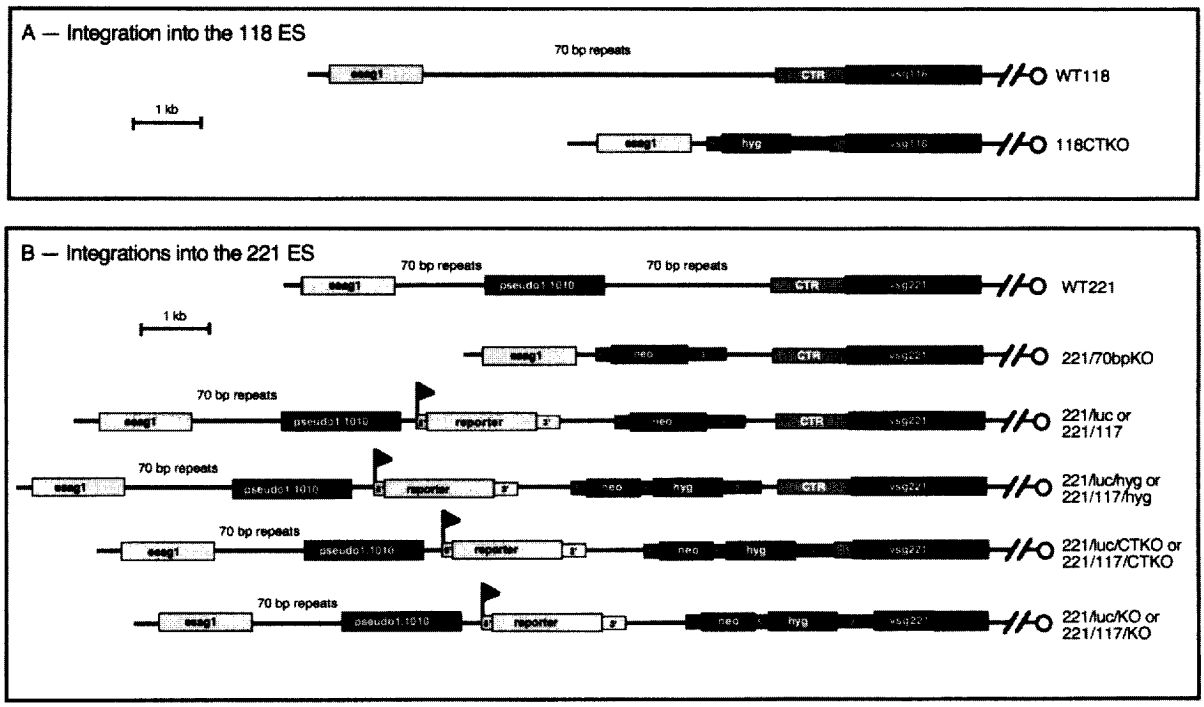

Fig. 1. Physical maps of (A) the 118 [63] and (B) 221 [64] expression sites before and after targeted integrations. The *hyg* and *neo* genes are flanked by the splice acceptor site (SAS) and polyadenylation site (PAS) from a *T. brucei* aldolase gene. The reporter genes are either luciferase (*luc*) or *vsg*117 flanked by the SAS and PAS from a *T. brucei* actin gene. The flag represents the core of the *parp* promoter. A detailed description of the plasmids used to generate these cell lines is given in Section 2. The following convention was used in naming the transformants. 118/ or 221/ denote integrations into the 118 or 221 ES respectively; CTKO denotes deletion of the co-transposed region (CTR) with *hyg*. 70 bpKO denotes deletion of the entire region between *esag*1 and the CTR with *hyg*; *luc* or 117 denote integration of the reporter genes, *luc* or *vsg*117 respectively, with deletion of the 70 bp repeats between the *vsg* pseudogene and the CTR. *hyg* denotes functional replacement of *neo* by *hyg*; KO denotes deletion of the CTR and disruption of the *vsg*221 ORF with *hyg*.

region between *esag*1 and the CTR was deleted. This was achieved using the targeting plasmid pBEANA5′, which contains the Tn5 neomycin phosphotransferase gene (*neo*) flanked by the aldolase SAS and PAS fragments described above for pBEAHA5′(NKO118). The *neo* cassette was flanked upstream by the 5′ ES-targeting sequence from *esag*1, described above, and downstream by a 3′ ES-targeting sequence, polymerase chain reaction (PCR)-amplified from pTgB221.1 [47] and consisting of 989 bp of the CTR and 253 bp of the *vsg*221 amino-terminal coding sequence (from position 704 in GenBank locus TRBGP221 to position 273 in GenBank locus TBMITAT12: the gap in the GenBank entries was sequenced by us). These clones were called 221/70 bpKO (221 70-bp knock-out). Two constructs deleted the region between the *vsg*1.1010 pseudogene and the CTR by integration of a dual-reporter cassette containing either *vsg*117 or luciferase (*luc*), flanked by the SAS and PAS from a *T. brucei* actin (*act*) gene (GenBank locus TRBACTA; from positions 1951 to 2062 and 1673 to 1950 for SAS and PAS respectively), upstream of the *neo* cassette. The core of the *parp* promoter (from position −114 to +24, relative to transcription start site, in the *parp*-A locus), lacking the upstream activating sequence and showing about 15% of maximal promoter activity in transient plasmid-transfection assays [48], was also present in these constructs, immediately upstream of the actin SAS, and its intrinsic role in the success of these exper-

iments is discussed below. Construction of the *luc* plasmid (pKD4) is described in greater detail elsewhere (V.B. Carruthers, K.P. Davies, P.K. Patnaik and G.A.M. Cross, manuscript in preparation). In order to integrate *vsg*117 into the ES, a *vsg*117 cDNA was removed from pH3 [49], using *Eco*RI digestion, and inserted into pKD4 from which *luc* had been removed using the same enzyme. Cells expressing either luciferase or the additional Vsg117 were named 221/luc or 221/117, respectively. Several clones, in which various regions of the CTR were deleted, were subsequently derived from the 221/luc or 221/117 clones. For this second integration, *hyg* was used as the selectable marker and the 5′ targeting sequence was the 3′ region of the previously integrated *neo*. Three different downstream targeting sequences were used, resulting in deletion of different amounts of the CTR. A control plasmid, pKD10(NKO), containing the CTR and 5′ portion of *vsg*221 as the downstream targeting region (989 bp of the CTR and 253 bp of the 221 *vsg* amino-terminal coding sequence, as described above) was used to produce transformants 221/luc/hyg or 221/117/hyg, depending on the parent line transfected. To remove most of the CTR, pKD10(NKO) was manipulated to generate pKD10(CTKO). This plasmid has a 3′ 221 ES-targeting sequence consisting of 126 bp upstream of the *vsg*221 initiation codon (including the SAS) and 253 bp of the *vsg*221 amino-terminal coding sequence. Transfection of cell lines with this construct yielded the transformants 221/luc/CTKO or 221/117/CTKO, depending upon the parent line. In plasmid pKD10(KO), the 3′ targeting sequence consisted of the part of the *vsg*221 coding region (position 290–1028 in GenBank locus TBMITAT12) amplified from *T. brucei* clone 221 genomic DNA, resulting in deletion of the entire CTR, 3′ untranslated region (UTR) and 270 bp of *vsg*221 amino-terminal coding sequence, yielding transformants 221/luc/KO or 221/117/KO, depending upon the parent line transfected.

2.3. Calculation of switch frequency

The switch frequency was calculated in vitro or in vivo, in the absence of drug selection. The in vitro technique, which utilized immunolysis to kill cells expressing either Vsg118 or Vsg221, was based on a published protocol [50]. At various times during the growth of clones in vitro, 10 μl of culture was removed and 10 μl of guinea-pig serum, containing 1% rabbit anti-Vsg118 or 1% chicken anti-Vsg221, was added. After incubating for 2 h at 37°C, 5 ml of HMI-9 was added. If any parasites had switched to expression of a new Vsg, they were able to survive immunoselection and continue to grow. Controls included incubating cells in guinea-pig serum in the absence of the antibody (no killing) or incubating cell lines expressing another Vsg (VsgbR2, for example) with complement and heterologous antibody (no killing). By this method, switching of WT cells was below the level of detection (1×10^{-6}). In addition, the switch frequencies for WT118 and 118/CTKO were determined in mice immunized with a crude preparation of Vsg118. The first immunizing dose was given in Freund's complete adjuvant while the second dose was given in incomplete adjuvant 3 weeks later. After a further 3 weeks, mice were challenged with different numbers of trypanosomes to determine the switching frequency. The in vivo assay accommodated switching rates lower than 1×10^{-6}, which could not be determined in vitro because of breakthrough of non-switched survivors when large populations were tested.

2.4. RNA and DNA analysis

Genomic DNA for Southern analysis was isolated essentially as previously described [51]. Total RNA was isolated using RNAstat60 (Tel-Test). Southern and Northern analyses were performed according to standard protocols [52] and all post-hybridization washes were at 65°C in 0.2 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.2% SDS. The *vsgs* expressed in switched clones, including *vsg*bR2, were characterized by reverse-transcriptase-PCR (RT-PCR). cDNA was synthesized from total RNA extracted from 5×10^7 cells using an oligonucleotide (ccggtacCGTGTTAAAATATATC; lower case indicates nucleotides added to facilitate cloning in other vectors) hybridizing to the 16-mer that is

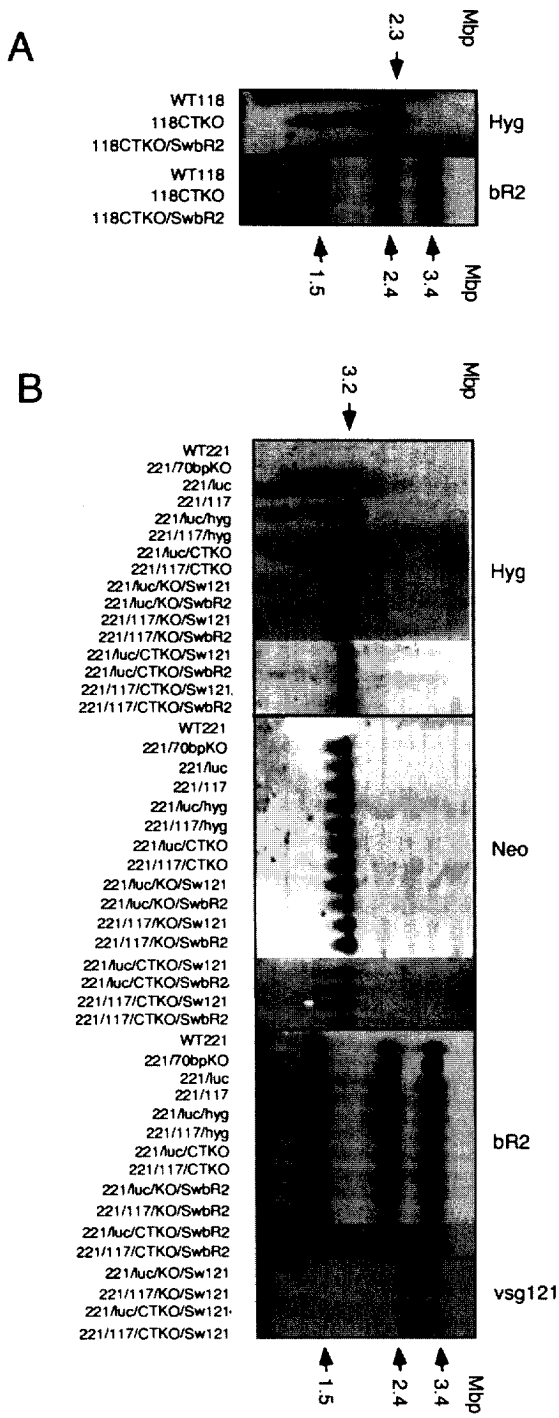

Fig. 2. Chromosome-sized DNA separated by rotating agarose gel electrophoresis on 0.9% agarose. Chromosome sizes were estimated by comparison with *S. pombe*, *S. cerevisiae* and *H. wingei* standards (not shown). (A) Cell lines derived from WT118 (B) cell lines derived from WT221. The derivation and naming of the cell lines is described in the legend to Fig. 1, with the addition of Sw121 or SwbR2, to denote switches in which *vsg*121 or *vsg*bR2, respectively are expressed. The hybridization probes consisted of the coding regions of the indicated genes, except for *vsg*bR2, where the probe was a 5′ region of the mRNA cloned by RT-PCR, as described in Section 2.

conserved in the 3′ UTR of all *vsgs*. The cDNA was amplified by PCR, using this primer and another (cgcgaattcTAGAACAGTTTCTGT) hybridizing to the RNA spliced leader. The single band obtained was cloned into the TA cloning vector (Stratagene) and confirmed to represent the expressed *vsg* by hybridizing a 5′ region of the cloned sequence to Northern blots. The cDNA clones were partially sequenced at the Rockefeller University Shared Technology Center.

2.5. Protein analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) separation of proteins and Western blot analysis were performed according to standard protocols. Immunofluorescence was performed essentially as previously described [27]. Primary antibodies were either rabbit antiserum to Vsg118, rabbit antiserum to Vsg121, and rabbit or chicken antiserum to Vsg221. For Western analysis, alkaline-phosphatase-conjugated anti-rabbit antibodies were visualized by reaction with 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine/nitroblue tetrazolium chloride. Secondary fluorescent antibodies were fluorescein isothiocyanate or rhodamine-conjugated goat anti-rabbit or goat anti-chicken, respectively (Sigma).

3. Results

The physical maps of the 118 and 221 ES, before and after transfection with the various constructs, are shown in Fig. 1. Transfection effi-

ciency was approximately 1×10^{-7} μg^{-1} DNA, similar to previously reported values [28,45]. Southern analysis (data not shown) and PFGE (Fig. 2) confirmed that the DNA cassettes inserted into the correct location: 118/CTKO transformants into a 2.3 Mbp chromosome (Fig. 2A, *hyg* probe) and 221 ES transformants into a 3.2 Mbp chromosome (Fig. 2B) *hyg* and *neo* probes). In the second generation 221 ES recombinants, where the CTR is altered, 544 bp of *neo* remained to react with the *neo* probe, though the gene is no longer functional.

3.1. Switching from the 118 ES

Experiments performed with WT118 cells deleted most of the region between *esag*1 and the *vsg*118. Only 199 bp of the CTR remained, including the *vsg* SAS. Ten 118/CTKO clones were selected from the cells transfected with plasmid pBEAHA118(NKO), in case of any variation in the switch rate between different clones. Immunofluorescence of fixed cells showed that >99.5% of the starting population of WT118 and the 118/CTKO transformants were expressing Vsg118. The average switch rate for the 10 transformant clones was $2.8 \pm 1.3 \times 10^{-4}$ when measured in vitro and 1×10^{-4} in vivo. This is an approximately 100-fold increase in the switch frequency compared to WT118, as determined here (Table 1) or reported previously [35].

Ten switched clones were selected for characterization. None of the cell lines now expressed Vsg118, as determined by immunofluorescence (data not shown). After SDS-PAGE, a new band of approximately 70 kDa could be seen after Coomassie staining or Western blotting (Fig. 3A), or after [^{14}C]myristic acid labeling of the Vsg glycosylphosphatidylinositol anchor (data not shown). RT-PCR was performed on one of the switches, selected at random, and the product was cloned and partly sequenced. The 3′ sequenced region included 77 nucleotides that were identical to the published 77-nucleotide sequence of *vsg*bR2 [53], albeit in a region that is highly conserved between *vsgs*. RNA analysis confirmed that the switched clones expressed *vsg*bR2 (Fig. 4A). The new clones continued to stably express *vsg*bR2, with no discernible reactivation of the 118 ES or switching to another ES. The clones were no longer tolerant of high levels of hygromycin (>30 μg ml^{-1}), and this was reflected by the barely detectable amounts of *hyg* RNA (Fig. 4). Northern blots demonstrated that *vsg*118 was not transcribed in any of the 118/CTKO switched clones analyzed, suggesting that the 118 ES was effectively silenced. Although some RNA in the 118/CTKO parent reacted with *vsg*bR2, even before immunoselection, this was attributed to the rapid switching and outgrowth of VsgbR2 clones, which grew 15% faster than WT118 cells, during propagation of parasites for RNA preparation.

As shown in Fig. 2A, there are three copies of *vsg*bR2, on chromosomal DNA bands of 3.4, 2.4 and 1.5 Mbp. The copy that was expressed in the switched clones must have been activated in situ, because the switch was not associated with the appearance of a new copy of *vsg*bR2. PFGE provided no evidence of telomeric conversion and restriction mapping demonstrated that the switched and unswitched variants had not undergone any discernible rearrangements of the 118 ES within 15 kb upstream of *vsg*118.

3.2. Instability of the 221 ES

In the 221 ES, deletion of either the region between the CTR and *esag*1 (221/70bpKO), or between the CTR and the *vsg*1.010 pseudogene, which included the tandem insertion of a second reporter gene (221/luc or 221/117), had no effect on the switching rate (Table 1). The stable simultaneous expression of two Vsgs (Fig. 3B), in the 221/117 recombinant, is interesting in itself and is more extensively explored elsewhere [20]. Replacement of *neo* by *hyg* (without disrupting the CTR), in a second round of transfection (clones 221/luc/hyg or 221/117/hyg), had no effect on stability (Table 1). ES stability was thus independent of the presence of the 70 bp repeats, the *vsg*1.010 pseudogene, or the inserted markers.

The switching frequencies upon removal of the CTR from the clones expressing either Vsg117 or luciferase (221/117/CTKO or 221/luc/CTKO, respectively) are shown in Table 1. Prior to the second round of transfection, cell lines 221/luc and 221/117 were confirmed to be >99.5% expressing Vsg221, by immunofluorescence. No cells

Table 1
Stability and switching frequency of clones with modified expression sites

Transformant	Stability and switch frequency	Number of independent cell lines and new *vsg*
WT118	Very stable (6×10^{-7} in vivo)	
118CTKO	Unstable (2.8×10^{-4} in vitro; 1.0×10^{-4} in vivo)	10 *vsg*bR2
WT221	Very stable ($< 1 \times 10^{-6}$ in vitro)	
221/70bpKO	Very stable ($< 1 \times 10^{-6}$ in vitro)	
221/luc	Very stable ($< 1 \times 10^{-6}$ in vitro)	
221/117	Very stable ($< 1 \times 10^{-6}$ in vitro)	
221/luc/hyg	Very stable ($< 1 \times 10^{-6}$ in vitro)	
221/117/hyg	Very stable ($< 1 \times 10^{-6}$ in vitro)	
221/luc/CTKO	Unstable ($< 1.3 \times 10^{-4}$ in vitro)	9 *vsg*bR2: 1 *vsg*121
221/117/CTKO	Unstable ($< 0.9 \times 10^{-4}$ in vitro)	9 *vsg*bR2: 1 *vsg*121
221/luc/KO	Very unstable (within a few cell divisions)	6 *vsg*bR2: 2 *vsg*121
221/117/KO	Very unstable (within a few cell divisions)	4 *vsg*bR2: 2 *vsg*121

expressing Vsg121 could be detected. The switching frequency of these clones, determined in vitro after CTR deletion, was similar to that observed with the 118/CTKO clones. Since the switch frequency of clones expressing 221 has been previously reported to lie in the range of 2×10^{-7} to 3×10^{-6} [35], CTR deletion again results in a more than 100-fold increase in switching frequency.

Ten switches derived from 221/luc/CTKO and ten from 221/117/CTKO were analyzed in detail. The switches again expressed a larger Vsg than the parent cell lines (Fig. 3B). By RT-PCR of clones chosen at random, we were able to demonstrate that, in each set of ten clones, one was expressing *vsg*121 and nine expressed *vsg*bR2. The expression of these genes was confirmed by Western and Northern analysis (examples are shown in Fig. 3 and Fig. 4). Apart from the potential for the 221/luc/CTKO or 221/117/CTKO cell lines to switch to expression of either *vsg*bR2 or *vsg*121, the results from these cell lines were similar to that of the 118 cell lines with CTR deletions. As for the 118/CTKP lines, the unstable 221/CTKO lines also contained bR2 RNA, due to outgrowth of switched cells during propagation of cells for RNA extraction. Activation of the new ES, in situ, was not accompanied by any detectable DNA rearrangements in the silenced 221 ES (Fig. 2 and Southern data not shown).

One of the most striking observations was that a deletion including the CTR and extending into the 5′ end of *vsg*221 (221/luc/KO or 221/117/KO) caused a very rapid switch to a new ES, again leading to expression of either *vsgbR2* or *vsg*121. Similar results were obtained with cell lines expressing luciferase (221/luc/KO), where disruption of the 221 SAS and 5′ coding sequence would have been expected to be lethal, or with a functional *vsg*117 (221/117/KO), which might have been expected to remain viable. Eight independently isolated clones of 221/luc/KO and six independently isolated clones of 221/117/KO were analyzed in detail. RT-PCR suggested that the clones were expressing either VsgbR2 or Vsg121. This was confirmed by Western (Fig. 3) and Northern (Fig. 4) analysis. Of the eight clones analyzed from 221/luc/KO, six were expressing VsgbR2, and two expressed Vsg121. From the six clones of 221/117/KO, four were expressing VsgbR2 and two expressed Vsg121. Immunofluorescence demonstrated that cell populations 221/luc/KO/Sw121 or 221/117/KO/Sw121 were homogeneously expressing Vsg121, and that no cells from the 221/luc/KO/SwbR2 or 221/117/KOSwbR2 populations could be detected which were expressing Vsg121. Because none of the transformant cell lines were a mixed population, the switch probably occurred instantaneously, during or shortly after transfection. We can also rule out the possibility that we are selecting for a sub-population of transformed cells that were already expressing a Vsg other than Vsg221 for the following reasons. We were never able to detect the expression of Vsg121 by immunofluorescence

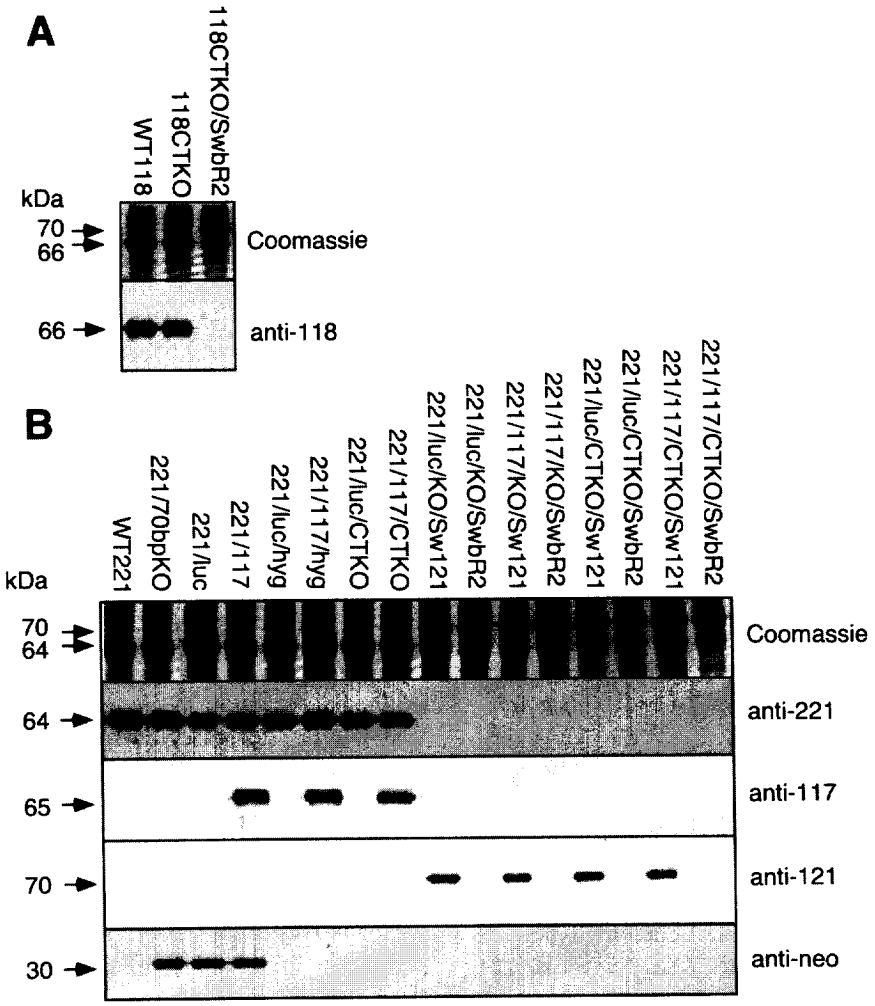

Fig. 3. Vsg expression in transformants and switches. Proteins were separated by SDS-PAGE, blotted onto nitrocellulose and reacted with the appropriate antibody. (A) Cell lines derived from WT118 (B) cell lines derived from WT221. For details of the derivation and nomenclature of the cell lines see Section 2 and legends to Fig. 1 and Fig. 2.

in the starting cell lines of 221/luc or 221/117, as was also the case for protein (Fig. 3) or RNA (Fig. 4) analyses of the cell populations. Furthermore, because the transfection efficiency is around 1×10^{-7} μg^{-1} DNA, and all of the isolated clones were expressing a new Vsg, a majority of cells in the starting population would have had to be expressing the new Vsg.

Again, PFGE analysis of the chromosomes of 221/luc/KO/Sw121, 221/117/KO/Sw121, 221/luc/KO/SwbR2 and 221/117/KO/SwbR2 (Fig. 2), confirmed that the new *vsg* was expressed from a new ES activated in situ, without any discernible rearrangement upstream of the *vsg*. RNA prepared from the switched variants indicated that the previously active 221 ES was now transcriptionally silent (Fig. 4: *vsg*221, *neo* and *hyg* probes). These transformants had apparently switched instantaneously to other ESs, but they had been selected by resistance to hygromycin. However, although these clones were resistant to 2.5 $\mu g\ ml^{-1}$ hygromycin, they would not tolerate concentrations higher than 20 $\mu g\ ml^{-1}$. In contrast, whereas expression of *hyg* from a fully

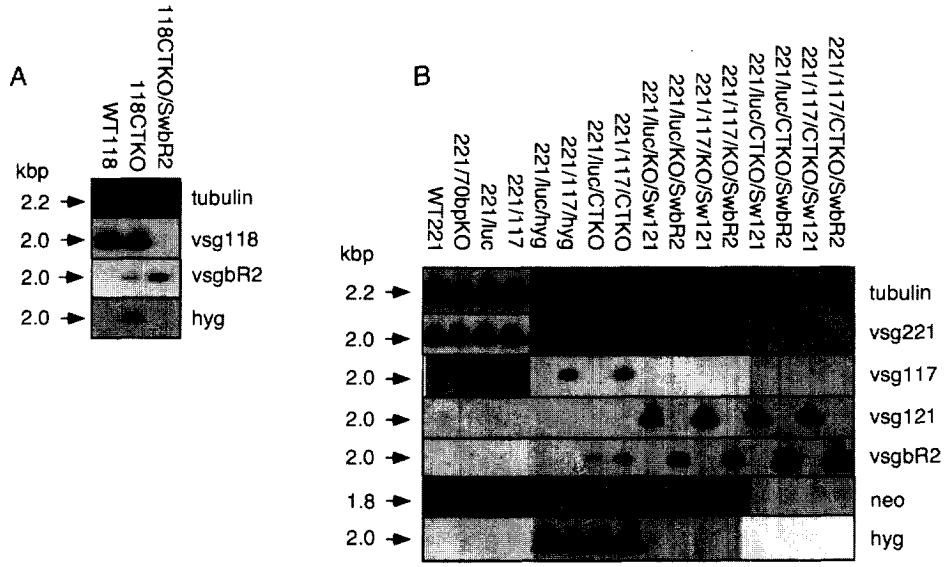

Fig. 4. Analysis of the RNA from the ES of the parent and transformed cell lines. (A) Cell lines derived from WT118 (B) cell lines derived from WT221. The derivation and naming of the cell lines, and the probes, are described in the legends to Fig. 1 and Fig. 2.

active ES confers resistance to >100 μg ml^{-1} hygromycin.

The presence of the *luc* reporter in some of these transformants was useful for confirming the residual transcriptional activity through the inactivated 221 ES. The 221/luc/CTKO clones initially expressed levels of luciferase similar to the 221/luc parent cell line. However, switches to a new expression site (either 221/luc/CTKO/Sw121 or 221/luc/CTKO/SwbR2) were accompanied by a 20-fold reduction in the luciferase levels (Table 2). A similar decrease in transcription through the silenced ES site was indicated by the luciferase activities observed in the 'instantaneous switch' clones 221/luc/KO/Sw121 or 221/luc/KO/SwbR2. This residual transcription was important to the success of these experiments as it allowed the selection of the switched clones in the presence of low levels of hygromycin.

Table 2
Luciferase activities in clones with modified expression sites containing *luc*

ES modification	Light units/10^6 cells
221/luc	538 ± 59
221/luc/hyg	475 ± 27
221/luc/CTKO	480 ± 76
221/luc/CTKOSwbR2	13 ± 12
221/luc/CTKOSw121	21 ± 10
221/luc/KOSwbR2	26 ± 11
221/luc/KOSw121	22 ± 5
Background	0.010

Light units are arbitrary instrument readings. (Turner Luminometer Model TD-20e).

4. Discussion

Deletion of most of the CTR in either the 118 ES or the 221 ES caused more than a 100-fold increase in the rate of switching to a new ES. In contrast, we could remove the entire region between *esag*1 and the CTR, which includes two sets of 70-bp repeats and a *vsg* pseudogene, with no effect on ES stability. Removal of the set of 70-bp repeats between the pseudogene and the CTR, with concomitant integration of a reporter gene and *neo*, also had no effect on ES stability. This agrees with other recent experiments, in which the 70-bp repeats in the 221 ES were also deleted and shown to have no effect on switching

frequency (R. McCulloch, G. Rudenko and P. Borst, Molecular Parasitology Meeting, Woods Hole, September 1996). We also have made several other ES recombinants for which this observation is also true (V.B. Carruthers, K.P. Davies, D.A. Horn and G.A.M. Cross, unpublished experiments).

The incorporation of the *vsg*117 into the 221 ES gave rise to cell lines that simultaneously expressed both Vsgs. These 'double expressors' are very stable in vitro. They express similar amounts of RNA from the two genes (K.P. Davies, unpublished data) and equal amounts of protein [20]. The amounts of RNA and protein are not measurably different to those found in wild-type cells expressing either Vsg117 or Vsg221.

To our surprise, in these 'double-expressor' cells, deletion of the CTR and part of the *vsg*221 (cell lines 221/117/KO and 221/luc/KO) was not lethal, but a new ES was activated, probably within a few divisions after transfection of the cell. The low transfection efficiency, and the continuous growth in the presence of drug selection, ensured that the selection of a sub-population from a contaminant in the starting cell line would be highly improbable. The rapidity of the switch argues against its dependence on a related event, such as base modification or rearrangement which have been proposed as mediators of antigenic variation in trypanosomes [5]. These observations implied that the presence of a second *vsg* was insufficient to stabilize a bloodstream-form ES.

An important control, if increased switch frequency were to be interpreted as being solely a consequence of CTR deletion, was provided by the second-generation constructs in which *hyg* was inserted by homology with *neo* and the CTR (clones 221/luc/hyg or 221/117/hyg). In these cell lines, the CTR was undisturbed and there was no difference in the switching rate, compared with the parental cell lines. It was only when *hyg* was inserted with concomitant deletion of the CTR that the switching rate increased. The process of switching was not accompanied by any detectable rearrangement of the ES.

In the switched clones, the inactivated ES was transcriptionally down-regulated. The residual transcription was attributed to the gratuitous presence, in the targeting constructs, of the core *parp* promoter (Section 2), rather than continuing weak transcription from the far-upstream 221 ES promoter. The prevalent evidence suggests that stage-specific gene expression in *T. brucei* is not mainly regulated at the level of transcription initiation, but rather at several stages along the road from gene to functional protein ([2]). Thus, although Parp (procyclic acidic repetitive protein, procyclin) is the major surface protein of the 'procyclic' Tsetse stage of the *T. brucei* life cycle, the *parp* promoter is active in bloodstream-form trypanosomes, but transcription is rapidly attenuated [11,54]. Other work has shown that a *parp* promoter, inserted into a silent expression site, upstream of the CTR, is considerably attenuated [27]. Taking these observations into consideration, we believe that the weak expression of *hyg* and *luc* reporters is more likely attributable to weak activity of the *parp* promoter rather than the ES promoter. This conclusion cannot be substantiated, but there is other circumstantial evidence. For example, attempts to perform similar ES insertions without the *parp* promoter resulted in no ES insertions, but yielded several recombinants in which the reporters had integrated via other homologous sequences (the *aldolase* SAS, for example) present in the targeting constructs (V.B. Carruthers, unpublished data).

Inactivation of the 221 ES favored expression of either *vsg*bR2 or *vsg*121. In WT118 cells, in which there is no telomeric copy of *vsg*121 [53], *vsg*bR2 appeared to be exclusively activated after CTR deletion. It is conceivable that the method of immunolysis used to kill the parental cells favors selection of clones expressing a particular Vsg, if others might cross-react with the selecting antibody. However, this would not explain the results observed when the CTR and open reading frame (ORF) of the *vsg*221 were simultaneously disrupted (clones 221/luc/KO and 221/117/KO), when a Vsg switch occurred in the absence of immune selection. Previous work has shown that several Vsg combinations are compatible with one another [20]. Therefore, the choice of newly expressed Vsg is unlikely to be constrained by intrinsic structural characteristics. This leads to the

suggestion that certain ESs are primed for activation, perhaps explaining the moderately ordered Vsg expression seen during infection [55,56].

In cell lines where the CTR was deleted (118/CTKO, 221/luc/CTKO or 221/117/CTKO), VsgbR2 RNA was detectable prior to the selection of switched clones (Fig. 4A,B). In contrast, VsgbR2 RNA was not detected in the parent cell lines (WT221 and WT118). There are two possible explanations for this observation, which are difficult to distinguish. The first possibility is that deletion of the CTR has 'primed' the cells for switching and they are already transcribing low levels of the VsgbR2. Immunofluoresence of the initial CTKO cell line demonstrated that the population was $>99.5\%$ homogeneously expressing the parent Vsg but, in the absence of a bR2 antibody, we cannot rule out the possibility that these cell lines co-expressed low levels of VsgbR2. A second explanation is that, because of the time required to obtain sufficient material for Northern analysis, clones have gone through several generations allowing expansion, in this unstable line, of a sub-population expressing VsgbR2. Given the high switch frequency determined for these clones, this seems a reasonable explanation. In a similar experiment, where the CTR was deleted from WT221 (221/luc/CTKO or 221/117/CTKO) cells switched to Vsg121. In this case, where antibody was available, Vsg121 could not be detected in the pre-switch population.

By what mechanism is CTR deletion increasing the rate of switching? It is possible that the partial deletion of the 221 ORF is giving rise to transcripts that are in some way harmful to the cell. Although this would not explain the switching frequency seen in the cell lines where the expressed *vsg*221 gene remains intact (cell lines 118/CTKO, 221/luc/CTKO or 221/117/CTKO), it is a possibility where the *vsg*221 ORF is disturbed following insertion of the *hyg* gene. In this case it is more likely that the toxic product would kill the parasite, rather than induce a switch to a new ES. Other work has shown that several constructs where a Vsg gene is either truncated, or alien genes are inserted into the Vsg reading frame, are not destabilizing the ES or toxic to the cell (M. Engstler and G.A.M. Cross, unpublished results).

Another possible explanation is that integration into the ES has in some way reduced the level of endogenous *vsg*221. In trypanosomes, *trans*-splicing is coupled to polyadenylation of the preceding gene [57,58]. *Trans*-splicing and maturation of *vsg*221 mRNA could have been negatively affected by the juxtaposition of the *hyg* mRNA polyadenylation site and the *vsg*221 splice-acceptor site when the CTR was deleted. If the amount of *vsg*221 mRNA was reduced, the quantity of Vsg117 expressed from the tandemly inserted gene, although substantial, might be insufficient for cell viability, forcing the cell to switch or die.

There are two obvious controls, unfortunately, that we could not obtain. Firstly, we have been unable to specifically delete the *vsg*221 from cell lines co-expressing *vsg*117 and *vsg*221 genes. This might have been because the inserted *vsg*117 alone yielded insufficient Vsg for cell viability. We also attempted, unsuccessfully, to insert an additional CTR upstream of the tandem *vsg*117, in which case, deletion of the endogenous CTR might not have resulted in switching. However, whatever the reason for the induced rapid switches, the fact that they happened was without precedent of observation or explanation.

By far the most intriguing possibility is that CTRs contain some kind of locus-stabilizing elements. The 221/117/CTKO clones, where most but not all of the CTR was deleted, switched more frequently than the parent cell lines (221/117), but not as rapidly as when the CTR and *vsg*221 ORF were disrupted together. This is an important observation of an intermediate situation in which ES disruption was compatible with survival. Most of the cells continued to use the 221 ES. In the case of the cell line 221/117/KO, the rapid switch to a new ES may be explained by a selection pressure exerted by an absolute requirement for the CTR to immediately precede the actively expressed *vsg*. Another possibility is that there are several stabilizing elements distributed throughout the CTR, with perhaps one lying very close to the *vsg*. These stabilizing elements could include the regions encoding several previously reported CTR-derived transcripts, containing short ORFs, that are polyadenylated, contain the spliced leader, and are found in polysomes [59,60]. The

transcripts are specific to a particular ES. Examination of CTR sequences deposited in GenBank shows no obvious patterns. However, despite the lack of sequence similarity, there may be motifs that affect DNA structure. It was previously noted [61] that mung-bean nuclease preferentially cleaves trypanosome DNA 300–800 bp 5′ to the start of the *vsg* coding sequence, suggesting the presence of a hypersensitive site, presumably determined by some anomaly in DNA coiling or chromatin structure. Alternatively, the locus–stabilizing elements might interact with nuclear matrix proteins associated with transcriptionally active regions of the nucleolus, or perhaps hold the ES in a structure that allows a high level of gene transcription, in a fashion similar to looping seen in other organisms [62]. Another possibility is that heterochromatinisation might be prevented by some factor binding to the CTR.

This is the first report of genetic manipulation of an ES causing a switch to another ES. If the effect is simply a by-product of the integration of foreign genes resulting in an inferior ES, which would not be consistent with the results of other extensive manipulations that have been performed in our laboratory, this possibility would need to be considered in all investigations where constructs are stably integrated into the ES. However, there are several other intriguing possibilities, the most interesting of which is that the CTR contains stabilizing elements for maintaining an active ES.

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