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VSG switching in *Trypanosoma brucei*: antigenic variation analysed using RNAi in the absence of immune selection

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Summary

***Trypanosoma brucei* relies on antigenic variation of its variant surface glycoprotein (VSG) coat for survival. We show that VSG switching can be efficiently studied *in vitro* using *VSG* RNAi in place of an immune system to select for switch variants. Contrary to models predicting an instant switch after inhibition of VSG synthesis, switching was not induced by *VSG* RNAi and occurred at a rate of 10^{-4} per division. We find a highly reproducible hierarchy of *VSG* activation, which appears to be capable of resetting, whereby more than half of the switch events over 12 experiments were to one of two VSGs. We characterized switched clones according to switch mechanism using marker genes in the active *VSG* expression site (ES). Transcriptional switches between ESs were the preferred switching mechanism, whereby at least 10 of the 17 ESs identified in *T. brucei* 427 can be functionally active *in vitro*. We could specifically select for switches mediated by DNA rearrangements by inducing *VSG* RNAi in the presence of drug selection for the active ES. Most of the preferentially activated *VSG*s could be activated by multiple mechanisms. This *VSG* RNAi-based procedure provides a rapid and powerful means for analysing VSG switching in African trypanosomes entirely *in vitro*.**

Introduction

The African trypanosome *Trypanosoma brucei* can successfully proliferate in the mammalian bloodstream as a consequence of a sophisticated strategy of antigenic variation of a homogeneous variant surface glycoprotein (VSG) coat. An infected host can effectively clear a given VSG variant via antibody-mediated lysis once it has mounted the appropriate antibody response. However, as VSG switch variants are continuously being generated, these temporarily escape destruction. As individual trypanosomes have many hundreds of *VSG* genes, cyclical waves of parasitaemia make up a chronic infection, which can persist for years. Antigenic variation in African trypanosomes is reviewed by Barry and McCulloch (2001), Vanhamme *et al*. (2001), Donelson (2003) and Pays *et al*. (2004).

The active *VSG* is transcribed in a mutually exclusive fashion from one of about 20 telomeric bloodstream form *VSG* expression sites (ES) (Pays *et al*., 1989; Chaves *et al*., 1999). The active ES is located in a discrete extranucleolar body (ESB), which appears to contain the transcription and RNA processing machinery necessary for high level expression of *VSG* (Navarro and Gull, 2001). Switching can involve transcriptional control, as the cell switches between ESs [reviewed by Borst and Ulbert (2001)]. Alternatively, DNA rearrangements can slot a previously inactive *VSG* into the active ES transcription unit via gene conversion or telomere exchange (Pays *et al*., 1983; 1985). Gene conversion is the most important mechanism during the course of an infection, as it allows access to a much larger pool of silent *VSG*s rather than just those at telomeres (Robinson *et al*., 1999). Segmental gene conversion reactions appear to play a predominant role later in an infection, allowing new mosaic *VSG*s to be formed (Roth *et al*., 1989; Thon *et al*., 1989; 1990).

Traditionally, VSG switching has been investigated using animals. Chronic infections can be established in rabbits, goats or cattle (Gray, 1965; Capbern *et al*., 1977; Barry, 1986). However, these experiments are very laborious to perform, as peaks of parasitaemia are typically too low to detect reliably by microscopy. Subsequent amplification steps in mice are normally necessary before variants can be characterized (Michels *et al*., 1983). In addition, experiments using chronic infections have the disadvantage that it is normally impossible to establish if a switch variant has arisen from the variant preceding it, or was already present as a minor variant in the infection. For this reason, ‘single relapse’ experiments in mice or

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rats are performed, whereby one defined switch away from a given VSG is analysed (Miller and Turner, 1981). However, it requires a large number of rodents to generate a panel of independent clonal switch variants. Multiple switch variants can be isolated from a single mouse (McCulloch *et al*., 1997; Rudenko *et al*., 1998), but it can be impossible to determine if superficially identical switch variants arose independently. All of these approaches to study VSG switching generate relatively few clonal *T. brucei* variants with a known pedigree.

We show here that *VSG* RNAi can be used in place of an immune system to rapidly select for large and statistically significant numbers of clonal *T. brucei* antigenic variants with a known parentage *in vitro*. We do not find any evidence that blocking VSG synthesis triggers a switch, as fluctuation test analysis showed that VSG switching occurred spontaneously at a rate of about 10^{-4} per cell division, and was not induced by the *VSG* RNAi. We identified the activated *VSG* expressed in a total of 168 switched clones by sequencing *VSG* cDNA amplified by reverse transcription polymerase chain reaction (RT-PCR), and show a highly reproducible preferential hierarchy of *VSG* activation. Most of the preferentially activated subset of *VSG*s were found to be activated by different switching mechanisms.

Results

Induction of *VSG221* RNAi in the *T. brucei* 221VG1.1 and 221VG2.1 strains results in the ablation of *VSG221* transcript down to 1–2% normal levels, and the cell-cycle arrest and subsequent death of all trypanosomes expressing VSG221 (Sheader *et al*., 2005). In these strains a puromycin resistance gene integrated behind the promoter of the active *VSG221* ES allows maintenance of homogeneous VSG221 expression in the presence of puromycin selection. However, after induction of *VSG221* RNAi for about 4 days, resistant trypanosomes invariably grow out. Immunofluorescence analysis (result not shown) and protein analysis of these revertants show that these resistant cells had switched their VSG (Fig. 1). VSG is normally the major band in Coomassie-stained gels of

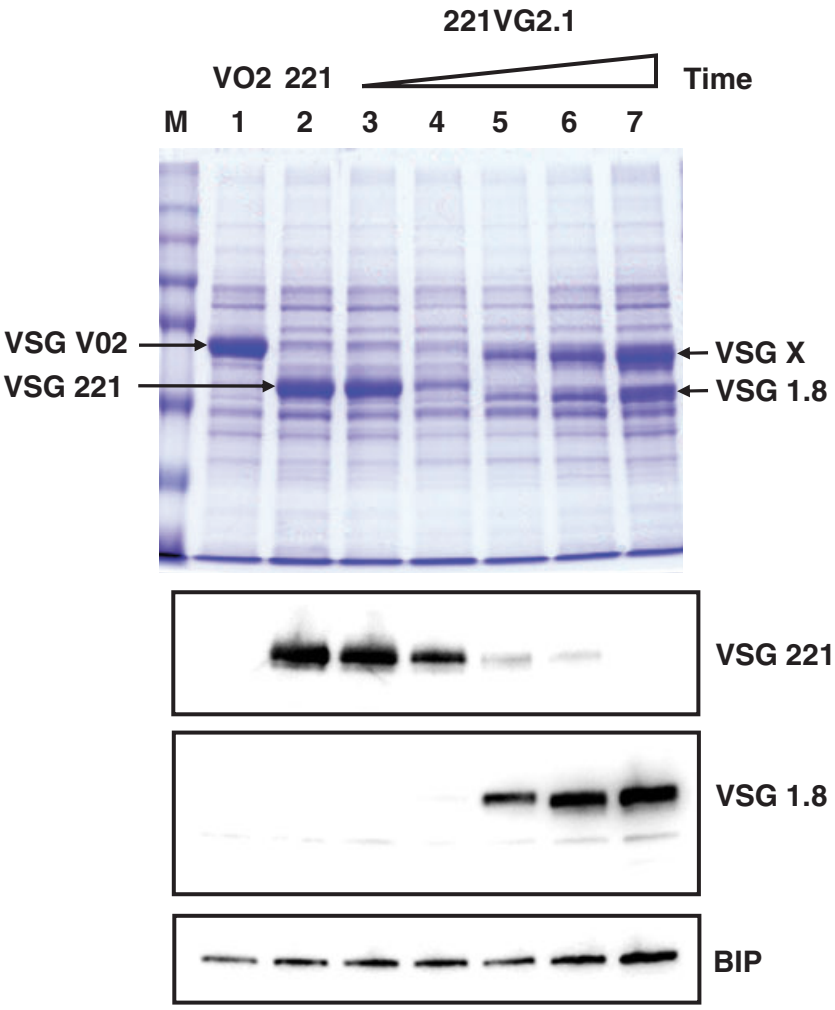

Fig. 1. Induction of *VSG221* RNAi allows selection of new VSG switch variants. Above is a Coomassie-stained SDS-PAGE gel with *T. brucei* total protein lysates, with the prominent band observed corresponding to VSG. Known VSGs are indicated with arrows, with VSGX putatively corresponding to an unknown VSG. Control protein lysates are from a *T. brucei* strain expressing VSGV02: *T. brucei* 221GP1(VO2+) (Sheader *et al*., 2004) (lane 1), or a *T. brucei* strain expressing VSG221: *T. brucei* HNI(221+) (Rudenko *et al*., 1998) (lane 2). Protein lysates isolated from *T. brucei* 221VG2.1 where *VSG* RNAi was induced with tetracycline for 0, 65, 112, 135 or 157 h are loaded in lanes 3–7 respectively. Below are panels from the Western blot of the same gel reacted with anti-VSG221 or VSG1.8 antibodies. As a control for protein loading, the blot was reacted with an antibody against BiP (Bangs *et al*., 1993). The lane with protein markers is indicated with M.

protein lysates from bloodstream form *T. brucei*. After over 4 days of induction of *VSG221* RNAi, cells expressing VSG221 had almost completely disappeared from the population, and other abundant proteins appeared, of which one of the identified bands corresponded to VSG1.8 (Fig. 1). This indicated that *VSG221* RNAi could be used as a novel means for selecting VSG switch variants in the absence of an immune system.

In order to investigate VSG switching in more detail, we designed an experimental procedure allowing us to select for very large numbers of independent VSG switch events *in vitro* using microtitre dishes (Fig. 2). Pilot experiments indicated that *VSG221* RNAi resistant trypanosomes were generated at a frequency of approximately 10^{-4} per division, and that these had indeed switched their VSG. In order to ensure that we were generating independent VSG switch events and not re-isolating VSG switch events already present in the parental population, we repeated the switching experiments with parallel cultures of trypanosomes too small to contain a switch variant. A range of dilutions were distributed over the wells of 96-well microtitre dishes (ranging from 15 to 120 cells per well). These plates were then incubated for typically three generations (7–8 h per generation) to allow independent switches to occur in the different wells. Tetracycline was then added to induce the *VSG221* RNAi, and plates were incubated for a further 6–8 days before scoring positive wells.

First, immunofluorescence microscopy was used to establish that the *VSG221* RNAi resistant clones had indeed switched their VSG coat away from VSG221. *T. brucei* can switch its VSG using different mechanisms based on DNA rearrangement (gene conversion or telomere exchange) or transcriptional control (*in situ* switch). Our parental *T. brucei* 221VG1.1 and 221VG2.1 cell lines contain the single copy *VSG221* in the active ES, in addition to genes encoding puromycin resistance and green fluorescent protein (GFP). Comparing the phenotypes and genotypes of the switched trypanosomes allows us to extrapolate which switching mechanism was used (Fig. 3). If the switched clone was still expressing GFP, it must have switched its VSG using DNA rearrangements (gene conversion or telomere exchange). PCR analysis allows us to differentiate between these mechanisms using the single copy genes located in the active *VSG221* ES. If the switched GFP expressing clones had lost *VSG221* sequences, they must have switched via gene

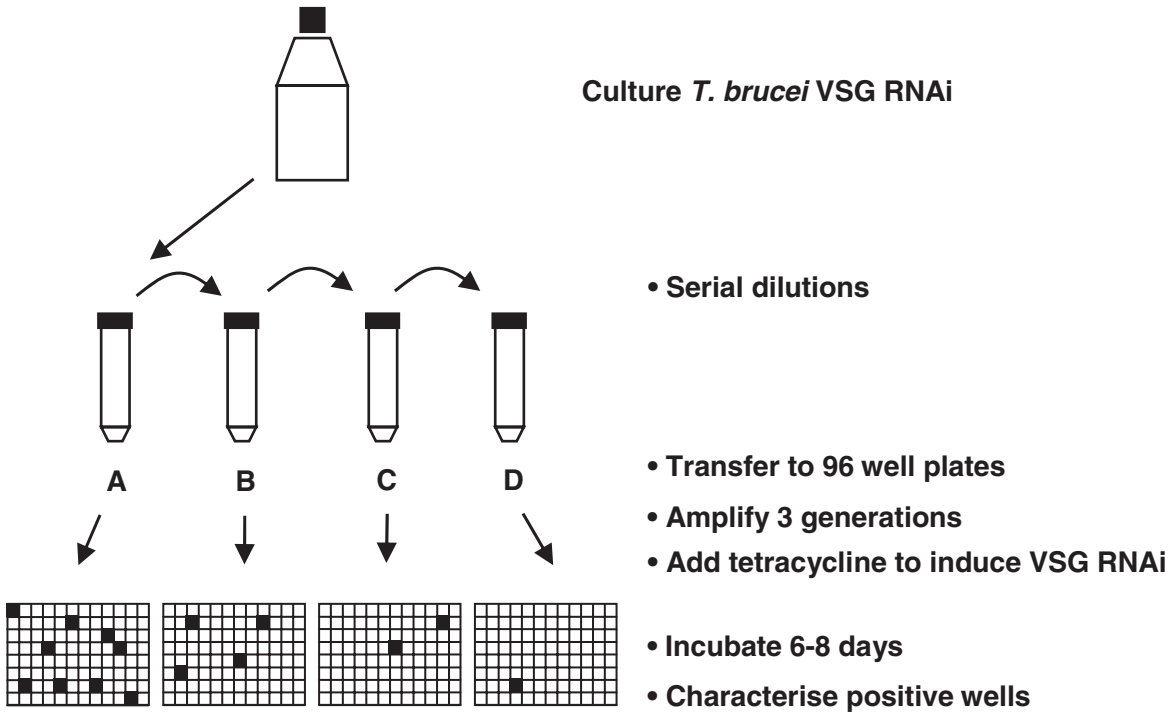

Fig. 2. Experimental procedure for selection of independent VSG switch events using *VSG* RNAi rather than an immune system. A culture of *T. brucei* 221VG1.1 or *T. brucei* 221VG2.1 was serially diluted, and each dilution was distributed over a 96-well plate at a concentration of 100–800 cells ml^{-1}. As the VSG switching frequency is about 10^{-4} in our strain using this procedure, individual wells would be highly unlikely to already contain a VSG switch variant. The wells were then expanded for typically three generations to allow a VSG switch to occur. Tetracycline was added to all of the wells to induce *VSG221* RNAi. This results in the cell-cycle arrest and subsequent death of all trypanosomes that had not switched to a new VSG. After incubation for 6–8 days clonal *T. brucei* variants were scored and analysed. See *Experimental procedures* for detailed protocol.

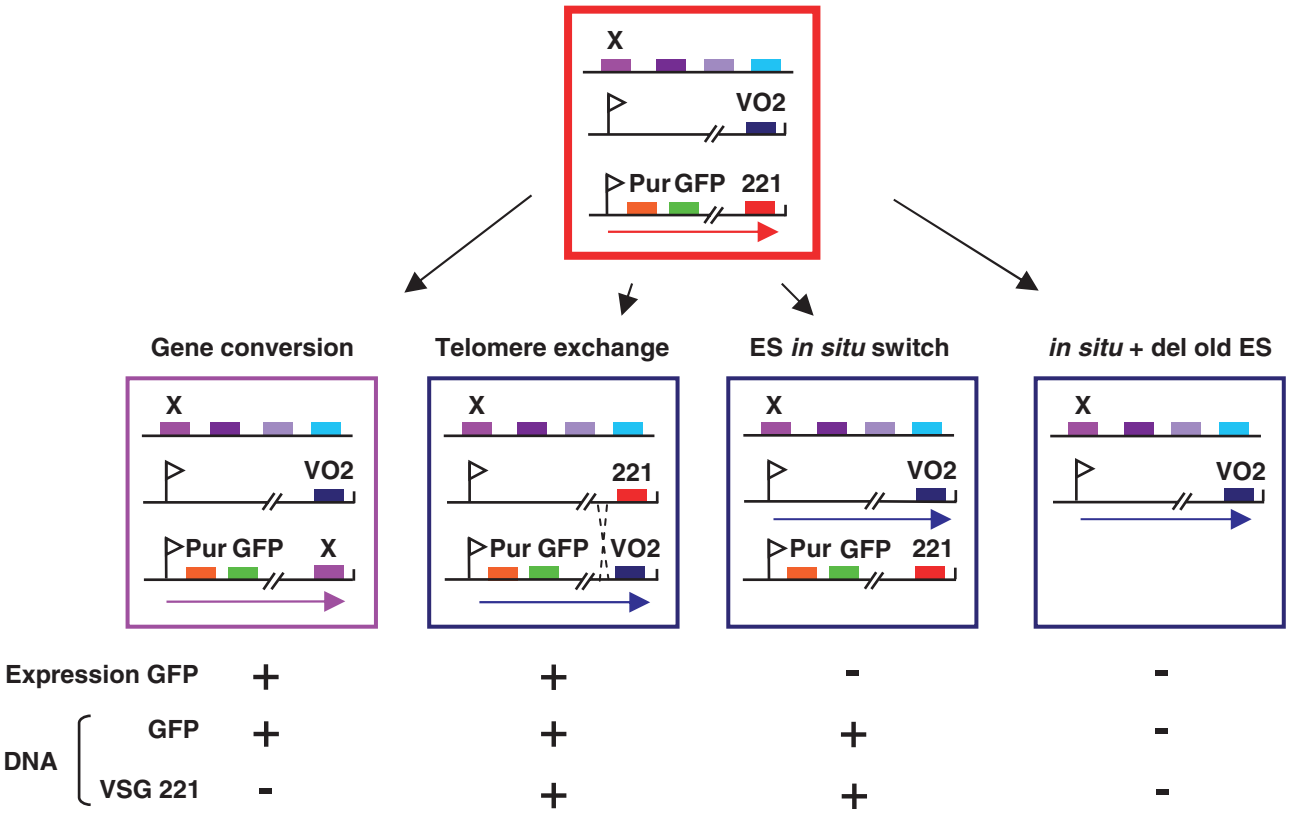

Fig. 3. Strategy for categorization of switched *T. brucei* clones according to the mechanism used for VSG switching. The large coloured boxes indicate trypanosomes, with the parental strain indicated above and the switch variants below. The *VSG* ES promoters are indicated with flags, transcription with arrows and various genes with solid coloured boxes. After the induction of *VSG* RNAi, cells that had switched away from VSG221 were characterized further. A comparison of the genotypes and phenotypes of the swiched *T. brucei* clones allowed the extrapolation of which VSG switching mechanism had mediated the switch. GFP expression was determined by microscopy. Cells that had retained GFP expression were categorized as having switched via gene conversion or telomere exchange, which could be differentiated by establishing the presence of *VSG221*. The presence (+) or absence (–) of *GFP* or *VSG221* sequences was determined using PCR. Cells that had retained *GFP* and *VSG221* but did not express either gene were established to have switched via an *in situ* switch. In the event that all marker genes in the inactivated ES were lost, the switched clone was categorized as having switched via an *in situ* switch plus deletion of the old ES (Cross *et al.*, 1998; Rudenko *et al.*, 1998).

conversion. If they had retained *VSG221*, they presumably switched via telomere exchange. Trypanosomes that did not express GFP, but contained all marker genes must have switched via an *in situ* switch. We have regularly found that trypanosomes that have undergone an *in situ* switch have lost the old ES (Cross *et al.*, 1998; Rudenko *et al.*, 1998). In this case the switched trypanosomes have lost all sequences present in the *VSG221* ES.

Using this strategy we analysed a total of 127 *VSG221* RNAi resistant trypanosome clones (Fig. 4A). In total, 3% of the *VSG221* RNAi resistant clones had not switched away from VSG221, and had instead presumably inactivated the inducible RNAi machinery. This reversion through mutation has been documented before (Chen *et al.*, 2003). However, 97% of clones had switched away from VSG221. Further analysis showed that these had used all expected VSG switching mechanisms. The most frequent switch event was an *in situ* switch to another ES (77% of total switch events). Switches mediated by gene conversion or telomere exchange were found relatively infrequently, and constituted, respectively, 4% or 2% of total switch events. During this experimental procedure *VSG221* RNAi was performed without drug selection pressure to maintain activity of the *VSG221* ES.

In order to select for VSG switches mediated by DNA rearrangements, we next performed *VSG221* RNAi in the presence of puromycin selection on the active *VSG221* ES (Fig. 4B). This would be expected to select against *in situ* switch events and leave those mediated by DNA rearrangements. This was indeed the case. A total of 41 *VSG221* RNAi resistant clones were analysed. The majority of these clones appeared to have switched via gene conversion (73%) or telomere exchange (7%), indicating that maintaining drug selection on the active ES was indeed a means for selectively isolating VSG switch events mediated by DNA rear-

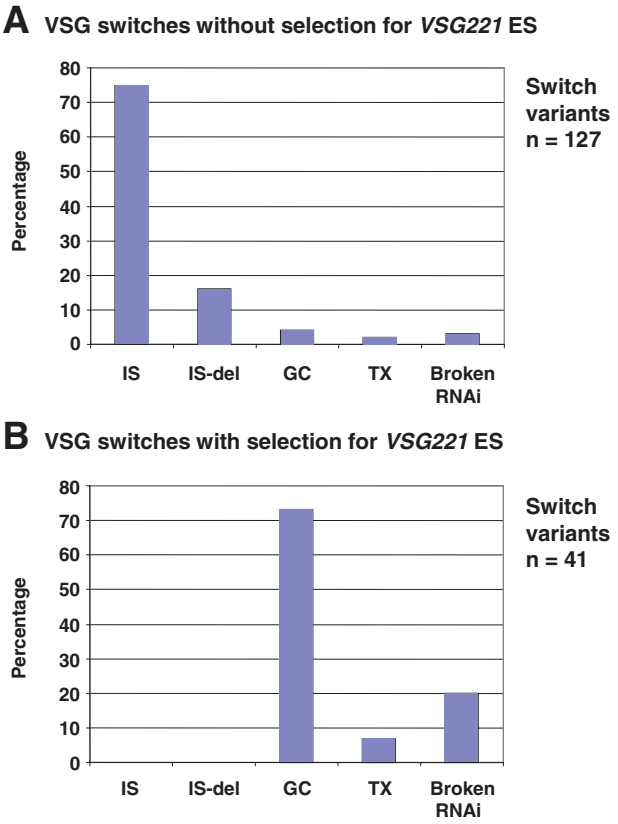

Fig. 4. Induction of *VSG221* RNAi allows the isolation of *T. brucei* which has switched its VSG coat.
A. Transcriptional switching between ESs (*in situ* switch) is the preferred switching mechanism in *T. brucei* 427. A total of 127 *T. brucei VSG221* RNAi resistant variants were characterized, with the percentage calculated to have switched by each mechanism indicated with bars. The mechanisms indicated are *in situ* switch (IS), *in situ* switch coupled with delection of the old ES (IS-del), gene conversion (GC) and telomere exchange (TX). *T. brucei* clones that were resistant to *VSG* RNAi but had retained the VSG221 coat are indicated in the category 'Broken RNAi'.
B. Induction of *VSG* RNAi in the presence of puromycin selection for activity of the *VSG221* ES results in the selective isolation of trypanosomes, which have switched via DNA rearrangements. In addition, there is increased isolation of clones that have inactivated the RNAi machinery. A total of 41 clones was analysed.

rangements. As expected, this modification also resulted in the relative enrichment for clones that had become *VSG221* RNAi resistant through inactivation of the RNAi machinery (20%).

We next determined the switch rate in our *T. brucei* 427 line with this *VSG* RNAi mediated procedure using fluctuation test analysis (Luria and Delbruck, 1943). In addition to a determination of the switch rate, this analysis also allowed us to establish if the VSG switch events that we were observing were occurring spontaneously, or were being induced by the *VSG* RNAi. Fluctuation tests rely on inoculation of many parallel cultures containing dilutions too low to contain a mutant, or in our case, switch variant. These cultures are then expanded to a predetermined concentration before the selection (*VSG* RNAi) is induced. These induced cultures are spread over 96-well plates to allow the scoring of individual clones (see *Experimental procedures* for details). The more generations a culture is expanded for, the more fluctuation will be seen in the percentage of clones that are resistant to the *VSG* RNAi depending on exactly when the switch occurred during the amplification step (Luria and Delbruck, 1943).

In this way, we determined the switching frequency in *T. brucei* 427 using *VSG221* RNAi to be approximately 1×10^{-4} per generation (Table 1). If a mutation or switch event is induced by the selection procedure, one would expect the variance in number of clones generated per culture divided by the mean to have a value of one. In contrast, in our experiments this value was consistently higher, arguing that we are indeed analysing spontaneous switch events. In a similar fashion, we performed fluctuation test analysis on VSG switching experiments performed in the presence of puromycin selection pressure for the active ES (Table 1). The frequency of generation of RNAi resistant clones after screening away *in situ* switches was approximately 7×10^{-6} per division. Similarly, there was no evidence that these were induced by the *VSG* RNAi, and we therefore appear to be isolating spontaneous switch events. In the experiments where *in situ* switches were screened away, the difference between the variance and the mean was greater, presumably as a consequence of the larger number of generations that the cultures were expanded for. An increased number of generations would be expected to increase the degree of fluctuation (Luria and Delbruck, 1943).

In order to determine whether or not preferential hierarchies of VSG activation were present, we identified the activated VSG in the switched clones. VSGs have a conserved carboxy terminus (Borst and Cross, 1982). We made cDNA for the new VSG variants using a primer against the conserved 3′ end of *VSG*. This cDNA was then amplified by PCR using this *VSG-3′* primer, and a primer targeted to the spliced leader present on all *T. brucei* transcripts. Both ends of the *VSG* cDNAs were then sequenced to determine their identity.

We found that there was a clear and reproducible preferential hierarchy in the *VSG*s that were activated in our switching experiments (Fig. 5A). *VSGbR2* and *VSG121* were highly preferentially activated, making up more than half of the total switch events. We have previously cloned 17 *T. brucei* 427 ESs in yeast, which presumably comprises the entire ES repertoire (Becker *et al.*, 2004). Nine of our activated *VSG*s are present in our cloned set of ESs (*VSGbR2*, *VSG121*, *VSG1.8*, *VSGJS1*, *VSGNA1*, *VSG224*, *VSG800*, *VSGT3*, *VSGV02*) (see *Experimental procedures* for sequence accession numbers). As these

Table 1. Determination of VSG switching rates using fluctuation test analysis *in vitro*.

	N_0	N_t	*C*	P_0	Mean	Var.	Var./Mean	Rate
Rates of VSG switching								
221VG1.1								
Ex. 1	50	1.5×10^4	16	0.170	3.13	6.53	2.09	1.20×10^{-4}
Ex. 2	50	8.4×10^3	24	0.250	2.42	5.83	2.41	1.65×10^{-4}
Ex. 3	10	1.5×10^4	16	0.563	1.94	10.1	5.19	3.84×10^{-5}
Ex. 4	5	2.5×10^4	16	0.125	7.69	16.7	2.17	8.32×10^{-5}
221VG2.1								
Ex. 1	50	1.0×10^4	24	0.625	0.75	1.52	2.03	4.70×10^{-5}
Ex. 2	50	1.0×10^4	24	0.333	1.46	2.42	1.66	1.10×10^{-4}
Ex. 3	10	8.0×10^3	16	0.313	2.88	5.86	2.04	1.45×10^{-4}
Ex. 4	5	3.0×10^4	16	0.125	6.69	14.3	2.14	6.93×10^{-5}
Rates of VSG switching in the presence of drug selection on the active ES								
221VG1.1								
Ex. 1	10	7.0×10^4	24	0.547	0.71	0.79	1.12	8.76×10^{-6}
Ex. 2	5	2.2×10^5	16	0.375	5.19	20.8	4.01	4.46×10^{-6}
221VG2.1								
Ex. 1	10	2.2×10^5	24	0.250	3.17	9.06	2.86	6.30×10^{-6}
Ex. 2	5	1.1×10^5	16	0.375	3.06	14.9	4.88	8.92×10^{-6}

Fluctuation analysis of separate VSG switching experiments (Ex.) using *T. brucei* 221VG1.1 and *T. brucei* VG2.1. The inoculation density (N_0) is indicated in cells ml^{-1}. The cell density at the point of induction of *VSG* RNAi with tetracycline (N_t) is indicated in cells ml^{-1}. The number of independent cultures is indicated with *C*. Cultures were distributed over wells in 96-well plates after induction of *VSG* RNAi. The proportion of cultures that did not contain *VSG* RNAi resistant trypanosomes after induction of *VSG* RNAi is indicated with P_0. The mean number of *VSG* RNAi resistant cells obtained per culture is indicated. The variance in these values is indicated, as is the variance divided by the mean. The rate of generation of *VSG* RNAi resistant cells per cell division is indicated in the last column. Fluctuation analysis was also performed on cells maintained on puromycin selection for the active ES to selectively isolate cells that have switched via DNA rearrangements. See *Experimental procedures* for further details and for how these calculations were performed.

*VSG*s were all activated by *in situ* switches, our experiments show that at least 10 of the previously characterized 17 ESs in *T. brucei* 427 can be functionally active *in vitro*. As expected, switching experiments selecting for DNA rearrangements also resulted in the activation of *VSG*s for which there is no evidence that they are linked to ESs (for example *VSGST1* and *VSGST2*). Both of these *VSG*s are located on *T. brucei* minichromosomes (result not shown), which could be a preferred location for *VSG*s activated by gene conversion early in an infection (Robinson *et al.*, 1999).

In general, *VSG*s that were preferentially activated (like *VSGbR2* or *VSG1.8*) were in the subset of *VSG*s preferentially activated by DNA rearrangements as well as transcriptional control (Fig. 5A). The striking exception to this was *VSG121*, which was frequently activated by *in situ* switches but infrequently activated by DNA rearrangements (Fig. 5A). The *T. brucei* 427 ESs are currently being sequenced, which could provide us with an explanation for the aberrant behaviour of this one ES. This preferential hierarchy of activation of a subset of *T. brucei* 427 *VSG*s was highly reproducible over a series of 12 experiments (Fig. 5B). If potential 'resetting' of the hierarchy is possible, this did not occur during the 6–8 weeks that the *T. brucei* parental strains were in culture after their rederivation by cloning (see *Experimental procedures* for details). Not all activated *VSG*s could be identified using our RT-PCR-based approach (indicated with a question mark in Fig. 5); however, this was a minor subset of total. Possibly some *VSG*s are more divergent at their 3′ ends, and are not recognized by our antisense *VSG* primer. These would have to be identified by other methods.

RNA analysis of a representative set of different *T. brucei* clones that had switched their VSG using different mechanisms, confirmed that GFP was only expressed in clones that had undergone DNA rearrangements, and in virtually all cases only the major VSG variant was detectable indicating clonality (Fig. 6). Pulsed Field gel analysis of a representative set of clones confirmed the absence of DNA rearrangements in clones that had undergone *in situ* switches (Fig. 7). In contrast, clones that had undergone a telomere exchange showed an exchange of *VSG221* with another telomeric *VSG* (Fig. 7). As expected, after having undergone a switch mediated by gene conversion, *VSG221* was lost from the active *VSG221* ES, and another *VSG* was inserted in its place.

Switches mediated via gene conversion led to a duplication of the new *VSG* into the active *VSG221* ES. However, in some cases (for example in some cases of gene conversion of *VSGbR2*) there was evidence for loss of copies of *VSGbR2*, as well as the duplication of *VSGbR2* into the active ES (Fig. 8). Multiple rearrangement events during switching in *T. brucei* that are not immediately

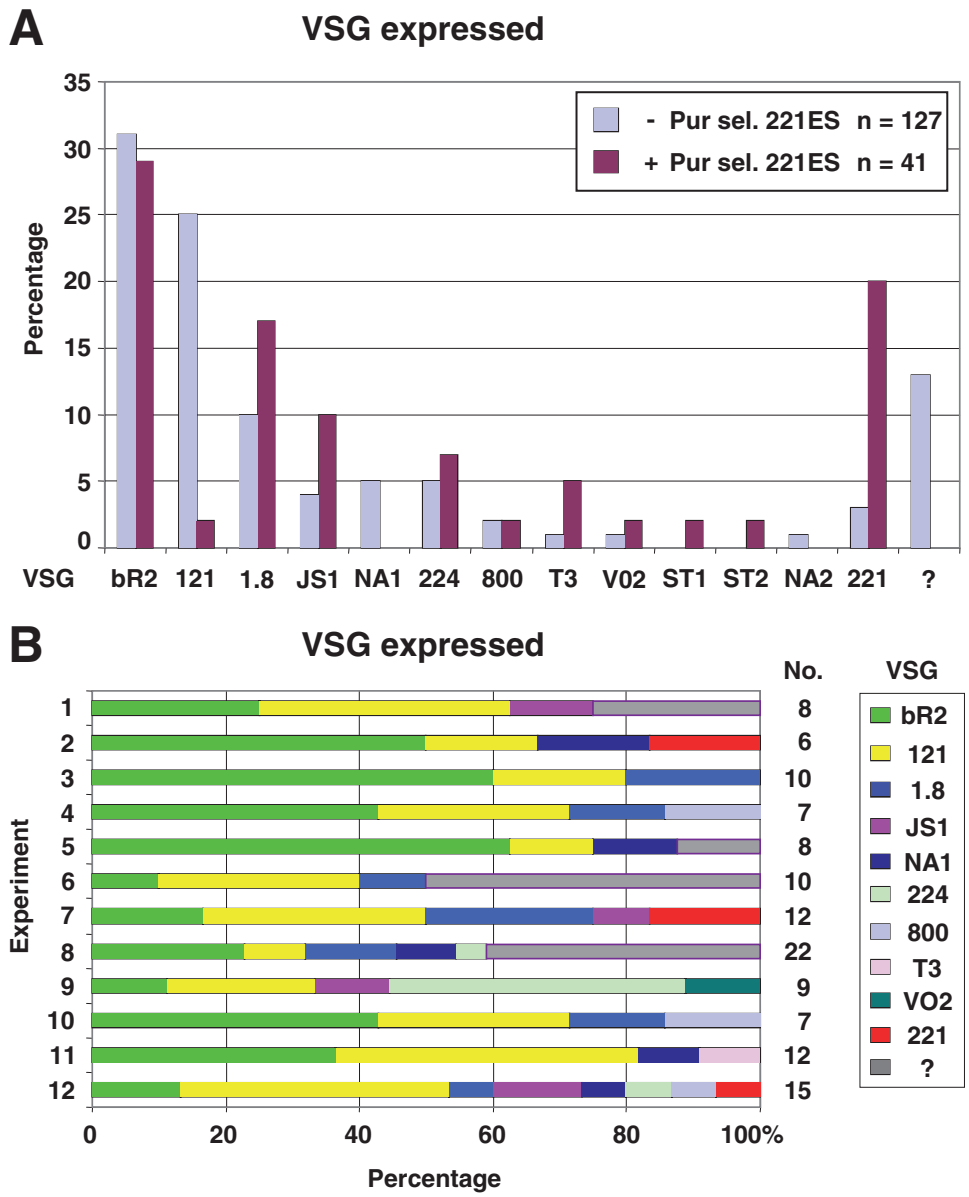

Fig. 5. Preferential hierarchies of VSG activation in *T. brucei* 427.
A. Frequency of activation of different VSGs after induction of *VSG* RNAi. Results were compared between experiments performed in the presence or absence of drug selection on the active *VSG221* ES. Results from experiments performed without drug selection for the active ES (light blue bars) are shown using a total of 127 *T. brucei* switch variants. These results are compared with those from experiments performed in the presence of drug selection for the active ES (dark purple bars) (41 variants analysed). The VSG type identified by cDNA sequencing is indicated below. Cells still expressing VSG221 after induction of *VSG* RNAi appeared to have inactivated the *VSG* RNAi machinery.
B. Preferential hierarchies of VSG activation plotted per experiment performed in the absence of puromycin selection for the active *VSG221* ES. The number of the experiment is indicated on the left. The number (No.) of switch variants isolated per experiment is indicated on the right. The relative percentage of the different activated VSGs per experiment are indicated with coloured bars.

related to the switch have been documented before (Van der Ploeg and Cornelissen, 1984; Myler *et al*., 1988; Aline *et al*., 1989; Navarro and Cross, 1996). This could indicate that the trypanosome undergoes a hyper-recombinogenic state during switching. Alternatively, these events could indicate high background rates of recombination between *VSG* genes.

Discussion

We show that *VSG* RNAi can be used instead of an immune system to rapidly generate clonal VSG switch variants completely *in vitro*. This has allowed us to investigate mechanisms of VSG switching. One possible model for how VSG switching operates, entails a decrease in

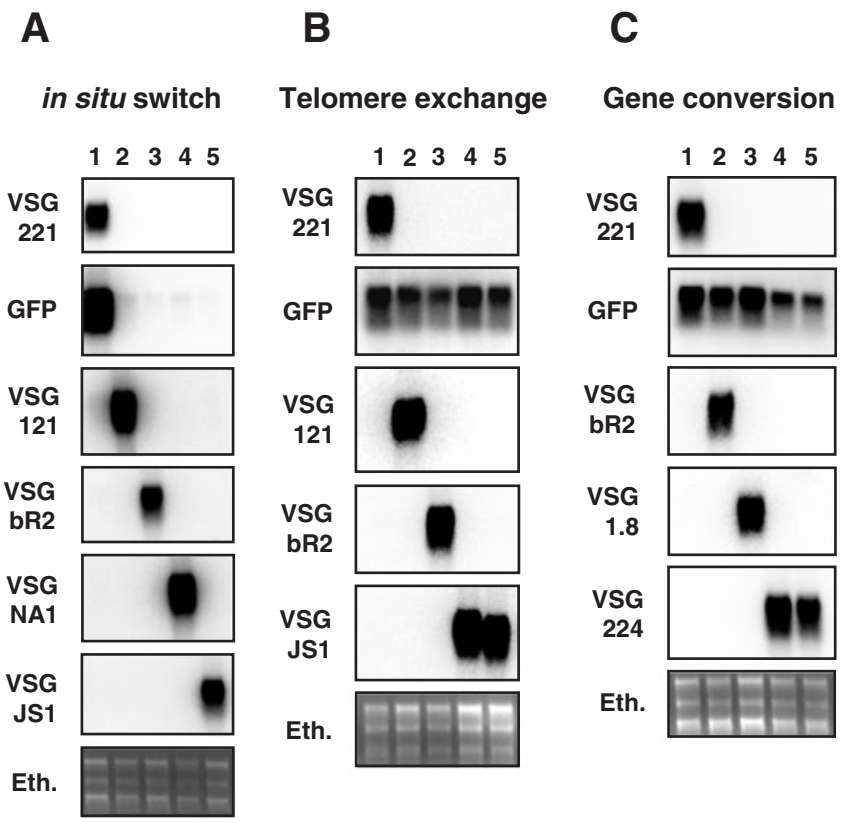

Fig. 6. RNA analysis of *T. brucei* VSG switch variants that have switched via an *in situ* switch (A), telomere exchange (B) or gene conversion (C).
A. VSG switch variants that have switched away from VSG221 via a transcriptional *in situ* switch. Total RNA from the parental *T. brucei* VG2.1 (lane 1) is compared with that from switch variants *T. b.*N19-2R2 (lane 2), *T. b.*N9-1R12 (lane 3), *T. b.*W3-1R2 (lane 4) and *T. b.*N9-1R15 (lane 5).
B. Variants that have switched via telomere exchange. Parental *T. brucei* VG2.1 RNA is compared with that from switch variant *T. b.*N9-1R1 (lane 2), *T. b.*N17-1R6 (lane 3), *T. b.*S2-1R2 (lane 4) and *T. b.*N17-1R11 (lane 5).
C. Transcriptional analysis of variants that have switched via gene conversion. Parental *T. brucei* VG2.1 RNA is compared with that from switch variant *T. b.*N17-1R2 (lane 2), *T. b.*S3-1R3 (lane 3), *T. b.*N17-1R3 (lane 4) and *T. b.*N17-1R4 (lane 5).

VSG mRNA or protein synthesis, which causes a 'stress' response triggering an immediate VSG switch (Borst and Ulbert, 2001). We do not find evidence in support of this, and show, using Luria–Delbruck fluctuation tests, that VSG switch variants are not induced by the *VSG* RNAi but arise spontaneously at a frequency of about 10^{-4} per division. The most frequent switch event is a transcriptional *in situ* switch away from the *VSG221* ES, although we could selectively isolate switches mediated by DNA rearrangements by performing the switch experiments in the presence of drug selection for the active *VSG221* ES. More than half of the *T. brucei* 427 ESs can be functionally active *in vitro*, as 10 of the 17 *T. brucei* 427 *VSG* ESs cloned (Becker *et al*., 2004) were active in our experiments. We show a highly preferential hierarchy of VSG activation, which was reproducible over a series of 12 experiments. With the exception of *VSG121*, *VSG*s that were preferentially activated by transcriptional control also appeared in the set of *VSG*s preferentially activated by DNA rearrangements.

Is our *VSG* RNAi-based system comparable to VSG switching experiments relying on immunized animals for selection against a given VSG? Both experimental approaches rely on effectively killing trypanosome variants expressing the old VSG (in our case VSG221). An antibody-based approach results in the lysis of all trypanosomes expressing a VSG with VSG221-like epitopes, while an RNAi-based method relies on sequence identity. RNAi-mediated ablation of transcript requires stretches of identity of between 21 and 23 nucleotides between the RNAi target fragment and the transcript to be targeted (Scherer and Rossi, 2003). Regions of identity between the *VSG221* RNAi target fragment and other related *VSG*s in *T. brucei* 427 could select against their activation. We do not think that this concern applies to our experiments, as *VSG221* is single copy in *T. brucei* 427, and does not appear to be a member of an extensive family of related genes (Frasch *et al*., 1982; Bernards *et al*.,1986). Our *VSG221* RNAi target fragment is from the divergent 5′ end of the *VSG221* gene, and does not include the conserved 3′ end common to all *VSG*s (Borst and Cross, 1982). Using a 5′ probe, which includes our *VSG221* RNAi fragment, only one very faintly hybridizing band was seen in *T. brucei* 427 under low stringency washing conditions (3× SSC at 65°C) (Frasch *et al*., 1982). We therefore find it highly unlikely that cross-hybridization with other *VSG*s is introducing bias into our results.

VSG switching has previously been studied using chronic infections typically in rabbits, or single relapses in mice or rats. In these approaches it can be impossible to establish the exact parentage of different switch variants

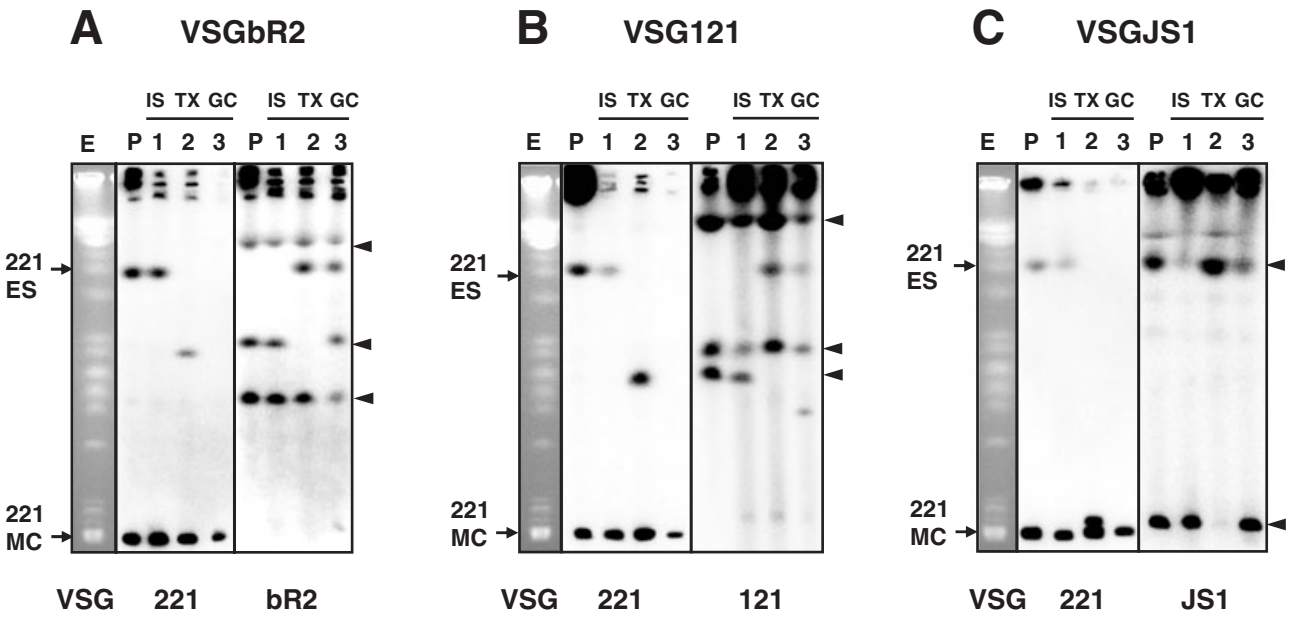

Fig. 7. Pulsed field gel analysis of selected VSG switch events away from VSG221.
A. Switches from VSG221 to VSGbR2. The ethidium stained gel is shown in panel E with the location of *VSG221* hybridizing bands marked with arrows. The *VSG221* ES is located on a 3 MB chromosome, and the *VSG221* RNAi construct hybridizing with the *VSG221* probe on a minichromosome (221 MC). The three *VSGbR2* genes separated under these conditions in the parental *T. brucei* line are indicated with arrowheads on the right. Lane P has DNA from the parental *T. brucei* VG1.1 strain. Lane 1 has DNA from a switched clone that has activated *VSGbR2* via an *in situ* switch (IS) (*T. b.*N9-1R10). Lane 2 has DNA from a clone that has activated *VSGbR2* via a telomere exchange (TX) (*T. b.*N17-1R6). Lane 3 has DNA from a clone that has switched via gene conversion (GC) (*T. b.*N17-1R5). The blots in the different panels are probed with the *VSG* indicated below.
B. Switches from VSG221 to VSG121. Lane P has DNA from the parental *T. brucei* VG1.1. The three *VSG121* genes separated under these conditions in the parental *T. brucei* line are indicated with arrowheads on the right. Lane 1 has DNA from a clone that has switched to VSG121 via an *in situ* switch (*T. b.*N9-1R7); lane 2 has DNA from a clone that has activated *VSG121* gene via a telomere exchange (*T. b.*N9-1R1); and lane 3 has DNA from a clone that has switched via gene conversion (*T. b.*S3-1R5).
C. Switches from VSG221 to VSGJS1. Lane P has DNA from the parental *T. brucei* VG1.1. The two copies of *VSGJS1* separated under these conditions in the parental *T. brucei* line are indicated with arrowheads on the right. Lane 1 shows DNA from a clone that has activated *VSGJS1* via an *in situ* switch (*T. b.*W5-2R9); lane 2 shows DNA from a switched clone that has activated *VSGJS1* gene via telomere exchange (*T. b.*N17-1R11); and lane 3 shows DNA from a clone that has switched via gene conversion (*T. b.*S3-1R3). Note that the *VSGJS1* ES appears to be located on the same chromosomal band as the *VSG221* ES.

in mixed infections. In contrast, we show that *VSG* RNAi can provide a much more manipulable means of studying VSG switching, as large and statistically significant numbers of clonal independent switch events are easily and rapidly generated. The manipulability of this system has allowed us to accurately determine rates of switching using Luria–Delbruck fluctuation tests, and establish that switching is not induced by the RNAi selection procedure. In addition, this approach can be used to investigate the effect of perturbations including gene knockouts, for their effect on VSG switching.

Various genetic modifications disrupting the active ES have resulted in increased rates of ES switching. These have included manipulation of the *VSG221* cotransposed region, which resulted in activation of a new ES within a few cell divisions (Davies *et al.*, 1997). Alternatively, replacement of the ES promoter by a T7 promoter in bloodstream form *T. brucei* prompted an ES switch (Navarro *et al.*, 1999). One model explaining these results, postulated that these ES modifications resulted in decreased synthesis of VSG mRNA or protein. This decrease could lead to a 'stress' response resulting in a switch (Borst and Ulbert, 2001). Our results disprove this model, as *VSG* RNAi did not trigger an instant switch. In contrast, there was no evidence that switching was induced by ablation of *VSG* transcript by *VSG* RNAi. This makes it unlikely that switching is coupled to a 'sensing' mechanism for either VSG protein or transcript.

Using immunized mice, the rate of switching in *T. brucei* 427 has been determined as being around 10^{-6}–10^{-7} per division (Lamont *et al.*, 1986), in contrast to rates of 10^{-2}–10^{-3} in pleomorphic strains of *T. brucei* (Turner and Barry, 1989). Using *T. brucei* 427 with a negative selectable marker inserted in the *VSG221* ES, the switch-off rate *in vitro* has been calculated as being between 1 and 3×10^{-5} per generation (Cross *et al.*, 1998). Our rate of 10^{-4} per division is higher, which could be a consequence of the experimental procedure. Selection using *VSG* RNAi

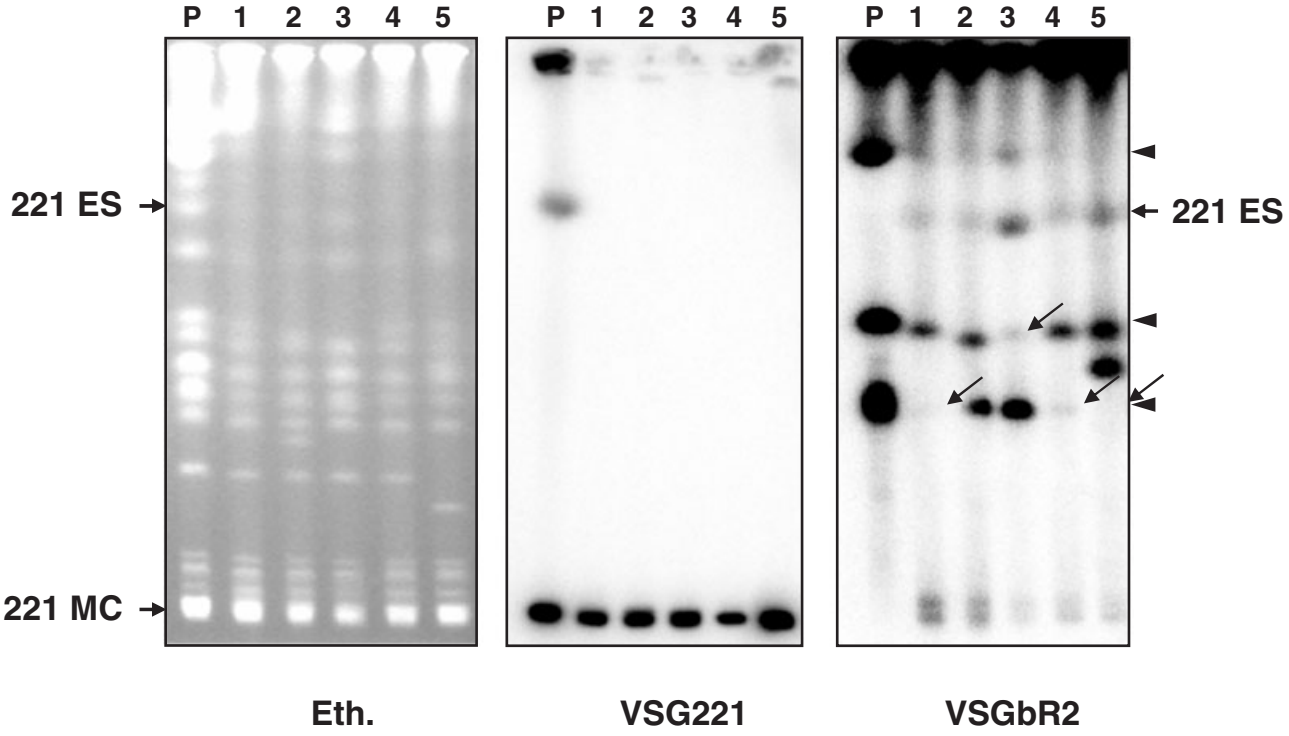

Fig. 8. Additional DNA rearrangements can occur during duplication of the donor *VSG* by gene conversion. During activation of *VSGbR2* by gene conversion, there is often evidence for loss of copies of *VSGbR2* in addition to duplication of *VSGbR2* into the *VSG221* ES. The ethidium stained gel (Eth) shows separation of *T. brucei* chromosomes, whereby bands containing the *VSG221* ES on a 3 MB chromosome and the *VSG221* construct on a minichromosome (221 MC) are indicated with arrows. The three *VSGbR2* genes separated under these conditions in the parental *T. brucei* line are indicated with arrowheads on the right. Lane P shows DNA from the parental *T. brucei* VG 1.1 line. Lanes 1–5 contains DNA from clones that have all switched via gene conversion of *VSGbR2* into the *VSG221* ES. In all of these cases *VSGbR2* is duplicated into the *VSG221* ES, and there is loss of *VSG221*. However, in many clones there is loss of additional *VSGbR2* copies, which appear unrelated to the switch. Lane 1 shows DNA from *T. b.*N17-2R1, lane 2 from *T. b.*N17-2R4, lane 3 from *T. b.*N17-2R5, lane 4 from *T. b.*S2-1R7 and lane 5 from *T. b.*S2-1R3. The chromosomal bands that miss copies of *VSGbR2* are indicated with arrows.

could be a more permissive procedure, whereby relatively recent switch events containing transcripts for both the old and the new *VSG* are still recovered. In contrast, in experiments using the thymidine kinase (*TK*) gene as a negative selectable marker (Cross *et al*., 1998), possibly only relatively older switch variants are rescued where *TK* transcript has been depleted down to low enough levels to allow resistance to the selective agent FIAU. However, an alternative explanation for our relatively high rates of switching, is that very low levels of VSG RNAi, attributed to 'leakiness' in the system, have given rise to a relative increase in VSG switching frequencies.

The majority of our switch events are a consequence of transcriptional activation of other ESs. This does not appear to be the consequence of *VSG* RNAi resulting in the disruption of a polycistronic precursor transcript derived from the *VSG221* ES transcription unit. RNAi against other genes present within the *VSG221* ES (either GFP or various expression site-associated genes) does not result in growth arrest (Sheader *et al*., 2005). This bias for switching by transcriptional activation of other ESs has been seen before in *T. brucei* 427, and could be a feature of monomorphic instead of pleomorphic *T. brucei* strains (Liu *et al*., 1985; McCulloch *et al*., 1997; Robinson *et al*., 1999). The extensive nature of our switching series has allowed us to establish that 10 different *VSG* ESs in *T. brucei* 427 can be individually functionally activated *in vitro*. Not all of these ES switch variants grow equally well under our *in vitro* culture conditions, highlighting the importance of generating and amplifying VSG switch events in independent parallel cultures.

The relative frequency of switches mediated by gene conversion or telomere exchange within our experiments was low (6%), which is lower than the relative frequency of telomere exchange or gene conversion from the 70 bp repeats reported for *T. brucei* 427 (12%) (McCulloch *et al*., 1997). However, a direct comparison between these experiments is not possible as McCulloch *et al*. documented a high rate of loss of all telomeric markers (65%). This could be explained by either telomere conversion, or loss of the old ES through telomere deletion events. ES deletion events are found frequently in *T. brucei* 427

(Cross *et al*., 1998; Rudenko *et al*., 1998). In our experiments, the majority of switch events isolated in the presence of drug selection for the *VSG221* ES, were gene conversions with *VSG*s presumably located in other ESs. This bias could be a consequence of the unusually short 70 bp repeat arrays in the *VSG221* ES, which would favour recombination on upstream *ESAG* sequences present in other ESs (Liu *et al*., 1985; Kooter *et al*., 1988). The 70 bp repeats provide upstream sequence homology facilitating the gene conversion of donor *VSG*s not located in ESs (Campbell *et al*., 1984). *VSGbR2* and *VSG1.8* are present in multiple copies within the cell, and we do not have proof that the donor *VSG* was the ES-located copy. However, this is highly likely a consequence of the predisposition of the *VSG221* ES for undergoing telomere conversions with other ESs (Kooter *et al*., 1988; McCulloch *et al*., 1997). It is likely that the relatively low rates of DNA rearrangement that we observe are a consequence of the *VSG221* ES sequence, as well as being a feature of monomorphic lines. Establishing these *VSG* RNAi switching experiments in a pleomorphic *T. brucei* line would provide a useful comparison point.

A striking and highly preferential hierarchy of VSG activation could be found reproducibly over a series of 12 switching experiments, whereby over half of the variants generated had switched to either VSGbR2 or VSG121. Earlier switching experiments with *T. brucei* 427 have documented preferred activation of specific VSGs. In the earliest *T. brucei* 427 switching experiments, up to half of the variants generated were VSG1.8 (Michels *et al*., 1984). Later experiments showed preferential activation of VSGV02 (Rudenko *et al*., 1995). This highly preferential activation of one or two ES-located *VSG*s indicates that at any point in time one or two ESs are in a 'preactive' state (Chaves *et al*., 1999; Ulbert *et al*., 2002). As *VSG1.8* and *VSGV02* were relatively infrequently activated in our experiments, this could indicate that resetting of the preferential hierarchy within *T. brucei* 427 can take place.

Our experiments were performed so as to minimize potential 'resetting' of this hierarchy within the experimental period. The parental *T. brucei* transformants used for switching were recloned to minimize potential heterogeneity, and thawed stabilates were not maintained in culture longer than 1 month (see *Experimental procedures* for details). At the moment we cannot exclude that this apparent shift in the preferential hierarchy of activated VSGs is a direct consequence of the *VSG* RNAi technique itself. However, we find this scenario very unlikely, as *VSG221* does not appear to be part of an extensive family of related genes that would be affected by *VSG221* RNAi (Frasch *et al*., 1982). Excluding this possibility will require further experiments in order to determine if 'resetting' of the preferentially activated subset of *VSG*s can occur within our experimental system.

Surprisingly, with the striking exception of *VSG121*, many *VSG*s that were preferentially activated by an *in situ* switch (for example *VSGbR2* or *VSG1.8*) were also present in the set of *VSG*s that were preferentially activated by DNA rearrangement. Some of these activated *VSG*s are present as multiple copies, and we cannot be sure which was the donor gene. However, as the *VSG221* ES frequently undergoes telomere conversions with other ESs (Kooter *et al*., 1988; McCulloch *et al*., 1997), we find it likely that the donor *VSG* was ES-located. The active ES has been shown to be present within the nucleus in a discrete extranucleolar body (ESB) presumably containing both transcription and RNA processing factories (Navarro and Gull, 2001). Possibly the 'preactive' state leading to preferential activation of an ES entails physical proximity to the active ES, whereby the 'preactive' ES hijacks the transcription and RNA processing machinery within the ESB. However, in addition, possibly proximity of these telomeres within the nucleus facilitates DNA recombination. Fluorescent *in situ* hybridization (FISH) experiments with ES specific sequences in *T. brucei* could allow us to test if ESs thought to be preferentially activated are indeed in physical proximity to the active ES within the ESB.

The striking exception to this observation is *VSG121*, which was frequently transcriptionally activated, but rarely moved into the active ES via DNA rearrangement. *VSG121* is present within an ES referred to as the dominant expression site (DES), as this was a preferred site for *VSG*s to move via duplicative gene conversion in *T. brucei* 427 (Michels *et al*., 1983; Liu *et al*., 1985; Timmers *et al*., 1987). We do not know why none of the *VSG121* genes were preferred *VSG* donors for the *VSG221* ES. The basic organization of both the *VSG221* ES and DES appears similar, although the DES lacks the large duplications present in the *VSG221* ES (Crozatier *et al*., 1990). The repertoire of *T. brucei* 427 ESs is currently being sequenced at the Sanger Institute, which should give us insight into what is causing this aberrant behaviour.

If the reason that some *VSG*s are preferentially activated is a consequence of subnuclear positioning, this would imply that chromosome ends within the nucleus of *T. brucei* could form relatively stable associations with each other. Telomeres in *T. brucei* have been shown to associate in clusters within the nuclei (Chung *et al*., 1990). It has been argued that these chromosome ends are predominantly in the central zone of the nucleus in long slender bloodstream form *T. brucei* (Perez-Morga *et al*., 2001), but it is not known if there is preferred clustering of specific ends. In *Plasmodium* the *VAR* genes are frequently located at telomeres, which form clusters within the nucleus presumably facilitating interchromosomal recombination (Freitas-Junior *et al*., 2000). However, in

Plasmodium there is no evidence that specific ends form stable interactions with each other (Freitas-Junior *et al*., 2000). Activation of particular *VAR* loci appears to be associated with movement to particular regions of the nuclear periphery (Duraisingh *et al*., 2005; Freitas-Junior *et al*., 2005; Ralph *et al*., 2005). Possibly, in contrast in *T. brucei*, 'preactive' ESs are in a more stable subnuclear structure within the cell whereby they enjoy privileged access to the active ES within the ESB.

Experimental procedures

Strains and culturing conditions

The trypanosomes used were modifications of *T. brucei* 90-13, a *T. brucei* 427 221a line (MiTat 1.2) containing genes encoding T7 RNA polymerase and tetracycline repressor [described in the study by Wirtz *et al.* (1999)]. The *VSG* RNAi cell lines *T. brucei* 221VG1.1 and 221VG2.1 are transformants of *T. brucei* 90-13 containing the MC^{177}*VSG221* RNAi construct targeted into minichromosomes (result not shown). This construct contains an 803 bp fragment of *VSG221* (MiTat 1.2) (Accession No. X56762, positions 122-925) cloned between the opposing T7 promoters of construct p2T7^{Ti}-177(Wickstead *et al*., 2002). Subsequently, the 221GP1 construct containing *eGFP* and the puromycin resistance gene (Sheader *et al*., 2004) was inserted immediately behind the *VSG221* ES promoter in these cells. Maintaining these transformants on puromycin selection allowed us to select for activity of the *VSG221* ES, and maintain the cultures as homogeneous VSG221 expressors. Immunofluorescence analysis of these trypanosomes indicated that non-VSG221 variants were not present at detectable levels (less than 10^{-3}).

Trypanosoma brucei 427 lines were cultured in HMI-9 medium (Sheader *et al*., 2004). The parental *T. brucei* 221VG1.1 and 221VG2.1 lines were maintained on 5 μg ml^{-1} hygromycin (Roche), 2 μg ml^{-1} G418 (Gibco), 2.5 μg ml^{-1} phleomycin (Sigma) and 0.2 μg ml^{-1} puromycin unless otherwise indicated. During normal VSG switching experiments puromycin selection for the *VSG221* ES was removed. Puromycin selection was maintained when VSG switches mediated by DNA rearrangements were specifically selected for.

Variant surface glycoprotein switching in vitro *using* VSG *RNAi rather than an immune system*

Selection for independent VSG switch events using RNAi was carried out by diluting a logarithmic growth culture (about 10^6 cells ml^{-1}) of *T. brucei* VG1.1 or *T. brucei* VG2.1 down to 100, 200, 400 or 800 cells ml^{-1} (15–120 cells per well) in medium. Each dilution was aliquoted over 96-well plates. As the switching frequency in our cell lines using this method *in vitro* was about 10^{-4} per cell division, cultures were so dilute that it was highly unlikely for individual wells to already contain a VSG switch event. The microtitre dishes were incubated for three generations resulting in approximately 800, 1600, 3200 or 6400 cells ml^{-1} (120–960 cells per well). This amplification step resulted in the generation of independent VSG switch events in individual wells. *VSG221* RNAi was then induced by the addition of tetracycline to a final concentration of 750 ng ml^{-1} and trypanosomes were not removed from tetracycline during the expansion of the *VSG221* RNAi resistant variants. *VSG221* RNAi resistant clones emerged 6–8 days following induction of *VSG221* RNAi. When VSG switch events mediated by DNA rearrangements were specifically selected for, cells were plated at the same density in the presence of puromycin selection. However, these cultures were allowed to grow for typically seven generations before induction of RNAi, to compensate for the lower frequency of these switch events.

Calculation of VSG *switching rate and Luria–Delbrück fluctuation analysis*

Fluctuation tests allow the determination of mutation or switching rates. An analysis of the variance compared with mean number of mutants or switch events generated during amplification of independent cultures also allows one to establish if generation of these variants has been induced by the experimental procedure (Luria and Delbruck, 1943; Rosche and Foster, 2000). We typically inoculated either 16 or 24 independent 1 ml cultures of *T. brucei* 221VG1.1 or 221VG2.1 at concentrations ranging from 5 to 50 cells ml^{-1}. A control culture was set in as a control for cell growth. Cells were amplified for typically eight or nine generations, and then *VSG221* RNAi was induced by adding tetracycline to an end concentration of 750 ng ml^{-1}. Each culture was spread over 12 wells of a 96-well plate and wells were allowed to grow out over 6–8 days before scoring.

The P_0 method was used to calculate the rates of VSG switching, whereby P_0 is the fraction of wells from a given culture that do not contain *VSG221* RNAi resistant cells (Luria and Delbruck, 1943; Rosche and Foster, 2000). The number of mutants per culture (m) was calculated from $-\ln P_0$. The switching rate was calculated as m divided by the number of cells per culture at the point of induction of *VSG* RNAi (N_i). The mean number of positive wells per culture, and the variance in the number of switchers per culture were also calculated. A value for variance divided by mean that is close to 1.0 is an indication that VSG switching has been induced by the procedure.

Phenotyping the switched T. brucei *after the induction of* VSG221 *RNAi*

All *VSG221* RNAi resistant clones were first analysed by immunofluorescence microscopy using rabbit anti-VSG221 specific antibodies to determine if they had indeed switched their VSG221 coat. If so, GFP expression was monitored by microscopy. Diagnostic PCR reactions were performed using specific primers for *VSG221*, *eGFP* or the large subunit of RNA polymerase I (*Pol I*). (Primer sequences are available on request.) RNA was isolated using RNeasy mini kits (Qiagen). To identify the newly expressed *VSG*, *VSG* cDNA was cloned using RT-PCR. *VSG* cDNA was generated using the Omniscript RT kit (Qiagen) and the primer VSG14XbaI-as (5′-CCCGCCTCTAGACGTGTTAAAATATATCAG-3′). PCR was performed using the same antisense primer and a primer

against the spliced leader: s-miniexRI (5′-CCGGAATTCG GCTATTATTAGAACAGTTTCTG-3′). Both ends of the *VSG* cDNA were sequenced to determine its identity. Alternative names for the *T. brucei* 427 genes listed plus their GenBank accession numbers are as follows: *VSGbR2* or MiTat 1.11 (AY935577), *VSG121* or MiTat 1.6 (X56764), *VSG1.8* or MiTat 1.8 (AY935574), *VSGNA1* or MiTat 1.13 (AY935576), *VSG224* or MiTat 1.3 (AY935575), *VSGT3* or MiTat 1.21 (AY935572), *VSGV02* or MiTat 1.9 (AY935573).

Protein and nucleic acid analysis

For protein analysis *T. brucei* 221VG2.1 was induced with tetracycline (750 ng ml^{-1}) in the absence of puromycin selection at a density of 8×10^5 cells ml^{-1}. Cells were washed twice, and then resuspended in cold lysis buffer [50 mM HEPES pH 7.5, 1.5 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1% Triton X-100 and one mini protease inhibitor cocktail tablet (Roche) per 10 ml mix] at 10^9 cells ml^{-1}. After microfuge centrifugation, supernatant from the equivalent of 5×10^6 cells was loaded per lane of an 8% SDS polyacrylamide gel. The gel was blotted to Hybond-P membrane (Amersham) and subsequently reacted with rabbit polyclonal antibodies for VSG221 and VSG1.8 (gift of P. Borst) or BiP (gift of Jay Bangs) (Bangs *et al*., 1993). Signal was visualized using an ECL Plus Western blotting detection system (Amersham).

Total *T. brucei* RNA was isolated using RNeasy RNA isolation kits (Qiagen). RNA was loaded on formaldehyde agarose gels, blotted onto Hybond-XL (Amersham) and probed using radiolabelled probes using standard procedures (Sambrook and Russell, 2001). Equivalent loading was determined using ethidium stained gels.

Pulsed field gel analysis was performed using a CHEF-DRIII system (Bio-Rad). DNA from approximately 2.5×10^7 cells was embedded in 1% low melting point agarose blocks. Resolution of megabase chromosomes was performed at 2.5 V cm^{-1} for 144 h, 16°C, 120° angle, 1400–700 s switching time on a 1.2% high strength agarose gel (Helena Biosciences) in 1× $TBE_{0.1}$ (Melville *et al*., 2000). Intermediate chromosomes (100–500 kb) were separated at 6 V cm^{-1}, 14°C, 120° angle, 25 s switching time, 20 h, on a 1% high strength agarose gel in 0.5× TBE. Chromosomal DNA was blotted to Hybond-XL membrane (Amersham) and probed with radiolabelled probes.

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