Consequences of Telomere Shortening at an Active *VSG* Expression Site in Telomerase-Deficient *Trypanosoma brucei*[▽][†]

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Trypanosoma brucei **evades the host immune response by sequential expression of a large family of variant surface glycoproteins (VSG) from one of ~20 subtelomeric expression sites (ES).** *VSG* **transcription is monoallelic, and little is known about the regulation of antigenic switching. To explore whether telomere length could affect antigenic switching, we created a telomerase-deficient cell line, in which telomeres shortened at a rate of 3 to 6 bp at each cell division. Upon reaching a critical length, short silent ES telomeres were stabilized by a telomerase-independent mechanism. The active ES telomere progressively shortened and frequently broke. Upon reaching a critical length, the short active ES telomere stabilized, but the transcribed** *VSG* **was gradually lost from the population and replaced by a new** *VSG* **through duplicative gene conversion. We propose a model in which subtelomeric-break-induced replication-mediated repair at a short ES telomere leads to duplicative gene conversion and expression of a new** *VSG*.

A variety of pathogens evade the host immune response by sequential expression of variant surface glycoproteins (VSG) (1, 4, 17, 32). In *Trypanosoma brucei*, the causative agent of sleeping sickness, the parasite surface consists of a homogeneous dense layer of ~10^7 VSG. A large repertoire of *VSG* genes is dispersed among many subtelomeric regions, but the single transcribed copy is located in a telomeric expression site (ES) (6). *T. brucei* contains ~20 similar ESs, and transcription occurs in a strictly monoallelic fashion (27, 37). An antigenic switch can be achieved by shifting transcription from one ES to another (in situ switch) (13), by a reciprocal translocation between two ESs (31), or when a previously silent *VSG* is copied into the active ES through duplicative gene conversion (24, 36). In pleomorphic wild-type strains, gene conversion-mediated switching predominates and occurs at a dramatically higher rate than in laboratory-adapted strains (29, 33, 34). Although we know a little about the molecular mechanisms underlying *VSG* switching, we do not know what precipitates switching events.

In this study, we tried to determine whether telomere length could influence *VSG* switching. Human and *T. brucei* telomeres consist of long tracts of TTAGGG repeats and, together with their associated factors, they protect chromosome ends from nucleolytic degradation and illegitimate DNA repair activities (3, 5, 21, 38). In contrast to any other known organism, *T. brucei* telomeres grow at a steady rate of 7 to 9 bp per population doubling (PD), and the telomere adjacent to the single transcribed *VSG* gene in *T. brucei* undergoes frequent truncations (2, 13, 23, 28, 35), which are rapidly elongated by the telomerase (8, 10, 15). It remains unclear whether telomere truncations play a role in antigenic variation. Deletion of

telomerase reverse transcriptase (TERT) from *T. brucei* causes progressive telomere shortening at a rate of 3 to 6 bp/PD, loss of minichromosomes, and genomic rearrangements among intermediate chromosomes (8, 9). At essential megabase chromosomes, silent ES telomeres stabilized within a discreet size range by a potentially novel telomerase-independent mechanism (8).

Here we report the consequences of telomere shortening at the actively transcribed *VSG* ES and propose a model for how this could precipitate a *VSG* switch.

MATERIALS AND METHODS

Trypanosome cell lines. The cell line used in these experiments, Lister 427 antigenic type MITat 1.2, designated single marker, expresses T7 RNA polymerase, a Tet repressor, and neomycin phosphotransferase and stably expresses VSG 221 (39). Generation of telomerase-deficient clones has been described previously (9). Parental single marker and mutant cells were cultured in HMI-9 containing 2.5 µg/ml G418 (Sigma) at 37°C (7, 11).

DNA isolation, Southern blotting, and telomere size distribution analysis. Genomic DNA was isolated as previously described (22). Terminal restriction fragments containing specific *VSG* were detected by genomic blotting, hybridization, and phosphorimaging (14). By use of ImageQuant software, each lane in Fig. 1A was partitioned into 30 equally sized rectangles. The signal intensity in each rectangle was measured as a percentage of the total signal in the entire lane and graphically represented as a function of telomere length.

Rotating agarose gel electrophoresis (RAGE). DNA agarose plugs were prepared as described previously by Navarro and Cross (26). Briefly, 2×10^8 cells were harvested, washed in TDB (5 mM KCl, 80 mM NaCl, 1 mM MgSO₄, 20 mM Na₂HPO₄, 20 mM glucose, pH 7.7) resuspended in 0.5 ml L buffer (0.1 M EDTA, pH 8.0, 10 mM Tris-HCl, pH 7.6, 20 mM NaCl), and incubated for 10 min at 42°C. Five-tenths milliliters of 1.6% low gelling agarose (Sigma) in L buffer was added to 0.5 ml cells, mixed, and poured into plug molds (Bio-Rad Laboratories). Plugs were treated in 3 ml L buffer for 2 days with 1 mg/ml proteinase K at 50°C. After two washes for 15 min with L buffer, proteinase K treatment and washing were repeated. Plugs were stored at 4°C until used. Plugs were embedded in 0.8% agarose in 0.5× Tris-borate-EDTA (TBE) gels. To separate megabase chromosomes, a two-window program was used, consisting of a 100- to 300-s linear ramp pulse time at 120 V for 10 h followed by a 1,000- to 2,500-s linear ramp pulse time at 50 V for 80 h. The rotation angle was 106° at a temperature of 12°C (26). Gels were stained for 45 min in TBE plus 0.02 µg/ml ethidium bromide and destained in TBE for 30 min prior to being photographed under UV light. Southern blotting and hybridization were performed as described above.

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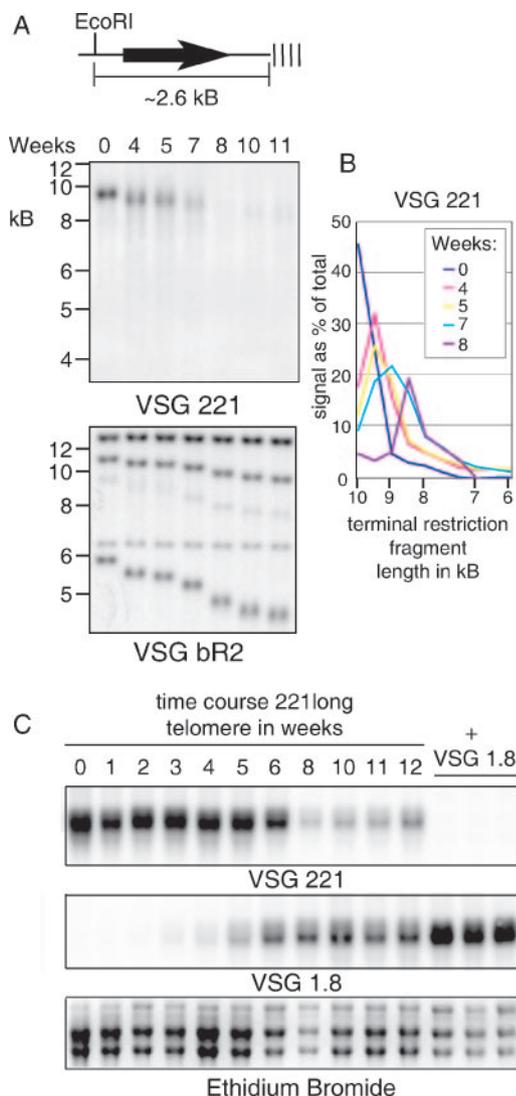
FIG. 1. Telomere length at the active *VSG* 221 ES in telomerase-deficient *T. brucei*. (A) (Upper panel) EcoRI telomere terminal restriction fragment hybridized with *VSG* 221. Progressive telomere shortening and breakage lead to loss of telomeric DNA at the active ES and smearing of the *VSG* 221 signal towards the bottom of the gel. (Lower panel) Reprobing with silent ES *VSG* bR2 verifies equal loading and progressive shortening of silent ES telomeres. (B) Signal intensity distribution in each lane of panel A is graphically represented as a function of telomere length. Smearing of the signal is apparent as peaks become broader and move towards lower molecular mass. (C) Northern blotting of *VSG* 221 (upper panel) and *VSG* 1.8 (middle panel) over 13 weeks. Positive controls for *VSG* 1.8 are in the right three lanes. Equal loading was verified by reprobing with a tubulin probe and ethidium bromide staining of the gel (lower panel). Phosphorimager quantification of the signal is shown in Fig. S1 in the supplemental material.

Long-range Southern blotting. Digestion of DNA in genomic plugs was performed as recommended by the enzyme manufacturer (New England Biolabs). Sixty units of ApaI was incubated in New England Biolabs buffer 4 plus bovine serum albumin at 25°C overnight. Plugs were subsequently washed in 0.1 M EDTA, pH 8.0, and 10 mM Tris-HCl, pH 7.6. Restriction fragments were separated on a 0.8% agarose gel in 0.5× TBE. We used a program consisting of a 1- to 12-s linear ramp at a constant 150 V for 15 h at 12°C. The rotation angle was 120°.
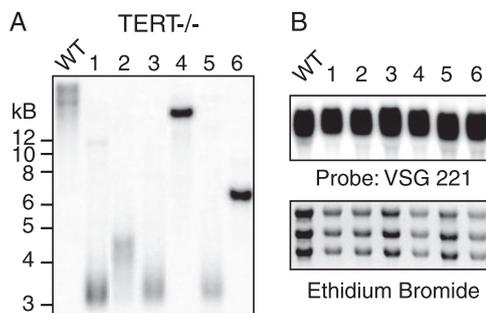

FIG. 2. Selection of clones with a short active ES telomere. (A) TERT-deficient (TERT$^{-/-}$) clones cannot repair telomere truncations, and successive rounds of cloning yield clones with very short active ES terminal restriction fragments (lanes 1, 3, and 5). In wild-type populations, telomere breakage and telomerase elongation lead to heterogeneity of the *VSG* 221 terminal restriction fragment (WT lane). (B) (Upper panel) Northern blotting of the cells from panel A confirms that they still express *VSG* 221. (Lower panel) Equal loading was verified by ethidium bromide staining.

Northern blotting and RT-PCR. *T. brucei* mRNA was isolated by using RNAstat60 according to the manufacturer's protocol. The dominant *VSG* expressed by the population was cloned by reverse transcriptase PCR (RT-PCR) using oligonucleotides corresponding to the *T. brucei* spliced leader sequence (5'-GACTAGTTTCTGTACTATAT-3') and a *VSG* C-terminal conserved region (5'-GACTAGTGTTAAAATATATCA-3'). PCR was carried out for 30 cycles of 1 min at 94°C, 1 min at 40°C, and 1 min at 72°C.

RESULTS

Telomere breakage and shortening at the active *VSG* 221 expression site. Telomere length changes at the active *VSG* 221 ES can be visualized by digestion with EcoRI and hybridization with a *VSG* 221 probe (Fig. 1A). The actively transcribed ES telomere is subject to frequent terminal deletions (2, 13, 23, 28, 35). These truncations and their subsequent repair by telomerase account for the telomere length heterogeneity we and others have observed at the active ES in wild-type cells (Fig. 2A, WT lane) (15). In telomerase-deficient cells, breakage and progressive telomere shortening occur. In a newly cloned population, the active ES *VSG* 221 terminal restriction fragment is a sharp band (Fig. 1A, upper panel, week 0). During subsequent culturing, as a result of telomere breakage in the absence of telomerase, the signal starts to smear towards the bottom of the gel. In contrast, wild-type *VSG* 221 signal is heterogeneous but remains within a high-molecular-size range throughout the time course (data not shown). To verify equal loading and to confirm the progressive moderate shortening at a silent ES, the blot was rehybridized with a probe against a silent *VSG* bR2 (Fig. 1A, lower panel). We quantified the extent of telomere loss at the active ES over 8 weeks by measuring signal intensity in each lane as a function of telomere length (Fig. 1B). In several independent experiments, the distribution of telomere signal changed reproducibly over time. We confirmed that loss of *VSG* 221 and decrease of its transcript levels (Fig. 1C, upper panel) coincided with a gradual increase in *VSG* 1.8 transcript levels (Fig. 1C, middle panel). By the end of the time course, the population expressed roughly equal amounts of *VSG* 1.8 and *VSG* 221, as judged by phosphorimager quantification (see Fig. S1 in the supplemental material), RT-PCR, and sequencing of 30 clones
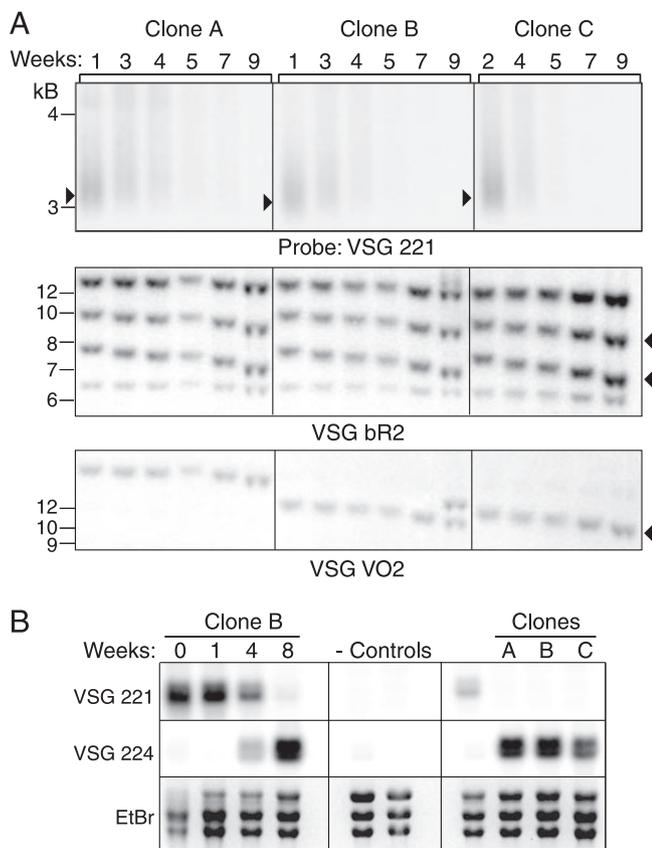
FIG. 3. A short telomere at the active ES leads to loss of the transcribed *VSG*. (A) *VSG* 221 loss in three individual TERT-deficient short active ES telomere clones (clones A, B, and C) during 9 weeks of continuous propagation. The size of the predominant *VSG* 221 band did not change over time, but signal intensity decreased, reflecting loss of the gene. Probes for silent ES *VSG* bR2 and *VSG* V02 (arrowheads) confirmed their stability and shortening and verified equal loading. (B) RT-PCR and Northern blotting show that *VSG* 224 transcripts replace *VSG* 221. EtBr, ethidium bromide.
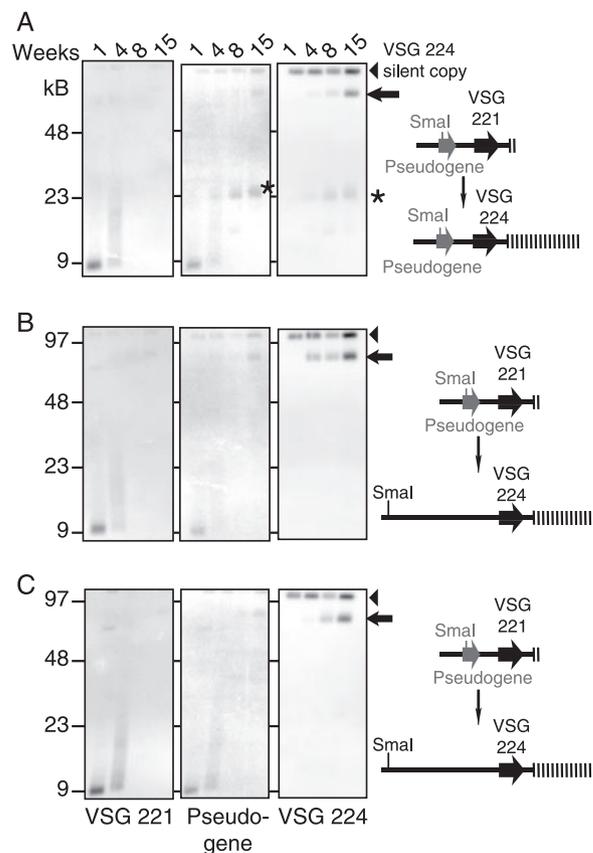
FIG. 4. Southern blotting with a unique upstream pseudogene (schematic representation on the right) demonstrates that *VSG* 224 replaced *VSG* 221 in the same active ES. (A) (Left panel) As shown in Fig. 3, the population lost *VSG* 221 by 8 weeks. (Middle panel) Reprobing of the blot with an ES-specific pseudogene probe. After *VSG* 221 loss, the size of the predominant terminal restriction fragment increases to ~23 kb (asterisk). (Right panel) Reprobing with the newly expressed *VSG* 224 probe. *VSG* 224 signal appeared after *VSG* 221 loss and colocalized precisely with the pseudogene signal (asterisk), indicating that *VSG* 224 translocated into the ES, previously occupied by *VSG* 221. An arrowhead marks the silent copy of *VSG* 224. The arrow and the asterisk indicate different means of *VSG* 224 activation within the population (see the text). (B and C) In two other clones, the pseudogene, the SmaI site, and *VSG* 221 were lost (left and middle panels). The duplicated copy of *VSG* 224 is indicated by an arrow (right panel).

(data not shown), although cells expressing *VSG* 221 appeared to have a slight growth advantage. In these cells, *VSG* 1.8 and *VSG* 221 appeared to be on opposite arms of chromosome VI. The fact that *VSG* 221 was lost as *VSG* 1.8 became transcribed suggests that the latter was activated through duplicative gene conversion (see Fig. S2 in the supplemental material). This result led us to investigate the consequences of a short telomere at an active ES in more detail by selecting clones that had lost large amounts of telomeric DNA from the active ES. Six telomerase-deficient clones are shown in Fig. 2A. Clones with very short telomeres, notably, clones 1, 2, 3, and 5, were obtained only after several rounds of continuous propagation and cloning. The sequence between *VSG* 221 and its telomere is known and indicates that an ~3-kb terminal restriction fragment contains ~200 to 400 bp of telomeric repeats (15). As judged by Northern blotting, telomere lengths in this range did not affect *VSG* 221 mRNA levels at this time (Fig. 2B).

Gradual loss of *VSG* 221 over time. Next, we determined whether further shortening of the active ES telomere would occur and whether it would affect the transcriptional status of *VSG* 221. We addressed these questions by keeping three in-

dependent clones (clones A to C), which already had a very short telomere at the active ES, in continuous culture for 15 weeks, during which we monitored population growth and isolated DNA and RNA every week. We also analyzed the status of *VSG* 221 at weeks 1, 2, 4, and 6 in an additional eight individual subclones of clone A. The results for weeks 2 and 4 are shown in Fig. S3 in the supplemental material. The short active *VSG* 221 ES terminal restriction fragment was heterogeneous and ended in ~200 to 400 bp of telomeric DNA (Fig. 3A, upper panel) but did not shorten further. In contrast to silent ES telomeres harboring *VSG* bR2 and *VSG* V02, which continue to shorten at a rate of 3 to 6 bp/PD (Fig. 3A, middle and lower panels) and which also act as equal-loading controls, the *VSG* 221 signal in the active ES almost disappeared by 5 weeks (Fig. 3A, upper panel). Analysis of individual subclones
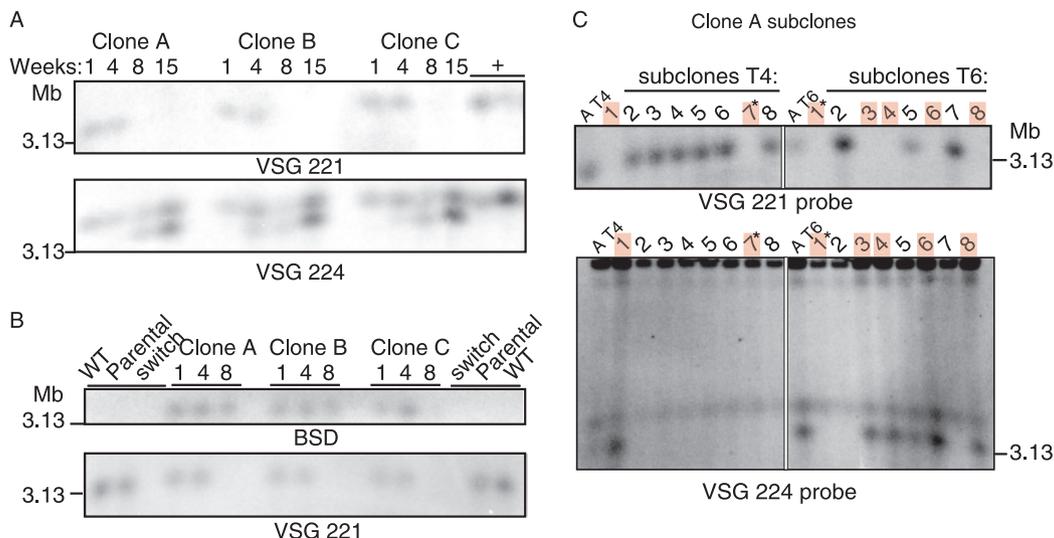
FIG. 5. Chromosomal DNA separation by RAGE confirms that *VSG* 224 replaced *VSG* 221 at the active ES through a duplicative gene conversion event. (A) (Upper panel) *VSG* 221 is lost by week 8. (Lower panel) Reprobing with *VSG* 224 shows that *VSG* 224 replaces *VSG* 221 on the same chromosome. Under the conditions used, the ~3.2-Mb chromosome VI band, harboring the 221 ES, is well separated from other chromosomes (20, 25). The entire gel is shown in Fig. S4 in the supplemental material. +, *VSG* 221 positive controls. (B) (Upper panel) ES integrity was verified by the retention of the blasticidin resistance marker (*BSD*) that was inserted immediately downstream from the ES promoter. All three clones retained *BSD* and were highly resistant to blasticidin. Lanes: switch, a switched clone from a previous experiment; parental, the telomerase-deficient cell line without *BSD* at the active ES; WT, wild type. (Lower panel) Similarly to results shown in panel A, *VSG* 221 is lost after 4 weeks of continuous culture. (C) RAGE of clone A subclones after 4 and 6 weeks. Red-shaded numbers indicate subclones that lost *VSG* 221. Asterisks indicate subclones that lost *VSG* 221 but did not duplicate *VSG* 224. AT4 and AT6 are the 4- and 6-week populations prior to subcloning. Hybridization probes are indicated below each panel.

revealed that some short active ES telomeres became elongated (see Fig S3A and C in the supplemental material), presumably due to repair by break-induced replication (BIR) using another telomere as a template.

The loss of *VSG* 221 poses two threats to the cell. First, the parasite needs a protective surface coat for survival. Second, loss of *VSG* 221 and its telomere results in an unprotected chromosome end that requires repair. Since we did not detect any growth retardation, we were interested to learn how the cell addressed these problems. RNA analysis of clone B confirmed that *VSG* 224 became dominant in the population as *VSG* 221 transcript levels diminished (Fig. 3B, left four lanes). Two independent clones (clones A and C) also lost *VSG* 221 over a period of 4 to 5 weeks and also activated *VSG* 224 (Fig. 3B, right three lanes). In other experiments, *VSG* 221 was replaced by a different *VSG* (Fig. 1C).

VSG 224 replaced VSG 221 in the active expression site. To clarify the events that led to *VSG* 224 expression, we generated an ~9-kb SmaI terminal restriction fragment that hybridized with both *VSG* 221 and an upstream *VSG* pseudogene (Fig. 4). After 4 weeks of continuous culture, *VSG* 221 was again lost from the population. By week 8, the size of the predominant restriction fragments containing the pseudogene increased to ~23 kb (Fig. 4A, middle panel) and colocalized with a new copy of *VSG* 224 (Fig. 4A, right panel), suggesting that *VSG* 224 and part or all of its associated telomere replaced *VSG* 221 at the previously transcribed ES. In addition to the ~23-kb bands, a higher molecular band that does not colocalize with the pseudogene appeared (Fig. 4A). We attribute this band to individual breaks in which the SmaI site and the pseudogene marker were deleted. Deletion of the pseudogene occurred

after 8 weeks in two analyzed clones, as the duplicated copy of *VSG* 224 appeared (Fig. 4B and C). To determine whether *VSG* 224 was activated by gene conversion or reciprocal translocation, we separated whole chromosomal DNA. In all three clones, *VSG* 221 was lost after ~4 weeks and *VSG* 224, initially present as a single-copy gene, was duplicated into the ES that previously carried *VSG* 221 (Fig. 5A and C).

We independently confirmed that the same ES remained active by using a blasticidin resistance gene that we had integrated immediately downstream of the active promoter (25) while *VSG* 221 was being expressed. No drug selection was applied during propagation and switching, but the population and individual subclones retained the marker and were highly resistant to blasticidin, indicating that the same ES was in use (Fig. 5B). This result was further confirmed by analyzing individual subclones from each time point. Subclones derived from clone A after 4 or 6 weeks are shown in Fig. 5C. By week 4 subclones 1 and 7 and by week 6 subclones 1, 3, 4, 6, and 8 had all lost *VSG* 221 (Fig. 5C, upper panel). Most subclones that lost *VSG* 221 had duplicated *VSG* 224 (Fig. 5C, lower panel). Subclone 7 from week 4 and subclone 1 from week 6 had lost the *VSG* 221 gene but did not duplicate *VSG* 224: these subclones must have undergone independent switch events that activated a different *VSG*.

Could a short telomere trigger an antigenic switch? Due to the low switching frequency and apparent growth advantage of VSG 221-expressing cells in vitro, our cell lines rarely lose *VSG* 221 or otherwise result in another VSG becoming dominant in the population, even after extensive propagation. Hence, the appearance of a new *VSG* at the active ES locus was unexpected and could be explained in two ways. As telomere length

at the active ES becomes very short, telomere breakage could lead to terminal truncations and gradual death of the VSG 221-expressing cells in the population. Switched cells, which normally arise at a low frequency, would progressively outgrow the dying cells and lead to the dominance of a new VSG in the population. Alternatively, telomere breaks could be repaired by BIR facilitated by other telomere-linked *VSG*, with a possible consequent increased rate of antigenic switching.

To address these questions, we attempted to measure switching frequency of strains with a very short active ES telomere by using immune selection in mice. However, due to extensive propagation in laboratory culture or unforeseen consequences of short telomeres in an animal infection that were not evident in culture, these clones did not grow well in mice. Readaptation to mice, during 3 weeks of sequential transfer, appeared to select for recombination-based elongation of the active ES telomere (data not shown).

We also made a mathematical model to predict the growth dynamics of a culture if a short telomere leads to gradual lethality and switchers arise at various frequencies (10^{-3} to 10^{-6}). Gradual death of cells with a short active ES telomere and concomitant appearance of switchers (at a rate of 10^{-6}, but with a growth advantage) would affect population growth dynamics only subtly over 4 to 5 weeks. In conclusion, unless the switching frequency is dramatically higher at short telomeres, switchers must have a clear growth advantage in order to outgrow the population within 4 weeks. However, we had to assume parameters, such as relative fitness of short telomere strains and growth advantage of switched parasites, for which we had no experimental data. Thus, we cannot exclude the possibility that switchers, at a frequency of 10^{-6}, could have outgrown dying parasites without giving rise to a significant decline in population fitness, which would mean that telomere break-induced repair did not necessarily increase the switching rate.

DISCUSSION

To investigate whether telomere length and telomere dysfunction could regulate antigenic switching, we created telomerase-deficient *T. brucei* mutant strains (9). As reported elsewhere, short telomeres at silent ESs were stabilized by a telomerase-independent lengthening mechanism and remained at a stable length over several months (8). Furthermore, dramatic telomere shortening at silent ESs did not affect *VSG* expression. In this report, we addressed the consequences of a short telomere at the actively transcribed ES. As with shortening at silent ES telomeres, the length of a short active ES telomere could be maintained. However, in sharp contrast to stabilized silent ES telomeres, the actively transcribed *VSG* 221 was gradually lost from the population and replaced by another *VSG* through a duplicative gene conversion event. What could account for the difference between silent and active ESs? One conspicuous difference is that the active ES undergoes frequent terminal truncations, which were observed many years ago (2, 13, 23, 28). Although the nature of these breaks remains unclear, they could be a consequence of transcription bubble destabilization and/or nucleolytic degradation as transcription reaches a DNA terminus. At long telomeres, terminal truncations delete telomeric repeats that can be elon-
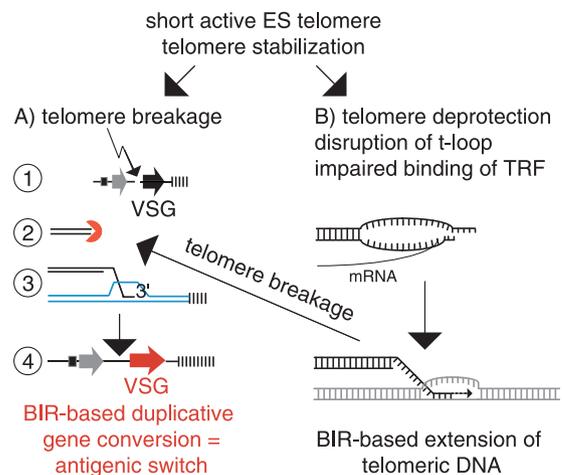

FIG. 6. (A) Model for telomere breakage and duplicative gene conversion through BIR. (B) BIR-based elongation of deprotected short active ES telomeres.

gated rapidly by telomerase (8, 10, 15). At short telomeres, we hypothesize that a truncation might frequently fall within the subtelomeric region, resulting in a double-stranded break. The double-stranded break could be repaired through BIR (Fig. 6A), a mechanism that has been well studied for *Saccharomyces cerevisiae* (16). During BIR, the centromere-proximal end of a break is processed into a 3′ overhang, which can invade the sister chromatid and use it as a template for repair. DNA polymerase-mediated synthesis completes the repair (12). In *T. brucei*, the repair template could be any ES or possibly just a *VSG*-containing telomere, depending on the location of the break (Fig. 6A) (12), and BIR could extend into the telomere, resulting in duplicative expression of a new *VSG* and seeding of a new telomere. Depending upon the circumstances, BIR might not effectively repair every active ES break: sometimes an entire ES might be deleted, resulting in an in situ ES switch or death (30). Additionally, transcription of a shortened active ES telomere could lead to telomere deprotection, possibly due to displacement of the *T. brucei* telomere binding factor (TbTRF) (19). Telomere deprotection could result in BIR-based elongation of the active ES telomere and contribute to the signal heterogeneity observed at the active ES (Fig. 6B).

In conclusion, within a few weeks of continuous culture, the *VSG* at a short active ES telomere in telomerase-deficient *T. brucei* is replaced by a new *VSG* through duplicative gene conversion. Analysis of individual subclones suggested that multiple switch events occurred within the population. We speculate that breakage of short telomeres at an active ES, followed by BIR, could accelerate *VSG* switching. Due to technical difficulties, we were unable to reliably measure the VSG switching rates in clones with short telomeres at the active ES, and mathematical modeling (data not shown) could not exclude the possibility that, when short active ES telomeres broke, *VSG* expression was compromised and these cells died. We could not determine the telomere breakage frequency in the population, and if it were low, VSG switches arising at the normal (for this strain) low frequency of $\sim 10^{-6}$/PD could dominate the population within a period of 4 to 5 weeks without an observable pause in population growth (13, 18). It

therefore remains to be determined whether short telomeres and preferential breakage at the active ES are at least partly responsible for the high rate of gene conversion-mediated antigenic variation in rapid-switching trypanosome isolates (29).

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