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Gene Conversions Mediating Antigenic Variation in *Trypanosoma brucei* Can Occur in Variant Surface Glycoprotein Expression Sites Lacking 70-Base-Pair Repeat Sequences

RICHARD MCCULLOCH, GLORIA RUDENKO, AND PIET BORST*

Division of Molecular Biology, The Netherlands Cancer Institute, 1066 CX Amsterdam, The Netherlands

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African trypanosomes undergo antigenic variation of their variant surface glycoprotein (VSG) coat to avoid immune system-mediated killing by their mammalian host. An important mechanism for switching the expressed VSG gene is the duplicative transposition of a silent VSG gene into one of the telomeric VSG expression sites of the trypanosome, resulting in the replacement of the previously expressed VSG gene. This process appears to be a gene conversion reaction, and it has been postulated that sequences within the expression site may act to initiate and direct the reaction. All bloodstream form expression sites contain huge arrays (many kilobase pairs) of 70-bp repeat sequences that act as the 5′ boundary of gene conversion reactions involving most silent VSG genes. For this reason, the 70-bp repeats seemed a likely candidate to be involved in the initiation of switching. Here, we show that deletion of the 70-bp repeats from the active expression site does not affect duplicative transposition of VSG genes from silent expression sites. We conclude that the 70-bp repeats do not appear to function as indispensable initiation sites for duplicative transposition and are unlikely to be the recognition sequence for a sequence-specific enzyme which initiates recombination-based VSG switching.

African trypanosomes such as *Trypanosoma brucei* are flagellated protozoan parasites that were the first organisms described to undergo antigenic variation, a general survival strategy employed by many pathogenic microorganisms (7–9, 46). Within the bloodstream of its mammalian host, *T. brucei* periodically changes its major surface protein, the variant surface glycoprotein (VSG) (14). In this way, a subpopulation of the parasites escapes the killing that is mediated by the host immune response directed against the previous VSG coat. The expressed VSG gene is always found in a discrete telomeric location named the expression site. These are large (40- to 60-kbp) polycistronic transcription units in which the VSG gene is always located adjacent to the telomere (26, 27, 44). In addition to the VSG gene, mRNAs from a number of expression site-associated genes (ESAGs) are generated from the same primary expression site transcript. Although each trypanosome contains approximately 20 expression sites (17, 38), normally only one is transcribed at a time, resulting in a VSG coat composed of a single protein species. Every trypanosome has about 1,000 different VSG genes (64). This enormous genetic reservoir is distributed over three locations: the various expression sites, at the telomeres of approximately 100 minichromosomes, and in large arrays in the interior of the larger chromosomes. For reviews on trypanosome antigenic variation, see references 8, 10, 15, 45, and 67.

A switch in the VSG species expressed on the trypanosome surface is accomplished in a number of ways (Fig. 1). The first two mechanisms, duplicative transposition and telomere conversion (Fig. 1A and B), are putative gene conversion reactions in which the VSG gene being transcribed is deleted and replaced by a copy made from another VSG gene (2, 24, 41, 42). Mechanistically, it is likely that these processes are identical, but they are distinguished because they involve different donors of the new VSG gene. The former involves duplicative movement of a chromosome-internal VSG gene, whereas in the latter the donor is at a telomere. During telomere conversion, the donor VSG gene may be resident in a silent expression site, in which case the amount of sequence duplicated can extend far upstream of the VSG gene to include ESAGs and can extend downstream of the VSG gene to perhaps include the telomere (18, 53). As the trypanosome has invested so much of its genome in silent VSG genes, it is probable that duplicative transposition is quantitatively the most significant route for VSG switching. Reciprocal recombination (Fig. 1C) is a simple genetic crossover. A number of such conservative recombination reactions have been documented (43, 50, 55). Finally, VSG switching can occur by activating transcription from a silent expression site and silencing transcription from the active expression site, a process termed an in situ switch (Fig. 1D). This process is distinct from all the previous mechanisms in that no DNA recombination appears to be involved (25, 49, 69).

Little is known about the specific mechanisms used by trypanosomes in VSG switching by duplicative transposition. Comparison of the substrates and products of switches has revealed blocks of sequence homology 5′ of the VSG gene (see below) and in 3′ regions extending from within the VSG open reading frame (ORF) to downstream of the end of the VSG mRNA (32, 36). Such comparisons have also shown that the extent of sequence transposed is variable, leading to the suggestion that the reaction is driven by homologous, rather than site-specific, recombination. The regions of sequence homology must therefore be used to align the two recombining DNA molecules during VSG switching.

One of the foremost questions that remain unresolved regarding recombination-based VSG switching is whether the process is mediated by unregulated general homologous recombination (7) or whether the reaction is initiated through cleavage of VSG sequences by a specific endonuclease. En-

* Corresponding author. Mailing address: Division of Molecular Biology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands. Phone: 31 20 512 2880. Fax: 31 20 669 1383.

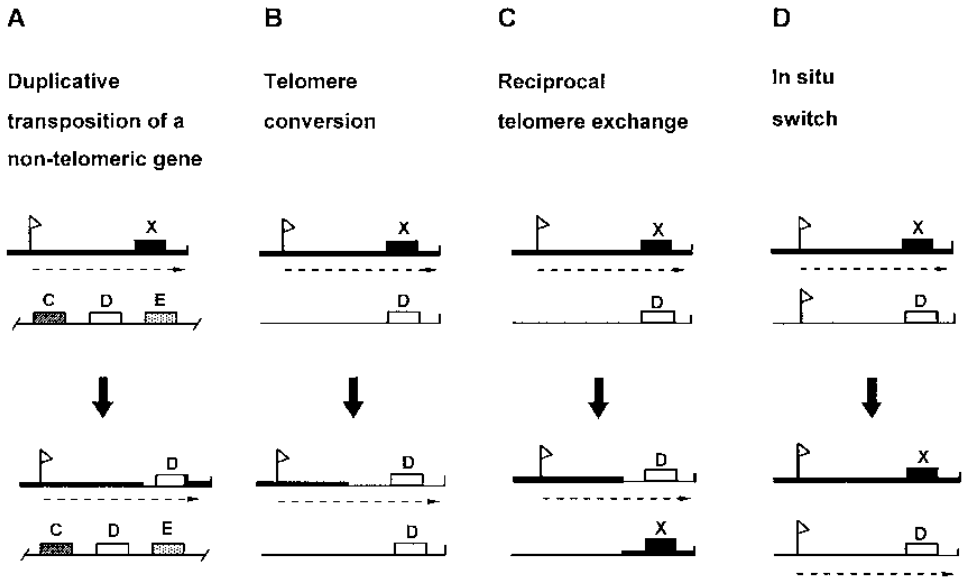


FIG. 1. Four modes of VSG switching. In each diagram, the VSG gene expressed prior to switching (gene X) is shown as a solid box and the VSG gene which becomes activated as a result of the switch (gene D) is shown as an open box; the promoter directing the transcription of the expression site is represented by a flag, and the dotted arrow represents the transcript that is expressed. (A) Duplicative transposition of a nontelomeric VSG gene. (B) Telomere conversion; this is duplicative transposition of a telomeric VSG gene, but since the switched VSG gene may be copied from another expression site, the amount of sequence duplicated during the reaction can be much larger than in panel A. (C) Reciprocal exchange between two telomeric VSG genes. (D) In situ switch between two VSG expression sites.

zymes that perform such an initiation function have been characterized in the processes of mating-type switching in yeast (57) and intron mobility in a range of organisms (28). One approach to examining this question in *T. brucei* is to modify the active bloodstream form expression site and thereby identify specific sequences that are essential for VSG switching. The sequences that we have tested in this role are the so-called 70-bp repeats, which were first described by Liu et al. (32). The 70-bp repeats are a striking feature of all characterized bloodstream form expression sites, where they are found 1 to 2 kbp 5′ of the VSG gene and are present in large arrays (varying in size from a few to tens of kilobase pairs [11, 21, 37]). The individual repeat units within these arrays vary in both their exact sequence and size. A few copies of the 70-bp repeats are found 5′ of most chromosome-internal VSG genes (1, 32), and longer stretches are similarly positioned relative to the VSG genes at the telomeres of the minichromosomes (48, 54). The repeats demarcate the 5′ boundary of the duplicative transposition of chromosome-internal VSG genes, demonstrating that they function as stretches of sequence homology during recombination (19). The 70-bp repeats are therefore a plausible initiation point for the gene conversion reaction (34, 39, 45). The repeats might be the recognition sequence for an unidentified endonuclease (34), or, alternatively, they might be intrinsically prone to DNA breaks because they adopt some nonstandard DNA conformation (11, 32).

Here we show that the 70-bp repeats can be removed from the active expression site in in vitro-cultured bloodstream form *T. brucei* and that gene conversions into this modified expression site can still occur. In addition, these changes had no significant effect on the VSG switching frequency. This suggests that the repeats are unlikely to act as an indispensable initiation sequence in VSG recombination and that their primary role is to create sequence homology 5′ of the VSG genes.

MATERIALS AND METHODS

Trypanosome strains and transformation. *T. brucei* 221a bloodstream form trypanosomes (MiTat1.2a) of strain 427 (16) were used throughout and were grown in vitro at 37°C in HMI-9 culture medium (23). Trypanosomes were transformed by electroporating 5×10^7 cells with approximately 5 μg of plasmid DNA that had been digested with *Not*I and then phenol-chloroform treated and ethanol precipitated. Electroporation was performed in 0.5 ml of Zimmerman postfusion medium (132 mM NaCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.5 mM magnesium acetate, 0.09 mM calcium acetate [pH 7.0]) supplemented with 1% glucose, using a Bio-Rad Gene Pulser II set at 1.4 kV and 25-μF capacitance (giving a delivered voltage of 1.63 to 1.69 kV and a time constant of 0.18 to 0.20 ms). Cells were allowed to recover for 18 h in HMI-9 medium before transformants were selected on semisolid agarose plates (12) containing 2.5 μg of hygromycin B per ml; transformant colonies were then recovered from the plates, and their resistance to G418 (2.0 μg/ml) was checked.

Plasmid constructions. All constructs were made in pBluescript II KS (Stratagene) by standard molecular biological techniques (51). The VSG221 targeting sequence was cloned as a 1.6-kbp *Pst*I fragment from the 221 expression site clone pTgB221.1 (4), creating plasmid p1.6; the cloned sequence comprises approximately 500 bp of VSG221 ORF and 1.1 kbp of upstream cotransposed region (including the characterized VSG221 splice acceptor site; [3]) but excludes any 70-bp repeats. The ESAG1 targeting sequence is a 1.0-kbp PCR fragment amplified from pTgB221.1 with primers SES1600 (5′ CAGGATCCAA GCTTATATCTTATGGAAATA 3′; *Bam*HI sequence underlined) and ESAG1 (AI) (5′ AACGGGCCCGCCAGAACCTCACAAGAAAAT 3′; *Apa*I sequence underlined); this corresponds to the 3′ 750 bp of the ESAG1 ORF and 250 bp of downstream sequence, again excluding any 70-bp repeats but including a conserved polypyrimidine tract putatively involved in processing the ESAG1 mRNA (17, 35, 52). The ESAG1 sequences were next recloned from pBluescript as a *Sac*II-*Apa*I fragment into the *Kpn*I and *Apa*I sites of p1.6, thus creating two flanking *Not*I sites that allowed the inserts shown in Fig. 2A to be liberated. The hygromycin resistance cassette has been described previously (31) and comprises a hygromycin phosphotransferase gene flanked by a 240-bp splice acceptor fragment and a 330-bp polyadenylation fragment, both derived from the intergenic region of the tubulin locus; it was cloned into a *Cla*I site between the VSG221 and ESAG1 sequences as a 1.7-kbp *Bam*HI-*Hin*dIII fragment. The neomycin resistance cassette comprises a neomycin phosphotransferase gene (from pNEO; Pharmacia) flanked at its 5′ end by a 400-bp intergenic region from the actin locus (this supplies splice acceptor signals [63]) and at its 3′ end by a 400-bp intergenic region from the calmodulin locus (supplying polyadenylation signals; a gift from M. Cross); the cassette was cloned as a 1.8-kbp *Bam*HI-*Hin*dIII fragment downstream of the hygromycin cassette, generating plasmid pRM3171. To make plasmids pRM3174 and pRM3176, a 3.0-kbp fragment containing 2.7 kbp of 70-bp repeats and 300 bp of downstream sequence was isolated from the

dominant expression site clone pGE117a.10 (19) after *Pst*I-*Ssp*I (partial) digestion and was cloned into pBluescript digested with *Pst*I and *Sma*I. The fragment was then recloned into a unique *Eco*RV site between the hygromycin and neomycin cassettes of pRM3171 as a *Bam*HI-*Eco*RI fragment, and the insert orientations were determined via an *Eco*RV site within the 300-bp downstream sequence.

Analysis of the *T. brucei* transformants. For mapping of the transformants, genomic DNA was restriction digested and separated on 0.7% agarose gels in 1× TBE (90 mM Tris borate, 2 mM EDTA). Intact chromosomes were separated by pulsed-field gel electrophoresis on 0.8% agarose gels with a Bio-Rad CHEF-DRIII system; the gels were run in 1× TAFE buffer (10 mM Tris, 0.5 mM EDTA, 4.4 mM acetic acid) for 72 h at 12°C and 2 V/cm with a fixed switching time of 900 s. Each gel lane contained approximately 2.5×10^7 cells embedded in 0.5% low-melting-point agarose (Bethesda Research Laboratories) (65). All DNA was blotted onto Hybond N (Amersham) as specified by the manufacturer. Hybridization of probes (labeled with [α-^{32}P]dATP by random priming) was as described by Sambrook et al. (51), and all blots were washed to a stringency of 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate at 65°C.

Correct integration of the transformed constructs into the 221 expression site was determined in two ways. (i) The *Xba*I-digested genomic DNA (see Fig. 2B) was probed with a 320-bp *Stu*I-*Hin*dIII fragment homologous to a region of VSG221 downstream of the targeting sequence cloned in the constructs. Each transformant shows a hybridizing band of 4.5 kbp, which is the expected size of the sequences encompassing the neomycin cassette and the complete VSG221 gene, and is distinct from the 7.0-kbp *Xba*I fragment recognized in the unaltered 221 expression site. (ii) The integrated hygromycin cassette of each transformant was shown to be linked to an *Xmn*I and *Cla*I site mapped within the 221 expression site upstream of the ESAG1 gene (reference 4 and data not shown), showing that the 5′ ends of the constructs were also correctly integrated.

To check that the hygromycin and neomycin cassettes were correctly linked and that no rearrangements had occurred within or around the 70-bp repeats, DNA from each transformant was digested with either *Nco*I alone or both *Xho*I and *Eco*RV and probed with a fragment containing the complete hygromycin phosphotransferase ORF (see Fig. 2C). In the *Nco*I digest, both 3174 and 3176 show a band of 5.1 kbp whereas 3171 shows a band of 2.1 kbp. These fragments are the expected size in each case, showing that no large rearrangements have occurred. (In this digest, each transformant also shows a common band of 3.3 kbp, which extends from the hygromycin cassette to an unmapped upstream *Nco*I site.) Double digestion with *Xho*I and *Eco*RV shows a band of 1.7, 2.0, and 4.4 kbp for transformants 3171, 3176, and 3174, respectively. This maps the *Eco*RV site separating the two resistance cassettes in 3171 and the *Eco*RV site at the end of the 300 bp of downstream sequence cloned in conjunction with the 70-bp repeats, and therefore it shows that the 70-bp inserts, when present, are of the expected size and orientation. As a more detailed check of the transformants, we used PCR to amplify the sequences separating the following coding regions: ESAG1 3′ to hygromycin 5′, hygromycin 3′ to neomycin 5′, and neomycin 3′ to VSG221 5′. On high-resolution agarose gels, the PCR product generated from each transformant was equivalent in size to the products derived from the plasmids (data not shown). As a final check, we sequenced the three PCR products generated from transformant pRM3171 by linear amplification sequencing (13) and confirmed that they all matched that of the plasmid construct (data not shown).

Generation and analysis of trypanosome variants that have switched their VSG coat. The approach adopted to isolate VSG switched variants is essentially that described by Rudenko et al. (50) but with some modifications. To generate mice immunized against VSG221, a number of BALB/c mice were each given intraperitoneal injections of 1×10^6 to 2×10^6 221a 427 trypanosomes. The infections were cured by injection of Berenil (10 mg/kg) 2 days later. After 10 days, this regimen of infection and curing was repeated, and the mice were left for 10 more days before being used. To allow trypanosome switch variants to arise, each transformant was passaged from medium containing hygromycin (12.5 μg/ml) and G418 (5 μg/ml) into nonselective medium at a density of 0.5×10^4 to 1.0×10^4 cells/ml. The cells were then grown for nine generations (approximately 72 h) until they reached a density of 2×10^6 to 5×10^6 /ml, the trypanosomes were counted, and 5×10^7 cells of each transformant in 300 μl of HMI-9 were intraperitoneally injected into an immunized mouse. Twenty-four hours later, the mice were bled by cardiac puncture. Switched variants were isolated from 0.4 ml of blood as described by Rudenko et al. (50) and were immediately cloned by being diluted in 20 ml of HMI-9; this volume was spread over a 96-well culture dish. Using this procedure, we found that a single trypanosome grew out to a visible population in 5 to 6 days.

To assay for the drug sensitivity of the switched trypanosomes, approximately 2×10^5 cells were passaged from the 96-well dish into 200 μl of HMI-9 medium alone or supplemented with 12.5 μg of hygromycin per ml or 5 μg of G418 per ml. Growth was scored 72 h later. Small-scale genomic DNA preparations for the PCR and dot blot assays were made from 2×10^6 to 3×10^6 cells by lysing the trypanosomes for 1 h at 60°C in 0.5 ml of 50 mM Tris.HCl (pH 8.0)–1 mM EDTA–100 mM NaCl–1% sodium dodecyl sulfate, 100 μg of proteinase K per ml, extracting the DNA two or three times with phenol-chloroform, and finally precipitating it with ethanol. The presence or absence of the hygromycin, neomycin, and VSG221 genes was assessed by PCR with the following primer pairs: Hygro 5′ (5′ ATGAAAAGCCTGAACTCACC 3′) and Hygro 3′ (5′ CTATTC CTTTGCCCTCGGAC 3′), Neo 5′ (5′ TTGCACGCAGGTTCTCCG 3′) and Neo 3′ (5′ GAACTCGTCAAGAAGGCG 3′), and VSG221 5′ (5′ CCGAATT CCGCATGCCTTCCAATCAGGAGGC 3′; *Eco*RI sequence underlined) and VSG221 3′ (5′ CGCGGATCCGCTGTATCGGCGACAACTGCAG 3′; *Bam*HI sequence underlined). The integrity of the DNA was assessed by a control amplification of the 5′ end of the gene encoding the large subunit of RNA polymerase I (50). PCR amplification was performed in a Perkin-Elmer Cetus Gene Amp PCR system 9600 apparatus; the reaction used 1/100 of the total DNA prepared as above and involved an initial 5-min denaturation at 95°C followed by 30 cycles of 94°C for 40 s, 55°C for 40 s, and 72°C for 1 min. For dot blots, the genomic DNA was denatured for 20 min on ice in 0.4 M NaOH, neutralized by adding 1 volume of ice-cold 2 M ammonium acetate, and blotted onto Hybond-N.

RESULTS

Generation of *T. brucei* transformants with an active expression site without 70-bp repeats or with inverted repeats. To examine the importance of the 70-bp repeats in VSG switching and to specifically test whether they are the initiation point for recombination-based switching, we made the constructs diagrammed in Fig. 2A. Upon transformation of the 427 VSG 221a trypanosome strain expressing the VSG221 expression site, these constructs should integrate by homologous recombination into the active expression site and delete all the naturally occurring 70-bp repeats between the VSG221 gene and the most telomere-proximal ESAG gene, ESAG1. In this region the 221 expression site contains approximately 4.5 kbp of 70-bp repeats interrupted by a VSG pseudogene (Fig. 2A) (4). This is a small number of repeats in comparison to the tens of kilobase pairs described in some other expression sites, but restriction mapping (27) has revealed no other stretches of 70-bp repeats within the 221 expression site. Integration of construct pRM3171 replaces the 70-bp repeats with resistance cassettes encoding hygromycin phosphotransferase and neomycin phosphotransferase, generating transformants without 70-bp repeats in their active expression site. Integration of constructs pRM3174 and pRM3176 should generate transformants with the same arrangement of resistance cassettes but separated by a 2.7-kbp stretch of 70-bp repeats in their normal or inverted orientation, respectively.

Transformation of each construct generated transgenic trypanosomes that were resistant to hygromycin B and G418. Note that the resistance cassettes used contain no associated promoter sequences, and therefore transcription of the genes is derived from the upstream expression site promoter. Southern blot analysis was performed to characterize the transformants. Figure 2B shows hybridization of a VSG221-specific DNA fragment (indicated in Fig. 2A) to *Xba*I-digested genomic DNA of each transformant, as well as to 221a parental trypanosomes. There is only a single copy of the 221 VSG gene in the 427 trypanosome strain, and the DNA fragments hybridizing indicate that the plasmid sequences have integrated in single copy into the expected region of the 221 expression site. Further mapping also demonstrated that the 5′ ends of the constructs were correctly integrated (Materials and Methods).

In two independent transformations, we observed that the transformants derived from electroporation of plasmids pRM3171 and pRM3176 took 2 to 3 days longer than the pRM3174-derived transformants to form visible colonies during selection on agarose plates and that they appeared to have longer generation times than the pRM3174 transformants during their initial growth in liquid medium after being recovered from the plates. Since these growth differences were no longer discernible after further culturing in vitro, we were concerned that the alterations made to the 70-bp repeats were deleterious and that we may have selected for changes in the integrated plasmid sequences. We therefore checked whether the orga-

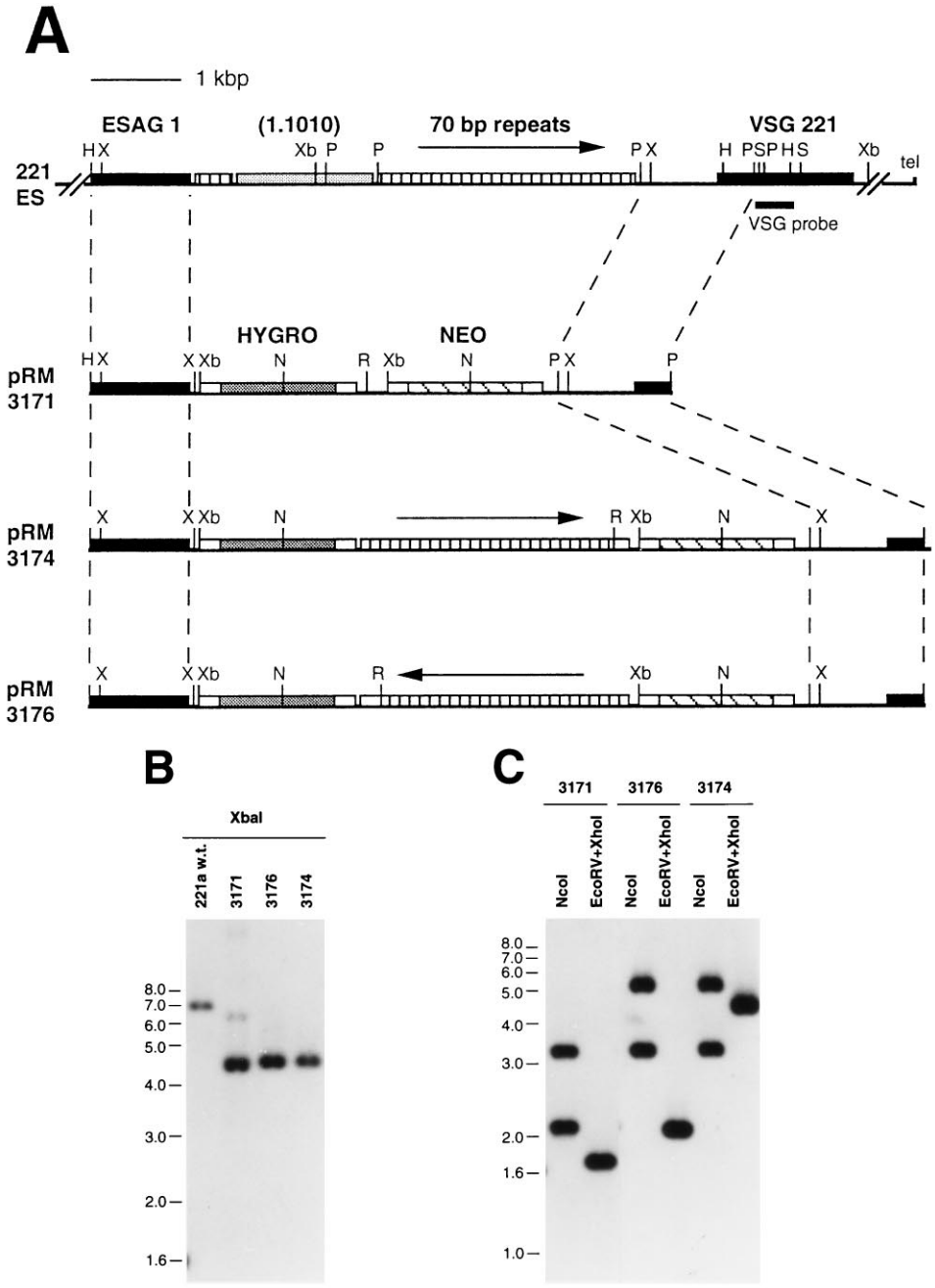


FIG. 2. Constructs used to examine the role of the 70-bp repeats in VSG gene switching and Southern blot analysis of trypanosome transformants. (A) The upper diagram shows the telomeric region of the 221a expression site: the VSG221 and ESAG1 genes are shown as solid boxes; the 70-bp repeats are shown as vertically striped boxes; a VSG pseudogene, named 1.1010, present within the 70-bp repeats is shown as a light grey box; and tel indicates the telomere. Each of the three constructs derived from the 221a expression site (shown below the 221a site) contains the same cloned regions of the ESAG1 and VSG221 genes (as indicated), and these serve as terminal homology regions that allow the inserts to recombine into the expression site. Resistance cassettes encoding hygromycin phosphotransferase (HYGRO; shown as a dark grey box) and neomycin phosphotransferase (NEO; shown as a diagonally striped box) are cloned between the ESAG1 and VSG221 sequences in each construct. The orientation of the 70-bp repeats separating the resistance cassettes in constructs pRM3174 and pRM3176 is indicated by an arrow. Restriction enzyme recognition sites are indicated as follows: H, *Hin*dIII; N, *Nco*I; P, *Pst*I; R, *Eco*RV; S, *Stu*I; X, *Xho*I; and Xb, *Xba*I. (B) Southern blot of *Xba*I-digested genomic DNA from the parental 221a trypanosome strain and from transformants generated with each construct. This was probed with a 320-bp *Stu*I-*Hin*dIII fragment (see panel A) derived from the VSG221 gene but outside the region cloned in the transformation constructs (note that the 3171 *Xba*I digestion is slightly partial). (C) Genomic DNA from the same transformants probed with the hygromycin gene after digestion with *Nco*I alone or with both *Eco*RV and *Xho*I.

nization of the integrated resistance markers was as expected and whether rearrangements had occurred in or around the 70-bp repeats separating the two markers. Figure 2C shows genomic mapping (see Materials and Methods), which demonstrates that the resistance cassettes are separated by the expected number and arrangement of repeats in each transformant and consequently that no large alterations in the intervening DNA had occurred during either transformation or subsequent culturing. More detailed characterization of the transformants was performed by PCR (data not shown; see

Materials and Methods), again showing that the sequence changes made within this telomeric region of the expression site were those dictated by the plasmid constructs and that the arrangement of the integrated resistance cassettes and 70-bp repeats is stable.

Effects of deleting or inverting the 70-bp repeats on the efficiency of VSG switching. Two alternative roles can be envisaged for the 70-bp repeats in VSG switching by duplicative transposition: either they passively act as 5′ stretches of sequence homology to align the recombining VSG sequences, or they act to initiate the homologous recombination reaction by the creation of DNA breaks, perhaps through an uncharacterised endonuclease. These alternative hypotheses can be distinguished by the three transformants described above.

If the repeats are an initiation sequence, then transformant 3171, which lacks 70-bp repeats in its active expression site, should inefficiently initiate recombination and should show a reduced frequency of VSG switching relative to transformant 3174. Since transformant 3176 contains the same 70-bp repeats in its expression site as 3174 does, and since there is no reason why these should fail to create DNA breaks in an inverted orientation, no such reduction in VSG switching should be observed. The VSG switching reactions used by the different transformants should also be distinguishable. In transformant 3171, the amount of duplicative transposition (and perhaps all recombination-based reactions) should be reduced, and switches should occur predominantly by in situ reactions. For transformant 3176, the DNA breaks at the 70-bp repeats should continue to initiate recombination, but their inverted orientation should preclude their alignment with repeats adjacent to other VSGs, and hence all duplicative transpositions should use upstream homology with other expression sites for recombination. In contrast, if the 70-bp repeats have no active role in the initiation of duplicative transposition, the VSG switching frequencies of all three transformants should be similar. The repeats occupy only 2.7 kbp of an expression site at least 50 kbp in size, leaving plenty of upstream sequence for homologous alignment in their absence. However, the removal of the 70-bp repeats in transformant 3171 and the inversion of the repeats in 3176 should again preclude the transposition of chromosome-internal or minichromosome VSG genes into these expression sites, and all gene conversion reactions should occur between expression sites in these trypanosomes.

To determine the frequency with which the various transformants undergo VSG switching and to allow us to isolate multiple switched trypanosomes from a single mouse without prolonged growth (since this may allow particular switched variants to outgrow and dominate a population), we modified the single relapse procedure that has been used previously (33, 50). In each experiment, a defined number of transformants was injected into mice immunized against the VSG221 coat, resulting in removal of unswitched trypanosomes from the population. Switched variants expressing a different VSG were isolated from the mouse blood 24 h later, diluted in culture medium, and distributed over a 96-well culture dish. This served two purposes. First, if the switched variants were present in sufficiently small numbers, only a fraction of the wells should contain a population of trypanosomes after 6 days of growth, with each population having arisen from a single trypanosome. The switched trypanosomes were therefore immediately cloned for subsequent analysis of the VSG switching event (see the next section). The second purpose was to allow us to estimate the numbers of switched trypanosomes present in the challenge populations and hence to make an estimate of the switching frequency for each transformant. Because we find that a population grows out from a single trypanosome with near 100% efficiency under these conditions (50), simply counting the number of wells with living trypanosomes after 6 days allows us to make this estimate (Table 1).

TABLE 1. VSG switching frequencies for the three transformants compared to the parental trypanosome strain

Trypanosome strain	No. of wells showing growth (6 days)	Estimated VSG switching frequency[a]	Mean switching frequency
3174	51	0.6×10^{-6}	0.3×10^{-6}
	16	0.2×10^{-6}	
	10	0.1×10^{-6}	
3171	30	0.4×10^{-6}	0.4×10^{-6}
	21	0.3×10^{-6}	
	28	0.4×10^{-6}	
3176	80	1.0×10^{-6}	1.1×10^{-6}
	80	1.0×10^{-6}	
	95	1.2×10^{-6}	
221a	25	0.3×10^{-6}	0.3×10^{-6}
	21	0.3×10^{-6}	

[a] The approach used to determine the frequency with which the different trypanosome strains undergo VSG switching is discussed in the text. In each experiment, 5×10^7 trypanosomes were injected into immunized mice, and the numbers of switched variants were estimated by isolating surviving trypanosomes from 0.4 ml of mouse blood after 24 h, culturing this population for 6 days in 96-well dishes, and then counting the number of wells containing living trypanosomes. For each transformant, this experimental analysis was performed three times from independently grown populations used to challenge the immunized mice (twice for the parental 221a trypanosomes). The stated number of wells with living trypanosomes in each experiment is an average derived from two 0.4-ml isolations from the same mouse. The estimated VSG switching frequencies were calculated from the number of wells that grew out and assume the following: the trypanosomes have a doubling time of 8 h in the mouse (i.e., the switched variants undergo three divisions in 24 h); a single trypanosome grows out to form a population in each well with 100% efficiency; and 0.4 ml represents one-fifth of the total blood volume of a mouse.

The estimated VSG switching frequencies for each transformant are shown in Table 1. The absence of 70-bp repeats within the active expression site of transformant 3171 clearly did not significantly change the efficiency of VSG switching relative to transformant 3174, which contains 2.7 kbp of correctly orientated repeats. Surprisingly, the frequency of VSG switching in transformant 3176 appeared to be at least threefold higher (this is probably a slight underestimate, since the populations in so many of the 96 wells grew out in these experiments, meaning that the populations in most wells are likely to have arisen from more than one switched variant). Since a higher switching frequency was also observed for an independently generated transformant of 3176 (see below), it is unlikely that this is simply the result of experimental fluctuation, but what feature of the altered expression site causes this is unknown.

It should be noted that the values shown in Table 1 are not a measure of the switching rate but simply an estimate of the number of switched variants in the challenge population. This number can be subject to fluctuations as a result of the switching events occurring at different times during growth prior to selection. It is unlikely, however, that potentially substantial fluctuations have caused us to significantly underestimate or overestimate the frequencies of VSG switching for any of the three transformants. For each transformant, the switching frequencies were determined for three separately grown challenge populations, and no significant differences were observed. Additionally, the experiments were repeated with independently generated clones of each transformant, yielding

very similar estimates of switching frequency: 0.5×10^{-6} for 3171, 0.8×10^{-6} for 3176, and 0.3×10^{-6} for 3174. Furthermore, the frequencies that were determined are approximately equal to that of the untransformed 221a trypanosomes (Table 1) and are in agreement with the more accurate rate measurements for laboratory-adapted trypanosomes of the 427 strain made by Lamont et al. (29).

Switching profiles of the three transformants. The principles of the assay that we used to determine the types of VSG switching event that had occurred in each switched variant are outlined in Fig. 3. A number of switched trypanosomes were passaged from the 96-well culture dishes and assayed for their sensitivity or resistance to hygromycin and to G418. PCR was then used on each of the trypanosome clones to assay for the presence or absence of each resistance marker gene and for the VSG221 gene (which is present as a single copy in this trypanosome variant genome [Fig. 2]). Combining these data, it is possible to predict whether the trypanosomes have switched the expressed VSG gene by reciprocal recombination or in situ switching (where the three genes analyzed are retained in the trypanosome genome) or by gene conversion (where some or all of the genes are deleted from the altered expression sites). Moreover, the position at which recombination reactions have occurred relative to the inserted markers can also be predicted. The results of these assays are shown in Fig. 4. We have assumed that each switched trypanosome population analyzed had arisen from one individual. However, we cannot exclude that in some cases we have analyzed a mixed population representing two or more types of switching event.

For transformant 3174, 55 switched variants from four independent experiments were analyzed (Fig. 4A). The majority (56%) of these trypanosomes had switched their VSG coat via putative gene conversion reactions that deleted the VSG221 gene and also the hygromycin and neomycin resistance genes within the expression site. The 5′ boundary of these homologous recombination reactions must have been upstream of the hygromycin marker gene, and hence the sequences donating the new VSG gene in these reactions must have been part of another expression site. Probably, this crossover was within the region containing the ESAG genes of the 221 expression site. A smaller number of putative gene conversion reactions (13%) were detected that had used the 2.7 kbp of 70-bp repeats as their 5′ homology sequence: these retained hygromycin resistance and the hygromycin gene but deleted both the neomycin and VSG221 genes. In these cases, the new VSG gene copied into the 221 expression site could have originated from the chromosome-internal arrays, the telomeres of the minichromosomes, or another expression site. Importantly, these data show that the cloned 70-bp repeats in these experiments are capable of acting as regions of sequence homology mediating VSG recombination and have not undergone rearrangements that would preclude their functioning. The remainder of the switching events (31%) had retained all three genes tested by PCR but were sensitive to both hygromycin and G418. These had either undergone in situ switches or reciprocal recombination events upstream of the hygromycin gene (i.e., within the ESAG sequences, and therefore between two expression sites).

Our primary conclusion from the analysis of transformant 3174 is that the alterations made to the 221 expression site do not generally impair the ability of the trypanosome to undergo VSG switching. Moreover, since examples of recombination-based switching were seen (putative gene conversions), comparison between this transformant and those lacking 70-bp repeats, or with the repeats inverted, allows us to examine the role of these repeats in this form of VSG switching. Since we have substantially remodelled the active expression site, and since this analysis could be prone to fluctuations due to particular switched variants arising early in the growth of the challenge populations, we do not wish to draw conclusions about the relative frequencies at which the different VSG switching mechanisms are employed. However, one type of switching reaction was not observed at all, suggesting that it is highly inefficient: a recombination event that occurs downstream of the neomycin marker and relies upon homology between the VSG221 gene and another VSG sequence. This absence is significant, because such an event would not be selected against even during passaging of the transformants on hygromycin and G418.

For transformant 3171, lacking any 70-bp repeats within its active expression site, 60 switched variants from five independent experiments were analyzed (Fig. 4B). Most of the switched trypanosomes (more than were seen for transformant 3174 [78 versus 31%]) had undergone VSG switching either by an in situ switch or by reciprocal exchange upstream of the hygromycin resistance marker. This is a 2.5-fold increase and may reflect inherent variations that can be present in the assay rather than any feature of this transformant type that predisposes it to use either of these mechanisms rather than gene conversion-based switching. A significant proportion (22%) had switched their coat by putative gene conversion reactions that deleted the VSG221, hygromycin resistance, and neomycin resistance genes. Deletion of all three genes suggests that the 5′ sequence homology used in all the recombination reactions was the ESAG sequences upstream of the hygromycin marker, and hence in each case the donor of the new VSG gene must have been another expression site.

The same profile of VSG switching was seen for transformant 3176 (which contains the same cloned 70-bp repeats as 3174 but in an inverted orientation) as for 3171. In common with 3171, this transformant showed an increased number of in situ or reciprocal exchange switching events relative to putative gene conversions when compared to transformant 3174 (78 versus 31%). Again, putative gene conversion reactions could be detected that had deleted all the marker genes within the expression site (22% of the 76 switched variants analyzed). None of the switched variants examined had retained the hygromycin resistance gene but deleted or inactivated the neomycin and VSG221 genes. This would be expected, since the 70-bp repeats in this transformant should be incapable of homologously aligning with the naturally orientated 70-bp repeats of incoming VSG genes during recombination reactions. These data demonstrate that the reduction in the number of putative gene conversion reactions seen in transformant 3171 relative to 3174 is not a consequence of the lack of 70-bp repeats in transformant 3171, since transformant 3176 contains exactly the same complement of repeats as transformant 3174.

Southern analysis of putative gene conversions in transformant 3171. The switching profiles detailed above are based on a combination of drug sensitivity and PCR. As PCR can lead to errors, we used Southern hybridization to check genomic DNA from 10 switched variants of transformant 3171 predicted to have deleted the three genes (Fig. 5A). The 10 variants analyzed were isolated from three independent switching experiments, using one of the two 3171 transformants that were generated. In all cases, the three single-copy genes within the expression site had indeed been deleted from the trypanosome genome. The absence of the three genes is most simply explained by their replacement during duplicative transposition of a silent VSG gene into the 221 expression site. To confirm this, chromosomes from two of the switched variants that were identified as expressing the previously characterized VO2 VSG (49) were electrophoretically separated and probed for the

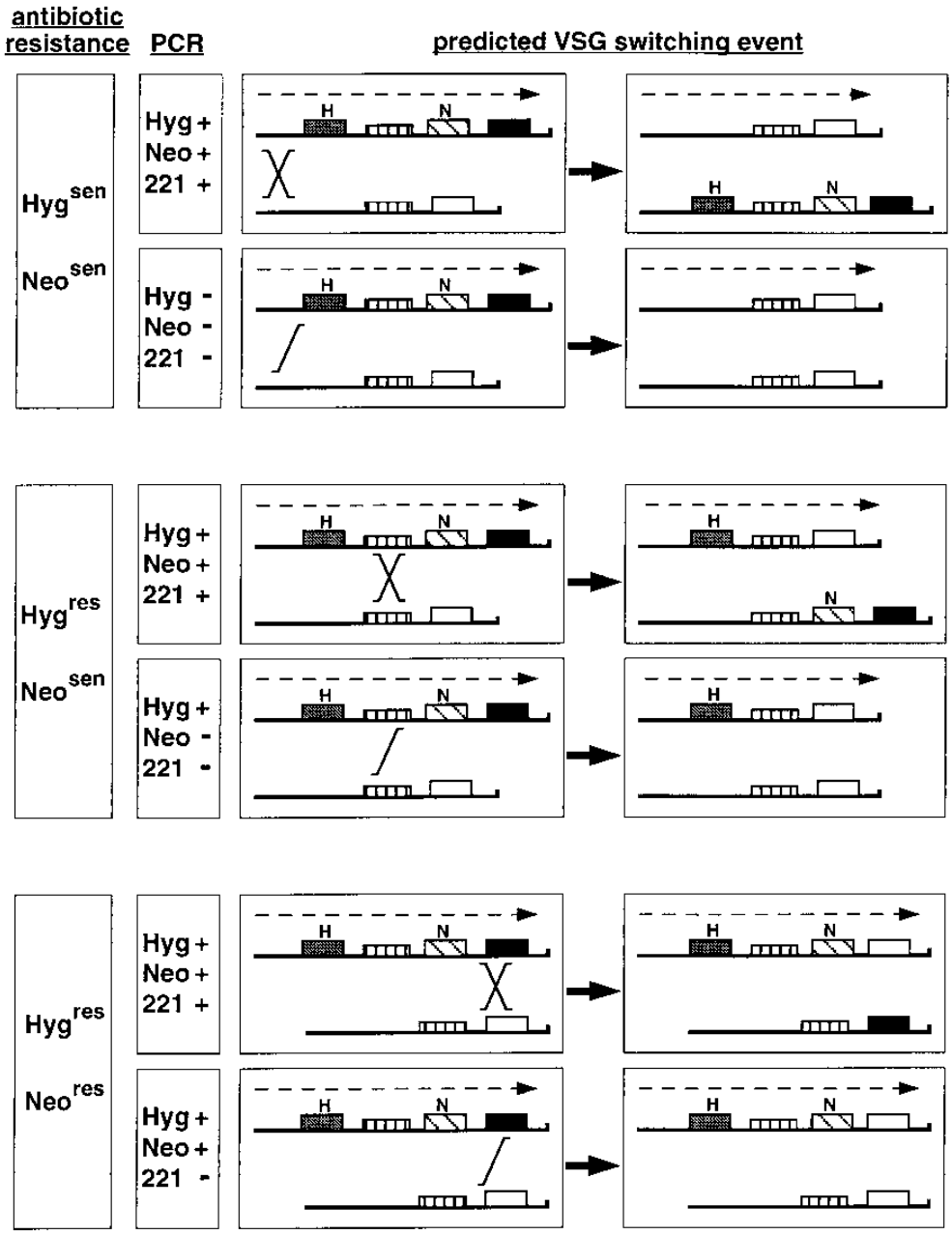
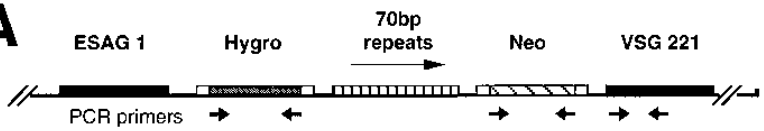


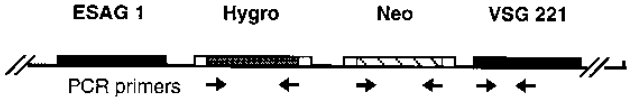
FIG. 3. Determining the type of recombination-based VSG switching reaction that has occurred in switched variants derived from transformant 3174. The genes and sequences within the active expression site of transformant 3174 are represented in the same way as in Fig. 2: the hygromycin cassette (H) is a grey box, the neomycin cassette (N) is a diagonally striped box, the VSG221 gene is a solid box, and the 70-bp repeats are a smaller vertically striped box (in this transformant, the repeats are present in their natural orientation). As in Fig. 1, the newly expressed VSG gene is always shown as an open box, and the transcription of the expression site is shown by a dotted arrow. Recombination reactions that switch the VSG gene can occur either through reciprocal exchange (indicated by a cross) or by nonreciprocal gene conversion (indicated by a half-cross representing the 5′ boundary of the recombinational exchange; the 3′ boundary is not indicated but must always be downstream of the VSG gene). These exchanges can theoretically occur either upstream of the hygromycin gene (within the ESAG sequences [top two boxes]), at the 70-bp repeats (middle two boxes), or downstream of the *neo* gene (i.e., on the VSG sequences [bottom two boxes]). The different positions of exchange can be determined by the resulting antibiotic resistances of switched variants (res indicates resistance, and sen indicates sensitivity). Furthermore, reciprocal exchanges can be distinguished from gene conversions by using PCR to assay for the presence of the three genes. In all cases of reciprocal exchange, the three genes are retained (PCR+). In gene conversions, the VSG221 gene is always deleted (PCR−), and the 5′ boundary of the recombination reaction is reflected in the presence or absence of the resistance markers. A gene conversion encompassing just the VSG gene retains both Hyg and Neo; if it extends up to the 70-bp repeats, then Hyg is retained but Neo is deleted; and if the reaction extends beyond Hyg and into the ESAGs, then both Hyg and Neo are deleted. This diagram only details VSG switching by recombination-based reactions. It should be noted that an in situ switch leading to the activation of another expression site would be indistinguishable from a reciprocal exchange upstream of the hygromycin gene in this analysis, since both reactions would lead to inactivation of the resistance markers while all the three genes being examined would be retained within the expression sites of the trypanosome.

A

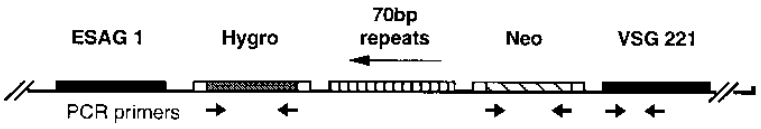


transformant (no. of clones analysed)	antibiotic resistances	PCR (presence or absence of gene)	predicted VSG switching event
3174 (55)	Hygro sen, Neo sen **48 / 55**	Hyg +, Neo +, 221 + **17 / 48**	in situ switch, or reciprocal exchange upstream of hygro
		Hyg -, Neo -, 221 - **31 / 48**	gene conversion from upstream of hygro
	Hygro res, Neo sen **7 / 55**	Hyg +, Neo +, 221 + **0 / 7**	reciprocal exchange at 70 bp repeats
		Hyg +, Neo -, 221 - **7 / 7**	gene conversion from 70 bp repeats
	Hygro res, Neo res **0 / 55**	N / A	recombination downstream of neo

B



transformant (no. of clones analysed)	antibiotic resistances	PCR (presence or absence of gene)	predicted VSG switching event
3171 (60)	Hygro sen, Neo sen **60 / 60**	Hyg +, Neo +, 221 + **47 / 60**	in situ switch, or reciprocal exchange upstream of hygro
		Hyg -, Neo -, 221 - **13 / 60**	gene conversion from upstream of hygro
	Hygro res, Neo sen **0 / 60**	N / A	recombination between hygro and neo
	Hygro res, Neo res **0 / 60**	N / A	recombination downstream of neo



3176 (76)	Hygro sen, Neo sen **76 / 76**	Hyg +, Neo +, 221 + **59 / 76**	in situ switch, or reciprocal exchange upstream of hygro
		Hyg -, Neo -, 221 - **17 / 76**	gene conversion from upstream of hygro
	Hygro res, Neo sen **0 / 76**	N / A	recombination between hygro and neo
	Hygro res, Neo res **0 / 76**	N / A	recombination downstream of neo

FIG. 4. Switching profiles of transformant 3174 (A) and transformants 3171 and 3176 (B). In each case, the genes within the altered 221a expression sites are represented as in Fig. 2, and only the telomeric portions of the sites are shown. The positions of the primers that were used to assay for the presence or absence of the hygromycin, neomycin, and VSG221 genes are schematically indicated. For the antibiotic resistance assays, res indicates resistance and sen indicates sensitivity. For the PCR analysis, + indicates that the amplification gave a product of the expected size, suggesting that the gene was present within the trypanosome genome, and − indicates that no product was generated, suggesting that it had been deleted. N/A, not applicable.

VO2 and 221 VSG genes (Fig. 5B). In each switched variant, an extra copy of the VO2 VSG gene (in addition to the two silent copies seen in the 3171 parental trypanosomes) had been created in chromosome 15, where the active 221 expression site is found. This clearly demonstrates for at least two of the switched variants that duplicative gene conversions can occur in the absence of 70-bp repeats within the active expression site.

DISCUSSION

We have generated transformants of *T. brucei* in which the 70-bp repeats of the active VSG expression site were removed or inverted. These alterations did not reduce the frequency of VSG switching, and reactions that deleted the 221 VSG gene within the active expression site were observed. We attribute such VSG switching events to gene conversion reactions in which sequence from an inactive expression site is copied into the active expression site. This was directly demonstrated for two switched variants by showing that a copy of the VSG gene derived from the VO2 expression site had been transferred into the 221 expression site. Our main conclusion, therefore, is that the 70-bp repeat sequences present in all characterized bloodstream form expression sites are not essential for VSG switching. In other words, they do not function as an indispensable sequence at which the process of VSG switching via homologous recombination is initiated in in vitro cultured trypanosomes. Although we have not formally demonstrated that VSG switching via reciprocal exchange can occur in the absence of 70-bp repeats, we find it unlikely that this form of homologous recombination could be specifically initiated by the repeats while gene conversions are not.

Our analysis excludes the possibility that the 70-bp repeats are utilized by *T. brucei* because they adopt an unusual structure or conformation that might predispose the expression site to cleavages that initiate duplex break repair recombination, leading to VSG switching (as has been suggested [11, 32, 66]). It is also unlikely that the 70-bp repeats are acted upon by a putative sequence-specific endonuclease (34). We cannot exclude the possibility that such an enzyme is present but that it recognizes another sequence that is unaltered by the expression site modifications made in these transformants. It might also act upon a more generalized feature of the expression site (such as specific chromatin structure [56, 62]). The chromatin of active expression sites is highly sensitive to nucleases specific for single-stranded DNA (22), and this might be the feature recognized by the putative nuclease (10). Alternatively, it is conceivable that such an endonuclease cutting the 70-bp repeats is present in some strains but that the gene encoding this enzyme has become mutated or its expression has become down-regulated during the extensive laboratory culturing of our parental 427-221a trypanosome strain. This possibility remains open, as differences in overall VSG switching rates have been observed between fly-transmitted trypanosome strains and laboratory-adapted strains (approximately 10^{-2} event/cell/generation versus 10^{-6} to 10^{-7} event/cell/generation, respectively [29, 61]). Direct evidence for such a labile endonuclease activity is lacking, however, and the higher VSG switching rate of fly-transmitted strains relative to laboratory-adapted trypanosomes has not been shown to be the consequence of an elevated level of recombination-based switching reactions.

A minor conclusion from this analysis is that the 70-bp repeats in active expression sites have no indispensable function for bloodstream form trypanosomes. This is tempered by the observation that the trypanosomes with altered repeats appeared, at least initially, to grow more slowly than those with a 2.7-kbp complement of correctly orientated repeats. However, transformants with alterations to the 70-bp repeats of

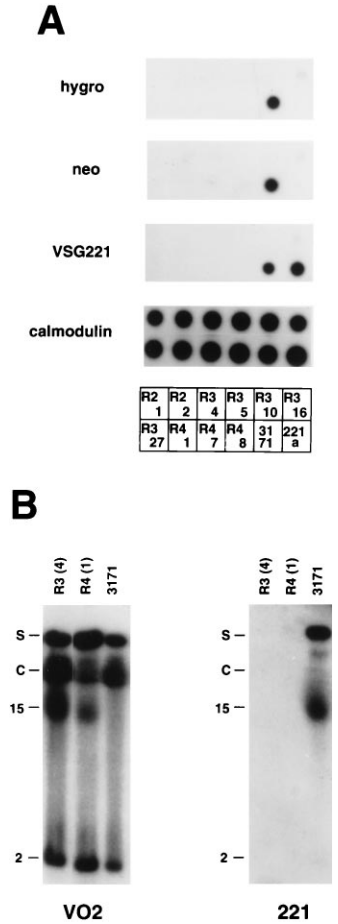


FIG. 5. Southern analysis of 3171 variants predicted to have switched by gene conversion. (A) Genomic DNAs isolated from 10 3171-derived switched variants, as well as from the 3171 transformant and the 221a parental strain, were replica blotted onto four nylon filters and probed for the presence of the hygromycin, neomycin, and VSG221 genes and also for the calmodulin locus (as indicated). Each switched variant was predicted, on the basis of PCR, to have deleted the resistance markers and VSG221 gene from the altered 221 expression site. The variants are identified in the lower panel by the clone number given to each during the switching analysis and were derived from three independent switching experiments (identified by R2, R3, and R4); DNA from the 3171 transformant and 221a trypanosomes is also indicated in the lower panel. (B) Intact chromosomes from switched variants R3(4) and R4(1) and from transformant 3171 were separated by pulsed-field gel electrophoresis, blotted, and probed for the VSG221 and VSG VO2 genes. S indicates the gel slot, 15 and 2 identify the respective chromosomes, and C indicates a signal within the gel compression zone.

their active site dictated by the plasmid constructs could demonstrably be generated, and there is no compelling evidence to suggest that the repeats have any other function than in VSG recombination.

Although our experiments show that 70-bp repeats are not essential for initiating gene conversions, they also confirm that these repeats are critical for efficient duplicative transposition of VSG genes outside an expression site. This follows from our demonstration that both integrated resistance markers were deleted in all characterized gene conversions involving an active expression site without 70-bp repeats or with inverted 70-bp repeats. This indicates that such crippled sites can use other expression sites as donors of new VSG genes exclusively during switching. That the extent of sequence homology between VSG221 and other VSGs is sufficiently low to preclude frequent recombination is also illustrated by the fact that we found no examples of switched variants that retained both the resistance markers but inactivated VSG221. In general, the level of sequence homology between VSGs is particularly low at the 5′ end (6), corresponding to the antigenically exposed N termini of the VSG proteins. Hence, the 70-bp repeats provide essential upstream homology to allow chromosome-internal and minichromosomal VSGs to be copied into the telomere of the active expression site (see below). This is in agreement with previous work that examined gene conversions between silent VSG genes lacking 70-bp repeats or that looked at gene conversions involving parts of VSG pseudogenes. These were relatively rare events, occurring only when a sufficiently homologous VSG gene is present within the active expression site (40, 47, 59, 60).

It is remarkable that in the transformant with naturally orientated 70-bp repeats, the number of putative gene conversions occurring between expression sites outweighed those occurring downstream of the integrated hygromycin resistance marker (and therefore probably using chromosome-internal or minichromosome VSG genes as sequence donors). Intuitively, one would expect the opposite ratio to allow the trypanosome to utilize the huge number of silent VSG genes available outside the ca. 20 expression sites. It is unlikely that our results are simply a consequence of the number of 70-bp repeats integrated into the active expression site, even though 2.7 kbp is a small stretch of repeats in comparison to the numbers found in most bloodstream form expression sites (where there can be over 20 kbp). Navarro and Cross also detected a high frequency of telomere conversion events into an expression site with a more extensive 70-bp repeat array: the 121ES or DES (38).

We consider it more likely that the most frequent gene conversion events are those in which substantial sequence homology exists around the recombining VSG genes, i.e., when the genes are both in expression sites. Due to the recombinogenic nature of telomeres (68), telomere conversion may be more frequent than expected. Recombination between VSG221 and chromosome-internal VSG genes relies upon the small number of 70-bp repeats at the 5′ flank of the latter, and hence these switches would represent lower-frequency events. This might mean that telomere conversion, and perhaps also telomere exchange and in situ switching, could have primary relevance early in an infection, as they result in a high frequency of switching among a relatively small pool of telomeric VSG genes. Once the host is immunized against these telomeric VSGs, less frequent events like gene conversion from chromosome-internal VSG genes could result in maintenance of a chronic infection (33).

Another remarkable result is that the apparent switching frequency is not affected by removal of the 70-bp repeats from the active expression site (Table 1), even though this removal prevents the use of around 99% of all potential donor VSG genes and restricts the donors to silent expression sites. This is also readily explained by the efficient use of other expression sites as donors in gene conversion, as illustrated by the results obtained with our transformant containing 70-bp repeats and in other transformants with wild-type 70-bp repeat arrays (50). We consider it likely that the rate-limiting step in VSG gene transposition is a cut in the acceptor DNA and that all subsequent steps in the transposition reaction are relatively fast. The initial cut is followed by a homology search. As long as there is at least one donor sequence left with substantial homology to the searching strand(s) (i.e., another expression site), the search can be successfully completed.

Why do trypanosomes use imperfect 70-bp repeats rather than perfect ones for VSG gene switching, given the preference of the recombination machinery of trypanosomes for relatively high levels of sequence identity during homologous alignment (5, 20, 30, 58)? Perfect 70-bp repeats might result in a (too) strong preference for donors with large repeat arrays. The use of imperfect-repeat arrays could allow the chromosome-internal VSG genes flanked by few 70-bp repeats to compete as donors with telomeric VSG genes flanked by several kilobase pairs of 70-bp repeats. A more detailed model of VSG gene transposition incorporating these features has been presented elsewhere (10).

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