DNA Rearrangements Associated with Multiple Consecutive Directed Antigenic Switches in *Trypanosoma brucei*

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Changes in variant surface glycoprotein (Vsg) expression allow *Trypanosoma brucei* to elude the immune response. The expressed *vsg* is always located at the telomeric end of a polycistronic transcription unit known as an expression site (ES). Although there are many ESs, only one is active at any particular time. The mechanisms regulating ES transcription and switching are unknown. Chromosome rearrangements within or upstream of the ES have been described to occur in occasional switch events, but no changes have been consistently associated with switching. We inserted the drug resistance genes *neo* and *ble*, conferring resistance to G418 and phleomycin, respectively, 1 kb downstream of “silent” ES promoters. This demonstrated that short-range transcription could be achieved from a silent ES promoter. From one initial transformant clone, panels of independent consecutive on-off-on switch clones were generated and analyzed. The first activation of the *neo*-targeted ES was always associated with deletion of the upstream tandem promoter in this ES, but no further rearrangements were detected in consecutive off-on switches of this ES. On the other hand, direct analysis of ES promoters showed that deletions and duplications occurred elsewhere. Activation of a *ble*-tagged 300-kb chromosome could not be achieved, but phleomycin-resistant clones could be obtained. One such clone arose from recombination between three ESs. Taken together, our experiments suggest that ES switching may occur after a period of chromosomal interactivity that may or may not leave tangible evidence in the form of detectable sequence changes.

Trypanosoma brucei is an extracellular protozoan parasite that multiplies in the tissue fluids and vascular system of its vertebrate host, causing major veterinary and medical problems in Africa. The entire surface of the bloodstream form trypanosome is covered by a dense coat, which consists largely of a single molecular species known as the variant surface glycoprotein (Vsg) (8). Sequential expression of *vsg* genes allows the parasite to elude the immune response of the host (reviewed in references 9, 10, and 51). The expressed *vsg* is always located adjacent to a telomere, at the end of a polycistronic expression site (ES) (15). Hybridization analysis suggests the presence of approximately 20 ESs, whose sequences are highly conserved (13, 54). Only one ES is active, yielding a single Vsg on the surface of the infective trypanosome. The ES contains a number of ES-associated genes (*esag*) (13, 51). ES transcription is driven by an α -amanitin-resistant polymerase (26, 27). The ES promoter is located 40 to 60 kb upstream of the telomeric *vsg* (25, 27). ES promoters sequenced so far show more than 90% sequence identity (18, 37, 53). The 5' end of the ES promoter is flanked by a region of imperfect direct repeats of 50 bp, which covers at least 10 kb in the *vsg221* ES (54).

Antigenic variation in *T. brucei* can occur by duplicative transposition of a new *vsg* into an already active ES, displacing the resident *vsg*. A switch in *vsg* expression can also be achieved by activation of another ES and concomitant inactivation of the previously active one (3). Among the different mechanisms for *vsg* switching, in situ ES activation remains most obscure. No sequence changes within 3.4 kb of the promoter region have been detected between active and inactive states (18, 37,

53). DNA rearrangements have been described to occur in the region upstream of the promoter, in a few ES inactivation events. Some of these rearrangements involved either the deletion of the upstream promoter, in an ES where the ES promoter, embracing a region of about 13 kb, was tandemly duplicated (18), or changes within the 50-bp repeat array upstream of the promoter (52). To determine the significance of these observations, we decided to search in detail, in panels of independent clones with consecutive on-off-on switches at a specific ES, for any DNA rearrangements that might occur consistently upon ES activation and inactivation. Only recently, with the application of DNA transformation techniques to bloodstream form *T. brucei* (5), has it become possible to target specific regions of an ES with drug resistance genes and to capture specific switch events in a representative manner.

Because homologous recombination occurs efficiently in *T. brucei* (16, 45), we were able to mark two inactive ESs with different drug resistance genes. We inserted marker genes 1 kb downstream of ES promoters to facilitate the capture of ES activation events and minimize the selection of switch events occurring by *vsg* gene conversion or partial ES recombination events. Drug selection in mice allowed us to obtain, in a single relapse, a panel of specific antigenic variants in which the tagged ES became active. The selected switch clones expressed high levels of the resistance marker as well as the *vsg* gene located at the telomeric end of the target ES. The marker genes could then be used as specific probes to map chromosomal organization, bypassing the problems associated with the high sequence similarity among ESs (1, 12, 13, 17, 33, 43, 44). The marker genes allowed us to monitor rearrangements linked to the ES promoter as well as interchromosomal duplication or recombination events. We analyzed DNA rearrangements upstream of the ES promoter associated with in situ (in)activation by separating chromosomes and large restriction fragments by pulsed-field gel electrophoresis (PFGE). More-

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detailed mapping of the upstream sequences of the ES promoter in active and inactive states was performed by partial digestion of these large restriction fragments containing the marker gene at the 3' end.

MATERIALS AND METHODS

Trypanosomes and switching experiments. *T. brucei* clone 221a parasites (Molteno Institute Trypanozoon antigenic type 1.2, [MITat 1.2], clone 221a) were derived from strain 427 (8). Trypanosomes were routinely cultured in HMI-9 at 37°C (21). All of the cell populations used in this study were cloned by limiting dilution in vitro. The homogeneity of trypanosome populations (based on counts of approximately 200 parasites) was verified by indirect immunofluorescence with specific antibodies against Vsg 121 and Vsg 221, together with 0.0025% Hoechst 33258 (Boehringer Mannheim) as a nuclear stain. For switching experiments, trypanosomes were grown in either female Swiss white mice or male Sprague-Dawley rats and then transferred to HMI-9 medium as described elsewhere (4). In vivo selection of resistant variants was carried out by intraperitoneal inoculation of 10^4 parasites per mouse. On the third day, when the parasitemia was approximately 10^6 /ml, and on each successive day, G418 or phleomycin was inoculated subcutaneously at a dose of 2 or 1 mg, respectively. On the fourth day the parasitemia continued to rise, to approximately 10^7 /ml, but trypanosomes were undetectable on day 5. On the sixth day a few parasites were detected in blood, and on day 8 the parasites were isolated, after 5 days of continuous drug treatment. The parasites were cloned under drug selection with 100 μ g of G418 or phleomycin per ml. Variants in which the tagged ES had switched off were obtained by using the immune response for negative selection. Ten cells were inoculated into rats, and parasitemia was monitored daily. The parasitemia peaked on the sixth or seventh day and then fell below a detectable level until day 9 or 10, when a second peak arose, upon which trypanosomes were isolated and cloned by limiting dilution. The clones were screened by immunofluorescence, by using antiserum to Vsg 221, to identify those in which the 221 ES was reactivated.

Plasmid constructions and probes. The ES-targeting sequences, derived from a 3.2-kb *SpeI*-*Bam*HI genomic clone containing the downstream promoter repeat and the 5' portion of *esag7* from the ES of *T. brucei* 118 clone 1, were kindly provided by Keith Gottesdiener (Columbia University, New York, N.Y.). This is the same ES that, in our current studies, contains *vsg121*. From this clone, the 1.5-kb *Sall*-*Pst*I fragment was subcloned into Bluescript (Stratagene) and named pBS1.5SP*Sall*-*Pst*I. The marker cassette was inserted into the *Stu*I site (located 962 bp downstream of the transcription initiation site) of pBS1.5*Sall*-*Pst*I. The *neo* cassette was derived from pHD30Neo by removing the *SpeI*-*Xho*I restriction fragment containing *neo* flanked by *T. brucei* actin splice acceptor and polyadenylation sequences (23). The 5' and 3' overhanging ends were filled or trimmed with Klenow fragment and T4 DNA polymerase, respectively, when necessary. To make a *ble* cassette, *neo* was replaced by the *Streptococcus hindustanensis* *ble* gene (24), derived from plasmid pUT56 (Cayla, Toulouse, France), by using the *Hind*III and *Bam*HI sites 5' and 3' of the coding region in the *neo* cassette.

Newly expressed *vsg* genes were cloned by reverse transcription-PCR (RT-PCR) by using an oligonucleotide (5'-CCGGTACCGTGTTAAATATATC3') hybridizing to the conserved 16-mer found at the 3' end of all *vsg* mRNAs and another (5'-CGCGAATTCTAGAACAGTTTCTGT3') hybridizing to the *T. brucei* mRNA spliced-leader sequence. The single band obtained was cloned into the TA cloning vector (Promega). The correct isolation of the expressed *vsg* was confirmed by Northern (RNA) analysis of RNA from the corresponding cloned trypanosomes. The genes were then sequenced at The Rockefeller University Shared Technology Resource Center. To generate *vsg*-specific probes, the 5' ends of the cloned genes were released by restriction digestion and gel purified. The *neo* probe was the *Rsa*I-*Bam*HI restriction fragment containing the 3' end of the gene. The core ES promoter probe was a 315-bp *Hpa*I-*Sall* fragment (positions 1359 to 1673 in the sequence described in reference 54) isolated from plasmid pRK8(+) (52).

Transformation and cloning. Plasmid DNA for transfection was prepared by using anion-exchange columns (Qiagen Inc.). To remove vector sequence from the targeting cassettes, the plasmid constructs were digested with *Bpu*1102I and *Alw*NI prior to electroporation. Trypanosomes were grown in Sprague-Dawley rats and transformed with a 1.75-kV pulse as previously described (5) by using the Electro Cell Manipulator 600 Electroporation System (BTX), except that Cytomix (47) replaced Zimmerman postfusion medium with glucose (ZPMG) and the linearized plasmid DNA (5 μ g for the *neo* construct or 30 μ g for the *ble* construct) was used in each cuvette. A 1- μ g/ml amount of G418 (Gibco BRL) or phleomycin (Cayla) was added to the growth medium on the day after electroporation. Clonal cell lines were derived from drug-resistant cultures as described elsewhere (4). Cultures were subsequently grown in the absence of drug selection unless otherwise stated.

RNA and DNA analysis. RNA was isolated by using RNastat60 (Tel-Test Inc). Northern and Southern analyses were carried out according to standard protocols (42). RNA and DNA were cross-linked to the membrane in a UV Stratalinker 1800 (Stratagene). Probes were generated by using the Prime-It II labeling kit (Stratagene) with Prep-A-Gene (Bio-Rad) gel-purified DNA. All hybridizations and washing of filters were done at 65°C in a hybridization oven (Hybaid).

Posthybridization washes were for 30 min at 65°C in $0.2\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate. RNA size was estimated according to the migration of an RNA ladder (Gibco-BRL). Hybridization signals were quantitated by using a Phosphorimager and Imagequant software (Molecular Dynamics).

PFGE. Parasite blocks were prepared as described for mammalian cells (42), at a concentration of 2×10^8 trypanosomes per ml. Chromosomal DNA separations were performed in 0.8% agarose gels, in a rotating agarose gel electrophoresis (RAGE) apparatus (Stratagene). The program consisted of two windows: a 100-s pulse at 120 V for 10 h followed by a ramp from pulse times of 1,000 to 2,500 s at 50 V for 80 h. The rotation angle was 106°, and a $0.5\times$ Tris-borate-EDTA recirculating buffer was run at 12°C. Digestion of chromosomal DNAs was carried out in agarose blocks (42) as recommended by the enzyme manufacturer (New England Biolabs). The partial-digestion analysis of the *Bam*HI restriction fragment containing the upstream region of the ES promoter was carried out by first digesting the DNA for 16 h with *Bam*HI, after which the agarose blocks were washed twice in Tris-EDTA and treated with different concentrations of the second restriction enzyme for 2 h. A preliminary digestion was analyzed, and the concentration that produced the optimum ladder of restriction fragments was chosen for analyzing all samples. Chromosomal DNA fragments between 2 and 100 kb were separated in 1% agarose RAGE gels by using a ramp from 1 to 2 s of pulse time at 150 V for 18 h with a 120° angle. To separate larger fragments, we used conditions described previously (36). DNA size was estimated from the migration of lambda DNA PFGE low-molecular-weight marker (New England Biolabs).

RESULTS

Insertion of selectable marker genes downstream of silent ES promoters. In clone 221a (MITat 1.2), the active ES (221 ES) is located on a chromosome of approximately 3.2 Mb (2, 11, 25, 27) and contains *vsg221*. Clone 221a has a "silent" ES (121 ES), on a 2.2-Mb chromosome (31, 32, 46, 48), which contains tandem promoters located 50 kb upstream of *vsg121* (19). We used sequences running from the 3' region of the downstream promoter to the 5' region of *esag7* as a target for integration into the 121 ES (Fig. 1B), inserting the Tn5 neomycin phosphotransferase gene (*neo*) at a *Stu*I site, 962 bp downstream of the transcription initiation site. The *neo* gene was flanked by splice acceptor and polyadenylation sequences from a *T. brucei* actin gene, to enable RNA processing (23). We selected transformants at a low drug concentration (1 μ g of G418 per ml) because we expected a low level of transcription from a silent ES promoter (41). We obtained three independent transformants. Southern analysis confirmed the correct insertion of the *neo* cassette by homologous recombination in all three clones (see below). The analysis confirmed the previously described map of this region (18, 20) and also confirmed that the promoter was duplicated in the silent 121 ES of clone 221a.

As the promoter regions are almost identical among different ESs (27, 38, 54), we tried to mark additional silent ESs of one of the G418-resistant transformants, using the same ES-targeting sequences but using the bleomycin resistance gene (*ble*) in place of *neo* (Fig. 1C). Four independent transformants were selected in medium containing phleomycin and G418 at low concentrations (1 μ g of each drug per ml). In different clones, the *ble* cassette inserted either into the 2.2-Mb chromosome or into chromosomes of 300 kb and 2.6 Mb (Fig. 2). The transformant clone *Pnb221* (promoter-proximal *neo ble* markers in cells expressing *vsg221*), which was the starting point for all experiments to be described, has *neo* inserted into the 121 ES and *ble* in a 300-kb chromosome, at a putative ES (see below). No other hybridization signals were detected. Immunofluorescence analysis of *Pnb221* showed that all cells were expressing Vsg 221.

First activation of the 121 ES. To obtain antigenic variants in which the *neo*-tagged 121 ES was activated, we used G418 plus immune selection in mice. Two mice were infected with 10^4 trypanosomes of clone *Pnb221*. After 3 days, all mice showing parasitemia were treated with G418 for 4 days (see Mate-

FIG. 1. Insertion of selectable markers downstream of silent ES promoters. (A) Schematic diagram of the 121 ES and 221 ES in *T. brucei* clone 221a. The expression-site-associated genes (*esag*) are indicated by boxes between the promoter (represented as a flag) and the *vsg* gene. The 50-bp repeats are represented by a hatched box upstream of the promoter. (B) Insertion of the *neo* cassette 962 bp downstream from the transcription initiation site of the 121 ES on a 2.2-Mb chromosome. The 3' targeting sequence, containing the 5' region of *esag7*, is only present at the 3' side of the downstream promoter. The cassettes contain the 5' and 3' untranslated regions from a *T. brucei* actin gene. (C) Insertion of the *ble* cassette used in the second transformation into an uncharacterized ES.

rials and Methods). This protocol allowed the trypanosome population to expand to a size at which switches would occur (the frequency of switching to a specific ES was expected to be between 10^{-6} and 10^{-8} (29). The mice developed an immune response against Vsg 221, which helped to eliminate the original variant, which otherwise overwhelmed the ability of G418 alone to control the already high level of parasitemia. G418 selected for switches to the 121 ES in the newly arising population, but the selection was imperfect, probably because blood G418 levels were low. The populations isolated from mice were analyzed by immunofluorescence before being grown in vitro. Approximately 20% of the cells expressed Vsg 121. Two percent of the cells still expressed Vsg 221, while approximately 80% expressed VsGs not recognized by either antibody. Trypanosomes isolated from blood were grown in 100 μ g of G418 per ml and cloned by limiting dilution. Two independent clones, sD1 and sD2, isolated from different mice, were homogeneously expressing Vsg 121, as judged by immunofluorescence analysis. RNA analysis confirmed that the *vsg221* transcript in the parent *Pnb221* was replaced by high levels of *vsg121* and *neo* mRNAs in switch clones sD1 and sD2 (Fig. 3).

To obtain a long-range restriction map of the *neo*-tagged 121 ES, we digested agar-embedded chromosomal DNA samples. Using restriction enzymes that produced large telomeric fragments hybridizing with both *neo* and *vsg121* probes, we confirmed the linkage of *vsg121* and *neo* in *Pnb221* (data not shown). The presence of a *Bam*HI site at the 3' end of the *neo* gene made it possible to map the region upstream of the promoter. This analysis showed that the 60-kb *Bam*HI restriction fragment from *Pnb221* had decreased by 15 kb during 121

ES activation (Fig. 4A). Loss of restriction sites between the tandem promoters in the parental clone *Pnb221* was detected in the switched clones (Fig. 4B). These results all suggested that the tandem promoter had been deleted. To analyze in detail where the deletion occurred, DNA from clones sD1 and sD2 was first digested with *Bam*HI and then partially digested with *Sau*3A or *Spe*I, for which a restriction map had been described (18). The results showed the loss of two *Spe*I sites and all but three *Sau*3A sites (Fig. 5). This deletion is identical or very similar to a deletion previously described to occur during inactivation of this ES (18).

To determine whether this deletion was consistently associated with activation of the 121 ES, we generated four additional independent switches from *Pnb221*, by G418 selection in mice. We also isolated three independent 121 ES activation variants from a clone that had previously switched, in a rat (see Materials and Methods), from the 221 ES to an uncharacterized ES. All seven switch clones, now expressing *vsg121*, showed the same deletion of the upstream promoter as described for the first two switches (Fig. 4 and 5 and data not shown).

Inactivation and reactivation of the 121 ES. Immune selec-

Chromosomal Location of Markers

Clone	<i>neo</i>	<i>ble</i>
2	121ES	---
2.1	121ES	121ES
<i>Pnb221</i>	121ES	0.3 Mb
2.3	121ES	121ES
2.4	121ES	2.6 Mb

FIG. 2. Chromosomal location of markers in the transformant clones. (Left) Ethidium bromide staining pattern of chromosomal DNA from the phleomycin- and G418-resistant clones obtained from the original *neo* transformant (clone 2) and separated by RAGE (see Materials and Methods for conditions). The chromosomal-DNA size markers were from *Saccharomyces cerevisiae* (Sc.) and *Schizosaccharomyces pombe* (Sp.). (Center and right) Southern analysis using the indicated probes. The table below summarizes the chromosomal locations of marker genes in each transformant.

FIG. 3. Activation of the 121 ES. RNA was isolated from two independent switch clones (sD1 and sD2) derived from *Pnb221*. A 4- μ g amount of RNA from each clone was separated in a gel and transferred to a nylon membrane. The blot was hybridized consecutively with *vsg221*, *vsg121*, *neo*, and β -tubulin (*tub* β) probes.

tion was used to obtain antigenic variants in which the 121 ES was once again switched off. Four rats were each infected with 10 cells of clone sD1 or sD2. After a second peak of parasitemia, following immune destruction of the infecting 121 variant, trypanosomes were isolated and cloned by limiting dilution in vitro without drug. As intended, none of the clones expressed Vsg 121. Two clones from each rat were maintained simultaneously in media with and without G418 (100 μ g/ml). In this study, we were interested only in switch events that occurred by in situ inactivation of the 121 ES and not in switch events occurring by replacement of *vsg121* at the same ES. Therefore, only the G418-sensitive clones were chosen for further analysis, on the presumption that these clones would have inactivated the 121 ES (Fig. 6). In some of the G418-sensitive clones, both *neo* and the expression-linked copy of *vsg121* had been lost (Fig. 7), suggesting that the entire *neo*-tagged ES had been deleted. The new *vsg* mRNAs expressed in these switches were cloned by RT-PCR. *vsgK* and *vsgR*, from switched clones sD1sK1 and sD2sR1, were partially sequenced. *vsgR* showed 97% sequence identity at the 5' end to the *Trypanosoma gambiense* AnTat 11.17 *vsg* (14) over a length of 388 bp, but *vsgK* was novel. Prior to activation, *vsgK* appeared to exist on a 1.8-Mb chromosome (Fig. 7). The presence of an expression-linked copy of *vsgK* and the deletion of *vsg121* and *neo*, in clones sD1sK1 and sD1sK2, suggested that *vsgK* had been activated by conversion of the entire 121 ES (Fig. 7). The single copy of *vsgR* resided on a large chromosome (>7 Mb), which was retained in the well during RAGE (data not shown), but its expression, in switched clone sD2R1, also resulted in the loss of both *neo* and the expression-linked copy of *vsg121* (Fig. 7). Neither switched clone was analyzed further.

Two G418-sensitive clones, sD1s221 and sD2s221, in which the 221 ES was reactivated without loss of the 121 ES were

isolated (Fig. 6). Mice were inoculated with these clones and again treated with G418, to select for reactivation of the 121 ES (Fig. 6). Immunofluorescence analysis of G418-treated trypanosome populations isolated from mice demonstrated that 80% of the cells expressed Vsg 121. Two clones from each parent were isolated in vitro and confirmed to be homogeneously expressing Vsg 121.

DNA from these consecutive switches, in which the 121 ES was inactivated and reactivated, was analyzed (data not shown). No rearrangements were detected within a 170-kb *Xba*I fragment encompassing the 121 ES. We compared the restriction fragment patterns from partial digestion of the *Bam*HI fragment among this panel of switched clones. Using a restriction enzyme with a 4-bp recognition sequence (*Sau*3A, *Rsa*I, or *Hin*II), we were unable to detect differences between active and silent states in the region upstream of the promoter. We also digested DNA with *Mse*I or *Tsp*509I, which cut the 50-bp imperfect direct repeats upstream of the ES promoter. No variation within the approximately 25-kb block of repeats was detected by partial digestion with these enzymes.

Attempted activation of the *ble*-targeted ES. The original transformant, *Pnb221*, contains marker genes at two inactive ES promoters: *neo* at the 121 ES and *ble* in a presumptive ES on a 300-kb chromosome (*ble* ES). Using phleomycin selection in mice, we now attempted to select antigenic variants in which the *ble* ES was activated. These switched clones were expected to show a high level of transcription of both *ble* and a new *vsg* located at the *ble* ES. However, we were unable to obtain the expected switch, even when using rats to screen a larger trypanosome population ($\sim 5 \times 10^9$ trypanosomes). We also tried to select phleomycin-resistant switch variants from clone sD1, in which the 121 ES, rather than the 221 ES, was active. Three days after inoculation of 10^4 cells, four mice were treated with phleomycin for 4 days. Seven days after inoculation, one mouse showed parasitemia resistant to phleomycin. Trypanosomes were cloned by limiting dilution in medium containing 100 μ g of phleomycin per ml. One clone obtained, sD1sB1, was first analyzed by RAGE. Once again, the previously active copies of *vsg121* and *neo* were missing, suggesting that the 121 ES had been largely replaced (Fig. 8 and *neo*-probed panel in Fig. 9A). Furthermore, the 2.2-Mb chromosome now contained a copy of *ble* (arrowed bands in Fig. 8).

The new *vsg* expressed in sD1sB1 (*vsgB*) was cloned by RT-PCR. Prior to its activation, *vsgB* was found on a 2.6-Mb chromosome but not on the 300-kb *ble*-tagged chromosome (Fig. 8). Southern analysis indicated that *vsgB* was telomeric linked in the 2.6-Mb chromosome. After activation in clone sD1sB1, a second telomeric copy of *vsgB* appeared on the 2.2-Mb chromosome. Restriction mapping confirmed that *ble* was linked to *vsgB* in clone sD1sB1 (data not shown).

Further analysis confirmed that *ble* was duplicated, together with at least 20 kb of downstream sequence from the 300-Mb chromosome (at least as far as the diagnostic *Sma*I site indicated on the restriction maps in Fig. 8). The additional *ble* *Xmn*I fragment in sD1sB1 (arrowed band in Fig. 9A) indicated that the upstream boundary of the 300-Mb chromosome-derived duplication must lie within the 4-kb *Xmn*I fragment containing the parental *ble*. Therefore, the switch to phleomycin resistance in trypanosome clone sD1sB1 results from replacement of the 121 ES by sequences from both 2.6-Mb and 300-kb chromosomes (restriction maps in Fig. 8). It cannot be determined whether these recombination events were simultaneous or sequential.

Analysis of promoter rearrangements and telomere length. In the first activation of the 121 ES, the upstream duplicated promoter was deleted. Other ESs also contain tandem promot-

FIG. 4. Southern analysis of activated 121 ES clones derived from *Pnb221*. After digestion of DNA with the indicated restriction enzymes, the products were separated by RAGE and hybridized with a *neo* probe. (A) *Bam*HI restriction fragments containing the upstream region of the 121 ES promoter. (B) Double digestion of the *Bam*HI restriction fragment with the indicated enzymes. The sDb and sDc series were obtained in additional switching experiments with mice and rats, respectively (see text). Only relevant restriction sites are indicated on the 121 ES map shown below the gels. The *Not*I, *Pme*I, and *Xba*I sites are unique in the 121 ES. Abbreviations: Ba, *Bam*HI; Bg, *Bgl*I; C, *Cla*I; E, *Eco*RI; S, *Spe*I; N, *Not*I; P, *Pme*I; X, *Xba*I.

ers (18, 20). We decided to investigate whether deletions or duplications of other ES promoters could be detected during switching events.

A Southern blot of *Xmn*I-digested DNAs was sequentially hybridized with *neo*, *ble*, and ES promoter probes. An *Xmn*I site lies between the tandem 121 ES promoters. Consequently, *Xmn*I digestion of the 121 ES in *Pnb221* generated 4.5- and 34-kb fragments that hybridized with the ES promoter, and the *neo* probe hybridized to the 4.5-kb band (Fig. 9A). During the first 121 ES activation and upstream promoter deletion, the intervening *Xmn*I site was lost, so that the 4.5-kb band moved to the 34-kb position, where it hybridized with the ES promoter and *neo* probes (Fig. 9A). We also probed with *ble*, which recognized a band derived from the 300-kb *ble* ES in all of the clones and one extra 34-kb band in the sD1sB1 switch clone (Fig. 9A, arrowheads), corresponding to displacement of *neo* by *ble* in the single-promoter 121 ES. Lack of *neo* hybridization confirmed that switches in sD1sK1, sD1sK2, sD2sR1, and sD2sR2 involved conversion of the entire 221 ES.

There are several points of interest in the patterns of hybridization to the ES promoter probe. Firstly, the lengths of the 50-bp repeat regions upstream of the promoter were not randomly distributed. The shortest fragments probably arise from ESs containing tandem promoters, although not all tan-

dem promoters may have the intervening *Xmn*I site. The intermediate sizes may represent a situation in which a single ES promoter is downstream of an array of 50-bp repeats spanning approximately 25 kb. The largest fragment sizes could represent ESs in which 50-bp repeats extend for approximately 50 kb (Fig. 9B). The bands hybridizing to the ES promoter and the *neo* or *ble* probes confirmed the linkage of the markers and ES promoters and also indicated that these bands contained a single ES promoter. By Phosphorimager quantitation of this blot, we detected $29 (\pm 3)$ ES promoters in the original transformant, *Pnb221*. In some switched clones, we detected variations in the relative abundance of some ES promoter bands, indicated by asterisks on the Phosphorimager profiles (Fig. 9C).

We also looked for variations in the lengths of the 121 ES and 221 ES telomeres, in a panel of consecutive switch clones. Digestion of genomic DNA with *Xmn*I separated the four copies of *vsg121* and exposed differences in the length of one band that identified it as the telomere-associated copy in the 121 ES (Fig. 10). Breakage of the 121 ES telomere was evident in three of six clones and breakage of the 221 ES telomere was evident in four of six clones (Fig. 10), but these changes could have occurred on just two occasions: at the *Pnb221*-to-sD2

FIG. 5. (Top) The chromosomal DNAs from independent activations of the 121 ES (see legend to Fig. 4) were first digested with *Bam*HI and then partially digested with *Sau*3A or *Spe*I. A ladder was visualized by *neo* hybridization to restriction fragments containing the marker gene at the 3' end. (Bottom) Detailed map showing the upstream promoter deletion during 121 ES activation. Abbreviations: Sp, *Spe*I; S, *Sau*3A.

switch or at the last activation of the 121 ES, in the sD1 series (Fig. 6).

DISCUSSION

An intrinsic problem in the study of antigenic variation in African trypanosomes has been the analysis of a limited number of nonsystematic switch events. Descriptive analyses of DNA rearrangements associated with individual in situ ES (in)activation events have been inconclusive, with respect to identifying the mechanism of switching. To investigate whether DNA rearrangements are responsible for or reproducibly associated with in situ (in)activation events, we tagged silent ES promoters with selectable marker genes. We found that selectable markers containing no supplementary promoters could be inserted 1 kb downstream of silent ES promoters. Expression of the marker gene was probably due to its proximity to the

promoter, since insertion of a promoterless *ble* gene close to the end of a silent ES, immediately upstream of the *vsg*, does not result in detectable expression (22). This result strongly suggests that a low level of transcription initiation occurs at silent ES promoters, and this is also suggested by the detection of transcripts from multiple unmodified ES promoters in bloodstream form trypanosomes (41). Therefore, transcription from silent ES promoters is not totally repressed, as previously proposed (3, 40).

The use of selectable markers allowed us to capture and analyze many independent but equivalent switch events. Insertion of marker genes did not appear to alter the inherent switching capacity of an ES. Low-frequency switching events could be selected from the large trypanosome populations that could be raised in mice or rats, but not in culture. Because of the exquisite sensitivity of bloodstream form *T. brucei* to G418 (34) and phleomycin, these drugs could be used in vivo to

FIG. 6. Lineage of switch clones. Each switch event is indicated by the prefix s. After the s, the active ES is indicated as follows: K, ES containing *vsgK* (1.8 Mb); B, ES containing *ble* (300 kb); R (located on a chromosome of >7 Mb), ES containing *vsgR*; D, 121 ES (2.2 Mb); 221, ES containing *vsg221* (3.2 Mb). A number following the ES designation indicates the individual mouse from which the switch clone was isolated. The procedures used to obtain the switch clones are indicated above the arrows. The *vsg* expressed in each switch clone is reiterated for clarification.

select for activation of the ES into which the resistance gene had been inserted, and the developing anti-Vsg immune response assisted in eliminating the large population of the initial variant, which otherwise overwhelmed the controlling ability of practical levels of the drugs. After drug selection in mice, we also detected cells not expressing *vsg* at the target ES, which we attributed to low-level transcription of the resistance gene in a silent ES. However, there appeared to be a growth advantage for cells that properly activated the targeted ES. Switched trypanosomes were cloned in vitro, where high drug concentrations ensured that only variants resulting from the intended activation events would survive. ES inactivation event clones could be isolated by using the immune response as a negative selection, as in earlier studies.

The insertion of marker sequences overcomes another major problem in analyzing ES rearrangements in trypanosomes, which is the high degree of similarity among ESs. Using the marker gene as a unique probe, we could analyze consecutive 121 ES activations and inactivations. Initially, in two independent switches, 121 ES activation was associated with deletion of the tandemly repeated ES promoter. Four additional independent clones with switches from *vsg221* to *vsg121* all showed the same deletion. To investigate if this deletion occurred only in direct switches from the 221 ES to the 121 ES, we used a variant that had previously switched from 221 to another uncharacterized ES. An identical upstream promoter deletion, in the 121 ES, was observed in the three independent activations of the 121 ES in this variant. A similar deletion of the duplicated 121 ES promoter and intervening sequences has been described previously (18, 20), but it occurred during inactivation of the 121 ES. Thus, the same deletion occurs if the 121 ES contains two promoters tandemly arranged and is involved in either an in situ activation or an in situ inactivation event. Interestingly, the percentage of cells expressing Vsg 121, detected in blood after the first 121 ES activation from the clone containing two promoters, was lower than it was after the second 121 ES activation, from clones containing one promoter. This suggests, very tentatively, that the 121 ES containing only one promoter might be activated more frequently than when the tandem promoter array is present. Deletion of the

upstream promoter sequences might tag the 121 ES for future switch events, explaining why some ESs are more frequently reactivated after certain switch events (30, 32).

We quantified the total number of ES promoters in the *T. brucei* genome. In the initial transformant (*Pnb221*), the estimated number was 29 (± 3), which is consistent with previous estimates of about 20 ESs (13, 54), of which about one-half contain duplicated promoters (20). However, if all of the tandemly repeated promoters were separated by an *XmnI* site, which is not certain, our data would imply that a smaller number are present as tandem repeats. Our observations suggest that there are frequent changes in ES promoters, presumably mainly involving deletion of tandem promoters or duplications and replacement of entire ESs. In the two initial

FIG. 7. Chromosomal locations of the *neo* and *vsg221* genes before and after inactivation of the 121 ES. Chromosomal DNAs of the G418-sensitive switch clones (sD1sK1, sD1sK2, sD1s221, sD2sR1, and sD2s221) derived from G18-resistant clones (sD1 and sD2) were separated by RAGE and transferred to a nylon membrane. The filter was hybridized to the probes indicated below each panel. The change in the apparent sizes of all the chromosomes in some clones is an artifact of gel loading. The *vsg121* basic copies (BC) and the expression-linked copy (ELC) are indicated.

FIG. 8. Multiple rearrangements yield a new composite expression site. Hybridization of a chromosomal blot with the indicated probes shows duplication (arrowheads) of *vsgB* and *ble* sequences from 2.6-Mb and 300-kb chromosomes to the 2.2-Mb ex-121 ES. The restriction maps of the three ESs implicated in the rearrangement are shown for parental clone sD1 and for switched clone sD1sB1. The flag on the 300-kb chromosome indicates the position of the putative promoter. For clarity, only informative restriction sites linked to specific probes are shown. Abbreviations: Ba, *Bam*HI; Bg, *Bgl*I; C, *Cl*aI; E, *Eco*RI; S, *Sma*I; Sp, *Spe*I; N, *Not*I; P, *Pme*I; X, *Xba*I; Xm, *Xmn*I. *Not*I, *Pme*I, and *Xba*I generate telomeric fragments.

switches the upstream promoter was deleted, but we also detected deletions and rearrangements of other ES promoters. Evidence consistent with deletion of tandem ES promoters independent of switching has been reported previously and can even occur in procyclic trypanosomes (20). Clearly, these recombination events cannot only involve promoter deletion, or tandem promoters would not be found.

After the first activation of the 121 ES, its restriction map did not change in consecutive off-on switches. The approximately 25-kb block of 50-bp imperfect direct repeats, located upstream of the 121 ES promoter, did not change. The ES promoter restriction fragment sizes were distributed in three groups, suggesting three patterns of upstream environments: promoter duplication and either 25 or 50 kb of 50-bp repeats.

Changes in telomere length have been associated with antigenic variation (35). Breakage of the 121 or 221 ES telomeres was evident in a few switch clones. However, the current experiments were not specifically designed to monitor telomere changes, and no conclusions can be made, from the present study, about their frequency or correlation with switching.

In three independent transformants, the *neo* cassette in-

serted at the downstream 121 ES promoter. In contrast, in four independent transformants, the *ble* cassette inserted into three chromosomes. These transformants were grown in the presence of both phleomycin and G418, to select against replacement of the *neo* cassette by *ble*, which might have been preferred because the targeting sequences came from the 121 ES. In two of these transformants, which were not further analyzed, *ble* inserted into the 2.2-Mb chromosome, probably at the opposite end from the 121 ES, or in a comigrating chromosome, or at the 121 ES tandem upstream promoter, via integration of contaminating circular plasmid. In the other two transformants, the *ble* cassette inserted into chromosomes of 2.6 Mb or 300 kb. Stable integration in *T. brucei* occurs only by homologous recombination (16, 45), suggesting that these two chromosomes have promoter sequences with a high degree of sequence similarity to the 121 ES promoter.

In the complex sDsB1 switch, recombination occurred between promoter regions on different chromosomes. In this switch, the *ble* cassette was duplicated from a 300-kb chromosome to the 2.2-Mb 121 ES chromosome. The restriction map of the 300-kb chromosome was consistent with the presence of a tandem ES promoter, upstream of the *ble* cassette. In addition, hybridization analysis has shown that the sequences between the 121 ES promoter repeats, which include *esag10*, are also present in a 300-kb chromosome (20). Thus, in clone sD1, the *ble* cassette was duplicated from the downstream tandem promoter in a putative ES to the single promoter present in the active 121 ES. These results indicate that interchromosomal recombination also occurs between ES promoter regions. Such events could result in transfer of some aspect of chromosomal conformational information between active and inactive states, achieving in situ ES (in)activation. Our observations provide further support to the earlier suggestions (50) that telomere interactions may precede switching. However, these interactions may extend over large regions of the ES and may or may not leave tangible evidence in the form of detectable rearrangements. This hypothesis is amenable to further experimental investigation.

Activation of *ble*, in the sD1sB1 switch, involved recombination among three chromosomes. We cannot determine whether the two recombination events occurred simultaneously or sequentially, but we favor the view of other investigators, who speculated that similar events occurred in a coordinated manner (19, 49). The putative ES on the *ble*-tagged 300-kb chromosome could not be activated in situ, suggesting that this putative ES is deficient in some essential sequence, which could be located either upstream of the *ble* cassette or downstream of the *Sma*I site that is duplicated in sD1sB1. It seems more likely that the missing sequences are located upstream of the *ble* cassette. Otherwise, if the putative *ble* ES were simply missing a *vsg* or other downstream sequences, these might have been readily donated by a recombination event similar to the duplication seen in sD1sB1. Similarly, the sD1sK switch clones showed a duplication of *vsgK* from a 1.8-Mb chromosome to the 121 ES, associated with deletion of *neo* and *vsg121*, suggesting that duplication of a large telomeric ES-like region had also occurred in this example, with concomitant deletion of the entire 121 ES. Thus, in several independent switch events, we observed duplications of other putative ESs (or combinations of them) into the 2.2-Mb chromosome, replacing the entire 121 ES. These results suggest that sequences located upstream of the ES promoter might be essential for ES activation. These sequences might maintain the activity of any ES linked to or duplicated downstream of them.

In the results shown here and by others (18, 20, 39, 52, 54),

FIG. 9. ES promoter deletions and duplications. (A) Southern analysis after *XmnI* digestion of DNAs from switched trypanosomes. The blot was hybridized sequentially with the indicated probes. The bands hybridizing to *ble* or *neo* that coincided with an ES promoter allowed us to identify single-copy ES promoter bands and estimate the number of ES promoters in every clone. Small arrows in the first lane indicate bands corresponding to the downstream (4.5-kb) and the upstream (34-kb) 121 ES tandem promoters in the original transformant *Pnb221*. The arrowheads indicate the duplication of *ble* to the former 121 ES. (B) Schematic representation of the ES promoter environments suggested by the Southern data: promoter duplication (line 1) and either 25 kb (line 2) or 50 kb (line 3) of 50-bp repeats. *Xm*, *XmnI*. (C) Phosphorimager profiles of ES promoter hybridization of selected switch clones. Small arrows indicate the bands arrowed in panel A. Asterisks indicate bands whose relative intensities vary.

FIG. 10. Telomere length variation. Chromosomal DNAs were digested with *Xmn*I, which distinguishes the *vsg121* basic (BC) and telomeric expression-linked (ELC) copies, and hybridized with probes for *vsg121* and *vsg221*. The silent (Sil) or active (Act) state of the 121 and 221 ESs in the consecutive switch clones is indicated above the lanes.

no rearrangements seem to be necessary for ES (in)activation. Moreover, ES core promoter sequences are not essential for ES switching control but can be replaced by rRNA promoter sequences (40). This suggests that a higher order of chromatin or nuclear organization may control ES activity. The discovery of specific DNA sequences responsible for attaching chromosomal DNA to the nuclear matrix provides a new paradigm for the functional organization of eukaryotic chromatin (reviewed in reference 28). Topological position effects in the nuclei of higher eukaryotes can modulate the accessibility of regulatory sequences to the transcription machinery (reviewed in reference 7). A model to explain the exclusive activity of an ES might involve nuclear localization by *cis*-acting matrix-attachment sequences upstream of the ES promoter, facilitating the accessibility to the transcription machinery. The suggestion that the active ES might be located at a single site in the nucleus is not new (6), but studies so far have failed to provide evidence of whether this is the mechanism that restricts transcription to a single ES. The cell lines described above, containing a marker gene close to the ES promoter in active and inactive states, may allow us to search for ES upstream *cis*-acting elements involved in transcriptional regulation as well as to localize the ES promoter in a particular region in the nucleus. Differential ES transcription in trypanosomes may provide a model system to investigate the role of the nuclear matrix in determining the transcriptional status of specific chromosomal domains. Studying the organization of chromatin domains and their sites of attachment to the nuclear matrix, in an early-branching eukaryote, may help us to understand more complex phenomena of gene regulation in higher eukaryotes.

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