Biological Variation Among African Trypanosomes: I. Clonal Expression of Virulence Is Not Linked to the Variant Surface Glycoprotein or the Variant Surface Glycoprotein Gene Telomeric Expression Site

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The potential association of variant surface glycoprotein (VSG) gene expression with clonal expression of virulence in African trypanosomes was addressed. Two populations of clonally related trypanosomes, which differ dramatically in virulence for the infected host, but display the same apparent VSG surface coat phenotype, were characterized with respect to the VSG genes expressed as well as the chromosome telomeric expression sites (ES) utilized for VSG gene transcription. The VSG gene sequences expressed by clones LouTat 1 and LouTat 1A of *Trypanosoma brucei rhodesiense* were identical, and gene expression in both clones occurred precisely by the same gene conversion events (duplication and transposition), which generated an expression-linked copy (ELC) of the VSG gene. The ELC was present on the same genomic restriction fragments in both populations and resided in the telomere of a 330-kb chromosome; a single basic copy of the LouTat 1/1A VSG gene, present in all variants of the LouTat 1 serodeme, was located at an internal site of a 1.5-Mb chromosome. Restriction endonuclease mapping of the ES telomere revealed that the VSG ELC of clones LouTat 1 and 1A resides in the same site. Therefore, these findings provide evidence that the VSG gene ES and, potentially, any cotranscribed ES-associated genes do not play a role in the clonal regulation of virulence because trypanosome clones LouTat 1 and 1A, which differ markedly in their virulence properties, both express identical VSG genes from the same chromosome telomeric ES.

Introduction

AFRICAN TRYPANOSOMES UNDERGO cellular differentiation and biological variation throughout their life cycle, in which the organisms change surface coat glycoproteins, alter biochemical pathways, remodel cellular morphology, and exhibit distinct host specificities. These changes are regulated by the expression of discrete genes and proteins. For example, bloodstream trypomastigotes display different surface antigens throughout infection in a mammalian host. This phenotypic variation has as its basis the differential expression of variant surface glycoprotein (VSG) genes, which permits trypanosomes to escape elimination by VSG-specific immune responses and is regulated primarily by a mobile expression body containing RNA polymerase I (Vickerman

and Luckins, 1969; Vickerman, 1978; Donelson, 1987; Cross, 1990; Van-der-Ploeg *et al.*, 1992; Borst and Rudenko, 1994; Borst *et al.*, 1998; Navarro and Gull, 2001; Borst, 2002; Mansfield *et al.*, 2002; Mansfield and Olivier, 2010) and participating proteins such as cohesin (Landeira *et al.*, 2009). Other types of biological variation that impact on parasite infectivity and host range occur in African trypanosomes. One of the most important variations for human infection is trypanosome susceptibility to trypanosome lytic factor (Rifkin, 1978; Duszenko *et al.*, 1985; Hajduk *et al.*, 1989, 1992, 1995; De Greef and Hamers, 1994; Hager *et al.*, 1994; Smith *et al.*, 1995; Hager and Hajduk, 1997) and, potentially, other elements in serum (Raper *et al.*, 1999, 2001; Molina Portela *et al.*, 2000). Differential expression of the SRA gene in *Trypanosoma brucei rhodesiense* appears to determine whether

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organisms are resistant to trypanocidal effects of serum and can infect humans (De Greef and Hamers, 1994; Xong *et al.*, 1998; Milner and Hajduk, 1999). In a related vein, the range of mammalian hosts that *Trypanosoma brucei brucei* can infect may also be influenced by transferrin receptor genes differentially expressed as expression site-associated gene (ESAG) 6 and 7 isoforms from within specific telomeric expression sites (ES) (Steverding *et al.*, 1994; Bitter *et al.*, 1998; Gerrits *et al.*, 2002; Musmann *et al.*, 2003). Thus, the VSG gene ES may play a significant role in trypanosome virulence for specific host species.

The different biological traits noted above can be defined as primary virulence factors for all African trypanosomes because parasites in which the relevant genes have been deleted, mutated, or knocked down are unable to infect or survive in specific mammalian hosts (Leal *et al.*, 2001; Hoek *et al.*, 2002; Wang *et al.*, 2003). A primary focus of this study, however, has been on relative virulence of trypanosomes for their mammalian hosts. It is well known that different isolates, species, subspecies, and laboratory lines of trypanosomes exhibit remarkable variation in pathogenicity and virulence for genetically defined host species (McNeillage and Herbert, 1968; Mulligan, 1970; Clayton, 1978; Barry *et al.*, 1979; Inverso and Mansfield, 1983). The underlying question has been whether such biological differences are immutable characteristics associated with genetically distinct populations of trypanosomes, or whether intracolonial biological variation occurs in trypanosomes, which impacts on the course of infection within an individual animal host.

This question was addressed in an earlier study from our laboratory in which inbred mice of a relatively resistant phenotype (Levine and Mansfield, 1981; DeGee and Mansfield, 1984; De Gee *et al.*, 1985, 1988) were each infected with a single *T. brucei rhodesiense* LouTat 1 trypanosome (Inverso and Mansfield, 1983; Inverso *et al.*, 1988). Several different variant antigenic types (VATs) were isolated from parasitemia peaks at intermediate and late time points during the ensuing infection; these VATs were subcloned and characterized as to VSG phenotype. Three different VATs, which represented antigenically distinct daughter cell populations clonally derived from a single LouTat 1 parental cell in a single mouse, were then used to infect resistant mice; the progression of infection was monitored in comparison with mice infected with the parental cell LouTat 1. The surprising result was that each daughter cell population exhibited a different virulence phenotype compared with the parental clone. For example, LouTat 1 caused death at approximately 62 days postinfection, whereas LouTat 1.3, 1.4, and 1.5 caused death at approximately 44, 30, and 28 days, respectively. These results demonstrated that VATs arising during infection initiated by a single cell expressed virulence phenotypes different from the infecting VAT. In essence, daughter cells arising within a trypanosome population expressed the capacity to transcend host genetic resistance characteristics and render a relatively resistant animal into a more susceptible one. As virulence expression appears to be a progressive phenotype during chronic trypanosome infections, this clonal characteristic recently has been termed a "virulence rheostat" (Mansfield, 2006).

Given that different VATs exhibited differences in virulence, and that other biological traits of trypanosomes may

be controlled by genes with VSG-like characteristics (e.g., SRA) or by ESAGs expressed within an active VSG gene ES (Borst *et al.*, 1998; Hoek *et al.*, 2000, 2002; Hoek and Cross, 2001; Berriman *et al.*, 2002), a logical question was whether the VSG gene, or the ES used by specific VSG genes, in virulent trypanosomes plays a role in relative virulence. Therefore, we initially hypothesized that trypanosome virulence may be determined directly by the VSG molecule itself. Such an hypothesis has an experimental basis because VSG molecules and their substituents have been shown to express or induce biological activities independent of their status as variant antigens (Musoke and Barbet, 1977; Mathias *et al.*, 1990; Lucas *et al.*, 1994; Magez *et al.*, 1997, 1998; Collier *et al.*, 2003; Leppert *et al.*, 2007; Lopez *et al.*, 2008). Alternatively, we proposed that the expression of virulence may be linked to the ES utilized by VSG genes. The rationale was that transcription of certain VSG genes may include cotranscription of hypothetical virulence regulatory genes, if such genes are ESAGs unique to a specific VSG ES.

We subsequently derived and characterized a subclone of LouTat 1, designated clone LouTat 1A, which was shown to be highly virulent for mice (Inverso *et al.*, 1988). Immunological and biochemical examinations of these organisms showed that *T. brucei rhodesiense* LouTat 1 and 1A both express a similar surface coat phenotype (Inverso *et al.*, 1988; Reinitz *et al.*, 1992). These findings suggested, but did not prove, that virulence is not associated with the VSG molecule. In this article, therefore, we address the hypothesis in greater detail, that is, the potential linkage of the expressed VSG gene or its ES with virulence regulation in LouTat 1 and LouTat 1A populations of *T. brucei rhodesiense*. To this end, VSG cDNA was sequenced from both organisms, and VSG probes were used to characterize the mechanism of LouTat 1/1A VSG gene expression, the genomic organization of this VSG gene, and the ES employed in members of the *T. brucei rhodesiense* LouTat 1 serodeme. Using these approaches, we determined that virulence expression in these populations was independent of both the VSG gene and the telomeric site from which the VSG gene is transcribed.

Materials and Methods

Trypanosomes

The isolation of *T. brucei rhodesiense* clone LouTat 1 and the derivation of antigenic variants 1.3, 1.4, and 1.5 have been described previously (Levine and Mansfield, 1981; Inverso and Mansfield, 1983; Inverso *et al.*, 1988). The highly virulent LouTat 1A subclone was obtained by serial subpassage of the less-virulent LouTat 1 clone through immunocompromised mice, as we have reported (Inverso *et al.*, 1988). Trypanosomes used as sources of RNA and DNA were grown by infection in immunosuppressed mice (cyclophosphamide treated; 300 mg/kg) (Smith *et al.*, 1982) with the relevant trypanosome stabilates. Mice were exsanguinated at 3–4 days postinfection and trypanosomes separated from blood by DEAE cellulose column chromatography (Lanham and Godfrey, 1970). Purified parasites were washed two times at 4°C with 10 mM phosphate-buffered saline (pH 8.0) containing 1% glucose. After the final wash, parasites were stored as frozen pellets in liquid nitrogen until use.

cDNA amplification and VSG cloning procedure

Cloning of full-length VSG cDNA was accomplished by methods previously described (Reinitz *et al.*, 1992). Polyadenylated RNA was isolated from frozen trypanosome pellets by conventional procedures of polytron homogenization in 8M guanidinium hydrochloric acid (HCl), centrifugation through 5.7M CsCl cushions, and poly(A+) RNA selection on oligo dT columns. RNA was reverse transcribed using 200 units moloney murine leukemia virus (M-MLV) reverse transcriptase and a 28-bp oligonucleotide primer based on a 3' conserved sequence present in all VSG transcripts (Merritt *et al.*, 1983) (GGGTAACCTACGTGTTAAAA TATATCAG). The following reaction conditions were used: 10 µg polyA RNA, 0.5 µg 28mer, 500 µM dNTPs, 2 µg BSA, 40 units RNasin, 1 µg actinomycin D, and 0.4 µg globin RNA in 10 mM Tris [pH 8.3], 0.15 mM KCl, 0.10 mM DTT, and 3 mM MgCl₂. The 50 µL reaction mixture was incubated at 37°C for 60 min, then 31 µL of 150 mM NaOH was added and incubated at 65°C for 1 h. The pH was adjusted to 8.0 and the DNA precipitated by adding 31 µL of 1 M Tris, 31 µL of 1 M HCl, 15 µL of 3 M NaOAc, and 388 µL ethanol. The chelating agent ethylenediaminetetraacetic acid (EDTA) was not added because of interference with Mg standardization in the subsequent PCRs.

First strand cDNA was resuspended in 60 µL water and 5 µL was added to PCRs containing 3 µM of each dNTP, 5 mM MgCl₂, 1 µM of each oligonucleotide (3' 28mer above, and a 5' 26mer [CGCTATTATTAGAACAGTTTCTGTAC]), derived from the trypanosome mini-exon-derived spliced leader sequence (Cross, 1990; Cross *et al.*, 1990), 2.5 units Amplitaq™ DNA polymerase (Perkin Elmer, Waltham, MA) in 10 mM Tris (pH 8.3), and 50 mM KCl in a total volume of 100 µL. Reaction mixtures were overlaid with 100 µL mineral oil, heated to 97°C for 5 min, slowly cooled to 45°C, and subjected to 30 cycles of amplification (MJR Thermal Cycler) with the following profile: 93°C for 1 min, 66.5°C for 1 min, and 72°C for 3 min. Samples were then concentrated 10-fold by ethanol precipitation and electrophoresed through 1.3% Sea Plaque GTG™ agarose (FMC Corp., Philadelphia, PA), and the full-length 1732-bp cDNA band was isolated by phenol extraction. This DNA was then ethanol precipitated and resuspended in 10 µL of 1× Klenow buffer (50 mM Tris [pH 7.7], 10 mM MgCl₂, 1 mM DTT, 50 µg/mL BSA, 42 mM dNTPs) and 5 units of the Klenow fragment of DNA polymerase I. The reaction was incubated at 30°C for 30 min, 75°C for 10 min, cooled to 4°C prior to adding 4 µL of 5× T4 polynucleotide kinase buffer (350 mM Tris [pH 8.0], 50 mM MgCl₂, 25 mM DTT), 1 µL of 10 mM ATP, 10 units polynucleotide kinase, and 13 µL water to give a final volume of 50 µL. Kinasing reactions were carried out by incubating for 1 h at 37°C and 10 min at 75°C, adjusted to 400 µL and 300 mM sodium acetate, extracted with phenol:chloroform:isoamyl alcohol (25:24:1), and ethanol precipitated. This was resuspended in 20 µL ligation mix containing 10 ng *Sma*I-restricted pBluescript II KS +™ (Stratagene), 8 units T4 DNA ligase (no. 600011; Stratagene), 500 µM ATP, 50 mM Tris (pH 8.0), 7 mM MgCl₂, and 1 mM DTT and was incubated overnight at 13°C. An aliquot of the ligation reaction (0.3 µL) was diluted 10-fold and used to transform competent *Escherichia coli* (strain DH5α F'), which were then selected by growth on Luria broth agar plates containing ampicillin

(100 µg/mL), isopropyl beta-D-thiogalactopyranoside (IPTG) (0.5 mM), and X-gal (80 µg/mL). White colonies were selected and grown overnight in Luria broth with ampicillin. Recombinant colonies harboring the 1732-bp full-length VSG cDNA were identified using an alkaline mini prep plasmid isolation procedure (Sambrook *et al.* 1989), followed by *Eco*RI and *Bam*HI double digestion and electrophoresis through 0.8% agarose.

DNA sequencing strategy

The VSG cDNA was sequenced primarily as a double-stranded (ds) template as originally described by Sanger (Sanger *et al.*, 1977; Sambrook *et al.*, 1989); however, certain regions were sequenced using single-stranded (ss) DNA. By a combination of these methods, both strands were completely sequenced. All reactions used the Sequenase™ enzyme and conditions were as described by the manufacturer (U.S. Biochemical, Cleveland, OH). A series of eight oligonucleotides (16–22 bp) were synthesized on an Applied Biosystems (Foster City, CA) 380B DNA synthesizer for use as primers in combination with various templates (as described below). Four oligonucleotides primed each strand. Their sequences and corresponding VSG gene positions (Fig. 1) were as follows:

- (1) AACAGCTATGACCATG, plasmid polylinker;
- (2) GGACGCAAGTCTACCTAGCAGC, 336–357;
- (3) TAAACATAGCGCACGAGACC, 519–536;
- (4) GACAGCAGAGCTAACAAACAG, 991–1010;

and on the complementary strand,

- (5) GTAAACGAACGGCCAGT, plasmid polylinker;
- (6) GTGTAACAGGCACACCAC, 1469–1452;
- (7) CTGCAAGTGTCTACCGATGCC, 1341–1321;
- (8) GTGGTCTGCGCTTCTGCTAG, 486–467.

The ds templates included cloned *Exo*III deletions and *Bss*HIII restriction fragments of the VSG cDNA, whereas the ss sequences were linear fragments obtained using selected pairs of the primers in asymmetric PCRs. The cDNA was found to possess a single *Bss*HIII site and the two fragments (742 and 990 bp) were subcloned separately into pBluescript and sequenced using the plasmid polylinker primers 1 and 5. The *Exo*III deletions were performed as described by the manufacturer (Stratagene). Briefly, susceptible 5' and protected 3' overhangs were created by digestion with *Eco*RI and *Kpn*I, respectively. Linearized templates were digested with 200 units *Exo*III and aliquots were removed every 30 s, frozen, and subsequently treated with S1 nuclease and Klenow prior to religation and transformation back into *E. coli* DH5α. Such techniques resulted in the isolation of two useful subclones representing nucleotides 649–1732 and 1180–1732, which were sequenced in both directions using primers 1 and 5. Generation of ss templates was accomplished using oligonucleotides 1 and 7 (1:50) with the *Exo*III deletion fragment (649–1732) as a template in the asymmetric PCR under the following conditions: 10 ng DNA, 20 nM oligo 1, 1 mM oligo 7, 5 mM MgCl₂, 10 mM Tris (pH 8.3), 50 mM KCl, 2.5 units Ampitaq, and 300 µM dNTPs. The 100 µL reactions were subjected to 30 cycles of 95°C for 1 min, 31°C for 30 s, and ramped over 90 s to 72°C for 1 min. Following amplification, ss cDNA templates were precipitated,

55 TAT TAT TAG AAC AGT TTC TGT ACT ATA TTG CCA CGG CAG CTC CAC TGT AAG ACC
 109 GGC TTT TTT TCT ATT TCG AGA GTC AGA TAA ACC GGT AGA GCA CAA AAA ACC AAC
 163 GAC AAG ATG CAA CCC AAT GGC GCA CAG CAC TTT ACT ATA CCG TTA ACA GCG GCG
 217 M Q P N G A Q H F T I P L T A A
 TTC CTT TTA CTC GCG CAC GAA ACG GCG GCT ACA GCA AAC CAC GGA CTC AAA CGG
 271 F L L L A H E T A A T A N H G L K R
 CAA ACA TGG GAG CCG GTG TGT CAA CTA AGT GAA GAA CTA GAC GGC GTC ACA CCA
 325 Q T W E P V C Q L S E E L D G V T P
 GGA ACG CTA GAG ACG ACC GTA AAC ATA ATG CAA ACA GCA ATA AAC ATG CAA AAG
 379 G T L E T T V N I M Q T A I N M Q K
 GAA GGA CTC AGG ACG CAA GTC TAC CTA GCA GCA AAG CCC AAT GAC CCC GAC ACC
 433 E G L R T Q V Y L A A K P N D P D T
 GAC AAA CTT GCA GCC CTA GGC GCG TAC CAA GCA ACA GCG GCG GGC GTG ATC GAA
 487 D K L A A L G A Y Q A T A A G V I E
 AAC AAT CTA AAG TCG ACA GGA ATT GAA AAA GCA CTA GCA GAA GCG CAG ACC ACA
 541 N N L K S T G I E K A L A E A Q T T
 AGC TAC CTC AAA GGC AAA ATT GAC GAA TGG CTA AAC ATA GCG CAC GAG ACC AAG
 595 S Y L K G K I D E W L N I A H E T K
 GGC GCC TCG CAC GGG TGC TTG ACG GTC GCA GCG GGG ACG ACA CCG GTC AAG AAG
 649 G A S H G C L T V A A G T T P V K K
 GTC GGC AAT ACT ATC GAC GGC GTC AAG TGC CCA CTA ACC CTC ACA AAA TTG GCG
 703 V G N T I D G V K C P L T L T K L A
 AGA AAA GCA AGG ACA CCG AAC AAG CTA ACG GCC GGC GGA TTC CAA GGG CTC AGG
 757 R K A R T P N K L T A G G F Q G L R
 GAG ACG ACA GCC GAC GGC AGG CGA CCA CCA AGA CAA CAG CGC GCA ATG CAG GCT
 811 E T T A D G R R P P R Q Q R A M Q A
 GAT GCA CTA CGA CAG TGC CAA CGA ATA GTA AAA ACA GAC GGC AAC ACC GAA GAC
 865 D A L R Q C Q R I V K T D G N T E D
 AGC AAG GTA ACC TAC GTA GAC GGC TAC ATA CAA GTA ACC GCA GCG GCA AAC TCA
 919 S K V T Y V D G Y I Q V T A A A N S
 AAA GCA GTT CTA GCG TTC GGA GGA GCT GAC GCG AAA CCA CAG ACC AGC AAT CAC
 973 K A V L A F G G A D A K P Q T S N H
 CCA GCT TGG GCA GCT GCA TAC GAG GCA GTT AAA GCA GCG CCG CTC GAA ACG GAA
 1027 P A W A A A Y E A V K A A P L E T E
 CCG GCC TAC AAA AAC AAG ACA GCA GAG CTA ACA ACA GAC GAG GAA GCC ACG ACT
 1081 P A Y K N K T A E L T T D E E A T T
 TTG TTC GCT TTA ATC GGC GCG GCA GAC AAG CCA AGG GAA GGA CAA GGC ATT GAA
 1135 L F A L I G A A D K P R E G Q G I E
 ACC TAC AAA AGA CAG CTG TTC TCG ACA ACC CCT GGC AAG GAA ATA GGC CGG CTA
 1189 T Y K R Q L F S T T P G K E I G R L
 ATG GCA AAA GTA GCG ACC TTC AAG CTG CCA CAC AAA TTT GGG GAC ACA AAA GAA
 1243 M A K V A T F K L P H K F G D T K E
 GGC ACC CTA CTA GGG GAC ATC ACT GAA CTA AGC GTA CTG GCA GCA CTA CTA GCG

	G	T	L	L	G	D	I	T	E	L	S	V	L	A	A	L	L	A
1297	GAC	TTA	CAG	CTA	AAA	AAT	GCA	CAA	AAT	CTA	AAA	ACC	TTA	AAC	GAC	AAG	GTA	GAA
	D	L	Q	L	K	N	A	Q	N	L	K	T	L	N	D	K	V	E
1351	GAA	GCT	TCG	AAG	AAC	AAT	CAA	AAG	GCA	TCG	GTA	GAC	ACT	TGC	AGT	AAA	ACA	GAA
	E	A	S	K	N	N	Q	K	A	S	V	D	T	C	S	K	T	E
1405	GAT	AAA	AAT	AAC	TGC	AAC	AGC	AAG	CCT	TTC	TGC	AGC	TAT	AAT	GAA	AGC	GCA	GCT
	D	K	N	N	C	N	S	K	P	F	C	S	Y	N	E	S	A	A
1459	GAG	GGT	GAC	AAA	AAG	TGC	CAC	TTT	AAT	GAA	ACA	AAA	GCC	TCA	AAA	AGT	GGT	GTG
	E	G	D	K	K	C	H	F	N	E	T	K	A	S	K	S	G	V
1513	CCT	GTA	ACA	CAA	ACT	CAA	ACT	GCA	GGA	GCC	GAC	ACG	ACA	GCA	GAA	AAG	TGC	AAA
	P	V	T	Q	T	Q	T	A	G	A	D	T	T	A	E	K	C	K
1567	GGC	AAA	GGA	GAG	AAA	GAT	TGC	AAA	TCT	CCG	GAT	TGC	AAA	TGG	GAG	GGC	GGA	ACT
	G	K	G	E	K	D	C	K	S	P	D	C	K	W	E	G	G	T
1621	TGC	AAA	GAT	TCC	TCT	ATT	CTA	GCA	AAC	AAA	CAA	TTT	GCC	CTC	AGC	GTG	GCT	TCT
	C	K	D	S	S	I	L	A	N	K	Q	F	A	L	S	V	A	S
1675	GCC	GCA	TTT	GTG	GCC	TTG	CTT	TTC	TAA	AAC	AAA	TAT	ATC	CCC	CCT	CAA	TTT	TCC
	A	A	F	V	A	L	L	F										
1729	TTA	AAA	AAT	TCT	TGC	TAC	TTG	AAA	ACT	TTC	TGA	TAT	ATT	TTA	ACA	CGT	AAG	TTA
	CCC																	

FIG. 1. (Continued).

resuspended in water, passed over CL-6B Sepharose™ spin columns, and sequenced as described (U.S. Biochemical) with primer 7.

Preparation of LouTat 1/1A VSG cDNA probes

A 395-bp VSG cDNA fragment, contained in the recombinant pGEM-3Z plasmid pTbrVSG^A-4 (Reinitz *et al.*, 1992), was isolated by double digestion with *Bam*HI and *Eco*RI, purified from 1.2% low-melting-temperature agarose gels, and digested with *Hpa*II to generate a 187-bp sequence representing an internal, variant specific sequence within the cDNA (Fig. 1). This fragment was further purified from 1.5% low-melting-temperature agarose gels and used as a probe for all subsequent northern blot analyses. The probe used in Southern blots was a 484-bp PCR fragment, which was generated using oligonucleotides annealing to nucleotides 336–357 (described in the sequencing strategy) and 800–819 (ACCTTGCTGTCTTCGGTGTT) of the VSG DNA sequence shown in Figure 1. The probe was purified from the PCR reactants by EtOH precipitation. ³²P-labeled cDNA probes were prepared to high specific activity (>1×10⁸ cpm/μg) by a standard nick translation protocol (Sambrook *et al.*, 1989) or by direct incorporation of α-³²P-dCTP in the PCR amplification reaction.

Northern blot analysis

Total RNA (5 μg/lane) was fractionated in 1.5% agarose gels containing 2.2 M formaldehyde (Lehrach *et al.*, 1977; Sambrook *et al.*, 1989). Following electrophoresis, gels were stained in ethidium bromide (0.5 μg/mL water), destained in dH₂O, soaked in 10× saline/sodium citrate (SSC) for 30–45 min to remove formaldehyde, and transferred to GeneScreen Plus (DuPont, Boston, MA) via capillary blotting in 10× SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0) for 16–20 h. After transfer was complete, membranes were baked at 80°C for 1.5 h. Hybridization and posthybridization washes were performed as stated (see figure legends).

Southern blot analysis

Genomic DNA was prepared from frozen cell pellets as described elsewhere (Milhausen *et al.*, 1983). Restriction enzyme digests of genomic DNA were performed by addition of 25 units restriction endonuclease to 10 μg DNA in 25 μL of 1× buffer provided by the supplier (BRL, Gaithersburg, MD). Digestion was carried out for 4 h at 37°C. The entire reaction mixture was loaded into a well of an agarose gel for electrophoresis in 1× TBE (tris/borate/EDTA buffer, pH 8.0) (89 mM Tris, 89 mM boric acid, 2 mM EDTA). Fractionated

FIG. 1. Complete nucleotide and deduced amino acid sequence of the variant surface glycoprotein (VSG) cDNA expressed by *Trypanosoma brucei rhodesiense* clones LouTat 1 and 1A. The precursor VSG molecule is 510 amino acids, which is processed to 461 residues by cleavage of a 26-amino acid hydrophobic leader and a 23-amino acid C-terminal extension (underlined). The 187-bp cDNA fragment derived from pTbr-VSG1a-4 as a probe for northern and selected Southern analyses is denoted by dotted underline. The 484-bp fragment generated by PCR for use as a probe in Southern analyses is denoted by solid underline within the DNA sequence. The accession number for the LouTat 1 VSG sequence is X56643 (Reinitz *et al.*, 1992).

DNA was visualized by ethidium bromide staining (0.5 µg/mL in water). Prior to transfer the DNA was partially depurinated (0.25 M HCl for 15 min) and denatured (30 min in 0.4 M NaOH–0.6 M NaCl). Gels were neutralized by soaking in 0.5 M Tris (pH 7.5) and 1.5 M NaCl for at least 30 min prior to transfer, and DNA was transferred onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH).

Filters containing DNA were prehybridized for 1 h at 42°C in buffer containing 5×SSPE (saline/sodium phosphate/EDTA buffer, 0.74 M NaCl, 0.05 M NaH₂PO₄·H₂O, 5 mM EDTA), 10× Denhardt's solution, 0.5% sodium dodecyl sulphate (SDS), and 50% formamide. Heat-denatured, nick-translated probe was added to the hybridization buffer at 10⁷ cpm/mL with 100 µg/mL denatured salmon sperm DNA. The blots were hybridized overnight at 55°C. Posthybridization washes were done at 65°C in 1× SSC and 0.1% SDS (2×, 30 min) followed by two washes at 65°C for 30 min each in 0.1× SSC and 0.01% SDS.

Bal31 exonuclease digestion

Genomic DNA was isolated as described above for Southern blot analysis. *Bal31* exonuclease digestions were performed by incubation of 1 unit enzyme/20 µg genomic DNA in digestion buffer (12 mM CaCl₂, 12 mM MgCl₂, 0.2 M NaCl, 20 mM Tris HCl [pH 8.0], 1 mM EDTA) at 30°C for different periods of time. Reactions were terminated by addition of EDTA (pH 8.0) to a final concentration of 20 mM. Samples were deproteinized immediately by phenol extraction and precipitated with ethanol. DNA recovered from the ethanol precipitation was suspended in the appropriate buffer and digested with excess *XmnI* (10 units/µg DNA) for 4 h at 37°C. The entire reaction mixture was loaded into a well of a 1.2% agarose gel and electrophoresed, transferred to nitrocellulose, and hybridized as described earlier for Southern blot analysis.

Pulsed-field gradient gel electrophoresis

Samples were prepared by embedding live trypanosomes in agarose blocks and subsequently lysing the embedded cells in proteinase K and SDS as described by Scholler *et al.* (1986). The pulsed-field gel electrophoresis (PFGE) approach was adapted from the methods of Schwartz and Cantor (1984). Gels containing 1% high-strength agarose (BioRad, Hercules, CA) in 0.5×TBE were cast in 18 cm×18 cm glass supports; agarose blocks containing approximately 3.8×10⁷ cells were loaded into the wells. Gels were subsequently run in a CHEF II apparatus (BioRad) in 0.5×TBE running buffer at 200 V and 12°C with equal alternating pulse times of 60 s for approximately 15 h and then for 6 h with a 90 s switch time. Following electrophoresis, the gels were stained with ethidium bromide to visualize chromosomal bands. The gels were transferred and hybridized as described for Southern blot analysis.

Results

Trypanosome clones LouTat 1 and 1A express the same VSG gene

Full-length cDNAs representing the VSG genes expressed in clones LouTat 1 and 1A were derived from poly(A⁺) RNA of the respective organisms by selective PCR amplification,

as previously described (Reinitz *et al.*, 1992). Briefly, a 26-bp oligonucleotide primer corresponding to the 5' spliced leader sequence (mini-exon-derived sequence) of trypanosome mRNAs and a 28-bp oligonucleotide primer corresponding to a conserved 3' sequence common to trypanosome VSG transcripts were used to amplify VSG cDNA. The resultant ds cDNA was treated to repair 5' and 3' overhangs, and a phosphate was added prior to blunt-ended ligation into a suitable vector. The full-length cDNA encoding LouTat 1 and LouTat 1A VSG molecules was sequenced in both directions, and the nucleotide sequences were shown to be identical for both clones (see Fig. 1 for nucleotide and deduced amino acid sequences). The LouTat 1/1A VSG cDNA encodes a precursor protein of 510 amino acids with a 26-residue N-terminal hydrophobic signal sequence and a C-terminal extension of 23 amino acids. Based on earlier partial amino acid sequence analyses of the VSG proteins from LouTat 1 and 1A (Inverso *et al.*, 1988), we predict that the precursor molecule is processed to a mature VSG of 461 amino acids, beginning at the N-terminus with threonine 27 and ending with conserved residues common to type I VSG molecules at the C-terminus. The LouTat 1/1A VSG molecule also contains conserved residues throughout the molecule, which are common to VSGs as a family (Rice-Ficht *et al.*, 1981; Reinitz *et al.*, 1992; Blum *et al.*, 1993).

To make a variant-specific probe, a recombinant plasmid, pTbrVSG^A-4, was generated, which contains a VSG cDNA fragment cloned into the *Bam*HI-*Eco*RI site of pGEM3Z. Probes for northern blot analyses were generated by digestion of the *Bam*HI-*Eco*RI purified insert with *Hpa*II to remove the 3' terminal 208-nucleotide sequence that contained conserved sequences common to VSG genes. The variant specific 5' 187-bp fragment isolated from this digestion was labeled for use in all subsequent northern blot and selected Southern blot analyses. An additional probe used in some Southern blots was a 484-bp PCR fragment that was generated by direct PCR using oligonucleotides annealing to nucleotides 336–358 (GGACGCAAGTCTACCTAGCAGC) and 800–819 (ACCTTGCTGTCTTCGGTGT) of the VSG DNA sequence shown in Figure 1.

To confirm that the VSG probe derived from pTbr-VSG^A-4 was variant specific, Northern blot analysis was performed. Total RNA from trypanosome populations was size-fractionated by agarose gel electrophoresis, transferred to nylon membranes, and hybridized to the labeled 187-bp VSG cDNA fragment. As shown in Figure 2, the probe hybridized equivalently to RNA from variants LouTat 1 and 1A but not to RNA from the heterologous variant LouTat 1.5 or from *T. brucei* procyclic forms, which do not express VSG genes. Additionally, the cDNA probe did not hybridize to RNA from other heterologous variants of the LouTat 1 serodeme such as LouTat 1.3 or 1.4 (not shown). The size of the RNA species that hybridized to the VSG cDNA probe was determined to be approximately 1.7 kb, which was a transcript size compatible with the predicted VSG molecule size. A control probe (NVS1a-12) was rehybridized to RNA from all populations, as shown in Figure 2. NVS1a-12 is a cDNA selected from a LouTat 1A cDNA library, which hybridized to an RNA species similar in size and abundance to that recognized by the VSG-specific cDNA probe; it does not display differential hybridization patterns among variants and, as such, represents a nonvariant specific sequence

FIG. 2. Variant specificity of the LouTat 1 VSG cDNA probe as shown by northern analysis. Five microliters of total RNA from LouTat clones 1, 1A, and 1.5 and *T. brucei* procyclic forms (Pro) were size fractionated in 1.5% formaldehyde agarose gels, transferred to nylon membranes by capillary blotting, and baked at 80°C for 1.5 h. Filters were prehybridized at 42°C for 1 h in buffer containing 50% formamide, 10% dextran sulfate, 1 M NaCl, and 1% SDS. To the hybridization buffer was added 100 µg/mL denatured salmon sperm DNA and 1×10⁵ cpm/mL of the ³²P-labeled 187-bp VSG1a-4 (LouTat 1 VSG) cDNA fragment (A), or the ³²P-labeled control nonvariant specific NVS1a-12 cDNA insert (B) after washing, stripping, and rehybridization of the membrane. Filters were hybridized at 42°C overnight. Post-hybridization washes were performed 2× (30 min) in 2× SSC and 1% SDS at 60°C and 2× (30 min) in 0.1× SSC at room temperature. SDS, sodium dodecyl sulphate; SSC, saline/sodium citrate.

(a similar pattern of control hybridization occurs with conventional probes such as α/β tubulin, as we have shown) (Reinitz *et al.*, 1992; Schopf and Mansfield, 1998; Demick *et al.*, 2010). Overall, therefore, these results support previous immunological and biochemical evidence that LouTat 1 and 1A express the same VSG surface coat, and that this coat is distinct from VSGs expressed by other variants of the LouTat 1 serodeme (Inverso *et al.*, 1988; Reinitz *et al.*, 1992; Schopf *et al.*, 1998).

LouTat 1 and 1A express their VSG gene by the same mechanism

The genomic organization of the VSG gene was examined by Southern blot analyses with the 187- and/or 484-bp VSG cDNA probes noted earlier. Hybridization of either probe to genomic DNA from clones LouTat 1, 1A, 1.3, 1.4, and 1.5 restricted by several enzymes is shown in Figure 3. The probes hybridized to two restriction fragments in DNA from expressor population LouTat 1. The smaller band of clone LouTat 1 was approximately 8.7 kb and the larger band was approximately 22 kb in size. Similarly, the probes hybridized

FIG. 3. Southern blot analysis of LouTat 1/1A VSG gene organization in different trypanosome populations shows that there are two gene copies in the expressor cells. Ten micrograms of genomic DNA from LouTat variants 1, 1A, 1.3, 1.4, and 1.5 were digested in 25 µL restriction digestion buffer with 25 units *SacI* for 4 h at 37°C. Digested DNA was size-fractionated in a 0.55% agarose gel and transferred to nitrocellulose membrane. The blots were prehybridized in a buffer containing 10× Denhardt's solution and 0.5% SDS, and 5×SSPE and 0.5% SDS at 42°C for 1 h. Denatured salmon sperm DNA (100 µg/mL) and ³²P-labeled 484-bp LouTat 1 VSG PCR-derived cDNA fragment (10⁷ cpm/mL hybridization buffer) were added and the blots incubated at 55°C overnight. Posthybridization washes were performed at 65°C in 1× SSC–0.1% SDS (2×, 30 min), followed by two high-stringency washes at 65°C for 30 min each in 0.1× SSC–0.01% SDS.

to two fragments in genomic DNA from clone LouTat 1A; these were a large fragment of approximately 23 kb and a smaller 7.6 kb band. Nonexpressor populations LouTat 1.4 and 1.5 each contained a single restriction fragment, 3 and 12.2 kb, respectively, which hybridized to these probes. The nonexpressor variant LouTat 1.3 also contained a single band of 10.3 kb, although a weakly hybridizing fragment was apparent directly below this band at approximately 9.8 kb. Because *SacI* does not cut within the cloned VSG cDNA, the bands detected in the Southern blot described earlier represent discrete copies of the VSG gene.

Differences in restriction fragment size of the VSG gene copies in expressor clones LouTat 1 and 1A were observed. This is not unexpected because actively expressed VSG genes located in telomeric ES often display size variability due to differences in subtelomeric sequences. However, we also observed differences in restriction fragment size among basic copy (BC) fragments of expressor and nonexpressor variants, suggesting that the BC gene may be located near a telomere. Therefore, we examined for a potential association of both VSG copies in the expressor populations with chromosome telomeres. Genomic DNA from several variants was digested with *Bal31* exonuclease, followed by digestion with selected restriction endonucleases. Shown in Figure 4 are the

FIG. 4. Relative *Bal31* exonuclease sensitivity of the VSG expression-linked copy (ELC) gene in LouTat 1/1A demonstrates that the ELC but not the basic copy (BC) gene in expressor trypanosomes is located near a chromosome telomere. Twenty micrograms of genomic DNA from LouTat 1 and 1A was digested with 1 unit *Bal31* exonuclease at 30°C for different lengths of time. Samples were deproteinized, precipitated with ethanol, and digested with *XmnI*. DNA was size-fractionated in 0.55% agarose gels, transferred to nitrocellulose, hybridized with a ³²P-labeled LouTat 1 VSG cDNA probe, and washed as described in Figure 3.

representative results of *Bal31* digestion studies with DNA from LouTat 1 and 1A. Genomic DNA from each variant was digested with *Bal31* for different periods of time, purified, and subsequently digested with *XmnI*. DNA was size fractionated in agarose gels, transferred to nitrocellulose, and hybridized with the 484-bp VSG cDNA probe described earlier. Only the lower bands of expressor variants LouTat 1 and 1A displayed relative sensitivity to *Bal31* exonuclease digestion (Fig. 4). The upper VSG fragment, presumably the BC, did not display similar sensitivity to *Bal31* exonuclease digestion; this was similar to other chromosome interior genes such as α/β tubulin, which were not affected by *Bal31* treatment (not shown). Thus, these results suggest that the expression-linked copy (ELC) but not BC of the LouTat 1/1A VSG gene is telomere associated.

Trypanosome clones LouTat 1 and 1A express their active VSG gene from the same chromosome telomeric site

We next determined if the expressed VSG ELC in LouTat 1 and 1A was on the same or different restriction fragments. Because of restriction fragment size variability of the VSG bands seen in expressor variants LouTat 1 and 1A as shown in Figure 3, it was difficult to determine the identity of ELC or BC by digestion with *SacI* or several other restriction enzymes. Therefore, to eliminate restriction fragment size variability related to telomere end sequence variability, we digested genomic DNA from all variants with *XmnI*, which, based on nucleotide sequence analysis, cuts at a single site within the 3'-region of VSG cDNA near the polyadenylation site. Genomic DNA from LouTat clones 1, 1A, and 1.5 was digested with *XmnI*, size fractionated by agarose gel electrophoresis, transferred to nitrocellulose, and hybridized with the 484-bp VSG probe (Fig. 5). Two bands were ap-

FIG. 5. Identification of ELC and BC fragments. Ten micrograms of genomic DNA from LouTat clones 1, 1A, and 1.5 were digested with *XmnI*, fractionated in a 0.55% agarose gel, transferred to nitrocellulose, hybridized with a ³²P-labeled LouTat 1 VSG cDNA probe, and washed as detailed in Figure 3.

parent in lanes containing the LouTat 1 or 1A DNA, representing a 2.4-kb ELC and 4.5-kb BC fragment. A single 4.5-kb BC band was apparent in *XmnI* digests from the nonexpressor variant LouTat 1.5. The presence of a single 4.5 kb band in Southern blots with *XmnI*-restricted genomic DNA from LouTat 1.3 (data not shown) suggests that the smaller weak band observed in Southern blots of *SacI*-digested DNA (Fig. 3) was due to telomere heterogeneity in the clonal population and does not represent a lingering ELC. The size of the ELC band in clones LouTat 1 and 1A is the same, and it therefore appears that the size variability previously observed in *SacI* restriction digests was also due to heterogeneity in telomere size between the two populations because *XmnI* digestion removes VSG subtelomeric sequences. Thus, these results suggest that the VSG ELC of LouTat 1 and 1A is present on the same restriction fragment and, potentially, within the same telomeric ES.

To determine whether the VSG ELC of LouTat 1 and 1A is expressed from the same chromosome, PFGE followed by Southern blot analysis was performed. LouTat 1A, the highly virulent trypanosome population, displays a chromosomal pattern identical to the VSG homologous but less virulent LouTat 1 and the heterologous variant LouTat 1.5. The chromosomal patterns are shown in ethidium bromide-stained gels (Fig. 6). When DNA in this gel was transferred to nitrocellulose and hybridized with a VSG cDNA probe, it was observed that the VSG BC gene, present in all VATs, is located on a chromosome of approximately 1.5 Mb (Fig. 6). The ELC of LouTat 1 and 1A was located on an intermediate-sized chromosome of approximately 330 kb, which appears to be the same in both populations. Thus, results of both *Bal31* digestion and PFGE analysis indicate that the ELC of the LouTat 1 and 1A VSG gene is expressed from a telomeric ES located on an intermediate-sized chromosome, whereas the BC of the VSG gene resides in the interior of a larger chromosome.

FIG. 6. Chromosomal location of LouTat 1/1A VSG ELC genes. Blocks containing approximately 3.8×10^7 trypanosomes from LouTat 1, 1A, and 1.5 were embedded in a 1% high-strength agarose gel and electrophoresed in $0.5 \times$ TBE for 15 h at 200 V and 12°C with equal alternating pulses of 60 s, followed by 6 h with a 90 s switch time. The gel was transferred to a nylon membrane, hybridized with a ^{32}P -labeled LouTat 1 VSG cDNA probe, and washed as described in Figure 3. Bands seen at the very top of the blot are from wells loaded with trypanosomes embedded in agarose blocks.

The foregoing restriction enzyme digestion studies and PFGE analysis demonstrated that the VSG gene ELC of LouTat 1 and 1A resides on a similar sized chromosome. However, more definitive evidence that both variants utilize the same ES is presented in Figure 7. Restriction mapping analysis of LouTat 1 and 1A ELC and BC genes was performed by standard restriction endonuclease digestion analyses combined with Southern blot analyses using the 5' VSG 484-bp cDNA probe. Results presented here demonstrate that the restriction map of the VSG gene and sequences 5' to the VSG gene are the same for LouTat 1 and 1A ELC. The upstream barren region of the ES, devoid of restriction sites, was determined to be approximately 10–15 kb. Additionally, the restriction map 5' to the upstream barren region was determined by digestion studies using eight different restriction endonucleases that cut in this region. No differences in restriction sites in this region were apparent between trypanosomes LouTat 1 and 1A. The BC restriction map of LouTat 1A, which is the same for LouTat 1 and 1.5, differs from the ELC map in regions immediately upstream of the *Xmn*I site 5' to the VSG gene. The barren region of the BC gene appears to be further upstream than the barren region of the ELC, and it could not be mapped accurately. Also, the BC was distal to a telomere and its position in relation to the telomere could not be determined. Overall, therefore,

these studies demonstrate that LouTat 1 and 1A utilize the same telomeric ES for transcription of their VSG genes.

Discussion

The central focus of this study was upon the potential linkage of virulence to the telomeric ES for VSG genes in African trypanosomes. Specifically, we have asked whether two clonally derived, but biologically very different, trypanosomes express the same VSG gene or utilize the same VSG gene chromosomal ES. To address these questions we have cloned and characterized the cDNA sequences specific for VSG gene transcripts present in genetically related high- and low-virulence trypanosome populations and have also characterized the chromosome telomeric ES used for VSG gene expression in both.

The organisms *T. brucei rhodesiense* LouTat 1 and LouTat 1A express low and high virulence phenotypes, respectively. In the LouTat serodeme (VATs derived from LouTat 1), infection of animals with relatively low-virulence trypanosomes such as LouTat 1 gives rise to VATs (daughter cell populations displaying different VSG surface coat phenotypes) that express progressively higher levels of virulence as infection progresses (Inverso and Mansfield, 1983). This observation was exploited to derive a series of highly virulent subclones of LouTat 1 by rapid subpassage through immunocompromised mice; one of several subclones selected for further study was clone LouTat 1A, which caused death of mice in 3–4 days compared with 60–70 days for the parent clone from which it was derived (Inverso *et al.*, 1988). Preliminary observations suggested that both LouTat 1 and 1A display similar surface coats, as seen from mAb- and pAb-binding tests and from partial protein sequence analysis (Inverso *et al.*, 1988). In this study, nucleotide sequence analysis revealed that VSG gene cDNAs from LouTat 1 and 1A are precisely identical for both organisms and encode a molecule with conserved C terminal sequences common to type 1 VSG gene isotypes, as well as other conserved VSG-associated residues (Rice-Ficht *et al.*, 1981; Reinitz *et al.*, 1992; Blum *et al.*, 1993). Variant specificity of the cDNA sequence was confirmed by northern blot hybridization to RNA from the homologous VATs LouTat 1 and 1A but not to RNA from the heterologous VAT LouTat 1.5 or to RNA from procyclic forms that do not express VSG genes. These results confirmed preliminary immunological and biochemical evidence that *T. brucei rhodesiense* clones LouTat 1 and 1A express the same VSG surface coat phenotype and VSG gene.

Genomic organization of the LouTat 1 VSG gene in the LouTat 1 serodeme was subsequently examined. In Southern blot analysis, VSG expressor populations LouTat 1 and 1A each contained two copies of the VSG gene, whereas non-expressor populations contained a single copy of the gene. Because the restriction enzymes used did not cut within the VSG sequence, these restriction fragments represented discrete copies of the VSG gene. The additional band present in Southern blots of genomic DNA from expressor LouTat 1 and 1A populations but not heterologous VATs suggests that the VSG gene is transcribed in both populations following a gene duplication and transposition event that generates an ELC.

Telomere linkage of VSG gene ELCs has been well documented and it is known that VSG genes are expressed only from telomeric ES in bloodstream forms (Borst and Cross,

The significance of the present findings stems from our observation that *T. brucei rhodesiense* clones LouTat 1 and 1A differ dramatically in their virulence expression for mice and other species (Inverso *et al.*, 1988) including nonhuman primates (Ndung'u and Mansfield, unpublished data). Because the VSG molecule plays a critical role in antigenic variation and because of the ability of the trypanosome to evade host immune responses during infection, we previously asked whether there was an association between expressed VSG molecules and virulence levels in the *T. brucei rhodesiense* LouTat 1 serodeme. Our preliminary observations, as well as unrelated studies by others (Musoke and Barbet, 1977; Mathias *et al.*, 1990; Lucas *et al.*, 1994; Magez *et al.*, 1997, 1998), suggested that the VSG molecule could play a direct role in the relative expression of trypanosome virulence expression for a host. This suggestion was derived from our observation that all daughter cell trypanosome populations that expressed elevated virulence in an infected host were derived from a less-virulent trypanosome parent cell population that expressed a surface coat distinct from each of the virulent populations (Inverso and Mansfield, 1983). However, comparative examinations within the subsequently developed model system of *T. brucei rhodesiense* clones LouTat 1 and 1A that express the same VSG surface coat (based on earlier limited biochemical and immunological analyses and the results of this work) but differ dramatically in their virulence for mice, indicates that the

VSG molecule itself is not associated with changes in relative virulence.

Also in this study, we have investigated further the potential relationship between VSG gene ES and virulence expression. As it is known that different ESAGs are coordinately transcribed with VSG genes as part of the polycistronic transcript generated within specific ES, and that ESAGs may regulate discrete aspects of trypanosome biology (Hoek and Cross, 2001; Berriman *et al.*, 2002; Hoek *et al.*, 2002), we searched for potential differences in the ES used by the VSG gene ELC in LouTat 1 compared with LouTat 1A. If the ES used by the VSG gene in two populations that differ in virulence was different, this finding would have provided a basis for further examination of the role of LouTat 1A ESAGs and their products in regulation of virulence. However, from the studies presented here it is apparent not only that the VSG molecules themselves are not virulence factors but also that the VSG gene and the VSG gene ES are not linked to virulence expression in the LouTat 1 serodeme of *T. brucei rhodesiense*. This conclusion is underscored by this work from our laboratory in which VATs derived from the highly virulent LouTat 1A expressed different VSG phenotypes and genes, as well as utilized different ES, but did not alter their virulence phenotypes (Inverso *et al.*, 1988; Schopf and Mansfield, 1998; Mansfield and Paulnock, 2005; Mansfield, 2006). Overall, therefore, we predict that the virulence phenotype of African trypanosomes, as well as clonal changes in relative virulence for a host species, are regulated by genes mapping outside the VSG gene ES and are expressed independently of VSG gene expression.

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Disclosure Statement

No competing financial interests exist.

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