INTRODUCTION

The protozoan parasite Trypanosoma brucei exists as three subspecies: T. b. brucei, T. b. rhodesiense, and T. b. gambiense, which differ in their geographical distribution and host specificity. T. b. brucei causes nagana in cattle and other livestock but is not pathogenic to humans because it is lysed by normal human serum (NHS) in contrast, T. b. rhodesiense and T. b. gambiense are resistant to NHS enabling them to infect humans. T. b. gambiense causes human African trypanosomiasis (HAT) in West and Central Africa, which is currently epidemic in Angola, Democratic Republic of Congo, and Sudan and prevalent in Cameroon, Central African Republic, Chad, Congo, Côte d’Ivoire, Guinea, Mozambique, Uganda, and United Republic of Tanzania (http://www.who.int/mediacentre/factsheets/fs259/en/index.html). The prominent feature that distinguishes T. b. gambiense from T. b. rhodesiense is that NHS resistance in T. b. gambiense is constitutive and independent on the presence of NHS, contrary to T. b. rhodesiense where resistant variants arise from sensitive ones by a mechanism of antigenic variation in the presence of NHS.¹

The T. b. gambiense genome has a DNA content that is 70–80% of the highest DNA content measured for T. b. brucei.²,³ This probably results from a reduced repertoire of variant surface glycoprotein (VSG) genes and expression sites (VSG EEs)³–⁶ and from a reduced compliment of minichromosomes that are also smaller (25 versus 100 kb).³,⁷ There are two distinct groups of T. b. gambiense strains, mainly based on iso-enzyme markers, VSG antigenic repertoire, and virulence to rodents and humans.⁸

The diagnosis of HAT or sleeping sickness caused by T. b. gambiense relies on serological detection of antibodies against the VSG-variant antigen type (VAT) LiTat 1.3 by the card agglutination trypanosomiasis test (CATT).⁹ Although very useful, the CATT has its limitations,¹⁰,¹¹ and PCR-based assays with increased sensitivity and specificity have been developed that can make this distinction. These tests are based on the presence of the SRA gene in T. b. rhodesiense and TGSgp in T. b. gambiense¹⁸,¹⁹ (see below).

We previously identified a VSG-like cDNA by RT-PCR amplification of T. b. gambiense RNA that we named TGSgp for T. b. gambiense-specific glycoprotein.²⁰ TGSgp was found to be specific to the T. b. gambiense subspecies, making it an excellent probe for diagnosis.¹⁹–²¹ It encodes a GPI-anchored VSG N-terminal domain similar to the ESAG6 subunit of the T. brucei transferrin receptor, and, similarly to this, TGSgp localizes to the flagellar pocket of the parasite.²⁰ To gain insight into possible mechanisms of TGSgp acquisition, given its telomeric location and its unusual RNA polymerase II-driven transcription, we isolated the genomic region containing TGSgp. We found that TGSgp was physically linked to a truncation of a gene homologous to AUT1 (APG3), a gene involved in internal vesicular formation and autophagy in Saccharomyces cerevisiae.²² We also found that T. b. gambiense is heterozygous for AUT1, with an AUT1/aut1 genotype, with each allele located on an independent chromosome II homologue. In all T. b. gambiense isolates analyzed, this genomic rearrangement appeared to be conserved. The size of the intergenic region between TGSgp and truncated aut1 varied among isolates but was similar in isolates of the same geographical area, and this observation may be used in epidemiology to trace the geographical origin of T. b. gambiense isolates.

MATERIALS AND METHODS

T. b. gambiense genomic library. The T. b. gambiense LiTat 1.3A genomic DNA was partially digested with Sau3A and the resulting DNA fragments fractionated in a 10–40% sucrose gradient by centrifugation for 20 h at 45,000g. Fractions containing 5–10 kb fragments were pooled, dialyzed against 1 mM EDTA and 10 mM Tris (pH 8), and cloned into the BamHI site of the lambda Express vector (Stratagene, La Jolla, CA). Clones containing TGSgp were identified by hybridization with the entire TGSgp cDNA probe.²⁰ The positive clones were processed to obtain the circular pBK-CMV plasmids version of the phage according to instructions of the manufacturer (Stratagene).

Southern blot analysis. Southern blot analyses were performed by digestion of 0.5 µg of DNA of the positive clones...
(or 1 μg of parasite gDNA) with various restriction enzymes, separation by electrophoresis on 1% agarose gels, blotting onto nitrocellulose membranes (Hybond N°, GE Healthcare), and hybridization with 32P-labeled probes (Rediprime II kit, GE Healthcare) at 65 °C in 6× SSC (0.3 M NaCl, 0.3 M NaOH), 5× Denhardt’s solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), and then washed at 65 °C with 0.1× SSC, before autoradiography.

**Sequencing and probes.** DNA sequencing was performed using the dideoxynucleotide chain-termination method. Hybridization probes for TGSGP, PLC, and the 5′ and 3′ regions of AUTO1 were obtained by PCR amplification with appropriate primers on 10–50 ng of purified gDNA or by random priming on purified cDNA fragments. TGSGP, TgsGP (fwd), 5′GCC GTA GCG GAG GCA TGG GC3′; TgsGP (rev), 5′GCC CTG TCT GAG TCT GC3′; PLC, cDNA24; AUTO1 5′, Δ Auto5′ (fwd), 5′GGG GCT GAG GCA TGG TTG AGG ATG AAC AAA CAA AGT TTG3′; AUTO1 3′ (rev), 5′GGG GTC TAG ATC ACA ACA TCT CGA GCG TGG ATG TTG AGG ATG AAC AAA CAA AGT TTG3′; AUTO1 3′ (fwd), 5′CAG GGA CCA AAC GGA GCG GC3′; AUTO1 3′ (rev), 5′CGC AGC TGG TCC ATT TTT AAA TCT CGA GAC CTG TCG ACA ACA AAT CAT ATT ATG CG3′.

**Trypanosomes.** Bloodstream forms of *T. b. gambiense* LiTat 1.3A and *T. b. brucei* AnTat 1.1A were injected intraperitoneally and grown in mice immunosuppressed with cyclophosphamide. The maintenance and care of experimental animals complied with Belgian guidelines for the humane use of laboratory animals. The resulting trypanosomes were purified from the blood by using an anion-exchange chromatography method (DE52, Whatman, Brentford, England) according to the method described in ref. 25. The origin of gDNAs is listed in Table 1.

**Pulsed-field gel electrophoresis.** Trypanosomes were prepared for pulsed-field gel electrophoresis as described. Chromosomes were separated using the following running conditions: 1% agarose, 1200–700 linear rayed pulse times for 22 hours at 6 V/cm in 0.5 mM EDTA, 22 mM Tris-borate at 14°C.

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**RESULTS**

**Isolation of the TGSGP genomic region.** The TGSGP genomic region was isolated from a phage library of genomic DNA partially digested with *Sal*I. The selected clone (accession number AM237444, database EMBL) had an insert size of 7,190 bp (Figure 1A). The first 3,223 bp corresponded to positions 348,058–351,280 of the published sequence from chromosome II of *T. b. brucei* (ChrII: 347536–389002). The homology between the *T. b. brucei* and the *T. b. gambiense* sequences ended in the middle of a gene (Tb927.2.1890) homologous to yeast AUTO1 (ATG3) (29% identity, 52% similarity at the amino-acid level). At this level, the open reading frame (ORF) corresponding to AUTO1 ended in a repeated sequence (G/A AT)14 encoding Asp or Asn. Hereafter we will refer to this broken AUTO1 gene as *aut1*. After this point, the DNA sequence rapidly changed into a simple, AT-rich region, similar to that found at subtelomeric VSG-proximal regions, which contain tandem 70-bp repeats flanking TAA-rich regions

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**TABLE 1** Origins of Trypanosoma gambiense isolates

<table>
<thead>
<tr>
<th>Stock</th>
<th>Identification code</th>
<th>Origin</th>
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<td>89/F.ST 10</td>
<td>Fontem focus, Cameroon</td>
</tr>
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<td>Kinshasa, DRC</td>
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<tr>
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<td>--/ITMAP 2228</td>
<td>DRC</td>
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<td>KEMLO</td>
<td>74/AnTAR 13, ITMAP 1821</td>
<td>Gemena, DRC</td>
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Key to stock origins: year of isolation/original name of isolate. All hosts are human. DRC: Democratic Republic of the Congo. The stocks in bold print are those used in the Northern Blot analysis.
gambiense, but in the latter case the hybridization signal was weaker and an extra fragment of 3.6 kb was present (Figure 2B, lanes 1 and 2). The 7.7-kb fragment was also revealed by the 3′/H11032 probe, which is able to detect \( \text{AUT1} \) but not the truncated \( \text{aut1} \) (Figure 2B, lanes 5 and 6). Therefore, the 7.7-kb band contained the \( \text{AUT1} \) allele, whereas the 3.6-kb fragment contained the truncated one (\( \text{aut1} \)). This result indicated that \( T. b. \) gambiense is heterozygous for \( \text{AUT1} \) with an \( \text{AUT1/aut1} \) genotype. Similarly, two \( \text{PstI} \) fragments of 2.4 and 3.6 kb were detected in \( T. b. \) gambiense only (Figure 2B, lanes 5 and 6). Therefore, the 7.7-kb band contained the \( \text{AUT1} \) allele, whereas the 3.6-kb fragment contained the truncated one (\( \text{aut1} \)). This result indicated that \( T. b. \) gambiense is heterozygous for \( \text{AUT1} \) with an \( \text{AUT1/aut1} \) genotype. Similarly, two \( \text{PstI} \) fragments of 2.4 and 3.6 kb were detected in \( T. b. \) gambiense only (Figure 2B, lanes 3, 4, 7, and 8), which corresponded to fragments derived from \( \text{AUT1} \) and \( \text{aut1} \) alleles, respectively. In \( T. b. \) gambiense, the 3′ probe revealed only fragments from \( \text{AUT1} \), indicating that the 3′ fraction of the \( \text{aut1} \) truncated allele was absent from this DNA. To confirm the presence of both \( \text{AUT1} \) and truncated \( \text{aut1} \) alleles in \( T. b. \) gambiense, we performed PCR on genomic DNA from \( T. b. \) brucei and \( T. b. \) gambiense, using primers designed to detect specifically each allele. As shown in Figure 2C, in \( T. b. \) gambiense DNA we detected PCR products corresponding to both \( \text{AUT1} \) (1 kb) and truncated \( \text{aut1} \) (0.5 kb), whereas in \( T. b. \) brucei DNA only the product from the \( \text{AUT1} \) allele was present. Altogether, these results showed that the isolated \( \text{TGSgp} \) genomic clone exhibited a restriction map identical to that in the genomic DNA of \( T. b. \) gambiense \( \text{TGSgp} \) locus. Restriction enzyme mapping followed by Southern analysis indicated that the \( \text{AUT1} \) locus in \( T. b. \) brucei and \( T. b. \) gambiense are similar (Figure 2 and data not shown).

Characterization of the \( \text{TGSgp} \)-containing chromosome. The recombination event that placed the \( \text{TGSgp} \)-containing telomere within a region transcribed by RNA polymerase II, thereby interrupting the \( \text{AUT1} \) gene, could have led to a major deletion in a chromosome II homologue of \( T. b. \) gambiense. To analyze this possibility, we separated the chromosomes of \( T. b. \) brucei and \( T. b. \) gambiense by pulsed-field gel electrophoresis (PFGE) and probed them for the presence of \( \text{TGSgp} \) and the phospholipase C gene (\( \text{PLC} \)), a marker of \( T. b. \) brucei chromosome II, as well as the 5′/H11032 and 3′/H11032 regions of \( \text{AUT1} \).

In our \( T. b. \) brucei AnTat 1.1A stock, the probes corresponding to the 5′ and 3′ regions of \( \text{AUT1} \) recognized the same 1.2-Mb band as the \( \text{PLC} \) probe (Figure 3A), confirming the presence of \( \text{AUT1} \) in chromosome II. For comparison, in the stock 427 of \( T. b. \) brucei, the two homologues of chromosome II could not be resolved given their size similarity (1.1 and 1.65 Mb). In \( T. b. \) gambiense the situation was different.

**Figure 1.** \( \text{TGSgp} \) genomic environment. (A) Schematic comparison between the \( \text{TGSgp} \) isolated genomic DNA fragment and the genomic region surrounding \( \text{AUT1} \) in \( T. b. \) brucei chromosome II (Tb927.2.1890). Vertical arrow: breakage point of homology. (B) Alignment between \( \text{AUT1} \) and \( \text{aut1} \), approximately 150 bp before and after the breakage point. Shared nucleotides are marked with asterisks. Nt position, nucleotide position in relation to the beginning of the \( \text{AUT1} \) ORF.
The PLC probe revealed two distinct chromosomes of ≈2.7 and 1.1 Mb (Figure 3A), indicating that one of the two chromosome II homologues was substantially larger than in the T. b. brucei stock analyzed. The aut1-TGSGP locus was localized on the 1.1-Mb chromosome because only the AUT15/H11032 and TGSGP probes— but not the AUT13/H11032 probe—were able to hybridize with it. The AUT1 allele was clearly in the 2.7-Mb chromosome given its recognition by both the AUT15/H11032 and AUT13/H11032 probes but not TGSGP. These results showed that the AUT1 and aut1 alleles are on two separate chromosome II homologues, confirming that the gene is heterozygous. In accordance with the Southern blot data, the AUT13 probes did not reveal any other chromosome than that containing the AUT1 gene. A possible interpretation of these data is given in Figure 3B, assuming the absence of polymorphism in the AUT1 loci of T. b. brucei and taking into consideration that only one chromosome II homologue has been sequenced in this subspecies (see below).

**Variation of the size of the aut1-TGSGP genomic region.**

Previous results showed that the TGSGP is a specific marker of T. b. gambiense.19,20 We examined if association between aut1 and TGSGP was a conserved characteristic of T. b. gambiense. We performed Southern blot analysis on 18 different strains of T. b. gambiense (Table 1). Hybridization with the TGSGP probe revealed a PstI fragment of 1.5 kb in all the T. b. gambiense strains except LIGO and ABBA (Figure 4A). These data confirmed the specificity of TGSGP for T. b. gambiense and confirmed that LIGO and ABBA are distinct from the conventional group 1 strains of this subspecies, in accordance with previous results showing that these parasites are not constitutively resistant to NHS.19 In all the strains studied, the AUT15 probe identified a fragment of 2.4 kb corresponding to the AUT1 allele together with a larger fragment of varying size (ranging from 3.6 to >8 kb) corresponding to the truncated allele aut1 (Figure 4A). As expected, the largest fragment corresponding to aut1 was not present in the strains that did not hybridize with TGSGP. These results showed that TGSGP is associated to the truncated aut1 allele in all the T. b. gambiense isolates tested. When classifying the stocks according to the size of the fragment containing the truncated aut1 allele, we observed a linkage with the geographical origin of the stock. As shown in Figure 4, with a few exceptions all isolates from a given region exhibited aut1-containing fragments of the same size, but this size differed between regions, ranging from 3.6 kb for the Ivory Coast stocks to 7 kb for the distant Ugandan stocks. Within neighboring countries, the aut1 fragment showed similar but distinct sizes: 6.5 kb for Congo, 6.5 and 8 kb for Cameroon, and 8 kb for the Democratic Republic of Congo.

**DISCUSSION**

We and others have previously shown that TGSGP is specific to T. b. gambiense; it has still not been found in other subspecies, such as T. b. brucei, T. b. rhodesiense, T. evansi, T. 
equiperdum, T. congolense, T. vivax, and T. theileri from several geographical origins.\textsuperscript{19–21} \textit{TGSGP} is a telomeric gene transcribed by a RNA polymerase II as shown by run-on and exonuclease digestion experiments.\textsuperscript{20} Even though it is not surprising for a \textit{VSG}-like gene to be telomeric, it is unusual for such a gene to be transcribed by an RNA polymerase II. Because all type I \textit{T. b. gambiense} isolates analyzed to date have conserved this gene presumably at a telomeric location, we cloned the genomic environment of \textit{TGSGP} to gain insight into its possible acquisition mechanism. We isolated a

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{T. b. gambiense contains two different chromosome II homologues. (A) PFGE analysis of \textit{T. b. brucei} (b) and \textit{T. b. gambiense} (g). The chromosomes of \textit{T. b. brucei} and \textit{T. b. gambiense} were separated by PFGE and probed as indicated. (B) Schematic representation of the position of the \textit{AUT1} region respective to the \textit{PLC} marker on the chromosome II homologues of \textit{T. b. brucei} and \textit{T. b. gambiense}. Designations IIA and IIB are arbitrary. The / / separator represents hundreds of thousands of bp.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{The \textit{aut1-TGSGP} rearrangement is conserved among \textit{T. b. gambiense} isolates. (A) Southern blot analysis of \textit{PstI} digests of genomic DNA from 18 \textit{T. b. gambiense} isolates sequentially probed with \textit{5' AUT1} (see Figure 2A) and \textit{TGSGP}. D.R.C., Democratic Republic of the Congo. (B) Relationship between size of the \textit{aut1-TGSGP} intergenic region and the geographical origin of the isolates.}
\end{figure}
7,190-bp fragment containing TGSGP and the associated genomic region. We identified a DNA rearrangement linked to the insertion of TGSGP, which interrupted a gene homologous to AUT1 from S. cerevisiae. In T. b. brucei, this gene is located on chromosome II, ≈ 0.35 Mb from its left-arm telomere and is transcribed away from this telomere. The rearrangement occurred in a simple AT-rich region resembling the repeats typically found upstream from telomeric VSG genes. Southern blot, PCR, and PFGE analyses indicated that the T. b. gambiense genome is heterozygous for AUT1; it contains AUT1 and a truncated aut1 allele, each present on different chromosome II homologues, as shown by the presence of the PLC marker. The size of the two chromosome II homologues from T. b. gambiense was quite different, 2.7 and 1.1 Mb, respectively. Such size differences are common in trypanosomes, because in the T. b. brucei stock 427 chromosome Ia is 1.85 Mb and chromosome Ib is 3.6 Mb. At the current status of the T. b. gambiense genome database (http://www.sanger.ac.uk/Projects/T_b_gambiense/), a sequence containing TGSGP is present (707f06.q1k). This sequence contains 1,057 bp of AT-rich sequence before the ATG codon of TGSGP, but it lacks the junction with the truncated aut1 allele. The main difference between 707f06.q1k (DAL 972) and the corresponding sequence in our LiTat 1.3A stock is a deletion of 98 bp in 707f06.q1k and five other nucleotide changes. At the protein level, the difference between the ORF (403 aa) of the two sequences is only three amino acids, of which only one constitutes a conservative change (data not shown).

As mentioned above, our previous results clearly showed that TGSGP was a telomeric gene located few kbp from a chromosomal end. This together with the fact that TGSGP is directly linked to a truncated AUT1 gene (Figure 2, A and B) indicated either: (i) a massive deletion (= 0.8 Mb, by comparison to the T. b. brucei’s 1.1-Mb chromosome II) between AUT1 and the TGSGP-containing telomere or (ii) an internal inversion implicating the 3’ region of AUT1 and upstream-linked region (≈ 0.35 Mb). Because the PLC chromosome II marker is still present in the TGSGP-containing chromosome (Figure 3), this suggests that no loss of downstream sequence from aut1 occurred but rather an internal inversion of the left arm of chromosome II (Figure 3). Alternative and more complex explanations would imply loss of chromosome II synteny between T. b. brucei and T. b. gambiense. The recombination target for this inversion was probably the G/A AT-rich region normally found within the ORF of the AUT1 allele, in a sequence coding for eight Asp and five Asn residues. Homologous recombination presumably occurred with a AT-rich barren region commonly found upstream of telomeric VSG genes. The recombination, possibly promoted by inverted repeats, caused inversion of a 0.35-Mb region without substantial loss of genetic information (except for an AUT1 allele) but with inversion of the direction of transcription (Figure 5). Interestingly, in T. b. brucei, the region downstream from AUT1 (Tb927_02_v4:347536–389002) shows a change in transcription direction, which may explain why such inversion was tolerated. A direct consequence of this inversion was the insertion of a RNA polymerase II-mediated transcription unit near a telomere that could have contained a VSG gene. It was probably this ancestral VSG gene that became TGSGP through evolution and selection of its modern function, or alternatively, it was acquired from the VSG reservoir by a recombination mechanism common to all VSG genes during antigenic variation. Actually, in T. b. brucei stock TREU 927/4 (genome project strain), there is a VSG gene highly homologous to TGSGP (81% identity at nucleotide level) (Tb10.v4.0178). It codes for a longer VSG than TGSGP and it may represent a common ancestor for both TGSGP and the

![Figure 5](https://via.placeholder.com/150)
LiTut 1.23 VSG. Tb10.v4.0178 is a VSG at one end of chromosome X surrounded by other VSG genes that together may be part of the VSG reservoir. What is compelling is the fact that TGSGP has been maintained in all type 1 T. b. gambiense isolates analyzed to date.\(^{19,20}\) This conservation goes beyond TGSGP and extends to the truncation of one AUT1 allele and inversion of a substantial part of one of the two chromosomes II homologues. The region bearing the breakpoint in aut1 varied from 3.6 to > 8 kb among 18 different T. b. gambiense isolates, presumably due to the simple nature of this AT-rich region and/or to the lack of coding genes. On the basis of this size variation, three major groups could be distinguished: isolates originating from the Ivory Coast region, from Uganda, and from the West Africa region (Congo, Democratic Republic of Congo, and Cameroon). This finding could be useful to determine the possible geographical origin of T. b. gambiense isolates.

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REFERENCES


