Construction of trypanosome artificial mini-chromosomes

Mary Gwo-Shu Lee*, Yaping E and Nancy Axelrod¹

Department of Pathology, New York University Medical Center, 550 First Avenue, New York, NY 10016, USA and

¹Biochemical Genetics, Sloan-Kettering Institute, New York, NY 10021, USA

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ABSTRACT

We report the preparation of two linear constructs which, when transformed into the procyclic form of *Trypanosoma brucei*, become stably inherited artificial mini-chromosomes. Both of the two constructs, one of 10 kb and the other of 13 kb, contain a *T.brucei* PARP promoter driving a chloramphenicol acetyltransferase (CAT) gene. In the 10 kb construct the CAT gene is followed by one hygromycin phosphotransferase (Hph) gene, and in the 13 kb construct the CAT gene is followed by three tandemly linked Hph genes. At each end of these linear molecules are telomere repeats and subtelomeric sequences. Electroporation of these linear DNA constructs into the procyclic form of *T.brucei* generated hygromycin-B resistant cell lines. In these cell lines, the input DNA remained linear and bounded by the telomere ends, but it increased in size. In the cell lines generated by the 10 kb construct, the input DNA increased in size to 20–50 kb. In the cell lines generated by the 13 kb constructs, two sizes of linear DNAs containing the input plasmid were detected: one of 40–50 kb and the other of 150 kb. The increase in size was not the result of *in vivo* tandem repetitions of the input plasmid, but represented the addition of new sequences. These Hph containing linear DNA molecules were maintained stably in cell lines for at least 20 generations in the absence of drug selection and were subsequently referred to as trypanosome artificial mini-chromosomes, or TACs.

INTRODUCTION

The African trypanosome *Trypanosoma brucei* is the causative agent of sleeping sickness in humans and nagana in cattle, both of which are diseases endemic to large parts of tropical Africa. *T.brucei*, a eukaryotic flagellate, exhibits many unusual biochemical properties, including antigenic variation, *trans*-splicing, RNA editing and polycistronic transcription (for review see 1–5). To advance the study of the mechanisms involved in these unusual properties, a number of stable DNA transformation systems have recently been developed for this organism (6–8). First, the use of homologous recombination allows drug resistance genes to be efficiently incorporated into specific regions of

chromosomes. This method allows expressing foreign genes as well as disrupting genes, including deletions and site specific mutations. The efficiency of this type of transformation can be as high as 1×10^{-3} , as has been calculated by using the tubulin locus as a target site (9). Secondly, to avoid detrimental effects or mutations at a specific chromosomal location, several plasmid DNA constructs have been created which allow the formation of extrachromosomal circular DNA molecules (episomes) in transformed trypanosomes and other trypanosomatids (10–15). One type of these plasmids, once inside the cell, forms large episomes containing tandemly linked input plasmid. These large episomes seem to be easily established in *Leishmania* and *Leptomonas* (12,16). Thus far, only one example of this type of large episome has been documented in the stably transformed *T.brucei* (15). Mechanisms and sequences involved in the DNA replication and segregation of these large episomes are unclear. Nevertheless, these large episomes can be stably maintained for a long period of time in a cell line in the absence of drug selection. However, the efficiency of generation of the large episomes in transformed *T.brucei* is only 10^{-6} . A second type of plasmid has been developed by selecting specific sequences which allow its autonomous replication as a monomeric circle in the procyclic form of trypanosome (13). This second type of plasmid (or episome) can also stably replicate in the absence of drug selection. However, after 15 generations grown in the absence of drug selection only 20–30% of the plasmid remains in the transformed trypanosomes. Additionally, Metzenberg and Agabian reported that mitochondrial mini-circle DNA can also support plasmid replication and maintenance in nuclei of *T.brucei* (14). Here, we report on plasmid DNA constructs which allow the formation of mini-chromosomes (MCs) in the procyclic form of trypanosome. These MCs generated by the input plasmid DNA are referred to as trypanosome artificial mini-chromosomes (TACs).

In *T.brucei*, there are ~100 MCs ranging in size from 50 to 150 kb (17,18). The exact function and the stability of the MCs are not clear and genes that encode functional proteins have not been found in these MCs. Since these MCs are confined to the trypanosome species that undergo antigenic variation, it has been speculated that they may function solely to expand the repertoire of the telomeric Variant cell Surface Glycoprotein (VSG) genes (2,4,5,19).

Thus far, only one trypanosome telomere clone has been well characterized which contains [GGGTAA] telomere repeats and subtelomeric sequences (17,20). Other functional units of

* To whom correspondence should be addressed

Figure 1. Physical maps of linearized pTAC1 and pTAC2 plasmids. Symbols: PARP (flag with black box), the PARP-B locus promoter; 3' sp (small white box), the PARP gene 3' splice acceptor site; Tub (shaded box), a portion of the intergenic region of $\beta\alpha$ tubulin gene; horizontal arrows, the telomere repeats GGGTTA; pBSK+ (white thin long box), the plasmid vector bluescript SK+; CAT, the chloramphenicol acetyltransferase gene; Hph, the hygromycin phosphotransferase gene. The subtelomere region (thick line) includes six 29 bp repeats and an additional 1.1 kb sequence containing three GC rich domains (20). Telomere repeats are horizontal arrowheads. The diagonally striped box at the end of each vector is the spacer DNA which is placed in between the two inverted telomere fragments in the circular form of the plasmid.

chromosomes such as centromere and autonomous replication origins (ARS) have not yet been fully characterized. One sequence may be an ARS as documented by Patnaik *et al.* (13). However, this potential putative ARS is only functional in the presence of the procyclic acidic repetitive gene (PARP) promoter (21). The TACs will potentially facilitate the analysis of genetic elements required for chromosomal function such as sequences required for chromosomal replication, segregation and stability. In addition, TACs may be of importance for the study of antigenic variation and positional control of gene expression, since all the VSG gene expression sites are telomerically located.

MATERIALS AND METHODS

Trypanosome strains

Procyclic *T.brucei* (stock 427-60), originally obtained from Dr Brun, was maintained at 27°C in SDM-79 medium (22).

DNA transformation

Transformation of procyclic form trypanosomes was carried out as previously described (7,23). Ten or 20 μ g of linearized plasmid DNA was electroporated into 3×10^7 trypanosomes in 0.4 ml, using a BTX electroporator. Forty hours post-electroporation, hygromycin B was added to a final concentration of 40 μ g/ml to select transformed trypanosomes. Simultaneously, limiting dilution cloning was performed. To facilitate the growth of transformed trypanosomes during the limiting dilution cloning, wild-type trypanosomes were added at a concentration of 2×10^6 cells/ml to each diluted sample.

Southern genomic DNA analysis

Trypanosome genomic DNA was prepared as described (24). Restriction enzyme digested genomic DNA was separated in a 0.8% agarose gel, transferred onto nitrocellulose filters and then

hybridized to 32 P-labeled probes. The final post-hybridizational wash was performed in 0.1 $\times$ SSC, 0.1% SDS at 65°C. For some of the experiments, the hybridization signal was quantitated using a Betage Betascope 603 blot analyzer.

Pulsed field gel electrophoresis (PFGE)

Preparation of chromosome sized DNA and PFGE analysis were as described (18,25). A 50 kb λ phage DNA ladder and *Hind*III digested λ phage DNA were used as size standards. The electrophoresis conditions were as indicated in each figure legend. The DNA of each PFG gel was transferred onto nitrocellulose filters and hybridized to 32 P-labeled probes. The final post-hybridizational wash was performed in 0.1 $\times$ SSC, 0.1% SDS at 65°C.

RESULTS

Construction of plasmid constructs and stable transformation

We have constructed two plasmids, pTAC1 and pTAC2, of 10 and 13 kb respectively (Fig. 1). Both constructs contain a *T.brucei* PARP coding gene promoter driving a chloramphenicol acetyltransferase (CAT) gene. In pTAC1, the CAT gene is followed by one hygromycin phosphotransferase (Hph) gene, which serves as a selectable marker for the transformation of trypanosomes. In pTAC2, three copies of tandemly linked Hph gene were inserted, which were originally designed to determine if the additional 3 kb of DNA would stabilize the DNA construct in transformed trypanosomes. To provide the signals for RNA processing, the PARP gene 3' splice acceptor site and a fragment from the $\beta\alpha$ tubulin intergenic region were linked to the 5' and 3' ends, respectively, of each CAT and Hph gene. Downstream of the 3' most copy of the Hph gene are two inverted copies of a ~1.5 kb DNA fragment derived from a trypanosome telomere. The two inverted telomere fragments were separated by a ~0.5 kb DNA

Figure 2. Pulsed field gel electrophoresis (PFGE) analysis of chromosome sized DNA from cell lines generated with pTAC1 and pTAC2. Cell lines R52, L57 and L20 were generated by pTAC1. Cell lines M16, M42, M43 and M45 were generated by pTAC2. The PFGE analysis was as described (18,25). Chromosome sized DNA from different cell lines were separated in a 1% agarose gel for 30 h at 16 V/cm and a pulse frequency of 35 s. The blot was hybridized with a ^{32}P -labeled Hph coding region probe.

fragment, which serves as a spacer, allowing the circular form of these plasmids to be stably amplified in *Escherichia coli*. The 1.5 kb telomere derived fragment consists of, proceeding from the tip of telomere, 55 copies of telomere repeats [GGGTAA], six copies of a 29 bp repeat and a 1.1 kb sequence containing several GC rich domains (17,20). When the pTAC1 and pTAC2 were cleaved at *Bgl*II restriction enzyme sites located at the end of the telomere sequence, the spacer sequence was released and linear molecules flanked by telomere sequences at each end were generated.

The linearized pTAC1 and pTAC2 were electroporated into the procyclic form of trypanosome and hygromycin B (Hyg-B) resistant cell lines were selected. The transformation efficiency was $\sim 10^{-3}$ to 5×10^{-4} with 10–20 μg linear pTAC1 (or pTAC2) as measured by limiting dilutions immediately following the transformations.

Analysis of stable transformants

Chromosome sized DNA was isolated from trypanosome cell lines transformed by pTAC1 and pTAC2 and was analyzed by PFGE. Figure 2 shows that, in cell lines that were generated by the 10 kb pTAC1 (Fig. 2, lanes R52, L57, L20), the input plasmid DNA increased in size to ~ 20 –50 kb. In the cell lines which were generated by the 13 kb pTAC2 (Fig. 2, lanes M16, M42, M43, M45), two sizes of DNA of ~ 40 –50 and 140–150 kb, contained the input plasmid. All of these input plasmid containing DNAs were sensitive to the BAL-31 exonuclease, indicating that, like the input molecules, the intracellular Hph containing DNAs were linear (data not shown). These lengthened, linear intracellular

DNAs generated by pTAC1 and pTAC2 have been named TACs (trypanosome artificial mini-chromosomes).

Restriction enzyme analysis of the TACs generated by pTAC1 showed that the DNAs fall into two classes, L (left) and R (right), which occurred at an equal frequency (Fig. 3). In both types of TACs, the structure of the input plasmid remained intact, except that an additional 10–30 kb sequence was added to the subtelomere region located at either end. The L type TACs, as exemplified by cell lines L22 and L57, contain additional 20–30 kb sequences at the subtelomeric region adjacent to the plasmid vector (pBSK+). The R type TACs, as exemplified by cell lines R52 and R68, contain additional 20–30 kb sequences at the subtelomeric region of the Hph side. Among these TACs, L20, derived from the L type cell line, was the smallest one where ~ 8 kb of DNA was added to the input plasmid. Figure 3, top panel, demonstrates examples of Southern blot analysis of TACs generated in these transformed cell lines. Restriction enzyme digestion of the TACs with *Hind*III separates the plasmid vector and the Hph gene (Fig. 3, panel H). *Hind*III digestion of the linearized pTAC1 results in a plasmid vector fragment of ~ 5.1 kb and a Hph fragment of ~ 4.3 kb. In the cell lines L20, L22 and L57, the sizes of the *Hind*III DNA fragments detected by the Hph probe and by the plasmid vector probe (pB) were ~ 5 –6 and >20 kb (or 13 kb for L20) respectively. The same probes detected *Hind*III restriction fragments of >20 kb (by Hph) and of 5–6 kb (by pB), respectively, in the cell lines R52 and R68. Similar banding patterns were also obtained with *Bam*HI digested genomic DNAs (Fig. 3, panel B). The structure of the short telomere arm in both types of TACs remained as that of input plasmid, but with a higher number of telomere repeats, determined by DNA sequence analysis and Southern blot analysis (data not shown). This explains the slightly larger sizes of the short arm of L and R TACs detected by the Hph probe or the plasmid, respectively, when compared with that of the input plasmid. It appeared that the length of telomeric fragments derived from TACs were relatively homogenous. However, when trypanosomes were maintained in the presence of a high concentration of hygromycin B, the length of the telomeric fragments of TACs rapidly became longer and more heterogeneous (see the following sections).

DNA of L20 was subcloned and recombinant clones were obtained that contained either the right or the left telomere end. DNA sequence analysis of these recombinant clones demonstrated that the number of telomere repeats at both ends increased to >55 copies (data not shown). In L20, sequence of the subtelomere domain adjacent to the Hph gene remained as that of the input plasmid pTAC1. However, in the subtelomere domain adjacent to the plasmid vector of L20, only ~ 1 kb DNA sequence was conserved, which was followed by new DNA sequences. Thus, the divergence occurred in the region spanning the upstream junction of the 29 bp subtelomere repeat. The complete DNA sequence analysis of the additional sequence in L20 TAC has not yet been completed. However, a 800 bp DNA fragment derived from the new sequence of L20 TAC hybridized to MCs and a subset of large chromosomes, indicating its multiplicity (data not shown).

Southern genomic blot analysis of cell lines generated with pTAC2 revealed three types of TACs. Figure 4 shows the physical maps of pTAC2 derived TACs. The TAC derived from the M43 cell line is ~ 45 kb long due to the addition of a 30–32 kb sequence to the subtelomeric region downstream of the Hph gene. The TAC

Figure 3. Southern genomic blot analysis of cell lines generated by pTAC1. Genomic DNA from the wild-type (untransformed) trypanosome (W) and cell lines L20, L22, L57, R52 and R68 were digested with *Hind*III (H) or *Bam*HI (B). These blots were hybridized with an Hph probe, and with a plasmid vector probe (pB) respectively. The final post-hybridizational wash was performed in 0.1× SSC, 0.1% SDS at 65°C. The bottom panel demonstrates the physical maps of pTAC1 and two types of TACs (L and R) generated from pTAC1. Bg is *Bgl*II. Double slashes represents a size of ~25–30 kb. Other symbols are as described in Figure 1.

from the M42 cell line is ~145 kb long as an additional 130–132 kb sequence was added to the subtelomeric region adjacent to the plasmid vector. In the cell line M16, the TAC is also ~145 kb. However, an additional 12 kb sequence was added into the subtelomeric region of the Hph gene side while a 120 kb sequence was added to the plasmid vector side. Among all cell lines analyzed (90 derived from pTAC1 and 50 derived from pTAC2), only one type of TAC was found to be present in each cell line.

Copy number and stability of the trypanosome artificial mini-chromosome

The copy number of the TAC in each cell line varies. Two examples are shown in Figure 5A. Blots containing *Hind*III digested genomic DNA from different cell lines were hybridized with an excess amount of Hph probe and the hybridization signals were quantitated with a Betagen Betascope blot analyzer. A control cell line in which *Hind*III generates two sizes of Hph fragments, one of one copy and the other of three copies (Fig. 5A; indicated by 1 and 3), was used as a standard for the calculation of the copy number of Hph containing fragments in each cell line. The hybridization with an endogenous gene probe (CRAM) was used as an internal control of the amount of DNA loaded in each lane. Comparison of the intensity of Hph signals demonstrated that the L57 cell line contained three to four copies of the 40 kb TAC and the M42 line contained only one copy of the 145 kb

TAC. M42 was generated with pTAC2 plasmid which has three tandemly linked Hph genes. The copy number of these TACs can be increased by increasing the concentration of the drug used. One example is shown in Figure 5B. After incubation of the L57 cell line in a medium containing 200 µg/ml of Hyg-B for 76 divisions, the amount of TACs present increased by ~8-fold. Additionally, the size of the Hph fragment derived from these TACs in L57 also increased and become less homogeneous. Further mapping confirmed that the formation of larger Hph fragment in L57 was due to an increase in the length of the telomere repeat (data not shown).

In transformed cell lines, TACs can be inherited for indefinite passages in the presence of Hyg-B. However, and interestingly, when the selective drug is removed, large TACs (~140–150 kb) are considerably more stable than the shorter ones (~50 kb) in transformed cell lines. We compared the stability of the Hph containing MC derived from three cell lines, D4, M42 and L57. The D4 cell line was generated by integration of a single copy of the Hph gene driven by a rRNA promoter into a MC of ~60 kb that contained rRNA promoter sequences, as described by Zomerdijs *et al.* (27) and Lee (unpublished). The sizes of the Hph containing MCs in cell lines, D4, M42 and L57 are ~60, 145 and 40 kb respectively, as shown in Figure 5C. When cell lines L57 (three to four copies of TAC/cell) and D4 were maintained in medium without drug selection for 32 generations, ~20% of

Figure 4. Physical maps of three types of TACs generated by pTAC2. Symbols are as described in Figure 1. The sizes of TACs in cell lines M43, M42 and M16 are ~45, 145 and 145 kb respectively. These maps were constructed based on Southern genomic and PFGE analysis (data not shown).

Figure 5. The copy number and the stability of TACs in transformed trypanosomes. (A) Determination of the copy number of TACs in two cell lines. Genomic DNAs from L57, M42 and a control cell line were digested with *Hind*III, separated in a 0.8% agarose gel and transferred to nitrocellulose filters. The blots then were sliced into two panels: top >4 kb and bottom <4 kb. The top and bottom panels were hybridized with an Hph probe and a CRAM probe (26) respectively, under probe excess as previously described (15). The control cell line (Lee, unpublished) was generated by integration of four tandem copies of an Hph plasmid construct into a large chromosome of procyclic form trypanosomes. Digestion of the integrated DNA of the control cell line yields three copies of the Hph hybridizing fragment of a size predicted for the input plasmid (indicated by 3) and one copy of the Hph hybridizing fragment of an altered size predicted for the integration event (indicated by 1). This cell line was used as a standard for the measurement of copy number of Hph genes in other cell lines. The CRAM hybridization was used as an internal control of the amount of DNA loaded in each lane. The hybridization signals were quantitated by a Betagen Betascope 603 blot analyzer. (B) Increase of the copy number of TACs by increasing the drug concentration during the selection. Genomic DNA was isolated from L57 cell line that has been maintained in medium containing 200 μ g/ml of Hyg-B for different periods of time, indicated on the top of the panel. The amount of Hph gene in these samples was compared by Southern blot analysis. The top panel was hybridized with the Hph probe, and the bottom with the CRAM probe. (C) PFGE analysis. Chromosome blocks from cell line D4, M42 and L57 were separated in a 1% PFGE for 30 h at 16 V/cm and a 35 s pulse frequency. The blot was hybridized with the Hph probe. The D4 cell line was generated by integrating a single copy of the Hph gene into a 60 kb MC that contains a rRNA gene promoter sequence as described by Zomerdijs *et al.* (27). (D) Stability of TACs in the absence of drug selection. Cell line L57, D4 and M42 were maintained in medium without Hyg-B over a period of time as indicated by 'Division'. At each indicated division time, DNA samples were isolated. The amount of Hph gene in these DNA samples were compared by Southern blot analysis. The top panel was hybridized with the Hph probe, and the bottom with a CRAM probe. The final post-hybridization wash was performed in 0.1 $\times$ SSC, 0.1% SDS at 65 $^{\circ}$ C.

TACs or of Hph containing MC remained (Fig. 5D, two left panels). However, in cell line M42 which contains only one copy of TAC per cell, no loss of TACs was observed, even 60

generations after the incubation without Hyg-B (Fig. 5D, right panel). This result strongly suggested that the size of the TAC may be a factor affecting the stability of MCs. It is not clear how MCs

and TACs replicate and segregate in trypanosomes. Since the loss of the TACs in L57 line only began between 8 and 20 generations after culture in the absence of drug (Fig. 5D), TACs were able to replicate for some time even without drug selection. The loss of TACs in L57 line may result from the inefficient replication of these TACs. Interestingly, the 60 kb endogenous MC containing the integrated Hph gene also disappeared from the D4 cell line after 32 generations growth in the absence of drug selection. This result suggests that perhaps some of the endogenous small MCs may not be stable and that the steady state level of MCs may be maintained by the continuous appearance of new MCs and disappearance of old MCs. Alternatively, the loss of the Hph tagged endogenous MC in the D4 line may result from some yet unidentified events which were generated in the process of the integration event. We are presently investigating these possibilities.

DISCUSSION

The genome of *T.brucei* contains ~20 large chromosomes, ranging in size from 200 kb to 5.7 Mb, and ~100 MCs of 50–150 kb (4,25). Pairs of homologous chromosomes have been identified among the large chromosomes (25). The genome has been proposed to be aneuploid for the MC, however evidence for the presence of homologous chromosomes for some MCs has also been documented (25,27). MCs in *T.brucei* appear to be mitotically stable and do not follow a Mendelian pattern of inheritance after a genetic cross (28). It is not clear whether these MCs are replicated and segregate at every division. Further analysis of the genetic content of these TACs will presumably provide insights into the requirements for chromosome stability. It has been shown that a subset of the MCs mainly consist of telomere repeats, subtelomere repeats, and 177 bp repeats which span over 90% of the length of the MCs (20,29). Additional sequences such as VSG genes and rRNA promoter sequences are also found in some of the MCs (27,29). The function of the 177 bp repeats in MCs is not yet clear, but it has been suggested that this repeat sequence may function to generate MCs that are of sufficient length to render the molecules mitotically stable (20). The nature of additional sequences in TACs generated by pTAC1 and pTAC2 is not completely characterized. However, sequences other than 177 bp repeats, VSG genes and rRNA promoter sequences are also present. These new sequences isolated from TACs originated from MCs and a subset of large chromosomes as determined by the PFGE analysis, indicating their multiplicity (data not shown). It is not yet clear whether single copy sequences may also be present in the newly acquired sequences of TACs. Attempts to recover intact the smallest 18.5 kb TAC of the L20 cell line in bacteria have failed. Extensive amounts of DNA sequence were deleted in the recovered TACs. This could be due to the repetitious nature of the DNA sequence in TACs as this type of DNA sequence is usually recombinogenic in bacteria. The structure of TACs and their DNA sequence complexity suggests a reciprocal exchange of pTAC1 and pTAC2 with endogenous MCs at a subtelomeric region, via homologous recombination which may have created TACs. A reciprocal recombination event has been previously documented as one way for the switching of VSG gene expression sites, which moves a telomeric VSG gene expression site to a different chromosome (30).

The basic functional units of chromosomes include centromeres, telomeres and ARSs. Thus far, in *T.brucei*, only one telomere clone has been well characterized and it is unclear

whether sequences of subtelomeric domain diversify among different chromosomes. Although one potential putative ARS was documented in *T.brucei*, the function of this ARS requires the presence of a functional PARP promoter (21). The structure of the centromere in *T.brucei* has not yet been determined and it is not clear if centromeres are present in MCs. However, only ~30 microtubules can be observed to be attached to the electron-dense, chromatin-containing plaques during trypanosome cell division (31). The number of mitotic spindles thus appears much lower than the number of chromosomes (~18 larger chromosomes and ~100 MCs; 18). In yeast *Saccharomyces cerevisiae*, fully functional centromeres, telomeres and ARSs have been well defined and are confined, at most, to a few hundred base pairs. When these elements are combined on yeast artificial chromosomes (YACs), which are ≥ 50 kb, the chromosomes display sufficient meiotic and mitotic stability (32,33). These YACs increase in mitotic stability through the size range up to 137 kb (34). The phenomena that mitotic stability of linear chromosomes is proportional to chromosome length was also observed in TACs. To further identify centromeres and ARSs of trypanosomes, we are presently characterizing the function of each of these putative DNA elements in TACs by complementing the function of pTAC1 and pTAC2.

An important reason to establish and study TACs is to gain insight into the 'switch' mechanism of antigenic variation. By repeatedly changing the expression of the antigenically different VSG protein, bloodstream form trypanosomes are able to survive the host's immune attack (for review see 2,4,5,19). *T.brucei* contains a reservoir of ~1000 different and transcriptionally silent VSG basic copy genes (BC) and ~20 potential VSG 'expression sites' (ES), which are telomerically located, and only one of which is active at a given time. Switching of the VSG gene expression can occur by translocation of a new BC VSG gene into a transcriptionally active ES or it can result from the differential transcriptional control of the VSG gene ES. The exact mechanisms involved in these processes are still unclear. TACs provide important tools for the study of the control mechanisms of telomerically-located gene expression in trypanosomes. Thus, this study may potentially facilitate the establishment of artificial VSG gene expression site for the further analysis of mechanisms involved in the antigenic variation of African trypanosomes.

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