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Cloning and expression of transgenes using linear vectors in *Trypanosoma cruzi*

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ABSTRACT

The identification of new targets for vaccine and drug development for the treatment of Chagas' disease is dependent on deepening our understanding of the parasite genome. Vectors for genetic manipulation in *Trypanosoma cruzi* basically include those that remain as circular episomes and those that integrate into the parasite's genome. Artificial chromosomes are alternative vectors to overcome problematic transgene expression often occurring with conventional vectors in this parasite. We have constructed a series of vectors named pTACs (Trypanosome Artificial Chromosomes), all of them carrying telomeric and subtelomeric sequences and genes conferring resistance to different selection drugs. In addition, one pTAC harbours a modified GFP gene (pTAC-gfp), and another one carries the ornithine decarboxilase gene from *Crithidia fasciculata* (pTAC-odc). We have encountered artificial chromosomes generated from pTACs in transformed *T. cruzi* epimastigotes for every version of the designed vectors. These extragenomic elements, in approximately 6–8 copies per cell, remained as linear episomes, contained telomeres and persisted after 150 and 60 generations with or without selection drugs, respectively. The linear molecules remained stable through the different *T. cruzi* developmental forms. Furthermore, derived artificial chromosomes from pTAC-odc could complement the auxotrophy of *T. cruzi* for polyamines. Our results show that pTACs constitute useful tools for reverse functional genetics in *T. cruzi* that will contribute to a better understanding of *T. cruzi* biology.

1. Introduction

Trypanosoma cruzi, the causative agent of Chagas' disease, exhibits many unusual genetic characteristics including polycistronic transcription, trans-splicing, post-transcriptionally regulated gene expression, mtRNA editing, lack of introns, absence of RNA polymerase II promoters (with the exception of the spliced leader (SL)-RNA promoter) and transcription of protein coding genes by RNA polymerase I (Martinez-Calvillo et al., 2010).

The nuclear genome of *T. cruzi* (approximate haploid size of 60 Mb) is distributed among an estimated number of 40 chromosomes; the majority of them are diploid and made up of large polycistronic transcriptional units (El-Sayed et al., 2005a).

Although *T. cruzi* telomeric and subtelomeric organisation has been well characterised (Chiurillo et al., 1999, 2002; Kim et al., 2005; Moraes Barros et al., 2012), other functional units of chromosomes such as centromeres and autonomous replication origins have remained elusive or could not be unequivocally assigned (Obado et al., 2005). Indeed, the completion of the Tritryps genome sequencing projects (El Sayed et al., 2005a; Berriman et al., 2005; Ivens et al., 2005) did not give new insights in this direction, especially for *T. cruzi*. DNA sequences potentially involved in mitotic stability and transmission of chromosomes have been reported in *Leishmania* (Tamar and Papadopoulou, 2001; Dubessay et al., 2002a,b) but these findings are yet to be confirmed.

The development of stable DNA transformation systems has advanced our understanding of the unique genetic features of trypanosomatids. In the case of *T. cruzi*, several types of expression vectors have been conceived. The first one to be constructed was pTEX (Kelly et al., 1992). Upon transfection of circular plasmid

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molecules, it is maintained as a multimeric episome of tandem repeat units, although circular extrachromosomal elements are generally lost in the absence of drug selection. Thereafter, the pRIBOTEX vector (Martinez-Calvillo et al., 1997) and its derivative, the pTREX vector (Vázquez and Levin, 1999), were developed. They carry a selection marker expressed under the Pol I rRNA promoter and are always inserted into the endogenous rRNA promoter locus, despite being introduced as circular molecules (Martinez-Calvillo et al., 1997; Lorenzi et al., 2003). Transfection of parasites with linear DNAs led to integration at homologous sites and gene replacement (La Flamme et al., 1996). Other vectors included sequences for integration in different regions of the genome (Da Rocha et al., 2004b; Araújo et al., 2011). Inducible vectors and high-throughput cloning systems have also been developed (Taylor and Kelly, 2006; Batista et al., 2010). Recently, a full set of plasmid vectors and molecular building blocks have been reported (Bouvier et al., 2013).

Our interest is to work with vectors that allow the expression of required genes in the absence of selective pressure, for example in experimental infection models, and to avoid possible detrimental effects due to insertion at specific chromosomal locations (Dhir et al., 2005). Furthermore, a system that remains as a linear episome and segregates faithfully offers an attractive model for recombination studies.

Trypanosoma cruzi exhibits great flexibility, accepting exogenous genetic material. In this context, artificial chromosomes could be of great value for the analysis of elements required for chromosomal replication, segregation and stability. Previously, linear vectors have been used in *Trypanosoma brucei* to express resistance genes or act as 'promoter-traps' (Lee et al., 1995; Patnaik et al., 1996). In *Leishmania* spp., linear constructs with synthetic telomeres have been used for protein over-expression (Kushnir et al., 2011) and vectors with added genomic fragments were conceived to study sequences conferring stability (Casagrande et al., 2005). In spite of a high degree of synteny between *Trypanosoma* spp and *Leishmania* spp (Tritryps) genomes, there are a growing number of examples that preclude direct extrapolation of characteristics among Tritryps (El-Sayed et al., 2005b; Obado et al., 2007). In fact, the majority of vectors designed for one trypanosomatid species have not worked properly in another member of the family (Wirtz et al., 1999; Tetaud et al., 2002; Taylor and Kelly, 2006). Unfortunately, the absence of an RNA interference (RNAi) mechanism further reduces the available toolkit for genetic manipulation in this parasite (Da Rocha et al., 2004a).

Artificial chromosomes have been largely used as cloning vectors in functional genomic studies (Lufino et al., 2008). Herein, we report a new generation of plasmid DNA constructs, termed pTACs (Trypanosome Artificial Chromosomes), which, after linearization, allow the formation of minichromosomes that remain stable in the absence of selection pressure through the different *T. cruzi* developmental forms. Furthermore, we show their utility as functional complementation tools, by reversion of a natural auxotrophy of this parasite for the ornithine decarboxylase (ODC) enzyme (Carrillo et al., 1999).

2. Materials and methods

2.1. Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, USA. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Federal University of Sao Paulo, Brazil (Permit Number: CEP09555-07). All surgery was performed under sodium pentobarbital anesthesia and all efforts were made to minimize suffering.

2.2. Parasite cultures

Trypanosoma cruzi epimastigotes from clone CL Brener (Zingales et al., 1997) were grown at 28 °C in liver infusion tryptose (LIT) medium supplemented with 10% heat-inactivated FBS, 20 mg/ml of hemin, 100 μg/ml of streptomycin and 100 U/ml of penicillin. Transfected parasites were cultured in LIT containing G418, puromycin or both (see Section 2.5). Trypanosomes transfected with pTAC-odc were also selected in SDM79 medium (Brun and Schonenberger, 1979) supplemented with 10% heat-inactivated FBS, 5 mg/l of hemin, 100 μg/ml of streptomycin and 100 U/ml of penicillin plus the appropriate selection drugs (see Section 2.5).

2.3. Nucleic acid isolation, electrophoresis and hybridization analyses

Total genomic DNA and chromosomal-sized DNA molecules were extracted from epimastigote cultures at the late logarithmic phase ($5–10 \times 10^6$/ml) as previously described (Lorenzi et al., 2003). Total RNA was isolated from epimastigotes bearing TACs in the exponential growth phase ($5–10^6$ cells/ml) using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. Chromosomal bands were separated using pulse-field gel electrophoresis (PFGE) in a Contour-clamped Homogeneous Electric Field electrophoresis (CHEF) apparatus (DR II, BioRad, USA) or in a Gene navigator apparatus (Pharmacia, Sweden) using conditions as previously described (Cano et al., 1995).

Separation of DNA fragments between 10 and 150 kb was performed by Field Inversion Gel Electrophoresis (FIGE Mapper, Bio Rad) under different conditions using programs 3, 4 or 10 of the manufacturer's protocol.

The following DNA fragments were used as [^{32}P] dCTP labelled probes: Gfp – 350 bp fragment from *gfp*; Neo – 500 bp fragment from *neo*, sz5 – 300 bp fragment from the *sz5* locus (Lorenzi et al., 2000), 189jc −189 bp fragment from the *T. cruzi* telomeric junction (Chiurillo et al., 1999), HX1T7-105 bp fragment spanning the HX1-T7 junction present in pTACs.

Hybridization conditions have been described elsewhere (Lorenzi et al., 2003). Densitometric analyses were performed with a Storm Phosphor Imager apparatus and quantified using Image Quant 5.2 software (Molecular Dynamics, USA).

2.4. Plasmid construction

2.4.1. pTAC vector

The pTAC vector was assembled by sequentially cloning several sequences into a pBluescript vector (pBS; Stratagene, USA). First, a 1 kb *Eco*RV-*Hin*dIII H64 fragment from the *T. cruzi* TcP2β locus (Vázquez et al., 1994) (GenBank accession No. X75031) was linked to the neomycin resistance cassette from pTREX (Vázquez and Levin, 1999) containing the neomycin phosphotranferase (NEO) gene flanked at both ends by the glyceraldehyde 3 phosphate dehydrogenase (GAPDH) intergenic region (GenBank accession No. AF005420). The H64 fragment inhibits recombination between the inverted telomeric repeats (Singer, 1988), allowing the circular vector to be stably amplified in *Escherichia coli*. The entire fragment was subcloned into a pBS vector (construct pH64Neo). A 900 bp *Xba*I-*Kpn*I fragment from pVATc13 (Chiurillo et al., 2002) containing *T. cruzi* telomeric and subtelomeric sequences (GenBank accession Nos. AF100650.1 and AY552588.1) was inserted at the *Nhe*I-*Kpn*I sites of pH64Neo (construct pH64NeoTel). A second fragment from pVATc13 was excised with *Sac*I and *Pvu*II and ligated with *Sac*I-*Eco*RV digested pH64NeoTel. This vector was named pTAC1.

A puromycin–acetyl transferase (PAC) unit harbouring the *pac* gene (GenBank accession No. AAK08635.1), flanked at 5′ end by the 220 bp HX1 intergenic region from the *TcP2β* gene (GenBank

accession No. X75031) and at 3′ end by the GAPDH intergenic region, was subcloned into pBS. The unit was excised by digestion with *Sac*I and *Nhe*I restriction enzymes, purified and ligated to the *Sac*I-*Xba*I digested pTAC1 vector in opposite orientation with respect to the NEO expression unit. The signals for correct RNA processing are provided by the GAPDH intergenic region (used in several previous vectors) and HX1, a highly efficient sequence to drive gene expression in *T. cruzi* (Vázquez et al., 1994). In this vector, a unique *Kpn*I recognition sequence provides a cloning site for the gene of interest, keeping the selection markers in both arms. Finally, and to increase the size of the vector, the H64 fragment was replaced by a 3.5 kb *Hin*dIII fragment from pYAC4 (Burke et al., 1987; GenBank accession No. U01086.1) yielding pTAC (Fig. 1A).

2.4.2. pTAC-derived constructs

To construct pTAC-gfp, an *Eco*RI site was introduced in the HX1-gfp cassette from pTREX-gfp by SOEing PCR (Horton et al., 1990). The 5′ moiety of *gfp* was amplified using GFP for and GFPrev primers and the 3'moiety using HX1 and GFPback primers (Supplementary Table S1). The 1 kb final product was obtained by amplification of the previous two SOEing-PCR products with HX1 and GFPrev primers, and was sub-cloned into the *Kpn*I site of pTAC. Sequences of primers and reaction conditions are detailed in Supplementary Table S1. The final version of pTAC-gfp contained a single *Eco*RI site at position 387 nucleotide (nt) of the *gfp* gene as the cloning site, allowing distinction of parasites which incorporated a recombinant pTAC-gfp vector (colourless) from those bearing empty artificial chromosomes (fluorescent) (Fig. 1B).

The fragment containing the ODC gene was obtained from pRibotex-odc (Carrillo et al., 1999) and inserted into the *Bam*HI site of the pBSHX1 vector (Lorenzi, unpublished data), generating a pBSHX1-odc construct. The HX1-odc cassette was excised and inserted into the *Kpn*I cloning site of pTAC, yielding pTAC-odc (Fig. 1C).

A plasmid containing *T. cruzi* subtelomeric sequences, termed pTACφGP85, was constructed by cloning subtelomeric sequences from the recombinant *T. cruzi* telomeric BAC D6C (Kim et al., 2005; GenBank accession No. AY551440) into pTAC. A 1.7 kb fragment from BAC D6C (positions nt 19806 to 21479) containing the truncated open reading frame (ORF) of a GP85 (trans-sialidase) pseudogene was amplified by PCR, cloned in fusion with the E-GFP gene from pEGFP (Clontech, USA) and the resulting fragment inserted into the *Kpn*I site of pTAC, generating the construct pTAC φGP85 (Fig. 1D).

All vectors were amplified in *E. coli* DH5α strain in Luria-Bertani medium containing ampicillin, extracted and purified with Qiagen maxiprep columns (Qiagen, USA).

2.5. Transfection and selection of T. cruzi epimastigotes

Electroporation of epimastigotes was performed as previously described (Vázquez and Levin, 1999). All constructs were linearized with *Hin*dIII before transfections, except pTREX-gfp which was used for control transfections.

The *neo* gene confers resistance to the aminoglycoside G418 and the *pac* gene confers resistance to the amynonucleoside puromycin. Cells were diluted in 10 ml of LIT medium and incubated for

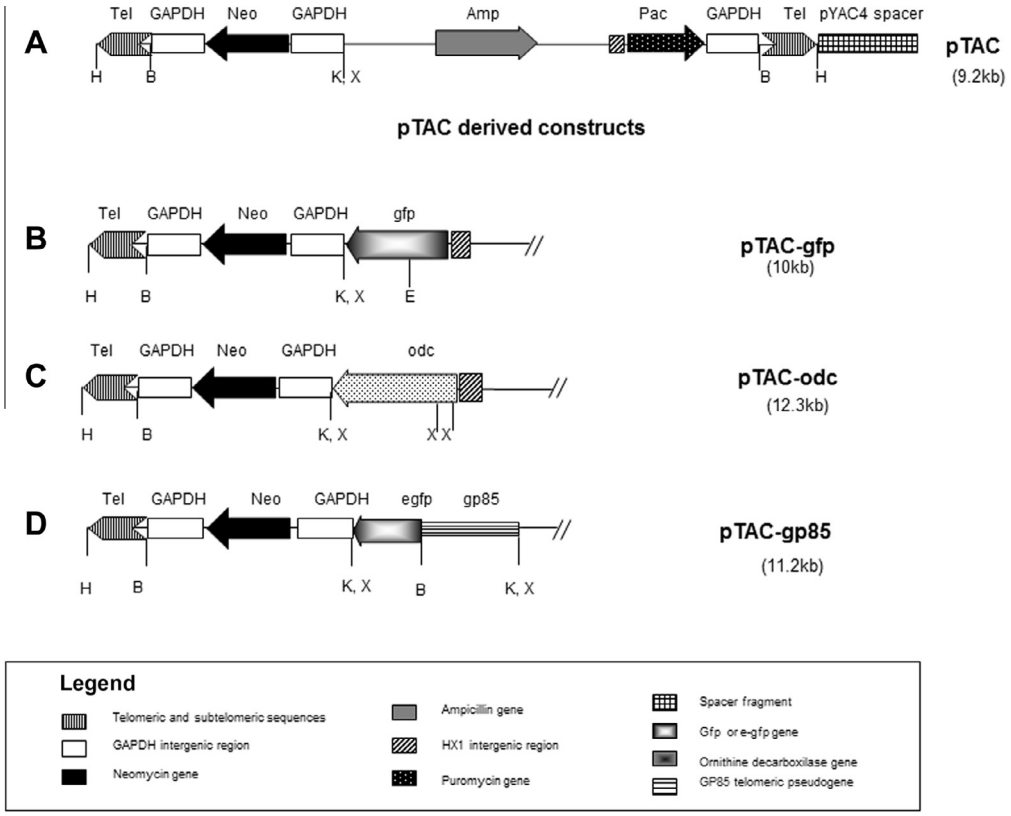

Fig. 1. Schematic representation of the pTAC (Trypanosoma Artificial Chromosomes) vector series. The constructs were built on a pBluescript (pBS) vector backbone (black solid line) and include subtelomeric and telomeric sequences (Tel) from *Trypanosoma cruzi*. All vectors include the neomycin resistance cassette (NEO), a puromycin resistance cassette (PAC) and a spacer fragment. (A) pTAC (9.2 kb). (B) pTAC-gfp (10 kb) harbours an engineered version of the GFP(gfp65s(T) gene containing an *Eco*RI restriction site. (C) pTAC-odc (12.3 kb) carries the *Crithidia fasciculata* ornithine decarboxylase (ODC) gene flanked by *T. cruzi* RNA processing signals. (D) pTAC-φgp85 (11.2 kb) includes a 1.7 kb fragment from BAC D6C. This vector contains a modified GFP gene (*e-gfp*). The names of the resulting vectors are indicated on the right of the schematic drawings. Sizes are given, excluding the spacer fragment (3.5 kb). Relevant restriction sites are indicated: X, *Xho*I; H, *Hin*dIII; E, *Eco*RI; B, *Bam*HI; and K, *Kpn*I.

48 h at 28 °C to allow recovery before the addition of 60 μg/ml of G418; 20 μg/ml of puromycin or 60 μg/ml of G418 plus 10 μg/ml of puromycin.

Selective pressure was kept at the lowest effective concentrations to avoid an increase in the copy number of the episomes due to excessive stress (Lee et al., 1995; Supplementary Fig. S1). Recovery of resistant parasites was achieved after 5 weeks of culture whereas mock controls stopped dividing after 3 weeks under these conditions. To monitor the selection process, wild type trypanosomes were also electroporated with circular pTREX-gfp (Da Rocha et al., 2004b) and after 5 weeks under selective pressure, the entire population became fluorescent. After selection, epimastigotes were cloned by limiting dilutions in LIT medium in the presence of selective pressure at 28 °C and, unless otherwise noted, only stable lines were used for the experiments. Cells were cultured in 96 well-plates and expanded to 24 well-plates or T25 bottles when necessary.

For functional complementation studies, two clones of parasites bearing pTAC-odc and one clone bearing pTAC-gfp, used as a control, were cultivated in SDM79 medium containing 60 μg/ml of G418 plus 10 μg/ml of puromycin. CL Brener wild type epimastigotes were kept in SDM79 medium without selection.

Cell growth curves were established as follows: cultures were seeded with 1–2 $\times$ 10^6 cells at day 0 and epimastigotes were counted daily on a hemocytometer from day 2 to day 12.

Observation of live parasites bearing pTAC-gfp was carried out directly on a slide after washing with PBS using an Olympus BX51 fluorescence microscope.

Estimation of expression efficiency of the pTAC-gfp vector was done by counting fluorescent cells over total cells in the same field. For that, cells were fixed with a solution of 4% paraformaldehyde in PBS for 10 min. Ten fields were counted for each clone, equating to approximately 600 cells per clone. The 95% confidence interval (CI) for expression efficiency was calculated using the standard formula: $a \pm (a\ (1\text{-}a)/n)^{1/2}$ where a = proportion of fluorescent cells and n = number of total cells.

2.6. Exonuclease assay

Genomic DNA was extracted from relevant clones and treated with 0.1 unit of Bal 31 exonuclease (New England Biolabs, USA) for different lengths of time. Digestions were run in agarose gels, transferred to Southern blots and hybridised with Gfp or Neo probes. To assess the activity of the enzyme in an endogenous chromosome, the DNA treated with Bal 31 was digested with *Bam*HI to allow running in a conventional gel, before Southern blotting and hybridization with a Sz5 probe.

To test the activity of Bal 31 over circular and linear targets, pTACgfp was used in its plasmid (circular) and linearized (HindIII digested) forms.

The action of Bal 31 on the endogenous chromosomes was addressed by digestion of agarose embedded DNA for 15 min; the reaction was stopped using 0.5 M EDTA pH 8 (final concentration: 40 mM EDTA) and the agarose blocks submitted to FIGE.

2.7. Stability of TACs in the absence of selective pressure

Transfected clones bearing pTAC-gfp were subcultured (1:100 dilution at each passage, equivalent to approximately seven generations between passages) from an initial concentration of 10^7 parasites/ml in media without G418 or puromycin. At different times, agarose blocks containing chromosome-sized DNA were submitted to PFGE, Southern-transferred and hybridised with Gfp, Neo or 189jc probes.

2.8. PCR and reverse transcription-PCR (RT-PCR) analysis of transfected parasites

The orientation of *gfp* and *pac* genes was addressed by PCR from whole genomic DNA obtained from pTAC-gfp transfected parasites (Supplementary Table S1). The SL acceptor site of *gfp* and *odc* transcripts was determined by RT-PCR. Total RNA (2 μg) was reverse-transcribed using the Retroscript system (Ambion, USA), using random decamers as primers. The cDNA was amplified using as a sense primer an oligonucleotide derived from the *T. cruzi* SL sequence and GFPr or ODCr as antisense oligonucleotides, respectively (Supplementary Table S1). PCR products were cloned into a pGem-T easy vector (Promega, USA) for sequencing.

2.9. Copy number estimation by real-time quantitative PCR (qPCR)

The copy number of TACs into pTAC-gfp transfected parasites was estimated by qPCR (Lee et al., 2006). In order to build a standard calibration curve, serial dilutions of pTAC-gfp plasmid (1, 2, 5, 10, 20, 50 and 100 copies/cell), were added to 10 ng of CL Brener genomic DNA. A Rotor-Gene 6000 device (Qiagen) was used for amplification and detection. The 20 μl reaction tube contained 0.5 mM of primers GFPup and GFPlow (Supplementary Table S1), 3 mM $MgCl_2$, 250 mM of each dNTP, 0.5 U of Platinum Taq polymerase (Invitrogen), SYBR Green (Invitrogen) at a final concentration of 0.5x and 10 ng of sample DNA. After 5 min of pre-incubation at 95 °C, PCR amplification was carried out for 40 cycles (94 °C for 10 s, 61 °C for 10 s and 72 °C for 10 s). The tubes were read at 72 °C at the end of each cycle. Amplification was immediately followed by a melt program with an initial denaturation step of 5 s at 95 °C and then a stepwise temperature increase of 0.1 °C/s from 72–90 °C.

2.10. Infection of mice with transfected parasites

Parasites were maintained alternately in mice and LIT medium containing 10% FBS at 28 °C. Metacyclic trypomastigotes from selected clones were harvested from cultures in the stationary growth phase and injected i.p. into female Swiss mice (12 weeks old). Subsequently, 3 weeks after infection, mice were bled by heart puncture and their blood seeded in LIT medium. To verify the presence of viable parasites, hemocultures were examined daily starting 2 weeks after seeding.

3. Results

On the basis of a pTAC construct that harbours telomeric and subtelomeric sequences at its ends and two cassettes for resistance to Neomycin and Puromycin in opposite orientations (Fig. 1A), two new versions of pTACs were constructed, namely pTAC-gfp (Fig. 1B) and pTAC-odc (Fig. 1C). The former carries a modified version of the GFP gene (*gfp*65t) containing an internal *Eco*RI recognition sequence as a cloning site, (Fig. 1B). The latter contains the *odc* gene from *Crithidia fasciculata*, allowing functional complementation assays (Fig. 1C).

3.1. Characterization of recombinant parasites carrying pTAC-gfp

CL Brener epimastigotes were transfected with linearized pTAC-gfp, grown under selection pressure, and selected clones were analysed by PFGE. The CHEF blot (Fig. 2A) revealed only one hybridising band of approximately 50 kb in clone C6, using a Gfp probe. The FIGE blot showed the presence of a 10 kb band hybridising with Gfp (Fig. 2B) and Neo (not shown) probes in clones E4, B5, B10 and D9, indicating the formation of artificial chromosomes of the

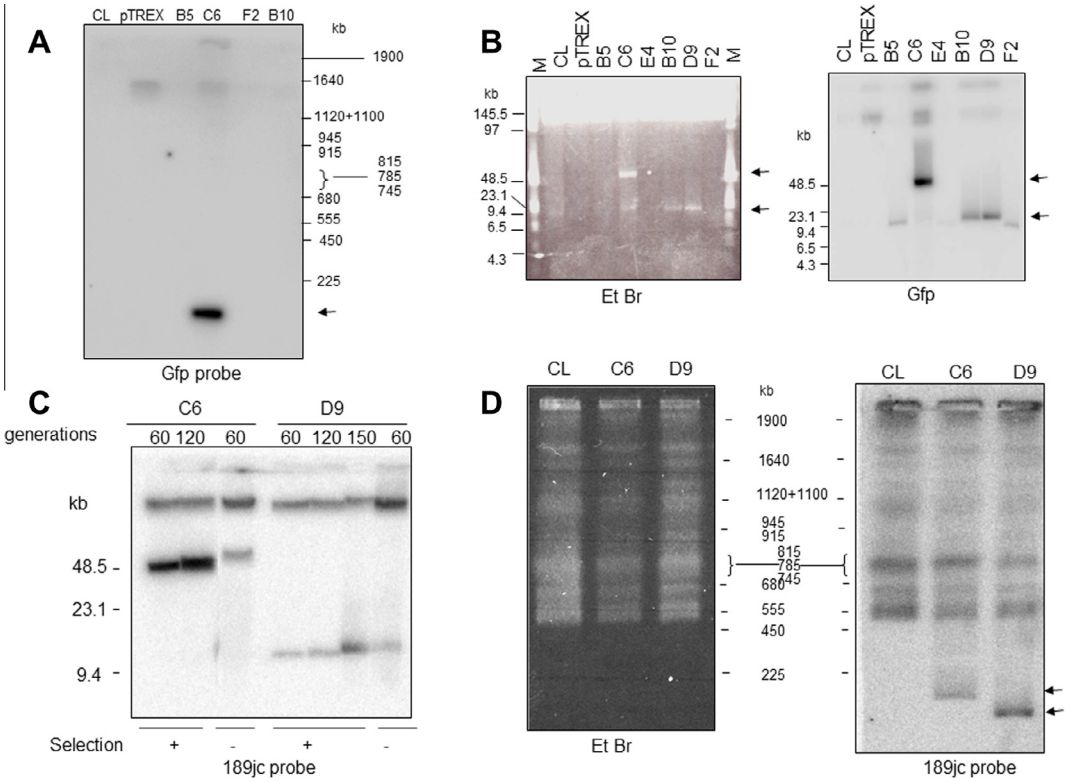

Fig. 2. Karyotypes of cell lines harbouring Trypanosoma Artificial Chromosomes (TAC)-gfp. Stability and presence of telomeres in derived TACs. (A, B) Contour-clamped Homogeneous Electric Field electrophoresis and Field Inversion Gel Electrophoresis analysis of clones bearing artificial chromosomes derived from pTAC-gfp. Clones were grown in liver infusion tryptose medium containing G418 and puromycin. CL, non-transfected cells; pTREX, cells transfected with pTREX-gfp, a vector that integrates into the ribosomal locus. DNA was Southern transferred and hybridised with a Gfp probe. For FIGE analysis, program 4 of Field Inversion Gel Electrophoresis Mapper (Bio Rad, USA) was used. (C) Stability of cell lines harbouring TAC-gfp. Clones were grown with and without selective pressure and genomic DNA prepared at indicated times. After electrophoresis and Southern transfer, the blot was hybridised with a probe spanning the 189 telomeric junction to assess the presence of telomeres. Program 10 of Field Inversion Gel Electrophoresis Mapper (Bio Rad) was used. (D) The pulse-field gel electrophoresis blot was hybridised with a 189jc probe to show TACs and endogenous chromosomes of *T. cruzi*. Arrows denote relevant bands. EtBr, ethidium bromide.

same size as the input vector, whereas clone C6 showed a TAC of higher molecular weight (Fig. 2B). For unknown reasons, agarose blocks from the E4 clone yielded a low concentration of embedded DNA, however a faint hybridising band with the Gfp probe was detected. Restriction analysis of genomic DNA from clone C6 revealed that the internal structure of the vector is conserved (data not shown).

The maintenance of TAC-gfp (Fig. 2C) and the corresponding fluorescence of cell lines (Supplementary Fig. S2) were demonstrated for more than 120 generations in selective medium and for more than 60 generations in the absence of antibiotics. The hybridization with the telomeric189jc probe (Fig. 2C) also demonstrated that telomeres were still present after 120 and 60 cell divisions in medium with or without antibiotics, respectively. These findings indicate that TACs are correctly replicated and segregated by the parasite.

The fluorescence was evenly displayed inside the cell, although parasites from the same clone exhibited different intensities (Supplementary Fig. S2). Parasites bearing TAC-gfp exhibited a lower intensity of fluorescence than a cell line transfected with pTREX-gfp, used as a control, which is likely due to the presence of the ribosomal promoter in the latter.

Two clones (D7 and G1) obtained in independent transfection experiments were used to estimate the expression efficiency of GFP. D7 clone (a sub-clone obtained from C6 clone) has been kept under continuous culture for 4 years and G1 clone for 18 months. (Karyotipes of D7 and G1 clones are shown in Supplementary Figs. S1B and S1C) The percentages of fluorescent cells reached 52 and 59%, respectively (Supplementary Fig. S3).

3.2. Linearity and copy number of TACgfp

In order to assess linearity of TACs, clones D9 and C6 were selected. Accordingly, genomic DNA from them was exposed to Bal 31 exonuclease digestion for different times, southern-transferred and hybridised with Neo and Gfp probes (Fig. 3A). Fig. 3A shows that hybridising bands disappeared after 8 min of exonuclease exposure. As a control, a probe spanning the Sz5 locus present in the *T. cruzi* genome was used to show the action of Bal 31 on natural chromosomes. An hybridising band was still visible after 20 min of digestion, reflecting the longer distance from telomeres with respect to fragments present in TACs. The activity of Bal 31 exonuclease on linear and circular forms of the vector is shown in Fig. 3B. The action of the enzyme on the endogenous chromosomes of the parasite is clearly noted in the compression zone of the ethidium bromide (EtBr) stained gel carrying agarose-embedded DNA from different clones (Fig. 3C).

Analysis of the mobility of TACs under different conditions of PFGE showed migration patterns that followed those of linear molecular weight markers, confirming their linear structure (Fig. 2B, C).

Comparative densitometric analysis of genomic Southern blots containing C6 and D9 DNA corresponding to approximately 75 generations yielded 11 ± 2 gfp gene copies/cell (Supplementary Fig. S4A). Estimation of the copy number of TACs was also performed by qPCR on genomic DNA extracted from the above-mentioned clones, 90 days later (approximately 75 additional cell divisions in medium with G418 and puromycin). A fragment of 170 bp from the *gfp* gene was used as a target for Real Time PCR

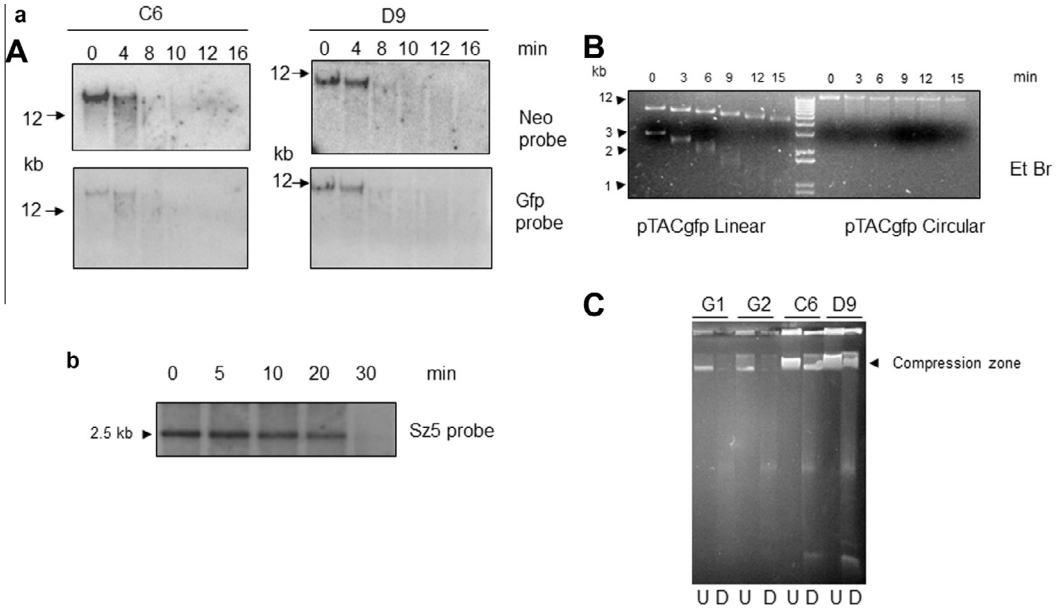

Fig. 3. Linear structure of pTAC-gfp-derived artificial chromosomes. (a) Total genomic DNA from clones C6 and D9 was exposed to Bal 31 exonuclease for different times, Southern transferred and hybridised with Neo or Gfp probes. (b) The *sz5* locus has an internal location on the *Trypanosoma cruzi* chromosome 20P (TriTrypDB), hence it is a positive control for digestion of a linear molecule. The time required by this fragment to be digested was evaluated by exposing genomic DNA to Bal 31. The products of Bal 31 action were further digested with *Bam*HI enzyme and Southern transferred. The blot was hybridised with a Sz5 probe. (B) Linear and circular forms of the original vector were included as controls. Linear pTAC-gfp: pTAC-gfp was previously digested with *Hin*dIII and the products (the 10 kb linear vector and the 3.5 kb spacer fragment) were exposed to Bal 31 exonuclease. Circular pTAC-gfp: pTAC-gfp in its circular form was exposed to Bal 31 exonuclease. Sizes of the bands and 1 kb Plus DNA ladder (Invitrogen, USA) are indicated. (C) Agarose-embedded DNA from selected clones was exposed to Bal 31 exonuclease for 15 min and blocks were subsequently submitted to Field Inversion Gel Electrophoresis. U, undigested control; D, Bal 31 digested samples; EtBr, ethidium bromide. TAC, *Trypanosoma* Artificial Chromosomes.

based quantification, allowing detection of 6.7 ± 1.6 gene copies/cell for clone C6 and 7.5 ± 1.3 for clone D9 (Supplementary Fig. S4B), in accordance with the estimates obtained from Southern blot analysis. Therefore, the persistence of TACs after 150 generations confirms their intrinsic stability and faithful segregation.

3.3. Structure of TACs in pTAC-gfp transformants

To check whether the TAC structures generated by pTAC-gfp are preserved with respect to the input vector, RT-PCR and PCR strategies were carried out in transfected cell lines. First, we mapped the 5′ splicing acceptor site of the *gfp* mature transcript. RT-PCR from total RNA extracted from clones D7 and D9 using SL forward and GFP reverse primers (Supplementary Table S1) amplified the expected band of 470 bp (Fig. 4B, oligos 1 and 2). Sequencing of this amplicon showed that the AG utilised as a 5′ acceptor site was the expected one, which is located within the HX1 region (data not shown). D7 is a sub-clone obtained after the re-cloning process of the C6 clone and its karyotype is shown in Supplementary Fig. S1B.

Second, a single primer PCR approach amplified a specific 3 kb band in clones C6 and D9 but not in CL Brener wild type parasites, demonstrating the presence of the inverted structure of both HX1 regions flanking the pBS backbone (Fig. 4C, oligo 3).

Third, we carried out PCR on genomic DNA from clone D9, to assess whether the structure of the HX1-pac region was conserved. UNI forward and PAC reverse primers (Supplementary Table S1) produced the approximately 700 bp expected band (Fig. 4D, oligo 4 and 5) and the fragment conserved structure was confirmed by sequence analysis (data not shown).

3.4. TACs are maintained through different T. cruzi developmental stages

To analyse segregation and maintenance of TACs during parasite development in the mammalian stage, metacyclic trypomastigotes were obtained from cell lines carrying pTAC-CφGP85 (Fig. 1D; R. Moraes Barros, unpublished data) and mice were infected with three different clones (namely C4, C7 and C8). After 21 days of infection, parasite populations were isolated from murine blood and DNA was extracted for hybridization experiments using Neo and Gfp probes. A single hybridising band of ~12 kb was detected in clones C4 and C7 whereas two bands of 12 and 23 kb were seen in clone C8 (Fig. 5A), suggesting that the latter could be a dimeric TAC. When C8 was re-cloned by the dilution method, only clones showing the single 12 kb hybridising band were obtained (Fig. 5B).

These findings provide evidence that TACs are stable in an in vivo experimental model. Furthermore, on the basis of similar courses of parasitemia after infection by either wild-type or TAC-containing parasites, we found no growth disadvantage conferred by TACφGP85 during parasite mammalian-stage development.

3.5. Functional complementation expressing a transgene from C. fasciculata

To investigate the feasibility of TACs as exogenous gene expression vectors we cloned the coding region of the *C. fasciculata odc* gene into a pTAC vector to generate pTAC-odc (Fig. 1C; see Section 2.4.2). The ODC gene codes for the enzyme ornithine decarboxylase, that catalyses the decarboxylation of ornithine to form putrescine, a critical step in the synthesis of polyamines. *Trypanosoma cruzi* lacks this enzyme and, therefore, it cannot grow in vitro without the exogenous supplement of polyamines (Carrillo et al., 1999).

To evaluate whether pTAC-odc was capable of expressing the *odc* transgene at levels high enough to revert the natural polyamine auxotrophy of *T. cruzi*, epimastigotes were transfected with pTAC-odc and grown in the presence of G418, puromycin or both antibiotics.

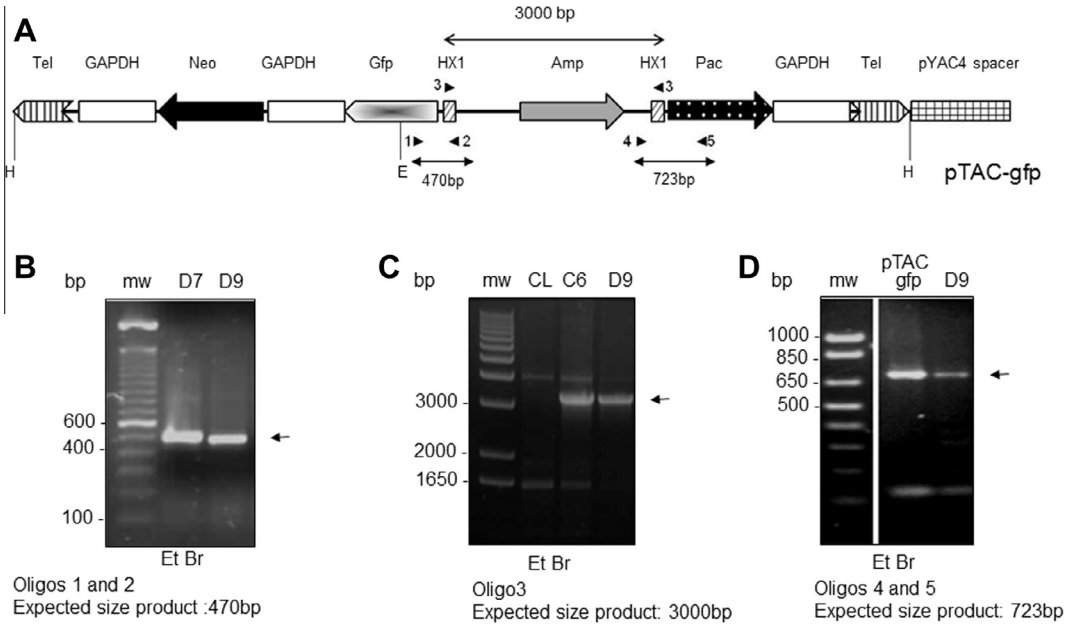

Fig. 4. Structure of Trypanosoma Artificial Chromosomes (TACs) in pTAC-gfp transformants. Total DNA and RNA were extracted from clones bearing TACs derived from pTAC-gfp. Reverse Transcription-PCR and PCR were carried out to assess the transcription direction. (A) Schematic representation of pTAC-gfp showing the position of primers used: 1, GFPrev; 2, HX1GF5; 3, HX1rev; 4, UNIrev and 5, PACshort. (B) Reverse Transcription-PCR. The expected fragment of 470 bp was obtained by PCR over cDNA with spliced leader sequence (SL) and GFPrev primers. (C) Genomic PCR. Single-primer PCR with HX1rev oligonucleotide over genomic DNA from non-transformed parasites (CL) and selected clones (C6,D9) gave the 3 kb expected product only with transfected cell lines. (D) PCR with UNIrev and PACshort primers over DNA from the input vector and D9 clone shows that the structure is conserved in artificial chromosomes. Tel: telomere, GAPDH: glyceraldehyde 3′ phosphate deshidrogenase, Neo: Neomycin, Gfp: Green Fluorescent Protein, HX1: intergenic region from the *TcP2β* gene, Amp: ampicillin, pYAC4: 3.5 kb spacer fragment from pYAC4.

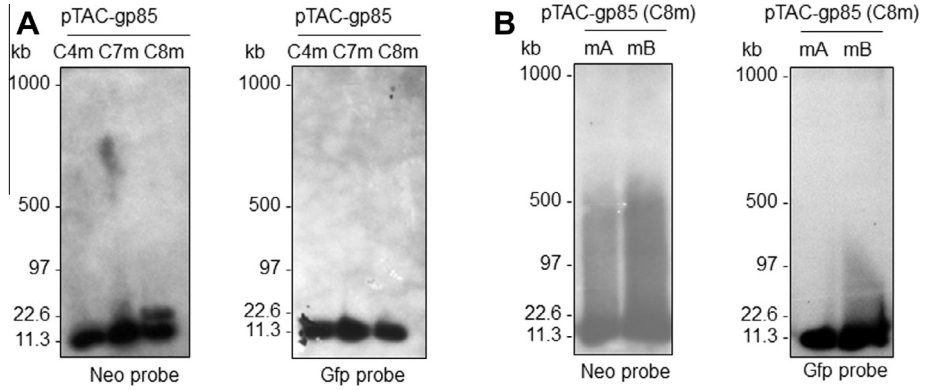

Fig. 5. pTACφgp85 is stably maintained in parasites during the developmental cycle in the vertebrate host. A region of 1.7 kb from the recombinant *Trypanosoma cruzi* telomeric BAC D6C was subcloned into a pTAC vector, generating pTACφgp85. Epimastigotes were transfected with *Hin*dIII linearized vectors and single clones were obtained. Mice were infected with three different clones (namely C4, C7 and C8). Clones C4, C7 and C8 carrying pTACφgp85 were recovered after passage in mice, genomic DNA was separated by electrophoresis and hybridised with Neo and Gfp probes. (A) Pulse-field gel electrophoresis blot hybridization of chromosomes from three cell populations derived from clones C4, C7 and C8. (B) Parasites derived from clone C8 recovered from mice were recloned and genomic DNA from two subclones (mA and mB) was separated by electrophoresis and hybridised with Neo and Gfp probes.

Analysis of transferred PFGE blots showed, for the three tested conditions, positive hybridising bands with both Neo and Pac probes in cell lines harbouring TAC-odc (data not shown). Fig. 6A shows a blot containing genomic DNA from cell lines exhibiting extrachromosomal elements generated by pTAC-odc as well as clones containing TAC-gfps included as size markers. Two of the clones selected in the presence of both G418 and puromycin, named ODC3 and ODC5, were grown in SDM79, a semi-defined culture medium almost free of polyamines, including the same selection drugs. Controls included CL Brener wild type parasites and a pTAC-gfp transformant cell line.

Fig. 6B shows that, contrary to wild type or pTAC-gfp transfected control trypanosomes, clones carrying TAC-odc could survive in SDM79, thus overcoming *T. cruzi* natural polyamine auxotrophy. Transcription of the *odc* gene was investigated by RT-PCR carried out over RNA extracted from epimastigotes from the entire population or from selected cell lines (Fig. 6C).

In all pTAC-odc transformants, the sequencing of the amplified DNA fragment showed the addition of the 39 nt SL to the predicted AG site following the polypirimydinic tract of the HX1 region (Fig. 6D).

The above-mentioned results provide evidence of TACs as useful tools to stably express exogenous genes in *T. cruzi* with functional activity.

4. Discussion

The identification of new targets for vaccine and drug development for the treatment of Chagas' disease is dependent on deepening our understanding of the parasite genome. In this context,

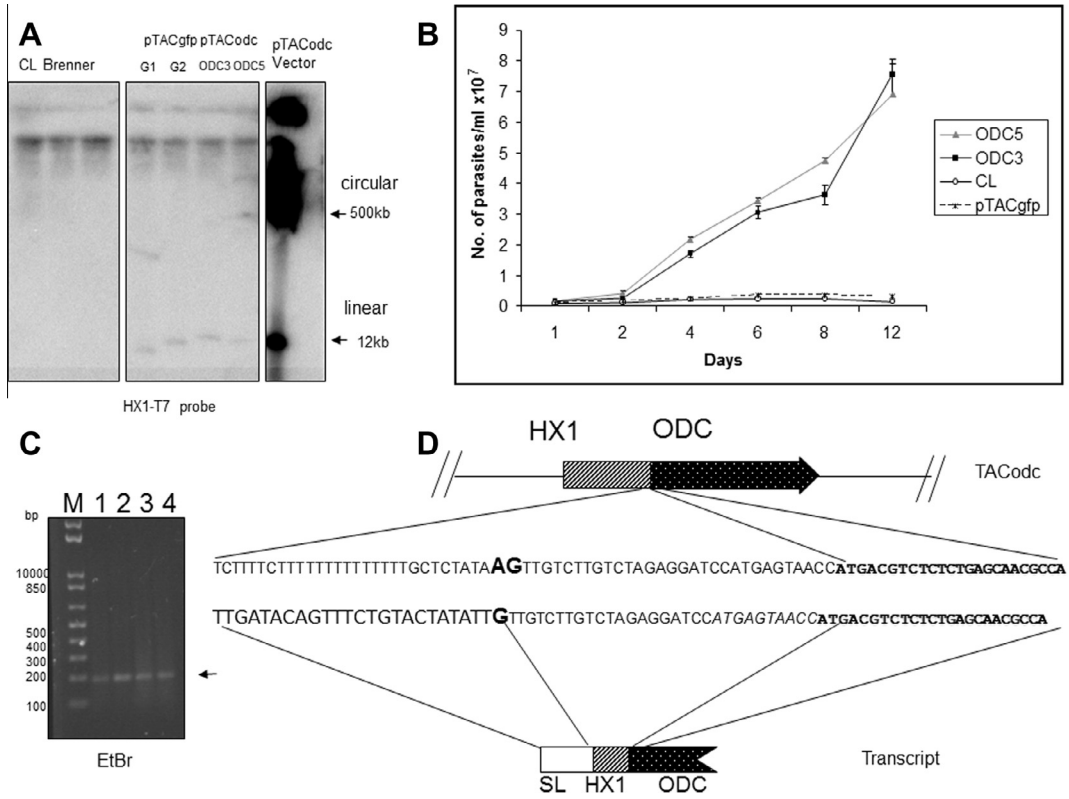

Fig. 6. pTAC-odc complements ornithine decarboxylase (ODC) activity in *Trypanosoma cruzi*. Karyotyping of pTAC-odc-transfected clones. Genomic DNA was submitted to pulse-field gel electrophoresis, transferred and hybridised with the vector probe HX1-T7. pTAC-gfp-transfected clones were included, as well as linear and circular forms of the input vector. The positive signals of large molecular weight are due to the HX1 portion of the probe, present in the genome of wild type parasites (note the signals in the CL Brener lanes). (B) Functional complementation of ODC activity in *T. cruzi* by a pTAC-odc vector. Growth curves of pTAC-odc-bearing parasites (clones C5 and B5) and pTAC-gfp in a G418 + puromycin semi-defined medium (SDM 79) with low polyamine content. Wild type parasites (clone CL Brener, CL) were grown in SDM 79 without selective drugs. Values are the means ± S.D. of three assays performed in triplicate. (C) Transcription of *odc* by pTAC-odc transformants. ReverseTranscription-PCR was carried out over total RNA of pTAC-odc transfected cell lines with spliced leader sequence (SL) and ODCrev primers (see Supplementary Table S1). Lanes: M, 1 kb Plus DNA marker (Invitrogen, USA); 1, Population TAC-odc grown in SDM 79 without selection; 2, Population TAC-odc grown in SDM79 plus G418 + Puromycin; 3, clone ODC3 grown in SDM79 plus G418 + Puromycin; 4, clone ODC5 grown in SDM79 plus G418 + Puromycin. (D) Mapping of the trans-splicing site in pTAC-odc transformants. Sequencing of the fragments allowed identification of the AG acceptor site from HX1 used for processing the mRNA by pTAC-odc transfected parasites. TAC, *Trypanosoma* Artificial Chromosomes.

vectors for genetic manipulation are needed and in particular those generating artificial chromosomes may be particularly useful to overcome problematic transgene expression often occurring when conventional vectors are used. Accordingly, we have developed a series of TAC constructs that once inside the parasite cells remained as linear molecules both in the presence and absence of selective pressure. Indeed, the maintenance of pTAC-gfp (Fig. 2) derived molecules and the corresponding fluorescence of cell lines (Supplementary Fig. S2) were demonstrated for more than 150 generations in selective medium and for more than 60 generations in the absence of antibiotics. These findings indicated that TACs are correctly replicated and segregated by the parasite. Similar constructs made for *T. brucei* were lost after no more than 15 generations (Patnaik et al., 1996). This is a remarkable feature of *T. cruzi* TACs, which may constitute an useful tool for long-term experiments in the absence of selective drugs or for in vivo imaging studies in murine models (Guevara et al., 2005), where the linear molecules were stable for more than 1 year (Fig. 5; Moraes Barros, unpublished data).

Several clones harbored TACs of the same size as the input vector, whereas clone C6 TAC showed an increase in size, probably due to an oligomerization of the input vector, given that the internal structure was conserved (data not shown). These results are in agreement with previous works that show that the amplification of extrachromosomal elements occurs either spontaneously or in response to drug selection (Alsford et al., 2003) and is a frequent event in transformation experiments in trypanosomatids, both with circular or linear molecules (Kelly et al., 1992; Dubessay et al., 2002a). This clone also presented faint bands of high molecular weight, which might be indicative of integration events, that seem to be absent in clones with smaller TACs. The moderate size increase of the TACs detected over time (Fig. 2C) is probably due to telomeric growth, as observed in *T. brucei* TACs (Patnaik et al., 1996).

Fluorescence in pTAC-gfp transformants appeared evenly displayed in the whole cell, although parasites from the same clone exhibited different intensities. Similar observations were made for other trypanosomatids with different vectors carrying fluorescent markers (Tetaud et al., 2002; Da Rocha et al., 2004b; Taylor and Kelly, 2006; Bouvier et al., 2013). The percentages of fluorescent cells reached 52 and 59% in clones that had been continuously cultured for approximately 1200 and 460 generations (D7 and G1 respectively, assuming a doubling time of 28 h). These figures further confirm the stability of pTACs vectors. Previous studies suggested that stochasticity may play an important role in gene expression and that the concept of a clone is a statistical oversimplification, representing a series of individuals having the same genome but capable of exhibiting wide phenotypic variation (Veitia, 2005). This is particularly important with heterologous gene expression, due to the lack of redundant genes or alternative ways to compensate for stochastic events.

The linear molecules generated from pTACφgp85 exhibited a similar behaviour to those from pTAC-gfp with regard to stability, segregation and conserved structure. Although the inclusion of

additional subtelomeric sequences did not confer them with different characteristics, these molecules replicated and segregated during both the insect and the mammalian stages. One of the clones (C8 m) obtained from infected mice presented a double band when analysed by hybridization experiments that was not seen in clones C8 mA or C8mB (Fig. 6) A possible explanation is that cells carrying dimeric TACs were lost during the re-cloning process. Alternatively, the double band present in the Neo probing experiment, but not with the Gfp probe, could correspond to a rearranged form of the vector that was not stable enough and was consequently lost.

In *T. brucei*, it has been shown that the loss of TbAGO 1 activity is responsible for growth and segregation defects (Durand-Dubief et al., 2007); a protein exhibiting some characteristics of AGO/PIWI proteins has been found in *T. cruzi*, but its biological function has not yet been revealed (Garcia Silva et al., 2010). Other studies have suggested that centromeric functions may be related with subtelomeric sequences (Dubessay et al., 2002a). The only sequence shared by TACs and natural chromosomes of *T. cruzi* are the telomeric repeated hexamers, the 189 bp junction and to some extent, subtelomeric sequences; hence, unless the above-mentioned elements are involved in TAC segregation, no other ones can be functionally ascribed in our constructs. The pBS backbone used to build TACs could be regarded as another potential source of stability and of ARS sequences; clearly more experiments are needed to address these questions.

All of these facts might also reflect a lack of specific requirements for functional centromere and/or ARS, or the existence of another mechanism to achieve the faithful segregation of chromosomes in *T. cruzi*. Indeed, it has been proposed that centromere identity and function are regulated epigenetically through the formation of a specialized chromatin structure rather than by a specific DNA sequence or chromosomal region (Elias and Faria, 2009; Torras-Llort et al., 2009).

Fig. 3 shows that, contrary to wild type or pTAC-gfp transfected trypanosomes, clones carrying TAC-odc could survive in SDM79 and thus overcame *T. cruzi* natural polyamine auxotrophy. This result provides evidence of TACs as useful tools for exogenous gene expression in *T. cruzi* at levels that are physiologically functional for the parasite.

Another characteristic of this type of molecule is its independence of Discrete Typing Unit (DTU) specific sequence requirements, so we reasoned that TACs could perform efficiently in strains belonging to different DTUs. As an example, transgenic populations of Sylvio X10 parasites (DTU I) harbouring pTAC-gfp and pTAC-rfp were obtained (data not shown) and are currently in the selection process. Preliminary results give us confidence to extend our studies to different strains.

In summary, we have demonstrated that TACs behave as linear extrachromosomal elements similar to natural chromosomes of *T. cruzi*. A lack of chromatin condensation during mitosis prevents the use of classical approaches to study chromosome behaviour and, therefore, TACs may provide an excellent model to study putative elements for proper replication, maintenance and segregation of natural chromosomes and to further investigate mechanisms of genetic exchange in *T. cruzi* (Gaunt et al., 2003; Andersson, 2011; Minning et al., 2011). TACs remained stable and segregated faithfully even in the absence of drug selection. Moreover, we have shown their utility as cloning and functional complementation tools, thus expanding the available toolbox for genetic manipulation in *T. cruzi*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpara.2014.03.009.

References

Alsford, S., Navarro, M., Jamnadass, H., Dunbar, H., Murphy, N.B., Gull, K., Ersfeld, K., 2003. The identification of circular extrachromosomal DNA in the nuclear genome of *Trypanosoma brucei*. Mol. Microbiol. 47, 277–289.

Andersson, B., 2011. The *Trypanosoma cruzi* genome; conserved core genes and extremely variable surface molecule families. Res. Microbiol. 162, 619–625.

Araújo, P., Burle Caldas, G., Silva Pereira, R., Bartholomeu, D., Da Rocha, W., Teixeira, S., 2011. Development of a dual reporter system to identify regulatory cis-acting elements in untranslated regions of *Trypanosoma cruzi* mRNAs. Parasitol. Int. 60, 161–169.

Batista, M., Marchini, F., Celedon, P., Fragoso, S., Probst, C., Preti, H., Ozaki, L., Buck, G., Goldenberg, S., Krieger, M., 2010. A high-throughput cloning system for reverse genetics in *Trypanosoma cruzi*. Microbiology 10, 259.

Berriman, M., Ghedin, E., Hertz-Fowler, C., Blandin, G., Renauld, H., Bartholomeu, D.C., Lennard, N.J., Caler, E., Hamlin, N.E., Haas, B., Böhme, U., Hannick, L., Aslett, M.A., Shallom, J., Marcello, L., Hou, L., Wickstead, B., Alsmark, U.C., Arrowsmith, C., Atkin, R.J., Barron, A.J., Bringaud, F., Brooks, K., Carrington, M., Cherevach, I., Chillingworth, T.J., Churcher, C., Clark, L.N., Corton, C.H., Cronin, A., Davies, R.M., Doggett, J., Djikeng, A., Feldblyum, T., Field, M.C., Fraser, A., Goodhead, I., Hance, Z., Harper, D., Harris, B.R., Hauser, H., Hostetler, J., Ivens, A., Jagels, K., Johnson, D., Johnson, J., Jones, K., Kerhornou, A.X., Koo, H., Larke, N., Landfear, S., Larkin, C., Leech, V., Line, A., Lord, A., Macleod, A., Mooney, P.J., Moule, S., Martin, D.M., Morgan, G.W., Mungall, K., Norbertczak, H., Ormond, D., Pai, G., Peacock, C.S., Peterson, J., Quail, M.A., Rabbinowitsch, E., Rajandream, M.A., Reitter, C., Salzberg, S.L., Sanders, M., Schobel, S., Sharp, S., Simmonds, M., Simpson, A.J., Tallon, L., Turner, C.M., Tait, A., Tivey, A.R., Van Aken, S., Walker, D., Wanless, D., Wang, S., White, B., White, O., Whitehead, S., Woodward, J., Wortman, J., Adams, M.D., Embley, T.M., Gull, K., Ullu, E., Barry, J.D., Fairlamb, A.H., Opperdoes, F., Barrell, B.G., Donelson, J.E., Hall, N., Fraser, C.M., Melville, S.E., El-Sayed, N.M., 2005. The genome of the African Trypanosome *Trypanosoma brucei*. Science 309, 416–422.

Bouvier, L.A., MdlM, Cámara., Canepa, G.E., Miranda, M.R., Pereira, C.A., 2013. Plasmid vectors and molecular building blocks for the development of genetic manipulation tools for *Trypanosoma cruzi*. PLoS One 8, e80217. http://dx.doi.org/10.1371/journal.pone.0080217.

Brun, R., Schonenberger, M., 1979. Cultivation and in vitro cloning of procyclic culture forms of *Trypanosoma brucei* in a semi-defined medium. Acta Trop. 36, 289–292.

Burke, D., Carle, G., Olson, M., 1987. Cloning of large segments of exogenous DNA into yeast by means of artificial chromosome vectors. Science 236, 806–812.

Cano, M.I., Gruber, A., Vazquez, M., Cortes, A., Levin, M., González, A., Degrave, W., Rondinelli, E., Zingales, B., Ramírez, J., Alonso, C., Requena, J., Da Silveira, J., 1995. Molecular karyotype of clone CL Brener chosen for the *Trypanosoma cruzi* genome project. Mol. Biochem. Parasitol. 71, 273–278.

Carrillo, C., Cejas, S., Gonzalez, N., Algranati, I., 1999. *Trypanosoma cruzi* epimastigotes lack ornithine decarboxylase but can express a foreign gene encoding this enzyme. FEBS Lett. 454, 192–196.

Casagrande, L., Ruiz, J., Beverley, S., Cruz, A., 2005. Identification of a DNA fragment that increases mitotic stability of episomal linear DNAs in *Leishmania major*. Int. J. Parasitol. 35, 973–980.

Chiurillo, M., Cano, I., Da Silveira, J., Ramírez, J., 1999. Organization of telomeric and subtelomeric regions of chromosomes from the protozoan parasite *Trypanosoma cruzi*. Mol. Biochem. Parasitol. 100, 173–183.

Chiurillo, M., Santos, M., Da Silveira, J., Ramírez, J., 2002. An improved general approach for cloning and characterizing telomeres: the protozoan parasite *Trypanosoma cruzi* as model organism. Gene 294, 197–204.

Da Rocha, W., Otsu, K., Teixeira, S., Donelson, J., 2004a. Test of cytoplasmatic RNA interference (RNAi) and construction of a tetracycline- inducible T7 promoter system in *Trypanosoma cruzi*. Mol. Biochem. Parasitol. 133, 175–186.

Da Rocha, W.D., Silva, R.A., Bartholomeu, D.C., Pires, S.F., Freitas, J.M., Macedo, A.M., Vazquez, M.P., Levin, M.J., Teixeira, S.M., 2004b. Expression of exogenous genes in *Trypanosoma cruzi:* improving vectors and electroporation protocols. Parasitol. Res. 92, 113–120.

Dhir, V., Allen, C., Field, M., 2005. Perturbation of local endogenous expression by insertion of Pol I expression constructs into the genome of *Trypanosoma brucei*. Exp. Parasitol. 109, 198–200.

Dubessay, P., Ravel, C., Bastien, P., Stuart, K., Dedet, J.P., Blaineau, C., Pagès, M., 2002a. Mitotic stability of a coding DNA sequence-free version of *Leishmania major* chromosome 1 generated by targeted chromosome fragmentation. Gene 289, 151–159.

Dubessay, P., Ravel, C., Bastien, P., Crobu, L., Dedet, J.P., Pagès, M., Blaineau, C., 2002b. The switch region on *Leishmania major* chromosome 1 is not required for mitotic stability or gene expression, but appears to be essential. Nucleic Acids Res. 30, 3692–3697.

Durand-Dubief, M., Absalon, S., Menzer, L., Ngwabyt, S., Ersfeld, K., Bastin, P., 2007. The Argonaute protein TbAGOl contributes to large and mini-chromosome segregation and is required for control of RIME retroposons and RHS pseudogene associated transcripts. Mol. Biochem. Parasitol. 156, 144–153.

Elias, M.C., Faria, M., 2009. Are there epigenetic controls in *Trypanosoma cruzi?* natural genetic engineering and natural genome editing. Ann. N.Y. Acad. Sci. 1178, 285–290.

El-Sayed, N.M.1., Myler, P.J., Bartholomeu, D.C., Nilsson, D., Aggarwal, G., Tran, A.N., Ghedin, E., Worthey, E.A., Delcher, A.L., Blandin, G., Westenberger, S.J., Caler, E., Cerqueira, G.C., Branche, C., Haas, B., Anupama, A., Arner, E., Aslund, L., Attipoe, P., Bontempi, E., Bringaud, F., Burton, P., Cadag, E., Campbell, D.A., Carrington, M., Crabtree, J., Darban, H., da Silveira, J.F., de Jong, P., Edwards, K., Englund, P.T., Fazelina, G., Feldblyum, T., Ferella, M., Frasch, A.C., Gull, K., Horn, D., Hou, L., Huang, Y., Kindlund, E., Klingbeil, M., Kluge, S., Koo, H., Lacerda, D., Levin, M.J., Lorenzi, H., Louie, T., Machado, C.R., McCulloch, R., McKenna, A., Mizuno, Y., Mottram, J.C., Nelson, S., Ochaya, S., Osoegawa, K., Pai, G., Parsons, M., Pentony, M., Pettersson, U., Pop, M., Ramirez, J.L., Rinta, J., Robertson, L., Salzberg, S.L., Sanchez, D.O., Seyler, A., Sharma, R., Shetty, J., Simpson, A.J., Sisk, E., Tammi, M.T., Tarleton, R., Teixeira, S., Van Aken, S., Vogt, C., Ward, P.N., Wickstead, B., Wortman, J., White, O., Fraser, C.M., Stuart, K.D., Andersson, B., 2005a. The genome sequence of *Trypanosoma cruzi*, etiologic agent of Chagas disease. Science, 309, 409–415.

El-Sayed, N.M.1., Myler, P.J., Blandin, G., Berriman, M., Crabtree, J., Aggarwal, G., Caler, E., Renauld, H., Worthey, E.A., Hertz-Fowler, C., Ghedin, E., Peacock, C., Bartholomeu, D.C., Haas, B.J., Tran, A.N., Wortman, J.R., Alsmark, U.C., Angiuoli, S., Anupama, A., Badger, J., Bringaud, F., Cadag, E., Carlton, J.M., Cerqueira, G.C., Creasy, T., Delcher, A.L., Djikeng, A., Embley, T.M., Hauser, C., Ivens, A.C., Kummerfeld, S.K., Pereira-Leal, J.B., Nilsson, D., Peterson, J., Salzberg, S.L., Shallom, J., Silva, J.C., Sundaram, J., Westenberger, S., White, O., Melville, S.E., Donelson, J.E., Andersson, B., Stuart, K.D., Hall, N., 2005b. Comparative genomics of trypanosomatids parasitic protozoa. Science, 309, 404–409.

Garcia Silva, M., Tosar, J., Frugier, M., Pantano, S., Bonilla, B., Esteban, L., Serra, E., Rovira, C., Robello, C., Cayota, A., 2010. Cloning, characterization and subcellular localization of a *Trypanosoma cruzi* argonaute protein defining a new subfamily distinctive of trypanosomatids. Gene 466, 26–35.

Gaunt, M.W., Yeo, M., Frame, I.A., Stothard, J.R., Carrasco, H.J., Taylor, M.C., Mena, S.S., Veazey, P., Miles, G.A., Acosta, N., de Arias, A.R., Miles, M.A., 2003. Mechanism of genetic exchange in American trypanosomes. Nature 421, 936–939.

Guevara, P., Dias, M., Rojas, A., Crisante, G., Abreu-Blanco, M.T., Umezawa, E., Vazquez, M., Levin, M., Añez, N., Ramirez, J.L., 2005. Expression of fluorescent genes in *Trypanosoma cruzi* and *Trypanosoma rangeli* (Kinetoplastida: Trypanosomatidae): its application to parasite-vector biology. J. Med. Entomol. 42, 48–56.

Horton, R., Cai, Z., Ho, S., Pease, L., 1990. Gene splicing by overlap extension: tailor made genes using the polymerase chain reaction. Biotechniques 8, 528–535.

Ivens, A.C., Peacock, C.S., Worthey, E.A., Murphy, L., Aggarwal, G., Berriman, M., Sisk, E., Rajandream, M.A., Adlem, E., Aert, R., Anupama, A., Apostolou, Z., Attipoe, P., Bason, N., Bauser, C., Beck, A., Beverley, S.M., Bianchettin, G., Borzym, K., Bothe, G., Bruschi, C.V., Collins, M., Cadag, E., Ciarloni, L., Clayton, C., Coulson, R.M., Cronin, A., Cruz, A.K., Davies, R.M., De Gaudenzi, J., Dobson, D.E., Duesterhoeft, A., Fazelina, G., Fosker, N., Frasch, A.C., Fraser, A., Fuchs, M., Gabel, C., Goble, A., Goffeau, A., Harris, D., Hertz-Fowler, C., Hilbert, H., Horn, D., Huang, Y., Klages, S., Knights, A., Kube, M., Larke, N., Litvin, L., Lord, A., Louie, T., Marra, M., Masuy, D., Matthews, K., Michaeli, S., Mottram, J.C., Muller-Auer, S., Munden, H., Nelson, S., Norbertczak, H., Oliver, K., O'Neil, S., Pentony, M., Pohl, T.M., Price, C., Purnelle, B., Quail, M.A., Rabbinowitsch, E., Reinhardt, R., Rieger, M., Rinta, J., Robben, J., Robertson, L., Ruiz, J.C., Rutter, S., Saunders, D., Schafer, M., Schein, J., Schwartz, D.C., Seeger, K., Seyler, A., Sharp, S., Shin, H., Sivam, D., Squares, R., Squares, S., Tosato, V., Vogt, C., Volckaert, G., Wambutt, R., Warren, T., Wedler, H., Woodward, J., Zhou, S., Zimmermann, W., Smith, D.F., Blackwell, J.M., Stuart, K.D., Barrell, B., Myler, P.J., 2005. The genome of the kinetoplastid parasite, *Leishmania major*. Science 309, 436–442.

Kelly, J., Ward, H., Miles, M., Kendall, G., 1992. A shuttle vector which facilitates the expression of transfected genes in *Trypanosoma cruzi* and *Leishmania*. Nucleic Acids Res. 20, 3963–3969.

Kim, D., Chiurillo, M.A., El-Sayed, N., Jones, K., Santos, M.R., Porcile, P.E., Andersson, B., Myler, P., da Silveira, J.F., Ramírez, J.L., 2005. Telomere and subtelomere of *Trypanosoma cruzi* chromosomes are enriched in pseudogenes of retrotransposon hot spot and transialidase-like gene families: the origins of *T. cruzi* telomeres. Gene 346, 153–161.

Kushnir, S., Cirstea, I.C., Basiliya, L., Lupilova, N., Breitling, R., Alexandrov, K., 2011. Artificial linear episome-based protein expression system for protozoon *Leishmania tarentolae*. Mol. Biochem. Parasitol. 176, 69–79.

La Flamme, A., Buckner, F., Swindle, J., Ajioka, J., Van Voorhis, W., 1996. *Trypanosoma cruzi*: expression of Interleukin-2 utilizing both supercoiled plasmids and linear DNAs. Exp. Parasitol. 83, 159–163.

Lee, C., Siak Wei Ow, D., Weng, Oh.S., 2006. Quantitative real-time polymerase chain reaction for determination of plasmid copy number in bacteria. J. Microbiol. Methods 65, 258–267.

Lee, M., Yaping, E., Axelrod, N., 1995. Construction of trypanosome artificial chromosomes. Nucleic Acids Res. 23, 4893–4899.

Lorenzi, H., Vázquez, M., Levín, M., 2000. The genes for a DEAH RNA helicase, a NifU like protein and the translation factor eIF6 constitute the *SZ5 locus* of *Trypanosoma cruzi*. Mol. Biochem. Parasitol. 111, 207–211.

Lorenzi, H., Vazquez, M., Levin, M., 2003. Integration of expression vectors into the ribosomal locus of *Trypanosoma cruzi*. Gene 310, 91–99.

Lufino, M., Edser, P., Wade-Martins, R., 2008. Vector technology: episomal maintenance, vector delivery, and transgene expression. Mol. Ther. 16, 1525–1538.

Martinez-, Calvillo.S., Lopez, I., Hernandez, R., 1997. PRIBOTEX expresion vector: pTEX derivative for a rapid selection of *Trypanosoma cruzi* transfectants. Gene 199, 71–76.

Martinez-Calvillo, S.,Vizuet-de- Rueda, I., Florencio-Martfnez, L., Manning-Cela, R., Figueroa-Angulo, E., 2010. Gene expression in trypasomatid parasites. J. Biomed. Biotechnol. doi:10.155/2010/525241.

Minning, T., Weatherly, D., Flibotte, S., Tarleton, R., 2011. Widespread, focal copy number variations (CNV) and whole chromosome aneuploidies in *Trypanosoma cruzi* strains revealed by array comparative genomic hybridization. BMC Genomics 12, 139.

Moraes Barros, R., Marini, M., Antonio, C., Cortez, D., Miyake, A., Lima, F., Ruiz, J., Bartholomeu, D., Chiurillo, M., Ramírez, J., Franco da Silveira, J., 2012. Anatomy and evolution of telomeric and subtelomeric regions in the human protozoan parasite *Trypanosoma cruzi*. BMC Genomics, 13, 229.

Obado, S., Bot, C., Nilsson, D., Andersson, B., Kelly, J., 2007. Repetitive DNA is associated with centromeric domains in *Trypanosoma brucei* but not *Trypanosoma cruzi*. Genome Biol. http://dx.doi.org/10.1186/gb-2007-8-3-r37.

Obado, S., Taylor, M., Wilkinson, S., Bromley, E., Kelly, J., 2005. Functional mapping of a trypanosome centromere by chromosome fragmentation identifies a 16-kb GC-rich transcriptional strand switch domain as a major feature. Genome Res. 15, 36–43.

Patnaik, P., Axelrod, N., Van der Ploeg, L., Cross, G., 1996. Artificial linear mini chromosomes for *Trypanosoma brucei*. Nucleic Acids Res. 4, 668–675.

Singer, B., 1988. On the role of homologous sequences in chromosomal rearrangements. Genes Dev. 2, 1800–1811.

Tamar, S., Papadopoulou, B., 2001. A telomere-mediated chromosome fragmentation approach to assess mitotic stability and ploidy alterations of *Leishmania* chromosomes. J. Biol. Chem. 276, 11662–11673.

Taylor, M., Kelly, J., 2006. PTc INDEX: a stable tetracycline-regulated expresi6n vector for *Trypanosoma cruzi*. BMC Biotechnol. http://dx.doi.org/10.1186/1472-6750-6-32.

Tetaud, E., Lecuix, I., Sheldrake, T., Baltz, T., Fairlamb, A., 2002. A new expression vector for *Crithidia fasciculata* and *Leishmania*. Mol. Biochem. Parasitol. 120, 195–204.

Torras-Llort, M., Moreno-Moreno, O., Azorín, F., 2009. Focus on the centre: the role of chromatin on the regulation of centromere identity and function. EMBO J. 28, 2337–2348.

Vázquez, M., Levin, M., 1999. Functional analysis of the intergenic regions of TcP2B gene loci allowed the construction of an improved *Trypanosoma cruzi* expression vector. Gene 239, 217–225.

Vázquez, M., Schijman, A., Levin, M., 1994. A short interspersed repetitive element provides a new 3acceptor site for trans-splicing in certain ribosomal P2B protein genes of *Trypanosoma cruzi*. Mol. Biochem. Parasitol. 64, 327–336.

Veitia, R., 2005. Stochasticity or the fatal imperfection of cloning. J. Biosci. 30, 21–30.

Wirtz, E., Leal, S., Ochatt, C., Cross, G., 1999. A tightly regulated inducible expression system for conditional gene knock-outs and dominant-negative genetics in *Trypanosoma brucei*. Mol. Biochem. Parasitol. 99, 89–101.

Zingales, B., Pereira, M.E., Oliveira, R.P., Almeida, K.A., Umezawa, E.S., Souto, R.P., Vargas, N., Cano, M.I., da Silveira, J.F., Nehme, N.S., Morel, C.M., Brener, Z., Macedo, A., 1997. *Trypanosoma cruzi* genome project: biological characteristics and molecular typing of clone CL Brener. Acta Trop. 68, 159–173.