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**Wanderson D. DaRocha · Rosiane A. Silva
Daniella C. Bartholomeu · Simone F. Pires
Jorge M. Freitas · Andrea M. Macedo
Martin P. Vazquez · Mariano J. Levin
Santuza M. R. Teixeira**

Expression of exogenous genes in *Trypanosoma cruzi*: improving vectors and electroporation protocols

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Abstract To improve transfection efficiency in *Trypanosoma cruzi*, we developed a new electroporation protocol and expression vectors which use *luciferase* and *green* and *red fluorescent proteins* as reporter genes. In transient transfections, the electroporation conditions reported here resulted in *luciferase* expression 100 times higher than the levels obtained with previously described protocols. To verify whether sequences containing different *trans*-splicing signals influence reporter gene expression, we compared DNA fragments corresponding to 5′ untranslated plus intergenic (5′ UTR plus Ig) regions from *GAPDH*, *TcP2β*, α- and *β-tubulin* and *amastin* genes. Vectors containing sequences derived from the first four genes presented similar efficiencies and resulted in *luciferase* expression in transiently transfected epimastigotes that was up to 10 times higher than that for a control vector. In contrast, the *amastin* 5′ UTR plus Ig resulted in lower levels of reporter gene expression. We also constructed a vector containing an expression cassette designed to be targeted to the *tubulin* locus of the parasite.

Introduction

The protozoan parasite *Trypanosoma cruzi* is the causative agent of Chagas' disease, a debilitating disease endemic in many Latin American countries, where 16–18 million people are affected (World Health Organization 1999). Being part of a group that diverged early in eukaryotic evolution, *T. cruzi*, as well as other members of the kinetoplastid family, present many distinctive features regarding the mechanisms controlling gene expression. Some of these features include polycistronic transcription, *trans*-splicing processing of the pre-mRNA, mitochondrial RNA editing and transcription of a set of protein-coding genes carried on by RNA polymerase I (for a recent review see Clayton 2002). Since primary transcripts are polycistronic, cleavage of the pre-mRNA has to occur in the nucleus in order to produce monocistronic mRNAs. These cleavage reactions are linked to the addition of the 39-nucleotide miniexon [or spliced leader (SL)] at the 5′-end and the poly(A) tail at the 3′-end of each mRNA. SL addition results from a *trans*-esterification reaction called *trans*-splicing, which requires a conserved AG dinucleotide as SL addition site. Studies in various trypanosomatids have provided strong evidence demonstrating that SL addition and polyadenylation are not independent events. Instead, they are part of a "cut-and-paste" mechanism that occurs simultaneously or immediately after transcription; poly(A) selection is governed by the location of the SL-addition site of the downstream gene in the polycistronic primary transcript. In addition to the correct distance, the presence of a polypyrimidine-rich motif is also crucial, since only AG dinucleotides located downstream from a polypyrimidine tract are used as the SL acceptor site (Lebowitz et al. 1993; Matthews et al. 1994).

Most of the knowledge about the mechanisms of gene expression in these parasites resulted from the development of transfection protocols. In the first transfection experiments reported for *T. cruzi*, vectors containing a segment of the *SL* gene (Lu and Buck 1991) or the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene (Kelly et al. 1992) were placed upstream from the bacterial chloramphenicol acetyl transferase (*CAT*) gene. In the pTEX vector, described by Kelly et al. (1992), the Neo^R selectable marker was also inserted.

W. D. DaRocha · R. A. Silva · D. C. Bartholomeu · S. F. Pires
J. M. Freitas · A. M. Macedo · S. M. R. Teixeira (✉)
Departamento de Bioquímica e Imunologia, ICB,
Universidade Federal de Minas Gerais,
MG 31270-010 Belo Horizonte, Brazil
E-mail: santuzat@icb.ufmg.br
Tel.: +55-31-34992665
Fax: +55-31-34992614

M. P. Vazquez · M. J. Levin
Laboratorio de Biologia Molecular de la Enfermedad de Chagas,
INGEBI-CONICET, School of Sciences,
University of Buenos Aires,
1428 Buenos Aires, Argentina

Although these earlier vectors contained all the sequences necessary for the expression of the exogenous mRNA, the lack of promoter elements resulted in relatively low levels of expression of the *CAT* gene. The introduction of sequences derived from rRNA promoter, as described by Teixeira et al. (1995), Tyler-Cross et al. (1995) and Martinez-Calvillo et al. (1997), resulted in a new generation of vectors, yielding expression of reporter genes at least two orders of magnitude greater in transfected epimastigotes. Besides the rRNA promoter, the only other promoter characterized in *T. cruzi* is the *SL-RNA* gene promoter, which yields only weak expression of reporter genes in epimastigotes (Nunes et al. 1997). Remarkably, transfection assays using promoter-trap plasmids and, more recently, genomic analyses have failed to reveal sequences with characteristics of the RNA polymerase II promoter in any trypanosomatid, even though transcription initiated by RNA polymerase II has been clearly described in these organisms.

To improve transfection efficiency in *T. cruzi* further, sequences placed downstream from the rRNA promoter should also be tested. These sequences may influence the correct processing of the pre-mRNA as well as the steady-state levels of the mature message. Various authors have described sequences derived from the 3′ untranslated region (UTR) plus intergenic (Ig) region of several genes, which contribute decisively to modulating gene expression in the parasite. (Nozaki and Cross 1995; Teixeira et al. 1995; Weston et al. 1999; Bartholomeu et al. 2003). In most cases, it has been demonstrated that these sequences are involved in mRNA stability control, but the presence of elements affecting the efficiency of polyadenylation must also be considered. On the other hand, the effect of sequences derived from the 5′ UTR and upstream Ig regions has been poorly investigated. These sequences may also contribute to mRNA stability and translation efficiency but, most importantly, they may contain signals determining *trans*-splicing efficiency. Here we evaluate the effect of various sequences containing the SL acceptor site and 5′ UTR derived from the surface protein *amastin*, α-and *β*-tubulin, the ribosomal protein *TcP2β* and *GAPDH* genes. These fragments were placed upstream from the *luciferase* reporter gene and tested in transiently transfected epimastigotes. In addition, we developed a vector that promotes the integration of an expression cassette into the *tubulin* locus of the parasite genome. This new vector allowed the generation of stable cell lines from transfected epimastigotes, which can be used in studies requiring differentiation into the other stages of the parasite's life cycle.

Materials and methods

Parasite

Epimastigote forms of CL Brener and Colombiana (Col.1.7G2) clones and the Tulahuén strain of *T. cruzi* were grown at 28°C in liver infusion tryptose (LIT) medium supplemented with 10% fetal calf serum (FCS) as described by Camargo (1964).

Plasmids

All constructs used in transfection experiments were derived from the pGEM-luc vector (Promega). The pLR-Tub plasmid was generated by cloning the 600-bp *Xho*I-*Sac*I 3′ UTR of α-tubulin gene fragment present in the pLATub vector (Bartholomeu et al. 2003) in the pGEM-luc vector downstream from the *luciferase* gene. A fragment amplified by the polymerase chain reaction (PCR) and containing the *T. cruzi* rRNA gene promoter was inserted upstream of *luciferase*. This fragment was generated by PCR amplification using total DNA purified from the CL Brener strain as template, with the forward primer (5′-CGGAGAAGCTTTTTGTAA-3′) and the reverse primer (5′-CTTGGGATCCCACGGTACCTTTGC-3′). The primer sequences were based on the analyses of the rRNA locus described by Dietrich et al. (1993). The forward primer has a *Hin*dIII site and the reverse primer has *Kpn*I and *Bam*HI sites. The 540-bp fragment corresponding to rRNA gene promoter was inserted into the *Hin*dIII and *Bam*HI sites of pLR-Tub. The resulting plasmid contains two sites, *Kpn*I and *Bam*HI, between the rRNA promoter and the *luciferase*-coding region, which facilitate the insertion of the different 5′ UTR fragments.

The various 5′ UTR plus Ig regions were amplified using primers based on the sequences of α- and *β*-tubulin, *GAPDH* and *TcP2β* genes. To generate the plasmid pLR5′alphaTub, a 237-bp fragment containing the 5′ UTR plus Ig of *T. cruzi* α-tubulin gene was obtained by PCR amplification using total DNA from the Tulahuén strain as template with the forward primer (5′-CGCTGGGTACCGGGTGCACC-3′) and reverse primer (5′-AGAAGGATCCTATTTGAGGG-3′). The plasmid pLR5′beta-Tub was constructed by inserting into pLR-Tub a 304-bp fragment, which contained the 5′ UTR plus Ig of *T. cruzi* *β*-tubulin gene obtained by PCR amplification using total DNA from the Tulahuén strain as template with the forward primer (5′-GCCTGGTACCATCGTCCGCT-3′) and the reverse primer (5′-CTTGTCTGGATCCTGATGTT-3′). These primers contain *Kpn*I and *Bam*HI sites. The plasmid pLR5′gapdh was obtained by inserting a 450-bp fragment, derived from the 5′ upstream region of the *T. cruzi gGAPDH I* gene, into the *Kpn*I and *Bam*HI sites of the plasmid pLR-Tub. This fragment was isolated from the pTEX vector (Kelly et al. 1992) by digestion with *Sac*I followed by treatment with T4 DNA polymerase. After this treatment, the same plasmid was digested with *Bam*HI. The fragment obtained was inserted into the pLR-Tub vector previously digested with *Kpn*I followed by treatment with T4 DNA polymerase and posterior *Bam*HI digestion. To construct the plasmid pLR5′HX1, the fragment corresponding to the HX1 5′ upstream region of the *TcP2β* gene was obtained from pTREX (Vazquez and Levin 1999) after *Bam*HI digestion. It was inserted into pLR-Tub, previously digested with *Bam*HI. To generate the plasmid pLR5′Ama1, a modification in the vector pLRT previously described by Teixeira et al. (1995) was introduced by replacing the 3′ UTR plus Ig region from the *TCR27* gene by a 600-bp *Xho*I-*Sac*I fragment containing the 3′ UTR of the α-tubulin gene. The 3′ UTR plus Ig region of the α-tubulin gene fragment was generated by PCR amplification using total DNA from the Tulahuén strain as template and the forward primer (5′-AGTACTCGAGTGGCCGCTCCCGCGT-3′) and the reverse primer (5′-CGAGGAGCTCCACTAAATAGATCTT-3′). These primers have *Xho*I and *Sac*I sites, respectively, to facilitate cloning (Bartholomeu et al. 2003). To ensure that all the plasmids used for the comparison of the influence of the 5′ sequences on reporter gene expression have the same vector background, we generated a second *amastin* plasmid, named pLR5′Ama2 by inserting a PCR fragment containing the *amastin* 5′ UTR from the pTCRGFPNeo vector (Teixeira et al. 1999a) into the *Bam*HI site of the pLR-Tub vector. Sequences from all of these constructs were confirmed by automated sequencing using the MegaBace 1000 (Amersham-Pharmacia Biotech).

To create the pROCKGFPNeo vector, a *β*-tubulin cDNA cloned in pBluescript (Bartholomeu et al. 2003) was completely digested with *Eco*RI and partially with *Pst*I. After filling in the *Eco*RI site with T4 DNA polymerase, the 1.0-kb *tubulin* fragment was cloned into the *Not*I/*Pst*I digested pBluescript vector,

generating pTUB plasmid. A cassette for foreign gene expression containing the neomycin resistance gene (*Neo*^{R}) was obtained by *Kpn*I digestion of the pTREX vector (Vazquez and Levin 1999), followed by filling the ends. This 3.1-kb fragment was cloned into pTUB, which was previously digested with *Hin*dIII/*Kpn*I and treated with T4 DNA polymerase. A fragment containing the expression cassette plus the β-tubulin sequence upstream was obtained by *Sac*I/*Nhe*I digestion of pTUB and was transferred to a modified pBluescript plasmid (pSk1) after digestion with *Not*I/*Kpn*I and T4 DNA polymerase treatment. pSk1 does not carry the T7 and T3 promoters (LaCount et al. 2002). In this final construct we inserted into the *Xba*I/*Xho*I sites the *GFP* (green fluorescent protein) or the *RFP* (red fluorescence protein) genes, generating pROCKGFPNeo (Fig. 5A) or pROCKRFPNeo, respectively. The *GFP* coding sequence was derived from pTCRGFPNeo (Teixeira et al. 1999a) and the *RFP* coding sequence was derived from pDsRed vector (Clontech).

Electroporation

Epimastigotes growing at a density of 5×10^6–10×10^6 parasites/ml in LIT plus10% FCS were harvested, washed once with phosphate buffered saline (PBS) and resuspended at a density of 10^8 parasites/ml in the electroporation buffer (120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4, 25 mM Hepes, 2 mM EDTA, 5 mM $MgCl_2$, pH 7.6). Aliquots (0.4 ml) of cell suspension were mixed with 50 µl DNA (25, 50 or 100 µg) on ice cold 0.2-cm cuvettes and electroporated using a Bio-Rad gene pulser set at 0.3 kV and 500 µF, with two pulses (10 s between pulses). The time constants were always between 3.0 and 6.0 ms. For comparison, we also used the following protocol, which had been described by Ramirez et al. (2000): epimastigote cultures were harvested, washed once with PBS and resuspended to 2×10^8parasites/ml in electroporation buffer III (137 mM NaCl, 21 mM Hepes, 5 mM KCl, 5.5 mM Na_2HPO_4, 0.77 mM glucose, pH 7.0), and aliquots (0.7 ml) of cell suspension were mixed with 25 µg DNA in 0.4-cm cuvettes and electroporated using a Bio-Rad gene pulser set at 0.3 kV and 500 µF with two pulses. In both cases, the transfected cells were transferred to 5 ml LIT plus 10% FCS and incubated at 28°C for 48 h. All plasmid samples used in electroporation experiments were obtained by alkaline lysis using Qiagen columns (Qiagen).

Luciferase and β-galactosidase assays

Parasites were harvested 48 h after transfection, washed once with PBS, transferred to microtubes and centrifuged at 10,000 *g* for 10 s. Cell pellets were resuspended in 200 µl lysis buffer (100 mM potassium phosphate, pH 7.8, 0.4% Triton X-100 and 2 µg/ml leupeptin). After centrifugation for 1 min at 10,000 *g*, 4 µl of epimastigote lysates in the supernatants was diluted 1:5 and assayed as described previously (de Wet et al. 1987). In the transfection experiments using different 5′ UTR constructs, we used a plasmid containing the β-galactosidase reporter gene constructed by Coughlin et al. (2000) as a control plasmid, in order to avoid any possible artifact due to differences in transfection efficiency. In these cases, 10 µg control plasmid was co-transfected with each of the *luciferase* constructs and β-galactosidase assays were performed with 25 µl of the same cell lysate using *O*-nitrophenyl β-D-galactopyranoside, as described previously (Sambrook et al. 1989).

Flow cytometry analyses

Parasites (5×10^6) were washed with PBS plus 3% FCS and fixed for 30 min with MFF, freshly prepared by mixing equal volumes of solution I [1% (w/v) paraformaldehyde, 1% (w/v) sodium cacodylate, 0.67% (w/v) NaCl] with PBS. Fixed parasites were analyzed using a FACScan (Becton Dickinson), with 100,000 gated events acquired for analysis. Untransfected control cells, which show a significant amount of auto-fluorescence were used to standardize the parameters employed.

Pulse-field gel electrophoresis

Epimastigotes were included in agarose blocks as described by Engman et al. (1987). Pulse-field gel electrophoresis (PFGE) was carried out as reported by Cano et al. (1995) with the following modifications: the chromosomes were separated in 0.8% agarose gels using a program with 5 phases of homogeneous pulses (north/south, east/west) with interpolation for 135 h at 83 V. Phase 1 had pulse time of 90 s (run time 30 h); phase 2 200 s (30 h); phase 3 350 s (25 h); phase 4 500 s (25 h); phase 5 800 s (25 h). Chromosomes from *Hansenula wingei* (Bio-Rad) were used as molecular mass standards. Separated chromosomes were transferred to nylon filters and hybridized with ^{32}-P labeled *tubulin* and *GFP* probes as described by Teixeira et al. (1994).

Results and discussion

Aiming to improve transient transfection efficiency of *T. cruzi*, we tested several published and a newly developed protocol based on the electroporation conditions used to transfect *T. brucei* and *Leishmania*. Here we compare our protocol with the electroporation conditions described by Ramirez et al. (2000), who used *GFP* as a reporter gene to test several parameters. Log phase cultures of epimastigotes were transfected with a construct carrying the *luciferase* gene driven by the rRNA promoter and containing 5′ and 3′ flanking sequences derived from *amastin* and α-tubulin genes, respectively. This vector, pLR5′Ama1, was derived from pLRT (Teixeira et al. 1995) from which the 3′ UTR plus Ig of the *TCR27* gene were replaced by a fragment corresponding to the α-tubulin 3′ UTR and Ig. Previous experiments have shown that the presence of the α-tubulin 3′ UTR plus Ig resulted in *luciferase* expression in transfected epimastigotes that was 48% higher than in epimastigotes transfected with pLRT (Bartholomeu et al. 2003). As shown in Fig. 1, with our protocol we generated more than 18 times the luciferase activity achieved when the protocol described by Ramirez et al. (2000) was employed, using the same amount of DNA (25 µg). When we increased the DNA quantity up to 100 µg, the reporter activity was almost 100 times higher than before (Fig. 1). Thus, the presence of the α-tubulin 3′ UTR plus Ig, together with the electroporation conditions described here, resulted in a significant improvement in the efficiency of the transfection of epimastigotes.

Although the luciferase assays indicated that overall transfection efficiency had been improved, we prepared a plasmid vector containing the *GFP* reporter gene to determine the actual number of transfected parasites using our protocol. Using the pTREX vector (Vazquez and Levin 1999), we inserted the *GFP* coding region in the *Xba*I/*Xho*I sites generating the pTREXGFP plasmid. As shown in Fig. 2B, 24 h after transfection, 4% and 8% of cells transfected with 30 and 60 µg plasmid,

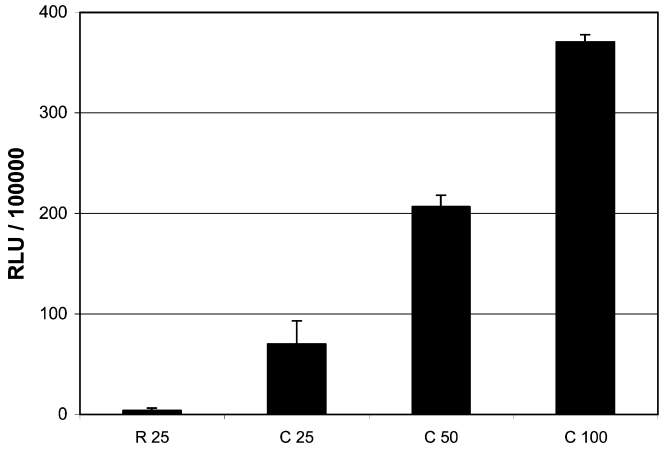
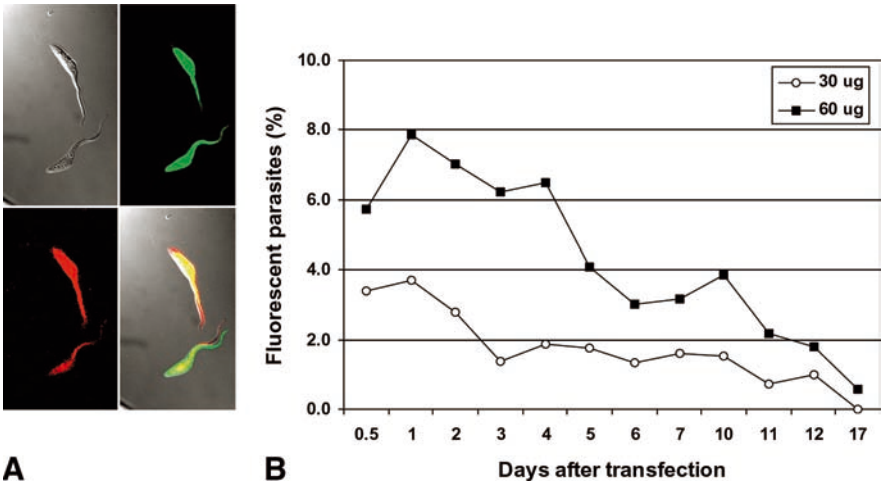


Fig. 1 Transfection efficiency in *Trypanosoma cruzi* cells. Epimastigote cultures of the CL Brener strain were transfected with different amounts of the pLR5′Ama1 plasmid. *C25*, *C50* and *C100* indicate luciferase activity in extracts of transfected parasites using 25, 50 and 100 μg of pLR5′Ama1, respectively, and the protocol described in "Material and methods". *R25* refers to the activity obtained with the protocol described by Ramirez et al. (2000) using 25 μg of the same plasmid. Data were derived from three independent experiments

respectively, were expressing high levels of *GFP*. These numbers are significantly higher than the transfection efficiency reported by Ramirez et al. (2000), who, after testing several strains, never observed a percentage of fluorescent parasites above 0.3% using 25 μg of a *GFP*-containing vector. Using pTREXGFP and the new transfection protocol, we were able to detect *GFP*-positive cells as soon as 12 h after transfection and even after 12 days in the absence of drug selection (Fig. 2B). Thus, it was possible to follow transiently transfected parasites for longer periods of time, in the absence of drug selection. To test whether the same efficiency of transient transfection can be achieved with a different reporter and also to verify whether a parasite can express two reporters at the same time using transient transfection assays, a similar plasmid was generated by replacing the *GFP* gene by the *RFP* coding region. Similarly to the expression of *GFP*, transfection of epimastigotes with 60 μg of the *RFP* plasmid (pTREXRFP) resulted in approximately 7% of positive parasites, detected 12 h post-transfection (not shown). As shown by the confocal microscopy analyses, parasites that were co-transfected with 30 μg of each *GFP* and *RFP* plasmids express high levels of green and red fluorescence distributed throughout the cell (Fig. 2A). The transfection efficiency with both markers is equivalent to the efficiency attained with each plasmid separately.

To test whether different 5′ UTR plus Ig regions may also influence the expression of a foreign gene in *T. cruzi* epimastigotes, we prepared the following constructs: the pLR5′alphaTub and pLR5′betaTub plasmids, which contain the 5′ UTR plus upstream sequences from *T. cruzi* α- and β-tubulin genes, respectively; the pLR5′gapdh plasmid with the sequence derived from the 5′ upstream region of the *T. cruzi gGAPDH I* gene; the pLR5′HX1 plasmid, which contains the fragment HX1 corresponding to the 5′ UTR and Ig region of the *T. cruzi TcP2β* gene (Vazquez and Levin 1999) and the pLR5′Ama2 plasmid in which the 5′ UTR plus Ig sequences from *amastin* gene were cloned upstream of *luciferase* in the same plasmid. In addition, we included in our experiments a plasmid pLucSL-control, which contains no *T. cruzi* sequences between the rRNA promoter and *luciferase* gene. Again, the 3′ UTR plus Ig region of α-tubulin was chosen to be placed down-

Fig. 2A, B Transient expression of green fluorescent protein (*GFP*) and red fluorescent protein (*RFP*) in *T. cruzi* epimastigotes. Epimastigotes from the CL Brener strain were transfected with 30 or 60 μg of pTREXGFP or pTREXRFP, as described in "Material and methods". **A** Differential interference contrast (DIC) and fluorescent images of epimastigotes expressing *GFP* and *RFP*, simultaneously, 24 h after co-transfection with 30 μg of each plasmid. These images were captured simultaneously on separate channels using a Zeiss LSM 510 laser scanning confocal microscope. **B** Kinetics of *GFP* expression after parasite transfection without drug selection, as determined using a fluorescent microscope. Each point corresponds to the percentage of green parasites in various fields with a total of 700 cells. Similar curves were obtained with parasite transfected with pTREXRFP and with both plasmids (not shown)



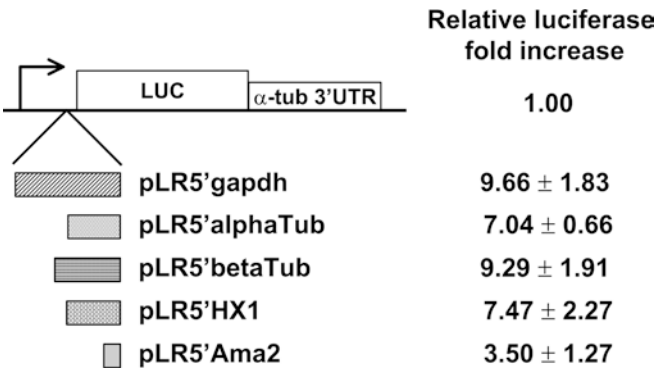


Fig. 3 The influence of 5′ untranslated region (UTR) sequences from different *T. cruzi* genes on transient gene expression. Different 5′ UTR sequences were cloned upstream from the *luciferase* reporter gene and 50 μg of each plasmid were used to transfect CL Brener epimastigotes using the protocol described in Fig. 1. The results are presented as the relative increases in luciferase activity compared to that of parasites transfected with a control plasmid, which has no *T. cruzi* sequences between the *luciferase* and the rRNA promoter. These results correspond to averages of three independent experiments, which were normalized using the values obtained from a co-transfected plasmid containing the *β*-galactosidase gene

stream from *luciferase* in all constructs. The results of transient transfection experiments, shown in Fig. 3, demonstrated that the presence of sequences containing signals for addition of SL resulted in up to 10-fold increase in the levels of luciferase, as compared to the plasmid containing no sequences between the rRNA promoter and *luciferase*. Even though the control plasmid contains no upstream sequences directing *trans*-splicing of the *luciferase* mRNA, we detect significant levels of luciferase activity in parasites transfected with this plasmid. In three independent experiments, the mean value of luciferase activity in cells transfected with 50 μg of the control plasmid were close to 25,000 relative light units per10^6 cells. This result is in agreement with previous reports suggesting that the rRNA promoter sequence may contain elements that could potentially function as cryptic acceptor sites for *trans*-splicing reactions (Martinez-Calvillo et al. 1997).

Only small differences in relative luciferase activities were observed when cells were transiently transfected with four out of the five different plasmids (Fig. 3). The results of three independent experiments show that luciferase activities vary between 7- and 9-fold when sequences derived from α- and *β*- tubulin, *TcP2β* and *GAPDH* genes are compared. On the other hand, the construct containing the 5′ UTR plus Ig region of *amastin* genes is much less efficient, and resulted in luciferase levels that are only 3-fold higher than the control plasmid. In order to search for elements that could modulate the level of luciferase activity in the transfected parasites, we analyzed the different 5′ UTR and Ig sequences that were cloned upstream the *luciferase* gene. Polypyrimidine tracts present in the Ig regions act as bifunctional elements recognized by the processing machinery, which affect both polyadenyla-

Fig. 4A–E The 5′ UTR sequences from different *T. cruzi* genes cloned upstream from the *luciferase* reporter gene. **A** α-Tubulin. **B** *β*-Tubulin. **C** *gGAPDH I*. **D** Ribosomal protein *TcP2β*. **E** *Amastin*. The different 5′ UTR sequences were cloned upstream from the *luciferase* coding region as described in "Material and Methods". *Highlighted sequences* are polypyrimidine tracts; *underlined sequences* are potential AG splice-leader acceptor sites as determined by Teixeira et al. (1994) for the *amastin* gene, by Vazquez and Levin (1999) for *TcP2β* and by Bartholomeu et al. (2003) for α- and *β*-tubulin; and the *doubly underlined* codon is the *luciferase* start codon

A- Alpha-tubulin

GGTACCGGGTGCACCCGCTCTTATATAAATATAAATATATATATATATATATATATATG
TTTACTTGGCACTTTTGCCTTCCTCTTTCCTTCTCTCTCTCTCTCTGTGTGTATGCCTGCG
TGTGCGAAATGCTGTGGCCTTTTCTCTTACTCTCCCCGTCTTGCGCGTTTGGTAGTTCGTT
CTAGCTAGGCTTTCGAGCTTCTCTTCAAACACCCTCAAATAggatccaaATG...

B- Beta-tubulin

GGTACCATCGTCCGCTTCTTCTCGCCGGGTGGCCCCTCTAAAAAGAAGGAAAAAAAAGAAA
TGAACGTCTCCGTTTCTTTTTCTCTCTTTCCTTTTCTTTTTGTTTGCCTTGTCTTTTCCCC
GCGCGCGCGGTTTGCGGCCGACTGCGCCGGTGGAACGGGGATCGAGTATCTCACTTGGGGA
AAAGATCTATTTAGTGGAATTACTCGTTCAGCTACTGAGCCAAGGAGAGCAGGGAAAAAGT
AATACATACATAAAGAAAGCAAACAACAAAAACATCAggatccaaATG...

C- gGAPDH I

CCCCGCGGCAGTAGCTGCCCCTACCCCAACAACTACTCTAGTCTAGGCACCCTCATTTCAT
CTATGCGTATCCGCATTCAGGAAGTGAGCGAAGTTCAACAGTTGCCAGTTTCTTCAGGAGA
CTCAAAAAAACAGACACAACAGTCTTATTAGACAAGAGCACAAACAAATTCTCATTGCATT
CAGAAGCAAACAAACAAACAAAAACAAAATGCGGAAAATACCTCAAATTTATTTTATGTCT
GTCCTCTACAAATAATAATAATAATAATAATTACAGCGGCCGTAAATGAATGCAAGAA
AAGAAACACAAAACCCACAATTGTCAACCTCCCCTTCATTCCACTTGTCTCTCTTTTCCCA
CGTTTCCTGCACGAATGCAGAAAGTGATATTTTTACTTTGAAAGCCATCTACCAACAACAA
TTACATTGAACAGAATTctagaactagtggatccaaATG...

D- TcP2beta (HX1 fragment)

CCCAACACGAGAGGCGGTTAACGAGTTTCTTCAAAATATGCAGCGGATTCACTAAGAAACA
TTTTCACGCACGAAAGCGAAATTATTATGATTGTTATTATAATACTTTTTCTTTGTTGTTT
TATCCACTTATTATTGTTGTGTTAAATTGTTTTTACCTTTTTTCTTTTCCAACTTCTTTTA
TGATGTCTTTTCTTTTTTTTTTTTTTGCTCTATAAGTTGTCTTGTCTAGAactagtggatc
caaATG...

E- Amastin

GGATCCTGCTCTCACTGACAGCTTTCTTTCCCCTGCTTTCAACCCTAGCTCTCACGTAGGA
AGggcccggatccaaATG...

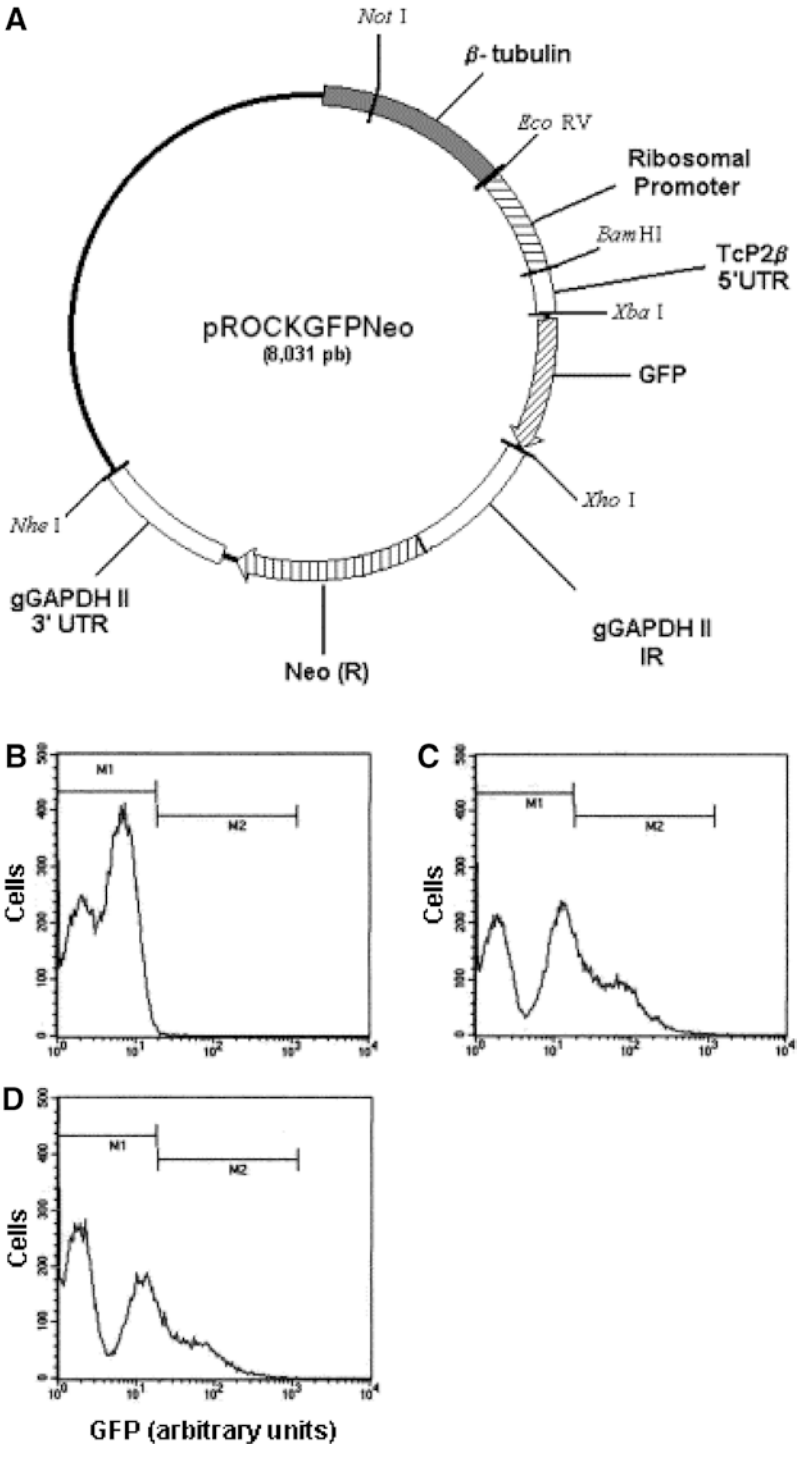


Fig. 5A–D An integrative vector for stable expression of *GFP* in *T. cruzi*. **A** Schematic representation of the pROCKGFPNeo, a vector that was used for integration of an expression gene cassette at the *β*-tubulin locus. Cultures of epimastigote of the Tulahúen strain were transfected with 50 μg of the linearized vector and submitted to drug selection in the presence of 200 μg/ml of G418 for 6 months. After this period, one aliquot of the culture was kept in the absence of drug for 5 weeks. **B** Fluorescence-activated cell sorter (FACS) profile of non transfected cells growing in liver infusion tryptose (LIT) medium without drug. **C** Profile of G418-resistant populations expressing green fluorescent protein (*GFP*) 6 months after transfection. **D** Profile of the culture shown in C after cultivation for 5 weeks in the absence of drug selection

tion and spliced-leader addition of the genes located upstream and downstream (Matthews et al. 1994; Schurch et al. 1994; revised by Vanhamme and Pays 1995). Deletion analyses and block substitution mutagenesis of the pyrimidine-rich sequence of *α*- and *β*-tubulin genes result in decreased expression of both *CAT* and *luciferase* reporter genes cloned upstream and downstream from this element (Matthews et al. 1994). In our experiments, a correlation between the polypyrimidine content present in the Ig regions and the values of luciferase activity can be observed if we compare the results obtained with the pLR5′Ama2 construct and the other four plasmids. As shown in Fig. 4, the 5′ flanking sequence of the *amastin* construct, which generated the lowest values of luciferase activity, presents the smallest number of consecutive pyrimidine residues.

In addition to the effect due to splicing signals, we must also consider the possibility that the sequences corresponding to the 5′ UTRs of these various genes may influence the fate of the *luciferase* mRNA by

affecting its stability and/or the efficiency of translation. The transfection results are consistent with previous observations showing that among the genes from which the 5′ UTR sequences were tested, *amastin* is the only one whose expression is down regulated in epimastigotes as compared to amastigotes (Teixeira et al. 1995). However, for all genes analyzed here, the sizes of the 5′ UTR are extremely short, with an average of 46 nt between the translation start codon and the dinucleotide AG used as SL addition site (Fig. 4). There are only few reports in the literature describing regulatory elements in the 5′ UTR in trypanosomes, one being the study of the nuclear replication protein A and the kinetoplast topoisomerase II, from *Crithidia fasciculata* (Mahmood et al. 1999). Another study indicates that a fragment containing the 5′ UTR plus upstream Ig sequences derived from the low-abundance mRNA *tuzin* gene may contribute to suppress the expression of this multicopy gene by both inefficient RNA processing and poor translation initiation (Teixeira et al. 1999b). On the other hand, various groups have described the influence of 3′ UTR plus Ig sequences on *T. cruzi* gene expression. In their study, Nozaki and Cross (1995) suggested that the highest number of pyrimidine residues present in the Ig region of the *GADPH* gene or its structure could be responsible for the highest values of luciferase activity when compared to the corresponding region of the *HSP60* and *GP85* genes.

Finally, as an alternative approach to generating stable expression of transfected genes in *T. cruzi*, we constructed a new vector designed to allow integration by homologous recombination in the *tubulin* locus of the parasite's genome. Only few reports have described the generation of stable transfected cell lines in *T. cruzi* using integrative vectors, which are transfected as linear plasmids containing sequences homologous to endogenous genes. In the case of pTCR27-2::Neo (Teixeira et al. 1999a), low efficiency results in generating stable transfectants were obtained, possibly because the target gene is a single copy gene. We decided to test whether targeting to a region containing a multicopy gene other than the rRNA could be used as a more efficient method to generate stable transfectants in *T. cruzi*. A vector containing an expression cassette flanked by *β*-tubulin sequences, named pROCKGFPNeo was constructed in such a way that any gene of interest can be inserted in place of *GFP* by double digestion with the enzymes *Xba*I/*Xho*I. To facilitate homologous recombination of *tubulin* sequences, the plasmid is linearized by *Not*I digestion, which has a unique site within the *β*-tubulin fragment (Fig. 5A). Using 50 μg of this vector, 0.4×10^8 epimastigotes of the Tulahuén strain were electroporated and selected in culture media containing 200 μg/ml G418. Drug selection for 4 weeks resulted in no parasites being detected in mock-transfected cultures and approximately 10% of G418-resistant parasites expressing *GFP*. To evaluate the stability of the *GFP* marker we performed analysis by fluorescence-activated cell sorter, which showed that 5 months after electroporation, the percentage of *GFP*-positive cells cultivated in the presence of 200 μg/ml G418 reached 40% (Fig. 5B, C). Moreover, after being cultivated for another 5 weeks in the absence of G418, 33% of the transfected population was still expressing high levels of *GFP* (Fig. 5D). To verify whether the *GFP* marker has been integrated in the *tubulin* locus, we analyzed a transformed population as well one isolated *GFP*-positive clone by pulse field gel electrophoresis. As shown on Fig. 6, Southern blot hybridizations indicate that *GFP*

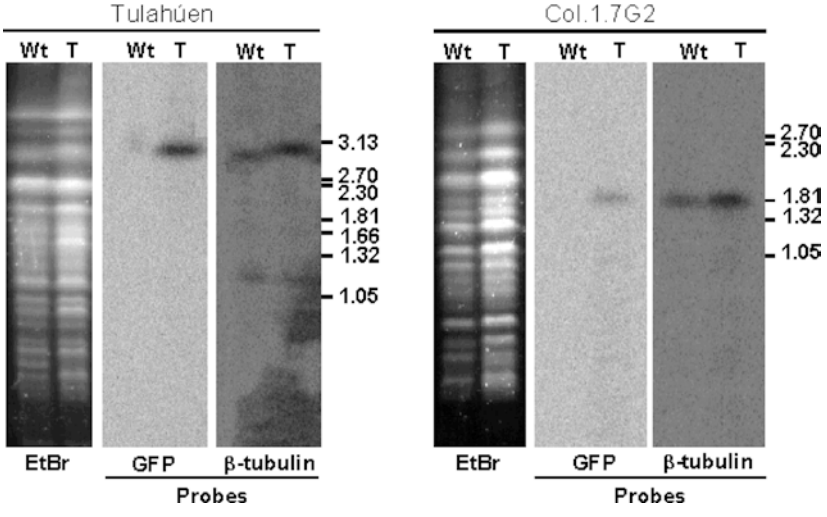


Fig. 6 Integration of pROCKGFPNeo into the *T. cruzi* genome, showing chromosomal bands from wild-type epimastigotes (*Wt*) and from transfected cells (*T*) stained with ethidium bromide (*EtBr*) or hybridized with ^{32}P-labeled probes for *GFP* and *β*–tubulin. In the *left panel*, the results with the Tulahuén strain, wild-type and a cloned, G418-resistant cell line generated after the transfection are shown. In the *right panel* we show the chromosomal separation and the hybridization results of wild type strain and transfected, G418-resistant population of the Col.1.7G2 *T. cruzi* cell line. The *numbers on the right* correspond to the sizes of a chromosomal molecular weight marker

sequences were integrated in the *tubulin* cluster. Both *GFP* and β-tubulin probes hybridized with the same chromosomal band (approximately 3 Mb and 1.8 Mb in the Tulahuén and Col.1.7G2 strains, respectively), which were identified as the β-tubulin locus in the wild type parasites. However we cannot exclude the possibility that some of the plasmids were also maintained as episomal elements. We were able to transfect at least two different strains of *T. cruzi* with (Tulahuén and Col.1.7G2) and in both strains the construct has integrated in the predicted locus of the parasite genome. We have also determined that the cloned cell lines expressing *GFP* are infective in tissue culture cells (S. Pires, unpublished data). These and other pROCKGFPNeo transfected parasites are now available for biological studies in animal models of *T.cruzi* infections.

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