SHORT COMMUNICATION

Hyg^R and Pur^R Plasmid Vectors for Episomal Transfection of *Trypanosoma cruzi*

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This work describes the development and functional testing of two episomes for stable transfection of Trypanosoma cruzi. *pHygD contained the 5'- and 3'- flanking regions of the gene encoding the cathepsin B-like protease of* T. cruzi *as functional trans-splicing and polyadenylation signals for the hyg^R ORF. Evidence is presented to support extrachromosomal maintenance and organization as tandem repeats in transfected parasites. pPac was derived from pHygD by replacement of the entire hyg^R ORF with a pur^R coding region. The ability to modify pHygD and the availability of the complete DNA sequence make these plasmids useful tools for the genetic manipulation of* T. cruzi.

Key words: *Trypanosoma cruzi* - selectable markers - stable transfection

Studies on kinetoplastid protozoa have been facilitated by advances in techniques to manipulate their genomes. The methods are based on plasmid constructions that (1) use dominant selectable markers since auxotrophs are generally not available, and (2) provide the elements needed to accomplish mRNA processing in the cell. Most episomes for *Trypanosoma cruzi* use the neomycin-resistance gene (Kelly et al. 1992, Buckner et al. 1996, Martinez-Calvillo et al. 1997). The hygromycin B phosphotransferase (*HPH*) gene has been used as marker for *T. cruzi* transfectants in experiments involving integration (Cooper et al. 1993, Buckner et al. 1997, Thomas & Gonzalez 1997). There are no reports of puromycin N-acetyltransferase (*PAC*) use in transfection of *T. cruzi*. Here we describe two plasmids that were maintained extrachromosomally in *T. cruzi* and provided resistance to hygromycin (hyg) or puromycin (pur) for potential use in genetic manipulation of this parasite.

The sensitivity of the CL Brener strain of *T. cruzi* to each drug was determined experimentally. The concentration necessary to produce 50% inhibition of growth (IC_{50}) was 100 µg/ml for hyg and 1 µg/ml for pur (data not shown). Both plasmids were originally generated for targeted replacement of the single-copy cathepsin B-like protease gene (*TCCB*) of *T. cruzi* (Nóbrega et al. 1998). Both selectable markers were flanked by PCR-amplified untranslated regions (UTRs) of a *TCCB* plasmid previously isolated from a Berenice strain genomic DNA library. Oligonucleotide primers Kpn and Cla were designed to produce a 1260-bp fragment upstream of the *TCCB* coding region. Primers Not and Sac were used to amplify an 872-bp region downstream of the gene (Fig. 1A, B). Each primer was named for the restriction site added to its 5' end. Following digestion with the cognate enzymes, both 5' and 3' UTRs were cloned into compatible sites present on the starting plasmid pBS HygA (Freedman & Beverly 1993), a pBluescript II SK (pBS) derivative containing the *HPH* open reading frame (ORF), generating the pHygD plasmid (Fig. 1C). The subsequent restriction and sequencing analysis of the construction revealed the loss of the *Cla*I site along with 175 bp from the 3' end of the 5' UTR. A second plasmid, pHygC, had a 234 bp deletion that removed the majority of a 28-bp polypyrimidine tract present 285 bp upstream of the ATG initiation codon of *TCCB* (Fig. 1A). These deletions were likely artifacts of the cloning procedure.

To favor targeted integration of the constructs, both pHygD and pHygC (50 µg) were digested with *Bss*HII, which cuts twice in pBS (Fig. 1C). Each digestion was used to transfect the CL Brener strain of *T. cruzi* without prior isolation of linear cassettes. Electroporation settings were as described previously (Saito et al. 1994) and selection was performed in LIT growth medium with hyg at 250 µg/ml. Cells transfected with pHygD developed resistance to hyg (hyg^R); pHygC did not yield living transfectants under a variety of buffer and electroporation conditions. This result suggested that the 28-bp polypyrimidine tract absent from pHygC might function as the *trans*-splicing site of the *TCCB* mRNA. This was corroborated by analy-

Financial support: CNPq
The sequence data reported herein have been submitted to Genbank^{TM} and assigned the accession numbers AF529182 and AF529183.
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Received 23 December 2003
Accepted 26 May 2004

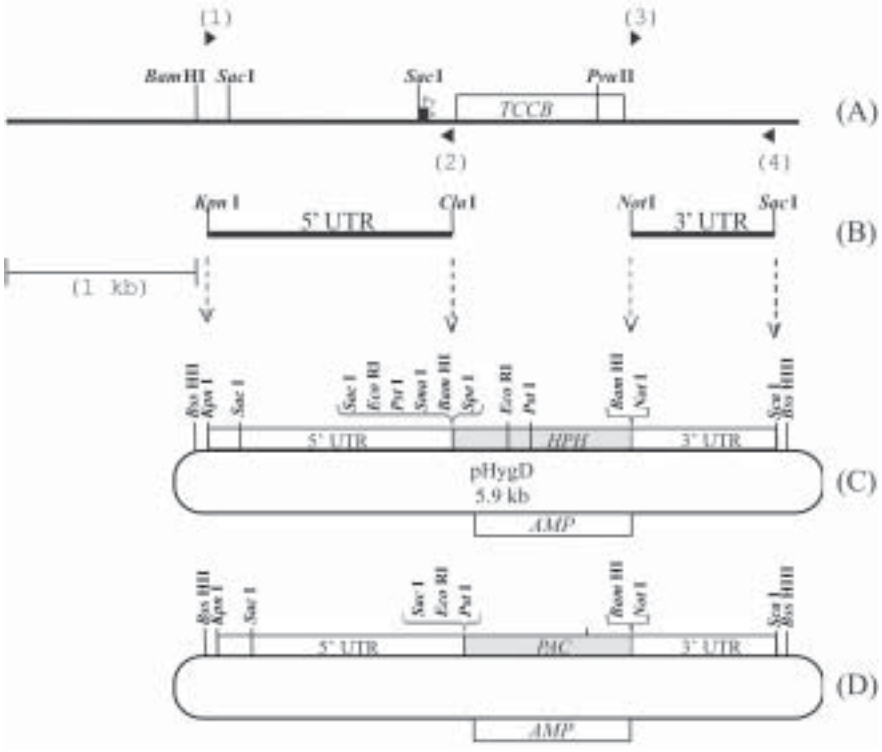

Fig 1: construction of transfection vectors. A map of the different untranslated regions of the *TCCB* locus is shown and schemes of the plasmids generated are presented, showing selected relevant restriction sites. A: map of the insert of the plasmid p2.11A2. The relative position and orientation of the primers used are depicted by arrowheads. Primers Kpn (1) (CGTCG GGGTA CCGGA CTGTG TCTCT GCGTG) and Cla (2) (ACGTG GATCG ATCCT TTTAA GCAGT TGTAC C) were designed to amplify a 1.2 kb segment of the 5' UTR, and primer Not (3) (AAATA AGAAT GCGGC CGCCT CTGTG GGTGT TGAGA AG) and Sac (4) (TAGCC GAGCT CGGAT CGATA TTGCC TGCC), to amplify a 0.8 kb 3' UTR (synthetic restriction site are underlined). The putative splicing acceptor site is indicated by an asterisk (*) and the 28-bp polypyrimidine tract by a thick line (Py); B: the length and position of the PCR products amplified with the primers above are indicated by solid lines relative to the map in A; C: construction of pHygD. As indicated by dashed arrows, the segments shown in B were ligated to pBS HygA to generate plasmid pHygD. All the synthetic restriction sites were maintained in the resulting construction except for *Cla*I; D: construction of pPac. The *HPH* ORF was replaced by the *PAC* ORF, generating plasmid pPac. The *HPH* ORF was excised from pHygD through *Sma*I-*Bam*HI digestion, and a blunted-*Bam*HI preparation of the *PAC* coding region originated from pVN 3.1 was ligated to it. The *TCCB* UTR regions employed in both constructions remain identical.

sis of the 5' UTR of the *TCCB* transcript by reverse transcriptase-PCR using the *T. cruzi* spliced leader sequence and part of the ORF as annealing sites for sense (TATTG CTACA GTTTC TGTAC TATAT TG) and antisense (CTCAT GATTC CAGCT GTTCG C) primers, respectively (data not shown). The splice-acceptor site was present 199 bp upstream of the ATG initiation codon of *TCCB*.

From the pool of hyg^R parasites, clonal lines pHygD1, pHygD2, and pHygD3 were derived by limiting dilution. To assess the organization of pHygD in these lines, total DNA was digested with *Pst*I and screened by Southern blotting using the PCR-amplified *TCCB* 5' UTR as probe. In addition to the chromosomal signal present in the untransfected DNA sample, the resulting profile showed an intense 5.6-kb band in all clonal lines (Fig. 2A), consistent with the linear size of the plasmid and suggesting episomal maintenance rather than the anticipated targeted integration event. No integration events were observed in either clonal or non-clonal (data not shown) populations. The presence of multiple copies of the plasmid was inferred based on the hybridization signal of the episome relative to the genomic signal in each clonal line. Densitometric comparison of the hybridization signals suggested that 20 to 70 copies of the plasmid were present per diploid genomic copy.

Arrangement in multiple, covalently-linked monomers is a common type of episomal structure in transfected trypanosomatids (Kelly et al. 1992, Biebinger & Clayton 1996). Southern blot analysis of pHygD1 DNA partially digested with *Kpn*I was performed using the *HPH* gene as probe. A digestion time course showed a pattern of high molecular weight forms of episome (uncut lane) that were resolved into bands of multiple sizes, consistent with the linear plasmid as well as dimeric (11.8 kb), trimeric (17.7 kb), tetrameric (23.6 kb) and pentameric (29.5 kb) forms (Fig. 2B). Thus, pHygD was maintained extrachromosomally and organized as tandem repeats in *T. cruzi*. Since electroporations were carried out with digested plasmid, the unexpected episomal organization could have arisen from religation of the DNA fragments in the intracellular milieu. Circularization of linear DNA fragments carrying cohesive ends have been described in vivo as an

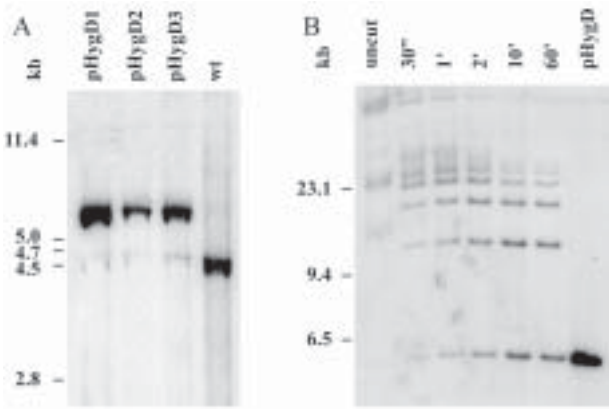

Fig. 2: analysis of the hygromycin-resistant (hyg^R) lines. DNA isolated from wild type *Trypanosoma cruzi* and from clonal populations of hyg^R cells (Medina-Acosta & Cross 1993) were submitted to Southern blot analysis in order to determine presence, organization and number of copies of the pHygD plasmid in transfected lines. A: episomal organization of pHygD in cloned populations. 5 µg of total DNA from three different clonal lines and 30 µg from wild type (wt) cells were digested with *Pst*I and probed with the *TCCB* 5' UTR. Six fold excess of wt DNA was used to allow the precise determination of the band corresponding to the genomic copy of the *TCCB* locus; B: clone pHygD1 contains multimeric copies of the episome. 10 µg of total DNA was digested with 5 units of *Kpn*I for the times indicated, electrophoresed in a 0.4% gel and probed with a *Bam*HI fragment of pBS HygA which contains the *HPH* coding region. Undigested DNA as well as 500 pg of linear pHygD were used as controls. All Southern blots were performed at high stringency. Marker sizes are indicated.

event that may precede targeted integration in *T. cruzi* (Chung & Swindle 1997). However, since the electroporated DNA was not gel purified, we cannot rule out the possibility that uncut plasmid was present at the time of electroporation.

The second vector was derived from pHygD by replacing the entire *HPH* ORF with the *PAC* ORF isolated from pVN3.1 (Lacalle et al. 1989). To ligate the *PAC* ORF between the single *Sma*I site and the *Bam*HI site downstream of *HPH*, pVN3.1 was first digested with *Not*I (which cuts upstream of *PAC*), blunt ended using Klenow fragment and then digested with *Bam*HI (which cuts downstream of *PAC*). The 0.8-kb blunted-*Bam*HI fragment was gel purified and ligated to the sites referred to above generating plasmid pPac (Fig. 1D). Due to the results obtained with pHygD transfections, the ability of pPac to generate episomes was tested using the uncut construction. Pur^R populations were obtained following electroporation of the CL Brener strain of *T. cruzi* with the circular plasmid (50 mg) and selection with pur at 3 µg/ml.

Efficient expression of heterologous genes in trypanosomatids is dependent on functional *trans*-splicing sites upstream of the gene (Laban & Wirth 1989, Bellofatto et al. 1991). In some cases, providing downstream *trans*-splicing acceptor sequences was required for polyadenylation of the transcripts (LeBowitz et al. 1993). All these requirements were met for the expression of the selectable markers developed in this study. The strain from which regulatory sequences were derived (Berenice) and the strain into which the plasmids were introduced (CL Brener) belong to different discrete typing units defined among the *T. cruzi* isolates (Brisse et al. 2000), possibly accounting for the lack of integration due to insufficient UTR homology, as seen in *T. brucei* (Blundell et al. 1996). The ability to modify pHygD to contain alternative selectable markers or to include reporter genes indicated that both vectors constitute potentially useful tools for the genetic manipulation of *T. cruzi*.

ACKNOWLEDGMENTS

To Dr Stephen Beverley (Washington University) and Dr Juan Ortín (UAM, Spain) for providing us with plasmids pBS HygA and pVN3.1, respectively. To Dr T Guy Roberts and Dr Michael C Yu for comments and suggestions.

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