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A shuttle vector which facilitates the expression of transfected genes in *Trypanosoma cruzi* and *Leishmania*

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ABSTRACT

A *Trypanosoma cruzi* expression vector has been constructed using sequences derived from the flanking regions of the glyceraldehyde 3-phosphate dehydrogenase (gGAPDH) genes. The neomycin phosphotransferase (*neo*^{r}) gene was incorporated as a selectable marker. Using electroporation we have introduced this vector into both *T.cruzi* and *Leishmania* cells and conferred G418 resistance. Transformation is mediated by large extrachromosomal circular elements composed of head-to-tail tandem repeats of the vector. The transformed phenotype is stable for at least 6 months in the absence of G418 and can be maintained during passage through the *T.cruzi* life-cycle. Foreign genes inserted into an expression site within the vector (pTEX) can be expressed at high levels in transformed cells. To our knowledge this paper describes the first trypanosome shuttle vector and the first vector which functions in both trypanosomes and *Leishmania*.

INTRODUCTION

The kinetoplastid protozoan parasite *Trypanosoma cruzi* is the causative agent of Chagas' disease, a serious public health problem in many Central and Southern American countries with up to 20 million people affected (1). The disease has a complex pathology, is often unresponsive to chemotherapy and there is no immediate prospect for the development of a vaccine (2). Research on many aspects of *T.cruzi* would greatly benefit from the development of a genetic transformation system as has recently been described for the other parasitic trypanosomatids *Leishmania* (3–8) and *Trypanosoma brucei* (9–11).

The mechanisms of trypanosomatid gene expression are complex and include several unique or unusual features (for review 12). For example, many trypanosomatid genes are arranged as direct tandem repeats and evidence suggests that expression involves multicistronic transcription followed by rapid processing to yield mature mRNA (13–15). The inability until recently, to identify trypanosome promoters, has complicated the design of expression vectors suitable for use with these organisms. However a variety of techniques including transient transfection assays have now been used to localise the promoter sequences for variant surface glycoprotein (VSG) genes (15,17) and the procyclin genes (18,19) of the African trypanosome, *T.brucei*. No regulatory sequences have been identified for any *T.cruzi* protein encoding gene and it is not known if a large separation occurs between a gene and its promoter as has been inferred for some *T.brucei* genes.

We initiated the development of transformation vectors for *T.cruzi* after isolation of the genes which encode the glycosomal enzyme glyceraldehyde 3-phosphate dehydrogenase (gGAPDH) (20). In *T.cruzi* these genes are arranged as a direct tandem repeat, of identical nucleotide sequence, separated by a short intergenic region (517 nucleotides). Tandemly repeated genes are located at two genetic loci in the X10/6 strain (0.92 and 0.95 megabase pair chromosomes). This paper describes the construction of *T.cruzi* expression vectors using fragments derived from the flanking regions of the gGAPDH genes. These constructs which replicate episomally in transformed cells, allow the expression of foreign genes into both trypanosomes and *Leishmania*. They will have widespread application to many areas of trypanosomatid research.

MATERIALS AND METHODS

Parasites

Culture form (epimastigote) *T.cruzi* (MHOM/BR/78/Sylvio-X10-CL6) were grown at 28°C as described previously (20). Although metacyclic (infective) trypomastigotes were observed within *T.cruzi* cultures, microscopically detectable peripheral blood parasitaemias were not produced in Balb/C mice inoculated directly with organisms from that source (either transformed or non-transformed). Accordingly *T.cruzi* transformed lines, and the parental controls were first passaged onto Vero cells. 1 ml of a stationary phase *T.cruzi* culture was added to an 80cm^2 50% confluent monolayer of Vero cells for 5hr and epimastigotes then removed by vigorous washing (×3). 15 to 20 days later emergent trypomastigotes were harvested by centrifugation and inoculated i.p. into S.C.I.D. mice. Parasitaemias were microscopically patent after 17 to 24 days and 3rd to 5th stage nymphs of the triatomine bug *Rhodnius prolixus* were then fed on anaesthetized mice. Parasites were also re-isolated by culture from cardiac blood. Fed bugs were dissected 20 to 25 days later, after washing in White's solution (22). Parasites were cultured from the intestinal contents, which were infected with epimastigotes and metacyclic trypomastigotes. They were layered

onto Difco medium (23) before re-establishment in liquid culture and subsequent analysis. *L.mexicana* (MHOM/BZ/82/BEL21) and *L.donovani* (MHOM/ET/67/HU3) promastigotes were maintained in culture at 23°C as described (24).

Construction of vectors

(i) *pTGAP (Fig. 1b).* Vector pTGAP contains a 1.7kb *Bam*HI/*Dra*I fragment derived from the 5′-upstream region of gGAPDH I (20) (Fig. 1a). The 0.8kb *neo*^{r} component was isolated from pMC1neo (Stratagene) after *Mlu*I/*Sal*I digestion. The 3′-region of the construct was derived from the downstream region of the gGAPDH II gene by *Pst*I/*Bam*HI digestion. These fragments were subcloned sequentially into pBluescript SK (Stratagene) to produce pTGAP (5.2kb) (Fig. 1b).

(ii) *pTGAN (Fig. 1c).* Construction of this vector began with partial *Dra*I digestion of the 5kb *Bam*HI fragment which contains both gGAPDH genes (20) (Fig. 1a). A 3.3kb *Bam*HI/*Dra*I fragment was isolated which contained 1.7kb of 5′-upstream sequence, the gGAPDH I gene and the intergenic region. This was subcloned into the *Bam*HI/*Sma*I site of pBluescript SK. The *neo*^{r} gene and the 3′-downstream region were isolated as described above and subcloned sequentially into this vector to yield pTGAN (7.8kb).

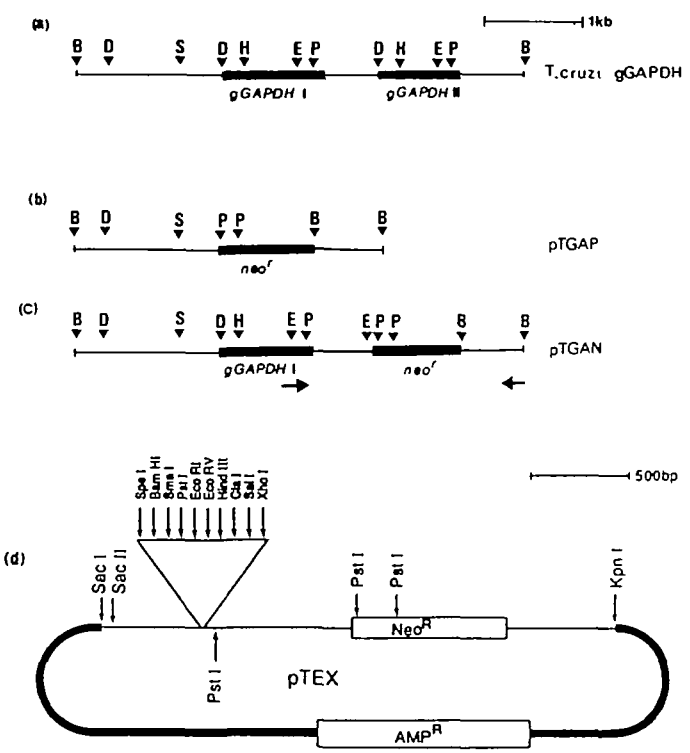

Fig. 1. Constructs used in transformation experiments. (a) Restriction map of the *T.cruzi* gGAPDH genes (20). B, *Bam*HI; D, *Dra*I; H, *Hind*III; E, *Eco*RI; P, *Pst*I; S, *Sac*II. There is a *Dra*I recognition site (TTTAAA) immediately adjacent to both start codons. (b,c) First-generation transformation vectors pTGAP and pTGAN (see Methods for details of construction). The sequences illustrated were subcloned into plasmid DNA (pBluescript) to allow propagation in *E. coli* (as for pTEX). (d) Shuttle vector pTEX (5.6kb). This vector was derived from pTGAN as described in Methods. The arrows beneath vector pTGAN indicate the location of primers used in the construction of pTEX. The expression site polylinker contains 9 unique restriction sites. The gGAPDH flanking sequences ensure that DNA cloned into the expression site can be transcribed to yield functional mRNA.

(iii) *pTEX (Fig. 1d).* The polymerase chain reaction (PCR) was used to amplify a 2.3kb fragment from vector pTGAN (Fig. 1c). The oligonucleotides used were 5′-GGCTCGAGCCATTTACG-ACTCCAAG$_{1241}$ and 5′-CCGGTACCCACACGGCTAGCAT-ACT$_{3573}$ (see ref 20 for precise location). The amplified fragment contained the entire intergenic region, the *neo*^{r} gene and the 3′-downstream region. Addition of *Xho*I and *Kpn*I sites to the termini of the fragment during amplification facilitated subcloning into the corresponding site of pBluescript KS to yield construct pT1. A 450 bp fragment (*Sac*II/*Dra*I) was derived from the 5′-upstream region of the gGAPDH I gene (Fig. 1a). This was subcloned into the *Sac*II/*Xba*I (blunt-ended) site of pT1 to produce construct pTEX-5. The final version of the pTEX vector (5.6kb) was achieved after removal of the *Eco*RI and *Bam*HI sites flanking the *neo*^{r} gene using partial digestion, end-fill in and religation.

Transfection procedures

Logarithmic-phase *T.cruzi* epimastigotes were washed once with phosphate buffered saline and resuspended at 10^8 cells/ml in electroporation buffer (272mM sucrose, 7mM sodium phosphate, pH 7.2.). After 10 minutes on ice, 10^8 cells were added to vector DNA (5−100μg) in a 24 well microtitre plate and left for a further 20 minutes. The cells were electroporated using a Hoefner Progenitor I apparatus set at 400V/99ms. In different experiments between 2 and 12 pulses have been used, all of which yielded transformants. The cells were left on ice for a further 10−20 minutes and then 200μl aliquots added to 1ml of normal growth medium. 16−18 hr later, 1ml of these cultures was added to 10ml of growth medium + G418 (100μg/ml). The cells were subcultured 1:3 after 3 days and then 1:10 after a further 5 days in the continued presence of G418. Drug resistant cells were usually observed 7−11 days later. As a matter of course, these cells are continuously subcultured in the presence of G418 for 4−6 weeks prior to preliminary analysis. A similar protocol was used for transfection of *Leishmania* promastigotes.

Analysis of DNA and RNA

High molecular weight genomic DNA was isolated from parasite cells by the proteinase K method (25). Preparation of chromosome-sized DNA was as described (26). Clamped, homogenous electric field (CHEF) electrophoresis (27) was performed using a BioRad CHEF DRII system (28). RNA was extracted from parasite cells after lysis with 4M guanidinium thiocyanate and pelleted by centrifugation through a 5.7M caesium chloride gradient. Northern analysis was performed after fractionation of glyoxalated RNA through 1.2% agarose gels (29).

Chloramphenicol acetyl transferase (CAT) assay

Parasite lysates were produced by freeze-thawing (×3) the cells in CAT extraction buffer (250mM Tris/HCl, pH7.6) (200μl per 10^8 cells) Lysates were stored at −20°C until analysis and assays were carried out as described previously (30). To stay within the linear range of the assay, 500-fold dilutions of the lysates were required. Reactions were incubated at 37°C for 16 hr, extracted once with ethyl acetate and spotted onto silica TLC plates. The TLC plates were developed in chloroform/methanol (95:5) and autoradiographed. CAT activity was established after scraping both acetylated and non-acetylated regions of the TLC plate and determining the amount of radioactivity by liquid scintillation counting. Results obtained (as cpm acetylated ^{14}C-chloramphenicol) were converted to units of CAT enzyme by

comparison with a titration of *E. coli* CAT (Pharmacia). Protein estimations were performed on the cell lysates (31) which allowed the CAT activity to be expressed as a percentage of the total cell protein.

GAPDH assay

GAPDH assays were performed as described by Misset and Opperdoes (32).

RESULTS

Stable transformation of *T.cruzi*

Initially we constructed two *T.cruzi* transformation vectors (see Methods for details). In construct pTGAP (Fig. 1b) the *neo*^{r} gene was fused to the 5′-upstream (1.7kb) and 3′-downstream region (0.7kb) of the gGAPDH tandem repeat (Fig. 1a); i.e. it was positioned to replace both of the gGAPDH genes and the intergenic sequence. In construct pTGAN (Fig. 1c) the *neo*^{r} gene was positioned to replace only gGAPDH II. *T.cruzi* cells were transfected with circular vector DNA by electroporation and transformants selected by the ability to grow in the presence of G418 (100μg/ml). Drug-resistant organisms, transformed with multiple copies of the vector DNA, were obtained in all experiments using both constructs. Attempts to transform cells with various linearised forms of pTGAP or pTGAN have not so far been successful. Some transformed lines contained 2−3 copies of vector DNA per cell whereas others had more than 50 [*Fig. 2a;* compare the endogenous gGAPDH signal (at least 4 copies per cell) with that derived from the gGAPDH component of the pTGAN vector].

The vectors pTGAP and pTGAN (Fig. 1b and c) were originally designed to facilitate targeted integration into the gGAPDH locus. In all cases so far investigated, transformation of the African trypanosome *T.brucei* has involved homologous recombination (9−11). We have now examined more than 20 drug-resistant *T.cruzi* cell lines and found no evidence for such an event. Instead the data are consistent with the transfected DNA being located extrachromosomally as large circular elements formed from head-to-tail tandem repeats of the vector sequence. The evidence is as follows:

(i) Junction fragments indicative of integration (either homologous or non-homologous) could not be detected on Southern blots of DNA from transformed lines. In the examples shown (Fig. 3a, b) DNA from a transformed line containing 3 or 4 vector copies per cell (see also track 1, Fig. 2a, b) was cut with *EcoRI* and *Pst*I. Targeted integration of the pTGAN sequences would have resulted in the appearance of a 5.8 kb band with both enzymes (as a result of sites downstream of the locus) when the blot was hybridised with the gGAPDH and *neo*^{r} probes (Fig. 3a, b). No such bands were apparent even after long exposure. Instead the pattern of hybridisation was identical to that observed when vector DNA alone was analyzed. The data further suggests that transfected DNA does not occur as linear molecules either in the form of single copies or tandem arrays; fragments derived from the termini of episomal linear molecules could not be detected (Fig. 3b as example).

(ii) Southern analysis of uncut DNA obtained from transformed lines demonstrated that the vector DNA sequences did not migrate as single circular copies, rather they co-migrated with high molecular weight genomic DNA. When the DNA was cut with *Hind*III (Fig. 3c) (which cuts once within the vector) in a time-course experiment, a series of fragments (multiples of 7.8kb) were detected by the *neo*^{r} probe. This suggested that multiple covalently joined copies of the linear vector were present arranged in a head-to-tail conformation.

(iii) Southern blots were prepared after chromosomal DNA from transformed cells had been fractionated by CHEF electrophoresis. A complex pattern was usually observed with extensive hybridisation to material close to the slot (Fig. 4). Bands detected with the *neo*^{r} probe did not correlate with the gGAPDH locus and frequently did not correspond with chromosomes visualised by ethidium bromide staining. Further CHEF analysis has indicated that the bands detected by hybridisation migrate independently of pulse time, a characteristic of circular DNA molecules.

Fig. 2a and b illustrate another phenomenon which was observed in approximately 15% of transformed lines, namely the presence of recombined or truncated forms of the vector. For example the 3.4kb band highlighted in Fig. 2a and the 2.5kb band highlighted in Fig. 2b (track 5 in both cases) are derived from pTGAN sequences which have undergone rearrangement.

Maintenance of a transformed phenotype throughout the parasite life-cycle

Transformed cells have been continuously cultured for more than one year in the presence of G418 with no reduction in the copy number of vector molecules. Additionally, transformed cells remained drug-resistant for at least 6 months when grown in the absence of G418. However after 10 months drug-resistant organisms could not be rescued. This reflected the loss of detectable vector sequences within the parasite population. We therefore sought to determine whether a transformed phenotype could be maintained after passage through the infectious cycle of the parasite.

Triatomine bugs were fed on mice which had been infected by inoculation with transformed *T.cruzi* (Methods). Parasites were subsequently cultured from the intestinal contents of the insects. The insect derived parasites were G418 resistant and were

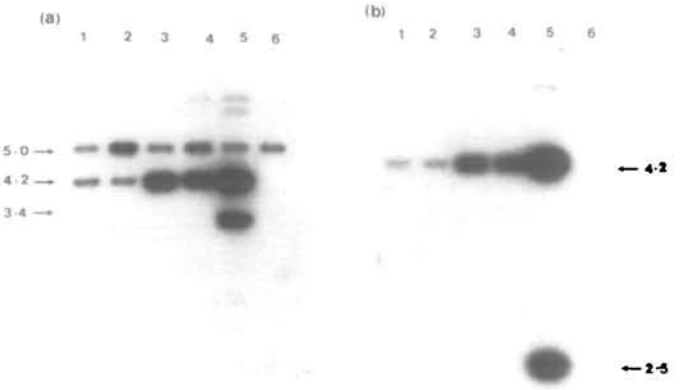

Fig. 2. (a) Southern analysis of *Bam*HI digested DNA obtained from independently derived transformed *T.cruzi* lines which had been transfected with vector pTGAN (tracks 1−5) or from non-transformed *T.cruzi* cells (track 6). The blot was hybridised with the 0.8kb radiolabelled *DraI/EcoR*I gGAPDH fragment (Fig. 1a). The fragment sizes (indicated by arrows) are given in kilobase pairs (kb). The 5kb band in each track corresponds to the endogenous gGAPDH genes. The intensity of this band acts as an internal control for the relative amount of DNA in each track. (b) The same as (a) except the blot was hybridised with a radiolabelled 0.7kb *neo*^{r} probe.

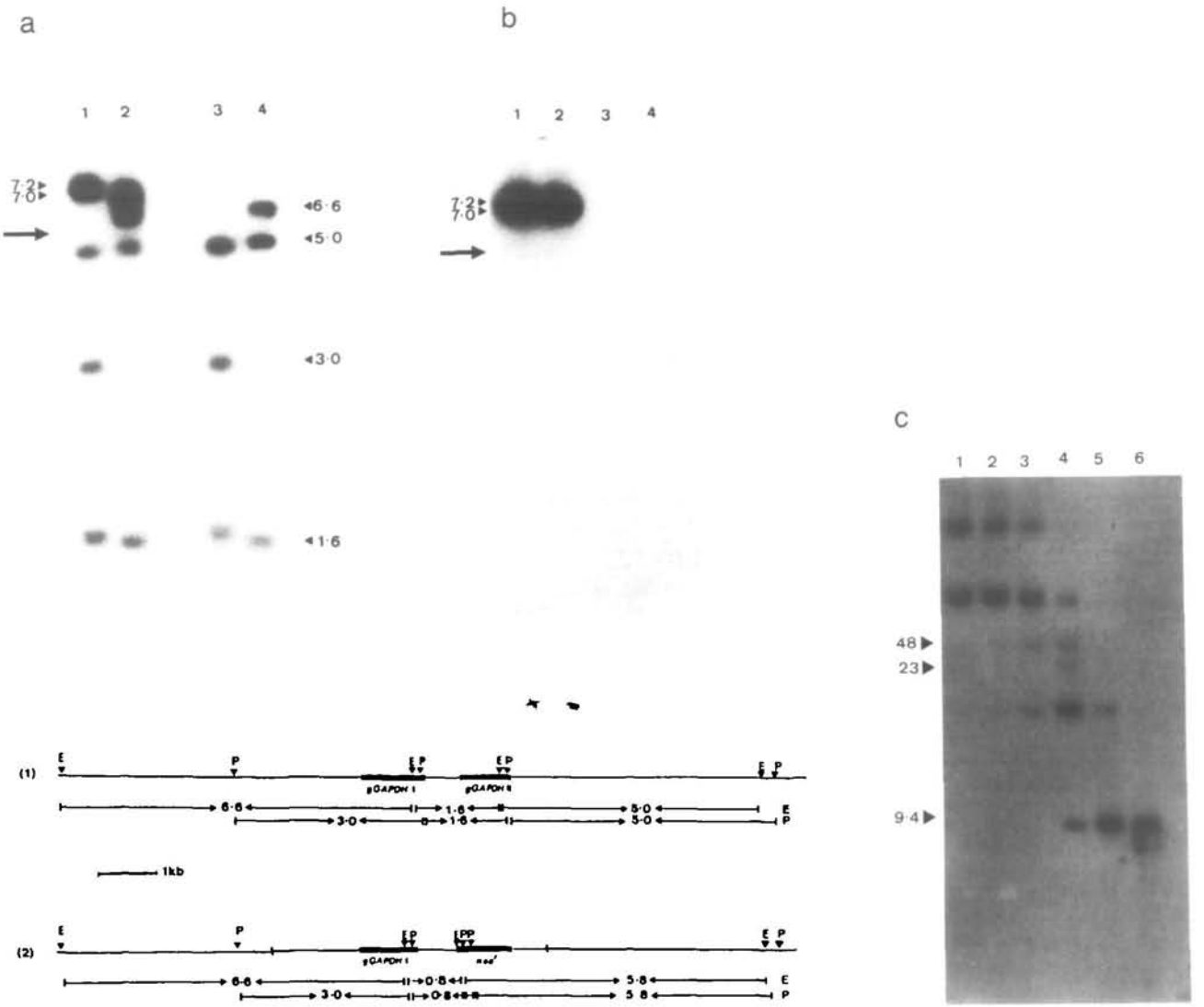

Fig. 3. Analysis of DNA from a *T.cruzi* cell line which had been transformed with vector pTGAN. (a) Autoradiograph of blot hybridised with a gGAPDH probe (1.6kb *Hind*III fragment (Fig. 1a) (b) Autoradiograph of blot hybridised with the 0.7kb *neo*^{r} probe. Tracks 1, 2 contain DNA from a transformed *T.cruzi* line (the same as track 1, Fig. 2a, b). Tracks 3, 4 contain DNA from non-transformed *T.cruzi*. Tracks 1 and 3 are *Pst*I digests. Tracks 2 and 4 are *EcoR*I digests. Restriction map (1) shows the location of *EcoR*I (E) and *Pst*I (P) sites flanking the gGAPDH genes with the sizes of the resultant fragments (kb) marked (compare with tracks 3 and 4, Fig. 3a). Restriction map (2) shows the hypothetical situation after targeted integration of the pTGAN sequences with the predicted restriction fragments. Note the absence of the 5.8kb *Pst*I and *EcoR*I bands (highlighted by arrows) in autoradiographs (a) and (b). The gGAPDH probe contains sequences from the 3'-untranslated region of the gene and would hybridise to this band. Bands of 0.8kb have migrated beyond the area covered by this autoradiograph. (c) Southern analysis of the same DNA as in Fig. 3a and b which has been digested with *Hind*III for 5 min (track 1), 10 min (track 2), 20 min (track 3), 30 min (track 4), 1hr (track 5) and 2hr (track 6). Complete digestion yields a single 7.8kb band. Uncut DNA runs as a band of >50 kb. In some experiments there was also hybridisation to a band which migrates slower than uncut genomic DNA; this probably represents concatenated forms of the vector. The gel was 0.4% agarose and hybridisation was with the *neo*^{r} probe. Size markers are in kb.

found to have retained a high copy number of vector DNA sequences (data not shown). Similar results were obtained when transformed *T.cruzi* were isolated from infected Vero cells grown in culture. Since the transformed phenotype does not affect infectivity and can be maintained after passage through mammalian and insect hosts it should be feasible to adapt these vectors to study aspects of expression and function of transfected genes at each stage of the parasite life cycle.

Expression of transfected genes in *T.cruzi*

A *neo*^{r} transcript of the predicted size (1.2kb) was detected on Northern blots of RNA from transformed cells (Fig. 5b). There was also an increase in the level of gGAPDH RNA in cells transfected with pTGAN (Fig. 5a). The abundance of both transcripts could be increased by raising the G418 concentration. In most cases examined (e.g. Fig. 5) the increase in the level of gGAPDH RNA was greater than that observed for the *neo*^{r} RNA; the reason for this remains unclear. The increase correlated with an increase in the copy number of vector DNA sequences (Fig. 7 as example). In cells cultured at 5000μg/ml the level of gGAPDH RNA was up to 100-fold greater than that constitutively expressed (Fig. 5). At this G418 concentration the growth rate is only slightly reduced from that of wild-type cells in drug-free medium.

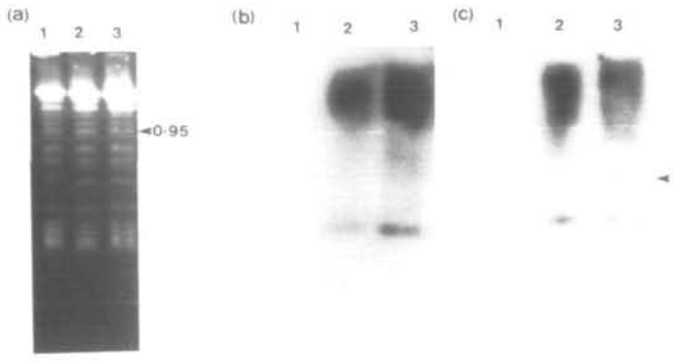

Fig. 4. CHEF electrophoresis analysis of DNA from transformed *T.cruzi*. Chromosomal DNA from non-transformed *T.cruzi* (track 1) and *T.cruzi* transformed with construct pTGAN (track 2, 3) was fractionated by CHEF electrophoresis on a 1.2% agarose gel run at 160v with switching times 1 sec: 30 min then 90 sec: 42 hr. (a) Ethidium bromide stained gel. (b and c) Autoradiographs of Southern blots hybridised with *neo*^{r} (b) and gGAPDH (c) probes (as in Fig. 3). The location of the gGAPDH locus is indicated by an arrow. Two closely migrating gGAPDH chromosome bands can be resolved after extended electrophoresis (not shown). Sizes are given in megabase pairs (mb).

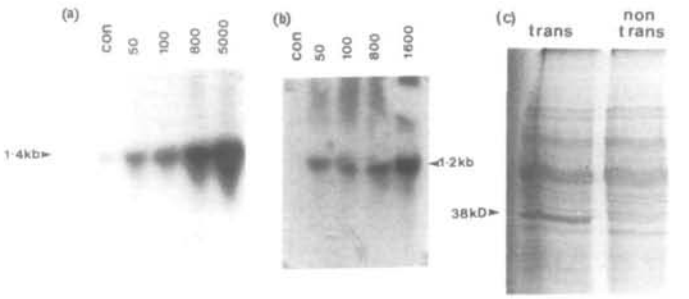

Fig. 5. Expression of transfected genes in *T.cruzi* (a, b) Northern analysis of RNA obtained from a *T.cruzi* cell line which had been transformed with pTGAN (Fig. 1c). The blots were hybridised with gGAPDH (a) and *neo*^{r} (b) probes (see Fig. 3). The cells, were continuously cultured at different G418 concentrations (50−5000μg/ml) for 2 months prior to RNA preparation and Northern analysis. The control (con) tracks contain RNA from non-transfected *T.cruzi* cells. (c) Protein lysates of the same transformed line (grown at 100μg/ml G418) and a non-transformed control were fractionated by SDS-PAGE (7%). The 38kD band prominent in transformed cells is indicated.

The enzymatic activity of GAPDH (Methods) in cells transformed with pTGAN was up to 5−10 fold greater than that in non-transformed cells when measured in 3 separate experiments (average values 1.03 and 0.16 μmoles/min/mg respectively). A 38 kD protein (the size of gGAPDH) constitutes up to 10% of the total cellular protein in these cells (Fig. 5c).

Stable transformation of *Leishmania*

Promastigotes of *L.mexicana* (a causative agent of cutaneous leishmaniasis) were transfected with pTGAN DNA (Fig. 1c) using the same conditions for electroporation and for selection of recombinants as described for *T.cruzi* (Methods). G418 resistant parasites were obtained in all experiments with the growth rate of transformed cells (100μg/ml G418) being similar or only slightly reduced when compared to wild type cells. Increasing the drug concentration from 100 to 2000μg/ml resulted

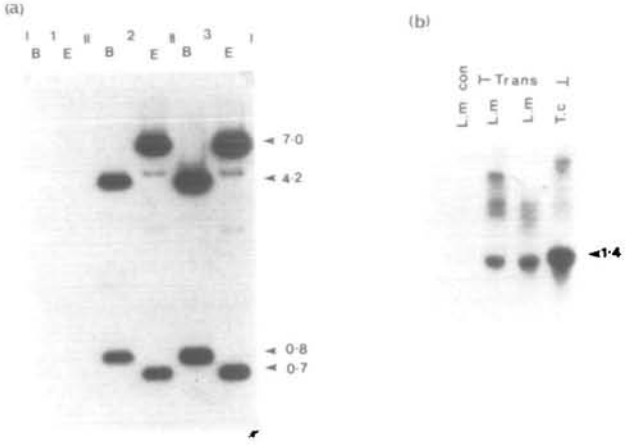

Fig. 6. Expression of foreign genes in transformed *L.mexicana*. (a) Southern analysis of DNA obtained from non-transformed (1) and transformed *L.mexicana* (2,3). The blot was hybridised with a *T.cruzi* gGAPDH probe under conditions in which there is no cross-hybridisation with the corresponding *L.mexicana* gene (Track 1B, E). The signal in tracks 2 and 3 corresponds to approx. 50 copies of vector DNA per cell as judged by comparison with the extent of hybridisation to comparable amounts of DNA from transformed *T.cruzi* cells (see Fig. 2a as example). B, *Bam*HI; E, *EcoR*I. (b) Northern analysis of RNA isolated from *L.mexicana* (L.m) and *T.cruzi* (T.c); transformed (trans) or control (con). The *L.mexicana* cells had been maintained at 200μg/ml G418 and the *T.cruzi* at 1600μg/ml G418. The probe used was *T.cruzi* gGAPDH. Sizes are in kb.

in a short lag in growth rate (3−4 days) followed by a return to the normal level. Southern analysis of DNA from transformed cells demonstrated that transfected DNA (Fig. 6a) was present in multiple copies and that the hybridisation pattern was the same as that produced by vector DNA alone. Further experiments (not shown), similar to those described for *T.cruzi* (above), suggest that in *Leishmania*, the transfected DNA is also arranged as direct tandem repeats within circular DNA elements. There is no evidence for integration. Similar conclusions can be drawn from analysis of transformed *L.donovani*, the causative agent of visceral leishmaniasis.

Northern analysis of RNA from transformed *L.mexicana* identified the presence of RNA transcripts of the predicted size (see gGAPDH RNA; *Fig. 6b*). This corresponded with a level of GAPDH activity (in cells grown at 200μg/ml G418) which was 5 times greater than in non-transformed cells (0.58 compared with 0.11 μmoles/min/mg). These experiments demonstrate that foreign genes, flanked by *T.cruzi* sequences, can be transcribed and translated in *Leishmania* cells, at least in the context of the pTGAN vector.

Construction of shuttle vector pTEX

The aim in constructing pTEX (Fig. 1d) was to produce a vector which would simplify the introduction of foreign genes into *T.cruzi* and *Leishmania*. The protocol used is detailed in the methods section. Briefly, the gGAPDH component of pTGAN (Fig. 1c) was removed and replaced by a polylinker and the 5′-upstream region was reduced from 1.7kb to 0.45kb. Where feasible, restriction sites were deleted to increase to nine the number of unique sites in the expression site polylinker.

The potential of pTEX to function as an efficient expression vector was examined using the bacterial *cat* gene as a model. The *cat* gene was cloned into the *Hind*III/*Sma*I site in the polylinker (Fig. 1d) to produce the construct pTEX-*cat*. After

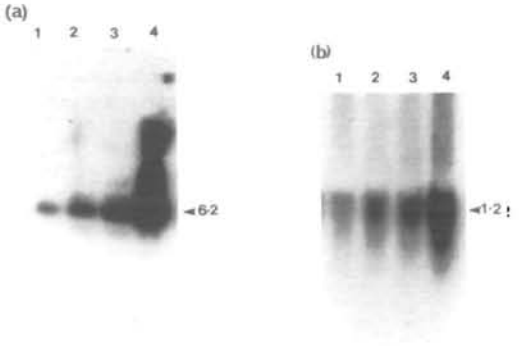

Fig. 7. Expression of CAT in stably transformed *T.cruzi*. *T.cruzi* cells were transfected with pTEX-*cat* and selected for G418 resistance (at 100μg/ml). When a drug-resistant line had been established, the cells were subcultured and grown continuously for 2 months in the presence of either 100 (track 1), 400 (track 2), 1000 (track 3) or 2000μg/ml G418 (track 4) and the DNA and RNA were prepared. (a) Southern analysis of *Hind*III cut DNA. (3μg per track) using a *cat* gene probe. The 6.2kb band identified is the linearised vector. (b) Northern analysis of RNA using the *cat* probe (20μg per track) with the 1.2 kb *cat* RNA indicated. Both autoradiographs were exposed for 3hr at −70°C.

transfection with this vector, using the same conditions as before (Methods), G418-resistant *T.cruzi* cells were obtained. The properties, as regards drug-resistance, of these cells were similar to cells transfected with pTGAP or pTGAN, i.e. the drug-pressure could be increased to high levels (>2000μg/ml) without significantly affecting the growth rate. Analysis of DNA from transformed cells revealed that as with pTGAN, pTEX-*cat* was maintained at high copy number and was arranged as direct tandem repeats. Furthermore the copy number of pTEX-*cat* could be increased by raising the G418 concentration (Fig. 7a). This parallels an increase in the abundance of *cat* RNA (Fig. 7b) and CAT activity (Table 1). In cells cultured at 2000μg/ml CAT constitutes more than 1% of the total cellular protein. Vector DNA could also be rescued from transformed parasites and propagated in *E. coli*. This required restriction digestion of total DNA with an enzyme which linearises the vector (*Sac*I was used) followed by religation and transfection. Direct transfection with total DNA did not produce ampicillin-resistant colonies suggesting the absence of single circular copies of the vector within transformed *T.cruzi* cells.

pTEX vectors have also been used to successfully introduce foreign genes into *L.mexicana* and *L.donovani* (data not shown). The resultant level of expression is similar to that obtained with *T.cruzi*. These results show that pTEX and related vectors can now be exploited as a means of investigating biochemical and genetic features of both trypanosomes and *Leishmania*.

DISCUSSION

The vectors described in this report fulfil several desirable criteria which suggest that they will have widespread application in the functional analysis of trypanosomatid genes; (i) transformation of both *T.cruzi* and *Leishmania* is reproducible with a success rate of close to 100%, (ii) the transformed phenotype can be maintained throughout the entire *T.cruzi* life cycle or in the prolonged absence of G418, (iii) a high copy number of introduced genes can be achieved, (iv) the level of expressed transfected gene products can be increased by raising the G418

Table 1. Expression of CAT in stably transformed *T.cruzi* as a function of G418 concentration

G418 concentration (μg/ml)[a]	CAT (% of total cellular protein)[c]
100	0.14[b] ± 0.04
400	0.35 ± 0.07
1000	0.83 ± 0.10
2000	1.17 ± 0.30

[a]The cell line used was the same as described in legend to Fig. 7.
[b]In two other independently derived transformed lines values of 0.19% and 0.22% were obtained for cells cultured at 100μg/ml G418.
[c]Average of three experiments.

concentration; this reflects an increased copy number of vector molecules (Fig. 7a). In the two examples presented (gGAPDH and CAT) the level of expression in transfected cells represents between 1% and 10% of total cellular protein. It may be possible to obtain higher levels after a systematic study of the variables which affect expression. However for most experiments involving functional analysis of transfected gene products, (or engineered forms thereof) expression at the levels already achieved should be sufficient.

Previous papers have reported the inability of *Leishmania*-derived extrachromosomal vectors to function in electroporated *T.cruzi* cells (8, 33) and it has been suggested that this may reflect a fundamental difference in the operation of at least one essential genetic signal between trypanosomes and *Leishmania*. We have now demonstrated that *Leishmania* are capable of expressing genes flanked by *T.cruzi* sequences and that the resultant transcripts can be processed sufficiently to direct the synthesis of functional proteins. Although the data presented do not address directly the localisation of the gGAPDH promoter they do indicate that expression can occur when 450bp of 5'-flanking sequence, derived from the gGAPDH locus, is placed upstream of a transfected gene. The precise definition of sequences involved in transcriptional regulation and RNA processing in the context of these vectors will require further analysis. Preliminary experiments involving primer extension suggest that the same splice-leader addition site, upstream of the gGAPDH sequence, is used in both *T.cruzi* and transformed *L.mexicana* (G.K. unpublished data).

Transfection experiments with the African trypanosome, *T.brucei* indicate that stable transformation results from targeted integration of introduced sequences (9−11). It has been proposed that the enzymatic machinery involved in such homologous recombination may also have a role in the generation of the tandemly repeated genes which are common in these organisms and may also be involved in maintaining sequence integrity within such arrays (7). We have examined more than 20 transformed *T.cruzi* cell lines and found no evidence of integration targeted to the gGAPDH locus. Transformation was found to be mediated by circular extrachromosomal elements composed of direct tandem repeats similar to the situation which has been observed in the insect trypanosomatid *Leptomonas seymouri* (34). This may imply that the length or nature of the flanking sequences in pTGAP, pTGAN and pTEX vectors are insufficient to efficiently recombine at this locus. Alternatively it could reflect a selective advantage, in G418-containing medium, of cells containing multiple extrachromosomal copies of the *neo*^{r} gene, over cells where a single integrative event has occurred. It may therefore require the use of defective or altered constructs to enable the isolation of cells in which the gGAPDH locus has been targeted.

The mechanism responsible for generating the perfect head-to-tail tandem arrays within transfected cells has yet to be defined. It is unlikely that these structures were formed by ligation of linearised vector molecules resulting in concatamers. To conform to the restriction digest data this mechanism would only be valid if all circular DNA molecules transfected into the cells were then cleaved at exactly the same point. It is also unlikely that the tandem arrays arose as a result of direct amplification due to G418 selection. This model would require the cell to recognise and specifically amplify only single vector units to achieve perfect tandem arrays. One possible mechanism which is consistent with the data is insertional duplication. This model has been implicated in the formation of tandem arrays in transfected mammalian (35) and yeast cells (36) and implies the presence of the machinery required for homologous recombination.

In summary, this paper describes the stable transformation of *T.cruzi*, a human pathogen of major importance in Central and South America. The vectors produced should have many applications including the identification and functional analysis of gene products involved in virulence and drug-resistance. They will also provide the basis for the development of other vectors with the ability to shuttle large genomic DNA fragments between trypanosomatids.

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