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Extrachromosomal, homologous expression of trypanothione reductase and its complementary mRNA in *Trypanosoma cruzi*

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ABSTRACT

Trypanothione reductase (TR), a flavoprotein oxidoreductase present in trypanosomatids but absent in human cells, is regarded as a potential target for the chemotherapy of several tropical parasitic diseases caused by trypanosomes and leishmanias. We investigated the possibility of modulating intracellular TR levels in *Trypanosoma cruzi* by generating transgenic lines that extrachromosomally overexpress either sense or antisense TR mRNA. Cells overexpressing the sense construct showed a 4–10-fold increase in levels of TR mRNA, protein and enzyme activity. In contrast, recombinant *T.cruzi* harbouring the antisense construct showed no significant difference in TR protein or catalytic activity when compared with control cells. Although increased levels of TR mRNA were detected in some of the antisense cells neither upregulation nor amplification of the endogenous trypanothione reductase gene (*tryA*) was observed. Instead, a proportion of plasmid molecules was found rearranged and, as a result, contained the *tryA* sequence in the sense orientation. Plasmid rescue experiments and sequence analysis of rearranged plasmids revealed that this specific gene inversion event was associated with the deletion of small regions of flanking DNA.

INTRODUCTION

The trypanosomatids, members of the order Kinetoplastida, include parasitic protozoa of relevant importance to public health such as *Trypanosoma spp.* (sleeping sickness and Chagas' disease) and *Leishmania spp.* (visceral, cutaneous and mucocutaneous leishmaniasis). They are characterised by complex life cycles comprising various developmental stages that alternate between insect and human hosts. Once inside the human host they proliferate rapidly to cause severe illness and in extreme cases, death (1). Since no satisfactory vaccines against trypanosomatid infections are yet available, chemotherapy remains the only means of treatment for the millions of infected individuals worldwide. In many cases however, available drugs are few and their efficacy is limited due mainly to developed parasite resistance and/or lack of specificity. Other drugs are highly toxic and cause severe side effects (2). Thus the need for more efficient antiparasitic drugs.

In recent years, a rational approach to the development of new pharmaceuticals has emerged as an alternative to random testing. It is based on the identification of structural or metabolic cellular components present in the target organism which are either absent in the host or are sufficiently different to their host's counterpart to be treated as unique. In the case of the trypanosomatids one of the most promising examples of such unique features is the trypanothione system (3,4). These ancient eukaryotes differ from their human hosts in their ability to conjugate two molecules of the tripeptide glutathione with one molecule of spermidine to form the dithiol N^1,N^8-bis(glutathionyl)-spermidine, trivially known as trypanothione (5). Together with its corresponding oxido-reductase, trypanothione reductase (TR), trypanothione is thought to fulfil important physiological functions including maintenance of a reduced intracellular environment and defense against heavy metals, oxidants and xenobiotics (4), roles ascribed to the glutathione system in most other organisms. Since the trypanothione system appears to be shared between most members of the trypanosomatid family it is likely that any rationally designed inhibitor of TR will be potentially useful in the treatment of a wide variety of parasitic diseases.

In attempting to develop new antiparasitic drugs against a potential cellular target it is important to gather genetic evidence that the target in question is essential for cell proliferation and survival. Reverse genetics techniques such as antisense RNA and gene replacement are ideal for this purpose; they have proved invaluable in the elucidation of the regulation of gene expression and of gene function in a variety of biological systems (reviewed in ref. 6–9). We and others have previously reported the cloning, characterisation, mutagenesis and heterologous overexpression of the trypanothione reductase gene (*tryA*) from *Trypanosoma cruzi* (10,11). Here we report its homologous overexpression and, in an attempt to interfere with the expression of the endogenous *tryA* gene, the extrachromosomal overexpression of its complementary mRNA.

MATERIAL AND METHODS

Trypanosomes

A cloned epimastigote line of *Trypanosoma cruzi* (MHOM/BR/78/Silvio X10-clone 6) was grown at 28°C in RPMI-1604

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medium (Gibco) supplemented with 20 mM HEPES, 0.03 mM haemin, 0.4 % (w/v) trypticase, 10 % (v/v) foetal calf serum, 50 U/ml penicillin and 50 µg/ml streptomycin. Trypanosomes were maintained by subculture and kept at cell densities ranging between 1×10^6 and 3×10^7 cells/ml.

Transfection and cloning of parasites

Transfection of *T.cruzi* was performed mainly as described previously (12). Briefly, late-log phase epimastigotes were washed in PBS solution (13) and resuspended in electroporation buffer (10 mM sodium phosphate pH 7.1, 0.27 M sucrose) at a concentration of 1×10^8 parasites/ml. Three to eight pulses from a Hoefner Progenitor I electroporator (400 V, 99 ms) were applied to 1 ml aliquots of the cell suspension in the presence or absence of 25 or 50 µg supercoiled plasmid DNA. Cells were diluted 5-fold with culture medium and allowed to recover for 24 h; a further 10-fold dilution with medium containing 0.1 mg/ml G418 was used to select for drug-resistant trypanosomes. Clones of transgenic trypanosomes were derived by limited dilution from established drug-resistant lines obtained in different transfection experiments.

Plasmid construction

Plasmid pBTR (11) was digested with *Eco*RI and *Pst*I to release a 1.5 kb fragment that contains the entire *tryA* coding sequence. Ends were rendered blunt by treatment with T4 and *E.coli* (Klenow fragment) DNA polymerases and the fragment was ligated to the *Eco*RV-linearised expression vector pTEX (12), which had been previously treated with alkaline phosphatase. Following transformation, *E.coli* JM109 clones containing plasmids with the *tryA* insert in either possible orientation were identified and used to purify plasmids pTTcTR (sense) and pTTcTRAS (antisense). The purity of plasmid DNA solutions used in transfection experiments was confirmed by control PCR reactions using increasing amounts of template DNA (10 pg to 500 ng) and appropriate combinations of sense- and antisense-specific oligonucleotides.

Enzyme assays

Trypanothione reductase and alanine aminotransferase (ALAT) activities were assayed in cell-free extracts prepared as follows: $1–3 \times 10^8$ *T.cruzi* epimastigotes were harvested by centrifugation, washed in PBS solution and lysed in 0.25–0.35 ml lysis buffer (10 mM potassium phosphate pH 7.2, 10 mM EDTA, 1 mM DTT, 1% Triton X-100, 5 mM benzamidine, 5 mM phenanthroline, 0.1 mM phenylmethylsulphonyl fluoride). Following three cycles of freeze (liquid N_2)–thawing, crude extracts were cleared by centrifugation at 4°C and 14 000 r.p.m. for 5 min. Supernatants were transferred to fresh tubes and kept on ice until assayed for enzymatic activity.

TR activity was monitored spectrophotometrically by following the trypanothione disulphide-dependent oxidation of NADPH at 340 nm (14). The reaction was initiated by adding 0.05 mM trypanothione disulphide (Bachem) to a reaction mixture (0.5 ml final volume) containing 0.1 M HEPES, pH 7.8, 0.5 mM EDTA, 0.2 mM NADPH and 20 µl of the appropriate cell-free extract. ALAT activity was assayed spectrophotometrically by a modification of the method of Segal *et al.* (15). The assay is based on the ALAT-dependent synthesis of pyruvate from L-alanine and α-ketoglutarate coupled to the pyruvate-dependant oxidation of NADH by lactate dehydrogenase, this being monitored at 340 nm. A standard reaction mixture (0.5 ml) contained 0.1 M HEPES pH 7.8, 0.5 mM EDTA, 0.17 mM NADH and 20 µl cell-free extract. The reaction was initiated by the addition of L-alanine and α-ketoglutarate to final concentrations of 30 and 2 mM respectively, plus 2.5 U lactic dehydrogenase (Sigma). Both TR and ALAT assays were performed using a Beckman DU-70 spectrophotometer fitted with a cell temperature regulator. One unit of ALAT or TR activity is defined as the amount of enzyme required to oxidise 1 µmol NADH or NADPH (respectively) per min at 27°C. Protein concentrations were determined by the method of Bradford (16).

Western blotting

Twenty five µg of total protein from cell-free extracts prepared as described above were fractionated by SDS–PAGE on a 10% gel and electroblotted onto nitrocellulose using a Mini-Protean II system (BioRad). A 1:100 dilution of a TR polyclonal antibody prepared as described previously (17) was used to probe the blot; bands were visualised using an alkaline phosphatase-coupled immunoassay (13).

Nucleic acids manipulations

Total DNA was prepared using the proteinase K method essentially as described (18), except that crude extracts were incubated at 37°C for 1 h in the presence of 70 µg/ml DNAse-free RNAse prior to the addition of proteinase K. Concentration of DNA was determined spectrophotometrically. For DNA blot analysis, 2 µg aliquots were digested with appropriate restriction enzymes and, following electrophoresis through 0.8% agarose gels, blotted onto nylon membranes (Hybond N, Amersham) using a Vacu-aid apparatus (Hybaid). RNA was extracted in the presence of guanidinium thiocyanate and purified by centrifugation through a 5.7 M CsCl cushion as described (18). For northern blot analysis, total RNA aliquots (10 µg) were electrophoresed through 1.1% formaldehyde-containing agarose gels and blotted onto nylon membranes by capillarity. Crosslinking of nucleic acids to nylon membranes was by exposure to UV light in a crosslinking oven (Stratagene). Hybridisation to radiolabelled probes was carried out using standard techniques (13). DNA probes were labelled to high specific activity by random priming (19). Labelled, orientation-specific RNA probes were generated with a TransProbe T Kit (Pharmacia) using T3 or T7 RNA polymerases and linear pBTR as a template.

Plasmid rescue

Since pTEX-derived vectors replicate as DNA multimers in *T.cruzi* (12; our own unpublished observations) unit-size plasmid molecules (7.1 kb) were recovered from recombinant parasites by digestion with *Xho*I, an endonuclease with a single recognition site within plasmids pTTcTR and pTTcTRAS (Fig. 1A). DNA aliquots (2 µg) were digested with *Xho*I and size-fractionated by electrophoresis through 0.8 % agarose gels. DNA fragments in the 6–8 kb size range were recovered from the gel by centrifugation through a glass fibre cushion and, after concentration, treated with T4 DNA ligase for 3 h at 16°C. Self-ligated DNA was transformed into *E.coli* JM109 or XL1-Blue competent cells. Ampicillin-resistant clones were then used for plasmid preparation and analysis.

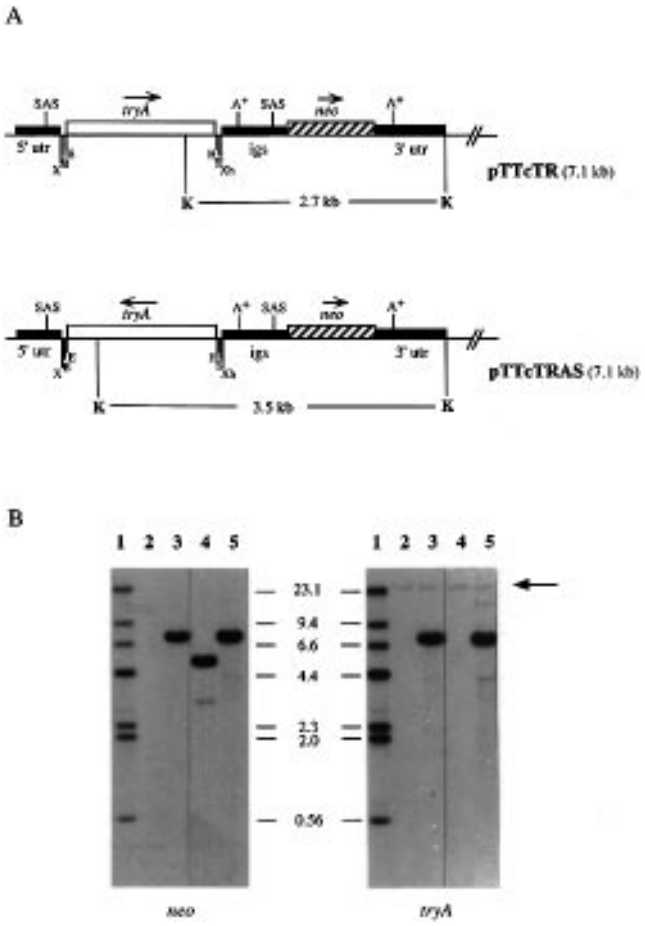

Figure 1. Structure of transfecting plasmids pTTcTR and pTTcTRAS. (**A**) Recombinant constructs used for the homologous overexpression of TR and its complementary mRNA in *T.cruzi*. Hollow boxes represent the parasite's *tryA* coding region; hatched boxes represent the bacterial neomycin phosphotransferase (*neo*) gene; arrows indicate the orientation of coding regions relative to the translation initiation codon. Untranslated regulatory sequences are represented by filled boxes; 5′ and 3′ utr, *T.cruzi GAPDH* 5′ and 3′ untranslated regions respectively; igs, *GAPDH* intergenic sequence; the location of polyadenylation (A^+) and splice acceptor sites (SAS) within these regions is also indicated. Unique polylinker restriction sites flanking *tryA* are shown: X, *Xba*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; Xh, *Xho*I. *Kpn*I sites (K) used as diagnostic of *tryA* orientation as well as the expected size of *Kpn*I restriction fragments highlighted by a *neo* probe are shown. (**B**) DNA blot analysis of recombinant and untransfected *T.cruzi*. *Eco*RI-digested DNA (2 µg aliquots) was size-fractionated by electrophoresis and blotted onto a nylon membrane. This was probed with a 250 bp *Pst*I fragment corresponding to the 5′-end of the *neo* gene and, after removal of the first probe, with the entire *tryA* coding sequence. Lane 1, molecular size markers (lambda *Hin*dIII, Promega); lane 2, untransfected *T.cruzi*; lane 3, pTTcTRAS transfected clone; lane 4, pTEX transfected control; lane 5, pTTcTR transfected clone. Arrow shows the position of chromosomal *tryA* alleles. Marker sizes are given in kilobases.

Sequencing of *tryA* flanking regions

The nucleotide sequence of *tryA* junctions in plasmid DNA was determined by the double-stranded DNA sequencing method using a Sequenase 2.0 kit (USB). DNA synthesis was primed by synthetic oligodeoxynucleotides TcTR19(–) [5′-GCGCCAATGACAACCAAATC-3′] and TcTR1441(+) [5′-GGTGAGAAGATGGAAAAGCC-3′]. The former is complementary to the DNA sequence 19 bp downstream from the translation initiation codon, the latter lies 19 bp upstream from the translation termination codon and were used to read into the 5′ and 3′ junctions respectively.

Cloning of AS1 cells

Antisense AS1 cells were seeded at a density of 0.5×10^6 cells/ml in culture medium supplemented with 0.5 mg/ml G418 and incubated at 28°C to a final density of 2×10^7 cells/ml. Cells were diluted in drug-free medium to a density of 2×10^5 cells/ml and serial 1:1 dilutions were then made in a 24-well culture dish until a theoretical population density of 0.025 parasites/ml was achieved. Plates were sealed and incubated at 28°C for 6–8 weeks. AS1–0.5 clones obtained in this manner were cultured for at least ten generations in the presence of G418 (0.1 mg/ml) before further analyses.

RESULTS

Construction of recombinant plasmids and clones

Plasmid pBTR has been described previously (11) and was used as the source of the *T.cruzi tryA* gene. The non-integrative shuttle vector pTEX (12) was used to clone the entire *tryA* coding region in both possible orientations. The resulting plasmids, pTTcTR (sense) and pTTcTRAS (antisense) are depicted in Figure 1A. Expression of *tryA* and antisense-*tryA* in these plasmids is linked to the constitutive expression of the drug resistance marker and utilises the polyadenylation and splice acceptor signals of the tandemly repeated glyceraldehyde phosphate dehydrogenase (GAPDH) genes from *T.cruzi* (12,20). The orientation of *tryA* was established by restriction with the diagnostic enzyme *Kpn*I (see Fig. 1A) and was confirmed by DNA sequencing. Following plasmid purity tests (Materials and Methods) *T.cruzi* epimastigotes were transfected with these constructs in various independent experiments and recombinant cells were selected in the presence of G418. Clones TR4 (sense) and AS1 and AS2 (antisense) were randomly chosen for analysis.

To test for the integrity of transfected plasmids and to estimate plasmid copy number per cell, DNA blot analysis of selected G418-resistant clones was carried out using *Eco*RI-digested DNA (Fig. 1B); the target site of *Eco*RI is present once in plasmids pTEX, pTTcTR and pTTcTRAS (see Fig. 1A). Figure 1B confirms the presence of major, unit-size plasmid bands in the corresponding *T.cruzi* recombinant clones, which is in agreement with the drug resistant phenotype. Comparison of band intensities between the single chromosomal *tryA* locus (Tovar *et al.*, submitted) and plasmid-borne *tryA* in Figure 1B, lanes 3 and 5 (*tryA* panel) reveals the presence of ≥20 plasmid molecules per cell in both sense and antisense recombinant clones. Equivalent amounts of plasmid DNA were estimated for the pTEX control clone when a *neo*-specific sequence was used to probe the same blot (Fig. 1B).

Phenotypic characterisation of transgenic trypanosomes

To assess the effect of the extrachromosomal expression of sense and antisense TR mRNA on TR activity levels in *T.cruzi*, we assayed for enzymatic activity in log phase epimastigotes. ALAT, another soluble, house-keeping enzyme in this organism was assayed in cell-free extracts to control for the general metabolic state of cells. As shown in Figure 2A, a 4-fold increase in TR specific activity was found in cells harbouring the sense construct.

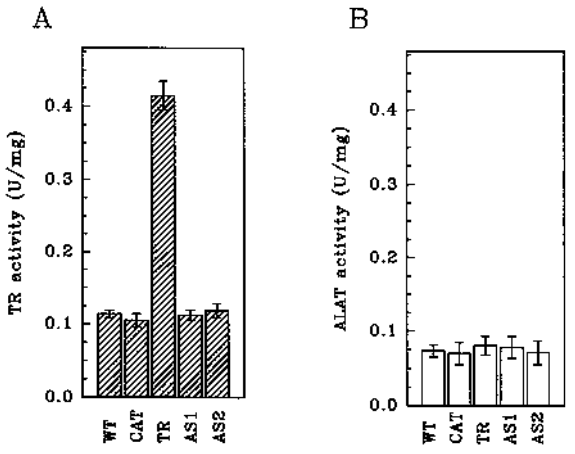

Figure 2. Enzymatic activity in recombinant *T.cruzi*. Cell-free extracts from indicated recombinants grown in the presence of G418 (0.1 mg/ml) were assayed for (**A**) TR and (**B**) ALAT catalytic activities. Plotted values represent the mean and standard deviations of triplicate measurements from two independent experiments.

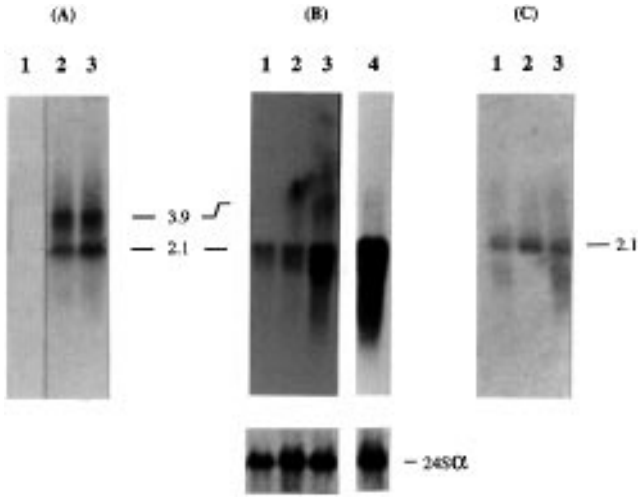

Figure 3. Northern blot analyses of untransfected and antisense AS1 recombinant cells. Aliquots (10 µg) of total RNA isolated from untransfected and recombinant *T.cruzi* were size fractionated and blotted onto nylon membranes. (**A**) Blot probed with a radiolabelled single-stranded RNA, antisense-specific *tryA* probe and exposed to X-ray film for 4 h. Lane 1, untransfected *T.cruzi*; lanes 2 and 3, recombinant AS1 grown in the presence of 0.1 and 0.5 mg/ml G418 respectively. (**B**) Blot probed with a single-stranded RNA, sense-specific *tryA* probe and exposed to X-ray film for 18 h; lanes 1–3, as blot A; lane 4, recombinant TR overexpressor. (**C**) As blot A but probed with a 500 bp cDNA corresponding to the 3′ untranslated region of the chromosomal *tryA* gene. Sense- and antisense-specific RNA probes of equivalent specific radioactivities were used. In all cases equivalent loading of RNA was checked by ethidium bromide staining (not shown) and, in the case of (B), confirmed by hybridisation to a 24Sα ribosomal DNA probe (43). Apparent mobilities of RNA species based on RNA size markers (Promega) are given in kilobases.

However, activity levels observed in both antisense clones were not significantly different to those of control cells suggesting either that the expression of antisense TR mRNA is not harmful to these cells or that any potentially deleterious effect of antisense RNA is efficiently neutralised. No significant difference in ALAT activity was found between untransfected and recombinant clones (Fig. 2B) indicating that replication of recombinant plasmids *per se* does not affect the general metabolic state of the cell.

It has been reported that regulation of gene expression by antisense RNA transcribed from transforming plasmid vectors usually requires a considerable excess of the antisense transcript over its complementary mRNA (reviewed in ref. 21). Since increased selective pressure has been used previously to boost steady-state mRNA levels of chloramphenicol acetyltransferase (CAT) expressed from a pTEX-derived vector in *T.cruzi* (12), we sought to increase the antisense/sense TR transcript ratio in AS clones by culturing cells in medium with increased G418 concentrations. No change in specific TR catalytic activity was observed in these cells even at drug concentrations as high as 1 mg/ml (data not shown).

Transgene expression

To investigate whether the lack of apparent changes on TR phenotype in recombinant antisense clones could be due to inefficient expression of antisense RNA, total RNA was isolated from antisense clone AS1 grown under two different selection regimes and analysed by northern blotting. High levels of apparently correctly processed antisense TR mRNA were observed in these cells using an antisense-specific *tryA* probe. This is indicated by the presence of a 2.1 kb transcript band in lanes 2 and 3 of Figure 3A which is of the expected size if the trans-splicing and polyadenylation sites present in plasmid pTTcTRAS (Fig. 1A) were used during RNA processing. Moreover, the fact that plasmid-derived sense and antisense TR transcripts are of equivalent sizes (Fig. 3A and B) also suggests that these antisense transcripts are correctly processed. However, the presence in the same lanes of an extra band in the 3.9 kb region, which is also highlighted by a *neo* probe (not shown) and most likely represents bicistronic pre-mRNA, suggests that antisense RNA processing is inefficient in these cells. Such accumulation of pre-mRNA could result from specific blocking of antisense RNA processing or, alternatively, from inefficient RNA processing due to high expression levels. The latter appears unlikely since cells that overexpress TR to a high level do not accumulate multicistronic transcripts (Fig. 3B, lane 4).

Unexpectedly, increased TR mRNA levels (3–5-fold) in AS1 cells grown at the higher drug concentration were detected with a single-stranded, sense-specific RNA probe (Fig. 3B, lane 3). Such an increase could be due to either endogenous gene amplification, chromosomal transcriptional upregulation or to structural rearrangement of plasmid DNA. To distinguish between these possibilities a cDNA sequence spanning the 3′ untranslated region of the wild-type TR mRNA was used as a probe. Since this sequence is not present in plasmid DNA, it should only recognise chromosome-derived TR transcripts. No significant difference in steady-state levels of TR mRNA was found between untransfected control and recombinant cells (Fig. 3C) indicating that the observed increased levels of TR mRNA in antisense AS1 cells was not due to the expression of chromosomal *tryA* alleles but rather to transcription of the plasmid-borne *tryA* sequence.

The lack of correlation between TR activity and observed TR mRNA levels prompted us to investigate whether excess TR transcripts might be translated, perhaps into non-functional protein. Western blot analysis using a polyclonal antibody raised

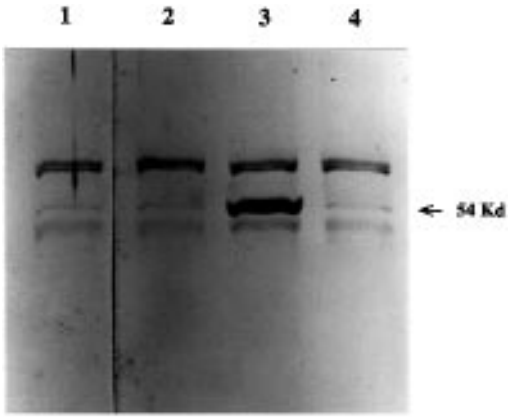

Figure 4. Western blot analysis of recombinant and untransfected *T.cruzi*. Cell-free extract aliquots (25 µg protein/lane) were size-fractionated by SDS–PAGE, blotted onto nitrocellulose and probed with a TR polyclonal antibody (17). Lane 1, untransfected control; lane 2, AS1 cells grown at 0.1 mg/ml G418; lane 3, TR4 control cells overexpressing TR; lane 4, AS1 cells grown at 0.5 mg/ml G418. The protein species indicated by the arrow is of the expected size for TR (based on protein size markers, BioRad) and co-migrated with TR protein purified as described in ref. 11 (not shown).

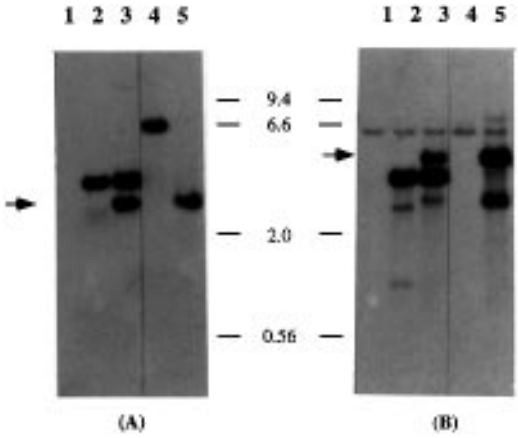

Figure 5. Structural rearrangement of plasmid DNA in antisense clone AS1. DNA aliquots (2 µg) were digested with *Kpn*I and, following electrophoresis, blotted onto a nylon membrane. This was then probed with radiolabelled *neo*- and *tryA*-specific sequences (A and B respectively) as described in legend to Figure 1B. Lane 1, untransfected control; lanes 2 and 3, antisense AS1 cells grown at 0.1 and 0.5 mg/ml G418 respectively; lane 4, control cells harbouring pTEX-CAT; lane 5, sense clone TR4. Arrows point at new major plasmid fragment bands appearing in AS1 cells grown at 0.5 mg/ml G418. Molecular size markers are given in kilobases.

against denatured TR (17) failed to detect any difference in TR protein content between antisense and untransfected control cells (Fig. 4). This result is in agreement with the activity data and suggests that only a minor proportion of those TR transcripts seen in antisense cells (Fig. 3B, lane 3) is efficiently translated.

Structural rearrangement of plasmid DNA in antisense clones AS1 and AS2

Extensive restriction analysis of total DNA isolated from antisense AS1 cells grown at different selection levels suggested that, at high selective pressure, structural changes had occurred in a proportion of plasmid molecules. This is best illustrated by restriction analysis with *Kpn*I which allows determination of the orientation of *tryA* in a plasmid (see Fig. 1A). Antisense plasmid pTTcTRAS (7.1 kb) should yield *Kpn*I fragment bands of about 3.5 and 3.6 kb; this is observed when a DNA digest from cells grown in the presence of 0.1 mg/ml G418 is probed with either *neo*- or *tryA*-specific probes (lane 2 of Fig. 5A and B respectively). However, digestion of DNA from cells grown at a higher drug concentration revealed an additional set of restriction fragments, one of ~2.7 kb which hybridises to *neo* and another of ~4.4 kb which hybridises to the *tryA* probe (lane 3 of Fig. 5A and B respectively). The fact that both new fragment bands co-migrated with those observed for pTTcTR transfected cells (Fig. 5A and B, lane 5) suggested the presence, in this antisense clone, of rearranged plasmid DNA carrying the *tryA* insert in the sense orientation.

To investigate this possibility, plasmid DNA isolated from antisense clones AS1 and AS2 was cloned back into *E.coli* as described in Material and Methods and its structure analysed by restriction mapping and DNA sequencing. The structure of plasmids rescued from antisense AS1 cells (Table 1) confirmed the restriction pattern observed in Figure 5. About one third of plasmid molecules rescued from cells grown at 0.5 mg/ml G418 contained the *tryA* insert in the sense orientation whilst none of those rescued from cells grown at 0.1 mg/ml G418 was found rearranged. Interestingly, a proportion of plasmids rescued from antisense AS2 cells were also rearranged, in this case at both drug concentrations. No structural rearrangement was observed in plasmid DNA rescued from TR4 cells.

Table 1. Structure of plasmid DNA rescued from transgenic trypanosomes

Clone	G418 (mg/ml)	Orientation of *tryA*[a]	
		Sense	Antisense
AS1	0.1	0/43	43/43
	0.5	10/28	18/28
AS2	0.1	13/30	17/30
	0.5	8/40	32/40
TR4	0.1	16/16	0/16
	0.5	20/20	0/20

Plasmid DNA from *T.cruzi* was cloned back into *E.coli* as described in Materials and Methods.

[a]Orientation of *tryA* determined by *Kpn*I restriction analysis of plasmid DNA purified from randomly selected bacterial colonies.

Analysis of rearranged plasmid DNA

In an attempt to understand the mechanics of the observed DNA rearrangements the nucleotide sequence of the *tryA* flanking regions of selected rescued plasmids was determined. Four apparently unmodified, and four rearranged plasmids from each of the antisense clones were analysed. As a control, four plasmids rescued from clone TR4 were also sequenced. All of the apparently unrearranged antisense plasmids were identical in sequence to the original transforming antisense plasmid. Likewise, the control sense plasmids were identical in sequence to the original transforming sense plasmid. However, of the eight rearranged plasmids analysed which originally contained the *tryA* gene in the antisense orientation, all were confirmed to contain *tryA* in the sense orientation and, additionally, were found to

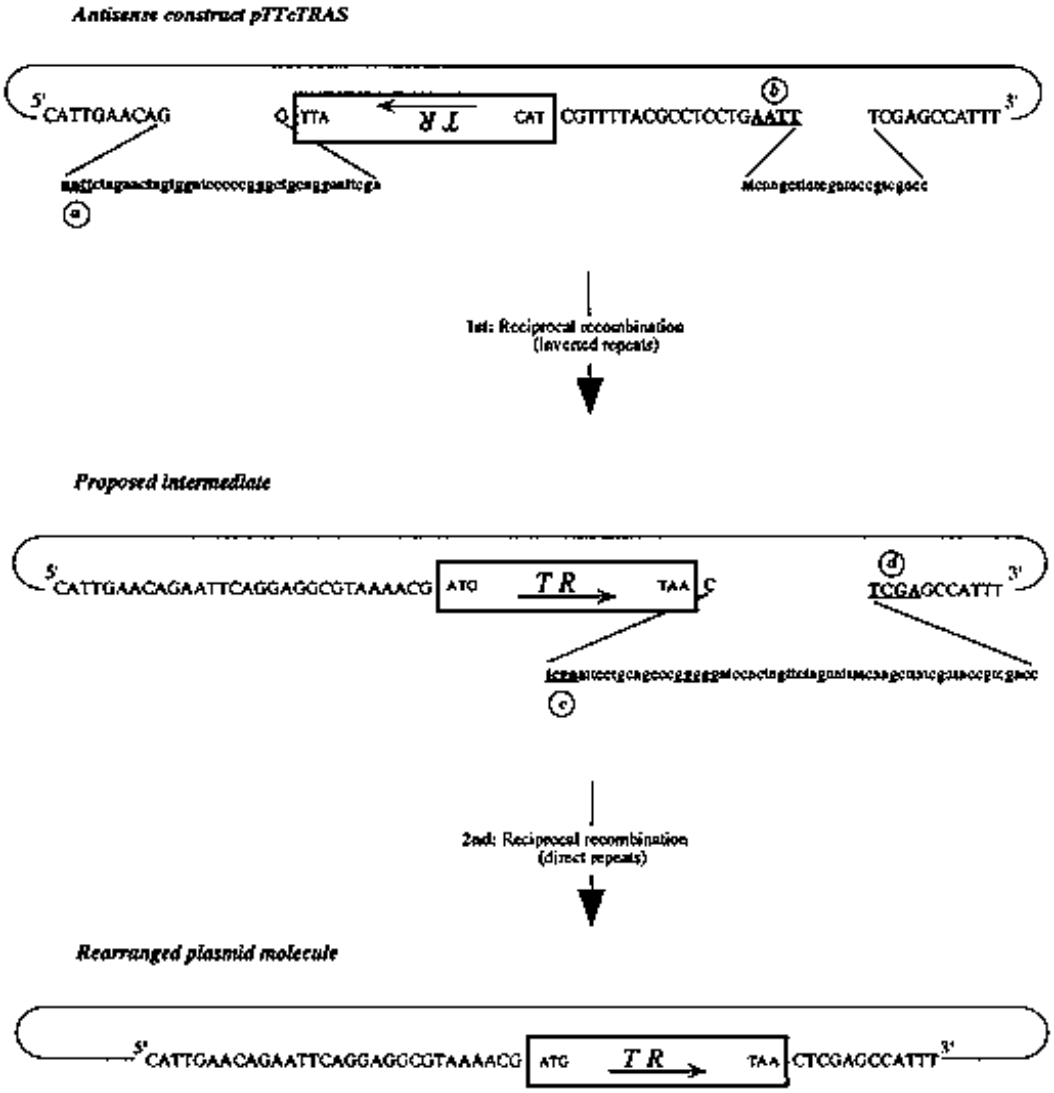

Figure 6. Proposed model of plasmid DNA rearrangement. The structural arrangement and relevant nucleotide sequence of antisense construct pTTcTRAS as well as that of rearranged plasmid DNA and a proposed recombination intermediate are shown. Polylinker sequences (lost during rearrangement) are shown in lower case. Small repeated sequences thought to be involved in the recombination process (see text for details) are underlined and marked with letters a–d. For simplicity, only the top DNA strand is shown in all cases.

contain a 41 bp deletion at the 5′ junction and a 25 bp deletion 3′ to the *tryA* translation termination codon. Such deletions span the entire polylinker sequence but do not affect the flanking untranslated regions present in the expression vector (Fig. 6). The accurate, identical nature of the DNA rearrangements observed in both antisense clones AS1 and AS2 hints at the involvement of homologous recombination.

Plasmid rescue experiments and DNA blot analyses of AS1 cells grown at high drug concentrations indicated that about one third of plasmid molecules were rearranged. Cloning of these cells by limited dilution was used to investigate whether this cell population was composed of a mixture of cells exclusively carrying either unmodified or rearranged plasmid DNA (the latter would behave as TR overexpressors) or, alternatively, whether each cell in the population harboured a mixture of rearranged and unmodified plasmid DNA. Of fifteen clones analysed phenotypically all were found to contain TR activity levels indistinguishable from those measured in untransfected control cells (not shown) indicating that the majority of cells in the population carried a mixture of both rearranged and unmodified plasmid DNA.

DISCUSSION

Regulation of gene expression by antisense RNA occurs naturally in both prokaryotic (22,23) and eukaryotic cells (24–26). Antisense RNA transcribed from transforming plasmid vectors has been used to regulate endogenous gene expression in a number of eukaryotic systems, including the slime mould *Dyctiostelium discoideum*, mammalian cells and plants (27–29). In parasitic protozoa however, no equivalent studies have yet been reported, although the effect of synthetic antisense oligonucleotides on parasite proliferation and survival has been documented (30–33).

In this study we investigated the possibility of modulating intracellular TR levels by extrachromosomal, homologous ex-

pression of sense and antisense TR mRNA in *T.cruzi*. Although TR specific activity was readily upregulated in cells harbouring the sense construct, cells transformed with the antisense construct proved recalcitrant to TR downregulation despite the presence of ≥20 copies of the antisense plasmid per cell and high expression of antisense TR mRNA. Antisense RNA is thought to work in eukaryotic cells by formation of hybrid, double-stranded RNA molecules that have been proposed to affect transcript stability and/or processing (splicing/nuclear export/ribosome binding) thus interfering with the expression of specific gene products (reviewed in ref. 34). Various possibilities could account for our failure to downregulate the expression of *tryA* in *T.cruzi*. Antisense transcripts may not be able to form duplex RNA hybrids with their target molecules due to the non-complementarity of their 5′ and 3′ untranslated regions, including the spliced leader sequence and poly A tail. Cellular compartmentalisation, well documented in eukaryotes (reviewed in ref. 35,36) may also play a part; formation of heteroduplex RNA may be prevented by the absence, in the appropriate cellular compartment, of one or more of a number of proteins that have been proposed to play a role in the process of antisense RNA regulation, including hybrid-promoting proteins, winding and unwinding activities, and double-stranded RNAses and their modulators (ref. 34 and references therein). At a different level, other cellular mechanisms such as transcriptional upregulation, gene amplification, or structural rearrangement of plasmid DNA could also explain failure to regulate gene expression by transforming antisense gene constructs. In this respect, a peculiar plasmid DNA rearrangement event was observed in our investigation. From its original antisense orientation, the *tryA* insert in plasmid pTTcTRAS was flipped over to the sense orientation. Although such an event lead to increased intracellular TR mRNA levels this was not reflected phenotypically by increased TR catalytic activity nor TR protein suggesting that only a proportion of these transcripts were translated into functional enzyme either as a result of RNA degradation (perhaps following heteroduplex formation with antisense RNA) or due to inefficient or incorrect RNA processing.

Rearrangement of plasmid DNA must have occurred in *T.cruzi* since control PCR experiments failed to detect the presence of contaminating sense plasmid molecules in antisense plasmid DNA solutions used in transfection experiments. This is further supported by the fact that the structure of rearranged molecules has been shown to be different from those of the original sense and antisense transfecting constructs and by the observation that no such rearranged molecules are detected in DNA blots of the originally selected antisense AS1 clone. Instead, structural analysis of rearranged plasmid molecules suggests the involvement of short repeated sequences in the inversion of *tryA* and associated deletion of flanking regions. In Figure 6 a sequence of events that could explain the conversion of an original antisense plasmid into a rearranged molecule is depicted. In this model a first round of homologous reciprocal recombination between the inverted duplications marked a and b in the diagram would lead to inversion of the intervening sequence. As a result the *tryA* coding region would now be in the sense orientation and the 41 bp 5′ polylinker fragment would have been removed from its original upstream location and fused to the 25 bp 3′ polylinker sequence. A second round of homologous reciprocal recombination between the newly created direct repeats marked c and d in the diagram would then lead to the deletion of the 66 bp intervening sequence, thus removing the entire polylinker region and producing a plasmid of structure identical to that observed for rearranged plasmid molecules. The proposed model faithfully accounts for our experimental observations and implies that repeated DNA sequences as short as 4 bp can be used as substrates for homologous recombination in *T.cruzi*. Eight base pair direct repeats have been shown to support homologous recombination in *Leishmania mexicana* (37), but the minimum homology requirement for reciprocal and non-reciprocal homologous recombination in trypanosomatids has yet to be determined experimentally.

High molecular weight plasmid concatemers that appear to be linked in a head to tail arrangement accumulate in transgenic *T.cruzi* harbouring pTEX-derived vectors (12; our own unpublished observations). This type of extrachromosomal plasmid multimers have also been observed (38) and extensively characterised in *T.brucei* (39), *Leishmania* (40,41) and *Leptomonas* (42). It is likely that plasmid DNA replication occurs in trypanosomatids by a mechanism in which DNA synthesis and recombination are tightly interrelated. Such a mechanism could facilitate the generation of plasmid DNA diversity in a given cell population and lead to selection of advantageous plasmid variants. At present it is not possible to test directly whether the observed plasmid rearrangements arose solely as a result of the expression of antisense TR mRNA since an inducible expression system for *T.cruzi* has not yet been developed. However, the high frequency of rearrangement, its accuracy and exclusivity to the antisense clones warrant further investigation.

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