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Trypanosoma cruzi: Exogenously Regulated Gene Expression

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Department of Microbiology and Immunology, Medical College of Virginia Campus, Box 980678, Virginia Commonwealth University, Richmond, Virginia 23298-0678, U.S.A.

Wen, L.-M., Xu, P., Benegal, G., Carvaho, M. R. C., Butler, D. R., and Buck, G. A. 2001. *Trypanosoma cruzi:* Exogenously regulated gene expression. *Experimental Parasitology* **97,** 196–204. A regulated expression vector would provide a strong tool for the dissection of gene function in *Trypanosoma cruzi*. Herein, we establish a system in which genes in *T. cruzi* expression vectors can be exogenously regulated by tetracycline. We first generated strains of *T. cruzi* that stably express the repressor of the bacterial tetracycline resistance gene and T7 RNA polymerase. Based on these strains, we developed two *T. cruzi* expression systems regulated by tetracycline—the first by use of a regulated rRNA promoter and the second by use of a regulated T7 promoter. In the former, we constructed an expression vector in which tetracycline resistance gene operators flank the transcription start point of the *T. cruzi* rRNA gene promoter. Reporter gene activity from this modified promoter was regulated up to 20-fold in the presence of different concentrations of tetracycline. In the T7 system, tetracycline resistance gene operators flank the transcription start point of the T7 promoter. Reporter gene activity from this modified promoter was regulated up to 150-fold in the presence of different concentrations of tetracycline. Expression in these systems was repressed when tetracycline was removed even after full induction for extended periods in the presence of tetracycline. We are now using these two systems to test protein function in *T. cruzi*.

Index Descriptors and Abbreviations: tetracycline-regulated gene expression; rRNA promoter; T7 promoter; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assays; rRNA, ribosomal RNA; Tet, tetracycline; TetO, tetracycline operator; TetR, tetracycline repressor; *Trypanosoma cruzi*; PBS, phosphate-buffered saline; PMSF, phenylmethyl sulfonyl fluoride.

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INTRODUCTION

A regulated gene expression system that permits controlled expression of exogenous genes provides an important tool for examining the functional role of the encoded proteins. A strict regulatory system that can completely eliminate expression under some conditions but that permits high levels of expression in others is essential to study the effects of genes that are expressed at very low levels or for which the gene product is toxic. The bacterial tetracycline resistance gene (*tetA*) regulatory system has proven adaptable for the generation of such tightly regulated expression systems in a wide variety of eukaryotes (Rossi and Blau 1998).

The tetracycline resistance gene is regulated by a 25-kDa repressor protein, TetR. In the absence of tetracycline, TetR binds 19-bp operators to effectively block transcription initiation (Hillen *et al.* 1984). Derepression occurs when TetR dissociates from the operators to preferentially bind tetracycline. The affinity of TetR is greater for tetracycline (k_a = 10^9) than for the operator sites (Takahashi *et al.* 1986), and *tetA* is strongly induced in the presence of the antibiotic. The regulatory components of the Tet operon have been successfully used to regulate promoters in a variety of eukaryotes, including plants (Gatz *et al.* 1994; Gatz and Quail 1988), fungi (Dingermann *et al.* 1992a, b; Faryar and Gatz 1992), mammalian cells (Deuschle *et al.* 1995; Gossen and Bujard 1993; Paulus *et al.* 1996), and parasitic protozoa (Biebinger *et al.* 1997; Ramakrishnan *et al.* 1997; Wirtz *et*

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al. 1998, 1999; Wirtz and Clayton 1995). To achieve regulation of an eukaryotic promoter with the Tet regulatory system, the promoter must be modified with *tetA* operators positioned such that binding of TetR will block transcription initiation. Wirtz and Clayton (1995) first used this system to establish a regulated expression system in a trypanosomatid using the PARP promoter of *Trypanosoma brucei* (Wirtz and Clayton 1995; Biebinger *et al.* 1996; Wirtz *et al.* 1998, 1999).

Only two promoters are known in *Trypanosoma cruzi*, the SL RNA promoter (Nunes *et al.* 1997a) and the rRNA promoter (Tyler-Cross *et al.* 1995). Ribosomal RNA promoters have also been mapped in other trypanosomatids, including *T. brucei* (White *et al.* 1986), *Crithidia fasciculata* (Grondal *et al.* 1990), and *Leishmania* (Uliana *et al.* 1996). In *T. cruzi*, a 508-bp region ~1.5 kb upstream from the 5′ end of 18S rRNA coding sequence contains a gene promoter that is at least 100-fold stronger than the promoter of the SL RNA gene (Lu and Buck 1990; Tyler-Cross *et al.* 1995). Moreover, as might be expected, the rRNA gene promoter stably expresses exogenous genes in at least the epimastigote, trypomastigote, and amastigote life cycle stages of *T. cruzi* (Santos and Buck 1999, 2000). Thus, the rRNA promoter is an optimal promoter for use in construction of a regulated gene expression system in *T. cruzi*.

T7 RNA polymerase is a single polypeptide of molecular weight 98,800. This enzyme is specific for its own promoter, a highly defined and conserved 23-bp sequence. The TetR-regulated T7 promoter has been succesfully used in other trypanosomatids in the development of regulated expression systems (Wirtz *et al.* 1994, 1998, 1999).

Herein, we utilize the *T. cruzi* rRNA promoter, the T7 promoter, and the bacterial tetracycline resistance gene regulatory system to generate exogenously controlled gene expression systems for *T. cruzi*.

MATERIALS AND METHODS

T. cruzi *culture, nucleic acid isolation, transfection and selection, and CAT assay.* Epimastigotes of *T. cruzi* CL Brener were grown in LIT medium essentially as previously described (Zwierzynski *et al.* 1989). Plasmid DNAs were prepared by CsCl density gradient centrifugation (Maniatis *et al.* 1982). Trypanosomes were transfected by electroporation with a GenePulser (Bio-Rad) essentially as previously described (Tyler-Cross *et al.* 1995). To generate permanent cell lines, 25 μg of each construct was linearized at a unique restriction enzyme site and used to transfect 5×10^7 epimastigotes from the TetR-expressing cell line. Selection at 500 μg/ml G418 (Sigma) or 800 μg/ml *zeocin* (Invitrogen) was applied 48 h after transfection. For transient assays in stable cell lines, 50–100 μg of reporter plasmid was used to transfect 5×10^7 cells. All transient transfections and CAT assays were performed three times with each data point represented in triplicate essentially as previously described (Lu and Buck 1990; Tyler-Cross *et al.* 1995). Tetracycline (Sigma) was added after the transfection at various concentrations (5–20 μg/ml). After 48 h tetracycline induction, cells were washed twice with PBS, transferred to fresh medium with or without tetracycline, incubated again for 48 h, and assayed for CAT. RNA and DNA were isolated from *T. cruzi* epimastigotes essentially as previously described (Auffray and Rougeon 1980; Swindle *et al.* 1988).

Primer extension mapping of the transcription start point (tsp) of rRNA promoter. To map the tsp, 60 μg total *T. cruzi* RNA was mixed with ~10^6 cpm ^{32}P-labeled oligonucleotide 5′-GCGATGCCAAACAAAAGACCG-3′ in RTase buffer and extended with AMV Reverse transcriptase (USB) as previously described (McCarthy-Burke *et al.* 1989). Plasmid pTcpaCAT (Tyler-Cross *et al.* 1995) was sequenced in parallel as a size marker with the same primer. The extension reactions were analyzed in parallel on a polyacrylamide–urea sequencing gel by standard techniques (Maniatis *et al.* 1982).

Plasmid constructions for transient and stable transfections. Plasmid pPATAN, containing the *T. cruzi* rRNA promoter driving the expression of TetR and the bacterial neomycin resistance gene, was constructed as follows. The rRNA promoter fused to the SA-35.2 splice acceptor from the *Kpn*I and *Sal*I fragments of pTc-paCAT (Tyler-Cross *et al.* 1995) was inserted into pBS SK+ phagemid (Stratagene), followed by the insertion of the *neo*R gene from pSV2neo (Santos *et al.* 2000) after digestion with *Sal*I and *Eco*RI. A polycloning site (PCS), which was synthesized as two complementary oligonucleotides PCS1 5′-CATGATCTAGGCCTCCTAGGAGCTCATATGACTAG-3′ and PCS2 5′-TCGACTAGTCATATGAGCTCCTAGGAGGCCTAGAT-3′, was inserted into the*Xba*I and *Sal*I sites upstream of neoR to facilitate subsequent cloning of genes. The tetR gene was PCR amplified with primers TET1 5′-CGGAAGATCTTATGTCTAGATTAGATAA-3′ and TET2 5′-AAAAGGCCTTTAGGACCCACTTTCACATTTAAG-3′ from pUHD15-1 (Gossen and Bujard 1992), cleaved with*Bgl*II and *Stu*I, and cloned into the *Bgl*II and *Stu*I sites of the PCS. Finally, a second acceptor site, SA-35.1 (McCarthy-Burke *et al.* 1989), which was synthesized as two complementary oligonucleotides, 35.1F 5′-TATGAATTATGGGGATAATATTCTTGTCTTTAAACTTCTTATAACCAATTGTGCTTTAGAGA-3′ and 35.1R 5′-CTAGTCTCTAAAGCACAATTGGTTATAAGAAGTTTAAAGACAAGAATATTATCGCCATAATTCA-3′, was cloned into the *Nde*I and *Spe*I sites of the PCS upstream of neoR (Fig. 1a).

Plasmid prRNA-Tet-CAT was constructed with the rRNA promoter containing two tandem Tet operators (TetO) driving expression of the bacterial chloramphenicol acetyltransferase gene. The Tet operators were inserted 1 bp upstream from the rRNA transcription start point as follows. The prRNA-Tet-CAT was constructed from pTc-paCAT by *inverse* PCR (Ochman *et al.* 1990) with primers R-Tet-OP3′ 5′-CTCTATCACTGATAGGGAGAATTTACTAAACCAAAACGAACGGCTA-3′ and R-Tet-OP5′ 5′-ATCTCCCTATCAGTGATAGAGATCATGGAGCGGTGGTCTCTCCG-3′ (the Tet operators are underlined). *Inverse* PCR was performed for 30 cycles with *Pfu* polymerase (Stratagene) under the following cycling conditions: 94°C, 1 min; 94°C, 10 s; 65°C, 30 s; 72°C, 5 min; 30 cycles. The PCR fragment was phosphorylated with T4 polynucleotide kinase (Promega), gel purified, and ligated to generate plasmid prRNA-Tet-CAT (Fig. 1b).

Plasmid prRNA-T7P-Zeo consisted of the rRNA promoter, the SA35.2 splice acceptor, the T7 polymerase gene, the SA35.1 splice acceptor, and the *Zeocin* gene (zeoR) for resistance to the antibiotic (Calmels *et al.* 1991). The plasmid was derived from pPATAN (see

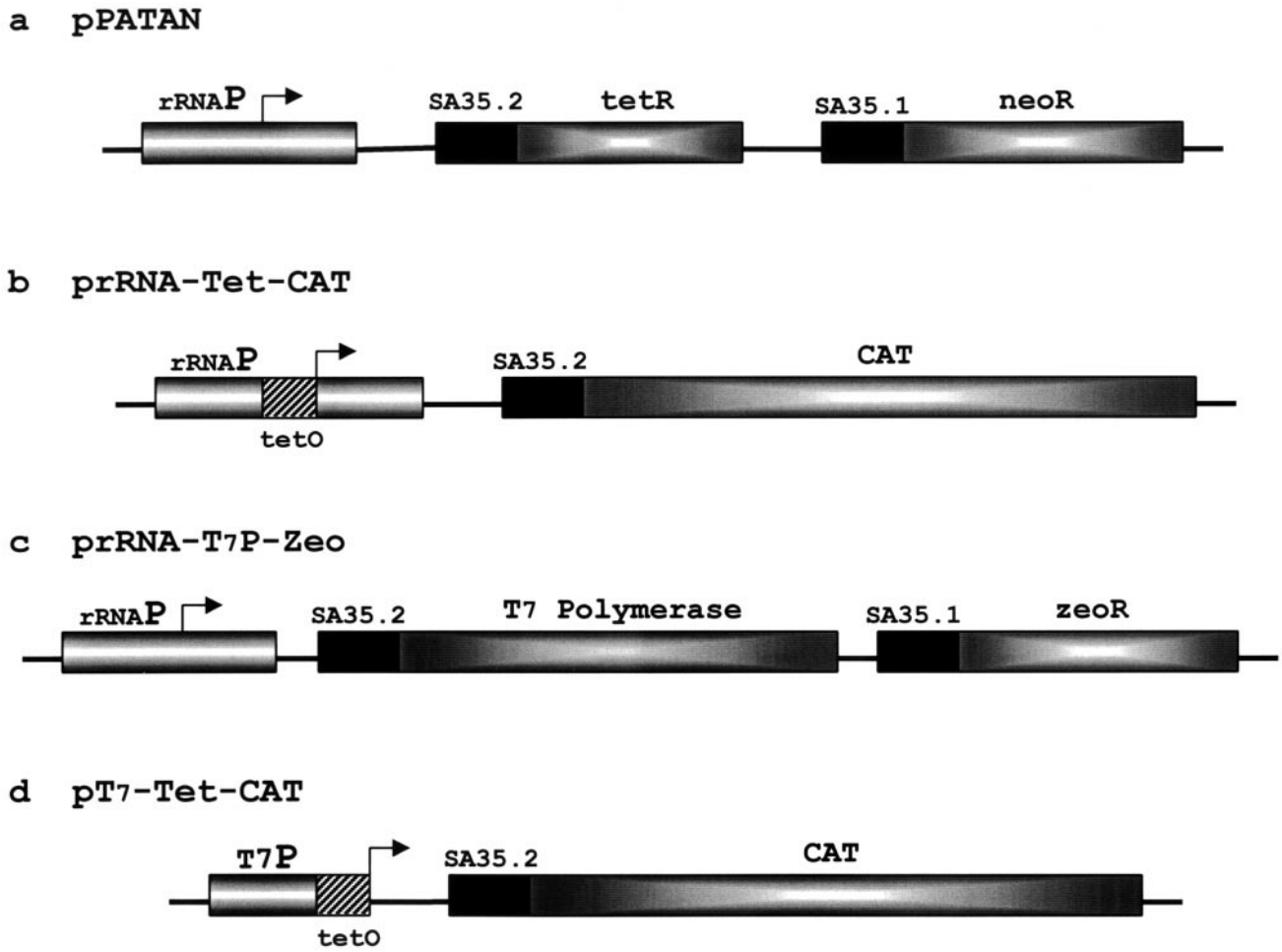

FIG. 1. Vector constructions. (a) tetR-expressing vector pPATAN, containing the *T. cruzi* rRNA promoter driving expression of TetR and the bacterial neomycin resistance gene. (b) rRNA-Tet regulatory expressing vector prRNA-Tet-CAT was constructed with the rRNA promoter containing two tandem Tet operators driving expression of the bacterial chloramphenicol acetyl transferase gene. (c) T7 polymerase-expressing vector prRNA-T7P-*Zeo*, consisting of the rRNA promoter driving expression of the T7 polymerase gene and the bacterial zeocin resistance gene. (d) T7-Tet regulatory expressing vector pT7-Tet-CAT was constructed with the bacteriophage T7 promoter containing two tandem Tet operators driving expression of the bacterial chloramphenicol acetyltransferase gene. Constructions were performed as described under Materials and Methods. Promoters (▬) rRNA**P** and *T7***P**; splice acceptors (■) SA35.1 and SA35.2; tetO, tetracycline operators (▨); protein-coding genes (▬) for the tetracycline repressor (tetR), *neomycin* resistance (neoR), *zeocin* resistance (zeoR), chloramphenicol acetyltransferase (CAT), and T7 polymerase.

above) by replacement of the TetR gene with the T7 polymerase gene and the neoR gene with the zeoR gene. The T7 polymerase gene was PCR amplified from plasmid pGP1-2 (Tabor and Richardson 1985) with primers T7-*Xba*I 5′-CTAGTCTAGAATGAACACGATTAACATC-3′ and T7-*Nde*I 5′-GGGAATTCCATATGTTACGCGAACGCGAAGT CC-3′, digested with *Xba*I and *Nde*I, and ligated into *Xba*I–*Nde*I-treated (to remove the TetR gene) pPATAN vector. The resultant vector, prRNA-T7P-Neo, was digested with *Spe*I and *Eco*RI to remove the *neo*R gene, ligated with an *Spe*I–*Eco*RI-digested PCR product generated by amplification of the *Zeocin* resistance gene from vector pCMV/*Zeo* with primers Zeo-*Spe*I 5′-GGACTAGTATGGCCAAGTTGAC CAGT-3′ and Zeo-*EcoR*I 5′-CGGAATTCTCAGTCCTGCTCCTCG GC-3′ to generate vector prRNA-T7P-Zeo (Fig. 1c).

Plasmid pT7-Tet-CAT was constructed with the bacteriophage T7 promoter containing two tandem TetO driving expression of the bacterial chloramphenicol acetyltransferase gene. The Tet operators were located 1 bp upstream from the transcription start point. The vector was generated as follows. A pGEM-derived T7 promoter bearing two Tet operators upstream from the transcription start point was generated by the annealing of primers Tet1 5′-CCCAAGCTTTAATACGACT CACTATAATCTCCCTATCAG-3′, Tet2 5′-TCACTGATAGGGTG ATCTCTATGACTGATAGGGAGATTATAG-3′, Tet3 5′-AGATC TCCCTATCAGTGATAGAGAGGGCGATAGAGAGGGCGAAA GCTTGGG-3′, and Tet4 5′-CCCAAGCTTTCGCCC-3′ and extending with *Taq* polymerase (PE BioSystems) in 30 cycles at 94°C, 1 min; 60°C, 1 min; 72°C, 30 s. This product was digested with *Hin*dIII and

ligated into *Hin*dIII-digested plasmid pRibCL (Nunes *et al.* 1997a) to generate pT7-Tet-CAT (Fig. 1d).

Protein extraction from T. cruzi. Protein extracts were prepared from *T. cruzi* for EMSA by modifications of previously published protocols (Gatz and Quail 1988). Briefly, 10-ml log-phase cultures of *T. cruzi* epimastigotes were pelleted, washed once in PBS, and resuspended in 0.5 ml protein extraction buffer (50 mM sodium phosphate [pH 7.5], 1 mM EDTA, 0.1% Triton X-100, 10 mM β-mercaptoethanol, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin, 2 mM sodium bisulfite, 2 mM PMSF, 1% polyvinylpyrolidone). The suspensions were supplemented with 0.1 g of glass beads (Sigma) and vortexed for 10 min at 4°C. The glass beads were removed by passage through a 3-cc syringe packed at the tip with sterile glass wool. The extract was centrifuged for 10 min at 4°C to pellet debris and any remaining beads. The supernatants were removed and stored at −70°C.

Electrophoretic mobility shift assays. A target double-stranded oligonucleotide containing the TetO element was prepared by the annealing of oligos OP3A 5′-GAATACACAAGGACACAGCTCTCTATCACTGATAGGGACACAGCATGACACTCTATCAG-3′ and OP3B 5′-GTGATAGAGTGTCATGCTGTGTCCCTATCAGTGATAGAGAGCTGTGTCCTTGTGTATTCCAG-3′. A target double-stranded labeled oligonucleotide containing T7 promoter was prepared by the annealing of oligos T7F 5′-TAATACGACTCACTATAGGGCGA-3′ and T7R 5′-TCGCCCTATAGTGAGTCGTATTA-3′. The oligos were labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP and used for EMSA as previously described (Nunes *et al.* 1997b). Similarly, we prepared double-stranded TetO nonspecific competitor oligos from 35.1F and 35.1R (see above). Approximately 1, 5, and 10 μl of protein extract were used in TetO binding reaction. Approximately 10 μl of protein extract was used in T7 promoter binding reaction. For competitions, the indicated quantities of unlabeled oligonucleotide were added to the reaction mixture prior to the addition of protein.

RESULTS

T. cruzi *stably expressing the bacterial tetracycline gene repressor TetR.* The first step in construction of a regulated gene expression vector in *T. cruzi* based on the bacterial tetracycline gene regulatory system was the establishment of a *T. cruzi* strain constitutively expressing TetR. Thus, we constructed vector pPATAN (Fig. 1a) containing a *T. cruzi* rRNA promoter driving expression of the TetR gene preceded by a homologous splice acceptor site. Downstream from the TetR gene, we inserted the selectable bacterial neomycin resistance gene (*neo*R) also preceded by a homologous acceptor site. This gene is expressed by read-through transcription from the rRNA promoter upstream from the TetR gene. *T. cruzi* epimastigotes were transfected with *Kpn*I-linearized pPATAN, and epimastigotes resistant to G418 were selected as described under Materials and Methods. Southern analyses with standard agarose and pulsed field gels of DNA from transfected epimastigotes indicated that the linearized pPATAN was integrated into a ~1.5-Mb chromosome previously shown (Cano *et al.* 1995) to bear the rRNA genes (data not shown).

Northern and electrophoretic mobility shift analyses were performed to verify expression of TetR protein in these stably transfected *T. cruzi* epimastigotes, termed *T. cruzi* CL-TetR. Northern analysis (Fig. 2a) identified a ~700-base transcript, the expected size for the TetR transcript. Total protein extracts (Gatz and Quail 1988) from these cells were used in electrophoretic mobility shift assays with the Tet operators as labeled probes (Fig. 2b). In these experiments, the Tet operator oligonucleotides shifted only when incubated in extracts from *T. cruzi* expressing TetR. The interaction was specific since the shifted band was competed in the presence of excess unlabeled Tet operator oligonucleotide (panel SC), and no competition was observed when excess random oligonucleotides were incubated in the *T. cruzi* extracts (panel NC). These results show that TetR is transcribed and translated in the stably transfected *T. cruzi* CL-TetR cells.

Mapping of the transcription start point of the T. cruzi *rRNA gene.* The 19-bp Tet operators to which TetR binds to repress transcription must generally be localized within a few basepairs of the tsp to efficiently block transcription initiation (Wirtz and Clayton 1995; Ramakrishnan *et al.* 1997). Therefore, we carefully mapped the transcription start point of the *T. cruzi* strain CL Brener rRNA gene promoter by primer extension as previously described (Martinez-Calvillo and Hernandez 1994). The results showed that the CL Brener strain transcription start point is a "C" at position 165 of a previously characterized DNA fragment (GenBank Accession No. U89779) bearing the rRNA promoter (Tyler-Cross *et al.* 1995) and located ~1.5 kb upstream from the first base of the 18S rRNA gene (data not shown).

Tetracycline-regulated gene expression mediated by rRNA promoter. In our first TetR-regulated system, we attempted to directly regulate the *T. cruzi* rRNA gene promoter. Thus, an expression construct prRNA-Tet-CAT (Fig. 1b) bearing a pair of 19-bp Tet operators substituted one base upstream from the mapped transcription start point of the rRNA gene (Fig. 3a) was generated as described under Materials and Methods. Downstream of the promoter–operator cassette, we inserted the bacterial CAT gene reporter preceded by a homologous splice acceptor. This vector was transfected into *T. cruzi* CL-TetR epimastigotes and examined for reporter gene expression in the presence of various concentrations of tetracycline.

The results (Fig. 4a) showed that incubation of these epimastigotes in 5 μg/ml of tetracycline generated a ~7-fold increase in CAT activity compared to the no-tetracycline control. Increasing concentrations of tetracycline up to 20

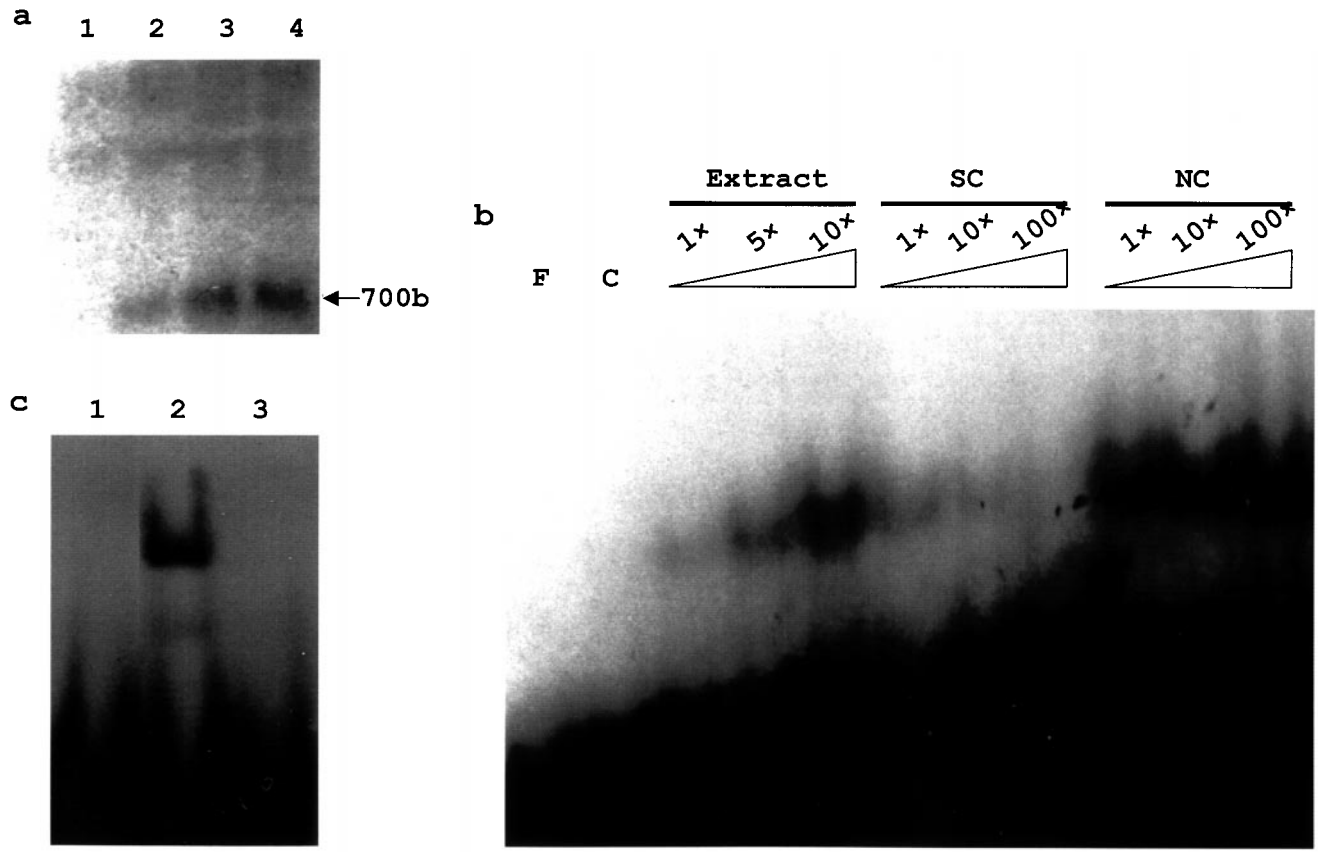

FIG. 2. Establishment of tetR and T7 RNA polymerase expressing *T. cruzi* strains. (a) Northern blot showing the tetR transcript in transfected *T. cruzi* epimastigotes. Lanes: 1, *T. cruzi* strain without transfected tetR-expressing vector pPATAN (control); 2–4, three independent clones of pPATAN-transfected *T. cruzi*. Northern analysis was performed by standard methodology (Maniatis *et al.* 1982) with 10 μg total RNA with a randomly radiolabeled tetR probe amplified from plasmid pPATAN with PCR primers TET1 and TET2 as described under Materials and Methods. (b) EMSA of tetR-expressing *T. cruzi* strain. A radiolabeled double-stranded oligonucleotide bearing TetO sites was incubated in *T. cruzi* protein extracts and subjected to EMSA as described under Materials and Methods. F, free probe; C, protein extract from *T. cruzi* strain without transfected tetR-expressing vector pPATAN (control); Extract, protein extracts (1, 5, 10 μl) from *T. cruzi* strain transfected with tetR-expressing vector pPATAN; SC, as above with 10 μl of extract and increasing amounts (1:1, 1:10, 1:100) of unlabeled specific competitor; NC, as above but with increasing amounts (1:1, 1:10, 1:100) of nonspecific competitor. (c) EMSA of T7 polymerase expressing *T. cruzi* strain. Protein extract (10 μl) from *T. cruzi* CL-TetR/T7P strain was incubated with radiolabeled T7 promoter probe in an EMSA as described under Materials and Methods. Lanes: 1, free probe; 2, protein extract from *T. cruzi* CL-TetR/T7P incubated with labeled T7 promoter probe; 3, protein extract from *T. cruzi* CL-TetR strain incubated with labeled probe (control).

μg/ml increased CAT expression up to ~20-fold over background levels. Higher levels of tetracycline were toxic to the cells (not shown). Removal of tetracycline from a cell line that was previously fully induced with 20 μg/ml tetracycline dropped expression, "re-repressed," close to expected levels; i.e., ~5-fold repression was observed compared with 20 μg/ml tetracycline-induced expression. Re-induction with 10 μg/ml tetracycline showed ~13-fold higher expression compared with the control in the absence of tetracycline. The results indicated that the TetR can bind and block expression from this promoter.

Maximal expression from this vector was less than 10% of the levels detected from similar constructs lacking the Tet operators, even when transfected into cells lacking the TetR (data not shown). Thus, substitution of the Tet operators in the rRNA promoter decreased transcription from this construct. More important, some expression of the reporter gene was detectable in the absence of tetracycline,

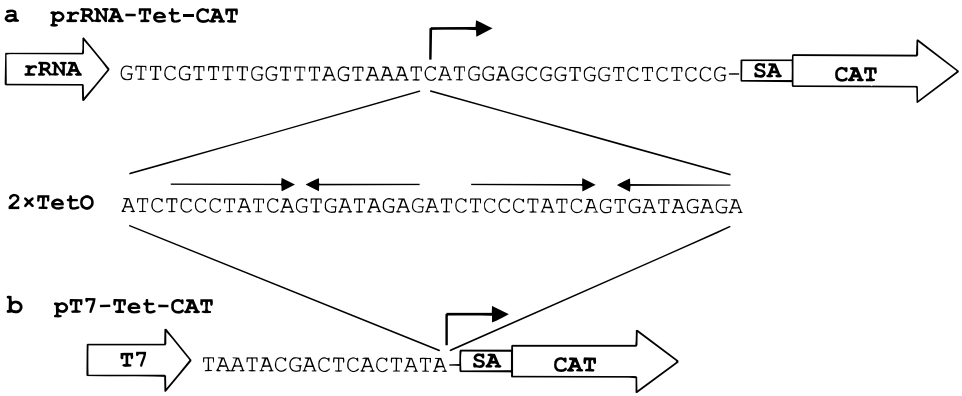

FIG. 3. Tet operator insertion sites. The Tet operators were inserted into the rRNA and T7 promoters as described under Materials and Methods. (a) Location of the two copies of the Tet operator immediately upstream of the transcription start point "C" in the prRNA-Tet-CAT vector. (b) Location of two copies of the Tet operator immediately upstream of the transcription start point of the T7 promoter in the pT7-Tet-CAT. Symbols: rRNA, *T. cruzi* ribosomal RNA promoter; T7, bacteriophage T7 promoter; SA, *T. cruzi* splice acceptor; CAT, chloramphenicol acetyltransferase gene; 2 × TetO, two copies of the Tet operator.

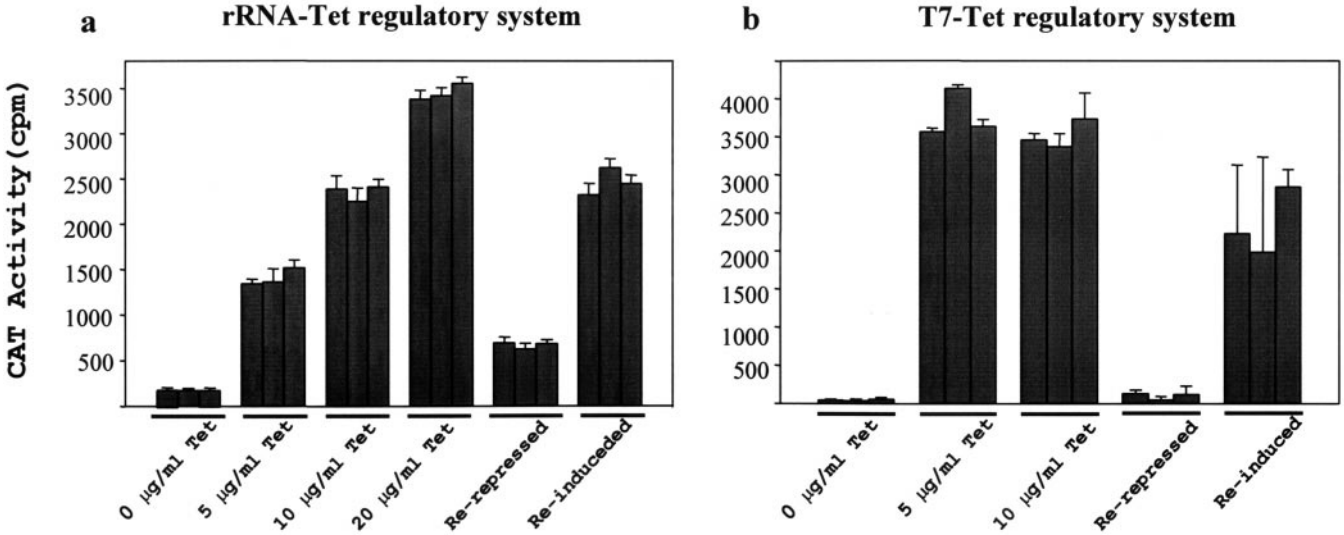

FIG. 4. rRNA-Tet and T7-Tet regulatory systems. The Tet repressor expressing *T. cruzi* strain was transiently transfected with the prRNA-Tet-CAT vector or the pT7-Tet-CAT vector in the absence of tetracycline or in increasing concentrations of tetracycline. Relative CAT activity was measured as described under Materials and Methods. Each condition was examined in three different experiments and each data point was performed in triplicate in each experiment. The error bars indicate the standard error of each triplicate assay. (a) 0 μg/ml (control), CAT expression from the Tet repressor-expressing *T. cruzi* strain (*T. cruzi* CL-TetR) transfected by prRNA-Tet-CAT vector and cultured for 48 h in the absence of tetracycline; 5 μg/ml, expression from the *T. cruzi* CL-TetR strain transfected by prRNA-Tet-CAT vector and cultured for 48 h in the presence of 5 μg/ml tetracycline; 10 μg/ml, as for 5 μg/ml but with 10 μg/ml tetracycline; 20 μg/ml, as for 5 μg/ml but with 20 μg/ml tetracycline; Re-repressed, expression from *T. cruzi* CL-TetR strain transfected by prRNA-Tet-CAT vector, cultured for 48 h in the presence of 20 μg/ml tetracycline, washed twice in PBS, and re-cultured for 48 h in the absence of tetracycline; Re-induced, as for re-repressed but the cells re-cultured for 48 h in the presence of 10 μg/ml tetracycline. (b) 0 μg/ml (control), the Tet repressor-expressing *T. cruzi* strain (*T. cruzi* CL-TetR) transfected by pT7-Tet-CAT vector and cultured for 48 h in the absence of tetracycline; 5 μg/ml, *T. cruzi* CL-TetR strain transfected by pT7-Tet-CAT vector and cultured for 48 h in the presence of 5 μg/ml tetracycline, 10 μg/ml, as for 5 μg/ml but with 10 μg/ml tetracycline; Re-repressed, *T. cruzi* CL-TetR strain transfected by pT7-Tet-CAT vector, cultured for 48 h in the presence of 10 μg/ml tetracycline, washed twice in PBS, and re-cultured for 48 h in the absence of tetracycline; Re-induced, as for re-repressed by the cells re-cultured for 48 h in the presence of 10 μg/ml tetracycline.

suggesting that some leaky transcription is occurring from the repressed rRNA promoter.

Tetracycline-regulated gene expression mediated by the bacteriophage T7 promoter. Although the tetracycline-regulated rRNA promoter provided ~20-fold regulation in the presence or absence of tetracycline, the leaky expression observed could negatively impact analysis of genes expressed at low levels. Thus, we developed a T7 promoter-*tet* regulatory expression system for more stringent control of gene expression.

The first step in development of this system was establishment of a *T. cruzi* strain that expresses both the TetR and the T7 RNA polymerase. Thus, *T. cruzi* CL-TetR was transfected with vector prRNA-T7P-Zeo (Fig. 1c), containing the rRNA promoter driving expression of the T7 RNA polymerase gene preceded by a homologous splice acceptor. Downstream from the T7 polymerase gene is a gene for *zeocin* resistance, also preceded by a splice acceptor. Thus, read-through transcription from the rRNA promoter transcribes the T7 RNA polymerase gene and *zeo*. Therefore, stable G418 and zeocin resistant *T. cruzi*, termed *T. cruzi* CL-TetR/T7P, express both the TetR and the T7 RNA polymerase. Electrophoretic mobility shift analysis demonstrated that *T. cruzi* CL-TetR/T7P protein extracts contained a protein that specifically binds to and shifts oligonucleotide probes containing the T7 promoter sequence (Fig. 2c). Thus, we concluded that *T. cruzi* CL-TetR/T7P epimastigotes express both TetR and T7 RNA polymerase.

Expression vector pT7-Tet-CAT, in which the bacteriophage T7 promoter containing Tet operators flanking the transcription start site (Fig. 3b) drives expression of the bacterial CAT gene, was constructed as described under Materials and Methods. This vector was used to transfect *T. cruzi* CL-TetR/T7P epimastigotes, and CAT activity was assayed under different concentrations of tetracycline as described under Materials and Methods (Fig. 4b). Significantly, levels of CAT expression in the absence of tetracycline, i.e., maximally repressed, approached background levels in untransfected *T. cruzi*. These results indicate that under conditions of maximal repression, the T7 promoter in the pT7-Tet-CAT vector is effectively shut down.

In contrast, low levels of tetracycline (e.g., 5 μg/ml) maximally induce the T7 promoter in this system. Expression is induced ~150-fold over background in the presence of 5, 10 μg/ml (Fig. 4b), or higher (not shown) levels of tetracycline. This 150-fold induction is ~7-fold greater than the ~20-fold induction seen with the prRNA-Tet-CAT construct, and absolute levels of expression of the fully induced pT7-Tet-CAT vector were slightly greater than that seen from the prRNA-Tet-CAT construct (not shown). When tetracycline was removed from cells maximally expressing CAT, transcription was "re-repressed" to background levels. When 10 μg/ml tetracycline was re-added to the washed cells, expression levels returned or were "re-induced" to nearly fully induced levels. These observations show that repression or re-induction can be restored in this system even when the T7 promoter has been very actively utilized.

DISCUSSION

The rRNA promoter of *T. cruzi* has proven to be robust and extremely useful for the expression of exogenous genes in this parasite. With this promoter, expression has been obtained in both transient (Tyler-Cross *et al.* 1995) and stable (Santos and Buck 1999, 2000) transfections at levels ~100 fold higher than that provided by the SL RNA gene promoter (Tyler-Cross *et al.* 1995), the only other *T. cruzi* promoter characterized in any detail (Lu and Buck 1990; Nunes *et al.* 1997b; Wen *et al.* 2000). Exogenous genes have been shown to be expressed in both epimastigotes and amastigotes and to remain expressed after transmission through insects and mice (Santos and Buck 2000). Thus, we believe that a regulated expression system based on the rRNA promoter will be extremely valuable for a dissection of gene function in *T. cruzi*.

Previous versions of *T. cruzi* expression vectors (Martinez-Calvillo and Hernandez 1994; Tyler-Cross *et al.* 1995; Nunes *et al.* 1997a) have used the rRNA gene promoter to express exogenous genes constitutively. However, in many cases, e.g., for essential genes, determination of the function of a gene will require conditional expression. For example, essential genes cannot be studied by knockout or deletion. A system providing regulated expression is necessary to study the function of these genes. The bacterial tetracycline resistance gene regulatory system has provided such a tool in plants, mammals, fungi, and *T. brucei*. Herein, we have adapted this system for function in *T. cruzi* using the rRNA gene promoter as a basis.

In the first system that we developed, the rRNA promoter was modified with Tet operators flanking the transcription start point. This system showed ~20-fold regulation in the presence or absence of tetracycline. Not surprisingly, when fully induced, this promoter generated <10% of expression of control constructs lacking the Tet operators. Some CAT expression was also detected from this construct in the absence of tetracycline, indicating that repression by the TetR

was not absolute. In other systems, regulation of the operator-modified promoters is largely affected by the proximity of the operators to the transcription start point. In *T. brucei*, optimal regulation of the RNA polymerase I transcribed PARP promoter required that the operators be located within 2 bp of the transcription start point (Wirtz and Clayton 1995), and in *Entamoeba histolytica*, operators positioned greater than 4 bp from the transcription start point were ineffectively repressed by TetR (Ramakrishnan *et al.* 1997). Although it is possible that a more optimal location for the operators in the *T. cruzi* rRNA promoter could be found, multiple other constructs tested yielded little or no regulation (unpublished data).

Although the regulated rRNA promoter system will be useful for many studies, it may not be helpful for the study of genes that are expressed at low levels because of the leaky expression from the fully repressed system. Rather than performing an exhaustive analysis to optimize down-regulation of this promoter, we elected to construct an alternative system based on expression of T7 polymerase in *T. cruzi* and use of T7 promoters in the expression vectors. Other systems (Wirtz *et al.* 1999; Ramakrishnan *et al.* 1997) using the T7 promoter show stringent regulation. Therefore, we constructed a *T. cruzi* system in which TetR and T7 polymerase are stably expressed in the same cells. Our vector was modified with a T7 promoter, containing Tet operators flanking the transcription start point. This system exhibited both excellent repression in the absence of tetracycline and excellent induction in the presence of low concentrations of tetracycline. This system will permit the study of toxic genes or genes that are expressed at very low levels. Moreover, we anticipate that it will be possible to induce genes in intracellular amastigotes and probably even *in vivo* in animal models. Finally, we anticipate that development of the regulatory system in the CL Brener strain will permit the functional analysis of both known genes and novel sequences identified in the *T. cruzi* genome project. Thus, this regulated gene expression system should have wide applications for *T. cruzi* research.

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