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***Leishmania chagasi*: a tetracycline-inducible cell line driven by T7 RNA polymerase**

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Abstract

Trypanosomatid protozoa lack consensus promoters for RNA polymerase (RNAP) II. However, the artificial insertion of the T7 promoter (P^{T7}) and the tetracycline repressor into *Trypanosoma brucei* cell lines expressing T7RNAP allows P^{T7}-driven gene expression to be tetracycline-inducible. These cell lines provide a molecular tool to address protein function by several recombinant approaches. We describe here the development of an analogous *Leishmania chagasi* cell line bearing the genes for exogenous T7RNAP and the tetracycline repressor inserted in the multi-gene α -tubulin locus. A plasmid construct with P^{T7} and the tetracycline operator upstream of a reporter gene, when introduced into this cell line as episomal plasmids or chromosomal insertion into the non-coding strand of an *18S rRNA* gene, resulted in tetracycline-inducible expression of the reporter as much as 16 and 150 fold, respectively. The reporter was under a much tighter control when chromosomally inserted than extra-chromosomally born. Furthermore, P^{T7} augmented the reporter's expression 2 fold more in comparison to P^{T7}-less constructs. This cell line is the first *Leishmania* spp. that allows the exogenous T7RNAP-driven gene expression to be tetracycline-inducible; and may provide a useful tool for addressing protein function by manipulating expression levels of *Leishmania* endogenous genes.

Keywords

Leishmania; repressor; operator; trypanosomatid; tetracycline induction

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1. Introduction

Eukaryotic cell genes are transcribed by one of three classes (I, II, III) of RNA polymerase (RNAP). Each RNAP class recognizes its own specific promoter(s) with the help of accessory initiation protein factors during transcription (Zawel and Reinberg, 1995; Tjian, 1996). Similar to other eukaryotes, the trypanosomatid protozoa such as *Trypanosoma* spp. and *Leishmania* spp. contain all three RNAP classes. Unlike other eukaryotes, they do not possess endogenous RNAPII promoters that can be conveniently used to artificially manipulate gene expression. Bacteriophage T7 or T3 promoters have been used to drive gene expression in *T. brucei* engineered to express T7 or T3 RNAP, respectively. These promoters augment gene expression up to five fold in comparison to endogenous RNAPI promoters (Wirtz et al., 1994). In addition, a tetracycline-inducible *T. brucei* cell line driven by T7RNAP promoter (P^{T7}) was generated, resulting in an additional 10⁴-fold increase in expression of a reporter gene in the presence of tetracycline (Wirtz et al., 1998; Wirtz et al., 1999). A similar cell line has been developed in the related protozoan *T. cruzi*, resulting in 150-fold increased expression of a reporter gene upon tetracycline induction (Wen et al., 2001). Furthermore, a tetracycline-inducible cell line of *L. donovani* was developed by inserting the tetracycline repressor (*TETR*) and luciferase (*LUC*) genes into the α -tubulin (α -*TUB*) or the *rRNA* locus. Tetracycline caused a 50-fold increase in LUC activity when the *TETR* and *LUC* genes were inserted in the α -*TUB* locus, and an additional 5-fold increase when inserted at the *rRNA* locus (Yan et al., 2001). An improved cell line that maintained 10³-fold expression of *LUC* gene with a much reduced background was reported later (Yan et al., 2002).

The advantages of using tetracycline-induction of gene expression driven by T7RNAP versus endogenous RNAPs in both *T. brucei* and *T. cruzi* include an increase in transcription to an additional order of magnitude (Clayton, 1999; Wen et al., 2001). Additionally, these cell lines have been used to facilitate RNA interference (RNAi) in *T. brucei* using oppositely oriented cassettes of P^{T7} and tetracycline operator (O^{tet}) surrounding a sequence of the targeted gene (LaCount et al., 2000; Wang et al., 2000). We describe here the generation of a *L. chagasi* cell line expressing both *T7RNAP* and *TETR* inserted into the multi-gene α -*TUB* locus, in which expression of specific genes can be driven by P^{T7} and induced by tetracycline. This is the first cell line of *Leishmania* spp. that allows tetracycline-inducible gene expression driven by T7RNAP. We propose to use this system to address functions of *Leishmania* spp. gene products.

2. Materials and Methods

2.1. Parasites and DNA amplification

A Brazilian strain of *L. chagasi* (MHOM/BR/00/1669) was continuously passaged in golden hamsters to maintain its virulence as previously described (Yao et al., 2002; Yao et al., 2004; Yao et al., 2005). All cell lines described herein descended from this parental cell line.

DNA fragments were amplified by the polymerase chain reaction (PCR) using *Pfu* DNA polymerase (Stratagene, Carlsbad, CA) and primers synthesized by IDT Inc. (Coralville, IA) as listed in Table 1. Templates used for PCR amplifications were as follows: (a) *L. chagasi* genomic DNA was used as a template for the 220 base pair (bp) regulatory region upstream of the major surface protease (*MSP*) genes, as well as the intercoding regions (ICs) of *MSPC*, α -*TUB* and *18SrRNA*. IC is defined as the region between the stop codon of an upstream gene and the start codon of a downstream gene, including both the 3'- and 5'-untranslated region (Purdy et al., 2005). (b) Genomic DNA of the transgenic *T. brucei* cell line 29-13 (Wirtz et al., 1999) was used to amplify *T7RNAP* and hygromycin phosphotransferase (*HPT*). (c) The pEGFP-1 Vector (Clontech, Palo Alto, CA) was the template for enhanced green fluorescent protein (eGFP). (d) The p220S vector (Ramamoorthy et al., 1996) was used to amplify *LUC*.

(e) The pX- β GAL vector was used to amplify neomycin phosphotransferase (*NPT*) and the dihydrofolate reductasethymidylate synthase (*DHFR-TS*) IC. Gel-purified PCR products of the expected sizes were first cloned into TOPO vector (Invitrogen, La Jolla, CA) and sequenced prior to cloning into final constructs.

2.2. Plasmids

All plasmids used in this study are listed in Table 2. pX- β GAL was generously provided by Dr. Stephen M. Beverley of Washington University at St. Louis and was described previously (LeBowitz et al., 1990). pTupactet was a generous gift from Dr. Kenneth Stuart of University of Washington. It was originally designed to introduce the *TETR* gene into α -*TUB* locus of *L. donovani* using puromycin N-acetyl transferase (*PAC*) gene as selectable marker (Yan et al., 2001) (Figure 1A). The α -*TUB* genes of *L. chagasi* (GenBank accession no. [DQ129864](#)) and *L. donovani* share more than 99% identity (data not shown). Since the minimal identity for homologous replacement is approximately 80% (Clayton, 1999), we expected that pTupactet would recombine with the α -*TUB* locus of *L. chagasi*.

pTuT7RpolHyg was engineered to introduce *T7RNAP* into the α -*TUB* locus of *L. chagasi* using *HPT* gene as a selection marker (Figure 1A). It was made from two separate plasmids, pT7Rpol and pHyg, using pBluescript SK- (Invitrogen, San Diego, CA) as a cloning vector. pT7Rpol was made by first cloning *T7RNAP*, generated by PCR with primers T7XbaF and T7BamAscR (See Table 1 for primer sequences), using genomic DNA of the transgenic *T. brucei* cell line 29-13 as template. This cell line contains a chromosomal copy of *T7RNAP* (LaCount et al., 2000). α -*TUB* IC was amplified from *L. chagasi* genomic DNA using primers TubIGSacBamF and TubIGXbaR. *T7RNAP* was cloned into *Bam*HI and *Xba*I sites, and the α -*TUB* IC was cloned into *Xba*I and *Sac*II sites, respectively, of pBluescript. The pHyg construct was similarly made by cloning three individual fragments into pBluescript: (a) *L. chagasi* *MSPC* IC (C-S4IRSacAscF and C-S4IRXbaR primers) (GenBank accession no. [DQ129865](#)) into *Sac*II and *Xba*I sites, (b) the *HPT* gene (HygXbaF and HygSmaR primers) into *Xba*I and *Sma*I sites, and (c) the *L. chagasi* α -*TUB* IC (TubIRSmaF and TubIRHindAscR primers) into *Sma*I and *Hind*III sites. The pHyg insert was cloned into the *Asc*I site of pT7Rpol; the resultant plasmid was named pTuT7RpolHyg. The linear pTuT7RpolHyg construct (Figure 1A) was excised from this plasmid vector with *Bam*HI. Constructs pTupactet and pTuT7RpolHyg were used to generate the background *L. chagasi* cell line T7-tet for insertion of tetracycline-inducible genes driven by P^{T7}.

Plasmids P^{T7}O^{tet}eGFP and P^{T7}O^{tet}Luc were designed to express the reporter genes for *eGFP* and *LUC*, respectively, under the control of P^{T7} and O^{tet} on an extra-chromosomal plasmid. A pP^{T7}O^{tet} construct was made first, and contained a P^{T7}, an O^{tet} and the 220 bp region upstream of *MSP* genes including the upstream regulatory polypyrimidine tract, the splice leader acceptor site and the 5'UTR (T7pToMSP5UEcoRF and MSP5UClaR primers), all of which were cloned into the *Eco*RI and *Cla*I sites of pBluescript SK- vector (Ramamoorthy et al., 1996). The P^{T7}, O^{tet}, and part of the *MSP* sequences were included in the forward primer for this construct. Second, peGFP and pLuc were individually constructed by cloning *eGFP* into *Cla*I and *Kpn*I sites (GFPClaF and GFPKpnBamR) or *LUC* into the *Xho*I and *Kpn*I sites (LucXhoF and LucKpnBamR) of plasmid pP^{T7}O^{tet}, respectively. Finally, plasmids P^{T7}O^{tet}eGFP and P^{T7}O^{tet}Luc were generated by cloning inserts from peGFP or pLuc into the *Bam*HI site in the same orientation as *NPT* gene using the *Escherichia coli*-*Leishmania* shuttle vector pX (LeBowitz et al., 1990). Plasmid O^{tet}Luc was made in a similar way but omitting the P^{T7} sequence from the forward primer of 5'UTR of *MSP* genes (ToMSP5UEcoRF and MSP5UClaR).

The 18SLuc construct was designed to insert *LUC* gene into the *18S*rRNA locus in the non-coding strand using *NPT* gene as a selectable marker. The following individual fragments were

cloned into the pBluescript SK- vector. The upstream 18S rRNA intergenic bottom strand sequence and the T7RNAP terminator (T7t) was cloned into *SacII* and *NotI* sites (NotT18SF and Sac18SR primers). The P^{T7}, two copies of O^{tet}, and the 220 bp *MSP* upstream regulatory elements were cloned in the *NotI* and *SpeI* sites (NotTptOMSPF and SpeMSP5UTRR primers). The *LUC* gene was cloned into *SpeI* and *BamHI* sites (SpeLucF and HindBamLucR primers). α -*TUB* IC was cloned into *BamHI* and *HindIII* sites (BamTubIRF and HindTubIRR primers). The *NPT* gene along with *DHFR-TS* intergenic regions were amplified from the pX- β GAL vector and cloned into *HindIII* and *XmaI* sites (HindNeoF and XmaKphDHFRTSR primers). Finally, the downstream 18S rRNA sequence with a T7t was cloned into the *KpnI* site (Kpn18SF and KpnT18SR primers). A second construct named 18SLucC lacking the P^{T7} was generated from 18SLuc by replacing the P^{T7}-O^{tet}-220 bp upstream *MSP* elements and *LUC* gene of 18SLuc with O^{tet}-220 bp upstream *MSP* elements and *LUC* but lacking a P^{T7} (NottOMSPF and HindBamLucR primers) in the *NotI* and *BamHI* sites. These circular plasmids were cut with *SacII* to remove the vector backbones. Gel-purified linear 18SLuc and 18SLucC inserts were used to transfect T7-tet *L. chagasi* cells. Colonies were selected on semi-solid M199 medium as described (Kapler et al., 1990). Since both the selectable marker (*NPT*) and the reporter *LUC* genes were in a tandem head-to-tail array downstream of P^{T7} and O^{tet}, it was necessary to include both doxycycline and G418 in the plates to select the transfected colonies.

2.3. Transfections

Leishmania transfections were performed according to a published technique (Kapler et al., 1990) modified as described (Yao et al., 2005). During transient transfections, cells were incubated in selection antibiotics for 24 h before induction with 0.1 μ g doxycycline/ml. To obtain stable episomal transfectants, cells were selected on semi-solid M199 plates with the appropriate antibiotics at 26°C until single colonies appeared, usually in 2 to 4 weeks. Chromosomal replacements were accomplished by electroporation of 20 μ g of purified linear insert DNA prior to application to M199 plates with antibiotic. The T7-tet cell line of *L. chagasi* containing both *T7RNAP* and *TETR* was raised by two rounds of homologous replacements, first with pTuT7RpolHyg using hygromycin as a selection antibiotic, and second with pTupactet using both puromycin and hygromycin as selection antibiotics. Antibiotic concentrations in M199 plates were 30 and 40 μ g/ml of puromycin, G418 and hygromycin. Liquid cultures contained 100 μ g of puromycin, G418 or hygromycin/ml for chromosomal transfectants. For episomal transfectants cells were cultured in 200 μ g of G418/ml.

2.4. Reporter analysis

Live promastigotes expressing GFP in logarithmic growth phase were washed in Hank's Balanced Salt Solution (GIBCO-BRL, Rockville, MD) and resuspended in the same solution with 50% glycerol at 4°C to minimize mobility. GFP was detected by an epifluorescence Olympus BX51 microscope equipped with a digital camera. The same exposure was used for all the samples. Furthermore, GFP was quantified by FACS using FACScan (Becton Dickinson). For each sample, 1×10^5 events were analyzed.

Promastigotes designated for LUC and β GAL assays were collected and washed in phosphate buffered saline. Afterwards, they were suspended in suitable buffers and underwent freeze-thaw cycles, and the soluble fractions were collected and analyzed for LUC and β GAL according to the manufacturer's protocols of Promega (Madison, WI) and Clontech (Mountain View, CA), respectively. Samples were either used right away or stored in -70°C for later use within a week. Cellular equivalences were used in most assays. In some cases, protein concentrations in the soluble fractions were determined by a BCA Protein Assay Kit (Pierce, Rockford, IL). Triplicate samples were analyzed by a Monolight 2010 Luminometer™ (Analytical Luminescence Laboratory, San Diego, CA).

2.5. Southern blots

L. chagasi genomic DNA (5 µg) was subjected to restriction enzyme digestion, and resultant fragments were separated on 0.8% agarose gels. After DNA transference to Nylon (Roche, Mannheim, Germany), the filters were probed with [³²P]-DNA probes generated with a Random Amplification Kit (Roche) using gel purified DNA fragments as templates. Blots were washed three times under high stringency conditions (0.1 × SSC, 0.1% SDS, 65°C, 30 min) and exposed to X-ray films.

3. Results

3.1. Generation of *L. chagasi* T7-tet cell lines

L. chagasi cell lines expressing T7RNAP and TETR were generated by sequential insertion of pTuT7RpolHyg and pTupactet into different locations in the multi-gene α -*TUB* locus (Figure 1A). Logarithmic growth phase promastigotes were transfected with linearized, gel-purified pTuT7RpolHyg and selected with hygromycin. Control cells were mock transfected without DNA. Southern blots of DNAs from ten pTuT7RpolHyg transfectants (designated as T7) hybridized with the *T7RNAP* probe, whereas two mock controls did not (examples in Figure 1B). *SacI* and *SacII* cut once in α -*TUB* IC and coding regions, respectively, each producing a prominent 2.3 kilobase (kb) fragment corresponding to the size of the tandem α -*TUB* repeat seen in lanes 2 and 3 of Figure 1B. Flanking sequences produced two less prominent bands hybridizing to both the coding and IC sequences, seen in single digestions in mock transfectants. The *SacI*-*SacII* double digest resulted in prominent 0.8 and 1.5 kb fragments corresponding to the digested repeat, seen in the lane 4. In the T7 cells, bands of 6.4, 6.5 and 5.7 kb seen after cleavage with *SacI*, *SacII*, or both, correspond to the expected sizes upon integration of TuT7RpolHyg into the genome (lanes 2, 3, and 4 in Figure 1B, T7RNAP panel). These bands were recognized by the *T7RNAP* and α -*TUB* IC probes, but not by the α -*TUB* coding region probe. Furthermore, correct integration was confirmed by PCR amplification of genomic DNA with primers flanking the conjunction between the insert and the chromosome (a-b and c-d) as shown in Figure 1D. These results demonstrate that *T7RNAP* was successfully inserted in the α -*TUB* locus.

One of the above ten T7 transfectants was further transfected with pTupactet to introduce *TETR* into this T7RNAP-expressing cell. The resultant clonal cells, designated as T7-tet, were selected with both hygromycin and puromycin to ensure pTupactet did not merely replace pTuT7RpolHyg. Control T7 cells transfected with no DNA were selected only with hygromycin. A Southern blot of genomic DNA digested with *KpnI* plus *NotI*, *BamHI* plus *NotI*, or *SacII* resulted in unique band patterns distinguishing non-integrated pTupactet from its chromosomal integration, as detected by loss of the *KpnI* and *NotI* sites at the ends of the plasmid insert sequence, and inclusion of a new *SacII* site in the *PAC* gene (Figure 1C). Furthermore, PCR amplification of sequences crossing the insert-chromosomal border yielded DNA fragments of the expected sizes (Figure 1D, primer pairs a-f and e-d). These data indicate *TETR* was inserted into the α -*TUB* locus of T7 cells.

3.2. Inducible expression of extra-chromosomal reporter genes

To determine whether gene expression could be placed under control of P^{T7} and O^{tet} in the *L. chagasi* T7-tet cells, reporter genes were cloned downstream of a P^{T7} and a O^{tet} in the pX shuttle vector (Figure 2A). The eGFP reporter construct P^{T7}O^{tet}eGFP was stably transfected into T7-tet cells, selected with puromycin, hygromycin and G418, induced with doxycycline for 24 h, and examined by epifluorescence microscopy. GFP was homogeneously distributed throughout promastigotes including the flagellum, whereas cells cultured under the same conditions without doxycycline did not contain a detectable level of GFP (Figure 2B). Further, GFP was quantified by FACS analysis of live promastigotes. T7-tet cells transfected with the

parental pX vector yielded 3.5% positive cells with an average fluorescence intensity of 8.8 units, whereas uninduced T7-tet cells containing the P^{T7}O^{tet}eGFP plasmid generated a mean fluorescence intensity of 20.7 with 92% positive cells. The mean fluorescence intensity increased to 60.4 and 76.4 after 24 h induction with 0.1 and 1.0 doxycycline, respectively, with ~ 95% positive cells in both cases (Table 3). These data indicate that GFP expression is induced 3-4 fold upon doxycycline induction even though leakiness exists in the absence of doxycycline.

A second reporter gene, *LUC*, yielded similar results. *LUC* was cloned downstream of P^{T7} and O^{tet} in pX (Figure 2A) and stably transfected into T7-tet cells. After doxycycline induction for 24 h, LUC activity increased approximately 16 fold (Figure 2C). To determine whether P^{T7} contributed to the expression of episomal *LUC*, pX-βGAL was co-transfected with either P^{T7}O^{tet}Luc or O^{tet}Luc as a control lacking P^{T7} in transient transfections. The pX-βGAL plasmid lacks both P^{T7} and O^{tet}. As expected, there was no substantive change in βGAL activity, i.e., only 1.1 fold (5929±599 vs. 6318±158 units, co-transfected with O^{tet}Luc) and 1.3-fold (4341±87 vs. 5661±169 units, co-transfected with P^{T7}O^{tet}Luc) differences after 24 h doxycycline induction. In contrast, there was a 8.7- and a 2.6-fold increase in LUC expression after doxycycline induction of the constructs O^{tet}Luc and P^{T7}O^{tet}Luc, respectively (Figure 2D). The basal uninduced LUC activity of the construct with the P^{T7} was six-fold higher than that of the construct lacking the promoter (Figure 2D). These data collectively demonstrate that: 1) P^{T7} enhances, but is not necessary for, the expression of extra-chromosomal reporter genes. 2) Recombinant TETR in T7-tet cells suppresses gene expression in either the presence or the absence of a close upstream promoter. Interestingly, a much better suppression was observed in the absence of than in the presence of the P^{T7} promoter.

There was a noticeable leakiness for TERT to suppress transcription of episomal reporter genes in the absence of doxycycline (Figure 2, Table 3). One possible explanation is read-through transcription from episomal DNAs. It appears that this read-through transcription is much higher in the presence of P^{T7} promoter than in the absence of the promoter. Indeed, the *Leishmania* spp. characteristically transcribe episomal DNAs in the absence of promoters (Ramamoorthy et al., 1996). To test this hypothesis, we cloned *LUC* into a chromosome of the T7-tet cell line.

3.3. Inducible expression of intra-chromosomal LUC

The non-coding strand of the *18S*rRNA locus was chosen for 'reverse' insertion of the coding strand of exogenously added genes because nuclear run-on assays have shown that this strand is not naturally transcribed (Martinez-Calvillo et al., 2001), potentially minimizing leakiness from read-through transcription (Yan et al., 2001; Yan et al., 2002), and because this chromosomal locus is expected to have a chromatin-open configuration of transcriptionally active regions. Two tandem copies of the O^{tet} were included in constructs in an effort to further tighten the control of transcription (Figure 3A). Genomic insertions containing insert 18SLuc or 18SLucC were generated in T7-tet cells. Transfected cells were selected with hygromycin, puromycin, G418, and doxycycline to ensure no elements were lost from the genome. Southern blots demonstrated that a single copy of 18SLuc was inserted into one of the many *18S*rRNA loci in three clonal transfectants. However, the single clone resulting from 18SLucC transfection contained two copies of the insert (Figure 3B).

LUC activity was monitored in all three 18SLuc clones and the one control 18SLucC clone (Figure 3C). The parental T7-tet cell line yielded very low background in either the absence or presence of doxycycline. Furthermore, "leaky" background LUC activity in the uninduced state for the cell line harboring 18SLucC or for the three lines harboring 18SLuc (designated 18SLuc.1, 18SLuc.2, and 18SLuc.3) was 1.7-, 1.7-, 1.7-, and 1.4-fold, respectively, increased activity compared to the parental T7-tet cell line. Induction with 0.1 µg doxycycline/ml for 24

h caused an increase in LUC activity of 65.1-, 142.0-, 149.5- and 155.2-fold, respectively, compared to the same cells before induction (Figure 3C. Please note the logarithmic scale). These data indicate, first, that genes inserted in the reverse orientation at the *18SrRNA* locus under control of the tetracycline repressor-operator have low background expression in the absence of doxycycline. This is contrary to the results observed in the experiments with episomal reporters. Second, the presence of the P^{T7} in T7RNAP-expressing cells only potentiates the effect of tetracycline induction on the order of two fold. This is similar to the observations in the experiments with episomal reporters.

4. Discussion

In this study, we have established the first tetracycline-inducible (T7-tet) cell line of *L. chagasi* driven by P^{T7}. These cells stably express *T7RNAP* and *TETR* integrated into different α -*TUB* genes at the α -*TUB* locus. To demonstrate that gene expression in these cells is inducible, we assayed for expression of the reporter genes *eGFP* and *LUC*. When these genes were introduced episomally or inserted into the chromosome by homologous recombination, expression of both reporters was increased upon induction with doxycycline. However, expression of the episomal reporter genes was less efficient compared to expression of their chromosomal counterparts. For example, LUC activity increased only 16- and 3-fold after doxycycline induction in stable and transient transfectants carrying episomal plasmids, respectively. In contrast, recombinant parasites harboring coding-strand *LUC* on the non-coding strand of the *18SrRNA* locus experienced a ~150-fold increase in LUC activity upon induction with doxycycline. This potent increase in LUC activity in the transfectants bearing chromosomally-inserted reporters apparently reflects a decrease in leakiness to levels that approach those of cells that do not contain a *LUC* reporter.

Both episomally and chromosomally localized reporter genes were apparently accessible to the recombinant *TETR* and *T7RNAP* gene products, suggesting these proteins were present in the nucleus despite the fact there are no nuclear localization signals engineered into these genes. This is consistent with earlier results reported for *L. donovani* (Yan et al., 2001) and *T. cruzi* (Wen et al., 2001) in which no sequences for nuclear localization signals were included in the coding sequences of *TRNAP* and/or *TETR*. Inclusion of a nuclear localization signal upstream of the coding sequence for *T7RNAP* in *T. brucei* (Wirtz et al., 1994) or *TETR* in *Giardia lamblia* (Sun et al., 2005) augments localization to the nucleus, although there is still residual *TETR* protein in the *G. lamblia* cytosol detected by indirect immunofluorescence assay (Sun et al., 2005). During future studies with T7-tet *L. chagasi*, it may prove worthwhile to include a nuclear localization signal upstream of the *T7RNAP* and/or *TETR* to enhance control of genes inserted in a chromosomal locus.

T7RNAP has been shown to function in *T. brucei*, and genes transcribed by T7RNAP are accurately *trans*-spliced. Furthermore, P^{T7}-driven gene expression was up to five times higher than transcription mediated by endogenous *T. brucei* RNAPI (Wirtz et al., 1994). A similar result was reported for *T. cruzi* (Wen et al., 2001). It is shown herein that an adjacent promoter is not required for *TETR* to suppress transcription to some extent in *Leishmania* spp. However, compared to a promoter-less construct, P^{T7} in the promoter-containing construct accounted for approximately 2-fold of the induced reporter gene expression (Figure 2D and 3C). Collectively, these data indicate that T7RNAP is able to replace transcription by endogenous RNAPs of the trypanosomatids.

The T7-tet cell line in *T. brucei* which is comparable to the *L. chagasi* reported herein has proven invaluable for studies of gene expression. Not only can genes be overexpressed, but expression of many genes has been successfully knocked out by RNAi in a tetracycline-inducible manner (Ngo et al., 1998; Bastin et al., 2000; Bringaud et al., 2000; LaCount et al.,

2000; Durand-Dubief et al., 2003; Sheader et al., 2005). Unfortunately, attempts at RNAi have been unsuccessful in the related protozoa *T. cruzi* (DaRocha et al., 2004) and *Leishmania* spp. (Robinson and Beverley, 2003). However, several groups have found that introduction of antisense RNA can lead to functional gene knockout in the *Leishmania* spp. similar to double stranded RNAi in *T. brucei*. Examples include A2 protein (Zhang and Matlashewski, 1997, 2000), MSP (Chen et al., 2000), cathepsin B-like cysteine protease (Somanna et al., 2002), and superoxide dismutase (Ghosh et al., 2003). This tetracycline-inducible cell line may provide a valuable tool for controllably manipulating gene expression in *Leishmania* spp.

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abbreviations

RNAP, RNA polymerase
 P^{T7}, T7RNAP promoter
 bp, base pair
 TETR, tetracycline repressor
 LUC, luciferase
 βGAL, β-galactosidase
 α-TUB, α-tubulin
 RNAi, RNA interference
 O^{tet}, tetracycline operator
 IC, intercoding region
 PAC, puromycin N-acetyl transferase
 HPT, hygromycin phosphotransferase
 eGFP, enhanced green fluorescence protein
 NPT, neomycin phosphotransferase
 PCR, polymerase chain reaction
 MSP, major surface protease
 T7t, T7 RNAP terminator

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Figure 1.

T7-tet cell line of *Leishmania chagasi*. **A.** Schematic depiction of DNA constructs used to generate T7-tet cells, and insertion sites at different α -TUB genes in the *L. chagasi* α -TUB locus. Restriction enzymes are indicated by vertical lines: B: *Bam*HI; K: *Kpn*I; N: *Not*I; SI: *Sac*I; and S: *Sac*II. Coding sequences are represented by rectangles, whereas intercoding sequences (IC) including UTRs and intergenic regions, are depicted by thick lines. Arrowheads a-f represent the locations and directions of PCR primers. **B.** Southern blot showing integration of *T7RNAP* gene into the α -TUB locus. Genomic DNA from one mock transfectant (M) or one pTuT7RpolHyg transfectant (T7) was digested with *Bam*HI (lanes 1), *Sac*I (lanes 2), *Sac*II (lanes 3) or a combination of *Sac*I and *Sac*II (lanes 4). The same filter was sequentially probed with the α -TUB coding sequence, the α -TUB IC, and the *T7RNAP* sequence. DNA fragments that are unique to T7 transfectants but negative in Mock transfectants, which are recognized by the α -TUB IC and *T7RNAP* probes, are marked by arrows. **C.** Southern blot showing integration of *TETR* into a *T7RNAP*-containing cell. The T7 cells from panel B carrying *T7RNAP* were transfected with pTupactet (T7-tet) or no DNA (T7, data not shown). Genomic DNA was digested with *Kpn*I and *Not*I (lanes 1), *Bam*HI and *Not*I (lanes 2), *Sac*I (lanes 3), *Sac*II (lanes 4), or *Sac*I and *Sac*II (lane 5). The filter was probed with the *TETR* coding sequence.

No signals were detected from T7 cells (data not shown). **D.** PCR amplification showing integration of pTuT7RpolHyg and pTupactet into α -*TUB* locus. Primer locations are depicted in A. The expected sizes of PCR products are: a-b: 1,064 bp; c-d: 2,050 bp; e-d: 1,603 bp; a-f: 2,220 bp.

Figure 2.

Induction of extra-chromosomal reporter genes. **A.** Schematic representation of the plasmids carrying reporter genes. **B:** *Bam*HI restriction enzyme site. The flag represents P^{T7}. Ω represents O^{tet}. **B.** Inducible eGFP expression. The promastigotes stably transfected with P^{T7}O^{tet}eGFP were incubated with (panels a and b) or without (panels c and d) 0.1 μ g doxycycline/ml for 24 h. Live cells were photographed for GFP (panels a and c) under same exposure conditions. Panels b and d show differential-interference contrast (DIC) images of a and c, respectively. Magnification was 400 $\times$. **C-D.** Inducible *LUC* expression. The promastigotes stably transfected with P^{T7}O^{tet}Luc were incubated with (induced) or without (uninduced) 0.1 μ g doxycycline/ml for 24 h. Triplicate samples with soluble proteins ranging from 3 to 300 ng were analyzed for LUC activity (C). Alternatively, T7-tet promastigotes were

transiently transfected with both pX- β GAL and O^{tet}Luc, or both pX- β GAL and P^{T7}O^{tet}Luc as described in the section of Materials and Methods. Triplicate samples of soluble proteins in 5×10^5 and 5×10^6 cell equivalences were used for LUC and β GAL analyses, respectively (**D**). The fold increase in LUC and β GAL activity after induction is shown on the top of induced sample bars.

Figure 3.

Induction of intra-chromosomally located *LUC*. **A.** Schematic depiction of *18SrRNA* locus and the 18SLuc construct. Thick arrows represent coding sequences and their transcription direction. Thin lines represent the intercoding regions (IC). The flag represents P^{T7}. Ω represents O^{tet}. T7t is represented by long striped vertical lines. Restriction enzymes are indicated by short solid vertical lines: E: *EcoRI*; H: *HindIII*. **B.** Southern blot showing *LUC* insertion into the genome. Genomic DNAs from T7-tet, 18SLucC or 18SLuc cells were digested with *EcoRI* (Lane 1) or *EcoRI* plus *HindIII* (Lane 2). The probe was the *NPT* gene. **C.** Luciferase activity of 18SLuc and 18SLucC. Promastigotes were induced with 0.1 μg/ml doxycycline for 24 h. Soluble proteins of 4 × 10⁴ cell equivalences in triplicates were used.

Table 1

Primers used in the study.

Primer Name	Sequences
T7XbaF	gctctagagcATGAACACGATTAACATC
T7BamAscR	cgggatcccgccgcccTTACGCGAACGCGAAGTC
TubIGSacBamF	tcccccgccggacgggacccGGTACACTCGTGCCGCG
TubIGXbaR	gctctagagcGGCTGAAAAAGAAGAAAG
C-S4IRSacAscF	tcccccgccggagccgcccGTTACGACGGTCGGCGAG
C-S4IRXbaR	gctctagagcGGCTCTGCAGGCGCGGG
^c HygXbaF	gctctagagcATGAAAAAGCCTGAAGTC
HygSmaR	tcccccgccggaCTGTTCCTTTGCCCTCGG
TubIRSmaF	tcccccgccggaGGTACACTCGTGCCGCG
TubIRHindAscR	cccaagcttggggcgcccGGCTGAAAAAGAAGAAAG
^a TubCF	GGACTACGAGGAGGTTGG
^d TubNR	CACGCGTTACCGACCTGG
^b T7NR	AGCGATGTTAATCGTGTTCAT
^e TetRF	AGAAGCTAGGTGTAGAGCAG
^f PACNR	CGTGGGCTTGTACTCGGTCAT
T7pToMSP5UEcoRF	ggaattcgTAATACGACTCACTATAGGGAGATCTCCCTATCA GTGATAGCTCTCCCTCCCTCACCACCTC
ToMSP5UEcoRF	ggaattcgGAGATCTCCCTATCAGTGATAGCTCTCCCTCCCTCAC CACCTC
MSP5UClaR	ccatcgatGGCTCTGCAGGCGCGGGG
GFPClaF	ccatcgatgATGGTGAGCAAGGCGAGG
GFPKpnBamR	ggggatcccgccgcccTTACTTGTACAGCTCGTCC
LucXhoF	ccgctcgagcggATGGAAGACGCCAAAAAC
LucKpnBamR	ggggatcccgccgcccTTACAATTGGGACTTTCC
NotT18SF	gcggccgcCTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGG <i>GGTTTTTGAACCGCAAAGCGCCCC</i>
Sac18SR	ccgcggTCAATTGTGTGACAACGTGAG
NotTptOMSPF	gcggccgcTAATACGACTCACTATAGGTCCCTATCAGTGATAGA GATCCCTATCAGTGATAGAGACTCTCCCTCCCTCACCACCTC
SpeMSP5UTRR	actagtCTCTGCAGGCGCGGGG
NottOMSPF	Gcggccgc TCCCTATCAGTGATAGAGATCCCTATCAGTGATAG AGACTCTCCCTCCCTCACCACCTC
SpeLucF	actagtATGGAAGACGCCAAAAAC
HindBamLucR	aagcttgatccTCGAGTTACAATTGGACTTTCCGCC
BamtubIRF	cgggatcccgGGTACACTCGTGCCGCG
HindTubIRR	aagcttGGCTGAAAAAGAAGAAAG
HindNeoF	aagcttATGGGATCGGCCATTGAAC
XmaKpnDHFRS	cccggtaccACAGGGGGAGGGCTACGGT
Kpn18SF	ggtaccAACC CGGAAAGCGCCCC
KpnT18SR	ggtaccCTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGG <i>TTTTTGTCAATTGTGTGACAACGTGAG</i>

T7: T7 RNA polymerase; Hyg: Hygromycin phosphotransferase; 18S: 18S ribosomal RNA gene; TubIR: intercoding regions of α -TUB including 3'-UTR, intergenic region and 5'-UTR; C-S4IR: Intercoding regions between the stop codon of *MSPC* and starting codon of *MSPS4*; Luc: luciferase; TetR: tetracycline repressor; PAC: puromycin N-acetyl transferase; eGFP: enhanced green fluorescent protein. Italicized *F* and *R* in the primer names represent forward (Top) and reverse (Bottom) primers, respectively. Superscripts a-f in the primer names represent the primers used in PCR as indicated in Figure 1A. The underlined sequence represents the promoter for T7 RNA polymerase, the bold letters are designated for tetracycline operator, the italicized capital letters represent the terminator for T7 RNA polymerase.

Table 2

Plasmids used in the study.

Plasmids	Regulatory Elements [#]		Target Gene Expression			Selection markers	Reference
	P ¹⁷	O ^{1e1}	Gene	Location [*]	Expression Pattern ^{&}		
pTupactet	0	0	<i>TETR</i>	Ch	C	PAC	(Yan et al., 2002)
pTut7RpolHvg	0	0	<i>T7RNAP</i>	Ch	C	<i>HPT</i>	This study
pX-βGAL	0	0	<i>βGAL</i>	Ep	C	<i>NPT</i>	(LeBowiz et al., 1990)
P ¹⁷ O ^{1e1} Luc	1	1	<i>LUC</i>	Ep	I	<i>NPT</i>	This study
O ^{1e1} Luc	0	1	<i>LUC</i>	Ep	I	<i>NPT</i>	This study
P ¹⁷ O ^{1e1} eGFP	1	1	<i>eGFP</i>	Ep	I	<i>NPT</i>	This study
p18SLuc	1	2	<i>LUC</i>	Ch	I	<i>NPT</i>	This study
p18SLucC	0	2	<i>LUC</i>	Ch	I	<i>NPT</i>	This study

[#] The copy numbers are shown.

^{*} Ep, Episomal; Ch, Chromosomal.

[&] C, constitutive expression; I: inducible expression.

Table 3Inducible GFP expression in T₇-tet cells determined by FACS analysis.

Plasmids	Doxycycline ($\mu\text{g/ml}$)	Percentage Positive (%)	Intensity of Positive Cells (Arbitrary units)
pX	1.0	3.5	8.8
P ^{T7} O ^{tet} eGFP	0.0	91.8	20.7
	0.1	93.9	60.4
	1.0	94.8	76.4