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A doubly inducible system for RNA interference and rapid RNAi plasmid construction in *Trypanosoma brucei*

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Abstract

The most rapid method for the generation of conditional mutants in *Trypanosoma brucei* is the use of RNA interference. A single copy of the target sequence is cloned between two opposing T7 promoters bearing *tet* operators, and the resulting plasmid is integrated into the genome of cells expressing both the *tet* repressor and T7 RNA polymerase. Upon addition of tetracycline, double-stranded RNA is synthesised from the two T7 promoters. Unfortunately, repression of T7 promoter activity may sometimes be insufficient to prevent expression of toxic amounts of double-stranded RNA. We describe here cell lines in which the expression of T7 polymerase is under tetracycline control, and show that regulation of polymerase expression can modulate transcription from a constitutive T7 promoter. In addition we describe a construct containing two copies of the *tn*10 Tet repressor for easy creation of repressor-expressing trypanosomes, and an RNA interference vector which allows "TA" cloning of unmodified PCR products and blue/white selection.

Keywords: *Trypanosoma brucei*; T7 polymerase; RNA interference vector; Tet repressor

1. Introduction

The study of the functions of essential genes in *Trypanosoma brucei* has been greatly facilitated by the use of inducible gene expression. The system relies on trypanosomes that stably express the Tn10 *Tet* repressor [1]. Plasmids containing the gene or RNA that is to be inducibly expressed are designed to integrate into a region of the genome that is usually transcriptionally silent, such as the ribosomal RNA spacer. The plasmids contain one promoter which drives expression of a selectable marker, and a second, inducible promoter bearing two copies of the *tet* operator very close to the transcriptional start site [2,3]. The inducible promoter that has been used most so far is an *EP*1 promoter (from the *EP* procyclin locus).

Down-regulation of the expression of essential genes in *T. brucei* is most easily achieved via RNA interference. A double-stranded RNA containing the sequences of interest is inducibly expressed, and causes destruction of the homologous mRNA [4–8]. Double-stranded RNAs can be obtained in two ways. One option is to insert, downstream of the inducible *EP* promoter, two copies of the specific DNA sequence in opposite orientations, separated by a "stuffer" fragment which enables the plasmid to replicate stably in *E. coli* [4]. Transcription of the (target)-stuffer-(reverse-target) construct generates a stem-loop RNA, and the stem mediates RNA interference. Promoter regulation is relatively tight but several cloning steps are required and some sequence com-

Abbreviations: 3′-UTR, 3′-untranslated region; TETR, Tetracycline-sensitive repressor from transposon Tn10; VSG, Variant surface glycoprotein; EP1, EP1 procyclin; ΔALD, deleted version of aldolase 3′-untranslated region; *CAT*, chloramphenicol acetyltransferase; LUC, firefly luciferase; T7POL, T7 polymerase; TUB, tubulin

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binations resist cloning. Alternatively, the double-stranded RNAs can be synthesised from a single template which is flanked by opposing T7 promoters [5,6]. The latter approach has the advantage of requiring only one cloning step, but "background" expression from the "repressed" T7 promoter is usually higher than for the *EP* promoter [9]. In the worst case, the background dsRNA expression may be so deleterious as to prevent selection of suitable transformants.

We here describe attempts to improve all aspects of RNAi in trypanosomes: creation of repressor-expressing lines, the cloning of PCR products, and the selection of trypanosomes exhibiting tightly-regulated RNAi.

2. Methods

2.1. Plasmid construction

To test 3′-untranslated regions, the plasmid pHD 1034 [10] was used. This contains a chloramphenicol acetyltransferase (*CAT*) cassette linked to a puromycin resistance cassette (*PURO*); transcription is from the ribosomal RNA (*RRNA*) promoter. In pHD 1034 the *CAT* cassette has an actin (*ACT*) 3′-UTR flanked by *Bam* HI and *Sal* I sites. The new 3′-UTRs were amplified from cloned cDNAs [11] using specific oligonucleotides and cloned as *Bam* HI–*Sal* I fragments into similarly cut pHD1034, except for the S83′-UTR which was cloned as a blunted *Bam* HI–*Kpn* I fragment. The boundary sequences (with restriction sites, underlined) for the different 3′-UTRs were as follows: Ribosomal protein S18 (pHD 1201)–GGATCCGAAAGGCTGCCAAGAAAC. . . .. TCTTTCTTTTTACTGAAAGTCGAC; Ribosomal protein L30 (pHD 1202)–-GGATCCTAGGTGTATGCCGTCATG. . .. ATTCAAACCAGCTTAAAAGTCGAC; Ribosomal protein L28 (pHD 1203)–GGATCCAGACAGTCCGCCCAAAGC. . .. CTTTTCGTTTACATTGAGGTCGAC; Ribosomal protein S8 (pHD 1204, blunt-end cloning)–GAGTGGAGAGCCGACCTG. . .. AAACACTACGGTATCGAC; Ribosomal protein S18 (pHD 1205)–GGATCCGCGTGCTCCTCGCAGTGT. . .. CCACCCTGTTGACAGTTTGTCGAC.

The plasmids were transfected into bloodstream trypanosomes and at least 3 clones selected for *CAT* activity measurement.

RNA interference plasmids directed against tubulin, PEX2 and histone H3 were as follows. The PEX2 and XRNB primers were as specified by the Trypanofan programme [12]: PEX2: CTAggatccGCTGTATCCGTTTCGTGGAT and ACCctcgagCAACGCAAGCATCTAAACGA; XRNB: AGGAagatctgcatgcTGCCAAAGTTCGCCTCTTGG and GTgaattcgtcgacCATAATTCAATCATCTGTGCTCC. The Histone H3 primers were TCGAGGACCAAGGAAACCG and ATGCACGTTCACCGCGTAG (the 397 bp target encompasses almost the entire H3 gene) and the tubulin primers were CC*CTCGAG*CCACACCTTCATTGGCAACAACA and CG*GGATCC*CCACCTCTGCGATGGCCG (the 2027 bp target encompasses parts of the α- and β-tubulin genes).

The construction of the vector plasmids used in the remainder of the study are described in the legends to Figs. 1, 2 and 4. Reconstructed sequences are available from the authors and the sequence of p2T7^{Ti}:TAblue is on the trypanofan Web site (http://www.trypanofan.org/).

2.2. Trypanosome culture

Culture and transfection of *T. brucei* lines 427, 927 was as described [11]. In all transfections 10 μg *Not* I-linearised DNA were transfected into 10^7 cells. Selection was initiated the following day and at the same time cells were cloned by limiting dilution. Selecting drug concentrations for bloodstream forms were: hygromycin 5 μg ml^{-1}, G4182 μg ml^{-1}, phleomycin 20 μg ml^{-1} (0.2 μg ml^{-1} for maintenance), puromycin 0.2 μg ml^{-1}. Drug concentrations for procyclic forms were: hygromycin 50 μg ml^{-1}, G41815 μg ml^{-1}, phleomycin 5 μg ml^{-1}, puromycin 2 μg ml^{-1}. Tetracycline was added at 100 ng ml^{-1}.

To test growth of clones, bloodstream trypanosomes were diluted to 10^4 to 10^5 ml^{-1} every 2 days, and procyclics were diluted to 10^5 to 5×10^5 ml^{-1}. Clones were allowed to grow and the number relative to the control counted before the next dilution.

2.3. Westerns, CAT and luciferase assays

Western blots were developed with antibody to the *tet* repressor (obtained from Prof. Dominique Soldati, then at the ZMBH, as a supernatant from a previous blot) and to T7 polymerase (rabbit anti-T7 RNA polymerase antibody affinity purified and used at 1:1000, obtained from Dr. Achim Schnaufer, Seattle Biomedical Research Institute). The control was a cytosolic marker described in [13]. Detection was with ECL (Amersham, Braunschweig) as described by the manufacturer.

CAT and luciferase assays were as previously described [14]. For *CAT* assays, 2×10^6 cells were freeze-thawed in 250 μl assay buffer and 2–20 μl were assayed. Slightly different protein concentrations were adjusted using Bradford Assay. Negative controls of trypanosome homogenate (no *CAT* gene) and buffer were used for background subtraction.

3. Results

3.1. A search for 3′-untranslated regions giving high constitutive expression

Anecdotal reports from various sources suggest that the initial plasmids designed to express the tetracycline repressor, pHD 360 [1] and pHD 449 [2], do not always yield sufficient repressor when transfected into new trypanosome lines. (The lines that are in routine use in our and other laboratories fortuitously integrated two copies

of the plasmid.) To solve this problem, Wirtz et al. constructed a cell line in which the transcription of the repressor was driven by a mutated T7 promoter, in cells expressing T7 polymerase [3]. These cells function very efficiently but require the application of continuous selection pressure to maintain the two transgenes (see http://tryps.rockefeller.edu/protocol_vector1.html). More importantly, two transfection steps are required to make such cells. Trypanosomes which can be transmitted by Tsetse are usually relatively difficult to maintain as bloodstream forms in culture, and their properties are adversely affected by prolonged culture and selection of transformed lines. It would therefore be useful to have a vector which would, after a single transfection, allow reliable expression of adequate levels of *tet* repressor through the trypanosome life cycle.

The plasmid we have used so far to express the tetracycline repressor, pHD 449, contains a single *TETR* gene with a truncated aldolase 3′-untranslated region, Δ*ALD*-3′, linked to the *BLE* resistance cassette. The efficiency of gene expression in trypanosomes is strongly influenced by the sequences of the 3′-untranslated regions (3′-UTR) of the mRNAs [15]. We first searched for 3′-untranslated regions that might give better expression in both bloodstream forms and procyclic forms. We chose cDNA sequences that were represented several times in the EST databases, coding for ribosomal proteins: S18; L30; L28; S8; and 60S P1 (see methods). The 3′-UTRs were cloned downstream of a *CAT* cassette, and transfected into bloodstream and procyclic trypanosomes to make permanent cell lines. *CAT* activities were between 50% and 80% of those for the *ACT* 3′-UTR (data not shown). We therefore abandoned this approach.

3.2. A double-tet-repressor plasmid

We next made a "double-tetR plasmid", pHD 1313, containing two copies of the *tet* repressor (*TETR*) gene (Fig. 1A). One copy of *TETR* has a *VSG* 3′-UTR [16], which is very short (facilitating cloning) and gives good protein expression in bloodstream forms. The second *TETR* copy has the Δ*ALD* 3′-UTR which gives good protein expression in procyclic forms. The two genes are separated by the *BLE* resistance cassette. Selection for phleomycin resistance favours retention of both copies of *TETR*, as homologous recombination between them would eliminate the *BLE* gene. To test pHD1313, we transfected it into *T. brucei* 927 bloodstream forms, and measured the levels of repressor by Western blotting. *T. brucei* 427 cells that contain two integrated copies of plasmid pHD 449 [2], and which reliably can be used for inducible expression of toxic genes (see e.g. [17]) were used as a control. The results showed that the 927–1313 cells expressed at least as much repressor as the 427–449 cells (Fig. 1B). To see whether expression was retained in procyclic forms, the 927–1313 parasites were allowed to differentiate. The repressor levels in all three lines were similar to those in bloodstream forms (Fig. 1B).

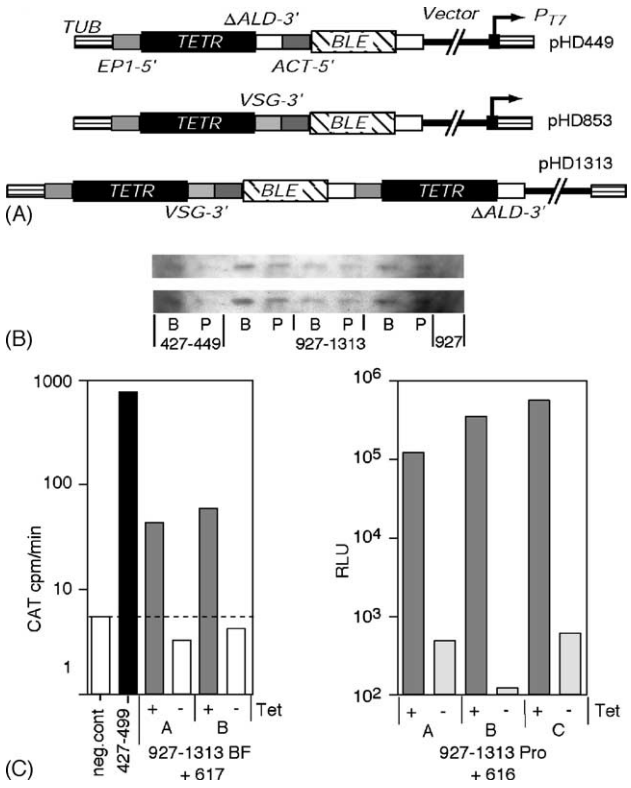

Fig. 1. (A) To construct pHD 853, the *VSG* 3′-UTR cassette (as in pHD 615) was first placed downstream of the *TETR* gene of pHD 449, replacing the truncated aldolase 3′-UTR. The T7 promoters in both pHD 449 and pHD 853 were removed by *Bgl* II digestion and re-ligation. Next, the cassette containing the *EP*15′-UTR, *TETR* gene and Δ*ALD* 3′-UTR was excised from pHD 449 and inserted in the *Sph*I site of pHD 853 [2] after end-filling, to make pHD 1313. (B) Three independent 927 bloodstream-form (B) lines containing pHD 1313 were induced to differentiate into procyclic forms (P). Expression of the *tet* repressor was assayed by Western blotting, with untransformed 927 and 427–449 [2] cells as controls. Two different exposures are shown. (C) 927–1313 bloodstream forms were transfected with pHD 617, two clones were selected and *CAT* activity was measured in the presence and absence of tetracycline. Means for at least two independent assays are shown. (D) 927–1313 procyclic forms were transfected with pHD 616, three clones were selected and luciferase activity was measured in the presence and absence of tetracycline. Means for at least two independent assays are shown.

To check repressor function, we transfected the bloodstream-form cells with a plasmid with a tetracycline-inducible *EP* promoter and *CAT* gene (pHD 617), generated permanent cell lines, and measured *CAT* activity in the presence and absence of tetracycline. The positive control line expressed *CAT* from a constitutive rRNA promoter, (plasmid pHD 499) [18]. *CAT* activity in the new *T. brucei* 927–1313–617 transformants was present in the presence of tetracycline, but was about 10 times lower than has been previously seen in 427 bloodstream forms carrying a similar plasmid (Fig. 1C). We do not know the reason for this discrepancy. *CAT* was undetectable in the absence of tetracycline. The procyclic 927–1313 cells were tested using a luciferase reporter (pHD 616) [2] (Fig. 1D). The regulation in 3 independent cell lines varied from 100- to 5000-fold

and the luciferase activity in the absence of tetracycline was similar to instrumental background. These results are similar to those obtained with the 427–449 line containing two integrated repressor copies [2].

The TREU927 strain is very useful for studies of trypanosome differentiation and genetics [11], but can grow only to relatively low densities in bloodstream-form culture [11] and may be human-infective [19]. The *T. brucei* STIB427 strain is more suitable for routine work on gene function and especially for high-throughput approaches. We therefore, next transfected pHD1313 into *T. brucei* STIB427 procyclic and bloodstream forms to generate 427–1313 double-repressor lines. The bloodstream-form cells have since been transfected independently with plasmids carrying inducible stem-loop constructs that cause RNA interference against four different essential genes and RNA interference was successful in all cases [20].

Transfection of the bloodstream and procyclic 427–1313 cells with a plasmid, pHD 514, bearing a *NEO* resistance marker and a T7 polymerase gene and targeted to the tubulin locus, yielded lines constitutively expressing both the polymerase and the repressor whose properties are described later.

From this point on, all experiments were done with STIB427-derived cell lines.

3.3. Inducible EP promoters with low maximal activity

The tetracycline-inducible T7 promoter exhibits much higher background than the inducible *EP*1 promoter [9]. We wondered whether it would be possible to lower the background from T7 promoters by making transcription of the T7 polymerase gene dependent on tetracycline addition. In all lines used so far, the T7 polymerase gene is integrated in the tubulin locus and transcribed by polymerase II. In earlier studies it was not possible to generate procyclic trypanosomes stably expressing T7 polymerase from the *EP*1 promoter [21], perhaps because the amounts of polymerase produced were toxic to the cells. Our first step was therefore to develop an inducible *EP*1 promoter with reduced activity at maximally inducing levels of tetracycline. To do this we made two different 5′deletions (Fig. 2A). One deletion (ΔEP^{Ti} promoter, pHD 1331) was expected to reduce activity by about 30%, whereas a longer deletion ($\Delta\Delta EP^{Ti}$ promoter, pHD 1330) was expected to reduce promoter activity by at least 70% [22,23]. The $\Delta\Delta EP^{Ti}$ promoter, when turned on by tetracycline, should give roughly the same activity as is normally seen by polymerase II read-through. To check inducibility of the crippled *EP*1 promoters, we used them to express *CAT* in repressor-expressing bloodstream trypanosomes (Strain STIB 427, either containing pHD449 [2] or pHD1313 (this work), using plasmids pHD 1332 and pHD1341, Fig. 2). For the $\Delta\Delta EP^{Ti}$ promoter construct, 7 out of 15 lines showed inducible expression. The mean activity after induction was 11% of the full *EP* promoter and inducibility was 10-fold. For the ΔEP^{Ti} promoter construct, 7 out of 8 clones showed an average of 45-fold inducible *CAT* with a 46% maximum (not shown). Thus the maximal activity, but not (unfortunately) the background, was affected as expected by the promoter mutation. We concluded that it should be possible to use these plasmids for inducible expression of T7 polymerase.

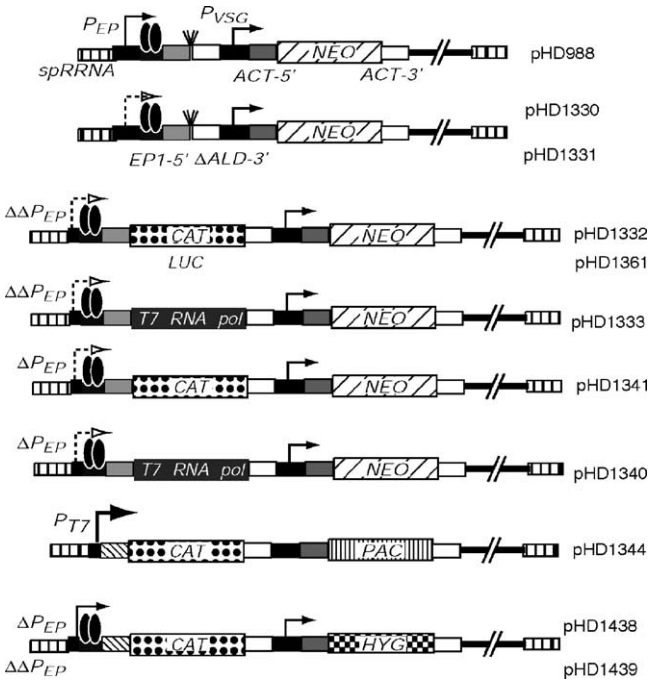

Fig. 2. Plasmid constructs for T7 polymerase expression and testing. Plasmid pHD1330 ("long" deletion) was generated by *Kpn* I and *Spe* I digestion of pHD 988 [26] followed by end-filling and re-ligation. The *CAT* (pHD 1332), *LUC* (pHD 1361) or T7 polymerase (*T7POL*) (pHD1333) genes were then inserted into the polylinker (*Apa* I site). (The T7 polymerase gene was excised from pAR 3283 [27] with *Bam* HI and *Bgl* II.) Plasmid pHD1331 ("short" deletion) was generated by *Kpn* I and *Nsi* I digestion of pHD 988 [26] followed by end-filling and re-ligation. The *CAT* (pHD 1341) or *T7POL* (pHD1340) genes were then inserted into the polylinker.

3.4. Inducible expression of T7 polymerase

We cloned the T7 polymerase (*T7POL*) gene into both deletion constructs, giving pHD 1333 ($\Delta\Delta EP^{Ti}$ promoter-*T7POL*) and pHD 1340 (ΔEP^{Ti} promoter-*T7POL*) (Fig. 2). These plasmids were then transfected into 427–1313 bloodstream and procyclic forms and several cell lines selected.

Fig. 3A shows T7 polymerase levels in all lines chosen for routine use. The bloodstream and procyclic cell lines expressing T7 polymerase constitutively from the tubulin locus (1313–514) had equivalent levels of the protein (lanes 2 and 6). T7 polymerase was barely detectable in the inducible bloodstream-form line (1313–1333) without tetracycline (lane 3) and was still low with tetracycline (lane 4). In contrast, the procyclic inducible line expressed as much T7 polymerase as the constitutive line in the presence of tetracycline (lane 8), but the "leakage" without tetracycline was also correspondingly higher (lane 7).

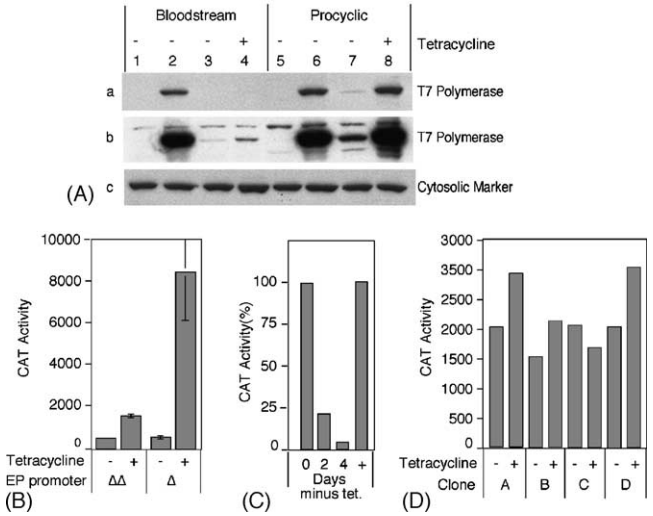

Fig. 3. (A) Western blot of lysates of 2×10^6 bloodstream or procyclic cells containing pHD 1313 (Lanes 1 and 5), pHD 1313 and pHD514 (Lanes 2 and 6); and pHD1313 and pHD1333 (Lanes 3, 4, 7, 8). The 1313–1333 cells were induced for 24 h with 100 ng/ml tetracycline. (a) The blot was probed with rabbit anti-T7 RNA polymerase antibody (1:1000). Panel b is an over-exposure of Panel a. Panel c is the cytosolic marker used as a loading control. (B) *CAT* activity from a constitutive T7 promoter in 1313–1333 and 1313–1340 bloodstream forms. Cells with the $\Delta\Delta EP^{Ti}$ or ΔEP^{Ti} promoter driving T7 polymerase expression were transfected with pHD 1344 and between 2 and 5 independent 1344 transformants for each line were selected with puromycin and tetracycline. Extracts were made before tetracycline removal (+) and 96 h after tet and puromycin removal. For each individual $\Delta\Delta EP^{Ti}$-*T7* or ΔEP^{Ti}-*T7* line, the mean of results for multiple *CAT*-expressing clones was calculated. The graph shows the mean and standard deviation for three 1313–1333 and five 1313–1340 cell lines, in cpm min^{-1} $(10^5$ cells$)^{-1}$. (C) Behaviour of the chosen 1313–1333 bloodstream-form cell line (T7 expression illustrated also in panel A). Two independent clones from this line containing pHD 1344 (T7-*CAT*) were assayed. Extracts were made before tetracycline removal (time 0), 48, and 96 h after tet and puromycin removal, and 48 h after re-addition of tetracycline (+). The level of *CAT* seen when tetracycline was added back was not affected if puromycin was added back as well (not shown). *CAT* activity is shown as the mean percent of the time 0 value. (D) *CAT* activity from a constitutive T7 promoter in 1313–1333 procyclic forms. Cells with the $\Delta\Delta EP^{Ti}$ promoter driving T7 polymerase expression (shown in panel A) were transfected with pHD 1424 (similar to 1344 but with a polylinker downstream of the *CAT* gene instead of the 3′-UTR) and selected with puromycin and tetracycline. Extracts were made before, and 3 days after, tet and puromycin removal. Results from 4 lines are shown, in cpm min^{-1} $(10^5$ $cells^{-1})$.

To see whether inducibility of the T7 polymerase could actually result in regulation of transcription from a single, integrated, constitutive T7 promoter, we transfected several bloodstream cell lines with a plasmid, pHD 1344, which has a constitutive T7 promoter driving a *CAT* gene and a puromycin resistance (*PAC*) selectable marker (P_{T7}-*CAT-PAC*) [20] (Fig. 2). We selected with puromycin in the presence of tetracycline. Both drugs were washed out to test T7 polymerase and *CAT* inducibility. Fig. 3B shows the average *CAT* activities for several cell lines derived from the bloodstream inducible T7 cells by transfection with the P_{T7}-*CAT-PAC* plasmid. For lines carrying the ΔEP^{Ti} promoter-*T7POL* plasmid, maximal *CAT* activity in the presence of tetracycline was extremely high, on 25 times that from a control containing a *CAT* gene driven by an *RRNA* promoter (not shown and [18]); maximal activity from lines carrying the $\Delta\Delta EP^{Ti}$ promoter-*T7POL* plasmid was five times lower. Backgrounds were quite variable. We chose, for future use, a line which consistently gave the lowest background and had moderate maximal *CAT* activity. The behaviour of this line is illustrated in Fig. 3C. *CAT* activity was reduced only one day after tetracycline withdrawal (not shown), and fell to a minimum of 5–10% of the induced level within 2 days (Fig. 3C).

We also transfected a similar P_{T7}-*CAT-PAC* plasmid into the chosen procyclic cells with inducible T7 polymerase. In this case, removal of tetracycline for 3 days had only a minor effect on *CAT* activity (Fig. 3D). This is consistent with the fact that the level of T7 polymerase (Fig. 3A) in the uninduced procyclic cells (lane 7) is higher than in the induced bloodstream forms (lane 4).

Addition of tetracycline had no effect at all on growth of the bloodstream and procyclic inducible T7 cell lines, confirming that the amounts of T7 polymerase expressed were not toxic.

3.5. Modification of the double-T7 vector

The $p2T7^{Ti}B$ vector [8] contains two tetracycline-inducible T7 promoters in opposite orientations. The T7 transcription should terminate at the terminators placed just upstream of each promoter (Fig. 4). The plasmid also contains a bleomycin resistance marker (*BLE*) driven by a ribosomal RNA promoter.

In our hands, the generation of clones expressing bleomycin resistance using $p2T7^{Ti}B$ requires two rounds of drug selection. This may be because bleomycin resistance is mediated by a protein which stoichiometrically binds the drug. In contrast, the hygromycin resistance cassette encodes an enzyme which inactivates the drug. We therefore, replaced the *BLE* cassette with a gene encoding *HYG*.

We also replaced the GFP cassette with a *lacZ* gene flanked by *Eam* 1105I sites. The *Eam* 1105I sites allow rapid TA-cloning of RNAi targets and a *LacZ* fragment serves as a stuffer between opposing promoters and also allows blue/white colour selection for recombinant clones. This vector, $p2T7^{Ti}$:TAblue (Fig. 4) is being successfully used in the TrypanoFAN chromosome I RNAi project (http://www.trypanofan.org/) results of which will be presented elsewhere. It is important to use *Eam* 1105I as this enzyme is very reliable.

A version of the plasmid with blasticidin resistance is also available (V.-D. Luu and Clayton, ZMBH, not shown).

3.6. Inducible T7 polymerase for RNA interference

The critical test of cells which inducibly expressed T7 RNA polymerase was to see if they could be used for RNA interference. We therefore transfected the different cell lines with a variety of potentially lethal p2T7 constructs. As a

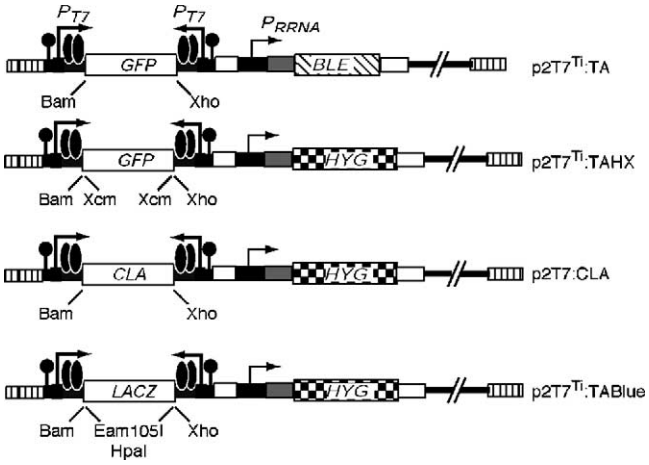

Fig. 4. Construction of a modified double-T7 vector. The primers, Phyg5 (5′-cgctagctttccacccagcgcggg-3′) and Phyg3 (5′-aaggcctctattcctttgccctcg-3′) were used to amplify an rRNA promoter-*HYG* cassette from pHD617 [2]. The PCR product and p2T7^{Ti}B were digested with *Nhe*I-*Stu*I and ligated to generate p2T7$^{Ti:HYG}$. A *Not* I site was inserted into the rDNA spacer by annealing an oligonucleotide, 5′-cgcggccg-3′, and ligating the product to *Cla* I-digested vector to generate p2T7$^{Ti:HN}$. A PCR product generated using the primers EamD1 (cccgtggtgtagataactacgatacg) and EamD2 (tagacgtcaggtggcactt) was then inserted into *Eam*1105I/T4RNAP/*Ssp* I digested p2T7$^{Ti:HN}$ to destroy an *Eam*1105I site within the *Amp* gene. Two primers, LacZ1: (5′-aattcctcgagttaacgaccttgcgtccgagatagggttgagtgt-3′) and LacZ2: (5′-agcttggatccgttaacgaccttgcgtcgcgcaacgcaattaatgtga-3′), were then used to amplify a *LacZ* fragment from pGEM5Zf(+) (Promega). The PCR product and p2T7$^{Ti:HN\Delta E}$ were digested with *Bam*HI-*Xho*I and ligated to generate p2T7^{Ti}:TAblue. A clathrin gene fragment (*CLA*) [24] was used to generate p2T7$^{Ti:ClH}$.

comparison we also included the “single marker” repressor-polymerase line which was previously described and is being used in the Trypanofan project [3]. We first targeted clathrin (Fig. 4), which is essential in *T. brucei* bloodstream forms [24]. Nineteen clones were obtained from two cuvettes (2×10^7 cells). Tetracycline was added to all nineteen clones. Eighteen out of 19 clones showed a reduction of growth which was quantitatively remarkably reproducible between lines (not shown). As usually happens in such experiments, the RNA interference effect was lost after about 5 days. If RNA interference is “leaky” in the absence of tetracycline, there is continuous selection for lines which are no longer capable of expressing the dsRNA. This results in loss of the inducible phenotype upon continuous culture or freezing. To check the stability of the clathrin RNAi lines, they were cultivated continuously for three weeks then re-induced with tetracycline. The growth phenotype was identical to that seen before.

Procyclic clones containing the p2T7 clathrin RNAi vector and expressing T7 polymerase either constitutively (1313–514) or inducibly (1313–1333), showed a 1.5- to 2-fold increase in generation time upon induction of clathrin RNAi.

Although the growth inhibition in the bloodstream-form clathrin RNAi cells was extremely reproducible and the inducibility was preserved over several weeks, the phenotype was much less dramatic than was reported for lines in which

Table 1
Efficiency of clone generation after transfection of different cell lines with RNAi constructs

Cell line	1313–1333		1313–514		Single-marker
RNAi target	B	P	B	P	B
Clathrin	19/48	11/24	ND	9/24	ND
Tubulin	1/48	23/24	0/48	0/24	0/48
Histone H3	10/48	21/24	2/48	3/24	9/48
PEX 2	4/48	5/24	0/48	1/24	6/48
XRNB	12/48	10/24	3/48	20/24	2/48
p2T7TA vector only	ND	ND	2/48	ND	ND

Five different bloodstream-form (B) or procyclic-form (P) trypanosomes expressing the *tet* repressor and T7 polymerase were transfected with p2T7 plasmids designed for down-regulation of five different essential genes. The next day selection was applied and the culture was distributed into 24-well plates. The number of wells giving stable trypanosome growth is given relative to the total number of wells. The cell lines are shown in the top two lines and the genes targeted by RNAi are listed in the left-hand column. The “single-marker” bloodstream-form cells contain pHD 328 and pLEW114hyg5′, and were made and described by Wirtz et al. [3]. ND: not done.

T7 polymerase is constitutively expressed. Cells lacking clathrin have a dramatically enlarged flagellar pocket (“big-eye” phenotype) and in later stages show dispersed nuclei [24]. In our induced cultures, “big-eye” cells were present, but relatively rare, whereas cells with dispersed nuclei were more common (not shown). The rather minor effects seen in the bloodstream trypanosomes could be a consequence of the low T7 polymerase levels in this $\Delta\Delta EP^{Ti}$ promoter-*T7POL* line; it may in the future be worthwhile to re-investigate bloodstream ΔEP^{Ti} promoter-*T7POL* cells.

The results from several other transfections are shown in Tables 1 and 2. The “tubulin” RNAi plasmid is p2T7 containing a 2 kb fragment from the tubulin locus. This fragment is considerably longer than those previously described as giving effective inducible RNAi, and is therefore probably more efficient in targeting the tubulin mRNA. The plasmid had repeatedly given no clones at all in the single-marker bloodstream-form line. We were able to get clones only from the cells with inducible T7 polymerase. Upon addition of tetracycline, the typical “FAT” phenotype of cells with RNAi against tubulin was seen, with growth retardation. With dsRNA targeting the histone H3 RNA, it was again easiest to get clones with the cells inducibly expressing T7 polymerase (Table 1); tetracycline addition resulted in growth retardation in both types of procyclic trypanosome line (Table 2). Cell lines showing growth effects also had a reduction in the targeted RNA (Table 2 and data not shown). It was notable that the transfection efficiency of the bloodstream-form cells expressing T7 polymerase was very low even when empty vector was used.

Induction of stem-loop-type RNAi against the glycosomal membrane protein PEX2 was rapidly lethal in bloodstream and procyclic forms [25], although the procyclic forms survived for longer than the bloodstream forms. We therefore tested this also with a p2T7 approach. Cells containing a p2T7 RNAi construct targeting PEX2 were generated with low efficiency, but none showed any growth effect upon RNAi

Table 2
Characterisation of selected cell lines from the experiment described in Table 1

RNAi target	Phenotype	1313–1333		1313–514		Stem-loop	
		B	P	B	P	B	P
Clathrin	Growth	Slow (18/19)	Slow (8/8)		Slow (6/6)	–	–
α- and β-tubulin	Growth	Died without tet	Slow, fat (3/3)	–		–	–
	RNA		Decreased (2/2)				
Histone H3	Growth	No effect (3/3)	Slow (3/3)	Slow (2/2)	Slow (3/3)	–	–
	RNA		Decreased (2/2)		Decreased (2/2)		
	Growth	No effect (2/2)					
PEX 2	Growth	No effect (3/3)	No effect (3/3)	–	No effect (1)	Lethal	Lethal 3–4 days [25]
XRNB	Growth	No effect (3/3)	Slow (3/3)		No effect (3/3)		Lethal 3–4 days

For selected lines (single-well populations) derived from the 1313–514 and 1313–1333 parents, RNA interference was induced by tetracycline addition. Cells were grown for up to a week, with dilution as required, and the effects of tetracycline observed by cell counting. "Slow" means that there was slow growth–a 1.5- to 2-fold increase in division time after 48 h. Northern blots were also made after 48 h for a subset of lines: "RNA decreased" means that the amount of the targeted mRNA was visibly decreased on the blot relative to the un-induced control. For each phenotype we show: (number of lines showing the phenotype/total number of lines tested). The "fat" phenotype is also noted for parasites with tubulin RNAi [4]. For the lines with RNAi targeting *XRNB* or *PEX2*, the result from RNAi using a stem-loop is also shown. The *PEX2* results are published [25]; *XRNB* stem-loop result: Freese, Li, Clayton et al., unpublished.

induction. Similar results were obtained by targeting an exoribonuclease, XRNB, which is essential in procyclic cells (Freese, Li, Estevez and Clayton, unpublished) except that a slowing of growth was seen in bloodstream forms. Clearly in these cases the stem-loop construct was more effective.

4. Discussion

We have made a number of modifications to RNAi vector and host strains in an attempt to increase the efficiency with which RNA interference screens can be conducted in trypanosomes.

To facilitate rapid cloning of large numbers of PCR products during RNAi screens, a vector bearing two T7 promoters was modified by adding restriction sites that allow TA cloning of unmodified *Taq* polymerase PCR products, and by including a *lacZ* gene for blue/white colony screening. In addition, we replaced the original *BLE* marker with a *HYG* marker as this should facilitate selection of recombinant trypanosomes. We find that this vector enables RNAi lines to be generated with much greater ease and efficiency than previous versions.

The p2T7^{Ti}:TAblue vector can be used with cells constitutively expressing T7 polymerase and the *tet* repressor which are not hygromycin-resistant. From the quantitative comparisons shown in the tables, it is clear that the new bloodstream forms described in this paper show no advantages at all over the "single marker" line described by Wirtz et al. [3]; and the latter has the additional advantage of containing a single selectable marker. In contrast, the procyclic forms with inducible T7 polymerase (1313–1333) are ideal for use with the new p2T7blue vector: cell lines showing inducible phenotypes are obtained much more easily than with alternative lines. Nevertheless, our failure to generate lines with p2T7 showing RNAi against PEX2 and XRNB demonstrate that the use of opposing T7 promoters will not always give reliable results: if an RNAi cell line is to be studied in detail, frozen, or re-transfected with additional plasmids, it will probably usually be necessary to use a stem-loop for RNAi.

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