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# A tightly regulated inducible expression system for conditional gene knock-outs and dominant-negative genetics in *Trypanosoma brucei*

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**Abstract**

First-generation inducible expression vectors for *Trypanosoma brucei* utilized a single tetracycline-responsive promoter to drive expression of an experimental gene, in tandem with a drug-resistance marker gene to select for integration (Wirtz E, Clayton CE. Science 1995; 268:1179–1183). Because drug resistance and experimental gene expression both depended upon the activity of the regulated promoter, this approach could not be used for inducible expression of toxic products. We have now developed a dual-promoter approach, for expressing highly toxic products and generating conditional gene knock-outs, using back-to-back constitutive T7 and tetracycline-responsive PARP promoters to drive expression of the selectable marker and test gene, respectively. Transformants are readily obtained with these vectors in the absence of tetracycline, in bloodstream or procyclic *T. brucei* cell lines co-expressing T7 RNA polymerase and Tet repressor, and consistently show tetracycline-responsive expression through a $10^3$–$10^4$-fold range. Uninduced background expression of a luciferase reporter averages no more than one molecule per cell, enabling dominant-negative approaches relying upon inducible expression of toxic products. This tight regulation also permits the production of functional gene knock-outs through regulated expression of an experimental gene in a null-mutant background. 

*Keywords:* Trypanosoma brucei; Tet-repressor; T7 RNA polymerase; Expression vector; Gene regulation

*Abbreviations:* BLE, product of the *Streptoalloteichus hindustanus BLE* gene; ES, variant surface glycoprotein gene expression site; *HYG*, hygromycin phosphotransferase gene; *NEO*, neomycin phosphotransferase gene; PARP, procyclic acidic repetitive protein; Pol I, RNA polymerase I; Pol II, RNA polymerase II; *RNP1*, Pol I large-subunit gene; T7RNAP, bacteriophage T7 RNA polymerase; TetR, Tet repressor; Tc, tetracycline; *TUB*, αβ-tubulin locus; UTR, untranslated region; VSG, variant surface glycoprotein.

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## 1. Introduction

Inducible expression of transgenes in *Trypanosoma brucei*, mediated by the prokaryotic Tet repressor (TetR) [1] binding to a pair of start-site-proximal *tet* operator sites inserted into the strong

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*T. brucei* PARP promoter, has been described previously [2,3]. Activity of this promoter was responsive to tetracycline (Tc) concentration, in *T. brucei* expressing TetR. Expression of genes linked to this Tc-responsive PARP promoter could be controlled with nanomolar amounts of Tc, over a 10 000-fold range [2].

In the prototypal inducible expression vectors, the Tc-responsive PARP promoter drives expression of both the test gene and a tandemly linked selectable marker [2]. This single-promoter design made drug resistance and test gene expression dependent upon the activity of the regulated promoter, requiring that cell lines be established under conditions of Tc-induction. This precluded their use for regulated expression of toxic products, limiting the utility of this system for dominant-negative genetic approaches. Subsequent attempts to construct non-inducing targeting vectors, which could be used for regulated expression of toxic products, relied upon insertion of a second unregulated *T. brucei* promoter, upstream of the resistance gene, to drive its expression constitutively [3]. The resulting dual-promoter vectors typically yielded 35–700-fold lower regulation factors, largely as a result of higher background expression than was observed in the first-generation single-promoter constructs, for reasons that were not then apparent ([4] and E. Wirtz, unpublished observations). This compressed regulatory range and high background present a problem for conditional gene knock-out strategies based upon regulated expression of an ectopic copy of a gene in a null-mutant background. There is also a potential problem for any dominant-negative approach based upon expression of a highly toxic product.

We have redesigned the inducible vector and the genetic background of the host cell line in ways that tighten regulation and permit conditional knockouts and inducible expression of toxic products. The test gene, with a regulatable promoter, and the marker gene, with a constitutive promoter, are positioned back-to-back. We also avoided driving marker gene expression with trypanosome polymerases, which could be subject to endogenous regulation, by creating a genetic background of T7 RNA polymerase (T7RNAP) expression. This background was also exploited to yield predictable levels of repressor protein, independent of endogenous transcription levels at the integration site. TetR expression was originally established using a promoter-less cassette targeted to the *TUB* locus [2]. Optimal expression of the repressor depended upon fortuituous integration of multiple copies of the TetR cassette and polycistronic read-through transcription, by RNA polymerase II (Pol II), at this strongly transcribed locus. We have now linked TetR transcription to a bacteriophage T7 promoter, crippled by a -10 point mutation that reduces its activity by 90% [5]. This cassette yields appropriate TetR levels for tight repression, with complete preservation of inducibility, whether targeted to loci that are transcribed weakly (RNA polymerase I large subunit; *RNP1*) or strongly (*TUB*). In frequently encountered schemes, relying upon regulated ectopic expression of an endogenous gene in a null mutant background, this confers the distinct advantage, from the standpoint of conservation of selectable markers, of making it possible to use the TetR cassette to disrupt one allele of the gene of interest.

Transformants obtained with these vectors, in the absence of Tc, in cell lines expressing T7RNAP and TetR, consistently give comparable regulation factors and even lower background expression than those generated using the original single-promoter vectors. The dual-promoter arrangement permits modulation of test gene expression by Tc during drug selection. Selection in the absence of Tc allows the introduction of toxic genes. Selection under inducing conditions permits the generation of conditional gene knockouts, where it is desirable to approximate the natural expression level of the targeted gene, while disrupting its native alleles.

## 2. Materials and methods

### 2.1. Construction of inducible expression vectors

The inducible expression vectors are illustrated in Fig. 1A. Sequence files of all constructs are available upon request, or can be downloaded

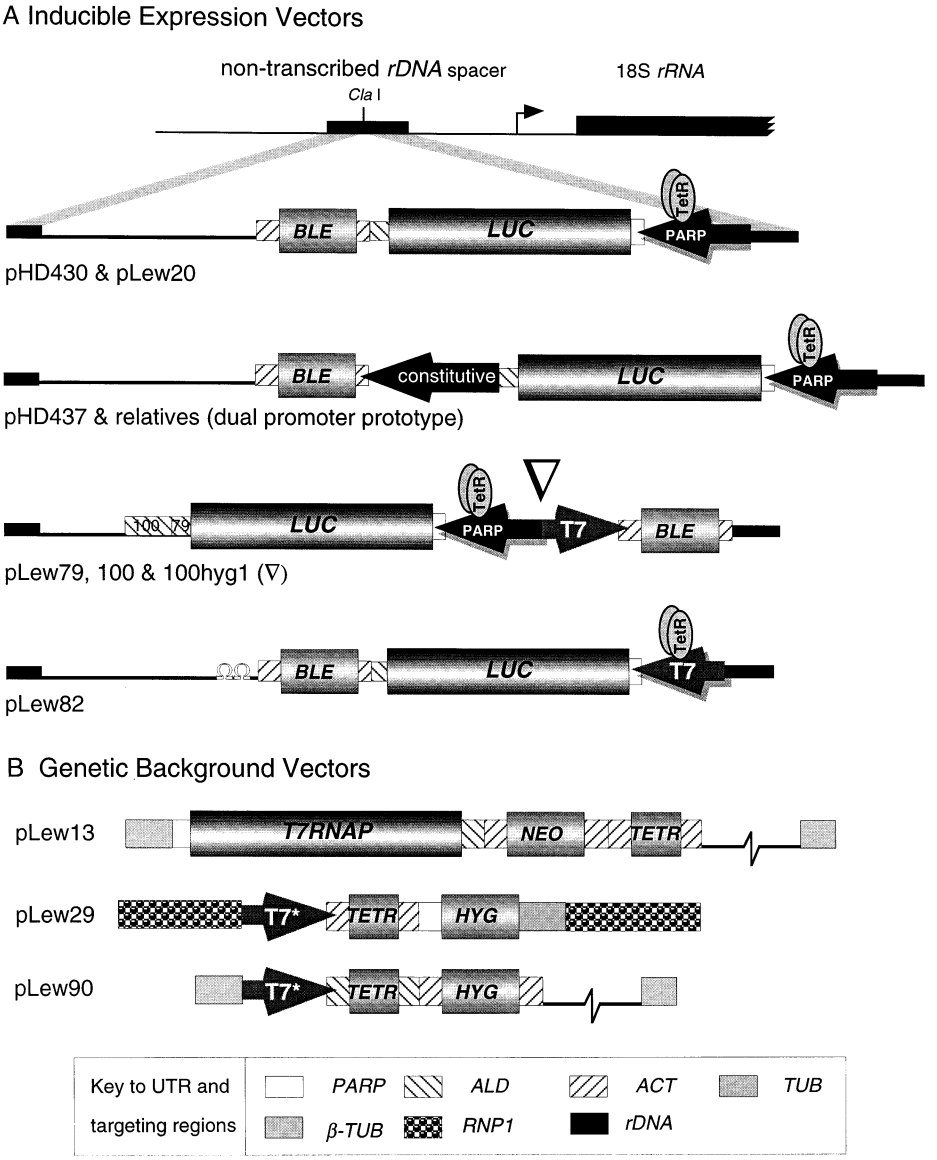


Fig. 1. Strategy for establishing inducible high-level expression. (A) Anatomy and targeting of inducible expression vectors, designed for integration into the non-transcribed spacer of the *rDNA* locus. pHD430 and pHD437 have been described previously [2,3]. pLew79 and pLew100 differ only in the length of the 3′ *ALD* region that flanks the luciferase gene. pLew100hyg1 has a *HYG* gene flanked by transposon sequences inserted between the PARP and T7 promoters, indicated by the white triangle, which is irrelevant to the experiments described here and does not affect the activity of the promoters or the luciferase reporter. In pLew82, the unregulated T7-directed transcription is terminated downstream of the *ACT*-derived 3′ UTR by tandem T7 terminators. (B) Vectors used to establish T7RNAP and TetR-expressing cell lines. T7* indicates a mutant (10% wild-type activity) T7 promoter.

from www.rockefeller.edu/labheads/cross/cross-lab.html. The starting point for construction of pLew20 was pLew2, a derivative of the published inducible vector pHD430 [2], in which the polylinker of pHD308 [6] replaces the *LUC* gene. The pGEM-derived T7 promoter of pLew2 was

deleted by *Pvu* II-*Eco* RI digestion and self-ligation, yielding pLew5. The luciferase coding sequence was then subcloned, as a *Hin* dIII-*Bam* HI fragment from pHD430, into the polylinker of pLew5, yielding pLew20. The starting point for pLew79 was pLew26 [5], a pLew5 derivative containing a T7 promoter-bearing complementary oligonucleotide cassette inserted in the filled *Asp* 718 site. pLew26 was digested with *Srf* I and *Stu* I and religated to release the *BLE* gene and flanking *ACT*-derived UTRs, yielding pLew77. A BLE expression cassette, consisting of the *Srf* I-*Nhe* I fragment of pLew5, was then cloned in the *Age* I site of pLew77, after filling of 5′ overhangs, yielding pLew78. The luciferase coding region was again subcloned as a *Hin* dIII-*Bam* HI fragment from pHD430, in the linker of pLew78, yielding pLew79. pLew100 was made by exchanging the truncated *ALD* 3′ UTR of pLew79 for the longer *ALD* intergenic region of pHD103 [7], using *Bam* HI and backbone site *Pvu* I. pLew100hyg1 contains a *HYG* insertion, which is not relevant to the present work, in the *Kpn* I site of pLew100.

### 2.2. Construction of targeting vectors for expressing prokaryotic regulatory factors

The targeting vectors are illustrated in Fig. 1B. To build pLew1, the *HYG* cassette of pHD328 [6] was released by *Spe* I-*Msc* I digestion and replaced with a *Hin* dIII-*Bam* HI fragment of pHD360, bearing the TetR open reading frame, after filling 5′ overhangs. pLew13 was made from pLew1 by insertion of a *Sma* I-*Stu* I fragment from pHD102 [7], bearing a *NEO* expression cassette, into the *Sma* I site. pLew29 was made by exchange of the *Age* I fragment of pTHrnp1KO (an *RNP1* replacement vector containing a TetR-*HYG* cassette (E. Wirtz and K.M. Gottesdiener, unpublished)) for annealed oligonucleotide pair, EW14 and EW15 [5], effectively inserting a T7 promoter, bearing a -10 point mutation, into the 5′ targeting segment of this knock-out vector. In the parental knock-out vector, this segment consisted of a *Bam*HI-*Tth* 111I fragment spanning the upstream region (from $-74$ to $-1134$ relative to the first ATG codon) of *RNP1*. The 3′ segment of the parental vector and derivative vector, pLew29, consists of a *Sal* I-*Fsp* I fragment from 5052–5985. pLew90 was made by inserting the same oligonucleotide pair, comprising a T7 promoter with a -10 mutation, into the *Mlu* I site of pLew88, a derivative of pHD360 in which the pGEM-derived T7 promoter had been deleted by *Pvu* II digestion and self-ligation, after filling of 5′ overhangs. The *HYG* cassette of pLew90 was replaced with a *NEO* cassette from pHD102 by exchange of *Sma* I-*Stu* I fragments to give pLew114. The *Hin* dIII-*Eco* RI fragment of *HYG* in p72hyg72 [8] was blunt-end cloned into pLew114 cut with *Xho* I and *Afl* III, replacing the *TUB* targeting segment and generating pLew114hyg5′.

### 2.3. Generation of transgenic procyclic-form cell lines

The procyclic host cell line 29–13, co-expressing T7RNAP and TetR, was derived from wild-type *T. brucei* 427 procyclic forms by sequential stable transfection with pLew13 and pLew29 (Fig. 1B), essentially as described previously [2]. Procyclic-form *T. brucei* were harvested from a log phase culture ($4–8 \times 10^6$ ml$^{-1}$ in SDM-79 [9]) with a gentle spin ($< 700 \times g$) at 4°C, washed once in ice-cold Zimmerman post-fusion medium, resuspended in $1–3 \times 10^7$ aliquots in 0.5 ml Zimmerman post-fusion medium and transfected with 10 μg of *Not* I-linearized pLew13 DNA per cuvette,using one pulse from a BTX electroporator set for peak discharge at 1.6 kV and resistance-timing mode R2 (24Ω). Time constants were typically about 0.3 msec. Cell suspensions and cuvettes were kept on ice. After electroporation,cells were transferred to 9.5 ml of SDM-79 medium and incubated overnight. After 24 h, transformants were cloned by serial dilution in conditioned SDM-79 containing 15 μg ml$^{-1}$ G418.

G418-resistant clones were evaluated for T7RNAP expression using transient reporter plasmids pHD54 or pHD216, described previously [6], where luciferase is driven by a T7 promoter. All clones examined expressed levels of T7RNAP leading to luciferase activities equivalent to or exceeding those produced by the PARP promoter (transient reporters pHD77 or pHD422) [6] in the same cell line. One of these clones served as the parental cell line for subsequent transfection with

pLew29. Approximately 2.5 μg of a gel-purified *Not* I-*Ssp* I cassette of pLew29 was used to transfect cell line 13 clone 3. Five clones, resistant to 25μg $ml^{-1}$ hygromycin, were assayed for TetR expression by transient assays using pLew20 in the presence or absence of Tc as previously described [2]. All clones showed more than 100-fold repression. Clone 6, which routinely showed more than 500-fold repression of the Tc-responsive PARP promoter in transient assays, served as the procyclic host cell line for subsequent transfections. This host cell line is designated 29-13. pLew29 was simultaneously used to transform double-resistant cell line 20-13, derived by stable transfection of cell line 13, clone 3 by *Not* I-linearized pLew20. Hygromycin-resistant clones were checked for Tc-dependent expression of luciferase. Line 29-20-13, clone 3 was used for further analysis.

Additional cell lines were generated using similar transfection protocols. After electroporation, cells were transferred to 9.5 ml of SDM-79 medium with appropriate inhibitors, according to host background, and incubated overnight. Selection was applied the following day: 15 μg $ml^{-1}$ G418 for pLew13; 2.5 μg $ml^{-1}$ phleomycin plus 100 ng $ml^{-1}$ Tc for pLew20; 2.5 μg $ml^{-1}$ phleomycin without Tc for pLew79 and derivatives; 25 μg $ml^{-1}$ hygromycin for pLew29; 50 μg $ml^{-1}$ hygromycin for pLew90 and derivatives. At this time, cells were serially diluted in 24-well microtiter plates, using conditioned medium, to speed the cloning process and ensure that clones are independent.

### 2.4. Generation of transgenic bloodstream-form cell lines

To generate a bloodstream-form host cell line co-expressing T7RNAP and TetR, wild-type 427 cells expressing VSG 221 were harvested from a log-phase culture with a gentle room-temperature spin ($< 700 \times g$ in a tabletop centrifuge), washed once in Cytomix [10] at 37°C, and resuspended at $2 \times 10^7$ $ml^{-1}$. Aliquots of $1 \times 10^7$ cells were electroporated with 10 μg of *Not* I-linearized pLew13, as for the procyclic form, except that the cuvettes were kept at room temperature. Following electroporation, cells were transferred to 9.5 ml of HMI-9 and survivors, typically 10% or less, were counted. 24-well microtiter plates were seeded with $3–10 \times 10^4$ living cells per well, in a volume of 0.5–1 ml. Selection was applied the following day by adding the same volume of HMI-9 containing 5 μg $ml^{-1}$ of G418, for a final concentration of 2.5 μg $ml^{-1}$ of G418. Using pLew82 (see Fig. 1A and [5]), a small number of G418-resistant clones were tested for T7RNAP expression in a transient transfection. The clone yielding the highest level of T7-mediated luciferase activity was transfected with *Not* I-linearized pLew90 and transformants were selected with 5 μg $ml^{-1}$ hygromycin. One double-resistant clone, 90-13 clone 4, showing greater than 1000-fold regulation of luciferase expression from an integrated pLew20 reporter (selected with 2.5 μg $ml^{-1}$ phleomycin plus 100 ng $ml^{-1}$ Tc, in addition to 5 μg $ml^{-1}$ hygromycin and 2.5 μg $ml^{-1}$ G418, to ensure retention of the T7RNAP and TetR transgenes) served as the original bloodstream-form host cell line. For transformation of this cell line and a single-marker host (see below) with pLew79 or pLew100 and derivatives, selection was carried out as for pLew20 but without Tc.

To generate a bloodstream-form host cell line co-expressing TetR and T7RNAP, with the expenditure of a single selectable marker, wild-type 427 cells expressing VSG 221 were transfected with *Not* I-linearized pHD328 [6]. Hygromycin-resistant transformants were checked for T7RNAP texpression in transient assays using pLew82 [5]. One clone, in which transient luciferase activities surpassed the levels achieved, in parallel transfec-ions, by the PARP and ES promoters of pLew20 and pLew92 (an analog of pLew20 containing an ES promoter in place of the PARP promoter), respectively, was stably transfected with *Aat* II-digested pLew114hyg5′. Neomycin-resistant clones were checked for hygromycin sensitivity. In roughly 25% of the clones, hygromycin resistance had been lost. Three such clones were chosen at random for stable transfection with pLew82 or pLew100hyg1 reporters, to assess regulation of Tc-responsive promoters.

### 2.5. Luciferase assays of stable cell lines

For luciferase assays from stable expressers, a Promega luciferase assay system was used. $2 \times 10^6$ cells were gently pelleted and lysed with 100 μl of the Promega lysis buffer on ice. Cell debris was removed by a 3–5 min microfuge spin at 4°C. 5 μl of the cleared supernatant was added to 45 μl of Promega assay buffer containing luciferin, at room temperature, and samples were read immediately in a Turner model TD-20e luminometer. All stable cell lines were assayed in duplicate multiple times and results from representative experiments are shown.

## 3. Results

### 3.1. Altering genetic background of a host cell line for optimal and reproducible TetR expression via the T7RNAP

To conserve drug-resistance genes, when generating conditional null mutants, it became necessary to use the TetR cassette to disrupt one allele of the gene of interest. Tight repression could not be achieved by integrating the TetR gene into weakly transcribed loci like *RNP1* [11] (E. Wirtz, unpublished observations). Indeed, single-copy integrations into *TUB*, as with pHD360 [2] or pLew13 (Fig. 1B) led to repression factors of less than 13-fold. This became an increasing concern as we and other investigators sought to regulate ectopic copies of different genes in null-mutant backgrounds. The need for reproducible levels of TetR expression, independent of local transcriptional read-through levels at the integration site, made it necessary to incoporate a promoter into the design of the TetR cassette. The lack of trypanosome Pol II promoters and the desire not to use *T. brucei* Pol I promoters, which are very strong, subject to endogenous regulation, and appeared to generate toxic levels of TetR (Wirtz, unpublished observations), led us to explore the use of bacteriophage promoters.

We have recently shown that T7RNAP can efficiently transcribe *T. brucei* chromatin templates, driving very high-level gene expression in bloodstream and procyclic forms, and that T7 promoters of different intrinsic strength could be generated, using point mutations [5]. Based on the original tightly regulating cell line, bearing 2–4 copies of the TetR cassette in the *TUB* locus [2], and our observations that a single-copy was inadequate, transcription equivalent to about twice Pol II read-through appeared to be necessary for adequate TetR expression. A bacteriophage T7 promoter, reduced to 10% of wild-type activity by a -10 A to T point mutation, was shown to drive luciferase expression, in trypanosomes expressing T7RNAP, at approximately twice the level of Pol II read-through at the *TUB* locus [5].

The 10% T7 promoter was inserted upstream of the 5′ UTR of the TetR expression cassette, in a vector designed for targeted disruption of *RNP1* (Fig. 1B; pLew29). This construct was transfected into a procyclic cell line in which T7RNAP expression had been established by transfection with pLew13 (Fig. 1B). Hygromycin-resistant 29-13 transformants were checked for regulation, by transient transfection with the luciferase reporter construct pLew20 (Fig. 1A). pLew29 was simultaneously introduced into cell line 20-13 (T7RNAP-expressing cell line 13 containing the stably integrated luciferase reporter pLew20), in order to monitor regulation of the Tc-responsive PARP promoter in a chromosomal context. Compared to using a promoter-less TetR cassette in the *RNP1* locus, in which regulation factors did not exceed 10-fold with transient or stably integrated reporters, pLew29 gave 200–800-fold regulation in transient assays (cell line 29-13) and $>1000$-fold regulation in stably transformed (29-20-13) clones (Fig. 2). Background expression remained low and Tc-inducibility remained high during prolonged subculture. Under the conditions of this experiment, luciferase returns to baseline levels within 5 days of removing Tc from the medium. Although there appears to be some variation in the uninduced levels, it should be noted that these levels represent about 1 molecule of luciferase per cell (see later). Driving TetR expression with a T7 promoter furnished predictable and effective levels of TetR, regardless of the low level of endogenous transcription of the targeted locus. It resulted in dramatic increase in repression rates,

without loss of inducibility or development of toxicity. This also confirmed that the low regulation in some earlier cell lines (13, for example) was a result of insufficient TetR expression.

### 3.2. Regulation and kinetics of Tc-induced gene expression for a back-to-back dual-promoter vector

Previous efforts to develop dual-promoter vectors, for expression of toxic products, relied upon insertion of ES or *rRNA* promoters into pHD430 [2], to yield pHD437 and derivatives (Fig. 1A) [3]. This approach resulted in a marked loss of regulation and led to speculation that interaction between two strong endogenous promoters [12] interfered with repression [3]. However, inserting a heterologous promoter in the same position, or using two tandem heterologous promoters (E. Wirtz, unpublished observations), led to the same dramatic reduction in repression rate for the upstream Tc-responsive promoter. This implicated the tandem arrangement rather than an interaction between endogenous promoters as the basis for the loss of repression. We therefore tried a back-to-back configuration of the constitutive and inducible promoters (Fig. 1A). The new vector, pLew79, was tested in procyclic forms with the 29-13 background. Transformants were selected in the absence of Tc. The regulation afforded by this vector is shown in Fig. 3, for four independent 5 randomly selected clones. Examples of unregulated clones probably represent cases where reporter cassettes failed to integrate into the transcriptionally silent target.

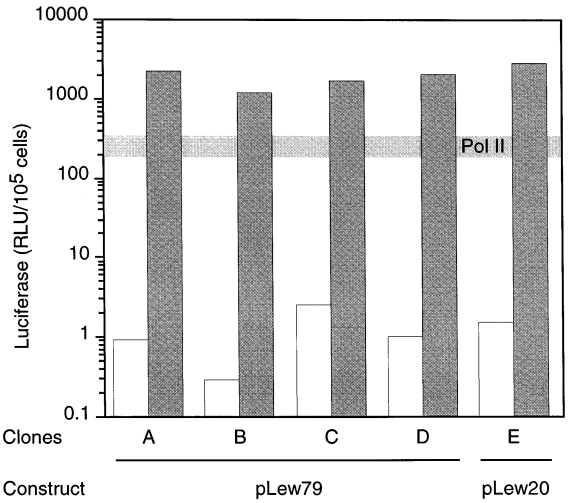


Fig. 3. Regulation of dual-promoter reporter constructs by Tc in procyclic-form T7RNAP/TetR background. pLew79-transfected 29-13 host cells were selected with phleomycin in the absence of Tc. Luciferase activities are shown for the first four randomly selected independent clones (A–D), cultured for 3 days in the presence or absence of Tc. Data for one 29-13 cell line, stably transformed with pLew20, the prototypical single-promoter construct [2], are shown for comparison (E). The horizontal gray bar labeled Pol II indicates the range of luciferase activity obtained by Pol II read-through transcription in the *TUB* array.

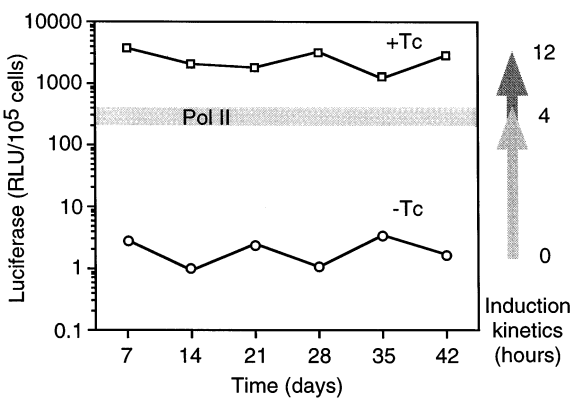


Fig. 2. Regulation of the Tc-sensitive PARP promoter in 29-13 procyclic *T. brucei*. The 20-29-13 triple-transformed cell line was continuously cultivated in SDM-79 in the absence of Tc and inhibitor selection for 6 weeks. At intervals, Tc was added and luciferase measured. The side panel indicates the luciferase levels induced 4 h and 12 h after Tc addition. The horizontal gray bar labeled Pol II indicates the range of luciferase activity obtained by Pol II read-through transcription in the *TUB* array (for details see [5]). The background RLU for wild-type cells is 0.

Induction kinetics for the Tc-responsive PARP promoter in the context of the new vector were investigated. Fig. 4 shows results for representative procyclic clones derived from stable transfection of 29-13 with either pLew79 or, for comparison, the single-promoter reporter pLew20. Induction kinetics for the two vectors were similar. Luciferase increased about 1000-fold in 12 h and levels did not increase after 48 h, at high Tc concentrations. Expression levels equiva-

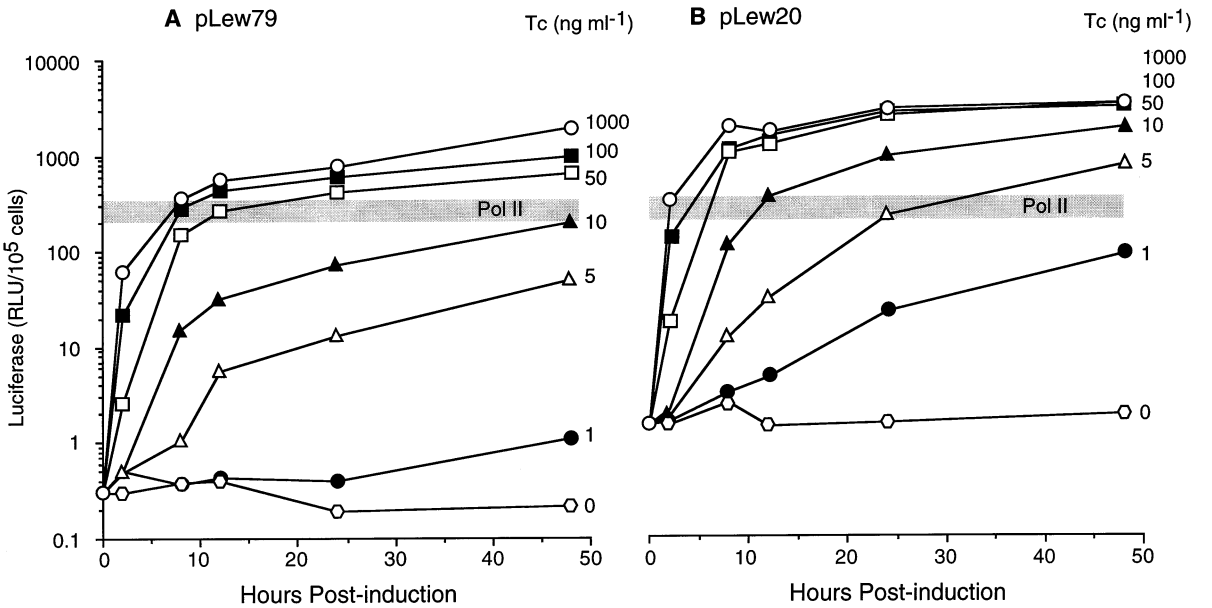


Fig. 4. Kinetics of luciferase induction by Tc concentrations ranging from 1 ng ml$^{-1}$ to 1 μg ml$^{-1}$, in transformed procyclic *T. brucei*. (A) One of the four dual-promoter pLew79 clones from Fig. 3. (B) Single-promoter pLew20. The horizontal gray bar labeled Pol II indicates the range of luciferase activity obtained by Pol II read-through transcription in the *TUB* array.

lent to Pol II transcription were reached in about 4 h. Steady-state expression in the Pol II range was induced by 10 ng ml$^{-1}$ Tc for pLew79 and about 3 ng ml$^{-1}$ Tc for pLew20.

A bloodstream-form host cell line co-expressing TetR and T7RNAP was constructed by sequential transfection of wild-type *T. brucei* with pLew13 and pLew90 (Fig. 1B). In this bloodstream-form line (90-13), harboring essentially the same expression cassettes as 29-13 procyclic host, but with the TetR cassette targeted to *TUB* instead of *RNP1*, luciferase expression from stably integrated pLew79 (cell line 79-90-13) was strictly Tc dependent (Fig. 5). Regulation factors were again in the range of $10^4$-fold. Thus regulation was very tight, but the maximum expression level that can be attained in bloodstream forms is constrained by the developmental regulation of the PARP promoter [13]. pLew79 also contains a short *ALD* 3′ UTR that is unfavorable (relative to the longer one used in pLew100) in the bloodstream form [14], giving about 6-fold lower expression (compare this with pLew100hyg1 in Fig. 8A). The initial overshoot, after adding Tc, is often seen. Transcriptional repression by TetR is expected to be fast, but will depend upon the rate at which Tc is eliminated from the cells, and may be atypically

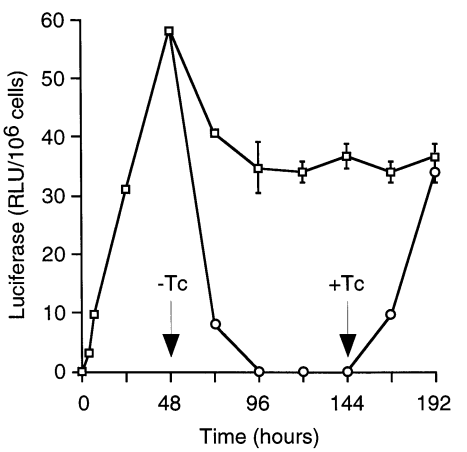


Fig. 5. Kinetics of luciferase induction and decay in bloodstream-form 79-90-13 *T. brucei* following addition and removal of Tc. Cells were initially grown in the presence of 1 μg ml$^{-1}$ Tc. After 48 h, the culture was split and half was transferred to medium lacking Tc. After 144 h, 1 μg ml$^{-1}$ Tc was added back to this culture.

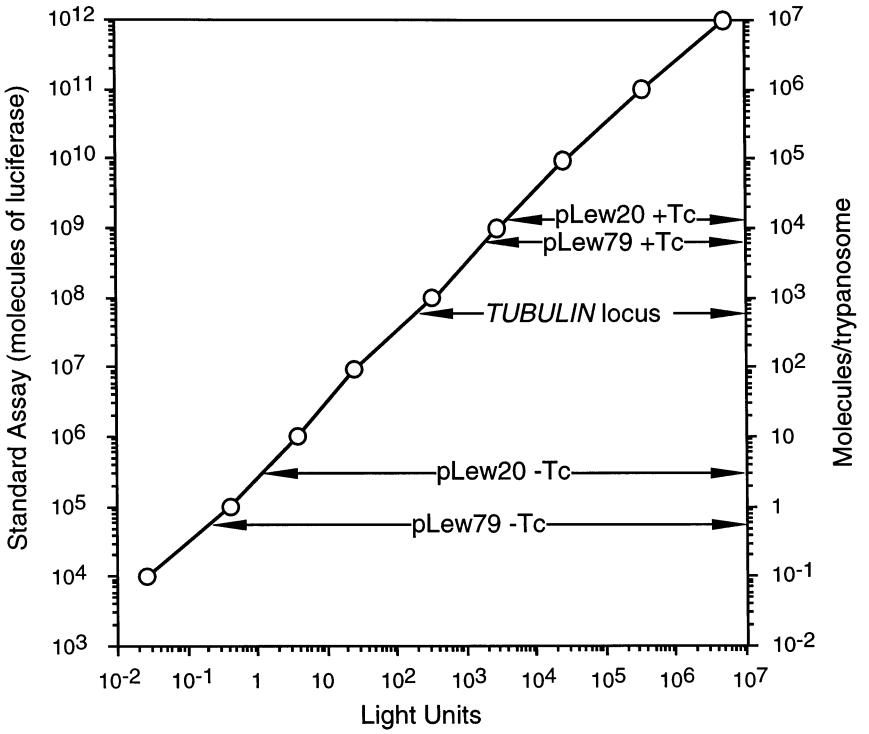


Fig. 6. Relation of luciferase light units to molecules per cell. Standard curves were constructed by adding known amounts of luciferase to extracts of 90-13 bloodstream-form (data not shown) or 29-13 procyclic-form *T. brucei* and measuring luciferase as described in Materials and Methods. Standard deviations lie within the range embraced by the point markers (○). Expression levels achieved +/− Tc by (A) dual-promoter pLew79 and (B) single-promoter pLew20, in molecules per cell, are indicated. The expression level of luciferase achieved by read-through transcription in *TUB*, using the construct pHD421 [2] in procyclic forms is also indicated.

slow in this experiment, where a large excess (1 μg $ml^{-1}$) of Tc was used. The rate at which the reporter disappears will depend upon several additional factors.

### *3.3. Background expression in the absence of Tc in terms of molecules per cell*

To anticipate whether functional knock-out of genes can be achieved, using a conditional-null-mutant strategy, we needed to quantify background expression levels for these vectors in terms of molecules per cell. We generated standard curves by adding known amounts of luciferase to extracts of 90-13 bloodstream or 29-13 procyclic cell lines in order to translate luciferase light units into molecules per cell (Fig. 6). Although there could be factors that we are unaware of, such as cell-specific posttranslational modifications, leading to differences between the specific activity of luciferase synthesized in *T. brucei* and that added to the cell extracts, this was the best approach that we could conceive for estimating molecules per cell. The results confirmed that regulation of the dual-promoter (pLew79 and derivatives) constructs is actually better than the original (pHD430 [2] and pLew20) single-promoter system, with respect to background expression in the absence of Tc. The 'off' state corresponded to less than one molecule of luciferase per cell and the fully induced level to around $10^4$ molecules per cell. The activity of standard amounts of luciferase was reduced about two-fold in bloodstream-form extracts (data not shown). This may contribute to the lower levels of luciferase in bloodstream forms. We attribute the majority of

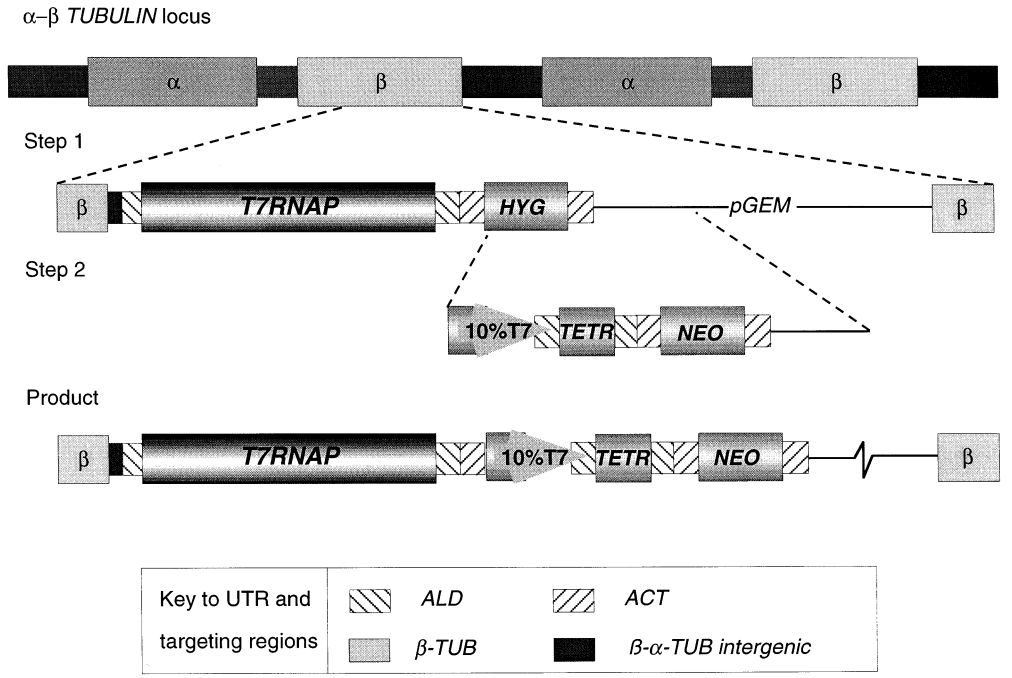


Fig. 7. Targeting strategy for constructing a cell line expressing T7RNAP and TetR with the final expenditure of only one selectable marker. In the example shown, a T7RNAP cassette was first targeted to *TUB* in bloodstream-form *T. brucei*. In the second step, after cloning the T7RNAP cell line, the TetR cassette was targeted to the *HYG* and plasmid sequences inserted in the first step, eliminating hygromycin resistance, but not the entire *HYG* gene.

this effect to thermolability of luciferase [15,16] at 37°C relative to 27°C.

### 3.4. Co-expression of T7RNAP and TetR using a single-marker

Establishment of T7RNAP/TetR co-expression has been streamlined for the expenditure of a single selectable marker. The previously described strategy for establishing T7RNAP/TetR co-expression, by sequential transfection with pLew13 and pLew29, relied upon Pol II read-through transcription of the T7RNAP gene, in the *TUB* locus. pLew13 (Fig. 1B) represented our original attempt to establish T7RNAP/TetR co-expression simultaneously, using a single-marker. This appears to produce adequate amounts of T7RNAP but, as previously discussed, this read-through transcription of TetR in pLew13 was inadequate for tight repression. Efforts to insert the 10% T7 promoter into pLew13 were complicated by difficulties propagating the plasmid in *Escherichia coli*, where circular transcription brought T7RNAP under the control of its own promoter, a situation that is toxic to *E. coli* [17]. These difficulties could not be overcome by inserting T7 terminators downstream of T7RNAP or by cloning in a background of T7 lysozyme expression. We therefore decided to link the T7RNAP and TetR expression cassettes directly in *T. brucei*, by a two-step transfection approach (Fig. 7). The second transfection eliminates the marker used in the first step. In principle, the same strategy can be used to target other loci. Regulation in these bloodstream-form single-marker clones remained tight, as illustrated in Fig. 8A, for luciferase expressed via the PARP promoter (pLew100hyg1 expression cassette). Introduction of pLew82, in which *LUC* is driven by a full-strength T7 promoter, showed that sufficient TetR was produced to adequately regulate this strong promoter (Fig. 8B). Targeting strategies

that placed T7RNAP downstream from its own promoter (even the 10% promoter) were not compatible with stable expression of the polymerase in *T. brucei* (S. Leal, unpublished observations), and appeared to result in mutations in either the T7RNAP or the T7 promoter.

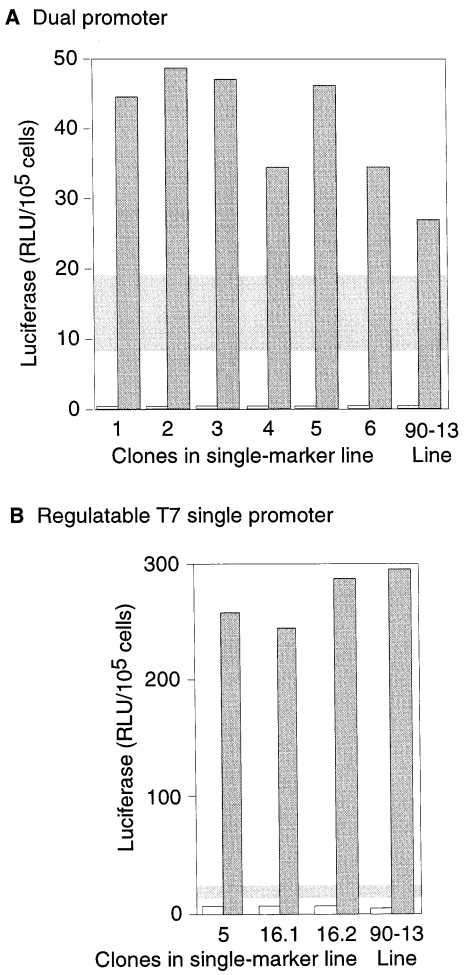


Fig. 8. Regulation of luciferase activity in the single-marker T7RNAP/TetR bloodstream-form cell line. (A) Activities are shown for 6 independent clones, in which pLew100hyg1 was transfected into the single-marker cell line described in Fig. 7. Clones were selected in the absence of Tc then grown for 2 days with or without Tc. Regulation of pLew100hyg1 in the bloodstream-form 90-13 cell line, in which T7RNAP and 10% T7-driven TetR cassettes analogous to those shown in Fig. 1, but both targeted independently to *TUB*, is shown for comparison. (B) Regulation of luciferase driven by a full-strength T7 promoter of pLew82 in the single-marker cell line. Results are shown for three independent clones and, for comparison, for pLew82 in the 90-13 line. The horizontal gray bars indicate the range of luciferase activity obtained by Pol II read-through transcription in the *TUB* array in bloodstream forms.

Single-marker bloodstream-form cells were subsequently differentiated to procyclic forms, which could be cultured indefinitely in DTM [18]. However, during several months of continuous cultivation, the transfection efficiency of these derived procyclic forms remained similar to that of bloodstream forms, and much lower than procyclic forms that have been maintained in continuous culture for several years. Tc regulation was lower and variable. We are unable to explain this behavior, which makes subsequent transformation of this host cell line extremely difficult, when low transfection efficiency is combined with the difficulties of cloning procyclic forms. One feature of procyclic forms that has been reported to vary, during culture, is the relative abundance of the two distinct forms of the surface coat protein PARP (also known as Procyclin) [19,20]. Differences in surface coats could affect membrane stability and influence killing by electroporation, to which bloodstream forms were previously thought to be more susceptible [21]. We are therefore attempting to establish a single-marker procyclic cell line by directly transfecting procyclic cells that have a long history of continuous culture.

## 4. Discussion

Producing adequate levels of the TetR was the critical factor in obtaining tight regulation of Tc-inducible promoters. Providing the TetR cassette with a promoter made expression levels predictable and independent of endogenous transcription at the target locus. This permits integration at loci where read-through transcription is very low, making it more useful for gene knock-outs, where using this cassette to disrupt one allele helps to conserve marker genes. Generation of a conditional knock-out, using the TetR and T7RNAP cassettes of pLew90 and pLew13, will be described elsewhere (Ochatt, C et al., manuscript in preparation).

Earlier dual-promoter vectors for inducible expression of toxic products [3] were re-designed,

leading to significantly tighter regulation and less variability among clones. The previous tandem arrangement, of the Tc-responsive promoter driving test gene expression and the constitutive promoter driving selectable marker expression, was abandoned for a back-to-back arrangement, in which constitutive T7 and Tc-responsive PARP promoters drive selectable marker and test gene expression, respectively, making marker expression independent of Tc. In a genetic background of TetR/T7RNAP co-expression, transformants were readily obtained in the absence of Tc in both procyclic and bloodstream-form host cell lines. These consistently showed regulation factors in the $10^3$–$10^4$-fold range and very low background expression. Steady-state expression levels can be modulated by adjusting Tc concentration. In the absence of inducer, all clones examined expressed less than 1 molecule of luciferase per cell. This is more than an order of magnitude below backgrounds observed with the previous tandem promoter arrangement [3] (E. Wirtz, unpublished observations). Regulation appears even tighter in bloodstream forms, but the maximum induced level of luciferase is lower, being constrained by the developmental down-regulation of the PARP promoter.

Compared to the original single-promoter vectors, maximum expression levels are somewhat lower and induction kinetics slower (about half as fast as for pHD430 or pLew20). The presence of the T7 promoter appears to debilitate the PARP promoter and facilitate its repression. Propagation of torsional changes from the initiation region of the constitutive T7 promoter into the oppositely oriented Tc-responsive PARP promoter seems to favor formation of repressor-operator complexes at the expense of competent preinitiation complexes, in the competition for binding sites in the promoter. On the other hand, a downstream promoter in the same orientation as the Tc-responsive PARP promoter, as in the tandem arrangement of pHD437, seems to enhance the capacity of the operator-bearing promoter for transcription relative to repression [3]. The basis for this reduced repression has been the subject of speculation invoking an interaction between two Pol I promoters, akin to that described for the PARP and VSG expression site promoters [12]. However, a similar effect was seen when vectors were engineered with two tandem T7 promoters (E. Wirtz, unpublished observations). In any case, repression is tighter for the new back-to-back promoter vector than for either the single or dual-promoter vector prototypes.

Ultimately, the maximum expression levels that can be achieved under induction, and the background expression that will remain under repression, will vary from one gene to another, as different coding sequences and 3′ untranslated regions confer different stability characteristics on RNA and protein products. Depending upon the extent to which signals mediating endogenous regulation are embedded in the coding sequence, or any other sequence included in the expression cassette, a gene product may or may not be amenable to over-expression or rapid depletion.

To address the issue of down regulation of the PARP promoter in bloodstream forms, we have engineered vectors based on an inducible T7 promoter, which are described elsewhere [5]. T7RNAP-driven expression in procyclic forms is 2 to 5-fold higher than that driven by the PARP promoter. In both stages, T7 and *rRNA* promoters have similar activities, more than 20-fold above expression levels that can be attained with the PARP promoter in bloodstream forms. While the T7 system was designed for high-level expression, it is not appropriate for regulated expression of highly toxic products, because of transcriptional leakage. In both procyclic and bloodstream-form cultures, T7-driven expression levels under repression amounted to about 20% of Pol II read-through [5]. While this will not present a problem for many dominant-negative approaches, it could preclude establishment of cell lines bearing genes encoding highly toxic products. For these sorts of applications, the dual-promoter vector will be required. The appropriate vector and host combination for a particular application will depend upon the maximum level of expression desired, the developmental stage(s) in which analyses are to be carried out, and the level of background expression that can be tolerated.

The best strategy for achieving a conditional knock-out will depend upon genomic organization.

Modular design of the T7RNAP and TetR expression cassettes, pLew13 and pLew90, facilitates their use for replacement of any gene or locus of interest, by adding appropriate targeting sequences. For single-copy genes or multi-copy genes that are linked in tandem arrays, it should be possible to establish regulated ectopic expression in a null mutant background. For multi-copy genes present at dispersed loci, dominant-negative approaches based on toxic RNA or protein products must be relied upon. In some cases, even with single-copy genes, a dominant-negative approach will be valuable in identifying possible functions of the targeted gene.

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## References

[1] Hillen W, Berens C. Mechanisms underlying expression of TN10 -encoded tetracycline resistance. Annu Rev Microbiol 1994;48:345–69.
[2] Wirtz E, Clayton C. Inducible gene expression in trypanosomes mediated by a prokaryotic repressor. Science 1995;268:1179–83.
[3] Biebinger S, Wirtz LE, Lorenz P, Clayton C. Vectors for inducible expression of toxic gene products in bloodstream and procyclic *Trypanosoma brucei*. Mol Biochem Parasitol 1997;85:99–112.
[4] Biebinger S, Rettenmaier S, Flaspohler J, Hartmann C, Penadiaz J, Wirtz LE, Hotz HR, Barry JD, Clayton C. The PARP promoter of *Trypanosoma brucei* is developmentally regulated in a chromosomal context. Nucl Acids Res 1996;24:1202–11.
[5] Wirtz E, Hoek M, Cross GAM. Regulated processive transcription of chromatin by T7 RNA polymerase in *Trypanosoma brucei*. Nucl Acids Res 1998;26:4626–34.
[6] Wirtz E, Hartmann C, Clayton C. Gene expression mediated by bacteriophage T3 and T7 RNA polymerases in transgenic trypanosomes. Nucl Acids Res 1994;22:3887–94.
[7] Häusler T, Stierhof Y-D, Wirtz E, Clayton C. Import of a DHFR hybrid protein into glycosomes in vivo is not inhibited by the folate-analogue aminopterin. J Cell Biol 1996;132:311–24.
[8] Cooper R, de Ribeiro Jesus A, Cross GAM. Deletion of an immunodominant *Trypanosoma cruzi* surface glycoprotein disrupts flagellum-cell adhesion. J Cell Biol 1993;122:149–56.
[9] Brun R, Schonenberger M. Cultivation and in vitro cloning of procyclic culture forms of *Trypanosoma brucei* in a semi-defined medium. Acta Trop 1979;36:289–92.
[10] van den Hoff MJB, Moorman AFM, Lamers WH. Electroporation in 'intracellular' buffer increases cell survival. Nucl Acids Res 1992;20:2902.
[11] Rudenko G, Blundell PA, Taylor MC, Kieft R, Borst P. VSG gene expression site control in insect form *Trypanosoma brucei*. EMBO J 1994;13:5470–82.
[12] Urmenyi TP, van der Ploeg LHT. PARP promoter-mediated activation of a VSG expression site promoter in insect form *Trypanosoma brucei*. Nucl Acids Res 1995;23:1010–8.
[13] Pays E, Coquelet H, Tebabi P, Pays A, Jefferies D, Steinert M, Koenig E, Williams RO, Roditi I. *Trypanosoma brucei*: constitutive activity of the VSG and procyclin gene promoters. EMBO J 1990;9:3145–51.
[14] Hug M, Carruthers V, Sherman D, Hartmann C, Cross GAM, Clayton CE. A possible role for the 3′-untranslated region in developmental regulation in *Trypanosoma brucei*. Mol Biochem Parasitol 1993;61:87–96.
[15] Nguyen VT, Morange M, Bensaude O. Protein denaturation during heat shock and related stress. *Escherichia coli* beta-galactosidase and *Photinus pyralis* luciferase inactivation in mouse cells. J Biol Chem 1989;264:10487–92.
[16] Schroder H, Langer T, Hartl FU, Bukau B. DnaK, DnaJ and GrpE form a cellular chaperone machinery capable of repairing heat-induced protein damage. EMBO J 1993;12:4137–44.
[17] Dubendorff JW, Studier FW. Creation of a T7 autogene. Cloning and expression of the gene for bacteriophage T7 RNA polymerase under control of its cognate promoter. J Mol Biol 1991;219:61–8.
[16] Schroder H, Langer T, Hartl FU, Bukau B. DnaK, DnaJ and GrpE form a cellular chaperone machinery capable of repairing heat-induced protein damage. EMBO J 1993;12:4137–44.
[17] Dubendorff JW, Studier FW. Creation of a T7 autogene. Cloning and expression of the gene for bacteriophage T7 RNA polymerase under control of its cognate promoter. J Mol Biol 1991;219:61–8.
[18] Overath P, Czichos J, Haas C. The effect of citrate/cis-aconitate on oxidative metabolism during transformation of *Trypanosoma brucei*. Eur J Biochem 1986;160:175–82.
[19] Treumann A, Zitzmann N, Hulsmeier A, Prescott AR, Almond A, Sheehan J, Ferguson MAJ. Structural characterisation of two forms of procyclic acidic repetitive protein expressed by procyclic forms of *Trypanosoma brucei*. J Mol Biol 1997;269:529–47.
[20] Butikofer P, Ruepp S, Boschung M, Roditi I. GPEET procyclin is the major surface protein of procyclic culture forms of *Trypanosoma brucei brucei* strain 427. Biochem J 1997;326:415–23.
[21] Carruthers VB, van der Ploeg LHT, Cross GAM. DNA-mediated transformation of bloodstream-form *Trypanosoma brucei*. Nucl Acids Res 1993;21:2537–8.