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Short communication

Targeting of a tetracycline-inducible expression system to the transcriptionally silent minichromosomes of *Trypanosoma brucei*

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1. Introduction

Tetracycline-regulated ectopic gene expression [1,2] has come to form the backbone of transgenic manipulation of *Trypanosoma brucei*. It has been used for the expression of toxic gene products [3] for conditional knock outs [4] and in RNA interference [5,6]. The system has been used to induce expression levels higher even than those of the most active endogenous loci and has been shown to regulate protein levels 10^4-fold in the most favourable cases [2].

In spite of many successes, tet-responsive gene expression in trypanosomes has also encountered limitations. Gene regulation over the huge range seen in the initial report has been difficult to recapture—most notably, lower levels of regulation have been observed in the cell lines now most commonly used in transgenic studies. Secondly, the levels of non-induced and, to a lesser extent, induced expression appear to be acutely clone-specific. For example, Biebinger et al. [7] described vectors for inducible expression of toxic gene products; the vector producing the clone with greatest regulation (700-fold at the protein level) also showed 6-fold or lower regulation in five of ten clones analysed. Finally, in inducible RNAi knock-down studies (in which endogenous mRNAs are specifically ablated upon induction of ectopic double-stranded RNA production), some groups report bleed-through of characteristics of RNAi-induction in non-induced cells. Others have noted a failure to obtain stable transformants when inducible RNAi is directed against certain genes. Both these phenomena are thought be the result of ‘leaky’ (i.e. undesirably high) expression of the regulated genes in the absence of induction.

Here we report that loci on the endogenous minichromosomes of *T. brucei* may be targeted for transgenics as a means to improve the regulation of a tet-inducible construct.

2. Ectopic expression from minichromosomes

In Trypanosomatidae, choice of integration site for inducible vectors is hampered by an unusual gene organisation and the promiscuous nature of RNA polymerase II (pol II). The majority of genes of these organisms are transcribed processively by pol II from very long polycistrons found on the megabase-sized chromosomes (MBCs). To facilitate this, the polymerase seems able to initiate transcription from very many sites in the genome, with an apparently low level of DNA sequence specificity. As a result—for tight down-regulation of non-induced cells—integration must avoid not only active genes, but also chromatic regions, which may initiate pol II transcription, or be party to its read-through.

The standard target sequence for inducible vectors in *T. brucei* has come to be the region upstream of the promoter for the 18S rRNA gene [8]—a site referred to here as the rDNA spacer. This MBC region has been shown to be quiescent by nuclear run-on analysis [9] and

Abbreviations: BSF, bloodstream-form; GFP, green fluorescent protein; IC, intermediate chromosome; MBC, megabase-sized chromosome; MC, minichromosome; PCF, procyclic-form; PCR, polymerase chain reaction; pol I, RNA polymerase I; pol II, RNA polymerase II; tet, tetracycline; rDNA, ribosomal RNA genes; *TUB*, α/β-tubulin gene locus; VSG, variable surface glycoprotein.

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is targeted such that inducible genes run in the opposite direction to that of neighbouring endogenous genes [2]. Even with this level of theoretical protection, in some instances the level of regulation of constructs targeted to this site is low.

There are, of course, other potential integration sites within the *T. brucei* genome which might reasonably be expected to give good down-regulation of inducible vectors. Prominent amongst these are loci on the minichromosomes (MCs): the population of 100 or so small linear chromosomes maintained by the organism as a reservoir for VSG genes (review [10]). Unlike the gene-dense MBCs, MCs are 30–150 kbp in size and consist predominantly of a repeat of ~177 bp [11] which is highly enriched in MCs and also found on the intermediate chromosomes (ICs; 200–700 kbp). This sequence is believed to form a large repetitive central core to MCs which is flanked by non-repetitive subtelomeric regions containing VSG genes (*VSG*s) and capped by telomeres [12,13]—a model we have confirmed by mapping several MCs (data not shown).

MCs are potentially good sites for inducible vector integration for several reasons. Most importantly, they are transcriptionally silent—no active genes are present along the entire chromosome. It follows that insertion of ectopic DNAs to MCs will not disrupt endogenous gene expression (the same can not be said of insertion into MBCs). MCs contain a large amount of repetitive DNA which is likely to be heterochromatic in nature. It is anticipated that a more closed chromatin structure might promote better regulation of integrated vectors. Finally, endogenous MCs are stable with respect to mitosis [14,15].

We have targeted integrative vectors to five sequences—the commonly used rDNA spacer and α/β-tubulin gene array locus (*TUB*), both found on MBCs, and three minichromosomal sites: the 177 bp repeat, *VSG*-G4 and *VSG*-S8. Both minichromosomal *VSG*s are subtelomeric and can be found on different MCs [15].

Fig. 1A shows the structure of the inducible vectors used in this study. The plasmids are essentially derivatives of pHD430 described by Wirtz and Clayton [2], though a number of changes have been made. They were designed for expression of GFP and a hygromycin drug-resistance marker under the action of a single tet-responsive promoter. In PCF cells this was a derivative of an endogenous procyclin EP gene promoter, recruiting endogenous pol I [2]. In BSF cells a tet-inducible derivative of a T7 RNA polymerase promoter was used [16]. A further vector was made lacking either promoter (not shown). This vector was not inducible and was targeted to the *TUB* locus, relying on pol II read-through for expression. We can supply further sequence data and full lineage for these vectors on request.

We used these vectors to stably transform *T. brucei* strain 427-derived cell lines expressing tetracycline repressor (PCF) or tetracycline repressor and T7 RNA polymerase (BSF)—'PTP cell line' (details from authors) or the 'single-marker cell line' described by Wirtz et al. [17], respectively. Transformation was carried out as in [16]. Positive transformants were selected by growth for 14 days in the presence of induction (1 μg ml^{-1} tetracycline PCF; 1 μg ml^{-1} doxycycline BSF) and selective drug (20 μg ml^{-1} hygromycin B PCF; 10 μg ml^{-1} hygromycin B BSF), as well as drugs for maintenance of background (2 μg ml^{-1} phleomycin PCF; 2 μg ml^{-1} G418 BSF). For analysis, independent clones were grown with background selection for a further 7 days with or without induction.

Each targeting sequence tested yielded positive transformants in both PCF and BSF cells. Southern hybridisation to whole chromosomes separated by pulsed-field gel electrophoresis demonstrated that *VSG*-targeted constructs were integrated into MCs in all clones analysed ($n = 14$). Constructs directed toward the 177 bp repeat were integrated into MCs (~80%, 30 of 38) or ICs (~15%, six of 38)—consistent with the distribution of the repeat in the genome—with the remainder integrated into MBCs or as episomes (~3%, one of 38, in both cases).

These data illustrate that loci on normally silent MCs can support ectopic expression in both PCF and BSF lifecycle stages.

3. Regulation of inducible expression from different genomic sites

To assess the transcriptional activity of vectors integrated into different genomic locations, we employed the technique of quantitative real-time reverse-transcription PCR. Total mRNA was harvested from actively dividing trypanosomes (High-Pure RNA isolation kit, Roche) and cDNA created by reverse-transcription (Omniscript kit, Qiagen) using an oligo-(dT)$_{15}$ primer. Relative amounts of *GFP* cDNA were then assessed by quantitative PCR employing a 5′-nuclease assay, in which the extent of amplification is assessed by release of a fluorophore from a labelled oligonucleotide probe (Taqman probes, Applied Biosystems). *GFP* mRNA levels were normalised against a separate assessment of constitutive γ-tubulin mRNA to control for loading, RNA quality and RT efficiency. No-reverse-transcription and no-template controls were included to check for genomic DNA contamination and mis-priming. We estimate this system to be capable of quantitation across more than six orders of magnitude of mRNA concentration in trypanosomes.

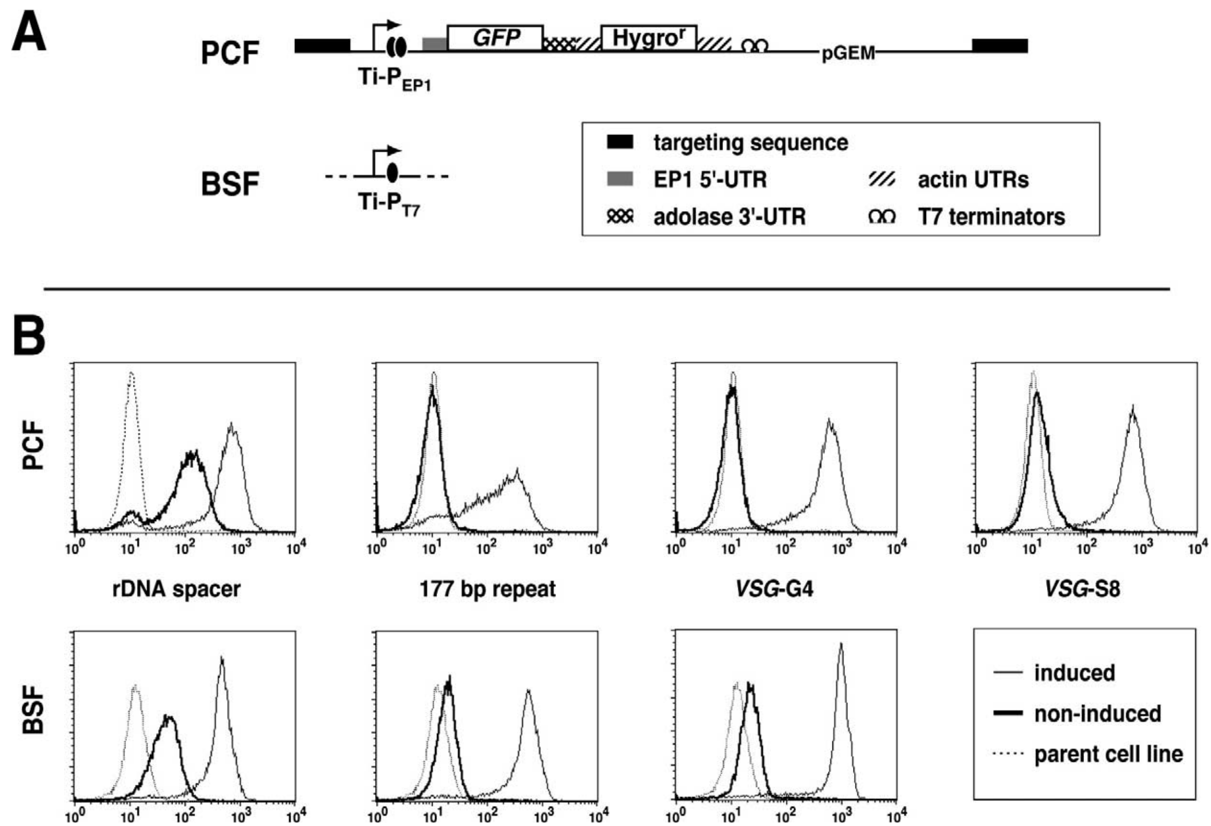

Fig. 1. Inducible expression from different sites in the *T. brucei* genome. (A) Anatomy of inducible expression vectors. Targeting sequences are: rDNA spacer, 177 bp repeat, and the minichromosomal *VSG*s G4 and S8. Vectors for expression in procyclic-form (PCF) and bloodstream-form (BSF) cells differ only in the inducible promoters used: tetracycline-inducible procyclin EP1 promoter (Ti-P_{EP1}) for PCF and tetracycline-inducible T7 polymerase promoter (Ti-P_{T7}) for BSF. (B) Flow cytometric analysis of GFP levels for stably transformed cell lines grown for 7 days in the presence (thin solid line) or absence (thick solid line) of induction (1 μg ml^{-1} tetracycline PCF; 1 μg ml^{-1} doxycycline BSF). Untransformed cells (dotted line) are also shown.

We investigated levels of expression in three independent randomly-selected clones from each of the stable transformations of PCF cells targeting the sites: rDNA spacer, 177 bp repeat, *VSG*-G4 and *VSG*-S8. Three clones expressing GFP by read-through at the *TUB* locus were also included in the analysis for PCF cells. For BSF cells, only rDNA spacer, 177 bp repeat and *VSG*-G4 were targeted.

The effect of different integration targets on the relative cellular concentration of *GFP* mRNA is shown in Fig. 2A. In PCF cells containing tet-inducible EP1 promoter, fully-induced constructs targeted to the rDNA spacer resulted in *GFP* mRNA levels about 5-fold above those produced by pol II transcription from the *TUB* locus—consistent with the results of other studies. These mRNA levels were matched in induced clones in which minichromosomal *VSG*s had been targeted. The concentration of *GFP* mRNA in induced 177 bp-tagged clones was somewhat lower—at or just above the level of pol II read-through—indicative, perhaps, of the more condensed nature of chromatin at these loci.

To our surprise, the regulation of constructs targeted to the rDNA spacer was very poor (on average 7-fold). Two out of three clones failed to be down-regulated even to the level of the *TUB* read-through construct (3-fold regulation) and the best regulated clone only showed 16-fold induction. For all clones analysed, regulation was vastly improved when minichromosomal loci were targeted instead of the rDNA spacer—mean regulation of ∼170-fold regulation was observed for 177 bp-targeted clones; targeting minichromosomal *VSG*-G4 and -S8 resulted in around 600-fold and 120-fold regulation, respectively.

BSF cells were stably transformed using constructs similar to those used for PCF cells except that a tetracycline-responsive T7 promoter was used in place of the procyclin EP1 promoter. Again three independent clones were selected at random for each site targeted. These clones showed a slightly different pattern of fully-induced mRNA levels to those observed in PCF equivalents; the rDNA spacer was in this case the target site displaying weakest transcription, followed by the 177 bp repeat and with *VSG*-G4 the most active site.

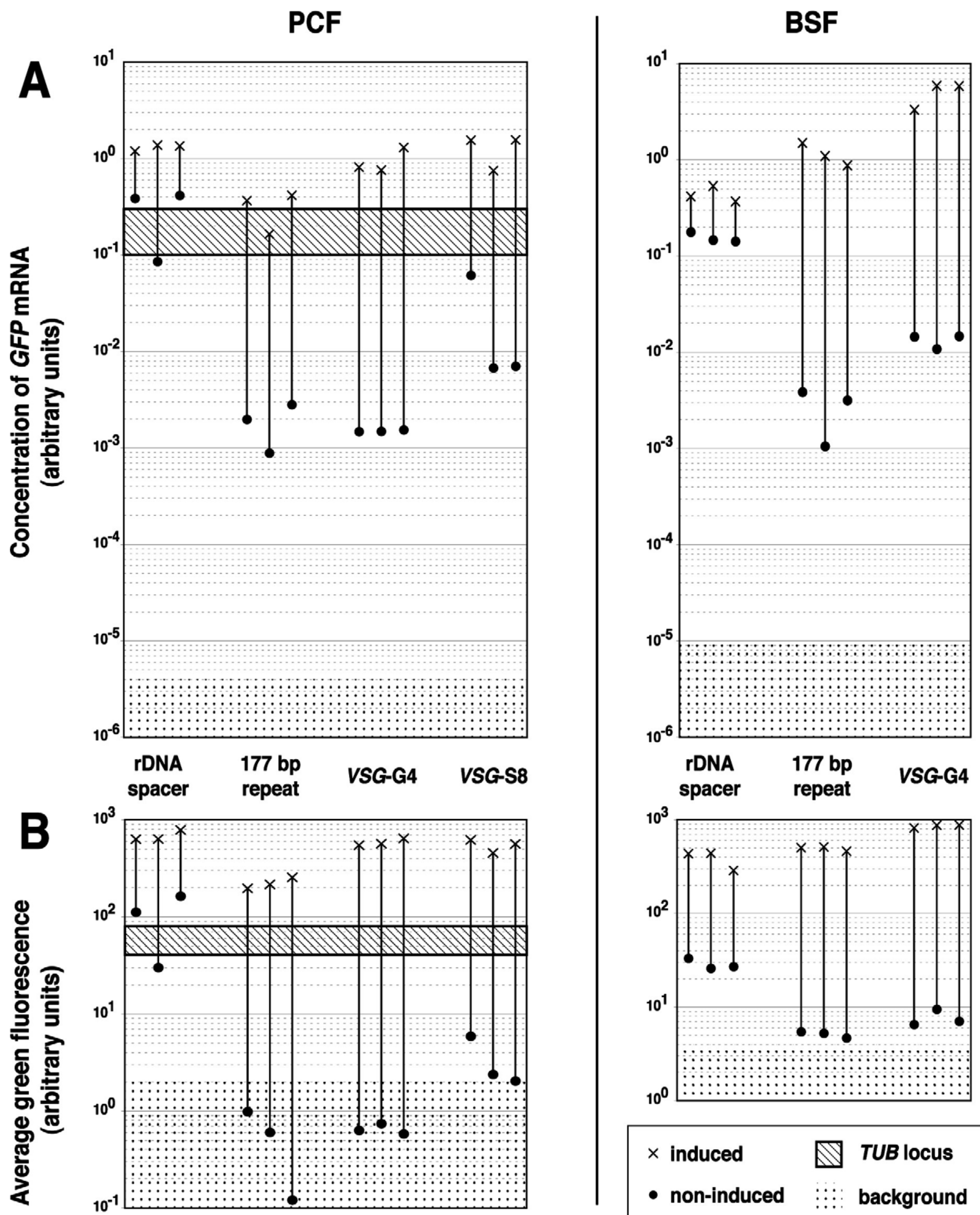

Fig. 2. Regulation of tetracycline-responsive expression from different sites in the *T. brucei* genome. Procyclic-form (PCF) and bloodstream-form (BSF) cells were stably transformed with the vectors shown in Fig. 1. Results are shown for three independent randomly-selection clones for each integration target site grown for 7 days in the presence (×) or absence (●) of induction (1 μg ml^{-1} tetracycline PCF; 1 μg ml^{-1} doxycycline BSF). Hatched box represents levels of expression from three independent clones tagged with a promoter-less construct targeted to the α/β-tubulin gene array (*TUB*). (A) Effect of integration target site on *GFP* mRNA levels. Relative mRNA concentration was assessed by quantitative real-time reverse-transcription PCR. Background represents the maximum amplification from no-RT and no template controls. (B) Effect of integration target site on GFP levels. Green fluorescence was assessed by flow cytometry and is displayed as units above fluorescence of untransformed (non-GFP expressing) cells. Background represents the error in measurement of fluorescence of untransformed cells.

Unfortunately, little can be inferred from the differences in expression levels between PCF and BSF cells in which the same sequences were targeted, since the inducible genes in the two life-cycle stages are transcribed by different polymerases. It is interesting, however, that in neither life-cycle stage did the minichromosomal *VSG*s show a noticeable degree of telomeric-silencing. Moreover, high-level ectopic expression could be induced from a normally silent MC-*VSG* locus (although not, it should be noted, of the *VSG* itself) in BSF cells expressing a different *VSG* from an expression-site on a MBC.

In BSF clones, as with PCF clones, the regulation of *GFP* mRNA from the rDNA spacer was extremely poor ($\sim$3-fold) and again regulation was greatly increased by targeting integrative constructs to minichromosomal loci. Regulation was not significantly different between 177 bp and *VSG*-G4 targeted constructs (mean induction of 600- and 400-fold, respectively), but targeting the 177 bp repeat gave lower levels of mRNA in the non-induced state.

We corroborated the results of the regulation of *GFP* mRNA by looking at regulation of the protein by flow cytometry. Fig. 1B shows the levels of green fluorescence seen in seven representative clones in which different genomic sites have been targeted for the insertion of inducible vectors. Fig. 2B shows the effect of integration target site on the average cellular green fluorescence of the clones analysed in Fig. 2A. For clones showing greater regulation of mRNA, levels of GFP in the non-induced state were difficult to assess since fluorescence was very close to that of untransformed cells ('background' in Fig. 2B). Interestingly, however, for those clones safely within the window of resolution, regulation of protein levels was in all cases better than that seen for mRNA. This is particularly pronounced in BSF cells, where rDNA spacer-tagged clones which are regulated only $\sim$3-fold at the mRNA level, show a $\sim$14-fold change in average cellular green fluorescence. The origin of this difference is unknown. None-the-less, patterns of regulation observed at the level of mRNA were also seen for protein levels—greatest regulation was observed when constructs were integrated into minichromosomal loci rather than the rDNA spacer.

In summary, constructs were developed to test a tet-inducible system integrated at different genomic locations in *T. brucei*. Ectopic expression could be achieved not only from MBC loci (rDNA spacer and *TUB*), as described previously, but also 177 bp repeat and *VSG* loci on MCs. Regulation of vectors incorporated into MCs was found to be much greater than for those targeted to the rDNA spacer, due to a better down-regulation of expression in the non-induced state. The system has yet to prove itself in a sufficiently large number of transgenic studies, but its potential for many applications can be clearly seen. We are particularly interested in the application of MC-targeting to the burgeoning field of RNAi in African trypanosomes. To this end we have developed a modified version of the p2T7^{Ti} plasmid [5] which replaces the rDNA spacer targeting sequence with 177 bp repeat sequence (p2T7^{Ti}-177). Initial data obtained using this construct are promising. We have used integration at 177 bp repeat loci in BSF cells to drive inducible lethal RNAi knock-down of a protein involved in transcriptional regulation. Our previous attempts to generate similar RNAi cell lines by integration at the rDNA spacer had failed. The vector p2T7^{Ti}-177 can be obtained from the authors on request.

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