Tagging a *T. brucei RRNA* locus improves stable transfection efficiency and circumvents inducible expression position effects

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Abstract

In *Trypanosoma brucei,* RNA interference (RNAi) and recombinant protein expression are established as powerful approaches for functional genomics, particularly when combined with inducible expression. The favoured methods involve exploiting homologous recombination to target expression cassettes to a chromosome sub-set to establish stable cell-lines. Unfortunately, bloodstream-form cells, those that cause disease in mammals, exhibit low efficiency stable transfection. Current expression systems can also exhibit other undesirable features, including variable position effects and leaky, inducible expression. We have developed systems in bloodstream-form cells that alleviate these problems. Using constructs for RNAi and expression of (GFP) tagged proteins, we target a (*hyg*) tagged ribosomal RNA (*RRNA*) locus which circumvents position effects and allows increased targeting efficiency. We also report a compatible double-inducible system for tight regulation of highly toxic products. This system exploits a new inducible *RRNA* promoter to drive T7 RNA polymerase (T7RNAP) transcription which then drives expression from inducible *T7* promoters. The developments described should facilitate functional analysis and increased throughput.

Keywords

bloodstream-form; functional genomics; GFP; leaky; position effect; RNAi; transfection; T7 RNA polymerase

1. Introduction

Genetic manipulation and inducible expression, using tetracycline (Tet) or an analogue, are widely used in the African trypanosome, *Trypanosoma brucei*. Inducible expression is particularly useful since it allows the effect of altering the expression of a particular gene product to be assessed in a single cell-line. RNA interference (RNAi), described in organisms ranging from trypanosomes to humans, is a conserved biological response to double-stranded RNA (dsRNA) that involves 'knock-down' of homologous mRNA. Thus, strains can be established that express toxic proteins or, for RNAi, dsRNA against an essential gene [1]. Inducible expression of dsRNA is a particularly convenient method for examining gene function and the absence of RNAi in the South American trypanosome, *Trypanosoma cruzi* [2] and *Leishmania major* [3] has focussed emphasis on *T. brucei* as a model for trypanosomatid functional analysis.

Most of the *T. brucei* genome appears to be arranged in large polycistronic gene clusters. In most cases, it is desirable that integrated genetic elements should be isolated from native genes and regulatory elements and non-transcribed *RRNA* loci [4] and mini-chromosomes

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[5] have consequently been popular for targeting. The mechanisms limiting transcription at these loci are not fully understood however. In excess of 1000-fold induction has been reported using either locus but much lower levels of regulation have also been reported at *RRNA* loci [5]. Because multiple copies of the non-transcribed *RRNA* loci are present in the genome [6] the site of integration is unpredictable and can influence reporter gene expression [7]. In addition, the generation of recombinant bloodstream-form *T. brucei* cell-lines is laborious, largely due to the low efficiency of DNA-mediated transformation. Current protocols require ~10μg of construct DNA and ~2.5×10^7 cells to generate a single transformant with DNA integrated at an *RRNA* locus. This low efficiency represents a bottleneck for routine studies and a significant bottleneck for high throughput studies.

Widely used inducible promoters for *T. brucei* are the *T. brucei EP/GPEET* and phage *T7* promoters [4]. These promoters have Tet operators (*TetO*) immediately downstream of the transcription start site and can constitute a simple 'Tet-on' system when imported into cells expressing the Tet-responsive repressor (TetR) [8]. RNA Polymerase (RNAP) I can transcribe mRNA in *T. brucei* due to trans-splicing of an RNAP II derived leader sequence onto every mRNA. Indeed the *EP/GPEET* promoter recruits RNAP I [9]. To use the phage *T7* promoter, T7RNAP must be expressed [10]. T7RNAP does not require co-factors for activity and is used in many heterologous gene expression systems. The protein expressed in *T. brucei* has an SV40 large T antigen nuclear localisation signal (PKKKRKV) engineered at the *N*-terminus [10].

Although a range of inducible expression tools are available for functional analyses in *T. brucei*, current systems exhibit a range of problems in generation and maintenance of recombinant cell lines, some of which prove prohibitive when attempting high-throughput studies. With the completion of the core genome sequences for several trypanosomatids (http://www.genedb.org/), there is an urgent need for optimised genetic tools to improve efficiency, provide time and cost savings and improve data quality and consistency. Here, we report efforts that go some way towards alleviating the problems outlined above. All constructs have been tested in bloodstream-form cells but should work equally well in insect-stage cells.

2. Materials & Methods

2.1 Plasmid constructs

pHD1313 [4] was used for *TetR* expression at the *TUB* locus. p2T7^{TAblue} [4] (see http://homepages.lshtm.ac.uk/~ipmbdhor/dhhome.htm - Resources) was used as the 'back-bone' for the production of all other constructs reported here. pRP^{cMyc} is similar to pRP^{GFP} but with a cMyc-tag instead of a GFP-tag. All constructs were mapped using restriction enzymes and the ligated junctions were checked by DNA sequencing. *In silico* assembled sequences are available upon request.

2.2 T. brucei growth and manipulation

Bloodstream-form Lister 427 cells, clone 221a, were grown in HMI-11 [11]. For all transfections, 2.5×10^7 cells were electroporated with 10μg ml^{-1} linearised construct DNA as described [12]. Drugs were added <6 hours post-transfection at the following concentrations: blasticidin (Invitrogen), 10 μg ml^{-1}; hygromycin (Sigma), 2.5μg ml^{-1}; phleomycin (CayLa), 2 μg ml^{-1}; puromycin (Calbiochem) 2 μg ml^{-1}. Cell counts were carried out using a haemocytometer. Tet was from Sigma.

2.3 Western blotting and microscopy

Western blots were developed using primary mouse -T7RNAP (Molecular Probes), mouse -cMyc (Upstate) or rabbit -GFP (Novagen) according to the manufacturers' instructions, and signals were detected using an ECL+ kit (Amersham).

For microscopy, cells were washed in PBS and fixed overnight in 2% (v/v) formaldehyde in PBS at 4°C. Prior to analysis, cells were washed in PBS and 1% (w/v) BSA in water. After drying onto glass slides, cells were mounted in Vectashield (Vector Laboratories) containing the DNA counterstain, 4 ,6-diamidino-2-phenylindole (DAPI). Slides were analysed on a Nikon Eclipse E600 epifluorescence microscope. Phase contrast and fluorescence images were captured using a Coolsnap FX (Photometrics) CCD camera and processed in Metamorph (Universal Imaging).

3. Results

3.1. A Tet-inducible ribosomal RNA promoter

Although fully active in insect-stage cells, the *EP/GPEET* promoter is down-regulated in the mammal-infective bloodstream stage [7]. In order to generate an inducible promoter that was not dependent on heterologous polymerase and that would be expected to function efficiently in both major life cycle stages we cloned a single *TetO1* operator [13] adjacent to the transcription start site of a ribosomal RNA (*RRNA*) promoter ([14], see Fig. 1A). A similar inducible *RRNA* promoter has been used in *Leishmania donovani* [15]. Using pRP^{GFP} (see Fig. 2A) which contains this promoter, we targeted a number of *N*-terminal GFP-tagged genes to *RRNA* loci in cells expressing TetR from the tubulin array [4]. The *RRNA* promoter system demonstrated robust inducible expression and can therefore be used as part of a T7RNAP-independent inducible expression system (Fig. 2) or for inducible expression of T7RNAP itself (Fig. 1).

A potential solution to problems associated with constitutive T7RNAP expression was 'double-inducible' expression whereby an inducible but truncated *EP/GPEET* promoter drives T7RNAP expression which then drives expression from inducible T7-promoters. This approach was successful in insect-stage cells but was unsuccessful in bloodstream-form cells, apparently due to down-regulation of the *EP/GPEET* promoter [4]. We therefore decided to test the inducible *RRNA* promoter, considered to be a 'strong' promoter, for expression of T7RNAP in bloodstream-form cells.

We replaced the GFP gene downstream of the Tet-inducible *RRNA* promoter in pRP^{GFP} with the T7RNAP gene and targeted the resulting pRP^{T7pol} construct (Fig. 1A) to an *RRNA* locus in cells expressing TetR. Robust inducible T7RNAP expression was possible but independent clones expressed different maximal levels of T7RNAP (Fig. 1B). We selected clone 1 for further analysis and the data show that induction was Tet dose-dependent (Fig. 1C) as expected. In the absence of Tet, cell line 1 exhibits the same growth dynamics as wild-type cells (not shown), increasing ~10-fold every 24h (Fig. 1D, left-hand plot). Induction with 5ng ml^{-1} does not affect growth rate while induction with 50ng ml^{-1} Tet leads to a significant decrease in growth rate (right-hand plot). Thus, T7RNAP is expressed at a sub-toxic level when induced with 5ng ml^{-1} Tet but cell growth is severely retarded following induction with 50ng ml^{-1} Tet. Tet is generally used between 0.1 and 1μg ml^{-1} in *T. brucei* cultures and no Tet-toxicity has been reported under these conditions so we conclude that T7RNAP expression is responsible for toxicity.

3.2. Circumventing position effect using cells with a tagged locus

We observed highly variable maximal expression levels in individual clones when targeting the non-transcribed *RRNA* loci using a target sequence spanning the region from 1595-1031 nt upstream of the transcription start site (Fig. 1B, 2B and our unpublished observations). Several clones could be generated and tested using each construct but clearly this approach is time-consuming and incompatible with high-throughput analysis. Since DNA transformation is predominantly via homologous recombination in *T. brucei* [16], a locus tagged with a unique sequence should allow constructs to be reproducibly inserted into the same genomic location, thus circumventing position effects. This would be useful when analysing putative regulatory elements and should also improve reproducibility for a range of other experiments, including those involving inducible expression. We first used pRP^{GFP} to integrate an inducible reporter into *RRNA* loci in cells expressing TetR (Fig. 2A, top). We selected a cell line with no evidence of leaky expression and robust inducible expression (i.e. no evidence of position effect repression) and using ph3E replaced the reporter cassette at the chosen target with a '*hyg*-tag', a portion of the *HYG* selectable marker (Fig. 2A, middle). These cells (*TetR^{BLE}:Tag^{PAC}*) can then be used for specific targeting to a locus validated for robust inducible expression by including the remainder of the *HYG* gene in the construct of choice (Fig. 2A, bottom). Tag-compatible constructs contain a truncated *HYG* gene that lacks the last four codons and shares 604 bp with the tag. We added *Asc*I sites to pRP^{GFP} to generate pRPa^{GFP} which is now compatible with *hyg*-tagged cells. Figure 2B again shows that inducible reporter expression varies between clones when several different *RRNA* loci are targeted. In contrast, reporter expression is reproducible among independent clones when the *hyg*-tagged *RRNA* locus is targeted (Fig. 2C and our unpublished observations with a number of different GFP-tagged proteins).

3.3. Improved targeting efficiency in cells with a tagged locus

There are ~10 *RRNA* loci in the *T. brucei* genome but target copy number is not thought to be proportional to targeting efficiency [6] so we were not in a position to predict transformation efficiency when targeting a single *hyg*-tagged locus. Remarkably, comparison of transformation efficiency indicated that the single tagged locus was targeted at significantly increased efficiency relative to ~10 un-tagged loci (Fig. 3). This increase in efficiency facilitates scaling in that *T. brucei* recombinants should now be reliably generated using only ~5μg of construct DNA, less than the typical yield from a plasmid Miniprep (Qiagen).

In cells with a tagged *RRNA* locus, only correct targeting was expected to generate a complete *HYG* gene while integration elsewhere in the genome was not expected to generate hygromycin-resistant cells. Indeed, we were unable to transform cells lacking a tagged locus using pT7a^{GFP} (data not shown). Correct targeting in tagged cells is expected to remove the *PAC* gene (see Fig. 2A) and this was confirmed by sensitivity to puromycin in >60% of the hygromycin resistant clones (Hyg^R:Pac^S). Using these clones, the *PAC* selectable marker is available for further manipulation. Loss of the *PAC* gene from the genome confirms correct integration circumventing the need to map the integration site and also avoiding problems associated with unexpected targeting (see [17] for example).

Interestingly, Hyg^R:Pac^R clones showed no evidence of position effects (data not shown) suggesting recombination with the tag and a second, possibly tandem, *RRNA* target and/or duplication of the tagged locus in some cells prior to assembly of an intact *HYG* gene. If the former explanation is correct, it should be possible to increase the proportion of Hyg^R:Pac^S clones by using constructs with unique targeting sequences at both ends. Regardless of the explanation for the generation of Hyg^R:Pac^R clones, the system described significantly improves transfection efficiency and circumvents position effects.

3.4. Double-inducible expression in cells with a tagged locus

To generate cells for double-inducible expression that are not prone to position effects, we introduced pRP^{T7pol} (see section 3.1) into cells with a *hyg*-tagged *RRNA* locus (see section 3.2). The combination should allow inducible T7RNAP to drive inducible *T7* promoters. We selected a clone with undetectable T7RNAP when un-induced and expressing the highest level of T7RNAP when induced. We used 5ng ml^{-1} Tet for these experiments since we knew that T7RNAP would be toxic if Tet were applied at significantly higher concentration (see Fig. 1D). These (*TetR^{BLE}:Tag^{PAC}:T7RNAP^{BLA}* or T7^i:Tag) cells showed no growth retardation or other discernable phenotype following induction for one week in 5ng ml^{-1} Tet (data not shown).

We first tested pT7a^{GFP}, a construct similar to pRPa^{GFP} (Fig. 2A) but with a *T7* promoter instead of an *RRNA* promoter. A GFP-tagged gene in this construct, demonstrated tight regulation, robust induction (Fig. 4A) and, as expected, nuclear localisation of the product (Fig. 4B). We then modified the p2T7^{TAblue} inducible RNAi construct [4] to generate p2T7a^{TAblue} (Fig. 4C), an RNAi construct compatible with the tagged locus. We easily generated clones with this construct containing a large ∕-tubulin insert which previously failed to transform 'single inducible' cells [4]. In the double-inducible system, this construct exhibited the expected [18] FAT cell phenotype (Fig. 4D) in the majority of cells and generated a severe growth defect when induced (Fig. 4E). RNAi revertants begin to grow out on the third day following induction (Fig. 4E) consistent with the idea that ~10^{-3} cells escape the RNAi phenotype. Using a similar system, this has been shown to occur predominantly through deletion of the RNAi target sequence [19].

Although Tet at 5ng ml^{-1} is sufficient for the expression cassettes described above, this is not sufficient to induce the expected phenotype for several other RNAi constructs (data not shown). Significantly lower constitutive T7RNAP expression does give the expected phenotypes so this limitation is probably due to insufficient activation of the inducible *T7* promoters by 5ng ml^{-1} Tet. Since increased Tet will lead to toxic T7RNAP expression, the utility of the current double-inducible system is probably limited to expression of particularly potent products. We have clones that express less T7RNAP (see Fig. 1B) but a truncated *RRNA* promoter (see [20]) should facilitate the construction of a more versatile system that tolerates more Tet.

4. Discussion

Clearly, technology enables scientific discovery. Now that several trypanosomatid genomes have been sequenced, there is an urgent need for new and optimised genetic tools. We identified a number of problems and bottlenecks associated with genetic manipulation and inducible expression procedures in *T. brucei* and have systematically endeavoured to overcome each in turn.

We have established cells with a unique target sequence at a single *RRNA* locus that has been validated for robust inducible expression and we show that a single modified locus is targeted at higher efficiency relative to ~10 unmodified loci. The system circumvents position effects and the need to map the insertion site. The parameters that influence targeting efficiency in *T. brucei* are not completely understood but it could be increased transcription of the tagged target that is responsible for increased efficiency (see Fig. 2A). Whatever the explanation, the system should facilitate increased throughput genetic manipulation.

High-level T7RNAP expression is thought to be toxic in *T. brucei* [4] but this interpretation was indirect, based on the inability to establish clones with T7 transcription driven by a

'strong' promoter. We use a new inducible *RRNA* promoter to directly demonstrate T7RNAP toxicity. Fortunately, lower, sub-toxic level expression is sufficient to drive significant transcription and strains have been established that constitutively express T7RNAP using 'weakened' promoters [4]. These strains have been used for a wide range of analyses but the consequences of long term constitutive expression of T7RNAP have not been assessed. It remains possible that low-level T7RNAP toxicity confers a selective advantage on altered cells. Leaky inducible expression can also compromise the ability to maintain cells transfected with toxic 'un-induced' expression cassettes [4]. Inducible expression of T7RNAP, as part of a double-inducible system, could help alleviate problems associated with toxicity and leaky expression. Our demonstration T7RNAP toxicity however underscores the importance of achieving the correct RNAP expression level in a double-inducible system. Alternatively, the inducible *RRNA* promoter can be used to circumvent the need for heterologous polymerase altogether.

Although there are clearly a number of outstanding issues to be addressed if we are to develop an optimised toolkit for manipulation of gene expression in *T. brucei*, the new constructs reported here should help to alleviate some current problems. We demonstrate utility at several levels. Position effects can be circumvented, targeting efficiency can be significantly increased and cells expressing highly toxic products can be generated. These tools should facilitate the application of scaleable protocols for functional genomics in *T. brucei.*

Acknowledgments

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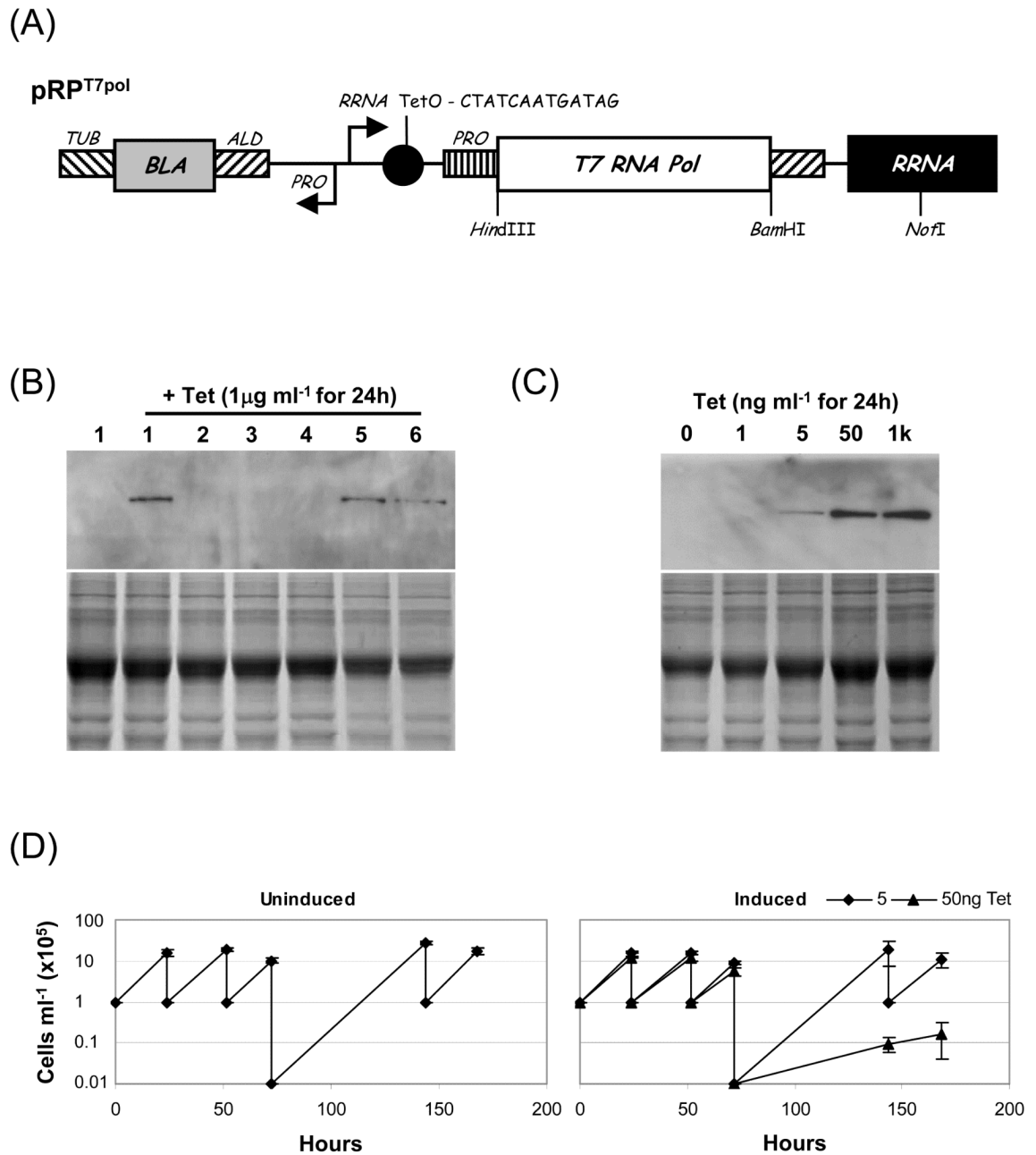
(A)

(B)

(C)

(D)

Fig 1.
Expression of T7RNAP from a Tet-inducible *RRNA* promoter. (A) *Not*I-digestion targets
pRP^{T7pol} to an *RRNA* locus. *BLA*, blasticidin resistance. Arrows represent promoters. RNA
processing regions and promoters: ALD, aldolase; PRO, procyclin; TUB, tubulin. (B and C)
Western blot analysis of T7RNAP expression. The blots were probed with primary mouse
-T7RNAP (1:5000). The lower panels show coomassie-stained gels as loading controls.
(B) Six independent cell-lines expressing TetR and transfected with pRP^{T7pol} express
different levels of T7 polymerase upon induction with 1μg ml^{-1} Tet. Lane 1: un-induced cell
line 1. (C) Induction of cell line 1 with 1ng to 1μg ml^{-1} Tet showed that T7 polymerase
expression was dose dependent, peaking in the presence of 50ng ml^{-1} Tet. (D) Growth

analysis was carried out in triplicate and populations were diluted as shown. Error bars: standard deviation.
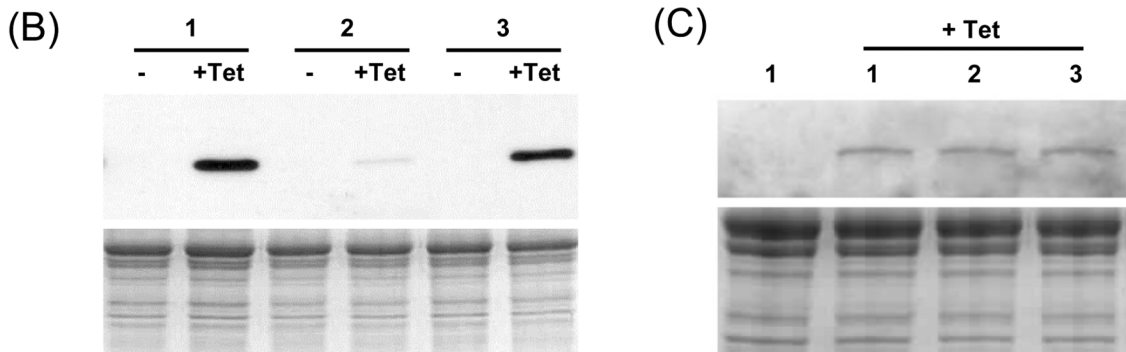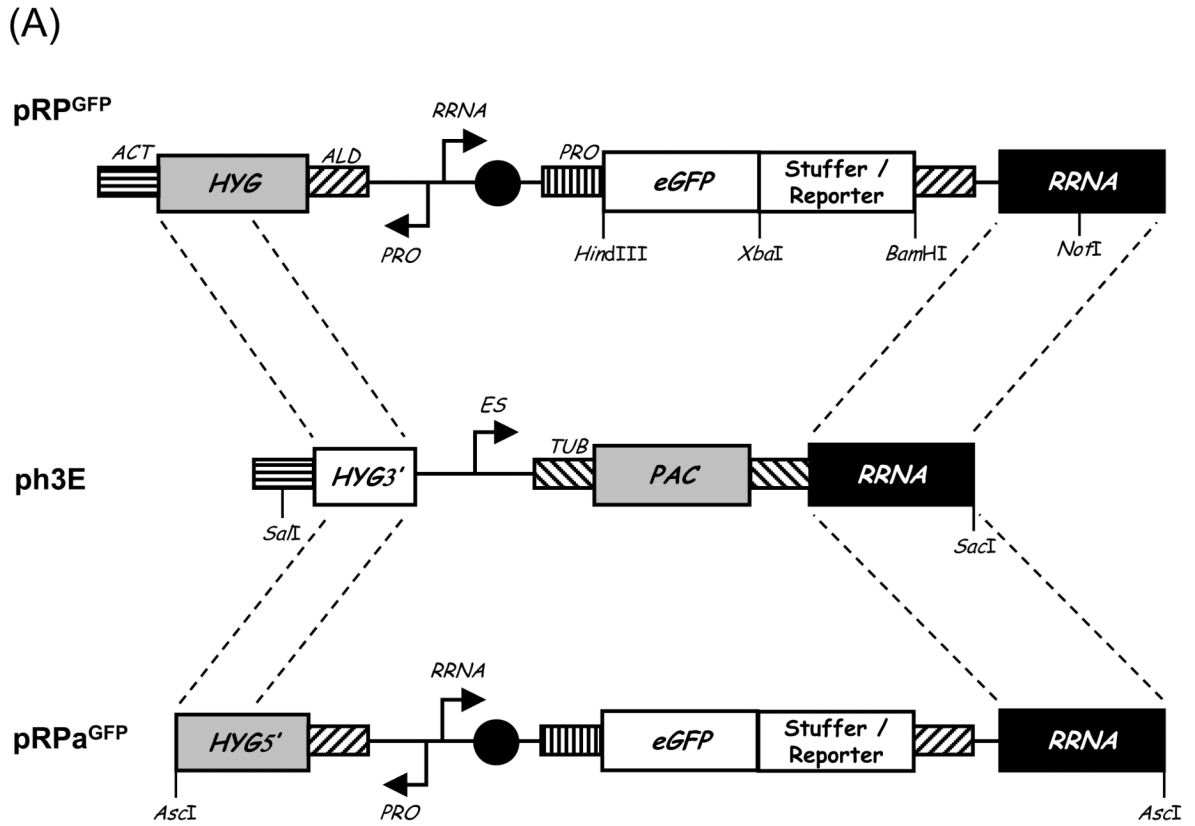
(A)

(B)

(C)

Fig. 2.
Tagging an *RRNA* locus. (A) *Not*I-digested pRP^GFP and *Sal*I/*Sac*I-digested ph3E were used to identify an *RRNA* locus with no evidence of position effect repression and to add the *hyg*-tag to that locus respectively. The resultant tag can then be replaced using *Asc*I-digested pRPa^GFP. *HYG*, hygromycin resistance; *PAC*, puromycin resistance; ACT, actin; ES, variant surface glycoprotein Expression-Site. (B) Western blot analysis of inducible expression in three independent clones generated using non-tagged cells and pRP^cMyc with a reporter gene (GeneDB: Tb927.4.2520). The blot was probed with primary mouse -cMyc (1:2000). (C) Western blot analysis of induced expression in three independent and representative clones generated using tagged *RRNA* locus cells and pRPa^GFP with a reporter

gene (GeneDB: Tb11.01.3380). Lane 1: un-induced cell line 1. The blot was probed with primary rabbit -GFP (1:4000). Other details as in Fig. 1.
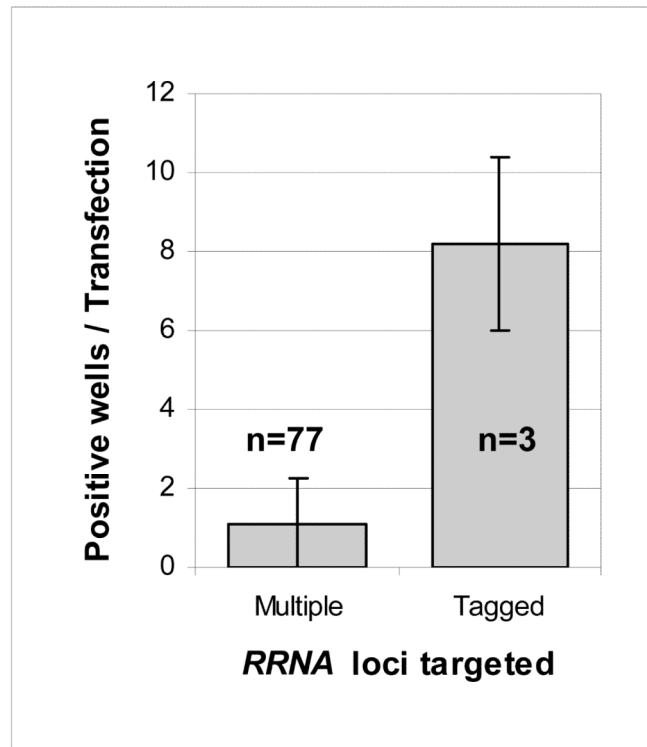
Fig. 3.
Increased transfection efficiency in cells with a *hyg*-tagged *RRNA* locus. *HYG* selection
was administered <6h following electroporation to minimise cell division prior to
distribution of cells in 96-well plates. Data for multiple targets was derived over several time
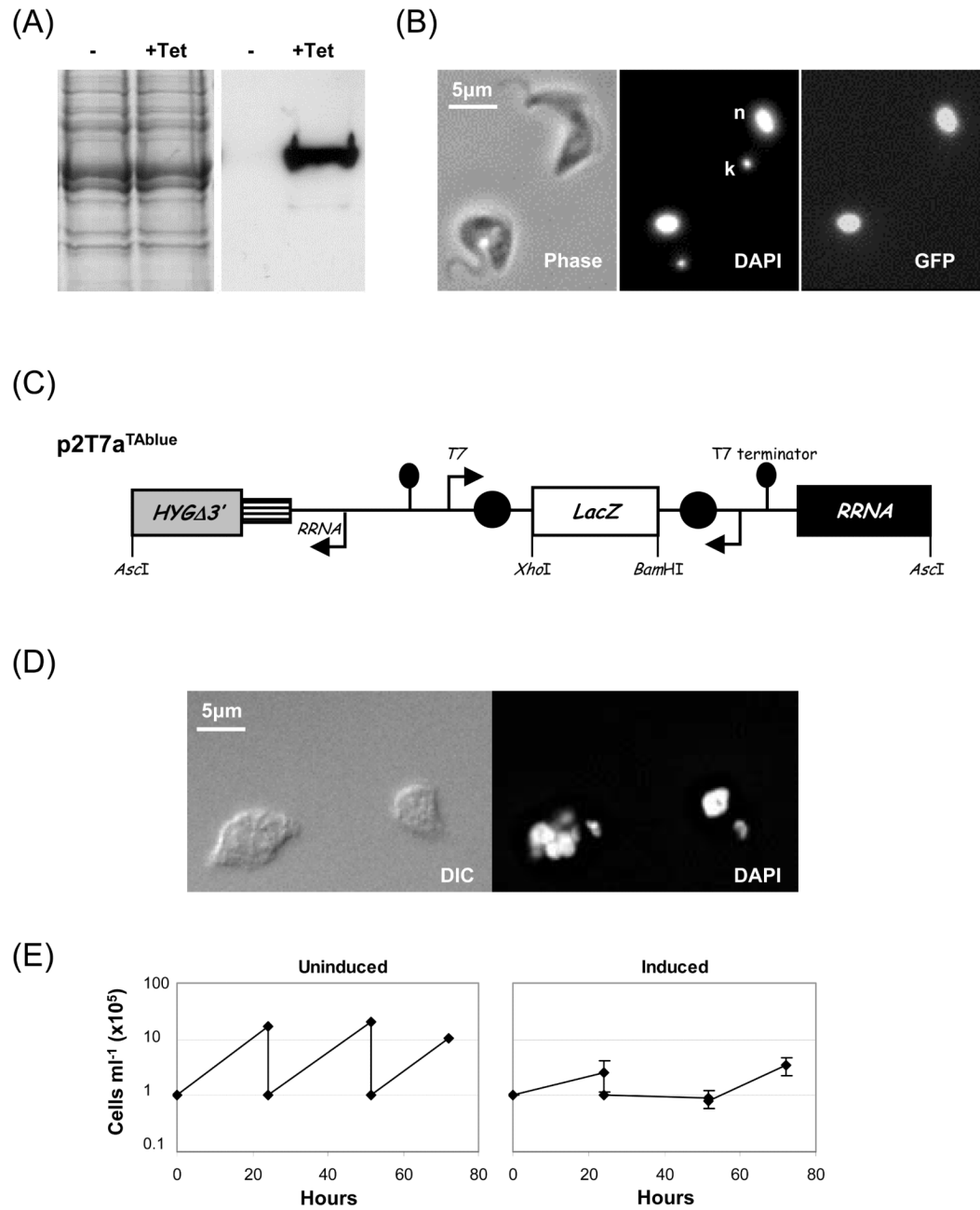points. Error bars: standard deviation.

(A)

(B)

(C)

(D)

(E)

Fig. 4.
Double-inducible expression in cells with a *hyg*-tagged *RRNA* locus. (A and B) A reporter gene (GeneDB: Tb10.6k15.3240) was cloned in pT7a^GFP (similar to pRPa^GFP but with a T7 promoter in place of an *RRNA* promoter) and the nuclear GFP-tagged protein was expressed in T7^i:Tag cells. (A) Induction was with 5ng ml^{-1} Tet for 24h. The western blot was probed with primary rabbit -GFP (1:4000). The left hand panel shows a coomassie-stained gel as a loading control. (B) Direct detection of the GFP-tagged protein. DNA counterstained with (DAPI); k, kinetoplast; n, nucleus. (C) The p2T7a^TAblue RNAi construct. Details as in Fig. 1A. (D and E) dsRNA against ∕ -tubulin was expressed in T7^i:Tag cells using p2T7a^TUB, a

derivative of p2T7a^{TAblue}. Details as above. (D) Microscopy. Induction was with 5ng ml^{-1} Tet for 20h. (E) Growth analysis. Details as in Fig. 1D.