

ANALYSIS OF SMALL GTPASE FUNCTION IN TRYPANOSOMES

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Abstract

Trypanosomatids are protozoan parasites, of interest due to both their disease burden and deeply divergent position within the eukaryotic lineage. The African trypanosome, *Trypanosoma brucei*, has emerged as a very amenable model system, with a considerable toolbox of methods available, including inducible overexpression, RNA interference, and a completed genome. Here we describe some of the special considerations that need to be addressed when studying trypanosome gene function, and in particular small GTPases; we provide protocols for transfection, RNA interference, overexpression and basic transport assays, in addition to an overview of available vectors, cell lines, and strategies.

1. OVERVIEW

Trypanosomatids comprise a protist lineage that includes many infectious species alongside numerous free-living taxa. Trypanosomes infect plants, arthropods, fish, and mammals; the latter category is of the most

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interest as these species are responsible for significant morbidity and mortality in humans and domestic animals (Barrett *et al.*, 2003). There are three major trypanosome lineages involved, the African trypanosome, *Trypanosoma brucei* sp., the South American trypanosome, *T. cruzi*, and the Leishmanias, both Old and New World. These organisms are responsible for a spectrum of disease affecting the majority of the human population across Africa, South America, Europe, and Asia. Genome sequencing indicates a remarkable degree of relatedness between these three lineages (Berriman *et al.*, 2005).

Small GTPases are highly important regulators of function in trypanosomes, and because of the absence of heterotrimeric GTPases from trypanosomatid genomes, Ras-like GTPases likely shoulder a larger proportion of the signal transduction burden than in higher eukaryotes. Over 40 small GTPases are encoded by the *T. brucei* genome (Field, 2005); considerable work has been done on the Rab subfamily (Field and Carrington, 2004), and a lesser amount on the Arf proteins (Price *et al.*, 2005), but essentially nothing is known of the functions of the GTPases more closely related to the Ras and Rho families (Field, 2005). Interest in intracellular trafficking is mainly prompted by the fact that the surfaces of trypanosomes are dominated by GPI-anchored proteins and glycoconjugates, and that in *T. brucei* endocytosis is likely a component of the immune evasion machinery (Field *et al.*, 2007). We remain almost completely ignorant of the downstream molecules that interact with trypanosome small GTPases; informatics is able to identify candidate molecules—for example, the nine TBC domain-containing proteins encoded in the trypanosome genome are likely Rab GTPase activators—but no information is available on function (A. J. O'Reilly, C. Gabernet-Castello, and M. C. Field, unpublished).

Much of the analytical tool kit exploited by the wider community is applicable to trypanosome GTPases, such as overexpression of dominantly active forms of the proteins, use of two hybrid libraries for screening for interacting partners, and epitope tagging, and these topics and strategies have been well discussed elsewhere. However, several aspects are unique; in particular, the trypanosome transcriptional machinery is predominantly polycistronic, and the basal transcriptional apparatus appears rather divergent (Ivens *et al.*, 2005). Further, a dismally low transfection efficiency, at less than 10^{-6} transformants/ μ g DNA for bloodstream forms, which has so far resisted all attempts at significant improvement, means that creation of transgenic trypanosomes must be done in a carefully considered manner, and that forward genetic screening methods are currently very cumbersome; a recent advance in technology bodes well for increased efficiency (Burkard *et al.*, 2007). The consequence is that specific vector systems have been developed, and reliable constitutive and inducible systems are now available primarily for reverse genetics approaches. The latter require use of engineered cell lines harboring the tetracycline repressor. Due to the diploid

genome and the comparative cumbersome nature of inducible expression systems in trypanosomes, initial analysis of small GTPase function by gene deletion is not recommended; at least three rounds of transfection are required—to remove both endogenous alleles and to provide a conditional copy in the case of essentiality—and under some circumstances the ability to control expression of inducible ectopic genes can prove challenging. Further, such approaches result in overexpression, as induction requires T7 or RNA pol I–driven transcription. As an overall strategy, we favor a combination of knockdown by RNAi, coupled with constitutive overexpression of wildtype or mutant forms of the relevant GTPase; such approaches have been highly successful (Hall *et al.*, 2004, 2005a,b,c).

This article focuses on analysis of small GTPases in *T. brucei* on account of this system being more technically advanced than other trypanosomatids; we will consider relevant vectors and their use and describe the various reporter lines that are required for specific approaches. We also describe general assays for analysis of endocytosis and exocytosis.

2. CULTURING

Trypanosomes require specialized media for culturing. These can be made *de novo*, and are straightforward to make, but media can also be purchased direct as custom orders from several companies (e.g., Invitrogen, Sigma, JRH Biosciences). In addition, workers may wish to contact the authors as the community places bulk orders with companies on an occasional basis. The most commonly used laboratory strains of *T. brucei* are derived from the Lister 427 isolate. The procedures outlined below have been optimized using Lister 427, and it may be necessary to modify some of these conditions for the growth of other isolates in culture.

For the bloodstream form, *T. brucei* HMI-9 is used (Hirumi and Hirumi, 1989). To the basic medium, add the following per liter: 3 g sodium bicarbonate (Sigma), 10 ml penicillin/streptomycin (100 × 5000 U to 5000 µg/ml, Life Tech, #15070-063), and 14 µl β-mercaptoethanol. Filter, sterilize, and store at 4° for up to 6 months. Add 10% heat inactivated (56°, 30 min) fetal calf serum before use. Grow cells at 37° with 5% CO₂, in nonadherent culture flasks, with vented caps. Do not allow the cells to exceed 2 × 10⁶/ml.

For procyclic forms, the routine culture medium is SDM-79 and can be made up from its components (Brun and Schonenberger, 1979). When making SDM-79, add the following fresh materials: 2 g/l of sodium hydrogen carbonate. Once made, the basal medium is stable for several months at 4°. To complete the medium, 3 ml of 2.5 mg/ml hemin dissolved in 50 mM sodium hydroxide and 100 ml fetal bovine serum are added per

liter. Once complete, the medium is stable for 1 month at 4°. Plastic tissue culture flasks are used: 10 ml culture in a 25-cm² flask, 30 ml in a 75-cm² flask, and 70 ml in a 175-cm² flask. Lids are adjusted to allow free gaseous exchange. Cultures are maintained at 27°; it is not necessary to provide a CO₂ source. Procyclic trypanosomes do not grow well if diluted to less than 1 × 10⁵/ml and reach a stationary phase at around 4 × 10⁷/ml. Routine cultures are maintained between 5 × 10⁵/ml and 1 × 10⁷/ml, a 20-fold range that can be conveniently maintained by making a 1-in-20 dilution every 2 or 3 days, depending on the strain. Subculturing using a dilution of more than 1 in 20 is not routinely used, as there can be a long lag before growth resumes.

The procyclic cell line Lister 427 29-13 (containing pLEW29 and pLEW13) or the Single Marker Bloodstream (SMB, *T7RNAP::TETR::NEO*) line are routinely used for the expression of tetracycline-inducible transgenes and tetracycline-inducible RNAi. Both contain integrated transgenes that constitutively express the tet repressor and T7 RNA polymerase. The 29-13 cell line does not grow as well as the wildtype; the doubling time is increased and cultures contain a higher percentage of cells with aberrant morphologies. An alternative with superior morphology and similar inducibility is the PTT line, also derived from 427; again the antibiotic selection must be maintained at all times. The morphology of procyclics can often be improved by rapid passage, and if this is unsuccessful, by cloning.

If performing RNAi knockdown experiments is intended, then additional consideration is advisable in testing fetal bovine serum batches. Normally, batches require testing for procyclic forms as occasional batches do not support growth. However, in addition, some batches of serum may be contaminated with oxytetracycline at sufficient concentration to induce low levels of RNAi. If the RNAi is deleterious to growth, and then there is rapid selection for cells that cannot perform the desired ablation for one of several reasons. This may be avoided by testing serum batches for the ability to support the growth of a Lister 427 29-13 or SMB cell line containing a rapidly lethal RNAi construct such as eIF4A1 or clathrin heavy chain, respectively.

3. TRANSFECTION AND CLONING OF T. BRUCEI

The production of recombinant trypanosomes is dependent on antibiotic selection. The antibiotics routinely used at present are geneticin (G418), hygromycin, phleomycin (zeocin), blasticidin, and puromycin (Tables 5.1 and 5.3). Antibiotics can be stored as 100× or 1000× stocks in sterile water. If you suspect an issue with antibiotic selection, such as antibiotic resistance in the absence of expression of the expected transgene

Table 5.1 Plasmid-Based Vectors Commonly Used for Manipulation of *Trypanosoma brucei* Gene Expression

Name	Features	Integration site	Applications	Selection ^a	Reference
p2T7 ^b	T7 promoter/TET operator	rRNA spacer	RNA interference	H	La Count et al., 2000
pXS5 ^c	rRNA promoter	rRNA spacer	Constitutive expression in BSF	GPH	Alexander et al., 2002
pXS2	Procyclin promoter	Tubulin intergenic	Constitutive expression in PCF	G	Bangs et al., 1996
pLEW100	Procyclin promoter	rRNA spacer	Inducible expression in BSF or PCF	P	Wirtz et al., 1999
pRPa ^{GFP}	rRNA promoter	rRNA spacer	Inducible expression in 2T1 cells	ZH	Alsford et al., 2005
pRPa ^{Con}	rRNA promoter	rRNA spacer	Constitutive expression in 2T1 cells	ZH	Alsford and Horn, unpublished
pRPa ^{SL}	rRNA promoter	rRNA spacer	RNAi in 2T1 cells	ZH	Alsford and Horn, unpublished

^a G, G418/neomycin; H, hygromycin; P, phleomycin; Z, zeocin.

^b A new version of this construct, p2T7^{T^{Ab}lue}, with increased efficiency for PCR cloning and selection is described in [Subramaniam et al. \(2006\)](#).

^c A version of both pXS2 and pXS5, designated pXS219 and pXS519, with a few additional cloning sites, is also available from the Field lab.

or unexpectedly high transfection efficiencies, it may be advisable to titer the concentration of the antibiotic, as the effective concentration may be influenced by the batch of fetal calf serum. To do this, it is suggested that a concentration range of 0.25 to $4.0\times$ of the concentration given in [Table 5.1](#) is assayed for efficient killing of cultures. For procyclics select the concentration that restricts growth to less than 2×10^6 /ml, and causes cell death (loss of motility) within 8 days. Phleomycin sometimes takes up to 10 days to kill the cells. For bloodstream forms, efficient killing should be obtained after 2 days. Antibiotic-mediated killing does not occur with stationary phase cells.

BSF trypanosomes are comparatively challenging to transfect as the efficiency is extremely low. Therefore adherence to the protocol is essential, as even a small decrease in efficiency can compromise the experiment. It is recommended that all transfection experiments include a positive control to ensure that the protocol has been correctly carried out, either a strong RNAi (e.g., clathrin heavy chain/eIF4A) or a cytoplasmic eGFP expression vector. The protocol described in the following is for the p2T7 series of RNAi vectors, but can be adapted for other constructs by altering the restriction enzyme used for linearization, and the selecting antibiotic, as required.

For transfection, a Bio-Rad Gene pulser II is used; other models can work but the pulse conditions will need to be parameterized for the specific instrument. For inducible expression, the SMB cell line described by [Wirtz *et al.* \(1999\)](#) is typically used, while a wildtype 427 line is preferable for a simple overexpression experiment, as the genetic background is unmanipulated. Use $10 \mu\text{g}$ of digested DNA for each electroporation, and usually perform two independent procedures per construct. The DNA must be completely linearized to prevent contamination with circular plasmid, which can both mis-integrate and provide a transient transfection. For p2T7, digest the DNA overnight with twofold excess of *NotI*, and check the plasmid digestion on an agarose gel with undigested p2T7^{TAb_{blue}} as a control. It is most convenient if the electroporation is performed in the morning. At least 2.5×10^7 cells are required for each electroporation. Harvest the cells by centrifugation, at $1000\times g$ for 10 min at ambient temperature and resuspend them in ~ 25 ml of cytomix¹ at 37° in a falcon tube. Pellet again and resuspend at $\sim 6 \times 10^7$ /ml in cytomix. While the cells are pelleting, aliquot sterilized linear DNA ($10 \mu\text{g}$ in $10 \mu\text{l}$) into microfuge tubes; the DNA is best sterilized by ethanol precipitation prior to use. Mix 0.45 ml of the cells with DNA, and transfer to a 2-mm gap electrocuvette and administer one pulse (1.4 kV, 25 μF). Five to 15% of the cells

¹ Cytomix: 2 mM EGTA, pH 7.6, 5 mM MgCl₂, 120 mM KCl, 120 mM, 0.5% glucose (dextrose), 0.15 mM CaCl₂, 0.1 mg/ml BSA, 10 mM K₂HPO₄/KH₂PO₄, pH 7.6, 1 mM hypoxanthine, 25 mM HEPES, pH 7.6. Adjust to pH 7.6 with KOH, filter sterilize, and store at 4° (adapted from Hoff, *et al.*, 1992).

should survive the pulse. After electroporation, place all contents of the cuvette into 36 ml of HMI-9 in a 25-cm flask, and 6 hr later add 25 μ l of a mixture of HygromycinB/G418² to each flask and transfer to 24-well plates. For transfection of wildtype 427 cells, the addition of G418 is omitted. On day 6, expand the positive wells and freeze samples³ as required. It is usually unnecessary to consider cloning of bloodstream trypanosomes, as the frequency of transfection will normally ensure that each well that grows up is a clone. Cloning by limiting dilution, that is, plating a theoretical 0.5 cells per well, is also possible should the need to clone arise. No adaptations to media are required for this procedure.

Procyclic cells are substantially more efficient to transfect, and hence for some purposes may be the stage of choice. Preparation of DNA is essentially the same as for bloodstream cells. For transfection into the PCF 29-13 cell line (*T7RNAP::TETR::NEO::HYG*), 20 μ g of linearized plasmid is added to 2×10^7 -log phase cells that have been resuspended in a total volume of 0.45 ml of Opti-MEM (Invitrogen). Thoroughly mix the sample with a P200 Gilson pipette, and then transfer to a precooled 2-mm gap electrocuvette (Bio-Rad), and chill on ice for 5 min prior to electroporation. Deliver a single pulse from a Bio-Rad Gene Pulser II electroporator (1.5 kV, 25 μ F). Immediately following electroporation, transfer the cells into 10 ml of SDM79/10%FCS containing 25 μ g/ml G418 and 25 μ g/ml hygromycin, and leave the culture to recover at 27° overnight. Approximately 16 h following electroporation, add phleomycin at 2.5 μ g/ml to select for transformants. Drug-resistant cell lines typically grow within 2 to 3 weeks. For transfection of wildtype 427 cells, the addition of G418 and hygromycin is omitted.

Procyclic trypanosomes can also be cloned by limiting dilution. Prepare 25 ml each of cells at one cell per milliliter and 0.2 cell per milliliter. Plate out as 24 1-ml cultures in a 24-well tissue culture plate, and inspect from 8 days onward up to 14 days. If eight wells or less contain trypanosomes on one of the plates, the well can be considered clonal. Cloning presents particular problems as procyclic trypanosomes do not tolerate dilution well, and either the use of conditioned medium or increased fetal bovine serum concentration and growth in CO₂ is required. Conditioned medium is prepared from a mid-log phase culture by centrifugation to remove most of the cells and filtering through a 0.22- μ m filter to remove residual cells and to resterilize. For cloning, use a mix of 60% fresh medium/40%

² Drug selection mix: hygromycin B stock; 5 mg/ml, G418 stock; 10 mg/ml. Store at -20°. For 10 flasks, make a working stock of 200 μ l hygromycin B and 80 μ l G418; 25 μ l in each flask gives a final concentration of 2.5 μ g/ml and 2 μ g/ml, respectively.

³ Cryopreservation mix: Mix cells in HMI9 plus 10% glycerol (sterile). Place in a suitable cryo-box or polystyrene box sealed with tape and place at -70° overnight. Move vials to a liquid nitrogen vessel for long-term storage.

conditioned medium. The alternative is to increase the fetal bovine serum to 15% and to grow in 5% CO₂. If this latter approach is used, then the selective concentration of any antibiotic may require retitering.

4. VECTOR SYSTEMS FOR OVEREXPRESSION AND RNA INTERFERENCE

In common with most biological systems, trypanosomes have specific elements that are required for gene transcription, and these elements must be included in ectopic expression systems. In addition, for inducible expression the T7 polymerase/tet-on system that has been frequently exploited has been successfully imported into trypanosomes (Wirtz and Clayton, 1995); this feature is particularly valuable for RNA interference, where tight control of potentially lethal dsRNAs is essential.

Unique features of trypanosome transcription include the complete lack of characterized RNA polymerase II promoters for protein-coding genes, the requirement for *trans*-splicing, and the importance of the 3' end in mRNA stability. For ectopic expression, these considerations have been incorporated into many vector designs (Fig. 5.1A). The pXS2 and pXS5 vectors (Alexander *et al.*, 2002; Bangs *et al.*, 1996) exploit RNA polymerase I promoters, together with intergenic regions from high-abundance transcripts/protein products, which provide the essential signals for *trans*-splicing and polyadenylation. pXS2 and pXS5 are also targeted to repetitive arrays within the genome, the tubulin locus on chromosome I, and the rRNA repeats. The expression cassettes are built into pBluescript backbones for easy propagation in *Escherichia coli*. In our lab, these vectors are highly reliable, producing between 3- and 10-fold overexpression of small GTPases compared to endogenous levels (Pal *et al.*, 2003), and such expression appears to be highly stable over a great number of generations. Overexpression of other constructs is also possible (Alexander *et al.*, 2002; Bangs *et al.*, 1996; Chung *et al.*, 2004). One possible complication with pXS2 is that variable transcriptional read-through occurs into the body of chromosome I, and in some cell lines increased levels of both tubulin mRNA and protein are observed, indicating that read-through transcripts can be processed and translated, although no obvious phenotype results (Dhir and Field, 2004; V. Koumandou and M. C. Field, unpublished data). It is not clear if this is a general phenomenon or specific to pXS2, and certainly care should be taken when selecting a vector to avoid potential artifacts. It should also be borne in mind that targeting to these arrays has the potential to result in differential expression depending on the precise context of the integration event. For this reason, we recommend that at least three clones or lines should be analyzed to ensure that the desired overexpression has been achieved.

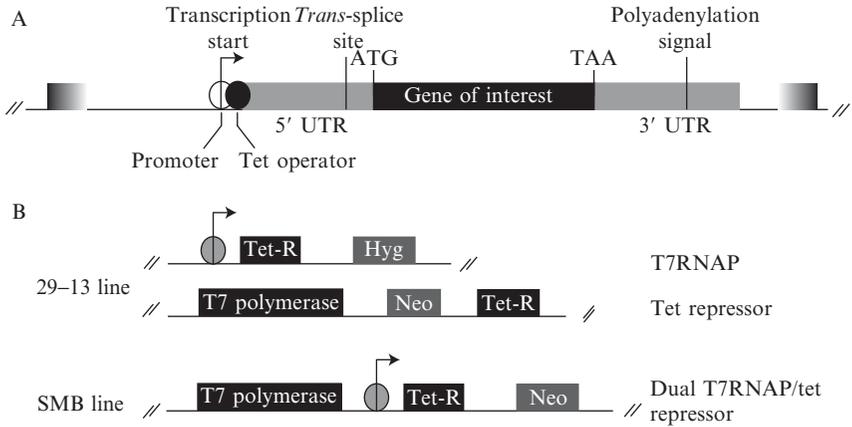


Figure 5.1 Features of expression systems in trypanosomes. (A) General features of trypanosome ectopic expression cassettes. Due to the polycistronic expression of most trypanosome protein-coding genes, a specialized set of vectors has been developed. These include a promoter (open oval), typically the Pol I promoter for VSG, procyclin, or rRNA, 5' sequence derived from a well-characterized gene (light gray), such as tubulin, which provides a 5' *trans*-splicing site for addition of the 39 nucleotide minixon, and a 3' sequence from a stable transcript (light gray), which also donates the polyadenylation site. In some instances, these promoters have been modified to include the Tet operator to facilitate conditional expression. The 3' end sequence is chosen carefully as this region also contains elements responsible for control of mRNA levels, and while these elements are poorly characterized, the behavior of such elements for the most part is predictable in terms of mRNA levels and developmental regulation. In addition, the promoter may be the T7 polymerase promoter, with the tet repressor binding site (operator, black oval) to facilitate inducible expression. Regions of more than 200 base pairs are normally used to facilitate faithful integration into the genome (gradient gray) and the gene of interest is shown as a black box. The double slash (//) indicates region of the construct that is not shown. The elements are not shown to scale. (B) Features of the tet-on inducible system for 90-13, SMB, and 29-13 lines. The 90-13 and 29-13 lines incorporate two elements, one carrying the tet repressor and the other the T7 RNA polymerase. Production of T7 pol and the downstream tet repressor ORF relies on read-through transcription. Production of tet repressor is also facilitated through a second element, which uses a crippled T7 promoter, with about 10% of normal transcriptional activity (gray oval). The SMB line was created by a two-step procedure to remove one of the selectable markers. The resulting cassette contains both the T7 polymerase and the tet repressor, and is maintained by neomycin/G418 selection. Again, transcription of the T7 polymerase relies on endogenous transcription, while the tet repressor is transcribed primarily from a 10% T7 promoter (gray oval). All of these elements are integrated into the tubulin repeat array on chromosome I, except the tet repressor construct in 29-13, which is in the RNP1 locus. The elements are not shown to scale; selectable markers are shown in a light gray box, and ORFs required for functioning of the transcription system in black boxes. The double slash (//) indicates sequence features not shown. (Based on data in [Wirtz, E., \(1999\)](#).)

The expression of transgenes is occasionally deleterious to the cell, a problem that can be overcome with a tightly regulated inducible promoter. This technology requires the use of a cell line engineered to produce T7 RNA polymerase and the tet repressor; several of these are now available and selections are described in [Tables 5.1 and 5.2](#). It should be noted that the inducible cell lines are suitable for both overexpression and RNAi experiments, as the basic expression cassettes are common (see [Fig. 5.1](#)). In all instances the various combinations of antibiotic must be continually maintained as expression of the T7 polymerase is not well tolerated and tends to be lost rapidly in the absence of selection.

A transgene cloned into the pLEW100 vector is expressed from an EP procyclin promoter that itself is regulated by two tet repressor binding sites ([Wirtz *et al.*, 1999](#)). The promoter is activated by the addition of tetracycline or doxycycline to the culture medium, the concentration can be used to titer expression, although this is technically challenging to achieve in a reproducible manner ([Wirtz *et al.*, 1999](#)). Routinely, an open reading frame is cloned into the HindIII and BamHI sites of pLEW100; the resultant chimeric mRNA has the EP procyclin 5'UTR, the inserted open reading frame, and a modified aldolase 3'UTR. Prior to electroporation, pLEW100-derived plasmids are linearized with the restriction enzyme NotI, and then targeted to the nontranscribed spacer in one of the rRNA gene loci. At maximum expression levels using 1 $\mu\text{g}/\text{ml}$ tetracycline, the transgene is usually expressed at about the level of an abundant cytoplasmic protein, such as the translation initiation factor eIF4A1 ([Dhalia *et al.*, 2006](#)). The promoter is silent in the absence of tetracycline and pLEW100, and its derivatives have been used for the regulated expression of several lethal dominant mutants ([Dhalia *et al.*, 2006](#)). Several labs have developed derivatives of pLEW100 to express transgenes with N- or C-terminal tags; epitope tags for detection and fluorescent proteins tag for subcellular localization. One such set of vectors is described at <http://web.mac.com/mc115/iWeb/mclab/resources.html>. The ability to use doxycycline also provides the possibility of using such systems in animal models, specifically mice and rats, as induction can be achieved by adding doxycycline to drinking water ([Lecordier *et al.*, 2005](#)).

A number of second-generation vectors and cell lines are available or are in development. These provide a range of options, a variety of epitope tags for N- or C-terminal tagging and improvements in ease of plasmid construction and transfection efficiency. A few examples are outlined below.

The p2T7 RNAi vector was modified for direct cloning of PCR products ([Alibu *et al.*, 2005](#)). This p2T7^{TAb_{luc}} vector has been used to screen more than 200 genes on *T. brucei* chromosome I ([Subramaniam *et al.*, 2006](#)), and is now available with a variety of selectable marker genes. The cloning site comprises a *LacZ* stuffer flanked by *Eam1105I* sites engineered for T-A cloning. The vector is digested with *Eam1105I* at 37° for

Table 5.2 Some Common *Trypanosoma brucei* Cell Lines Used in Expression and/or RNA Interference Studies

Name	Features	Applications	Used markers	Reference
927	Genome strain	Not commonly used	None	van Deursen <i>et al.</i>, 2001
Lister 427 SMB (328.114)	Common lab strain Bloodstream line	Overexpression, knockout Inducible expression/RNAi	None G418	Melville <i>et al.</i>, 2000 Wirtz <i>et al.</i>, 1999
29-13 ^a	Procyclic line	Inducible expression/RNAi	G418 and Hygro	Wirtz <i>et al.</i>, 1999
90-13	Bloodstream line	Inducible expression/RNAi	G418 and Hygro	Wirtz <i>et al.</i>, 1999
PTT	Procyclic line	Inducible expression/RNAi	G418 and Hygro	Bastin, P, unpublished
PTH	Procyclic line	Inducible expression (not T7)	Hygro	Bastin <i>et al.</i>, 1999
PTP	Procyclic line	Inducible expression (not T7)	Phleo	Wickstead <i>et al.</i>, 2003
2T1	Bloodstream line with tagged <i>rRNA</i> spacer	Inducible/constitutive expression/RNAi	Phleo and Puro ^b	Alsford <i>et al.</i>, 2005

^a If this strain is not cultured carefully, it can have poor morphology that may be problematic for morphometric and cell cycle analysis. The PTT cell line is superior for this type of analysis, and is essentially identical in all other regards.

^b The *PAC* gene is lost from the genome upon correct integration of the expression/RNAi vector.

Table 5.3 Selectable Markers in Use for Transfection of Trypanosomes

Antibiotic	Concentration ($\mu\text{g/ml}$)	
	Procyclic form	Bloodstream form
Geneticin (neomycin/G418)	15	1
Hygromycin	25	5
Phleomycin (zeocin/bleomycin)	2.5	1
Nourseothricin	100	25
Puromycin	1.0	0.1
Blasticidin	10	10

Note: These concentrations are based on experiments with *Trypanosoma brucei brucei* Lister 427 lines cultured in HMI-9 (bloodstream form) or SDM79 (procyclic form), and hence may require adapting for other strains, subspecies or media.

1 to 2 h in preparation for ligation with RNAi target fragments with 3' A-overhanging ends. We typically use *Taq* DNA polymerase for target fragment amplification. In *E. coli*, on plates containing X-gal and IPTG, uncut or re-ligated vector-containing cells form blue colonies, while the desired recombinants generate white colonies.

Targeted integration at a tagged *rRNA* spacer locus in the 2T1 cell line has three significant advantages (Alsford *et al.*, 2005). Transformation efficiency is increased approximately four-fold, such that multiple clones are derived using $\sim 5 \mu\text{g}$ of linearized DNA. Faithful double-crossover integration is rapidly verified by screening for puromycin-sensitivity in multiwell plates, and a *PAC* gene is removed from the genome upon correct integration. And finally, all clones analyzed to date exhibit equivalent expression; consequently, screening multiple clones for differential expression is not required (D. Horn and S. Alsford, unpublished data). Recovery of cells with expression cassettes integrated at the correct locus is ensured since complementing portions of the selectable marker are located on the vector and within the 2T1 genome. We typically use a 2T1 strain with a tagged *rRNA* spacer to support the highest expression level because, in most cases, it is desirable to express the maximum quantity of recombinant mRNA or dsRNA in the case of RNAi. There are now a number of 2T1-compatible expression constructs available. These are mostly driven by the *rRNA* promoter, and therefore do not require parallel expression of T7 RNA polymerase; the vector series includes constructs for constitutive or inducible expression and for RNAi. The cloning sites are versatile, and they are linearized for integration using *AscI*.

For Tet-inducible expression of a tagged protein, we typically allow 24 h induction prior to analysis at which point expression is usually maximal with rare cases of toxicity resulting from overexpression. The

situation is different for RNAi experiments, where it is important to carefully consider the timeframe between Tet-addition (RNAi-induction) and specific phenotype assay(s). “Growth curve” analysis can act as a guide if a growth defect is seen. In these circumstances, it is desirable to carry out specific phenotype assays prior to, or immediately after, the defect is seen to reveal primary rather than secondary phenotypes. Monitoring mRNA and/or protein knockdown is also important and can act as a guide where no growth defect is seen. If more rapid and efficient knockdown is required, this can be achieved with “stem-loop” dsRNA vectors, probably due to the more stable intramolecular interaction, but assembly of these vectors is more complex. It is also important to note that the RNAi effect may be lost over time using any of these systems. If RNAi is deleterious to growth, cells that lose a component of the RNAi machinery will come to predominate in the population following an extended period of induction (Chen *et al.*, 2003).

5. CLONING AND MANIPULATION OF TRYPANOSOME SMALL GTPASE SEQUENCES

Trypanosome small GTPases fully conserve the overall primary structure of the Ras superfamily, retaining G boxes one to five, the hypervariable region, and, in most cases, the C-terminal prenylation motif. Some deviation from this structure is apparent for several GTPases (see Field, 2005 and Fig. 5.2), but no functional analysis is available on these factors at present.

Construction and overexpression of either wildtype or mutant forms of trypanosome small GTPases is best achieved by constitutive ectopic expression, and provides a convenient method for both localization and analysis of function. The pXS2 or pXS5 vector series have proven to be highly reliable for this purpose, and in the vast majority of cases transformation and overexpression are readily achieved; no specific selection of host strain is required for these vectors. The constitutive approach is preferred over inducible systems here as precise control of the levels of induction, together with epigenetic effects, as the parasite adjusts to an increase or decrease in activity of the transgene make reproducible control of the system difficult using induction; a serious issue if detailed analysis of function is intended, for example, by trafficking assays. Recent work indicates that manipulation of Rab5 expression levels also influences the expression of clathrin heavy chain (CLH), without altering the levels of CLH mRNA, indicating a post-translational effect that would be challenging to control in an inducible format (Hall *et al.*, 2004; S. Natesan, V. Koumandou, and M. C. Field, unpublished data). Monitoring of overexpression is important, as occasional mistargeting or other factors can compromise function of the introduced transgene—this is

	G1 (P loop) GXXXGK [S/T] S - N	G3 (switch 3) DXXG Q - L
TbX1	KIIVIGDSGVGKSSLTVRLSEDEV	----LQIWDTAGQERFQSVATAFYR
TbX2	KIIVIGDVGVGKSNISSRFCDSI	--ILLQIWDTSQODRFVSLTTAYYR
Tb1A	KLLLIGDSGVGKSCLLLRFADDS	KVIKIQIWDTAGQERFRTITSSYYR
Tb18	KIVLLGESGVGKSSLLLSFSLGT	KKLKHLHWDTAGQERFRTLTSYYR
Tb2B	KYIIIGDSGVGKSCLLLQFTDKR	KNIKLQIWDTAGQESFRSITRSYYR
Tb4	KLIVVGDSTGKSSLLHRFVEDT	RRIKLQIWDTAGQERYKSVTRSYR
Tb11	KVVIVGDSGVGKSNLMTRYTADE	RDAKVQIWDTAGQERFRAISRSIYH
Tb5B	KIVLLGDSGVGKSSLVQRLAKNE	--VNFDIWDTAGQERYKSLASMYR
Tb5A	RTVLLGESAVGKSSIALRFARNE	GTIKFEIWDTAGQERYRSLAPIYYR
Tb7	KIIILGDSGVGKTALVHQYVNKN	--VTLQIWDTAGQERFQSLGSIFYR
Tb21	KVLLGEGRVGKTSLSISRNVNDT	KTVNLMVWDTAGQERFHALGPIYYR
Tb6	KIVLLGDQAVGKTSLITRFMYDT	RATRLHVWDTAGQERFRSLIPSYIR
Tb1B	KIILVGDSEYVGTTRFLKNLVGAI	KTVQVLMYDTCGQERFRAMTAQFYR
Tb23	KVIVIGDENVGKSSLLRRFVTGT	TTVNSFLWDTAG-ETVSSVKDIYYL
TbX3	KVILLGDSAVGKSKLVERFLMQR	-AIDVDIWDTAGQERFSTMPHAYYH
Tb28	KVIVVGDGAVGKTSLIRRYCVAD	-DVKMEIWDIGGQQIGGTMIDNYIM
TbRX3	KIAVVDYNVGKTSIVKRLLDIP	QHVRLQFWDTAGLERYASVHD----
TbRX2	KVAIVGDYVGVKTSMVKRLLDIP	QHVRLQLWDTAGLERYASVDK----
TbRHP	KVLLGEGRVGKTSLSISRNVNDT	KTVNLMVWDTAGQERFHALGP----
TbRX1	QVAVVGAPTGVGKTAFFVQMLHSNG	NTVEMIIFDVSGQREYEPMVS----
TbRLJ	KIVSLGDSGVGKSCLIKQYCEGR	ISTRVNFWDMSGCEEYLEIRN----
TbRLP	NLVVLGDGGVGKSSLI IQYVNRN	QPTVLTIVDTSQODVFGGMRV----
TbRHP	NVVVLGCERVGKSTFIDQVMKGT	RNYVLHLCDSGSEAFVRHRL----
TbGTR	KLLLMGPARAGKTSMSRIIFDNY	NMYYNLWDCGGQQQYVAEYLNQR
TbGRP	KVLLMGLRKSQKTSIQKVFEGM	DFVNFEVWDFPGQTDPFDLNNTVHY
TbSAR	KILFLGLDNAGKTTLLGKLATDQ	--IKLKTIDMGHLEARRLWK----
TbNST	IPALRGSNKIAAASGEKEPQEQG	IGESENINDMDDVVERRGVDRSLR-
TbEAR	RIAIVGRMNSGKSLFNLLRLEP	RMVEEAFRTVETADAIAIFVTAVDED
TbFRP	RVCGRVFCASACCSETVNALVQSA	QQGQVQFMNPTTVAAYRVCFACHYE

Figure 5.2 Key residues in trypanosome Rab and signaling class GTPases. Portions of a ClustalX alignment are shown highlighting the GVGKS (G1) and WDTAGQ (G3) boxes that are highly conserved between small GTPases—the critical residues are shaded. The complete Rab family together with the majority of the signaling GTPases are included (see [Ackers *et al.*, 2005](#); and [Field, 2005](#)). These regions may be exploited for the construction of S- to N-dominant negative “GDP-locked,” and Q to L constitutively active “GTP-locked” forms of the proteins for overexpression studies (e.g., [Pal *et al.*, 2003](#)). A minority of the GTPases are divergent within the WDTAGQ region, but most Ras-, Rho-, Rac-, and Rab-related GTPases retain the consensus sequence.

most conveniently done either by introducing an N-terminal tag, using an antibody raised against the recombinant protein, or by qRT-PCR.

By contrast, RNAi experiments are always conducted using inducible systems, and hence must be performed in the SMB, 29-13, or equivalent cell background. It is critical that the expression level of the targeted gene of interest is monitored. This is required both for ensuring specificity, that is, that the intended target has been suppressed and to eliminate off-target effects,

and also for monitoring the progress of the emergence of a phenotype. In bloodstream stage cells, effects from RNAi normally manifest within 1 to 2 days, but longer times of up to 5 days are not uncommon (see [Subramaniam et al., 2006](#), and trypanofan.path.cam.ac.uk for examples), and in procyclics knockdown can be even more protracted. Monitoring knockdown by Western blot is preferable, as it is the level of protein that is important for phenotype, but in the absence of such a reagent, qRT-PCR is rapid, convenient, and accurate; however, such data need to be considered carefully as the half-life of small GTPases in trypanosomes can vary considerably. For the majority of trypanosome small GTPases, RNAi-mediated knockdown results in severe growth inhibition ([Dhir and Field, 2004](#); [Hall et al., 2004, 2005a, b](#); K Abbassi, and M. C. Field, unpublished data).

Construction of expression constructs can be achieved by PCR very readily. As trypanosome open reading frames lack introns, recovery of sequences is most easily achieved by amplification direct from genomic DNA, and subcloning into a convenient vector, followed by the required mutagenesis. It is recommended that a prolonged hot start for up to 30 min is employed when using trypanosome genomic DNA, but because of the small size of the GTPase open reading frame, a proofreading polymerase is not necessary and Taq can be used, providing that the resulting construct is fully sequenced. Sequences can be retrieved from GeneDB at www.genedb.org/genedb/tryp/. As the genome strain (TREU 927) is not the same as the common laboratory strain, it is to be expected that a small number of single nucleotide polymorphisms will be detected between the database sequence and that obtained by PCR from strain 427 DNA. The vast majority are noncoding, and any concerns may be allayed by also sequencing the PCR product directly. There is no significant base bias or abnormal codon usage in the African trypanosome, and hence design of mutagenic oligonucleotides can follow standard protocols. In addition, expression of trypanosome small GTPases as GST-fusion proteins is also standard, and there is no need for specialized host strains for production of recombinant material, either for immunization or for biochemistry on purified protein; however, we do recommend the use of BL21 (DE5) or Rosetta strains of *E. coli* for expression to improve both yield and solubility. Yields of 1 mg/liter of the GTPase can be easily attained.



6. TRAFFICKING ASSAYS TO MONITOR EXOCYTOSIS AND ENDOCYTOSIS

The analysis of bulk exocytosis in trypanosomes is comparatively straightforward as the GPI-anchored surface antigen, variant surface glycoprotein (VSG), is very highly expressed, and represents ~80% of exported

protein. For endocytosis only, the transferrin receptor represents a well-characterized system for receptor-mediated endocytosis, while a second assay, uptake of the mannose-binding lectin concanavalin A is also useful. As VSG represents 90% of surface molecules and is bound by conA, this assay mainly reports on VSG uptake. Using the lectin has the advantage that VSG-specific antibodies are not required, which for the majority of VSG isoforms are unavailable. Protocols for analysis by fluorescence microscopy can be found in [Field *et al.* \(2004\)](#).

Export of VSG to the cell surface uses accessibility of surface VSG to GPI-phospholipase C (GPI-PLC) ([Allen *et al.*, 2003](#); [Bangs *et al.*, 1986](#)); the supernatant following GPI-PLC autodigestion contains soluble VSG (sVSG) but internal (mf) VSG remains in the pellet fraction. Take 5×10^7 mid-log phase BSF cells, wash in labeling medium (DMEM without methionine or cysteine) prewarmed to 37°. After centrifugation (800×g, 10 min), resuspend in 1 ml labeling medium, and incubate for 15 min to starve the cells. Pulse label at 37° for 5 to 10 min with [³⁵S] methionine/cysteine ProMix (Amersham Biosciences, >1000 Ci/mm) to a final concentration of 200 μCi/ml, and chase by adding prewarmed complete HMI9. Withdraw 1-ml aliquots and place on ice. Pellet cells in a microfuge (20,000×g, 20 s, 4°), wash once in ice-cold PBS/1 mg/ml BSA, and resuspend in 920 μl of hypotonic lysis buffer (10 mM Tris-HCl, pH 7.5). Incubate for 5 min on ice followed by 10 min at 37° to enable GPI-PLC autodigestion. Separate mfVSG from sVSG by centrifuging for 10 min in a microfuge (20,000×g, 4°), and remove 900 μl of supernatant (sVSG) to a new eppendorf. Wash the pellet (mfVSG) with 1 ml ice-cold hypotonic buffer, and resuspend in 1 ml ice-cold solubilization buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40), and incubate on ice for 25 min. Add 90 μl of 10× the solubilization buffer to the supernatant to bring samples to equivalence. Clear the lysates by centrifugation for 15 min (20,000×g, 4°) and transfer to new microfuge tubes. Include 5 mM iodoacetamide, 0.1 mM N α-p-tosyllysine chloromethyl ketone (TLCK), and 1 μg/ml leupeptin as peptidase inhibitors. Add 10 μl of a 50% slurry of ConA-sepharose 4B in Con A wash buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM CaCl₂ and 1 mM MnCl₂) to each sample, and rotate for 1 h at 4°. Pellet the beads and wash three times in ice-cold Con A wash buffer, and then once in ice-cold PBS. Following the final wash, resuspend the beads in SDS-PAGE, reducing sample buffer at 1×10^5 cell equivalents per microliter. Analyze by electrophoresis and autoradiography on 12% polyacrylamide gels.

Quantitation of FITC-transferrin (Molecular Probes) accumulation, that is, receptor-mediated endocytosis, may be conveniently performed using smaller numbers of cells. Serum (including transferrin) must be removed prior to the assay by washing the cells at least once in serum-free HMI9 medium containing 1% BSA. The cells can be resuspended in HMI9/BSA

at 1×10^7 ml⁻¹ and preincubated at 37° for 20 min. FITC-transferrin (100 µg/ml) is added, and cells are incubated at 37°. Uptake is stopped by either the addition of 1 ml ice-cold HMI9 medium or by withdrawing an aliquot from the labeling culture and adding this to 1 ml ice-cold HMI9 medium. Cells are then washed once in PBS at 4° before fixing for 1 h at 4° in 4% paraformaldehyde, and processing for immunofluorescence (Field *et al.*, 2004). The procedure for conA accumulation is essentially the same. Following washing, biotin or FITC-conjugated conA (100 µg/ml) (Vectalabs) is added and the cells incubated at 37° for up to 1 h. Uptake is stopped by placing cells on ice at relevant time points. Labeled cells are washed in HMI9/BSA at 4°, then fixed with 4% paraformaldehyde as described above. For biotinylated probes, the lectin is visualized using FITC-streptavidin (Molecular Probes). Quantitation for both of these assays is done by defining regions of interest in captured digital images and determining the fluorescence intensity. Typically, at least 20 individual cells need to be examined for each data point. The assay has the distinct advantage that analysis is at the single-cell level; as RNAi knockdown is not synchronous, and cell populations are therefore heterogeneous, this flexibility is critical and allows interrogation of cells specifically where a morphological defect or loss of a marker protein (probed by co-staining with an antibody, for example) can be used to verify the degree of knockdown.

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WEB RESOURCES

Useful sites for details on vectors, protocols and genome data:

<http://trypanofan.path.cam.ac.uk>. Maintained by Mark Field. A functional genomics website describing the data obtained from RNAi analysis of T. brucei chromosome I, together with several protocols.

- <http://trysps.rockefeller.edu/>. Maintained by George A. M. Cross. This laboratory developed a number of important aspects of transfection and inducible expression, and the website offers extensive details on a number of available vectors and protocols.
- <http://homepages.lshmt.ac.uk/~ipmbdhor/dhhome.htm>. Maintained by David Horn. Contains details of a number of second-generation vectors.
- <http://web.mac.com/mc115/iweb/mclab/home.html>. Maintained by Mark Carrington. Contains details of a number of second-generation vectors designed using the pLEW100 inducible system.
- <http://www.genedb.org/genedb/tryp/>. Maintained by the Sanger Institute. An excellent and well-curated resource for trypanosome genome annotation and sequence data.
- <http://www.izb.unibe.ch/res/seebeck/taggingvectors.php>. Maintained by Thomas Seebeck. Provides details of vectors for in situ tagging. Only suitable for procyclic (insect) form due to transfection efficiency.