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Research Brief

Cell density-dependent ectopic expression in bloodstream form *Trypanosoma brucei*

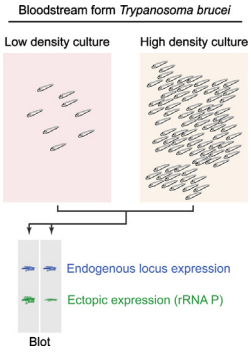
Moazzam Ali, Mark C. Field *

Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, UK

HIGHLIGHTS

- Ectopic expression is a standard tool for molecular cell biology in trypanosomes.
- Ectopic expression of a range of proteins derived from rRNA promoters depends on cell density.
- The effect on expression is potentially mediated by a diffusible factor.
- Density-dependent expression was not observed in insect stage parasites.

GRAPHICAL ABSTRACT



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ABSTRACT

Ectopic expression of either wild type or mutant proteins is a standard method in cell biology, and a vital part of the tool kit of molecular parasitology. During study of protein expression levels mediating intracellular trafficking, we became aware of highly variable expression between experiments. When investigated systematically it became apparent that ectopic expression of proteins from a ribosomal promoter diminished at high cell culture density in bloodstream form *Trypanosoma brucei*. This phenomenon was not restricted to expression of a specific protein or cell line or the vector backbone. While procyclic form cells did not exhibit detectable density-related expression changes, bloodstream form cells manifest significant reduction in expression at high density, confirmed by qRT PCR, Western blotting and fluorescence microscopy. Culturing in conditioned media unveiled a similar reduction in expression at lower cell densities. Taken together we concluded that this effect is likely related to the influence of a diffusible factor present in conditioned media and has implications for accurate quantification of ectopic expression using transgenic expression systems.

1. Introduction

Ectopic expression is one of the most widely exploited tools for the functional analysis of gene products in cells, for example, by over-expression of a protein to elicit a phenotype or expression of mutant forms of proteins to specifically block processes and map sequence to function. Further, this is a strategy that is convenient where antibodies are unavailable and/or genomic tagging is not possible due to low endogenous expression or other issues. Ectopic expression is generally assumed to be constitutive under most experimental conditions, but is rarely analysed in detail. Frequently, such expression is driven by constitutive promoters inserted into the genome in regions distinct from their original location, and the assumption is that these elements act as essentially isolated units, being designed to encompass all splicing sites and mRNA processing signals required. If variable expression is required inducible systems are employed.

In trypanosomes, while the range of reagents available is substantially fewer than mammalian cells, an impressive range of plasmids have been produced by many groups to facilitate both

* Corresponding author. Present address: Institute of Biochemistry and Biotechnology, University of the Punjab, Lahore, Pakistan.
E-mail address: mcf34@cam.ac.uk (M. Ali).

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constitutive and inducible ectopic expression (Quijada et al., 2002; Wirtz et al., 1998; LaCount et al., 2002; Kelly et al., 2007). This strategy is part of the standard toolbox for the study of gene and protein function in trypanosomes, and has been exploited extensively. As part of recent work we have become specifically interested in analysis of the effects of over-expression or RNAi on proteins in trans, i.e. analysing the effect of alteration of copy number of one gene product on copy number of a second (e.g. Lumb et al., 2011) to infer functional interaction. We have also been addressing effects on turnover and protein stability resulting from manipulation of a second protein (e.g. Leung et al., 2011). In many cases the analysed protein is ectopically expressed to overcome issues with poor detection from suboptimal antibodies or low endogenous copy number, as well as to avoid, in many cases, generating an antibody reagent for a limited set of experiments. Again, this is also a standard approach in studies in model organisms and parasites.

During investigations of the functions of small GTPases in the endosomal system, and Rab21 in particular (MA and MCF, in preparation), we observed highly variable expression of various reporter proteins. When analysing localisation such issues are either unimportant or non-apparent. For example, in mammalian cells transient transfection is frequently used and results in heterogenous expression levels across a population of cells. However, in trypanosomes, transfectants are frequently cloned, so that clonal variability becomes more prominent. As this phenomenon was of potential significance to our ongoing studies we chose to investigate this phenomenon in using a systematic approach. Specifically, we asked if the effect depended on the specific protein being expressed, if this were due to a specific vector or if the phenomenon depended on the life stage being considered. Finally we sought to determine the conditions that led to the effect.

We found that ectopic expression in *Trypanosoma brucei* bloodstream stages, driven by an rRNA promoter, was reduced significantly with increasing culture density. This was not because of a generalised transcription/translation arrest, as endogenous short half-life control protein expression levels remained unchanged. The rRNA promoter is widely used for constitutive ectopic expression and this observation is therefore of general interest to the field of *T. brucei* cell biology and genetics.

2. Materials and methods

2.1. Culturing of T. brucei cell lines

T. brucei bloodstream form cells (Lister 427) were cultured in HMI-9 supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 U/ml streptomycin and 2 mM L-glutamine) at 37 °C with 5% CO_2 in flasks with vented caps (Hirumi and Hirumi, 1989). Procyclic form cells were cultured in SDM79 supplemented with 10% fetal bovine serum, penicillin, streptomycin, L-glutamine and haemin (Brun and Schonenberger, 1979). Ectopic expression was maintained using antibiotic selection. The CELLSPIN system from Integra Biosciences, Switzerland was used to agitate BSF cultures. 1 l spinner flasks were used to spin the cell culture at 50 rpm in a standard cell culture incubator. Cell density was counted using haemocytometer or Z2 Coulter Counter (Beckman Coulter), averaging three measurements.

2.2. Nucleic acid extraction

For genomic DNA isolation from BSF cell, 1.5×10^7 cells were harvested by centrifugation, followed by washing with PBS. The cell pellet was resuspended in 300 l of TELT buffer (50 mM TrisCl pH 8.0, 62.5 mM EDTA, 2.5 M LiCl and 4% v/v Triton X-100) and mixed with 300 l of phenol:chloroform:isoamyl alcohol mix (25:24:1, v/v/v Sigma). The aqueous phase, separated by centrifugation was mixed with 0.7 volumes of isopropanol to precipitate DNA. For RNA extraction 2×10^7 *T. brucei* (BSF or PCF) cells were harvested by centrifugation at 800*g* for 10 min. The cell pellet was washed once with PBS and snap frozen in dry ice. Total RNA was purified using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA concentration and purity was determined using a NanoDrop spectrophotometer (Thermo Fischer Scientific).

2.3. Quantitative reverse transcription PCR

First strand cDNA was synthesized using SuperScript™ III Reverse Transcriptase (Invitrogen). Quantitative real time PCR was performed using iQ™ SYBR Green Supermix set on a MiniOpticon Real-Time PCR System, both from Bio-Rad. Primers were; qRT YFP F CGACCACTACCAGCAGAACA, qRT YFP R GAACTCCAGCAGGACCATGT, qRT mCherry F CCCCGTAATGCAGAAGAAGA, qRT mCherry R CTTGGCCATGTAGGTGGTCT, qRT Rab 21 F CGTTCCATTGCTTAACAGTGAC, qRT Rab21 R CACATCGTAGACGAGGATTGC. All other primers were as described previously; TbVps23 (Leung et al., 2008), TERT and PRF2 (Brenndorfer and Boshart, 2010). The normalised expression (ΔΔCt) of mRNA was determined using Bio-Rad CFX manager software.

2.4. Generation of transgenic T. brucei

For ectopic expression pHD1034 or pXS5 were used to clone fluorescent protein genes or N-terminal epitope tagged proteins. pHD1034 was used for overexpression in both life forms while pXS5 is specific for BSF. For ectopic expression TbRab21 was amplified from genomic DNA using primers RAB 21f TAAAGCTTCTCGGTCGTCGGTGCCTAGT, RAB 21r CGGATCCTCAGGAACAACAGCGGTTC and cloned into pHD1034 containing an N terminal HA-tag. Expression constructs for YFP, mCherry and HA-TbVps23 were described earlier (Leung et al., 2008; Lumb et al., 2011). *T. brucei* bloodstream form cells were transfected with 10 μg NotI linearised plasmids. An Amaxa Nucleofector™ device was used for transfection together with the Amaxa Human T cell nucleofector kit. Cells were selected for 5–6 days using puromycin 0.2 g/ml, and/or phleomycin 1 g/ml as appropriate (Field et al., 2008). PCF cells were transfected as described (Hall et al., 2004).

2.5. Protein electrophoresis and western blotting

For lysate preparation, cells were harvested by centrifugation at 800*g* for 10 min at 4 °C, washed in PBS and resuspended in SDS sample buffer before heating at 95 °C for 10 min. Samples were electrophoresed on 10% SDS–PAGE minigels at 5×10^6 cell equivalents per lane and then transferred to PVDF membranes (Millipore) by wet blot in transfer buffer (192 mM glycine, 25 mM Tris and 20% (v/v) methanol). Ponceau S staining was performed to monitor loading, by shaking membranes in Ponceau S solution (0.1% Ponceau S in 5% (v/v) acetic acid, Sigma) for 5 min and washing off excess stain with Milli-Q water.

Membranes were blocked and processed following standard procedures. Primary antibodies were used at the following dilutions; Rabbit anti-GFP (Abcam) 1:20,000, mouse anti-HA (Santa Cruz Biotechnology, Inc) 1:2,000, rabbit anti-ISG75 (from M Carrington, University of Cambridge, UK) 1:10,000 and rabbit anti TbRab11 (Jeffries et al., 2001) 1:2,000. Secondary antibodies; peroxidase goat anti-rabbit conjugate or peroxidase rabbit anti-mouse conjugate (Sigma) were used at 1:20,000. Bound antibodies were detected by reaction with luminol and visualized by exposure to Kodak BioMax MR X-ray film or using a G:BOX CCD chemiluminescence imaging