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Short communication

A novel epitope tag system to study protein targeting and organelle biogenesis in *Trypanosoma brucei*

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African trypanosomes are now susceptible to study by a variety of genetic tools permitting the analysis of their gene expression, regulation and function [1]. Genes may be overexpressed [2], switched on or switched off using inducible expression vectors [3] or knocked-out by homologous recombination [4]. However, two major drawbacks of these techniques are (1) the difficulty of distinguishing the protein products of the transfected gene from the endogenous cellular protein components, (2) the definition of the precise cytological location of the protein products of the transfected gene. We have therefore developed an effective and versatile epitope tag system which allows the identification and the cellular location of the protein products of transfected genes. This system has great potential in the future for the dissection of protein localisation signals and the analysis of organelle biogenesis.

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We have chosen to develop as an epitope tag a 10 amino acid sequence from the immunologically well characterised major structural protein of the *Saccharomyces cerevisiae* Ty1 virus-like particle [5] for a number of reasons. Firstly, previous study in this laboratory has shown that it is a linear epitope, whose amino acid sequence has been defined [5], and that the epitope is recognised in different mutant forms of the protein suggesting that it can be recognised in various environments [6]. Secondly, this epitope is recognised by two different monoclonal antibodies with overlapping binding sites: BB2, which is an immunoglobulin G (IgG1) and TYG 5, which is an immunoglobulin A [5]. This is quite infrequent and promises to be a very useful combination for double labelling in immunofluorescence or immunoelectron microscopy. Moreover, both monoclonal antibodies have been shown to recognise well their epitope in a wide variety of immunological assays including immunofluorescence, immunoblotting, immuno-precipitation and ELISA [5]. Finally, neither antibody cross-reacts with trypanosomal proteins.

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In an initial evaluation of this system, we have chosen to introduce the epitope tag sequence into two different trypanosome genes: the β-tubulin gene and the paraflagellar rod (PFR) gene A. The proteins encoded by these genes represent components of well defined organelles in trypanosomes (microtubules and the paraflagellar rod respectively [7,8]) with a precisely characterised cellular location. A variety of monoclonal and polyclonal antibodies recognising these structures are available, as well as the genetic information for the major protein components [9,10]. The complete nucleotide coding sequence (start codon to stop codon) of the β-tubulin gene and of the paraflagellar rod (PFR) gene A were amplified by polymerase chain reaction (PCR) and each cloned in a pBluescriptII KS vector. For the insertion of the epitope tag, two oligonucleotides were designed based on the amino acid sequence (GluValHisThrAsnGlnAspProLeuAsp) [5] of the chosen Ty1 epitope: 5′GAGGTCCATACTAACCAGGATCCACTTGAC3′ (sense) and 5′GTCAAGTGGATCCTGGTTAGTATGGACCTC3′ (antisense). These two complementary oligonucleotides were annealed and the resulting double stranded 30-mer was ligated into the unique *Eco*RV site of the β-tubulin gene (position 1065) or of the PFR gene A (position 75). These plasmids have been named pKSTUBTAG1 and pKSPFRATAG1 respectively. In both cases, the insertion site is between codons, such that there is no disruption of the reading frame. These tagged genes were subsequently cloned in a trypanosome transient expression vector containing the promoter and the 3′ untranslated region of the procyclin gene [11] (pKMTUBTAG1 for β-tubulin and pKMPFRATAG1 for PFR A, Fig. 1). The tag sequence was also cloned at the 3′ end of the β-tubulin gene by PCR using a hybrid oligonucleotide containing the last 20 nucleotides of the β-tubulin gene and the tag sequence followed by a stop codon. This plasmid was called pKMTUBTAG2 (not shown).

To assay expression of tagged proteins, 3×10^7 procyclic *T. brucei* (strain 427) were transiently transfected [12] with 50 μg of pKMTUBTAG1, containing the tagged β-tubulin gene. After overnight growth, the cells were spread onto slides, air dried, fixed in methanol at −20°C and processed for immunofluorescence using the BB2 anti-tag antibody as the primary antibody. Fig. 1(A) shows a representative image: whilst most cells are negative with BB2, a small proportion ($\approx 0.1\%$) are strongly labelled. The same result was obtained when the tag was placed at the 3′ end of the gene (plasmid pKMTUBTAG2, not shown). Far more striking was the result obtained with cells transfected with the plasmid pKMPFRATAG1 containing the tagged PFR A gene (Fig. 1B). In these cells labelled with the monoclonal antibody BB2, an intense staining was observed exclusively localised in the flagellum, the expected localisation of the paraflagellar rod proteins. Clearly the tagged protein was strongly expressed and associated closely with the PFR. This was confirmed by detergent treatment of such slides, a method that removes soluble proteins leaving only trypanosome cytoskeletons [13]. In these conditions, the PFR staining is still present, indicating that the tagged PFR protein was associated within the PFR structure. Double labelling of these cells with the monoclonal antibody TYG 5 (also against the tag) and a monoclonal antibody against the main PFR proteins demonstrated perfect co-localisation of the tagged and normal PFR proteins (not shown). Finally, when trypanosomes were transfected with pKSTUBTAG1 or pKSPFRATAG1 (i.e. the tagged genes without promoter or 3′UTR) as control, no positive cells at all were detected (not shown). This demonstrates that the tag can be correctly incorporated into cellular proteins, its expression readily visualised, and can be used to follow correct localisation of exogenous manipulated expression products. It also confirms that the tag sequence itself does not contain any cryptic addressing signal for any organelles in trypanosomes.

Analyses of the construction of cellular organelles may require both stable and inducible expression of intact and genetically altered components. We therefore assessed whether the tag had any toxic effect on the cell. Thus we cloned the tagged PFR gene into the expression vector pHD 451 (kind gift from C. Clayton, Heidelberg) [3], that contains the gene for resistance to the

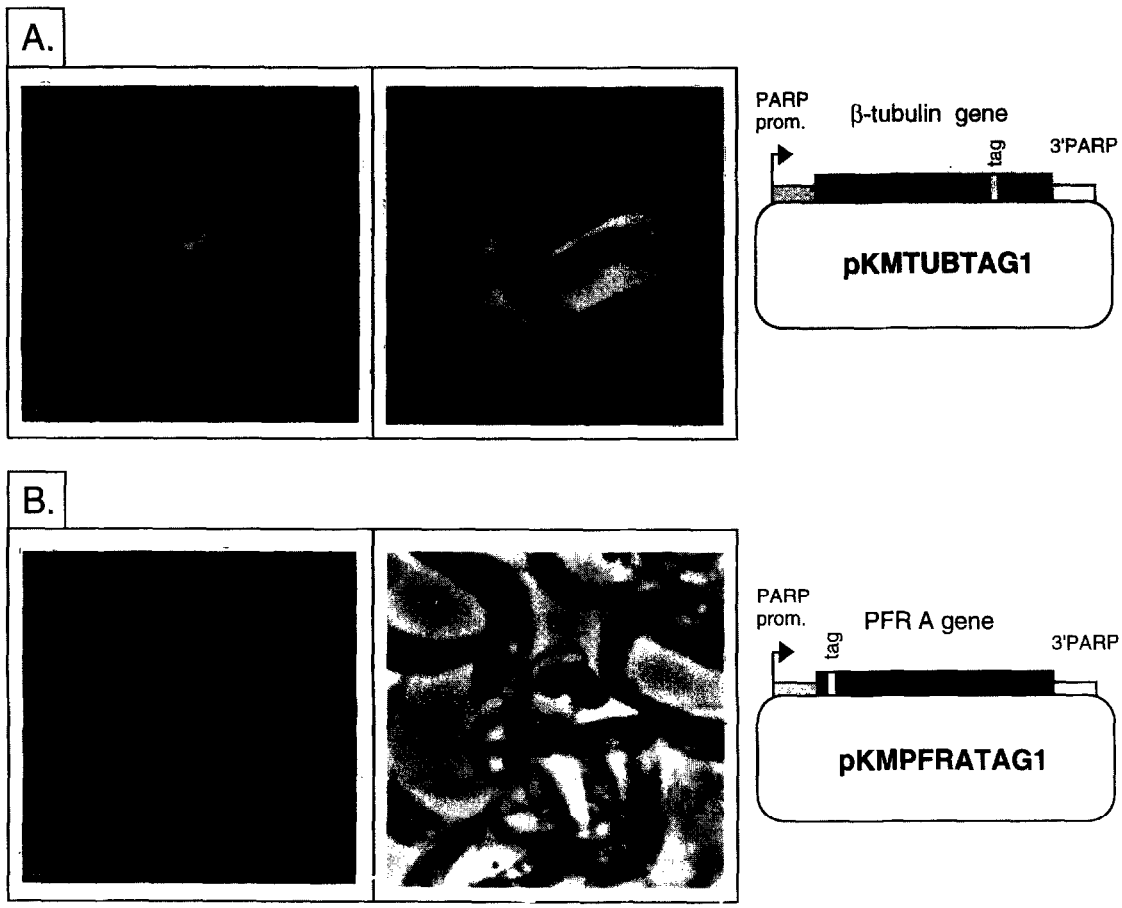

Fig. 1. Transient transfection of trypanosomes with pKMTUBTAG1 (upper panel, A) or with pKMPFRATAG1 (lower panel, B), grown overnight, fixed and processed for immunofluorescence with the monoclonal antibody BB2. Left, fluorescein isothiocyanate pattern; right, corresponding phase pattern. Bar represents 10 μm. Schematic representation of the transfected plasmids is shown on the right.

antibiotic hygromycin as a selectable marker (Fig. 2). For technical reasons, the last 42 nucleotides of the PFR coding sequence had to be deleted. The plasmid was called p451PFRATAG1, linearised through the *Not* I site of the inverted rRNA spacer and transfected into procyclic *T. brucei*. Cell lines resistant to hygromycin were obtained, cloned by limiting dilution and analysed by immunofluorescence and immunoblotting (Fig. 2). For every cell line tested with the monoclonal antibody BB2, all cells were almost equally labelled and exclusively in the flagellum, the expected localisation of the PFR protein (Fig. 2A). Fig. 2(B) shows an immunoblot of total protein samples from untransfected and from transfected trypanosomes, probed with the monoclonal antibody BB2 recognising the epitope tag. A single band is detected, at the expected Mr of PFR A ($\approx$ 70,000) only in the transfected cell line. These cells express high level of the tagged PFR protein but grow at the same rate and show the same morphology as the untransfected cell line. Thus, expression of the tag *per se* is not toxic to the cells.

In conclusion, we have developed a novel epitope tag system that allows rapid identification of

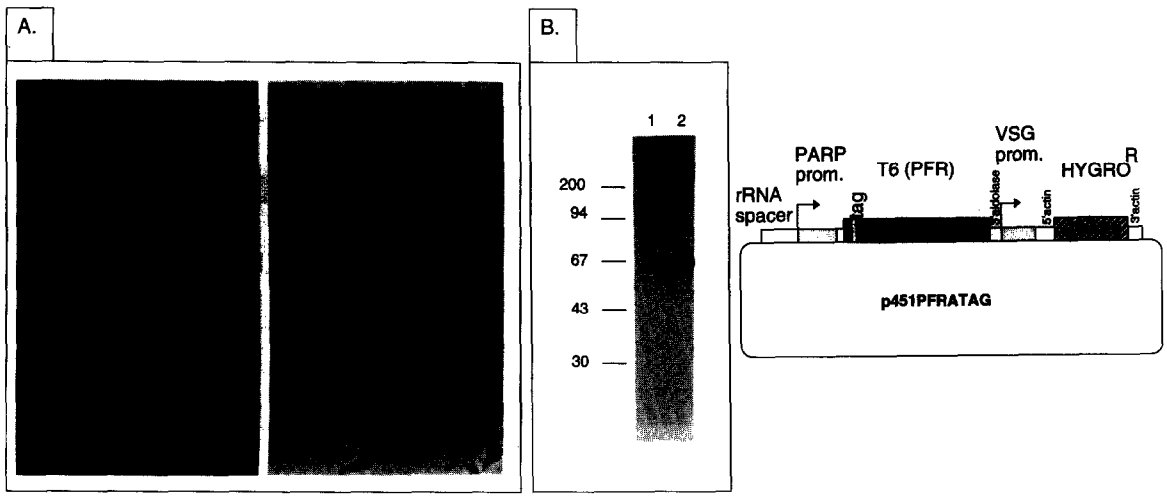

Fig. 2. Stable transformation of trypanosomes with p451PFRATAG (schematic representation of the transfected plasmid is shown on the right) after selection with the antibiotic hygromycin. (A) Immunofluorescence with the monoclonal antibody BB2. Left, fluorescein isothiocyanate pattern; right, corresponding phase pattern. Bar represents 10 μm. (B) Immunoblot of total protein samples of untransfected trypanosomes (1) or of a hygromycin-resistant cell line (2) probed with the monoclonal antibody BB2.

tagged proteins. Our initial experiments suggest this will be very valuable in the future for the study of addressing signals, of organelle organisation and replication.

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