

Short communication

In vivo epitope tagging of *Trypanosoma brucei* genes using a one step PCR-based strategy

Shuiyuan Shen^a, George K. Arhin^a, Elisabetta Ullu^{a,b}, Christian Tschudi^{a,*}

^a Department of Internal Medicine, Yale University School of Medicine, P.O. Box 208022, 333 Cedar Street, New Haven, CT 06520-8022, USA

^b Department of Cell Biology, Yale University School of Medicine, P.O. Box 208022, 333 Cedar Street, New Haven, CT 06520-8022, USA

Received 10 October 2000; accepted in revised form 11 December 2000

Keywords: *Trypanosoma brucei*; Gene targeting; Epitope tagging

With the accumulation of sequence information on the *Trypanosoma brucei* genome, techniques for the systematic analysis of gene function are urgently needed. One important advance in the functional analysis of *Saccharomyces cerevisiae* genes has been the development of a one step polymerase chain reaction (PCR)-mediated technique for the creation of chromosomal gene disruption and modification [1]. The approach is based on the PCR amplification of a reporter cassette using two primers containing flanking sequences specific to the target gene followed by transformation of the PCR product into an appropriate yeast strain. A number of different modules have been described for either gene deletion, conditional expression or the generation of epitope-tagged fusion proteins [2–4]. The major advantage of this method is that it allows rapid gene manipulation without requiring plasmid clones of the gene of interest. In *T. brucei*, there is one report that PCR-based gene disruption is possible [5] using about 42 nt of genomic sequences flanking a reporter gene. Here, we have further developed the power of this strategy and constructed a series of modules for the introduction of either N- or C-terminal epitope tags at discrete chromosomal loci.

In initial experiments, we tested the PCR methodology by replacing one allele of the *TbMT40* gene, which codes for a subunit of the 300 kDa cap 4-specific methyltransferase complex (unpublished results), with

the drug selection marker conferring resistance to blasticidin following the strategy described by Gaud et al. [5]. Briefly, each oligonucleotide primer contained a sequence required for amplification of the resistance gene linked to 90 nt from the *TbMT40* locus to target specific integration. Then 2 µg of PCR fragments were transfected into procyclic *T. brucei* cells [6] and cloned stable transformants were assayed by diagnostic PCRs to verify replacement of the endogenous gene (data not shown). In our hands, gene replacement occurred with a rate of one in 10⁵ cells, which is comparable to one in 4 × 10⁴ as measured by Gaud et al. [5]. So far, we have not investigated whether changing the size of the target sequence has any effect on the efficiency of gene replacement. However, it should be noted that it might be necessary to increase the extent of flanking homology in certain circumstances, since in *S. pombe* transcriptionally silent genes were targeted with a low efficiency (4%) using 80 nt of homology, but integration efficiencies of upto 100% were obtained with flanking homologies of 250 bp or larger [7].

Next, we tested whether we could extend the above technique to allow epitope tagging of trypanosome genes in the genome. Epitope tagging of proteins has become a method of choice for the analysis of function, interaction and the subcellular localization of proteins. However, one problem often encountered with overexpression of tagged proteins is mislocalization in the cell and the assembly into nonphysiological complexes (Fig. 1D). Thus, it is necessary to establish procedures that allow expression of the tagged protein at, or close to, its natural expression levels. As a first step, we constructed

* Corresponding author. Tel.: +1-203-7857332; fax: +1-203-7853864.

E-mail address: christian.tschudi@yale.edu (C. Tschudi).

a module for N-terminal tagging of chromosomal genes with the epitope recognized by the BB2 monoclonal antibody (*neo*/BB2 module; Fig. 1A). This epitope tag corresponds to 10 amino acids from the immunologically well-characterized major structural protein of the *S. cerevisiae* Ty1 virus-like particle [8,9]. We chose this particular epitope, since the monoclonal antibody BB2

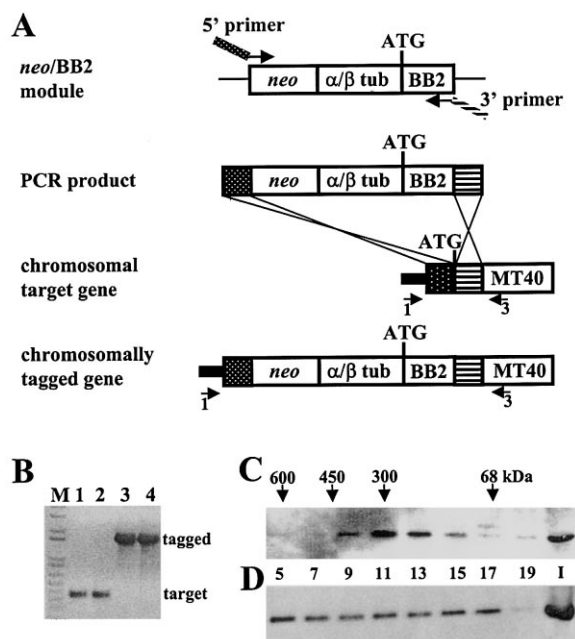


Fig. 1. In vivo epitope tagging in *T. brucei*. (A) Strategy for N-terminal epitope tagging of chromosomal open reading frames. The structure of the *neo*/BB2 module is shown at the top. The *neo* resistance marker (*neo*) is followed by the α/β tubulin intergenic region (α/β tub) and the epitope recognized by the BB2 monoclonal antibody (BB2, [9]). This module was cloned into pBluescript II KS (Stratagene). Following PCR amplification with the 5' and 3' oligonucleotide primer, the PCR product is transfected into *T. brucei* cells resulting in an in-frame fusion of the BB2 tag with the MT40 target gene as shown at the bottom. Drawings are not to scale. (B) PCR analysis of genomic DNA from wild-type cells (lane 1), a clonal cell line, where one allele of the MT40 gene was replaced with a marker gene giving resistance to blasticidin (lane 2), and two independent cloned cell lines selected for *neo* resistance (lanes 3 and 4) with oligonucleotide primers 1 and 3 as indicated in (A). Note that, as predicted, only the tagged MT40 gene is detectable in lanes 3 and 4. Additional PCRs were performed to confirm the predicted genomic structure (data not shown). M, 1 kb plus DNA ladder (GibcoBRL). (C) Western blot analysis with BB2 monoclonal antibodies. S-100 extracts were prepared from the clonal cell line shown in lane 3 of panel (B) expressing the BB2-MT40 fusion, fractionated on a Superdex-200 gel filtration column (Amersham Pharmacia Biotech) and every other fraction, labeled 5–19, was probed with the BB2 antibody. Only the relevant fractions are shown here. Approximate molecular weights of known protein standards are indicated above the blot. I, S-100 input fraction. (D) An S-100 extract was prepared from a cell line expressing BB2 epitope-tagged MT40 under the control of the PARP promoter and analyzed as described in (C). Note that under these conditions of overexpression epitope-tagged MT40 assembled into complexes ranging in size from 68 to over 600 kDa, which most likely represent nonphysiological complexes.

shows little or no cross-reactivity with trypanosome proteins [9].

The *neo*/BB2 module contains in a 5' to 3' direction the neomycin resistance gene (*neo*) to serve as a selectable marker for stable transformants in *T. brucei* followed by the intergenic region separating the translated regions of the α - and β -tubulin genes to provide RNA processing signals for polyadenylation of the *neo* gene and trans-splicing of the downstream target gene, and an ATG initiation codon followed by the epitope recognized by the BB2 antibody. To demonstrate the functionality of this module, we tagged the *Tb*MT40 gene in a single knockout cell line. The 5' primer included 90 nt of the *Tb*MT40 5' UTR immediately upstream of the ATG initiation codon, and the 3' primer included 90 nt of the *Tb*MT40 translated region adjacent to the ATG initiation codon. The expectation was that the module will integrate at the *Tb*MT40 ATG initiation codon, thus generating the chromosomal configuration shown in Fig. 1A. Following transfection, stable transformants were selected for *neo* resistance and integration into the correct genomic locus was verified by PCR (Fig. 1B). Furthermore, expression and assembly of the tagged protein into a 300 kDa complex was checked by gel filtration analysis and subsequent Western blotting with BB2 antibodies (Fig. 1C). The rate of integration by homologous recombination was approximately one in 10^5 cells with 90 nt of target sequences. Reducing the size of the homologous sequences to 75 or 50 nt did not significantly change the integration efficiency, and even 30 nt of target sequences allowed the selection of cell lines with epitope-tagged *Tb*MT40, albeit with a 5-fold lower efficiency. Taken together, these assays showed that it is indeed possible to introduce epitope tags into the trypanosome genome at discrete loci and that the tagged protein appears to closely mimic expression of the endogenous one.

To further extend the usefulness of this strategy, we constructed a series of modules for either N- or C-terminal tagging, containing different combinations of selectable markers and epitope tags (Fig. 2). The constructs for N-terminal tagging contain two new selectable markers (*BSR* and *BLE*), which confer resistance to blasticidin or phleomycin (Fig. 2A). The created modules allow use of either selectable marker to generate fusion proteins with a BB2, V5, or Xpress epitope. The latter two tags were chosen because they showed little cross-reactivity with trypanosome proteins (data not shown) and commercially available antibodies work for immunofluorescence, Western blot analysis and immunoprecipitations. Finally, we have generated a module to fuse the BB2 tag to the C-terminus of proteins (Fig. 2B).

In conclusion, we have described a set of modules for in vivo epitope tagging of *T. brucei* genes. The straight-

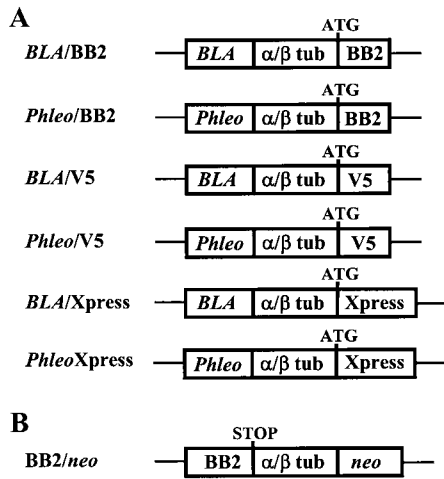


Fig. 2. Modules for use as PCR templates to generate fragments for N- and C-terminal epitope tagging. The selectable markers confer resistance to blasticidin (*BLA*), phleomycin (*Phleo*) or G418 (*neo*). The epitope tags are BB2 [9], V5, a 14 amino acid sequence (GKPIP-NPLLGLDST) found in the P/V proteins of paramyxovirus SV5 (Invitrogen) and Xpress, a peptide of eight amino acids (DLYD-DDDK, Invitrogen) with an enterokinase cleavage site. α/β tub, α/β tubulin intergenic region. (A) Modules to be used for N-terminal protein tagging. (B) Module to be used for C-terminal protein tagging. Drawings are not to scale.

forward and easily applicable PCR-based methodology makes them useful for studying several trypanosome genes in parallel.

Acknowledgements

This study received support from National Institutes

of Health Grants AI28798 to E.U and AI43594 to C.T. E. Ullu is the recipient of a Burroughs Wellcome Fund Scholar Award in Molecular Parasitology and C. Tschudi is the recipient of a Burroughs Wellcome Fund New Investigator Award in Molecular Parasitology.

References

- [1] Baudin A, Ozier-Kalogeropoulos O, Denouel A, Lacroute F, Cullin C. A simple and efficient method for direct gene deletion in *Saccharomyces cerevisiae*. *Nucleic Acids Res* 1993;21:3329–30.
- [2] Wach A, Brachat A, Pohlmann R, Philippsen P. New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast* 1994;10:1793–808.
- [3] Wach A, Brachat A, Alberti-Segui C, Rebischung C, Philippsen P. Heterologous HIS3 marker and GFP reporter modules for PCR-targeting in *Saccharomyces cerevisiae*. *Yeast* 1997;13:1065–75.
- [4] Schneider BL, Seufert W, Steiner B, Yang QH, Futcher AB. Use of polymerase chain reaction epitope tagging for protein tagging in *Saccharomyces cerevisiae*. *Yeast* 1995;11:1265–74.
- [5] Gaud A, Carrington M, Deshusses J, Schaller DR. Polymerase chain reaction-based gene disruption in *Trypanosoma brucei*. *Mol Biochem Parasitol* 1997;87:113–5.
- [6] Fantoni A, Dare AO, Tschudi C. RNA polymerase III-mediated transcription of the trypanosome U2 small nuclear RNA gene is controlled by both intragenic and extragenic regulatory elements. *Mol Cell Biol* 1994;14:2021–8.
- [7] Krawchuk MD, Wahls WP. High-efficiency gene targeting in *Schizosaccharomyces pombe* using a modular, PCR-based approach with long tracts of flanking homology. *Yeast* 1999;15:1419–27.
- [8] Brookman JL, Stott AJ, Cheeseman PJ, Burns NR, Adams SE, Kingsman AJ, Gull K. An immunological analysis of Ty1 virus-like particle structure. *Virology* 1995;207:59–67.
- [9] Bastin P, Bagherzadeh Z, Matthews KR, Gull K. A novel epitope tag system to study protein targeting and organelle biogenesis in *Trypanosoma brucei*. *Mol Biochem Parasitol* 1996;77:235–9.