

Transposon Mutagenesis in the Parasitic Protozoa *Trypanosoma brucei* with the EZ::TN™ Transposome™

Elisabetta Ullu^{1,2}, Huafang Shi¹, Steven Wormsley¹, and Christian Tschudi^{1,3}

¹ Departments of Internal Medicine, ² Cell Biology, and

³ Epidemiology and Public Health, Yale University Medical School, New Haven, CT

Introduction

Trypanosoma brucei, a flagellate protozoa and the causative agent of African trypanosomiasis, belongs to the family *Trypanosomatidae*, which includes other important human pathogens, like the South American trypanosomes and Leishmanias. Ever since their discovery at the beginning of the last century, research on these organisms has focused primarily on their parasitic lifestyles. However, the application of recombinant DNA methodologies, as well as cell and molecular biological techniques led to the discovery of a number of unique biological properties, including trans-splicing, mitochondrial RNA editing, and polycistronic transcription units. Although first described in trypanosomes, many of these properties have subsequently been shown to be present in other members of the eukaryotic kingdom and thus have provided paradigms of eukaryotic biology. Another reason for a resurgence of interest into trypanosomes stems from the realization that many of the protozoan parasites are among the most deeply divergent eukaryotic organisms and therefore represent model systems for the study of eukaryotic evolution. The importance of trypanosomatid protozoa as model systems for unicellular pathogens is further underscored by many ongoing genome sequencing projects.

Although we have come a long way, there is still an urgent need for the development of techniques for the systematic analysis of gene function in trypanosomes. One recent advance in the functional analysis of *T. brucei* genes has been the development of a one step polymerase chain reaction (PCR)-mediated approach 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 electroporation of the PCR product into trypanosomes.

An alternative tool to study protein function, generate gene fusions and mutations, and provide physical landmarks for subsequent analysis, is the insertion of transposable elements into the genome.

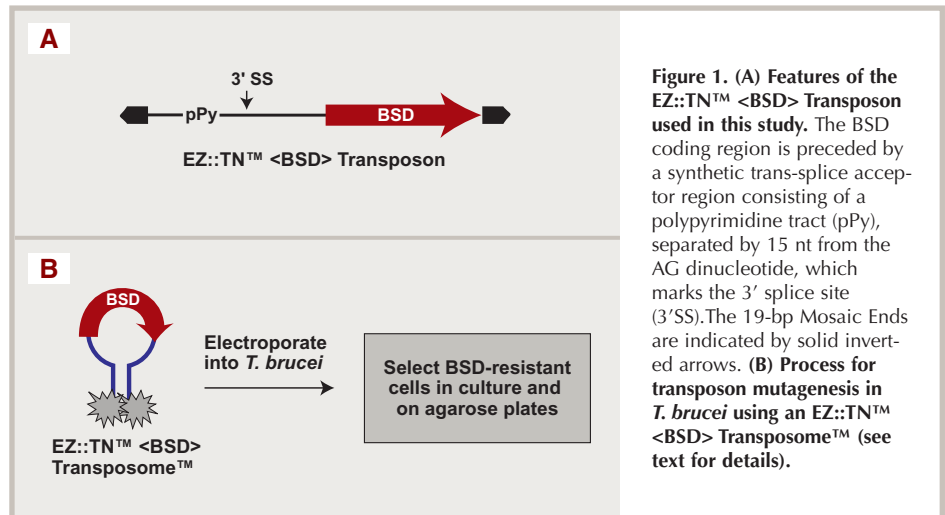


Figure 1. (A) Features of the EZ::TN™ <BSD> Transposon used in this study. The BSD coding region is preceded by a synthetic trans-splice acceptor region consisting of a polypyrimidine tract (pPy), separated by 15 nt from the AG dinucleotide, which marks the 3' splice site (3' SS). The 19-bp Mosaic End sequences are indicated by solid inverted arrows. **(B) Process for transposon mutagenesis in *T. brucei* using an EZ::TN™ <BSD> Transposome™** (see text for details).

Reznikoff and colleagues developed a technique for *in vivo* transposition using extraordinarily stable transposon-transposase complexes, referred to as EZ::TN™ Transposomes™.³ Key features of this system are: i) the transposon is defined by two 19-bp Mosaic End sequences that form an inverted repeat and the DNA between the repeat plays no role in transposition; ii) Transposomes can be assembled in the test tube and are resistant to harsh conditions, such as electroporation; and iii) there is no need to generate a transposase expression system for the organism of interest and transposon insertions are irreversible.

Electroporation of EZ::TN Transposomes has found wide applications in a variety of bacteria for the generation of insertional mutants. However, the extension of this system to eukaryotic organisms has lagged behind, barring one report of its use in *Saccharomyces cerevisiae*.³ Here we describe the adaptation of the EZ::TN Transposome system to *T. brucei*² and highlight its possible application to other parasites, like *Plasmodium*.

Methods and Results

To test the feasibility of using the EZ::TN Transposome for genetic studies in *T. brucei*, we first constructed a transposon for the expression of a drug resistance marker (blasticidin S deaminase or BSD, in our case). Generally, genes that encode housekeeping proteins in trypanosomes are transcribed into polycistronic pre-mRNAs.

Individual mature mRNAs are then generated by the addition of the spliced leader sequence at the 5' end through trans-splicing and by cleavage/polyadenylation at the 3' end. Thus, we used PCR to assemble a promoterless BSD expression cassette by attaching to the 5' end the trans-splicing signals described in Figure 1A. To avoid possible homologous recombination with trypanosome DNA, no trypanosome-derived 3' untranslated region nor sequences required for poly(A) addition were provided for the BSD gene. The BSD cassette was then cloned into the *EcoR* I and *Bam*H I sites of the EZ::TN™ pMOD™<MCS> Transposon Construction Vector. This plasmid contains a multiple cloning site flanked by the Mosaic Ends recognized by EZ::TN™ Transposase. To generate an EZ::TN <BSD> Transposome, the transposon was amplified by PCR, purified and incubated with EZ::TN Transposase in the absence of Mg⁺⁺ ions following the manufacturer's instructions (Figure 1B).

Next, we electroporated different amounts of EZ::TN <BSD> Transposomes into 10⁸ procyclic YTaT 1.1 trypanosomes using our standard conditions,⁴ except that cells were washed and electroporated in cytomix without Mg⁺⁺ ions. Blasticidin-resistant trypanosomes, which became apparent after approximately 10 days, were initially selected as populations. The genomic DNA was digested with *Kpn* I, which does not cut in the BSD cassette, and analyzed by Southern blot

hybridization with a BSD-specific gene probe (Figure 2). The hybridization pattern, with bands ranging in size from low to high molecular weight, was superimposed over a smear of hybridization and consistent with integration events at random sites in the genome.

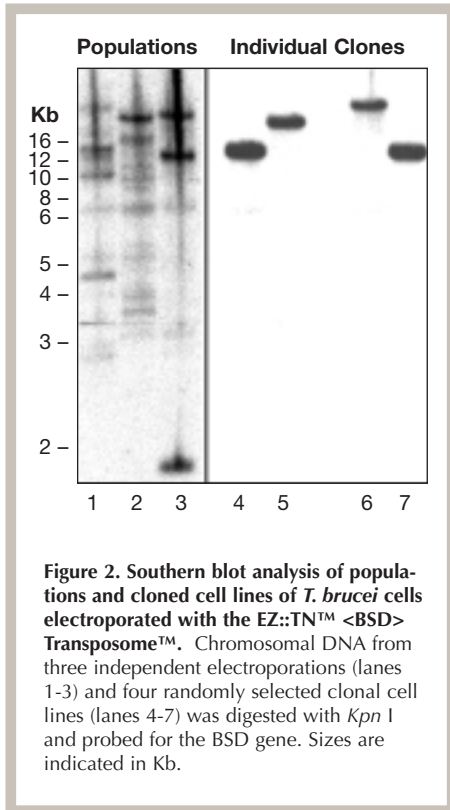


Figure 2. Southern blot analysis of populations and cloned cell lines of *T. brucei* cells electroporated with the EZ::TN™ <BSD> Transposome™. Chromosomal DNA from three independent electroporations (lanes 1-3) and four randomly selected clonal cell lines (lanes 4-7) was digested with *Kpn* I and probed for the BSD gene. Sizes are indicated in Kb.

We then generated clonal cell lines from one of the populations by plating the trypanosomes on agarose plates as described.⁵ The analysis of four such cell lines is shown in Figure 2 and revealed a single hybridizing band demonstrating that each line contained a single BSD gene insertion. Sequencing four insertion sites revealed that the transposon inserted as described for bacterial systems by a cut-and-paste mechanism and therefore generated a 9 bp duplication at the insertion site. Two insertions occurred in known genes, whereas the other two insertions were in predicted open reading frames.²

Blastocystin resistant trypanosomes arose at a frequency of about 10^{-4} to 10^{-3} using 20 to 100 ng of Transposome. This is most certainly an underestimation of the true insertion frequency since generation of a functional BSD mRNA and hence the isolation of drug resistant trypanosomes was dependent on insertion into an actively transcribed chromosomal region. Furthermore, the BSD gene had to be inserted in the same orientation as the

direction of transcription. Despite these limitations, the trypanosome insertion frequency is fairly comparable to what has been obtained in bacterial systems. Indeed, we have now generated a library of about 200,000 transposon-tagged trypanosomes, making it feasible to saturate the genome.

Discussion

Among potential applications, we envision using Transposomes as a means to identify genes and to generate fusions with the green fluorescent protein to localize protein components of different subcellular structures. Since trypanosomes are diploid, it will be necessary to generate homozygous organisms to obtain useful mutants. One possibility would be to select for loss of heterozygosity or alternatively, to chemically mutagenize cells before transposon insertion. However, for parasites with a haploid genome, like *Plasmodium* and *Toxoplasma*, this system has a great potential to provide a simple and easy way to generate mutants for functional analysis.

The EZ::TN™ pMOD™<MCS> Transposon Construction Vector used in this study has been replaced by the EZ::TN™ pMOD™-2<MCS> and pMOD™-3<R6Kγori /MCS> Transposon Construction Vectors which incorporate unique primer binding sites at each end of a custom EZ::TN Transposon for bidirectional sequencing. The latter vector as well as the EZ::TN™ <R6Kγori /KAN-2> Tnp Transposome™ also include a conditional origin of replication (R6Kγori) which can be used for "rescuing" DNA flanking the transposon insertion site. See the center insert for more information.

Ready-to-use EZ::TN Transposomes are available containing either a kanamycin selectable marker (<KAN-2> or <R6Kγori /KAN-2>) or a dihydrofolate reductase gene (<DHFR-1>) that can be selected on plates containing trimethoprim. The number of transposition clones obtained is highly dependent on the transformation efficiency of the host cell. The higher the transformation efficiency of the cell, the more clones will be produced. Electroporation of *E. coli* with a transformation efficiency for pUC19 DNA of $>10^9$ cfu/ug typically results in $>10^5$ independent insertion clones when 1 μl of EZ::TN Transposome is used.

Acknowledgements

This work was supported by grant AI28798 from the NIH.

References

- Shen, S. et al. (2001) *Mol. Biochem. Parasitol.* **113**, 171.
- Shi, H. et al. (2002) *Mol. Biochem. Parasitol.* in press.
- Goryshin, I. Y. et al. (2000) *Nat. Biotechnol.* **18**, 97.
- Ngo, H. et al. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 14687.
- Carruthers, V. B., and Cross, G. A. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 8818.

www.epicentre.com/transposomics.asp

EZ::TN™ pMOD™-2<MCS> Transposon Construction Vector
MOD0602 20 μg

EZ::TN™ pMOD™-3<R6Kγori /MCS> Transposon Construction Vector
MOD1503 20 μg

Each includes Vector and the pMOD™<MCS> Forward and Reverse PCR Primers for amplification of transposon DNA. Primers for bidirectional sequencing of an insertion site are available separately.

EZ::TN™ Transposase
TNP92110 10 Units

www.epicentre.com/transposomics.asp

EZ::TN™<KAN-2>Tnp Transposome™ Kit
TSM99K2 10 Reactions

EZ::TN™<DHFR-1>Tnp Transposome™ Kit
TSM99D1 10 Reactions

EZ::TN™<R6Kγori /KAN-2>Tnp Transposome™ Kit
TSM08KR 10 Reactions

Each Kit contains the specific EZ::TN™ Transposome™ and forward and reverse primers for bidirectional sequencing from the transposon.