

Short communication

# Efficient transposition of preformed synaptic Tn5 complexes in *Trypanosoma brucei*

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Insertion of transposable elements into the genome of bacterial or eukaryotic organism is a powerful genetic tool to generate mutations or gene fusions, to study protein function, and to provide physical landmarks for sequencing and cloning. In parasites, insertion of the Mariner transposon has been achieved in *Leishmania* and *Plasmodium* [1,2].

In vivo transposition usually requires engineering the organism of choice to express the transposase, as well as in most instances the construction of an appropriate donor plasmid. Furthermore, transposon insertions might be unstable in the presence of transposase. To circumvent many of the limitations of in vivo transposition systems, Reznikoff and coworkers developed a technique for transposition of in vitro generated Tn5 synaptic transposition complexes, referred to as EZ::TN Transposomes (Ref. [3] and Fig. 1A). The technology is based on the following key observations: (i) the transposon is defined by two specific inverted 19 bp repeats and the DNA between the repeats plays no role in transposition; (ii) synaptic complexes can be assembled in the test tube by incubating purified transposase with DNA containing the 19 bp inverted repeats at the ends of the molecule; and (iii) the EZ::TN Transposomes are extraordinarily stable DNA–protein complexes. Thus, DNA substrates can be generated by restriction enzyme digestion after cloning in a suitable plasmid vector, or by PCR amplification with appropriate oligonucleotide primers. Synaptic complexes are then assembled in vitro

and electroporated into cells. Thus, the EZ::TN Transposome technology avoids the need to construct a transposase expression system and suicide vector specific for the organism of interest, and has the added advantage that the transposon insertions cannot be reversed by the action of transposase, as in vivo expression of this enzyme is not required.

Electroporation of EZ::TN Transposomes has been so far used in a variety of bacteria to generate insertional mutants [3]. However, the application of this technology to eukaryotic organisms has lagged behind, although it has been shown that Tn5 synaptic complexes can transpose in the yeast *Saccharomyces cerevisiae* [3]. Here we have engineered a Tn5-like DNA substrate containing the blasticidin resistance gene (blasticidin S deaminase or BSD) and assayed for EZ::TN <BSD> Transposomes to insert into genomic DNA following electroporation of *Trypanosoma brucei* procyclic cells.

To generate EZ::TN Transposomes for expression and selection in *T. brucei*, we followed the strategy depicted in Fig. 1A. A promoterless BSD expression cassette was constructed by including a synthetic trans-splicing acceptor region, namely a polypyrimidine tract plus an AG dinucleotide, and a short 5' untranslated region, immediately upstream of the ATG initiation codon of the BSD coding region. Purposely, no trypanosome-derived 3' untranslated sequences, nor sequences required for poly(A) addition were included in the cassette, in order to minimize the possibility of homologous recombination with trypanosome DNA. The BSD cassette was cloned in between the *Eco*RI and *Bam*HI sites of the pMOD<sup>TM</sup><MCS> plasmid vector (EPICENTRE) that contains the mosaic ends of Tn5 separated by multiple cloning sites. The insert DNA was amplified by PCR, purified and incubated with Tn5

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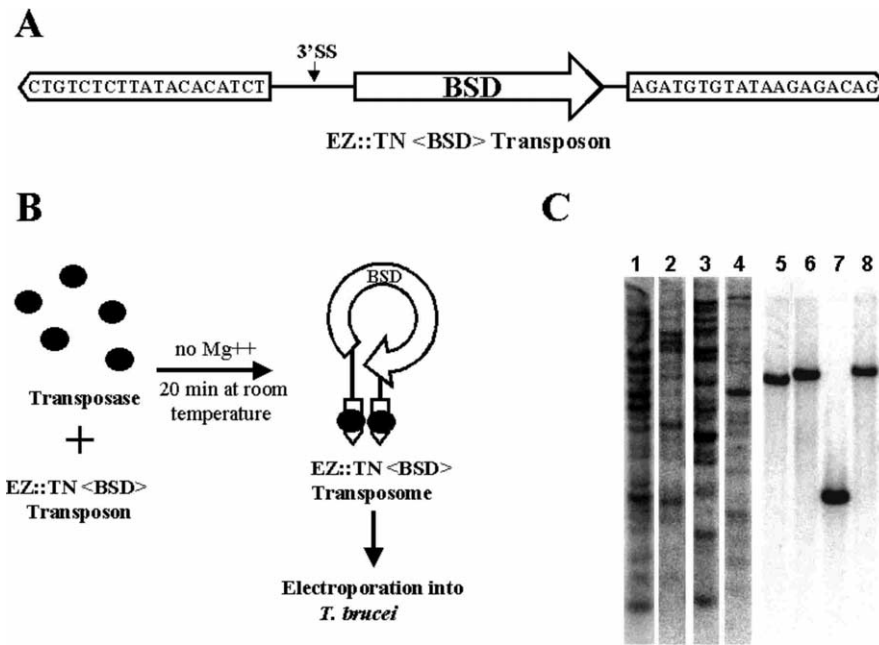


Fig. 1. (A) Structural features of the EZ::TN <BSD> Transposon. The BSD coding region is preceded by a synthetic trans-splice acceptor region consisting of a polypyrimidine tract, separated by 15 nt from the AG dinucleotide, which marks the 3' splice site (3' SS). The 19 bp mosaic end sequences of the Tn5 transposon are shown inside inverted open arrows. (B) Strategy for in vitro assembly of EZ::TN <BSD> Transposomes (see text for details). (C) Southern blot analysis of genomic DNA from populations of blasticidin-resistant trypanosomes (lanes 1–4) or from clonal cell lines (lanes 5–8). Genomic DNA was digested with *Hind*III, fractionated by electrophoresis through a 1% agarose gel, transferred to a nitrocellulose membrane and hybridized to a radiolabelled BSD gene-specific probe.

transposase in the absence of  $Mg^{++}$  ions following the manufacturer's instructions. Next, we electroporated different amounts of EZ::TN <BSD> Transposomes into  $10^8$  procyclic trypanosomes using cytomix without  $Mg^{++}$  ions. Following selection with blasticidin we initially isolated populations of resistant trypanosomes and analyzed their genomic DNA by Southern blot hybridization using a BSD-specific gene probe (Fig. 1C, lanes 1–4). Numerous hybridizing bands ranging in size from low to high molecular weight, superimposed over a smear of hybridization, were detected. This suggested that the EZ::TN <BSD> Transposomes had inserted at multiple sites in the genome. To determine whether resistant trypanosomes contained single or multiple insertions, we generated a number of clonal cell lines by plating on agarose plates. Southern hybridization analysis of four clonal cell lines is shown in Fig. 1C (lanes 5–8) and demonstrated that each line contained a single BSD gene insertion.

To estimate the frequency of insertion of the EZ::TN <BSD> Transposomes we then repeated the experiment except that one day after electroporation the cells were cloned by limited dilution in microtiter dishes. From this we calculated that about one out of 10,000 cells acquired blasticidin resistance, when electroporating 20 ng of EZ::TN <BSD> Transposomes into  $10^8$  procyclic trypanosomes.

Expression of the BSD cassette into a functional mRNA required insertion into an actively transcribed

chromosomal region and with the BSD gene in the same orientation as the direction of transcription. To characterize some of the target sites of BSD transposon insertion, we performed 3' end RACE on RNA isolated from clonal trypanosome cell lines using BSD-specific primers and oligo(dT)-containing adaptors (data not shown). Amplified cDNAs were sequenced and searching the current trypanosome databases identified the 3' end of the insertion sites. The result of this analysis is presented in Fig. 2A and can be summarized as follows. In three clonal cell lines (EU4, EU8 and EU11) the transposon inserted into the translated region of genes: two of them representing known mitochondrial proteins, namely the ADP–ATP carrier protein and Hsp60, whereas the third one is an ORF of unknown function located on chromosome 1. Polyadenylation occurred a few hundred nucleotides downstream of the protein coding regions. In the case of cell lines EU6 and EU2 insertion occurred in non-coding DNA, a few hundred nucleotides downstream of putative ORFs of unknown function. As expected, in the five cell lines analyzed the BSD gene was in the same orientation as the direction of transcription of the targeted chromosomal region. Lastly, the EU13 insertion site was located a few hundred nucleotides upstream of a RIME repetitive sequence located on chromosome 10, but we could not identify any ORFs in the vicinity. Nevertheless, transcription of this locus must occur, since the transposon produces functional BSD mRNA.

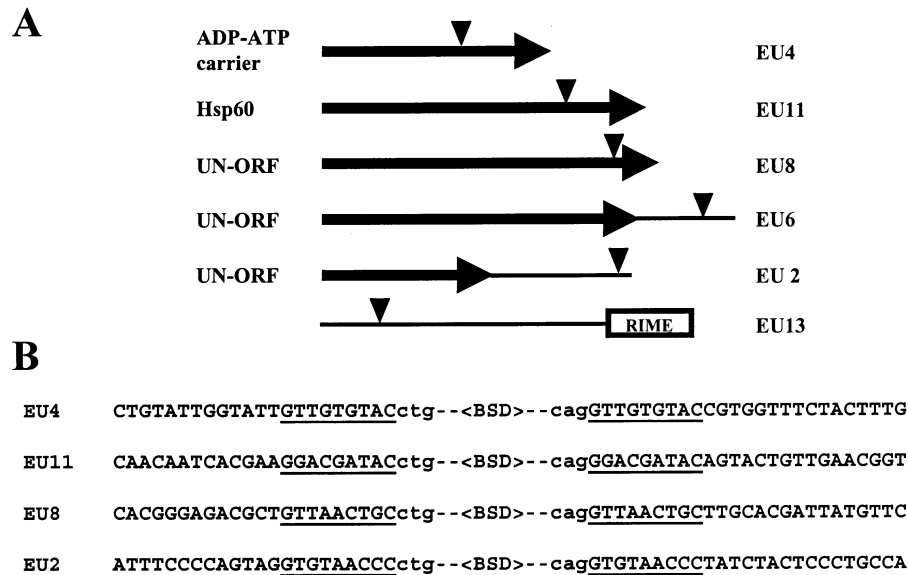


Fig. 2. (A) Schematic representation of transposon insertion sites. Arrows represent open reading frames. The location of the transposon insertion sites is indicated by a solid arrow head. The identity of the genes is shown on the left. UN-ORF, ORF of unknown function. (B) Sequence of transposon insertion sites. Only the first and last three nucleotides of the mosaic ends of the EZ::TN <BSD> Transposon are shown (small letters). The 9 bp duplications at the insertion sites are underlined. The accession numbers for the insertion sequences are: EU4, AF049130; EU11, L43797; EU8, AC091702; EU2, AL482796.

On the basis of the information obtained from 3' end RACE, we generated locus-specific primers that were then used to amplify the genomic DNA flanking the site of insertion in cell lines EU4, EU11, EU8 and EU2. In all cases two PCR fragments were obtained: one corresponding to genomic DNA containing the insertion and the other to the wild-type locus, indicating that the insertion occurred only in one of the two alleles (data not shown). Sequencing of the PCR products revealed that all four insertion events contained the predicted Tn5 end sequences, flanked by a 9 bp duplication of the target sequence (Fig. 2B). The latter result was as expected for a Tn5 transposon that operates via the so called 'cut-and-paste mechanism' [4].

The results presented here demonstrate that in vitro preformed Tn5 synaptic complexes can insert into the genome of *T. brucei* and generate 9 bp duplication at the target site, as expected from a true transposition event mediated by the Tn5 transposase. In a standard electroporation experiment using 20 ng of preformed EZ::TN <BSD> Transposomes the frequency of insertion was calculated to be approximately  $1 \times 10^{-4}$ . This is probably an underestimation of the true insertion frequency considering that expression of the *BSD* gene is restricted to transcribed regions of the genome, and that the transposon needs to be colinear with the direction of transcription at the insertion site. Nevertheless, the trypanosome insertion frequency is quite comparable to what has been obtained in bacterial systems [3], and thus large number of insertions can be easily obtained making it feasible to saturate the genome.

At least in three cases the insertion of the *BSD* transposon occurred within coding regions demonstrating that this approach can be used to disrupt genes. However, since trypanosomes are diploid, it is necessary to generate homozygous organisms in order to obtain useful mutants. One way to achieve this would be to select for loss of heterozygosity, as it has been shown in *Leishmania* [5,6], but to our knowledge this has not yet been described in *T. brucei*. An alternative approach would be to chemically mutagenize cells before transposon insertion. However, one should keep in mind that dominant mutations, although rare, do occur in diploid organisms and that loss of heterozygosity can be selected for, if the selection pressure is stringent enough.

With appropriately engineered EZ::TN Transposomes, lacking independent trans-splicing and translation signals, it will be possible to generate gene fusions for functional analysis, as it has been done in other systems most notably in bacteria and yeast [7,8]. Furthermore, the application of the EZ::TN Transposome method to haploid parasites, like *Plasmodium*, could provide an easy way to generate and tag mutants for functional analysis, and to insert genes of interest into the parasite genome.

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