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A Transposon-Based Tool for Transformation and Mutagenesis in Trypanosomatid Protozoa

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Abstract

The ability of transposable elements to mobilize across genomes and affect the expression of genes makes them exceptional tools for genetic manipulation methodologies. Several transposon-based systems have been modified and incorporated into shuttle mutagenesis approaches in a variety of organisms. We have found that the Mos1 element, a DNA transposon from *Drosophila mauritiana*, is suitable and readily adaptable to a variety of strategies to the study of trypanosomatid parasitic protozoa. Trypanosomatids are the causative agents of a wide range of neglected diseases in underdeveloped regions of the globe. In this chapter we describe the basic elements and the available protocols for the in vitro use of Mos1 derivatives in the protozoan parasite *Leishmania*.

Keywords

Mariner; *Leishmania*; Transposon; Mutagenesis; In vitro transposition

1 Introduction

Trypanosomatid parasitic protozoa of the *Leishmania* and *Trypanosoma* genera are the causative agents of leishmaniasis, Chagas disease, and African trypanosomiasis. These major neglected diseases of humans and domestic animals cause high rates of morbidity in underdeveloped areas of the globe [1, 2]. These parasites efficiently circumvented the defense strategies mounted by their hosts and chemotherapy and vaccination strategies are either inadequate or unavailable [3]. The interaction between trypanosomatids and their diverse environments is deeply influenced by the parasite peculiar control of gene expression, which is not fully understood. Canonical promoter sequences have not been described and functionally unrelated genes are transcribed as large polycistrons and independently regulated mainly at the post-translational level [4–7]. A better comprehension of these parasites unique gene expression apparatus will not only shed light on the evolutionary history of trypanosomatids but also contribute to the rational design of more effective therapeutic strategies. The collection of tools available for the manipulation of trypanosomatids has a bearing on the kind of biological question that can be addressed. In fact, many advances in our understanding of the basic biology of these protozoa have come from studies of the sequence, organization and expression of these organisms' genomes using a relatively limited repertoire of genetic tools [8–11].

In this chapter, we focus on the use of transposon-based mutagenesis in the trypanosomatid *Leishmania*. Transposable elements (TEs) are mobile DNA sequences with the ability to relocate and entail changes in genome structure and the expression [12]. Such remarkable feature makes TEs exceptional tools for genetic manipulation methodologies. The structure and transposition mechanisms of these mobile DNA sequences are highly diverse and determine their classification [13]. TEs are normally divided into Class I, or retrotransposons and Class II, which are the DNA transposons. Current transposon technology is mostly based on Class II DNA TEs due to the relative simplicity of transposon structure and mode of transposition. Nonetheless, the design of TE mutagenesis protocols heavily depends on the transposon system used, as well as on the intended outcome.

The most common application of TEs as a genetic tool is found in the determination of gene function where transposon mutagenesis mediating gain or loss of function can be easily explored either in gene-to-gene strategies or in genome-wide approaches. TE-mediated insertional mutagenesis and/or protein tagging can be adapted and explored in the study of trypanosomatids. Among the main classes of TEs found in eukaryotes, only retroelements have been described in trypanosomatids [14], making them amenable to transposon-based mutagenesis approaches. Heterologous TE systems can be imported into parasites and mobilization can be carried out in vivo [15, 16]. In this approach, the expression of the transposase activity within the parasite and the introduction of modified transposons tend to be less manageable due to difficulties in attaining high transfection efficiencies and in controlling the level of transposase expression. An efficient alternative for the constraints of in vivo transposition in trypanosomatids is the use of shuttle mutagenesis strategies, in which the transposon is mobilized prior to the introduction into the parasite [17]. The majority of shuttle mutagenesis protocols make use of non-autonomous TEs in which the transposase gene has been replaced with reporter genes or selectable markers and the transposase activity is supplemented in *trans* [18, 19]. Shuttle mutagenesis can be carried out in *Escherichia coli* or in in vitro reactions. The in vitro mobilization reaction not only avoids some of the shortcomings that are inherent of in vivo transposition systems, but also constitutes a manageable and practical strategy to introduce genetic alterations into protozoa parasites.

Several TEs have been manipulated and tailored to the point that they are easily incorporated into shuttle mutagenesis strategies [18, 20, 21]. One of such example is the *mariner/Tc1* superfamily of transposons, which is widely distributed in nature and includes various Class II TEs. The in vivo mobilization of mariner-based TEs has been described in a wide range of organisms [15, 22–25]. The heterologous mobilization of the mariner element Mos1 from *Drosophila mauritiana* within the *Leishmania* genome set the bases for the use of transposon shuttle mutagenesis strategies in trypanosomatids [15]. We have found that this element is suitable and readily adaptable to be employed in a wide range of in vitro approaches to investigate gene function in *Leishmania*.

A major characteristic of the element Mos1 from *D. mauritiana*, which is a defining member of the *mariner/Tc1* transposon family, is its minimal *cis* requirements for transposition [26]. This feature makes Mos1 an especially useful element in transposon-based approaches. The terminal inverted repeats (TIRs) that define the boundaries of mariner transposons contain

the binding sites for the transposase and are essential for mobilization [27, 28]. However, the 28 bps TIRs of Mos1 alone does not suffice for optimal transposition of modified versions of the element in vitro. The retention of a few base pairs internal to the TIRs is necessary for proper transmobilization by the active transposase [19]. Tolerance for cargo DNA length varies among different TEs and greatly affects their functionality. Different from other TEs that can carry longer sequences [29], *mariner* elements can be rendered unmovable by the increase in cargo length [18, 30].

The Mos1 mobilization in *Leishmania* emphasizes its usefulness as a tool for probing gene function in this parasitic protozoan. However, the estimate frequency of in vivo transposition of Mos1 within the *Leishmania* genome is as low as 10^{-6} for a single allele inactivation. Besides the difficulties in modulating the levels of expression of the heterologous transposase within the parasite, the diploid nature of the *Leishmania* genome plays an important part in the intrinsic limitations of an in vivo transposition approach. Considering that the efficiency of Mos1 in vitro transposition can be as high as 10^{-3} /target DNA molecule [19], the in vitro mobilization of Mos1-derived elements constitutes a fine alternative for the in vivo strategy. Higher mobilization efficiency and the possibility to control the transposition reaction not only facilitate the construction of insertion libraries into a variety of targets but also expand the applicability of these exceptional tools.

The *mariner* in vitro transposition reaction developed for use in trypanosomatids includes the recombinant Mos1 transposase and a variety of modified elements cloned into a donor plasmid. The modified *mariner* elements available for use in *Leishmania* promote the inactivation of the target gene upon insertion and can also be expressed in bacteria. Some of them mediate the expression of translational or transcriptional fusions and, therefore, are adequate for subcellular localization studies or gene trapping strategies. As illustrated in Fig. 1 and revised elsewhere [21], some TEs bear eukaryotic selectable markers such as Neomycin Phosphotransferase (NPT), Streptothricin Acetyl Transferase (SAT), and the Bleomycin binding protein (PHLEO) and their use is limited to gene disruption protocols. Other elements mediate the selection of protein fusions in the parasite. Most of these elements use the Green Fluorescent Protein (GFP) as reporter of translational fusions. Some of these elements lack the reporter stop codon and maintain an open reading frame across the element sequence and allow the recovery of products that preserve both amino and carboxy termini of the target protein [18]. Other trapping reporters, such as β -glucuronidase or NPT are also available [20]. In this chapter we describe the available protocol for in vitro use of modified Mos1 elements in *Leishmania* studies.

2 Materials

2.1 The Mos1 Transposase Expression and Purification

1. Ca^{2+} -competent *E. coli* strain BL21 (DE3)/pLysS.
2. The pET3a vector bearing the *D. mauritiana* Mos1 transposase (pET3a-TPase construct; [19]).
3. LB medium, 1 % tryptone, 0.5 % yeast extract, 1 % NaCl.

4. 1 M isopropyl- β -D-thio-galactopyranoside (IPTG).
5. Cell Resuspension Buffer, 20 mM Tris-HCl (pH 7.6), 2 mM MgCl₂, 25 % sucrose, 0.6 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine (BZA), 1 mM dithiothreitol (DTT).
6. Liquid nitrogen.
7. Cell Lysis Buffer, 20 mM Tris-HCl (pH 7.6), 4 mM EDTA, 200 mM NaCl, 1 % deoxycholate, 1 % nonylphenoxy polyethoxy ethanol (NP-40), 0.6 mM PMSF, 1 mM BZA, 1 mM DTT.
8. DNase I.
9. 1 M MgCl₂.
10. Lysozyme.
11. Inclusion Bodies Wash Buffer, 100 mM Tris-HCl (7.6), 4 M deionized urea.
12. Column Buffer, 20 mM Tris-HCl (pH 7.6), 50 mM NaCl, 4 M guanidine-HCl, 1 mM PMSF, 1 mM BZA, 5 mM DTT.
13. DEAE-Sephadex equilibrated in Column Buffer.
14. SDS-PAGE apparatus.
15. 10 kDa cut-off Slide-A-Lyzer dialysis cassette (Pierce).
16. Dialysis Buffer A, 10 % glycerol, 25 mM Tris-HCl (pH 7.6), 50 mM NaCl, 5 mM MgCl₂, 2 mM DTT.
17. Dialysis Buffer B, 10 % glycerol, 25 mM Tris-HCl (pH 7.6), 50 mM NaCl, 5 mM MgCl₂, 0.5 mM DTT.

2.2 The In Vitro Transposition Assay and Selection

1. 10 $\times$ Transposition Reaction Buffer, 250 mM HEPES (pH 7.9), 1 M NaCl, 20 mM DTT, 50 mM MgCl₂.
2. 100 % glycerol.
3. 10 mg/ml purified acetylated BSA.
4. Reaction Stop Buffer, 50 mM Tris-HCl (pH 7.6), 0.5 mg/ml proteinase K, 10 mM EDTA, 250 mg/ml yeast tRNA.
5. 25:24:1 Phenol-chloroform-isoamyl alcohol.
6. 3 M sodium acetate.
7. 100 % ethanol (EtOH).
8. 70 % EtOH.
9. 10 mM Tris-HCl pH 7.5.
10. *Pir-E. coli* electrocompetent cells (DH10B).

11. Electroporator.
12. Liquid and semisolid LB medium.
13. Selection drugs at appropriate concentration, Ampicillin (100 mg/ml), hygromycin (30 mg/ml), nourseothricin (50 mg/ml), and Zeocin (100 mg/ml). *See* Note 1.

3 Methods

3.1 The Expression of the Mos1 Transposase

1. Transform the pET3a-TPase construct into Ca^{2+} -competent *E. coli* strain BL21 (DE3)/pLysS, plate transformed cells onto semisolid LB medium containing ampicillin (100 $\mu\text{g/ml}$) and incubate overnight at 37 °C. *See* Note 2.
2. Pick one colony and inoculate into LB medium containing 100 $\mu\text{g/ml}$ ampicillin and incubate overnight at 37 °C under vigorous shaking.
3. Make a 1:100 dilution of the saturated culture into 100 ml of fresh LB medium containing 100 $\mu\text{g/ml}$ ampicillin. Further incubate at 37 °C under vigorous shaking to an OD_{600} of 0.7–0.8.
4. Add IPTG to a final concentration of 0.5 mM in order to induce expression of the transposase. Incubate at 37 °C with vigorous shaking for 1 h. *See* Note 3.
5. Harvest cells by centrifugation at $1,300 \times g$ for 10 min at 4 °C.
6. Resuspend cell pellet in 1/100 of induced cell culture volume in Cell Resuspension Buffer.
7. Quick freeze in liquid nitrogen. *See* Note 4.

3.2 The Purification of the Mos1 Transposase

1. Thaw the sample at room temperature.
2. Add lysozyme to a final concentration of 1 mg/ml and incubate for 5 min at room temperature with gentle agitation.
3. Add 1 ml of Cell Lysis Buffer and incubate for 15 min at room temperature with gentle agitation.
4. Add 60 μg of DNaseI and MgCl_2 to a final concentration of 10 mM. Pipette up and down until the sample is no longer viscous and Incubate for 20 min at room temperature.
5. Pellet inclusion bodies by centrifuging for 15 min at $14,000 \times g$ at 4 °C. Discard the supernatant.

¹The target DNA and modified elements used in the transposition reaction will determine the appropriate selection drug and/or the adequate combination of drugs, as exemplified in Fig. 1.

²In our hands, the use of electrocompetent *E. coli* BL21 strain did not allow the selection of the pET3a-TPase transformant.

³Save an aliquot of cell suspension before addition of IPTG. Process this aliquot according to the described protocol scaling down buffers and reagents. The final protein lysate will be used as the pre-Induction control.

⁴This step can be omitted if the samples are to be processed immediately. In this case proceed to **step 2** in the next section. When performing large scale induction, divide induced resuspended cells into 1 ml aliquots, freeze in liquid nitrogen and stored at -80°C . Take one aliquot at a time to perform the protein purification.

6. Resuspend pellet in 1 ml of ice cold Wash Buffer by vortexing (or pipetting up and down). Centrifuge for 15 min at $14,000 \times g$ at 4°C and discard the supernatant.
7. Repeat **step 5** two more times.
8. Resuspend the final inclusion bodies-containing pellet in 0.5 ml of ice cold Column Buffer. Vortex vigorously to completely dissolve the pellet.
9. Centrifuge 3 min at $14,000 \times g$ at 4°C . Take the supernatant, save a 50 μl aliquot and proceed to the next step.
10. Apply the sample from the previous step onto a 10 ml DEAE-Sephadex column previously equilibrated with ice-cold Column Buffer. Carry on this step at cold-room temperature.
11. Elute the DEAE-Sephadex column using the Column Buffer and collect up to ten 0.5 ml fractions. Carry on this step at cold-room temperature.
12. Analyze 20 μl of each eluted fraction by SDS-PAGE. Also include an aliquot with equivalent volume of the pre induced control (**step 4**; Subheading 3.1) and input material before loaded into DEAE-Sephadex column (**step 8**; this section). *See Note 5.*
13. Pool together the transposase-containing fractions up to 2 ml and dilute the sample to 12 ml using ice cold Column Buffer. *See Note 6.*
14. Transfer the sample to the dialysis slide. Perform dialysis against 1 l of Dialysis Buffer A for 6 h at 4°C .
15. Discard Dialysis Buffer A and perform a second round of dialysis at 4°C overnight, using 1 l of Dialysis Buffer B. *See Note 7.*
16. Discard the dialysis buffer and remove insoluble material from dialyzed sample by centrifugation at $10,000 \times g$ for 10 min at 4°C .
17. Take the supernatant, add glycerol to a final concentration of 50 % and estimate protein concentration by BCA method.
18. Store 100 μl aliquots at -80°C . *See Note 8.*

3.3 The In Vitro Transposition Reaction

1. Prepare a typical transposition reaction, which is carried out in 20 μl in 0.6 ml microfuge tubes and contains 2 μl of $10\times$ Transposition Reaction Buffer, 2 μl of 100 % glycerol, 0.5 μl of purified acetylated BSA, 30 fmol of donor plasmid, and 10 fmol of target plasmid. *See Note 9.*

⁵Unbound transposase will normally be eluted between fractions 3 and 7 from a 10 ml column. However, it is advisable to analyze all eluted fraction using SDS-PAGE. The exclusion of the ion exchange chromatography step yielded inactive transposase after refolding suggesting that the column purification eliminates an inhibitory factor.

⁶Omission of the dilution step resulted in precipitation of inactive protein during the following dialysis step.

⁷This is a limiting step in refolding active transposase. The recovering of active enzyme is very sensitive to the refolding conditions used. Eventually, It may be necessary empirically determine the optimal conditions for this step.

⁸Transposase activity is sensitive to freeze and thaw. Therefore, it is important to aliquot the sample before freezing. Use one aliquot at a time for transposition reaction.

2. Add 100 ng of recombinant Mos1 transposase. *See* Note 10.
3. Incubate reaction for 1 h at 25 °C.
4. Add 80 µl of Reaction Stop Buffer and incubate for 30 min at 30 °C.
5. Add 100 µl of 25:24:1 phenol–chloroform–isoamyl alcohol and vortex vigorously.
6. Centrifuge at 14,000 × *g* for 15 min and transfer 90 µl of the aqueous phase (upper layer) into a 1 ml microfuge tube.
7. Add 10 µl of 3 M sodium acetate, 250 µl of 100 % EtOH and incubate at –80 °C for 1 h.
8. Precipitate DNA by centrifugation at 14,000 × *g* for 30 min at 4 °C. Discard the supernatant.
9. Wash precipitated DNA with 1 ml of 70 % EtOH and centrifuge at 14,000 × *g* for 15 min at 4 °C. Discard the supernatant and remove residual liquid with a pipette.
10. Resuspend the pellet in 10 µl of 10 mM Tris–HCl pH 7.5.
11. Transform 2 µl of the purified transposition reaction into *pir-E. coli* DH10B electrocompetent cells. *See* Note 11.
12. Plate transformed cells onto semisolid LB medium containing the appropriated selective drugs and incubate overnight at 37 °C. *See* Note 12.

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⁹Transposition efficiency may vary depending on the amount and purity of target and donor DNA. The maximum activity can be reached using 150 ng of donor plasmid. Efficiency of transposition can also be improved by using DNA preparations containing a high proportion of supercoiled DNA.

¹⁰Due to variations in the refolding process, the transposition efficiency may vary among different transposase preparations. It is advisable to test each preparation prior to conducting transposition reactions. Excessive transposase (above 100 nM) does not increase transposition efficiencies.

¹¹Incubate transformed cells for 1 h at 37 °C with vigorous agitation. Plate 10 µl of a 1:100 dilution onto medium containing the appropriate antibiotic for selection of the target DNA to determine the transformation efficiency. Plate the undiluted suspension onto medium containing the antibiotics required to double-select the expression of resistance markers found on both target and donor plasmids. Determine transposition efficiency by dividing the number of colonies grown in double-selection medium by the transformation efficiency. Control transposition efficiencies should range from 10^{–4} to 10^{–3}.

¹²Depending on the planned application, insertions events can be further characterized and used for functional studies within the parasite. These include the transfection of either tagged genes for subcellular localization of its product, or interrupted versions of genes for knockout generation. This can be done using individual insertion events or a library of transposition product in mass transfection into the parasite.

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Fig. 1.

The *in vitro mariner* transposition assay. The major components of the transposition reaction include a modified version of the mariner element (a), which is carried in the donor plasmid (b), the target plasmid (c) and the recombinant Mos1 transposase. Following the reaction, the transposition reaction products are transformed into bacteria and selection is carried out with the appropriate drugs according to the selection markers in target plasmid and in the modified Mos1 element (d). (a) The Mos1 modified transposons available for use in Gene Tagging and/or Gene disruption protocols in *Leishmania* [21]. These elements contain variable elements according to the application intended; all modified elements bear a drug resistance marker for selection of integration events. The selection markers include SAT (Nourseothricin resistance marker), Km (Kanamycin resistance marker), PHLEO (Phleomycin/zeocin resistance marker) and NEO (G418 resistance marker). The generation of transcriptional or translational fusions is mediated by tag protein genes such as GFP (Green Fluorescent Protein), GUS (β -glucuronidase) or NEO (Neomycin Phosphotransferase II); *black arrowheads*, *E. coli* promoters; “/,” indicates that the gene lacks a start or stop codon; “*” the gene contains a stop codon; AG, trans splice acceptor site. (b) The transposon donor plasmid contains an R6K replication origin (*oriR6K*) and will not

propagate in *pir*-bacteria strains used to select the transposition production. The *arrowheads* represent the Terminal Inverted Repeats (TIR) and the internal sequence at 5' and 3'-ends that contain the required *cis-elements* for transposition and define the minimal Mos1 element; the *dashed line* represents the different markers and/or reporter genes that may compose the modified elements