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A transposon toolkit for gene transfer and mutagenesis in protozoan parasites

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Abstract

Protozoan parasites affect millions of people around the world. Treatment and control of these diseases are complicated partly due to the intricate biology of these organisms. The interactions of species of *Plasmodium*, *Leishmania* and trypanosomes with their hosts are mediated by an unusual control of gene expression that is not fully understood. The availability of the genome sequence of these protozoa sets the stage for using more comprehensive, genome-wide strategies to study gene function. Transposons are effective tools for the systematic introduction of genetic alterations and different transposition systems have been adapted to study gene function in these human pathogens. A *mariner* transposon toolkit for use *in vivo* or *in vitro* in *Leishmania* parasites has been developed and can be used in a variety of applications. These modified *mariner* elements not only permit the inactivation of genes, but also mediate the rescue of translational gene fusions, bringing a major contribution to the investigation of *Leishmania* gene function. The *piggyBac* and Tn5 transposons have also been shown to mobilize across *Plasmodium* spp. genomes circumventing the current limitations in the genetic manipulation of these organisms.

1. Introduction

Plasmodium spp. and the trypanosomatids *Leishmania* and *Trypanosoma* are some of the major protozoan parasites causing deadly diseases around the globe. The recent determination of the complete nucleotide sequence of the genome of these pathogens has improved our understanding on how these organisms have structured and transferred their genetic information throughout evolution. The identification of new genes and cellular processes in the parasite as well as a better comprehension of their genome maintenance and expression is expected to contribute to the development of new tools for disease control, such as targets for new drugs, antigens for vaccine design and more adequate diagnostic approaches.

The focus of this review is to present and discuss the use of transposon technology as a tool to better understand the intricate biology of protozoan pathogens. Here, we present the transposition systems that have been adapted for the study of trypanosomatids and malaria protozoans. Particular attention is given to the *mariner* 'toolkit' developed for functional studies in *Leishmania*.

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2. Major diseases caused by protozoan parasites

Some of the most prevalent and lethal diseases that affect the residents of developing countries are caused by parasitic protozoa. Malaria, Leishmaniasis and African and American trypanosomiasis are the major diseases caused by these parasites and cause the death of over 2 million people each year. The high rates of morbidity and mortality associated to these pathogens can be attributed not only to socio-economical factors of the afflicted populations but also to intricate host-parasite interactions. Parasite survival is guaranteed by several sophisticated mechanisms allowing the evasion from the immune system of the host. Moreover, the genetic complexity of parasitic protozoa has hampered the development of vaccines or effective drugs for the treatment of the diseases they cause.

Malaria is a deadly disease caused by three different species of *Plasmodium*. The disease affects over 400 million people around the globe with an estimated death toll of about 2,5 million per year (Greenwood and Mutabingwa, 2002). The control of malaria has been held back by widespread drug resistance, insecticide-resistant mosquitoes as well as by the lack of an effective vaccine (Bremar et al., 2004; Targett and Greenwood, 2008). The most deadly species, *P. falciparum*, infects host erythrocytes and promotes its adhesion to the linings of small blood vessels. The compromised tissue perfusion is believed to be an important pathophysiological feature underlining metabolic acidosis and the cerebral form of the disease. However, the clinical outcome of *Plasmodium* infections depends on many other factors including parasite drug resistance, host immunity and access to treatment by affected populations.

Leishmaniasis is a spectrum of diseases that affect more than 12 million people around the world. About 350 million people are at risk in endemic areas (Croft et al., 2006). These diseases can be categorized according to their clinical manifestations. Visceral leishmaniasis is the most serious and lethal form of the disease in which the infection spreads to the liver, spleen and bone marrow. The cutaneous form of the disease is characterized by localized, rarely diffused, long-term lesions of the dermis. In mucocutaneous leishmaniasis, mucosal tissues are affected and destroyed. At least 20 *Leishmania* species have been shown to infect humans. The genetic variability of the host and insect vector factors are also determinants of the clinical manifestations of leishmaniasis. There is no effective vaccine against these diseases (Noazin et al., 2008) and the available therapeutic arsenal is extremely limited.

The genus *Trypanosoma* comprises species and subspecies that cause distinct pathologies. African trypanosomes are the causative agents of sleeping sickness in man and nagana in domestic livestock. The World Health Organization (WHO) estimates that nearly half a million people are currently infected with these parasites in Sub-Saharan Africa. In most cases, the symptoms include fever, severe headaches and neurological disorders. African trypanosomiasis is usually fatal if left untreated (Simarro et al., 2008). Chagas disease is a human illness caused by the infection with *Trypanosoma cruzi*. Clinical symptoms include cardiopathy, heart failure and digestive-tract abnormalities. Although natural transmission has been considerably diminished in large areas of Latin America, the WHO estimates an annual death toll of about 13,000.

3. Transposition based genome manipulations

The information generated by sequencing the genome of the different protozoan parasites allows the study of the relationship between gene structure and function. It also permits the determination of differential gene expression and protein localization throughout the cell life cycle of the parasite. The systematic introduction of genetic alterations and the study of their effects also constitute an important platform for gene function investigation. However, despite

the development of many molecular and genetic tools over the past few years, there is still a large proportion of gene modifications that does not lead to obvious phenotype changes.

Among the available tools for genome-wide studies, transposon mutagenesis mediating gain or loss of function is one of the most straightforward strategies. Pioneering studies on the heterologous mobilization of a *mariner* transposon within the *Leishmania* genome set the stage for genome-wide mutagenesis approaches in protozoan parasites (Gueiros-Filho and Beverley, 1997). Mobile elements can be used for the determination of the role and importance of genes in different cellular processes. The transposon technology constitutes a remarkable tool for the design of genetic manipulation approaches. (Hamer et al., 2001a; Hamer et al., 2001b; Ross-Macdonald et al., 1999a; Ross-Macdonald et al., 1999b). Successful use of mobile sequences in studies of gene function and transgenesis has been demonstrated in plants, animals and microbial systems.

Other examples of the application of transposable elements (TEs) in various organisms have been reviewed elsewhere (Mates et al., 2007). For instance, in important vectors of pathogens, such as *Aedes aegypti*, TEs like Mos1 (Coates et al., 1998), *piggyBack* (Adelman et al., 2002) and *Hermes* (Jasinskiene et al., 1998) have been shown to mobilize. In these vectors, this technology could be used for carrying and spreading genes of interest throughout natural populations of the insect (Adelman et al., 2002; Arensburger et al., 2005). In vertebrates, the Sleeping Beauty (SB) transposon and the *piggyBac* element have been reported as highly active in human and mouse genomes (Collier et al., 2005; Collier and Largaespada, 2005). The SB element has already been used in mammals as gene therapy and germline and somatic mutagenesis (Collier et al., 2005; Collier and Largaespada, 2005). Different TEs have been widely used as genetic tools across a broad range of microbes as in the combination of *mariner* and Tn10 elements for *in vivo* mutagenesis of *Bacillus anthracis* (Wilson et al., 2007). Large-scale T-DNA and transposon insertion libraries, as well as maize Ac-Ds transposable elements, have been engineered for the study of functional genomics in different plants (Cooper et al., 2004; Jeon et al., 2000; Singh et al., 2006). Altogether, these examples illustrate the usefulness of TEs as an effective tool for the study of genome structure and expression in a wide range of organisms.

The TEs can be classified based on the necessity for a reverse transcriptase activity to transpose. Type I TEs, or retrotransposons, require a reverse transcription step for mobilization. Type II TEs, or DNA transposons, are flanked by terminal inverted repeats (TIR) that are recognized by the transposase during the transposition event (Figure 1). Type II TEs encoding an intact transposase gene is considered an autonomous DNA transposon and is able to mediate the transposition event. Transposition systems used in shuttle mutagenesis protocols employ modified TEs in which the transposase gene is absent. In these cases the TE is called non-autonomous DNA transposon and will require the transposase activity in *trans* for adequate mobilization.

The element Mos1 from *Drosophila mauritiana* is a defining member of the Tc1/*mariner* transposon family. Elements of this TE group have been engineered into a broad range of organisms from bacteria to vertebrates. Active Tc1/*mariner* TEs typically contain short terminal repeats flanking a single gene encoding the transposase polypeptide. Mobilization occurs by a cut and paste mechanism catalyzed by the transposase through the recognition of the terminal inverted repeats and insertion into a specific target site. The *piggyBac* mobile element was originally identified in the genome of the cabbage looper moth *Trichoplusia ni*. This element encodes a 68 KDa transposase flanked by 13 bps terminal inverted repeats. *PiggyBac* inserts into specific sites, which are duplicated upon insertion and are essential in the excision reaction (Fraser et al., 1985).

The use of these TE-based systems in phenotype driven screenings presents numerous advantages because the molecular analysis of the resulting mutations is uncomplicated. This tool can be used either *in vivo* or *in vitro* depending on the TE or the target organism (Figure 2). However, the control of an *in vivo* transposition reaction can be a challenge in an asexual diploid organism such as the protozoan parasite *Leishmania*. The use of shuttle mutagenesis *in vitro* has been shown to be a more adequate and powerful tool for a wide range of applications in these organisms. In this approach, a TE is previously mobilized *in vitro*. A genomic library, constructed in shuttle vectors, is subjected to the transposition and insertion events can be transfected back into the parasite. The mariner *Mos1* transposable element is a well adapted tool for gene function studies from sequencing to phenotypic inactivation in *Leishmania* (Gueiros-Filho and Beverley, 1997; Beverley, 2003a; Beverley 2003b; Marchini et al., 2003; Squina et al., 2007; Tosi and Beverley, 2000).

Transposon technology can also be associated with proteomic studies (Beverley et al, 2002). When used in proteomic approaches, transposon-based gene fusion libraries have the ability to report on protein levels. This valuable feature is especially interesting in trypanosomatid parasites whose expression regulation is likely to occur at the post transcriptional level.

4. Relevant features of TEs used as DNA delivery tools

The use of mobile elements for the introduction of new phenotypes as well as the destruction of endogenous loci within genomes may be profoundly affected by some of its characteristics, such as host range functionality and integration site specificity. Members of the Tc1/*mariner* family are especially useful due to their minimal *cis* requirements for transposition (Mates et al., 2007). The 5' and 3' TIRs sequences of *mariner* transposons contain the binding sites for the enzyme and are essential for transposition (Auge-Gouillou et al., 2001a; Auge-Gouillou et al., 2001b). The presence of the 28 bps TIRs alone is insufficient to mediate the mobilization of modified *Mos1* elements *in vitro* and the retention of a few base pairs internal to the TIRs guarantees the *trans*-mobilization by the active transposase in *trans* (Tosi and Beverley, 2000).

The selection of the integration site varies among different TEs and may also affect their usefulness. With few exceptions, such as the hAT family element *Tol2* whose insertion sites are highly heterogenic (Urasaki et al., 2006), primary DNA sequence is the most relevant factor in site selection. Tc1/*mariner* transposons insert specifically into dinucleotides TA while the *piggyBac* element uses the tetra nucleotide TTAA sequence as target of integration. Integration site preference is a decisive feature in the choice of a transposon system to be used in mutagenesis or gene delivery protocols. Preferred integration within genes may be either beneficial or detrimental depending on the intended application.

Although the primary DNA sequence seems to be the major determinant of target site preference, the chromatin structure may also play a role in the integration process when it is conducted *in vivo*. The possible interaction between the transposase and specific chromatin-associated proteins may dictate the integration site. As a result, there are transposons that insert mostly into intergenic regions (Martin et al., 2002), others prefer 5' regulatory sequences (Spradling et al., 1999; Thibault et al., 2004), while other elements show preference for transcription units (Wilson et al., 2007). For instance, the *P* element inserts preferably into the 5' regulatory regions of transcription units (Spradling et al., 1999; Thibault et al., 2004). On the other hand, Tc1/*mariner* elements do not seem to have such biased integration preference when used in *in vitro* reaction (Tosi and Beverley, 2000).

Another relevant feature of TEs used as genetic tools is their cargo capacity. The ability to disrupt regular gene function can be achieved by relative small sequences. However, the expression of transgene constructs may require large fragments bearing genes and/or regulatory

sequences. Increasing the size of the DNA sequence placed within the transposon may greatly affect its functionality. Tolerance for cargo DNA length will depend on the TE and has to be suitable to the application planned. While some TEs support large cargo sizes (Spradling et al., 1999; Thibault et al., 2004) the inhibition of mobilization by the increase in cargo length has been characterized for the elements belonging to the Tc1/*mariner* family (Goyard et al., 2001; Balciunas et al., 2006; Fischer et al., 1999; Izsvak et al., 2000; Lozovsky et al., 2002; Urasaki et al., 2006).

The efficiency of transposition can be further affected by a phenomenon known as over-production inhibition, in which efficiency is decreased as the concentration of transposase rises within the cell. It has been shown that high levels of Tc1/*mariner* transposase expression result in the formation of inactive oligomers (Lohe and Hartl, 1996). This effect has been described for a variety of TEs (Zayed et al., 2004) and modulation of transposase expression is believed to circumvent the inhibitory effect of enzyme levels. However, the *in vitro* reaction mediated by the recombinant Mos1 enzyme does not seem to be subjected to this kind of modulation (Tosi and Beverley, 2000).

5. Application of transposons in parasitic protozoa

The Leishmanias

The *Leishmania* species genomes comprise ~33Mb and contain about 8,300 genes (Ivens et al., 2005). So far, the majority of these genes has been classified as genes of unknown function. A striking feature is that, despite the major differences in the diseases the three different *Leishmania* species cause, there is minor variation in their gene content (Peacock et al., 2007). This fact reveals that the differences in the clinical manifestations of the disease might be a consequence of differential gene expression between these species.

Leishmania genes are arranged in directional gene clusters (DGCs) across the chromosomes resembling the distribution pattern of prokaryotic polycistronic transcription units (Martinez-Calvillo et al., 2004). However, the genes within *Leishmania* DGCs are not functionally related. This type of gene organization is reflected in a polycistronic transcriptional mode with a profound implication in gene expression regulation (Holzer et al., 2006; Leifso et al., 2007). Control of expression is possibly conducted at the post-transcriptional level (Boucher et al., 2002; Myung et al., 2002). In fact, a variety of DNA microarray studies addressing the identification of *Leishmania* genes presenting patterns of stage-specific expression revealed limited differences between species or life stages of the parasite (Akopyants et al., 2004; Almeida et al., 2004; Holzer et al., 2006; Saxena et al., 2007). These data suggest that regulation is likely to occur at the level of stability and translation of mRNA and/or protein modification. The elucidation of expression regulation and gene function provides a unique insight into this parasite's biology, allowing a better understanding of the host-parasite interaction and the development of new strategies to combat leishmaniasis.

Several transposon systems have been engineered and can be readily incorporated into mutagenesis strategies within a variety of organisms. These systems include Mu (Lavoie and Chaconas, 1996), Ty1 (Merkulov and Boeke, 1998), Tn5 (Goryshin and Reznikoff, 1998), and the Tc1/*mariner* family elements (Fischer et al., 1999; Lampe et al., 1996; Gueiros-Filho and Beverley, 1997; Tosi and Beverley, 2000). This last group of TEs is a family of DNA transposons within eukaryotic genomes presenting especially interesting properties, which include the ability to transpose across a variety of genomes regardless of host-specific factors (Lampe et al., 1996). The Mos1 *mariner* element from *Drosophila mauritiana* is 1.3-kb long and contains a single open reading frame (ORF) encoding the transposase flanked by 28-base pair inverted repeats. Recognition of the TE ends is crucial for mobilization into the TA nucleotide target site (Auge-Gouillou et al., 2001b; Medhora et al., 1991).

In vivo mobilization of *mariner*-based elements has been observed in organisms ranging from prokaryotes to vertebrates (Gueiros-Filho and Beverley, 1997; Coates et al., 1998; Fadool et al., 1998; Fischer et al., 2001; Rubin et al., 1999; Sherman et al., 1998; Zhang et al., 2000). The wide evolutionary distance traversed by the *Drosophila mariner* element Mos1, for instance, is confirmed by its mobilization in *Leishmania*, which is separated by an evolutionary distance of probably more than 1 billion years (Gueiros-Filho and Beverley, 1997). Previous studies showed heterologous Tc1/*mariner* transposition only within members of the same taxonomic order (Loukeris et al., 1995). The Mos1 mobilization in *Leishmania* underscores its usefulness as a tool for probing the genome of this human parasite. The estimate frequency of transposition of Mos1 within the *Leishmania* genome is about of 10^{-6} for the inactivation of a single allele. This relatively low efficiency of transposition is due to the fact that, in this diploid organism, a loss of function mutation requires two genetic events (Figure 2). Moreover, difficulties in controlling the expression of the transposase within the parasite's cell represent constraints in the use of Mos1 *in vivo* transposition in large scale studies in *Leishmania*. For a variety of applications, *in vitro* transposition is a suitable and powerful tool. The efficiency of Mos1 *in vitro* transposition can be as high as 10^{-3} /target DNA molecule (Tosi and Beverley, 2000). Higher mobilization efficiency and a controllable transposition reaction, inherent of an *in vitro* system, expand its effectiveness and have facilitated the construction of insertion libraries into a variety of targets (Figure 2).

The *mariner* toolkit developed for use in *Leishmania* contains transposons designed to be efficiently mobilized in an *in vitro* transposition reaction. The majority of these elements can mediate the inactivation of genes upon insertion. Modified elements also allow the recovery of a variety of translational or transcriptional gene fusions to reporters such as the Green Fluorescent Protein (GFP), β -glucuronidase, or eukaryotic selectable markers such as Neomycin Phosphotransferase (NPT), Streptothricin Acetyl Transferase (SAT) and the PHLEO cassette. The insertion and activation of a reporter or marker can be used not only as a insertional mutagen, but also to trap genes of unknown function and determine its expression profile or subcellular localization of its product (Augusto et al., 2004; Goyard et al., 2001; Robinson et al., 2004).

The definition of Mos1 *cis* requirements for *in vitro* transposition allowed the development of a minimal element that can be used as a starting point for the construction of transposons with a variety of cargos (Goyard et al., 2001). These elements are carried in a donor plasmid and, with the purified transposase and a target DNA, constitute the main components of an *in vitro* transposition reaction (Figure 2). The different applications in *Leishmania* studies of some of the available modified transposons are presented in Table 1. All of these elements are adequate for use in gene disruption protocols and, with one exception, can mediate the expression of their markers in bacteria.

Some of these transposons can be used exclusively in gene disruption or Primer Island sequencing protocols. These include the elements MosHYG, employed in the initial *in vivo* studies (Gueiros-Filho and Beverley, 1997), and ELSAT used in the disruption of a telomeric essential gene of the parasite (Squina et al., 2007). The elements K1 and ELNEO present similar characteristics and have been applied to characterize the *mariner in vitro* transposition reaction (Goyard et al., 2001; Tosi and Beverley, 2000).

Considering that this parasite might depend on post-transcriptional regulatory mechanisms for most of its control of protein expression, a few other elements, which facilitate the recovery of protein fusions, can be efficiently used. Elements from the /GEP series, and the /GFP*K and /GFP*ELSAT transposons are suitable for recovery of translational fusions to GFP (Goyard et al., 2001). The reporter GFP in these elements does not have an initiating ATG codon and GFP expression will only occur if the insertion event places the reporter in frame

within a parasite's gene. Unlike the other GFP transposons, the reporter gene in the elements /GEP2/ and /GEP3/ lack the stop codon preserving an open reading frame across the transposon. Protein fusions resulting from insertion of these elements will carry both the amino and carboxy terminus of the target protein. This feature brings obvious advantages in studies of protein localization, for instance.

Other elements, such as /GUS*ELSAT and /NEO*ELSAT contain the alternative reporter genes β -glucuronidase and NPT, respectively (Augusto et al., 2004). We have integrated /NEO*ELSAT-tagged genes into the genome and observed that the expression of a fusion product was only possible after the amplification of the locus as an extrachromosomal amplicon. As summarized in Figure 3, the characterization of a NPT-tagged gene revealed that the expression of the fusion protein occurs exclusively in cell lines bearing amplicons of this particular locus, irrespective of its copy number. These data showed that the expression of the tagged gene is under strict control when encoded within the genome and that gene amplification may be an important means to regulate the expression of genes in these parasites. Therefore, the use of TEs to tag genes throughout the genome can be explored as a reliable tool to study not only gene function, but also general mechanisms of gene expression regulation.

Other components of the *mariner* toolkit include the transposon /-2 $\times$ 5 that allows for linker-insertional mutagenesis. In this protocol, the excision of the transposon by restriction endonucleases generates libraries of short insertions across a given gene enabling subsequent functional studies of the protein. The rescue of insertion events from the parasite after transposition *in vivo* can be mediated by the element TK-PG, which contains a prokaryotic origin of replication (Robinson et al., 2004).

The NEO*ELSAT cassette described above has been imported into an *in vitro* transposable system based on the bacterial Tn5 transposon. The system is highly efficient and has been used to characterize large cosmids bearing *Leishmania braziliensis* genomic DNA (Laurentino et al., 2007).

The Plasmodia

Unlike in *Leishmania*, the use of *mariner* transposons has been limited in the malaria pathogen. *In vivo* mobilization of an autonomous Mos1-derived element carrying the Dihydrofolate Reductase (DHFR) marker in *Plasmodium falciparum* has been reported (Mamoun et al., 2000). Despite the relative low frequency of the integration, which might be improved by increasing transposase expression using plasmodium-specific regulatory sequences, the integration occurred into the expected TA sites. Interestingly, a possible growth advantage led to a biased integration into the coding region of protein kinase A.

The *Plasmodium* genome comprises 23-26 Mb which encode more than 5,000 genes (Carlton, 2003; Gardner et al., 2002). This is a notably A/T-rich genome which poses a major challenge to conventional recombinant DNA technology. Only a restricted percentage of *Plasmodium* genes present a significant similarity to hypothetical proteins in other organisms and the majority of the predicted proteins are unique to this organism. These features call attention to the need for genetic tools that will permit gene function studies.

Gene expression regulation in these Apicomplexa is also peculiar. Although *P. falciparum* has many of the characteristics common to eukaryotic transcription, it also presents unique patterns of gene expression that are critical to parasite virulence and involve highly regulated patterns of gene expression and silencing. For instance, the expression control of the *var* gene family, which encodes a protein expressed in the surface of infected erythrocytes, is crucial to the process of antigenic variation. Only one member of the *var* family is expressed in a parasite cell, while the other *var* genes are kept silenced. The control of this mutually exclusive

transcription permits the parasite to switch expression to a different *var* gene, which guarantees its chronicity and virulence (Freitas-Junior et al., 2000). The relative scarcity of transcription-associated proteins and specific *cis*-regulatory elements may reflect a reduced role for transcription factors in the control of gene expression in these parasites (Coleman and Duraisingh, 2008). However, recent studies have identified a family of putative transcriptional regulators in *Plasmodium falciparum* (Balaji et al., 2005; Silva et al., 2008). These findings can lead to the exploration of the role of transcription regulators throughout the parasite life cycle.

Functional analysis of the *Plasmodium spp.* genomes is restricted because of the relatively limited ability to genetically manipulate these protozoa. The generation of mutants is problematic, due not only to the low transfection efficiency (O'Donnell et al., 2002) but also to the difficulty in manipulating sequences with A/T-content of about 80%. (Balu and Adams, 2006). The use of TEs may not only circumvent the need for *in vitro* recombinant DNA technology, but also generate a collection of mutants in a single transfection experiment.

Two different DNA transposon systems have been adapted to be used as a genetic tool for the study of malaria parasites. The Tn5 derived mini-Tn5 transposon consists of a kanamycin resistance marker flanked by two 24-bp long terminal repeats from the original Tn5 transposon, which are enough for efficient mobilization of any cargo DNA sequence. In this shuttle mutagenesis protocol, developed for the rodent parasite *P. berghei*, the insertion reaction is carried out *in vivo* and is based on the conjugation between bacteria cell lines bearing either a modified mini-Tn5, carrying the human Dihydrofolate Reductase (*hDHFR*) selectable marker, or the plasmid containing the parasite target DNA. The transconjugates are selected and a pool of insertional events can be purified and electroporated back into the parasite cells in a straightforward mass-transfection experiment. The wild type copy of the targeted gene is replaced for the transposon-disrupted copy, carried in the transfected linear fragment, by double cross-over homologous recombination. Replacement events can be rescued when transfected parasites are inoculated in mice treated with pyrimethamine. Resulting phenotypes can be studied in detail (Sakamoto et al., 2005).

Another well characterized transposition system adapted to *Plasmodium spp.* is the lepidopteran *piggyBac* transposon. *In vivo* integration of a modified *piggyBac* element within the parasite genome occurs at a high frequency, in the range of 10^{-3} and insertion events are stable. As expected, integration into the tetra nucleotide TTAA is highly specific within the parasite (Balu et al., 2005). *piggyBac* transformation of *P. falciparum* was carried out in the blood-stage of the parasite. In this *in vivo* protocol erythrocytes are loaded with independent plasmids carrying either the *piggyBac* transposase or the *hDHFR*-containing transposon prior to the infection by mature blood-stage parasites. DHFR inhibitor-resistant parasites emerge in the culture after four generations (Balu et al., 2005). As for the Tn5 system discussed above, the use of this TE prevents major difficulties in manipulating plasmodia sequences and generating designed gene disruption reagents. Further characterization of the *piggyBac* system in plasmodia revealed a tendency for insertions within the 5' UTRs of predicted parasite genes (Balu and Adams, 2006). This pattern of integration is possibly mediated by a more accessible chromatin structure, which facilitates the study of promoter and regulatory sequences. In fact, the ability to trap a *P. falciparum* promoter, using a *piggyBac* transposon carrying the GFP reporter gene, has already been demonstrated (Balu and Adams, 2006). Therefore, the set-up of the *piggyBac* system in plasmodia guarantees efficient and convenient integration events across the genome, which is essential for large-scale, whole-genome mutagenesis of malaria parasites.

The trypanosomes

The *Trypanosoma* species genomes comprise about 30-60 Mbp and the estimated number of genes is nearly 22,000 (Berriman et al., 2005; El-Sayed et al., 2005). The genome organization of the *Trypanosome* genus is the same as that of other kinetoplastids, as described above for *Leishmania*. The genes are organized into long DGCs, with batteries of genes oriented in the same direction. With a few exceptions, trypanosome genes do not contain introns (Reviewed by (Pays, 2005).

The transposition of a Mos1-derived mariner element was also demonstrated for one locus of *T. brucei* (Leal et al., 2004). In this study, the transposon was provided transiently after electroporation and *mariner* transposase was expressed from an inducible chromosomal locus. Selection of mutants was based in a defective expression of a surface EP-procyclins marker as detected by concanavalin A (ConA) binding. Remarkably, a homozygous *mariner* insertion into a homologue of the human *ALG12*, which encodes a mannosyltransferase, was observed. Since trypanosomes are diploid, this requires either two independent insertion events or a transposition followed by LOH (see Figure 2). In this study however, insertions into this locus were characterized by an unusually high frequency of LOH, on the order of 10^{-3} . Unfortunately, this frequency of LOH appears unique to this study, as the LOH at other loci in Trypanosomes is more typical of other eukaryotes, eg $\sim 10^{-5}$ (Scahill et al., 2008), in agreement with results from *L. major* (Gueiros-Filho and Beverley, 1996). Perhaps for this reason, other attempts to associate *mariner* transposition and gene conversion events to generate *T. brucei* or *Leishmania* mutants have not been successful (Alvaro Acosta-Serrano, personal communication; SMB, unpublished data).

5. Concluding Remarks

Transposon mutagenesis provides a significant addition to the repertoire of genetic tools for tackling the vast information unveiled with the completion of genome projects on protozoan pathogens. Transposition systems that are suitable for studying genes of each of these human pathogens have been identified. In some cases, as the *mariner* system adapted for *Leishmania*, an extensive collection of reagents is available. However, this is an ongoing process and further improvement is required for the generation, maintenance and sharing of mutant libraries. The availability of these tools is a starting point for the major challenge of studying in detail as many disruption events and trapped genes and their resulting phenotypes as possible. The exploration of such libraries offers a unique opportunity to a better understanding not only of these parasites' biology, but also of the interaction they establish with their hosts. The unveiling of mechanisms of gene expression and protein function will certainly help the identification of new drug targets and the development of vaccination strategies minimizing the misery these pathogens inflict to millions of people around the globe.

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Figure 1. DNA transposons mobilization

Scheme of the general cut-and-paste transposition mechanism. Expressed transposase (blue spheres) recognizes Terminal Inverted Repeats (TIRs; dashed arrowhead) which are flanked by a duplicated target site characteristic for each DNA transposon family (TA in the case of Tc1/*mariner* superfamily). TEs are excised by transposase that cleaves at TIRs and the double strand break is appropriately repaired leaving a footprint, also typical of each transposon family. The transposase-TE complex recognizes the DNA target site which is duplicated upon insertion. Different TEs can be mobilized. Autonomous elements encode a functional transposase that can be expressed upon integration (1). Non-autonomous transposons rely on transposase activity in *trans* for proper mobilization since its transposase gene is either absent or truncated (2).

Figure 2. TE-based approaches for protozoan parasites genome manipulation

A **Transposon donor plasmid** carrying a suitable TE (as summarized in Table 1) can be used in two types of TE-based approaches. **A.** In an *in vitro* **shuttle mutagenesis** the purified transposase mediates the insertion event into a **Genomic DNA library**. The **Insertion library** can be used to perform **Episome transfection** into the parasite followed by screening for gene fusion expression. Insertion library can also generated fragments that can be used for **Transfection/Double crossover** into the parasite. In a diploid organism, targeting fragments can disrupt one (**1-hit**) or both (**2-hits**) alleles of the locus of interest by homologous recombination. While 1-hit double crossover events allow for trapping strategies, the 2-hits event occurs at a lower frequency and may be influenced by the selection efficiency and gene essentiality. Alternatively, double targeting can be achieved through an LOH event. **B.** Mobilization can also be mediated *in vivo* by providing a transposon donor plasmid to a **parasite bearing an active transposase**. Following **Transposase expression** and **Transposition into chromosome**, the selection of 1-hit or 2-hits insertion events will greatly depend not only on the factors listed above, but also on levels of transposase activity, loci accessibility to the enzyme and target site preference. The double mutant can be also selected after the induction of LOH in a 1-hit-insertion selected parasite.

Figure 3. Expression of a NEO-tagged gene depends on genomic context

(A) Schematic representation of the insertion event of the modified mariner element / NEO*ELSAT that was used to disrupt the wild gene copy. (B) Phenotypes observed from cell lines bearing the ORF::/NEO*ELSAT construct in different contexts. The cell INT, which expresses this construct from the chromosome, is resistant to Nourseothricin (SAT^R) and the expression of a NEO-tagged protein is absent. The cell line EPI, which bears a plasmid containing the ORF::/NEO*ELSAT construct, is also resistant to Nourseothricin and expresses the NEO-tagged protein, as confirmed by western blot analysis (α -Neo). The growth of the cell line INT under Nourseothricin pressure elicited the formation of an amplicon. As for the cell line supplied with the plasmid, the AMPL strain expresses the NEO-tagged protein. These data underscore the utility of this transposon-based approach and suggests that gene expression control in *Leishmania* is dependent on the genomic context.

Table1
Representative *mariner* transposons available for functional studies in *Leishmania*.

Transposon	Schematic Representation
ELSAT
/GFP* ^K
/GEP3*
/GEP3/ /GEP2/
/NEO* ^{ELSAT}
/-2x5

Transposon

Schematic Representation

TK-PG

Grey arrowheads, 5' and 3' *mariner* inverted repeats; black arrowhead, *E.coli* promoter; '/', the gene lacks a start or a stop codon;

* the gene contains a stop codon; AG, trans-splicing acceptor site. SAT, nourseothricin resistance marker; ; GFP, green fluorescent protein; Km, kanamycin resistance marker; PHLEO, phleomycin/zeocin resistance marker; NEO, G418 resistance marker; GUS, β -glucuronidase.