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Size matters: versatile use of *PiggyBac* transposons as a genetic manipulation tool

Adele Kim · Ilmari Pyykko

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Abstract Transposons have been promising elements for gene integration, and the *Sleeping Beauty* (SB) system has been the major one for many years, although there have been several other transposon systems available, for example, *Tol2*. However, recently another system known as *PiggyBac* (PB) has been introduced and developed for fulfilling the same purposes, for example, mutagenesis, transgenesis and gene therapy and in some cases with improved transposition efficiency and advantages over the *Sleeping Beauty* transposon system, although improved hyperactive transposase has highly increased the transposition efficacy for SB. The PB systems have been used in many different scientific research fields; therefore, the purpose of this review is to describe some of these versatile uses of the *PiggyBac* system to give readers an overview on the usage of *PiggyBac* system.

Keywords *PiggyBac* · *Sleeping Beauty* · Transposon · Vector · Forward genetics · Genetic tool

Abbreviations

TR	Terminal repeat
IR	Internal repeat
ORF	Open reading frame
PEI	Polyethylenimine
ES	Embryonic stem
MLV	Murine leukemia virus
HIV	Human immunodeficiency virus
DBD	DNA-binding domain
NLS	Nuclear localization signal

A. Kim (✉) · I. Pyykko
Department of Otorhinolaryngology, University of Tampere Medical School, Tampere, Finland
e-mail: nam.kim@uta.fi

Introduction

DNA transposons and retrotransposons constitute a major component of repetitive sequences in eukaryotes [1, 2]. Their movement around the host genome has been partially responsible for the current genomes. DNA transposons are genetic elements that can relocate between genomic sites by a 'cut and paste' mechanism and these elements have been used for functional genomics in different organisms [3, 4]. Many different types of transposon systems for mammalian gene transfer are as follows: *hAT*-like *Tol2*, the only naturally active vertebrate transposon, isolated from the genome of the Japanese medaka fish; two *Tcl*-like transposons, *Sleeping Beauty* (SB) and *Frog Prince*, reconstructed from inactive transposons of fish and frog genomes, respectively; and the *PiggyBac* isolated from the cabbage looper moth *Trichoplusia ni* [5–8]. Among these transposon systems, SB has gained until recently the leading position in transgenesis and mutagenesis.

Sleeping Beauty was the first DNA transposon system shown to be functional in mammalian cells, and it has been used for insertional mutagenesis in some rodent germ lines [9, 10]; however, its tendency for strong 'local hopping' and also leaving footprints seem to have made SB to share its popularity with *PiggyBac*. SB, however, has successfully been used to screen for new cancer genes [11, 12].

The *PiggyBac* element was originally discovered by Fraser et al. [13] from the *Trichoplusia ni* cell line TN-368 as a repetitive element, and it was isolated by Cary et al. in 1989 [14]. Subsequently, it was found to efficiently transpose in many different species [15, 16]. One of the advantages of PB transposon over SB transposon is that it does not leave any footprint as it is precisely excised [8, 17] when a transposition takes place. For instance, the TA dinucleotides used by the *Sleeping Beauty* transposon

as integration sites can be altered after excision; however, the TTAA integration sites used by *piggyBac* transposons are repaired to its original sequence on excision. This allows removal of transposons from the host genome without changing any nucleotide sequence. PB was demonstrated by Ding et al. [18] and also by Wu et al. [19] that it was very efficient for germ line mutagenesis in mice and also confirmed later that it has significantly higher transposition activity in mammalian cell lines than SB or *Tol2* [19], although more recently SB has shown a very high transposition efficacy in CD34$^+$ [20]. PB seems to have many advantages compared to several other transposon systems and some of them are discussed in this review later.

In parallel with the use of PB system in the studies of functional genomics, new PB-related elements and sequences are also being found in a variety of insect species and other organisms [21]. Finding sequences related to PB elements in different organisms also allows researchers to study the lineage of PB and also contributes in understanding how and why PB elements have existed for so long; however, the main purpose of this review is not to discuss this but to deploy how PB system can be adapted for performing gene integration in several different organisms. Therefore, PB-related sequence will not be discussed in detail.

This review is quite unique in its own way as to compare with several other reviews published in the past on the subject of PB, for instance, many of the previous reviews have specified the use of PB to a certain specific type of insects or mammals, i.e., *Drosophila* or mouse. However, in this review, the scope is much broader and therefore maybe shallower but still gives readers to have a general overview of what PB is capable of doing as a genetic manipulation tool and how it may be modified to function more efficiently.

Molecular structure of *PiggyBac* element

The initial sequence of PB element is a 2,475 bp with short-inverted repeats; it has an asymmetric terminal repeat structure with a 3-bp spacer between the 5′ 13-bp TR (terminal repeat) and 19-bp IR (internal repeat) and a 31-bp spacer between the 3′ TR and IR [14], and the single 2.1-kb open reading frame (ORF) encodes a functional transposase [22].

The PB element is capable of precise excision from baculovirus insertion sites, and this process involves site-specific recombination [8] and this precise excision is possible in plasmid-based assays [17, 23]. In 2005, Li et al. [24] published an article on the minimum internal and external sequences of PB required for sufficient transposition, and they have found out that a minimum of 55 bp of intervening sequence is necessary for optimal transposition, whereas the length less than 44 bp result in a dramatic decrease in transposition frequency. Several years after this discovery, the same group has found out that a 5′-terminal repeat of 313 bp and a 3′-terminal repeat of 235 bp are the minimum PB terminal repeats that are essential for transformation of target genome. Cadinanos and Bradley [25] have also published a paper where they stated that the minimum inverted flanking sequences may be able to transpose between different plasmids in insect cells; however, those are not sufficient to allow transposition from a donor plasmid to genomic DNA.

In the same year, another group of researchers discovered something unexpectedly. This group used the PB transposon for constructing vectors that were used for inducing heterologous gene expression in lepidopteran insect cells, and from this they have found out that the position where the heterologous genes placed within the vector is affecting the levels of gene expression, for instance, the rightwards-oriented heterologous genes were expressed in higher levels than the leftwards-oriented ones. They have revealed that there is stimulation by an activator element in the 3′-TRD (terminal repeat domain) of PB [26].

The correct molecular structure of PB terminals is not the only important factor when PB element is used in a vector but the molecular structure of transposase is also an important factor as this phenomenon was proven several times in several articles by Keith et al. [27]. They have applied a bioinformatics tool and shown that by performing a PSORTLL analysis with the transposase amino acid sequence, they predicted NLS is required for the transposase to enter the nucleus of S2 cells in *D. melanogaster*, and they have also proved in the same year that a highly conserved aspartate residues are essential in PB transposase when mutants tested had significantly decreased excision frequency when compared with the wild type transposase.

Mechanism of DNA integration by PB system

PB transposons, after being used in a vector construction with some essential sequences, are capable of integrating its cargo gene into chromosomes of a host; however, this is not possible unless an enzyme called PB transposase is present. When a transposition is taking place, either the vector consisting the cargo has also the ORF of PB transposase or a separate vector known as helper plasmid is often used as a co-transfecting plasmid, and this helper vector consists of an ORF of PB transposase, therefore, the expression of transposase allows the cargo to be cut and integrated into host genome.

In some cases, instead of using the helper plasmids expressing PB transposase, it has been suggested that the

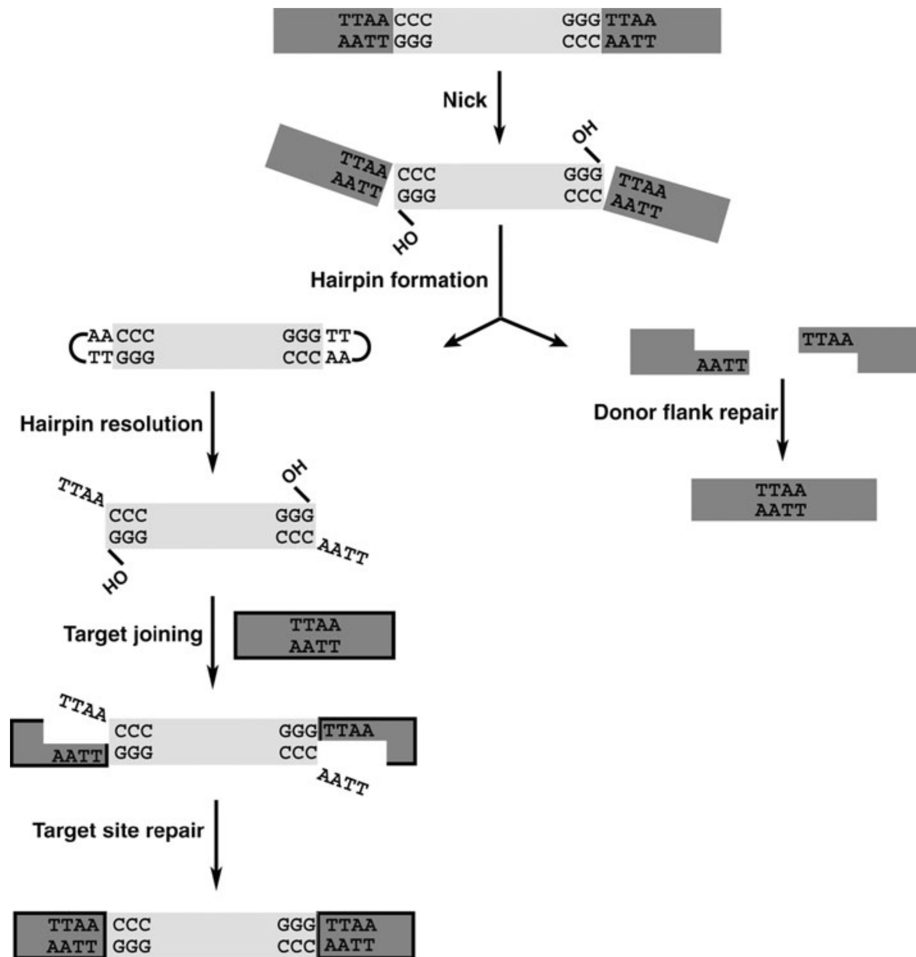

Fig. 1 Schematic representation of the *piggyBac* cut and paste transposition. *piggyBac* transposition initiates with nicks at the 3′ ends of the transposon, exposing 3′OHs. These 3′OHs then attack the complementary strand 4nt into the flanking donor DNA, thereby forming hairpins on the transposon ends with the concomitant release of the transposon ends. Donor site repair can occur by ligation of the complementary 5′ TTAA overhangs on the flanking donor ends, precisely reforming the TTAA target sequence. Transposon end hairpins are resolved by transposase, re-exposing the 3′OH transposon ends generating 4nt TTAA overhangs on the 5′ ends of the excised transposon. The 3′ OH transposon ends join to the staggered positions at the 5′ T's of the TTAA/AATT target sequence. Repair of the single-strand gaps flanking the newly inserted transposon gives rise to the 4 bp TTAA target sequence duplication. Figure adapted from Mitra et al. [29]. [Figure adapted and reprinted with permission from the publisher]

enzyme itself could be used or even mRNAs encoding transposase have been suggested as a source of the enzyme [28] and this may reduce the potential genotoxicity induced by the helper plasmids. Unlike the characteristics of most of the other transposition reactions by several other transposon systems, the DNA cut and paste transposon PB from cabbage looper moth *Trichoplusia ni* consistently shows precise excision on element transposition [14, 22]. The exclusive use of TTAA target sites has been discovered by Fraser et al. [8]. The self-explanatory schematic representation of the PB cut and paste transposition is shown here reproduced from Mitra et al. [29] (Fig. 1).

Benefits and versatile use of PB

As briefly mentioned in the Introduction, PB is better than several other transposons available nowadays, and one of the significant reasons for it is the size of the cargo that it can carry, for example, PB can carry up to 14 kb of foreign genes in the mouse germ line [18], and even more recently, Lacoste et al. [30] have designed a *PiggyBac* system named *ePiggyBac* and this was capable of delivering up to 18-kb inserts in hES cells. In this article, unfortunately there is no comparison study on copy number of short and long transposons; however, the large size of cargo being inserted by a PB may open up more possibilities for PB capacity as a genetic tool. Besides for SB, transposition efficiency seems to have been reduced in a size-dependent manner (about 50% when the size of transposon reaches 6 kb) [31].

In 2007, Wilson et al. [32] compared transposition activity of PB with SB, and they have shown that PB did not have any overproduction inhibition of transposase, which can be a limitation of SB system although titration method partially solves this limitation. If there is no overproduction problem, it is possible to construct one vector consisting of both PB transposase and transposon; however, this type of vector exhibited only a twofold increase in transposition activity [32].

Recently, PB is also found to be capable of mediating stable integration of up to four independent transposons concurrently in human cells following a single transfection [33] and by doing this, a stable cell line useful for high-throughput electrophysiological analysis was possible. This

technology has potential to enable genetic engineering of cellular models for use in drug discovery applications targeting multiprotein complexes, for simultaneous production of multiple recombinant proteins and for treatment of genetic disorders that require the stable delivery of multiple genes.

Initially, PB was used for gene transfer and germ line transformation in a wide range of invertebrate species and later on, PB was also used for vertebrates. The Mediterranean fruit fly was the very first invertebrate target organism to be successfully transformed using the PB system [34], and a wide variety of insects have been subsequently transformed and a review on this was published in 2002 [35]. Later on, even more variety of organisms including vertebrates has been transformed and some of them are discussed in detail in the following in this section. The PB, therefore, has been recognized as an important tool for genetic transformation in many different biological systems.

PB has been used mainly in the medical-related fields as described in the following nevertheless, PB has also been useful for industry in few cases, for instance, Wen et al. [36] have recently produced transgenic silkworms to produce silk with higher tensile strength and elasticity by using PB. The next part is divided into three sections, namely, Mutagenesis, Transgenesis and Gene Therapy, and in each section, examples of different species are described.

Mutagenesis

Drosophila

In *Drosophila melanogaster*, the use of PB in insertional mutagenesis experiments has greatly increased our understanding of fruitfly biology, leading to the identification of genes involved in biological functions as diverse as development, immunity, tissue modelling and embryogenesis [16, 37–39]. The genus *Drosophila* has an evolutionary history of exposure to alcohols; therefore, the power of joining genetics and molecular analysis has made *Drosophila* an established model in studies of alcohol metabolism and tolerance. In 2009, Eanes et al. [40] used PB to create deletions and duplications to study the two genes, namely, *Gpdh* and *Mdh1*, that affect ethanol tolerance in adult *D. melanogaster*.

Transgenesis

Mosquitos

In mosquitos, PB has been shown to be functional in germ line transformation experiments, and *Anopheles* species have shown to be transformed efficiently by PB [41, 42].

Malaria is a leading infectious disease that affects 400–600 million people, causing 2–3 million deaths, every year [43], and female *Anopheles* mosquitos carrying *Plasmodium falciparum*, most dangerous species among four *Plasmodium* species, are responsible for much of the mortality associated with the disease in young children in sub-Saharan Africa. Recently, Balu et al. [44] used PB as a molecular genetic tool for functional characterization of the *Plasmodium falciparum* genome and clearly demonstrated that PB is an indispensable tool for forwards functional genomics in *Plasmodium falciparum* and that helps to understand parasite biology better and eventually accelerate drug and vaccine development for malaria.

Human blood fluke, *Schistosoma mansoni*

Schistosomiasis is considered as the most important of the human helminthiases in terms of morbidity and mortality [45–47]. Treatment for people acquired of this pathogen is the use of praziquantel; however, this medication may eventually develop drug resistance [48]. PB transposon has been used by Morales et al. [49] to mediate transgenesis of this human blood fluke, and they have found out that the PB transposon had integrated into numerous sites within the parasite chromosomes. Their findings suggest and open up the path towards reverse genetics, heritable transgenesis and functional genomics of this parasite and eventually these allow human to tackle and combat this neglected tropical disease.

Chicken

The chicken embryo has been widely used for studying early embryogenesis because of its ready availability and accessibility to various manipulations [50]. During the past decade, early neural developments such as neurogenesis, patterning and migrations have been able to be studied [51–53], but studying the progress in the late development of axon targeting and synapse formation have been difficult because of technical limitations, and this is where PB can be manipulated and unlike recombinant retroviral systems that have been used before, PB can accommodate relatively large DNA fragments without compromising transfection efficiency [18]. Using PB transgenic in chicks, Lu et al. [54] managed to achieve temporal and spatial regulation of transgene expression and performed stable RNA interference (RNAi).

Human

Immunotherapy with antigen-specific T cells can control and eliminate virus infections and Epstein–Barr virus-associated

lymphoma after hematopoietic stem cell transplantation [55, 56], and has shown promises for the treatment of lymphoma, melanoma and carcinoma arising in the immunocompetent host [57, 58]. T cells are modified by retrovirus and lentivirus vectors, both have limited transgene capacity that allows maximum expression of two to three transgenes [59, 60]. Recently, however, using the PB, it was possible to transduce human T lymphocytes with multiple genes [61]. Another more recent work where PB was used for genetically manipulating T cells for the treatment of B-lineage malignancies is shown by Manuri et al. [62].

Another group has also been able to show that PB is very promising as a non-viral gene delivery system in T cells, and Galvan et al. [63] have also assessed the potential genotoxicity of PB in T cells by performing genome-wide analysis of integration sites. They suggested that PB is less genotoxic than currently and clinically used viral-based vectors, such as MLV and HIV-based lentiviral vectors.

Transgenic expression of just four different transcription factors (c-Myc, Klf4, Oct4 and Sox2) is sufficient to reprogram somatic cells to a pluripotent state [64–67], and the resulting induced pluripotent stem (iPS) cells resemble embryonic stem cells in their properties and potential to differentiate into a range of adult cell types. Currently, reprogramming strategies involve retroviral [64], lentiviral [68], adenoviral [69] and plasmid [70] transfection to deliver reprogramming factor transgenes. In 2009, Woltjen et al. [71] demonstrated successfully that instead of using any of those existing transfection methods, PB was used to transpose murine and human embryonic fibroblasts to induce pluripotent stem cells.

Human immunodeficiency virus type 1 (HIV-1) is responsible for the current infection of more than 20 million people and the death of more than 2 million living in sub-Saharan Africa (UNAIDS 2007). Therefore, developing a safe and effective vaccine is essential, and a PB transposon was once again used to create HIV-1 gag transgenic insect cell lines for VLP production. This method has replaced the baculovirus system of which has some drawbacks, such as contamination [72].

Swine

Swine is important in agriculture as well as a biomedical model. Manipulation of the pig genome improves production efficiency, and the pigs are also used for modelling human disease so it can contribute towards developing treatments and also xenotransplantation. Because the PB transposon has already been proven to be a useful tool for genetic manipulation, once again this was used with cells derived from the swine tissue and found that the PB is functioning efficiently once again [73].

Gene therapy

Murine

Ovarian carcinoma is a common cancer worldwide, and there is still no effective therapy for such disease. Gene therapy may represent an attractive answer as a treatment, and many experimental approaches have shown potential promise in ovarian cancer treatment [74, 75]. Last year, Kang et al. [76] have demonstrated that PB coupled with polyethylenimine (PEI) encoding herpes simplex thymidine kinase (HSV-tk) have reduced the size of tumour in ovarian carcinoma in mice. Their results show that a non-viral gene delivery system coupling PB transposon with PEI can be used as an efficient tool for gene therapy in ovarian cancer.

The transposability of PB transposon in mice was demonstrated by Nakanishi et al. at the beginning of 2010 [77] for the very first time, and the gene expressions were sustained more than 2 months. Gene trap system based on PB transposon for mutagenesis in mice has several advantages, for instance, it has higher transposition frequency and because it does not leave any foot prints that itself can be a mutagenic. Another advantage of the PB gene trap is that it uses breeding to generate new multipurpose alleles without in vitro ES cell manipulations or in vivo blastocyst injections [19].

Discovery of PB-related sequences

Many groups of researchers are trying to find sequences that are similar to PB in many different organisms, and the importance of this work is not only gaining insight into its evolution but also it is very much concerned with the stability of PB transposons as some endogenous transposases may mobilize transgenes introduced by related exogenous transposases. One approach is to avoid this cross-mobilization problem by using exogenous transposons that have no related endogenous transposons in the target species [78].

There are around 2,000 PB-like elements dispersed throughout human chromosomes. Two major families (MER85 and MER75) are among the most recently amplified DNA transposon families recognizable in the human genome [1]. However, these two families are almost entirely represented by short copies with no transposase ORF, and there is no evidence that any endogenous PB-like transposase remains in the human genome [79]. This finding indicates that PB may be used for human cells without any cross-mobilization problems.

Perspectives

As described above, PB has been adopted in many different research fields; however, there is one serious drawback which is that PB transposition is specific as it only targets TTAA sequence [14] but not specific enough where the integration is concerned. In other words, PB cannot target a specific position within the genome but randomly inserted into chromosomes because there are hundreds of TTAA sites available. Methods to integrate the transposon to a unique genomic site have been proposed [80]. Theoretically, transposon site-specific integration can be achieved by increasing the DNA-binding specificity of its transposase [80, 81]. It seems that fusing the DNA-binding domains (DBD) that only recognizes a unique chromosomal sequence can be attached onto the transposase. In 2006, Maragathavally et al. [81] have shown that a chimeric *PiggyBac* transposase with GAL4 DBD attached to its N terminus resulted in targeting a specific site upstream of the UAS (GAL4-binding) site in a plasmid assay system in *Aedes aegypti* embryos. Another problem regarding the site specificity is that the PB seems to favour transcription start sites as targets for integration, and this raises concerns about its suitability for gene therapy applications [82].

Another problem with PB is controlling the remobilization, for instance, if a gene was inserted into a right position of a chromosome by a PB where it can exist for a long time, this gene must not again be translocated but this seems to be happening in many cases. To avoid this, one possible suggestion is to degrade all the transposases that are required for integration; however, this is not so easy as it is said. In one article by Sethuraman et al. [83], they said that they did not observe any remobilization after the gene delivered by PB to chromosomes in *Aedes aegypti* mosquitoes, unlike many other cases with the insects. One suggested reason for this stability was that the chromatin structure, such as the degree of DNA packing and the extent to which the chromosomes are in 'open' and 'closed' configuration, may limit access of transposase to the sequence where this enzyme must react with. Perhaps if we study this organism further, we will be able to find out why this organism offers more stability than many other organisms.

As an author for this review, I am still at an early stage of my research with PB; therefore, until now I do not have any published data to support my ideas on how PB is manipulated at molecular level to target only a specific genomic site, however, to reduce any harms caused by unspecific integration; I personally thought that telomere could be a target site for integration because telomeres degrade as the chromosomes age and do not contain any genes; however, the telomeres in humans are mostly composed of a repeated sequence of TTTAGGG, and the third T should be an A for a *PiggyBac* element to be inserted and therefore, manipulation of the specific binding enzymes such as tankyrase (a poly polymerase) and telomerase on the DNA to target the telomeres is not so feasible after all.

Finally, potential genotoxicity of PB is a big issue that needs addressing. The presence of transposase ORF within a separate vector in cells after transfection is not only capable of integrating the cargo (gene) flanked by the PB terminal repeat element sequences but also itself has the potential to be integrated into the host genome, and this is considered as a potential genotoxic effect. Therefore, a vector that consists of both transposons cargo and the PB transposase with a function where once the cargo is released, the transposase gene is rendered inactive [84] can be a very useful mechanism.

The potential genotoxicity of PB depends on the frequency of integration into genes and the expression level of the cargo [85, 86], and its genotoxicity can be reduced perhaps by adapting insulator elements [87].

Conclusions

As the first part of the title of this review emphasized, PB has gained its place as a genetic manipulation tool by its size: The size of the cargo it can carry and also the size of the animal kingdom where PB was adopted.

As once SB was the leading system as a genetic tool before PB was discovered, maybe one day in the near future PB will be replaced by even more efficient system. However, until then, the best option as a tool for genetic manipulation is the PB. The PB has proven to be more advantageous compared with the SB; however, there are still some major obstacles to overcome, such as specific targeting for integration and also whether it will be eventually possible to be used for gene therapy in human. To achieve these purposes, much more intensive ongoing researches are needed, and one day this goal is feasible for the benefit of many human beings.

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