Microbiology Spectrum
American Society for Microbiology Press

piggyBac Transposon

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ABSTRACT The *piggyBac* transposon was originally isolated from the cabbage looper moth, *Trichoplusia ni*, in the 1980s. Despite its early discovery and dissimilarity to the other DNA transposon families, the *piggyBac* transposon was not recognized as a member of a large transposon superfamily for a long time. Initially, the *piggyBac* transposon was thought to be a rare transposon. This view, however, has now been completely revised as a number of fully sequenced genomes have revealed the presence of *piggyBac*-like repetitive elements. The isolation of active copies of the *piggyBac*-like elements from several distinct species further supported this revision. This includes the first isolation of an active mammalian DNA transposon identified in the bat genome. To date, the *piggyBac* transposon has been deeply characterized and it represents a number of unique characteristics. In general, all members of the *piggyBac* superfamily use TTAA as their integration target sites. In addition, the *piggyBac* transposon shows precise excision, i.e., restoring the sequence to its preintegration state, and can transpose in a variety of organisms such as yeasts, malaria parasites, insects, mammals, and even in plants. Biochemical analysis of the chemical steps of transposition revealed that *piggyBac* does not require DNA synthesis during the actual transposition event. The broad host range has attracted researchers from many different fields, and the *piggyBac* transposon is currently the most widely used transposon system for genetic manipulations.

INTRODUCTION

The *piggyBac* transposon superfamily is a relatively recently recognized transposon superfamily. The original *piggyBac* transposon was isolated from the genome of the cabbage looper moth, *Trichoplusia ni* in the 1980s. However, the second member of the *piggyBac*-like element superfamily was not identified until 2000. It was not described as a transposon superfamily in the previous edition of *Mobile DNA*. In the last decade or so, a number of sequenced genomes have revealed that *piggyBac*-like elements are actually widespread DNA transposons. Active copies of the transposon have also been identified from another moth species, from frogs, and for the first time, from a mammal. Moreover, because the *piggyBac* transposon has a broad host spectrum from yeast to mammals, this mobile element has been widely used for a variety of applications in a diverse range of organisms. In this chapter, we will describe the discovery and diversity of the *piggyBac* transposon, its mechanism of transposition, and its application as a genetic tool. We will also provide two examples of genetic screening that the *piggyBac* transposon has enabled.

DISCOVERY OF THE *piggyBac* TRANSPOSON

It was known in the late 1970s that when insect DNA viruses, namely *Galleria mellonella* or *Autographa californica* nuclear polyhedrosis viruses (species of Baculovirus), were serially passaged in *T. ni* cell line TN-368, mutant viruses that showed the Few Polyhedra (FP) plaque morphology phenotype appeared spontaneously but reproducibly (1, 2, 3). Analyses of these mutant viruses revealed that the host cell DNA had inserted into the viral genome, resulting in the loss of the 25 KDa viral protein (2). These mutant viruses could revert to the wild-type by serial passage in the TN-368 cell line. This was associated with the loss of the inserted DNAs in the revertant viral genome (2). The sizes of these inserted

Received: 15 May 2014, **Accepted:** 14 August 2014, **Published:** 5 March 2015
Editors: Mick Chandler, Université Paul Sabatier, Toulouse, France, and Nancy Craig, Johns Hopkins University, Baltimore, MD
Citation: Yusa K. 2014. *piggyBac* transposon. *Microbiol Spectrum* 3(2):MDNA3-0028-2014. doi:10.1128/microbiolspec.MDNA3-0028-2014.
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fragments varied between 0.5 and 2.8 kb (2). One of the most frequently inserted fragments was 2.5 kb in size and was present as multiple copies in the *T. ni* genome (2). These observations strongly indicated that the insertions in the viral genome are mobile elements that reside in the *T. ni* genome. Sequencing analyses of the inserted DNA revealed that the integration sites had typical DNA transposon signatures such as perfect duplication of the 4-bp viral DNA (TTAA) and terminal inverted repeats (TIRs) within the inserted fragments (4). In 1989, the entire sequence of the inserted DNA was reported, revealing the structure of the *piggyBac* transposon for the first time (5).

The *T. ni piggyBac* transposon is a 2,475-bp-long autonomous mobile element (GenBank Accession number. J04364.2; Fig. 1). It has 13-bp TIRs located at both ends and 19-bp subterminal inverted repeats located 3-bp and 31-bp 5′ and 3′ internally from the TIRs, respectively. Between the subterminal inverted repeats, there is a single 1.8-kb open reading frame, which encodes the 594-amino acid *piggyBac* transposase with a molecular weight of 64 kDa. The functionality of the coded protein as a transposase was first confirmed in 1996 with the successful mobilization of a nonautonomous *piggyBac* element (6).

DIVERSITY AND DOMESTICATION OF THE *piggyBac* SUPERFAMILY TRANSPOSONS

Since its discovery in 1989, *T. ni piggyBac* had for a long time remained the only member of the currently-known *piggyBac* superfamily. However, this view has now changed completely owing to the discovery of a number of *piggyBac*-like elements in a variety of organisms. The *piggyBac* transposon superfamily is now recognized as a widespread DNA transposon superfamily. Surprisingly, a currently active copy of the *piggyBac* transposon was recently isolated from mammals. Furthermore, genome sequencing has revealed many genes that were derived from transposable elements. It has been shown that domesticated *piggyBac* transposases play an essential role in cellular functions in ciliates (7, 8). This section describes the diversity of the *piggyBac* transposon and the domestication of *piggyBac* transposases.

piggyBac Transposable Elements

In the 1980s and 1990s, several genomic DNA segments were observed to contain target site duplication of the tetranucleotide TTAA and an addition of 2-to-3 Cs. These included *Tx1* derived from *Xenopus* (9, 10) and TFP3 (4, 11) from *T ni.*, *Pokey* from *Daphnia*, and host DNA integration into the genome of *Autographa californica* nuclear polyhedrosis virus passaged in a *Spodoptera frugiperda* cell line (12, 13, 14). All these elements were nonautonomous and the transposases responsible for the mobilization of these nonautonomous elements could not be identified. It was thus not clear whether these TTAA-specific transposons shared molecular characteristics with *T. ni piggyBac* and belonged to the same transposon superfamily.

In the mid-1990s, transgenesis of nondrosophilid insects using *T. ni piggyBac* transposon-based vectors was successful, which popularized the approach (13, 14). Detection and genetic stability of the integrated transposon were often examined by Southern blot analysis. None of the early transgenesis work identified genetic elements that cross-hybridized with the *T. ni piggyBac* transposon in the test insects' genomes (15, 16), suggesting that *piggyBac* might be restricted in *T. ni*. However, surprising results were reported in 2000 in a paper describing the transgenesis of the Oriental fruit fly, *Bactrocera dorsalis* (17). The genome of the Oriental fruit fly contains sequences, some of which are apparently full-length elements, that cross-hybridized with *T. ni piggyBac*. Sequencing analysis of a transposase-coding region revealed that the cross-hybridizing elements are indeed *piggyBac*-like elements with 95% nucleotide identity to *T. ni piggyBac* (17). This remarkable similarity and the absence of the *piggyBac*-like elements in another bactrocerid species, *B. cucuribitae*, or Mediterranean fruit fly, *Ceratitis capitata*, suggested a very recent horizontal transmission between *T. ni* and *B. dorsalis*; this

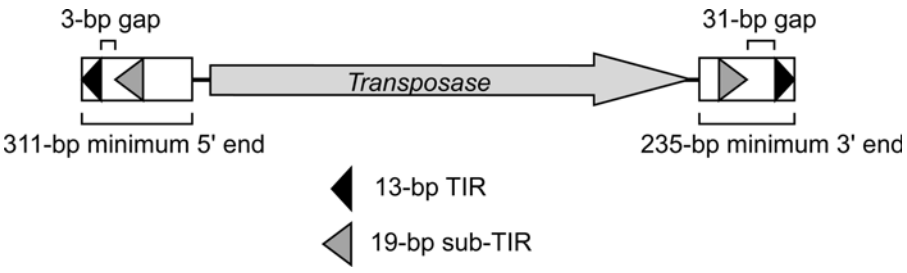


FIGURE 1 Structure of the *T. ni piggyBac* transposon (GenBank accession number J04364.2). TIR, terminal inverted repeat. The minimum TIR sequences are based on ref. (61). doi:10.1128/microbiolspec.MDNA3-0028-2014.f1

was probably via one or more intermediate species since these species are geographically distinct (17).

In the following several years, whole genome sequences were published for human (18), pufferfish *Fugu rubripes* (19), and the African malaria mosquito *Anopheles gambiae* (20). Analyses of these genome sequences revealed a number of *piggyBac*-like repetitive sequences. Furthermore, the full-length transposase-coding sequence of the *Pokey* element was isolated in 2002 and showed a clear similarity to *T. ni piggyBac* transposase (21). Thus far, *piggyBac*-like sequences have been found in the genomes of a wide range of organisms including fungi, plants, insects, crustaceans, urochordates, amphibians, fishes, and mammals (22). In addition, active or apparently intact full-length *piggyBac*-like transposons have been identified in another moth *Macdunnoughia crassisigna* (23), silkworm (24), ants (25), *Xenopus* (26), and the bat *Myotis lucifugus* (27).

piggyBat, The First Active DNA Transposon Isolated from Mammals

Around 35 to 50% of the mammalian genome is repetitive elements and a few percent typically comprise fossil DNA transposons derived from various transposon superfamilies (18, 28, 29, 30, 31, 32). A detailed analysis of the evolutionary history of DNA transposons found in the human genome revealed that DNA transposons were active during early primate evolution (approximately 80 to 40 million years ago); however, all these elements are now extinct and there is no evidence of transposition in the last 40 million years (33). A similar trend is also observed in the mouse genome (28). Like other organisms, the *M. lucifugus* genome contains numerous DNA transposons derived from almost every transposon superfamily (34). However, the DNA transposons in the *M. lucifugus* genome have clear differences from those found in the human genome. Firstly, the sequences of the bat DNA transposons are minimally diverged from their consensus sequence. Secondly, the bat genome contains potentially functional full-length *hAT* and *piggyBac* transposons (34). Finally, preintegration sites have also been identified in a related species *M. austroriparius* (34, 35), which separated from *M. lucifugus* approximately 6 million years ago. This strongly suggests that the bat genome was recently invaded by the DNA transposons. Among these transposons, *piggyBac*-like elements are markedly young. For example, a large fraction of the nonautonomous *piggyBac1_ML* element (*npiggy_156* and *npiggy_239*) is identical in sequence (34). Thirty-four full-length copies of the *piggyBac1_ML* element identified in the *M. lucifugus* genome show >99% nucleotide identity (27). Recently, the full-length *piggyBac1_ML* element was cloned and tested for its transposition activity. Similar to *T. ni piggyBac*, the bat-derived *piggyBac* transposon (named *piggyBat*) is able to transpose in yeast, bat, and human cells, albeit at a 2-to-10 times lower frequency than *T. ni piggyBac* (27). Cloning of the *piggyBat* element represented the first isolation of mammalian *piggyBac* derivative. Further analysis of this active mammalian transposon would give us deeper insight into how these parasitic genetic elements contribute to the diversity and evolution of the host species.

Domesticated *piggyBac* Transposases

Various organisms have occasionally utilized transposase genes to create new genes with beneficial cellular functions. This is called "domestication" of transposases, the process of which is well exemplified by RAG1 in V(D)J recombination (derived from *Transib* transposases [36, 37]) and the CENPB centromere protein (derived from *pogo* transposases [38]). The genome sequences of various species have also revealed a number of previously unrecognized genes that were derived from various transposable elements including *piggyBac* transposases (18, 22). For example, the human genome contains 5 genes that were apparently derived from *piggyBac* transposases, *PGBD1-5*. While *piggyBac*-derived sequences in *PGBD1-4* are found in one coding exon, those of *PGBD5* are separated by multiple introns. Furthermore, *PGBD5* can be found in all vertebrates including the lamprey *Petromyzon marinus* and also in the lancelet *Branchiostoma floridae*. Surprisingly, both amino acid sequences and synteny are highly conserved, indicating that *PGBD5* was domesticated more than 500 million years ago. Given that *PGBD5* is expressed mainly in the brain and central nervous system, this gene may have played an important role in the evolution of the central nervous system (39).

Most of the domesticated *piggyBac* transposases have substitutions in the catalytic core residues DDD (see below). They are thus unlikely to have transposase activity, suggesting that their DNA-binding capacity may be utilized by the host (22). Intriguing instances of domestication of *piggyBac* transposase can be found in the ciliates *Paramecium tetraurelia* (7) and *Tetrahymena thermophila* (8). Ciliates are unicellular eukaryotes and have two functionally distinct versions of nuclei in the cytoplasm, namely a micronucleus as the germ-line genome and a macronucleus that is derived from a micronucleus and responsible for somatic gene transcription. During macronucleus development, the genome undergoes massive

DNA amplification and extensive genome rearrangement including elimination of internal eliminated sequences (IESs) and transposable elements. The precise excision of IESs is particularly essential as this is required for reconstruction of functional genes. Domesticated *piggyBac* transposases encoded by *piggyMac* in *P. tetraurelia* or *TPB2* in *T. Thermophila* play a central role in the genome rearrangement, as shown in RNA interference experiments (7, 8). Animals in which expression of the domesticated gene was knocked down by RNAi exhibited deficiency in initiating genome rearrangement in the developing macronucleus. Furthermore, both proteins carry a conserved DDD motif and the *TPB2* gene product has been shown to have an endonuclease activity *in vitro* (27). In addition to RAG1 recombinase, the domesticated *piggyBac* transposases in ciliates provide another example that the catalytic activity of transposases is utilized by the host organisms.

MECHANISM OF TRANSPOSITION

piggyBac Transposase

Many transposases and retroviral integrases contain a DDE/D domain, which includes two highly conserved aspartic acid (D) residues and either a glutamic acid (E) residue or a third D. This domain is also known to form an RNase H-like fold (40, 41). The essential amino acid triad coordinates divalent metal ions such as Mg^{2+}, which catalyses transposition/integration activities (40, 41). The disruption of any one of the triad completely abolishes transposase activity. An alignment of *piggyBac* transposases from *piggyBac*-like elements found in various species shows several highly conserved blocks of amino acids in the core region between positions 130 and 522 of the *T. ni piggyBac* transposase, which contains several conserved aspartic acid (D) and glutamic acid (E) residues (22, 42, 43). Although this region does not readily show similarity to the widespread DDE domains, a weak similarity to the IS4 family protein was identified (22), leading to predictions that D268 and D346 in the *T. ni piggyBac* transposase are the conserved aspartic acid in the DDE/D domain. Mutational analyses of D268, D346 as well as another highly conserved D447 of *T. ni piggyBac* transposase revealed that these residues are absolutely required for all steps of transposition including nicking, hairpin resolution, and target joining (see below). This allows us to conclude that the *piggyBac* transposase is a member of the DDE/D recombinase family (43).

The C-terminal region is variable but contains several conserved cysteine residues with regular spacing, forming a putative zinc-binding plant homeodomain (PHD) finger (43). A *T. ni piggyBac* transposase mutant lacking the C-terminal domain can show *in vitro* transposition activity at the level equivalent to the wild-type transposase. Given that PHD fingers bind to chromatin (44), the C-terminus of *piggyBac* transposases may facilitate binding to transposon DNA in a chromatin context.

Chemical Steps

The *piggyBac* transposon has a broad host range, suggesting that its mobilization could be host-factor independent. In 2008, Craig and colleagues purified bacterially expressed *piggyBac* transposase and reconstituted excision and integration reactions *in vitro* using the purified transposase and substrate DNA (43). Their detailed analyses revealed the unique chemical steps that *piggyBac* employs.

Figure 2 illustrates the chemical steps of the mechanism of *piggyBac* transposition. When the *piggyBac* transposase binds to the transposon end, it initiates the excision reaction by nicking the 3′ end of each strand of the transposon ends. The free 3′ OH then attacks the complementary strand of the 5′ end of the flanking TTAA, resulting in the formation of a hairpin on the transposon end and the release of the transposon from the host genome. The hairpin structures in the transposition intermediates are quickly resolved by the transposase and the exposed 3′ OH is then used for target joining. During the integration reaction, the exposed 3′ OH at the transposon end first attacks the 5′ end of target TTAA sites. This joins one strand of the transposon to the target site. Integration is completed when the ends of the transposon ligate to the complementary genomic strand.

At the excised genomic sites, 5′ overhangs of the tetranucletide TTAA are produced at both ends. These are simply paired and re-ligated by host factors, which restores the sequence to its original state. *piggyBac* thus shows precise excision and does not usually leave a footprint. However, there are occasional failures of donor site repair, which result in small insertions and/or deletions, at a frequency of around 1% of excision events during chromosomal transposition in mouse embryonic stem (ES) cells (45).

There are two unique features in *piggyBac* transposition. Firstly, it requires no DNA synthesis. Mobilization of many conventional DNA transposons is associated with DNA synthesis by host repair proteins, which results in target site duplication and a “footprint” (Fig. 3 right). However, *piggyBac* produces 5′ TTAA overhang

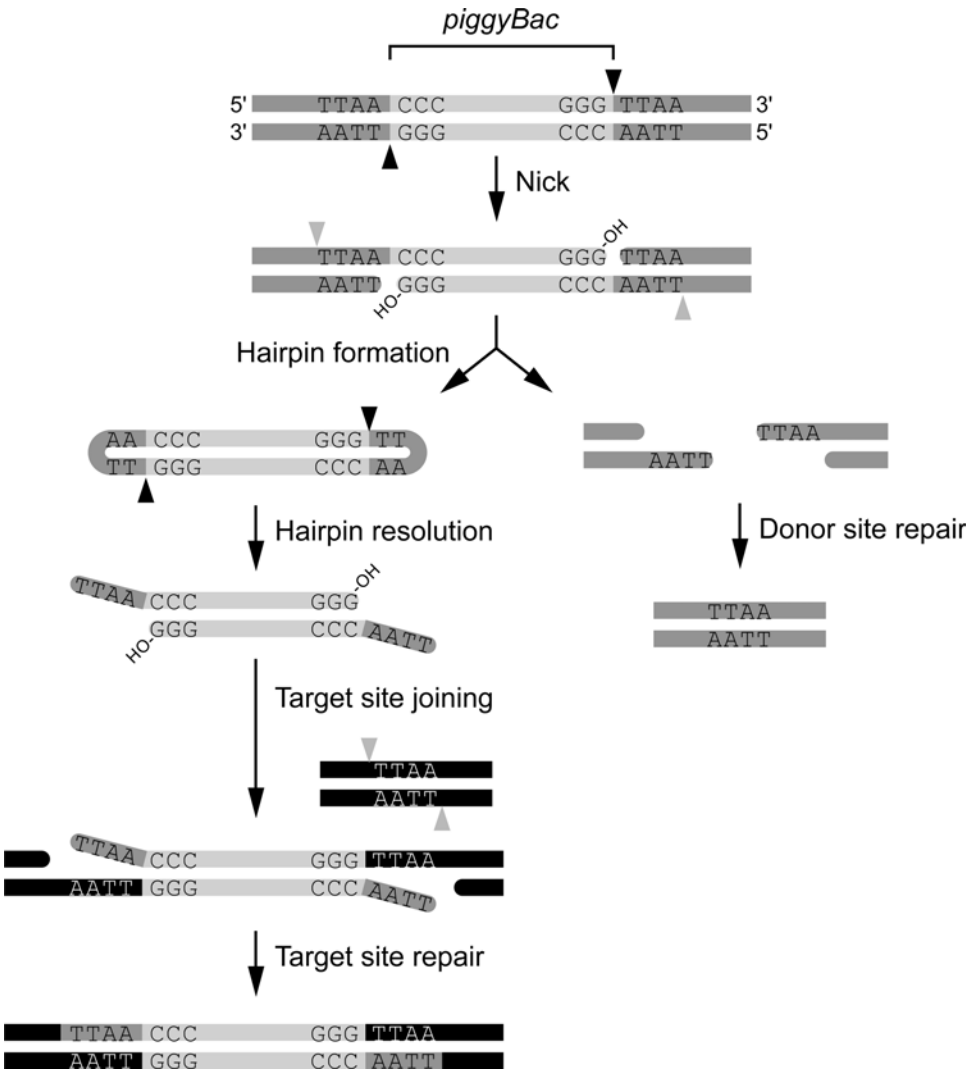


FIGURE 2 The chemical steps of *T. ni piggyBac* transposition. Black and grey arrowheads indicate positions of nicks or sites where 3′ OH groups attack, respectively. Modified from ref. (43). doi:10.1128/microbiolspec.MDNA3-0028-2014.f2

attached to the transposition intermediates and uses it to base-pair with the 5′ TTAA single-strand gaps on the target DNA during integration (Fig. 3 left). Therefore, *piggyBac* does not require DNA synthesis. Secondly, the excised transposon ends form a hairpin structure. This mechanism was the first to be observed in eukaryotic transposons although it has been seen in bacterial NA transposons that belong to the IS*4* family, namely Tn*5* and Tn*10*. In the Tn*5* and Tn*10* transposons, an essential step of hairpin processing is base flipping, which is often observed in reactions involving DNA modifying enzymes such as DNA methylases and DNA glycosylases. The cocrystal structure of Tn*5* synaptic complex revealed for the first time that base flipping is employed in transposition reactions. Bischerour and Chalmers further characterized base flipping at the molecular level and revealed the involvement of two W residues in Tn*5* and one W and one E residue in Tn*10*. These residues are located at the equivalent position near the third E residue of the DDE triad (46, 47). Although these amino acids are not conserved in their corresponding positions in *T. ni piggyBac* transposase, there is one W residue (position 465 in *T. ni piggyBac* transposase, downstream of the third D residue of the DDD triad) that is highly conserved among *piggyBac* transposases and has been suggested to be involved in base flipping (48). However, W465A mutant showed much reduced nicking activity as well as deficiency in

FIGURE 3 Comparison of target site joining and repair in *piggyBac* (left) and *Tc1* (right). Grey arrowheads indicate sites where 3′ OH groups attack. Modified from ref. (136).
doi:10.1128/microbiolspec.MDNA3-0028-2014.f3

every subsequent step of transposition reaction: hairpin formation, hairpin resolution, and target joining (43). Therefore, W465 plays a central role in the transposase activity and cannot have a role only in DNA hairpin formation and resolution. Given that Tn*5*/Tn*10* transposons produce a blunt intermediate molecule (38, 39) whereas *piggyBac* intermediate carries 4-nucleotide 5′ overhangs (43), *piggyBac* may therefore use different amino acids or mechanism(s) to facilitate hairpin processing.

Integration Site Preference

The *piggyBac* target site, TTAA, is fairly frequent in the genome but the epigenetic status of the target sites may affect integration site preference of the *piggyBac* transposon. Understanding such a preference is important, especially when *piggyBac* is used as an insertional mutagen or a gene therapy vehicle.

To analyze site preference with an adequate statistical power, a large number of integration sites are required. Ligation-mediated PCR is the most frequently used method to identify transposon integration sites (49). In this method, genomic DNA is typically digested with a 4-bp cutter restriction enzyme, ligated with an adaptor (known as a splinkerette) and amplified by PCR using transposon-specific and splinkerette-specific primers. The resulting PCR products, which contain junctions between the transposon end and the flanking genomic regions, are determined by capillary sequencing, thereby identifying the integration sites. An issue is that the throughput of this method is too low to collect statistically significant numbers of transposon integration sites. Recent advances in sequencing technologies, however, have transformed the way we identify transposon integration sites (50). It is now possible to identify tens of thousands of sites simultaneously. These can then be compared with an accumulating genomic dataset, such as those of the histone modification and DNase hypersensitive sites, allowing us to comprehensively analyze and identify the epigenetic elements that affect integration site preference.

Using the method described above, Li *et al.* identified more than 30 thousand *T. ni piggyBac* integration sites in mouse embryonic stem cells (51). When mobilized from chromosomal donor sites, the *piggyBac* transposon showed strong local hopping patterns; these are commonly observed in DNA transposon mobilization. Approximately 10 to 15% of integrations were found within 5 Mb from the donor sites. This also increased the frequency of integration into the chromosome that contains the donor sites, resulting in 25% of reintegrations in the original donor chromosome. When compared to

the epigenetic features, the *piggyBac* integration sites are clearly associated with accessible chromatin structures. These sites include DNase hypersensitive sites, sites with trimethylated lysine 4 on histone H3 and sites with pol II binding. In sharp contrast, the *piggyBac* integration sites are negatively correlated with lamin-associated domains, which are heterochromatin domains. These trends are more obvious when the *piggyBac* transposons are mobilized from genomic donor sites rather than from transfected plasmid DNA. *piggyBac* preferentially integrates in genic regions, especially regions that contain expressed genes.

These preferential integrations into the open chromatin structure were also observed in *piggyBat* transposon (the *M. lucifugus*-derived *piggyBac*-like element) in both human cells and bat fibroblasts (27). Interestingly, the *piggyBat* transposons are accumulated in genic regions of the bat genome, indicating that the *in vitro* studies using human or mouse cell lines truly reflect native transposition patterns.

piggyBac AS GENETIC TOOLS

DNA transposons have been used as versatile genetic tools in a wide range of organisms. For example, the *Tc*1 element was used in *Caenorhabditis elegans* mutagenesis (52) and the *P* element was used in *Drosophila melanogaster* (53, 54). Earlier studies revealed that *Drosophila*-derived transposons such as the *P* element and the *hobo* transposon were either not functional or limited in nondrosophilid insects (55). There was therefore a need to develop alternative transposon-based transgenesis systems for nondrosophilid insects. The Lepidopteran transposon, *piggyBac*, was one such candidate. An engineered *piggyBac*-based vector was first shown to be able to mobilize in a nonhost *S. frugiperda* cell line in 1995 (56, 57). Subsequently, it was shown that the *piggyBac* was able to mobilize in non-Lepidopteran insects (15). Today, *T. ni piggyBac* transposition has been confirmed in 5 orders of insects (Table 1) and in organisms including plants, yeasts, protozoa, and vertebrates (Table 2). The *piggyBac*-based transposon

TABLE 1 Studies in which *piggyBac* transposition has been confirmed in insect species

Order	Common name	Scientific name	Reference
Coleoptera	Ladybird beetle	*Harmonia axyridis*	(81)
Coleoptera	Red flour beetle	*Tribolium castaneum*	(82)
Diptera	La Crosse encephalitis vector mosquito	*Aedes triseriatus*	(83)
Diptera	Yellow fever mosquito	*Aedes aegypti*	(84)
Diptera	Dengue vector mosquito	*Aedes albopictus*	(83)
Diptera	Mosquito	*Aedes fluviatilis*	(85)
Diptera	Mexican fruit fly	*Anastrepha ludens*	(86)
Diptera	Malaria mosquito	*Anopheles albimanus*	(87)
Diptera	Malaria mosquito	*Anopheles gambiae*	(88)
Diptera	Mosquito	*Anopheles gambiae*	(89)
Diptera	Malaria mosquito	*Anopheles stephensi*	(90)
Diptera	Oriental fruit fly	*Bactrocera dorsalis*	(17)
Diptera	Queensland fruit fly	*Bactrocera tryoni*	(91)
Diptera	Mediterranean fruit fly	*Ceratitis capitata*	(15)
Diptera	Fruit fly	*Drosophila melanogaster*	(16, 84)
Diptera	Spotted wing drosophilid	*Drosophila suzukii*	(92)
Diptera	House fly	*Musca domestica*	(93)
Diptera	Stalk-eyed fly	*Teleopsis dalmanni*	(94)
Hymenoptera	Sawfly	*Athalia rosae*	(95)
Lepidoptera	Butterfly	*Bicyclus anynana*	(96)
Lepidoptera	Silkworm	*Bombyx mori*	(59)
Lepidoptera	Codling moth	*Cydia pomonella*	(97)
Lepidoptera	Tobacco budworm	*Heliothis virescens*	(98)
Lepidoptera	Cabbage moth	*Mamestra brassicae*	(99)
Lepidoptera	Asian corn borer	*Ostrinia furnacalis*	(100)
Lepidoptera	Pink bollworm	*Pectinophora gossypiella*	(101)
Lepidoptera	Potato tuber moth	*Phthorimaea operculella*	(102)
Lepidoptera	Diamondback moth	*Plutella xylostella*	(103)
Lepidoptera	Fall armyworm	*Spodoptera frugiperda*	(56)
Orthoptera	Two-spotted cricket	*Gryllus bimaculatus*	(104)

vector system is the most widely used transposon system for a variety of applications such as transgenesis and mutagenesis. Furthermore, *piggyBac* has potential as a gene therapy vehicle (58). The next section describes the *piggyBac* transposon in the context of genetic tools.

The Engineered Nonautonomous *piggyBac* Transposon System

Similar to the other transposon systems such as *P* element and *Tc*1 transposon, the autonomous *piggyBac* transposon can be separated into 2 components: a DNA segment flanked by *piggyBac* TIR and a transposase. The initial transgenesis experiments using the *piggyBac* transposon were conducted in a conservative vector system in which genetic marker genes were simply inserted into the middle of the transposase coding region (13, 14). Alternatively, the short fragment (0.75 kb) in the transposase coding region was replaced with marker genes (59), retaining most of the original transposon sequences. To improve and increase the versatility of the vector system, the minimum terminal sequences needed to be identified. This was done by analysis of a series of internal deletion constructs. The originally identified minimum terminal sequences were 125 bp for the 5′ terminus and 162 bp for the 3′ terminus; such a vector had transposition activities that were comparable to the full-length transposon in *T. ni* embryos (60). However, this minimal *piggyBac* vector had a dramatic reduction in transformation efficiency in *Drosophila*. Further analysis revealed the necessity of internal domain sequences for efficient transposition and identified the optimal terminal length as 311 bp for the 5′ terminus and 235 bp for the 3′ terminus (61). This is the most commonly used vector configuration today.

Any DNA fragments can be inserted as a transposon cargo between these terminal repeats and mobilized by the transposase. Various elements have been used depending on the experimental purpose. To express a gene of interest exogenously, a transcription unit, containing a suitable promoter, a coding sequence, and a polyadenylation signal sequence, is inserted. For disrupting gene function, a gene trap element consisting of a splice acceptor site and a polyadenylation signal sequence can be used. The cargo capacity of the *piggyBac* transposon is fairly large; transposons with a cargo of up to 10 kb can be mobilized without losing transposition efficiency (62). Recently, it has been shown that *piggyBac* can transpose bacterial artificial chromosomes (BACs), which are 150 to 300 kb in length, in mouse and human pluripotent stem cells (50, 63) and in mouse zygotes (64). In the best result in the zygote injection, 45% of F0 mice carried a BAC transgene and most of them have transposon signature (64).

TABLE 2 Studies in which *piggyBac* transposition has been confirmed in noninsect species

Organism	Common name (cell type)	Scientific name	Reference
Yeast	Budding yeast	*Saccharomyces cerevisiae*	(43)
Yeast	Fission yeast	*Schizosaccharomyces pombe*	(105)
Protozoa	Malaria parasite	*Plasmodium falciparum*	(79)
Protozoa	Human blood fluke parasite	*Schistosoma mansoni*	(106)
Protozoa	Malaria parasite	*Plasmodium berghei*	(107)
Protozoa	Apicomplexan parasite	*Eimeria tenella*	(108)
Protozoa	Rat gastrointestinal parasite	*Strongyloides ratti*	(109)
Pranarian	Pranarian	*Girardia tigrina*	(110)
Fish	Zebrafish (Zygote)	*Danio rerio*	(111)
Bird	Chicken (PGC, spinal cord)	*Gallus gallus*	(112, 113, 114, 115)
Mammal	Mouse (ES cells, *in vivo*)	*Mus musculus*	(49, 62, 64)
Mammal	Rat (Zygote, *in vivo*)	*Rattus norvegicus*	(116, 117)
Mammal	Goat (Fetal fibroblasts)	*Capra aegagrus*	(118)
Mammal	Pig (Fetal fibroblasts)	*Sus scrofa*	(119)
Mammal	Horse (Fetal fibroblasts)	*Equus ferus*	(120)
Mammal	Human (Cancer cell lines, primary T lymphocyte, $CD34^+$ hematopoietic stem cells, ES cells, iPS cells)	*Homo sapiens*	(63, 70, 121, 122, 123)
Mammal	Macaque (ES cells)	*Macaca fascicularis* *M. mulatta*	(65)
Plant	Rice	*Oryza sativa*	(124)

Transposase/Transposon Variants

Increasing transposition efficiency is the key to improving efficiency of transposon-mediated genetic manipulation. This has been done recently by a series of mutagenesis of the TIRs and the transposase, followed by screening for hyperactive variants.

(i) Transposon

Transposon TIR sequences were randomly mutagenized by error-prone PCR. These sequences were then screened for higher transposition activity. A mutant 5′ TIR carrying two substitutions, T53C and C136T, showed a 59% increase in overall transposition compared to the wild-type TIR (65). The mechanisms by which these substitutions increase transposition frequency remain elusive.

(ii) Transposase

Since *piggyBac* is derived from insects, the codon usage may not be suitable for expression in mammalian cells. One simple idea to increase the transposition efficiency is to optimize codon usage and increase the expression level of the transposase. Indeed, optimization of the transposase-coding sequences to the codon usage of mouse (66) or human (65) increased the transposition efficiency by several fold when compared to the original insect sequences. Another approach is random mutagenesis of the transposase by error-prone PCR followed by screening for hyperactive mutants (45). The yeast-based transposition assay system is particularly useful for large-scale screening. A total of 10,000 clones were screened and 17 hyperactive mutants were identified. The activity of each mutant was further tested in mouse ES cells and 5 mutants showed higher activity (I30V/G165S, S103P, M282V, S509G/N570S and N538K). All seven amino acid substitutions that were found in the five mutants were then combined into one coding sequence and the resulting transposase, named hyPBase, showed increases of approximately 20-fold in excision and 10-fold in integration in mouse ES cells (45). The hyPBase was also able to increase transposition efficiency in human cells (67). The mechanisms by which these mutations alter transposition activity remain elusive.

In addition to the developments described above, a variant called $Exc^{+}Int^{-}$ transposase that can excise a transposon but cannot integrate it back into the host genome has recently been generated (68). This variant was identified by site-directed mutagenesis of potentially DNA-interacting amino acids. Given that *piggyBac* can be excised seamlessly, this new variant transposase is useful for the removal of transgenes carried by *piggyBac*. The variant was found to have the R372A/K375A double mutation and was further mutagenized by error-prone PCR to screen for hyperactive variants. Two mutations, namely M194V and D450N, were found to increase the excision activity of the $Exc^{+}Int^{-}$ (R372A/K375A) transposase. When R372A/K375A/D450N mutations were introduced into the hyPBase background, the transposase showed a marked increase in its excision activity with no change in its integration activity.

In addition, transposase variants have also been generated by fusing functional protein domains. The ERT2 domain (a mutated version of the ligand-binding domain of the human estrogen receptor) allows temporal regulation of enzymatic activity of the fusion proteins by tamoxifen administration (69). The *piggyBac* transposase-ERT2 fusion protein can be activated upon induction, but remains inactive without tamoxifen (66). DNA-binding domains such as the Gal4 DNA-binding domain and custom-made zinc finger DNA-binding domains have also been used. These fusion transposes are not only active but also able to integrate transposons into sites that are in close proximity to their respective binding sites, thereby allowing site-directed transposition (65, 68, 70). *Sleeping Beauty* and *Tol2* transposases are not amenable to protein fusion (70). The flexibility of transposase modification by protein domain fusion is therefore another unique feature of the *piggyBac* transposon system.

piggyBac-MEDIATED GENETIC SCREENING

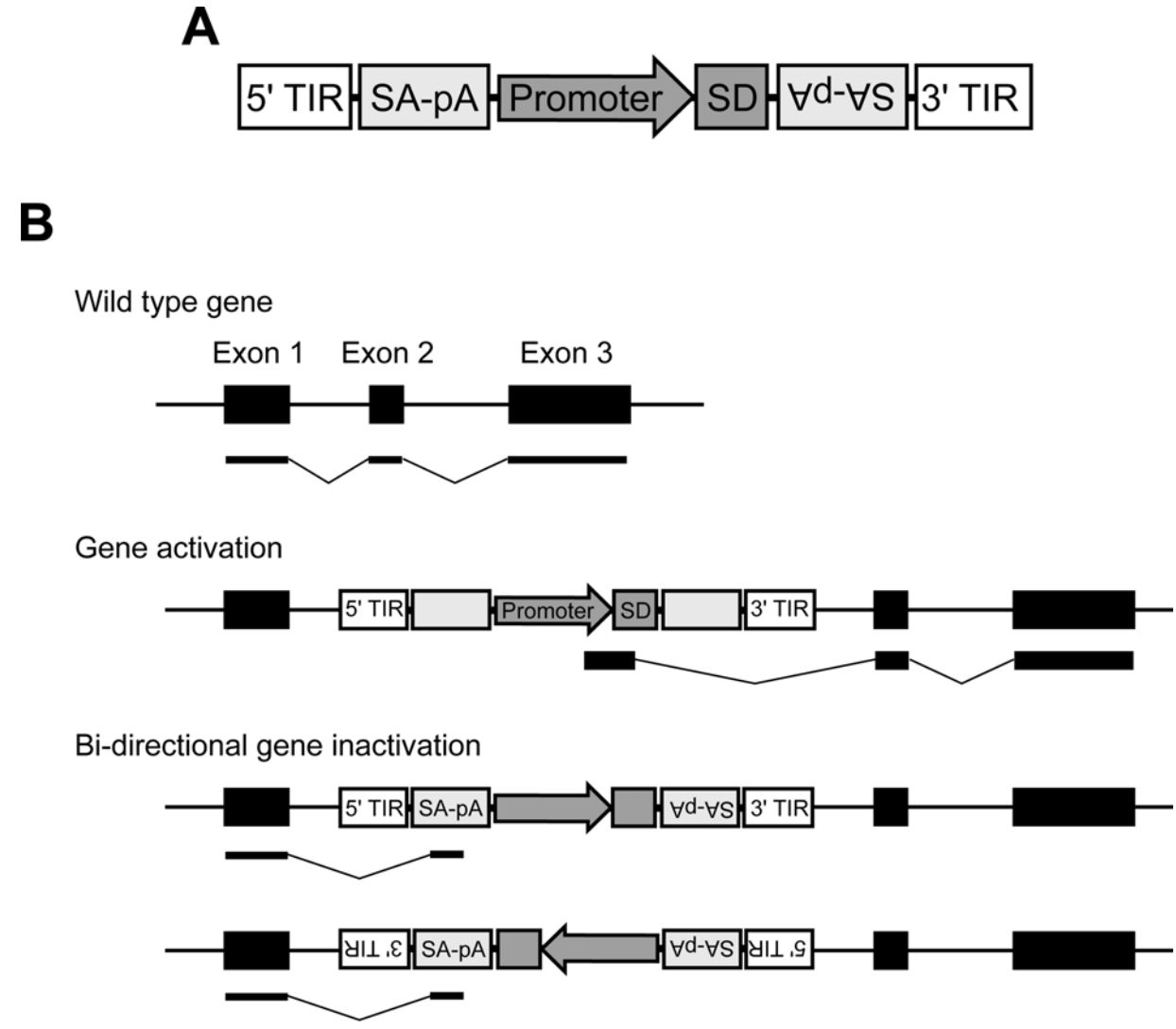
One major application of DNA transposons is insertional mutagenesis. The *piggyBac* transposon vectors have been used as an insertional mutagen in several organisms (Table 3). One of the best examples of mutagenesis using *piggyBac* is *in vivo* mutagenesis in mice for cancer gene discovery. Traditionally, murine leukemia virus and murine mammary tumor virus were used

TABLE 3 Studies using the *piggyBac* transposon as an insertional mutagen

Organisms	Purpose	Species	Reference
Insect	Mutagenesis	*Tribolium castaneum*	(125, 126)
Insect	Mutagenesis	*Drosophila melanogaster*	(127, 128, 129)
Mouse	*In vivo* cancer gene discovery	*Mus musculus*	(76, 130, 131)
Mouse	*In vitro* screening in ES cells	*Mus musculus*	(132, 133, 134)
Yeast	Mutagenesis	*Fission yeast*	(105)
Malaria	Mutagenesis and phenotype-based screen	*Plasmodium berghei* *Plasmodium falciparum*	(80, 107, 135)

for oncogene discovery in hematopoietic cells (71, 72) and mammary tissues (73), respectively. However, such experiments were not possible in a large fraction of solid tumors due to the limited accessibility of these retroviruses. Analyses of such tumors therefore required the development of active transposon systems in mammalian cells. *Sleeping Beauty* is the first transposon system that has sufficient transposition efficiency for use in mutagenesis (74). Subsequently, *piggyBac* was also shown to be able to transpose efficiently in mice (62). The vector configuration that is typically used in mice for *in vivo* mutagenesis is shown in Fig. 4. It contains both gene inactivation and activation elements to identify tumor suppressors and oncogenes, respectively. Mobilization of this mutagenic transposon in mice could either significantly increase tumor formation (75, 76) or promote tumorigenesis when used in conjunction with cancer-predisposing genetic backgrounds (77). These transposon-induced tumors typically hosted *piggyBac* integration events within the tumor suppressors and/or upstream of the oncogenes (76). Detailed analyses successfully identified novel oncogenes (76).

FIGURE 4 Transposon-mediated cancer gene discovery in mice. (**A**) Commonly used genetic elements. TIR, terminal inverted repeat; SA, splice acceptor site; pA, polyadenylation signal sequence; SD, splice donor site. (**B**) In gene activation, a strong constitutive promoter ectopically expresses or overexpresses a trapped gene. The transposon carries two splice acceptor sites in both directions; the trapped genes will be inactivated in spite of the transposon orientation relative to the gene. doi:10.1128/microbiolspec.MDNA3-0028-2014.f4



Another example of the use of *piggyBac* in mutagenesis is the malaria parasite, *Plasmodium falciparum*. Genetic manipulation is a fundamental experimental method for gene function studies in any organism. However, it is extremely difficult in *P. falciparum* for a number of reasons (78). The transfection efficiency is extremely low since DNA has to travel through multilayers of membranes to get into the nucleus of blood-stage parasites. Linear DNA is degraded before reaching the parasite nucleus, whereas circular DNA stays as episomes in the parasite nucleus. The *piggyBac* transposon can circumvent these issues and a *piggyBac*-based transformation system has recently been developed (79). By scaling up the experimental scale, one can generate a library of mutant parasites. A small library consisting of 189 parasites with *piggyBac* insertion was recently generated and, from this library, 29 parasites that were deficient in gametocytogenesis were successfully isolated (80).

CONCLUDING REMARKS

Recent advances in genome sequencing of various organisms have unveiled a number of previously unrecognized transposable elements and domesticated transposases, which has allowed us to greatly widen our understanding of these elements. The *piggyBac* transposon is one such good example, as exemplified by the discoveries of *piggyBat* and *piggyMac*. Genome sequence data will continue to accumulate and may provide even more surprising characteristics of these parasitic DNA elements. In terms of the molecular biology of *piggyBac*, a number of efforts have been made and the chemical steps of *piggyBac* transposition have been characterized. However, its uniqueness has left some unsolved questions; for instance, the molecular mechanisms of hairpin processing. Further biochemical characterization of *piggyBac* transposase and domesticated enzymes will reveal its unique DNA processing mechanisms. In particular, crystal structures of *piggyBac* transposases in a free or in a DNA-binding form will be of great interest to unveil the mechanisms of hairpin processing. In addition, the versatility of the *piggyBac* transposon system has allowed us to achieve previously difficult-to-perform transgenesis and mutagenesis. This will further expand our ability to address biological questions.

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