Cell, Vol. 122, 473–483, August 12, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.cell.2005.07.013

Efficient Transposition of the *piggyBac* (*PB*) Transposon in Mammalian Cells and Mice

Resource

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Summary

Transposable elements have been routinely used for genetic manipulation in lower organisms, including generating transgenic animals and insertional mutagenesis. In contrast, the usage of transposons in mice and other vertebrate systems is still limited due to the lack of an efficient transposition system. We have tested the ability of *piggyBac* (*PB*), a DNA transposon from the cabbage looper moth *Trichoplusia ni*, to transpose in mammalian systems. We show that *PB* elements carrying multiple genes can efficiently transpose in human and mouse cell lines and also in mice. *PB* permits the expression of the marker genes it carried. During germline transposition, *PB* could excise precisely from original insertion sites and transpose into the mouse genome at diverse locations, preferably transcription units. These data provide a first and critical step toward a highly efficient transposon system for a variety of genetic manipulations including transgenesis and insertional mutagenesis in mice and other vertebrates.

Introduction

Transposable elements or transposons are mobile genetic units identified in many metazoa, including worms, insects, and humans. In humans and mice, transposon-derived sequences account for more than 40% of the genome (Lander et al., 2001; Waterston et al., 2002), indicating the importance of transposition in evolution. Since the discovery of the first transposon in maize by McClintock (McClintock, 1950), transposable elements have become invaluable tools for genetic analysis in many organisms. In prokaryotes, transposon-based mutagenesis has led to discovery of genes important for microbial pathogenesis (Hutchison et al., 1999; Vilen et al., 2003). In eukaryotes, the introduction of P element-mediated transgenesis and insertional mutagenesis dramatically advanced *Drosophila* genetics (Rubin and Spradling, 1982). Many transposons, including P elements, are nonfunctional outside their natural hosts, suggesting host factors are involved in transposition (Handler et al., 1993).

Transposition systems, including members from the *Tc1/Mariner* family, have been used in mouse and the zebrafish *Danio rerio*. By using a comparative phylogenetic approach, a synthetic Tc1-like transposon *Sleeping Beauty* (*SB*) has been proven active in mouse and human cells (Ivics et al., 1997; Luo et al., 1998). Although transposons such as *SB* and *Minos* have been tested for insertional mutagenesis in mouse (Dupuy et al., 2001; Fischer et al., 2001; Horie et al., 2001; Zagoraiou et al., 2001), general application of these transposons in mouse genetics was limited due to the fact that new transposon insertions were heavily concentrated near the original site, occurred with low efficiencies, and had a limited capacity to carry DNA fragments (Drabek et al., 2003; Dupuy et al., 2001; Fischer et al., 2001; Horie et al., 2001, 2003; Zagoraiou et al., 2001).

PB elements are 2472 bp transposons with 13 bp inverted terminal repeats (ITRs) and a 594 amino acid transposase (Cary et al., 1989; Fraser et al., 1995, 1996). These elements have been successfully used for genetic analysis in *Drosophila melanogaster* and other insects. It was found that *PB* transposons insert into the tetranucleotide TTAA site, which is duplicated upon insertion (Fraser et al., 1995, 1996). Because of the unique transposase and TTAA target-site sequences, the transposon has been suggested as the founding member of a new DNA transposon family, called the *piggyBac* family (Robertson, 2002). *PB* elements have been used to transform the germline of more than a dozen species spanning four orders of insects (Handler, 2002; Sumitani et al., 2003). As a mutagen, *PB* transposes at least as effectively as the P element in *Drosophila* (Thibault et al., 2004). In the red flour beetle *Tribolium castaneum*, *piggyBac* transposition also efficiently occurred between nonhomologous chromosomes (Lorenzen et al., 2003). Many *PB* element-like sequences have been found in the genomes of phylogenetically diverse species from fungi to mammals, further indicating that their activity may not be restricted to insects (Sarkar et al., 2003). In fact, *PB* has recently been shown capable of transposition in the planarian *Girardia tigrina* (Gonzalez-Estevez et al., 2003).

In order to develop efficient transgenesis and insertional mutagenesis tools for the mouse, we have explored a variety of transposons and retroviruses. We reasoned that *PB*-mediated transposition may be less dependent on host factors and thus may also work in

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mammalian systems. We therefore tested the transpositional ability of *PB* in mammals. *PB* transposition was found not only in human and mouse cell lines, but also in mouse germline cells. Both in vitro and in vivo studies showed that *PB* transposition occurs almost exclusively at TTAA sites. When introduced into fertilized eggs, *PB* could integrate into the mouse genome without obvious chromosome regional preferences and preferably be inserted into transcriptional units. Also, *PB* elements can carry multiple marker genes and allow the expression of these genes at various insertion sites. Our studies suggest that the *PB* transposon system can be a useful new tool for efficient genetic manipulation and analysis in mice and other vertebrate organisms.

Results

Transposition Activity of *piggyBac* in Cultured Mammalian Cells

We designed a binary cotransfection assay system consisting of both a donor and a helper plasmid to detect *PB*-mediated chromosomal integration events in tissue culture cells. The donor plasmid contained the *PB* elements in which the *PB* transposase (PBase) was replaced by a drug selection marker (Figure 1A). The helper plasmid carried the PBase fragment but lacked the terminal sequences required for transposition (Figure 1B). In the absence of the helper plasmid, the donor plasmid may randomly integrate into the genome, but these random integration events can be minimized if the plasmid is kept in circular form. Thus, an increase of drug-resistant clones in the presence of the helper plasmid would indicate transposition events.

We first examined *PB* transposition in human 293 cells. Cotransfection of the donor *PB[SV40-neo]* element carrying an *SV40* promoter-driven neomycin resistance (*neo*) gene and the helper *CMV-PBase* plasmid carrying a ubiquitously expressed PBase (Figure 1) produced neomycin-resistant clones at a 10-fold higher rate than transfection with the donor plasmid alone (Figure 2A). To test whether the elevated integration of donor was due to transposition, inverse PCR was performed to recover sequence adjacent to the right end (PBR) of integrated *PB[SV40-neo]* (Figure 1A). PCR products from a true transposition event should result in genomic DNA sequence outside the PBR rather than plasmid sequence. Eighteen independent genomic sequences were recovered from five drug-resistant clones. All of these sequences contained genomic DNA outside of the PBR, with the signature TTAA sequence at the integration site (see Table S1 in the Supplemental Data available with this article online). TTAA duplication was confirmed by sequencing several junction fragments at the other end of the transposon (data not shown). In contrast, inverse PCR analysis of neomycin-resistant clones stably transfected with *PB[SV40-neo]* alone only detected junction plasmid sequences, which is consistent with random insertion events (data not shown). This experiment demonstrated that *PB* transposition occurred in human cells with the same site preference as in insect cells.

We next tested the ability of *PB* to transpose in mouse *W4/129S6* embryonic stem (ES) cells. In this test, the donor *PB[PGK-neo]* element carried a *PGK* promoter-driven *neo* gene and the helper plasmid *Act-PBase* provided PBase under the control of a hybrid *actin* promoter (Figure 1B). In three repeated transfection experiments, *PB[PGK-neo]* and *Act-PBase* cotransfection produced drug-resistant clones on average 50-fold higher than *PB[PGK-neo]* transfection alone (Figures 2B and 2C). Inverse PCR analysis confirmed that the enhanced clone production was due to transposition (Table S2).

piggyBac Transposes Efficiently in the Mouse Germline

Efficient transposition in mouse ES cells encouraged us to test the feasibility of *PB* transposition in the mouse germline. Pronuclei coinjection of transposon donor and transposase helper plasmids was performed to generate transgenic mice. To facilitate the analysis of transposition in transgenic mice, we used visible markers (red fluorescent protein, RFP) instead of drug-resistance markers in donor plasmids. Donor *PB[Act-RFP]* elements and the helper plasmid *Act-PBase* were coinjected in circular forms into the pronuclei of *FVB/Nj* mouse embryos. PCR analysis showed that 34.8% (62/184) of the founders were *PB[Act-RFP]* single positive, 0.5% (1/184) were *Act-PBase* single positive, and 2.7% (5/184) were double positive. In comparison, only 10.4% (10/96) of the founders were positive when injection was carried out with *PB[Act-RFP]* alone. Similar results were obtained when a *PB* element with a different marker gene, *tyrosinase*, which affects skin pigmentation (*PB[K14-Tyr]*), was coinjected with the same helper construct (Figures 1 and 3A).

To analyze the structures of integrated transgenes in *RFP*-positive founders, Southern hybridization with a transposon-specific probe was performed (Figure 1A). The majority of the founders carried multiple integration events (Figure 3B). We then performed inverse PCR to recover genomic sequences flanking the transposon termini. A total of 85 transposition events were recovered from 42 RFP-positive founders (Table S3). Most of these transpositions were mapped to the mouse genome according to genomic sequence flanking the right terminal repeat of the integrated transposon. We randomly selected nine transposition events and amplified the genomic junction sequences on the opposite side of the transposon. In each case, transposon insertion was found to produce a precise TTAA duplication of the integration site (data not shown). These results indicate that most of the transgene integrations produced from coinjection are likely to be due to transposition.

To test the capability of integrated transposons to transmit through the germline, several *PB[Act-RFP]*-positive but helper plasmid-negative founders were mated with wild-type *FVB/Nj* mice to generate transgenic lines. One of the founders (AF0-61) that had at least eight *PB[Act-RFP]* integrations was analyzed in detail. PCR-based genotyping showed that 15 out of 16 progenies of this founder retained the transposon DNA. Southern analysis of PCR-positive individuals showed that all of them inherited at least one copy of

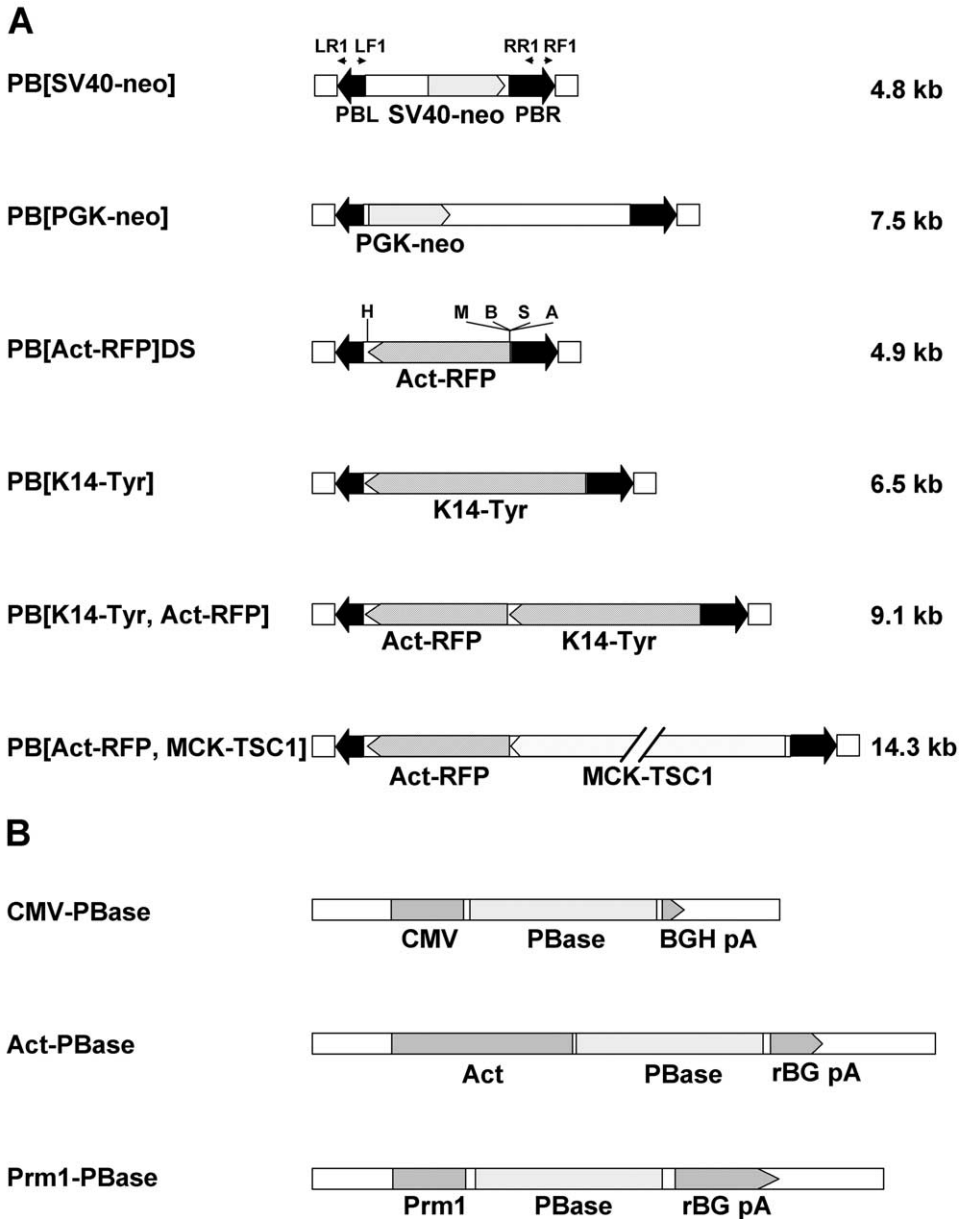

Figure 1. Transposon Vectors and Transposase Constructs of the *piggyBac* Binary Transposition System for Mammalian Cells and Mice

(A) *PB* donor constructs. Marker or endogenous genes (shaded boxes with arrows denoting transcription direction) driven by various promoters were placed between a pair of *PB* repeat termini (PBL and PBR, black arrows). Arrowheads above the termini show the relative positions of primers used for inverse PCR. Total lengths of the transposons are also indicated. Open boxes represent the plasmid backbone sequences. M: MfeI, B: BamHI, S: SwaI, A: AscI, H: HindIII.

(B) *PB* transposase helper constructs. The *piggyBac* transposase gene (*PBase*) driven by cytomegalovirus (*CMV*), β-actin (*Act*), or Protamine 1 (*Prm1*) promoters were followed by either bovine growth hormone polyA (*BGH pA*) or rabbit β-globin polyA (*rBG pA*).

the transposed *PB[Act-RFP]* (Figure 3C and data not shown). The random segregation of these transgenes suggested a diverse chromosomal distribution of the initial transposition events in the founder. Progeny analysis of a second founder (AF0-47) that carried a single transposon indicated that two out of eight F1s in a single litter inherited the transposon (Figure 3C and data not shown). PCR-based genotyping with primers targeting several individual transposon integration sites also confirmed stable inheritance of the integrated transposons from founders to the F1 generation (data not shown). The insertion in the AF0-47 founder has been stably transmitted for five generations (AF0-47T6, Table S3). Taken together, the high frequency of transposition-mediated gene integration and the capability of integrated transgenes to transmit through the germline demonstrates the feasibility of using *PB* elements as a gene transfer tool in mouse.

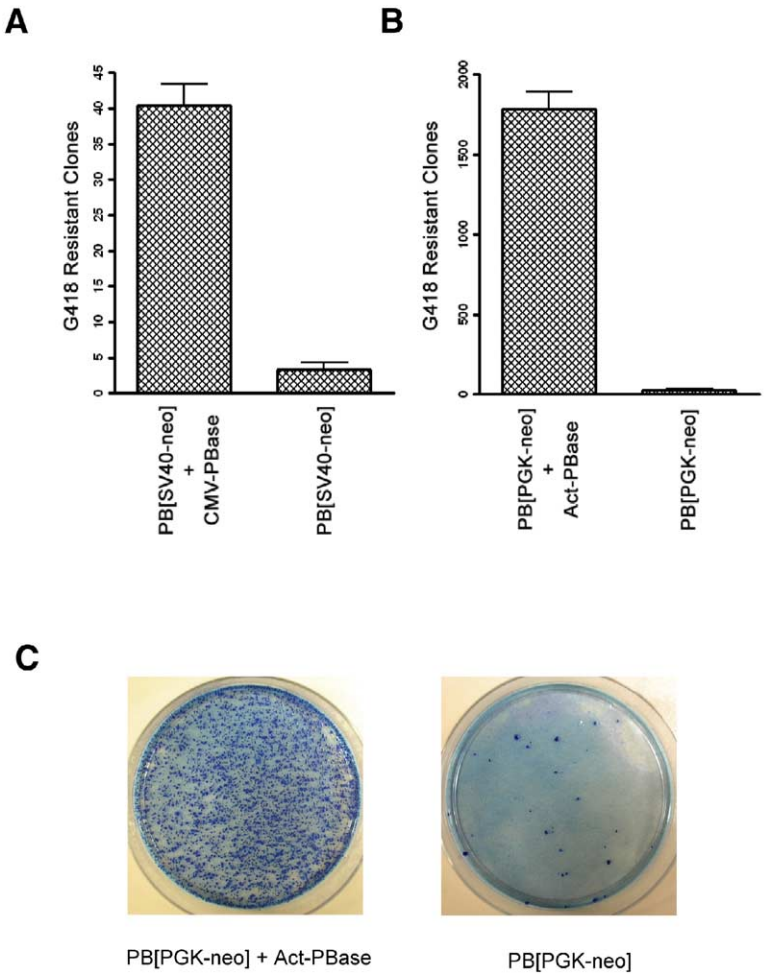

Figure 2. *piggyBac* Integration in Mammalian Cultured Cells

(A) Statistical results of enhanced transgene integration in 293 cells. The numbers of G418-resistant clones were scored from transfections of donor transposon construct with or without helper plasmids. Each number is the average obtained from three transfection experiments. The bar shows the standard deviation ($p < 0.0001$).

(B) Statistical results of enhanced transgene integration in mouse *W4/129S6* ES cells. Clones were counted as in (A).

(C) An example of mouse ES cell transfection experiments. Surviving clones were stained with methylene blue after G418 selection.

Precise Excision and Transposition of *piggyBac* in the Mouse Germline

We further tested the transposition behavior of *PB* in the mouse germline with the classical breeding strategy of "jumpstarter" and "mutator" stocks (Cooley et al., 1988; Horn et al., 2003). In this procedure, a mutator line carrying a nonautonomous transposon is crossed with a jumpstarter line that expresses transposase in the male germline. Active transposition is expected to occur exclusively in the germ cells of males carrying both transposon and transposase DNA. These males are subsequently mated with wild-type females to produce lines with new transposon insertions. We revised this procedure and used a coinjection method to directly produce mice doubly positive for a nonautonomous transposon and a helper transposase gene. Transgenic animals were produced by conventional pronuclei injection of linear plasmids, which assured the cointegration of both donor and helper plasmids in the same locus. Several transgenic mouse lines carrying both *PB[Act-RFP]* and *protamine 1* (*prm1*) promoter-driven *PB* transposase transgenes (*Prm1-PBase*) were generated (Figure 1). The *prm1* promoter was expected to be active during spermiogenesis (O'Gorman et al., 1997). Thus, in such double-positive transgenic lines, male mice were expected to produce new transposition events whereas female mice could be used as breeders.

One of these double-transgenic lines, referred as BF0-33, was tested for transposition in its progeny. Southern hybridization with the transposon-specific probe (Figure 1A) revealed new transposon integrations in 67.8% (19/28) of the transposon-positive progenies (Figure 4A and data not shown). On average, 1.1 new insertions were generated per gamete. The new insertions seemed not to be regional since three of those new insertions were sequenced and found to be located on three separate chromosomes (BF1-29T6, BF1-30T43, and BF1-44T10 in Table S3).

Primers targeting *PB[Act-RFP]* plasmid sequences flanking the transposon were used to explore the transposition behavior of *PB* in the germline (Figure 4B). If *PB* transposed through a cut-and-paste manner, a 273 bp PCR product would be detected. Indeed, this PCR product was detected in 10 out of 17 offspring from line BF0-33 (Figure 4C). Seven of these samples have been sequenced and revealed the existence of a single TTAA target site (data not shown), demonstrating that *PB* transposed through a precise cut-and-paste mechanism in the mouse male germline. Because the founder carried a transgene array and the possibility of repair involved in the sister chromatid, it is expected that

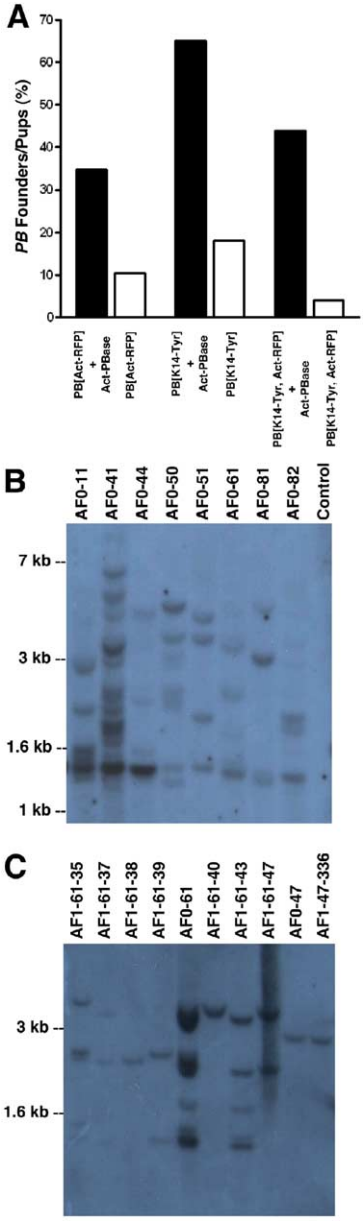

Figure 3. *piggyBac* Elements Transposition in Mice

(A) Ratio of the transposon-positive founders determined by PCR genotyping among all pups resulting from injection of circular plasmids. The solid bars and open bars represent the results from coinjections of the donor and helper plasmids or injections of the donor plasmid alone, respectively. The presence of the *PB* transposase resulted in an elevated transgenic efficiency.

(B) Southern analysis of *PB[Act-RFP]*-positive founders (AF0). In some cases, more than 10 integrations in a single founder mouse (AF0-41) were observed, while no signals were found in the wild-type control.

(C) Southern analysis indicated germline transmission of *PB* elements. After mating with wild-type animals, founders (AF0) and their progenies (AF1) were analyzed. Multiple *PB[Act-RFP]* integrations in a male founder (AF0-61) were segregated in its offspring. A female *PB[Act-RFP]* founder (AF0-47) that carried a single *PB[Act-RFP]* transposition integration (judged by the Southern and the inverse PCR result, AF0-A47T6 in Table S3) also transmitted its transposon to one of its progeny (AF1-47-336).

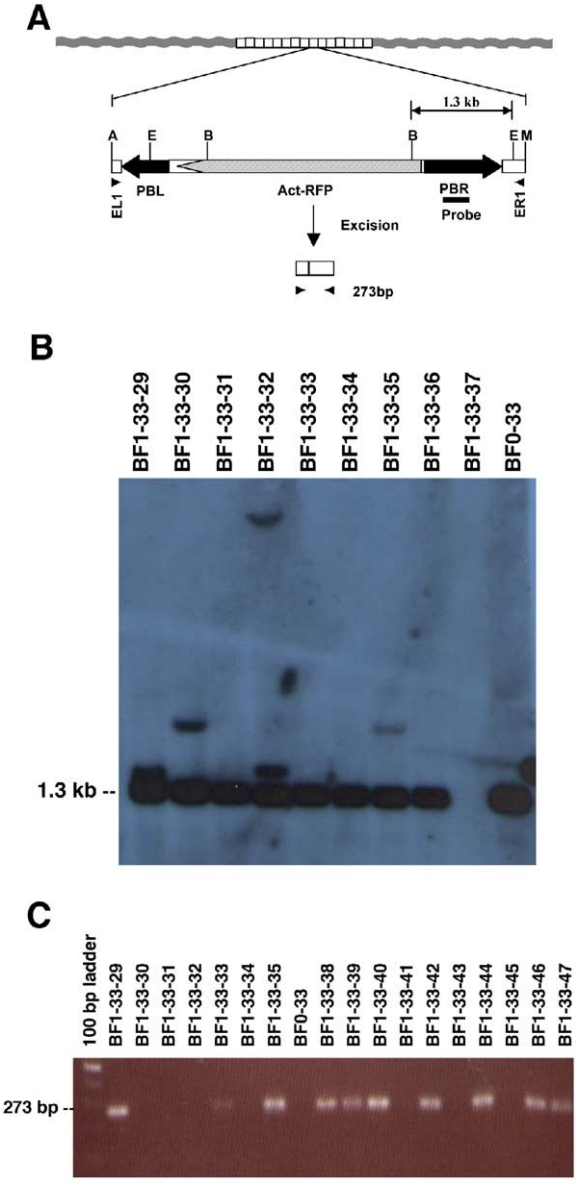

Figure 4. Precise Excision and Transposition of *piggyBac* in Mouse Germline

A male founder mouse coinjected with *Prm1-PBase* and *PB[Act-RFP]* (BF0-33) was used for analyzing germline transposition.

(A) Scaled structure of the *PB[Act-RFP]* transposon. Genomic DNA is represented by curved lines, while the *PB* transposon-containing plasmid concatamer is shown in aligned boxes. Restriction sites: M: MluI, E: EcoRV, B: BglII, A: Acc65I. Position of the probe for Southern analysis is illustrated by the solid line. Primers used to detect excision events are shown as arrowheads.

(B) Southern analysis of a founder (BF0-33) and its progeny (BF1) revealed bands other than the 1.3 kb concatamer signal, thus implying the occurrence of germline transposition.

(C) Positive bands with expected length from precise excision were observed in several progenies (BF1) after PCR amplification with the primers shown in (A).

some transposition events might not coupled with the detection of this 273 bp product (progeny BF1-33-30 and BF1-33-32 in Figures 4B and 4C).

piggyBac Transposition System as a Unique Transgenic Tool

It has been shown previously that transposition efficiency significantly decreases with increasing the length of some transposons, which hampers their utility as a genetic tool. For example, in HeLa cells, *SB* transposons were shown to have an approximately 30% decrease in efficiency of transposition with each kb increase in length in addition to its 1.7 kb original length (Izsvak et al., 2000). To determine the size limitation of *PB* transposition in mice, several *PB* elements ranging from 4.8 to 14.3 kb were used in making transgenic mice (Figure 1A). These transposons carried either a *RFP* reporter cassette and/or a separate transcription unit. The integration rate of these *PB* elements in circular plasmids was tested in the absence or presence of *Act-PBase* helper plasmid (Figure 3A). Results indicated that *PB* elements can carry up to 9.1 kb of foreign sequence without significantly reducing integration efficiency. PCR analysis confirmed the presence of transposition events in 83.9% (26/31) of the founders with the *PB[K14-Tyr, Act-RFP]* element, which carries two marker genes.

Helper-assisted integration dropped significantly using the 14.3 kb *PB[Act-RFP, MCK-TSC1]* element. Eleven *PB[Act-RFP, MCK-TSC1]*-positive founders were analyzed by Southern hybridization and inverse PCR, and four were found to carry transposition integration (Table S3 and data not shown). Thus, *PB* is able to transpose sequence up to 14 kb, albeit at reduced frequency.

Next, we evaluated the behavior of transgene expression from integrated *PB* elements. Among the mice that carried *PB[Act-RFP]*, 98% (39/40) expressed the RFP marker. In our experiment, even one copy of *PB[Act-RFP]* transposon produced a visible red signal under UV illumination (Figure 5A). The RFP transgene expression of the *PB[Act-RFP]* lines does not vary among siblings or individuals of different generations. One of the first single copy *PB[Act-RFP]* lines has been stably expressing RFP through five generations of breeding (Figure 5A). These data indicate that *PB* element permits transgene expression and is not significantly affected by silencing or epigenetic modification. Some of the founders exhibit mosaic RFP signals, a phenomenon most likely due to the transposition in embryonic development after the one-cell stage (Figure 5B). Coexpression of both *RFP* and *tyrosinase* markers was observed in 29% (9/31) of the founders carrying *PB[K14-Tyr, Act-RFP]*, a transposon containing both a *K14* promoter-driven *tyrosinase* gene (*K14-Tyr*) and a *RFP* expression cassette (Figures 1A, 5C, and 5D).

The ability of simultaneous expression of two separate transcription units and high frequency integration events suggests that *PB* transposition can be used as an effective method to generate transgenic mice. Furthermore, the *PB[Act-RFP]DS* construct, which contains unique cloning sites and a RFP expression cassette, serves as a universal transgenic vector that permits the generation of animals carrying a single-copy transgene that can be followed with a visible marker.

piggyBac Transposition System as an Insertional Mutagenesis Tool

To test the feasibility of *PB* as an insertional mutagenesis tool in vertebrates, we evaluated 104 transposition events produced in mice (Table S3). First, the TTAA sequence was found at all *PB* integration sites except one. Second, we compared the genomic sequences flanking the TTAA sites of integration with randomly sampled TTAA sites in the mouse genome and found enrichment of Ts and As surrounding the core TTAA sequence (Figure 6A). This is similar to the integration sites found in insects (Li et al., 2005). Finally, genomic locations of these transposition sites were analyzed against the Ensembl mouse genome database. Although some of the sites could not be mapped due to the presence of repetitive sequences and sequence gaps in the database, the exact locations of 93 transposon integration sites were determined (Table S3; Figure 6E). A wide range of chromosomal distribution was observed among these transposition sites. All mouse chromosomes except two (chromosome 19 and chromosome Y) were hit by *PB* transpositions (Figure 6E).

Sixty-seven percent (70/104) of all transposition sites were mapped to known or predicted transcription units. Among these integrations, about 97% (68/70) hit introns, while 3% (2/70) hit exons (Figure 6B). The preference of integration within transcription units still remained high even if unvalidated (i.e., predicted) genes were excluded from analysis (48% [50/104]). Furthermore, more than 40% of the "intergenic" transpositions were mapped within 50 kb of known genes or ESTs (Figures 6C and 6D). When a 10 kb interval was set as an arbitrary threshold for regulatory regions at 5′ and 3′ ends of a transcription unit, the frequency of genes hit by *PB* transposition were about 80% (83/104) for known or predicted transcription units (Figure 6B).

The wide chromosomal distribution and the preference of transposition into transcription units indicates that *PB* elements can be used as a highly effective mutagen for genome-wide genetic screens.

Discussion

We have shown that *PB* elements can actively transpose in mouse and human cells. *PB* transposition has been thought to be less dependent on host factors than other transposons, for it is the only known transposon capable of transposition in more than a dozen different insect species (Handler, 2002; Sumitani et al., 2003). The fact that *PB* can effectively transpose in both insects and mammals indicates that this transposon system can have broad applications for genetic studies in both invertebrates and vertebrates. It further suggests that the transposition mechanism of *PB* elements may be significantly different from other naturally existing transposons, which only work in highly restricted species.

piggyBac as a Tool for Transgenesis

Our studies suggest that *PB* is a practical tool for generating transgenic mice. First, *PB* can be introduced into the mouse germline with high efficiency. Pronuclear coinjecting of helper and donor plasmids results in more than 30% of the founders carrying integrated

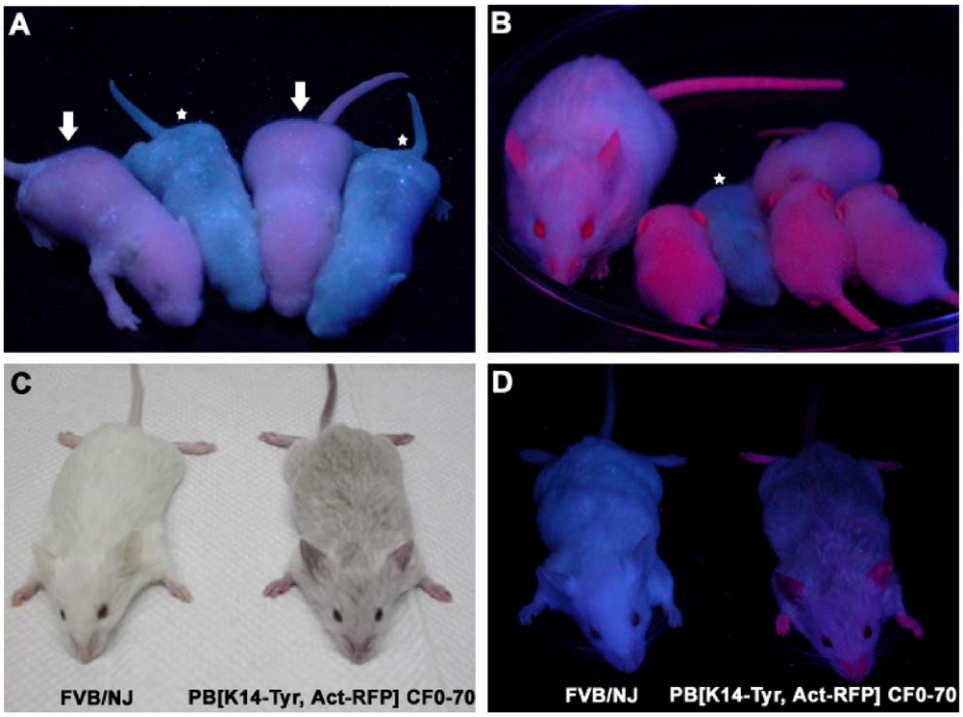

Figure 5. Expression of Transgenes in *piggyBac* Vectors

(A) *PB[Act-RFP]* expression in the progenies resulted in red fluorescence under the illumination of a portable long-wave UV light. Two positive mice (arrows) carrying the same single copy transposon (AF0-47T6) and two negative littermates (asterisks) are shown.

(B) *PB[Act-RFP]* expression in a founder mouse and her progeny. Red fluorescence was mosaic in the founder. Segregation of transposons in the progeny resulted in different intensities of RFP signal. The star marks the transgene-negative littermate.

(C and D) Coexpression of two transgenes in the same *piggyBac* vector. As a result of tyrosinase expression, a *PB[K14-Tyr, Act-RFP]* founder shows gray coat color under white light, while the transgene-negative littermate remains albino ([C], right and left, respectively). When illuminated by UV, red fluorescence was observed from this founder (D).

donor plasmids in their germline (Figure 3A). The insertions are stably integrated in the genome when they are not exposed to PBase. Second, the approach produces single copies of integrated transgenes. In most cases, classical pronuclear injection of linear DNA into mice results in the formation of transgene concatamers (Nagy et al., 2003), which usually result in transgenes expressed higher than normal physiological levels. The *TSC* genes regulate growth, and it has been difficult to generate *TSC* transgenic mice with traditional methods (Potter et al., 2001; data not shown). We had no difficulty to obtain *TSC* transgenic mice with the *PB* system (*PB[Act-RFP, MCK-TSC1]*), although the frequency of the transgenic animals was lower, which could due to the size of the construct or the deleterious effect of the *MCK-TSC1* transgene. Third, we showed that individual transposon integration sites can be quickly defined by inverse PCR. Thus, the location of the insertions and the effect of the chromatin environment on integrated transgenes can be estimated. Fourth, the *PB* element allows the expression of the transgene it carries. The overall frequency of mice showing the expected transgenic expression pattern was comparable to conventional transgenic experiments. Finally, our results indicate that *PB* can carry transgenes up to 9.1 kb without a significant reduction of the transposition frequency. Transposition was observed for transgenes as big as 14.3 kb, which allows insertions much bigger than retroviral vectors can carry. Thus, a single *PB* element can carry multiple genes, which allows one to perform complex transgenic experiments such as identifying positive transgenic animals with the help of a visible marker.

Given that the *PB* system works in both human cells and mice, it could be a powerful tool for generating transgenic animals for other vertebrates and for gene therapy.

piggyBac as a Genomics Tool for Deciphering Gene Function

In the postgenome era, systematic gene inactivation is one of the most powerful approaches to decipher the function of the genome. This approach has been proven to be successful in the study of single-cell organisms like bacteria and yeast, as well as of multicellular organisms such as *C. elegans*, *Drosophila*, zebrafish, and *Arabidopsis*. Unfortunately, efficient methods for genome-wide gene inactivation in mammals are still limited. ENU mutagenesis is one of the few available methods for genome-scale gene inactivation in the mouse; however, mapping ENU-induced mutations and cloning the genes defined by the mutations is usually laborious and time consuming (Herron et al., 2002). Retroviral-mediated insertional mutagenesis has also been widely used to produce mutations throughout the

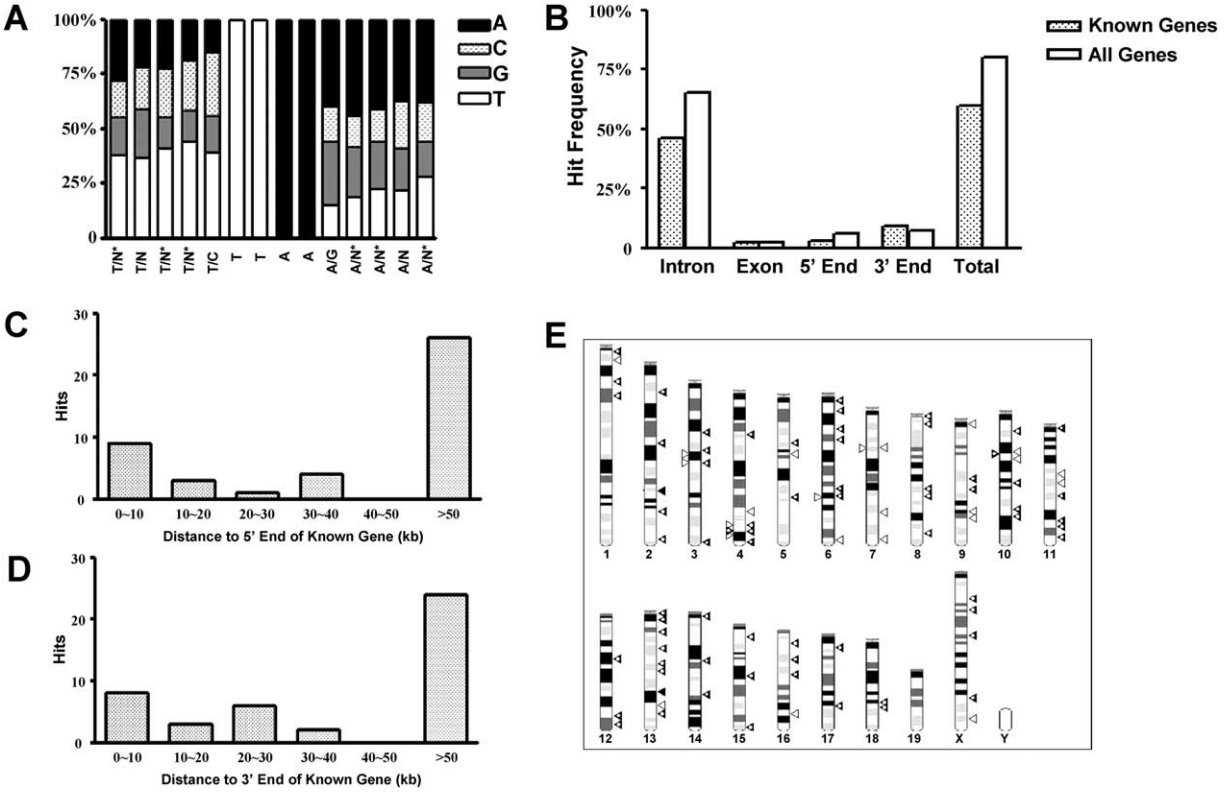

Figure 6. *piggyBac* Integration Sites in Mouse

(A) Nucleotide composition of flanking sequences from 100 *PB* integration sites. In addition to the TTAA target site specificity, an enrichment of Ts and As in the flanking sequences was observed. Asterisks denote $p < 0.05$ when compared with flanking sequence of the randomly sampled TTAA control.

(B) Distribution of *PB* insertions in genes. Percentages of the *PB* insertions located in exons, introns, 5′ regulatory sequences (10 kb adjacent to transcription start site), 3′ regulatory sequences (10 kb adjacent to poly(A) site), and in all four regions (total) are illustrated. Empty bars indicate data from all known and predicted genes and solid bars indicated data from the known genes or ESTs.

(C) Distribution of *PB* insertions in 5′ regions.

(D) Distribution of *PB* insertions in 3′ regions.

(E) Analysis of 93 integration sites in mice showed that *PB* integrations appeared to hit all but the two smallest chromosomes (19 and Y). Filled arrowheads indicate hits in exons, dark arrowheads indicate hits in introns, empty arrowheads indicate hits in predicted intergenic regions.

mouse genome. While this method indeed produces a large number of mutations, most of these mutations are generated in mouse ES cells, and a significant amount of additional effort is needed to transmit these gene-specific mutations into live animals before phenotypes can be examined. Recently, SB has been tested for the insertional mutagenesis in the mouse. However, local hopping and a relatively low efficiency of transposition into transcription units prevent it from being widely used. In contrast, *PB* provides a new and attractive choice for screening recessive mutations in the mouse. The success of efficient *PB* transposition in the mouse germline suggests the suitability of this transposon for insertional mutagenesis. Furthermore, preliminary phenotypic characterization of several *PB* insertions has revealed that the insertion alleles produce phenotypes similar to those of the traditional knockout alleles, e.g., *PKD2* (Table S3; S.D., X.W., T.X., unpublished data). Several unique properties of *PB* could greatly facilitate insertional mutagenesis studies in mice.

One important consideration of insertional mutagenesis experiments is whether the mutagen can hit every gene in the genome in an unbiased fashion. Our experiments have shown that *PB* integrations have a diverse distribution in the mouse genome, which is consistent with a recent study in *Drosophila* showing that *PB* hits genes more randomly than the widely used P element (Thibault et al., 2004). Interestingly, our study has revealed a high preference of *PB* transposition for transcriptional units. Sixty-seven percent of the transposon integrations were found within known or predicted transcriptional units. Including insertions in the regulatory regions adjacent to the transcriptional initiation and termination sites, the frequency of *PB* transposition in genes is even higher. Given that only ~15% of the mouse euchromatin sequence encodes genes, *PB* transposition is highly selective for transcription units. It is not clear whether this integration property is influenced by the transcriptional activities of the genome or the exogenous sequence carried by the *PB* elements. Nevertheless, this integration preference makes *PB* a potential dream tool for genome-wide insertional mutagenesis.

Another important aspect in the analysis of mutations obtained from random mutagenesis is the verification of the relationship between mutations and the pheno-

types they cause. This is particularly important in the analysis of novel genes. Verification of genotype/phenotype correlation is usually done by introducing a wild-type gene into the mutant background and looking for phenotypic "rescue" (ideally, reversion of the induced mutation back to wild-type). Another way to determine genotype/phenotype correlation is to excise insertional mutations and look for phenotypic reversion. The ability of transposons to excise has thus always been considered as an important advantage over retriviral vectors. However, most transposons leave a small deletion or insertion after excision from the original site. Interestingly, *PB* often leaves no footprint after excision, making it ideal for producing revertants. This feature also makes *PB* less likely to cause genomic damage during mutagenesis, in which multiple transposition events occur in a single genome. Our studies have demonstrated that *PB* excision can be easily achieved with germline expression of the transposase.

The fact that *PB* can carry multiple genes during transposition offers great advantages for many genetic manipulations, including insertional mutagenesis and phenotypic characterization. It allows one to follow the insertion/mutation and the status of the mutation, such as heterozygous versus homozygous and single mutant versus double mutants, by visible markers such as RFP and Tyrosinase. Given the long generation time and the high animal housing cost associated with mouse breeding, this will dramatically cut down the cost for many types of experiments and will make some unrealistic experiments become practical. Furthermore, *PB* transposons for insertional mutagenesis could also carry reporter genes for enhancer/promoter detection, or "gene trapping," which can greatly facilitate the effort of functional annotation of the mouse genome and provide reagents for many types of biological analyses.

In conclusion, our experiments provide a first step toward a highly efficient transgenesis and insertional mutagenesis system in mouse and suggest that the *PB* system could also be used as a powerful tool for genetic manipulations in other vertebrate organisms.

Experimental Procedures

Plasmid Construction

Construction of *PB[SV40-neo]* was as follows: the BamHI-KpnI fragment of *pSLfa1180fa* (Horn and Wimmer, 2000) was replaced by the BamHI-KpnI fragment from *pCLXSN* (IMGENEX). The neomycin cassette was then cut out with AscI and inserted into the AscI site of *pBac{3xP3-EGFPafm}* (Horn and Wimmer, 2000).

Construction of *CMV-PBase* was as follows: the coding sequence of the *piggyBac* transposase was PCR amplified from *phsp-Bac* (Handler and Harrell, 2001) with primers BacEN-F (5′-GCCACCATGGGATGTTCTTTAG-3′) and BacEN-B (5′-GTACTCA GAAACAACTTTGGC-3′), and cloned into the SpeI and SphI sites of *pSLfa1180fa* to generate *pSL-BacEN*. A HindIII-EcoRI fragment containing the transposase gene was isolated from *pSL-BacEN* and inserted into *pcDNA4/HisA* (Invitrogen) to generate the final construct.

Construction of *PB[PGK-neo]* was as follows: the *PGK-neo* gene from *pPNT* (Tybulewicz et al., 1991) was cloned into the BglII site of *pBac-AB*, a modified *PB* construct (X.W. and T.X., unpublished data) to generate *PB[PGK-neo]*.

Construction of *PB[Act-RFP] and PB[Act-RFP]DS* was as follows: the 0.7 kb EcoRI fragment of *pCX-EGFP* (Okabe et al., 1997) was replaced by the coding sequence of mRFP (Campbell et al., 2002) to make *pCX-RFP*. The SalI-BamHI fragment of *pCX-RFP*, including the intact RFP expression cassette, was further cloned into the BglII site of *pBac-AB* to generate *PB[Act-RFP]*. The SmaI fragment from *PB[Act-RFP]* that consists of the RFP expression cassette and the left terminus (PBL) was used to replace the SalI-EcoRV fragment of *pBluescript* to generate *pBS-BLRFP*. The SmaI-EcoRV fragment of *PB[Act-RFP]* that consists of the right terminus (PBR) was then cloned into the PmeI site of *pBS-BLRFP* to generate *PB[Act-RFP]DS*, which serves as a universal *PB*-based transgenic vector.

Construction of *Prm1-PBase* was as follows: the *Prm-1* promoter and the BamHI-SalI fragment from *pPrm1-SB10* (Fischer et al., 2001) were cloned into the HindIII site and the BamHI-XhoI site of *pSL-BacEN,* respectively, to generate this testis-specific transposase helper plasmid.

Construction of *Act-PBase* was as follows: Using a NheI-NotI linker, the EcoRI fragment of *pCX-EGFP* was replaced by the SpeI-EagI transposase fragment of *pSL-BacEN* to generate this ubiquitously expressed transposase helper plasmid.

Construction of *PB[K14-Tyr]* was as follows: the SmaI fragment of the *K14* promoter in *pInK14-Albino* (Saitou et al., 1995), a *tyrosinase* cDNA amplified from a skin sample of a 129S mouse by RT-PCR and the SV40 poly(A), were inserted into the BglII site of *pBac-AB* to generate *PB[K14-Tyr]*.

Construction of *PB[K14-Tyr, Act-RFP]* was as follows: the SalI-BamHI fragment from *pCX-RFP* was cloned into the AscI site of *PB[K14-Tyr]* to generate this construct.

Construction of *PB[Act-RFP, MCK-TSC1]* was as follows: the BssHII fragment of the *MCK-TSC1 gene* (Inoki et al., 2002) and a hGH poly(A) (Nguyen et al., 1998) were cloned into the SwaI site of *PB[Act-RFP]DS*.

Cell Transfections

293 cells were cultured in DMEM (GIBCO-BRL) supplemented with 10% fetal bovine serum at 37°C and 5% CO_2. 1.5×10^5 cells were seeded into each well of a 24-well-plate 1 day prior to transfection. For each well, 0.5 μg circular *PB[SV40-neo]* and 0.5 μg circular *CMV-PBase* in the test group or 0.5 μg circular *pcDNA4/HisA* in the control group were transfected using LipofectAMINE 2000 according to standard protocols (Invitrogen). One day after transfection, the cells in each well were trypsinized and seeded onto one 10 cm plate in medium containing 500 μg/ml G-418 (GIBCO-BRL). Drug selection continued for 2 weeks.

The conditions for culture and electroporation of *W4/129S6* mouse ES cells were described in the manufacturer-recommended protocols (Taconic). Twenty-four micrograms of circular *PB[PGK-neo]* and 6 μg *Act-PBase* in the test group or 6 μg herring sperm DNA (Promega) in the control group were used for electroporation of 10 million cells. Immediately after electroporation, cells in each group were seeded onto three 10 cm plates containing mitomycin C-treated mouse embryonic fibroblast feeder cells. Selection was initiated 48 hr after electroporation with medium containing 200 μg/ml G-418. Drug selection continued for two weeks.

At the end of drug selection, cells were fixed with PBS containing 4% paraformaldehyde for 10 min and then stained with 0.2% methylene blue for 1 hr. Clones were counted after extensive washing with deionized water.

PCR and Sequence Analysis

HaeIII or MspI digests of genomic DNA were self-ligated to serve as the template for inverse PCR. Primers used to recover the flanking sequence of the left side of the *PB* transposon were LF1 (5′-CTT GAC CTT GCC ACA GAG GAC TAT TAG AGG-3′) and LR1 (5′-CAG TGA CAC TTA CCG CAT TGA CAA GCA CGC-3′). Primers used to recover the flanking sequence of the right side of *piggyBac* transposon were RF1 (5′-CCT CGA TAT ACA GAC CGA TAA AAC ACA TGC-3′) and RR1 (5′-AGT CAG TCA GAA ACA ACT TTG GCA CAT ATC-3′).

PCR detection of the excision site was carried out with primer EL1 (5′-CCA TAT ACG CAT CGG GTT GA-3′) and primer ER1 (5′-TTA AAG TTT AGG TCG AGT AAA GCG C-3′).

PCR products were cloned into *pGEM-T* (Promega) for subsequent sequencing. Sequencing results were analyzed with NCBI BLAST searches (www.ncbi.nlm.nih.gov) and Ensembl human or mouse genome databases (www.ensembl.org).

To detect additional sequence preference of *PB* insertion events,

five base pairs upstream and downstream of the TTAA target site were analyzed for 100 *PB* insertions in mice. At the same time, 100 randomly selected TTAA sites were analyzed as the control. One-sided probabilities were calculated between two proportions with STATISTICA 6.0.

Generation of Transgenic Mice

Circular *PB* donor constructs were mixed with a helper plasmid at a ratio of 2:1. Mixed DNA samples (2 ng/μl) were microinjected into fertilized *FVB/Nj* oocytes as described (Nagy et al., 2003).

Southern Blot

Genomic DNA was isolated from tail samples, digested with EcoRV and BglII, and then fractionated in 0.7% agarose gels prior to Southern analysis. The probe was a 499 bp fragment SacII digest of *PB[Act-RFP]*.

Supplemental Data

Supplemental Data include three tables and can be found with this article online at http://www.cell.com/cgi/content/full/122/3/473/DC1/.

Acknowledgments

We thank Ying BeiBei, Zhu Huaxing, Wang Jiajing, Yang Yanling, Tan Yanfeng, and Raymond Pagliarini for assistance and R. Plasterk, E. Fuchs, and K.L. Guan for reagents. Special thanks to Qin Shaode, Wang Shenghong, Yang Yuliang, Chen Xiaoman, Zhu Zuoyan, and Jiang Hujun for encouragement and support. We are grateful to Tan Jiazhen (C.C. Tan), Sheng Zujia, Liu Zudong, and Li Ruqi, who taught Mendel/Morgan Genetics during the difficult years in China and first introduced Genetics to us, and to the US scientific community and the advisors to T.X., M.H., and Y.Z., who hosted and trained Chinese students. The work is partially supported by the National Natural Science Foundation of China.

Received: February 17, 2005
Revised: June 7, 2005
Accepted: July 6, 2005
Published online: July 21, 2005

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