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A complementary transposon tool kit for *Drosophila melanogaster* using *P* and *piggyBac*

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With the availability of complete genome sequence for *Drosophila melanogaster*, one of the next strategic goals for fly researchers is a complete gene knockout collection. The *P*-element transposon[1], the workhorse of *D. melanogaster* molecular genetics, has a pronounced nonrandom insertion spectrum[2]. It has been estimated that 87% saturation of the ~13,500-gene complement of *D. melanogaster*[3] might require generating and analyzing up to 150,000 insertions[2]. We describe specific improvements to the lepidopteran transposon *piggyBac*[4] and the *P* element that enabled us to tag and disrupt genes in *D. melanogaster* more efficiently. We generated over 29,000 inserts resulting in 53% gene saturation and a more diverse collection of phenotypically stronger insertional alleles. We found that *piggyBac* has distinct global and local gene-tagging behavior from that of *P* elements. Notably, *piggyBac* excisions from the germ line are nearly always precise, *piggyBac* does not share chromosomal hotspots associated with *P* and *piggyBac* is more effective at gene disruption because it lacks the *P* bias for insertion in 5′ regulatory sequences.

We incorporated three technological improvements to increase the efficiency of transposon mutagenesis in *D. melanogaster*, with an emphasis on generating stronger loss-of-function alleles. First, we used an additional mobile genetic element. Alternative transposons have been used in *D. melanogaster*[5], but for various technical reasons, none had been applied for large-scale gene knockouts when we began our studies. We selected *piggyBac* as the most promising transposon for this application. Owing to the low remobilization rates observed with a heat shock–inducible transposase (data not shown), we reengineered the transposase with a constitutive promoter and germline-stabilizing 3′ untranslated region. With this construct, we and others[6,7] observed remobilization frequencies of 60–80% (data not shown). Second, we constructed a series of different *P* (XP) and *piggyBac* (PB, RB, WH) vectors carrying 'splice-trap' and transcriptional silencing elements (**Supplementary Fig. 1** online) for more effective gene disruption. Third, although previous screens typically mobilized transposons in the male germ line, differences in transposon activity between male and female germ lines[8] led us to use the female germ line, with the hope of altering the spectrum of genes accessible to tagging. An ongoing challenge for

Table 1 Distribution of *P* and *piggyBac* transposons across the genome

	Chromosome arm[a]							
Transposon	X	2L	2R	3L	3R	4	U[b]	Total
P element XP	1,803	1,280	1,528	1,800	1,848	22	34	8,315
piggyBac PB	658	1,018	1,130	1,582	1,931	45	434	6,798
piggyBac RB	814	769	820	892	965	53	35	4,348
piggyBac WH	1,470	1,264	1,172	1,407	1,604	77	85	7,079

[a]Transposons mapped to Release 2 sequence assembly. [b]Unmapped scaffold sequence.

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Table 2 Summary of deficiency complementation and excision of lethal *piggyBac* insertions

Lethal *piggyBac*[a]	Chromosome	Noncomplementing deficiency[b]	Independent excisions	Viable excisions	Excisions complementing deficiency	Precise molecular excisions
c00080	2	B-3084, *Df(2L)ast2*	10	10/10	10/10	10/10
c00093	2	B-442, *Df(2R)CX1*	10	10/10	10/10	10/10
c00146	2	B-384, *Df(2L)N22-5*	10	10/10	10/10	10/10
c00221	2	B-1006, *Df(2R)nap1*	10	10/10	10/10	10/10
c00119	3	B-1910, *Df(3R)TI-P*	10	10/10	10/10	10/10
c00083	3	B-3011, *Df(3R)Cha7*	5	5/5	5/5	5/5
c00136	2	B-3138, *Df(2L)b87e25*	NA			
c00014	3	B-1011, *Df(3R)faf-BP*	NA			
c00064	3	B-3007, *Df(3R)ry615*	NA			
c00213	3	B-3627, *Df(3L)31A*	NA			

[a]Exelixis stock number. [b]Bloomington *Drosophila* Stock Center number and deficiency name.
NA, not applicable.

fruit fly researchers is that mutants and transgenic strains come from a variety of genetic backgrounds that may harbor pre-existing mutations[9], thus precluding or complicating highly sensitized or behavioral screens. We generated all our transposon reagents and associated stocks in a freshly derived isogenic background; the insertion collection reported here is fully isogenic.

With these new tools and strategies we generated 29,682 *piggyBac* and XP insertion lines, of which 89% were molecularly placed on the genome (**Table 1**). We observed higher frequencies of recessive lethal mutations for both XP (17%) and *piggyBac* (22%) than the 10–15% reported from previous *P* screens[10]. Molecular and genetic analysis suggests that this was not simply due to multiple insertions. Eighty-nine percent of the lines yielded unique flanking sequence by inverse PCR. In addition, 97 of 100 *piggyBac* lines analyzed by genomic Southern blotting had single insertions (data not shown). The increased lethal frequency observed for *piggyBac* was not the result of elevated background mutagenic effects ('hit and runs') at sites independent of the final insertion. Because we started with a clean isogenic strain, we were able to assess the background mutagenic rate with high accuracy using the following tests. We selected ten lethal *piggyBac* lines and confirmed that each did not complement a corresponding deficiency (**Table 2**), indicating these mutations are at least closely linked to the insert. We then used six of these lines to generate 55 excision alleles; each was molecularly confirmed as a precise event that also reverted the chromosome to a viable phenotype (**Table 2**). Previous studies also found a majority of molecularly precise excision events, although lethal alleles (generated in a nonisogenic background) were not always reverted. Additionally, separate genetic screens using our collection found that only 0.3–0.5% of *piggyBac* stocks contained unlinked background mutations (H.L.F.-L., unpublished data); thus; hit and run events do not account for the high percentage of recessive lethal *piggyBac* insertions. Finally, secondary mutations may arise if transposase expression activates cryptic elements or induces genomic instability. The insertion and transposase strains have been stable for 4 years with no observed breakdown.

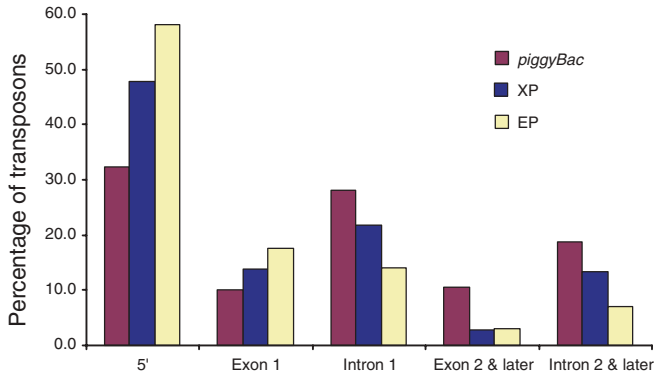

Figure 1 Distribution within genes of 6,196 *piggyBac* elements, 2,462 XP elements and 1,109 EP[11] transposons tagging DGC (r1.0) genes. 5′ of start = 1,000-bp window upstream of the transcriptional start.

Taken together, these data support the conclusion that *piggyBac* acts as an effective mutagen and that the mutations observed are directly caused by the transposon insertion. The higher frequency of lethal mutations generated by *piggyBac* versus *P* cannot be explained by secondary mutations but must result from some other property of the transposon, such as its insertion pattern or the ability of the transposon itself to locally perturb gene function. To investigate these possibilities, we compared the local and global patterns of *piggyBac* and XP insertions.

After associating insertions with a highly curated set of 5,849 genes (the Berkeley *Drosophila* Genome Project's *Drosophila* Gene Collection (DGC) r1.0), we determined their relative positions within the genes (**Fig. 1**). We also analyzed an unbiased public set of *P* insertions (EP element[11]) for comparison. We found that a larger fraction of *piggyBac* elements inserted after the transcriptional start site (67.5% versus 52.1% of XP and 41.9% of EP lines). Unlike *P*, which preferentially inserts at the 5′ end of genes[12], only one-third of *piggyBac* inserts were found upstream of the transcriptional start site (**Fig. 1**). The XP collection was also less biased than EP by this metric, possibly because we used female dysgenesis in our screen. We also observed that *piggyBac* inserted into coding exons at a higher rate than either XP or EP. Excluding first exons, *piggyBac* tagged remaining exons more than three times more frequently. Because initial exons in *D. melanogaster* are often non–protein coding, insertions in later exons are more likely to fully disrupt gene function by interrupting the open reading frame. Intronic insertions may also be disruptive by interfering with regulatory sequences or proper gene splicing[13,14]. Thus, because *piggyBac* insertions occur more frequently between transcriptional start and stop, they should create null alleles more commonly.

Examining molecular placements identified hot-spot regions for both XP and *piggyBac* (**Fig. 2**). Defining a hot spot as a 50-kb interval containing 30 or more insertions, we found 23 XP hot spots (**Supplementary Table 1** online). In contrast, from more than twice as many *piggyBac* insertions, we found only 26 *piggyBac* hot spots (**Supplementary Table 1** online), none of which overlapped XP hot

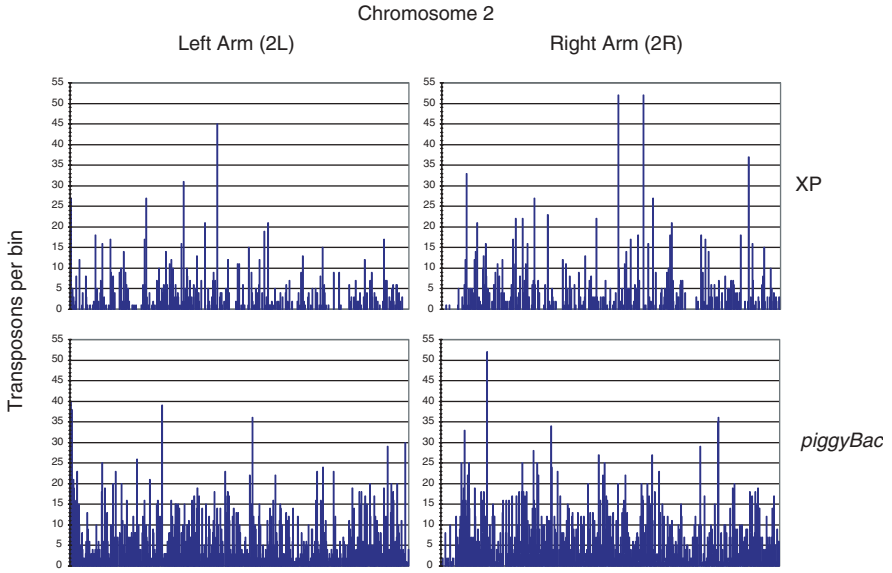

Figure 2 Distribution of XP (2,661) and *piggyBac* (6,765) inserts across the left and right arms of *D. melanogaster* chromosome 2. Chromosome sequence was binned into 50-kb intervals and the number of transposons in each bin is plotted. Hot-spot and cold-spot regions are apparent for both transposons. All hot spots are nonoverlapping between *P* and *piggyBac*. Results were similar across remaining chromosomes.

spots. The mean number of *piggyBac* insertions per hot-spot bin (38) was also less than that for XP (43). Neither transposon had a random (Poisson) distribution (**Fig. 3**), but the spectrum of *piggyBac* insertions was different from and broader than the spectrum of XP insertions.

To assess relative gene tagging frequencies for *P* and *piggyBac*, we plotted the cumulative number of DGC genes tagged against recovery of new insertions (**Fig. 4**). *piggyBac* was much more efficient, yielding a high rate of new hits even after 17,000 insertions. Therefore, to achieve 87% gene saturation with *piggyBac* should require substantially fewer insertions than to do so using *P* alone, perhaps 75,000–100,000. Our combined *piggyBac* and XP collection tagged 53% of the DGC genes and, by extension, all *D. melanogaster* genes.

After generating and characterizing thousands of *piggyBac* and *P*-element insertions, we find *piggyBac* to be an efficient and practical gene tagging system in *D. melanogaster* on par with the *P* element. These reagents and the associated transposon tool kit will be a useful complement to existing *D. melanogaster* gene knockout resources. In fact, this collection has already been extensively used by us and our collaborators for biological analysis of gene function in pharmaceutically relevant disease pathways[15,16].

METHODS

***D. melanogaster* strains and plasmid vectors.** We maintained flies on standard cornmeal-molasses-agar medium at 25 °C (ref. 9). We used three classes of *piggyBac* vectors and one *P* element in the screen (**Supplementary Fig. 1** online). The *piggyBac* vector (PB) is the simplest, comprising a complete *piggyBac* transposon with the open reading frame interrupted by the *D. melanogaster* gene mini-white (w^{+mC})[17] flanked by short (48-bp) FRT recombination sites from the yeast 2 plasmid[18]. The *piggyBac* vector (RB) contains the mini-white marker gene and a single long (199-bp) FRT site. Because exons in *P* elements can disrupt gene function by acting as ectopic splice acceptors[13,14], we designed the RB vector with an exon from the *D. melanogaster* gene *Rbp1* (ref. 19) as a second splice trap

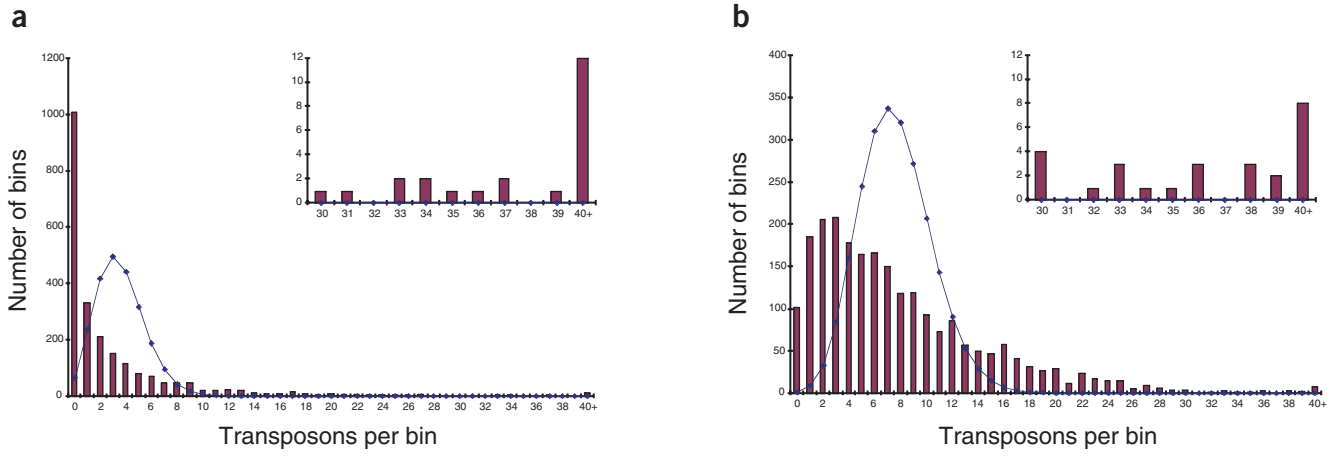

Figure 3 The assembled *D. melanogaster* genome sequence was divided into 50-kb bins and the number of transposons in each bin is represented as a histogram for XP (**a**) and all *piggyBac* insertions (**b**). Overlaid on each histogram is a line representing the predicted Poisson distribution for 8,281 XP and 17,671 *piggyBac* inserts (transposons placed on unassembled Chromosome U are excluded from this analysis). Both XP and *piggyBac* distributions were significantly different from Poisson (χ^2 analysis, $P < 0.001$). The number of bins containing 30 or more transposons is highlighted in the inset histograms. XP and *piggyBac* elements had comparable numbers of hot spots, although more than twice as many *piggyBac* inserts were generated.

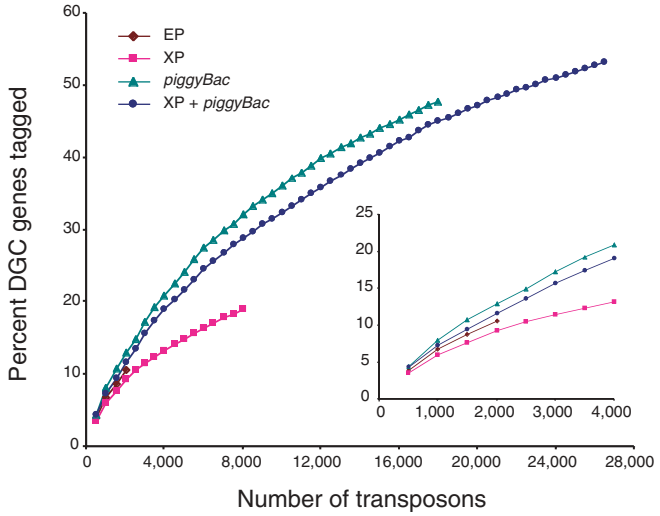

Figure 4 Gene-tagging frequency of *P*-element (XP) and *piggyBac* transposons. The EP[11] set is included for comparison. Percentage of DGC-tagged genes was plotted by random sampling of the set with increasing numbers of transposons. At an equal number of transposons (8,000), *piggyBac* tagged 69% more genes than XP. The combined collection of 26,540 XP and *piggyBac* molecularly localized insertions tagged ~53% of DGC genes. The initial gene tagging rate is highlighted in the inset graph.

in the opposite orientation of mini-white. The *piggyBac* vector (WH) contains the gene mini-white, a long FRT site, Su(*Hw*) insulator sequences[20] and a terminal UAS site for Gal4-driven misexpression of adjacent genes[11]. The *P* element (XP) used in our screen contains the gene mini-white, *Su(Hw)* insulator sequences and dual terminal UAS sites with one flanked by long FRT sites. In this issue, Parks *et al.*[21] describe the use of FRT sites within XP, RB and WH for FLP-mediated recombination[22] between two insertions, enabling a rapid method for generating molecularly defined deficiencies.

We cloned *piggyBac* transposase sources into the improved *P*-element vector pExPress (derived from pCasPeR) and introduced them into *D. melanogaster* by standard methods. We remobilized transposase sources onto the CyO balancer chromosome and characterized them for activity before selecting the isolates used in our screen. We constructed the inducible *piggyBac* transposase by placing the *piggyBac* coding sequence under control of the *D. melanogaster Hsp70* promoter and regulatory sequences. We constructed the constitutive *piggyBac* transposase by cloning the *piggyBac* open reading frame under control of the *D. melanogaster* α*Tub84B* promoter[23] into a variant of pExPress in which the gene mini-white is flanked by short FRT sequences. We further modified this *piggyBac* transposase source by adding the *fs(1)K10* 3′ untranslated region to lend germline stability to the transcript[24,25]. The FRT-flanked gene mini-white allowed for FLP-mediated removal of the marker gene from transformed lines to generate a w^- *piggyBac* transposase strain.

Remobilization. We remobilized the PB element using *Hsp70:piggyBac* transposase from a single ammunition element on either the X or third chromosome. We induced transposase expression by immersing bottles in a circulating 37 °C water bath for a daily (days 3–10 after egg-laying) 1-h heat shock. We outcrossed the resulting dysgenic males to an isogenic *w* strain. New insertions were identified on the basis of a change in eye color (third chromosome ammunition) or the appearance of w^+ male progeny (X chromosome ammunition). We remobilized both RB and WH elements using the constitutive α-1 tubulin:*piggyBac* transposase source. We remobilized the RB element from a single X chromosome insertion in dysgenic males and the WH element from a single ammunition element on the Binsinscy balancer chromosome in dysgenic females. We outcrossed dysgenic males or virgin females in vials to the isogenic *w* strain and selected new hops in the following generation. Only a single new insertion was retained per vial. For the *P* element, XP, we selected an easily mobilized ammunition element among inserts hopped onto the Binsinscy balancer. New insertions were collected in vials from dysgenic females using the standard chromosomal source of transposase, Δ2-3 (ref. 26). All lines were mapped to a chromosome by standard genetic methods, examined for homozygous viability and used for recovery of flanking genomic sequence.

Localization by sequence. We obtained flanking genomic sequence from 5′ and 3′ *piggyBac* ends by inverse PCR using a method adapted from J. Rehm (Berkeley Drosophila Genome Project). We purified DNA from five flies per line in 96-well format. DNA was digested separately with *Sau*3A1 (5′) and *Hin*P1 (3′) and then diluted and self-ligated. We obtained PCR products for *piggyBac* 5′ flanks by a nested reaction with primers 5F1 and 5R1 followed by dilution (1:100) and a second PCR with primers 5F2 and 5R2. We obtained PCR products for 3′ flanks with nested primers 3F1 and 3R1 for the first round and 3F2 and 3R2 for the second round. Similarly, we recovered 5′ flanking sequence for XP with primers 51A and 51B for the first round and 52A and 52B for the second round. For 3′ XP inverse PCR, we used the primers 31A and 31B twice. We treated products with shrimp alkaline phosphatase and DNA exonuclease I. We determined sequences using fluorescent label dye-terminator chemistry (BigDye, ABI) with primers pB-5SEQ and pB-3SEQ for *piggyBac* 5′ and 3′ ends, respectively, and XP-5SEQ and XP-3SEQ for XP 5′ and 3′ ends, respectively. All primer sequences are available on request.

Association with genome and genes. The sequences recovered from transposon flanks were trimmed for quality and masked of any vector. We assembled 5′ and 3′ ends assembled and searched them against the *D. melanogaster* genomic sequence deposited in GenBank. Eighty-nine percent of all lines could be associated with a unique region of the genome. The remaining stocks either failed sequencing or could not be uniquely placed on the genome (sequence too short, repetitive, chimeric).

Availability of reagents and accessions. We made the fly strains described in this report available to public stock centers. The Bloomington *Drosophila* Stock Center selected ~2,100 stocks (**Supplementary Table 2** online) to complement their existing collection. Harvard University (S. Artavanis-Tsakonas, Department of Cell Biology, Harvard Medical School, Charlestown, Massachusetts, USA) selected ~16,500 stocks (**Supplementary Table 3** online), which were culled from the total to reduce redundant hits and provide a diversity of insert type across the genome. Stocks deposited at Harvard University may be requested by e-mail (drosophila@hms.harvard.edu). Resource constraints did not allow either repository to accept all the stocks reported here. We deposited genomic DNA sequence flanking insertion sites (**Supplementary Tables 2** and **3** online) with the Berkeley *Drosophila* Genome Project and vector maps and plasmid DNA with the *Drosophila* Genomics Resource Center.

URLs. The method for obtaining flanking genomic sequence from 5′ and 3′ *piggyBac* ends by inverse PCR is available at http://www.fruitfly.org/about/methods/inverse.pcr.html. The Bloomington *Drosophila* Stock Center is available at http://flystocks.bio.indiana.edu. The *Drosophila* Genomics Resource Center is available at http://dgrc.cgb.indiana.edu.

GenBank accession numbers. Vector sequences for PB, AY515146; RB, AY515147; WH, AY515148; and XP, AY515149.

Note: Supplementary information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Genetics* website for details).

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