

# *piggyBac*-based insertional mutagenesis in the presence of stably integrated *P* elements in *Drosophila*

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*P* element-mediated mutagenesis has been used to disrupt an estimated 25% of genes essential for *Drosophila* adult viability. Mutation of all genes in the fly genome, however, poses a problem, because *P* elements show significant hotspots of integration. In addition, advanced screening scenarios often require the use of *P* element-based tools like the generation of germ-line mosaics using FLP recombinase-mediated recombination or gene misexpression using the UAS/Gal4 system. These techniques are *P* element-based and can therefore not be combined with the use of *P* elements as mutagenic agents. To circumvent these limitations, we have developed an insertional mutagenesis system using non-*P* element transposons. An enhanced yellow fluorescent protein-marked *piggyBac*-based mutator element was mobilized by a *piggyBac* specific transposase source expressed from a *Hermes*-based jump-start transposon marked with enhanced cyan fluorescent protein. In a pilot screen, we have generated 798 *piggyBac* insertions on *FRT* bearing third chromosomes of which 9% have sustained a putatively *piggyBac*-related lethal hit. The *FRTs* present on the target chromosome remained stably integrated during the screen and could subsequently be used to generate germ-line clones associated with maternal and zygotic phenotypes. PCR-based analysis of insertion loci shows that 57% of the insertions are in genes for which no *P* element insertions have been reported. Our data demonstrate the potential of this technique to facilitate the quest for saturation mutagenesis of the *Drosophila* genome. The system is *Drosophila* nonspecific and potentially applicable in a broad spectrum of nonmodel organisms.

At a time when whole genome sequences for the most important developmental biology model organisms have become available, the focus of developmental biologists is turning toward the functional analysis of whole genomes. The most straightforward strategy for the identification of gene functions relies on the inactivation of individual transcription units, by either random or site-directed mutagenesis. In *Drosophila*, an estimated 25% of all vital genes have been mutated to date (1, 2). Mutagenesis efforts have been based largely on chemically induced random mutagenesis by using ethane methyl sulfonate (3) or on transposon-mediated insertional mutagenesis by using *P* elements (4, 5). Unfortunately, both of these methods have limitations, which make the mutation of all genes in the *Drosophila* genome difficult to attain.

Mapping of chemically induced mutations at the DNA level is labor intensive and time consuming and therefore not practical on a genome-wide basis. In contrast, *P* element-induced mutations can easily and quickly be identified on a large scale. However, the tendency of *P* elements to integrate preferentially into certain hotspots (1) makes saturation mutagenesis difficult, if not impossible. Moreover, genetic screens based on the isolation of zygotic lethal mutations allow only the detection of the earliest function of a given gene. The analysis of later gene functions requires the generation of mosaic clones of homozygous mutant tissue in an otherwise phenotypically wild-type (heterozygous) background. Such mosaics are generated by

using the site-specific recombination system based on the yeast FLP recombinase (FLP) and its recombination target sequences (*FRTs*) (6). Because genome-integrated *FRTs* are contained in *P* elements, they would be mobilized and lost during *P* element-mediated mutagenesis. Chromosomes carrying *FRTs* can therefore not be used in *P* element-based screens. Instead, preexisting *P* element insertions must be recombined with *FRT* containing chromosomes by meiotic recombination to make them accessible to clonal analysis, which is time consuming and labor intensive.

To overcome these limitations and combine the advantages of transposon-based mutagenesis with direct access to the FLP/*FRT* system [and other *P* element-based genetic tools like the UAS/Gal4 system (7)], we have adapted a non-*P* element-based germ-line transformation system (8, 9) to allow insertional mutagenesis in *Drosophila*. The system is based on the use of *Hermes* and *piggyBac* transposable elements (10, 11) that carry different spectral variants of the GFP as dominant transformation markers (8, 9, 12). In contrast to *P* elements, GFP-marked *Hermes* and *piggyBac* transposons are *Drosophila* nonspecific and are therefore expected to be applicable in a broad spectrum of nonmodel organisms (13).

Here, we show that a *piggyBac*-based system can be used to generate transposon insertions in the *Drosophila* genome with efficiency similar to *P* elements. *P* introduced *FRTs* present on the target chromosome remain stably integrated and can be used subsequently to induce FLP-mediated recombination. New *piggyBac* insertions are stable in the *Drosophila* genome but can be reverted when transposase is reintroduced. A Gal4 reporter gene in the mutator transposon can be used for misexpression studies and enhancer trapping (14–17). Analysis of insertion loci shows that *piggyBac* is less susceptible to hotspots and has an insertion preference significantly different from *P* elements. This *piggyBac*-based insertional mutagenesis system is therefore a valuable tool for a broad spectrum of genetic applications.

## Materials and Methods

**Genetics.** Fly strains were reared under standard laboratory conditions. *Drosophila* germ-line transformation with *piggyBac* and *Hermes* vectors was performed as described (9). Filter sets used for the identification of the different fluorescent transformation markers have been described (13). Embryonic enhancer traps were visualized by crossing mutator-carrying males to *UAS-lacZ*-carrying virgins and performing a 5-bromo-4-chloro-3-indolyl  $\beta$ -D-galactoside staining (14). Germ-line clones were generated by crossing mutator-carrying virgins to *P*{*hsFLP*}22, *y*[1] *w*[\*]; *P*{*ovoD1-18*}3L *P*{*FRT(w[hs])*}2A/*TM3* males for 3L or *P*{*hsFLP*}22, *y*[1] *w*[\*]; *P*{*neoFRT*}82B *P*{*ovoD1-18*}3R/

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Abbreviations: EYFP, enhanced yellow fluorescent protein; ECFP, enhanced cyan fluorescent protein.

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TM3 males for 3R (6). Heat shocks were performed at the third larval instar stage for 2 h at 37°C on 2 consecutive days.

**Molecular Biology.** Construction of *pBac*{*3xP3-EYFP*, *p-GAL4Δ-K10*}, and *pHer*{*3xP3-ECFP*, *atub-piggyBacK10*} is described in detail in ref. 18. DNA sequences flanking recessive lethal *piggyBac* transposon insertions were amplified by inverse PCR as described (19). In brief, five fly equivalents of genomic DNA were cleaved with *Hae*III and ligated. Flanking sequences were amplified by PCR [5 min, 95°C; 35× (30 s, 95°C; 1 min, 65°C; 2 min, 72°C); 7 min, 72°C] by using primers PLF (5'-CTTGACCTTGCCACAGAGACTATTAGAGG-3') and PLR (5'-CAGTGACACTTACCGCATTGACAAGCACGC-3') for the 5' junction and PRF (5'-CCTCGATATACAGACCGATAAACACATGC-3') and PRR (5'-AGTCAGTCAGAAA-CACTTTGGCACATATC-3') for the 3' junction. Amplified DNA fragments were directly sequenced by using primers PLR and PRF, respectively. Sequences were analyzed by using BLAST searches of the *Drosophila* Genome Database at [www.ncbi.nlm.nih.gov/BLAST](http://www.ncbi.nlm.nih.gov/BLAST).

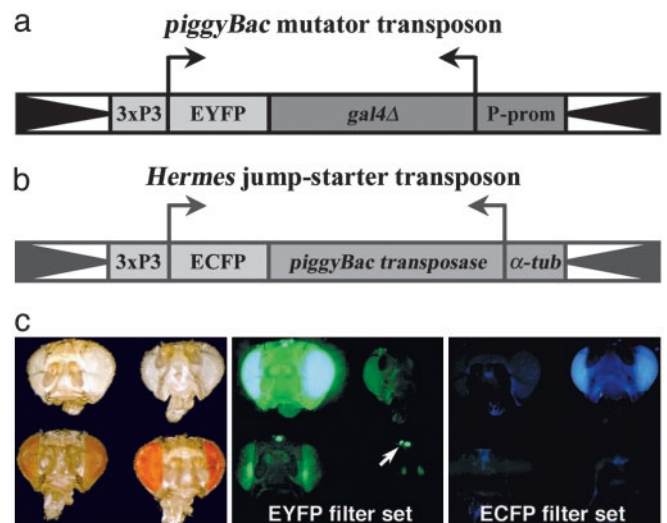
Insertion sites of viable *piggyBac* insertions were mapped to the genomic sequence by the Berkeley *Drosophila* Genome Project, funded by National Institutes of Health Grant P50 HG00750 to G. M. Rubin and by the Howard Hughes Medical Institute through its support of work in the laboratories of G. M. Rubin and A. C. Spradling. Sequences flanking insertion sites were amplified by inverse PCR by G. Tsang, M. Evans, and G. Davis in the laboratory of G. M. Rubin at the University of California, Berkeley. PCR products were sequenced by S. Park, K. Wan, and R. A. Hoskins at Lawrence Berkeley National Laboratory under Department of Energy Contract DE-AC0376SF00098, University of California. Mapping information was determined by using software written by G. Liao in the laboratory of G. M. Rubin, followed by manual curation by B. Levis and A. C. Spradling at the Carnegie Institution.

## Results

**Mutator and Jump-Starters Transposons.** To establish a transposon insertion system in *Drosophila* that can function independently of *P* element-based systems, we made use of the *Hermes* and *piggyBac* transposons, which had previously been shown to allow germ-line integration and transposition in *Drosophila* (20, 21). To be able to distinguish the *Hermes* and *piggyBac* transposons from each other and from *P* elements simultaneously present in the genome, we used the spectral variants of GFP, enhanced yellow fluorescent protein (EYFP) and enhanced cyan fluorescent protein (ECFP), as dominant markers (12). The mutator transposon (Fig. 1a) was marked with *3xP3-EYFP*, which is expressed under control of the endogenous *Pax6* gene of the fly in the larval light sensory organ, the adult compound eye and ocelli (Fig. 1c) (8), and parts of the nervous system (9). In addition, we included the heterologous transcriptional activator Gal4 to allow enhancer trapping (14–17).

Mobilization of the mutator transposon requires the expression of a stable *piggyBac* transposase source in the germ line. To avoid mobilization of the *piggyBac* transposase source itself, we integrated the *piggyBac* transposase coding region into a *Hermes* transposon marked with *3xP3-ECFP* (Fig. 1b). ECFP can be readily distinguished from the EYFP of the mutator transposon and allows selection against the jump-starter transposon in progeny flies carrying new transposon insertions (Fig. 1c). The *piggyBac* transposase was placed under control of the constitutively active *α1-tubulin* promoter (Fig. 1b) (22). The generation of *Drosophila* lines carrying these mutator and jump-starter constructs was described previously (18).

***piggyBac*-Mediated Mutagenesis in *Drosophila*.** The *piggyBac* transposon insertion system was tested in a pilot mutagenesis screen.



**Fig. 1.** (a) *piggyBac*-based mutator transposon marked with *3xP3-EYFP*. (b) *Hermes*-based jump-starter transposon marked with *3xP3-ECFP*. (c) Expression of *3xP3-EYFP* and *3xP3-ECFP* in unpigmented and pigmented eyes. (Left) Bright-field view of eyes expressing *3xP3-EYFP* (upper left) or *3xP3-ECFP* (upper right) in a *white<sup>-</sup>* background, and eyes expressing *3xP3-EYFP* in the presence of one (lower left) or two copies of a *white* transgene (lower right). (Center) Same as *Left* viewed through the EYFP filter set; *3xP3-EYFP* is detectable in *white<sup>-</sup>* eyes (upper left) or in eyes expressing one copy of a *white* transgene (lower left); in eyes expressing two copies of a *white* transgene (lower right), *3xP3-EYFP* is detectable in the ocelli (arrow) and the pseudopupil of the compound eye (not visible in frontal view); *3xP3-ECFP* is not detectable (upper right). (Right) Same as *Left* viewed through the ECFP filter set. *3xP3-EYFP* is not detected (left and lower right), but *3xP3-ECFP* is detected (upper right).

A *piggyBac* mutator transposon located on the X chromosome was mobilized from a *piggyBac* transposase source integrated on the second chromosome. Because one feature of the system was to function in the presence of *P* elements without interfering with their stability, we targeted third chromosomes carrying insertions of *FRT*-bearing *P* elements at the base of the left and right arms (6). A list of available stocks generated for this purpose is shown in Table 2, which is published as supporting information on the PNAS web site, [www.pnas.org](http://www.pnas.org), and the respective crossing scheme is given in Fig. 5, which is published as supporting information on the PNAS web site.

We established a total of 2,500 single dysgenic male crossings (SMCs), of which 1,741, corresponding to a jumping rate of 70%, yielded new autosomal *piggyBac* insertions. This rate depended on the number of progeny obtained from each SMC and climbed to >90% among vials, which produced 40 males or more. Therefore, the rate of recovery of *piggyBac* insertions is at least equal to that observed for *P* elements (23). A segregation analysis established that 798 (46%) new insertions were located on the third chromosome, suggesting that *piggyBac* insertions are distributed evenly between the autosomes. The data of the pilot screen are summarized in Table 1.

***piggyBac* and *P* Elements Have Distinct Insertion Preferences.** An inherent problem in *P* element-based mutagenesis screens is the existence of hotspots of integration (1). To investigate whether the *piggyBac* system can be used to mutagenize genes that are not good targets for *P* elements, we determined the genomic flanking sequences of 604 *piggyBac* insertions. Mapping of these insertions to the annotated *Drosophila* genome sequence showed that the insertions are distributed evenly throughout the third chromosome (Fig. 2a; Table 3, which is published as supporting information on the PNAS web site). Three hundred and four of

**Table 1. Statistical overview over *piggyBac* insertions on the third chromosome**

Category	n	%
Single male crosses	2,500	
Independent insertions recovered	1,741	70
Insertions on third chromosome	798	46
Insertions sequenced	604	
Repetitive	46	
Unique	558	
Intergenic insertions	254	
Insertions in transcripts	304	
Previously hit by <i>P</i> elements	132	43
No <i>P</i> element hit reported	172	57
Lethal insertions	131 (72)	16 (9)
Excised	39	
Reverted	22	56

Numbers in parentheses represent values corrected for insertions likely to be due to *piggyBac*-associated lethal mutations based on excision data.

the 604 insertions are located within putative transcripts. Of those transcripts, 83 were hit two times or more, accounting for 27% of the insertions. In similar *P* element-mediated screens, genes hit six times or more have been shown to account for 39% of insertions integrated into vital genes (1). Conversely, 72% of the *piggyBac* insertions were single hits compared with 29% for *P* elements. The maximum number of *piggyBac* insertions found in the same transcription unit or intergenic region was four, equaling  $\approx 1\%$  of insertions. By comparison, *P* element hotspots account for up to 7% of insertions in *P* element-mediated mutagenesis (1). These data strongly suggest that *piggyBac* is significantly less susceptible to hotspots of integration.

Of the 10 transcription units most frequently hit by *P* elements (1), seven were not hit at all in our *piggyBac* screen, two were hit twice, and one was hit once (Fig. 2a). Using the data available in FLYBASE (<http://flybase.bio.indiana.edu>), we also determined that no *P* element insertions have been reported to date for 172 (57%) of the 304 *piggyBac* insertions located in putative genes. These data suggest that, in addition to the lack of obvious hotspots, the insertion preference of *piggyBac* is significantly different from *P* elements.

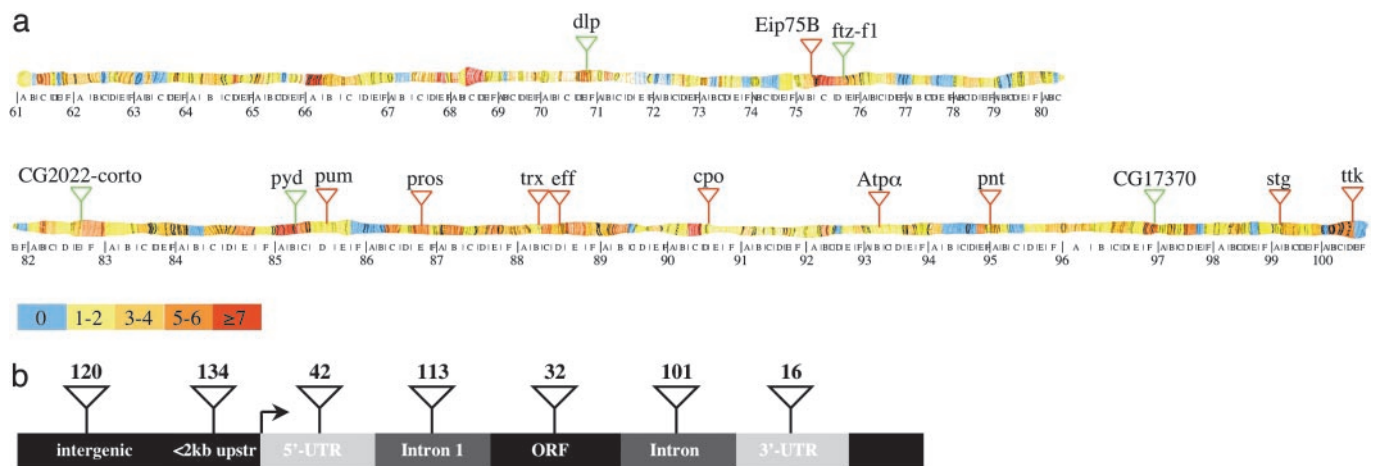
Forty-one (6.8%) of the 604 analyzed *piggyBac* transposons were found integrated into repetitive sequences and five inser-

tions mapped to “Arm U,” which is mainly heterochromatic and could therefore not be assigned to unique chromosomal locations. Of the 558 *piggyBac* insertions, which mapped to unique loci, 254 (45%) are in intergenic regions (Fig. 2b). Among the latter, 134 (24% of all insertions) are integrated within 2 kb of putative transcripts. Together with the insertions located inside putative genes (54%), 78% of all mapped insertions were in the vicinity of genes, indicating that *piggyBac* has a strong preference to integrate into or near transcribed sequences.

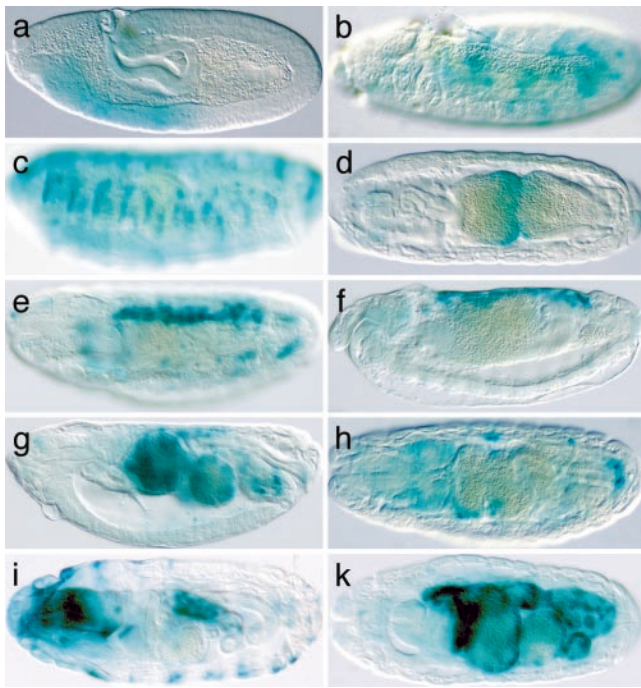
We also investigated the insertion preference of *piggyBac* within putative transcription units. Among the 304 *piggyBac* transposons located inside predicted transcripts, 42 (8%) were in the 5'-UTR, 32 (6%) were in putative ORFs, 214 (38%) were in introns, and 16 (3%) were in the 3'-UTR (Fig. 2b). One hundred thirteen insertions, corresponding to 53% of intronic insertions and 19% of all insertions, were found in the first intron of a given gene. These data show that *piggyBac* has a strong preference to insert into introns near the 5'-end of a transcription unit.

**Generation of Recessive Lethal Alleles.** The value of *piggyBac* as a mutagenesis tool depends significantly on its ability to introduce mutations efficiently. In 131 (16%) of the 798 third chromosomal insertions, the target chromosome had sustained a recessive lethal hit (see Table 1). We selected 39 of the lethal lines and reintroduced the jump-starter transposon to determine whether the transposons could be remobilized and whether the lethality was associated with the *piggyBac* insertion. Excision of the *piggyBac* transposon was observed in all assays, indicating that *piggyBac* excision is highly efficient. Among 10 parallel assays set up for each line, the result was always identical, suggesting that imprecise excisions of *piggyBac* are rare. However, excision of the *piggyBac* transposon reversed the lethality of the target chromosome only in 22 (56%) of the 39 tested lines. Consequently, the rate of lethal hits caused by *piggyBac* must be corrected to 9%, which is comparable to the rate obtained with *P* elements (2).

**Enhancer Trapping.** The *piggyBac* mutator transposon used contains the coding sequence of the heterologous transcriptional activator Gal4 to allow enhancer trapping (18). To confirm the Gal4 reporter gene function of the mutator transposon, we randomly selected 20 insertion lines from the lethal collection, crossed them to a *P{UAS-lacZ}* line, and performed 5-bromo-4-chloro-3-indolyl  $\beta$ -D-galactoside stainings on embryos.



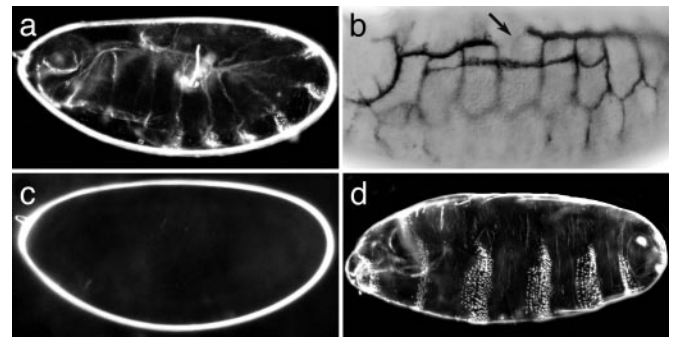
**Fig. 2.** (a) Distribution of lethal *piggyBac* insertions on chromosome 3. Color code represents number of *piggyBac* insertions isolated for each letter division of the third chromosome. Transposon insertions in red show the 10 genes most frequently hit by *P* elements (1). Transposon insertions in green show the genes most frequently hit by *piggyBac* in this screen. *dlp*, *ftz-f1*, and *CG17370* were hit three times each, and *pyd* and the intergenic region between *CG2022* and *corto* were hit four times. (b) Insertion preference of *piggyBac* within a schematic transcription unit.



**Fig. 3.** Enhancer trap expression patterns associated with *piggyBac* insertions. Shown are 5-bromo-4-chloro-3-indolyl  $\beta$ -D-galactoside stainings of embryos from *piggyBac* insertion lines crossed to *P{UAS-LacZ}*. (a) Line *l(3)PL00759* inserted in *hb*. (b and c) Line *l(3)PL00790* inserted in *bnl*. (d) Line *l(3)PL00757* inserted in *lab*. (e) Line *l(3)PL00779* inserted in *srp*. (f and g) Line *l(3)PL00799* inserted in *Mpk2*. (h) Line *l(3)PL00795* inserted in *CG10823*. (i) Line *l(3)PL00724* inserted in *CG10522*. (j) Line *l(3)PL00749* inserted in *CG12546*. Anterior is to the left, dorsal side up.

Tissue-specific *lacZ* expression was detected in 10 of the tested lines (Fig. 3) and was found associated with transposons inserted in introns, 5'-UTRs, and upstream of genes. Several lines reproduced expression patterns of previously described genes. The earliest *lacZ* expression was detectable at stage 9 in embryos produced by line *l(3)PL00759*. This insertion lies in the first intron of *hunchback* (*hb*) (24) (Fig. 3a) and detects the early anterior expression domain of *hb*. Line *l(3)PL00790* is inserted in the second intron of *branchless* (*bnl*) (25) and reproduces the epidermal expression of the *Drosophila* FGF homolog starting at stage 10 (Fig. 3b). Due to the stability of the Gal4 protein, the pattern remains visible until the end of embryogenesis (Fig. 3c). In *l(3)PL00757*, a *piggyBac* is found in the second intron of *labial* (*lab*) (26) and recapitulates the expression of this homeodomain protein in the second midgut constriction (Fig. 3d). In *l(3)PL00779*, the mutator transposon is inserted in the first intron of the GATA transcription factor gene *serpent* (*srp*) (27) and shows expression in the fat body and the amnioserosa (Fig. 3e). A common feature of all these expression patterns is a significant delay in the appearance of the *lacZ* expression compared with expression of the endogenous genes.

We also discovered tissue-specific enhancers of several previously uncharacterized genes. *l(3)PL00799* is inserted into the 5'-UTR of the *Mpk2* gene, which has been implicated in regulation of the *Drosophila* immune response by *in vitro* studies (28). The insertion in *l(3)PL00799* detects an enhancer driving expression in the amnioserosa (Fig. 3f) and later in the embryonic midgut (Fig. 3g). These patterns are strikingly reminiscent of *srp*, which is expressed in a similar pattern in the amnioserosa and has been shown to be essential for the development of the embryonic midgut (27). In addition, *srp* is also involved in mediating the *Drosophila* immune response (29), suggesting a possible link between *Mpk2* and *srp* during this process.



**Fig. 4.** Embryonic phenotypes associated with *piggyBac* insertions. (a) Cuticle phenotype of a homozygote of line *l(3)PL00779* inserted in *srp*. (b) Embryo homozygous for line *l(3)PL00790* inserted in *bnl* stained with 2A12 antibodies. (c) Cuticle phenotype of an embryo derived from a female carrying germ-line clones of line *l(3)PL00784* inserted in *14-3-3* crossed to a wild-type male. (d) Cuticle phenotype of embryo maternally and zygotically mutant for line *l(3)PL00739* inserted in *ftz-f1*.

*l(3)PL00795* is inserted in the first intron of *CG10823* (30), a putative G protein-coupled receptor encoding gene and shows enhancer trap expression in a small ring of cells surrounding the hindgut and in small groups of cells throughout the embryo (Fig. 3h). *l(3)PL00724* is inserted near the 5'-end of *CG10522* (31), encoding a putative serine-threonine kinase and shows *lacZ* expression in epidermal stripes and in the hindgut epithelium (Fig. 3i). *l(3)PL00749* is inserted just upstream of *CG12546* and shows strong *lacZ* expression throughout the midgut and hindgut at stage 16 of embryogenesis (Fig. 3k). These data confirm the function of *Bac{3xP3-EYFP, p-Gal4Δ-K10}* as a tool for enhancer trapping studies in *Drosophila*.

**Stability of P Elements in the Presence of *piggyBac* Transposase.** An important question is whether the *FRTs* present on the target chromosomes remain stably integrated during *piggyBac* mobilization. During the course of the screen, several strains carrying *FRT* bearing *P* elements (see Table 2) and *Bac{3xP3-EYFP, p-Gal4Δ-K10}* or *Her{3xP3-ECFP, atub-piggyBac-K10}* were kept for >50 generations. Spontaneous loss or mobilization of *P* elements or *piggyBac* was never observed. Furthermore, mobilization or loss of *P{FRT}* was not observed during the screen when *P* elements were combined with both *Bac{3xP3-EYFP, p-Gal4Δ-K10}* and *Her{3xP3-ECFP, atub-piggyBac-K10}* at the same time. *P* elements therefore appear to be insensitive to the presence of *piggyBac* transposase. In contrast, spontaneous loss of *Her{3xP3-ECFP, atub-piggyBac-K10}* was observed on several occasions, suggesting that *Hermes* transposons may not be stable in *Drosophila*. *Hermes* transposons have been reported to be unstable in the presence of *hobo* elements (32), which could explain this observation. This, however, poses no problem during *piggyBac* mutagenesis, because the transposase source is introduced only transiently during the screen.

***piggyBac*-Induced Zygotic Lethal Mutations with Maternal Effect Phenotypes.** To determine whether *piggyBac* insertions are associated with embryonic phenotypes, we performed cuticle preparations on 77 lethal insertion lines. Surprisingly, we found consistent phenotypes in only two lines. Homozygotes of *l(3)PL00779*, which is inserted in the first intron of *srp*, die with the u-shaped cuticle phenotype previously reported for *srp* mutants (Fig. 4a) (33). Immunohistochemical stainings using the trachea-specific antibody 2A12 confirmed that insertion *l(3)PL00790* frequently shows defects in tracheal development similar to *bnl* alleles (Fig. 4b). These data indicate that insertions of *Bac{3xP3-EYFP, p-Gal4Δ-K10}* do create recessive lethal

mutations associated with zygotic phenotypes even when inserted into introns. It may be noteworthy, however, that many *piggyBac* insertions in embryonic lethal genes appeared to create hypomorphic alleles. The insertions in *bnl* and *14-3-3ε* (34) are semilethal, and homozygotes of the insertions in the embryonic lethal genes *crumbs* (*crb*) (35), *lab*, and *hb* die only at the larval or pupal stages, respectively.

We then asked whether the *FRTs* can be used to remove a potential maternal contribution of the mutated genes by inducing germ-line clones for the left or the right arm of the third chromosome by using the dominant female sterile-flipase (DFS-FLP) technique (6). In this technique, all females are heterozygous for the *piggyBac* insertion and for the dominant sterile mutation *ovo<sup>D</sup>*, which aborts oogenesis at an early stage. Therefore, females can not lay eggs unless a *FLP*-mediated recombination event occurs at the *FRTs* and creates germ-line cells, which have lost the *ovo<sup>D</sup>* mutation. We tested a total of 30 lines and found that in each case, germ-line clone females that laid eggs could be produced for at least one arm of the third chromosome, showing that the *FRTs* on the target chromosome remain functional after *piggyBac* mutagenesis.

In several cases, germ-line clones of the chromosome arm bearing a zygotic lethal *piggyBac* insertion were associated with maternal effect phenotypes. Line *l(3)PL00784* has a *piggyBac* insertion in the first intron of the *14-3-3* gene. Germ-line clone females of this line produce embryos that die before they produce a larval cuticle and appear as “empty eggs” (Fig. 4c), as previously reported (36). Line *l(3)PL00739* has a *piggyBac* insertion in the third intron of the gene *ftz transcription factor 1* (*ftz-fl*) (37). *ftz-fl* encodes a steroid hormone receptor like protein, which is required as a cofactor for the pair-rule transcription factor *fushi tarazu* (*ftz*) (38, 39). Embryos homozygous for *l(3)PL00739* die at the end of embryogenesis without obvious cuticle defects (not shown). However, embryos maternally and zygotically mutant for *l(3)PL00739* develop with a pair-rule type segmentation defect (Fig. 4d). Several other insertions causing maternal effect phenotypes were obtained in noncharacterized genes whose detailed analysis will be given elsewhere. These examples confirm that the *FRTs* remained functional after *piggyBac* mutagenesis and that *piggyBac* insertions can cause mutations associated with maternal effect phenotypes.

## Discussion

In this study, we present a transposon mutagenesis system alternative to the existing *P* element-based system. We show that this *piggyBac*-based system functions efficiently to create new mutations in the presence of *P* elements and can therefore be applied with *P* element-based tools in the background. This feature allows the combination of insertional mutagenesis screens with UAS/Gal4 misexpression and FLP/*FRT* systems. Mutagenesis screens designed for mosaic tissue analysis or for the identification of genetic modifiers of misexpression phenotypes (40) are therefore no longer limited to chemical mutagens, which hamper the identification and characterization of the mutated genes. In addition, *piggyBac* is less susceptible to hotspots and has a significantly different insertion preference, which makes genes accessible to mutation that have not been hit by *P* elements.

**The Mutator and Jump-Starters Transposons.** *piggyBac* belongs to the TTAA-specific transposon family and was originally found in cell lines of the cabbage looper moth *Trichoplusia ni* (11). We chose *piggyBac* as a mutator transposon because no endogenous transposase sources are present in the *Drosophila* genome, and *piggyBac* insertions are stable (21). This was confirmed by our results, because we did not observe spontaneous mobility of *piggyBac* in the absence of a *piggyBac*-specific transposase source. Moreover, *P* element insertions were stable in the

presence of *piggyBac* transposase. *piggyBac* seems therefore well suited as a tool for transposon tagging in *Drosophila*. To facilitate detection of the mutator transposon, we used the highly sensitive transformation marker *3xP3-EYFP* (12). This marker allows easy distinction from the *3xP3-ECFP* marker used in the jump-starter transposon and also from the *white<sup>+</sup>* marker used in many *P* elements, which can be present in the genome at the time of *piggyBac* mobilization. One limitation of this marker is that it is difficult to detect in pigmented eyes. This problem can be remedied, however, by introducing a *cn bw* chromosome, which results in white eye color independent of the *white* locus, or the use of a *UASw-RNAi* transgene (41), which inactivates expression of *Drosophila white* genes.

The *piggyBac* mutator transposon is mobilized by a transposase source introduced into the genome via a *Hermes* transposon. The use of different transposons for mutator and transposase sources, which belong to different transposon superfamilies that cannot crossmobilize each other, makes it unnecessary to disable the transposon carrying the transposase source like in the case of the  $\Delta 2-3$  *P* element transposase source (5). We marked the *Hermes* transposon with the less-sensitive *3xP3-ECFP*, because it is not necessary to screen through large numbers of individuals for the transposase source. We also selected *Hermes* for the jump-starter transposon and not as a mutator, because *Hermes* has been reported to be unstable in the presence of *hobo* elements in the genome (32). We did, in fact, observe spontaneous loss of *Hermes* transposons from the jump-starter stock on several occasions. This, however, is not a significant problem during mutagenesis screens, because the transposase source is needed only transiently.

***piggyBac*-Mediated Mutagenesis on *FRT* Chromosomes.** In this screen, insertions were recovered from 70–90% of all single dysgenic male crossings. This rate is equal or superior to the rate usually obtained in *P* element-mediated mutagenesis (23) and shows that *piggyBac* mutator elements are easily remobilized and that the *piggyBac* transposase source is highly effective.

Analysis of the flanking sequences of the *piggyBac* insertions showed that the insertions are distributed evenly over the third chromosome. We did not observe any obvious hotspots of integration, and 57% of the insertions are in genes that have not been targeted by *P* elements. This result suggests that the insertion preference of *piggyBac* is significantly different from *P* elements and promises that *piggyBac* will be a valuable tool to increase the coverage of genes tagged by transposon insertions in the *Drosophila* genome.

We also find that 70% of the insertions hitting putative genes are in introns, clearly distinguishing *piggyBac* from *P* elements, which preferentially insert in 5'-UTRs (42). This observation could suggest that the rate at which *piggyBac* induces lethal mutations might be low, because many transposons may be removed from the pre-mRNA during splicing, and a functional transcript may be generated. However, we find lethal insertions at a rate similar to that observed for *P* elements (1, 2). It is possible that the efficiency of *piggyBac* transposons to inactivate genes could even be increased by using vectors carrying additional splice donor and acceptor sites that lead to aberrant splicing or by including strong transcriptional terminators that abort a transcript before it can be spliced. The preference to insert into introns can also be an advantage in certain instances: *piggyBac* could serve as an excellent tool for the generation of gene trap (43) or protein trap (44) insertions and could make these approaches significantly more efficient than they are with *P* elements.

Another difference between *piggyBac* and *P* elements appears to be the precision of excision. Despite extensive efforts, we could not find evidence for imprecise excisions when we tried to revert *piggyBac* insertions. This result may preclude the possi-

bility of converting nonlethal *piggyBac* insertions into lethal mutations unless special measures are taken, for example, by incorporating *P* element sequences into a *piggyBac* transposon. On the other hand, the precise excision mechanism may harbor advantages as well. *piggyBac* transposons leave no target site duplication (footprint) when they are excised (45) and, therefore, allow restoration of insertion loci to wild type even when the transposon is inserted in the ORF. In contrast to *P* elements, *piggyBac* is therefore less likely to cause secondary mutations by a hit-and-run mechanism, which can severely complicate the identification of mutations.

A major advantage is that *piggyBac* can be mobilized in the presence of *P* elements in the genome, which opens the possibility of combining existing *P* element-based tools with *piggyBac*-based mutagenesis screens. As we have shown here, *piggyBac*-mediated mutagenesis can easily be carried out to target *FRT*-bearing chromosomes, which makes the new insertions directly accessible to mosaic analyses using FLP/*FRT*-based recombination. This possibility avoids time- and labor-intensive recombination of preexisting *P* element insertions onto *FRT* chromosomes and facilitates easy identification of the mutated genes. Similarly, *piggyBac*-based mutagenesis could be combined with other *P* element-based tools like the UAS/Gal4 system. In a screen for new Gal4 insertions, like the one presented here, dysgenic males can be crossed directly to a UAS-GFP reporter, allowing the identification of enhancer traps

at the larval or adult stages in the F<sub>1</sub> generation (18). F<sub>1</sub> screens for genetic modifiers of a UAS/Gal4-generated overexpression phenotype could be done by *piggyBac* mutagenesis, allowing quick identification of the mutated loci. Similarly, once the repertoire of available *piggyBac* transposons has been expanded, misexpression screens using an EP-type (46) *piggyBac* transposon could be conducted, crossing dysgenic flies directly to a Gal4 reporter, allowing the identification of UAS-tagged insertions without the prior establishment of individual lines. We expect that the availability of the *piggyBac*-based alternative insertional mutagenesis system will open the field to a variety of new applications, while at the same time decreasing the time and effort involved in the successful identification of novel gene functions.

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