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Mutagenesis by imprecise excision of the *piggyBac* transposon in *Drosophila melanogaster*

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ABSTRACT

Mutagenesis by transposon-mediated imprecise excision is the most extensively used technique for mutagenesis in *Drosophila*. Although *P*-element is the most widely used transposon in *Drosophila* to generate deletion mutants, it is limited by the insertion coldspots in the genome where *P*-elements are rarely found. The *piggyBac* transposon was developed as an alternative mutagenic vector for mutagenesis of non-*P*-element targeted genes in *Drosophila* because the *piggyBac* transposon can more randomly integrate into the genome. Previous studies suggested that the *piggyBac* transposon always excises precisely from the insertion site without initiating a deletion or leaving behind an additional footprint. This unique characteristic of the *piggyBac* transposon facilitates reversible gene-transfer in several studies, such as the generation of induced pluripotent stem (iPS) cells from fibroblasts. However, it also raised a potential limitation of its utility in generating deletion mutants in *Drosophila*. In this study, we report multiple imprecise excisions of the *piggyBac* transposon at the *sepiapterin reductase* (*SR*) locus in *Drosophila*. Through imprecise excision of the *piggyBac* transposon inserted in the 5′-UTR of the *SR* gene, we generated a hypomorphic mutant allele of the *SR* gene which showed markedly decreased levels of *SR* expression. Our finding suggests that it is possible to generate deletion mutants by *piggyBac* transposon-mediated imprecise excision in *Drosophila*. However, it also suggests a limitation of *piggyBac* transposon-mediated reversible gene transfer for the generation of induced pluripotent stem (iPS) cells.

1. Introduction

Transposons are mobile DNA segments that insert in and excise from the genome through the action of transposase. Transposons can escape from the genome with or without genomic alteration at the original insertion site, termed imprecise and precise excision, respectively. In imprecise excision, transposons usually generate a genomic deletion by excising a local genomic region near the original insertion [1,2]. Transposon-mediated imprecise excision is the most extensively used technique for mutagenesis in *Drosophila* [3]. Among a variety of transposable elements, the *P*-element is the most popular vehicle to disrupt genes in *Drosophila* because its excision is imprecise at high rates and is completely dependent on *P*-transposase, which allows the control of its excision [4,5]. However, genomic coldspots, where *P*-elements are rarely inserted, limit its utility [6]. To overcome this limitation of *P*-elements, the *piggyBac* transposable element was developed as an alternative. The *piggyBac* transposon is more randomly integrated into the *Drosophila* genome and inserts successfully even in the coldspots of *P*-element integration [7–9]. Exelixis Inc. (San Francisco, CA) has generated genome-wide *piggyBac* transposon-inserted *Drosophila* libraries, which are available through Harvard Medical School [9].

The *piggyBac* transposon was first discovered in the cabbage looper moth, *Trichoplusia ni* [10]. It is a Class II DNA transposable element, which includes the *P*, *hobo* and *mariner* elements [11]. The *piggyBac* transposon is 2472 bp flanked by 13-bp inverted terminal repeats [10,12,13]. It integrates into the genome at the tetranucleotide TTAA site, which is duplicated upon insertion [14]. In contrast to *P*-elements, the *piggyBac* transposon is also functional in mammalian cells and mice [15]. Despite these attractive advantages, the *piggyBac* transposon is not widely used for gene disruption because it was thought that the *piggyBac* transposon always excised precisely by the *piggyBac*-specific transposase and generated no local deletions [11,12,16]. This feature limits the use of the *piggyBac* transposon in generating deletion mutants by imprecise excision [5]. Consequently, only the flies carrying a *piggyBac*

Abbreviations: *SR*, *sepiapterin reductase*; iPS cells, induced pluripotent stem cells; UTR, untranslated region; BL, Bloomington; REV, revertant.

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transposon insertion that directly disrupts a gene or alters specific gene expression have been used as mutant alleles [3].

In addition to the utility of the *piggyBac* transposon as a mutagenic vector in *Drosophila*, the *piggyBac* transposon has recently been used as a gene delivery vector. The *piggyBac* transposon was demonstrated to transfer exogenous genes successfully into human and mouse cell lines as well as into mice [15,17]. Moreover, the unique features of the *piggyBac* transposon enable reversible gene transfer through precise excision of the transposon after gene delivery, leaving no transgene and no genomic alteration at the original integration locus [18,19]. Due to these advantages of the *piggyBac* vector compared with viral vectors, the *piggyBac* transposon has been recently utilized to establish transgene-free induced pluripotent stem (iPS) cells [20,21]. Fibroblasts were successfully reprogrammed to iPS cells by *piggyBac* transposon-mediated introduction of four key genes (*c-Myc*, *Klf4*, *Oct4* and *Sox2*) that were clearly removed from the genome without genomic alteration after pluripotent cells were established [20].

In this study, we report imprecise excisions of *piggyBac* transposons in *Drosophila melanogaster* and the generation of *sepiapterin reductase* (*SR*, EC1.1.1.153) mutants by *piggyBac* transposon-mediated imprecise excision.

2. Materials and methods

2.1. Drosophila strains

All stocks were maintained and raised on standard fly food at 25 °C unless otherwise specified. The following fly stocks were obtained from Bloomington Stock Center: $SR^{piggyBac}$ (No. 18753); *CyO,PBAC\T* (No. 8285); *FM6* (No. 4327). W^{1118} was used as a wild-type control strain.

2.2. Southern blot analysis

Genomic DNA was extracted from 1-day-old male flies using G-spin™ (iNtRON Biotechnology, Korea). Genomic DNA (40 μg) was digested with *Hin*dIII (Fermentas), separated by 1% agarose gel, and transferred onto Nytran SuPerCharge membrane (Schleicher and Schuell). Hybridization was performed in ExpressHyb hybridization solution (Clontech) with [α-^{32}P]dCTP-labeled DNA probes specific for the *piggyBac* transposon. The *piggyBac* transposon fragment was amplified using *piggyBac* transposon-specific primers (forward, 5′-GTCTGCGTAAAATTGACGCATGCATTC-3′ and reverse, 5′-CTACCGCTTGACGTTGGCTGCAC-3′) from *BL18753* genomic DNA. Radioactive probes were prepared using Megaprime DNA labeling systems (GE Healthcare). Radioactive signals were visualized using a BAS-2500 Bio-image analyzer (Fujifilm).

2.3. Sequence analysis of piggyBac transposon integration loci

Genomic DNA of *BL18753* was prepared using DNAzol (Invitrogen). Genomic PCR was performed with specific primer sets. For the *piggyBac* transposon 5′ end, 5′-TTGGCTGTCTTTTTGCCTTAACTCG CTTTG-3′ and 5′-TCCAAGCGGCGACTGAGATG-3′ primers were used. For the *piggyBac* transposon 3′ end, 5′-CCTCGATATACAGACCGA-TAAAAC-3′ and 5′-GCTGACCTTGACATTGACATTCGATCACACA-3′ primers were used [9]. PCR products were separated by 1% agarose gel, extracted from the agarose gel, and sequenced to confirm the exact insertion site.

2.4. Induction of piggyBac transposon excision in Drosophila

piggyBac transposon excision was induced in *Drosophila* by following the cross scheme under 25 °C culture conditions (Fig. 2).

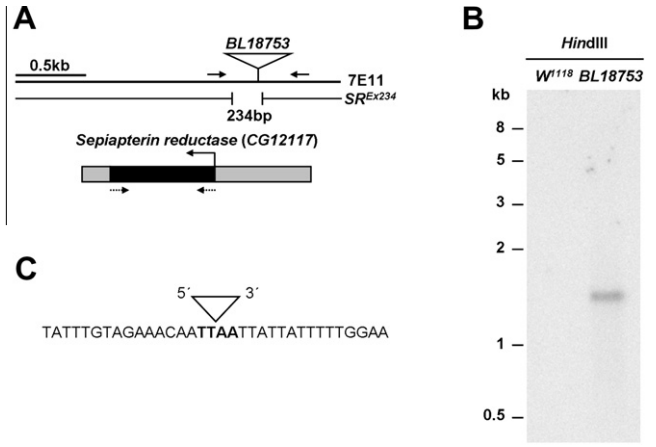

Fig. 1. Characterization of *BL18753*, a fly line carrying the *piggyBac* transposon insertion at the *sepiapterin reductase* locus. (A) Schematic representation of the *sepiapterin reductase* genomic locus. The original *piggyBac* transposon insertion site is indicated by a triangle above the map. SR^{Ex234} was obtained by *piggyBac* transposon-mediated imprecise excision, and the excised region is represented by vertical lines. (B) Southern blot analysis of W^{1118} and *BL18753* hybridized with radioactive DNA probes specific for *piggyBac* transposon. (C) Genomic sequence of *piggyBac* transposon insertion locus in *BL18753*. A triangle indicates the *piggyBac* transposon insertion. Bold letters (TTAA) designate the insertion site of the *piggyBac* transposon.

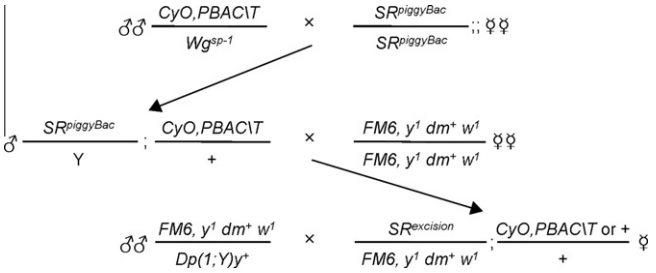

Fig. 2. Cross scheme used to induce *piggyBac* transposon excision at the *SR* locus in *Drosophila*. ♂, a male; ♀, a virgin female; ♂♂, males; ♀♀, virgin females.

Male flies carrying *piggyBac*-specific transposase (*BL8285*) were mated to virgin flies carrying the *piggyBac* transposon insertion at the *SR* locus (*BL18753*) in bottles. Male progenies carrying both the *piggyBac* transposon insertion and *piggyBac*-specific transposase were selected by a phenotypic selection marker and mated to *FM6* first balancer (*BL4327*) virgin flies in vials. A total of 208 matings were established, each with one male and five virgins. From the cross, *piggyBac* transposon excised virgins were collected and maintained by mating to *FM6* first balancer male flies. To test the effect of culture temperature on *piggyBac* transposon-mediated imprecise excision, the same cross scheme was also performed at 29 °C. A total of 193 matings were established.

2.5. Analyses of piggyBac transposon excision type at sepiapterin reductase locus

Fly genomic DNA was prepared using DNAzol (Invitrogen). PCR was performed with the forward primer sequence 5′-TTGGCTGTCT TTTTGCCTTAACTCGCTTTG-3′ and the reverse primer sequence 5′-GCTGACCTTGACATTGACATTCGATCACACA-3′, to obtain a 542-bp product using wild-type genomic DNA as the template. Used primers are indicated by solid arrows in Fig. 1A. PCR products were analyzed on 2.5% agarose gel. Shifted bands compared with the control band were extracted from agarose gel and sequenced to verify the exact

deletion region. Sequencing was performed using a 3730 DNA analyzer (Applied Biosystems) at the Genome Research Facility in Seoul National University.

2.6. RT-PCR

For RT-PCR, total RNA was isolated using easy-spin™ (iNtRON Biotechnology, Korea) and reverse transcribed using M-MLV reverse transcriptase (Promega) and oligo-dT (18mer) primers. The resulting cDNA was amplified by PCR with specific primer sets. For *sepiapterin reductase*, 5′-ATGGACCTGAAACAGCGCACATA-3′ and 5′-CTAGAACTGCTCATCCCTGTAAT-3′ primers were used to obtain a 786-bp product. Used primers are indicated by dashed arrows in Fig. 1A. For the *GAPDH* control, 5′-GTCAACGATCCCTTCATCGA-3′ and 5′-TGTACGATAGTTTTGGCTAG-3′ primers were used [22].

2.7. Real-time RT-PCR

cDNA was prepared by the same method described in the RT-PCR section. Real-time RT-PCR was conducted using *sepiapterin reductase* specific primers with the forward sequence 5′-AACGGATCAAAGCCGAGGGTTCC-3′ and the reverse sequence 5′-TTTCCACCGGATGCCTCCAGAAT-3′. The *rp49* control was amplified with the forward primer 5′-AGATCGTGAAGAAGCGCACCAAG-3′ and the reverse primer 5′-CACCAGGAACTTCTTGAATCCGG-3′ [23]. The values were normalized to *rp49*. The results are expressed as arbitrary units. *SR* gene expression in W^{1118} was taken arbitrarily as 10.

3. Results

3.1. Characterization of BL18753, a fly line with piggyBac transposon insertion at the sepiapterin reductase locus

The *D. melanogaster* ortholog of the human *sepiapterin reductase* is encoded by *CG12117* and was named *SR* [24]. *Sepiapterin reductase* is a key enzyme in the biosynthesis of tetrahydrobiopterin, an essential cofactor required for the synthesis of catecholamine neurotransmitters [25]. The *SR* gene is located at 7E11 on the X chromosome in *Drosophila* (Fig. 1A). The *BL18753* fly line carrying a *piggyBac* transposon insertion at the *SR* locus was obtained from the Bloomington Stock Center. The *piggyBac* transposon is the only transposable element available at the *SR* locus. To determine the number of *piggyBac* transposon integrations in *BL18753*, we performed Southern blot analysis. We confirmed that *BL18753* carries only one *piggyBac* transposon integration in the genome (Fig. 1B). Next, we examined the exact *piggyBac* transposon insertion site and flanking sequence. The *piggyBac* transposon integration locus was sequenced by PCR product sequencing. This revealed the *piggyBac* transposon insertion at a TTAA tetranucleotide in the 5′-UTR of the *sepiapterin reductase* gene (Fig. 1A and C), which is consistent with the fly line information provided from the Bloomington Stock Center. No further mutation was observed near the integration locus (Fig. 1C).

3.2. Induction of piggyBac transposon excision in Drosophila

To induce *piggyBac* transposon excision in *Drosophila*, we designed a cross scheme (Fig. 2) to obtain male flies carrying both the *piggyBac* transposon insertion and the *piggyBac*-specific transposase. *piggyBac* transposon excision occurs in the germlines of these flies that constitutively express a *piggyBac*-specific transposase under the control of the *αTub84B* promoter. The excision of the *piggyBac* transposon is easily detected in the next generation by loss of eye color, a selection marker of the *piggyBac* transposon. In total, we obtained 131 *piggyBac* transposon excised lines from 208 independent male germlines at 25 °C and 74 *piggyBac* transposon excised lines from 193 independent male germlines at 29 °C (data not shown).

3.3. Identification of piggyBac transposon-mediated imprecise excision

To determine whether the excisions occurred precisely or imprecisely, we conducted genomic PCR, as described in the Materials and methods section. We identified three imprecise excisions (SR^{Ex17a}, SR^{Ex135} and SR^{Ex198}) and 66 precise excisions (data not shown) from the 25 °C culture condition (Fig. 3A). We also identified three imprecise excisions (SR^{Ex17b}, SR^{Ex22} and SR^{Ex41}) and 43 precise excisions (data not shown) from the 29 °C culture condition (Fig. 3B). The percentage of imprecise excision to total excision was 4.3% at 25 °C and 6.5% at 29 °C. The SR^{Ex234} line from 29 °C culture condition was excluded from statistical analysis because it was obtained from an experiment without statistical control. Multiple precise excisions and identical imprecise excisions obtained from the same male germline were counted as one because one gonialblast develops into 64 spermatids during *Drosophila* spermatogenesis [26]. We then determined genomic lesions by sequence analyses of the genomic PCR products. Sizes of deletions varied from 17 to 234 bp (Tables 1 and 2). Among seven imprecise excision alleles, six alleles displayed bidirectional deletions from their insertion site, and one allele displayed an unidirectional deletion (Tables 1 and 2). Intriguingly, two alleles gained random footprints after excision. SR^{Ex17a} has a single nucleotide change (A to T) at the 4th nucleotide position downstream from the breakpoint (Table 1), and SR^{Ex234} has a new TAT trinucleotide sequence at the breakpoint (Table 2).

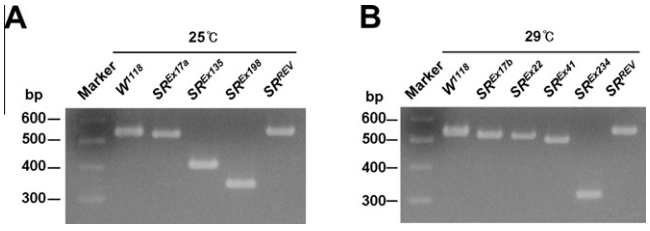

Fig. 3. *piggyBac* transposon-mediated imprecise excisions at the *SR* locus in *Drosophila*. (A and B) Genomic PCR analyses of imprecise excisions were carried out under 25 and 29 °C culture conditions, respectively. W^{1118} and SR^{REV} were used as controls. REV, revertant. Superscript numbers on *SR* excision lines indicate size of deletion (bp) in each line. 100-bp DNA ladder (Bioneer) was used as the size marker.

Table 1
Analysis of alleles created by *piggyBac* transposon-mediated imprecise excision under 25 °C culture conditions.

Allele	Size of deletion (bp)	Direction of deletion	Footprint
SR^{Ex17a}	17	Bidirectional	*
SR^{Ex135}	135	Unidirectional	None
SR^{Ex198}	198	Bidirectional	None

* Single nucleotide change (A to T) at 4th nucleotide position downstream from the breakpoint.

Table 2
Analysis of alleles created by *piggyBac* transposon-mediated imprecise excision under 29 °C culture conditions.

Allele	Size of deletion (bp)	Direction of deletion	Footprint
SR^{Ex17b}	17	Bidirectional	None
SR^{Ex22}	22	Bidirectional	None
SR^{Ex41}	41	Bidirectional	None
SR^{Ex234}	234	Bidirectional	#

Gain of new TAT trinucleotide sequence at the breakpoint.

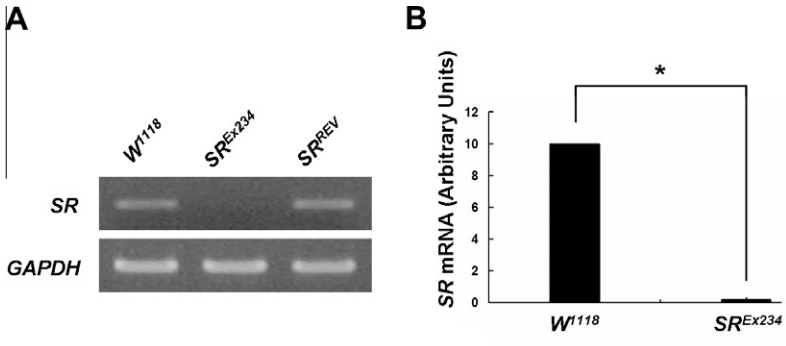

Fig. 4. Characterization of the SR^{Ex234} mutant generated by imprecise excision of the *piggyBac* transposon. (A) *SR* mRNA expression analysis by RT-PCR in 3-day-old male flies. *GAPDH* was used as a loading control. (B) The expression level of *SR* in 3-day-old male flies was measured by real-time RT-PCR analysis. mRNA levels are expressed by arbitrary units. Error bar indicates the standard deviation (S.D.) of three independent experiments. Asterisk indicates significance by Student's *t*-test ($^*p < 0.0001$).

3.4. Characterization of the SR^{Ex234} mutant

We characterized the SR^{Ex234} mutant, the allele carrying the longest (234-bp) deletion at the *SR* locus among the seven genome-deleted lines. The genomic lesion in the SR^{Ex234} mutant disrupts the 5′-UTR of the *SR* gene but not the coding sequence of the *SR* gene (Fig. 1A). To characterize the SR^{Ex234} mutant, we examined the transcript level of *SR* by RT-PCR. In the SR^{Ex234} mutant, the transcript level of *SR* was markedly reduced (Fig. 4A), suggesting that SR^{Ex234} is a strong hypomorphic mutant of *SR*. We also confirmed the expression of *SR* mRNA by real-time RT-PCR. Consistent with RT-PCR data, the expression of *SR* mRNA was significantly reduced in SR^{Ex234} (Fig. 4B).

4. Discussion

Mutagenesis by *P*-element-mediated imprecise excision has contributed greatly to elucidate the function of genes in *Drosophila* [2,3]. However, *P*-elements have a critical limitation because of their insertion biases in the genome [6]. Newly developed insertional mutagenesis by the *piggyBac* transposon allows us to study genes in the coldspots of *P*-elements due to its random insertion into the genome [9]. Previous studies suggested that the *piggyBac* transposon always excises precisely from the genome without deleting the genomic region around the original insertion site [11,12,16]. In this study, we clearly demonstrate that the *piggyBac* transposon induces imprecise excision at the *sepiapterin reductase* (*SR*) locus in *Drosophila*. The deletion size of *piggyBac* transposon-mediated imprecise excision is much smaller than that of *P*-elements, which ranges from a few base pairs to several kilobases [1]. Nevertheless, *piggyBac* transposable elements still have an advantage in generating deletion mutants of the genes that are located in genomic regions where *P*-elements are rarely found. The frequency of imprecise excision by the *piggyBac* transposon at the *SR* locus (4.3% at 25 °C and 6.5% at 29 °C, respectively) is higher than by *P*-elements on average (approximately 1%) [5].

Sepiapterin reductase is a key enzyme in the biosynthesis of tetrahydrobiopterin, an essential coenzyme required for the synthesis of important biogenic amines, including catecholamines and serotonin [25]. In an effort to establish tetrahydrobiopterin-deficient fly models, we successfully generated *SR* mutants by *piggyBac* transposon-mediated imprecise excision. However, it remains to be determined whether *piggyBac* transposon-mediated imprecise excision generally occurs at other loci in *Drosophila*. Although other groups used the same transgenic fly line that constitutively expresses the *piggyBac*-specific transposase under the *αTub84B* promoter, they failed to obtain imprecise excision of the *piggyBac* transposon [9,16]. We believe that it was due to the difference of integration loci of the *piggyBac* transposon that may affect the frequency of *piggyBac* transposon-mediated imprecise excision.

The *piggyBac* transposon has been recently utilized to establish transgene-free iPS cells [20,21]. Four key genes (*c-Myc*, *Klf4*, *Oct4* and *Sox2*) introduced by the *piggyBac* transposon were clearly removed from the genome without any genomic alteration after fibroblasts had been successfully reprogrammed to iPS cells [20]. Our findings raise the limitation of the use of the *piggyBac* transposon for reversible gene delivery because of the possibility of imprecise excision and thereby, unexpected mutations in the reprogrammed iPS cells. Therefore, the reversible gene transfer strategy using the *piggyBac* transposon should be more carefully monitored.

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