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Insertional mutagenesis of the mouse germline with *Sleeping Beauty* transposition

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ABSTRACT | Efficient linking of primary DNA sequence information to gene functions in vertebrate models requires that genetic modifications and their effects are analyzed in an efficacious, controlled, and scalable manner. Thus, to facilitate analysis of gene function, new genetic tools and strategies are currently under development. Transposable elements, by virtue of their inherent ability to insert into DNA, can be developed into useful tools for chromosomal manipulations. Mutagenesis screens based on transposable elements have numerous advantages as they can be applied in vivo and are therefore phenotype-driven, and molecular analysis of the mutations is straightforward. Current progress in this field indicates that transposable elements will serve as indispensable tools in the genetic toolkit of vertebrate models. Here, we provide experimental protocols for the construction, functional testing, and application of the *Sleeping Beauty* transposon for insertional mutagenesis of the mouse germline.

KEYWORDS | Functional Genomics ; Gene Trap ; Insertional Mutagenesis ; Poly A-Trap ; Transgenesis ; Transposon.

1. Introduction

DNA transposons are natural, nonviral "vehicles" that are able to move a defined DNA segment from one genetic location to another. Transposons have been successfully used in lower metazoan model species and in plants for transgenesis and insertional mutagenesis, but until the reactivation of the *Sleeping Beauty* (SB) transposon system in 1997 [1], there was no indication of DNA-based transposons sufficiently active for these purposes in vertebrates. SB exhibits high transpositional activity in a variety of vertebrate-cultured cell lines [2], embryonic stem cells [3], and in both somatic [4] and germline [5-9] cells of the mouse and rat [10] in vivo. Therefore, SB is a valuable tool for functional genomics in several model organisms (reviewed in [11]).

SB transposes through a conservative, cut-and-paste mechanism, during which the transposable element is excised from its original location by the transposase, and is integrated into a new location (Fig.1A). SB represents a two-component gene transfer vector system consisting of a transposase protein and a gene-of-interest (GOI) cloned between the terminal inverted repeats (IRs) containing binding sites for the transposase (Fig.1B). This enables the generation of transgenic stocks, each containing a separate component of the binary transposon system in its genome: one component, encoding the transposase, is carried by the "jumpstarter" strain, which on intercrossing, efficiently mobilizes the second component, a nonautonomous transposon in the genome of the "mutator" strain (Fig.2) [12]. Most transposon-based experimental strategies in vertebrates have been utilizing this two-component, binary approach in which transposition is controlled by *trans*-supplementation of the transposase. This experimental setup is especially useful for directing transposition events to particular tissues or organs by tissue-specific promoters driving transposase expression. Importantly, once integrated, transposase-deficient nonautonomous transposons are stable in the absence of the transposase.

Insertional mutagenesis using engineered transposable elements can be one of the most productive and versatile approaches toward disrupting and manipulating genes on a genome-wide scale. Transposon insertion into a gene can itself be mutagenic, if the insertion disrupts the transcriptional regulatory or coding region of a gene. However, most intronic insertions are not expected to be mutagenic (Fig.3A). Thus, several features to enhance the mutagenicity as well to add reporting capabilities of insertional vectors by trapping transcription units were developed; these are summarized in Fig.3. Gene trapping (often referred to as promoter or exon trapping) is based on the activation of a promoter-less reporter gene whose expression is dependent on splicing between the exons of the trapped gene and a splice acceptor (SA) site carried by the transposon (Fig.3B). Thus, gene trap vectors both report the insertion of the transposon into an expressed gene, and have a mutagenic effect by truncating the transcript through imposed splicing.

More sophisticated vectors have been developed that contain a polyadenylation (polyA) trap cassette that reports insertion into a Pol II transcription unit (Fig.3C). The marker gene lacks a polyA signal (pA), but contains a splice donor (SD) site. Thus, when integrating into an intron, a fusion transcript can be synthesized consisting the marker and the downstream exons of the trapped gene. Because polyA trap cassettes have their own promoters, they can report the insertion into genes irrespective of their expression status in a given cell type. The gene trap and polyA trap cassettes can be combined. Such dual tagging systems (Fig.3D) allow the isolation of both upstream and downstream fusion transcripts of the trapped gene, and have been used in the mouse [13,14].

The mutagenicity of gene trap vectors is higher than that of simple insertional vectors, and they enable easy identification of the hit gene by reverse transcriptase-polymerase chain reaction (PCR) targeting the composite transcripts made up by sequences of the insertional vector and the endogenous gene. In cell culture, drug resistance markers are generally in use whereas in animal systems reporters offer the possibility to visualize spatial and temporal expression patterns of the mutated genes by using *LacZ* or fluorescent proteins (Fig.3). SB can be equipped with gene trap cassettes [8,9,15], which significantly enhances its utility as a tool for functional genomics in vertebrate models. Furthermore, similar to the GAL4 transcriptional factor and its upstream activating sequences system in *Drosophila*, a conditional, tetracycline-regulated system has been shown to be applicable to transposon-mediated insertional mutagenesis in mice [16].

SB has been successfully used for forward genetics approaches in the mouse. Double transgenic mouse lines were generated bearing chromosomally present transposons and either an ubiquitously [6-9] or male germline-specifically [5] expressed transposase gene. Segregating the transposition events by mating the founder males to wild-type females revealed that up to 90% of the progeny can carry transposon insertions [7], and a single sperm of a founder can contain, on average, two insertion events [6]. The germline of such a founder was estimated to harbor approximately 10,000 different mutations [8]. Importantly, transposition of gene trap transposons identified mouse genes with ubiquitous and tissue-specific expression patterns, and mutant/lethal phenotypes were easily obtained by generating homozygous animals [8,9,17].

New transposon insertions tend to cluster around the original transposon donor locus, a phenomenon termed "local hopping." Keng et al. [18] took advantage of SB's local hopping behavior to provide proof-of-concept that transposon technology can be utilized to mutagenize mouse genes at a saturation level within a certain chromosomal interval.

The major advantage of transposon-mediated insertional mutagenesis in the mouse lies in the ability to generate and maintain whole libraries of insertional mutants in vivo, in the testes of founder animals. The phenotypic effects of these mutations can then be easily analyzed by simple breeding of the founders. In order to take full advantage of local hopping for saturation mutagenesis, libraries of transposon donors in chromosomal regions of interest (e.g., quantitative trait loci or syntenic regions of certain disease loci where genes of interest are located in clusters) can be generated.

2. Materials

2.1. Tissue Culture

1. Dulbecco's phosphate-buffered saline (PBS) (1X) without Mg^+ and Ca^+ (PAA Laboratories GmbH, Cölbe, Germany).
2. Dulbecco's modified Eagle's medium + GlutaMax (+4.5 g/L D-glucose; + pyruvate) (Gibco [Invitrogen Corporation, Carlsbad, CA, USA]).
3. JetPEI RGD transfection reagent (Biomol [BIOMOL GmbH, Hamburg, Germany]).
4. Antibiotic-antimycotic solution (100X) (Gibco).

2.2. PCR Assay for Transposon Excision in Cell Culture

1. Primer pUC1 5'-CAG TAA GAG AAT TAT GCA GTG CTG CC.
2. Primer pUC2 5'-GCG AAA GGG GGA TGT GCT GCA AGG.
3. Primer pUC3 5'-CGA TTA AGT TGG GTA ACG CCA GGG.
4. Primer pUC4 5'-CAG CTG GCA CGA CAG GTT TCC CG.
5. Primer pUC5 5'-TCT TTC CTG CGT TAT CCC CTG ATT C.
6. Primer pUC6 5'-CCA TTC GCC ATT CAG GCT GCG CAA C.
7. *Taq* polymerase (InviTek, Berlin, Germany).

2.3. PCR Assay for Transposon Excision In Vivo

1. HotStarTaq DNA polymerase (Qiagen GmbH, Hilden, Germany).
2. GeneAmp PCR system 9700 (PE Applied Biosystem, Forster City, CA USA).

2.4. Detection of poly(A) Trapped Events in Mice

1. Fluorescence microscope (WILD M10; Leica Geosystems AG, St. Gallen, Switzerland).

2.5. Detection of Promoter-Trapped Events in Mice

1. 4-Chloro-5-bromo-3-indolyl-β-D-galactopyranoside (X-Gal) (Nakalai tesque, Kyoto, Japan) is dissolved at 40 mg/mL in dimethyl sulfoxide and stored at −30°C.
2. 25% of Glutaraldehyde solution (Nakalai tesque) stored at 4°C.
3. 4% of Paraformaldehyde solution is dissolved in 0.001 *N* of NaOH.
4. 10% of Nonidet P-40 (NP-40) dissolved in H_2O.

2.6. Ligation-Mediated PCR (LM-PCR) to Determine Integration Sites of the SB Transposon

1. HotStarTaq DNA polymerase (Qiagen).
2. GeneAmp PCR system 9700 (PE Applied Biosystem).
3. Splinkerette linkers:
    a. Spl-top (molecular weight 19361.8) 5′-CGA ATC GTA ACC GTT CGT ACG AGA ATT CGT ACG AGA ATC GCT GTC CTC TCC AAC GAG CCA AGG.
    b. SplB-Sau (molecular weight 9154.1) 5′-GAT CCC TTG GCT CGT TTT TTT TTG CAA AAA.
    c. SplB-BLT (molecular weight 7918.2) 5′-CCT TGG CTC GTT TTT TTT TGC AAA AA.
    d. Linker for blunt end: 10.6 µg of Spl-top and 4.4 µg of SplB-BLT are combined in 50 µL of a solution containing 10 m*M* of Tris-HCl pH 7.5 and 5 m*M* of $MgCl_2$. The mixture is soaked in 95°C followed by gradual cooling to room temperature.
    e. Linker for cohesive end: 10.2 µg of Spl-top and 4.8 µg of SplB-Sau are combined in 50 µL of a solution containing 10 m*M* of Tris-HCl pH 7.5 and 5 m*M* of $MgCl_2$. The mixture is soaked in 95°C followed by gradual cooling to room temperature. The prepared linkers are kept at ™30°C until use.
4. Oligonucleotides for PCR:
    a. T/direct repeat (DR) 5′-CTG GAA TTG TGA TAC AGT GAA TTA TAA GTG.
    b. T/BAL 5′-CTT GTG TCA TGC ACA AAG TAG ATG TCC.
    c. Spl-P1 5′-CGA ATC GTA ACC GTT CGT ACG AGA A.
    d. Spl-P2 5′-TCG TAC GAG AAT CGC TGT CCT CTC C.
5. TaKaRa Ligation Kit version 1 (TAKARA Bio, Shiga, Japan).

Methods

3.1. Construction of Mutagenic Transposon Vectors

To generate pTrans-SA-IRES*LacZ*-CAG-GFP-SD:*Neo* construct (Fig.4), the following steps are performed.

1. *Unique restriction enzyme (RE) sites are introduced just outside of the transposon vector*: multicloning sites of pBluescript II were replaced with *Asc*I, *Xho*I, *Not*I, and *Swa*I sites by PCR amplification with primers 5′ GCC GCT CGA GGG CGC GCC AGA TTT AAA TC AGC TTT TGT TCC CTT TAG TGA G 3′ and 5′ CGC AGC GGC CGC ATT TAA ATG AGG CGC GCC GCT CCA ATT CGC CCT ATA GTG 3′ using pBluescriptII as a template. A 2.9-kb *Xho*I-*Not*I fragment of the PCR product was ligated to a 0.8-kb *Xho*I-*Not*I fragment of IR/DR(R,L) from pBS-IR/DR(R,L), resulting in pBS-IR/DR-AS, which contains *Asc*I and *Swa*I sites flanking the IRs and DRs.
2. *Introduction of unique enzyme sites into the old transposon vector*: linkers containing *Asc*I-*Kpn*I-*Swa*I sites and *Pme*I-*Pac*I sites were created by annealing oligonucleotides 5′ GTA CGG CGC GCC GGT ACC ATT TAA AT 3′ and 5′ GTA CAT TTA AAT GGT ACC GGC GCG CC 3′ and oligonucleotides 5′ CGT TTA AAC TTA ATT AAG AGC T 3′ and 5′ CTT AAT TAA GTT TAA ACG AGC T 3′, respectively. Each linker was inserted into the unique *Kpn*I and *Sac*I sites, respectively, of pTransCX-*GFP*:*Neo* after the removal of the TransCX-*GFP* fragment, resulting in pAKS:*Neo*:PP.
3. *Construction of GFP-SD unit for poly(A) trap*: the *Sal*I-*Bam*HI fragment of pCXenhanced green fluorescent protein (*EGFP*) *PigA*, containing

CAG-*EGFP*, and the 256-bp fragment of the *Neo* cassette, consisting of SD sequences from the mouse *hprt* gene exon 8/intron 8 region and the mRNA instability signal derived from the 3′ untranslated region of the human granulocyte-macrophage colony-stimulating factor cDNA were inserted into *Sal*I-blunted *Not*I sites of pBluescript II, resulting in pCAG-*GFP*-SD.

4. *Addition of SA-LacZ-poly(A) unit for promoter trap*: an *Xba*I-blunted-*Hin*dIII fragment of the rabbit ®-globin poly(A) addition signal and a *Sac*II-*Not*I fragment of the *lacZ* gene, containing the nuclear localizing signal were isolated and were inserted at *Xba*I and *Sma*I sites and *Sac*II and *Not*I sites of pBluescriptII, respectively, resulting in p*LacZ*-BS. A *Sal*I-*Xho*I fragment of CAG-*GFP*-SD from pCAG-*GFP*-SD was inserted at the *Xho*I site of p*LacZ*-BS, resulting in p*LacZ*-CAG-*GFP*-SD. The human *bcl-2* intron 2/exon 3 SA sequence was amplified by using primers 5′ CGG CAA GCT TCT CGA GCT GTA TCT CTA AGA TGG CTG G 3′ and 5′ GCC ACG GTC GAC GCC TGC ATA TTA TTT CTA CTG C 3′, with the removable exon trap [19] (RET) vector as a template. The internal ribosome entry site (IRES) sequence was amplified with primers 5′ GGA GCG TCG ACT ACG TAA ATT CCG CCC CTC TCC CTC 3′ and 5′ GGA GCG TCG ACT ACG TAA ATT CTC CCT CCC C 3′, with the RET vector as a template. A *Hin*dIII-*Sal*I SA-containing fragment and a *Sal*I-*Bam*HI IRES-containing fragment were simultaneously cloned into the *Hin*dIII and *Bam*HI sites of p*LacZ*CAG-*GFP*-SD, resulting in pSA-IRES*LacZ*-CAG-*GFP*-SD.

5. *Generation of the final construct*: the *Xho*I fragment of pSA-IRES-*LacZ*-CAG-*GFP*-SD containing SA, IRES, *lacZ*, poly(A), and CAG-*GFP*-SD was blunted and inserted at the *Eco*RI and *Bam*HI sites of the pBS-IR/DR-AS after both sites were blunted, resulting in pTrans-SA-IRES*LacZ*-CAG-*GFP*-SD. The *Asc*I-*Swa*I fragment of pTrans-SA-IRES*LacZ* CAG-*GFP*-SD was inserted at the *Asc*I and *Swa*I site of pAKS:*Neo*:PP, resulting in pTrans-SA-IRES*LacZ*-CAG-*GFP*-SD:*Neo* (*see* **Note 1**).

3.2. Testing Transposon Excision In Vitro

The main steps of the procedure are illustrated in Fig.5. The following procedure is applicable for any transposon that is cloned into the multiple cloning regions of pUC and pBluescript-derived plasmid vectors.

3.2.1. Transfection of Human HeLa Cells

1. Trypsinize a 10-cm HeLa plate when the plate is 80–85% confluent.
2. Resuspend the cells in 4 mL of medium.
3. Plate out 50 µL in a 6-well plate (TPPAG, Trasadingen, Switzerland) or 1–1.5 × 10^5 cells/well (*see* **Note 2**).
4. Let the cells grow for 24 h.
5. Transfect cells using jetPEI RGD as transfection reagent with 100 µg of transposase-expressing helper plasmid DNA and 1 µg of transposon donor plasmid.
6. Put together the plasmid DNAs and then add 150 m*M* of NaCl to 50 µL.
7. Prepare a mastermix with the transfection reagent: 2 µL of jetPEI with 48 µL of 150 m*M* NaCl; add this to the prepared plasmid mix.
8. Incubate for 30 min and then pipet the mixture put it to the cells.
9. Incubate the cells in the presence of the transfection reagent for 2 d.

3.2.2. Harvest of the Cells

1. Aspirate medium and wash the cells with PBS.
2. Trypsinize the cells.
3. Resuspend the cells in 1 mL of serum-containing medium and transfer them into an 2-mL Eppendorf tube.
4. Pellet cells for 3 min at 800*g*.
5. Aspirate medium and wash with 1 mL of PBS.
6. Repeat centrifugation step and aspirate PBS.
7. Pellet is used for DNA preparation or stored at −80°C for later use.

3.2.3. Preparation of Plasmids From Transfected Cells

This method is based on the Qiagen Spin Miniprep Kit (Qiagen).

1. Resuspend harvested cells in 300 µL buffer P1.
2. Add 300 µL 1.2% sodium dodecyl sulfate and 5 µL of proteinase K (10 mg/mL), mix well but do not vortex.
3. Incubation at 55°C for 30 min.
4. Add 400 µL of buffer N3, mix well but do not vortex.
5. Incubate on ice for 30 min.

6. Centrifuge for 10 min at 16,000*g*.
7. Pipet the supernatant into the spin column.
8. Follow the subsequent steps (washing and elution) exactly as specified in the Qiagen protocol for Spin Prep.
9. Measure DNA concentration.

3.2.4. Excision PCR

3.2.4.1. PCR-I

DNA	1.5 µL (50ng)
10X PCR buffer	2.0 µL
$MgCl_2$ (25 m*M*)	1.2 µL
dNTPs (10 m*M*)	0.4 µL
Primer pUC (10 µ*M*)	1.0 µL
Primer pUC6 (10 µ*M*)	1.0 µL
Taq DNA polymerase	0.25 µL
H_2O	To a final volume of 20 µL

PCR-cycle:

95°C-5´
95°C-30´´
65°C-30´´ } 30X
72°C-1´
72°C-5´
4°C

3.2.4.2. PCR-II

Dilute products 1:100 for PCR-II:

DNA	3 µL diluted PCR product
10X PCR buffer	5 µL
$MgCl_2$ (25 m*M*)	2.5 µL
dNTPs (10 m*M*)	1 µL
Primer pUC5 (10 µ*M*)	2.5 µL
Primer pUC2 (10 µ*M*)	2.5 µL
Taq DNA polymerase	0.5 µL
H_2O	To a final volume of 50 µL

• PCR cycle as above.

• Run PCR products on a 1.5% agarose gel.

• If your product is to weak you can perform a third PCR as followed.

• Dilute products of PCR-II with sterile distilled water (DW) 1:100.

3.2.4.3. PCR-III

DNA	3 µL diluted PCR product
10XPCR buffer	5 µL
$MgCl_2$ (25 m*M*)	2.5 µL
dNTPs (10 m*M*)	1.0 µL
puc3 Primer (10 µ*M*)	2.5 µL
puc4 Primer (10 µ*M*)	2.5 µL
Taq DNA polymerase	0.5 µL
H_2O	To a final volume of 50 µL

• PCR cycle as in PCR-1.

• Run PCR products on a 1.5% agarose gel.

3.3. Generation of Transgenic Mice: SB Transposase-Expressing (SB Transgenic) Mice and Transposon-Containing (GFP Transgenic) Mice

To generate pCAG-SB construct, a blunt-ended *Sac*II SB fragment pSB10 was inserted at the blunt end of *Eco*RI site of pCX-EGFP, after removal of an *Eco*RI EGFP fragment. The *Sal*I-*Bam*HI fragment of pCAG-SB was gel purified and injected into fertilized eggs obtained from the mating of BCF1(C57BL/6 × C3H) × BCF1 mice to generate SB transgenic mice (*see* **Note 3**). The SB transgenic line was established by mating the founder mice with C57BL/6 mice (*see* **Note 4**). pTrans-SA-IRES*LacZ*-CAG-*GFP*-SD:*Neo* was linearized with *Pac*I and injected into BDF1 (C57BL/6 × DBA) × BDF1 fertilized eggs to generate GFP mice (*see* **Note 5**).

3.4. Selection of GFP Transgenic Mice

As most transpositions occur locally, close to the donor sites in GFP-transgenic mice, mutant mice homozygous for a new transposon insertion often contain the donor site at both alleles. Selection of GFP transgenic mice in which the donor site does not affect phenotype when homozygous is important for phenotype screening by the SB transposon system.

3.5. Breeding, Generation of Double-Transgenic "Seed" Mice

As illustrated in Fig.2, SB transgenic mice are mated with GFP-transgenic mice to generate double-transgenic male "seed" mice (*see* **Note 6**).

3.6. Testing Transposon Excision In Vivo

Excision of SB transposon was examined by PCR with following primers: TgTP-2L, 5'-ACA CAG GAA ACA GCT ATG ACC ATG ATT ACG - 3' and TGTP-1U,

5′ GAC CGC TTC CTC GTG CTT TAC GGT ATC - 3′. Each primer is located just outside of the IR/DR-R and IR/DR-L of pTrans-SA-IRES*LacZ*-CAG-*GFP*-SD:*Neo*. PCR conditions were 95°C for 15 min, 50 cycles 94°C for 1 min, 59°C for 1 min, and 72°C for 1 min, followed by 72°C for 10 min. As this PCR condition detected approximately one excision event, particular GFP transgenic line could be evaluated by frequency of excision in "seed" mice generated by mating with SB mice.

3.7. Detection of poly(A)-Trap Events in Mice

Newborn mice are checked with a fluorescence microscope with GFP-specific filter before the appearance of hair. GFP-positive mice (Fig.6) are candidates of gene-trapped mice.

3.8. Detection of Gene-Trap Events in Mice

To examine the expression patterns of trapped genes, tissues or embryos are fixed with 1% paraformaldehyde, 0.2% glutaraldehyde, and 0.02% NP-40 in PBS (pH 7.3) for 30 min at room temperature, washed with PBS containing 0.02% NP-40 for three times, and then stained in a solution of 1 mg of X-Gal/mL, 2 m*M* $MgCl_2$, 4 m*M* $K_3Fe(CN)_6$, and 4 m*M* $K_4Fe(CN)_6$ in PBS (pH 7.3). A ®-galactosidase-expressing tissue sample is illustrated in Fig.7.

3.9. LM-PCR to Determine Integration Sites of SB Transposon

1. Isolate genomic DNA from mouse tail using 500 µL of DNA extraction buffer (1 m*M* EDTA, 1X SSC, 1% sodium dodecyl sulfate, and 10 m*M*Tris-HCl pH 7.4) with 10 µL of proteinase K (10 mg/mL) and incubate at 56°C overnight.
2. Centrifuge at 15,000 rpm for 5 min at 4°C to separate undissolved tissue and transfer liquid phase to fresh Eppendorf tube using blue pipet tips cut at the end to avoid genomic DNA shearing.
3. Add equal volume (500 µL) of phenol:chloroform and mix by rotation for 15–30 min.
4. Centrifuge at 15,000 rpm for 5 min at 4°C and transfer aqueous phase to fresh Eppendorf tube using blue pipet tips cut at the end to avoid genomic DNA shearing.
5. Add 0.7 vol (350 µL) of isopropanol and mix by gentle inversion.
6. Centrifuge at 18,000*g* rpm for 10 min at 4°C to pellet genomic DNA.
7. Discard supernatant and wash with 500 µL of 80% ethanol.
8. Centrifuge at 15,000 rpm for 5 min at 4°C to pellet genomic DNA.
9. Discard supernatant and dissolve genomic DNA in 50 µL TE.
10. Incubate at 56–60°C for 15–30 min to dissolve genomic DNA. *Note*: **steps 1–10** can be done using automated DNA isolation equipment.
11. Measure DNA concentration using Nano-Drop spectrophotometer (NanoDrop Technologies, Wilmington, DE USA).
12. Dilute genomic DNA to a 10 ng/µL concentration using DW.
13. Proceed to digest 100 ng of diluted genomic DNA at 37°C for 3 h using one of the following RE (4-base cutters) in a final volume of 50 µL.
    a. *Alu*I
    b. *Mbo*I
    c. *Hae*III
    d. *Rsa*I;

    For IF or IR vectors, use *Mbo*I or *Alu*I, respectively, as the first choice RE for initial screening, followed by remaining enzymes. If sample quantity is small, use all three enzymes (*Alu*I, *Mbo*I, and *Hae*III). Use *Rsa*I only if all other enzymes fail.
14. Heat inactivation for 20 min after incubation:
    a. *Alu*I, *Mbo*I, or *Rsa*I at 65°C.
    b. *Hae*III at 80°C.
15. Linker ligation at 16°C for at least 2 h:
    a. *Alu*I, *Rsa*I, and *Hae*III-use Spl-top/blunt.
    b. *Mbo*I-use Spl-top/Sau.

    Linker ligation reaction:

RE digested genomic DNA	2 µL
Appropriate linker	1 µL
Takara ligation buffer A	12 µL
Takara ligation buffer B	3 µL
Total	18 µL

16. Purify using Qiagen PCR purification kit (using manufacturer's instructions)-resuspend in 38 µL DW. For each sample, proceed to digest at 37°C for 3 h using *Kpn*I.

    RE digest reaction:

Linker-ligated/purified genomic DNA	38 µL
Buffer (10X)	5 µL
BSA (10X)	5 µL
*Kpn*I (in excess)	2 µL
Total	50 µL

17. Purify using Qiagen PCR purification kit (using manufacturer's instructions) and resuspend in 50 µL DW (may omit if sample quantity is large—proceed directly to nested-PCR).

18. Nested-PCR using 1 µL template with the following primer sets (first PCR):

    First PCR:

DNA (from **step 17**)	1 µL
Buffer (10X)	5 µL
dNTP (10 m*M*)	1 µL
Primer 1 T/DR (10 µ*M*)	1 µL
Primer 2 Sp1-P1 (10 µ*M*)	1 µL
Hot start *Taq*	0.25 µL
DW	40.75 µL
Total	50 µL

    PCR condition:
    95°C
    94°C
    55°C } 30X
    72°C
    72°C
    Cool to 25°C

    Second PCR using the following primer sets:

First PCR	1 µL
Buffer (10X)	5 µL
dNTP (10 m*M*)	1 µL
Second primer 1 T/BAL (10 µ*M*)	1 µL
Second primer 2 SpI-P2 (10 µ*M*)	1 µL
Hot start *Taq*	0.25 µL
DW	40.75 µL
Total	50 µL

    PCR condition: Similar to first PCR.

19. Check PCR product by running 5 µL of each sample in a 2% agarose gel (ethidium bromide added) (Fig.8). *Note*: for samples with no visible PCR product, proceed to repeat using other 4-base RE and start from **step 13** again.

20. Proceed to run preparative 2% agarose gel (ethidium bromide added).

21. Gel extraction of bands under ultraviolet and proceed to purification using Qiagen gel extraction kit (using manufacturer's instructions)–resuspend in either 30 µL or 50 µL DW, depending on the intensity of the PCR band.

22. Recheck for purity by running 5 µL of resuspended PCR-band product in a 2% agarose gel (ethidium bromide added).

23. Proceed to cycle sequencing using 1 µL of the purified PCR-band product with the appropriate primer.

4. Notes

1. When the blunted SA-IRES-*LacZ*-CAG-GFP-SD was inserted into the pBS-IR/DR-AS, both orientations were obtained. IF was that, transcriptional orientation of *LacZ*, GFP, and *Neo* was same in the final construct. On the other hand, IR was that *Neo* and other units were reverse.

2. Because there is always some variation from one transfection to another, it is good practice to transfect two wells with the same plasmid combination. The transfected cells harvested from the two wells can be combined for subsequent DNA preparation.

3. SB10 transposase could be replaced by hyperactive versions of the SB transposase [20-23]. C57BL/6 fertilized eggs can be used for generation of SB transgenic mice.

4. In our experience, it took a long time (6 mo) for the SB transgene in the founder mouse to be transmitted to the next generation.

5. The vector backbone was included for suppression of *LacZ* and GFP expression, because the SD of GFP and downsteam of SA of *LacZ* within tandem array of transgenes may allow expression of GFP. As predicted, GFP signal was not detected in most founder mice (seven out of eight).

6. Transposition efficiencies in male or female germ cell from double-positive mice were examined by comparing GFP-positive progeny. Male double-positive (seed) mice could generate much higher percentage of GFP-positive mice, suggesting higher transpositions in male germ cells.

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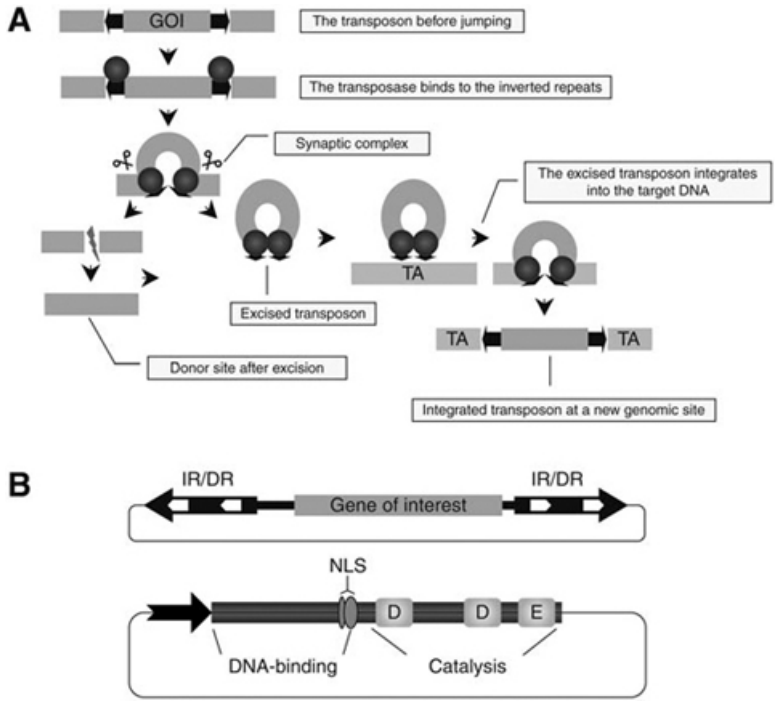

Fig.1. The SB transposon system. (**A**) Mechanism of SB transposition. The transposable element carrying a GOI (orange box) is maintained and delivered as part of a DNA vector (blue DNA). The transposase (purple circle) binds to its sites within the transposon IRs (black arrows). Excision takes place in a synaptic complex. Excision separates the transposon from the donor DNA, and the double-strand DNA breaks that are generated during this process are repaired by host factors. The excised element integrates into a TA dinucleotides site in the target DNA (green DNA) that will be duplicated and will be flanking the newly integrated transposon. **(B**) Components and structure of a two-component gene transfer system based on SB. A GOI (orange box) to be mobilized is cloned between the terminal (IR/DR, black arrows) that contain binding sites for the transposase (white arrows). The transposase gene (purple box) is physically separated from the IR/DRs, and is expressed in cells from a suitable promoter (black arrow). The transposase consists of an N-terminal DNA-binding domain, a nuclear localization signal, and a catalytic domain characterized by the D aspartic acid and glutamic acid signature.

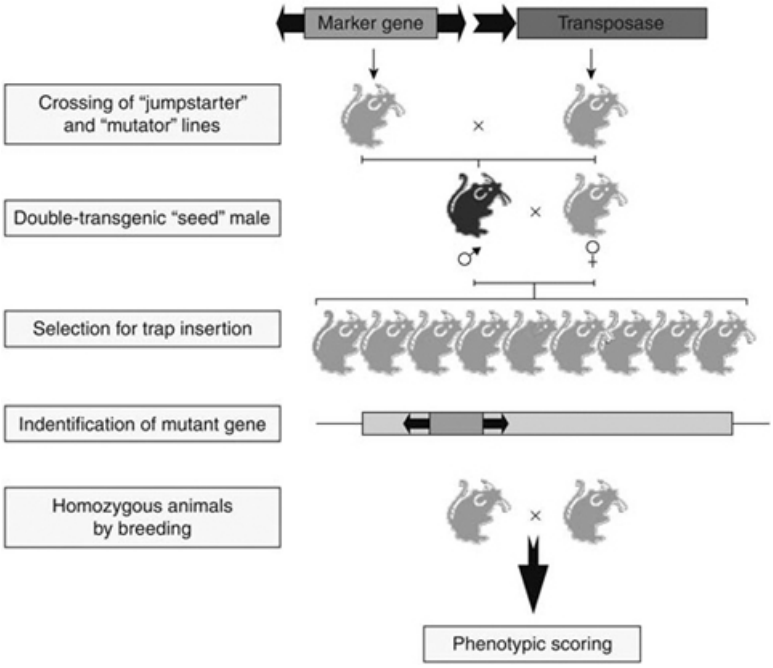

Fig.2. In vivo germline mutagenesis of the mouse with transposable elements. Breeding of "jumpstarter" and "mutator" stocks induces transposition in the germline of double-transgenic "seed" males. The transposition events that take place in germ cells are segregated in the offspring. Animals with transposition events need to be bred to homozygosity in order to visualize the phenotypic effects of recessive mutations. Mutant genes can easily be cloned by different PCR methods making use of the inserted transposon as a unique sequence tag.

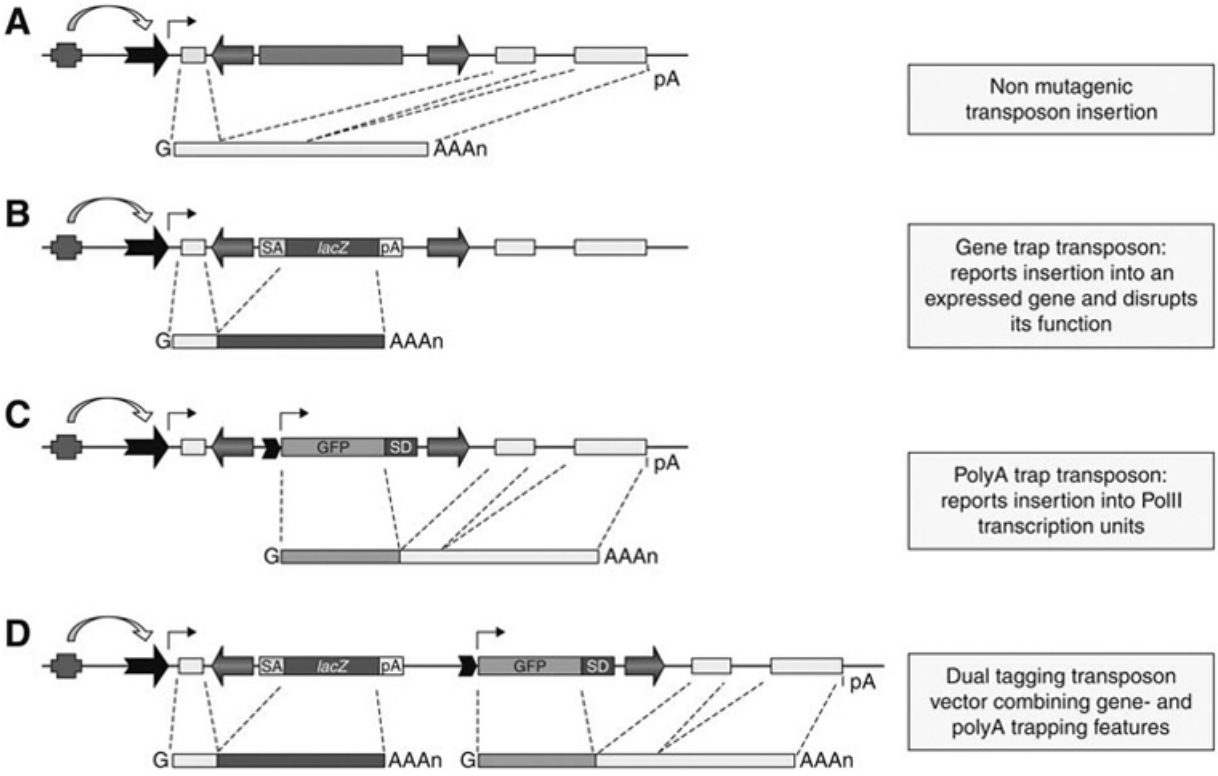

Fig.3. Transposon-based vectors for insertional mutagenesis. (**A**) A hypothetical transcription unit is depicted with an upstream regulatory element (purple box), a promoter (black arrow), three exons (yellow boxes), and a pA. Transposon insertion into one of the introns is not expected to be mutagenic, because the element is spliced out of the pre-mRNA together with the intron sequences. Major classes of transposon-based trapping constructs and spliced transcripts are shown in the figure. Transposon IRs are indicated by gray arrows. (**B**) The conventional gene-trapping cassettes contain a SA followed by a reporter gene such as *lacZ* and a pA. The reporter is only expressed when transcription starts from the promoter of an endogenous transcription unit. Thus, the expression of the reporter follows the expression pattern of the trapped gene. (**C)** poly(A) traps contain a promoter followed by a reporter gene such as green fluorescent protein (GFP) and a SD site, but they lack a pA. Therefore, reporter gene expression depends on splicing to downstream exon/s of a Pol II transcription unit containing a pA. (**D**) The "dual tagging" vectors are based on both gene- and poly(A) trapping of a targeted transcription unit.

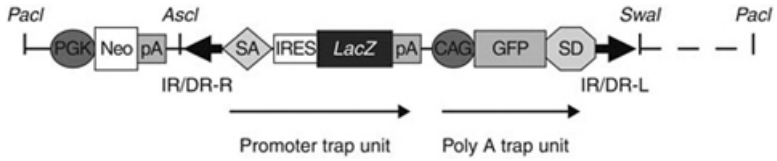

Fig.4. Schematic representation of a "dual tagging" gene trap transposon construct. The orientations of transcription are shown by an arrow. Some of unique RE sites (*Pac*I, *Swa*I, and *Asc*I) are also shown. Dashed line indicates the vector backbone. SD, splice donor; SA, splice acceptor; pA, polyA signal.

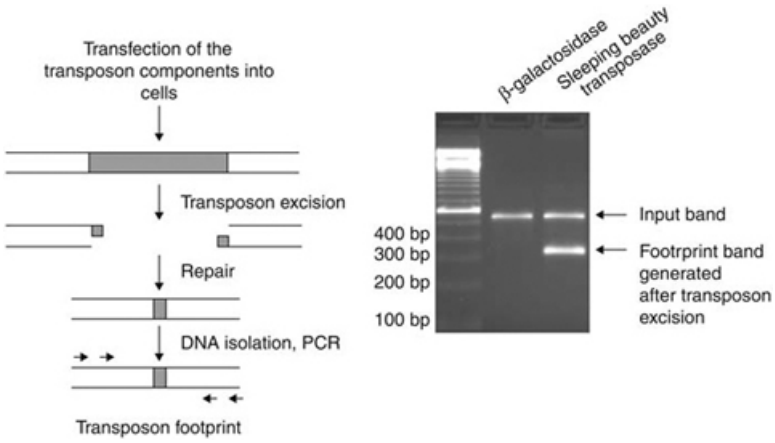

Fig.5. Transposon excision assay in transfected cells. HeLa cells were cotransfected with a *neo*-marked transposon plasmid and vectors expressing the proteins indicated. Transposon excision is assayed with PCR that amplifies a footprint product. PCR-amplification of the *neo* marker inside the transfected transposon donor serves as a loading control.

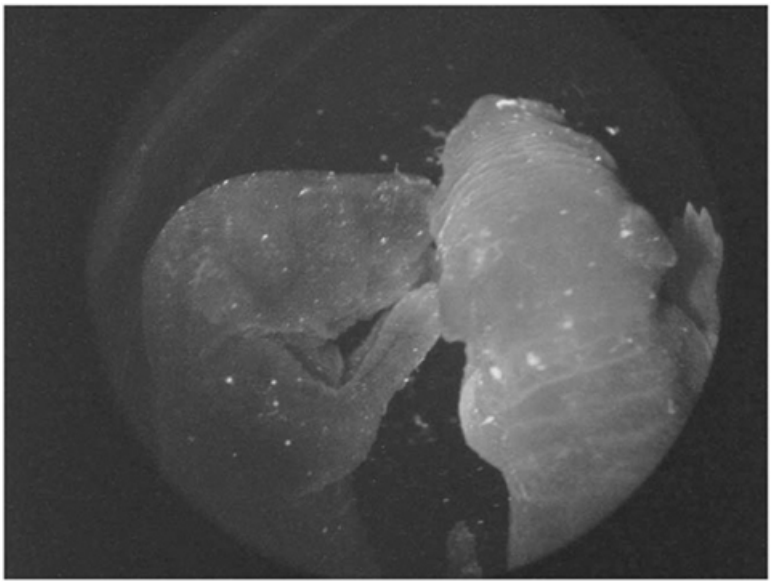

Fig.6. Examination of polyA trap events using fluorescence stereomicroscopy in newborn mice. The mouse at the left is GFP-negative and the mouse at the right is GFP-positive.

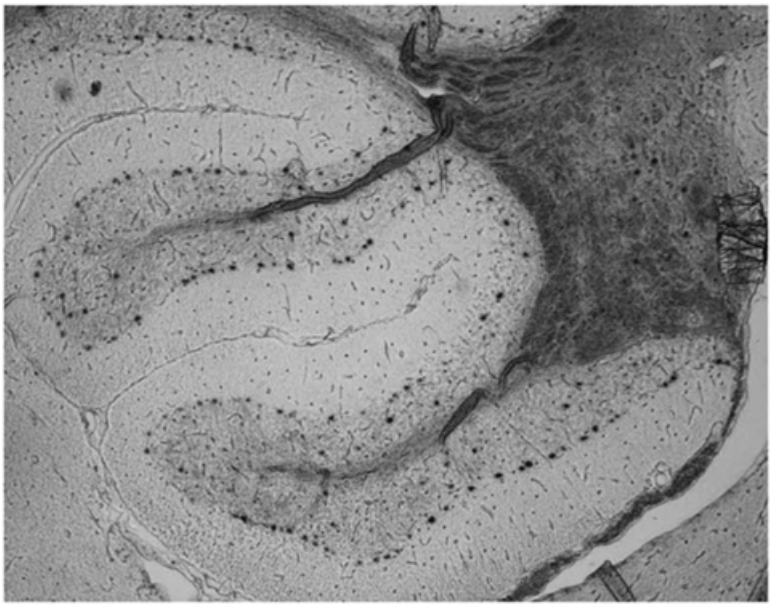

Fig.7. Detection of promoter trap events using X-Gal staining. The cross-section of the cerebellum derived from one of the SB transposon-inserted lines is shown. Purkinje cells are positive for X-Gal staining.

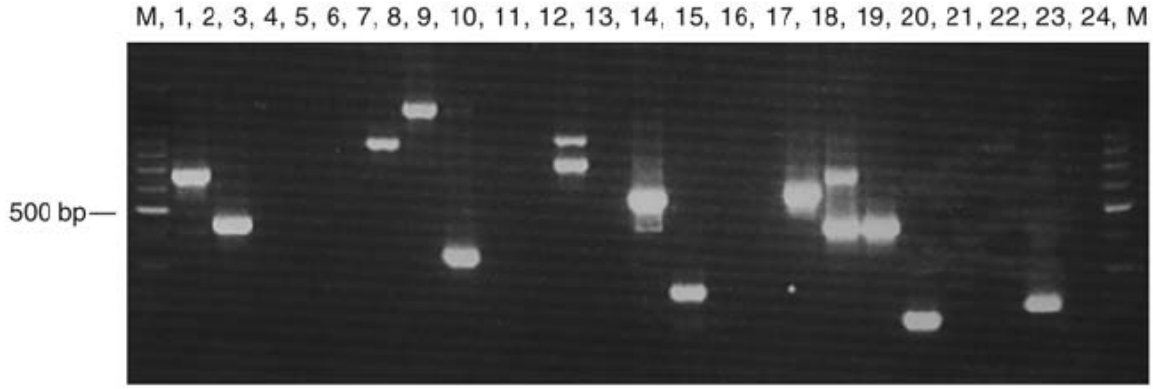

Fig.8. LM-PCR analysis of transposon insertions. The agarose gel shows products of LM-PCR on DNA samples prepared from mice with transposon insertions. M indicates a marker of a 100-bp DNA ladder.