Insertional Mutagenesis by a Hybrid PiggyBac and Sleeping Beauty Transposon in the Rat

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ABSTRACT A hybrid piggyBac/Sleeping Beauty transposon-based insertional mutagenesis system that can be mobilized by simple breeding was established in the rat. These transposons were engineered to include gene trap sequences and a *tyrosinase* (*Tyr*) pigmentation reporter to rescue the albinism of the genetic background used in the mutagenesis strategy. Single-copy transposon insertions were transposed into the rat genome by co-injection of plasmids carrying the transposon and RNA encoding piggyBac transposase into zygotes. The levels of transgenic *Tyr* expression were influenced by chromosomal context, leading to transgenic rats with different pigmentation that enabled visual genotyping. Transgenic rats designed to ubiquitously express either piggyBac or Sleeping Beauty transposase were generated by standard zygote injection also on an albino background. Bigenic rats carrying single-copy transposons at known loci and transposase transgenes exhibited coat color mosaicism, indicating somatic transposition. PiggyBac or Sleeping Beauty transposase bigenic rats bred with wild-type albino rats yielded offspring with pigmentation distinct from the initial transposon insertions as a consequence of germline transposition to new loci. The germline transposition frequency for Sleeping Beauty and piggyBac was ~10% or about one new insertion per litter. Approximately 50% of the insertions occurred in introns. Chimeric transcripts containing endogenous and gene trap sequences were identified in *Gabrb1* mutant rats. This mutagenesis system based on simple crosses and visual genotyping can be used to generate a collection of single-gene mutations in the rat.

THE laboratory rat (*Rattus norvegicus*) is an important organism for modeling human biology and disease. These relatively large rodents are especially useful for physiological, neurobehavioral, transplantation, and toxicological studies. Many genomic resources for the rat have become available (Aitman *et al.* 2008). The genome has been sequenced, assembled, and annotated (Rat Genome Sequencing Project Consortium 2004), leading to current predictions of >22,000 genes (www.ensembl.org/Rattus_norvegicus). The rat model system is now primed for functional genomic studies. Unfortunately, relatively few single-gene mutations in the rat have been isolated or generated compared to the

other predominant laboratory rodent, the mouse. A large collection of mutations in the rat and a continuous source of new mutations would be a valuable resource to facilitate studies of gene function and model human biology and disease when mouse models are inadequate.

There are a variety of relatively new mutagenesis strategies that have been developed to generate mutations in rats. Chemical mutagenesis using *N*-ethyl-*N*-nitrosourea (ENU) was used to generate mutations in rats (Zan *et al.* 2003; Smits *et al.* 2005, 2006; Homberg *et al.* 2007; Olivier *et al.* 2008; van Bostel *et al.* 2008; Homberg *et al.* 2009; van Bostel *et al.* 2010). These studies have used reverse genetics approaches to identify point mutations in specific genes of interest. In addition, an ENU-mutagenized cryopreserved rat sperm library was generated carrying random point mutations (Mashimo *et al.* 2008). Somatic tissues from the sperm donors used to generate these libraries can be screened by molecular methods to identify point mutations in any gene. If the point mutation is of interest, then the sperm can be used for *in vitro* fertilization to recover the mutation for study. Spermatogonial stem cells (SCCs) are present in postnatal testes that can self-renew

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or differentiate into spermatozoa (Oatley and Brinster 2008). SCCs can also be used to generate random mutations in rats (Izsvák *et al.* 2010). These cells are isolated from testes, expanded and genetically modified *in vitro*, and then injected directly into the seminiferous tubules of recipients to complete spermatogenesis. Sperm carrying the mutations can then be used to create mutant rats (Izsvák *et al.* 2010). Zinc-finger nucleases (ZFNs) are bifunctional DNA-binding proteins designed to recognize and cleave specific gene sequences. They can be expressed in rat zygotes by pronuclear injection to generate targeted mutations (Geurts *et al.* 2009; Mashimo *et al.* 2010; Cui *et al.* 2011). Recently, germline competent rat embryonic stem (ES) cell lines have been isolated (Buehr *et al.* 2008; Li *et al.* 2008; Kawamata and Ochiya 2010; Blair *et al.* 2012). In addition, the first demonstration of a targeted germline mutation created using rat ES cells was reported (Tong *et al.* 2010). All of these mutagenesis methods are relatively new in the rat model. It is currently unclear which of these approaches will be the most productive for generating single-gene mutations in the rat (Dolgin 2010).

Transposon-mediated insertional mutagenesis is another approach that has been established in rodents, including the mouse and rat (Carlson *et al.* 2003; Horie *et al.* 2003; Kitada *et al.* 2007; Lu *et al.* 2007; Izsvák *et al.* 2010). Engineered transposons contain terminal inverted repeats (TIRs) at both ends that are recognized by their corresponding transposase (Curcio and Derbyshire 2003). These TIRs can flank cargo sequences for delivery into the genome by transposition. Most DNA transposons are mobilized through a “cut-and-paste” mechanism (Reznikoff *et al.* 1999). Transposon transposition into the genome or mobilization within the genome can result in insertional mutation, providing a molecular tag of the genetic lesion. Sleeping Beauty transposase inserts Sleeping Beauty transposons into highly abundant TA sequences in the genome (Ivics *et al.* 1997). PiggyBac transposase inserts piggyBac transposons in TTAA sequences and has been shown to have a higher activity for transposon mobilization than Sleeping Beauty, Tol2, and Mos1 in mammalian cells (Wu *et al.* 2006). However, Grabundzija *et al.* (2010) showed that the transpositional efficiencies in human HeLa and hematopoietic stem cells is Sleeping Beauty > piggyBac > Tol2. Sleeping Beauty transposon excision is not precise, frequently leaving a 5-base pair (bp) footprint, whereas piggyBac leaves no molecular footprint (Ivics *et al.* 1997; Izsvák *et al.* 2004; Lu *et al.* 2007; Woltjen *et al.* 2009). Sleeping Beauty has a higher frequency of local hops (within 4 Mb), whereas piggyBac tends to mobilize and reinsert on other chromosomes (Liang *et al.* 2009). In the mouse and rat, transposons are typically introduced into the germline by standard pronuclear injection of purified DNA fragments that results in multicopy tandem arrays usually integrated randomly at a single locus (Dupuy *et al.* 2002). Transposons can be mobilized in the germline by transposase expression from a second transgene (Dupuy *et al.* 2002; Carlson *et al.* 2003; Horie *et al.* 2003). One disadvantage of mobilizing multiple copies of transposons in tandem arrays is chromosomal damage (Geurts *et al.* 2006). Single-

copy transposon mobilization for mutagenesis has yet to be reported in the rat.

We have developed and validated a dual transposon mutagenesis system in the rat that can be mobilized by either Sleeping Beauty or piggyBac transposase. A gene trap module for insertional mutagenesis is combined with a pigmentation reporter gene (*tyrosinase*, *Tyr*) for visual genotyping. In this system, single-copy transposons integrated at known loci are mobilized to new chromosomal locations by crosses with transgenic rats designed to ubiquitously express Sleeping Beauty or piggyBac transposase. Expression of the *Tyr* reporter is influenced by chromosomal context. Thus, transposition to a new site in the genome is usually accompanied by a visible change in pigmentation, greatly simplifying the identification of transposition events. In addition, rats hemizygous for a transposon insertion can be distinguished from homozygotes by coat color intensity. This system using either Sleeping Beauty or piggyBac transposase results in ~10% frequency of germline transposition events. Truncated endogenous transcripts containing vector sequences demonstrate the activity of the gene trap modules. This simple mutagenesis system can facilitate the generation of a collection of new mutant rat strains to model human biology and disease.

Materials and Methods

Rats

Sprague-Dawley outbred and Wistar Kyoto (WKY/NCrl) partially inbred rats were purchased from Charles Rivers. WKY/NHsd inbred rats were purchased from Harlan Laboratories. Transposon-carrying rats were generated using Sprague-Dawley zygotes and then backcrossed to WKY/NCrl and subsequently WKY/NHsd. *UBC-SB* and *UBC-PB* transgenic rats were generated using WKY/NHsd zygotes and maintained on the same genetic background. All animal manipulations were conducted in accordance with the National Research Council Guide for Care and Use of Laboratory Animals and approved by the University of Texas M. D. Anderson Cancer Center Institutional Animal Care and Use Committee.

Construction of the *Bhr2* and *Bhr7* transposons and transposase gene constructs

Bhr2 is shown in Figure 1A. *Bhr2* was constructed by inserting two copies of a gene-trap cassette, which consists of an adenovirus splice acceptor (Ad2) and SV40 poly(A) signal (SA-pA) in opposite orientations flanking a mouse *Tyr* minigene in pXL-BacII piggyBac (Li *et al.* 2001). The SA-pA fragment was subcloned by PCR from pT2/Bart3 (Lu *et al.* 2007). The *Tyr* minigene was isolated from pT-TyBS (P. Overbeek, unpublished data), and modified by PCR cloning a 290-bp minimal promoter, and ligating it to the *PmeI* site. The Sleeping Beauty transposon 5' arm was PCR-cloned from pT3 (Yant *et al.* 2004), to flank the SA-pA cassettes and *Tyr* minigene. *AttP/attB* sites were inserted in between piggyBac and Sleeping Beauty arms using oligonucleotides.

Bhr7 is shown in Figure 1B. Bhr7 was constructed by inserting two copies of the Sleeping Beauty 5' arm, PCR cloned from pT3, into pXL-BacII to flank a gene-trap module and *Tyr* minigene. rTA2S-M2 coding sequence led by an IRES sequence from the hXIAP 5'-UTR (Holcik *et al.* 1999) was inserted into the SA-pA cassette mentioned above to create the gene-trap module. The *Tyr* minigene coding sequence was followed by an encephalomyocarditis virus IRES sequence (Borovjagin *et al.* 1991) and the splice donor site from rabbit β -globin intron (van Ooyen *et al.* 1979). The *Tyr* minigene is similar to the one in Bhr2 except that it lacks a pA.

UBC-SB and *UBC-PB* transposase gene constructs are shown in Figure 5A. The human *UBC* promoter is used to direct Sleeping Beauty or piggyBac transposase expression. The constructs are flanked by chicken transcriptional insulators (cHS4) (two copies on each side). The *UBC* promoter was isolated from the UI4_GFP_SIBR plasmid (provided by D. Turner, University of Michigan, Ann Arbor, MI). The Sleeping Beauty transposase coding sequence was isolated from pCMV-HSB3 (Yant *et al.* 2004). The piggyBac transposase coding sequence was isolated from pcDNA3-mPB (Cadinanos and Bradley 2007). A rabbit β -globin intron and poly(A) signal (van Ooyen *et al.* 1979) was subcloned downstream of the transposase coding sequences. The cHS4 insulator sequence was isolated from pNI-CD (Bell *et al.* 1999).

Generation of transgenic rats

Transgenic rats carrying transposons were generated by co-injecting circular plasmids with the transposons and piggyBac transposase RNA synthesized from pcDNA3-mPB, into zygotes (Jang and Behringer 2007). Transgenic rats expressing transposases were generated using standard methods (Filipiak and Saunders 2006).

Southern blot analysis

Toe DNA was used for Southern blot analysis as described (Sambrook *et al.* 1989). Genomic DNA was cut with *PvuII* for Bhr2 or *ApaLI/BamHI* for Bhr7, separated by electrophoresis through a 1% agarose gel, and transferred to Hybond-XL (GE Healthcare). A 909-bp *HindIII/ScaI* fragment of mouse *Tyr* was isolated from Bhr2 and used as a probe for detecting Bhr2. A 487-bp *ApaLI/PacI* fragment containing IRES and SD sequences from Bhr7 was used as a probe for detecting Bhr7. Probes were labeled with 32 P-dCTP using the Rediprime II Random Prime Labeling system (GE Healthcare).

Inverse polymerase chain reaction

To identify the genomic locations of the transposon integration events in the rat genome, we used an iPCR strategy (Chen *et al.* 2010). The locations of primers within the transposon and their sequences used for iPCR are shown (Figure 1, A and B, Table 1). We used I1/I2 and N1/N2 primer sets to identify transposon locations after initial pronuclear injections or after mobilization by piggyBac transposase by crosses. The primer sets of I1/IS2 and N1/NS2 were used to identify transposon locations after mobilization by Sleeping Beauty trans-

Figure 1 Transposons for transgenic rat production. (A) Bhr2 and (B) Bhr7 transposon constructs. Bhr2 and Bhr7 contain terminal inverted repeat sequences recognized by piggyBac transposase (light gray arrowheads) and the inverted/direct repeat sequences recognized by Sleeping Beauty transposase (dark gray arrowheads). Pink and green circles are *attB* and *attP* sequences, respectively, not exploited in this study. The primers for initial iPCR are I1, I2, and IS2 (black arrowheads) and for nested iPCR are N1, N2, and NS2 (blue arrowheads). The primer for RT-PCR for Bhr2 is B2-RT (red arrowhead). The primers, Tyr-F and Tyr-R (green arrowheads), are used for detecting the transposon in the rat genome. A portion of the *Tyr* reporter gene in Bhr2 and the splice donor and a subregion of the 3' IRES sequence in Bhr7 were used as probes for Southern blot analysis. Primer sequences for iPCR, genotyping, and RT-PCR are described in Table 1. SA, Ad2 splice acceptor; pA in Bhr2, SV40 polyadenylation signal; pA in Bhr7, human growth hormone polyadenylation signal; Pro, *Tyr* promoter; pA after *Tyr*, *Tyr* polyadenylation signal; IRES, internal ribosomal entry site; rTA, reverse tetracycline transactivator; SD, splice donor of rabbit β -globin intron; A, *ApaI*; AL, *ApaLI*; B, *BamHI*; E, *EcoRI*; P, *PvuII*; and X, *XbaI*.

posase. A total of 1.5 μ g of genomic DNA was digested by *ApaI*, *BamHI*, *EcoRI*, or *XbaI* and heated at 70° for 10 min to inactivate the enzyme. The digested genomic DNA was self-ligated using T4 DNA ligase (Roche) and purified by ethanol precipitation. The precipitate was dissolved in 25 μ l of TE and 2 μ l of the self-ligation mixture was used as template for a first-round PCR reaction. The first-round PCR was performed using the following conditions: 94° for 1 min followed by 35 cycles at 94° for 30 sec, 55° for 30 sec, and 72° 3 min. A second round of PCR was performed using 2 μ l of the PCR products from the first round of amplification as the template using an annealing temperature of 58°. Amplified DNA fragments were fractionated and purified for direct sequencing using primers N2 or NS2. The chromosomal location of the transposon was analyzed using the BLAST Search program provided by National Center for Biotechnology Information (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and Ensembl (<http://www.ensembl.org/Multi/blastview>).

PCR genotyping

Transposon detecting primers were Tyr-F and Tyr-R, which recognized the *Tyr* minigene. The *UBC-SB* or *UBC-PB* transposase rats were identified by primers UBC-F1 and SB-R or UBC-F1 and PB-R, respectively. After the transposon locus was identified by iPCR, the transposon rats were confirmed by locus-specific primers. *Gabrb1*^{Bhr2} rats were genotyped using wild-type allele primers Gab-F and Gab-R and transposon allele

Table 1 Primer sequences for iPCR, genotyping transgenic and Tn rats, and RT-PCR

iPCR		
I1	5'-CAGTAGTCCAGGGTTTCCTTGATG-3'	
I2	5'-AGTGACACTTACCGCATTGACAAG-3'	
IS2	5'-CACAGGTACACCTCCAATTGACTC-3'	
N1	5'-TGAGGACAAACTCTTCGCGGTCTT-3'	
N2	5'-TCCTAAATGCACAGCGACGGATTG-3'	
NS2	5'-CTTCTGACCCACTGGAATTGTGATAC-3'	
Genotyping		
Gene	Primer	Size (bp)
Tn	(Tyr-F) 5'-GCTCAGTCTATGTCATCCCCACAGG-3' (Tyr-R) 5'-GTGGTCCCTCAGGTGTCCATCG-3'	453
T'ase (SB)	(Ubc-F) 5'-CTGAAGCTCCGGTTTGAACATATGCG-3' (SB-R) 5'-GTACAGATGAACGTGGTACCGCCAG-3'	351
T'ase (PB)	(Ubc-F) 5'-CTGAAGCTCCGGTTTGAACATATGCG-3' (PB-R) 5'-GATCACGTTCTGCTCGTCCAGGATC-3'	428
<i>Gabrb1</i> (WT)	(Gab-F) 5'-ATTTCATGCAGGACGTGATGGGCAGC-3' (Gab-R) 5'-GGCAAAGCCTTGGTACAATAGCAGGC-3'	450
<i>Gabrb1</i> (Tn)	(Gab-F) 5'-ATTTCATGCAGGACGTGATGGGCAGC-3' (B2-3) 5'-CCCATCTGGTCCATCATGATGAACGG-3'	628
<i>LOC685774</i> (WT)	(LOC-F) 5'-GCTCACAATCTACCATTATCTCTGGTGGCC-3' (LOC-R) 5'-AGCACATCTGACATTCCTCACCTCC-3'	406
<i>LOC685774</i> (Tn)	(LOC-F) 5'-GCTCACAATCTACCATTATCTCTGGTGGCC-3' (PBIR-5.2) 5'-TCCTAAATGCACAGCGACGGATTGCG-3'	319
RT-PCR		
<i>Beta-actin</i>	(b-act F) 5'-CCAGTTCGCCATGGATGACGATATCG-3' (b-act R) 5'-TCCATCACAATGCCAGTGGTACGACC-3'	471
<i>Gabrb1</i> (5')	(G-RT5F) 5'-CCATGGTTTGTGTGCACACAGCTCC-3' (G-RT5R) 5'-GTTGGTCAGCTACCCTGTTGTCAAGG-3'	288
<i>Gabrb1</i> (Tn (-))	(G-RTIF) 5'-ACAACAGGGTAGCTGACCAACTCTGG-3' (G-RTIR) 5'-ACTGATGGTTGTCATGGTCAGCACCG-3'	545
<i>Gabrb1</i> (3')	(G-RT3F) 5'-TGTGTTCTGTTCTCTGGCTACTGG-3' (G-RT3R) 5'-CGCTGTCGTATGAGTACATAGTGGCC-3'	257
<i>Gabrb1</i> (Tn (+))	(G-RTIF) 5'-ACAACAGGGTAGCTGACCAACTCTGG-3' (B2-RT) 5'-CACTGCATTCTAGTTGTGGTTGTCCAACTCATC-3'	278

WT, wild type; Tn, transposon; T'ase, transposase.

primers Gab-F and B2-3, *LOC685774*^{Bhr7} rats were genotyped using wild-type allele primers LOC-F and LOC-R and transposon allele primers LOC-F and PB-IR5.2. The sizes of the amplification products and primer sequences are listed in Table 1.

Gabrb1^{Bhr2} RT-PCR

One microgram of total RNA isolated using the TRIzol Reagent (Invitrogen) from adult brain was subjected to RT-PCR analysis, using the SuperScript II First-Strand Synthesis system (Invitrogen), according to the protocol provided by the manufacturer. The purity and quantity of cDNAs were analyzed with rat β -actin-specific primers B-act F and B-act R. Primers used to detect the gene transcripts are: Gab-RT5F, Gab-RT5R, Gab-RTIF, Gab-RTIR, B2-RT, Gab-RT3F, and Gab-RT3R. The locations for each primer are indicated in Figure 6A. The sequence and size of the DNA fragments amplified by the primers are listed in Table 1.

Results

Generation of hybrid piggyBac and Sleeping Beauty transposons

Two types of transposon vectors, Bhr2 and Bhr7, were constructed (Figure 1, A and B). Both vectors contain

a mouse *Tyr* minigene expression cassette flanked by the inverted repeat/direct repeat (IR/DR) sequences recognized by Sleeping Beauty transposase and the terminal inverted repeat (TIR) sequences recognized by piggyBac transposase, i.e., a Sleeping Beauty transposon within a piggyBac transposon. The *Tyr* pigmentation gene was used as a visual reporter for transposon integration on a genetic background that is albino, e.g., Sprague-Dawley, Wistar, or F344 rats (Overbeek *et al.* 1991; Overbeek 2002; Blaszczyk *et al.* 2005; Lu *et al.* 2007).

The Bhr2 transposon (Figure 1A) contains two copies of a gene trap cassette that consists of splice acceptor (SA) and polyadenylation (pA) signals in opposite orientations flanking the *Tyr* minigene. Thus, integration of Bhr2 into an intron should trap endogenous transcripts independent of orientation. *attP* and *attB* sites that are recognized by PhiC31 integrase were placed between the piggyBac TIRs and Sleeping Beauty IR/DRs for potential DNA fragment exchange but were not exploited in the current study.

The Bhr7 transposon (Figure 1B) contains a gene trap cassette that consists of a SA-internal ribosomal entry site (IRES)-reverse tetracycline-controlled transactivator (rtTA)-pA module placed 5' of the *Tyr* minigene. The gene trap function of Bhr7 requires integration in one specific orientation

and should result in the expression of rtTA in the pattern of the trapped gene. In contrast to Bhr2, the *Tyr* minigene expression cassette in Bhr7 does not contain a pA sequence but rather an IRES splice donor (SD) sequence. This was designed so that *Tyr* expression would depend on integration within an intron and splicing into a downstream endogenous exon to provide a pA signal, increasing the mutagenesis potential of Bhr7. However, there appear to be many cryptic pA signals within the genome not necessarily residing in exons (C.-W. Jang and R. R. Behringer, unpublished observations). Thus, in practice, the IRES-SD strategy was not very specific for identifying insertions within introns of genes.

Transposition of single-copy transposons into the rat genome

To eliminate the problems associated with transposons in multicopy tandem arrays created by the injection of purified DNA fragments, we mobilized Bhr2 and Bhr7 into the rat genome as single-copy integrants using piggyBac transposase. The Bhr2 and Bhr7 transposons were introduced into the genome of Sprague-Dawley outbred albino rats by injection of plasmids carrying the transposons and *in vitro* synthesized RNA encoding piggyBac transposase into the pronuclei of zygotes (Jang and Behringer 2007). Fourteen (14/22 born, 64%) Bhr2 and four (4/11 born, 36%) Bhr7 founder rats were generated. All of the resulting transposon positive founders identified by PCR had darkly pigmented heads and dorsa (Figure 2A). Thus, the *Tyr* reporter expressed from both types of transposons rescued the albinism and revealed the *hooded* mutant coat color phenotype present in the genetic background of the albino strain. If integration of the transposons was by transposition, then the transposons should be present as single-copy insertions but could integrate on more than one chromosome. Therefore, founders were bred with Sprague-Dawley albino mates to generate progeny. Interestingly, 100% of the progeny from all of the founders were pigmented and pigmentation varied between individual progeny (Figure 2B). These results suggest that each founder contained transposon insertions at multiple loci and that each insertion contributes to the total levels of *Tyr* expression. Southern blot analysis of the founders and progeny demonstrated that the founders had five to eight transposon copies and that all transposons were present at one copy per locus (Figure 2C and data not shown).

To facilitate our mutagenesis strategy, we bred the founders with wild-type albino rats and segregated the transposon insertions assessed by Southern blot (data not shown) to generate pedigrees with single-transposon insertions (Figure 3A). This was initiated using the Kyoto Wistar (WKY/NCrl) partially inbred rat but subsequently we used the Kyoto Wistar (WKY/NHsd) inbred strain. Each pedigree had a unique coat color, including light ash gray, medium gray, dark gray, tan, or brown (Figure 3B). In addition, two pedigrees with Bhr7 insertions were albino, indicating that

Figure 2 Generation of transgenic rats by piggyBac transposition. (A) Litter generated from piggyBac transposon Bhr2 injected to albino Sprague-Dawley rat zygotes. Bhr2 piggyBac transposon founder transgenic rats are pigmented. Sprague-Dawley rats are homozygous for the *hooded* mutation that restricts pigmentation to the head and dorsum. (B) Offspring from a piggyBac transposon founder male crossed with a Sprague-Dawley female. All progeny were pigmented and each individual had a different coat color. (C) Southern blot analysis using a Bhr7-specific probe, showing multiple transposon integrations in a founder (F) and their segregation in progeny (1–8) generated by crossing with a Sprague-Dawley female.

Figure 3 Segregation and analysis of initial piggyBac transposon insertions. (A) Southern blot analysis, showing the isolation of single Bhr7 transposon gene insertions in separate pedigrees. L, *LOC685774*; P, *Phex*; +, wild type. Bhr2 transposon insertion in the *Gabrb1* locus (G). The *Tyr* probe used for Bhr2 Southern analysis recognizes a single band of ~7.4 kb for the endogenous *Tyr* locus. The Bhr2 transposon insertion in the *Gabrb1* locus shows a single band of ~7.6 kb and therefore appears the same as wild type. (B) Coat color differences in rats with different single-transposon insertions. The rats with insertions in *LOC685774* or *Phex* loci remained albino. (C) Rats with the Bhr2 transposon in the *Gabrb1* locus. Tn/Tn rats are darker than Tn/+ rats. (D) Rats with the Bhr7 transposon in the *Zbtb20* locus. Tn/Tn rats are darker than Tn/+ rats. (E) PCR genotyping of progeny from a cross between *Gabrb1*^{Bhr2} hemizygotes.

either the transposons were physically damaged or epigenetically silenced perhaps by chromosomal position effects (Figure 3B). The unique level of pigmentation associated with each transposon insertion site remained stable through subsequent generations. These results suggest that the level of expression of the *Tyr* pigmentation reporter from each transposon insertion site varied depending upon influences at the locus of integration.

The genomic locations of some of the single-copy transposon insertions generated from the original pronuclear injections were identified by inverse PCR (iPCR), DNA sequencing, and verified by PCR genotyping (Table 2, Figure 4). One of the Bhr2 transposon insertions was located in intron 4 of the *Gamma-aminobutyric acid A receptor, subunit beta 1* (*Gabrb1*) locus (see below). Hemizygotes (Tn/+) had tan pigmentation and intercrosses between hemizygotes yielded albino (+/+), tan (Tn/+), and brown (putative Tn/Tn) progeny (Figure 3C). The brown progeny were viable with no obvious abnormal phenotypes. These genotypes were verified by PCR using specific primers for the wild-type and mutant alleles (Figure 3E). A second example of the correlation of coat color with genotype is shown for a Bhr7 insertion in the *Zbtb20* locus (Figure 3D). Thus, the *Tyr* pigmentation reporter can be used for visual genotyping of the transposon insertion site without the need for molecular analysis (e.g., PCR). The two pedigrees with the Bhr7 transposon insertions that remained albino had insertions upstream of the first methionine-encoding exon of *LOC685774* and ~212 bp upstream of *Phex* (Figure 3B).

Interestingly, both *LOC685774* and *Phex* are located on the X chromosome. *LOC685774*^{Bhr7} and *Phex*^{Bhr7} males appeared normal and were fertile. The rats with known positions for Bhr2 in the *Gabrb1* locus and Bhr7 in *LOC685774* served as genetic reagents to develop our mutagenesis strategy.

Generation and characterization of piggyBac and Sleeping Beauty transposase transgenic rats

To mobilize the single-copy Bhr2 and Bhr7 transposon insertions to create new insertional mutations by simple crosses, we generated transgenic rats to ubiquitously express either Sleeping Beauty or piggyBac transposase by standard pronuclear injection of albino zygotes. The human *UBIQUITIN-C* (*UBC*) promoter was used to express the transposase genes (Figure 5A) (Lois *et al.* 2002). For Sleeping Beauty transposase, an improved variant designated HSB3 was used (Yant *et al.* 2004). The transposase gene constructs were flanked by pairs of transcriptional insulators from the chicken *beta-globin* locus to reduce the influence of the site of chromosomal integration. Founders were identified by PCR using primers to *UBC* and transposase cDNA (Figure 5A). One (1/15, 7%) *UBC-SleepingBeauty* (*UBC-SB*) and three (3/8, 38%) *UBC-piggyBac* (*UBC-PB*) founders were generated.

The *UBC-SB* and *-PB* transgenic rats were screened for their ability to mobilize single-copy Bhr2 and Bhr7 transposon insertions. *Gabrb1*^{Bhr2} (tan) and *LOC685774*^{Bhr7} (albino) hemizygous rats were bred with *UBC-SB* and *UBC-PB*

Table 2 Summary of initial transposon insertions in founders

Tn	Locus	Chr
Bhr2	<i>Gabrb1</i>	14
Bhr2	7 IRs	Multiple loci
Bhr7	<i>Ataxn7</i>	15
Bhr7	<i>LOC685774</i>	X
Bhr7	<i>Mug^a</i>	4
Bhr7	<i>Phex^b</i>	X
Bhr7	<i>Smurf2</i>	10
Bhr7	<i>Sucg2</i>	4
Bhr7	<i>Zbtb20</i>	11
Bhr7	3 IRs	Multiple loci

Chr, chromosome; IR, intergenic region; Tn, transposon.

^a iPCR sequence data for this single-copy Tn insertion matches four genes (*LOC297568*, *Mug1*, *Mug2*, and *F1M4L8_RAT*) linked within a ~500-kb region. *F1M4L8_RAT* is *Cpamd8*, which is *Mug2* in the mouse. The precise location of the Tn is currently ambiguous because the genes have high similarity with each other. Only in the case of *F1M4L8_RAT* is the Tn in the correct orientation for gene trapping.

^b Tn insertion located in promoter region in reverse orientation relative to the direction of transcription.

transgenic rats to generate bigenic progeny carrying both the transposon and transposase transgenes designated as “seed” rats. All of the seed rats generated using *Gabrb1^{Bhr2}*, *LOC685774^{Bhr7}*, *UBC-SB*, and *UBC-PB* showed coat color mosaicism, suggesting somatic transposition to new loci (Figure 5, B and C). The pigmentation observed in the seed rats generated using *LOC685774^{Bhr7}* demonstrated that Bhr7 was functionally intact and could express the *Tyr* pigmentation reporter when mobilized to a permissive site within the genome. These results suggest that the *UBC* promoter can direct transposase expression during development to mobilize and reinsert Bhr2 and Bhr7 transposons at new genomic locations that are revealed by higher or lower levels of *Tyr* expression relative to the site of the initial transposon insertion.

Mobilization of single-copy transposons in the rat germline

The coat color mosaicism in the seed rats indicated that transposon mobilization might also occur in their germ cells. To determine whether transposition events occurred in the germ cells of the seed rats, we crossed the seed rats with albino mates. If transposition events occurred in the germ cells of the seed rat, then the progeny that inherited insertions at new loci would be revealed by pigmentation that is different from that of the original transposon insertion.

Male *Gabrb1^{Bhr2}*; *UBC-SB* or *-PB* seed rats were bred with wild-type albino females. PCR genotyping for *Gabrb1^{Bhr2}* and the transposase transgene was performed on the resulting progeny. Albino progeny were either wild type or transposase positive. Pigmentation mosaic progeny carried *Gabrb1^{Bhr2}* and the transposase transgene. Tan progeny were *Gabrb1^{Bhr2}* hemizygotes without the transposase transgene (Figures 3C and 6, A–C). We also obtained pups with a homogenous color that was different from the original *Gabrb1^{Bhr2}* insertion (Figure 6B). These pigmented rats

Figure 4 Diagram of transposon insertions in the rat genome. Yellow circles, initial transposon insertions; blue circles, insertions mobilized from *Gabrb1^{Bhr2}* or *LOC685774^{Bhr7}* by piggyBac transposase; red circles, insertions mobilized from *Gabrb1^{Bhr2}* or *LOC685774^{Bhr7}* by Sleeping Beauty transposase.

were genotyped as wild allele for *Gabrb1^{Bhr2}* and lacked the transposase transgene, indicating that Bhr2 had transposed to a new locus (Figure 6C). Southern blot analysis using a *Tyr* fragment probe fortuitously produces similar sized bands for the endogenous *Tyr* locus and *Gabrb1^{Bhr2}* when digested with *PvuII* (Figures 3A and 6D). Two of the progeny (1 and 3) showing new coat colors had a hybridizing band distinct in size from the endogenous *Tyr* locus and the original *Gabrb1^{Bhr2}* transposon insertion (Figure 6D). In one of the progeny (2), the size of the hybridizing band was very similar to the size of the endogenous *Tyr* locus and *Gabrb1^{Bhr2}* even though the rat was much darker and genotyped by PCR as wild type for *Gabrb1^{Bhr2}* (Figure 6, B–D). These results demonstrate that new transposition events occurred in germ cells of the male seed rats and became fixed when segregated from the transposase transgene. In addition, these new transposition events corresponded with a change in coat color.

Male *LOC685774^{Bhr7}*; *UBC-SB* or *-PB* seed rats with coat color mosaicism were bred with wild-type albino females. Albino progeny from this cross were either (1) wild type, (2) transposase positive, or (3) *LOC685774^{Bhr7}*, whereas (4) coat color mosaics carried *LOC685774^{Bhr7}* and the transposase transgene. Some of the progeny had homogenous pigmentation (Figure 7B) and were genotyped as wild allele for *LOC685774^{Bhr7}* and lacked the transposase transgene (Figure 7C). Southern blot analysis using a Bhr7-specific probe showed that the transposon in the pigmented progeny had moved to a new locus (Figure 7D). Similar to the *Gabrb1^{Bhr2}* results above, the *LOC685774^{Bhr7}* insertion can be mobilized in the germline and segregated away from the transposase by a simple cross. In the case of the *LOC685774^{Bhr7}* insertion, however, transposon mobilization events are more easily recognized by the presence of homogeneously pigmented progeny (i.e., albino vs. pigmented) rather than a coat color

Figure 5 Generation and functional analysis of piggyBac and Sleeping Beauty transposase-expressing transgenic rat lines. (A) Diagram of *UBC-SB* and *UBC-PB* gene constructs. The Sleeping Beauty or piggyBac transposase expression is driven by the human *UBC* promoter and flanked by chicken hypersensitive site 4 transcriptional insulators (cHS4) to reduce the influence of chromosomal integration sites. Transposase transgenic rats were identified by PCR using *UBC-F* and *SB-R* or *PB-R* primers (black arrowheads). Primer sequences for genotyping are described in Table 1. pA, rabbit β -globin intron and polyadenylation signal. (B) Male containing the Bhr2 transposon in the *Gabrb1* locus (left) and seed male containing the Bhr2 transposon in the *Gabrb1* locus and Sleeping Beauty (center) or piggyBac (right) transposase transgenes. All seed rats exhibited coat color mosaicism. (C) Male containing the Bhr7 transposon in *LOC685774* locus (left) and seed male containing the Bhr7 transposon in the *LOC685774* locus and Sleeping Beauty transposase (center) or piggyBac transposase (right) transgene. All seed rats were pigmented.

change compared to the original insertion (i.e., tan vs. a different color). Based on the above results, we designed two simple transposon mutagenesis strategies to generate mutant rats (Figures 6E and 7E).

Transposon mutagenesis and isolation of rat mutants

Using our coat color-based mutagenesis system we generated a set of rats with new transposon insertions. The transposition frequency in the germline using Sleeping Beauty transposase (*UBC-SB*) was 8.5% (12 new insertions/141 progeny screened) and using piggyBac transposase (*UBC-PB*) was

11.1% (9/81). The combined frequency was 9.5%. At this frequency about one new insertion per litter is expected.

iPCR and DNA sequencing identified the location of 16 of the 21 new insertions (Table 3, Figure 4). Twelve of these 16 (75%) new insertions occurred on a different chromosome relative to the initial transposon insertion site, whereas 4 were on the same chromosome (Table 3). Two of the new insertions mobilized from *Gabrb1*^{Bhr2} by piggyBac transposase were 4 and 11 kb away from the initial transposon insertion site. 8 of the 16 (50%) transposon insertions were in introns of genes. For Sleeping Beauty transposase, one of the new insertions mobilized from *Gabrb1*^{Bhr2} remained on chromosome 14 and one of the new insertions mobilized from *LOC685774*^{Bhr7} remained on the X chromosome. Five of the intron insertions were generated by Bhr2 (Table 3). Three of the intron insertions were generated by Bhr7 and all of these were in the desired orientation relative to the direction of endogenous gene transcription (Table 3). Progeny with transposon insertions at different loci were obtained from the same male seed rat, demonstrating that multiple unique transposition events can be recovered from an individual seed rat. In addition, we did not recover progeny with new transposon insertions at the same locus from an individual male seed rat. Combining the insertions generated by the initial pronuclear injections mediated by piggyBac transposase and the insertions generated by crosses, we have generated 12 rat lines with gene-trap insertions in the following genes: *Adam9*, *Ataxin7*, *Col4a3bp*, *Gabrb1*, *LOC685774*, *Plcb1*, *Prrxl1*, *RGD1310778*, *RGD1311463*, *Smurf2*, *Sucg2*, and *Zbtb20* (Tables 2 and 3, Figure 4).

To determine whether the Bhr2 transposon can trap endogenous mRNA, we analyzed rats homozygous for *Gabrb1*^{Bhr2}. RT-PCR was performed using total RNA from adult brain (Figure 8). Bhr2 is located in intron 4 of *Gabrb1*. Primers specific to *Gabrb1* exon 4 and the SA of Bhr2 amplified cDNA from the homozygous mutants, indicating that the gene trap sequences were functional (Figures 1A, 6A, and 8). In addition, primers were designed to determine whether wild-type transcripts could also be generated by the mutant locus. These experiments demonstrated that some wild-type transcripts were also generated from the mutant locus, indicating that splicing was occurring around the transposon sequences (Figure 8). Thus, the Bhr2 transposon located in intron 4 of the *Gabrb1* locus could trap the mRNA transcribed by the locus but not completely, suggesting that this is a hypomorphic allele.

Discussion

We have developed a hybrid transposon mutagenesis system in rats that uses simple crosses to mobilize single-copy transposons. Our transposons located in known genomic locations can be mobilized by matings to rats that express either Sleeping Beauty or piggyBac transposase. New integration sites in the genome are identified by changes in coat color. The Sleeping Beauty transposon

Figure 6 Structure of the Bhr2 transposon insertion in the *Gabrb1* locus and germline mobilization. (A) Diagram of the Bhr2 transposon insertion site in the *Gabrb1* locus. Brown arrow shows the direction of *Tyr* transcription. Primers for genotyping, arrowheads. Gab-F and Gab-R can detect the wild-type allele and Gab-F and B2-3 can detect the transposon allele. Primers for RT-PCR analysis, red arrowheads; Ex, exon. (B) Coat colors of the progeny with new transposon insertions originating from the *Gabrb1* Tn locus generated by crosses of seed males and transposase-expressing rats. Rats 1–3 have different pigmentation compared to the original Bhr2 transposon *Gabrb1* rat G, *Gabrb1* Tn/+ rat. (C) PCR analysis of genomic DNA. The Sleeping Beauty transposase transgene is no longer present in the progeny (1–3) carrying the transposon at a new position (top). The rat with the Bhr2 transposon in the *Gabrb1* locus has a wild-type (450 bp) and transposon (628 bp) allele but the rats with the new transposition events only have the wild-type band, demonstrating that the Bhr2 transposon has transposed away from the *Gabrb1* locus (bottom). Rats 1–3 and G are the same as in B. PC, positive control; Wt, wild type. (D) Transposition events revealed by Southern blot analysis of offspring from seed males using *Tyr* probe. The Bhr2 transposon in the *Gabrb1* locus and the endogenous *Tyr* locus digested with *PvuII* resulted in 7.6 kb (G) and 7.4 kb (Wt) bands, respectively. Progeny generated from seed male with different coat colors compared to *Gabrb1*^{Bhr} rats have an additional band (*). Progeny 2 likely has a new insertion that yields a band of similar size to the *Gabrb1* insertion. Progeny 1–3 and G are the same as in C. (E) Mutagenesis strategy using Bhr2 transposon insertion in the *Gabrb1* locus. The seed male carrying both the transposon and transposase transgene that has coat color mosaicism is bred with an albino female. Progeny carrying the transposon at a different locus should have a different coat color compared to the original Bhr2 *Gabrb1* insertion. Primer sequences for genotyping and RT-PCR are described in Table 1.

system has been used for mutagenesis in rats but until now there have been no reports using piggyBac transposons to generate mutations (Kitada *et al.* 2007; Lu *et al.* 2007; Izsvák *et al.* 2010). PiggyBac/Sleeping Beauty hybrid transposons have been previously reported and shown to function *in vitro* (Wang *et al.* 2008; Liang *et al.* 2009). Here we show that hybrid transposons can be mobilized by both types of transposases in rat somatic and germ cells *in vivo*. Once rats with new insertions are identified by coat color, the precise site of integration can be determined quickly, using standard molecular techniques, *i.e.*, PCR and DNA sequencing. Thus, using our system requires no specialized skills to generate mutant rats in contrast to the unique methods required by other rat mutagenesis strategies, *e.g.*, *in vitro* fertilization, rat ES cell culture and chimera generation, spermatogonial stem cell culture, and seminiferous tubule transplantation (Buehr *et al.* 2008; Li *et al.* 2008; Mashimo *et al.* 2008; Oatley and Brinster 2008; Izsvák *et al.* 2010; Kawamata and Ochiya 2010; Tong *et al.* 2010). It may be more efficient to generate transposon mutant rats by zygote pronuclear injection rather than by breeding but again this requires special skills and resources for micromanipulation.

Our transposon vectors contain a *Tyr* minigene that can rescue albinism (Yokoyama *et al.* 1990; Overbeek *et al.* 1991). Nearly all of our transgenic rats with single-copy transposon insertions had detectable pigmentation, although even the darkest pigmented pedigrees were lighter than wild type. Thus, the *Tyr* minigene can express at many locations within the genome (Yokoyama *et al.* 1990; Overbeek *et al.* 1991; Lu *et al.* 2007). In addition, as the level of expression of the minigene is influenced by chromosomal context, different transposon insertion sites in the genome resulted in different levels of pigmentation (Yokoyama *et al.* 1990; Overbeek *et al.* 1991; Lu *et al.* 2007). For most transposon integrations, there was a gene dosage effect. Rats that are homozygous for the transposon insertion usually were obviously darker than hemizygotes. Thus, the *Tyr* minigene allows visual genotyping and greatly simplifies the identification of homozygous, hemizygotes, and wild-type rats (Yokoyama, *et al.* 1990; Overbeek *et al.* 1991; Lu *et al.* 2007). Therefore, no molecular analyses are required to genotype the rats once the pedigrees are established, greatly simplifying their maintenance and analysis.

We isolated two transposon insertion sites (*LOC685774*^{Bhr7} and *Phex*^{Bhr7}) that were albino either because *Tyr* expression is

Figure 7 Structure of the Bhr7 transposon insertion in the LOC685774 locus and germline remobilization. (A) Diagram of Bhr7 transposon insertion site in the LOC685774 locus. Brown arrow shows the direction of *Tyr* transcription. Primers for genotyping, arrowheads. LOC-F and LOC-R can detect the wild-type allele and LOC-F and PB-IR-5.2 can detect the transposon allele. Ex, exon. (B) Coat color of progeny 1 and 2 with new transposition insertion sites. (C) PCR analysis of genomic DNA for the piggyBac transposase transgene (top) and transposon insertion in LOC685774 (bottom). Top shows that the piggyBac transposase transgene is not present in the rats carrying the Bhr7 transposon at a new position (1 and 2). Bottom shows the transposon in the LOC685774 locus is not present in the pigmented progeny (1 and 2). Progeny 1 and 2 are the same as in B. L, Bhr7 transposon in the LOC685774 locus of a Tn/+ female; Wt, wild type, S, seed rat. (D) Transposition events revealed by Southern blot analysis of offspring from seed males using a Bhr7-specific probe. The seed male (S) has multiple bands because of multiple transposition events in somatic tissues. The progeny that are pigmented have single bands of different size compared to the Bhr7 transposon in the LOC685774 (1 and 2). 1, 2, L, Wt, and S are the same as in C. (E) Mutagenesis strategy using Bhr7 transposon insertion in the LOC685774 locus. Rats carrying the Bhr7 transposon in the X chromosome-linked LOC685774 locus are albino. The seed male rat carrying both the transposon and transposase transgene that shows coat color mosaicism is bred with an albino female. Progeny carrying the transposon at a different locus can become pigmented. Primer sequences for genotyping are described in Table 1.

repressed by chromosomal context or the *Tyr* minigene was damaged upon integration (Karpen 1994; Pikaart *et al.* 1998). Bigenic rats carrying *LOC685774^{Bhr7}* and either the *UBC-SB* or *UBC-PB* transgenes showed mosaic pigmentation and outcrosses to albino females yielded pigmented progeny. Therefore, we conclude that Bhr7 in the LOC685774 locus is structurally intact but *Tyr* expression is repressed at this chromosomal location. These transposon insertions whose expression is repressed and therefore albino proved to be very useful because the mutagenesis screen becomes simply looking for any pigmented progeny in crosses between seed males and albino females rather than distinguishing one color from another.

Previous transposon mutagenesis studies have generated initial transgenic mice or rats by standard pronuclear injection to create single integrations with transposon concatemers (Dupuy *et al.* 2001; Fischer *et al.* 2001; Horie *et al.* 2001; Dupuy *et al.* 2002; Ding *et al.* 2005; Kitada *et al.* 2007; Lu *et al.* 2007; Urschitz *et al.* 2010). These have been crossed with transgenic animals that express the corresponding transposase in the germline, using male germ

cell-specific regulatory sequences. One problem with mobilizing transposons in a concatemer is chromosomal damage and scattering of the transposons to multiple sites within the genome, complicating the molecular identification of the new insertions (Geurts *et al.* 2006). To circumvent these problems, we introduced our transposons into the rat genome by transposition to obtain single-copy insertions, albeit at multiple chromosomal locations (Jang and Behringer 2007). It might be possible to generate founders with single-transposon insertions, bypassing the subsequent breeding required to generate single-transposon pedigrees by titrating the transposon-containing plasmid or transposase RNA injected into zygotes. Our founders had five to eight transposon insertions and were very darkly pigmented but their progeny that inherited varying numbers of the transposons had lighter pigmentation. This suggests that multiple transposons are expressing *Tyr*, and the pigmentation of the founder is a collation of *Tyr* expression. Therefore, the generation of transgenic founder animals by piggyBac transposition may be useful in certain species when expression is desired and germline

Table 3 Summary of transposon insertions after transposition from the initial transposon insertions

Tn	Initial locus (Chr)	New locus	Chr	Transposase
Bhr2	<i>Gabrb1</i> (14)	<i>Gabrb1</i> ^a	14	PB
Bhr2	<i>Gabrb1</i> (14)	<i>Gabrb1</i> ^a	14	PB
Bhr2	<i>Gabrb1</i> (14)	<i>Col4a3bp</i>	2	SB
Bhr2	<i>Gabrb1</i> (14)	<i>Plcb1</i>	3	PB
Bhr2	<i>Gabrb1</i> (14)	<i>Prrxl1</i>	16	SB
Bhr2	<i>Gabrb1</i> (14)	4 IRs	5, 7, 10, 14	SB
Bhr7	<i>LOC685774</i> (X)	<i>Adam9</i>	3	PB
Bhr7	<i>LOC685774</i> (X)	<i>RGD1311463</i>	3	PB
Bhr7	<i>LOC685774</i> (X)	<i>RGD1310778</i>	11	SB
Bhr7	<i>LOC685774</i> (X)	4 IRs	1, 1, 9, X	SB

Chr, chromosome; IR, intergenic region; PB, piggyBac; SB, Sleeping Beauty; Tn, transposon.

^aIndependent transposition events within the same intron 3' of the original Tn insertion.

transmission is not required or difficult (e.g., nonhuman primates) (Chan *et al.* 2001). This can also be achieved using lentiviral vectors (Lois *et al.* 2002; Sasaki *et al.* 2009).

Our initial attempts to generate transgenic rats that express Sleeping Beauty or piggyBac transposase specifically in the germline using *Oct4* sequences were not successful (Yeom *et al.* 1996). We therefore, decided to express these transposases ubiquitously using the human *UBC* regulatory sequences. This proved profitable because the seed males could be visually identified very easily because they developed coat color mosaicism, indicating transposon mobilization in melanocytes or melanocyte precursors. Crosses of the seed males containing single-copy transposons with albino females demonstrated germline transposition for both Sleeping Beauty and piggyBac transposases.

The frequency of new insertions by germline mobilization of single-copy transposons in our system using *UBC-PB* or *UBC-SB* was ~10%. Considering the average litter size of the rat strains we used, this would translate to about one new insertion per litter. There have been a few reports in mice, demonstrating the remobilization and insertion of single-copy Sleeping Beauty transposons by crosses with Sleeping Beauty transposase transgenic lines. In one report, one remobilization/insertion event was obtained in ~100 progeny screened, using a CAGGS-SB10 transgene (Geurts *et al.* 2006). In another study, remobilization/insertion was much more efficient ranging from 17 to 50%, using a *Prrxl1*-*HSB16* transgene (Ruf *et al.* 2011). The variation in the frequency of remobilization/insertion of single-copy piggyBac or Sleeping Beauty transposons may be due to the different transposase-expressing transgenic lines used. Until now there has been no report of mobilizing single-copy transposons by simple crosses in rats. We obtained transposition from the *Gabrb1*^{Bhr2} and *LOC685774*^{Bhr7} loci to sites on the same and different chromosomes. Although the numbers are small, it appears that both Sleeping Beauty and piggyBac transposition onto the same chromosome occur at similar frequencies in our system. For the transpositions onto the same chromosome, we generated two additional alleles for *Gabrb1*. These

Figure 8 Expression analysis in *Gabrb1* mutant rats. Adult brain mRNA of wild-type (left) and *Gabrb1* (Tn/Tn) (right) rats. Primers for RT-PCR (red arrow-heads) are shown in Figures 1A and 6A. Primers G-RT5F/G-RT5R, G-RT1F/G-RT1R, and G-RT3F/G-RT3R amplify mRNA before (5), between (I), and after (3) the transposon insertion site, respectively. Primers G-RT1F/B2-RT amplify mRNA trapped by the transposon in the presence of reverse transcriptase (+RT). All primer sets do not amplify DNA in the absence of RT (–RT). The Tn/Tn rat shows trapped mRNA (Tn) and wild-type mRNA (I and 3). Primers b-act F/b-act R were used to detect β -actin expression (C). Primer sequences for RT-PCR are described in Table 1. m, 100-bp ladder DNA marker.

were located 4 and 11 kb from the original intron 4 insertion site into the same intron, respectively. Therefore, perhaps for larger genes, our system could be used to generate an allele series for a particular gene of interest.

In this initial screen, we generated insertions in 12 genes in the rat genome. Six of these genes have corresponding mouse mutants, including *Adam9*, *Col4a3bp*, *Plcb1*, *Prrxl1*, *Smurf2*, and *Zbtb20*. Thus, at this point in time about half of our rat mutants do not have an equivalent mouse mutant. *Prrxl1* knockout mice have skin and pain sensitivity abnormalities and die at about 3 weeks of age (Chen *et al.* 2001). Our *Prrxl1* homozygous mutant rats appear normal. It is not clear whether this is because our allele is a hypomorph or that the rat genetic background is not permissive for expression of the skin or postnatal viability phenotype.

For the initial Bhr2 insertion into the *Gabrb1* locus, molecular analysis demonstrated that the gene trap sequences were functional; however, wild-type transcripts could also be detected in the homozygotes most likely due to splicing around the gene trap sequences (McClive *et al.* 1998; Voss *et al.* 1998). The *Gabrb1* homozygous mutant rats appeared normal and were fertile. There is currently no mouse knockout for this gene; however, other GABA receptor mutants have been reported to be overtly normal perhaps because of the complexity of the GABA receptor system (Homanics *et al.* 1997; Sur *et al.* 2001). Molecularly, this *Gabrb1* mutation is a hypomorphic allele. Hypomorphic alleles for certain types of genes could be very useful in cases when a null allele causes lethality at early embryonic stages or before adulthood. Perhaps Bhr2 and Bhr7 could be modified to generate more efficient gene traps, e.g., different splice acceptor sequences. However, our current system has been used by others to create a protein null allele in the rat *Bobby Sox* homolog, *Bbx* (C.-M. Chen, personal communication).

Our initial results indicate that germline transposition can produce new integrations sites that are dispersed throughout the genome, implying that mutations can be created at many different loci. This is also true for chemical

mutagenesis strategies (Zan *et al.* 2003; Smits *et al.* 2005; 2006; Homberg *et al.* 2007; Olivier *et al.* 2008; van Boxtel *et al.* 2008; Mashimo *et al.* 2008; Homberg *et al.* 2009; van Boxtel *et al.* 2010) and transposon mutagenesis in spermatogonial stem cells (Izsvák *et al.* 2010). Current methods to generate targeted mutations in specific genes include gene targeting in rat ES cells, zinc finger nucleases, and TALE nucleases (Geurts *et al.* 2009; Mashimo *et al.* 2010; Tesson *et al.* 2011; Tong *et al.* 2012). Many of these systems required very specialized technical skills not available in most laboratories. We are cryopreserving our transgenic rat strains, including *Gabrb1*^{Bhr2}, *LOC685774*^{Bhr7}, *UBC-SB*, and *UBC-PB*. Because our system requires breeding rats, visual genotyping, and standard molecular techniques most biomedical laboratories should be able to perform a mutagenesis screen using these rat strains. This system will lead to the generation of a large collection of single-gene mutations for new rat models of human biology and disease.

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