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Efficient mammalian germline transgenesis by cis-enhanced *Sleeping Beauty* transposition

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Abstract

Heightened interest in relevant models for human disease increases the need for improved methods for germline transgenesis. We describe a significant improvement in the creation of transgenic laboratory mice and rats by chemical modification of *Sleeping Beauty* transposons. Germline transgenesis in mice and rats was significantly enhanced by in vitro cytosine-phosphodiester-guanine methylation of transposons prior to injection. Heritability of transgene alleles was also greater from founder mice generated with methylated versus non-methylated transposon. The artificial methylation was reprogrammed in the early embryo, leading to founders that express the transgenes. We also noted differences in transgene insertion number and structure (single-insert versus concatemer) based on the influence of methylation and plasmid conformation (linear versus supercoiled), with supercoiled substrate resulting in efficient transpositional transgenesis (TnT) with near elimination of concatemer insertion. Combined, these substrate modifications resulted in increases in both the frequency of transgenic founders and the number of transgenes per founder, significantly elevating the number of potential transgenic lines. Given its simplicity, versatility and high efficiency, TnT with enhanced *Sleeping Beauty* components represents a compelling non-viral approach to modifying the mammalian germline.

Keywords

Sleeping Beauty; Transposon; Transgenesis; Mouse; Rat; Methylation

Introduction

The rate of transgenesis by pronuclear injection (PNI) of linearized plasmid fragments in various strains of mice, rats, pigs, and cattle ranges from 1 to 4% per embryo injected, 5–20% of live-born animals, depending on species (Brem and Muller 1994; Wolf et al. 2000; Tesson et al. 2005; Filipiak and Saunders 2006). When successful, the typical result of injecting these DNA fragments is the random nonhomologous integration of a repetitive, multicopy transgene concatemer. Repetitive sequences such as these are prone to destabilization and transgene silencing (Dorer and Henikoff 1997; Garrick et al. 1998; Geurts et al. 2006a, b), frequently leading to transgenic animals that do not faithfully express the desired transgene. Despite significant enhancement (up to 10-fold) of transgenesis rates per embryo by the perivitelline injection of lentiviruses (Lois et al. 2002; Hofmann et al. 2003, 2004), limits in cargo size, technical challenges in viral production, and the proclivity of proviral integration into genes (He et al. 2005; Hofmann et al. 2006; Michalkiewicz et al. 2007), have limited its widespread application. As an alternative, two binary DNA-based transposons, *Sleeping Beauty* (SB) (Dupuy et al. 2002; Mátés et al. 2009) and *piggyBac* (Ding et al. 2005), have been successfully applied to mammalian transgenesis by PNI. In such two component systems, a transgene of interest can be flanked by specific terminal repeats that function as transposase-recognition sites. The *trans* provision of transposase protein results in excision of the transgene from vector DNA and subsequent integration into the target genome. Co-injection of SB transposon donor and a source of transposase into the pronucleus of mouse zygotes led to transpositional transgenesis (TnT) at rates exceeding injection of the donor transposon alone (up to 50%) (Dupuy et al. 2002; Mátés et al. 2009), while pronuclear co-injection of *piggyBac* components resulted in a transgenesis frequency of 36–65%, with rates apparently dependent on the sequence or size of the transposon cargo (Ding et al. 2005). Several improvements have since been made to the SB transposon (Cui et

al. 2002; Zayed et al. 2004) and transposase sequences (Geurts et al. 2003; Mátés et al. 2009). Furthermore, modification of SB transposons by cytosine-phosphodiester-guanidine (CpG) methylation was observed to significantly enhance transposition in cultured cells (Yusa et al. 2004; Ikeda et al. 2007). With improved SB components and a better understanding of the modulators of transposition, we demonstrate here a dramatic enhancement in the production efficiency of transgenic rodents and describe parameters critical to the structure of transgenes.

Results

Efficient rodent transgenesis by enhanced TnT

Mouse germline transgenesis by SB transposition was previously reported to result in a significant, but modest improvement over standard pronuclear injection techniques (Dupuy et al. 2002). A newly developed hyperactive SB100X transposase was recently found capable of efficient mouse transgenesis, although a genomic and heritability analyses were not presented (Mátés et al. 2009). We revisited SB mediated transgenesis using improved cis-acting components with SB11 (Cui et al. 2002; Geurts et al. 2003), with a focus on investigating the influence of transposon CpG methylation and vector conformation on transgenesis. The T2/sh_mCFTR1, KT2/HSACCTG₃₀₀, and KT2/KD^{Rab38} SB transposon-based transgenes (Fig. 1a, Supplementary Figs. 1, 2) were generated for modeling human single-gene disorders in transgenic laboratory mice and rats, while the KT2H-CD40Ig transposon was designed to direct β -cell specific expression of the fusion protein CD40Ig (Noelle et al. 1992). Cocktails for the various methylated or nonmethylated linear transposon plasmid DNA substrates plus in vitro transcribed, capped SB11 transposase mRNA were injected into wild type mouse or rat pronuclei to determine the effects of CpG methylation on gene transfer to the early embryo.

We characterized the rate of transgenesis (% transgenic per liveborn) and the structure of transgenes for founders generated with methylated and nonmethylated SB transposons using PCR and Southern analysis. Injection with transposons plus transposase mRNA resulted in both transgenic and nontransgenic founders (Fig. 1). Southern analysis of both T2/sh_mCFTR1 and KT2H-CD40Ig animals revealed founders containing transgenes with structure-profiles corresponding to transgenesis by transposition only (TO), transposition plus multicopy concatemer integration (TMC), and rarely by multicopy concatemer integration only (MCO) (Fig. 1).

The rate of mouse transgenesis was significantly (Z -test for proportions; $p = 0.0001$) and suggestively (Z -test for proportions; $p = 0.0537$) enhanced by methylation of T2/sh_mCFTR1 and KT2H-CD40Ig, respectively (Tables 1, 2). Notably, transgenesis with nonmethylated T2/sh_mCFTR1, which contains a short hairpin RNA expression cassette, was suggestively lower (Z -test for proportions; $p = 0.0909$) than that observed using nonmethylated transposons in this and previous studies (Dupuy et al. 2002), suggesting that transgenesis is somehow compromised when this construct is not methylated. Transgenesis with either the methylated or nonmethylated KT2/HSA-CCTG₃₀₀ transposon was quite efficient considering its large size, although CpG methylation did not significantly enhance the rate of transgenesis or the number of transposition mediated insertions per founder for this transposon (Supplementary Fig. 1; Tables 1, 2). While previous reports have indicated that large SB transposons do not transpose efficiently (Izsvak et al. 2000; Geurts et al. 2003) transposition is clearly evident in 13 of the 23 transgenic founders with a total of 33 independent insertions and an average of 2.5 ± 1 insertions per transposition positive founder (the number following “ $\pm$ ” sign is the 95% confidence interval and is used throughout the manuscript).

The structure of transgenes in founders was also significantly influenced by CpG methylation. While linear methylated or nonmethylated transposons resulted in transgenic founders with each structure-profile (TO, TMC, and MCO, Fig. 1; Table 2), more TO animals were observed using linear methylated KT2H-CD40Ig (56% of liveborns $\pm$ 13) and T2/sh_mCFTR1 (75% of liveborns $\pm$ 19) versus nonmethylated KT2H-CD40Ig (32% of liveborns $\pm$ 18) and T2/sh_mCFTR1 (19% of transgenics $\pm$ 19). Beyond enrichment for TO founders, the use of methylated transposons appears to significantly increase the number of independent transposon integrations per transgenic founder (Fig. 1; Table 2). TO founders generated with methylated KT2H-CD40Ig and T2_sh_mCFTR1 transposons harbored approximately 180 individual insertions (3.9 ± 0.7 per TO founder) compared to only 17 (1.5 ± 0.5 per TO founder) from non-methylated transposons. Inexplicably, analysis of the TMC founders revealed a high and nearly equivalent number of transposon integrations when either methylated (4.5 ± 1.6) and nonmethylated (4 ± 1) substrate was used. Since concatemers have been demonstrated as effective substrates for transposition (Horie et al. 2003; Geurts et al. 2006b), this raised the possibility that the observed transposition events were secondary to concatemer integration. Were this to be the case, we would expect transposons in the same founder to be closely linked, since mobilization from integrated concatemers results in a phenomenon referred to as local hopping (Horie et al. 2003; Geurts et al. 2006b). We therefore determined the location of transposon insertions from four different KT2HCD40Ig TMC founders by linker-mediated PCR and sequencing (LM-PCR, Supplementary Table 1). Among the 39 insertions mapped from four founders local hopping was not apparent (Supplementary Table 1). This suggests that concatemer integration and transposition are parallel not serial events. Notably, the number of independent transposition events per founder identified by LM-PCR closely matched estimates made by Southern analysis.

Germline mosaicism is commonly observed in transgenic animals generated by PNI (Wall and Burdon 1997). Southern analysis revealed differences in intra-founder band intensity in both methylated and nonmethylated founders, suggesting transgene mosaicism (Fig. 1a, b). To determine if transgene methylation had an impact on mosaicism, heritability analysis was conducted by outcrossing several founders generated with methylated and nonmethylated T2/sh_mCFTR1 and KT2H-CD40Ig transposons. Though germline mosaicism was observed in both groups, the heritability of individual transposase mediated insertions was significantly higher from founders generated with methylated transposon ($33\% \pm 9$) versus those generated with nonmethylated transposon ($13\% \pm 4.5$, Fig. 2a, b). Improvements in the rate of transgenesis, transgene copy-number and heritability thus demonstrate that methylation-enhanced TnT can significantly reduce the number of founders required to generate a transgenic mouse line.

While methylation-enhanced TnT is a valuable tool for generating transgenic mice, even greater value might be realized in working with mammals where transgenesis is not routine. Therefore, we tested our ability to generate transgenic rats using our most efficient condition for TnT. A linear, methylated pKT2/KD^{Rab38} transposon was injected into pronuclear staged rat embryos. This resulted in 7 of 11 ($64\% \pm 28$) transgenics among liveborn rats (Supplementary Fig. 2; Table 1). To verify that rat transgenesis was a result of SB transposition, LM-PCR was used to identify transposition events in each founder. Fourteen independent transposition events were identified among six of the seven founder animals, while the remaining founder demonstrated random, nonhomologous integration of the donor transposon plasmid (data not shown). Transgene expression was observed in 4 out of 6 founders (Supplementary Fig. 2C), and efficient germline transmission was observed for 4 out of 4 transgenic rats, each of which passed at least one copy of the KT2/KD^{Rab38} transposon through the germline (data not shown). These results clearly demonstrate the potential of this approach for gene supplementation in rats, and potentially other species.

Precise TnT with supercoiled DNA

Given the ability of concatemers to result in chromosomal rearrangements and sub-Mendelian transmission (Gordon and Ruddle 1985; Covarrubias et al. 1986; Overbeek et al. 1986; Wilkie and Palmiter 1987; Rohan et al. 1990; Mark et al. 1992; Hamada et al. 1993; Chen et al. 1995; Pravtcheva and Wise 1995; Bishop 1997; Takano et al. 1997; Nakanishi et al. 2002; Geurts et al. 2006a, b), and the fact that transgenes in concatemers are often silenced (Dorer and Henikoff 1997; Garrick et al. 1998), we sought to identify conditions that favored the generation of TO founders. It has previously been demonstrated that PNI of supercoiled DNA results in decreased concatemer formation, albeit with at least a fivefold reduction in transgenesis (Brinster et al. 1985). We reasoned that TnT with supercoiled transposons might nonetheless permit efficient mouse transgenesis. We injected mouse embryos with both methylated and nonmethylated supercoiled KT2H-CD40Ig transposon along with SB11 mRNA (Fig. 3a). Transgenesis using nonmethylated supercoiled DNA resulted in liveborn transgenesis of $24\% \pm 16$ whereas methylation of the supercoiled transposon resulted in $47\% \pm 16$ transgenesis (Fig. 3b; Tables 1, 2). Despite a reduction in overall transgenesis frequency compared to a linear transposon (by ~30%), concatemer formation was significantly reduced (*Z*-test for proportions; $p < 0.0001$). Among the 67 liveborn animals generated by injection with either methylated or nonmethylated supercoiled transposon, only one contained a transgene concatemer ($1.5\% \pm 3$). In contrast, 52 of the 158 liveborn animals injected with a linear transposon contained a transgene concatemer ($33\% \pm 8$). Interestingly, the reduction in transgenesis frequency by the use of supercoiled transposon is nearly equivalent to the rate of concatemer formation for linear transgenes. In addition, the difference between the number of transposase-mediated insertions per founder using supercoiled versus linear KT2H-CD40Ig ($\pm$ methylation, Fig. 3b; Table 2) was insignificant (two tailed *t*-test; $p > 0.60$). These observations further support the hypothesis that concatemer and transposon integrations occur in parallel. Thus, injection of supercoiled transposons results in the efficient production of TO transgenic mice without altering the number of transposon insertions per founder.

We further explored the statistical significance of the effects of transposon vector, methylation, and concatemerization on transposon copy number by fitting all mouse insert data to a generalized linear model. We considered an interaction between methylation and concatemerization since the enhancement of copy number in the presence of a concatemer appears to be restricted to founders generated with nonmethylated transposons. Since the majority (67%) of founders harbored 4 integrations or fewer (Supplementary Fig. 5), a Poisson distribution was assumed. Linear regression analysis confirmed that insert copy number varies significantly between transgene vectors and is positively affected by both methylation (despite insignificant enhancement KT2/HSA-CCTG₃₀₀ transposon) and the presence of a concatemer (Table 3). However, as noted above, the difference in the number of transposon integrations from linear and supercoiled forms of KT2H-CD40Ig is insignificant (A versus B, Table 3). Additionally, the analysis verified our suspicion that methylation and concatemerization interact to influence transposon copy number.

Erasure of transgene methylation with transcriptional activation

Cytosine methylation is an essential epigenetic modification to many eukaryotic genomes and is developmentally regulated. The genomes of both gametes have unique methylation patterns prior to fertilization; the paternal genome undergoes active, nonspecific demethylation, while methylation of the maternal genome depletes with every cell division until the morula stage (Armstrong et al. 2006). We therefore hypothesized that the methylation pattern of transposons integrated into the early embryo would be largely erased during development. Nevertheless, since hypermethylation of CpG residues within transgene sequences can cause gene silencing in transgenic animals (Betzel et al. 1996; Schumacher et

al. 2000; Chevalier-Mariette et al. 2003; Hofmann et al. 2006), we analyzed transposon transgenes in several founder mice to assess both their methylation status and transcriptional activity.

Two CpG-rich regions of T2/sh_mCFTR1 (SV and P, Fig. 4a) were examined by bisulfite sequencing on DNA extracted from liver. Since the methylation patterns could vary from transgene to transgene, we sequenced multiple (5) independently isolated PCR fragments for each animal. The raw data for several founders and their transgenic offspring is shown (Fig. 4b, Supplementary Fig. 3). If methylation was not reset during embryogenesis, we would expect the artificial in vitro methylation pattern to persist in adult animals injected with methylated transgenes. In contrast, over 50% of all reads from animals injected with methylated transgene were hypomethylated (<20% of CpGs) clearly indicating that methylation status of the transgene is reset in the early embryo. Consistent with the mammalian epigenome globally (Rakyan et al. 2004; Eckhardt et al. 2006), the percent methylation of transposons was bi-modal, with the vast majority of loci being either hyper (>80% of CpGs) or hypomethylated (Fig. 4b, c). The status of five independent transposon insertions was examined in F1 siblings (from separate founders) to assess the consistency of specific transgene methylation. We found significant conservation of the degree of methylation in siblings segregating the same transgene alleles (Fig. 4b), suggesting not only that in vitro methylated transposons are reprogrammed, but that they assume and maintain an epigenetic status consistent with their site of integration. Transgene expression analysis from both F0 and F1 reveals a broad range of Puro and shCFTR1 expression (Supplementary Fig. 6) in animals generated with both methylated and nonmethylated transposons, suggesting integration site is the primary determinant of transgene expression. Puromycin expression in the small intestine of single-copy F1 offspring from multiple founders reveals the expected relationship between genome methylation of the SV region (Fig. 4d) and Puro gene expression. No correlation was observed between methylation of the SV region and shCFTR1 expression within the same individuals (data not shown), suggesting independent regulation of the H1 promoter (Pol III). Taken together, these data demonstrate that in vitro methylated transposon substrate is efficiently integrated into the host genome and is reprogrammed and expressed in a locus specific manner.

Discussion

Although reasonable unfacilitated frequencies of germline transgenesis are possible with naked DNA in the mouse, previous studies have demonstrated that the use of transposons as a molecular adjuvant can significantly improve the efficiency of transgenic founder production (Dupuy et al. 2002; Ding et al. 2005; Mátés et al. 2009). We have described a significant improvement to germline TnT by the use of methylated and supercoiled *Sleeping Beauty* transposons that provide a platform for highly efficiency TnT in mice and rats. CpG methylation of transposon substrate resulted in a significant increase in TnT compared to nonmethylated transposon, accompanied by an increase in integrations per founder. This observation is consistent with previous observations that substrate methylation and heterochromatinization enhance SB transposition in vitro (Yusa et al. 2004; Ikeda et al. 2007). We also observed a significant increase in the transmission of transgenes using methylated versus nonmethylated transposon substrate. We hypothesize that this improvement could derive from enhanced transposition earlier in embryonic development.

Transposition with the T2/sh_mCFTR1 transposon was most significantly influenced by CpG methylation. Although this T2/sh_mCFTR1 contains a modest sized cargo (2.3 Kb) and ITRs identical to the other transposons used in our study, TnT using nonmethylated transposon was only 25%, an efficiency significantly lower than previous observations (Dupuy et al. 2002) and for other transposons used in our study. This inefficiency did not

derive from an intrinsic inability of this transposon to mobilize since methylated substrate was capable of extremely efficient TnT (90%). Perhaps nonmethylated T2/sh_mCFTR1 is subject to a transient burst of shRNA expression that is toxic to embryos. Complications have indeed been observed for the production and propagation of rodent models based on RNAi by pronuclear injection, perhaps due to activation of the interferon response (Cao et al. 2005). Additionally, the machinery responsible for processing small RNAs is prone to saturation by excess synthetic shRNA (Grimm et al. 2006). Critical roles for small RNAs in the transition from maternal to zygotic expression (Giraldez et al. 2006) and stem cell maintenance and differentiation (Kanellopoulou et al. 2005; Marson et al. 2008; Sinkkonen et al. 2008) underscore the importance of their processing in early embryonic development. Indeed, mice deficient in the miRNA processing enzyme dicer succumb to embryonic lethality due to the lack of mature short RNAs (Bernstein et al. 2003). Taken together, it is not unreasonable to propose that gross overexpression of synthetic shRNA can swamp the processing of endogenous miRNA and contribute to embryonic lethality. Therefore, along with enhanced transposition efficiency, methylation of transgenes prior to injection may help to attenuate transient gene expression until epigenetic reprogramming is complete. Regardless of the precise mechanism, CpG methylation of T2/sh_mCFTR1 led to highly efficient transgenesis, germline transmission, and knockdown of endogenous CFTR that resulted in phenotypes consistent with CFTR knockout mice (Carlson et al., manuscript in preparation).

Surprisingly, we observe a statistically significant increase in transposition in TMC mice, a phenomenon that interacts with substrate methylation (Table 3). This is reminiscent of high frequency transposition in the germline of transgenic mouse lines that contain concatemers (Geurts et al. 2006a, b) that have a tendency for epigenetic modification (Dorer and Henikoff 1997; Garrick et al. 1998). Such a burst of transposition from multicopy concatemers in founder embryos could potentially induce chromosomal rearrangements (Geurts et al. 2006a) and confound transgenic animal propagation. However, an absence of local hopping does not support transposition from integrated concatemers. Indeed, the use of supercoiled substrate eliminated concatemer integrations without suppressing transposition, suggesting that concatemer integration and transposition are parallel, not serial events. Why founders containing concatemers also displayed significant transposition (TMC) remains a mystery, although it is possible that concatemer integration could stimulate components of the nonhomologous end joining (NHEJ) pathway to indirectly enhance parallel transposition (Izsvak et al. 2004).

Using methylated and supercoiled KT2H-CD40Ig transposon (to avoid concatemers) resulted in a two to threefold increase in transgenic liveborn founders compared to traditional methods, and retained an average of four integrations per genome, overall resulting in an 8–12-fold enhancement. Although we observed higher rates of TnT using linearized vs supercoiled substrate, the use of supercoiled DNA avoided the generation of animals containing multi-copy concatemers, arguing for the use of supercoiled substrate as the most effective method for generating animals with multiple independent, single-copy integrations that can be reliably transmitted through the germline. Single copy insertions are particularly preferable to concatemers for stable transmission and for targeted manipulation such as recombinase mediated cassette elimination, inversion and exchange (Lewandoski and Martin 1997; Lewandoski 2001).

We have also reported the first example of rat transgenesis using a transposon system. A $64\% \pm 28$ transgenesis rate by methylation-enhanced TnT in SD rats is a marked enhancement over using naked DNA, which can result in transgenesis efficiencies ranging from 17 to 41% per live born animal in this strain (Filipiak and Saunders 2006). While this efficiency is similar to that achieved by perivitelline injection of lentiviruses, the *Sleeping*

Beauty components can be easily manufactured and injected in any laboratory system with minimal biosafety considerations. The rat has been the preferred model of physiologists, though the majority of phenotypes/strains studied have been the result of spontaneous or ENU mutagenesis (Jacob and Kwitek 2002). Given its high efficiency and ease of preparation, TnT may be an important tool for establishing a link between genotype and phenotype by gene supplementation in the rat.

Although an increased affinity for heterochromatin has been observed for SB transposase (Ikeda et al. 2007) the precise mechanism for enhancement of SB transposition by CpG methylation is not clear. In order to better understand the mechanism for CpG enhancement it may be valuable to address the conservation of CpG-mediated enhancement of transposition for other transposable elements. Transposition of *piggyBac* was actually inhibited by CpG methylation (Wang et al. 2008). *piggyBac* may have evolved in its insect host to transpose most effectively into and out of euchromatin, a preference perhaps related to its affinity for genes (Ding et al. 2005) and the absence of CpG methylation of repeated DNA and transposons in certain insects (Field et al. 2004). It remains to be seen whether CpG enhancement of transposition is common to other Tc1/Mariner transposons such as Frog Prince and Passport (Miskey et al. 2003; Clark et al. 2009) or is a characteristic unique to SB.

Applications of highly efficient TnT

With a routine transgenesis frequency of 50–90% and a transgene copy number ranging from 1 to 14 per transgenic founder (majority with four or fewer alleles), one can envision several applications of highly efficient TnT. When the generation of a line containing a single allele is desired, Mendelian segregation from founders with multiple independent alleles offers multiple opportunities to identify F1 animals that properly express the gene of interest. However, segregation by breeding comes at a cost in time and logistics, with the ideal number of alleles dependent on the expense of founder generation, litter size, generation interval, and the cost of animal husbandry. Considering the contemporary cost of transgenic swine production we've determined that founders with 2–4 independent insertions provides the greatest efficiency (data not shown) for monogenic line development. We anticipate a similar ideal for any mammal with large litters. Similar considerations also suggest that microinjection of a cocktail of transposons containing different cargos could permit the efficient generation of different transgenic lines by simple segregation from a multi-transgenic founder.

It is also conceivable with this efficiency of founder transgenesis that a single set of microinjections could permit the analysis of traits of interest in the founder itself, without the cost of segregating each allele into independent lines. Screening founders has already been demonstrated as useful for mis-expression of signaling molecules in early embryos to study their roles in cell differentiation (Lammert et al. 2001) and developmental fate (Zwijssen et al. 2000). Similarly, constructs containing reporter molecules can be used to identify cis-acting gene regulatory elements in transient transgenics (MacKenzie and Quinn 1999; Germain et al. 2001). Founders generated by TnT with multiple integrated transgenes would not only decrease the impact of position effect on analysis, but could also provide a range in transgene expression among a cohort of founders. Perhaps most importantly, high-frequency delivery and expression of dominant acting alleles in founders could provide a rapid and inexpensive method for determining the suitability of large animal models of specific human diseases.

Materials and methods

Injection DNA/RNA preparation

Methylated transposon plasmids were propagated in TOP10 *E. coli* (Invitrogen, Carlsbad CA) and treated with *SssI* CpG methylase (New England Biolabs, Ipswich, MA) according to the manufacturer's recommendations. Complete methylation was confirmed by cutting 100 ng of treated sample with one unit of *HinPII* endonuclease for 1 h at 37°C and assayed by agarose gel electrophoresis.

For animal transgenesis, the pT2/sh_mCFTR1 and pKT2/KD^{Rab38} transposon plasmids were propagated in TOP10 *E. coli* (Invitrogen, Carlsbad CA), linearized with *ApaI* endonuclease, and the pKT2/HSA-CCTG₃₀₀ transposon with *AseI* after methylase treatment. These transgenes were purified after gel electrophoresis using the UltraClean 15 DNA Purification Kit (MoBio, Carlsbad, CA), ethanol precipitated twice, and resuspended in injection buffer (5 mM Tris–Cl pH 7.5, 0.1 mM EDTA) before serial dialysis three times against 500 mL of injection buffer using Slide-ALyzer cassettes (10,000 MWCO, Pierce, Rockford, IL). The methylated or nonmethylated KT2H-CD40Ig transposons were pre-treated with RNasequre® (Ambion, Austin, TX) and either cut with *SspI* to linearize or left supercoiled prior to cleanup with the Qiagen MiniPrep Kit (Valencia, CA) and eluted in injection buffer prior to serial dialysis as performed above. The rat transgene was purified using a Nucleospin kit (Clontech, Mountain View, CA) as previously described (Filipiak and Saunders 2006). SB11 mRNA was prepared as using the Ambion (Austin, TX) mMessage mMachine® T3 kit as previously described (Wilber et al. 2006). Transposon DNA and transposase mRNA were diluted in injection buffer to a final concentration of 5 ng/uL DNA and 15 ng/uL RNA and maintained on ice before injection into FVB/N strain mouse embryos or Sprague–Dawley strain rat embryos (both rodent strains from Charles River Laboratories) using standard techniques.

Southern blot analysis

Mouse tail biopsy DNA was extracted using standard procedures. 10 µg of DNA was subjected to restriction endonuclease digestion as indicated in the figure legend, resolved and transferred to nylon membranes using standard methods. Membranes were probed with a random-primed, α-³²P labeled restriction fragment of each transposon (probe location indicated in figures).

LM-PCR for cloning transposon junctions

Blocked linker-mediated PCR was performed as described (Clark et al. 2007) on genomic DNA extracted from mouse or rat tail biopsy. Briefly, genomic DNA was digested with a cocktail of restriction enzymes, including *XbaI*, *NheI*, *AvrII*, and *SpeI*. The DNA was ligated to a blocked linker made by annealing the oligos primerette-long [CCTCCACTACGACTCACTGAAGGGCAAGCAGTCCTAACAACCATG] and blink-XbaI [5'P-CTAGCATGGTTGTTAGGACTGCTTGC-3'P]. Nested PCR was performed on the ligated DNA to specifically amplify junctions between the SB transposon and genomic DNA. The transposon-specific primers for the primary and secondary PCR are included in Supplementary Table 2. Resulting PCR fragments were shotgun cloned and sequenced.

Transgenic rat PCR genotyping and RT-PCR

Tail biopsy DNA from Sprague–Dawley founder animals were screened by PCR for presence of the KT2/KD^{Rab38} transposon yielding a 339-bp product (primer sequences are listed in Supplementary Table 2). Trizol® (Invitrogen, Carlsbad CA) isolated and DNase treated RNA was subjected to RT-PCR using the Superscript™ III One-Step RT-PCR

system (Invitrogen, Carlsbad CA) yielding a 277-bp product after splicing of the 140-bp synthetic intron (Supplementary Fig. 2A).

Bisulfite-mediated genomic sequencing

Bisulfite sequencing of transposon integrations was performed as previously described (Park et al. 2005, 2006). For the present study, liver genomic DNA was digested with restriction endonuclease *EcoRI* to fragment the genome and ensure complete DNA denaturation during bisulfite treatment prior to PCR amplification (primer sequences are available in Supplementary Table 2), cloning and sequencing of SV and P amplicons.

Transcript quantification

Small intestine RNA was reverse transcribed with SuperscriptTM III according to the manufacturer's protocol (Invitrogen, Carlsbad CA). Puromycin expression was measured by RT-PCR normalized to HPRT expression levels using iQ SYBR Green Supermix (Biorad, Hercules, CA). Primer sequences are listed in Supplementary Table 2.

Statistical analysis

Confidence intervals (CI) for proportions and means were calculated using standard methods. Any value within this manuscript following the “±” symbol is the 95% confidence interval for a given mean or proportion. The *z*-test for difference between proportions was used to determine if two proportions were significantly different. Resulting *z*-values were used to derive *p*-values for each test.

The effect of transgene vector, methylation and concatemerization was fit to a generalized linear model using the (GLM) procedure of R considering a Poisson distribution of insert copy number. The statistical model was: number of transposon integrations = transposon vector ID/conformation + substrate methylation + presence of a concatemer + substrate methylation × presence of a concatemer + random residual; family = Poisson.

The significance of allelic effect and sibling variation within the same allele were tested for SV and P regions separately using one-way nested ANOVA analysis implemented by the GLM procedure of SAS (Cary, NC). The statistical model was: percentage methylation = allele + (sib within allele) + random residual. For both SV and P regions, difference across alleles was highly significant (*p*-value < 0.0001) but difference among sibs sharing a transgene allele was insignificant (*p*-value = 0.999 for SV region and *p*-value = 0.25 for P region) rejecting the null hypothesis that the methylation status of a shared allele differs between siblings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

SB	Sleeping Beauty
TnT	Transpositional transgenesis
CpG	Cytosine-phosphodiester-guanine

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Fig. 1.

Efficient transposition of chemically modified transposons after PNI. **a** The T2/sh_mCFTR1 transposon expresses puromycin N-acetyltransferase (Puro) from the SV40 viral promoter and enhancer (E) with the SV40 polyA signal (pA) following the coding sequence. A hairpin designed to silence the mouse *Cftr* mRNA is shown as a grey box downstream of the human H1 promoter (indicated as a *left-facing arrow*). Both expression cassettes are located within the SB 5' and 3' inverted terminal repeats (ITRs). Below are Southern blots on founder animals obtained after PNI with linear CpG-methylated (+CpG) or nonmethylated (–CpG) T2/sh_mCFTR1. *Bam*HI (*B*) digestion of genomic DNA will result in a predictable 2.3-kb and (black arrowhead) in animals harboring a concatemer insertion while transposase-

mediated events are evident as slower-migrating fragments. Four classes of founders can be observed animals generated with either methylated or nonmethylated transposons including: (1) non transgenic (for example, founders 1, 10, 71–73); (2) founders containing transposition events only (TO) (for example, founders 4–7, 16–20); (3) founders containing transposition events plus multicopy concatemers (TMC) (for example, founders 3, 8 and 15); (4) and rarely, animals containing multicopy concatemers only (MCO) (founder 80). A nonspecific hybridizing band near 4.8-kb (*open arrowhead*) is evident in all lanes, whether wild type or transgenic. Animals were not pre-screened for the presence of transgene prior to Southern blotting. **b** The KT2H-CD40Ig transposon drives expression of CD40Ig from the human insulin promoter (HIP). *Bam*HI-*Eco*RV (*B* and *E*) digestion of genomic DNA result in a predictable 2.7-kb fragment (black arrowhead) from random, nonhomologous integrants while transposase-mediated events are evident as slower-migrating fragments. Similar to the T2/sh_mCFTR1 transposon, three of four classes of founders can be observed animals generated with either methylated or nonmethylated transposons including: (1) non transgenic (for example, founders 079, 080, 195, 196); (2) TO founders (for example, founders 085–087, 089–092); (3) TMC founders (for example, founders 081, 082, 097–101, 209–211); (4) although several founders have hybridizing bands at 2.7 kb, a vector hybridization did not reveal any MCO founders from the KT2H-CD40ig transposon (data not shown). A nonspecific hybridizing band near 4.8-kb (*open arrowhead*) is also evident in both wild type and transgenic animals

Fig. 2. Increased heritability from methylated TnT founders. Transposition only founders carrying 2–6 independent transposase mediated insertions generated with either methylated or nonmethylated transposon substrate were randomly chosen and crossed to wild type mice to determine transgene heritability. Southern blotting of F1's resulting from T2/sh_mCFTR1 (a) and KT2H-CD40Ig (b) founder outcrosses reveal greater heritability from founders generated with a methylated transposon. All offspring (transgenic and non-transgenic) from T2/sh_mCFTR1 outcross (a) were subjected to Southern analysis while only PCR positive transgenic offspring ($n = 8$ per family) were included on KT2H-CD40Ig Southern blots (b). The F0 insertion pattern for animals CF 4, 13, 74, 79 and 82 can be observed in Fig. 1a.

*Bam*HI (*B*) (T2/sh_mCFTR1 founders) or *Bam*HI-*Eco*RV (*B* and *E*) (KT2H-CD40Ig founders) digestion of genomic DNA would result in a predictable 2.3 and 2.7-kb fragments (*black arrowhead*) from concatemer insertions while transposase-mediated events are evident as slower-migrating fragments. A nonspecific hybridizing band near 4.8-kb (*open arrowhead*) is evident in all lanes

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Fig. 3.

Efficient transposition from a supercoiled transposon. **a** Pronuclear stage embryos were injected with either methylated or nonmethylated supercoiled KT2H-CD40Ig transposon. **b** Genomic DNA from transgene-positive founders was digested with *Bam*HI (*B*) plus *Eco*RV (*E*). Only one of the transgenic founders contains a dense band indicative of a concatemer at 2.7-kb (compared to several in Fig. 1b) indicating a significant reduction in concatemer formation while maintaining an average of four transposon insertions per founder (Table 2). A nonspecific hybridizing band near 4.8-kb (*open arrowhead*) is evident in all lanes

Fig. 4. Methylation of T2/sh_mCFTR1 transposons in founders and offspring. **a** Three CpG rich regions are found in the T2/sh_mCFTR1 transposon (*hatched boxes*). Two amplicons, SV and P, overlapping the SV40/H1 promoter and Puro coding regions were analyzed for methylation in transgenic founder and F1 DNA. **b** This plot displays individual measurements of methylation from the SV region (*black circles*) and the P regions (*black triangles*) from a number of F1 animals harboring a single transposon transgene. F1's harboring a single insert have consistent methylation patterns between siblings with identical transgene alleles (see statistical analysis section of the "Materials and methods" section), but differ from sibling pairs from separate founders containing a different transgene allele (One way ANOVA, p -value < 0.0001). Allelic information was determined by Southern blotting of F1 animals (see Fig. 2a) and assigned a unique letter designation. **c** Methylation data appeared to follow a bimodal pattern. This is clearly evident when data accumulated from founders and F1's was divided into two groups: greater than 50% methylation (*upward arrow*) or less than 50% methylation (*downward arrow*). Mean and the 95% confidence interval is shown from 52 SV and 67 P amplicons from founder animals and 112 SV and

170 P amplicons from F1 animals revealing little variation from this bimodal pattern (See Supplementary Fig. 3 for plots of individual founders). **d** Among F1 animals that carried a single transgene insertion, a significant correlation between percent methylation in the liver and normalized Puro expression in the small intestine was found ($n = 9$, $p < 0.05$, linear regression analysis)

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Table 1

Germline transgenesis by SB and *piggyBac* transposons and lentivirus

Vector	Host	Size (kb)	Transgenic (% liveborn)	Copy number ^a	Concatemer free # (% transgenic) ^d	Reference
<i>SB transposon</i>						
Methylated						
T2/sh_mCFTR1	Mouse	2.3	18 (90 ± 13)	1-8 SC, MC	15 (83 ± 17)	Here
KT2/HSA-CCTG ₃₀₀	Mouse	9.1	12 (57 ± 21)	1-4 SC, MC	1 (8 ± 16)	Here
KT2H-CD401g	Mouse	3	44 (80 ± 11)	1-11 SC, MC	31 (70 ± 13)	Here
<i>KT2H-CD401g</i> (sc) ^f	Mouse	3	18 (47 ± 16)	1-11 SC, MC	17 (94 ± 11)	Here
KT2/KD ^{Rab38}	Rat	3.5	7 (64 ± 28)	1-4 SC, MC	NA	Here
Nonmethylated						
T2/sh_mCFTR1	Mouse	2.3	4 (25 ± 21)	1 SC, MC	3 (75 ± 44)	Here
KT2/HSA-CCTG ₃₀₀	Mouse	9.1	11 (52 ± 21)	1-4 SC, MC	1 (9 ± 17)	Here
KT2H-CD401g	Mouse	3	15 (60 ± 19)	1-9 SC, MC	8 (53 ± 25)	Here
<i>KT2H-CD401g</i> (sc) ^f	Mouse	3	7 (24 ± 16)	1-4 SC	NA	Here
T/K14-Agouti	Mouse	4.3	19 (45 ± 14)	1-5 SC, MC	6 (32 ± 21)	Dupuy et al. (2002)
<i>PiggyBac transposon</i>						
PB[Act-RFP]	Mouse	4.5	67 (36 ± 7)	4-10 + SC, MC ^b	86 ^c	Ding et al. (2005)
PB[K14-Tyr]	Mouse	6.5	NR (65) ^b	NR	NR	Ding et al. (2005)
PB[K14-Tyr,Act-RFP]	Mouse	9.1	NR (45) ^b	NR	NR	Ding et al. (2005)
PB[Act-RFP,MCK-TSCI]	Mouse	14.3	4 (36 ± 28)	NR	NR	Ding et al. (2005)
<i>Lentivirus</i>						
FUGW	Mouse		49 (86 ± 9)	1-21 SC	100	Lois et al. (2002)
FMHGW	Mouse		11 (73 ± 22)	2-15 SC	100	Lois et al. (2002)
FUGW	Rat		13 (59 ± 21)	1-7 SC	100	Lois et al. (2002)
LV-GFP	Rat		13 (22 ± 10)	1-4 SC	100	Michalkiewicz et al. (2007)
LV-GFP	Cattle		10 (83 ± 21) ^e	5-10 SC	100	Hofmann et al. (2004)
LV-PGK	Pig		32 (70 ± 13)	1-20 SC	100	Hofmann et al. (2003)

Numbers reported following the “±” symbol are the 95% confidence interval of the proportion

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- ^aSC- single copy mediated by transposition or proviral insertion, MC- multicopy (non-transposition mediated) with or without additional transposition events, NR- not reported
- ^bEstimated from bar graph and Southern in Ding et al. 2005; Fig. 3
- ^cThe frequency of animals co-transgenic for the PB transposon plus PB transposase indicative of concatemers
- ^dAnimals with single copy transposase or integrase mediated insertion only; NA—not assayed, NR—not reported
- ^eTransgenesis was determined by GFP visualization and only GFP positive embryos were transferred to pseudopregnant females
- ^fKT2H-CD40lg (sc)—supercoiled transposon substrate, all other transposons were linearized within the plasmid backbone

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Table 2

Summary of mouse transgenesis data

Transposon	CpG ^a	SB11 ^b	Tg/Liveborn (%)	MCO (% Tg)	# TMC founders liveborn	% TMC founders liveborn	#TI in TMC founders (TI/TMC)	#TO founders (% liveborn)	# TI in TO founders (TI/TO)
KT2H-CD40lg	+	+	44/55 (80 ± 11)	0	13 (24 ± 11)		58 (4.5 ± 2)	31 (56 ± 13)	137 (4.4 ± 1)
	-	+	15/25 (60 ± 19)	0	7 (28)		27 (4 ± 1)	8 (32 ± 18)	13 (2 ± 1)
	+	-	5/34 (15 ± 12)	5 (100)	5		0	NA	0
	-	-	2/11 (18 ± 23)	2 (100)	2		0	NA	0
KT2H-CD40lg (sc) ^c	+	+	18/38 (47 ± 16)	0	1 (3 ± 5)		3 (3)	17 (45 ± 16)	75 (4.4 ± 1.5)
	-	+	7/29 (24 ± 16)	0	1		0	7 (24 ± 16)	13 (2 ± 1)
T2/sh_mCFTRl	+	+	18/20 (90 ± 13)	0	3 (15 ± 16)		14 (5 ± 3)	15 (75 ± 19)	43 (2.9 ± 0.6)
	-	+	4/16 (25 ± 21)	1 (25 ± 42)	1 (6 ± 12)		0	3 (19 ± 19)	4 (1 ± 1)
KT2-HSA CCTG ₃₀₀	+	+	12/21 (57 ± 21)	0	11 (52 ± 21)		14 (2.8 ± 2)	1 (5 ± 9)	1 (1)
	-	+	11/21 (52 ± 21)	0	10 (48 ± 21)		17 (2.8 ± 1)	1 (5 ± 9)	1 (1)
T/K14 Agouti ^d	-	+	19/42 (45 ± 15)	0	13 (31 ± 14)		NR	6 (14 ± 11)	18 (3 ± NR)

Tg transgene positive regardless of insertion type, MCO Multicopy concatemer insertion only, TMC Transposition plus multicopy concatemers, TO insertion by transposition only, TI independent transposase mediated insertions determined by Southern analysis, NR not reported, NA not assayed

Numbers reported following the “±” symbol are the 95% confidence interval of the proportion

^aIn vitro GpG methylation of the injected transposon

^bSB11 mRNA added to injection cocktail

^cKT2H-CD40lg (sc)—Supercoiled transposon substrate, all other transposons have been linearized

^dDupuy et. al. (2002)

Table 3

Linear regression analysis of factors influencing transposition

Effect	Estimate of effect	Std. error	p-value
A versus B	0.01894	0.12976	0.883961
A versus C	-0.30884	0.14641	0.034907 [*]
A versus D	-0.48842	0.19902	0.014123 [*]
Methylation	0.25465	0.06724	0.000152 ^{***}
Concatemers	0.23668	0.07327	0.001238 ^{**}
Methylation × Concatemers [†]	-0.19796	0.06691	0.003088 ^{**}

A = Linear KT2H-CD40Ig

B = Supercoiled KT2H-CD40Ig

C = Linear T2/sh_mCFTR1

D = Linear KT2/HAS-CCTG300

[†] Interaction between methylation and concatemers^{*}
 $p < 0.05$ ^{**}
 $p < 0.01$ ^{***}
 $p < 0.001$