Somatic integration and long-term transgene expression in normal and haemophilic mice using a DNA transposon system

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The development of non-viral gene-transfer technologies that can support stable chromosomal integration and persistent gene expression *in vivo* is desirable. Here we describe the successful use of transposon technology for the nonhomologous insertion of foreign genes into the genomes of adult mammals using naked DNA. We show that the *Sleeping Beauty* transposase can efficiently insert transposon DNA into the mouse genome in approximately 5–6% of transfected mouse liver cells. Chromosomal transposition resulted in long-term expression (>5 months) of human blood coagulation factor IX at levels that were therapeutic in a mouse model of haemophilia B. Our results establish DNA-mediated transposition as a new genetic tool for mammals, and provide new strategies to improve existing non-viral and viral vectors for human gene therapy applications.

Introduction

There is considerable interest in the development of new technologies for somatic gene transfer in mammals, especially for the treatment of inherited and acquired disorders. Many viral-based vectors have shown potential as vehicles for *in vivo* gene delivery, including adenovirus[1], herpesvirus[2], retroviruses[3,4] and adeno-associated virus[5]. The latter two vectors can integrate into the chromosomes of transduced cells and support long-term transgene expression in treated animals[6–10]. Viral vectors, however, have been fraught with problems related to production and safety. Specifically, the preparation and purification of many of these viral vectors can be hard to achieve, laborious, cost-prohibitive and not amenable to industrial-scale manufacture. In addition, although viral vectors are designed to be replication-defective, there have been instances in which recombination events occurred, giving rise to unwanted by-products[11]. Furthermore, some viral vectors induce immune responses that diminish the efficacy and biosafety of repeated administration[12–14]. Therefore, efforts have been made to develop alternative approaches for *in vivo* gene transfer.

An alternative currently in development is the use of naked DNA, either alone[15–20] or in conjuction with a variety of molecular conjugates, such as liposomes[21,22], polymers[23] and polypeptides[24]. Unlike the preparation of most viral carriers, large-scale DNA manufacture is cost-effective, reproducible and the final product does not require sophisticated storage conditions. In the past decade, these vectors have been successfully administered *in vivo* and have produced transgene expression in liver[15,16], lung[17], muscle[18], skin[19] and heart[20]. A limitation of this approach is the absence of persistent and/or therapeutic gene expression *in vivo* because the vector genome fails to integrate with plasmid-based vectors.

One approach to increase the integration frequency of nonviral vectors in animals to prolong transgene expression is to incorporate components of a eukaryotic DNA transposon into the vector. Transposons are naturally occurring genetic elements capable of moving from one chromosomal location to another. The Tc1/*mariner* superfamily of transposable elements transpose by a 'cut-and-paste' mechanism that requires the binding of an element-encoded enzyme, the transposase, to short inverted repeat (IR) sequences flanking the element[25]. Most of these elements integrate into a TA target dinucleotide, which is duplicated upon insertion. Transposition can occur *in vitro*[26,27], suggesting that the transposase can function independently of host-specific factors. Consistent with this hypothesis, both *mariner*-like and Tc1-like elements have been used to generate non-mammalian transgenic animals following microinjection into mosquito[28], zebrafish[29] and chicken[30] embryos.

Here we describe the activity of the *Sleeping Beauty* transposon system in adult mammals. *Sleeping Beauty* is a synthetic transposable element made from defective copies of an ancestral Tc1-like fish element[31]. It is a 1.6-kb element flanked by 250-bp terminal IRs and encodes a single protein, the *Sleeping Beauty* transposase, that catalyses its transposition from one genomic loci to another. Previous studies have shown that *Sleeping Beauty* can insert foreign genes into the chromosomes of cultured vertebrate cell lines, including mouse embryonic stem (ES) cells[32] and human cells[31]. Here we demonstrate that *Sleeping Beauty* can facilitate somatic integration of naked DNA into mouse chromosomes, resulting in long-term therapeutic transgene expression in normal and haemophilic adult mice.

Results

Transposition in cultured mammalian cells

We produced three *Sleeping Beauty*-based vectors (Fig. 1*a*) and tested their functional competency in cultured mammalian cells. We transiently transfected HeLa cells with a plasmid containing a

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neomycin-phosphotransferase (*neo*) expression cassette flanked by *Sleeping Beauty* inverted repeats (pTnori) and selected for G418 drug-resistant growth ($G418^R$) following co-transfection with different helper plasmids. Although cells expressed similar levels of wild-type and mutant transposase (data not shown), results indicated that cells receiving the pCMV-SB plasmid encoding transposase form approximately 80-fold more $G418^R$ transformants than cells receiving control plasmids encoding either the green flourescent protein (pCMV-GFP) or a transposase harbouring a missense mutation in its carboxy-terminal aspartate-aspartate-glutamate (D,D(35)E) motif (pCMV-mSB; Fig. 1*b*). Similar mutations also abolished the catalytic activity of the *mariner* transposase in *Drosophila melanogaster*[33], indicating that this conserved motif is necessary for transposase function. Therefore, *Sleeping Beauty* can enhance the integration of plasmid-encoded transgenes *in vitro*.

Chromosomal transposition in adult mice

To determine whether *Sleeping Beauty* functions in adult mammals, we administered the plasmid pTnori to mice in a manner shown to transfect mouse hepatocytes *in vivo*[15,16] and induced transposition by co-injecting animals with pCMV-SB or pCMV-mSB as a control. We identified 11 independent transposition events using a plasmid recovery strategy that facilitates the cloning and sequencing of cellular-transposon junction fragments (Fig. 2*a*). This genetic approach exploits the power of bacterial growth selection to isolate kanamycin-resistant (Kan^R) transposons that have been mobilized from the ampicillin-resistant (Amp^R) donor plasmid pTnori (Fig. 2*b*). When we analysed total liver DNA from mice injected with pTnori and pCMV-SB, we obtained bacteria that were predominantly Amp^S and Kan^R (90% or 167/185; n=5 mice), suggesting efficient transposon excision from the Amp^R donor. Plasmid DNA analysis from 11 randomly chosen Amp^S/Kan^R colonies showed that each clone contained two bands (2.4-kb and 0.4-kb) corresponding to internal transposon sequences, as well as a variety of novel bands indicative of transposition-mediated integration (Fig. 2*c*). Sequence analyses showed that each end of the transposon was flanked by TA dinucleotides, followed by sequences that were different from the plasmid vector sequences originally flanking the transposon in pTnori (Fig. 2*d*). Vector-specific sequences were still present in the region flanking the transposon in clone 2, which is consistent with the presence of the 0.2-kb vector-specific *Hin*dIII fragment (Fig. 2*c*, lanes D, 1). Sequence analysis of the donor and target sites in this clone indicated intraplasmid transposition of the transposon into a TA target dinucleotide within the donor plasmid Amp gene. This event resulted in an Amp^S phenotype and resulted in a 2-bp (TA) duplication at the site of integration. These results indicate that the fidelity of the cut-and-paste mechanism of *Sleeping Beauty* transposition is maintained in mammals. We screened the sequences flanking the transposon in the remaining 10 Amp^S clones for homologies in GenBank by an advanced BLAST search. These searches revealed that cellular sequences contained in one clone possessed 100% homology to the mouse WSB-1 mRNA (clone 12; positivity, 170 of 170), whereas another clone harboured partial homology to the mouse gene *H2-M10.1* on chromosome 17 encoding MHC class Ib antigen (clone 14, positivity, 459 of 520 (88%)). The integration sites for the transposon were within an intron of the gene encoding WSB-1 and the 3′ UTR of *H2-M101*. No homology was found in the remaining eight sequences. These data demonstrate that *Sleeping Beauty* can promote the integration of foreign genes into the genome of mouse hepatocytes *in vivo*.

In contrast to the injection of pCMV-SB, liver DNA from mice injected with pCMV-mSB produced very few Amp^S/Kan^R bacterial colonies (2.7% or 8/299; n=2 mice), and DNA from these eight clones did not contain any transposon-specific fragments. The absence of detectable transgene integration in the presence of mutant transposase is consistent with the results of our *in vitro* studies and indicates that chromosomal transposition in mice is strictly helper-dependent. In addition, these data suggest that endogenous sources of functional transposase were either not present in the mouse genome or not expressed at levels sufficient for transposition in the absence of exogenous sources of functional transposase.

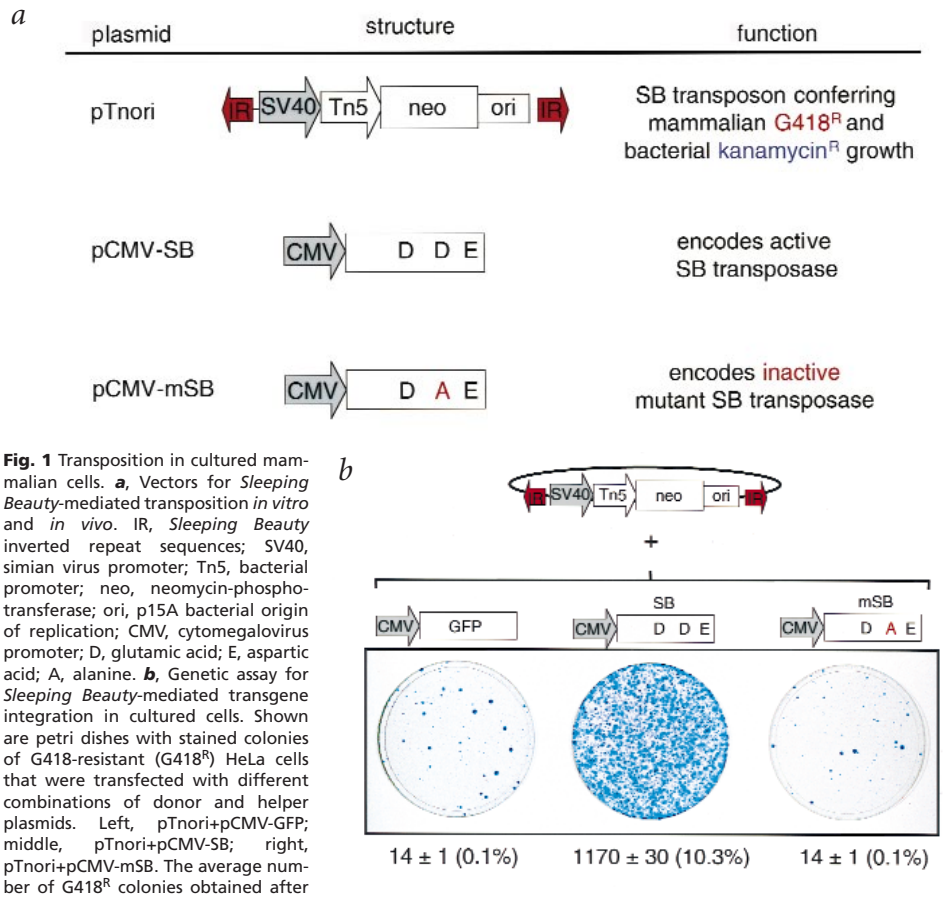

Fig. 1 Transposition in cultured mammalian cells. ***a***, Vectors for *Sleeping Beauty*-mediated transposition *in vitro* and *in vivo*. IR, *Sleeping Beauty* inverted repeat sequences; SV40, simian virus promoter; Tn5, bacterial promoter; neo, neomycin-phosphotransferase; ori, p15A bacterial origin of replication; CMV, cytomegalovirus promoter; D, glutamic acid; E, aspartic acid; A, alanine. ***b***, Genetic assay for *Sleeping Beauty*-mediated transgene integration in cultured cells. Shown are petri dishes with stained colonies of G418-resistant ($G418^R$) HeLa cells that were transfected with different combinations of donor and helper plasmids. Left, pTnori+pCMV-GFP; middle, pTnori+pCMV-SB; right, pTnori+pCMV-mSB. The average number of $G418^R$ colonies obtained after three independent transfections is shown below each panel. Estimated transposition efficiencies for each experimental condition are shown in parentheses.

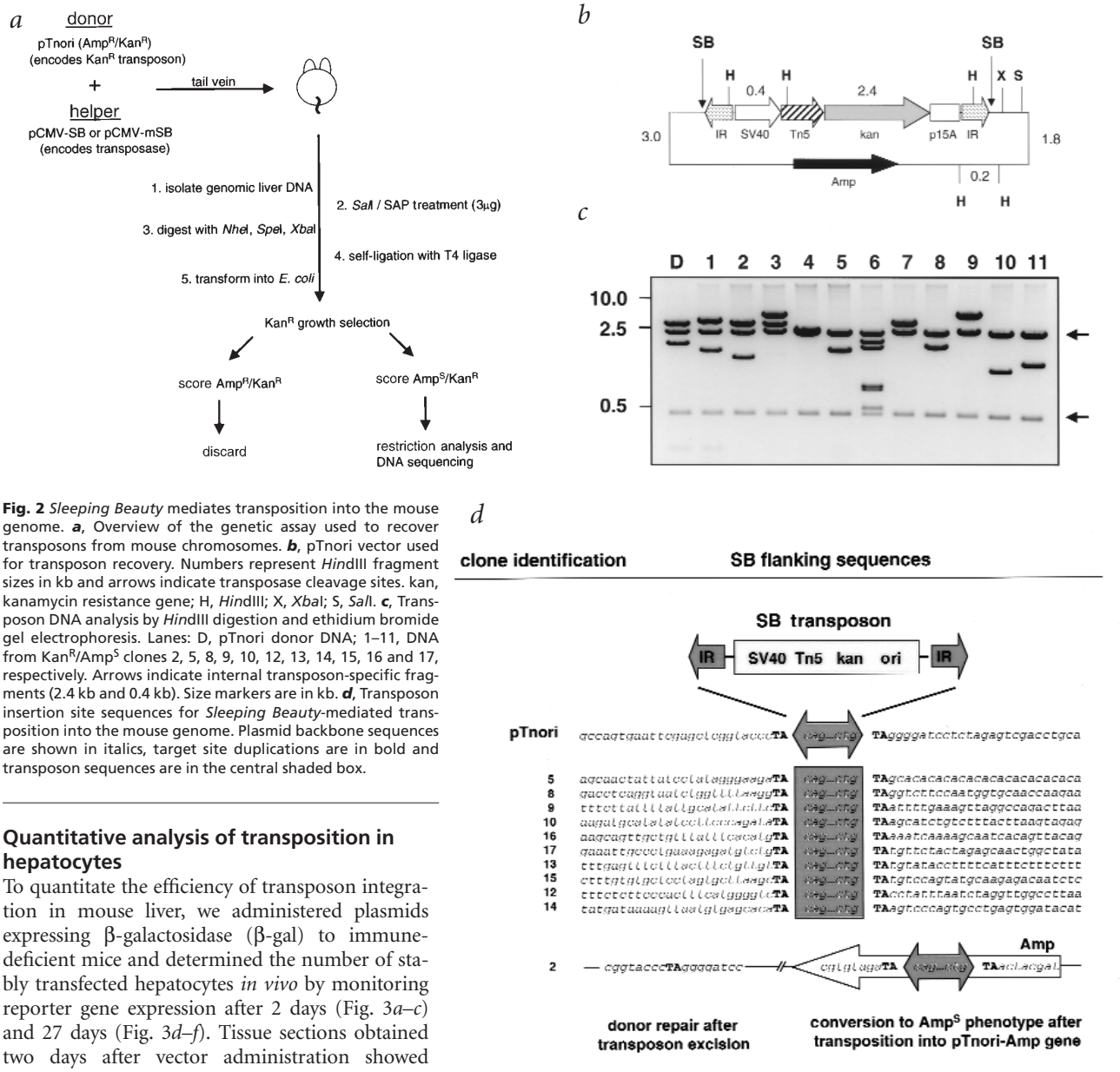

Fig. 2 *Sleeping Beauty* mediates transposition into the mouse genome. ***a***, Overview of the genetic assay used to recover transposons from mouse chromosomes. ***b***, pTnori vector used for transposon recovery. Numbers represent *Hin*dIII fragment sizes in kb and arrows indicate transposase cleavage sites. kan, kanamycin resistance gene; H, *Hin*dIII; X, *Xba*I; S, *Sal*I. ***c***, Transposon DNA analysis by *Hin*dIII digestion and ethidium bromide gel electrophoresis. Lanes: D, pTnori donor DNA; 1–11, DNA from Kan^R/Amp^S clones 2, 5, 8, 9, 10, 12, 13, 14, 15, 16 and 17, respectively. Arrows indicate internal transposon-specific fragments (2.4 kb and 0.4 kb). Size markers are in kb. ***d***, Transposon insertion site sequences for *Sleeping Beauty*-mediated transposition into the mouse genome. Plasmid backbone sequences are shown in italics, target site duplications are in bold and transposon sequences are in the central shaded box.

Quantitative analysis of transposition in hepatocytes

To quantitate the efficiency of transposon integration in mouse liver, we administered plasmids expressing β-galactosidase (β-gal) to immune-deficient mice and determined the number of stably transfected hepatocytes *in vivo* by monitoring reporter gene expression after 2 days (Fig. 3*a–c*) and 27 days (Fig. 3*d–f*). Tissue sections obtained two days after vector administration showed reporter gene expression primarily in the liver (~40% of mouse hepatocytes were X-gal$^+$ with (Fig. 3*a*,*c*) or without (Fig. 3*b*) functional transposase expression), with little or no reporter gene expression in heart, kidney, lung, spleen or brain (<0.01%; data not shown). Gene expression in the liver persisted in 2.3±0.7% of mouse hepatocytes in the presence of active transposase (Fig. 3*f*; n=6) compared with less than 0.01% in mice that received either inactive transposase (Fig. 3*e*; n=6) or a reporter gene lacking terminal repeats (Fig. 3*d*, n=6). This value corresponds to a transposition efficiency in transfected mouse hepatocytes equal to approximately 5–6% and was similar to that obtained with safe doses of integrating viral vectors such as lentivirus[10,34] and rAAV vectors[7,35,36]. This estimation is a conservative value because the use of cytoplasmic β-gal as a reporter for integrating vectors tends to underestimate the actual number of hepatocytes that stably express the transgene product[10,37].

Transposition mediates long-term transgene expression *in vivo*

To ascertain whether *Sleeping Beauty*-mediated gene transfer supports stable gene expression in an adult animal, we inserted expression cassettes encoding human serum marker proteins into vectors containing or lacking IR sequences (Fig. 4*a*) and injected these plasmids into the tail veins of immune-competent mice. Results indicate that co-administration of pCMV-SB and a plasmid encoding the human α–1-antitrypsin (AAT) cDNA flanked by intact terminal repeat sequences (pThAAT) produced approximately 40-fold more serum hAAT for more than 6 months (2,810 ng/ml±604 ng/ml) compared with mice receiving control plasmids encoding either no transposase (pcDNA3, 65 ng/ml±38 ng/ml) or inactive transposase (pCMV-mSB, 43 ng/ml±14 ng/ml; Fig. 4*b*). Reporter levels obtained with an identical donor plasmid lacking IR sequences (phAAT) were not significantly different in mice receiving pcDNA3, pCMV-SB or pCMV-mSB (Fig. 4*c*), indicating that intact terminal repeat sequences are required for transposition. Similarly, co-injection of pCMV-SB and a plasmid encoding the human faxtor IX (FIX) cDNA flanked by IR sequences (pTEF1α-hFIX) produced approximately 80-fold more serum FIX for more than 5 months (164 ng/ml±64 ng/ml) compared with mice receiving pCMV-mSB (2 ng/ml±3 ng/ml; Fig. 4*d*). This level of plasma FIX was

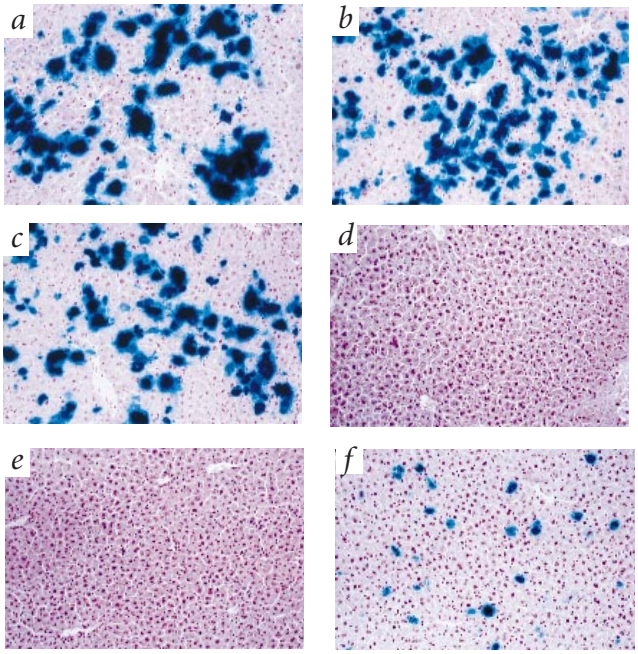

Fig. 3 β-galactosidase expression in mouse liver following administration of transposition vectors. C57Bl/6-*scid* mice received plasmids encoding a β-galactosidase reporter gene with (pTβgeo) or without (pβgeo) flanking transposon repeats, as well as plasmids expressing either functional (pCMV-SB) or nonfunctional mutant (pCMV-mSB) transposase. Mice were injected into the tail vein with 25 μg pβgeo and 2.5 μg pCMV-SB (***a***,***d***), 25 μg pTβgeo and 2.5 μg pCMV-mSB (***b***,***e***), or 25 μg pTβgeo and 2.5 μg pCMV-SB (***c***,***f***). Representative sections are shown from liver 2 d (*a–c*; n=3 mice per group) and 27 d (*d–f*; n=6 mice per group) after vector administration. Original magnification ×200. Our previous studies indicate that the number of X-gal–positive cells obtained with cytoplasmic β-gal is likely an underestimation of the true hepatocyte transfection efficiencies[10,37].

approximately 3% of normal human levels, and would convert a severely affected patient with haemophilia B to one with a much milder phenotype[38]. Therefore, *Sleeping Beauty* maintains the expression of transgenes flanked by intact terminal repeat sequences, resulting in therapeutic levels of foreign gene expression *in vivo*.

The effects of increased transposase gene dosage in mice

In a previous study, we injected immune-competent mice with pThAAT and increasing concentrations of pCMV-SB and monitored long-term serum human AAT levels over time as an indicator of the relative transposition efficiency. Results showed approximately tenfold less serum human AAT in the long-term with a 25-μg dose of pCMV-SB (225 ng/ml±85 ng/ml) compared with a 2.5-μg dose of pCMV-SB (2,810 ng/ml±604 ng/ml; Fig. 4*b*, and data not shown). One possible explanation for this result is that high doses of pCMV-SB increased the presentation of vector-specific antigens on the surface of transfected hepatocytes. In an immune-competent animal, this may have promoted the clearing of transfected cells by cytotoxic T lymphocytes, resulting in reduced transgene levels over time. When we monitored reporter gene expression in immune-deficient *Prkdc*^{scid} mice, however, we observed a similar decrease in stable transgene levels in animals receiving high doses of pCMV-SB (Fig. 4*b*,*e*). Alternatively, it is possible that transposase activity itself was cytotoxic in mice. To test this, we injected mice with different doses of pCMV-SB or pCMV-mSB and then monitored serum glutamic pyruvic transaminase (SGPT), a sensitive measure of liver injury. During the two-week period immediately following plasmid administration, we found no evidence for any transposase-dependent toxicity in mice regardless of transposase activity (pCMV-SB versus pCMV-mSB) or gene dosage (we compared 0 μg, 1 μg and 25 μg pCMV-SB and pCMV-mSB; data not shown). A third consideration is that net transposase activity might be reduced at high transposase concentrations. This phenomenon, called overproduction inhibition, has been recently described for the reconstructed *Himar1* transposase[39] and the *Mos1* element[40,41] and can effectively downregulate transposition of these *mariner*-like elements *in vitro* and in *Drosophila*, respectively. Determining whether or not *Sleeping Beauty* is subject to overproduction inhibition will require further investigation. Nevertheless, our data indicate that a low dose of transposase facilitates more efficient transposition *in vivo* than a higher dose.

Correction of the bleeding diathesis in haemophilic mice

To determine if transposon-based gene expression is therapeutic in an animal model, we studied transposition of a human FIX-marked transposon in haemophilia B-C57Bl/6 mice (Table 1). These mice have a deficiency of functional coagulation FIX and exhibit a characteristic bleeding defect similar to human haemophilia B (ref. 42). We injected pTEF1α-hFIX and either pCMV-SB or pCMV-mSB into mouse tail veins and determined their serum human FIX levels after 60 days. Human FIX levels were undetectable (<1.5 ng/ml) in mice injected with pCMV-mSB (n=3), whereas mice receiving pCMV-SB (n=3) had human FIX levels ranging from 77 ng/ml to 105 ng/ml. To ascertain whether vector re-administration enhances stable human FIX levels, we re-injected these mice as before and determined the serum human FIX concentrations after seven weeks (day 102 of this study). Mice re-injected with pCMV-SB now had stable human FIX levels ranging from 189 ng/ml to 452 ng/ml, corresponding to a fourfold increase in stable human FIX, whereas

Table 1 • Phenotypic correction of mouse haemophilia B

Mouse strain	Treatment[a]	Human factor IX (ng/ml)[b] before	day 102	Bleeding times (min)[c]
C57Bl/6	none	NA[d]	NA	2–3
haemophilia B-C57Bl/6	1 μg pCMV-mSB + 25 μg pT-EF1α-hFIX	UD[e]	UD	>30
		UD	UD	>30
		UD	UD	>30
	1 μg pCMV-SB + 25 μg pT-EF1α-hFIX	UD	189	7
		UD	452	4
		UD	395	6

[a]Factor IX-deficient animals were injected via the tail vein at day 0 and day 60 with the indicated dose of donor and helper plasmids. The secondary administration was performed to determine the efficacy of vector re-administration in an immune-competent animal. [b]Plasma factor IX levels in each mouse was determined by an ELISA assay at the indicated time after the primary injection. [c]Bleeding times were determined at day 102 by transecting the tail and measuring the time required to clot. Bleeding times before plasmid treatment were >30 min for all haemophilic mice (n=6) and 2–4 min for untreated C57Bl/6 mice (n=5). [d]NA, not applicable. [e]UD, undetectable by ELISA (<1.5 ng/ml).

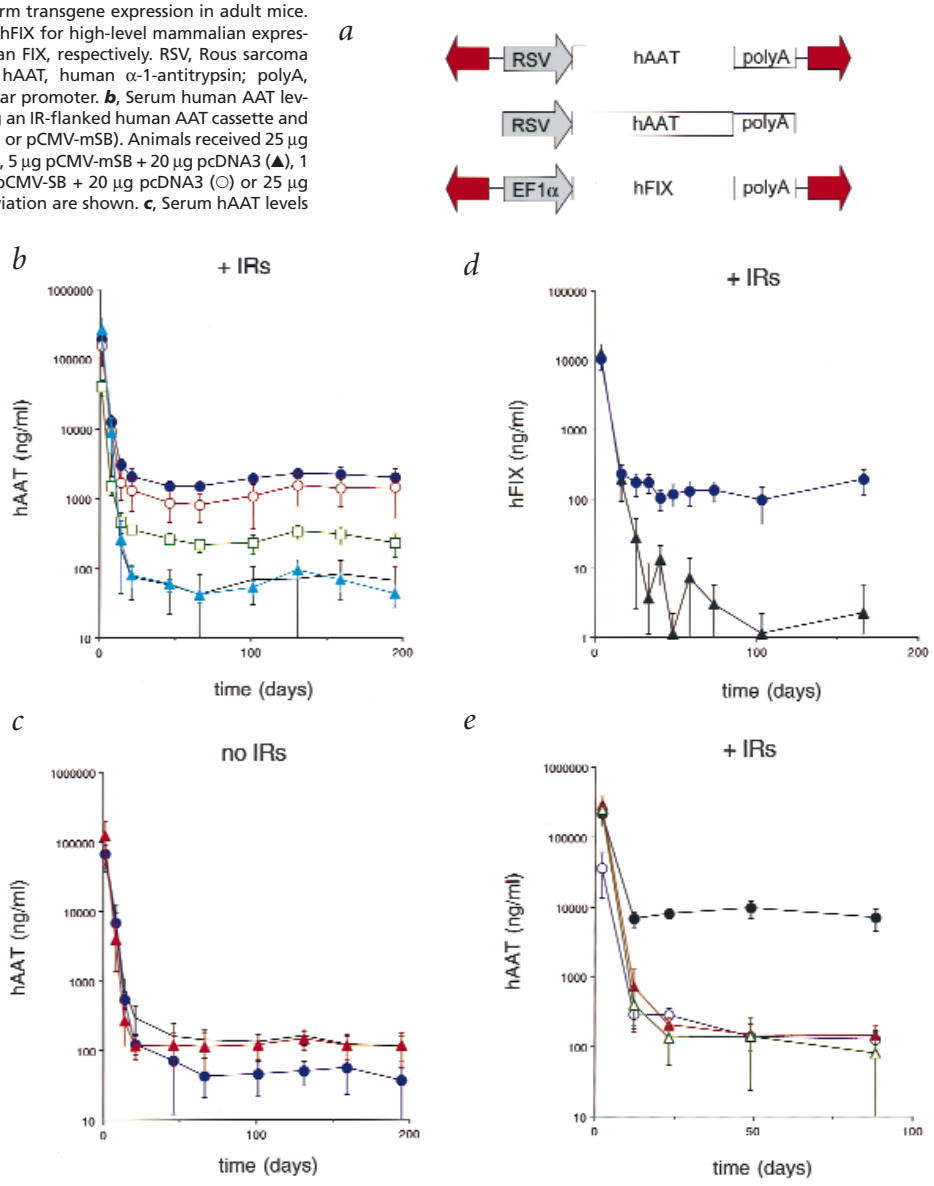

Fig. 4 *Sleeping Beauty* mediates long-term transgene expression in adult mice. ***a***, Plasmids pThAAT, phAAT and pTEF1α-hFIX for high-level mammalian expression of human α-1-antitrypsin and human FIX, respectively. RSV, Rous sarcoma virus long terminal repeat promoter; hAAT, human α-1-antitrypsin; polyA, polyadenylation signal; EF1α, *Eif1a* cellular promoter. ***b***, Serum human AAT levels in mice (n=5 mice per group) receiving an IR-flanked human AAT cassette and plasmids encoding transposase (pCMV-SB or pCMV-mSB). Animals received 25 μg pThAAT together with 25 μg pcDNA3 (—), 5 μg pCMV-mSB + 20 μg pcDNA3 (▲), 1 μg pCMV-SB + 24 μg pcDNA3 (●), 5 μg pCMV-SB + 20 μg pcDNA3 (○) or 25 μg pCMV-SB (□). Mean values±standard deviation are shown. ***c***, Serum hAAT levels from a transgene lacking terminal repeat sequences. 25 μg phAAT was delivered systemically to mice (n=5 mice per group) in combination with 25 μg pcDNA3 (—), 5 μg pCMV-SB + 20 μg pcDNA3 (●), or 5 μg pCMV-mSB + 20 μg pcDNA3 (▲). ***d***, Serum hFIX concentrations in mice following transposon DNA injection. We administered 25 μg pTEF1α-hFIX via the tail vein to C57Bl/6 mice (n=5 mice per group) with 1 μg pCMV-SB (●) or 1 μg pCMV-mSB (▲). ***e***, Serum human AAT levels in immune-deficient mice following plasmid administration. C57Bl/6-*scid* mice (n=3 or 4 mice per group) received 1 μg pCMV-SB + 24 μg pcDNA3 (●), 25 μg pCMV-SB (○), 1 μg pCMV-mSB + 24 μg pcDNA3 (▲) or 25 μg pCMV-mSB (△).

human FIX levels in mice re-injected with pCMV-mSB remained undetectable. Therefore, vector re-administration enhances persistent transgene expression *in vivo*.

We assessed bleeding times in treated mice after transection of their tails (Table 1). Results showed that mice treated with pCMV-SB exhibited a long-term reduction in their bleeding times (4–7 min; n=3) compared with animals treated with pCMV-mSB (>30 min; n=3). These data indicate that transposition mediated by *Sleeping Beauty* can sustain the production of biologically active FIX protein, resulting in partial correction of the bleeding diathesis in a mouse model of haemophilia B.

Discussion

Here we used a delivery technique that specifically targets the mouse liver[15,16], demonstrating that transgenes encoded by plasmid-based vectors can be efficiently inserted into mammalian chromosomes *in vivo*. This is the first demonstration of transposition-mediated gene transfer in an adult animal and represents an advance in the development of stable non-viral gene transfer systems. Furthermore, our data suggest that members of the Tc1/*mariner* family of transposable elements may have widespread use as transformation vectors in mammals. Within the liver, the integration of exogenous DNA in these long-lived cells may permit life-long gene expression with a single administration of vector[43]. Depending on the turnover rate in other tissues, however, integrating vector systems may require multiple administrations to maintain persistence of gene expression. Studies with viral-based vectors have shown that re-administration can promote unwanted immune responses that can severely limit gene-transfer efficacy and biosafety in immune-competent animals[12–14]. Our results indicate that secondary administrations of DNA-based transposition vectors enhance persistent transgene expression in immune-competent mice. The immunological or cytological consequences of repeat administration, especially with respect to transposon stability, remains to be determined. It is also necessary to study transposition in other mouse strains and tissues using alternative non-viral delivery strategies such as lipofection and electroporation. Alternatively, this technology could be packaged into viral vectors (for example, adenovirus[44] and herpesvirus) to produce an improved integrating viral-based system for *in vivo* gene delivery.

In addition to demonstrating helper-dependent integration of a non-viral vector, we have shown that a single vector administration can result in therapeutic serum levels of human FIX for more than five months in adult mice. Our observation that transposon-based gene expression can improve the clotting defect in a small animal model of haemophilia B indicates that this technology may have potential as a therapeutic agent. These integrating vectors are widely amenable to industrial-scale manufacture and should prove useful in human gene therapy applications. Indeed, the relative ease with which these plasmid-based vectors can be

produced, purified and maintained pathogen-free in any laboratory at very little cost to the investigator presents some advantages over viral vectors currently used. Additionally, although our plasmid-based vectors already achieve stable chromosomal integration at levels comparable with the use of many viral-based technologies, basic research into the mechanisms of DNA-mediated transposition may suggest new ways to further improve this novel integrating system. Finally, research into the use of other transposable elements that can function in cultured vertebrate cells[44–46] could prove useful in inserting multiple regulatory sequences *in vivo*.

Methods

Animals studies. We obtained 6–8-week C57Bl/6 and C57Bl/6-*scid* mice from Jackson Laboratory and housed them under SPF conditions. Adult haemophilia B-C57Bl/6 mice[42] were provided by D.W. Stafford. Animals were treated according to the NIH Guidelines for Animal Care and the Guidelines of Stanford University. We injected plasmid DNA in 0.85% saline (2 ml) into the tail vein over 5–8 s (refs 17,18). We periodically bled mice by the retro-orbital technique.

Blood analysis. We analysed mouse serum for total human AAT or human FIX antigens by an ELISA assay[47,48]. We assessed liver injury in mice following plasmid administration by analysing serum glutamic pyruvic transaminase (SGPT) levels every other day for 14 d as described[49]. We determined bleeding times by clipping a section of tail 2–3 mm in length and measuring the time required to clot. For mice whose blood did not clot, we collected blood for 30 min or until 300 µl had been collected, at which time the tails were cauterized.

Cell culture studies. We used a transient transfection assay to determine the functional competency of the plasmids pTnori, pCMV-SB and pCMV-mSB in cultured mammalian cells. We seeded 5×10^5 HeLa cells in 6-cm-diameter dishes 24 h before transfection. We transfected cells with 1.5 µg pTnori and 1.5 µg pCMV-GFP, 1.5 µg pCMV-SB or 1.5 µg pCMV-mSB using Superfect (Qiagen). We trypsinized cells after 48 h and seeded 3.8×10^4 cells on 10-cm-diameter dishes for growth in DMEM containing G418 (500 µg/ml) for 14 d. To determine the relative transposition frequencies, we counted the number of G418-resistant ($G418^R$) foci on each plate after fixing in formaldehyde and staining with methylene blue.

Protein analysis. We prepared total cell lysates from HeLa cells transfected with pCMV-SB or pCMV-mSB and determined transposase expression levels by western-blot analysis. We made cell lysates in the presence of a protease inhibitor mix (Boehringer), separated them by 10% SDS–PAGE and transferred proteins to nitrocellulose. We incubated blots for 1 h at RT in blocking buffer (1×PBS containing 0.1% Tween 20 and 1% milk), followed by a 1 h RT incubation in blocking buffer containing polyclonal anti-*Sleeping Beauty* transposase antibody (Z.I. and Z.I., unpublished data) at a 1:1,000 dilution. We washed blots three times for 15 min each in washing buffer (blocking buffer with 0.5% BSA), then incubated them for 1 h at RT in blocking buffer containing HRP-labelled goat anti-rabbit IgG (Pierce) at a 1:2,500 dilution. We washed blots as before and developed them using the ECL kit (Amersham).

Plasmid construction. The plasmid pCMV-SB, expressing the *Sleeping Beauty* transposase from the CMV promoter, has been described[31]. To generate the plasmid pCMV-mSB encoding nonfunctional transposase, we introduced an aspartate→alanine (D244A) mutation into the catalytic D,D(35)E domain of *Sleeping Beauty* by PCR. Using oligonucleotide primers DA1 (5′–CAAATGGCCAATGACCCCAAGCAT–3′) and DA2 (5′–GTCATTG GCCATTTGGAAGACCCA–3′) in conjunction with CL1 (5′–CGCATC GATGACGGCCAGTGAATT–3′) and CL2 (5′–GCCATCGATCAAGCTTG CATGCCT–3′), we generated a 1-kb product. We digested PCR products with *Bam*HI and *Eco*RI, cloned them into a derivative of pcDNA3 (Invitrogen) containing a 2.2-kb *Pvu*II *neo* deletion (pcDNA3-N) and confirmed the mutation by sequence analysis. We constructed the control plasmid pCMV-GFP expressing the green fluorescent protein by inserting the 0.8-kb *Pst*I-*Not*I fragment from pEGFP-N1 (Clontech) into pcDNA3-N.

We generated the plasmid pThAAT, which encodes a 2.8-kb human AAT transposon under the control of the *RSV* promoter, by replacing the *Nsi*I-*Bsm*I region of pTNeo (ref. 31) with the 2-kb *Xho*I fragment from pBS-RSV-hAAT-bpA (ref. 47). We constructed the pTEF1α-hFIX plasmid encoding a 5.4-kb transposon expressing human FIX from the ubiquitous *Eif1a* promoter by *Not*I-*Spe*I ligation of the 4.7-kb fragment from pAAV-EF1α-hFIX (ref. 9) with the plasmid pT-MCS. To make the pT-MCS plasmid, which contains 9 unique restriction sites between the transposon terminal repeats, we replaced the 1.4-kb *Nsi*I-*Bsm*I fragment in pTNeo with annealed oligonucleotide primers MCS1 (5′–TATCGATACTAGTT TAATTAAGATCTCGAGCTAGCGGCCGCTG–3′) and MCS2 (5′–GCG GCCGCTAGCTCGAGATCTTAATTAAACTAGTATCGATATGCA–3′). We produced the plasmid pTβgeo, which encodes a 6-kb transposon expressing β-galactosidase from the RSV promoter, by first replacing the 1.4-kb *Hin*dIII-*Xma*I human ATT cDNA in pBS-RSV-hAAT-bpA with the 4.3-kb *Hin*dIII fragment from pSAβGeolox2dta (provided by P. Soriano) to create pβgeo, from which a 5-kb *Xho*I fragment was excised and inserted into the *Nsi*I-*Bsm*I region of pTNeo. We made the transposon recovery plasmid pTnori by replacing the origin-minus *neo* cassette in pTNeo with the 2.2-kb *Avr*II-*Bsm*I fragment from pAAV-Snori[50].

β-galactosidase expression. Mouse liver, spleen, kidney, heart, lung and brain were frozen in OCT buffer on dry ice. We stained sections (10 µm) for β-galactosidase (β-gal) expression using 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal). For liver tissue, we counted ~2,000 total hepatocyte nuclei located within 30–40 random fields from the sections of 2–3 liver lobes from each animal.

Transposon rescue from the mouse genome. We injected C57Bl/6 mice with pTnori (25 µg) and a helper plasmid (2.5 µg) expressing either functional (pCMV-SB) or nonfunctional (pCMV-mSB) transposase and killed them after 30 d. We digested total liver DNA (3 µg) with *Sal*I and treated it with shrimp alkaline phosphatase (SAP) to minimize recovery of the supercoiled donor plasmid in bacteria. To release the transposon and its flanking genomic sequences from mouse chromosomes, we digested DNA with *Nhe*I, *Spe*I and *Xba*I. Each of these endonucleases produces compatible cohesive ends and does not cut within the transposon or the plasmid-encoded ampicillin gene. We ligated with T4 DNA ligase under dilute conditions to form circular plasmids. We transformed DH10B electrocompetent cells with ligations and growth-selected by replica plating on LB agar containing ampicillin (100 µg/ml) or kanamycin (30 µg/ml). We analysed transposon DNA from Amp^S/Kan^R bacteria by *Hin*dIII digestion and gel electrophoresis. We sequenced the DNA flanking the 5′ and 3′ terminal transposon repeats using primers IR-1 (5′–AGATGTCCTAACTGACTTGCC–3′) and IR-2 (5′–GTG GTGATCCTAACTGACCTT–3′), respectively.

GenBank accession numbers. Mouse WSB-1, mRNA AF033186; mouse *H2-M10.1*, AF016309.

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