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Review

A Broad Range of Dose Optima Achieve High-level, Long-term Gene Expression After Hydrodynamic Delivery of *Sleeping Beauty* Transposons Using Hyperactive SB100x Transposase

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The *Sleeping Beauty* (SB) transposon system has been shown to enable long-term gene expression by integrating new sequences into host cell chromosomes. We found that the recently reported SB100x hyperactive transposase conferred a surprisingly high level of long-term expression after hydrodynamic delivery of luciferase-encoding reporter transposons in the mouse. We conducted dose-ranging studies to determine the effect of varying the amount of SB100x transposase-encoding plasmid (pCMV-SB100x) at a set dose of luciferase transposon and of varying the amount of transposon-encoding DNA at a set dose of pCMV-SB100x in hydrodynamically injected mice. Animals were immunosuppressed using cyclophosphamide in order to prevent an antiluciferase immune response. At a set dose of transposon DNA (25 µg), we observed a broad range of pCMV-SB100x doses (0.1–2.5 µg) conferring optimal levels of long-term expression (>10^{11} photons/second/cm^2). At a fixed dose of 0.5 µg of pCMV-SB100x, maximal long-term luciferase expression (>10^{10} photons/second/cm^2) was achieved at a transposon dose of 5–125 µg. We also found that in the linear range of transposon doses (100 ng), co-delivering the CMV-SB100x sequence on the same plasmid was less effective in achieving long-term expression than delivery on separate plasmids. These results show marked flexibility in the doses of SB transposon plus pCMV-SB100x that achieve maximal SB-mediated gene transfer efficiency and long-term gene expression after hydrodynamic DNA delivery to mouse liver.

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The *Sleeping Beauty* transposon system (SB) is a nonviral vector that overcomes the limitation of transient expression by integration of new gene sequences into the host cell genome. SB is made up of two components[1]: (i) the transposon, which consists of a sequence of interest flanked by transposon inverted repeat/direct repeats and (ii) SB transposase, which binds to the inverted repeat/direct repeats and through a concerted cut and paste mechanism integrates the transposon sequence into the host cell genome. SB has been tested for stable gene delivery *in vitro* in a wide variety of cell types and *in vivo* in several different tissues,[2,3] most extensively after hydrodynamic delivery to the liver.[4–10] Since it was originally reconstructed, several hyperactive forms of SB transposase have been generated by introducing amino acid substitutions into the catalytic and DNA-binding domains of the transposase. Modifications have also been introduced into the transposon inverted repeat/direct repeat sequence, rendering a more effective substrate for transposition.[11–15] Hyperactive SB100x, generated by a combination of site-directed mutagenesis and *in vitro* evolution, exhibits a considerable increase in transposition frequency compared with the first-generation SB10 transposase[16] and confers a remarkably high level of long-term expression after hydrodynamic delivery to the liver.[16,17] However, a detailed study of *in vivo* SB transposon and transposase dose responses has not been reported to date.

To further characterize the *in vivo* effectiveness of hyperactive SB100x transposase, we started by varying the amount of pCMV-SB100x transposase-encoding plasmid infused hydrodynamically along with a fixed amount (25 µg) of transposon-encoding plasmid into C57BL/6 mice. We recently reported[17] that high-level, long-term expression of luciferase in immunocompetent C57/BL6 mice results in a cellular antiluciferase immune response and a 4 log-fold reduction in luciferase expression, so in these experiments all animals were immunosuppressed by administration of cyclophosphamide (unless otherwise noted). As shown in **Figure 1a**, animals that were administered 2.5 µg of pCMV-SB100x expressed the highest level of sustained luciferase activity, 4×10^{10} photons/second/cm^2 at week 14. We observed a marked reduction in luciferase expression starting 1 day post injection and a 10-fold reduced level of long-term expression for animals administered 12.5 µg of pCMV-SB100x relative to other doses. Animals administered 0.5 µg of pCMV-SB100x expressed high levels of luciferase out to week 5, but then had to be euthanized due to poor health most likely associated with an aggressive (twice weekly) cyclophosphamide administration schedule (**Figure 1a**). **Figure 1b** shows that a second set of animals co-injected with 500 or 100 ng of pCMV-SB100x along with 25 µg of pT2/CaL expressed similarly high levels of luciferase long term, 3–4×10^{10}

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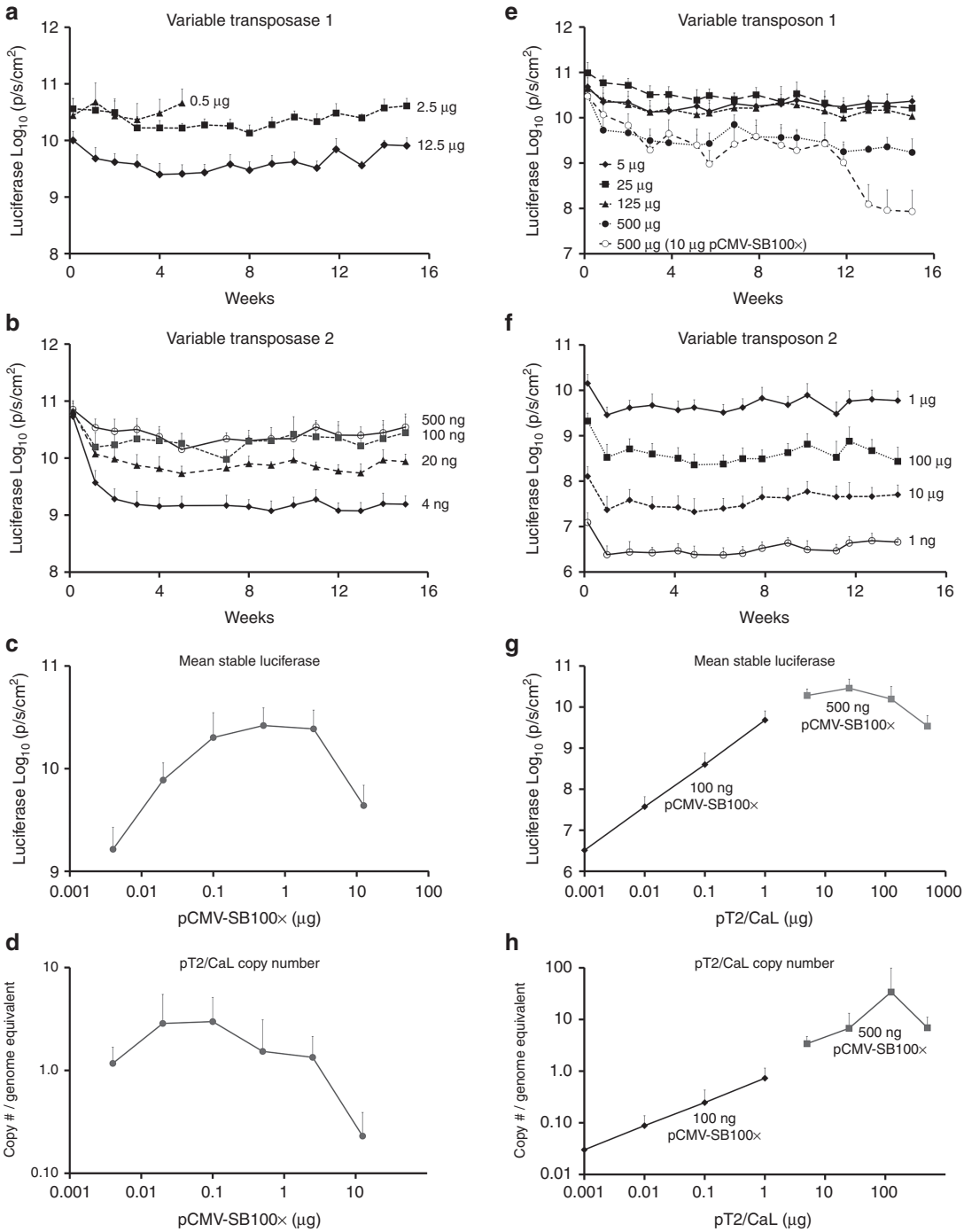

photons/second/cm^2 at week 14. A significant drop in long-term expression was not observed until the dose of pCMV-SB100X was reduced to 20 ng (8×10^9 photons/second/cm^2), and even at a dose as low as 4 ng of pCMV-SB100X, we observed a long-term expression level of >10^9 photons/second/cm^2. We have reported that animals receiving no pCMV-SB100x maintained long-term luciferase expression levels at around 10^8 photons/second/cm^2 (ref. 17). The mean level of stable, long-term luciferase expression from weeks 4 through 14 is plotted against the dose of pCMV-SB100X in **Figure 1c**, demonstrating that (i) only low doses of transposase-encoding plasmid (as low as 20 and even 4 ng) are required for significant levels (>10^9 photons/second/cm^2) of long-term luciferase expression and (ii) optimally effective doses of pCMV-SB100x (*i.e.*, conferring >10^{10} photons/second/cm^2 long-term luciferase expression) ranged broadly, from 100 ng up to 2.5 µg, when co-delivered along with 25 µg of pT2/CaL transposon.

We also tested the effectiveness of hyperactive SB100x transposase expressed after administration at a set dose of 0.5 µg of pCMV-SB100x, systematically varying the dose of pT2/CaL transposon DNA. Under these conditions, mice infused with 5, 25, and 125 µg of transposon DNA all

expressed similar, optimally high levels of luciferase long term, around 1–2.3×10^{10} photons/second/cm^2 at 14 weeks post-infusion (**Figure 1e**). The level of long-term luciferase expression was reduced in animals administered 500 µg of pT2/CaL, whether co-administered with 0.5 or 10 µg of pCMV-SB100x. In contrast, for animals administered lower doses of pT2/CaL at a set dose of 0.1 µg of pCMV-SB100X, there was a one-to-one correspondence between transposon dose (from 1 µg down to 1 ng) and the level of long-term luciferase expression (from 6×10^9 down to 4.6×10^6 photons/second/cm^2; **Figure 1f**). We thus observed optimal levels of long-term luciferase expression at a broad range of transposon doses (*i.e.*, 5 –125 µg), but even doses as low as 1 ng were able to confer long-term luciferase expression (mean levels of long-term expression plotted in **Figure 1g**). In combination with the broad range of pCMV-SB100X dose optima described above, the SB system exhibited marked flexibility in relative doses of transposon plus transposase-encoding DNA that can be used to achieve maximal gene expression after hydrodynamic DNA delivery.

After 14–15 weeks of monitoring luciferase expression, the animals were euthanized, and DNA extracted from whole liver for quantitative PCR analysis of luciferase gene copy number (**Figure 1d**,**h**). Animals infused with 25 µg of pT2/CaL at variable pCMV-SB100X all had an average of one to three luciferase copies per genome equivalent, except that animals administered the highest dose of transposase-encoding plasmid (12.5 µg) had a reduced number of sequences (0.2 copies/genome equivalent; **Figure 1d**). These results imply significant retention of unexpressed transposon DNA in the liver of animals administered lower doses (4 and 20 ng) of pCMV-SB100X that becomes converted to expressed DNA, assumedly by chromosomal transposition[18] in animals administered optimum levels (0.1, 0.5, and 2.5 µg) of pCMV-SB100X. At set doses of 100 and 500 ng of pCMV-SB100X, luciferase copy number corresponded directly with the dose of transposon DNA, ranging from a mean of 0.03 copies/genome equivalent in animals administered 1 ng of pT2/CaL up to a mean of 34 copies/genome equivalent in animals administered 125 µg of pT2/CaL (**Figure 1h**). Animals that received the highest dose (500 µg) of pT2/CaL had fewer luciferase copies (mean of 7.0 per genome equivalent), although this was not significantly different from animals administered the 25 or 125 µg dose. We thus observed maximally effective doses of pT2/CaL (125 µg) and pCMV-SB100X (5 µg) which if superseded became detrimental to retention of transgene copy number (pCMV-SB100X) as well as the long-term level of luciferase expression (both pCMV-SB100X and pT2/CaL).

The linear correspondence between long-term gene expression and the dose of transposon DNA delivered below 1 µg provides an opportunity to test different conditions of delivery for the effect on transposition and long-term expression *in vivo*. In this way, we tested the effect of delivering the transposon and transposase coding sequences on the same plasmid (*cis*-configuration) in comparison with delivery on two separate plasmids (*trans*-configuration). Animals were hydrodynamically injected with 100 ng of pT2/CaL plus 100 ng of pCMV-SB100x (*trans*-configuration), with 100 ng of pT2/CaL//CMV-SB100X (*cis*-configuration, **Figure 2a**) or with 100 ng of pT2/CaL alone. These amounts are in the linear range of the transposon dose response (**Figure 1g**), so the relative effectiveness of *cis* versus *trans* delivery can be gauged by the difference in long-term expression level observed. This lower transposon dose also eludes a cellular antiluciferase immune response elicited at higher levels (>10^9 photons/second/cm^2) of luciferase expression.[17] Animals that received the *trans*-configuration maintained a stable luciferase expression level of 2.3×10^8 photons/second/cm^2 (**Figure 2b**), similar to that observed in **Figure 1f** for immunosuppressed animals, verifying lack of immunogenicity at this dose of transposon. Although the *cis* plasmid pT2/CaL//CMV-SB100X showed a higher burst of expression on day 1 post-injection, luciferase activity subsequently dropped about 10-fold, stabilizing at around 5.5×10^7 photons/second/

Figure 1 Effect of variable transposon and transposase plasmid dose on the level of long-term expression after hydrodynamic delivery. (**a**) C57Bl/6 mice were hydrodynamically injected at a set dose of 25 µg of pT2/CaL plus 12.5, 2.5, or 0.5 µg of pCMV-SB100x and then assayed weekly for luciferase expression by *in vivo* bioluminescence imaging. (**b**) Luciferase expression of animals that received 500, 100, 20, or 4 ng of pCMV-SB100x at a fixed dose of 25 µg of pT2/CaL by hydrodynamic injection. (**c**) Mean level of luciferase expression starting on week 1 to the end of the experiment for animals depicted in **a** and **b**. (**d**) Luciferase DNA copy number in total liver DNA extracted from animals in **c**. (**e**) Luciferase imaging of animals hydrodynamically injected with 500 ng of pCMV-SB100x plus 5, 25, 125, or 500 µg of pT2/Cal with an additional group of mice receiving 500 µg of pT2/CaL plus 10 µg of pCMV-SB100x. (**f**) Luciferase expression of mice hydrodynamically injected at a fixed dose of 100 ng of pCMV-SB100x along with 1 µg, 100 ng, 10 ng, or 1 ng of pT2/CaL. (**g**) Mean levels of luciferase expression starting at week 1 to the end of the experiment for animals depicted in **f** and animals that received 500 ng of pCMV-SB100x in **e**. (**h**) Mean luciferase DNA copy number as determined by real-time quantitative PCR on total liver DNA extracted from animals depicted in **g**. Methods: C57BL/6NCr females (National Cancer Institute, Fredrick, VA) were housed under specific pathogen-free conditions and provided food and water *ad libitum*. The University of Minnesota Institutional Animal Care and Use reviewed and approved all animal procedures. Previously described transposon pT2/CaL (containing the CAGs promoter regulating firefly luciferase[23]) and SB transposase-encoding plasmid pCMV-SB100X[16] were hydrodynamically injected into female mice. Animals were immunosuppressed using 120 mg/kg cyclophosphamide (CP; Sigma-Aldrich, St Louis, MO) infused i.p. on the day before, the day of, daily for 2 days after hydrodynamic injection, and then weekly thereafter. Briefly, for hydrodynamic injections, animals were administered 0.03 ml of analgesic cocktail consisting of 8 mg/ml of ketamine (Phoenix Scientific, St Joseph, MO), 0.01 mg/ml of butorphanol tartrate (Fort Dodge Animal Health, Fort Dodge, IA), and 0.1 mg/ml of acepromazine maleate (Phoenix Scientific) in 0.9% sodium chloride by i.p. injection. As previously described, plasmid was diluted in lactated Ringer's solution to 10% volume per animal weight and then infused through the lateral tail vein of the mouse in less than 10 seconds.[19,20,24] Starting on day 1 and then weekly after hydrodynamic injection, luciferase expression was assessed by *in vivo* bioluminescence imaging. The mice were anesthetized with 0.2 ml of analgesic cocktail i.p., followed by 0.1 ml of 28.5-mg/ml D-luciferin substrate (Gold Biotechnology, St Louis, MO) i.p. The animals were then imaged for a duration of 0.5 seconds to 5 minutes using the Xenogen IVIS imaging system (Xenogen, Alameda, CA). Luciferase activity levels are expressed as photons emitted per second per square centimeter (p/s/cm^2). For real-time quantitative PCR, total DNA was extracted from pulverized livers using phenol-chloroform. Luciferase sequences in DNA samples were quantified by real-time PCR using the Applied Biosystems TaqMan Gene Expression Assay kit (Life Technologies, Grand Island, NY) and an Eppendorf thermocycler realplex (Eppendorf North America, Hauppauge, NY). Linearized pT2Cal was diluted into naive C57BL/6 DNA to generate luciferase copy number standards. Results are expressed as luciferase sequence copies per genome equivalent.

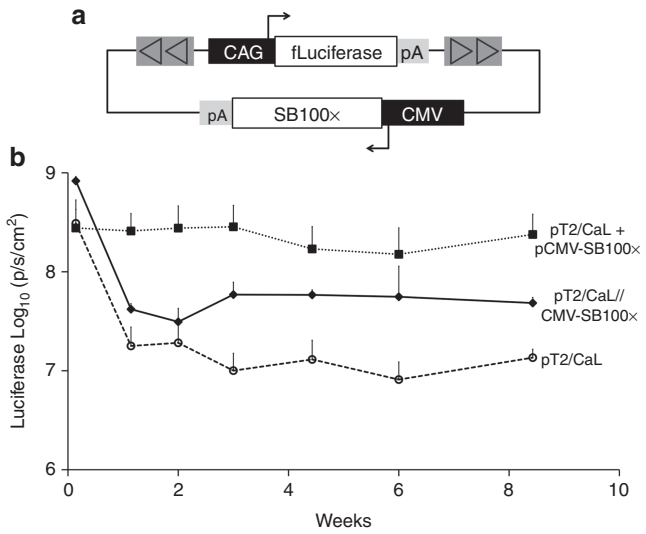

Figure 2 Comparative level of luciferase expression after *cis* versus *trans* delivery of transposon and transposase-encoding sequences. (**a**) Plasmid pT2/CaL/CMV-SB100x was engineered from pT2/CaL[22] to contain both the CAG's regulated firefly luciferase transposon and the CMV's regulated SB100X. (**b**) B6 mice were hydrodynamically injected with 100 ng of pT2/CaL plus 100 ng of pCMV-SB100x (*trans*-configuration), 100 ng of pT2/CaL//CMV-SB100x (*cis*-configuration), or 100 ng of pT2/CaL alone, then assayed on day 1 and weekly thereafter for luciferase expression. Methods: Plasmid pT2/CaL//CMV-SB100X was constructed to contain the T2/CaL transposon from pT2/CaL, with the addition of: (i) a CMV promoter sequence from pCMV-SB11[11] starting 33 bp downstream of the T2/CaL transposon IR/DR, (ii) the SB100x coding sequence[25] from 810 to 1,833 bp downstream of the T2/CaL transposon sequence, and (iii) an SV40 polyadenylation signal from 1,849 to 2,087 bp downstream of the T2/CaL sequence. Other methods are as described in Figure 1. IR/DR, inverted repeat/direct repeat.

cm^2. This is about five times higher than the expression level in animals injected with pT2CaL alone (~1.1×10^7 photons/second/cm^2; **Figure 2b**) but is about fivefold less than the level achieved by co-delivery of SB100X-encoding DNA in *trans*. It should be noted that on a molar basis, there was about 50% less transposon and transposase-encoding DNA injected in *cis*, but this does not explain the fivefold reduced level of long-term expression observed in comparison with delivery in *trans*.

Effectiveness of the *Sleeping Beauty* transposase system to achieve long-term gene expression following hydrodynamic delivery into murine liver has been widely reported.[4,7,10,17] The maximal effective dose of hydrodynamically delivered DNA in mice is in the range of 10–25 µg, and while there is no apparent benefit in gene expression associated with larger doses of DNA (several hundred micrograms), neither does there appear to be notable toxicity associated with these larger doses.[19,20] We similarly observed little difference in the level of long-term expression after delivery of 5–125 µg of pT2/CaL transposon DNA. However, at higher doses (500 µg) of pT2/CaL transposon, we observed an inhibitory effect on the level of long-term expression achieved, as well as a reduced level of maintained transposon sequence. This reduced level of transposition was not likely the result of an imbalance with the level of transposase since it was also observed at a higher relative dose (10 µg) of pCMV-SB100X DNA (**Figure 1e**). This reduction in both long-term luciferase expression and transposon copy number could be due to either a cytotoxic response or transposon degradation.

As originally reported by Mátés *et al.*,[16] we found that by using the hyperactive pCMV-SB100x transposase plasmid, expression of hydrodynamically delivered transposons was maintained at levels largely undiminished from the initial peak of expression (**Figures 1a**,**e** and **2b**; ref. 17). We further observed that a broad range of transposon and transposase DNA doses achieved a high level of stable long-term expression. Doses ranging from 100 ng to 2.5 µg of SB100x transposase-encoding plasmid DNA and from 5 to 125 µg of transposon plasmid DNA achieved sustained, high levels of luciferase expression (over 10^{10} photons/second/cm^2). This wide range of optimal conditions allows the user of the *Sleeping Beauty* transposase system considerable flexibility in DNA dosing while maintaining high-level gene expression in mice. Long-term expression at somewhat reduced levels were observed not only at surprisingly low doses (as little as 4 ng) of SB100X transposase-encoding plasmid, but also at higher doses of pCMV-SB100X, consistent with the concept of transposase overexpression inhibition,[21,22] whereby an excess of transposase compromises formation of the synaptic complex during the process of transposition.[22] Reduced transposon copy number in animals administered the higher dose of pCMV-SB100X also implies either cytotoxicity or DNA degradation associated with the reduced level of long-term luciferase expression. Mikkelsen *et al.*[9] reported a similarly reduced level of SB-mediated long-term expression when the CMV promoter was used to regulate expression of SB10 in the context of bifunctional transposon–transposase vectors. However, improved levels of long-term expression were observed when more moderate strength promoters were used for SB transposase expression. We similarly observed that more moderate doses of pCMV-SB100X conferred optimal levels of long-term SB-mediated expression *in vivo*. These results show the marked flexibility in relative doses of SB transposon plus hyperactive pCMV-SB100x that can be used to achieve maximal SB-mediated gene transfer efficiency and gene expression after hydrodynamic DNA delivery to the liver.

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