

RNA as a Source of Transposase for *Sleeping Beauty*-Mediated Gene Insertion and Expression in Somatic Cells and Tissues

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***Sleeping Beauty* (SB) is a DNA transposon capable of mediating gene insertion and long-term expression in vertebrate cells when co-delivered with a source of transposase. In all previous reports of SB-mediated gene insertion in somatic cells, the transposase component has been provided by expression of a co-delivered DNA molecule that has the potential for integration into the host cell genome. Integration and continued expression of a gene encoding SB transposase could be problematic if it led to transposon re-mobilization and re-integration. We addressed this potential problem by supplying the transposase-encoding molecule in the form of mRNA. We show that transposase-encoding mRNA can effectively mediate transposition *in vitro* in HT1080 cells and *in vivo* in mouse liver following co-delivery with a recoverable transposon or with a luciferase transposon. We conclude that *in vitro*-transcribed mRNA can be used as an effective source of transposase for SB-mediated transposition in mammalian cells and tissues.**

Key Words: non-viral integration, RNA delivery, bioluminescent imaging, *Sleeping Beauty*, liver

The *Sleeping Beauty* (SB) transposon system was generated by “repairing” an evolutionarily decayed *Tc1*-like sequence found in the genomes of salmonid fish [1]. When expressed, the SB transposase is able to insert sequences flanked by engineered binding sites into cultured human cells more efficiently than any other transposable element [2], making SB the most active, currently identified system for non-viral gene insertion. Several laboratories have used the SB system to demonstrate chromosomal integration and long-term expression of reporter genes and therapeutic transgenes in tissues of experimental mice, suggesting SB as a good candidate for non-viral gene therapy applications (reviewed in [3,4]).

All reported studies of SB-mediated gene transfer in somatic cells and tissues have thus far supplied SB-encoding plasmid DNA as the source of the transposase component of the system. This strategy of delivery has proven to be quite effective in mediating *in vivo* transposition. However, provision of the transposase-encoding sequence as a co-deliverable DNA molecule may be problematic. Continued expression of transposase after

the initial transposition event raises the possibility of subsequent transposon excision and re-integration, with associated risk of genotoxicity. There is also the possibility that some transposase-encoding DNA sequences will become integrated into the host genome, potentially resulting in stable transposase expression with destabilizing effects with respect to the integrated transgene. Messenger RNA (mRNA) may be an effective alternative to DNA as a source of SB transposase for targeting somatic cells and tissues. Although it is possible for RNA to undergo reverse transcription followed by cDNA integration, this is very unlikely, making RNA a safer alternative to DNA as a source of SB transposase for somatic gene therapy applications. Here we report the use of *in vitro*-transcribed mRNA as a transient source of transposase and subsequent transposition in cultured human cells and in live mice.

Current use of mRNA as a source of transposase has been limited to micro-injection studies in single-cell embryos of zebrafish and mice (reviewed in [5,6]). To test the usefulness of mRNA as a transient source of trans-

posase in somatic cells, we transfected a transposon-donor plasmid consisting of a puromycin resistance gene transcriptionally regulated by the human PGK promoter (pT2/PGK-Puro) into cultured HT1080 human epithelial cells in combination with *in vitro*-transcribed, 7-methylguanosine (m₇G)-capped RNAs encoding the SB11 transposase stabilized with 5' and 3' untranslated sequences from the *Xenopus laevis* β -globin gene. We included cultures transfected with SB11-encoding plasmids in which transposase expression was regulated by the human PGK or UbC promoters as controls (Fig. 1A). Fig. 1B shows that co-delivery of a DNA-encoded source of transposase resulted in a 23- to 40-fold increase in puromycin-resistant colonies relative to controls receiving an equimolar amount of a luciferase-encoding plasmid (used here as an irrelevant DNA control). Importantly, when we used SB11-encoding mRNA as a transposase source, we observed a 9-fold increase in puromycin-resistant colony formation relative to co-transfection with an equal amount of UTR-stabilized luciferase-encoded mRNA (Fig. 1B).

To confirm that the increased frequency of stable gene insertion using mRNA as a source of transposase was the result of transposition rather than an increased rate of random recombination, we used a genomic recovery strategy to examine chromosome:transposon junction sequences from puromycin-resistant colonies. We inserted a rescue cassette consisting of the pUC19 bacterial origin of replication and the prokaryotic EM7 promoter regulating the blasticidin-resistance gene (Bsd^R) into pT2/PGK-Puro just upstream of the PGK promoter (Fig. 2). We mixed this transposon with SB11 transposase-encoding mRNA and co-transfected them into cultured HT1080 cells. We selected stable clones in growth medium supplemented with puromycin. We isolated genomic DNA from a pooled population of puromycin-resistant colonies, digested it with *SspI* (an enzyme that does not cut inside the transposon), and then used a plasmid rescue procedure to recover transposon integrants along with both chromosomal flanking sequences. Direct sequencing of recovered plasmids revealed integration of transposon sequences into human chromosomes and duplication of a target TA dinucleotide (Fig. 2A). These results demonstrate that *in vitro*-transcribed, UTR-stabilized transposase-encoding mRNA can be used as a source of transposase for *Sleeping Beauty*-mediated transposition in cultured somatic cells.

Rapid injection of naked DNA or RNA in a large volume of solution through the tail vein of mice can lead to high-level gene expression in the liver [7–9], providing a convenient method for studying gene expression in live animals. Using this *in vivo* transfection technique, McCaffrey *et al.* established several important criteria essential to achieving efficient transfer of mRNA to the livers of living mice [9]. Based on these criteria, we established conditions (data not shown) that achieve

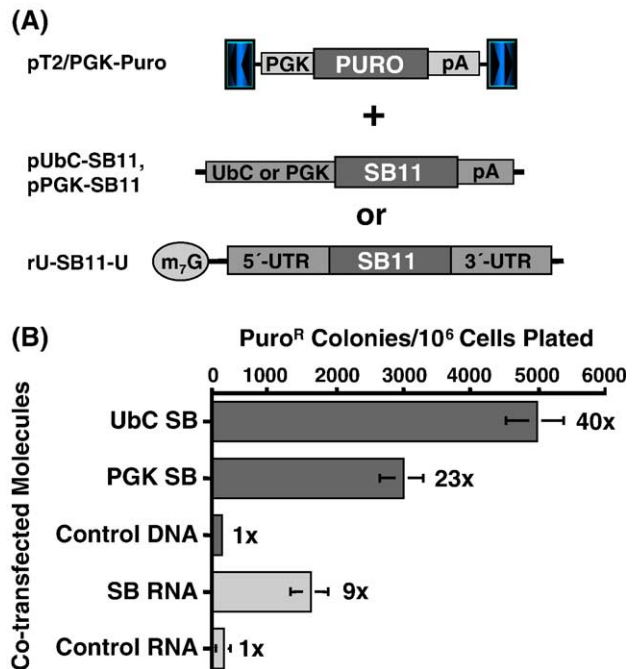


FIG. 1. *In vitro*-transcribed mRNA encoding *Sleeping Beauty* transposase mediates transposition in cultured human cells. (A) Schematic diagram of transposon and transposase combinations used for *in vitro* colony-forming transposition assay. A transposon that confers resistance to puromycin was constructed using T2 inverted terminal repeat sequences as described [27]. A puromycin resistance expression cassette was excised from pNEB193/PGK-Puro by digestion with *SmaI*-*PmeI* and then cloned into the *EcoRV* site of pT2/BH to generate pT2/PGK-Puro. Both DNA and mRNA were used to provide a transient source of transposase protein. PGK, human phosphoglycerate kinase promoter; UbC, human ubiquitin C promoter; SB11, *Sleeping Beauty* transposase; pA, bovine growth hormone polyadenylation signal; UTR, 5' and 3' untranslated sequences from the *X. laevis* β -globin gene; m₇G, 5' 7-methylguanosine cap. A plasmid template for *in vitro* transcription of *Sleeping Beauty* transposase was constructed by insertion of an *NcoI* (blunt)-*BamHI* SB11 sequence obtained from pCMV-SB11 [28] into pT3/TS between *SmaI* and *BamHI* (described in Davidson *et al.* [29]) to create rU-SB11-U. Capped RNA transcripts were generated from *BamHI*-digested rU-SB11-U using T3 RNA polymerase and the mMessage Machine Kit from Ambion (Austin, TX, USA), subsequently treating the RNA product with DNase I to remove DNA template. RNA was purified by lithium chloride precipitation and washed twice with 70% ethanol. To generate pPGK-SB11, the luciferase sequence was excised from pPGK-Luc between *BglII* and *XbaI* and replaced with the SB11 coding sequence [30]. pUbC-SB11 was generated by removing the luciferase gene between *BamHI* and *XbaI* and inserting SB11 as a *BglII*-*XbaI* fragment. Plasmid DNA was prepared using an Endofree Maxi Prep Kit (Qiagen, Valencia, CA, USA). (B) Puromycin-resistant colony formation when mRNA is used as a transient source of transposase. $4\text{--}5 \times 10^5$ human fibrosarcoma HT1080 cells were seeded into 6-cm tissue culture plates. Cells were co-transfected the following day with pT2/PGK-Puro (500 ng) plus DNA or RNA sources of transposase (500 ng) as well as equimolar amounts of control (luciferase-encoding) DNA or RNA using SuperFect reagent (Qiagen). 48 h later, 50,000 viable cells (trypan blue negative) were plated into 100-mm dishes containing complete growth medium supplemented with 2 $\mu\text{g}/\text{ml}$ puromycin (Invivogen, San Diego, CA, USA). After 14 days of selection, puromycin-resistant colonies were fixed and stained with 70% methanol/1% crystal violet to determine the frequency of puromycin-resistant colony formation. Three independent transfections were performed for each pair of constructs and average colony numbers \pm SE are reported for every 10^6 cells plated.

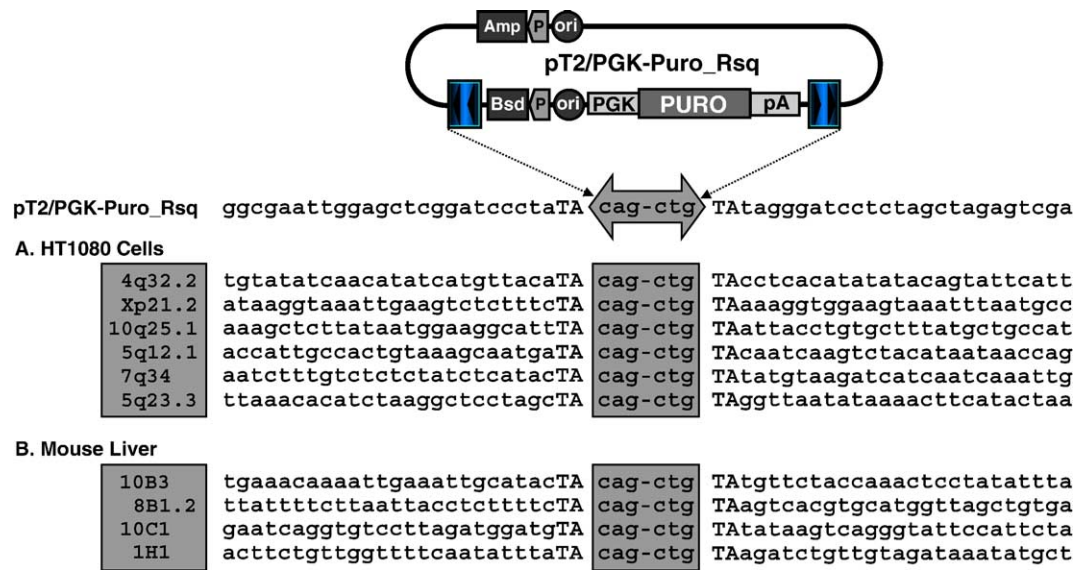
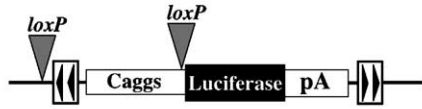


FIG. 2. Molecular evidence of transposition in cultured HT1080 cells and mouse liver after co-delivery of *Sleeping Beauty* transposase-encoding mRNA. To recover chromosome:transposon junction sequences, a rescue cassette consisting of the pUC bacterial origin of replication, EM7 bacterial promoter, and blasticidin-resistance gene was cloned as a *Sna*I fragment into the *Eco*RV site upstream of the PGK promoter in pT2/PGK-Puro to generate pT2/PGK-Puro_Rsq. (A) HT1080 cells were co-transfected with 500 ng of pT2/PGK-Puro_Rsq along with an equal concentration of *in vitro*-transcribed SB11 transposase-encoding RNA using Superfect (Qiagen). DNA was extracted from approximately 300 pooled puromycin-resistant colonies using the Puregene DNA purification kit (Gentra Systems, Minneapolis, MN, USA; Protocol 00970). (B) C57BL/6 mice ($N = 2$) were co-administered 20 μ g of pT2/PGK-Puro_Rsq along with 50 μ g of *in vitro*-transcribed SB11 transposase-encoding RNA supplemented with 800 units of native RNasin (Promega, Madison, WI, USA). One month after injection, animals were killed and total DNA was prepared from whole liver homogenates digested overnight by incubation in 10 ml cell lysis buffer (Gentra Systems) supplemented with 100 μ g/ml proteinase K followed by organic extraction with phenol/chloroform/isoamyl alcohol and precipitation with 2 volumes of 100% isopropanol. For genomic recovery of transposon insertion site sequences, 2 μ g of total DNA was digested with *Ssp*I and precipitated in 100% isopropanol and the recovered DNA ligated under dilute conditions (500 μ l) with 20 units of T4 DNA ligase (New England BioLabs, Beverly, MA, USA). The ligated DNA was precipitated with 100% isopropanol and washed twice with 70% ethanol before being resuspended in 10 μ l sterile H₂O. Two microliters of reconstituted DNA was electroporated into DH10B electrocompetent *Escherichia coli* (Promega), which were allowed to recover before being plated on LB agar containing 100 μ g/ml blasticidin. Blasticidin-resistant colonies were counter-selected on agar supplemented with 50 μ g/ml ampicillin. Plasmid DNA was isolated from Bsd^R/Amp^S colonies and sequenced at the Advanced Genetics Analysis Center at the University of Minnesota using pT2 inverted terminal repeat (ITR)-specific primers: ITR right, 5'-CCACTGGGAATGTGATGAAAG; ITR left, 5'-GACTTGTGTCATGCACAAAGTAG. Plasmid backbone sequences of pT2/PGK-Puro_Rsq are shown. TA dinucleotide target site duplications are in capital letters. Transposon-specific sequences are in the center box. Several of the recovered sequences were subjected to BlastN analysis against the human or mouse genome using the ENSEMBL database (locations indicated in shaded box on the left).

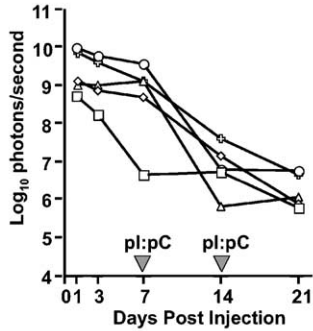
expression of newly introduced mRNA at levels that mimic those observed using DNA containing moderate strength promoters and that have been used to provide levels of transposase optimal for effective transposition in mouse liver [10,11]. To test the ability of transposase-encoding mRNA to mediate transposition *in vivo*, we co-administered the recoverable transposon pT2/PGK-Puro_Rsq (Fig. 2) with 50 μ g of m⁷G-capped RNA encoding UTR-stabilized transposase into normal C57BL/6 mice by rapid, high-volume injection. One month later, we sacrificed the animals, prepared genomic DNA from liver tissue, digested it with *Ssp*I, and then used a plasmid rescue procedure to recover transposon integrants along with chromosomal flanking sequences. Direct sequencing of recovered plasmids revealed transposon integration into mouse chromosomes and duplication of the target TA dinucleotide (Fig. 2B). This result demonstrates that *in vitro*-transcribed, transposase-encoding mRNA can be used as a source of transposase for *Sleeping Beauty*-mediated transposition in live mice.

The effect of SB-mediated transposition can be evaluated *in vivo* by comparing long-term expression levels after transposon delivery in the presence versus the absence of a source of transposase. To restrict gene expression to transposition events, we engineered plasmids to contain genetic elements that efficiently silence expression from episomal or randomly integrated transgenes, as recently reported by Score *et al.* [11]. To distinguish between transient expression and expression mediated by transposition, we engineered a transposon-containing plasmid for luciferase expression (pT2(flox)/Caggs-Luc) with *loxP* sites positioned such that Cre-mediated recombination excises a sequence that includes the promoter (Fig. 3A), unless it has been segregated from the plasmid by the SB transposase. We delivered pT2(flox)/Caggs-Luc either alone (Fig. 3B) or in combination with *in vitro*-transcribed, UTR-stabilized SB11 transposase mRNA (Fig. 3C) to the livers of Mx1Cre transgenic mice [12] containing a Cre recombinase transgene transcriptionally regulated by an interferon-

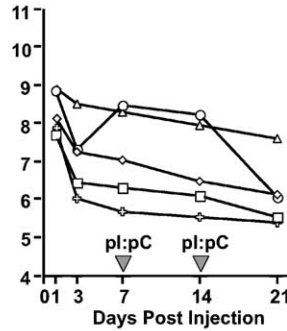
(A) pT2(flox)/Caggs-Luc



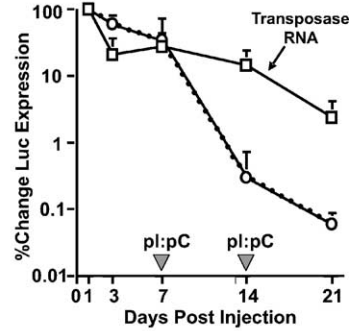
(B) No Transposase



(C) Plus Transposase RNA



(D) Mean Signal Intensity



(E) Excision PCR

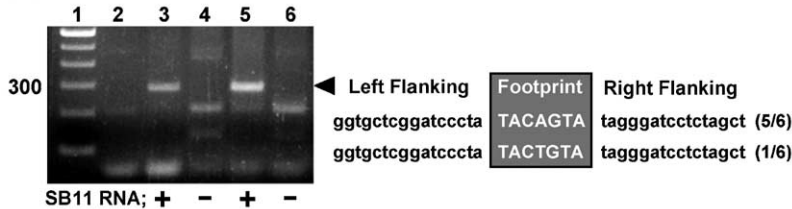


FIG. 3. *In vitro*-transcribed mRNA encoding *Sleeping Beauty* transposase mediates transposition in live mice. 25 μ g of pT2(flox)/Caggs-Luc transposon DNA \pm 100 μ g of UTR-stabilized RNA (rU-SB11-U) was supplemented with 800 units of native RNasin (Promega) and administered by rapid, high-volume tail vein injection as described [7,8] into sedated Mx1Cre Tg mice ($N = 5$ per group). Expression of Cre recombinase was induced for 3 successive days beginning on days 7 and 14 in each group by i.p. injection of 250 μ l poly(I)-poly(C) (Sigma Aldrich, St. Louis, MO, USA; 1 mg/ml solution in normal saline). At each indicated time point, animals were anesthetized by i.p. injection of 160 mg/kg ketamine plus 0.8 mg/kg acepromazine and 0.08 mg/kg butorphanol, and then 100 μ l of 28.5 mg/ml luciferin substrate was injected i.p. At 4–5 min post-injection, the live anesthetized mice were imaged for 1 s to 2 min using an intensified, charge-coupled device camera (Series 100; Xenogen Corp.) as described [30]. Raw values were recorded as photons of light emitted per second. (A) Schematic diagram of pT2(flox)/Caggs-Luc transposon engineered to contain loxP sites by partial digest of pT2F/Cage [11] with *Xba*I, replacing the human erythropoietin coding sequence with an *Xba*I luciferase fragment isolated from pT2/Caggs-Luc [31]. The resulting vector contains loxP sites positioned such that Cre-mediated recombination excises a sequence that includes the left IR/DR and the Caggs promoter, thus silencing luciferase expression. Time courses of *in vivo* luciferase enzyme activity are shown before and after induction of Cre recombinase for individual mice infused (B) with pT2(flox)/Caggs-Luc alone or (C) with rU-SB11-U. (D) The mean percentage luciferase activity remaining at any given time point relative to that observed at 24 h is shown for each group (error bar: SEM). (E) Transposon excision assay. On day 22, animals infused with DNA/RNA solutions were killed and genomic DNA was prepared from whole liver as previously described. PCR was carried out using primers ExF, 5'-TGTGCTGCAAGCGGATTA, and ExR, 5'-GGTACTAATCGGTTTCGAGC, located outside of the left and right ITR sequences and internal to the loxP site engineered into the plasmid backbone of pT2(flox)/Caggs-Luc. PCR conditions were 95°C for 10 min followed by 40 cycles of 95°C for 30 s, 61°C for 30 s, and 72°C for 90 s, with a final extension at 72°C for 10 min. The expected 300-bp products were isolated on a 2% agarose gel, extracted, cloned into pCR2.1 TOPO TA cloning vector (Invitrogen, Carlsbad, CA, USA), and sequenced at the Advanced Genetics Analysis Center at the University of Minnesota. The ethidium bromide-stained gel demonstrates excision products amplified from DNA extracts from the livers of mice infused with SB mRNA. Lane 1, 100 bp marker; lane 2, no DNA control; lanes 3 and 5, two mice infused with pT2(flox)/Caggs-Luc and rU-SB11-U; lanes 4 and 6, two mice infused with pT2(flox)/Caggs-Luc alone. The brightest band in the marker lane is 600 bp. On the right are shown the sequences identified in several plasmid clones of the 300-bp PCR product, compared against pT2(flox)/Caggs-Luc by direct alignment to define footprints (i.e., sequence remaining after excision), and the fraction of the total observations for that particular sequence.

inducible promoter. We induced expression of Cre recombinase 7 and 14 days later by i.p. injection of poly(I)-poly(C) and evaluated mRNA-mediated transposition by monitoring for persistent luciferase expression as a discrete signal upon whole-body *in vivo*

bioluminescence imaging of individual living mice [13]. There was a substantial reduction in the initial level of luciferase expression observed for animals co-injected with SB RNA in comparison with control animals, perhaps due to RNA-mediated induction of interferon.

Nevertheless, our data show that induction of Cre recombinase resulted in a 1400-fold reduction in luciferase expression for animals infused with the transposon alone, while there was only a 45-fold reduction in luciferase expression in animals receiving SB11 transposase mRNA. To account for animal-to-animal variability in expression, we calculated the percentage luciferase activity remaining at different time points relative to that observed at 24 h for each individual animal, and the mean \pm SE is presented in Fig. 3D. After two rounds of Cre recombinase induction, animals infused with the transposase mRNA maintained 2.3% of their initial luciferase activity compared to 0.06% for animals receiving the transposon alone ($P < .05$ by Student's *t* test). To confirm the ability of SB mRNA to serve as a source of transposase, we performed an excision assay, which has previously been used to determine SB-mediated transposition activity in mouse liver [14], on total genomic DNA extracted from the livers of animals in each treatment group (Figs. 3B and 3C). The excision assay generates a 300-bp PCR product detected only in animals infused with both the transposon and the transposase RNA (Fig. 3E). We cloned and sequenced these PCR products and identified canonical footprints (TACAGTA or TACTGTA) that are consistent with repair of the excision site in the plasmid (Fig. 3E). Based on this evidence, we conclude that luciferase expression remaining in these animals after induction of Cre recombinase is attributable to SB-mediated transposition. mRNA-encoded SB transposase can thus direct the transposition of SB transposons from plasmids into mouse chromosomes, thereby providing an effective alternative to DNA as a source of SB transposase.

For SB-based gene therapy applications, the use of mRNA offers a number of important advantages over delivery of a transposase-encoding DNA molecule. There is improved control with respect to the duration of transposase expression, minimizing persistence in the tissue and the potential for transgene re-mobilization and re-insertion following the initial transposition event. Furthermore, the transposase-encoding RNA sequence is likely incapable of integrating into the host genome, thereby eliminating concerns about long-term transposase expression and destabilizing effects with respect to the gene of interest. Our results show that liver-directed systemic delivery of stabilized and protected mRNA can indeed provide levels of transposase expression sufficient for transposition.

In vivo administration of mRNA has been used for therapeutic vaccination and interference with endogenous gene expression. Hoerr *et al.* reported that a specific cytotoxic T cell response and circulating antigen-specific antibodies were detected after administration of *in vitro*-transcribed, UTR-stabilized, and protamine-condensed bacterial lacZ mRNA into the ear pinna of Balb/C mice [15]. Additional studies showed that mRNA condensed

with protamine could induce maturation of cultured mouse dendritic cells, suggesting Toll-like receptors 7 and 8 as promising candidates for recognition of stabilized mRNA [16]. Interestingly, short double-stranded RNAs have been shown to be specific inhibitors of gene expression in mouse oocytes [17] and in adult mice [18] independent of global translational silencing. Recently, however, Kim *et al.* reported induction of interferon- α and - β in cultured mammalian cells transfected with single-stranded GFP-encoding mRNA transcribed *in vitro* using T3, T7, or Sp6 phage polymerases, an effect that was shown to be mediated by the free 5' triphosphate [19]. The Mx1Cre transgenic mice used for our *in vivo* studies contain the inducible promoter of the *Mx1* gene to control the expression of Cre recombinase. The *Mx1* promoter can be transiently activated upon induction of interferon with synthetic double-stranded RNA (poly(I)-poly(C)). We did observe a 10-fold decrease in luciferase activity immediately following co-delivery of transposase-encoding mRNA along with transposon DNA, likely due to interferon induction resulting from systemic delivery of mRNA transcribed *in vitro* using T3 RNA polymerase.

The use of RNA as a source of enzyme activity is potentially applicable to other integrating systems, including the *Caenorhabditis elegans* Tc1 [20], *Drosophila* Minos [21], horn fly Himar1 [22], cabbage looper moth piggyBac [23], and *Xenopus* Frog Prince [24] Tc1-type transposons as well as retrotransposons such as L1 [25]. The ϕ C31 phage integrase system has been shown to mediate successful insertion and long-term expression of therapeutic transgenes in somatic tissues of mice [26]. Similar to SB, the ϕ C31 phage integrase can be delivered and expressed separately from the plasmid that contains the integrating sequence and thus could be provided through expression of co-delivered mRNA. Use of mRNA could also provide a more regulated source of recombinase activity for experimental manipulations employing the Cre/LoxP or FLIP recombinase systems, thus preventing inadvertent prolonged recombinase expression.

In summary, our data indicate that mRNA can be used as a transient source of transposase for SB-mediated gene insertion both *in vitro* and *in vivo*. Because co-delivery of transposase-encoding DNA could result in prolonged or even indefinite transposase expression with subsequent transposon instability, the use of RNA as a source of transposase addresses a significant safety concern with respect to clinical application of the SB transposon system. Expression of co-delivered RNA may also be a more effective way of providing transposase for SB-mediated transposition in cells and tissues. Studies are currently in progress to evaluate the effectiveness of RNA as a transposase source for integration and long-term expression of therapeutic genes, with implications for SB-mediated transposition for genetic therapy of human disease.

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