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# *Cis*-Acting Gene Regulatory Activities in the Terminal Regions of *Sleeping Beauty* DNA Transposon-Based Vectors

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## ABSTRACT

***Sleeping Beauty* (SB) DNA transposon-based vectors belong to a growing family of nonviral integrating vectors that represent attractive alternatives to conventional virus-based integrating gene vehicles. Because of concerns related to mutagenesis and/or activation of cellular genes by integrating vectors, much attention has been paid to integration site preferences and the ability of vectors to influence expression of neighboring genes. Here, we test the hypothesis that terminal repeats of transposons carry *cis*-acting regulatory sequences. In transient gene expression studies, we demonstrate that the inverted repeats of SB direct gene expression in HeLa cells to levels that are 3-fold higher than in promoter-deficient controls. Inverted repeats pointing toward the transposon center consistently facilitate the highest levels of activity in a number of cell lines. We show that transposon sequences flanking the inverted repeats of SB are required for positive effects on gene expression and, moreover, that these regions contain both stimulatory and inhibitory *cis*-acting elements. In the context of an integrated SB vector the regulatory activities of the transposon termini are sufficient to drive expression of selectable marker genes carried by the transposon, indicating that opposing transcriptional activities originating from the transposon termini may influence expression of its genetic cargo. Finally, detection of regulatory properties of the terminal repeats of the active Tc*3* element from *Caenorhabditis elegans* leads to the suggestion that transcriptional activities of the inverted repeats are conserved among Tc*1*/*mariner* transposons in nature. Our data suggest that SB-based gene vectors may carry ancient properties of self-regulation with potential relevance for SB-directed therapeutic gene transfer.**

## INTRODUCTION

With the revived *Sleeping Beauty* DNA transposon as a pioneer, transposable DNA elements have emerged as new promising nonviral vehicles for therapeutic gene delivery (Ivics *et al*., 1997; Yant *et al*., 2000). DNA transposons are small mobile units of DNA encoding typically a single protein, the transposase, which facilitates excision and reinsertion of the element, allowing continuous proliferation throughout the genome of a host. The simple gene integration machinery of cut-and-paste DNA transposons has provided nonviral gene delivery systems—in their simplest form, naked plasmid DNA-based gene carriers—the ability to insert genes into the genome of transfected cells. The long-term therapeutic potential of such vectors has been demonstrated in liver (Yant *et al*., 2000; Mikkelsen *et al*., 2003; Ehrhardt *et al*., 2005; Ohlfest *et al*., 2005b; Balciunas *et al*., 2006; Keravala *et al*., 2006; Aronovich *et al*., 2007; Wilber *et al*., 2007), lung (Liu *et al*., 2004, 2006), skin (Ortiz-Urda *et al*., 2003), and brain (Ohlfest *et al*., 2004, 2005a) of adult mice treated with plasmid DNA. Transposon-containing plasmid DNA, administered in synthetic capsids composed of cationic polylysines or hydrophobic lipids, may eventually mimic integrating viral vectors and facilitate efficacious gene insertion in a format that exhibits low toxicity and immunogenicity as well as easy large-scale vector manufacturing.

*Sleeping Beauty* (SB), a member of the Tc*1*/*mariner* superfamily of transposable elements, was genetically regenerated from fossil genetic elements in teleost fish (Ivics *et al*., 1997). SB transposition in human cells is catalyzed by the SB transposase protein and further stimulated by conserved high mobility group proteins (Zayed *et al*., 2003). The element contains

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two terminal 225-bp inverted repeats (IRs), each harboring two 30-bp direct repeat sequences that serve as binding sites for a total of four transposase subunits during transposition. A synaptic complex is formed by DNA bending and interactions of transposase subunits bound to the two IRs, leading to element excision and reinsertion in an alternative acceptor site. SB-based vector systems typically consists of two plasmids: one that carries the transposon in which the original transposase gene has been replaced by a transgene driven by a promoter of choice, and a helper plasmid that contains a transposase expression cassette. Alternatively, the transposon and the transposase expression cassette can be placed on the same plasmid, allowing higher levels of transposition in difficult-to-transfect cells (Mikkelsen *et al*., 2003). Vector transposition *in vitro* and *in vivo* is inhibited by elevated levels of transposase by a process known as "overproduction inhibition" (OPI) (Izsvak *et al*., 2000; Yant *et al*., 2000; Geurts *et al*., 2003; Mikkelsen *et al*., 2003; Balciunas *et al*., 2006). Successful SB-based gene transfer may therefore require that the transposase expression level be optimized either by regulating the amount of transposase-encoding DNA (Yant *et al*., 2000) or by altering the strength of the promoter driving the transposase gene (Mikkelsen *et al*., 2003). Despite potential discrepancies between regulatory mechanisms that have shaped transposable elements during evolution and mechanisms in play during short-term laboratory experiments, there is solid evidence that the activity of members of the Tc*1*/*mariner* superfamily and other transposon families is influenced by self-inhibitory regulatory mechanisms including OPI. Hence, a negative dosage effect is one of several natural regulatory mechanisms that may affect use of transposons as gene carriers. Other potential mechanisms include DNA methylation, epigenetic modifications, and RNA interference, all pushing the inserted elements toward pre- or posttranscriptional silencing.

Insertional mutagenesis is a major safety concern in any integrating vector technology. The apparent lack of gene-regulatory sequences in the SB transposon and other Tc*1*/*mariner* elements suggests that expression of flanking genes may only rarely be upregulated by an inserted DNA transposon, although it cannot be excluded that an internal promoter driving the transgene of a transposon-based vector may affect neighboring genes by transcriptional read-through. The lack of known SB-derived regulatory elements fuels the idea that expression of the transposase gene in original elements depends on endogenous regulatory sequences flanking the inserted transposon, thereby giving rise to read-through RNA transcripts that may serve as templates for transposase synthesis. Notably, generation of such read-through RNA molecules may trigger a self-regulatory RNA interference response, as shown for Tc*1* elements in *Caenorhabditis elegans* (Sijen and Plasterk, 2003). Also, work suggests that LINE-1 (long interspersed nuclear element-1) retrotransposons contain sense and antisense promoters with the ability to drive synthesis of bidirectional RNA transcripts (Yang and Kazazian, 2006). Interestingly, such transcripts are processed to small interfering RNAs that suppress retrotransposition in human cells. Of additional interest, bidirectional promoters have also been identified in adeno-associated virus (AAV) (Flotte *et al*., 1993; Haberman *et al*., 2000; Qiu *et al*., 2002). In this paper we investigate potential regulatory activities of the terminal regions of standard SB-derived gene vehicles. We show that the terminal repeat sequences of SB possess moderate promoter activities and that these residual activities involve sequences flanking the inner direct repeat of both the left and right IRs. Interestingly, both left and right IRs oriented against the center of the transposon have the highest stimulatory effects on gene expression and the right IR is consistently the strongest inducer of the IRs in SB. We speculate that *cis*-acting regulatory activities leading to opposing transcriptional activities driven by the SB termini may influence transgene expression of integrated transposon-based vectors by directly affecting gene expression or by mechanisms that may possibly involve transcriptional interference pathways.

## MATERIALS AND METHODS

### *Plasmid construction*

All oligonucleotides used for the generation of plasmid constructs are presented in Table 1. To generate a panel of firefly luciferase expression plasmids driven by the *Sleeping Beauty* inverted repeats, a construct with a promoter insertion linker, pPIL-luc, was created by end-polishing of the *Hin*dIII/*Nhe*I-digested pGL3-Control vector (Promega, Madison, WI) before religation. Segments containing the inverted repeats were amplified by polymerase chain reaction (PCR) from pT/MCS (Yant *et al*., 2000) and inserted into *Kpn*I- or *Kpn*I/*Mlu*I-digested pPIL-luc. To generate pLIRin-luc the LIRin fragment was amplified with primers P1 and P2. The LIRout sequence was amplified and cloned with primers P3 and P2, whereas constructs pLIRin.LIFR-luc and pLIRout.LIFR-luc, containing the inner flanking region of the left inverted repeat, were generated by amplification with primer sets P1/P4 and P4/P5, respectively. To generate pRIRin-luc and pRIRout-luc the right inverted repeat was amplified with P6 and P1. To generate pRIRin.RIFR-luc and pRIRout.RIFR-luc, containing the inner flanking region of the right inverted repeat, primers P7 and P1 were used for amplification of the IR sequence before cloning. A construct containing only the RIR inner flanking region (pRIFR-luc) was generated with primers P8 and P7. pRIRin.RIFR-luc.Δenh was made by replacing the *Sal*I/*Xba*I fragment from pRIRin.RIFR-luc with an enhancer-deficient PCR fragment containing the simian virus 40 (SV40) pA signal (primers available on request). A panel of 28-bp deletions within the inner flanking region of the right inverted repeat was made by overlap PCR, creating pRIRin.RIFRΔ1-luc, pRIRin.RIFRΔ2-luc, pRIRin.RIFRΔ3-luc, pRIRin.RIFRΔ4-luc, and pRIRin.RIFRΔ5-luc (primers available on request). A plasmid expressing *Renilla* luciferase (pR-luc) was generated by end-polishing of *Xba*I/*Mlu*I-digested psiCHECK-2 (Promega) and subsequent religation. A database search for transcriptional factor-binding sites was performed with Transcription Element Search Software (TESS) (Schug and Overton, 1997; available at http://www.cbil.upenn.edu/cgi-bin/tess/tess).

The *Frog Prince*, Tc*1*, and Tc*3* inverted repeats were inserted into the *Nco*I and *Xho*I sites of the pGL3.Enhancer vector (Promega) to generate (1) pFP.IRin-luc and pFP.IRout-luc, (2) pTc1.IRin-luc and pTc1.IRout-luc, and (3) pTc3.IRin-luc and pTc3.IRout-luc, respectively. The *Frog Prince* 214-bp IR was amplified from pFPT/PGK-Puro, using primers P9 and P10 for the inward direction and primers P11 and P12 for the op-

TABLE 1. OLIGONUCLEOTIDES USED TO GENERATE PLASMID CONSTRUCTS

| *Primer no.* | *Oligonucleotide sequence* |
|---|---|
| P1 | 5′-AAAGGTACCTACAGTTGAAGTCGGAAGT |
| P2 | 5′-ATAGGTACCCAGTCAACTTAGTGTATG |
| P3 | 5′-AAAACGCGTTACAGTTGAAGTCGGAAGT |
| P4 | 5′-AAAGGTACCCCTTGAAATACATCCACAGG |
| P5 | 5′-AAAACGCGTTACAGTTGAAGTCGGAAGT |
| P6 | 5′-AAAGGTACCTTGAGTGTATGTTAACTTCTG |
| P7 | 5′-AAAGGTACCCATCACAAAGCTCTGACCTC |
| P8 | 5′-AAAGGTACCTCAATTAGTATTTGGTAGC |
| P9 | 5′-GTGCTCGAGTACAGTGGTGTGAAAAAG |
| P10 | 5′-GTGCCATGGACAAGGGGGGCAATCACT |
| P11 | 5′-GTGCCATGGTACAGTGGTGTGAAAAAG |
| P12 | 5′-GTGCTCGAGACAAGGGGGGCAATCACT |
| P13 | 5′-tcgagTACAGTGCTGGCCAAAAAGATATCCACTTTTGGTTTTTTGTGTGTAACTTTTTTCTc |
| P14 | 5′-catggAGAAAAAAGTTACACACAAAAAACCAAAAGTGGATATCTTTTTGGCCAGCACTGTAc |
| P15 | 5′-catggTACAGTGCTGGCCAAAAAGATATCCACTTTTGGTTTTTTGTGTGTAACTTTTTTCTc |
| P16 | 5′-tcgagAGAAAAAAGTTACACACAAAAAACCAAAAGTGGATATCTTTTTGGCCAGCACTGTAc |
| P17 | 5′-GTGCCATGGGCTAGGAATTTTAGTAGG |
| P18 | 5′-GTGCTCGAGTACAGTGTGGGAAAGTTC |
| P19 | 5′-GTGCTCGAGGCTAGGAATTTTAGTAGG |
| P20 | 5′-GTGCCATGGTACAGTGTGGGAAAGTTC |
| P21 | 5′-CCAACCAGTTATTAGTTTAGGGGGCAATCACTTTTTCAC |
| P22 | 5′-GTAAAACGACGGCCAGTG |
| P23 | 5′-AAAGGTACCCGAAAACCCTCTAATGTAAC |
| P24 | 5′-TAAACCTAATAACTGGTTGG |
| P25 | 5′-GTAAAACGACGGCCAGTG |
| P26 | 5′-GATGGCGGCCGCCCTTGAAATACATCCACAGG |
| P27 | 5′-AAGGGCGGCCGCCATCACAAAGCTCTGACCTC |
| P28 | 5′-GGAAACAGCTATGACCATG |
| P29 | 5′-ATAGCGGCCGCTACCATGACCGAGTACAAGC |
| P30 | 5′-CCGTGGGTTACCAGCTTCTGATGGAATTAG |
| P31 | 5′-ATAGCGGCCGCAGATATGAAAAAGCCTGAAC |
| P32 | 5′-CAGAAGCTGGTAACCCACGGGGCCAGTGGC |
| P33 | 5′-ACGGCCCGGGGATATCGATTCG |
| P34 | 5′-TTGGCTCATTCCTCCTTTCTGCGGCCGCTACCATGACCGA |
| P35 | 5′-AGAAAGGAGGAATGAGCCAA |

posite orientation. The Tc*1* IR (inward) was generated by annealing and cloning of oligonucleotides P13 and P14. The Tc*1* IR (outward) was obtained by annealing P15 and P16 before cloning. The Tc*3* inverted repeat was amplified from pRP749 (van Luenen *et al*., 1994), using primer sets P17/P18 and P19/P20 for the inward and outward orientations, respectively. The IRin.RIFR and IRout.RIFR fragments of pFP.IRin.RIFR-luc and pFP.IRout.RIFR-luc were generated by overlap extension PCR. Primers P21 and P22 were used to amplify the FP-RIR fragment, using pFPT/PGK-Puro as template. The FP-RIFR fragment was amplified with primers P23 and P24, using pFV-FP (Miskey *et al*., 2003) as template. The overlap extension reaction was performed with equivalent amounts of the two fragments and primers P22 and P23. The full-length fragment was digested with *Kpn*I and inserted into the *Kpn*I site of pPIL-luc.

To investigate the effects of SB inverted repeats in integrated transposons, pSBT/hygro.puro*rev* was generated. The SB transposon from pT/MCS was removed by *Eco*RI/*Bam*HI digestion and replaced by a transposon, generated by overlap extension PCR, containing only the inverted repeats and the inner flanking regions separated by a *Not*I site. The LIR.LIFR and RIR.RIFR fragments were amplified from pT/MCS with primer sets P25/P26 and P27/P28, respectively. The overlap extension reaction was performed with primers P25 and P28 and the full-length transposon vector was digested with *Eco*RI/*Bam*HI and ligated into the pT/MCS vector, generating pSBT/*Not*I. A head-to-head double-selection cassette was made by overlap extension PCR. The puro-pA fragment was amplified from pSBT/PGK-Puro with P29 and P30 and the hygro-pA fragment was amplified from pCEP4 (Invitrogen, Carlsbad, CA) with P31 and p32. The amplified overlap fragment was made with P29 and P31 and inserted into the *Not*I site of pSBT/*Not*I. pSBT/hygro.puro*rev*.ΔSS1 was generated by *Xba*I/*Cla*I digestion of pSBT/hygro-puro*rev*, removing the puromycin resistance (puro$^R$) gene and RIR.RIFR region. A PCR fragment containing the puro$^R$ gene and the ΔRIFR1-deleted RIR.RIFR sequence was generated by overlap extension PCR, using in initial PCRs P33/P34 for the puro$^R$ fragment and P35/P28 for the RIR.ΔRIFR1 fragment before overlap extension PCR with the primer set P33/P35. The resulting fragment was inserted into *Xba*I/*Cla*I-cleaved pSBT/hygro.puro*rev*.

### *Cell culture*

HeLa, Huh-7, HT-1080, CHO-K1, and 208F cells were cultured at 37°C in 5% (v/v) $CO_2$ and maintained in Dulbecco's modified Eagle's medium (Cambrex, Verviers, Belgium) with D-glucose (4.500 mg/liter) supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (0.1 mg/ml), and L-glutamine (265 mg/liter).

### *Transfections and Dual-Luciferase reporter assays*

Cells were seeded at $1 \times 10^4$ cells/cm$^2$ in 12-well dishes 1 day before transfection. Transfections were performed according to the manufacturer's instructions, using 2 $\mu$l of FuGENE-6 (Roche, Basel, Switzerland) and a total of 1 $\mu$g of DNA (900 ng of firefly luciferase reporter plasmid plus 100 ng of pR-luc, the latter serving as a transfection control). Forty-eight hours after transfection cells were analyzed with the Dual-Luciferase reporter assay system (Promega). Briefly, cells were incubated with 250 $\mu$l of passive lysis buffer for 15 min, and then 50 $\mu$l of the lysate was added to 100 $\mu$l of luciferase assay reagent II for firefly luciferase detection followed by the addition of 100 $\mu$l of Stop & Glo reagent for *Renilla* luciferase detection. Firefly luciferase expression was normalized to *Renilla* luciferase expression and is presented relative to the no-promoter control (pPIL-luc). All transfections were performed in triplicate unless otherwise noted.

### *Transposition assays*

HeLa cells ($2 \times 10^4$ cells/cm$^2$ in 6-well dishes) were seeded 1 day before transfection with 4 $\mu$l of FuGENE-6 and a total of 2 $\mu$g of DNA (1.5 $\mu$g of transposon plasmid and 0.5 $\mu$g of transposase-expressing plasmid). Two days posttransfection cells were trypsinized and reseeded at appropriate dilutions. Diluted cells were grown in medium containing either puromycin (0.5 $\mu$g/ml; Sigma, St. Louis, MO) or hygromycin B (200 $\mu$g/ml; Invitrogen). For analysis of double resistance, puromycin-resistant clones were isolated and expanded and subjected to selection with hygromycin B. All transfections were performed in triplicate.

## RESULTS

### *Regulatory activities of* Sleeping Beauty *inverted repeats depend on IR-flanking sequences*

The architecture of currently used SB transposon-derived vectors is based on the original vector generated during molecular reconstruction of SB by Ivics and coworkers (1997). In the process of creating a transposable substrate vector, a transgene expression cassette was inserted between the terminal inverted repeats of a salmonid-type Tc*1*-like element (TcE) derived from the genome of *Tanichthys albonubes*, leaving 352 and 372 bp of TcE-derived sequences on the left and right side, respectively, of the transposon (Fig. 1). As TcE naturally possesses imperfect 225-bp inverted repeats, the IRs of the original SB vector are not perfectly inverted and differ at several positions outside the perfectly matched direct repeats. Importantly, this design entails that the left inverted repeat (LIR) is flanked on the inner side by a 127-bp left inner flanking region (LIFR) derived from the TcE, whereas the right inverted repeat (RIR) is flanked internally by a 147-bp right inner flanking region (RIFR). Notably, the RIFR sequence contains the 3′ 143 bp of the TcE transposase-coding region, including the stop codon.

In early studies of promoterless gene transfer vectors based on the SB transposon, we found evidence suggesting that the terminal IR sequences were able to stimulate gene expression of downstream genes (data not shown). To investigate the potential regulatory functions of the terminal repeat sequences of SB, we carried out transient expression studies in HeLa cells based on pGL3-derived reporter constructs containing the firefly luciferase (*luc*) gene. In separate constructs, the 225-bp LIR and RIR of SB were inserted in front of the *luc* reporter gene. The effects of LIR and RIR were analyzed in both orientations, either "inward" pointing toward the transposon center (LIRin and RIRin) or "outward" pointing away from the body of the transposon (LIRout and RIRout). Of the four constructs tested, only pRIRout-luc demonstrated a level of *luc* expression that was consistently higher than the background level obtained with the promoter-deficient control construct (Fig. 2A). To test the terminal regions of SB vectors in their entirety, we created a panel of *luc* vectors that, in addition to the LIR or RIR sequences, contained the inner flanking regions LIFR and RIFR. The effect was an elevated level of *luc* expression. For both LIRin- and RIRin-containing constructs *luc* expression was stimulated more than 3-fold by including the IFR sequences, compared with constructs containing the IRs only (Fig. 2A). The activity of IRs in the outward orientation was increased only about 1.5-fold by including the IFRs. On the basis of these findings, we created pRIFR-luc, an expression construct containing only the inner flanking sequence of the right terminal repeat. As this construct did not express *luc* at levels exceeding background (Fig. 2A), we conclude that the regulatory activities of the SB termini require sequences within both the terminal repeats and the inner flanking regions. In addition, the SV40-derived enhancer, situated in the backbone of the construct, was found to be required for the regulatory processes driving IR-directed *luc* expression (data not shown). Moreover, the strongest IR-containing promoter (RIRin.RIFR) was found to be approximately 60 times less active than a standard SV40-derived promoter (data not shown), demonstrating that active terminal SB promoters are considerably weaker than commonly used promoters.

### *Terminal SB promoters are active in a variety of cell lines*

To further investigate the *cis*-acting regulatory activities of the terminal regions of SB (IR plus IFR), we carried out transient *luc* expression analyses in four additional cell types including human hepatoma cells (Huh-7), human fibrosarcoma cells (HT-1080), Chinese hamster ovary cells (CHO-K1), and rat fibroblasts (208F). In all analyzed cell lines, the strongest stimulatory effects were again obtained with expression constructs driven by LIRin.LIFR and RIRin.RIFR promoters (Fig. 2B). In contrast, constructs with IRs inserted in the opposite direction did not support expression above the background level of the promoter-deficient control construct. In all cell lines, except CHO-K1, the RIRin.RIFR context supported the highest

level of *luc* expression, reaching in HT-1080 and 208F cells levels that were 18-fold higher than were obtained with the LIRout.LIFR sequence. These data were concordant with our findings in HeLa cells and, thus, further stress that (1) the terminal regions of SB contain promoter activities that are oriented primarily toward the center of the transposon and (2) the right terminal sequence in the context found in SB vectors contains the strongest gene-regulatory activity.

### *RIFR of the SB RIR contains both expression stimulatory and inhibitory sequences*

To characterize in more detail the stimulatory activities of the TcE-derived sequence flanking the RIR of SB vectors we carried out a deletion analysis of the 147-bp RIFR sequence. In a panel of five *luc* expression constructs, we introduced 28-bp deletions (designated ΔRIFR1 to ΔRIFR5) spanning the RIFR sequence next to the SB RIR as illustrated in Fig. 3. In three of these deletion variants the regulatory activity was strongly diminished, facilitating low levels of expression that were comparable to the RIFR- and RIR-deficient variants. However, to our surprise the variants carrying the ΔRIFR3 and ΔRIFR5 deletions were far more active than the unmodified RIRin.RIFR promoter (Fig. 3). Hence, the most active deletion variant, harboring the ΔRIFR5 deletion on the border of RIFR and RIR, induced levels of expression that were more than 3-fold higher than the wild-type sequence and almost 8-fold above the background level. Because these regions of the RIFR appear to contain sequences with inhibitory effects on gene expression we have designated these regulatory sequence elements as inhibitory sequences 1 and 2 (IS1 and IS2). A TESS database search confirmed that binding sites for known transcription factors were not inadvertently created at the junctions of the deletions. We conclude that the residual SB promoter activity relies on sequences within both IR and IFR and that the partial TcE transposase sequence that constitutes the RIR-flanking region in SB vectors contains both stimulatory and repressive elements with effects on inward IR-driven transcription.

### *Strong inward promoter activity of terminal inverted repeats of Tc3 transposable element from Caenorhabditis elegans*

In consideration of the gene-regulatory activities of the terminal regions of the genetically reconstructed SB element, we set out to investigate the *cis*-acting regulatory activities of naturally occurring Tc*1*/*mariner* family members. In contrast to elements such as *Mos1*, *Himar1*, and Tc*1*, both SB and the active *C. elegans* Tc*3* element contain inverted terminal repeats of a substantial length. The 462-bp perfectly inverted Tc*3* IR

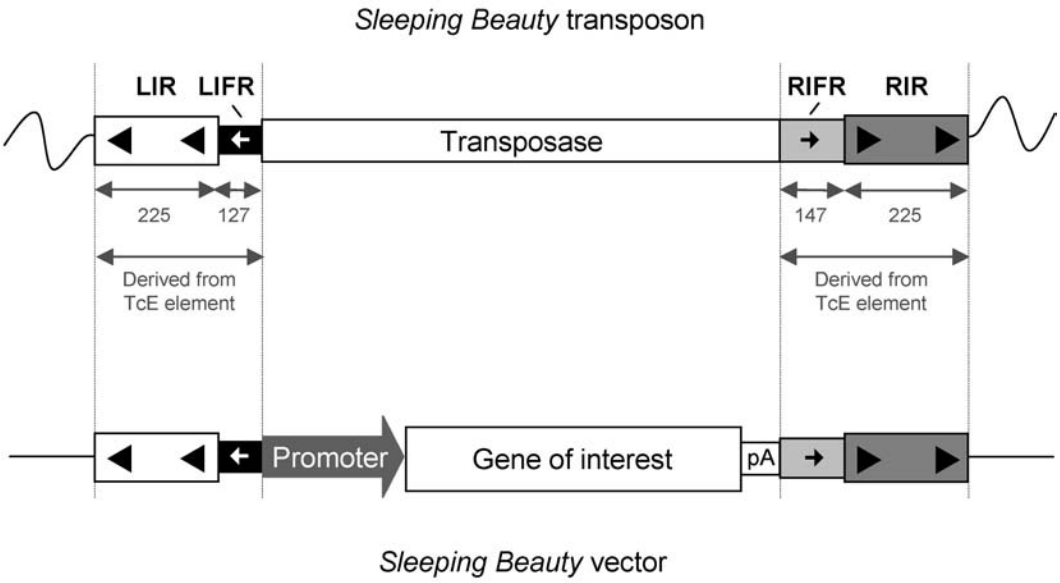


**FIG. 1.** Schematic representation of the *Sleeping Beauty* DNA transposable element and the derived pT vector. Currently used SB transposon-derived vectors are based on the genetically reconstructed SB element (Ivics *et al*., 1997). In creating an SB-based transposable vector, a transgene expression cassette is inserted between the terminal inverted repeats of a salmonid-type TcE element derived from the genome of *Tanichthys albonubes*, leaving 352 and 372 bp of TcE-derived sequences on the left and right side, respectively, of the transposon. The 225-bp TcE inverted repeats are flanked on the inner side by 127-bp (left IR) and 147-bp (right IR) inner flanking regions (designated LIFR or RIFR) containing part of the untranslated region and the 3′ coding region of the TcE transposase gene, respectively. Left and right inverted repeats are indicated by white and gray boxes, respectively. Direct repeats are indicated by black arrowheads within the IRs, and illustrate the orientation of the inverted repeats. Inward and outward orientations of the IRs, as referred to in text, and plasmid names refer to the orientation of the IRs in the context of the transposon. Inwardly directed IRs point toward the transposon center (as in LIRin and RIRin) whereas an "outward" direction refers to IRs pointing away from the body of the transposon (as in LIRout and RIRout). The orientation of LIFR and RIFR with respect to the flanking IR sequence is indicated by white and black arrows, respectively. Lengths (in base pairs) of relevant regions are indicated below the transposon. The original SB element does not contain a known promoter driving expression of the transposase gene; a promoter driving expression of the gene of interest in the vector is indicated by the dark gray arrow.

sequence was therefore inserted in the *luc* reporter construct and assayed for the ability to drive *luc* expression in HeLa cells. Interestingly, the Tc*3* IR oriented against the center of the transposon promoted high levels of *luc* expression that were more than 20-fold above the level of the promoter-deficient control construct (Fig. 4). When inserted in the opposite orientation, however, the IR did not stimulate *luc* expression, indicating that the residual promoter activities of Tc*3* IRs were orientation dependent. In contrast to these findings, we were not able to demonstrate any positive regulatory activities of the short 54-bp IR

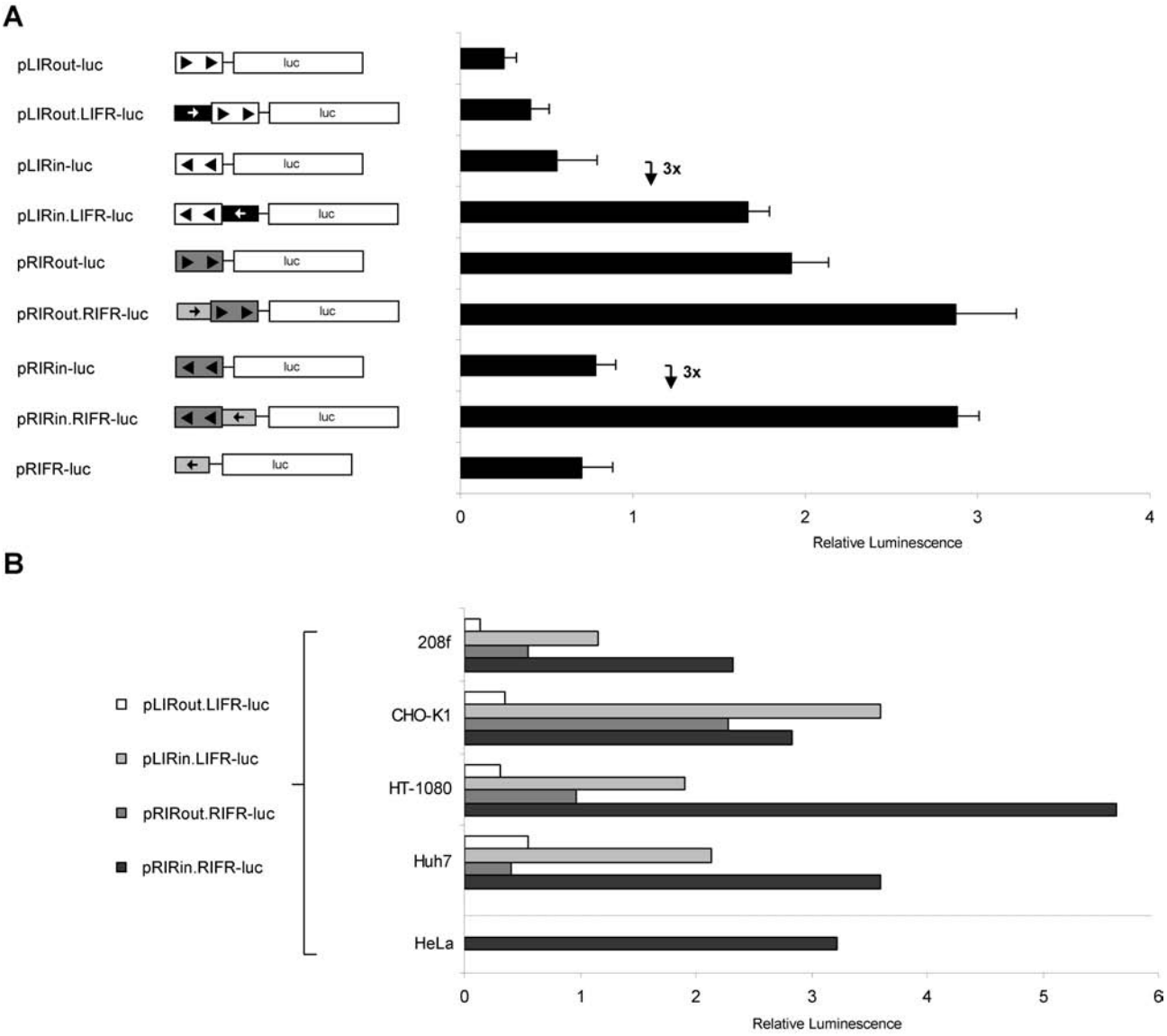


**FIG. 2.** Reporter gene expression driven by inverted repeat-based promoters. Transient expression studies in HeLa cells were carried out by cotransfection of 900 ng of pGL3-based reporter plasmid containing the firefly luciferase gene (*luc*) driven by promoter candidates and 100 ng of plasmid encoding the *Renilla* luciferase gene as transfection control. Measurement of *luc* expression was performed as described in Materials and Methods. Levels of expression, presented as relative luminescence, have been normalized for transfection variations and are presented relative to the level of expression obtained with the promoter-deficient pPIL-luc construct. (**A**) Transient expression of *luc* driven by left and right inverted repeats excluding or including the inner flanking regions LIFR and RIFR. The extent of the IFR-directed increase in *luc* expression is indicated. Inverted repeats, inner flanking regions, and the orientation of these sequences are indicated by symbols described in the caption to Fig. 1. Results represent the mean of three independent transfections. Absolute relative light unit (RLU) values measured in this experiment ranged from $3 \times 10^3$ to $4 \times 10^4$. (**B**) *Cis*-acting regulatory activities of the SB terminal regions in human and rodent cell lines. *luc* reporter constructs containing the SB inverted repeats and the inner flanking regions were transfected into the indicated cell lines together with plasmid encoding the *Renilla* luciferase gene for normalization. For a schematic representation of all constructs used, see (**A**). Levels of expression have been normalized to the level of expression obtained with a promoter-deficient promoter construct. Results are derived from a single round of transfections. Transfection of pRIRin.RIFR-luc in HeLa cells was performed as a control.

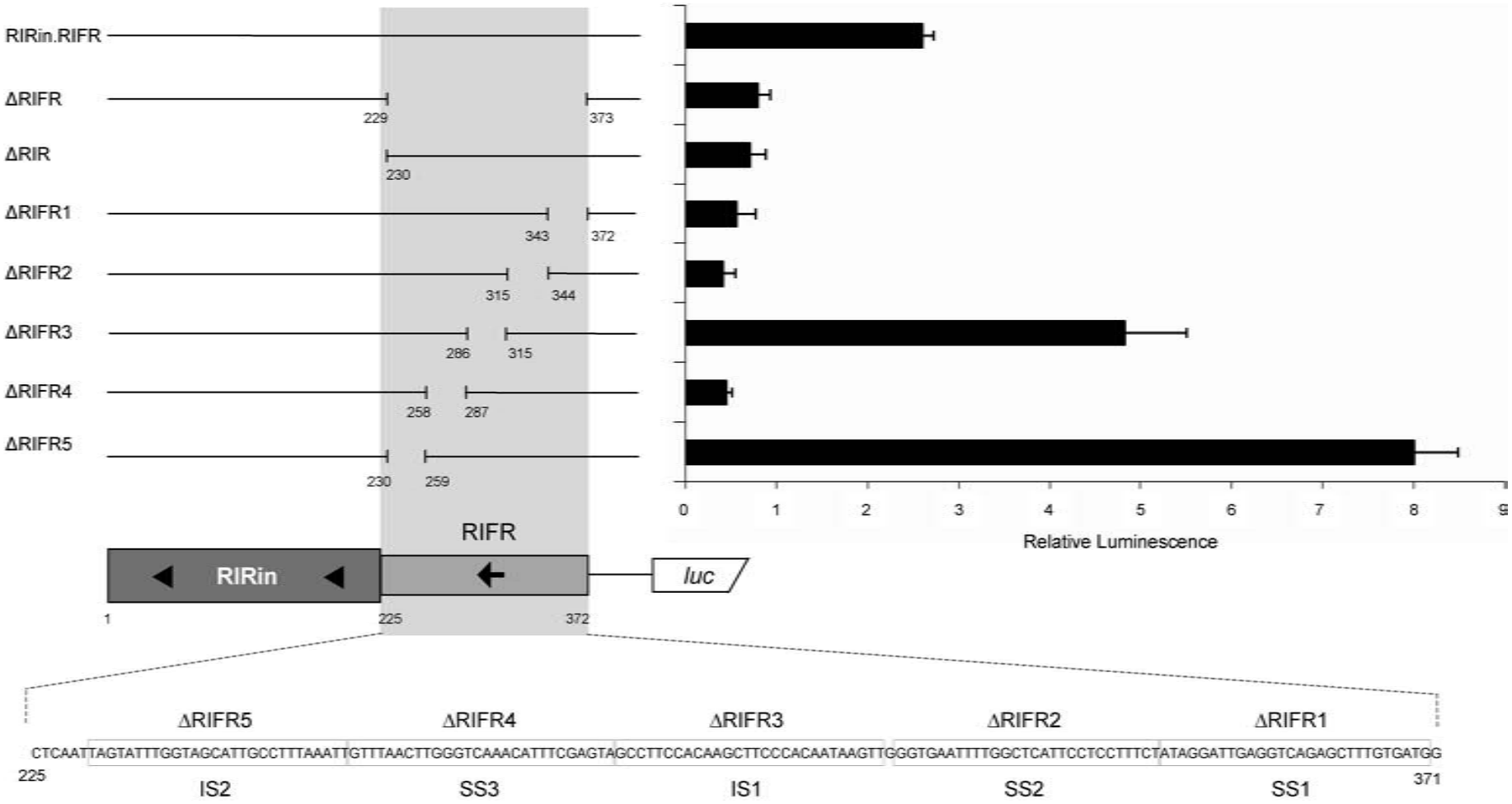


**FIG. 3.** Identification of stimulatory and repressive sequences within the inner flanking region of the RIRin.RIFR promoter. Deletion variants were generated by overlap PCR, as described in Materials and Methods, and transfected into HeLa cells. *luc* expression driven by each promoter variant was measured as described in the caption to Fig. 2. The sequence covered by each deletion (ΔRIFR1 through ΔRIFR5) is indicated near the bottom. Inhibitory sequence elements are designated IS1 and IS2, whereas sequences with a stimulatory effect on expression have been designated SS1, SS2, and SS3. Known transcriptional factor-binding sites were not generated at junctions between deletion-flanking sequences. Results are given as the mean of three independent transfections.

derived from Tc*1*, the most prevalent DNA transposable element in *C. elegans* (Fig. 4).

The *Frog Prince* (FP) transposon was genetically reconstructed from transposable elements in the leopard frog genome (Miskey *et al*., 2003), leading to the creation of a vector with high activity in human cells. Although FP and SB are structurally similar they share only approximately 50% sequence similarity and may therefore possess different functional properties. In contrast to SB, FP contains two perfectly inverted terminal repeats. By inserting the 214-bp FP IR in both orientations upstream from the *luc* reporter gene, we demonstrated that FP IRs are unable to drive *luc* expression and therefore do not possess positive regulatory sequences (data not shown). In an attempt to mimic the RIR promoter structure of SB, we included the RIFR of the original FP element (the 3′ 150-bp region of the FP transposase gene) in our vectors but were, nevertheless, not able to detect any stimulatory effects on expression from either the inward or the outward directed sequences (data not shown). Our results thus suggest that IR-directed stimulatory effects on transcription are partially conserved properties among Tc*1*/*mariner* transposons.

### *Inverted terminal repeats of integrated SB vectors influence expression of genetic cargo*

The importance of positive regulatory effects of the SB terminal repeats within the context of integrated SB vectors is currently not clear. To investigate the impact of terminal repeat sequences on the expression of integrated transgenes, we generated the SB transposon vector pSBT/hygro.puro*rev*, containing head-to-head promoter-deficient gene cassettes encoding the hygromycin B resistance (hygro$^R$) and puromycin resistance (puro$^R$) genes (see schematic representation of vectors in Fig. 5A). In this vector, the hygro$^R$ and puro$^R$ genes, both including poly(A) sequences, were immediately flanked and driven by the LIRin.LIFR and RIRin.RIFR sequences, respectively. To verify that expression of genes regulated by the right terminal region did in fact depend on activities of the RIRin.RIFR promoter, we also generated the pSBT/hygro.puro*rev*.ΔSS1 vector, which harbored a deletion of stimulatory sequence 1 (SS1) within the RIFR sequence (Fig. 3). By comparing the ability of this vector and pSBT/hygro.puro*rev* (cotransfected with an SB transposase expression plasmid) to confer hygromycin B resistance in HeLa cells, we verified that this deletion did not affect transposition negatively. In fact, transposition from pSBT/hygro.puro*rev*.ΔSS1 produced about 500 hygromycin B-resistant colonies more per transfection than transposition from transfected pSBT/hygro.puro*rev* (Fig. 5B). In contrast, deletion of the SS1 sequence dramatically reduced the number of puromycin-resistant colonies from 300 to 10 clones per transfection, demonstrating that puro$^R$ gene expression was negatively affected by the deletion (Fig. 5B). Control transfections, including an inactive version of the SB transposase, resulted in few drug-resistant colonies (data not shown).

To identify potential double-resistant clones resulting from simultaneous LIRin.LIFR- and RIRin.RIFR-driven gene expression, we subjected puromycin-resistant clones (obtained from pSBT/hygro.puro*rev* transfections) to hygromycin B selection. Among 12 individual clones that were initially selected for expression of the puro$^R$ gene we were able to detect 10 clones that were also resistant to hygromycin B (data not shown). Although it cannot be excluded that some of these clones contain more than one transposon, these data are consistent with the notion that the terminal regions of integrated SB vectors facilitate opposing transcriptional activities, leading to simultaneous expression of the two marker genes. This suggests that SBT/hygro.puro*rev* acts as an enhancer-trapping vector in which the residual regulatory activities of the SB termini

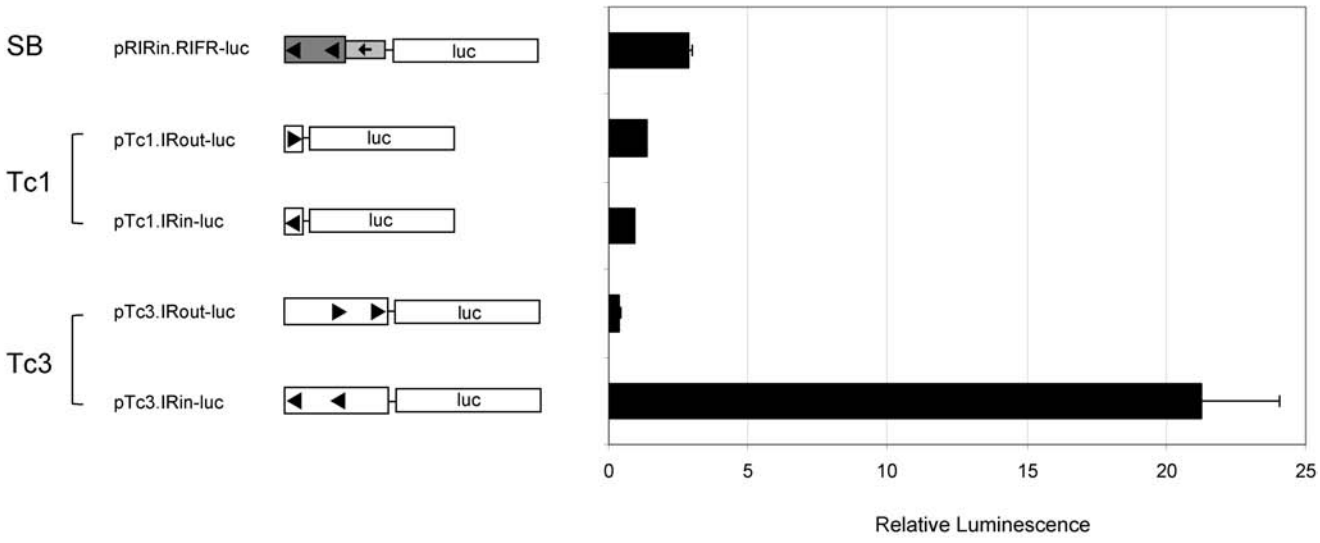


**FIG. 4.** Analysis of promoter activity within the inverted repeat of Tc*1*/*mariner* transposons. All constructs were tested for activity in transiently transfected HeLa cells. Levels of *luc* expression were normalized for variations in transfection efficiency and related to the activity of a promoter-deficient negative control. The promoter activities of the *Sleeping Beauty* (SB) terminal sequences were compared with terminal regions of the Tc*1* and Tc*3* elements from *C. elegans*. IR sequences derived from Tc*1* (54 bp) and Tc*3* (462 bp) were inserted upstream of the luciferase reporter gene and tested for activity in both orientations.

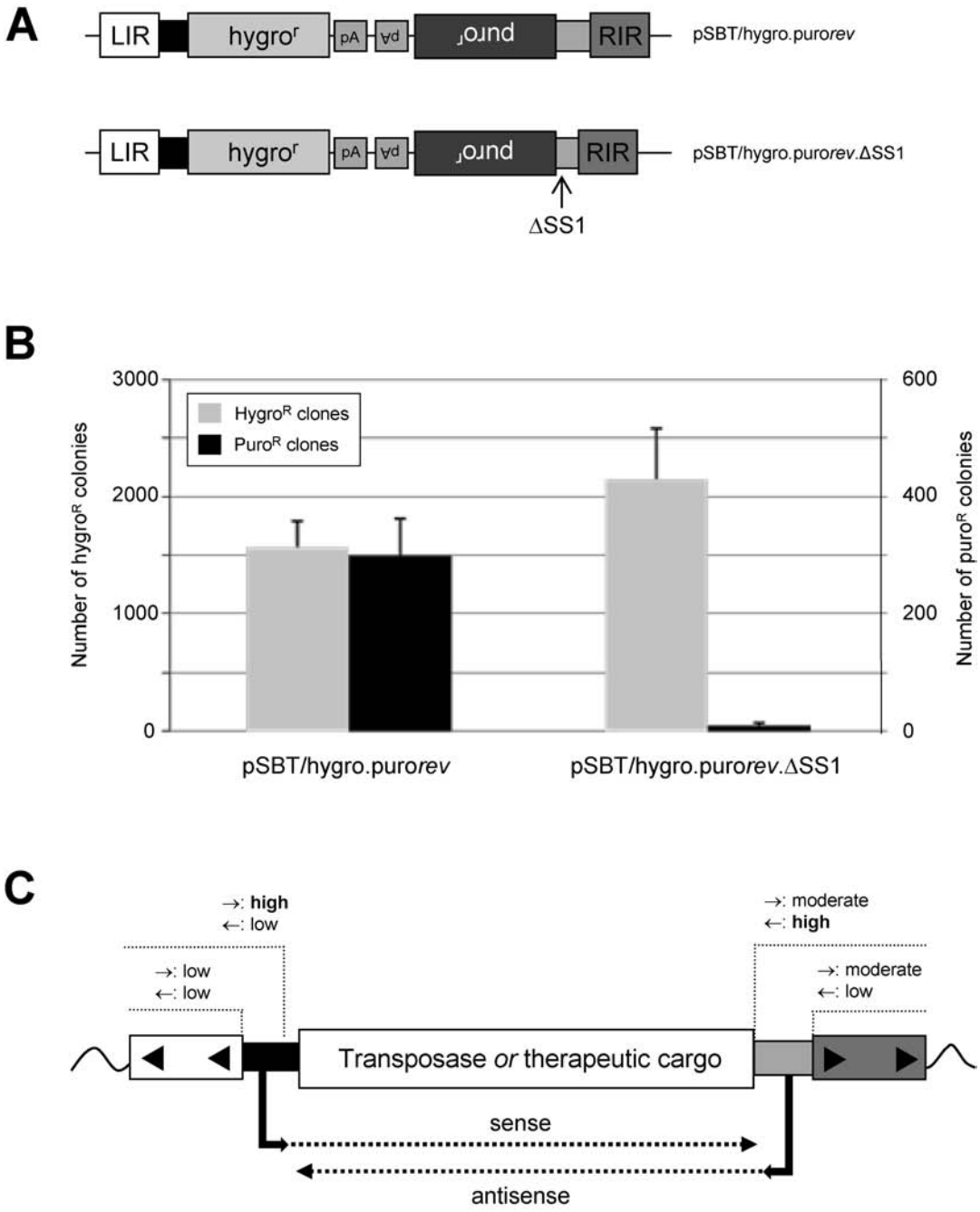


**FIG. 5.** Opposing transcriptional activities driven by the terminal sequences of integrated SB transposon vectors. (**A**) Schematic representation of SB-based vectors containing head-to-head promoter-deficient gene cassettes. Vectors encode the hygromycin B resistance (hygro$^R$) and puromycin resistance (puro$^R$) genes, both including poly(A) sequences. In pSBT/hygro.puro*rev* and pSBT/hygro.puro*rev*.ΔSS1 the hygro$^R$ gene is flanked upstream by the LIRin.LIFR sequence (LIFR is represented by the black box), whereas the puro$^R$ gene is flanked by the RIRin.RIFR sequence (RIFR is indicated by the light gray box). pSBT/hygro.puro*rev*.ΔSS1 was generated by deleting the SS1 sequence (see Fig. 3) from the RIFR sequence flanking RIR. (**B**) Gene expression directed by the right terminal region of integrated SB requires sequence elements within the RIFR sequence. SB transposon vector pSBT/hygro.puro*rev* or pSBT/hygro.puro*rev*.ΔSS1 was transfected into HeLa cells together with a plasmid encoding the SB transposase. Transfected cells were grown both in hygromycin B- and puromycin-containing media and resistant colonies, appearing as a result of transposase-mediated transposition, were counted 2 weeks after transfection. Cotransfection of SB vectors with a plasmid encoding an inactive version of the SB transposase resulted in about 100-fold fewer drug-resistant colonies (data not shown). Gray columns indicate the number of hygromycin B-resistant colonies (as indicated on the left *y* axis), whereas solid columns indicate the number of puromycin-resistant colonies (as indicated on the right *y* axis). (**C**) Schematic model demonstrating potential implications of bidirectional promoter activities of the SB termini. Sense and antisense RNAs driven by *cis*-acting regulatory activities of the terminal transposon regions are indicated by hatched lines. Relative promoter strengths of inverted repeats with and without the inner flanking sequences are indicated above the transposon. The direction of the transcriptional activities is indicated by arrows pointing toward the center of transposon (inward activity) or toward the flanking genomic DNA (outward activity).

are context dependent and/or stimulated by endogenous enhancers. On the basis of these findings we conclude that transposon sequences flanking the terminal repeat are required for the promoter activity of the right inverted repeat of integrated SB transposons. Furthermore, our data propose that the minimal promoter residing in the terminal region of SB is active in the context of the integrated vector but may act under the influence of neighboring genomic sequences. In summary, our findings strongly indicate that bidirectional transcriptional activities driven by the SB termini, including sequences within the IRs and IFRs, may trigger synthesis of sense and antisense transposon RNA with potential implications for transcriptional interference and pathways of element regulation (Fig. 5C).

## DISCUSSION

On the basis of a panel of transient luciferase expression assays we demonstrate in this paper that the inverted terminal repeats of *Sleeping Beauty* DNA transposon-based vectors possess residual *cis*-acting gene regulatory activities. Much to our surprise, we also discovered that transposon sequences flanking both the left and right inverted repeats are required for the regulatory properties of these terminal promoters. In a set of human and rodent cell lines we consistently measured robust luciferase production driven by terminal transposon sequences pointing toward the center of the element. Still, the most potent of these promoters, RIRin.RIFR, was about 60 times less active than a standard SV40 promoter. This suggests that the internal regulatory *cis* elements of integrated SB vectors, in comparison with the long terminal repeat (LTR) promoters of retro- and lentiviral vectors, are less likely to influence expression of flanking cellular genes. In support of this finding, it has been demonstrated that integrating *Sleeping Beauty* vectors may induce loss-of-function or gain-of-function mutations primarily as a result of actions of the internal expression cassette, and not of the transposon itself (Collier *et al*., 2005; Dupuy *et al*., 2005). Deletion analysis of the region flanking the RIR sequence illustrated that this region contains sites that either stimulate or repress the regulatory functions. Hence, three of five 28-bp deletions in the RIFR sequence resulted in disruption of promoter function, whereas the remaining two deletion variants resulted in reporter expression levels that were up to 3-fold higher than the unmodified sequence. By searching appropriate databases we screened these regions for candidate regulatory sequences but were not able to identify sites (e.g. transcription factor-binding sites) with obvious relevance for the properties of the deletion variants. More detailed mapping of the sequences involved therefore will require additional mutational analyses. In addition, attempts to map a potential preferred transcriptional start site by 5′ RACE (rapid amplification of 5' complementary DNA ends) on RNA transcripts directed by the RIRin.RIFR promoter were not successful (data not shown), stressing the fact that the residual regulatory activities of the SB terminal repeats are of low efficiency.

In attempts to examine whether gene-regulatory properties of the IR sequences are conserved properties of transposons, we searched for promoter activities in the terminal regions of other Tc*1*/*mariner* transposons including the active Tc*3* element from *C. elegans*. Interestingly, the Tc*3* IR oriented toward the center of the transposon supported substantial expression of a downstream gene in HeLa cells, providing partial support of the notion that inward transcription driven by the IRs may play a part in transposon replication and regulation. Along these lines, studies claim that the *piggyBac* transposon within the internal domain sequence of the 3′-terminal repeat contains regulatory *cis*-sequences with effects on upstream genes (Shi *et al*., 2007). We were not able to demonstrate gene-regulatory properties of the IR from *Frog Prince*, another genetically reconstructed element. Although a significant phylogenetic distance between FP and SB may explain such differences (Miskey *et al*., 2003), it cannot be excluded that the reconstruction processes of SB and FP, respectively, may have somehow affected the inherent regulatory properties of these elements. Moreover, we cannot leave out the possibility that the observed regulatory properties of SB are affected negatively, or positively, by mutations that have accumulated in the salmonid-type Tc*1*-like transposable element, the natural ancestor of SB-based vectors (Ivics *et al*., 1997).

Our analyses demonstrate no stimulatory effects of the short IR derived from the Tc*1* element. Although possible suppression of such activity in human cells could explain this finding it is supported by previous findings demonstrating that transcription of Tc*1* sequences in *C. elegans* is initiated in the flanking sequences and not within the inverted repeats (Sijen and Plasterk, 2003). Interestingly, such read-through transcripts may form double-stranded RNA (dsRNA) by folding back on themselves, allowing complementary intramolecular IR sequences to anneal. These dsRNA regions are processed by the cellular RNA interference (RNAi) machinery, triggering silencing of the transposable element by transposon-specific RNA degradation (Sijen and Plasterk, 2003). Yang and Kazazian reported that sense and antisense promoters within the LINE-1 retrotransposon guide the production of bidirectional RNA transcripts. As a result, double-stranded RNA is processed to short interfering RNAs (siRNAs) that can suppress LINE-1 retrotransposition in human cells (Yang and Kazazian, 2006). As an interesting analogy, AAV-based viral vectors possess transcriptional activities in both inverted terminal repeat (ITR) sequences (Flotte *et al*., 1993). Although AAV ITRs appear sufficient to produce low-level transcriptional activity the exact role of such bidirectional transcription in AAV replication remains elusive (Haberman *et al*., 2000; Qiu *et al*., 2002). In summary, supported by characteristics of other mobile elements, our data suggest that SB-based gene vectors may carry ancient properties of self-regulation with obvious importance for models of element evolution. The question remains, however, whether these findings may have direct relevance for SB-based gene transfer.

To study the promoter effects in the context of an integrated vector, we generated SB vectors containing head-to-head selection genes driven by the SB LIRin.LIFR and RIRin.RIFR sequences, respectively. In accordance with the fact that this vector reproducibly produced a significant number of drug-resistant colonies, more than 80% of the analyzed clones were resistant to both drugs, as demonstrated by subjecting puromycin-selected clones to hygromycin B selection. These findings demonstrate that both LIR and RIR promoters are active in the integrated form of the vectors but may suggest, because not all clones were double-resistant, that these vectors

may act as enhancer-trapping vectors. Although it cannot be excluded that at least some of these colonies were the result of cryptic events of gene trapping, we suggest, in accordance with our findings in transient expression assays, that residual regulatory activities of the SB termini may be influenced by the context of the insertion site and in some incidences rely on stimulation by endogenous enhancers. In accordance with this explanation, we found that SB vectors harboring a deletion of the stimulatory SS1 sequence produced fewer stably transfected colonies based on RIR-driven drug resistance gene expression, whereas LIR-driven expression, and hence overall transposition, was unaffected in these vectors. This finding strongly argues that sequences affected by the ΔRIFR1 deletion are crucial for inward promoter activity of integrated SB elements, resulting in the production of transposon antisense RNA transcripts. However, this does not exclude the possibility that transcriptional activities of the SB IRs require assistance from flanking enhancers, as indicated by the importance of the SV40 enhancer in our plasmid-based experiments. In previous work by Clark and co-workers a designed SB gene trap vector containing an IRES-Neo$^R$ cassette was used to generate a panel of G418-resistant HeLa clones (Clark *et al*., 2004). Despite the obvious selection for insertion within genes in these clones, Northern blot analysis showed that short RNA transcripts, indicative of initiation of transcription within the transposon, were produced in numerous clones. Although the origin of these transcripts is unknown, our data support the notion that IR-driven (and, in the case of the gene trap, LIR-driven) transcription may facilitate production of such short transcripts.

The functional properties of the terminal transposon sequences discussed in this paper refer to properties of the inverted repeats and inner flanking regions found in the original TcE derived from the genome of *Tanichthys albonubes* (Ivics *et al*., 1996). Since the development of the first generation of SB vectors (often designated pT) harboring the unmodified terminal sequences of TcE, attempts have been made to optimize the *cis*-acting elements of the IRs in SB-based vectors. In pT2 vectors few nucleotide changes within the IR were found to increase transposition almost 4-fold relative to the pT standard vector (Cui *et al*., 2002), whereas replacing the RIR with the LIR in pT3 vectors was found to improve activity of the vector more than 2-fold (Izsvak *et al*., 2002; Yant *et al*., 2004). These optimized substrates were created without paying special attention to the inner flanking regions of the RIR and therefore different sets of vectors contain different versions of the RIFR sequence. As the terminal sequences of pT2 vectors pT2/HB or pT2/SVneo (Cui *et al*., 2002) do not contain the complete RIFR sequence, and in particular do not harbor the sequence deleted in the ΔRIR1 variant, we suggest that the pT2 context, rather than pT or pT3, be used as a standard transposition substrate in future applications of the SB system.

Ancient functional properties of transposable elements, perhaps including mechanisms of self-regulation, may influence expression of the genetic cargo of vectors as well as flanking cellular genes and therefore will need to be considered in applications of transposon-based gene transfer systems. In virtually all living organisms transposons have colonized and shaped the genome, resulting in a diverse genomic collection of fossil genetic relics. We speculate that IR-directed generation of sense and antisense transposon RNA transcripts may be one of several evolutionarily conserved self-regulatory mechanisms with possible implications for SB transposon-based gene transfer.

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## AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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