viral *versus* non-viral vectors

The ideal vector

1. to produce a therapeutic response, expression of therapeutic gene(s) in target cells/tissue must result in physiologically effective levels of the gene products for an adequate duration

1. expression of transferred genes and its products must not cause serious adverse side effects

in more details the ideal gene therapy vector should ensure

- low invasivness
- target selectivity
- no immunogenic response
- high cloning capacity
- small size
- high stability
- low copy number

the viral vectors display several disadvantages

1. the transduction protocols require laborious and time-consuming preparations of recombinant vectors.

1. the **genetic design** of the vector **is restricted** due to intrinsic structural and size constraints. Moreover, the normal regulation of gene expression may be compromised.

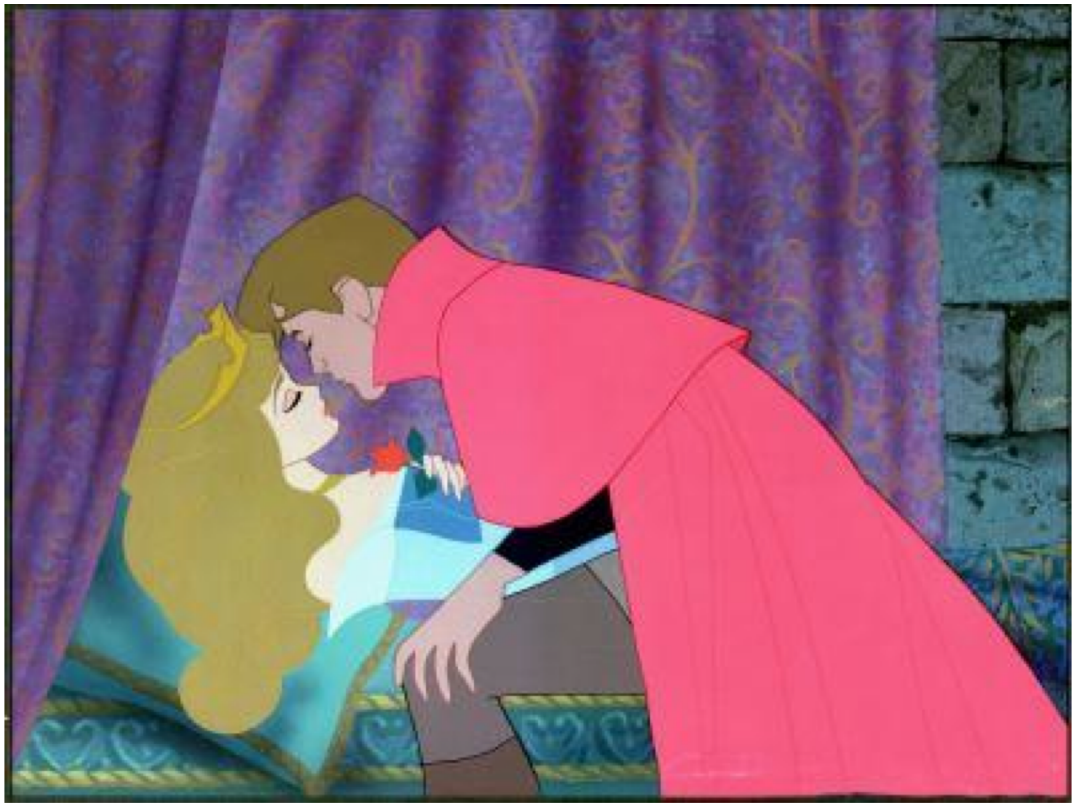
1. a serious concern working with viral vector is **biosafety**. In addition to the risk this technology poses to the researcher, the retroviral constructs are mutagenic at the site of genomic integration, mainly due to the risk of inadvertent oncogene activation and congruent development of cancer. Both retroviral and lentiviral vectors exhibit preferential integration into expressed genes or regulatory elements.

Non viral vectors

By non-viral vectors the therapeutic genes are delivered to target cells using nonviral carriers such as synthetic polymers, liposomes, or polyamines which form DNA-polymer complexes.

The therapeutic gene is typically inserted into a plasmid; the plasmid may be equipped with elements ensuing integration (transposon-based, site-specific recombination) or episomal maintenance (S/MAR, Scaffold/Matrix Attached Region).

sleeping beauty



The trasposon toolkit for mammalian genome technology

- **Sleeping Beauty (SB)**
- Frog prince
- Hsmar1
- piggyBac
- Tol2

1) weak enhancer/promoter activity
2) hypercative transposase
3) random integration

STRUCTURE OF SLEEPING BEAUTY TRANSPOSON SYSTEM

The SB transposon was awakened from inactive Tc1-like transposable elements originally found in fish genomes in 1997

SB was awakened from the fish

Sleeping beauty features

Table 1
Main features of the non-viral *Sleeping Beauty* transposon system.

Mechanism	Cut and paste (no reverse transcription)
Cargo capacity	~10kb (with sandwich > 10kb)
Immunogenicity	Similar to non-viral plasmids
Tropism	Somatic, germinal, dividing & non-dividing cells
Integration profile	Close to random, can be targeted
Stable expression	Silencing is cargo-dependent
Transcriptional act.	Benign promoter/enhancer activity (100x<MLV LTR)
OPI	Sensitive to high transposase concentrations
Efficacy	In certain cell types comparable to retroviruses

OPI: overproduction inhibition

Cell, Vol. 91, 501–510, November 14, 1997, Copyright ©1997 by Cell Press

Molecular Reconstruction of *Sleeping Beauty*, a *Tc1*-like Transposon from Fish, and Its Transposition in Human Cells

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Hungary

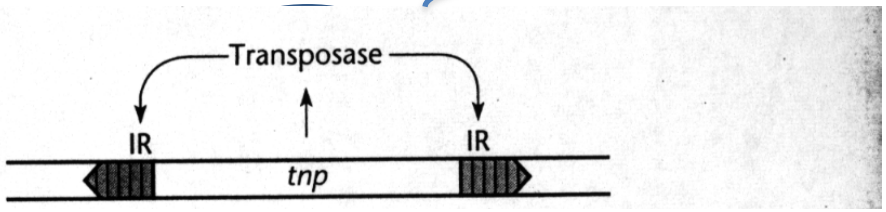
AUTONOMOUS TRASPOSONS: trasposons having all the trans and cis elements for transposition

NON-AUTONOMOUS TRASPOSOS: trasposons having lost the trans elements for transposition. They may eventually transpose when trans- factors are provided

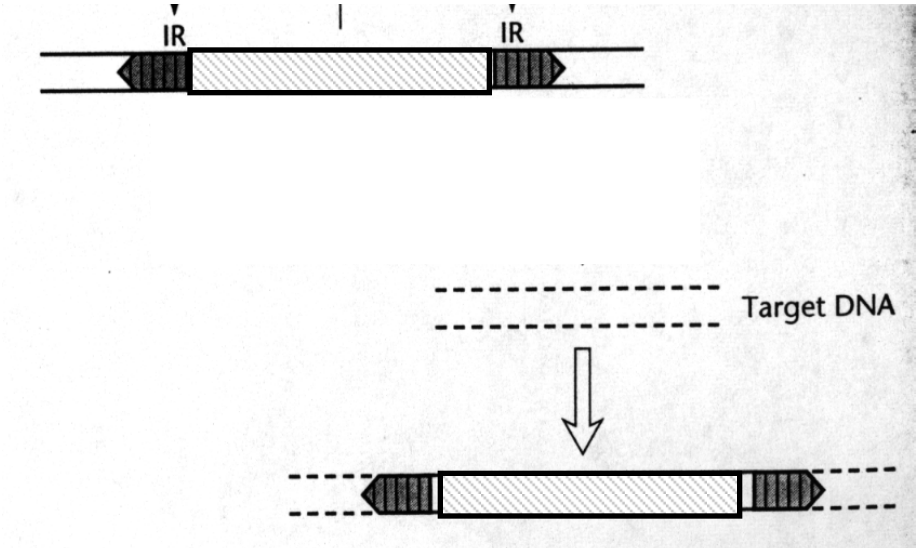
Not a single autonomous element has been isolated from vertebrates; they seem to be defective for having mutations as a result of a process called “**vertical inactivation**”

Inactive transposon can be mobilized by an active trasposon/trasnsposase

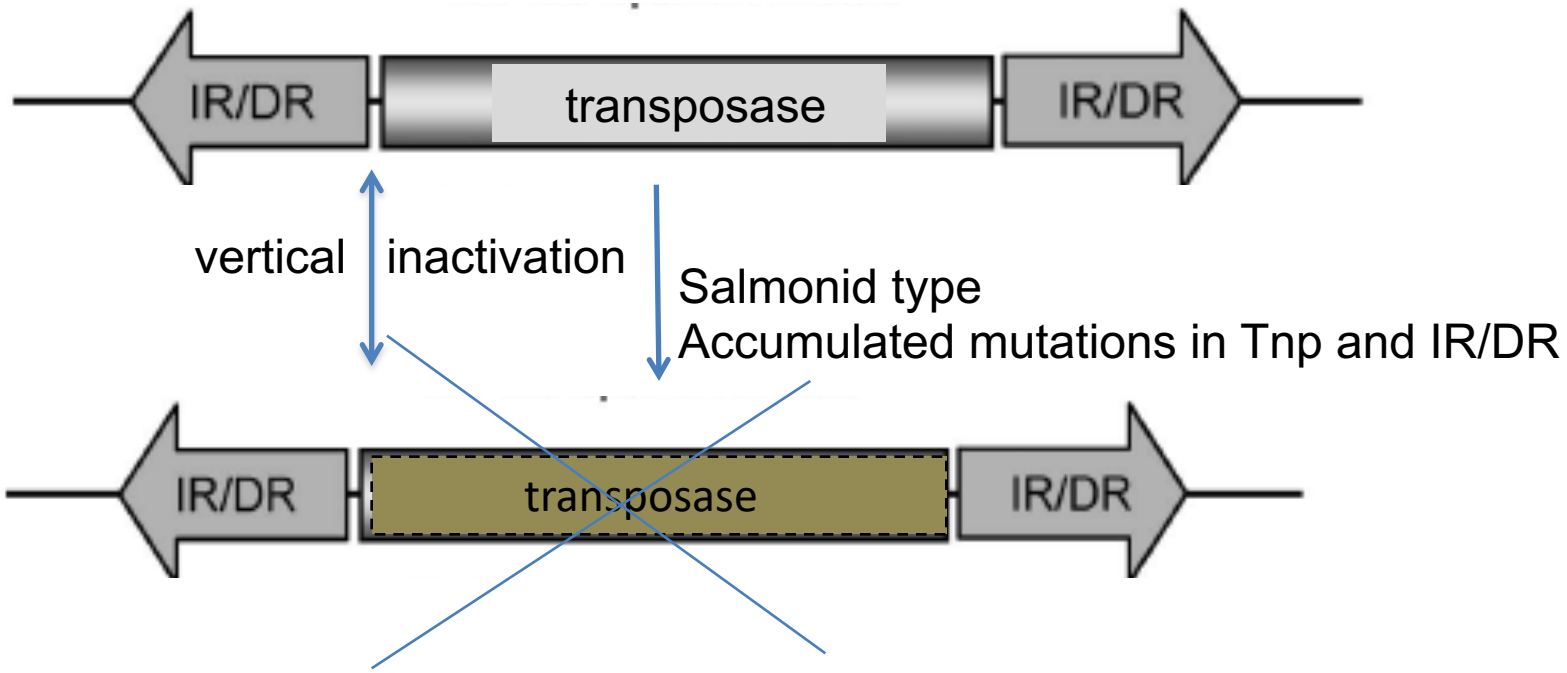
active transposase



inactive transposase

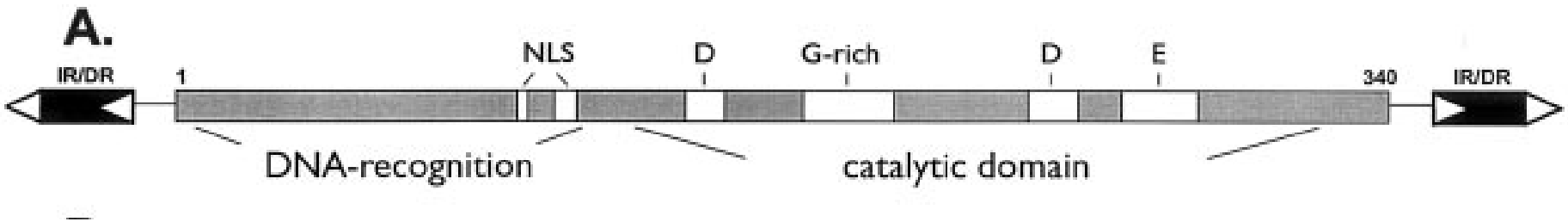


Tc1/mariner superfamily



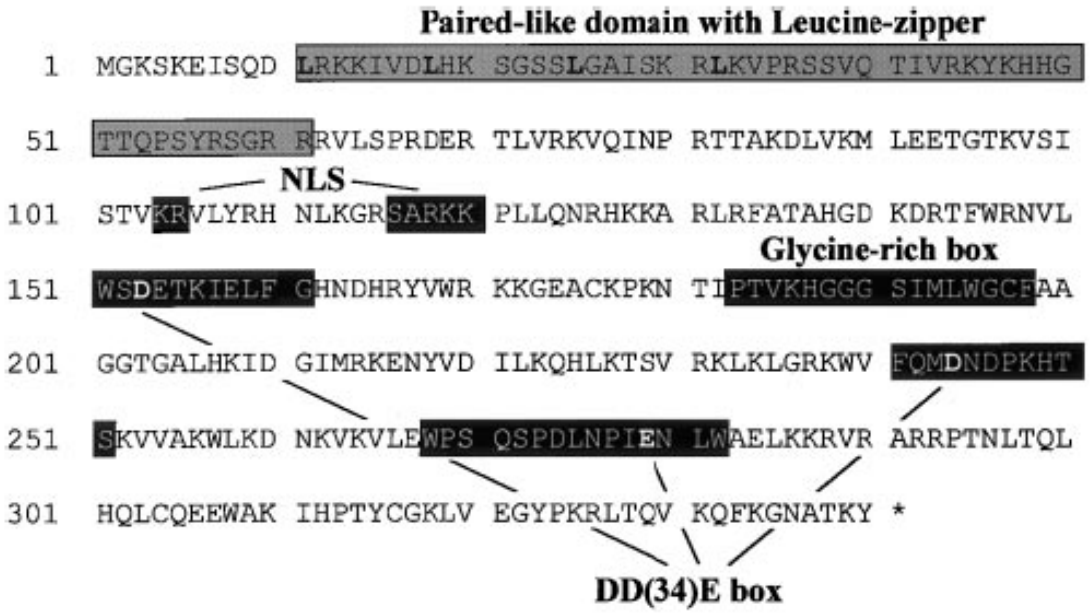
INACTIVE

Molecular reconstruction of a salmonid Tc1-like transposase

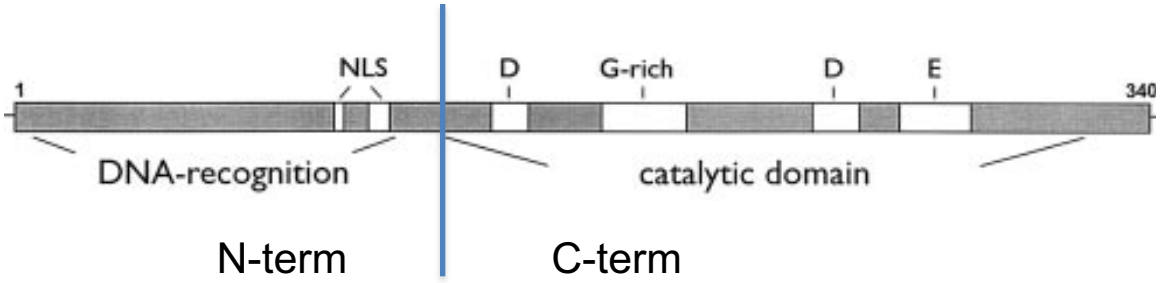


Schematic map of a salmonid TcE with the conserved domains in the transposase and IR/DR flanking sequences.

Tc transposase



SB trasposase (360 aa): two major functional domains



(i) an N-terminal: DNA-binding domain, which consists of two helix-turn-helix motifs;

→ The helix-turn-helix motifs: contact DR sequence in the inverted terminal repeats; mediate the protein-protein interactions between transposase subunits to facilitate the formation of a synaptic complex,

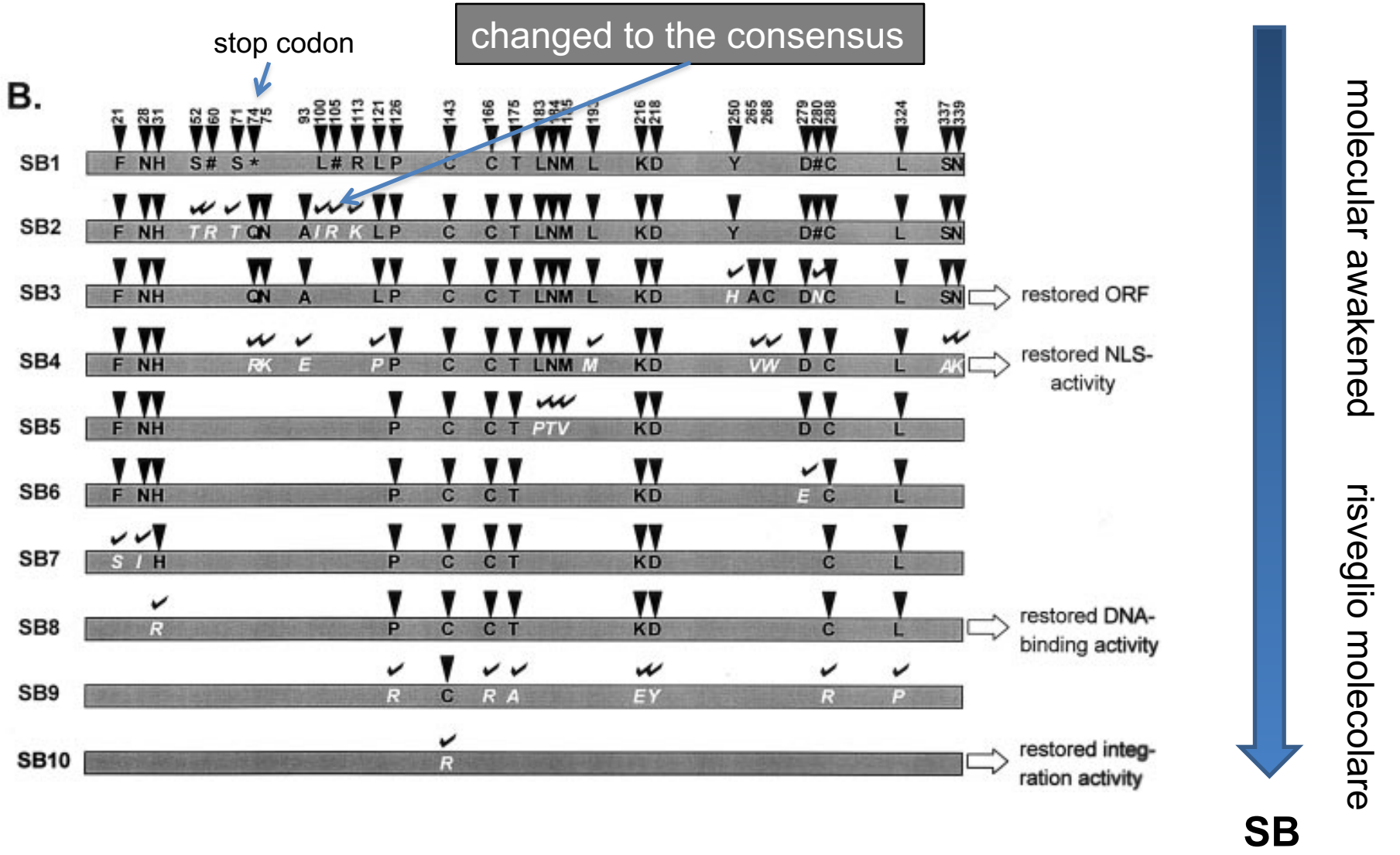
a bipartite nuclear localization sequence (NLS)

→ The nuclear localization sequence is essential for translocation of the transposon from the cytoplasm to the nucleus of target cells

(ii) a C-terminal catalytic domain characterized by the DDE motif: two aspartic acids (D) and one glutamic acid (E). There is a 34-35 amino acid space separating the second D and E residue.

→ identify TA insertion sites and catalyze DNA breakage and paste reactions during the transposition process

Molecular reconstruction of a salmonid Tc1-like transposase



The strategy of first constructing an open reading frame for a salmonid transposase and then systematically introducing amino acid replacements into this gene is illustrated. Amino acid residues are typed black when different from the consensus, and their positions within the transposase polypeptide are indicated with arrows. Translational termination codons appear as asterisks; frame shift mutations are shown as number signs. Amino acids changed to the consensus are check- marked and typed white italic. In the right margin, the various functional tests that were done at certain stages of the reconstruction procedure are indicated.

The first SB-based vector: Integration actvity of sleeping beauty in HeLa cells

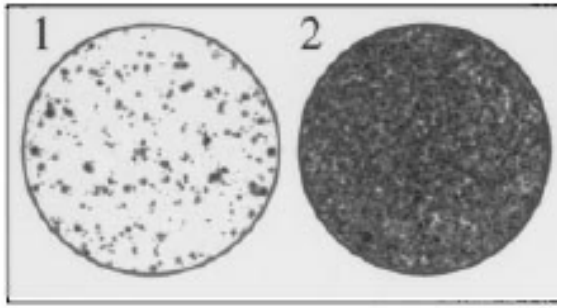
pT-neo, the SB vectors with the selectable marker

pSB, the plasmid providing in trans the SB-transposase

G418 R cells

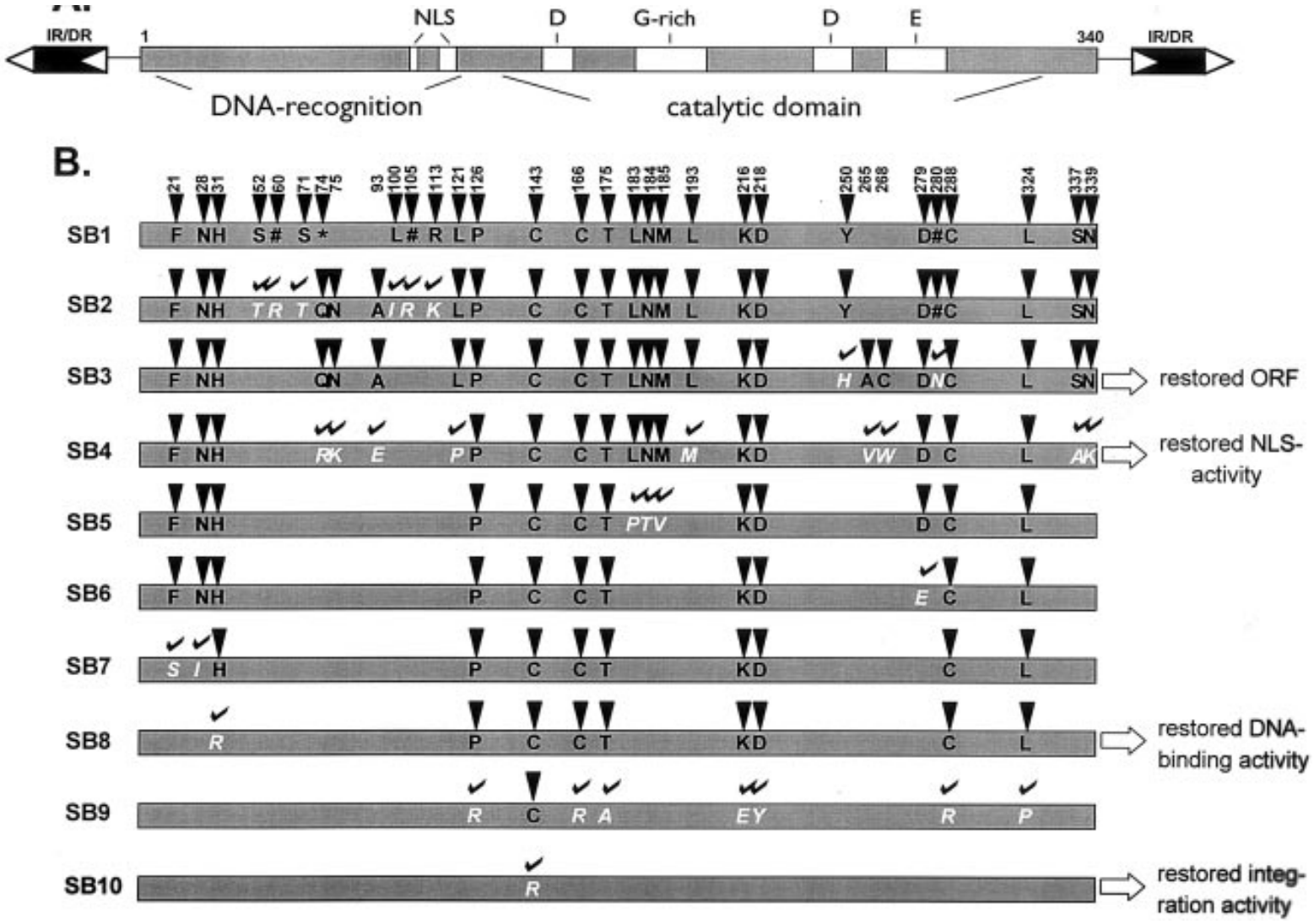
transfection

selection for G418 resistance



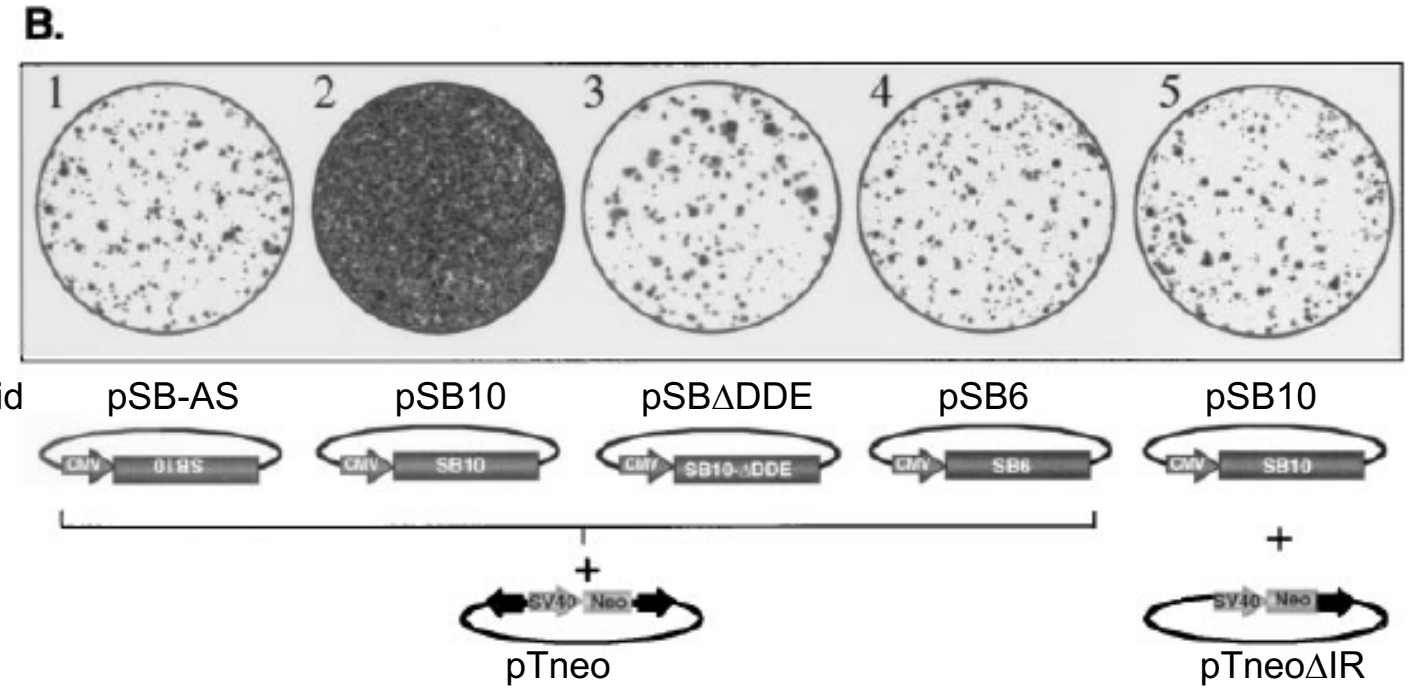
1. Control, w/o transposase
2. Tnp + IR/DR-neo

mutant transposase activity



Activity assay: testing the different components of the SB vector in HeLa cells

G418 R cells

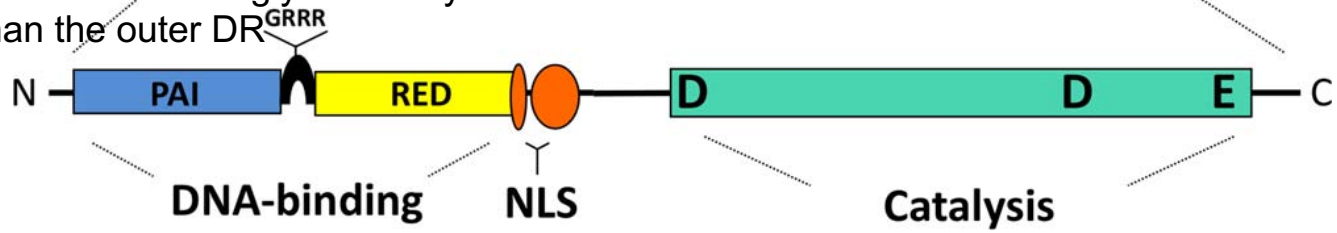


sleeping beauty transposition

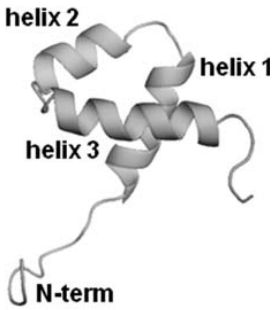
structure and function of the SB elements

The terminal IR/DR (black arrows) contain two binding sites for the transposase (white arrows). The element contains a single gene encoding the transposase (purple box).

The inner DRs are more strongly bound by the transposase than the outer DR



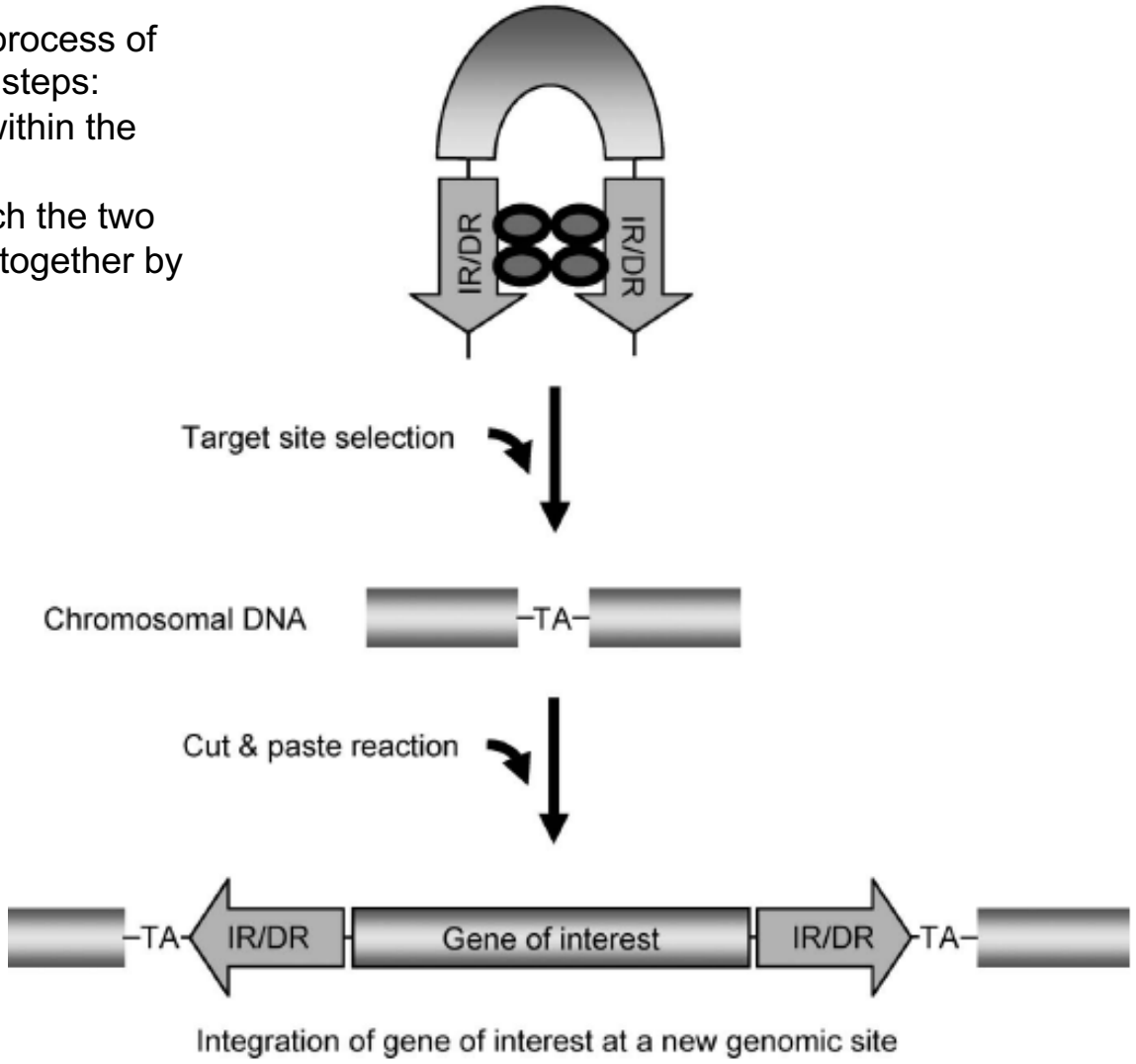
Substrate recognition is mediated by the DNA-binding domain which is predicted to form a paired-like structure with two sub-domains (PAI + RED= PAIRED) joined by a flexible linker (GRRR hook motif).
Each subdomain possess three alpha helices, two of which form a helix-turn-helix (HTH) motif



The majority of known transposases, including SB, and retroviral integrases possess a well conserved triad of amino acids, known as the aspartate–aspartate–glutamate, in short the DDE signature in their C-terminal catalytic domain. These amino acids play an essential role in catalysis by coordinating two divalent cations (Mg++). One metal ion acts as a Lewis acid, and stabilizes the transition state of the penta-coordinated phosphate, the other one acts as a general base and deprotonizes the incoming nucleophile during trans-esterification and strand transfer.

The SB transposition

The typical “cut-and-paste” transposition process of SB can be divided into at least four major steps:
(1)binding of the transposase to its sites within the transposon IRs;
(2)formation of a synaptic complex in which the two ends of the elements are paired and held together by transposase subunits;
(3)excision from the donor site;
(4)reintegration at a target site



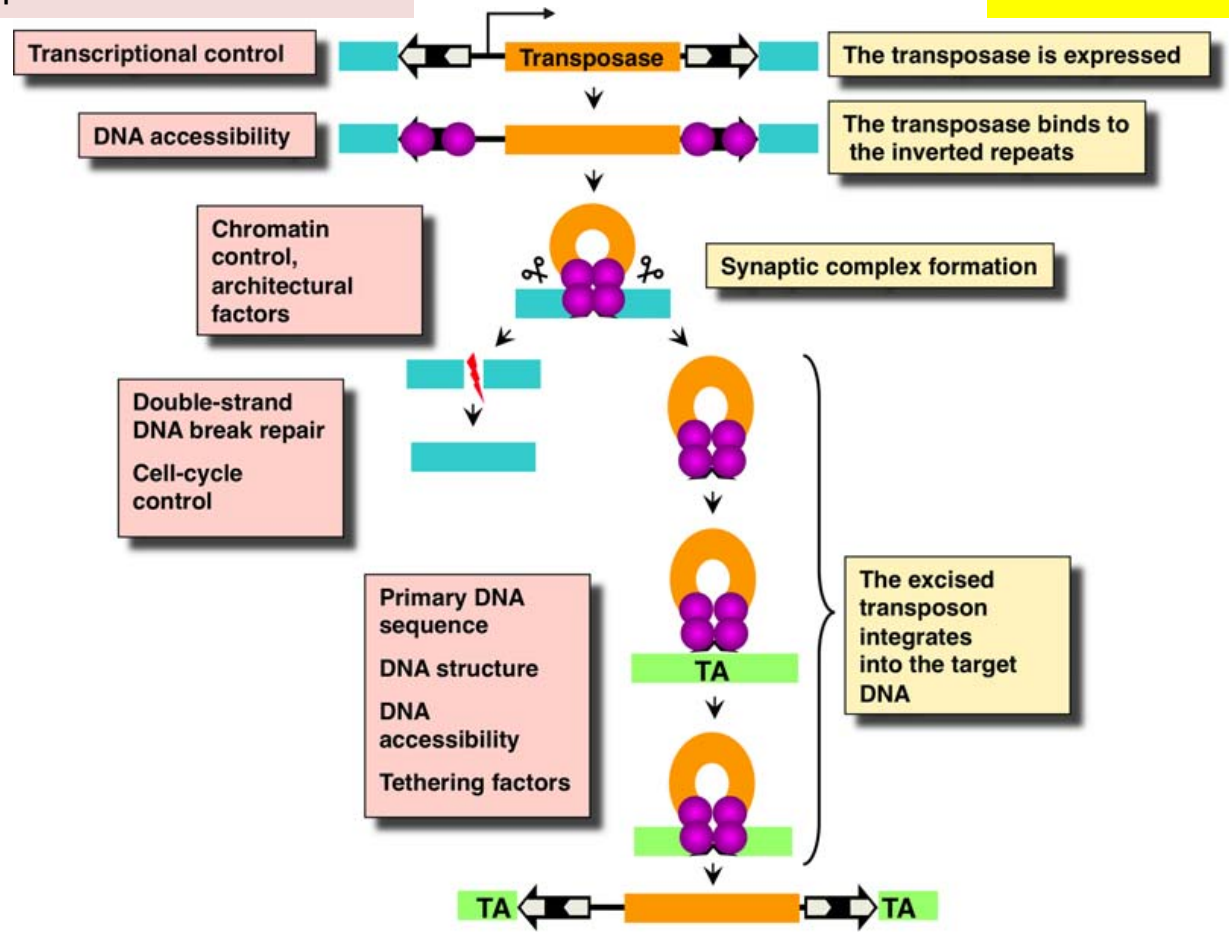
Integration of gene of interest at a new genomic site

Mechanism and regulation of SB transposition

SB can transpose in a wide range of vertebrate cells from fish to human, although the efficiency of transposition varies significantly, suggesting that host-encoded factors may affect activity and eventually limit the host range.

mechanisms and host factors regulating each step of transposition

the various steps of transposition



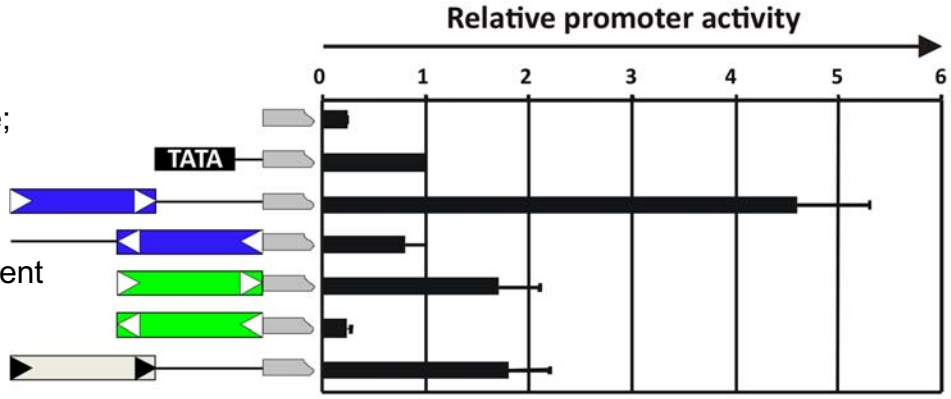
The transposase gene (orange box) bracketed by terminal IRs (solid black arrows) that contain binding sites of the transposase (white arrows) and flanking donor DNA (blue boxes). Transcriptional control elements in the 5'UTR of the transposon drive transcription (arrow) of the transposase gene. The transposase (purple spheres) binds to its sites within the transposon IRs. Excision takes place in a synaptic complex, and separates the transposon from the donor DNA. The excised element integrates into a TA site in the target DNA (green box) that is duplicated and flanks the newly integrated transposon.

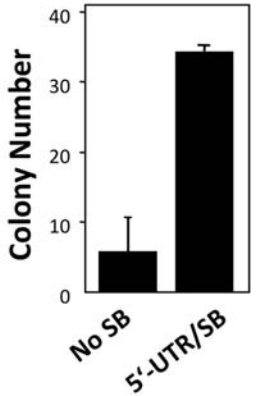
Transcriptional regulation of the SB transposon

The UTRs of the SB transposon exhibit moderate, directional promoter activities.

Blue box: left IR of SB;
green box: right IR of SB;
grey box: left IR of Frog Prince;

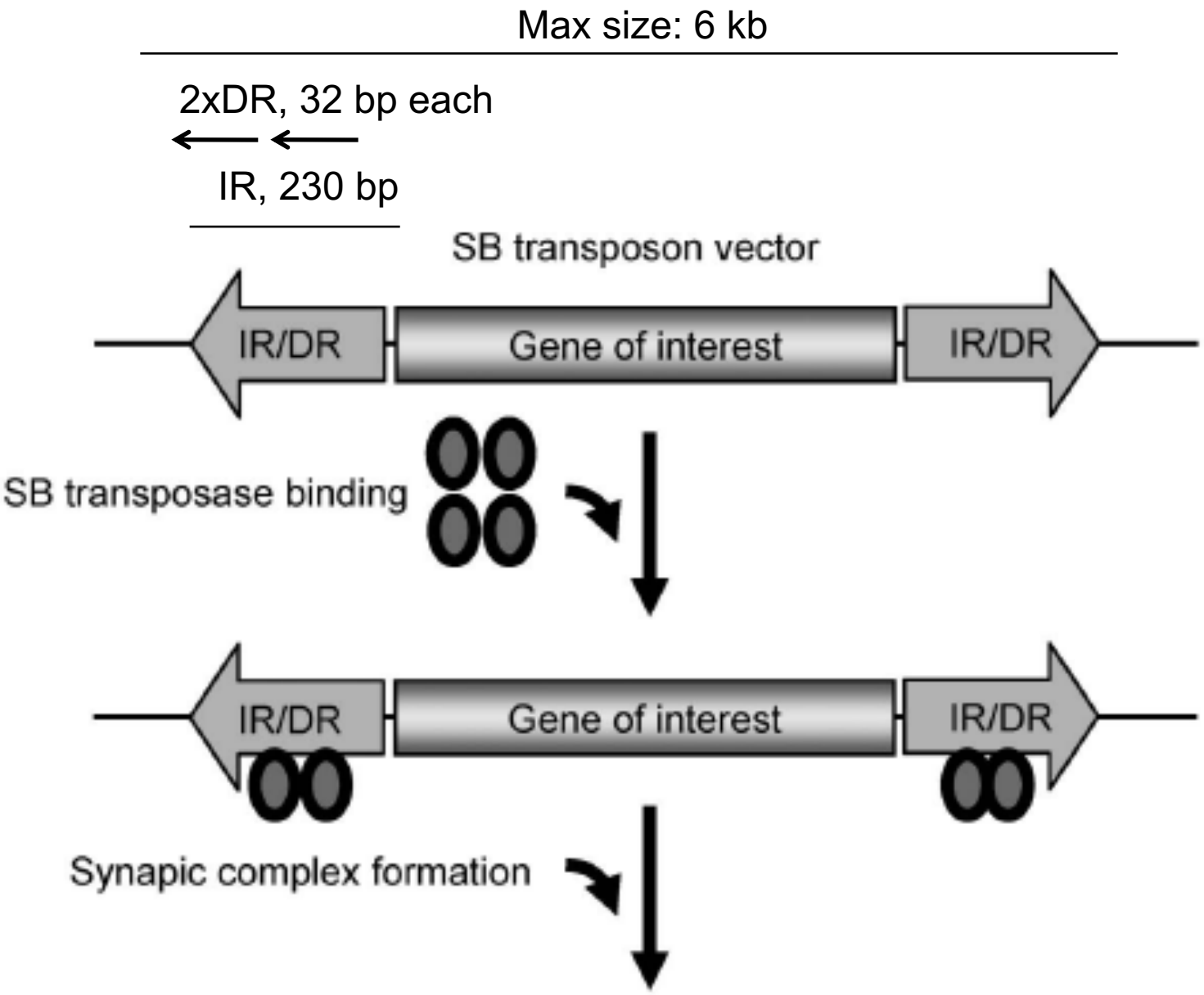
black lines connecting the IRs and the luciferase gene represent transposon sequences directly upstream of the transposase coding regions.



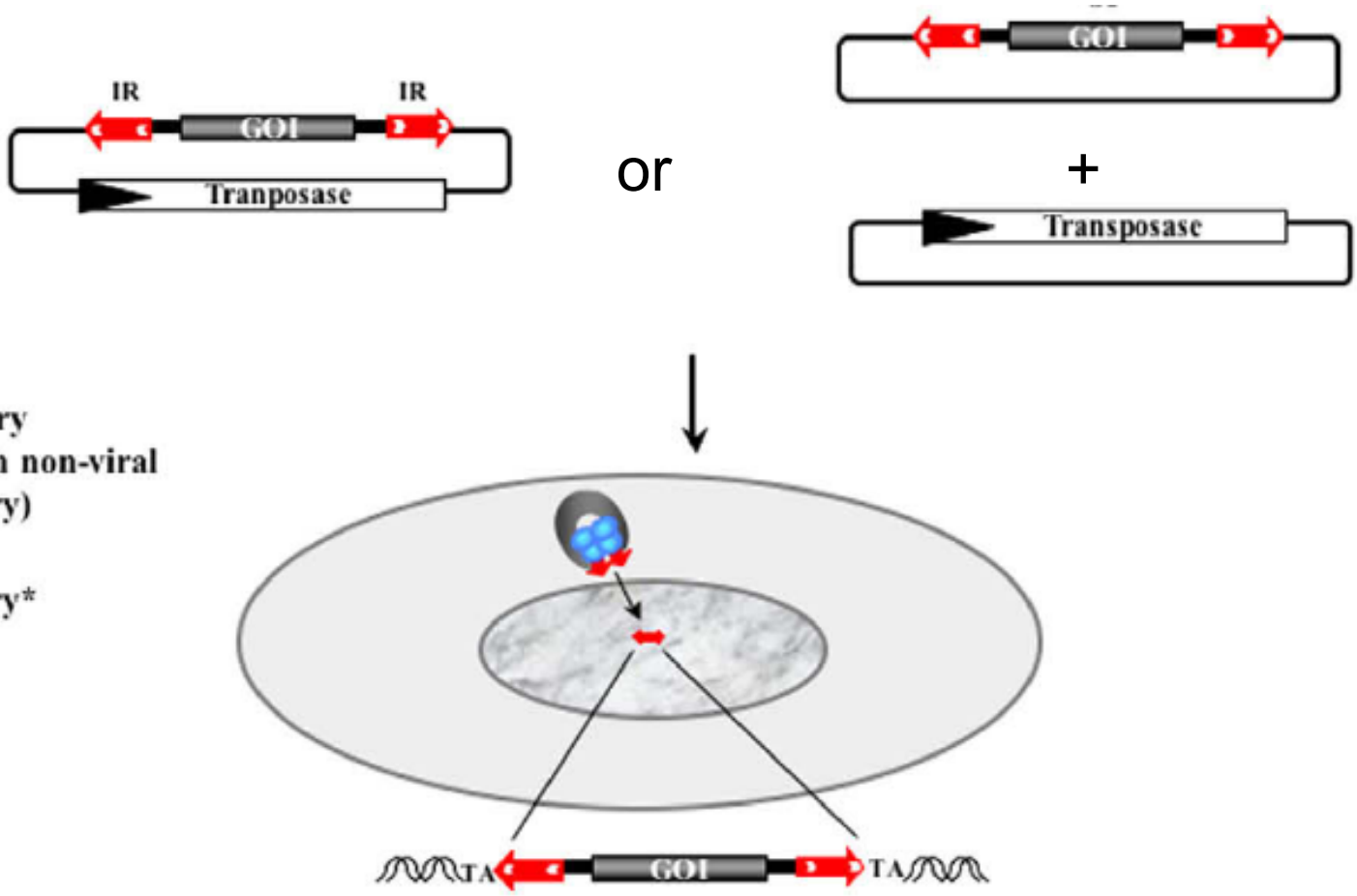


Relative promoter activities as determined by transient luciferase assays in HeLa cells. Activity of a minimal promoter (TATA-box) control was arbitrarily set to value 1. Transposon sequences flanking the transposase gene were placed in front of a luciferase reporter gene in two possible orientations (in the case of the 5'UTR, the luciferase gene precisely replaces the transposase coding region). The 5!-UTR of SB can drive transposase expression at a level sufficient for the detection of chromosomal transposition events in cultured cells. A neo-tagged SB transposon plasmid was cotransfected together with an SB expression construct, in which the transposase is expressed from the 5!-UTR of the transposon or with an empty cloning vector. The difference in numbers of G418-resistant cell colonies is evidence for trans- position.

The SB vector



the Sleeping beauty transposon system



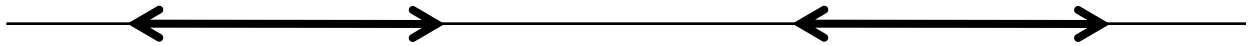
SLEEPING BEAUTY
cargo capacity

Transposon size limits the efficiency of transposition

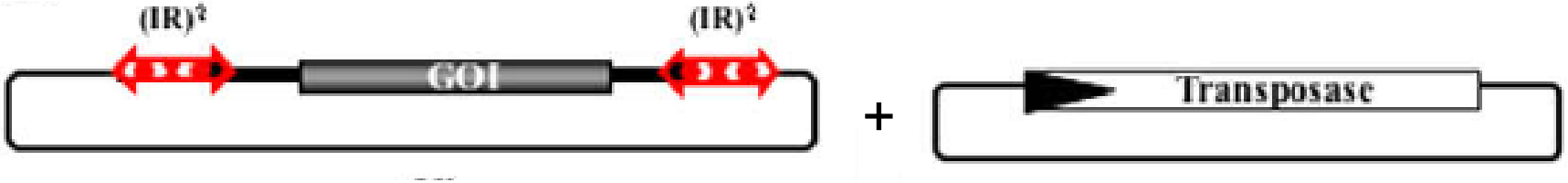
Similar to other transposable elements, transposition efficiency is inversely proportional to the size of the transposon and thus the size of the 'gene of interest' within the SB transposon may affects the efficiency of integration.

A transposon of 6 kb in size, which is sufficient to accommodate about 80% of coding sequences in the human genome, only retained half of the maximal transposition activity provided by a 2-kb transposon.

Sandwich SB >10 kb



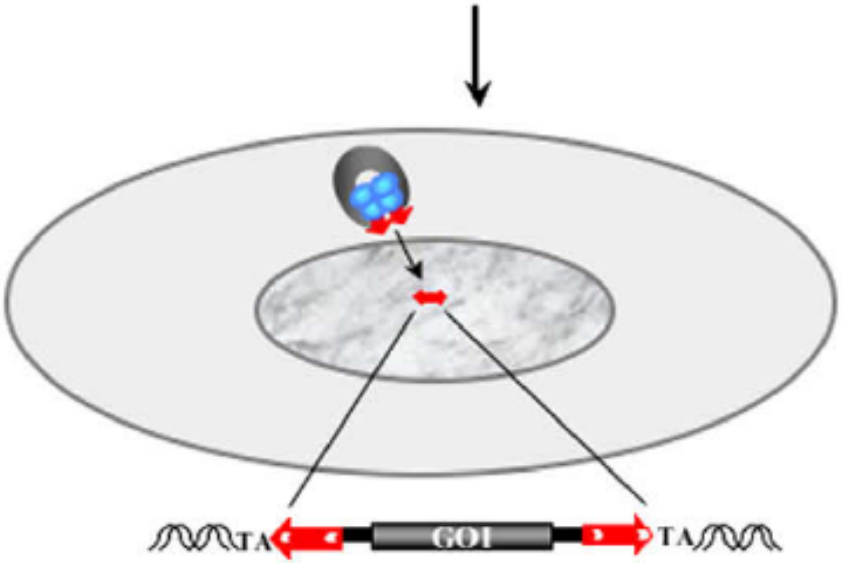
a. the sandwich vector, the Transposase is expected to target the out-side ends



b. Cellular entry
(combined with non-viral
or viral delivery)

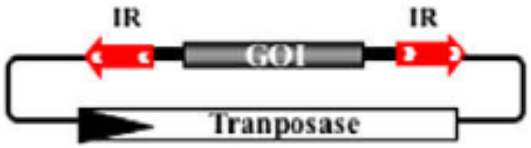
c. Nuclear entry*

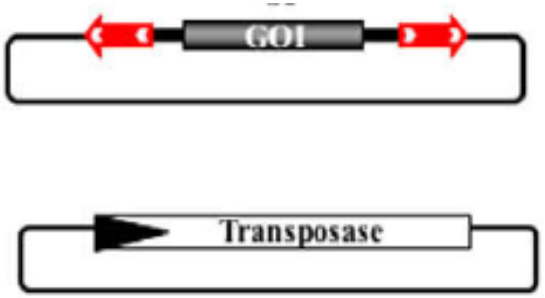
d. Integration



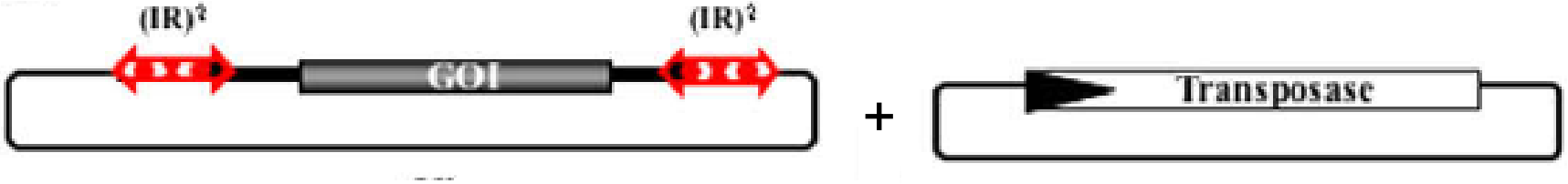
GOI: gene of interest

the SB vector systems





high cloning capacity



the sleeping beauty efficiency

acting on the transposase to increase trasposone integration efficiency

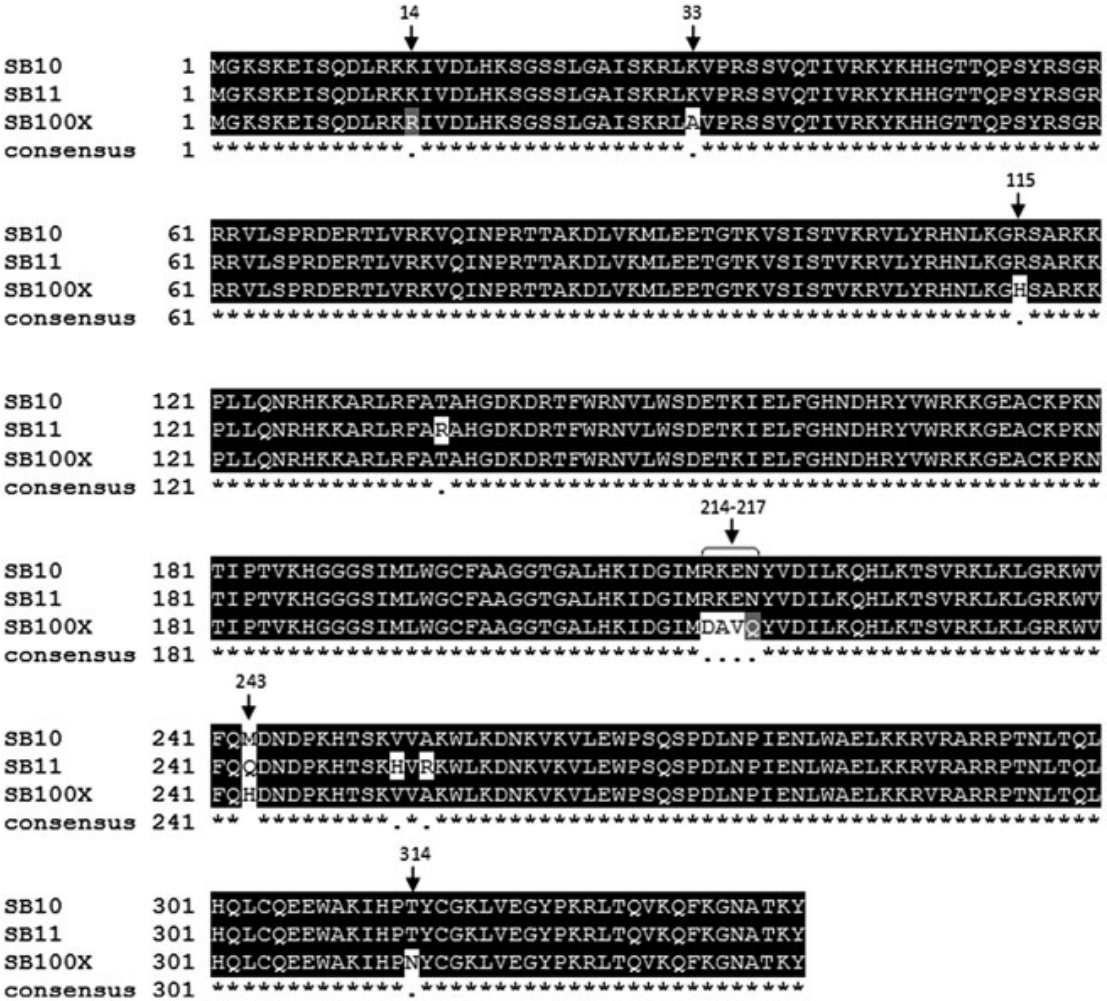
hSB17: 17-fold higher transposase activity

Zayed, H., Izsvak, Z., Walisko, O., and Ivics, Z. (2004) Development of hyperactive sleeping beauty transposon vectors by mutational analysis.
Mol.Ther. 9, 292 – 304

SB100X: hyperactive transposase

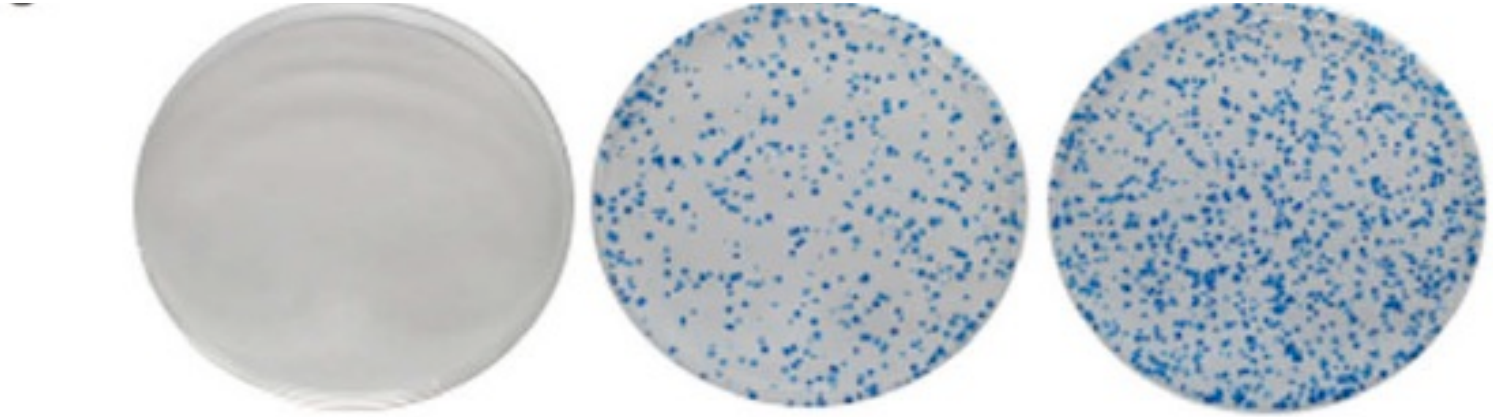
L. Mates, M.K. Chuah, E. Belay, B. Jerchow, N. Manoj, A. Acosta-Sanchez, D.P. Grzela, A. Schmitt, K. Becker, J. Matrai, L. Ma, E. Samara-Kuko, C. Gysemans, D. Pryputniewicz, C. Miskey, B. Fletcher, T. Vandendriessche, Z. Ivics, Z. Izsvak, Nat. Genet. 41 (2009) 753–761 (Epub 2009 May 3).

aminoacids substitution to make hyperactive SB transposases



Multiple sequence alignment of Sleeping Beauty transposase sequences. Hyperactive amino acid mutations (in white and/or gray) compared to SB10 the original version of the SB transposase, SB10. Hyperactive SB11 and SB100X are targeted for clinical applications. Multiple sequence alignment was performed using EBI Clustal omega (Sievers et al., 2011) and shading was performed using BOXSHADE server version 3.21 (http://www.ch.embnet.org/software/BOX_form.html).

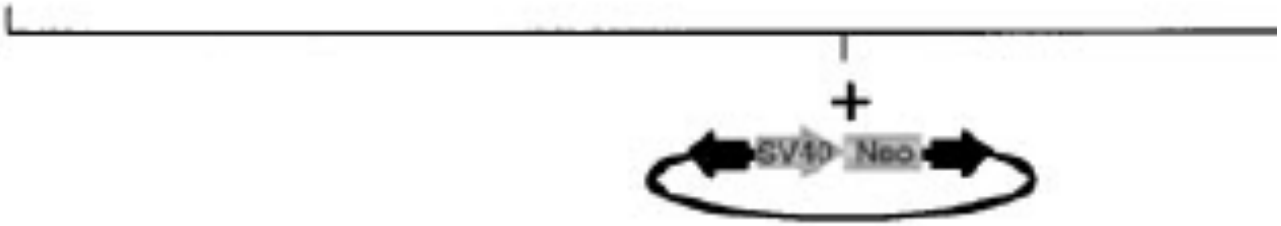
HeLa neoR colonies



Tp	50ng	50ng	500ng
Copy #	–	3-4	~12

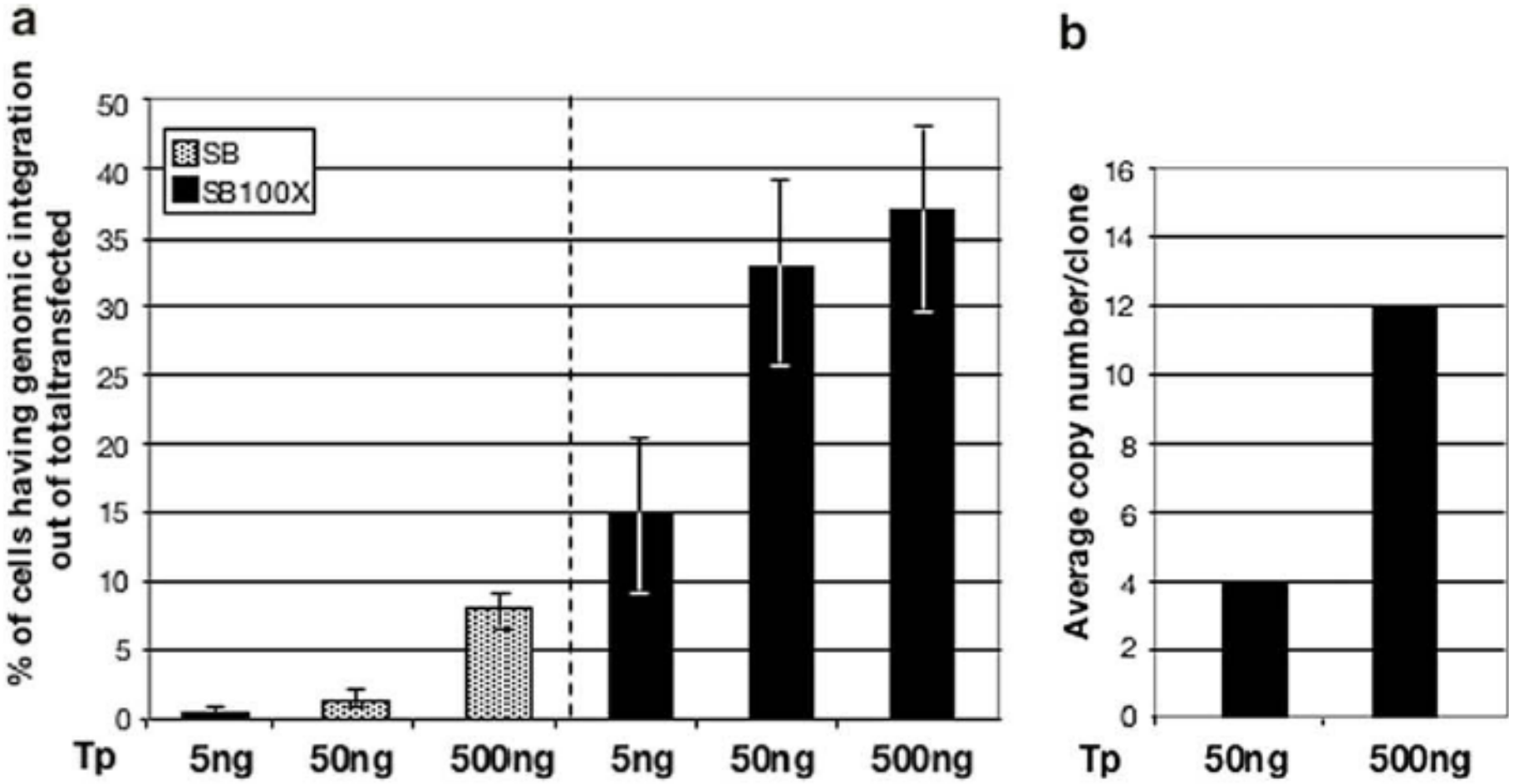
Trasposase vector

Transposone vector



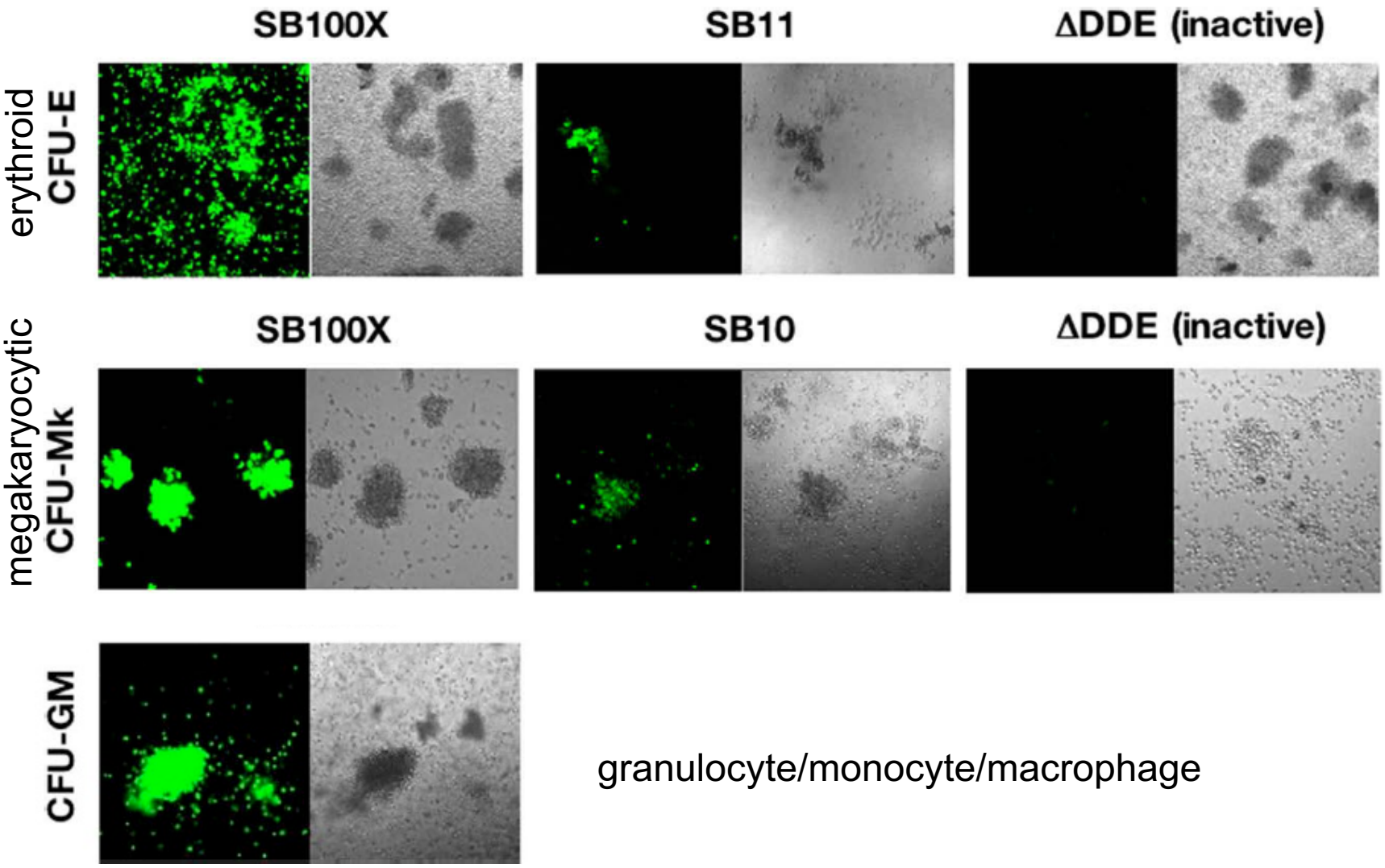
neo-resistant colonies (plating dilution 1:100); colonies stained with methylene-blue

SB100X efficacy in HeLa



SB efficacy in stem cells

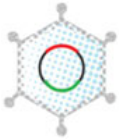
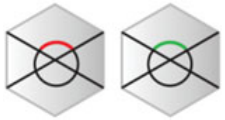
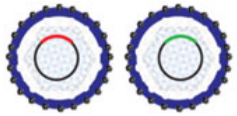
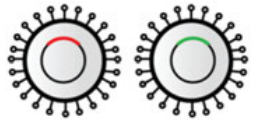
CD34+ transfected with pT2/CAGGS-GFP
In vitro differentiation into erythroid (CFU-E), granulocyte/monocyte/macrophage (CFU-GM), magakaryocytic (CFU-MK)



granulocyte/monocyte/macrophage

SB-hybrid systems

Table 1. Various sleeping beauty-viral hybrid technologies.

Hybrid technology	Delivering vehicle	Integration machinery	Advantages	References
Adeno/SB 	Recombinant adenovirus	SB transposase	• High transduction efficiency • SB-mediated unbiased, random integration profile • Stable long term expression • Capable of integrating large genetic cargos	(Yant *et al.*, 2002)
AAV/SB 	Recombinant AAV			(Zhang *et al.*, 2013)
HSV-1 amplicon/SB 	HSV-1			(Bowers *et al.*, 2006; de Silva *et al.*, 2010a; de Silva *et al.*, 2010b; Peterson *et al.*, 2007)
Baculo/SB 	Baculovirus			(Luo *et al.*, 2012; Turunen *et al.*, 2014)
IDLV/SB 	IDLV			(Moldt *et al.*, 2011; Staunstrup *et al.*, 2009; Vink *et al.*, 2009;)

Adeno: adenovirus; AAV: adeno associated virus; IDLV: integrase defective lentivirus; HSV-1: herpes simplex virus 1 amplicon; baculo: baculovirus

Note: The transposase (highlighted in green) and the transposon (highlighted in red) plasmids can be packaged into various recombinant viruses. A colored version is available online (www.informahealthcare.com/bmg)

SB - application

Sleeping Beauty Transposition From Nonintegrating Lentivirus

Conrad A Vink[1], H Bobby Gaspar[1], Richard Gabriel[2], Manfred Schmidt[2], R Scott McIvor[3], Adrian J Thrasher[1] and Waseem Qasim[1]

Molecular Therapy vol. 17 no. 7, 1197–1204 july 2009

Rationale: Lentiviral vectors enter cells with high efficiency and deliver stable transduction through integration into host chromosomes, but their preference for integration within actively transcribing genes has been reported.
Sleeping Beauty (SB) transposase does not exhibit a preference for integration within active genes.

Hypothesis: it has been hypothesized that SB transposase may change the integration profile of lentiviral vectors

Aim: to change lentiviral integration profile by using the SB integration profile

changing the integration profile of lentiviral vectors

SB has been developed as a vector by substituting the transposase coding sequence with a transgene expression cassette (**SB-vector**). Following delivery to cells, the transposase protein is provided *in trans* to mediate cut-and-paste transposition of the transgene into the target cell genome (**Tp provided in trans**).

Cleavage is dependent upon the presence of flanking TA dinucleotides and is enhanced when the transposon is flanked by TATA motifs.

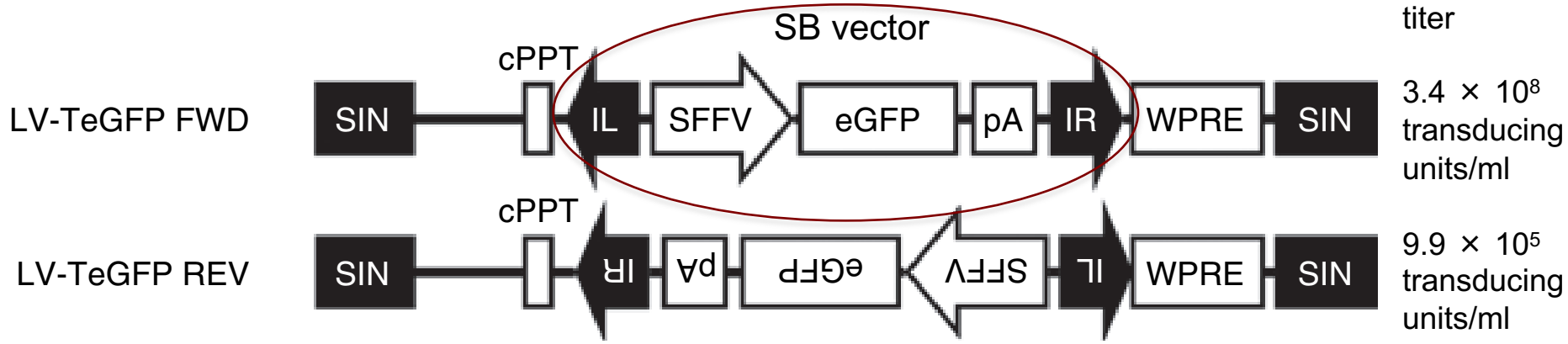
SB integration occurs exclusively at TA dinucleotides, and DNA repair following integration results in a duplication signature with TA dinucleotides on either side of the transposon.

Importantly, **integration occurs within genes at a frequency close to that expected from random integration** and is not biased toward actively transcribing genes.

generation of integrasedeficient lentiviral vectors (IDLVs) to carry SB transposon and transposase expression cassettes.

to establish the optimal orientation of the SB within the IDVL vector

eGFP, expression cassette was inserted in either the forward or reverse orientation with respect to the lentiviral back- bone (IDLV-TeGFP forward and IDLV-TeGFP reverse.

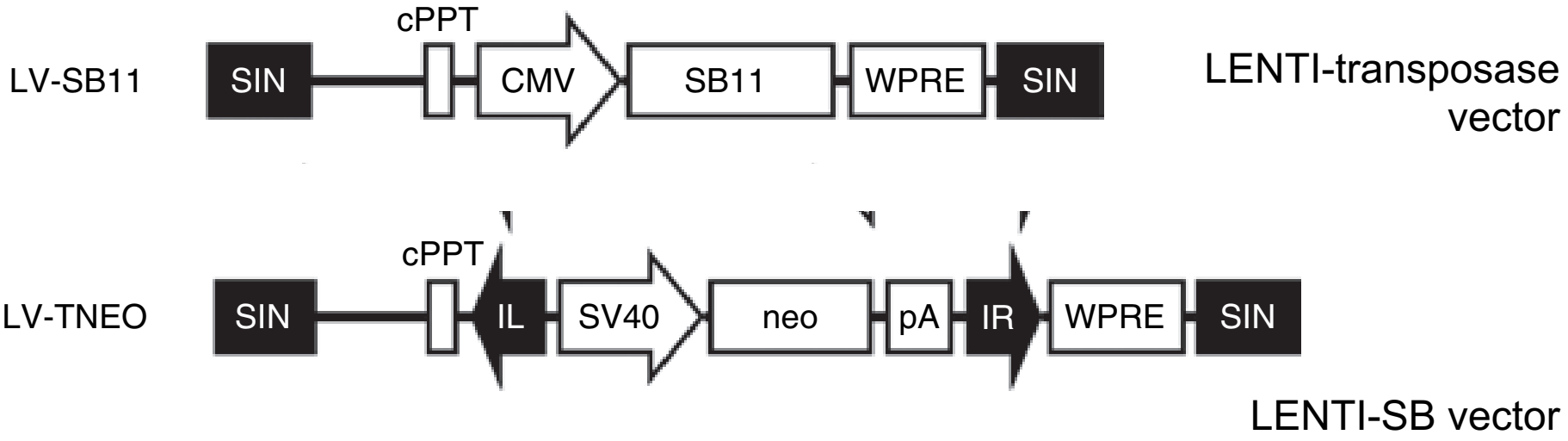


vector titers were notably reduced when the transposon was in the reverse orientation (9.9 × 10^5 transducing units/ml) relative to the lentiviral backbone compared to the forward orientation (3.4 × 10^8 transducing units/ml), and thus the forward orientated constructs were used in all subsequent experiments

cPPT, central polypurine tract;

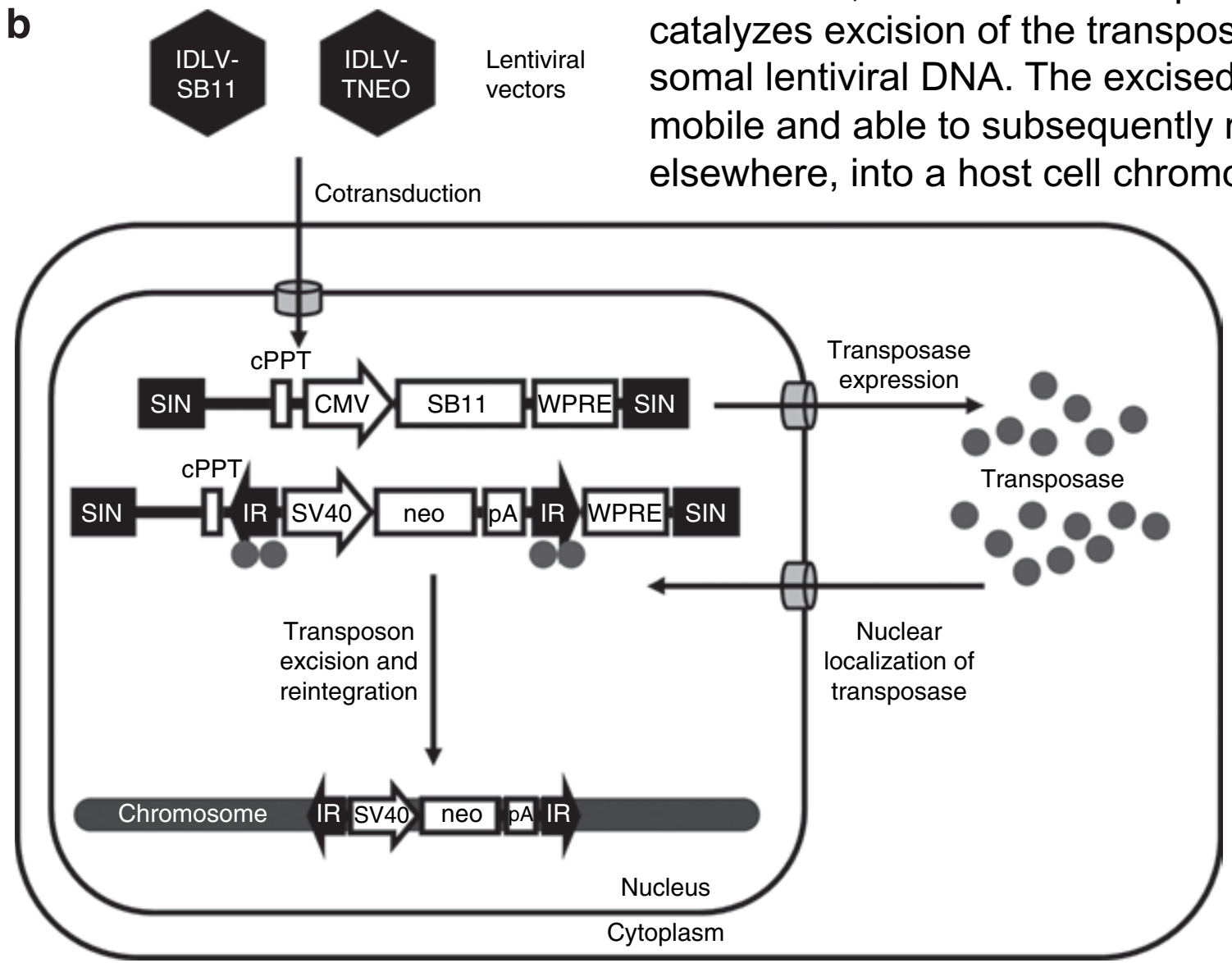
generation of integrasedeficient lentiviral vectors (IDLVs) to carry SB transposon and transposase expression cassettes.

The transposon vector and transposase are provided by separate, independent lentiviral vectors

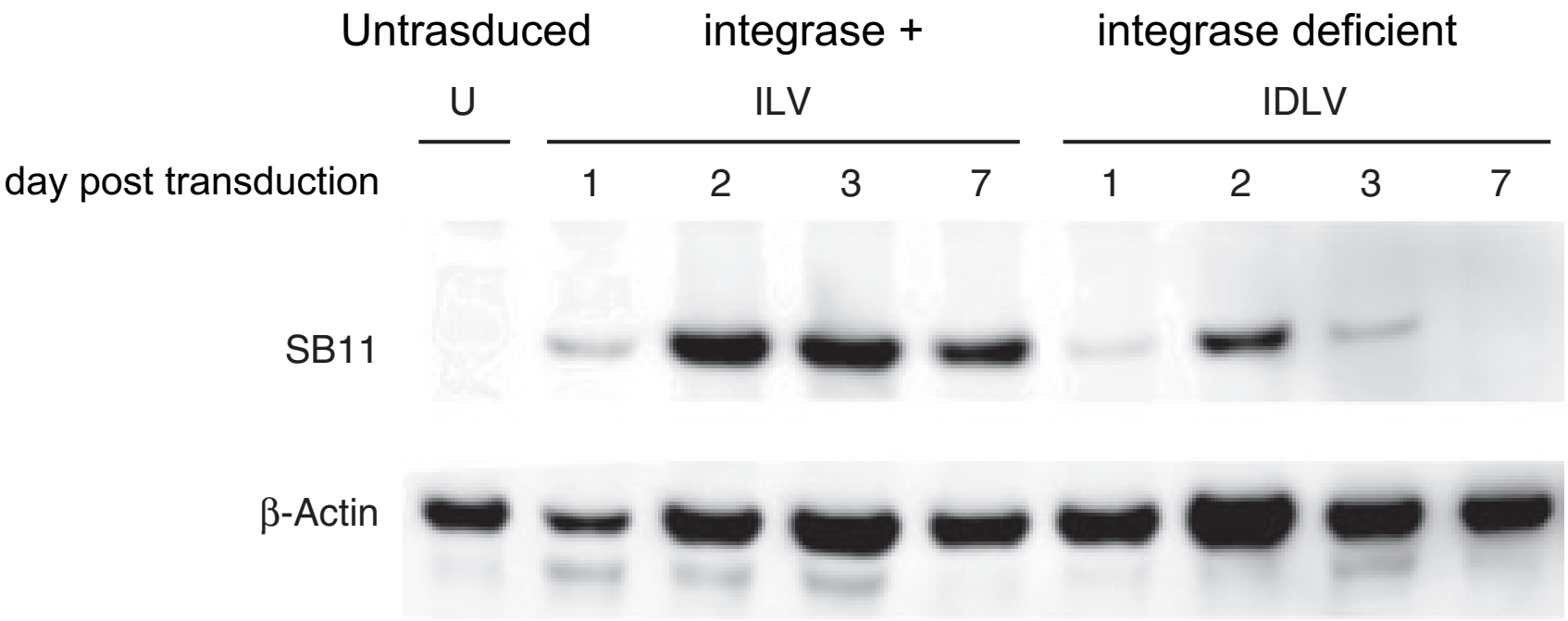


Cells are cotransduced with integrasedeficient lentiviral vectors carrying the Sleeping Beauty transposase and transposon.

Transposase protein is expressed, localized to the nucleus, binds to the transposon IR and catalyzes excision of the transposon from epi somal lentiviral DNA. The excised transposon is mobile and able to subsequently reintegrate elsewhere, into a host cell chromosome



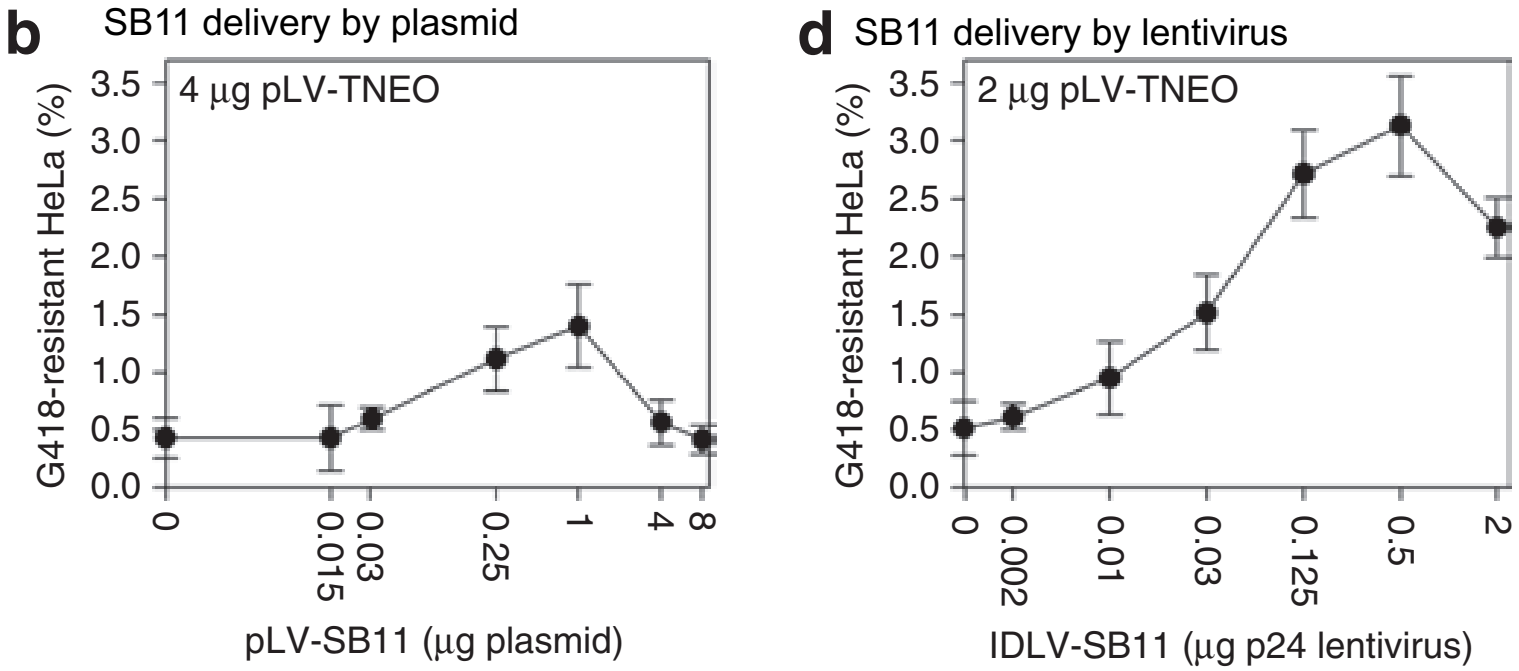
transposase expression in HeLa transduced cells



Integraseproficient ILVSB11 and integrasedeficient IDLVSB11 transposase expression vectors were prepared in parallel and concentrated by ultracentrifugation. 10^6 HeLa cells were transduced with 0.5µg of vector DNA per well. At 1, 2, 3, and 7 days posttransduction, cells were trypsinized and pellets of equal cell number were frozen for subsequent determination of protein expression by western blot.
IDLV, integrasedeficient lentiviral vector; **ILV**, integraseproficient lentiviral vector; **U,** untransduced.

10^5 HeLa cells were transduced with Sleeping Beauty components in a double titration integration assay.

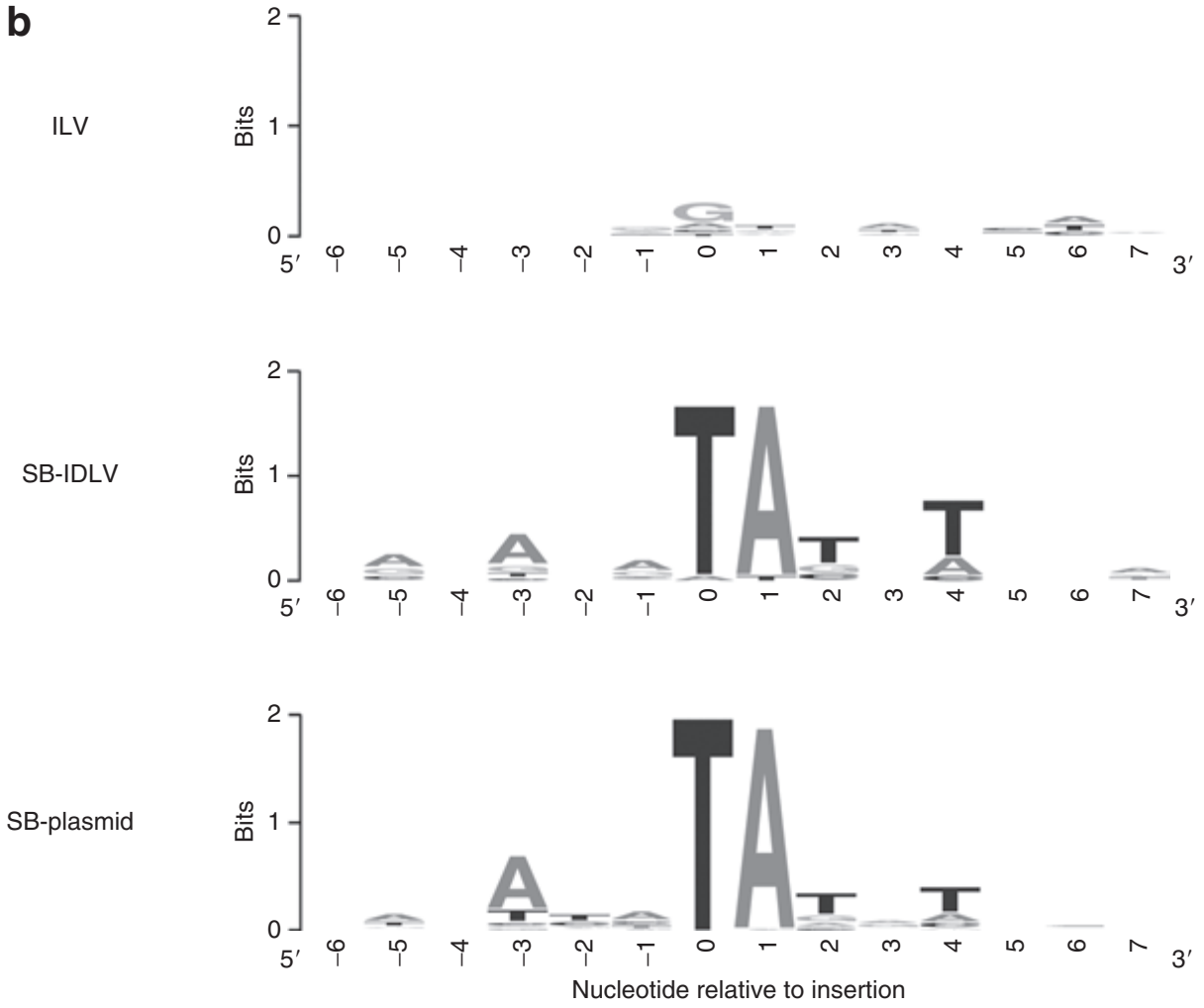
When both transposon and transposase were delivered by IDLV transduction, the highest rate of gene marking observed was 2.6 ± 0.2%



The rate of integration was assessed by the number of G418resistant colonies formed. Data are expressed as a percentage of $G418^R$ cells.

integration profile

G418-resistant HeLa cell colonies were produced by transduction with ILV-TNEO or IDLV-TNEO alone, IDLV-TNEO plus IDLV- SB11, or transfection with plasmids pLV-TNEO plus pLV-SB11 under the previously optimized conditions. Integration sites were recovered from surviving colonies using ligation-mediated PCR



integration profiles in Hela cells

Table 1 Integration profiles of vectors relative to RefSeq genes

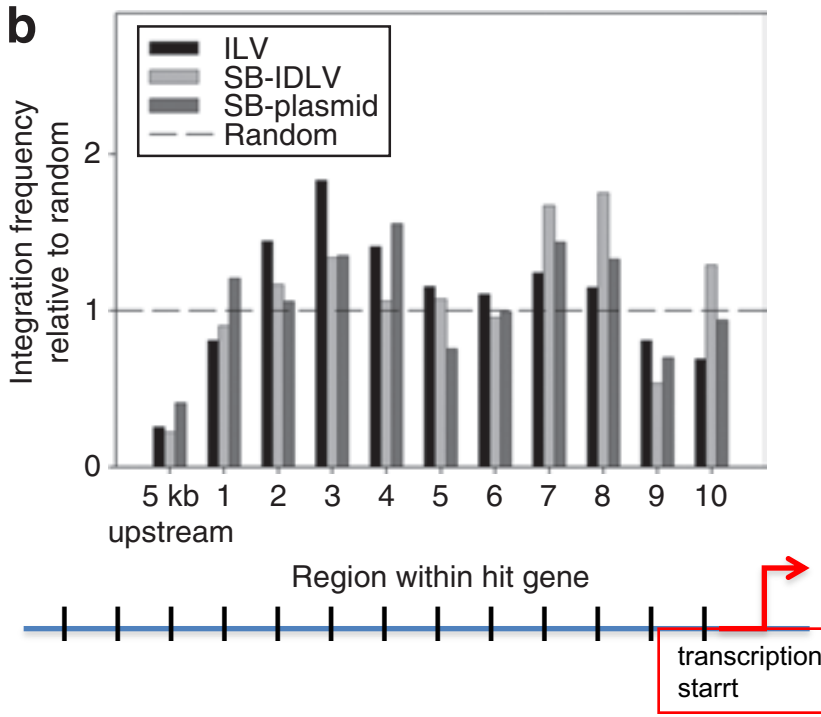
Vector type	Number of integration sites	Sites within genes (%)
ILV	976	76.9
SB-IDLV	161	53.4
SB-plasmid	752	42.7
Random	1,000	34.2

Abbreviations: IDLV, integrase-deficient lentiviral vector; ILV, integrase-proficient lentiviral vector; SB, Sleeping Beauty.

RANDOM, the integration of the Neo gene is not driven by any specific mechanisms (no lentiviral, nor SB)

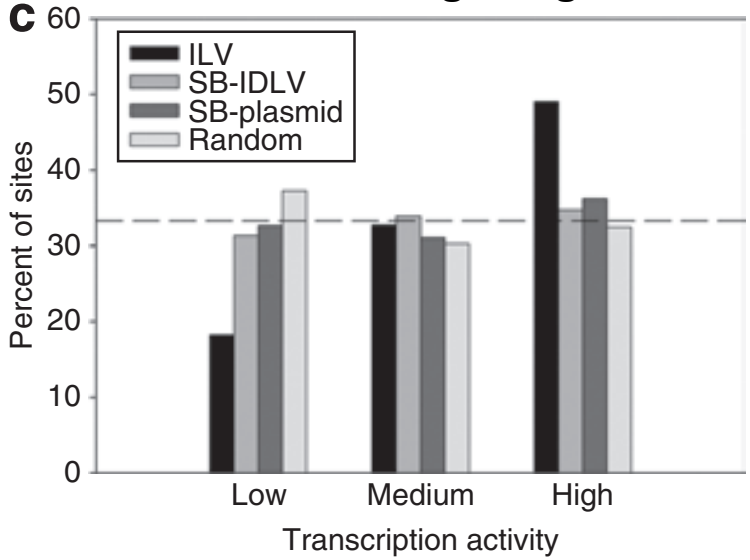
intragenic position and transcriptional activity

Intragenic position of integration sites within genes



RefSeq genes containing integration sites were divided by length into 10 equally sized regions and a 5 kb upstream region, and the proportion of integration sites within each region was counted.

Transcriptional activity of genes containing integration sites



All RefSeq genes were scored for transcription in HeLa cells using a published microarray dataset. All genes were then assigned to one of three transcription levels (containing equal numbers of genes) to give low, medium, and highly transcribed genes. Integration sites within genes were then scored according to whether the hit gene was transcribed at a low, medium, or high level. For each vector type, the number of intragenic sites per transcription level is expressed as a percentage of the total number of intragenic sites.

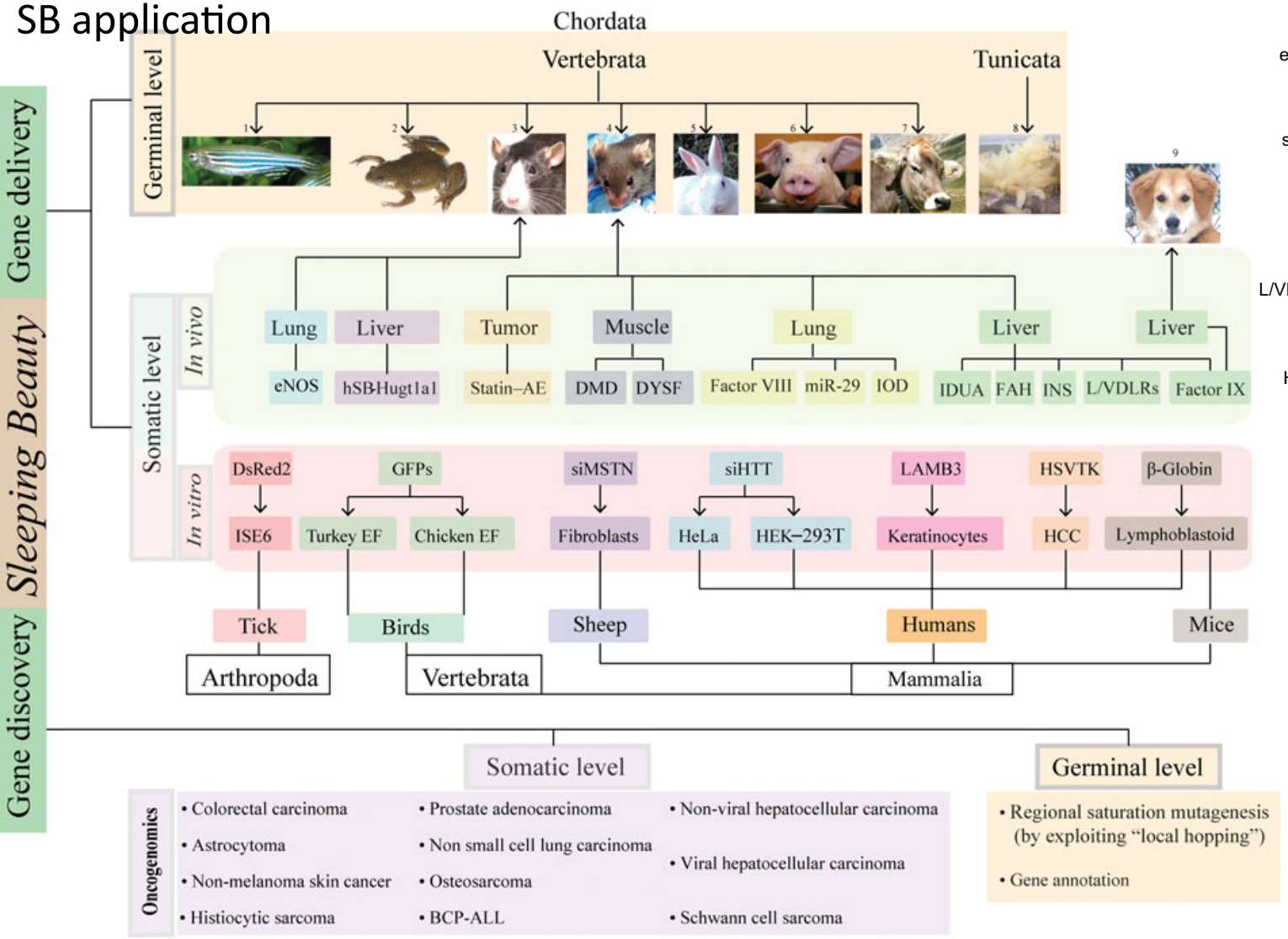
SUMMARY

The proportion of ILV (integrating lentiviral vectors) integration sites within RefSeq genes was (77%) greater than that for SB-IDLV (53%) or SB-plasmid (43%) ($P < 0.01$). Random integration was 34%.

When integrations within genes were mapped relative to their position within the gene or upstream region **no bias toward transcription start sites** was detected and **no significant variation** in integration pattern was observed **along the length of the gene.**

Lentiviral vector integration occurs preferentially within transcriptionally active genes. When considering only genes containing integration sites**, ILV integration exhibited a clear preference for genes with high levels of transcriptional activity (P < 0.01)**, whereas ***SB-IDLV and SB-plasmid integration showed no bias toward any particular level of transcriptio***n, and resembled the profile generated for random integration events.

SB application



eNOS: endothelial nitric oxide synthase; hUGT1A1: human uridinediphosphoglucuronate glucuronosyltransferase-1A1; statin-AE: angiostatin–endostatin fusion cassette; DMD: Duchenne muscular dystrophy; DYSF: dysferlin; IDUA: a-L-iduronidase; FAH: fumarylacetoacetate hydrolase; INS: insulin; L/VDLRs: low-density lipoprotein and very-low-density lipoprotein receptors; IOD: indoleamine-2,3 dioxygenase; LAMB3: laminin subunit beta-3; HSVTK: herpes simplex virus thymidine kinase type 1 gene; BCP-ALL: B cell precursor acute lymphoblastic leukemia).

Sleeping Beauty transposon-based applications. SB was successfully used for germline transgenesis, in various models (fish1, frog2, rat3, mouse4, rabbit5, pig6, cow7 and sea squirt8). The SB system has been employed for somatic gene delivery in various vertebrates, but also in a tick (insect) cell line, ISE6. SB-based gene delivery has been used in several preclinical animal models. Alternatively, the mutagenic version of the SB can be employed in functional genomics. Insertional mutagenesis screens can be used to annotate genes in somatic cells (oncogenomics) or in the germline. Abbreviations: eNOS: endothelial nitric oxide synthase; hUGT1A1: human uridinediphosphoglucuronate glucuronosyltransferase-1A1; statin-AE: angiostatin–endostatin fusion cassette; DMD: Duchenne muscular dystrophy; DYSF: dysferlin; IDUA: a-L-iduronidase; FAH: fumarylacetoacetate hydrolase; INS: insulin; L/VDLRs: low-density lipoprotein and very-low-density lipoprotein receptors; miR–29: micro RNA 29; IOD: indoleamine-2,3 dioxygenase; DsRed2: red fluorescent protein 2; GFPs: green fluorescent proteins; siMSTN: siRNA against myostatin; siHTT: siRNA against Huntington; LAMB3: laminin subunit beta-3; HSVTK: herpes simplex virus thymidine kinase type 1 gene; BCP-ALL: B cell precursor acute lymphoblastic leukemia). A color version of the figure is available online (see color version of this figure at www.informahealthcare.com/bmg).

SB in clinical trials

Vector	Gene Therapy Clinical Trials	
	Number	%
Sleeping Beauty transposon	**10**	**0.5**

Da consultare

Zolta´n Ivics, Perry B. Hackett, Ronald H. Plasterk and Zsuzsanna Izsva´ “Molecular Reconstruction of Sleeping Beauty, a Tc1-like Transposon from Fish, and Its Transposition in Human Cells” Cell (1997) Vol. 91: 501–510. (optional)

Suneel A. Narayanavari, Shreevathsa S. Chilkunda, Zoltán Ivics & Zsuzsanna Izsvák (2017) Sleeping Beauty transposition: from biology to applications, Critical Reviews in Biochemistry and Molecular Biology, 52:1, 18-44, DOI: 10.1080/10409238.2016.1237935