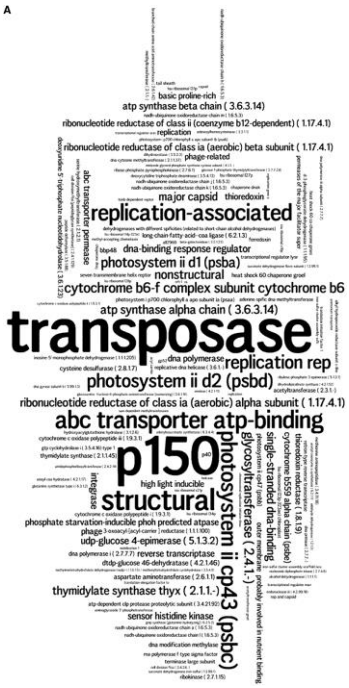


DNA Integrating Vectors (Transposon, Integrase)

- Viral vector → immunogenicity problem, oncogenicity problem, production of a self-replicating vector integrating DNA

- Plasmid DNA → simpler and safer alternative than viral vector; easier to engineer and produce ➔ Integration using transposon and integrase systems

- The primary considerations used to evaluate these nonviral vectors have been their
  1) activity to insert specific genetic sequences into chromosomes,
  2) genetic cargo capacity,
  3) sensitivity to different levels of the relevant recombinase,
  4) integration-site preferences, and
  5) their propensity to induce chromosomal rearrangements.

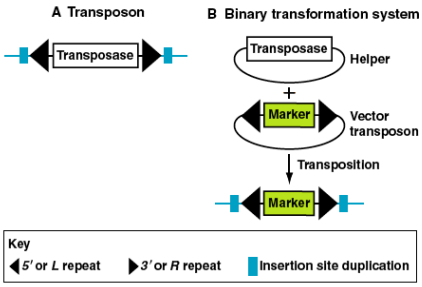
Nonviral integrating gene therapy

- Transposon systems
- Sequence specific phage integrases
- Zinc finger nucleases

Transposons

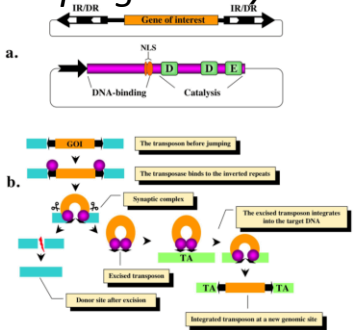
Barbara McClintock
The Nobel Prize in Physiology or Medicine 1983

- Mobile genetic elements that can "jump" around genome
- DNA transposons utilize "Cut and paste" mechanism for jumping (remain relatively low copy #)
- Retrotransposons utilize RNA intermediate and "copy and paste" tranposition (increases copy # --LINE transposon alone makes up 21% of human genome!)
- Transposons can be separated from transposase so transposition is controlled independently
- Transposons can be vehicles for transgene delivery and mobilization



Transposon Systems

- two major classes of mobile DNA elements:
  - Retrotransposons and
  - DNA transposons ➔ have been used for gene therapy
- transposons for gene therapy: mostly *Tc1/mariner* family including *Sleeping Beauty* (SB)



Transposon Systems

- The system consists of :
  - DNA sequence to be inserted (transposon)
  - enzyme that accomplishes integration into the genome (transposase).
- Natural transposons :
  - code for the transposase and
  - contain the sequences required for its insertion, called terminal inverted repeat (IR)/direct repeat (DR) elements
- In engineered systems,
  - the transgene cassette → between the two IR/DR elements so that it will be inserted
  - The transposase gene may either be placed on the outside of the IR/DR elements, or on a separate plasmid

Transposons as Mutagens

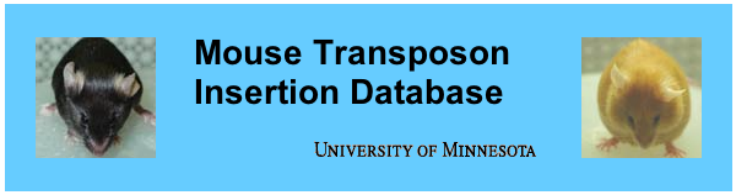
Advantage: mutated gene is "tagged" by transposon for easy identification

Disadvantage: transposons do not insert randomly
-some genes hit easily, others not

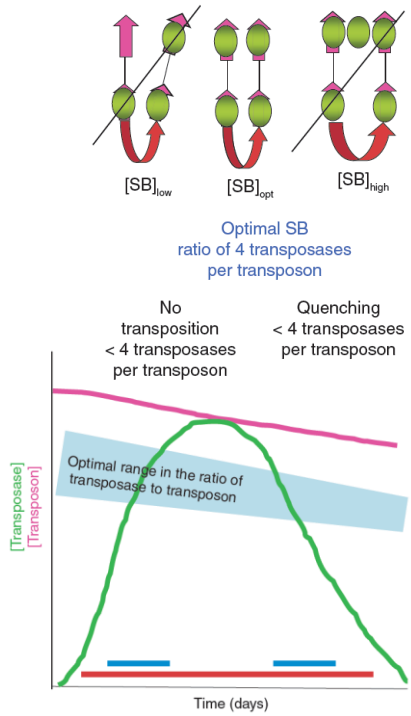
DOI: 10.1534/genetics.104.026427

The BDGP Gene Disruption Project: Single Transposon Insertions Associated With 40% of Drosophila Genes

Hugo J. Bellen,* Robert W. Levis,† Guochun Liao,‡,1 Yuchun He,* Joseph W. Carlson,§ Garson Tsang,‡ Martha Evans-Holm,‡ P. Robin Hiesinger,* Karen L. Schulze,* Gerald M. Rubin,‡ Roger A. Hoskins§ and Allan C. Spradling†,2



- SB transposon has two perceived limitations:
  1) the highest transposition rates require an optimal ratio of transposase to transposon and
     (called *overexpression inhibition*),
  2) transposition rates appear to be inversely proportional to genetic cargo size.



Main features of the Sleeping Beauty transposon system

Genetic material	DNA
Delivering capacity	~ 10 kb
Tropism	Somatic, embryonic, germinal cells, dividing/nondividing cells (?)
Immunogenic potential	The transposase is not toxic, even at high concentrations; no dramatic immune response to repeated administration Similar to non-viral plasmid
Integration sites	Fairly random integration, reduced insertion into other repetitive elements, no rearrangements t the integration sites, no signs of proliferative transformation Can be targeted
Integrated DNA	The gene integrates in a single-copy form; the gene remains intact, no rearrangements
Stable expression	Silencing is Cargo dependent
Transcriptional act	Benign promoter/enhancing activity (100 x < MLT LTR)
Vector	Can be combined with nonviral or with viral approaches; plasmid-based technology with integrating feature
Safely	SB is less likely to integrate into genes than HIV- or AVV-based vectors; no interference with endogeneous Transposons

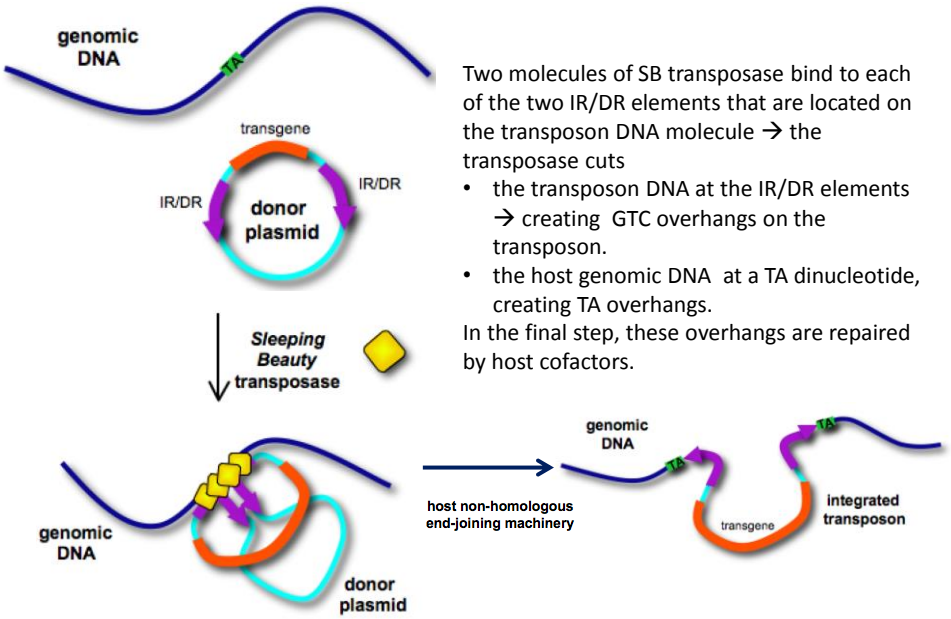
TABLE 17.1

Categorization of Transposable Elements by Transposition Mechanism

Category	Examples	Host Organism
I. Cut-and-paste transposons	IS elements (e.g., IS*50*)	Bacteria
	Composite transposons (e.g., Tn*5*)	Bacteria
	Ac/*Ds* elements	Maize
	P elements	*Drosophila*
	hobo elements	*Drosophila*
	piggyBac	moth
	Sleeping Beauty	salmon
II. Replicative transposons	Tn*3* elements	Bacteria
III. Retrotransposons		
A. Retroviruslike elements (also called long terminal repeat, or LTR, retrotransposons)	Ty*1*	Yeast
	copia	*Drosophila*
	gypsy	*Drosophila*
B. Retroposons	*F*, *G*, and *I* elements	*Drosophila*
	Telomeric retroposons	*Drosophila*
	LINEs (e.g., *L1*)	Humans
	SINEs (e.g., *Alu*)	Humans

Commonly Used Transposons

Species of Study	Transposon	Original source
Drosophila	P element	D. melanogaster
	piggy bac*	Insects (cabbage looper)
	minos*	D. hydei
	*works in many species--including mouse	
C. Elegans	Tc1	C. Elegans
	note: used for mutagenesis but not transgenesis	
Zebrafish	Tol2	Fish (medaka)
Mouse	Sleeping Beauty	Fish (salmon/trout)

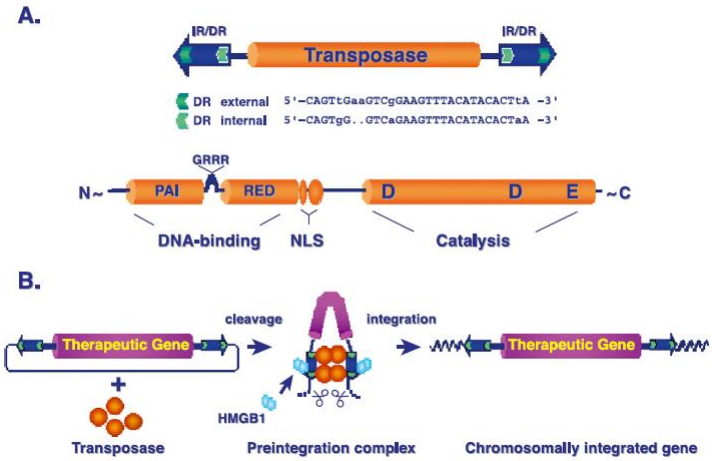


Two molecules of SB transposase bind to each of the two IR/DR elements that are located on the transposon DNA molecule → the transposase cuts

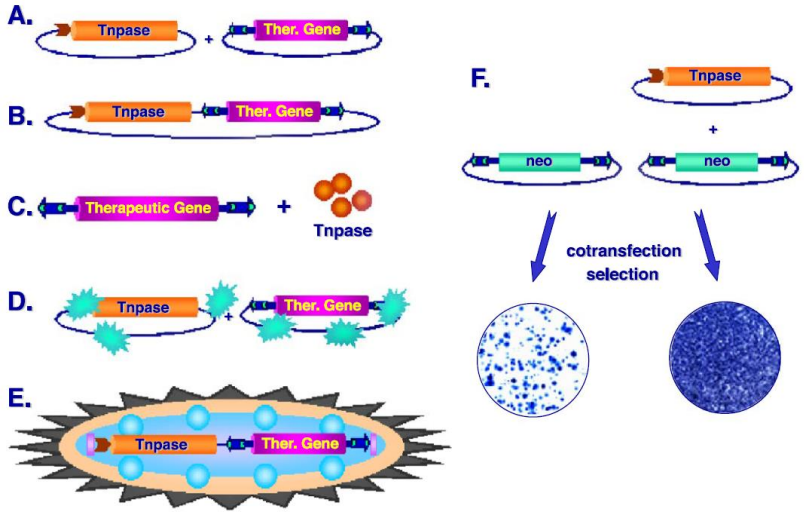
- the transposon DNA at the IR/DR elements → creating GTC overhangs on the transposon.
- the host genomic DNA at a TA dinucleotide, creating TA overhangs.

In the final step, these overhangs are repaired by host cofactors.

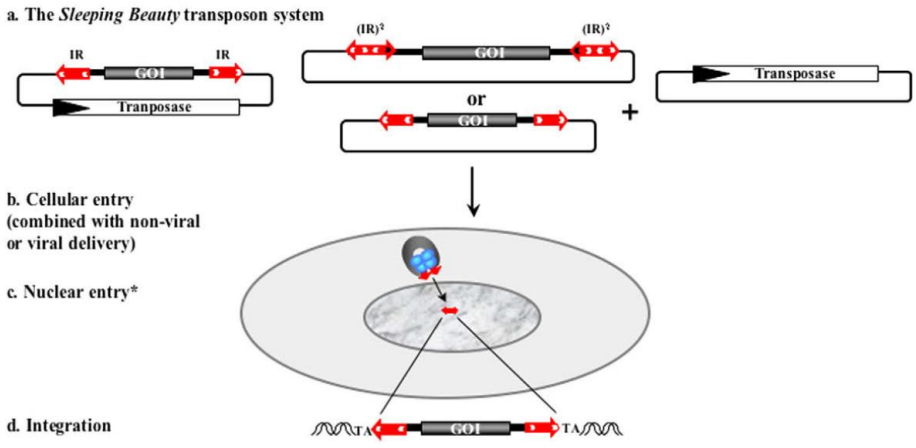
Transposition using *Sleeping Beauty* for gene therapy. The transposase binds IR/DR elements in the transposon and mediates recombination at TA dinucleotides in the genome.



The Sleeping Beauty transposable element and its transposition. (A) Components of the element. On top, a schematic drawing of the transposon is shown. The element has a single gene encoding the transposase, which is flanked by terminal inverted repeats (IR/DRs, blue arrows), each containing two binding sites for the transposase (small green arrows). A sequence alignment shows the actual sequences of the external and internal transposase binding sites. The transposase has an N-terminal, bipartite, paired-like DNA-binding domain containing a GRRR AT-hook motif, a nuclear localization signal (NLS), and the DDE catalytic domain. (B) Transposition. The transposase gene within the element can be replaced by a therapeutic gene, and the resultant transposon can be maintained in a simple plasmid vector. The transposase is supplied in trans. The transposase binds to its binding sites within the IR/DR repeats and, together with host factors such as HMGB1, forms a synaptic complex, in which the ends of the transposon are paired. The transposon is excised from the donor molecule and integrates into a new location MOLECULAR THERAPY Vol. 9, No. 2,



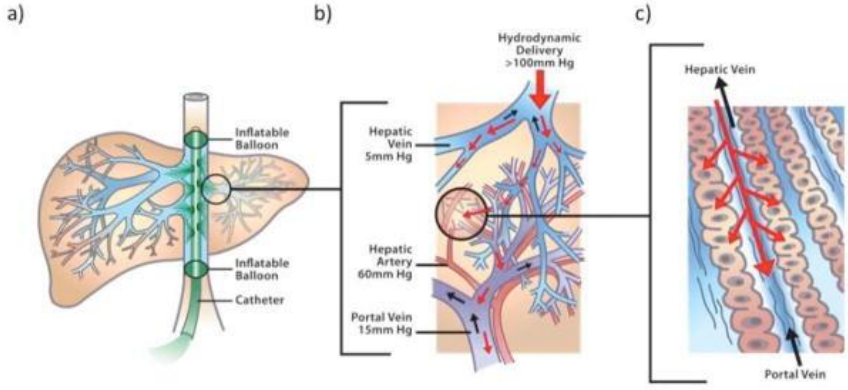
Delivery of the Sleeping Beauty system into cells. (A) Two-component system, in which the transposon containing a therapeutic gene and the transposase gene are maintained on separate plasmids. (B) cis-vector, in which the transposon containing a therapeutic gene and the transposase gene are maintained on the same plasmid. (C) The transposon containing the therapeutic gene is supplied with recombinant transposase protein. Possibly, ''transpososomes'' consisting of transposon DNA precomplexed with transposase can be formulated, but this technology is not established yet. (D) Combination of the transposon plasmids with nonviral gene transfer. The plasmid vectors can be complexed with liposomes, PEI, or any other reagent (green, amorphous objects) used for gene transfer. (E) The components of the transposon system are incorporated into a virus vector. (F) Demonstration of transposition in tissue culture. The transposon containing a selectable antibiotic resistance gene is transfected either with or without a transposase-expressing helper plasmid. Transfected cells are placed under antibiotic selection. The dramatic increase in the number of resistant cell colonies in the presence of transposase is the result of transposition of the element from the plasmid vector into chromosomes.



Trangene delivery by Sleeping Beauty (SB) transposition.
(a) The Sleeping Beauty transposon system. the transposon and the transposase can be provided on the same molecule (cis configuration, left); or on different molecules (trans configuration, middle and right). GOI is designated for Gene of Interest. (b) Cellular entry. The transposon is a non-viral system, it needs to be combined by either viral or non-viral delivery. The nuclear localization signal (NLS) of the transposase promotes active nuclear entry. The pre-integration complex consists of the cargo flanked by IRs and at least four transposase molecules (blue balls) recognizing the IRs. SB transposon integrates into TA-dinucleotides, otherwise the integration profile is fairly random. The targeted TA is duplicated at the site of integration, framing the de novo integration. The cleavage and integration reactions are concerted and precise, no deletions or rearrangements of the cargo occur. Stable integration of the integration cassette into a chromosome result in long-term expression of the GOI.

TABLE 2. Summary of gene therapeutic approaches using *Sleeping Beauty*

Approach	Vector	Cell type	Method of delivery	Transferred gene	Model	Special features
Nonviral	Plasmid	Hepatocyte/ mouse	Hydrodynamic tail-vein injection	*lacZ* *α1-antitrypsin* *factor IX*	Wild type Wild type Hemophilia B	5–6% integration, therapeutic-level long-term expression
Nonviral (*ex vivo*)	Plasmid	Keratinocyte/ human patients	Primary cell transfection/ selection	*LAMB3*	Junctional epidermolysis bullosa	Therapeutic-level long-term expression
Nonviral	Plasmid	Hepatocyte/ mouse	Hydrodynamic tail-vein injection	*FAH*	Tyrosinemia I	4% integration, long-term expression
Nonviral	Plasmid	Lung/ mouse	PEI/hydrodynamic tail-vein injection	*luciferase*	Wild type	2–3% integration, long-term expression
Viral	Adenovirus/ transposon hybrid	Hepatocyte/ mouse	Tail-vein injection/ infection	*lacZ* *factor IX*	Wild type Hemophilia B	Therapeutic-level long-term expression



Hydrodynamic delivery of transposon DNA to liver.

Table 1. Examples of successful DNA transposon-based gene delivery in disease models.

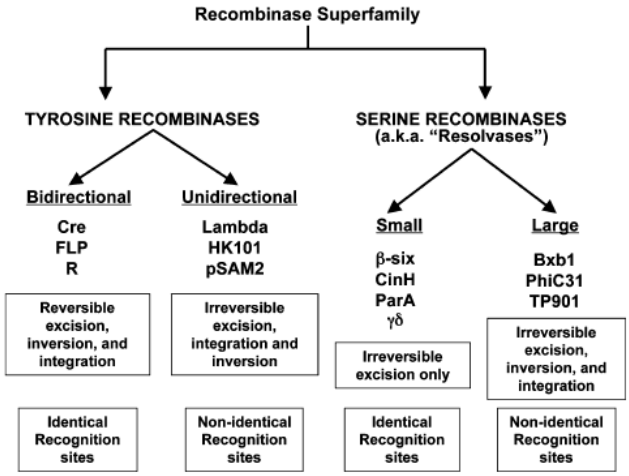
Disease	Delivered gene	Model organism or cell type	DNA-transposon used	References
Hemophilia A	Human clotting factor VIII	Mouse	*Sleeping Beauty*	[60-62]
Hemophilia B	Human clotting factor IX	Mouse	*Sleeping Beauty*	[57]
Inherited type I tyrosinemia	hFAH[a]	Mouse	*Sleeping Beauty*	[58]
Glioblastoma	sEGF-R[b] and AE-FP[c]	Mouse	*Sleeping Beauty*	[69]
Junctional epidermolysis bullosa	hLAMB-3[d]	Human patient derived cells	*Sleeping Beauty*	[59]
Pulmonary hypertension	eNOS[e]	rat	*Sleeping Beauty*	[97]
Lung allograft fibrosis	hIDO[f]	rat	*Sleeping Beauty*	[63]
Mucopolysaccharidosis, type I and VII	hIDUA[g] and/or hGUSB[h]	Mouse	*Sleeping Beauty*	[64,65]
Crigler-Najjar syndrome, type I	hUGT1A[i]	rat	*Sleeping Beauty*	[66]
Ovarian cancer	suicide gene[j]	Mouse ovarian tumor	*PiggyBac*	[70]
Ovarian cancer	suicide gene[j]	Human ovarian adenocarcinoma cell line	*PiggyBac*	[72]
B-lymphoid malignancies	CAR[k]	Mouse primary T cells	*Sleeping Beauty*	[77]
B-lymphoid malignancies	CAR[k]	Primary human T cells/mouse tumor	*Sleeping Beauty*	[98]
Solid tumor (murine xenograft of)	HER2[l]-CAR[k]	Human peripheral blood mono-nuclear cells	*PiggyBac*	[71]
Fanconi anemia, type C	FANC-C[m]	Human lymphoblastoid cells	*Sleeping Beauty*	[67]
Skin inflammation	Human β1 and α2 integrin	Pig	*Sleeping Beauty*	[99]
Osteosarcoma lung metastase	CAR[k] and IL-11Rα[n]	T cells/mouse tissue	*Sleeping Beauty*	[100]
Pulmonary fibrosis	miR-29[o]	Mouse lung tissue	*Sleeping Beauty*	[101]

a. hFAH, human fumaryl acetoacetate hydroxylase. b. sEGF-R, soluble endothelium growth factor receptor. c. AE-FP, angiotensin-endothelin fusion protein. d. hLAMB-3, human laminin-β3. e. eNOS, endothelial nitric oxide synthase. f. hIDO, human indoleamine-2-3-dioxygenase. g. hIDUA, human α-L-iduronase. h. hGUSB, human β-glucuronidase. i. hUGT1A, uridinediphosphoglucuronate glucuronosyl transferase-1A1. j. Herpes simplex thymidine kinase. k. CAR, chimeric antigen receptor. l. HER2, human epidermal growth factor 2. m. FANC-C, Fanconi anemia gene C. n. IL-11Rα, interleukin-11 receptor α; o. miR-29, microRNA-29.

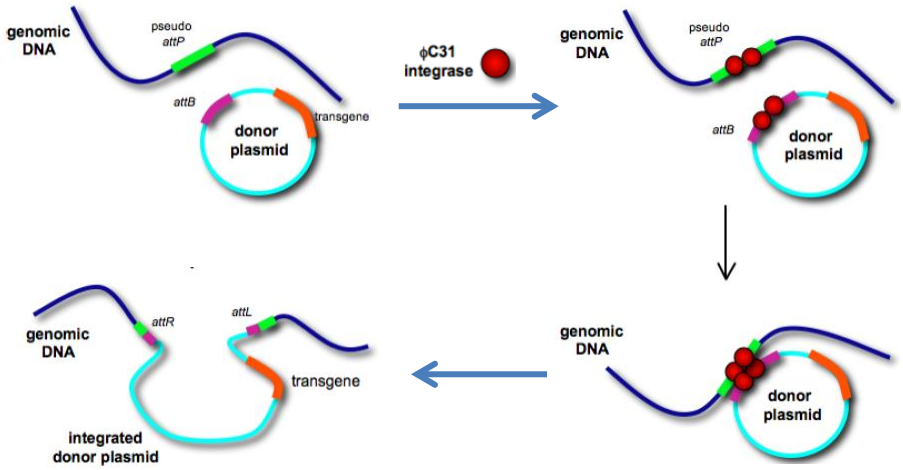
Integrase Systems

- An integrase is a recombinase enzyme that is capable of integrating DNA, usually that of a virus, into another piece of DNA, i.e the host chromosome
- Bacteriophage DNA can be integrated into the host chromosome by an integrase.

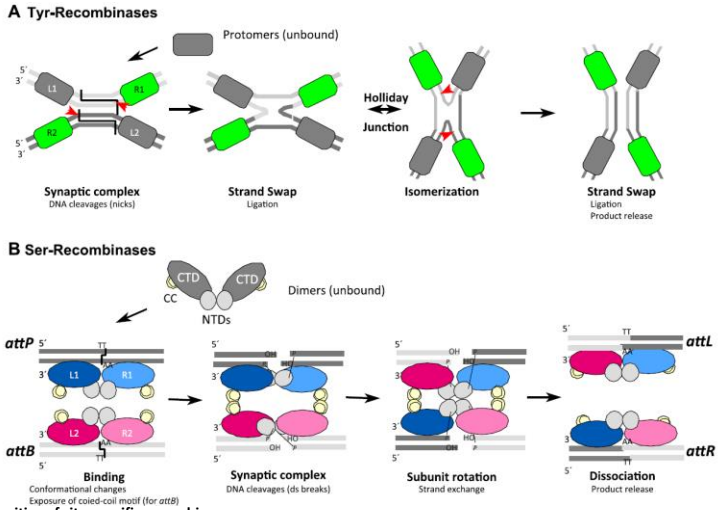
- two categories of recombinases →dependent on the amino acid responsible for cleaving the DNA, either a tyrosine or serine residue



- Integrases recombine two distinct sequences to create hybrid sites that can no longer be recognized and recombined by the integrase, making integration irreversible.
- *φ*C31 integrase → most favorable properties for gene therapy.



Integration using *φ*C31 integrase for gene therapy. The integrase binds to both the *attB* sequence supplied on the donor plasmid and native pseudo *attP* sequences in the genome, then recombines the DNA to integrate the transgene permanently.
The DNA is cleaved at a core sequence in the *att* sites, and the DNA-integrase complex rotates to form the recombination products. The resulting hybrid *att* sites are termed *attL* and *attR*, because they are found to the left (5) and right (3) of the phage genome.
Wild-type *φ*C31 integrase cannot perform the excision reaction between *attL* and *attR* → integration is irreversible



Mechanistic particularities of site-specific recombinases

A) Tyrosine recombinase protomers form dimers only after associating with the left- and right-hand arms of the participating recognition target sites, here, minimal sites with two 13-bp inverted repeats surrounding the 8-bp spacer. Guided by interprotomer contacts, association of two 34-bp sites occurs in an antiparallel fashion, yielding the synaptic complex. SSBs are introduced into the 5 margin of the spacers at either 8-bp (Flp) or 6-bp (Cre) distances.

B) Serine recombinases recombine two educt-recognition sites, *att*P and *att*B. Each of these is characterized by two different protomer-binding arms (L and R) surrounding the 2-bp crossover region. Although not strictly palindromic, these arms resemble inverted repeats. Formation of recombinase dimers is dominated by NTD contacts before synapsis. Synapsis occurs *via* coiled-coil (CC) motifs residing in the CTDs. Within the tetrameric synaptosome complex synchronous staggered DSBs are induced, followed by strand exchange. This is achieved by subunit rotation to induce new interarm (L1:R2) contacts for protomers that remain bound to dsDNA portions. Product sites are *att*L (structure L-O-L) and *att*R (structure R-O-R).

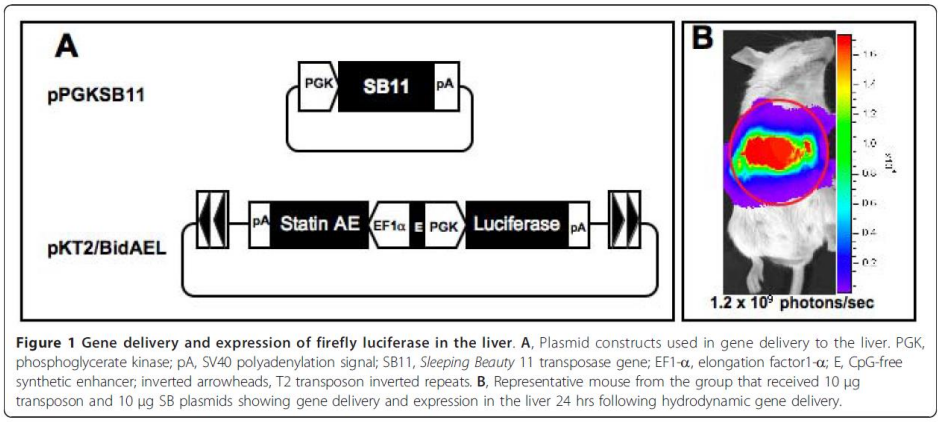


Figure 1 Gene delivery and expression of firefly luciferase in the liver. **A**, Plasmid constructs used in gene delivery to the liver. PGK, phosphoglycerate kinase; pA, SV40 polyadenylation signal; SB11, *Sleeping Beauty* 11 transposase gene; EF1-α, elongation factor1-α; E, CpG-free synthetic enhancer; inverted arrowheads, T2 transposon inverted repeats. **B**, Representative mouse from the group that received 10 μg transposon and 10 μg SB plasmids showing gene delivery and expression in the liver 24 hrs following hydrodynamic gene delivery.

Belur et al. Molecular Cancer 2011, 10:14
Inhibition of angiogenesis and suppression of colorectal cancer metastatic to the liver using the Sleeping Beauty Transposon System

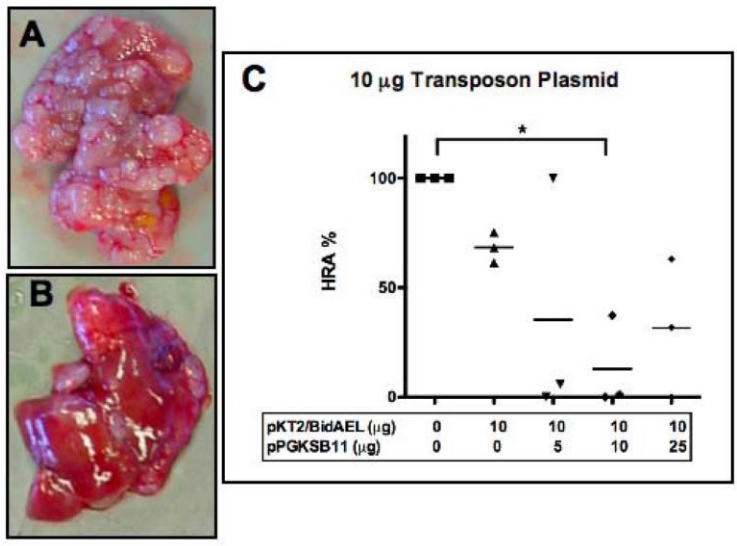


Figure 5 Effects of transposon antiangiogenic therapy on macroscopic appearance and tumor hepatic replacement area (HRA) of resected livers. Livers from sacrificed animals were evaluated macroscopically for clinical appearance, and histologically, for replacement of hepatic tissue by tumor tissue. Transposon (pKT2/BidAEL), and transposase (pPGKSB11) plasmid DNA amounts infused into animals are summarized below the graph. **A**, Liver from untreated tumor bearing control animal; **B**, Liver from animal treated with 10 μg low dose transposon + 25 μg SB transposase; **C**, % HRA in cohorts of animals treated with low dose of transposon plasmid (bar indicates mean of n = 3 per group). *P < 0.05.