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MASTERARBEIT

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„Untersuchung der Transposon vermittelten Transgenese unter Verwendung von Bacterial Artificial Chromosomes (BAC) in der Maus“

verfasst von

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Abstract

Transgenesis is a cornerstone of biomedical research. The alteration and manipulation of the genome is essential for understanding biological mechanisms and exploring medical aberrations. In the last few decades, different methods were developed to generate transgenic animals, where the focus was on designing a technique for efficient and simple transgenesis, which could be applied to a variety of model systems.

In contrast to classical techniques such as pronuclear injection and lentiviral transfection of early embryos, transposon mediated transgenesis using bacterial artificial chromosomes (BAC) represents a relatively new method to generate transgenic founders. BAC transgenesis has many advantages over classical approaches, like full-length integration, reduced position effect variation and a decreased gene silencing. However, the efficiency of BAC transgenesis is low and requires the development of new techniques. A possible approach is BAC transgenesis mediated by transposases, which has been shown to be successful in mice, zebrafish and embryonic stem cells. BAC plasmids of up to 160kb have been successfully transposed and the limit of cargo capacity is still undetermined.

The first aim of this study was to find out if the hyperactive transposase *Sleeping Beauty100X* is able to integrate a large BAC (over 200kb) in mice. Therefore, a ~220kb plasmid was generated by recombineering using the BAC RP23-190D1 (backbone vector: pBACe3.6). The target gene cassette is flanked by two ITR sites, suitable for transposition and includes a diphtheria toxin receptor (DTR), an internal ribosome entry site (IRES) and the red fluorescent reporter gene tdTomato. The expression of the target cassette is controlled by the regulatory elements of the mouse S100a4 gene. Pups were analyzed by fluorescence screening and PCR genotyping. However, no transgene positive mice could be generated in this study.

The second aim of this study was to compare the capability and efficiency of cytoplasmic with pronuclear microinjection in the *SB100X* transposon system. A 6.3kb Venus plasmid, which encodes for a green fluorescence protein was co-injected with *SB100X* mRNA into the cytoplasm of murine zygotes and compared to a previous study where the same mixture was injected into the pronucleus. The pronuclear injection resulted in 62.3% transgenic offspring, whereas only 31.1% of the cytoplasmic injected animals generated in this study were transgenic. Integration sites of the transposon were identified by linker-mediated PCR followed by sequencing.

1. Introduction

The generation of genetically modified animals has become a key tool in molecular biology and is a driving force for basic and applied research. The attempt to understand how modification of genetic information alters the phenotype of an organism dates back to Gregor Mendel. However, the modification of genetic material is associated with a variety of different problems and still remains inefficient.

A variety of different methods for the modification of genetic material in different organisms have been developed during the last century and range from easily achievable chemical mutagenesis associated with random genetic alterations, to sophisticated stem cell methods, where an exogenous gene is inserted or an endogenous gene is modified or removed.

The aim of genetic modification is the stable expression of an altered or inserted gene in a global or cell type specific way and the germline transmission of this genetic modification. The integration of the genetic modification in cells belonging to the germline, is the precondition for heritability of the mutation or the exogenous gene. Therefore, the genetic modification has to be performed at a very early developmental stage to ensure that every cell, especially cells of the future reproductive tissue, harbors this modification.[2]

Therefore, the choice of method for transgenic engineering is determined by the complexity of the used model organism as well as the ability to modify embryonic tissue, which is the limiting factor to date.[3]

The genome of *Mus musculus*, the house mouse, has over 95% similarity with the human genome. In addition, mice are also characterized by significant anatomical, histological and physiological similarity with humans. Therefore, mice represent an excellent model to study the regulation and function of mammalian genes and to identify disease specific targets to develop novel therapeutic approaches.[4]

The relatively short developmental time of 6-8 weeks from birth to sexual maturity, comparatively low husbandry costs (0.50-0.70 EUR/day), the availability of different well-established and commonly used technologies and disease models as well as the relatively easy manipulation has made *M. musculus* the most commonly used model organism in biomedical research worldwide.[5] The mouse genome was first successfully genetically manipulated more than 30 years ago. Retroviral transduction and microinjection of short DNA constructs into pronuclei of fertilized oocytes were initially used to introduce foreign DNA into early embryos.

The rate of transgene animals was extremely low due to degradation or silencing of the inserted transgene.[6, 7]

A technical breakthrough was the establishment of lentivirus vectors as gene transfer vehicles. Lentiviruses are advantageous over retroviruses for a number of reasons: Retroviruses are flanked by viral long-terminal-repeats (LTR), which are recognized and subsequently silenced by host factors, leading to suppressed expression of the transgene. Lentiviruses have modified LTRs and are therefore less prone to silencing events. Furthermore, in contrast to retroviruses, lentiviruses are cell cycle independent, and can also infect cells that are not actively replicating at the time of infection.[8]

Another milestone in transgenesis was the development of embryonic stem cell (ESC) technologies for gene targeting via homologous recombination. Using this technique a part of a gene locus harboring the target (for example an exon) is replaced by electroporated DNA, which is flanked by homology arms at the 5' and 3' end. ES cells carrying correctly targeted mutations as confirmed by PCR and Southern blotting technologies are then injected into host blastocysts. The resulting chimeric animals are mated to obtain mice with germline integration.[9] In addition to replacement of endogenous genes, predictable and constitutive expression of an exogenous transgene is possible by homologous recombination into the well characterized ROSA26 locus, which was first isolated by Friedrich et al in 1991.[10]

Presently, the two most commonly used methods to generate transgenic animals with random integrations are pronuclear microinjection and lentiviral transfection. However, both methods have their advantages as well as limitations as outlined and discussed below.

1.1 Generation of transgenic animals using pronuclear microinjection (PNI)

PNI was the first successful method to produce transgenic animals. DNA is injected into the pro-nucleus of fertilized oocytes, where it randomly integrates via non-homologous end-joining into the host genome. Depending on the size of the transgene, every zygote receives ten to one hundred copies of the plasmid. In many model organisms the male pronuclei are typically used due to their larger size and better visibility. In mice the male pro-nucleus is visible five to seven hours after the entry of the sperm and disappears before the first cell division approximately 30 hours after fertilization.[11, 12, 13, 14]

However, pronuclear microinjection can only be performed in mammals because in

lower vertebrates and invertebrates the pronucleus is not visible.[15] However, transgenesis can also be performed in these organisms through injection of DNA into the cytoplasm of embryos, which is less difficult and invasive than targeting the pronucleus. Cytoplasmic microinjection can also be successful in mammals such as cows and swine, where pronuclear injection is not feasible due to the opaque cytoplasm.[16, 17]

Classical microinjection requires specialized equipment and trained personal staff, but is otherwise easy to perform and is associated with founder frequencies ranging from 15-25%.[14] The main problems of this method are genomic disruptions, low integration frequency and transgene silencing. The random integration of the transgene into the mouse genome results in a highly variable expression pattern of the transgene between founders due to the integration loci. Additionally the expression pattern can vary within one organism, due to heterochromatization of the integration site in different tissues. This phenomenon is called position effect varigation (PEV), which describes the inactivation of genes due to their location in the genome. PEV is caused by the integration of the transgene into or near a transcriptionally inactive site. It can result in heterochromatization of the transgene according to the surrounding DNA and therefore to its silencing. Random integration can also result in disruption of the host genome via integration within an endogenous gene or within the regulatory elements of that gene. [18]

Another major problem of pronuclear microinjection is concatemeric multicopy integration, which consists of the insertion of up to a hundred copies of the transgene per chromosome in a head-to-tail manner. These concatemeres can induce aberrant splicing, heterochromatin formation and consequent silencing of the transgene. Furthermore, the pairing of non-homologous chromosomes followed by chromosomal rearrangements and missegregation in cytokinesis has severe consequences for the organism.[11, 14]

1.2 Generation of transgenic animals using viral vectors

Lentiviruses belong to the family of retroviruses but have the unique ability to also infect non-proliferating cells and are therefore are considered to be the most efficient gene delivery vectors. For this reason they are commonly used for gene transfer in either hard to transfect cells (e.g. cells of the immune system) or non-proliferating cells (e.g. neural cells).[19]

When lentiviral vectors are used to generate transgenic animals, a recombinant

lentiviral vector is injected into the perivitelline space between the *Zona pellucida* and the cell membrane of the zygote.[20] The viral RNA is reverse transcribed into DNA by the enzyme reverse transcriptase, which was packed together with the viral RNA in the infectious particles. The reverse transcriptase generates a second DNA strand leading to the formation of a double-stranded DNA copy of the viral genome. This DNA double helix is then integrated into the host genome as a defined integration cassette by a virus-encoded enzyme called integrase. This leads to the generation of mice, which may inherit the modification.[21]

Lentiviral transfection has some advantages over pronuclear injection. First, injection of lentiviral particles into the perivitelline space is not as mechanically invasive. Second, this method leads to the integration of a single copy (compared to the concatemeres observed in pronuclear injected transgenes). Third, the integration rate following lentiviral transduction is significantly higher (70-90% compared to about 20% for PNI).[22]

However, this infectious gene transfer system is also associated with a number of problems and limitations. The major limiting factor remains the recognition of viral genetic elements by different host mechanisms. This can either result in transcriptional silencing of the transgene or even viral toxicity induced through activation of an immune response. Furthermore, the cargo capacity of lentiviral vector is limited to ~8kb, restricting the size of the transgene. Another concern is that these systems are genotoxic as a consequence of mutagenic effects caused by the insertion of the transgene into or near host genes. Finally, handling of lentiviral vectors requires a biosafety level II laboratory, which is associated with special safety requirements.[23, 24]

Consequently, there is an interest in alternative methods that are simple to use and result in a high germline transmission rate and a predictable transgene expression pattern. A method which could improve the generation and investigation of transgenic organisms, is transgenesis induced by Bacterial Artificial Chromosomes (BAC) combined with a transposon system.

1.3 Generation of transgenic animals using Bacterial artificial chromosomes (BAC)

The BAC cloning system was developed in 1992 by Shizuya et al. and is based on the fertility plasmid (F plasmid) of *Escherichia coli*. The F plasmid belongs to the class of conjugative plasmids controlling sexual functions of bacteria and is able to carry DNA fragments as large as 1Mb, which makes them suitable for the cloning of very large DNA fragments. In fact, BACs were successfully used to sequence the genome of various species, such as the Human Genome Project or the Mouse Genome Project. As a consequence, there are several libraries of BACs spanning the entire human and mouse genome, which can be used for research purposes.[25] The F plasmid encodes for genes essential for the regulation of replication and the control of copy number. These regulatory genes include *oriS* and *repE*, which mediate the unidirectional replication of the plasmid and *ParA* and *parB*, which maintain the characteristic low copy number of only one to two copies per cell. This reduces the potential risk for recombination or re-arrangement between DNA fragments of the plasmid and ensure the structural maintenance of BACs.

The structure of a BAC is relatively simple and includes a multiple cloning site flanked by phage promoters, like SP6 or T7, and GC-rich sites for rare-cutting restriction enzymes such as intron encoded nuclease PI-SceI. CosN and loxP sites can be used for linearization of the plasmid for mapping techniques. There is also an antibiotic resistance gene, most commonly chloramphenicol acetyltransferase, for positive selection of bacteria carrying the vector **(Figure 1)**.[26]

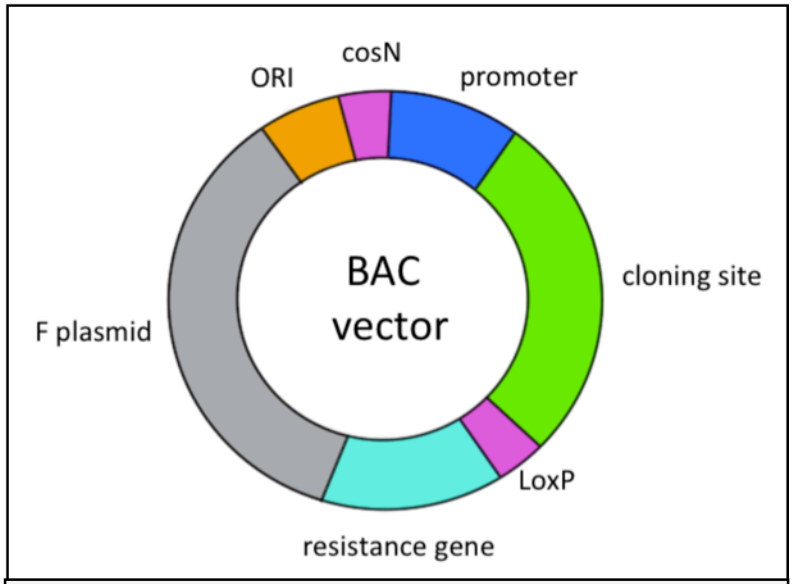

Figure 1: Schematic map of a typical BAC plasmid. A basic BAC vector is composed of a BAC backbone based on the F plasmid, cloning sites, a promoter, different restriction sites, sequences suitable for enzyme mediated linearization (LoxP, cosN) and an origin of replication (ORI).

BACs can hold DNA segments as large as 350kb. Due to their size, BACs are able to contain large parts of chromosomes, which can include several entire genes including all cis-regulatory elements in their native configuration. The availability of the promoter, several enhancers and repressor sequences >100kb upstream and downstream of the transcriptional start site assures that the transgene expression pattern will better mimic the natural expression of the gene. This assures that the binding affinity of regulatory DNA-binding proteins is maintained, which is essential for regulation of gene expression.[27, 28]

Technical progress in recent years has led to the development of methods that allow for the simple manipulation and modification of BACs via recombineering. Recombineering is based on recombinases cloned from phages, which facilitate the integration of a DNA cassette of interest, flanked by short homology arms, via homologous recombination. The challenge of finding a suitable restriction site is thus circumvented.

Generally, the final BAC construct is injected into the pronucleus of embryos and randomly integrates via non-homologous end-joining.

In summary, BAC transgenesis is not prone to the position effects and silencing typical for conventional transgenesis approaches and is therefore characterized by stable integration and physiological expression pattern of transgenes. However, classical BAC transgenesis still suffers from inefficient integration. Therefore, the higly effective transposon system seemed like a promising approach for improving the integration rate of BACs.

1.4 Transposable elements

Transposons, also called transposable elements (TEs), are small pieces of DNA, which can change their position within the genome. They were first described in maize by Barbara McClintock in the 1940ies, and earned her the Nobel Prize in Medicine in 1983. Transposable elements were subsequently identified in almost all prokaryotes and eukaryotes, including higher vertebrates such as mammals and humans. Due to their ability to change their position within the genome (a process called transposition) they are also known as “jumping genes” and “selfish elements”. Their ability to duplicate and rearrange the genome has made TEs a considerable driver of genomic evolution. The duplicated genes can then undergo point mutations and single nucleotide polymorphisms without affecting cellular function, leading to the development of new genes and to genome divergence within species followed by

phylogenetic drift. Therefore, transposons represent a major perpetrator of evolution.[29,30]

The structure of a transposon is simple: it is composed of a DNA sequence, which is flanked by inverted terminal repeats (ITR). The ITRs are recognized by the corresponding transposase, which is the essential mediator of transposition and catalyzes the copy or excision of the transposon and its random reintegration into the genome.[31]

The origin of transposons has been a matter of debate and still remains poorly understood. Some evidence suggests that their historical ancestry might be viral, since viruses and TE share similarities in their genome structure and biochemical properties.[32] Some transposons encode their own transposase enzyme, and hence their transposition, which further supports the theory of a viral origin. These transposons are therefore classified as "autonomous". On the other hand there are also transposons consisting of only non-coding sequences and which rely on transposase activity from other transposons and are termed "non-autonomous".[33, 1]

Generally, transposons can be divided into two classes depending on their mechanism of transposition:

1) Class I transposable elements:

The transposon is transcribed from DNA to RNA by polymerase II, which is then transcribed to cDNA by a reverse transcriptase and inserted at a new position of the genome. This process is also called retrotransposition and results in duplication of the transposable element via *copy and paste transposition.* The reverse transcriptase is often encoded by the transposable element itself. Accordingly, this class of transposons is also termed retrotransposons and share characteristics of retroviruses. Retrotransposons are ubiquitous in many eukaryotic organisms and can be distinguished as autonomous and non-autonomous elements:

- *Non-autonomous retrotransposons* lack a transposase and reverse transcriptase and rely on the transposition machinery of autonomous retroelements for their mobilization, e.g. short interspersed elements (SINEs).[30, 34]

- *Autonomous retrotransposons* encode for their own reverse transcriptase and are further classified as:

  - *TE with Long terminal repeats (LTRs)* are flanked by two LTR sites, which range from 100bp to 5kb. The orientation of the LTRs is crucial for the direction of the transcription

start and integration of the cDNA.

- *TE with non long terminal repeats (non-LTRs*) have an endonuclease related to restriction enzymes that recognizes specific sequences and mediates their re-integration into the genome, e.g. long interspersed nuclear elements (LINEs).[35]

2) Class II transposable elements:

Transposition of these elements is catalyzed by several different transposases, which integrate the transposon at random target sites within the DNA. This class is termed "DNA transposons" referring to *cut and paste transposition* due to the direct transposition of DNA sequences. DNA transposons are further divided into:

- *DDE transposons* are related to integrases and includes a protein domain containing an acidic amino acid motif consisting of two or three aspartic acids (DDE or DDD) that catalyzes the "cut and paste" transposition. They are autonomous transposons and have two inverted terminal repeats (ITRs) flanking the coding sequence for the DDE transposase. The transposase induces cuts with single strand overhangs, which are the ligation sites for the transposon. A DNA polymerase and a DNA ligase are then required for ligation. DDE transposons are typical "cut and paste" transposons and are widespread in vertebrates and several superfamilies, such as Tc1/Mariner, P element and PiggyBac have been discovered.[36]

- *Helitrons* encode a rolling-cycle recombinase, which inserts one strand into the target site and uses it as a template for replication.[29]

- *Polintons/Mavericks* include an integrase and several elements which are functionally similar to double stranded DNA viruses.[30]

- *Non-autonomous DNA transposons* use the enzymatic machinery of autonomous transposons for their transposition e.g. miniature inverted transposable elements (MITE).[29]

The unpredictable and random integration of transposons renders them potentially mutagenic. As a consequence the "host" has developed different strategies for the recognition and silencing of transposons via heterochromatization. DNA transposons constitute about 3% of the human genome and are mostly inactivated by the accumulation of mutations due to "vertical inactivation", the absence of proofreading systems for new mutations in transposons due to to the lack of selection pressure.[37] In contrast, retrotransposons escape silencing mechanisms and remain active. Due to their prevalence of

~40% in the human genome, epigenetic control mechanisms like methylation are necessary for silencing. However, some diseases including hemophilia, severe combined immunodeficiency, and a predisposition to cancer due to the disruption of tumor suppressor genes can be caused by transposons.[30, 38]

Furthermore, autonomous transposons with a highly active promoter can alter the transcription rate of an endogenous gene, if they are integrated in its promoter region.[39]

1.5 Transposon mediated transgenesis

A new strategy is the use of class II DNA transposons where any DNA sequence of interest can be cloned between two ITR sites and mobilized by transposition. The transposase can be provided as an expression plasmid, in vitro synthesized mRNA (non-autonomous system), or as part of the transgene plasmid (autonomous system). However, a bicomponent non-autonomous transposon system provides better control of transposition frequency and activity. The transposase enzyme recognizes the transposon by its ITR sites, where it binds **(Figure2)**. Then it mediates the excision of the target sequence from the donor plasmid and brings the ends of the transposons together. Depending on its type, the transposase then re-integrates the transgene at a specific or non-specific site within the genome of the host.[1,36]

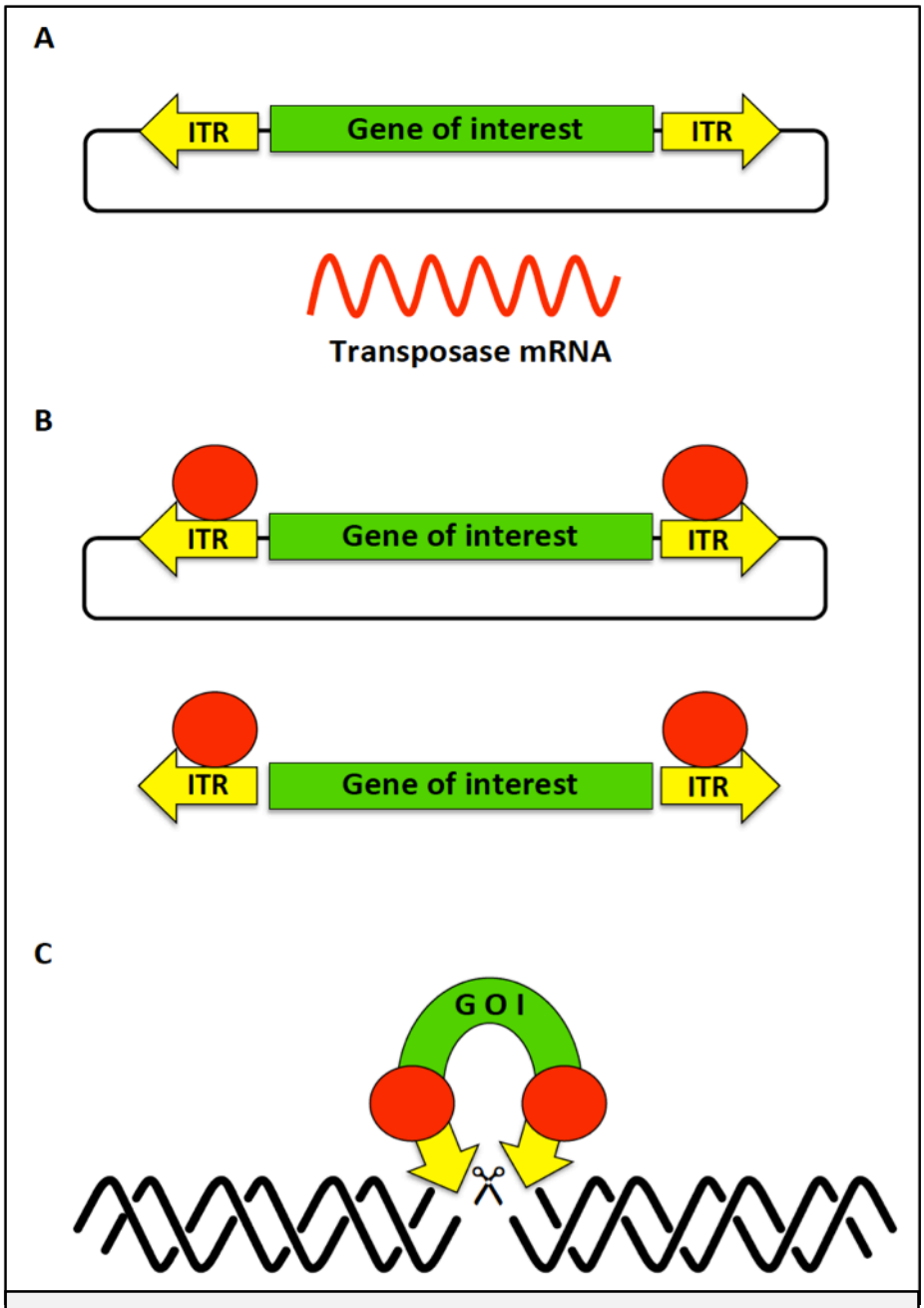

Figure 2: Schematic illustration of transposition. A bicomponent transposable system is composed of a transposon plasmid, which contains the gene of interest flanked by two ITR sites and a mRNA encoding for a transposase (**A**). After microinjection into host cells the transposase recognizes the transposon by its ITR sites and mediates the excision from the plasmid (**B**). Integration of the transposon occurs at a random locus in the host genome (**C**).[1]

Transposon technology was first successfully used in plants and invertebrates for forward genetic screens 25 years ago. Especially in *Caenorhabitis elegans* and *Drosophila melanogaster* transposons have become an important tool in mutagenesis and therefore in basic research of gene function.[33] Although many different organisms have similar transposon

fragments in their genome, successful transposition seems to be species dependent. For example, the P element, the most common transposon in Drosophila, is inactive in other species, indicating that host factors are also required for transposition. However recent evidence suggests that the human P element homolog THAP9 is capable of transposition of P element dependent transposons in Drosophila. These data indicate that transposons of more complex eukaryotic organisms displays conserved activity during evolution and that the use of the transposon system across species is possible.[40]

Another limiting fact is that all isolated DNA transposases in vertebrates with the exception of *Tol2* are inactivated by the accumulation of mutations, which represents the host defense against uncontrollable transposition combined with poor evolutionary pressure.

The development and successful establishment of sleeping beauty (*SB*) in 1997, the first entirely synthetic transposon system, has overcome this restriction. To date, a number of different synthetic transposon systems, which differ in cargo size and preferences for integration site, are available, creating a toolbox of multiple alternative vector systems (see Table 1).[1]

Transposon	Origin	Family	Cargo size	Target site
Minos	*D. hydei*	Tc1	<10kb	TA
Mos1/Mariner	*D. mauritania*	Mariner	<10kb	TA
P element	*D. melanogaster*	P	20 kb	Heterogenic
PiggyBac	*Trichoplusia*	Tc1	>100kb	TTAA
Sleeping beauty	*Teleost fish*	Tc1	>100kb	TA
Tc1	*C. elegans*	Tc1	<10kb	TA
Tol2	*Medaka*	hAT	>100kb	Heterogenic

Table 1: The most frequently used transposon systems. The main characteristics of the most commonly used transposon systems are listed in this table. *Origin* describes the species in which the transposase was discovered and first isolated. *Cargo size* indicates the maximal amount of DNA transposed so far. *Target site* represents the preferred sequence in the host genome for the integration of the transposon.[33]

In transposons belonging to the Tc1/mariner family the efficiency of transposition decreases exponentially with increasing cargo size. In contrast, no limit regarding the cargo size was observed using P elements so far.[41]

The integration pattern of commonly used transposons is variable, but is not random

and displays some characteristic preferences for insertion sites. The *piggyBac* transposon preferentially targets TTAA sequences, with a bias to integrate into transcription units, mainly transcriptional start sites. This characteristic of *piggyBac* was used in in forward genetic screens.[42]

Tol2, the only active cut-and-paste transposase in vertebrates, and the *P element*, have a preference for 5´ regulatory sequences and typically integrate into transcriptional start sites.[43] This characteristic renders both transposons eligible for enhancer trapping. *Minos* and *Mos1* seem to have no preference for active sites of a gene and predominantly cause transposition into introns. *Sleeping Beauty* and other Tc1/mariner members display a preference for TA motives but typically avoid 5' regulatory regions and have a minor preference for introns.[33, 44]

Since the preference for integration site greatly influences safety and efficiency of a transposon vector, the choice of the suitable transposon system is essential in respect to the scientific effort.

Previous studies have documented the wide applicability of transposon-based vector systems including the transposition of fluorescent reporter genes, small hairpin RNA expression cassettes and gene therapy. In addition to their multiple applications, transposon mediated transgenesis benefits from many other advantages. Similar to viral vectors the transposon system leads to permanent and efficient genomic integration of transgenes with continuous expression. In contrast to viral vectors, transposons can be easily cloned as DNA plasmids, rendering their production simple and cheap. Also the cargo capacity of transposons is greater than that of viruses. In fact, the limit in cargo size is not yet fully examined. Manipulation and adaption to different scientific questions is simply achieved by exchanging of the target cassette between the two ITR sites. Additionally the ITR sites display lower enhancer/promoter activity than the LTRs of retrotransposons, and hence do not disrupt the expression of the target gene.[38]

Transposons are not recognized by the immune system and therefore are not prone to toxicity mediated by the immune system or silencing by other host mechanisms.

Furthermore, the design of transposon vectors with cell type-specific promoters allows for genetic manipulation of individual cell types. This includes overexpression as well as conditional deletion of genes of interest using the Cre-lox system. Additionally, cell-type specific expression of the diphtheria toxin receptor encoded by the human hbEGF gene allows

for ablation of specific cells in vivo, thus revealing their function. Following binding of diphtheria toxin (DT) to its receptor, the DT-hbEGF complex translocate to the cytoplasm where it causes ADP-ribosylation of the eukaryotic elongation factor 2 (eEF2) resulting in inhibition of protein synthesis and apoptosis. Mouse and human/ simian hbEGF display 80% amino acid sequence identity. However, mice do not express a functional receptor for DT because the EGF-like domain is significantly different from human/simian hbEGF. This offers the possibility to ablate specific cells in mice by expressing the human/simian hbEGF under the control of a cell type specific promoter.[45]

Nevertheless, transposon mediated transgenesis is associated with some risks. The main problem is the dosage of the transposases, which can lead to multiple integrations. This can be prevented by the cloning of non-autonomous vector systems where the transposases are not integrated into the transgene plasmid but added as mRNA and or as an individual plasmid. The ratio between the target plasmid and transposase is critical for successful transposition.[31] Accordingly the degradation of transposase mRNA by RNAses and the exceeding transcription of the plasmid can interfere with the functionality of this system. Generally the amount of co-injected transposase mRNA needs to be adjusted depending on the type of transposase and the amount of the target plasmid. Another risk in transposon-mediated transgenesis is the risk of mobilization of cryptic transposons within the host genome.[1, 46]

A methodological pitfall for successful transgenesis during pronuclear injection is the degradation of large constructs like BACs due to the shearing forces during the injection procedure.[23] Recent studies have indicated that cytoplasmic injection is an alternative to pronuclear microinjection. Pronuclear microinjection is almost impossible in larger animals like cows, sheep and swine due to the high concentration of colored lipids, which cover the pronucleus. It was recently reported that cytoplasmic microinjection of plasmids is capable of efficient transposition in the zygotes of these animals. It was shown that 50–60% of the bovine blastocysts expressed a fluorescent marker gene, which was delivered by cytoplasmic injection of a 220kb BAC plasmid. The plasmid is able to migrate into the nucleus where it integrates into the genome.[17] In another study cytoplasmic injection of the *SB100X* transposon system and a Venus carrying plasmid in swine zygotes resulted in 42% transgene piglets.[16]

At present it remains elusive if cytoplasmic microinjection of SB transposase is also suitable for transgenesis of BAC plasmids in mice.

1.6 The transposase Sleeping Beauty

In 1997 Ivics and colleagues engineered a synthetic transposase based on transposon remnants found in eight different teleost fish genomes, and named it Sleeping beauty (*SB*).[47] Sleeping beauty is based on the Tc1/mariner transposon superfamily, which is conserved in evolution and can be found in vertebrate but also invertebrate cells, making this transposon system suitable for the manipulation of many different genomes. The *SB* system consists of a target gene plasmid flanked by two ITR sites, and an mRNA encoding for the transposase. It represents a non-autonomous DNA transposon system, which can be easily experimentally controlled by the separation of the two functional components. Since *SB* was synthetically engineered it lacks silencing problems and limitations in the targeted host. The absence of sequence similarity also decreases the risk of mobilizing endogenous transposons within the host genome. *SB* shows a preference for the integration into TA dinucleotide sequences and does not display a genomic bias with respect to insertions into genes or intergenic regions. Furthermore, it also avoids transcription units, 5' regulatory regions and exons.[33] Recent evidence suggests that target selection is rather defined by the chromatin structure itself and not by its sequence.[48]

To increase the transposase activity Mates et al subjected *SB* to high-throughput PCR-based DNA-shuffling and generated a variant with 100-fold increased insertion potency than the original enzyme, which was named *SB100X*. This increased activity was confirmed using over 2000 mammalian gene variants. *SB100X* has been successfully used for efficient gene transfer in commonly used vertebrate model systems such as mouse and zebrafish, xenopus and also human cells.[49, 50] *SB100X* leads to a stable gene transfer in ~50% of cells, including troublesome cell types such as human hematopoietic stem cells (hEHEC) and progenitor cells.[51, 52] Furthermore, the stable integration of a target gene could be increased from 15-20% up to 45% in mice by pronuclear microinjection into zygotes.[46] Thus *SB100X* represents a highly efficient, non-viral, transposon-based gene delivery system suitable for functional genomics and gene therapy, whereby the restrictions regarding the cargo size of SB100X still remains elusive.[53]

1.7 Transposon mediated transgenesis using BAC

Bacterial artificial chromosomes (BACs) are large transgenes of up to 350kb harboring several cis-regulatory sequences and avoid the typical problems of plasmids including position effect varigation, concatemeres and problems associated with integration of multiple copies on different chromosomes.

The majority of naturally occurring transposons are <2kb in size and it was reported that increasing cargo size is associated with a reduction in frequency of transposition. However, successful transposition of large transgenes of up to 60kb were reported using different transposon systems in in zebrafish, mice, human ES cells and also in generation of induced pluripotent stem cells (iPSC).[54, 55, 23, 56]

It has been shown that BAC transgenesis via transposition has some considerable advantages. After pronuclear microinjection the circular plasmid has to be opened by fragmentation in order to integrate into the host genome. Although the circular plasmid is the more stable conformation, the breakage is uncontrolled and may result in degradation of the plasmid. Accordingly, the BAC plasmid is linearized by restriction enzymes before the injection, which makes it prone to sharing.[48]

These problems can be circumvented by the insertion of ITR sites in circular BAC, which serve as defined restriction sites and increase the probability of successful integration of the entire transgene. The ITR sites also determine the recognition sites for the transposase and lead to a single copy integration, which ensures a physiological expression of the transgene. Furthermore, the formation of concatemeres is prevented and the integration site of the transgene can easily be detected by transposon display via linker-mediated PCR.[48] Consequently, it can be evaluated where the transgene integrated and if an endogenous gene was disrupted, what is essential for the selection of an operative founder line.

Some transposon systems seem to be more suitable to transfer larger cargos than others. *Tol2* was successfully used for transposition of BAC plasmids containing 120kb DNA in zebrafish and 66kb in mice and *piggyBac* was shown to transpose BAC of 150kb size in murine zygotes.[48, 54]

PiggyBac was also used for transposon-mediated transgenesis of a 161kb BAC in human embryonic stem cells (hESC) which are known to be difficult to transfect showing that *piggyBac* transposases works efficiently in human cell lines.[55, 57]

Our laboratory has previously demonstrated that SB causes efficient transposition of a

6.3kb plasmid containing a fluorescent marker protein (Venus) with 62.3% of pups having a stable integration of the transposon.[58]

In the present study, we engineered a ~220kb sized BAC to investigate the efficiency of the Sleeping Beauty system for transposition of large plasmids.

2. Materials and Methods

2.1 Materials

All appliances, enzymes, kits, oligo nucleotides and software packages used in experiments at the Institute for Laboratory Animal Science and Biomodels Austria, University for Veterinary Medicine and the Institute for Pharmacology of the Medical University of Vienna are summarized in the appendix. Recipes for all buffers, reagents and stocks are listed there in detail.

2.1.1 Animals

Female C57BL/6N mice were used to isolate zygotes for microinjection to generate transgenic animals. Eight weeks old mice were super ovulated by i.p. injection of 5.0 IU PMSG (pregnant mare serum gonadotropin) at 10:00 a.m. followed by injection of 5.0 IU hCG (human chorionic gonadotropin) 46-48 hours later. Mice were then mated with males of the same strain and mating was checked by plug control.

Zygotes injected with plasmid were transferred into the right horn of the uterus pseudo-pregnant surrogate CD1 females.

All animals were housed under specific pathogen-free conditions (SPF) at the mouse facility of the Institute for Laboratory Animal Science and Biomodels Austria, University for Veterinary Medicine, Vienna.

Animals were free of bacterial, viral, and parasitic pathogens listed in the Federation of European Laboratory Animal Science Associations recommendations.[59]

Up to ten female donor mice were housed in groups in Makrolon cages Type III and male mice were housed individually in Makrolon cages Type II. Pregnant females were also housed separately under SPF conditions in Type II cages. Pups were weaned at the age of 3 weeks. Cages were cleaned once a week. Sterile Pur-Zellin and a carton lodge were provided as nesting material. Mice were maintained on a 12 hour light cycle (06:00 am to 06:00 pm) with a light source of 200 lux at a height of 3,5 meters. Room air was changed 8 times per hour. The humidity ranged between 55 % and 65 %, the temperature was fluctuating between 19.5 °C and 22 °C. All mice were supplied with untreated water and fed with breeding diet ssniff® M-Z Extrudat ad libitum.

2.2. Methods

2.2.1 Principals of BAC recombineering

Recombineering of BAC follows a uniform working course, whereby the working steps are repeated until all desired target cassettes are integrated into the BAC:[60]

1. Inoculation: *E.coli* carrying BAC are enriched by culture in lysogeny broth media (LB) with constant shaking at 37°C.

2. Preparation of electrocompetent cells: The membranes of bacteria have to be transiently permeable for the uptake of nucleic acids and modification of plasmid DNA. For this purpose bacteria have to be in a state of competence, which is a time-limited response to an environmental condition like starvation or cell density. Bacteria cell growth is monitored by photometry and cells have to be isolated at a state of logarithmic growth, which is the case when the optical density at 600nm (OD_{600}) has reached a value of ~0.5. At this point cells are immediately put on ice to stabilize the membranes and prevent cells from further proliferation. Pelleted cells are finally washed with ice-cold 10% glycerol to reinforce the effect on membranes. Bottles, cuvettes and solutions have to be chilled at 4°C to maintain the electrocompetence of bacterial cells.

3. Preparation of the target plasmid: The target plasmid has to be linearized to assure efficient integration into the BAC by recombineering. In addition it prevents replacement of the BAC by the considerably smaller circular plasmid, which contains the target sequence. Suitable restriction sites are identified using the program “A plasmid Editor” (ApE). The plasmid is then digested by these restriction enzymes and the DNA fragment is separated by agarose gel electrophoresis.

4. Electroporation: Electrocompetent cells and the linearized target plasmid are placed in a cuvette and chilled on ice. An electroporation device delivers a pulse of electric current which transiently changes the permeability of bacterial cell membranes allowing the linearized plasmid to enter. The combination of voltage and pulse length depends on the density and strain of bacteria as well as on the size and concentration of the plasmid in the

electroporation mix. A 1mm standard cuvette, a pulse length of 5.8 milliseconds and a voltage of 2.5kV resulted in efficient transformation of cells.

5. Hit check: Every plasmid contains a specific antibiotic resistance gene. Bacterial cells are cultivated on agar plates and in growth media containing specific antibiotics in order to enrich for cells with correct integration of the target plasmid.

2.2.2 Rationale and design of a DTR-tdTomato expressing BAC suitable for transposon mediated transgenesis

Preparation of the BAC was performed at the Institute of Pharmacology at the Medical University of Vienna and the Ludwig Boltzmann Institute for Cancer Research with the kind support of Dr. Christoph Österreicher and Dr. Emilio Casanova.

We decided to generate a novel BAC transgenic mouse line in which the expression of the diphtheria toxin receptor (DTR) encoded by the simian heparin-binding EGF-like growth factor (hbEGF) and a red fluorescent reporter gene is controlled by the regulatory elements of the mouse S100a4 gene. The BAC RP23-190D1 (backbone vector: pBACe3.6) was chosen for this purpose based on a BLAST search. The BAC RP23-190D1 harbors all exons as well as 100kb of the 5' and 3' flanking regions of the *S100a4* gene and was purchased from the Children Hospital Oakland Research Institute. A cDNA encoding the simian hbEGF, kindly provided by Richard A. Lang, Cincinnati Children's Hospital, followed by an Internal Ribosome Entry Site (IRES) sequence and tdTomato cDNA (kindly provided by Roger Tsien from the University of California San Diego), encoding for a red fluorescent reporter protein, were inserted at the translational start by homologues recombination/ET cloning.

Successful transgenesis should result in detectable red fluorescence due to the expression of S100a4 and hence to the tdTomato reporter gene in the skin.

The pIntron poly A plasmid described by Casanova was used for recombineering.[61] First, simian hbEGF and tdTomato separated by an IRES sequence were cloned into this vector using conventional PCR cloning techniques. 5' and 3' homology arms corresponding to ~200bp up- and downstream of the translational start of the S100a4 gene (located in exon 2) were added. The pIntron poly A plasmid also contains a FRT flanked ampicillin cassette located between the homology arms, which is used for positive selection of correctly recombined

clones. Finally a linearized kanamycin cassette flanked by ITR sites was inserted in the backbone by recombineering finally resulting in a ~220kb sized BAC.

Two types of helper plasmids are required for recombineering. The pSC101αβγ plasmid encodes for a recombinase enzyme, which catalyze recombination by DNA exchange of homologous sequences. The other helper plasmid is the FLP recombinase, which recognizes short flippase recognition target (FRT) sites (34bp). Depending on the orientation of the FRT sites FLP mediates either reverse orientation or excision of the FRT flanked sequence. FLP was used for excision of an FRT flanked ampicillin resistance gene used for positive selection of correctly recombined BAC plasmids. Although the pSC101αβγ recombinase is essential for the recombination of homologue sequences and the FLP recombinase catalyze the crucial excision of an obsolete antibiotic resistance genes, both plasmids have to be inactivated after fulfilling their function. This is accomplished by the temperature sensitivity of both plasmids (**Figure 3**).

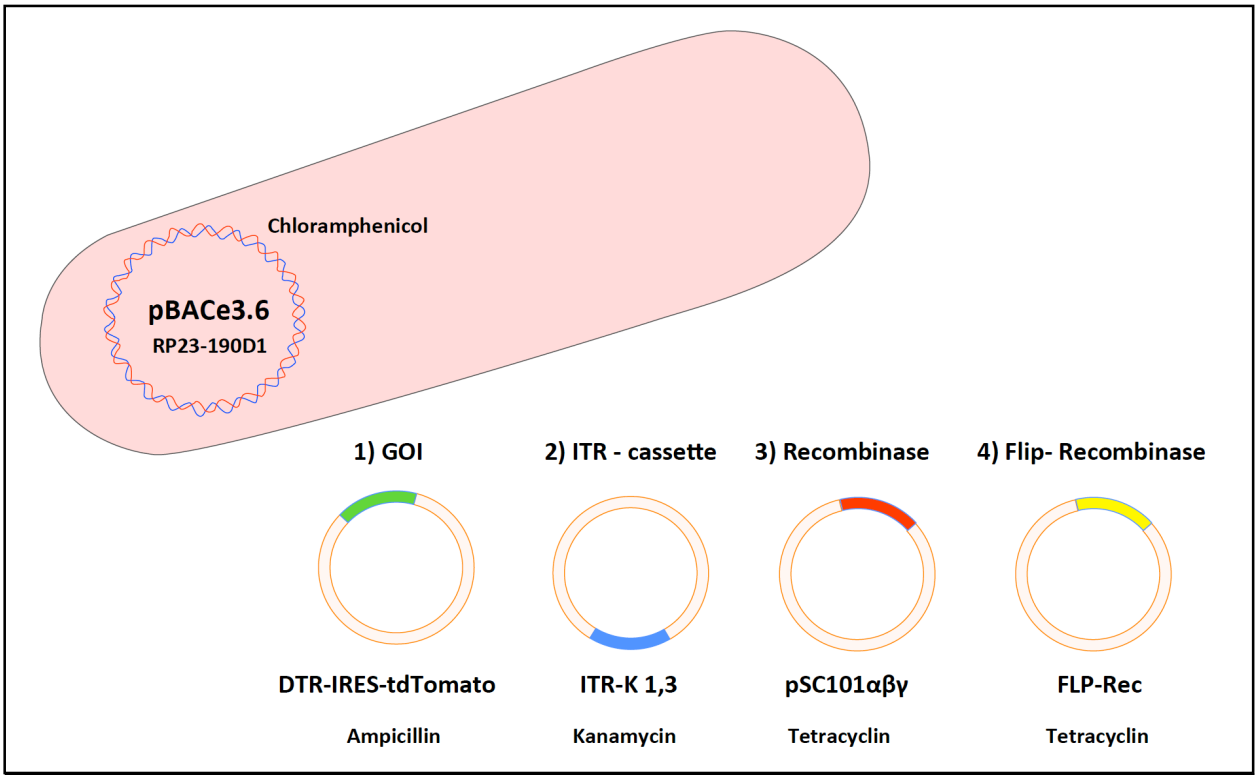

Figure 3: Toolbox for BAC recombineering. For the modification of a BAC plasmid, *E.coli* carrying the desired genomic locus are obtained from a BAC library (e.g. pBACe3.6 – RP23-190D1) and are cultivated. In addition, 4 plasmids are required. The GOI plasmid carries the target gene cassette (e.g.DTR-IRES-tdTomato) and the ITR cassette harbours a Kanamycin resistance gene flanked by ITR sites. *E coli* are electroporated with a helper plasmid, encoding for a recombinase, which mediates integration of these elements into the BAC. Antibiotic resistance mediates specific growth of *E. coli* with BACs with successful recombination. The ampicillin resistance cassette of the GOI plasmid is later excised by a FLP-recombinase, another helper plasmid, which is temperature sensitive and accordingly lost by changes in temperature. The kanamycin resistance gene of the ITR-K plasmid is lost during transposition.

2.2.3 Generation of a DTR-tdTomato BAC with ITR sites by recombineering

Inoculation and electrocompetence of bacteria

Five mL of LB containing chloramphenicol (12.5µ/mL) were inoculated with bacterial cells containing the BAC (RP23-190D1) using a sterile pipette tip and shaken over night at 37°C. The backbone vector (pBACe3.6) contains a chloramphenicol resistance gene, which guarantees selective growth of bacteria harboring the BAC. The next day the OD was measured and the culture was diluted to an OD ~0.1-0.2 in a total volume of 50mL LB containing chloramphenicol (12.5µg/mL). Cells were continuously grown and the OD was monitored regularly using a photometer. When the OD reached a value of ~0.5, cells were placed on ice for 10 minutes and then centrifuged in pre-chilled, ice-cold 50 mL Falcon tube in a pre-chilled centrifuge at 5000rpm for 5 minutes. Cells were washed with ice-cold ddH_2O twice and re-suspended in 500-1000µL of 10% glycerol. Aliquots of 100µL were frozen at -80°C.

Integration of the recombinase helper plasmid

One vial of competent cells was thawed on ice and 1µL containing 100ng of pSC101αβγ recombinase plasmid containing a tetracycline resistance gene was added. The mix was kept on ice for 10 minutes to ensure agglomeration of the plasmid DNA to the bacterial membrane. Then the electroporation mix was transferred to a 1mm cuvette and electroporated at 2.5kV (pre-set program) with a Bio-Rad Gene Pulser. After electroporation cells were re-suspended in 1mL of LB media, transferred to a fresh Eppendorf tube and incubated at 30°C for 1 hour due to the temperature sensitivity of the plasmid. Then different amounts of the bacterial suspension were plated on up to 5 LB plates containing chloramphenicol and tetracycline (12.5µg/mL and 3µg/mL) and incubated at 30°C over night.

The next day, a single colony was picked and inoculated in 5mL of LB containing chloramphenicol and tetracycline (12.5µg/mL and 3µg/mL) with constant shaking at 30°C over night. The OD of the culture was measured and again diluted to an OD of <0.1 in a total volume of 50mL LB. Bacteria were incubated at a bacterial shaker and arabinose (20% stock) was added to a final concentration of 0.3% when the OD reached 0.2 to activate transcription of the recombinase. Then the temperature was switched to 37°C to assure loss of the recombinase plasmid. Cells were incubated until the OD reached a value of ~0.5 and then made competent as described above.

Preparation and transfection of the targeting plasmid

Linearization of the final targeting plasmid (DTR-IRES-tdTomato) was performed with enzymes cutting immediately next to the 5' and 3' homology region (*MfeI* and *AscI*) in an appropriate buffer containing BSA for 2 hours at 37°C. Then the target sequence was separated from the plasmid by agarose gel electrophoresis (1%). The ~4.9kb band was cut out using a sterile scalpel and purified using the Omega Gel Extraction Kit. Ten µl of linearized plasmid were pipetted into one vial of competent cells (pBAC3.6e-αβγ) and kept on ice for 10 minutes. Cells were electroporated as outlined above and then plated on LB plates containing chloramphenicol and ampicillin (12.5µg/mL and 50µg/mL). Successful integration of the targeting plasmid confers resistance to ampicillin (**Figure 3**), which was used for positive selection. After incubation at 37°C over night correct integration was confirmed by Southern blotting.

Alkaline lysis and Southern blotting

Two mL of bacterial suspension were harvested by centrifugation and re-suspended in 300µL of Buffer 1 (50mM Tris, 10mM EDTA, 100µg/mL RNase, pH 8.0) from the Qiagen Midi Kit. For cell lysis 300µL of Buffer 2 (200mM NaOH, 1% SDS) were added. After 5 minutes of incubation the solution was neutralized with 300µL of Buffer 3 (3M acetic acid, pH 5.5) and centrifuged for 10 minutes. The supernatant was transferred to a new Eppendorf tube and 1mL isopropanol was added to precipitate DNA. Tubes were centrifuged, and pelleted DNA was washed with 75% EtOH. The pellet was then dried at a heat block at 37°C to evaporate the remaining ethanol. Then DNA was dissolved in 50µL TE buffer. DNA samples were subjected to phenol-chloroform extraction to reduce the amount of protein contamination. For this purpose 450µL of DNA buffer was added to each sample and an equal amount of phenol:chloroform:isoamyl alcohol (25:24:1, v/v, Sigma Aldrich) was added. Samples were inverted several times and then centrifuged for 10 minutes at 13 000 rpm. The upper phase (~500µL) was transferred to a fresh Eppendorf tube and 400µL isopropanol were added to precipitate DNA.

DNA was digested with HindIII over night at 37°C. The next day DNA fragments were separated by agarose gel electrophoresis (0.8%). The gel was rinsed in distilled water, incubated in 0.25 M HCL for 30 minutes under constant gentle agitation, rinsed in distilled

water again and then washed in denaturation buffer (1.5M NaCl, 0.5 NaOH) and neutralization buffer (1.5 M NaCl, 0.5 M Tris, pH 7.0). Separated DNA fragments were transferred to a nylon membrane (Amersham) through upward capillary transfer using a high salt buffer (20x SSC: 3 M NaCl, 0.3 M trisodium citrate, pH 7.0). The membrane was dried at 80°C and DNA was then cross-linked using a DNA cross linker (Stratagene).

An oligo nucleotide (5'-GAGGCACAGAAAGGTGCTGGCATGG-3'), which binds upstream of the 5' homology arm used for recombination, was labeled with ^{32}P-γ-ATP (New England Nucleotides). The kinase reaction was performed using T4 polynucleotide kinase (Fermentas) applying 50µCurie according to the recommendation of the manufacturer. The kinase reaction was performed at 37°C for 30 minutes and inactivated by incubation at 75°C for 10 minutes. The blot was then carefully rolled and put into a hybridization tube and then incubated with Church blocking buffer containing 100µg/mL salmon sperm for 30 minutes at 65°C. Finally, the ^{32}P-labeled oligo nucleotide was added and the blot incubated over night at 65°C. After two washing steps with Church wash buffer at room temperature for 5 minutes and one wash step for 30 minutes at 65°C the pattern of hybridization was visualized on X-ray film (Kodak BioMax MR film) by autoradiography. For this purpose the blot was kept at -80°C for 1, 3, and 7 days and the film developed.

Successful recombination induced an additional HindIII site in the BAC creating a shorter fragment. Accordingly, a 2.4kb fragment indicates correctly recombined clones, whereas the initial BAC displays a 5.6kb fragment.

Excision of the ampicillin cassette by FLP recombinase

The ampicillin resistance gene of the recombined BAC needed to be excised with FLP recombinase. The cells were made electrocompetent as usual to integrate the helper-plasmid. One aliquot of pBAC3.6e-DTR-IRES-tdTomato cells were incubated with 1µL FLP recominase 606 plasmid (0.66µg/µL) for 10 minutes and electroporated as outlined above. Cells were then incubated at 30°C for 1 hour and plated on LB plates containing chloramphenicol and tetracycline (12.5µg/mL and 3µg/mL), according to the temperature sensitivity and antibiotic resistance of the FLP plasmid. After visible colonies were formed, plates were transferred to 37°C for 1 hour to induce loss of the FLP plasmid. Then up to four colonies were picked and inoculated in 2mL LB media containing chloramphenicol (12.5µg/mL) and shaked at 37°C at over night. The next day cultures were plated on an agar plate containing chloramphenicol

(12.5µg/mL) and incubated at 37°C over night. Four colonies were picked again from each plate and inoculated in 2mL of LB media containing chloramphenicol (12.5µg/mL) for 37°C at the shaker. The next day, each culture was plated on dishes containing chloramphenicol (12.5µg/mL) and ampicillin (50µg/mL) and incubate at 37°C over night. Clones with successful excision of the ampicillin cassette by the FLP recombinase did not grow on these plates and were chosen for further cloning steps.

Integration of the recombinase helper plasmid

The recombinase plasmid pSC101αβγ was again required to insert the ITR cassette. Therefore pBAC3.6e-DTR-IRES-tdTomato bacterial cells were made competent and transfected with 100ng of pSC101αβγ recombinase plasmid via electroporation, cultured and made competent for recombination as outlined above.

Preparation and transfection of the ITR-Kanamycin plasmid

The ITR plasmid was digested with *NotI*, *KpnI* and *PvuI* to isolate the ITR flanked kanamycin cassette and to cause fragmentation of the remaining backbone vector assuring appropriate separation by agarose gel electrophoresis. A 40µL digestion reaction was set up and incubated at 37°C for one hour as follows:

- 5µL plasmid (1,3 λ)
- 3µL 10x buffer
- 1µL enzyme
- 20µL ddH_2O

After each digestion step, DNA was purified using the Omega Gel Extraction Kit according to the manufactures instructions.

For the transfection, electrocompetent pBAC3.6e-DTR-IRES-tdTomato bacteria were thawed on ice and incubated with 5µL of linearized ITR plasmid and electroporated as described in detail above. Bacteria were incubated on LB plates containing chloramphenicol and kanamycin (12.5µg/mL and 50µg/mL) at 37°C over night to select for correctly recombined clones. Three clones were picked and analyzed by sequencing.

Purification of the final BAC plasmid

The final construct was obtained by incubating 500mL of LB media containing chloramphenicol and kanamycin (12.5µg/mL and 50µg/mL) at 37°C over night. Cells were collected by centrifugation and BAC plasmid DNA isolated using the NucleoBond BAC 100 kit from Machery Nagel following the manufacturer's instruction.

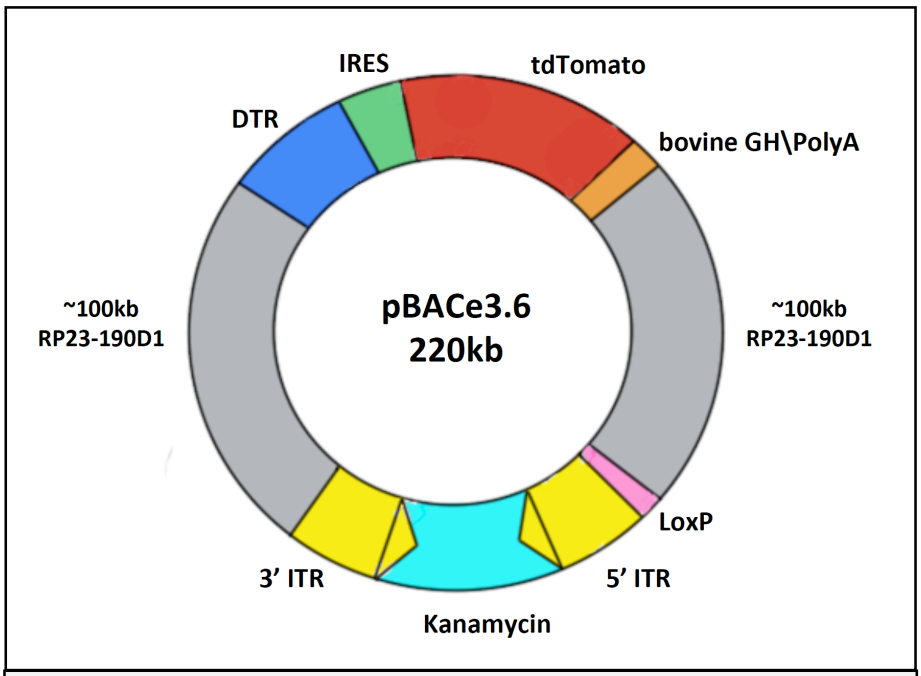

Figure 4: Schematic overview of the modified BAC RP23-190D1. For BAC transposon mediated transgenesis a BAC (RP23-190D1, backbone vector pBACe36) was modified by recombineering with a target gene cassette and an ITR-Kan cassette. The resulting modified BAC consists of a DTR-IRES-tdTomato cassette flanked by 100kb of genomic DNA of the RP23-190D1 and ITR sites. Resistance against Kanamycin was used as a selection marker.

2.2.4 Venus plasmid used as a positive control

As a positive control a plasmid expressing the green fluorescent protein Venus, under the control of a CAGGS promoter was used. The Venus plasmid was generated in cooperation with Prof. Dr. Mates from the Institute of Genetics, Biological Research Centre, Szeged, Hungary and was previously used in our laboratory for transposon-mediated transgenesis.[58] The optimal composition of injection mixes was tested by ex vivo experiments at the Max Delbrück Centre in Berlin. Zygotes were co-injected with different amounts of plasmids, maintained in culture, and reporter gene expression was evaluated after seven days.[46]

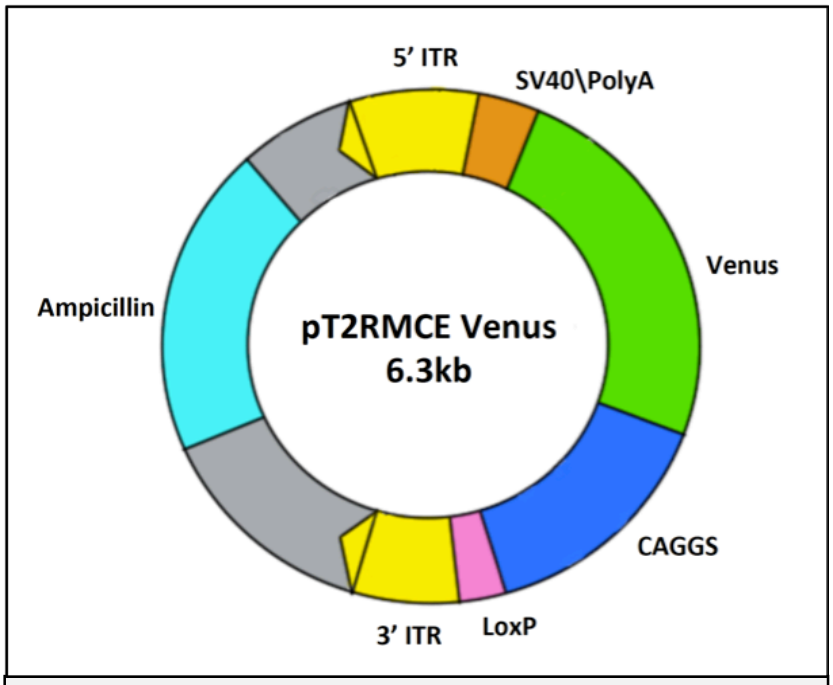

Figure 5: Schematic map of the Venus plasmid. The pT2RMCE plasmid carries the green fluorescent protein Venus under the control of the CAGGS promoter flanked by two ITR sites. Resistance against ampicillin was used as a selection marker.

2.2.5 Generation of Transgenic Mice

Fertilized eggs from C57BL/6N mice were isolated and washed with M16 media. All microinjections were performed by Prof. Dr. Thomas Rülicke. The following experimental conditions were applied:

I)	Plasmid	Injected Zygotes	Transferred Embryos
Co-Injection PNI	BAC 1.0	41	20 (48.7%)
	BAC 2.0	45	39 (86.6%)
Injection PNI/CP	BAC 1.0	52	41 (91.0%)
Injection PNI/CP	Venus	40	24 (60.0%)
II)			
Co-Injection CP	Venus	92	86 (93.4%)

Table 2: Microinjection conditions for transposon mediated transgenesis. 2pL of microinjection solution containing 1ng/µL or 2ng/µL BAC plasmid DNA were co-injected into the pronucleus of C57BL/6N zygotes. In another group, 2pL of microinjection mix containing 5ng/µL SB100X mRNA were injected into the cytoplasm and then 2pL containing 1ng/µL BAC plasmid were injected into the pronucleus As a control, zygotes were injected with the same conditions except that the Venus (0.4 ng/µL) instead of the BAC plasmid was used. To investigate the efficiency of cytoplamatic compared to pronuclear microinjection, 2pL microinjection solution containing 0.4ng/µL Venus plasmid and 5ng/µL of SB100X mRNA were co-injected in the cytoplasm.

In conditions called “co-injection”, the plasmids as well as the mRNA were injected at the same time into the male pronucleus or into the cytoplasm. When combined pronuclear and cytoplasmic injection were performed (PNI/CP), the mRNA was first injected into the cytoplasm and about 30 minutes later the plasmid was injected into the pronucleus. The Venus plasmid and mRNA of the *SB100X* transposase were kindly provided by Prof. Dr. Mates (Institute of Genetics, Biological Research Centre, Szeged, Hungary).

For all approaches using the Venus plasmid, 2pL of solution mix containing 0.4ng/µL plasmid was used, while for BAC transgenesis 1ng/µL or 2ng/µL plasmid DNA were injected. In all experiments 5ng/µL *SB100X* transposase mRNA were applied. The most efficient concentration of the plasmid and the mRNA and their relation were determined previously.[46] In the series of cytoplasmic injections of the Venus plasmid the pronucleus was hit in four cases. Transgenesis induced by pronuclear injection cannot be excluded in these four cases. Two hours after injections, viable embryos were selected and up to 20 embryos were transferred into the right horn of the uterus of CD1 pseudo-pregnant recipient mice.

2.2.6 Detection of Fluorescence Marker Gene Expression in Born Animals

Positive integration of the construct was evaluated as soon as possible by exposing newborn mice to a light source (BLS, Budapest, H) and a color specific emission filter. The green fluorescence of Venus was visualized at a wavelength of λ= 460-495nm. Furthermore a light source with a wavelength of λ= 560-585nm was used to detect the red fluorescence of tdTomato. Only transgene positive mice with appropriate expression of reporter gene display green fluorescence for Venus and a red fluorescence for BAC integration.

2.2.7 Tail Sample Taking

At the age of three weeks pups were weaned from their mothers and separated according to sex. Furthermore, mice received an ear-tag and a tail biopsy of 1 to 2mm tail was taken for isolation of DNA for genotyping purposes.

2.2.8 DNA Isolation from Tail Biopsy

Tail biopsies were incubated in 100µL TNES buffer and 2µL proteinase K (20mg/mL) at 55°C over night. After vortexing, 35µL of 5M NaCl were added to denature and precipitate proteins. Then tubes were centrifuged at 12000rpm for two minutes at room temperature. The supernatant was transferred to a new Eppendorf tube and DNA was precipitated by adding 100µL ice-cold 100% EtOH. Tubes were inverted until the typical white haze of precipitating DNA became visible. Samples were then centrifuged at maximum speed for 2 minutes. The supernatant was removed, the pellet washed with 70µL EtOH (70%). After centrifugation ethanol was aspirated and the pellets were dried with an open lid to ensure evaporation of all residual ethanol. DNA was dissolved with 60µL TE-buffer and stored at 4°C or at -20°C for long time storage.

2.2.9 Genotyping PCR

I) BAC:

Detection of BAC positive mice was done by PCR amplifying a 743bp fragment located in the tdTomato sequence. A PCR using primers binding to the mouse collagen14a1 gene amplifying a 687bp fragment was performed as an endogenous control.

Primer Sequences:

tdTomato fw: ATGGTGAGCAAGGGCGAGG

tdTomato rev: ATGTTGTTGTCCTCGGAGGAGG

Coll14a1 fw: GGGGAAATGTCACCTTCAAA

Coll14a1 rev: TGGGAGGATGGCTGTGTA

Table 3: Reaction mix for BAC genotyping

µL/reaction		Stocks
2.5	PCR buffer	10x
3.4	$MgCl_2$	15mM
2.5	dNTPs	2mM
1	tdTomato fw	10µM
1	tdTomato rev	10µM
1	Coll14a1 fw	10µM
1	Coll14a1 rev	10µM
0.1	Taq Polymerase	5units/µL
11.5	ddH_2O	
1	genomic DNA	

II) Venus:

A PCR reaction amplifying a 363bp fragment of the *Venus* sequence was performed to confirm integration of Venus in fluorescent founders. As an endogenous control the same PCR reaction as for BAC genotyping was performed.

Primer Sequences:

Venus Fwd: CTCTTCTCGTTAGGGTCCTT

Venus Rev: GGAGAGAACCATCTTCTTCA

Table 4: Reaction mix for Venus genotyping

µL/reaction		Stocks
2	PCR buffer	10x
2	$MgCl_2$	15mM
2	dNTPs	2mM
0.5	Venus fw	10µM
0.5	Venus rev	10µM
0.5	Coll14a1 fw	10µM
0.5	Coll14a1 rev	10µM
0.1	Taq Polymerase	5units/µL
9.5	ddH_2O	
2	genomic DNA	

Annealing temperatures of primers were optimized by temperature gradient PCR. The 743bp fragment for tdTomato was reproducibly amplified at 63°C and the 363 bp fragment of Venus at 55°C. Each PCR included a positive control consisting of DNA from a mouse expressing tdTomato for genotyping of possible BAC transgenic animals and DNA from a mouse expressing Venus for genotyping of possible Venus transgenic animals. DdH_2O was used as a negative control.

Table 5: PCR conditions for BAC

96°C	2 min	
92°C	40 sec	35 cycles
63°C	40 sec	
72°C	1 min	
72°C	10 min	

Table 6: PCR conditions for Venus

96°C	2 min	
92°C	40 sec	30 cycles
55°C	40 sec	
72°C	1 min	
72°C	10 min	

PCR products were diluted with loading buffer and loaded on a 2% agarose gel containing 3µL ethidium bromide (10mg/mL). As DNA ladder GeneRuler 100kb (Fermentas) was used.

2.2.10 Transposon Display via linker-mediated PCR

Transposon display via linker-mediated PCR was performed to determine the number of transposon integrations in the genome of positive founder animals. Since no BAC transgenic mice were generated, this section refers only to Venus positive animals.

Linker-mediated PCR is single-sided PCR method, which requires only one primer annealing sequence; the second one is provided by the ligation based binding of a unique DNA linker. The linker-binding primer in collaboration with the gene specific primer allows the amplification of the genomic host DNA in-between, which is then applicable for sequencing.

For linker-mediated PCR the DNA is digested with a suitable frequently cutting restriction enzyme (e.g. *FspBI*). The restriction enzyme FspB1 cuts at the palindromic sequence 5'-CTAG-3' 381bp away of the integration junction of the 5' ITR, which marks the end of the integration site of the transposon. In addition it cuts at the closest 5'-CTAG-3' sequence in the genomic DNA of the host. Two annealed BfaI linker oligonucleotide sequences are ligated to the overhanging restriction ends to prepare a unidirectional ligation site for the Linker Primer. The 3'hydroxyl end of the long linker (+) ligates to the 5'phosphate of the genomic DNA and the short linker (-) hybridizes to the long overhang within the ITR site, finally generating TA overhangs. The following two PCRS's purify the amplifications and reduce the background. For the first PCR round Linker primer, witch binds to the linker oligonucleotide sequences and the transposon specific Tba rev3 primer were used, where Tba rev3 binds 255bp away from the 5' ITR sequence end. The second PCR requires the Nested primer; also binding at the linker oligonucleotide sequences in the genomic DNA of the host, and the TbaI primer, which binds 109bp away from the end of the 5'ITR sequence end. PCR products of different lengths and amounts are amplified in each round corresponding to the number and sites of integration. These amplified sequences are separated by agarose gel electrophoresis, purified and used for sequencing, to determine the integration site of the transposon **(Figure 6)**.[62]

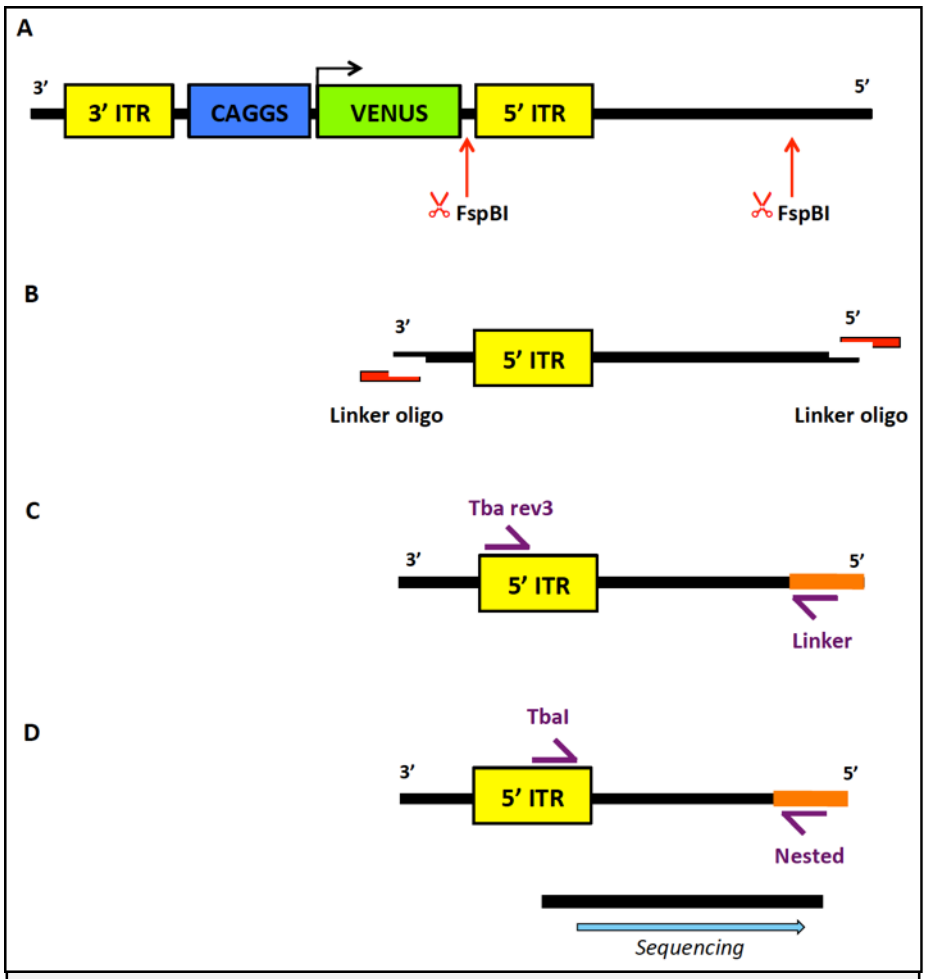

Figure 6: Transposon Display via linker-mediated PCR. The restriction enzyme FspBI cuts at the palindromic sequence 5'-C^TAG-3 (**A**). Oligonucleotides containing linker sequences are annealed and create binding sites for specific primers (**B**). The first linker-mediated PCR was performed using a primer pair, which anneal in the linker and in the transposon (TbaI rev3) (**C**). The second linker-mediated PCR was performed with a nested primer, which also binds to the linker sequence and the primer TbaI, which binds in the 5' ITR.[36, 40]

Genomic DNA (300ng) was digested with *FspBI*, which cuts at the palindromic sequence 5'-CTAG-3' 381bp away from the integration junction of the 5' ITR, which marks the end of the integration site.

Table 7: Digestion of genomic DNA using FspBI

300ng	genomic DNA
2µL	10x buffer
0.5µL	FspBI (10U/µL)
up to 20µL	ddH_2O

DNA was digested for three hours at 37°C and then the FspBI was inactivated by incubation at 65°C for 30 minutes.

Two partly complementary oligo nucleotides were annealed to generate a synthetic linker sequence for both ends of digested fragments.

Oligo sequences:

BfaI linker (+) 5'-GTAATACGACTCACTATAGGGCTCCGCTTAAGGGAC-3'

BfaI linker (-) 5'-TAGTCCCTTAAGCGGAG-3'

The oligo nucleotides BfaI linker (+) and BfaI linker (-) were mixed to a final concentration of 10pmol/µL with TE buffer containing 1M NaCl. Annealing of these oligo nucleotides was achieved by gradual of temperature in a PCR machine using the following conditions:

Table 8: PCR conditions for oligo annealing

94°C	2 min
80°C	5 min
75°C	10 min
70°C	10 min
65°C	10 min
60°C	10 min
55°C	10 min
50°C	10 min
45°C	10 min
37°C	10 min

Annealed BfaI linker (+/-) oligo nucleotides were ligated to DNA digested with *FspB1* using T4 ligase in a reaction mixture at 16°C over night as outlined below.

Table 9: Reaction for linker ligation

10µL (150 ng)	FspBI digested genomic DNA
2µL	annealed linker (10pmol/µL)
5µL	T4 Ligase buffer 10x
6 units	T4 Ligase
up to 50µL	ddH_2O

T4 ligase was inactivated by incubation at 65°C for 10 minutes.

Then a nested PCR was performed consisting of two consecutive PCR reactions. Primers, which bind to a sequence within the linker (Linker and Nested), and primers, which bind to the 5`ITR of the transposon sequence (TbaI rev3 and TbaI), were used for this purpose. The first PCR was accomplished using the primer pair Linker and TbaI rev3, the second PCR with the Nested and the TbaI primer. Ten µl of the first PCR reaction was analyzed by 1% agarose gel electrophoresis to test the functionality of the first PCR. Loading dye, DNA ladder and EtBr were used as described above. Another ten µl was diluted 1:100 with H_2O and used in the second PCR step.

<u>Primer Sequences:</u>

I. Linker-PCR:

Linker: 5'-GTAATACGACTCACTATAGGGC-3'

TbaI rev3: 5'-AAAGCCATGACATCATTTTCTGGAATT-3'

II. Nested-PCR:

Nested: 5'-AGGGCTCCGCTTAGGGGAC-3'

TbaI: 5'-CTTGTGTCATGCACAAAGTAGATCGTCC-3'

Linker and Nested PCR were performed according to the same protocol.

Table 10: Reaction mix for linker-mediated PCR

µL/reaction		Stocks
5	PCR buffer	10x
5	$MgCl_2$	15mM
5	dNTPs	2mM each
1	Linker/ Nested primer	10µM
1	TbaI/TbaI rev3	10µM
0.5	Taq Polymerase	5units/µL
31	ddH_2O	
1.5	Template 1:100	

Table 11: PCR conditions for linker-mediated PCRs

96°C	2 min	
92°C	40 sec	30 cycles
55°C	40 sec	
72°C	1 min	
72°C	10 min	

PCR products were separated by electrophoresis using a 1 % agarose gel. Bands were excised with a scalpel under UV light and DNA was purified by gel extraction using peqGOLD GEL Extraction Kit from PeqLab according the manufacturers recommendations. The DNA content of each sample was measured by spectrometry and samples were sent to Microsynth for sequencing.

3. Results

A ~220kb large BAC carrying the red fluorescence reporter gene tdTomato was generated to examine the cargo capacity of *SB100X* based transposon-mediated transgenesis. The successful recombination of the targeting cassette into the BAC plasmid was controlled by a Southern blot.

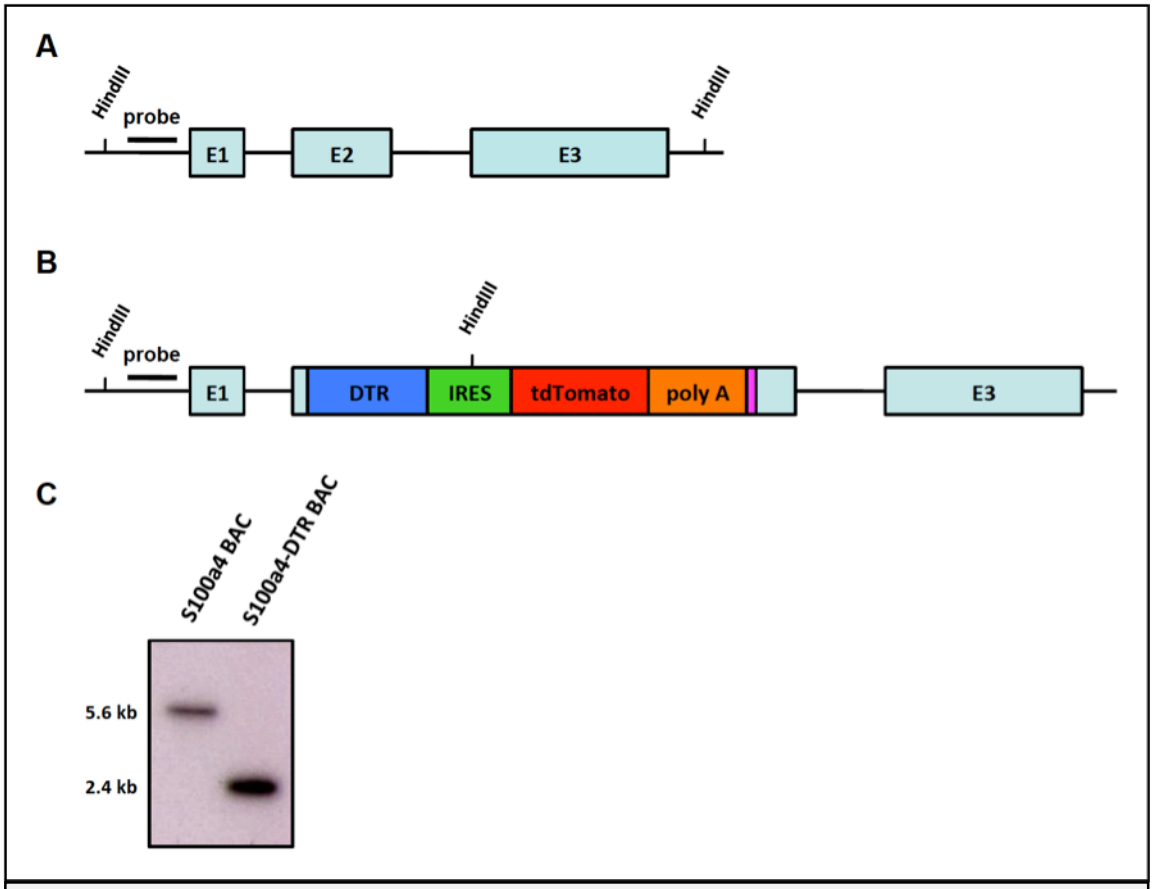

Figure 7: Recombineering of the mouse S100a4 gene in a BAC. The mouse S100a4 gene consists of 3 exons and is flanked by 2 HindIII sites (**A**). A third HindIII site is integrated following successful integration of a DTR-IRES-tdTomato cassette (**B**). Southern blotting confirmed correct recombination of a BAC containg the mouse S100a4 gene. An oligonucleotide binding upstream of Exon 1 was labelled with P32γ and used as a probe (**C**).

Zygotes of C57BL/6N mice were injected with *SB100X* transposase mRNA and different amounts of BAC plasmid in order to generate BAC transgenic mice. A previously used and well-established plasmid containing the green fluorescent reporter gene Venus served as a positive control **(Table 12).** In all approaches 2pL containing 5ng/µL SB100X transposase were injected.

For the first experiment, 2pL of 1ng/µL BAC plasmid and 5ng/µL *SB100*X were co-injected into the pronuclei of 41 zygotes. Only 20 zygotes (48.7%) survived and could be transferred into surrogate mothers, which gave birth to 16 (80%) pups. However, none of these newborn mice expressed the transgene. The concentration of the BAC plasmid was increased to 2ng/µL and 45 zygotes were injected. Thirty-nine embryos (86.7%) were transferred, but only one mouse maintained pregnancy until term and gave birth to four pups (10.2%). Three of these pups were dead and only one survived. Integration of the BAC could not be detected in any animal.

In the next experiment, 2pL containing 5ng/µL *SB100X* mRNA was injected into the cytoplasm and pronucleus of 52 zygotes. After 2 hours, 2pL of 1ng/µL BAC plasmid were injected. Forty-one zygotes (78.8%) survived and could be transferred and 17 pups (41.5%) were born alive. No integration of the BAC was documented.

As a control experiment, 40 zygotes were injected with 2pL containing 5ng/µL *SB100X* into the cytoplasm. After 2 hours 2pL containing 0.4ng/µL Venus plasmid were injected into the pronucleus. Twenty-four embryos survived and could be transferred (60%) 12 of these embryos were born alive (50%) and two pups were found dead. Only one pup (8.3%) had a detectable integration of the Venus plasmid **(Table 12)**.

	Plasmid	Injected Zygotes	Transferred Embryos	Born	Positive	Dead
Co-Injection PNI	BAC 1.0	41	20 (48.7%)	16 (80.0%)	0 (0%)	0
	BAC 2.0	45	39 (86.6%)	4 (7.6%)	0 (0%)	3
Injection PNI/CP	BAC 1.0	52	41 (91.0%)	17 (41.0%)	0 (0%)	0
Injection PNI/CP	Venus	40	24 (60.0%)	12 (50.0%)	1 (8.3%)	2

Table 12: Results for BAC transposon mediated transgenesis. Efficiency of transgenesis following microinjection of the BAC plasmid at different concentrations. The Venus plasmid served as a control.

The second aim of this study was to compare the efficiency of cytoplasmic versus pronuclear microinjection. The results of pronuclear microinjection of the Venus plasmid performed in our previous study served as a reference.[58] The same experimental conditions were used for cytoplasmiatic injection. Ninety-two zygotes were co-injected with 2pL of injection buffer containing 5ng/µL *SB100X* mRNA and 0.4ng/µL Venus plasmid into the cytoplasm. Eighty-six zygotes (93.5%) were transferred into CD1 surrogate mothers and 45 (52.3%) of these embryos were born, but 3 pups died after birth. Fourteen out of the 45 mice

(31.1%) displayed the green fluorescence of the Venus plasmid. Integration of Venus was confirmed by PCR **(Table 13)**.

Classical pronuclear microinjection resulted in 38 founders (62.3%). Out of 157 transferred embryos 61 pups were born (38.8 %) and eight mice died within the first three days, whereby 4 of them were Venus positive.

	Plasmid	Injected Zygotes	Transferred Embryos	Born	Positive	Dead
Co-Injection PNI[58]	Venus	D.n.p.	157	61 (38.8%)	38 (62.3%)	8
Co-Injection CP	Venus	92	86 (93.4%)	45 (52.3%)	14 (31.1%)	3

Table 13: Results for transposon mediated transgenesis comparing pronucleus and cytoplasmatic microinjection. Embryos were co-injection with the Venus plasmid and SB100X mRNA into cytoplasm and the efficiency of transgenesis was compared to classical pronucleus microinjection of a previous approach. Both experiments were performed under the same conditions. D.n.p.: data not provided.

Successful integration of the BAC transgene carrying the red fluorescence protein tdTomato was evaluated by exposing newborn pups to a light source with a wavelength of λ= 560 - 585 nm and an emission filter. However, no fluorescence was detectable in the skin of any mice.

Venus positive mice were examined at a wavelength of λ= 460-495 nm to visualize the green fluorescence of the Venus protein. Venus expression was detectable in 14 mice with varying intensity. In founders #1, 3, 5, 7, 8, 14, 16 and 17 the signal was intense and included the entire body. In contrast, dim or mosaic fluorescence was observed in mice #4, 6, 15, 18, 19 and the dead pup **(Figure 8)**.

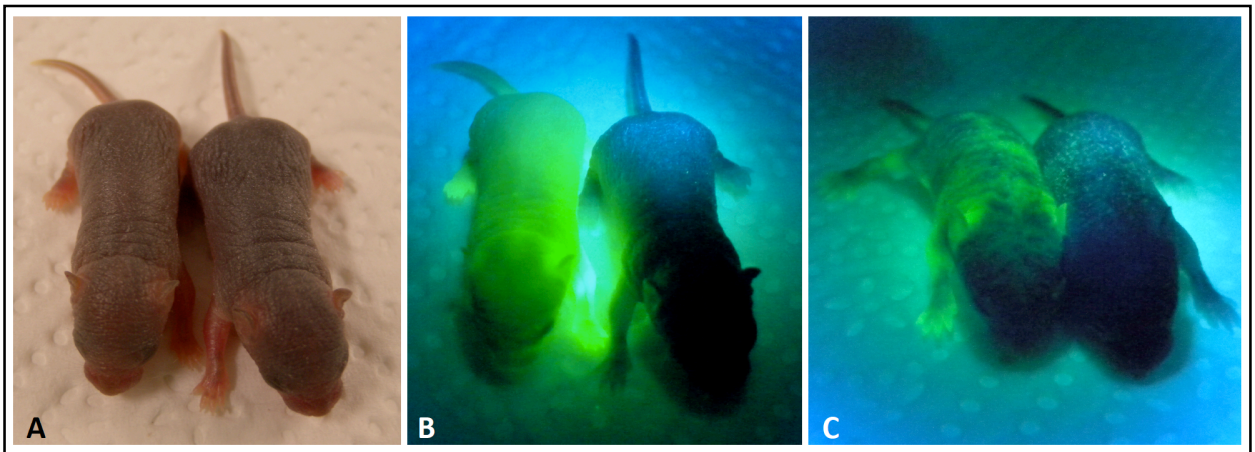

Figure 8: Detection of fluorescence in newborn mice. No fluorescence can be detectable in newborn pups under normal light conditions (A). Animals were exposed to a light source with a wavelength of λ= 460-495nm to visualize the green fluorescence of the Venus reporter gene in transgene positive mice. The pattern and intensity of green fluorescence differs among transgene positive founders.

In addition, all born pups were genotyped by PCR using primers specific for fluorescence marker genes. The PCR products were separated by agarose gel electrophoresis and visualized by UV light. Collagen14a1 was used as an endogenous control (687bp) in both approaches.

The absence of a 743bp PCR product using primers binding in the tdTomato sequence confirmed the negative results of the optical fluorescence check in all samples. In **Figure 9** results for 7 mice generated by co-injection of 1ng/µL BAC plasmid are shown.

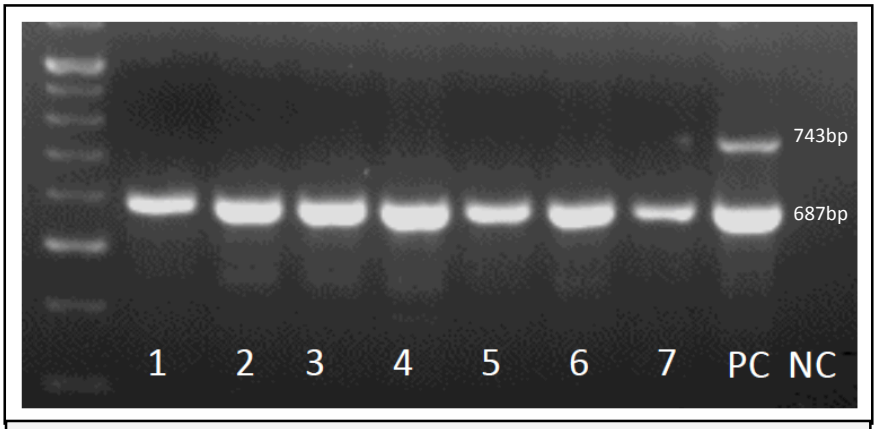

Figure 9: Genotyping of BAC founders. Genotyping results for founders co-injected with BAC plasmid (1.0 ng/µL) and SB100 mRNA (5.0 ng/µL). PCR products were separated by a 2% agarose gel. tdTomato: 743bp, Collagen14a1: 687bp (endogenous control), PC= positive control, NG= negative control.

Primers used for genotyping of Venus generate a 363bp PCR product. Genotyping PCR confirmed results for the all 14 mice, which were previously identified by fluorescence (Figure 9). This indicates that PCR positive mice actively express the integrated reporter gene and that the transgene was not subjected to silencing. Overall 11 mice generated by cytoplasmic injection had a successful integration of the transgene and only one PCR positive pup died after birth. In the group of mice with pronuclear microinjection (PNI) 3 mice displayed an integration of the Venus. Mouse #8 is the only positive founder of the control group of the BAC experiment. DNA from two fluorescent negative mice was subjected to PCR analysis to address potential contaminations (**Figure 10**).

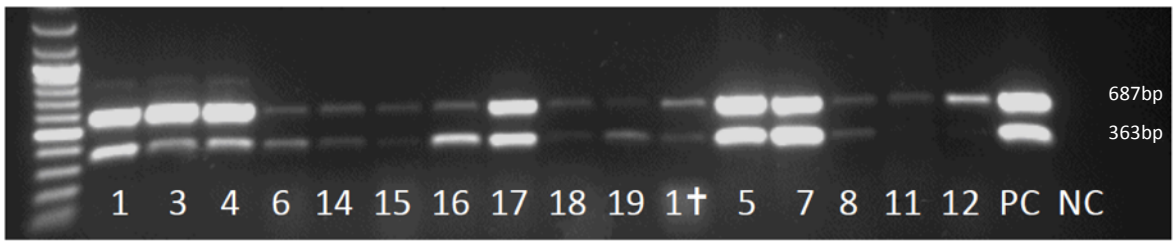

Figure 10: Genotyping of Venus founders. Genotyping results of all green fluorescent founders generated by co-injection of Venus plasmid (0.4ng/µL) and SB100 mRNA (5.0ng/µL) into the cytoplasm of C57BL/6 zygotes. Venus: 363bp, Collagen14a1: 687bp (endogenous control), sample 11 and 12 serve as fluorescence negative reference, 1†= born dead, PC= positive control, NG= negative control

Transposon display via linker-mediated PCR was performed to identify the integration sites of the transgenes and determine the number of integrations. Numerous PCR products were observed after the first PCR (data not shown), which can be explained by binding of the Linker primer at every ligated linker sequence on both sides of different sized DNA fragments. In the second PCR step, only the sequence between the Nested primer and the TbaI primer binding to the 5'ITR end, which marks the end of the integration site, were amplified. Amplicons were separated by agarose gel (1%) electrophoresis **(Figure 11**).

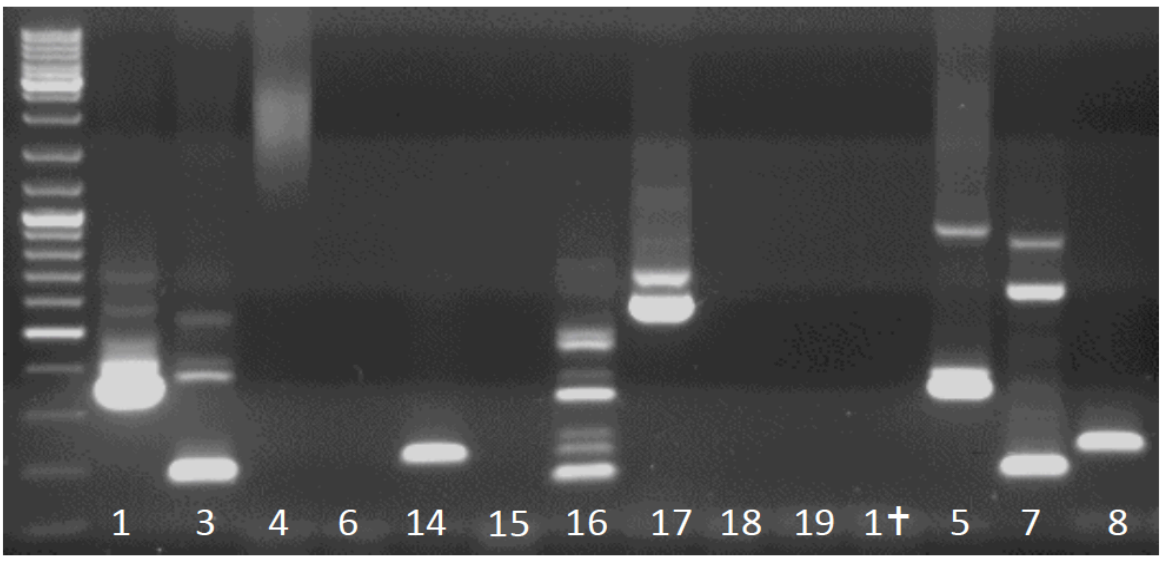

Figure 11: Linker-mediated PCR of Venus positive founders. In the second PCR reaction, the sequence between the nested primer and the TbaI primer at the 5' end of the ITR were amplified. PCR products were separated by agarose gel electrophoresis (1%). Founders had different numbers of integrations of the transposon. It was not possible to perform transposon display in founders with weak fluorescence (#4, 6, 15,18 and 19).

After separation every band for each founder was cut out. No PCR product was obtained for mice with dim fluorescence. The remaining founders showed different numbers and sites of integration as represented by different sizes of PCR products. Three animals displayed a single integration of the transgene (#1, 14, 8), three showed integration at two different sites (#3, 17, %), one mouse had three distinct integrations (# 7), and one had four integration sites (# 16) **(Table 14)**.

Founder	Integration sites	Approximate Band size (bp)
1	1	350
3	2	200
		350
14	1	200
16	4	200
		250
		300
		500
17	2	600
		700
5	2	350
		1000
7	3	180
		600
		950
8	1	200

Table 14: Results for Transposon display. In each founder different numbers and integration sites were detected by band size.

PCR products were purified using a gel extraction kit and sent to Microsynth (Vienna) for sequencing. Not all integration sites for each founder could be sequenced due to insufficient length. The preference of *SB100X* for TA sites was confirmed in all samples (Data not shown). The integration site of the transgene was identified by BLASTing sequencing results **(Table 15)**. Sequencing results were obtained for 8 samples and indicated three integrations at chromosome 19, two at chromosome 1, two on chromosome 6, and single integrations on chromosome 7 and 14. Almost all integrations were intragenic, confirming the

preference of *SB100X* for non-coding areas. The only exceptions were integrations on chromosome 1.

In founder #17 one transposon integrated in an intron of Tram2 and in founder #5 the transgene integrated in an intron of Dpp10. Furthermore, founder #7 had an integration within an alternative splice site of the AK211759 mRNA at the chromosome 3.

Founder	Integration numbers	Approx. band size (bp)	Size (bp)	Chr	Start	End	Location
1	1	350	311	19	19447034	19447247	Intergenic
3	2	350	81	19	19447194	19447247	Intergenic
14	1	200	152	14	11829096	11829102	Intergenic
16	4	300	265	6	19127124	19127303	Intergenic
17	2	600	512	1	21034650	21035073	Tram2, Intron
5	2	350	288	1	12381113	12381132	Dpp10, Intron
		1000	62	6	19534984	19535004	Intergenic
7	3	950	359	19	45220362	45220650	AK211759
8	1	200	158	7	23198429	23198488	Intergenic

Table 15: Sequencing results. Identification of the integration site of the Venus plasmid using BLAST. Chr: chromosome

4. Discussion

Transposons have been proven to be an efficient tool for the genetic manipulation of different cell types and organisms. Various recent studies disapproved the suspicion that transposon-mediated transgenesis is only feasible for pronucleus microinjection of small plasmids. Indeed, it was demonstrated that larger sized plasmids such as BACs can be subjected to transposition. Transposition of BAC is associated with a number of advantages such as single, full-length integrations. This reduces the influence of position effects and is associated with expression patterns of the transgene in the host organism mimicking endogenous gene expression. BACs can contain up to 350kb, which increases the opportunities of DNA engineering regarding the size of the transgene. It also offers the possibility to integrate large parts of a chromosome including several, including potential regulatory elements. This stabilizes the expression of the transgene and allows for the addressing of a plethora of different scientific questions.

A number of different transposase systems are available and some of these have been shown to be more suitable for the transposition of the large cargos such as BAC. *Tol2* was shown to enable transposition of a 120kb BAC in mice and zebrafish.[27] Furthermore, transposition of a 100kb sized BAC was reported in hEHEC using the *piggyBac* transposase.[29]

The versatility of Sleeping Beauty (*SB100X*), a synthetic transposase belonging to the Tc1/mariner super family, has been documented in the genetic manipulation of different vertebrates. Previous data in our laboratory has indicated that *SB100X* is efficient for the transposition of a 6.3kb sized plasmid expressing the green fluorescence reporter gene Venus. In previous experiments, murine zygotes were co-injected with 2pl injection mix containing Venus plasmid (0.4ng/µL) and *SB100X* mRNA (5ng/µL) into the pronucleus. 62.3% of mice born displayed stable genomic integration and efficient expression of the Venus protein as evaluated by green fluorescence. Germline transmission was confirmed by inheritance analysis. Every founder animal transmitted the transgene to some pups within its litter. Silencing events were only observed in two out of nearly 300 analyzed F1 animals.[58]

With the kind support of Dr. Christoph Österreicher and Dr. Emilio Casanova from the Institute of Pharmacology at the Medical University of Vienna and the Ludwig Boltzmann Institute for Cancer Research a ~220kb sized BAC containing a red fluorescent reporter gene

was generated by recombineering. This BAC was used to investigate the ability of *SB100X* to transpose also large plasmids in murine zygotes.

The BAC plasmid contains a cDNA cassette composed of the simian diphtheria toxin receptor (DTR) and tdTomato separated by an IRES sequence. This construct is flanked by 100kb of genomic DNA of the S100a4 gene on each side. The 5' as well as the 3' are completed by an ITR, the binding sites for the transposase. The ITRs also flanked a kanamycin resistance gene, which was used as a plasmid selection marker. Successful transposition of the BAC at the ITR sites would accordingly result in loss of the kanamycin cassette **(Figure 4)**.

To investigate the capability of SB100X to catalyze transposition of this modified BAC, more then 90 murine zygotes were co-injected with 1.0ng/µL BAC and 5.0ng/µL *SB100X* mRNA by pronuclear microinjection. The BAC plasmid was also injected into 45 zygotes at a higher concentration (2.0ng/µL BAC plasmid). As a control, another group of zygotes were co-injected with *SB100X* mRNA together with a Venus plasmid, which was recently successfully used in our laboratory **(Figure 5)**.

All approaches to generate BAC transgenic animals failed. The lack of red fluorescence was confirmed by PCR using genomic DNA **(Figure 9)**. The majority of embryos survived the injection (48-91%) and could be transferred into pseudo-pregnant mothers suggesting that the failure of the BAC to transpose is not related to mechanical manipulation or other technical pitfalls. In an additional approach using 2.0ng/µL BAC plasmid, only 4 pups were born from initially 39 transferred embryos and three of them died after birth **(Table 12)**. This suggests that large amounts of bulky plasmid DNA interfere with the development of the embryo. Furthermore, only one transgene positive animal was born in the Venus control group suggesting a decrease in SB100X activity. The most likely explanation is the degradation of *SB100X* mRNA by RNAses, because the functionality of the Venus plasmid was demonstrated previously in our laboratory.

Another limiting fact might be the distance of the two ITR sites in the plasmid, which mediate binding of the transposase. The transposase binds at the ITRs, cuts the transposon according to the orientations of the ITRs, brings the ITR sites together creating a loop in the transgene and then inserts this DNA loop into the host DNA. Consequently, the spatial distance of the ITRs is essential for proper function of the transposase **(Figure 2)**. Rostovskaya et al. have previously demonstrated that the distance between the two ITR sites on the outside of the transgene is the limiting factor for the transposition of BACs with a cargo size

over 100kb. The authors performed transposition of two different ~160kb sized BAC carrying an eGFP-IRES-hNANOG or mCerry-IRES-hOCT4 cassette into hESC using *piggyBac*. An increased distance of the ITR sites of more than 1kb resulted in a 2-3 fold reduction of transposition efficiency.[48] In my approach the ITR sites of the BAC plasmid are separated by an 1820bp sized kanamycin cassette. This raises the hypothesis that the lack of SB100X mediated transposition is related to the spatial distance of the ITRs and the failure to excise and bring the ITRs together resulting in lack of integration into the host genome. Further studies will address this hypothesis in cell culture, which is easier than in murine zygotes and considerably faster and cheaper. If transgenesis also fail in cell culture the design of the BAC design has to be re-considered.

The same experimental conditions were applied to investigate the efficiency of transposition following cytoplasmic co-injection compared to classical pronucleus injection. Accordingly, 2pl injection mix containing 5ng/µL *SB100X* mRNA and 0.4ng/µL of the Venus plasmid (6.3kb), were injected into the cytoplasm of 92 C57BL/6N zygotes. Eighty-six viable embryos (93.5%) were transferred into surrogate mothers compared to 85-90% following pronuclear microinjection. This suggests that cytoplasmic injection leads to the same frequency of viable embryos as a classical approach. However, this method is less technically demanding and can be more easily performed. Furthermore, an increased percentage of pups born are born following cytoplasmic injection (52.3%) compared to 38.8% following pronuclear injection, suggesting that this method is less invasive and less harmful to the early developing embryo.

Fourteen pups generated by cytoplasmic injection displayed expression of Venus as evaluated by green fluorescence of newborn mice corresponding to an efficiency of transgenesis of 31.1%. This is efficiency is lower than in our previous study (62.3%) but still exceeds the performance of ~25% transgenesis following classical pronuclear injection of a non-transposon plasmid published in literature **(Table 13)**.

In 8 founders (#1, 3, 5, 7, 8 14, 16, 17) the green fluorescence was intense and included the entire body. Six animals (#4, 6, 15, 18, 19 and 1†) presented scattered fluorescence of lower intensity. The integration sites and its heterochomatization might explain varieties in the intensity of the fluorescence.

The integration of the transposon was verified by genotyping using PCR and the results matched with the observed fluorescence. This suggests that no transposon was prone to positional effect variegation and no integration site was silenced.

Eight founders could be analyzed by transposon display, which revealed multiple integrations for two animals as indicated by three or more bands. Three mice displayed two integration sites and another 3 animals displayed a single integration. These results are in accordance with the published results, indicating an integration bias of *SB100X* for single or double integration events **(Table 14)**. Exons were spared from integration verifying the preference of *SB100X* for non-coding sequences. All 9 analyzed integration sites were different. Six of these were intergenic and only 2 were located within an intron. One transposon was integrated into an alternative splice site. The integration locus was consistently on the 5' or 3' side of a duplicated TA sequence, another characteristic of *SB100X*. No preference for integration in a particular chromosome was detected **(Table 15)**.

The ITR sites in the Venus plasmid are separated by 2.8kb, but successful transposition was observed in 14 animals. This suggests that lack of transgenesis in BAC attributed to the spatial distance of the ITRs only affects larger plasmids.

In summary, cytoplasmic co-injection of a transposon plasmid together with the *SB100X* mRNA is less invasive than the classical pronucleus injection resulting in increased viability of injected zygotes (93.5%) and birth rate (52.3% versus 38.8%). Successful integration of the transgene was detectable in only 31.1% of the born animals compared to 62.3% following pronuclear injection.

5. Appendix

Summary

The generation of transgenic animals is an indispensible tool in biomedical research for the examination gene functions, diseases and pharmaceutical research. Therefore different techniques and methods like viral transduction and pronucleus microinjection were developed, whereby all this techniques suffer someway in efficiency, handling or safety. Nowadays the preferred method to generate transgenic mice is the pronuclear microinjection of bacterial artificial chromosomes. BACs circumvent the common problems of small plasmids like concatemerization and silencing due to position effect variegation. BACs carry intact genomic regions with cis–regulatory elements, which lead to a stable expression of the transgene. Furthermore, their size extends the opportunities in plasmid design and target genes cassettes. To increase their efficiency in transgenesis, BACs were combined with a transposable system. Transposons have been proven to be a highly successful tool in the generation of transgenic animals like Drosophila, Xenopus, Zebrafish and mice but are also efficient in different cell lines and embryonic stem cells. The transposon system is based on DNA transposons and is composed of a vector plasmid where the target gene is flanked by two inverted terminal repeats (ITR). These sites are recognized by a transposase, which is injected as mRNA or as individual plasmid. The transposase cuts the transposon due to its ITRs and mediates the integration into the host genome.

Recent studies have shown the capability different transposons in transposon mediated transgenesis with BACs, whereby some seems to be more competent then others.

To investigate the transposition capacity of sleeping beauty 100X (*SB100X*), a hyperactive synthetic transposase, a 220kb large BAC was design by recombineering. The BAC carries a DTR-IRES-tdTomato cassette flanked by ITR sites and was co-injected with 2pl of 5ng/µL *SB100X* mRNA into the pronucleus of C57BL/6N zygotes, whereby two different concentrations of BAC (1.0ng/µL and 2.0ng/µL) were used. As control group 0.4ng/µL of a 6.3kb large plasmid carrying the green fluorescence gene Venus, which was previously established in our laboratory, was injected with 5ng/µL *SB100X* mRNA into the pronucleus. All manipulated embryos were carried to term by CD1 pseudo-pregnant surrogate mothers.

In total, 37 mice were born in the BAC approach, but none had successful integration of the BAC plasmid, as determined by fluorescence check and genotyping PCR.

Also in the control group only one mouse exhibit an integration of the Venus transposon. This bad outcome of transgene animals might be caused by degradation of the transposon mRNA. Furthermore the design of the BAC could be adverse, since the ITR sites are spaced by more than 1kb, which has been proven to be a limiting factor in the transposition of large plasmids.

The other approach of my master thesis was the examination of the transposition efficiency in cytoplasmic microinjection compared to classical pronucleus injection. The same Venus plasmid as in the BAC experiment was used.

The co-injection of 2pL injection mix containing 0.4ng/µL Venus plasmid and 5.0ng/µL *SB100X* mRNA into the cytoplasm results in 14 transgene positive mice, which corresponds to 31.1%. Compared to 62.3% transgene animals generated by pronuclear microinjection, in our previous experiments by the same concentrations and conditions, the cytoplasmic injection clearly displays a lower efficiency.

Zusammenfassung

Die Herstellung transgener Tiermodelle ist in der Molekular Biologie ein unverzichtbares Werkzeug zur Erforschung von Genfunktionen, Krankheiten und für die Entwicklung neuer Therapeutika.

Unter den vielen vertebraten Modellorganismen stellt die Maus aufgrund ihrer hohen genetischen Ähnlichkeit zum Menschen, der relativ einfachen genetischen Manipulierbarkeit und des kurzen Generationszyklus das meist verwendete Modell für die Genetik und Molekularbiologie der Säugetiere dar. In den letzten Jahrzehnen wurden diverse Methoden zur Manipulation des Mäusegenoms und zur Herstellung transgener Tiere entwickelt, wobei die Vorkerninjektion rekombinanter DNA Sequenzen und virale Transduktion lange Zeit die bevorzugten Methoden für die Erzeugung transgener Mäuse mit stabil vererbbarer genomischer Integration des Transgenes darstellten. Obwohl beide Methoden von ihren jeweiligen Eigenheiten profitieren, konnten die gleichzeitig daraus entstehenden Probleme wie geringe Integrationseffizienz, technischer Aufwand und die hohen Sicherheitsanforderungen im Umgang mit Viren nicht überwunden werden. In den letzten Jahren wurde daher die Verwendung von Artifiziellen Bakterienchromosomen sogenannten bacterial artificial chromosomes (BAC) etabliert. BACs sind ringförmige DNA Plasmide über 100kb und bieten daher die Möglichkeit auch große Transgene und regulative Sequenzen in das Transgen zu inkludieren. Weiters überwinden sie auf Grund ihrer Größe die typischen Probleme kleiner Plasmide wie Konkatermerisation, und Stilllegung durch Integration in heterochromatische Bereiche des Genoms. BACs enthalten eigene cis- regulative Elemente, die die Expression des Transgens stabilisieren. Um die Integrationseffizienz zu erhöhen wurden BACs mit dem Transposonsystem kombiniert. Transposon vermittelte Transgenese basiert auf DNA Transposons, wobei die essentiellen Bestandteile eine Transposase und ein Vektorplasmid dessen Transgen von zwei ITR Elementen flankiert ist, darstellen. Die Transposase, ein Enzym das die ITR Seiten erkennt, bindet und sowohl das Ausschneiden des Transgens aus dem Vektor wie auch dessen Integration in das Wirtsgenom moderiert, wird entweder als eigenständiges Plasmid oder aber als mRNA in den Modellorganismus eingebracht. Der Erfolg dieser Methode wurde bereits mit kleineren Plasmiden zur Herstellung von transgenen Tieren und Zelllinien aufgezeigt. Von den verschiedenen etablierten Transposonsystemen scheinen mache geeigneter für die Transposition von großen Transgenen zu sein als andere.

Die hyperaktive Transposase Sleeping Beauty 100X (*SB100X*) wurde aus verschiedenen ausgestorbenen Knochenfischgenomen synthetisiert und hat sich in diversen Experimenten als sehr effizient in der Transposition von kleinen Plasmiden in Zebrafisch und Maus erwiesen. Um die Funktionalität von *SB100X* auch für große Plasmide zu ermitteln, wurde ein 220kb großes BAC das eine ITR flankierte DTR-IRES-tdTomato Kassette trägt, per Rekombination hergestellt. Das BAC wurde gemeinsam mit 2pl Pufferlösung die 5ng/µL *SB100X* mRNA enthielt, in den Vorkern von C57BL/6N Zygoten co-injiziert, wobei zwei verschiedene Konzentrationen verwendet wurden (1.0ng/µL und 2.0ng/µL). Als Positivkontolle wurde ein 6.3kb großes Venus Plasmid unter denselben Voraussetzungen injiziert. Das Venus Plasmid kodiert für ein grün fluoreszierendes Reportergen. Seine Transpositionsfunktionalität in Kombination mit *SB100X* war in unseren vorangegangenen Experimenten bereits bewiesen worden. Wobei eine Transpositionseffizienz von 62.3% in Mäusen erreicht werden konnte. Von den insgesamt 37 geborenen Tieren die durch die Vorkerninjektion des BAC Plasmides hergestellt wurden, wies keine Maus die erfolgreiche Integration des Transgenes auf. Auch in der Kontrollgruppe war nur eine Maus Venus positiv. Dieses unerwartet negative Resultat, lässt ein technisches Problem, wie die Degradierung der mRNA durch RNAsen vermuten, zumal die hohe Transpositionsrate von Venus durch *SB100X* bereits bewiesen wurde.

Ein weiteres Ziel der Masterarbeit was der Vergleich von Vorkerninjektion und zytoplasmatischer Injektion zur Herstellung von Transposon vermittelter transgener Tiere. Die zytoplasmatische Injektion wird inzwischen in großen Säugetieren wie Schwein und Kuh routinemäßig durchgeführt, und ist technisch weniger aufwendig und weniger invasiv als die Injektion von Plasmiden in den kleinen Vorkern. Für dieses Experiment wurden wiederum das bereits etablierte Venus Plasmid verwendet, wobei 2pL Injektionsmix bestehend aus 0.4ng/µL Venus Plasmid und 5.0ng/µL *SB100X* in das Zytoplasma von C57BL/6N Zygoten co-injiziert wurde. Als Referenz wurden die Ergebnisse der Vorkerninjektion von Venus aus dem vorangegangen Experiment verwendet, da beide Versuche unter denselben Bedingungen durchgeführt wurden. Insgesamt konnten 14 venustransgene Mäuse hergestellt werden, was einer Integrationsrate von 31.1% entspricht. Damit weist die zytoplasmatische Injektion nur halb so viel Erfolg wie die Vorkerninjektion auf (62.3% transgenene Tiere).

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Equipment, Chemicals, Reagents and Stocks

Axiovert 200, Zeis, Vienna, A

BioPhotometer®, Eppendorf, Hamburg, D

Centrifuge 5415 R®, Eppendorf, Hamburg, D

DNA Engine Dyad® Thermal Cycler. Peltier Thermal Cycle, BIO-RAD, Berkeley, CA

Emission Filters FHS/EF-4Y2, BLS Ltd., Budapest, H

Exitation Light Source FBL/Basic-B&N-01, BLS Ltd., Budapest, H

FemtoJet, Eppendorf, Hamburg, D

Gel iX Imager, INTAS, Göttingen, D

Gene Pulser MXcell®, Electroportion System, BIO-RAD, Berkeley, CA

Light Head FHS/LS-1B, BLS Ltd., Budapest, H

Mini-Sub Cell GT, BIO-RAD, Berkeley, CA

Thermomixer Compact®, Eppendorf, Hamburg, D

Power Supply PowerPac Basic, BIO-RAD, Berkeley, CA

Shaking Incubator 3031, GFL GmbH, Burgwedel, D

Sub-Cell GT, Bio-Rad, Vienna, A

Thermomixer Compact, Eppendorf, Hamburg, D

TransferMan® NK2, Eppendorf, Hamburg, D

UV Crosslinker – Stratalinker®, Stratagene/Agilent, Vienna, A

Vortex Mixer neoLab 7-2020®, Heidelberg, D

Agarose, Sigma-Aldrich, Steinheim, D

Ampicillin, Sigma-Aldrich, Steinheim. D

Bacteriological Agar, Sigma-Aldrich, Steinheim, D

Bacto-tryptone, Roth, Karlsruhe, D

Bromphenol blue, Merck, Darmstadt, D

dNTPs, MBI Fermentas, St. Leon-Rot, D

Ethanol absolute, Sigma-Aldrich, Seelze, D

Ethidium bromide, Sigma-Aldrich, Steinheim, D

EDTA (Ethylene diamine tetracetic acid), Roth, Karlsruhe, D

Glycerol, Sigma-Aldrich, Steinheim, D

Isopropanol, Sigma-Aldrich, Seelze, D

PBS, Sigma, Vienna, A

Peptone, Roth, Karlsruhe, D

Tris, Roth, Karlsruhe, D

Water biochemical, Molecular Biology Grade, VWR International GmbH, Darmstadt, D

Xylene Cyanol FF, Sigma-Aldrich, Steinheim, D

Yeast exctract, Roth, Karlsruhe, D

Enzymes, Kits, Oligos

BioTaq DNA- Polymerase, Dialat Ltd., Moskau, RU

GeneRulerTM DNA ladder mix, Fermentas GmbH, St. Leon-Rot, D

Oligo Sequences, Fisher Scientific, Vienna, A

Proteinase K, Fermentas GmbH, St. Leon-Rot, D

^{32}P-γ-ATP, New Englands BioLabs,

peqGOLG GEL Extraction Kit, Peqlab

Restriction Endonuclease FspBI (BfaI), Fermentas GmbH, St. Leon-Rot, D

Restriction Endonuclease BglI, Fermentas GmbH, St. Leon-Rot, D

T4 DNA Ligase, Fermentas GmbH, St. Leon-Rot, D

Software

Digital Imaging KS300/KS400, Zeiss, Vienna, A

NCBI-BLAST (nucleotide-nucleotide, blastn) http://www.ncbi.nlm.nih.gov/blast/

NCBI-Pubmed http://www.ncbi.nlm.nih.gov/

Screenshot Gel iX and Gel Jet Imager Acquisition Software INTAS

ApE – A plasmit Editor

BioEdit Sequence Alignment

TierBase version 3.8.5 Nielson and Mossmann, 2003

Recipes for Buffers and Reagents

Church blocking buffer containing 2L

1000mL 1M NaP (pH 7,2) = 0,5M

700 mL 20% SDS = 7%

2mL 0,5M EDTA = 0,5mM

100µg/mL salmon sperm

Church Wash Buffer

0.5M NaP

1% SDS

Glycerine stock 10%

54mL mQ (dd H_2O)

6mL Glycerole

10x Loading Buffer for Agarose gel electrophoresis

50% glycerol

0.1M EDTA pH 8.0

0.05% Bromophenol Blue

0.05% Xylene Cyanol FF

Lysogeny Broth medium (LB), 1L

10g peptone

5g yeast extract

5g NaCl

1mL NaOH (1mol)

H_2O up to 1L

LB Agar dishes

1.5g Bacteriological Agar /100mL LB media

Chloramphenicol	100 mg/mL	12.5ug/mL
Ampicillin	34mg/mL	50ug/mL

Tetracyclin	10mg/mL	3ug/mL
Kanamycin		50ug/mL

TE pH 8.0, 50mL

10mM Tris-Cl

1mM EDTA

pH 8.0

TNES buffer 100mL:

5mL 1M Tris (pH 7.5)

8mL 5M NaCl

20mL 0.5M EDTA

5mL 10% SDS

62mL dd H_2O

1M Tris

121g Tris base

dissolve in 800mL H_2O

adjust to desired pH with concentrated HCl

autoclave

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Curriculum vitae

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Education

2010 -2013: Master: Molecular Medicine, University of Vienna

Master thesis: 1.9 2012 – 30.6.2013

„Investigation of transposon mediated transgenesis using a Bacterial Artificial Chromosome (BAC) in mice“
Institute of Laboratory Animal Science, University of Veterinary Medicine Vienna, Supervisor: Prof. Dr. Thomas Rülicke

2007-2010: Bachelor: Biomedicine and Biotechnology, University of Veterinary Medicine, Vienna

Bachelor thesis: 1. 2. - 30.6.2010

„Analysis of DNA-Methylation in patients with colorectal cancer “, Institute for Clinical Pathology, Medical University Vienna, Supervisor: Dr. Gerda Egger

2002-2007: Institution of higher education for Ecology and Economy Yspertal, Matriculation June 2007

1998-2002: Secondary School II Grieskirchen

1994-1998: Elementary School Gallspach

KNOW-HOW AND EXPERTISE

University:

Internships:

- „ Expression of histone deacetylase 8 in embryonic chicken brain “ Institute for Embryology, Medical University of Vienna; Supervisor: Dr. Christian Schoefer; March – July 2012

- „Establishment of a mouse model for human CML per intrabone marrow transplantation “ Institute of Laboratory Animal Science, University of Veterinary Medicine Vienna; Supervisor: Dr. Ullrike Scherer, September – December 2011

- „Establishment of an erythropoietin knock-out mouse“ Institute of Laboratory Animal Science, University of Veterinary Medicine Vienna; Supervisor: Dr. Thomas Kolbe, September - December 2008

- „Establishment of a murine stem cell line“ Institute of Laboratory Animal Science, University of Veterinary Medicine Vienna; Supervisor: Dr. Susanne Klinger, May – June 2008

- „The role of pax5 during embryonic development of medaka“ Institute for animal husbandry and genetics, University of Veterinary Medicine Vienna; Supervisor: Dr. Thomas Cerny, February 2008

Technical skills:

- FELASA-B course
- Cryo conservation
- Embryo transfer
- Microinjection
- Vasectomy
- Genotyping
- Bisulfite conversion
- qPCR
- Cell culture
- Microsurgery (embryo transplantation)
- Preparation of chicken embryo
- Live imaging
- Isolation of primary cells from mouse liver
- Immunofluorescence and Immunohistochemistry

School:

Languages: English, fluent

- French, basic knowledge

School projects:

- Project week Austria 2007
- Project week Netherlands 2006
- Project week Tokaj, Hungary 2005
- Project week Matrei, Austria 2004
- "European Youth Parliament", Kreissau, Poland, 2004
- "Eco-Expert Project", Vesselí, Czech Republic, 2004

Class projects:

- Waste management concept, 2007
- Food analytics, 2006
- Analysis of drinking water, 2005
- Alternative forms of energy, 2004

Further skills:

- QM- Manager
- waste management adviser
- dangerous goods safety adviser

Work experience:

Jobs:

- Since December 2012: research volunteer, Institute of Pharmacology Medical University of Vienna
- December 2012 –April 2013: Laboratory for milk hygienic, Clinic for Ruminants, University of Veterinary Medicine Vienna

Ferial internships:

- August 2012: Pöttinger Agricultural engineering GesmbH, Department for Quality management and Safety, Grieskirchen
- August 2011: Pöttinger Agricultural engineering GesmbH, Department for Quality management and Safety, Grieskirchen
- July 2009: Laboratory for Microbiology and Hygiene, Clinical center Wels – Grieskirchen
- July 2008: Merkur, Wels
- Summer 2006 and 2007: Reiding stable Aicherhof; Hofkrichen
- September 2005: Pöttinger Agricultural engineering GesmbH, Department for technical construction
- July 2005: Cobbins Nursery Ldt., Worthing, England
- June 2005: Pöttinger Agricultural engineering GesmbH, Department for disposal engineering, Grieskirchen
- July 2004: Bio winery Nikolaihof, Mautern/Krems.