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SB100X-mediated Mouse Transgenesis

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1. Introduction

1.1 The Mouse as a Model Organism

Model organisms are widely used in basic research to get insight into biological phenomena. Due to anatomic, physiologic and genetic similarity, the laboratory mouse (*Mus musculus*) is an excellent mammalian model for studying human gene function and regulation (van der Weyden et al., 2002). Also the short life cycle, the small size and the fully sequenced genome makes the mouse an attractive model system. Sequencing both, the mouse and the human genome showed that we share 99 % of our encoded sequences. The field of functional genomics is now interested in studying how these genes act and what pathways and processes they regulate in the physiological setting. (Nguyen and Xu, 2008)

The first functional analyses in the mouse were limited to spontaneous mutations which occur in a very low frequency (~ 5 x 10^{-6} per locus) (Stanford et al., 2001). Therefore, several mutagenesis strategies have been developed through the last century trying to detect certain genes associated with interesting phenotypes.

1.2 Mutagenesis Strategies

1.2.1 Physical and Chemical Mutagenesis

In the 1930s the first X-ray mutagenesis experiments were carried out. The interest increased when larger centres began to study the effects of radiation fall-out on genetic stability. Exposure to X-ray causes a mutation frequency which is 20 – 100 times higher than that of spontaneously occurring mutations (Stanford et al., 2001). However, the resulting mutations consist mainly of chromosomal aberrations which are not suitable for the analysis of single genes.

Another mutagen that leads to such chromosomal rearrangements, especially smaller deletions and translocations, is the chemical chlorambucil. It shows a higher

mutagenesis frequency than X-ray. Both, X-ray and chlorambucil are useful for genetic screens and mapping studies but they do not lead to identification of individual gene function. Therefore, they are not used in high- throughput approaches. (Stanford et al., 2001)

A preferred approach to the mutagenesis strategies described above is chemical mutagenesis with the laboratory-synthesized mutagenic agent N-ethyl-N-nitrosourea (ENU). It is a highly effective compound to induce random, single base pair mutations (Kennedy and O'Bryan, 2006). ENU transfers its ethyl group directly to oxygen or nitrogen radicals in DNA, which results in mispairing leading to point mutations if not repaired (Justice et al., 1999). More than 82 % of sequenced mutations show AT to TA transversion and AT to GC transition. This preference for AT base pairs results in a higher mutation rate for AT-rich regions in contrast to GC-rich regions. Also the length of a gene affects the rate at which it will be mutated because larger genes provide a longer target for mutations. (Kennedy and O'Bryan, 2006)

To create mutants, male mice are injected intraperitoneally with ENU resulting in random mutations in spermatogonial stem cells which can be passed on to the offspring (Kennedy and O'Bryan, 2006). Pre-meiotic spermatogonial stem cells show the highest rate of mutation of any cell type examined with a single locus mutation frequency of 6-1.5 x 10^{-3}. In a single gene of choice the desired mutation is obtained in one out of every 175-655 screened gametes. (Justice et al., 1999)

ENU can cause different mutated alleles of one gene (allelic series) which may have different effects on the protein product enabling the identification of the critical domains within a protein. Most of the induced mutations are loss-of function variants but also gain-of function mutants can be obtained. (Kennedy and O'Bryan, 2006)

1.2.2 Generating Transgenic Mice

Transgenic mice are genetically modified organisms which carry genome integrations of *in vitro* recombined DNA sequences and are able to inherit the mutation to the following generation (Rülicke, 2001). In mice there are different strategies known to produce transgenic founders. The first method to introduce foreign DNA into early

mouse embryos was retroviral infection (Jaenisch, 1976) followed by microinjection of DNA constructs into pronuclei of fertilized oocytes (Gordon et al., 1980). A later developed alternative is the introduction of foreign coding sequences into cultured embryonic stem (ES) cells followed by blastocyst injection of selected cells carrying a targeted mutation. The resulting chimeric animals can give rise to transgenic founder lines in the next generation (Carlson and Largaespada, 2005).

There are many applications for transgenic animals. In basic research they are used to obtain information on gene function and regulation. Moreover, nowadays transgenic animals of several other mammalian species than mice are used to obtain high value products like recombinant proteins and xeno-organs for humans but also to improve animal products for human consumption (Houdebine, 2005).

1.2.2.1 Viral Gene Transfer

In 1976 the first transgenic mouse was produced by infection of 4-8 cell embryos with exogenous Moloney leukemia virus (MLV). The born chimeric animals were able to pass on the integrated virus DNA to their offspring (Jaenisch, 1976). However, the retrovirally delivered genes were frequently not expressed in the newborn mice probably due to recruitment of host factors which recognize viral long-terminal-repeats (LTRs) and repress viral gene expression (Pfeifer, 2004). This phenomenon of gene silencing has clearly reduced the utility of retroviral transgenesis. Another disadvantage of using retroviruses is their dependence on cell cycling. That means that gene transfer can only occur in host cells that are actively replicating at the time of infection (Pfeifer, 2004).

The great breakthrough for viral transgenesis was the switch to lentiviruses. Lois et al. (2002) and Pfeifer et al. (2002) used HIV derived SIN vectors to transfer a transgene to murine preimplantation embryos at the zygote and morula stage, resulting in the generation of transgenic mice. For the first time satisfying transgene integration *and* expression could be demonstrated for the use of viral vector systems. Also germ line transmission could be shown. (Pfeifer, 2004)

Lentiviruses belong to the large family of retroviruses and are characterized by a complex genome and morphology. The best studied lentivirus is the human immunodeficiency virus (HIV). Like the simple retrovirus MLV, lentiviruses are enveloped viruses with a RNA genome carrying *gag*, *pol* and *env* genes, encoding for internal structure proteins, viral enzymes like reverse transcriptase and envelope glycoproteins. After infection of host cells, the viral RNA genome is reverse transcribed into DNA and gets integrated into the host genome where it serves as a template for the production of progeny virions. Lentiviruses carry at least three additional genes necessary for their more complex life cycle which allows them to also infect non dividing cells because of active transport of virus genome to the nucleus of host cells. (Pfeifer, 2004)

When using viral vector systems for the generation of transgenic mice, the zona pellucida, a physical barrier that shields the embryo from viral infection has to be overcome. This can be performed either by removing the zona pellucida or by injection of viral particles into the space between zona pellucida and cell membrane of the zygote – the perivitelline space (Pfeifer, 2004). These techniques are less invasive for zygotes than the routinely used DNA pronuclear microinjection and, therefore, leading to an 8-fold increase in the number of transgenics per embryo treated and transferred to a surrogate mother. If just taking the offspring into account, lentiviral transgenesis is about 4-fold more efficient than pronuclear injection of recombinant DNA (Pfeifer, 2004). Recent studies could show enhanced transgenesis by intracytoplasmic injection of envelope free lentiviruses into mouse zygotes, leading to a transgenic rate of 97 % with number of transgene insertions ranging from one to 32 (Yang et al., 2007).

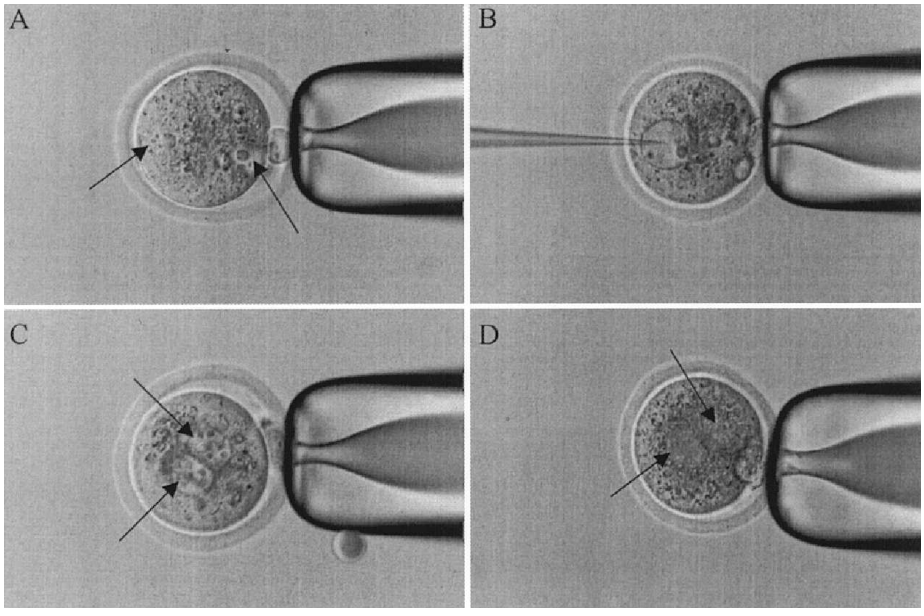
Yang et al. (2008) analysed lentiviral integration sites to determine if there are preferable sites for lentiviral integration in the early embryonic genome. They found no integration preference within specific chromosomes, repetitive elements or CpG islands but for integration into intragenic regions, especially in introns.

Early generations of lentiviral vectors were able to produce functional vector particles up to a genome size of 16 kb, however, with low efficacy. It was found to increase the transgenic efficiency when using smaller vectors with genome sizes of 5-7 kb. When calculating the cloning capacity one has to include the backbone of lentiviral vectors necessary for appropriate infection which is generally 1.6-2.2 kb (Park, 2007).

1.2.2.2 Pronuclear Microinjection

The possibility to introduce foreign DNA sequences into the one cell mouse embryo by microinjection has become a reliable and powerful method to study mammalian gene function. The male pronucleus is formed five to seven hours after fertilization, the female one is formed six to eight ours after entry of the sperm (Rülicke, 2001). Usually, a small volume containing 100 to 500 copies of the linear DNA-construct (Houdebine, 2005) is microinjected into the more-visible, male pronucleus (Stanford et al., 2001). Pronuclear injection can only be performed in mammals, because in lower vertebrates and invertebrates the pronuclei are not visible. Nevertheless, transgenesis is even possible in those species by DNA injection into the cytoplasm of the embryo (Houdebine, 2005).

Figure 1: Mouse zygotes – pronuclear microinjection



Development of pronuclei A) Male and female pronucleus becomes visible. B) Injection of DNA into the male pronucleus. C) Pronuclei draw near each other. D) Pronuclei disappear before the first cell division. (Rülicke and Hübscher, 2000)

In some of the injected zygotes the exogenously added DNA that consists of a protein coding sequence and regulatory elements integrates randomly into the host genome and becomes a stably heritable genetic trait. The desired integration event occurs sometimes already in the zygote. Frequently observed integration events in later developmental stages results in mosaic founder animals (Brinster et al., 1985). Mostly, the foreign DNA is inserted at one integration site as a head-to-tail concatemere with up to hundred of copies, but also examples of multiple integration sites have been observed (Brinster et al., 1985; Woychik and Alagramam, 1998). Concatemerization of foreign DNA increases the possibility of chromosomal rearrangements, aberrant splicing, heterochromatin formation or gene silencing (Miskey et al., 2005).

Integration into the host genome is just the first step. Once the transgene is integrated it should be expressed efficiently. A certain number of conditions have to be fulfilled to optimize transgene expression. Basically, the construct has to carry a promoter, splice sites, start- and stop-codon and a polyA-site. Long DNA fragments (100 kb or more) also contain further regulatory elements which are often located in a great distance from the transgene and its promoter. For correct RNA maturation and transfer of mature mRNA to the cytoplasm, the transgene should contain at least one intron. Additionally, the transgene must not contain too many GC-rich regions because they are often recognized as foreign elements and further their C becomes methylated (Houdebine, 2005). The successful expression of transgenes is also dependent on the integration locus. Heterochromatic regions consist of highly condensed DNA and are transcriptionally inactive. The transgene will not be expressed properly when integrated into heterochromatin or even in its nearness. This phenomenon is called “position effect variegation” (Rülicke, 2001).

However, just 1-3 % of microinjected embryos become transgenic animals (Houdebine, 2005). This estimated frequency is based on losses during injection and prenatal lethality. In mice an average number of up to 25 % of born animals carry the transgene (Rülicke and Hübscher, 2000).

Insertional mutations generated after pronuclear DNA injection are molecularly tagged by the transgene. This big advantage can be used to identify the integration locus by cloning of flanking regions. This fact enables insights to the genomic region disrupted

by the insertion and to identify endogenous genes that are possibly linked with the mutant phenotype (Woychik and Alagramam, 1998).

1.2.2.3 Gene Targeting by Homologous Recombination

The development of mouse embryonic stem (ES) cells in 1981 opened a new field of creating mouse mutants. It has become feasible to create mice carrying genetic alterations ranged from small point mutations and single gene disruption to large genomic deletions or even to generate specifically engineered chromosomal translocations (Muller, 1999).

Embryonic stem cells are isolated from the inner cell mass of cultured pre-implanted blastocysts. Because of their pluripotency, they are able to differentiate into every single cell type of an individual including the germ cells, when reinjected into a host blastocyst. This capacity depends on the culture conditions that keep the cell in an undifferentiated state. The inhibition of differentiation can be obtained by feeder cells that additionally serve as a matrix for ES cell adherence and/or by adding leukemia inhibitory factor (LIF) to the culture medium (Muller, 1999). Injection of ES cells into a host blastocyst leads to the formation of a chimeric animal with somatic and germ cells emanated from both sorts of cells, the host inner cell mass cells and the injected ES cells. Through breeding one can obtain animals heterozygous for the genetic modification in every cell (Carlson and Largaespada, 2005).

In 1986 it could be shown, that ES cells can be genetically modified *in vitro* by introduction of a transgene. Three years later the groups of Smithies and Capecchi had demonstrated, that recombination of incoming DNA and the homologous sequence present in the genome is possible (Babinet and Cohen-Tannoudji, 2001), even if this event is relatively rare compared to random integration which is 1000-fold more likely to occur (Vasquez et al., 2001). Recombination frequency is affected by many factors like length of total homology between vector and targeted locus or chromatin structure (Babinet and Cohen-Tannoudji, 2001).

Homologous recombination of introduced DNA and the genome offers the possibility to create targeted mutants at a chosen locus. This method is widely used for creating

null mutants with at least one knocked out gene. For the creation of so called knock out mice two types of vectors can be used. (I) When using a replacement vector, one exon of an endogenous gene is disrupted by a neomycin resistance marker after homologous recombination with the linearized targeting vector. The vector is also flanked by a HSV thymidin kinase gene which gets lost upon homologous recombination but not upon random integration. (II) In contrast, insertion vectors are linearized within the region of homology between one exon and the marker gene that allows positive selection. Homologous recombination events lead to integration of vector sequences and partial duplication of genomic sequences. (Muller, 1999)

Before the presumed phenotype of a knock out mutant can be analyzed, one must assess whether any residual protein is expressed by the targeted locus. As long as remaining coding sequences of the targeted gene are still present in the genome, truncated or mutant forms of the protein may still be expressed. Truncated polypeptides may acquire new properties like transdominant interactions with other proteins. (Muller, 1999)

Removal of all coding sequences of a gene may circumvent this problem of residual protein expression. But deletion of large genomic regions can lead to loss of yet unknown genes resided in introns or encoded by the opposite strand, or regulatory elements controlling the expression of unrelated endogenous genes (Muller, 1999).

An interesting variation of the targeting vectors for creating null mutants can be obtained by the so called “knock in” technology. This method uses the introduction of a given cDNA in frame with the coding sequence of the targeted gene. Homologous recombination between the vector and the endogenous gene results in expression of cDNA instead of the targeted gene. When using the coding sequence for a reporter gene like *lacZ* from *E.coli*, encoding β-galactosidase, the expression pattern of the targeted gene gets perfectly mimicked. This can be useful for studying expression patterns but also for monitoring the fate of cells that normally express the targeted gene. (Babinet and Cohen-Tannoudji, 2001)

1.2.2.4 Gene Trapping

Gene trapping takes the middle path between random and target-oriented mutations. Trapping vectors are used to either disrupt transcription of chromosomal genes by random insertion or to report expression patterns of endogenous genes (Stanford et al., 2001). There are several types of trapping vectors with different applications. On the one hand, trapping vectors are used to disrupt endogenous gene expression and, therefore, create null mutantions. On the other hand, special types of trapping vectors can be used to identify regulatory elements or to report expression patterns of endogenous genes (Stanford et al., 2001; Carlson and Largaespada, 2005). They are introduced into ES cells by electroporation or retroviral infection. Mutated cells are then selected and injected into blastocysts before they are transferred to pseudo pregnant foster mice (Nagy Andras et al., 2003).

All three types of trapping vectors contain a reporter gene (e.g. *lacZ*) and a neomycin resistance gene driven by an autonomous promoter which allows selection in ES cell culture (Stanford et al., 2001). Promoter traps are the commonly used trapping vectors. They contain a splice acceptor followed by the reporter gene and a polyadenylation signal (pA site). The promoter trap vector needs to be inserted in the coding sequence of an endogenous gene for reporter gene expression. The resulting gene product is a fusion transcript and protein between the upstream sequence of the trapped gene and the reporter (Stanford et al., 2001; Carlson and Largaespada, 2005).

The advantage of using promoter traps is that the insertion site is in transcribed DNA, so the disrupted gene can be identified by cloning. On the other side, the disadvantage of this strategy is the low integration frequency into exons (Stanford et al., 2001).

In comparison, gene trap vectors contain a splice acceptor immediately upstream of the reporter gene and a polyadenylation signal. They are also lacking an own promoter. It will, like the promoter trap, also lead to a fusion gene product if integrated into an intron. This avoids the expression of the trapped gene but reports its expression pattern. Because insertion appears in an intron, alternative splicing can lead to a lower level of transcripts. (Stanford et al., 2001)

Another useful trapping vector to identify regulatory elements of endogenous genes is the enhancer trap system. This type of vector includes a full expression cassette with a

minimal promoter which is not active unless affected by a positive *cis* regulatory element. (Carlson and Largaespada, 2005; Stanford et al., 2001)

The drawback with the trapping vectors described above is that the reporter genes are not expressed when the trapped gene or promoter is not active in undifferentiated ES cells. To prevent this problem a so called polyA trap vector can be used. It contains a promoter, a reporter gene and a splice donor, but lacks a polyA signal. The reporter is expressed if inserted into an exon in the correct orientation. In this case the endogenous gene will also be disrupted. (Carlson and Largaespada, 2005)

Trapping vectors are very useful tools to identify genes or regulatory elements and to study their gene function or expression patterns.

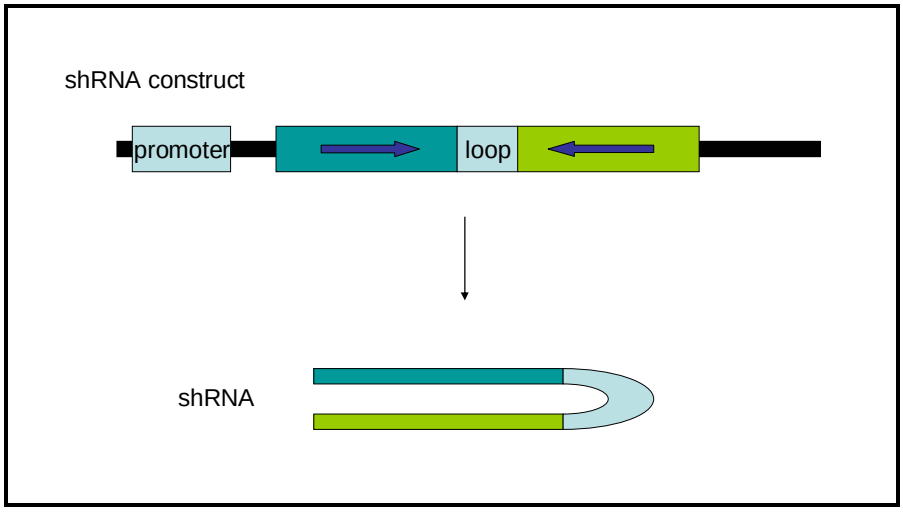
1.2.2.5 Gene Targeting by RNAi: the Knock Down Approach

Another valuable alternative for studying gene function in vivo is the RNA interference-based mutagenesis approach where expression of a chosen gene can be down regulated to a minimum. In 1998, Fire et al. described the phenomenon of post-transcriptional gene repression by injection of double stranded (ds) RNA in adult *C. elegans*.

RNA interference is a highly conserved mechanism throughout all kinds of organisms. When dsRNA is present in a cell, a complex cascade of molecular events gets started. During this pathway, the cellular enzyme Dicer binds to dsRNA and catalyzes the cleavage into short pieces of about 20 nucleotides in length known as small interfering RNAs (siRNAs). siRNAs are then incorporated in a RNA-induced silencing complex (RISC). This enzyme uses the incorporated siRNA to find and degrade the homologous mRNA. (Mocellin and Provenzano, 2004)

RNA interference can be induced in mammalian cells either by transfection of dsRNA molecules or by expression of small hairpin RNAs (shRNAs). Both strategies will lead to Dicer catalyzed cleavage into siRNAs (Peng et al., 2006).

Figure 2: Schematic illustration of a small hairpin RNA construct



ShRNA constructs consist of a promoter and a sense and antisense region separated by the loop sequence. Sense and antisense region are reverse complementary and form a hairpin structure through self pairing after transcription.

For in vivo models, transgenic RNAi mice or rats can be produced either by ES cell manipulation, or directly by modification of the one-cell embryo by lentiviral infection or pronuclear injection of shRNA expression constructs. Upon genomic integration, the construct becomes stably inheritable and leads to long-term down-regulation of the chosen gene. (Peng et al., 2006)

RNAi knock down technologies offer a lot of new possibilities especially for studying gene function in rats and other organisms where gene knock out is rather difficult compared to the well established ES cell based approach in mice.

1.2.2.6 Gene Targeting by Zinc Finger Nucleases

Zinc finger nucleases (ZNFs) are artificial restriction endonucleases which consist of two domains, a target site specific DNA binding domain fused to an unspecific nuclease domain. The binding domain is called zinc finger array and can be engineered to recognise any DNA sequence of interest. Once it binds to the target site, a double strand break is induced. Such a break can be repaired by non homologous DNA end

joining (NHEJ), an error-prone process which often leads to the creation of insertions or deletions at the site of the break. (Foley et al., 2009)

ZFN induced DNA double strand breaks and the resulting non homologous end joining mediated repair can be used as a tool for highly efficient genome manipulations. They were already established for experiments in mammalian cell culture, in fruit flies and zebrafish and were recently used for the creation of knock out rats. (Foley et al., 2009; Geurts et al., 2009)

For elimination of specific rat gene function, Geurts et al. (2009) delivered ZFNs to embryos by pronuclear or intracytoplasmic injection of either ZFN encoding DNA or mRNA. Best results were obtained by intracytoplasmic injection of high amounts of ZFN mRNA to disrupt *IgM* gene function. 75 % of born animals showed to carry mutations in the targeted genomic sequence but no gene disruption at any of 20 predicted off-target sites indicating that ZFNs are highly specific for their target site.

The strategy of ZFN-mediated genome manipulation opens up a range of new experiments especially in laboratory rats where targeted genome modifications are largely intractable.

1.3 Transposable Elements

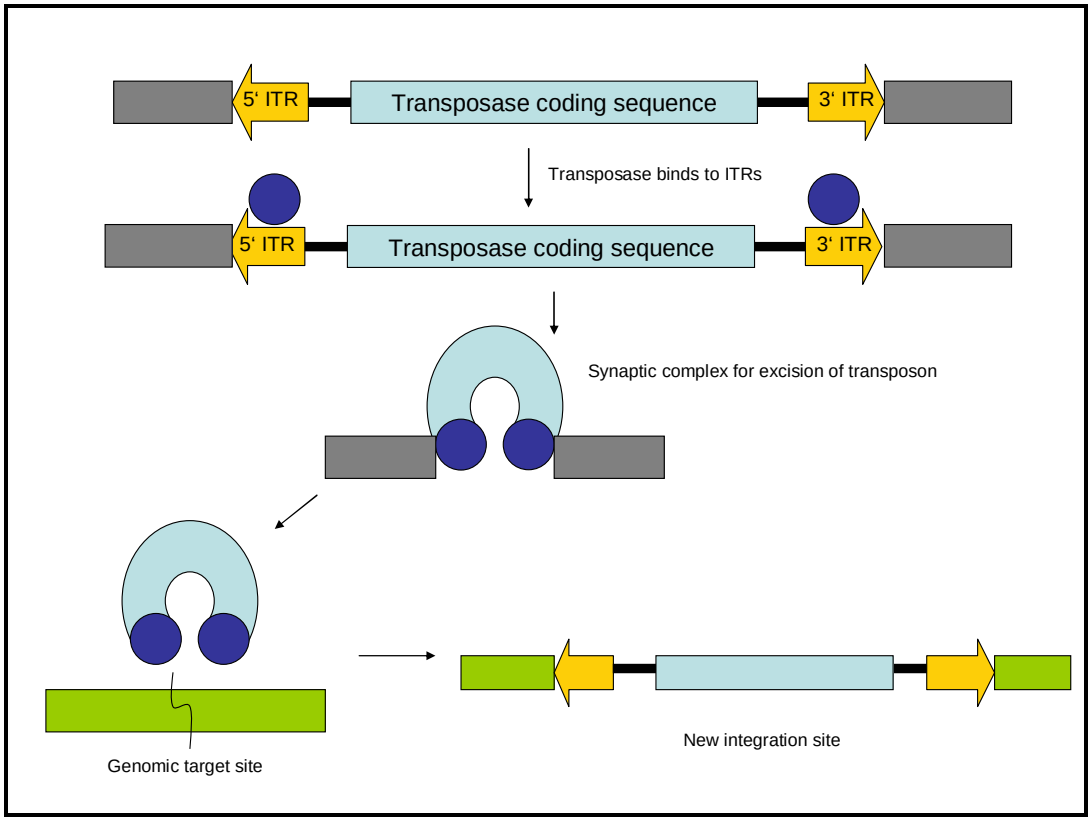
Transposable elements (TEs) are DNA sequences that possess an intrinsic capability to change their genomic position. In the 1940s Barbara McClintock first discovered chromosomal elements with the ability to move from place to place in maize (Comfort, 2001). Nowadays, transposable elements have been detected in all organisms from bacteria to humans and form a major fraction of eukaryotic genomes (Miskey et al., 2005). 35 % of the human genome is recognizable as transposon DNA compared to exons of cellular genes which represent about 5 % (Yoder et al., 1997). Nevertheless, most of these TEs are inactive because in the absence of selection pressure, “vertical inactivation” leads to accumulation of mutations in the transposon sequence (Ivics et al., 2004).

TEs are distinguished whether their movement involves an RNA intermediate like retroelements (class I transposable elements) or relies exclusively on DNA intermediates like DNA transposons (class II transposable elements) (Miskey et al., 2005).

DNA transposons move in the host genome via a “cut and paste” mechanism. They are simply organized DNA sequences with a coding sequence for a protein called transposase flanked by inverted terminal repeats (ITRs) (Mates et al., 2007).

Transpositon is catalyzed by the transposase which recognizes binding sites within the ITRs leading to excision and following reintegration (Miskey et al., 2005).

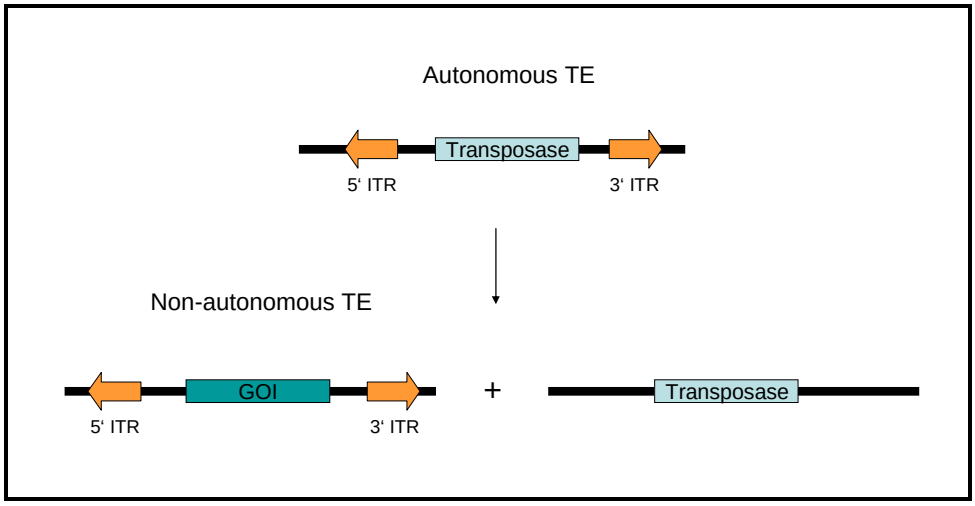
Figure 3: Schematic illustration – transposon excision and reintegration



Transposase binds to ITRs and catalyzes excision from genomic dondor site. Also following transposition and integration of transposon to genomic target site is catalyzed by the transposase.

Transposition can easily be controlled by separating the source of transposase from the transposable DNA harbouring the ITRs, thereby creating a non-autonomous TE. In this case the transposon will only be able to move when the transposase is offered in *trans* (Mates et al., 2007).

Figure 4: Autonomous and non-autonomous TE



Non-autonomous TEs can be created easily by separating the transposase conding sequence from transposon DNA that harbours the ITRs. Every gene of interest (GOI) can be placed between the ITRs.

The sequence between the ITRs plays no role for transposition. Any sequence of interest can be positioned between the ITRs, depending on experimental needs (Mates et al., 2007). Due to the ability to cause mutations by inserting into genes, transposable elements became a great tool for genetic manipulations, including the generation of transgenic animals and insertional mutants (Ding et al., 2005).

1.3.1 Transposon-based Mutagenesis

Since their discovery, TEs became invaluable tools for genetic analysis in many organisms. In prokaryotes, transposon-based mutagenesis has led to discovery of microbial pathogenesis genes. In eukaryotes, transposons have already been found to facilitate functional genetics research greatly in funghi, plants, lower metazoan models and invertebrates and have recently been established for their use in vertebrate genome manipulations (Ding et al., 2005; Mates et al., 2007; Miskey et al., 2005).

Especially in *Drosophila melanogaster* transposons are widely used in many applications as important mutagenesis tools. The predominantly used transposable element in this species is the *P* element which is a currently active resident TE in

Drosophila. *P* elements are not active outside the *Drosophila* genus, indicating that host factors are required for transpositional events. Because they are present in recently wild caught strains but not in laboratory stocks established during the first half of the 20th century, *P* elements are thought to be very recent invaders (Mates et al., 2007). This and also the high transpositional activity are the great advantages of this transposable element. In contrast to the *Tc1* element used in *Caenorhabditis elegans* mutagenesis where mutation identification is very difficult because of multiple *Tc1* copies which are present in the genome, it was possible in laboratory *Drosophila* stocks to create transgenic flies each containing a separate component of the binary transposon system (Mates et al., 2007). One stock ("jump-starter") was generated to carry the coding sequence for *P* element transposase, the other stock ("mutator") was created to harbour a non-autonomous transposon in the genome. Upon inter-crossing, double transgenic flies are obtained. Through outcrossing to wild type animals the transposon can be mobilized and this leads to new integration sites. This system is extremely suitable for forward genetics applications and has also been tested for vertebrates like zebrafish (Ivics et al., 2004) and even for mice (Dupuy et al., 2001; Fischer et al., 2001; Ivics et al., 2004; Horie et al., 2001).

Because of fairly inefficient homologous recombination in *Drosophila*, TEs can also be used here for reverse genetic approaches. For generating targeted gene replacement, TE excision induced double strand breaks resulting in DNA repair is used to enhance recombination frequency (Mates et al., 2007).

When performing transposon-based mutagenesis, it is important to know the insertion patterns of different transposon systems. Chromatic integration patterns and tolerance for cargo size vary greatly between elements. While the efficiency of transposition exponentially decreases with increased cargo size of *Tc1/mariner* elements, for *P* elements the cargo size is not limiting utility (up to 100 kb are possible) (Mates et al., 2007). Preference of integration sites should be checked before using a certain transposon system for mutagenesis purpose. Members of the *Tc1/mariner* family target their integration into TA dinucleotides and show no or weak preference for transcription units. The 5' regulatory regions are disfavoured; most hits in genes are localized in introns. On the contrary, the *P* element shows a clear preference for 5'

regulatory regions of genes. Also *piggyBac* which names an own transposon family, the *piggyBac* family, shows high preference for transcription units (Mates et al., 2007). Nowadays, several transposon systems are used for transgenesis and insertional mutagenesis in vertebrates, expanding our abilities in genome manipulations in vertebrate model organisms.

1.3.2 Sleeping Beauty

The best characterized DNA transposons found in vertebrates are members of the *Tc1/mariner* superfamily from teleost fish. *Tc1/mariner* elements are extremely widespread in nature, in contrast to other transposable elements like the *P* element (*Drosophila*) which is restricted to one genus. This indicates that host requirements for *Tc1/mariner* transposition are not that tight. All of the isolated TEs in vertebrates accumulated several mutations in their transposase genes and are therefore inactive (Ivics et al., 2004). In 1997, Ivics et al. engineered an active transposon system on the basis of a consensus sequence obtained from 12 remnant *Tc1/mariner* elements from eight different fish species and named it *Sleeping Beauty* (SB).

The SB transposon system consists of two functional components: the transposase encoded by a synthetic gene, and a second, nonautonomous element carrying ITRs with recognition sites for the transposase. For transposition, both components have to at least temporarily co-exist in a cell (Ivics et al., 2004).

Most transposable elements do not integrate randomly into the genome. Target selection can depend on DNA sequences or chromatin structure. SB shows a relatively random insertion profile. Transposition assays performed in human HeLa cells showed that although some chromosomes were hit more frequently than others, no clear preference for any chromosome could be detected. 35 % of the transposition events occurred in transcribed regions. About one third of the human genome is transcriptionally active, so this frequency suggests no preference for or against transcription units. (Ivics et al., 2004)

However, all *Tc1/mariner* elements, and so *Sleeping Beauty*, target their integration into TA dinucleotides. Upon integration the TA dinucleotide gets duplicated and flanks the newly inserted transposon (Mates et al., 2007).

The activity of *Sleeping Beauty* transposase has been demonstrated in cultured mammalian cells, mouse embryonic stem cells, mouse hepatocytes, the one-cell mouse embryo and the mouse germline, declaring this transposon system as a useful tool for different approaches in vertebrate mutagenesis (Carlson et al., 2003).

1.4 Aim of the Study

Mates et al. (2009) developed a hyperactive *Sleeping Beauty* transposase by incorporating phylogenetically conserved amino acids from related transposons belonging to the *Tc1/mariner* family into the SB transposase. Several variants were tested in a cell culture based transposition assay. Best results were obtained by an engineered transposase that showed 100-fold transposition activity as compared to the original SB transposase and was therefore named *SB100X*.

To investigate the activity of *SB100X in vivo,* circular plasmids containing the reporter gene *Venus* (fluorescent protein) flanked by ITRs were coinjected with *SB100X* mRNA into the pronucleus of mouse zygotes. To determine the optimal concentration between the two injected components, embryos were cultured until day seven post injection and were then checked for fluorescence. The optimal injection solution was used for *in vivo* studies. 37 % of the born animals were transgenic and showed an average integration amount of one or two transgene copies per genome.

In this study, the *SB100X* transposon system should be established for the routinely use in the production of transgenic mice. Therefore the *in vitro* optimized injection mix containing 0.4 ng/µl donor plasmid and 5 ng/µl *SB100X* mRNA should be used to generate a mutant mouse strain with the name *B6;D2-Tn(Venus)Biat* (short name: *Venus-SB100X*).

The frequency of transgenic founders and the number of integrations per genome should be compared to the results from Lajos Mátès and his group at the Max Delbrück Center in Berlin. Furthermore, it should be cleared if all transgenic animals show expression of the reporter gene *Venus* which can easily be detected by fluorescence. An important part of the study is to show if the transgene is passed to the next generation and if silencing occurs. Additionally, it should be verified if integration events are truly catalyzed by the transposase to exclude cases of insertion by non homologous DNA end joining.

2. Material and Methods

2.1 Material

Equipment, chemicals, reagents and stocks, enzymes, kits, oligos and software used for the investigation are summarised in the appendix. Moreover, recipes for buffers and reagents are integrated there in detail.

2.1.1 Animals

Animals were free of all bacterial, viral, and parasitic pathogens listed in the Federation of European Laboratory Animal Science Associations recommendations and were maintained under specific pathogen-free conditions. Housing and experimental protocols were in accordance with the Austrian Animal Protection Law.
For generation of transgenic mice super ovulated *B6D2F1* females were mated with males of the same hybrid strain and used as donors to isolate zygotes for microinjection. *CD1* females were used as surrogate mothers for transfer of manipulated zygotes. After genotyping of the resulting offspring, the transgenic founder animals were outcrossed with wild type mice of *129S5 origin.*

All animals were housed in the mouse facility at the Institute for Laboratory Animal Science and Biomodels Austria, University for Veterinary Medicine, Vienna. They were supplied with untreated tap water and breeding diet (SSNIFF Zuchtfutter V1126). Mice were held alone or in groups up to ten fully grown adults in Makrolon cages Type II or III filled with bedding type FS14 (Rettenmaier & Söhne). Cages were cleaned once a week. Sterile Pur-Zellin was provided as nesting material. From 6 – 18 o'clock the rooms were lighted up with 200 lux in a height of two meters. Air was changed 8 times every hour. The humidity was between 30 % and 86 %, the temperature was fluctuating between 19.5 °C and 24 °C.

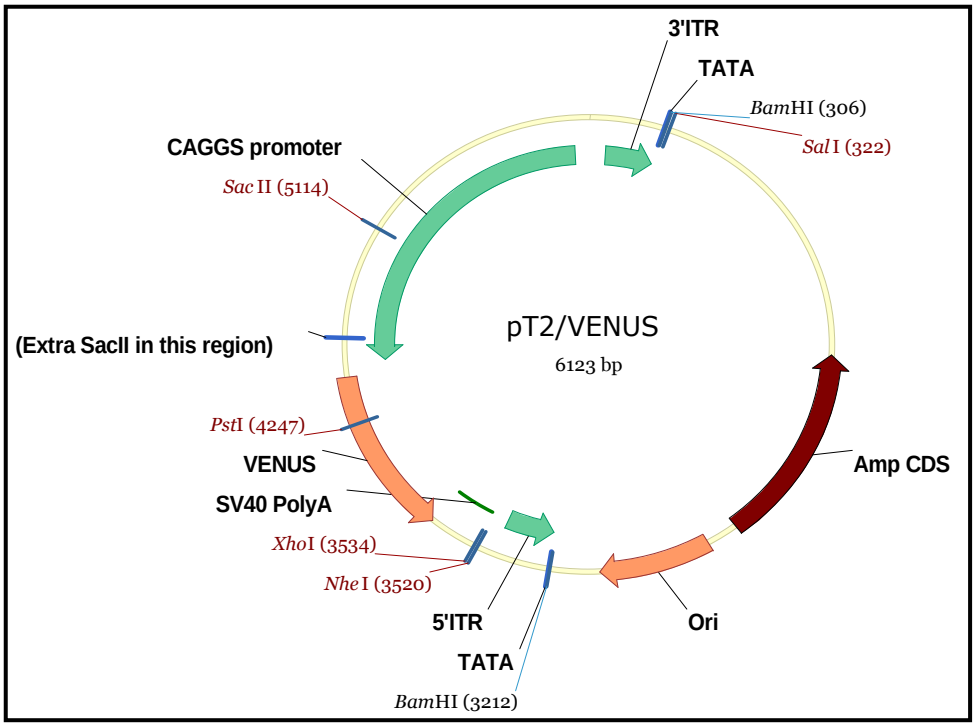
2.2 Methods

2.2.1 Generation of Transgenic Mice

Fertilized eggs from *B6D2F1* mice were isolated and used for pronuclear microinjection of the circular vector pT2/Venus plus mRNA for *SB100X* transposase to generate the transgenic founders designated as *B6;D2-Tn(Venus)Biat*. The concentration of the injection solution was 0.4 ng/µl plasmid DNA and 5 ng/µl transposase mRNA. Treated zygotes were transferred to pseudo pregnant *CD1* recipients.

The optimal concentration for injection mixes was tested before at the Max Delbrück Centre in Berlin Buch through *ex vivo* experiments. For that purpose, treated zygotes were cultured and checked for reporter gene expression at day seven post injection (Mates et al., 2009).

Figure 5: pT2/Venus plasmid map



2.2.2 Cloning of pT2/Venus

For using *SB100X* transposon system for the routinely production of transgenic mice, the plasmid has to be cloned and stored. For later use, every sequence of interest can be cloned into the space between 5'ITR and 3'ITR.

Chemically competent *E.coli* DH5α cells were transformed with the injection mix used for pronuclear microinjection (0.4 ng/µl pT2/Venus and 5 ng/µl *SB100X* transposase mRNA). The cells were put on ice for 30 minutes and then incubated at 42 °C for 45 seconds. After heating, the cells were put on ice again for two minutes. After adding 900 µl of SOC Medium cells were incubated on a shaker for one hour at 37 °C. Bacteria were plated on LB agar plates containing 50 µg ampicillin per ml agar for selection of plasmid carrying cells. Plates were incubated at 37 °C over night.

One colony was picked and put in 3 ml liquid LB medium containing 150 µg ampicillin and incubated on a shaker for four hours at 37 °C. Then the cell solution was poured into 50 ml LB medium containing 2.5 mg ampicillin and shaked over night at 37 °C.

Bacterial cells were centrifuged at 4 °C with maximum speed for 15 minutes. The supernatant was discarded; the pellet was stored at -20 °C.
For reisolation of the amplified vector Plasmid DNA Purification (Midi Prep) for high-copy plasmids (Machery Nagel, Düren, D) was performed like described in the user manual.

The purified plasmid was digested with *BglI* to make sure that the correct plasmid was isolated.
Number of fragments and their lengths after restriction digest was calculated by NEBcutter (New England BioLabs, Ipswich, UK).

Table 1: Reaction mix for *BglI* digest of isolated plasmid

2 µg	Plasmid DNA
4 µl	10x buffer
5 units	*BglI*
add ddH_2O to a total volume of 40 µl	
37 °C for 2 hours	
80 °C for 20 minutes to inactivate the enzyme	

2.2.3 Analysis of Born Animals

One to three day old mice were exposed to a lightsource (BLS, Budapest, H) with a wavelength of λ = 460-495 nm and checked for fluorescence. Only transgene positive mice with appropriate expression of reporter gene show yellow fluorescence.

2.2.4 Sample Taking

Three weeks old mice were marked by ear clip for identification. For tail biopsy 1 to 2 mm tail tips were taken and stored at -20 °C.

2.2.5 DNA Isolation

2.2.5.1 DNA Isolation from Tail Samples (HOM)

For "HOM" Isolation of DNA, tail samples were incubated over night at 60 °C with 3 µl Proteinase K (20 mg/ml) and 500 µl HOM buffer. After vortex and 3 minutes of

centrifugation at 13200 rpm the supernatant was transferred to a new tube and 200 µl 5M NaCl and 700 µl CIA (Chloroform:Isoamylalcohol 24:1) were added. After 10 minutes of mixing by inverting, samples were centrifuged at 13200 rpm for 10 minutes. The upper phase was carefully transferred to a new tube and charged with 1 volume of isopropanol for DNA precipitation. Again it was mixed by inverting for 10 minutes. After 10 minutes of centrifugation at 13200 rpm the supernatant was discarded and the pellet was washed once with 70 % (v/w) ethanol. The supernatant was removed and the pellet was air dried. 30 µl ddH_2O were added to the pellet and incubated over night at room temperature for complete dissolving of DNA. DNA samples were stored at 4 °C or at -20 °C.

2.2.5.2 DNA Extraction with PKII Buffer

For diagnostic use only PKII extraction was performed. Tail tips were incubated for at least four hours or over night with 100 µl PKII buffer and 2 µl Proteinase K (20 mg/ml) at 60 °C. To inactivate the Proteinase samples were cooked for 10 minutes at 95 °C. After three minutes of centrifugation at 13200 rpm, 2 µl of the supernatant (from surface area) were directly used for PCR. This method leads to a poor purity of isolated DNA and lacks storage capabilities.

2.2.6 Determination of DNA Concentration

The concentration of dissolved DNA was analyzed with BioPhotometer by measuring the extinction (optical density, OD) at λ = 260 nm of a 1:25 dilution with ddH_2O. For analysis a disposal cuvette with a path length of 10 mm was used. DNA concentration was calculated from the OD at λ = 260 nm, the dilution factor (d) and the dsDNA specific multiplication factor (f = 50) according to following formula:

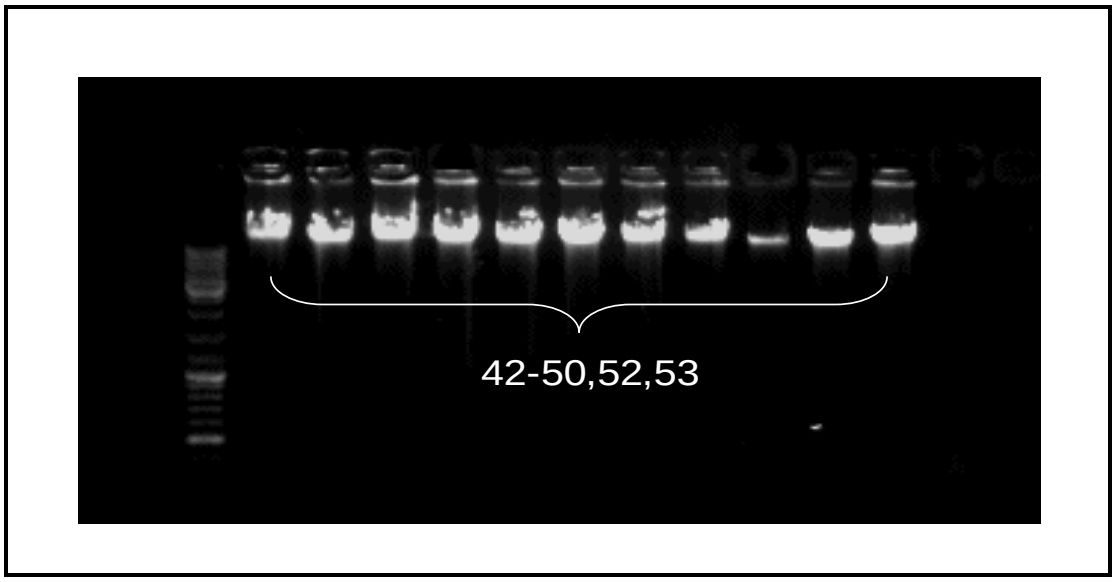
$$C\ [ng/\mu l] = OD_{260nm} \times d \times f$$

Additionally the 260/280 nm ratio was determined to check protein contamination of the samples. The dynamic range is between 0.1 and 2 OD.

2.2.7 Degradation Check by Agarose Gel Electrophoresis

To make sure that genomic DNA is in good conditions and not degraded it was analyzed via agarose gel electrophoresis. Therefore 2 µl of isolated DNA were diluted with 8 µl ddH2O and charged with 1 µl 10x loading dye. The whole sample was loaded on a 1 % agarose gel containing 0.05 µg/ml ethidium bromide. Electrophoresis was performed for 30 minutes to 1 hour with 5 V/cm in 0.5 % Tris/borate/EDTA (TBE). DNA was visualized with Gel iX Imager.
As a length marker GeneRuler^{TM} DNA ladder mix was loaded.

Figure 6: Visualizing genomic DNA



Genomic DNA from *Venus-SB100X* animals with the numbers 42-50, 52 and 53 was loaded to see if DNA is degraded. Degraded DNA would be visible as a long smear in the whole lane. Non-degraded DNA runs very high in the 1% agarose gel and does not show a smear.

2.2.8 PCR Diagnostics

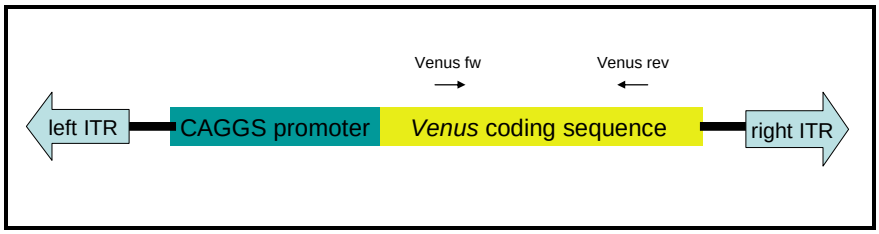
2.2.8.1 Genotyping PCR

For detection of *Venus* positive mice, genomic DNA was analyzed via touchdown PCR. Therefore primers were used to amplify a 501 bp fragment from the *Venus* coding sequence.
As an endogenous control primers were used to amplify a 678 bp fragment from the *Collagen14a1* gene.

Primer sequences:

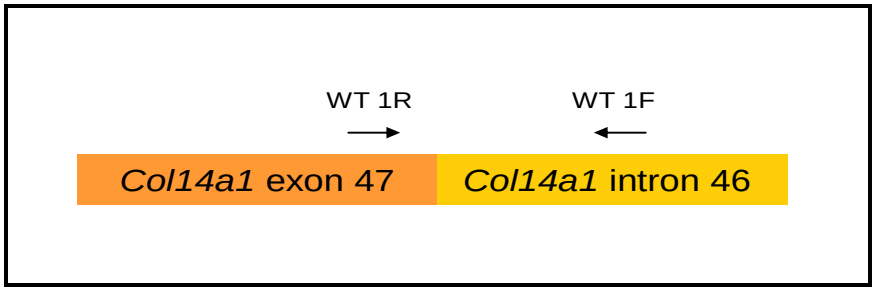
Venus fw	5'-ACCTACGGCAAGCTGACCCTGAA-3'
Venus rev	5'-CTGGTAGCTCAGGTAGTGGTTGT-3'
WT 1F	5'-GGGGAAATGTCACCTTCAAA-3'
WT 1R	5'-TGGTTGAGGATGGCTGTGTA-3'

Figure 7: Schematic illustration of the transposon fragment



This fragment gets integrated into the mouse genome during transposase-mediated excision from the pT2/Venus plasmid and integration into the genome. Venus fw and Venus rev are primers which bind within the *Venus* coding sequence.

Figure 8: Schematic illustration of exon 47 and intron 46 of the gene *Collagen14*



WT 1R binds within exon 47 of the *Collagen14* gene, WT 1F binds to intron 46 resulting in a 678 bp fragment in the PCR reaction.

Table 2: Reaction mix for genotyping PCR

µl/reaction		stocks
0.5	Venus fw	10 µM
0.5	Venus rev	10 µM
0.5	WT 1F	10 µM
0.5	WT 1R	10 µM
2	dNTPs	2 mM each
2	PCR buffer	10x
2	$MgCl_2$	15 mM
0.1	Taq Polymerase	5 units/µl
2	genomic DNA	
9.9	ddH_2O	

Table 3: PCR conditions

	1 min	94 °C
	45 sec	92 °C
6 cycles → -1 °C/cycle	1 min	61-55 °C
	3 min	72 °C
	45 sec	92 °C
26 cycles	1 min	54 °C
	2 min	72 °C

PCR products were diluted with 10x loading buffer. 10µl were loaded on a 2 % agarose gel containing ethidium bromide for visualizing.

2.2.8.2 Backbone-Specific PCR

To certain that only the transposon got integrated and not the whole plasmid, primers were used which bind within the ampicillin resistance cassette present on the vector pT2/Venus and lead to a 854 bp amplicon in the PCR.

Primer sequences:
Amp long fw 5'-ATGAGTATTCAACATTTCCGTGTC-3'
Amp long rev 5'-TGCTTAATCAGTGAGGCACCTA-3'

Table 4: Reaction mix for backbone specific PCR

µl/reaction		stocks
0.2	Amp long fw	10 µM
0.2	Amp long rev	10 µM
2	dNTPs	2 mM each
2	PCR buffer	10x
2.6	$MgCl_2$	15 mM
0.1	Taq Polymerase	5 units/µl
2	genomic DNA	
10.9	ddH_2O	

Table 5: PCR conditions

	4 min	95 °C
40 cycles	30 sec	95 °C
	30 sec	63 °C
	30 sec	72 °C
	7 min	72 °C

PCR products were diluted with 10x loading buffer. 10 µl were loaded on a 1 % agarose gel for visualizing.

2.2.8.3 PCR for the Detection of *SB100X* Coding Sequence

To detect if the injected *SB100X* mRNA got transcribed into DNA and integrated to the host genome, PCR was performed. Therefore, primers that bind within the *SB100X*

coding sequence were used. In the PCR, a fragment with a length of 576 bp was amplified.

Primer sequences:

SB100X fw 5’-TCAGCAAGGAAGAAGCCACT-3’

SB100X rev 5’-TTCCTCCTGACAGAGCTGGT-3’

Table 6: Reaction mix for SB100X-PCR

µl/reaction		stocks
0.5	SB100X fw	10 µM
0.5	SB100X rev	10 µM
2	dNTPs	2 mM each
2	PCR buffer	10x
2	$MgCl_2$	15 mM
0.1	Taq Polymerase	5 units/µl
2	genomic DNA	
10.9	ddH_2O	

Table 7: PCR conditions for SB100X-PCR

	5 min	95 °C
35 cycles	30 sec	95 °C
	40 sec	65 °C
	40 sec	72 °C
	7 min	72 °C

PCR products were analysed by agarose gel electrophoresis.

2.2.9 Histology

Cerebrum, cerebellum, myocardium, skeletal muscle, pancreas, stomach, liver, spleen, kidney, testis, epididymis and lung were fixed in 4 % phosphate-buffered formalin for 24 hours at room temperature. Following embedding was performed over night by an automatic embedding equipment system Shandon Excelsior Tissue Processor (Thermo Fisher Scientific, USA). Solvents and times were according to the following protocol:

1.	Ethanol 75 % (v/w)	30 °C	60 minutes
2.	Ethanol 80 % (v/w)	30 °C	60 minutes
3.	Ethanol 96 % (v/w)	30 °C	60 minutes
4.	Ethanol 96 % (v/w)	30 °C	60 minutes
5.	Ethanol 100 % (v/w)	30 °C	60 minutes
6.	Ethanol 100 % (v/w)	30 °C	60 minutes
7.	Xylen	30 °C	40 minutes
8.	Xylen	30 °C	40 minutes
9.	Xylen	30 °C	40 minutes
10.	Paraffin (Histo-Comp)	61 °C	80 minutes
11.	Paraffin	61 °C	80 minutes
12.	Paraffin	61 °C	80 minutes

Sections of 3 µm were made and put on coated slides.

2.2.9.1 Staining of Sections

The samples were stained with DAPI according to following protocol:

1.	Xylen	2 minutes
2.	Xylen	2 minutes
3.	Ethanol 100 % (v/w)	1 minutes
4.	Ethanol 100 % (v/w)	1 minutes
5.	Ethanol 96 % (v/w)	1 minutes
6.	Ethanol 70 % (v/w)	1 minutes
7.	Aqua dest.	1 minutes
8.	PBS	1 minutes
9.	DAPI	3 minutes
10.	washing 3 times for 20 seconds with PBS	
11.	mounting with Mowiol (Hoechst, Frankfurt)	

Stained sections were examined with a confocal laser scanning microscope (Zeiss).

2.2.10 Dephosphorylation of GeneRuler™ DNA ladder mix

For later labeling of 5' termini of the GeneRuler™ DNA ladder mix with ^{32}Phosphor, the 5' phosphate had to be removed.

Table 8: Reaction to remove 5' phosphate from GeneRuler™DNA ladder mix

1 µl	DNA ladder mix (0.5 µg/µl)
2.5 µl	10x buffer
2 µl	Calf Intestine Alkaline Phosphatase (1 unit/µl)
19.5 µl	ddH_2O
37 °C for 1 hour	
85 °C for 15 minutes to inactivate the enzyme	

2.2.11 Labelling the 5' termini of DNA with ^{32}Phosphor

Table 9: Reaction to label GeneRuler^{TM}DNA ladder mix

5 µl	GeneRuler^{TM}DNA ladder from phosphatase reaction
33 µl	ddH_2O
96 °C for 5 minutes to denature possible secondary structures then put on ice	
5 µl	10x PNK buffer A
5 µl	γ^{32}P ATP
2 µl	PNK (10 units/µl)
37 °C for 1 hour	
70 °C for 12 minutes to inactivate the enzyme	

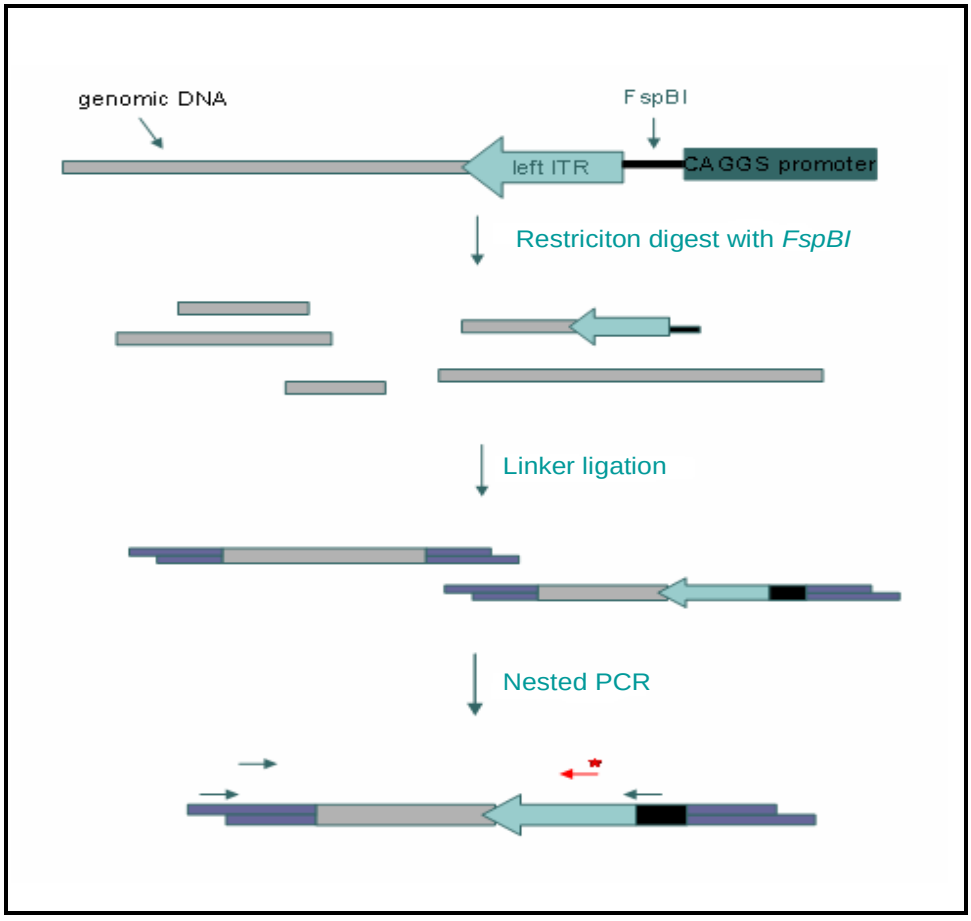
Table 10: Reaction to label the primer Tbal

1 µl	Tbal (50 µM)
37 µl	ddH_2O
96 °C for 5 minutes to denature possible secondary structures then put on ice	
5 µl	10x PNK buffer A
5 µl	γ^{32}P ATP
2 µl	PNK (10 units/µl)
37 °C for 1 hour	
70 °C for 12 minutes to inactivate the enzyme	

2.2.12 Transposon Display

To determine the number of transposon integrations in the genome of *Venus-SB100X* founder animals, Transposon Display was performed with 12 randomly selected founder animals.

Figure 9: Schematic illustration of Transposon Display



In the first step genomic DNA is digested with *FspBI*. Linker sequences are ligated to every fragment. Nested PCR is performed with primers binding within the linker region and transposon specific primers. The transposon specific primer for the second PCR is ^{32}P labelled to make only specific products visible.

In the first step 300 ng of genomic DNA were digested with *FspBI (BfaI)*. This restriction enzyme cuts at the palindromic sequence: 5'-C^TAG-3'

3'-GAT^C-5'

Within the integrated transposon sequence, there is a *FspBI* restriction site 299 bp next to the beginning of the left ITR.

Table 11: Restriction digest of genomic DNA

300 ng	genomic DNA
2 µl	10x buffer
5 units	*FspBI*
add ddH_2O to a total volume of 20 µl	
37 °C for 2-3 hours	
65 °C for 20 minutes to inactivate the enzyme	

After restriction digest, a linker sequence was ligated on both sides of every fragment. To generate this linker, two complement oligos were annealed.

Oligo sequences:
BfaI linker (+) 5'-GTAATACGACTCACTATAGGGCTCCGCTTAAGGGAC-3'
BfaI linker (-) 5'-TAGTCCCTTAAGCGGAG-3' (5' phospho, 3' amino)

The 3' amino modification of BfaI linker (-) is necessary to avoid polymerase-mediated elongation in later PCRs.

For the annealing of the linker, oligos BfaI linker (+) and BfaI linker (-) were mixed to a final concentration of 10 pmol/µl each in TE buffer containing 50 mM NaCl. Slow annealing was performed in the PCR machine.

94 °C	2 minutes
80 °C	5 minutes
75 °C	10 minutes
70 °C	10 minutes

65 °C	10 minutes
60 °C	10 minutes
55 °C	10 minutes
50 °C	10 minutes
45 °C	10 minutes
37 °C	10 minutes

Table 12: Reaction for linker ligation

10 µl (150 ng)	*FspBI* digested genomic DNA
2 µl	annealed linker (10 pmol/µl)
5 µl	T4 Ligase buffer 10x
6 units	T4 Ligase
add ddH_2O to a total volume of 50 µl	
16 °C over night	
65 °C for 10 min to inactivate the enzyme	

Nested PCR was performed using primers which bind to a sequence within the linker (Linker Primer, Nested Primer) and primers which bind to the transposon sequence (Tbal rev3, Tbal).

Table 13: Reaction mix for the first PCR

µl/reaction		stocks
1.5	ligated DNA	
5	PCR buffer	10x
5	$MgCl_2$	15 mM
5	dNTPs	2 mM each
1	Linker Primer	10 µM
1	TbaI rev3	10 µM
0.5	Taq Polymerase	5 units/µl
31	ddH_2O	

Table 14: PCR conditions

	2 min	96 °C
	40 sec	92 °C
5 cycles → -1 °C/cycle	40 sec	68-63 °C
	1 min	72 °C
	40 sec	92 °C
10 cycles → -0.5 °C/cycle	40 sec	63-58 °C
	1 min	72 °C
	40 sec	92 °C
20 cycles	40 sec	57 °C
	1 min	72 °C
	10 min	72 °C

10 µl of the PCR product were mixed with 10x loading buffer and loaded on a 1 % agarose gel to see if the PCR worked.

For further use the PCR product was diluted 1:100 with ddH_2O and stored at -20 °C.

Table 15: Reaction mix for the second PCR

µl/reaction		stocks
1	100x diluted first PCR product	
5	PCR buffer	10x
5	$MgCl_2$	15 mM
5	dNTPs	2 mM each
1	Nested Primer	10 µM
1	Tbal (^{32}P labeled)	~ 1 µM
0.5	Taq Polymerase	5 units/µl
31.5	ddH_2O	

Table 16: PCR conditions

	2 min	96 °C
	40 sec	92 °C
10 cycles → -1 °C/cycle	40 sec	66-56 °C
	1 min	72 °C
	40 sec	92 °C
10 cycles	40 sec	56 °C
	1 min	72 °C
	10 min	72 °C

To visualize the second PCR product, a denaturing 4 % polyacrylamid gel was prepared:

22.7 ml H_2O
22.5 g Urea
5 ml TBE 10x
5 ml Polyacrylamid 40 % (19:1)
400 µl APS 10 %
20 µl TEMED

Before loading the samples, the gel pre ran for 1 hour at 200 Volt in 1x TBE. Slots had to be rinsed out with buffer directly before the samples were loaded.
50 µl of 2x formamide loading buffer were added to the reaction of the second PCR. After 4 minutes of incubation at 95 °C samples were put on ice and loaded directly from ice. 10 µl were loaded on the gel.
GeneRuler^{TM}DNA ladder mix from the labelling reaction was diluted 1:5 with water and charged with 2x formamide loading buffer. 5 µl were loaded on the gel.
The gel ran for about 2 hours at 250 Volt and was afterwards dried on a 3 MM Wattman Paper under vacuum and 80 °C.
The dried gel was exposed to an X-ray film. After 4-7 days the film could be developed.

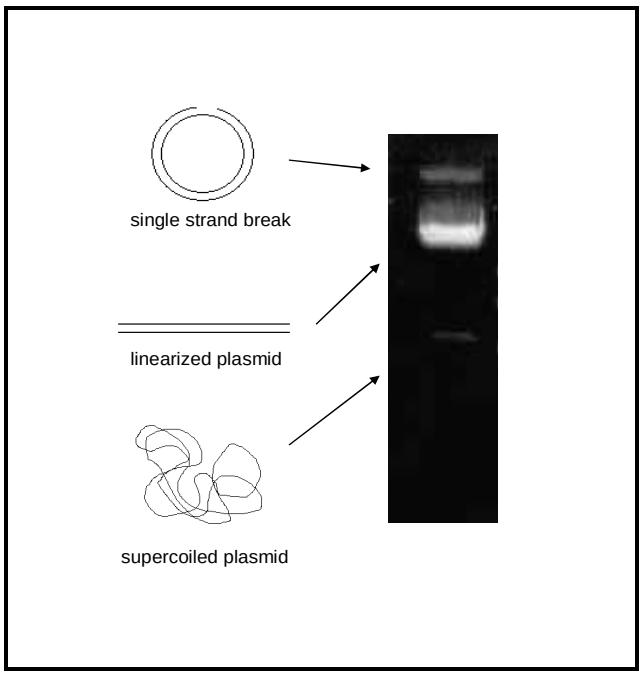
3. Results

3.1. Cloning the Transposon Carrying Vector pT2/Venus

Plasmid DNA can occur in three conformations, which should all be detectable by agarose gel electrophoresis after isolation from bacterial cells. To check isolated pT2/Venus DNA after amplification in *E.coli* and reisolation, 1 µg of plasmid was loaded on a 1 % agarose gel.
Supercoiled plasmid DNA runs faster in the gel than linearized plasmids and plasmids with single strand breaks.

When the undigested plasmid had been loaded, all three conformations could be detected. Most of the plasmid DNA is linearized which could have happened during dissolving the precipitated plasmid with too much vortexing.

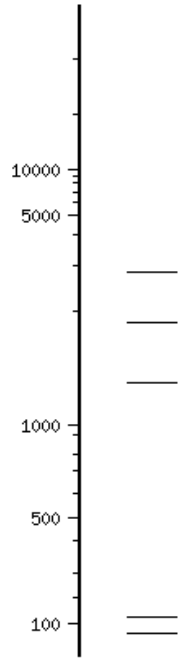
Figure 10: Three conformations of undigested plasmid DNA



1 µg of undigested pT2/Venus DNA was analysed by agarose gel electrophoresis.

BglI digest was made to make sure that the isolated plasmid shows the expected pattern of fragment lengths. First, pT2/Venus sequence was digested *in silico* using the online programme NEBcutter (New England Biolabs). In Figure 11 detailed information about coordinates of restriction sites is given and the predicted pattern of fragment lengths in a 0.8 % agarose gel after a real digest is shown.

Figure 11: Expected results for *BglI* digested pT2/Venus DNA

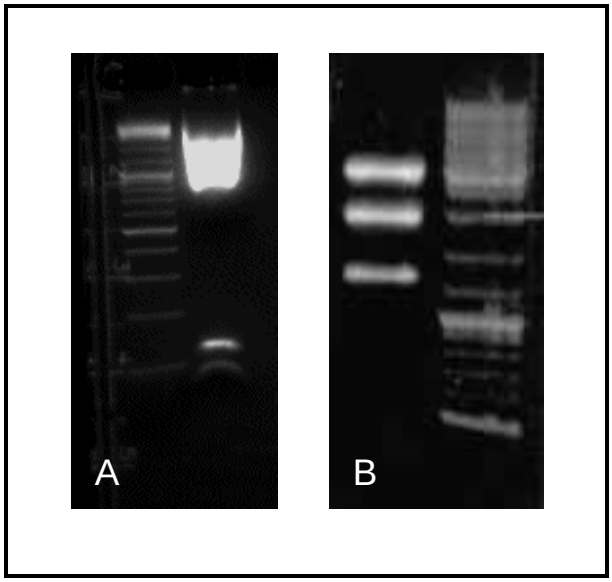


#	Ends	Coordinates	Length (bp)
1	BglI-BglI	3025-5842	2818
2	BglI-BglI	6036-1757	1845
3	BglI-BglI	1758-3024	1267
4	BglI-BglI	5914-6035	122
5	BglI-BglI	5843-5913	71

NEBcutter from New England BioLabs was used to show the pattern of digested pT2/Venus DNA in a 0.8 % agarose gel and coordinates of restriction sites on the plasmid.

When loading *BglI* digested pT2/Venus DNA on a 1 % agarose gel (like in Fig), only the three large fragments were visible. To detect the small fragments with 122 bp and 71 bp, a higher amount of plasmid DNA (at least 10 µg) had to be digested and ran on a 2 % agarose gel with high speed (1.5 times higher Voltage than usual).

Figure 12: pT2/Venus after *BglI* digest



A) 10µg of pT2/Venus has been digested and was loaded on a 2 % agarose gel with high speed to make the smaller fragments (122 and 71 bp) visible. Picture B shows the three larger fragments in a 1 % agarose gel after *BglI* digest of pT2/Venus.

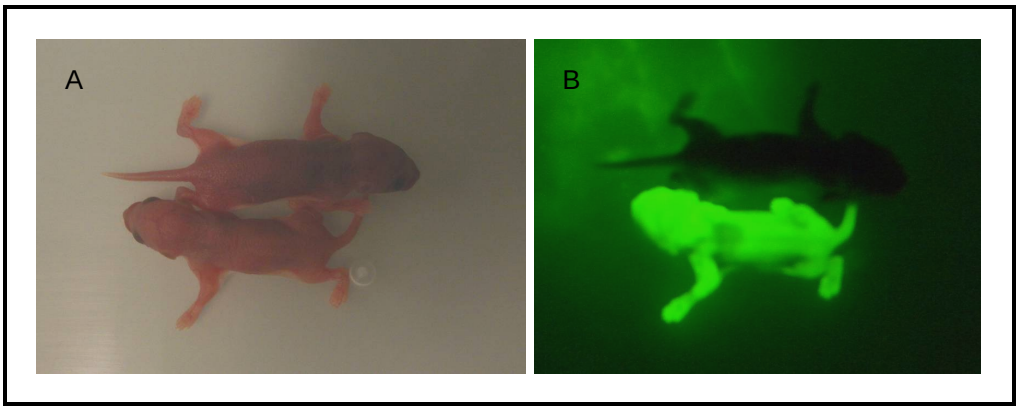
3.2 Generation and Analysis of Transgenic Mice

All together 157 treated zygotes were implanted into pseudo pregnant *CD1* foster mothers after microinjection of circular plasmid pT2/Venus and *SB100X* mRNA.
After three weeks of pregnancy 61 mice were born which means that 38.8 % of implanted zygotes evolved into living animals.
8 animals died within the first three days. 53 remaining mice were marked by ear clip when they were three weeks old and tail tips for DNA isolation could be taken.

3.2.1 Macroscopical Fluorescence Analysis

Newborn mice were checked for fluorescence. 37 out of 61 animals showed overall fluorescence, one animal showed signs of mosaicism.
These numbers lead to a rate of 62.3 % out of newborn animals that showed reporter gene expression.
4 fluorescent animals died within their first days.

Figure 13: Fluorescent and non-fluorescent siblings

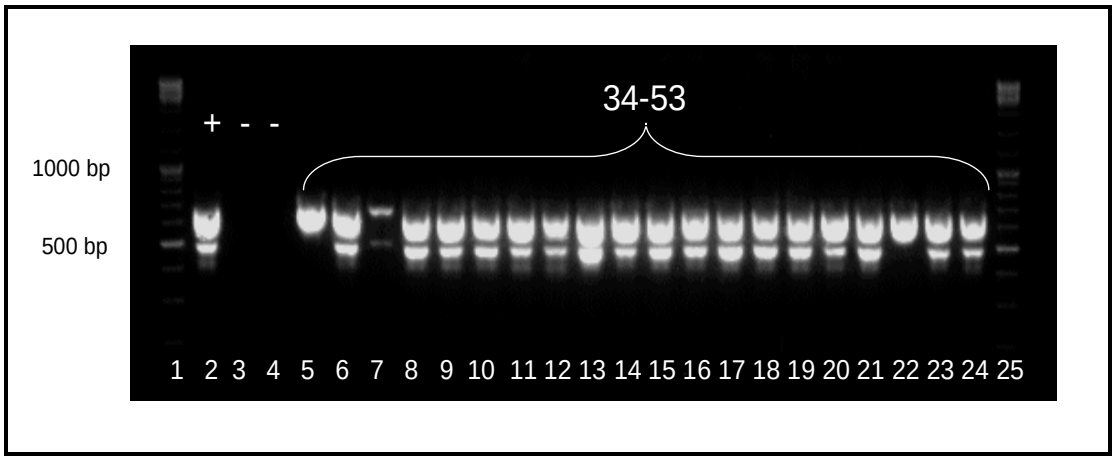


A) Under normal light conditions no fluorescence emission is visible. B) Animals were exposed to a light source (BLS, Budapest, H) with a wavelength of λ = 460-495 nm to detect expression of the fluorescent protein *Venus* in one animal.

3.2.2 Genotyping PCR

PCR with transgene specific primers was performed to detect transgene carrying animals. In Venus positive animals a 501 bp amplicon could be detected. The amplicon from the *Collagen14* gene used as an endogenous control is 678 bp long.
All animals (53) that survived until day 21, when tail tips for DNA isolation could be taken, were tested and 34 (64 %) of them were positive for the *Venus* coding sequence. All genotypic confirmed founder animals correspond with the animals that showed fluorescence which means that no transgene silencing took place in the founder animals.

Figure 14: Detection of *Venus* positive mice



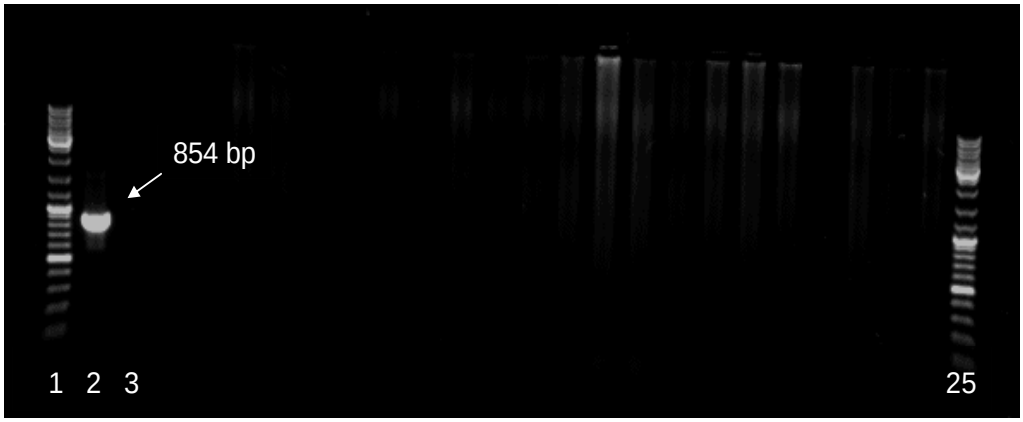
In lane 1 and 25 DNA ladder was loaded. PCR products from samples of *Venus-SB100X* animals 34 to 53 were loaded in lanes 5 to 24. The positive control is in lane 2, two non-template controls are in lane 3 and 4. Animals number 34 and 51 don't carry the *Venus* coding sequence.

3.2.3 Backbone-Specific PCR

To make sure that integration into the mouse genome was transposase mediated, backbone specific PCR was performed. If the whole injected plasmid had been integrated via the cell machinery, it could be detected with the primers Amp long fw

and Amp long rev which bind within the ampicillin resistance gene present on the pT2/Venus vector. During PCR a 854 bp fragment gets amplified.
All *Venus-SB100X* animals (number 1-53) were tested. The ampicillin resistance gene was not found in any of these animals.

Figure 15: Detection of vector integration



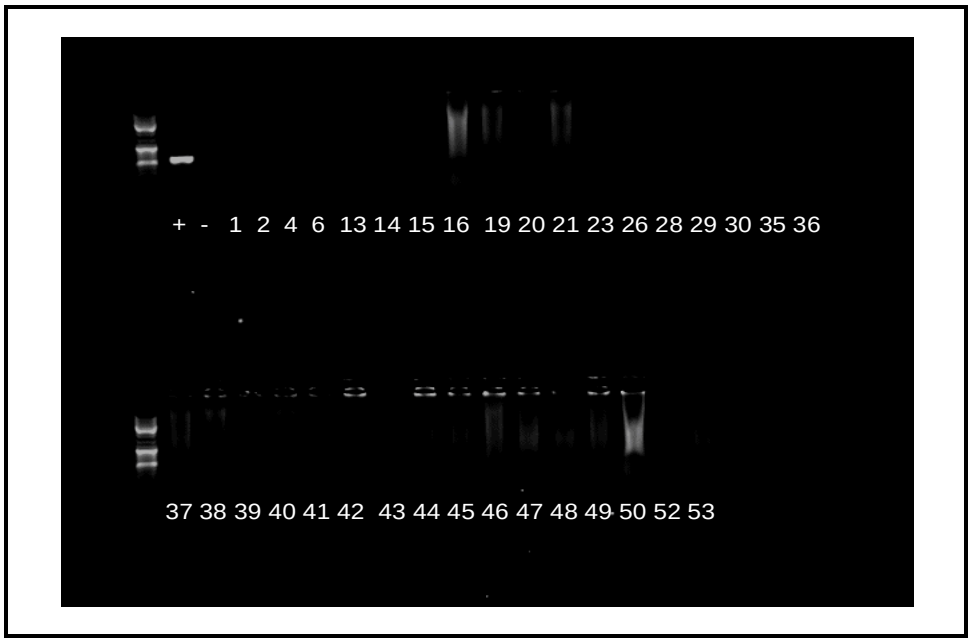
DNA ladder was loaded in lane 1 and 25. The pT2/Venus plasmid was used as a positive control, the PCR product was loaded in lane 2. In lane 3 a non-template-control was loaded. PCR products from the *Venus-SB100X* animals with the numbers 34 to 53 were loaded in lanes 5 to 24. None of these loaded samples carries the ampicillin resistance gene.

3.2.4 SB100X-PCR

SB100X-PCR was performed to detect genomic integrations of the coding sequence for the *SB100X* transposase. Such integration events could have occurred after possible transcription of mRNA into DNA. This process is comparable to the formation of pseudo genes where endogenous genes get duplicated during retrotransposition which includes an RNA intermediate.
SB100X fw and SB100X rev are primers that bind within the coding region for *SB100X* and lead to amplification of a 576 bp fragment in the PCR.

Venus-SB100X founder animals were tested but none of them gave a positive result in the PCR.

Figure 16: Detection of *SB100X* integration by PCR



All *Venus-SB100X* founder animals were analysed via SB100X-PCR for genomic integrations of *SB100X* coding sequence. None of them gave a positive signal in the PCR.

3.2.5 Histology

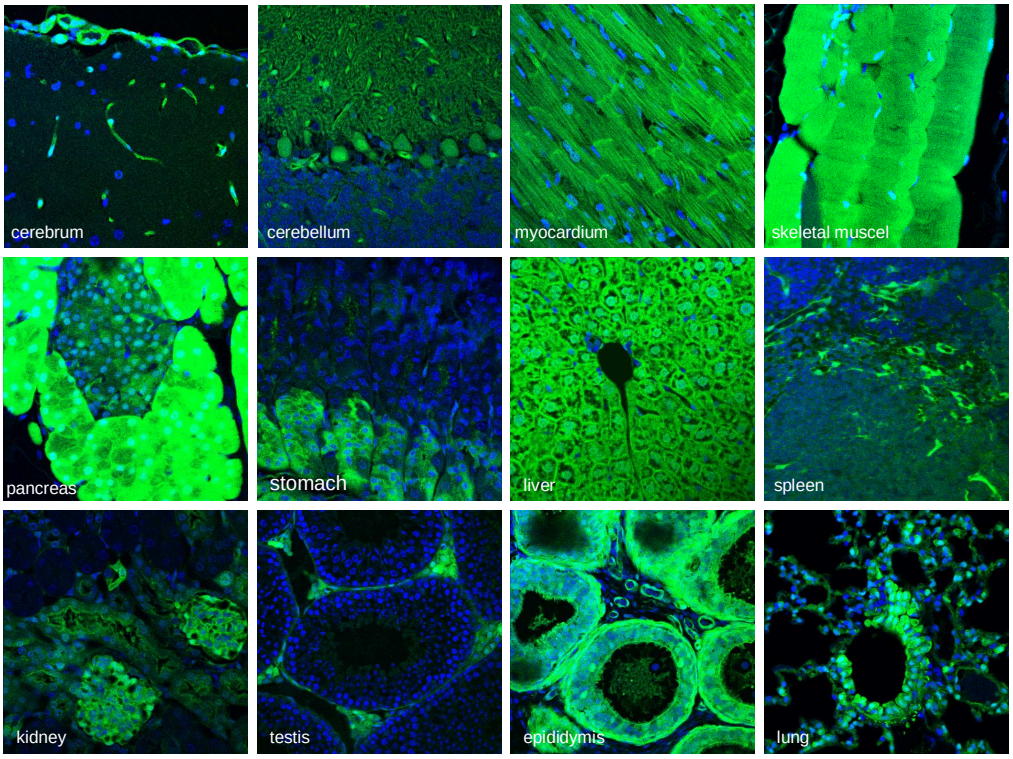
To show that reporter gene expression takes place in every organ a *Venus-SB100X* animal from the F1 generation which showed overall fluorescence as a newborn has been analyzed via histology.
When the abdomen had been opened, all the inner organs except the spleen showed strong fluorescence.

Histology of 12 different organs showed that the fluorescent protein *Venus* is expressed in every organ but not in every cell within an organ. In some cells the protein seems to be located in either the nucleus or the cytoplasm, in other cells it was found in both.

Figure 17: Fluorescent abdomen of a *Venus-SB100X* animal

The opened abdomen has been macroscopically checked for fluorescence. Except for the spleen, the whole abdomen shows fluorescence.

Figure 18: Sections of 12 organs from a *Venus-SB100X* mouse



Venus expression could be detected in sections of all 12 analysed organs including the spleen. Nuclei were stained with DAPI.

3.2.6 Inheritance Analysis

To find out if the transgene is passed to the next generation and if silencing occurs during germline passage, every founder was mated with a *129S5* wild type animal.
The number of transgene carrying animals in the F1 generation additionally gives the possibility to demonstrate if the transgenic founder is either a genetic mosaic or carries more than one independent integration sites of the transgene.
A non-mosaic animal which carries just one transgene integration site should statistically pass it onto 50 % of its progeny when mated to wild type. More than 50 % of transgenic mice in the F1 generation would mean that the transgenic mother/father carries more than one transgene copy.
F1 animals were checked for fluorescence and were analyzed via genotyping PCR.
Within all F1 animals two non-fluorescent mice were determined as transgenics via genotyping PCR, pointing out that gene silencing can happen with transposon mediated transgenes.

Table 17: Results from inheritance analysis

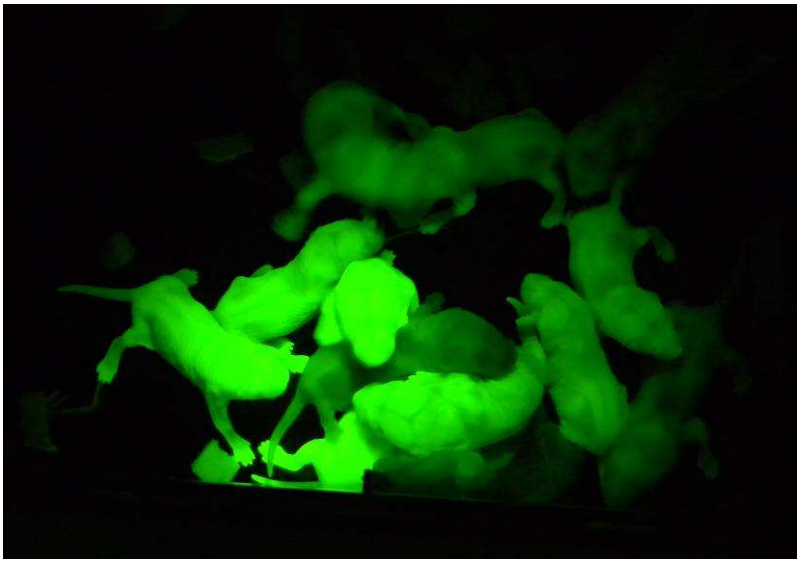
number/name of founder animal	n progeny	n fluorescent animals among progeny	n transgenics among progeny (%)
1	9	5	5 (56)
2	10	7	7 (70)
4	16	4	4 (25)
6	14	3	3 (21)
13	8	3	3 (38)
14	9	8	8 (89)
15	9	8	8 (89)
16	9	9	9 (100)
19	7	6	6 (86)
20	9	8	8 (89)
21	8	3	3 (38)
23	7	3	3 (43)
26	8	4	4 (50)
28	6	3	3 (50)
29	9	3	3 (33)
30	8	5	5 (62)
35	4	3	3 (75)
36	3	3	3 (100)
37	12	12	12 (100)
38	7	5	5 (71)

39	8	8	8 (100)
40	9	7	7 (78)
41	5	4	4 (80)
42	22	22	22 (100)
43	4	1	1 (25)
44	8	7	7 (88)
45	15	1	1 (7)
46	5	5	5 (100)
47	8	8	8 (100)
48	6	4	5 (83)
49	12	6	6 (50)
50	10	9	10 (100)
52	6	1	1 (17)
53	7	5	5 (71)

One third of founder animals had 50 % or less of transgenic pups within their litters. Two third had 50-100 % transgenic progeny.

When more than 50 % of animals within a litter showed fluorescence, intensity was often uneven (Figure 19). On the opposite, the intensity between siblings was comparable, when 50 % or less of the litter showed fluorescence.

Figure 19: Offspring of founder Nr.37



Litter of founder Nr. 37 with 12 pups were checked for fluorescence one day after birth. Every single animal in this litter shows fluorescence but intensities are uneven.

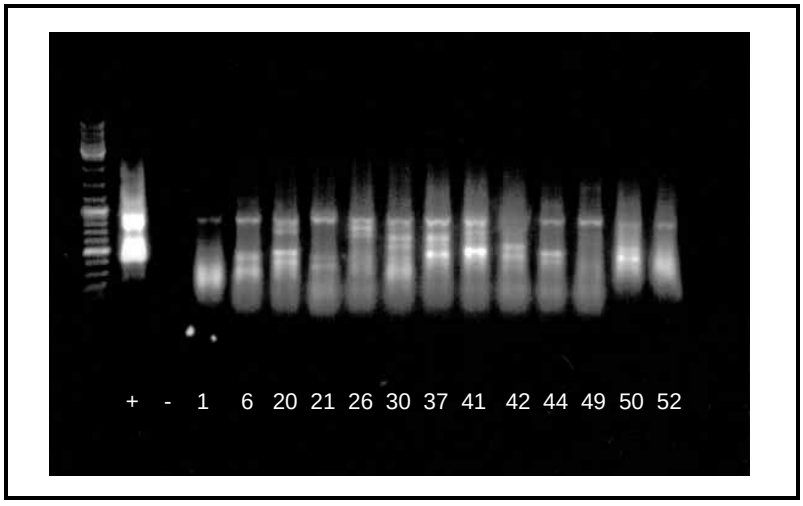
3.2.7 Transposon Display

For determination of transgene integration numbers via Transposon Display, 6 female and 6 male founder animals were randomly selected.

pT2/Venus was used as a positive control. The expected fragment after the second PCR should be 742 bp long.

After the first PCR, products were analysed on a 1 % agarose gel containing ethidium bromide before they were diluted 1:100 for the second PCR.
During the first PCR step a lot of unspecific products accumulate. This happens because of the Linker primer who binds to all the linker sequences which were ligated to every genomic fragment on both sides.

Figure 20: Visualizing the first PCR product on an agarose gel

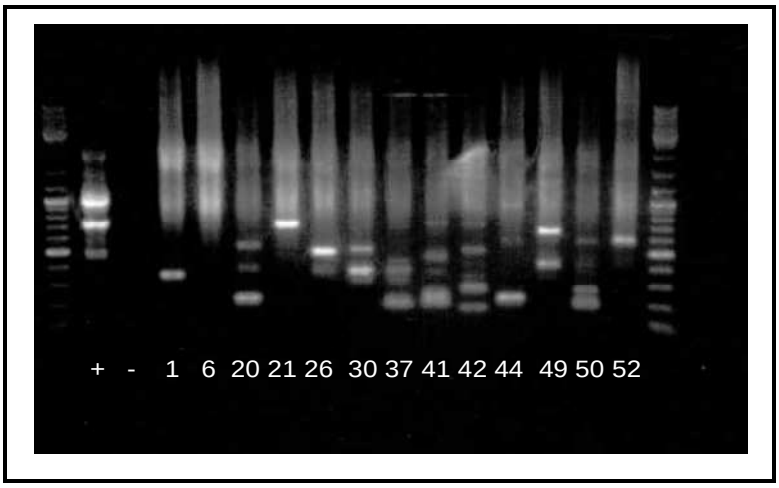


GeneRuler^{TM}DNA ladder mix has been used as a lenght marker. 10 µl of the first PCR products were loaded on a 1 % agarose gel.

In the second PCR, a ^{32}P labelled, transgene specific primer (Tbal), was used to make only transgene specific products visible. Products were analysed via polyacrylamid gel electrophoresis. The gel was dried on a 3MM Wattmanpaper and exposed to an X-ray film where radioactive PCR products become visible as black dots.

As a comparison, the second PCR was also performed with non-labelled TbaI and products were loaded on a 1 % agarose gel.

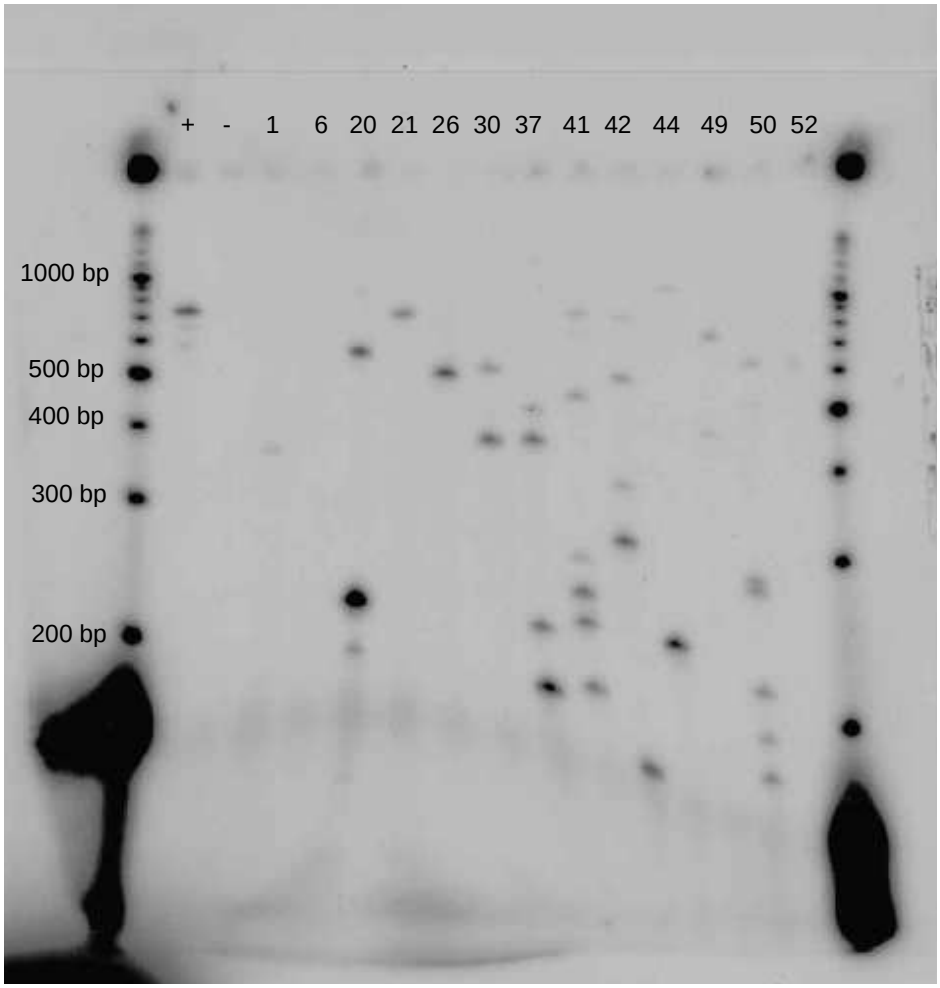
Figure 21: Visualizing the second PCR product on an agarose gel



GeneRuler^{TM}DNA ladder mix has been used as a length marker. Samples from the second PCR were loaded on a 1 % agarose gel. Animal number 6 does not show a specific band in this picture.

In the second PCR the unspecific products get still amplified (visible as a smear in figure 21) but transgene specific products dominate and are visible as clear bands on the agarose gel.

Figure 22: Detection of transgene copy numbers by Transposon Display



The polyacrylamid gel was dried after electrophoresis and exposed to an X-ray film. ^{32}P labelled PCR products from nested PCR are visible. Labelled GeneRuler^{TM}DNA ladder mix was used as a length marker and has been loaded in the first and the last lane. Animals with only one transgene integration in their genome give one signal on the film. Several signals per sample would mean that the analysed animal carries more than one transgene integration. In this picture animal number 6 shows no signal.

Table 18: Analysed founder animals and number of transgene integrations

Number/name of founder	Transgene integrations	Transgenic offspring in %
1	one	56
20	several	89
21	one	38
26	one	50
30	several	62
37	several	100
41	several	80
42	several	100
44	one or two	88
49	several	50
50	several	100
52	one	17

♀

♂

One third of analyzed animals showed to carry only one copy of the transgene. Two third showed multiple integrations from 2 copies per genome up to possible 7 copies.

4. Discussion

Transgenic model organisms are important tools to study gene function and regulation. Especially the mouse became an important mammalian model organism.

The commonly used method to generate transgenic mice is microinjection of recombinant DNA into the pronucleus of fertilized oocytes. Treated zygotes are then transferred to a pseudo pregnant foster mother.

This method leads to a rate of up to 25 % transgenic founders out of born animals (Rülicke and Hübscher, 2000). Usually, the injected linear DNA is integrated into the murine genome by the cell machinery as a head-to-tail concatemere with up to hundred of copies (Brinster et al., 1985). This phenomenon can lead to diverse types of chromosomal rearrangements, abberant splicing or gene silencing by heterochromatin formation (Miskey et al., 2005).

Attractive new tools for transgenic technologies are so called cut-and-paste transposons. They are simply organized DNA sequenced with inverted terminal repeats (ITRs) flanking the coding sequence for the transposase which is necessary for excision of transposon sequence and reintegration to a target site (Miskey et al., 2005; Mates et al., 2007). When the source for the transposase is separated from the ITR carrying sequence, the created two component system can be easily used for insertional mutagenesis. For integration of transposon sequence to the genome, the two components have to at least temporarily coexist in the cell (Mates et al., 2007).

Depending on the research purpose there are many tranpsoson systems which can be used for transgenesis and insertional mutagenesis. The activity of *Sleeping Beauty*, a member of the best characterized transposon superfamily, the *Tc1/mariner* family, has been demonstrated in several approaches for vertebrate mutagenesis. Even more attractive for mouse transgenesis is a hyperactive *Sleeping Beauty* transposase (*SB100X*) that has been developed at the Max Delbrück Centre in Berlin. This mutated transposase was a 100 times more active in cell culture experiments than the original enzyme. First in vivo experiments showed that *SB100X* can be used to produce transgenic mice by pronuclear microinjection of a donor plasmid carrying a gene of interest flanked by ITRs and *SB100X* mRNA. In a first attempt 37 % of born animals were transgenic and showed one or two transgene integrations. (Mates et al., 2009)

With the use of a circular plasmid instead of the normally used linear fragments, host factor mediated integration can be reduced to a minimum. When transgene integration occurs it is very likely that integration is catalyzed by the transposase.

The transposase could also be provided by an expression plasmid, but transcription in the one-cell mouse embryo is limited, therefore mRNA was used to guarantee proper expression.

In this study, a comprehensive analysis of *SB100X* transgenic mice should be made to clearify the way for the routinely use of this hyperactive transposon system for the production of transgenic mouse models.

Injection experiments with the *in vitro* optimized ratio between donor plasmid and transposase mRNA have been performed at the Institute for Laboratory Animal Science, University for Veterinary Medicine, Vienna. Briefly, 62.3 % of born animals were transgenic and all of them showed expression of the transgene *Venus* which could easily be detected by fluorescence. Only one animal showed signs of mosaicism as a baby but due to lacking tagging possibilities it was not possible to seek this animal out later. Dissection and histological analysis of possible candidates would give us more information about the mosaic but transgenic founder animals are still used for breeding studies.

Transposase-mediated transgene integration has been verified by PCR where primers were used to detect the ampicillin resistance gene present on the backbone of the injected vector pT2/Venus. A positive signal would have meant that either the backbone alone got integrated by the cell machinery after excision of the transposon or that the whole vector (backbone + transposon) got integrated after injection. None of the transgenic animals showed to carry the ampicillin resistance gene indicating that every integration event was catalyzed by the transposase. These results are not astonishing when we think about the small amount of plasmid DNA that had been injected. 0.4 ng/µl is very low compared to the commonly used concentration of 2 ng/µl of linear DNA in an injection buffer used for pronuclear microinjection. Maybe the likeliness for vector integration increases when a higher amount of plasmid DNA is injected.

Additionally, we analysed if the injected transposase mRNA got transcribed into DNA and integrated into the genome afterwards. This very unlikely process is comparable to

the formation of pseudo genes which are present in eukaryotic genomes and accrue by retrotransposition of endogenous genes (Thibaud-Nissen et al., 2009). The coding sequence for *SB100X* was not found in any of the genomes of our founder animals. If the transposase would have been provided by an expression plasmid, genomic integrations would have been more likely. This is another reason why the use of mRNA as a transposase source should be preferred over expression plasmids.

Histology was performed to check transgene expression in several organs and to detect differences in fluorescence intensities within these organs. All analysed organs showed trangene expression but not every cell within an organ was fluorescent. Sometimes the fluorescent protein was located in the nucleus, sometimes in the cytoplasm and sometimes in both. Comparison of animals with different transgene integration sites would be interesting and would possibly show if expression patterns differ with the integration locus, a phenomenon known as position effect variegation.

Very important parts of this project were inheritance analysis. All 34 founder animals were mated with *129S5* wild type animals. Analysis of progeny gives information about silencing events and about the number of transgene integrations in the genome of the founder mother/father. Only two out of nearly 300 tested F1 animals were determined as transgenic via PCR but did not show transgene expression. Breeding studies with these animals would be interesting to see if transgene expression can be reactivated in the following generations.

Comparison of breeding results and results from Transposon Display, where number of trangene integrations were determined, showed that animals with one copy of the transgene had 50 % or less of transgenic pups within their litters. Animals with several transgene copies had 50 to 100 % of transgenic pups. An interesting fact was that fluorescence intensities were not even within litters where all the pups showed fluorescence. This phenomenon can have two reasons. Either the intensity of fluorescence correlates with the number of transgene copies or with the integration locus or with both.

Both methods, matings and Transposon Display, led to the cognition that one third of founder animals carry only one copy of the transgene, the others showed several integration sites with a varying number of two to possibly seven independent integrations. The disadvantage of Transposon Display is that you can not be a 100 %

sure that the bands you see on the X-ray film are all specific. An animal which gives only one signal on the X-ray film carries only one integration but it is not possible to distinguish between unspecific and specific signals when an animal shows for example five signals on the film. Therefore, it was important to verify the results from the Transposon Display with those from the breeding studies. For establishing a transgenic line the founder animal has to carry one stable genomic integration. More than one integration would mean that independent segregation is very likely and progeny will be genetically unequal. Unfortunately, the majority of our created founder animals carries more than one transgene copy which can be explained by the high activity of *SB100X*. Using a lower amount of transposase for microinjection would possibly reduce the number of multiple integration events.

Hyperactivity of *SB100X* makes this transposase a very attractive tool for the production of transgenic mice by pronulear microinjection. The very high transgenic rate of 62.3 % that could be reached and the possibility to create mutants with single transgene integrations instead of concatemeres are big advantages of using transposons for mouse mutagenesis.

Still uncleared questions are cargo size capacity and chromatic integration patterns for *SB100X*. The original *Sleeping Beauty* transposase shows decreasing efficiency with increasing cargo size. In transposition assays performed in HeLa cells, no preference for or against transcription units could be detected (Ivics et al., 2004). Before *SB100X* is routinely used for mouse transgenesis it should be cleared if the hyperactive derivate shows similar characteristics as the original *Sleeping Beauty* transposase concerning these questions.

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6. Appendix

6.1 Acknowledgments

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6.2 Summary

Transgenic mouse models became indispensable tools for studying gene function and regulation. Pronuclear microinjection of *in vitro* recombinant DNA is the commonly used method to create mutants with stably inheritable genomic integrations of introduced DNA sequences.

DNA transposons came to the focus of researchers mainly through their use in *Drosophila* genetics but have also been proved of value for mutagenesis approaches in prokaryotes or lower metazoans and have been established recently for their use in vertebrate genome manipulations. In this study, the newly developed hyperactive transposase *SB100X* was used to create transgenic mice with transposase mediated stable genomic integrations of a reporter gene. A donor plasmid containing the reporter gene coding sequence flanked by inverted repeats (cognition sites for the transposase) was co-injected in the pronucleus of a one-cell mouse embryo with the *SB100X* mRNA which should catalyze excision of transposon from the plasmid and integration to the murine genome. Manipulated embryos were carried to term by a pseudopregnant surrogate mother. 62.3 % of born animals showed to carry stable transposase-mediated genomic integrations of transposon DNA. All transgenic founder animals showed proper expression of the reporter gene *Venus* which could be easily detected by fluorescence. Germ line transmission was proved by inheritance analysis and showed that every founder animal had transgene carrying pups within their litters. Silencing events were found only in two out of nearly 300 analysed F1 animals.

The hyperactivity of *SB100X* led to multiple integration events in about two third of founder animals. Only one third of founders carried single integrations which are essential for establishing a transgenic mouse model. Compared to the conventional production of transgenic mice via pronuclear microinjection where injected linear fragments often form concatemeres, transposase catalyzed insertion leads to only one transgene copy per integration locus. This is a great advantage, because concatemeres can lead to chromosomal rearrangements with fatal consequences.

This study shows that the generation of transgenic mouse models with transposons, especially with the *SB100X* system, is possible and has several advantages compared to the conventional method of pronuclear injection.

Zusammenfassung

Transgene Tiermodelle sind unverzichtbare Werkzeuge geworden um Genfunktionen und deren Regulationsmechanismen zu untersuchen. Besonders die Maus ist aufgrund ihres kurzen Lebenszyklus und der genetischen Ähnlichkeit zum Menschen ein viel verwendetes Modell für die Genetik und Molekurarbiologie der Säugetiere. Die Vorkerninjektion von rekombinanten DNA Sequenzen ist die gängige Methode um transgene Mäuse mit stabil vererbbaren genomischen Integrationen zu erzeugen.

DNA Transposons wurden durch ihre zahlreichen Anwendungsmöglichkeiten in der *Drosophila*-Genetik als Werkzeuge für Mutagenesestudien bekannt. Auch für Manipulationen am Genom von Prokaryoten oder niederen Metazoen hat sich die Verwendung von Transposons als nützlich erwiesen. Kürzlich wurden die mobilen DNA Elemente auch für Anwendungen am Vertebratengenom bis hin zur Verwendung bei der Erstellung transgener Mäuse etabliert.

Für diese Studie wurde eine hyperaktive Transposase, *SB100X*, verwendet um transgene Mäuse mit stabilen genomischen Integrationen des Reportergens *Venus* zu erzeugen. Dazu wurde ein Donor-Plasmid mit der Sequenz für *Venus*, welche von so genannten Inverted Repeats flankiert ist, gemeinsam mit der mRNA als Transposasequelle in den Vorkern einer murinen Zygote injiziert. Die manipulierten Embryonen wurden von Ammentieren ausgetragen. 62,3 % der geborenen Tiere zeigten Transposase-vermittelte stabile Integrationen der eingebrachten Transposonsequenz. Expression des Reportergens bringt ein fluoreszierendes Protein hervor, wodurch die Genexpression des Transgens leicht untersucht werden konnte. Die Genexpression von *Venus* konnte in allen transgenen Foundertieren nachgewiesen werden. Durch Verpaarung der Founder mit Wildtyptieren konnte die Keimbahngängigkeit der fremden Sequenzen gezeigt werden. Jedes Foundertier hatte unter seiner Nachkommenschaft mehr oder weniger transgene Jungtiere, wobei auch beinahe alle davon das Reportergen *Venus* exprimieren. Bei nur zwei von beinahe 300 analysierten Tieren der F1 Generation wurde keine *Venus*-Expression nachgewiesen.

Aufgrund der großen katalytischen Aktivität von *SB100X* trägt die Mehrheit (zwei Drittel) der transgenen Tiere Mehrfachintegrationen. Bei einem Drittel der

Foundertiere wurden Einfachintegrationen gefunden, welche essentiell für die Etablierung eines transgenen Tiermodells sind.

Bei der konventionellen Vorkerninjektion werden lineare DNA Fragmente injiziert, die sich aneinanderlagern und so genannte Konkatemere bilden, die im Mausgenom zu chromosomalen Umlagerungen mit fatalen Folgen führen können. Die Verwendung von Transposons schließt die Konkatemerisierung aus und führt zu nur einer Kopie des Transgens pro Integrationsstelle.

In dieser Studie konnte gezeigt werden, dass die Herstellung transgener Mausmodelle mittels Transposons, insbesondere mittels *SB100X*, nicht nur möglich ist, sondern auch diverse Vorteile im Vergleich zur herkömmlichen Vorkerninjektion mit sich bringt.

6.3 Curriculum Vitae

Personal Data:

Name:	Katharina Katter
Date of Birth:	10.04.1986
Place of Birth:	Eisenstadt, Austria
Marital Status:	unmarried
Nationality:	Austrian

Education:

1992-1996:	Elementary School in St. Margarethen, Burgenland (Austria)
1996-2000:	Secondary School Theresianum Eisenstadt (Austria)
2000-2004:	Upper School Theresianum Eisenstadt
July 2004:	School Leaving Examination (Matura) passed with distinction
October 2004:	Entering the Diploma Study of Genetics and Microbiology as a branch of Biology at the University of Vienna
March 2006:	Finishing first part of Study in minimum time Receiving the first Diploma Entering second part of Study with the chosen special field of genetic engineering and biotechnology
October 2008 – July 2009:	Diploma Thesis at the University for Veterinary Medicine Vienna, Institute for Laboratory Animal Science on "*SB100X*-mediated Mouse Transgenesis" (Supervisor: Prof. Dr. Thomas Rülicke)

Internships:

- Practical course at the Medical Diagnostic Laboratory Dr. Böhm – Bacteriology in Vienna (July 2006)
- Molecular biological internship at the Ludwig Boltzmann Institute for Osteology Vienna (September 2007)
- Internship in the cancer research lab at Boehringer Ingelheim Pharma Ges. m. b. H. Vienna (August and September 2008)
- Participation on the course "Einführung in die Labortierkunde" FELASA B (Introduction to the handling of laboratory animals)

Additional Skills:

- Violin Study from 1992 to 2006 at the Joseph Haydn Conservatory Eisenstadt
- Several prizes won at music competitions in Austria
- Played in international orchestras

Command of language:

German as Mother Tongue
Fluent in English (iBT TOEFL, March 2009)
Basic Knowledge in Italian

Lebenslauf

Persönliche Daten

Name:	Katharina Katter
Geburtsdatum bzw. -ort:	10.04.1986 in Eisenstadt
Familienstand:	ledig
Staatsbürgerschaft:	Österreich

Schulische Ausbildung:

1992-1996:	Volksschule in St. Margarethen, Burgenland
1996-2000:	Hauptschule Theresianum Eisenstadt
2000-2004:	Oberstufenrealgymnasium Theresianum Eisenstadt
Juli 2004:	Matura mit ausgezeichnetem Erfolg
Oktober 2004:	Beginn des Diplomstudiums Biologie an der Universität Wien
Februar 2006:	Abschluss des ersten Studienabschnitts in Mindeststudienzeit und Beginn des Studienzweigs Genetik und Mikrobiologie mit Spezialisierung auf Gen- und Biotechnologie
Oktober 2008-Juli 2009:	Praktische Arbeit an der Diplomarbeit mit dem Thema „*SB100X*-mediated Mouse Transgenesis" am Institut für Labortierkunde, Veterinärmedizinische Universität Wien unter der Leitung von Prof. Dr. Thomas Rülicke

Außeruniversitäre Praktika

- Praktikum im Medizinisch Diagnostischen Labor Dr. Böhm, Bakteriologie im Sommer 2006

- Molekularbiologisches Praktikum im Ludwig Boltzmann Institut für Osteologie im Hanuschkrankenhaus Wien im September 2007

- Ferialpraktikum bei Boehringer Ingelheim Pharma Ges. m. b. H. im Bereich der Krebsforschung im Sommer 2008

- Teilnahme am Kurs „Einführung in die Labortierkunde" FELASA B

Zusätzliche Fähigkeiten:

- Violinstudium von 1992 bis 2006 am Joseph Haydn Konservatorium Eisenstadt

- Zahlreiche Auszeichnungen und Preise bei bundesweiten Musikwettbewerben

- Erfahrungen in internationalen Orchestern

Sprachkenntnisse:

- Deutsch als Muttersprache
- Fließendes Englisch (iBT TOEFL, März 2009)
- Basiskenntnisse in Italienisch

6.4 Equipment, Chemicals, Reagents and Stocks

Amersham Hyperfilm™ MP 18x24 cm, GE Healthcare, Buckinghamshire, UK

Axiovert 200, Zeiss, Vienna, A

BioPhotometer, Eppendorf, Hamburg, D

Cell Tram Oil, Eppendorf, Hamburg, D

Centrifuge 5415 R, Eppendorf, Hamburg, D

Centrifuge 5810 R, Eppendorf, Hamburg, D

Coolpix P6000, Nikon GmbH, Vienna, A

Ecocell, MMM Medcenter Einrichtungen GmbH, Gräfelfing, D

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 Dryer 583, Bio-Rad, Vienna, A

Gel iX Imager, INTAS, Göttingen, D

GeneAmp®PCR System 9700, Applied Biosystems, Foster City, USA

Gibco BRL Vertical Gel Electrophoresis Apparatus Model V15.17, Invitrogen, Lofer, A

Goggles FHS/F-00, BLS Ltd., Budapest Hungary

Hypercassette™, Amersham Biosciences, Buckinghamshire, UK

Incucell, MMM Medcenter Einrichtungen GmbH, Gräfelfing, D

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

LSM 510 META^{MK4}, Zeiss, Vienna, A

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

MJ Research DNA Engine DYAD, Bio-Rad, Vienna, A

MJ Research PTC 200 thermal cycler, Bio-Rad, Vienna, A

OPTIMAX X-Ray Film Processor, Protec Medizintechnik GmbH & Co. KG, Oberstenfeld, G

Power Supply PowerPac Basic, Bio-Rad, Vienna, A

Power Supply PowerPac 300, Bio-Rad, Vienna, A

Shaking Incubator 3031, GFL GmbH, Burgwedel, D

Shandon Excelsior Tissue Processor, Thermo Fisher Scientific, Waltham, USA

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

Thermomixer Compact, Eppendorf, Hamburg, D

TransferMan® NK2, Eppendorf, Hamburg, D

Vacuum Pump, Bio-Rad, Vienna, A

Vapor Trap, Bio-Rad, Vienna, A

Vortex Mixer, neoLab, Heidelberg, D

Whatman 3MM Chr, Schleicher & Schuell GmbH, Dassel, G

Acetic acid (CH_3COOH), Roth, Karlsruhe, D

Acrylamid/Bis 19:1 (40 %), Gibco BRL, UK

Agarose, Sigma-Aldrich, Steinheim, D

Ampicillin, Sigma-Aldrich, Steinheim. D

APS (Ammonium persulfate), JT Baker, Phillipsburg, USA

Bacteriological Agar, Sigma-Aldrich, Steinheim, D

Bacto-tryptone, Roth, Karlsruhe, D

Borate (boric acid), Sigma-Aldrich, Steinheim, D

Bromphenol blue, Merck, Darmstadt, D

Chloroform isoamyl alcohol 24:1, Sigma-Aldrich, Steinheim, D

DAPI, Molecular Probes®, Invitrogen, Lofer, A

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

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

Ethanol absolute, Sigma-Aldrich, Seelze, D

Ethidium bromide, Sigma-Aldrich, Steinheim, D

Formamide, Merck, Darmstadt, D

Glycerol, Sigma-Aldrich, Steinheim, D

Glucose, Roth, Karlsruhe, D

Histo-Comp, Vogel, Giessen, D

Isopropanol, Sigma-Aldrich, Seelze, D

LB EZ Mix, Sigma-Aldrich, Steinheim, D

Magnesium chloride ($MgCl_2$), Roth, Karlsruhe, D

Magnesium sulphate ($MgSO_4$), Roth, Karlsruhe, D

Mowviol 4-88, Hoechst, Frankfurt, D

PBS, Sigma, Vienna, A

Phenol, Sigma-Aldrich, Steinheim, D

Potassium chloride (KCl), Roth, Karlsruhe, D

Sodium chloride (NaCl), Roth, Karlsruhe, D

Sodium dodecyl sulphate (SDS), JT Baker, Phillipsburg, USA

Sucrose, Sigma-Aldrich, Steinheim, D

TEMED (Tetramethylethylendiamin), Invitrogen, Lofer, A

Tris, Roth, Karlsruhe, D

Urea, Fluka, CH

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

Xylene, Sigma,Vienna, A

Xylene Cyanol FF, Sigma-Aldrich, Steinheim, D

Yeast exctract, Roth, Karlsruhe, D

6.5 Enzymes, Kits, Oligos

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

Calf Intestine Alkaline Phosphatase (CIAP), Fermentas GmbH, St. Leon-Rot, D

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

NucleoBond® PC100, Machery-Nagel, Düren, D

Oligo Sequences, Fisher Scientific, Vienna, A

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

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

T4 Polynucleotide Kinase (PNK), Fermentas GmbH, St. Leon-Rot, D

6.6 Software

Digital Imaging KS300/KS400, Zeiss, Vienna, A

in silico PCR http://insilico.ehu.es/PCR/

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

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

NEBcutter, New England BioLabs V2.0 http://tools.neb.com/NEBcutter2/index.php (Vincze et al., 2003)

Primer 3 version 0.4.0 http://frodo.wi.mit.edu/

Screenshot Gel iX and Gel Jet Imager Acquisition Software INTAS

TierBase version 3.8.5 Nielson and Mossmann, 2003

6.7 Recipes for Buffers and Reagents

50x TAE, 1 l

242 g Tris base
57.1 ml glacial acetic acid
37.2 g $Na_2EDTA \times 2H_2O$
H_2O to 1 l

10x TBE, 1 l

108 g Tris base (890 mM)
55 g boric acid (890 mM)
40 ml 0.5M EDTA, pH 8.0
H_2O to 1 l

0.5 M EDTA, 1 l

186.1 g $Na_2EDTA \times 2H_2O$
dissolve in 700 ml H_2O
adjust pH to 8.0 with 10 M NaOH (~50 ml)
add H_2O to 1 l
autoclave

TE pH 8.0, 50ml

10 mM Tris-Cl
1 mM EDTA pH 8.0

1 M Tris-Cl, 1 l

121 g Tris base
dissolve in 800 ml H_2O
adjust to desired pH with concentrated HCl
mix and add H_2O to 1 l
autoclave

10x Loading Buffer for Agarose Electrophoresis

50 % glycerol
0.1 M EDTA pH 8.0
0.05 % Bromophenol Blue
0.05 % Xylene Cyanol FF

LB Medium, 1 l

20.6 g LB EZ Mix
H_2O to 1 l
autoclave

LB Agar, 1 l

20.6 g LB EZ Mix
15 g Bacteriological Agar
H_2O to 1 l
autoclave

SOC-Medium

2 % Bacto-tryptone
0.5 % Yeast exctract
10 mM NaCl
2.5 mM KCl
10 mM $MgCl_2$
10 mM $MgSO_4$
20 mM Glucose*
dissolve in H_2O and adjust pH to 7.0 NaOH
autoclave

*Add sterile filtered glucose after autoclaving.

10x PCR Buffer

200 mM Tris-HCl pH 8.0
500 mM KCl

HOM Buffer

160 mM Sucrose
100 mM Tris-HCl pH 8.0
80 mM EDTA pH 8.0
0.5 % SDS

PKII Buffer

25 mM Tris-HCl pH 8.4
37 mM KCl
1.5 mM $MgCl_2$

2x Formamide Loading Buffer 10 ml

9.5 ml Formamide
0.4 ml 0.5M EDTA, pH 8.0
2.5 mg Xylene Cyanol FF
2.5 mg Bromphenol Blue