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(71) Applicant(s)
Minos Biosystems Limited

(72) Inventor(s)
Savakis, Charalambos;Craig, Roger;Grosveld, Frank

(74) Agent / Attorney
Wray & Associates, Level 4, The Quadrant 1 William Street, Perth, WA, 6000

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(71) Applicant *(for all designated States except US)*: **MINOS BIOSYSTEMS LIMITED** [GB/GB]; Jubilee House Farm, Spen Green, Smallwood, Sandbach, Cheshire CW11 2XB (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **CRAIG, Roger** [GB/GB]; Jubilee House Farm, Spen Green, Smallwood, Sandbach, Cheshire CW11 2XB (GB). **SAVAKIS, Charalambos** [GR/GR]; IMBB-Forth, P.O. Box 1527, GR-711 10 Heraklion (GR). **GROSVELD, Frank** [NL/NL]; Maaike Weezenaar Straat 27, NL-3065 GE Rotterdam (NL).

(74) Agents: **FURLONG, Isla, Jane** et al.; D Young & Co, 21 New Fetter Lane, London EC4A 1DA (GB).

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(54) Title: INDUCIBLE TRANSPOSITION IN TRANSGENIC ORGANISM USING TRANSPOSON VECTOR

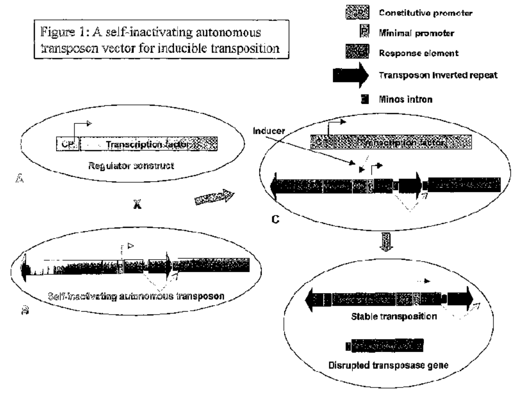

Figure 1: A self-inactivating autonomous transposon vector for inducible transposition

(57) **Abstract:** A method of inducing transposition in a transgenic embryo is described, comprising the steps of (a) generating a first adult transgenic organism comprising within its genome one or more copies of a transposon; (b) generating a second adult transgenic organism comprising within its genome one or more copies of a gene encoding a transposase cognate for said transposon and/or a sequence capable of regulating expression of said gene encoding the transposase; (c) crossing the first adult transgenic organism with the second transgenic adult organism to provide a progeny which comprises, in the genome of one or more of its cells, both (i) one or more copies of the transposon and (ii) a gene encoding a transposase cognate for said transposon, wherein the gene encoding the transposase is under the control of one or more inducible regulatory sequences which permit expression of the transposase, and (d) inducing expression of said gene encoding the transposase in said embryo to cause mobilisation of said transposon within at least a portion of the tissues or cells of the progeny. Using the method, mobilisation of a transposon can advantageously be induced at predetermined stages of development of an embryo and the mutated gene of a single cell may be replicated in subsequent cell divisions, resulting in groups of cells which are essentially homogeneous for the transposed gene.

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Genetic Manipulation Method

Field of the Invention

The present invention relates to a method for the transfer of genetic information in an organism using transposons. In particular, it relates to a method of inducing genetic modification of cells at a predetermined stage of development.

Background to the Invention

The development of high through-put DNA sequencing technology, and sophisticated data-capture and computational analysis has resulted in the sequence determination of entire genomes including *Drosophila melanogaster* and *Homo sapiens*. This has identified novel "predicted" gene sequences but no associated biology ascribing function. Functional information is a prerequisite to delineate which genes may prove to be therapeutic targets for disease management and diagnosis in man.

The identification of individual gene function and the functional relationship of genes to disease states is now a pre-occupation of the Biotechnology and Pharmaceutical industry. The identification of disease related genes will allow the development of new drugs or targets for drug discovery, provide diagnostic or prognostic markers for disease and provide prescriptive guides for physicians. The latter of these will be particularly useful in diseases having complex genetics. Where genetic variation between patients can be measured, personalised medicine programs can be developed where defined patient responses to drug action are identified.

Many methods for identifying gene function are being applied, most with a dependency on the comparative analysis of gene structure and gene expression profiles in healthy and diseased states. The approach is expensive and time consuming, and the outcomes often subjective, lacking hard evidence relating a variation in gene expression to a functional disease related event *in vivo*. Validation of gene function requires studies in animal model systems which directly relate cause (i.e. a mutation in a gene sequence, a deletion or an insertion) with a measurable effect (i.e. behavioural, developmental, metabolic etc) in the whole animal.

Gene function studies in mice and other mammals are presently restricted to:

A) Painstaking mutational analysis of individual genes in "knock-out" transgenic mice derived from libraries of embryonic stems cells (ES cells) each cell containing one or more tagged genes often introduced by viral infection.

B) The random mutation *in vivo* of mouse genes by alkylating agents, and subsequently whole genome sequence analysis to identify multiple mutations

The knockout approach is valid where a function can be surmised based on sequence homology with closely related genes of known function but this approach is time consuming and labour intensive.

The alkylation approach relies entirely on whole genome sequencing to identify sites of mutation and the cataloguing of changes in previously determined behavioural traits and metabolic read-outs. Identification of a phenotypic change must then be correlated to one of perhaps one hundred alkylation events in the target mouse genome. The approach is also time consuming and requires the generation and maintenance of large mouse libraries, and is limited to inbred strains of mice (for comparative review see Abuin et al.(2002) TIB 20, 36-42).

Another method for obtaining mutations is through the introduction of exogenous DNA into the genome.

Transposons are natural genetic elements capable of jumping or transposing from one position to another within the genome of a species. Mobilisation of a transposon is dependant on the expression of a tranposase enzyme which binds to sequences flanking the transposon DNA leading to the excision of DNA from one position in the genome and reinsertion elsewhere in the genome. Insertion into a gene sequence will lead to a change in gene function which may, in turn, result in a measurable phenotypic change in the whole organism.

Of the three "classical" model animals, the fly, the worm and the mouse, efficient transposon based insertion methodologies have been developed for *D. melanogaster* and for *C. elegans*. The introduction of *P* element mediated transgenesis and insertional mutagenesis in *Drosophila* (Spradling & Rubin (1982) *Science* 218, 341-347) transformed *Drosophila* genetics and formed the paradigm for developing equivalent methodologies in other eukaryotes. However, the *P* element has a very restricted host range, and therefore other elements have been employed in the past decade as vectors for gene transfer and/or mutagenesis in a variety of complex eukaryotes, including nematodes, plants, mammals, fish e.g. zebrafish and birds.

Minos, a type 2 transposon and member of the Tc1 family of elements, was isolated from *D. hydei* and has been used for the germ line transformation of *D. melanogaster*, *C. capitata*, and *Anopheles stephensi* (Loukeris, T. G. et al (1995) *Proc Natl Acad Sci U S A*, **92,** 9485-9; Loukeris, T. G. et al (1995) *Science*, **270,** 2002-5, Catteruccia F. *et al.* (2000) Nature **405** 959-962) and using transient mobilisation assays it has also been shown to be active in embryos of *D. melanogaster*, *Aedes aegypti*, *Anopheles stephensi* and *Bombyx mori* and in cell lines of *D. melanogaster*, *Aedes aegypti*, *Anopheles gambiae* and *Spodoptera frugiperda* (Catteruccia, F. et al (2000) *Proc Natl Acad Sci U S A*, **97,** 2157-2162., Klinakis et al (2000) EMBO Reports 1:416-421; Shimizu et al : Insect Mol Biol 2000 Jun;9(3):277-81).

The *hobo* element of *Drosophila melanogaster* has been described by Gelbart WM, Blackman RK, Prog Nucleic Acid Res Mol Biol (1989);36:37-46.

Salmonid type transposons such as the *Sleeping Beauty* (*SB*) transposon, a *Tc1/mariner*-like transposable element reconstructed from fish have been described by Ivics et al (1997) *Cell* **91**, 501-510 and Horie et al (2001), Proc. Natl. Acad. Sci. USA, Vol. 98, Issue 16, 9191-9196.

Mariner is a transposon originally isolated from *Drosophila mauritiana*, but since discovered in several invertebrate and vertebrate species. The use of *mariner* to transform organisms is described in International patent application WO99/09817.

Hermes is derived from the common housefly. Its use in creating transgenic insects is described in US patent 5,614,398, incorporated herein by reference in its entirety.

PiggyBac is a transposon derived from the baculovirus host *Trichplusia ni.* Its use for germ-line transformation of Medfly has been described by Handler *et al.*, (1998) PNAS (USA) 95:7520-5 and US patent 6,218,185.
European Patent Application 0955364 (Savakis *et al.*, the disclosure of which is incorporated herein by reference) describes the use of Minos to transform cells, plants and animals. The generation of transgenic mice comprising one or more Minos insertions is also described.

International Patent Application WO99/07871 describes the use of the Tc1 transposon from *C. elegans* for the transformation of *C. elegans* and a human cell line.

The use of *Drosophila* P-elements in *D. melanogaster* for enhancer trapping and gene tagging has been described; see Wilson *et al.*, (1989) Genes dev. 3:1301; Spradling *et al.*, (1999) Genetics 153:135.

In the techniques described in the prior art, the use of the cognate transposase for inducing transposon jumping (or transposition) is acknowledged to be necessary. Transgenic animals, where described, have the transposase provided in *cis* or *trans*, for example by cotransformation with transposase genes.

The standard methodology for transposable element mediated transformation is by co-injecting into pre-blastoderm embryos a mixture of two plasmids: one expressing transposase (Helper) but unable to transpose, and one carrying the gene of interest flanked by the inverted terminal repeats of the element (Donor). Transformed progeny of injected animals are detected by the expression of dominant marker genes.

PCT/EP01/03341 (WO 01/71019) describes the generation of transgenic animals using transposable elements. According to this method, the transposase function is provided by crossing of transgenic organisms, one of which provides a transposon function and the other providing a transposase function in order to produce organisms containing both transposon and transposase in the required cells or tissues. The use of tissue specific chromatin opening domains directs transposase activity in a tissue specific manner and gives rise to multiple independent transposition events in somatic tissues (see Zagoraiou et al (2001) P.N.A.S. 98 11474-11478).

Transpositions can be "tagged" allowing positional changes within complex genomes to be rapidly determined and flanking genes determined by sequence analysis. This allows an immediate link between cause (i.e. an insertional event in a specific gene or regulatory element) and effect (i.e. a phenotypic of measurable change). However, conventional methods of inducing genetic modifications by transposition suffer from the disadvantage that the tissue in which transposition has occurred will be a mosaic of individual cells each with unique transpositions. As a result, analysis of phenotypic results of the transposition event may be difficult to perform as each transposition event is unique. Thus it can be seen that a method of controlling a transposition event so as to provide the same genetic modification in a number of cells would provide a valuable contribution to the art.

Summary of the Invention

According to a first aspect of the present invention, there is provided a method of generating a transgenic progeny and inducing transposition, comprising the steps of:

(a) generating a first adult transgenic organism comprising within its genome one or more copies of a transposon;

(b) generating a second adult transgenic organism comprising within its genome one or more copies of a gene encoding a transposase cognate for said transposon and/or an element capable of regulating expression of said gene encoding the transposase;

(c) crossing the first adult transgenic organism with the second transgenic adult organism to provide a progeny which comprises, in the genome of one or more of its cells, both (i) one or more copies of the transposon and (ii) a gene encoding a transposase cognate for said transposon, wherein the gene encoding the transposase is under the control of one or more regulatory sequences which permit expression of the transposase; and

(d) inducing expression of said gene encoding the transposase in said progeny to cause mobilisation of said transposon within at least a portion of the tissues or cells of the progeny.

Alternatively expressed, the invention thus provides a method of generating a transgenic progeny by transposon mobilisation, comprising the steps of:

(a) providing a progeny which comprises, in the genome of one or more of its cells, both (i) one or more copies of a transposon and (ii) one or more genes encoding a transposase cognate for said transposon, wherein the gene encoding the transposase is under the control of one or more regulatory sequences which permit expression of the transposase, and

(b) inducing expression of said transposase in said progeny to cause mobilisation of said transposon within at least a portion of the tissues or cells of the progeny.

Suitably, the first adult transgenic organism can be transgenic lines comprising stably integrated "dormant" transposons. Such transgenic lines can be generated using standard ES cells technologies. Dormant transposons can be induced to transpose through crossing with the second adult transgenic organism. Accordingly, the invention provides a method which can allow the rapid generation of thousands of mutant progeny, such as mouse mutants.

By "progeny" is meant the result of reproduction between the first transgenic organism and the second transgenic organism.

In a preferred embodiment, the one or more regulatory sequences which permit expression of the transposase are sequences which allow specific expression of the transposase during germline development. Accordingly, the germ cells of the progeny have transposition events.

Figure 13 is a schematic diagram showing *in vivo* transposition with the female contributing the transposon, and transposase. Transposition takes place in the oocyte. Mutants are obtained after crossing to male.

Accordingly, in one embodiment, the progeny is a female transgenic organism resulting from reproduction between the first and second transgenic organisms referred to above. In this embodiment, the one or more regulatory sequences which permit expression of the transposase are sequences which allow specific expression of the transposase during oogenesis in the female progeny. Thus, transposase expression is induced upon oogenesis. This, in turn leads to germline transposition events taking place in oocytes to generate oocytes having inserted sequences.

In this embodiment, the one or more regulatory sequences which permit expression of the transposase are derived from regulatory sequences for genes which are expressed in developing oocytes. Suitable regulatory sequences include those which control the expression of oocytes genes such as Zp3, Zp1, Zp2, Gdf9, Bmp15, Figla and Mater (see, for example, Rajkovic and Matzuk, Molecular and Cellular Endocrinology 187(2002), 5-9). Other suitable regulatory sequences may be derived from the regulatory sequences of Oct-4,

Figure 14 is a schematic diagram showing *in vivo* transposition with the female contributing the transposon, the male the transposase. Transposition takes place in the sperm.

Accordingly, in another embodiment, the progeny is a male transgenic organism resulting from reproduction between the first and second transgenic organisms referred to above. In this embodiment, the one or more regulatory sequences which permit expression of the transposase are sequences which allow specific expression of the transposase during spermatogenesis in the male progeny. Thus, transposase expression is induced upon spermatogenesis. This, in turn leads to germline transposition events taking place in spermatocytes to generate spermatocytes having inserted sequences.

In this embodiment, the one or more regulatory sequences which permit expression of the transposase are derived from regulatory sequences for genes which are expressed in developing spematocytes. Suitable regulatory sequences include those which control the expression of spematocyte specific mRNAs such as the transcript of the H1t gene (Bartell et al. Biol Of Reproduction 2000; Aug; 63(2); 409-16).

Suitably, the progeny which have transposition events taking place in the germline are then mated to produce offspring in which the transposition events can be characterised. A progeny having germline transposition can be mated to a normal mate or to a mate which, itself has been generated to have germline transposition.

In a further embodiment the invention, the "progeny" is an embryo. Accordingly, in this embodiment, there is provided a method of generating a transgenic embryo and inducing transposition, comprising the steps of:

a) generating a first adult transgenic organism comprising within its genome one or more copies of a transposon;

b) generating a second adult transgenic organism comprising within its genome one or more copies of a gene encoding a transposase cognate for said transposon and/or an element capable of regulating expression of said gene encoding the transposase;

c) crossing the first adult transgenic organism with the second transgenic adult organism to provide an embryo which comprises, in the genome of one or more of its cells, both (i) one or more copies of the transposon and (ii) a gene encoding a transposase cognate for said transposon, wherein the gene encoding the transposase is under the control of one or more regulatory sequences which permit expression of the transposase; and

d) inducing expression of said gene encoding the transposase in said embryo to cause mobilisation of said transposon within at least a portion of the tissues or cells of the embryo.

Alternatively expressed, the invention thus provides a method of generating a transgenic embryo by transposon mobilisation, comprising the steps of:

a) providing an embryo which comprises, in the genome of one or more of its cells, both (i) one or more copies of a transposon and (ii) one or more genes encoding a transposase cognate for said transposon, wherein the gene encoding the transposase is under the control of one or more regulatory sequences which permit expression of the transposase, and

b) inducing expression of said transposase in said embryo to cause mobilisation of said transposon within at least a portion of the tissues or cells of the embryo.

"Embryo" as herein described should be understood to refer to the structure developing from a single fertilised egg or zygote to the time of birth or hatching in the case of vertebrates or invertebrates or germination in the case of plants. Thus, in the context of the present invention, "embryo" should be understood to also encompass a mammalian fetus.

Figure 15 is a schematic diagram showing *in vivo* transposition with the male contributing the transposon, the female the transposase. Transposition takes place in the egg or early embryo.

Mobilisation of a transposon in embryonic cells or tissues may be induced at any time during the development of the embryo, for example at predetermined stages of development of an embryo. By inducing transposition during such stages of development, the mutated gene of a single cell may be replicated in subsequent cell divisions, resulting in a group or groups of cells which are essentially homogeneous for the transposed gene. Thus the transposed gene may be present in some or all of the cells of a particular tissue or group of tissues. The invention thus enables the generation of transgenic embryos and organisms comprising one or more clonal populations of cells homogeneous for one or more individual mutations.

Thus, a second aspect of the invention provides a method of generating a transgenic organism having a plurality of cells or tissues homogeneous for a gene modified by transposon mobilisation, the method comprising generating a transgenic embryo and inducing transposition therein according to the method of the first aspect of the invention.

By enabling the regulation of transposition at different times during development, the method of the present invention also increases the likelihood of genome-wide transposition since chromatin domains accessible to transcriptional complexes and, in all probability, transposition events vary in different cell tissue types at different times during embryonic development and moreover in adult life and in abnormal growth situations such as tumours.

The likelihood of achieving transposition in particular regions of the genome may be increased further by the use of chromatin opening domains, for example ubiquitously-acting chromatin opening elements (UCOEs) (PCT/GB99/02357 (WO 0005393)), locus control regions (LCRs) (Fraser, P. & Grosveld, F. (1998). Curr. Opin. Cell Biol.10, 361-365), CpG islands or insulators to control expression of the transposon and/or the gene encoding the transposase.

In preferred embodiments of the invention, the transposon and/or the gene encoding the transposase and/or a sequence regulating expression of the gene encoding the transposase are incorporated within chromatin opening domains to increase the likelihood of achieving transposition in a target tissue of the embryo, thus enabling the generation of a population of cells of that tissue homogeneous for a transposition event. For example, where it is desired to induce transposition in a defined tissue, the transposon and/or gene encoding the transposase and/or a sequence regulating expression of the gene encoding the transposase are incorporated within a locus control region, conferring tissue specific control on the expression of a transgene in that tissue. Where it is desired to induce transposition in a tissue for which specific LCRs are not available and/or where it is desired to induce early induction of transposition in a particular tissue, the transposon and/or the gene encoding the transposase and/or a sequence regulating expression of the gene encoding the transposase may be incorporated within a UCOE. In particularly preferred embodiments of the invention, both the transposon and the gene encoding the transposase are incorporated within chromatin opening domains. This advantageously enhances the efficiency of transposition in particular loci throughout the genome during embryo development, enabling the production of one or more populations of cells homogeneous for a particular transposition event.

Thus the invention further provides in a third aspect a method of generating a transgenic embryo and inducing transposition, comprising the steps of:

(a) generating a first adult transgenic organism comprising within its genome one or more copies of a transposon;

(b) generating a second adult transgenic organism;

(c) crossing the first adult transgenic organism with the second transgenic adult organism to provide an embryo which comprises, in the genome of one or more of its cells, both (i) one or more copies of the transposon and (ii) a gene encoding a transposase cognate for said transposon, wherein the gene encoding the transposase is under the control of one or more regulatory sequences which permit expression of the transposase and wherein the transposon and/or the gene encoding the transposase and/or a gene regulating expression of the gene encoding the transposase lie within a chromatin opening domain; and

(d) inducing expression of said gene encoding the transposase in said embryo to cause mobilisation of said transposon within at least a portion of the tissues or cells of the embryo.

In preferred embodiments of the invention, the embryo is produced by crossing a first organism, which is a transgenic organism comprising one or more copies of the transposon, with a second organism, which is a transgenic organism which comprises, in its genome one or more copies of the regulatable gene encoding a cognate transposase. In an alternative embodiment, the embryo may be produced by crossing a first organism, which is a transgenic organism comprising one or more copies of both the transposon and the gene encoding the cognate transposase with a second organism comprising one or more copies of regulatory elements necessary to permit transposase expression.

In a preferred embodiment, the transposon and the gene encoding the transposase may be provided as a single construct such that the gene encoding the transposase is disrupted when the transposon mobilises, thus limiting further mobilisation of the transposon. This may be achieved by placing one of the inverted repeats of the transposon in an intron which interrupts the transposase gene in such orientation that the transposase gene is disrupted when the transposon is mobilised. This vector enables a single cross step to be used to generate a transgenic organism that contains regulator, transposase gene and transposon. Further, transposition leads to complete inactivation of the transposase source, resulting in stability of the new insertion even in the presence of inducer. Figure 1 illustrates schematically the use of such a vector in the present invention.

The incorporation of Cre/lox functions (details of which are reviewed in Sauer, Methods of Enzymology; 1993, Vol. 225, 890-900) and different transposon/transposase combinations may also be used to eliminate primary transposase function. In further embodiments of the invention, however, the transposase gene is not destroyed on transposition, thus allowing further transposon mobilisation on, for example, administration of inducer.

In methods of the invention, transposition may be induced using any system known to the skilled person. Transposition may be induced by induction of transposase gene expression via application of an endogenous substance or via operation of an endogenous signal, such as a developmental regulated signal.

The one or more regulatory sequences of which the gene encoding the transposase is under the control may be inducible regulatory sequences. For example, suitable induction systems include tet based systems, the lac operator-repressor system, ecdysone based systems and oestrogen based systems, details of which are provided *infra.* Exogenous inducers may be provided in any convenient fashion, e.g. by injection to the maternal animal or embryo or as an additive to the food or water supply to the maternal animal. Transposition may be induced at one or more times during embryo development. Thus inducers may be administered only once or repeatedly during one or more stages of development.

In alternative embodiments of the invention, expression of the gene encoding the transposase may be induced in response to a gene regulatory signal produced at a particular stage of embryo development. Such control may be achieved by placing the gene encoding the transposase under the control of a gene regulatory sequence such as a developmental regulated sequence or promoter, for example, a developmental regulated specific promoter responsive to a particular gene regulatory signal such as a transiently expressed development regulated protein. Where the gene encoding the transposase is under such control, expression of the gene encoding the transposase will only occur when the gene regulatory signal, for example a transiently expressed developmental regulated protein, is produced, or, alternatively, when such a signal protein is introduced to the embryo, for example by injection or in the feed of the maternal animal.

Using the methods of the invention, the timing of the expression of the gene encoding the transposase and hence the timing of the induction of transposition in an embryo may be controlled.

In embodiments where it is desired to tightly control the duration and effect of expression of the transposase gene and thus further restrict the timing of transposition, the gene encoding the transposase may be provided within the same construct as the transposon such that, on transposon mobilisation, the gene encoding the transposase is disrupted, preventing the production of further transposase and thus limiting further transposition.

By thus selecting the time of induction, mobilisation of transposons may be induced at a predetermined stage of embryo development. For example, transposition may be induced at very early stages of development such as at the zygote stage, a four cell embryo, sixty-four cell embryo etc. or at later stages of development.

In one embodiment, induction of transposition by placing the transposase under the control of one or more regulatory sequences that will drive transposase gene expression in the early fertilised egg e.g. at the two or four cell stage is desirable. Suitable regulatory sequences for controlling transposase expression at this stage may be derived from the regulatory sequences of genes whose expression is activated at this stage. Such genes include ZP3, Oct-4 (Kirchof et al. Biol Reprod 2000, Dec; 63(6):1698-705), and maternal effect genes such as Zp1, Zp2, Gdf9, Bmp15, Figla and Mater. Other suitable genes include hsp70.1 (Bevilacqua et al. Development 2000; Apr; 127(7):1541-51).

Depending on the stage of development, cells in which transposition has occurred may divide with further rounds of cell divisions resulting in a population of cells homogeneous for the initial transposition event. Where transposition has been induced more than once, a population of cells may be homogeneous for each of two or more transposition events. Thus, depending on the stage of development, an insertional event may be present in populations of cells within one or more tissues, complete tissues or groups of tissues. The precise nature of the insertional event will determine whether it will influence functional gene expression in some or all embryonic and adult tissues. Thus, gene expression patterns of modified genes can be monitored during embryo development and in adult cells and tissues.

Moreover, similar populations of cells homogeneous for an initial transposition event can also be generated in rapidly growing adult cells and tissue derived from stem cells, typically during tissue regeneration or during cell and tissue maintenance. Examples of such cells and tissues include but are not limited to cells of the gut lining, the liver and blood, which are subject to rapid turnover and/or regeneration from stem cells in the adult. Similarly, populations of cells homogeneous for a transposition event may be generated in tumours. The methods of the invention may be adapted to provide populations of cells homogeneous for an initial transposition event in such adult cells.

Thus, in further embodiments of the invention, step (d) of the first or third aspect of the invention may be adapted by inducing expression of the gene encoding the transposase in a neonatal, young adult or adult organism to cause mobilisation of the transposon within at least a portion of the tissues or cells of the organism instead of or, preferably, in addition to inducing expression of the transposase in the embryo. Thus, the method of the invention enables the generation of a population of cells homogeneous for a transposition event induced at a predetermined stage of development in a neonate, young adult or adult organism. In such embodiments, the transposon or the gene encoding the transposase is preferably under the control of a locus control region to enable tissue specific control of the transgene. For example, where it is desired to induce transposition in liver cells, the transposon and/or the gene encoding the transposase may be under the control of a locus control region associated with expression in liver cells.

Where transposition events are induced in the early development of the zygote, it is possible to develop ES cell lines having transpositions. These ES cell lines can be sequenced, characterised and stored for future use.

The generation of genetic mutations in transgenic organisms as a result of transposon insertion according to the invention may give rise to novel phenotypic variations in the organisms. Using the methods of the invention, transgenic embryos may be produced in which one or more clusters of cells or tissues are each homogeneous for a different transposition event, each of which may or may not have a phenotypic effect. Thus using the methods of the invention, embryos and adults developed therefrom comprising one or more clusters or groups of cells each displaying a phenotypic variation compared to the phenotype of corresponding cells or tissues in which no insertional event has occurred may be produced.

The effect of a transposition event on the phenotype of the transgenic embryo will, of course, depend to some extent on the developmental stage at which transposition occurs. Where transposition is induced, for example at the single zygote stage, all cells of the embryo developed therefrom will be homogeneous for the insertional event. Thus, if the transposition event, e.g. insertional event, results in change in phenotype which is lethal to each cell, the embryo will not develop. Where the transposition event(s) has been induced at a later stage of development, each insertional event will be present in the cluster of cells or tissues derived from cell divisions of the cell in which the transposition event(s) occurred. This may therefore result in each of those cells displaying the same phenotypic variation. For example, if the transposition event has been induced in a cell from which some or all cells of a particular tissue of a particular organ is derived, the phenotypic consequences of the insertional event may be limited to the cells, the particular tissue or the particular organ. Where the transposition event has been induced in a cell from which only some of the cells of a particular tissue of a particular organ are derived, a milder phenotype may result from the transposition event than might be observed if all cells of the tissue display the transposition event. Where the transposition event is lethal to a cell, the cells will not survive. If the insertional event is present in all cells from which a particular tissue or organ is composed, that tissue or organ may not function or develop and the embryo may not be viable. Alternatively, the transposition event may have non-lethal phenotypic consequences. For example, the transposition event may have the effect of modulating the function of an enzyme in the affected cells, resulting in a relative change in metabolism compared to the unaffected cells. This may therefore result in an organ such as the liver, in which a sector of tissue of the variant phenotype is present adjacent to a sector of the same tissue of the normal phenotype or even a second variant phenotype. The distribution of the variant phenotype within an organism will thus depend on the stage of embryonic development at which transposition is induced. Moreover, in some embodiments of the invention induction of a "second round" of transposition may be useful in detecting either inversion of the phenotype, caused by excision of an element, or, more importantly, modification of the phenotype, caused by a new insertion to an interacting gene.

Phenotypic variations in cells, tissues or organs of the transgenic organisms may be traced back to transposition events in the genome of those cells, tissues or organs.

Accordingly, in a fourth aspect the invention provides a method for detecting and characterising a genetic mutation in a transgenic organism, comprising the steps of:

(a) generating a transgenic embryo and inducing transposition therein by a method according to the first or third aspect of the invention or a transgenic organism according to the second aspect of the invention;

(b) identifying in the transgenic embryo or offspring developed therefrom the presence of a plurality of cells displaying a variant phenotype;

(c) detecting the position of one or more transposon transposition events in the genome of one or more of said cells; and correlating the position of the transposition events with the observed variant phenotype, the position of the transposition events being indicative of the location of one or more genetic loci associated with the observed variant phenotype.

A "transposition event" is a change in genomic sequence caused by transposon mobilisation and includes insertion events, excision events or chromosomal breaks.

Insertion events may be detected by screening for the presence of the transposon by probing for the nucleic acid sequence of the transposon. Excisions may also be identified by the "signature" sequence left behind upon excision.

A fifth aspect of the invention provides a method for isolating a gene which is correlated with a phenotypic characteristic in a plurality of cells in a transgenic animal, comprising the steps of:

(a) generating a transgenic embryo and inducing transposition therein by a method according to the first or third aspect of the invention or a transgenic organism according to the second aspect of the invention;
(b) identifying in the transgenic embryo or offspring developed therefrom the presence of a plurality of cells displaying said phenotypic characteristic;
(c) detecting the position of one or more transposon transposition events in the genome of one or more of said cells; and
(d) cloning the genetic loci comprising the insertions.

The locus of the modification may be identified precisely by locating the transposon insertion. Sequencing of flanking regions allows identification of the locus in databases, potentially without the need to sequence the locus.

In preferred embodiments of the invention, the transposon may be a natural transposon. Preferably, it is a type 2 transposon, such as Minos. Most advantageously, it is Minos. Alternative transposons include *mariner, Hermes, piggyBac, hobo* and salmonid -type transposons such as *SleepingBeauty*.

Modified transposons, which incorporate one or more heterologous coding sequences and/or expression control sequences may also be used in the invention. Such coding sequences may include selectable and/or unselectable marker genes, which may facilitate the identification of transposons in the genome and cloning of the loci into which the transposons have been integrated. Suitable markers include fluorescent and/or luminescent polypeptides, such as GFP and derivatives thereof, luciferase, β-galactosidase, or chloramphenicol acetyl transferase (CAT).

Such markers may be used in *in vivo* enhancer or silencer traps and exon traps, by, for example inserting transposons which comprise marker genes which are modulated in their expression levels by proximity with enhancers or exons. Constructs for use in exon and enhancer traps are described in EP 0955364. Using the methods of the invention, the plurality of cells or tissues homogeneous for a transposition event may display modulation of expression of marker gene(s), thus enabling efficient trapping of enhancers and/or silencers and/or exons. Moreover, in embodiments where only a proportion of cells or tissues of a particular type are homogeneous for the transposition event, modulation of the expression of a marker gene may be identified by comparison with cells or tissues of the same type in the same transgenic animal which does not display such modulation.

Accordingly, the invention further provides in a sixth aspect a method for isolating an enhancer or a silencer in a transgenic animal, comprising the steps of:

(a) generating a transgenic embryo and inducing transposition therein by a method according to the first or third aspect of the invention or a transgenic organism according to the second aspect of the invention, wherein the transposon comprises a reporter gene under the control of a minimal promoter such that it is expressed at a basal level;
(b) assessing the level of expression of the reporter gene in one or more cells or tissues of the of the transgenic embryo or offspring derived therefrom;
(c) identifying and cloning genetic loci in one or more of said cells or tissues in which the modulation of the reporter gene is increased or decreased compared to the basal expression level; and
(d) characterising the cloned genetic loci in said cell or tissue.

In a seventh aspect, there is provided a method for isolating an exon in a transgenic animal, comprising the steps of:

(a) generating a transgenic embryo and inducing transposition therein by a method according to the first or third aspect of the invention or a transgenic organism according to the second aspect of the invention, wherein the transposon comprises a reporter gene which lack translation initiation sequences but includes splice acceptor sequences;
(b) identifying in the embryo or offspring derived therefrom one or more cells or tissues in which the reporter gene is expressed; and
(c) cloning the genetic loci comprising the expressed reporter gene from said cells or tissues.

Figures 2, 3 and 4 schematically illustrate gene trap constructs which may be used in generating embryos for use in these aspects of the invention.

Figure 2 illustrates a transposase construct which is under the control of the inducible TetO promoter and a transposon comprising a marker gene encoding an autofluorescent protein (AFP) under the control of a minimal promoter. In transgenic embryos comprising both constructs, expression of the transposase may be induced by activation of the inducible TetO promoter, such that the transposition of the transposon construct may be achieved. Integration into the genome at or near an enhancer site can be detected by expression of the marker gene.

Figure 3 illustrates a transposase construct which is under the control of the inducible TetO promoter and a transposon comprising an AFP fluorescent reporter gene which lacks translation initiation sequences but includes splice acceptor sequences. In transgenic embryos comprising both constructs, expression of the transposase may be induced by activation of the inducible TetO promoter, such that the transposition of the transposon construct may be achieved. Integration into an intron in the appropriate orientation can be detected by expression of the marker gene.

In a preferred embodiment, transposons may be used to upregulate the expression of genes. For example, a transposon may be modified to include an enhancer or other transcriptional activation element. Mobilisation and insertion of such a transposon in the vicinity of a gene upregulates expression of the gene or gene locus. This embodiment has particular advantage in the isolation of oncogenes, which may be identified in clonal tumours by localisation of the transposon.

Figure 4 illustrates a gene activation system which may be used in generating embryos for use in this aspect of the invention. Figure 4 illustrates a transposase construct which is under the control of the inducible TetO promoter and a transposon comprising an AFP fluorescent marker gene similarly under the control of the inducible TetO promoter. In transgenic embryos comprising both constructs, expression of the transposase may be induced by activation of the inducible TetO promoter, such that the transposition of the transposon construct may be achieved. Further, if the transposed construct comprising the AFP is inserted upstream of an ectopic gene, the gene may be activated and its phenotypic effect observed upon induction of the TetO promoter.

In conventional methods of inducing genetic modifications by transposition, in which a mosaic of cells each of which may have unique transposition induced genetic modifications is produced, the study of the phenotypic results of each transposition event is difficult. Similarly, the study of the effects of natural and artificial stimuli on the transposed cells is difficult to perform and to interpret. However, in contrast, as the methods of the invention enable the production of transgenic embryos and organisms in which one or more cluster of cells or tissues is homogeneous for a single transposition event, the effect of a pharmacological or natural stimulus may be easily observed, e.g. by comparison of reporter gene expression in a homogeneous cluster of cells with that in surrounding cells or tissues. Thus, the methods of the invention may be used to study the response of cells in which transposition events have occurred to natural stimuli such as physiological stimuli, for example, hormones, cytokines and growth factor or artificial stimuli such as drugs in drug discovery approaches, toxicology studies and the like. Indeed, the methods of the invention enable the response of a cluster of cells or tissues to a stimulus to be observed in real time.

Accordingly, in an eighth aspect of the invention, there is provided a method for identifying a gene responsive to a stimulus in a transgenic animal comprising the steps of :

(a) generating a transgenic embryo and inducing transposition therein by a method according to the first or third aspect of the invention or a transgenic organism according to the second aspect of the invention, wherein the transposon comprises a reporter gene under the control of a minimal promoter such that it is expressed at a basal level;

(b) assessing the level of expression of the reporter gene in one or more cells or tissues of the of the transgenic embryo or offspring derived therefrom in the absence of the stimulus;

(c) providing the stimulus;

(d) identifying and cloning genetic loci in one or more of said cells or tissues in which the modulation of the reporter gene is increased or decreased in response to the stimulus compared to the basal expression level.

The method may further comprise the additional step:

(e) characterising the cloned genetic loci in said cell or tissue.

This aspect of the invention may thus be used in the identification of novel targets for molecular intervention, including targets for disease therapy in humans, plants or animals, development of insecticides, herbicides, antifungal agents and antibacterial agents.

One further application is the discovery of genes responsible for pathogenesis (for example, in mouse disease models). If activation of a gene, (e.g. a kinase or a receptor) is involved in the pathogenesis of the disease, it is possible that, for example, 50% inactivation of the gene will alleviate or reverse one or more phenotypes of the disease. Therefore, groups of cells with an insertion of a transposon which inactivates one of the two copies of such a gene will be detectable as healthy clusters in a diseased background.

The transposon may be inserted into a gene. Preferably, the transposon is inserted into a transcribed gene, resulting in the localisation of said transposon in open chromatin. The transposon may be flanked by chromatin opening domain elements, such as locus control regions which provide tissue specific expression (Fraser, P. & Grosveld, F. (1998). Curr. Opin. Cell Biol.10, 361-365) or ubiquitously-acting chromatin opening elements - (UCOEs), which enable non-tissue specific expression (for example see WO 0005393). Other chromatin opening domains which may be used in methods of the invention include CpG rich islands, which may normally be associated with housekeeping genes or tissue specific genes, or insulators.

Moreover, the transposon may itself comprise, between the transposon ends, chromatin opening domains. This will cause activation of the chromatin structure into which the transposon integrates, facilitating access of the inducible transposase in a cell or tissue specific manner thereto.

Similarly the transposase construct may comprise or be flanked by chromatin opening domain elements.

The ability to regulate the transposition event during embryo development and adult life increases the likelihood of transposition events in multiple chromatin domains present in different tissues during different times of development.

The methods of the invention may advantageously be used in the generation of a library of genetically modified organisms, in each of which one or more populations of cells or tissues are homogeneous for a genetic modification produced by transposon mobilisation at a predetermined stage of development. Thus, in a further aspect of the invention, there is provided a method for producing a library of transgenic organisms in each of which one or more populations of cells or tissues are homogeneous for a gene modified by transposon mobilisation, comprising modifying cells by transposon mobilisation by the method according to a first or second or third aspect of the invention, wherein step (d) is performed at said predetermined stage of embryo development.

A library of transgenic organisms produced by such a method forms a further aspect of the invention.

Brief Description of the Figures

Figure 1 illustrates schematically, the use of a self-inactivating autonomous transposon construct in an embodiment of the invention. A first transgenic organism (A), the genome of which comprises a regulator construct, is crossed with a second transgenic organism (B), the genome of which comprises a transposon comprising a gene of interest. One of the inverted repeats of the transposon is positioned in an intron which interrupts the transposase gene. On inducing mobilisation of the transposon in the progeny of the cross (C) the transposase gene is disrupted, resulting in stabilised transposition of the gene of interest with no further transposition events, even in the presence of inducer.

Figure 2 shows schematically a transposase encoding construct in which the transposase gene is under the control of the inducible TetO promoter and a transposon comprising an AFP fluorescent marker gene under the control of a minimal promoter for use in enhancer trap methods of the invention.

Figure 3 illustrates a transposase construct which is under the control of the inducible TetO promoter and a transposon comprising an AFP fluorescent reporter gene which lacks translation initiation sequences but includes splice acceptor sequences for use in exon trap methods of the invention.

Figure 4 shows schematically a transposase construct and a transposon for use in gene traps to identify ectopic genes.

Figure 5 shows the Minos derived vector pMiCMVGFP. Minos inverted terminal repeats are shown as thick black arrows. White blocks outside these arrows indicate the sequences flanking the original Minos element in the D. hydei genome. Arrowheads indicate the positions of primers used to detect Minos excisions. Small arrows indicate the direction of transcription of the GFP and transposase genes. Black bars represent fragments used as probes.

Figure 6 illustrates the structure of the *Minos* transposase expression cassette which may be used in an embodiment of the invention as described in Example 4. The 6.5kb 5' flanking region and promoter of the ZP3 gene were joined to *Minos* transposase cDNA (ILMi) and the second intron and polyadenylation site of the human β globin gene. The relevant restriction sites are indicated.

Figure 7 illustrates detection of the *Minos* transposase transcription by RT-PCR analysis. The different tissues that were analysed are indicated. The murine hypoxantine phosphoribosyltransferase (HPRT) was used as an internal control. pUC 18 DNA *Msp*I digested was used as a marker (M) and H_2O in the negative control.

Figure 8 illustrates Southern blot analysis of different offspring showing transposition events. All DNA samples were *Bgl* II digested and probed with a ^{32}P labelled GFP probe. Lane 1 is a control (female mouse carrying the GFP transposon as a multicopy on Chr 14 and expressing *Minos* transposase in developing oocytes). Lanes 2, 5, 6,7,8,10 and 11 correspond to progeny of a double transgenic female and wt male. They all represent different transposition events. Lanes 2 and 5: mice with 2 transposition events. Lanes 3 and 4 correspond to the offspring of the mouse in lane 2 with 2 transposition events, showing segregation of the insertions (lane 3 on chromosome 2, lane 4 on chromosome 14 near the centromere). Lane 9: Offspring of the mouse shown in lane 8, showing segregation.

Figure 9 illustrates the sequence of the parental and four different *Minos* insertions in the *Mus musculus* genome. Chromosomal sequences flanking the new inserted transposon are represented by capital letters, transposon sequence in small letters and the target site duplication in red. The chromosomal locations of insertions and scaffold numbers from the Celera database are indicated

Figure 10 illustrates FISH analysis of *Minos* transposition events. Chromosomes were stained with 4', 6-diamino-2-phenylindole and probed with a GFP probe as described in Example 4 Materials and Methods. Panel A: mouse 8218-01 with two transposition events (on chromosome 2 and at the centromere of chromosome 14; see Figure 8 lane 2); Red colour (indicated by arrow head) : staining after tyramide amplification (see Example 4 Materials and Methods). Panel B: mouse 8218-02 with two transposition events (one on chromosome 18 and one near the telomere of chromosome 14 close to the initial position of the transposon concatemer (Figure 8 lane 5). Green colour (indicated by arrow head):FITC staining. Yellow arrows indicate transposition events.

Figure 11 illustrates knock-in (A) and regular constructs (B) for specific expression of transposase in sperm or egg or for ubiquitous expression. In Figure 11B, the beta globin 3'second exon (red), intervening sequences (green) and third exon (red) are added.

Figure 12 illustrates a trap construct for transgenic mice and/or cloning into retroviral plasmid.

Figure 13 is a diagram showing *in vivo* transposition with the female contributing the transposon, and transposase. Transposition takes place in the oocyte.

Figure 14 is a diagram showing *in vivo* transposition with the female contributing the transposon, the male contributing the transposase. Transposition takes place in the sperm.

Figure 15 is a diagram showing *in vivo* transposition with the male contributing the transposon, the female contributing the transposase. Transposition takes place in the fertilised egg or embryo.

Detailed Description of the Invention

Although in general the techniques mentioned herein are well known in the art, reference may be made in particular to Sambrook *et al.*, Molecular Cloning, A Laboratory Manual (1989) and Ausubel *et al.*, Short Protocols in Molecular Biology (1999) 4th Ed, John Wiley & Sons, Inc.

Transposons

Any transposon may be used in the method of the invention. Preferably, the transposon is type 2 transposon, more preferably selected from the group consisting of *Minos*, *mariner*, *Hermes*, *piggyBac,* and *Sleeping Beauty*. Advantageously, the transposon is *Minos*. Each transposon is advantageously employed with its natural cognate transposase, although the use of modified and/or improved transposases is envisaged. Minos transposons, and their cognate transposase, are described in detail in US patent 5,840,865 and European patent application EP 0955364.

The transposon preferably comprises a nucleic acid sequence encoding a heterologous polypeptide. This sequence will be integrated, together with the transposon, into the genome of the cell on transposon integration. Moreover, it will be excised, together with the transposon, when the latter excises on remobilisation. In a preferred embodiment, the heterologous polypeptide is a selectable marker. This allows cells having integrated transposons to be identified and the site of integration to be accurately mapped.

Marker Genes

Preferred marker genes include genes which encode fluorescent polypeptides. For example, green fluorescent proteins ("GFPs") of cnidarians, which act as their energy-transfer acceptors in bioluminescence, can be used in the invention. A green fluorescent protein, as used herein, is a protein that fluoresces green light, and a blue fluorescent protein is a protein that fluoresces blue light. GFPs have been isolated from the Pacific Northwest jellyfish, *Aequorea victoria*, from the sea pansy, *Renilla reniformis*, and from *Phialidium gregarium*. (Ward *et al.*, 1982, Photochem. Photobiol., 35: 803-808; Levine *et al.*, 1982, Comp. Biochem. Physiol.,72B: 77-85). Fluorescent proteins have also been isolated recently from Anthoza species (accession nos. AF168419, AF168420, AF168421, AF168422, AF168423 and AF168424).

A variety of *Aequorea*-related GFPs having useful excitation and emission spectra have been engineered by modifying the amino acid sequence of a naturally occurring GFP from Aequorea victoria (Prasher *et al.*, 1992, Gene, 111: 229-233; Heim *et al.*, 1994, Proc. Natl. Acad. Sci. U.S.A., 91: 12501-12504; PCT/US95/14692). *Aequorea*-related fluorescent proteins include, for example, wild-type (native) *Aequorea victoria* GFP, whose nucleotide and deduced amino acid sequences are presented in Genbank Accession Nos. L29345, M62654, M62653 and others *Aequorea*-related engineered versions of Green Fluorescent Protein, of which some are listed above. Several of these, i.e., P4, P4-3, W7 and W2 fluoresce at a distinctly shorter wavelength than wild type.

Examples of other marker genes which may be used include selectable marker genes such as genes encoding neomycin, puromycin or hygromycin or counter-selection genes such as the genes for cytosine deaminase or nitroreductase.

Those skilled in the art are aware of a multitude of marker genes which may be used. Any suitable marker gene may be used and it should be appreciated that no particular choice is essential to the present invention.

Identification of insertion and excision events

Transposons, and sites from which transposons have been excised, may be identified by sequence analysis. For example, Minos typically integrates at a TA base pair, and on excision leaves behind a duplication of the target TA sequence, flanking the four terminal nucleotides of the transposon. The presence of this sequence, or related sequences, may be detected by techniques such as sequencing, PCR and/or hybridisation.

Inserted transposons may be identified by similar techniques, for example using PCR primers complementary to the terminal repeat sequences.

Transposases

Effective transposon mobilisation depends on both efficient delivery of the transposable element itself to the host cell and the presence of an effective cognate transposase in the cell in order to catalyse transposon jumping. A "cognate" transposase, as referred to herein, is any transposase which is effective to activate transposition of the transposon, including excision of the transposon from a first integration site and/or integration of the transposon at a second integration site. Preferably, the cognate transposase is the transposase which is naturally associated with the transposon in its *in vivo* situation in nature. However, the invention also encompasses modified transposases, which may have advantageously improved activities within the scope of the invention. For example, the sequence of the gene encoding the transposase may be modified to optimise codon usage and thus increase transposition frequencies. Optimisation of codon usage is a method well known in the art to increase the expression levels of a given gene. Alternatively the transposase may comprise one or more insertions, substitutions or deletions of amino acids to provide enhanced activity in the host organism.

The gene encoding the transposase may be provided in the genome of a second organism which is crossed with a first organism comprising, in its genome, the transposon to produce an embryo for use in the methods of the invention. In an alternative embodiment, one or more copies of both the transposon and the gene encoding the cognate transposase are provided in the genome of a first organism, which may be crossed with a second organism comprising one or more copies of regulatory elements necessary to permit inducible transposase expression to produce an embryo.

A number of methods are known in the art for introduction of a gene into the genome of a host cell, and may be employed in the context of the present invention. For example, transposase genes may be inserted into the host cell genome by transgenic techniques. Such methods are discussed further below.

Regulation of Transposase Expression

Coding sequences encoding the transposase may be operatively linked to regulatory sequences which modulate transposase expression as desired. Control sequences operably linked to sequences encoding the transposase include promoters/enhancers and other expression regulation signals. These control sequences may be selected to be compatible with the host organism in which the expression of the transposase is required. The term promoter is well known in the art and encompasses nucleic acid regions ranging in size and complexity from minimal promoters to promoters including upstream elements and enhancers.

The promoter is typically selected from promoters which are functional in cell types homologous to the organism in question, or the genus, family, order, kingdom or other classification to which that organism belongs, although heterologous promoters may function – e.g. some prokaryotic promoters are functional in eukaryotic cells. The promoter may be derived from promoter sequences of viral or eukaryotic genes. For example, it may be a promoter derived from the genome of a cell in which expression is to occur. With respect to eukaryotic promoters, they may be promoters that function in a ubiquitous manner (such as promoters of α-actin, β-actin, tubulin) or, alternatively, a tissue-specific manner (such as promoters of the genes for pyruvate kinase). In the generation of germline transposition events, the promoters may be derived from genes whose expression is induced during gametogenesis, either oogenesis or spermatogenesis. Alternatively, for developmentally regulated transposition events such as transposition during zygote development, the promoters may be derived from genes whose expression is developmentally regulated. For expression in the early zygote, promoters from maternal effect genes may be used. They may also be promoters that respond to specific stimuli, for example promoters that bind steroid hormone receptors. Viral promoters may also be used, for example the Moloney murine leukaemia virus long terminal repeat (MMLV LTR) promoter, the rous sarcoma virus (RSV) LTR promoter or the human cytomegalovirus (CMV) IE promoter.

According to the invention, the gene encoding the transposase is under the control of one or more regulatory sequences, meaning that the levels of expression obtained using e.g. a promoter can be regulated. For example the regulatory sequence may be an inducible regulatory sequence. Inducible systems for gene expression are known in the art, and include tetracycline, ecdysone and estrogen-inducible systems or the lac operator–repressor system.

A widely used system of this kind in mammalian cells is the tetO promoter-operator, combined with the tetracycline/doxycycline-repressible transcriptional activator tTA, also called Tet-Off gene expression system (Gossen, M. & Bujard, H. (1992) Tight control of gene expression in mammalian cells by tetracycline responsive promoters.

Proc. Natl. Acad. Sci. USA 89:5547–5551), or the doxycycline-inducible rtTA transcriptional activator, also called Tet-On system (Gossen, M., Freundlieb, S., Bender, G., Muller, G., Hillen, W. & Bujard, H. (1995) Transcriptional activation by tetracycline in mammalian cells. Science 268:1766–1769).

In the Tet-Off system, gene expression is turned on when tetracycline (Tc) or doxycycline (Dox; a Tc derivative) is removed from the culture medium. In contrast, expression is turned on in the Tet-On system by the addition of Dox. Procedures for establishing cell lines carrying the transcriptional activator gene and the Tet-regulatable gene stably integrated in its chromosomes have been described. For example see http://www.clontech.com/techinfo/manuals/PDF/PT3001-1.pdf. For example, the Tet-On system may be employed for tetracycline-inducible expression of Minos transposase in a transgenic animal.

A doubly transgenic animal may be generated by standard homologous recombination ES cell technology. Two constructs are used: first, a construct containing the rtTA gene under a constitutive promoter. An example of such construct is the pTet-On plasmid (Clontech) which contains the gene encoding the rtTA activator under control of the Cytomegalovirus immediate early (CMV) promoter. The rtTA transcriptional activator encoded by this construct is active only in the presence of Doxycycline. The second construct contains the Minos transposase gene under control of the tetracycline-response element, or TRE. The TRE consists of seven direct repeats of a 42-bp sequence containing the tet operator (tetO), and is located just upstream of the minimal CMV promoter, which lacks the enhancer elements normally associated with the CMV immediate early promoter. Because these enhancer elements are missing, there is no "leaky" expression of transposase from the TRE in the absence of binding by rtTA. An example of such construct is the pTRE2 plasmid (Clontech) in the MCS of which is inserted the gene encoding Minos transposase. In cells stably transformed with the two constructs, rtTA is expressed but does not activate transcription of Minos transposase unless Doxycycline is administered to the animal.

Therefore, when, according to a method of the invention, a transgenic animal comprising both pTet-On and the pTRE2 constructs is crossed with another animal, the genome of which comprises a transposon, mobilisation of transposons in resulting embryos comprising within their genomes both the transposon and the gene encoding the transposase will not occur in the absence of Doxycycline. Administration of Doxycycline may thus be used to induce transposition.

Inducers, e.g. Doxycycline, may be administered to embryos by any suitable method. In a preferred embodiment of the invention, inducers of transposase expression are administered via the food or water of the parent organism.

Alternative inducible systems include tamoxifen inducible transposase [a modified oestrogen receptor domain (Indra et al., Nucl Acid Res. 27, 4324-27, 1999) coupled to the transposase which retains it in the cytoplasm until tamoxifen is given to the culture], an RU418 inducible transposase (operating under the same principle with the glucocorticoid receptor; see Tsujita et al., J. Neuroscience, 19, 10318-23, 1999), or an ecdysone-inducible system.

The ecdysone-inducible system is based on the heterodimeric ecdysone receptor of Drosophila, which is induced by the insect hormone, ecdysone and its derivatives. During metamorphosis of Drosophila melanogaster, a cascade of morphological changes is triggered by the steroid hormone 20-OH ecdysone, generally referred to as "ecdysone", via the ecdysone receptor. Ecdysone responsiveness may be transferred to mammalian cells by the stable expression of a modified ecdysone receptor that regulates an optimized ecdysone responsive promoter. Transgenic organisms, e.g. mice expressing the modified ecdysone receptor can activate an integrated ecdysone responsive promoter upon administration of hormone or its derivatives e.g. Once the receptor binds ecdysone or muristerone, an analog of ecdysone, the receptor activates the ecdysone-responsive promoter to give controlled expression of the gene of interest. Ecdysone-based inducible systems are reported to exhibit lower basal activity and higher inducibility than tetracycline based systems. Further details of ecdysone based inducible systems can be found, for example, in US 6,245,531 and in No D, Yao TP, Evans RM Ecdysone-inducible gene expression in mammalian cells and transgenic mice, Proc Natl Acad Sci U S A 1996 Apr 93:3346-51, the contents of each of which are herein incorporated by reference.

The lac operator–repressor system has recently been shown to be functional in mammals, in particular the mouse. Cronin et al, Genes and Development, 15, 1506-1517 (2001), the contents of which are herein incorporated by reference, describes the use of a *lac* repressor transgene that resembles a typical mammalian gene both in codon usage and structure and that expresses functional *lac* repressor protein ubiquitously in mice to control the expression of a reporter gene under the control of the *lac* promoter. Expression of the reporter gene is reversible using the lactose analog IPTG provided in the drinking water of the mouse or mother of the embryo or nursing pup. The *lac* operator–repressor system may thus be adapted for use to regulate expression of transposase by placing the transposase gene under the control of a *lac* promoter.

In addition, any of these promoters may be modified by the addition of further regulatory sequences, for example enhancer sequences. Chimeric promoters may also be used comprising sequence elements from two or more different promoters described above.

The use of locus control regions (LCRs) is also envisaged. LCRs are capable of conferring tightly-regulated tissue specific control on transgenes, and to greatly increase the fidelity of transgene expression. A number of LCRs are known in the art. These include the β-globin LCR (Grosveld *et al.*, (1987) Cell 51:975-985); α-globin (Hatton *et al.*, (1990) Blood 76:221-227; and CD2 (Festenstein *et al.*, (1996) Science 271:1123-1125) the T cell specific CD4 (Boyer et al J Immunol 1997, 159:3383-3390), and TCR loci (Diaz P, et al Immunity 1994, 1:207-217; Ortiz et al EMBO J 1997,16:5037-5045; Hong et al Mol Cell Biol 1997, 17:2151-2157.) the B-cell-specific MHC class II Ea (Carson et al Nucleic Acids Res 1993,21:2065-2072), the macrophage-specific lysozyme gene (Bonifer et al EMBO J 1990,9:2843-2848), the neuron-specific S100 gene (Friend et al J Neurosci 1992, 12:4337-4346), the liver-specific LAP gene (Talbot et al Nucleic Acids Res 1994, 22:756-766), the human growth hormone locus (Jones et al Mol Cell Biol 1995, 15:7010-7021), plus immunoglobulins, muscle tissue, and the like. Further details on LCRs are provided in Fraser, P. & Grosveld, F. (1998). Curr. Opin. Cell Biol.10, 361-365 and Li, Q., Harju, S. & Peterson, K.R. (1999). Trends Genet.15: 403-408.

Alternatively, gene domains that need to be open and switched-on in all cells of the body; i.e. gene domains whose proteins (such as enzymes for generating energy from sugars), are needed by all cells for survival and which are therefore ubiquitously expressed may be exploited to enable expression of the transposon and/or transposase in any tissue. Examples of such ubiquitously-acting chromatin opening elements - (UCOEs) include the human genes known as TBP and hnRNPA2. Further details of the use of such UCOEs may be found in Antoniou, M. and Grosveld, F. (1999). (Genetic approaches to therapy for the haemoglobinopathies. in: Blood Cell Biochemistry, Volume 8: Hematopoiesis and Gene Therapy Fairbairn and Testa eds. Kluwer Academic/Plenum Publishers, New York. pp 219-242) and in PCT/GB99/02357 (WO 0005393), the contents of both of which are herein incorporated by reference.

Regulation of transposase and/or transposon expression may also be achieved through the use of ES cells. Using transformed ES cells to construct chimeric embryos, it is possible to produce transgenic organisms which contain the transposase genes or transposon element in only certain of their tissues. This can provide a further level of regulation.

Maximising efficiency of transposition

As described above and for example, in WO 01/71019 and WO 02/062991, transposition is achieved by the action of the transposase enzyme on the terminal repeat sequences of the integrated transposon, resulting in excision of the transposon from its original position in the "host" genome and reinsertion of the transposon at a different position in the genome.

As with most biochemical processes, this process can be made to be more efficient by simply improving the concentration of substrates, high levels of the terminal repeats sequences, i.e. an increase in copy number and high levels of the transposase enzyme.

An increase in copy number can be achieved by generating multiple copy arrays at the original insertion site. For example, 10 to 100 copies can be generated through standard transgenesis or using a PAC vector. Alternatively, multiple copies can be generated by the presence of multiple insertions at different sites in the genome.

The sequence of the transposase may be modified to optimise codon usage and thus, increase transposition frequencies. Optimisation of codon usage is a method well known in the art to increase the expression levels of a given gene.

Thus, the efficiency of the fly transposase in mammalian cells or animals may be increased by increasing its concentration as a result of a more efficient translation from mRNA by replacing the fly codon usage to mammalian codon usage.

Assays for determining transposase efficiency can include a standard transposition assay as described, for example, by Klinakis et al.; Insect Molecular Biology, 9 (3), 269-275, 2000.

The concentration of transposase mRNA can also be increased by including in the transposase mRNA sequence 5' and 3' sequences found in abundant stable mRNAs such as those encoding growth hormone, globin, actin or albumin.

Transgenic Organisms

Methods of the invention may employ one or more transgenic organisms having integrated in the genome the transposon, a gene encoding the cognate transposase or both.

The introduction of the transposon or gene encoding the transposase may be accomplished by any available technique, including transformation/transfection, delivery by viral or non-viral vectors and microinjection. Each of these techniques is known in the art. The transposon and the gene encoding the transposase may be inserted using the same or different methods. For example, the Drosophila P-element may be used to introduce a Minos transposase construct into Drosophila.

In a preferred embodiment, the transposon or gene encoding the transposase may be inserted into the host cell genome by transgenic techniques, for example to produce a transgenic animal comprising a transposon, a gene encoding a cognate transposase or both. Where the transgenic animal comprises both the transposon and the gene encoding the transposase, both constructs can be inserted using the same or different methods. Where delivery of the construct is by viral vector, a composite vector comprising both the transposon and the gene encoding the transposase under the control of a control sequence such as the Tet operator may be used. Alternatively, separate vectors may be used.

Any suitable transgenic animal may be used in the present invention. Animals include animals of the phyla cnidaria, ctenophora, platyhelminthes, nematoda, annelida, mollusca, chelicerata, uniramia, crustacea and chordata. Uniramians include the subphylum hexapoda that includes insects such as the winged insects. Chordates include vertebrate groups such as mammals, birds, fish, reptiles and amphibians. Particular examples of mammals include non-human primates, cats, dogs, ungulates such as cows, goats, pigs, sheep and horses and rodents such as mice, rats, gerbils and hamsters.

Techniques for producing transgenic animals which may be used in the method of the invention are well known in the art. A useful general textbook on this subject is Houdebine, Transgenic animals – Generation and Use (Harwood Academic, 1997) – an extensive review of the techniques used to generate transgenic animals.

In a preferred embodiment, the animal is an insect. Methods for producing transgenic insects which may be used in the method of the invention are well known (see for example Loukeris et al (1995), Science 270 2002-2005). Briefly, a transposable element carrying the gene of interest is inserted into a preblastoderm embryo using e.g. microinjection. Preferably, the new genetic material is placed at the polar plasm, which is the section of egg destined to become the still nascent insect' s own egg or sperm cells. After many divisions of the nuclear material, most of it segregates to the periphery where it will become the nuclei of the insect's body. A small number of nuclei migrate to the pole to become egg cells on maturity. If these cells incorporate the transgene, progeny will be transgenic. Further details of producing transgenic insects are provided in Loukeris et al (1995), Science 270 2002-2005 and O'Brochta and Atkinson (1998) Scientific American 279 60-65.

In another preferred embodiment, the animal is preferably a mammal. Advances in technologies for embryo micromanipulation now permit introduction of heterologous DNA into, for example, fertilised mammalian ova. For instance, totipotent or pluripotent stem cells can be transformed by microinjection, calcium phosphate mediated precipitation, liposome fusion, retroviral infection or other means, the transformed cells are then introduced into the embryo, and the embryo then develops into a transgenic animal. In a highly preferred method, developing embryos are infected with a retrovirus containing the desired DNA, and transgenic animals produced from the infected embryo. In a most preferred method, however, the appropriate DNAs are coinjected into the pronucleus or cytoplasm of embryos, preferably at the single cell stage, and the embryos allowed to develop into mature transgenic animals. Those techniques as well known (see reviews of standard laboratory procedures for microinjection of heterologous DNAs into mammalian fertilised ova, including Hogan *et al.*, Manipulating the Mouse Embryo, (Cold Spring Harbor Press 1986); Krimpenfort *et al.*, Bio/Technology 9:844 (1991); Palmiter *et al.*, Cell, 41: 343 (1985); Kraemer *et al.*, Genetic manipulation of the Mammalian Embryo, (Cold Spring Harbor Laboratory Press 1985); Hammer *et al.*, Nature, 315: 680 (1985); Wagner *et al.*, U.S. Pat. No. 5,175,385; Krimpenfort *et al.*, U.S. Pat. No. 5,175,384, the respective contents of which are incorporated herein by reference.).

Another method used to produce a transgenic animal involves microinjecting a nucleic acid into pro-nuclear stage eggs by standard methods. Injected eggs are then cultured before transfer into the oviducts of pseudopregnant recipients.

Transgenic animals may also be produced by nuclear transfer technology as described in Schnieke, A.E. *et al.*, 1997, Science, 278: 2130 and Cibelli, J.B. *et al.*, 1998, Science, 280: 1256. Using this method, fibroblasts from donor animals are stably transfected with a plasmid incorporating the coding sequences for a polypeptide of interest under the control of regulatory sequences. Stable transfectants are then fused to enucleated oocytes, cultured and transferred into female recipients.

Analysis of animals which may contain transgenic sequences would typically be performed by either PCR or Southern blot analysis following standard methods.

By way of a specific example for the construction of transgenic mammals, such as cows, nucleotide constructs comprising a sequence encoding a DNA binding molecule are microinjected using, for example, the technique described in U.S. Pat. No. 4,873,191, into oocytes which are obtained from ovaries freshly removed from the mammal. The oocytes are aspirated from the follicles and allowed to settle before fertilisation with thawed frozen sperm capacitated with heparin and prefractionated by Percoll gradient to isolate the motile fraction.

The fertilised oocytes are centrifuged, for example, for eight minutes at 15,000 g to visualise the pronuclei for injection and then cultured from the zygote to morula or blastocyst stage in oviduct tissue-conditioned medium. This medium is prepared by using luminal tissues scraped from oviducts and diluted in culture medium. The zygotes must be placed in the culture medium within two hours following microinjection.

Oestrous is then synchronized in the intended recipient mammals, such as cattle, by administering coprostanol. Oestrous is produced within two days and the embryos are transferred to the recipients 5-7 days after oestrous. Successful transfer can be evaluated in the offspring by Southern blot.

Alternatively, the desired constructs can be introduced into embryonic stem cells (ES cells) and the cells cultured to ensure modification by the transgene. The modified cells are then injected into the blastula embryonic stage and the blastulas replaced into pseudopregnant hosts. The resulting offspring are chimeric with respect to the ES and host cells, and nonchimeric strains which exclusively comprise the ES progeny can be obtained using conventional cross-breeding. This technique is described, for example, in WO91/10741.

Alternative methods for delivery and stable integration of transposons and/or genes encoding transposases into the genome of host animals include the use of viral vectors, such as adenoviral vectors, retroviral vectors, baculoviral vectors and herpesviral vectors. Such techniques have moreover been described in the art, for example by Zhang *et al.* (Nucl. Ac. Res., 1998, 26:3687-3693).

Suitable viral vectors may be retroviral vectors, and may be derived from or may be derivable from any suitable retrovirus. A large number of different retroviruses have been identified. Examples include: murine leukaemia virus (MLV), human immunodeficiency virus (HIV), simian immunodeficiency virus, human T-cell leukaemia virus (HTLV). equine infectious anaemia virus (EIAV), mouse mammary tumour virus (MMTV), Rous sarcoma virus (RSV), Fujinami sarcoma virus (FuSV), Moloney murine leukaemia virus (Mo-MLV), FBR murine osteosarcoma virus (FBR MSV), Moloney murine sarcoma virus (Mo-MSV), Abelson murine leukaemia virus (A-MLV), Avian myelocytomatosis virus-29 (MC29), and Avian erythroblastosis virus (AEV). A detailed list of retroviruses may be found in Coffin et al., 1997, "retroviruses", Cold Spring Harbour Laboratory Press Eds: JM Coffin, SM Hughes, HE Varmus pp 758-763.

Details on the genomic structure of some retroviruses may be found in the art. By way of example, details on HIV and Mo-MLV may be found from the NCBI GenBank (Genome Accession Nos. AF033819 and AF033811, respectively).

Retroviruses may be broadly divided into two categories: namely, "simple" and "complex". Retroviruses may even be further divided into seven groups. Five of these groups represent retroviruses with oncogenic potential. The remaining two groups are the lentiviruses and the spumaviruses. A review of these retroviruses is presented in Coffin et al., 1997 (ibid).

Host range and tissue tropism varies between different retroviruses. In some cases, this specificity may restrict the transduction potential of a recombinant retroviral vector. For this reason, many gene therapy experiments have used MLV. A particular MLV that has an envelope protein called 4070A is known as an amphotropic virus, and this can also infect human cells because its envelope protein "docks" with a phosphate transport protein that is conserved between man and mouse. This transporter is ubiquitous and so these viruses are capable of infecting many cell types.

Replication-defective retroviral vectors are typically propagated, for example to prepare suitable titres of the retroviral vector for subsequent transduction, by using a combination of a packaging or helper cell line and the recombinant vector. That is to say, that the three packaging proteins can be provided in trans.

A "packaging cell line" contains one or more of the retroviral gag, pol and env genes. The packaging cell line produces the proteins required for packaging retroviral DNA but it cannot bring about encapsidation due to the lack of a psi region. The helper proteins can package a psi-positive recombinant vector to produce the recombinant virus stock. This virus stock can be used to transduce cells to introduce the vector into the genome of the target cells. A summary of the available packaging lines is presented in Coffin et al., 1997 (ibid).

The lentivirus group can be into "primate" and "non-primate". Examples of primate lentiviruses include human immunodeficiency virus (HIV), and simian immunodeficiency virus (SIV). The non-primate lentiviral group includes the prototype "slow virus" visna/maedi virus (VMV), as well as the related caprine arthritis-encephalitis virus (CAEV), equine infectious anaemia virus (EIAV) and the more recently described feline immunodeficiency virus (FIV) and bovine immunodeficiency virus (BIV). See, for example, Rovira *et al.*, Blood. 2000;96:4111-4117; Reiser *et al.*, J Virol. 2000 Nov;74 (Mulder, M.P *et al.* (1995). *Hum Genet* **96(2)**:133-141):10589; Lai *et al.*, Proc Natl Acad Sci U S A 2000 Oct 10;97(Southern, E.M. (1975). *J. Mol. Biol* **98**,503-517):11297-302; and Saulnier *et al.*, J Gene Med 2000 Sep-Oct;2(5):317-25.

A distinction between the lentivirus family and other types of retroviruses is that lentiviruses have the capability to infect both dividing and non-dividing cells. In contrast, other retroviruses - such as MLV - are unable to infect non-dividing cells such as those that make up, for example, muscle, brain, lung and liver tissue

A number of vectors have been developed based on various members of the lentivirus sub-family of the retroviridae and a number of these are the subject of patent applications (WO-A-98/18815; WO-A-97/12622). Preferred lentiviral vectors are based on HIV, SIV or EIAV. The simplest vectors constructed from HIV-1 have the complete HIV genome except for a deletion of part of the env coding region or replacement of the nef coding region. Notably these vectors express gag/pol and all of the accessory genes hence require only an envelope to produce infectious virus particles. Of the accessory genes vif, vpr, vpu and nef are non-essential.

One preferred general format for HIV-based lentiviral vectors is, HIV 5'LTR and leader, some gag coding region sequences (to supply packaging functions), a reporter cassette, the rev response element (RRE) and the 3'LTR. In these vectors gag/pol, accessory gene products and envelope functions are supplied either from a single plasmid or from two or more co-transfected plasmids, or by co-infection of vector containing cells with HIV.

The adenovirus is a double-stranded, linear DNA virus that does not go through an RNA intermediate. There are over 50 different human serotypes of adenovirus divided into 6 subgroups based on the genetic sequence homology all of which exhibit comparable genetic organisation. Human adenovirus group C serotypes 2 and 5 (with 95% sequence homology) are most commonly used in adenoviral vector systems and are normally associated with upper respiratory tract infections in the young.

Adenoviruses/adenoviral vectors which may be used in the invention may be of human or animal origin. As regards the adenoviruses of human origin, preferred adenoviruses are those classified in group C, in particular the adenoviruses of type 2 (Ad2), 5 (Ad5), 7 (Ad7) or 12 (Ad12). Among the various adenoviruses of animal origin, canine adenovirus, mouse adenovirus or an avian adenovirus such as CELO virus (Cotton et al., 1993, J Virol 67:3777-3785) may be used.

HSV vectors may be derived from, for example, HSV1 or HSV2 strains, or derivatives thereof. Attenuated strains may be used for example strain 1716 (MacLean et al., 1991, J Gen Virol 72: 632-639), strains R3616 and R4009 (Chou and Roizman, 1992, PNAS 89: 3266-3270) and R930 (Chou et al., 1994, J. Virol 68: 8304-8311) all of which have mutations in ICP34.5, and d27-1 (Rice and Knipe, 1990, J. Virol 64: 1704-1715) which has a deletion in ICP27. Alternatively strains deleted for ICP4, ICP0, ICP22, ICP6, ICP47, vhs or gH, with an inactivating mutation in VMW65, or with any combination of the above may also be used to produce HSV strains of the invention.

The terminology used in describing the various HSV genes is as found in Coffin and Latchman, 1996. Herpes simplex virus-based vectors. In: Latchman DS (ed). Genetic manipulation of the nervous system. Academic Press: London, pp 99-114.

Baculovirus vectors may moreover be employed in the invention. The baculovirus Autographa californica multiple nuclear polyhedrosis virus (AcMNPV) is a DNA virus which can replicate only in cells of certain lepidopteran insects and has been used widely for expression of recombinant proteins in insect cells. Baculoviruses such as

AcMNPV have been used recently for introducing heterologous DNA with high efficiency in a variety of mammalian cells, such as a hepatoma cell line and primary liver cells, and endothelial cells (Boyce FM, Bucher NL (1996) *Baculovirus-mediated gene transfer into mammalian cells.* Proc Natl Acad Sci U S A 93, 2348-52; Airenne KJ, Hiltunen MO, Turunen MP, Turunen AM, Laitinen OH, Kulomaa MS, Yla-Herttuala S (2000) *Baculovirus-mediated periadventitial gene transfer to rabbit carotid artery.* Gene Ther 7,1499-1504). Moreover, baculovirus vectors for gene transfer, methods for introducing heterologous DNA into their genome and procedures for recombinant virus production in insect cell cultures are available commercially; furthermore, baculoviruses cannot normally replicate in mammalian cells, so there is no need to engineer them for this use.

Construction of vectors for use in methods of the invention may employ conventional ligation techniques. Isolated viral vectors, plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to generate the plasmids required. If desired, analysis to confirm correct sequences in the constructed vectors is performed in a known fashion. Transposon presence and/or mobilisation may be measured in a cell directly, for example, by conventional Southern blotting, dot blotting, PCR or in situ hybridisation, using an appropriately labelled probe which may be based on a sequence present in the transposon. Those skilled in the art will readily envisage how these methods may be modified, if desired. Vectors useful in the present invention are advantageously provided with marker genes to facilitate transposon identification and localisation as described above.

Uses of the Invention

The methods of the present invention enables the generation of transgenic embryos and organisms comprising one or more clonal populations of cells homogeneous for one or more individual mutations. Thus, transgenic embryos and animals can be produced in which a cluster of cells, a tissue or tissues, or an organ or group of organs each share the same genetic modification. The presence of the same genetic modification in a number of cells, tissues or organs enables convenient phenotypic and genotypic analysis of the modification and moreover enables the comparison of the effects of a particular gene modification to be compared with corresponding wild-type genes or indeed other gene modifications in the same type of cell, tissue or organ within the same organism.

The invention may further be used to monitor gene expression patterns of modified genes can be monitored during embryo development and in adult cells and tissues.

Transposons, and sites from which transposons have been excised, may be identified by sequence analysis. For example, Minos typically integrates at a TA base pair, and on excision leaves behind a footprint, consisting of duplication of the target TA sequence, flanking the four terminal nucleotides of the transposon. The presence of the sequence of Minos, its footprint, or related sequences, may be detected by techniques such as sequencing, PCR or hybridisation.

Inserted transposons may be identified by similar techniques, for example using PCR primers complementary to the terminal repeat sequences.

The invention allows functional mapping of a genome by permitting precise gene modulation at predetermined stages of development and subsequent detection using transposons. Thus, the invention provides for efficient intracellular transposon mobilisation and insertion into the cell genome of one or more cells or tissues of an organism, providing exon trapping functionality in cells of transgenic organisms at early stages of development. The induction of transposon mobilisation at such stages of embryonic development enables the generation of clusters of cells or tissues which are homogeneous for a transposed gene. This may therefore enable rapid and efficient detection of a change in phenotype and/or identification of the modified gene.

An example of a suitable trap construct is given in Figure 12.

The invention, in an advantageous embodiment, allows genes to be marked for functional genetic analysis in a group of cells or tissues, or knocked out, by transposon insertion and then specifically identified through the transposon “tag” without requiring costly and time-consuming genetic analyses, and frequently without significant amounts of sequencing.

A further embodiment of the invention provides for the generation of libraries of transgenic organisms, such as transgenic mice. Target genes may be identified phenotypically according to the phenotype of one or more cells, tissues or organs, and identified genetically by determination of the transposon insertion site. Inducible expression systems, as described above, may be used to regulate the switch between partial and antisense-induced complete knockout of a gene. Somatic cells carrying transposon insertions can be immortalized, for example by deriving immortal cell lines by standard methodologies, or by generating transgenic animal lines by nuclear implantation methodologies.

Such libraries can be used for phenotypic analysis and identification of gene associations. The present methods allow advantages over the current methods.

In inherited diseases such as the haemoglobinopathies, haemophilia, cystic fibrosis, and muscular dystrophy, well defined mutations in single genes or their regulatory elements result in inappropriate gene expression with clinical consequences which profoundly effect the life style and life expectancy of affected individuals.

However in other diseases particularly those related to aging, such as the dementias and psychoses; psychiatric disorders such as schizophrenia and manic depression; bone and joint related inflammatory conditions; obesity, insulin resistance, type 2 diabetes and related vascular and cardiovascular conditions; the genetic component is more complex and still poorly understood (see Lander and Schork, Science (1994) 265, 2037-2048).

Where multiple mutations in differing targets determines factors such as the time of disease onset (eg post menopausal insulin resistance) and severity of the disease state (eg pre-disposition to vascular disease and stroke) strategies based on random alkylation of inbreed mouse strains, and transgenic mice laboriously developed from single gene deletions in vast ES cell libraries seem destined to fail.

The present invention provides a more attractive strategy through the rapid generation of randomly "tagged" mouse strains starting with a background known to be prone to a disease state, and therefore likely to have existing mutations in interrelated disease causing genes. The background can be selected by using founder cell lines carrying multiple "dormant" transposons at known integration sites. Regulatable transposase activity will allow the rapid generation of mouse libraries in different genetic backgrounds reflecting differing models of disease.

For example it has been shown that the mutations in the insulin receptor cause mild to severe hyperinsulinemia dependent on the mouse genetic background (see Kido (2000) Diabetes 49, 589-596) indicating the involvement of other genes in the predisposition to insulin resistance. Thus generation of a tagged mouse library using *in vivo* gene transposition technology in the "mild phenotype" mouse background should lead to selection of animals with a more severe phenotype. Sequence analysis of DNA flanking new transposition events will then identify new candidate disease causing genes which contribute to the onset of the severe phenotype.

Candidate disease genes can then become the focus of further studies to determine their precise role in animal models, and validation of a disease related role in man

Target validation in man will utilise existing clinical and genetic databases, containing DNA and clinical information on relevant patient and control groups.

Phenotypic analysis of the transgenic organisms created can be through simple and rapid measurements including changes in a metabolite, protein (e.g. insulin), lipid or carbohydrate (e.g. when measuring glucose tolerance) present in urine, blood, spinal fluid or tissue. Measurements in body fluids can be made by any one of a number of techniques known to those skilled in the art including measurement by NMR, Elisa, GMS and so forth.

Other phenotypic characteristics can be analysed by measuring behavioural patterns or responses to external stimuli by using tests such as light, sound, memory and stress tests.

Other measurable phenotypic characteristics include growth and ageing parameters, tumour growth, obesity and so forth which can be measured by assessing, for example, weight, fat content and growth rate. Furthermore changes in other measurable features such as blood pressure, heart rate, lung function and so forth can be assessed.

Advantageously, the transposon technology of the present invention allows the production of libraries of transgenic animals without the requirement for extensive storage. Previous methods of generating transgenic animals involve methods such as chemical mutagenesis in which mutations are generated. These methods involve multiple mutations per animal (e.g. mouse). The animals, once generated are analysed phenotypically and then need to be archived/stored for use in the future. The present invention allows the generation of starter cell lines or starter transgenic organisms having different transposons inserted into the genome. These cell lines or organisms can then be used by breeding with transgenic animals carrying a transposase to generate a new library.

In another embodiment, the methods of the invention may be used to generate libraries of ES cells with different transposon insertions distributed throughout the genome. These can be sequenced and characterised. The ES cells can conveniently be stored for future use.

In an alternative embodiment, the methods of the invention may be used to "mark" genes whose expression is modulated by external stimuli. Thus, an embryo, organism, or tissue or cell derived from either, which has been exposed to transposon mobilisation with a marked transposon is subjected to treatment with an external stimulus, such as a candidate drug or other test agent, and modulation of the expression of the marker observed. Cells in which the marker is over or under-expressed are likely to have the transposon inserted in or near a gene which is upregulated or downregulated in response to the stimulus. The invention may thus be used to provide *in vivo* enhancer trap and exon trap functions, by inserting transposons which comprise marker genes which are modulated in their expression levels by the proximity with enhancers or exons.

This approach is useful for the study of gene modulation by drugs in drug discovery approaches, toxicology studies and the like. Moreover, it is applicable to study of gene modulation in response to natural stimuli, such as hormones, cytokines and growth factors, and the identification of novel targets for molecular intervention, including targets for disease therapy in humans, plants or animals, development of insecticides, herbicides, antifungal agents and antibacterial agents.

The invention is further described, for the purpose of illustration, in the following examples.

Examples

A: Activation of Minos *in vivo* using Doxycycline

Example 1: Generation of Transposon Carrying Mice and Transposase carrying Mice

Two transgenic mouse lines are generated. The transposon-carrying line (line MCG) contains a tandem array of a fragment containing a *Minos* transposon containing the GFP gene under the control of the cytomegalovirus promoter. The transposon is engineered such that almost all sequence internal to the inverted repeats is replaced by the CMV/GFP cassette. Not containing the transposase-encoding gene, this transposon is non-autonomous, and can only be mobilized when a source of transposase is present. The second transgenic mouse line contains the *Minos* transposase gene expressed under the control of the inducible promoter

Transposon MiCMVGFP was constructed as follows: The plasmid pMILRTetR (Klinakis *et al.* (2000) *Ins. Mol. Biol.* **9,** 269-275 (2000b) was cut with *BamH* I and re-ligated to remove the tetracycline resistance gene between the *Minos* ends, resulting in plasmid pMILRΔ*Bam*H1. An *Asp*718/*Sac*I fragment from pMILRΔ*Bam* H1, containing the *Minos* inverted repeats and original flanking sequences from *D. hydei*, was cloned into plasmid pPolyIII-I-lox (created by insertion of the *loxP* oligo:

ATAACTTCGTATAGCATACATTATACGAAGTTAT

into the *Asp*718 site of the vector pPolyIII-I (accession No. M18131), resulting in plasmid ppolyMILRΔ*BamH.* The final construct (pMiCMVGFP, Figure 5) used for the generation of transgenic mice, was created by inserting into the *Spe* I site of ppolyMILRΔ*Bam*H1 the 2.2 kb *Spe*I fragment from plasmid pBluescriptGFP, containing a humanised GFP gene (from Clontech plasmid pHGFP-S65T) driven by the CMV promoter and followed by the SV40 intervening sequence and polyadenylation signal.

The transposon-carrying MCG line was constructed by microinjecting the 3.2 kb *Xho*I fragment from the pMiCMVGFP plasmid into FVB X FVB fertilized oocytes. Transgenic animals were identified by Southern blotting of DNA from tail biopsies, using GFP DNA as a probe.

The transposase-expressing line is generated via introduction of the transposase gene into embryonic stem cells via standard homologous recombination ES cell technology. The ES cells are injected into blastocysts to obtain transgenic animals via standard procedures (Manipulating the mouse embryo, Hogan et al., Cold Spring Harbor Press, 1994). Two constructs are used: First, a construct containing the rtTA gene under a constitutive promoter expressed in the target cells. The construct used is the pTet-On plasmid (Clontech) which contains the gene encoding the rtTA activator under control of the Cytomegalovirus immediate early (CMV) promoter. The rtTA transcriptional activator encoded by this construct is active only in the presence of Doxycycline. The second construct contains the Minos transposase gene under control of the tetracycline-response element, or TRE. The TRE consists of seven direct repeats of a 42-bp sequence containing the tet operator (tetO), and is located just upstream of the minimal CMV promoter, which lacks the enhancer elements normally associated with the CMV immediate early promoter. Because these enhancer elements are missing, there is no "leaky" expression of transposase from the TRE in the absence of binding by rtTA. The second construct used is the pTRE2 plasmid (Clontech) in the multiple cloning site (MCS) of which is inserted the gene encoding Minos transposase. In cells stably transformed with the two constructs, rtTA is expressed but does not activate transcription of Minos transposase unless Doxycycline (0.1-1 micrograms/ml) is added in the medium.

Transgenic animals are identified by Southern blotting of DNA from tail biopsies, using a transposase cDNA fragment as a probe.

Example 2: Activation of Minos *in vivo*

A transgenic mouse of the transposon-carrying MCG line is crossed with a transgenic mouse of the transposase carrying line. Mobilisation of transposons in resulting embryos comprising within their genomes both the transposon and the gene encoding the transposase will only occur in the presence of Doxycycline. Doxycycline is administered to the embryos in the water of the maternal organism. Doxycycline is only administered for a limited amount of time (one day - day 2 of gestation) in order to restrict the potential number of transposition events.

On birth, the transgenic offspring developed from the embryos are isolated and various cells and tissues are used for genotyping.

Example 3: Detection of Transposition

A PCR assay for transposon excision is used to detect active transposition by *Minos* transposase in the mouse tissues, using primers that hybridise to the non-mobile *Drosophila hydei* sequences which flank the *Minos* transposon in the constructs

(Klinakis *et al.* (2000) *Ins. Mol. Biol.* 9, 269-275). In *Drosophila* cells, transposase-mediated excision of *Minos* is followed by repair of the chromatid which usually leaves a characteristic 6-base pair footprint (Arca *et al.* (1997) *Genetics* 145, 267-279). With the specific pair of primers used in the PCR assay this creates a diagnostic 167 bp PCR fragment (Catteruccia F. *et al.* (2000) *Proc. Natl. Acad. Sci. U S A* 97, 2157-2162).

Genomic DNA from different tissues is isolated with the DNeasy Tissue-Kit (QIAGEN) according to the manufacturers instructions. PCR reactions are performed using primers 11DML:

(5'AAGTGTAAGTGCTTGAAATGC-3')

and GOUM67:

(5'-GCATCAAATTGAGTTTTGCTC-3').

PCR conditions are as follows: 10 mM Tris-HCI (pH 8.8), 50 mM KCI, 1.5 mM $MgCl_2$, 0.001% gelatin; 1.2 units Taq 2000^{TM} DNA Polymerase (STRATAGENE), 200 g template DNA and 10 pmol of each primer per 25 μl reaction. 43 or 60 cycles of 30" at 94°C, 30" at 59 °C and 30" at 72 °C were performed. PCR products are cloned into the PCRII TA cloning vector (Invitrogen) and are sequenced using the T7 primer.

The diagnostic band is present in certain tissues of the transgenic offspring. The identity of the fragment is confirmed by Southern blot analysis using a labelled DNA probe specific for the amplified sequence (data not shown). Clusters of cells are shown to be homogeneous for the same transposed gene.

B: Activation of Minos *in vivo* using transposase under the control of the ZP3 promoter

Example 4

In this example, it is demonstrated that, by placing expression of the gene encoding the transposase under the control of a gene regulatory signal produced at a particular stage of development, transposition can be induced at that stage of embryo development. In this example, the gene encoding the transposase was placed under the control of the ZP3 promoter, and so the transposase was only expressed in growing oocytes during a 2-to-3-week period of oogenesis. The *Minos* transposase expressed in growing oocytes catalysed the excision of a modified, non autonomous *Minos* transposon and promoted its re-integration into new sites of the genome.

Figure 11 shows alternative constructs (constructs for knock-in and for regular transgenesis) in which the transposase is inserted in the endogenous sperm specific H1t gene for transposition in sperm. Alternative constructs are made for egg specific expression or ubiquitous expression by replacing H1t sequences with equivalent sequences flanking the start of the Zp3 (egg specifically expressed) or hnRNP (ubiquitously expressed) gene.

C: Mammalian codon usage in Minos sequence

Example 5

Improving transposase

One of the ways to improve the efficiency of the fly transposase in mammalian cells or animals is to increase its concentration as a result of a more efficient translation from mRNA by replacing the fly codon usage to mammalian codon usage. To this end we replaced the coding sequence of the fly *Minos* transposase with the sequence:

- SEQ New: 1026 bp;

Composition 321 A; 235 C; 261 G; 209 T; 0 OTHER

Percentage: 31% A; 23% C; 25% G; 20% T; 0%OTHER

Molecular Weight (kDa): ssDNA: 317.79 dsDNA: 632.5

ORIGIN

```
   1 ATGGTGCGCG GTAAGCCTAT CTCTAAGGAG ATCAGAGTAC TGATCAGGGA CTATTTTAAG
  61 TCTGGGAAGA CACTCACTGA GATAAGCAAG CAGTTAAACT TGCCTAAGAG CTCTGTGCAT
 121 GGGGTGATAC AGATTTTCAA GAAAAATGGG AACATTGAGA ATAACATCGC GAATAGAGGC
 181 CGAACATCCG CAATAACCCC CCGCGACAAG AGACAGCTGG CCAAAATTGT GAAGGCTGAC
 241 CGCCGCCAAT CCCTGAGAAA CTTGGCTTCC AAGTGGTCGC AGACCATTGG CAAGACTGTC
 301 AAGCGGGAGT GGACCCGGCA GCAATTAAAG AGTATTGGCT ACGGTTTTTA TAAGGCCAAG
 361 GAAAAACCCC TGCTTACGCT TCGGCAAAAA AAGAAGCGTC TGCAATGGGC TCGGGAAAGG
 421 ATGTCTTGGA CTCAAAGGCA GTGGGATACC ATCATCTTCA GCGATGAGGC TAAATTTGAT
 481 GTGAGTGTCG GCGACACGAG AAAGCGCGTC ATCCGTAAGA GGTCCGAGAC ATACCATAAG
 541 GACTGCCTGA AAAGAACAAC CAAGTTTCCT GCAAGCACTA TGGTATGGGG ATGTATGTCT
 601 GCCAAAGGAC TCGGAAAGCT TCACTTCATC GAAGGGACCG TTAATGCCGA AAAATACATT
 661 AACATTCTCC AGGATAGTTT GCTGCCCTCA ATACCAAAAC TATCCGATTG TGGTGAATTC
 721 ACTTTTCAGC AGGACGGAGC ATCATCGCAC ACCGCCAAGC GGACCAAAAA CTGGCTGCAG
 781 TACAATCAGA TGGAGGTGCT CGATTGGCCC TCAAATAGTC CGGATCTAAG CCCAATCGAA
 841 AATATCTGGT GGCTAATGAA AAACCAGCTG CGAAACGAGC CACAGAGGAA CATTTCCGAC
 901 TTGAAAATCA AGCTGCAAGA GATGTGGGAC TCAATCTCTC AGGAGCACTG CAAAAACCTG
 961 CTCAGCAGCA TGCCTAAACG AGTGAAATGC GTGATGCAGG CCAAGGGCGA CGTTACACAG
1021 TTCTGA
```

This sequence corresponds to the normal mammalian codon usage and results in a protein sequence after translation that is identical to the fly transposase protein sequence. The gene (cDNA) synthesized as oligonucleotides (upper, sense strand, lower antisense strand print) in three part, each part was cloned, and subsequently put together in one cDNA:

- part A plus Kozak preceding the start codon

Linker A Kozak: CCACCATGG

```
         AatII    NcoI
-15 CCCCGACGTCCCACCATGGTGCGCG GTAAGCCTAT CTCTAAGGAG ATCAGAGTAC TGATCAGGGA CTATTTTAAG
                    TACCACGCGC CATTCGGATA GAGATTCCTC TAGTCTCATG ACTAGTCCCT GATAAAATTC

61                  TCTGGGAAGA CACTCACTGA GATAAGCAAG CAGTTAAACT TGCCTAAGAG CTCTGTGCAT
                    AGACCCTTCT GTGAGTGACT CTATTCGTTC GTCAATTTGA ACGGATTCTC GAGACACGTA
```

```
121                GGGGTGATAC AGATTTTCAA GAAAAATGGG AACATTGAGA ATAACATCGC GAATAGAGGC
                   CCCCACTATG TCTAAAAGTT CTTTTTACCC TTGTAACTCT TATTGTAGCG CTTATCTCCG

181                CGAACATCCG CAATAACCCC CCGCGACAAG AGACAGCTGG CCAAAATTGT GAAGGCTGAC
                   GCTTGTAGGC GTTATTGGGG GGCGCTGTTC TCTGTCGACC GGTTTTAACA CTTCCGACTG

241                CGCCGCCAAT CCCTGAGAAA CTTGGCTTCC AAGTGGTCGC AGACAATTGG CAAGACTGTC
                   GCGGCGGTTA GGGACTCTTT GAACCGAAGG TTCACCAGCG TCTGTTAACC TGATCAGGGG
                                                                   MfeI       SpeI
```

Linker B

```
                                                           MfeI
                                                    GGGGCAATTGG CAAGACTGTC
        GCGGCGGTTA GGGACTCTTT GAACCGAAGG TTCACCAGCG TCTGTTAACC GTTCTGACAG

301     AAGCGGGAGT GGACCCGGCA GCAATTAAAG AGTATTGGCT ACGGTTTTTA TAAGGCCAAG
        TTCGCCCTCA CCTGGGCCGT CGTTAATTTC TCATAACCGA TGCCAAAAAT ATTCCGGTTC

361     GAAAAACCCC TGCTTACGCT TCGGCAAAAA AAGAAGCGTC TGCAATGGGC TCGGGAAAGG
        CTTTTTGGGG ACGAATGCGA AGCCGTTTTT TTCTTCGCAG ACGTTACCCG AGCCCTTTCC

421     ATGTCTTGGA CTCAAAGGCA GTGGGATACC ATCATCTTCA GCGATGAGGC TAAATTTGAT
        TACAGAACCT GAGTTTCCGT CACCCTATGG TAGTAGAAGT CGCTACTCCG ATTTAAACTA

481     GTGAGTGTCG GCGACACGAG AAAACGCGTC ATCCGTAAGA GGTCCGAGAC ATACCATAAG
        CACTCACAGC CGCTGTGCTC TTTTGCGCAG TAGGCATTCT CCAGGCTCTG TATGGTATTC

541     GACTGCCTGA AAAGAACAAC CAAGTTTCCT GCAAGCACTA TGGTATGGGG ATGTATGTCT
        CTGACGGACT TTTCTTGTTG GTTCAAAGGA CGTTCGTGAT ACCATACCCC TACATACAGA

601     GCCAAAGGAC TCGGAAAGCT TCACTTCATC GAAGGGACCG TTAATGCCGA AAAATACATT
        CGGTTTCCTG AGCCTTTCGA ACCCCTACGTACCCC
               HindIII       NsiI
```

Linker C

```
                HindIII
601                  CCCCAAGCT TCACTTCATC GAAGGGACCG TTAATGCCGA AAAATACATT
                         TTCGA AGTGAAGTAG CTTCCCTGGC AATTACGGCT TTTTATGTAA

661     AACATTCTCC AGGATAGTTT GCTGCCCTCA ATACCAAAAC TATCCGATTG TGGTGAATTC
        TTGTAAGAGG TCCTATCAAA CGACGGGAGT TATGGTTTTG ATAGGCTAAC ACCACTTAAG

721     ACTTTTCAGC AGGACGGAGC ATCATCGCAC ACCGCCAAGC GGACCAAAAA CTGGCTGCAG
        TGAAAAGTCG TCCTGCCTCG TAGTAGCGTG TGGCGGTTCG CCTGGTTTTT GACCGACGTC

781     TACAATCAGA TGGAGGTGCT CGATTGGCCC TCAAATAGTC CGGATCTAAG CCCAATCGAA
        ATGTTAGTCT ACCTCCACGA GCTAACCGGG AGTTTATCAG GCCTAGATTC GGGTTAGCTT

841     AATATCTGGT GGCTAATGAA AAACCAGCTG CGAAACGAGC CACAGAGGAA CATTTCCGAC
        TTATAGACCA CCGATTACTT TTTGGTCGAC GCTTTGCTCG GTGTCTCCTT GTAAAGGCTG

901     TTGAAAATCA AGCTGCAAGA GATGTGGGAC TCAATCTCTC AGGAGCACTG CAAAAACCTG
        AACTTTTAGT TCGACGTTCT CTACACCCTG AGTTAGAGAG TCCTCGTGAC GTTTTTGGAC

961     CTCAGCAGCA TGCCTAAACG AGTGAAATGC GTGATGCAGG CCAAGGGCGA CGTTACACAG
        GAGTCGTCGT ACGGATTTGC TCACTTTACG CACTACGTCC GGTTCCCGCT GCAATGTGTC

1021    TTCTGAGGAT CC
        AAGACTCCTA GGCCCCGGGG AGATCTCCTA CGTAGGGG
              BamHI            XbaI    NsiI
```

Materials and methods

Plasmid construction

The construction of the modified *Minos* transposon pMiCMVGFP (Figure 5),which was used for the generation of transgenic mice, is described in Example 1. In short, a 2.2 kb fragment, containing a humanised GFP gene driven by a CMV promoter and followed by an intervening sequence and an SV40 poly A signal, was positioned between *Minos* inverted repeats. A lox P site was included in front of the left inverted repeat for the generation of single copy transgenic animals if needed.

The *Minos* transposase cDNA was cloned as a 1 kb *ClaI/ NotI* fragment in the vector Pev3 (Clare Gooding, Biotechnology Dept, Zeneca, Macclesfield, UK; Pev3 is further described in Needham et al, Nucl. Acids Res., 20, 997-1003, 1992) A 3.8 kb *ClaI* / *Asp*718 fragment from the resulting plasmid (containing the *Minos* transposase cDNA followed by an intron and a polyadenylation signal from the human β globin gene) was cloned in pBluescript SK $^{+}$(Stratagene, La Jolla, Ca, USA) creating the plasmid pBlue/ILMi/3$^{'}$β. A 6.5 kb blunt *Asp*718 fragment from plasmid ZP3/ 6.5Luc (Lira, S. *et al* (1990) *Proc. Natl. Acad. Sci. USA* **87**, 7215-7219.) containing the 5$^{'}$flanking region and promoter of the zona pellucida 3 (ZP3) gene was cloned into the *Eco*RV site of pBlue/ILMi/3$^{'}$β, resulting in plasmid ZP3/ILMi (Figure 6), which was used for the generation of transgenic mice expressing the transposase in developing eggs.

Generation of transgenic mice

To generate *Minos* transposase expressing lines, a 10.3 kb SmaI/Asp718 fragment was excised from pZP3/ILMi (Figure 6), separated from plasmid sequences by gel electrophoresis (Sambrook, J et al. Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989)), purified and concentrated using an ELUTIP-d column (Schleicher & Schuell GmbH, Dassel, Germany) and injected into fertilised oocytes (FVBxFVB) at a concentration of 4 ng/μl. Injected eggs were transferred into pseudopregnant mice and transgenic offspring was identified by Southern blot analysis of tail DNA (Southern, E.M. (1975). *J. Mol. Biol* 98,503-517).

The transposon carrying (MCG) line was generated as described above and in (Zagoraiou, L et al (2001) *Proc. Natl. Acad. Sci. USA* **98**,11474-11478).

RT-PCR

For RT-PCR analysis, total RNA was isolated from different organs of ZP3/ILMi transgenic mice using the Ultraspec RNA isolation system (Biotech Laboratories, Houston, TX, USA). From 1 μg of total RNA, cDNA was synthesised in a 20 μl reaction using Reverse Transcriptase (Super RT; HT Biotechnology, Cambridge, UK) and oligo(dT) primer. PCR reactions were performed in a volume of 50 μl PCR buffer (Life Technologies, Paisley, UK) containing 1 μl of the cDNA from the RT reaction, 1.5 mM $MgCl_2$, 100 ng of each primer, 0.2 mM dNTPs and 2.5 U *Taq* DNA polymerase (Pharmacia). A total number of 25 cycles were performed with denaturation at 94° C for 45 seconds, annealing at 55° C for 30 seconds and extension at 72° C for 45 seconds. PCR products were visualised by electrophoresis on a 2% agarose gel. The *Minos* transposase specific primers Minos1: 5'-CAGCTTCGAAATGAGCCAC-3' and beta EX: 5'-TGGACAGCAAGAAAGCGAG-3' were used. Primers specific for murine hypoxanthine phosphoribosyltransferase (HPRT) were: 5'CACAGGACTAGAACACCTGC-3' and 5'-GCTGGTGAAAAGGACCTCT-3'.

Breeding program

Transposon carrying (MCG) females were bred with ZP3/ILMi line 15 males. Double positive females obtained from these crosses were bred with wild type (WT) males and their offspring analysed by Southern blot analysis for possible transposition events. Genomic DNA was digested either with *Eco*RV *or Bgl*II, separated on a 0.7 or 1% agarose gel (Sigma, Steinheim, Germany), blotted onto a nylon membrane (Hybond-N^+, Amersham Pharmacia, Buckinghamshire England) and probed with a ^{32}P labelled 737 bp *SacI/Not*I GFP fragment from pMiCMVGFP.

DNA Fluorescent in Situ Hybridisation (FISH) analysis

Mouse metaphase spreads were prepared according to routine procedures from peripheral white blood cells (Mulder, M.P *et al.* (1995). *Hum Genet* **96(2)**:133-141). The 737-bp *SacI/ NotI* GFP fragment from the pMiCMVGFP construct was used as a probe. The probe was either labelled with biotin (Boehringer Mannheim) and immunochemically detected directly with FITC or a tyramide based step was included to improve signal detection (Raap, A.K. et al (1995) *Human Molecular Genetics* **4**, 529-534). The DNA was counterstained with DAPI.

Cloning of the insertion sites

Mouse DNA from animals with a new *Minos* insertion site was cut with *Eco*RV or *Bgl*II and resolved in a 0.7% agarose gel The gel regions containing transposition events were cut out and the DNA was isolated. Depending on the fragment size, inverse PCR was performed either directly on self-ligated fragments using *Minos* primers IMio1 (5' AAGAGAATAAAATTCTCTTTGAGACG 3') for the first PCR and IMio2 (5' GATAATATAGTGTGTTAAACATTGCGC 3') for the nested PCR (Klinakis, A.G., Zagoraiou, L., Vassilatis, D.K & Savakis, C. (2000) EMBO Reports 11, 416-421.), or the obtained *Eco*RV or *Bgl*II fragments were further digested with *Alu*I and then circularised. Inverse PCR was performed with Minos primers IMio1 and IMii1 (5' CAAAAATATGAGTAATTTATTCAAACGG 3'), followed by nested PCR with primers IMio2 and IMii2 (5' GCTTAAGAGATAAGAAAAAAGTGACC 3') as previously described (Klinakis, A.G., Zagoraiou, L., Vassilatis, D.K & Savakis, C. (2000) *EMBO Reports* 11, 416-421). In this way, left and right flanks were amplified separately. The PCR fragments were either sequenced directly or after cloning into the pGEM T easy vector (Promega), or PCRII vector (Invitrogen). With the sequences obtained, a BLAST search was performed against the mouse genome sequences available at the time in the Celera (www.celera.com) database.

Results

The transposon carrying transgenic mouse line (MCG) was generated. It contains 6 copies of *Minos* transposon MiCMVGFP (Fig.5) integrated in mouse chromosome 14.

The transposon is nonautonomous, i.e. it cannot transpose on its own, since it lacks the transposase gene. In parallel, two transposase expressing mouse lines were generated. These expressed the *Minos* transposase specifically in growing oocytes due to the use of a 6.5 Kb 5'flanking region and promoter of the ZP 3 gene (Fig.6). In both of the *Minos* transposase (ZP3/ILMi) lines, the transgene integrated as a tandem array. In this example we used ZP3/I LMi line 15 with the higher number of copies integrated (data not shown). As expected, transposase expression in this line was restricted to the ovaries (Fig.7). RT-PCR performed on RNA samples from different tissues of transgenic ZP3/ILMi line showed that the ~360 bp fragment, corresponding to the correctly spliced transposase RNA, was restricted to the ovary. The amplification of contaminating DNA to a similar sized fragment was prevented by using a primer which spans an exon/intron junction (beta globin IVSII-Fig. 6).

Since the *Minos* transposase expression from the transgene is driven by the ZP3 promoter, it should be expressed only in growing oocytes during a 2-to-3-week period of oogenesis.

Normally, zona pellucida transcripts cannot be detected in primordial oocytes (10-15 μm), and maximum levels are observed in 50 μm-diameter oocytes. As the oocytes reach maximum size (70-80 μm), the level of ZP3 transcripts begins to decline. Ovulated eggs contain less than 5% of the peak levels of all zona pellucida transcripts (Millar, et al (1991) *Molecular and Cellular Biology* **11**, 6197-6204; Liu, C et al (1996) *Proc. Natl. Acad. Sci. USA.* **93**, 5431-5436). To exclude the possibility that some transposase activity remains in mature oocytes to mediate transposition of a paternally contributed transposon transgene, the Zp3/ ILMi males (that do not express the transposase) were mated to females of the multycopy transposon carrying line MCG. Female progeny positive for both transgenes were selected for further study. We analysed 307 progeny of the double positive females and wild type males by Southern blot analysis. *Eco*RV and/or *Bgl*II digested tail DNA was blotted and hybridised with the GFP probe. Since neither of these two enzymes cuts within the transposon, there is one single band that hybridises to the transposon probe in an MCG line. If transposition occurs and the transposon inserts outside the genomic *Eco*RV or

*Bgl*II fragment where it was initially present, a new band will be detected after hybridisation with the transposon probe. Out of 307 mice, 146 mice were transposon (GFP) positive. Among these 146 mice, 12 transposition events were observed that resulted in a novel restriction fragment on the blot giving a transposition frequency of 8.2%. In two mice, two independent transposition events were found (Fig 8 lanes 2 and 5). The observation that some offspring carry *Minos* mediated insertions but did not inherit the transposase transgene strongly suggests that transposition occurred in the germ cells of the mother prior to meiosis II.

To prove that the observed transposition events had indeed occurred in the germ line, animals with transposition events were further crossed to wild type mice. Analysis of their progeny showed that these mice stably transmitted the reinserted *Minos* element (Fig 8). The segregation of a transposon concatemer and a new insertion into two different lines in the F1 generation was clear evidence that transposition had occurred to another chromosome (Fig. 8, lane 2 versus 3 and 4, and lane 8 versus 9). In all transposition events except one, a single copy of the transposon was mobilised.

It has been noted previously that the size of a transposon influences its transposition frequency; longer elements tend to transpose with a lower frequency (Lampe, D. J. et al (1998). *Genetics* **149**, 179-187; Fischer, S. E., et al (1999) *Mol. Gen. Genet.* **262**, 268-274.), suggesting that when transposase binding sites (inverted repeats) are closer to each other they are recognised more efficiently.

Minos transpositions are characterised by a precise integration of the element without mobilisation of flanking DNA. In *Drosophila* and in HeLa cells, the transposon inserts into TA dinucleotide causing target site duplication upon insertion (17, 29). To investigate the structure of the insertions in the mouse genome, we cloned the flanking regions (as described in Materials and Methods) from five different transposition events. As is observed in *Drosophila*, the *Minos* ends were flanked by the diagnostic TA dinucleotide followed by sequences unrelated to the sequence that flanks the element in the founder mouse line MCG (Fig. 9). BLAST searches with the obtained sequences in the Celera mouse genome database showed that all five of the novel flanking sequences (in one case only one flanking sequence was obtained) correspond to widely scattered genomic locations (Fig. 9). Out of five transposition events analysed only one was on chromosome 14. It is a single copy of the transposon integrated into a centromeric region without the presence of a transposon concatamer on tip of chromosome 14. Thus this transposition event occurred into a different chromosome (See Fig. 10). The DNA Fluorescent in Situ Hybridisation (FISH analysis) performed on metaphase spreads from peripheral white blood cells confirmed the results obtained from sequencing of the flanking regions (data not shown). However, transposition events that were clearly detectable by Southern blot analysis but were located close to the original site on the same chromosome would not be identified by FISH. It is actually very difficult to detect single copy (3kb) transpositions by FISH and this may partially explain the low frequency of transposition (0.61%) we obtained previously with the same transposon containing transgenic line (MCG) and *Minos* transposase expressed specifically in T cells (Zagoraiou, L., et al (2001) *Proc. Natl. Acad. Sci. USA* **98**,11474-11478) where FISH was the only method of detection used. Since "local insertions", close to the original site of the element, are quite frequent at least with the *Drosophila* P element (Zhang, P.& Sprading, A.C. (1993) *Genetics* **133**, 361-373) and *Sleeping Beauty* (Luo, G et al(1998) *Proc. Natl. Acad. Sci. USA*. 95, 10769-10773; Fisher, S.E.J., et al (2001) *Proc.Natl. Acad. Sci. USA*. 98, 6759-6764.), it is unlikely that this would not be the case with the *Minos* element.

In order to determine the size of the EcoRV/BglII flanking regions we generated a single copy transposon line from the MCG line using the loxP/Cre system (data not shown). On the basis of Southern blot data, there is approximately 10kb of genomic sequence flanking the transposon, within the *Eco*RV and *Bgl*II diagnostic digests. Local reinsertions that occur within this area (but which are not interesting for mutagenesis purposes), will escape detection and are not included in our estimation of the frequency of transposition. Thus, the frequency of 8.2% represents the "useful" transposition frequency rather than the actual transposition frequency. The reported frequency of transpositions in the mouse male germ line with *Sleeping Beauty* was approximately 20%, but only 2% had transposed to a different chromosome (Fisher, S.E.J., Wienholds, E. & Plasterk, R.H.A. (2001) *Proc.Natl. Acad. Sci. USA*. 98, 6759-6764). In contrast we find for *Minos* approx. 6% transposition to a different chromosome (8 out of 11 analysed and 1 unknown, out of 146 offspring), while only 3 out of 11 transpositions were to the same chromosome (e.g. Fig 10B). This suggests that the *Minos* system has a preference for transpositions to a different chromosome (provided there weren't many transpositions close to the original site that went undetected). It should be noted however that a direct comparison of the different transposon systems published to date may not be very valid. The size, the copy number and the initial chromosomal position of the transposon might all affect the transposition efficiency and none of these were comparable in the different systems.

In conclusion, these results show that transposition can be achieved in the mouse germ line and that, by selecting the time of induction, mobilisation of transposons may be induced at a predetermined stage of embryo development. Furthermore, the results demonstrate that systems using, for example, *Minos*, are excellent tools for insertional gene inactivation, gene tagging, enhancer trapping and exon trapping in organisms, for example, mice.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are apparent to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.

Claims

1. A method of generating a transgenic non-human mammalian progeny, comprising the steps of:

a) generating a first adult transgenic non-human mammal by inserting into its genome one or more copies of a transposon;

b) generating a second adult transgenic non-human mammal by inserting into its genome one or more copies of a gene encoding a transposase cognate for said transposon and an element capable of regulating expression of said gene encoding the transposase;

wherein the first and second adult transgenic non-human mammals have a background known to be prone to a disease state;

c) crossing the first adult transgenic non-human mammal with the second transgenic adult non-human mammal to provide a progeny which comprises, in the genome of one or more of its cells, both (i) one or more copies of the transposon and (ii) a gene encoding transposase cognate for said transposon, wherein the gene encoding the transposase is under the control of one or more regulatory sequences which permit expression of the transposase; and

d) inducing expression of said gene encoding the transposase in the germ cells of said progeny to cause mobilisation of said transposon during germline development in the progeny

2. A method as claimed in claim 1 wherein the progeny is a female transgenic non-human mammal and mobilisation of said transposon is during oogenesis.

3. A method as claimed in claim 2 wherein the one or more regulatory sequences which permit expression of the transposase during oogenesis are derived from ZP3 regulatory sequences.

4. A method as claimed in claim 1 wherein the progeny is a male transgenic non human mammal and mobilisation of said transposon is during spermatogenesis.

5. A method as claimed in claim 4 wherein the one or more regulatory sequences which permit expression of the transposase are sequences which allow specific expression of the transposase during spermatogenesis such as the Hlt gene regulatory sequences.

6. A method as claimed in any of claims 1 to 5 further comprising mating the progeny to produce offspring in which the transposition events can be characterised.

7. The method according to any of claims 1 to 6 wherein said first transgenic non-human mammal comprises within its genome one or more copies of both the transposon and the gene encoding the cognate transposase and the second transgenic non-human mammal comprises one or more copies of a regulatory sequence necessary to permit transposase expression in any defined tissue or tissues during spermatogenesis or oogenesis.

8. The method according to claim 7 wherein said transposon and said gene encoding the cognate transposase are provided as a single construct within the genome of said first mouse, wherein, on mobilisation of the transposon, the gene encoding the transposase is disrupted.

9. The method according to any preceding claim, wherein the transposon and/or the gene encoding the transposase lie within a chromatin opening element such that the transposon and/or the gene encoding the transposase and/or a gene regulating expression of the gene encoding the transposase lie in open chromatin structure.

10. The method according to claim 9 wherein the chromatin opening element is a ubiquitously-acting chromatin opening element (UCOE), a locus control region (LCR), a CpG island or an insulator.

11. The method according to any one of the preceding claims wherein the regulatory sequence is a developmental control sequence, activatable at a predetermined stage of development.

12. The method according to any one of claims 1 to 11 wherein the regulatory sequence is an inducible regulatory sequence selected from the group including a tetracycline inducible expression system, an oestrogen inducible expression system, an ecdysone inducible expression system, and a *lac* operator repressor system.

13. The method according to any one of the preceding claims wherein expression of the gene encoding the transposase is eliminated by i) transposase gene excision as a result of transposition or ii) excision of the transposase gene after the mobilisation of said transposon.

14. The method according to any preceding claim, wherein the transposon and/or the gene encoding the transposase is inserted into or adjacent to an expressed gene.

15. The method according to any one of the preceding claims wherein the transposon is a type 2 transposon.

16. The method according to claim 15 wherein the transposon is Minos, mariner, Hermes, piggyBac or Sleeping Beauty.

17. The method according to claim 16 wherein the transposon is Minos.

18. The method according to any preceding claim, wherein the transposon has been modified to include a heterologous nucleic acid sequence, flanked by inverted terminal repeats homologous to the transposon.

19. The method according to any one of claim 18, wherein the transposon comprises a nucleic acid sequence encoding a selectable marker.

20. The method according to claim 19, wherein the selectable marker is a fluorescent or luminescent polypeptide.

21. The method according to any one of the preceding claims wherein the nucleotide sequence of the gene encoding the transposase has been modified to optimise codon usage.

22. The method according to any one of the preceding claims wherein the transposase gene is flanked by sequences recognisable by enzymes capable of excising said transposase gene.

23. The method according to claim 22 wherein the excision enzymes and cognate recognition sequences is cre/lox.

24. A method according to any one of the preceding claims further comprising generating, by mating the progeny, a transgenic non-human mammal, wherein all cells of the non-human mammal are homogeneous for a gene modified by transposon mobilisation.

25. A transgenic non-human mammal obtainable by the method of claim 24, wherein the non-human mammal has a background known to be prone to a disease state and all cells of the non-human mammal are homogeneous for a gene modified by transposon mobilisation.

26. A method for isolating a gene which is correlated with a phenotypic characteristic in a transgenic non-human mammal comprising the steps of

a) taking a progeny obtained by a method as defined in any of the claims 1 to 23,

b) identifying in the transgenic progeny developed therefrom the presence of a plurality of cells displaying said phenotype characteristic;

c) detecting the position of one or more transposon transposition events in the genome of one or more of said cells; and

d) cloning the genetic loci comprising the insertions.

27. A method according to any proceeding claims, wherein the transgenic non-human mammal is a mouse.

Figure 1: A self-inactivating autonomous transposon vector for inducible transposition

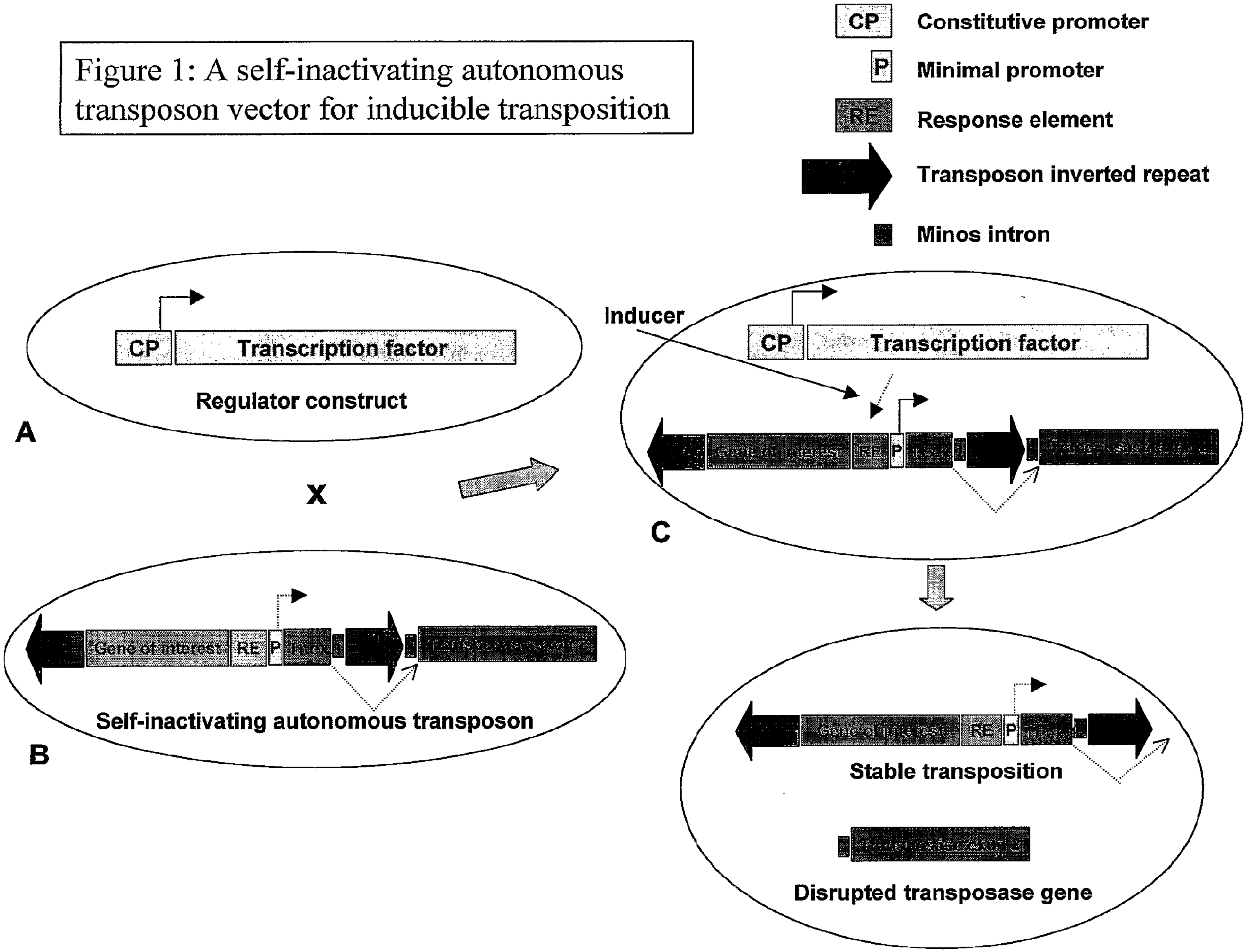


Figure 2. Inducible enhancer trap system

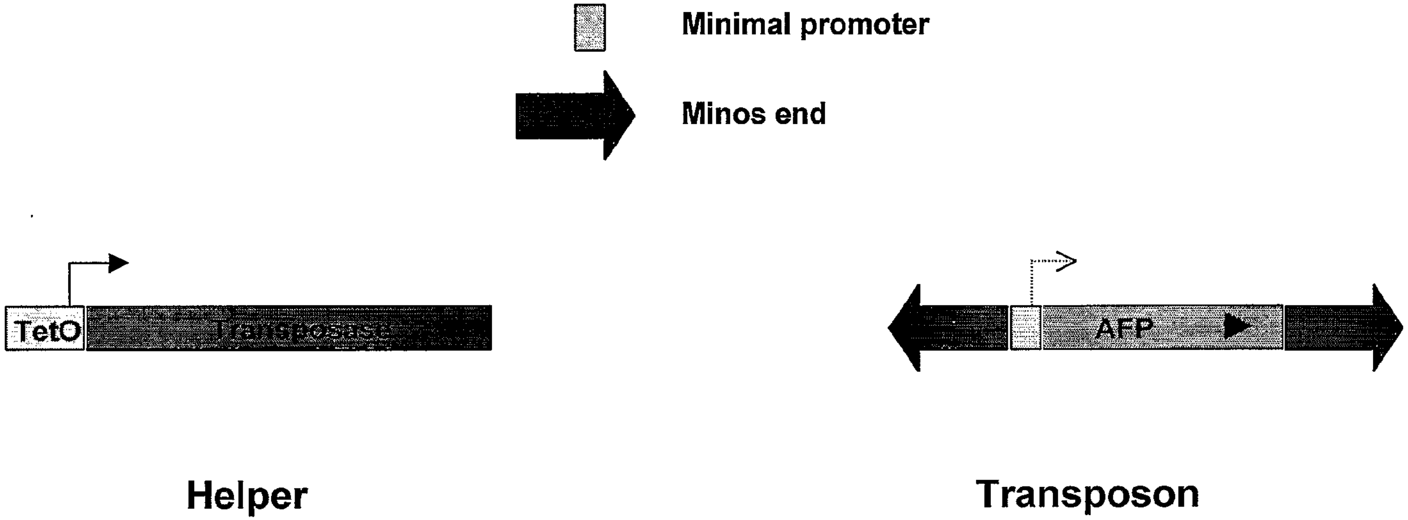


Figure 3. Inducible exon trap system

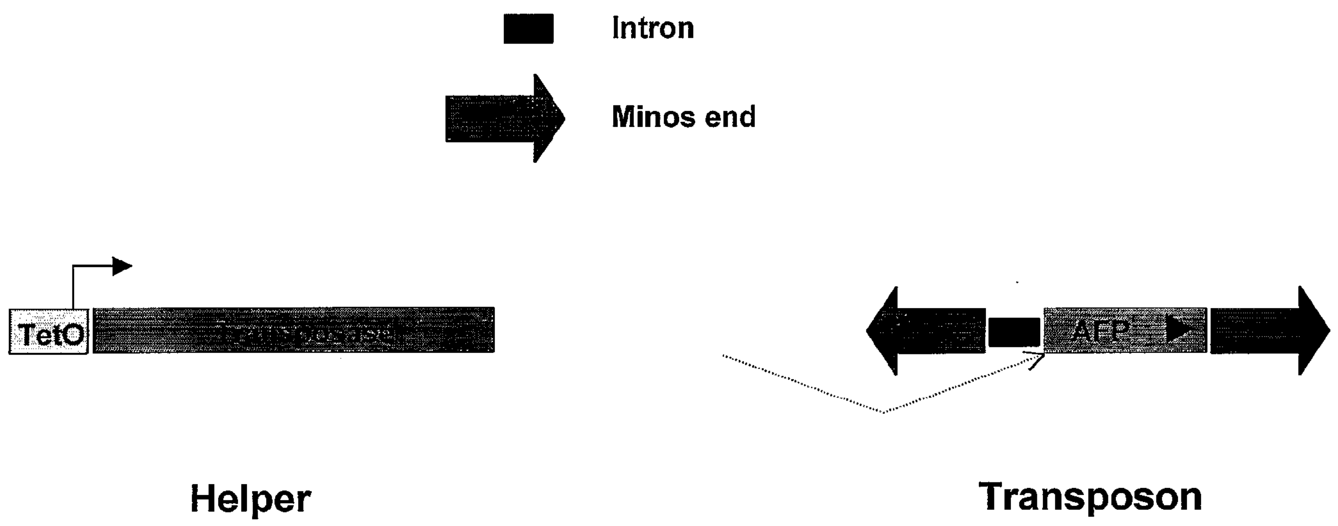


Figure 4. Inducible gene activation system

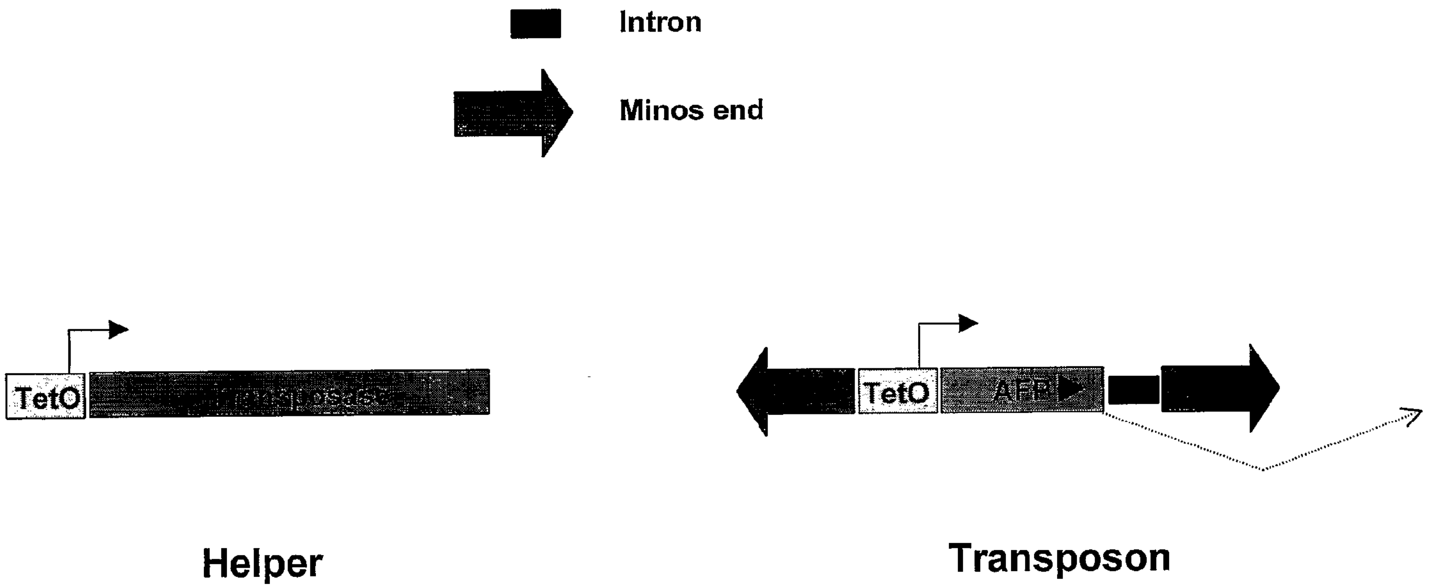
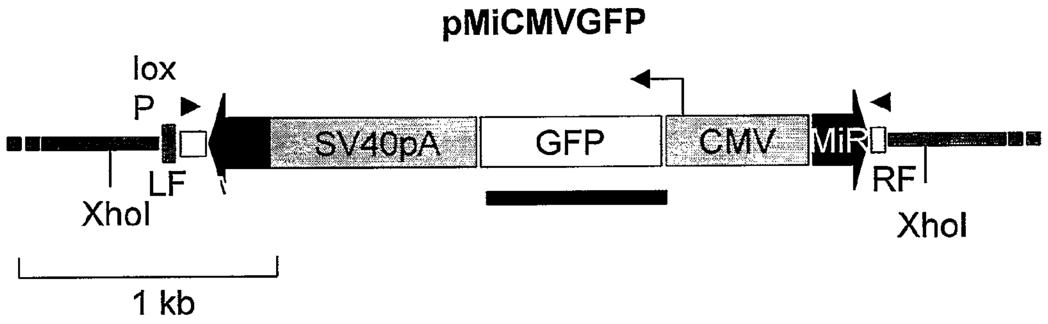
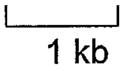


Figure 5





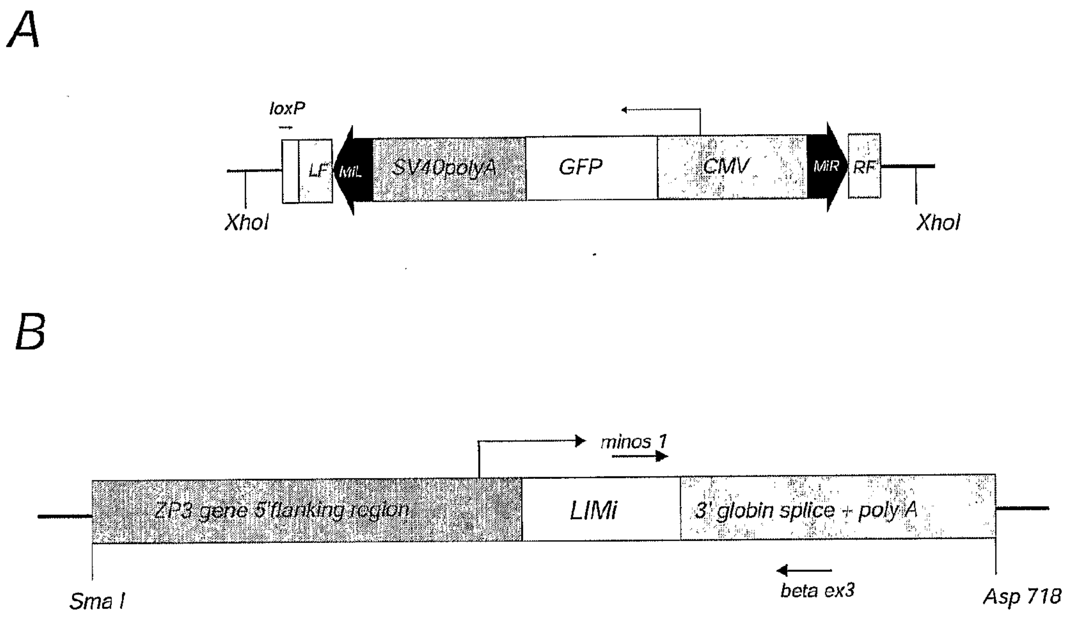

A
loxP
LF
MiL
SV40polyA
GFP
CMV
MiR
RF
XhoI
XhoI
B
minos 1
ZP3 gene 5'flanking region
LIMi
3' globin splice + poly A
beta ex3
Sma I
Asp 718

FIGURE 6

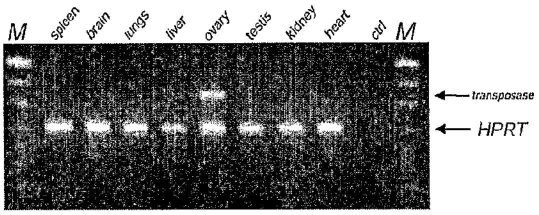

M
spleen
brain
lungs
liver
ovary
testis
kidney
heart
ctrl
M
transposase
HPRT

FIGURE 7

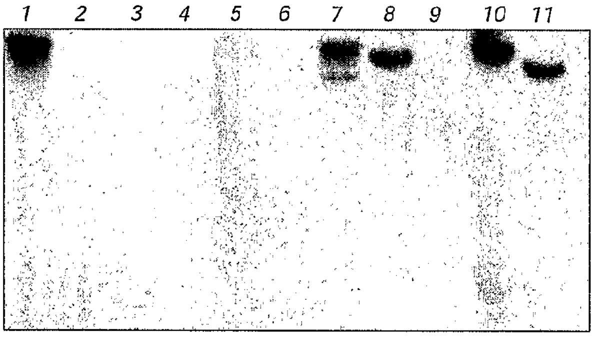
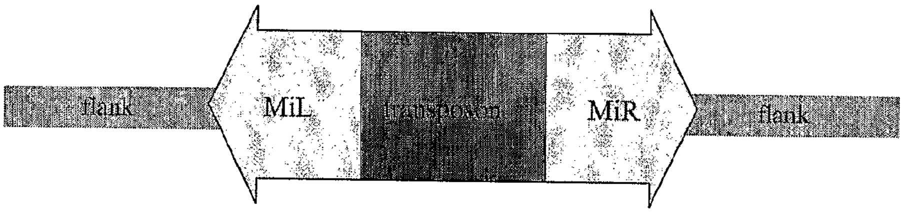


FIGURE 8



	GCACGTTAGCAGCTTCTacga		Original integration	tcgtAGAAAACGAAAAATATC
TE1	ATAGCTATAGTGGTAA	Ta cga	Chr 10 (GA_5J8B7W3239)	tcg tAGAGGTATGATTCCTAT
TE2	ATTTTTCTTACCTACA	Ta cga	Chr 7 (GA_5J8B7W8 9HK)	tcg tACATGTGCATATATGGC
TE3	ACACACAAAATAACAT	Ta cga	Chr 2 (GA_X5JB7W5LKQ)	tcg tATTCTATCTTCTTAATAT
TE4	TTAGACAGCTCAATGT	Ta cga	Chr 14 (GA_5J8B7W)	tcg tATCATTCATGATGCATT

FIGURE 9

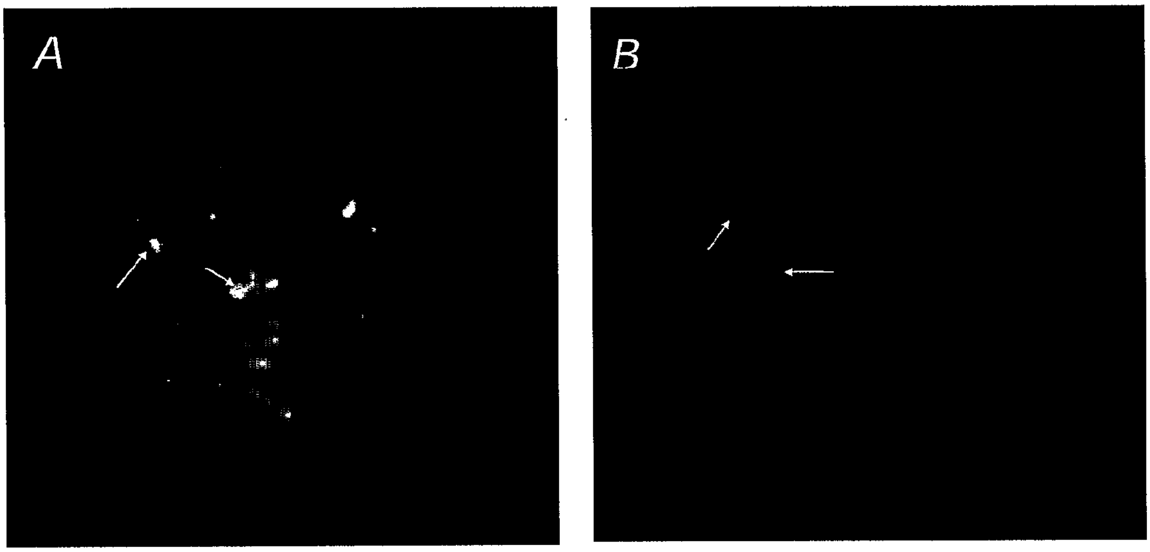
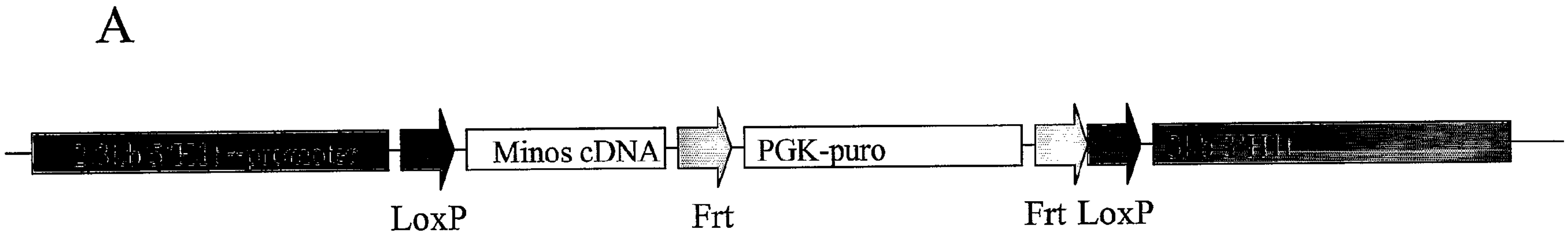


FIGURE 10

Figure 11



- •Kozak sequence in constructs at the ATG
- •*3' homology incudes TGA stem loop and Pu rich sequence

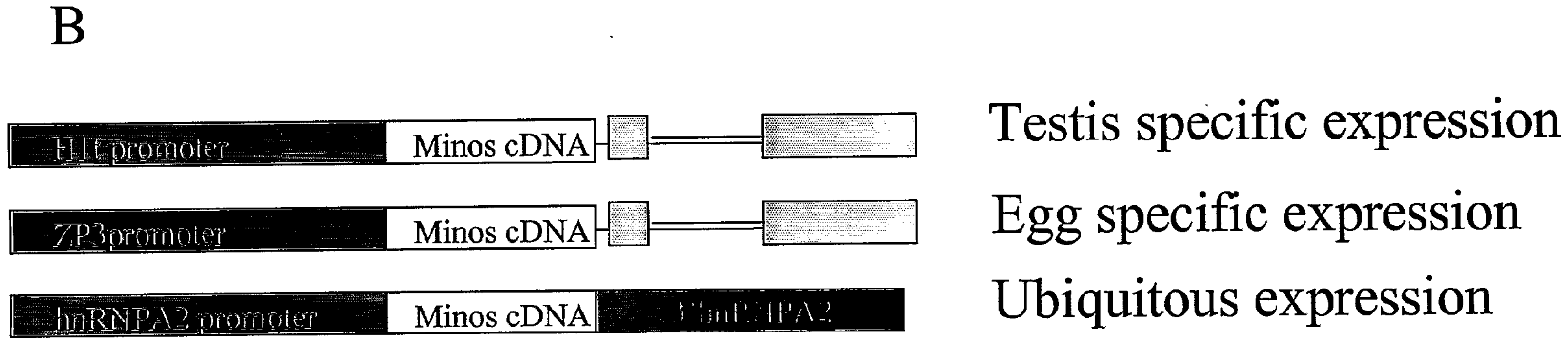
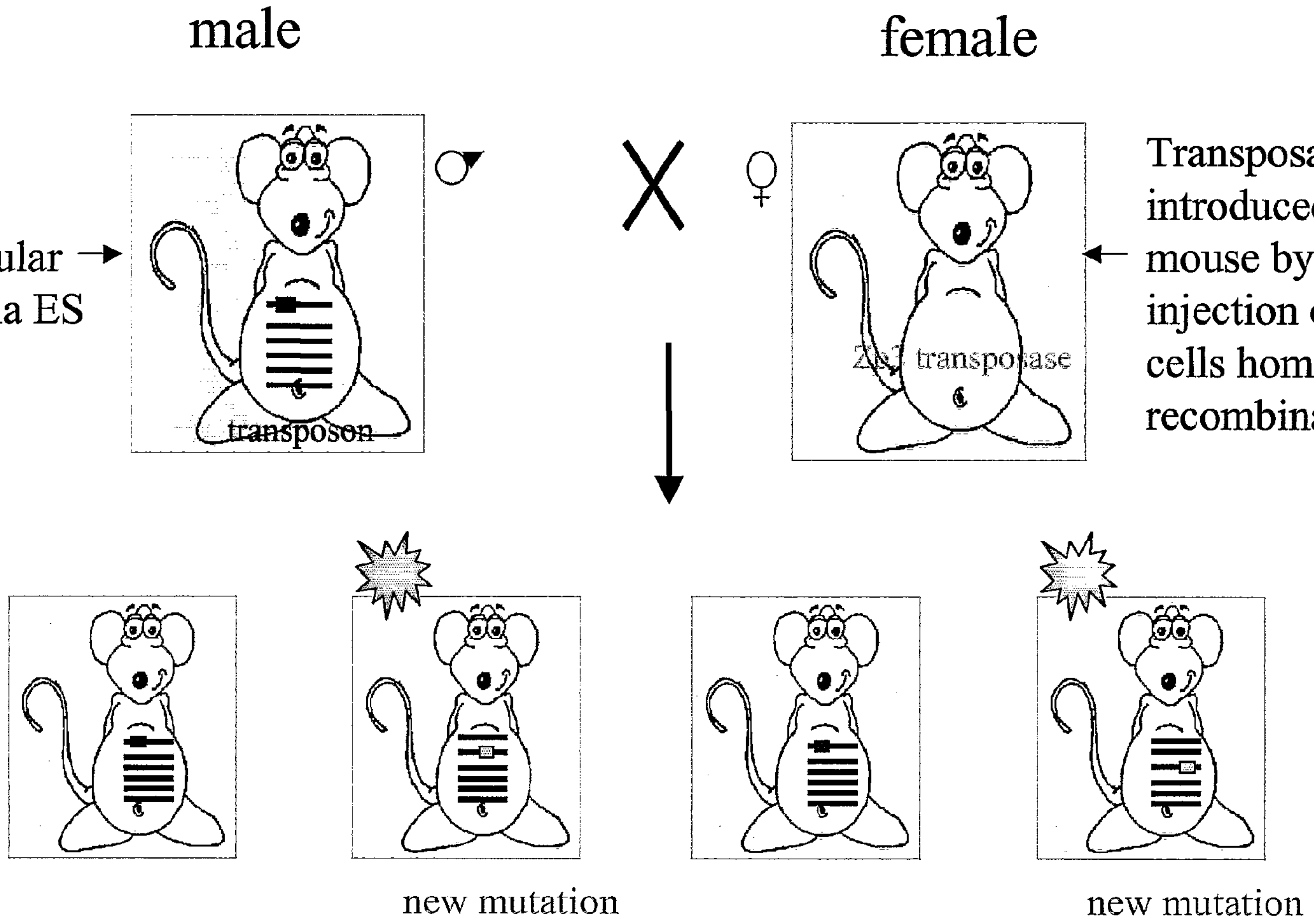


Figure 12

Trap construct for transgenic mice and /or cloning into retroviral plasmid

Dflank: small pieces of Drosophila DNA
RIR, LIR: Left and right inverted repeat sequence
TRE: Tet inducible CMV promoter
En2 SA: intron-splice acceptor
IRES/EGFP: internal ribosome binding site, flueorescent marker gene

In Vivo Transposition Technology with the male contributing the transposon, the female the transposase. Transposition takes place in the egg or early embryo.

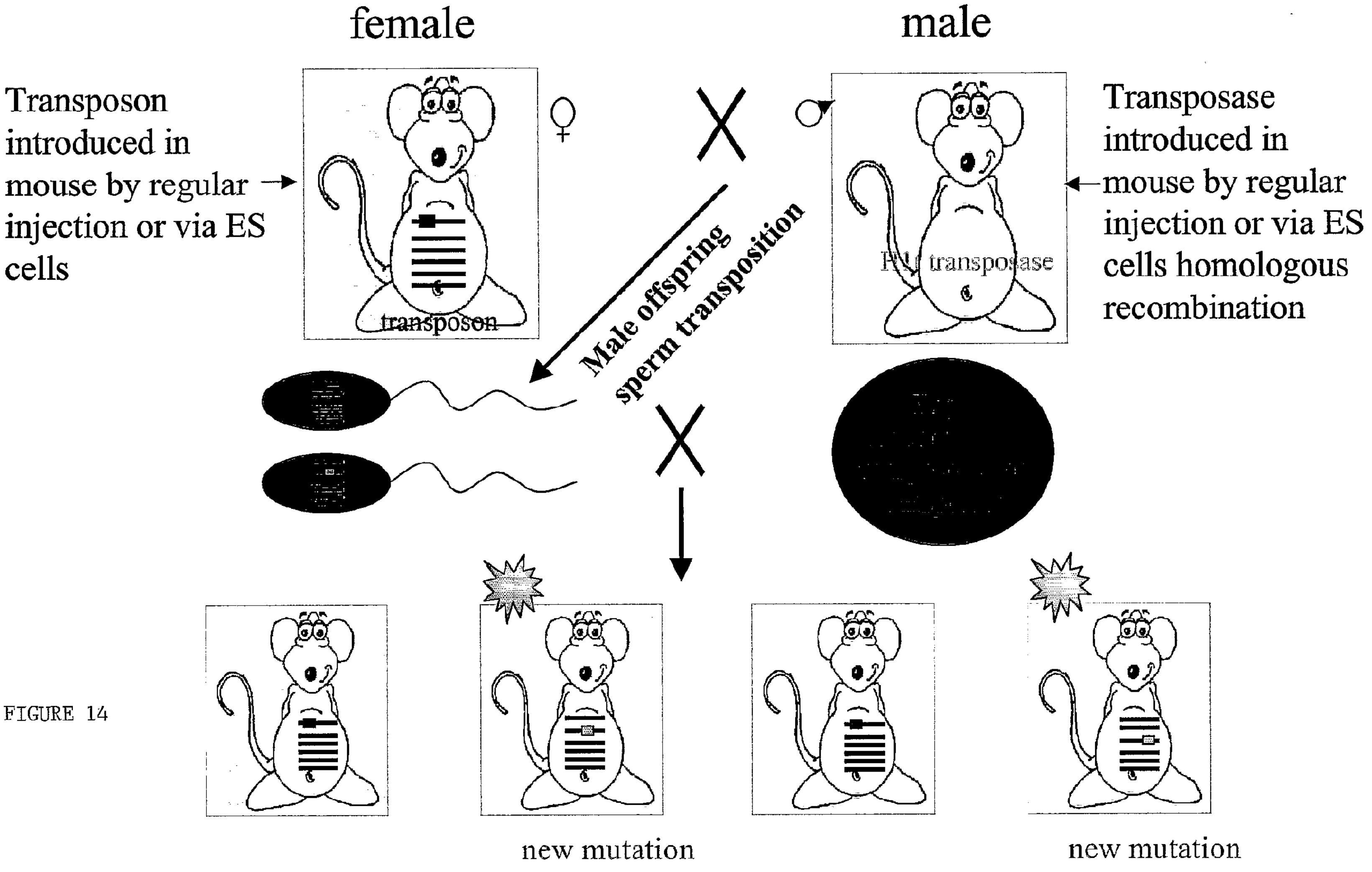


Transposon introduced in mouse by regular injection or via ES cells

Transposase introduced in mouse by regular injection or via ES cells homologous recombination

FIGURE 13

In Vivo Transposition Technology with the female contributing the transposon, the male the transposase. Transposition takes place in the sperm



female

male

Transposon introduced in mouse by regular injection or via ES cells

Transposase introduced in mouse by regular injection or via ES cells homologous recombination

Male offspring sperm transposition

new mutation

new mutation

FIGURE 14

In Vivo Transposition using ubiquitous expression. Males and females are interchangeable with respect to the transposon or transposase

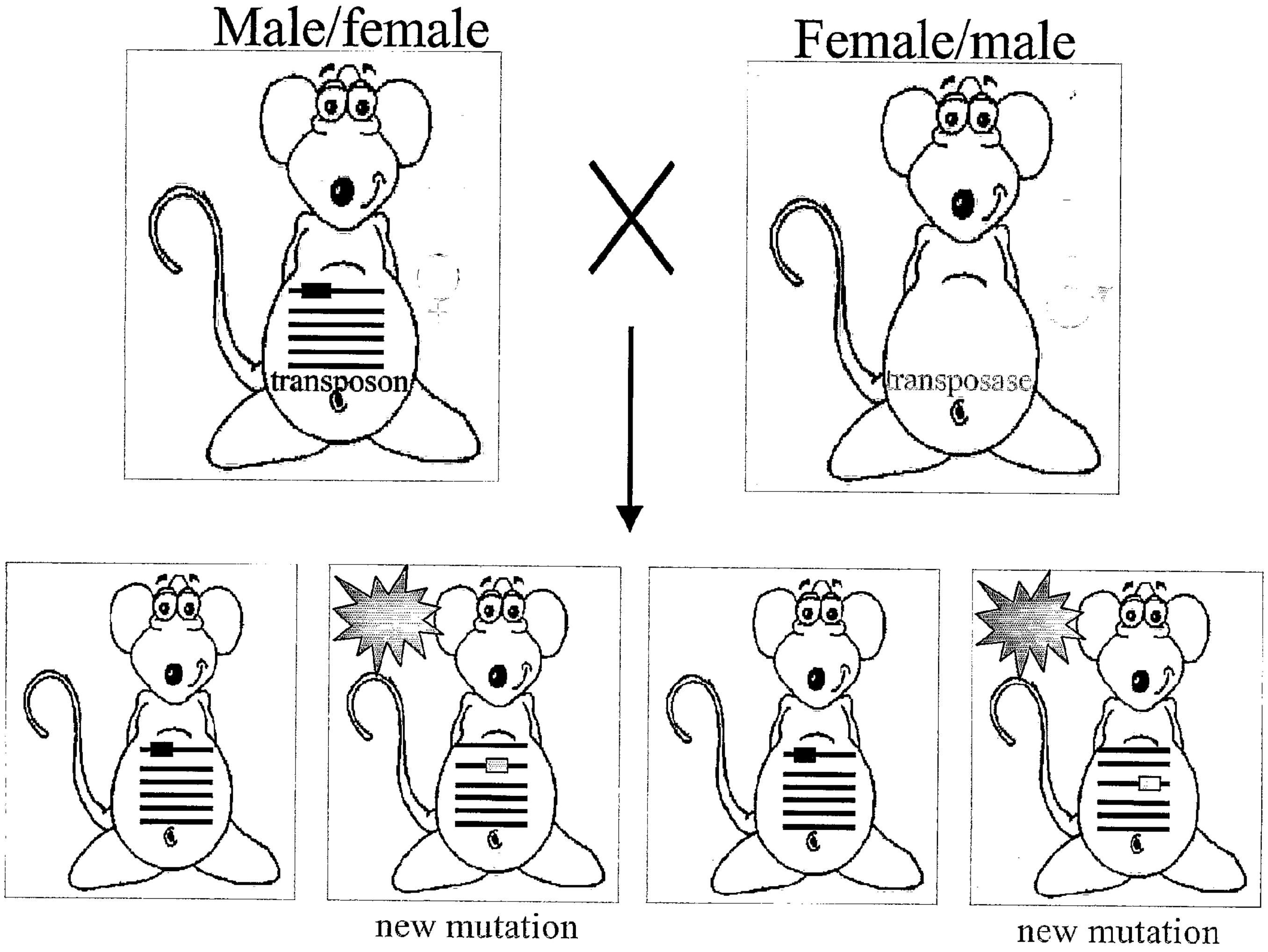


FIGURE 15