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(54) **Title:** TRANSPOSOME TETHERED TO A GENE DELIVERY VEHICLE

(57) **Abstract:** The present invention provides methods and compositions for improved gene delivery using transposases. A complex is provided that contains a transposome comprising at least one transposase bound to a nucleic acid sequence of interest by way of transposase recognition sequences. The transposome is bound, by way of a releasable or cleavable linker, to a targeting element, which assists in delivery of the transposome to a pre-selected DNA-containing organelle (nucleus or mitochondria). Once delivered to the organelle, the transposome is typically released from the targeting element, and inserts the nucleic acid of interest into the host cell genome. The complex provides for many uses, including creation of transgenic plants and animals, as well as therapeutic and prophylactic treatment of animals and humans.



TRANSPOSOME TETHERED TO A GENE DELIVERY VEHICLE

CROSS REFERENCE TO RELATED APPLICATION

This application claims priority of U.S. Provisional Application No. 61/779,623 filed on March 13, 2013, the content of which is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to the field of molecular biology. More specifically, the invention relates to use of transposomes bound to a gene delivery vehicle to improve gene delivery into plant and animal cells, such as for treatment of diseases, production of useful proteins, and generation of genetically modified plants and animals for other reasons.

Description of Related Art

Unlike DNAase, a single molecule of which can generate numerous breaks in a target DNA, the transposase complex is believed to create only one DNA cleavage per complex. Therefore, unlike with DNase I, the degree of DNA fragmentation is easily controlled during transposase fragmentation by controlling the ratio of transposase complex to target DNA in the reaction mixture. Despite obvious advantages in cost, time and labor, the transposase method is less frequently used as compared to sonication because it does not result in entirely random fragmentation (bias) of target DNA.

The most commonly used transposase for DNA fragmentation is a modified (mutated) Tn5 transposase. From the onset of its use, Tn5 transposase has been problematic in several respects. First of all, the native transposase was practically impossible to produce, as it is toxic for *E. coli* when expressed from a strong promoter. However, this difficulty was overcome by deleting several N-terminal amino acids (Weinreich et al., J. Bacteriol., 176: 5494-5504, 1994). Though this solved the toxicity problem, and the N-terminally truncated transposase was produced at high yield, it possessed very low activity. Therefore, several other mutations were introduced to increase its activity (see, for example, U.S. Patent 5,965,443; U.S. Patent 6,406,896; and U.S. Patent 7,608,434). However, this did not solve all of the problems with the enzyme.

For example, the mutated enzyme is stable only in high salt, such as 0.7M NaCl, (Steiniger et al., Nucl. Acids Res., 34: 2820-2832, 2006); it quickly loses its activity at the lower salt conditions that are required for the transposase reaction, with a half-life only 2.4 minutes in the reaction mixture. Thus, DNA fragmentation reactions using this
5 transposase are typically performed in five minutes and very large amounts of enzyme are used. Despite the fact that high salt concentration is maintained throughout the purification process, the purified enzyme is largely inactive; thus, 9.4 times excess of enzyme over oligonucleotides is typically used to form Tn5 transposase-oligonucleotide complexes (Naumann and Reznikoff, J. Biol. Chem., 277:17623-17629, 2002). In
10 addition, the transposase is prone to proteolytic degradation. To address this problem, the degradation-prone sites were mutated. Interestingly, these mutations resulted in drastic reduction of the *in vivo* activity of the enzyme, but had little effect on the *in vitro* activity (Twining et al., J. Biol. Chem., 276: 23135-23143, 2001). Overall, Tn5 transposase is difficult to produce, it is required in large amounts, and it is very expensive.

15 It is generally believed that native unmutated transposases are inherently inactive because high activity would be incompatible with the host cell survival (Reznikoff, W.S., Mol. Microbiol., 2003, 47, 1199-206). Because native transposases are believed to possess low activity, they are believed to be unsuitable for many DNA fragmentation reactions. Further, in view of the fact that it took many years of mutagenesis and
20 biological selection to render purified Tn5 transposase active, for years the task of providing another transposase that has suitable activity seemed problematic.

However, recently the present inventor devised new reaction media for DNA fragmentation with transposases, which is useful for both native and mutant transposases. For example, U.S. patent application number 13/470,087, filed 11 May 2012 (incorporated
25 herein by reference), discloses among other things that transposases, such as the *Vibrio harvei* transposase ("Vibhar"), can have relatively high DNA fragmenting activity if manganese (Mn⁺⁺) ions are included in the fragmentation buffer.

Further, the present inventor recently devised a way to purify transposases in an active form, active enough for use in many DNA fragmentation reactions. The solution
30 obviated the need for conventional transposase purification by first forming a complex of a transposase, such as the Vibhar transposase, with oligonucleotides in crude cell lysates, which is a more physiological environment than employed in prior schemes, and then

purifying the complex. (See, for example, U.S. provisional patent application number 61/708,332, filed 1 October 2012, and non-provisional applications based on that document, which is incorporated herein by reference in its entirety.)

Transposases are well established as tools for gene delivery. However, despite
5 their numerous advantages, which will be discussed below, they are rarely used as compared to viral gene delivery systems. The reason for that is low efficiency of the transposase system, *e.g.*, many more cell clones have to be screened to obtain clones with a desired genotype.

In stem cell research, somatic cells have been reprogrammed into pluripotent stem
10 cells by introducing a combination of several transcription factors, such as Oct3/4, Sox2, Klf4, and c-Myc. Induced pluripotent stem (iPS) cells from a patient's somatic cells could be a useful source for drug discovery and cell transplantation therapies. However, most human iPS cells are made using viral vectors, a practice that raises serious safety concerns. Several methods have been reported to overcome the safety concerns associated with the
15 generation of iPS cells, including use of transposons, transient expression of the reprogramming factors using plasmids, and direct delivery of reprogramming proteins. However, studies of gene expression, epigenetic modification, and differentiation revealed that there was insufficient reprogramming of iPS cells using non-viral methods, thus suggesting the need for improvement of the reprogramming procedure not only in quantity
20 but also in quality (Keisuke and Yamanaka, *Philos Trans R Soc Lond B Biol Sci.* 201 1 August 12; 366(1575): 21 98-2207).

Improved gene delivery technologies are also needed for the treatment of disease in animals. Many diseases and conditions can be treated with gene-delivery technologies, which provide a gene of interest to an animal suffering from the disease or the condition.
25 An example of such a disease is Type 1 diabetes. Type 1 diabetes is an autoimmune disease that ultimately results in destruction of the insulin producing beta-cells in the pancreas. Although animals with Type 1 diabetes may be treated adequately with insulin injections or insulin pumps, these therapies are only partially effective. In addition, hyper- and hypoglycemia occurs frequently despite intensive home blood glucose monitoring.
30 Finally, careful dietary constraints are needed to maintain an adequate ratio of calories consumed. Development of gene therapies providing delivery of the insulin gene into the

pancreas of diabetic animals could overcome many of these problems and result in improved life expectancy and quality of life.

Several of the prior art gene delivery technologies employed viruses that are associated with potentially undesirable side effects and safety concerns. The majority of
5 current gene delivery technologies useful for gene therapy rely on virus-based delivery vectors, such as adeno and adeno-associated viruses, retroviruses, and other viruses, which have been attenuated to no longer replicate. (Kay, M. A., et al., 2001, Nature Medicine 7:33-40).

There are multiple problems associated with the use of viral vectors. First, they are
10 not tissue-specific. In fact, a gene therapy trial using adenovirus was halted because the vector was present in a patient's sperm ("Gene trial to proceed despite fears that therapy could change child's genetic makeup. The New York Times, Dec. 23, 2001). Second, viral vectors are likely to be transiently incorporated, which necessitates re-treating a patient at specified time intervals. (Kay, M. A., et al. 2001. Nature Medicine 7:33-40). Third, there
15 is a concern that a viral-based vector could revert to its virulent form and cause disease. Fourth, viral-based vectors require a dividing cell for stable integration. Fifth, viral-based vectors indiscriminately integrate into various cells, which can result in undesirable germline integration. Sixth, the required high titers needed to achieve the desired effect have resulted in the death of one patient, and they are believed to be responsible for
20 induction of cancer in a separate study. (Science, News of the Week, Oct. 4, 2002).

RNAi has been targeted as a tool for several uses, including treatment of genetic abnormalities, disease, and cancer, and in study of development. There are mainly two types of short RNAs that target complementary messengers in animals: small interfering
25 RNAs and micro-RNAs. Both are produced by the cleavage of double-stranded RNA precursors by Dicer, a member of the RNase III family of double-stranded specific endonucleases, and both guide the RNA-induced silencing complex to cleave specifically RNAs sharing sequence identity with them.

RNAi technology can be used in therapeutic approaches to treat disease and various conditions. However, a major drawback to RNAi therapy has been the lack of a
30 reliable delivery method of the short RNA sequences. Most researchers working in the field rely on producing short double stranded RNA (dsRNA) in the laboratory and then delivering these short dsRNAs by direct injection, electroporation, by complexing with a

transfecting reagent, or other methods. The result is gene silencing, but only as long as the dsRNA remains present in the cell, which generally begins to decrease after about 20 hours. In order to obtain lasting therapeutic effects, the RNAi sequence must be expressed long term, preferably under a constitutive promoter. In order to accomplish RNAi
5 expression in a plasmid-based vector and subsequent recognition by RNA-induced silencing complex (RISC), the RNA must be double stranded. To obtain dsRNA from a vector, it must be expressed as a short hairpin RNA (shRNA), in which there is a sense strand, a hairpin loop region and an antisense strand (M. Izquierdo. 2004. Short interfering RNAs as a tool for cancer gene therapy. Cancer Gene Therapy pp 1- 1; Miyagishi et al. 2004. J Gene Med 6:715-723). The hairpin region allows the antisense strand to loop back
10 and bind to the complimentary sense strand.

Unlike RNAi, transposases can facilitate stable insertion of genetic material into the genomes and are useful for generation of knockout mutants as well as for expression of additional genes in the cells of interest. However, for some applications they can be
15 engineered to provide transient integration. Furthermore, unlike with virus-based vectors, no introduction of potentially dangerous viral genetic material into the cells is necessary, *i.e.*, genetic material can be strictly limited to a gene of interest that is operably linked with gene regulatory elements, *e.g.*, an appropriate promoter and poly-adenylation signal. Despite these advantages, transposases are rarely used because of their low efficiency.

20 Preformed transposase complexes of superactive Tn5 transposase and genes of interest (*i.e.*, preformed transposomes) have been extensively used for gene delivery into bacterial cells using the method of electroporation. Electroporation is a rather harsh method that is suitable for bacterial cells, but is typically damaging to animal cells. Therefore, the transposase complex was used very rarely for gene delivery into eukaryotic
25 cells, *e.g.*, mammalian (Suganuma et al., Biol Reprod. 2005 Dec;73(6):1157-63), insect (Rowan et al., Insect Biochem Mol Biol. 2004 Jul;34(7):695-705.) and plant (Wu et al., Plant J. 2011 Oct;68(1):186-200; Wu et al., Plant Mol Biol. 2011 Sep;77(1-2):117-27). These publications demonstrated applicability of prokaryotic transposase (superactive Tn5 transposase) for gene delivery into eukaryotic cells. However, its application on animal
30 cells was limited to microinjection, which is laborious and is usually performed on large cells (spermatozoa or oocytes). With the plant cells another harsh method was used, *i.e.*, particle bombardment, which is also damaging to animal cells.

A typical transposase gene delivery system does not comprise a transposome, but instead comprises two plasmids, one donor plasmid encoding a gene of interest that is operably linked to a promoter, and another encoding a transposase. (See, for example, Meir and Wu, MChang Gung Med J. 2011 Nov-Dec;34(6):565-79; Yusa et al., Proc. Natl. Acad. Sci. USA, Vol. 108, No.4, 1531-1536, 2011; Germon et al., genetic, Vol. 173, No. 3: 265-276, 2009; De Silva et al., Human Gene Therapy, 21: 1603-1613, 2010). The general configuration is shown in Fig. 1A. Most commonly used transposases for gene delivery are provided on a plasmid and are derivatives of either piggyback transposase, mariner transposase, or sleeping beauty transposase (*ibid*). In either case, the gene of interest and the promoter are flanked by transposase recognition sequences that are inverted with respect to each other. Another plasmid, *i.e.*, a helper plasmid, encodes a transposase that is operably linked to a promoter. Upon co-transfection into the cytoplasm of eukaryotic host cells (I), some amount of the helper plasmid should reach the nucleus (II) where the transposase gene can be transcribed into mRNA (III). Next, mRNA should be transported into the cytoplasm (IV) where it is translated by ribosomes into transposase protein (V), and then transposase binds to its recognition sequences in the donor plasmid DNA (VI) (which hopefully remained for all this time in the cytoplasm and was not transported to other cell compartments or damaged by nucleases), excises extra plasmid DNA sequences (VII), and forms a complex with a gene of interest flanked by the recognition sequences, *i.e.*, transposome (VIII). Next, the complex has to be transported into the nucleus (IX) where it can act on genomic DNA and become integrated into it (X).

Evidently, traditional technology is a multi-stage process which is problematic at many steps. For instance, transporting donor plasmid across the cell membrane and then across the nucleus membrane is problematic, because while a typical transfection reagent (*e.g.*, liposomes) can efficiently deliver DNA across the cell membrane, because the liposome lipid bilayer shell fuses with the cell membrane and empties the liposome contents into the cytoplasm, it is difficult for a charged molecule such as DNA to pass another membrane barrier, *e.g.*, the nuclear or mitochondrial membrane.

An even larger problem is associated with forming the donor plasmid-transposase complexes. Transposase complexes are efficiently formed in the absence of divalent cations such as Mg^{++} or Mn^{++} . However, these ions serve as co-factors for hundreds of various enzymes, and the cytoplasm of animal and plant cells always contain these ions

(Valberg et al (1965), Journal of Clinical Investigation 44 (3): 379-389; Seiler et al (1966), American Journal of Cardiology 17 (6): 786-791. doi: 10.1016/0002-9149(66)90372-9; Walser, M. (1967), Ergebnisse der Physiologie Biologischen Chemie und Experimentellen Pharmakologie 59: 185-296; Iyengar et al., (1978), The Elemental Composition of Human
5 Tissues and Body Fluids. Weinheim, New York: Verlag Chemie; Stelzer, R.; Lehmann et al (1990) Botanica Acta 103: 415-423; Shaul et al. (1999), EMBO Journal 18 (14): 3973-3980).

Another problem is that usual methods rely on completely double-stranded transposomes for gene delivery. However, as was discovered at Agilent Technologies,
10 Inc., and as will be discussed below, transposome activity can be severely inhibited by a double-stranded DNA region adjacent to the recognition sequences, and optimal activity has been observed only when the dsDNA region was minimized to the size of the transposase recognition sequence, *e.g.*, 19 bp for the Vibhar transposase.

Furthermore, once the complex is formed in the cytoplasm, and because Mg⁺⁺
15 ions are plentiful there, there is nothing to prevent it from inserting the gene of interest into the donor or helper plasmid DNA. Therefore, a majority of the complex, (even if some was formed) is likely to be wasted for insertion into these plasmids before it has any chance of reaching the nucleus environ and the target DNA, or any other environ of interest, *e.g.*, mitochondria or chloroplasts.

Accordingly, what is needed is a new method to produce transgenic animals and
20 humans with stably incorporated genes, in which the vector containing those genes does not cause disease or other unwanted side effects. There is also a need for DNA constructs that would be stably incorporated into the tissues and cells of animals and humans, including cells in the resting state that are not replicating. There is a further recognized
25 need in the art for DNA constructs capable of delivering genes to specific tissues and cells of animals and humans and for producing proteins in those animals and humans. By recognizing problems that plague extant transposase gene delivery technologies, the author has invented methods and compositions for improved gene delivery using transposases.

SUMMARY OF THE INVENTION

30 The present invention provides technical solutions that expand the application field for transposases from the current relatively small field of sample preparation for Next Generation Sequencing (NGS) to a much larger field of gene delivery into live cells. It is

widely recognized that improvements in gene delivery technologies are essential for stem cell research, gene therapy, generation of transgenic plants and animals, animal models for drug discovery, genetic screens for functional gene annotation in model species, genetically modified insects for controlling their populations in the environment, and other
5 uses. The present invention provides such an improvement by overcoming the general low efficiency of transposase systems for gene delivery into animal and plant cells.

At its core, the invention provides complexes comprising a transposome (*i.e.*, at least one transposase bound to a nucleic acid having two binding sites for the transposase(s)) reversibly or releasably linked to a targeting element that targets the
10 transposome to a pre-selected DNA-containing organelle of a cell of interest (also called "host cell" or "target cell" at times herein). In use, the complex is delivered to cells of interest, taken up by the cells, and transported within the cells to the pre-selected DNA-containing organelle. Once inside the pre-selected DNA-containing organelle, the transposome and targeting elements are typically separated, and the transposome inserts
15 the nucleic acid into the DNA of the host cell.

The invention further provides methods of making a complex. In general, the method includes: attaching a targeting element to a linker; forming a transposome from a nucleic acid and at least one transposase; and attaching the transposome to the linker, thus making a complex comprising a transposome linked to a targeting element. The order in
20 which the steps are performed is not critical. Thus, for example, in embodiments, the nucleic acid of the transposome element can be attached to the linker prior to creation of the transposome. However, because the inventor has recently discovered that purification of active transposase is improved if the transposase is bound to its cognate binding sequences on a DNA molecule, it is preferred that creation of the transposome occurs prior
25 to attaching the transposome to the linker.

The invention additionally provides a method of delivering a nucleic acid to a pre-selected DNA-containing organelle. The method of this aspect of the invention generally comprises contacting a cell of interest with a complex according to the invention under conditions that allow for uptake of the complex by the cell of interest and movement of the
30 complex to the pre-selected DNA-containing organelle. For *in vitro* embodiments of the invention, the step of contacting typically comprises adding the complex to the medium in which the cells are growing, and the conditions are standard conditions known in the art as

useful for delivery and uptake of exogenously supplied elements. For example, animal host cells can be incubated at 37°C in 5% CO₂ in an appropriate salt medium containing serum, and the complex added to that medium. For *in vivo* embodiments of the invention, the step of contacting typically comprises either directly delivering the complex to a cell of interest or indirectly delivering it by delivering it to a tissue or organ of the animal or plant of which the cell is a part and, if possible, allowing the animal or plant to transport the complex to the cell of interest. Furthermore, the conditions for *in vivo* practice of the method are those of the animal or plant into which the complex is delivered. Once the act of contacting the cells with the complex has been accomplished, the cells are maintained under conditions that permit intracellular movement of the complex to the pre-selected DNA-containing organelle. Typically, those conditions are the same as for contacting of the cells with the complex.

In this respect, the invention provides a method of delivering a nucleic acid to a particular site in an organism, such as tumor, particular organ, or certain cell type, and having it transported to a DNA-containing organelle. By way of example, a transposome that encodes VEGF (vascular endothelial growth factor) can be attached to nanoparticles with magnetic properties, injected into the bloodstream, concentrated in the heart area using a magnet, and delivered into heart muscle cells to facilitate their regeneration, similarly to as described for adenoviral vectors (July Zhang et al., Targeted Delivery of Human VEGF Gene via Complexes of Magnetic Nanoparticle-Adenoviral Vectors Enhanced Cardiac Regeneration, Plos ONE, 2012, Volume 7, Issue 7. Organ or tumor delivery of magnetic nanoparticles can be controlled by magnetic resonance imaging (see U.S. Patent No. 8,323,618; U.S. patent application publication number 20120010499), incorporated herein by reference. Furthermore, delivery into certain cell types, for instance into tumor cells, can be by functionalizing the surface of nanoparticles with targeting agents (U.S. patent application publication number 20110123629), *e.g.*, polyethyleneimine polymer, for delivery into brain cells (U.S. patent application number 20110054236), peptides (U.S. patent application publication number 20040126900), *e.g.*, RGD peptide (U.S. patent application publication number 20090087493) or antibodies (U.S. patent application publication numbers 20120156686, 20120263739, 20110143417, 20100086585, and 20090087493), incorporated herein by reference.

The method of delivering a nucleic acid to a pre-selected DNA-containing organelle can be extended to provide a method of inserting a nucleic acid into the DNA of a pre-selected DNA-containing organelle. That is, the method can be practiced such that once the complex is delivered to a pre-selected DNA-containing organelle, the cells are maintained for an additional amount of time to allow for the transposome to insert the nucleic acid into the DNA of the host cell.

In view of the fact that the invention provides methods of delivering a nucleic acid to a pre-selected DNA-containing organelle and insertion of the nucleic acid into the DNA of that organelle, it should be evident that the invention provides a method of treating a disease or disorder having a genetic basis. The method generally includes contacting a cell, *in vivo* or *ex vivo*, with a complex of the invention and maintaining the cell under conditions where the complex can be taken up by the cell and transported to a pre-selected DNA-containing organelle. Once in the cell organelle, the transposome inserts the nucleic acid of the complex into the cell's DNA. In the method of treating, the nucleic acid contains a coding region for a therapeutic polyamino acid, a coding region for a therapeutic RNA, or, by way of insertion into a gene, disrupts the gene to provide a therapeutic effect.

In addition, the invention provides kits. The kits can comprise some or all of the components and reagents needed to practice a method of the invention. In some embodiments, a kit according to the invention comprises all of the components and reagents used to make a complex of the invention, with the exception of the nucleic acid to be used in the transposome.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate embodiments of the invention, and together with the written description, serve to explain certain principles, aspects, and features of the invention.

Figs. 1A and B show a comparison of the prior art (A) and the present methods (B) for delivery of a nucleic acid to the nucleus of a target cell using a transposome.

Figs. 2A and B show examples of recognition sequences (A) suitable for creation of transposomes in embodiments of the invention and their comparable activity (B) on

human DNA in sample preparation for next generation sequencing (NGS). The four pairs of recognition sequences shown in Fig. 2A are those for native Vibhar (SEQ ID NOs: 1 and 2), typically used for NGS (SEQ ID NOs: 1 and 3), for Phil A1/Vibhar (SEQ ID NOs: 4 and 5), and for Phil C1/Vibhar (SEQ ID NOs: 6 and 7).

5 Fig. 3A schematically depicts a complex according to an embodiment of the invention in which a gene of interest is linked to a nanoparticle by way of a PNA/DNA tether that has a photo switch for separation of the gene and nanoparticle once inside a pre-selected DNA-containing organelle.

10 Fig. 3B schematically depicts the process of delivery of the complex of Fig. 3A to a cell nucleus and photoseparation of the gene of interest from the nanoparticle.

 Fig. 3C schematically depicts an embodiment of the invention that uses a tether that contains a photocleavable linker.

 Fig. 3D schematically depicts the process of nucleic acid insertion into a host cell DNA by the transposome of an embodiment of a complex of the invention.

15 Fig. 4A schematically depicts formation of a complex according to an embodiment of the invention in which a gene of interest in single-stranded form is linked to a silica particle by way of a biotinylated oligonucleotide bound to the single-stranded gene sequence and a streptavidin-Si tag fusion protein.

20 Fig. 4B schematically depicts the process of delivery of the complex of Fig. 4A to a cell nucleus.

 Fig. 4C schematically depicts insertion of the gene of interest into the host DNA, and removal of the biotinylated oligonucleotide/streptavidin element as a result of filling-in of the complementary strand of the gene of interest.

25 Fig. 5A schematically depicts an embodiment of a complex according to the invention in which a transposome is linked to a peptide containing a Nuclear Localization Signal (NLS) by way of a biotinylated oligonucleotide that binds to a sequence on a single-stranded gene of interest.

30 Fig. 5B schematically depicts the process of movement of the complex of Fig. 5A from the cellular surface through the cytoplasm, and into the nucleus of a target cell.

 Fig. 6A schematically depicts a complex according to another embodiment of the invention, in which a single-stranded gene of interest is linked to a dexamethasone nuclear

targeting element by way of an oligonucleotide that binds to a sequence on the gene of interest.

Fig. 6B schematically depicts the process of movement of the complex of Fig. 6B through the cytoplasm and into the nucleus of a target cell as a result of binding of the dexamethasone to a glucocorticoid receptor.

Fig. 7 shows that the type of nucleic acid inserted between two recognition sequences can vary and can include fully dsDNA, fully ssDNA, partially dsDNA and partially ssDNA, as well as other structures.

Fig. 8 schematically depicts a complex according to an embodiment of the invention, in which a single-stranded gene of interest is linked, via oligonucleotide tethers, to a cell penetrating peptide (CPP) and a nuclear targeting element (NLS).

Fig. 9 schematically depicts a complex according to yet another embodiment of the invention, in which a single-stranded gene of interest is bound, via oligonucleotide tethers, to multiple short amphipathic peptides (MPG and Pep-1; SEQ ID NOs: 18 and 19), where the positively charged ends of the peptides bind to the negatively charged nucleic acid and the hydrophobic ends of the peptides form a hydrophobic shell around the nucleic acid.

Fig. 10 schematically depicts formation of a complex according to an embodiment of the invention, in which a transposome is bound to a nanoparticle for delivery to the cytoplasm of a cell, and is further bound to an NLS to effect highly efficient nuclear localization.

Fig. 11 schematically depicts formation of a complex according to another embodiment of the invention, in which a single-stranded gene of interest is linked to multiple nuclear targeting elements and multiple cell penetrating peptides by way of single-stranded DNA binding protein tethers.

DETAILED DESCRIPTION OF VARIOUS EMBODIMENTS OF THE INVENTION

Reference will now be made in detail to various exemplary embodiments of the invention, examples of which are illustrated in the accompanying drawings. The following detailed description is not intended to be limiting of the invention, but instead is provided to give the reader a better understanding of certain features and details of embodiments of the invention.

Before embodiments of the present invention are described in detail, it is to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting. Further, where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit, unless the context clearly dictates otherwise, between the upper and lower limits of that range is also specifically disclosed. Each smaller range between any stated value or intervening value in a stated range and any other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included or excluded in the range, and each range where either, neither, or both limits are included in the smaller ranges is also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the term belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. The present disclosure is controlling to the extent it conflicts with any incorporated publication.

As used herein and in the appended claims, the singular forms "a", "an", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a cell" includes a plurality of such cells and reference to "the complex" includes reference to one or more complexes. Furthermore, the use of terms that can be described using equivalent terms include the use of those equivalent terms. Thus, for example, the use of the term "subject" is to be understood to include the terms "animal", "human", "patient", and other terms used in the art to indicate one who is subject to a medical or therapeutic treatment.

The most widely used prior art method for gene delivery using transposases is schematically represented in Fig. 1A. To assist the reader in recognizing the differences between that method and the present method, Fig. 1B schematically depicts the method of

the present invention. As can be seen, the prior art method includes cut-and-paste transposition of a gene of interest into genomic DNA comprising two plasmids. As a genetic manipulation tool, transposome elements are divided into two parts, one part on a donor plasmid and the other part on a helper plasmid. The donor plasmid contains genes of interest embraced by terminal inverted repeats (TIRs). The helper plasmid expresses transposase, which binds to the TIRs and excises the transposon from the donor plasmid. The excised transposon is then brought to the target site by the transposase, followed by integration. A more detailed description of this process is provided in the Background section of this document.

There are severe problems that reduce the efficiency of the prior art method. For example, co-transfection of a cell with two plasmids (step I) is a somewhat inefficient step in which some cells will take up one, but not both, plasmids, and other cells will take up neither. Further, once inside the cells, a much greater inefficiency is encountered. First, only the helper plasmid must make its way to the nucleus so that the transposase can be transcribed, then the mRNA transported from the nucleus to the cytoplasm for translation. After translation, the transposase must find a donor plasmid that is still in the cytoplasm intact, bind to its recognition sequences in the donor plasmid, and cleave the plasmid DNA to remove the sequence located between its two recognition sequences to form a transposome. Once the transposome has formed, it must be transported to the nucleus so that it can integrate the nucleic acid of interest into the host cell genome.

In contrast to the prior art method, the present method creates a transposome (*i.e.*, at least one transposase bound to a sequence of interest flanked by transposase recognition sequences) *in vitro*, and links that transposome to a targeting element that targets the complex to a pre-selected DNA-containing organelle (step I of Fig. 1B). As has been shown for formation of transposomes *in vitro* for NGS, the transposome of the present invention is efficiently formed *in vitro*. Therefore, the present invention reduces the complexity and inefficiencies of *in vivo* steps II-VIII of the prior art method into a straightforward, simple *in vitro* process of combining four elements (*i.e.*, nucleic acid having a sequence of interest, transposase, linker/tether, and targeting element), all of which have been pre-designed to interact with another element of the complex in known, proven ways to form a fully defined complex that is capable of being taken up by a target cell and delivered to a pre-selected DNA-containing organelle. The method of the present

invention resolves inefficiency problems of the prior art and reduces the number of steps that are required for insertion of a desired sequence into a target cell DNA from ten to four (*i.e.*, formation of complex *in vitro*, delivery to target cells, transport into a DNA-containing organelle, and integration of sequence of interest into host DNA).

5 One advantage provided by the present invention is that a transposome is attached to a vehicle (*i.e.*, targeting element) for efficient delivery through cell barriers. There are numerous suitable delivery vehicles known in the art, such as, but not limited to, nanoparticles, micells, lysosomes, dendrimers, or even individual molecules, such as protein molecules, peptides, or ligands, which can deliver transposase complexes into the
10 cells, into specific types of cells and into the nucleus or into another organelle, such as the mitochondria. In brief, this invention takes advantage of many vehicles that are known to deliver substances (*e.g.*, DNA, RNA, proteins, nucleoprotein complexes, and drugs), and discloses methods of attaching transposomes to those vehicles via a tether that on one end is attached to the vehicle and on the other end is attached to the transposome. Attachment
15 to the transposome is typically reversible and is achieved either via hybridization of oligomer sequences, *e.g.*, oligonucleotide or peptide nucleic acid (PNA), complementary to a sequence of the nucleic acid of interest of the transposome or through electrostatic interactions with the nucleic acid of interest. Attachment of the tether to the vehicle can be reversible or irreversible, as will become clear from Examples below. In brief, in
20 exemplary embodiments, transposome-vehicle complexes are delivered through cell barriers (*e.g.*, lipid bilayer membranes) to the target genomes, and the transposomes of the complexes are allowed to insert genes of interest into the genomes in the cell environment. Genes of interest are linked in an operable manner to transposases to achieve insertion of these genes into host cell genomes or into episomal genomes and modify the cells via
25 expression of genes, and/or by destroying the host cell genes into which they are inserted.

A complex according to the invention comprises a nucleic acid having a sequence of interest flanked by two recognition sequences for transposase(s) and at least one transposase that binds to the recognition sequences. When bound together, this unit is referred to as a transposome, in accordance with the art-recognized term. As used herein,
30 the term "nucleic acid having a sequence of interest" means a nucleic acid molecule that has a known sequence over at least a portion of the molecule, where that sequence has a desired functional characteristic. The functional characteristic can be any characteristic of

a nucleic acid, including, but not necessarily limited to, encoding a polyamino acid (*i.e.*, peptide, polypeptide, protein) having a desired function, encoding an RNA having a desired function (*e.g.*, siRNA, microRNA), and encoding a DNA having a desired function (*e.g.*, introducing a mutation in a host gene to alter the function of the encoded gene product or to abolish production of a functional gene product). For brevity, the term "nucleic acid having a sequence of interest" is at times herein referred to as a "gene of interest". However, it is to be understood that when "gene of interest" is used, the phrase is not limited to the use of a full gene, but instead the term encompasses all sequences of interest.

The nucleic acid having a sequence of interest also includes, at each end, a recognition sequence for a transposase. Numerous recognition sequences are known in the art for various transposases, and the practitioner is free to select any combination of recognition sequence/transposase combination. Exemplary recognition sequences for the Vibhar transposase are presented in Fig. 2. As shown in the figure, nucleotide changes can be designed (Panel A) without considerable drop in activity (Panel B). Those skilled in the art will immediately recognize that by mutagenizing the recognition sequence the sequence can be optimized contingent upon the cell type, organelle type, and gene delivery vehicle, and tailor it to the needs of specific disease treatment.

Although embodiments encompass the use of a single transposase per complex, preferably the nucleic acid having a sequence of interest comprises two recognition sequences for transposases and, in the complex, each recognition sequence is attached to a transposase. In some embodiments, the two recognition sequences are the same, while in other embodiments, the two differ. Likewise, in some embodiments, a transposome of the complex comprises two of the same transposase, while in other embodiments, the transposome comprises two different transposases.

In addition to the transposome, the complex of the invention comprises a linker or tether (used interchangeably) that links the transposome to a targeting element that targets the transposome to a pre-selected DNA-containing organelle of a cell of interest. The linker provides another advantage to practice of the invention as compared to prior art methods. That is, there are numerous, commercially-available and well characterized molecules and chemical/biochemical systems for linking nucleic acids and proteins to any number of other substances, including substances known to have the ability to target

substances to cells and to membrane-bound organelles, such as the nucleus, (referred to at times herein and in the art as "delivery vehicles" or simply "vehicles"). The practitioner is free to select any linker that is suitable for the desired goal of practice of the invention. Several linkers of the same or different type or of different types can be used to connect a

5 transposome to one or more gene delivery vehicles. Further, because linkers known and used in the art are well characterized, the skilled artisan will be able to select and implement an appropriate linker without any undue experimentation. It is to be understood that the linker is not defined by any one overall general structure under which each different individual linker fits. Rather, a linker according to the invention is a

10 functional unit that may take any number of physical shapes and have any number of physical sizes. The skilled artisan, having an understanding of this disclosure and skill in the art, will immediately recognize what can, and what cannot, be used as a linker according to the invention. In exemplary embodiments, the linker includes one end that binds, attaches, associates, etc. to nucleic acid, and preferably specifically binds to a

15 sequence present on the nucleic acid having a sequence of interest. Binding to the nucleic acid should be reversible in some way such that the linker does not become a permanent modification to the host cell DNA once the nucleic acid is inserted into the host cell DNA. In other embodiments, the linker binds to one or both of the transposases of the transposome. The linker includes another end that binds, attaches, associates, etc., with an

20 element that delivers itself and substances bound to it into the cytoplasm of a cell, into a nucleus or mitochondria of a cell, or both (*i.e.*, a "targeting element", "delivery vehicle", or "vehicle").

Examples of linkers useful in practicing the present invention include, but are not limited to, single molecules such as an oligonucleotide having a sequence on one end that

25 hybridizes to a sequence on the nucleic acid of the transposome and having on its other end a sequence that hybridizes to a nucleic acid coated on a nanoparticle; an oligonucleotide having a peptide nucleic acid (PNA) sequence on one end that hybridizes to a sequence on the nucleic acid of the transposome and having on its other end a sequence that hybridizes to a nucleic acid coated on a nanoparticle, wherein the two

30 sequences of the tether are linked via a photo-cleavable chemical or peptide sequence; a modified oligonucleotide having a sequence on one end that hybridizes to a sequence on the nucleic acid of the transposome and having on its other end a dexamethasone tag that

can bind directly to a glucocorticoid receptor; or a modified oligonucleotide having a sequence on one end that hybridizes to a sequence on the nucleic acid of the transposome and having on its other end a sequence that is modified to include a poly-histidine tag that binds to a nickel nanobead. Numerous other suitable linkers and methods of their attachment to nanoparticles are known to those skilled in the art (*e.g.*, U.S. Patent Nos. 8,377,714; 8,357,424; 8,333,822; 8,013,048; 7,993,749; 7,115,688; and 6,767,635; U.S. patent application publications 20100260676 and 20040126900; incorporated herein by reference). Alternatively, the linker can be a multi-part system, such as one using a biotin-streptavidin linkage or some other known specific binding pair (*e.g.*, antibody-antigen, enzyme-substrate). For example, the linker can comprise a first part, which is a modified oligonucleotide having a sequence on one end that hybridizes to the nucleic acid of the transposome and having biotin attached to the other end, and a second part, which has a streptavidin molecule on one end bound to a silica-binding moiety on the other end. In practice the first part of the system binds to the nucleic acid of the transposome, the second part binds to a silica nanoparticle, and the two are linked via a biotin-streptavidin linkage. As another non-limiting example, the silica-binding moiety can be replaced by a short peptide conferring a nuclear localization signal (NLS).

Although not required, it is preferred that the linker have the ability to allow the transposome and targeting element to be released from, or otherwise separate from each other once inside the pre-selected DNA-containing organelle of a cell of interest. This reduces bulk and minimizes the possibility of interference of the targeting element on the transposome. Many mechanisms are known in the art for providing releasable linkers, and any such linker can be used according to the invention. Non-limiting examples include single-stranded or double-stranded linkers, which are susceptible to endonucleolytic cleavage by cellular nucleases, peptide linkages having cleavage sequences for proteolytic enzymes, disulfide bridges that can be broken based on pH conditions, linkages that can be broken by application of mechanical energy, such as ultrasound, and linkages that can be broken by application of electromagnetic radiation, such as photo-cleavable linkages and linkages that can be broken using magnetic resonance imaging (MRI) or nuclear magnetic resonance (NMR), or X-ray irradiation, or linker dissociated from transposome or from nanoparticle by a pH change.

In addition to the transposome and the linker, the complex of the invention comprises a targeting element. In general, the targeting element functions to deliver the transposome to a pre-selected DNA-containing organelle of a cell of interest. As mentioned above, and similar to the linkages used in the complex, there are numerous substances available to act as targeting elements, having widely diverse compositions and structures. A targeting element according to the invention is thus a functional element. The skilled artisan, having an understanding of this disclosure and having skill in the art, will immediately recognize what can, and what cannot, be used as a targeting element according to the invention. Non-limiting examples of targeting elements include:

nanoparticles having on their surface one or more ligands for internalization of substances by nuclear membrane, mitochondrial membrane receptors, or nuclear shuttle proteins; molecules presenting a hydrophobic surface for interaction with lipid bi-layer membranes; peptides; and antibodies.

In exemplary embodiments, the targeting element is a nanoparticle. There are many nanoparticles that are known to those skilled in the art that can deliver genetic material through cell barriers and into the nucleus. For example, TAT peptide-conjugated monodisperse mesoporous silica nanoparticles (Pan et al., *J Am Chem Soc.* 2012 Apr 4;134(13):5722-5); iron oxide nanoplates and nanoflowers (Palchoudhury et al., *Chem Commun (Camb)*. 2012 Oct 1;48(85):10499-501); superparamagnetic iron oxide nanoparticles (Wahajuddin and Arora, *Int J Nanomedicine*. 2012;7:3445-71; Mesarosova et al., *Neoplasma*. 2012;59(5):584-97); NLS-functionalized polyplexes (Larsen et al., *J Gene Med*. 2012 Sep 13. doi: 10.1002/jgm.2669); Hydroxyapatite nanoparticles (Do et al., *Ther Deliv*. 2012 May;3(5):623-32); negatively charged silicon carbide nanoparticles (Serdiuk et al., *Nanotechnology*. 2012 Aug 10;23(31):315101); silver nanoparticles (*J Appl Toxicol*. 2012 Nov;32(11):867-79); CuO nanoparticles (Wang et al., *Chem Res Toxicol*. 2012 Jul 16;25(7):1512-21); lactosyl-norcantharidin -associated N-trimethyl chitosan nanoparticles (Guan et al., *Int J Nanomedicine*. 2012;7:1921-30); Multifunctional envelope-type nano device (MEND) for organelle targeting via a stepwise membrane fusion process (Yamada et al., *Methods Enzymol*. 2012;509:301-26); Polyethylene glycol-based protein nanocapsules (Biswas et al., *Biomaterials*. 2012 Jul;33(21):5459-67); gold nanoparticles with attached cell penetrating peptide (Sousa et al., *Small*. 2012 Jul 23;8(14):2277-86); nitrocellulose nanoparticles (Oh et al., *Recent Pat Nanotechnol*. 2012

Jun 26); and lipid-DNA particles (Hope et al., Mol Membr Biol. 1998 Jan-Mar;15(1):1-14).

The complex of the invention is made using standard molecular biology and biochemistry techniques and routine laboratory practices. As such, the skilled artisan should have no difficulty making any embodiment of a complex encompassed by the invention. Making a complex according to the invention includes binding one or two transposases to recognition sequences present on a nucleic acid having a sequence of interest, binding a linker to the nucleic acid, the transposase(s), or both, and binding the linker to a targeting element, thus making a complex comprising a transposome linked to a targeting element through a linker element. Typically, the transposase(s) are bound to the nucleic acid prior to binding to the linker. Further, typically, one end of the linker is bound to the transposome while the other end of the linker is bound to the targeting element. Yet further, in some embodiments, the targeting element can be considered a modifying moiety for the linker (*e.g.*, a protein tag fused to an oligonucleotide). In such embodiments, it is often preferred that the modified linker be created then bound to the nucleic acid having a sequence of interest, the transposase(s), or both. In some embodiments, the method comprises forming a transposome from the nucleic acid containing the sequence of interest, forming a linker-targeting element combination, and linking the transposome and the linker-targeting element.

While it is envisioned that the practitioner will make the complex using a pre-formed nucleic acid containing a sequence of interest, it is envisioned that the method can be practiced to include the step of preparing the nucleic acid. Standard molecular biology and/or chemical synthesis methods can be used to create the nucleic acid. The general outline for making the nucleic acid is to insert a sequence that includes a sequence of interest between two pre-defined transposase recognition sequences. Typically, this is accomplished by cloning the recognition sequences into an expression vector (*e.g.*, plasmid, phage, phagemid), then cloning the nucleic acid containing the sequence of interest between the two recognition sequences. However, the skilled artisan is well aware of alternative procedures for inserting nucleic acid elements into expression vectors at desired sites, including, but not limited to, inserting the recognition sequences at the ends of the nucleic acid prior to incorporation into the expression vector, using mutagenesis,

such as site-directed mutagenesis, to create recognition sequences in a vector already containing the sequence of interest, among other ways.

Of course, the reaction conditions for binding the various possible transposomes, linkers, and targeting elements will vary from embodiment to embodiment depending on which particular substances are selected for each of the three components. However, the conditions will be similar enough that a complex according to the invention can be made without excessive experimentation. Generally, the complexes can be formed at about the range of 0° - 37°C; 50 -750 mM KCl; about neutral or slightly alkaline pH (*e.g.*, pH 6 - 9); any buffers suitable for this pH range can be used, at about the concentration sufficient to stabilize the pH, typically in the concentrations range 10-50 mM. About 10-50% glycerol and surfactants or non-ionic detergents, *e.g.*, Triton-X-100, NP40, at concentrations typically slightly above critical micelle concentration (CMC), *e.g.*, about 2xCMC can be used if transposase aggregation is a problem. Time can vary from few minutes to several hours or even longer contingent upon the properties of the transposase of choice and composition of the reaction mixture. Optimally, the reaction is performed in the absence of Mn⁺⁺ or Mg⁺⁺, and a chelating reagent, such as EDTA can be added to about 1 mM concentration to bind trace amounts of divalent cations that might be present in the reaction mixture.

One aspect of the invention that provides a significant advantage over prior art methods is the ability to efficiently delivery a nucleic acid of interest to a pre-selected DNA-containing organelle using transposases. As mentioned above, the method avoids the need to create transposomes *in vivo* and rely on cellular machinery to move elements of a transposome complex into and out of the nucleus and cytoplasm. Instead, the present method delivers a pre-formed transposome to a target cell attached to a targeting element that assists in transporting the transposome to the cell nucleus (or, if desired, mitochondria). The present method is much more straightforward than prior art methods and much more efficient at delivering desired nucleic acids to target DNA molecules. The method of the invention comprises contacting a target cell with a complex according to the invention under conditions that allow for uptake of the complex by the target cell and transport of the complex to the pre-selected DNA-containing organelle. In general, the step of contacting does not require direct physical contact of the construct with the cell of interest. Rather, it allows for introduction of the complex into the general environment of

the cell, and permitting natural movement of the environment to effect physical contact of the two. Thus, for example, for *in vivo* practice of the method, the step of contacting can be accomplished by introducing (*e.g.*, injecting, infusing, inhaling, ingesting) the complex into the body of the subject, and allowing bodily processes (*e.g.*, blood circulation) to
5 deliver the complex to the target cell, thus effecting physical contact. Similarly, for *in vitro* and *ex vivo* practice of the method, contacting can be accomplished indirectly by adding the complex to the growth media and allowing diffusion of the complex through the media to effect physical contact of the complex with the target cell. In another embodiment, external manipulation of magnetic nanoparticles with attached transposomes
10 in a fluid medium is achieved using permanent magnet-based or electromagnetic field-generating stator sources, similar to those described in U.S. Patent No. 8,313,422, incorporated herein by reference.

It is to be noted that, as plants move material between different tissues and cells by way of a vascular system, delivery and contact can be effected in much the same way that
15 it occurs in animals. Furthermore, whereas in humans, germline alterations are generally avoided in therapeutic treatments due to ethical concerns, such concerns are not present with non-human animals and plants. Therefore, in embodiments, the method of the invention uses as target cells, germ cells.

The amount of the nucleic acid containing the sequence of interest that is delivered
20 to a subject, such as a human, depends on various factors, as is recognized in the art. These factors include the size/weight of the subject, the route of delivery, the efficiency of the delivery into a certain type of cell, the type of targeted cell, whether the subject is in a diseased state, or has a diseased organ, etc. However, determining the amount to deliver is routinely established through typical experimentation using methods well known to those
25 skilled in the art. For example, Hahnewald et al., Genetic Vaccines and Therapy 2009, 7:9, incorporated herein by reference, describes a method for establishing doses for treatment of metabolic diseases using adeno-associated virus-mediated gene therapy in a murine model, including extrapolation of the animal trials data dosage to treatment of a one year old child with a body weight of 10 kg.

30 Once the act of contacting the cells with the complex has been accomplished, the cells are maintained under conditions that permit intracellular movement of the complex to the pre-selected DNA-containing organelle. Typically, those conditions are the same as

for contacting of the cells with the complex. The time provided for effecting physical contact with target cells will vary between *in vivo* practice of the method and *in vitro* practice of the method. For *in vivo* practice of the method, the amount of time can range from about one minute (for delivery directly to tissues containing the target cell) to up to
5 24 hours or more (for systemic delivery via the blood system). In contrast, for *in vitro* or *ex vivo* practice of the method, the amount of time is typically on the lower end of the range for *in vivo* practice, such as from about one minute to about 30 minutes.

While delivering a complex to a pre-selected DNA-containing organelle can, in some situations, be adequate to allow for expression of a sequence present on the nucleic
10 acid having a sequence of interest, in many instances, it will be preferred to insert the sequence of interest into the host cell DNA. As such, in embodiments, the method of delivering a nucleic acid to a pre-selected DNA-containing organelle further includes inserting the nucleic acid of the transposome into the DNA, and in most embodiments into genomic DNA, of the pre-selected DNA-containing organelle. As the act of inserting is a
15 fully intracellular activity, the only action required by the practitioner to effect this action is to maintain the host cell in a viable state for a sufficient period of time to allow for insertion of the nucleic acid into the host cell DNA. In general, the amount of time is widely variable depending on the identity of the host cell. However, typically one can expect to see the effect of insertion anywhere from two hours to 72 hours after contact of
20 the target cell with the complex.

The methods of the invention can be advantageously used to provide methods of treating a subject suffering from, or at a high likelihood of developing a disease or disorder. The method may be a therapeutic method of treating a subject suffering from a disease or disorder. Alternatively, the method may be a prophylactic method of treating a
25 subject suspected of having a high likelihood of developing a disease or disorder in the future. In yet other embodiments, the method is method of preventing a disease or disorder in a subject, such as through a DNA vaccine. By delivering nucleic acids encoding desired genes, and under certain control elements, into the cells of a subject, certain proteins or nucleic acids can be expressed in the cells, or expression of certain
30 proteins or nucleic acids can be reduced or abolished, thus effecting treatment of the subject.

In yet other embodiments, the method involves removing cells from a tissue of a subject, introducing or delivering into the cells nucleic acids of interest in the transposome complex as described in the present invention, culturing the cells *in vitro* and subsequently transplanting the cells back into the subject. As used herein, such methods are referred to as *ex vivo* methods.

In yet other embodiments, the method involves introducing or delivering into stem cells nucleic acids of interest in the transposome complex as described in the present invention, growing the stem cells in culture conditions that direct the stem cells to differentiate towards a desired somatic cell lineage, and subsequently transferring the differentiated cells into the desired tissues of a subject. The stem cells can be undifferentiated or partially differentiated cells, such as pluripotent, progenitor, or precursor cells, etc. Examples of such cells include embryonic stem (ES) cells, induced pluripotent stem (iPS) cells, MUSE cells, adult stem cells (*e.g.*, hematopoietic stem cells, neural stem cells, and mesenchymal stem cells), neural progenitor cells, and cardiac progenitor cells. Methods for *in vitro* culturing these cells and inducing the cells to differentiate towards a more mature cell lineage are known in the art. See, *e.g.*, US Patent No. 8318951, US Patent No. 7029913, US Patent No. 6200806, US 20110086379, US 20100183570, US 20090227032, and US 20090304646. The concepts of using the stem cells in treating diseases have been tested and validated in the state of art by various sources. See Singec, I. *et al.*, "The leading Edge of Stem Cell Therapeutics," Annual Review of Medicine, 58:313-328 (2007), WO2008042174A2, WO199740137A1, US8158120B2, WO2010108665A1 and US20120093832A1. The disclosures of these references herein are incorporated by reference.

The skilled artisan can immediately recognize numerous diseases and disorders that can be treated using the complex of the present invention. Non-limiting examples include hereditary and acquired metabolic disorders, *e.g.*, diabetes, urea cycle disorder, hyperlipidemia, paroxysmal nocturnal hemoglobinuria, porphyria, Wilson's disease, Fanconi syndrome, myopathy, amyloidosis, mucopolysaccharidosis, glycogen storage diseases, fatty oxidation disorders, mitochondrial disorders, hypertrichosis, hemochromatosis, thrombotic disorders, galactosemia, carnitine-acylcarnitine translocase deficiency, chronic granulomatous disease Landau-Kleffner syndrome, myoadenylate deaminase deficiency, glucose-6-phosphate dehydrogenase deficiency, calcium

homeostasis disorders, metabolic bone disease, Schindler disease, pyruvate kinase deficiency, sickle-cell anemia, auto-immune diseases, allergies, developmental abnormalities, cancers, and cardiovascular diseases. The complex also can be used in generation of pluripotent cells for regenerative medicine, *i.e.*, for replacing or regenerating human cells, tissues, or organs to restore or establish normal function.

Yet again, the ability to deliver a nucleic acid having a sequence of interest to a pre-selected DNA-containing organelle provides a method for creating transgenic non-human animals and plants, which have one or more desired phenotypic characteristics, such as immunity to certain viruses or bacteria, resistance to certain drugs or toxins, drought resistance, increased protein-to-fat ratio, increased production of a nutrient, expression of a pharmaceutically active substance, and reduced production of a harmful substance.

To more easily provide embodiments of the invention to the practitioner, the invention contemplates kits. In general, kits of the invention include, in packaged combination, some or all of the components and reagents needed to practice a method of the invention. In a basic form of a kit according to some embodiments, the kit includes all of the components and reagents used to make a complex of the invention, with the exception of the nucleic acid to be used in the transposome.

Generally, each of the components of the kit are packaged separately, then combined at the appropriate time by the practitioner. However, in some embodiments, two or more components are provided together, for example in the same vial, tube, vessel, etc. For example, where two pre-selected transposases or two pre-selected recognition sequences are provided in a kit, the two transposases can be provided in the same vial or the two recognition sequences are provided in the same vial.

In some embodiments, a kit will provide all of the components and reagents necessary to create a genetically modified cell or genetically modified organism. In such embodiments, the kit will contain one or more nucleic acids having one or more sequences of interest, flanked by recognition sequences for pre-selected transposases, the pre-selected transposases, one or more targeting elements, and a linker element that can link the nucleic acids and/or transposases to the targeting elements. In some embodiments, the nucleic acids and transposases are provided in the form of transposomes. In some embodiments, the linker element is provided in a form bound to the targeting element or

bound to the transposome or its constituent elements. In some embodiments, kits of the invention provide a fully-formed complex according to the invention. Typically, where a kit does not provide a fully-formed complex, reagents for making binding reaction conditions are provided.

5

EXAMPLES

The invention will be further explained by the following Examples, which are intended to be purely exemplary of the invention, and should not be considered as limiting the invention in any way.

Example 1: Gene delivery using double-stranded transposome attached to
10 nanoparticles

First, a gene of interest is amplified in PCR and two inverted transposase recognition sequences are introduced at the ends of the fragment. By the way of example and not limitation, transposase recognition sequence, *e.g.*, 19 bp for Vibhar (US patent applications number 13/470,087, filed 11 May 2012) or 19 bp "mosaic" recognition
15 sequence for hyperactive Tn5 transposase (Steiniger-White et al., J Mol Biol. 2002 Oct 4;322(5):971-82) are incorporated into PCR primers and introduced at the ends of the PCR fragment. As it was recently discovered, Vibhar transposase can efficiently recognize substantially different 19 bp recognition sequences, whereas only one such sequence, *i.e.*, "mosaic", is known for Tn5 transposase (Fig. 2). It is preferred that substantially different
20 recognition sequences, such as shown on Fig. 2, are incorporated into PCR primers that are designed for different ends of the fragment. Otherwise generation of undesirable PCR products (*i.e.*, concatemers and primer-dimers) could lead to a low yield of the PCR product. Thermostable polymerases that do not add any nucleotides that are not encoded in the template (*e.g.*, PfuUltra) are preferred as using polymerases that add extra
25 nucleotides to the 3' ends of PCR fragments (*e.g.*, Taq polymerase) would reduce the activity of the transposome as this would alter the transposase recognition sequences.

Next, the PCR fragment is purified from unused primers, *e.g.*, using Agencourt[®] AMPure[®] XP magnetic beads (Beckman-Coulter, Brea, CA) according to the manufacturer's instructions. Then it is mixed with the transposase and incubated at
30 conditions that allow formation of transposase-DNA complex, and with a tether, *e.g.*, an oligomer comprising a peptide nucleic acid (PNA) portion that is complementary to the amplified PCR fragment and a DNA oligonucleotide portion that can bind nanoparticle

(Fig. 3A). PNA invades DNA duplex and forms a triplex by hybridizing to complementary DNA strand. The major requirement for invading dsDNA and for forming a triplex is that hybridization should occur at a polypyrimidine stretch of the DNA sequence. One of skill in the art can either find such stretch in the native gene sequence or change codons in the ORF of the gene of interest to encode the same amino acids, but comprising pyrimidines, or by introducing such stretch into the PCR fragment, *e.g.*, after the stop codon. Conditions that favor formation of the triplex and PNA sequence requirements are well established (see, for example, Nielsen PE. *Curr Opin Mol Ther.* 2010 Apr;12(2):184-91). Next, the transposome with tether can be attached to a nanoparticle. If, as represented in Fig. 3A, the tether is a PNA-DNA chimera, then, via the DNA portion, it can be attached to a nanoparticle that is coated with oligonucleotides. Apart from PNA-DNA chimeras, PNA-Morpholino (PMO) or PNA-DNA-PMO chimeras can be used also. For simplicity, only one PNA strand is shown in Fig. 3, when in reality two PNA strands bind to the DNA strand and displace another DNA strand forming so called P-loop or D-loop (Frank-Kamenetskii and Mirkin, *Annu Rev Biochem.* 1995;64:65-95; Wang and Xu, *Cell Res.* 2004 Apr;14(2):111-6). PNA-DNA chimeras and nanoparticles coated with oligonucleotides can be manufactured by the methods well known to those skilled in the art (Borgatti et al., *Curr Drug Targets.* 2004 Nov;5(8):735-44; Liu et al., *Anal Chem.* 2005 Apr 15;77(8):2595-600; Thomson et al., *Biomacromolecules.* 2012 Jun 11;13(6):1981-9; Niikura et al., *J Am Chem Soc.* 2012 May 9;134(18):7632-5; Wang et al., *Biomaterials.* 2012 Jun;33(19):4872-81; Ma et al., *Biosens Bioelectron.* 2012 Feb 15;32(1):37-42; Sarkar and Irudayaraj, *Anal Biochem.* 2008 Aug 1;379(1):130-2) incorporated herein by reference. It should be noted, and as will be presented below, tethering to the nanoparticles or other vehicles does not necessarily have to be via DNA-DNA hybridization, but can be provided with a variety of methods, whereas one member of binding pair is provided on the tether and another on the vehicle, *e.g.*, nanoparticle.

As shown in Fig. 3B, nanoparticles are delivered into the nucleus, and as was discussed above, there are many types of nanoparticles that are suitable for delivery into a nucleus. Optionally, the PNA part of the tether can be equipped with a photoswitch, *e.g.*, a single or multiple azobenzene photoswitch (Stafforst and Hilvert, *Angewandte Chemie*, Vol. 49, No. 51, 9998-10001, 2010). Irradiation with ultraviolet light results in cis-trans

conformational change of azobenzene moiety, making the triplex structure sterically unfavorable, and release of the tether from DNA (Fig. 3B). To avoid undesirable effects of UV light on host cells, substitution of all four ortho positions with methoxy groups in an amidoazobenzene derivative are preferred as this results in an about 35 nM red shift, thus green light rather than UV light can be used (Beharry et al., J. Am. Chem. Soc., 2011, 133 (49), pp 19684–19687). Alternatively, as shown on Fig. 3C, the tether can be equipped with a photocleavable linker (Dmochowski and Tang, Biotechniques. 2007 Aug;43(2):161, 163, 165; Ball et al., Artif DNA PNA XNA. 2010 Jul;1(1):27-35), incorporated herein by reference. A variety of photocleavable linkers is available from Amberg, Watertown, MA.

Next, as shown in Fig. 3D, the transposome inserts nucleic acid material into a target DNA. This process is effected by transposase activity, resulting in stable insertion of the gene of interest. Importantly, no extra DNA is incorporated, except for 19 bp inverted repeats flanking the insertion. Use of 19 bp repeats that differ in several nucleotide positions, is preferred to eliminate any possibility of recombination at the repeats and instability of the insertion.

Example 2: Gene delivery using a predominantly single-stranded transposome attached to nanoparticles

The main difference between this embodiment and the embodiment described in Example 1 is the use of predominantly single-stranded transposome, as represented in Fig. 4, rather than the double-stranded transposome represented in Fig. 3. The disadvantage of double-stranded transposomes is that before they reach the nucleus, or another organelle containing a target genome, they could be exposed to Mg^{++} and Mg^{++} ions while passing through the cytoplasm. These ions activate transposases that can act on the double-stranded DNA portion of the transposome before it arrives at its destination (*e.g.*, the nucleus), largely incapacitating it. Indeed, in our *in vitro* experiments forming transposase complexes of Vibhar transposase with partially double-stranded oligonucleotides where the dsDNA portion was limited to 19 bp recognition sequence resulted in the most active transposase complexes. However, three extra bp, *i.e.*, a dsDNA portion of 21 bp, significantly reduced activity of the complex on target DNA. Furthermore, using oligonucleotides that were double-stranded for about 40 nucleotides reduced the activity to barely detectable. On the contrary, transposome activity was not significantly impacted if

one of the strands was about 60 nucleotides long, and another only 19, *i.e.*, the size of the dsDNA stretch was limited to 19 bp. Transposomes that carry a gene of interest would typically carry a DNA fragment of at least several hundred nucleotides in length and would be extremely inefficient if all this DNA is double-stranded. However, they can be rendered much more efficient if the size of the dsDNA stretches is limited to 19 bp, *i.e.*, two inverted dsDNA transposase recognition sequences connected with a long ssDNA stretch.

First, as shown in Fig. 4A, a single-stranded copy of gene of interest is generated, preferably using asymmetric PCR (Marimuthu et al., Analyst. 2012 Mar 21;137(6):1307-15; Millican and Bird, Methods Mol Biol. 1998;105:337-50), incorporated herein by reference. At the same time, 19 bp transposase recognition sequences are introduced at the ends of the PCR fragment by incorporating these sequences into the PCR primers. Using substantially different recognition sequences, such as represented in Fig. 2 is preferred, one for forward, and another for the reverse primer. Using the same recognition sequence for both primers is less preferred as this could lead to formation of concatemers and primer-dimers and low yield of the desired PCR product.

By way of example, and not a limitation, a biotinylated DNA oligonucleotide could be optionally provided with a photocleavable linker, and combined with a streptavidin-Si tag fusion protein to form a two-part tether. The Si-tag can strongly bind to silica nanoparticles (Ikeda and Kuroda, Colloids Surf B Biointerfaces. 2011 Sep 1;86(2):359-63) which are widely used as delivery vehicles (Mai and Meng, Integr Biol (Camb). 2012 Oct 5; Petkar et al., Crit Rev Ther Drug Carrier Syst. 2011;28(2):101-64; Popat et al., Nanoscale. 2011 Jul;3(7):2801-18; Zhao et al., Expert Opin Drug Deliv. 2010 Sep;7(9):1013-29; Liu et al., Curr Cancer Drug Targets. 2011 Feb;11(2):156-63; Zhao et al., Expert Opin Drug Deliv. 2010 Sep;7(9):1013-29). As shown in Fig. 4A, predominantly single-stranded gene of interest, transposase, tether and silica nanoparticle are combined to form transposome tethered to a silica nanoparticle.

Next, as shown in Fig. 4B, transposomes that were tethered to silica nanoparticles are applied to the target cells and delivered into the nucleus. During this journey, the DNA component of the transposome is protected in a vesicle from nucleases that could be present in the cytoplasm of the target eukaryotic cells (Liao et al., Nucleic Acids Res. 2011 Aug;39(14):5967-77).

As shown in Fig. 4C, once in the nucleus, the gene of interest is inserted into the host genome. The single-stranded region is converted to dsDNA by cellular machinery, causing the linker to be displaced.

5 Example 3: Gene delivery using Nuclear Localization Signal (NLS) and liposomes or micells

There are many suitable liposome and micelle systems, *e.g.*, cationic lipid-based gene delivery systems (Mahato et al., Pharm Res. 1997 Jul;14(7):853-9; lipid modified polyion complex micelles (Sun et al., Int J Pharm. 2012 Apr 4;425(1-2):62-72); and proton-actuated membrane-destabilizing polyion complex micelles (Yessine et al.,
10 Bioconjug Chem. 2007 May-Jun;18(3):1010-4), incorporated herein by reference.

As shown in Fig. 5A, a PCR fragment encoding a gene of interest flanked by transposase recognition sequences is combined with a transposase, biotinylated DNA oligonucleotide and streptavidin-NLS fusion protein. Next, the complex is formed and delivered into the cytoplasm using a liposome or micelle system, preferably the one that
15 facilitates release into the cytoplasm and protects DNA from degradation by binding to it and shielding it from nucleases, *e.g.*, biomimetic lipid-polycation copolymer, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-graft-poly(l-lysine)-block-polyethylene glycol (Sun et al., Int J Pharm. 2012 Apr 4;425(1-2):62-72). Next, the NLS facilitates targeting the transposome through a nuclear pore into the nuclear membrane, as shown in Fig. 5B. In
20 the nucleus, the transposome effects insertion of the gene of interest, similar to as shown in Fig. 4C in Example 2.

Example 4: Gene delivery using a small molecule vehicle

In eukaryotic cells, some proteins reside in the cytoplasm or could be anchored at the cell membrane, but are transported into the nucleus upon binding to specific ligands.
25 Embodiments of the present invention link transposomes to such ligands and use native cell proteins for ligand-transposome complex delivery into the nucleus. The rationale is that native cell proteins can be more efficient than artificial delivery systems because intracellular translocation machinery has evolved over a long time, which is difficult to match with an artificial system. Furthermore, using a native delivery system could be
30 more physiological, resulting in less cytotoxicity, faster delivery into the nucleus, less exposure to nucleases during the delivery, and better efficiency of gene transfer.

By way of example and not limitation, dexamethasone, which is a ligand for the glucocorticoid receptor, can be used as a small molecule vehicle for transposome delivery. The glucocorticoid receptor is a nuclear receptor that, in its un-liganded state, resides in the cytoplasm, but it is promptly transported into the nucleus upon binding to its ligand, *e.g.*, dexamethasone. It is known that dexamethasone can be linked to large molecules, *e.g.*, fluorescein, without significant loss of activity, which allows its use in functional assays, such as glucocorticoid PolarScreen assay (Life Technologies, Carlsbad, CA). Similarly, dexamethasone is used in embodiments of the present invention as a modification to a DNA oligonucleotide. As shown in Fig. 6A, such a dexamethasone-tagged oligonucleotide can be hybridized to a single-stranded transposome, and the transposome can be delivered into the cytoplasm, *e.g.*, using liposomes or micells (Fig. 6B).

Next, dexamethasone binds to endogenous glucocorticoid receptors that reside in the cytoplasm, and transport of the liganded receptor that is tethered to the transposome is facilitated by natural nuclear translocation machinery (Vandevyver et al., Traffic. 2012 Mar;13(3):364-74). In the nucleus, the transposome effects insertion of the gene of interest, similar to as shown in Fig. 4C in Example 2.

Example 5: Generation of transposomes with variable double-stranded DNA (dsDNA) content

Depending on the application, dsDNA content can vary (Fig. 7). By way of example, in different embodiments of this invention, genes of interest that are flanked with transposase recognition sequences can be completely double-stranded (A), predominantly single-stranded with a dsDNA region comprising only transposase recognition sequences (B), or any variation of a dsDNA component in between these two states (C-E). Methods for generating and using transposomes with completely dsDNA were described in Example 1 and transposomes with predominantly single stranded DNA that is double stranded only at its ends were described in Examples 2-4. However, there are certain advantages in using a transposome with DNA components that are single-stranded in areas that are adjacent to the transposase recognition sequences, *e.g.*, 19 bp inverted repeats for Vibhar and Tn5 transposases, but at least partially double-stranded in the central part (Fig. 7C, D, E). These advantages would be immediately recognized by those skilled in the art

contingent upon the transposase used, its delivery method, target cells and overall costs constraints.

An advantage of completely dsDNA (A) is that it is more resilient to nucleases than predominantly ssDNA (B). Breaks on one strand of dsDNA would not lead to
5 disintegration of the gene of interest unless two breaks occur sufficiently close to each other. Therefore, less sophisticated delivery methods that do not require direct delivery into the nucleus or protection of DNA with biocompatible polymers can be used. The main disadvantage of completely dsDNA transposomes as compared to largely ssDNA transposomes is that dsDNA immediately adjacent to the recognition sequences drastically
10 inhibits transposase activity. Furthermore, a transposome can integrate into a dsDNA moiety of another transposome, or even into its own dsDNA moiety and incapacitate itself as well as another transposome into which it integrates. A cost-related disadvantage of completely dsDNA (A) is that rather expensive and harder to work with photocleavable PNA/DNA tethers should be used with the dsDNA (Example 1), whereas inexpensive
15 DNA oligonucleotides can be hybridized to the ssDNA (B), (Examples 2-4). Furthermore, for completely dsDNA (A) a polypyrimidine tract has to be engineered as a landing site for PNA, unless it is already present in the ORF of the gene of interest, whereas with ssDNA (B) a landing site for DNA oligonucleotide can be easily found in the natural genes of interest by those skilled in the art, *e.g.*, using Primer 3 program.

An advantage of the transposase DNA moiety presented in Fig. 7C is that it is
20 nearly as resilient to the nuclease action as the one represented in Fig. 7A, but the transposase activity is higher as the regions immediately adjacent to transposase recognition sequences are single-stranded. Furthermore, single-stranded DNA regions can be used as landing sites for DNA oligonucleotides obviating the need for a PNA-based
25 tether. Alternatively, a single stranded region can be specifically provided away from the transposase recognition sequences to generate a landing site for a tether comprising DNA oligonucleotide that hybridizes to that region (Fig. 7D). In any case (A-E), positioning tethers in about the middle of the genes of interest is preferred as it keeps the tether and comparatively bulky delivery vehicle (*e.g.*, nanoparticle) far away from the active site of
30 the transposome, thus providing better accessibility of the transposome active site to the target DNA in a crowded environment of the nucleus or another organelle.

Finally, multiple single-stranded DNA fragments, *e.g.*, PCR fragments or oligonucleotides, can be hybridized to a single-stranded gene of interest (E). These can comprise modified nucleotides resilient to nucleases as well as to transposase insertion, and can cover the single stranded region nearly entirely. Alternatively, unmodified

5 oligonucleotides can be hybridized fairly sparsely and serve as multiple primers for DNA polymerase when the ssDNA gap is being repaired by polymerase in the nucleus (Fig. 4C).

Those skilled in the art would know how to generate any of the DNA types represented in the various panels of Fig. 7. By way of example, generation of DNA types A and B were described in Examples 1-5. DNA types C-E can be generated from DNA

10 type B. To this end, the lower strand of the type C can be generated using method of asymmetric PCR with primers complementary to the gene of interest. Next, it is hybridized to the DNA type B, leaving single stranded gaps between transposase recognition sequences (adapters) and central double-stranded region. Similarly, two or more lower strand PCR fragments can be generated and hybridized to the DNA type B,

15 thus leaving suitable single stranded gaps in between themselves and the adapters. Synthetic oligonucleotides can be used instead of the PCR fragments to generate DNA of type E, or a combination of PCR fragments and oligonucleotides.

Example 6: Gene delivery using cell penetrating peptides

Cell-penetrating peptides (CPPs) are short peptides that facilitate cellular uptake of

20 various molecular cargo (from nanosize particles to small chemical molecules and large fragments of DNA). The "cargo" is associated with the peptides either through chemical linkage via covalent bonds or through non-covalent interactions. The function of the CPPs is to deliver the cargo into cells, a process that commonly occurs through endocytosis with the cargo delivered to the endosomes of living mammalian cells (Koren and Torchilin,

25 Trends Mol Med. 2012 Jul;18(7):385-93; Lehto et al., Expert Opin Drug Deliv. 2012 Jul;9(7):823-36). Cell-penetrating peptides are able to transport different types of cargo molecules across the plasma membrane; thus, they act as molecular delivery vehicles.

In one embodiment, CPP (*e.g.* Tat PGRKKRRQRRPPQ/SEQ ID NO: 8; Penetratin RQIKIWFQNRRMKWKK/SEQ ID NO: 9; Transportan

30 GWTLNSAGYLLGKINLKALAALAKKIL/SEQ ID NO: 10; VP-22 DAATATRGRSAASRPTERPRAPARSASRRPVD/SEQ ID NO: 11; MAP Chimeric KALAKALAKALA/SEQ ID NO: 12; SAP Proline-rich motif

VRLPPPVRLLLLPRLPPP/SEQ ID NO: 13; PPTG1

GLFRALLRLLRSLWRLLLRA/SEQ ID NO: 14; Oligoarginine Agr8 or Arg9; hCT (9–32) LGTYTQDFNKTFPQTAIGVGAP/SEQ ID NO: 15; SynB

RGGRLSYSRRRFSTSTGR/SEQ ID NO: 16; or Pvec LLILRRRIRKQAHAAHSK/SEQ

5 ID NO: 17, (Heitz et al., Br. J. Pharmacol., 157(2), 195-206, 2009)) is connected either to a DNA or PNA moiety of the tether via stable or cleavable conjugation involving mainly disulfide or thio-esters linkages. The DNA or PNA moiety of the tether is hybridized to the DNA component of transposomes as described in Examples 1-5. Some CPPs comprise nuclear localization signals. Additional NLSs can be either fused with CPP or
10 independently tethered through PNA or DNA to the DNA component of transposome (Fig. 8).

In another embodiment, stable non-covalent attachment of CPP to a DNA component of a transposome is achieved via electrostatic interaction of negatively charged DNA and positively charged amino acids of CPP. For example, MPG

15 GALFLGFLGAAGSTMGAWSQPKKKRKV /SEQ ID NO: 18 or Pep-1

KETWWETWWTEWSQPKKKRKV SEQ ID NO: 19 peptides can be used for this

purpose. The hydrophobic N-terminal portion facilitates penetration of a transposome-peptide complex through the cell membrane and the positively charged C-terminal tail

interacts with DNA and contains NLS motifs that can target a cargo to a nucleus (Morris
20 et al., Cell, Vol. 100, 201-217, 2008). MPG or Pep-1 can condense DNA and facilitate formation of nanoparticles, which have a hydrophobic shell, thus protecting the DNA from nuclease degradation (Fig. 9). Additional NLS motifs can be either fused with CPP or independently tethered through PNA or DNA to the DNA component of the transposome.

Example 7: Gene delivery using different combination of tethers, CPPs, NLSs and
25 nanoparticles

Elements of the transposome delivery methods described in Examples 1-6 can be used in different combinations contingent upon a particular application, target cells, and chromosomal or episomal genomes. By way of example, in this embodiment elements of embodiments presented in Examples 2 and 3 are combined, as represented in Fig. 10.

30 In another embodiment, predominantly single-stranded transposomes can be combined with CPP, NLS, and a single stranded DNA binding protein, *e.g.*, *E.coli* Single-Strand DNA Binding Protein (SSB) (Fig. 9). *E. coli* SSB binds ssDNA with high

specificity and can partially protect it from nuclease degradation (Krauss et al. (1981) Biochemistry 20, 5346; Weiner et al. (1975) J. Biol. Chem. 250, 1972). *In vivo*, the protein is involved in DNA replication, recombination, and repair. *In vitro*, SSB enhances several molecular biology applications by destabilizing DNA secondary structure and increasing the processivity of polymerases. SSB-NLS-CPP fusion proteins can be constructed, purified, bound to a single-stranded transposome, and facilitate its delivery into the nucleus through the cell barriers, shield it from possible nuclease degradation in the cytoplasm, and facilitate repair of a single-stranded gap in the target genome after the transposon insertion (Fig. 11).

Example 8: Kits for generation of stem cells

It is now possible to generate cells with many properties of pluripotent embryonic stem cells by retroviral transduction of differentiated cells with only four transcription factors: Oct3/4, Sox2, Klf4 and c-Myc (Lewitzky and Yamanaka, Curr Opin Biotechnol. 2007 Oct;18(5):467-73). However, retroviral vectors, like other viral vectors, have safety issues which were extensively discussed above. Therefore, according to methods of this invention described in Examples 1-7, transposases are used for compiling the kits.

Kits according to the present exemplary embodiment of the invention comprise at least one gene of interest (*i.e.*, Oct3/4, Sox2, Klf4 or c-Myc) flanked by at least one adapter, each adapter comprising a double-stranded nucleotide sequence that a pre-selected transposase can recognize as a site for binding, and a transposase bound to the adapter, *i.e.*, a transposome. Genes of interest could be provided using one transposome comprising all of the genes, or several transposomes, *e.g.*, four transposomes comprising one gene each. In some embodiments, the kits comprise two different adapters, both of which are recognized by a pre-selected transposase. In some embodiments, the kit comprises a delivery vehicle bound to the transposome(s). The practitioner is free to select any appropriate target host cell and expression system, and such a choice, and design of appropriate control elements, is a routine activity for the skilled artisan. The kits of the invention provide the genes of interest flanked with adapters, the plasmid and/or host cell, in containers. The containers can be any suitable vessels for nucleic acids and host cells, such as plastic microfuge tubes. The containers are provided in packaged combination in a suitable package, such as a box made of cardboard, plastic, metal, or a combination thereof. Suitable packaging materials for biotechnology reagents are known

and widely used in the art, and thus need not be specified herein. In another embodiment, vectors that are suitable for cloning any genes of interest in a context suitable for gene delivery using transposomes are included with the kits.

5 It will be apparent to those skilled in the art that various modifications and variations can be made in the practice of the present invention without departing from the scope or spirit of the invention. Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention. It is intended that the specification and examples be considered as exemplary only.

10

CLAIMS

1. A complex comprising:

a transposome element comprising at least one nucleic acid containing a sequence of interest bound to at least one transposase molecule by way of two transposase
5 recognition sequences;

a linker element; and

at least one targeting element, which delivers the transposome element through cell membranes and into a pre-selected DNA-containing organelle,

wherein the linker element is bound at one end to the transposome element and at
10 the other end to the targeting element, and wherein the linker element releasably connects the transposome element to the targeting element.

2. The complex of claim 1, wherein the pre-selected DNA-containing
organelle is a cell nucleus.

15

3. The complex of claim 1 or 2, wherein the targeting element is: a
nanoparticle, a liposome, a dendrimer, a Q dot, a nuclear localization signal, a cell
penetrating peptide, or a ligand that can target the transposome into the pre-selected DNA-
containing organelle via attachment to an indigenous receptor.

20

4. The complex of any one of claims 1-3, wherein the linker element
comprises a polynucleotide that is hybridized to the nucleic acid sequence of interest, said
polynucleotide comprising: PNA, DNA, or both PNA and DNA.

25

5. The complex of claim 4, wherein hybridization of the polynucleotide to the
nucleic acid sequence of interest is light sensitive.

6. The complex of any one of claims 1-5, wherein the linker element can be
cleaved by exposure to light.

30

7. The complex of any one of claims 1-6, wherein the linker element is
attached at one end to the transposome element and at the other end to the targeting
element via covalent bonding to the targeting element.

8. The complex of any one of claims 1-6, wherein the linker element is attached to the targeting element via non-covalent binding in one of the following ways:

a) hybridization of an oligonucleotide that is part of the linker element to an oligonucleotide disposed on the surface of the targeting element;

5 b) hydrophobic interaction between a hydrophobic binding pair, one member of the binding pair being attached to the linker element and another to the targeting element;

c) charge interaction between a binding pair, wherein one charged member of the binding pair is provided on the linker element and another, oppositely charged member, is provided on the targeting element;

10 d) interaction of binding pair members comprising specific tags selected from the group consisting of: reduced glutathione-glutathione S transferase, biotin-streptavidin or biotin-avidin, and Si tag-silica particles.

9. The complex of any one of claims 1-8, wherein attachment of the linker
15 element to the targeting element is contingent upon a photo switch.

10. The complex of any one of claims 1-8, wherein attachment of the linker element to the transposome element is achieved through electrostatic interactions.

20 11. The complex of any one of claims 1-10, wherein the sequence of interest is a gene that encodes a therapeutic protein.

12. The complex of any one of claims 1-10, wherein the sequence of interest contains a stop codon which, when inserted into a host cell target DNA, disrupts
25 expression of a target gene.

13. The complex of any one of claims 1-11, wherein the sequence of interest is a gene that encodes one or more proteins conferring a beneficial phenotype to a cell into which the sequence is inserted.

30 14. The complex of claim 13, wherein the phenotype is: resistance or immunity to a disease or disorder in an animal or human, correction of a deleterious genetically-based disease or disorder in an animal or human, up-regulation or down-

regulation of a metabolic pathway in an animal, human, or plant, or production of a pharmaceutically active substance in a plant.

15 15. A method of inserting a nucleic acid sequence of interest into the genome of a target cell, said method comprising contacting the cell with the complex of claim 1 under conditions that permit uptake of the complex by the cell, intracellular transport of the complex to a pre-selected DNA-containing organelle, and insertion of the sequence of interest by the transposase of the transposome.

10 16. The method of claim 15, which is a therapeutic method of treating a subject suffering from a disease or disorder.

 17. The method of claim 15, which is a prophylactic method of vaccinating a subject at risk of developing a disease or disorder.

15 18. The method of claim 15, wherein the method is a method of generating a pluripotent cell for regenerative medicine.

 19. The method of claim 15, wherein the target cell is a stem cell.

20 20. The method of claim 19 further comprising growing the stem cell in culture conditions that direct the stem cell to differentiate towards a desired somatic cell lineage and generate one or more differentiated cells.

25 21. The method of claim 20 further comprising subsequently transferring said differentiated cells into desired tissues of a subject.

Fig. 1

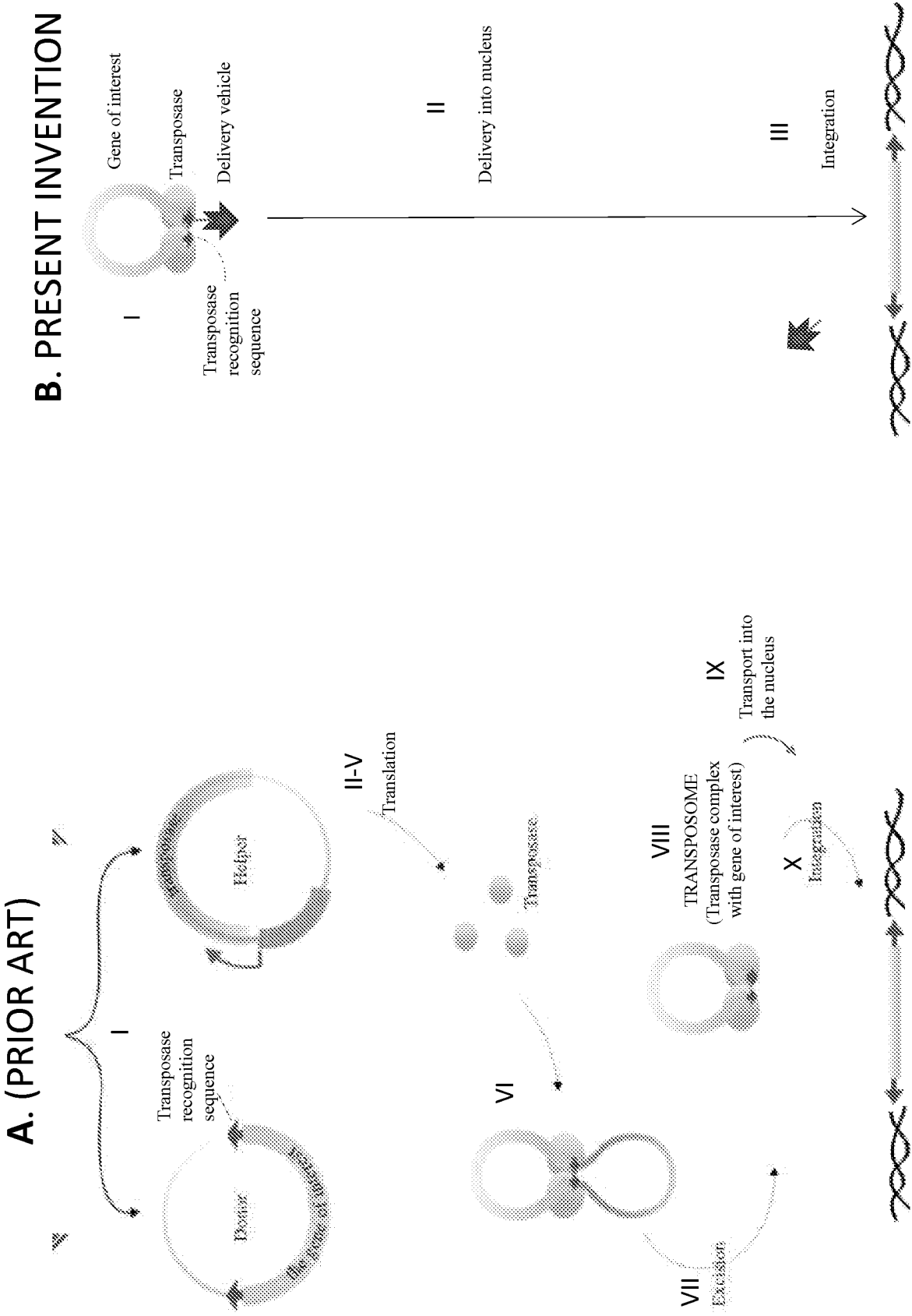
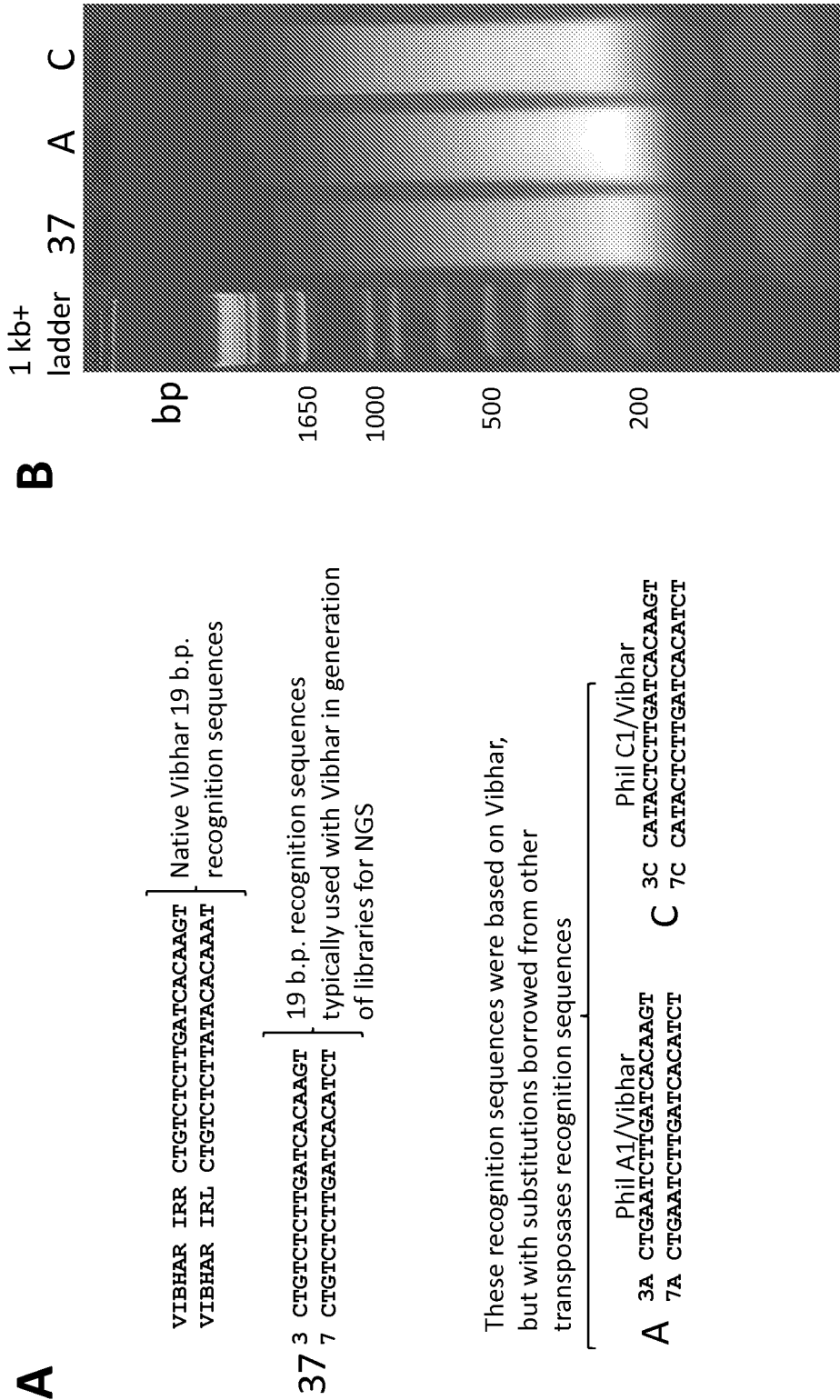


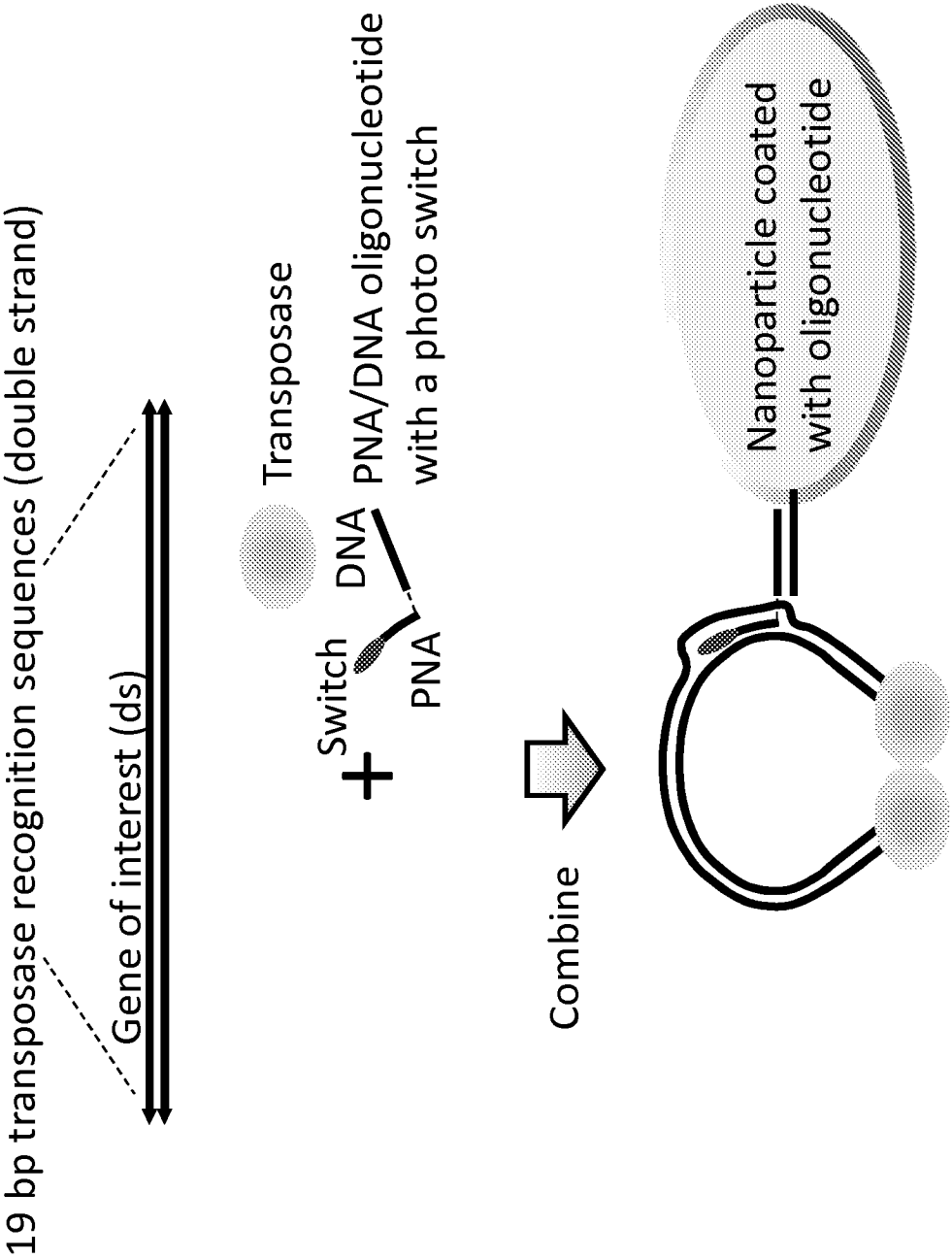
Fig. 2

RECOGNITION OF DIFFERENT 19 B.P. SEQUENCES* BY VIBHAR



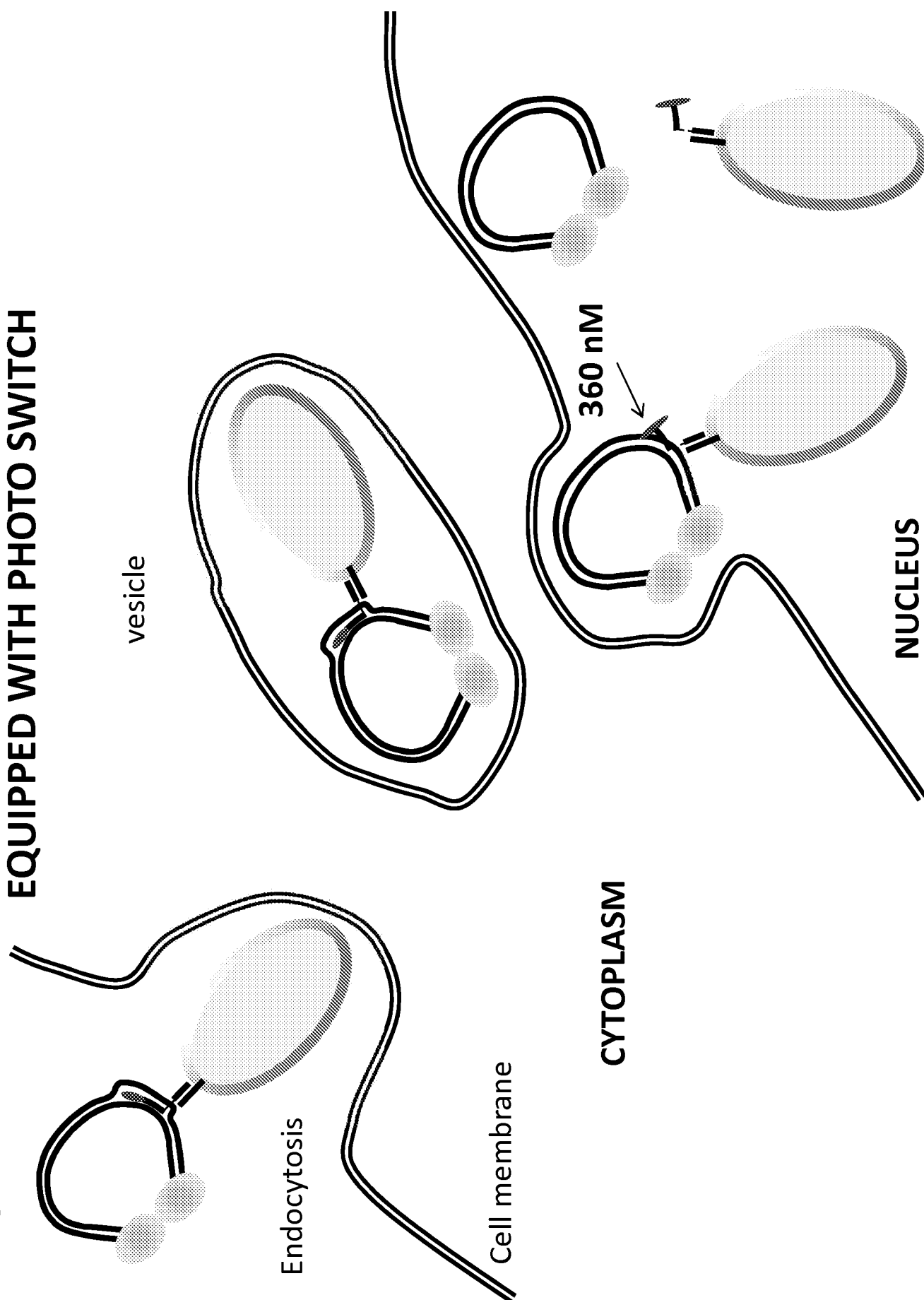
DS TRANSPOSOME TETHERED TO NANOPARTICLE

Fig. 3A



NANOPARTICLE DELIVERY INTO NUCLEUS EQUIPPED WITH PHOTO SWITCH

Fig. 3B



CLEAVABLE LINKER

Fig. 3C

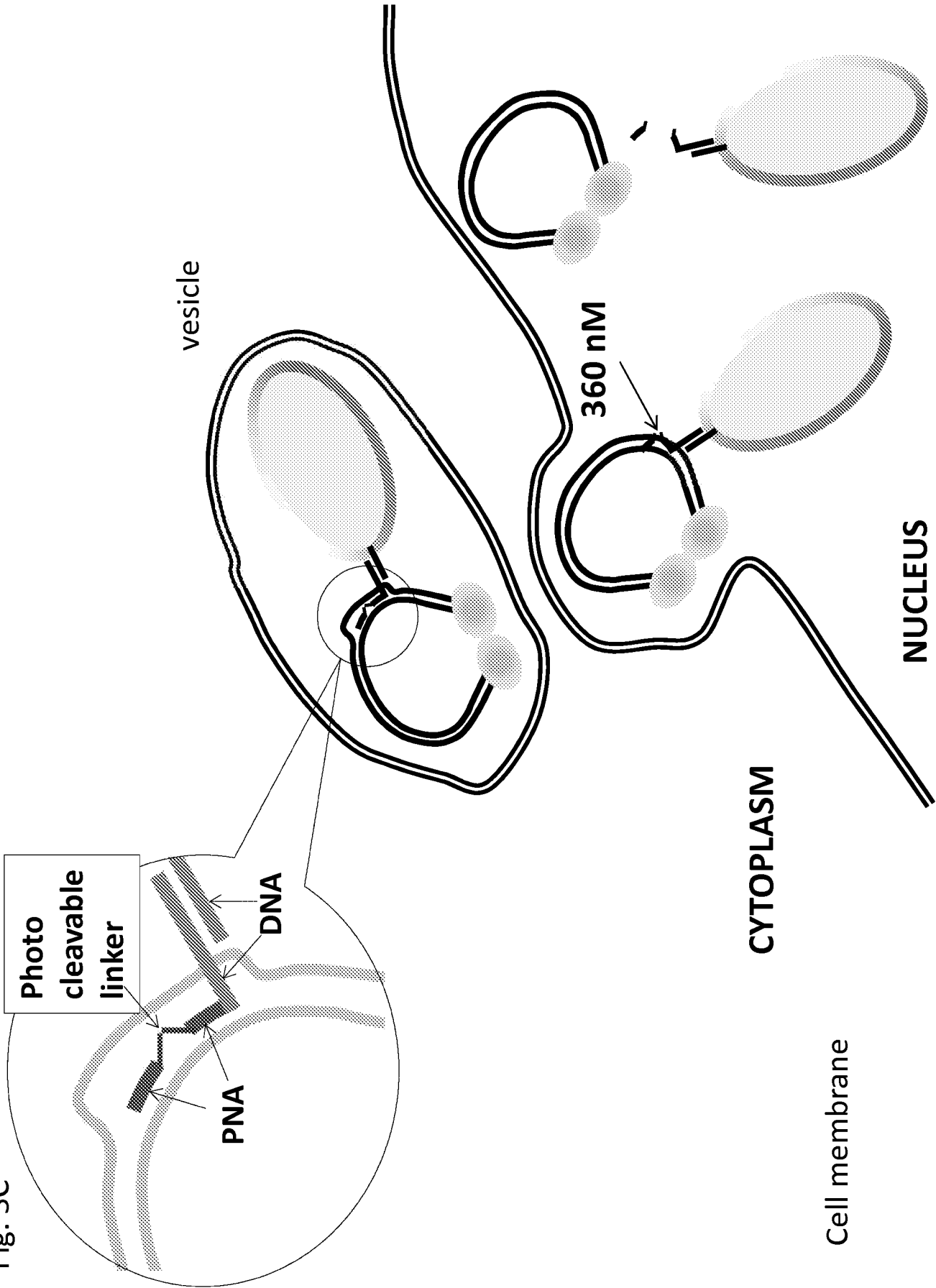


Fig. 3D

IN THE NUCLEUS

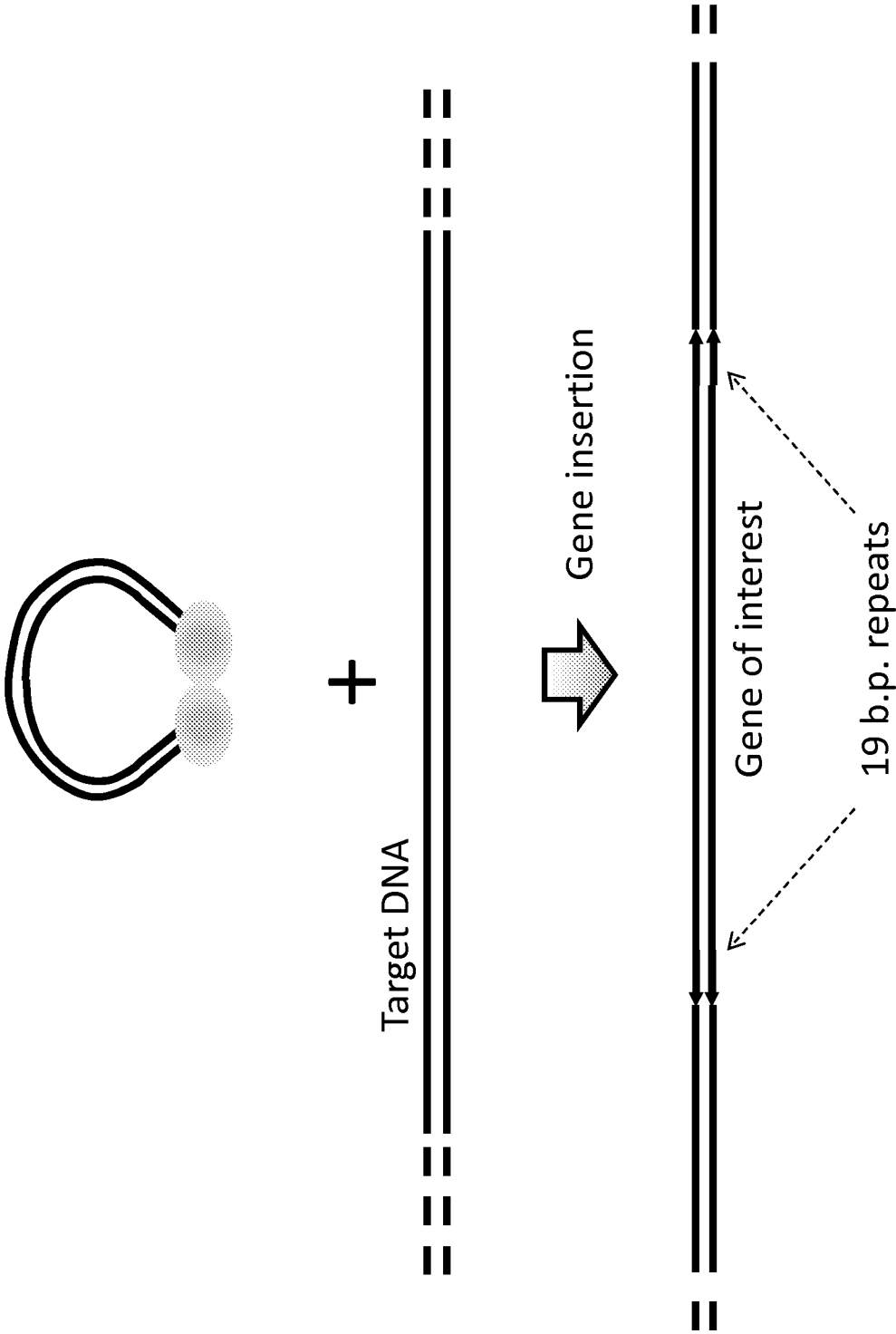
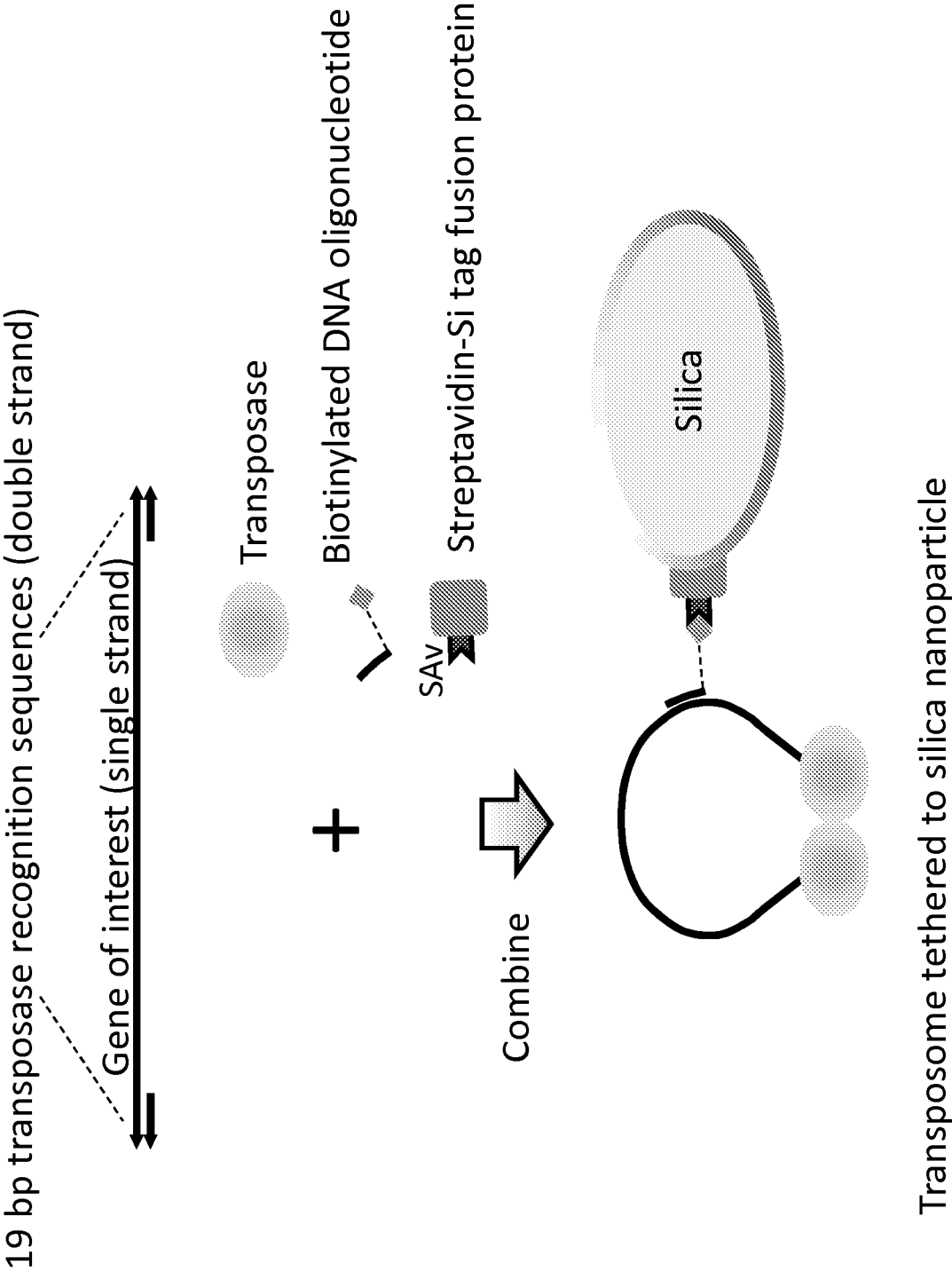


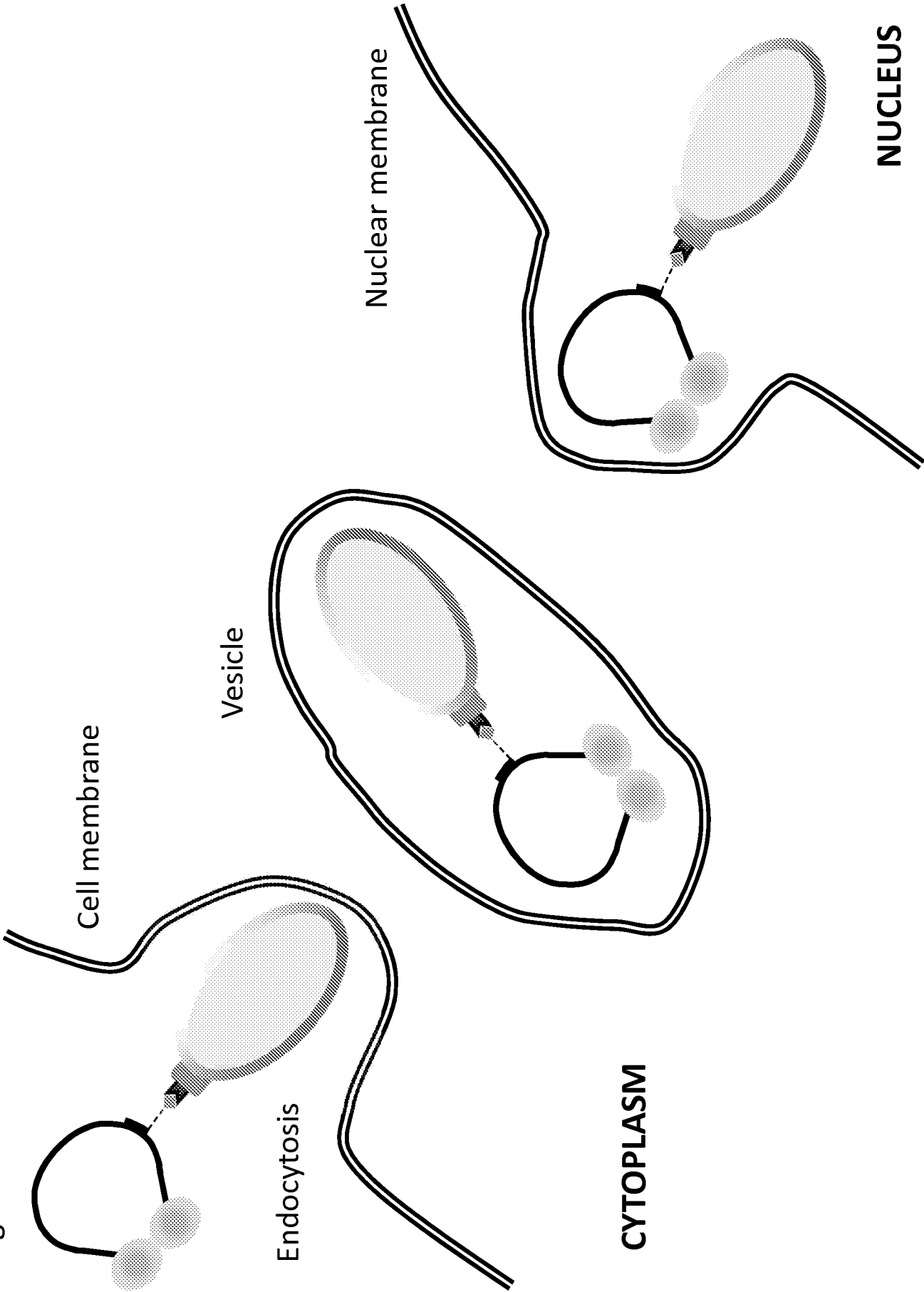
Fig. 4A



SINGLE-STRANDED TRANSPOSOME TETHERED TO NANOPARTICLE

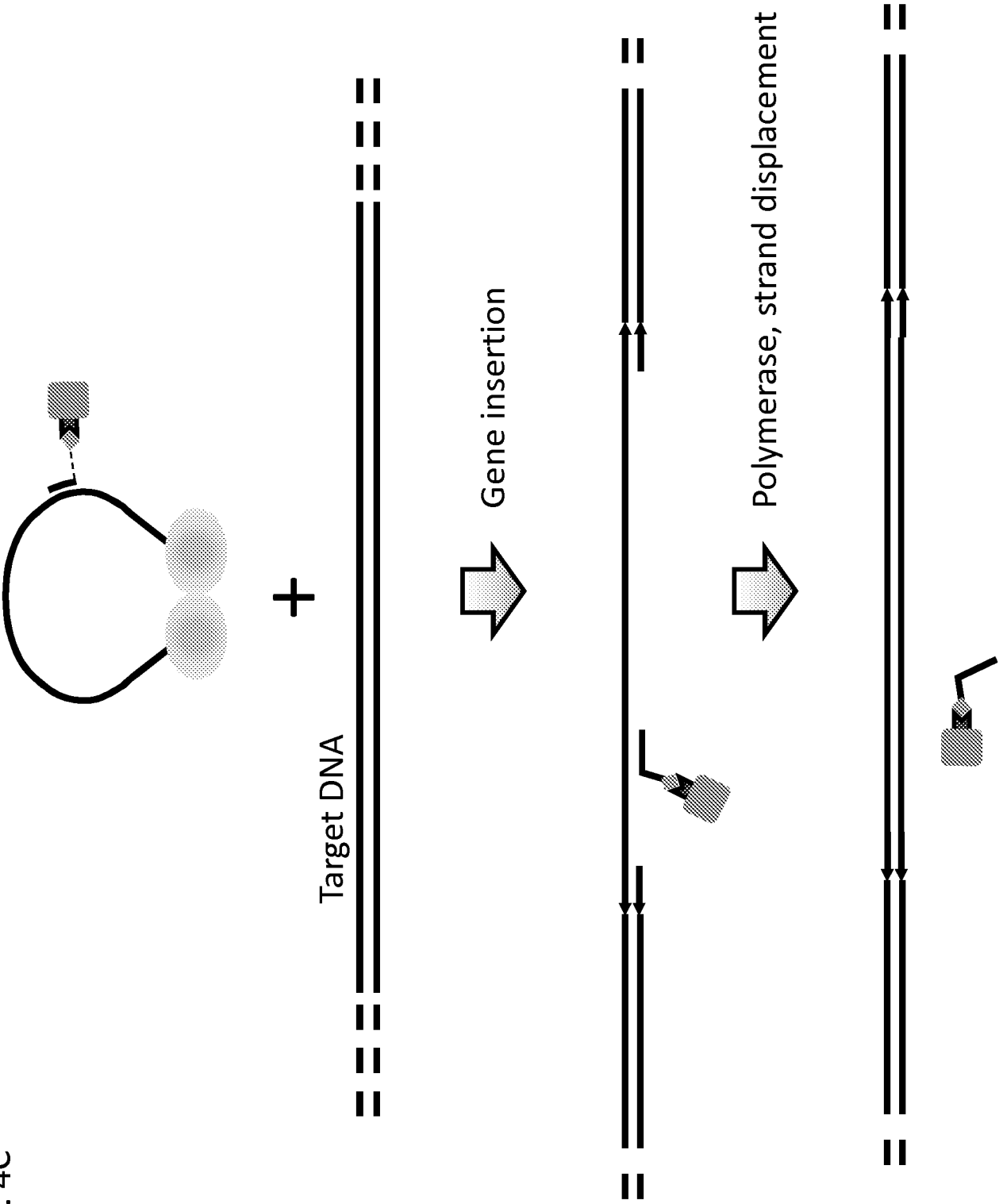
NANOPARTICLE DELIVERY INTO NUCLEUS

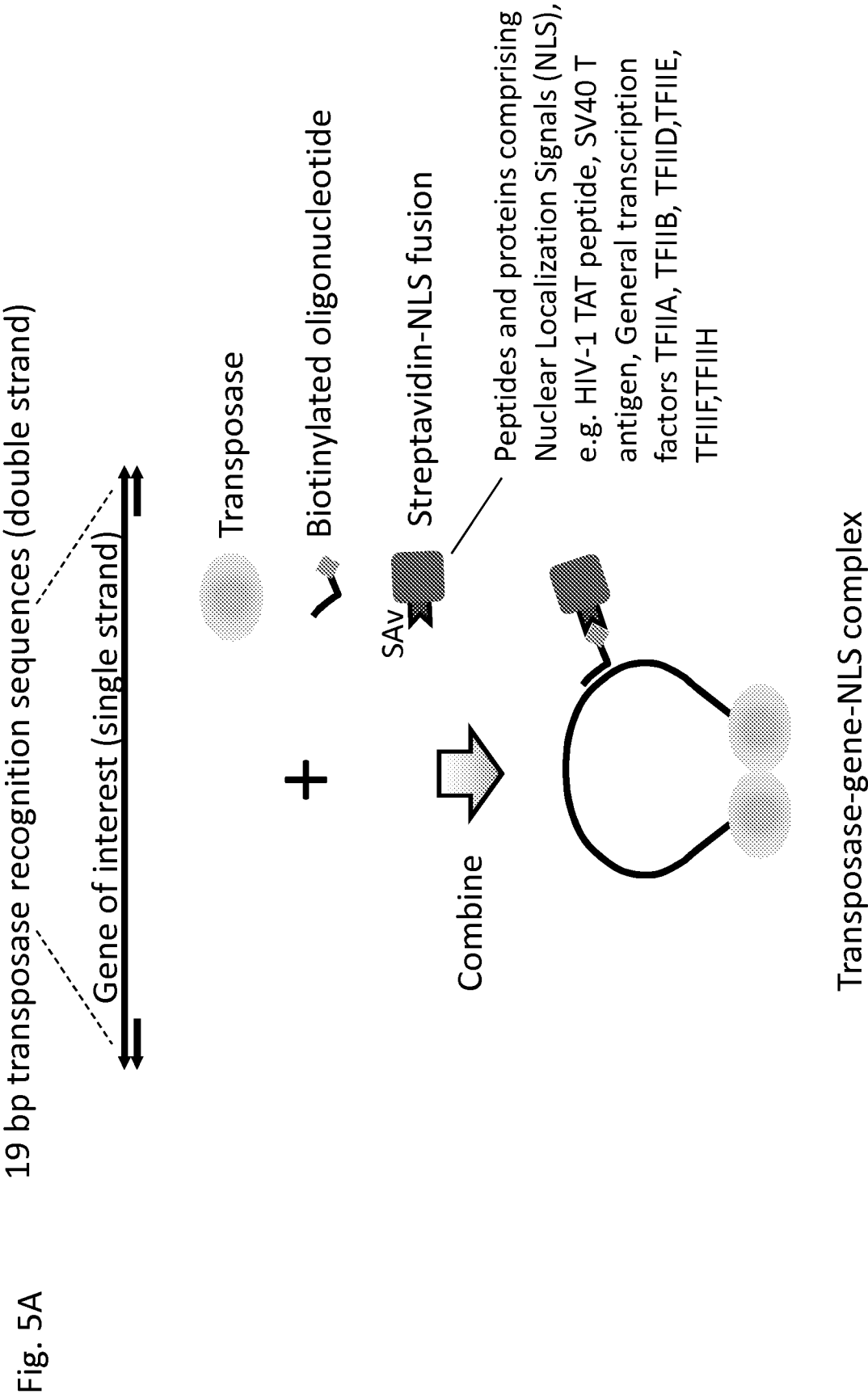
Fig. 4B



IN THE NUCLEUS

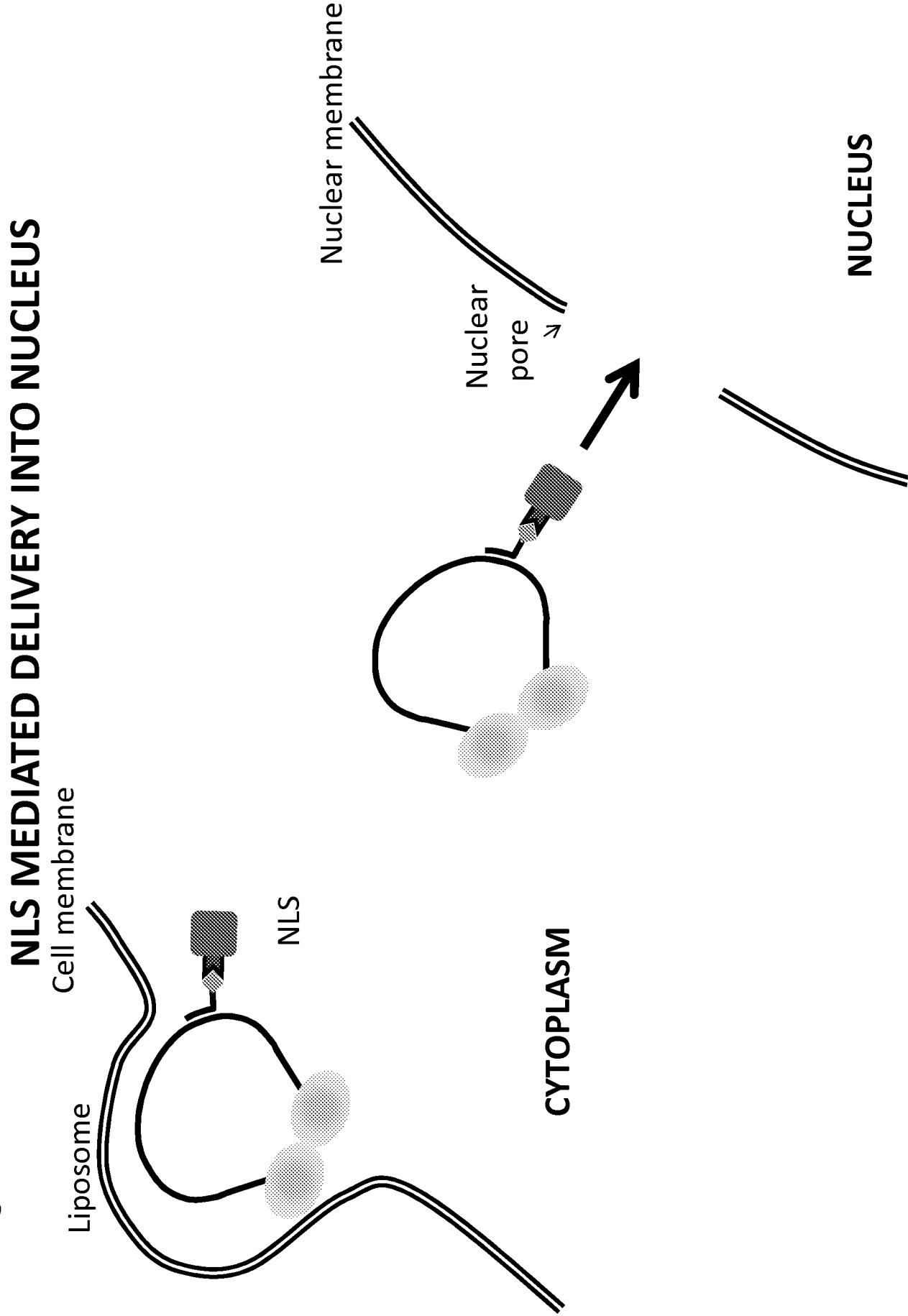
Fig. 4C





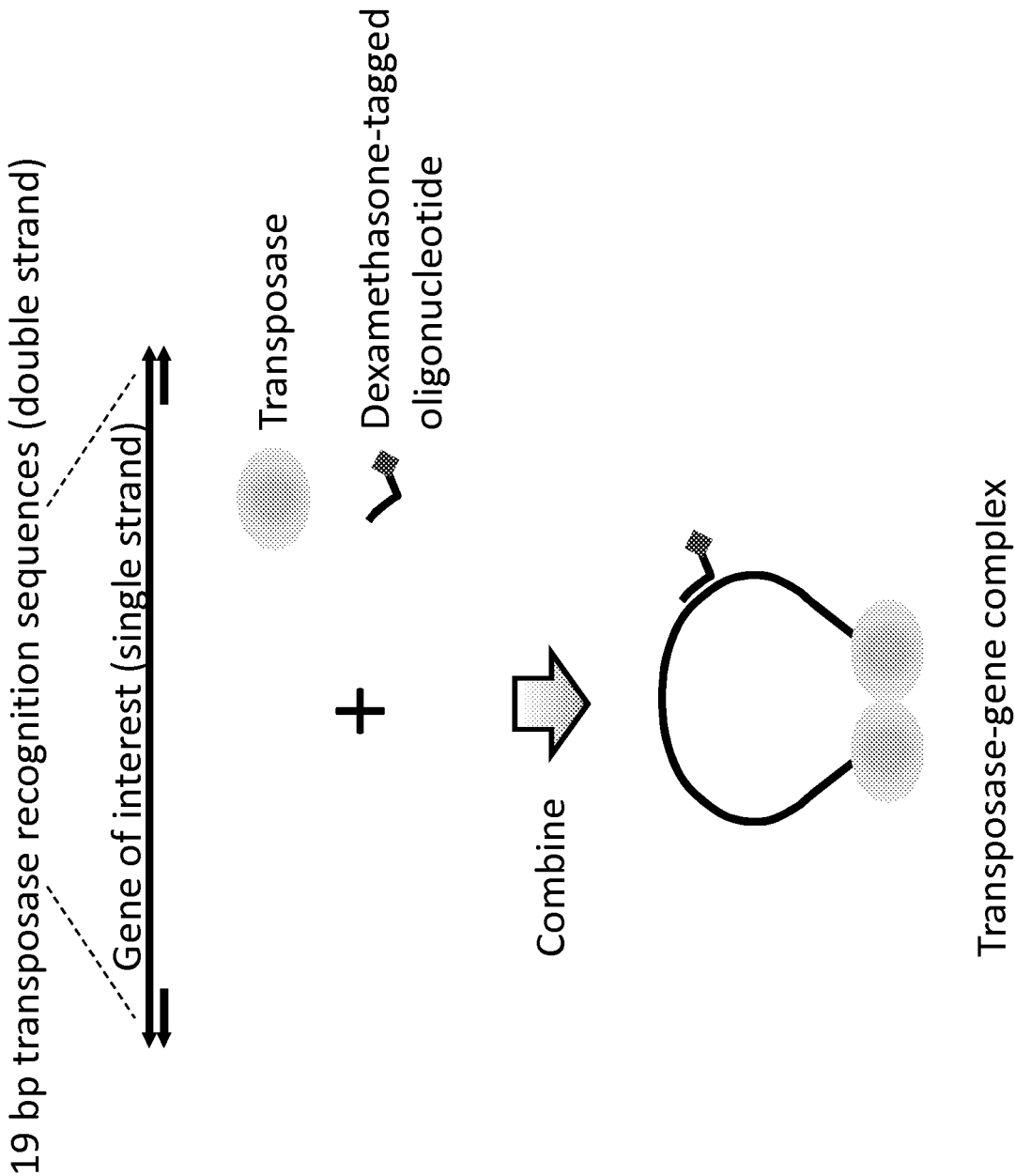
TRANSPOSOME TETHERED TO NUCLEAR LOCALIZATION SIGNAL (NLS)

Fig. 5B



TRANSPOSOME TETHERED TO A LIGAND

Fig. 6A



DELIVERY INTO HOST CELL NUCLEUS

Fig. 6B

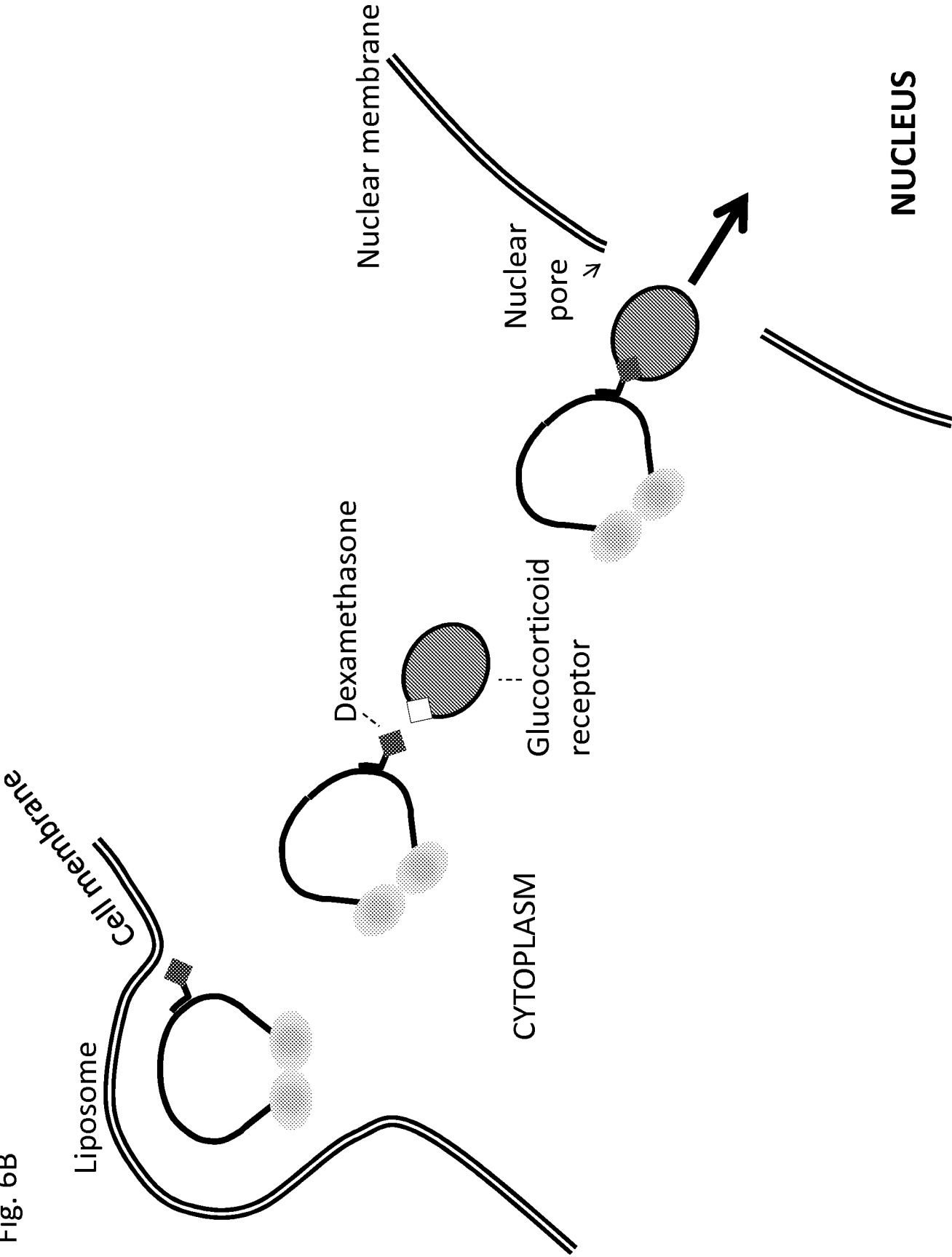


Fig. 7

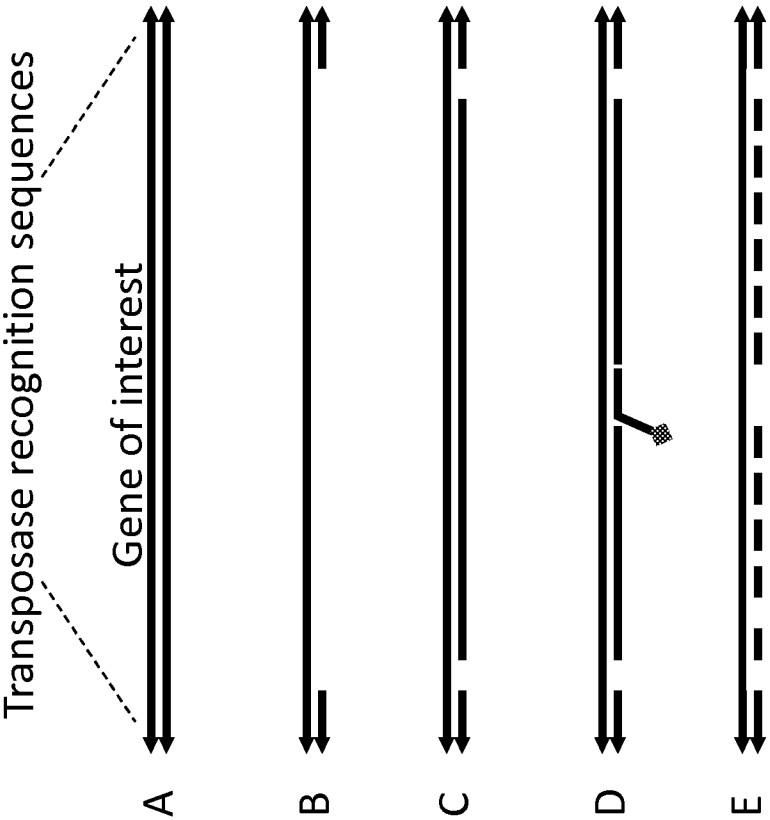


Fig. 8 TRSANSPOSOME TETHERED TO NLS AND CPP

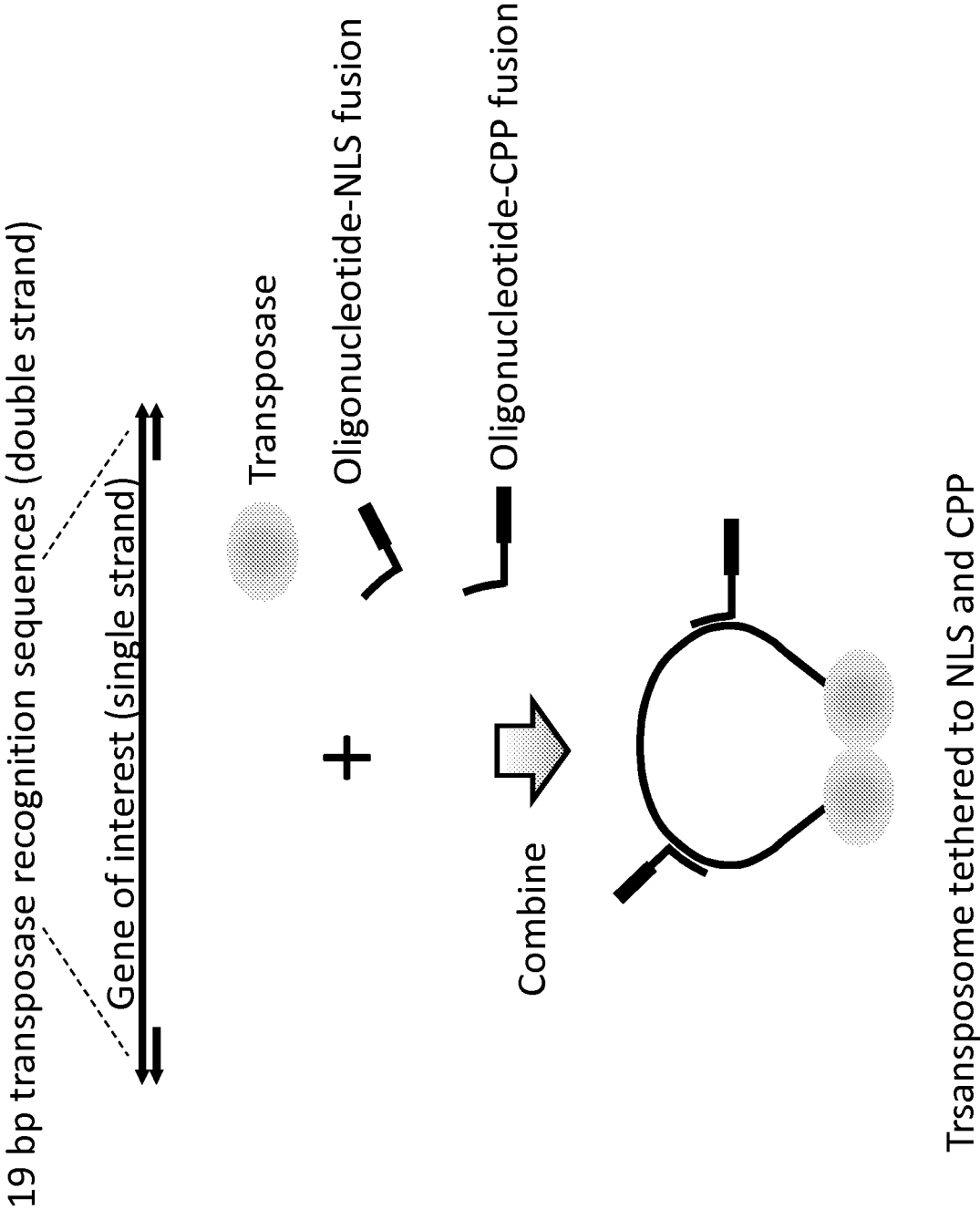


Fig. 9 AMPHIPATHIC PEPTIDE-BASED NANOPARTICLES

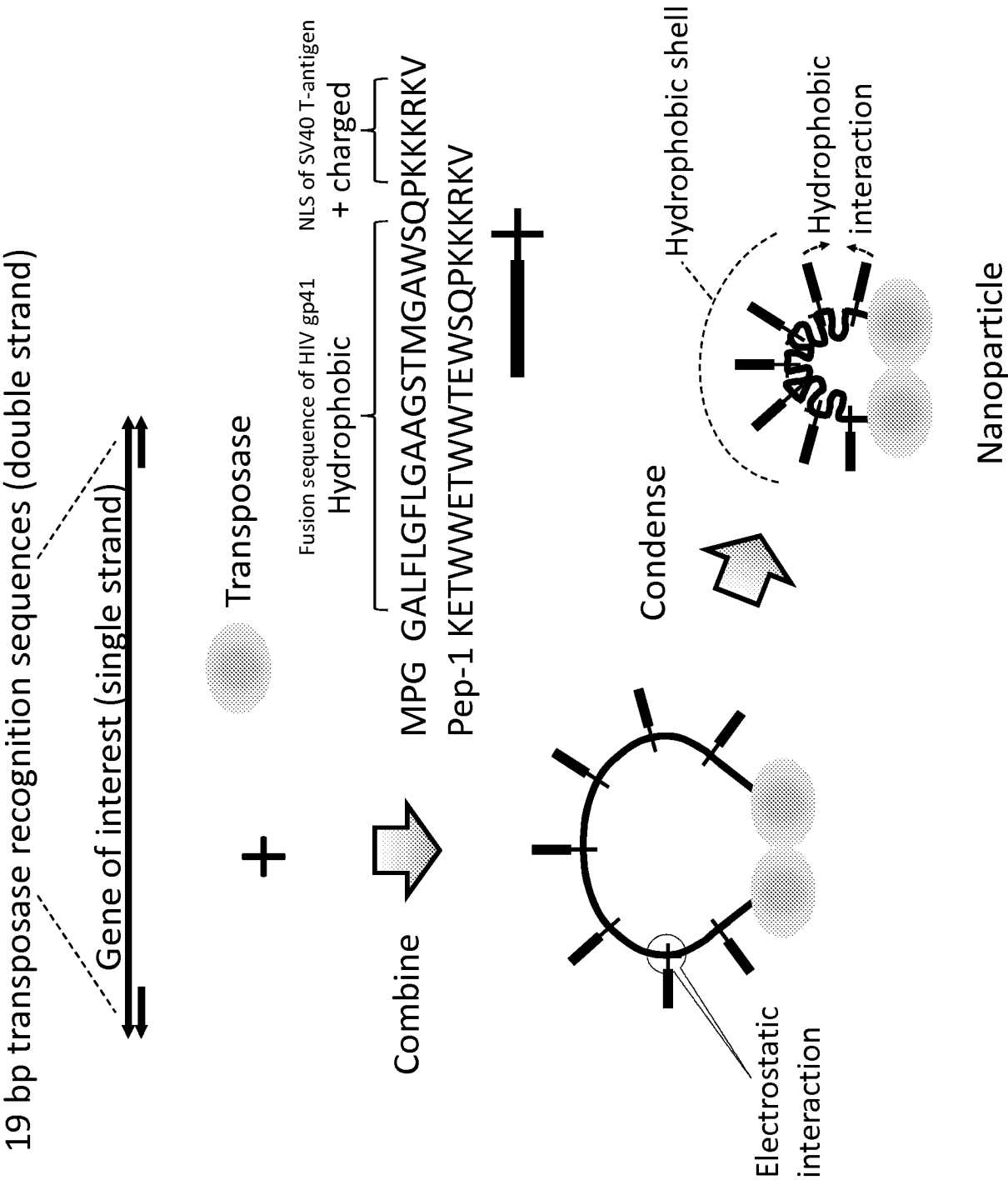


Fig. 10 PREFORM TRANSPOSASE GENE VEHICLE COMPLEX

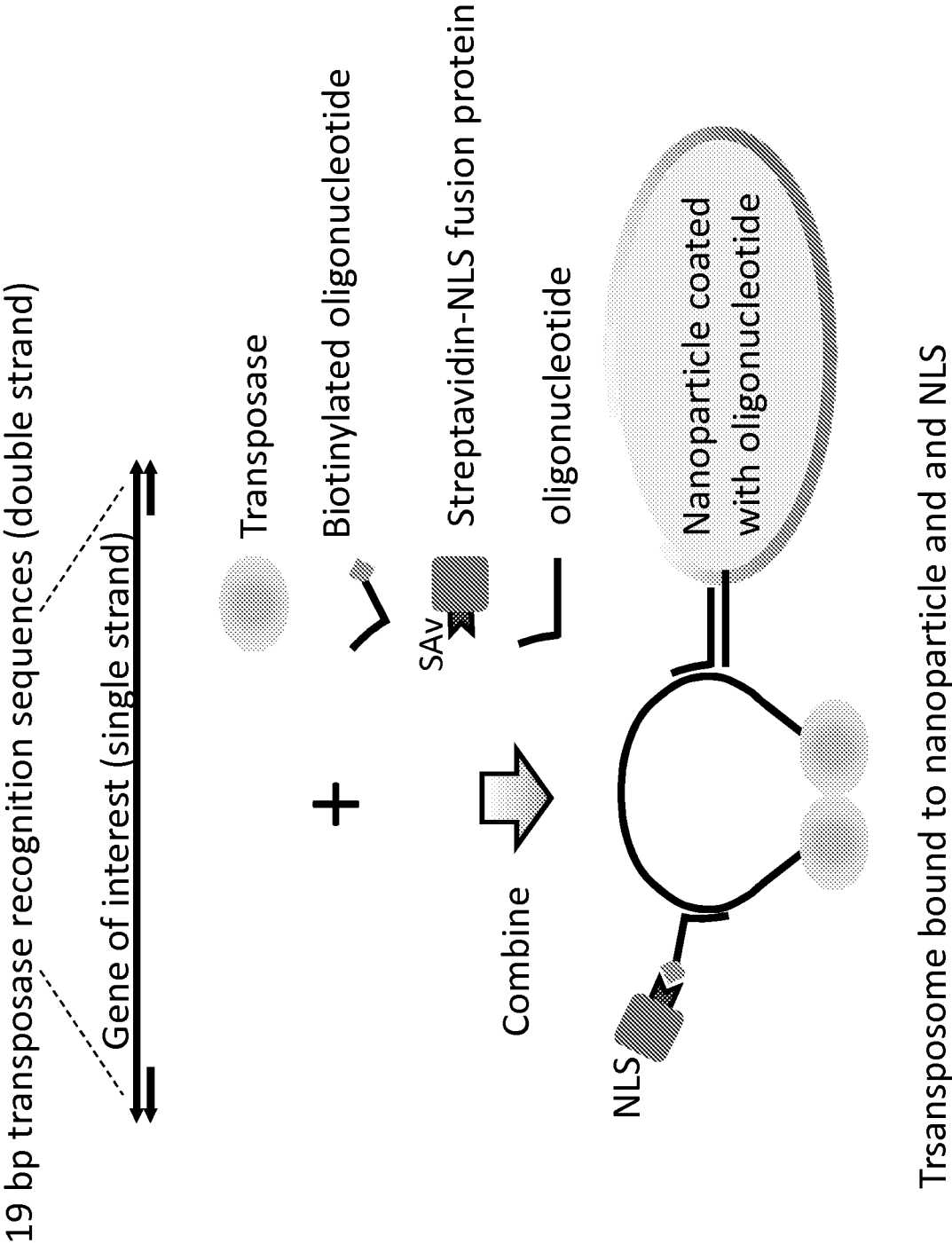
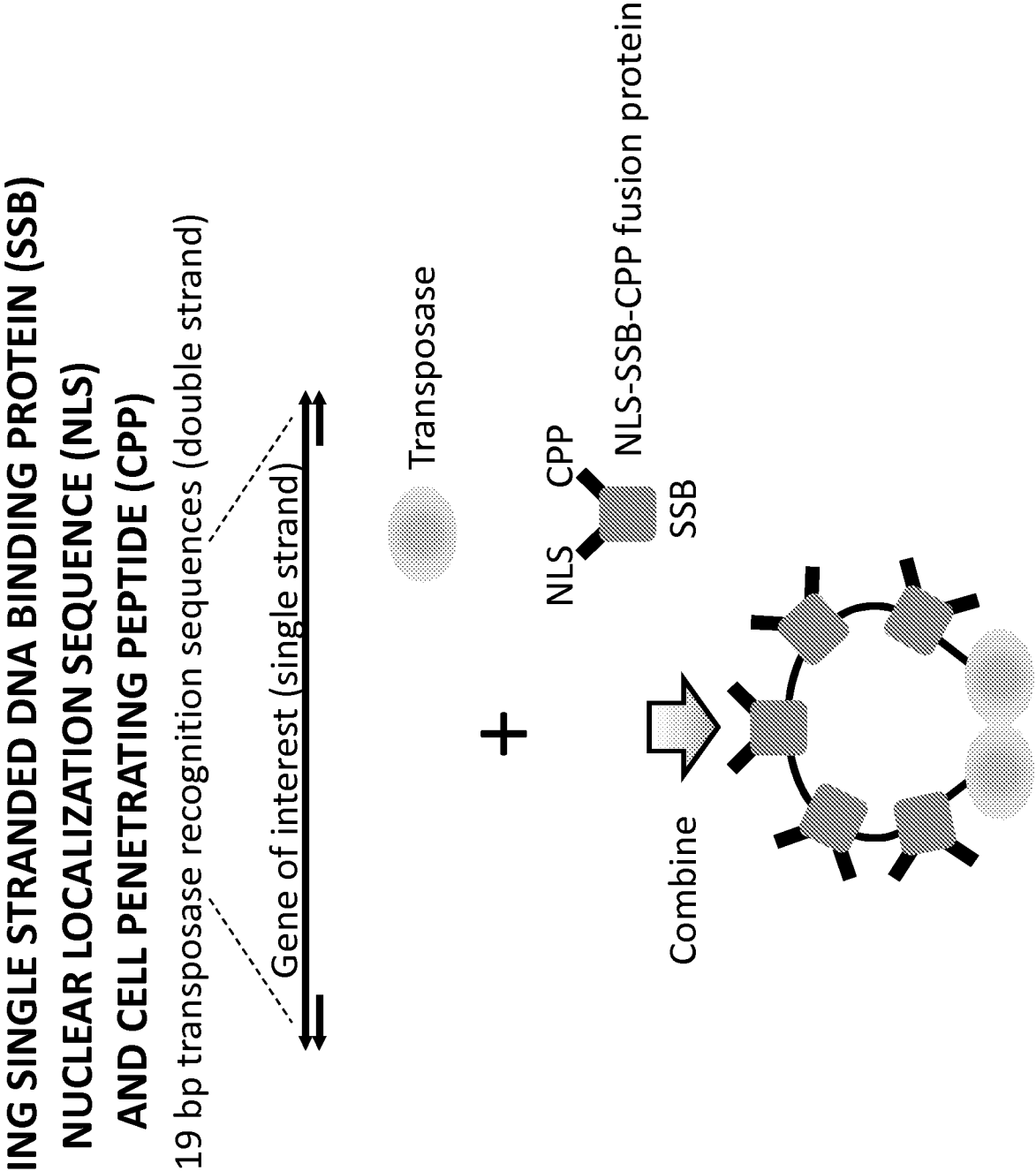


Fig. 11



INTERNATIONAL SEARCH REPORT

International application No
PCT/US2014/010858

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/90 C12N9/22
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, COMPENDEX, EMBASE, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2004/069994 A2 (MAX DELBRUECK CENTRUM [DE]; IVICS ZOLTAN [DE]; IZSVAK ZSUZSANNA [DE]) 19 August 2004 (2004-08-19)	1-17
Y	the whole document, in particular p.10, last paragr.; paragr. bridging p.13/14; p.14, 5th paragr.; p.17, last paragr.; claims; Fig. 1	18-21
X	WO 2004/069995 A2 (MAX DELBRUECK CENTRUM [DE]; IVICS ZOLTAN [DE]; IZSVAK ZSUZSANNA [DE]) 19 August 2004 (2004-08-19)	1-17
Y	the whole document, in particular p.11, last paragr.; paragr. bridging p.12/13; p.14, 2nd paragr.; p.18, 4th paragr.; claims; Fig. 1	18-21
	----- -/-	



Further documents are listed in the continuation of Box C.



See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search

31 March 2014

Date of mailing of the international search report

04/04/2014

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Authorized officer

Bassias, Ioannis

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2014/010858

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	the whole document, in particular p.11, last paragr.; paragr. bridging p.12/13; p.14, 2nd paragr.; p.15, 2nd paragr.; p.18, 3rd paragr.; claims; Fig. 1	18-21
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