

TRANSPPOSITION OF MAIZE AC/DS ELEMENTS IN VERTEBRATES

BACKGROUND OF THE INVENTION

[0001] The present invention is directed to the use of the maize *Ac/Ds* transposable elements in vertebrates, including fish, birds, and other animals including mammals and humans.

[0002] The publications and other materials used herein to illuminate the background of the invention, and in particular, cases to provide additional details respecting the practice, are incorporated by reference, and for convenience are referenced in the following text by author and date and are listed alphabetically by author in the appended bibliography.

[0003] Transgenic animals, including fish, provide an excellent vertebrate model for studying many facets of development, physiology and disease. A wide variety of fish may be utilized for this purpose. Exemplary fish include teleost fish, such as zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), mummichog (*Fundulus heteroclitus*), killifish (Genus *Fundulus*), catfish (Genus *Ictalurus*), such as channel catfish; carp (Genus *Cyprinus*), such as common carp; and trout or salmon (e.g., Genus *Salvelinus*, *Salmo*, and *Oncorhynchus*). Zebrafish have become an established model for investigating many facets of development, physiology and disease.

[0004] Zebrafish are particularly useful for studying many facets of development, physiology and disease. They are small, develop *ex utero*, and have a short generation time. At 5 days of age each fish is a free swimming/feeding organism complete with most of the organ systems employed by mammals, such as heart, brain, blood, and pancreas. Within the last 10 years, mutant zebrafish lines isolated from large-scale mutagenesis screens have led to a greater understanding of vertebrate development (Driever et al., 1996; Haffter et al., 1996; Gollig et al., 2002). Although these studies have shown that zebrafish mutants can serve as good models for human diseases, zebrafish have not been widely used in this capacity. So far the biggest limitation in zebrafish research has been determining the identity of causative genes disrupted from these mutagenesis screens, as the vast majority of mutants have been created using the chemical mutagen *N*-Ethyl-*N*-nitrosourea (ENU). Identification of ENU-derived point mutations requires laborious and slow positional cloning efforts. Insertional mutagenesis using retrovirus, on the other hand, is effective, and a gene mutated in this way can take as little as 2 weeks to identify with minimal resources (Gollig et al., 2002). By creating a bank of retroviral insertions in zebrafish sperm, a library can be created where at least one insert into roughly every gene is housed in a small freezer space. Characterization and determination of the genomic location of all the inserts will make it possible to easily perform reverse genetics in the zebrafish by using the sperm sample with the mutation of interest to fertilize eggs *in vitro*. In

this way, researchers could save a great deal of time and money by ordering mutations of interest instead of random screening. An even more powerful use of the library would lie in forward genetic screens.

[0005] To date, the number of cloned ENU mutants in zebrafish remains extraordinarily low considering the number of labs currently working on the hundreds of mutant lines. About 100 genes mutated with ENU have been published since the completion of two large-scale ENU mutagenesis screens in 1996. In fact, many of these genes were not identified using strictly positional cloning efforts. Rather, they were found by recognizing that certain mutant phenotypes were similar to known *Drosophila* or mouse mutants whose genes and pathways had already been decoded (Talbot and Hopkins, 2000). In these instances a "candidate gene" approach was taken whereby each gene in the presumed developmental pathway was examined individually for mutations to correctly isolate the disruption. While this method has proven extremely successful in cloning mutant genes, it has not led to the discovery of novel genes/pathways and has mainly recapitulated that which is known about development from other organisms.

[0006] A more successful approach to cloning mutant genes in zebrafish has been to use pseudo-typed mouse retroviruses as the mutagen. These retroviruses have the vesicular stomatitis virus G coat protein that allows infection of a broad range of host cells including zebrafish (Yee et al., 1994; Emi et al., 1991; Burns et al., 1993). The viral DNA inserts into the genome as a single-copy entity in a mostly random fashion without altering its junctional sequence, although retroviruses have been seen to prefer 5' ends of genes as their insertion site (Vijaya et al., 1986; Rohdewohld et al., 1987; Mooslehner et al., 1990; Scherdin et al., 1990). Further, the retroviral insert serves as a molecular beacon, making it a rather simple process to link a mutant phenotype to a disrupted gene. One drawback is that the virus needs to be injected into zebrafish embryos at the 1000- to 2000-cell stage when the germ cells are still dividing (a necessary event for retrovirus integration). This requires more work up front than the traditional ENU mutagenesis method to create mutagenized founder fish. Since there are, on average, fewer retroviral insertions than ENU lesions per gamete, the mutagenic frequency of the retrovirus is less than that of chemical mutagens (1/1 ENU mutagenized F2 families produce a visible recessive mutation compared to 1/7 retroviral F2 families (Golling et al., 2002).

[0007] Recently, the retroviral method of mutagenesis has been used to conduct a large-scale developmental screen. That screen generated more than 500 mutants affecting zebrafish development; more than half of these disrupted genes were cloned (Golling et al., 2002;

Amsternam et al., 1999). Results of this work have shown that all of the identified disrupted genes have homologues in human, but approximately 20% of these disrupted genes do not contain any obvious motifs or features that would allow one to classify the biochemical function of the resultant protein (Golling et al., 2002). In contrast, the genes so far cloned from ENU mutants show little degree of novelty.

[0008] One approach which has shown a great deal of promise for reverse genetics in zebrafish is the generation of gene knock-downs using morpholino based oligonucleotides (Heasman, 2002; Nasevicius and Ekker, 2000; Ekker, 2000). This technique relies on generating a short (24mer) morpholino oligonucleotide that is complimentary to the translation start site of the gene of interest (Summerton et al., 1997, Summerton and Weller, 1997). Injection of a morpholino oligonucleotide at the 1-2 cell stage inhibits translation of the endogenous target gene's mRNA. In this way many ENU derived mutant phenotypes have been phenocopied by injecting morpholino oligonucleotides specific to the mutated gene, establishing the proof in principle of the technique (Heasman, 2002; Nasevicius and Ekker, 2000; Ekker, 2000). These oligonucleotides have also been successfully used to examine unknown gene function of genes identified from *in situ* hybridization screens (Sakaguchi et al., 2001; Tsang et al., 2002).

[0009] While morpholino antisense technology is widely used in the zebrafish community it has some severe limitations. For instance, the window of opportunity for studying a knock-down of a favorite gene is only 2-3 days, thus limiting the technique primarily to the study of early development. Since the morpholino is not a stable, heritable element the amount of morpholino oligonucleotides in each cell is diminished by degradation and dilution with every round of division. Hence, if the desired gene is not expressed within this time period, which may presumably be the case with many disease genes, then this method will not work. The ability to perform suppressor or enhancer modifier screens would also be limited with morpholino oligonucleotides as each embryo in the screen would need to be injected with the oligonucleotide, a very time consuming effort.

[0010] Recent advances in producing high-titer retrovirus have greatly simplified its use in zebrafish. Some of the first retroviral constructs used for insertional mutagenesis in zebrafish generated injected (founder) fish that transmitted proviral integrations to only 5% of their F1 progeny (Lin et al., 1994). The number of inserts carried by these F1 fish was also low, usually one insert per gamete and fewer than 5 total insertions per germ-line (Lin et al., 1994). To do any meaningful mutagenesis screens with these constructs one would have to inject zebrafish

embryos for a period of many years to generate 500,000 inserts. As seen in Chen et al. (2002), new retroviral vectors have significantly reduced this time frame. Now two people injecting retrovirus could make enough founder fish to harbor a half-million inserts in two months time (Chen et al., 2002). Furthermore, the efficiency of the retroviral system now allows more than 25 different insertions on average for each founder fish. These fish can easily be raised, tagged for individual identification system with a novel marking system, and stored in approximately 200 fish tanks, which is a small sized aquatic facility for a researcher.

[0011] Insertional mutagenesis is now the quickest method for cloning mutated genes in the zebrafish. Not only does the retroviral insert serve as a molecular tag for the disrupted gene, it also serves as a valuable marker to establish genetic linkage with the mutant phenotype. Chemical mutagenesis methods must rely on establishing tight linkage of the disrupted gene to a marker, thereby narrowing the genomic region of interest to a size small enough that it can be managed by sequencing of a BAC or PAC clone. The complication with chemical mutagenesis derives from the fact that there are hundreds of markers to test and they must be tested on a significant number (a few thousand) of recombinant fish to show that the linked marker does not segregate from the mutant locus. In screening for embryonic mutations, generating a few thousand mutant embryos is not usually a problem. However, if one were to look for adult phenotypes, such as those particular to certain diseases (diabetes, Parkinson's, obesity, etc.), it would take an inordinate amount of time, space, and resources to raise all of the required recombinants to find a linked locus. With insertional mutagenesis, linkage is established simply by running a Southern blot of the restriction enzyme digested DNA from the mutant fish and the pairs of adult fish that generated the mutants. Using a labeled portion of the retrovirus as a probe, one would expect all of the mutant fish and their parents to have one band migrating at the same molecular size, while pairs of fish that did not produce phenotypic clutches would not have the same insert (i.e., both fish would not have the linked band). In this way one would only have to look at a handful of affected fish to generate a probable lead on the disrupted gene.

[0012] The most time consuming aspect of cloning genes disrupted by insertional mutagenesis has been in cases where the genomic DNA flanking the retroviral insert does not contain exonic or known gene sequences. Usually when the candidate linked insertion is identified and its flanking sequence is cloned, it is no longer than a few kilobases in length due to the ineffectiveness of PCR to amplify larger fragments. In previous work, it was found that about one-third of the flanking DNA cloned in this way did not have useful information in that their sequences did not reveal any homology or identity in database searches (G. Golling,

unpublished result). This will no longer be a problem as the first draft of the zebrafish genome will be completed soon and it should be possible to identify which gene is disrupted by sequencing less than 50 bases of DNA flanking the retroviral insert.

[0013] Reverse genetic approaches in mouse have provided many insights into human diseases. Researchers have been able to take a gene of interest and disrupt its expression by homologous recombination in ES cells, then re-introduce those cells back into an embryo to create targeted transgenic knock-outs. While this approach has not yet proven successful in zebrafish or other vertebrate model organisms other than the mouse and rat, another method, called target-selected mutagenesis, has been developed to create targeted disruptions in specific genes of interest. Target-selected mutagenesis is accomplished by first mutagenizing germ-line DNA of an organism and then using PCR to amplify the gene of interest. Sequencing the PCR product for comparison to the wild type gene then identifies samples containing mutations in the gene of interest. Wienholds et al. have recently used this technique in zebrafish to isolate mutations in the *Rag1* gene. By sequencing two exons of *Rag1* from a sperm library consisting of nearly 2700 randomly ENU mutagenized males, the researchers found 15 mutations, one of which was a premature stop codon (Wienholds et al., 2002). While the methodology does in fact work, it may be cumbersome for large-scale screening. To identify the single stop mutation approximately 12,500 sequencing reactions were carried out over a period of two months (Wienholds et al., 2002). Extrapolating this method to the entire zebrafish genome (~40,000 genes) would take approximately 500 million sequencing reactions. A more efficient method would be to create a mutant sperm library from retroviral insertions. Since the retrovirus can serve as a tag for the disrupted locus many fewer sequencing reactions would be needed. The actual number of bases to be sequenced would also be smaller given the imminent completion of the zebrafish genome. Over a period of 4-5 months, three injectors could routinely generate the approximately 40,000 male founders necessary to harbor more than 1 million inserts. This would give an insert density on average of 1 every 1800bp, essentially at least one insertion for every gene. Space requirements for housing the frozen library would be smaller than what is needed for most cDNA or genomic library arrays.

[0014] Forward genetics has been an invaluable approach in many model organisms; however, almost all of the forward and reverse genetics taking place in zebrafish are in the form of loss-of-function alleles. Forward genetic approaches based on the proposed retroviral zebrafish library would be enhanced with the addition of functional genetic elements. Retroviruses can provide additional functions aside from the obvious loss-of-function gene

disruptions. For instance, in one large scale screen the predominant retroviral vector used had a gene-trapping cassette (Golling et al., 2002; Amsterdam et al., 1999). Of the founders injected with this construct, Chen et al. (2002) found there was at least one trapping event in the germ-line of each fish. While the trap vector itself did not prove to be more mutagenic than the previous non-trapping vectors (Chen et al., 2002), it did show the possibilities for other creative screens in the zebrafish that have previously been performed in *Drosophila*. Among these are enhancer trap constructs where fish could be screened for particular gene expression patterns, and promoter-containing vectors for mis-expression/over-expression screens. This latter approach has been used successfully in flies where Gal4 binding sites within a P-element drive expression of genes located downstream of the randomly located insert (Rorth et al., 1998; Hay et al., 1997). Transgenic fly lines expressing Gal4 protein in a controlled manner, for example under the control of an eye specific enhancer or tetracycline operator, would cause the gene downstream of the P-element to be mis-expressed according to the researcher's preference. A function such as this would provide a valuable resource for studying the function of genes that do not display obvious loss-of-function phenotype. It is estimated that such genes constitute more than two-thirds of genes in flies, worm, and yeast (Sulston et al., 1992; Dujon et al., 1994; Miklos and Rubin, 1996). It is likely that an even higher percentage of vertebrate genes have no obvious loss of function phenotype. These genes are often biologically important. For example, although loss of either NPY and/or AGRP function in mice display no detectable abnormalities (Qian et al., 2002; Erickson et al., 1996), NPY and AGRP have been found to play a key role in regulation of food-intake by gain-of-function studies (Levine and Morley, 1984); Clark et al., 1984; Graham et al., 1997; Ollmann et al., 1997). Furthermore, their pathways have been drug discovery targets for obesity and diabetes in several pharmaceutical and biotech companies (Halford, 2001).

[0015] It is estimated that half of the genes identified in the first draft of the human genome have no function ascribed to them (Lander et al., 2001; Venter et al, 2001). The ability to rapidly examine the biological roles of these unknown genes is the goal of many research institutions and pharmaceutical companies. Currently, the best vertebrate model organism for conducting these genetic studies is the mouse. Homologous recombination and random retroviral mutagenesis have made the mouse a viable resource for functional genomics research. However, several drawbacks inherent in the mouse's biology have stymied the development of quick, large-scale approaches towards gene and gene function identification. These include significantly large space requirements, small litter sizes, development *in utero*, high cost of

breeding and maintenance, and vast regulatory/animal handling requirements. In zebrafish nearly the opposite is the case concerning these issues. Zebrafish have the added advantage of being transparent through most of their development, allowing easy visualization of the morphology and function of internal organs by light microscopy using a variety of techniques involving fluorescent, luminescent or colorimetric labeling. Thus, developing zebrafish as a forward genetic model would significantly enhance the understanding of vertebrate gene and protein function.

[0016] Recently an effort has been made by at least two companies to generate a zebrafish sperm library and, in fact, one loss-of-function gene has been published from this library (Nasevicius and Ekker, 2000). Since the library was made from fish mutagenized with ENU, several thousand PCR and sequencing reactions were required to isolate the lone mutant. Using retroviral insertional mutagens would vastly improve the speed with which a zebrafish library could be generated and then used by researchers. Stored as sperm samples and/or as approximately 20,000 or fewer fish, possibly as few as 2,000 fish or fewer, this insertional library occupies little space. The sperm samples can easily be reconstituted into living fish for studies of gene and/or protein function. The fish can easily be reproduced for studies of gene and/or protein function. A large repository of cloned retroviral mutations in the zebrafish would be a valuable resource for the study of the function of a specific gene of interest, for screening the library for phenotypes relevant to disease so as to identify putative drug targets, for screening the library for fish that do not respond to significant drugs, toxins or other chemicals so as to identify the gene and protein that are the site(s) of action of such compounds, and for screening for compounds that might alter the expression or activity of known disease genes, or other genes/proteins of interest.

[0017] DNA transposons are mobile elements that can move from one position in a genome to another. Naturally, transposons play roles in evolution as a result of their movements within and between genomes. Geneticists have used transposons as tools for both gene delivery and insertional mutagenesis or gene tagging in lower animals (Shapiro, 1992) but not, until recently, in vertebrates. Transposons are relatively simple genetic systems, consisting of some genetic sequence bounded by inverted terminal repeats and a transposase enzyme that acts to cut the transposon out of one source of DNA and paste it into another DNA sequence (Plasterk, 1993). Autonomous transposons carry the transposase gene inside the transposon whereas non-autonomous transposons require another source of transposase for their mobilization.

[0018] One well known transposable element is the maize *Ac/Ds* element (Shure et al., 1983; Fedoroff et al., 1983; Pohlman et al., 1984). Maize *Ac/Ds* elements can transpose in a wide variety of plant species (Osborne and Baker, 1995). Moreover, successful *Ds* transposition catalyzed by modified transposase was demonstrated in *Saccharomyces cerevisiae* (Weil and Kunze, 2000), implying that plant-specific proteins were not essential for transposition. Transposition in heterologous animal hosts has been reported for a number of other transposons e.g. *mariner* element from *Drosophila mauritiana* in zebrafish (Fadool et al., 1998), nematode *Caenorhabditis elegans* *Tc3* element (Raz et al., 1998) in zebrafish, synthetic transposon *Sleeping Beauty* in mammals and zebrafish (Horie et al, 2001; Davidson et al, 2003; Balciunas et al., 2004), *Tol2* from *Oryzias latipes* in zebrafish, *Xenopus* and mouse (Kawakami et al., 2000; Kawakami et al., 2004; Kawakami and Noda 2004) etc. However, none of the known transposons has yet been demonstrated to transpose in both plants and animals.

[0019] The use of heterologous transposons has been a powerful tool for genetic research in a number of model species (Parinov et al., 1999; Spradling et al., 1995). The advantage of using heterologous elements is that there is no transposase in a new host once inserted a genomic copy of non-autonomous element is immobile but can be mobilized if transposase is delivered into the cells.

[0020] Methods for introducing DNA into a cell are known. These include, but are not limited to, DNA condensing reagents such as calcium phosphate, polyethylene glycol, and the like), lipid-containing reagents, such as liposomes, multi-lamellar vesicles, and the like, and virus-mediated strategies. These methods all have their limitations. For example, there are size constraints associated with DNA condensing reagents and virus-mediated strategies. Further, the amount of nucleic acid that can be introduced into a cell is limited in virus strategies. Not all methods facilitate integration of the delivered nucleic acid into cellular nucleic acid and while DNA condensing methods and lipid-containing reagents are relatively easy to prepare, the incorporation of nucleic acid into viral vectors can be labor intensive. Moreover, virus-mediated strategies can be cell-type or tissue-type specific and the use of virus-mediated strategies can create immunologic problems when used in vivo.

[0021] There remains a need for new methods for introducing DNA into a cell, particularly methods that promote the efficient integration of nucleic acid fragments of varying sizes into the nucleic acid of a cell, particularly the integration of DNA into the genome of a cell. There also remains a need to develop a vertebrate (e.g., a zebrafish, mouse, etc.) insertional mutation library

that could be used to screen for genetic defects, to study genes of interest, to screen for drugs useful for treating or preventing a disease condition associated with a gene of interest.

SUMMARY OF THE INVENTION

[0022] The present disclosure is directed to the use of the modified maize *Ac/Ds* transposable elements in vertebrates, including fish, birds, and other animals, including mammals and humans. The transposon system of this disclosure has applications to many areas of biotechnology. Development of transposable elements for vectors in animals permits the following: 1) efficient insertion of genetic material into animal chromosomes using the methods given in this application; 2) identification, isolation, and characterization of genes involved with growth and development through the use of transposons as insertional mutagens; 3) identification, isolation and characterization of transcriptional regulatory sequences controlling growth and development; 4) use of marker constructs for quantitative trait loci (QTL) analysis; 5) identification of genetic loci of economically important traits, besides those for growth and development, i.e., disease resistance; and 6) nonviral vectors for gene therapy.

[0023] Thus, in a first aspect, there is provided a gene transfer system to introduce a polynucleotide into the DNA of a vertebrate cell comprising:

a modified *Ac* transposase or a nucleic acid encoding the modified *Ac* transposase, wherein the modified *Ac* transposase comprises a nuclear localization signal (NLS) at the N-terminus of a truncated *Ac* transposase, wherein the NLS localizes the transposition reaction to the nucleus of the vertebrate cell and wherein the truncated *Ac* transposase comprises an amino acid sequence having at least 80% identity to SEQ ID NO:8 and catalyzes the integration of a polynucleotide into DNA in a vertebrate cell; and

a nucleic acid fragment comprising a polynucleotide positioned between inverted repeats that can bind to the modified *Ac* transposase.

[0023a] In a second aspect, there is provided a method for introducing a polynucleotide into the DNA of a vertebrate cell comprising the steps of:

introducing into the cell a nucleic acid fragment comprising a polynucleotide positioned between inverted repeats that can bind to a modified *Ac* transposase;

introducing a modified *Ac* transposase or a nucleic acid encoding a modified *Ac* transposase into the cell, wherein the modified *Ac* transposase comprises a nuclear localization signal (NLS) at the N-terminus of a truncated *Ac* transposase, wherein the NLS localizes the transposition reaction to the nucleus of the vertebrate cell and wherein the truncated *Ac* transposase comprises an amino acid sequence having at least 80% identity to SEQ ID NO:8 and catalyzes the integration of the polynucleotide into DNA in a vertebrate cell; and

culturing the cell to stably incorporate the polynucleotide into the genome of the cell to produce a transgenic vertebrate cell.

[0023b] In a third aspect, there is provided a transgenic vertebrate cell line produced by the method as defined above, wherein the genome of the cell line comprises the polynucleotide flanked by Ds 5' and 3' ends.

[0023c] In a fourth aspect, there is provided a method for producing a transgenic non-human vertebrate comprising the steps of:

introducing a nucleic acid fragment comprising a polynucleotide positioned between inverted repeats that can bind to a modified *Ac* transposase into a vertebrate cell;

introducing a modified *Ac* transposase or a nucleic acid encoding a modified *Ac* transposase into a vertebrate cell, wherein the modified *Ac* transposase comprises a nuclear localization signal (NLS) at the N-terminus of a truncated *Ac* transposase, wherein the NLS localizes the transposition reaction to the nucleus of the vertebrate cell and wherein the truncated *Ac* transposase comprises an amino acid sequence having at least 80% identity to SEQ ID NO:8 and catalyzes the integration of the polynucleotide into DNA in a vertebrate cell;

culturing the vertebrate cell to stably incorporate the polynucleotide into the genome of the cell to produce a transgenic vertebrate cell; and

growing the transgenic vertebrate cell into a transgenic vertebrate.

[0023d] In a fifth aspect, there is provided a method for identifying the function of a gene which comprises:

(a) preparing a vertebrate cell having a polynucleotide insert in a gene, wherein the polynucleotide comprises a portion of the inverted repeats that can bind to a modified *Ac* transposase and wherein the vertebrate cell is prepared by

(i) introducing into the cell a nucleic acid fragment comprising a polynucleotide positioned between inverted repeats that can bind to a modified *Ac* transposase;

(ii) introducing a modified *Ac* transposase or a nucleic acid encoding a modified *Ac* transposase into the cell, wherein the modified *Ac* transposase comprises a nuclear localization signal (NLS) at the N-terminus of a truncated *Ac* transposase, wherein the NLS localizes the transposition reaction to the nucleus of the vertebrate cell and wherein the truncated *Ac* transposase comprises an amino acid sequence having at least 80% identity to SEQ ID NO:8 and catalyzes the integration of the polynucleotide into DNA in a vertebrate cell;

(iii) culturing the cell to stably incorporate the polynucleotide into the genome of the cell to produce a transgenic vertebrate cell;

(b) screening the vertebrate cell for an insertional mutation for a phenotype or biochemical characteristic of interest; and

(c) correlating the phenotype or biochemical characteristic with the nucleotide sequence of the gene, whereby the function of the gene is identified.

[0023e] In a sixth aspect, there is provided a method for identifying a putative drug target comprising:

- (a) preparing a vertebrate cell having a polynucleotide insert in a gene, wherein the polynucleotide comprises a portion of the inverted repeats that can bind to a modified *Ac* transposase and wherein the vertebrate cell is prepared by
 - (i) introducing into the cell a nucleic acid fragment comprising a polynucleotide positioned between inverted repeats that can bind to a modified *Ac* transposase;
 - (ii) introducing a modified *Ac* transposase or a nucleic acid encoding a modified *Ac* transposase into the cell, wherein the modified *Ac* transposase comprises a nuclear localization signal (NLS) at the N-terminus of a truncated *Ac* transposase, wherein the NLS localizes the transposition reaction to the nucleus of the vertebrate cell and wherein the truncated *Ac* transposase comprises an amino acid sequence having at least 80% identity to SEQ ID NO:8 and catalyzes the integration of the polynucleotide into DNA in a vertebrate cell;
 - (iii) culturing the cell to stably incorporate the polynucleotide into the genome of the cell to produce a transgenic vertebrate cell;
- (b) identifying a vertebrate cell o having an insertional mutation for a phenotype or biochemical characteristic of interest; and
- (c) identifying a nucleotide sequence of the gene and an amino acid sequence of a protein encoded by the gene for the identified vertebrate cell, whereby a putative drug target is identified.

[0023f] In a seventh aspect, there is provided a method for identifying a site of action of a compound comprising:

(a) preparing a vertebrate cell having a polynucleotide insert in a gene, wherein the polynucleotide comprises a portion of the inverted repeats that can bind to a modified *Ac* transposase and wherein the vertebrate cell is prepared by

(i) introducing into the cell a nucleic acid fragment comprising a polynucleotide positioned between inverted repeats that can bind to a modified *Ac* transposase;

(ii) introducing a modified *Ac* transposase or a nucleic acid encoding a modified *Ac* transposase into the cell, wherein the modified *Ac* transposase comprises a nuclear localization signal (NLS) at the N-terminus of a truncated *Ac* transposase, wherein the NLS localizes the transposition reaction to the nucleus of the vertebrate cell and wherein the truncated *Ac* transposase comprises an amino acid sequence having at least 80% identity to SEQ ID NO:8 and catalyzes the integration of the polynucleotide into DNA in a vertebrate cell;

(iii) culturing the cell to stably incorporate the polynucleotide into the genome of the cell to produce a transgenic vertebrate cell;

(b) exposing a vertebrate cell having an insertional mutation to a compound of interest;

(c) exposing a wildtype vertebrate cell to the compound of interest;

(d) assessing the effect of the compound on the vertebrate cell having an insertional mutation and the wildtype vertebrate cell; and

(e) identifying a nucleotide sequence of a gene in the vertebrate cell having an insertional mutation which does not respond to the compound compared to the wildtype vertebrate cell, whereby a site of action of the compound is identified.

[0023g] In an eighth aspect, there is provided a vertebrate insertional mutation library wherein each member of the library has a

polynucleotide insert in a gene, wherein the polynucleotide comprises a portion of the inverted repeats that can bind to a modified *Ac* transposase and wherein the library has a polynucleotide insert in at least 15% of the genes of the vertebrate genome.

[0023h] In a ninth aspect, there is provided a method for identifying a function of a gene comprising:

screening at least one member of the vertebrate insertional mutation library as defined above for a phenotype of interest; and

correlating the phenotype of interest with a DNA sequence of the gene of said library, whereby the function of said gene is identified.

[0023i] In a tenth aspect, there is provided a method for identifying a putative drug target comprising:

identifying a member of the vertebrate insertional mutation library as defined above associated with a phenotype of interest; and

identifying a DNA sequence of the gene and an amino acid sequence of a protein encoded by the gene for the identified member of the library, whereby a putative drug target is identified.

[0023j] In an eleventh aspect, there is provided a method for identifying a site of action of a compound comprising:

exposing at least one member of the vertebrate insertional mutation library as defined above to a compound of interest;

exposing wildtype vertebrate individuals to the compound of interest;

assessing the effect of the compound on the member of library and the wildtype vertebrate individuals;

identifying a member of the library which does not respond to the compound compared to the wildtype vertebrate individuals; and

identifying a DNA sequence of a gene in the identified member of the library, whereby a site of action of the compound is identified.

[0023k] In a twelfth aspect, there is provided a method for identifying a gene of interest comprising:

screening members of the vertebrate insertional mutation library as defined above for a characteristic of interest;

correlating the presence of the gene of interest in a specific member of the library having the characteristic of interest; and

identifying the gene of interest from the DNA sequence of the gene associated with the specific member of the library.

Disclosed are modified *Ac* and *Ds*

transposable elements for transposition in vertebrates. In one embodiment, a modified *Ds* element contains a polynucleotide of interest and can be used to prepare transgenic vertebrates having the polynucleotide of interest stably integrated in its genome. In a second embodiment, a modified *Ds* element can be used to prepare insertional mutations in vertebrates. In one embodiment, the modified *Ds* element (also known as a *Ds* construct) comprises the polynucleotide of interest positioned between the 5'- and 3'-ends of the *Ds* element.

[0024] Disclosed are transgenic vertebrates that contain either a modified *Ac* transposase or a modified *Ds* element stably integrated in their genome.

[0025] Disclosed are methods for transposition in vertebrates using modified maize *Ac/Ds* transposable elements. In one embodiment, the modified *Ds* element or a vector containing the modified *Ds* element is introduced into a vertebrate. In one facet of this embodiment, the modified *Ds* element or a vector containing the modified *Ds* element is introduced into a vertebrate along with the RNA for the modified *Ac* transposase. In a second facet, the modified *Ds* element or a vector containing the modified *Ds* element is introduced into a transgenic vertebrate containing a modified *Ac* transposase gene as described herein stably incorporated into its genome. In a third facet, the modified *Ds* element or a vector containing the modified *Ds* element is introduced into a vertebrate along with the

modified *Ac* transposase protein. In a fourth facet, the modified *Ds* element or a vector containing the modified *Ds* element is introduced into a vertebrate along with a vector containing a modified transposase gene. In each instance, the modified *Ac* transposase protein drives transposition of the modified *Ds* element in the vertebrate.

[0026] In a second embodiment, the modified *Ds* element is stably incorporated into the vertebrate genome to make a transgenic vertebrate. Transgenic vertebrates are prepared as described herein using the modified *Ds* elements described herein. In one facet of this embodiment, the RNA for the modified *Ac* transposase is introduced into the transgenic vertebrate containing the modified *Ds* element. In a second facet, a transgenic vertebrate containing a coding sequence for the modified *Ac* transposase stably incorporated in its genome is crossed with a transgenic vertebrate containing the modified *Ds* element. In a third facet, the modified *Ac* transposase protein is introduced into the transgenic vertebrate containing the modified *Ds* element. In a fourth facet, a vector containing a modified transposase gene is introduced into the transgenic vertebrate containing the modified *Ds* element. In each instance, the modified *Ac* transposase protein drives transposition of the modified *Ds* element in the vertebrate.

[0027] Disclosed are insertional mutations in genes of the target vertebrate to provide an indexed library of genetically altered cells and animals. The present disclosure also provides methods of organizing the cells and animals into an easily manipulated and characterized library. In one embodiment, the genetically altered cells are a vertebrate, such as zebrafish, that are produced by treating vertebrate embryos, growing the embryos to adults and collecting the adult vertebrates. The adult vertebrates containing insertional mutations are termed founder vertebrates. In a second embodiment, the genetically altered cells are vertebrate sperm cells that are produced by treating vertebrate embryos, growing the embryos to adults and collecting sperm from adult males. Male vertebrate containing insertional mutations are also termed founder males. In a third embodiment, the original sperm from a founder male is thawed and then used for *in vitro* fertilization on eggs from wild type females to generate vertebrates that harbor the desired inserts. Secondary sperm samples are collected from all of the newly created males once they have reached adulthood. Using these methods the library allows for the completion of numerous genetic screens. The library comprises a set in which each member of the set possesses at least one mutation in at least one gene, and the set collectively comprises at least 15% of the genes

[0028] Disclosed is the characterization of the inserts, e.g. by sequencing of the DNA flanking the inserts.

[0029] Disclosed is a sequence database. The sequence database subsequently serves as an index for the library. In essence, every sperm cell line and/or founder vertebrates or offspring thereof in the library is individually catalogued using the sequence information. The resulting sequence is specific for the insertional mutation. From this database, a gene of interest can be identified. Once identified, the corresponding mutant sperm cell or fish may be withdrawn from the library based on cross reference to the sequence data.

[0030] Disclosed are methods for rapidly identifying the function of genes. Vertebrates containing mutations of genes of interest, generated as described above, may be examined for specific phenotypes, much as knockout mice are studied to determine the same. As used herein, "phenotype" denotes a definable detectable heritable trait of a cell or organism that is caused by the presence and action of at least one gene or mutant gene.

[0031] Disclosed are methods for carrying out genetic screens. As a first step in the methods, vertebrates (or vertebrates encompassing the library), or their offspring, may be used, or alternatively, sperm is thawed and then used for *in vitro* fertilization on eggs from wild type females to generate vertebrates that harbor the desired inserts. Diploid vertebrates may be screened from offspring of the injected females with the mutations in either the homozygous or heterozygous state, or screening may be performed in haploid embryos, known to survive and develop for three days post fertilization. Vertebrates homozygous for the mutation may be created by conventional breeding methods, creation of haploid embryos, or creation of gynogenetic diploid embryos. These methods are all well described in the prior art (for example, see Westerfield (2000) with respect to zebrafish). The vertebrates harboring the desired inserts, prepared using any of the methods described above, are used to study gene function by studying the effects of the insertional mutation *in vivo*. In this manner, phenotypes can be associated with the insertional mutation and the sequence data, thus allowing the identification, for example, of novel collections of putative drug targets. The sequence data also identifies the gene of interest and is used to search for homologs or orthologs in other organisms, including humans.

[0032] Disclosed are methods for screening candidate compounds. Vertebrates that harbor a desired insert, i.e., vertebrates that have a desired mutation, are generated as described above. These transgenic vertebrates can be exposed to

compounds to assess the effect of the compound on a phenotype or assay outcome of interest. For example, test compounds can be administered to transgenic vertebrates harboring the desired insert or representing all the inserts. By screening and subsequently identifying vertebrates that, by virtue of a specific mutation or mutation(s), no longer respond to a compound, one will have identified a gene encoding the protein required for the action of said compound. Test compounds may act as either inhibitors or activators of a protein encoded by the gene of interest. In this manner, compounds which are useful as drugs for treating or preventing disease conditions associated with the gene of interest are identified.

[0033] Disclosed is the use of modified maize *Ac/Ds* transposable elements for gene therapy in vertebrates.

BRIEF DESCRIPTION OF THE FIGURES

[0034] Figures 1A-1C show constructs made in accordance with the present invention. Fig. 1A shows a *Ds* donor construct carrying 3.1 kb reporter fragment (EGFP gene under the zebrafish *keratin 8* promoter), inserted between 5'- and 3'-*Ds cis*-sequences (250 bp and 370 bp correspondingly). Black arrowheads indicate the primers for excision PCR, gray arrowheads – specific primers for TAIL-PCR. Fig. 1B shows a TPase construct containing SP6 promoter for in vitro transcription, coding sequence for a truncated *Ac* transposase (TPase₁₀₃₋₈₀₇) fused to a synthetic nuclear localization signal. Dashed lines represent 5'- and 3'-UTRs of *Xenopus β-globin* gene. Fig. 1C shows the N-terminal amino acid sequences of the NLS-, NLS^{KSE}- and NoNLS-TPase. NLS signals are shown in bold and highlighted. The sequence for NLS is SEQ ID NO:2. The full amino acid sequence shown for NLS is shown by amino acid residues 1-15 of SEQ ID NO:20. The sequence for NLS^{KSE} is SEQ ID NO:9. The full amino acid sequence shown for NLS^{KSE} is shown by amino acid residues 1-15 of SEQ ID NO:44. The sequence for NoNLS is SEQ ID NO:31, and represents the first four amino acid residues of TPase₁₀₃₋₈₀₇, e.g., amino acid residues 12-15 of SEQ ID NO:20.

[0035] Figures 2A-2C show the TPase-specific excision and insertion of *Ds* element. Fig. 2A shows a *Ds* excision assay. Zebrafish embryos were injected with *Ds* construct and NoNLS-, NLS^{KSE}- and NLS-TPase RNA (lanes 2, 3 and 4 correspondingly) and *Ds* construct only (lane 1). DNA was isolated from injected embryos at 10 hours post injection and subjected to PCR using primers flanking the *Ds* donor site. (M) 1 kb DNA Ladder (NEB). Fig. 2B shows predominant excision footprints from two different donor vectors. Missing and changed nucleotides of the flanking donor vector are in bold or underlined respectively. Lower case

letters indicates the borders of the *Ds* sequence. For before excision of Donor 1, the nucleotide sequence is the sequence shown in SEQ ID NO:1 from nucleotide 29 to 3917, inclusive. For after excision for Donor 1, the top line sequence is the sequence shown in SEQ ID NO:3 and the bottom line sequence is the sequence shown in SEQ ID NO:4. For before excision of Donor 2, the nucleotide sequence 5' of *Ds* is SEQ ID NO:68, and the nucleotide sequence 3' of *Ds* is SEQ ID NO:69. The "*Ds*" sequence is the sequence shown in SEQ ID NO:1 from nucleotide 54 to nucleotide 3892, inclusive. For after excision for Donor 2, the top line sequence is the sequence shown in SEQ ID NO:5 and the bottom line sequence is the sequence shown in SEQ ID NO:6. Fig. 2C shows representative examples of sequences flanking the *Ds* insertion sites from two different transgenic F₁ fish, demonstrating specific transposition mechanism. *Ds* end sequences are shown in lower case, flanking sequences – in upper case. Classic 8 bp direct target duplication is in bold and underlined. The sequence 5' of *Ds* in the top line is the sequence shown in SEQ ID NO:32 and the sequence 3' of *Ds* in the top line is the sequence shown in SEQ ID NO:33. The sequence 5' of *Ds* in the bottom line is the sequence shown in SEQ ID NO:34 and the sequence 3' of *Ds* in the bottom line is the sequence shown in SEQ ID NO:35. The "*Ds*" sequence is the sequence shown in SEQ ID NO:1 from nucleotide 54 to 3892, inclusive.

[0036] Figures 3A-3F show the effects of different NLS sequences on the intracellular localization of TPase. Figs. 3A-3C show subcellular localization of the GFP-tagged versions of NoNLS-TPase, NLS^{KSE}-TPase and NLS-TPase zebrafish epithelial cells. Photographs were overexposed to highlight the cellular outline. Figs. 3D-3F show intracellular localization of NoNLS-, NLS^{KSE}- and NLS-EGFP fusion proteins in zebrafish epithelial cells.

[0037] Figure 4 shows evaluation of the *Ds* copy number in F₁ fish. Individual F₁ fish were out-crossed to the *wt* and DNA from 12 randomly selected GFP-positive embryos was used for Southern blot analysis. The DNA samples were digested with EcoRI (the *Ds* construct contains a single EcoRI site) and hybridized with DIG-labeled probe for the EGFP sequence. (Lane 1-4) progenies of four different F₁ fish from the same family (from the same founder F₀); (Lanes 5-11) progenies of F₁ fish from different families (originated from different F₀ founders), (Lane 2) GFP negative control.

[0038] Figure 5 shows transposition of genomic *Ds* insertions. Top: mock-injected transgenic fish with a single *Ds* insertion in genome exhibiting even and weak GFP expression pattern in the skin epithelia and the gut. Middle: a representative example of a fish carrying the same *Ds* insertion injected with TPase RNA demonstrating ectopic GFP expression in the brain, spinal chord, ears, muscles, gonadal region, and variegating mosaic expression in the skin

(presumably due to increased number of copies). Bottom: Example of a novel expression pattern found among F₁ generation - expression in notochord is not present in the control; moreover the dotted pattern on the skin of the control fish is not present in the F₁ fish indicating that the donor *Ds* copy is lost during transposition.

[0039] Figures 6A-6C show the results of the analysis of *Ds* in three examples of transposed HEK293 cells. The "*Ds*" sequence shown in Fig. 6B is the sequence shown in SEQ ID NO:70 from nucleotide 17 to nucleotide 4819, inclusive. The *Ds* (lower case letters) in these three examples is flanked by human DNA (upper case letters) (not the vector DNA of the donor) starting from the first nucleotide immediately adjacent to the *Ds* end sequence. This demonstrates that *Ds* integrated into human genome via transposase-mediated mechanism. In case of DNA 2, the insertion site is surrounded by classic 8 bp direct repeat that is often created when hAT transposons integrate into new locations.

DETAILED DESCRIPTION OF THE INVENTION

[0040] The present invention describes the use of the modified maize *Ac/Ds* transposable elements in vertebrates, including fish, birds, and other animals including mammals. As used herein, fish refers to any member of the classes collectively referred to as Pisces. It is preferred that fish belonging to species and varieties of fish of commercial or scientific interest be used. Such fish include, but are not limited to, salmon, trout, tuna, halibut, catfish, zebrafish, medaka, carp, tilapia, goldfish, and loach. Mammals include, but are not limited to rodents, such as rats and mice, ungulates, such as cows, goats, sheep or pigs, and primates, such as monkeys, apes and humans.

[0041] The use of the modified maize *Ac/Ds* transposable elements in accordance with the present invention has a significant advantage for the production of stable genomic integration, i.e., eliminating the risk of re-transposition or recombination due to transposase activity produced by the host. For example, insertions of the *Tol2* element originally extracted from medaka fish will be unstable in the same fish because of the host transposase (Koga et al.). Maize *Ac/Ds* elements have no significantly similar homologs in vertebrates.

[0042] In the description which follows, the aspects of the invention are described with reference to zebrafish for convenience only. It is understood that other fish and other animals can be used in place of zebrafish. As demonstrated in the Examples, the present invention is applicable for all vertebrates ranging from fish to humans. Thus, the modified maize *Ac/Ds* transposable element of the present invention can be used to introduce DNA into both

pluripotent (i.e., a cell whose descendants can differentiate into several restricted cell types, such as hematopoietic stem cells or other stem cells) and totipotent cells (i.e., a cell whose descendants can become any cell type in an organism, e.g., embryonic stem cells). Suitable cells include oocytes, eggs, and one or more cells of an embryo are also considered in this invention. For gene transfer, the modified maize *Ac/Ds* transposable element of the present invention can be used to introduce DNA into mature cells from a variety of organs or tissues. Suitable cells include, but are not limited to, lymphocytes, hepatocytes, neural cells, muscle cells, a variety of blood cells, and a variety of cells of an organism.

[0043] The most preferred fish for use with the present invention is zebrafish, *Danio rerio*. Zebrafish are an increasingly popular experimental animal since they have many of the advantages of popular invertebrate experimental organisms, and include the additional advantage that they are vertebrates. Another significant advantage of zebrafish is that, like *Caenorhabditis*, they are largely transparent (Kimmel, 1989). General zebrafish care and maintenance are described by Streisinger (1984) and Westerfield (2000).

[0044] In accordance with the present invention, *Ds* transposition in fish is driven by a modified *Ac* transposase. It has been demonstrated earlier that a truncated form of *Ac* transposase lacking 102 N-terminal amino acids (TPase₁₀₃₋₈₀₇) (SEQ ID NOs:7 and 8) is highly active (Houba-Herlin et al., 1990). In one embodiment of the present invention, this modified *Ac* transposase is further modified to contain a synthetic nuclear localization signal (NLS). The synthetic NLS is added to the N-terminus of TPase₁₀₃₋₈₀₇. The synthetic NLS is added in order to localize the transposition reaction to the nucleus. In one embodiment, the synthetic NLS is analogous to that of the SV40 large T antigen. In a preferred embodiment, this synthetic NLS has the amino acid sequence MGPPKKRKVE (SEQ ID NO:2). Other synthetic NLSs can be used in place of the synthetic NLS analogous to the SV40 antigen. In one embodiment, the synthetic NLS is a modified NLS. One embodiment of a modified NLS is NLS^{KSE} that has the amino acid sequence MGPPEKKRKVE (SEQ ID NO:9). A nucleotide sequence coding for the synthetic NLS is added to the 5' end of the coding sequence for TPase₁₀₃₋₈₀₇ to produce a coding sequence for the modified *Ac* transposase. One embodiment of a coding sequence for NLS-TPase₁₀₃₋₈₀₇ is set forth in SEQ ID NO:10. One embodiment of a coding sequence for NLS^{KSE}-TPase₁₀₃₋₈₀₇ is set forth in SEQ ID NO:36.

[0045] In a further embodiment of the present invention, the modified *Ac* transposase utilized in the invention may have a nucleotide sequence that has at least about 60%, preferably at least about 70%, more preferably at least about 80%, and most preferably at least about 90% identity

to the nucleotide sequence of the modified *Ac* transposase discussed herein when optimally aligned (with appropriate nucleotide insertions or deletions). A skilled artisan recognizes that the nucleotide sequence can be modified on the basis of the genetic code to produce a different nucleotide sequences that encode the identical protein. Alternatively, the modified *Ac* transposase utilized in the invention may have an amino acid sequence that is at least about 75%, preferably at least about 85%, more preferably at least about 90% and most preferably at least about 95% identity to the amino acid sequence disclosed herein for the modified *Ac* transposase.

[0046] Identity means the degree of sequence relatedness between two polypeptides or two polynucleotides sequences as determined by the identity of the match between two strings of such sequences. Identity can be readily calculated. While there exist a number of methods to measure identity between two polynucleotide or polypeptide sequences, the term "identity" is well known to skilled artisans (*Computational Molecular Biology*, Lesk, A. M., ed., Oxford University Press, New York, 1988; *Biocomputing: Informatics and Genome Projects*, Smith, D. W., ed., Academic Press, New York, 1993; *Computer Analysis of Sequence Data*, Part I, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; *Sequence Analysis in Molecular Biology*, von Heinje, G., Academic Press, 1987; and *Sequence Analysis Primer*, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991). Methods commonly employed to determine identity between two sequences include, but are not limited to those disclosed in *Guide to Huge Computers*, Martin J. Bishop, ed., Academic Press, San Diego, 1994, and Carillo, H., and Lipman, D., *SIAM J Applied Math.* 48:1073 (1988). Preferred methods to determine identity are designed to give the largest match between the two sequences tested. Such methods are codified in computer programs. Preferred computer program methods to determine identity between two sequences include, but are not limited to, GCG (Genetics Computer Group, Madison Wis.) program package (Devereux, J., et al., *Nucleic Acids Research* 12:387 (1984)), BLASTP, BLASTN, FASTA (Altschul et al. (1990); Altschul et al. (1997)). The well-known Smith Waterman algorithm may also be used to determine identity.

[0047] As an illustration, by a polynucleotide having a nucleotide sequence having at least, for example, 95% "identity" to a reference nucleotide sequence of is intended that the nucleotide sequence of the polynucleotide is identical to the reference sequence except that the polynucleotide sequence may include up to five point mutations per each 100 nucleotides of the reference nucleotide sequence. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of

nucleotides up to 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence. These mutations of the reference sequence may occur at the 5 or 3 terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence.

[0048] Additionally, the modified *Ac* transposase gene may include nucleotide sequences having substantial similarity to the modified *Ac* transposase nucleotide sequence discussed herein. By "substantial similarity", it is meant herein that the nucleotide sequence is sufficiently similar to a reference nucleotide sequence that it will hybridize therewith under moderately stringent conditions. This method of determining similarity is well known in the art to which the invention pertains. Briefly, moderately stringent conditions are defined in Sambrook et al. (*Molecular Cloning: A Laboratory Manual*, 2nd Ed., Vol. 1, pp. 101-104, Cold Spring Harbor Laboratory Press (1989)) as including the use of a prewashing solution of 5 X SSC (a sodium chloride/sodium citrate solution), 0.5% sodium dodecyl sulfate (SDS), 1.0 mM ethylene diaminetetracetic acid (EDTA) (pH 8.0) and hybridization and washing conditions of 55° C., 5 X SSC.

[0049] The modified *Ac* transposase may be either synthesized *in vitro* or isolated from a biological source. Such methods of synthesis and isolation are well known to the skilled artisan.

[0050] The modified *Ac* transposase is introduced into fish. In one embodiment, a modified *Ac* transposase protein is introduced into fish. The protein is introduced into fish using conventional techniques, such as microinjection.

[0051] In a second embodiment, RNA for the modified *Ac* transposase is introduced into fish. In accordance with this embodiment, the modified *Ac* transposase transcript may be synthesized *in vitro* or isolated from a biological source. In one aspect, a nucleic acid construct is prepared which contains an RNA polymerase promoter and the coding sequence for the modified *Ac* transposase. The RNA polymerase promoter is preferably the SP6 promoter. However, other RNA polymerase promoters can be used, including the T7 promoter. The nucleic acid construct further comprises 5'- and 3'-UTRs and a polyA tail. Any 5'- and 3'-UTRs may be used, although it is preferred to use the 5'-and 3'-UTRs and polyA tail of the *Xenopus β -globin* gene. Alternatively, 5'-and 3'-UTRs native to fish may be used in place of the 5'-and 3'-UTRs of the *Xenopus β -globin* gene. Similarly, a polyA tail native to fish may be used in place of the polyA tail of the *Xenopus β -globin* gene. One embodiment of such a nucleic acid construct is set forth in SEQ ID NO:11. A second embodiment of such a nucleic acid is set forth in SEQ ID NO:37.

[0052] In a third embodiment, the modified *Ac* transposase gene is stably incorporated into the fish genome to make a transgenic fish. As used herein, transgenic fish refers to fish, or progeny of a fish, into which an exogenous construct has been introduced. A fish into which a construct has been introduced includes fish which have developed from embryonic cells into which the construct has been introduced. As used herein, an exogenous construct is a nucleic acid that is artificially introduced, or was originally artificially introduced, into an animal. The term artificial introduction is intended to exclude introduction of a construct through normal reproduction or genetic crosses. That is, the original introduction of a gene or trait into a line or strain of animal by cross breeding is intended to be excluded. However, fish produced by transfer, through normal breeding, of an exogenous construct (that is, a construct that was originally artificially introduced) from a fish containing the construct are considered to contain an exogenous construct. Such fish are progeny of fish into which the exogenous construct has been introduced. As used herein, progeny of a fish are any fish which are descended from the fish by sexual reproduction or cloning, and from which genetic material has been inherited. In this context, cloning refers to production of a genetically identical fish from DNA, a cell, or cells of the fish. The fish from which another fish is descended is referred to as a progenitor or founder fish. As used herein, development of a fish from a cell or cells (embryonic cells, for example), or development of a cell or cells into a fish, refers to the developmental process by which fertilized egg cells or embryonic cells (and their progeny) grow, divide, and differentiate to form an adult fish.

[0053] A transgene construct containing a coding sequence for the modified *Ac* transposase is used to prepare transgenic fish. Transgene constructs are the genetic material that is introduced into fish to produce a transgenic fish. Such constructs are artificially introduced into fish. The manner of introduction, and, often, the structure of a transgene construct, render such a transgene construct an exogenous construct. Although a transgene construct can be made up of any nucleic acid sequences, for use in the disclosed transgenic fish it is preferred that the transgene constructs combine expression sequences operably linked to a sequence encoding an expression product. The transgenic construct also preferably includes other components that aid expression, stability or integration of the construct into the genome of a fish. As used herein, components of a transgene construct referred to as being operably linked or operatively linked refer to components being so connected as to allow them to function together for their intended purpose. For example, a promoter and a coding region are operably linked if the promoter can function to result in transcription of the coding region.

[0054] In a one aspect, the transgene construct is the RNA polymerase promoter construct described above. In a second aspect, a transgene construct containing a coding sequence for the modified *Ac* transposase is prepared to include expression sequences. The expression sequences are used to mediate expression of an expression product encoded by the construct. As used herein, expression sequences include promoters, upstream elements, enhancers, and response elements. It is preferred that the expression sequences used in the disclosed constructs be homologous expression sequences. As used herein, in reference to components of transgene constructs used in the disclosed transgenic fish, homologous indicates that the component is native to or derived from the species or type of fish involved. Conversely, heterologous indicates that the component is neither native to nor derived from the species or type of fish involved.

[0055] As used herein, expression sequences are divided into two main classes, promoters and enhancers. A promoter is generally a sequence or sequences of DNA that function when in a relatively fixed location in regard to the transcription start site. A promoter contains core elements required for basic interaction of RNA polymerase and transcription factors, and may contain upstream elements and response elements. Enhancer generally refers to a sequence of DNA that functions at no fixed distance from the transcription start site and can be in either orientation. Enhancers function to increase transcription from nearby promoters. Enhancers also often contain response elements that mediate the regulation of transcription. Promoters can also contain response elements that mediate the regulation of transcription.

[0056] For expression of encoded peptides or proteins, a transgene construct also needs sequences that, when transcribed into RNA, mediate translation of the encoded expression products. Such sequences are generally found in the 5' untranslated region of transcribed RNA. This region corresponds to the region on the construct between the transcription initiation site and the translation initiation site (that is, the initiation codon). The 5' untranslated region of a construct can be derived from the 5' untranslated region normally associated with the promoter used in the construct, the 5' untranslated region normally associated with the sequence encoding the expression product, the 5' untranslated region of a gene unrelated to the promoter or sequence encoding the expression product, or a hybrid of these 5' untranslated regions. Preferably, the 5' untranslated region is homologous to the fish into which the construct is to be introduced. Preferred 5' untranslated regions are those normally associated with the promoter used.

[0057] Transgene constructs for use in the disclosed transgenic fish may encode a reporter protein (for detection and quantitation of expression). As used herein, a reporter protein is any protein that can be specifically detected when expressed. Reporter proteins are useful for detecting or quantitating expression from expression sequences. For example, operatively linking nucleotide sequence encoding a reporter protein to a tissue specific expression sequences allows one to carefully study lineage development. In such studies, the reporter protein serves as a marker for monitoring developmental processes, such as cell migration. Many reporter proteins are known and have been used for similar purposes in other organisms. These include enzymes, such as β -galactosidase, luciferase, and alkaline phosphatase, that can produce specific detectable products, and proteins that can be directly detected. Virtually any protein can be directly detected by using, for example, specific antibodies to the protein.

[0058] The use of reporter proteins that are directly detectable without requiring the addition of exogenous factors are preferred for detecting or assessing gene expression during zebrafish embryonic development. A transgenic zebrafish embryo, carrying a construct encoding a reporter protein and a tissue-specific expression sequences, can provide a rapid real time *in vivo* system for analyzing spatial and temporal expression patterns of developmentally regulated genes.

[0059] The disclosed transgene constructs preferably include other sequences which improve expression from, or stability of, the construct. For example, including a polyadenylation signal on the constructs encoding a protein ensures that transcripts from the transgene is processed and transported as mRNA. The identification and use of polyadenylation signals in expression constructs is well established. It is preferred that homologous polyadenylation signals be used in the transgene constructs.

[0060] In accordance with the above principles, the coding sequence for the modified *Ac* transposase is operably linked to a promoter. Any promoter active in the fish species may be used. Since most mammalian promoters are found not to work well in fish, then the genomic regulatory sequences of the zebrafish, fugu or other fish species often must be specifically cloned upstream, within, and downstream of the coding sequence of interest, which may be accomplished by procedures routine to those skilled in the art.

[0061] As defined herein, a nucleotide sequence is "operably linked" to another nucleotide sequence when it is placed in a functional relationship with another nucleotide sequence. For example, if a coding sequence is operably linked to a promoter sequence, this generally means that the promoter may promote transcription of the coding sequence. Operably linked means

that the DNA sequences being linked are typically contiguous and, where necessary join two protein coding regions, contiguous and in reading frame. Since enhancers may function when separated from the promoter by several kilobases and intron sequences may be of variable lengths, some nucleotide sequences may be operably linked but not contiguous.

[0062] The construct further preferably comprises a marker or a reporter gene. In a preferred embodiment, the oncogene is preceded by a reporter gene, such as a fluorescent protein gene (e.g., EGFP, GFP, RFP, BFP, YFP, or dsRED2) or a luciferase protein gene. In a most preferred embodiment, the marker is enhanced green fluorescent protein (EGFP) (Zhang et al., 1996). EGFP is preferred because of the high sensitivity of the reporter protein. In the preferred embodiment, a fusion of the marker and the oncogene is prepared such that the fused gene is under control of the promoter.

[0063] Although, the use of specific markers has been disclosed and discussed herein, the present invention is in no way limited to the specifically disclosed markers. Many additional reporter proteins are known and have been used for similar purposes. These include enzymes, such as β -galactosidase, luciferase, chloramphenicol acyltransferase, β -glucuronidase and alkaline phosphatase, that can produce specific detectable products, and proteins that can be directly detected. Virtually any protein can be directly detected by using, for example, specific antibodies to the protein. Any reporter which can be readily detected may be used in place of the EGFP. Additional markers (and associated antibiotics) that are suitable for either positive or negative selection of eukaryotic cells are disclosed, *inter alia*, in Sambrook and Russell (2001), *Molecular Cloning*, 3rd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, and Ausubel et al. (1992), *Current Protocols in Molecular Biology*, John Wiley & Sons, including periodic updates. Any of the disclosed markers, as well as others known in the art, may be used to practice the present invention.

[0064] Transgenic fish are prepared using the constructs described herein. In one embodiment, a method includes introducing the nucleic acid, i.e., construct or vector described herein, into a fertilized fish egg (i.e., including a fish embryo) or an unfertilized fish egg nucleic acid. When a fertilized fish egg is used, the method includes developing the fish embryo into a transgenic fish. When the nucleic acid is introduced into a non-fertilized egg, the method includes fertilizing the egg and developing the fish embryo into a transgenic fish. The nucleic acid may be introduced into the egg by a variety of methods known to the art, including mechanical methods, chemical methods, lipophilic methods, retroviral infection methods, and electroporation. Exemplary mechanical methods include, for example, microinjection.

Exemplary chemical methods include, for example, use of calcium phosphate or DEAE-Dextran. Exemplary lipophilic methods include use of liposomes and other cationic agents for lipid-mediated transfection. Such methods are generally well known to the art and many of such methods are described in, for example, *Gene Transfer Methods: Introducing DNA into Living Cells and Organisms*, (P. A. Norton and L. F. Steel, eds., Biotechniques Press, 2000); and *Current Protocols in Molecular Biology* (Ausubel et al., eds., John Wiley & Sons). Microinjection techniques involving fish are further more fully described in, for example, Chen and Powers (1990) and Fletcher and Davis (1991). Electroporation techniques involving fish are further more fully described in, for example, Powers et al. (1992) and Lu et al. (1992). Techniques for introducing DNA into fish eggs or embryos by infection with retroviral vectors, such as pantropic retroviral vectors, are further described in, for example, Burns et al. (1993).

[0065] The vector or other nucleic acid comprising the transgene may be introduced into an unfertilized egg or a fertilized egg at a desired stage of development. Multiple vectors, each encoding different transgenes as described herein may be used. When using a fertilized egg, or embryo, it is preferred to introduce the nucleic acid into the embryo (i.e., at the one-cell stage of development). However, the nucleic acid may also be administered at later stages of development, including the two-cell stage, four-cell stage, etc. Therefore, the nucleic acid may be introduced into the morula, blastula, etc. At least one isolated nucleic acid molecule incorporating the above-described transgenic construct is introduced into the zygote. Additionally, when the nucleic acid is introduced into an egg at later stages of development, at least one isolated nucleic acid molecule incorporating the above-described transgenic construct is introduced into at least one cell of the, for example, morula, blastula, etc.

[0066] Fish eggs may be obtained from the appropriate fish by standard methods. Many of the fish may be purchased commercially from, for example, pet stores. Fertilized eggs may be obtained by methods known to the art. For example, a desired number of appropriately aged fish, such as about three to about twelve month old fish, with a desired ratio of females to males (such as about 2:1) may be placed in an appropriately sized container, such as a tank. Eggs may be collected by, for example, placing the fish in a nuptial chamber in the tank for an appropriate time after mating, such as about 10 to 60 minutes. Such methods are described in, for example, Culp et al. (1991). Alternatively, fish eggs may be artificially fertilized by methods known to the skilled artisan. One skilled in the art is familiar with other methods of obtaining such fertilized fish eggs.

[0067] After introducing the nucleic acid construct into the fish egg or embryo, the fish egg or embryo is provided with an environment conducive to development into an adult fish. Such an environment may include, for example, growth at 28.5° C. in E3 egg water for 15 days followed by introduction into circulating system water by day 16 (Westerfield, 2000).

[0068] Fish harboring a transgene can be identified by any suitable means. The use of reporter proteins that, like fluorescent proteins (such as EGFP, GFP, RFP, BFP, YFP, or dsRED2), are directly detectable without requiring the addition of exogenous factors are preferred for detecting or assessing gene expression during zebrafish embryonic development. A transgenic zebrafish embryo, carrying a construct encoding a reporter protein can provide a rapid real time *in vivo* system for analyzing spatial and temporal expression patterns of developmentally regulated genes. Alternatively, the genome of potential transgenic fish can be probed for the presence of construct sequences. To identify transgenic fish actually expressing the transgene, the presence of an expression product can be assayed. Several techniques for such identification are known and used for transgenic animals and most can be applied to transgenic fish. Probing of potential or actual transgenic fish for nucleic acid sequences present in or characteristic of a transgene construct is preferably accomplished by Southern or Northern blotting. Also preferred is detection using polymerase chain reaction (PCR) or other sequence-specific nucleic acid amplification techniques. Preferred techniques for identifying transgenic zebrafish are described in the examples.

[0069] The transgene may be included in a vector for delivery. A vector, as used herein and as known in the art, refers to a nucleic acid construct that includes genetic material designed to direct transformation (i.e., the process whereby genetic material of an individual cell is altered by incorporation of exogenous DNA into its genome) of a targeted cell. A vector may contain multiple genetic elements positionally and sequentially oriented, i.e., operably linked with other necessary or desired elements such that the nucleic acid in a cassette can be transcribed and, if desired, translated in the microinjected, single-cell fertilized embryo.

[0070] Recombinant expression vectors may be constructed by incorporating the above-recited nucleotide sequences within a vector according to methods well known to the skilled artisan and as described, for example, in references cited herein. A wide variety of vectors are known that have use in the invention. Suitable vectors include plasmid vectors, viral vectors, including retrovirus vectors (e.g., see Miller et al., 1993), adenovirus vectors (e.g., see Erzurum et al., 1993; Zabner et al., 1994; Davidson et al., 1993) adeno-associated virus vectors (e.g., see

Flotte et al., 1993), herpesvirus vectors (e.g., see Anderson et al., 1993), and lentivirus vectors (e.g., see Lever, 2000).

[0071] The disclosed constructs and methods can be used with any type of fish. As used herein, fish refers to any member of the classes collectively referred to as Pisces. It is preferred that fish belonging to species and varieties of fish of commercial or scientific interest be used. Such fish include, but are not limited to, salmon, trout, tuna, halibut, catfish, zebrafish, medaka, carp, tilapia, goldfish, and loach.

[0072] The most preferred fish for use with the disclosed constructs and methods is zebrafish, *Danio rerio*. Zebrafish are an increasingly popular experimental animal since they have many of the advantages of popular invertebrate experimental organisms, and include the additional advantage that they are vertebrates. Another significant advantage of zebrafish is that, like *Caenorhabditis*, they are largely transparent (Kimmel, 1989). General zebrafish care and maintenance are described by Streisinger (1984) and Westerfield (2000).

[0073] Zebrafish embryos are easily accessible and nearly transparent. Given these characteristics, a transgenic zebrafish embryo, carrying a construct encoding a reporter protein and tissue-specific expression sequences, can provide a rapid real time *in vivo* system for analyzing spatial and temporal expression patterns of developmentally regulated genes. In addition, embryonic development of the zebrafish is extremely rapid. In 24 hours an embryo develops rudiments of all the major organs, including a functional heart and circulating blood cells (Kimmel, 1989). Other fish with some or all of the same desirable characteristics are also preferred.

[0074] Identifying the pattern of expression in the disclosed transgenic fish can be accomplished by measuring or identifying expression of the transgene in different tissues (tissue-specific expression), at different times during development (developmentally regulated expression or developmental stage-specific expression), in different cell lineages (cell lineage-specific expression). These assessments can also be combined by, for example, measuring expression (and observing changes, if any) in a cell lineage during development. The nature of the expression product to be detected can have an effect on the suitability of some of these analyses. On one level, different tissues of a fish can be dissected and expression can be assayed in the separate tissue samples. Such an assessment can be performed when using almost any expression product. This technique is commonly used in transgenic animals and is useful for assessing tissue-specific expression. Expression can be determined biochemically, enzymatically, phenotypically or in a model fish.

[0075] This technique can also be used to assess expression during the course of development by assaying for the expression product at different developmental stages. Where detection of the expression product requires fixing of the sample or other treatments that destroy or kill the developing embryo or fish, multiple embryos must be used. This is only practical where the expression pattern in different embryos is expected to be the same or similar. This is the case when using the disclosed transgenic fish having stable and predictable expression. A more preferred way of assessing the pattern of expression of a transgene during development is to use an expression product that can be detected in living embryos and animals.

[0076] In zebrafish, the nervous system and other organ rudiments appear within 24 hours of fertilization. Since the nearly transparent zebrafish embryo develops outside its mother, the origin and migration of lineage progenitor cells can be monitored by following expression of an expression product in transgenic fish. In addition, the regulation of a specific gene can be studied in these fish.

[0077] In a fourth embodiment, a vector containing a gene encoding a modified *Ac* transposase is introduced into fish. In one aspect, the gene encoding a modified *Ac* transposase is a transgene construct such as described above. Any suitable vector, e.g., a plasmid vector, a viral vector, and the like can be used to introduce the modified *Ac* transposase gene into fish using conventional techniques, such as by microinjection.

[0078] *Ds* transposition in fish is accomplished by introducing a modified *Ds* element carrying DNA (also referred to as a polynucleotide) of interest and the modified *Ac* transposase into fish. In one embodiment, the modified *Ds* element or a vector containing the modified *Ds* element is introduced into fish. In one aspect of this embodiment, the modified *Ds* element or a vector containing the modified *Ds* element is introduced into fish along with the RNA for the modified *Ac* transposase. In a second aspect, the modified *Ds* element or a vector containing the modified *Ds* element is introduced into a transgenic fish containing a coding sequence for a modified *Ac* transposase gene as described herein stably incorporated into its genome. In a third aspect, the modified *Ds* element or a vector containing the modified *Ds* element is introduced into fish along with the modified *Ac* transposase protein. In a fourth aspect, the modified *Ds* element or a vector containing the modified *Ds* element is introduced into fish along with a vector containing a modified transposase gene. In each instance, the modified *Ac* transposase protein drives transposition of the modified *Ds* element in fish.

[0079] In a second embodiment, the modified *Ds* element is stably incorporated into the fish genome to make a transgenic fish. Transgenic fish are prepared as described herein using the

modified *Ds* elements described herein. In one aspect of this embodiment, the RNA for the modified *Ac* transposase is introduced into the transgenic fish containing the modified *Ds* element. In a second aspect, a transgenic fish containing a coding sequence for the modified *Ac* transposase is crossed with a transgenic fish containing the modified *Ds* element. In a third aspect, the modified *Ac* transposase protein is introduced into the transgenic fish containing the modified *Ds* element. In a fourth aspect, a vector containing a modified transposase gene is introduced into the transgenic fish containing the modified *Ds* element. In each instance, the modified *Ac* transposase protein drives transposition of the modified *Ds* element in fish.

[0080] The *Ds* element is modified to contain a polynucleotide of interest. The modified *Ds* element (also referred to as a *Ds* construct) comprises the polynucleotide of interest positioned between the 5'- and 3'-ends of a native *Ds* element. The *Ds* 5'- and 3'-ends contain sequences required for transposition including two short terminal inverted repeat sequences and sequences that can bind the modified *Ac* transposase. Following transposition, the polynucleotide that is incorporated into the fish DNA comprises both *Ds* 3'- and 5'-ends and the polynucleotide of interest that was placed between them. In one embodiment, the short terminal repeat sequences are: 5'-terminal repeat: TTTCATCCCTG (SEQ ID NO:12) and 3'-terminal repeat: TTTCATCCCTA (SEQ ID NO:13). In other embodiments, modifications of the 5'- and 3'-ends of the wildtype *Ds* element are made that are useful for transposition with the modified *Ac* transposase or native *Ac* transposase. The polynucleotide of interest may contain another transposable element, for example, *Tol2*, *Sleeping Beauty* and the like.

[0081] In one embodiment, the *Ds* 5' end *cis*-required sequence is shown in SEQ ID NO:45. In a second embodiment, the *Ds* 5' end *cis*-required sequence is shown as nucleotides 3657-3903 of SEQ ID NO:1. In one embodiment, the *Ds* 3' end *cis*-required sequence is shown in SEQ ID NO:49. In a second embodiment, the *Ds* 3' end *cis*-required sequence is shown as nucleotides 43-412 of SEQ ID NO:1.

[0082] In a further embodiment of the present invention, the *Ds* 5' and 3' ends utilized in the invention may have a nucleotide sequence that has at least about 60%, preferably at least about 70%, more preferably at least about 80%, and most preferably at least about 90%, 95% or 98% identity to the nucleotide sequence of the modified *Ds* 5' and 3' ends discussed herein when optimally aligned (with appropriate nucleotide insertions or deletions). Additionally, the sequences of the *Ds* 5' and 3' ends may include nucleotide sequences having substantial similarity to the *Ds* 5' and 3' ends nucleotide sequences discussed herein. The *Ds* 5' and 3' ends are capable of being recognized by the modified *Ac* transposase described herein. The modified

Ac transposase binds to the *Ds* 5' and 3' ends and catalyzes the integration of the nucleic acid located between the *Ds* 5' and 3' ends into the genome of the fish cell.

[0083] The *Ds* 5' and 3' ends may be either synthesized *in vitro* or isolated from a biological source. Such methods of synthesis and isolation are well known to the skilled artisan.

[0084] The polynucleotide of interest may be a marker or reporter gene, such as described herein. Alternatively, the polynucleotide of interest may be any gene or nucleic acid that is desired to be inserted into the genome of the target fish. Such polynucleotide of interest can be selected for use in promoter-trapping, enhancer-trapping, gene-trapping, activation tagging, RNA or protein expression and the like, as well known to persons skilled in the art. For example, the polynucleotide of interest may be a coding sequence operatively linked to a promoter for expression of a protein in the target transgenic fish made in accordance with the present invention, i.e., a target fish subjected to transposition of the coding sequence from the *Ds* construct using the modified *Ac* transposase. Alternatively, the polynucleotide of interest could be a marker sequence operatively linked to a weak promoter for enhancer-trapping. Alternatively, the polynucleotide of interest gene could be a marker sequence without a promoter for identifying a previously uncharacterized promoter element (the technique referred to as promoter trapping). Alternatively, the polynucleotide of interest could be a 3-frame His-tag DNA sequence that can be used for activation tagging such as disclosed in U.S. Patent No. 6,709,863.

[0085] A *Ds* construct useful for insertional mutations contains the necessary features required for making a functional transposable element, such as the 5'- and 3'-ends of the *Ds* element, and a selectable marker, such as those described herein. Additional functional elements are included as desired for specific mutagenic capabilities, such as inducible over-expression of downstream genes. Such additional functional elements may include a cell or tissue specific promoter, Gal4 operators, tetracycline operators, *Xenopus* basal promoter, such as the *efl- α* gene, a rabbit β -globin intron, MAZ transcriptional pause site, loxP homologous recombination site T7 RNA polymerase promoter, basal TATA box. The use of a cell or tissue specific promoter results in the mis-expression of genes in specific cells or tissue.

[0086] Mis-expression can be regulated through the use of a Gal4/VP16 transactivator, a Tet-on (rtTA) transactivator that would activate transcription upon doxycycline induction, or a Tet-off (tTA) transactivator whose constitutive activation could be shut off by addition of tetracycline. Yeast and bacterial derived enhancers are used not only because these modules provide versatility, but also because enhancers that can be recognized by vertebrate transcription

factors may decrease the titer of the virus. However, in appropriate circumstances, vertebrate transcription factors may also be utilized. The second intron of the rabbit β -globin gene is included to enhance the expression of the downstream genes and to splice out any endogenous intron where the virus may integrate. In a preferred aspect, these gene products are expressed under different zebrafish promoters or under homologs or orthologs of these zebrafish promoters, as they may be in any of the other vectors described herein. As used herein, a "homolog" is a nucleic acid or polypeptide similar in sequence to other nucleic acids or polypeptides among a single species, and an "ortholog" is a nucleic acid or polypeptide obtained from one species that is the functional counterpart of a nucleic acid or polypeptide from a different species. One example, among many, is the zebrafish tyrosine hydroxylase promoter or its orthologs. By expressing Gal4 protein under this promoter it is possible to mis-express genes, in combination with the sperm library, only in those tyrosine hydroxylase expressing neurons, allowing the examination of tissue-specific effects in the fish while excluding other areas that may complicate phenotypic analysis.

[0087] The inclusion of a loxP site for targeted homologous recombination may be very useful in characterizing genes from the insertional library. For example, a gene that is disrupted by a transposable insert could have its expression restored by using the wild type gene flanked by loxP sites and Cre recombinase. It is also possible to examine expression patterns of the gene by inserting a loxP flanked GFP reporter. As described previously, in a preferred aspect, these gene products are expressed under different zebrafish promoters or under orthologs of these zebrafish promoters.

[0088] It may also be useful to include a transcriptional pause site and a synthetic, strong polyadenylation site in the opposite orientation of transcription. Although a spectrum of alleles varying in strength is desirable in some situations, it is desirable to construct a library with complete loss-of-function mutations. The addition of transcriptional pause sites and a polyadenylation signal should stop the transcription at the insertion site and result in truncated transcripts, even if the insertions are in introns. In this manner, all essential genes should be included in the library. As described previously, in a preferred aspect, these gene products are expressed under different zebrafish promoters or under orthologs of these zebrafish promoters.

[0089] Enhancers often determine the regulation of expression of a gene. This effect has been seen in so-called enhancer trap constructs where introduction of a construct containing a reporter gene operably linked to a promoter is expressed only when the construct inserts into the domain of an enhancer (O'Kane et al., 1987; Allen et al., 1988; Kothary et al., 1988; Gossler et

al., 1989). In such cases, the expression of the construct is regulated according to the pattern of the newly associated enhancer. Thus, a *Ds* construct having only a minimal promoter, such as a carp β -actin promoter, and a reporter gene can be used to identify enhancers in the target fish. The *Sleeping Beauty* transposon has been used for exon trapping in zebrafish (Balciunas et al., 2004).

[0090] Simple microinjection of nucleic acids into fish embryos is generally ineffective in order to produce stable transgenic fish. The use of transposon vector for transgene delivery facilitates germ-line integration of the polynucleotide of interest. Moreover, unlike the unspecific integrations (simple nucleic acid microinjection) that occur at random positions within vector sequences and are usually concatemeric, transposon insertions into host DNA are usually single and have specific boundaries. It simplifies identification of their surrounding regions (using TAIL-PCR, inversed PCR, and other suitable techniques) and is optimal for stable expression of the transposon-transmitted transgenes.

[0091] The present invention can be used for insertional mutagenesis. *Ds* can produce somatic and heritable germ-line mutations upon insertion into the fish genome. Unlike retroviral or non-specific insertions, transposons can be remobilized if the modified *Ac* transposase is supplied. It can be used to produce insertions into surrounding genes or deletions. The *Ds* element can carry various constructs for use in RNA or protein expression, promoter-trapping, enhancer-trapping, gene-trapping or activation tagging. Such methods are well known to skilled artisans.

[0092] A *Ds* element can integrate into either of two types of chromatin, functional DNA sequences where it may have a deleterious effect due to insertional mutagenesis or non-functional chromatin where it may not have much of a consequence. This power of "transposon tagging" has been exploited in simpler model systems for nearly two decades (Bingham et al., 1981; Bellen et al., 1989). Transposon tagging is an old technique in which transgenic DNA is delivered to cells so that it will integrate into genes, thereby inactivating them by insertional mutagenesis. In the process, the inactivated genes are tagged by the transposable element which then can be used to recover the mutated allele. Insertion of a transposable element may disrupt the function of a gene which can lead to a characteristic phenotype. Because insertion is approximately random, the same procedures that generate insertional, loss-of-function mutants can often be used to deliver genes that will confer new phenotypes to cells. Gain-of-function mutants can be used to understand the roles that gene products play in growth and development as well as the importance of their regulation.

[0093] There are several ways of isolating the tagged gene. In all cases genomic DNA is isolated from cells from one or more tissues of the mutated animal by conventional techniques (which vary for different tissues and animals). The DNA is cleaved by a restriction endonuclease that may or may not cut in the transposon tag (more often than not it does cleave at a known site). The resulting fragments can then either be directly cloned into plasmids or phage vectors for identification using probes to the transposon DNA (for references, see Kaiser et al., 1995). Alternatively, the DNA can be PCR amplified in any of many ways; we have used the LM-PCR procedure of Izsvak and Ivics (1993) and a modification by Devon et al. (1995) and identified by its hybridization to the transposon probe. Alternative methods include inverse-PCR (e.g., Allende et al., 1996) TAIL-PCR (Liu & Whittier, 1995), etc. Alternatively, other methods of amplification can be used in place of PCT. Regardless of method for cloning, the identified clone is then sequenced. The sequences that flank the transposon (or other inserted DNA) can be identified by their non-identity to the insertional element. The sequences can be combined and then used to search the nucleic acid databases for either homology with other previously characterized gene(s), or partial homology to a gene or sequence motif that encodes some function. In some cases the gene has no homology to any known protein. It becomes a new sequence to which others will be compared. The encoded protein will be the center of further investigation of its role in causing the phenotype that induced its recovery.

[0094] The presently described invention allows for large-scale genetic analysis of the genome of zebrafish. The library is constructed by transfecting embryos by standard techniques, or preferably, by injecting embryos with the *Ds* elements described herein. The injected embryos also receive proper care and feeding once they have fully consumed their yolk at day 5. This approach generates enough fish to harbor, on average, more than one *Ds* element per gene in the zebrafish.

[0095] Characterization of the inserts, in terms of where in the genome (i.e., specific sequence) they have landed, takes place once the founder males have reached adulthood and can produce significant quantities of sperm (about 4 months). Briefly, two days before sperm collection, each injected male is mated with wild-type females. Successfully mated males are kept individually for two days in disposable 16 oz cups while the overall health of their clutch is examined. Fecund males then have their testes removed for sperm freezing and storage. DNA from the 5-day old offspring of each male is extracted and analyzed using conventional techniques, such as TAIL-PCR, inversed PCR and the like.

[0096] Completion of sequencing the DNA flanking the inserts for all the founder fish, yields a library of the inserts. These samples are easily contained in cryovials in a liquid nitrogen storage unit. For safety purposes, each sample is split for storage in multiple liquid nitrogen units. When a sample is needed for testing it is thawed and then used for *in vitro* fertilization on eggs from wild type females to generate fish that harbor the desired inserts. Since multiple freeze/thaw cycles can decrease the viability of the sperm sample, secondary sperm samples are generated from all of the newly created males once they have reached adulthood. The library allows for the completion of numerous genetic screens. The library comprises a set or population of zebrafish sperm in which each member of the set possesses at least one mutation in at least one gene, and the set or population of zebrafish sperm collectively comprises at least 15% of the genes, or at least 20% of the genes, or at least 25% of the genes, or at least 30% of the genes, or at least 35% of the genes, or at least 40% of the genes, or at least 45%, or at least 50%, or at least 55% of the genes, or at least 60% of the genes, or at least 65% of the genes, or at least 70%, at least 75% of the genes, or at least 80% of the genes, or at least 85% of the genes, or at least 90% of the genes, or at least 95% of the genes, or at least 98% of the genes, or at least 99% of the genes, or 100% of the genes contain a mutation.

[0097] Alternatively, a set or population of fish is generated from the founder fish or their progeny, in which the set of fish collectively comprises a substantially comprehensive library of the inserts. The fish and their progeny are easily maintained in an automated aquarium system. These fish can easily be raised, tagged for individual identification system, and stored in approximately 200 fish tanks, which is a small sized aquatic facility for a researcher. The library allows for the completion of numerous genetic screens. The library comprises a set or population of zebrafish in which each member of the set possesses at least one mutation in at least one gene, and the set or population of zebrafish collectively comprises at least 15% of the genes, or at least 20% of the genes, or at least 25% of the genes, or at least 30% of the genes, or at least 35% of the genes, or at least 40% of the genes, or at least 45%, or at least 50%, or at least 55% of the genes, or at least 60% of the genes, or at least 65% of the genes, or at least 70%, at least 75% of the genes, or at least 80% of the genes, or at least 85% of the genes, or at least 90% of the genes, or at least 95% of the genes, or at least 98% of the genes, or at least 99% of the genes, or 100% of the genes contain a mutation.

[0098] On the basis of the draft of the zebrafish genome, it should be possible to identify which gene is disrupted by sequencing less than 50 bases of DNA flanking the insert. The sequences produced during the characterization of the inserts, provide a means to identify and

catalogue the genes mutated in each sperm sample or in each fish of the library. Such a database provides both an index for the presently disclosed libraries, and a resource for using the library. The library can be used to (a) identify the function(s) of genes of interest, (b) screen for genes that may encode useful or putative drug targets, (c) screen for genes that may encode the proteins acting as site(s) of action of drugs, toxins and other chemicals, and (d) screen compounds for modulating effects on proteins encoded by genes of interest. Alternatively, various comparisons can be made between the library database sequences and any other sequence database as would be familiar to those practiced in the art.

[0099] The novel utility of the library lies in the ability to search the library database for a gene of interest based upon some knowledge of the nucleic acid or amino acid sequence. Alternatively, the database could be searched for a gene of interest on the basis of knowledge of desired phenotype. With library of insertional mutations, it is possible to identify a sperm sample or a fish containing an insertion in any gene which is known or thought to produce a disease condition. The identified sperm can be used to breed progeny fish bearing the mutation and thereby enabling a means to study the gain-of-function or loss-of-function of the encoded gene product. Alternatively, the identified fish can be used to study the gain-of-function or loss-of-function of the encoded gene product. The library can be surveyed for mutations in specific genes by polymerase chain reaction methods using an oligonucleotide primer specific for the designated gene with another oligonucleotide specific for the insertion sequence. Alternatively the library can be searched by sequence homology using a standard BLAST query of the database composed of all library insertions. Alternately, the library can be screened to recover all insertions within a gene family for which one member is known to contribute to a disease condition. In addition, the library can be used to generate site-specific deletions using irradiation, for example, where the identified retroviral insertion is located near the gene, but is not disrupting the gene. Once a sequence is identified, the specific sperm or the specific fish in the library can be accessed and used to (a) identify the function(s) of genes of interest, (b) screen for genes that may encode useful or putative drug targets, (c) screen for genes that may encode the proteins acting as site(s) of action of drugs, toxins and other chemicals, and (d) screen compounds for modulating effects on proteins encoded by genes of interest. These studies are accomplished by means familiar to those practiced in the art. Transgenic zebrafish are directly generated from the sperm found in the library as is familiar to those practiced in the art.

[0100] Genetic mutations often correlate with disease (e.g., breast cancer, Parkinson's, obesity, ataxia telangiectasia, etc.). Given that the present invention allows for directed gene

discovery, additional embodiments of the present invention include methods for identifying the genetic basis of disease. For example, genetic mutations can often contribute to the disease state by altering the normal regulatory processes of the cell. As such, once a given transcription factor or regulatory protein has been associated with a given disease, the entire protein, or a relevant domain therefrom, may be used to identify genes directly or indirectly regulated by the protein or which interact with the protein. Consequently, the present invention may be used to identify the various proteins involved in a given disease or disease pathway. Diseases of particular interest include, but are not limited to: autoimmune disease, systemic lupus erythematosus, rheumatoid arthritis, inflammatory response, post-angioplasty vascular inflammatory response, bacterial or viral infection, inflammatory bowel disease, diabetes, multiple sclerosis, cancer, asthma, muscular dystrophy, Alzheimer's disease, dementia and other neuropathologies, hypertension, hemochromatosis, porphyrias, galactosemia, hyperlipoproteinemia, gout, interstitial lung disease, platelet disorders, myasthenia gravis, congenital heart disease, cystic fibrosis, and obesity. In addition, given that the present invention allows for directed gene discovery, additional embodiments of the present invention include methods for identifying gene function.

[0101] In addition to disease, the presently described methods and libraries are equally well suited for identifying the molecular basis for genetically determined advantages such as prolonged life-span, low cholesterol, low blood pressure, low cancer risk, low diabetes, low obesity, and attenuation of severity or prevention of all inflammatory disorders, including, but not limited to coronary artery disease, multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, and inflammatory bowel disease.

[0102] Additionally, the sequence information may be used to generate a highly specific probe for isolating both genomic clones from existing databases, as well as a full length cDNA or promoters or other regulatory sequences. Additionally, the probe may be used to isolate the homologous gene from sufficiently related species, including humans. Once isolated, the gene may be over expressed, or used to generate a targeted knock-out vector that may be used to generate cells and animals that are homozygous for the mutation of interest. Such animals and cells are deemed to be particularly useful as disease models (i.e., cancer, genetic abnormalities, AIDS, etc.), for developmental study, to assay for toxin susceptibility or the efficacy of therapeutic agents, and as hosts for gene delivery and therapy experiments (e.g., experiments designed to correct a specific genetic defect *in vivo*).

[0103] With a library of insertional mutations, it is possible to identify a sperm sample or a fish containing an insertion in any gene which is known to produce a disease condition. The identified sperm can be used to breed progeny fish bearing the mutation, thereby enabling a means to study the gain-of-function or loss-of-function of the encoded gene product. The fish can be used directly to study the gain-of-function or loss-of-function of the encoded gene product. The library can be surveyed for mutations in specific genes by polymerase chain reaction methods using an oligonucleotide primer specific for the designated gene with another oligonucleotide specific for the insertion sequence. Alternatively the library can be searched by sequence homology using a sequence alignment analysis of the database composed of all library insertions. For example, humans bearing mutations in ferrochelatase, an enzyme in the heme biosynthesis pathway, experience erythropoietic protoporphyria, a condition which shows light-dependent hemolysis and liver disease. While it is difficult to study damage initiated indirectly by light to non-cutaneous organ in mammals, it is possible to use zebrafish to study this condition in a non-invasive manner. Further it is possible to identify similarly acting mutations in paralogous genes and to study the resulting disease phenotypes in zebrafish. (Childs et al., 2000).

[0104] Transgenic animals produced using the information available from the presently described library are useful to (a) identify the function(s) of genes of interest, (b) screen for genes that may encode useful or putative drug targets, (c) screen for genes that may encode the proteins acting as site(s) of action of drugs, toxins and other chemicals, and (d) screen compounds for modulating effects on proteins encoded by genes of interest. These animals are also useful for the study of basic biological processes and diseases including, but not limited to, aging, cancer, autoimmune disease, immune disorders, alopecia, glandular disorder, inflammatory disorders, diabetes, arthritis, high blood pressure, atherosclerosis, cardiovascular disease, pulmonary disease, degenerative diseases of the neural or skeletal systems, Alzheimer's disease, Parkinson's disease, asthma, developmental disorders or abnormalities, infertility, epithelial ulcerations, and microbial pathogenesis.

[0105] One use of the library of the present invention is to rapidly identify the function of genes. Fish containing mutations of genes of interest, generated as described herein, may be examined for specific phenotypes, much as knockout mice are studied to determine the same. In accordance with this aspect of the invention, this use of the library is capable of identifying the function of genes that have been or can be missed using conventional and currently available cloning techniques. By using the library described herein, unknown and/or uncharacterized

genes and their function can be rapidly identified. The proteins encoded by these genes have use as, among other things, human therapeutics and diagnostics and as targets for drug discovery.

[0106] A "known" gene is directed to the level of characterization of a gene. The invention allows identification of genes that have been characterized, as well as identification of genes that have not been characterized. Different levels of characterization are possible. These include detailed characterization, such as cloning, DNA, RNA, and/or protein sequencing, and relating the regulation and function of the gene to the cloned sequence (e.g., recognition of promoter and enhancer sequences, functions of the open reading frames, introns, and the like). Characterization can be less detailed, such as having mapped a gene and related function, or having a partial amino acid or nucleotide sequence, or having purified a protein and ascertained a function. Characterization may be minimal, as when a nucleotide or amino acid sequence is known or a protein has been isolated but the function is unknown. Alternatively, a function may be known but the associated protein or nucleotide sequence is not known or is known but has not been correlated to the function. Finally, there may be no characterization in that both the existence of the gene and its function are not known. The invention allows identification of any gene at any of these or other specific degrees of characterization.

[0107] A gene of interest can be identified based on a phenotype conferred by the gene having the insert. Examples of selectable phenotypes include cellular proliferation, growth factor independent growth, colony formation, cellular differentiation (e.g., differentiation into a neuronal cell, muscle cell, epithelial cell, etc.), anchorage independent growth, activation of cellular factors (e.g., kinases, transcription factors, nucleases, etc.), expression of cell surface receptors/proteins, gain or loss of cell-cell adhesion, migration, cellular activation and phenotypes of disease conditions. Identification of gene function is important because the protein encoded by the gene or the gene itself is presumably responsible for the observed phenotype. Thus, the protein encoded by the gene or the gene itself may be an important therapeutic drug or drug target for treating or inducing the observed phenotype. In addition, this use of the library can identify all of the genes and proteins involved with a particular pathway or disease condition of interest, e.g., diabetes. The identification of such genes provides a collection of genes and/or proteins which are putative drug targets for the pathway or disease of interest. Since these genes are associated with specific zebrafish sperm or fish in the library, transgenic fish prepared as described herein can be used to screen potential drugs for use in treating or preventing the disease of interest.

[0108] For such screens and other screening methods described herein, the transgenic fish harboring the desired insert or representing all inserts can be screened using a number of assays. Depending on the characteristics of the protein(s) of interest (e.g., secreted versus intracellular proteins), the library can be screened for biochemical activity, enzymatic activity, gene regulation, phenotypic characteristic(s) and activity of a disease models, e.g., is the cell or organism resistant to an inserted transgene associated with a disease model. Other assay formats can also be used as known in a skilled artisan. In addition, the screening can be performed on different genetic states of the fish. In one embodiment, screening may be performed on fish produced by fertilizing zebrafish eggs with at least one sperm cell of the zebrafish sperm insertional library. In a second embodiment, screening may be performed on at least one founder fish or its progeny of the zebrafish insertional library. In one aspect of this embodiment, the progeny are heterozygote. In a second aspect of this embodiment, the progeny have been bred to be homozygote. In third embodiment, the screening may be performed on at least one haploid embryo. Haploid embryos may be prepared as described by, for example, Westerfield (2000). In a fourth embodiment, the screening may be performed on at least one gynogenetic diploid embryo. Gynogenetic diploid embryos may be prepared as described by, for example, Westerfield (2000). As used herein, screening is intended to include each of these embodiments, as well as isolated cells or secreted or excreted products as described further herein, and is intended to include *in vivo* and *in vitro* screening methods as described herein and as well known to skilled artisans.

[0109] A transgenic fish having a gene of interest can be exposed to compounds to assess the effect of the compound on the modulation of the protein encoded by the gene of interest and the biochemical pathway in which the protein encoded by the gene of interest is involved. For example, test compounds can be administered to transgenic fish harboring the gene of interest. Alternatively and preferably, the compound can be dosed in the water holding the transgenic fish, with the fish taking up substances via their gills. The compounds can also be dosed in the water holding the haploid embryos or the gynogenetic diploid embryos. By comparing the expression of the gene or protein encoded by the gene in fish exposed to a test compound to those that are not exposed, the effect of the compound on the modulation of the protein encoded by the gene of interest can be assessed. Test compounds can act as either inhibitors or activators of the gene. In this manner, compounds which are useful as drugs for treating or preventing disease conditions associated with gene of interest are identified. Similarly, toxins, potential therapeutic products or other chemicals can be screened using the present library to identify the

site of action of these compounds and to effect of these compounds on the genes or proteins of the library. By identifying fish that, by virtue of a specific mutation or mutation(s), no longer respond to a compound, a gene encoding the protein required for the action of said compound is identified.

[0110] The invention also encompasses zebrafish model-based assays for the identification of compounds exhibiting the ability to alter or correct phenotypes associated with the various genotypes identified and constructed using the present methods. Such model-based assays can also be used as a standard to assay for purity and potency of the compounds, including recombinantly or synthetically produced proteins or compounds.

[0111] In addition to the zebrafish based systems for identifying compounds of interest, other *in vitro* systems can also be used to identifying compounds that inhibit, activate or bind to proteins encoded by a gene of interest. The identified compounds may be useful, for example, in modulating the activity of wild type and/or mutant gene products. *In vitro* systems may also be utilized to screen for compounds that disrupt normal regulatory interactions.

[0112] The assays used to identify compounds that bind to proteins involve preparing a reaction mixture of a given protein and the test compound under conditions and for a time sufficient to allow the two components to interact and bind, thus forming a complex which can be removed and/or detected in the reaction mixture. The protein used can vary depending upon the goal of the screening assay. For example, where agonists of the natural ligand are sought, a full length protein, or a fusion protein containing a protein or polypeptide that affords advantages in the assay system (e.g., labeling, isolation of the resulting complex, etc.) can be utilized. In addition, *in vitro* assays may involve substances, enzymes, and the like which are secreted from the fish, which are then assayed.

[0113] The screening assays can be conducted in a variety of ways. For example, one method to conduct such an assay would involve anchoring the protein, polypeptide, peptide or fusion protein or the test substance onto a solid phase and detecting binding between the protein and test compound or mutant cell. In one embodiment of such a method, the receptor protein reactant may be anchored onto a solid surface, and the test compound, which is not anchored, may be labeled, either directly or indirectly. In another embodiment of the method, the test protein is anchored on the solid phase and is complexed with labeled antibody (and where a monoclonal antibody is used, it is preferably specific for a given region of the protein). Then, a test compound could be assayed for its ability to disrupt the association of the protein/antibody complex.

[0114] Alternatively, a reaction can be conducted in a liquid phase, the reaction products separated from unreacted components, and complexes detected; e.g., using an immobilized antibody specific for the test protein, polypeptide, peptide or fusion protein, or the test compound to anchor any complexes formed in solution, and a labeled antibody specific for the other component of the possible complex to detect anchored complexes.

[0115] The basic principle of the assay systems used to identify compounds that interfere with the interaction between a protein and its binding partner or partners involves preparing a reaction mixture containing the test protein, polypeptide, peptide or fusion protein as described above, and the binding partner under conditions and for a time sufficient to allow the two to interact and bind, thus forming a complex. In order to test a compound for inhibitory activity, the reaction mixture is prepared in the presence and absence of the test compound. The test compound can be initially included in the reaction mixture, or may be added at a time subsequent to the addition of the test protein and its binding partner. Control reaction mixtures are incubated without the test compound or with a placebo. The formation of any complexes between the test protein and the binding partner is then detected. The formation of a complex in the control reaction, but not in the reaction mixture containing the test compound, indicates that the compound interferes with the interaction of the test protein and the binding partner.

[0116] Further details concerning the above described *in vitro* systems and additional *in vitro* systems can be found in U.S. Patent No. 6,080,576.

[0117] A variety of test compounds can be evaluated in accordance with the present invention. In certain embodiments, the compounds to be tested can be derived from libraries (i.e., are members of a library of compounds). While the use of libraries of peptides is well established in the art, new techniques have been developed which have allowed the production of mixtures of other compounds, such as benzodiazepines (Bunin and Ellman, 1992; DeWitt et al., 1993), peptoids (Zuckermann, 1994), oligocarbamates (Cho et al., 1993), and hydantoins (DeWitt et al., 1993). An approach for the synthesis of molecular libraries of small organic molecules with a diversity of 10⁴-10⁵ as been described (Carell et al., 1994a; Carell et al., 1994b).

[0118] The compounds of the present invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries, synthetic library methods requiring deconvolution, the 'one-bead one-compound' library method, and synthetic library methods using affinity chromatography selection. The biological library approach is limited to

peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, 1997). Other exemplary methods for the synthesis of molecular libraries can be found in the art, for example in Erb et al. (1994), Horwell et al. (1996) and Gallop et al. (1994).

[0119] Libraries of compounds may be presented in solution (e.g., Houghten et al., 1992), or on beads (Lam et al., 1991), chips (Fodor et al., 1993), bacteria (U.S. Pat. No. 5,223,409), spores (U.S. Pat. No. 5,223,409), plasmids (Cull et al., 1992) or on phage (Scott and Smith, 1990; Devlin et al., 1990; Cwirla et al., 1990; Felici et al., 1991). In still another embodiment, the combinatorial polypeptides are produced from a cDNA library.

[0120] Exemplary compounds which can be screened for activity include, but are not limited to, peptides, nucleic acids, carbohydrates, small organic molecules, and natural product extract libraries.

[0121] The goal of rational drug design is to produce structural analogs of biologically active polypeptides of interest or of small molecules with which they interact (e.g., agonists, antagonists, inhibitors) in order to fashion drugs which are, for example, more active or stable forms of the polypeptide, or which, e.g., enhance or interfere with the function of a polypeptide *in vivo*. Several approaches for use in rational drug design include analysis of three-dimensional structure, alanine scans, molecular modeling and use of anti-id antibodies. These techniques are well known to those skilled in the art. Such techniques may include providing atomic coordinates defining a three-dimensional structure of a protein complex formed by said first polypeptide and said second polypeptide, and designing or selecting compounds capable of interfering with the interaction between a first polypeptide and a second polypeptide based on said atomic coordinates.

[0122] Following identification of a substance which modulates or affects polypeptide activity, the substance may be further investigated. Furthermore, it may be manufactured and/or used in preparation, i.e., manufacture or formulation, or a composition such as a medicament, pharmaceutical composition or drug. These may be administered to individuals.

[0123] A substance identified as a modulator of polypeptide function may be peptide or non-peptide in nature. Non-peptide "small molecules" are often preferred for many *in vivo* pharmaceutical uses. Accordingly, a mimetic or mimic of the substance (particularly if a peptide) may be designed for pharmaceutical use.

[0124] The designing of mimetics to a known pharmaceutically active compound is a known approach to the development of pharmaceuticals based on a "lead" compound. This approach

might be desirable where the active compound is difficult or expensive to synthesize or where it is unsuitable for a particular method of administration, e.g., pure peptides are unsuitable active agents for oral compositions as they tend to be quickly degraded by proteases in the alimentary canal. Mimetic design, synthesis and testing is generally used to avoid randomly screening large numbers of molecules for a target property.

[0125] Once the pharmacophore has been found, its structure is modeled according to its physical properties, e.g., stereochemistry, bonding, size and/or charge, using data from a range of sources, e.g., spectroscopic techniques, x-ray diffraction data and NMR. Computational analysis, similarity mapping (which models the charge and/or volume of a pharmacophore, rather than the bonding between atoms) and other techniques can be used in this modeling process. Such techniques include those disclosed in U.S. Patent No. 6,080,576.

[0126] A template molecule is then selected, onto which chemical groups that mimic the pharmacophore can be grafted. The template molecule and the chemical groups grafted thereon can be conveniently selected so that the mimetic is easy to synthesize, is likely to be pharmacologically acceptable, and does not degrade *in vivo*, while retaining the biological activity of the lead compound. Alternatively, where the mimetic is peptide-based, further stability can be achieved by cyclizing the peptide, increasing its rigidity. The mimetic or mimetics found by this approach can then be screened to see whether they have the target property, or to what extent it is exhibited. Further optimization or modification can then be carried out to arrive at one or more final mimetics for *in vivo* or clinical testing.

[0127] With regard to intervention, any compounds which reverse any aspect of a given phenotype or expression of any gene *in vivo* and which modulates protein activity or binding with binding partner *in vitro* should be considered as candidates for further development or potential use in humans. Dosages of test agents may be determined by deriving dose-response curves using methods well known in the art.

[0128] As previously noted, the present invention has been described with reference to zebrafish for convenience. It is understood that other fish and other animals can be used in place of zebrafish. Thus, it is understood that the present invention pertains to the use of the maize *Ac/Ds* transposable elements in vertebrates, including fish, birds, and other animals including mammals. Vertebrate cells can also incorporate the modified *Ds* element of this invention in the presence of the modified *Ac* transposase protein. Cells from fish, birds and other animals can be used, as can cells from mammals including, but not limited to, rodents, such as rats or mice, ungulates, such as cows, goats, sheep and pigs or cells from a human.

[0129] In the gene transfer system of this invention the modified *Ac* transposase protein can be introduced into the cell as a protein or as nucleic acid encoding the protein. In one embodiment the nucleic acid encoding the protein is RNA and in another, the nucleic acid is DNA. Further, nucleic acid encoding the modified *Ac* transposase protein can be incorporated into a cell through a viral vector, cationic lipid, or other standard transfection mechanisms including electroporation or particle bombardment used for eukaryotic cells. Following introduction of nucleic acid encoding modified *Ac* transposase, the modified *Ds* element of this invention can be introduced into the same cell. Alternatively, the modified *Ds* element of this invention can be introduced into the cell simultaneously with the modified *Ac* transposase protein or nucleic acid encoding the modified *Ac* transposase protein.

[0130] In a further aspect, the modified maize *Ac/Ds* transposable elements described above are useful for gene therapy in vertebrates, such as through the use of the above described gene transfer system for gene therapy. Gene therapy has the potential to improve the clinical outcome of many diseases, including cancers, by transferring therapeutic genes into diseased cells, including tumor cells, or normal host tissue. Gene transfer into tumor cells or tumor-associated stroma is being employed to induce tumor cell death, stimulate anti-tumor immune response, inhibit angiogenesis, and control tumor cell growth. Viral vectors have been used to achieve this proof of principle in animal models and, in select cases, in human clinical trials. Nevertheless, there has been considerable interest in developing nonviral vectors for cancer gene therapy. Nonviral vectors are simpler, more amenable to large-scale manufacture, and potentially safer for clinical use. Nonviral vectors were once limited by low gene transfer efficiency and transient or steadily declining gene expression. However, recent improvements in plasmid-based vectors and delivery methods are circumventing these obstacles. Nonviral vector technology for use in gene therapy includes transposons (Liu et al., 2006; Ohlfest et al., 2005; Essner et al., 2005; Hackett et al., 2005; Converse et al., 2004; Liu et al., 2004; Izsvak and Ivics, 2004; Kaminski et al., 2002; Richardson et al., 2002). Thus, the gene transfer system with the modified maize *Ac/Ds* transposable elements described herein is useful for gene therapy in vertebrates.

[0131] The practice of the present invention employs, unless otherwise indicated, conventional techniques of chemistry, molecular biology, microbiology, recombinant DNA, genetics, immunology, cell biology, cell culture and transgenic biology, which are within the skill of the art. See, e.g., Maniatis et al., 1982, *Molecular Cloning* (Cold Spring Harbor

Laboratory Press, Cold Spring Harbor, New York); Sambrook *et al.*, 1989, *Molecular Cloning*, 2nd Ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); Sambrook and Russell, 2001, *Molecular Cloning*, 3rd Ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); Ausubel *et al.*, 1992), *Current Protocols in Molecular Biology* (John Wiley & Sons, including periodic updates); Glover, 1985, *DNA Cloning* (IRL Press, Oxford); Anand, 1992; Guthrie and Fink, 1991; Harlow and Lane, 1988, *Antibodies*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); Jakoby and Pastan, 1979; *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. 1984); *Transcription And Translation* (B. D. Hames & S. J. Higgins eds. 1984); *Culture Of Animal Cells* (R. I. Freshney, Alan R. Liss, Inc., 1987); *Immobilized Cells And Enzymes* (IRL Press, 1986); B. Perbal, *A Practical Guide To Molecular Cloning* (1984); the treatise, *Methods In Enzymology* (Academic Press, Inc., N.Y.); *Gene Transfer Vectors For Mammalian Cells* (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); *Methods In Enzymology*, Vols. 154 and 155 (Wu *et al.* eds.), *Immunochemical Methods In Cell And Molecular Biology* (Mayer and Walker, eds., Academic Press, London, 1987); *Handbook Of Experimental Immunology*, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); Riott, *Essential Immunology*, 6th Edition, Blackwell Scientific Publications, Oxford, 1988; Hogan *et al.*, *Manipulating the Mouse Embryo*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986); Westerfield, M., *The zebrafish book. A guide for the laboratory use of zebrafish (Danio rerio)*, (4th Ed., Univ. of Oregon Press, Eugene, 2000).

EXAMPLES

[0132] The present invention is described by reference to the following Examples, which are offered by way of illustration and are not intended to limit the invention in any manner. Standard techniques well known in the art or the techniques specifically described below were utilized. The Examples demonstrate that the invention is applicable to and functions in all vertebrates as shown by studies utilizing zebrafish and a human cell line.

EXAMPLE 1

Materials and Methods

[0133] *Plasmid Constructs*

[0134] The construct containing *EGFP* (Clontech Laboratories, USA) under 2.25 kb promoter of *keratin 8 (krt8)* gene (GenBank accession number AF440690) was obtained from

Dr. Zhiyuan Gong from the National University of Singapore. The 3.1kb *krt8:EGFP* fragment was placed into a 0.6kb *miniDs* construct (Weil and Kunze, 2000).

[0135] The NLS-TPase cDNA was PCR-amplified using primers "Ac5'-1": CCAAAGAAG AAGCGTAAGGTAGAAATGGCTATTGTTTCATGAACCACA (SEQ ID NO:14) and "Ac3": GTATCGAT AAGCTTGATATCGAATTCC (SEQ ID NO:15) and pWL80 plasmid (Weil and Kunze, 2000) as a template. The product was used as a template in the secondary PCR using primers "Ac5'-2": CGCGGATCCGCCACCATGGGTCCTCCAAAGAAGAAGCGTAAGGT AG (SEQ ID NO:16) and "Ac3": GTATCGATAAGCTTGATATCGAATTCC (SEQ ID NO:17). The product containing nuclear localization sequence (NLS) (MGPPKKKRKVE (SEQ ID NO:2)) fused to a truncated *Ac* TPase₁₀₃₋₈₀₇ and Kozak sequence was digested with *Bam*HI and cloned into *Bgl*III site of the pSP64T vector (Krieg and Melton, 1984). The NLS^{K5E}-TPase was obtained by chance during cloning of the NLS-TPase construct due to a random mismatch in the primer. To produce the NoNLS construct, the corresponding NLS sequence was removed using QuikChangeTM Site Directed Mutagenesis Kit (Stratagene) and primers: CTCAACTTTG GCAGATCCGCCACCATGGCTATTGTTTCATGAACCACAACC (SEQ ID NO:38) and GGT TGTGGTTCATGAACAATAGCCATGGTGGCGGATCTGCCAAAGTTGAG (SEQ ID NO:39).

[0136] To produce NLS-TPase-EGFP and NLS^{K5E}-TPase-EGFP fusion constructs PCR amplified NLS-TPase and NLS^{K5E}-TPase fragments using primers AGAGGGATCCAGCTCA GAATAAACGCTCAAC (SEQ ID NO:40) and AGAGACCGGTCCTGGAGAGGAGCCAC TTGCTA (SEQ ID NO:41) and cloned it into *krt8-EGFP* plasmid (Gong et al., 2002) using *Age*I and *Bam*HI. To produce NLS-EGFP and NLS^{K5E}-EGFP constructs the *Ac* TPase₁₀₃₋₈₀₇ CDS sequence was deleted from the NLS-TPase-EGFP and NLS^{K5E}-TPase-EGFP constructs correspondingly using QuikChangeTM Site Directed Mutagenesis Kit (Stratagene) and primers: AGAAGAAGCGTAAGGTAGAAATGGTGAGCAAGGGCGAGGAGC (SEQ ID NO:42) and GCTCCTCGCCCTTGCTCACCATTCTACCTTACGCTTCTTCT (SEQ ID NO:43).

[0137] RNA Preparation and Injections

[0138] The TPase plasmid were linearized with *Bam*HI (cut downstream of the polyA tail) and used for *in vitro* transcription of capped transposase RNA. The mMMESSAGE mMACHINE SP6 kit (Ambion) was used. The product was purified using RNeasy Mini Kit (QIAGEN, Germany). 5-10 pg of plasmid DNA with 25-50 pg of *in vitro* synthesized transposase mRNA were co-injected into zebrafish embryos at the 1-2-cell stage. The actual amount of injected RNA was empirically adjusted to produce 50% embryo survival rate.

[0139] *Zebrafish*

[0140] Zebrafish was maintained according to established protocols (Westerfield, 2000).

[0141] *Analysis of the Ds Excision Sites*

[0142] Two primers flanking the *Ds* donor site that enclosed 3.7 kb long *Ds* and 120 bp of the flanking sequences were designed. The primers have the following sequences: GAGAATTT CACTTGTTGACTAGA (SEQ ID NO:18) and GCGCATGAACTCCTTGATGAC (SEQ ID NO:19). The PCR conditions without extension and with a short annealing time were used to prevent amplification of the long donor product: 94° C for 30 sec and 55° C for 10 sec for 35 cycles. Under these conditions only the 120 bp *Ds*-excision product could amplify but not 3.7 kb-long donor site that was present in excess. Products were separated using 1.8% agarose gel. The bands were cut from the gel, purified using QIAquick Gel Extraction Kit (QIAGEN, Germany), and sequenced using ABI Cycle Sequencing chemistry (PE Applied Biosystems, CA) and an ABI Prism 310 Genetic Analyzer with Data Collection Software (PE Applied Biosystems, USA) supplied by the producer.

[0143] *Analyses of Ds Flanking Sequences*

[0144] TAIL-PCR (thermal asymmetric interlaced PCR) was performed as described previously (Liu and Whittier, 1995; Parinov et al., 2004) using the following set of primers: Ds5'-1: CCGTTTACCGTTTTGTATATCCCG (SEQ ID NO:21); Ds5'-2: CGTTCCGTTTTTC GTTTTTTACC (SEQ ID NO:22); Ds5'-3: CGGTCGGTACGGGATTTTCC (SEQ ID NO:23); Ds3'-1: CGATTACCGTATTTATCCCGTTCG (SEQ ID NO:24); Ds3'-2: CCGGTATATCCC GTTTTTCG (SEQ ID NO:25); Ds3'-3: GAAATTGAAAACGGTAGAGGT (SEQ ID NO:26); AD-1: WGTGNAGNANCANAGA (SEQ ID NO:27); AD-2: WCAGNTGWTNGTNCTG (SEQ ID NO:28); AD-3: STTGNTASTNCTNTGC (SEQ ID NO:29); AD-4: NCASGAWAGNCSW CAA (SEQ ID NO:30). Products of the secondary and tertiary reactions were separated using 1.8% agarose gel. The individual bands from the "band shift" pairs were cut from the gel and purified using QIAquick Gel Extraction Kit (QIAGEN, Germany), and sequenced with Ds5'-3 and Ds3'-3 primers using ABI Cycle Sequencing chemistry (PE Applied Biosystems, CA) and an ABI Prism 310 Genetic Analyzer with Data Collection Software (PE Applied Biosystems, USA) supplied by the producer.

[0145] *Southern Blot Hybridization*

[0146] *EcoRI*-digested genomic DNA was fractionated by gel electrophoresis transferred to positively charged nylon membrane (Roche Applied Science, USA) by capillary blotting (Sambrook et al., 1989) and crosslinked by UV irradiation. The DNA probe for EGFP was

labeled with digoxigenin (Roche Applied Science, USA) using PCR DIG synthesis kit. We used DIG EasyHyb DIG Wash and Block Buffer Set for hybridization, alkaline phosphatase labeled anti-DIG antibody and CDP-Star chemiluminescent substrate (Roche Applied Science, USA) for detection of the hybridized probe. Hybridization and detection was carried out as described in the manufacture user's guide.

EXAMPLE 2

Experimental Design

[0147] A two-component system consisting of a donor construct with a non-autonomous *Ds* element, and a messenger RNA encoding a modified *Ac* transposase was utilized with an aim to produce *Ds* insertions in the zebrafish genome. The *Ds* construct carried EGFP gene under the zebrafish 2.25kb *keratin 8* (*krt8*) promoter (Gong et al., 2002), confined between 5'- and 3'-end *cis*-required sequences of *miniDs* element (Weil and Kunze, 2000) (Fig. 1A). The second construct harbored the coding sequence of a truncated *Ac* transposase (TPase₁₀₃₋₈₀₇) (Houba-Herlin et al., 19990) fused to an animal-specific synthetic nuclear localization signal (NLS; MGPPKKKKRKVE (SEQ ID NO:2)) analogous to that of SV40 large T antigen (Fig. 1B). The gene encoding such a chimeric NLS-TPase₁₀₃₋₈₀₇ fusion (NLS-TPase) was cloned into the pSP64T plasmid (Krieg and Melton, 1984) containing the SP6 promoter for *in vitro* transcription. This plasmid also contained the 5'- and 3'-UTRs of the *Xenopus β-globin* gene and a dA₃₂ polyA tail. Two similar TPase constructs were additionally made (Figs. 1B and 1C): one containing only the TPase₁₀₃₋₈₀₇ sequence without NLS (NoNLS-TPase) and the other containing an amino acid substitution (K to E) at the 5th position of the NLS (NLS^{K5E}-TPase).

[0148] Table 1 shows the nucleotide sequence of the modified *Ac* transposase constructs. Table 1A shows the NLS-containing construct and Table 1B shows the NLS^{K5E}-containing construct. Plasmid sequences are shown in lower case. The SP6 promoter (nucleotides 70-89 of SEQ ID NO:11 or 37) is shown in lower case bold italics. The Kozak sequence (nucleotides 152-157 of SEQ ID NO:11 or 37) is shown in caps preceding the coding sequence for the synthetic NLS (upper case, bolded and underlined; nucleotides 158-190 of SEQ ID NO:11 or 37). The coding sequence for truncated *Ac* TPase₁₀₃₋₈₀₇ is shown in upper case and bolded (nucleotides 191-2305 of SEQ ID NO:11 or 37) and the stop codon and 3'-UTR of *Ac* transposase is shown in upper case (nucleotides 2306-2477 of SEQ ID NO:11 or 37). The 5'-UTR of *β-globin* is represented by nucleotides 89-150 of SEQ ID NO:11 or 37. The 3'-UTR of *β-globin* is represented by nucleotides 2478-2624 of SEQ ID NO:11 or 37.

[0149] Table 2 shows the amino acid sequence of the modified *Ac* TPase. Table 2A shows the NLS-*Ac* TPase and Table 2B shows the NLS^{KSE}-*Ac* TPase. The synthetic NLS (bolded; amino acids 1-11 of SEQ ID NO:20) or NLS^{KSE} (bolded; amino acids 1-11 of SEQ ID NO:44) is fused to truncated *Ac* TPase₁₀₃₋₈₀₇ (Weil and Kunze, 2000; Houba-Herlin et al., 1990; amino acids 12-716 of SEQ ID NO:20 or 44).

TABLE 1

Nucleotide Sequence of the Modified *Ac* Transposase Construct

A. NLS-Containing Construct

...attaggcttgatcatatgtngtttagaagcggtgctacaattaatacacaaccttatgtatcatcacacatacgatt
taggtgacactatagaataacaagcttgctgttctttttgcagaagctcagaataaacgctcaactttggcagatcc
GCCACCATGGGTCTCTCCAAAGAAGAAGCGTAAGGTAGAAATGGCTATTGTTTCATGAACCACAACCACAACCACAACC
ACAACCAGAACCAACCAACCAAGCCACAACCTGAACCCGAAGAAGAAGCACCACAGAAGAGGGGCAAAGAAGTGCACAT
CGGATGTATGGCAGCATTTCACCAAGAAGGAAATTGAAGTGGAGGTCGATGGAAAGAAATACGTTTCAGGTATGGGGG
CATTGCAACTTTCCTAATTGCAAGGCTAAGTATAGGGCTGAGGGTCATCATGGAACAAGCGGATTTGAAATCACTT
GAGAACATCACATAGTTTAGTTAAAGGTCAGTTGTGTCTAAAAAGTGAAAAGGATCATGGCAAAGACATAAATCTCA
TTGAGCCCTTATAAGTACGATGAAGTGGTTAGCCCTAAAGAAGCTTCATTTGGCAATAATCATGCATGAATACCCCTTC
AATATTGTAGAACATGAGTACTTTGTTGAGTTTGTAAAGTCTCTGCGCCCTCACTTTCCAATAAAGTCCCGTGTCTC
TGCTAGAAAATATATCATGGATTGTATTTGGAAGAAAAAGAAAGTTGTATGGAAAACATAAAGATGTTTCAGTCTC
GCTTCAGTACAACATATGGATATGTGGACATCTTGTCAAATAAGTCATACATGTGTGTCCACATCCATTGGATTGAT
GATGATTGGTGTCTCCAAAAAGAATTGTTGGCTTTTTTCATGTTGAAGGGCGCCACACTGGCCAAAGGTTATCACA
AACCCTCACTGCAATCATGGTTAAGTGGAAACATTGAGAAAAAATTGTTTGGCTTGTCTTTGGATAATGCTAGTGCAA
ATGAAGTAGCTGTGCAGCATATAATTGAGGATTGTCAGGACACTGATTCAAATCTAGTTTGTGATGGTGTCTTCTTT
CATGTGAGGTGTGCTTGTACATACTGAACCTGGTTGCAAGGATGGCTTGGCTGTAATTGCAGGAACAATTGAGAA
AATCAAAGCGATTGTTCTTGTCTGTAATACTTCTCCTTTGCAGTGGGAAGAACTAATGAAGTGTGCTAGTGAATGTG
ACTTGGATAAATCTAAAGGGATCTCATATGATGTCTCACTAGATGGAATTCACCTATTGATGTTGAGGGATGCC
TTATATTATAAGCCTGCCTAATAAGGCTTAAACAAGTGATCCTCGCAGGTACGATGCAATTTGTCTTAAAGCCGA
GGAGTGAAGATGGCATTAACTCTTTTAAAGTGTGTTGAAGAAGTTTGTGATCTCACTGAACCTCTATCTGGTACTC
AATATTCCACTGCAAAATTTATTTTACAAAGGTTTCTGTGAGATAAAGGATTTGATTGACCAATGGTGTGTTTCATGAA
AAATTTGTTCATTAGGAGAATGGCCGTTGCAATGAGTGAAGGTTTGAAGAAATATTGGAAAGTGTCTAATATTGCACT
AGCTGTAGCATGCTTCTTGCACCTAGGTACAAGAAATATTGATTGAGTTCTATATGAAAAAATTTTCATGGTGATT
CATACAAAGTTCATGTAGATGACTTTGTTAGGGTCATTAGAAAATTTGTATCAATTCATTTCTAGTTGTAGTCTTCA
GCTCCAAAGACAAAGACAATACTAATGATAGTATGGATGATACCTTGATGGAAATGAAGATGATGAATTTCAAAA
CTATTTGCATGAGTTGAAGGATTATGATCAAGTAGAGTCAATGAATGGAATAAATATATGTCTGAACCCCTTTTGA
AGCATAGTGGTCAGTTTGTATTTTATCATGGTGGAGGGGAAGGGTTGCAGAAATATCTATTTCTACCCAAATTTGCA
AGGGATGTGCTAGCAATACAAGTGTCAACTGTTGCTTCTGAGTCTGCGTTTCAGTGTGCTGGTGGTGGTGGTGGTGGT
TTACCGCAATCGTCTTGGTTCGGAGATTGTTGAAGCTTTGATATGCACAAAAGATTGGGTAGCAGCATCTAGAAAAG
GTGCTACATATTTTCCAACAATGATTGGTGATCTCGAGGTGCTAGACTCTGTTATTTGCTGCTGCAACAAATCATGAG
AATCATATGGATGAGGATGAAGACGCAATAGAATTTTCTAAGAATAATGAAGATGTAGCAAGTGGCTCCTCTCCATC
AGCAATGTGTCTTATGTTTGTGACAGATGAGCCTTGGTTGTAATAGTTTATGTCATGCTAAGTGCTCCAGATGTGAG
CAAGTGATTATGAATATGTGTTTTAACTTTATATTGTGTGTCATGTGTGCTAGTAGACTTATATGGCTTCTTATGTTA
GCCAAGGGGGCCCCGGatctggttaccactaaaccagcctcaagaacacccgaatggagtctctaaagctacataat
accaacttacactttacaaaatgttggtccccaagatgtagccattcgtatctgctcctaataaaaaagaaagtttct
tcacattctaaaaaataaa
(SEQ ID NO:11)

B. NLS^{KSE}-Containing Construct

...attaggcttgatcatatgtngtttagaagcggtgctacaattaatacacaaccttatgtatcatcacacatacgatt
taggtgacactatagaataacaagcttgctgttctttttgcagaagctcagaataaacgctcaactttggcagatcc
GCCACCATGGGTCTCTCCAGAGAAGAAGCGTAAGGTAGAAATGGCTATTGTTTCATGAACCACAACCACAACCACAACC

Amino Acid Sequence of the Modified *Ac* TPase

MGPPKKKKRKVEMATVHEPQPQPQPQPEPQPQPQPEPEEEAPQKRAKCKTSDVWQHFTKKEIEVEVDGKKYVQVWGHC
NFPNCKAKYRAEGHHGTSGFRNHLRTSHSLVKGQLCLKSEKDHGKDINLIEPKYKDEVVSLKKLHLAIIIMHEYPFNI
VEHEYFVEFVKSLRPHFPIKSRVTARKYIMDLYLEEKEKLYGKLKDVQSRFSTTMDMWTSQNKSYMCVTIHWIDDD
WCLQKRIVGFFHVEGRHTGQRLSQTTATIMVKWNIKKLFLALSLDNASANEVAVHDIIEDLQDTSNLVCDGAFFHV
RCACHILNLVAKDGLAVIAGTIEKIKATVLAVKSSPLQWEELMKCASECDLDSKSGISYDVSTRWNSTYLMRLDALY
YKPALIRLKTSDPRRYDAICPKAEWKMALTLFKCLKFFDLTELLSGTQYSTANLFYKGFCEIKDLIDQWCVHEKF
VIRMAVAMSEKFEKYWKVSNIALAVACFLDPYKILIEFYMKKFHGDSYKVHVDDFVRVIRKLYQFYSSCSPSAP
KTKT'TTNDSDMTLMENEDDEFQNYLHELKDYDQVESNELDKYMSPELLKHSGQFDILSWWRGRVAEYPILTQIARD
VLAIQVSTVASESAFSAGGRVDPYRNRLGSEIVEALICTKWVAASRKGATYFPTMIGDLEVLDSVIAAATNHENH
MDEDEDAIEFSKNEDVASGSSP (SEQ ID NO:20)

MGPPPEKKRKVEMAIIVHEPQPQPQPQPEPQPQPQPEPEEEAPQKRAKKCTSDVWQHFTTKEIEVEVDGKKYVQVWGHC
NFPNCKAKYRAEGHHGTSGFRNHLRTSHSLVKQLCLKSEKDHGKDINLIEPYKYDEVVSLKKLHLAIIIMHEYPFNI
VEHEYFVEFVKSLRPHFPPIKSRVTARKYIMDLYLEEKEKLYGKLDVQSRFSTTMDMWTSLCKNKSIMCVTIHWIDDD
WCLQKRIVGFFHVGEGRHTGQRLSQTFTAIMVKWNIKKLFALSLDNASANEVAVHDIIEDLQDTSNLVCDGAFFHV
RCACHILNLVAKDGLAVIAGTTEKIKAIVLAVKSSPLQWEELMKCASECDLDSKSGISYDVSTRWNSTYLMRLDALY

YKPALIRLKTSDPRRYDAICPKAEWKMALTLFKCLKKFFDLTELLSGTQYSTANLFYKGFCEIKDLIDQWCVHEKF
 VIRRMAMSEKFEKYWKVSNIALAVACFLDPYKILIEFYMKKPHGDSYKVHVDDFVRVIRKLYQFYSSCSPSAP
 KTKTTTNDSDMTLMENEDDEFQNYLHELKDYDQVESNELDKYMSEPLLKHSQGFDILSWWRGRVAEYPILTQIARD
 VLAIQVSTVASESAFSAGGRVDPYRNRLGSEIVEALICTKDWVAASRKGATYFPTMIGDLEVLDSVIAAATNHNH
 MDEDEDAIEFSKNNEDVASGSSP (SEQ ID NO:44)

[0150] Table 3 shows the nucleotide sequence of the modified *Ds* construct. The EGFP gene under zebrafish *keratin 8* (*krt8*) promoter was confined between 247 bp of 5'-*Ds* end sequence (nucleotides 3657-3903 of SEQ ID NO:1) and 370 bp of 3'-*Ds* end sequence (nucleotides 43-412 of SEQ ID NO:1) (Weil and Kunze, 2000), shown in bolded, underlined upper case letters. These two *Ds* end sequences are also called "minimal *Ds*" implying that shortening it even further will negatively affect transposition efficiency. The enhanced green fluorescent protein (EGFP) gene was used for selection of transgenic fish. The *krt8*-promoter – EGFP (lower case) was confined between *Ds* 5'- and 3'-end sequences. The *krt8* promoter includes nucleotides 436-2674 of SEQ ID NO:1. The coding sequence for EGFP includes nucleotides 2669-3644 of SEQ ID NO:1.

TABLE 3

The *Ds* Construct Carrying *krt8*-promoter – EGFP.

CCATGGTGGCGACCGGTGCTCGACTCTAGAGGATCCCCGACATAGGGATGAAAACGGTCCGGTAAACGGTCCGGTAAAAAT
ACCTCTACCGTTTTCATTTTCATATTTAACTTGCGGGACGGAAACGAAACGGGATATACCGGTAAACGAAACGAAAC
GGGATAAATACGGTAATCGAAAACCGATACGATCCGGTTCGGTTAAAGTCGAAATCGGACGGGAACCGGTATTTTTC
TTCCGGTAAATCACACATGAAAACATATATTTCAAACTTAAAAACAATATATAAAAAATTGTAAACACAAGTCTTAAT
TAAACATAGATAAAATCCATATAAATCTGGAGCACACATAGTTTAAATGTAGCACATAAGTGATAAGTCTTGGGCTCT
TGGCTAACATAAGAAGCCATATAAGTCcctcgacctctagcttcgaattcccttcccttctacttttgacgtccttt
 taagagcttgtgcatgaaagcagatttggagctgattactcatctcaaacacccatacaaaagggatgattgcccgtac
 catgatctcacacctttcacacctgggtttatactatgatagttgtagacgattgcgtaattgctattaaatgcccatc
 agtgcctggctgtgacacccaactgctgccatttcgtgttgacttgcacgagaaatgagaaattgtctgactatgcag
 ggtgtctatgcgtgggaacatttatcagtggtcattaaatactatagtttacagtttagacaaagtgtgctgtatatt
 ttgtgttagcttagctgcagttttttgtgtgtgaagtaacaaatgacaaatactcaaacatttgtaattaagtagtatt
 ttctcagaaattgtaatttactaagtagtttaaaaatgtgtacttttactttcccttgagtacatttttagtgcaggt
 gttggtacttttatttccacttcttccactcctgcagtcactgttatttatttcttctgtctatgtggattagaca
 aatcagtcctgtgattcctgtocaaatcaaatgacacatgagaaggtaaatcacatcataatgaactaccttaagacat
 gggccattttataattgcagcaaaactgtttgccagcattaaaaagaagatgtcaaaaatatttcacacgcattaaaccag
 agactgcttagatgcatgtcactgatgagaagatgatggatgtttactgtatgatgaccgaaataaactttaaacgca
 cacaagacggcacaagacgtcaacatggcgttaggttgacgttgtaccccaacgcagtggggacgttgcattttgtt
 tagaaatgaaaattaggttgacgtcagaactcaacgtcaggtcgatgtcaatgttcaacatccaatctaaaatcata
 tatcaatgtctaatgatgttacagcttgatgttatgaggatgttaccctatgacgtctatcagacgttggattatg
 gttgccatacctgatgaataaatgtcattatttgacgttgggttaagatgttggttcogacattggatttttggtcgt
 ttccaacacaacctaaatccaccaaataattaacttccatgacatcggttattggacgtcaaaaataacaatatcctta
 gatgctggctagactttgaatttaggtcaccacaacctatatttaacctaatattaacatcttatgatgttgtgtgc
 ctgctgggcaataactaaatgcactacagaatgttacgtttcacacacatgtaaatcacatgtaaatgcacagcttt
 tcacagcataatactcacttactactcttgagtacttttaaaaaagctacttttactcactacttttgagtaata
 tttaacactgatacttttactgcactacatttttaggcatttttagatatttttactatgatttttactactctt
 tccactactgcagccctcccatataatcgatgtttacacatatgggtggagtttagagccataatctcactatg
 ctttgttagccgctagcattactgtgcagaattgtgtgtgtgcacattttccaatatcaatacagaaggaaactgtg
 ttccctgttcccttgtaaatctcaacaatgcaactgttcagctcagggggaaaaatgccctgocagatccaaacggc
 tggcaaaagtgaatggaaaaaagcctttcattaatgtgaaagtgtgcgcgccccaccagataaaaagagcagag

gtaaacaatgctctctacggctgtccagccaaccagatactgagggcagaaacacaccccgtggcagatgggtgagagct
acactgtctttttccagagtttctactggaatgctgtcctcaagtctcaagcctctccttgacattctctcattccac
ctggggcaaagccccaggctgggtgtgacaacatttatcttaccactttctctctgtacctgtctaacaggtaggggt
gtgtgtgagagtgcgatgtgtgcaagtgcgtgtgtgtgtgagagcagtcagctccaccctctcaagagtgtgtata
aaattgggtcagccagctgctgagagacacgcagagggactttgactctcctttgtgagcaacctcctccactcactc
ctctctcagagagcactctcgtacctccttctcagcaactcaaagacacaggatccaccgggtcgccaccatgggtgag
caagggcgaggagctgttcacccgggtgggtgcccatcctgggtcgagctggacggcgacgtaaaccggccacaagttca
gcgtgtccggcgagggcgagggcgatgccacctacggcaagctgacctgaagttcatctgcaccaccgggaagctg
cccgtgccctggccaccctcgtgaccaccctgacctacggcggtgcagtgcttcagccgctaccccgaccacatgaa
gcagcacgacttcttcaagtccgccatgcccggaaggctacgtccaggagcgaccatcttcttcaaggacgacggca
actacaagaccgcgcccagggtgaagttcgagggcgacacccctgggtgaaccgcacatcgagctgaagggcatcgacttc
aaggaggacggcaacatcctggggcacaagctggagtacaactacaacagccacaacgtctatatcatggccgacaa
gcagaagaacggcatcaaggtgaacttcaagatccgccacaacatcgaggacggcgagcgtgcagctcgccgaccact
accagcagaacacccccatcgggcgacggccccgtgctgctgcccgaacaaccactacctgagcaccagtcgcccctg
agcaagacccccaacgagaagcgcgatcacatgggtcctgctggagttcgtgacccgcccgggatcactctcggcat
ggacgagctgtacaagtaagcgccgacgtctagatcataatcagccataccacattttagaggttctacttgc
tttaaaaaacctcccacacctccccctgaacctgaacataaaaatgaatgcaattgttgttgaactgtttattg
cagcttataatggttacaaataaagcaatagcatcacaaatttcacaaataaagcatttttttctactgcattctagt
tgtggtttgtccaaactcatcaatgggtcgagggatctgt**TTGCAGTCATCCCGAATTAGAAAAACGGTAACGAAACG**
GGATCATCCCGATTAAAAACGGGATCCCGGTGAAACGGTCGGGAAACTAGCTCTACCGTTTCGGTTTCGGTTTACCG
TTTTGTATATCCCGTTTCGGTTCCGTTTTCGTTTTTACCTCGGGTTCGAAATCGATCGGGATAAACTAACAAAAT
CGTTTATACGATAACGGTCGGTACGGGATTTTCCCATCTTACTTTTCATCCCTGCGTCGGGTATCGAGCTCGAATTAA
GCTTGATATCGAATTCCTGCAGGAAAAAAAACAATTAGAATTAATTTTATATTATACAATTTATGGTGC (SEQ
ID NO:1)

EXAMPLE 3

Co-Injection of Ds Donor Construct with TPase mRNA Causes Specific Ds Excision

[0151] *In vitro* transcribed, capped and polyadenylated TPase mRNA was microinjected together with the non-linearized *Ds* donor plasmid, into zebrafish embryos at the one-cell stage. The embryos were incubated for 10 hours at 28°C and the genomic DNA was analyzed by excision PCR with primers flanking the *Ds* sequence (see methods for description). The excision products were detected only in the embryos injected with both the TPase mRNA (NLS^{KSE}-TPase or NoNLS-TPase) and the *Ds* construct, whereas the control embryos injected with the *Ds* construct alone produced no PCR fragments of the expected lengths (Fig. 2A). Surprisingly the NLS-TPase failed to produce *Ds* excision products in contrast to the NLS^{KSE}-TPase that produced the highest yield. The NoNLS-TPase required 5 times more RNA to induce excision at a level similar to the NLS^{KSE}-TPase. Based on these preliminary excision data the NLS^{KSE}-TPase was selected as the most productive, and it was used in the majority of the experiments. The TPase-mediated excision of the *Ds* element from its donor site was further confirmed by nucleotide sequencing of the PCR-amplified excision derivatives. Analysis of these sequences revealed that excision occurred specifically at the *Ds*-termini consistent with transposition. Since excision PCR products were expected to contain a mixture of various excision-repair events, we anticipated mixed sequencing patterns beginning at the junction of the

Ds and the adjacent vector. However, dominant sequence patterns were observed in products from two vectors with different *Ds* flanking sequence, indicative of preferential excision (Fig. 2B). The predominant excision footprints involved deletion of a flanking nucleotide immediately adjacent to one *Ds*-end accompanied by change or deletion of a flanking nucleotide at the other *Ds*-end.

EXAMPLE 4

Nuclear Localization Signals Affect Intracellular Localization of *Ac* TPase and Aggregation

[0152] To investigate the effects of different NLSs on intracellular localization, *krt8*:TPase-EGFP fusion constructs for all three TPases (NoNLS-, NLS- and NLS^{KSE}-TPase) were produced. Promoter *krt8* drives expression into single layer of epithelial tissue that consists of large and flat cells that are very convenient model to observe cellular localization. These constructs were injected into zebrafish embryo at 1 cell stage and GFP fluorescence was observed at 24 hpf stage. High level of expression of any of the three TPase constructs was toxic to the embryos. Injection of 15 pg of plasmid caused over 50% lethality during the first 24 hours of development and the surviving embryos were mostly devoid of *krt8*-specific GFP expression. Injection of 3 pg of plasmid produced mainly GFP-negative embryos. We assumed that expression of high (easily visible by GFP fluorescence) amount of the TPase fusion proteins was toxic for the cells. Nevertheless, in each case we found a small proportion of GFP-positive cells that retained their epithelial shape, thus allowing the identification of the intracellular localization of the GFP-tagged TPase therein (Figs. 3A-3C). The NoNLS-TPase-EGFP was found to localize mainly to the cytoplasm, whereas NLS-TPase-EGFP and NLS^{KSE}-TPase-EGFP were predominantly nuclear. The NoNLS-TPase-EGFP and NLS-TPase-EGFP showed a strong tendency to form aggregates in the cytoplasm and nucleus respectively (Figs. 3A-3C), that resembled the *Activator* TPase aggregates reported in plants (Boehm et al., 1995; Heinlein et al., 1994). On the contrary, the NLS^{KSE} rarely gave rise to aggregates even at visibly higher expression levels. To confirm that both NLS and NLS^{KSE} are functional in zebrafish cells, we analyzed subcellular localization of NLS-EGFP and NLS^{KSE}-EGFP fusion proteins in the similar experiments (Figs. 3D-3F). We observed a gradual increase in the nucleus to cytoplasm ratio in the distribution of NoNLS-EGFP, NLS^{KSE}-EGFP and NLS-EGFP respectively.

EXAMPLE 5

Modified *Ac* Transposase Induces High Rates of
Germinal *Ds* Insertions That Require Nuclear Localization

[0153] The injected embryos were raised to adulthood and out-crossed to the *wt* fish. We made absolutely no selection based on intensity and/or abundance of the GFP signal in the injected embryos: all the injected embryos were raised regardless of their GFP expression. Approximately 60% of the founders (F_0) injected with NLS-TPase or NLS^{K5E}-TPase produced offspring (F_1) containing GFP-fluorescent embryos (Table 4). The ratios of EGFP-positive embryos among the progeny was also striking: ~10% of positive founders produced progenies containing more than 50% of GFP-positives with multiple expression patterns including one F_0 fish which produced 100% GFP-positive progeny. The ratios were counted at 4 dpf to avoid possible maternal expression (Parinov et al., 2004). This was also considered as an indication of high transposition activity and it also suggested that *Ac/Ds* transposition probably occurred early in development. Founders injected with NoNLS-TPase produced significantly lower transgenesis rate. We did not observe any GFP-positive offspring in the control population injected with the *Ds* construct alone (integration of circular DNA being ineffective in zebrafish).

TABLE 4

Transgenesis Efficiencies of Different TPase Constructs.

	No NLS	NLS ^{K5E}	NLS
F_0 screened:	26	91	20
F_0 producing GFP-positive F_1 :	2	52	12
Transgenesis rate:	8%	57%	60%
Highest F_1 GFP ratio:	14/67 (21%)	133/138 (96%)	250/250 (100%)

Transgenesis rates are calculated as percent of founders producing GFP-positive offspring among the total number of screened founders. The last row shows the highest ratios of GFP-positive embryos among the F_1 progenies.

EXAMPLE 6

Integration of *Dissociation* Element into Zebrafish Genome

[0154] We isolated DNA sequences flanking *Ds* insertions in the F_1 fish using thermal asymmetric interlaced PCR "TAIL-PCR" (Liu and Whittier, 1995). Twenty-eight nonredundant flanking sequences thus obtained perfectly matched zebrafish nucleotide sequences from GenBank or Ensembl databases. The match in each instance started from the first nucleotide

adjacent to *Ds* 5'- or 3'-termini. Moreover, *Ds* insertions were flanked by the classic 8 bp direct duplication of the target site, typically accompanying *Ac/Ds* insertions in plants and other hAT transposons (Fig. 2C). Therefore, *Ds* integrated into the zebrafish genome through a specific TPase-mediated transposition mechanism. In a few F₁ fish, we isolated flanking sequences corresponding to the original *Ds* donor vector that were, however, accompanied by additional non-vector flanking sequence in the same F₁ fish. Twenty-one out of twenty eight identified *Ds* insertion sites were found within genes, suggestive of a potential preference for actively transcribed regions.

[0155] Southern blot hybridization with EGFP-specific probe revealed predominantly multiple insertions in different F₁ progeny (Fig. 4). The copy number ranged from 1 to 7 or more insertions per F₁ fish with an average of 4 insertions per genome. Different F₁ progeny from the same family (descendents from the same F₀ founder) often carried different independent insertions (Fig. 4, lanes 1-4).

[0156] Such an unusually high frequency of germline transposition suggests, firstly, that original host factors are dispensable and, secondly, that the new host environment does not suppress *Ds* transposition.

EXAMPLE 7

Transposition of the Genomic *Ds* Elements

[0157] To further validate transposition activity of the *Ac/Ds*, we remobilized the genomic *Ds* insertions by injecting TPase mRNA into the embryos of the transgenic fish carrying a single *Ds* insertion showing EGFP expression in the skin epithelia and the gut (Fig. 5). Over 90% of the embryos injected with NLS-TPase (80 out of 85) and NLS^{KSE}-TPase (69 out of 72) exhibited ectopic EGFP expression in various organs including brain, spinal chord, muscles, heart, liver, gonadal region etc. (Fig. 5). Ectopic expression of the EGFP reporter could be attributed to the enhancer-trap effect created as a consequence of the successful re-insertion of the excised *Ds* copy in each instance. No such effect was observed in the mock-injected control embryos. For mock injections we used RNA of *To12* transposase (Kaeakami et al., 2000) that apparently does not recognize the *Ds* sequence. Interestingly, injection of NoNLS-TPase caused a much lower rate of ectopic GFP expression (9 out of 168 injected embryos) compared to NLS-TPase and NLS^{KSE}-TPase. The novel patterns induced by NoNLS-TPase were simpler, usually affecting only a single cluster of same cell type. This experiment is additional supporting evidence that TPase required nuclear localization for genomic transposition. The embryos injected with

NLS^{KSE}-TPase were raised to maturity and out-crossed to *wt* fish. We found F₁ embryos with novel expression patterns among the progenies of 10 out of 13 screened founders (77%) (Fig. 5 and Table 5). Note that the real number of transpositions should be higher since we only detected the insertions that generated new distinguishable patterns. Sequences amplified from the F₁ embryos carrying such new expression patterns revealed novel *Ds* insertion sites that were not present in the original fish line. Hence, the modified *Ac* transposase is clearly capable of effectively transposing not only the *Ds* carried by the vector construct supplied via pan-embryonic injection, but also the *Ds* elements stably integrated into zebrafish nuclear genome. In addition to the unusually high re-insertion rate we observed frequently altered GFP segregation ratios (Table 5). One out of the thirteen founders produced GFP segregation ratio significantly higher than 1:1 (the expected ratio for an outcross of a single allele heterozygous parent) manifesting the increase of *Ds* copy number. Five out of thirteen founders demonstrated GFP segregation significantly lower than 1:1 indicative of a loss of the donor *Ds*. Altogether in 11 out of 13 (85%) screened founders we were able to detect the TPase activity by observing the presence of novel GFP expression pattern or/and by altered segregation ratio.

TABLE 5

Re-Transposition and Loss of *Ds* Donor
in the Transgenic Line Injected with RNA

F ₀ fish	GFP+/GFP-	New GFP patterns	GFP segregation
1	144/151	-	
2	70/78	+1	
3	118/130	+1	
4	175/93	+3	<i>Ds</i> copies increase
5	120/187	+2	loss of <i>Ds</i>
6	55/314	-	loss of <i>Ds</i>
7	56/170	+1	loss of <i>Ds</i>
8	41/122	+1	loss of <i>Ds</i>
9	225/201	+1	
10	74/253	+2	loss of <i>Ds</i>
11	165/172	-	
12	126/124	+2	
13	153/134	+1	

Transgenic embryos carrying a single heterozygous *Ds* insert were injected with NLS^{KSE}-TPase, raised to maturity and out-crossed to wild type fish. In F₁ generation we observed the occurrence of novel expression patterns and altered GFP segregation.

EXAMPLE 8

Ds Transposition of Human Embryonic Kidney Cell Line

[0158] We have also demonstrated *Ds* transposition in human cells. We utilized a different strategy compared to the one used in fish. Instead of a messenger RNA encoding a modified *Ac* transposase we used a plasmid DNA construct containing ORF of the modified NLS^{K5E}-*Ac* transposase under the CMV promoter. The *Ds* construct was also different: The *Ds* element carried EGFP gene under the CMV promoter, it also contained a blasticidin-resistance gene (BSD) and internal plasmid replication origin. Two plasmids were co-transfected into human embryonic kidney cell line HEK293, selected on blasticidin. The cells were harvested and their DNAs were analyzed by TAIL-PCR and sequencing for the presence of TPase-mediated *Ds* integrations in the human genome. We have successfully obtained such flanking sequences that perfectly matched human nucleotide sequences starting from the first nucleotide immediately adjacent to *Ds* 5'- or 3'-termini (Figure 6). Moreover, in one case the *Ds* insertions were flanked by the classic 8 bp direct duplication of the target site, typically accompanying hAT transposons. Therefore, intracellular environment of human cells is also suitable for the *Ds* transposition.

[0159] **Materials and Methods**[0160] *Transfection of Ac/Ds into HEK293 Cell Line*

[0161] 1) 2.5×10^5 HEK293 (ATCC# CRL-1573) cells per 6-well plate were seeded 24 hours before transfection in 2 ml DMEM/10%FBS at 5%CO₂.

[0162] 2) Cells were co-transfected with 0.5 mg each plasmid (pDs-CMV-EGFP-Ub-Bsd and pCMV-Ac). The GenePorter2 transfection reagent (GTS, CA, USA; Cat. #T202096) was used for transfection according to the recommended protocol.

[0163] 3) 24 hours after transfection, single-cell diluted cells were seeded in 96-well plate in DMEM/10%FBS containing 10 mg/ml Blasticidin (Invitrogen; #R210-01). The antibiotic selection of resistant to antibiotic colonies was continued during 6 days. After selection, the media was changed to DMEM/10%FBS and GFP positive colonies continue grow till confluence in 6-well plate.

[0164] 4) DNA from the cells was obtained using phenol/chloroform extraction followed by ethanol precipitation.

[0165] 5) Flanking sequences were obtained by TAIL-PCR.

[0166] *Constructs Used in Human Embryonic Kidney Cell Line*

[0167] *Ds Construct (pDS-CMV-EGFP-Ub-Bsd) (SEQ ID NO:70)*

[0168] bp 1-292, *Ds* 5'-end *cis*-required sequence:

[0169] CAGGGATGAAAGTAGGATGGGAAAATCCCGTACCGACCGTTATCGTATAACCGATTTTGTAGTTTTA
TCCCGATCGATTTGAAACCCGAGGTAAAAACGAAAACGGAACGGAAACGGGATATACAAAACGGTAAACGGAAACG
GAAACGGTAGAGCTAGTTTCCCGACCGTTTACCGGGATCCCGTTTTTAATCGGGATGATCCCGTTTCGTTACCGTA
TTTTCTAATTCGGGATGACTGCAACAGATCCCTCGAGCGCTTAAGTTTAAACGCGTTAACAAATTGGCCAT (SEQ
ID NO:45)

[0170] bp 293-1687, pEGFP-N3 plasmid fragment (bp 9-1403) containing pCMV-EGFP:

[0171] AATAGTAATCAATTACGGGGTCATTAGTTTCATAGCCCATATATGGAGTTCCGCGTTACATAACTTACG
GTAAATGGCCCGCTGGCTGACCGCCCAACGACCCCGCCCATTTGACGTCAATAATGACGTATGTTCCCATAGTAAC
GCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCCACTTGGCAGTACATCAAGTGT
ATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCTGGCATTATGCCAGTACATGACC
TTATGGGACTTTCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTGGCAGTA
CATCAATGGGCGTGGATAGCGGTTTGACTCAGCGGGATTTCCAAGTCTCCACCCCATTTGACGTCAATGGGAGTTTGT
TTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAACAACCTCCGCCCCATTGACGCAAAATGGGCGGTAGGCGT
GTACGGTGGGAGTCTATATAAGCAGAGCTGGTTTAGTGAACCGTCAGATCCGCTAGCGCTACCGGACTCAGATCTC
GAGCTCAAGCTTCGAATTTCTGCAGTCGACGGTACCGCGGGCCCGGGATCCATCGCCACCATGGTGAGCAAGGGCGAG
GAGCTGTTTACCGGGGTGGTGCCCATCCTGGTTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTTACGCGTGTCCGG
CGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTTCATCTGCACCACCGGCAAGCTGCCCGTGCCTT
GGCCACCCCTCGTGACCAACCTGACCTACGGCGTGCAGTGCTTACGCGCGTACCCCGACCATGAAGCAGCACGAC
TTCTTCAAGTCCGCCATGCCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGAC
CCGCGCCGAGGTGAAGTTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACG
GCAACATCTTGGGCGACAAGCTGGAGTACAACACTACAACAGCCACAACGTCATATCATGGCCGACAAGCAGAAGAAC
GGCATCAAGTCAAGCTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAA
CACCCCATCGGCGACGGCCCCGTGCTGCTGCCGACAACCACTACCTGAGCACCCAGTCCGCCCCGAGCAAGACCC
CCAACGAGAAGCGGATCACATGGTCTCTGCTGGAGTTCTGTGACCGCCCGGGATCACTCTCGGCATGGACGAGCTG
TACAAGTAAAGCGGCCGC (SEQ ID NO:46)

[0172] bp 1688-3403, pUBBSD1 plasmid fragment (bp 1917-3632) containing blasticidin-
resistance gene (BSD):

[0173] GATATCGCTAGCTCGAGATCGGGAGATCTGGCCTCCGCGCCGGGTTTTGGCGCCCCCGCGGGCGCC
CCTCCTCACGGCGAGCGCTGCCACGTACAGACGAAGGGCGCAGAGCGTCTGATCCTTCCGCCCCGACGCTCAGGAC
AGCGGCCCGCTGCTCATAAGACTCGGCCTTAGAACCCAGTATCAGCAGAAGGACATTTTAGGACGGGACTTGGGTG
ACTTAGGGCACTGGTTTTCTTTCCAGAGAGCGGAACAGGCGAGGAAAAGTAGTCCCTTCTCGGCGATTCTGCGGAG
GGATCTCCGTGGGGCGGTGAACGCCGATGATTATATAAGGACGCGCCGGGTGTGGCACAGCTAGTTCCGCTCGCAGCC
GGGATTTGGGTGCGGTTCTTGTGTTGTTGGATCGCTGTGATCGTCACTTGGTGAGTAGCGGGCTGCTGGGCTGGCCG
GGCTTTTCGGCCGCGCGCCGCTCGGTGGGACGGAAGCGTGTGGAGAGACCGCCAAAGGGCTGTAGTCTGGGTCCG
GAGCAAGGTTGCCCTGAAGTGGGGGTTGGGGGAGCGCAGCAAAATGGCGGCTGTTCCCGAGTCTTGAATGGAAGAC
GCTTGTGAGGCGGGCTGTGAGGTCGTTGAAACAAGGTGGGGGCGATGGTGGGCGGCAAGAACCCAAAGGTCTTGAGGC
CTTCGCTAATGCGGGAAGCTCTTATTCGGGTGAGATGGGCTGGGGCACCATCTGGGGACCCGACGTGAAGTTTGT
CACTGACTGGAGAAGCTCGGTTTGTCTGCTGTTGCGGGGCGCGAGTTATGGCGGTGCCGTTGGGCGAGTGCAACCCGTA
CCTTTGGGAGCGCGCGCCCTCGTCTGCTGCTGACGTCACCCGTTCTGTTGGCTTATAATGCAGGGTGGGGCCACCTG
CCGGTAGGTGTGCGGTAGGCTTTTCTCCGTCGACGAGCGCAGGGTTCGGGCTAGGGTAGGCTCTCCTGAATCGACA
GGCGCCGACCTCTGGTGGGGGAGGGATAAGTGGGCGTCAGTTCTTTGGTGGTTTTATGTACCTATCTTCTT
AGTAGCTGAAGCTCCGGTTTTGAAGTATGCGCTCGGGGTTGGCGAGTGTGTTTTGTGAAGTTTTTTAGGCACCTTTT
GAAATGTAATCATTTGGGTCAATATGTAATTTTCAAGTGTAGACTAGTAAATTTGTCGCTAAATTTTGGCCGTTTTT
GGCTTTTTTGTAGACCGGACCGTGTGACAATTAATCATCGGCATAGTATATCGGCATAGTATAATACGACAAGGT
GAGGAATAAACCATGGCCAAGCCTTTGTCTCAAGAAGAATCCACCTCATTGAAAGAGCAACGGCTACAATCAACA
GCATCCCCATCTCTGAAGACTACAGCGTCGCCAGCGCAGCTCTCTTAGCGACGGCCGATCTTCACTGGTGTCAAT
GTATATCATTTTACTGGGGGACCTTGTGCAAGACTCGTGGTCTGGGCACTGCTGCTGCTGCGGCGAGCTGGCAACCT
GACTGTATCTGTCGCGATCGGAAATGAGAACAGGGGCATCTTGAGCCCCGCGGACGGTGGCGACAGGTGCTTCTCG
ATCTGCATCTGGGATCAAAGCCATAGTGAAGGACAGTGTGAGACACCGGACGGCAGTTGGGATTCTGTGAATTGCTG
CCCTCTGGTTATGTGTGGGAGGGCTAAGCAC (SEQ ID NO:47)

[0174] bp 3404-4465, cloning vector fragment containing plasmid replication origin:

[0175] TTCGTGGCCGAGGAGCAGGACTGACACTCGACCTCGAAACTTGTATTATGACGCTTATAATGGTTACA
AATAAAGCAATAGCATCACAAATTTACAAATAAAGCATTTTTTCACTGCATTCTAGTTGTGGTTGTCCAAACTC
ATCAATGTATCTTATCATGTCTGAATTCGCGGGATCCTCTAGACTGTGACACCAAGTTTACTCATATATACTTTAG

ATTGATTTTAAACCTTCATTTTAAATTTAAAGGATCTAGGTGAAGATCCTTTTGTATAATCTCATGACCAAAATCCCC
TTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTC
TGCGCGTAATCTGCTGCTTGCACAAACAAAAAACACCGCTACCAGCGGTGGTTTGTGTCGGGATCAAGAGCTACCA
ACTCTTTTCCGAAGGTAAGTGGCTTCAGCAGAGCGCAGATACCAATACTGTTCTTCTAGTGTAGCCGTAGTTAGG
CCACCACCTTCAAGAACTCTGTAGCACCCGCTACATACCTCGCTCTGCTAATCCTGTTACCAAGTGGCTGCTGCCAGTG
GCATAAGTCTGCTTACCCGGTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTGCGGCTGAACGGGG
GGTTCGTGACACAGCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAG
CGCCACGCTTCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGACAGGTCGGAACAGGAGAGCGCACGAGGG
AGCTTCCAGGGGAAACGCCTGGTATCTTTATAGTCCTGTGCGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTGT
TGATGCTCGTCAGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCCTTTTACGGTTCCCGGCCCTTTGCTG
GCCCTTGTCTCACATGCTGGGCCCCAGCCGGCCAGATCTGAGCTCGCGGCCGATATCGCTAGCTCGAGG (SEQ
ID NO: 48)

[0176] bp 4466-4835, *Ds* 3'-end *cis*-required sequence:

[0177] GACTTATATGGCTTCTTATGTTAGCCAAGAGCCCAAGACTTATCACTTATGTGCTACATTAACTATG
TGTGCTCCAGATTTATATGGATTTTATCTATGTTTAAATTAAGACTTGTGTTTACAATTTTTTATATTTGTTTTAAG
TTTTGAATATATGTTTTCATGTGTGATTTTACCGAACAAAAATACCGGTTCCCGTCCGATTTTCGACTTTAACCCGAC
CGGATCGTATCGGTTTTCGATTACCGTATTTATCCCGTTCGTTTTCTGTTACCGGTATATCCCGTTTTCTGTTCCGTC
CCGCAAGTTAAATATGAAAATGAAAACGGTAGAGGTATTTTACCGACCGTTACCGACCGTTTTTCATCCCTA (SEQ
ID NO: 49)

[0178] bp 4836-6495, cloning vector fragment containing ampicillin resistance gene:

[0179] TGCATTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTTCGTTCA
TCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAAT
GATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACAGCCAGCCGAGCGGCGAGCGCAGAA
GTGGTCCCTGCAACTTTATCCGCTCCATCCAGTCTATTAATTGTTGCCGGAAGCTAGAGTAAGTAGTTTCGCCAGTT
AATAGTTTGCAGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTACGCTCGTCTGTTGGTATGGCTTCATTCAG
CTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCCTC
CGATCGTTGTGCAAGTAAGTTGGCCGCGAGTGTATCACTCATGTTTATGGCAGCACTGCATAATTTCTCTACTGTC
ATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACC
GAGTTGCTCTTGGCCCGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAA
AACGTTCTTCGGGGCGAAAACCTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTTCGATGTAAACCACTCGTGCACCC
AACTGATCTTCAGCATCTTTTACTTTTACCAGCGTTTCTGGGTGAGCAAAAAACAGGAAGGCAAAATGCCGCAAAAA
GGGAATAAGGGCGACACGGAATGTTGAATACTCATACTCTTCCCTTTTCAATATAATTGAAGCATTTATCAGGGTT
ATTGCTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGA
AAAGTGCCACCTAAATTTGTAAGCGTTAATATTTTGTAAAAATTCGCGTTAAATTTTGTAAATCAGCTCATTTTTT
AACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAGAATAGACCGAGATAGGGTTGAGTGTGTTCCAGT
TTGGAACAAGAGTCCACTATTTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAAACCGTCTATCAGGGCGATGGCC
CACTACGTGAACCATCACCTAATCAAGTTTTTTTGGGGTCGAGGTGCCGTAAAGCACTAAATCGGAACCTTAAAGGG
AGCCCCCGATTTAGAGCTTGACGGGGAAGCCGGCGAACGTGGCGAGAAAGGAAGGGAAGAAAGCGAAAGGAGCGGG
CGCTAGGGCGCTGGCAAGTGTAGCGGTACGCTGCGCGTAAACCAACACCCCGCGCTTAATGCGCCGCTACAGG
GCGCGTCCCATTGCGCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAG
CTGGCGAAAGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAAACGCCAGGGTTTTCCAGTCACGACGTTGTAAAC
GACGGCCAGTGAATTGATACGACTCACTATAGGGCGAATTGGGTACCCGACG (SEQ ID NO: 50)

[0180] *Ac*-TPase Construct (*pCMV-Ac*) (SEQ ID NO: 71)

[0181] DNA construct carrying NLS^{KSE}-TPase ORF under CMV promoter was made by cloning the PCR amplified NLS^{KSE}-TPase fragments using primers: Ac5Bam: gcgcGGATCCatagcatttagtgacactatag (SEQ ID NO: 51) and Ac3Not: cgatcgatgcGGCCgCCTT GGCTAACATAAGAAG (SEQ ID NO: 52) into *Bam*HI and *Not*I restriction sites of the pEGFP-N1 construct.

[0182] bp 1-665, pEGFP-N1 plasmid fragment (bp 1-665) containing promoter CMV:

[0183] tagttattaatagtaatacaattacgggggtcattagttcatagcccatatatggagttccgcgttacat
aacttacggttaaatggccccgcctggctgaccgccccaacgacccccgccattgacgtcaataatgacgtatgttccc
atagtaacgccaatagggactttccattgacgtcaatgggtggagttatttacggttaaaactgccacttggcagtaga
tcaagtgtatcatatgccaagtacgccccctattgacgtcaatgacggttaaatggccccgcctggcattatgccagt
acatgaccttatgggactttcctacttggcagtagatcactacgtattagtcacgtattaccatgggtgatggcgttt
tggcagtagatcactaattggcggtggatagcggtttgactacggggattttccaagtctccacccattgacgtcaatgg
gagtttgttttggcaccataatcaacgggactttccaaaatgtcgtaacaactccgccccattgacgcaaatggcg

gtaggcgtgtacggtgggaggtctatataagcagagctggttttagtgaaccgctcagatccgctagcgtaccggact
cagatctcgagctcaagcttcgaattctgcagtcgacggtaccgcgccggccgggatcc (SEQ ID NO:53)

[0184] bp 665-3066, amplified fragment containing NLS^{K5E}-TPase ORF sequence:

[0185] atacgatttaggtgacactatagaatacaagcttgcttgttctttttgcagaagctcagaataaaacgc
tcaacttttggcagatccGCCACCATGGGTCTCCAGAGAAGAAGCGTAAGGTAGAAATGGCTATTGTTTCATGAACCA
CAACCACAACCACAACCACAACCAGAACCACAACCACAGCCACAACCTGAACCCGAAGAAGAAGCACCACAGAAGAG
GGCAAAGAAGTGCACATCGGATGTATGGCAGCATTTACCAAGAAGGAAATTGAAGTGGAGGTCGATGGAAAGAAAT
ACGTTTCAGGTATGGGGGCATTGCAACTTTCTTAATTGCAAGGCTAAGTATAGGGCTGAGGGTCATCATGGAACAAGC
GGATTTGGAATCACTTTGAGAACATCACATAGTTTAGTTAAAGGTCAGTTGTGTCTAAAAAGTGAAAAGGATCATGG
CAAAGACATAAATCTCATTTGAGCCTTATAAGTACGATGAAGTGGTTAGCCTAAAGAAGCTTCATTTGGCAATAATCA
TGCATGAATACCCCTTCAATATTGTAGAACATGAGTACTTTGTTGAGTTTGTAAAGTCTCTGCGCCCTCACTTTCCA
ATAAAGTCCCGTGTCACTGCTAGAAAATATATCATGGATTGTATTGGAAGAAAAAGAAAGTTGTATGGAAAACT
ATAAGATGTTTCAGTCTCGCTTCAGTACAACATGATGATGTTGGACATCTTGTCAAAATAAGTCATACATGTGTGTCA
CCATCCATTGGATTGATGATGATTGGTGTCTCCAAAAAGAATTGTTGGCTTTTTTCATGTTGAAGGGCGCCACACT
GGCCAAAGGTTATCACAAACCTTCACTGCAATCATGGTTAAGTGGAAACATTGAGAAAAAATTTGCTTGTCTTTT
GGATAATGCTAGTGCAAATGAAGTAGCTGTGCACGATATAATTGAGGATTGTCAGGACACTGATTTCAAATCTAGTTT
GTGATGGTGTCTTCTTTCATGTGAGGTGTGCTTGTACATACTGAACCTGGTTGCAAAGGATGGCTTGGCTGTAAAT
GCAGGAACAATTGAGAAAAATCAAAGCGATTGTTCTTGTCTGTAATAATCTTCTCTTTGTCAGTGGGAAGAACTAATGAA
GTGTGCTAGTGAATGTGACTTGGATAAATCTAAAGGGATCTCATATGATGTCTCAACTAGATGGAATTCACCTATT
TGATGTTGAGGGATGCTTATATTATAAGCCTGCATAATAAGGCTTAAACAAGTGATCCTCGCAGGTACGATGCA
ATTTGTCCTAAAGCCGAGGAGTGAAGATGGCATTAACCTCTTTTAAAGTGTGTTGAAGAAGTTTGTGATCTCACTGA
ACTCTATCTGGTACTCAATATTCCTACTGCAATTTATTTTACAAAGGTTTCTGTGAGATAAAGGATTTGATTGACC
AATGGTGTGTTTCATGAAAAATTTGTCTATTAGGAAGATGGCCGTTGCAATGAGTGAAAAAGTTTGAGAAATATTGGAAA
GTGTCTAATATTGCCTAGCTGTAGCATGCTTCTTTCACCTTAGGTACAGTACAAGAAAAATATTGATTGAGTTCTATATGAA
AAAATTTTCATGGTGTATTCATACAAAGTTTCATGTAGTAGTACTTTGTTAGGGTCATTAGAAAAATTTGATCAATCTATT
CTAGTTGTAGTCTTTCAGCTCCAAAGACAAAGACAACCTACTAATGATAGTATGGATGATACCTTGATGGAAAAATGAA
GATGATGAATTTCAAACTATTTGCATGAGTTGAAGGATTATGATCAAGTAGAGTCAAATGAATTTGGATAAATATAT
GTCTGAACCCCTTTTGAAGCATAGTGGTCACTTTGATATTTTATCATGGTGGAGGGGAAGGGTTCAGAAATATCCTA
TTCTCACCCAAATTCGAAGGGATGTGCTAGCAATACAAGTGTCAACTGTTGCTTCTGAGTCTGCGTTCAGTGTCTGGT
GGTCTGTGTTGTTGATCCTTACCGCAATCGTCTTGGTTCGGAGATTGTTGAAGCTTTGATATGCACAAAAGATTGGGT
AGCAGCATCTAGAAAAGGTGCTACATATTTTCCAACAATGATTGGTGTATCTCGAGGTGCTAGACTCTGTTATTGCTG
CTGCAACAAATCATGAGAATCATATGGATGAGGATGAAGACGCAATAGAAATTTCTAAGAATAATGAAGATGTAGCA
AGTGCTCCCTCTCCATGAGCAATGTGTCTTATGTTTGTGACAGATGAGCCTTGGTTGTAATAGTTTATGCATGCTA
AGTGCTCAGTGTGAGCAAGTGATTATGAATATGTGTTTAACTTTATATTGTGTCTATGTGTCTAGTAGACTTA
TATGGCTTCTTATGTTAGCCAAG (SEQ ID NO:54)

[0186] bp 3067-6400, pEGFP-N1 plasmid fragment (bp 1400-4733):

[0187] gggggcgcgactctagatcataatcagccataccacattttagtagaggttttacttggcttttaaaaaaac
tcccacacccctcccccgaacctgaacataaaatgaatgcaattgtgtgtgtaacttggttattgcagcttataat
ggttacaaataaagcaatagcatcacaaatttcacaaataaagcatttttttactgcatcttagttgtggtttgtc
caaactcatcaatgtatcttaaggcgtaaatgtgaagcgtaaatattttgttaaaatttcgcttaaatttttgttaa
atcagctcatttttttaaccaataggccgaatcgccgaatcccttataaatcaaaagaatagaccgagataggggtt
gagtgttgttccagtttggacaagagtcactattaaagaacgtggactccaacgtcaaagggcgaaaaaccgtct
atcagggcgatggccactacgtgaacctcaccccaatcaagttttttggggtcgaggtgcccgtaaagcactaaat
cggaaccctaaagggagcccccgatttagagcttgacggggaaagccggcgaaacgtggcgagaaaggaaggaagaa
agcgaaagggagcgggcgctagggcgctggcaagtgtagcggtcacgctgcgcgtaaccaccacacccgcgcgctta
atgcgcgcgtacagggcgcgctcaggtggcacttttcggggaaatgtgcgcggaacccctatttgtttatttttctaa
atacattcaaatatgtatccgctcatgagacaataaccctgataaatgcttcaataatattgaaaaaggaagagtc
tgaggcggaagaaccagctgtggaatgtgtgtcagtttaggggtgtggaagtcgccaggtctcccgagcaggaag
tatgcaaagcatgcatctcaattagtcagcaaccatagtcgccgccctaaactccgcccataccgcccctaaactccgcccagt
tccgcccatttccgcccctaggtgactaatttttttttattttagcagagggccgagggccgctogggcctctgagct
attccagaagttagtgaggaggttttttggaggcctaggtttttgcaaagatcgatcaagagacaggatgaggatcg
tttcgcatgattgaacaagattgacgcaggttctccggccgcttgggtggagaggtctattccgctatgactg
ggcacaacagacaatcggtgctctgatgcgcgcgtgttcgggctgtcagcgagggggcgccggttcttttgtca
agaccgacctgtccggtgccctgaatgaactgcaagacgaggcagcgcggtatcggtggctggccacgacggggcgtt
ccttgcgcagctgtgctcgacgttgtcactgaagcgggaaggggactggctgctattgggggaagtgccggggcgagga
tctcctgtcacttcccttgcctcctgcccagaaaagtatccatcatggctgatgcaatgcgggggctgcatacgtt
atccggctaccttccgcttgcaccaccaagcgaaacatcgcatcgagcgagcagctactcggatggaagcggctctt
gtcgatcaggatgatctggacgaagagcatcaggggctcgccgagccgaactgttcgacaggtccaggcagcat
gcccagcgggcaggatctcgtcgtgacctatggcgatgcttggcgaatatcatggtggaaaatggccgctttt

ctggattcatcgactgtggcggcgtgggtgtggcggaccgctatcaggacatagcgttggctaccogtgcatttgc
gaagagcttggcggcgaatgggctgacgcttccctcgctgtttacgggtatcgccgctcccgattcgcagcgcacatgc
cttctatcgcttcttgacgagttcttctgagcgggactctgggggttcgaaatgaccgaccaagcgcagcggccaaacct
gccatcacgagatttgcattccacgcgccttctatgaaagggttgggcttcggaatcggttttcggggacgcgggct
ggatgatcctccagcgcggggtatctcatgctggagttcttcgcccaccctagggggaggttaactgaaacacggaag
gagacaataaccggaaggaaaccgcgctatgacggcaataaaaagacagaataaaaacgcacggtgttgggtcggttgt
tcataaacgcggggttcgggtcccagggtggcactctgtcgataccccaccgagaccccatgggggccaatacgccc
gcgtttcttcttcttccccaccccccaagttcgggtgaaggcccagggtcgcagccaacgtcggggcgga
ggccttgcctatagcctcaggttactcatatatacttttagattgattttaaaacttcatttttaattttaaaggatcta
ggtgaagatcctttttgataatctcatgacaaaaatcccttaacgtgagttttcgttccactgagcgtcagaccccg
tagaaaagatcaaaggatcttcttgagatccttttttctgcgcgtaactctgctgcttgcaaaaaaaaccacccg
ctaccagcgggtggtttgtttgcgggatcaagagctaccaactctttttccgaaggtaactggcttcagcagagcgca
gataccaaaactgtcttctagtgttagccgttagttaggccaccacttcaagaactctgtagcaccgcctacatacc
tcgctctgctaactcctgttaccagtggtgctgctgacgtggcgataagtcgtgtcttaccgggttggactcaagacga
tagttacccgataaggcgcagcgggtcgggctgaacggggggttcgtgcacacagcccagcttggagcgaacgacct
caccgaactgagatacctacagcgtgagctatgagaaagcgccacgcttcccgaaggagaaaggcggacaggtatc
cggtgaagcggcaggggtcggaacaggagagcgacaggggagcttcagggggaaacgcctgggtatctttatagtcct
gtcgggttttcgccacctctgacttgagcgtcgatttttgtgatgctcgtcagggggcgaggcctatggaaaaacgc
cagcaacgcggcctttttacgggttcctggccttttgcgtggccttttgcacatgttcttctcgttgcgttatccctg
attctgtggataaccgtattaccgccatgcat (SEQ ID NO:55)

[0188] The results of the analysis of Ds in three examples of transfected HEK293 cells are shown in Figure 6. The Ds (lower case letters) in these three examples is flanked by human DNA (upper case letters) (not the vector DNA of the donor) starting from the first nucleotide immediately adjacent to the Ds end sequence. This demonstrates that Ds integrated into human genome via transposase-mediated mechanism. In case of DNA 2 insertion site is surrounded by classic 8 bp direct repeat that is often created when hAT transposons integrate into new locations.

[0189] The use of the terms “a” and “an” and “the” and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms “comprising,” “having,” “including,” and “containing” are to be construed as open-ended terms (i.e., meaning “including, but not limited to,”) unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., “such as”) provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

[0190] Embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

BIBLIOGRAPHY

- [0191] Allen et al. (1988). "Transgene as probes for active chromosomal domains in mouse development." *Nature* 333:852-855.
- [0192] Allende, M.L. et al. (1996). "Insertional mutagenesis in zebrafish identifies two novel genes, pescadillo and dead eye, essential for embryonic development." *Genes Dev* 10:3141-3155.
- [0193] Altschul, S.F. et al., (1990). "Basic local alignment search tool." *J Mol Biol* 215:403-410.
- [0194] Altschul, S.F. et al. (1997). "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs." *Nucleic Acids Res* 25:3389-3402.
- [0195] Amsterdam, A. et al. (1999). "A large-scale insertional mutagenesis screen in zebrafish." *Genes Dev* 13:2713-2724.
- [0196] Andersen, J.K. et al. (1993). "Herpesvirus-mediated gene delivery into the rat brain: specificity and efficiency of the neuron-specific enolase promoter." *Cell Mol Neurobiol* 13:503-515.
- [0197] Balciunas, D. et al. (2004). "Enhancer trapping in zebrafish using the *Sleeping Beauty* transposon." *BMC Genomics* 5:62(1-15).
- [0198] Bellen, H.J. et al. (1989). "P-element-mediated enhancer detection: a versatile method to study development in *Drosophila*." *Genes Dev* 3:1288-1300.
- [0199] Bingham, P.M. et al. (1981). "Cloning of DNA sequences from the white locus of *D. melanogaster* by a novel and general method." *Cell* 25:693-704.
- [0200] Boehm, U. et al. (1995). "One of three nuclear localization signals of maize Activator (Ac) transposase overlaps the DNA-binding domain." *Plant J* 7:441-451.
- [0201] Bunin, B.A. and Ellman, J.A. et al. (1992). "A general and expedient method for the solid-phase synthesis of 1,4-benzodiazepine derivatives." *J. Am. Chem. Soc.* 114:10997-10998.
- [0202] Burns, J.C. et al., (1993). "Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells." *Proc Natl Acad Sci USA* 90:8033-8037.

- [0203] Carell, E. et al. (1994a). "A Novel Procedure for the Synthesis of Libraries Containing Small Organic Molecules." *Angew. Chem Int. Ed. Engl.* 33:2059-2061.
- [0204] Carell, E. et al. (1994b). "A Solution-Phase Screening Procedure for the Isolation of Active Compounds from a Library of Molecules." *Angew. Chem. Int. Ed. Engl.* 33:2061-2064.
- [0205] Chen, T.T. and Powers, D.A. (1990). "Transgenic fish." *Trends Biotechnol* 8:209-215.
- [0206] Chen, W. et al. (2002). "High-throughput selection of retrovirus producer cell lines leads to markedly improved efficiency of germ line-transmissible insertions in zebra fish." *J Virol* 76:2192-2198.
- [0207] Childs, S. et al. (2000). "Zebrafish dracula encodes ferrochelatase and its mutation provides a model for erythropoietic protoporphyria." *Curr Biol* 10:1001-1004.
- [0208] Cho, C.Y. et al. (1993). "An Unnatural biopolymer." *Science*. 261:1303-1305.
- [0209] Clark, J.T. et al. (1984). "Neuropeptide Y and human pancreatic polypeptide stimulate feeding behavior in rats." *Endocrinology* 115:427-429.
- [0210] Converse, A.D. et al. (2004). "Counterselection and co-delivery of transposon and transposase functions for Sleeping Beauty-mediated transposition in cultured mammalian cells." *Biosci Rep* 24:577-594.
- [0211] Cull, M.G. et al. (1992). "Screening for Receptor Ligands Using Large Libraries of Peptides Linked to the C Terminus of the lac Repressor." *Proc Natl Acad Sci USA* 89:1865-1869.
- [0212] Culp, P. et al. (1991). "High-frequency germ-line transmission of plasmid DNA sequences injected into fertilized zebrafish eggs." *Proc Natl Acad Sci USA* 88:7953-7957.
- [0213] Cwirla, S.E. et al. (1990). "Peptides on Phage: A Vast Library of Peptides for Identifying Ligands." *Proc. Natl. Acad. Sci. USA* 87:6378-6382.
- [0214] Davidson, A.E. (2003). "Efficient gene delivery and gene expression in zebrafish using the Sleeping Beauty transposon." *Dev Biol* 263:191-202.
- [0215] Davidson, B.L. et al. (1993). "A model system for in vivo gene transfer into the central nervous system using an adenoviral vector." *Nature Genetics* 3:219-223.
- [0216] Devlin, J.L. et al. (1990). "Random Peptide Libraries: A Source of Specific Protein Binding Molecules." *Science* 249:404-406.
- [0217] Devon, R.S. et al. (1995). "Splinkerettes--improved vectorettes for greater efficiency in PCR walking." *Nucl Acids Res* 23:1644-1645.
- [0218] DeWitt, S.H. et al. (1993). "Diversomers": An Approach to Nonpeptide, Nonoligomeric Chemical Diversity." *Proc. Natl. Acad. Sci. USA* 90:6909-6913.
- [0219] Driever, W. et al. (1996). "A genetic screen for mutations affecting embryogenesis in zebrafish." *Development* 123:37-46.
- [0220] Dujon, B. et al. (1994). "Complete DNA sequence of yeast chromosome XI." *Nature* 369:371-378.
- [0221] Ekker, S.C. (2000). "Morphants: a new systematic vertebrate functional genomics approach." *Yeast* 17:302-306.

- [0222] Emi, N. et al. (1991). "Pseudotype formation of murine leukemia virus with the G protein of vesicular stomatitis virus." *J Virol* 65:1202-1207.
- [0223] Erb, E. et al. (1994). "Recursive Deconvolution of Combinatorial Chemical Libraries." *Proc. Natl. Acad. Sci. USA* 91:11422-11426.
- [0224] Erickson, J.C. et al. (1996). "Sensitivity to leptin and susceptibility to seizures of mice lacking neuropeptide Y." *Nature* 381:415-421.
- [0225] Erzurum, S.C. et al. (1993). "Protection of human endothelial cells from oxidant injury by adenovirus-mediated transfer of the human catalase cDNA." *Nucleic Acids Res* 21:1607-1612.
- [0226] Essner, J.J. et al. (2005). "Awakening gene therapy with Sleeping Beauty transposons." *Curr Opin Pharmacol* 5:513-519.
- [0227] Fadool, J.M. et al. (1998). "Transposition of the *mariner* element from *Drosophila mauritiana* in zebrafish." *Proc Natl Acad Sci USA* 95:5182-5186.
- [0228] Fedoroff, N. et al. (1983). Isolation of the transposable maize controlling elements *Ac* and *Ds*. *Cell* 35:235-242.
- [0229] Felici, F. et al. (1991). "Selection of antibody ligands from a large library of oligopeptides expressed on a multivalent exposition vector." *J Mol. Biol.* 222:301-310.
- [0230] Fletcher, G.L., and Davis, P.L. (1991). "Transgenic fish for aquaculture." In *Genetic Engineering*, Setlow, J.K., ed., Plenum Press.
- [0231] Flotte, T.R. et al. (1993). "Stable in vivo expression of the cystic fibrosis transmembrane conductance regulator with an adeno-associated virus vector." *Proc Natl Acad Sci USA* 90:10613-10617.
- [0232] Fodor, S. et al. (1993). "Multiplexed biochemical assays with biological chips." *Nature* 364:555-556.
- [0233] Gallop, M.A. et al. (1994). "Applications of combinatorial technologies to drug discovery. 1. Background and peptide combinatorial libraries." *J. Med. Chem.* 37:1233-1251.
- [0234] Golling, G. et al. (2002). "Insertional mutagenesis in zebrafish rapidly identifies genes essential for early vertebrate development." *Nat Genet* 31:135-140.
- [0235] Gong, Z. et al. (2002). "Green fluorescent protein expression in germ-line transmitted transgenic zebrafish under a stratified epithelial promoter from keratin8." *Dev Dyn* 223:204-215.
- [0236] Gossler et al. (1989). "Mouse Embryonic Stem Cells and Reporter Constructs to Detect Developmentally Regulated Genes." *Science* 244:463-465.
- [0237] Graham, M., et al. (1997). "Overexpression of *Agrt* leads to obesity in transgenic mice." *Nat Genet* 17:273-274.
- [0238] Hackett, P.B. et al. (2005). "Sleeping beauty transposon-mediated gene therapy for prolonged expression." *Adv Genet* 54:189-232.
- [0239] Haffter, P. et al. (1996). "The identification of genes with unique and essential functions in the development of the zebrafish *Danio rerio*." *Development* 123:1-36.
- [0240] Halford, J.C. (2001). "Pharmacology of appetite suppression: implication for the treatment of obesity." *Curr Drug Targets* 2:353-370.

- [0241] Hay, B.A. et al. (1997). "P element insertion-dependent gene activation in the *Drosophila* eye." *Proc Natl Acad Sci USA* 94:5195-5200.
- [0242] Heasman, J. (2002). "Morpholino oligos: making sense of antisense?" *Dev Biol* 243:209-214.
- [0243] Heinlein, M. et al. (1994). "In vivo aggregation of maize Activator (Ac) transposase in nuclei of maize endosperm and *Petunia* protoplasts." *Plant J* 5:705-714.
- [0244] Horie, K. et al. (2001). "Efficient chromosomal transposition of a *Tc1/mariner*-like transposon *Sleeping Beauty* in mice." *Proc Natl Acad Sci USA* 98:9191-9196.
- [0245] Horwell, D. et al (1996). "Targeted' molecular diversity: design and development of non-peptide antagonists for cholecystokinin and tachykinin receptors." *Immunopharmacology* 33:68-72.
- [0246] Houba-Herlin, N. et al. (1990). "Excision of a *Ds*-like maize transposable element (*Ac delta*) in a transient assay in *Petunia* is enhanced by a truncated coding region of the transposable element *Ac*." *Mol Gen Genet* 224:17-23.
- [0247] Houghten, R.A. et al. (1992). "The use of synthetic peptide combinatorial libraries for the identification of bioactive peptides." *Biotechniques* 13:412-421.
- [0248] Izsvak, Z. and Z. Ivics (1993). "Two-stage ligation-mediated PCR enhances the detection of integrated transgenic DNA." *Biotechniques* 15:814-818.
- [0249] Izsvak, Z. and Z. Ivics (2004). "Sleeping beauty transposition: biology and applications for molecular therapy." *Mol Ther* 9:147-156.
- [0250] Kaiser, K. et al. (1995). "Eukaryotic transposable elements as tools to study gene structure and function." In *Mobile Genetic Elements*, Sheratt, D.J., ed., Oxford University Press, Oxford, pp. 69-100.
- [0251] Kaminski, J.M. et al. (2002). "Design of a nonviral vector for site-selective, efficient integration into the human genome." *FASEB J* 16:1242-1247.
- [0252] Kawakami, K. and T. Noda (2004). "Transposition of the *Tol2* element, an *Ac*-like element from the Japanese medaka fish *Oryzias latipes*, in mouse embryonic stem cells." *Genetics* 166:895-899.
- [0253] Kawakami, K. et al. (2000). "Identification of a functional transposase of the *Tol2* element, an *Ac*-like element from the Japanese medaka fish, and its transposition in the zebrafish germ lineage." *Proc Natl Acad Sci USA* 97:11403-11408.
- [0254] Kawakami, K. et al. (2004). "Excision of the *Tol2* transposable element of the medaka fish *Oryzias latipes* in *Xenopus laevis* and *Xenopus tropicalis*." *Gene* 338:93-98.
- [0255] Kimmel, C.B. (1989). "Genetics and Early Development of Zebrafish." *Trends Genet* 5:283-288.
- [0256] Koga, A. et al. (2002). "Gene transfer and coining of flanking chromosomal regions using the medaka fish *Tol2* transposable element." *Mar Biotechnol (NY)* 4:6-11.
- [0257] Kothary et al. (1988). "A transgene containing lacZ inserted into the dystonia locus is expressed in neural tube." *Nature* 335:435-437.
- [0258] Krieg, P.A. and D.A. Melton (1984). "Functional messenger RNAs are produced by SP6 in vitro transcription of cloned cDNAs." *Nucleic Acids Res* 12:7057-7070.

- [0259] Lam, K.S. (1997). "Application of combinatorial library methods in cancer research and drug discovery." *Anticancer Drug Des.* 12:145-167.
- [0260] Lam, K.S. et al. (1991). "A new type of synthetic peptide library for identifying ligand-binding activity." *Nature* 354:82-84.
- [0261] Lander, E.S. et al. (2001). "Initial sequencing and analysis of the human genome." *Nature* 409:860-921.
- [0262] Levine, A.S. and J.E. Morley (1984). "Neuropeptide Y: a potent inducer of consummatory behavior in rats." *Peptides* 5:1025-1029.
- [0263] Lever, A.M. (2000). "Lentiviral vectors: progress and potential." *Curr Opin Mol Ther* 2:488-496.
- [0264] Lin, S. et al. (1994). "Integration and germ-line transmission of a pseudotyped retroviral vector in zebrafish." *Science* 265:666-669.
- [0265] Liu, L. et al. (2005). "Endothelial targeting of the Sleeping Beauty transposon within lung." *Mol Ther* 10:97-105.
- [0266] Liu, L. et al. (2006). "Sustained FVIII Expression and Phenotypic Correction of Hemophilia A in Neonatal Mice Using an Endothelial-Targeted Sleeping Beauty Transposon." *Mol Ther* Feb 3; [Epub ahead of print].
- [0267] Liu, Y.G. and R.F. Whittier (1995). "Thermal asymmetric interlaced PCR: automatable amplification and sequencing of insert end fragments from P1 and YAC clones for chromosome walking." *Genomics* 25:674-81.
- [0268] Lu, J.K. et al. (1992). "Integration, expression and germ-line transmission of foreign growth hormone genes in medaka (*Oryzias latipes*)." *Molec Mar Biol Biotechnol* 1:366-375.
- [0269] Miklos, G.L. and G.M. Rubin (1996). "The role of the genome project in determining gene function: insights from model organisms." *Cell* 86:521-529.
- [0270] Miller, A.D. et al., (1993). "Use of retroviral vectors for gene transfer and expression." *Methods of Enzymology* 217:581-599.
- [0271] Mooslehner, K. et al. (1990). "Retroviral integration sites in transgenic Mov mice frequently map in the vicinity of transcribed DNA regions." *J Virol* 64:3056-3058.
- [0272] Nasevicius, A. and S.C. Ekker (2000). "Effective targeted gene 'knockdown' in zebrafish." *Nat Genet* 26:216-220.
- [0273] Ohlfest, J.E. et al. (2005). "Nonviral vectors for cancer gene therapy: prospects for integrating vectors and combination therapies." *Curr Gene Ther* 5:629-641.
- [0274] Ollmann, M.M. et al. (1997). "Antagonism of central melanocortin receptors *in vitro* and *in vivo* by agouti-related protein." *Science* 278:135-138.
- [0275] O'Kane, et al. (1987). "Detection in situ of Genomic Regulatory Elements in *Drosophila*." *Proc. Natl. Acad. Sci. USA* 84:9123-9127.
- [0276] Osborne, B.I. and B. Baker (1995). "Movers and shakers: maize transposons as tools for analyzing other plant genomes." *Curr Opin Cell Biol* 7:406-413.
- [0277] Parinov, S. et al. (1999). "Analysis of flanking sequences from *Dissociation* insertion lines: a database for reverse genetics in *Arabidopsis*." *Plant Cell* 11:2263-2270.

- [0278] Parinov, S. et al. (2004). "Enhancer trap transposable element as a tool for identification of developmentally regulated genes in zebrafish *in vivo*." *Dev Dyn* 231:449-459.
- [0279] Plasterk, R.H. (1993). "Molecular mechanisms of transposition and its control." *Cell* 74:781-786.
- [0280] Pohlman, R.F. et al. (1984). The nucleotide sequence of the maize controlling element *Activator*. *Cell* 37:635-643.
- [0281] Powers, D.A. et al. (1992). "Electroporation: a method for transferring genes into the gametes of zebrafish (*Brachydanio rerio*), channel catfish (*Ictalurus punctatus*), and common carp (*Cyprinus carpio*)." *Molec Mar Biol Biotechnol* 1:301-308.
- [0282] Qian, S. et al. (2002). "Neither agouti-related protein nor neuropeptide Y is critically required for the regulation of energy homeostasis in mice." *Mol Cell Biol* 22:5027-5035.
- [0283] Raz, E. et al. (1998). "Transposition of the nematode *Caenorhabditis elegans* *Tc3* element in the zebrafish *Danio rerio*." *Curr Biol* 8:82-88.
- [0284] Richardson, P.D. et al. (2002). "Gene repair and transposon-mediated gene therapy." *Stem Cells* 20:105-118.
- [0285] Rohdewohld, H. et al. (1987). "Retrovirus integration and chromatin structure: Moloney murine leukemia proviral integration sites map near DNase I-hypersensitive sites." *J Virol* 61:336-343.
- [0286] Rorth, P. (1996). "A modular misexpression screen in *Drosophila* detecting tissue-specific phenotypes." *Proc Natl Acad Sci USA* 93:12418-12422.
- [0287] Rorth, P. et al. (1998). "Systematic gain-of-function genetics in *Drosophila*." *Development* 125:1049-1057.
- [0288] Sakaguchi, T. et al. (2001). "A novel sox gene, 226D7, acts downstream of Nodal signaling to specify endoderm precursors in zebrafish." *Mech Dev* 107:25-38.
- [0289] Sambrook et al. (1989). *Molecular Cloning*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- [0290] Scherдин, U. et al. (1990). "Transcriptionally active genome regions are preferred targets for retrovirus integration." *J Virol* 64:907-912.
- [0291] Scott, J.K. and J.P. Smith (1990). "Searching for Peptide Ligands with an Epitope Library." *Science* 249:386-390.
- [0292] Shapiro, J.A. (1992). "Natural genetic engineering in evolution." *Genomics* 86:99-111.
- [0293] Shure, M. et al. (1983). Molecular identification and isolation of the *Waxy* locus in maize. *Cell* 35:225-233.
- [0294] Spradling, A.C. et al. (1995). "Gene disruptions using *P* transposable elements: an integral component of the *Drosophila* genome project." *Proc Natl Acad Sci USA* 92:10824-10930.
- [0295] Streisinger (1984). Attainment of Minimal Biological Variability and Measurements of Genotoxicity: Production of Homozygous Diploid Zebra Fish. *Natl. Cancer Inst. Monogr.* 65:53-58.

- [0296] Sulston, J. et al. (1992). "The C. elegans genome sequencing project: a beginning." *Nature* 356:37-41.
- [0297] Summerton, J. and D. Weller (1997). "Morpholino antisense oligomers: design, preparation, and properties." *Antisense Nucleic Acid Drug Dev* 7:187-195.
- [0298] Summerton, J. et al. (1997). "Morpholino and phosphorothioate antisense oligomers compared in cell- free and in-cell systems." *Antisense Nucleic Acid Drug Dev* 7:63-70.
- [0299] Talbot, W.S. and N. Hopkins (2000). "Zebrafish mutations and functional analysis of the vertebrate genome." *Genes Dev* 14:755-762.
- [0300] Tsang, M. et al. (2002). "Identification of Sef, a novel modulator of FGF signalling." *Nat Cell Biol* 4:165-169.
- [0301] Venter, J.C. et al. (2001). "The sequence of the human genome." *Science* 291:1304-1351.
- [0302] Vijaya, S. et al. (1986). "Acceptor sites for retroviral integrations map near DNase I-hypersensitive sites in chromatin." *J Virol* 60:683-692.
- [0303] Weil, C.F. and R. Kunze (2000). "Transposition of maize *Ac/Ds* transposable elements in the yeast *Saccharomyces cerevisiae*." *Nat Genet* 26:187-190.
- [0304] Westerfield, M. (2000). *The Zebrafish Book: A guide for the laboratory use of Zebrafish (Danio rerio)*, 4th ed., University of Oregon Press, Eugene.
- [0305] Wienholds, E. et al. (2002). "Target-Selected Inactivation of the Zebrafish *rag1* Gene." *Science* 297:99-102.
- [0306] Yee, J.K. et al. (1994). "A general method for the generation of high-titer, pantropic retroviral vectors: highly efficient infection of primary hepatocytes." *Proc Natl Acad Sci USA* 91:9564-9568.
- [0307] Zabner, J. et al. (1994). "Safety and efficacy of repetitive adenovirus-mediated transfer of CFTR cDNA to airway epithelia of primates and cotton rats." *Nature Genetics* 6:75-83.
- [0308] Zhang, G. et al. (1996). "An enhanced green fluorescent protein allows sensitive detection of gene transfer in mammalian cells." *Biochem Biophys Res Commun* 227:707-711.
- [0309] Zuckermann, R.N. (1994). "Discovery of nanomolar ligands for 7-transmembrane G-protein-coupled receptors from a diverse N-(substituted)glycine peptoid library." *J. Med. Chem.* 37:2678-2685.

WHAT IS CLAIMED IS:

1. A gene transfer system to introduce a polynucleotide into the DNA of a vertebrate cell comprising:
 - a modified *Ac* transposase or a nucleic acid encoding the modified *Ac* transposase, wherein the modified *Ac* transposase comprises a nuclear localization signal (NLS) at the N-terminus of a truncated *Ac* transposase, wherein the NLS localizes the transposition reaction to the nucleus of the vertebrate cell and wherein the truncated *Ac* transposase comprises an amino acid sequence having at least 80% identity to SEQ ID NO:8 and catalyzes the integration of a polynucleotide into DNA in a vertebrate cell; and
 - a nucleic acid fragment comprising a polynucleotide positioned between inverted repeats that can bind to the modified *Ac* transposase.

2. The gene transfer system of claim 1, wherein the inverted repeats are selected from the group consisting of:
 - (a) inverted repeats that are part of a *Ds* element;
 - (b) inverted repeats that are part of the *Ds* 5' end *cis*-required sequence and part of the *Ds* 3' end *cis*-required sequence;
 - (c) inverted repeats that are part of *Ds* 5' end *cis*-required sequence having the nucleotide sequence set forth in SEQ ID NO:45 and part of the *Ds* 3' end *cis*-required sequence having a nucleotide sequence set forth in SEQ ID NO:49;
 - (d) inverted repeats that are part of *Ds* 5' end *cis*-required sequence having the nucleotide sequence set forth in nucleotides 3657-3903 of SEQ ID NO:1 and part of the *Ds* 3' end *cis*-required sequence having a nucleotide sequence set forth in nucleotides 43-412 of SEQ ID NO:1; and
 - (e) inverted repeats that are part of a *Ds* 5' end *cis*-required sequence having at least 80% identity to the *Ds* 5' end *cis*-required sequence of (c) or (d) and part of a *Ds* 3' end *cis*-required sequence having at least 80% identity to the *Ds* 3' end *cis*-required sequence of (c) or (d), wherein the *Ds* 5' end *cis*-required sequence and *Ds* 3' end *cis*-required sequence can bind to the modified *Ac* transposase.

3. The genetic transfer system of claim 2, wherein the NLS comprises an amino acid sequence set forth in SEQ ID NO:2 or SEQ ID NO:9.

4. The gene transfer system of claim 3, wherein the modified *Ac* transposase comprises an amino acid sequence having at least 80% identity to SEQ ID NO:20 or SEQ ID NO:44 and catalyzes the integration of the polynucleotide into DNA in a vertebrate cell.
5. The gene transfer system of any one of claims 1 to 4 which comprises the modified *Ac* transposase.
6. The gene transfer system of any one of claims 1 to 4 which comprises the nucleic acid encoding the modified *Ac* transposase and wherein the nucleic acid is an RNA or DNA.
7. The gene transfer system of any one of claims 1 to 4 which comprises the nucleic acid encoding the modified *Ac* transposase and the nucleic acid is stably integrated into the genome of a non-human vertebrate.
8. The gene transfer system of any one of claims 1 to 4, wherein the nucleic acid fragment is part of a vector.
9. The gene transfer system of any one of claims 1 to 4, wherein the nucleic acid fragment is stably incorporated in the genome of a non-human vertebrate.
10. The gene transfer system of any one of claims 1 to 4, wherein the polynucleotide comprises at least a portion of an open reading frame.
11. The gene transfer system of claim 10, wherein the polynucleotide comprises a promoter operably linked to at least a portion of an open reading frame.
12. The gene transfer system of any one of claims 1 to 4, wherein the polynucleotide comprises at least one expression control region.
13. The gene transfer system of claim 12, wherein the expression control region is selected from the group consisting of a promoter, an enhancer or a silencer.

14. The gene transfer system of any one of claims 1 to 4, wherein the DNA into which the polynucleotide is capable of integrating is the cell genome.
15. A method for introducing a polynucleotide into the DNA of a vertebrate cell comprising the steps of:
 - introducing into the cell a nucleic acid fragment comprising a polynucleotide positioned between inverted repeats that can bind to a modified *Ac* transposase;
 - introducing a modified *Ac* transposase or a nucleic acid encoding a modified *Ac* transposase into the cell, wherein the modified *Ac* transposase comprises a nuclear localization signal (NLS) at the N-terminus of a truncated *Ac* transposase, wherein the NLS localizes the transposition reaction to the nucleus of the vertebrate cell and wherein the truncated *Ac* transposase comprises an amino acid sequence having at least 80% identity to SEQ ID NO:8 and catalyzes the integration of the polynucleotide into DNA in a vertebrate cell; and
 - culturing the cell to stably incorporate the polynucleotide into the genome of the cell to produce a transgenic vertebrate cell.
16. The method of claim 15, wherein the inverted repeats are selected from the group consisting of:
 - (a) inverted repeats that are part of a *Ds* element;
 - (b) inverted repeats that are part of the *Ds* 5' end *cis*-required sequence and part of the *Ds* 3' end *cis*-required sequence;
 - (c) inverted repeats that are part of *Ds* 5' end *cis*-required sequence having the nucleotide sequence set forth in SEQ ID NO:45 and part of the *Ds* 3' end *cis*-required sequence having a nucleotide sequence set forth in SEQ ID NO:49;
 - (d) inverted repeats that are part of *Ds* 5' end *cis*-required sequence having the nucleotide sequence set forth in nucleotides 3657-3903 of SEQ ID NO:1 and part of the *Ds* 3' end *cis*-required sequence having a nucleotide sequence set forth in nucleotides 43-412 of SEQ ID NO:1; and
 - (e) inverted repeats that are part of a *Ds* 5' end *cis*-required sequence having at least 80% identity to the *Ds* 5' end *cis*-required sequence of (c) or (d) and part of a *Ds* 3' end *cis*-required sequence having at least 80% identity to the *Ds* 3' end *cis*-required

sequence of (c) or (d), wherein the *Ds* 5' end *cis*-required sequence and *Ds* 3' end *cis*-required sequence can bind to the modified *Ac* transposase.

17. The method of claim 16, wherein the NLS comprises an amino acid sequence set forth in SEQ ID NO:2 or SEQ ID NO:9.
18. The method of claim 17, wherein the modified *Ac* transposase comprises an amino acid sequence having at least 80% identity to SEQ ID NO:20 or SEQ ID NO:44 and catalyzes the integration of the polynucleotide into DNA in the vertebrate cell.
19. The method of any one of claims 15 to 18 wherein a nucleic acid encoding the modified *Ac* transposase is introduced into the cell and stably integrated into its genome prior to the introduction of the nucleic acid fragment.
20. The method of any one of claims 15 to 18, wherein the modified *Ac* transposase is introduced into the cell.
21. The method of any one of claims 15 to 18, wherein a nucleic acid encoding the modified *Ac* transposase is introduced into the cell and wherein the nucleic acid is an RNA or a DNA.
22. The method of any one of claims 15 to 18, wherein the nucleic acid fragment is part of a vector.
23. The method of any one of claims 15 to 18, wherein the polynucleotide comprises at least a portion of an open reading frame.
24. The method of claim 23, wherein the polynucleotide comprises a promoter operably linked to at least a portion of an open reading frame.
25. The method of any one of claims 15 to 18, wherein the polynucleotide comprises at least one expression control region.

26. The method of claim 25, wherein the expression control region is selected from the group consisting of a promoter, an enhancer or a silencer.
27. The method of any one of claims 15 to 18, wherein the DNA into which the polynucleotide is capable of integrating is the cell genome.
28. The method of any one of claims 15 to 18, wherein introducing the polynucleotide to the cell comprises using a method selected from the group consisting of microinjection, electroporation, combining the nucleic acid fragment with cationic lipid vesicles or DNA condensing reagents, and incorporating the nucleic acid fragment into a viral vector and contacting the viral vector with the cell.
29. The method of any one of claims 15 to 18, wherein the vertebrate is a fish.
30. A transgenic vertebrate cell line produced by the method of any one of claims 15 to 18, wherein the genome of the cell line comprises the polynucleotide flanked by *Ds* 5' and 3' ends.
31. A method for producing a transgenic non-human vertebrate comprising the steps of:
 - introducing a nucleic acid fragment comprising a polynucleotide positioned between inverted repeats that can bind to a modified *Ac* transposase into a vertebrate cell;
 - introducing a modified *Ac* transposase or a nucleic acid encoding a modified *Ac* transposase into a vertebrate cell, wherein the modified *Ac* transposase comprises a nuclear localization signal (NLS) at the N-terminus of a truncated *Ac* transposase, wherein the NLS localizes the transposition reaction to the nucleus of the vertebrate cell and wherein the truncated *Ac* transposase comprises an amino acid sequence having at least 80% identity to SEQ ID NO:8 and catalyzes the integration of the polynucleotide into DNA in a vertebrate cell;
 - culturing the vertebrate cell to stably incorporate the polynucleotide into the genome of the cell to produce a transgenic vertebrate cell; and
 - growing the transgenic vertebrate cell into a transgenic vertebrate.
32. The method of claim 31, wherein the inverted repeats are selected from the group consisting of:
 - (a) inverted repeats that are part of a *Ds* element;

(b) inverted repeats that are part of the *Ds* 5' end *cis*-required sequence and part of the *Ds* 3' end *cis*-required sequence;

(c) inverted repeats that are part of *Ds* 5' end *cis*-required sequence having the nucleotide sequence set forth in SEQ ID NO:45 and part of the *Ds* 3' end *cis*-required sequence having a nucleotide sequence set forth in SEQ ID NO:49;

(d) inverted repeats that are part of *Ds* 5' end *cis*-required sequence having the nucleotide sequence set forth in nucleotides 3657-3903 of SEQ ID NO:1 and part of the *Ds* 3' end *cis*-required sequence having a nucleotide sequence set forth in nucleotides 43-412 of SEQ ID NO:1; and

(e) inverted repeats that are part of a *Ds* 5' end *cis*-required sequence having at least 80% identity to the *Ds* 5' end *cis*-required sequence of (c) or (d) and part of a *Ds* 3' end *cis*-required sequence having at least 80% identity to the *Ds* 3' end *cis*-required sequence of (c) or (d), wherein the *Ds* 5' end *cis*-required sequence and *Ds* 3' end *cis*-required sequence can bind to the modified *Ac* transposase.

33. The method of claim 32, wherein the NLS comprises an amino acid sequence set forth in SEQ ID NO:2 or SEQ ID NO:9.
34. The method of claim 33, wherein the modified *Ac* transposase comprises an amino acid sequence having at least 80% identity to SEQ ID NO:20 or SEQ ID NO:44 and catalyzes the integration of the polynucleotide into DNA in the vertebrate cell.
35. The method of any one of claims 31 to 34, wherein a nucleic acid encoding the modified *Ac* transposase is introduced into the cell and stably integrated into its genome prior to the introduction of the nucleic acid fragment.
36. The method of any one of claims 31 to 34, wherein the modified *Ac* transposase is introduced into the cell.
37. The method of any one of claims 31 to 34, wherein a nucleic acid encoding the modified *Ac* transposase is introduced into the cell and wherein the nucleic acid is an RNA or a DNA.

38. The method of any one of claims 31 to 34, wherein the nucleic acid fragment is part of a vector.
39. The method of any one of claims 31 to 34, wherein the polynucleotide encodes at least a portion of an open reading frame.
40. The method of claim 39, wherein the polynucleotide comprises a promoter operably linked to the portion of an open reading frame.
41. The method of any one of claims 31 to 34, wherein the polynucleotide encodes an expression control region.
42. The method of claim 41, wherein the expression control region is selected from the group consisting of a promoter, an enhancer or a silencer.
43. The method of any one of claims 31 to 34, wherein the vertebrate is a fish.
44. A method for identifying the function of a gene which comprises:
 - (a) preparing a vertebrate cell having a polynucleotide insert in a gene, wherein the polynucleotide comprises a portion of the inverted repeats that can bind to a modified *Ac* transposase and wherein the vertebrate cell is prepared by
 - (i) introducing into the cell a nucleic acid fragment comprising a polynucleotide positioned between inverted repeats that can bind to a modified *Ac* transposase;
 - (ii) introducing a modified *Ac* transposase or a nucleic acid encoding a modified *Ac* transposase into the cell, wherein the modified *Ac* transposase comprises a nuclear localization signal (NLS) at the N-terminus of a truncated *Ac* transposase, wherein the NLS localizes the transposition reaction to the nucleus of the vertebrate cell and wherein the truncated *Ac* transposase comprises an amino acid sequence having at least 80% identity to SEQ ID NO:8 and catalyzes the integration of the polynucleotide into DNA in a vertebrate cell;
 - (iii) culturing the cell to stably incorporate the polynucleotide into the genome of the cell to produce a transgenic vertebrate cell;

(b) screening the vertebrate cell for an insertional mutation for a phenotype or biochemical characteristic of interest; and

(c) correlating the phenotype or biochemical characteristic with the nucleotide sequence of the gene, whereby the function of the gene is identified.

45. The method of claim 44, wherein the correlation comprises identifying the gene with the insertional mutation; and determining the nucleotide sequence of the gene.

46. The method of claim 44 or 45 which further comprises the step of searching a sequence database for homologs or orthologs of the gene.

47. A method for identifying a putative drug target comprising:

(a) preparing a vertebrate cell having a polynucleotide insert in a gene, wherein the polynucleotide comprises a portion of the inverted repeats that can bind to a modified *Ac* transposase and wherein the vertebrate cell is prepared by

(i) introducing into the cell a nucleic acid fragment comprising a polynucleotide positioned between inverted repeats that can bind to a modified *Ac* transposase;

(ii) introducing a modified *Ac* transposase or a nucleic acid encoding a modified *Ac* transposase into the cell, wherein the modified *Ac* transposase comprises a nuclear localization signal (NLS) at the N-terminus of a truncated *Ac* transposase, wherein the NLS localizes the transposition reaction to the nucleus of the vertebrate cell and wherein the truncated *Ac* transposase comprises an amino acid sequence having at least 80% identity to SEQ ID NO:8 and catalyzes the integration of the polynucleotide into DNA in a vertebrate cell;

(iii) culturing the cell to stably incorporate the polynucleotide into the genome of the cell to produce a transgenic vertebrate cell;

(b) identifying a vertebrate cell o having an insertional mutation for a phenotype or biochemical characteristic of interest; and

(c) identifying a nucleotide sequence of the gene and an amino acid sequence of a protein encoded by the gene for the identified vertebrate cell, whereby a putative drug target is identified.

48. The method of claim 47 which further comprises the step of searching a sequence database for homologs or orthologs of the gene.
49. A method for identifying a site of action of a compound comprising:
- (a) preparing a vertebrate cell having a polynucleotide insert in a gene, wherein the polynucleotide comprises a portion of the inverted repeats that can bind to a modified *Ac* transposase and wherein the vertebrate cell is prepared by
 - (i) introducing into the cell a nucleic acid fragment comprising a polynucleotide positioned between inverted repeats that can bind to a modified *Ac* transposase;
 - (ii) introducing a modified *Ac* transposase or a nucleic acid encoding a modified *Ac* transposase into the cell, wherein the modified *Ac* transposase comprises a nuclear localization signal (NLS) at the N-terminus of a truncated *Ac* transposase, wherein the NLS localizes the transposition reaction to the nucleus of the vertebrate cell and wherein the truncated *Ac* transposase comprises an amino acid sequence having at least 80% identity to SEQ ID NO:8 and catalyzes the integration of the polynucleotide into DNA in a vertebrate cell;
 - (iii) culturing the cell to stably incorporate the polynucleotide into the genome of the cell to produce a transgenic vertebrate cell;
 - (b) exposing a vertebrate cell having an insertional mutation to a compound of interest;
 - (c) exposing a wildtype vertebrate cell to the compound of interest;
 - (d) assessing the effect of the compound on the vertebrate cell having an insertional mutation and the wildtype vertebrate cell; and
 - (e) identifying a nucleotide sequence of a gene in the vertebrate cell having an insertional mutation which does not respond to the compound compared to the wildtype vertebrate cell, whereby a site of action of the compound is identified.
50. The method of claim 49 which further comprises the step of searching a sequence database for homologs or orthologs of the gene.

51. A vertebrate insertional mutation library wherein each member of the library has a polynucleotide insert in a gene, wherein the polynucleotide comprises a portion of the inverted repeats that can bind to a modified *Ac* transposase and wherein the library has a polynucleotide insert in at least 15% of the genes of the vertebrate genome.
52. The library of claim 51, wherein the modified *Ac* transposase comprises a nuclear localization signal (NLS) at the N-terminus of a truncated *Ac* transposase, wherein the NLS localizes the transposition reaction to the nucleus of the vertebrate cell and wherein the truncated *Ac* transposase comprises an amino acid sequence having at least 80% identity to SEQ ID NO:8 and catalyzes the integration of the polynucleotide into DNA in a vertebrate cell
53. The library of claim 51 or 52, wherein the members of the library are vertebrate sperm cells.
54. The library of claim 51 or 52, wherein the members of the library are individual vertebrates.
55. The library of claim 54, wherein the vertebrates are heterozygotic for the polynucleotide insert.
56. The library of claim 54, wherein the vertebrates are homozygotic for the polynucleotide insert.
57. The library of claim 51 or 52, wherein the members of the library are haploid vertebrate embryos.
58. The library of claim 51 or 52, wherein the members of the library are vertebrate gynogenetic diploid embryos.
59. The library of claim 51 or 52 which further comprises a DNA sequence for the gene associated with each polynucleotide insert.

60. The library of claim 51 or 52 which further comprises an amino acid sequence for a protein encoded by said gene.
61. The library of claim 59 which further comprises an amino acid sequence for a protein encoded by said gene.
62. The library of claim 51 or 52, wherein the vertebrate is a fish.
63. A method for identifying a function of a gene comprising:
 - screening at least one member of the vertebrate insertional mutation library of claim 51 or 52 for a phenotype of interest; and
 - correlating the phenotype of interest with a DNA sequence of the gene of said library, whereby the function of said gene is identified.
64. The method of claim 63 which further comprises the step of searching a sequence database for homologs or orthologs of said gene.
65. The method of claim 63, wherein the members of the library are selected from the group consisting of vertebrate sperm cells, vertebrate individuals heterozygotic for the polynucleotide insert, vertebrate individuals homozygotic for the polynucleotide insert, vertebrate haploid embryos and vertebrate gynogenetic diploid embryos.
66. The method of claim 63, wherein the vertebrate is a fish.
67. A method for identifying a putative drug target comprising:
 - identifying a member of the vertebrate insertional mutation library of claim 51 or 52 associated with a phenotype of interest; and
 - identifying a DNA sequence of the gene and an amino acid sequence of a protein encoded by the gene for the identified member of the library, whereby a putative drug target is identified.
68. The method of claim 67, which further comprises the step of searching a sequence database for homologs or orthologs of said gene.

69. The method of claim 67, wherein the members of the library are selected from the group consisting of vertebrate sperm cells, vertebrate individuals heterozygotic for the polynucleotide insert, vertebrate individuals homozygotic for the polynucleotide insert, vertebrate haploid embryos and vertebrate gynogenetic diploid embryos.
70. The method of claim 67, wherein the vertebrate is a fish.
71. A method for identifying a site of action of a compound comprising:
 exposing at least one member of the vertebrate insertional mutation library of claim 51 or 52 to a compound of interest;
 exposing wildtype vertebrate individuals to the compound of interest;
 assessing the effect of the compound on the member of library and the wildtype vertebrate individuals;
 identifying a member of the library which does not respond to the compound compared to the wildtype vertebrate individuals; and
 identifying a DNA sequence of a gene in the identified member of the library, whereby a site of action of the compound is identified.
72. The method of claim 71, which further comprises the step of searching a sequence database for homologs or orthologs of said gene.
73. The method of claim 71, wherein the members of the library are selected from the group consisting of vertebrate sperm cells, vertebrate individuals heterozygotic for the polynucleotide insert, vertebrate individuals homozygotic for the polynucleotide insert, vertebrate haploid embryos and vertebrate gynogenetic diploid embryos.
74. The method of claim 71, wherein the effect is assessed on a biochemical characteristic, an enzyme, a phenotype or a transgene.
75. The method of claim 71, wherein the vertebrate is a fish.
76. A method for identifying a gene of interest comprising:

screening members of the vertebrate insertional mutation library of claim 51 or 52 for a characteristic of interest;

correlating the presence of the gene of interest in a specific member of the library having the characteristic of interest; and

identifying the gene of interest from the DNA sequence of the gene associated with the specific member of the library.

77. The method of claim 76, which further comprises the step of searching a sequence database for homologs or orthologs of said gene.
78. The method of claim 76, wherein the members of the library are selected from the group consisting of vertebrate sperm cells, vertebrate individuals heterozygotic for the polynucleotide insert, vertebrate individuals homozygotic for the polynucleotide insert, vertebrate haploid embryos and vertebrate gynogenetic diploid embryos.
79. The method of claim 76, wherein the vertebrate is a fish.

1/48

SEQUENCE LISTING

<110> Temasek LifeSciences Laboratory
 Parinov, Sergey
 Emelyanov, Alexander

<120> Transposition of Maize AC/DS Elements in Vertebrates

<130> 2577-171

<150> US 60/681,447

<151> 2005-05-17

<160> 71

<170> PatentIn version 3.3

<210> 1

<211> 3998

<212> DNA

<213> Artificial

<220>

<223> Ds Construct

<400> 1

```

ceatggtggc gaccggtgct cgactctaga ggatccccga catagggatg aaaacggtcg      60
gtaacggtcg gtaaaatacc tctaccgttt tcattttcoat atttaacttg cgggacggaa      120
acgaaaacgg gatataccgg taacgaaaac gaacgggata aatacggtaa tcgaaaaccg      180
atacgatccg gtcgggttaa agtcgaaatc ggacgggaac cgggtattttt gttcggtaaa      240
atcacacatg aaaacatata ttcaaaactt aaaaacaaat ataaaaaatt gtaaacacaa      300
gtottaatta aacatagata aaatccatat aaatctggag cacacatagt ttaatgtagc      360
acataagtga taagtottgg gctcttggct aacataagaa gccatataag tccctcgacc      420
tctagcttcg aattcccttc ccttctaact ttgaogtcoct ttttaagagct tgtgcatgaa      480
agcagatttg gagctgatta ctcatctcaa acaoccatac aaagggatga ttgccgtacc      540
atgatctcac acctttcaca cctggtttat actatgatag ttgtagacga ttgcgtaatg      600
ctattaaatg cccatcagtg ctggctgtga caoccaaactg ctgccatttc gtgttgactt      660
gcacgagaaa tgagaaattg tctgactatg cagggtgtct atgcgtggga acatttatca      720
gtggtcatta aatactatag ttacagttta gaocaaagtg tgctgtatit ttgtgttagc      780
ttagctgcag tttttgtgtg tgaagtaaca aatgacaaat actcaaaacta ttgtaattaa      840
gtagtttttc tcagaaattg taatttaact agtagtttaa aaatgtgtac ttttactttc      900
ccttgagtac atttttagtg cagtgttggg acttttattt caettcocttc cttcaacctg      960
cagtcactac tttatttatt cttgtctatg tggattagac aaatcagtcg tgtgattcct      1020

```

2/48

gtccaatcaa attgcacata gaaggtaa	cacatcataa tgaactac	ct taagacat	gg 1080		
gccatttata attgcagcaa actgtttgcc	agcattaaaa gaagatgt	ca aaaatat	ttta 1140		
caogcattaa cccagagact gcttagat	gc atgtcactga tgagaagat	g atggat	gttt 1200		
actgtatgat gaccgaaata actttaa	acg cacacaagac ggcacaag	ac gtcaacat	gg 1260		
cgttaggttg acgttgtacc ccaacgc	cagt ggggacgttg cattttgt	ttt agaaat	gaaa 1320		
attaggttga cgtcagaact caacgtc	cagg tcgatgtcaa tgttcaac	at ccaatc	taaa 1380		
atcatatata aatgtcta	at gatgttacag cttgatgt	tta tgoggat	gtt accoct	atga 1440	
ogtctatcag acgttggatt atggttg	cca tacctgatga ataaatgt	ca ttatttg	acg 1500		
ttggtttaag atgttggttc gacattg	gat tttggctgct ttccaac	aca acctaa	atcc 1560		
accaaata	tt aacttcctat gacatcgt	tta ttggacgt	ca aaataa	caat atcctt	agat 1620
gctggctaga ctttga	at	aggtcaccac aacctata	tt taacct	aata ttaacat	ctt 1680
atgatgttgt gtgcctgctg ggcaata	act aaatgcacta cagaatgt	tta cgtttac	aca 1740		
catgtaaat acatgtaaat gcatcag	cct ttcacagcat aatactc	act acttact	act 1800		
cttgagtact tttaaaaag ctacttt	tca ctcatacttt gagtaata	tt tacaact	gat 1860		
acttttactc gcactacatt tttagg	catt gat tattgat	att tttact	atga tttttc	cagta 1920	
ctctttccac tactgcagcc ctcccc	atac ataactcgtat gtttac	acat atgg	tgag	1980	
ttagagccat aatctacatt agcttt	gtta gccgctagca ttactgt	gca gaattgt	gtg 2040		
tgtgcacatt ttccaatata aatacaga	ag gaaactgtgt tccctgt	tcc cttgt	aaatc 2100		
tcaacaatgc aactgttcag ctcagg	ggga aaaatgc	ctt gccagat	cca aacgg	cctggc 2160	
aaaagtgaat ggaaaaaagc ctttc	attaa tgtgaa	gtt gctgcgcgc	cc ccaocc	cagat 2220	
aaaaagagca gaggttaaca tgctct	ctac ggctgtocag ccaacc	cagat actgag	gcag 2280		
aaacacaccc gctggcagat ggtgag	agct acactgt	cct ttccag	agtt tctact	ggaa 2340	
tgctgtcct caagtctcaa gcctct	cctt gcattctctc attccac	ctg gggcaa	agcc 2400		
ccaggctggg tgtgacaaca tttatc	ttac cactttctct ctgtac	cctgt ctaac	aggta 2460		
gggtgtgtgt gagagtgcgt atgtgt	gcaa gtgcgtgtgt gtgtgag	agc agtcag	ctcc 2520		
acctctcaa gagtgtgtat aaaatt	ggtc agccagctgc tgagag	acac gcagag	gggac 2580		
tttgactctc ctttgtgagc aaoc	ctctoc actcactcct ctctcag	aga gcactct	cgt 2640		
acctccttct cagcaactca aagac	acagg atccacc	gggt cgcacc	atg gtgag	caagg 2700	
gcgaggagct gttcaccggg gtgg	tgccca tccgtgtcga gctgg	acggc gacgt	aaacg 2760		
gccacaagtt cagcgtgtcc ggcg	agggog agggcgatgc cacct	acggc aagct	gaccc 2820		

3/48

tgaagttcat ctgcaccacc ggcaagctgc cCGtgccctg gcccaaccctc gtgaccaccc 2880
 tgacctacgg cgtgcagtgC ttcagccgct accccgacca catgaagcag caccgacttct 2940
 tcaagtcgcg catgcccgaA ggctacgtcc aggagcgcac catcttcttc aaggacgacg 3000
 gcaactacaa gaccgcgcgc gaggtgaagt tcgagggcga caccctgggtg aacgcgcatcg 3060
 agctgaaggg catcgacttc aaggaggacg gcaacatcct ggggcacaag ctggagtaca 3120
 actacaacag ccacaacgtc tatatcatgg ccgacaagca gaagaacggc atcaagggtga 3180
 acttcaagat ccgccacaac atcgaggacg gcagcgtgca gotcgcgcac cactaccagc 3240
 agaacacccc catcggcgac ggccccgtgc tgctgcccga caaccactac ctgagcaccc 3300
 agtcgcgcct gagcaaagac cccaacgaga agcgcgatca catggtcctg ctggagtctg 3360
 tgaccgcgcg cgggatcact ctcggcgatgg acgagctgta caagtaaagc ggccgcgact 3420
 ctagatcata atcagccata ccacatttgt agaggttcta ctgcttttaa aaaacctccc 3480
 acacctcccc ctgaacctga aacataaaat gaatgoaatt gttgttggtta acttgtttat 3540
 tgcagcttat aatgggttaca aataaagcaa tagcatcaca aatttcacaa ataaagcatt 3600
 tttttcactg cattctagtt gtggtttgtc caaactcatc aatggtcgag ggatctgttg 3660
 cagtcatccc gaattagaaa atacggtaac gaaacgggat catcccgatt aaaaacggga 3720
 tcccggtgaa acggtcggga aactagctct acogtttccg tttccgttta ccgttttgta 3780
 tatcccgttt ccgttccggt ttcgtttttt acctcgggtt cgaaatcgat cgggataaaa 3840
 ctaacaaaat cggttatacg ataacggtcg gtacgggatt ttcccatcct actttcatcc 3900
 ctgcgtcggg tatcgagctc gaattaagct tgatatcgaa ttctgcagg aaaaaaaaaac 3960
 aattagaatt aattttatat ttatacaatt tatgggtgc 3998

<210> 2

<211> 11

<212> PRT

<213> Artificial

<220>

<223> Synthetic Nuclear Localization Signal

<400> 2

Met Gly Pro Pro Lys Lys Lys Arg Lys Val Glu
 1 5 10

<210> 3

<211> 27

<212> DNA

<213> Artificial

4/48

<220>
 <223> vector DNA

<400> 3
 gaggatcccc gacggtcggg tatcgag

27

<210> 4
 <211> 27
 <212> DNA
 <213> Artificial

<220>
 <223> vector DNA

<400> 4
 gaggatcccc gactgtcggg tatcgag

27

<210> 5
 <211> 26
 <212> DNA
 <213> Artificial

<220>
 <223> vector DNA

<400> 5
 tggagctccc cgagtcgggt acccaa

26

<210> 6
 <211> 28
 <212> DNA
 <213> Artificial

<220>
 <223> vector DNA

<400> 6
 tggagctccc cgacggtcgg gtacccaa

28

<210> 7
 <211> 2115
 <212> DNA
 <213> Zea mays

<220>
 <221> CDS
 <222> (1)..(2115)

<400> 7
 atg gct att gtt cat gaa cca caa cca caa cca caa cca caa gaa
 Met Ala Ile Val His Glu Pro Gln Pro Gln Pro Gln Pro Gln Pro Glu
 1 5 10 15

48

cca caa cca cag cca caa cct gaa ccc gaa gaa gaa gca cca cag aag
 Pro Gln Pro Gln Pro Gln Pro Glu Pro Glu Glu Glu Ala Pro Gln Lys

96

5/48

20	25	30	
agg gca aag aag tgc aca tgc gat gta tgg cag cat ttc acc aag aag Arg Ala Lys Lys Cys Thr Ser Asp Val Trp Gln His Phe Thr Lys Lys 35 40 45			144
gaa att gaa gtg gag gtc gat gga aag aaa tac gtt cag gta tgg ggg Glu Ile Glu Val Glu Val Asp Gly Lys Lys Tyr Val Gln Val Trp Gly 50 55 60			192
cat tgc aac ttt cct aat tgc aag gct aag tat agg gct gag ggt cat His Cys Asn Phe Pro Asn Cys Lys Ala Lys Tyr Arg Ala Glu Gly His 65 70 75 80			240
cat gga aca agc gga ttt cga aat cac ttg aga aca tca cat agt tta His Gly Thr Ser Gly Phe Arg Asn His Leu Arg Thr Ser His Ser Leu 85 90 95			288
gtt aaa ggt cag ttg tgt cta aaa agt gaa aag gat cat ggc aaa gac Val Lys Gly Gln Leu Cys Leu Lys Ser Glu Lys Asp His Gly Lys Asp 100 105 110			336
ata aat ctc att gag cct tat aag tac gat gaa gtg gtt agc cta aag Ile Asn Leu Ile Glu Pro Tyr Lys Tyr Asp Glu Val Val Ser Leu Lys 115 120 125			384
aag ctt cat ttg gca ata atc atg cat gaa tac cct ttc aat att gta Lys Leu His Leu Ala Ile Ile Met His Glu Tyr Pro Phe Asn Ile Val 130 135 140			432
gaa cat gag tac ttt gtt gag ttt gtt aag tct ctg cgc cct cac ttt Glu His Glu Tyr Phe Val Glu Phe Val Lys Ser Leu Arg Pro His Phe 145 150 155 160			480
cca ata aag tcc cgt gtc act gct aga aaa tat atc atg gat ttg tat Pro Ile Lys Ser Arg Val Thr Ala Arg Lys Tyr Ile Met Asp Leu Tyr 165 170 175			528
ttg gaa gaa aaa gaa aag ttg tat gga aaa cta aaa gat gtt cag tct Leu Glu Glu Lys Glu Lys Leu Tyr Gly Lys Leu Lys Asp Val Gln Ser 180 185 190			576
cgc ttc agt aca act atg gat atg tgg aca tct tgt caa aat aag tca Arg Phe Ser Thr Thr Met Asp Met Trp Thr Ser Cys Gln Asn Lys Ser 195 200 205			624
tac atg tgt gtc acc atc cat tgg att gat gat gat tgg tgt ctc caa Tyr Met Cys Val Thr Ile His Trp Ile Asp Asp Asp Trp Cys Leu Gln 210 215 220			672
aaa aga att gtt ggc ttt ttt cat gtt gaa ggg cgc cac act ggc caa Lys Arg Ile Val Gly Phe Phe His Val Glu Gly Arg His Thr Gly Gln 225 230 235 240			720
agg tta tca caa aac ttc act gca atc atg gtt aag tgg aac att gag Arg Leu Ser Gln Thr Phe Thr Ala Ile Met Val Lys Trp Asn Ile Glu 245 250 255			768
aaa aaa ttg ttt gcc ttg tct ttg gat aat gct agt gca aat gaa gta Lys Lys Leu Phe Ala Leu Ser Leu Asp Asn Ala Ser Ala Asn Glu Val			816

6/48

260	265	270	
gct gtg cac gat ata att gag gat ttg cag gac act gat tca aat cta Ala Val His Asp Ile Ile Glu Asp Leu Gln Asp Thr Asp Ser Asn Leu 275 280 285			864
gtt tgt gat ggt gct ttc ttt cat gtg agg tgt gct tgt cac ata ctg Val Cys Asp Gly Ala Phe Phe His Val Arg Cys Ala Cys His Ile Leu 290 295 300			912
aac ttg gtt gca aag gat ggc ttg gct gta att gca gga aca att gag Asn Leu Val Ala Lys Asp Gly Leu Ala Val Ile Ala Gly Thr Ile Glu 305 310 315 320			960
aaa atc aaa gcg att gtt ctt gct gta aaa tct tct cct ttg cag tgg Lys Ile Lys Ala Ile Val Leu Ala Val Lys Ser Ser Pro Leu Gln Trp 325 330 335			1008
gaa gaa cta atg aag tgt gct agt gaa tgt gac ttg gat aaa tct aaa Glu Glu Leu Met Lys Cys Ala Ser Glu Cys Asp Leu Asp Lys Ser Lys 340 345 350			1056
ggg atc tca tat gat gtc tca act aga tgg aat tca acc tat ttg atg Gly Ile Ser Tyr Asp Val Ser Thr Arg Trp Asn Ser Thr Tyr Leu Met 355 360 365			1104
ttg agg gat gcc tta tat tat aag cct gca cta ata agg ctt aaa aca Leu Arg Asp Ala Leu Tyr Tyr Lys Pro Ala Leu Ile Arg Leu Lys Thr 370 375 380			1152
agt gat cct cgc agg tac gat gca att tgt cct aaa gcc gag gag tgg Ser Asp Pro Arg Arg Tyr Asp Ala Ile Cys Pro Lys Ala Glu Glu Trp 385 390 395 400			1200
aag atg gca tta act ctt ttt aag tgt ttg aag aag ttt ttt gat ctc Lys Met Ala Leu Thr Leu Phe Lys Cys Leu Lys Lys Phe Phe Asp Leu 405 410 415			1248
act gaa ctc cta tct ggt act caa tat tcc act gca aat tta ttt tac Thr Glu Leu Leu Ser Gly Thr Gln Tyr Ser Thr Ala Asn Leu Phe Tyr 420 425 430			1296
aaa ggt ttc tgt gag ata aag gat ttg att gac caa tgg tgt gtt cat Lys Gly Phe Cys Glu Ile Lys Asp Leu Ile Asp Gln Trp Cys Val His 435 440 445			1344
gaa aaa ttt gtc att agg aga atg gcc gtt gca atg agt gaa aag ttt Glu Lys Phe Val Ile Arg Arg Met Ala Val Ala Met Ser Glu Lys Phe 450 455 460			1392
gag aaa tat tgg aaa gtg tct aat att gca cta gct gta gca tgc ttc Glu Lys Tyr Trp Lys Val Ser Asn Ile Ala Leu Ala Val Ala Cys Phe 465 470 475 480			1440
ctt gac cct agg tac aag aaa ata ttg att gag ttc tat atg aaa aaa Leu Asp Pro Arg Tyr Lys Lys Ile Leu Ile Glu Phe Tyr Met Lys Lys 485 490 495			1488
ttt cat ggt gat tca tac aaa gtt cat gta gat gac ttt gtt agg gtc Phe His Gly Asp Ser Tyr Lys Val His Val Asp Asp Phe Val Arg Val 500 505 510 515			1536

7/48

500	505	510	
att aga aaa ttg tat caa ttc tat tct agt tgt agt cct tca gct cca			1584
Ile Arg Lys Leu Tyr Gln Phe Tyr Ser Ser Cys Ser Pro Ser Ala Pro			
515	520	525	
aag aca aag aca act act aat gat agt atg gat gat acc ttg atg gaa			1632
Lys Thr Lys Thr Thr Thr Asn Asp Ser Met Asp Asp Thr Leu Met Glu			
530	535	540	
aat gaa gat gat gaa ttt caa aac tat ttg cat gag ttg aag gat tat			1680
Asn Glu Asp Asp Glu Phe Gln Asn Tyr Leu His Glu Leu Lys Asp Tyr			
545	550	555	560
gat caa gta gag tca aat gaa ttg gat aaa tat atg tct gaa ccc ctt			1728
Asp Gln Val Glu Ser Asn Glu Leu Asp Lys Tyr Met Ser Glu Pro Leu			
565	570	575	
ttg aag cat agt ggt cag ttt gat att tta tca tgg tgg agg gga agg			1776
Leu Lys His Ser Gly Gln Phe Asp Ile Leu Ser Trp Trp Arg Gly Arg			
580	585	590	
gtt gca gaa tat cct att ctc acc caa att gca agg gat gtg cta gca			1824
Val Ala Glu Tyr Pro Ile Leu Thr Gln Ile Ala Arg Asp Val Leu Ala			
595	600	605	
ata caa gtg tca act gtt got tct gag tct gcg ttc agt gct ggt ggt			1872
Ile Gln Val Ser Thr Val Ala Ser Glu Ser Ala Phe Ser Ala Gly Gly			
610	615	620	
cgt gtt gtt gat cct tac cgc aat cgt ctt ggt tcg gag att gtt gaa			1920
Arg Val Val Asp Pro Tyr Arg Asn Arg Leu Gly Ser Glu Ile Val Glu			
625	630	635	640
gct ttg ata tgc aca aaa gat tgg gta gca gca tct aga aaa ggt gct			1968
Ala Leu Ile Cys Thr Lys Asp Trp Val Ala Ala Ser Arg Lys Gly Ala			
645	650	655	
aca tat ttt cca aca atg att ggt gat ctc gag gtg cta gac tct gtt			2016
Thr Tyr Phe Pro Thr Met Ile Gly Asp Leu Glu Val Leu Asp Ser Val			
660	665	670	
att gct gct gca aca aat cat gag aat cat atg gat gag gat gaa gac			2064
Ile Ala Ala Ala Thr Asn His Glu Asn His Met Asp Glu Asp Glu Asp			
675	680	685	
gca ata gaa ttt tct aag aat aat gaa gat gta gca agt ggc tcc tct			2112
Ala Ile Glu Phe Ser Lys Asn Asn Glu Asp Val Ala Ser Gly Ser Ser			
690	695	700	
cca			2115
Pro			
705			

<210> 8
 <211> 705
 <212> PRT
 <213> Zea mays

8/48

<400> 8

```

Met Ala Ile Val His Glu Pro Gln Pro Gln Pro Gln Pro Gln Pro Glu
1          5          10          15

Pro Gln Pro Gln Pro Gln Pro Glu Pro Glu Glu Glu Ala Pro Gln Lys
          20          25          30

Arg Ala Lys Lys Cys Thr Ser Asp Val Trp Gln His Phe Thr Lys Lys
          35          40          45

Glu Ile Glu Val Glu Val Asp Gly Lys Lys Tyr Val Gln Val Trp Gly
          50          55          60

His Cys Asn Phe Pro Asn Cys Lys Ala Lys Tyr Arg Ala Glu Gly His
          65          70          75          80

His Gly Thr Ser Gly Phe Arg Asn His Leu Arg Thr Ser His Ser Leu
          85          90          95

Val Lys Gly Gln Leu Cys Leu Lys Ser Glu Lys Asp His Gly Lys Asp
          100          105          110

Ile Asn Leu Ile Glu Pro Tyr Lys Tyr Asp Glu Val Val Ser Leu Lys
          115          120          125

Lys Leu His Leu Ala Ile Ile Met His Glu Tyr Pro Phe Asn Ile Val
          130          135          140

Glu His Glu Tyr Phe Val Glu Phe Val Lys Ser Leu Arg Pro His Phe
          145          150          155          160

Pro Ile Lys Ser Arg Val Thr Ala Arg Lys Tyr Ile Met Asp Leu Tyr
          165          170          175

Leu Glu Glu Lys Glu Lys Leu Tyr Gly Lys Leu Lys Asp Val Gln Ser
          180          185          190

Arg Phe Ser Thr Thr Met Asp Met Trp Thr Ser Cys Gln Asn Lys Ser
          195          200          205

Tyr Met Cys Val Thr Ile His Trp Ile Asp Asp Asp Trp Cys Leu Gln
          210          215          220

Lys Arg Ile Val Gly Phe Phe His Val Glu Gly Arg His Thr Gly Gln
          225          230          235          240

Arg Leu Ser Gln Thr Phe Thr Ala Ile Met Val Lys Trp Asn Ile Glu
          245          250          255

Lys Lys Leu Phe Ala Leu Ser Leu Asp Asn Ala Ser Ala Asn Glu Val
          260          265          270

Ala Val His Asp Ile Ile Glu Asp Leu Gln Asp Thr Asp Ser Asn Leu
          275          280          285

Val Cys Asp Gly Ala Phe Phe His Val Arg Cys Ala Cys His Ile Leu
          290          295          300

Asn Leu Val Ala Lys Asp Gly Leu Ala Val Ile Ala Gly Thr Ile Glu

```

9/48

305		310		315		320
Lys Ile Lys Ala Ile Val Leu Ala Val Lys Ser Ser Pro Leu Gln Trp						
		325		330		335
Glu Glu Leu Met Lys Cys Ala Ser Glu Cys Asp Leu Asp Lys Ser Lys						
		340		345		350
Gly Ile Ser Tyr Asp Val Ser Thr Arg Trp Asn Ser Thr Tyr Leu Met						
		355		360		365
Leu Arg Asp Ala Leu Tyr Tyr Lys Pro Ala Leu Ile Arg Leu Lys Thr						
		370		375		380
Ser Asp Pro Arg Arg Tyr Asp Ala Ile Cys Pro Lys Ala Glu Glu Trp						
		385		390		400
Lys Met Ala Leu Thr Leu Phe Lys Cys Leu Lys Lys Phe Phe Asp Leu						
		405		410		415
Thr Glu Leu Leu Ser Gly Thr Gln Tyr Ser Thr Ala Asn Leu Phe Tyr						
		420		425		430
Lys Gly Phe Cys Glu Ile Lys Asp Leu Ile Asp Gln Trp Cys Val His						
		435		440		445
Glu Lys Phe Val Ile Arg Arg Met Ala Val Ala Met Ser Glu Lys Phe						
		450		455		460
Glu Lys Tyr Trp Lys Val Ser Asn Ile Ala Leu Ala Val Ala Cys Phe						
		465		470		475
Leu Asp Pro Arg Tyr Lys Lys Ile Leu Ile Glu Phe Tyr Met Lys Lys						
		485		490		495
Phe His Gly Asp Ser Tyr Lys Val His Val Asp Asp Phe Val Arg Val						
		500		505		510
Ile Arg Lys Leu Tyr Gln Phe Tyr Ser Ser Cys Ser Pro Ser Ala Pro						
		515		520		525
Lys Thr Lys Thr Thr Thr Asn Asp Ser Met Asp Asp Thr Leu Met Glu						
		530		535		540
Asn Glu Asp Asp Glu Phe Gln Asn Tyr Leu His Glu Leu Lys Asp Tyr						
		545		550		555
Asp Gln Val Glu Ser Asn Glu Leu Asp Lys Tyr Met Ser Glu Pro Leu						
		565		570		575
Leu Lys His Ser Gly Gln Phe Asp Ile Leu Ser Trp Trp Arg Gly Arg						
		580		585		590
Val Ala Glu Tyr Pro Ile Leu Thr Gln Ile Ala Arg Asp Val Leu Ala						
		595		600		605
Ile Gln Val Ser Thr Val Ala Ser Glu Ser Ala Phe Ser Ala Gly Gly						
		610		615		620
Arg Val Val Asp Pro Tyr Arg Asn Arg Leu Gly Ser Glu Ile Val Glu						

10/48

625					630					635					640
Ala	Leu	Ile	Cys	Thr	Lys	Asp	Trp	Val	Ala	Ala	Ser	Arg	Lys	Gly	Ala
				645					650					655	
Thr	Tyr	Phe	Pro	Thr	Met	Ile	Gly	Asp	Leu	Glu	Val	Leu	Asp	Ser	Val
			660					665					670		
Ile	Ala	Ala	Ala	Thr	Asn	His	Glu	Asn	His	Met	Asp	Glu	Asp	Glu	Asp
		675					680					685			
Ala	Ile	Glu	Phe	Ser	Lys	Asn	Asn	Glu	Asp	Val	Ala	Ser	Gly	Ser	Ser
690						695					700				

Pro
705

```
<210> 9
<211> 11
<212> PRT
<213> Artificial
```

<220>
<223> Modified Synthetic Nuclear Localization Signal

<400> 9

Met Gly Pro Pro Glu Lys Lys Arg Lys Val Glu
1 5 10

```
<210> 10
<211> 2148
<212> DNA
<213> Artificial
```

<220>
<223> NLS-TPase103-807, CDS

<400>	10							
atggggtcctc	caaagaagaa	gcgtaaggta	gaaatggcta	ttgttcatga	accacaacca			60
caaccacaac	cacaaccaga	accacaacca	cagccacaac	ctgaacccca	agaagaagca			120
ccacagaaga	gggcaaagaa	gtgcacatcg	gatgtatggc	agcatttcac	caagaaggaa			180
attgaagtgg	aggtcgatgg	aaagaaatac	gttcagggtat	gggggcattg	caactttcct			240
aattgcaagg	ctaagtatag	ggctgagggt	catcatggaa	caagcggatt	tcgaaatcac			300
ttgagaacat	cacatagttt	agttaaaggt	cagttgtgtc	taaaaagtga	aaaggatcat			360
ggcaaagaca	taaatctcat	tgagcccttat	aagtacgatg	aagtggttag	cctaaagaag			420
cttcatttgg	caataatcat	gcatgaatac	cctttcaata	ttgtagaaca	tgagtacttt			480
gttgagtttg	ttaagtctct	gcgccctcac	tttccaataa	agtcccgtgt	cactgctaga			540
aaatatatca	tggattttgta	tttggaagaa	aaagaaaagt	tgtatggaaa	actaaaagat			600

11/48

gttcagtctc gcttcagttac aactatggat atgtggacat cttgtcaaaa taagtcatatc 660
atgtgtgtca ccatccattg gattgatgat gattgggtgc tccaaaaaag aattgttggc 720
ttttttcatg ttgaagggcg ccacactggc caaagggttat cacaaacctt cactgcaatc 780
atgggttaagt ggaacattga gaaaaaattg tttgccttgt ctttggataa tgctagtgc 840
aatgaagtag ctgtgcaoga tataattgag gatttgcagg aactgattc aaatctagtt 900
tgtgatgggtg ctttcttttca tgtgaggtgt gcttgtcaca tactgaactt ggttgcaaaag 960
gatggcttgg ctgtaattgc aggaacaatt gagaaaatca aagcgattgt tcttgctgta 1020
aaatcttctc ctttgcagtg ggaagaacta atgaagtgtg ctagtgaatg tgaottggat 1080
aaatctaaag ggatctcata tgatgtctca actagatgga attcaacctt tttgatgttg 1140
agggatgcot tatattataa gcctgcacta ataaggctta aaacaagtga tcttcgcagg 1200
tacgatgcaa tttgtcctaa agccgaggag tggaagatgg cattaactct ttttaagtgt 1260
ttgaagaagt tttttgatct cactgaactc ctatctggta ctcaatattc cactgcaaat 1320
ttatttttaca aagggtttctg tgagataaag gatttgattg accaatgggtg tgttcatgaa 1380
aaattttgtca ttaggagaat ggccgttgca atgagtgaag agtttgagaa atattggaaa 1440
gtgtctaata ttgcactagc tgtagcatgc ttccttgacc ctaggtacaa gaaaatattg 1500
attgagttct atatgaaaaa atttcatggt gattcataca aagttcatgt agatgaactt 1560
gttaggggtca ttagaaaaatt gtatcaattc tattctagtt gtagtccttc agctocaaag 1620
acaaagacaa ctactaatga tagtatggat gataccttga tggaaaatga agatgatgaa 1680
tttcaaaact atttgcata gttgaaggat tatgatcaag tagagtcaaa tgaattggat 1740
aaatatatgt ctgaaccctt tttgaagcat agtggtcagt ttgatatttt atcatgggtg 1800
aggggaaggg ttgcagaata tctattctc acccaaattg caagggatgt gctagcaata 1860
caagtgtcaa ctgttgcttc tgagtctgcg ttcagtgtgt gtggtogtgt tgttgatcct 1920
taccgcaatc gtottgggtc ggagattgtt gaagctttga tatgcacaaa agattgggtg 1980
gcagcatcta gaaaagggtc tacatatttt ccaacaatga ttggtgatct cgagggtgcta 2040
gaotctgtta ttgctgctgc aacaaatcat gagaatcata tggatgagga tgaagaogca 2100
atagaatttt ctaagaataa tgaagatgta gcaagtgggt cctctoca 2148

<210> 11
<211> 2687
<212> DNA
<213> Artificial

<220>

12/48

<223> Modified Ac Transposase Construct

<220>

<221> misc_feature

<222> (1)..(2685)

<223> n may be any nucleotide

<400> 11

attagggttg tacatatgtn gttagaacgc ggctacaatt aatacataac ottatgtatc	60
atacacatac gatttaggtg acactataga atacaagott gottgttctt tttgcagaag	120
ctcagaataa acgctcaact ttggcagatc cgcaccatg ggtcctocaa agaagaagcg	180
taaggtagaa atggctattg ttcatgaacc acaaccacaa ccacaaccac aaccagaacc	240
acaaccacag ccacaacctg aacccgaaga agaagcacca cagaagaggg caaagaagtg	300
cacatcggat gtatggcagc atttcaccaa gaaggaaatt gaagtggagg togatggaaa	360
gaaatacgtt caggtatggg ggcatgtcaa ctttctaat tgcaaggcta agtatagggc	420
tgagggtcat catggaacaa goggatttcg aaatcacttg agaacatcac atagttagt	480
taaaggtcag ttgtgtctaa aaagtgaaaa ggatcatggc aaagacataa atctcattga	540
gccttataag tacgatgaag tggttagcct aaagaagctt catttggtcaa taatcatgca	600
tgaataccct ttcaatattg tagaacatga gtactttggt gagtttggtta agtctctgcg	660
ccctcacttt ccaataaagt cccgtgtcac tgcctagaaaa tatatcatgg atttgtattt	720
ggaagaaaaa gaaaagttgt atggaaaact aaaagatggt cagtctcgct tcagtacaac	780
tatggatatg tggacatctt gtcaaaaataa gtcatacatg tgtgtcacca tcattggat	840
tgatgatgat tgggtgtctc aaaaaagaat tgttggcttt ttcatgttg aagggcgcca	900
cactggccaa aggttatcac aaacottcac tgcaatcatg gttaagtgga acattgagaa	960
aaaattgttt gccttgtctt tggataatgc tagtgcaaat gaagtagctg tgcacgatat	1020
aattgaggat ttgcaggaca ctgattcaaa totagtttgt gatggtgott tctttcatgt	1080
gaggtgtgct tgtcacatac tgaacttggt tgcaaaggat ggcttggctg taattgcagg	1140
aacaattgag aaaatcaaag cgattgttct tgcgtgtaaaa tcttctcctt tgcagtggga	1200
agaactaatg aagtgtgcta gtgaatgtga cttggataaa totaaaggga tctcatatga	1260
tgtctcaact agatggaatt caacctatct gatgttgagg gatgcottat attataagcc	1320
tgcactaata aggcttaaaa caagtgatcc togcaggtag gatgcaattt gtcctaaagc	1380
cgaggagtgg aagatggcat taactctttt taagtgtttg aagaagtttt ttgatctcac	1440
tgaactccta tctggtactc aatattccac tgcaaattta ttttaciaag gtttctgtga	1500
gataaaggat ttgattgacc aatggtgtgt tcatgaaaaa tttgtcatta ggagaatggc	1560

13/48

```

cgttgcaatg agtgaaaagt ttgagaaata ttggaaagtg tctaattattg cactagctgt 1620
agcatgcttc cttgacccta ggtacaagaa aatattgatt gagttctata tgaaaaaatt 1680
tcatgggtgat tcatacaaag ttcattgtaga tgactttgtt agggtcatta gaaaattgta 1740
tcaattctat tctagttgta gtcccttcagc tccaaagaca aagacaacta ctaatgatag 1800
tatggatgat accttgatgg aaaatgaaga tgatgaattt caaaactatt tgcattgagtt 1860
gaaggattat gatcaagtag agtcaaatga attggataaa tatatgtctg aacccttttt 1920
gaagcatagt ggtcagtttg atatttttatc atgggtggagg ggaaggggtg cagaatatcc 1980
tattctcacc caaattgcaa gggatgtgct agcaatacaa gtgtcaactg ttgcttctga 2040
gtctgcgttc agtgctggtg gtctgtttgt tgatccttac cgcaatcgtc ttggttcgga 2100
gattgttgaa gctttgatat gcacaaaaga ttgggttagca gcatctagaa aagggtgctac 2160
atatttttcca acaatgattg gtgatctoga ggtgctagac tctgttattg ctgctgcaac 2220
aaatcatgag aatcatatgg atgaggatga agacgcaata gaattttcta agaataatga 2280
agatgtagca agtggctcct ctccatgagc aatgtgtctt atgtttgttg acagatgagc 2340
cttggttgta atagtttatg catgctaagt gctccagatg tgagcaagtg attatgaata 2400
tgtgttttaa acttttatatt gtgtcatgtg tgctagtaga cttatatggc ttcttatgtt 2460
agccaagggg gccccgggat ctggttacca ctaaaccagc ctcaagaaca cccgaatgga 2520
gtctctaagc tacataatac caacttacac tttaaaaaat gtgtgtcccc aagatgtagc 2580
cattcgtatc tgctcctaatt aaaaagaaag tttcttcaca ttctaaaaaa aaaaaaaaaa 2640
aaaaaaaaaa aaaaaaaaaa cccccccccc ccccctgca ggtcgac 2687

```

<210> 12
<211> 11
<212> DNA
<213> Zea mays

<400> 12
tttcatccct g 11

<210> 13
<211> 11
<212> DNA
<213> Zea mays

<400> 13
tttcatccct a 11

<210> 14
<211> 47

14/48

<212> DNA
<213> Artificial

<220>
<223> PCR Primer

<400> 14
ccaaagaaga agcgtaaggt agaaatgggt attgttcattg aaccaca

47

<210> 15
<211> 27
<212> DNA
<213> Artificial

<220>
<223> PCR Primer

<400> 15
gtatcgataa gottgatattc gaattcc

27

<210> 16
<211> 46
<212> DNA
<213> Artificial

<220>
<223> PCR Primer

<400> 16
cgcggtatccg ccaccatggg tcttccaaag aagaagcgta aggtag

46

<210> 17
<211> 27
<212> DNA
<213> Artificial

<220>
<223> PCR Primer

<400> 17
gtatcgataa gottgatattc gaattcc

27

<210> 18
<211> 23
<212> DNA
<213> Artificial

<220>
<223> Primer

<400> 18
gagaatttca cttgttgact aga

23

<210> 19
<211> 21

15/48

<212> DNA
<213> Artificial

<220>
<223> Primer

<400> 19
gcgcatgaac tccttgatga c

21

```
<210> 20
<211> 716
<212> PRT
<213> Artificial
```

<220>
<223> NLS-TPase103-807

<400> 20

Met	Gly	Pro	Pro	Lys	Lys	Lys	Arg	Lys	Val	Glu	Met	Ala	Ile	Val	His
1				5					10					15	
Glu	Pro	Gln	Pro	Gln	Pro	Gln	Pro	Gln	Pro	Glu	Pro	Gln	Pro	Gln	Pro
			20					25					30		
Gln	Pro	Glu	Pro	Glu	Glu	Glu	Ala	Pro	Gln	Lys	Arg	Ala	Lys	Lys	Cys
		35					40					45			
Thr	Ser	Asp	Val	Trp	Gln	His	Phe	Thr	Lys	Lys	Glu	Ile	Glu	Val	Glu
	50					55					60				
Val	Asp	Gly	Lys	Lys	Tyr	Val	Gln	Val	Trp	Gly	His	Cys	Asn	Phe	Pro
65					70					75					80
Asn	Cys	Lys	Ala	Lys	Tyr	Arg	Ala	Glu	Gly	His	His	Gly	Thr	Ser	Gly
				85					90					95	
Phe	Arg	Asn	His	Leu	Arg	Thr	Ser	His	Ser	Leu	Val	Lys	Gly	Gln	Leu
			100					105					110		
Cys	Leu	Lys	Ser	Glu	Lys	Asp	His	Gly	Lys	Asp	Ile	Asn	Leu	Ile	Glu
		115					120					125			
Pro	Tyr	Lys	Tyr	Asp	Glu	Val	Val	Ser	Leu	Lys	Lys	Leu	His	Leu	Ala
	130					135						140			
Ile	Ile	Met	His	Glu	Tyr	Pro	Phe	Asn	Ile	Val	Glu	His	Glu	Tyr	Phe
145					150					155					160
Val	Glu	Phe	Val	Lys	Ser	Leu	Arg	Pro	His	Phe	Pro	Ile	Lys	Ser	Arg
				165					170					175	
Val	Thr	Ala	Arg	Lys	Tyr	Ile	Met	Asp	Leu	Tyr	Leu	Glu	Glu	Lys	Glu
		180						185					190		
Lys	Leu	Tyr	Gly	Lys	Leu	Lys	Asp	Val	Gln	Ser	Arg	Phe	Ser	Thr	Thr
		195					200					205			
Met	Asp	Met	Trp	Thr	Ser	Cys	Gln	Asn	Lys	Ser	Tyr	Met	Cys	Val	Thr

16/48

210	215	220
Ile His Trp Ile Asp Asp Asp Trp Cys Leu Gln Lys Arg Ile Val Gly 225 230 235 240		
Phe Phe His Val Glu Gly Arg His Thr Gly Gln Arg Leu Ser Gln Thr 245 250 255		
Phe Thr Ala Ile Met Val Lys Trp Asn Ile Glu Lys Lys Leu Phe Ala 260 265 270		
Leu Ser Leu Asp Asn Ala Ser Ala Asn Glu Val Ala Val His Asp Ile 275 280 285		
Ile Glu Asp Leu Gln Asp Thr Asp Ser Asn Leu Val Cys Asp Gly Ala 290 295 300		
Phe Phe His Val Arg Cys Ala Cys His Ile Leu Asn Leu Val Ala Lys 305 310 315 320		
Asp Gly Leu Ala Val Ile Ala Gly Thr Ile Glu Lys Ile Lys Ala Ile 325 330 335		
Val Leu Ala Val Lys Ser Ser Pro Leu Gln Trp Glu Glu Leu Met Lys 340 345 350		
Cys Ala Ser Glu Cys Asp Leu Asp Lys Ser Lys Gly Ile Ser Tyr Asp 355 360 365		
Val Ser Thr Arg Trp Asn Ser Thr Tyr Leu Met Leu Arg Asp Ala Leu 370 375 380		
Tyr Tyr Lys Pro Ala Leu Ile Arg Leu Lys Thr Ser Asp Pro Arg Arg 385 390 395 400		
Tyr Asp Ala Ile Cys Pro Lys Ala Glu Glu Trp Lys Met Ala Leu Thr 405 410 415		
Leu Phe Lys Cys Leu Lys Lys Phe Phe Asp Leu Thr Glu Leu Leu Ser 420 425 430		
Gly Thr Gln Tyr Ser Thr Ala Asn Leu Phe Tyr Lys Gly Phe Cys Glu 435 440 445		
Ile Lys Asp Leu Ile Asp Gln Trp Cys Val His Glu Lys Phe Val Ile 450 455 460		
Arg Arg Met Ala Val Ala Met Ser Glu Lys Phe Glu Lys Tyr Trp Lys 465 470 475 480		
Val Ser Asn Ile Ala Leu Ala Val Ala Cys Phe Leu Asp Pro Arg Tyr 485 490 495		
Lys Lys Ile Leu Ile Glu Phe Tyr Met Lys Lys Phe His Gly Asp Ser 500 505 510		
Tyr Lys Val His Val Asp Asp Phe Val Arg Val Ile Arg Lys Leu Tyr 515 520 525		
Gln Phe Tyr Ser Ser Cys Ser Pro Ser Ala Pro Lys Thr Lys Thr Thr		

17/48

530 535 540
 Thr Asn Asp Ser Met Asp Asp Thr Leu Met Glu Asn Glu Asp Asp Glu
 545 550 555 560
 Phe Gln Asn Tyr Leu His Glu Leu Lys Asp Tyr Asp Gln Val Glu Ser
 565 570 575
 Asn Glu Leu Asp Lys Tyr Met Ser Glu Pro Leu Leu Lys His Ser Gly
 580 585 590
 Gln Phe Asp Ile Leu Ser Trp Trp Arg Gly Arg Val Ala Glu Tyr Pro
 595 600 605
 Ile Leu Thr Gln Ile Ala Arg Asp Val Leu Ala Ile Gln Val Ser Thr
 610 615 620
 Val Ala Ser Glu Ser Ala Phe Ser Ala Gly Gly Arg Val Val Asp Pro
 625 630 635 640
 Tyr Arg Asn Arg Leu Gly Ser Glu Ile Val Glu Ala Leu Ile Cys Thr
 645 650 655
 Lys Asp Trp Val Ala Ala Ser Arg Lys Gly Ala Thr Tyr Phe Pro Thr
 660 665 670
 Met Ile Gly Asp Leu Glu Val Leu Asp Ser Val Ile Ala Ala Ala Thr
 675 680 685
 Asn His Glu Asn His Met Asp Glu Asp Glu Asp Ala Ile Glu Phe Ser
 690 695 700
 Lys Asn Asn Glu Asp Val Ala Ser Gly Ser Ser Pro
 705 710 715

<210> 21
 <211> 24
 <212> DNA
 <213> Artificial

<220>
 <223> Primer

<400> 21
 ccgtttaccg ttttgtatat ccgc

24.

<210> 22
 <211> 22
 <212> DNA
 <213> Artificial

<220>
 <223> Primer

<400> 22
 cggtccggtt tcgtttttta cc

22

18/48

<210> 23
<211> 20
<212> DNA
<213> Artificial

<220>
<223> Primer

<400> 23
cggtcggtac gggattttcc

20

<210> 24
<211> 24
<212> DNA
<213> Artificial

<220>
<223> Primer

<400> 24
cgattaccgt atttatcccg ttcg

24

<210> 25
<211> 19
<212> DNA
<213> Artificial

<220>
<223> Primer

<400> 25
ccggtatatc cggttttcg

19

<210> 26
<211> 21
<212> DNA
<213> Artificial

<220>
<223> Primer

<400> 26
gaaattgaaa acggtagagg t

21

<210> 27
<211> 16
<212> DNA
<213> Artificial

<220>
<223> Primer

<220>
<221> misc_feature
<222> (1)..(16)

19/48

<223> n may be any nucleotide

<400> 27
wgtgnagnan canaga

16

<210> 28
<211> 16
<212> DNA
<213> Artificial

<220>
<223> Primer

<220>
<221> misc_feature
<222> (1)..(16)
<223> n may be any nucleotide

<400> 28
wcagntgwn gtnctg

16

<210> 29
<211> 16
<212> DNA
<213> Artificial

<220>
<223> Primer

<220>
<221> misc_feature
<222> (1)..(16)
<223> n may be any nucleotide

<400> 29
sttgntastn ctntgc

16

<210> 30
<211> 16
<212> DNA
<213> Artificial

<220>
<223> Primer

<220>
<221> misc_feature
<222> (1)..(16)
<223> n may be any nucleotide

<400> 30
ncasgawagn cswcaa

16

20/48

<210> 31
<211> 4
<212> PRT
<213> Zea mays

<400> 31

Met Ala Ile Val
1

<210> 32
<211> 25
<212> DNA
<213> Artificial

<220>
<223> Zea mays Ds flanked by zebrafish DNA

<400> 32
catgttgaac tgactaggga tgaaa

25

<210> 33
<211> 25
<212> DNA
<213> Artificial

<220>
<223> Zea mays Ds flanked by zebrafish DNA

<400> 33
tttcatccct ggaactgacc gtcac

25

<210> 34
<211> 25
<212> DNA
<213> Artificial

<220>
<223> Zea mays Ds flanked by zebrafish DNA

<400> 34
tatatgcttt tcttttaggga tgaaa

25

<210> 35
<211> 25
<212> DNA
<213> Artificial

<220>
<223> Zea mays Ds flanked by zebrafish DNA

<400> 35
tttcatccct gctttttcttc tgctg

25

<210> 36

21/48

<211> 2148
<212> DNA
<213> Artificial

<220>
<223> NLS(K5E)-TPase103-807 CDS

<400> 36

```
atgggtcctc cagagaagaa gcgtaaggta gaaatggcta ttgttcatga accacaacca      60
caaccacaac cacaaccaga accacaacca cagccacaac ctgaaccoga agaagaagca      120
ccacagaaga gggcaaagaa gtgcacatcg gatgtatggc agcatttcac caagaaggaa      180
attgaagtgg aggtcgatgg aaagaaatac gttcaggtat gggggcattg caactttcct      240
aattgcaagg ctaagtatag ggctgagggt catcatggaa caagcggatt tcgaaatcac      300
ttgagaacat cacatagttt agttaaaggt cagttgtgtc taaaaagtga aaaggatcat      360
ggcaaagaca taaatctcat tgagcettat aagtacgatg aagtggttag cctaaagaag      420
cttcatttgg caataatcat gcatgaatac cttttcaata ttgtagaaca tgagtacttt      480
gttgagtttg ttaagtctct gcgccctcac tttccaataa agtcccggtg cactgctaga      540
aaatatatca tggatttcta tttggaagaa aaagaaaagt tgtatggaaa actaaaagat      600
gttcagtctc gtttcagtac aactatggat atgtggacat cttgtcaaaa taagtcatac      660
atgtgtgtca ccatccattg gattgatgat gattggtgtc tccaaaaaag aattgttggc      720
ttttttcatg ttgaaggggc ccacactggc caaaggttat cacaaacctt cactgcaatc      780
atggttaagt ggaacattga gaaaaaattg tttgccttgt ctttggataa tgctagtgca      840
aatgaagtag ctgtgcacga tataattgag gatttgcagg aactgattc aaatctagtt      900
tgtgatggtg ctttctttca tgtgaggtgt gottgtcaca tactgaactt ggttgcaaaag      960
gatggccttg ctgtaattgc aggaacaatt gagaaaatca aagcgattgt tcttgctgta     1020
aaatcttctc ctttgcagtg ggaagaacta atgaagtgtg ctagtgaatg tgacttggat     1080
aaatctaaag ggatctcata tgatgtctca actagatgga attcaaccta tttgatgttg     1140
agggatgcct tatattataa gcctgcacta ataaggctta aaacaagtga tcctcgcagg     1200
tacgatgcaa tttgtcctaa agccgaggag tggaagatgg cattaactct ttttaagtgt     1260
ttgaagaagt tttttgatct cactgaactc ctatctggta ctcaatattc cactgcaaat     1320
ttattttaca aaggtttctg tgagataaag gatttgattg accaatggtg tgttcatgaa     1380
aaatttgtca ttaggagaat ggccgttgca atgagtgaag agtttgagaa atattggaaa     1440
gtgtctaata ttgcactagc tgtagcatgc ttccttgacc ctaggtacaa gaaaatattg     1500
attgagttct atatgaaaaa atttcatggg gattcataca aagttcatgt agatgacttt     1560
```

22/48

```

gttagggtca ttagaaaatt gtatcaattc tattctagtt gtagtccttc agctccaaag 1620
acaaagacaa ctactaatga tagtatggat gatacottga tggaaaatga agatgatgaa 1680
tttcaaaact atttgcatga gttgaaggat tatgatcaag tagagtcaaa tgaattggat 1740
aaatatatgt ctgaaccocot tttgaagcat agtggtcagt ttgatatttt atcatgggtgg 1800
aggggaaggg ttgcagaata tcctattctc acccaaattg caagggatgt gctagcaata 1860
caagtgtcaa ctgttgcttc tgagtctgog ttcagtgctg gtggtcgtgt tgttgatcct 1920
taccgcaatc gtcttggttc ggagattggt gaagctttga tatgcacaaa agattgggta 1980
gcagcatcta gaaaagggtgc tacatatattt ccaacaatga ttggtgatct cgagggtgcta 2040
gactctgtta ttgctgctgc aacaaatcat gagaatcata tggatgagga tgaagacgca 2100
atagaatttt ctaagaataa tgaagatgta gcaagtggct cctctcca 2148

```

```

<210> 37
<211> 2687
<212> DNA
<213> Artificial

```

```

<220>
<223> Modified Ac Transposase Construct

```

```

<220>
<221> misc_feature
<222> (20)..(20)
<223> n is a, c, g, or t

```

```

<400> 37
attaggcttg tacatatgtn gttagaacgc ggctacaatt aatacataac cttatgtatc 60
atacacatac gatttaggtg acactataga atacaagctt gcttggttctt tttgcagaag 120
ctcagaataa acgctcaact ttggcagatc cgccaccatg ggtcctccag agaagaagcg 180
taaggtagaa atggctattg ttcattgaacc acaaccacaa ccacaaccac aaccagaacc 240
acaaccacag ccacaacctg aaccogaaga agaagcacca cagaagaggg caaagaagtg 300
cacatcggat gtatggcagc atttcaccaa gaaggaaatt gaagtggagg tcgatggaaa 360
gaaatacgtt caggtagggg ggcattgcaa otttcctaatt tgcaaggcta agtatagggc 420
tgagggtcat catggaacaa gcggatttcg aaatcacttg agaacatcac atagtttagt 480
taaaggtcag ttgtgtctaa aaagtgaaaa ggatcatggc aaagacataa atctcattga 540
gccttataag taogatgaag tggttagcct aaagaagctt catttggaac taatcatgca 600
tgaataccct ttcaatattg tagaacatga gtactttggt gagtttgta agtctctgog 660
ccctcacttt ccaataaagt ccggtgtcad tgctagaaaa tatatcatgg atttgtattt 720

```

23/48

ggaagaaaaa gaaaagttgt atggaaaact aaaagatggt cagtctcgct tcagtacaac 780
tatggatatg tggacatctt gtcaaaaataa gtcatacatg tgtgtcacca tccattggat 840
tgatgatgat tgggtgtctcc aaaaaagaat tgttggcttt tttcatgttg aagggcgcca 900
cactggccaa aggttatcac aaacottcac tgcaatcatg gttaagtgga acattgagaa 960
aaaattgttt gccttgtctt tggataatgc tagtgcaaat gaagtagctg tgcaogatat 1020
aattgaggat ttgcaggaca ctgattcaaa tctagtttgt gatggtgctt tctttcatgt 1080
gaggtgtgct tgtcacatac tgaacttggg tgcaaaggat ggcttggctg taattgcagg 1140
aacaattgag aaaatcaaag cgattgttct tgotgtaaaa tcttctctt tgcaagtggga 1200
agaactaatg aagtgtgcta gtgaatgtga cttggataaa tctaaaggga tctcatatga 1260
tgtctcaact agatggaatt caacctatct gatgttgagg gatgccttat attataagcc 1320
tgcaactaata aggttataaa caagtgatcc tgcagggtac gatgcaattt gtctaaagc 1380
cgaggagtgg aagatggcat taactctttt taagtgttg agaagtttt ttgatctcac 1440
tgaactccta tctggtactc aatattccac tgcaaattta ttttaciaag gtttctgtga 1500
gataaaggat ttgattgacc aatggtgtgt tcatgaaaaa tttgtcatta ggagaatggc 1560
cgttgcaatg agtgaaaagt ttgagaaata ttggaaagtg tctaattattg cactagctgt 1620
agcatgcttc cttgacccta ggtacaagaa aatattgatt gagttctata tgaaaaaatt 1680
tcatggtgat tcatacaaag ttcattgtga tgaotttgtt agggtcatta gaaaattgta 1740
tcaattctat tctagttgta gtccttcagc tccaaagaca aagacaacta ctaatgatag 1800
tatggatgat accttgatgg aaaatgaaga tgatgaattt caaaactatt tgcattgatt 1860
gaaggattat gatcaagtag agtcaaatga attggataaa tatatgtctg aacocctttt 1920
gaagcatagt ggtcagtttg atattttatc atggtggagg ggaaggggtg cagaatatcc 1980
tattctcacc caaattgcaa gggatgtgct agcaatacaa gtgtcaactg ttgcttctga 2040
gtctgcgttc agtgctggtg gtcgtgttgt tgatccttac cgcaatcgtc ttggttcgga 2100
gattgttgaa gctttgatat gcacaaaaga ttgggttagc gcatctagaa aagggtgtac 2160
atattttcca acaatgattg gtgatctoga ggtgctagac tctgttattg ctgctgcaac 2220
aatcatgag aatcatatgg atgaggatga agacgcaata gaattttcta agaataatga 2280
agatgtagca agtggctcct ctccatgagc aatgtgtctt atgtttgttg acagatgagc 2340
cttggttgta atagtttatg catgctaagt gctccagatg tgagcaagtg attatgaata 2400
tgtgttttaa actttatatt gtgtcatgtg tgctagtaga cttatatggc ttcttatgtt 2460
agccaagggg gccccgggat ctggttacca ctaaaccagc ctcaagaaca cccgaatgga 2520

24/48

gtctctaaagc tacataatac caacttacac tttaacaaat gttgtccccc aagatgtagc 2580

cattcgtatc tgctcctaataaaaaagaaag tttcttcaca ttctaaaaaa aaaaaaaaaa 2640

aaaaaaaaaa aaaaaaaacc oooooooooo ccccccctgca ggtcgac 2687

<210> 38
<211> 50
<212> DNA
<213> Artificial

<220>
<223> Primer

<400> 38
ctcaactttg gcagatccgc caccatggct attgttcacg aaccacaacc 50

<210> 39
<211> 50
<212> DNA
<213> Artificial

<220>
<223> Primer

<400> 39
ggttgtgggt catgaacaat agccatgggt gcggatctgc caaagttgag 50

<210> 40
<211> 31
<212> DNA
<213> Artificial

<220>
<223> PCR Primer

<400> 40
agagggatcc agctcagaat aaacgctcaa c 31

<210> 41
<211> 32
<212> DNA
<213> Artificial

<220>
<223> PCR Primer

<400> 41
agagaccggt cctggagagg agccacttgc ta 32

<210> 42
<211> 42
<212> DNA
<213> Artificial

25/48

<220>

<223> Primer

<400> 42

agaagaagcg taaggtagaa atggtgagca agggcgagga gc

42

<210> 43

<211> 42

<212> DNA

<213> Artificial

<220>

<223> Primer

<400> 43

gctcctcgcc cttgctcacc atttctacct taogcttctt ct

42

<210> 44

<211> 716

<212> PRT

<213> Artificial

<220>

<223> NLS(K5E)-TPase103-807

<400> 44

Met	Gly	Pro	Pro	Glu	Lys	Lys	Arg	Lys	Val	Glu	Met	Ala	Ile	Val	His
1				5					10					15	

Glu	Pro	Gln	Pro	Gln	Pro	Gln	Pro	Gln	Pro	Glu	Pro	Gln	Pro	Gln	Pro
			20					25					30		

Gln	Pro	Glu	Pro	Glu	Glu	Glu	Ala	Pro	Gln	Lys	Arg	Ala	Lys	Lys	Cys
		35					40					45			

Thr	Ser	Asp	Val	Trp	Gln	His	Phe	Thr	Lys	Lys	Glu	Ile	Glu	Val	Glu
	50					55					60				

Val	Asp	Gly	Lys	Lys	Tyr	Val	Gln	Val	Trp	Gly	His	Cys	Asn	Phe	Pro
65					70					75					80

Asn	Cys	Lys	Ala	Lys	Tyr	Arg	Ala	Glu	Gly	His	His	Gly	Thr	Ser	Gly
				85					90					95	

Phe	Arg	Asn	His	Leu	Arg	Thr	Ser	His	Ser	Leu	Val	Lys	Gly	Gln	Leu
			100						105					110	

Cys	Leu	Lys	Ser	Glu	Lys	Asp	His	Gly	Lys	Asp	Ile	Asn	Leu	Ile	Glu
		115					120						125		

Pro	Tyr	Lys	Tyr	Asp	Glu	Val	Val	Ser	Leu	Lys	Lys	Leu	His	Leu	Ala
		130					135					140			

Ile	Ile	Met	His	Glu	Tyr	Pro	Phe	Asn	Ile	Val	Glu	His	Glu	Tyr	Phe
145						150					155				160

Val	Glu	Phe	Val	Lys	Ser	Leu	Arg	Pro	His	Phe	Pro	Ile	Lys	Ser	Arg
-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----

26/48

165										170					175				
Val	Thr	Ala	Arg	Lys	Tyr	Ile	Met	Asp	Leu	Tyr	Leu	Glu	Glu	Lys	Glu				
			180					185					190						
Lys	Leu	Tyr	Gly	Lys	Leu	Lys	Asp	Val	Gln	Ser	Arg	Phe	Ser	Thr	Thr				
		195					200					205							
Met	Asp	Met	Trp	Thr	Ser	Cys	Gln	Asn	Lys	Ser	Tyr	Met	Cys	Val	Thr				
	210					215					220								
Ile	His	Trp	Ile	Asp	Asp	Asp	Trp	Cys	Leu	Gln	Lys	Arg	Ile	Val	Gly				
225					230						235				240				
Phe	Phe	His	Val	Glu	Gly	Arg	His	Thr	Gly	Gln	Arg	Leu	Ser	Gln	Thr				
			245						250					255					
Phe	Thr	Ala	Ile	Met	Val	Lys	Trp	Asn	Ile	Glu	Lys	Lys	Leu	Phe	Ala				
			260					265						270					
Leu	Ser	Leu	Asp	Asn	Ala	Ser	Ala	Asn	Glu	Val	Ala	Val	His	Asp	Ile				
		275					280					285							
Ile	Glu	Asp	Leu	Gln	Asp	Thr	Asp	Ser	Asn	Leu	Val	Cys	Asp	Gly	Ala				
	290					295					300								
Phe	Phe	His	Val	Arg	Cys	Ala	Cys	His	Ile	Leu	Asn	Leu	Val	Ala	Lys				
305					310						315				320				
Asp	Gly	Leu	Ala	Val	Ile	Ala	Gly	Thr	Ile	Glu	Lys	Ile	Lys	Ala	Ile				
				325					330					335					
Val	Leu	Ala	Val	Lys	Ser	Ser	Pro	Leu	Gln	Trp	Glu	Glu	Leu	Met	Lys				
			340					345						350					
Cys	Ala	Ser	Glu	Cys	Asp	Leu	Asp	Lys	Ser	Lys	Gly	Ile	Ser	Tyr	Asp				
		355					360					365							
Val	Ser	Thr	Arg	Trp	Asn	Ser	Thr	Tyr	Leu	Met	Leu	Arg	Asp	Ala	Leu				
	370					375					380								
Tyr	Tyr	Lys	Pro	Ala	Leu	Ile	Arg	Leu	Lys	Thr	Ser	Asp	Pro	Arg	Arg				
385					390						395				400				
Tyr	Asp	Ala	Ile	Cys	Pro	Lys	Ala	Glu	Glu	Trp	Lys	Met	Ala	Leu	Thr				
				405					410					415					
Leu	Phe	Lys	Cys	Leu	Lys	Lys	Phe	Phe	Asp	Leu	Thr	Glu	Leu	Leu	Ser				
			420					425						430					
Gly	Thr	Gln	Tyr	Ser	Thr	Ala	Asn	Leu	Phe	Tyr	Lys	Gly	Phe	Cys	Glu				
		435					440					445							
Ile	Lys	Asp	Leu	Ile	Asp	Gln	Trp	Cys	Val	His	Glu	Lys	Phe	Val	Ile				
	450					455					460								
Arg	Arg	Met	Ala	Val	Ala	Met	Ser	Glu	Lys	Phe	Glu	Lys	Tyr	Trp	Lys				
465					470						475				480				
Val	Ser	Asn	Ile	Ala	Leu	Ala	Val	Ala	Cys	Phe	Leu	Asp	Pro	Arg	Tyr				

485

490

495

Lys Lys Ile Leu Ile Glu Phe Tyr Met Lys Lys Phe His Gly Asp Ser
500 505 510

Tyr Lys Val His Val Asp Asp Phe Val Arg Val Ile Arg Lys Leu Tyr
515 520 525

Gln Phe Tyr Ser Ser Cys Ser Pro Ser Ala Pro Lys Thr Lys Thr Thr
530 535 540

Thr Asn Asp Ser Met Asp Asp Thr Leu Met Glu Asn Glu Asp Asp Glu
545 550 555 560

Phe Gln Asn Tyr Leu His Glu Leu Lys Asp Tyr Asp Gln Val Glu Ser
565 570 575

Asn Glu Leu Asp Lys Tyr Met Ser Glu Pro Leu Leu Lys His Ser Gly
580 585 590

Gln Phe Asp Ile Leu Ser Trp Trp Arg Gly Arg Val Ala Glu Tyr Pro
595 600 605

Ile Leu Thr Gln Ile Ala Arg Asp Val Leu Ala Ile Gln Val Ser Thr
610 615 620

Val	Ala	Ser	Glu	Ser	Ala	Phe	Ser	Ala	Gly	Gly	Arg	Val	Val	Asp	Pro
625					630					635					640

Tyr Arg Asn Arg Leu Gly Ser Glu Ile Val Glu Ala Leu Ile Cys Thr
645 650 655

Lys Asp Trp Val Ala Ala Ser Arg Lys Gly Ala Thr Tyr Phe Pro Thr
660 665 670

Met Ile Gly Asp Leu Glu Val Leu Asp Ser Val Ile Ala Ala Ala Thr
675 680 685

Asn His Glu Asn His Met Asp Glu Asp Glu Asp Ala Ile Glu Phe Ser
690 695 700

Lys Asn Asn Glu Asp Val Ala Ser Gly Ser Ser Pro
705 710 715

<210> 45

<211> 292

<212> DNA

<213> Zea mays

<400> 45

caggggatgaa agtaggatgg gaaaatcccg taccgaccgt tatcgtataa ccgattttot 60

tagttttatc ccgatcgatt tcgaacccga ggtaaaaaaac gaaaacggaa cggaaacggg 120

atatacaaaa cggtaaacgg aaacggaaac ggtagagcta gtttcccgac cgtttcaccg 180

ggatcccggtt tttaatcggg atgatcccggt ttcgttaccg tattttctaa ttcgggatga 240

ctgcaacaga tccctcgagc gcttaagttt aaacgcgtta acaattggcc at 292

28/48

<210> 46
 <211> 1395
 <212> DNA
 <213> Artificial

<220>
 <223> pEGFP-N3 fragment

<400> 46
 aatagtaatc aattacgggg tcattagttc atagcccata tatggagttc cgcgttacat 60
 aacttacggt aaatggcccg cctggctgac cgcccaaoga ccccgccca ttgaogtcaa 120
 taatgaogta tgttcccata gtaacgcaa tagggacttt ccattgacgt caatgggtgg 180
 agtatttacg gtaaaactgc cacttggcag tacatcaagt gtatcatatg ccaagtacgc 240
 cccctattga cgtcaatgac ggtaaatggc ccgcctggca ttatgccag tacatgacct 300
 tatgggactt toctacttgg cagtacatct acgtattagt catogctatt accatggtga 360
 tgcggttttg gcagtacatc aatgggcgtg gatagcgggt tgactcaagg ggatttccaa 420
 gtctccaccc cattgacgtc aatgggagtt tgttttggca ccaaaatcaa cgggactttc 480
 caaaatgtcg taacaactcc gcccattga cgcaaattgg cggtaggcgt gtacggtggg 540
 aggtctatat aagcagagct ggttttagtga accgtcagat ccgctagcgc taccggactc 600
 agatctcgag ctcaagcttc gaattctgca gtgcacggta ccgcggggcc gggatccatc 660
 gccaccatgg tgagcaaggg cgaggagctg ttcaacgggg tggtgcccat cctggtcgag 720
 ctggacggcg acgtaaacgg ccacaagttc agcgtgtccg gcgagggcga gggcgatgcc 780
 acctacggca agctgacct gaagttcatc tgcaaccacc gcaagctgcc cgtgccctgg 840
 cccacctcg tgaccacct gacctacggc gtgcagtgtc tcagccgcta ccccgaccac 900
 atgaagcagc acgaattott caagtccgc atgcccgaag gctacgtcca ggagcgcacc 960
 atcttcttca aggacgacgg caactacaag accgcgcgcg aggtgaagtt cgagggcgac 1020
 accctggtga accgcacoga gctgaagggc atcgacttca aggaggacgg caacatcctg 1080
 gggcacaagc tggagtacaa ctacaacagc cacaacgtct atatcatggc cgacaagcag 1140
 aagaacggca tcaaggtgaa cttcaagatc cgcacacaac tcgaggacgg cagcgtgcag 1200
 ctgcgcgacc actaccagca gaacaccccc atcggcgacg gcccgtgtgt gctgcccgac 1260
 aaccactacc tgagcaccga gtccgccctg agcaaagacc ccaacagaga gcgcgatcac 1320
 atggtcctgc tggagttcgt gaccgcgcgc gggatcactc tcggcatgga cgagctgtac 1380
 aagtaaagcg gcgcg 1395

29/48

<210> 47
<211> 1716
<212> DNA
<213> Artificial

<220>

<223> pUBBSD1 plasmid fragment

<400> 47

```
gatatcgcta gctcgagatc gggagatctg gcctccgcgc cgggttttgg cgcggccggc 60
ggggggcccc tcctcacggc gagcgctgcc acgtcagacg aagggcgcac gagcgctcctg 120
atccttccgc ccggacgctc aggacagcgg ccgctgctc ataagactcg gccttagaac 180
cccagtatca gcagaaggac attttaggac gggacttggg tgactctagg gcactggttt 240
tctttccaga gagcggaaca ggcgaggaaa agtagtccct tctcgccgat tctgcggagg 300
gatctccgtg gggcggtgaa cgccgatgat tatataagga cgcgcggggt gtggcacagc 360
tagttccgtc gcagccggga tttgggtcgc ggttcttgtt tgtggatcgc tgtgatcgtc 420
acttggtgag tagcgggctg ctgggctggc cggggctttc gtggccggcg ggccgctcgg 480
tgggacggaa gcgtgtggag agaccgcaa gggctgtagt ctgggtccgc gagcaaggtt 540
gccctgaact ggggggttggg gggagcgcag caaaatggcg gctgttcccg agtcttgaat 600
ggaagacgct tgtgaggcgg gctgtgaggt cgttgaaaca aggtgggggg catggtgggc 660
ggcaagaacc caaggtcttg aggccttcgc taatgcggga aagctcttat tcgggtgaga 720
tgggctgggg caccatctgg ggaccctgac gtgaagtttg tcaactgactg gagaactcgg 780
tttgtcgtct gttgcggggg cggcagttat ggcgggtgocg ttgggcagtg caccggtacc 840
tttgggagcg cgcgccctcg tcgtgtcgtg acgtcacccg ttctgttggc ttataatgca 900
gggtggggcc acctgcgggt aggtgtgcgg taggcctttc tccgtcgcag gacgcagggt 960
tcgggcctag ggtaggctct cctgaatcga caggcgccgg acctctggtg aggggaggga 1020
taagtgaggc gtcagtttct ttggtcgggt ttatgtacct atcttcttaa gtagctgaag 1080
ctccggtttt gaactatgcg ctccggggtg gcgagtgtgt tttgtgaagt tttttaggca 1140
ccttttgaaa tgtaatcatt tgggtcaata tgtaattttc agtgttagac tagtaaattg 1200
tccgctaaat tctggccggt tttggctttt ttgttagacc ggaccgtgtt gacaattaat 1260
catoggcata gtatatcggc atagtataat acgacaaggt gaggaactaa accatggcca 1320
agcctttgtc tcaagaagaa tccaccctca ttgaaagagc aacggctaca atcaacagca 1380
tcccatctc tgaagactac agcgctgcga gcgcagctct ctctagcgac ggccgcatct 1440
tcactggtgt caatgtatat cattttaactg ggggaccttg tgcagaactc gtggtgctgg 1500
gcactgctgc tgctcgggca gctggcaacc tgacttgtat cgtcgcgac ggaaatgaga 1560
```

30/48

acaggggcat cttgagcccc tgcggacggt gccgacaggt gcttctcgat ctgcatcctg 1620
 ggatcaaagc catagtgaag gacagtgatg gacagccgac ggcagttggg attcgtgaat 1680
 tgctgccctc tggttatgtg tgggagggt aagcac 1716

<210> 48
 <211> 1062
 <212> DNA
 <213> Artificial

<220>
 <223> Cloning vector fragment with plasmid replication origin

<400> 48
 ttctgtggcgg aggagcagga ctgacactcg acctcgaaac ttgtttattg cagcttataa 60
 tggttacaaa taaagcaata gcatcacaaa tttcacaaat aaagcatttt tttcactgca 120
 ttctagttgt ggtttgtcca aactcatcaa tgtatcttat catgtctgaa ttccogggga 180
 tcctctagac tgtcagacca agtttactca tatatacttt agattgattt aaaacttcat 240
 ttttaattta aaaggatcta ggtgaagatc ctttttgata atctcatgac caaaatcctt 300
 taaogtgagt tttcgttcca ctgagcgtca gaccccgtag aaaagatcaa aggatcttct 360
 tgagatcctt tttttctgcg cgtaatctgc tgcttgcaaa caaaaaaac accgctacca 420
 ggggtgggtt gtttgcogga tcaagagcta ccaactcttt ttccgaaggt aactggcttc 480
 agcagagcgc agataccaaa tactgttctt ctagtgtagc cgtagttagg ccaccacttc 540
 aagaactctg tagcacccgc tacatacctc gctctgctaa tcctgttacc agtggctgct 600
 gccagtggcg ataagtcgtg tcttacccgg ttggactcaa gaogatagtt accggataag 660
 gcgcagcggg cgggctgaac ggggggttcg tgcacacagc ccagcttgga gcgaacgacc 720
 tacaccgaac tgagatacct acagcgtgag ctatgagaaa gcgccacgct tccogaaggg 780
 agaaaggcgg acaggtatcc ggttaagcggc agggtcggaa caggagagcg cacgaggag 840
 cttccagggg gaaacgcctg gtatctttat agtctgtcg ggtttcgcca cctctgactt 900
 gagcgtcgat ttttgtgatg ctgcgcaggg gggcggagcc tatggaaaaa cgccagcaac 960
 gcggcctttt tacggttccc gcccttttgc tggccttttg ctacatgct gggcccagcc 1020
 ggccagatct gagctcgcgg ccgcgatatc gctagctcga gg 1062

<210> 49
 <211> 370
 <212> DNA
 <213> Zea mays

<400> 49

31/48

```

gacttatatg gotttattatg ttagccaaga gccaagact tatcacttat gtgtacatt      60
aaactatgtg tgctccagat ttatatggat tttatctatg ttttaattaag acttgtgttt      120
acaatttttt atatttggtt ttaagttttg aatatatggt ttcattgtgtg attttacoga      180
acaaaaatac cggttcccg cggatttoga ctttaaccgg accggatcgt atcgggtttc      240
gattacogta tttatcccg tcgttttcgt taccgggtata tcccgttttc gtttccgctc      300
cgcaagttaa atatgaaaat gaaaacggta gaggtatttt accgaccggt accgaccggt      360
ttcatcccta                                     370

```

```

<210> 50
<211> 1660
<212> DNA
<213> Artificial

```

<220>

<223> Cloning vector fragment with Amp resistance gene

<400> 50

```

tgcatcttga cagttaccaa tgcttaatca gtgaggcacc tatctcagcg atctgtctat      60
ttcgttcctc catagttgcc tgactccccg togtgtagat aactacgata cgggagggct      120
taccatctgg cccagtgct gcaatgatac cgcgagacc acgctcacgg gctccagatt      180
tatcagcaat aaaccagcca gcggaaggg cgcgagcag aagtggctct gcaactttat      240
ccgcctccat ccagtctatt aattgttgcc gggaagctag agtaagtagt tcgccagtta      300
atagtttgcg caacgttggt gccattgcta caggcatcgt ggtgtcagcg tcgtcgtttg      360
gtatggcttc attcagctcc ggttcccaac gatcaaggcg agttacatga tccccatgt      420
tgtgcaaaaa agcgggttagc tccttcgggc ctccgatcgt tgtcagaagt aagttggccg      480
cagtgttctc actcatggtt atggcagcac tgcataatc tcttactgtc atgccatccg      540
taagatgctt ttctgtgact ggtgagtact caaccaagtc attctgagaa tagtgtatgc      600
ggcgaccgag ttgctcttgc cggcgctcaa taogggataa taccgggcca catagcagaa      660
ctttaaaagt gctcatcatt ggaaaacgtt cttcggggcg aaaactctca aggatcttac      720
cgctgttgag atccagttcg atgtaaccca ctctgcacc paactgatct tcagcatctt      780
ttactttcac cagcgtttct ggttgagcaa aaacaggaag gcaaaatgcc gcaaaaaagg      840
gaataagggc gacacggaaa tggtgaatac tcatactctt cctttttcaa tataattgaa      900
gcatttatca gggttattgt ctcatgagcg gatacatatt tgaatgtatt tagaaaaata      960
aacaatatag ggttccgcgc acatttcccc gaaaagtgcc acctaaattg taagcgttaa      1020
tattttgtta aaattcgcgt taaatttttg ttaaatacgc tcatttttta accaataggc      1080

```

32/48

cgaaatcggc aaaatccctt ataaatcaaa agaatagacc gagatagggg tgagtgttgt 1140
 tccagtttgg aacaagagtc cactattaaa gaacgtggac tocaacgtca aagggcgaaa 1200
 aacggtctat cagggcgatg gccactacg tgaaccatca ccctaataca gttttttggg 1260
 gtcgaggtgc ogtaaagcac taaatcgga ccctaaaggg agccccgat ttagagcttg 1320
 acggggaaaag cgggcgaacg tggcgagaaa ggaagggaag aaagcgaaag gagcggggcg 1380
 tagggcgctg gcaagtgtag cggtcacgct gcgcgtaacc accacacccg ccgcgcttaa 1440
 tgcgcgcgta cagggcgctg ccattcgcc attcaggctg cgcaactgtt gggaagggcg 1500
 atcgggtgcgg gcctcttcgc tattacgcca gctggcgaaa gggggatgtg ctgcaaggcg 1560
 attaagttgg gtaacgccag ggttttccca gtcacgacgt tgtaaaacga cggccagtga 1620
 attgatacga ctactatag ggccaattgg gtaccgcagc 1660

<210> 51
 <211> 33
 <212> DNA
 <213> Artificial

<220>
 <223> PCR Primer

<400> 51
 ggcgggatcc atacgattta ggtgacacta tag 33

<210> 52
 <211> 34
 <212> DNA
 <213> Artificial

<220>
 <223> PCR Primer

<400> 52
 cgatcgatgc ggccgccttg gctaacataa gaag 34

<210> 53
 <211> 665
 <212> DNA
 <213> Artificial

<220>
 <223> pEGFP-N1 plasmid fragment

<400> 53
 tagttattaa tagtaatcaa ttacgggggtc attagttcat agcccatata tggagttccg 60
 cgttacataa cttacggtaa atggcccgcc tggctgaccg cccaacgacc cccgcccatt 120
 gacgtcaata atgacgtatg ttcccatagt aacgccaata gggactttcc attgacgtca 180

33/48

```

atgggtggag tatttacggt aaactgccca cttggcagta catcaagtgt atcatatgcc 240
aagtaagccc cctattgacg tcaatgacgg taaatggccc gcctggcatt atgccagta 300
catgaacctta tgggactttc ctacttggca gtacatctac gtattagtoa tcgctattac 360
catggtgatg cggtttttggc agtacatcaa tgggcgtgga tagcggtttg actcacgggg 420
atttccaagt ctccacccca ttgacgtcaa tgggagtttg ttttggcacc aaaatcaacg 480
ggactttoca aaatgtcgta acaactccgc cccattgacg caaatgggcg gtaggcgtgt 540
acggtgggag gtctatataa gcagagctgg tttagtgaac cgtcagatcc gctagcgcta 600
cgggactcag atctcgagct caagcttcga attctgcagt cgacgggtacc gcgggcccgg 660
gatcc 665

```

<210> 54
 <211> 2401
 <212> DNA
 <213> Artificial

<220>
 <223> Amplified fragment with NLS(K5E)-TPase ORF

```

<400> 54
atacgatttta ggtgacacta tagaatacaa gotttgcttgt tcttttttgca gaagctcaga 60
ataaacgctc aacttttggca gatccgccac catgggtcct ccagagaaga agcgtaaggt 120
agaaatggct attgttcatg aaccacaacc acaaccacaa ccacaaccag aaccacaacc 180
acagccacaa cctgaacccg aagaagaagc accacagaag agggcaaaga agtgcacatc 240
ggatgtatgg cagcatttca ccaagaagga aattgaagtg gaggtcgatg gaaagaaata 300
cgttcaggta tgggggcatt gcaactttcc taattgcaag gctaagtata gggctgaggg 360
tcacatgga acaagcggat ttcgaaatca cttgagaaca tcacatagtt tagttaaagg 420
tcagtttgtg ctaaaaagtg aaaaggatca tggcaaagac ataaatctca ttgagcetta 480
taagtacgat gaagtggta gcctaaagaa gotttcatttg gcaataatca tgcataaata 540
ccctttcaat attgtagaac atgagtactt tgttgagttt gttaagtctc tgcgccctca 600
ctttccaata aagtcccgtg tcaactgctag aaaatatatc atggatttgt atttggaaga 660
aaaagaaaag ttgtatggaa aactaaaaga tgttcagtct cgcttcagta caactatgga 720
tatgtggaca tcttgtcaaa ataagtcata catgtgtgtc accatccatt ggattgatga 780
tgattgggtg ctccaaaaaa gaattgttgg cttttttcat gttgaagggc gccacactgg 840
ccaaagggtta tcacaaacct tcaactgcaat catgggttaag tggaacattg agaaaaaatt 900
gtttgcottg tcttttgata atgctagtgc aaatgaagta gctgtgcacg atataattga 960

```

34/48

```

ggatttgcag gacactgatt caaatctagt ttgtgatggg gctttctttc atgtgaggtg 1020
tgcttgtcac atactgaact tggttgcaaa ggatggcttg gctgtaattg caggaacaat 1080
tgagaaaatc aaagcgattg ttcttgctgt aaaatcttct cctttgcagt gggaagaact 1140
aatgaagtgt gctagtgaat gtgacttgga taaatctaaa gggatctcat atgatgtctc 1200
aactagatgg aattcaacct atttgatgtt gagggatgcc ttatattata agcctgcaact 1260
aataaggctt aaaacaagtg atcctcgag gtaogatgca atttgtccta aagccgagga 1320
gtggaagatg gcattaactc tttttaagtg tttgaagaag ttttttgatc tcaactgaact 1380
cctatctggg actcaatatt ccactgcaaa tttattttac aaaggtttct gtgagataaa 1440
ggatttgatt gaccaatggg gtgttcataa aaaatttgtc attaggagaa tggccgttgc 1500
aatgagtga aagtttgaga aatattggaa agtgtctaatt attgcactag ctgtagcatg 1560
cttcottgac cctaggtaca agaaaatatt gattgagttc tatatgaaaa aatttcattg 1620
tgattcatac aaagttcatg tagatgactt tgttaggggc attagaaaaat tgtatcaatt 1680
ctattctagt tgtagtcctt cagctccaaa gacaaagaca actactaatg atagtatgga 1740
tgataccttg atggaaaatg aagatgatga atttcaaaac tatttgcatg agttgaagga 1800
ttatgatcaa gtagagtcaa atgaattgga taaatatatg tctgaacccc ttttgaagca 1860
tagtggtcag tttgatattt tatcatgggt gaggggaagg gttgcagaat atcctattct 1920
cacccaaatt gcaagggatg tgctagcaat acaagtgtca actgttgctt ctgagttctgc 1980
gttcagtgcg ggtggctcgt ttgttgatcc ttaccgcaat cgtcttggtt cggagattgt 2040
tgaagctttg atatgcacaa aagattgggt agcagcatct agaaaagggt ctacatattt 2100
tccaacaatg attggtgatc tcgaggtgct agactctggt attgctgctg caacaaatca 2160
tgagaatcat atggatgagg atgaagacgc aatagaattt totaagaata atgaagatgt 2220
agcaagtggc tcctctccat gagcaatgtg tcttatgttt gttgacagat gagccttggt 2280
tgtaatagtt tatgcatgct aagtgtcca gatgtgagca agtgattatg aatatgtgtt 2340
ttaaacttta tattgtgtca tgtgtgctag tagacttata tggcttctta tgttagccaa 2400
g 2401

```

<210> 55
 <211> 3334
 <212> DNA
 <213> Artificial

<220>
 <223> pEGFP-N1 plasmid fragment

<400> 55

35/48

gcgggcgcga ctctagatca taatcagcca taccacattt gtagaggttt tacttgcttt	60
aaaaaacctc ccacacctcc ccctgaacct gaaacataaa atgaatgcaa ttgtttgttgt	120
taaottgttt attgcagctt ataattggtta caaataaaagc aatagcatca caaatttcac	180
aaataaagca tttttttcac tgcattctag ttgtggtttg tccaaactca tcaatgtatc	240
ttaaggcgta aattgtaagc gttaatat ttgttaaaatt cgcgttaaatt ttttgttaaa	300
tcagctcatt ttttaaccaa taggcgaaa tcggcaaaat cccttataaa tcaaaagaat	360
agaccgagat aggggttgagt gttgttccag tttggaacaa gagtccacta ttaaagaacg	420
tggactccaa cgtcaaaggc cgaaaaaccg totatcaggc cgatggccca ctacgtgaac	480
catcaccta atcaagtttt ttggggctga ggtgcgtaa agcactaaat cggaaacctc	540
aaggagagccc cagatttaga gcttgacggc gaaagccggc gaacgtggcg agaaaggaag	600
ggaagaaagc gaaaggagcg ggcgctaggc cgctggcaag tgtagcggtc acgctgocg	660
taaccaccac acccgccgcg cttaatgcgc cgctacaggc cgcgtcaggt ggcacttttc	720
ggggaaatgt ggcggaacc cctatttgtt tatttttcta aatacattca aatatgtatc	780
cgctcatgag acaataaacc tgataaatgc ttcaataata ttgaaaagg aagagtcctg	840
aggcggaag aaccagctgt ggaatgtgtg tcagttaggc tgtggaaagt cccaggctc	900
cccagcaggc agaagtatgc aaagcatgca totcaattag tcagcaacca ggtgtgaaa	960
gtcccaggc tcccagcag gcagaagtat gcaaagcatg catctcaatt agtcagcaac	1020
catagtcccg cccctaactc cgcccatccc gcccctaact ccgcccagtt ccgcccattc	1080
tccgccccat ggctgactaa ttttttttat ttatgcagag gccgaggccg cctcggcctc	1140
tgagctattc cagaagtagt gaggaggctt ttttgaggc ctaggctttt gcaaagatcg	1200
atcaagagac aggatgagga tcgtttcgca tgattgaaca agatggattg cacgcagggt	1260
ctccggccgc ttgggtggag aggcatttcg gctatgactg ggcacaacag acaatcggct	1320
gctctgatgc cgccgtgttc cggctgtcag cgcaggggcg cccggttctt tttgtcaaga	1380
ccgacctgtc cgggtccctg aatgaactgc aagacgaggc agcgggcta tcgtggctgg	1440
ccacgacggc cgttccctgc gcagctgtgc tcgacgttgt cactgaagcg ggaagggact	1500
ggctgctatt gggcgaagtg ccggggcagg atctcctgtc atctcacctt gctectgccg	1560
agaaagtatc catcatggct gatgcaatgc ggcggctgca tacgcttgat ccggctacct	1620
gccattcga ccaccaagcg aaacatcgca tcgagcgagc acgtactcgg atggaagccg	1680
gtcttgctga tcaggatgat ctggacgaag agcatcaggc gctcgcgcca gccgaactgt	1740
tcgccaggct caaggcgagc atgcccagc gcgaggatct cgtcgtgacc catggcgatg	1800

36/48

```

cctgottgoc gaatatcatg gtggaaaatg gcogettttc tggattcatc gactgtggcc 1860
ggctgggtgt ggcggaacgc tatcaggaca tagcgttggc taccogtgat attgotgaag 1920
agcttggcgg cgaatgggct gaccgcttcc tcgtgcttta cggtatogoc gctcccgatt 1980
cgcagogcat cgccttctat cgccttcttg acgagttott ctgagcggga ctctggggtt 2040
cgaaatgacc gaccaagcga cgcaccaact gccatcacga gatttcgatt ccaccgccgc 2100
cttctatgaa aggttgggct tcggaatcgt tttcogggac gccggctgga tgatcctcca 2160
gcgcggggat ctcatgctgg agttcttcgc ccaccctagg gggaggctaa ctgaaacacg 2220
gaaggagaca ataccggaag gaaccgcgc tatgacggca ataaaaagac agaataaac 2280
gcacggtgtt gggctgcttg ttcataaacg cgggggttcgg toccagggct ggcactctgt 2340
cgatacccca ccgagacccc attggggcca atacgccgc gtttcttctt tttcccccacc 2400
ccacccccca agttcgggtg aaggcccagg gctcgcagcc aacgtogggg cggcaggccc 2460
tgccatagcc tcaggttact catatatact ttagattgat ttaaaaottc atttttaatt 2520
taaaaggatc taggtgaaga tcttttttga taatctcatg accaaaatcc cttaacgtga 2580
gttttcgttc cactgagcgt cagaccccggt agaaaagatc aaaggatott cttgagatcc 2640
ttttttctg cgcgtaatct gctgcttgca aacaaaaaaa ccaccgctac cagcgggtgg 2700
ttgtttgcg gatcaagagc taccaactct ttttcogaag gtaactggct tcagcagagc 2760
gcagatacca aatactgtcc ttctagtgtg gccgtagtta ggccaccact tcaagaactc 2820
tgtagcaccg cctacatacc tcgctctgct aatcctgtta ccagtggctg ctgccagtgg 2880
cgataagtgc tgtcttaccg ggttggactc aagacgatag ttaccggata aggcgcagcg 2940
gtcgggctga acgggggggt cgtgcacaca gccagcttg gagcgaacga cctacacoga 3000
actgagatac ctacagcgtg agctatgaga aagcgccacg cttcccgaag ggagaaaggc 3060
ggacaggat ccggtaaagc gcagggtcgg aacaggagag cgcacgaggg agcttccagg 3120
gggaaacgcc tggatatctt atagtcctgt cgggtttcgc cacctctgac ttgagcgtcg 3180
atttttgtga tgctcgtcag gggggcggag cctatggaaa aacgccagca acgcggcctt 3240
tttacggttc ctggcctttt gctggccttt tgcacacatg ttctttctg cgttatcccc 3300
tgattctgtg gataaccgta ttaccgcat gcat 3334

```

<210> 56

<211> 259

<212> DNA

<213> Artificial

<220>

<223> Zea mays Ds flanked by human DNA

37/48

<400> 56
atcgggttata cgatacggtc ggtacgggat tttcccatcc tacttttcato cctgagtgag 60
actctgacgg cctcctgctt cccccacact gtggaagggtg gggctcccca agcctgcact 120
cctacotgag atgccagtct ctttgtctac ttttagacacc aggttcctaaa gaccctgtgc 180
tctcagatac cttttatgtc taaagcagag gccctacccc agcactcccc atctcagtag 240
acagtaactc catttacca 259

<210> 57
<211> 253
<212> DNA
<213> Artificial

<220>
<223> Zea mays Ds flanked by human DNA

<400> 57
agcacaggggt ctttggaacc tgggtgtctaa agtagacaaa gagactggca tctcaggtag 60
gagtgcaggc ttggggagcc ccaccttcca cagtgtgggg gaagcaggag gccgtcagag 120
tctcactcag ggatgaaagt aggatgggaa aatcccgtac cgaccgttat cgtataaccg 180
attttgtttag ttttatcccg atcgatttcg aacccgaggt aaaaaacgaa aacggaacgg 240
aaacggatat aca 253

<210> 58
<211> 260
<212> DNA
<213> Artificial

<220>
<223> Zea mays Ds flanked by human DNA

<400> 58
gggcctctgc tttagacata aaaggtatct gagagcacag ggtcttttga acctggtgtc 60
taaagtagac aaagagactg gcattctcagg taggagtgca ggcttgggga gcccacctt 120
ccacagtgtg ggggaagcag gaggccgtca gagtctcact cagggatgaa agtaggatgg 180
gaaaatcccg taccgaccgt tatcgtataa ccgattttgt tagttttatc ccgatcgatt 240
tcgaaccoga ggtaaaaacg 260

<210> 59
<211> 265
<212> DNA
<213> Artificial

<220>
<223> Zea mays Ds flanked by human DNA

38/48

<400> 59
 gcttttagaca taaaaggtat ctgagagcac agggctctttg gaacctggtg totaaagtag 60
 acaaagagac tggcatotca ggtaggagtg caggcttggg gagccccacc ttccacagtg 120
 tgggggaagc aggaggoögt cagagtctca ctcagggatg aaagtaggat gggaaaatcc 180
 cgtaccgacc gttatogtat aaccgatttt gttagtttta tcccgatoga tttcgaaccc 240
 gaggtaaaaa acgaaaacgg aacga 265

<210> 60
 <211> 250
 <212> DNA
 <213> Artificial

<220>
 <223> Zea mays Ds flanked by human DNA

<400> 60
 actggcaaca ccgcctcttg ttctcctcta tttccagcat ccagaaaagt gtctggctta 60
 tccttggtgc tcataaatt tttactgaat gaatgaataa atgaattcat gaatgaacag 120
 gatggctagt gagactaggg atgaaaacgg tcggtaacgg tcggtaaaat acctctaccg 180
 ttttcatttt catatttaac ttgcgggacg gaaacgaaaa cgggatatac cggtaacgaa 240
 aacgaacggg 250

<210> 61
 <211> 218
 <212> DNA
 <213> Artificial

<220>
 <223> Zea mays Ds flanked by human DNA

<400> 61
 gactggcaac accgcctctt gttctcctct atttccagca tccagaaaag tgtctggctt 60
 atccttggtg ctccataaat ttttactgaa tgaatgaata aatgaattca tgaatgaaca 120
 ggatggctag tgagactagg gatgaaaacg gtcggtaacg gtcggtaaaa tacctctacc 180
 gttttcattt tcatatttaa cttgcgggac ggaaacga 218

<210> 62
 <211> 44
 <212> DNA
 <213> Artificial

<220>
 <223> Insertion site of Ds construct in human DNA

<400> 62

39/48

ggaagcagga ggccgtcaga gtctcactca gggatgaaag tagg

44

<210> 63
<211> 44
<212> DNA
<213> Artificial

<220>

<223> Insertion site of Ds construct in human DNA

<400> 63

accgtttttca tccctagtct cactagccat cctgttcatt catg

44

<210> 64
<211> 479
<212> DNA
<213> Artificial

<220>

<223> Zea mays Ds flanked by human DNA

<400> 64

aatatgaaaa tgaaaacggt agaggtatct taccgaccgt taccgaccgt tttcatccct 60

atatagatgc atgttttagag aggactgagg gggcaactga atgtgtttta tttcaagcct 120

actatgtgat catgaaatac ccaacacatt atgctggtac aaatccagag ttctaaaactg 180

agaccgaggt ggcataatgca tatttgagag ttagcagcat aaagatacta attgaagcta 240

tgaaacagat gtgattaatt tggaggagaa taattagaga aaaataatag gtcaaagaca 300

gactcctgag gaacccaac attttaaggg acacaaaaga ggttgaaaag agacagccag 360

agaggtaaaa ggaaaaccag aaatgcttgg agtgagaaaa gccaaagtaa gagggatctt 420

caagcaaaag tagtcaacag tgtgttttct aaacacgtgc agactgattg gaagotaga 479

<210> 65
<211> 501
<212> DNA
<213> Artificial

<220>

<223> Zea mays Ds flanked by human DNA

<400> 65

ctgttgacta cttttgcttg aataccctct ttacttggct tttctcactc caagcatttc 60

tggttttctt tttacctctc tggctgtctc ttttcaacct cttttgtgtc ccttaaaatg 120

ttgggggttc tcaggagtct gtctttgacc tattatcttt ctctaatat tctctccaa 180

attaatcaca totgtttcat agcttcaatt agtatcttta tgctgctaac totcaaatat 240

gcataatgcca cctcggtctc agtttacaac tctggatttg taccagcata atgtgttggg 300

40/48

tattttcatga tcacatagta agcttgaaat taaacacatt cagttgcccc ctcaagtcctc 360
 tctaaacatg catctatata gggatgaaaa cggtcggtaa cggtcggtaa aatacctcta 420
 ccgttttcat tttcatatatt aacttgoggg acggaaacga aaacgggata tacoggtaac 480
 gaaaacgaac gggataaata c 501

<210> 66
 <211> 393
 <212> DNA
 <213> Artificial

<220>
 <223> Zea mays Ds flanked by human DNA

<400> 66
 tccgtcccgcc aagttaaata tgaaaatgaa aacggtagag gtatttttacc gacogttacc 60
 gacogttttc atccctagtg agagtctgct cttctgtaaa cagacggctg aaattgctga 120
 aaatcagaca caagctgtta ctatgcaagt gattgacctg caacgaaagg tacatgctca 180
 gcctcaactgg atgtctactg ttaaagggag gacattgatt ggaaaagagt gggaccctga 240
 aagttgagat ggggacacgt cgagggggccc taatgaagct gggaatactg agataataaa 300
 ttctgatgag ctttttttagc aagaggggaat cgctcccca cccccagtgg tagaaacatg 360
 cccttgccca tctactgcag taccaggctt tca 393

<210> 67
 <211> 362
 <212> DNA
 <213> Artificial

<220>
 <223> Zea mays Ds flanked by human DNA

<400> 67
 atatgaaaat gaaaacggta gaggtatttt accgaccgtt accgaccgtt ttcattcccta 60
 gtgagagtct gctcttctgt aaacagacgg ctgaaattgc tgaaaatcag acacaagctg 120
 ttactatgca agtgattgac ctgcaacgaa aggtacatgc tcagcctcac tggatgtcta 180
 ctgttaaagg gaggacattg attggaaaag agtgggaccc tgaaagttga gatggggaca 240
 cgtcgagggg cctaataatgaa gctgggaata ctgagataat aaattotgat gagccttttt 300
 agcaagaggg aatcgccctcc ccaccccccag tggtagaaac atgcoccttgc ccactactg 360
 ca 362

<210> 68
 <211> 25
 <212> DNA

41/48

<213> Artificial

<220>

<223> Zea mays Ds flanked by vector DNA

<400> 68

tggagctccc cgactaggga tgaaa

25

<210> 69

<211> 25

<212> DNA

<213> Artificial

<220>

<223> Zea mays Ds flanked by vector DNA

<400> 69

tttcatccct gogtcgggta cccaa

25

<210> 70

<211> 6495

<212> DNA

<213> Artificial

<220>

<223> Ds Construct

<400> 70

cagggatgaa agtaggatgg gaaaatcccg taccgaccgt tatcgtataa ccgattttgt	60
tagttttatc ccgatogatt tcgaaccoga ggtaaaaaac gaaaacggaa cggaaacggg	120
atatacaaaa cggtaaacgg aaacggaaac ggtagagcta gtttcccgac cgtttcaccg	180
ggatcccgtt tttaatcggg atgatcccg ttcgttacgg tattttctaa ttogggatga	240
ctgcaacaga tccctcgagc gcttaagttt aaacgcgtta acaattggcc ataatagtaa	300
tcaattacgg ggtcattagt tcatagccca tatatggagt tccgcgttac ataacttacg	360
gtaaatggcc cgcttggtg accgcccaac gaccccgcc cattgacgtc aataatgacg	420
tatgttccca tagtaacgcc aatagggact ttccattgac gtcaatgggt ggagtattta	480
cggtaaaactg cccacttggc agtacatcaa gtgtatcata tgccaagtac gcccctatt	540
gacgtcaatg acggtaaatg gccgcctgg cattatgcc agtacatgac cttatgggac	600
tttctacttt ggcagtacat ctacgtatta gtcacgcta ttaccatggt gatgogggtt	660
tggcagtaca tcaatgggcg tggatagcgg ttgactcac ggggatttcc aagtctcac	720
cccattgacg tcaatgggag tttgttttgg caccaaaatc aacgggactt tccaaaatgt	780
cgtaacaact cggcccatc gacgcaaatg ggcggtaggc gtgtacggtg ggaggtctat	840
ataagcagag ctggttttagt gaaccgtcag atccgctagc gctaccggac tcagatctcg	900

42/48

agctcaagct tcgaattctg cagtcgacgg taccgcgggc ccgggatoca tgcgccaccat	960
ggtgagcaag ggcgaggagc tgttcacogg ggtggtgccc atcctggtog agctggacgg	1020
cgacgtaaac ggccacaagt tcagcgtgtc cggcgagggc gagggcgatg ccacctacgg	1080
caagctgacc ctgaagttca tctgcaccac cggcaagctg cccgtgccct ggcccacct	1140
ogtgaccacc ctgacctacg gcggtgcagtg cttcagcgcg taccocgacc acatgaagca	1200
gcacgacttc ttcaagtccg ccatgccga aggctacgtc caggagcgc caatcttctt	1260
caaggacgac ggcaactaca agaccgcgc cgaggtgaag ttcgagggcg acacctggt	1320
gaaccgcac gagctgaagg gcatcgactt caaggaggac ggcaacatcc tggggcacaa	1380
gctggagtac aactacaaca gccacaacgt ctatatcatg gccgacaagc agaagaacgg	1440
catcaaggtg aacttcaaga tccgccacaa catcgaggac ggcagcgtgc agctcgccga	1500
ccactaccag cagaacaccc ccatcggcga cggccccgtg ctgctgcccg acaaccacta	1560
cctgagcacc cagtcggccc tgagcaaaga ccccaacgag aagcgcgac acatggtcct	1620
gctggagttc gtgaccgccc ccgggatcac tctcggcatg gacgagctgt acaagtaaag	1680
cggccgcgat atcgctagct cgagatcggg agatctggcc tccgcgcggg gttttgggc	1740
cccccgggg cggccctcc tcacggcgag cgtgccacg tcagacgaag ggcgcacgag	1800
cgtcctgatc cttccgccc gacgctcagg acagcggccc gctgctcata agactcggcc	1860
ttagaacccc agtatcagca gaaggacatt ttaggacggg acttgggtga ctctagggca	1920
ctggttttct ttccagagag cggaacaggc gaggaaaagt agtcccttct cggcgattct	1980
gcggagggat ctccgtgggg cggtgaaacg cgatgattat ataaggacgc gccgggtgtg	2040
gcacagctag ttccgtcgca gccgggattt gggctcgggg tcttggttgt ggatocgtgt	2100
gatcgtcact tggtagtag cgggctgctg ggctggccgg ggctttcgtg gccgccgggc	2160
cgtcgggtgg gacggaagcg tgtggagaga ccgccaaggg ctgtagtctg ggtccgcgag	2220
caaggttgcc ctgaactggg ggttgggggg agcgcagcaa aatggcggct gttcccagat	2280
cttgaatgga agacgcttgt gaggcgggt gtgaggtcgt tgaaacaagg tggggggcat	2340
ggtgggcggc aagaacccaa ggtcttgagg ccttcgctaa tgcgggaaag ctcttattcg	2400
ggtgagatgg gctggggcac catctgggga ccctgacgtg aagtttgtca ctgactggag	2460
aactcggttt gtcgtctgtt gggggggcgg cagttatggc ggtgccgttg ggcagtgcac	2520
cgtacctttt gggagcgcgc gccctcgtcg tgcgtgacg tcaccogtcc tgttggetta	2580
taatgcaggg tggggccacc tgccggtagg tgtgcggtag gcttttctcc gtgcaggac	2640
gcagggttcg gccctagggg aggtctctct gaatcgacag gcgccggacc tctggtgagg	2700

43/48

ggagggataa gtgaggcgctc agtttctttg gtcggtttta tgtacctatc ttcttaagta 2760
gctgaagctc cggttttgaa ctatgcgctc ggggttgccg agtgtgtttt gtgaagtttt 2820
ttaggcacct tttgaaatgt aatcatttgg gtcaatatgt aattttcagt gttagactag 2880
taaatgtgoc gctaaattct ggccgttttt ggcttttttg ttagaccgga cagtgttgac 2940
aattaatcat cggcatagta tatcggcata gtataatacg acaaggtgag gaactaaacc 3000
atggccaagc ctttgtctca agaagaatcc accctcattg aaagagcaac ggctacaatc 3060
aacagcatcc ccatctctga agactacagc gtcgccagcg cagctctctc tagcgacggc 3120
cgcatcttca ctggtgtcaa tgtatatcat tttactgggg gaccttgtgc agaactcgtg 3180
gtgctgggca ctgctgctgc tggggcagct ggcaacctga cttgtatcgt cgcgatcgga 3240
aatgagaaca ggggcatctt gagccctgc ggacggtgcc gacaggtgct tctcgatctg 3300
catcctggga tcaaagccat agtgaaggac agtgatggac agccgacggc agttgggatt 3360
cgtgaattgc tgcctcttgg ttatgtgtgg gagggctaag cacttcgtgg ccgaggagca 3420
ggactgacac tcgacctga aacttgttta ttgcagctta taatggttac aaataaagca 3480
atagcatcac aaatttcaca aataaagcat ttttttact gcattctagt tgtggtttgt 3540
ccaaactcat caatgtatct tatcatgtct gaattccogg ggatcctcta gactgtcaga 3600
ccaagtttac tcatatatac tttagattga tttaaaactt catttttaat ttaaaaggat 3660
ctaggtgaag atcctttttg ataatctcat gacccaaatc cottaacgtg agttttcgtt 3720
ccactgagcg tcgaccccc tagaaaagat caaaggatct tcttgagatc ctttttttct 3780
ggcgctaata tgctgcttgc aaacaaaaaa accacogcta ccagcggtagg tttgtttgcc 3840
ggatcaagag ctaccaactc tttttccgaa ggttaactggc ttcagcagag cgcagatabc 3900
aaatactgtt cttctagtgt agccgtagt aggccaccac ttcaagaact ctgtagcacc 3960
gcctacatac ctgctctgc taatcctgtt accagtggct gctgccagtg gcgataagtc 4020
gtgtcttacc ggggttgact caagacgata gttaccgat aaggcgcagc ggtcgggctg 4080
aacggggggg tctgtcacac agcccagctt ggagcgaacg acctacaccg aactgagata 4140
cctacagcgt gagctatgag aaagcgccac gcttcocgaa gggagaaagg cggacaggta 4200
tccggtaagc ggcagggctg gaacaggaga gcgcacgagg gagcttcag ggggaaacgc 4260
ctggtatctt tatagtctg tgggttttg ccacctctga cttgagcgtc gatttttgtg 4320
atgctgtca ggggggcgga gcctatggaa aaacgccagc aacgcggcct ttttacggtt 4380
cccggccttt tctgtgacct ttgtcacat gctgggccca gcggccaga tctgagctog 4440
cggccgcgat atcgtagct cgagggaactt atatggcttc ttatgttagc caagagccca 4500

44/48

agaattatca cttatgtgct acattaaaact atgtgtgctc cagatttata tggattttat	4560
ctatgtttta ttaagacttg tgtttacaat tttttatatt tgtttttaag ttttgaatat	4620
atgttttcat gtgtgatttt accgaacaaa aataccgggt cccgtccgat ttcgacttta	4680
accogaccgg atcgtatcgg ttttogatta cgtattttat cccgttcggt ttcgttacgg	4740
gtatatcccg ttttcgtttc cgtcccgcaa gttaaataatg aaaatgaaaa cggtagagggt	4800
attttacoga cgtttacoga cgttttcat cctatgcat tctgacaggt accaatgctt	4860
aatcagttag gcacctatct cagcgatctg totatttcgt tcatccatag ttgcctgact	4920
cccgtcgtg tagataacta cgatacggga gggcttacca tctggcccca gtgctgcaat	4980
gataccgga gaccacgct caccggctcc agatttatca gcaataaacc agccagccgg	5040
aagggccgag cgcagaagtg gtcctgcaac tttatccgcc tccatccagt ctattaattg	5100
ttgcgggaa gctagagtaa gtagttcgcc agttaatagt ttgcgcaacg ttgttgccat	5160
tgtacaggc atcgtggtgt cacgctcgtc gtttggtatg gcttcattca gtcocggttc	5220
ccaacgatca aggcgagtta catgatcccc catgttgtgc aaaaaagcgg ttagctcctt	5280
cggctcctcg atcgttgtca gaagtaagtt ggccgcagtg ttatcactca tggttatggc	5340
agcactgcat aattctctta ctgtcatgoc atccgtaaga tgcttttctg tgactggtga	5400
gtactcaacc aagtcattct gagaatagtg tatgcggcga ccgagttgct cttgcccggc	5460
gtcaatacgg gataataccg cgccacatag cagaacttta aaagtgtca tcattggaaa	5520
acgttcttcg gggcgaaaac totcaaggat cttaccgctg ttgagatcca gttogatgta	5580
accactcgt gcacccaact gatcttcagc atcttttact ttcaccagcg tttctgggtg	5640
agcaaaaaa ggaaggcaaa atgccgcaaa aaagggaata agggcgacac ggaaatgttg	5700
aatactcata ctcttccttt ttcaatataa ttgaagcatt tatcagggtt attgtctcat	5760
gagcggatac atatttgaat gtatttagaa aaataaacia ataggggttc cgcgcacatt	5820
tccccgaaaa gtgccacctt aattgtaagc gttaatattt tgttaaaatt cgcgttaaat	5880
ttttgttaaa tcagctcatt ttttaaccaa taggcgaaa tcggcaaaat ccttataaa	5940
tcaaaagaat agaccgagat agggttgagt gttgttcag tttggaacia gactccacta	6000
ttaaagaacg tggactcaa cgtcaaagg cgaaaaaccg totatcaggg cgatggccca	6060
ctacgtgaac catcaccta atcaagtttt ttggggtcga ggtgcggtaa agcactaaat	6120
cggaacctta aaggagacc cgtatttaga gcttgacggg gaaagccggc gaacgtggcg	6180
agaaaggaag ggaagaaagc gaaaggagcg ggcgctaggg cgtggcaag ttagcggtc	6240
acgtgcgcg taaccaccac accgcgcgc cttaatgcgc cgtacaggg cgcgtccat	6300

45/48

tcgccattca ggctgcgcaa ctgttgggaa gggcgatcgg tgcggggcctc ttogetatta 6360
 cgccagctgg cgaaaggggg atgtgctgca aggcgattaa gttgggtaac gccagggttt 6420
 tcccagtcac gacgttgtaa aacgacggcc agtgaattga tacgactcac tatagggcga 6480
 attgggtacc cgacg 6495

<210> 71
 <211> 6400
 <212> DNA
 <213> Artificial

<220>
 <223> Ac-TPase Construct

<400> 71
 tagttattaa tagtaatcaa ttacggggtc attagttcoat agcccatata tggagttccg 60
 cgttacataa cttacggtaa atggcccgcc tggctgaccg cccaacgacc ccgcccatt 120
 gacgtcaata atgacgtatg ttcccatagt aacgccataa gggactttcc attgacgtca 180
 atgggtggag tatttacggt aaactgccc a ttggcagta catcaagtgt atcatatgcc 240
 aagtaacccc cctattgacg tcaatgacgg taaatggccc gcctggcatt atgcccagta 300
 catgacctta tgggactttc ctacttggca gtacatctac gtattagtca tcgctattac 360
 catggtgatg cggttttggc agtacatcaa tgggcgtgga tagcggtttg actcacgggg 420
 atttccaagt ctccacccca ttgacgtcaa tgggagtttg ttttggcacc aaaatcaacg 480
 ggactttcca aaatgtcgta acaactccgc ccattgacg caaatgggcg gtaggcgtgt 540
 acggtgggag gtctatataa gcagagctgg tttagtgaac cgtcagatcc gctagcgcta 600
 ccggactcag atctcgagct caagcttoga attctgcagt cgacgggtacc gcgggcccgg 660
 gatccatacg atttaggtga cactatagaa tacaagcttg cttgttcttt ttgcagaagc 720
 tcagaataaa cgctcaactt tggcagatcc gccaccatgg gtccctocaga gaagaagcgt 780
 aaggtagaaa tggctattgt tcatgaacca caaccacaac cacaaccaca accagaacca 840
 caaccacagc cacaacctga acccgaagaa gaagcaccac agaagagggc aaagaagtgc 900
 acatcggatg tatggcagca tttcaccaag aaggaaattg aagtggaggt cgatggaaaag 960
 aaatacgttc aggtatgggg gcattgcaac tttcctaatt gcaaggctaa gtatagggt 1020
 gagggtcac atggaacaag cggatttoga aatcacttga gaacatcaca tagtttagtt 1080
 aaaggtcagt tgtgtctaaa aagtgaagag gatcatggca aagacataaa tctcattgag 1140
 ccttataagt acgatgaagt ggttagccta aagaagcttc atttggcaat aatcatgcat 1200
 gaataccctt tcaatattgt agaacatgag tactttgttg agtttggtta gtctctgcgc 1260

46/48

oetcaactttc caataaagtc cegtgtcact gotagaaaat atatcatgga tttgtattttg 1320
gaagaaaaag aaaagttgta tggaaaacta aaagatgttc agtctcgott cagtacaact 1380
atggatatgt ggacatcttg tcaaaataag tcatacatgt gtgtcaccat ccattggatt 1440
gatgatgatt ggtgtctcca aaaaagaatt gttggctttt ttcattgttgaggggcgccac 1500
actggccaaa gggtatcaca aaccttcact gcaatcatgg ttaagtggaa cattgagaaa 1560
aaattgtttg ccttgtcttt ggataatgct agtgcaaatg aagtagctgt gcacgatata 1620
attgaggatt tgcaggacac tgattcaaat ctagtttgtg atgggtgcttt ctttcatgtg 1680
aggtgtgctt gtcacatact gaacttggtt gcaaaggatg gcttggtgtt aattgcagga 1740
acaattgaga aaatcaaagc gattgttctt gotgtaaaat cttctccttt gcagtgggaa 1800
gaactaatga agtgtgctag tgaatgtgac ttggataaat cttaaaggat ctcatatgat 1860
gtctcaacta gatggaattc aacctatttg atgttgaggg atgccttata ttataagcct 1920
gcactaataa ggcttaaaac aagtgatcct cgcaggtacg atgcaatttg tcctaaagcc 1980
gaggagtggga agatggcatt aactcttttt aagtgtttga agaagttttt tgatctcact 2040
gaactcctat ctggtactca atattccact gcaaatttat tttacaaagg tttctgtgag 2100
ataaaggatt tgattgacca atgggtgtgtt catgaaaaat ttgtcattag gagaatggcc 2160
gttgcaatga gtgaaaagtt tgagaaatat tggaaagtgt ctaattattgc actagctgta 2220
gcatgcttcc ttgacctag gtacaagaaa atattgattg agttctatat gaaaaaattt 2280
catggtgatt catacaaagt tcatgtagat gactttgtta gggtcattag aaaattgtat 2340
caattctatt ctagttgtag tccttcagct ccaaagacaa agacaactac taatgatagt 2400
atggatgata ccttgatgga aaatgaagat gatgaatttc aaaactattt gcatgagttg 2460
aaggattatg atcaagtaga gtcaaatgaa ttggataaat atatgtctga accccttttg 2520
aagcatagtg gtcagtttga tattttatca tgggtggaggg gaagggttgc agaatatcct 2580
attctcacc ccaattgcaag ggatgtgcta gcaatacaag tgtcaactgt tgcttctgag 2640
tctgcgttca gtgctgggtg tcgtgttgtt gatccttacc gcaatcgtct tggttcggag 2700
attgttgaag ctttgatatg cacaaaagat tgggttagcag catctagaaa aggtgctaca 2760
tattttocaa caatgattgg tgatctcgag gtgctagact ctgttattgc tgctgcaaca 2820
aatcatgaga atcatatgga tgaggatgaa gacgcaatag aattttctaa gaataatgaa 2880
gatgtagcaa gtggctctc tccatgagca atgtgtctta tgtttgttga cagatgagcc 2940
ttggttgtaa tagtttatgc atgctaagt ctccagatgt gagcaagtga ttatgaatat 3000
gtgtttttaa ctttatattg tgtcatgtgt gctagtagac ttatatggct tcttatgtta 3060

47/48

gccaaaggcgg cgcgcactct agatcataat cagccatacc acatttgtag aggttttact	3120
tgcttttaaaa aacctccac acctccccct gaacctgaaa cataaaatga atgcaattgt	3180
tgttgttaac ttgtttattg cagcttataa tggttacaaa taaagcaata gcatcacaaa	3240
tttcacaaat aaagcatttt tttcactgca ttctagttgt ggtttgtcca aactcatcaa	3300
tgtatcttaa ggcgtaaatt gtaagcgta atattttgtt aaaattcgcg ttaaattttt	3360
gttaaatcag ctcatTTTTT aaaccaatag cggaaatcgg caaaatccct tataaatcaa	3420
aagaatagac cgagataggg ttgagtgttg ttccagtttg gaacaagagt ccactattaa	3480
agaacgtgga ctccaacgtc aaaggcgga aaacgtcta tcagggcgat ggcccactac	3540
gtgaaccato acctaatca agtttttttg ggtcgaggtg ccgtaaagca ctaaatcgga	3600
accctaaagg gagccccga tttagagctt gacggggaaa gccggcgaac gtggcgagaa	3660
aggaaggga gaaagcgaaa ggagcggcg ctagggcgct ggcaagtgt gcggtcacgc	3720
tgcgcgtaac caccacaccc gccgcgctta atgcgcgct acagggcgcg tcaggtggca	3780
cttttcgggg aaatgtgcgc ggaaccoccta tttgtttatt tttctaaata cattcaaata	3840
tgtatccgct catgagacaa taaccctgat aaatgottca ataattattga aaaaggaaga	3900
gtcctgaggc ggaaagaacc agctgtggaa tgtgtgtcag ttaggggtgtg gaaagtcccc	3960
aggctcccca gcaggcagaa gtatgcaaag catgcatctc aattagtcag caaccaggtg	4020
tggaaagtcc ccaggctccc cagcaggcag aagtatgcaa agcatgcato tcaattagtc	4080
agcaaccata gtcccgcccc taactccgcc catcccgccc ctaactccgc ccagttccgc	4140
ccattctccg ccccatggct gactaatttt ttttatttat gcagaggccg aggcgcctc	4200
ggcctctgag ctattccaga agtagtgagg aggtttttt ggaggcctag gcttttgcaa	4260
agatcgatca agagacagga tgaggatcgt ttccgatgat tgaacaagat ggattgcacg	4320
caggttctcc ggccgcttgg gtggagaggc tattcggcta tgactgggca caacagacaa	4380
tcgggtgctc tgatgcggc gtgttccggc tgtcagcgca gggcgcccg gttotTTTTTg	4440
tcaagaccga cctgtccggt gccctgaatg aactgcaaga cgaggcagcg cggctatcgt	4500
ggctggccac gacggcggtt ccttgccgag ctgtgctcga cgttgtcact gaagcgggaa	4560
gggactggct gctattgggc gaagtgcgg ggccagatct cctgtcatct cacttgctc	4620
ctgcgcagaa agtatccatc atggctgatg caatgcggcg gctgcatacg cttgatccgg	4680
ctacctgccc attcgaccac caagcgaaac atcgcatcga gcgagcacgt actcggatgg	4740
aagccggtct tgtcgatcag gatgatctgg acgaagagca tcaggggctc gcgcagccg	4800
aaactgttcgc caggctcaag gcgagcatgc ccgacggcga ggatctcgtc gtgacccatg	4860

48/48

gcgatgcctg cttgccgaat atcatggtgg aaaatggccg cttttotgga ttcatogact	4920
gtggccggct ggggtgtggcg gaccgctatc aggacatagc gttggctacc cgtgatattg	4980
ctgaagagct tggoggcgaa tgggctgacc gcttctcgt gctttacggc atogccgctc	5040
ccgattogca ggcacatgcc ttctatogcc ttcttgacga gttcttctga ggggactct	5100
ggggttogaa atgaccgacc aagcgacgoc caacctgcca tcacgagatt tcgattccac	5160
cgcgccttc tatgaaaggt tgggcttcgg aatcgttttc cgggaogccg gctggatgat	5220
cctccagcgc ggggatctca tgctggagtt cttcgccac octaggggga ggctaactga	5280
aacacggaag gagacaatac cggaaggaa cgcgctatg acggcaataa aaagacagaa	5340
taaaacgcac ggtgttgggt cgtttgttca taaacgcggg gttcgggtccc agggctggca	5400
ctctgtogat accccaccga gacccattg gggccaatac gcccggttt cttccttttc	5460
ccccccac ccccaagtt cgggtgaagg cccagggctc gcagccaacg tcggggcggc	5520
agggcctgcc atagcctcag gttactcata tatactttag attgatttaa aacttcattt	5580
ttaatttaaa aggatctagg tgaagatcct ttttgataat ctcatgacca aaatccctta	5640
acgtgagttt tcgttccact gagcgtcaga ccccgtagaa aagatcaaag gatcttcttg	5700
agatcccttt tttctgogcg taatctgotg cttgcaaaca aaaaaaccac cgctaccagc	5760
ggtggtttgt ttgcgggac aagagctacc aactcttttt ccgaaggtaa ctggcttcag	5820
cagagcgag ataccaaata ctgtccttct agtgtagccg tagttaggcc accacttcaa	5880
gaactctgta gcaccgccta cataoctcgc tctgctaato ctgttaccag tggctgctgc	5940
cagtggcgat aagtcgtgtc ttacogggtt ggactcaaga cgatagttac cggataaggc	6000
gcagcggctg ggctgaacgg ggggttcgtg cacacagccc agcttggagc gaacgacctc	6060
caccgaactg agatacctac agcgtgagct atgagaaagc gccacgcttc ccgaaggag	6120
aaaggcggac aggtatccgg taagcggcag ggtcggaaca ggagagcgca cgagggagct	6180
tccaggggga aacgcctggg atctttatag tcctgtcggg tttcgccacc totgacttga	6240
gcgtcgattt ttgtgatgct cgtcaggggg gcggagccta tggaaaaacg ccagcaacgc	6300
ggccttttta cggttcctgg ccttttgctg gccttttgct cacatgttct ttctgcgtt	6360
atccctgat tctgtggata accgtattac cgccatgcat	6400

the present invention is directed to the use of the maize *Ac/Ds* transposable elements in vertebrates.