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TRANSPOSASE UND VERFAHREN ZUR GENMODIFIZIERUNG

TRANSPOSASE ET PROCEDE DE MODIFICATION DE GENES

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Description

Technical Field

[0001] The present invention relates to a new protein having a transposase-like activity, a transposase composed of the above protein, a method of modifying the gene structure of a cellular gene by using these protein and transposase, a method of modifying the function of a cell by this method, a method of introducing a gene by this method, a plasmid used for this method, and a cell having been modified in function by this method.

Background Art

[0002] The medaka fish (Oryzias latipes) is a teleost inhabiting in East Asia and has been used for studying vertebrate genetics. The mutations at the i locus of the medaka fish cause amelanotic skin and red-colored eyes. This i locus is known to encode a gene for tyrosinase. From one of the i alleles, i^4, DNA of about 4.7-kb was cloned and found to have a transposon-like sequence; that is to say, it included open reading frames homologous to transposases of transposons of the hAT family including hobo of Drosophila, Ac of maize and Tam3 of snapdragon, and short terminal inverted repeats. This medaka element was named Tol2. The laboratory strains of the medaka fish contain about 10 copies of this element per haploid genome.

[0003] In the i^4 mutant fish, the Tol2 element found in the tyrosinase gene locus has been shown by PCR to be excised from the target locus during embryonic development (Koga et al., 1996).

[0004] Zebrafish (Danio rerio), as well as the medaka fish (Oryzias latipes) , is a small teleost and has been developed as a model animal to study vertebrate genetics and development (Takeuchi, 1966; Yamamoto, 1967; Streisinger et al., 1981). In zebrafish, large-scale chemical mutagenesis screens have been performed (Driever et al., 1996; Haffter et al., 1996), and, to facilitate cloning of the mutated genes, an insertional mutagenesis method using a pseudotyped retrovirus has been developed and performed (Lin et al., 1994; Gaiano et al., 1996; Amsterdam et al., 1997). Also, in an attempt to develop transposon technologies that would allow enhancer trap and gene trap screens to be performed, transposition of transposons of the Tc1/mariner family in fish has been tested and demonstrated (Ivics et al., 1997; Raz et al., 1997; Fadool et al., 1998). Although these results are encouraging, neither highly efficient transgenesis nor insertional mutagenesis methods using a transposon have not yet been developed.

[0005] The present inventors have been interested in developing novel transposon technologies using the Tol2 element. As a first step towards this goal, the present inventors developed a transient embryonic excision assay using zebrafish embryos, in which zebrafish fertilized eggs were injected with a plasmid DNA harboring the Tol2 element, showed that the Tol2 element was excisable from the injected plasmid DNA, and indicated that the Tol2 element is an autonomous member and is active in zebrafish (Kawakami et al., (1998) Gene **225**, 17-22). Although the DNA sequence of the Tol2 element is similar to those of transposases of transposons of the hAT family, neither an active enzyme, which can function in trans, nor cis-elements essential for the excision reaction have been identified. In order to develop the Tol2 element as a useful tool for transgenesis and insertional mutagenesis, it is necessary to dissect and characterize cis and trans requirements. The functional transposase encoded by the Tol2 element had not yet been identified prior to the present invention.

Disclosure of the Invention

[0006] The present invention first aims to identify mRNA transcribed from the Tol2 element injected in zebrafish embryos. Secondly, in order to determine whether the transcript encodes an active enzyme or not, the present invention develops a novel assay method, in which zebrafish fertilized eggs are co-injected with RNA synthesized in vitro using the Tol2 cDNA as a template and a plamid DNA harboring a nonautonomousTol2 element, which has a deletion in the transposase coding region.

[0007] The present invention also identifies the active trans-factor and essential cis-elements, that function in excision of the Tol2 element in zebrafish.

[0008] Consequently, the present invention results in a new protein encoded by the Tol2 element and a polynucleotide encoding the same. Also the present invention, by using the above protein, results in a method of modifying the gene structure of a cell, preferably the gene structure of a vertebrate, in a method of modifying the function of a cell by modifying the gene structure thereof, and in a cell having been modified in function by these methods. Furthermore, the present invention discloses the cis-element structures essential for transposition, and presents the same.

[0009] Accordingly the invention provides a method of introducing a Tol2 element into the genome of a cell having no Tol2 element comprising using a gene encoding a protein having a transposase-like activity which can insert a fragment of a gene into the genome, which protein

(i) has the amino acid sequence shown in sequence number 2 of the SEQUENCE LISTING, or
(ii) a substituted amino acid sequence with replacement or deletion in part of the original amino acid sequence of sequence number 2, or
(iii) a substituted amino acid sequence with addition of several other amino acids to the original amino acid sequence of sequence number 2.

[0010] The invention also provides a method of inserting any DNA into the genomic DNA of a cell having no Tol2 element by using the activity of a protein having a transposase-like activity, wherein a Tol2 element has been introduced into the genome of the cell by using a gene encoding a protein having a transposase-like activity which can insert a fragment of a gene into the genome, which protein

(i) has the amino acid sequence shown in sequence number 2 of the SEQUENCE LISTING, or
(ii) a substituted amino acid sequence with replacement or deletion in part of the original amino acid sequence of sequence number 2, or
(iii) a substituted amino acid sequence with addition of several other amino acids to the original amino acid sequence of sequence number 2. the said DNA, or is the corresponding RNA.

[0011] The present invention reveals that the said protein has a transposase-like activity which catalyzes transposition of the above transposon, and relates to a method of modifying the gene structure comprises the excision in part of a gene in a cell, preferably a vertebrate cell, or the insertion of the excised part into any other locus in the presence of the said protein or the nucleic acid which can produce the said protein. It is preferable that the said excised gene has nucleotide sequences containing at least one inverted repeat (the Angel elements) in forepart of its nucleotide sequence.
[0012] Further, the present invention relates to a method of inserting a foreign gene into a gene of a cell, and a method of modifying a function of a cell based on gene expression, and furthermore relates to a cell having been modified in function by the said method.
[0013] Also, the present invention relates to a plasmid used in these methods and, more in detail, a plasmid which contains DNA having a nucleotide sequence that includes at least one inverted repeat sequence in the forepart of its nucleotide sequence.
[0014] Furthermore, in a method of inserting any DNA into the genomic DNA of a vertebrate, the present invention relates to a method of inserting any DNA into the genomic DNA of a vertebrate which is characterized by operating the said insertion of DNA autonomously using the transposase activity, wherein a preferable DNA is the Tol2 element and the vertebrate is fish.

Brief Description of the Drawings

[0015]

Figure 1 shows the structure of the Tol2 plasmid and the transcript, and the structure of cDNAs described in the present invention. The dotted lines indicate introns. The inverted repeat (the Angel elements) in the first intron and positions of primers used in the present invention are shown by arrows.
Figure 2 shows a comparison of amino acid sequences of transposases of the Tol2 element described in the present invention and the Ac element.
Figure 3 shows a scheme for the transient embryonic excision assay by co-injection described in the present invention. Primers (tyr-ex4f and tyr-ex5r) used to detect the excision products are shown by arrows.
Figure 4 shows photos, substitutes for drawings, which show the results of the PCR analysis of the excision reaction in zebrafish embryos described in the present invention.
Figure 5 shows the structures of the (Tol2-tyr)ΔRV plasmid used for transposition of the Tol2 element into the genome, the Tol2-tyr plasmid and Tol2 cDNA. The black line in the upper part of Figure 5 shows the probe used for Southern blot analysis.
Figure 6 shows photos, substitutes for drawings, which show the results of Southern blot analysis of F1 progeny fish from each parental fish (ff-1 and ff-7) wherein the presence of Tol2 is identified (Figure 6,A) and show the results of PCR (Figure 6,B).
Figure 7 shows the nucleotide sequences surrounding the Tol2 element inserted in the genome of F1 progeny A, B and C from ff-7.

Best Mode for Carrying Out the Invention

[0016] Previously, the present inventors injected the Tol2-tyr plasmid, a plasmid harboring the Tol2 element cloned

from the tyrosinase gene locus, into zebrafish fertilized eggs and showed that the Tol2 element is excisable from the injected plasmid DNA (Kawakami et al., 1998). In order to identify a transcript encoding a putative transposase activity, total RNA from embryos injected with the Tol2-tyr plasmid were prepared. The present inventors first performed 3' RACE using four pairs of nested primers that annealed different parts of the Tol2 sequence.

[0017] Nested forward primers used to perform 3' RACE are:

Tol2f2; 5'-TTGGTCAGACATGTTCATTG-3' and
Tol2f3; 5'-ATGTTCATTGGTCCTTTGGA-3',
Tol2f4; 5'-ATAGCTGAAGCTGCTCTGATC -3' and
To12f5; 5'-CTGCTCTGATC ATGAAACAG-3',
Tol2f8; 5'-GCTTAATAAAGAAATATCGGCC -3' and
Tol2f9; 5'-AATATCGGCCTTCAAAAGTTCG-3', and
Tol2f12; 5'-CTGTAATCAGAGAGTGTATGTGTA -3' and
To12f13; 5'-ATTGTTACATTTATTGCATACAAT -3'.

[0018] cDNAs with polyadenylation were successfully amplified by 3' RACE using Tol2f8 and Tol2f9, and Tol2f4 and Tol2f5, but not by 3' RACE using Tol2f2 and Tol2f3, and Tol2f12 and Tol2f13.

[0019] Then, using nested reverse primers designed to perform 5' RACE,
Tol2r4; 5'-CTCAATATGCTTCCTTAGG -3' and
Tol2r5; 5'-CTTCCTTAGGTTTGATGGCG-3',
5' RACE was performed and the full-length Tol2 transcript of 2156 nucleotides was identified (Figure 1).

[0020] The cDNA sequence obtained is shown in sequence number 1 of SEQUENCE LISTING.

[0021] Figure 1 shows the structures of the Tol2 plasmids and its transcript. The top line of Figure 1 shows the full-length Tol2 (Tol2-tyr). Dotted lines in the figure show introns. The inverted repeat (the Angel elements) in the first intron and positions of the said primers are shown by arrows. The lower three lines of Figure 1 show the results of 3' RACE and 5' RACE. In each case, introns are shown as dotted lines.

[0022] The fifth line shows the structure of the full-length mRNA. The translated region corresponds to the nucleotide sequence between the 85th (ATG) and the 2032nd nucleotide (TAG) of cDNA of sequence number 1.

[0023] The two lines in the bottom show the structures of deletion mutants, (Tol2-tyr)ΔRV, and (Tol2-tyr) Δin1ΔRV.

[0024] In the 5' RACE analysis, aberrant transcripts that started from the plasmid sequence and jumped into cryptic splice acceptor sites in the first exon of the Tol2 element were also found (data not shown). These transcripts were not studied further.

[0025] DNA sequencing of the cDNA revealed the exon-intron structure of the Tol2 element (i.e., four exons and three introns) (as shown in the upper part of Figure 1). The cDNA encodes a protein of 649 amino acids. The amino acid sequence of this protein is shown in sequence number 2 of SEQUENCE LISTING.

[0026] Although the Tol2 element had been known to have a transposon-like sequence, the present invention for the first time identified that the Tol2 element encodes a protein and the expression of the protein described here generates the function. That is to say, the present invention results in a new protein encoded by the Tol2 element and also a polynucleotide which encodes the protein described here.

[0027] Figure 2 shows a comparison of amino acid sequences of the protein described in the present invention and a known transposase of a transposon of the hAT family. This comparison shows these proteins are similar, especially in the middle part (Figure 2). But the amino acid sequences of NH_2- and COOH-terminus rather varies.

[0028] In order to determine whether the protein (the Tol2 transcript) identified in the present invention encodes a functional enzyme, a new transient embryonic excision assay by co-injection was developed and, by using this method, identification of the enzymatic activity was performed.

[0029] Zebrafish fertilized eggs were co-injected with mRNA synthesized in vitro using the cDNA shown in the sequence number 1 as a template and the (Tol2-tyr)ΔRV plasmid containing (Tol2-tyr)ΔRV (see Figure 1), which has a deletion of the nucleotides between the EcoRV sites of the Tol2 element. About 8 hours after the co-injection, DNA was prepared from each embryo and analyzed by PCR using primers, tyr-ex4 f and tyr-ex5r,
tyr-ex4f 5'- GCTACTACATGGTGCCATTCCT-3'
tyr-ex5r: 5'-CACTGCCAGATCTGCTGGGCTT-3'
which were prepared based on the sequence adjacent to the Tol2 element. Figure 3 shows a scheme of this method and these primers are shown in Figure 4A.

[0030] PCR products of about 250bp, indicative of excision of the Tol2 element from (Tol2-tyr)ΔRV plasmid, was amplified in all embryos analyzed (56 out of 56, see Figure 4B lanes 1-10). This PCR product was never detected from embryos injected only with the (Tol2-tyr)ΔRV plasmid DNA (0 out of more than 50, see Figure 4B lanes 11-20).

[0031] The PCR products from six different embryos were cloned and sequenced. Three of them had the wild-type medaka fish tyrosinase gene sequence (Figure 4C, excision product a), indicating that precise excision had occurred,

and the other three had nearly wild type sequences with addition of a few nucleotides (Figure 4C, excision products b and c), characteristic to excision of transposons of the hAT family (Pohlman et al., 1984; Sutton et al., 1984; Koga et al., 1996; Kawakami et al., 1998), suggesting that the excision event in this experiment is catalyzed by a transposase-like activity.

[0032] These results, i.e., when co-injected with the mRNA which has the nucleotide sequence shown in sequence number 1 of the present invention, the PCR product characteristic to excision of the transposon was obtained and, when co-injected without the mRNA, such PCR products were not obtained, indicate that the protein (the Tol2 transcript) described in the present invention encodes a functional transposase, which can catalyze the excision. Further, these results show that the (Tol2-tyr)ΔRV plasmid contains sequences of cis-elements essential for the excision.

[0033] Figure 4 shows the results of this experiment and arrows in Figure 4A show positions and directions of the primers used in the analyses. The upper panel of Figure 4B is a photo, a substitute for a drawing, which shows the PCR products using primers tyr-ex4f and tyr-ex5r, and the lower panel shows the PCR products using primers Tol2f1 and Tol2r3. In lanes 1-10, zebrafish embryos were injected with both the (Tol2-tyr)ΔRV plasmid and the Tol2 mRNA, in lanes 11-20 the (Tol2-tyr)ΔRV plasmid alone was injected, and in lane G and P PCR products were amplified from 50ng of the zebrafish genomic DNA and from 10pg of the (Tol2-tyr)ΔRV plasmid DNA. Figure 4C shows the DNA sequences of the excision products obtained in the above experiments. The Tol2 sequence is shown in bold and 8bp direct repeat sequences flanking to the Tol2 element are underlined.

[0034] It was noted that, although the excision products could be detected after a single-round PCR amplification in the experiments in the present invention, two rounds of PCR were required in the previous analysis, in which fertilized eggs were injected with a sole plasmid DNA containing the full-length Tol2 element without mRNA prepared in vitro. The higher efficiencies of the excision reaction observed here can be explained as more transposases were supplied by RNA injection than those supplied by DNA injection.

[0035] The first intron of the Tol2 element contains about 300bp of large inverted repeats, and the repeat was recently identified as the Angel element (Izsvak et al., 1999) (see Figure 1). To test whether the sequence in the intron is essential for excision, we constructed the (Tol2-tyr) Δin1ΔRV plasmid, containing (Tol2-tyr) Δin1ΔRV (see the bottom of Figure 1), which completely lacked the sequences of the first intron, and its activity was analyzed by co-injection with the Tol2 mRNA as the said method. This result is shown in Figure 4D.

[0036] The upper panel of Figure 4D is a photo, a substitute for a drawing, which shows PCR products amplified using primers tyr-ex4f and tyr-ex5r and, the lower panel shows PCR products amplified using primers Tol2f1 and Tol2r3. In lanes 1-8, zebrafish embryos were injected with both the (Tol2-tyr) Δin1ΔRV plasmid and the Tol2 mRNA, in lanes 9-12 both the (Tol2-tyr)ΔRV plasmid and the Tol2 mRNA were injected, and, in lanes 13-16, the (Tol2-tyr) Δin1ΔRV plasmid alone was injected. Lane P shows the PCR product amplified from 10pg of the (Tol2-tyr) Δin1ΔRV plasmid DNA.

[0037] In lanes 9-12, the said experiment was conducted as controls and the PCR products indicating the excision could be detected, but the excision product could not be detected in lanes 1-8 when the plasmid lacking the intron part (0 out of 16, see Figure 4D lanes 1-8) was used, suggesting that the first intron contains cis-elements essential for excision.

[0038] Further, the (Tol2-tyr) Δin1 plasmid, containing (Tol2-tyr) Δin1 which restored theΔRV deletion and was about the same size as the (Tol2-tyr) ΔRV plasmid, i.e., which has a deletion between the 644th and 2163rd nucleotides of the Tol2 element, was also examined by the co-injection assay, but PCR product indicating the excision could not be obtained (0 out of 16, data not shown).

[0039] Although, further analyses using smaller deletions and point mutations in the first intron sequence will be needed to define the essential cis-sequences for excision precisely, since these results show that the intron part is essential for the excision and the intron contains the Angel elments as inverted repeats, it can be thought that the inverted repeats are essential sequences for the excision described in the present invention.

[0040] Thus, we successfully identified for the first time the transcript (the protein described in the present invention) encoded by the Tol2 element and also identified a transposase activity of this protein and cis-sequences essential for transposition. These discoveries will lead to a biochemical characterization of the Tol2 transposase.

[0041] On the other hand, transposition of transposons belonging to the Tc1/mariner family into the zebrafish genome has been reported (Raz et al., 1997; Fadool et al., 1998). In the experiments described in their reports, zebrafish one-cell-stage embryos were co-injected with transposase RNA transcribed in vitro and transposon vectors containing essential cis-sequences.

[0042] While a transposon belonging to a different family may have different specificities and efficiencies for insertion into the genome, by a method of present invention which is a novel transposon technology in fish using the Toi2 element, since its transposon excision procedure has been carried out in the way of Raz et al., it might be possible to transpose DNA such as the Tol2 element into the genome in the way conducted by using transposons of the Tc1/mariner family.

[0043] Therefore, we tested whether the Tol2 element can be inserted into the zebrafish genome by transposition. It is known that the zebrafish genome does not contain the Tol2 element.

[0044] To test whether the Tol2 element encodes a transposase that can catalyze transposition, zebrafish fertilized eggs were co-injected with RNA transcribed in vitro using the Tol2 cDNA as a template, which encoded a putative

transposase, and a plasmid DNA harboring the (Tol2-tyr)ΔRV element, which has a deletion in part of the region presumed to code the transposase.

[0045] The structures of (Tol2-tyr)ΔRV plasmid and Tol2 cDNA are shown in Figure 5. 3' and 5' indicate the direction of transcription.

[0046] The injected eggs were raised to adulthood and mated to non-injected fish. And the progeny fish were analyzed for the presence of theTol2 sequence.

[0047] Two out of eight injected fish could transmit the Tol2 sequence to their progeny. These two fishes were named ff-1 (founder fish-1) and ff-7 (founder fish-7).

[0048] Two fish out of 68 F1 fish from the ff-1 fish had the Tol2 sequence. These two fish had the sequence of the plasmid portion as well as the Tol2 sequence. On the other hand, 25 fish out of 50 F1 fish from the ff-7 fish had the Tol2 sequence. These 25 fish did not have the plasmid sequence and were classified into three groups, A, B and C, from the result of Southern blot shown in Figure 6A. 7 fish were grouped as A, 3 fish as B, and 15 fish as C.

[0049] Figure 6A is a photo, a substitute for a drawing, which shows the result of Southern blot analysis using a probe shown in Figure 5, in which DNA samples prepared from caudal fins of F1 fish from ff-1 and ff-7 were digested with EcoRV. Two samples from ff-1 showed the same pattern but samples from ff-7 showed three patterns, A, B and C.

[0050] Then, PCR analyses of F1 fish from ff-1 and ff-7 were performed. Primers used were shown in Figure 5 as PCR1, PCR2 and PCR3. As controls, zebrafish genomic DNA (G) and genomic DNA plus (Tol2-tyr)ΔRV plasmid DNA (G+P) were used. In F1 fish from ff-7, PCR products using PCR2 and PCR3 could not be amplified. This indicated that progeny fish from ff-7, unlike progeny fish from ff-1, did not have the plasmid sequence flanking to the Tol2 element.

[0051] From the ff-7 progeny fish, DNA fragments containing the Tol2 sequence and the flanking region were cloned by inverse PCR and sequenced. In each three case, A, B and C, the Tol2 sequence was surrounded by zebrafish genomic sequences and 8bp duplications were created adjacent to the insertion. 8bp duplications at both ends of the Tol2 element are characteristic to integration of transposons of the hAT family, indicating that the integration described here was catalyzed by a transposase.

[0052] Figure 7 shows the determined nucleotide sequences of three types, A, B and C. Tol2 in Figure 7 shows the Tol2 sequence. In A repeats of ⌜CTCAACTG⌟, in B repeats of ⌜TATAGAGA⌟, and in.C repeats of ⌜GTTTTCAG⌟ were created at both ends of and adjacent to the Tol2 sequence.

[0053] In the vertebrate cultured cells and the germ line, transposition activities of Sleeping Beauty which was reconstituted and activated artificially (Ivics, Z., et al., Cell, 91, 501-510 (1977)), Tc3 of C. elegans (Raz, E., et al., Current Biology, 8, 82-88 (1977)) and mariner of Drosophila (Fadool, J.M., et al., Proc. Natl. Acad. Sci. USA, 95, 5182-5186 (1988)), all belonging to the Tc1/mariner family, have been reported. No autonomous transposon activity residing endogenously in any vertebrate genome, however, has been reported.

[0054] The present invention is the first report that identified an autonomous element from a vertebrate genome and also for the first time reported a functional transposase activity in vertebrate.

[0055] Therefore, the present invention relates not only to a method to excise a gene autonomously in vertebrate but also to a method to insert the excised gene into any locus or any gene on the genome.

[0056] The protein in the present invention has the amino acid sequence shown in sequence number 2 of SEQUENCE LISTING, but all of the amino acids shown there are not necessarily required, and the protein in the present invention can include a protein having the transposase activity described in the present invention or similar activities described above (both of these are called transposase-like activities) and also can include a protein having replacement or deletion in part of amino acids of the above protein, or having addition of any other amino acids to the above protein. And preferably it has the amino acid sequence derived from the Tol2 element. Further, the protein in the present invention includes a protein which is produced from mRNA having the nucleotide sequence corresponding to sequence number 1 of SEQUENCE LISTING.

[0057] The nucleic acid in the present invention encodes the amino acid sequence which is related to the said protein, and preferably which has the polynucleotide having the sequence shown in sequence number 1 of SEQUENCE LISTING. The nucleic acid in the present invention includes not only the said nucleotide sequence but also a nucleotide sequence which can hybridize to the said nucleic acid, preferably under stringent conditions.

[0058] As for a method of modifying the gene structure of a gene in a cell in the presence of the protein in the present invention or the nucleic acid which can produce the said protein, by introducing the protein or the nucleic acid, for instance the mRNA which can produce the protein described here, and, at the same time, by introducing genes including a gene to be transposed, for instance a plasmid, the gene structure in a cell can be modified by the enzymatic activity of the protein in the present invention. The modification in the present invention is involved preferably in autonomous transposition. The cell is preferably an animal cell, more preferably a vertebrate cell, and much more preferably fish cell including a zebrafish cell.

[0059] Genes containing the said gene to be transposed can be substances which do not exist in a natural cell, such as a plasmid carrying a foreign gene to be transposed, and also can be a genomic gene existing in a natural cell. In this case, cis-elements required for transposition could be added to the gene if necessary. The gene to be transposed is

preferably a transposon, in certain circumstances, it may be a gene that insert a normal gene into the cell which has a disease caused by abnormalities of genes of various kinds.

[0060] Further, a method of modification in the present invention may only include the excision of part of a gene in a cell such as an inserted plasmid, however, may also include the insertion of all or part of the gene excised by this method, into any gene.

[0061] A gene excised in a method of modification in the present invention preferably has nucleotide sequences containing at least one inverted repeat in the forepart of its nucleotide sequence. The inverted repeat is thought as a cis-elment or part of cis-elments for transposition of the gene.

[0062] Further, the present invention, by using the said methods of modification, relates to a method of introducing a foreign gene into a gene of a cell and to a method of modifying a function of a cell based on expression of the gene. By performing the said methods, for example, it is possible for a foreign gene on a plasmid to be transposed into the genome in a cell, and for a new gene, which the cell concerned does not contain originally, to be inserted into a cell. Further, by expression of the newly inserted gene, it is possible to modify a function of a cell. Furthermore, the present invention can result in a cell, whose function has been modified by this method. The said cell is preferable as a cell described in this method.

[0063] As a plasmid in the present invention which contains the nucleotide sequence having at least one inverted repeat in the forepart of its nucleotide sequence, an optional substitute is to mediate transposition of a gene therein, contains a region containing at least one inverted repeat and a gene to be transposed near the repeat, and is easy to be inserted into a cell.

Examples

[0064] The present invention will be described by Examples below more precisely, but these Examples do not limit the present invention.

[0065] In the experiments in the present invention, eggs for injection were obtained from zebrafish strains, Tuebingen, TL and brass and were used for the following experiments.

Example 1 (cloning of cDNA)

[0066] Zebrafish fertilized eggs were injected with the (Tol2-tyr) plasmid and, 9 hours after the injection, total RNA was extracted from 50 of zebrafish embryos with Tri Zol Reagent (Life Technologies, Inc.) and about 3μg of the total RNA obtained was used for 3' RACE and 5' RACE, respectively.

[0067] Nested forward primers used to perform 3' RACE are:

Tol2f2; 5' -TTGGTCAGACATGTTCATTG-3 ' and
Tol2f3; 5' -ATGTTCATTGGTCCTTTGGA-3' ,
Tol2f4; 5' -ATAGCTGAAGCRGCTCTGATC -3' and
Tol2f5; 5' -CTGCTCTGATC ATGAAACAG-3',
Tol2f8; 5'-GCTTAATAAAGAAATATCGGCC -3' and
Tol2f9; 5'-AATATCGGCCTTCAAAAGTrCG-3', and
Tol2f12; 5'-CTGTAATCAGAGAGTGTATGTGTA -3' and
Tol2f13; 5'-ATTGTTACATTTATTGCATACAAT -3'.

[0068] Nested reverse primers used for 5' RACE are:

Tol2r4; 5'-CTCAATATGCTTCCTTAGG -3' and
Tol2r5; 5'-CTTCCTTAGGTTTGATGGCG-3'.

[0069] The 3' RACE and 5' RACE products were gel-extracted, cloned with TOPO TA Cloning Kit (Invitrogen, Inc.) and sequenced using the ABI PRISM 310 Genetic Analyzer.

[0070] The sequence determined is shown in sequence number 1 of SEQUENCE LISTING and the amino acid sequence of its translated region is shown in sequence number 2 of SEQUENCE LISTING.

[0071] Also, the summary is shown in Figure 1. The numbers in the parentheses are bp from the 5' end of the Tol2 element. DDBJ/EMBL/Genbank accession number for the cDNA sequence is AB032244.

Example 2 (Construction of the (Tol2-tyr) Δin1ΔRV plasmid)

[0072] The (Tol2-tyr)Δin1ΔRV plasmid was first constructed by replacing the NruI-NspV of the (Tol2-tyr) plasmid with

the NruI-NspV fragment of the cDNA and the resulting plasmid was digested with EcoRV and self-ligated.

Example 3 (mRNA synthesis, injection to embryos and PCR analysis)

[0073] The cDNA encoding the entire coding region of the transposase was cloned in pBluescript SK+ (Stratagene), linearized, digested with proteinase K and phenol/chloroform extracted. mRNA was generated by in vitro transcription by using T7 RNA polymerase and the mCAP mRNA Capping kit (Stratagene). The concentration and the size of the transcript were examined on agarose gel electrophoresis.

[0074] Zebrafish fertilized eggs were injected with 1-2nl of a DNA solution (~25ng/μl of a plasmid DNA) with or without the mRNA (~5ng/μl of theTol2 mRNA) and incubated at 28°C for ~8 hours. Each embryo was soaked in 50μl of 10mM EDTA, 10mM Tris-HCl (pH8.0), 200μg/ml proteinase K and incubated at 50°C for 3 hours.

[0075] Then 1 μl of the lysed embryo was used for PCR (35 cycles of 94°C 30sec, 55°C 30sec and 72°C 30sec) using tyr-ex4f and tyr-ex5r primers (Kawakami et al., 1998). The PCR products were analyzed on 2% agarose gel electrophoresis. The result is shown in Figure 4.

[0076] For the DNA sequencing analysis, the PCR products were gel-extracted, cloned with TOPO TA Cloning (Invitrogen) and sequenced. The presence of the injected plasmid DNA in each sample was verified by PCR (25 cycles of 94°C 30sec, 55°C 30sec and 72°C 30sec) using Tol2f1 (5'-TCCACCCATGCTTCCAGCAGTA-3') and Tol2r3 (5'-CGTTGTGGTTGCAATCCATTCAAC-3') primers.

Industrial Applicability

[0077] The present invention results in a new protein having a transposase-like activity of a gene and the nucleic acid encoding the same.

[0078] Further, the present invention discloses that a transposase of a different family is able to generate an enzymatic activity which can catalyze transposition of a gene in a vertebrate cell, and greatly contributes to the development of technologies concerning the transposition of a gene in vertebrate and the analyses of mutants generated by the said transposition. On the other hand however, since recent gene technologies are extending from modification of a cell to modification of an organism, a method of transposition of a gene in a cell in the present invention is expected not to be limited only to the modification of a cell but also applicable to modification of the structures and functions of genes of mammals in the medical and agricultural fields as one of the methods for modifying the organism of the traits. It can be expected to be a powerful method especially for the gene therapy and the improvement of fish breeding.

SEQUENCE LISTING

[0079]

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<120> TRANSPOSASE AND METHOD OF GENE MODIFICATION

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<151> 1999-12-03

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20 25 30

Phe Ser Leu Ser Gly Val Asn Lys Asp Ser Phe Lys Met Lys Cys Val
35 40 45

Leu Cys Leu Pro Leu Asn Lys Glu Ile Ser Ala Phe Lys Ser Ser Pro
50 55 60

Ser Asn Leu Arg Lys His Ile Glu Arg Met His Pro Asn Tyr Leu Lys
65 70 75 80

Asn Tyr Ser Lys Leu Thr Ala Gln Lys Arg Lys Ile Gly Thr Ser Thr
85 90 95

His Ala Ser Ser Ser Lys Gln Leu Lys Val Asp Ser Val Phe Pro Val
100 105 110

Lys His Val Ser Pro Val Thr Val Asn Lys Ala Ile Leu Arg Tyr Ile
115 120 125

Ile Gln Gly Leu His Pro Phe Ser Thr Val Asp Leu Pro Ser Phe Lys
130 135 140

Glu Leu Ile Ser Thr Leu Gln Pro Gly Ile Ser Val Ile Thr Arg Pro
145 150 155 160

Thr Leu Arg Ser Lys Ile Ala Glu Ala Ala Leu Ile Met Lys Gln Lys
165 170 175

Val Thr Ala Ala Met Ser Glu Val Glu Trp Ile Ala Thr Thr Thr Asp
180 185 190

Cys Trp Thr Ala Arg Arg Lys Ser Phe Ile Gly Val Thr Ala His Trp
195 200 205

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210 215 220

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Thr Asp Ser Gly Ser Asn Phe Met Lys Ala Phe Arg Val Phe Gly Val
260 265 270

Glu Asn Asn Asp Ile Glu Thr Glu Ala Arg Arg Cys Glu Ser Asp Asp
275 280 285

Thr Asp Ser Glu Gly Cys Gly Glu Gly Ser Asp Gly Val Glu Phe Gln
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Asp Ala Ser Arg Val Leu Asp Gln Asp Asp Gly Phe Glu Phe Gln Leu
305 310 315 320

Pro Lys His Gln Lys Cys Ala Cys His Leu Leu Asn Leu Val Ser Ser
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Val Asp Ala Gln Lys Ala Leu Ser Asn Glu His Tyr Lys Lys Leu Tyr

340 345 350

Arg Ser Val Phe Gly Lys Cys Gln Ala Leu Trp Asn Lys Ser Ser Arg

355 360 365

Ser Ala Leu Ala Ala Glu Ala Val Glu Ser Glu Ser Arg Leu Gln Leu

370 375 380

Leu Arg Pro Asn Gln Thr Arg Trp Asn Ser Thr Phe Met Ala Val Asp

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Arg Ile Leu Gln Ile Cys Lys Glu Ala Gly Glu Gly Ala Leu Arg Asn

405 410 415

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Phe Leu Thr Glu Trp Ala Asn Thr Met Arg Pro Val Ala Lys Val Leu

435 440 445

Asp Ile Leu Gln Ala Glu Thr Asn Thr Gln Leu Gly Trp Leu Leu Pro

450 455 460

Ser Val His Gln Leu Ser Leu Lys Leu Gln Arg Leu His His Ser Leu

465 470 475 480

Arg Tyr Cys Asp Pro Leu Val Asp Ala Leu Gln Gln Gly Ile Gln Thr

485 490 495

Arg Phe Lys His Met Phe Glu Asp Pro Glu Ile Ile Ala Ala Ala Ile

500 505 510

Leu Leu Pro Lys Phe Arg Thr Ser Trp Thr Asn Asp Glu Thr Ile Ile

515 520 525

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Lys Lys Glu Leu Ala Asn Ser Ser Ser Asp Asp Glu Asp Phe Phe Ala

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625                 630                 635                 640
Leu Asn Leu Arg Phe Tyr Asn Phe Glu
                645
```

Claims

1. A in vitro method of introducing a Tol2 element into the genome of a cell having no Tol2 element comprising using a gene encoding a protein having a transposase-like activity which can insert a fragment of a gene into the genome, which protein

   (i) has the amino acid sequence shown in sequence number 2 of the SEQUENCE LISTING, or
   (ii) a substituted amino acid sequence with replacement or deletion in part of the original amino acid sequence of sequence number 2, or
   (iii) a substituted amino acid sequence with addition of several other amino acids to the original amino acid sequence of sequence number 2.

2. The method according to claim 1, wherein the cell having no Tol2 element is derived from a vertebrate animal.

3. The method according to claim 2, wherein the vertebrate animal is a fish.

4. A in vitro method of inserting any DNA into the genomic DNA of a cell having no Tol2 element by using the activity of a protein having a transposase-like activity, wherein a Tol2 element has been introduced into the genome of the cell by using a gene encoding a protein having a transposase-like activity which can insert a fragment of a gene into the genome, which protein

   (i) has the amino acid sequence shown in sequence number 2 of the SEQUENCE LISTING, or
   (ii) a substituted amino acid sequence with replacement or deletion in part of the original amino acid sequence of sequence number 2, or
   (iii) a substituted amino acid sequence with addition of several other amino acids to the original amino acid sequence of sequence number 2.

5. The method according to 4, wherein the said any DNA contains a Tol2 element.

Patentansprüche

1. In-vitro-Methode zum Einführen eines Tol2-Elements in das Genom einer Zelle, die kein Tol2-Element besitzt,

umfassend die Verwendung eines Gens, das für ein Protein mit einer Transposase-Aktivität codiert, welche ein Fragment eines Gens in das Genom inserieren kann, welches Protein

(i) die Aminosäuresequenz aufweist, die in Sequenz Nummer 2 der SEQUENZLISTE gezeigt ist, oder
(ii) eine substituierte Aminosäuresequenz mit einer Ersetzung oder Deletion in einem Teil der ursprünglichen Aminosäuresequenz der Sequenz Nummer 2 aufweist, oder
(iii) eine substituierte Aminosäuresequenz mit einer Addition mehrerer weiterer Aminosäuren an die ursprüngliche Aminosäuresequenz der Sequenz Nummer 2 aufweist.

2. Methode nach Anspruch 1, wobei die Zelle, die kein Tol2-Element besitzt, von einem Säugetier stammt.

3. Methode nach Anspruch 2, wobei das Säugetier ein Fisch ist.

4. In-vitro-Methode zum Einführen irgendeiner DNA in die genomische DNA einer Zelle, die kein To12-Element besitzt, durch Ausnützen der Aktivität eines Proteins mit einer Transposase-Aktivität, wobei ein Tol2-Element in das Genom der Zelle durch Verwenden eines Gens eingeführt worden ist, das für ein Protein mit einer Transposase-Aktivität codiert, die ein Fragment eines Gens in das Genom inserieren kann, welches Protein

(i) die Aminosäuresequenz aufweist, die in Sequenz Nummer 2 der SEQUENZLISTE gezeigt ist, oder
(ii) eine substituierte Aminosäuresequenz mit einer Ersetzung oder Deletion in einem Teil der ursprünglichen Aminosäuresequenz der Sequenz Nummer 2 aufweist, oder
(iii) eine substituierte Aminosäuresequenz mit einer Addition mehrerer weiterer Aminosäuren an die ursprüngliche Aminosäuresequenz der Sequenz Nummer 2 aufweist.

5. Methode nach Anspruch 4, wobei die besagte irgendeine DNA ein Tol2-Element enthält.

Revendications

1. Méthode *in vitro* d'introduction d'un élément Tol2 dans le génome d'une cellule n'ayant pas d'élément Tol2 comprenant l'utilisation d'un gène codant pour une protéine ayant une activité de type transposase qui peut insérer un fragment d'un gène dans le génome, protéine qui

(i) a la séquence d'acides aminés présentée dans la séquence numéro 2 de la liste de séquences, ou
(ii) une séquence d'acides aminés substituée par un remplacement ou une délétion partielle de la séquence d'acides aminés originale de la séquence numéro 2, ou
(iii) une séquence d'acides aminés substituée par l'addition de plusieurs autres acides aminés à la séquence d'acides aminés originale de la séquence numéro 2.

2. Méthode selon la revendication 1, dans laquelle la cellule n'ayant pas d'élément Tol2 provient d'un animal vertébré.

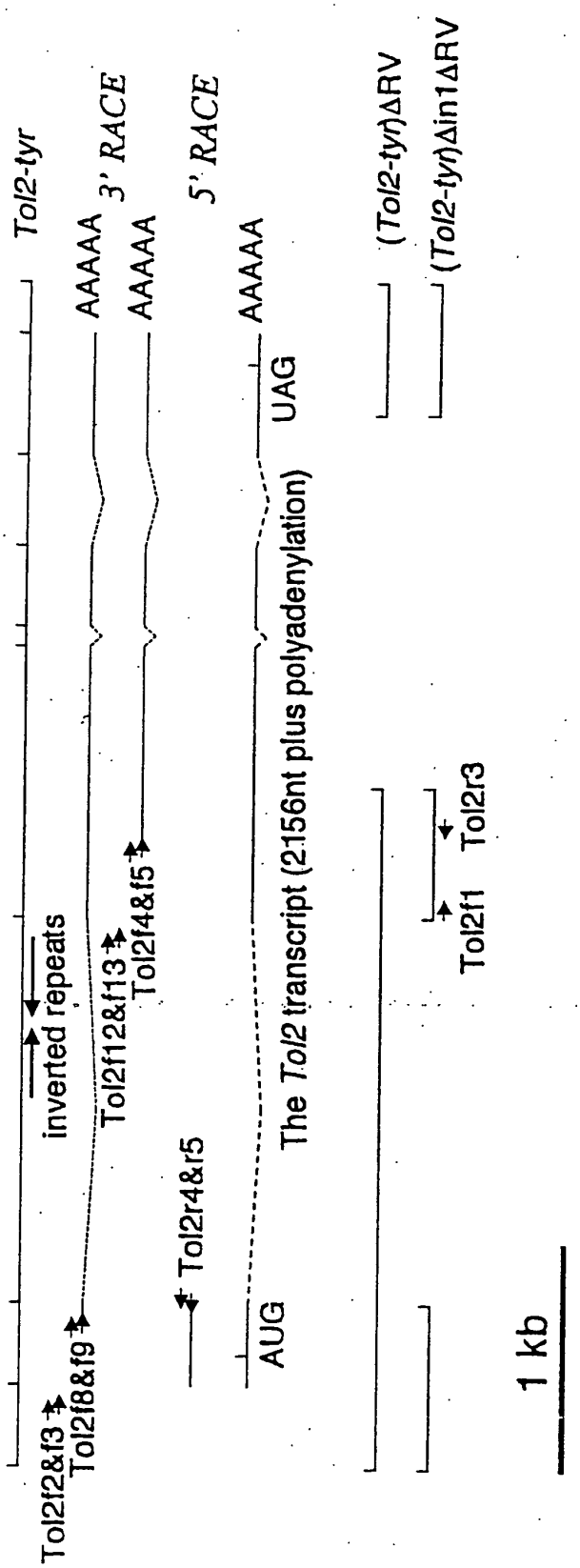
3. Méthode selon la revendication 2, dans laquelle l'animal vertébré est un poisson.

4. Méthode *in vitro* d'insertion d'un ADN quelconque dans l'ADN génomique d'une cellule n'ayant pas d'élément Tol2 en utilisant l'activité d'une protéine ayant une activité de type transposase, dans laquelle un élément Tol2 a été introduit dans le génome de la cellule en utilisant un gène codant pour une protéine ayant une activité de type transposase qui peut insérer un fragment d'un gène dans le génome, protéine qui

(i) a la séquence d'acides aminés présentée dans la séquence numéro 2 de la liste de séquences, ou
(ii) une séquence d'acides aminés substituée par un remplacement ou une délétion partielle de la séquence d'acides aminés originale de la séquence numéro 2, ou
(iii) une séquence d'acides aminés substituée par l'addition de plusieurs autres acides aminés à la séquence d'acides aminés originale de la séquence numéro 2.

5. Méthode selon la revendication 4, dans laquelle ledit ADN quelconque contient un élément Tol2.

FIG. 1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3' RACE
AAAAA
5' RACE
AAAAA
UAG
(Tol2-tyr)ΔRV
(Tol2-tyr)Δin1ΔRV
inverted repeats
Tol2f12&f13
Tol2f4&f5
Tol2f8&f9
Tol2r4&r5
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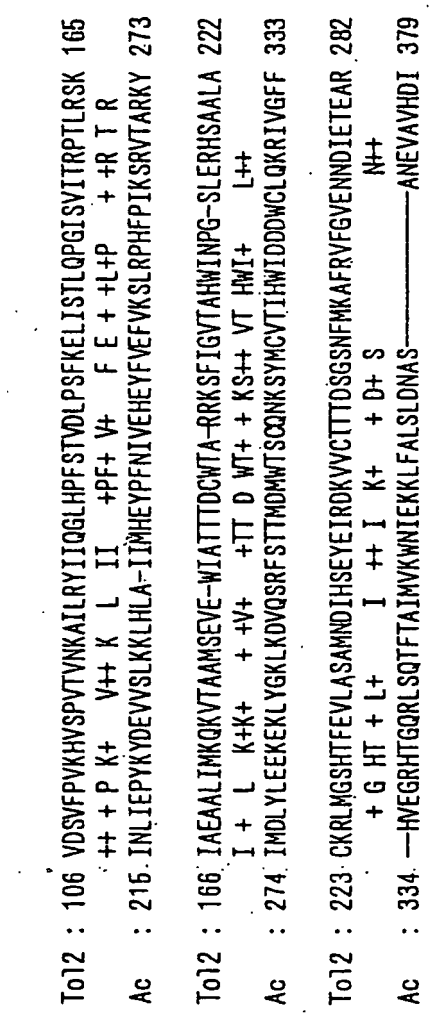
Tol2r3
Tol2f2&f3
1 kb

FIG. 2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Ac : 215 INLIEPYKYDEVVSLKKLHLA-IINHEYPFNIVEHEYFVEFVKSLRPHFPIKSRVTARKY 273
Tol2 : 166 IAEALINKQKVTAAMSEVE-WIATTTDCWTA-RRKSFIGVTAHWINPG-SLERHSAALA 222
I + L K+K+ + +V+ +TT D WT+ + KS++ VT HWI+ L++
Ac : 274 IMDLYLEEKEKLYGKLKDVQSRFSTTMDWTSQQNKSYMCVTIHWIDDDWCLQKRIVGFF 333
Tol2 : 223 CKRLMGSHTFEVLASAMNDIHSEYEIRDKVVCTTTDSGSNFMKAFRVFGVENNDIETEAR 282
+ G HT + L+ I ++ I K+ + D+ S N++
Ac : 334 —HVEGRHTGQRLSQTFTAIMVKWNIEKKLFALSLDNAS————————ANEVAVHDI 379
Tol2 : 283 RCESDDTDSEGCGEGSDGVEFQDASRVLDQDDGFEFQLPKHQKCACHLLNLVS 335
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Ac : 380 IEDLQDTDSNLV——CDGAFF————————————HVRCACHILNLVA 410

FIG. 3

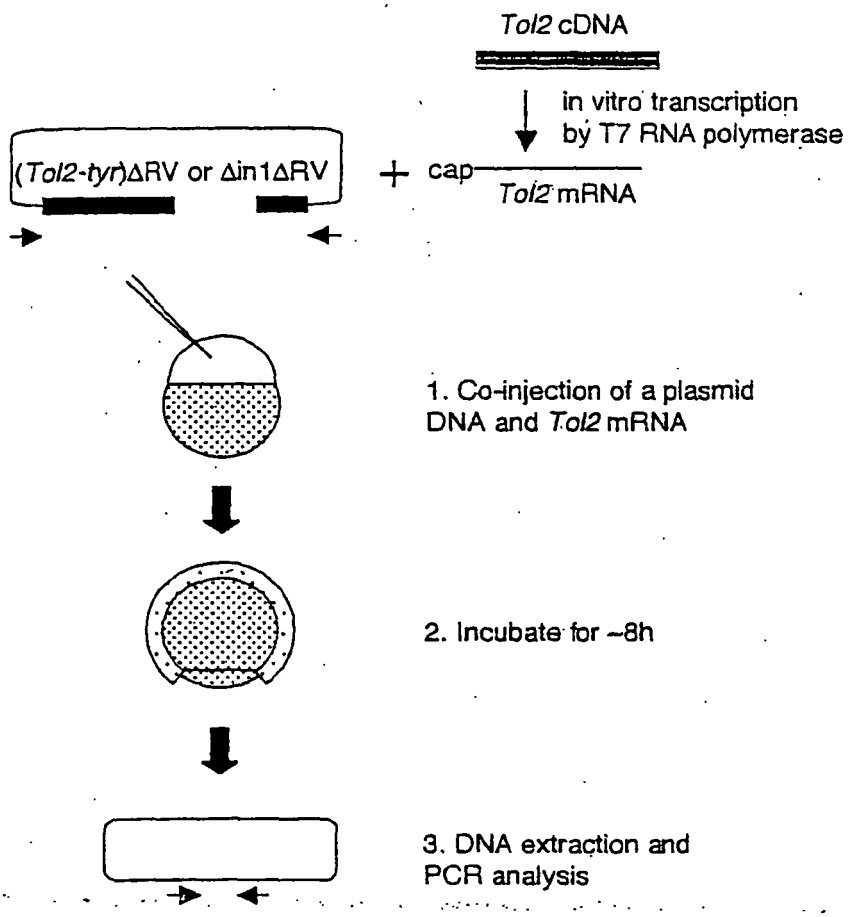
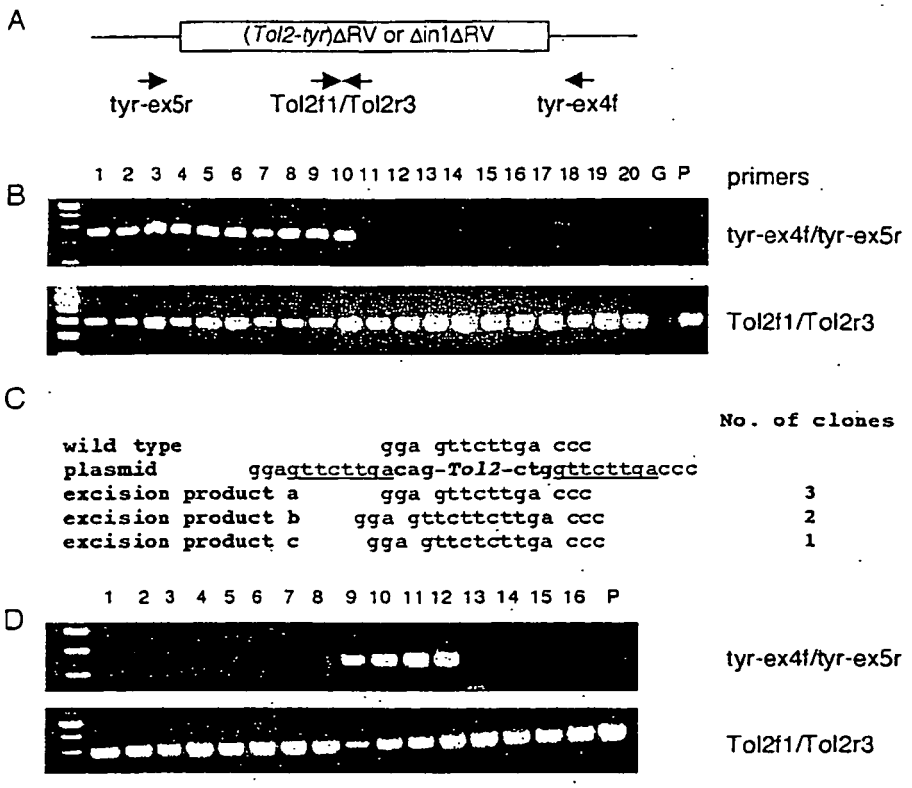

Tol2 cDNA
in vitro transcription
by T7 RNA polymerase
(Tol2-tyr)ΔRV or Δin1ΔRV
+ cap
Tol2 mRNA
1. Co-injection of a plasmid
DNA and Tol2 mRNA
2. Incubate for ~8h
3. DNA extraction and
PCR analysis

FIG. 4


A
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tyr-ex5r
Tol2f1/Tol2r3
tyr-ex4f
B
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primers
tyr-ex4f/tyr-ex5r
Tol2f1/Tol2r3
C
No. of clones
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plasmid ggagttcttgacag-Tol2-ctggttcttgaccc
excision product a gga gttcttga ccc 3
excision product b gga gttcttcttga ccc 2
excision product c gga gttctcttga ccc 1
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Tol2f1/Tol2r3

FIG. 5

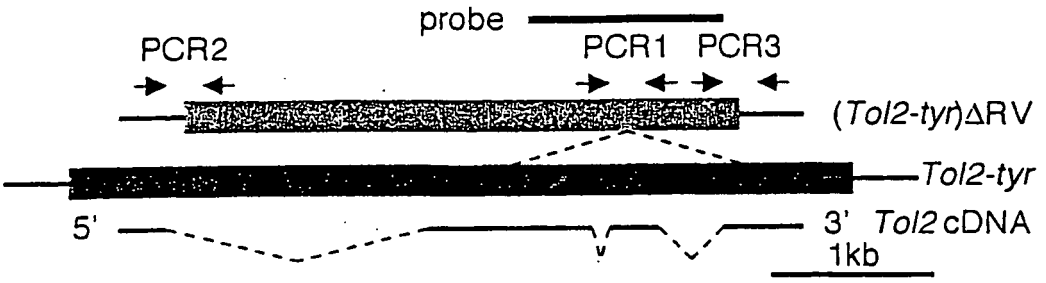

FIG. 6

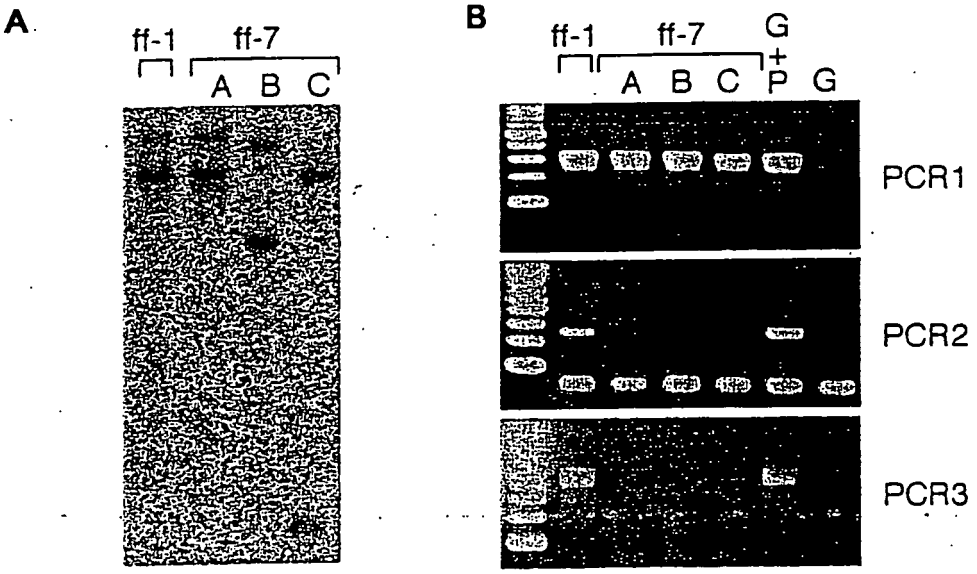

FIG. 7

A GTTTTTTTTTTACATCTCAACTG-Tol2-CTCAACTGATAGTCTAATCACAC
B CGCTGAGCTCTCTTATATAGAGA-Tol2-TATAGAGATGGCTGTTATACGAG
C AAGTGACGTCAATGTGTTTTCAG-Tol2-GTTTTCAGCTCATCTGTTCATTA

REFERENCES CITED IN THE DESCRIPTION

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Patent documents cited in the description

- JP 11345508 A **[0079]**
- JP 2000109033 A **[0079]**

Non-patent literature cited in the description

- **IVICS, Z. et al.** *Cell,* 1977, vol. 91, 501-510 **[0053]**
- **RAZ, E. et al.** *Current Biology,* 1977, vol. 8, 82-88 **[0053]**
- **FADOOL, J.M. et al.** *Proc. Natl. Acad. Sci. USA,* 1988, vol. 95, 5182-5186 **[0053]**