DESCRIPTION

Title of the Invention: METHOD FOR INCREASING MUTATION INTRODUCTION EFFICIENCY IN GENOME SEQUENCE MODIFICATION TECHNIQUE, AND MOLECULAR COMPLEX TO BE USED THEREFOR

[Technical Field]

[0001]

The present invention relates to a method for improving mutation introduction efficiency of a genome sequence modification technique that enables modification of a nucleic acid base in a particular region of a genome without cleaving double-stranded DNA, i.e., with no cleavage or single strand cleavage, or inserting a foreign DNA fragment, and a complex of a nucleic acid sequence-recognizing module, a nucleic acid base converting enzyme and a base excision repair inhibitor to be used therefor.

[Background Art]

[0002]

In recent years, genome editing is attracting attention as a technique for modifying the object gene and genome region in various species. Conventionally, as a method of genome editing, a method utilizing an artificial nuclease comprising a molecule having a sequence-independent DNA cleavage ability and a molecule having a sequence recognition ability in combination has been proposed (non-patent document 1).

For example, a method of performing recombination at a target gene locus in DNA in a plant cell or insect cell as a host, by using a zinc finger nuclease (ZFN) wherein a zinc finger DNA binding domain and a non-specific DNA cleavage domain are linked (patent document 1), a method of cleaving or modifying a target gene in a particular nucleotide sequence or a site adjacent thereto by using TALEN wherein a transcription activator-like (TAL) effector which is a DNA binding module that the plant pathogenic bacteria Xanthomonas has, and a DNA endonuclease are linked (patent document 2), a method utilizing

CRISPR-Cas9 system wherein DNA sequence CRISPR (Clustered Regularly interspaced short palindromic repeats) that functions in an acquired immune system possessed by eubacterium and archaebacterium, and nuclease Cas (CRISPR-associated) protein family having an important function along with CRISPR are combined (patent document 3) and the like have been reported. Furthermore, a method of cleaving a target gene in the vicinity of a particular sequence, by using artificial nuclease wherein a PPR protein constituted to recognize a particular nucleotide sequence by a continuation of PPR motifs each consisting of 35 amino acids and recognizing one nucleic acid base, and nuclease are linked (patent document 4) has also been reported.

These genome editing techniques basically presuppose double-stranded DNA breaks (DSB). However, since they include unexpected genome modifications, side effects such as strong cytotoxicity, chromosomal rearrangement and the like occur, and they have common problems of impaired reliability in gene therapy, extremely small number of surviving cells by nucleotide modification, and difficulty in genetic modification itself in primate ovum and unicellular microorganisms.

[0003]

On the other hand, as a method for performing nucleotide modification without accompanying DSB, the present inventors reported that, in the CRISPR-Cas system wherein at least one DNA cleavage ability of Cas is inactivated, a genome sequence was successfully modified without accompanying DSB and by nucleobase conversion in a region containing a specific DNA sequence. In the system, they used deaminase that catalyzes a deamination reaction, which was linked to a molecule having a DNA sequence recognition ability (patent document 5). According to this genome editing technique, since the technique does not involve insertion of foreign DNA or cleavage of DNA double strand, it is superior in safety, and the range of mutation introduction can theoretically be set widely from a single base pinpoint to several hundred bases. However, there

was a problem that efficiency of mutation introduction is low as compared to genome editing technique using Cas9 having normal DNA cleaving ability.

[0004]

In genome editing technique, moreover, a method for enhancing the efficiency of mutation introduction by shifting the culture temperature of the cell to a low temperature has not been reported. In addition, there is no report teaching that the activity of Petromyzon marinus-derived PmCDA1 (Petromyzon marinus cytosine deaminase 1), which is one kind of deaminase, is enhanced when the temperature is lower than about 37°C which is the optimal temperature of general enzymes.

[Document List]

[Patent documents]

[0005]

patent document 1: JP-B-4968498

patent document 2: National Publication of International Patent Application No. 2013-513389

patent document 3: National Publication of International Patent Application No. 2010-519929

patent document 4: JP-A-2013-128413

patent document 5: WO 2015/133554

[non-patent document]

[0006]

non-patent document 1: Kelvin M Esvelt, Harris H Wang (2013) Genome-scale engineering for systems and synthetic biology, Molecular Systems Biology 9: 641

[SUMMARY OF THE INVENTION]

[Problems to be Solved by the Invention]

[0007]

An object of the present invention is to provide a genome editing method for improving mutation introduction efficiency by modifying nucleic acid bases of a particular sequence of a gene by not cleaving double-stranded DNA or cleaving single strand, and a complex therefor of a nucleic acid sequence-

recognizing module, a nucleic acid base converting enzyme, and a base excision repair inhibitor.

[Means of Solving the Problems]

[0008]

The present inventors searched for the development of a method for improving the mutation introduction efficiency in the genome editing technique using a nucleic acid base converting enzyme. In the development of a method for improving mutation introduction efficiency, in general, the focus is placed on a method for increasing the nucleic acid base converting ability by artificially mutating a nucleic acid base converting enzyme or replacing same with other enzyme, or a method for increasing the nucleic acid recognizing ability of a nucleic acid sequence-recognizing module and the like. The present inventors changed these general ideas and assumed that, in the genome editing technique, one of the causes of the low mutation introduction efficiency might be that the mechanism of base excision repair by DNA glycosylase or the like works at the site where the base was converted by the nucleic acid base converting enzyme and the introduced mismatch is repaired. They then had an idea that the mutation introduction efficiency might be increased by inhibiting proteins acting on the base excision repair mechanisms. Thus, the present inventors coexpressed a uracil DNA glycosylase inhibitor (Ugi) that inhibits repair of deaminated bases, and found that the mutation introduction efficiency was strikingly improved.

[0009]

PmCDA1, which is one kind of nucleic acid base converting enzyme, is derived from Petromyzon marinus, a poikilothermic animal. Thus, they assumed that the optimal temperature for the enzyme activity of PmCDA1 might be lower than about 37°C, which is the optimal temperature for general enzymes, and had an idea that the enzyme activity might be enhanced by adjusting the culture temperature. In an attempt to enhance the enzyme activity of PmCDA1, therefore, they cultured the cells

transfected with PmCDA1 temporarily at a low temperature and found that the mutation introduction efficiency was improved.

The present inventor have conducted further studies based on these findings and completed the present invention.

[0010]

Therefore, the present invention is as described below.

[1] A method of modifying a targeted site of a double-stranded DNA, comprising a step of introducing a complex wherein a nucleic acid sequence-recognizing module that specifically binds to a target nucleotide sequence in a given double-stranded DNA and PmCDA1 are bonded, into a cell containing the double-stranded DNA, and culturing the cell at a low temperature at least temporarily to convert one or more nucleotides in the targeted site to other one or more nucleotides or delete one or more nucleotides, or insert one or more nucleotides into said targeted site, without cleaving at least one strand of said double-stranded DNA in the targeted site,

wherein the nucleic acid sequence-recognizing module is a CRISPR-Cas system wherein at least one DNA cleavage ability of Cas is inactivated.

[2] The method of [1], wherein the aforementioned Cas is deficient in two DNA cleavage abilities.

[3] The method of [1] or [2], wherein the aforementioned cell is a mammalian cell.

[4] The method of [3], wherein the low temperature is 20°C to 35°C.

[5] The method of [3], wherein the low temperature is 25°C.

[6] The method of any of [1] to [5], wherein the double-stranded DNA is contacted with the complex by introducing a nucleic acid encoding the complex into a cell having the double-stranded DNA.

[7] A method of modifying a targeted site of a double-stranded DNA, comprising a step of contacting a complex wherein a nucleic acid sequence-recognizing module that specifically

binds to a target nucleotide sequence in a given double-stranded DNA, a nucleic acid base converting enzyme and a base excision repair inhibitor are bonded, with said double-stranded DNA to convert one or more nucleotides in the targeted site to other one or more nucleotides or delete one or more nucleotides, or insert one or more nucleotides into said targeted site, without cleaving at least one strand of said double-stranded DNA in the targeted site,

wherein the nucleic acid sequence-recognizing module is a CRISPR-Cas system wherein at least one DNA cleavage ability of Cas is inactivated.

[8] The method of [7], wherein the aforementioned Cas is deficient in two DNA cleavage abilities.

[9] The method of [7] or [8], wherein the aforementioned nucleic acid base converting enzyme is cytidine deaminase.

[10] The method of [9], wherein the aforementioned cytidine deaminase is PmCDA1.

[11] The method of [9] or [10], wherein the base excision repair inhibitor is a uracil DNA glycosylase inhibitor.

[12] The method of any of [7] to [11], wherein the double-stranded DNA is contacted with the complex by introducing a nucleic acid encoding the complex into a cell having the double-stranded DNA.

[13] The method of [12], wherein the aforementioned cell is a mammalian cell.

[14] A nucleic acid-modifying enzyme complex wherein a nucleic acid sequence-recognizing module that specifically binds to a target nucleotide sequence in a given double-stranded DNA, a nucleic acid base converting enzyme and a base excision repair inhibitor are bonded, which complex converts one or more nucleotides in the targeted site to other one or more nucleotides or deletes one or more nucleotides, or inserts one or more nucleotides into said targeted site, without cleaving at least one strand of said double-stranded DNA in the targeted site,

wherein the nucleic acid sequence-recognizing module is a CRISPR-Cas system wherein at least one DNA cleavage ability of Cas is inactivated.

[15] A nucleic acid encoding the nucleic acid-modifying enzyme complex of [14].

[Effect of the Invention]

[0011]

According to the genome editing technique of the present invention, the mutation introduction efficiency is strikingly improved as compared to conventional genome editing techniques using nucleic acid base converting enzymes.

[Brief Description of the Drawings]

[0012]

Fig. 1 is a schematic showing of the genome editing plasmid used in the Examples.

Fig. 2 is a schematic showing of the evaluation method of the mutation introduction efficiency.

Fig. 3 shows the analysis results of the mutation pattern of the target gene region in the obtained mutant populations.

[Description of Embodiments]

[0013]

The present invention provides a method of improving mutation introduction efficiency in the genome editing technique including modifying a targeted site of a double stranded DNA by converting the target nucleotide sequence and nucleotides in the vicinity thereof in the double stranded DNA to other nucleotides, without cleaving at least one chain of the double stranded DNA to be modified. The method characteristically contains a step of introducing a complex wherein a nucleic acid sequence-recognizing module that specifically binds to the target nucleotide sequence in the double-stranded DNA and PmCDA1 are bonded into a cell having the double-stranded DNA, and culturing the cell at a low temperature at least temporarily to convert the targeted site, i.e., the target nucleotide sequence and nucleotides in the

vicinity thereof, to other nucleotides, or delete the targeted site, or insert nucleotide into the site.

[0014]

In another embodiment, the method characteristically contains a step of contacting a complex wherein a nucleic acid sequence-recognizing module that specifically binds to the target nucleotide sequence in the double-stranded DNA, a nucleic acid base converting enzyme, and a base excision repair inhibitor are bonded with the double-stranded DNA to convert the targeted site, i.e., the target nucleotide sequence and nucleotides in the vicinity thereof, to other nucleotides, or delete the targeted site, or insert nucleotide into the site.

[0015]

In still another embodiment, the method characteristically contains a step of introducing a complex wherein a nucleic acid sequence-recognizing module that specifically binds to the target nucleotide sequence in the double-stranded DNA, and a nucleic acid base converting enzyme are bonded, into a cell having the double-stranded DNA, and inhibiting base excision repair of the cell to convert the targeted site, i.e., the target nucleotide sequence and nucleotides in the vicinity thereof, to other nucleotides, or delete the targeted site, or insert nucleotide into the site.

[0016]

In the present invention, the "modification" of a double-stranded DNA means that a nucleotide (e.g., dC) on a DNA strand is converted to other nucleotide (e.g., dT, dA or dG), or deleted, or a nucleotide or a nucleotide sequence is inserted between certain nucleotides on a DNA strand. While the double-stranded DNA to be modified is not particularly limited, it is preferably a genomic DNA. The "targeted site" of a double-stranded DNA means the whole or partial "target nucleotide sequence", which a nucleic acid sequence-recognizing module specifically recognizes and binds to, or the vicinity of the target nucleotide sequence (one or both of 5′ upstream and 3′

downstream), and the range thereof can be appropriately adjusted between 1 base and several hundred bases according to the object.

[0017]

In the present invention, the "nucleic acid sequence-recognizing module" means a molecule or molecule complex having an ability to specifically recognize and bind to a particular nucleotide sequence (i.e., target nucleotide sequence) on a DNA strand. Binding of the nucleic acid sequence-recognizing module to a target nucleotide sequence enables a nucleic acid base converting enzyme and a base excision repair inhibitor linked to the module to specifically act on a targeted site of a double-stranded DNA.

[0018]

In the present invention, the "nucleic acid base converting enzyme" means an enzyme capable of converting a target nucleotide to other nucleotide by catalyzing a reaction for converting a substituent on a purine or pyrimidine ring on a DNA base to other group or atom, without cleaving the DNA strand.

[0019]

In the present invention, the "base excision repair" is one of the DNA repair mechanisms of living organisms, and means a mechanism for repairing damages of bases by cutting off damaged parts of the bases by enzymes and rejoining them. Excision of damaged bases is performed by DNA glycosylase, which is an enzyme that hydrolyzes the N-glycosidic bond of DNA. An abasic site (apurinic/apyrimidic (AP) site) resulting from the abasic reaction by the enzyme is treated by an enzyme at the downstream of the base excision repair (BER) pathway such as AP endonuclease, DNA polymerase, DNA ligase and the like. Examples of such gene or protein involved in the BER pathway include, but are not limited to, UNG (NM_003362), SMUG1 (NM_014311), MBD4 (NM_003925), TDG (NM_003211), OGG1 (NM_002542), MYH (NM_012222), NTHL1 (NM_002528), MPG

(NM_002434), NEIL1 (NM_024608), NEIL2 (NM_145043), NEIL3 (NM_018248), APE1 (NM_001641), APE2 (NM_014481), LIG3 (NM_013975), XRCC1 (NM_006297), ADPRT (PARP1) (NM_0016718), ADPRTL2 (PARP2) (NM_005484) and the like (parentheses indicate refseq number in which the base sequence information of each gene (cDNA) is registered).

[0020]

In the present invention, the "base excision repair inhibitor" means a protein that consequently inhibits BER by inhibiting any of the stages of the above-mentioned BER pathway, or inhibiting the expression itself of the molecule mobilized by the BER pathway. In the present invention, "to inhibit base excision repair" means to consequently inhibit BER by inhibiting any of the stages of the above-mentioned BER pathway, or inhibiting the expression itself of the molecule mobilized by the BER pathway.

[0021]

In the present invention, the "nucleic acid-modifying enzyme complex" means a molecular complex comprising a complex comprising the above-mentioned nucleic acid sequence-recognizing module and nucleic acid base converting enzyme are connected, and having nucleic acid base converting enzyme activity and imparted with a particular nucleotide sequence recognition ability. A base excision repair inhibitor may be further linked to the complex. The "complex" here encompasses not only one constituted of multiple molecules, but also one having a nucleic acid sequence-recognizing module and a nucleic acid base converting enzyme in a single molecule, like a fusion protein. In addition, "encoding the complex" encompasses both of encoding each molecule constituting the complex and encoding a fusion protein containing the constituting molecule in a single molecule.

[0022]

In the present invention, the "low temperature" means a temperature lower than the general culture temperature for cell

proliferation in cell culture. For example, when the general culture temperature of the cell is 37°C, a temperature lower than 37°C corresponds to the low temperature. On the other hand, the low temperature needs to be a temperature that does not damage cells since the cells are damaged when the culture temperature is too low. While the low temperature varies depending on the cell type, culture period and other culture conditions, for example, when the cell is a mammalian cell such as Chinese hamster ovary (CHO) cell and the like, it is typically 20°C to 35°C, preferably 20°C to 30°C, more preferably 20°C to 25°C, further preferably 25°C.

[0023]

In the present invention, "culturing at a low temperature at least temporarily" means culturing a cell under the above-mentioned "low temperature conditions" for at least a part of the whole culture period and encompasses culturing at a low temperature for the whole culture period. In addition, culturing cells intermittently at a low temperature in multiple times during the culture period is also encompassed in "culturing at a low temperature at least temporarily". While the timing and duration of the low temperature culture is not particularly limited, generally, low temperature culture is maintained for not less than one night after introduction of the complex of a nucleic acid sequence-recognizing module and PmCDA1 or a nucleic acid encoding same into the cell. The upper limit of the culture period is not particularly limited as long as it is the minimum period necessary for modification of the targeted site of the double-stranded DNA, and the cells may be cultured at a low temperature for the whole culture period. While the whole culture period varies depending on the cell type, culture period and other culture conditions, for example, when a mammalian cell such as CHO cell and the like is cultured at 25°C, it is typically about 10 days to 14 days. In a preferable one embodiment, a mammalian cell such as CHO cell and the like is cultured for not less than one night,

preferably one night to 7 days (e.g., overnight) after introduction of the complex at 20°C to 35°C, preferably 20°C to 30°C, more preferably 20°C to 25°C, further preferably 25°C.

[0024]

The nucleic acid base converting enzyme to be used in the present invention is not particularly limited as long as it can catalyze the above-mentioned reaction, and examples thereof include deaminase belonging to the nucleic acid/nucleotide deaminase superfamily, which catalyzes a deamination reaction that converts an amino group to a carbonyl group. Preferable examples thereof include cytidine deaminase capable of converting cytosine or 5-methylcytosine to uracil or thymine, respectively, adenosine deaminase capable of converting adenine to hypoxanthine, guanosine deaminase capable of converting guanine to xanthine and the like. As cytidine deaminase, more preferred is activation-induced cytidine deaminase (hereinafter to be also referred to as AID) which is an enzyme that introduces a mutation into an immunoglobulin gene in the acquired immunity of vertebrate or the like.

[0025]

While the derivation of nucleic acid base converting enzyme is not particularly limited, for example, PmCDA1 derived from Petromyzon marinus (Petromyzon marinus cytosine deaminase 1), or AID (Activation-induced cytidine deaminase; AICDA) derived from mammal (e.g., human, swine, bovine, horse, monkey etc.) can be used. For example, GenBank accession Nos. EF094822 and ABO15149 can be referred to for the base sequence and amino acid sequence of cDNA of PmCDA1, GenBank accession No. NM_020661 and NP_065712 can be referred to for the base sequence and amino acid sequence of cDNA of human AID. From the aspect of enzyme activity, PmCDA1 is preferable. As shown in the below-mentioned Examples, it was found that the risk of off-target mutation can be suppressed even when Ugi is used in combination in a particular embodiment using PmCDA1 as cytidine deaminase. Therefore, from the aspect of reduction of the risk

of off-target mutation, PmCDA1 is preferable.

[0026]

While the base excision repair inhibitor to be used in the present invention is not particularly limited as long as it consequently inhibits BER, from the aspect of efficiency, an inhibitor of DNA glycosylase located at the upstream of the BER pathway is preferable. Examples of the inhibitor of DNA glycosylase to be used in the present invention include, but are not limited to, a thymine DNA glycosylase inhibitor, an uracil DNA glycosylase inhibitor, an oxoguanine DNA glycosylase inhibitor, an alkylguanine DNA glycosylase inhibitor and the like. For example, when cytidine deaminase is used as a nucleic acid base converting enzyme, it is suitable to use a uracil DNA glycosylase inhibitor to inhibit repair of U:G or G:U mismatch of DNA generated by mutation.

[0027]

Examples of such uracil DNA glycosylase inhibitor include, but are not limited to, a uracil DNA glycosylase inhibitor (Ugi) derived from Bacillus subtilis bacteriophage, PBS1, and a uracil DNA glycosylase inhibitor (Ugi) derived from Bacillus subtilis bacteriophage, PBS2 (Wang, Z., and Mosbaugh, D.W. (1988) J. Bacteriol. 170, 1082-1091). The above-mentioned inhibiter of the repair of DNA mismatch can be used in the present invention. Particularly, Ugi derived from PBS2 is also known to have an effect of making it difficult to cause mutation, cleavage and recombination other than T from C on DNA, and thus the use of Ugi derived from PBS2 is suitable.

[0028]

As mentioned above, in the base excision repair (BER) mechanism, when a base is excised by DNA glycosylase, AP endonuclease puts a nick in the abasic site (AP site), and exonuclease completely excises the AP site. When the AP site is excised, DNA polymerase produces a new base by using the base of the opposing strand as a template, and DNA ligase finally seals the nick to complete the repair. Mutant AP

endonuclease that has lost the enzyme activity but maintains the binding capacity to the AP site is known to competitively inhibit BER. Therefore, these mutation AP endonucleases can also be used as the base excision repair inhibitor in the present invention. While the derivation of the mutant AP endonuclease is not particularly limited, for example, AP endonucleases derived from Escherichia coli, yeast, mammal (e.g., human, mouse, swine, bovine, horse, monkey etc.) and the like can be used. For example, UniprotKB No. P27695 can be referred to for the amino acid sequence of human Apel. Examples of the mutant AP endonuclease that has lost the enzyme activity but maintains the binding capacity to the AP site include proteins having mutated activity site and mutated Mg (cofactor)-binding site. For example, E96Q, Y171A, Y171F, Y171H, D210N, D210A, N212A and the like can be mentioned for human Ape1.

[0029]

The base excision repair of the cell can be inhibited by introducing an inhibitor of the aforementioned BER or a nucleic acid encoding same or a low-molecular-weight compound inhibiting BER. Alternatively, BER of the cell can be inhibited by suppressing the expression of a gene involved in the BER pathway. Suppression of gene expression can be performed, for example, by introducing siRNA capable of specifically suppressing expression of a gene involved in BER pathway, an antisense nucleic acid or an expression vector capable of expressing polynucleotides of these into cells. Alternatively, gene expression can be suppressed by knockout of genes involved in BER pathway.

[0030]

Therefore, as one embodiment of the present invention, a method for improving the mutation introduction efficiency comprising a step of introducing a complex wherein a nucleic acid sequence-recognizing module that specifically binds to the target nucleotide sequence in the double-stranded DNA and a

nucleic acid base converting enzyme are bonded into a cell containing the double-stranded DNA and showing suppressed expression of a gene relating to the BER pathway to convert the targeted site, i.e., the target nucleotide sequence and nucleotides in the vicinity thereof, to other nucleotides, or delete the targeted site, or insert nucleotide into the site is provided.

[0031]

siRNA is typically a double-stranded oligo RNA consisting of an RNA having a sequence complementary to a nucleotide sequence of mRNA or a partial sequence thereof (hereinafter target nucleotide sequence) of the target gene, and a complementary strand thereof. The nucleotide sequences of these RNAs can be appropriately designed according to the sequence information of the genes involved in the BER pathway. It is a single-stranded RNA in which a sequence complementary to the target nucleotide sequence (first sequence) and a sequence complementary thereto (second sequence) are linked via a hairpin loop portion, and an RNA (small hairpin RNA: shRNA) in which the first sequence forms a double-stranded structure with the second sequence by adopting a hairpin loop type structure is also one of the preferable embodiments of siRNA.

[0032]

The antisense nucleic acid means a nucleic acid containing a nucleotide sequence capable of specifically hybridizing with the target mRNA under physiological conditions of cells expressing the target mRNA (mature mRNA or initial transcription product) and capable of inhibiting translation of polypeptide coded by the target mRNA while being hybridized. The kind of the antisense nucleic acid may be DNA or RNA, or DNA/RNA chimera. The nucleotide sequence of these nucleic acids can be appropriately designed according to the sequence information of a gene involved in the BER pathway.

[0033]

Knockout of genes involved in the BER pathway means that

all or a part of the genes involved in the BER pathway have been destroyed or recombined so as not to exhibit their original functions. The gene may be destroyed or mutated so that one allele on the genome will not function and plural alleles may be destroyed or mutated. Knockout can be performed by a known method. For example, a method of knocking out by introducing a DNA construct made to cause genetic recombination with the target gene into the cell, a method of knocking out by insertion, deletion, substitution introduction of bases by using TALEN, CRISPR-Cas9 system or the like can be mentioned.

[0034]

A target nucleotide sequence in a double-stranded DNA to be recognized by the nucleic acid sequence-recognizing module in the nucleic acid-modifying enzyme complex of the present invention is not particularly limited as long as the module specifically binds to, and may be any sequence in the double-stranded DNA. The length of the target nucleotide sequence only needs to be sufficient for specific binding of the nucleic acid sequence-recognizing module. For example, when mutation is introduced into a particular site in the genomic DNA of a mammal, it is not less than 12 nucleotides, preferably not less than 15 nucleotides, more preferably not less than 17 nucleotides, according to the genome size thereof. While the upper limit of the length is not particularly limited, it is preferably not more than 25 nucleotides, more preferably not more than 22 nucleotides.

[0035]

As the nucleic acid sequence-recognizing module in the nucleic acid-modifying enzyme complex of the present invention, CRISPR-Cas system wherein at least one DNA cleavage ability of Cas is inactivated (hereinafter to be also referred to as "CRISPR-mutant Cas" and also encompasses CRISPR-mutant Cpf1), zinc finger motif, TAL effector and PPR motif and the like, as well as a fragment containing a DNA binding domain of a protein that specifically binds to DNA, such as restriction enzyme,

transcription factor, RNA polymerase and the like, and free of a DNA double strand cleavage ability and the like can be used, but the module is not limited thereto. Preferably, CRISPR-mutant Cas, zinc finger motif, TAL effector, PPR motif and the like can be mentioned.

[0036]

As a genome editing technique using CRISPR, a case using CRISPR-Cpf1 has been reported besides CRISPR-Cas9 (Zetsche B., et al., Cell, 163:759-771 (2015)). Cpf1 has properties different from Cas9 in that it does not require tracrRNA, that the cleaved DNA is a cohesive end, that the PAM sequence is present on the 5'-side and is a T-rich sequence and the like. Cpf1 capable of genome editing in mammalian cells includes, but is not limited to, Cpf1 derived from Acidaminococcus sp. BV3L6, Cpf1 derived from Lachnospiraceae bacterium ND2006 and the like. Mutant Cpf1 lacking DNA cleavage ability includes a D917A mutant in which the 917th Asp residue of Cpf1 (FnCpf1) derived from Francisella novicida U112 is converted to an Ala residue, an E1006A mutant obtained by converting the 1006th Glu residue to an Ala residue, a D1255A mutant obtained by converting the 1255th Asp residue to an Ala residue and the like. The mutant is not limited to these mutants and any mutant Cpf1 lacking the DNA cleavage ability can be used in the present invention.

[0037]

A zinc finger motif is constituted by linkage of 3 - 6 different Cys2His2 type zinc finger units (1 finger recognizes about 3 bases), and can recognize a target nucleotide sequence of 9 - 18 bases. A zinc finger motif can be produced by a known method such as Modular assembly method (Nat Biotechnol (2002) 20: 135-141), OPEN method (Mol Cell (2008) 31: 294-301), CoDA method (Nat Methods (2011) 8: 67-69), Escherichia coli one-hybrid method (Nat Biotechnol (2008) 26:695-701) and the like. The above-mentioned patent document 1 can be referred to as for the detail of the zinc finger motif production.

[0038]

A TAL effector has a module repeat structure with about 34 amino acids as a unit, and the 12th and 13th amino acid residues (called RVD) of one module determine the binding stability and base specificity. Since each module is highly independent, TAL effector specific to a target nucleotide sequence can be produced by simply connecting the module. For TAL effector, a production method utilizing an open resource (REAL method (Curr Protoc Mol Biol (2012) Chapter 12: Unit 12.15), FLASH method (Nat Biotechnol (2012) 30: 460-465), and Golden Gate method (Nucleic Acids Res (2011) 39: e82) etc.) have been established, and a TAL effector for a target nucleotide sequence can be designed comparatively conveniently. The above-mentioned patent document 2 can be referred to as for the detail of the production of TAL effector.

[0039]

PPR motif is constituted such that a particular nucleotide sequence is recognized by a continuation of PPR motifs each consisting of 35 amino acids and recognizing one nucleic acid base, and recognizes a target base only by 1, 4 and ii(-2) amino acids of each motif. Motif constituent has no dependency, and is free of interference of motifs on both sides. Therefore, like TAL effector, a PPR protein specific to the target nucleotide sequence can be produced by simply connecting PPR motifs. The above-mentioned patent document 4 can be referred to as for the detail of the production of PPR motif.

[0040]

When a fragment of restriction enzyme, transcription factor, RNA polymerase and the like is used, since the DNA binding domains of these proteins are well known, a fragment containing the domain and free of a DNA double strand cleavage ability can be easily designed and constructed.

[0041]

Any of the above-mentioned nucleic acid sequence-recognizing module can be provided as a fusion protein with the above-mentioned nucleic acid base converting enzyme and/or base

excision repair inhibitor, or a protein binding domain such as SH3 domain, PDZ domain, GK domain, GB domain and the like and a binding partner thereof may be fused with a nucleic acid sequence-recognizing module and a nucleic acid base converting enzyme and/or base excision repair inhibitor, respectively, and provided as a protein complex via an interaction of the domain and a binding partner thereof. Alternatively, a nucleic acid sequence-recognizing module and a nucleic acid base converting enzyme and/or base excision repair inhibitor may be each fused with intein, and they can be linked by ligation after protein synthesis.

[0042]

The nucleic acid-modifying enzyme complex of the present invention may be contacted with a double-stranded DNA by introducing the complex or a nucleic acid encoding the complex into a cell having the object double-stranded DNA (e.g., genomic DNA). In consideration of the introduction and expression efficiency, it is desirable to introduce the complex in the form of a nucleic acid encoding same rather than the nucleic acid modifying enzyme complex itself, and express the complex in the cell.

Therefore, the nucleic acid sequence-recognizing module, the nucleic acid base converting enzyme and the base excision repair inhibitor are preferably prepared as a nucleic acid encoding a fusion protein thereof, or in a form capable of forming a complex in a host cell after translation into a protein by utilizing a binding domain, intein and the like, or as a nucleic acid encoding each of them. The nucleic acid here may be a DNA or an RNA. When it is a DNA, it is preferably a double-stranded DNA, and provided in the form of an expression vector disposed under regulation of a functional promoter in a host cell. When it is an RNA, it is preferably a single-stranded RNA.

[0043]

Since the complex of the present invention does not

accompany double-stranded DNA breaks (DSB), genome editing with low toxicity is possible, and the genetic modification method of the present invention can be applied to a wide range of biological materials. Therefore, the cells to be introduced with nucleic acid encoding the above-mentioned nucleic acid converting enzyme complex can encompass cells of any species, from bacterium of Escherichia coli and the like which are prokaryotes, cells of microorganism such as yeast and the like which are lower eucaryotes, to cells of vertebrate including mammals such as human and the like, and cells of higher eukaryote such as insect, plant and the like.

[0044]

A DNA encoding a nucleic acid sequence-recognizing module such as zinc finger motif, TAL effector, PPR motif and the like can be obtained by any method mentioned above for each module. A DNA encoding a sequence-recognizing module of restriction enzyme, transcription factor, RNA polymerase and the like can be cloned by, for example, synthesizing an oligoDNA primer covering a region encoding a desired part of the protein (part containing DNA binding domain) based on the cDNA sequence information thereof, and amplifying by the RT-PCR method using, as a template, the total RNA or mRNA fraction prepared from the protein-producing cells.

A DNA encoding a nucleic acid base converting enzyme and base excision repair inhibitor can also be cloned similarly by synthesizing an oligoDNA primer based on the cDNA sequence information thereof, and amplifying by the RT-PCR method using, as a template, the total RNA or mRNA fraction prepared from the enzyme-producing cells. For example, a DNA encoding PBS2-derived Ugi can be cloned by designing suitable primers for the upstream and downstream of CDS based on the DNA sequence (accession No. J04434) registered in the NCBI/GenBank database, and cloning from PBS2-derived mRNA by the RT-PCR method.

The cloned DNA may be directly used as a DNA encoding a protein, or prepared into a DNA encoding a protein after

digestion with a restriction enzyme when desired, or after addition of a suitable linker (e.g., GS linker, GGGAR linker etc.), spacer (e.g., FLAG sequence etc.) and/or a nuclear localization signal (NLS) (each organelle transfer signal when the object double-stranded DNA is mitochondria or chloroplast DNA). It may be further ligated with a DNA encoding a nucleic acid sequence-recognizing module to prepare a DNA encoding a fusion protein.

Alternatively, a DNA encoding a nucleic acid modification enzyme complex may be fused with a DNA encoding a binding domain or a binding partner thereof, or both DNAs may be fused with a DNA encoding a separation intein, whereby the nucleic acid sequence-recognizing conversion module and the nucleic acid modification enzyme complex are translated in a host cell to form a complex. In these cases, a linker and/or a nuclear localization signal can be linked to a suitable position of one of or both DNAs when desired.

[0045]

A DNA encoding a nucleic acid modification enzyme complex can be obtained by chemically synthesizing the DNA strand, or by connecting synthesized partly overlapping oligoDNA short strands by utilizing the PCR method and the Gibson Assembly method to construct a DNA encoding the full length thereof. The advantage of constructing a full-length DNA by chemical synthesis or a combination of PCR method or Gibson Assembly method is that the codon to be used can be designed in CDS full-length according to the host into which the DNA is introduced. In the expression of a heterologous DNA, the protein expression level is expected to increase by converting the DNA sequence thereof to a codon highly frequently used in the host organism. As the data of codon use frequency in host to be used, for example, the genetic code use frequency database (http://www.kazusa.or.jp/codon/index.html) disclosed in the home page of Kazusa DNA Research Institute can be used, or documents showing the codon use frequency in each host may

be referred to. By reference to the obtained data and the DNA sequence to be introduced, codons showing low use frequency in the host from among those used for the DNA sequence may be converted to a codon coding the same amino acid and showing high use frequency.

[0046]

An expression vector containing a DNA encoding a nucleic acid modification enzyme complex can be produced, for example, by linking the DNA to the downstream of a promoter in a suitable expression vector.

As the expression vector, Escherichia coli-derived plasmids (e.g., pBR322, pBR325, pUC12, pUC13); Bacillus subtilis-derived plasmids (e.g., pUB110, pTP5, pC194); yeast-derived plasmids (e.g., pSH19, pSH15); insect cell expression plasmids (e.g., pFast-Bac); animal cell expression plasmids (e.g., pA1-11, pXT1, pRc/CMV, pRc/RSV, pcDNAI/Neo); bacteriophages such as λphage and the like; insect virus vectors such as baculovirus and the like (e.g., BmNPV, AcNPV); animal virus vectors such as retrovirus, vaccinia virus, adenovirus and the like, and the like are used.

As the promoter, any promoter appropriate for a host to be used for gene expression can be used. In a conventional method accompanying DSB, since the survival rate of the host cell sometimes decreases markedly due to the toxicity, it is desirable to increase the number of cells by the start of the induction by using an inductive promoter. However, since sufficient cell proliferation can also be afforded by expressing the nucleic acid-modifying enzyme complex of the present invention, a constituent promoter can also be used without limitation.

For example, when the host is an animal cell, SRα promoter, SV40 promoter, LTR promoter, CMV (cytomegalovirus) promoter, RSV (Rous sarcoma virus) promoter, MoMuLV (Moloney mouse leukemia virus) LTR, HSV-TK (simple herpes virus thymidine kinase) promoter and the like are used. Of these,

CMV promoter, SRα promoter and the like are preferable.

When the host is Escherichia coli, trp promoter, lac promoter, recA promoter, λP_L promoter, lpp promoter, T7 promoter and the like are preferable.

When the host is genus Bacillus, SPO1 promoter, SPO2 promoter, penP promoter and the like are preferable.

When the host is a yeast, Gal1/10 promoter, PHO5 promoter, PGK promoter, GAP promoter, ADH promoter and the like are preferable.

When the host is an insect cell, polyhedrin promoter, P10 promoter and the like are preferable.

When the host is a plant cell, CaMV35S promoter, CaMV19S promoter, NOS promoter and the like are preferable.

[0047]

As the expression vector, besides those mentioned above, one containing enhancer, splicing signal, terminator, polyA addition signal, a selection marker such as drug resistance gene, auxotrophic complementary gene and the like, replication origin and the like on demand can be used.

[0048]

An RNA encoding a nucleic acid modification enzyme complex can be prepared by, for example, transcription to mRNA in a vitro transcription system known per se by using a vector containing a DNA encoding each protein as a template.

[0049]

The complex of the present invention can be intracellularly expressed by introducing an expression vector containing a DNA encoding a nucleic acid modification enzyme complex, and culturing the host cell.

As the host, genus Escherichia, genus Bacillus, yeast, insect cell, insect, animal cell and the like are used.

As the genus Escherichia, Escherichia coli K12・DH1 [Proc. Natl. Acad. Sci. USA, 60, 160 (1968)], Escherichia coli JM103 [Nucleic Acids Research, 9, 309 (1981)], Escherichia coli JA221 [Journal of Molecular Biology, 120, 517 (1978)], Escherichia

coli HB101 [Journal of Molecular Biology, 41, 459 (1969)], Escherichia coli C600 [Genetics, 39, 440 (1954)] and the like are used.

As the genus Bacillus, Bacillus subtilis MI114 [Gene, 24, 255 (1983)], Bacillus subtilis 207-21 [Journal of Biochemistry, 95, 87 (1984)] and the like are used.

As the yeast, Saccharomyces cerevisiae AH22, AH22R$^-$, NA87-11A, DKD-5D, 20B-12, Schizosaccharomyces pombe NCYC1913, NCYC2036, Pichia pastoris KM71 and the like are used.

[0050]

As the insect cell when the virus is AcNPV, cells of cabbage armyworm larva-derived established line (Spodoptera frugiperda cell; Sf cell), MG1 cells derived from the mid-intestine of Trichoplusia ni, High Five™ cells derived from an egg of Trichoplusia ni, Mamestra brassicae-derived cells, Estigmena acrea-derived cells and the like are used. When the virus is BmNPV, cells of Bombyx mori-derived established line (Bombyx mori N cell; BmN cell) and the like are used as insect cells. As the Sf cell, for example, Sf9 cell (ATCC CRL1711), Sf21 cell [all above, In Vivo, 13, 213-217 (1977)] and the like are used.

As the insect, for example, larva of Bombyx mori, Drosophila, cricket and the like are used [Nature, 315, 592 (1985)].

[0051]

As the animal cell, cell lines such as monkey COS-7 cell, monkey Vero cell, CHO cell, dhfr gene-deficient CHO cell, mouse L cell, mouse AtT-20 cell, mouse myeloma cell, rat GH3 cell, human FL cell, human fetal kidney-derived cells (e.g., HEK293 cell) and the like, pluripotent stem cells such as iPS cell, ES cell and the like of human and other mammals, and primary cultured cells prepared from various tissues are used. Furthermore, zebrafish embryo, Xenopus oocyte and the like can also be used.

[0052]

As the plant cell, suspend cultured cells, callus, protoplast, leaf segment, root segment and the like prepared from various plants (e.g., grain such as rice, wheat, corn and the like, product crops such as tomato, cucumber, egg plant and the like, garden plants such as carnation, Eustoma russellianum and the like, experiment plants such as tobacco, arabidopsis thaliana and the like, and the like) are used.

All the above-mentioned host cells may be haploid (monoploid), or polyploid (e.g., diploid, triploid, tetraploid and the like).

[0053]

An expression vector can be introduced by a known method (e.g., lysozyme method, competent method, PEG method, $CaCl_2$ coprecipitation method, electroporation method, the microinjection method, the particle gun method, lipofection method, Agrobacterium method and the like) according to the kind of the host.

Escherichia coli can be transformed according to the methods described in, for example, Proc. Natl. Acad. Sci. USA, 69, 2110 (1972), Gene, 17, 107 (1982) and the like.

The genus Bacillus can be introduced into a vector according to the methods described in, for example, Molecular & General Genetics, 168, 111 (1979) and the like.

A yeast can be introduced into a vector according to the methods described in, for example, Methods in Enzymology, 194, 182-187 (1991), Proc. Natl. Acad. Sci. USA, 75, 1929 (1978) and the like.

An insect cell and an insect can be introduced into a vector according to the methods described in, for example, Bio/Technology, 6, 47-55 (1988) and the like.

An animal cell can be introduced into a vector according to the methods described in, for example, Cell Engineering additional volume 8, New Cell Engineering Experiment Protocol, 263-267 (1995) (published by Shujunsha), and Virology, 52, 456 (1973).

[0054]

A cell introduced with a vector can be cultured according to a known method according to the kind of the host.

[0055]

For example, when Escherichia coli or genus Bacillus is cultured, a liquid medium is preferable as a medium to be used for the culture. The medium preferably contains a carbon source, nitrogen source, inorganic substance and the like necessary for the growth of the transformant. Examples of the carbon source include glucose, dextrin, soluble starch, sucrose and the like; examples of the nitrogen source include inorganic or organic substances such as ammonium salts, nitrate salts, corn steep liquor, peptone, casein, meat extract, soybean cake, potato extract and the like; and examples of the inorganic substance include calcium chloride, sodium dihydrogen phosphate, magnesium chloride and the like. The medium may contain yeast extract, vitamins, growth promoting factor and the like. The pH of the medium is preferably about 5 - about 8.

[0056]

As a medium for culturing Escherichia coli, for example, M9 medium containing glucose, casamino acid [Journal of Experiments in Molecular Genetics, 431-433, Cold Spring Harbor Laboratory, New York 1972] is preferable. Where necessary, for example, agents such as 3β-indolylacrylic acid may be added to the medium to ensure an efficient function of a promoter. Escherichia coli is cultured at generally about 15 - about 43°C. Where necessary, aeration and stirring may be performed.

The genus Bacillus is cultured at generally about 30 - about 40°C. Where necessary, aeration and stirring may be performed.

[0057]

Examples of the medium for culturing yeast include Burkholder minimum medium [Proc. Natl. Acad. Sci. USA, 77, 4505 (1980)], SD medium containing 0.5% casamino acid [Proc. Natl. Acad. Sci. USA, 81, 5330 (1984)] and the like. The pH of the

medium is preferably about 5 - about 8. The culture is performed at generally about 20°C - about 35°C. Where necessary, aeration and stirring may be performed.

[0058]

As a medium for culturing an insect cell or insect, for example, Grace's Insect Medium [Nature, 195, 788 (1962)] containing an additive such as inactivated 10% bovine serum and the like as appropriate and the like are used. The pH of the medium is preferably about 6.2 - about 6.4. The culture is performed at generally about 27°C. Where necessary, aeration and stirring may be performed.

[0059]

As a medium for culturing an animal cell, for example, minimum essential medium (MEM) containing about 5 - about 20% of fetal bovine serum [Science, 122, 501 (1952)], Ham's F12 medium, Dulbecco's modified Eagle medium (DMEM) [Virology, 8, 396 (1959)], RPMI 1640 medium [The Journal of the American Medical Association, 199, 519 (1967)], 199 medium [Proceeding of the Society for the Biological Medicine, 73, 1 (1950)] and the like are used. The pH of the medium is preferably about 6 - about 8. The culture is performed at generally about 30°C - about 40°C. Where necessary, aeration and stirring may be performed.

[0060]

As a medium for culturing a plant cell, for example, MS medium, LS medium, B5 medium and the like are used. The pH of the medium is preferably about 5 - about 8. The culture is performed at generally about 20°C - about 30°C. Where necessary, aeration and stirring may be performed.

[0061]

The culture period is not particularly limited as long as it is at least the period necessary for the targeted site of the double-stranded DNA to be modified, and can be appropriately selected according to the host cell. To avoid undesirable off-target mutation, preferably, the culture is not

performed beyond a period sufficient to modify the targeted site. The timing and duration of the low temperature culture when performing the step of culturing at a low temperature at least temporarily is as described above.

As mentioned above, nucleic acid modification enzyme complex can be expressed intracellularly.

[0062]

An RNA encoding a nucleic acid modification enzyme complex can be introduced into a host cell by microinjection method, lipofection method and the like. RNA introduction can be performed once or repeated multiple times (e.g., 2 - 5 times) at suitable intervals.

[0063]

When a complex of a nucleic acid sequence-recognizing module and a nucleic acid base converting enzyme is expressed by an expression vector or RNA molecule introduced into the cell, the nucleic acid sequence-recognizing module specifically recognizes and binds to a target nucleotide sequence in the double-stranded DNA (e.g., genomic DNA) of interest and, due to the action of the nucleic acid base converting enzyme linked to the nucleic acid sequence-recognizing module, base conversion occurs in the sense strand or antisense strand of the targeted site (whole or partial target nucleotide sequence or appropriately adjusted within several hundred bases including the vicinity thereof) and a mismatch occurs in the double-stranded DNA (e.g., when cytidine deaminase such as PmCDA1, AID and the like is used as a nucleic acid base converting enzyme, cytosine on the sense strand or antisense strand at the targeted site is converted to uracil to cause U:G or G:U mismatch). When the mismatch is not correctly repaired, and when repaired such that a base of the opposite strand forms a pair with a base of the converted strand (T-A or A-T in the above-mentioned example), or when other nucleotide is further substituted (e.g., U→A, G) or when one to several dozen bases are deleted or inserted during repair, various mutations are

introduced. By using inhibitors of base excision repair in combination, the BER mechanism in cells is inhibited, the frequency of repair error is increased, and mutation introduction efficiency can be improved.

[0064]

As for zinc finger motif, production of many actually functionable zinc finger motifs is not easy, since production efficiency of a zinc finger that specifically binds to a target nucleotide sequence is not high and selection of a zinc finger having high binding specificity is complicated. While TAL effector and PPR motif have a high degree of freedom of target nucleic acid sequence recognition as compared to zinc finger motif, a problem remains in the efficiency since a large protein needs to be designed and constructed every time according to the target nucleotide sequence.

In contrast, since the CRISPR-Cas system recognizes the object double-stranded DNA sequence by a guide RNA complementary to the target nucleotide sequence, any sequence can be targeted by simply synthesizing an oligoDNA capable of specifically forming a hybrid with the target nucleotide sequence.

Therefore, in a more preferable embodiment of the present invention, a CRISPR-Cas system wherein at least one DNA cleavage ability of Cas is inactivated (CRISPR-mutant Cas) is used as a nucleic acid sequence-recognizing module.

[0065]

Fig. 1 is a schematic showing of the genome editing plasmid of the present invention using CRISPR-mutant Cas as a nucleic acid sequence-recognizing module.

The nucleic acid sequence-recognizing module of the present invention using CRISPR-mutant Cas is provided as a complex of an RNA molecule consisting of a guide RNA (gRNA) complementary to the target nucleotide sequence and tracrRNA necessary for recruiting mutant Cas protein, and a mutant Cas protein.

[0066]

The Cas protein to be used in the present invention is not particularly limited as long as it belongs to the CRISPR system, and preferred is Cas9. Examples of Cas9 include, but are not limited to, Streptococcus pyogenes-derived Cas9 (SpCas9), Streptococcus thermophilus-derived Cas9 (StCas9) and the like. Preferred is SpCas9. As a mutant Cas to be used in the present invention, any of Cas wherein the cleavage ability of the both strands of the double-stranded DNA is inactivated and one having nickase activity wherein at least one cleavage ability of one strand alone is inactivated can be used. For example, in the case of SpCas9, a D10A mutant wherein the 10th Asp residue is converted to an Ala residue and lacking cleavage ability of a strand opposite to the strand forming a complementary strand with a guide RNA, or H840A mutant wherein the 840th His residue is converted to an Ala residue and lacking cleavage ability of strand complementary to guide RNA, or a double mutant thereof can be used, and other mutant Cas can be used similarly.

[0067]

A nucleic acid base converting enzyme and base excision repair inhibitor are provided as a complex with mutant Cas by a method similar to the coupling scheme with the above-mentioned zinc finger and the like. Alternatively, a nucleic acid base converting enzyme and/or base excision repair inhibitor and mutant Cas can also be bound by utilizing RNA aptamers MS2F6, PP7 and the like and RNA scaffold by binding proteins thereto. Guide RNA forms a complementary strand with the target nucleotide sequence, mutant Cas is recruited by the tracrRNA attached and mutant Cas recognizes DNA cleavage site recognition sequence PAM (protospacer adjacent motif) (when SpCas9 is used, PAM is 3 bases of NGG (N is any base), and, theoretically, can target any position on the genome). One or both DNAs cannot be cleaved, and, due to the action of the nucleic acid base converting enzyme linked to the mutant Cas,

nucleic acid base conversion occurs in the targeted site (appropriately adjusted within several hundred bases including whole or partial target nucleotide sequence) and a mismatch occurs in the double-stranded DNA. Due to the error of the BER system of the cell to be repaired, various mutations are introduced.

[0068]

When CRISPR-mutant Cas is used as a nucleic acid sequence-recognizing module, CRISPR-mutant Cas is desirably introduced, in the form of a nucleic acid encoding nucleic acid modification enzyme complex, into a cell having a double-stranded DNA of interest, similar to when zinc finger and the like are used as a nucleic acid sequence-recognizing module.

A DNA encoding Cas can be cloned by a method similar to the above-mentioned method for a DNA encoding a base excision repair inhibitor, from a cell producing the enzyme. A mutant Cas can be obtained by introducing a mutation to convert an amino acid residue of the part important for the DNA cleavage activity (e.g., 10th Asp residue and 840th His residue for Cas9, though not limited thereto) to other amino acid, into a DNA encoding cloned Cas, by a site specific mutation induction method known per se.

Alternatively, a DNA encoding mutant Cas can also be constructed as a DNA having codon usage suitable for expression in a host cell to be used, by a method similar to those mentioned above for a DNA encoding a nucleic acid sequence-recognizing module and a DNA encoding a DNA glycosylase, and by a combination of chemical synthesis or PCR method or Gibson Assembly method. For example, CDS sequence and amino acid sequence optimized for the expression of SpCas9 in eukaryotic cells are shown in SEQ ID NOs: 3 and 4. In the sequence shown in SEQ ID NO: 3, when "A" is converted to "C" in base No. 29, a DNA encoding a D10A mutant can be obtained, and when "CA" is converted to "GC" in base No. 2518-2519, a DNA encoding an H840A mutant can be obtained.

[0069]

A DNA encoding a mutant Cas and a DNA encoding a nucleic acid base converting enzyme may be linked to allow for expression as a fusion protein, or designed to be separately expressed using a binding domain, intein and the like, and form a complex in a host cell via protein-protein interaction or protein ligation. Alternatively, a design may be employed in which a DNA encoding mutant Cas and a DNA encoding a nucleic acid base converting enzyme are each split into two fragments at suitable split site, either fragments are linked to each other directly or via a suitable linker to express a nucleic acid-modifying enzyme complex as two partial complexes, which are associated and refolded in the cell to reconstitute functional mutant Cas having a particular nucleic acid sequence recognition ability, and a functional nucleic acid base converting enzyme having a nucleic acid base conversion reaction catalyst activity is reconstituted when the mutant Cas is bonded to the target nucleotide sequence. For example, a DNA encoding the N-terminal side fragment of mutant Cas9 and a DNA encoding the C-terminal side fragment of mutant Cas are respectively prepared by the PCR method by using suitable primers; a DNA encoding the N-terminal side fragment of a nucleic acid base converting enzyme and a DNA encoding the C-terminal side fragment of a nucleic acid base converting enzyme are prepared in the same manner; for example, the DNAs encoding the N-terminal side fragments are linked to each other, and the DNAs encoding the C-terminal side fragments are linked to each other by a conventional method, whereby a DNA encoding two partial complexes can be produced. Alternatively, a DNA encoding the N-terminal side fragment of mutant Cas and a DNA encoding the C-terminal side fragment of a nucleic acid base converting enzyme are linked; and a DNA encoding the N-terminal side fragment of a nucleic acid base converting enzyme and a DNA encoding the C-terminal side fragment of mutant Cas are linked, whereby a DNA encoding two partial complexes can also

be produced. Respective partial complexes may be linked to allow for expression as a fusion protein, or designed to be separately expressed using a binding domain, intein and the like, and form a complex in a host cell via protein-protein interaction or protein ligation. Two partial complexes may be linked to be expressed as a fusion protein. The split site of the mutant Cas is not particularly limited as long as the two split fragments can be reconstituted such that they recognize and bind to the target nucleotide sequence, and it may be split at one site to provide N-terminal side fragment and C-terminal side fragment, or not less than 3 fragments obtained by splitting at two or more sites may be appropriately linked to give two fragments. The three-dimensional structures of various Cas proteins are known, and those of ordinary skill in the art can appropriately select the split site based on such information. For example, since the region consisting of the 94th to the 718th amino acids from the N terminus of SpCas9 is a domain (REC) involved in the recognition of the target nucleotide sequence and guide RNA, and the region consisting of the 1099th amino acid to the C-terminal amino acid is the domain (PI) involved in the interaction with PAM, the N-terminal side fragment and the C-terminal side fragment can be split at any site in REC domain or PI domain, preferably in a region free of a structure (e.g., between 204th and 205th amino acid from the N-terminal (204..205), between 535th and 536th amino acids from the N-terminal (535..536) and the like) (see, for example, Nat Biotechnol. 33(2): 139-142 (2015)). A combination of a DNA encoding a base excision repair inhibitor and a DNA encoding a mutant Cas and/or a DNA encoding a nucleic acid base converting enzyme can also be designed in the same manner as described above.

[0070]

The obtained DNA encoding a mutant Cas and/or a nucleic acid base converting enzyme and/or base excision repair inhibitor can be inserted into the downstream of a promoter of

an expression vector similar to the one mentioned above, according to the host.

On the other hand, a DNA encoding guide RNA and tracrRNA can be obtained by designing an oligoDNA sequence linking a coding sequence of crRNA sequence containing a nucleotide sequence complementary to the target nucleotide sequence (e.g., when FnCpf1 is recruited as Cas, crRNA containing SEQ ID NO: 20; AAUUUCUACUGUUGUAGAU at the 5'-side of the complementary nucleotide sequence can be used, and underlined sequences form base pairs to take a stem-loop structure), or a crRNA coding sequence and, as necessary, a known tracrRNA coding sequence (e.g., as tracrRNA coding sequence when Cas9 is recruited as Cas, gttttagagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggc accgagtcggtggtgctttt; SEQ ID NO: 9) and chemically synthesizing using a DNA/RNA synthesizer. While a DNA encoding guide RNA and tracrRNA can also be inserted into an expression vector similar to the one mentioned above, according to the host. As the promoter, pol III system promoter (e.g., SNR6, SNR52, SCR1, RPR1, U6, H1 promoter etc.) and terminator (e.g., T_6 sequence) are preferably used.

[0071]

An RNA encoding mutant Cas and/or a nucleic acid base converting enzyme and/or base excision repair inhibitor can be prepared by, for example, transcription to mRNA in vitro transcription system known per se by using a vector encoding the above-mentioned mutant Cas and/or a nucleic acid base converting enzyme and/or base excision repair inhibitor as a template.

Guide RNA-tracrRNA can be obtained by designing an oligoDNA sequence linking a sequence complementary to the target nucleotide sequence and known tracrRNA sequence and chemically synthesizing using a DNA/RNA synthesizer.

[0072]

A DNA or RNA encoding mutant Cas and/or a nucleic acid

base converting enzyme and/or base excision repair inhibitor, guide RNA-tracrRNA or a DNA encoding same can be introduced into a host cell by a method similar to the above, according to the host.

[0073]

Since conventional artificial nuclease accompanies Double-stranded DNA breaks (DSB), inhibition of growth and cell death assumedly caused by disordered cleavage of chromosome (off-target cleavage) occur by targeting a sequence in the genome. The effect thereof is particularly fatal for many microorganisms and prokaryotes, and prevents applicability. In the method of the present invention, cytotoxicity is drastically reduced as compared to a method using a conventional artificial nuclease since mutation introduction is performed not by DNA cleavage but by nucleic acid base conversion reaction on DNA.

[0074]

When sequence-recognizing modules are produced corresponding to the adjacent multiple target nucleotide sequences, and simultaneously used, the mutation introduction efficiency can increase more than using a single nucleotide sequence as a target. As the effect thereof, similarly mutation induction is realized even when both target nucleotide sequences partly overlap or when the both are apart by about 600 bp. It can occur both when the target nucleotide sequences are in the same direction (target nucleotide sequences are present on the same strand), and when they are opposed (target nucleotide sequence is present on each strand of double-stranded DNA).

[0075]

Since the genome editing technique of the present invention shows extremely high mutation introduction efficiency, modification of multiple DNA regions at completely different positions as targets can be performed. Therefore, in one preferable embodiment of the present invention, two or more

kinds of nucleic acid sequence-recognizing modules that specifically bind to different target nucleotide sequences (which may be present in one object gene, or two or more different object genes, which object genes may be present on the same chromosome or different chromosomes) can be used. In this case, each one of these nucleic acid sequence-recognizing modules, a nucleic acid base converting enzyme and a base excision repair inhibitor form a nucleic acid-modifying enzyme complex. Here, a common nucleic acid base converting enzyme and a base excision repair inhibitor can be used. For example, when CRISPR-Cas system is used as a nucleic acid sequence-recognizing module, a common complex of a Cas protein, a nucleic acid base converting enzyme and a base excision repair inhibitor (including fusion protein) are used, and two or more kinds of chimeric RNAs of tracrRNA and each of two or more guide RNAs that respectively form a complementary strand with a different target nucleotide sequence are produced and used as guide RNA-tracrRNAs. On the other hand, when zinc finger motif, TAL effector and the like are used as nucleic acid sequence-recognizing modules, for example, a nucleic acid base converting enzyme and base excision repair inhibitor can be fused with a nucleic acid sequence-recognizing module that specifically binds to a different target nucleotide.

[0076]

To express the nucleic acid-modifying enzyme complex of the present invention in a host cell, as mentioned above, an expression vector containing a DNA encoding the nucleic acid-modifying enzyme complex, or an RNA encoding the nucleic acid-modifying enzyme complex is introduced into a host cell. For efficient introduction of mutation, it is desirable to maintain an expression of nucleic acid-modifying enzyme complex of a given level or above for not less than a given period. From such aspect, it is ensuring to introduce an expression vector (plasmid etc.) autonomously replicatable in a host cell. However, since the plasmid etc. are foreign DNAs, they are

preferably removed rapidly after successful introduction of mutation. Therefore, though subject to change depending on the kind of host cell and the like, for example, the introduced plasmid is desirably removed from the host cell after a lapse of 6 hr - 2 days from the introduction of an expression vector by using various plasmid removal methods well known in the art.

Alternatively, as long as expression of a nucleic acid-modifying enzyme complex, which is sufficient for the introduction of mutation, is obtained, it is preferable to introduce mutation into the object double-stranded DNA by transient expression by using an expression vector or RNA without autonomous replicatability in a host cell (e.g., vector etc. lacking replication origin that functions in host cell and/or gene encoding protein necessary for replication).

[0077]

The present invention is explained in the following by referring to Examples, which are not to be construed as limitative.

[Examples]

[0078]

In the below-mentioned Examples, experiments were performed as follows.

<Cell line•culture•transformation•expression induction>

CHO-K1 adherent cell derived from Chinese hamster ovary was used. The cell was cultured in a hamF12 medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Biosera, Nuaille, France) and 100 μg/mL penicillin-streptomycin (Life Technologies, Carlsbad, CA, USA). The cells were incubated under humidified 5% CO_2 atmosphere at 37°C. For transfection, a 24 well plate was used and the cells were seeded at 0.5×10^5 cells per well and cultured for one day. According to the manufacturer's instructions, 1.5 μg of plasmid and 2 μL of lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) were transfected into the cells. After 5 hr from the transfection, the medium was exchanged with hamF12 medium

containing 0.125 mg/mL G418 (InvivoGen, San Diego, CA, USA) and the cells were incubated for 7 days. Thereafter, the cells were used for the calculation of the following mutation introduction efficiency.

[0079]

The step of culturing at a low temperature temporarily included transfection similar to the above-mentioned, medium exchange with hamF12 medium containing 0.125 mg/mL G418 at 5 hr after the transfection medium, continuous overnight culture at 25°C, and culturing for 2 days at 37°C. Thereafter, the cells were used for the following calculation of the mutation introduction efficiency.

[0080]

<Calculation of mutation introduction efficiency>

The outline of the calculation of the mutation introduction efficiency is shown in Fig. 2. HPRT (Hypoxanthine-guanine phosophoribosyltransferase) is one of the purine metabolic enzymes, and the cells with destroyed HPRT gene acquires resistance to 6-TG (6-thioguanine). To calculate mutation introduction efficiency of HPRT gene, the cells were detached from plastic by using trypsin-EDTA (Life Technologies, Carlsbad, CA, USA) and 100-500 cells were spread on a dish containing hamF12 medium containing G418 or G418 + 5 g/mL 6-TG (Tokyo Chemical Industry, Tokyo, Japan). Seven days later, the number of resistant colonies was counted. The mutation introduction efficiency was calculated as a ratio of 6TG resistant colonies to the G418 resistant colonies.

[0081]

<Sequence analysis>

For sequence analysis, G418 and 6TG resistant colonies were treated with trypsin and pelletized by centrifugation. According to manufacturer's instructions, genomic DNA was extracted from the pellets by using NucleoSpin Tissue XS kit (Macherey-Nagel, Duren, Germany). PCR fragments containing the targeted site of HPRT was amplified from genomic DNA by using

the forward primer (ggctacatagagggatcctgtgtca; SEQ ID NO: 18) and the reverse primer (acagtagctcttcagtctgataaaa; SEQ ID NO: 19). The PCR product was TA cloned into Escherichia coli (E. coli) vector and analyzed by the Sanger method.

[0082]

<Nucleic acid operation>

DNA was processed or constructed by any of PCR method, restriction enzyme treatment, ligation, Gibson Assembly method, and artificial chemical synthesis. The plasmid was amplified with Escherichia coli strain XL-10 gold or DH5α and introduced into the cells by the lipofection method.

[0083]

<Construct>

The outline of the genome editing plasmid vector used in the Example is shown in Fig. 1. Using pcDNA3.1 vector as a base, a vector used for gene transfer by transfection into CHO cells was constructed. A nuclear localization signal (ccc aag aag aag agg aag gtg; SEQ ID NO: 11 (PKKKRKV; encoding SEQ ID NO: 12)) which was added to Streptococcus pyogenes-derived Cas9 gene ORF having a codon optimized for eucaryon expression (SEQ ID NO: 3 (encoding SEQ ID NO: 4)), the resulting construct was ligated to the downstream of a CMV promoter via a linker sequence, a deaminase gene (Petromyzon marinus Petromyzon marinus-derived PmCDA1) ORF having a codon optimized for human cell expression (SEQ ID NO: 1 (encoding SEQ ID NO: 2)) was added thereto, and then the obtained product was expressed as a fusion protein. In addition, a construct for fusion expression of Ugi gene (PBS2-derived Ugi was codon-optimized for eukaryotic cell expression: SEQ ID NO: 5 (encoding SEQ ID NO: 6)) was also produced. A drug resistant gene (NeoR: G418 resistant gene) was also ligated via a sequence encoding 2A peptide (gaa ggc agg gga agc ctt ctg act tgt ggg gat gtg gaa gaa aac cct ggt cca; SEQ ID NO: 13 (encoding EGRGSLLTCGDVEENPGP; SEQ ID NO: 14)). As the linker sequence, 2xGS linker (two repeats of ggt gga gga ggt tct; SEQ ID NO: 15

(encoding GGGGS; SEQ ID NO: 16)) was used. As the terminator, SV40 poly A signal terminator (SEQ ID NO: 17) was ligated.

In Cas9, mutant Cas9 (nCas9) into which a mutation to convert the 10th aspartic acid to alanine (D10A, corresponding DNA sequence mutation a29c) was introduced and mutant Cas9 (dCas9) into which a mutation to convert the 840th histidine to alanine (H840A, corresponding DNA sequence mutation ca2518 gc) was further introduced were used to remove cleavage ability of each or both sides of DNA strand.

gRNA was placed between the H1 promoter (SEQ ID NO: 10) and the poly T signal (tttttt) as a chimeric structure with tracrRNA (derived from Streptococcus pyogenes; SEQ ID NO: 9) and incorporated into a plasmid vector for expressing the above-mentioned deaminase gene and the like. As the gRNA-targeting base sequence, the 16th - 34th sequence (ccgagatgtcatgaaagaga; SEQ ID NO: 7) (site 1) from the start point of exon3 of the HPRT gene, and a complementary strand sequence (ccatgacggaatcggtcggc; SEQ ID NO: 8) (site 2R) to the -15th - 3rd sequence from the start point of exon1 of the HPRT gene were used. They were introduced into the cell, expressed in the cells to form a complex of gRNA-tracrRNA and Cas9-PmCDA1 or Cas9-PmCDA1-Ugi.

[0084]

Example 1 Various genome editing plasmids and evaluation of mutation introduction efficiency by conditions

The evaluation results of various genome editing plasmids and mutation introduction efficiency by conditions are shown in Table 1. In Example 1, site 1 (SEQ ID NO: 7) was used as the gRNA-targeting base sequence for all those not described as site 2R.

[0085]

[Table 1]

	Modifier plasmid	Transformants 6-TG resisntant	mutation frequency
	Cas	341 328	96.2%
	nCas (D10A)-2A-Neo	4745 3	0.06%
	nCas-PmCDA1-2A-Neo	282 101	35.9%
	dCas-PmCDA1-2A-Neo	384 8	2.08%
	dCas-2A-Neo site1	8066 0	0%
	dCas-2A-Neo site2R	6241 0	0%
	Neo only	15180 0	0%
+25°C pulse	dCas-2A-Neo	8900 0	0%
	nCas-PmCDA1-2A-Neo	480 297	61.9%
	dCas-PmCDA1-2A-Neo	240 30	12.5%
+Ugi	nCas-PmCDA1-2A-Neo	723 658	91.0%
	dCas-PmCDA1-2A-Neo	823 709	86.2%

[0086]

A plasmid using nCas9 as mutant Cas9 (nCas-PmCDA1-2A-Neo) showed mutation introduction efficiency of 35.9%, and a plasmid using dCas9 as mutant Cas9 mutant (dCas-PmCDA1-2A-Neo) showed mutation introduction efficiency of 2.08%. On the other hand, in cases using a plasmid with Ugi ligated to PmCDA1, a plasmid using nCas9 as mutant Cas9 (+Ugi nCas-PmCDA1-2A-Neo) showed

mutation introduction efficiency of 91.0%, and a plasmid using dCas9 as mutant Cas9 (+Ugi dCas-PmCDA1-2A-Neo) showed mutation introduction efficiency of 86.2%. Therefore, it was shown that mutation introduction efficiency is significantly improved by fusion expression of Ugi protein which inhibits repair of deaminated bases, and particularly, one using dCas9 showed a striking increase in the mutation introduction efficiency improving effect by the combined use of Ugi. In Table 1, Cas shows a plasmid using Cas9 without introduction of mutation, nCas(D10A)-2A-Neo, dCas-2A-Neo site 1 and dCas-2A-Neo site 2R show plasmids without ligation of a nucleic acid base converting enzyme, and they were each used as a control.

[0087]

In addition, it was shown that the cells cultured temporarily (overnight) at a low temperature of 25°C (+25°C pulse) after transfection and using any of nCas9 (nCas-PmCDA1-2A-Neo) and dCas9 (dCas-PmCDA1-2A-Neo) exhibit significantly improved mutation introduction efficiency (61.9% and 12.5%, respectively). In Table 1, dCas-2A-Neo shows a plasmid without fusion with nucleic acid base converting enzyme and used as a control.

[0088]

From the above, it was shown that, according to the genome editing technique of the present invention, the mutation introduction efficiency is strikingly improved as compared to the conventional genome editing techniques using nucleic acid base converting enzymes.

[0089]

Example 2 Analysis of mutation introduction pattern

Genome DNA was extracted from the obtained mutation introduction colonies, the target region of the HPRT gene was amplified by PCR, TA cloning was performed, and sequence analysis was performed. The results are shown in Fig. 3. The editing vectors used were Cas9, nCas9(D10A)-PmCDA1 and dCas9-PmCDA1, the base excision repair inhibitor was not expressed,

and the colonies from the cells cultured at 37°C were used. In the Figure, TGG enclosed in a black box shows PAM sequence.

In Cas9 free of introduction of mutation, large deletions and insertions centered on directly above the PAM sequence were observed. On the other hand, in nCas9 (D10A)-PmCDA1, a small scale deletion of about dozen bases was observed, and the region thereof contained a deamination target base. In dCas9-PmCDA1, a mutation from C to T was observed at 19 to 21 bases upstream of the PAM sequence, and a single base pinpoint mutation was introduced in 10 clones in total 14 clones subjected to sequence analysis.

[0090]

From the above, it was shown that pinpoint mutation introduction is possible even when a genome editing technique using a nucleic acid base converting enzyme is applied to mammalian cells.

[0091]

Example 3 Study using other mammalian cell

Using HEK293T cell, which is derived from human fetal kidney, mutation introduction efficiency was evaluated. The same vector as in Example 1 was used as a vector except that the gRNA target base sequence was the sequence of the EMX1 gene (shown in SEQ ID NO: 21) described in "Tsai S.Q. et al., (2015) Nat Biotechnol., 33(2): 187-197" and the off-target candidate sequences 1 to 4 (respectively correspond to the sequences of Emx 1 off target 1 - Emx 1 off target 4 in Tables 2, 3 and shown in SEQ ID NOs: 22 - 25). The vector was introduced into HEK293T cells by transfection. Without selection of the cells, the whole cells were recovered two days later and genomic DNA was extracted. Culture conditions other than cell selection and period up to total cell recovery and transfection conditions were the same as those of the above-mentioned CHO-K1 cells. Then, according to the method described in "Nishida K. et al. (2016) Science, 6: 353(6305)", the regions containing respective targets were amplified by PCR using the primers

shown in Table 2, and mutation introduction pattern was analyzed using a next-generation sequencer. The results are shown in Table 3. In the Table, the number under the sequence shows a substitution rate (%) of the nucleotide.

[0092]

[Table 2]

target name	SEQ ID NO:	Primer name	Primer sequence (5'→3')
Emx1	26	TA501 EMX 1st-3	GTAGTCTGGCTGTCACAGGCCATACTCTTCCACAT
	27	TA575 EMX 1st-6	GTGGGTGACCCACCCAAGCAGCAGGCTCTCCACCA
	28	TA576 EMX 2nd-3	TCTTTCCCTACACGACGCTCTTCCGATCTACTTAGCTGGAGTGTGGAGGCTATCTTGGC
	29	TA410 EMX 2nd-2	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGCTAGGGACTGGCCAGAGTCCAGC
Emx1 off target 1	30	TA502 EMX-off1 1st-3	CTGCCCATATCCACCACAAGCAAGTTAGTCATCAA
	31	TA412 EMX-off1 1st-2	AATCAAAATCTCTATGTGTGGGGCACAGGG
	32	TA413 EMX-off1 2nd-1	TCTTTCCCTACACGACGCTCTTCCGATCTCATTGGCTAGAATTCAGACTTCAAG
	33	TA414 EMX-off1 2nd-2	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTATGAGGGAGATGTACTCTCAAGTGA
Emx1 off target 2	34	TA503 EMX-off2 1st-3	CATGTTCCCTCACCCTTGGCATCTACACACTTTCT
	35	TA416 EMX-off2 1st-2	TAGTTTACCCTGAGGCAATATCTGACTCCA
	36	TA417 EMX-off2 2nd-1	TCTTTCCCTACACGACGCTCTTCCGATCTTCATTTTCAAATGCCTATTGAGCGG
	37	TA418 EMX-off2 2nd-2	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAAGGCTCCTTGCCTTTACATATAGG
Emx1 off target 3	38	TA419 EMX-off3 1st-1	TCACTTTTGTCAATTCATGCCACCATCAGT
	39	TA420 EMX-off3 1st-2	GCCACCTCCACTCTGCCAGGAATAGGTTCA
	40	TA421 EMX-off3 2nd-1	TCTTTCCCTACACGACGCTCTTCCGATCTATGGACTGTCCTGTGAGCCCGTGGC
	41	TA422 EMX-off3 2nd-2	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCTCGGTGGCCTGCAAGTGGAAAGCC
Emx1 off target 4	42	TA423 EMX-off4 1st-1	GGGACCACTTGAAGTGAGTAAAATTATAGG
	43	TA424 EMX-off4 1st-2	CCCAGCTGTTGCTAGCTTATGGCCAGTCCT
	44	TA425 EMX-off4 2nd-1	TCTTTCCCTACACGACGCTCTTCCGATCTCACTGCCTTTCGGGCTAGCCTCCAA
	45	TA426 EMX-off4 2nd-2	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTAGATGTTAATAGGTTATTGGGGTG

Fragments amplified from genomic DNA with a pair of 1st primers were amplified again with a pair of 2nd primers to add an adapter sequence for NGS.

[0093]

[Table 3]

target name each editing construct	NGS read number	detected indel (%) in total	target sequence																								PAM					
Emx1			-25	-24	-23	-22	-21	-20	-19	-18	-17	-16	-15	-14	-13	-12	-11	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1	0	1	2	3	4	5
	Read	Indel (%)	G	G	C	C	T	G	A	G	T	C	C	G	A	G	C	A	G	A	A	G	A	A	G	A	A	G	G	G	C	T	C
Cas9	143352	11.5																															
nCas9 PmCDA1	139289	0.15										T 1.49	T 0.44				T 0.38																
												G 0.52	G 0.32																				
												A 0.35																				
nCas9 PmCDA1 UGI	60714	0.16										T 7.38	T 2.29				T 1.23																
												G 0.31	G 0.35				G 0.14																
												A 0.11																				
dCas9 PmCDA1	104633	0.11																															
dCas9 PmCDA1 UGI	119871	0.13										T 1.82	T 0.47				T 0.36																

Emx1 off target 2				-25	-24	-23	-22	-21	-20	-19	-18	-17	-16	-15	-14	-13	-12	-11	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1	0	1	2	3	4	5
	Read		Indel (%)	G	A	C	A	A	G	A	G	T	C	T	A	A	G	C	A	G	A	A	G	A	A	G	A	A	G	A	G	A	G	C
Cas9	33354		1.51																															T 0.16
nCas9 PmCDA1	67209		0.49										T 0.19															G 0.19						T 0.18
													G 0.56																					
nCas9 PmCDA1 UGI	130203		0.48										T 0.77																					T 0.2
dCas9 PmCDA1	22108		0.5																															T 0.23
dCas9 PmCDA1 UGI	63161		0.46																															T 0.22

Emx1 off target 3				-25	-24	-23	-22	-21	-20	-19	-18	-17	-16	-15	-14	-13	-12	-11	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1	0	1	2	3	4	5
	Read		Indel (%)	A	T	G	A	G	G	A	G	G	C	C	G	A	G	C	A	G	A	A	G	A	A	A	G	A	C	G	G	C	G	A
Cas9	228515		2.85															T 0.24																	
nCas9 PmCDA1	338152		0.76										T 0.2				T 0.18	T 0.25																	
nCas9 PmCDA1 UGI	210475		0.34										T 0.14	T 0.17			A 0.14	T 0.29																	

dCas9 PmCDA1	258150		0.29															T 0.21																						
dCas9 PmCDA1 UGI	187272		0.31															T 0.21																						
Emx1 off target 4				-25	-24	-23	-22	-21	-20	-19	-18	-17	-16	-15	-14	-13	-12	-11	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1	0	1	2	3	4	5							
	Read		Indel (%)	G	A	C	C	T	G	A	G	T	C	C	T	A	G	C	A	G	G	A	G	A	A	G	A	A	G	A	G	G	C	A							
Cas9	93074		1.02											T 1.12																		A 0.14								
nCas9 PmCDA1	48271		0											T 1.25																										
nCas9 PmCDA1 UGI	71071		0											T 1.25				T 0.19																						
dCas9 PmCDA1	69603		0											T 1.13																			T 0.17							
dCas9 PmCDA1 UGI	66474		0											T 1.12																			T 0.17							

[0094]

From Table 3, a mutation introduction efficiency improving effect by the combined use of UGI was found even when human cells were used. Furthermore, it was suggested that the ratio of off-target mutation rate to on-target can be lowered, namely, the risk of off-target mutation can be suppressed. To be specific, when nCas9-PmCDA1-UGI was used, for example, the ratio of the substitution rate of cytosine at the corresponding position of the off-target candidate to the substitution rate of the -16th cytosine in the target sequence of EMX1 was not more than 1/10. When nCas9-PmCDA1-UGI was used, the mutation rate was improved in the target sequence of EMX1 as compared to that of nCas9-PmCDA1 without combined use of UGI; however, in the off-target candidate sequence, the mutation rate showed almost no difference and off-target mutation was suppressed. Similarly, when dCas9-PmCDA1-UGI was used, the mutation rate was improved in the target sequence of EMX1 as compared to that of dCas9-PmCDA1 without combined use of UGI; however, the mutation rate showed almost no difference in the off-target candidate sequence and off-target mutation was suppressed. As for the -15th cytosine in the off-target candidate sequence 4 (sequence name: Emx1 off target 4) in Table 3, substitution occurred at the same rate irrespective of the vector used. Since substitution is found similarly in Cas9 as well, these substitutions were highly possibly caused by sequencing errors.

[Industrial Applicability]

[0095]

The present invention makes it possible to safely introduce site specific mutation into any species highly efficiently without accompanying insertion of a foreign DNA or double-stranded DNA breaks, and is extremely useful.

[0096]

This application is based on a patent application No. 2016-085631 filed in Japan (filing date: April 21, 2016), the contents of which are incorporated in full herein.

Claims

1. A method of modifying a targeted site of a double-stranded DNA, comprising a step of introducing a complex wherein a nucleic acid sequence-recognizing module that specifically binds to a target nucleotide sequence in a given double-stranded DNA and PmCDA1 are bonded, into a cell containing the double-stranded DNA, and culturing the cell at a low temperature at least temporarily to convert one or more nucleotides in the targeted site to other one or more nucleotides or delete one or more nucleotides, or insert one or more nucleotides into said targeted site, without cleaving at least one strand of said double-stranded DNA in the targeted site,
wherein the nucleic acid sequence-recognizing module is a CRISPR-Cas system wherein at least one DNA cleavage ability of Cas is inactivated.

2. The method according to claim 1, wherein said Cas is deficient in two DNA cleavage abilities.

3. The method according to claim 1 or 2, wherein said cell is a mammalian cell.

4. The method according to claim 3, wherein the low temperature is 20°C to 35°C.

5. The method according to claim 3, wherein the low temperature is 25°C.

6. The method according to any one of claims 1 to 5, wherein the double-stranded DNA is contacted with the complex by introducing a nucleic acid encoding the complex into a cell having the double-stranded DNA.

7. A method of modifying a targeted site of a double-stranded DNA, comprising a step of contacting a complex wherein a nucleic acid sequence-recognizing module that specifically binds to a target nucleotide sequence in a given double-stranded DNA, a nucleic acid base converting enzyme and a base excision repair inhibitor are bonded, with said double-stranded DNA to convert one or more nucleotides in the targeted site to other one or more nucleotides or delete one or more nucleotides, or insert one or more nucleotides into said targeted site, without cleaving at least one strand of said double-stranded DNA in the targeted site,
wherein the nucleic acid sequence-recognizing module is a CRISPR-Cas system wherein at least one DNA cleavage ability of Cas is inactivated.

8. The method according to claim 7, wherein said Cas is deficient in two DNA cleavage abilities.

9. The method according to claim 7 or 8, wherein said nucleic acid base converting enzyme is cytidine deaminase.

10. The method according to claim 9, wherein said cytidine deaminase is PmCDA1.

11. The method according to claim 9 or 10, wherein the base excision repair inhibitor is a uracil DNA glycosylase inhibitor.

12. The method according to any one of claims 7 to 11, wherein the double-stranded DNA is contacted with the complex by introducing a nucleic acid encoding the complex into a cell having the double-stranded DNA.

13. The method according to claim 12, wherein said cell is a mammalian cell.

14. A nucleic acid-modifying enzyme complex wherein a nucleic acid sequence-recognizing module that specifically binds to a target nucleotide sequence in a given double-stranded DNA, a nucleic acid base converting enzyme and a base excision repair inhibitor are bonded, which complex converts one or more nucleotides in the targeted site to other one or more nucleotides or deletes one or more nucleotides, or inserts one or more nucleotides into said targeted site, without cleaving at least one strand of said double-stranded DNA in the targeted site,

wherein the nucleic acid sequence-recognizing module is a CRISPR-Cas system wherein at least one DNA cleavage ability of Cas is inactivated.

15. A nucleic acid encoding the nucleic acid-modifying enzyme complex according to claim 14.