DESCRIPTION

PROCESS FOR PRODUCTION OF PROTEIN

Technical Field

[0001]

This invention relates to a method for producing a protein of interest, comprising introducing a protein expression vector which comprises a gene fragment comprising a DNA encoding a protein of interest and a selectable marker gene and transposon sequences at both terminals of the gene fragment, into a suspension mammalian cell, integrating the gene fragment inserted between a pair of the transposon sequences into a chromosome of the mammalian cell to obtain a mammalian cell capable of expressing the protein of interest; and suspension-culturing the mammalian cell; and a suspension mammalian cell capable of expressing the protein of interest.

Background Art

[0002]

Production of exogeneous proteins by recombinant DNA techniques is used in various industries such as pharmaceutical industry and food industry. In most cases, production of recombinant proteins is carried out by introducing an expression vector comprising a nucleotide sequence encoding a protein of interest into a host, such as *Escherichia coli*, yeast, insect cell, plant cell, and animal cell, selecting a transformant in which the expression vector is integrated into the chromosome, and further culturing the cell line under appropriate culture conditions.

[0003]

However, in order to develop a host which can produce an exogeneous protein efficiently, it is necessary to select a host cell having good productivity for each protein of interest, so that a further technical innovation is desired on the exogeneous protein production techniques for individual host.

[0004]

In the bacteria systems, such as *Escherichia coli*, and yeast systems, different from animal cells, post-translational modifications, such as sugar chain modification, are difficult to attain in many cases and thus cause a problem in producing a protein having its activity.

[0005]

Since the produced protein is subject to a post-translational modification such as phosphrylation and addition of sugar chains in the insect system, this system has a merit that the protein having its original physiological activity can be expressed. However, since the sugar chain structure of the secreted protein is different from that of mammalians-derived cells, antigenicity and the like become a problem when the protein is applied to pharmaceutical use.

[0006]

In addition, since a recombinant virus is used in the insect cell system when an exogeneous gene is introduced, there is a problem that its inactivation and containment of the virus are required from the viewpoint of safety.

[0007]

In the animal cell system, post-translational modifications, such as phosphorylation, sugar chain addition, and folding, can be conducted to proteins derived from higher animals including human, in more similar manner to those produced in the living body. Such accurate post-translational modifications are necessary for recreating the physiological activity originally possessed by a protein in its recombinant protein, and a protein production system in which a mammalian cell is used as a host is usually applied to pharmaceutical products and the like that requires such physiological activity.

[0008]

However, a protein expression system in which a mammalian cell is used as the host is generally low in productivity, and also causes a problem of the stability of introduced genes in many cases. Improvement of productivity of a protein using a mammalian culture cell as a host is not only very important in producing medicaments for treatment, diagnostic agents and the like, but also greatly contributes to research and development of them. Thus, it is urgent to develop a gene expression system which easily makes it possible to obtain a cell line of a high productivity using a mammalian culture cell, particularly Chinese hamster ovary cell (CHO cell), as the host.

[0009]

A transposon is a transposable genetic element which can transfer from one locus to other locus on the chromosome. A transposon is a strong tool for the study on molecular biology and genetics and used for a purpose, such as mutagenesis, gene trapping, and preparation of transgenic individuals, in insects or nematode (e.g., *Drosophila melanogaster* or *Caenorhabditis elegans*) and plants. However, development of such a technique has been delayed for vertebral animals including mammalian cells.

[0010]

In recent years, however, transposons which have activities also in vertebral animals have been reported, and some of them were shown to have an activity in mammalian cells, such as cell derived from mouse and human. Typical examples include transposons Tol1 (Patent Reference 1) and Tol2 (Non-patent Reference 1) cloned from a medaka (killifish), Sleeping Beauty reconstructed from a non-autonomous transposon existed in *Onchorhynchus* fish genome (Non-patent Reference 2), an artificial transposon Frog prince (Non-patent Reference 3) which is derived from frog and a transposon piggyBac (Non-patent Reference 4) which is derived from insect.

[0011]

These DNA transposons have been used for mutagenesis, gene trapping, preparation of transgenic individuals, expression of drug-resistant proteins, and the like, as a gene transfer tool for bringing a new phenotype in a genome of a mammalian cell (Non-patent References 5 to 12).

[0012]

In the case of insects, a method in which an exogeneous gene is introduced into silkworm chromosome using the transposon piggyBac derived from a Lepidoptera insect to express the protein encoded by said exogeneous gene was studied, and a protein production method using the above techniques has been disclosed (Patent Reference 2).

[0013]

However, since the expressed protein of interest is not sufficient in expression level and is produced in the whole body of silkworm, it causes an economical problem due to the necessity of an advanced purification technique for recovering the expressed exogeneous protein in a highly purified form from the body fluid including a large amount of contaminated proteins.

[0014]

In addition, an example in which a protein relating to G418 resistance is expressed in a mammalian cell using the medaka-derived transposon Tol2 (Non-patent Reference 12) is known.

Citation List

Patent Literature

[0015]

Patent Literature 1 WO2008/072540

Patent Literature 2 Japanese Published Unexamined Patent Application No. 2001-532188

Non Patent Literature

[0016]

Non Patent Literature 1 *Nature* 383, 30 (1996)

Non Patent Literature 2 *Cell* 91, 501-510 (1997)

Non Patent Literature 3 *Nucleic Acids Res*, 31, 6873-6881 (2003)

Non Patent Literature 4 *Insect Mol.Biol.*5, 141-151 (1996)

Non Patent Literature 5 *Genetics.*166, 895-899 (2004)

Non Patent Literature 6 *PLoS Genet*, 2, e169 (2006)

Non Patent Literature 7 *Proc. Natl. Acad. Sci.* USA 95, 10769-10773 (1998)

Non Patent Literature 8 *Proc. Natl. Acad. Sci.* USA 98:6759-6764 (2001)

Non Patent Literature 9 *Nature* 436,221-22 6 (2005)

Non Patent Literature 10 *Nucleic Acids Res.*, 31, 6873-6881 (2003)

Non Patent Literature 11 *Nucleic Acids Res.*, 35, e87 (2007)

Non Patent Literature 12 *Proc Natl. Acad. Sci. USA*, 103, 15008-15013 (2006)

Summary of Invention

Technical Problem

[0017]

In order to produce and analyze a protein of interest, it is necessary to select a cell line which stably and highly expresses a protein of interest, using a mammalian-derived culture cell, but preparation and culture of the cell that produces the protein of interest require considerable labor and time.

[0018]

In addition, though it is known that a protein of interest is expressed in a mammalian cell using a transposon sequence, preparation of a cell which can highly express a protein of interest and thus can be used as a protein production system by using a transposon sequence; preparation method of a mammalian cell which can highly produce a protein of interest by using a transposon sequence; and a production method of a protein using the cell are not known.

[0019]

As described in the above, the expression of a protein of interest in a large amount by establishing a protein production system which can highly produce a protein of interest using a mammalian culture cell efficiently and within a short period has been required. Thus, the objects of the invention are to provide a cell capable of highly

expressing a protein of interest which can be efficiently established, and a method for producing the protein of interest using the cell.

Solution to Problems

[0020]

To solve the above-mentioned problems, the present inventors have conducted intensive studies and found as a result that a mammalian cell capable of highly expressing a protein of interest can be efficiently prepared by introducing a protein expression vector which comprises a gene fragment comprising a DNA encoding the protein of interest and a selectable marker gene and transposon sequences at both terminals of the gene fragment, into a suspension mammalian cell; and integrating the gene fragment inserted between a pair (two) of the transposon sequences into a chromosome of the mammalian cell. In addition, it was found that the protein of interest can be produced efficiently by using the cell, and thereby the invention was accomplished.

Detailed Description of the Invention

[0021]

Specifically, the invention is as follows:

1. A method for producing a protein of interest, comprising introducing a protein expression vector which comprises a gene fragment comprising a DNA encoding a protein of interest and a selectable marker gene and transposon sequences at both terminals of the gene fragment, into a suspension mammalian cell; integrating the gene fragment inserted between a pair of the transposon sequences into a chromosome of the mammalian cell to obtain a mammalian cell capable of expressing the protein of interest; and suspension-culturing the mammalian cell;

2. A method for producing a protein of interest, comprising the following steps (A) to (C):

(A) a step of simultaneously introducing the following expression vectors (a) and (b) into a suspension mammalian cell:

(a) an expression vector which comprises a gene fragment comprising a DNA encoding a protein of interest and transposon sequences at both terminals of the gene fragment,

(b) an expression vector which comprises a DNA encoding a transposase which recognizes the transposon sequences and has activity of transferring a gene fragment inserted between a pair of the transposon sequences into a chromosome,

(B) a step of expressing transiently the transposase from the expression vector introduced in the step (A) to integrate the gene fragment inserted between a pair of the transposon sequences into a chromosome of the mammalian cell to obtain a suspension mammalian cell capable of expressing the protein of interest, and

(C) a step of suspension-culturing the suspension mammalian cell capable of expressing the protein of interest obtained in the step (B) to produce the protein of interest;

3. A method for obtaining a suspension mammalian cell capable of expressing a protein of interest, comprising introducing a protein expression vector which comprises a gene fragment comprising a DNA encoding a protein of interest and a selectable marker gene and transposon sequences at both terminals of the gene fragment into a suspension mammalian cell; and integrating the gene fragment inserted between a pair of the transposon sequences, into a chromosome of the mammalian cell;

4. The method described in any one of the aforementioned items 1 to 3, wherein the suspension mammalian cell is a cell capable of surviving and proliferating in a serum-free medium;

5. The method described in any one of the aforementioned items 1 to 4, wherein the suspension mammalian cell is at least one selected from a suspension CHO cell in which a CHO cell is adapted to suspension culture, a PER.C6 cell, a rat myeloma cell YB2/3HL.P2.G11.16Ag.20 (or also called YB2/0) and a suspension mouse myeloma cell NS0 adapted to suspension culture;

6. The method described in the aforementioned item 5, wherein the CHO cell is at least one selected from CHO-K1, CHO-K1SV, DUKXB11, CHO/DG44, Pro-3 and CHO-S;

7. The method described in any one of the aforementioned items 1 to 6, wherein the selectable marker gene is a cycloheximide resistance gene;

8. The method described in the aforementioned item 7, wherein the cycloheximide resistance gene is a gene encoding a mutant of human ribosomal protein L36a;

9. The method described in the aforementioned item 8, wherein the mutant is a mutant in which proline at position 54 of the human ribosomal protein L36a is substituted with other amino acid;

10. The method described in the aforementioned item 9, wherein the other amino acid is glutamine;

11. The method described in any one of the aforementioned items 1 to 10, wherein a pair of the transposon sequences are nucleotide sequences derived from a pair of DNA-type transposons which function in a mammalian cell;

12. The method described in the aforementioned item 11, wherein the nucleotide sequences derived from a pair of DNA type transposons are nucleotide sequences derived from a pair of Tol1 transposons or nucleotide sequences derived from a pair of Tol2 transposons;

13. The method described in the aforementioned item 12, wherein the nucleotide sequences derived from a pair of Tol2 transposons are a nucleotide sequence comprising the nucleotide sequence shown in SEQ ID NO:2 and the nucleotide sequence shown in SEQ ID NO:3;

14. The method described in the aforementioned item 12, wherein the nucleotide sequences derived from a pair of Tol1 transposons are the nucleotide sequence shown in SEQ ID NO:14 and the nucleotide sequence shown in SEQ ID NO:15;

15. A suspension mammalian cell capable of producing a protein of interest, into which a protein expression vector comprising a gene fragment comprising a DNA encoding a protein of interest and a selectable marker gene and transposon sequences at both terminals of the gene fragment is introduced, to integrate the gene fragment inserted between a pair of the transposon sequences into a chromosome;

16. A suspension mammalian cell capable of producing a protein of interest, into which an expression vector (a) comprising a gene fragment comprising a DNA encoding a protein of interest and a selectable marker gene and transposon sequences at both terminals of the gene fragment, and an expression vector (b) comprising a DNA encoding a transposase (a transferase) which recognizes the transposon sequences and has activity of transferring the gene fragment inserted between a pair of the transposon sequences into a chromosome to integrate the gene fragment inserted between a pair of the transposon sequences into the chromosome;

17. The cell described in the aforementioned item 15 or 16, wherein the cell is a cell capable of surviving and proliferating in a serum-free medium;

18. The cell described in any one of the aforementioned items 15 to 17, wherein the cell is at least one suspension mammalian cell selected from a suspension CHO cell in which a CHO cell is adapted to suspension culture, a PER.C6 cell, a rat myeloma cell YB2/3HL.P2.G11.16Ag.20 (or also called YB2/0) and a suspension mouse myeloma cell NS0 adapted to suspension culture;

19. The cell described in the aforementioned item 18, wherein the CHO cell is at least one selected from CHO-K1, CHO-K1SV, DUKXB11, CHO/DG44, Pro-3 and CHO-S;

20. The cell described in any one of the aforementioned items 15 to 19, wherein the selectable marker gene is a cycloheximide resistance gene;

21. The cell described in the aforementioned item 20, wherein the cycloheximide resistance gene is a gene encoding a mutant of human ribosomal protein L36a;

22. The cell described in the aforementioned item 21, wherein the mutant is a mutant in which proline at position 54 of the human ribosomal protein L36a is substituted with other amino acid;

23. The cell described in the aforementioned item 22, wherein the other amino acid is glutamine;

24. The cell described in any one of the aforementioned items 15 to 23, wherein a pair of the transposon sequences are nucleotide sequences derived from a pair of DNA-type transposons which function in a mammalian cell;

25. The cell described in the aforementioned item 24, wherein the nucleotide sequences derived from a pair of the DNA-type transposons are nucleotide sequences derived from a pair of Tol1 transposons or nucleotide sequences derived from a pair of Tol2 transposons;

26. The cell described in the aforementioned item 25, wherein the nucleotide sequences derived from a pair of the Tol2 transposons are the nucleotide sequence shown in SEQ ID NO:2 and the nucleotide sequence shown in SEQ ID NO:3;

27. The cell described in the aforementioned item 25, wherein the nucleotide sequences derived from a pair of the Tol1 transposons are the nucleotide sequence shown in SEQ ID NO:14 and the nucleotide sequence shown in SEQ ID NO:15;

28. A protein expression vector, comprising a gene fragment comprising a DNA encoding a protein of interest and a selectable marker gene, and a pair of transposon sequences at both terminals of the gene fragment;

29. The protein expression vector described in the aforementioned item 28, wherein a pair of the transposon sequences are nucleotide sequences derived from a pair of Tol1 transposons or nucleotide sequences derived from a pair of Tol2 transposons.

30. The protein expression vector described in the aforementioned item 29, wherein the nucleotide sequences derived from a pair of theTol2 transposons are the nucleotide sequence shown in SEQ ID NO:2 and the nucleotide sequence shown in SEQ ID NO:3; and

31. The protein expression vector described in the aforementioned item 29, wherein the nucleotide sequences derived from a pair of the Tol1 transposons are the nucleotide sequence shown in SEQ ID NO:14 and the nucleotide sequence shown in SEQ ID NO:15.

Advantageous Effects of Invention

[0022]

According to the protein production method of the invention, a protein of interest can be efficiently produced by the use of a mammalian cell. In addition, the cell of the invention can be used as a protein production cell for producing a recombinant protein with a high efficiency.

Brief Description of the Drawings

[0023]

[Fig. 1] Fig. 1 shows a schematic illustration of a transposon vector for expressing an anti-human influenza M2 antibody. Tol2-L represents a left end Tol2 transposon (SEQ ID NO:2), Tol2-R represents a right end Tol2 transposon (SEQ ID NO:3), CMV represents a CMV promoter, poly A represents a polyadenylation site, Hc represents a human antibody H chain cDNA, Lc represents a human antibody L chain cDNA, and CHX-r represents a cycloheximide resistance gene.

[Fig. 2] Fig. 2 shows a schematic illustration of an anti-human influenza M2 antibody expression vector. CMV represents a CMV promoter, poly A represents a polyadenylation site, Hc represents a human antibody H chain cDNA, Lc represents a human antibody L chain cDNA and CHX-r represents a cycloheximide resistance gene.

[Fig. 3] Fig. 3 shows a schematic illustration of a Tol2 transposase expression vector. CAGGS represents a CAGGS promoter, poly A represents a polyadenylation site, and TPase cDNA represents a Tol2 transposase cDNA.

[Fig. 4A] Fig. 4A shows a result of examining expression level of an anti-human influenza M2 antibody in a suspension CHO-K1 cell when a Tol2 transposon vector for expressing an anti-human influenza M2 antibody was used. The ordinate shows the amount of antibody production (μg/ml), and the abscissa shows the number of transgenic clones of the suspension CHO-K1 cell.

[Fig. 4B] Fig. 4B shows a result of examining expression level of an anti-human influenza M2 antibody in an adhesive CHO-K1 cell when a Tol2 transposon vector for expressing an anti-human influenza M2 antibody was used. The ordinate shows the amount of antibody production (μg/ml), and the abscissa shows the number of transgenic clones of the adhesive CHO-K1 cell.

[Fig. 5] Fig. 5 shows a schematic illustration of a Tol1 transposon vector for expressing an anti-human influenza M2 antibody. Tol1-L represents a left end Tol1 transposon (SEQ ID NO:14), Tol1-R represents a right end Tol1 transposon (SEQ ID NO:15), CMV represents a CMV promoter, poly A represents a polyadenylation site, Hc represents a human antibody H chain cDNA, Lc represents a human antibody L chain cDNA, and CHX-r represents a cycloheximide resistance gene.

[Fig. 6] Fig. 6 shows a schematic illustration of a Tol1 transposase expression vector. CAGGS represents a CAGGS promoter, poly A represents a polyadenylation site, and TPase cDNA represents a Tol1 transposase cDNA.

[Fig. 7] Fig. 7 shows a result of examining expression level of an anti-human influenza M2 antibody in a suspension CHO-K1 cell when a Tol1 transposon vector for expressing an anti-human influenza M2 antibody was used. The ordinate shows the amount of antibody production (μg/ml), and the abscissa shows the number of transgenic clones of the suspension CHO-K1 cell.

[0024]

This invention relates to a method for producing a protein of interest, comprising introducing a protein expression vector comprising a gene fragment comprising a DNA encoding a protein of interest and a selectable marker gene and transposon sequences at both terminals of the gene fragment, into a suspension mammalian cell; integrating the gene fragment inserted between a pair (two) of the transposon sequences, into a chromosome of the mammalian cell to obtain a mammalian cell capable of expressing said protein of interest; and suspension-culturing the mammalian cell.

[0025]

Examples of the method for producing a protein of interest of the present invention include a method, comprising the following steps (A) to (C):

(A) a step of simultaneously introducing the following expression vectors (a) and (b) into a suspension mammalian cell:

(a) an expression vector which comprises a gene fragment comprising a DNA encoding a protein of interest and transposon sequences at both terminals of the gene fragment,

(b) an expression vector which comprises a DNA encoding a transposase which recognizes the transposon sequences and has activity of transferring a gene fragment inserted between a pair of the transposon sequences into a chromosome,

(B) a step of expressing transiently the transposase transiently from the expression vector introduced in the step (A) to integrate the gene fragment inserted between a pair

of the transposon sequences into a chromosome of the mammalian cell to obtain a suspension mammalian cell capable of expressing the protein of interest, and

(C) a step of suspension-culturing the suspension mammalian cell capable of expressing the protein of interest obtained in the step (B)to produce the protein of interest.

[0026]

In addition, the present invention relates to a suspension mammalian cell capable of producing a protein of interest, into which a protein expression vector comprising a gene fragment comprising a DNA encoding a protein of interest and a selectable marker gene and transposon sequences at both terminals of the gene fragment is introduced, to integrate the gene fragment inserted between a pair of the transposon sequences into a chromosome.

[0027]

Furthermore, the present invention relates to a suspension mammalian cell capable of producing a protein of interest, into which an expression vector (a) comprising a gene fragment comprising a DNA encoding a protein of interest and a selectable marker gene and transposon sequences at both terminals of the gene fragment, and an expression vector (b) comprising a DNA encoding a transposase (a transferase) which recognizes the transposon sequences and has activity of transferring the gene fragment inserted between a pair of the transposon sequences into a chromosome to integrate the gene fragment inserted between a pair of the transposon sequences into the chromosome.

[0028]

The term "transposon" in the present specification is a transposable genetic element and means a gene unit which moves on a chromosome or from a chromosome to other chromosome (transposition) while keeping a certain structure.

[0029]

The transposon comprises a gene unit of a repeating transposon sequences (also called inverted repeat sequence (IR sequence) or terminal inverted repeat sequence (TIR sequence)) which positions in the same direction or the reverse direction at both terminals of the gene unit and a nucleotide sequence encoding a transposase which recognizes the transposon sequence to transfer a gene existing between the transposon sequences.

[0030]

The transposase translated from the transposon can transfer a DNA by recognizing transposon sequences of both terminals of the transposon, cutting out the

DNA fragment inserted between a pair of the transposon sequences and inserting the fragment into the site to be transferred.

[0031]

The term "transposon sequence" in the present specification means the nucleotide sequence of a transposon recognized by a transposase and has the same meaning as the IR sequence or TIR sequence. A DNA comprising the nucleotide sequence may comprise an imperfect repeating moiety as long as it can be transferred (inserted into other position in the genome) by the activity of a transposase, and comprise a transposon sequence specific to the transposase.

[0032]

As the transposon sequence to be used in the invention, a nucleotide sequence derived from a DNA-type transposon is preferable, and a nucleotide sequence derived from a pair of natural or artificial DNA-type transposons, which can be recognized by a transposase and be transposed in mammalian cells, is more preferable.

[0033]

Examples of the nucleotide sequence derived from a DNA-type transposon include the nucleotide sequences derived from the medaka fish-derived Tol1 transposon and Tol2 transposon, the Sleeping Beauty reconstructed from a non-autonomous transposon existed in an *Onchorhynchus* fish genome, the frog-derived artificial transposon Frog prince and the insect-derived transposon PiggyBac.

[0034]

Particularly, among them, the nucleotide sequences derived from the medaka fish-derived Tol2 transposon comprising the nucleotide sequence shown in SEQ ID NO:6 and the medaka fish-derived Tol2 transposon comprising the nucleotide sequence shown in SEQ ID NO:13 are preferable.

[0035]

Examples of the nucleotide sequence derived from a pair of Tol2 transposons include the nucleotide sequence at positions 1 to 2229 and the nucleotide sequence at positions 4148 to 4682 in the Tol2 transposon nucleotide sequence shown in SEQ ID NO:6 of Sequence Listing.

[0036]

As the nucleotide sequence derived from a pair of Tol2 transposons, the nucleotide sequence at positions 1 to 200 (SEQ ID NO:2) (hereinafter referred to as "Tol2-L sequence") and the nucleotide sequence at positions 2285 to 2788 (SEQ ID NO:3) (hereinafter referred to as "Tol2-R sequence") in the Tol2 transposon nucleotide sequence shown in SEQ ID NO:1 of Sequence Listing are more preferable.

[0037]

Examples of the nucleotide sequence derived from a pair of Tol1 transposons include the nucleotide sequence comprising a nucleotide sequence at positions 1 to 157 and the nucleotide sequence at positions the 1748 to 1855 in the Tol1 transposon nucleotide sequence shown in SEQ ID NO:13 of Sequence Listing.

[0038]

As the nucleotide sequence derived from a pair of Tol1 transposons, the nucleotide sequence at positions 1 to 200 (SEQ ID NO:14) (hereinafter referred to as "Tol1-L sequence") and the nucleotide sequence at positions 1351 to 1855 (SEQ ID NO:15) (hereinafter referred to as "Tol1-R sequence") in the Tol2 transposon nucleotide sequence shown in SEQ ID NO:1 of Sequence Listing are more preferable.

[0039]

Examples of the transposon sequence to be used in the invention include transposon sequences of which transfer reactions are controlled by using a partial sequence of a transposon sequence derived from the above-mentioned transposon, by adjusting the length of the nucleotide sequence and by modifying the nucleotide sequence due to addition, deletion or substitution.

[0040]

Regarding the control of the transfer reaction of a transposon, the transfer reaction can be accelerated or suppressed by accelerating or suppressing recognition of the transposon sequence by a transposase, respectively.

[0041]

The term "transposase" in the present specification means an enzyme which recognizes nucleotide sequences having transposon sequences and transfers a DNA existing between the nucleotide sequences into a chromosome or from the chromosome to other chromosome.

[0042]

Examples of the transposase include the Tol1 and Tol2 which are derived from medaka fish, the Sleeping Beauty reconstructed from a non-autonomous transposon existed in an *Onchorhynchus* fish genome, the artificial transposon Frog prince which is derived from frog and the transposon PiggyBac which is derived from insect.

[0043]

As the transposase, a native enzyme may be used, and any transposase in which a part of its amino acids are substituted, deleted, inserted and/or added may be used as long as the same transfer activity as the transposase is maintained. By controlling the

enzyme activity of the transposase, the transfer reaction of the DNA existing between the transposon sequences can be controlled.

[0044]

In order to analyze whether or not it possesses a transfer activity similar to that of transposase, it can be measured by the 2-components analyzing system disclosed in Japanese Published Unexamined Patent Application No.235575/2003.

[0045]

Illustratively, whether or not a non-automatic Tol2 element can be transferred and inserted into a mammalian cell chromosome by the activity of a transposase can be analyzed by separately using a plasmid comprising a Tol2 transposase-deleted Tol2 transposon (Tol2-derived non-autonomous transposon) and a plasmid comprising Tol2 transposase.

[0046]

The term "non-autonomous transposon" in the present specification means a transposon which is lost a transposase existed inside the transposon and cannot therefore perform its autonomous transfer. The non-autonomous transposon can transfer the DNA inserted between transposon sequences of the non-autonomous transposon into the host cell chromosome, by allowing a transposase protein, an mRNA encoding the transposase protein or a DNA encoding the transposase protein to simultaneously present in the cell.

[0047]

The transposase gene means a gene encoding a transposase. In order to improve its expression efficiency in a mammalian cell, a sequence which adjusts a space between the Kozak's consensus sequence (Kozak M., *Nucleic Acids Res.*, 12, 857 - 872 (1984)) or a ribosome binding sequence, Shine-Dalgarno sequence and the initiation codon, to an appropriate distance (e.g., from 6 to 18 bases) may be connected to an upstream site of the translation initiation codon ATG of the gene.

[0048]

According to the method of the invention, in order to integrate a gene fragment comprising a DNA encoding the protein of interest and a selectable marker gene in an expression vector into the chromosome of a host cell, an expression vector which comprises the gene fragment comprising a DNA encoding the protein of interest and a selectable marker gene and transposon sequences at both terminals of the gene fragment is introduced into the host cell, and a transposase is allowed to act upon the transposon sequences comprised in the expression vector which is introduced into the cell.

[0049]

In order to allow a transposase to act upon the transposon sequences comprised in the expression vector which is introduced into the cell, the transposase may be injected into the cell, or an expression vector comprising a DNA encoding the transposase may be introduced into the host cell together with an expression vector comprising a DNA encoding the protein of interest and a selectable marker gene. In addition, by introducing an RNA encoding a transposase gene into the host cell, the transposase may be expressed in the cell.

[0050]

The expression vector is not particularly limited. Any expression vector can be used by optionally selecting from the expression vectors known to those skilled in the art, depending on a host cell into which an expression vector comprising a transposase gene is introduced; the use; and the like.

[0051]

In order that a protein constituted from two or more polypeptides is produced by the method of the invention, the DNA can be integrated into the chromosome of the cell by integrating a DNA encoding the two or more polypeptides into the same or different expression vectors and then introducing the expression vectors into a host cell.

[0052]

The transposase may be inserted into an expression vector to express together with the protein of interest or may be inserted into a vector different from the expression vector. The transposase may be allowed to act transiently or may be allowed to act continuously, but it is preferably to allow the transposase to act transiently in order to prepare a cell for stable production.

[0053]

As the method for allowing the transposase to act transiently, examples include a method comprising preparing an expression vector which comprises a DNA encoding the transposase and an expression vector comprising a DNA encoding a protein of interest and then introducing both of the expression plasmids simultaneously into a host cell.

[0054]

The term "expression vector" in the present specification means an expression vector to be used for introducing a mammalian cell in order to express a protein of interest. The expression vector used in the invention has a structure in which at least a pair of transposon sequences is present at both sides of an expression cassette.

[0055]

The term "expression cassette" in the present specification means a nucleotide sequence which has a gene expression controlling region necessary for expressing a protein of interest and a sequence encoding the protein of interest. Examples of the gene expression controlling region include an enhancer, a promoter, and a terminator. the expression cassette may contain a selectable marker gene.

[0056]

Any promoter can be used, so long as it can function in an animal cell. Examples include a promoter of IE (immediate early) gene of cytomegalovirus (CMV), SV40 early promoter, a promoter of retrovirus, a metallothionein promoter, a heat shock promoter, SRα promoter, moloney murine leukemia virus, an enhancer and the like. Also, the enhancer of the IE gene of human CMV can be used together with the promoter.

[0057]

The "selectable marker gene" means an arbital other marker gene which can be used for distinguishing a cell to which a plasmid vector is introduced from a cell lacking of the vector.

[0058]

Examples of the selectable marker gene include a drug resistance gene (a neomycin resistance gene, a DHFR gene, a puromycin resistance gene, a blasticidin resistance gene, a hygromycin resistance gene, and a cycloheximide resistance gene (Japanese Published Unexamined Patent Application No.262879/2002)), fluorescence and bio-luminescence marker genes (such as green fluorescent protein GFP) and the like.

[0059]

In the invention, preferable selectable marker is a drug resistance gene and particularly preferable selectable marker is a cycloheximide resistance gene. In addition, by carrying out a gene modification of the selectable marker gene, drug resistance performance and luminescence performance of the selectable marker protein can also be modified.

[0060]

Cycloheximide (hereinafter sometimes referred to as CHX) is a protein synthesis inhibitor, and as examples of the use of the CHX resistance gene as a selectable marker gene, the cases of yeast (Kondo K. *J. Bacteriol.*, 177, 24, 7171 - 7177 (1995)) and animal cells (Japanese Published Unexamined Patent Application No.262879/2002) are known.

[0061]

In the case of the animal cells, it has been found that the resistance to cycloheximide is provided by a transformant which expresses a protein encoded by the nucleotide sequence shown in SEQ ID NO:7 of Sequence Listing in which proline at position 54 in human ribosomal protein subunit L36a encoded by the nucleotide sequence shown in SEQ ID NO:5 of Sequence Listing is substituted with glutamine.

[0062]

The method for introducing the above-mentioned protein expression vector comprising a transposon sequence, a transposase expressing plasmid vector and RNA is not particularly limited. Examples include calcium phosphate transfection, electroporation, a liposome method, a gene gun method, lipofection and the like.

[0063]

Examples of the method for directly introducing a transposase in the form of a protein include by microinjection or endocytosis for supplying into a cell. The gene transfer can be carried out by the method described in *Shin Idenshi Kogaku Handbook* (New Genetic Engineering Handbook), edited by Masami Muramatsu and Tadashi Yamamoto, published by Yodo-sha, ISBN 9784897063737.

[0064]

The host cell may be any mammalian cell as long as it can be subcultured and stably express a protein of interest. Examples of the host cell include PER.C6 cell, human leukemia cell Namalwa cell, monkey cell COS cell, rat myeloma cell YB2/3HL.P2.G11.16Ag.20 (also referred to as YB2/0), mouse myeloma cell NS0, mouse myeloma cell SP2/0-Ag14, Syrian hamster cell BHK, HBT5637 (Japanese Unexamined Patent Application Publication No. 1998-000299), Chinese hamster ovarian cell CHO cell (*Journal of Experimental Medicine*, 108, 945 (1958); *Proc. Natl. Acad. Sci. USA.*, 601275 (1968); *Genetics*, 55, 513 (1968); *Chromosoma*, 41, 129 (1973); *Methods in Cell Science*, 18, 115 (1996); *Radiation Research*, 148, 260 (1997); *Proc. Natl. Acad. Sci. USA.*, 77, 4216 (1980); *Proc. Natl. Acad. Sci.*, 60, 1275 (1968); *Cell*, 6, 121 (1975); *Molecular Cell Genetics, Appendix I,II* (pp. 883-900)), CHO/DG44, CHO-K1 (ATCC CCL-61), DUKXB11 (ATCC CCL-9096), Pro-5 (ATCC CCL-1781), CHO-S (Life Technologies, Cat #11619), Pro-3 and substrain of CHO cell.

[0065]

In addition, the above-mentioned host cell can also be used in the protein production method of the invention by modifying it so as to be suitable for the protein production, by modification of chromosomal DNA, introduction of an exogeneous gene, and the like.

[0066]

Further, in order to control the sugar chain structure bound to a protein of interest to be produced, Lec13 which acquired lectin resistance [*Somatic Cell and Molecular Genetics*, 12, 55 (1986)] and CHO cell from which α1,6-fucosyltransferase gene is deleted (WO2005/35586, WO2002/31140) can also be used as the host cell.

[0067]

The protein of interest may be any protein so long as it can be expressed by the method of the invention. Specifically, examples include a human serum protein, a peptide hormone, a growth factor, a cytokine, a blood coagulation factor, a fibrinolysis system protein, an antibody and partial fragments of various proteins, and the like.

[0068]

Preferable examples of the protein of interest include a monoclonal antibody such as a chimeric antibody, a humanized antibody and a human antibody; Fc fusion protein; and albumin-bound protein; and a fragment thereof.

[0069]

An effector activity of a monoclonal antibody obtained by the method of the present invention can be controlled by various methods. For example, known methods are a method for controlling an amount of fucose (hereinafter, referred to also as "core fucose") which is bound N-acetylglucosamine (GlcNAc) through α-1,6 bond in a reducing end of a complex type N-linked sugar chain which is bound to asparagine (Asn) at position 297 of an Fc region of an antibody (WO2005/035586, WO2002/31140, and WO00/61739), a method for controlling an effector activity of a monoclonal antibody by modifying amino acid group(s) of an Fc region of the antibody, and the like. The effector activity of the monoclonal antibody produced by the method of the present invention can be controlled by using any of the methods.

[0070]

The "effector activity" means an antibody-dependent activity which is induced via an Fc region of an antibody. As the effector activity, an antibody-dependent cellular cytotoxicity (ADCC activity), a complement-dependent cytotoxicity (CDC activity), an antibody-dependent phagocytosis (ADP activity) by phagocytic cells such as macrophages or dendritic cells, and the like are known.

[0071]

In addition, by controlling a content of core fucose of a complex type N-linked sugar chain of Fc region of a monoclonal antibody, an effector activity of the antibody can be increased or decreased.

[0072]

As a method for lowering a content of fucose which is bound to a complex type N-linked sugar chain bound to Fc of the antibody, an antibody to which fucose is not bound can be obtained by the expression of an antibody using a CHO cell which is deficient in a gene encoding α1,6-fucosyltransferase. The antibody to which fucose is not bound has a high ADCC activity.

[0073]

On the other hand, as a method for increasing a content of fucose which is bound to a complex type N-linked sugar chain bound to Fc of an antibody, an antibody to which fucose is bound can be obtained by the expression of an antibody using a host cell into which a gene encoding α1,6-fucosyltransferase is introduced. The antibody to which fucose is bound has a lower ADCC activity than the antibody to which fucose is not bound.

[0074]

Further, by modifying amino acid residue(s) in an Fc region of an antibody, the ADCC activity or CDC activity can be increased or decreased. For example, the CDC activity of an antibody can be increased by using the amino acid sequence of the Fc region described in US2007/0148165.

[0075]

Further, the ADCC activity or CDC activity of an antibody can be increased or decreased by modifying the amino acid as described in US Patent Nos. 6,737,056, or 7,297,775 or 7,317,091.

[0076]

The term "suspension mammalian cell" in the present invention means a cell which does not adhere to a cell culture anchorage coated for facilitating adhesion of culture cells, such as microbeads, a culture container for tissue culture (also referred to as a tissue culture or adhesion culture container and the like) and the like, and can survive and grow by suspending in the culture liquid.

[0077]

When the cell does not adhere to the cell culture anchorage, it may survive and grow under a state of a single cell in the culture liquid or survive and grow under a state of a cell mass formed by the agglutination of two or more cells.

[0078]

In addition, as the suspension mammalian cell to be used in the present invention, a cell which can survive and grow in a serum-free medium that does not contain fetal calf serum (hereinafter referred to as FCS) and the like, while suspending in the culture liquid without adhering to the cell culture anchorage, is preferable, and a

mammalian cell which can survive and grow while suspending in a protein-free medium that does not contain protein is more preferable.

[0079]

As the culture container for tissue culture, it may be any culture container such as a flask, a Petri dish and the like, so long as coating for adhesion culture is applied thereto. Specifically, for example, whether or not it is a suspension mammalian cell can be confirmed by the use of commercially available tissue culture flask (manufactured by Greiner), adhesion culture flask (manufactured by Sumitomo Bakelite) and the like.

[0080]

As the suspension mammalian cell to be used in the present invention, it may be either a cell prepared by further adapting a cell originally having a suspension property to suspension culture or a suspension mammalian cell prepared by adapting an adhesive mammalian cell to suspension culture conditions.

[0081]

Examples of the cell originally having a suspension property include PER.C6 cell, a rat myeloma cell YB2/3HL.P2.G11.16Ag.20 (or also called YB2/0), CHO-S cell (manufactured by Invitrogen) and the like.

[0082]

The aforementioned "suspension mammalian cell prepared by adapting an adhesive mammalian cell to suspension culture conditions" can be prepared by the method described in *Mol. Biotechnol.*, 2000, 15(3), 249 - 57 or by the method shown in the following, and can be prepared by establishing a cell which shows proliferation property and surviving property similar to those before the suspension culture adaptation or superior to those before adapting to suspension culture (*J. Biotechnol.*, 2007, 130(3), 282 - 90).

[0083]

The term "similar to those before the suspension culture adaptation" means that survival ratio, proliferation rate (doubling time) and the like of the cell adapted to the suspension culture are substantially the same as those of the cell before adapting suspension culture.

[0084]

Examples of the method for adapting an adhesive mammalian cell to suspension culture conditions according to the present invention include the following method. The serum content of a serum-containing medium is reduced to 1/10 and sub-culturing is repeated at relatively high concentration of cell. When the mammalian cell

comes to be able to survive and proliferate, the serum content is further reduced and the sub-culturing is repeated. By this method, a suspension mammalian cell which can survive and proliferate under serum-free conditions can be prepared.

[0085]

In addition, a suspension mammalian cell can also be prepared by a method comprising culturing with the addition of an appropriate nonionic surfactant such as Pluronic-F68 or the like in the culture liquid.

[0086]

Examples of the adhesive mammalian cell which acquires suspension property by adapting to a suspension culture condition include a mouse myeloma cell NS0, a CHO cell and the like.

[0087]

In the present invention, as a property possessed by the suspension mammalian cell, when 2×10^5 cells/ml of the cell is suspension-cultured, the cell concentration after culturing for 3 or 4 days is preferably 5×10^5 cells/ml or more, more preferably 8×10^5 cells/ml or more, particularly preferably 1×10^6 cells/ml or more, most preferably 1.5×10^6 cells/ml or more.

[0088]

In addition, doubling time of the suspension mammalian cell of the present invention is preferably 48 hours or less, more preferably 24 hours or less, particularly preferably 18 hours or less, most preferably 11 hours or less.

[0089]

Examples of the medium for suspension culturing include commercially available media, such as CD-CHO medium (manufactured by Invitrogen), EX-CELL 325-PF medium (manufactured by SAFC Biosciences), SFM4CHO medium (manufactured by HyClone) and the like. In addition, it can also be obtained by mixing saccharides, amino and the like acids which are necessary for the culturing of mammalian cells.

[0090]

The suspension mammalian cell can be cultured using a culture container which can be used for suspension culturing under a culture condition capable of suspension culturing. Examples of the culture container include a 96 well plate for cell culture (manufactured by Corning), a T-flask (manufactured by Becton Dickinson), an Erlenmeyer flask (manufactured by Corning) and the like.

[0091]

Regarding the culture conditions, for example, it can be statically cultured in an atmosphere of 5% CO_2 at a culture temperature of 37°C. A shaking culture equipment, such as culturing equipment for suspension culture exclusive use, Wave Bioreactor (manufactured by GE Healthcare Bioscience), can also be used.

[0092]

Regarding the suspension culture conditions of a suspension mammalian cell using the Wave Bioreactor equipment, the cell can be cultured by the method described on the GE Healthcare Bioscience homepage http://www.gelifesciences.co.jp/tech-support/manual/pdf/cellcult/wave-03-16.pdf.

[0093]

In addition to the shaking culture, culturing by a rotation agitation equipment such as a bioreactor, can also be used. Culturing using a bioreactor can be carried out by the method described in *Cytotechnology*, (2006) 52: 199 - 207, and the like.

[0094]

In the present invention, when a cell line other than the suspension mammalian cells is used, any cell line can be used so long as it is a mammalian cell line adapted to the suspension culture by the above-mentioned method and is a cell line which can be used in the protein producing method of the present invention.

[0095]

Purification of the protein of interest produced by the suspension mammalian cell is carried out by separating the protein of interest from impurities other than the protein of interest in a culture liquid or cell homogenate containing the protein of interest. Examples of the separation method include centrifugation, dialysis, ammonium sulfate precipitation, column chromatography, a filter and the like. The separation can be carried out based on the difference in physicochemical properties of the protein of interest and impurities and based on the difference in their affinity for the column carrier.

[0096]

The method for purifying the protein of interest can be carried out, for example, by the method described in *Protein Experimentation Note* (the first volume) - *Extraction, Separation and Expression of Recombinant Protein* (translation of a textbook written in Japanese) (edited by Masato Okada and Kaori Miyazaki, published by Yodo-sha, ISBN 9784897069180).

[0097]

The entire contents of the references, such as the scientific documents, patents, patent applications cited herein are incorporated herein by reference to the same degree of those illustratively described, respectively.

[0098]

The present invention has been described in the foregoing by showing preferred embodiments thereof for the sake of easy understanding. Hereinafter, the present invention is further described specifically based on examples, but the above-mentioned explanations and the following examples are provided merely for the purpose of exemplifications and not provided for the purpose of limiting the invention. Accordingly, the scope of the invention is not limited to the embodiments and examples which are specifically described herein, but is limited by the claims alone.

[0099]

Various experimental techniques relating to genetic recombination described hereinafter, such as the cloning and the like were carried out in accordance with the genetic engineering techniques described in *Molecular Cloning* 2nd edition edited by J. Sambrook, E. F. Frisch and T. Maniatis, *Current Protocols in Molecular Biology* edited by Frederick M. Ausubel *et al*, published by Current Protocols, and the like.

EXAMPLES

[0100]

[Example 1]

Preparation of transposon vector for expressing anti-human influenza M2 antibody

A plasmid which contains a gene expression cassette for mammalian cells comprising an arbitrary human antibody gene and a drug resistance marker gene inserted between a pair of Tol2 transposon sequences was used as a plasmid vector for protein expression.

[0101]

Each DNA of the used genes was chemically and artificially synthesized based on a known nucleotide sequence or obtained by preparing primers for its both terminal sequences and then carrying out PCR using an appropriate DNA source as a template. In order to carry out the gene manipulation later, a restriction site for a restriction enzyme was added to the terminal of the primer.

[0102]

Among the nucleotide sequence of the non-autonomous Tol2 transposone disclosed by Japanese Published Unexamined Patent Application No.235575/2003 (SEQ ID NO:1), the nucleotide sequence at position 1 to 200 (Tol2-L sequence) (SEQ

ID NO:2) and the nucleotide sequence at positions 2285to 2788 (Tol2-R sequence) (SEQ ID NO:3) were used as the transposon sequences.

[0103]

Each synthetic DNA fragments comprising a pair of transposon sequences (manufactured by TAKARA BIO INC.) was prepared by the following method. A DNA fragment comprising a nucleotide sequence in which a recognition sequence of a restriction enzyme *Nru*I was attached to both of the 5'-terminal and 3'-terminal of the Tol2-R sequence was prepared. Then, a DNA fragment comprising a nucleotide sequence in which a recognition sequence of a restriction enzyme *Fse*I was attached to the 5'-terminal of the Tol2-L sequence and a restriction enzyme *Asc*I was attached to the 3'-terminal thereof was prepared.

[0104]

Next, the thus prepared DNA fragments comprising Tol2-R sequence and Tol2-L sequence were inserted into an expression vector N5LG1-M2-Z3 vector (WO2006/061723) comprising a nucleotide sequence encoding an amino acid sequence of anti-human influenza M2 antibody Z3G1.

[0105]

The N5LG1-M2-Z3 vector (WO2006/061723) into which a nucleotide sequence (SEQ ID NO:8) encoding the H chain of the anti-human influenza M2 antibody Z3G1 (ATCC Deposit No. PTA-5968: deposited March 13, 2004, American Type Culture Collection, Manassas, VA, USA) and a nucleotide sequence (SEQ ID NO:10 and SEQ ID NO:11) encoding the L chain (SEQ ID NO:9) of the same were inserted under the control of the CMV enhancer/promoter control was used as an antibody gene expression cassette.

[0106]

The DNA fragment comprising the Tol2-R sequence was inserted into the restriction enzyme *Nru*I site of the N5LG1-M2-Z3 vector, at the 5'-terminal side of a gene fragment comprising the antibody gene expression cassette and a resistance marker gene. Then, the DNA fragment comprising the Tol2-L sequence was inserted into the restriction enzyme *Fse*I and *Asc*I sites at the 3'-terminal side.

[0107]

In addition, a transposon vector for expressing an anti-human influenza M2 antibody was constructed (Fig. 1) by inserting a cycloheximide resistance gene expression cassette connected with a nucleotide sequence (SEQ ID NO:5) encoding a resistance gene for cycloheximide (a gene in which proline at position 54 of the human ribosomal protein L36a was substituted with glutamine) into the *Fse*I recognition site of

the N5LG1-M2-Z3 vector connected with the Tol2 transposon sequence, under the control of the CMV enhancer/promoter.

[0108]

On the other hand, a vector containing no transposon sequences was named anti-human influenza M2 antibody expression vector and used as the control vector (Fig. 2).

[0109]

[Example 2]

Preparation of transposase expression vector

The transposase was expressed using an expression vector independent of the expression vector of the antibody of interest. That is, a gene which is encoding a medaka fish-derived Tol2 transposase (SEQ ID NO:4) was inserted into a downstream of the CAGGS promoter of a pCAGGS vector (*Gene*, 108, 193 - 200, 1991) and used as the expression vector (Fig. 3).

[0110]

[Example 3]

(1) Preparation of suspension CHO cell

An adhesive CHO cell which had been cultured using an α-MEM medium (manufactured by Invitrogen) containing 10% serum (FCS) was peeled off and recovered by a trypsin treatment and shaking-cultured at 37°C in a 5% CO_2 incubator using fresh α-MEM medium containing 10% FCS. Several days thereafter, growth of these cells was confirmed and then shaking culture was carried out by seeding them into a α-MEM medium containing 5% FCS at a concentration of 2×10^5 cells/ml.

[0111]

Further several days thereafter, the inoculation was similarly carried out using the α-MEM medium containing 5% FCS. Finally, a cell adapted to the suspension culture was prepared by repeating the sub-culture and shaking culture using serum-free α-MEM medium and confirming that the cells have the same growing ability of the case of their culturing in the presence of serum.

[0112]

(2) Preparation of antibody-producing CHO cell

The transposon vector for expressing the anti-human influenza M2 antibody prepared in Example 1 and Example 2 (hereinafter referred to as transposon vector) and Tol2 transposase expression vector pCAGGS-T2TP (Fig. 3, Kawakami K. & Noda T., *Genetics*, 166, 895 - 899 (2004)) were used as the expression vectors. In addition, the

anti-human influenza M2 antibody expression vector having no transposon sequences was used as the control.

[0113]

By introducing the aforementioned expression vectors into the suspension culture-adapted CHO-K1 cell (American Type Culture Collection Cat. No. CCL-61) or HEK293 cell (FreeStyle 293F cell, manufactured by Invitrogen), the frequencies of obtaining cycloheximide-resistant clones were compared.

[0114]

Each cells (4×10^6 cells) was suspended in 400 μl of PBS, and the transposon vector for expressing the anti-human influenza M2 antibody (10 μg) and Tol2 transposase expression vector (25 μg) were co-transfected directly in the form of circular DNA by electroporation. In this connection, in order to express the Tol2 transposase transiently, the Tol2 transposase expression vector was directly introduced in the form of circular DNA for the purpose of preventing from integrateing into the host chromosome.

[0115]

In addition, as the control, the anti-human influenza M2 antibody expression vector (10 μg) was linearized by a restriction enzyme and then introduced into each cells, in accordance with the standard gene transfer method by electroporation.

[0116]

The electroporation was carried out using a cuvette of 4 mm in gap width (manufactured by Bio-Rad), using an electroporator (Gene Pulser Xcell System (manufactured by Bio-Rad)) under conditions of 300 V in voltage, 500 μF in electrostatic capacity and room temperature.

[0117]

After the transfection by electroporation, each cell was seeded into three 96-well plates and cultured in a CO_2 incubator for 3 days using the EX-CELL 325-PF medium manufactured by SAFC Biosciences for the CHO cell, and the FreeStyle-293 medium (manufactured by Invitrogen) for the HEK293 cell.

[0118]

Next, from the day of medium exchange on the 4th day of the transfection, 3 μg/ml of cycloheximide was added to the medium so that the cells were cultured in the presence of cycloheximide, followed by culturing for 3 weeks while carrying out the medium exchange in every week.

[0119]

After culturing for 3 weeks, the number of wells in which cycloheximide-resistant colonies were found was counted. The results are shown in Table 1 and Table 2.

[0120]

[Table 1]

Table 1 Comparison of the numbers of cycloheximide-resistant cells (CHO cell)

	Transposon vector	Conventional vector
Test 1	155 / 288	0 / 288
Test 2	100 / 288	0 / 288
Test 3	94 / 288	0 / 288

[0120]

[Table 2]

Table 2 Comparison of the numbers of cycloheximide-resistant cells (HEK293 cell)

	Transposon vector	Conventional vector
Test 1	0 / 288	0 / 288
Test 2	0 / 288	0 / 288
Test 3	0 / 288	0 / 288

[0122]

As shown in Table 1, each the anti-human influenza M2 antibody expression transposon vector or anti-human influenza M2 antibody expression vector was introduced into the suspension CHO-K1 cell. As a result, cycloheximide-resistant transformants were not obtained from the cell introduced with anti-human influenza M2 antibody expression vector like the case of other cell lines, but cycloheximide-resistant transformants were obtained from the cell introduced with transposon vector for expressing anti-human influenza M2 antibody with a high frequency.

[0123]

On the other hand, as shown in Table 2, cycloheximide-resistant transformants were not obtained when either of the transposon vector for expressing anti-human influenza M2 antibody and anti-human influenza M2 antibody expression vector was introduced into the HEK293 cell.

[0124]

Based on these results, it was found that the intended protein-encoded gene and cycloheximide resistance gene which were inserted between a pair of transposon

sequences are efficiently introduced into the chromosome of the host cell, namely a suspension mammalian cell.

[0125]

(3) Examination on the antibody production by suspension CHO cell and adhesive CHO cell

In order to examine antibody production efficiency by a suspension CHO cell or an adhesive CHO cell, the amounts of antibodies produced by respective cell lines were examined. As the suspension CHO cell, the suspension CHO-K1 cell adapted to suspension culture was used. In addition, as the adhesive CHO cell, the adhesive CHO-K1 cell before adaptation to suspension culture was used.

[0126]

The anti-human influenza M2 antibody expression transposon vector (10 μg) and Tol2 transposase expression vector (25 μg) were introduced into the suspension CHO-K1 cell and adhesive CHO-K1 cell by means of electroporation, respectively. Thereafter, the suspension CHO-K1 cell and the adhesive CHO-K1 cell were seeded into three 96-well plates for each cell.

[0127]

A medium for suspension cells (EX-CELL 325-PF, manufactured by SAFC Biosciences) was used for the suspension CHO-K1 cell, and the α-MEM medium containing 10% serum was used for the adhesive CHO-K1 cell. Each cell was cultured in a CO_2 incubator for 3 days. From the day of medium exchange on the 4th day of the transfection, 3 μg/ml of cycloheximide was added to the medium so that the cells were cultured in the presence of cycloheximide and the cells were further cultured for 3 weeks. In this case, the medium exchange was carried out every week.

[0128]

For the suspension CHO-K1 cell, 1×10^6 of the cells were seeded into a 6-well plate and shaking-cultured in a CO_2 incubator for 3 days, and the amount of the anti-human influenza M2 antibody protein was measured by HPLC using the culture supernatant.

[0129]

For the adhesive CHO-K1 cell, medium exchange was carried out when the cell reached confluent on a 6-well plate (2×10^6 cells), and 3 days after static culture, the amount of the antibody protein was measured by HPLC using the culture supernatant.

[0130]

The antibody concentration in the culture supernatant was measured in accordance with the method described in *Yeast Res.*, 7 (2007), 1307 - 1316. The results are shown in Fig. 4A and Fig. 4B.

[0131]

As shown in Fig. 4A, a large number of cells showing a markedly high antibody expression level were obtained when the CHO-K1 cell adapted to suspension culture was used. On the other hand, as shown in Fig. 4B, only the cells showing an expression level of the HPLC detection limit (5 μg/ml) or less were obtained when the adhesive CHO-K1 cell was used.

[0132]

Based on these results, it was found that, for the expression of a protein of interest using a transposon vector, the protein of interest can be expressed at a high level when a suspension mammalian cell is used.

[0133]

In addition, it was found from the results of Examples 1 to 3 that the method of the invention can be used as a novel method for producing a protein of interest, by efficiently preparing a production cell which can highly express an exogeneous gene using a suspension mammalian cell adapted to suspension culture.

[0134]

[Example 4]

Preparation of Tol1 transposon vector for expressing anti-human influenza M2 antibody

In the same manner as in Example 1, a plasmid which contains a gene expression cassette for mammalian cells, comprising an arbitrary human antibody gene and a drug resistance marker gene inserted between a pair of Tol1 transposon sequences, was used as a protein expression plasmid vector.

[0135]

Each DNA of the used genes was chemically synthesized artificially based on the known sequence information or obtained by preparing primers of its both terminal sequences and carrying out PCR using an appropriate DNA source as the template. For the gene manipulation to be carried out later, a site cleaved by a restriction enzyme was added to the end of the primer.

[0136]

Among the non-autonomous Tol1 transposon nucleotide sequence shown in SEQ ID NO:13 of Sequence Listing (WO2008/072540), the nucleotide sequence at positions 1 to 200 (Tol1-L sequence) (SEQ ID NO:14) and the nucleotide sequence at

positions 1351 to 1855 (Tol1-R sequence) (SEQ ID NO:15) were used as the transposon sequences.

[0137]

Each of the synthetic DNA fragments comprising each a pair of transposon sequences was prepared by the following method. A DNA fragment comprising a nucleotide sequence in which a recognition sequence of a restriction enzyme *Nru*I was connected to both of the 5'-terminal and 3'-terminal of the Tol1-R sequence. Then, a DNA fragment comprising a nucleotide sequence in which a recognition sequence of a restriction enzyme *Fse*I was connected to the 5'-terminal of the Tol1-L sequence and a restriction enzyme *Asc*I was connected to the 3'-terminal thereof.

[0138]

Next, the thus prepared DNA fragments comprising Tol1-R sequence and Tol1-L sequence were inserted into the expression vector N5LG1-M2-Z3 vector. The DNA fragment comprising the Tol1-R sequence was inserted into the restriction enzyme *Nru*I site of the N5LG1-M2-Z3 vector, existing on the 5'-terminal side of a gene fragment comprising the antibody gene expression cassette and a resistance marker gene, and the DNA fragment comprising the Tol1-L sequence was inserted into the restriction enzyme *Fse*I and *Asc*I sites existing on the 3'-terminal side.

[0139]

In addition, Tol1 transposon vector for expressing an anti-human influenza M2 antibody was constructed (Fig. 5) by inserting a cycloheximide resistance gene expression cassette connected with a resistance gene for cycloheximide (a gene in which proline at position 54 in the human ribosomal protein L36a was mutated to glutamine) into the *Fse*I recognition site of the N5LG1-M2-Z3 vector connected with the Tol1 transposon sequence,under the control of the CMV enhancer/promoter.

[0140]

[Example 5]

Preparation of Tol1 transposase expression vector

The transposase was expressed using an expression vector independent from the expression vector of the antibody of interest. That is, a Tol1 transposase gene expression cassette connected with a DNA fragment encoding a medaka fish-derived Tol1 transposase, containing the nucleotide sequence shown in SEQ ID NO:16 of Sequence Listing, was inserted into pBluescriptII SK (+) (manufactured by Stratagene) under the CMV enhancer/promoter control and used as the expression vector pTol1ase (Fig. 6).

[0141]

[Example 6]

(1) Preparation of antibody-producing CHO cell

The Tol1 transposon vector for expressing the anti-human influenza M2 antibody (hereinafter referred to as Tol1 transposon vector) and Tol1 transposase expression vector pTol1ase of Example 4 and Example 5 were used as the expression vectors. In addition, the CHO-K1 cell prepared by adapting to suspension culture in the same manner as in Example 3(1) was used as the cell.

[0142]

The aforementioned expression vectors were introduced into the CHO-K1 cell adapted to suspension culture, and the frequency of obtaining clones resistant to cycloheximide was measured. The CHO-K1 cell adapted to suspension culture (4 x 10^6 cells) were suspended in 400 μl of PBS, and the Tol1 transposon vector for expressing the anti-human influenza M2 antibody (10 μg) and Tol1 transposase expression vector (50 μg) were co-transfected directly in the form of circular DNA by electroporation. In order to effect transient expression of the Tol1 transposase, the Tol1 transposase expression vector was directly introduced in the form of circular DNA for the purpose of preventing from integrating into the host chromosome.

[0143]

The electroporation was carried out using a cuvette of 4 mm in gap width (manufactured by Bio-Rad), using an electroporator (Gene Pulser Xcell System (manufactured by Bio-Rad)) under conditions of 300 V in voltage, 500 μF in electrostatic capacity and room temperature.

[0144]

After the transfection by electroporation, each cell was seeded into two 96-well plates and cultured in a CO_2 incubator for 3 days using the EX-CELL 325-PF medium (manufactured by SAFC Biosciences) for the CHO cell. Next, from the day of medium exchange on the 4th day of the transfection, 3 μg/ml of cycloheximide was added to the medium so that the cells were cultured in the presence of cycloheximide, followed by culturing for 3 weeks while carrying out the medium exchange every week.

[0145]

After the culturing for 3 weeks, the number of wells in which cycloheximide-resistant colonies were found was counted. The results are shown in Table 3. Each of the tests 1 to 3 in Table 3 shows a result of carrying out the gene transfer three times.

[0146]

[Table 3]

	Tol1 transposon vector
Tests 1	133 / 192
Tests 2	67 / 192
Tests 3	122 / 192

[0147]

As shown in Table 3, when the Tol1 transposon vector for expressing the anti-human influenza M2 antibody was introduced into the suspension CHO-K1 cell, cycloheximide-resistant transformants were obtained at a high frequency similarly to Example 3 in which the Tol2 transposon vector for expressing the anti-human influenza M2 antibody was introduced.

[0148]

It was found based on these results that the antibody gene and cycloheximide resistance gene inserted between a pair of transposon sequences are efficiently transduced into the chromosome of the host cell, namely the suspension mammalian cell, in the case of using the Tol1 transposon, too.

[0149]

(2) Examination on antibody production by suspension CHO-K1 cell

Antibody production efficiency of the suspension CHO-K1 cell was examined using the suspension CHO-K1 cell. The transposon vector for expressing the anti-human influenza M2 antibody (10 μg) and Tol1 transposase expression vector (50 μg) were introduced by electroporation into the suspension CHO-K1 cell adapted to suspension culture.

[0150]

Thereafter, the cells were seeded into respective two 96-well plates and cultured for 3 days in a CO_2 incubator using the suspension culture medium EX-CELL 325-PF. From the medium exchange on the 4th days after the electroporation, the cells were cultured for 3 weeks in the presence of 3 μg/ml of cycloheximide. In this case, the medium exchange was carried out every week.

[0151]

For the suspension CHO-K1 cell, 1×10^6 of the cells were seeded into a 6-well plate and shaking-cultured in a CO_2 incubator for 3 days, and amount of the anti-human influenza M2 antibody protein was measured by HPLC using the culture supernatant.

[0152]

The antibody concentration in culture supernatant was measured in accordance with the method described in *Yeast Res.*, 7 (2007), 1307 - 1316. The results are shown in Fig. 7.

[0153]

As shown in Fig. 7, a large number of cells showing a markedly high antibody expression level were obtained in the case of the use of the Tol1 transposon, too. From this result, it was found that similar to the case of the use of the Tol2 transposon-derived nucleotide sequence, a suspension mammalian cell capable of highly expressing the protein of interest can also be obtained when a Tol1 transposon-derived nucleotide sequence is used as the transposon sequence.

[0154]

While the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skill in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof. This application is based on Japanese application No. 2009-140626, filed on June 11, 2009, and U.S. provisional application No. 61/186,138, filed on June 11, 2009, the entire contents of which are incorporated hereinto by reference and all references cited herein are incorporated in their entirety.

Industrial Applicability

[0155]

By the method for producing the protein of the present invention, a protein of interest can be efficiently produced using a suspension mammalian cell. The cell of the present invention can be used as a protein producing cell for producing a recombinant protein.

Sequence Listing Free Text

[0156]

SEQ ID NO:1 - Description of Artificial sequence: Nucleotide sequence of non-autonomous Tol2 transposon

SEQ ID NO:2 - Description of Artificial sequence: Tol2-L sequence

SEQ ID NO:3 - Description of Artificial sequence: Tol2-R sequence

SEQ ID NO:7 - Description of Artificial sequence: Nucleotide sequence of cycloheximide resistance gene

SEQ ID NO:8 - Description of Artificial sequence: Amino acid sequence of protein encoded by cycloheximide resistance gene

SEQ ID NO:9 - Description of Artificial sequence: Nucleotide sequence encoding H chain of M2Z3 antibody

SEQ ID NO:9 - Description of Artificial sequence: Nucleotide sequence encoding H chain of M2Z3 antibody

SEQ ID NO:10 - Description of Artificial sequence: Nucleotide sequence encoding H chain of M2Z3 antibody

SEQ ID NO:11 - Description of Artificial sequence: Nucleotide sequence encoding L chain of M2Z3 antibody

SEQ ID NO:12 - Description of Artificial sequence: Amino acid sequence encoding L chain of M2Z3 antibody

SEQ ID NO:13 - Description of Artificial sequence: Nucleotide sequence of non-autonomous Tol1 transposon

SEQ ID NO:14 - Description of Artificial sequence: Tol1-L sequence

SEQ ID NO:15 - Description of Artificial sequence: Tol1-R sequence

CLAIMS

1. A method for producing a protein of interest, comprising introducing a protein expression vector which comprises a gene fragment comprising a DNA encoding a protein of interest and a selectable marker gene and transposon sequences at both terminals of the gene fragment, into a suspension mammalian cell which is capable of surviving and proliferating in a serum-free medium; integrating the gene fragment inserted between a pair of the transposon sequences into a chromosome of the mammalian cell to obtain a mammalian cell capable of expressing the protein of interest; and suspension-culturing the mammalian cell,

wherein the suspension mammalian cell is a suspension CHO cell, and

wherein the transposon sequences are nucleotide sequences derived from a pair of Tol1 transposons or nucleotide sequences derived from a pair of Tol2 transposons.

2. A method for producing a protein of interest, comprising the following steps (A) to (C):

(A) a step of simultaneously introducing the following expression vectors (a) and (b) into a suspension mammalian cell which is capable of surviving and proliferating in a serum-free medium:

(a) an expression vector which comprises a gene fragment comprising a DNA encoding a protein of interest and a selectable marker gene and transposon sequences at both terminals of the gene fragment,

(b) an expression vector which comprises a DNA encoding a transposase which recognizes the transposon sequences and has activity of transferring a gene fragment inserted between a pair of the transposon sequences into a chromosome,

(B) a step of expressing transiently the transposase from the expression vector introduced in the step (A) to integrate the gene fragment inserted between a pair of the transposon sequences into a chromosome of the mammalian cell to obtain a suspension mammalian cell capable of expressing the protein of interest, and

(C) a step of suspension-culturing the suspension mammalian cell capable of expressing the protein of interest obtained in the step (B) to produce the protein of interest,

wherein the suspension mammalian cell is a suspension CHO cell, and

wherein the transposon sequences are nucleotide sequences derived from a pair of Tol1 transposon or nucleotide sequences derived from a pair of Tol2 transposons.

3. A method for obtaining a suspension mammalian cell capable of expressing a protein of interest, comprising introducing a protein expression vector which comprises a gene fragment comprising a DNA encoding a protein of interest and

a selectable marker gene and transposon sequences at both terminals of the gene fragment into a suspension mammalian cell which is capable of surviving and proliferating in a serum-free medium; and integrating the gene fragment inserted between a pair of the transposon sequences, into a chromosome of the mammalian cell,

wherein the suspension mammalian cell is a suspension CHO cell, and

wherein the transposon sequences are nucleotide sequences derived from a pair of Tol1 transposons or nucleotide sequences derived from a pair of Tol2 transposons.

4. The method according to any one of claims 1 to 3, wherein the CHO cell is at least one selected from CHO-K1, CHO-K1SV, DUKXB11, CHO/DG44, Pro-3 and CHO-S.

5. The method according to any one of claims 1 to 3, wherein the selectable marker gene is a cycloheximide resistance gene.

6. The method according to claim 5, wherein the cycloheximide resistance gene is a gene encoding a mutant of human ribosomal protein L36a.

7. The method according to claim 6, wherein the mutant is a mutant in which proline at position 54 of the human ribosomal protein L36a is substituted with other amino acid.

8. The method according to claim 7, wherein the other amino acid is glutamine.

9. The method according to any one of claims 1 to 3, wherein the nucleotide sequences derived from a pair of Tol2 transposons are a nucleotide sequence comprising the nucleotide sequence shown in SEQ ID NO:2 and the nucleotide sequence shown in SEQ ID NO:3.

10. The method according to any one of claims 1 to 3, wherein the nucleotide sequences derived from a pair of Tol1 transposons are the nucleotide sequence shown in SEQ ID NO:14 and the nucleotide sequence shown in SEQ ID NO:15.

11. A suspension mammalian cell capable of producing a protein of interest, into which a protein expression vector comprising a gene fragment comprising a DNA encoding a protein of interest and a selectable marker gene and transposon sequences at both terminals of the gene fragment is introduced, to integrate the gene fragment inserted between a pair of the transposon sequences into a chromosome,

wherein the cell is a CHO cell capable of surviving and proliferating in a serum-free medium, and,

wherein the transposon sequences are nucleotide sequences derived from a pair of Tol1 transposons or nucleotide sequences derived from a pair of Tol2 transposons.

12. A suspension mammalian cell capable of producing a protein of interest, into which an expression vector (a) comprising a gene fragment comprising a DNA encoding a protein of interest and a selectable marker gene and transposon sequences at both terminals of the gene fragment, and an expression vector (b) comprising a DNA encoding a transposase (a transferase) which recognizes the transposon sequences and has activity of transferring the gene fragment inserted between a pair of the transposon sequences into a chromosome to integrate the gene fragment inserted between a pair of the transposon sequences into the chromosome,

wherein the cell is a CHO cell capable of surviving and proliferating in a serum-free medium, and,

wherein the transposon sequences are nucleotide sequences derived from a pair of Tol1 transposons or nucleotide sequences derived from a pair of Tol2 transposons.

13. The cell according to claim 11 or 12, wherein the CHO cell is at least one selected from CHO-K1, CHO-K1SV, DUKXB11, CHO/DG44, Pro-3 and CHO-S.

14. The cell according to claim 11 or 12, wherein the selectable marker gene is a cycloheximide resistance gene.

15. The cell according to claim 14, wherein the cycloheximide resistance gene is a gene encoding a mutant of human ribosomal protein L36a.

16. The cell according to claim 15, wherein the mutant is a mutant in which proline at position 54 of the human ribosomal protein L36a is substituted with other amino acid.

17. The cell according to claim 16, wherein the other amino acid is glutamine.

18. The cell according to claim 11 or 12, wherein the nucleotide sequences derived from a pair of the Tol2 transposons are the nucleotide sequence shown in SEQ ID NO:2 and the nucleotide sequence shown in SEQ ID NO:3.

19. The cell according to claim 11 or 12, wherein the nucleotide sequences derived from a pair of the Tol1 transposons are the nucleotide sequence shown in SEQ ID NO:14 and the nucleotide sequence shown in SEQ ID NO:15.

5