DESCRIPTION

Title of Invention: PROCESS FOR PRODUCTION OF PROTEIN

Technical Field

[0001]

This invention relates to a method for integrating a gene fragment inserted between a pair of transposon sequences into a chromosome of a mammalian cell, comprising introducing at least one expression vector which comprises a gene fragment comprising a DNA encoding a protein of interest and also comprises a pair of transposon sequences at both terminals of the gene fragment, into a suspension mammalian cell; and a method for producing the protein comprising suspension-culturing a suspension mammalian cell which produces the protein, a suspension mammalian cell which expresses the protein; and an expression vector which comprises a gene fragment comprising a DNA encoding a protein of interest and also comprises a pair of transposon sequences at both terminals of the gene fragment.

Background Art

[0002]

Production of exogenous 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 transformed cell line under appropriate culture conditions.

[0003]

However, in order to develop a host which can produce an exogenous 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 exogenous protein production techniques for each 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 phosphorylation 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 exogenous 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 move 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) which are 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 introduction 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 exogenous gene is introduced into silkworm chromosome using the transposon piggyBac derived from a Lepidoptera insect to express the protein encoded by said exogenous gene has been studied, and a protein production method using the above techniques was disclosed (Patent Reference 2).

[0013]

However, since protein of interest is not expressed at sufficient levels 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 exogenous 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 References 12 and 13) is known.

[0015]

In the case of producing a protein drug for medical use using a mammal-derived cultured cell, it is important that an animal-derived component is not contained during its production process in order to prevent unexpected contamination of an unknown virus or pathogenic polypeptide. CHO cell is most frequently used as an animal cell for producing a protein drug, and due to the studies of recent years, a suspension CHO cell line capable of culturing in a safe medium which does not use a

serum or animal-derived component has also be established. However, productivity of a cell line into which a gene was introduced under a serum-free or protein-free condition is limited to half that of the cell line into which a gene was introduced under a serum-used condition (Non Patent Literature 14). It is shown that gene transduction under a serum-free or protein-free condition is technically difficult.

[0016]

It is general that a selectable marker for screening a cell expressing a protein of interest is arranged on the same gene expression vector. This is based on a hypothesis that there are a region where a gene existing in the genome is easily expressed and a region where a gene existing in the genome is hardly expressed (called as position effects, Non Patent Literature 15), and that the protein of interest is also expressed when the selectable marker is expressed.

[0017]

On the other hand, when a protein of interest, is comprised of two or more polypeptides such as an antibody and the like, it is also known that each polypeptide is expressed using different vectors. In the case of an antibody, it has been shown that the productivity is higher when expression of heavy chain of the antibody is higher than the expression of light chain (Non Patent Literature 16). Since it is predicted that expressions of heavy chain and light chain become constant on the same vector. It becomes possible to obtain a cell line which expresses the heavy chain and light chain at an optimum ratio by intentionally expressing the heavy chain and light chain using different vectors for the purpose of obtaining high productivity. However, when a protein is expressed using two or more different vectors, two or more selectable marker genes are also necessary.

[0018]

As a way for overcoming this, it was reported a case in which a dhfr gene originally consisting of one polypeptide chain was divided into two polypeptide chains and one of them was arranged on a heavy chain expression vector, and the other was arranged on a light chain expression vector (Non Patent Literature 17).

[0019]

However, the cell described in the Non Patent Literature 17 is a CHO cell in which the cell is dependent on the protein component added to the medium, and as described in the above, there is a possibility that the gene introduction efficiency is high different from the case of the gene introduction under a serum-free or protein-free condition. It is predicted that selecting a cell of high productivity is still difficult when a gene is introduced under a serum-free or protein-free condition having high safety and free from the danger of viral infection and the like.

[Citation List]

[Patent Literature]

[0020]

[Patent Literature 1] WO2008/072540

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

[Non Patent Literature]

[0021]

[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)

[Non Patent Literature 13] *Plos Genetics*, 2,1715-1724(2006)

[Non Patent Literature 14] *Biotech. Bioeng.* 96, 1118-1126 (2007)

[Non Patent Literature 15] *Nature Biotech.* 22, 1393-1398 (2004)

[Non Patent Literature 16] *Biotech. Bioeng.* 96, 337-348 (2007)

[Non Patent Literature 17] *Biotech. Bioeng.* 84, 439-444 (2003)

Disclosure of Invention

Problems to be Solved by the Invention

[0022]

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. However, preparing and culturing the cell that produces the protein of interest require considerable effort and time.

[0023]

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; a 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.

[0024]

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. In addition, establishment of a producing cell which does not require any components derived from an animal from the gene introduction to establishment of a producing cell has been desired.

[0025]

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.

Means for Solving the Problems

[0026]

To solve the above-mentioned problems, the present inventors have conducted intensive studies and found as a result that a protein of interest can be efficiently produced by introducing at least one expression vector which comprises a gene fragment comprising a DNA encoding the protein of interest and also comprises a pair of 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.

[0027]

Specifically, the invention relates to the followings:

1. A method for producing a protein of interest, comprising introducing at least one expression vector which comprises a gene fragment comprising a DNA encoding a protein of interest and also comprises a pair of transposon sequences at both terminals of the gene fragment, into a suspension mammalian cell; integrating the gene fragment comprising the DNA encoding the protein of interest inserted between a pair of the transposon sequences into a chromosome of the mammalian cell to obtain a mammalian cell which expresses 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) at least one expression vector which comprises a gene fragment comprising a DNA encoding a protein of interest and also comprises a pair of 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 obtaining a suspension mammalian cell which expresses the protein of interest by expressing transiently the transposase from the expression vector which is introduced into the suspension mammalian cell in the step (A) to integrate the gene fragment inserted between a pair of the transposon sequences into a chromosome of the mammalian cell, and

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

3. A method for obtaining a suspension mammalian cell which expresses a protein of interest, comprising introducing at least one expression vector which comprises a gene fragment comprising a DNA encoding a protein of interest and also comprises 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 above items 1 to 3, wherein at least one of the expression vectors which comprises a gene fragment comprising a DNA encoding the protein of interest and also comprises a pair of transposon sequences at both terminals of the gene fragment is an expression vector which comprises a gene fragment comprising a DNA encoding the protein of interest and a selectable marker gene and also comprises a pair of transposon sequences at both terminals of the gene fragment;

5. The method described in any one of the above items 1 to 4, comprising introducing an expression vector which comprises a gene fragment comprising a selectable marker and comprises a pair of transposon sequences at both terminals of the gene fragment into a mammalian cell in addition to the expression vector which comprises a gene fragment comprising a DNA encoding the protein of interest and also comprises a pair of transposon sequences at both terminals of the gene fragment,;

6. The method described in any one of the above items 1 to 5, wherein the DNA encoding the protein of interest is a DNA encoding an antibody.

7. The method described in any one of the above item 6, wherein the DNA encoding an antibody is at least one of a DNA encoding an H chain of the antibody and a DNA encoding a L chain of the antibody;

8. The method described in any one of the above items 4 to 7, wherein an expression vector selected from the following (a) to (d) is introduced into a suspension mammalian cell:

(a) an expression vector which comprises a gene fragment comprising a DNA encoding a H chain of an antibody and also comprises a pair of transposon sequences at both terminals of the gene fragment, an expression vector which comprises a gene fragment comprising a DNA encoding a L chain of an antibody and also comprises a pair of transposon sequences at both terminals of the gene fragment and an expression vector which comprises a gene fragment comprising a selectable marker gene and also comprises a pair of transposon sequences at both terminals of the gene fragment

(b) an expression vector which comprises a gene fragment comprising a DNA encoding a H chain of an antibody and a selectable marker gene and also comprises a pair of transposon sequences at both terminals of the gene fragment and an expression vector which comprises a gene fragment comprising a DNA encoding a L chain of an antibody and also comprises a pair of transposon sequences at both terminals of the gene fragment,

(c) an expression vector which comprises a gene fragment comprising a DNA encoding a L chain of an antibody and a selectable marker gene and also comprises a pair of transposon sequences at both terminals of the gene fragment and an expression vector which comprises a gene fragment comprising a DNA encoding a H chain of an antibody and also comprises a pair of transposon sequences at both terminals of the gene fragment, and

(d) an expression vector which comprises a gene fragment comprising a DNA encoding a H chain and a L chain of an antibody and a selectable marker gene and also comprises a pair of transposon sequences at both terminals of the gene fragment;

9. The method described in any one of the above items 1 to 8, wherein the suspension mammalian cell is a cell capable of surviving and proliferating in a serum-free medium;

10. The method described in any one of the above items 1 to 9, 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;

11. The method described in the above item 10, wherein the CHO cell is at least one selected from CHO-K1, CHO-K1SV, DUKXB11, CHO/DG44, Pro-3 and CHO-S;

12. The method described in any one of the above items 4 to 11, wherein the selectable marker gene is a cycloheximide resistance gene;

13. The method described in any one of the above item 12, wherein the cycloheximide resistance gene is a ribosome protein;

14. The method described in any one of the above items 1 to 13, wherein a pair of the transposon sequences are nucleotide sequences derived from a pair of DNA-type transposons which function in a mammalian cell;

15. The method described in the above item 14, 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;

16. The method described in the above item 15, 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;

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

18. A suspension mammalian cell, which has a chromosome into which a gene fragment inserted between a pair of the transposons is integrated and which produces a protein of interest obtainable by simultaneously introducing at least one of expression vector (a) which comprises the gene fragment comprising a DNA encoding a protein of interest and also comprises the pair of transposon sequences at both terminals of the gene fragment and an expression vector (b) which comprises a DNA encoding a transposase (transferase) capable of recognizing the transposon sequences and having the activity to transfer the gene fragment inserted between the pair of transposon sequences to the chromosome.

19. The mammalian cell described in the above item 18, wherein the at least one of expression vector (a) which comprises a gene fragment comprising a DNA encoding a protein of interest and also comprises a pair of transposon sequences at both terminals of the gene fragment is an expression vector which comprises a gene fragment comprising a DNA encoding a protein of interest and a selectable marker gene and also

comprises a pair of transposon sequences at both terminals of the gene fragment.

20. The mammalian cell described in the above item 18 or 19, which is a cell prepared by further introducing an expression vector (c) which comprises a gene fragment comprising a selectable marker gene and also comprises a pair of transposon sequences at both terminals of the gene fragment in addition to the expression vectors (a) and (b).

21. The mammalian cell described in any one of the above items 18 to 20, wherein the DNA encoding the protein of interest is a DNA which encodes an antibody.

22. The mammalian cell described in the above item 21, wherein the DNA which encodes an antibody is at least one of a DNA encoding a H chain of an antibody and a DNA encoding a L chain of an antibody.

23. The mammalian cell described in any one of the above items 18 to 22, into which an expression vector selected from the following (a) to (d) is introduced:

(a) an expression vector which comprises a gene fragment comprising a DNA encoding a H chain of an antibody and also comprises a pair of transposon sequences at both terminals of the gene fragment, an expression vector which comprises a gene fragment comprising a DNA encoding a L chain of an antibody and also comprises a pair of transposon sequences at both terminals of the gene fragment and an expression vector which comprises a gene fragment comprising a selectable marker gene and also comprises a pair of transposon sequences at both terminals of the gene fragment

(b) an expression vector which comprises a gene fragment comprising a DNA encoding a H chain of an antibody and a selectable marker gene and also comprises a pair of transposon sequences at both terminals of the gene fragment and an expression vector which comprises a gene fragment comprising a DNA encoding an L chain of an antibody and also comprises a pair of transposon sequences at both terminals of the gene fragment,

(c) an expression vector which comprises a gene fragment comprising a DNA encoding a L chain of an antibody and a selectable marker gene and also comprises a pair of transposon sequences at both terminals of the gene fragment and an expression vector which comprises a gene fragment comprising a DNA encoding a H chain of an antibody and also comprises a pair of transposon sequences at both terminals of the gene fragment, and

(d) an expression vector which comprises a gene fragment comprising a DNA encoding a H chain and a L chain of an antibody and a selectable marker gene and also comprises a pair of transposon sequences at both terminals of the gene fragment.

24. The mammalian cell described in any one of the above item 18 to 23, wherein the cell is a cell capable of surviving and proliferating in a serum-free medium;

25. The mammalian cell described in any one of the above items 18 to 24, wherein the cell is any 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;

26. The mammalian cell described in the above item 25, wherein the CHO cell is any one selected from CHO-K1, CHO-K1SV, DUKXB11, CHO/DG44, Pro-3 and CHO-S;

27. The mammalian cell described in any one of the above items 19 to 26, wherein the selectable marker gene is a cycloheximide resistance gene;

28. The mammalian cell described in the above item 27, wherein the cycloheximide resistance gene is a gene encoding a mutant of human ribosomal protein L36a;

29. The mammalian cell described in any one of the above items 19 to 28, wherein a pair of the transposon sequences are nucleotide sequences derived from a pair of DNA-type transposons which function in a mammalian cell;

30. The mammalian cell described in the above item 29, 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;

31. The mammalian cell described in the above item 30, 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;

32. The mammalian cell described in the above item 30, 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;

33. An expression vector, which comprises a gene fragment comprising a DNA encoding a protein of interest, and also comprises a pair of transposon sequences at both terminals of the gene fragment;

34. The expression vector described in the above item 33, 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.

35. The protein expression vector described in the above item 34, 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

36. The expression vector described in the above item 34, 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.

Effect of Invention

[0028]

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

Brief Description of the Drawings

[0029]

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

[Fig. 8] Fig. 8 shows a schematic illustration of a transposon vector for expressing an anti-human CD98 antibody heavy chain. Tol2-L represents a left end Tol2 transposon (SEQ ID NO:2), Tol2-R represents a right end Tol2 transposon (SEQ ID NO:3), Pmo represents a Moloney Murine Leukemia Virus promoter, poly A represents a polyadenylation site, and Hc represents an anti-human CD98 antibody heavy chain cDNA (SEQ ID NO:18).

[Fig. 9] Fig. 9 shows a schematic illustration of a transposon vector for expressing anti-human CD98 antibody light chain. 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, and Lc represents an anti-human CD98 antibody light chain cDNA (SEQ ID NO:21).

[Fig. 10] Fig. 10 shows a schematic illustration of a transposon vector for expressing a cycloheximide resistant gene. 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, and CHX-r represents a cycloheximide resistant gene (SEQ ID NO:7).

[Fig. 11] Fig. 11 shows production amount of anti-human TNFα antibody when TNFα-CHX tandem vector or TNFαH-CHX vector and TNFαL vector were gene-introduced into a CHO-K1 cell. The ordinate shows the concentration of the antibody (μg/ml) which is produced in the medium, the control plot is shown by Control, and the test plot is shown by Exp.

[Fig. 12] Fig. 12 shows production amount of anti-human CD20 antibody when CD20-CHX tandem vector or CD20H-CHX vector and CD20L vector were gene-introduced into a CHO-K1 cell. The ordinate shows the concentration of the antibody (μg /ml) which is produced in the medium, the control plot is shown by Control, and the test plot is shown by Exp.

[Fig. 13] Fig. 13 shows structure of the antibody expression vector A. In Fig. 13, Tol2-Lrepresents a DNA fragment comprising the Tol2-L sequence (SEQ ID NO:2), and Tol2-R represents a DNA fragment comprising the Tol2-R sequence (SEQ ID NO:3), CMV represents a CMV promoter, poly A represents a polyadenylation site, Hc represents a heavy chain gene of CD98 antibody, Lc represents an anti-human CD98 antibody light chain gene, SO represents an SV40 promoter, SV represents an SV40 polyadenylation site, and Neo-r represents a neomycin resistance gene.

Embodiments for Carrying Out the Invention

[0030]

This invention relates to a method for producing a protein of interest, comprising introducing at least one expression vector which comprises a gene fragment comprising a DNA encoding a protein of interest and also comprises 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 suspension mammalian cell which expresses said protein of interest; and suspension-culturing the mammalian cell.

[0031]

Examples of the method for producing a protein of interest in the present invention (hereinafter referred to as the method of the present invention) comprise a method for producing a protein of interest, which comprises 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) at least one expression vector which comprises a gene fragment comprising a DNA encoding a protein of interest and also comprises transposon sequences at both terminals of the gene fragment,

(b) a 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 (b) which is introduced into the suspension mammalian cell 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 which expresses the protein of interest, and

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

[0032]

In addition, the present invention relates to a suspension mammalian cell, into which at least one of expression vector which comprises a gene fragment comprising a DNA encoding a protein of interest and also comprises a pair of transposon sequences at both terminals of the gene fragment and an expression vector is introduced to integrate the gene fragment inserted between the pair of transposon sequences into chromosome, and which produces the protein of interest.

[0033]

In the present invention, the protein of interest is a protein comprised of one or more polypeptides, and according to the method of the invention, it can carry out any of the expression of at least one of the protein of interest and/or expression of at least one polypeptide.

[0034]

The at least one of expression vectors which comprises a gene fragment comprising a DNA encoding a protein of interest and also comprises a pair of transposon sequences at both terminals of the gene fragment means one or two or more species of the expression vector. Particularly, in order to express a protein of interest comprised of two or more polypeptides, it is necessary to use two or more expression vectors which comprise a gene fragment including a DNA encoding respective polypeptides and also comprise a pair of transposon sequences at both terminals of the gene fragment.

[0035]

More particularly, for example, when the above-mentioned protein of interest comprised of two or more polypeptides is an antibody, a H chain and a L chain of an antibody may be expressed using one expression vector or may be expressed using two expression vectors of a vector which expresses the H chain and a vector which expresses the L chain, respectively.

[0036]

According to the method of the present invention, it can produce a protein of interest using a suspension mammalian cell which produces the protein of interest, in which a gene fragment inserted between a pair of transposon sequences is integrated into chromosome, by introducing the expression vector which comprises a gene fragment including a DNA encoding the protein of interest and also comprises a pair of transposon sequences at both terminals of the gene fragment.

[0037]

The selectable marker gene to be used as an index of gene insertion may be integrated into the same vector as the expression vector which comprises the DNA

encoding the protein of interest or may be integrated into a different vector.

[0038]

That is, at least one of the expression vectors which comprise a gene fragment including a DNA encoding a the protein of interest and also comprise a pair of transposon sequences at both terminals of the gene fragment may be used as the expression vector which comprises a gene fragment including a DNA encoding a protein of interest and a selectable marker gene and also comprises a pair of transposon sequences at both terminals of the gene fragment.

[0039]

Also, in addition to the expression vector which comprises a gene fragment including a DNA encoding a the protein of interest and also comprises a pair of transposon sequences at both terminals of the gene fragment, an expression vector which comprises a gene fragment including a selectable marker gene and also comprises a pair of transposon sequences at both terminals of the gene fragment may be further introduced into a mammalian cell.

[0040]

Specifically, 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 also comprises a pair of transposon sequences at both terminals of the gene fragment,

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

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

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

[0041]

In addition, 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), (b)

and (c) into a suspension mammalian cell:

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

(b) an expression vector which comprises a selectable marker and a pair of transposon sequences at both terminals of the selectable marker,

(c) a vector which comprises a DNA encoding a transposase which recognizes the transposon sequences and has activity of transferring the 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 (c) which is introduced into the suspension mammalian cell in the step (A) to integrate the gene fragment inserted between a pair of the transposon sequences into a chromosome of the mammalian cell and obtaining a suspension mammalian cell which expresses the protein of interest, and

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

[0042]

The present invention relates to a suspension mammalian cell, into which at least one expression vector which comprises a gene fragment comprising a DNA encoding a protein of interest and also comprises a pair of transposon sequences at both terminals of the gene fragment and an expression vector which comprises a selectable marker and a pair of transposon sequences at both terminals of the selectable marker are introduced to integrate the gene fragment and the selectable marker inserted between a pair of the transposon sequences into a chromosome, and which produces a protein of interest.

[0043]

In addition, the present invention relates to a suspension mammalian cell into which a protein expression vector which comprises a gene fragment which comprising a DNA encoding a protein of interest and a selectable marker, and also comprises a pair of 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, and which produces a protein of interest.

[0044]

Furthermore, examples of the suspension mammalian cell which produces a protein of interest of the present invention include a suspension mammalian cell into which an expression vector (a) comprising a gene fragment comprising a DNA encoding a protein of interest and a selectable marker gene and also comprising

transposon sequences at both terminals of the gene fragment, and a 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 are simultaneously introduced and which produces the protein of interest.

[0045]

According to the present invention, the number of expression vectors which comprise a gene fragment including a DNA encoding the protein of interest and also comprise a pair of transposon sequences at both terminals of the gene fragment, to be introduced into a suspension mammalian cell, is not particularly limited as long as expression and production of the protein of interest can be carried out by the mammalian cell, and examples include preferably 1 to 20 species of expression vectors, more preferably 2 to 10 species of expression vectors, can be mentioned, and for example, 3 to 8 species of expression vectors, 4 to 7 species of expression vectors, 1 to 6 species of expression vectors, 1 to 5 species of expression vectors, 1 to 4 species of expression vectors and 1 to 3 species of expression vectors are preferable.

[0046]

In addition, examples of the embodiment of the present invention include a method for increasing integration of a gene fragment inserted between a pair of transposon sequences into chromosome of the mammalian cell, by simultaneously introducing into the suspension mammalian cell (a) at least one of expression vector which comprises a gene fragment comprising a DNA encoding a protein of interest and also comprises a pair of transposon sequences at both terminals of the gene fragment and (b) a vector which comprises a DNA encoding transposase capable of recognizing the transposon sequences and having the activity to introduce the gene fragment inserted between the pair of transposon sequences to chromosome, a method for integrating a DNA encoding a protein of interest into chromosome of the mammalian cell at a high frequency and a suspension mammalian cell which is obtained by the methods and can produce a protein of interest.

[0047]

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 another chromosome (transposition) while keeping a certain structure.

[0048]

The transposon comprises 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 a gene unit and a nucleotide sequence encoding a transposase which recognizes the transposon sequence to introduce a gene existing between the transposon sequences.

[0049]

The transposase translated from the transposon can introduce a DNA by recognizing transposon sequences of both terminals of the transposon, cleaving out the DNA fragment inserted between a pair of the transposon sequences and inserting the fragment into the site to be introduced.

[0050]

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 introduced (inserted into other position in the genome) by the activity of a transposase, and there is a transposon sequence specific to a transposase.

[0051]

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.

[0052]

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.

[0053]

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.

[0054]

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.

[0055]

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.

[0056]

As the transposon sequence derived from a pair of Tol1 transposons, example 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.

[0057]

As the transposon 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 Tol1 transposon nucleotide sequence shown in SEQ ID NO:13 of Sequence Listing are more preferable.

[0058]

Examples of the transposon sequence to be used in the invention include transposon sequences of which transposition 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.

[0059]

As the method for producing the protein of the interest of the present invention, examples also include a method in which at least one of the protein of interest is produced using at least two of transposon sequence and at least two of transposase.

[0060]

Specifically, examples include a protein production method which comprises the steps of introducing a vector comprising a DNA encoding a first protein of interest inserted into two Tol1 transposon sequences, a vector comprising a DNA encoding a second protein of interest inserted into two Tol2 transposon sequences, a Tol1 transposase expression vector and a Tol2 transposon expression vector, simultaneously or in order into chromosome of the mammalian cell and thereby obtaining a mammalian cell which produces the two proteins of interest.

[0061]

In addition, the first protein of interest and the second protein of interest may

be the same, and productivity of the protein of interest can also be improved by increasing the number of copies of the gene to be introduced into the cell.

[0062]

Regarding the control of the transposition reaction of a transposon, the transposition reaction can be accelerated or suppressed by accelerating or suppressing recognition of the transposon sequence by a transposase, respectively. In addition, with regard to the transposition reaction of transposon, the transposition reaction can be enhanced by shortening the length of the nucleotide sequence inserted between a pair (two) of the transposon sequences and the transposition reaction can be lowered by elongating the length. Therefore, when a protein of interest comprising plural proteins is expressed and prepared, the proteins of interest can be prepared by inserting DNA encoding each protein into a different expression vector, integrating the DNA in its chromosome of a host cell and can preparing a suspension mammalian cell which is able to prepare the protein of interest to produce the protein of interest by using the cell.

[0063]

The term "transposase" in the present specification means an enzyme which recognizes nucleotide sequences having transposon sequences and transfers a gene fragment existing between the nucleotide sequences on a chromosome or from the chromosome to another chromosome.

[0064]

Examples of the transposase include enzymes derived from Tol1 and Tol2 which are derived from medaka fish, the Sleeping Beauty (SB) reconstructed from a non-autonomous transposon existed in an *Onchorhynchus* fish genome, Sleeping Beauty 11 (SB11), the artificial transposon Frog prince (FP) which is derived from frog and the transposon PiggyBac (PB) which is derived from insect.

[0065]

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 transposition activity as the transposase is maintained. By controlling the enzyme activity of the transposase, the transposition reaction of the DNA existing between the transposon sequences can be controlled.

[0066]

In order to analyze whether or not it possesses a transposition 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.

[0067]

Particularly, whether or not a non-autonomous 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.

[0068]

The term "non-autonomous transposon" in the present specification means a transposon which is lost a transposase existed inside the transposon and can not therefore perform its autonomous transposition. 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.

[0069]

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.

[0070]

According to the method of the invention, in order to integrate a gene fragment comprising a DNA encoding the protein of interest in at least one 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 also comprises a pair of 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.

[0071]

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 at least one protein of interest or a DNA encoding a protein of interest 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.

[0072]

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.

[0073]

In the case where a protein of interest comprised of two or more polypeptides or two or more proteins of interest is produced by the method of the invention, a protein producing cell in which a DNA encoding each protein is integrated in to a chromosome of a host cell can be prepared by inserting the DNA encoding each of protein on the same expression vector or inserting the DNA into respective different expression vector and introducing the expression vector into a host cell.

[0074]

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.

[0075]

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.

[0076]

The term "expression vector" in the present specification means an expression vector to be used for introducing a mammalian cell and expressing 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.

[0077]

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 include a selectable marker gene.

[0078]

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.

[0079]

The "selectable marker gene" means an optional 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.

[0080]

Examples of the selectable marker gene include a drug resistance gene (a neomycin resistance gene, a dihydrofolate reductase (DHFR) gene, a puromycin resistance gene, a blasticidin resistance gene, a zeocin 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.

[0081]

In the invention, the preferable selectable marker is a drug resistance gene and particularly preferable selectable marker is a cycloheximide resistance gene. Further, drug resistance property and luminescence property of the selectable marker protein can also be changed by preparing an amino acid modified variant by genetically modifying the selectable maker gene or by controlling transcription or translation of the selectable marker gene (e.g., modification of a promoter, modification of an amino acid codon and the like). In addition, a selectable marker gene introduced cells having different drug resistance strengths can also be selected by adjusting the drug concentration.

[0082]

For controlling drug resistance property and luminescence property of the selectable marker protein, it is preferable to use an as the attenuated selectable marker gene. The attenuated selectable marker gene is a selectable marker gene which is modified in such a manner that activity of the protein encoded by the selectable marker gene inside the cell is lowered.

[0083]

Examples of the selectable marker gene which is modified in such a manner that the activity in the cell becomes low include (A) an selectable marker gene in which an amino acid sequence of a protein encoded by a selectable marker gene is modified so that activity of the protein in the cell is lowered and (B) an selectable marker gene in which a nucleotide sequence which controls expression of a selectable marker gene is modified or a nucleotide sequence inside of ORF (open reading frame) is modified so

that the expression of the selectable marker gene is lowered.

[0084]

Examples of the selectable marker gene in which an amino acid sequence of a protein encoded by a selectable marker gene is modified so that activity of the protein in the cell is lowered include the neomycin resistance gene described by Sauter et al. [*Biotech. Bioeng.*, 89, 530 - 538 (2005)] or Chen et al. [*Journal of Immunological Methods*, 295, 49 - 56 (2004)].

[0085]

Examples of the method for lowering expression level of a protein in the cell by modifying a nucleotide sequence which controls expression of the selectable marker gene include a method for modifying the sequence of promoter sequence, terminator sequence, enhancer sequence, kozak's consensus sequence or Shine-Dalgarno sequence, which controls expression of the selectable marker gene. More specifically, examples include a method in which a promoter sequence which controls expression of a selectable marker gene is replaced by a weaker promoter sequence.

[0086]

Examples of the method for lowering expression level of the protein in the cell by modifying a nucleotide sequence in the ORF of a selectable marker gene include a method in which a codon in the ORF is replaced by a synonymous codon having further lower frequency of codon usage in the cell.

[0087]

Examples of the attenuated selectable marker gene of the invention include a selectable marker in which the above codon in the ORF of the gene is replaced by a synonymous codon having further lower frequency of codon usage in the cell.

[0088]

In the cells of various biological species, the synonymous codon having further lower frequency of usage among each synonymous codon can be selected based on known literatures, data bases and the like.

[0089]

As such a replacement by a synonymous codon having lower frequency of usage, specifically in the case of CHO cell, examples include replacement of the codon of leucine with TTA, replacement of the codon of arginine with CGA or CGT, replacement of the codon of alanine with GCG, replacement of the codon of valine with GTA, replacement of the codon of serine with TCG, replacement of the codon of isoleucine with ATA, replacement of the codon of threonine with ACG, replacement of the codon of proline with CCG, replacement of the codon of glutamic acid with GAA, replacement of the codon of tyrosine with TAT, replacement of the codon of lysine with

AAA, replacement of the codon of phenylalanine with TTT, replacement of the codon of histidine with CAT, replacement of the codon of glutamine with CAA, replacement of the codon of asparagine with AAT, replacement of the codon of aspartic acid with GAT, replacement of the codon of cysteine with TGT and replacement of the codon of glycine with GGT.

[0090]

In an attenuated selectable marker gene, the number of codons to be placed compared to the selectable marker gene before the modification is not particularly limited as long as a protein producing cell can be efficiently obtained, but it is preferable to replace codons corresponding to 20 or more amino acid residues.

[0091]

In an attenuated selectable marker gene, the number of bases to be modified compared to the selectable marker gene before modification is not particularly limited, but it is preferable to modify 10% or more of the nucleotide sequence encoding the selectable marker gene.

[0092]

In addition, in an attenuated selectable marker gene, the amino acid residues encoded by the codons to be replaced is not particularly limited, but preferable examples include leucine, alanine, serine and valine.

[0093]

In the case of an attenuated selectable marker gene, in the case where the codons corresponding to leucine are replaced not particularly limited, but it is preferable to replace the codons corresponding to 70% or more of leucine residues among the codons corresponding to the total of the leucine residues contained in the selectable marker gene.

[0094]

Also, in the case of an attenuated selectable marker gene, when the codons corresponding to alanine are replaced not particularly limited, but it is preferable to replace the codons corresponding to 70% or more of alanine residues among the codons corresponding to the total of the alanine residues contained in the selectable marker gene.

[0095]

Specific examples of the attenuated selectable marker gene obtained by such as a modification in which codons are replaced with synonymous codons having lower frequency of usage include a neomycin resistance gene comprising the nucleotide sequence represented by SEQ ID NO:37, 38 or 39, a puromycin resistance gene comprising the nucleotide sequence represented by SEQ ID NO:41, 43 or 44, a Zeocin

resistance gene consisting of the nucleotide sequence represented by SEQ ID NO:45 or 46 and a hygromycin resistance gene comprising the nucleotide sequence represented by SEQ ID NO:47 or 48.

[0096]

In addition, it is possible to attenuate a selectable marker gene also by considerably increasing concentration of a drug in comparison with the conventionally used concentration when a drug-resistant cell is selected in preparing an antibody producing cell or by carrying out additional administration before the drug resistance gene metabolizes and degrades the drug.

[0097]

Cycloheximide (hereinafter, referred sometimes to as CHX) is a protein synthesis inhibitor, and examples of using a CHX resistance gene as the selectable marker gene include known cases of yeast [Kondo K., *J. Bacteriol.*, 177, 24, 7171 - 7177 (1995)] and animal cells (JP-A-2002-262879).

[0098]

In the case of animal cells, it has been revealed that a transformant expressing a protein encoded by the nucleotide sequence represented by SEQ ID NO:7 of SEQUENCE LISTING in which the 54-position proline of a human ribosomal protein subunit L36a encoded by the nucleotide sequence represented by SEQ ID NO:5 of SEQUENCE LISTING is replaced by glutamine provides resistance to cycloheximide. In addition, examples of the cycloheximide resistance marker include a mutant human ribosomal protein subunit L44 in which proline at position 54 of a human ribosomal protein subunit L44 is replaced by glutamine.

[0099]

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

[0100]

Examples of the method for directly introducing a transposase in the form of a protein include a microinjection technique or supply into a cell by endocytosis. The gene introduction 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.

[0101]

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 subclonal cell line of CHO cell.

[0102]

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

[0103]

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 a CHO cell from which α1,6-fucosyltransferase gene is deleted (WO2005/35586, WO2002/31140), a cell deficient in GDP-mannose 4,6-dehydratase (GMD) and a cell deficient in Fx protein can also be used as the host cell.

[0104]

In the present invention, the protein of interest also includes any one of a protein consisting of at least one polypeptide and a complex protein consisting of two or more polypeptides of protein. In addition, a protein and a polypeptide are synonymous in the invention, but a protein molecule having a relatively low molecular weight or a protein constituting a complex protein may be defined sometimes as a polypeptide.

[0105]

The protein of interest in the invention may be any protein or polypeptide as long as it can be expressed by the method of the invention. Particularly, examples of the protein of interest include a human serum protein, an albumin binding protein, a peptide hormone, a growth factor, a cytokine, a blood coagulation factor, a fibrinolytic protein, an antibody, a selectable marker protein, a membrane protein, partial fragments of various proteins and the like. Particularly, examples of the protein of interest include human vein immunoglobulin (IVIG), erythropoietin (EPO), albumin, growth

hormone (GH), follicle-stimulating hormone (FSH), hepatocyte growth factor (HGF), insulin, insulin-like growth factor-I (IGF-I), interferon (INF), Fas ligand, blood coagulation factors (II, VII, VIII, IX, X), prothrombin, fibrinogen, protein C, protein S, antithrombin III (ATIII), the tissue plasminogen activator (tPA), a monoclonal antibody, a polyclonal antibody and the like.

[0106]

The antibody is a molecule comprising of an antibody heavy chain (H chain) polypeptide and two antibody light chain (L chain) polypeptides, and as a subclass, IgA, IgD, IgE, IgG and IgM subclasses are known. Further, the IgG is classified into IgG1, IgG2, IgG3 and IgG4.

[0107]

The IgG antibody is a heterotetrameric molecule consisting of two H chain polypeptides and two L chain polypeptides. Each of the H chain and L chain consists of a variable region (V) which relates to the antigen binding and a constant region (C) and each of them is called VH, CH, VL or CL, respectively. The CH region is further classified into CH1, CH2 and CH3 regions, and the CH2 and CH3 regions are called in combination as Fc region or simply as Fc.

[0108]

The antibody includes a monoclonal antibody which reacts with a single epitope, a polyclonal antibody which reacts with two or more epitopes and a recombinant antibody.

[0109]

The monoclonal antibody is an antibody which is secreted by a single clonal antibody producing cell and recognizes only one epitope (also called an antigenic determinant), and the amino acid sequence (primary structure) constituting a monoclonal antibody is uniform.

[0110]

The polyclonal antibody is a mixture of monoclonal antibodies and can react with two or more epitopes.

[0111]

Examples of the recombinant antibody include a chimeric antibody, a humanized antibody, a human antibody, a Fc fusion protein, Fc amino acid modified antibody, and a multivalent antibody and a partial fragment thereof. An amino acid modified antibody may have an amino acid modification in either a variable region or a constant region and antibody activity is controlled.

[0112]

The multivalent antibody includes a multivalent antibody which reacts with

two or more different epitopes on one antigen, a multivalent antibody which react with two or more different antigens and the like, but it may include any multivalent antibody. In addition, the multivalent antibody may be any multivalent antibody having any structure as long as it retains the binding activity to the antigen (WO2001/77342, US Patent No. 7,612,181 and WO2009/131239).

[0113]

According to the producing method of the present invention, any of the above protein of interest and/or the peptide of interest can be expressed and produced.

[0114]

Examples of the cell into which a DNA encoding at least one protein of interest of the present invention include an antibody producing cell prepared by the following steps (A) and (B).

Step (A) a step of simultaneously introducing both of one combination of expression vector selected from the following (a) to (c) or expression vector (d) and expression vector (e) into a suspension mammalian cell:

(a) an expression vector which comprises a gene fragment comprising a DNA encoding a H chain of an antibody and also comprises a pair of transposon sequences at both terminals of the gene fragment, an expression vector which comprises a gene fragment comprising a DNA encoding a L chain of an antibody and also comprises a pair of transposon sequences at both terminals of the gene fragment and an expression vector which comprises a gene fragment comprising a selectable marker gene and also comprises a pair of transposon sequences at both terminals of the gene fragment

(b) an expression vector which comprises a gene fragment comprising a DNA encoding a H chain of an antibody and a selectable marker gene and also comprises a pair of transposon sequences at both terminals of the gene fragment and an expression vector which comprises a gene fragment comprising a DNA encoding a L chain of an antibody and also comprises a pair of transposon sequences at both terminals of the gene fragment,

(c) an expression vector which comprises a gene fragment comprising a DNA encoding a L chain of an antibody and a selectable marker gene and also comprises a pair of transposon sequences at both terminals of the gene fragment and an expression vector which comprises a gene fragment comprising a DNA encoding a H chain antibody of an antibody and also comprises a pair of transposon sequences at both terminals of the gene fragment, and

(d) an expression vector which comprises a gene fragment comprising a DNA encoding a H chain and a L chain of an antibody and a selectable marker gene and also comprises a pair of transposon sequences at both terminals of the gene fragment;

(e) a vector comprising 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; and

Step (B) a step of selecting a suspension mammalian cell which expresses an antibody in which the genes of the above H chain, L chain and selectable marker which are inserted between a pair of the transposon sequence are integrated into a chromosome of the above mammalian cell by transiently expressing the transposase from the expression vector (e) which is introduced into the suspension mammalian cell in the step (A).

[0115]

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

Step (A) a step of simultaneously introducing one combination of expression vector selected from the following (a) to (c) or expression vector (d), and expression vector (e) into a suspension mammalian cell:

(a) an expression vector which comprises a gene fragment comprising a DNA encoding a H chain of an antibody and also comprises a pair of transposon sequences at both terminals of the gene fragment, an expression vector which comprises a gene fragment comprising a DNA encoding a L chain of an antibody and also comprises a pair of transposon sequences at both terminals of the gene fragment and an expression vector which comprises a gene fragment comprising a selectable marker gene and also comprises a pair of transposon sequences at both terminals of the gene fragment

(b) an expression vector which comprises a gene fragment comprising a DNA encoding a H chain of an antibody and a selectable marker gene and also comprises a pair of transposon sequences at both terminals of the gene fragment and an expression vector which comprises a gene fragment comprising a DNA encoding a L chain of an antibody and also comprises a pair of transposon sequences at both terminals of the gene fragment,

(c) an expression vector which comprises a gene fragment comprising a DNA encoding a L chain of an antibody and a selectable marker gene and also comprises a pair of transposon sequences at both terminals of the gene fragment and an expression vector which comprises a gene fragment comprising a DNA encoding a H chain of an antibody and also comprises a pair of transposon sequences at both terminals of the gene fragment, and

(d) an expression vector which comprises a gene fragment comprising a DNA encoding a H chain and a L chain of an antibody and a selectable marker gene and also

comprises a pair of transposon sequences at both terminals of the gene fragment;

(e) a vector comprising 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;

Step (B) a step of obtaining a suspension mammalian cell which expresses an antibody in which the genes of the above H chain, L chain and selectable marker which are inserted between a pair of the transposon sequence are integrated into a chromosome of the above mammalian cell by transiently expressing the transposase from the expression vector (e) which is introduced into the suspension mammalian cell in the step (A); and

Step (C) a step of producing the antibody by suspension-culturing a suspension mammalian cell obtained in the step (B) which expresses an antibody.

[0116]

In addition, the present invention includes a method for producing a cell line which has a high antibody productivity and a method for screening the cell line comprising the following steps (A) and (B).

Step (A) a step of simultaneously introducing one combination of expression vector selected from the following (a) to (c) or expression vector (d), and expression vector (e) into a suspension mammalian cell:

(a) an expression vector which comprises a gene fragment comprising a DNA encoding a H chain of an antibody and also comprises a pair of transposon sequences at both terminals of the gene fragment, an expression vector which comprises a gene fragment comprising a DNA encoding a L chain of an antibody and also comprises a pair of transposon sequences at both terminals of the gene fragment and an expression vector which comprises a gene fragment comprising a selectable marker gene and also comprises a pair of transposon sequences at both terminals of the gene fragment

(b) an expression vector which comprises a gene fragment comprising a DNA encoding a H chain of an antibody and a selectable marker gene and also comprises a pair of transposon sequences at both terminals of the gene fragment and an expression vector which comprises a gene fragment comprising a DNA encoding a L chain of an antibody and also comprises a pair of transposon sequences at both terminals of the gene fragment,

(c) an expression vector which comprises a gene fragment comprising a DNA encoding a L chain of an antibody and a selectable marker gene and also comprises a pair of transposon sequences at both terminals of the gene fragment and an expression vector which comprises a gene fragment comprising a DNA encoding a H chain of an antibody and also comprises a pair of transposon sequences at both terminals of the

gene fragment, and

(d) an expression vector which comprises a gene fragment comprising a DNA encoding a H chain and a L chain of an antibody and a selectable marker gene and also comprises a pair of transposon sequences at both terminals of the gene fragment;

(e) a vector comprising 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; and

Step (B) a step of selecting a suspension mammalian cell which highly expresses an antibody in which the genes of the above H chain, L chain and selectable marker which are inserted between a pair of the transposon sequence are integrated into a chromosome of the above mammalian cell by transiently express the transposase from the expression vector (e) which is introduced into the suspension mammalian cell in the step (A).

[0117]

In addition, the present invention includes a method for producing an antibody comprising the following steps (A) , (B) and (C).

Step (A) a step of simultaneously introducing one combination of expression vector selected from the following (a) to (c) or expression vector (d) and expression vector (e) into a suspension mammalian cell:

(a) an expression vector which comprises a gene fragment comprising a DNA encoding a H chain of an antibody and also comprises a pair of transposon sequences at both terminals of the gene fragment, an expression vector which comprises a gene fragment comprising a DNA encoding a L chain of an antibody and also comprises a pair of transposon sequences at both terminals of the gene fragment and an expression vector which comprises a gene fragment comprising a selectable marker gene and also comprises a pair of transposon sequences at both terminals of the gene fragment

(b) an expression vector which comprises a gene fragment comprising a DNA encoding a H chain of an antibody and a selectable marker gene and also comprises a pair of transposon sequences at both terminals of the gene fragment and an expression vector which comprises a gene fragment comprising a DNA encoding a L chain of an antibody and also comprises a pair of transposon sequences at both terminals of the gene fragment,

(c) an expression vector which comprises a gene fragment comprising a DNA encoding a L chain of an antibody and a selectable marker gene and also comprises a pair of transposon sequences at both terminals of the gene fragment and an expression vector which comprises a gene fragment comprising a DNA encoding a H chain of an antibody and also comprises a pair of transposon sequences at both terminals of the

gene fragment, and

(d) an expression vector which comprises a gene fragment comprising a DNA encoding a H chain and a L chain of an antibody and a selectable marker gene and also comprises a pair of transposon sequences at both terminals of the gene fragment;

(e) a vector comprising 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;

Step (B) a step of obtaining a suspension mammalian cell which expresses an antibody in which the genes of the above H chain, L chain and selectable marker which are inserted between a pair of the transposon sequence are integrated into a chromosome of the above mammalian cell by transiently expressing the transposase from the expression vector (e) which is introduced into the suspension mammalian cell in the step (A); and

Step (C) a step of producing the antibody by suspension-culturing a suspension mammalian cell obtained in the step (B) which expresses an antibody.

[0118]

Examples of the mammalian cell into which a DNA encoding at least one protein of interest of the present invention include a polyclonal antibody producing cell into which several different antibody genes are introduced, a complex molecule producing cell and the like.

[0119]

Examples of a polyclonal antibody producing cell include a cell into which at least two or more different monoclonal antibody genes are introduced, a cell into which genes of several monoclonal antibodies against several antigens are introduced, a cell which is immunized by an antigen and into which a gene library of a non-human antibody is introduced, a cell into which a gene library of antibody derived from a patient is introduced and the like.

[0120]

The complex molecule producing cell may be any cell as long as DNAs encoding respective proteins which are co-expressed in a cell to form a complex molecule is introduced. Specific examples include a cell into which FcγRIII (CD16) and common γ chain are co-transfected, a cell into which neonatal Fc receptor (FcRn) and β2 macrogloblin are co-expressed, a cell into which CD98 and LAT1 are co-transfected (WO2007/114496) and the like

[0121]

The antibody which is produced by the antibody production method of the present invention can be any antibody and examples include an antibody which

recognize a tumor-related antigen, an antibody which recognizes an allergy- or inflammation-related antigen, an antibody which recognizes an cardiovascular disease-related antigen, an antibody which recognizes an antigen which relating to autoimmune diseases, an antibody which recognizes virus- or bacterial infection-related antigen and the like.

[0122]

Examples of the tumor-related antigen includes CD1a, CD2, CD3, CD4, CD5, CD6, CD7, CD9, CD10, CD13, CD19, CD20, CD21, CD22, CD25, CD28, CD30, CD32, CD33, CD38, CD40, CD40 ligand (CD40L), CD44, CD45, CD46, CD47, CD52, CD54, CD55, CD55, CD59, CD63, CD64, CD66b, CD69, CD70, CD74, CD80, CD89, CD95, CD98, CD105, CD134, CD137, CD138, CD147, CD158, CD160, CD162, CD164, CD200, CD227, adrenomedullin, angiopoietin related protein 4 (ARP4), aurora, B7-H1, B7-DC, integlin, bone marrow stromal antigen 2 (BST2), CA125, CA19.9, carbonic anhydrase 9 (CA9), cadherin, cc-chemokine receptor (CCR) 4, CCR7, carcinoembryonic antigen (CEA), cysteine-rich fibroblast growth factor receptor-1 (CFR-1), c-Met, c-Myc, collagen, CTA, connective tissue growth factor (CTGF), CTLA-4, cytokeratin-18, DF3, E-catherin, epidermal growth facter receptor (EGFR), EGFRvIII, EGFR2 (HER2), EGFR3 (HER3), EGFR4 (HER4), endoglin, epithelial cell adhesion molecule (EpCAM), endothelial protein C receptor (EPCR), ephrin, ephrin receptor (Eph), EphA2, endotheliase-2 (ET2), FAM3D, fibroblast activating protein (FAP), Fc receptor homolog 1 (FcRH1), ferritin, fibroblast growth factor-8 (FGF-8), FGF8 receptor, basic FGF (bFGF), bFGF receptor, FGF receptor (FGFR) 3, FGFR4, FLT1, FLT3, folate receptor, Frizzled homologue 10 (FZD10), frizzled receptor 4 (FZD-4), G250, G-CSF receptor, ganglioside (such as GD2, GD3, GM2 and GM3), globo H, gp75, gp88, GPR-9-6, heparanase I, hepatocyte growth factor (HGF), HGF receptor, HLA antigen (such as HLA-DR), HM1.24, human milk fat globule (HMFG), hRS7, heat shock protein 90 (hsp90), idiotype epitope, insulin-like growth factor (IGF), IGF receptor (IGFR), interleukin (such as IL-6 and IL-15), interleukin receptor (such as IL-6R and IL-15R), integrin, immune receptor translocation associated-4 (IRTA-4), kallikrein 1, KDR, KIR2DL1, KIR2DL2/3, KS1/4, lamp-1, lamp-2, laminin-5, Lewis y, sialyl Lewis x, lymphotoxin-beta receptor (LTBR), LUNX, melanoma-associated chondroitin sulfate proteoglycan (MCSP), mesothelin, MICA, Mullerian inhibiting substance type II receptor (MISIIR), mucin, neural cell adhesion molecule (NCAM), Necl-5, Notch1, osteopontin, platelet-derived growth factor (PDGF), PDGF receptor, platelet factor-4 (PF-4), phosphatidylserine, Prostate Specific Antigen (PSA), prostate stem cell antigen (PSCA), prostate specific membrane antigen (PSMA), Parathyroid hormone related protein/peptide (PTHrP), receptor activator of NF-kappaB ligand

(RANKL), receptor for hyaluronic acid mediated motility (RHAMM), ROBO1, SART3, semaphorin 4B (SEMA4B), secretory leukocyte protease inhibitor (SLPI), SM5-1, sphingosine-1-phosphate, tumor-associated glycoprotein-72 (TAG-72), transferrin receptor (TfR), TGF-beta, Thy-1, Tie-1, Tie2 receptor, T cell immunoglobulin domain and mucin domain 1 (TIM-1), human tissue factor (hTF), Tn antigen, tumor necrosis factor (TNF), Thomsen-Friedenreich antigen (TF antigen), TNF receptor, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), TRAIL receptor (such as DR4 and DR5), system ASC amino acid transporter 2 (ASCT2), trkC, TROP-2, TWEAK receptor Fn14, type IV collagenase, urokinase receptor, vascular endothelial growth factor (VEGF), VEGF receptor (such as VEGFR1, VEGFR2 and VEGFR3), vimentin, VLA-4 and the like, and antibodies against the above antigens.

[0123]

Further, examples of the antibody which recognizes a tumor-related antigen include [*AntiCancer Res.*, 13, 331 (1993)], anti-GD3 antibody [*Cancer Immunol. Immunother.*, 36, 260 (1993)], anti-GM2 antibody [*Cancer* Res., 54, 1511 (1994)], anti-CD52 antibody [*Proc. Natl. Acad. Sci. USA*, 89, 4285 (1992)], anti-MAGE antibody [British J. *Cancer*, 83, 493 (2000)], anti-HM1.24 antibody [*Molecular Immunol.*, 36, 387 (1999)], anti-parathyroid hormone related protein (PTHrP) antibody [*Cancer*, 88, 2909 (2000)], anti-bFGF antibody, anti-FGF-8 antibody [*Proc. Natl. Acad. Sci. USA*, 86, 9911 (1989)], anti-bFGFR antibody, anti-FGFR1 antibody (WO2005/037235), anti-FGF-8R antibody [*J. Biol. Chem.*, 265, 16455 (1990)], anti-IGF antibody [*J. Neurosci. Res.*, 40, 647 (1995)], anti-IGF-IR antibody [*J. Neurosci. Res*, 40, 647 (1995)], anti-PSMA antibody [J.Urology, 160, 2396 (1998)], anti-VEGF antibody [*Cancer* Res, 57, 4593 (1997), Avastin (R)], anti-VEGFR antibody [*Oncogene*, 19, 2138 (2000), WO96/30046], anti-CD20 antibody [*Curr. Opin. Oncol.*, 10, 548 (1998), US5, 736, 137, Rituxan (R), Ocrelizumab, Ofatumumab], anti-EGFR antibody (Erbitux (R), Vectivix (R)), anti-HER2 antibody (*Proc. Natl. Acad. Sci. USA*, 89, 4285 (1992), US5, 725, 856, Herceptin (R), Pertuzumab), anti-HER3 antibody (US2008/0124345), c-Met antibody (US6, 468, 529), anti-CD10 antibody, anti-EGFR antibody (WO96/402010), anti-Apo-2R antibody (WO98/51793), anti-ASCT2 antibody (WO2010/008075), anti-CEA antibody [*Cancer* Res., 55 (23 suppl): 5935s-5945s, (1995)], anti-CD38 antibody, anti-CD33 antibody, anti-CD22 antibody, anti-CD20 amino acid modified antibody (*Immunology*, 115, 4393, 2010.), anti-EpCAM antibody, anti-A33 antibody, anti-folate receptor antibody (MRAb-003) and the like.

[0124]

Examples of the antibody which recognizes an allergy- or inflammation-related antigen include anti-interleukin 6 antibody [*Immunol. Rev.*, 127, 5 (1992)],

anti-interleukin 6 receptor antibody [*Molecular Immunol.*, 31, 371 (1994)], anti-interleukin 5 antibody [*Immunol. Rev.*, 127, 5 (1992)], anti-interleukin 5 receptor antibody, anti-interleukin 4 antibody [*Cytokine*, 3, 562 (1991)], anti-interleukin 4 receptor antibody [*J. Immunol. Meth.*, 217, 41 (1998)], anti-tumor necrosis factor antibody [*Hybridoma*, 13, 183 (1994)], anti-tumor necrosis factor receptor antibody [*Molecular Pharmacol.*, 58, 237 (2000)], anti-CCR4 antibody [*Nature*, 400, 776 (1999)], anti-chemokine antibody [Peri *et al.*, *J. Immuno. Meth.*, 174, 249-257 (1994)], anti-chemokine receptor antibody [*J. Exp. Med.*, 186, 1373 (1997)] and the like.

Examples of the antibody which recognizes a cardiovascular disease-related antigen include anti-GpIIb/IIIa antibody [*J. Immunol.*, 152, 2968 (1994)], anti-platelet-derived growth factor antibody [*Science*, 253, 1129 (1991)], anti-platelet-derived growth factor receptor antibody [*J. Biol. Chem.*, 272, 17400 (1997)], anti-blood coagulation factor antibody [*Circulation*, 101, 1158 (2000)], anti-IgE antibody, anti-αVβ3 antibody, anti-α4β7 antibody, and the like.

[0125]

Examples of the antibody which recognizes virus- or bacterial infection-related antigen includes anti-gp120 antibody [*Structure*, 8, 385 (2000)], anti-CD4 antibody [*J. Rheumatology*, 25, 2065 (1998)], anti-CCR5 antibody, anti-verotoxin antibody [*J. Clin. Microbiol.*, 37, 396 (1999)], anti-M2 antibody (JP2003-235575) and the like.

[0126]

The effector activity of a monoclonal antibody produced by the method of the present invention can be controlled by various methods. Examples of the known methods include 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 by modifying amino acid residue(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.

[0127]

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.

[0126]

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

[0128]

As a method for lowering a content of fucose which is bound to a complex type N-linked sugar chain bound to Fc region 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.

[0129]

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.

[0130]

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 of the antibody described in US2007/0148165.

[0131]

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

[0133]

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 while suspending in the culture solution.

[0134]

As long as the cell does not adhere to the cell culture anchorage, the cell may survive and grow in a state of a single cell in the culture solution or survive and grow in a state of a mass of cells formed by the agglutination of two or more cells.

[0135]

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 solution 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.

[0136]

The culture container for tissue culture may be any one such as a flask, a Petri dish and the like as long as it is coated for adhesion culture is applied thereto. Particularly, for example, whether or not it is a suspension mammalian cell can be confirmed using commercially available tissue culture flask (manufactured by Greiner), adhesion culture flask (manufactured by Sumitomo Bakelite) and the like.

[0137]

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.

[0138]

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.

[0139]

The above "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 adapting the suspension culture or superior to those before adapting to suspension culture (*J. Biotechnol.*, 2007, 130(3), 282 - 90).

[0140]

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.

[0141]

In the present invention, examples of the method for adapting an adhesive mammalian cell to suspension culture conditions 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.

[0142]

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 solution.

[0143]

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.

[0144]

In the present invention, as a property possessed by the suspension mammalian cell, suspension culturing is carried out under the condition of 2×10^5 cells/ml, and then 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.

[0145]

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.

[0146]

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

[0147]

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 suspension cell culture (manufactured by Corning), a T-flask (manufactured by Becton Dickinson), a conical flask (manufactured by Corning) and the like.

[0148]

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, e.g., Wave Bioreactor (manufactured by GE Healthcare Bioscience), can be also used.

[0149]

Regarding the suspension culture conditions for 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.

[0150]

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.

[0151]

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 production method of the present invention.

[0152]

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 solution or cell homogenate containing the protein of interest. Examples of the separation method include centrifugation, dialysis, ammonium sulfate precipitation, column chromatography, a filtering and the like. The separation can be carried out based on the difference in physicochemical properties of the protein of interest and impurities or the difference in their avidity for the column carrier itself.

[0153]

As the method for purifying the protein of interest, the purification is carried out 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) and the like.

[0154]

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.

[0155]

The present invention has been described in the above 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 in the present specification, but is limited by the claims alone.

[0156]

Various experimental techniques relating to recombination described in the followings, 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

[0157]

[Example 1]

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

A plasmid which comprises 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.

[0158]

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.

[0159]

In the nucleotide sequence (SEQ ID NO:1)of the non-autonomous Tol2 transposon disclosed by Japanese Published Unexamined Patent Application No.235575/2003, 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.

[0160]

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.

[0161]

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.

[0162]

The N5LG1_M2_Z3 vector (WO2006/061723) into which a nucleotide sequence (SEQ ID NO:9) encoding the H chain (SEQ ID NO:10) and a nucleotide sequence (SEQ ID NO:11) encoding the L chain (SEQ ID NO:12) of the anti-human influenza M2 antibody Z3G1 (ATCC Deposit No. PTA-5968: deposited March 13, 2004, American Type Culture Collection, Manassas, VA, USA) were inserted under the control of the CMV enhancer/promoter control was used as an antibody gene expression cassette.

[0163]

The DNA fragment comprising the Tol2-R sequence was inserted into the restriction enzyme *Nru*I site positioned at the 5'-terminal side of a gene fragment comprising the antibody gene expression cassette and a selectable marker gene on the N5LG1_M2_Z3 vector. Then, the DNA fragment comprising the Tol2-L sequence was inserted into the restriction enzyme *Fse*I and *Asc*I sites positioned at the 3'-terminal side.

[0164]

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 in which 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) is connected under the control of the CMV enhancer/promoter into the *Fse*I recognition site of the N5LG1_M2_Z3 vector connected with the Tol2 transposon sequence.

[0165]

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

[0166]

[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) to prepare a Tol2 transposase expression vector (hereinafter referred to as Tol2 vector) (Fig. 3).

[0167]

[Example 3]

Preparation of transformant using mammal animal cell

(1) Preparation of suspension CHO cell

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

[0168]

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 the serum-free α-MEM medium and confirming that the cells have the same growing ability as the case of their culturing in the presence of serum.

[0169]

(2) Preparation of CHO cell which produces antibody

As the expression vector, the transposon vector for expressing the anti-human influenza M2 antibody prepared in Example 1 and Example 2 (hereinafter referred to as a transposon vector) and Tol2 vector pCAGGS-T2TP (Fig. 3, Kawakami K. & Noda T., *Genetics*, 166, 895 - 899 (2004)) were used. In addition, the anti-human influenza M2 antibody expression vector having no transposon sequences was used as the control.

[0170]

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

[0171]

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 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 vector was directly introduced in the form of circular DNA for the purpose of preventing from integrating into the host chromosome.

[0172]

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 introduction method by electroporation.

[0173]

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.

[0174]

After the gene introduction 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.

[0175]

Next, from the day of medium exchange on the 4th day of the gene introduction, 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.

[0176]

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.

[0177]

[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

[0178]

[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

[0179]

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 cells into which the anti-human influenza M2 antibody expression vector was introduced as in the other cell lines, but cycloheximide-resistant transformants were obtained from the cell into which the transposon vector for expressing anti-human influenza M2 antibody was introduced with a high frequency.

[0180]

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 cells.

[0181]

Based on these results, it was found that the gene encoding a protein of interest and cycloheximide resistance gene which were inserted between a pair of transposon sequences were efficiently introduced into the chromosome of the host cell in the suspension mammalian cell.

[0182]

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

In order to examine antibody production efficiency in a suspension CHO cell or an adhesive CHO cell, the amounts of antibodies produced by each cell line were

examined. As the suspension CHO cell, a suspension CHO-K1 cell adapted to suspension culture was used. In addition, as the adhesive CHO cell, an adhesive CHO-K1 cell before adaptation to suspension culture was used.

[0183]

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

[0184]

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 of the 4th day of the electroporation, 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.

[0185]

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

[0186]

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 after static culturing for 3 days, the amount of the antibody protein was measured by HPLC using the culture supernatant.

[0187]

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. 4.

[0188]

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.

[0189]

Based on these results, it was found that, in order to express a protein of interest using a transposon vector, the protein of interest could be expressed at a high level when a suspension mammalian cell is used.

[0190]

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

[0191]

[Example 4]

Preparation of antibody expression cell using Tol1 transposon and antibody preparation (1)

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

In the same manner as in Example 1, a plasmid which comprised 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.

[0192]

Each DNA of the used genes was chemically synthesized in the artificial way 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 restriction enzyme digestion site was added to the end of the primer.

[0193]

In the nucleotide sequence of the non-autonomous Tol1 transposon represented by SEQ ID NO:13 in 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.

[0194]

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.

[0195]

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, existing on the 5'-terminal side of a gene fragment comprising the antibody gene expression cassette and a selectable marker gene on the N5LG1_M2_Z3 vector, 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.

[0196]

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 in which a resistance gene for cycloheximide (a gene in which proline at position 54 in the human ribosomal protein L36a was mutated to glutamine) is connected under the control of the CMV enhancer/promoter into the *Fse*I recognition site of the N5LG1_M2_Z3 vector connected with the Tol1 transposon sequence.

[0197]

(2) 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 in which a DNA fragment encoding a medaka fish-derived Tol1 transposase (SEQ ID NO:17) comprised of the nucleotide sequence represented by SEQ ID NO:16 was connected under the CMV enhancer/promoter control was inserted into pBluescriptII SK (+) (manufactured by Stratagene) and used as the Tol1 transposase expression vector pTol1ase (Fig. 6).

[0198]

(3) Preparation of CHO cell which produces antibody

Using the expression vectors prepared in the above (1) to (3), the introduction efficiency of the expression vector by Tol1 transposon was examined in the same manner as Example 3. The result was shown in Table 3.

[0199]

[Table 3]

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

[0199]

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 as in the case with Example 3 in which the Tol2 transposon vector for expressing the anti-human influenza M2 antibody was introduced.

[0200]

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.

[0201]

(4) Examination on antibody production by suspension CHO cell

Antibody production efficiency of the suspension CHO cell was examined using the Tol1 transposon in the same manner as Example 3(3).

[0202]

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

[0203]

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

[0204]

[Example 5] Preparation of anti-human CD98 antibody

(1) Preparation of anti-human CD98 antibody heavy chain expression transposon vector and anti-human CD98 antibody light chain expression transposon vector

In order to prepare an anti-human CD98 antibody having the variable region H chain and L chain represented by the amino acid sequences of SEQ ID NOs:20 and 23, respectively, amino acid sequences of the H chain and L chain were prepared by connecting amino acid sequence of human IgG1 antibody constant region to each antibody variable region.

[0205]

Using the sequences integrated into a vector (N5KG1-Val C2IgG1NS/I117L)

disclosed in Japanese Patent No. 4324637 as the gene sequences (SEQ ID Nos:18 and 21, respectively) of the anti-human CD98 antibody heavy chain variable region and light chain variable region to which a signal sequence had been connected, and using the transposon sequence, and promoter similar to those used in Example 1, an anti-human CD98 antibody heavy chain expression transposon vector (hereinafter, referred to as CD98H vector) and an anti-human CD98 antibody light chain expression transposon vector (hereinafter, referred to as CD98L vector) were respectively constructed (Figs. 8 and 9).

[0206]

The DNA fragment to be used was chemically synthesized in the artificial way based on the conventionally known sequence or obtained by preparing primers of its both terminal sequences and carrying out PCR using an appropriate DNA source as the template. A restriction enzyme digestion site was attached to a terminal of each primer for the sake of the later gene recombination operations.

[0207]

(2) Preparation of cycloheximide resistance gene expression transposon vector

A cycloheximide resistance gene expression transposon vector (hereinafter, referred CHX vector) was constructed by connecting the sequence encoding a cycloheximide resistance gene (SEQ ID NO:7) under control of the CMV enhancer/promoter described in Example 1 and inserting a pair of transposon sequences (Tol-2L, Tol2-R) into both terminals of the cycloheximide resistance gene expression cassette (Fig. 10).

[0208]

The DNA fragment to be used was artificially chemically synthesized based on the conventionally known sequence or obtained by preparing primers of its both terminal sequences and then carrying out PCR using an appropriate DNA source as the template. A restriction enzyme digestion site was attached to a terminal of each primer for the sake of the later gene recombination operations.

[0209]

(3) Preparation of CHO cell which produces anti-human CD98 antibody

The CD98H vector (Fig. 8), CD98L vector (Fig. 9) and CHX vector (Fig. 10) prepared in the above-mentioned (1) and (2) and the Tol2 vector (Fig. 3) prepared in Example 2 were introduced into CHO-K1 cell which was adapted to suspension culture, and the number of appeared cells capable of highly expressing the antibody was compared.

[0210]

In the test plot, 4×10^6 cells of the CHO-K1 cell were suspended in 400 μl of

PBS, and CD98H vector (10 μg), CD98L vector (10 μg), CHX vector (10 μg) and Tol2 vector (10 μg) were directly co-transfected in a form of circular DNA by electroporation. In order to express Tol2 transposase transiently and to prevent integration into the host chromosome, the Tol2 vector was introduced directly in the form of circular DNA. The electroporation was carried out using an electroporator (Gene Pulser Xcell system, manufactured by Bio-Rad) under conditions of voltage of 300 V, electrostatic capacity of 500 μF and room temperature and using a cuvette of 4 mm in gap width (manufactured by Bio-Rad).

[0211]

Also, in the control plot, each of CD98H vector (10 μg), CD98L vector (10 μg) and CHX vector (10 μg) was linearlized using a restriction enzyme *Pci*I (Takara Bio Inc.) and then electroporation was carried out in the same manner as the above.

[0212]

After the gene introduction by electroporation, the cells in each cuvette were suspended in a CD OptiCHO medium supplemented with 0.5% soybean hydrolyzate (hereinafter, referred to as 0.5CD medium), inoculated onto one 96-well plate and cultured for 4 days in a CO_2 incubator. Next, from the medium exchange after 5 days of the gene introduction, culturing was carried out in the presence of cycloheximide using the 0.5CD medium supplemented with 3 μg/ml of cycloheximide (C4859, Sigma-Aldrich) followed by culturing for 4 weeks while carrying out the medium exchange at intervals of one week.

[0213]

After 4 weeks of the culturing, expression of the antibody was determined by a sandwich method (LENCETM, Perkin-Elmer Corp) using FRET (fluorescence resonance energy transfer). Regarding the antibody high expression cells, clones expressing the antibody at a concentration in culture supernatant of 5.0 μg/ml or more were counted as the antibody-expressing cells, with the results shown in Table 4.

[0214]

[Table 4]

	Control plot	Test plot
	The number of wells where the antibody is expressed	
Plate 1	10/96	29/96
Plate 1	20/96	49/96

[0215]

As shown in Table 4, large number of anti-human CD98 antibody expression cells were found in the test plot in which Tol2 vector was co-transfected into the suspension CHO-K1 cell together with anti-human CD98 heavy chain expression

transposon vector, anti-human CD98 light chain expression transposon vector and cycloheximide resistance gene vector, but the anti-human CD98 antibody expression cells were not found in the control plot in which Tol2 vector was not co-transfected in spite of making the vectors into linear chains.

[0216]

[Example 6] Production of anti-human CD98 antibody

(1) Preparation of expression transposon vector comprising anti-human CD98 antibody heavy chain gene fragment, anti-human CD98 antibody light chain gene fragment and cycloheximide resistance gene

An expression transposon vector containing anti-human CD98 antibody heavy chain gene fragment, anti-human CD98 antibody light chain gene fragment and cycloheximide resistance gene (hereinafter, referred to as CD98-CHX tandem vector) was constructed using a synthetic DNA and a PCR method in the same manner as in the above by connecting the anti-human CD98 antibody heavy chain expression transposon vector prepared in Example 5(1) with the anti-human CD98 antibody light chain expression gene cassette prepared in Example 5(1) and the cycloheximide resistance gene cassette prepared in Example 5(2).

[0217]

(2) Preparation of expression transposon vector comprising anti-human CD98 antibody heavy chain gene fragment and cycloheximide resistance gene

An expression transposon vector comprising anti-human CD98 antibody heavy chain gene fragment and cycloheximide resistance gene (hereinafter, referred to as CD98H-CHX expression transposon vector) was constructed using a synthetic DNA and a PCR method in the same manner as in the above by connecting the anti-human CD98 antibody heavy chain expression transposon vector prepared in Example 5(1) with the cycloheximide resistance gene cassette prepared in Example 5(2).

[0218]

(3) Preparation of CHO cell producing anti-human CD98 antibody

Using the expression transposon vectors prepared in the above Example 5(1) and (2) and the above Example 6(1) and (2), the incidence of cells capable of highly expressing anti-CD98 antibody were compared on the case of gene-transferring H chain and L chain of anti-human CD98 antibody using the same expression vector (control plot), on the case of gene-transferring H chain or L chain of anti-human CD98 antibody or cycloheximide resistance gene respectively using different expression vectors (test plot 1) and on the case of gene-transferring H chain or L chain using different expression vectors (test plot 2).

[0219]

In the test plot 1, 4×10^6 cells of the CHO-K1 cell were suspended in 400 μl of PBS, and CD98H vector (10 μg), CD98L vector (10 μg), CHX vector (10 μg) and Tol2 vector (10 μg) were directly co-transfected as circular DNA by electroporation.

[0220]

In the test plot 2, 4×10^66 cells of the CHO-K1 cell were suspended in 400 μl of PBS, and CD98H-CHX vector (10 μg), CD98L vector (10 μg) and Tol2 vector (10 μg) were directly co-transfected as circular DNA by electroporation.

[0221]

In the control plot, 4×10^6 cells of the CHO-K1 cell were suspended in 400 μl of PBS, and CD98-CHX tandem vector (10 μg) and Tol2 vector (20 μg) were directly co-transfected as circular DNA by electroporation. Also, in all of the tests, in order to express Tol2 transposase transiently and to prevent integration into the host chromosome, the Tol2 vector was introduced directly in the form of circular DNA.

[0222]

In the following method, the incidence of antibody producing cells was confirmed in the same manner as Example 5(3). Regarding the antibody producing cells, the clones in which the antibody concentration in culture supernatant was 3.0 μg/ml or more were counted as the antibody-expressing cells. The results are shown in Table 5.

[0223]

[Table 5]

	Control plot	Test plot 1	Test plot 2
	The number of wells where the antibody is expressed		
Plate 1	18/96	82/96	95/96
Plate 2	21/96	85/96	96/96
Total	39/192	167/192	191/192

[0224]

In the test plot 1 in which CD98H vector, CD98L vector and CHX vector were introduced and the test plot 2 in which CD98H vector and CD98L vector were introduced, the incidence of the cells capable of highly expressing the anti-human CD98 antibody was markedly increased.

[0225]

The above results show that cells having a high antibody productivity can be easily obtained and produced when different expression vectors in which the antibody heavy chain gene and antibody light chain gene are respectively inserted between transposon sequences are co-transfected into the suspension CHO cell, in comparison with a case in which an expression vector prepared by integrating the antibody heavy

chain gene and antibody light chain gene into the same expression vector is introduced to the suspension CHO cell. In addition, it was revealed from the results of test plot 1 and test plot 2 that even when the vector to be introduced is two or more, at least one drug resistance gene (selectable marker gene) is enough. Further, it was revealed that the drug resistance gene may be present on an expression vector into which the antibody heavy chain gene is integrated or on a different independent vector.

[0226]

The above results show that a transposon vector is effective as a means for efficiently introducing genes arranged on two or more vectors into a suspension of mammalian cells, which was conventionally difficult to achieve. Further, it is shown that for the purpose of achieving high productivity of a protein comprising more than one polypeptides or of more than one proteins, it is effective to introduce polypeptides and proteins using different transposon vectors.

[0227]

(4) Culturing of CHO cell which produces anti-human CD98 antibody

The top three cell lines having high antibody productivity were selected from each of the cells into which the CD98-CHX tandem vector obtained in the above-mentioned Example 6(3) was introduced and the cells into which the CD98H-CHX vector and CD98L vector were introduced, and their antibody expression levels were compared. Details of the tests are shown below.

[0228]

The CHO-K1 cell obtained in Example 6(3) which was selected based on the cycloheximide resistance and also expresses the anti-CD98 antibody, was expansion-cultured using a 96-well plate, a 24-well plate and a 6-well plate (Corning Glassworks) in that order. After the expansion culturing, antibody concentration in each culture supernatant was measured, and the top three cell lines CHO cells having high level of anti-CD98 antibody expression were selected. Next, each of the thus selected three cell lines were suspended in 3 ml of 0.5% CD medium (Invitrogen), namely 0.5 CD medium, to a density of 2×10^5 cells/ml, and cultured on a shaker for 5 days in an atmosphere of 37°C and 5% CO_2 using a 6-well plate. The amount of the antibody in the medium after 5 days of culturing was determined by HPLC (Waters Associates, Inc.). The results are shown in Table 6.

[0229]

[Table 6]

	Cells derived from control plot			Cells derived from test plot 2		
Expression level of antibody	70	67	41	196	87	67

(mg/L)

[0230]

As shown in Table 6, the CHO-K1 cell into which CD98H vector and CD98L vector were co-transfected has a high antibody production level in comparison with the CHO-K1 cell into which CD98-CHX tandem vector was introduced.

[0231]

The above results show that not only an antibody high producer cell line can be obtained and produced easily, but also the thus obtained cell has a high antibody productivity, when different expression vectors in which the antibody heavy chain gene and antibody light chain gene are respectively inserted between a pair of transposon sequences are co-transfected into the suspension CHO cell.

[0232]

[Example 7] Production of anti-human tumor necrosis factor-alpha (TNFα) antibody

(1) Preparation of expression transposon vector containing a TNFα antibody heavy chain gene fragment, a TNFα antibody light chain gene fragment and cycloheximide resistance gene

In order to prepare anti-human TNFα antibody having the amino acid sequence of SEQ ID NO:26 and SEQ ID NO:29, an anti-human TNFα antibody heavy chain gene fragment, an anti-human TNFα antibody light chain gene fragment and a cycloheximide resistance gene expression transposon vector (hereinafter, referred to as TNFα-CHX tandem vector) were constructed by replacing VH and VL gene fragments of the expression transposon vector comprising the anti-human CD98 heavy chain gene fragment and light chain gene fragment and cycloheximide resistance gene prepared in Example 6(1) (CD98-CHX tandem vector) by the anti-human TNFα antibody-derived VH and VL, respectively.

[0233]

The sequences of anti-human TNFα antibody heavy chain gene and light chain gene were prepared using a synthetic DNA, by preparing amino acid sequences (SEQ ID NOs:26 and 29) in which a signal sequence was connected to the amino acid sequences (SEQ ID NOs:25 and 28) of the heavy chain variable region subunit or light chain variable region subunit of Adalimumab (recombinant) described in Fig. 1 and Fig.2, respectively, of HUMIRA(R) subcutaneous injection 40 mg inspection report (Pharmaceutical and Medical Devices Agency, February 14, 2008) and determining the nucleotide sequences in such a manner that the amino acid sequences did not change (SEQ ID NOs:24 and 27) . For the sake of the latter gene manipulations, a restriction enzyme digestion site was added to the terminal of the artificial sequences.

[0234]

(2) Preparation of expression transposon vector comprising anti-human TNFα antibody heavy chain fragment and cycloheximide resistance gene

An expression transposon vector containing anti-human TNFα antibody heavy chain fragment and cycloheximide resistance gene (hereinafter, referred to as TNFαH-CHX vector) was constructed by modifying a VH gene fragment region of the expression transposon vector containing anti-human CD98 antibody heavy chain fragment and cycloheximide resistance gene (CD98H-CHX vector) prepared in Example 6(2) to an anti-human TNFα antibody VH gene fragment. As the anti-human TNFα antibody heavy chain gene, a sequence of the same sequence shown in this item (1) was used.

[0235]

(3) Preparation of anti-human TNFα antibody light chain gene expression transposon vector

An anti-human TNFα antibody light chain gene expression transposon vector (hereinafter, referred to as CD98L vector) was constructed by modifying the light chain gene region of the anti-human CD98 antibody light chain gene expression transposon vector prepared in Example 6(1) to anti-human TNFα antibody light chain. As the anti-human TNFα antibody VL gene, the same sequence as the sequence shown in this item (1) was used.

[0236]

(4) Preparation of CHO cell which produces anti-human TNFα antibody

In order to prepare CHO-K1 cell which produced anti-human TNFα antibody, the TNFα-CHX tandem vector (20 μg) prepared in the above-mentioned (1) and the Tol2 transposase expression vector (Tol2 vector) (10 μg) prepared in Example 2 were introduced into CHO-K1 cell adapted to suspension culturing prepared in Example 3 (control plot).

[0237]

In the same manner, the TNFαH-CHX vector (10 μg), TNFα L vector (10 μg) and Tol2 vector (10 μg) prepared in the above-mentioned (2) and (3) were directly co-transfected in the form of circular DNA (test plot). The incidences of cells capable of highly expressing the antibody were compared by carrying out the gene introduction, cell culturing and the like in the same manner as in Example 6 except that culturing of the gene-introduced cells was carried out on five plates of the 96-well plate. Regarding the cell having a high antibody productivity, the clones in which the antibody concentration in culture supernatant was 3.0 μg/ml or more were counted as the antibody-expressing cells. The results are shown in Table 7.

[0238]

[Table 7]

	Control plot	Test plot
	The number of wells where the antibody is expressed	
Plate 1	20/96	83/96
Plate 2	22/96	76/96
Plate 3	21/96	82/96
Plate 4	20/96	79/96
Plate 5	27/96	81/96
Total	110/480	401/480

[0239]

As shown in Table 7, as in the case of the anti-human CD98 antibody producing cell prepared in Example 6, the CHO-K1 cell into which TNFαH-CHX vector and TNFαL vector were co-transfected showed about 4 times higher incidence of cells in which the anti-human TNFα antibody was highly expressed, in comparison with the CHO-K1 cell into which TNFα-CHX tandem vector was introduced.

[0240]

This result shows that, regarding any case of the antibody, a cell line having a high antibody productivity can be easily obtained and produced by co-transfecting the antibody heavy chain gene and the antibody light chain gene which are respectively inserted between a pair of transposon sequences introduced into different expression vectors, in the suspension CHO cell.

[0241]

(5) Culturing of CHO cell which produces anti-human TNFα antibody

The cells which are selected based on the cycloheximide resistance from the TNFα-CHX tandem vector-introduced cells obtained in the above-mentioned (4) and the cells into which the TNFαH-CHX vector and TNFαL vector were co-transfected, and also expressing the anti-human TNFα antibody, were selected and expansion-cultured using 96-well plate, 24-well plate and 6-well plate in that order. Regarding 4 cell lines of the TNFα-CHX tandem vector-introduced cells succeeded in the expansion culturing and 52 cell lines into which the TNFαH-CHX vector and TNFαL vector were co-transfected, these cells were cultured in the same manner as in Example 6(4) except that the culturing period was 7 days, and the expression levels of the antibodies were measured. The results are shown in Fig. 11.

[0242]

As a result, the CHO-K1 cell into which the TNFαH-CHX vector and TNFαL vector were co-transfected showed about 2.4 times higher antibody productivity than that of the CHO-K1 cell into which the TNFα-CHX tandem vector was introduced.

[0243]

This result shows that, as in the case of Example 6(4), not only a cell having a high antibody productivity can be obtained and produced, but also the thus obtained cell has a high antibody productivity, when different expression vectors in which each of the antibody heavy chain gene and the antibody light chain gene are respectively inserted between a pair of transposon sequences are co-transfected into the suspension CHO cell.

[0244]

[Example 8] Production of anti-human CD20 antibody

(1) Preparation of expression transposon vector comprising anti-human CD20 antibody heavy chain gene fragment, anti-human CD20 antibody light chain gene fragment and cycloheximide resistance gene

In order to prepare an anti-human CD20 antibody comprising VH and VL represented by the amino acid sequences of SEQ ID NOs:32 and 35, respectively, an expression transposon vector comprising an anti-human CD20 antibody heavy chain gene fragment, an anti-human CD20 antibody light chain gene fragment and a cycloheximide resistance gene (hereinafter, referred to as CD98-CHX tandem vector) was constructed by replacing antibody VH and VL gene regions of the CD98-CHX tandem vector prepared in Example 6(1) by the anti-human CD20 antibody-derived VH and VL, respectively.

[0245]

The gene sequences of anti-human CD20 antibody VH region and VL region were prepared using a synthetic DNA, by preparing the nucleotide sequence described in GenBank accession No. AR000013 and amino acid sequences (SEQ ID NOs:31 and 34, respectively) in which a signal sequence was connected to the amino acid sequences (SEQ ID NOs:32 and 35, respectively) of the VH and VL of rituximab described in accompanying sheet of Rituxan(R) for injection 10 mg/ml inspection report (reported by National Institute of Health Sciences, No. 3395, August 28, 2003) and determining the nucleotide sequences in such a manner that the amino acid sequences did not change (SEQ ID NOs:30 and 33). For the sake of the latter gene manipulations, a restriction enzyme digestion site was added to the terminal of the artificial sequences.

[0246]

(2) Preparation of expression transposon vector comprising anti-human CD20 antibody heavy chain gene fragment and cycloheximide resistance gene

An expression transposon vector comprising anti-human CD20 antibody heavy chain gene fragment and cycloheximide resistance gene (hereinafter, referred to as CD20H-CHX vector) was constructed by modifying the antibody VH gene region of the CD98H-CHX vector prepared in Example 6(2) to an anti-human CD20

antibody-derived VH. As the anti-human CD20 antibody heavy chain gene, the same sequence as a sequence shown in the above-mentioned (1) was used.

[0247]

(3) Preparation of anti-human CD20 antibody light chain gene expression transposon vector

An anti-human CD20 antibody light chain gene expression transposon vector (hereinafter, referred to as CD20L vector) was constructed by modifying the VL gene regions of the anti-human CD98 antibody prepared in Example 6(1) to the anti-human CD20 antibody-derived VL. As the anti-human CD20 antibody heavy and light genes, the same sequences as a sequence shown in the above-mentioned (1) were used.

[0248]

(4) Preparation of CHO cell which produces anti-human CD20 antibody

In order to prepare CHO-K1 cell which produces anti-human CD20 antibody, the CD20-CHX tandem vector prepared in the above-mentioned (1) and the Tol2 transposase expression vector (Tol2 vector) prepared in Example 2 were introduced into CHO-K1 cell adapted to suspension culturing prepared in Example 3(1) (control plot).

[0249]

In the same manner, the CD20H-CHX vector (10 μg) and CD20L vector (10 μg) prepared in the above-mentioned (2) and (3) were co-transfected into CHO-K1 cell together with Tol2 vector (10 μg) (test plot). The incidences of cells capable of highly expressing the antibody were compared by carrying out the gene introduction, cell culturing and the like in the same manner as Example 6 except that culturing of the gene-introduced cells was carried out on five plates of the 96-well plate. Also, antibody concentrations of 3.0 μg/ml or more were counted as the antibody-expressing wells. The results are shown in Table 8.

[0250]

[Table 8]

	Control plot	Test plot
	The number of wells where the antibody is expressed	
Plate 1	2/96	4/96
Plate 2	2/96	9/96
Plate 3	4/96	4/96
Plate 4	1/96	8/96
Plate 5	2/96	5/96
Total	11/480	30/480

[0251]

As a result, the CHO-K1 cell into which the CD20H-CHX vector and CD20L

vector were co-transfected showed about 3 times higher incidence of cells which highly expresses the anti-human CD20 antibody in comparison with the CHO-K1 cell into which the CD20-CHX tandem vector was introduced.

[0252]

This result is similar to the result of the case of anti-human CD98 antibody and anti-human TNFα antibody carried out in Example 6(3) or Example 7(3) and shows that an antibody high level producer cell line can be easily obtained and produced regarding each case of the antibodies when different expression vectors in which each of the antibody heavy chain gene and the antibody light chain gene are respectively integrated between transposon sequences are co-transfected into the suspension CHO cell.

[0253]

(5) Culturing of CHO cell which produces anti-human CD20 antibody

The cells which are selected based on the cycloheximide resistance from the CD20-CHX tandem vector-introduced cells obtained in the above-mentioned (3) and the cells into which the CD20H-CHX vector and CD20L vector were co-transfected, and also expressing the anti-human CD20 antibody, were selected and expansion-cultured using 96-well plate, 24-well plate and 6-well plate in that order. Regarding 4 cell lines of the control plot cells succeeded in the expansion culturing and 50 cell lines of test plot cells, these cells were cultured in the same manner as in Example 6(4) except that the culturing period was 7 days, and their antibody expression levels were measured. The results are shown in Fig. 12.

[0254]

As shown in Fig. 12, it was revealed that the CHO-K1 cell into which the CD20H-CHX vector and CD20L vector were co-transfected had about 1.6 times higher antibody productivity than the CHO-K1 cell into which the CD20-CHX tandem vector was introduced.

[0255]

This result is similar to the result of the case of anti-human CD98 antibody and anti-human TNFα antibody carried out in Example 6(4) or Example 7(5) and shows that not only a cell line having a high antibody productivity can be easily obtained and produced when different expression vectors in which the antibody heavy chain gene and antibody light chain gene are respectively integrated between transposon sequences are co-transfected into the suspension CHO cell, but also the thus obtained cell has a high antibody productivity.

[0256]

[Example 9] Preparation of a transposon vector which expresses neomycin resistance gene and anti-human CD98 antibody

(1) Preparation of a transposon vector which expresses wild type neomycin resistance gene and anti-human CD98 antibody

A plasmid which comprised a gene expression cassette for mammalian cell use comprising an arbitrary human antibody gene and a drug resistance marker gene inserted between a pair of Tol2-derived nucleotide sequences was used as the plasmid vector for protein expression.

[0257]

The DNA of the gene to be used was obtained by carrying out chemical synthesis in the artificial way based on a conventionally known nucleotide sequence or by preparing primers of its both terminal sequences and thereby carrying out PCR using an appropriate DNA source. For the sake of the latter gene manipulations, a restriction enzyme digestion site was added to the primer terminal.

[0258]

In the non-autonomous Tol2 transposon nucleotide sequence (SEQ ID NO:1) disclosed by JP-A-2003-235575, a nucleotide sequence at positions 1 to 200 (Tol2-L sequence) (SEQ ID NO:2) and a nucleotide sequence at positions 2285 to 2788 (Tol2-R sequence) (SEQ ID NO:3) were used as the transposon sequences.

[0259]

A DNA fragment comprising either of the Tol2-R sequence and Tol2-L sequence was synthesized.

[0260]

A DNA fragment including a nucleotide sequence (SEQ ID NO:18) which encodes antibody H chain under control of CMV promoter, amplified based on the anti-human CD98 antibody N5KG1-Val C2IgG1NS/I117L vector (Japanese Patent No. 4324637), was prepared as the antibody heavy chain gene cassette, and a DNA fragment comprising a nucleotide sequence (SEQ ID NO:21) which encoded antibody light chain under control of SV40 promoter, amplified based on the anti-human CD98 antibody N5KG1-Val C2IgG1NS/I117L vector, as the antibody light chain gene cassette.

[0261]

As the neomycin resistance gene cassette, a DNA fragment comprising a DNA which comprises a nucleotide sequence encoding a neomycin resistance gene under control of SV40 promoter (a DNA which encodes a neomycin phosphotransferase consisting of the nucleotide sequence represented by SEQ ID NO:36 and GenBank Accession No. U47120.2) was prepared.

[0262]

An anti-CD98 antibody expression vector A was prepared by connecting the above-mentioned antibody heavy chain gene expression cassette, antibody light chain

gene expression cassette and neomycin resistance gene expression cassette and further connecting its both terminals with a DNA fragment comprising a Tol2-R sequence and a DNA fragment comprising a Tol2-L sequence (Fig. 13).

[0263]

(2) Preparation of anti-human CD98 antibody expression transposon vector comprising a modified type neomycin resistance gene 1

An anti-human CD98 antibody expression transposon vector B in which the neomycin resistance gene of the anti-human CD98 antibody expression transposon vector A obtained in (1) which comprises a wild type neomycin resistance gene was replaced by a modified type neomycin resistance gene 1 comprising the nucleotide sequence represented by SEQ ID NO:37 was prepared.

[0264]

The modified type neomycin resistance gene 1 encodes an amino acid sequence identical to that of the wild type neomycin resistance gene and was modified to have a nucleotide sequence in which 167 bases corresponding to 22% of the entire were modified. Specifically, among the total of 32 leucine residues, codons corresponding to 25 leucine residues were modified so as to be TTA.

[0265]

(3) Preparation of anti-human CD98 antibody expression transposon vector comprising a modified type neomycin resistance gene 2

An anti-human CD98 antibody expression transposon vector C in which the neomycin resistance gene of the anti-human CD98 antibody expression transposon vector A obtained in (1) which comprises a wild type neomycin resistance gene was replaced by a modified type neomycin resistance gene 2 comprising the nucleotide sequence represented by SEQ ID NO:38 was prepared.

[0266]

The modified type neomycin resistance gene 2 encoded the amino acid sequence identical to that of the wild type neomycin resistance gene and had a nucleotide sequence in which the 180 bases corresponding to 23% of the entire were modified. Specifically, among the total of 32 leucine residues, codons corresponding to 28 leucine residues were modified so as to be TTA.

[0267]

(4) Preparation of anti-human CD98 antibody expression transposon vector having a modified type neomycin resistance gene 3

An anti-human CD98 antibody expression transposon vector D in which the neomycin resistance gene of the anti-human CD98 antibody expression transposon vector A obtained in (1) which comprises a wild type neomycin resistance gene was

replaced by a modified type neomycin resistance gene 3 comprising the nucleotide sequence represented by SEQ ID NO:39 was prepared.

[0268]

The modified type neomycin resistance gene 3 encoded the amino acid sequence identical to that of the wild type neomycin resistance gene and had a nucleotide sequence in which 203 bases corresponding to 26% of the entire were modified. Specifically, among the total of 32 leucine residues, codons corresponding to 30 leucine residues were modified so as to be TTA.

[0269]

[Example 101 Antibody production by antibody producer CHO cells which expresses modified type neomycin resistance gene

Antibody producing cells A to D were prepared by introducing each of the anti-human CD98 expression transposon vectors A to D prepared in Example 9(1) to (4) into the suspension CHO-K1 cell together with a vector pCAGGS-T2TP which expresses a Tol2 transposase comprising the amino acid sequence represented by SEQ ID NO:40 [Kwakami K. & Noda T., *Genetics*, 166, 895 - 899 (2004)].

[0270]

Introduction of vectors into the suspension CHO cell was carried out by suspending the CHO cell ($4 \times l0^6$ cells) in 400 µl of PBS buffer and co-transfecting the anti-human CD98 antibody expression transposon vector (10 µg) and Tol2 transposase expression vector pCAGGS-T2TP (20 µg) directly in the form of circular DNA by electroporation.

[0271]

In this case, the Tol2 transposase expression vector was also introduced directly as circular DNA in order to transiently express Tol2 transposase.

[0272]

In addition, as a control which did not use Tol2 transposase, the anti-human CD98 antibody expression transposon vector D (10 µg) of Example 19(4) was linearlized using a restriction enzyme *Pci*I (TARA BIO INC.) and then introduced into suspension CHO-K1 cell by electroporation.

[0273]

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

[0274]

After the gene introduction by electroporation, the cells in each cuvette were

inoculated onto one 96-well plate and cultured for 3 days in a CO_2 incubator using a CD OptiCHO medium (Invitrogen) supplemented with 5% soybean hydrolyzate.

[0275]

Next, from the medium exchange after 4 days of the gene introduction, culturing was carried out in the presence of G418 (Geneticin(R), Invitrogen) by adding the G418 to give a final concentration of 500 μg/ml, and the culturing was carried out for 3 weeks while changing the medium at intervals of one week.

[0276]

After the culturing, expression of the antibody was determined using LANCE(R) assay (Perkin-Elmer Corp) by a sandwich method to which FRET (fluorescence resonance energy introduction) was applied. The results are shown in Table 9.

[0277]

[Table 9]

	Antibody producing cells				
	A (Wild Type)	B (Modified Type 1)	C (Modified Type 2)	D (Modified Type 3)	Control cell
Antibody expression level (mg/L) of cells showing maximum expression	0.5	2.0	1.6	5.1	-
Average antibody expression level (mg/L) of top 10 cells	0.5	0.7	0.7	1.7	-

[0278]

As shown in Table 9, expression levels of anti-human CD98 antibody of the cells B to D expressing the modified type neomycin resistance genes were higher than that of the cell A which expressed the wild type neomycin resistance gene.

[0279]

Particularly, in the case of the anti-human CD98 antibody producing cell D which expresses the modified type neomycin resistance gene 3, the cell line showing the 10 times higher expression level than that of the anti-human CD98 antibody producing cell A which expresses the wild type neomycin resistance gene was obtained.

[0280]

In addition, even when the modified type neomycin resistance gene 3 was used, it was not able to obtain a cell which expresses the anti-human CD98 antibody by the control cell into which the Tol2 transposase expression vector was not co-transfected in spite of making the vector into linear form.

[0281]

[Example 11] Preparation of transposon vector expressing puromycin resistance gene and anti-human CD98 antibody

(1) Preparation of anti-human CD98 antibody expression transposon vector comprising modified type puromycin resistance gene 1

An anti-human CD98 antibody expression transposon vector E in which the neomycin resistance gene of the anti-human CD98 antibody expression transposon vector A obtained in Example 9(1) which comprised wild type neomycin resistance gene, was replaced by a modified type puromycin resistance gene 1 consisting of the nucleotide sequence represented by SEQ ID NO:41 was prepared.

[0282]

The modified type puromycin resistance gene 1 encoded an amino acid sequence identical to that of the wild type puromycin resistance gene consisting of the nucleotide sequence represented by SEQ ID NO:42 (a puromycin-N-acetyltransferase gene, consists of the nucleotide sequence disclosed in GenBank Accession No. U07648.1) and had a nucleotide sequence in which 17 bases corresponding to the 3% of the entire bases are modified. Specifically, among the total of 28 alanine residues contained in the puromycin resistance gene, codons corresponding to 17 alanine residues were changed to GCG by the modification and, together with the codons which were already GCG in the wild type, the codons which correspond to all of the alanine residues were changed to GCG.

[0283]

(2) Preparation of anti-human CD98 antibody expression transposon vector comprising modified type puromycin resistance gene 2

An anti-human CD98 antibody expression transposon vector F in which the neomycin resistance gene of the anti-human CD98 antibody expression transposon vector A obtained in Example 9(1) which comprises wild type neomycin resistance gene was replaced by a modified type puromycin resistance gene 2 comprising the nucleotide sequence represented by SEQ ID NO:43 was prepared. The modified type puromycin resistance gene 2 encodes an amino acid sequence identical to that of the wild type puromycin resistance gene and had a nucleotide sequence in which 79 bases corresponding to the 14% of the entire bases are modified. Specifically, in addition to the modification of codons which correspond to the alanine residues of the modified

type puromycin resistance gene 1, the codons corresponding to leucine residues were changed so as to be TTA, and the codons corresponding to valine residues were changed so as to be GTA and the codon of serine were changed so as to be TCG.

[0284]

[Example 12] Antibody production by antibody producing CHO cell which expresses modified type puromycin resistance gene 1

Antibody producing cells E and F were prepared by introducing the anti-human CD98 antibody expression transposon vector E of Example 11(1) comprising the modified type puromycin resistance gene 1, the anti-human CD98 antibody expression transposon vector F of Example 11(2) comprising the modified type puromycin resistance gene 2 and the Tol2 transposase expression vector pCAGGS-T2TP into the suspension CHO-K1 cell.

[0285]

Introduction of the vectors into suspension cell was carried out by suspending the suspension CHO cell ($4 \times l0^6$ cells) in 400 µl of PBS buffer and co-transfecting the anti-human CD98 antibody expression transposon vector comprising the modified type puromycin resistance gene in the form of circular DNA (10 µg) and the pCAGGS-T2TP (20 µg) directly by electroporation.

[0286]

In this case, the Tol2 transposase expression vector pCAGGS-T2TP was also introduced directly in the form of circular DNA in order to transiently express Tol2 transposase.

[0287]

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

[0288]

After the gene introduction by electroporation, the cells in each cuvette were inoculated onto one 96-well plate and cultured for 3 days in a CO_2 incubator using a CD OptiCHO medium (Invitrogen) supplemented with 5% soybean hydrolyzate.

[0289]

Next, from the medium exchange after 2 days of the gene introduction, culturing was carried out for 4 weeks while adding puromycin (P9620, Sigma-Aldrich) to give a final concentration of 5 µg/ml and carrying out the medium exchange to the puromycin-containing medium at intervals of one week.

[0290]

After the culturing, expression level of the antibody was determined using LANCE(R) assay (Perkin-Elmer Corp) by a sandwich method to which FRET (fluorescence resonance energy transfer) was applied. The results are shown in Table 2.

[0291]

[Table 10]

	Antibody producing cells	
	E (Modification 1)	F (Modification 2)
Antibody expression level (mg/L) of cells showing maximum expression	1.0	2.2
Average antibody expression level (mg/L) of top 10 cells	0.7	1.6

[0292]

As shown in Table 10, the antibody producing cell F which expresses the modified type puromycin resistance gene 2 showed two times or more antibody productivity of the antibody producing cell E which expresses the modified type puromycin resistance gene 1.

[0293]

[Example 13] Antibody production by antibody producing CHO cell which expresses modified type puromycin resistance gene 2

The antibody producing cell F obtained in Example 12 which expresses the modified type puromycin resistance gene 2 was cultured using a conical flask to produce anti-human CD98 antibody.

[0294]

Specifically, the antibody producing cell F was expansion-cultured using 96-well plate, 24-well plate and 6-well plate in that order. Two cell lines of the antibody producing cell F in which the number of cell was sufficiently increased (cell line 1 and cell line 2) were selected, and respectively suspended in 35 ml of the CD OptiCHO medium (Invitrogen) supplemented with 5% soybean hydrolyzate so as to give a cell density of 2×10^5 cells/ml and cultured for 1 week on a shaker using a 125 ml capacity of conical flask (with a bent cap, Corning Glassworks) in an atmosphere of 37°C and 5% CO_2, thereby producing the anti-human CD98 antibody.

[0295]

Amount of the antibody in the medium after culturing was determined by HPLC (Waters Associates, Inc.). The results are shown in Table 11.

[0296]

[Table 11]

	Cell line 1	Cell line 2
Antibody expression level (mg/l)	15.6	14.8

[0297]

The above results show that in the suspension CHO cell, the antibody gene inserted between a pair of transposon sequences and the modified type drug resistance gene are introduced efficiently into the host chromosome and also are effective for the selection of a high expression cell. In addition, it was found that the thus obtained cell can be expansion-cultured and production of the protein of interest under a suspension culturing condition is possible.

[0298]

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]

[0299]

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 encoding Cycloheximide Resistance Gene

SEQ ID NO:9 - Description of Artificial sequence; Nucleotide Sequence encoding M2Z3 Antibody H chain

SEQ ID NO:10 - Description of Artificial sequence; Amino Acid Sequence of M2Z3 Antibody H chain

SEQ ID NO:11 - Description of Artificial sequence; Nucleotide Sequence encodingM2Z3 Antibody L chain

SEQ ID NO:12 - Description of Artificial sequence; Amino Acid Sequence of M2Z3 Antibody L chain

SEQ ID NO:13 - Description of Artificial sequence; Nucleotide Sequence of Non-autonomous Tol1

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

SEQ ID NO:15 - Description of Artificial sequence; Toll-R sequence

SEQ ID NO:18 - Description of Artificial sequence; Nucleotide Sequence encoding Anti-CD98 Antibody Heavy Chain Variable Region

SEQ ID NO:19 - Description of Artificial sequence; Amino Acid Sequence of Anti-CD98Antibody Heavy Chain Variable Region

SEQ ID NO:20 - Description of Artificial sequence; Amino Acid Sequence of Anti-CD98 Antibody Heavy Chain Variable Region

SEQ ID NO:21 - Description of Artificial sequence; Nucleotide Sequence encoding Anti-CD98 Antibody Light Chain Variable Region

SEQ ID NO:22 - Description of Artificial sequence; Amino Acid Sequence of Anti-CD98 Antibody Light Chain Variable Region

SEQ ID NO:23 - Description of Artificial sequence; Amino Acid Sequence of Anti-CD98 Antibody Light Chain Variable Region

SEQ ID NO:24 - Description of Artificial sequence; Nucleotide Sequence encoding Anti-human TNFα Antibody Heavy Chain Variable Region

SEQ ID NO:25 - Description of Artificial sequence; Amino Acid Sequence of Anti-human TNFα Antibody Heavy Chain Variable Region

SEQ ID NO:26 - Description of Artificial sequence; Amino Acid Sequence of Anti-human TNFα Antibody Heavy Chain Variable Region

SEQ ID NO:27 - Description of Artificial sequence; Nucleotide Sequence encoding Anti-human TNFα Antibody Light Chain Variable Region

SEQ ID NO:28 - Description of Artificial sequence; Amino Acid Sequence of Anti-human TNFα Antibody Light Chain Variable Region

SEQ ID NO:29 - Description of Artificial sequence; Amino Acid Sequence of Anti-human TNFα Antibody Light Chain Variable Region

SEQ ID NO:30 - Description of Artificial sequence; Nucleotide Sequence encoding Anti-human CD20 Antibody Heavy Chain Variable Region

SEQ ID NO:31 - Description of Artificial sequence; Amino Acid Sequence of Anti-human CD20 Antibody Heavy Chain Variable Region

SEQ ID NO:32 - Description of Artificial sequence; Amino Acid Sequence of Anti-human CD20 Antibody Heavy Chain Variable Region

SEQ ID NO:33 - Description of Artificial sequence; Nucleotide Sequence encoding Anti-human CD20 Antibody Light Chain Variable Region

SEQ ID NO:34 - Description of Artificial sequence; Amino Acid Sequence of Anti-human CD20 Antibody Light Chain Variable Region

SEQ ID NO:35 - Description of Artificial sequence; Amino Acid Sequence of Anti-human CD20 Antibody Light Chain Variable Region

SEQ ID NO:36 - Description of Artificial sequence; Nucleotide Sequence of Wild Type of Neomycin Resistance Gene

SEQ ID NO:37 - Description of Artificial sequence; Nucleotide Sequence of Modified Type of Neomycin Resistance Gene

SEQ ID NO:38 - Description of Artificial sequence; Nucleotide Sequence of Modified Type of Neomycin Resistance Gene

SEQ ID NO:39 - Description of Artificial sequence; Nucleotide Sequence of Modified Type of Neomycin Resistance Gene

SEQ ID NO:41 - Description of Artificial sequence; Nucleotide Sequence of Modified Type of Puromycin Resistance Gene

SEQ ID NO:42 - Description of Artificial sequence; Nucleotide Sequence of Wild Type of Puromycin Resistance Gene

SEQ ID NO:43 - Description of Artificial sequence; Nucleotide Sequence of Modified Type of Puromycin Resistance Gene

SEQ ID NO:44 - Description of Artificial sequence; Nucleotide Sequence of Modified Type of Puromycin Resistance Gene

SEQ ID NO:45 - Description of Artificial sequence; Nucleotide Sequence of Modified type of Zeocin resistance gene

SEQ ID NO:46 - Description of Artificial sequence; Nucleotide Sequence of Modified type of Zeocin resistance gene

SEQ ID NO:47 - Description of Artificial sequence; Nucleotide Sequence of Modified type of Hygromycin resistance gene

SEQ ID NO:48 - Description of Artificial sequence; Nucleotide Sequence of Modified type of Hygromycin resistance gene

Claims

[Claim 1]

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

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

wherein the pair of the transposon sequences are nucleotide sequences derived from a pair of Tol2 transposons which function in a mammalian cell.

[Claim 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) at least one expression vector which comprises a gene fragment comprising a DNA encoding a protein of interest and also comprises a pair of 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 obtaining a suspension mammalian cell which expresses the protein of interest by expressing transiently the transposase from the expression vector which is introduced into the suspension mammalian cell in the step (A) to integrate the gene fragment inserted between a pair of the transposon sequences into a chromosome of the mammalian cell, and

(C) a step of suspension-culturing the suspension mammalian cell which expresses 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 which is capable of surviving and proliferating in a serum-free medium,

wherein the pair of the transposon sequences are nucleotide sequences derived from a pair of Tol2 transposons which function in a mammalian cell.

[Claim 3]

A method for obtaining a suspension mammalian cell which expresses a protein of interest, comprising introducing at least one expression vector which comprises a gene fragment comprising a DNA encoding a protein of interest and also comprises 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,

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

wherein the pair of the transposon sequences are nucleotide sequences derived from a pair of Tol2 transposons which function in a mammalian cell.

[Claim 4]

The method according to any one of claims 1 to 3, wherein at least one of the expression vectors which comprises a gene fragment comprising a DNA encoding the protein of interest and also comprises a pair of transposon sequences at both terminals of the gene fragment is an expression vector which comprises a gene fragment comprising a DNA encoding the protein of interest and a selectable marker gene and also comprises a pair of transposon sequences at both terminals of the gene fragment.

[Claim 5]

The method according to any one of claims 1 to 4, comprising introducing an expression vector which comprises a gene fragment comprising a selectable marker and comprises a pair of transposon sequences at both terminals of the gene fragment into a mammalian cell in addition to the expression vector which comprises a gene fragment comprising a DNA encoding the protein of interest and also comprises a pair of transposon sequences at both terminals of the gene fragment.

[Claim 6]

The method according to any one of claims 1 to 5, wherein the DNA encoding the protein of interest is a DNA encoding an antibody.

[Claim 7]

The method according to any one of claim 6, wherein the DNA encoding an antibody is at least one of a DNA encoding a H chain of the antibody and a DNA encoding a L chain of the antibody.

[Claim 8]

The method according to any one of claims 4 to 7, wherein an expression vector selected from the following (a) to (d) is introduced into a suspension mammalian cell:

(a) an expression vector which comprises a gene fragment comprising a DNA encoding a H chain of an antibody and also comprises a pair of transposon sequences at both terminals of the gene fragment, an expression vector which comprises a gene fragment comprising a DNA encoding a L chain of an antibody and also comprises a

pair of transposon sequences at both terminals of the gene fragment and an expression vector which comprises a gene fragment comprising a selectable marker gene and also comprises a pair of transposon sequences at both terminals of the gene fragment

(b) an expression vector which comprises a gene fragment comprising a DNA encoding a H chain of an antibody and a selectable marker gene and also comprises a pair of transposon sequences at both terminals of the gene fragment and an expression vector which comprises a gene fragment comprising a DNA encoding a L chain of an antibody and also comprises a pair of transposon sequences at both terminals of the gene fragment,

(c) an expression vector which comprises a gene fragment comprising a DNA encoding a L chain of an antibody and a selectable marker gene and also comprises a pair of transposon sequences at both terminals of the gene fragment and an expression vector which comprises a gene fragment comprising a DNA encoding a H chain of an antibody and also comprises a pair of transposon sequences at both terminals of the gene fragment, and

(d) an expression vector which comprises a gene fragment comprising a DNA encoding a H chain and a L chain of an antibody and a selectable marker gene and also comprises a pair of transposon sequences at both terminals of the gene fragment.

[Claim 9]

The method according to claims 1-8, wherein the CHO cell is at least one selected from CHO-K1, CHO-K1SV, DUKXB 11, CHO/DG44, Pro-3 and CHO-S.

[Claim 10]

The method according to any one of claims 4 to 9, wherein the selectable marker gene is a cycloheximide resistance gene.

[Claim 11]

The method according to any one of claim 10, wherein the cycloheximide resistance gene is a ribosome protein.

[Claim 12]

The method according to claims 1-11, 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.