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(54) Titre : METHODE DE PRODUCTION DE PROTEINES
(54) Title: METHOD FOR PRODUCING PROTEINS

(57) Abrégé/Abstract:
This invention relates to a method for producing a protein of interest, comprising introducing 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, into a suspension mammalian cell; integrating the gene fragment inserted between the pair of transposon sequences into a chromosome of the mammalian cell; obtaining a suspension mammalian cell producing the protein of interest; and suspension-culturing the suspension mammalian cell, and a suspension mammalian cell which expresses the protein of interest by the method.

Abstract

[Abstract]
This invention relates to a method for producing a protein of interest, comprising
5 introducing 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, into a suspension mammalian cell; integrating the gene fragment inserted between the pair of transposon sequences into a chromosome of the mammalian cell; obtaining a suspension
10 mammalian cell producing the protein of interest; and suspension-culturing the suspension mammalian cell, and a suspension mammalian cell which expresses the protein of interest by the method.
[Selected Figure] Nil

DESCRIPTION

Title of Invention: METHOD FOR PRODUCING PROTEINS

5 Technical Field

[0001]

This invention relates to a method for producing a protein of interest, comprising introducing an expression vector which comprises a gene fragment comprising a DNA encoding the protein of interest and a selectable marker gene and 10 also comprises a pair of transposon sequences at both terminals of the gene fragment, into a suspension mammalian cell; integrating the gene fragment inserted between the pair of transposon sequences into a chromosome of the mammalian cell; obtaining a suspension mammalian cell producing the protein of interest; and suspension-culturing the mammalian cell, and a suspension mammalian cell which expresses the protein of 15 interest by the method.

Background Art

[0002]

Production of exogenous proteins by recombinant DNA techniques is used in 20 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 25 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 30 production techniques for each host.

[0004]

In the bacteria systems, such as *Escherichia coli*, or 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 35 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
5 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.

10 [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
15 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]

20 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
25 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
30 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
35 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 Reference 12) is known.

[0015]

As one method for efficiently screening high expression cells, attenuation of a selectable marker gene is known. As a method for attenuation, amino acid modification in a neomycin resistance gene (Non-patent References 13 and 14) and binding of a destabilization sequence in dhfr gene (Non-patent Reference 15) are known. Alternatively, it is shown that high expression cells can be obtained by using an attenuated selectable marker gene.

[0016]

On the other hand, it is also shown that the number of drug-resistant cells is drastically reduced by the attenuation and that, as a result, there is a possibility of not obtaining any drug-resistant cell. Thus, creation of a method for efficiently screening high expression cells is still desired.

5 [0017]

It is known that in protein coding genes, there is codon usage bias depending on species and that human erythropoiethin expression in a CHO cell is improved by optimizing this codon bias (Non-patent Reference 16).

[Citation List]

10 [Patent Literature]

[0018]

[Patent Literature 1] WO 2008/072540

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

15 [Non Patent Literature]

[0019]

[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)

20 [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)

25 [Non Patent Literature 9] *Nature* 436,221-226 (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] *Biotech. Bioeng.* 89, 530-538 (2005)

30 [Non Patent Literature 14] *Journal of Immunological Methods* 295, 49-56 (2004)

[Non Patent Literature 15] *Metabolic Engineering* 9, 304-316 (2007)

[Non Patent Literature 16] *Gene* 199, 293-301 (1997)

Disclosure of Invention

35 Problems to be Solved by the Invention

[0020]

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.

[0021]

In addition, though it is known that a protein is expressed in a mammalian cell using a transposon sequence, preparation of a cell highly expressing a protein and thus can be used as a protein production system by using a transposon sequence; a high production cell comprising a transposon sequence; and a production method of a protein using the cell are not known. Further, any example that a high expression cell can be obtained by modifying codon to suppress expression (translation) of a drug resistance gene is not known.

[0022]

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 consistently, from the gene introduction to establishment of a producing cell, has been desired.

[0023]

Thus, the objects of the present 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

[0024]

To solve the above-mentioned problems, the present inventors have conducted intensive studies and found as a result that a production cell which highly expressing a protein of interest can be efficiently produced by introducing an expression vector which comprises a gene fragment comprising a DNA encoding the protein of interest and an attenuated selectable marker gene 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 the pair of transposon sequences into a chromosome of the suspension mammalian cell. In addition, it was found that time for preparing a high expression cell line of the protein of interest could be drastically reduced, and thereby the invention was accomplished. Therefore, the object of the present invention is to provide a novel preparation method of a production cell

which can efficiently prepare the production cell which highly expresses a exogenous gene; and a production method of a recombinant protein.

[0025]

Specifically, the present invention relates to the followings:

1. A method for producing a protein of interest, comprising introducing an expression vector which comprises a gene fragment comprising a DNA encoding the protein of interest and an attenuated selectable marker gene 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 the pair of transposon sequences into a chromosome of the mammalian cell; obtaining 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) and (B):

(A) a step of simultaneously introducing the following expression vectors (a) and (b) into a suspension mammalian cell; integrating a gene fragment inserted between a pair of transposon sequences into a chromosome of the mammalian cell by a transiently expressed transposase; and obtaining a suspension mammalian cell which expresses the protein of interest:

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

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

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

3. The method described in above item 1 or 2, wherein the suspension mammalian cell is a cell capable of surviving and proliferating in a serum-free medium;

4. The method described in any one of the above items 1 to 3, wherein the suspension mammalian cell is any one of the cells 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;

5. The method described in the above item 4, wherein the CHO cell is any one of the cells selected from CHO-K1, CHO-K1SV, DUKXB11, CHO/DG44, Pro-3 and CHO-S;

6. The method described in any one of the above items 1 to 5, wherein the attenuated selectable marker gene is a selectable marker gene modified such that expression level in the mammalian cell is lowered;

7. The method described in the above item 6, wherein the selectable marker
5 gene modified such that expression level in the mammalian cell is lowered is a selectable marker gene modified to encode the same amino acid sequence as the selectable marker gene before the modification and to comprise codons used at a low frequency in the mammalian cell;

8. The method described in the above item 6 or 7, wherein the selectable
10 marker gene modified such that expression level in the mammalian cell is lowered is modified in 10% or more of the nucleotide sequence encoding the selectable marker gene before the modification;

9. The method described in any one of the above items 6 to 8, wherein the selectable marker gene modified such that expression level in the mammalian cell is
15 lowered is modified such that 70% or more of codons corresponding to leucine residue are TTA among the codons corresponding to leucine residue included in the gene;

10. The method described in any one of the above items 6 to 9, wherein the selectable marker gene modified such that expression level in the mammalian cell is lowered is modified such that 70% or more of codons corresponding to alanine residue
20 are GCG among the codons corresponding to alanine residue included in the gene;

11. The method described in any one of the above items 6 to 10, wherein the selectable marker gene modified such that expression level in the mammalian cell is lowered is modified such that all the codons corresponding to leucine residue included in the gene are TTA or all the codons corresponding alanine residue included in the gene
25 are GCG;

12. The method described in any one of the above items 1 to 11, wherein the selectable marker gene is one selectable marker gene selected from the group consisting of a neomycin resistance gene, a puromycin resistance gene, a hygromycin resistance gene, a zeocin resistance gene, and a blasticidin resistance gene;

30 13. The method described in any one of the above items 1 to 12, wherein the pair of transposon sequences are nucleotide sequences derived from a pair of transposons which function in a mammalian cell;

14. The method described in the above item 13, wherein the nucleotide sequences derived from the pair of transposons are nucleotide sequences derived from a
35 pair of Tol2;

15. The method described in the above item 14, wherein the nucleotide sequences derived from the pair of Tol2 are the nucleotide sequence shown in SEQ ID NO:2 and the nucleotide sequence shown in SEQ ID NO:3;

16. The method described in the above item 13, wherein the nucleotide sequences derived from the pair of transposons are the nucleotide sequences shown in SEQ ID NO:35 and the nucleotide sequence shown in SEQ ID NO:36;

17. A suspension mammalian cell, in which an expression vector which comprises a gene fragment comprising a DNA encoding a protein of interest and an attenuated selectable marker gene and also comprises a pair of transposon sequences at both terminals of the gene fragment is introduced, and, wherein the gene fragment inserted between the pair of transposon sequences is integrated into a chromosome of the suspension mammalian cell, and the suspension mammalian cell produces the protein of interest;

18. A suspension mammalian cell, which has a chromosome into which a gene fragment inserted between a pair of transposons is integrated and which produces a protein of interest obtainable by simultaneously introducing the following vectors (a) and (b):

(a) a protein expression vector which comprises a gene fragment comprising a DNA encoding the protein of interest and an attenuated selectable marker gene and also comprises the 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 the gene fragment inserted between the pair of transposon sequences into the chromosome;

19. The mammalian cell described in the above item 17 or 18, which is a mammalian cell capable of surviving and proliferating in a serum-free medium;

20. The mammalian cell described in any one of the above items 17 to 19, wherein the cell is any one of the cells 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;

21. The mammalian cell described in the above item 20, wherein the CHO cell is any one of the cells selected from CHO-K1, CHO-K1SV, DUKXB11, CHO/DG44, Pro-3 and CHO-S;

22. The mammalian cell described in any one of the above items 17 to 21, wherein the attenuated selectable marker gene is a selectable marker gene modified such that expression level in the mammalian cell is lowered;

23. The mammalian cell described in the above item 22, wherein the selectable marker gene modified such that expression level in the mammalian cell is lowered is a selectable marker gene modified to encode the same amino acid sequence as the selectable marker gene before the modification and to comprise codons used at a low
5 frequency in the mammalian cell;

24. The mammalian cell described in the above item 22 or 23, wherein the selectable marker gene modified such that expression level in the mammalian cell is lowered is modified in 10% or more of the nucleotide sequence encoding the selectable marker gene before the modification;

10 25. The mammalian cell described in any one of the above items 22 to 24, wherein the selectable marker gene modified such that expression level in the mammalian cell is lowered is modified such that 70% or more of codons corresponding to leucine residue are TTA among the codons corresponding to leucine residue included in the gene;

15 26. The mammalian cell described in any one of the above items 22 to 25, wherein the selectable marker gene modified such that expression level in the mammalian cell is lowered is modified such that 70% or more of codons corresponding to alanine residue are GCG among the codons corresponding to alanine residue included in the gene;

20 27. The mammalian cell described in any one of the above items 22 to 26, wherein the selectable marker gene modified such that expression level in the mammalian cell is lowered is modified such that all the codons corresponding to leucine residue included in the gene are TTA or all the codons corresponding alanine residue included in the gene are GCG;

25 28. The mammalian cell described in any one of the above items 17 to 27, wherein the selectable marker gene is one selectable marker gene selected from the group consisting of a neomycin resistance gene, a puromycin resistance gene, a hygromycin resistance gene, a zeocin resistance gene, and a blasticidin resistance gene;

29. The mammalian cell described in any one of the above items 17 to 28,
30 wherein the pair of transposon sequences are nucleotide sequences derived from a pair of transposons which function in a mammalian cell;

30. The mammalian cell described in the above item 29, wherein the nucleotide sequences derived from the pair of transposons are nucleotide sequences derived from a pair of Tol2;

35 31. The mammalian cell described in the above item 30, wherein the nucleotide sequences derived from the pair of Tol2 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 29, wherein the nucleotide sequences derived from the pair of transposons are the nucleotide sequences shown in SEQ ID NO:35 and the nucleotide sequence shown in SEQ ID NO:36;

33. An expression vector, which comprises a gene fragment comprising a DNA encoding a protein of interest and an attenuated selectable marker, 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 the pair of transposon sequences are nucleotide sequences derived from a pair of Tol2;

35. The expression vector described in the above item 34, wherein the nucleotide sequences derived from the pair ofTol2 are the nucleotide sequence shown in SEQ ID NO:2 and the nucleotide sequence shown in SEQ ID NO:3;

36. The vector described in any one of the above items 33 to 35, wherein the attenuated selectable marker gene is a selectable marker gene modified such that expression level in the mammalian cell is lowered;

37. The vector described in the above item 36, wherein the selectable marker gene modified such that expression level in the mammalian cell is lowered is a selectable marker gene modified to encode the same amino acid sequence as the selectable marker gene before the modification and to comprise codons used at a low frequency in the mammalian cell;

38. The vector described in the above item 36 or 37, wherein the selectable marker gene modified such that expression level in the mammalian cell is lowered is modified in 10% or more of the nucleotide sequence encoding the selectable marker gene before the modification;

39. The vector described in any one of the above items 36 to 38, wherein the selectable marker gene modified such that expression level in the mammalian cell is lowered is modified such that 70% or more of codons corresponding to leucine residue are TTA among the codons corresponding to leucine residue included in the gene;

40. The vector described in any one of the above items 36 to 39, wherein the selectable marker gene modified such that expression level in the mammalian cell is lowered is modified such that 70% or more of codons corresponding to alanine residue are GCG among the codons corresponding to alanine residue included in the gene;

41. The vector described in any one of the above items 36 to 40, wherein the selectable marker gene modified such that expression level in the mammalian cell is lowered is modified such that all the codons corresponding to leucine residue included in the gene are TTA or all the codons corresponding alanine residue included in the gene are GCG;

42. The vector described in any one of the above items 33 to 41, wherein the selectable marker gene is one selectable marker gene selected from the group consisting of a neomycin resistance gene, a puromycin resistance gene, a hygromycin resistance gene, a zeocin resistance gene, and a blasticidin resistance gene.

5

Effect of Invention

[0026]

According to the protein production method of the present invention, a protein of interest can be efficiently produced by using a mammalian cell. The cell of the
10 present invention can be used as a protein production cell for producing a recombinant protein.

Brief Description of the Drawings

[0027]

15 [Fig. 1] Fig. 1 shows structure of the antibody expression vector A. In Fig. 1, Tol2-L represents 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
20 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

[0028]

25 The present invention relates to a method for producing a protein of interest, comprising introducing 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 (two) of transposon sequences at both terminals of the gene fragment, into a suspension mammalian cell; integrating the gene fragment inserted
30 between the pair of transposon sequences into a chromosome of the mammalian cell; obtaining a suspension mammalian cell producing the protein of interest; and suspension-culturing the mammalian cell, and a suspension mammalian cell which expresses the protein of interest by the method.

[0029]

35 The examples of the cell producing a protein of interest of the present invention include a suspension mammalian cell, wherein 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 is introduced, the gene fragment inserted between the pair of transposon sequences is integrated into a chromosome, and the suspension mammalian cell produces the protein of interest.

5 [0030]

Further, the examples of the cell producing a protein of interest of the present invention include a suspension mammalian cell, which has a chromosome into which a gene fragment inserted between a pair of transposons is integrated and which produces the protein of interest obtainable by simultaneously introducing the following vectors 10 (a) and (b):

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

(b) an expression vector which comprises a DNA encoding a transposase which 15 recognizes the transposon sequences and has activity of transferring the gene fragment inserted between the pair of transposon sequences into the chromosome.

[0031]

The examples of the method for producing a protein of interest of the present invention include a method for producing a protein of interest, comprising the following 20 steps (A) and (B):

(A) a step of simultaneously introducing the following expression vectors (a) and (b) into a suspension mammalian cell and obtaining a suspension mammalian cell which expresses the protein of interest by integrating a gene fragment inserted between a pair of transposon sequences into a chromosome of the mammalian cell by a transiently 25 expressed transposase:

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

(b) an expression vector which comprises a DNA encoding the transposase 30 which recognizes the transposon sequences and has activity of transferring the gene fragment inserted between the pair of transposon sequences into the chromosome,

, and

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

35 [0032]

The terms used in the present specification include the following definitions.

[0033]

The term transposon 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.

[0034]

5 The transposon has 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.

10 [0035]

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 the pair of transposon sequences and inserting the fragment into the site to be introduced.

15 [0036]

The term transposon sequence means the nucleotide sequence of a transposon recognized by a transposase and has the same meaning as the IR sequence or TIR sequence. The 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

20 transposase, and there is a transposon sequence specific to a transposase.

[0037]

The transposon sequence to be used in the present invention may be any sequence as long as it is a nucleotide sequence derived from natural or artificial transposons which can be recognized by a transposase and be transposed in mammalian

25 cells. Examples thereof include the medaka fish-derived Tol1 and Tol2 transposons, 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.

[0038]

30 Particularly, among them, the nucleotide sequences derived from the medaka fish-derived Tol2 transposon comprising the nucleotide sequence shown in SEQ ID NO:6 is preferable. As the nucleotide sequence derived from a pair of Tol2 transposons, examples include the nucleotide sequence comprising a nucleotide sequence at positions 1 to 2229 and the nucleotide sequence at positions the 4148 to

35 4682 in the Tol2 transposon nucleotide sequence shown in SEQ ID NO:6 of Sequence Listing.

[0039]

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.

[0040]

As the transposon sequence of the present invention, the nucleotide sequence derived from the medaka fish-derived Tol1 transposon consisting of the nucleotide sequence shown in SEQ ID NO:37 of Sequence Listing can be used. As the nucleotide sequence derived from a pair of Tol1 transposons, examples include a nucleotide sequence at positions 1 to 157 and a nucleotide sequence at positions 1748 to 1855 in the nucleotide sequence derived from the medaka fish-derived Tol1 transposon consisting of the nucleotide sequence shown in SEQ ID NO:37 of Sequence Listing.

[0041]

As the nucleotide sequence derived from a pair of Tol1 transposons, the region at positions 1 to 200 (SEQ ID NO:35) (hereinafter referred to as "Tol1-L sequence") and the the region at positions 1351 to 1855 (SEQ ID NO:36) (hereinafter referred to as "Tol1-R sequence ") in the nucleotide sequence derived from Tol1 transposon consisting of the nucleotide sequence shown in SEQ ID NO:37 of Sequence Listing are more preferable.

[0042]

Examples of the transposon sequence of the present invention include transposon sequences of which transposition reactions are controlled by using a partial sequence of a transposon sequence specific to the above-mentioned transposon, by adjusting the length of the nucleotide sequence and by modifying the nucleotide sequence due to addition, deletion or substitution. Regarding the control of the transposition reaction of a transposon, the transposition reaction can either be accelerated or suppressed by raising or lowering recognition of the transposon sequence by a transposase, respectively.

[0043]

The term transposase 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.

[0044]

Examples of the transposase include enzymes derived from Tol1 and Tol2 which are derived from medaka fish, the Sleeping Beauty reconstructed from a

non-autonomous transposon existed in an *Onchorhynchus* fish genome, the artificial transposon Frog prince which is derived from frog and the transposon PiggyBac which is derived from insect.

[0045]

5 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.

10 [0046]

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. 2003-235575. Particularly, whether or not a non-autonomous Tol2 element can be transferred and

15 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.

[0047]

20 The term non-autonomous transposon in the present invention 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

25 encoding the transposase protein or a DNA encoding the transposase protein to simultaneously present in the cell.

[0048]

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

30 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.

[0049]

35 According to the present invention, in order to integrate an expression vector into the chromosome of a host cell, a transposase is allowed to act upon the expression vector. In order to allow a transposase to act upon a cell, the transposase enzyme may

be injected into the cell, or a DNA encoding transposase gene may be introduced into an intended expression vector and the vector may be transfected with the cell. In addition, by transfecting with an RNA encoding a transposase gene into the cell, the transposase may be expressed in the cell.

5 [0050]

The expression vector which can be used herein 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.

10 [0051]

In the case where a protein of interest comprised of two or more polypeptides is produced by the method of the present invention, the expression vector may be integrated into a chromosome of a host cell by inserting the DNA encoding each of two or more polypeptides on the same or different expression vector. Specifically, a heavy 15 chain and a light chain of an antibody may be inserted into different expression vectors and the expression vector may be integrated into a chromosome of a host cell.

[0052]

The transposase may be inserted into an expression vector to express together with the protein of interest or may be inserted into a vector different from the expression 20 vector. The transposase may be allowed to act transiently or may be allowed to act continuously, but it is preferably to allow the transposase to act transiently in order to prepare a cell for stable production.

[0053]

In order to allow the transposase to act transiently, for example, a transposase 25 gene may be inserted into an expression plasmid which is different from an expression vector having a protein of interest and a cell may be transfected with them.

[0054]

The term expression vector means an expression vector to be used for introducing into a mammalian cell. The expression vector used in the present 30 invention has a structure in which at least a pair of transposon sequences is present at both sides of an expression cassette.

[0055]

The term expression cassette means a nucleotide sequence which has a gene expression controlling region necessary for expressing a protein of interest and a 35 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.

[0056]

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

[0057]

The selectable marker gene means an optional marker gene which can be used
10 for distinguishing a cell to which a plasmid vector is introduced from a cell lacking of the vector. Examples of the selectable marker gene include a drug resistance gene (such as a neomycin resistance gene, DHFR gene, a puromycin resistance gene, a blasticidin resistance gene, a zeocin resistance gene, and a hygromycin resistance gene), fluorescence and bio-luminescence marker genes (such as green fluorescent protein
15 GFP) and the like.

[0058]

An 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.

20 [0059]

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 or (B) an selectable marker gene in
25 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.

[0060]

Examples of the selectable marker gene in which an amino acid sequence of a
30 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)].

[0061]

35 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.

[0062]

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.

[0063]

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.

[0064]

Examples of the attenuated selectable marker gene of the present 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.

[0065]

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.

[0066]

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.

[0067]

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.

[0068]

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.

[0069]

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.

[0070]

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. 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.

[0071]

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:9, 11 or 13, a puromycin resistance gene comprising the nucleotide sequence represented by SEQ ID NO:21, 23 or 25, a Zeocin resistance gene consisting of the nucleotide sequence represented by SEQ ID NO:27 or 29 and a hygromycin resistance gene comprising the nucleotide sequence represented by SEQ ID NO:31 or 33.

[0072]

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.

[0073]

The method for introducing the above-mentioned 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.
5 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.

10 [0074]

The host cell may be any mammalian cell as long as it can be subcultured and stably express a protein of interest.

[0075]

Examples of the particular host cell include PER.C6 cell, human leukemia cell
15 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);
20 *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 (ATCC CRL-9096), CHO-K1 (ATCC CCL-61), DUKXB11 (ATCC CCL-9096), Pro-5 (ATCC CCL-1781), CHO-S
25 (Life Technologies, Cat #11619), Pro-3 and subline cell line of CHO cell.

[0076]

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

[0077]

Further, in the present invention, 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)] or a CHO cell from which
35 α1,6-fucosyltransferase gene is deleted (WO2005/35586, WO2002/31140), can also be used as the host cell producing a protein of interest of the present invention.

[0078]

The protein of interest produced in the present invention may be any protein as long as it can be expressed by the method of producing a protein using a non-autonomous transposon of the present invention. Particularly, examples of the protein of interest include a human serum protein, a peptide hormone, a growth factor, a cytokine, a blood coagulation factor, a fibrinolytic protein, an antibody partial fragments of various proteins and the like.

[0079]

Examples of the protein of interest produced in the present invention include preferably a monoclonal antibody such as a chimeric antibody, a humanized antibody, and a human antibody, a Fc fusion protein, an albumin binding protein and a partial fragment thereof.

[0080]

The effector activity of a monoclonal antibody produced in 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 (WO 2005/035586, WO 2002/31140, and WO 00/61739), a method for controlling an effector activity by modifying amino acid residue(s) of an Fc region of the antibody, or the like. The effector activity of the monoclonal antibody produced in the present invention can be controlled by using any of the methods.

[0081]

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.

[0082]

In addition, by controlling a content of core fucose of a complex type N-linked sugar chain of Fc of a monoclonal antibody which is produced in the present invention, an effector activity of the antibody can be increased or decreased. As a method for lowering a content of fucose which is bound to a complex type N-linked sugar chain bound to Fc of the antibody, an antibody to which fucose is not bound can be obtained by the expression of an antibody using a CHO cell which is deficient in a gene encoding α1,6-fucosyltransferase.

[0083]

The antibody to which fucose is not bound has a high ADCC activity. 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.

[0084]

Further, by modifying amino acid residue(s) in an Fc region of an antibody, the ADCC activity or CDC activity can be increased or decreased. For example, the CDC activity of an antibody can be increased by using the amino acid sequence of the Fc region described in US 2007/0148165. Further, the ADCC activity or CDC activity 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.

[0085]

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. 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.

[0086]

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.

[0087]

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, 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.

[0088]

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. 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.

[0089]

The suspension mammalian cell prepared by adapting an adhesive mammalian cell to suspension culture conditions in the present invention 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).

[0090]

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.

[0091]

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.

[0092]

In addition, a suspension mammalian cell can also be prepared by culturing with the addition of an appropriate nonionic surfactant such as Pluronic™-F68 or the like in the culture solution. Examples of the suspension mammalian cell in which the adhesive mammalian cell is adapted to a suspension culture condition include a mouse myeloma cell NS0, a CHO cell or the like.

[0093]

In the present invention, the suspension CHO cell preferably possesses a property that when suspension culturing is carried out under the condition of 2×10^5

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

[0094]

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

[0095]

The suspension culturing can be conducted by using a culture container which
15 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.

[0096]

20 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.

[0097]

25 Regarding the suspension culture conditions for a suspension mammalian cell using the Wave Biorector equipment, the cell can be cultured according to the manufacturer's instructions.

[0098]

In addition to the shaking culture, culturing by a rotation agitation equipment
30 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.

[0099]

In the present invention, when a cell line other than the CHO cells is selected, any cell line can be applied so long as it is a cell line adapted to the suspension culture
35 by the above-mentioned method and the protein production method of the present invention can be used.

[0100]

Purification of the protein produced by the cultured 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. Examples of the separation method include centrifugation, dialysis, ammonium sulfate precipitation, column chromatography, a filtering or 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.

[0101]

As the method for purifying the protein, 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.

[0102]

[0103]

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 described 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 present invention is not limited to the embodiments and examples which are specifically described in the present specification, but is limited by the claims alone.

[0104]

Hereinafter, examples are shown to further describe the present specification specifically, but the present invention is not limited to the description of these examples.

[0105]

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

[0106]

[Example 1] 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
5 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.

10 [0107]

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
15 enzyme digestion site was added to the primer terminal. 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.

20 [0108]

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

[0109]

A DNA fragment including a nucleotide sequence (SEQ ID NO:15) which
25 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:17) which encoded antibody light chain under control of SV40 promoter, amplified based on the anti-human CD98 antibody
30 N5KG1-Val C2IgG1NS/I117L vector, as the antibody light chain gene cassette.

[0110]

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
35 consisting of the nucleotide sequence represented by SEQ ID NO:7 and GenBank Accession No. U47120.2) was prepared.

[0111]

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. 1).

[0112]

(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:9 was prepared.

[0113]

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.

[0114]

(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:11 was prepared.

[0115]

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.

[0116]

(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:13 was prepared.

5 [0117]

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

10 to 30 leucine residues were modified so as to be TTA.

[0118]

[Example 2] 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

15 anti-human CD98 expression transposon vectors A to D prepared in Example 1(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:5 [Kawakami K. & Noda T., *Genetics,* 166, 895 - 899 (2004)].

[0119]

20 Introduction of vectors into the suspension CHO cell was carried out by suspending the CHO cell (4×10^6 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.

25 [0120]

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

[0121]

In addition, as a control which did not use Tol2 transposase, the anti-human

30 CD98 antibody expression transposon vector D (10 μg) of Example 1(4) was linearlized using a restriction enzyme *Pci*I (TAKARA BIO INC.) and then introduced into suspension CHO-K1 cell by electroporation.

[0122]

The electroporation was carried out using an electroporator [Gene Pulser™

35 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).

[0123]

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.

5 [0124]

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.

10 [0125]

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 1.

15 [0126]

[Table 1]

	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	-

[0127]

As shown in Table 1, expression levels of anti-human CD98 antibody of the cells B to D expressing the modified type neomycin resistance genes were higher than

20 that of the cell A which expressed the wild type neomycin resistance gene.

[0128]

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.

[0129]

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.

[0130]

[Example 3] 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 1(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:21 was prepared.

[0131]

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:19 (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.

[0132]

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.

[0133]

(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 1(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:23 was prepared.

[0134]

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 5 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.

[0135]

10 [Example 4] 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 3(1) comprising the modified type puromycin resistance gene 1, the anti-human CD98 antibody expression 15 transposon vector F of Example 3(2) comprising the modified type puromycin resistance gene 2 and the Tol2 transposase expression vector pCAGGS-T2TP into the suspension CHO-K1 cell.

[0136]

Introduction of the vectors into suspension cell was carried out by suspending 20 the suspension CHO cell (4×10^6 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.

[0137]

25 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.

[0138]

The electroporation was carried out using an electroporator (Gene Pulser Xcell 30 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).

[0139]

After the gene introduction by electroporation, the cells in each cuvette were 35 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.

[0140]

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.

5 [0141]

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.

10 [0142]

[Table 2]

	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

[0143]

As shown in Table 2, the antibody producing cell F which expresses the modified type puromycin resistance gene 2 showed two times or more antibody

15 productivity of the antibody producing cell E which expresses the modified type puromycin resistance gene 1.

[0144]

[Example 5] Antibody production by antibody producing CHO cell which expresses modified type puromycin resistance gene 2

20 The antibody producing cell F obtained in Example 4 which expresses the modified type puromycin resistance gene 2 was cultured using a conical flask to produce anti-human CD98 antibody.

[0145]

Specifically, the antibody producing cell F was expansion-cultured using

25 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

30 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.

[0146]

Amount of the antibody in the medium after culturing was determined by HPLC (Waters Associates, Inc.). The results are shown in Table 3.

5 [0147]

[Table 3]

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

[0148]

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
10 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.

[0149]

15 [Reference Example] (1) Preparation of suspension CHO cell

An adhesive CHO-K1 cell EC85051005 (European Collection of Cell Cultures) 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
20 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.

[0150]

Further several days thereafter, the inoculation was similarly carried out using
25 the α-MEM medium containing 5% FCS. Finally, a cell adapted to the suspension culture was prepared by repeating the sub-culturing 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.

[0151]

30 While the present invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skill in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof. This application is based on the Japanese patent application (No. 2010-279850), filed on December 15, 2010.

Industrial Applicability

[0152]

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

[Sequence Listing]

[0153]

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 Wild Type of Neomycin Resistant Gene

SEQ ID NO:8 - Description of Artificial sequence; Amino Acid Sequence encoded by Wild Type of Neomycin Resistant Gene

SEQ ID NO:9 - Description of Artificial sequence; Nucleotide Sequence Modified Type of Neomycin Resistant Gene

SEQ ID NO:10 - Description of Artificial sequence; Amino Acid Sequence of Synthetic Construct

SEQ ID NO:11 - Description of Artificial sequence; Nucleotide Sequence of Modified Type of Neomycin Resistant Gene

SEQ ID NO:12 - Description of Artificial sequence; Amino Acid Sequence of Synthetic Construct

SEQ ID NO:13 - Description of Artificial sequence; Nucleotide Sequence of Modified Type of Neomycin Resistant Gene

SEQ ID NO:14 - Description of Artificial sequence; Amino Acid Sequence of Synthetic Construct

SEQ ID NO:15 - Description of Artificial sequence; Nucleotide Sequence encoding Anti-Human CD98 Antibody Heavy Chain Variable Region

SEQ ID NO:16 - Description of Artificial sequence; Amino Acid Sequence of Synthetic Construct

SEQ ID NO:17 - Description of Artificial sequence; Nucleotide Sequence encoding Anti-Human CD98 Antibody Light Chain Variable Region

SEQ ID NO:18 - Description of Artificial sequence; Amino Acid Sequence of Synthetic

Construct
SEQ ID NO:19 - Description of Artificial sequence; Nucleotide Sequence of Wild Type of Puromycin Resistance Gene
SEQ ID NO:20 - Description of Artificial sequence; Amino Acid Sequence encoded by
5 Wild Type of Puromycin Resistance Gene
SEQ ID NO:21 - Description of Artificial sequence; Nucleotide Sequence of Modified Type of Puromycin Resistance Gene
SEQ ID NO:22 - Description of Artificial sequence; Amino Acid Sequence of Synthetic Construct
10 SEQ ID NO:23 - Description of Artificial sequence; Nucleotide Sequence of Modified Type of Puromycin Resistance Gene
SEQ ID NO:24 - Description of Artificial sequence; Amino Acid Sequence of Synthetic Construct
SEQ ID NO:25 - Description of Artificial sequence; Nucleotide Sequence of Modified
15 Type of Puromycin Resistance Gene
SEQ ID NO:26 - Description of Artificial sequence; Amino Acid Sequence of Synthetic Construct
SEQ ID NO:27 - Description of Artificial sequence; Nucleotide Sequence of Modified Type of Zeocin Resistance Gene
20 SEQ ID NO:28 - Description of Artificial sequence; Amino Acid Sequence of Synthetic Construct
SEQ ID NO:29 - Description of Artificial sequence; Nucleotide Sequence of Modified Type of Zeocin Resistance Gene
SEQ ID NO:30 - Description of Artificial sequence; Amino Acid Sequence of Synthetic
25 Construct
SEQ ID NO:31 - Description of Artificial sequence; Nucleotide Sequence of Modified Type of Hygromycin Resistance Gene
SEQ ID NO:32 - Description of Artificial sequence; Amino Acid Sequence of Synthetic Construct
30 SEQ ID NO:33 - Description of Artificial sequence; Nucleotide Sequence of Modified Type of Hygromycin Resistance Gene
SEQ ID NO:34 - Description of Artificial sequence; Amino Acid Sequence of Synthetic Construct

SEQ ID NO:35 - Description of Artificial sequence; Tol1-L transposon sequence

SEQ ID NO:36 - Description of Artificial sequence; Tol1-R transposon sequence

SEQ ID NO:37 - Description of Artificial sequence; nonautologous Tol1 transposon

Claims

[Claim 1]

A method for producing a protein of interest, comprising

5 (a) introducing an expression vector which comprises a gene fragment comprising a DNA encoding the protein of interest and an attenuated selectable marker gene and also comprises Tol1 or Tol2 transposon sequences at both terminals of the gene fragment, into a suspension Chinese hamster ovary (CHO) cell;

(b) integrating the gene fragment comprising the DNA encoding the protein of
10 interest inserted between the transposon sequences into a chromosome of the CHO cell;

(c) obtaining a CHO cell which expresses the protein of interest; and

(d) suspension-culturing the CHO cell,

wherein the attenuated selectable marker gene is a modified version of a corresponding unmodified selectable marker gene, wherein the attenuated selectable marker gene and
15 the unmodified selectable marker gene encode the same amino acid sequence, wherein the attenuated selectable marker gene is modified relative to the unmodified selectable marker gene to comprise codons used at a low frequency in the CHO cell such that expression level in the CHO cell is lowered, and wherein the selectable marker gene is a neomycin resistance gene, a puromycin resistance gene, a hygromycin resistance gene, a
20 zeocin resistance gene, or a blasticidin resistance gene.

[Claim 2]

A method for producing a protein of interest, comprising the following steps (A) and (B):

25 (A) a step of simultaneously introducing the following expression vectors (a) and (b) into a suspension Chinese hamster ovary (CHO) cell; integrating a gene fragment inserted between Tol1 or Tol2 transposon sequences into a chromosome of the CHO cell by a transiently expressed transposase; and obtaining a suspension CHO cell which expresses the protein of interest:

30 (a) an expression vector which comprises the gene fragment comprising a DNA encoding the protein of interest and an attenuated selectable marker gene and also comprises the transposon sequences at both terminals of the gene fragment, wherein the attenuated selectable marker gene is a modified version of a corresponding unmodified selectable marker gene, wherein the attenuated selectable marker gene and the
35 unmodified selectable marker gene encode the same amino acid sequence, wherein the attenuated selectable marker gene is modified relative to the unmodified selectable marker gene to comprise codons used at a low frequency in the CHO cell such that

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expression level in the CHO cell is lowered, and wherein the selectable marker gene is a neomycin resistance gene, a puromycin resistance gene, a hygromycin resistance gene, a zeocin resistance gene, or a blasticidin resistance gene,

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

(B) a step of suspension-culturing the suspension CHO cell which expresses the protein of interest to produce the protein of interest.

10 [Claim 3]

The method according to claim 1 or 2, wherein the suspension CHO cell is a cell capable of surviving and proliferating in a serum-free medium.

[Claim 4]

15 The method according to any one of claims 1 to 3, wherein the suspension CHO cell is a CHO cell adapted to suspension culture.

[Claim 5]

The method according to any one of claims 1 to 4, wherein the CHO cell is a
20 CHO-K1 cell, a CHO-K1SV cell, a DUKXB11 cell, a CHO/DG44 cell, a Pro-3 cell or a CHO-S cell.

[Claim 6]

The method according to any one of claims 1 to 5, wherein the difference in
25 sequence identity between the nucleotide sequence of the attenuated selectable marker gene and the nucleotide sequence of the unmodified selectable marker gene is 10% or more.

[Claim 7]

30 The method according to any one of claims 1 to 6, wherein 70% or more of the codons encoding leucine residues in the nucleotide sequence of the attenuated selectable marker gene are TTA.

[Claim 8]

35 The method according to any one of claims 1 to 7, wherein 70% or more of the codons encoding alanine residues in the nucleotide sequence of the attenuated selectable marker gene are GCG.

[Claim 9]

The method according to any one of claims 1 to 8, wherein all the codons encoding leucine residues in the nucleotide sequence of the attenuated selectable marker gene are TTA, or all the codons encoding alanine residue in the nucleotide sequence of the attenuated selectable marker gene are GCG.

[Claim 10]

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

[Claim 11]

The method according to any one of claims 1 to 9, wherein the Tol1 transposon sequences are a nucleotide sequence comprising the nucleotide sequence shown in SEQ ID NO:35 and a nucleotide sequence comprising the nucleotide sequence shown in SEQ ID NO:36.

[Claim 12]

A suspension Chinese hamster ovary (CHO) cell, in which an expression vector which comprises a gene fragment comprising a DNA encoding a protein of interest and an attenuated selectable marker gene and also comprises Tol1 or Tol2 transposon sequences at both terminals of the gene fragment is introduced, wherein the attenuated selectable marker gene is a modified version of a corresponding unmodified selectable marker gene, wherein the attenuated selectable marker gene and the unmodified selectable marker gene encode the same amino acid sequence, wherein the attenuated selectable marker gene is modified relative to the unmodified selectable marker gene to comprise codons used at a low frequency in the CHO cell such that expression level in the CHO cell is lowered, wherein the selectable marker gene is a neomycin resistance gene, a puromycin resistance gene, a hygromycin resistance gene, a zeocin resistance gene, or a blasticidin resistance gene, and wherein the gene fragment inserted between the transposon sequences is integrated into a chromosome of the suspension CHO cell, and the suspension CHO cell produces the protein of interest.

[Claim 13]

A suspension Chinese hamster ovary (CHO) cell, which has a chromosome into

which a gene fragment inserted between Tol1 or Tol2 transposon sequences is integrated, and which produces a protein of interest obtainable by simultaneously introducing the following vectors (a) and (b):

(a) a protein expression vector which comprises a gene fragment comprising a DNA encoding the protein of interest and an attenuated selectable marker gene and also comprises the transposon sequences at both terminals of the gene fragment, wherein the attenuated selectable marker gene is a modified version of a corresponding unmodified selectable marker gene, wherein the attenuated selectable marker gene and the unmodified selectable marker gene encode the same amino acid sequence, wherein the attenuated selectable marker gene is modified relative to the unmodified selectable marker gene to comprise codons used at a low frequency in the CHO cell such that expression level in the CHO cell is lowered, and wherein the selectable marker gene is a neomycin resistance gene, a puromycin resistance gene, a hygromycin resistance gene, a zeocin resistance gene, or a blasticidin resistance gene,

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

[Claim 14]

The CHO cell according to claim 12 or 13, which is a CHO cell capable of surviving and proliferating in a serum-free medium.

[Claim 15]

The CHO cell according to any one of claims 12 to 14, wherein the suspension CHO cell is a CHO cell adapted to suspension culture.

[Claim 16]

The CHO cell according to any one of claims 12 to 15, wherein the CHO cell is a CHO-K1 cell, a CHO-K1SV cell, a DUKXB11 cell, a CHO/DG44 cell, a Pro-3 cell or a CHO-S cell.

[Claim 17]

The CHO cell according to any one of claims 12 to 16, wherein the difference in sequence identity between the nucleotide sequence of the attenuated selectable and the nucleotide sequence of the unmodified selectable marker gene is 10% or more.

[Claim 18]

The CHO cell according to any one of claims 12 to 17, wherein 70% or more of the codons encoding leucine residues in the attenuated selectable marker gene are TTA.

5 [Claim 19]

The CHO cell according to any one of claims 12 to 18, wherein 70% or more of the codons encoding alanine residues in the attenuated selectable marker gene are GCG.

[Claim 20]

10 The CHO cell according to any one of claims 12 to 19, wherein all the codons encoding leucine residues in the nucleotide sequence of the attenuated selectable marker gene are TTA, or all the codons encoding alanine residues in the nucleotide sequence of the attenuated selectable marker gene are GCG.

15 [Claim 21]

The CHO cell according to any one of claims 12 to 20, wherein the Tol2 transposon sequences are a nucleotide sequence comprising the nucleotide sequence shown in SEQ ID NO:2 and a nucleotide sequence comprising the nucleotide sequence shown in SEQ ID NO:3.

20

[Claim 22]

The CHO cell according to any one of claims 12 to 20, wherein the Tol1 transposon sequences are a nucleotide sequence comprising the nucleotide sequence shown in SEQ ID NO:35 and a nucleotide sequence comprising the nucleotide
25 sequence shown in SEQ ID NO:36.

[Claim 23]

An expression vector, which comprises a gene fragment comprising a DNA encoding a protein of interest and an attenuated selectable marker gene, and also
30 comprises Tol1 or Tol2 transposon sequences at both terminals of the gene fragment, wherein the attenuated selectable marker gene is a modified version of a corresponding unmodified selectable marker gene, wherein the attenuated selectable marker gene and the unmodified selectable marker gene encode the same amino acid sequence, wherein the attenuated selectable marker gene is modified relative to the unmodified selectable
35 marker gene to comprise codons used at a low frequency in the CHO cell such that expression level in the CHO cell is lowered, and wherein the selectable marker gene is a

neomycin resistance gene, a puromycin resistance gene, a hygromycin resistance gene, a zeocin resistance gene, or a blasticidin resistance gene.

[Claim 24]

5 The expression vector according to claim 23, wherein Tol2 transposon sequences are a nucleotide sequence comprising the nucleotide sequence shown in SEQ ID NO:2 and a nucleotide sequence comprising the nucleotide sequence shown in SEQ ID NO:3.

[Claim 25]

10 The vector according to claim 23 or 24, wherein the difference in sequence identity between the nucleotide sequence of the attenuated selectable marker gene and the nucleotide sequence of the unmodified selectable marker gene is 10% or more.

[Claim 26]

15 The vector according to any one of claims 23 to 25, wherein 70% or more of the codons encoding leucine residues in the attenuated selectable marker gene are TTA.

[Claim 27]

The vector according to any one of claims 23 to 26, wherein 70% or more of the 20 codons encoding alanine residues in the attenuated selectable marker gene are GCG.

[Claim 28]

The vector according to any one of claims 23 to 27, wherein all the codons encoding leucine residues in the nucleotide sequence of the attenuated selectable marker 25 gene are TTA, or all the codons encoding alanine residues in the nucleotide sequence of the attenuated selectable marker gene are GCG.

Fig. 1