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(54) **METHOD OF ENHANCING OF BINDING
ACTIVITY OF ANTIBODY COMPOSITION
TO FCGAMMA RECEPTOR IIIA**

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(57) **ABSTRACT**

A method for enhancing a binding activity of an antibody composition to Fc γ receptor IIIa, which comprises modifying a complex N-glycoside-linked sugar chain which is bound to the Fc region of an antibody molecule; a method for enhancing an antibody-dependent cell-mediated cytotoxic activity of an antibody composition; a process for producing an antibody composition having an enhanced binding activity to Fc γ receptor IIIa; a method for detecting the ratio of a sugar chain in which fucose is not bound to N-acetylglucosamine in the reducing end in the sugar chain among total complex N-glycoside-linked sugar chains bound to the Fc region in an antibody composition; an Fc fusion protein composition produced by using a cell resistant to a lectin which recognizes a sugar chain in which 1-position of fucose is bound to 6-position of N-acetylglucosamine in the reducing end through α -bond in a complex N-glycoside-linked sugar chain; and a process for producing the same.

FIG. 2

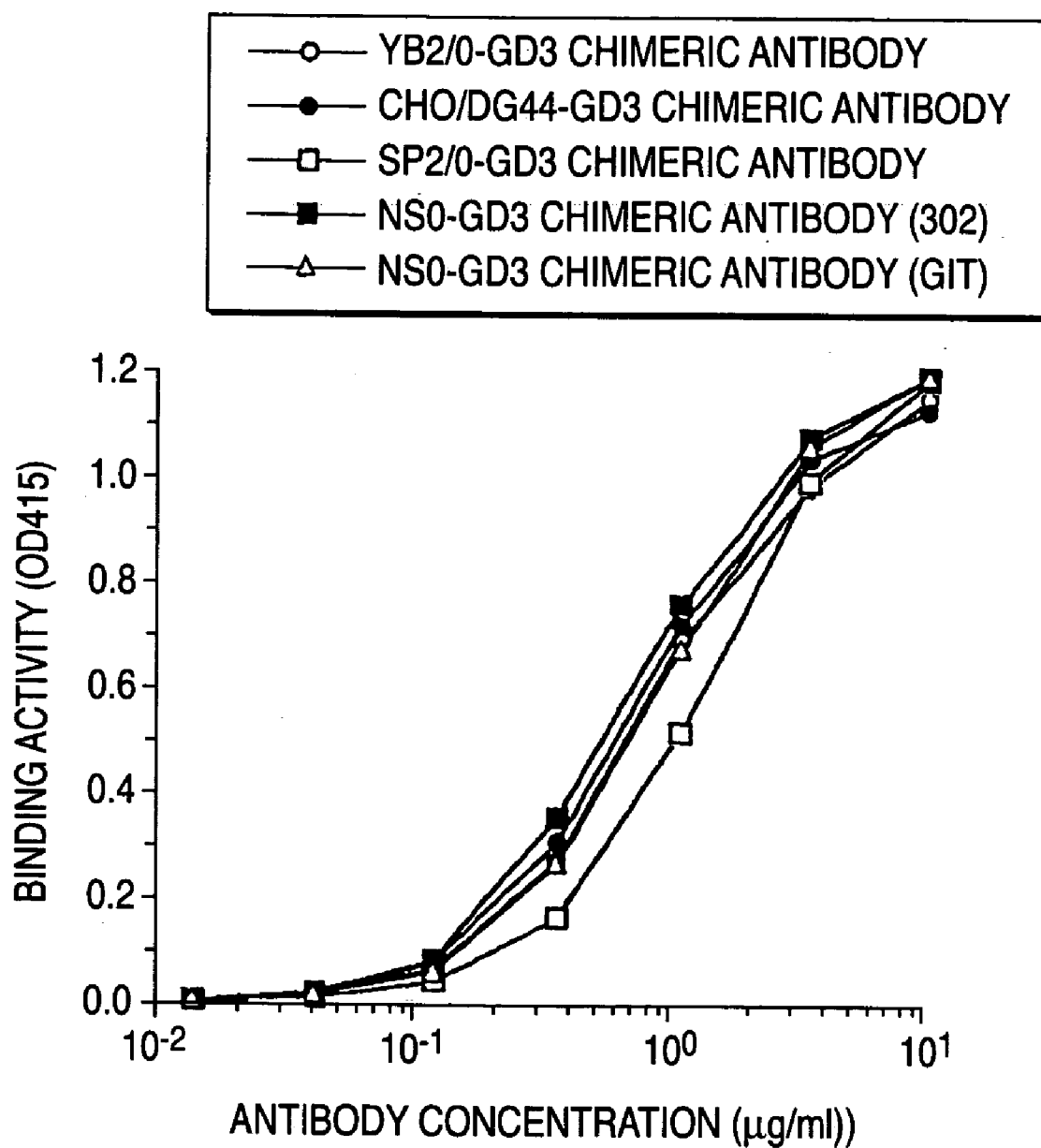


FIG. 3

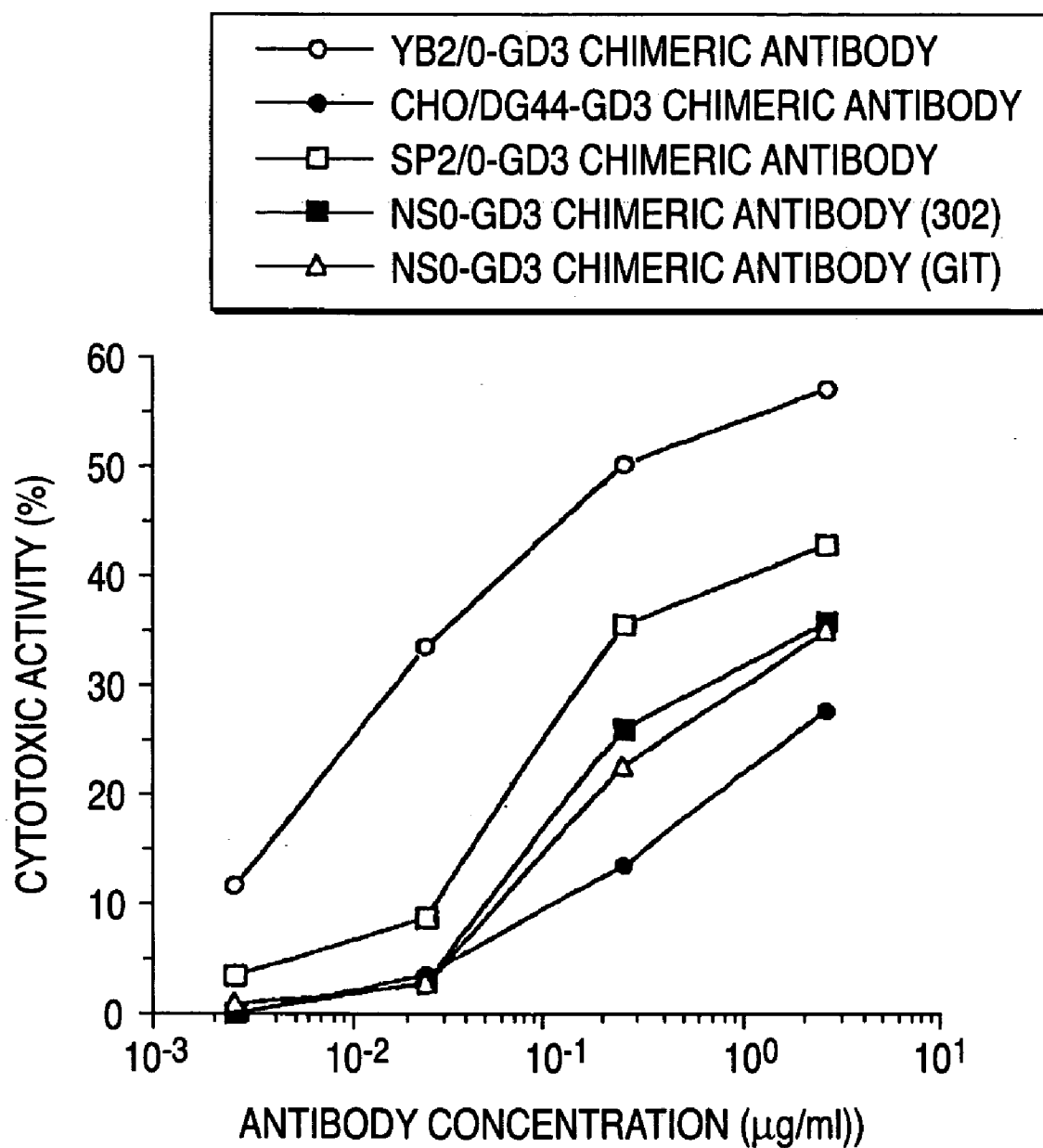


FIG. 4

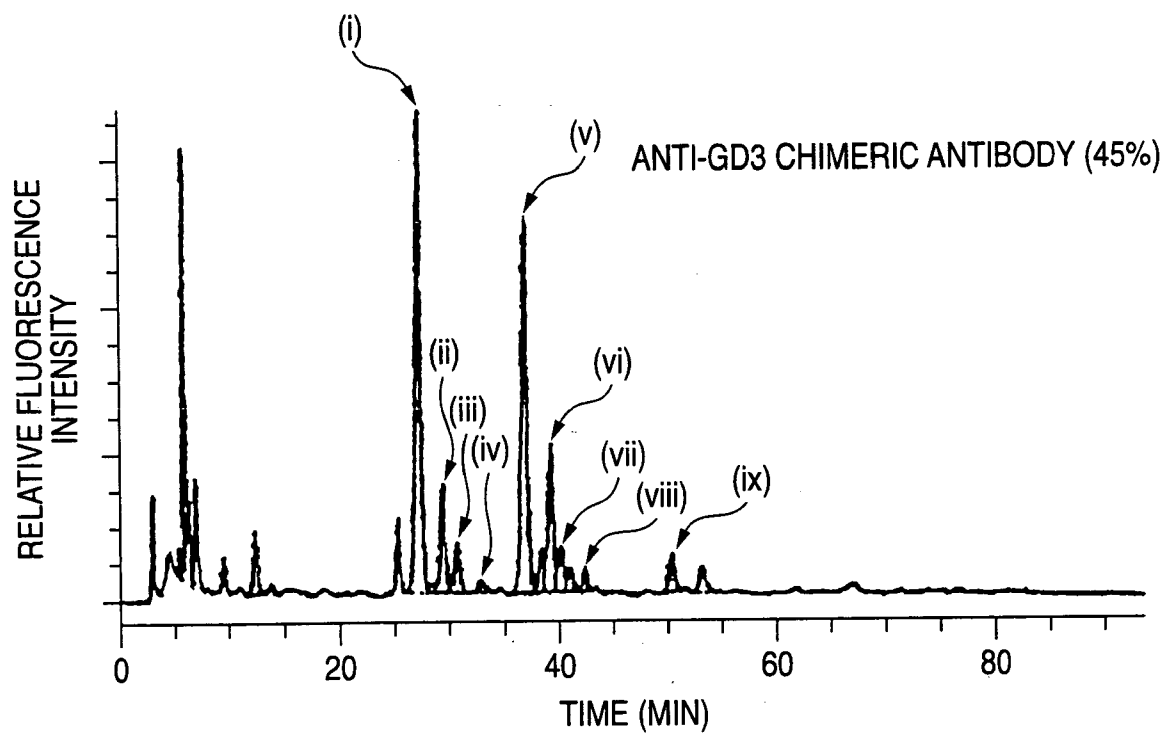
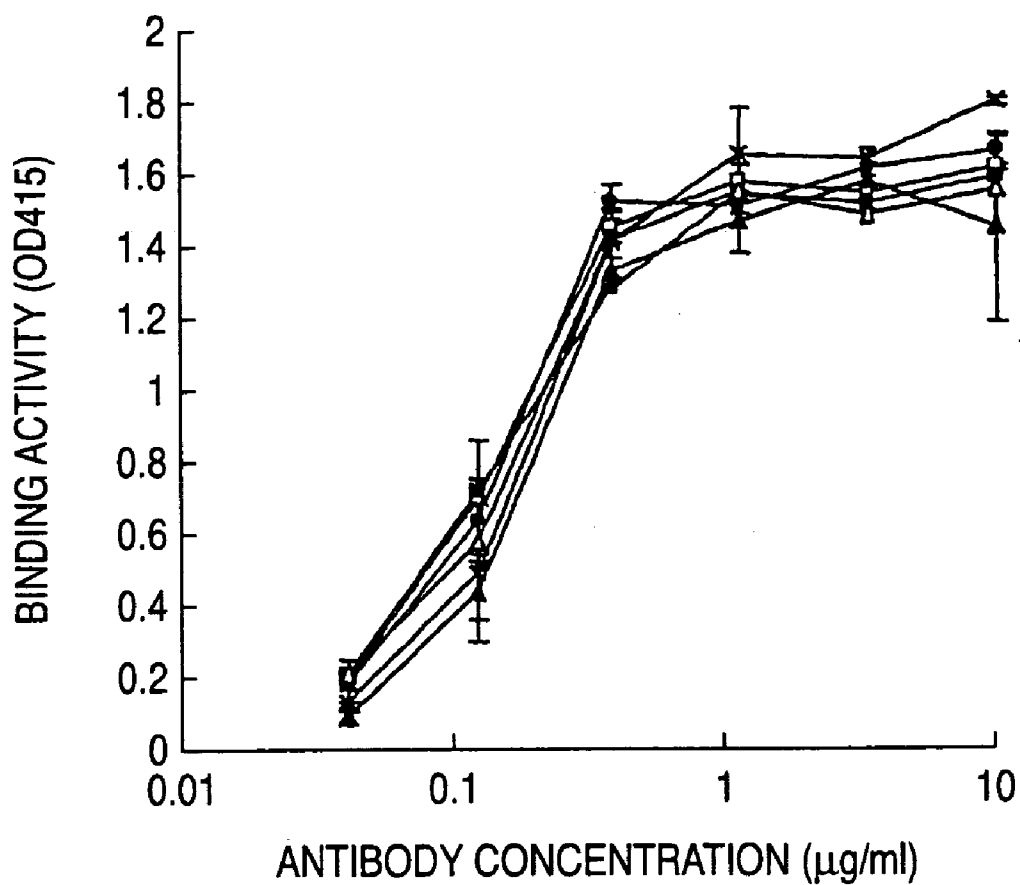


FIG. 5



- ANTI-GD3 CHIMERIC ANTIBODY (50%)
- ANTI-GD3 CHIMERIC ANTIBODY (45%)
- ANTI-GD3 CHIMERIC ANTIBODY (29%)
- △— ANTI-GD3 CHIMERIC ANTIBODY (24%)
- ▲— ANTI-GD3 CHIMERIC ANTIBODY (13%)
- ×— ANTI-GD3 CHIMERIC ANTIBODY (7%)

FIG. 6A

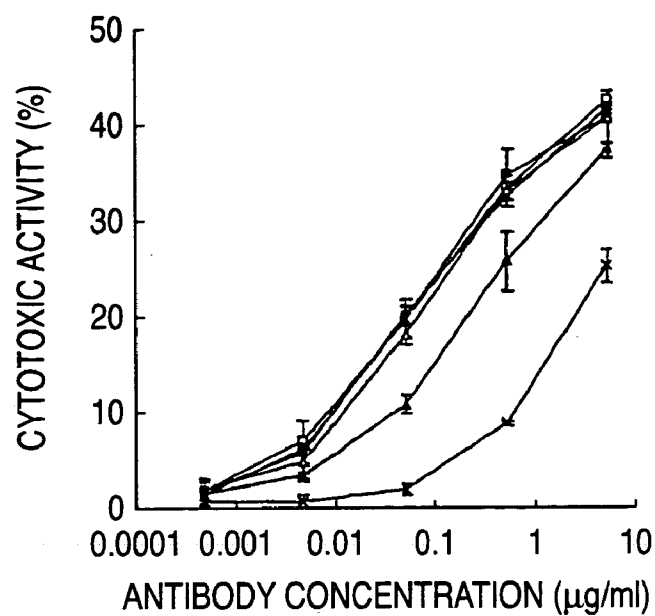
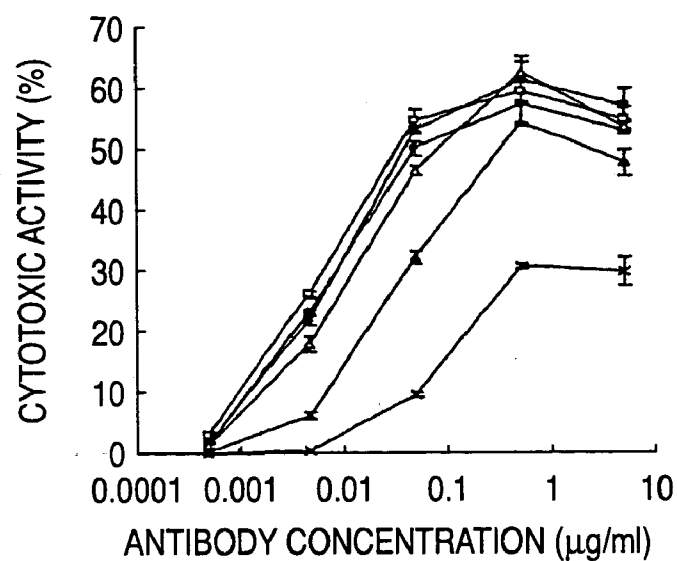


FIG. 6B



- ANTI-GD3 CHIMERIC ANTIBODY (50%)
- ANTI-GD3 CHIMERIC ANTIBODY (45%)
- ANTI-GD3 CHIMERIC ANTIBODY (29%)
- △— ANTI-GD3 CHIMERIC ANTIBODY (24%)
- ▲— ANTI-GD3 CHIMERIC ANTIBODY (13%)
- *— ANTI-GD3 CHIMERIC ANTIBODY (7%)

FIG. 7

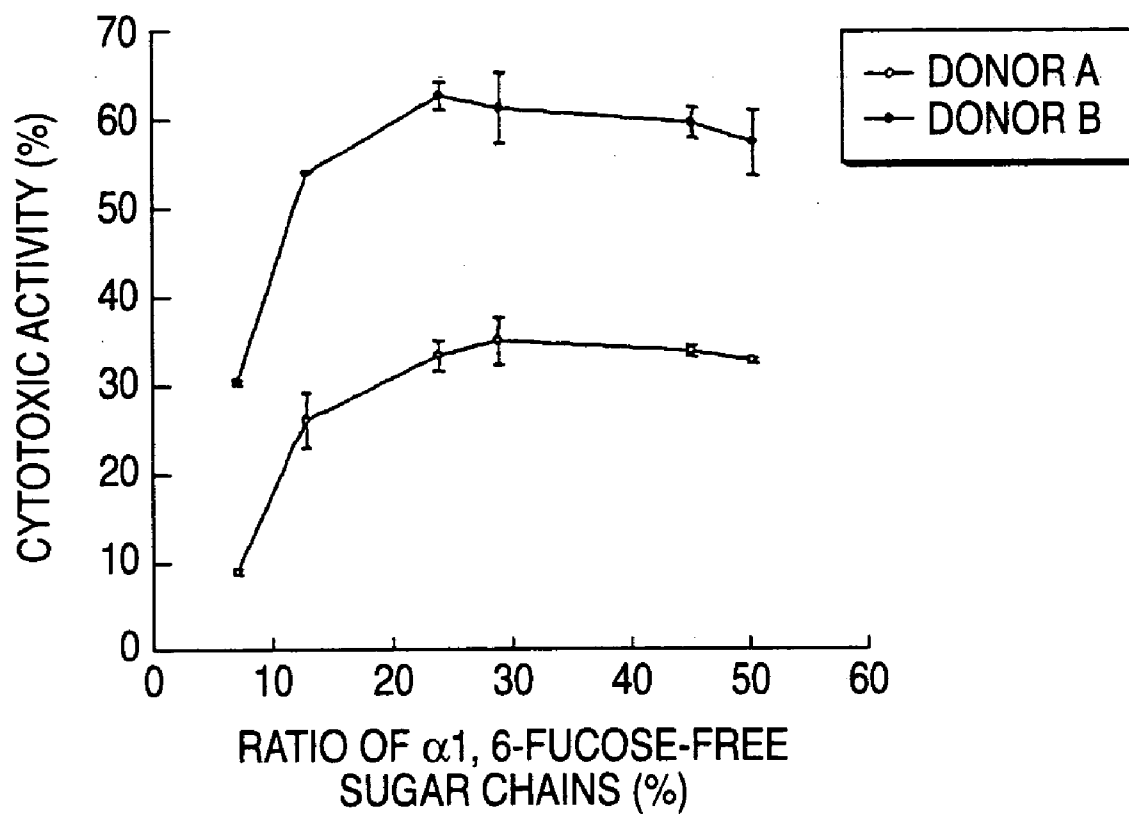


FIG. 8

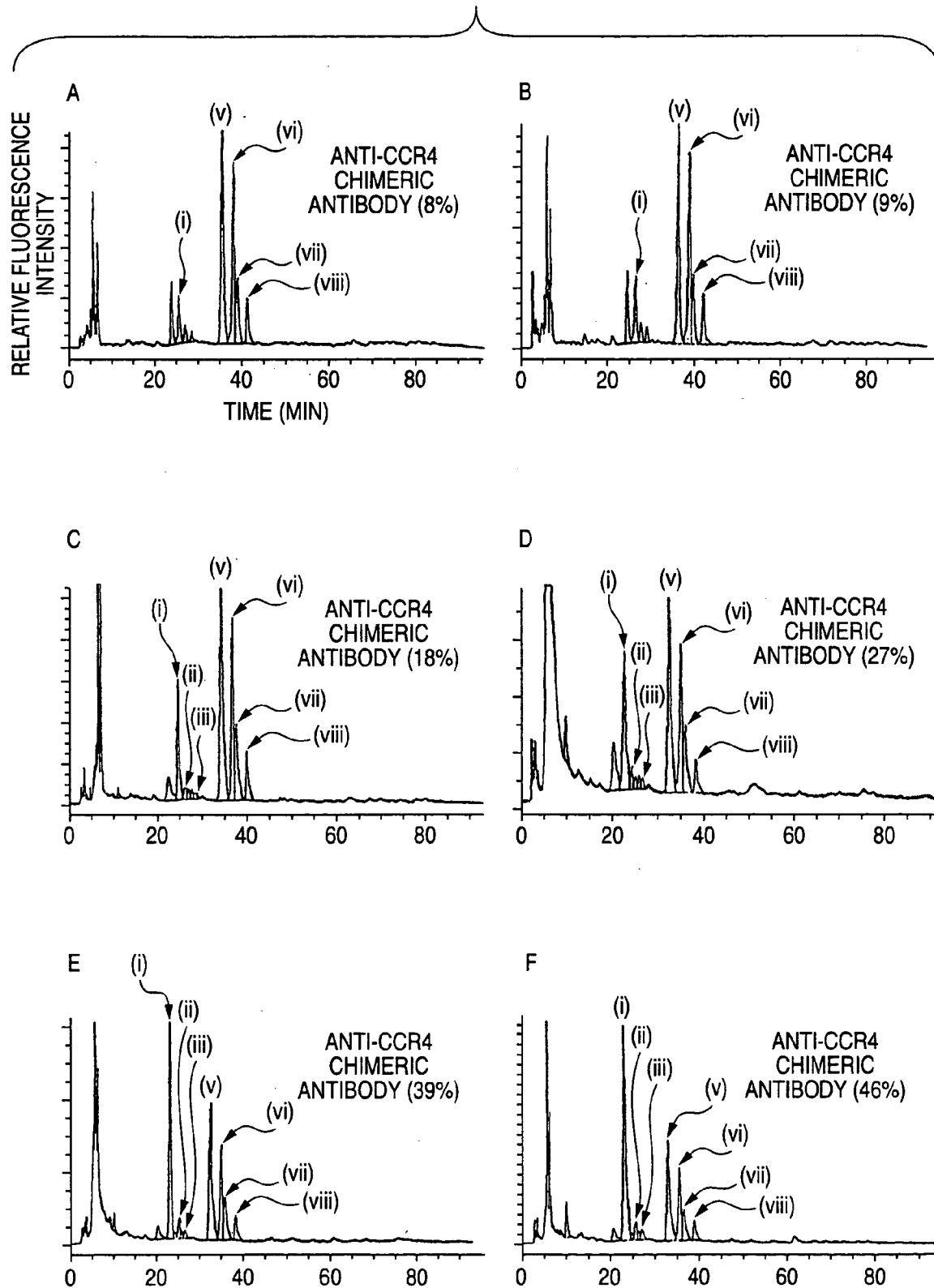


FIG. 9

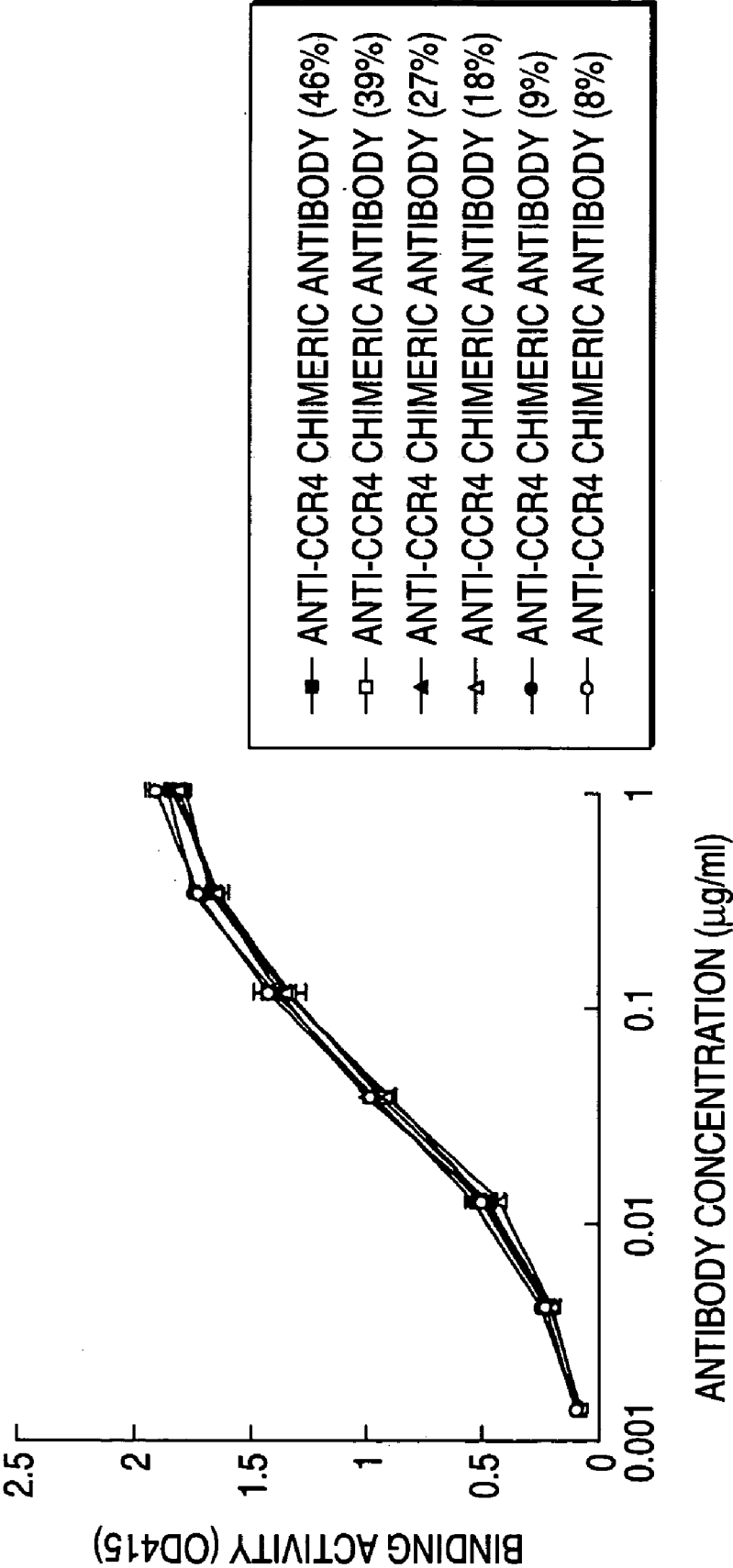


FIG. 10

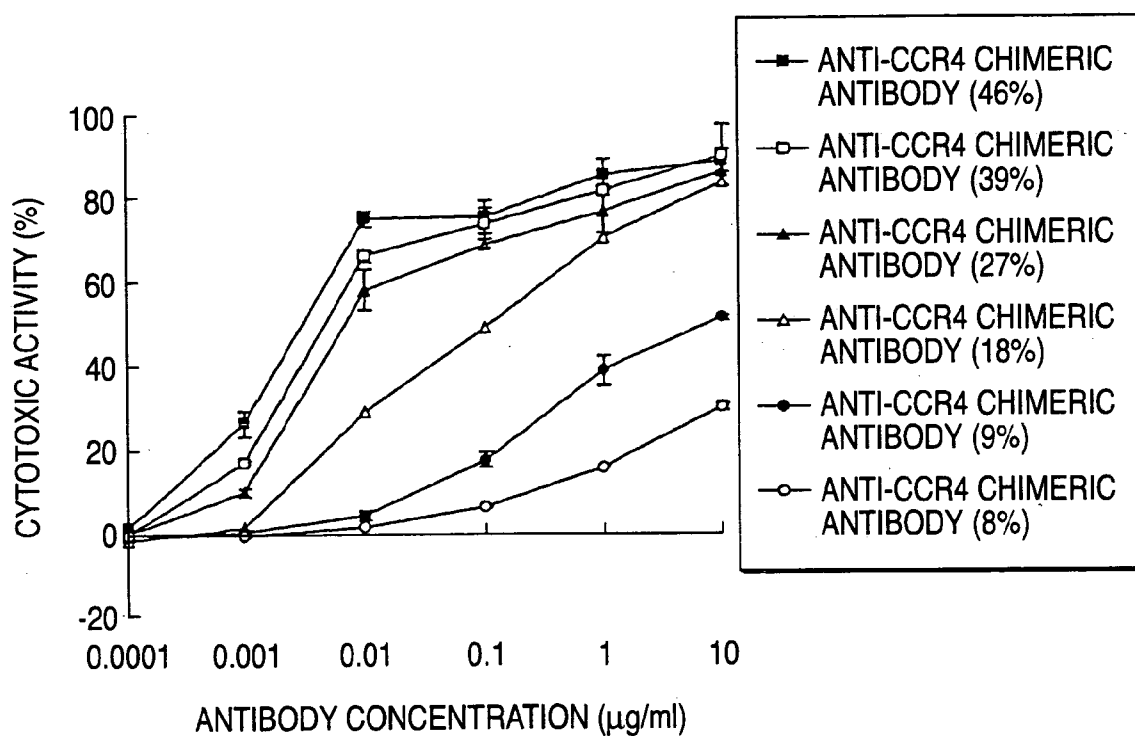


FIG. 11

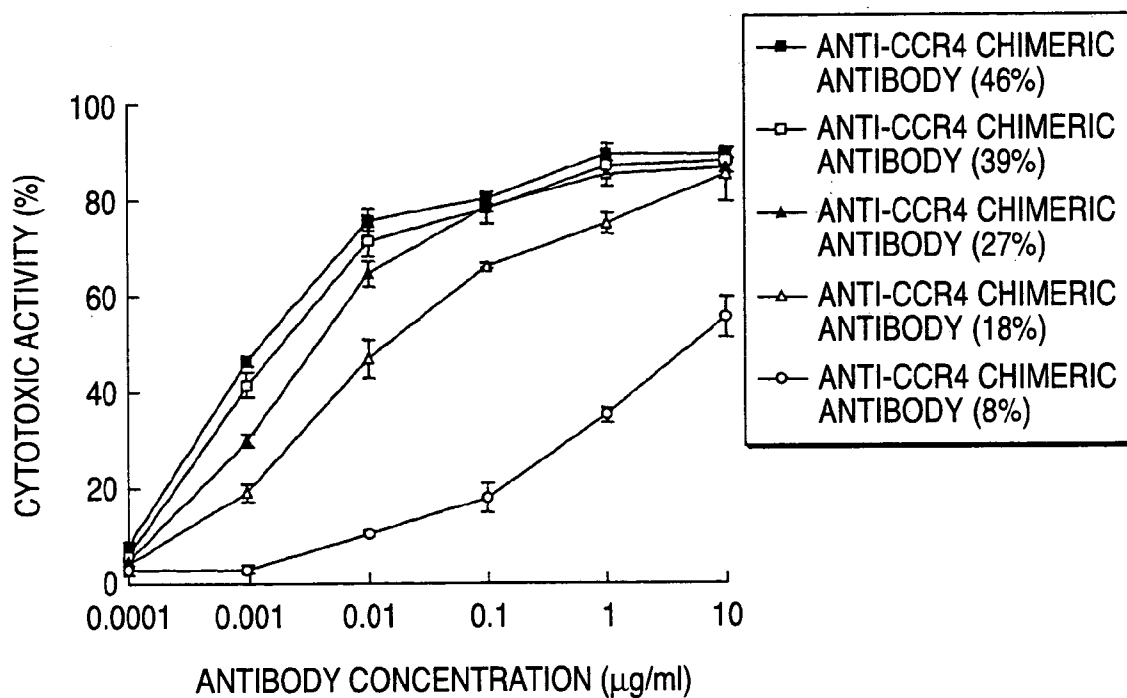


FIG. 12

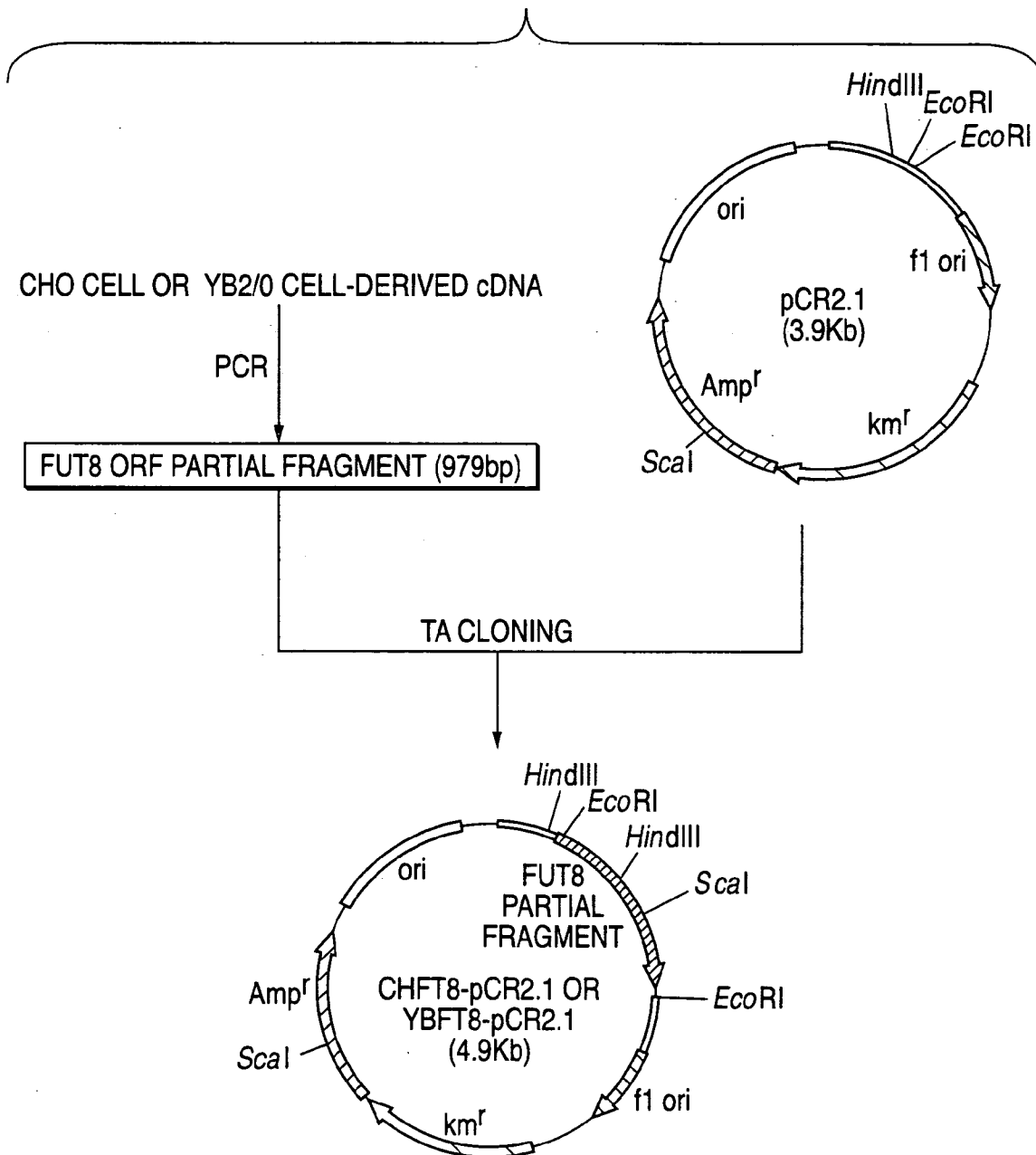


FIG. 13

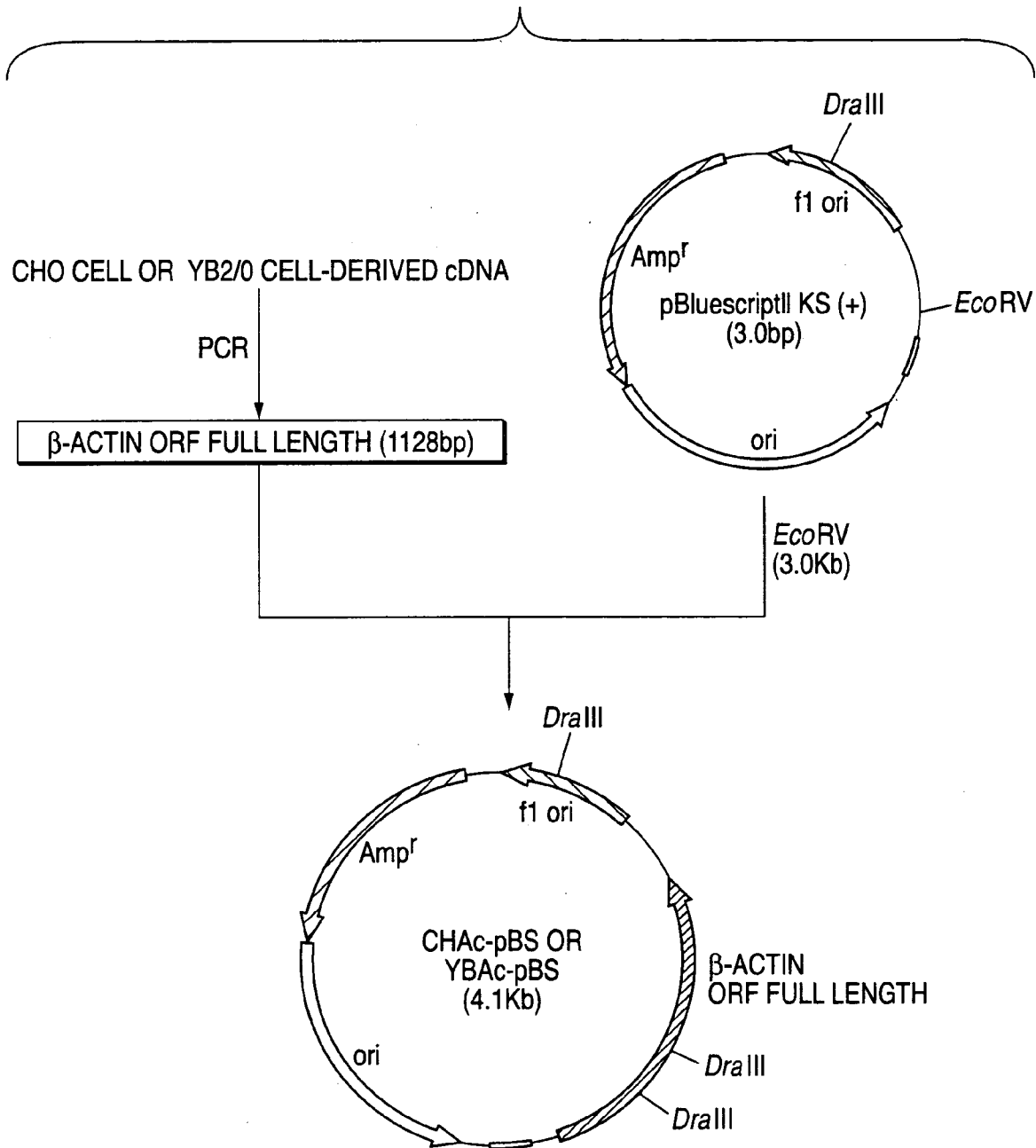


FIG. 14

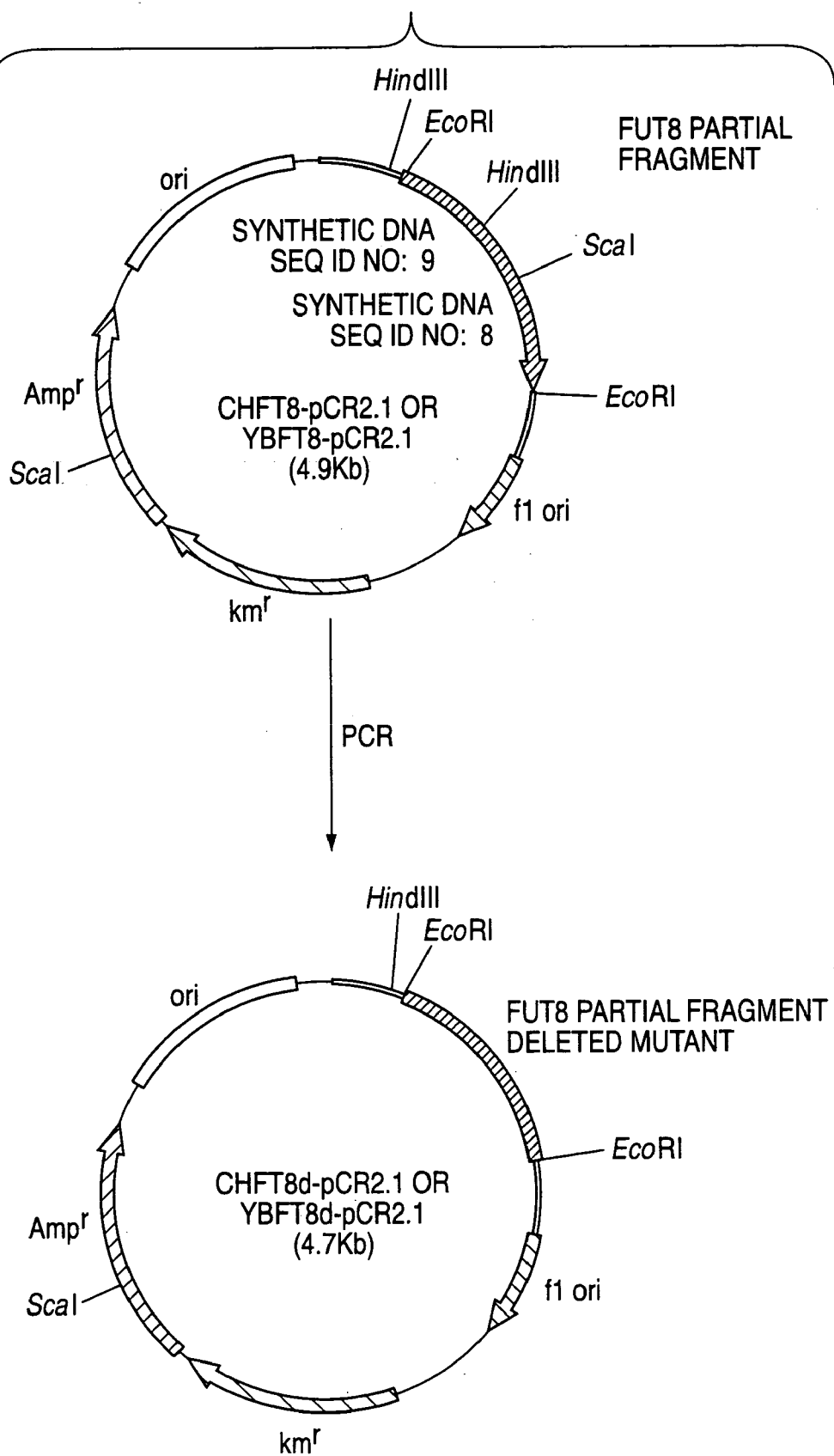


FIG. 15

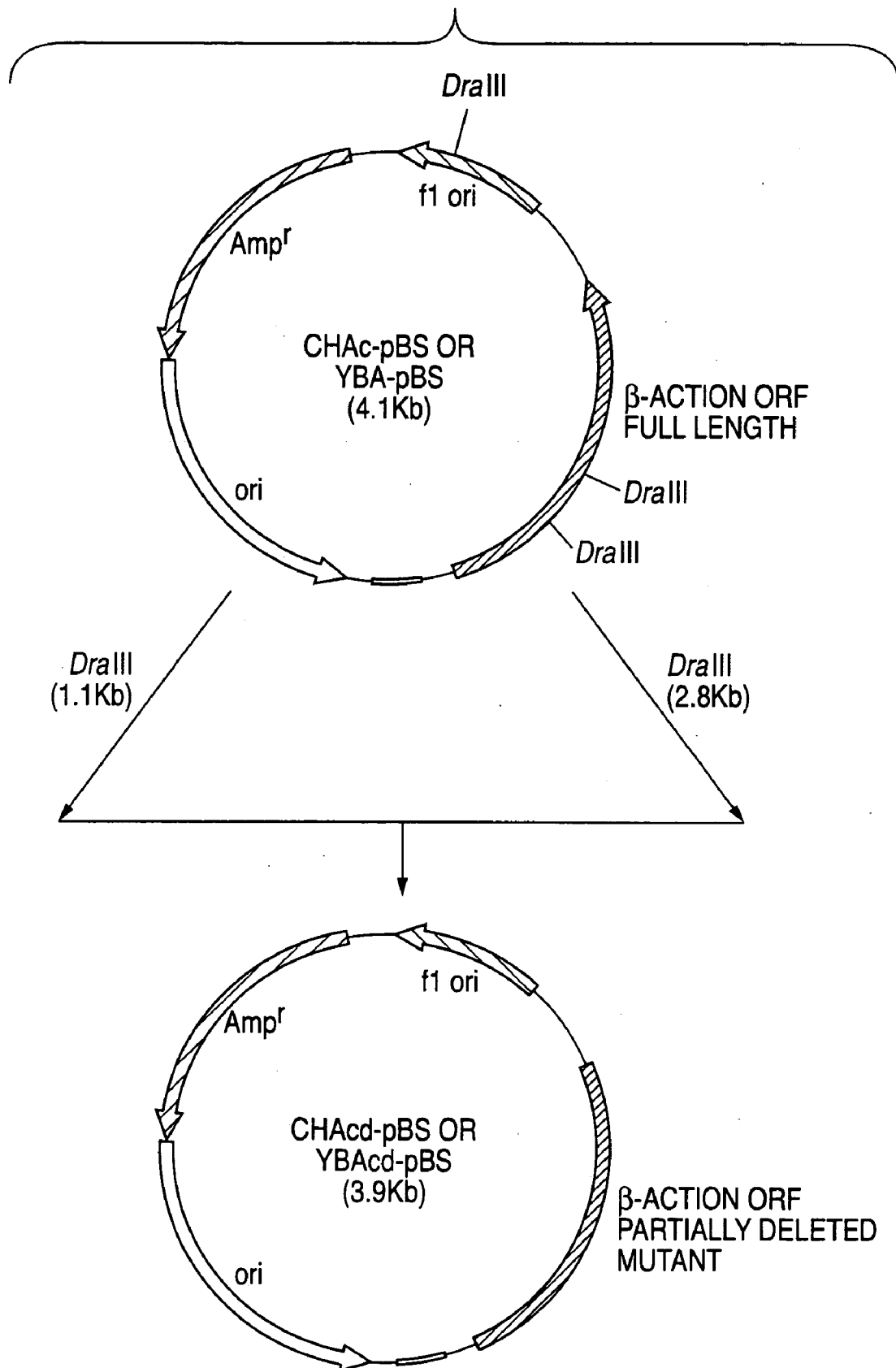


FIG. 16

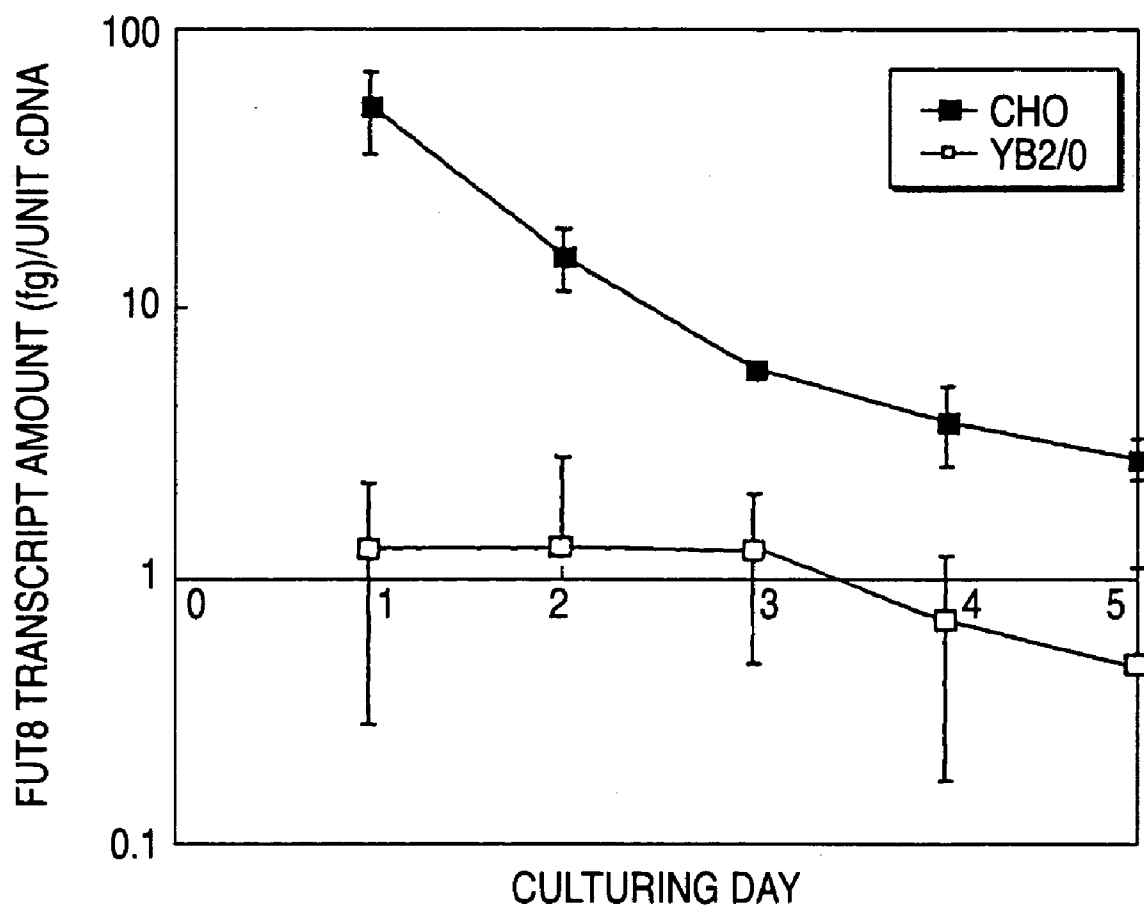


FIG. 17

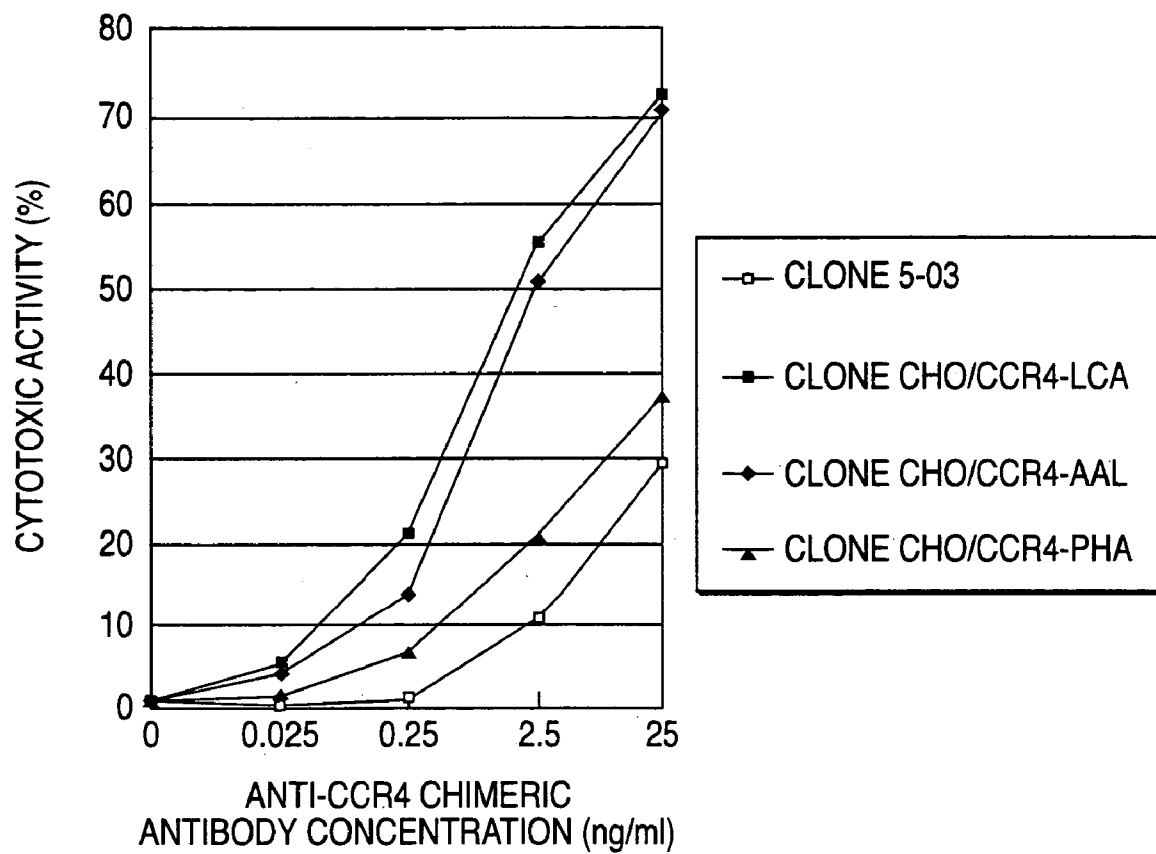


FIG. 18

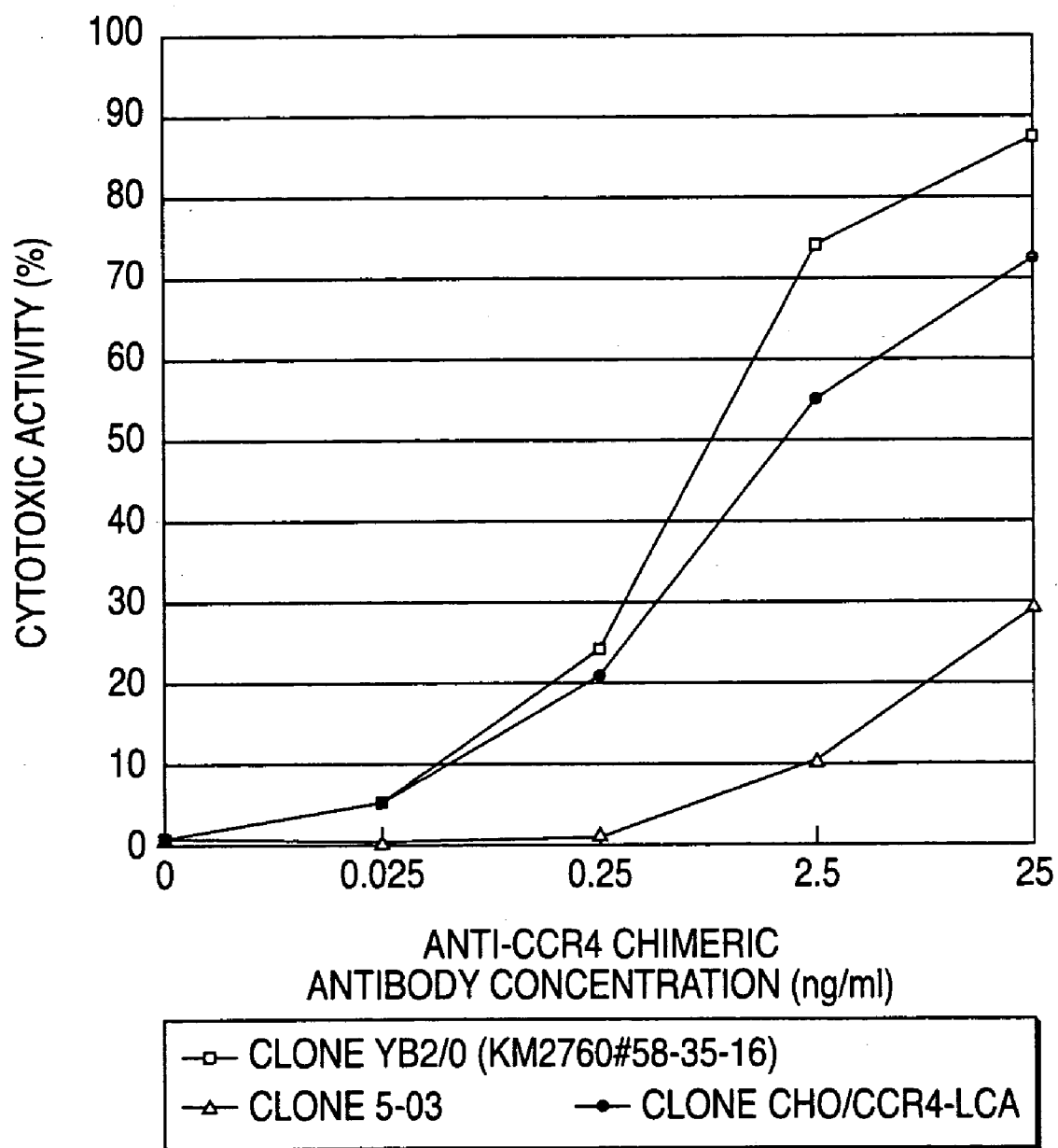


FIG. 19

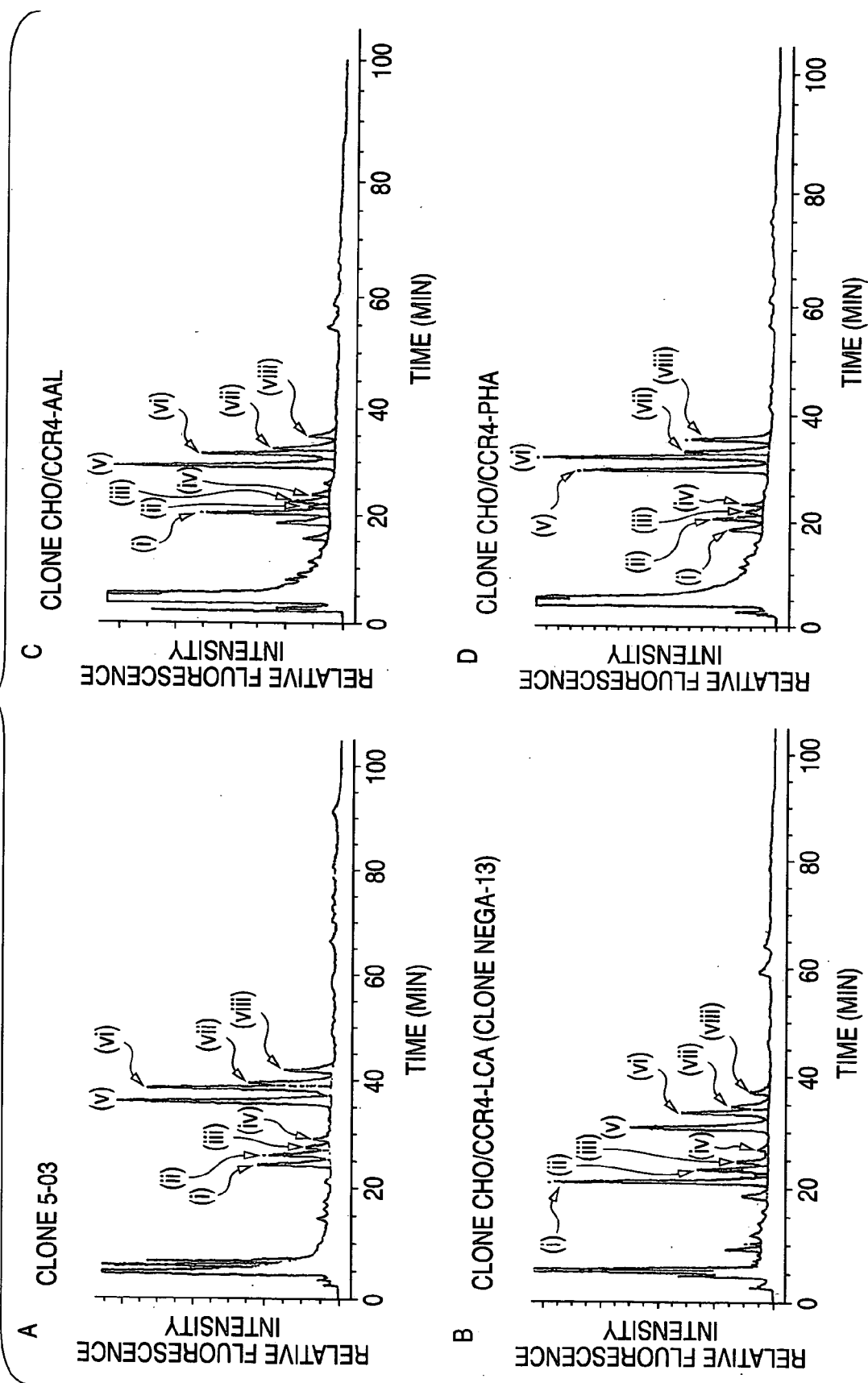


FIG. 20

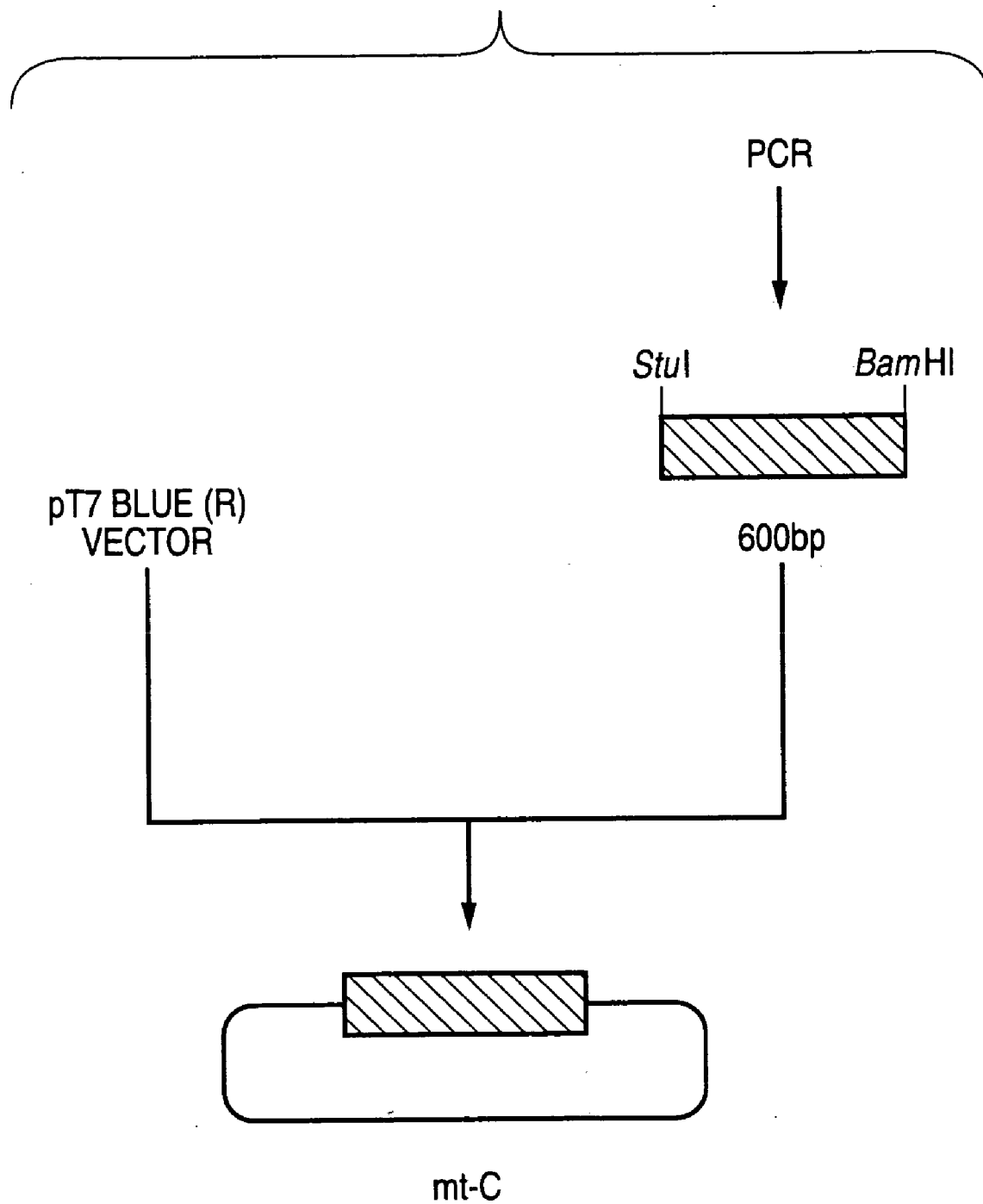


FIG. 21

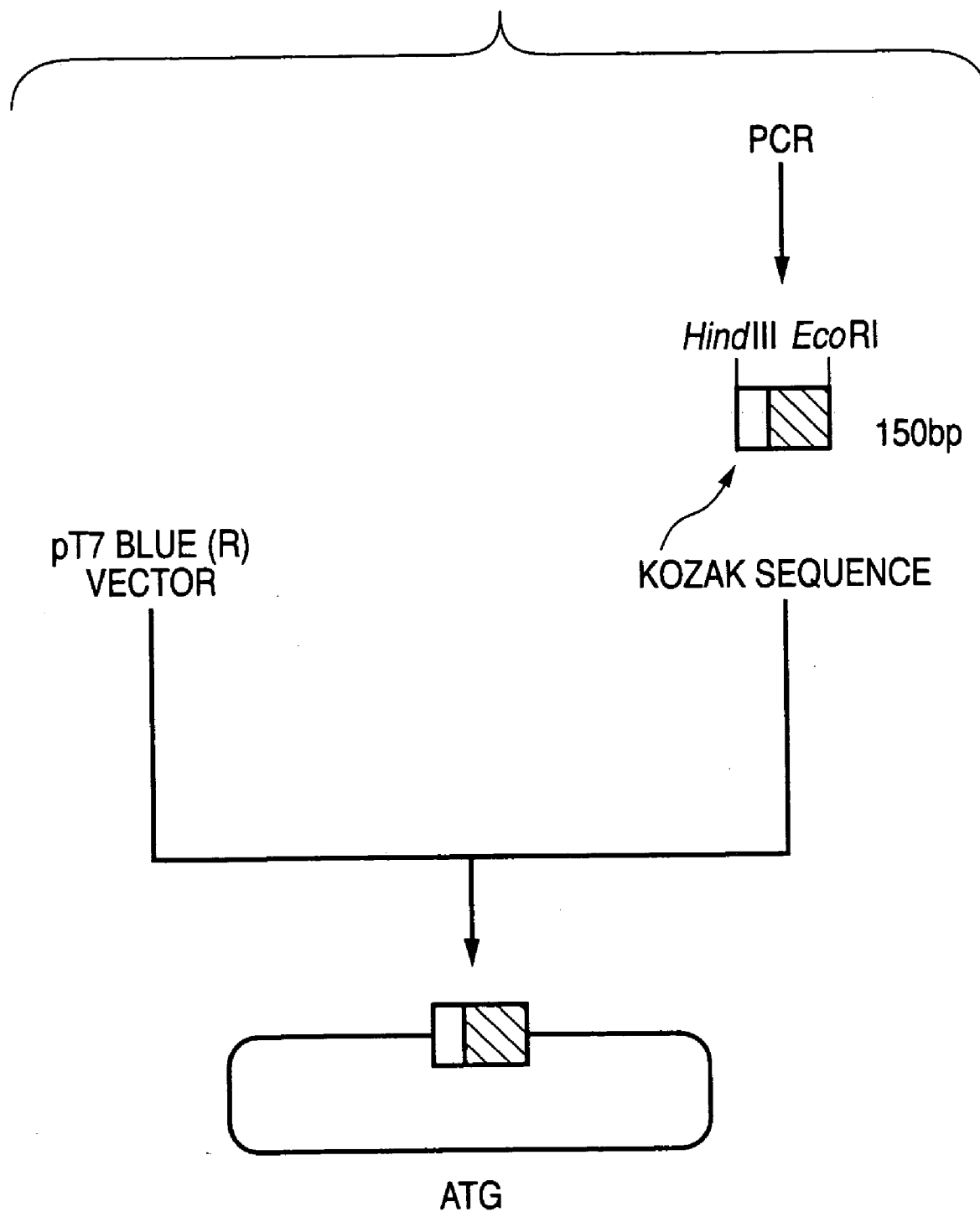


FIG. 22

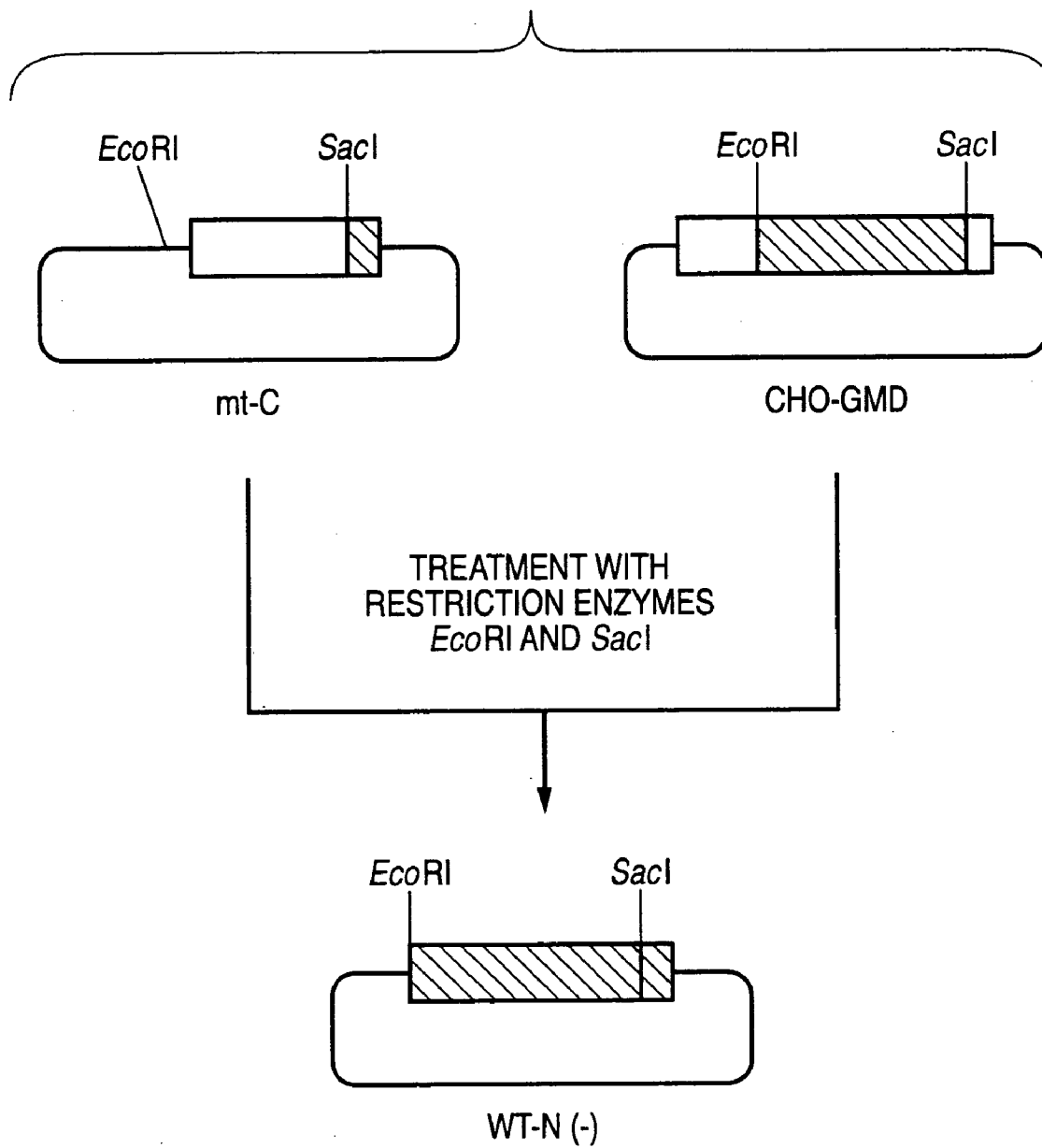


FIG. 23

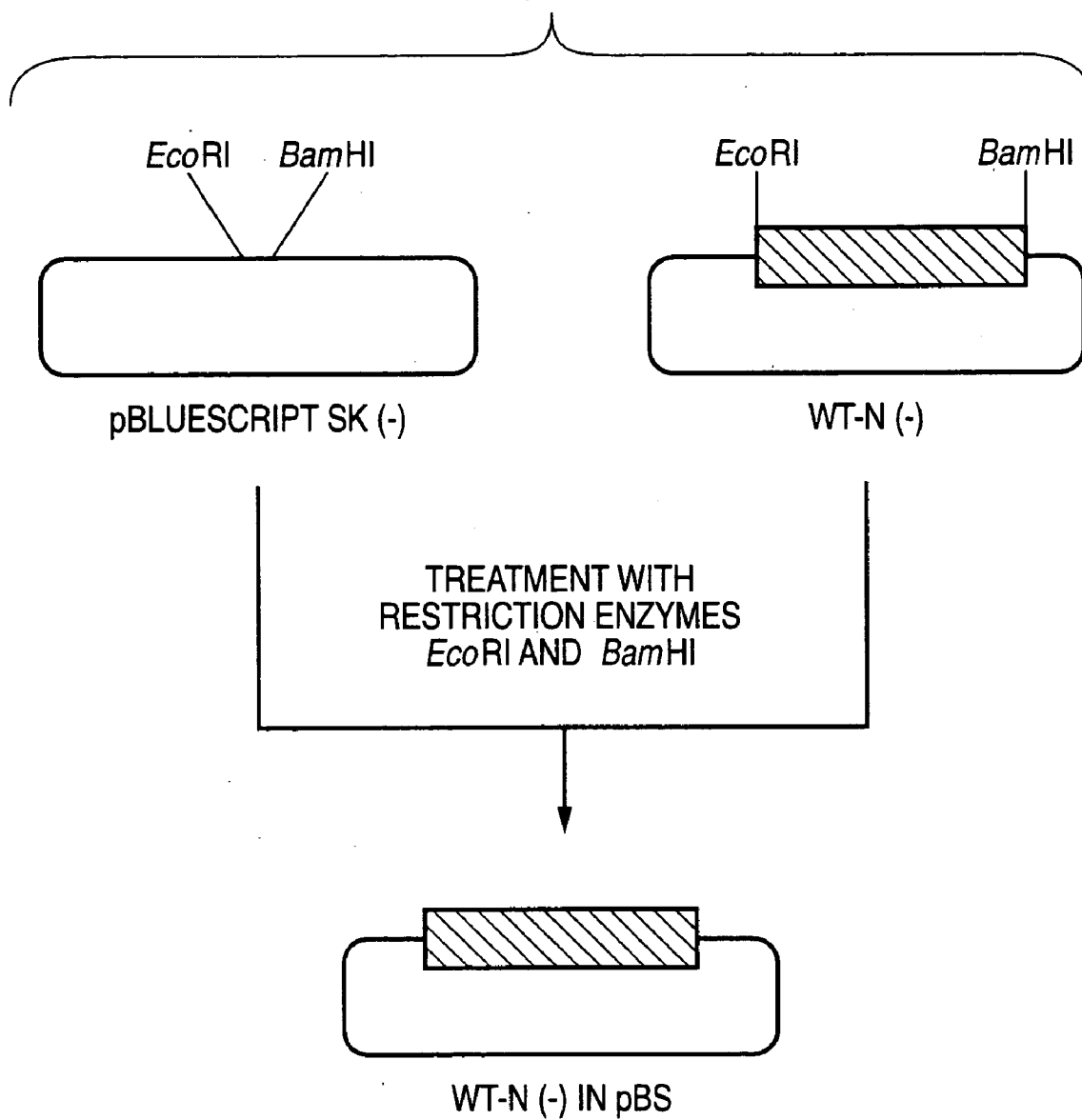


FIG. 24

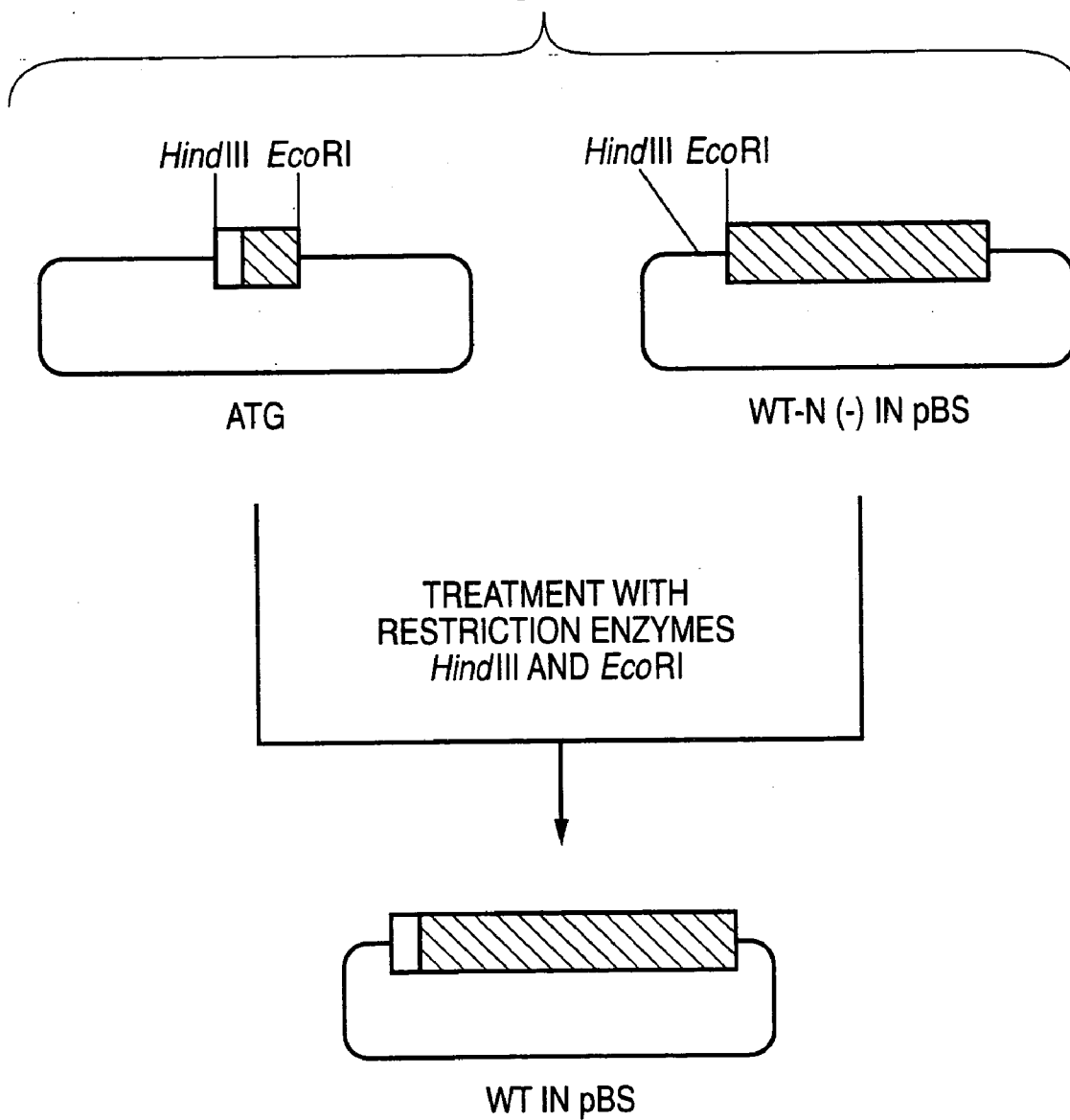


FIG. 25

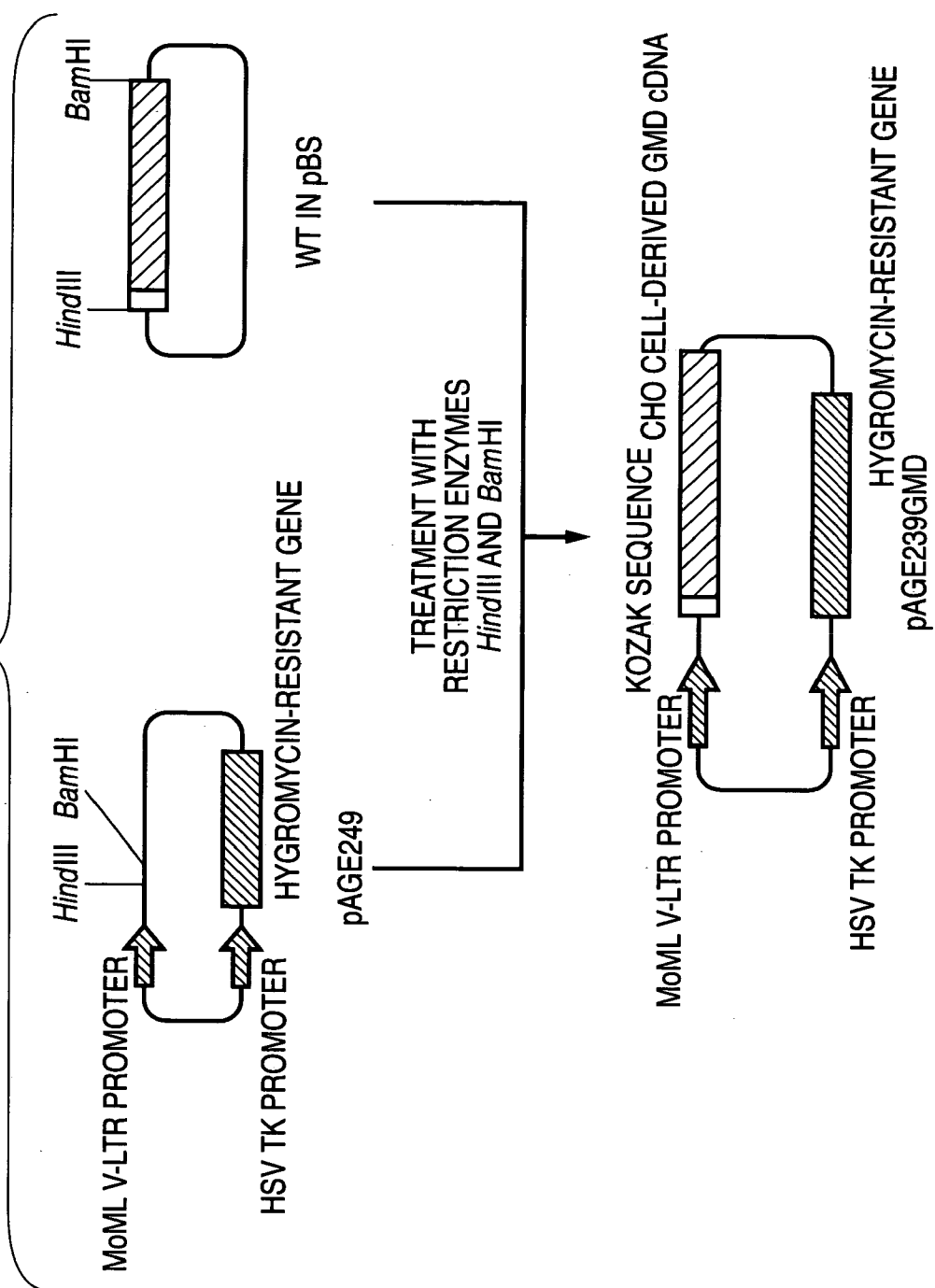


FIG. 26

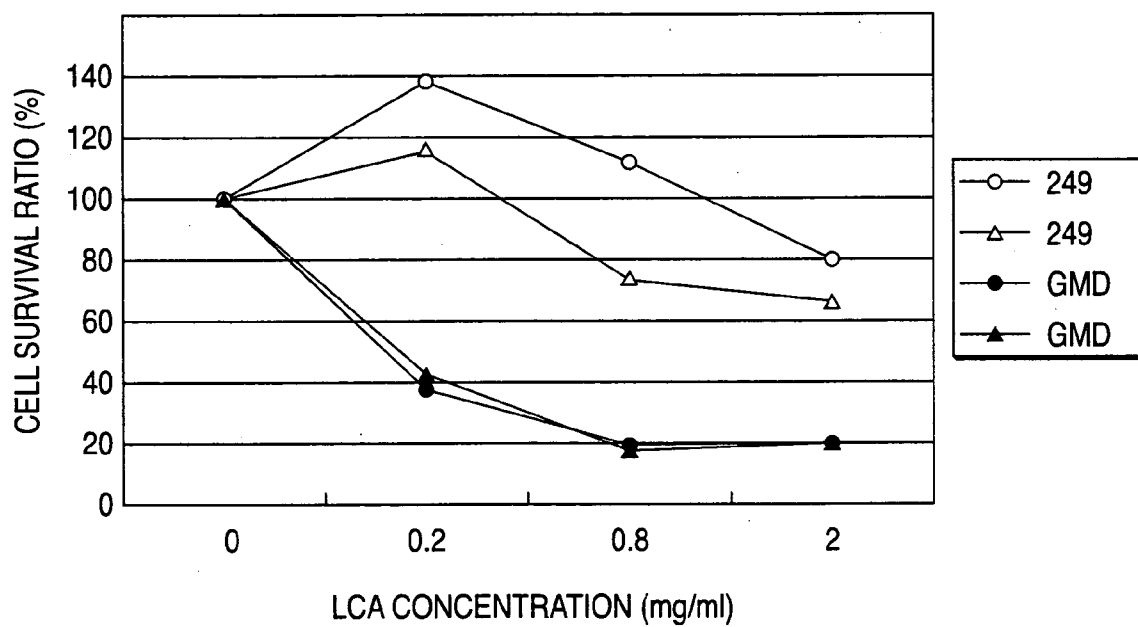


FIG. 27

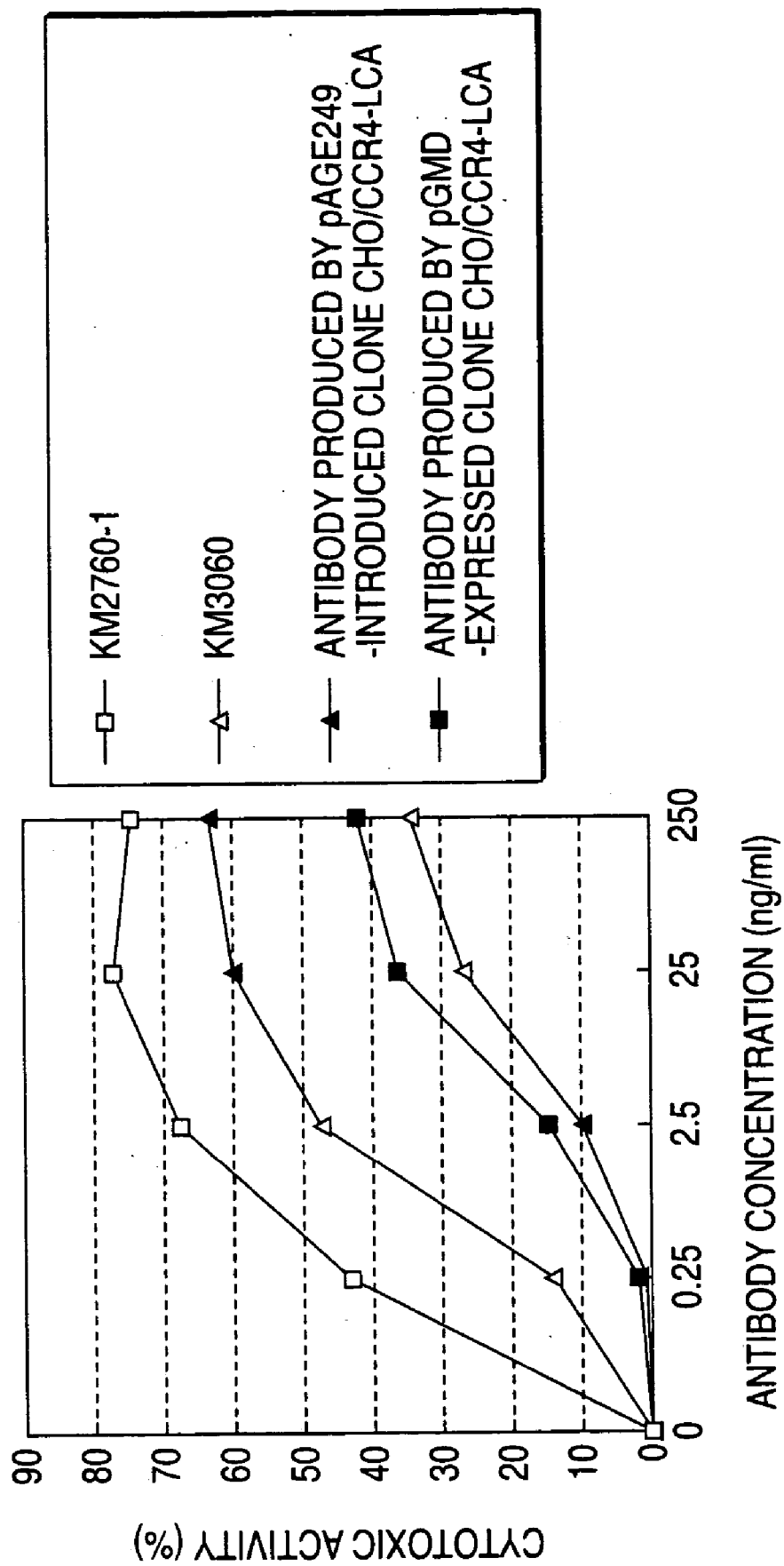


FIG. 28

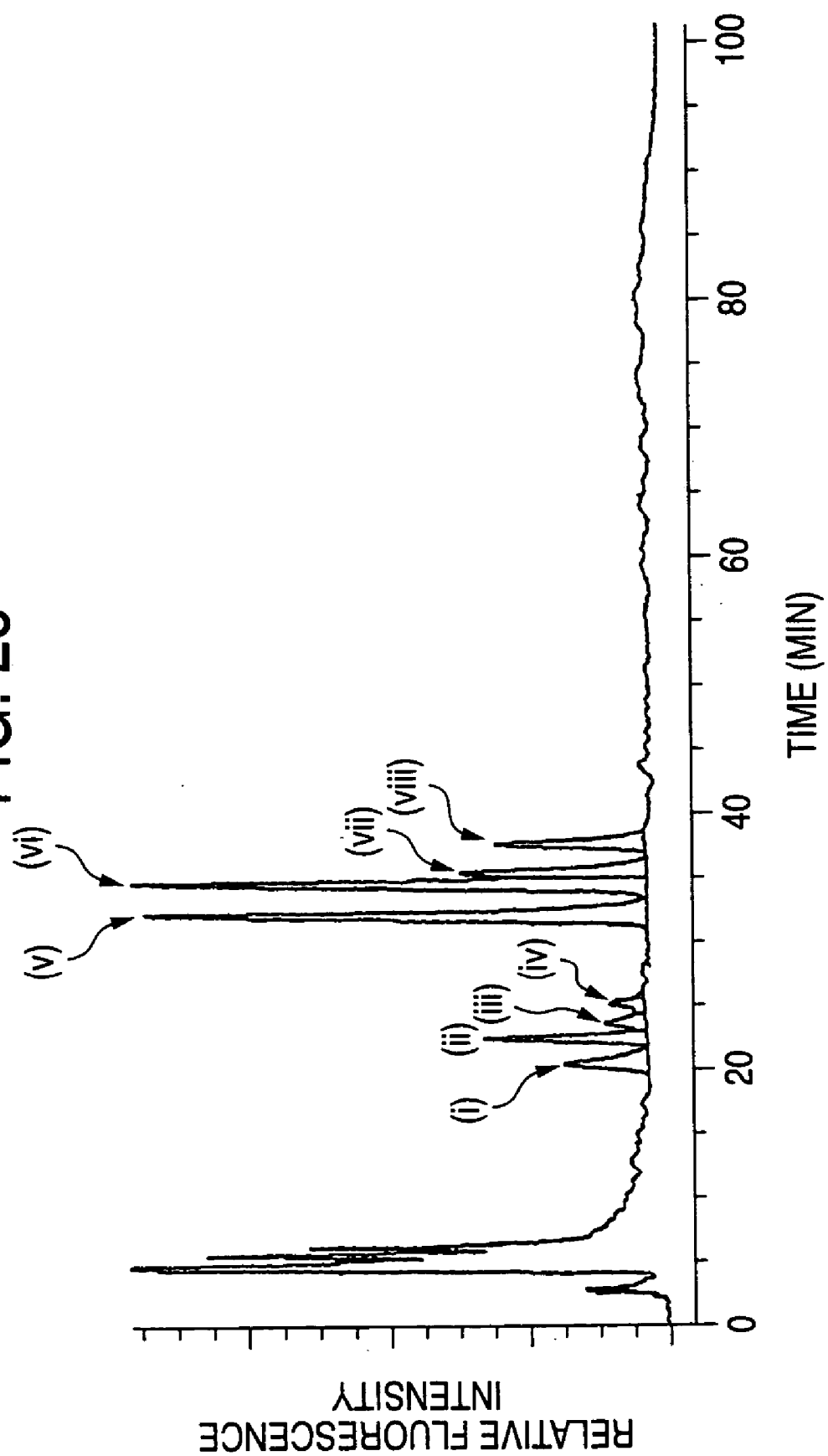


FIG. 29

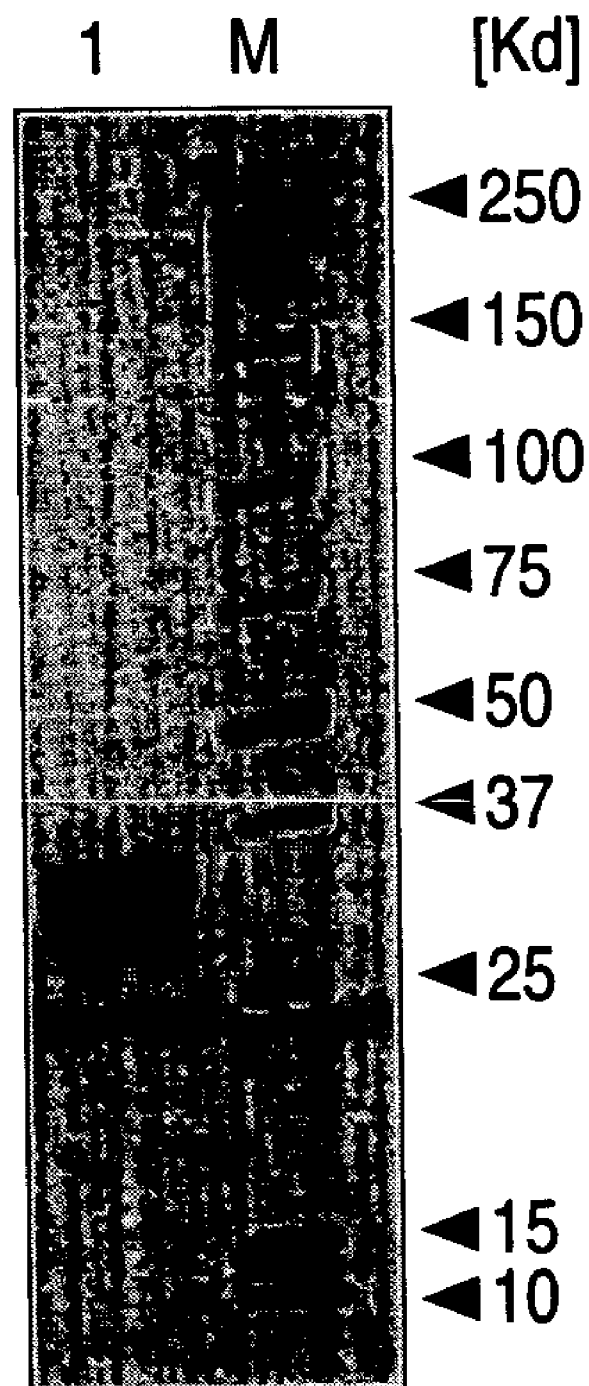


FIG. 30

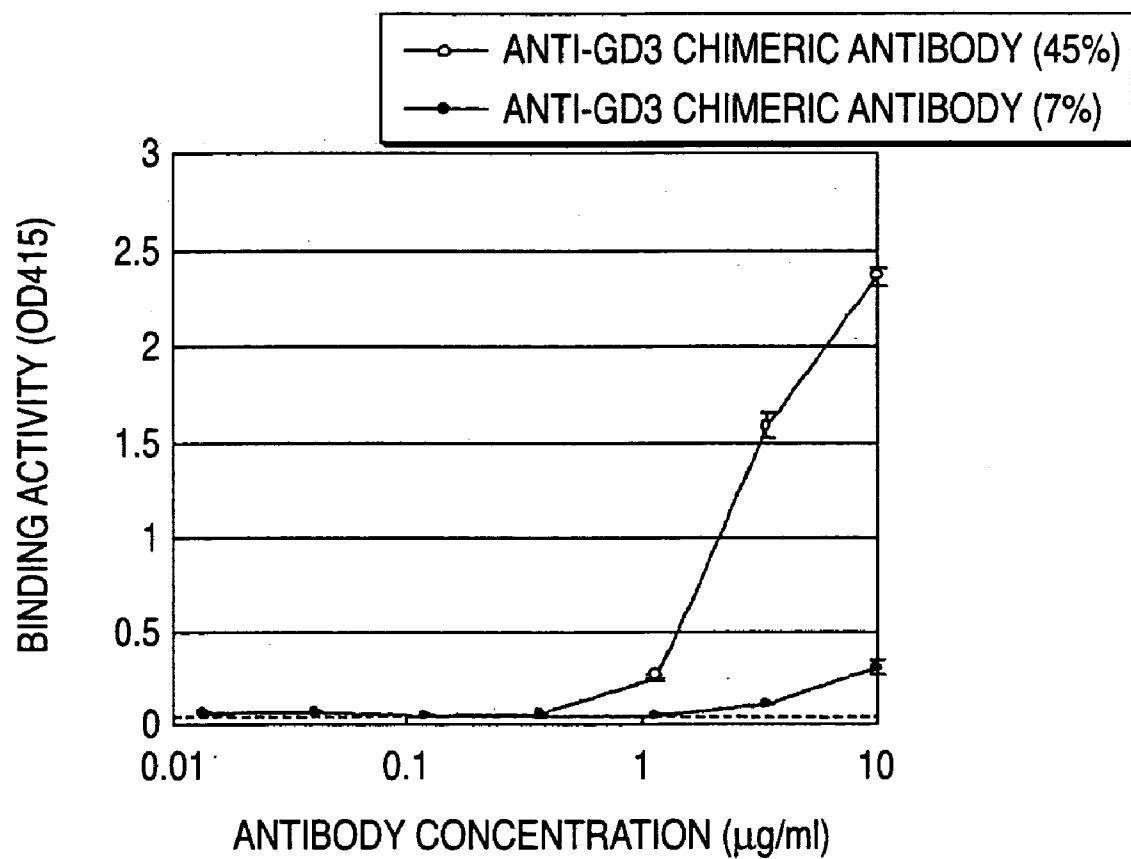


FIG. 31

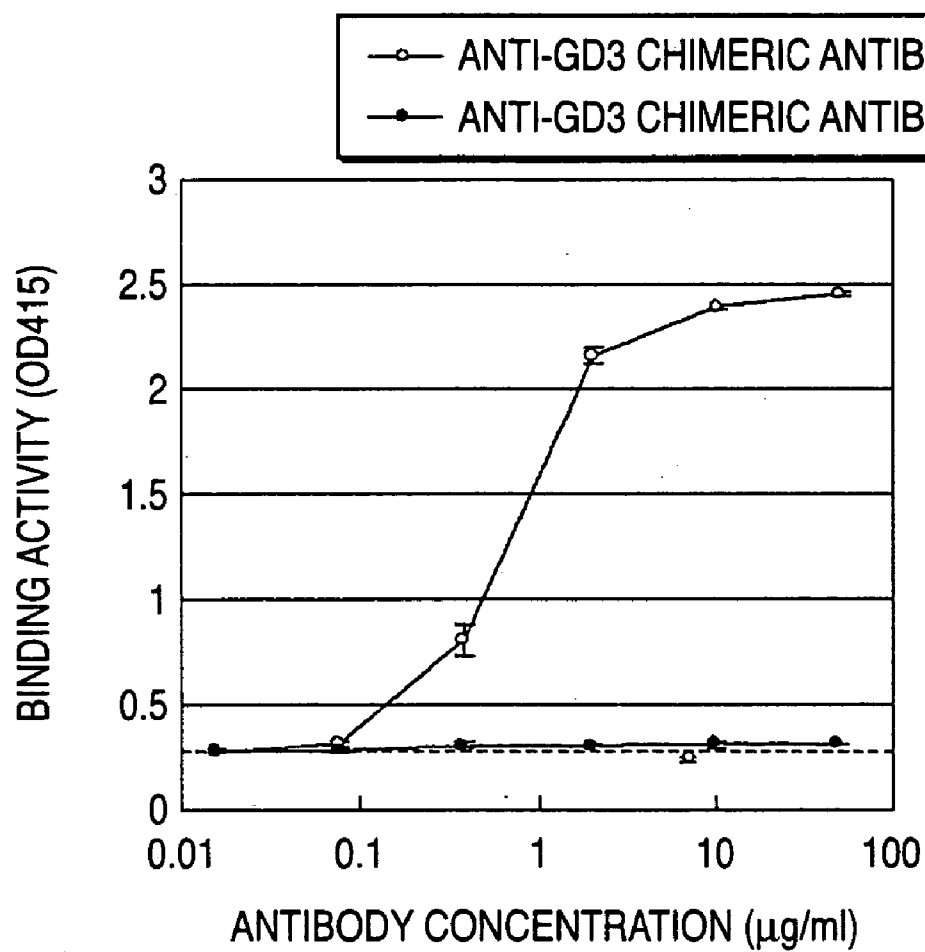


FIG. 32A

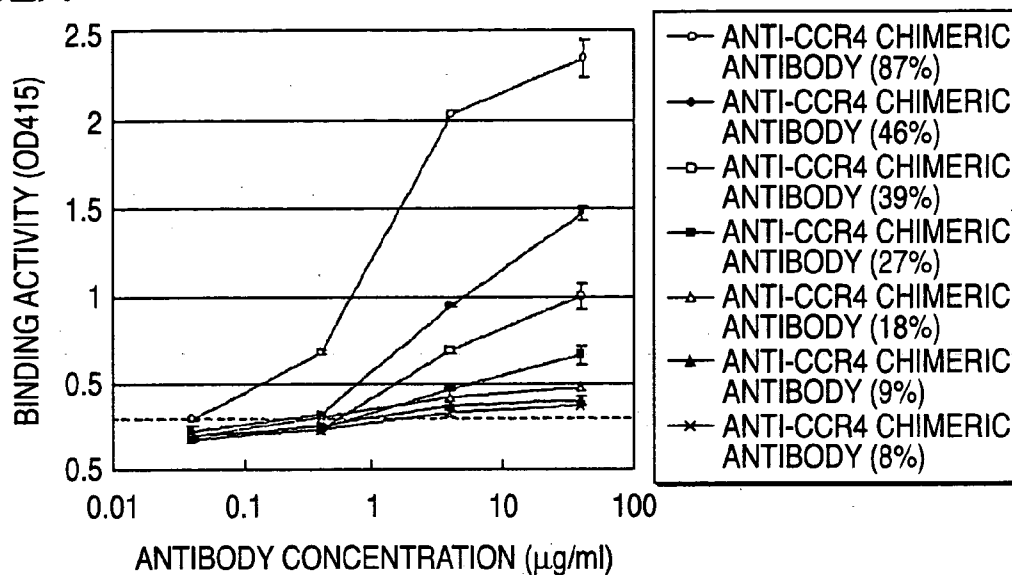


FIG. 32B

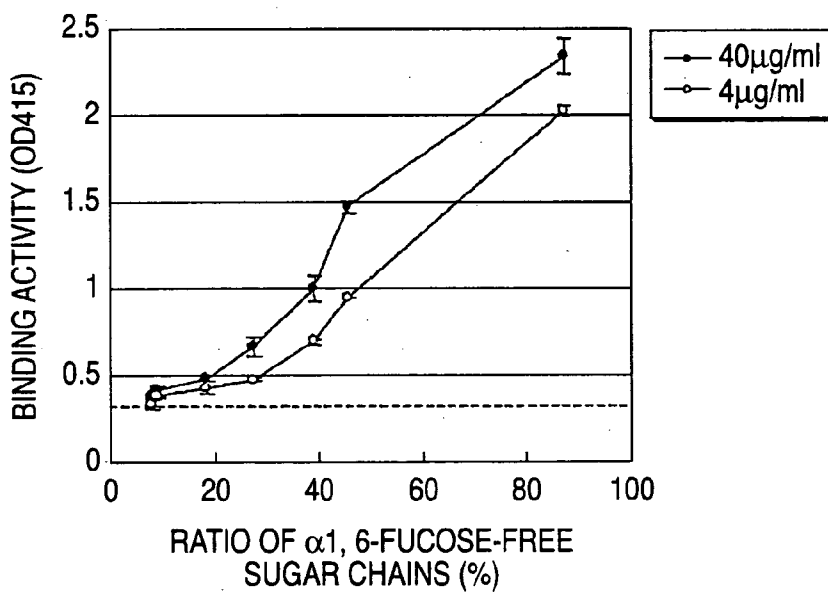


FIG. 33

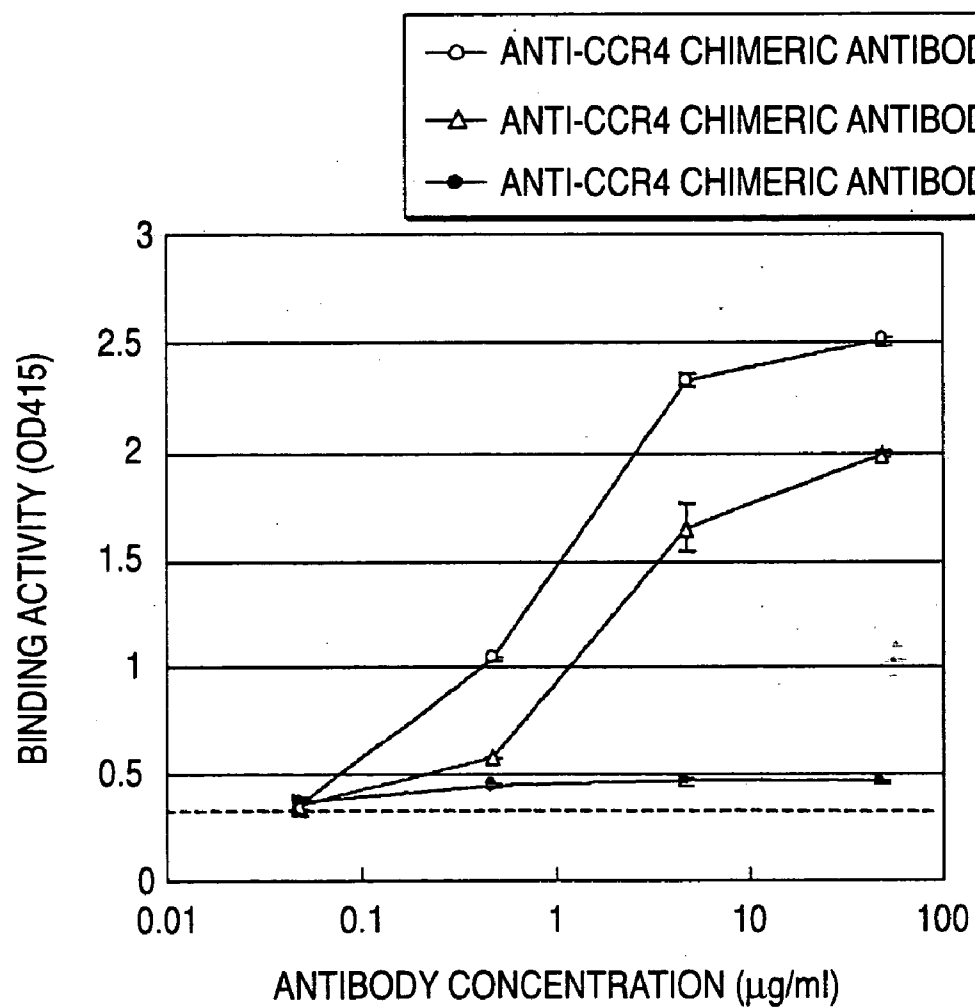


FIG. 34

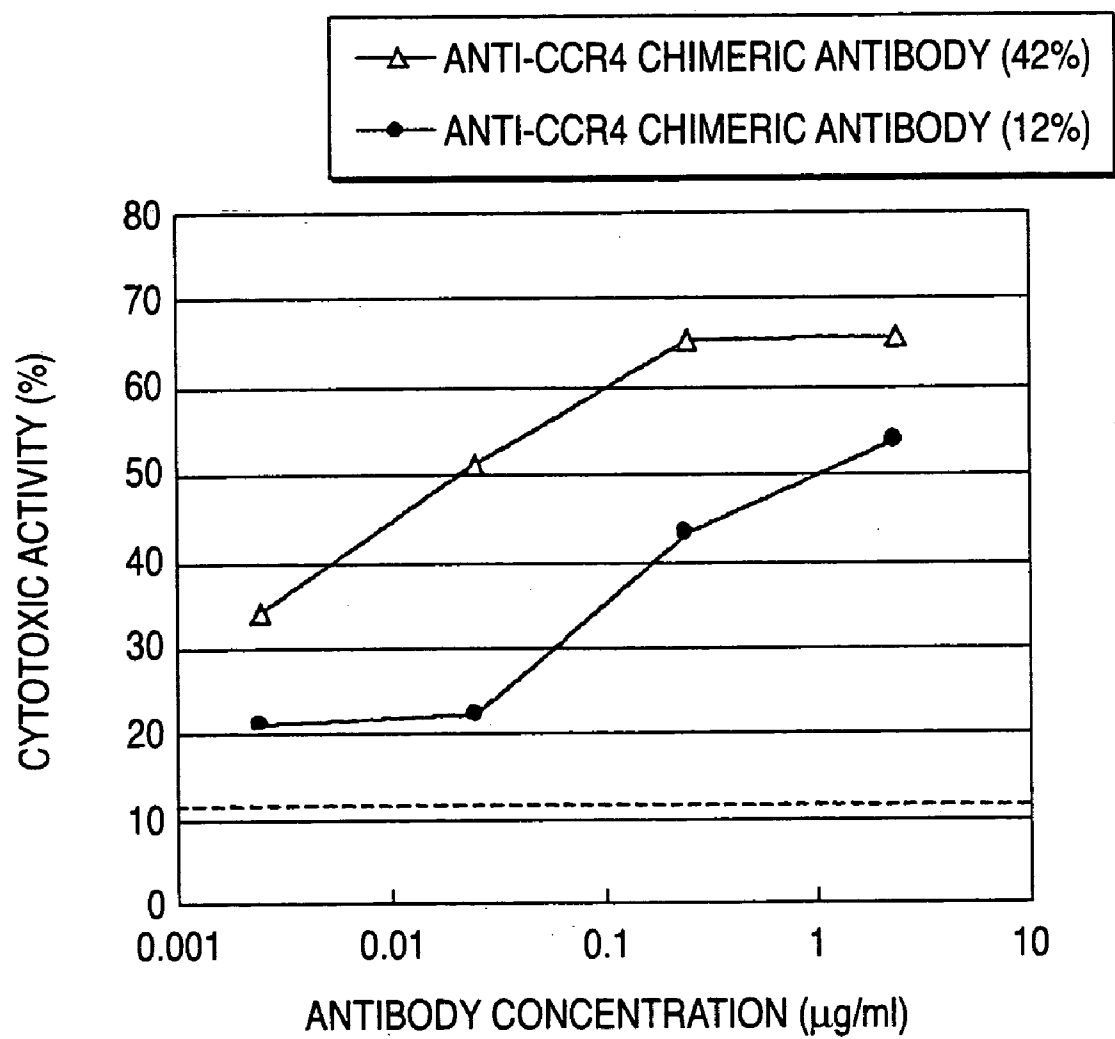


FIG. 35

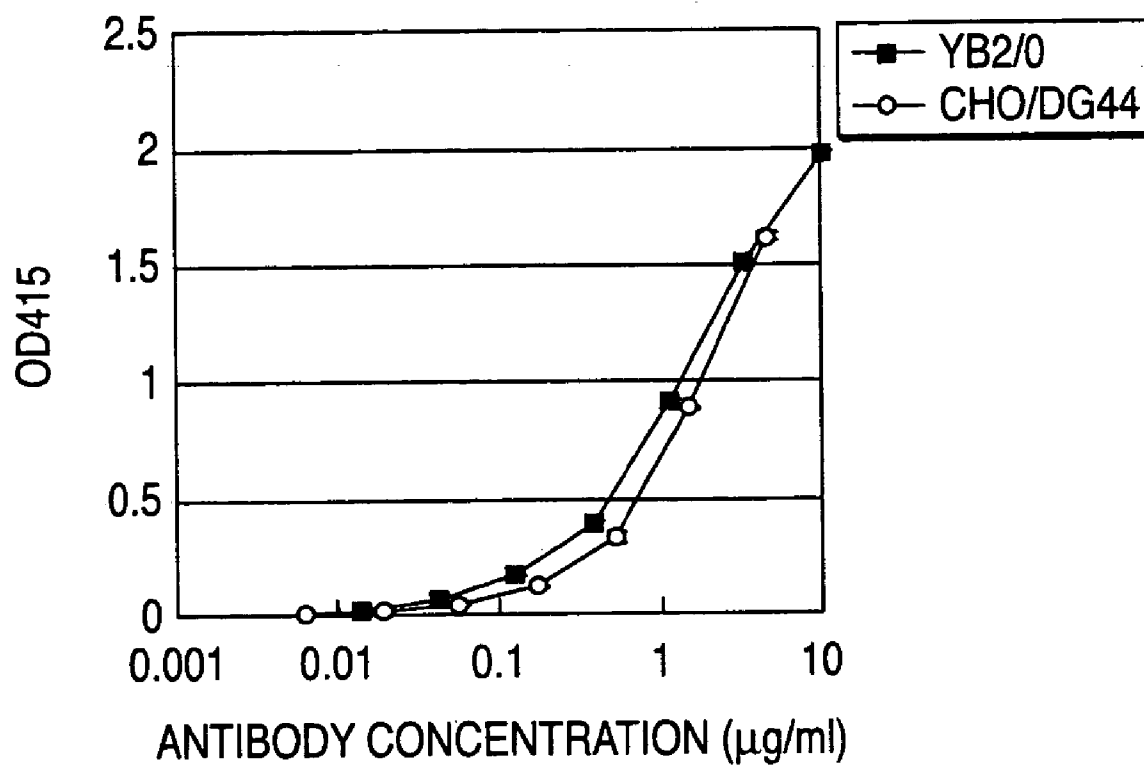


FIG. 36

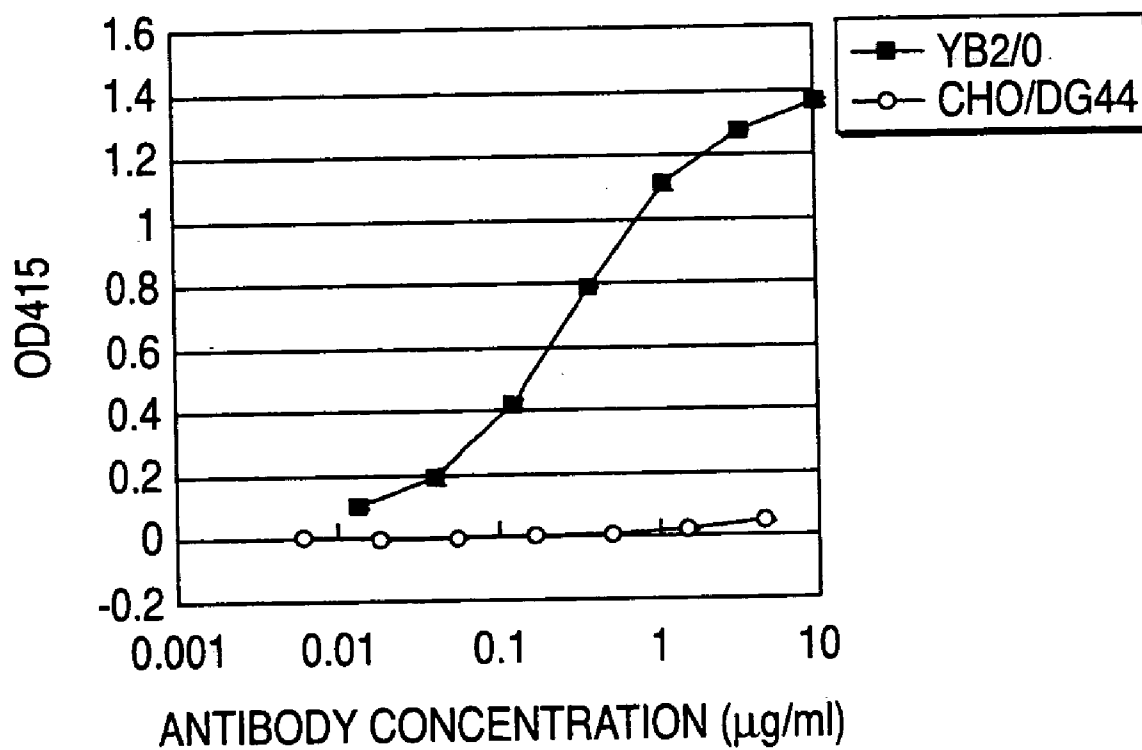


FIG. 37

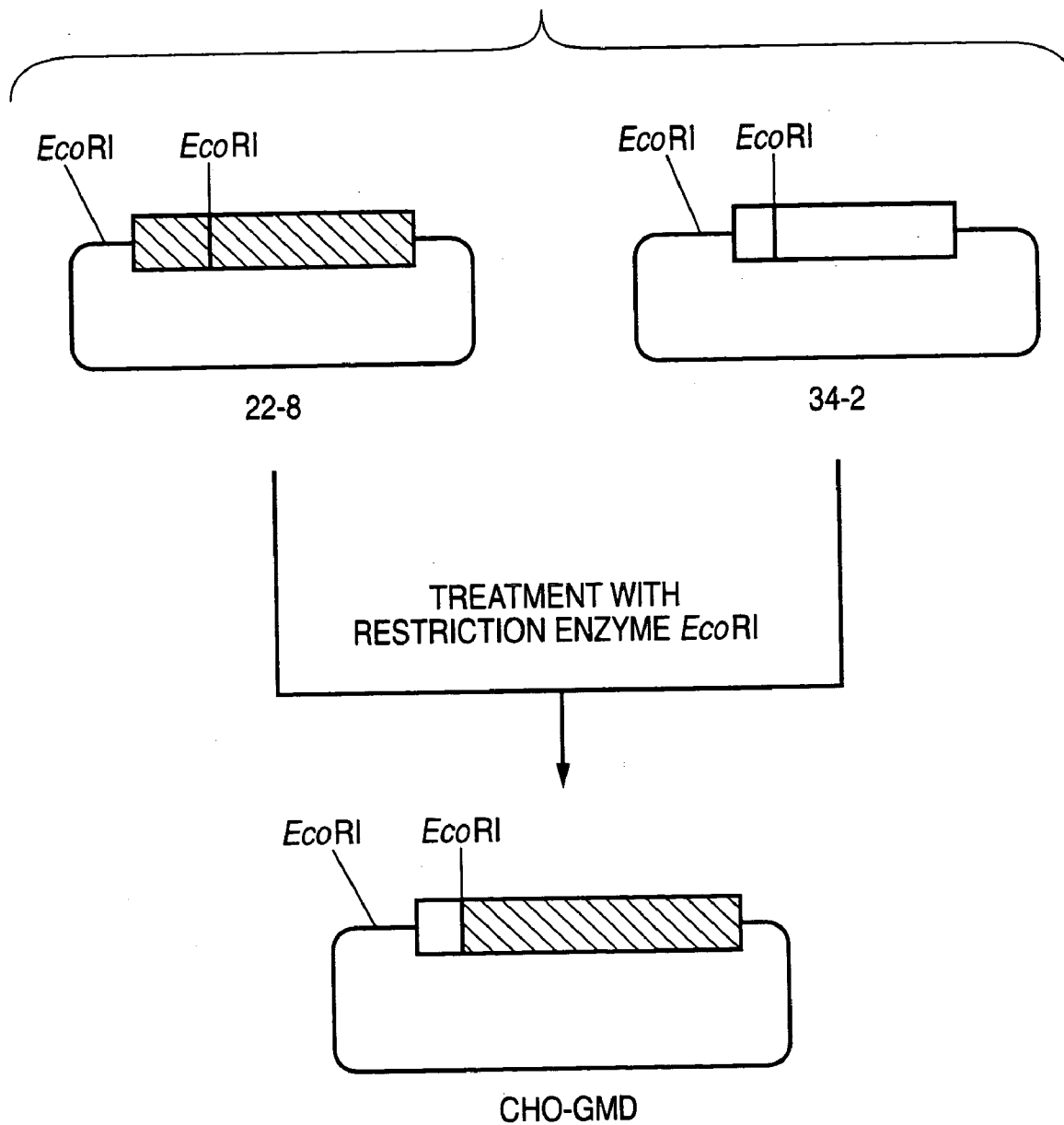
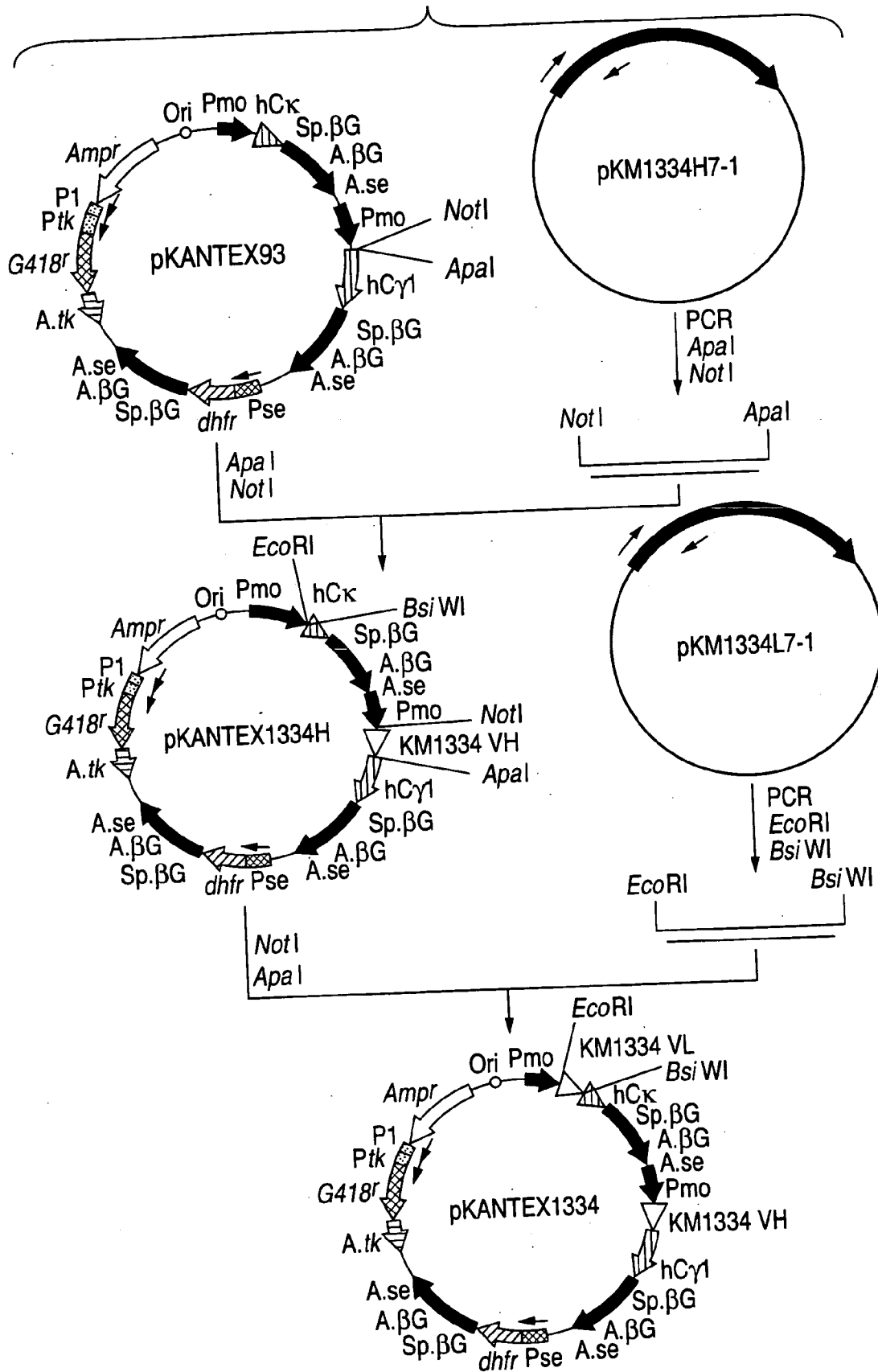


FIG. 38



METHOD OF ENHANCING OF BINDING ACTIVITY OF ANTIBODY COMPOSITION TO FCGAMMA RECEPTOR IIIA

BACKGROUND OF THE INVENTION

[0001] 1. Field of the Invention

[0002] The present invention relates to a method for enhancing a binding activity of an antibody composition to Fc γ receptor IIIa, which comprises modifying a complex N-glycoside-linked sugar chain which is bound to the Fc region of an antibody molecule; a method for enhancing an antibody-dependent cell-mediated cytotoxic activity of an antibody composition; a process for producing an antibody composition having an enhanced binding activity to Fc γ receptor IIIa; a method for detecting the ratio of a sugar chain in which fucose is not bound to N-acetylglucosamine in the reducing end in the sugar chain among total complex N-glycoside-linked sugar chains bound to the Fc region in an antibody composition; an Fc fusion protein composition produced by using a cell resistant to a lectin which recognizes a sugar chain in which 1-position of fucose is bound to 6-position of N-acetylglucosamine in the reducing end through α -bond in a complex N-glycoside-linked sugar chain; and a process for producing the same.

[0003] 2. Brief Description of the Background Art

[0004] Since antibodies have high binding activity, binding specificity and high stability in blood, their applications to diagnosis, prevention and treatment of various human diseases have been attempted [*Monoclonal Antibodies: Principles and Applications*, Wiley-Liss, Inc., Chapter 2.1 (1995)]. Also, production of a humanized antibody such as a human chimeric antibody or a human complementarity determining region (hereinafter referred to as "CDR")-grafted antibody from a non-human animal antibody have been attempted by using genetic recombination techniques. The human chimeric antibody is an antibody in which its antibody variable region (hereinafter referred to as "V region") is derived from a non-human animal antibody and its constant region (hereinafter referred to as "C region") is derived from a human antibody. The human CDR-grafted antibody is an antibody in which the CDR of a human antibody is replaced by CDR derived from a non-human animal antibody.

[0005] It has been revealed that five classes, namely IgM, IgD, IgG, IgA and IgE, are present in mammal antibodies. Antibodies of human IgG class are mainly used for the diagnosis, prevention and treatment of various human diseases because they have functional characteristics such as long half-life in blood and various effector functions [*Monoclonal Antibodies: Principles and Applications*, Wiley-Liss, Inc., Chapter 1 (1995)]. The human IgG class antibody is further classified into the following 4 subclasses: IgG1, IgG2, IgG3 and IgG4. A large number of studies have so far been conducted for antibody-dependent cell-mediated cytotoxic activity (hereinafter referred to as "ADCC activity") and complement-dependent cytotoxic activity (hereinafter referred to as "CDC activity") as effector functions of the IgG class antibody, and it has been reported that among antibodies of the human IgG class, the IgG1 subclass has the highest ADCC activity and CDC activity [*Chemical Immunology*, 65, 88 (1997)]. In view of the above, most of the anti-tumor humanized antibodies, including commercially

available Rituxan and Herceptin, which require high effector functions for the expression of their effects, are antibodies of the human IgG1 subclass.

[0006] Expression of ADCC activity and CDC activity of the human IgG1 subclass antibodies requires binding of the Fc region of the antibody to a receptor for an antibody (hereinafter referred to as "Fc γ R") existing on the surface of effector cells such as killer cells, natural killer cells or activated macrophages and various complement components are bound. Regarding the binding, it has been suggested that several amino acid residues in the hinge region and the second domain of C region (hereinafter referred to as "C γ 2 domain") of the antibody are important [*Eur. J. Immunol.*, 23, 1098 (1993), *Immunology*, 86, 319 (1995), *Chemical Immunology*, 65, 88 (1997)] and that a sugar chain in the C γ 2 domain [*Chemical Immunology*, 65, 88 (1997)] is also important.

[0007] Regarding the sugar chain, Boyd et al. have examined effects of a sugar chain on the ADCC activity and CDC activity by treating a human CDR-grafted antibody CAMPATH-1H (human IgG1 subclass) produced by a Chinese hamster ovary cell (hereinafter referred to as "CHO cell") or a mouse myeloma NS0 cell (hereinafter referred to as "NS0 cell") with various glycosidases, and reported that elimination of sialic acid in the non-reducing end did not have influence upon both activities, but the CDC activity alone was affected by further removal of galactose residue and about 50% of the activity was decreased, and that complete removal of the sugar chain caused disappearance of both activities [*Molecular Immunol.*, 32, 1311 (1995)]. Also, Lively et al. have analyzed the sugar chain bound to a human CDR-grafted antibody CAMPATH-1H (human IgG1 subclass) which was produced by CHO cell, NS0 cell or rat myeloma Y0 cell (hereinafter referred to as "Y0 cell"), measured its ADCC activity, and reported that the CAMPATH-1H derived from Y0 cell showed the highest ADCC activity, suggesting that N-acetylglucosamine (hereinafter sometimes referred to as "GlcNAc") at the bisecting position is important for the activity [*Glycobiology*, 5, 813 (1995), WO99/54342]. These reports indicate that the structure of the sugar chain plays an important role in the effector functions of human antibodies of IgG1 subclass and that it is possible to prepare an antibody having much higher effector function by modifying the structure of the sugar chain. However, structures of sugar chains are various and complex actually, and it cannot be said that an important structure for the effector function was completely identified.

[0008] Thus, the sugar chain bound to the CH2 domain of an IgG class antibody has great influence on the induction of effector functions of an antibody. As described above, some of effector functions of an antibody are exerted via interaction with Fc γ R present on the effector cell surface [*Annu. Rev. Immunol.*, 18, 709 (2000), *Annu. Rev. Immunol.*, 19, 275 (2001)].

[0009] It has been found that 3 different types are present in Fc γ R, and they are respectively called Fc γ RI (CD64), Fc γ RII (CD32) and Fc γ RIII (CD16). In human, Fc γ RII and Fc γ RIII are further classified into Fc γ RIIIa and Fc γ RIIIb, and Fc γ RIIIa and Fc γ RIIIb, respectively. Fc γ R is a membrane protein belonging to the immunoglobulin super family, Fc γ RII and Fc γ RIII have an α chain having an extracellular region containing two immunoglobulin-like domains, Fc γ RI

has an α chain having an extracellular region containing three immunoglobulin-like domains, as a constituting component, and the α chain is involved in the IgG binding activity. In addition, Fc γ RI and Fc γ RIII have a γ chain or ζ chain as a constituting component which has a signal transduction function in association with the α chain [*Annu. Rev. Immunol.*, 18, 709 (2000), *Annu. Rev. Immunol.*, 19, 275 (2001)].

[0010] Fc γ RI is a high affinity receptor having a binding constant (hereinafter referred to as " K_A ") value of 10^8 to 10^9 M $^{-1}$ and also has high binding activity for monomeric IgG [*Ann. Hematol.*, 76, 231 (1998)]. On the other hand, Fc γ RII and Fc γ RIII are low affinity receptors which have a low K_A value of 10^5 to 10^7 M $^{-1}$ for monomeric IgG and efficiently bind to an IgG immune complex polymerized by binding to an antigen or the like 8*Ann. Hematol.*, 76, 231 (1998)]. Based on its functions, Fc γ R is classified into an activating receptor and an inhibitory receptor [*Annu. Rev. Immunol.*, 19, 275 (2001)].

[0011] In the activating receptor, there is a sequence consisting of 19 amino acid residues, called immunoreceptor tyrosine-based activation motif (hereinafter referred to as "ITAM") in the intracellular region of the α chain or the associating γ chain or ζ chain. According to the binding of an IgG immune complex, a tyrosine kinase such as Src or Syk which interacts with ITAM is activated to induce various activation reactions.

[0012] In the inhibitory receptor, there is a sequence consisting of 13 amino acid residues, called immunoreceptor tyrosine-based inhibitory motif (hereinafter referred to as "ITIM") in the intracellular region of the CL chain. When ITIM is phosphorylated via its association with the activating receptor, various reactions including activation of a phosphatase called SHIP are induced to suppress activation signal from the activation receptor.

[0013] In human, the high affinity Fc γ RI and the low affinity Fc γ RIIa and Fc γ RIIIa function as activating receptors. In Fc γ RI, an ITAM sequence is present in the intracellular region of the associated γ chain. Fc γ RI is expressed on macrophages, monocytes, dendritic cells, neutrophils, eosinophils and the like. Fc γ RIIa comprises a single α chain, and an ITAM-like sequence is present in the intracellular region. Fc γ RIIa is expressed on macrophages, mast cells, monocytes, dendritic cells, Langerhans cells, neutrophils, eosinophils, platelets and a part of B cells. Fc γ RIIIa has an ITAM sequence present in the intracellular region of the associated γ chain or ζ chain and is expressed on NK cells, macrophages, monocytes, mast cells, dendritic cells, Langerhans cells, eosinophils and the like, but is not expressed on neutrophils, B cells and T cells.

[0014] On the other hand, the low affinity receptor Fc γ RIIb comprises a single α chain, and the amino acid sequence in the extracellular region has homology of about 90% with Fc γ RIIa. However, since an ITMI sequence is present in the intracellular region, it functions as a suppressing receptor. Fc γ RIIb is expressed on B cells, macrophages, mast cells, monocytes, dendritic cells, Langerhans cells, basophils, neutrophils and eosinophils, but is not expressed on NK cells and T cells. Fc γ RIIb comprises a single α chain, and the amino acid sequence in the extracellular region has a homology of about 95% with Fc γ RIIIa. However, it is specifically expressed on neutrophils as a glyco-

syolphosphatidylinositol (hereinafter referred to as "GPI")-anchored membrane protein. Fc γ RIIIb binds to an IgG immune complex but cannot activate cells by itself, and it is considered to function via its association with a receptor having an ITAM sequence such as Fc γ RIIa. Thus, in vivo effector functions of IgG class antibodies are obtained as the result of complex interaction with activating and suppressing Fc γ Rs expressed on various effector cells.

[0015] It is considered that ADCC activity as one of the effector functions of IgG class antibodies is generated as a result of activation of effector cells such as NK cells, neutrophils, monocytes and macrophages, and among these, NK cells play an important role [*Blood*, 76, 2421 (1990), *Trends in Immunol.*, 22, 633 (2001), *Int. Rev. Immunol.*, 20, 503 (2001)].

[0016] Fc γ R expressed on NK cells is Fc γ RIIIa. Accordingly, it is considered that the ADCC activity can be enhanced by enhancing the activation signal from Fc γ RIIIa expressed on the NK cells.

[0017] As Fc fusion protein, Etanercept (trade name: Enbrel, manufactured by Immunex) (U.S. Pat. No. 5,605, 690) and Alefacept (trade name: Arneive, manufactured by Biogen) (U.S. Pat. No. 5,914,111) and the like are known. Also, it is known that it has no ADCC activity when CH2 domain of an antibody is absent.

SUMMARY OF THE INVENTION

[0018] The present invention relates to the following (1) to (48).

[0019] (1) A method for enhancing a binding activity of an antibody composition to Fc γ receptor IIIa, which comprises modifying a complex N-glycoside-linked sugar chain which is bound to the Fc region of an antibody molecule.

[0020] (2) The method according to (1), wherein the modification of a complex N-glycoside-linked sugar chain which is bound to the Fc region of an antibody molecule is to bind a sugar chain in which 1-position of fucose is not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond in the complex N-glycoside-linked sugar chain to the Fc region in the antibody molecule.

[0021] (3) The method according to (1) or (2), wherein the sugar chain is synthesized by a cell in which the activity of a protein relating to modification of a sugar chain in which fucose is bound to N-acetylglucosamine in the reducing end in the complex N-glycoside-linked sugar chain is decreased or deleted.

[0022] (4) The method according to (3), wherein the protein relating to modification of a sugar chain in which fucose is bound to N-acetylglucosamine in the reducing end in the complex NV-glycoside-linked sugar chain is selected from the group consisting of the following (a), (b) and (c):

[0023] (a) a protein relating to synthesis of an intracellular sugar nucleotide, GDP-fucose;

[0024] (b) a protein relating to modification of a sugar chain in which 1-position of fucose is bound to

6-position of N-acetylglucosamine in the reducing end through α -bond in a complex N-glycoside-linked sugar chain;

[0025] (c) a protein relating to transport of an intracellular sugar nucleotide, GDP-fucose to the Golgi body.

[0026] (5) The method according to (3) or (4), wherein the cell is resistant to a lectin which recognizes a sugar chain structure in which 1-position of fucose is bound to 6-position of N-acetylglucosamine in the reducing end through α -bond in a complex N-glycoside-linked sugar chain.

[0027] (6) The method according to any one of (3) to (5), wherein the cell is resistant to at least one lectin selected from the group consisting of the following (a) to (d):

[0028] (a) a *Lens culinaris* lectin;

[0029] (b) a *Pisum sativum* lectin;

[0030] (c) a *Vicia faba* lectin;

[0031] (d) an *Aleuria aurantia* lectin.

[0032] (7) The method according to any one of (3) to (6), wherein the cell is selected from the group consisting of a yeast, an animal cell, an insect cell and a plant cell.

[0033] (8) The method according to any one of (3) to (7), wherein the cell is selected from the group consisting of the following (a) to (i):

[0034] (a) a CHO cell derived from a Chinese hamster ovary tissue;

[0035] (b) a rat myeloma cell line YB2/3HL.P2.G11.16Ag.20 cell;

[0036] (c) a BHK cell derived from a Syrian hamster kidney tissue;

[0037] (d) a mouse myeloma cell line NS0 cell;

[0038] (e) a mouse myeloma cell line SP2/0-Ag14 cell;

[0039] (f) a hybridoma cell;

[0040] (g) a human leukemic cell line Namalwa cell;

[0041] (h) an embryonic stem cell;

[0042] (i) a fertilized egg cell.

[0043] (9) The method according to any one of (1) to (8), wherein the antibody molecule is selected from the group consisting of the following (a) to (d):

[0044] (a) a human antibody;

[0045] (b) a humanized antibody;

[0046] (c) an antibody fragment comprising the Fc region of (a) or (b);

[0047] (d) a fusion protein comprising the Fc region of (a) or (b).

[0048] (10) The method according to any one of (1) to (9), wherein the antibody molecule belongs to an IgG class.

[0049] (11) The method according to any one of (1) to (10), wherein, in the complex N-glycoside-linked sugar chain which is bound to the Fc region of an antibody molecule, the ratio of a sugar chain in which 1-position of fucose is not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond is 20% or more of total complex N-glycoside-linked sugar chains.

[0050] (12) A method for enhancing an antibody-dependent cell-mediated cytotoxic activity of an antibody composition, which comprises using the method according to any one of (1) to (11).

[0051] (13) A process for producing an antibody composition having an enhanced binding activity to Fc γ receptor IIIa, which comprises modifying a complex N-glycoside-linked sugar chain which is bound to the Fc region of an antibody molecule.

[0052] (14) The process according to (13), wherein the modification of a complex N-glycoside-linked sugar chain which is bound to the Fc region of an antibody molecule is to bind a sugar chain in which 1-position of fucose is not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond in the complex N-glycoside-linked sugar chain to the Fc region in the antibody molecule.

[0053] (15) The process according to (13) or (14), wherein the sugar chain is synthesized by a cell in which the activity of a protein relating to modification of a sugar chain in which fucose is bound to N-acetylglucosamine in the reducing end in the complex N-glycoside-linked sugar chain is decreased or deleted.

[0054] (16) The process according to (15), wherein the protein relating to modification of a sugar chain in which fucose is bound to N-acetylglucosamine in the reducing end in the complex N-glycoside-linked sugar chain is selected from the group consisting of the following (a), (b) and (c):

[0055] (a) a protein relating to synthesis of an intracellular sugar nucleotide, GDP-fucose;

[0056] (b) a protein relating to modification of a sugar chain in which 1-position of fucose is bound to 6-position of N-acetylglucosamine in the reducing end through α -bond in a complex N-glycoside-linked sugar chain;

[0057] (c) a protein relating to transport of an intracellular sugar nucleotide, GDP-fucose to the Golgi body.

[0058] (17) The process according to (15) or (16), wherein the cell is resistant to a lectin which recognizes a sugar chain structure in which 1-position of fucose is bound to 6-position of N-acetylglucosamine in the reducing end through α -bond in a complex N-glycoside-linked sugar chain.

[0059] (18) The process according to any one of (15) to (17), wherein the cell is resistant to at least one lectin selected from the group consisting of the following (a) to (d):

[0060] (a) a *Lens culinaris* lectin;

[0061] (b) a *Pisum sativum* lectin;

- [0062] (c) a *Vicia faba* lectin;
- [0063] (d) an *Aleuria aurantia* lectin.
- [0064] (19) The process according to any one of (15) to (18), wherein the cell is selected from the group consisting of a yeast, an animal cell, an insect cell and a plant cell.
- [0065] (20) The process according to any one of (15) to (19), wherein the cell is selected from the group consisting of the following (a) to (i):
- [0066] (a) a CHO cell derived from a Chinese hamster ovary tissue;
- [0067] (b) a rat myeloma cell line YB2/3HL.P2.G11.16Ag.20 cell;
- [0068] (c) a BHK cell derived from a Syrian hamster kidney tissue;
- [0069] (d) a mouse myeloma cell line NS0 cell;
- [0070] (e) a mouse myeloma cell line SP2/0-Ag14 cell;
- [0071] (f) a hybridoma cell;
- [0072] (g) a human leukemic cell line Namalwa cell;
- [0073] (h) an embryonic stem cell;
- [0074] (i) a fertilized egg cell.
- [0075] (21) The process according to any one of (13) to (20), wherein the antibody molecule is selected from the group consisting of the following (a) to (d):
- [0076] (a) a human antibody;
- [0077] (b) a humanized antibody;
- [0078] (c) an antibody fragment comprising the Fc region of (a) or (b);
- [0079] (d) a fusion protein comprising the Fc region of (a) or (b).
- [0080] (22) The process according to any one of (13) to (21), wherein the antibody molecule belongs to an IgG class.
- [0081] (23) The method according to any one of (13) to (22), wherein, in the complex N-glycoside-linked sugar chain which is bound to the Fc region of an antibody molecule, the ratio of a sugar chain in which 1-position of fucose is not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond is 20% or more of total complex N-glycoside-linked sugar chains.
- [0082] (24) A process for producing an antibody composition having an increased antibody-dependent cell-mediated cytotoxic activity, which comprises using the process according to (12).
- [0083] (25) An antibody composition produced by the process according to any one of (13) to (24).
- [0084] (26) A method for detecting the ratio of a sugar chain in which fucose is not bound to N-acetylglucosamine in the reducing end in the sugar chain among total complex N-glycoside-linked sugar chains bound to the Fc region in an antibody composition, which comprises: reacting an antigen with a tested antibody composition to form a complex of the antigen and the antibody composition, contacting the complex with an Fc γ receptor IIIa to measure the binding activity to the Fc γ receptor IIIa; and detecting the ratio of a sugar chain in a standard antibody composition with a standard curve showing the binding activity to the Fc γ receptor IIIa.
- [0085] (27) A method for detecting the antibody-dependent cell-mediated cytotoxic activity in an antibody composition, which comprises: reacting an antigen with a tested antibody composition to form a complex of the antigen and the antibody composition; contacting the complex with an Fc γ receptor IIIa to measure the binding activity to the Fc γ receptor IIIa; and detecting the ratio of a sugar chain in a standard antibody composition with a standard curve showing the binding activity to the Fc γ receptor IIIa.
- [0086] (28) A method for detecting the ratio of a sugar chain in which fucose is not bound to N-acetylglucosamine in the reducing end in the sugar chain among total complex N-glycoside-linked sugar chains bound to the Fc region in an antibody composition, which comprises: contacting a tested antibody composition with a Fc γ receptor IIIa to measure the binding activity of the antibody composition to the Fc γ receptor IIIa; and detecting the ratio of a sugar chain in a standard antibody composition with a standard curve showing the binding activity to the Fc γ receptor IIIa.
- [0087] (29) A method for detecting the antibody-dependent cell-mediated cytotoxic activity in an antibody composition, which comprises: contacting a tested antibody composition with a Fc γ receptor IIIa to measure the binding activity of the antibody composition to the Fc γ receptor IIIa, and detecting the ratio of a sugar chain in a standard antibody composition with a standard curve showing the binding activity to the Fc γ receptor IIIa.
- [0088] (30) An Fc fusion protein composition produced by using a cell resistant to a lectin which recognizes a sugar chain structure in which 1-position of fucose is bound to 6-position of N-acetylglucosamine in the reducing end through α -bond in a complex N-glycoside-linked sugar chain.
- [0089] (31) The Fc fusion protein composition according to (30), wherein the cell is selected from the group consisting of the following (a), (b) and (c):
- [0090] (a) an enzyme protein relating to synthesis of an intracellular sugar nucleotide, GDP-fucose;
- [0091] (b) an enzyme protein relating to modification of a sugar chain in which 1-position of fucose is bound to 6-position of N-acetylglucosamine in the reducing end through α -bond in a complex N-glycoside-linked sugar chain;
- [0092] (c) a protein relating to transport of an intracellular sugar nucleotide, GDP-fucose to the Golgi body,
- [0093] wherein the activity of the protein is decreased or deleted.
- [0094] (32) The Fc fusion protein composition according to (30) or (31), wherein the cell is resistant to at least one lectin selected from the group consisting of the following (a) to (d):

- [0095] (a) a *Lens culinaris* lectin;
- [0096] (b) a *Pisum sativum* lectin;
- [0097] (c) a *Vicia faba* lectin;
- [0098] (d) an *Aleuria aurantia* lectin.
- [0099] (33) The Fc fusion protein composition according to any one of (30) to (32), wherein the cell is a cell into which a gene encoding an Fc fusion protein is introduced.
- [0100] (34) The Fc fusion protein composition according to (33), wherein the Fc is derived from an IgG class of an antibody molecule.
- [0101] (35) The Fc fusion protein composition according to any one of (30) to (34), wherein the cell is selected from the group consisting of a yeast, an animal cell, an insect cell and a plant cell.
- [0102] (36) The Fc fusion protein composition according to any one of (30) to (35), wherein the cell is a mouse myeloma cell.
- [0103] (37) The Fc fusion protein composition according to (36), wherein the mouse myeloma cell is NS0 cell or SP2/0-Ag14 cell.
- [0104] (38) The Fc fusion protein composition according to any one of (30) to (37), wherein the cell is selected from the group consisting of the following (a) to (g):
- [0105] (a) a CHO cell derived from a Chinese hamster ovary tissue;
- [0106] (b) a rat myeloma cell line YB2/3HL.P2.G11.16Ag.20 line;
- [0107] (c) a BHK cell derived from a Syrian hamster kidney tissue;
- [0108] (d) an antibody-producing hybridoma cell;
- [0109] (e) a human leukemic cell line Namalwa cell;
- [0110] (f) an embryonic stem cell;
- [0111] (g) a fertilized egg cell.
- [0112] (39) An Fc fusion protein composition comprising an Fc fusion protein having an complex N-glycoside-linked sugar chain at the Fc region of an antibody molecule, wherein the ratio of a sugar chain in which fucose is not bound to N-acetylglucosamine in the reducing end in the sugar chain is 20% or more of total complex N-glycoside-linked sugar chains which are bound to the Fc region in the composition.
- [0113] (40) The Fc fusion protein composition according to (39), wherein the sugar chain in which fucose is not bound is a sugar chain in which 1-position of fucose is not bound to 6-position of V-acetylglucosamine in the reducing end in the complex N-glycoside-linked sugar chain through α -bond.
- [0114] (41) The Fc fusion protein composition according to (39) or (40), wherein the antibody molecule belongs to an IgG class.
- [0115] (42) The Fc fusion protein composition according to any one of (30) to (41), wherein the Fc fusion protein composition is Fc-fused fibroblast growth factor-8.
- [0116] (43) A cell which produces the Fc fusion protein composition according to any one of (30) to (42).
- [0117] (44) The cell according to (43), which is selected from the group consisting of a yeast, an animal cell, an insect cell and a plant cell.
- [0118] (45) The cell according to (43) or (44), which is a mouse myeloma cell.
- [0119] (46) The cell according to (45), wherein the mouse myeloma cell is NS0 cell or SP2/0-Ag14 cell.
- [0120] (47) The cell according to any one of (43) to (46), which is selected from the group consisting of the following (a) to (g):
- [0121] (a) a CHO cell derived from a Chinese hamster ovary tissue;
- [0122] (b) a rat myeloma cell line YB2/3HL.P2.G11.16Ag.20 line;
- [0123] (c) a BHK cell derived from a Syrian hamster kidney tissue;
- [0124] (d) an antibody-producing hybridoma cell;
- [0125] (e) a human leukemic cell line Namalwa cell;
- [0126] (f) an embryonic stem cell;
- [0127] (g) a fertilized egg cell.
- [0128] (48) A process for producing an Fc fusion protein composition, which comprises culturing the cell according to any one of (43) to (47) in a medium to form and accumulate an Fc fusion protein composition in the culture, and recovering the Fc fusion protein composition from the culture.

BRIEF DESCRIPTION OF THE DRAWINGS

[0129] **FIG. 1** shows photographs of electrophoresis patterns of SDS-PAGE of five kinds of purified anti-GD3 chimeric antibodies (using gradient gel from 4 to 15%). **FIG. 1A** and **FIG. 1B** show results of the electrophoresis under non-reducing conditions and under reducing conditions, respectively. Lanes 1 to 7 show electrophoresis patterns of high molecular weight markers, YB2/0-GD3 chimeric antibody, CHO/DG44-GD3 chimeric antibody, SP2/0-GD3 chimeric antibody, NS0-GD3 chimeric antibody (302), NS0-GD3 chimeric antibody (GIT) and low molecular weight markers, respectively.

[0130] **FIG. 2** shows binding activities of five kinds of purified anti-GD3 chimeric antibodies to GD3, measured by changing the antibody concentration. The ordinate and the abscissa show the binding activity to GD3 and the antibody concentration, respectively. "○", "●", "□", "■" and "Δ" show the activities of YB2/0-GD3 chimeric antibody, CHO/DG44-GD3 chimeric antibody, SP2/0-GD3 chimeric antibody, NS0-GD3 chimeric antibody (302) and NS0-GD3 chimeric antibody (GIT), respectively.

[0131] **FIG. 3** shows ADCC activities of five kinds of purified anti-GD3 chimeric antibodies to a human melanoma

cell line G-361. The ordinate and the abscissa show the cytotoxic activity and the antibody concentration, respectively. “○”, “●”, “□”, “■” and “Δ” show the activities of YB2/0-GD3 chimeric antibody, CHO/DG44-GD3 chimeric antibody, SP2/0-GD3 chimeric antibody, NS0-GD3 chimeric antibody (302) and NS0-GD3 chimeric antibody (GIT), respectively.

[0132] FIG. 4 shows elution patterns of PA-treated sugar chains prepared from the anti-GD3 chimeric antibody of lot 2, obtained by analyzing them with reverse phase HPLC. The ordinate and the abscissa show the relative fluorescence intensity and the elution time, respectively.

[0133] FIG. 5 shows binding activities of six kinds of anti-GD3 chimeric antibodies having a different ratio of a sugar chain in which 1-position of fucose is not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond to GD3, measured by changing the antibody concentration. The ordinate and the abscissa show the binding activity to GD3 and the antibody concentration, respectively. “●”, “□”, “■”, “Δ”, “▲” and “x” show the activities of anti-GD3 chimeric antibody (50%), anti-GD3 chimeric antibody (45%), anti-GD3 chimeric antibody (29%), anti-GD3 chimeric antibody (24%), anti-GD3 chimeric antibody (13%) and anti-GD3 chimeric antibody (7%), respectively.

[0134] FIG. 6 shows results of ADCC activities using an effector cell of each donor. FIG. 6A and FIG. 6B show ADCC activities of six kinds of anti-GD3 chimeric antibodies having a different ratio of a sugar chain in which 1-position of fucose is not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond against a human melanoma cell line G-361, using effector cells of the donor A and the donor B, respectively. The ordinate and the abscissa show the cytotoxic activity and the antibody concentration, respectively. “●”, “□”, “■”, “Δ”, “▲” and “x” show the activities of anti-GD3 chimeric antibody (50%), anti-GD3 chimeric antibody (45%), anti-GD3 chimeric antibody (29%), anti-GD3 chimeric antibody (24%), anti-GD3 chimeric antibody (13%) and anti-GD3 chimeric antibody (7%), respectively.

[0135] FIG. 7 shows the relationship between sugar chain contents in which 1-fucose is not bound to N-acetylglucosamine at the reduced end in the donor A and the donor B and the ADCC activities.

[0136] FIG. 8 shows elution patterns of PA-treated sugar chains prepared from six kinds of anti-GD3 chimeric antibodies, obtained by analyzing them with reverse phase HPLC. The ordinate and the abscissa show the relative fluorescence intensity and the elution time, respectively.

[0137] FIG. 9 shows binding activities of six kinds of anti-CCR4 chimeric antibodies having a different ratio of a sugar chain in which 1-position of fucose is not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond measured by changing the antibody concentration to CCR4. The ordinate and the abscissa show the binding activity to CCR4 and the antibody concentration, respectively. “■”, “□”, “▲”, “Δ”, “●” and “○” show the activities of anti-CCR4 chimeric antibody (46%), anti-CCR4 chimeric antibody (39%), anti-CCR4 chimeric antibody (27%), anti-CCR4 chimeric antibody (18%), anti-CCR4 chimeric antibody (9%) and anti-CCR4 chimeric antibody (8%), respectively.

[0138] FIG. 10 shows ADCC activities of anti-CCR4 chimeric antibodies having a different ratio of a sugar chain in which 1-position of fucose is not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond against CCR4/EL-4 cell, using an effector cell of the donor A. As effector cells, the effector cells derived from donor A were used. The ordinate and the abscissa show the cytotoxic activity and the antibody concentration, respectively. “■”, “□”, “▲”, “Δ”, “●” and “○” show the activities of anti-CCR4 chimeric antibody (46%), anti-CCR4 chimeric antibody (39%), anti-CCR4 chimeric antibody (27%), anti-CCR4 chimeric antibody (18%), anti-CCR4 chimeric antibody (9%) and anti-CCR4 chimeric antibody (8%), respectively.

[0139] FIG. 11 shows ADCC activities of anti-CCR4 chimeric antibodies having a different ratio of a sugar chain in which 1-position of fucose is not bound to 6-position N-acetylglucosamine in the reducing end through α -bond against CCR4/EL-4 cell, using an effector cell of the donor B. As effector cells, the effector cells derived from donor B were used. The ordinate and the abscissa show the cytotoxic activity and the antibody concentration, respectively. “■”, “□”, “▲”, “Δ”, “●” and “○” show the activities of anti-CCR4 chimeric antibody (46%), anti-CCR4 chimeric antibody (39%), anti-CCR4 chimeric antibody (27%), anti-CCR4 chimeric antibody (18%), anti-CCR4 chimeric antibody (9%) and anti-CCR4 chimeric antibody (8%), respectively.

[0140] FIG. 12 shows construction of plasmids CHFT8-pCR2.1 and YBFT8-pCR2.1.

[0141] FIG. 13 shows construction of plasmids CHAc-pBS and YBAc-pBS.

[0142] FIG. 14 shows construction of plasmids CHFT8d-pCR2.1 and YBFT8d-pCR2.1.

[0143] FIG. 15 shows construction of plasmids CHAc-d-pBS and YBAc-d-pBS.

[0144] FIG. 16 shows results of determination of an α 1,6-fucosyltransferase (FUT8) transcription product in each host cell line using competitive RT-PCR. Amounts of the FUT8 transcription product in each host cell line when rat FUT8 sequence was used as the standard and internal control are shown. “■” and “□” show results when CHO cell line and YB2/0 cell line, respectively, were used as the host cell.

[0145] FIG. 17 shows results of evaluation of ADCC activities of anti-CCR4 human chimeric antibodies produced by lectin-resistant cell lines. The ordinate and the abscissa show the cytotoxic activity and the antibody concentration, respectively. “□”, “■”, “◆” and “▲” show the activities of antibodies produced by the clone 5-03, the clone CHO/CCR4-LCA, the clone CHO/CCR4-AAL and the clone CHO/CCR4-PHA, respectively.

[0146] FIG. 18 shows results of evaluation of ADCC activities of anti-CCR4 human chimeric antibodies produced by lectin-resistant clones. The ordinate and the abscissa show the cytotoxic activity and the antibody concentration, respectively. “□”, “Δ” and “●” show activities of antibodies produced by the clone YB2/0 (KM2760#58-35-16), the clone 5-03 and the clone CHO/CCR4-LCA, respectively.

[0147] FIG. 19 shows elution patterns of PA-treated sugar chains prepared from purified anti-CCR4 human chimeric antibodies, obtained by analyzing them with reverse phase HPLC. The ordinate and the abscissa show the relative fluorescence intensity and the elution time, respectively. FIG. 27A, FIG. 27B, FIG. 27C and FIG. 27D show results of analyses of antibodies produced by the clone 5-03, the clone CHO/CCR4-LCA, the clone CHO/CCR4-AAL and the clone CHO/CCR4-PHA, respectively.

[0148] FIG. 20 shows the 1st step of construction of an expression vector of CHO cell-derived GMD (6 steps in total).

[0149] FIG. 21 shows the 2nd step of construction of the expression vector of CHO cell-derived GMD (6 steps in total).

[0150] FIG. 22 shows the 3rd step of construction of the expression vector of CHO cell-derived GMD (6 steps in total).

[0151] FIG. 23 shows the 4th step of construction of the expression vector of CHO cell-derived GM (6 steps in total).

[0152] FIG. 24 shows the 5th step of construction of the expression vector of CHO cell-derived GMD (6 steps in total).

[0153] FIG. 25 shows the 6th step of construction of the expression vector of CHO cell-derived GMD (6 steps in total).

[0154] FIG. 26 shows resistance of GMD-expressed clone CHO/CCR4-LCA for LCA lectin. The measurement was carried out twice by defining the survival rate of a group of cells cultured without adding LCA lectin as 100%. In the drawing, "249" shows the survival rate of the clone CHO/CCR4-LCA introduced with an expression vector to LCA lectin. GMD shows resistance of the clone CHO/CCR4-LCA introduced with a GMD expression vector pAGE249GMD to LCA lectin.

[0155] FIG. 27 shows ADCC activities of an anti-CCR4 chimeric antibody produced by cells of GMD-expressed clone CHO/CCR4-LCA. The ordinate and the abscissa show the cytotoxic activity and the antibody concentration, respectively.

[0156] FIG. 28 shows elution patterns of PA-treated sugar chains prepared from an anti-CCR4 human chimeric antibody purified from GMD gene-expressed clone CHO/CCR4-LCA, obtained by analyzing them with reverse phase HPLC. The ordinate and the abscissa show the relative fluorescence intensity and the elution time, respectively.

[0157] FIG. 29 shows a photograph of SDS-PAGE (using 4 to 15% gradient gel) electrophoresis pattern of purified shFcγRIIIa under reduced conditions. Lane 1 and lane M show electrophoresis patterns of shFcγRIIIa and molecular weight markers, respectively.

[0158] FIG. 30 shows binding activities of various anti-GD3 chimeric antibodies to shFcγRIIIa. The ordinate and abscissa show the cytotoxic activity and the antibody concentration, respectively. "○" and "●" show the activities of anti-GD3 chimeric antibody (45%) and anti-GD3 chimeric antibody (7%), respectively.

[0159] FIG. 31 shows binding activities of various anti-fibroblast growth factor-8 (FGF-8) chimeric antibodies to shFcγRIIIa. The ordinate and abscissa show the cytotoxic activity and the antibody concentration, respectively. "○" and "●" show the activities of anti-FGF-8 chimeric antibody (58%) and anti-FGF-8 chimeric antibody (13%), respectively.

[0160] FIG. 32 shows binding activities of various anti-CCR4 chimeric antibodies to shFcγRIIIa. In FIG. 32A, the ordinate and abscissa show the cytotoxic activity and the antibody concentration, respectively. "○", "●", "□", "■", "Δ", "▲" and "x" show the activities of anti-CCR4 chimeric antibody (87%), anti-CCR4 chimeric antibody (46%), anti-CCR4 chimeric antibody (39%), anti-CCR4 chimeric antibody (27%), anti-CCR4 chimeric antibody (18%), anti-CCR4 chimeric antibody (9%) and anti-CCR4 chimeric antibody (8%), respectively. In FIG. 32B, the ordinate and abscissa show the cytotoxic activity and the ratio of a sugar chain in which 1-position of fucose is not bound to 6-position of N-acetylglucosamine in the reducing end through α-bond, respectively. "●" and "○" show the activities at 40 μg/mL and 4 μg/mL, respectively.

[0161] FIG. 33 shows binding activities of various anti-CCR4 chimeric antibodies to shFcγRIIIa. The ordinate and abscissa show the cytotoxic activity and the antibody concentration, respectively. "○", "Δ" and "●" show the activities of anti-CCR4 chimeric antibody (87%), anti-CCR4 chimeric antibody (48%) and anti-CCR4 chimeric antibody (8%), respectively.

[0162] FIG. 34 shows ADCC activities of various anti-GD3 chimeric antibodies to human myeloma cell line G-361. The ordinate and abscissa show the cytotoxic activity and the antibody concentration, respectively. "▲" and "●" show the activities of anti-GD3 chimeric antibody (42%) and anti-GD3 chimeric antibody (7%), respectively.

[0163] FIG. 35 shows results of measurement of binding activities of FGF-8/Fc fusion proteins to KM1334. The ordinate and abscissa show the binding activity and the antibody concentration, respectively. "■" and "○" show the activities of FGF-8/Fc fusion proteins produced by the cell line YB2/0 and the cell line CHO/DG44, respectively, as the host cell.

[0164] FIG. 36 shows results of measurement of binding activities of various FGF-8/Fc fusion proteins to shFcγRIIIa(V). The ordinate and abscissa show the binding activity and the antibody concentration, respectively. "■" and "○" show the activities of FGF8/Fc fusion proteins produced by the cell line YB2/0 and the cell line CHO/DG44, respectively, as the host cell.

[0165] FIG. 37 show a construction step of a plasmid CHO-GMD in which the 5'-terminal of a clone 34-2 is introduced into the 5'-terminal of a CHO cell-derived GMD cDNA clone 22-8.

[0166] FIG. 38 shows construction steps of plasmid pKANTEX1334H and pKANTEX 1334.

DETAILED DESCRIPTION OF THE INVENTION

[0167] The present invention relates to a method for enhancing a binding activity of an antibody composition to

Fc γ receptor IIIa, which comprises modifying a complex N-glycoside-linked sugar chain which is bound to the Fc region of an antibody molecule.

[0168] In the present invention, the antibody molecule may be any antibody molecule, so long as it is a molecule comprising the Fc region of an antibody. Examples include an antibody, an antibody fragment, a fusion protein comprising an Fc region, and the like.

[0169] An antibody is a protein, which is produced in vivo by immunization as the result of extra-antigen stimulation. The antibody has a specific binding activity to an antigen. The antibody includes an antibody secreted by a hybridoma cell prepared from a spleen cell of an animal immunized with an antigen, an antibody prepared by genetic engineering technique, i.e., an antibody obtained by introducing an antibody gene-inserted antibody expression vector into a host cell; and the like. Examples include an antibody produced by a hybridoma, a humanized antibody, a human antibody and the like.

[0170] A hybridoma is a cell which is obtained by cell fusion between a B cell obtained by immunizing a non-human mammal with an antigen and a myeloma cell derived from mouse or the like and can produce a monoclonal antibody having the desired antigen specificity.

[0171] The humanized antibody includes a human chimeric antibody, a human CDR-grafted antibody and the like.

[0172] A human chimeric antibody is an antibody which comprises an antibody heavy chain V region (hereinafter referred to heavy chain as "H chain", and referred to as "HV" or "VH") and an antibody light chain V region (hereinafter referred to light chain as "L chain", and referred to as "LV" or "VL"), both of a non-human animal, a human antibody H chain C region (hereinafter also referred to as "CH") and a human antibody L chain C region (hereinafter also referred to as "CL"). The non-human animal may be any animal such as mouse, rat, hamster or rabbit, so long as a hybridoma can be prepared therefrom.

[0173] The human chimeric antibody can be produced by obtaining cDNAs encoding VH and VL from a monoclonal antibody-producing hybridoma, inserting them into an expression vector for host cell having genes encoding human antibody CH and human antibody CL to thereby construct a vector for expression of human chimeric antibody, and then introducing the vector into a host cell to express the antibody.

[0174] The CH of human chimeric antibody may be any CH, so long as it belongs to human immunoglobulin (hereinafter referred to as "hIg") can be used. Those belonging to the hIgG class are preferred and any one of the subclasses belonging to the hIgG class such as hIgG1, hIgG2, hIgG3 and hIgG4 can be used. Also, as the CL of human chimeric antibody, any CL can be used, so long as it belongs to the hIg class, and those belonging to the κ class or λ class can also be used.

[0175] A human CDR-grafted antibody is an antibody in which amino acid sequences of CDRs of VH and VL of a non-human animal antibody are grafted into appropriate positions of VH and VL of a human antibody.

[0176] The human CDR-grafted antibody can be produced by constructing cDNAs encoding V regions in which CDR

sequences of VH and VL of a non-human animal antibody are grafted into CDR sequences of VH and VL of a desired human antibody, inserting them into an expression vector for host cell having genes encoding human antibody CH and human antibody CL to thereby construct a vector for expression of human CDR-grafted antibody, and then introducing the expression vector into a host cell to express the human CDR-grafted antibody.

[0177] The CH of human CDR-grafted antibody may be any CH, so long as it belongs to the hIg. Those of the hIgG class are preferred and any one of the subclasses belonging to the hIgG class, such as hIgG1, hIgG2, hIgG3 and hIgG4, can be used. Also, as the CL of human CDR-grafted antibody, any CL can be used, so long as it belongs to the hIg class, and those belonging to the κ class or λ class can also be used.

[0178] A human antibody is originally an antibody naturally existing in the human body, but it also includes antibodies obtained from a human antibody phage library, a human antibody-producing transgenic non-human animal and a human antibody-producing transgenic plant, which are prepared based on the recent advance in genetic engineering, cell engineering and developmental engineering techniques.

[0179] Regarding the antibody existing in the human body, a lymphocyte capable of producing the antibody can be cultured by isolating a human peripheral blood lymphocyte, immortalizing it by its infection with EB virus or the like and then cloning it, and the antibody can be purified from the culture.

[0180] The human antibody phage library is a library in which antibody fragments such as Fab and single chain antibody are expressed on the phage surface by inserting a gene encoding an antibody prepared from a human B cell into a phage gene. A phage expressing an antibody fragment having the desired antigen binding activity can be recovered from the library based on the activity to bind to an antigen-immobilized substrate. The antibody fragment can be converted further into a human antibody molecule comprising two full H chains and two full L chains by genetic engineering techniques.

[0181] A human antibody-producing transgenic non-human animal is an animal in which a human antibody gene is introduced into cells. Specifically, a human antibody-producing transgenic mouse can be prepared by introducing a human antibody gene into ES cell of a mouse, transplanting the ES cell into an early stage embryo of other mouse and then developing it. By introducing a human antibody-gene into a fertilized egg of an animal and developing it, the transgenic non-human animal can be also prepared. Regarding the preparation method of a human antibody from the human antibody-producing transgenic non-human animal, the human antibody can be produced and accumulated in a culture by obtaining a human antibody-producing hybridoma by a hybridoma preparation method usually carried out in non-human mammals and then culturing it.

[0182] The transgenic non-human animal includes cattle, sheep, goat, pig, horse, mouse, rat, fowl, monkey, rabbit and the like.

[0183] Moreover, in the present invention, the antibody is preferably an antibody which recognizes a tumor-related antigen, an antibody which recognizes an allergy- or inflam-

mation-related antigen, an antibody which recognizes cardiovascular disease-related antigen, an antibody which recognizes autoimmune disease-related antigen or an antibody which recognizes a viral or bacterial infection-related antigen. Also, the class of the antibody is preferably IgG.

[0184] An antibody fragment is a fragment which comprises at least part of the Fc region of the above antibody. The Fc region is a region at the C-terminal side of H chain of an antibody, such as CH2 region and CH3 region, and includes a natural type and a mutant type. The at least part of the Fc region is preferably a fragment comprising CH2 region, and more preferably a region comprising aspartic acid at position 1 existing in CH2 region. The Fc region of the IgG class is from Cys at position 226 to the C-terminal or from Pro at position 230 to the C-terminal according to the numbering of EU Index of Kabat et al. [*Sequences of Proteins of Immunological Interest*, 5th Ed., Public Health Service, National Institutes of Health, Bethesda, Md. (1991)]. The antibody fragment includes an H chain monomer, an H chain dimer and the like.

[0185] A fusion protein comprising a part of Fc region is a substance in which an antibody comprising the part of Fc region of an antibody or the antibody fragment is fused with a protein such as an enzyme or a cytokine (hereinafter referred to as "Fc fusion protein").

[0186] In the present invention, N-glycoside-linked sugar chain bound to the Fc region of the antibody molecule includes a complex type in which the non-reducing end side of the core structure has one or more parallel branches of galactose-N-acetylglucosamine (hereinafter referred to as "Gal-GlcNAc") and further the non-reducing end side of Gal-GlcNAc has a structure of sialic acid, bisecting N-acetylglucosamine or the like.

[0187] Since the Fc region in the antibody molecule has positions to which N-glycoside-linked sugar chains are separately bound, two sugar chains are bound per one antibody molecule. Since many sugar chains having different structures are present for the two N-glycoside-linked sugar chains bound to the antibody, homology of antibody molecules can be judged in view of the sugar chain structure bound to the Fc region.

[0188] The antibody composition is a composition which comprises an antibody molecule having complex N-glycoside-linked sugar chains in the Fc region, and may comprise an antibody molecule having the same sugar chain structure or, an antibody molecule having different sugar chain structures.

[0189] Modification of the N-glycoside-linked sugar chain bound to the Fc region of an antibody molecule is preferably carried out by binding a sugar chain in which fucose is not bound to N-acetylglucosamine in the reducing end in the complex N-glycoside-linked sugar chain to the Fc region of an antibody molecule.

[0190] In the present invention, the sugar chain in which fucose is not bound to N-acetylglucosamine in the reducing end in the complex N-glycoside-linked sugar chain is a complex M-glycoside-linked sugar chain in which 1-position of fucose is not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond in the complex N-glycoside-linked sugar chain.

[0191] The sugar chain can be synthesized by a cell in which the activity of an enzyme protein relating to the modification of a sugar chain in which fucose is not bound to N-acetylglucosamine in the reducing end in the complex N-glycoside-linked sugar chain is decreased or deleted.

[0192] In the present invention, the enzyme protein relating to the modification of a sugar chain in which 1-position of fucose is not bound to 6-position of N-acetylglucosamine in the reducing end in the complex N-glycoside-linked sugar chain includes:

[0193] (a) an enzyme protein relating to synthesis of an intracellular sugar nucleotide, GDP-fucose (hereinafter referred to as "GDP-fucose synthase");

[0194] (b) an enzyme protein relating to modification of a sugar chain in which 1-position of fucose is bound to 6-position of N-acetylglucosamine in the reducing end through α -bond in a complex N-glycoside-linked sugar chain (hereinafter referred to as " α 1,6-fucose modifying enzyme"); and

[0195] (c) a protein relating to transport of an intracellular sugar nucleotide, GDP-fucose, to the Golgi body (hereinafter referred to as "GDP-fucose transport protein").

[0196] In the present invention, the GDP-fucose synthase may be any enzyme, so long as it is an enzyme relating to the synthesis of the intracellular sugar nucleotide, GDP-fucose, as a supply source of fucose to a sugar chain, and includes an enzyme which has influence on the synthesis of the intracellular sugar nucleotide, GDP-fucose.

[0197] The intracellular sugar nucleotide, GDP-fucose, is supplied by a de novo synthesis pathway or a salvage synthesis pathway. Thus, all enzymes relating to the synthesis pathways are included in the GDP-fucose synthase.

[0198] The GDP-fucose synthase relating to the de novo synthesis pathway includes GDP-mannose 4-dehydratase (hereinafter referred to as "GMD"), GDP-keto-6-deoxymannose 3,5-epimerase, 4-reductase (hereinafter referred to as "Fx") and the like.

[0199] The GDP-fucose synthase relating to the salvage synthesis pathway includes GDP-beta-L-fucose pyrophosphorylase (hereinafter referred to as "GFPP"), fucokinase and the like.

[0200] As the enzyme which has influence on the synthesis of an intracellular sugar nucleotide, GDP-fucose, an enzyme which has influence on the activity of the enzyme relating to the synthesis pathway of the intracellular sugar nucleotide, GDP-fucose, and an enzyme which has influence on the structure of substances as the substrate of the enzyme are also included.

[0201] In the present invention, the GMD includes a protein encoded by a DNA selected from the group consisting of (a) and (b), a protein selected from the group consisting of (c), (d) and (e), and the like:

[0202] (a) a DNA comprising the nucleotide sequence represented by SEQ ID NO:65;

[0203] (b) a DNA which hybridizes with the DNA comprising the nucleotide sequence represented by

SEQ ID NO:65 under stringent conditions and encodes a protein having GMD activity;

[0204] (c) a protein comprising the amino acid sequence represented by SEQ ID NO:71;

[0205] (d) a protein which comprises an amino acid sequence in which at least one amino acid is deleted, substituted, inserted and/or added in the amino acid sequence represented by SEQ ID NO:71 and has GMD activity, and

[0206] (e) a protein which comprises an amino acid sequence having a homology of 80% or more with the amino acid sequence represented by SEQ ID NO:71 and has GMD activity.

[0207] Fx includes a protein encoded by a DNA selected from the group consisting of (a) and (b), a protein selected from the group consisting of (c), (d) and (e), and the like:

[0208] (a) a DNA comprising the nucleotide sequence represented by SEQ ID NO:48;

[0209] (b) a DNA which hybridizes with the DNA comprising the nucleotide sequence represented by SEQ ID NO:48 under stringent conditions and encodes a protein having Fx activity;

[0210] (c) a protein comprising the amino acid sequence represented by SEQ ID NO:19;

[0211] (d) a protein which comprises an amino acid sequence in which at least one amino acid is deleted, substituted, inserted and/or added in the amino acid sequence represented by SEQ ID NO:19 and has Fx activity, and

[0212] (e) a protein which comprises an amino acid sequence having a homology of 80% or more with the amino acid sequence represented by SEQ ID NO:19 and has Fx activity.

[0213] GFPP includes a protein encoded by a DNA selected from the group consisting of (a) and (b), a protein selected from the group consisting of (c), (d) and (e), and the like:

[0214] (a) a DNA comprising the nucleotide sequence represented by SEQ ID NO:51;

[0215] (b) a DNA which hybridizes with the DNA comprising the nucleotide sequence represented by SEQ ID NO:51 under stringent conditions and encodes a protein having GFPP activity;

[0216] (c) a protein comprising the amino acid sequence represented by SEQ ID NO:20;

[0217] (d) a protein which comprises an amino acid sequence in which at least one amino acid is deleted, substituted, inserted and/or added in the amino acid sequence represented by SEQ ID NO:20 and has GFPP activity, and

[0218] (e) a protein which comprises an amino acid sequence having a homology of 80% or more with the amino acid sequence represented by SEQ ID NO:20 and has GFPP activity.

[0219] In the present invention, the α 1,6-fucose modifying enzyme includes any enzyme, so long as it is an enzyme

relating to the reaction of binding of 1-position of fucose to 6-position of N-acetylglucosamine in the reducing end through α -bond in the complex N-glycoside-linked sugar chain. The enzyme relating to the reaction of binding of 1-position of fucose to 6-position of N-acetylglucosamine in the reducing end through α -bond in the complex N-glycoside-linked sugar chain includes an enzyme which has influence on the reaction of binding of 1-position of fucose to 6-position of N-acetylglucosamine in the reducing end through α -bond in the complex N-glycoside-linked sugar chain.

[0220] The α 1,6-fucose modifying enzyme includes α 1,6-fucosyltransferase, α -L-fucosidase and the like.

[0221] Also, an enzyme which has influence on the activity of the above enzyme relating to the reaction of binding of 1-position of fucose to 6-position of N-acetylglucosamine in the reducing end through α -bond in the complex N-glycoside-linked sugar chain and an enzyme which has influence on the structure of substances as the substrate of the enzyme are included.

[0222] The α 1,6-fucosyltransferase includes a protein encoded by a DNA selected from the group consisting of the following (a), (b), (c) and (d), a protein selected from the group consisting of the following (e), (f), (g), (h), (i) and (j), and the like:

[0223] (a) a DNA comprising the nucleotide sequence represented by SEQ ID NO:1;

[0224] (b) a DNA comprising the nucleotide sequence represented by SEQ ID NO:2;

[0225] (c) a DNA which hybridizes with the DNA comprising the nucleotide sequence represented by SEQ ID NO:1 under stringent conditions and encodes a protein having α 1,6-fucosyltransferase activity;

[0226] (d) a DNA which hybridizes with the DNA comprising the nucleotide sequence represented by SEQ ID NO:2 under stringent conditions and encodes a protein having α 1,6-fucosyltransferase activity;

[0227] (e) a protein comprising the amino acid sequence represented by SEQ ID NO:23;

[0228] (f) a protein comprising the amino acid sequence represented by SEQ ID NO:24;

[0229] (g) a protein which comprises an amino acid sequence in which at least one amino acid is deleted, substituted, inserted and/or added in the amino acid sequence represented by SEQ ID NO:23 and has α 1,6-fucosyltransferase activity;

[0230] (h) a protein which comprises an amino acid sequence in which at least one amino acid is deleted, substituted, inserted and/or added in the amino acid sequence represented by SEQ ID NO:24 and has α 1,6-fucosyltransferase activity;

[0231] (i) a protein which comprises an amino acid sequence having a homology of 80% or more with the amino acid sequence represented by SEQ ID NO:23 and has α 1,6-fucosyltransferase activity, and

- [0232] (j) a protein which comprises an amino acid sequence having a homology of 80% or more with the amino acid sequence represented by SEQ ID NO:24 and has α 1,6-fucosyltransferase activity.
- [0233] The GDP-fucose transport protein may be any protein, so long as it is a protein relating to the transportation of the intracellular sugar nucleotide, GDP-fucose to the Golgi body or a protein which has an influence on the reaction to transport the intracellular sugar nucleotide, GDP-fucose to the Golgi body.
- [0234] The GDP-fucose transport protein includes a GDP-fucose transporter and the like.
- [0235] Furthermore, the protein which has an influence on the reaction to transport the intracellular sugar nucleotide, GDP-fucose to the Golgi body includes a protein which has an influence on the above GDP-fucose transport protein or has an influence on the expression thereof.
- [0236] In the present invention, the GDP-fucose transporter includes a protein encoded by a DNA selected from the group consisting of the following (a) to (h), and the like:
- [0237] (a) a DNA comprising the nucleotide sequence represented by SEQ ID NO:91;
- [0238] (b) a DNA comprising the nucleotide sequence represented by SEQ ID NO:93;
- [0239] (c) a DNA comprising the nucleotide sequence represented by SEQ ID NO:95;
- [0240] (d) a DNA comprising the nucleotide sequence represented by SEQ ID NO:97;
- [0241] (e) a DNA which hybridizes with the DNA comprising the nucleotide sequence represented by SEQ ID NO:91 under stringent conditions and encodes a protein having GDP-fucose transporter activity;
- [0242] (f) a DNA which hybridizes with the DNA comprising the nucleotide sequence represented by SEQ ID NO:93 under stringent conditions and encodes a protein having GDP-fucose transporter activity;
- [0243] (g) a DNA which hybridizes with the DNA comprising the nucleotide sequence represented by SEQ ID NO:95 under stringent conditions and encodes a protein having GDP-fucose transporter activity; and
- [0244] (h) a DNA which hybridizes with the DNA comprising the nucleotide sequence represented by SEQ ID NO:97 under stringent conditions and encodes a protein having GDP-fucose transporter activity.
- [0245] Furthermore, the GDP-fucose transporter of the present invention includes a protein selected from the group consisting of the following (i) to (t), and the like:
- [0246] (i) a protein comprising the amino acid sequence represented by SEQ ID NO:92;
- [0247] (j) a protein comprising the amino acid sequence represented by SEQ ID NO:94;
- [0248] (k) a protein comprising the amino acid sequence represented by SEQ ID NO:96;
- [0249] (l) a protein comprising the amino acid sequence represented by SEQ ID NO:98;
- [0250] (m) a protein which comprises an amino acid sequence in which at least one amino acid is deleted, substituted, inserted and/or added in the amino acid sequence represented by SEQ ID NO:92 and has GDP-fucose transporter activity,
- [0251] (n) a protein which comprises an amino acid sequence in which at least one amino acid is deleted, substituted, inserted and/or added in the amino acid sequence represented by SEQ ID NO:94 and has GDP-fucose transporter activity,
- [0252] (o) a protein which comprises an amino acid sequence in which at least one amino acid is deleted, substituted, inserted and/or added in the amino acid sequence represented by SEQ ID NO:96 and has GDP-fucose transporter activity,
- [0253] (p) a protein which comprises an amino acid sequence in which at least one amino acid is deleted, substituted, inserted and/or added in the amino acid sequence represented by SEQ ID NO:98 and has GDP-fucose transporter activity,
- [0254] (q) a protein which comprises an amino acid sequence having a homology of 80% or more with the amino acid sequence represented by SEQ ID NO:92 and has GDP-fucose transporter activity,
- [0255] (r) a protein which comprises an amino acid sequence having a homology of 80% or more with the amino acid sequence represented by SEQ ID NO:94 and has GDP-fucose transporter activity,
- [0256] (s) a protein which comprises an amino acid sequence having a homology of 80% or more with the amino acid sequence represented by SEQ ID NO:96 and has GDP-fucose transporter activity, and
- [0257] (t) a protein which comprises an amino acid sequence having a homology of 80% or more with the amino acid sequence represented by SEQ ID NO:98 and has GDP-fucose transporter activity.
- [0258] A DNA which hybridizes under stringent conditions is a DNA obtained, e.g., by a method such as colony hybridization, plaque hybridization or Southern blot hybridization using a DNA such as the DNA having the nucleotide sequence represented by SEQ ID NO:1, 2, 48, 51, 65, 91, 93, 95 or 97 or a partial fragment thereof as the probe, and specifically includes a DNA which can be identified by carrying out hybridization at 65° C. in the presence of 0.7 to 1.0 mol/l sodium chloride using a filter to which colony- or plaque-derived DNAs are immobilized, and then washing the filter at 65° C. using 0.11 to 2×SSC solution (composition of the 1×SSC solution comprising 150 mmol/l sodium chloride and 15 mmol/l sodium citrate). The hybridization can be carried out in accordance with the methods described, e.g., in *Molecular Cloning, A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press (1989) (hereinafter referred to as "*Molecular Cloning, Second Edition*"), *Current Protocols in Molecular Biology*, John Wiley & Sons, 1987-1997 (hereinafter referred to as "*Current Protocols in*

Molecular Biology"); *DNA Cloning 1: Core Techniques, A Practical Approach*, Second Edition, Oxford University (1995) and the like. The hybridizable DNA includes a DNA having at least 60% or more, preferably 70% or more, more preferably 80% or more, still more preferably 90% or more, far more preferably 95% or more, and most preferably 98% or more, of homology with the nucleotide sequence represented by SEQ ID NO:1, 2, 48, 51, 65, 91, 93, 95 or 97.

[0259] The protein which comprises an amino acid sequence in which at least one amino acid is deleted, substituted, inserted and/or added in the amino acid sequence represented by SEQ ID NO:19, 20, 23, 24, 71, 92, 94, 96 or 98 and has α 1,6-fucosyltransferase activity, GMD activity, Fx activity, GFPP activity or GFP-fucose transporter activity can be obtained, e.g., by introducing a site-directed mutation into a DNA encoding a protein having the amino acid sequence represented by SEQ ID NO:1, 2, 48, 51 or 65, respectively, using the site-directed mutagenesis described, e.g., in *Molecular Cloning, Second Edition; Current Protocols in Molecular Biology; Nucleic Acids Research*, 10, 6487 (1982); *Proc. Natl. Acad. Sci. USA*, 79, 6409 (1982); *Gene*, 34, 315 (1985); *Nucleic Acids Research*, 13, 4431 (1985); *Proc. Natl. Acad. Sci. USA*, 82, 488 (1985); and the like. The number of amino acids to be deleted, substituted, inserted and/or added and the number is not particularly limited, but is a number which can be deleted, substituted or added by a known technique such as the site-directed mutagenesis, e.g., it is 1 to several tens, preferably 1 to 20, more preferably 1 to 10, and most preferably 1 to 5.

[0260] Also, in order to maintain the α 1,6-fucosyltransferase activity, GMD activity, Fx activity, GFPP activity or GDP-fucose transporter activity of the protein to be used in the present invention, it has at least 80% or more, preferably 85% or more, more preferably 90% or more, still more preferably 95% or more, far more preferably 97% or more, and most preferably 99% or more, of homology with the amino acid sequence represented by SEQ ID NO:19, 20, 23, 24, 71, 92, 94, 96 or 98, when calculated using an analyzing soft such as BLAST [*J. Mol. Biol.*, 215, 403 (1990)] or FASTA [*Methods in Enzymology*, 183, 63 (1990)].

[0261] As the method for obtaining the above cells, any technique can be used, so long as it can decrease or delete the enzyme activity of interest. The technique for decreasing or deleting the enzyme activity includes:

- [0262] (a) a gene disruption technique which comprises targeting a gene encoding the enzyme;
- [0263] (b) a technique for introducing a dominant negative mutant of a gene encoding the enzyme;
- [0264] (c) a technique for introducing mutation into the enzyme, and
- [0265] (d) a technique for surprising transcription and/or translation of a gene encoding the enzyme, and the like.

[0266] Also, the method includes a method for selecting a cell having resistance to lectin which recognizes the structure of a sugar chain in which 1-position of fucose is bound to 6-position of N-acetylglucosamine in the reducing end through α -bond in a complex N-glycoside-linked sugar chain.

[0267] The growth of lectin-resistant cell is not inhibited in the presence of a lectin at an effective concentration during cell culturing.

[0268] In the present invention, the effective concentration of a lectin which does not inhibit the growth can be decided depending on the cell line, and is generally 10 μ g/ml to 10.0 mg/ml, preferably 0.5 to 2.0 mg/ml. The effective concentration of lectin in the case where mutation is introduced into a parent cell is a concentration in which the parent cell cannot normally grow or higher than the concentration, and is a concentration which is preferably similar to, more preferably 2 to 5 times, still more preferably 10 times, and most preferably 20 times or more, higher than the concentration in which the parent cell cannot normally grow.

[0269] The lectin which recognizes a sugar chain structure in which 1-position of fucose is bound to 6-position of N-acetylglucosamine through α -bond includes any lectin, so long as it is a lectin which is capable of recognizing the sugar chain structure. Examples include lentil agglutinin derived from *Lens culinaris* (Lentil lectin LCA), pea lectin derived from *Pisum sativum* (pea lectin PSA), (agglutinin derived from *Vicia faba* (broad bean lectin VFA), lectin derived from *Aleuria aurantia* (Aleuria aurantia lectin AAL) and the like.

[0270] The parent cell is a cell before a certain treatment is applied, namely a cell before the step for selecting the lectin-resistant cell used in the present invention is carried out or a cell before genetic engineering techniques for decreasing or deleting the above enzyme activity is carried out.

[0271] Although the parent cell is not particularly limited, the following cells are exemplified.

[0272] The parent cell of NS0 cell includes NS0 cells described in literatures such as *BIO/TECHNOLOGY*, 10, 169 (1992) and *Biotechnol. Bioeng.*, 73, 261 (2001). Furthermore, it includes NS0 cell line (RCB 0213) registered at RIKEN Cell Bank, The Institute of Physical and Chemical Research, sub-cell lines obtained by acclimating these cell lines to media in which they can grow, and the like.

[0273] The parent cell of SP2/0-Ag14 cell includes SP2/0-Ag14 cells described in literatures such as *J. Immunol.*, 126, 317 (1981), *Nature*, 276, 269 (1978) and *Human Antibodies and Hybridomas*, 3, 129 (1992). Furthermore, it includes SP2/0-Ag14 cell (ATCC CRL-1581) registered at ATCC, sub-cell lines obtained by naturalizing these cell lines to media in which they can grow (ATCC CRL-1581.1), and the like.

[0274] The parent cell of CHO cell derived from Chinese hamster ovary tissue includes CHO cells described in literatures such as *Journal of Experimental Medicine*, 108, 945 (1958), *Proc. Natl. Acad. Sci. USA*, 60, 1275 (1968), *Genetics*, 55, 513 (1968), *Chromosoma*, 41, 129 (1973), *Methods in Cell Science*, 18, 115 (1996), *Radiation Research*, 148, 260 (1997), *Proc. Natl. Acad. Sci. USA*, 77, 4216 (1980), *Proc. Natl. Acad. Sci. USA*, 6, 1275 (1968), *Cell*, 6, 121 (1975) and *Molecular Cell Genetics*, Appendix I, II (p. 883-900). Furthermore, it includes cell line CHO-K1 (ATCC CCL-61), cell line DUXB11 (ATCC CRL-9060) and cell line Pro-5 (ATCC CRL-1781) registered at ATCC, commercially available cell line CHO-S (Cat # 11619 of Life

Technologies), sub-cell lines obtained by acclimating these cell lines to media in which they can grow, and the like.

[0275] The parent cell of a rat myeloma cell line YB2/3HL.P2.G11.16Ag.20 cell includes cell lines established from Y3/Ag1.2.3 cell (ATCC CRL-1631) such as YB2/3HL.P2.G11.16Ag.20 cell described in literatures such as *J. Cell. Biol.*, 93, 576 (1982) and *Methods Enzymol.* 73B, 1 (1981). Furthermore, it include YB2/3HL.P2.G11.16Ag.20 cell (ATCC CRL-1662) registered at ATCC, sub-lines obtained by acclimating these cell lines to media in which they can grow, and the like.

[0276] In the present invention, Fc γ R means an Fc receptor (hereinafter also referred to as "FcR") against an IgG class antibody. FcR means a receptor which binds to the Fc region of an antibody [*Annu. Rev. Immunol.*, 2, 457 (1991)]. Furthermore, Fc γ R includes Fc γ RI, Fc γ RII and Fc γ RIII subclasses and their allele mutants and isoforms formed by alternative splicing. In addition, Fc γ RII includes Fc γ RIIa and Fc γ RIIb, and Fc γ RII includes Fc γ RIIIa and Fc γ RIIIb [*Annu. Rev. Immunol.*, 9, 457 (1991)].

[0277] The binding activity to Fc γ RIIIa can be increased by binding sugar chains to the Fc region of an antibody molecule so as to adjust the ratio of a sugar chain in which fucose is not bound to N-acetylglucosamine in the reducing end in the sugar chain among the total complex N-glycoside-linked sugar chains bound to the Fc region to preferably 20% or more, more preferably 30% or more, still more preferably 40% or more, particularly preferably 50% or more, and most preferably 100%.

[0278] The ratio of a sugar chain in which fucose is not bound to N-acetylglucosamine in the reducing end in the sugar chain among the total complex N-glycoside-linked sugar chains bound to the Fc region contained in the antibody composition is a ratio of the number of a sugar chain in which fucose is not bound to N-acetylglucosamine in the reducing end in the sugar chain to the total number of the complex N-glycoside-linked sugar chains bound to the Fc region contained in the composition. Also, the ratio of a sugar chain is preferably a ratio of a sugar chain in which 1-position of fucose is not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond in the sugar chain.

[0279] The sugar chain in which fucose is not bound to N-acetylglucosamine in the reducing end in the complex N-glycoside-linked sugar chain is a sugar chain in which fucose is not bound to AF-acetylglucosamine in the reducing end through α -bond in the complex N-glycoside-linked sugar chain. Preferably, it is a sugar chain in which 1-position of fucose is not bound to 6-position of N-acetylglucosamine in the complex N-glycoside-linked sugar chain through α -bond.

[0280] The ratio of a sugar chain in which fucose is not bound to N-acetylglucosamine in the reducing end in the sugar chain contained in the composition which comprises an antibody molecule having complex N-glycoside-linked sugar chains in the Fc region can be determined by releasing the sugar chain from the antibody molecule using a known method such as hydrazinolysis, enzyme digestion or the like [*Biochemical Experimentation Methods 23—Method for Studying Glycoprotein Sugar Chain* (Japan Scientific Societies Press), edited by Reiko Takahashi (1989)], carrying out

fluorescence labeling or radioisotope labeling of the released sugar chain, and then separating the labeled sugar chain by chromatography. Also, the released sugar chain can be determined by analyzing it with the HPAED-PAD method [*J. Liq. Chromatogr.*, 6, 1577 (1983)]. The antibody composition in which binding activity to Fc γ RIIa has been enhanced by the method of the present invention has high ADCC activity.

[0281] In the present invention, the ADCC activity is a cytotoxic activity in which an antibody bound to a cell surface antigen on a cell such as a tumor cell in the living body activates an effector cell mediated the antibody Fc region and an Fc receptor existing on effector cell surface and thereby injures the tumor cell and the like [*Monoclonal Antibodies: Principles and Applications*, Wiley-Liss, Inc., Chapter 2.1 (1995)]. The effector cell includes killer cells, natural killer cells, monocytes, macrophages, and the like.

[0282] A process for producing a host cell in which the activity of a protein relating to modification of a sugar chain in which fucose is bound to N-acetylglucosamine in the reducing end in the complex NV-glycoside-linked sugar chain is decreased or deleted used in the method of the present invention is explained below in detail.

[0283] 1. Preparation of Host Cell Used in the Method of the Invention

[0284] The host cell used in the method of the present invention can be prepared by the following techniques.

[0285] (1) Gene Disruption Technique which Comprises Targeting a Gene Encoding an Enzyme

[0286] The host cell used in the method of the present invention can be prepared by targeting a gene encoding a GDP-fucose synthase, α 1,6-fucose modifying enzyme or a GDP-fucose transport protein by using a gene disruption technique. The GDP-fucose synthase includes GMD, Fx, GFPP, fucokinase and the like. The α 1,6-fucose modifying enzyme includes α 1,6-fucosyltransferase, α -L-fucosidase and the like. The GDP-fucose transport protein includes GDP-fucose transporter.

[0287] The gene as used herein includes DNA and RNA.

[0288] The gene disruption method may be any method, so long as it can disrupt the gene encoding the target enzyme. Examples include an antisense method, a ribozyme method, a homologous recombination method, an RNA-DNA oligonucleotide (RDO) method, an RNA interference (RNAi) method, a method using retrovirus, a method using transposon and the like. The methods are specifically described below.

[0289] (a) Preparation of Host Cell Used in the Present Invention by the Antisense Method or the Ribozyme Method

[0290] The host cell used in the method of the present invention can be prepared by targeting the GDP-fucose synthase, α 1,6-fucose modifying enzyme or the GDP-fucose transport protein according to the antisense or ribozyme method described in *Cell Technology*, 12, 239 (1993); *BIO/TECHNOLOGY*, 17, 1097 (1999), *Hum. Mol. Genet.*, 5, 1083 (1995); *Cell Technology*, 13, 255 (1994); *Proc. Natl. Acad. Sci. USA*, 96, 1886 (1999); or the like, e.g., in the following manner.

[0291] A cDNA or a genomic DNA encoding GDP-fucose synthase, α 1,6-fucose modifying enzyme or the GDP-fucose transport protein is prepared.

[0292] The nucleotide sequence of the prepared cDNA or genomic DNA is determined.

[0293] Based on the determined DNA sequence, an antisense gene or ribozyme construct of an appropriate length comprising a DNA moiety which encodes the GDP-fucose synthase, α 1,6-fucose modifying enzyme or the GDP-fucose transport protein, a part of its untranslated region or an intron is designed.

[0294] In order to express the antisense gene or ribozyme in a cell, a recombinant vector is prepared by inserting a fragment or total length of the prepared DNA into downstream of the promoter of an appropriate expression vector.

[0295] A transformant is obtained by introducing the recombinant vector into a host cell suitable for the expression vector.

[0296] The host cell used in the method of the present invention can be obtained by selecting a transformant based on the activity of the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein. The host cell of the present invention can also be obtained by selecting a transformant based on the sugar chain structure of a glycoprotein on the cell membrane or the sugar chain structure of the produced antibody molecule.

[0297] As the host cell used for preparing the host cell used in the method of the present invention, any cell such as yeast, an animal cell, an insect cell or a plant cell can be used, so long as it has a gene encoding the target GDP-fucose synthase, α 1,6-fucose modifying enzyme or GDP-fucose transport protein. Examples include host cells described in the following item 3.

[0298] As the expression vector, a vector which is autonomously replicable in the host cell or can be integrated into the chromosome and comprises a promoter at such a position that the designed antisense gene or ribozyme can be transferred is used. Examples include expression vectors described in the following item 3.

[0299] As the method for introducing a gene into various host cells, the methods for introducing recombinant vectors suitable for various host cells described in the following item 3, can be used.

[0300] The method for selecting a transformant based on the activity of the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein includes biochemical methods or genetic engineering techniques described in *New Biochemical Experimentation Series (Shin-Jikken Kagakii Koza) 3—Saccharides (Toshitsu) I*, Glycoprotein (Totanpakushitu) (Tokyo Kagaku Dojin), edited by Japanese Biochemical Society (1988); *Cell Engineering (Saibo Kogaku)*. Supplement, Experimental Protocol Series, Glycobiology Experimental Protocol, Glycoprotein, Glycolipid and Proteoglycan (Shujun-sha), edited by Naoyuki Taniguchi, Akemi Suzuki, Kiyoshi Furukawa and Kazuyuki Sugawara (1996); *Molecular Cloning*, Second Edition; *Current Protocols in Molecular Biology*; and the like. The biochemical method includes a method in which the enzyme activity is evaluated using enzyme-specific substrate and the like. The genetic engineering technique

include the Northern analysis, RT-PCR and the like which measures the amount of mRNA of a gene encoding the enzyme.

[0301] The method for selecting a transformant based on the sugar chain structure of a glycoprotein on the cell membrane includes the methods described in the following item 1(5). The method for selecting a transformant based on the sugar chain structure of a produced antibody molecule includes the methods described in the following items 6 and 7.

[0302] As the method for preparing cDNA encoding the GDP-fucose synthase, α 1,6-fucose modifying enzyme or the GDP-fucose transport protein, the following method is exemplified.

[0303] Preparation of DNA:

[0304] A total RNA or RNA is prepared from a human or non-human animal tissue or cell.

[0305] The mRNA of a human or non-human tissue or cell may be a commercially available product (e.g., manufactured by Clontech) or may be prepared from a human or non-human animal tissue or cell as follows. The method for preparing a total RNA from a human or non-human animal tissue or cell includes the guanidine thiocyanate-caesium trifluoroacetate method [*Methods in Enzymology*, 154, 3 (1987)], the acidic guanidine thiocyanate phenol chloroform (AGPC) method [*Analytical Biochemistry*, 162, 156 (1987); *Experimental Medicine*, 9, 1937 (1991)] and the like.

[0306] Also, the mRNA can be prepared as poly(A)⁺ RNA from a total RNA by the oligo(dT)-immobilized cellulose column method (*Molecular Cloning*, Second Edition) and the like.

[0307] In addition, mRNA can be prepared using a kit such as Fast Track mRNA Isolation Kit (manufactured by Invitrogen) or Quick Prep mRNA Purification Kit (manufactured by Pharmacia).

[0308] A cDNA library is prepared from the prepared mRNA of a human or non-human animal tissue or cell. The method for preparing cDNA library includes the methods described in *Molecular Cloning*, Second Edition; *Current Protocols in Molecular Biology*; and the like, or methods using a commercially available kit such as SuperScript Plasmid System for cDNA Synthesis and Plasmid Cloning (manufactured by Life Technologies) or ZAP-cDNA Synthesis Kit (manufactured by STRATAGENE).

[0309] As the cloning vector for the preparation of the cDNA library, any vector such as a phage vector or a plasmid vector can be used, so long as it is autonomously replicable in *Escherichia coli* K12. Examples include ZAP Express [manufactured by STRATAGENE, *Strategies*, 5, 58 (1992)], pBluescript II SK(+) [*Nucleic Acids Research*, 172, 9494 (1989)], Lambda ZAP II (manufactured by STRATAGENE), λ gt10 ad λ gt11 [*DNA Cloning, A Practical Approach*, 1, 49 (1985)], λ TriplEx (manufactured by Clontech), λ ExCell (manufactured by Pharmacia), pT7T318U (manufactured by Pharmacia), pcD2 [*Mol. Cell. Biol.*, 3, 280 (1983)], pUC18 [*Gene* 33, 103 (1985)] and the like.

[0310] Any microorganism can be used as the host microorganism for the preparation of the DNA library, and *Escherichia coli* is preferably used. Examples include

Escherichia coli XL1-Blue MRF⁺ [manufactured by STRAT-AGENE, *Strategies*, 5, 81 (1992)], *Escherichia coli* C600 [Genetics, 39440 (1954)], *Escherichia coli* Y1088 [Science, 222, 778 (1983)], *Escherichia coli* Y1090 [Science, 222, 778 (1983)], *Escherichia coli* NM522 [J. Mol. Biol., 166, 1 (1983)], *Escherichia coli* K802 [J. Mol. Biol., 16, 118 (1966)], *Escherichia coli* JM105 [Gene, 38, 275 (1985)] and the like.

[0311] The cDNA library can be used as such in the following analysis, and in order to obtain a full length cDNA as efficient as possible by decreasing the ratio of an infull length cDNA, a cDNA library prepared by using the oligo cap method developed by Sugano et al. [Gene, 138, 171 (1994), Gene, 200, 149 (1997)] Protein, Nucleic Acid and Protein 41, 603 (1996)], *Experimental Medicine*, 12491 (1993); *cDNA Cloning* (Yodo-sha) (1996), *Methods for Preparing Gene Libraries* (Yodo-sha) (1994)] can be used in the following analysis.

[0312] Based on the amino acid sequence of the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein, degenerative primers specific for the 4'-terminal and 3'-terminal nucleotide sequences of a nucleotide sequence presumed to encode the amino acid sequence are prepared, and DNA is amplified by PCR [PCR Protocols, Academic Press (1990)] using the prepared cDNA library as the template to obtain a gene fragment encoding the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein.

[0313] It can be confirmed that the obtained gene fragment is a DNA encoding the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein by a method generally used for analyzing a nucleotide such as the dideoxy method of Sanger et al. [Proc. Natl. Acad. Sci. USA, 74, 5463 (1977)] or by using a nucleotide sequence analyzer such as ABIPRISM 377 DNA Sequencer (manufactured by PE Biosystems).

[0314] A DNA encoding the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein can be obtained by carrying out colony hybridization or plaque hybridization (*Molecular Cloning*, Second Edition) for the cDNA or cDNA library synthesized from the mRNA contained in the human or non-human animal tissue or cell, using the gene fragment as a DNA probe.

[0315] Also, using the primers used for obtaining the gene fragment encoding the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein, a DNA encoding the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein can also be obtained by carrying out screening by PCR using the cDNA or cDNA library synthesized from the rRNA contained in a human or non-human animal tissue or cell as the template.

[0316] The nucleotide sequence of the obtained DNA encoding the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein is analyzed from its terminus and determined by a method generally used for analyzing a nucleotide such as the dideoxy method of Sanger et al. [Proc. Natl. Acad. Sci. USA, 74, 5463 (1977)] or by using a nucleotide sequence analyzer such as ABIPRISM 377 DNA Sequencer (manufactured by PE Biosystems).

[0317] A gene encoding the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein can also be determined from genes in data bases by searching nucleotide sequence data bases such as GenBank, EMBL and DDBJ using a homology retrieving program such as BLAST based on the determined cDNA nucleotide sequence.

[0318] The nucleotide sequence of the gene encoding the GDP-fucose synthase obtained by the above method includes the nucleotide sequence represented by SEQ ID NO:48, 51 or 65. The nucleotide sequence of the gene encoding the α 1,6-fucose modifying enzyme includes the nucleotide sequence represented by SEQ ID NO:1 or 2. The nucleotide sequence of the gene encoding the GDP-fucose transport protein includes the nucleotide sequence represented by SEQ ID NO:91, 93, 95 or 97.

[0319] The cDNA encoding the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein can also be obtained by chemically synthesizing it with a DNA synthesizer such as DNA Synthesizer model 392 manufactured by Perkin Elmer using the phosphoramidite method, based on the determined DNA nucleotide sequence.

[0320] The method for preparing a genomic DNA encoding the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein includes known methods described in *Molecular Cloning*, Second Edition; *Current Protocols in Molecular Biology*; and the like. Furthermore, the genomic DNA can be prepared by using a kit such as Genome DNA Library Screening System (manufactured by Genome Systems) or Universal GenomeWalkerTM Kits (manufactured by CLONTECH).

[0321] The nucleotide sequence of the genomic DNA encoding the GDP-fucose synthase obtained by the method includes the nucleotide sequence represented by SEQ ID NO:67 or 70. The nucleotide sequence of the genomic DNA encoding the α 1,6-fucose modifying enzyme includes the nucleotide sequence represented by SEQ ID NO:3. The nucleotide sequence of the genomic DNA encoding the GDP-fucose transport protein includes the nucleotide sequence represented by SEQ ID NO:99 or 100.

[0322] In addition, the host cell can also be obtained without using an expression vector, by directly introducing an antisense oligonucleotide or ribozyme into a host cell, which is designed based on the nucleotide sequence encoding the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein.

[0323] The antisense oligonucleotide or ribozyme can be prepared in the usual method or by using a DNA synthesizer. Specifically, it can be prepared based on the sequence information of an oligonucleotide having a corresponding sequence of continued 5 to 150 bases, preferably 5 to 60 bases, and more preferably 5 to 40 bases, among nucleotide sequences of a cDNA and a genomic DNA encoding the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein by synthesizing an oligonucleotide which corresponds to a sequence complementary to the oligonucleotide (antisense oligonucleotide) or a ribozyme comprising the oligonucleotide sequence.

[0324] The oligonucleotide includes oligo RNA and derivatives of the oligonucleotide (hereinafter referred to as "oligonucleotide derivatives").

[0325] The oligonucleotide derivatives includes oligonucleotide derivatives in which a phosphodiester bond in the oligonucleotide is converted into a phosphorothioate bond, an oligonucleotide derivative in which a phosphodiester bond in the oligonucleotide is converted into an N3'-P5' phosphoamidate bond, an oligonucleotide derivative in which ribose and a phosphodiester bond in the oligonucleotide are converted into a peptide-nucleic acid bond, an oligonucleotide derivative in which uracil in the oligonucleotide is substituted with C-5 propynyluracil, an oligonucleotide derivative in which uracil in the oligonucleotide is substituted with C-5 thiazoleuracil, an oligonucleotide derivative in which cytosine in the oligonucleotide is substituted with C-5 propynylcytosine, an oligonucleotide derivative in which cytosine in the oligonucleotide is substituted with phenoxazine-modified cytosine, an oligonucleotide derivative in which ribose in the oligonucleotide is substituted with 2'-O-propylribose and an oligonucleotide derivative in which ribose in the oligonucleotide is substituted with 2'-methoxyethoxyribose [*Cell Technology (Saibo Kogaku)*, 16, 1463 (1997)].

[0326] (b) Preparation of Host Cell Used in the Method of the Present Invention by Homologous Recombination

[0327] The host cell used in the method of the present invention can be prepared by targeting a gene encoding the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein and modifying the target gene on chromosome through a homologous recombination technique.

[0328] The target gene on the chromosome can be modified by using a method described in *Manipulating the Mouse Embryo, A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press (1994) (hereinafter referred to as "*Manipulating the Mouse Embryo, A Laboratory Manual*"), *Gene Targeting, A Practical Approach*, IRL Press at Oxford University Press (1993); *Biomaterial Series 8, Gene Targeting, Preparation of Mutant Mice using ES*, Yodo-sha (1995) (hereinafter referred to as "*Preparation of Mutant Mice using ES Cells*"); or the like, for example, as follows.

[0329] A genomic DNA encoding the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein is prepared.

[0330] Based on the nucleotide sequence of the genomic DNA, a target vector is prepared for homologous recombination of a target gene to be modified (e.g., structural gene encoding the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein or a promoter gene).

[0331] The host cell used in the method of the present invention can be produced by introducing the prepared target vector into a host cell and selecting a cell in which homologous recombination occurred between the target gene and target vector.

[0332] As the host cell, any cell such as yeast, an animal cell, an insect cell or a plant cell can be used, so long as it has a gene encoding the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein. Examples include the host cells described in the following item 3.

[0333] The method for preparing a genomic DNA encoding the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein includes the methods described in "Preparation method of genomic DNA" in the item 1(1)(a) and the like.

[0334] The nucleotide sequence of the genomic DNA encoding the GDP-fucose synthase obtained by the above method includes the nucleotide sequence represented by SEQ ID NO:67 or 70. The nucleotide sequence of the genomic DNA of the α 1,6-fucose modifying enzyme includes the nucleotide sequence represented by SEQ ID NO:3. The nucleotide sequence of the genomic DNA of the GDP-fucose transport protein includes the nucleotide sequence represented by SEQ ID NO:99 or 100.

[0335] The target vector for the homologous recombination of the target gene can be prepared in accordance with a method described in *Gene Targeting, A Practical Approach*, IRL Press at Oxford University Press (1993); *Preparation of Mutant Mice using ES Cells*; or the like. The target vector can be used as either a replacement type or an insertion type.

[0336] For introducing the target vector into various host cells, the methods for introducing recombinant vectors suitable for various host cells described in the following item 3 can be used.

[0337] The method for efficiently selecting a homologous recombinant includes a method such as the positive selection, promoter selection, negative selection or polyA selection described in *Gene Targeting, A Practical Approach*, IRL Press at Oxford University Press (1993); *Preparation of Mutant Mice using ES Cells*; or the like. The method for selecting the homologous recombinant of interest from the selected cell lines includes the Southern hybridization method for genomic DNA (*Molecular Cloning*, Second Edition), PCR [*PCR Protocols*, Academic Press (1990)], and the like.

[0338] (c) Preparation of Cell of the Present Invention by RDO Method

[0339] The host cell used in the method of the present invention can be prepared by targeting a gene encoding the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein according to an RDO method, for example, as follows.

[0340] A cDNA or a genomic DNA encoding the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein is prepared.

[0341] The nucleotide sequence of the prepared cDNA or genomic DNA is determined.

[0342] Based on the determined DNA sequence, an RDO construct of an appropriate length comprising a DNA encoding the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein, a DNA encoding an untranslated region or a DNA encoding an intron, is designed and synthesized.

[0343] The host cell used in the method of the present invention can be obtained by introducing the synthesized RDO into a host cell and then selecting a transformant in which a mutation occurred in the target enzyme, i.e., the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein.

[0344] As the host cell, any cell such as yeast, an animal cell, an insect cell or a plant cell can be used, so long as it has a gene encoding the target GDP-fucose synthase, α 1,6-fucose modifying enzyme or GDP-fucose transport protein. Examples include the host cells described in the following item 3.

[0345] The method for introducing RDO into various host cells includes the methods for introducing recombinant vectors suitable for various host cells described in the following item 3.

[0346] The method for preparing cDNA encoding the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein includes the methods described in "Preparation of DNA" in the item 1(1)(a) and the like.

[0347] The method for preparing a genomic DNA encoding the GDP-fucose synthase, α 1,6-fucose modifying enzyme or the GDP-fucose transport protein includes the methods described in "Preparation method of genomic DNA" in the item 1(1)(a) and the like.

[0348] The nucleotide sequence of the DNA can be determined by digesting it with appropriate restriction enzymes, cloning the fragments into a plasmid such as pBluescript SK(-) (manufactured by Stratagene), subjecting the clones to the reaction generally used as a method for analyzing a nucleotide sequence such as the dideoxy method of Sanger et al. [*Proc. Natl. Acad. Sci. USA*, 74, 5463 (1977)] or the like, and then analyzing the clones using an automatic nucleotide sequence analyzer such as ABI PSISM 377DNA Sequencer (manufactured by PE Biosystems) or the like.

[0349] The RDO can be prepared in the usual method or by using a DNA synthesizer.

[0350] The method for selecting a cell in which a mutation occurred, by introducing the RDO into the host cell, in the gene encoding the targeting enzyme, the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein includes the methods for directly detecting mutations in chromosomal genes described in *Molecular Cloning*, Second Edition, *Current Protocols in Molecular Biology* and the like.

[0351] Furthermore, the method described in the item 1(1)(a) for selecting a transformant based on the activity of the introduced GDP-fucose synthase, α 1,6-fucose modifying enzyme or GDP-fucose transport protein and the method for selecting a transformant based on the sugar chain structure of a glycoprotein on the cell membrane described later in the item 1(5), and the method for selecting a transformant based on the sugar structure of a produced antibody molecule described later in the item 6 or 7 can also be used.

[0352] The construct of the RDO can be designed in accordance with the methods described in *Science*, 273, 1386 (1996); *Nature Medicine*, 4, 285 (1998); *Hepatology*, 25, 1462 (1997); *Gene Therapy*, 5, 1960 (1999) *J. Mol. Med.*, 75, 829 (1997); *Proc. Natl. Acad. Sci. USA*, 96, 8774 (1999); *Proc. Natl. Acad. Sci. USA*, 96, 8768 (1999); *Nuc. Acids. Res.*, 27, 1323 (1999); *Invest. Dermatol.*, 111, 1172 (1998); *Nature Biotech.*, 16, 1343 (1998); *Nature Biotech.*, 18, 43 (2000); *Nature Biotech.*, 18, 555 (2000); and the like.

[0353] (d) Preparation of Host Cell Used in the Method of the Present Invention by RNAi Method

[0354] The host cell used in the method of the present invention can be prepared by targeting a gene encoding the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein according to the RNAi method, for example, as follows.

[0355] A cDNA encoding the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein is prepared.

[0356] The nucleotide sequence of the prepared cDNA is determined.

[0357] Based on the determined DNA sequence, an RNAi gene construct of an appropriate length comprising a DNA encoding the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein or a DNA encoding a untranslated region, is designed.

[0358] In order to express the RNAi gene in a cell, a recombinant vector is prepared by inserting a fragment or full length of the prepared DNA into downstream of the promoter of an appropriate expression vector.

[0359] A transformant is obtained by introducing the recombinant vector into a host cell suitable for the expression vector.

[0360] The host cell used in the method of the present invention can be obtained by selecting a transformant based on the activity of the introduced GDP-fucose synthase, α 1,6-fucose modifying enzyme or GDP-fucose transport protein, or the sugar chain structure of the produced antibody molecule or of a glycoprotein on the cell membrane.

[0361] As the host cell, any cell such as yeast, an animal cell, an insect cell or a plant cell can be used, so long as it has a gene encoding the target GDP-fucose synthase, α 1,6-fucose modifying enzyme or GDP-fucose transport protein. Examples include the host cells described in the following item 3.

[0362] As the expression vector, a vector which is autonomously replicable in the above host cell or can be integrated into the chromosome and comprises a promoter at such a position that the designed RNAi gene can be transferred is used. Examples include the expression vectors described in the following item 3.

[0363] As the method for introducing a gene into various host cells, the methods for introducing recombinant vectors suitable for various host cells described in the following item 3 can be used.

[0364] The method for selecting a transformant based on the activity of the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein includes the methods described in the item 1(1)(a).

[0365] The method for selecting a transformant based on the sugar chain structure of a glycoprotein on the cell membrane includes the methods described in the following item 1(5). The method for selecting a transformant based on the sugar chain structure of a produced antibody molecule includes the methods described in the following item 6 or 7.

[0366] The method for preparing cDNA of the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the

GDP-fucose transport protein includes the methods described in "Preparation of DNA" in the item 1(1)(a) and the like.

[0367] In addition, the host cell used in the method of the present invention can also be obtained without using an expression vector, by directly introducing an RNAi gene designed based on the nucleotide sequence of the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein.

[0368] The RNAi gene can be prepared in the usual method or by using a DNA synthesizer.

[0369] The RNAi gene construct can be designed in accordance with the methods described in *Nature*, 39, 806 (1998); *Proc. Natl. Acad. Sci. USA*, 95, 15502 (1998); *Nature*, 395, 854 (1998); *Proc. Natl. Acad. Sci. USA*, 96, 5049 (1999); *Cell*, 95, 1017 (1998); *Proc. Natl. Acad. Sci. USA* 96, 1451 (1999); *Proc. Natl. Acad. Sci. USA*, 95, 13959 (1998), *Nature Cell Biol.*, 2, 70 (2000), and the like.

[0370] (e) Preparation of Host Cell Used in the Method of the Present Invention by Method Using Transposon

[0371] The host cell used in the method of the present invention can be prepared by selecting a mutant based on the activity of the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein or the sugar chain structure of a produced antibody molecule or of a glycoprotein on the cell membrane by using a transposon system described in *Nature Genet.*, 25, 35 (2000) or the like.

[0372] The transposon system is a system in which a mutation is induced by randomly inserting an exogenous gene into chromosome, wherein an exogenous gene interposed between transposons is generally used as a vector for inducing a mutation, and a transposase expression vector for randomly inserting the gene into chromosome is introduced into the cell at the same time.

[0373] Any transposase can be used, so long as it is suitable for the sequence of the transposon to be used.

[0374] As the exogenous gene, any gene can be used, so long as it can induce a mutation in the DNA of a host cell.

[0375] As the host cell, any cell such as yeast, an animal cell, an insect cell or a plant cell can be used, so long as it has a gene encoding the targeting GDP-fucose synthase, α 1,6-fucose modifying enzyme or GDP-fucose transport protein. Examples include the host cells described in the following item 3. For introducing the gene into various host cells, the method for introducing recombinant vectors suitable for various host cells described in the following item 3, can be used.

[0376] The method for selecting a mutant based on the activity of the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein includes the methods which will be described above in the item 1(1)(a).

[0377] The method for selecting a mutant based on the sugar chain structure of a glycoprotein on the cell membrane includes the methods described in the following item 1(5). The method for selecting a transformant based on the sugar chain structure of a produced antibody molecule includes the methods described in the following item 6 or 7.

[0378] (2) Method for Introducing Dominant Negative Mutant of Enzyme

[0379] The host cell used in the method of the present invention can be prepared by targeting a gene encoding the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein according to a technique for introducing a dominant negative mutant of the enzyme. The GDP-fucose synthase includes GMD, Fx, GFPP, fucokinase and the like. The α 1,6-fucose modifying enzyme includes α 1,6-fucosyltransferase, α -L-fucosidase and the like. The GDP-fucose transport protein includes GDP-fucose transporter and the like.

[0380] The enzymes catalyze specific reactions having substrate specificity, and dominant negative mutants of the enzymes can be prepared by disrupting the active center of the enzymes which have the catalytic activity having substrate specificity. The method for preparing a dominant negative mutant is specifically described as follows with reference to GMD among the target enzymes.

[0381] As a result of the analysis of the three-dimensional structure of *E. coli*-derived GMD, it has been found that 4 amino acids (threonine at position 133, glutamic acid at position 135, tyrosine at position 157 and lysine at position 161) have an important function on the enzyme activity [Structure, 8, 2 (2000)]. That is, when mutants were prepared by substituting the 4 amino acids with other different amino acids based on the three-dimensional structure information, the enzyme activity of all of the mutants was significantly decreased. On the other hand, changes in the ability of GMD to bind to GMD coenzyme NADP and its substrate GDP-mannose were hardly observed in the mutants. Accordingly, a dominant negative mutant can be prepared by substituting the 4 amino acids which control the enzyme activity of GMD. A dominant negative mutant can be prepared by comparing the homology and predicting the three-dimensional structure using the amino acid sequence information based on the results of the *E. coli*-derived GMD. For example, in GMD (SEQ ID NO:65) derived from CHO cell, a dominant negative mutant can be prepared by substituting threonine at position 155, glutamic acid at position 157, tyrosine at position 179 and lysine at position 183. Such a gene into which amino acid substitution is introduced can be prepared by the site-directed mutagenesis described in *Molecular Cloning*, Second Edition, *Current Protocols in Molecular Biology* or the like.

[0382] The host cell can be prepared by using the above prepared dominant negative mutant gene of the target enzyme according to the method described in *Molecular Cloning*, Second Edition, *Current Protocols in Molecular Biology*, *Manipulating the Mouse Embryo*, Second Edition or the like, for example, as follows.

[0383] A gene encoding the dominant negative mutant of the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein (hereinafter referred to as "dominant negative mutant gene") is prepared.

[0384] Based on the full length DNA of the prepared dominant negative mutant gene, a DNA fragment of an appropriate length containing a DNA encoding the antibody molecule is prepared, if necessary.

[0385] A recombinant vector is prepared by inserting the DNA fragment or full length DNA into downstream of the promoter of an appropriate expression vector.

[0386] A transformant is obtained by introducing the recombinant vector into a host cell suitable for the expression vector.

[0387] The host cell used in the method of the present invention can be prepared by selecting a transformant based on the activity of the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein, or the sugar chain structure of a produced antibody molecule or of a glycoprotein on the cell membrane.

[0388] As the host cell, any cell such as yeast, an animal cell, an insect cell or a plant cell can be used, so long as it has a gene encoding the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein. Examples include the host cells described in the following item 3.

[0389] As the expression vector, a vector which is autonomously replicable in the host cell or can be integrated into the chromosome and comprises a promoter at a position where transcription of the DNA encoding the dominant negative mutant of interest can be effected is used. Examples include the expression vectors described in the following item 3.

[0390] For introducing the gene into various host cells, the method for introducing recombinant vectors suitable for various host cells described in the following item 3, can be used.

[0391] The method for selecting a mutant based on the activity of the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein includes the methods which will be described in the above item 1(1)(a).

[0392] The method for selecting a mutant based on the sugar chain structure of a glycoprotein on the cell membrane includes the methods described in the following item 1(5). The method for selecting a transformant based on the sugar chain structure of a produced antibody molecule includes the methods described in the following item 6 or 7.

[0393] (3) Method for Introducing Mutation into Enzyme

[0394] The host cell used in the method of the present invention can be prepared by introducing a mutation into a gene encoding the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein, and then selecting a cell line of interest in which the mutation occurred in the enzyme.

[0395] The GDP-fucose synthase includes GMD, Fx, GFPP, fucokinase and the like. The α 1,6-fucose modifying enzyme includes α 1,6-fucosyltransferase, α -L-fucosidase and the like. The GDP-fucose transport protein includes GDP-fucose transporter and the like.

[0396] The method for introducing mutation into an enzyme includes 1) a method in which a desired clone is selected from mutants obtained by a mutation-inducing treatment of a parent cell line with a mutagen or spontaneously generated mutants, based on the activity of the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein, 2) a method in which a desired clone is selected from mutants obtained by a mutation-inducing treatment of a parent cell line with a mutagen or spontaneously generated mutants, based on the sugar

chain structure of a produced antibody molecule, 3) a method in which a desired clone is selected from mutants obtained by a mutation-inducing treatment of a parent cell line with a mutagen or spontaneously generated mutants, based on the sugar chain structure of a glycoprotein on the cell membrane, and the like.

[0397] As the mutation-inducing treatment, any treatment can be used, so long as it can induce a point mutation or a deletion or frame shift mutation in the DNA of cells of the parent cell line.

[0398] Examples include treatment with ethyl nitrosourea, nitrosoguanidine, benzopyrene or an acridine pigment and treatment with radiation. Also, various alkylating agents and carcinogens can be used as mutagens. The method for allowing a mutagen to act upon cells includes the methods described in *Tissue Culture Techniques*, 3rd edition (Asakura Shoten), edited by Japanese Tissue Culture Association (1996), *Nature Genet.*, 24, 314 (2000) and the like.

[0399] The spontaneously generated mutant includes mutants which are spontaneously formed by continuing subculture under general cell culture conditions without applying special mutation-inducing treatment.

[0400] The method for measuring the activity of the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein includes the methods described above in the item 1(1)(a). The method for distinguishing the sugar chain structure of a produced antibody molecule includes the methods described in the following item 6 or 7. The method for distinguishing the sugar chain structure of a glycoprotein on the cell membrane includes the methods described in the following item 1(5).

[0401] (4) Method for Suppressing Transcription and/or Translation of Enzyme

[0402] The host cell used in the method of the present invention can be prepared by targeting a gene encoding the GDP-fucose synthase or the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein and suppressing transcription and/or translation of the target gene according to the antisense RNA/DNA technique [*Bioscience and Industry*, 50, 322 (1992); *Chemistry*, 46, 681 (1991), *Biotechnology*, 9, 358 (1992), *Trends in Biotechnology*, 10, 87 (1992), *Trends in Biotechnology*, 10, 152 (1992), *Cell Engineering*, 16, 1463 (1997)], the triple helix technique [*Trends in Biotechnology*, 10, 132 (1992)] or the like.

[0403] The GDP-fucose synthase includes GMD, Fx, GFPP, fucokinase and the like. The α 1,6-fucose modifying enzyme includes α 1,6-fucosyltransferase, α -L-fucosidase and the like. The GDP-fucose transport protein includes GDP-fucose transporter and the like.

[0404] (5) Method for Selecting Clone Resistant to Lectin which Recognizes Sugar Chain Structure in which 1-Position of Fucose is Bound to 6-Position of N-acetylglucosamine in the Reducing End through α -Bond in the N-glycoside-linked Sugar Chain

[0405] The host cell used in the method of the present invention can be prepared by using a method for selecting a clone resistant to a lectin which recognizes a sugar chain structure in which 1-position of fucose is bound to 6-position of N-acetylglucosamine in the reducing end through α -bond in the N-glycoside-linked sugar chain.

[0406] The method for selecting a clone resistant to a lectin which recognizes a sugar chain structure in which 1-position of fucose is bound to 6-position of N-acetylglucosamine in the reducing end through α -bond in the N-glycoside-linked sugar chain includes the methods using lectin described in *Somatic Cell Mol. Genet.*, 12, 51 (1986) and the like.

[0407] As the lectin, any lectin can be used, so long as it is a lectin which recognizes a sugar chain structure in which 1-position of fucose is bound to 6-position of N-acetylglucosamine in the reducing end through α -bond in the N-glycoside-linked sugar chain. Examples include a *Lens culinaris* lectin LCA (lentil agglutinin derived from *Lens culinaris*), a pea lectin PSA (pea lectin derived from *Pisum sativum*), a broad bean lectin VFA (agglutinin derived from *Vicia faba*), an *Aleuria aurantia* lectin AAL (lectin derived from *Aleuria aurantia*) and the like.

[0408] Specifically, the clone of the present invention resistant to a lectin which recognizes a sugar chain structure in which 1-position of fucose is bound to 6-position of N-acetylglucosamine in the reducing end through α -bond in the NA-glycoside-linked sugar chain can be selected by culturing cells by using a medium comprising the lectin at a concentration of 1 μ g/ml to 1 mg/ml for 1 day to 2 weeks, preferably 1 day to 1 week, subculturing surviving cells or picking up a colony and transferring it into a culture vessel, and subsequently continuing the culturing using the lectin-containing medium.

[0409] The method for confirming that the cell is a lectin-resistant cell includes a method for confirming expression of the GDP-fucose synthase, α 1,6-fucose modifying enzyme or the GDP-fucose transport protein, a method for culturing the cell in a medium to which lectin is directly added. Specifically, when the expression amount of the mRNA of α 1,6-fucosyltransferase which is one of α 1,6-fucose modifying enzymes in the cell is measured, a lectin-resistant cell decreases in an amount of the mRNA expressed.

[0410] 2. Preparation of Transgenic Non-Human Animal or Plant or the Progenies

[0411] The cell used in the method of the present invention can be prepared by using a transgenic non-human animal or plant or the progenies thereof in which a genomic gene is modified in such a manner that the activity of the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein is decreased or deleted. The transgenic non-human animal or plant or the progenies thereof can be prepared by targeting a gene encoding the above protein according to the method similar to that in the item 1.

[0412] In a transgenic non-human animal, the embryonic stem cell used in the process of the present invention in which the activity of the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein is decreased or deleted can be prepared applying the method similar to that in the item 1 to an embryonic stem cell of the intended non-human animal such as cattle, sheep, goat, pig, horse, mouse, rat, fowl, monkey or rabbit.

[0413] Specifically, a mutant clone is prepared in which a gene encoding the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein is inactivated or substituted with any nucleotide sequence, by

a known homologous recombination technique [e.g., *Nature*, 326, 6110, 295 (1987); *Cell*, 51, 3, 503 (1987); etc.]. Using the prepared mutant clone, a chimeric individual comprising an embryonic stem cell clone and a normal cell can be prepared by an injection chimera method into blastocyst of fertilized egg of an animal or by an aggregation chimera method. The chimeric individual is crossed with a normal individual, so that a transgenic non-human animal in which the activity of the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein is decreased or deleted in the whole body cells can be obtained.

[0414] The target vector for the homologous recombination of the target gene can be prepared in accordance with a method described in *Gene Targeting, A Practical Approach*, IRL Press at Oxford University Press (1993); *Preparation of Mutant Mice using ES Cells*, or the like. The target vector can be used as any of a replacement type an insertion type and a gene trap type.

[0415] As the method for introducing the target vector into the embryonic stem cell, any method can be used, so long as it can introduce DNA into an animal cell. Examples include electroporation [*Cytotechnology*, 3, 133 (1990)], the calcium phosphate method (Japanese Published Unexamined Patent Application No. 227075/90), the lipofection method [*Proc. Natl. Acad. Sci. USA*, 84, 7413 (1987)], the injection method [*Manipulating the Mouse Embryo*, Second Edition], a method using particle gun (gene gun) (Japanese Patent No. 2606856, Japanese Patent No. 2517813), the DEAE-dextran method [*Biomanual Series 4—Gene Transfer and Expression Analysis* (Yodo-sha), edited by Takashi Yokota and Kenichi Arai (1994)], the virus vector method [*Manipulating a Mouse Embryo*, Second Edition] and the like.

[0416] The method for efficiently selecting a homologous recombinant includes a method such as the positive selection, promoter selection, negative selection or poly A selection described in *Gene Targeting, A Practical Approach*, IRL Press at Oxford University Press (1993), *Preparation of Mutant Mice using ES Cells*, or the like. Specifically, in the case of the target vector containing hprt gene, it is introduced into the hprt gene-defected embryonic stem cell, the embryonic stem cell is cultured in a medium containing aminopterin, hypoxanthine and thymidine, and positive selection which selects the homologous recombinant of the hprt gene can be carried out by selecting an a homogenous recombinant containing an aminopterin-resistant clone. In the case of the target vector containing a neomycin-resistant gene, the vector-introduced embryonic stem cell is cultured in a medium containing G418, and positive selection which selects a homogenous recombinant containing a neomycin-resistant gene can be carried out by selecting a G418-resistant clone. In the case of the target vector containing DT gene, the vector-introduced embryonic stem cell is cultured, and negative selection selecting a DT gene-free homogenous recombinant can be carried out by selecting the grown clone (in the recombinants introduced into a chromosome at random rather than the homogenous recombination, since the DT gene is expressed while integrated in the chromosome, the recombinants cannot grow because of the toxicity of DT). The method for selecting the homogenous recombinant of interest among the selected clones include the Southern hybridization for genomic DNA (*Molecular Cloning*, Second Edition), PCR [*PCR Protocols*, Academic Press (1990)] and the like.

[0417] When the embryonic stem cell is introduced into a fertilized egg by using an aggregation chimera method, in general, a fertilized egg at the development stage before 8-cell stage is preferably used. When the embryonic stem cell is introduced into a fertilized egg by using an injection chimera method, in general, it is preferred that a fertilized egg at the development stage from 8-cell stage to blastocyst stage is preferably used.

[0418] When the fertilized egg is transplanted into a female mouse, it is preferred that a fertilized egg obtained from a pseudopregnant female mouse in which fertility is induced by mating with a male non-human mammal which is subjected to vasoligation is artificially transplanted or implanted. Although the pseudopregnant female mouse can be obtained by natural mating, the pseudopregnant female mouse in which fertility is induced can be obtained by mating with a male mouse after administration of a luteinizing hormone-releasing hormone (hereinafter referred to as "LHRH") or its analogue thereof. The analogue of LHRH includes [3,5-Dil-Tyr5]-LHRH, [Gln8]-LHRH, [D-Ala6]-LHRH, des-Gly10-[D-His(Bzl)6]-LHRH ethylamide and the like.

[0419] Also, a fertilized egg cell of the present invention in which the activity of the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein is decreased or deleted can be prepared by applying the method similar to that in the item 1 to fertilized egg of a non-human animal of interest such as cattle, sheep, goat, pig, horse, mouse, rat, fowl, monkey, rabbit or the like.

[0420] A transgenic non-human animal. An which the activity of the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein is decreased or deleted can be prepared by transplanting the prepared fertilized egg cell into the oviduct or uterus of a pseudopregnant female using the embryo transplantation method described in *Manipulating Mouse Embryo*, Second Edition or the like, followed by childbirth by the animal.

[0421] In a transgenic plant, the callus of the present invention in which the activity of the GDP-fucose synthase or the activity of an enzyme relating to modification of a sugar chain in which 1-position of fucose is bound to 6- or 3-position of N-acetylglucosamine in the reducing end in the N-glycoside-linked complex sugar chain is decreased or deleted can be prepared by applying the method similar to that in the item 1 to a callus or cell of the plant of interest.

[0422] A transgenic plant in which the activity of the GDP-fucose synthase, the α 1,6-fucose modifying enzyme or the GDP-fucose transport protein is decreased or deleted can be prepared by culturing the prepared callus in a medium comprising auxin and cytokinin to redifferentiate it in accordance with conventional methods [*Tissue Culture (Soshiki Baiyo)*, 20 (1994); *Tissue Culture (Soshiki Baiyo)*, 21 (1995); *Trends in Biotechnology*, 15, 45 (1997)].

[0423] 3. Method for Producing Antibody Composition

[0424] The antibody composition can be obtained by expressing it in a host cell by using the methods described in *Molecular Cloning*, Second Edition; *Current Protocols in Molecular Biology*; *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988 (hereinafter sometimes referred to as "Antibodies"); *Monoclonal Antibodies: Principles and Practice*, Third Edition, Acad. Press, 1996 (here-

inafter sometimes referred to as "Monoclonal Antibodies"); and *Antibody Engineering, A Practical Approach*, IRL Press at Oxford University Press, 1996 (hereinafter sometimes referred to as "Antibody Engineering"), for example, as follows.

[0425] A full length cDNA of an antibody molecule is prepared, and a DNA fragment of an appropriate length comprising a DNA encoding the antibody molecule is prepared.

[0426] A recombinant vector is prepared by inserting the DNA fragment or the full length cDNA into downstream of the promoter of an appropriate expression vector.

[0427] A transformant which produces the antibody molecule can be obtained by introducing the recombinant vector into a host cell suitable for the expression vector.

[0428] As the host cell, the host cell of any cell such as yeast, an animal cell, an insect cell, a plant cell or the like can be used, so long as it can express the gene of interest.

[0429] A cell such as yeast, an animal cell, an insect cell, a plant cell or the like into which an enzyme relating to the modification of an N-glycoside-linked sugar chain which binds to the Fc region of the antibody molecule is introduced by a genetic engineering technique can also be used as the host cell.

[0430] As the expression vector, a vector which is autonomously replicable in the host cell or can be integrated into the chromosome and comprises a promoter at such a position that the DNA encoding the antibody molecule of interest can be transferred is used.

[0431] The cDNA can be prepared from a human or non-human tissue or cell by using a probe primer specific for the antibody molecule of interest and the like according to the methods described in "Preparation of DNA" in the item 1(1)(a).

[0432] When yeast is used as the host cell, the expression vector includes YEP13 (ATCC 37115), YEP24 (ATCC 37051), YCP50 (ATCC 37419) and the like.

[0433] As the promoter, any promoter can be used so long as it can function in yeast. Examples include a promoter of a gene relating to the glycolytic pathway such as a hexose kinase, PHOS promoter, PGK promoter, GAP promoter, ADH promoter, gal 1 promoter, gal 10 promoter, heat shock protein promoter, MF α 1 promoter, CUP 1 promoter and the like.

[0434] The host cell includes yeast belonging to the genus *Saccharomyces*, the genus *Schizosaccharomyces*, the genus *Kluyveromyces*, the genus *Trichosporon*, the genus *Schwanniomyces* and the like, such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Trichosporon pullulans* and *Schwanniomyces alluvius*.

[0435] As the method for introducing the recombinant vector, any method can be used, so long as it can introduce DNA into yeast. Examples include electroporation [*Methods in Enzymology*, 194, 182 (1990)], spheroplast method [*Proc. Natl. Acad. Sci. USA*, 84, 1929 (1978)], lithium acetate method [*J. Bacteriol.*, 153, 163 (1983)], a method described in *Proc. Natl. Acad. Sci. USA*, 75, 1929 (1978) and the like.

[0436] When an animal cell is used as the host cell, the expression vector includes pcDNA1, pcDM8 (available from Funakoshi), pAGE107 [Japanese Published Unexamined Patent Application No. 22979/91, *Cytotechnology*, 3, 133 (1990)], pAS3-3 (Japanese Published Unexamined Patent Application No. 227075/90), pCDM8 [*Nature*, 329, 840 (1987)], pcDNA1/Amp (manufactured by Invitrogen), pREP4 (manufactured by Invitrogen), pAGE103 [*J. Biochemistry*, 101, 1307 (1987)], pAGE210 and the like.

[0437] As the promoter, 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), an early promoter of SV40, a promoter of retrovirus, a promoter of metallothionein, a heat shock promoter, an SR α promoter and the like. Also, an enhancer of the IE gene of human CMV may be used together with the promoter.

[0438] The host cell includes a human cell such as Namalwa cell, a monkey cell such as COS cell, a Chinese hamster cell such as CHO cell or HBT5637 (Japanese Published Unexamined Patent Application No. 299/88), a rat myeloma cell, a mouse myeloma cell, a cell derived from Syrian hamster kidney, an embryonic stem cell, a fertilized egg cell and the like.

[0439] As the method for introducing the recombinant vector, any method can be used, so long as it can introduce DNA into an animal cell. Examples include electroporation [*Cytotechnology*, 3, 133 (1990)], the calcium phosphate method (Japanese Published Unexamined Patent Application No. 227075/90), the lipofection method [*Proc. Natl. Acad. Sci. USA*, 84, 7413 (1987)], the injection method [*Manipulating the Mouse Embryo, A Laboratory Manual*], a method using particle gun (gene gun) (Japanese Patent No. 2606856, Japanese Patent No. 2517813), the DEAE-dextran method [*Biomanual Series 4—Gene Transfer and Expression Analysis* (Yodo-sha), edited by Takashi Yokota and Kenichi Arai (1994)], the virus vector method [*Manipulating Mouse Embryo*, Second Edition] and the like.

[0440] When an insect cell is used as the host cell, the protein can be expressed by the method described in *Current Protocols in Molecular Biology, Baculovirus Expression Vectors, A Laboratory Manual*, W.H. Freeman and Company, New York (1992), *Bio/Technology*, 6, 47 (1988) or the like.

[0441] That is, the protein can be expressed by co-introducing a recombinant gene-introducing vector and a baculovirus into an insect cell to obtain a recombinant virus in an insect cell culture supernatant and then infecting the insect cell with the recombinant virus.

[0442] The gene introducing vector used in the method includes pVL1392, pVL1393, pBlueBacIII (all manufactured by Invitrogen) and the like.

[0443] The baculovirus includes *Autographa californica* nuclear polyhedrosis virus which is infected by an insect of the family Barathra.

[0444] The insect cell includes *Spodoptera frugiperda* oocytes Sf9 and Sf21 [*Current Protocols in Molecular Biology, Baculovirus Expression Vectors, A Laboratory Manual*, W.H. Freeman and Company, New York (1992)], a *Trichoplusia ni* oocyte High 5 (manufactured by Invitrogen) and the like.

[0445] The method for co-introducing the above recombinant gene-introducing vector and the baculovirus to the insect cells for preparing the above recombinant virus includes the calcium phosphate method (Japanese Published Unexamined Patent Application No. 227075/90), the lipofection method [*Proc. Natl. Acad. Sci. USA*, 84, 7413 (1987)] and the like.

[0446] When a plant cell is used as the host cell, the expression vector includes Ti plasmid, tobacco mosaic virus and the like.

[0447] As the promoter, any promoter can be used, so long as it can function in a plant cell. Examples include cauliflower mosaic virus (CaMV) 35S promoter, rice actin 1 promoter and the like.

[0448] The host cell includes plant cells of tobacco, potato, tomato, carrot, soybean, rape, alfalfa, rice, wheat, barley and the like.

[0449] As the method for introducing the recombinant vector, any method can be used, so long as it can introduce DNA into a plant cell. Examples include a method using *Agrobacterium* (Japanese Published Unexamined Patent Application No. 140885/84, Japanese Published Unexamined Patent Application No. 70080/85, WO94/00977), electroporation (Japanese Published Unexamined Patent Application No. 251887/85), a method using a particle gun (gene gun) (Japanese Patent No. 2606856, Japanese Patent No. 2517913) and the like.

[0450] As the method for expressing an antibody gene, secretion production, expression of a fusion protein of the Fc region with other protein and the like can be carried out in accordance with the method described in *Molecular Cloning*, Second Edition or the like, in addition to the direct expression.

[0451] When a gene is expressed by a microorganism, yeast, an animal cell, an insect cell or a plant cell into which a gene relating to the synthesis of a sugar chain is introduced, an antibody molecule to which a sugar or a sugar chain is added by the introduced gene can be obtained.

[0452] An antibody composition can be produced by culturing the obtained transformant in a medium to produce and accumulate the antibody molecule in the culture and then recovering it from the resulting culture. The method for culturing the transformant in a medium can be carried out in accordance with a general method which is used for the culturing of host cells.

[0453] As the medium for culturing a transformant obtained using yeast as the host cell, the medium may be either a natural medium or a synthetic medium, so long as it comprises materials such as a carbon source, a nitrogen source and an inorganic salt which can be assimilated by the organism and culturing of the transformant can be efficiently carried out.

[0454] As the carbon source, those which can be assimilated by the organism can be used. Examples include carbohydrates such as glucose, fructose, sucrose, molasses thereof, starch and starch hydrolysate; organic acids such as acetic acid and propionic acid, alcohols such as ethanol and propanol; and the like.

[0455] The nitrogen source includes ammonia; ammonium salts of inorganic acid or organic acid such as ammo-

nium chloride, ammonium sulfate, ammonium acetate and ammonium phosphate; other nitrogen-containing compounds; peptone; meat extract; yeast extract; corn steep liquor; casein hydrolysate; soybean meal; soybean meal hydrolysate; various fermented cells and hydrolysates thereof; and the like.

[0456] The inorganic salt includes potassium dihydrogen phosphate, dipotassium hydrogen phosphate, magnesium phosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, copper sulfate, calcium carbonate, and the like.

[0457] The culturing is carried out generally under aerobic conditions such as a shaking culture or submerged-aeration stirring culture. The culturing temperature is preferably at 15 to 40° C., and the culturing time is generally 16 hours to 7 days. During the culturing, the pH is maintained at 3.0 to 9.0. The pH is adjusted using an inorganic or organic acid, an alkali solution, urea, calcium carbonate, ammonia or the like.

[0458] Furthermore, if necessary, an antibiotic such as ampicillin or tetracycline can be added to the medium during the culturing.

[0459] When yeast transformed with a recombinant vector obtained by using an inducible promoter as the promoter is cultured, an inducer can be added to the medium, if necessary. For example, when yeast transformed with a recombinant vector obtained by using lac promoter is cultured, isopropyl- β -D-thiogalactopyranoside and the like can be added to the medium, and when yeast transformed with a recombinant vector obtained by using trp promoter is cultured, indoleacrylic acid and the like can be added to the medium.

[0460] When a transformant obtained by using an animal cell as the host is cultured, the medium includes generally used RPMI 1640 medium [*The Journal of the American Medical Association*, 199, 519 (1967)], Eagle's MEM medium [*Science* 122, 501 (1952)], Dulbecco's modified MEM medium [*Virology*, 8, 396 (1959)], 199 medium [*Proceeding of the Society for the Biological Medicine* 73, 1 (1950)] and Whitten's medium [*Developmental Engineering Experimentation Manual—Preparation of Transgenic Mice* (Kodan-sha), edited by M. Katsuki (1987)], the media to which fetal calf serum, etc. are added, and the like.

[0461] The culturing is carried out generally at conditions under pH of 6 to 8 and 30 to 40° C. for 1 to 7 days in the presence of 5% CO₂ and the like, for 1 to 7 days.

[0462] Furthermore, if necessary, an antibiotic such as kanamycin or penicillin can be added to the medium during the culturing.

[0463] The medium for culturing a transformant obtained by using an insect cell as the host includes generally used TNM-FH medium (manufactured by Pharmingen), Sf-900 II SFM medium (manufactured by Life Technologies), ExCell 400 and ExCell 405 (both manufactured by JRH Biosciences), Grace's Insect Medium [*Nature*, 195, 788 (1962)] and the like.

[0464] The culturing is carried out generally at conditions under pH of 6 to 7 and 25 to 30° C. and the like, for 1 to 5 days.

[0465] Furthermore, if necessary, an antibiotic such as gentamicin can be added to the medium during the culturing.

[0466] A transformant obtained by using a plant cell as the host can be cultured as a cell or by differentiating it into a plant cell or organ. The medium for culturing the transformant includes generally used Murashige and Skoog (MS) medium and White medium, the media to which a plant hormone such as auxin or cytokinin is added, and the like.

[0467] The culturing is carried out generally at conditions under pH of 5 to 9 and 20 to 40° C. for 3 to 60 days.

[0468] Furthermore, if necessary, an antibiotic such as kanamycin or hygromycin can be added to the medium during the culturing.

[0469] As described above, an antibody composition can be produced by culturing a transformant derived from yeast, an animal cell, an insect cell or a plant cell, which comprises a recombinant vector into which a DNA encoding an antibody molecule is inserted, in accordance with a general culturing method, to thereby produce and accumulate the antibody composition, and then recovering the antibody composition from the culture.

[0470] As the method for expressing the gene encoding an antibody, secretion production, expression of a fusion protein and the like can be carried out in accordance with the method described in *Molecular Cloning*, Second Edition in addition to the direct expression.

[0471] The method for producing an antibody composition includes a method of intracellular expression in a host cell, a method of extracellular secretion from a host cell, and a method of production on a host cell membrane outer envelope. The method can be selected by changing the host cell used or the structure of the antibody composition produced.

[0472] The method for producing an antibody composition includes a method of intracellular expression in a host cell, a method of extracellular secretion from a host cell, and a method of production on a host cell membrane outer envelope. The method can be selected by changing the host cell used or the structure of the antibody composition produced.

[0473] When the antibody composition is produced in a host cell or on a host cell membrane outer envelope, it can be positively secreted extracellularly in accordance with the method of Paulson et al. [*J. Biol. Chem.*, 264, 17619 (1989)], the method of Lowe et al. [*Proc. Natl. Acad. Sci. USA*, 86, 8227 (1989), *Genes Develop.*, 4, 1288 (1990)], the methods described in Japanese Published Unexamined Patent Application No. 336963/93 and Japanese Published Unexamined Patent Application No. 823021/94 and the like.

[0474] That is, an antibody molecule of interest can be positively secreted extracellularly from a host cell by inserting a DNA encoding the antibody molecule and a DNA encoding a signal peptide suitable for the expression of the antibody molecule into an expression vector according to a gene recombination technique and then expressing the antibody molecule.

[0475] Also, its production amount can be increased in accordance with the method described in Japanese Published

Unexamined Patent Application No. 227075/90 according to a gene amplification system using a dihydrofolate reductase gene.

[0476] In addition, the antibody composition can also be produced by using a gene-introduced animal individual (transgenic non-human animal) or a plant individual (transgenic plant) which is constructed by the redifferentiation of an animal or plant cell into which the gene is introduced.

[0477] When the transformant is an animal individual or a plant individual, an antibody composition can be produced in accordance with a general method by rearing or cultivating it to thereby produce and accumulate the antibody composition and then recovering the antibody composition from the animal or plant-individual.

[0478] The method for producing an antibody composition using an animal individual includes a method in which the antibody composition of interest is produced in an animal constructed by introducing a gene in accordance with a known method [*American Journal of Clinical Nutrition*, 63, 639S (1996); *American Journal of Clinical Nutrition*, 63, 627S (1996), *Bio/Technology*, 9, 830 (1991)].

[0479] In the case of an animal individual, an antibody composition can be produced, for example, by rearing a transgenic non-human animal into which a DNA encoding an antibody molecule is introduced to thereby produce and accumulate the antibody composition in the animal, and then recovering the antibody composition from the animal. The place of the animal where the composition is produced and accumulated includes milk (Japanese Published Unexamined Patent Application No. 309192/88) and eggs of the animal. As the promoter used in this case, any promoter can be used, so long as it can function in an animal. Preferred examples include mammary gland cell-specific promoters such as α casein promoter, β casein promoter, β lactoglobulin promoter, whey acidic protein promoter and the like.

[0480] The method for producing an antibody composition using a plant individual includes a method in which an antibody composition is produced by cultivating a transgenic plant into which a DNA encoding an antibody molecule is introduced by a known method [*Tissue Culture (Soshiki Baiyo)*, 20 (1994); *Tissue Culture (Soshiki Baiyo)*, 21 (1995); *Trends in Biotechnology*, 15, 45 (1997)] to produce and accumulate the antibody composition in the plant, and then recovering the antibody composition from the plant.

[0481] Regarding purification of an antibody composition produced by a transformant into which a gene encoding an antibody molecule is introduced, for example, when the antibody composition is intracellularly expressed in a dissolved state, the cells after culturing are recovered by centrifugation, suspended in an aqueous buffer and then disrupted by using ultrasonic oscillator, French press, Mantou Gaulin homogenizer, dynamill or the like to obtain a cell-free extract. A purified product of the antibody composition can be obtained from a supernatant obtained by centrifuging the cell-free extract according to a general enzyme isolation purification techniques such as solvent extraction; salting out or desalting with ammonium sulfate; precipitation with an organic solvent; anion exchange chromatography using a resin such as diethylaminoethyl (DEAE)-Sepharose or DIAION HPA-75 (manufactured by

Mitsubishi Chemical); cation exchange chromatography using a resin such as S-Sepharose FF (manufactured by Pharmacia), hydrophobic chromatography using a resin such as butyl-Sepharose or phenyl-Sepharose, gel filtration using a molecular sieve; affinity chromatography, chromatofocusing; electrophoresis such as isoelectric focusing, and the like which may be used alone or in combination.

[0482] Also, when the antibody composition is expressed intracellularly by forming an insoluble body, the cells are recovered, disrupted and centrifuged in the same manner, and the insoluble body of the antibody composition is recovered as a precipitation fraction. The recovered insoluble body of the antibody composition is solubilized by using a protein denaturing agent. The antibody composition is made into a normal three-dimensional structure by diluting or dialyzing the solubilized solution, and then a purified product of the antibody composition is obtained by the same isolation purification method.

[0483] When the antibody composition is secreted extracellularly, the antibody composition or derivatives thereof can be recovered from the culture supernatant. That is, the culture is treated according to a technique such as centrifugation as described above to obtain a soluble fraction, and a purified preparation of the antibody composition can be obtained from the soluble fraction by the same isolation purification method as described above.

[0484] The thus obtained antibody composition includes an antibody, the fragment of the antibody, a fusion protein comprising the Fc region of the antibody, and the like.

[0485] As an example for obtaining the antibody composition, a method for producing a composition of a humanized antibody and Fc fusion protein is described below in detail, but other antibody compositions can also be obtained in a manner similar to the method.

[0486] A. Preparation of Humanized Antibody Composition

[0487] (1) Construction of Vector for Expression of Humanized Antibody

[0488] A vector for expression of humanized antibody is an expression vector for animal cell into which genes encoding CH and CL of a human antibody are inserted, which can be constructed by cloning each of genes encoding CH and CL of a human antibody into an expression vector for animal cell.

[0489] The C regions of a human antibody may be CH and CL of any human antibody. Examples include the C region belonging to IgG1 subclass in the H chain of a human antibody (hereinafter referred to as "hCy1"), the C region belonging to κ class in the L chain of a human antibody (hereinafter referred to as "hCk"), and the like.

[0490] As the genes encoding CH and CL of a human antibody, a chromosomal DNA comprising an exon and an intron can be used, and a cDNA can also be used.

[0491] As the expression vector for animal cell, any vector can be, used, so long as a gene encoding the C region of a human antibody can be inserted therein and expressed therein. Examples include pAGE107 [*Cytotechnology*, 3, 133 (1990)], pAGE103 [*J. Biochem.*, 101, 1307 (1987)], pHSG274 [*Gene*, 27, 223 (1984)], pKCR [*Proc. Natl. Acad.*

Sci. USA, 78, 1527 (1981), pSG1 β d2-4 [*Cytotechnology*, 4, 173 (1990)] and the like. The promoter and enhancer in the expression vector for animal cell includes SV40 early promoter and enhancer [*J. Biochem.*, 101, 1307 (1987)], Moloney mouse leukemia virus LTR promoter [*Biochem. Biophys. Res. Commun.*, 149, 960 (1987)], immunoglobulin H chain promoter [*Cell*, 41, 479 (1985)] and enhancer [*Cell*, 33, 717 (1983)], and the like.

[0492] The vector for expression of humanized antibody may be either of a type in which genes encoding the H chain and L chain of an antibody exist on separate vectors or of a type in which both genes exist on the same vector (hereinafter referred to "tandem type"). In respect of easiness of construction of a vector for expression of humanized antibody, easiness of introduction into animal cells, and balance between the expression amounts of the H and L chains consisting of an antibody in animal cells, a tandem type of the vector for humanized antibody expression is more preferred [*J. Immunol. Methods*, 167, 271 (1994)].

[0493] The constructed vector for expression of humanized antibody can be used for expression of a human chimeric antibody and a human CDR-grafted antibody in animal cells.

[0494] (2) Preparation Method of cDNA Encoding V Region of Non-Human Animal Antibody

[0495] cDNAs encoding VH and VL of a non-human animal antibody such as mouse antibody can be obtained in the following manner.

[0496] A cDNA is synthesized from mRNA extracted from a hybridoma cell which produces the mouse antibody of interest. The synthesized cDNA is cloned into a vector such as a phage or a plasmid to obtain a cDNA library. Each of a recombinant phage or recombinant plasmid comprising a cDNA encoding VH and a recombinant phage or recombinant plasmid comprising a cDNA encoding VL is isolated from the library by using a C region part or a V region part of an existing mouse antibody as the probe. Full nucleotide sequences of VH and VL of the mouse antibody of interest on the recombinant phage or recombinant plasmid are determined, and full length amino acid sequences of VH and VL are deduced from the nucleotide sequences.

[0497] As the non-human animal, any animal such as mouse, rat, hamster or rabbit can be used, so long as a hybridoma cell can be produced therefrom.

[0498] The method for preparing a total RNA from a hybridoma cell includes the guanidine thiocyanate-caesium trifluoroacetate method [*Methods in Enzymology*, 154, 3 (1987)] and the like, and the method for preparing mRNA from total RNA includes an oligo(dT)-immobilized cellulose column method (*Molecular Cloning*, Second Edition) and the like. In addition, a kit for preparing mRNA from a hybridoma cell includes Fast Track mRNA Isolation Kit (manufactured by Invitrogen), Quick Prep mRNA Purification Kit (manufactured by Pharmacia) and the like.

[0499] The method for synthesizing a cDNA and preparing a cDNA library includes the usual methods (*Molecular Cloning*, Second Edition, *Current Protocols in Molecular Biology*, Supplement 1-34), methods using a commercially available kit such as SuperScript™, Plasmid System for

cDNA Synthesis and Plasmid Cloning (manufactured by GIBCO BRL) or ZAP-cDNA Synthesis Kit (manufactured by Stratagene), and the like.

[0500] In preparing the cDNA library, the vector into which a cDNA synthesized by using mRNA extracted from a hybridoma cell as the template is inserted may be any vector, so long as the cDNA can be inserted. Examples include ZAP Express [*Strategies*, 5, 58 (1992)], pBluescript II SK(+) [*Nucleic Acids Research*, 17, 9494 (1989)], λ zapII (manufactured by Stratagene), λ gt10 and λ gt11 [*DNA Cloning, A Practical Approach*, I, 49 (1985)], Lambda BlueMid (manufactured by Clontech), λ ExCell, pT7T318U (manufactured by Pharmacia), pcD2 [*Mol. Cell. Biol.*, 3, 280 (1983)], pUC18 [*Gene*, 33, 103 (1985)] and the like.

[0501] As *Escherichia coli* into which the cDNA library constructed from a phage or plasmid vector is introduced, any *Escherichia coli* can be used, so long as the cDNA library can be introduced, expressed and maintained. Examples include XL1-Blue MRF [*Strategies*, 5, 81 (1992)], C600 [*Genetics*, 39, 440 (1954)], Y1088 and Y1090 [*Science*, 222, 778 (1983)], NM522 [*J. Mol. Biol.*, 166, 1 (1983)], K802 [*J. Mol. Biol.*, 16, 118 (1966)], JM105 [*Gene*, 38, 275 (1985)] and the like.

[0502] As the method for selecting a cDNA clone encoding VH and VL of a non-human animal antibody from the cDNA library, a colony hybridization or a plaque hybridization using an isotope- or fluorescence-labeled probe can be used (*Molecular Cloning*, Second Edition). The cDNA encoding VH and VL can also be prepared by preparing primers and carrying out polymerase chain reaction (hereinafter referred to as "PCR"; *Molecular Cloning*, Second Edition *Current Protocols in Molecular Biology*, Supplement 1-34) using a cDNA synthesized from mRNA or a cDNA library as the template.

[0503] The nucleotide sequences of the cDNAs can be determined by digesting the selected cDNAs with appropriate restriction enzymes, cloning the fragments into a plasmid such as pBluescript SK(-) (manufactured by Stratagene), carrying out the reaction of a generally used nucleotide sequence analyzing method such as the dideoxy method of Sanger et al. [*Proc. Natl. Acad. Sci. USA*, 74, 5463 (1977)], and then analyzing the clones using an automatic nucleotide sequence analyzer such as A.L.F. DNA Sequencer (manufactured by Pharmacia).

[0504] Whether or not the obtained cDNAs encode the full length amino acid sequences of VH and VL of the antibody comprising a secretory signal sequence can be confirmed by deducing the full length amino acid sequences of VH and V from the determined nucleotide sequence and comparing them with the full length amino acid sequences of VH and VL of known antibodies [*Sequences of Proteins of Immunological Interest*, US Dep. Health and Human Services (1991), hereinafter referred to as "*Sequences of Proteins of Immunological Interest*"].

[0505] (3) Analysis of Amino Acid Sequence of V Region of Non-Human Animal Antibody

[0506] Regarding the full length amino acid sequences of VH and VL of the antibody comprising a secretory signal sequence, the length of the secretory signal sequence and the N-terminal amino acid sequences can be deduced and subgroups to which they belong can also be found, by compar-

ing them with the full length amino acid sequences of VH and VL of known antibodies (*Sequences of Proteins of Immunological Interest*). In addition, the amino acid sequences of each CDR of VH and VL can also be found by comparing them with the amino acid sequences of VH and VL of known antibodies (*Sequences of Proteins of Immunological Interest*).

[0507] (4) Construction of Human Chimeric Antibody Expression Vector

[0508] A human chimeric antibody expression vector can be constructed by cloning 4 cDNAs encoding VH and VL of a non-human animal antibody into upstream of genes encoding CH and CL of a human antibody in the vector for expression of humanized antibody constructed described in the item 3(1). For example, a human chimeric antibody expression vector can be constructed by linking each of cDNAs encoding VH and VL of a non-human animal antibody to a synthetic DNA comprising nucleotide sequences at the 3'-terminals of VH and VL of a non-human animal antibody and nucleotide sequences at the 5'-terminals of CH and CL of a human antibody and also having a recognition sequence of an appropriate restriction enzyme at both terminals, and by cloning them into upstream of genes encoding CH and CL of a human antibody contained in the vector for expression of humanized antibody constructed described in the item 3(1) in such a manner that they can be expressed in a suitable form.

[0509] (5) Construction of cDNA Encoding V Region of Human CDR-Grafted Antibody

[0510] cDNAs encoding VH and VL of a human CDR-grafted antibody can be obtained as follows. First, amino acid sequences of the frameworks (hereinafter referred to as "FR") of VH and VL of a human antibody for grafting CDR of VH and VL of a non-human animal antibody is selected. As the amino acid sequences of FRs of VH and VL of a human antibody, any amino acid sequences can be used so long as they are derived from a human antibody. Examples include amino acid sequences of FRs of VH and VL of human antibodies registered at databases such as Protein Data Bank, amino acid sequences common in each subgroup of FRs of VH and VL of human antibodies (*Sequences of Proteins of Immunological Interest*) and the like. In order to produce a human CDR-grafted antibody having enough activities, it is preferred to select an amino acid sequence having homology as high as possible (at least 60% or more) with amino acid sequences of VH and VL of a non-human animal antibody of interest.

[0511] Next, the amino acid sequences of CDRs of VH and VL of the non-human animal antibody of interest are grafted to the selected amino acid sequences of FRs of VH and VL of a human antibody to design amino acid sequences of VH and VL of the human CDR-grafted antibody. The designed amino acid sequences are converted into DNA sequences by considering the frequency of codon usage found in nucleotide sequences of antibody genes (*Sequences of Proteins of Immunological Interest*), and the DNA sequences encoding the amino acid sequences of VH and VL of the human CDR-grafted antibody are designed. Based on the designed DNA sequences, several synthetic DNAs having a length of about 100 bases are synthesized, and PCR is carried out by using them. In this case, it is preferred in each of the H chain and the L chain that 6 synthetic DNAs are

designed in view of the reaction efficiency of PCR and the lengths of DNAs which can be synthesized.

[0512] Also, they can be easily cloned into the vector for expression of humanized antibody described in the item 3(1) by introducing recognition sequences of an appropriate restriction enzyme into the 5'-terminals of the synthetic DNA on both terminals. After the PCR, the amplified product is cloned into a plasmid such as pBluescript SK(-) (manufactured by Stratagene) and the nucleotide sequences are determined by the method in the item 3(2) to thereby obtain a plasmid having DNA sequences encoding the amino acid sequences of VH and VL of the desired human CDR-grafted antibody.

[0513] (6) Construction of Human CDR-Grafted Antibody Expression Vector

[0514] A human CDR-grafted antibody expression vector can be constructed by cloning the cDNAs encoding VH and VL of the human CDR-grafted antibody constructed in the item 3(5) into upstream of the gene encoding CH and CL of a human antibody in the vector for expression of humanized antibody described in the item 3(1). For example, recognizing sequences of an appropriate restriction enzyme are introduced into the 5'-terminals of both terminals of a synthetic DNA fragment, among the synthetic DNA fragments which are used in the item 3(5) for constructing the VH and VL of the human CDR-grafted antibody, so that they are cloned into upstream of the genes encoding CH and CL of a human antibody in the vector for expression of humanized antibody described in the item 3(1) in such a manner that they can be expressed in a suitable form, to thereby construct the human CDR-grafted antibody expression vector.

[0515] (7) Stable Production of Humanized Antibody

[0516] A transformant capable of stably producing a human chimeric antibody and a human CDR-grafted antibody (both hereinafter referred to as "humanized antibody") can be obtained by introducing the vectors for humanized antibody expression described in the items 3(4) and (6) into an appropriate animal cell.

[0517] The method for introducing a humanized antibody expression vector into an animal cell includes electroporation [Japanese Published Unexamined Patent Application No. 257891/90, *Cytotechnology*, 3, 133 (1990)] and the like.

[0518] As the animal cell into which a humanized antibody expression vector is introduced, any cell can be used so long as it is an animal cell which can produce the humanized antibody.

[0519] Examples include mouse myeloma cells such as NS0 cell and SP2/0 cell, Chinese hamster ovary cells such as CHO/dhfr⁻ cell and CHO/DG44 cell, rat myeloma such as YB2/0 cell and IR983F cell, BHK cell derived from a syrian hamster kidney, a human myeloma cell such as Namalwa cell, and the like, and a Chinese hamster ovary cell CHO/DG44 cell, a rat myeloma YB2/0 cell and the host cells of the present invention described in the item 5 are preferred.

[0520] After introduction of the humanized antibody expression vector, a transformant capable of stably producing the humanized antibody can be selected by using a medium for animal cell culture comprising an agent such as G418 sulfate (hereinafter referred to as "G418"; manufac-

tured by SIGMA) and the like in accordance with the method described in Japanese Published Unexamined Patent Application No. 257891/90. The medium to culture animal cells includes RPMI 1640 medium (manufactured by Nissui Pharmaceutical), GIT medium (manufactured by Nihon Pharmaceutical), EX-CELL 302 medium (manufactured by JRH), IMDM medium (manufactured by GIBCO BRL), Hybridoma-SFM medium (manufactured by GIBCO BRL), media obtained by adding various additives such as fetal bovine serum (hereinafter referred to as "FBS") to these media, and the like. The humanized antibody can be produced and accumulated in the culture supernatant by culturing the obtained transformant in a medium. The amount of production and antigen binding activity of the humanized antibody in the culture supernatant can be measured by a method such as enzyme-linked immunosorbent assay (hereinafter referred to as "ELISA"; *Antibodies, Monoclonal Antibodies*) or the like. Also, the amount of the humanized antibody produced by the transformant can be increased by using a DHFR gene amplification system in accordance with the method described in Japanese Published Unexamined Patent Application No. 257891/90.

[0521] The humanized antibody can be purified from a culture supernatant culturing the transformant by using a protein A column (*Antibodies*, Chapter 8; *Monoclonal Antibodies*). In addition, purification methods generally used for the purification of proteins can also be used. For example, the purification can be carried out through the combination of gel filtration, ion exchange chromatography and ultrafiltration. The molecular weight of the H chain, L chain or antibody molecule as a whole of the purified humanized antibody can be measured, e.g., by polyacrylamide gel electrophoresis [hereinafter referred to as "SDS-PAGE", *Nature*, 227, 680 (1970)], Western blotting (*Antibodies, Monoclonal Antibodies*) or the like.

[0522] B. Preparation of Fc Fusion Protein

[0523] (1) Construction of Fc Fusion Protein Expression Vector

[0524] An Fc fusion protein expression vector is an expression vector for animal cells into which genes encoding the Fc region of a human antibody and a protein to be fused are inserted, which can be constructed by cloning each of genes encoding the Fc region of a human antibody and the protein to be fused into an expression vector for animal cell.

[0525] The Fc region of a human antibody includes those containing a part of a hinge region and/or CH1 in addition to regions containing CH2 and CH3 regions. Also, it can be any Fc region, so long as at least one amino acid of CH2 or CH3 may be deleted, substituted, added or inserted, and substantially has the binding activity to the Fc γ receptor.

[0526] As the genes encoding the Fc region of a human antibody and the protein to be fused, a chromosomal DNA comprising an exon and an intron can be used, and a cDNA can also be used. The method for linking the genes and the Fc region includes PCR using each of the gene sequences as the template (*Molecular Cloning*, Second Edition, *Current Protocols in Molecular Biology*, Supplement 1-34).

[0527] As the expression vector for animal cell, any vector can be used, so long as a gene encoding the C region of a human antibody can be inserted thereinto and expressed therein. Examples include pAGE107 [*Cytotechnology*, 3,

133 (1990)], pAGE103 [*J. Biochem.*, 101, 1307 (1987)], pHSG274 [*Gene*, 27, 223 (1984)], pKCR [*Proc. Natl. Acad. Sci. USA* 78, 1527 (1981)], pSG1 β d2-4 [*Cytotechnology*, 4, 173 (1990)] and the like. The promoter and enhancer in the expression vector for animal cell include SV40 early promoter and enhancer a [*J. Biochem.*, 101, 1307 (1987)], Moloney mouse leukemia virus LTR [*Biochem. Biophys. Res. Commun.*, 149, 960 (1987)], immunoglobulin H chain promoter [*Cell*, 41, 479 (1985)] and enhancer [*Cell*, 33, 717 (1983)], and the like.

[0528] (2) Preparation of DNA Encoding Fc Region of Human Antibody and Protein to be Fused

[0529] A DNA encoding the Fc region of a human antibody and the protein to be fused can be obtained in the following manner.

[0530] A cDNA is synthesized from mRNA extracted from a cell or tissue which expresses the protein of interest to be fused with Fc. The synthesized cDNA is cloned into a vector such as a phage or a plasmid to obtain a cDNA library. A recombinant or recombinant plasmid comprising cDNA encoding the protein of interest is isolated from the library using the gene sequence part of the protein of interest as the probe. A full nucleotide sequence of the antibody of interest on the recombinant phage or recombinant plasmid is determined, and a full length amino acid sequence is deduced from the nucleotide sequence.

[0531] As the non-human animal, any animal such as mouse, rat, hamster or rabbit can be used, so long as a cell or tissue can be removed therefrom.

[0532] The method for preparing a total RNA from a cell or tissue includes the guanidine thiocyanate-cesium trifluoroacetate method [*Method in Enzymology*, 154, 3 (1987)] and the like, and the method for preparing mRNA from total A includes an oligo (dT)-immobilized cellulose column method (*Molecular Cloning*, Second Edition) and the like. In addition, a kit for preparing mRNA from a cell or tissue includes Fast Track mRNA Isolation Kit (manufactured by Invitrogen), Quick Prep mRNA Purification Kit (manufactured by Pharmacia) and the like.

[0533] The method for synthesizing a cDNA and preparing a cDNA library includes the usual methods (*Molecular Cloning*, Second Edition; *Current Protocols in Molecular Biology*, Supplement 1-34); methods using a commercially available kit such as SuperScriptTM, Plasmid System for cDNA Synthesis and Plasmid Cloning (manufactured by GIBCO BRL) or ZAP-cDNA Synthesis Kit (manufactured by Stratagene); and the like.

[0534] In preparing the cDNA library, the vector into which a cDNA synthesized using mRNA extracted from a cell or tissue as the template is inserted may be any vector so long as the DNA can be inserted. Examples include ZAP Express [*Strategies*, 5, 58 (1992)], pBluescript II SK(+) [*Nucleic Acids Research*, 17, 9494 (1989)], λ zapII (manufactured by Stratagene), λ gt10 and λ gt11 [*DNA Cloning, A Practical Approach*, I, 49 (1985)], Lambda BlueMid (manufactured by Clontech), λ ExCell, pT7T318U (manufactured by Pharmacia), pcD2 [*Mol. Cell. Biol.*, 3, 280 (1983)], pUC18 [*Gene*, 33, 103 (1985)] and the like.

[0535] As *Escherichia coli* into which the cDNA library constructed from a phage or plasmid vector is introduced,

any *Escherichia coli* can be used, so long as the cDNA library can be introduced, expressed and maintained. Examples include XL1-Blue MRF [*Strategies*, 5, 81 (1992)], C600 [*Genetics*, 39, 440 (1954)], Y1088 and Y1090 [*Science*, 222, 778 (1983)], NM522 [*J. Mol. Biol.*, 166, 1 (1983)], K802 [*J. Mol. Biol.* 16, 118 (1966)], JM105 [*Gene*, 38, 275 (1985)] and the like.

[0536] As the method for selecting a cDNA clone encoding the protein of interest from the cDNA library, a colony hybridization or a plaque hybridization using an isotope- or fluorescence-labeled probe can be used (*Molecular Cloning*, Second Edition). The cDNA encoding the protein of interest can also be prepared by preparing primers and using a cDNA synthesized from mRNA or a cDNA library as the template according to PCR.

[0537] The method for fusing the protein of interest with the Fc region of a human antibody includes PCR. For example, synthesized oligo DNAs (primers) are designed at the 5'-terminal and 3'-terminal of the gene sequence encoding the protein of interest, and PCR is carried out to prepare a PCR product. In the same manner, primers are designed for the gene sequence encoding the Fc region of a human antibody to be fused to prepare a PCR product. At this time, the primers are designed in such a manner that the same restriction enzyme site or the same gene sequence is present between the 3'-terminal of the PCR product of the protein to be fused and the 5'-terminal of the PCR product of the Fc region. When it is necessary to modify the amino acids around the linked site, mutation is introduced by using the primer into which the mutation is introduced. PCR is further carried out by using the two kinds of the obtained PCR fragments to link the genes. Also, they can be linked by carrying out ligation after treatment with the same restriction enzyme.

[0538] The nucleotide sequence of the DNA can be determined by digesting the gene sequence linked by the above method with appropriate restriction enzymes, cloning the fragments into a plasmid such as pBluescript SK(-) (manufactured by Stratagene), carrying out analysis by using a generally used nucleotide sequence analyzing method such as the dideoxy method of Sanger et al. [*Proc. Natl. Acad. Sci. USA*, 74, 5463 (1977)] or an automatic nucleotide sequence analyzer such as ABI PRISM 377 DNA Sequencer (manufactured by Pharmacia).

[0539] Whether or not the obtained cDNA encodes the full length amino acid sequences of the Fc fusion protein containing a secretory signal sequence can be confirmed by deducing the full length amino acid sequence of the Fc fusion protein from the determined nucleotide sequence and comparing it with the amino acid sequence of interest.

[0540] (3) Stable Production of Fc Fusion Protein

[0541] A transformant capable of stably producing an Fc fusion protein can be obtained by introducing the Fc fusion protein expression vector described in the item (1) into an appropriate animal cell.

[0542] The method for introducing the Fc fusion protein expression vector into an animal cell include electroporation [Japanese Published Unexamined Patent Application No. 257891/90, *Cytotechnology*, 3, 133 (1990)] and the like.

[0543] As the animal cell into which the Fc fusion protein expression vector is introduced, any cell can be used, so long as it is an animal cell which can produce the Fc fusion protein.

[0544] Examples include mouse myeloma cells such as NS0 cell and SP2/0 cell, Chinese hamster ovary cells such as CHO/dhfr⁻ cell and CHO/D44 cell, rat myeloma such as YB2/0 cell and IR983F cell, BHK cell derived from a syrian hamster kidney, a human myeloma cell such as Namalwa cell, and the like, and preferred are a Chinese hamster ovary cell CHO/DG44 cell, a rat myeloma YB2/0 cell and the host cells used in the method of the present invention described in the item 1.

[0545] After introduction of the Fc fusion protein expression vector, a transformant capable of stably producing the Fc fusion protein expression vector can be selected by using a medium for animal cell culture comprising an agent such as G418 and the like in accordance with the method described in Japanese Published Unexamined Patent Application No. 257891/90. The medium to culture animal cells includes RPMI 1640 medium (manufactured by Nissui Pharmaceutical), GIT medium (manufactured by Nihon Pharmaceutical), EX-CELL 302 medium (manufactured by JRH), IMDM medium (manufactured by GIBCO BRL), Hybridoma-SFM medium (manufactured by GIBCO BRL), media obtained by adding various additives such as fetal bovine serum to these media, and the like. The Fc fusion protein can be produced and accumulated in the culture medium by culturing the obtained transformant in a medium. The production amount and antigen binding activity of the Fc fusion protein in the culture medium can be measured by a method such as ELISA. Also, the amount of the Fc fusion protein produced by the transformant can be increased by using a dhfr gene amplification system in accordance with the method described in Japanese Published Unexamined Patent Application No. 257891/90.

[0546] The Fc fusion protein can be purified from a culture supernatant culturing the transformant using a protein A column (*Antibodies*, Chapter 8, *Monoclonal Antibodies*). In addition, purification methods generally used for purifying proteins can also be used. For example, the purification can be carried out through the combination of a gel filtration, an ion exchange chromatography and an ultrafiltration. The molecular weight as a whole of the purified Fc fusion protein molecule can be measured by SDS-PAGE [*Nature*, 227, 680 (1970)], Western blotting (*Antibodies*, Chapter 12; *Monoclonal Antibodies*) or the like.

[0547] Thus, methods for producing an antibody composition using an animal cell as the host cell have been described, but, as described above, it can also be produced by yeast, an insect cell, a plant cell, an animal individual or a plant individual by the same methods on the animal cell.

[0548] When the host cell is capable of preparing the antibody molecule, the antibody composition of the present invention can be prepared by culturing the cell capable of expressing an antibody molecule according to the method described in the above item 1, culturing the cell, and recovering the antibody composition of interest.

[0549] 4. Measurement of Binding Activity to Human FcγRIIIa

[0550] Binding activity of the antibody composition to FcγRIIIa can be measured by the following technique.

[0551] (1) Preparation of Human FcγRIIIa

[0552] FcγRIIIa which can be used includes FcγRIIIa present on the cell surface of peripheral blood lymphocyte of a human or non-human animal, FcγRIIIa obtained by preparing a gene encoding FcγRIIIa and introducing the gene into a host cell and expressing the FcγR on the cell surface, FcγRIIIa secreted from the cell, and the like.

[0553] A method for preparing a gene encoding FcγRIIIa, introducing the gene into a host cell and expressing the FcγRIIIa on the cell surface, and a method obtaining FcγRIIIa by secreting it from the cell are described below.

[0554] A total RNA or mRNA is prepared from human or non-human animal tissues or cells.

[0555] A commercially available product (e.g., manufactured by Clontech) can be used as the mRNA of human or non-human animal tissues or cells, or it may be prepared from human or non-human animal tissues or cells as follows. The method for preparing a total RNA from human or non-human animal tissues or cells includes the guanidine thiocyanate-cesium trifluoroacetate method [*Methods in Enzymology*, 154, 3 (1987)], the acidic guanidine thiocyanate phenol chloroform (AGPC) method [*Analytical Biochemistry*, 162, 156 (1987); *Experimental Medicine (Jikken Igaku)*, 9, 1937 (1991)] and the like.

[0556] Also, the method for preparing mRNA as poly(A)⁺ RNA from a total RNA includes an oligo(dT)-immobilized cellulose column method (*Molecular Cloning*, Second Edition) and the like.

[0557] In addition, mRNA can be prepared by using a kit such as Fast Track mRNA Isolation Kit (manufactured by Invitrogen) or Quick Prep mRNA Purification Kit (manufactured by Pharmacia).

[0558] A cDNA library is prepared from a full mRNA or mRNA of the prepared human or non-human animal tissue or cell.

[0559] The method for preparing a cDNA library include methods described in *Molecular Cloning*, Second Edition, *Current Protocols in Molecular Biology*, and the like, methods using a commercially available kit such as SuperScript Plasmid System for cDNA Synthesis and Plasmid Cloning (manufactured by Life Technologies) or ZAP-cDNA Synthesis Kit (manufactured by STRATAGENE), and the like.

[0560] As the cloning vector for the preparation of the cDNA library, any vector such as a phage vector or a plasmid vector can be used, so long as it is autonomously replicable in *Escherichia coli* K12. Examples include ZAP Express (manufactured by STRATAGENE, *Strategies*, 5, 58 (1992)), pBluescript II SK(+) [*Nucleic Acids Research*, 17, 9494 (1989)], Lambda ZAP II (manufactured by STRATAGENE), λgt10 and λgt11 [*DNA Cloning, A Practical Approach*, 1, 49 (1985)], λTriplEx (manufactured by Clontech), λExCell (manufactured by Pharmacia), pcD2 [*Mol. Cell. Biol.*, 3, 280 (1983)], pUC18 [*Gene*, 33, 103 (1985)] and the like.

[0561] Any microorganism can be used as the host microorganism, and *Escherichia coli* is preferably used. Examples include *Escherichia coli* XL1-Blue MRF⁺ [manufactured by STRATAGENE, *Strategies*, 5, 81 (1992)], *Escherichia coli* C600 [*Genetics*, 39, 440 (1954)], *Escherichia coli* Y1088

[*Science*, 222, 778 (1983)], *Escherichia coli* Y1090 [*Science*, 222, 778 (1983)], *Escherichia coli* NM522 [*J. Mol. Biol.* 166, 1 (1983)], *Escherichia coli* K802 [*J. Mol. Biol.* 16, 118 (1966)], *Escherichia coli* JM105 [*Gene*, 38, 275 (1985)] and the like.

[0562] The cDNA library may be used as such in the succeeding analysis, and in order to obtain a full length cDNA as efficient as possible by decreasing the ratio of an infull length cDNA, a cDNA library prepared using the oligo cap method developed by Sugano et al. [*Gene*, 138, 171 (1994); *Gene*, 200, 149 (1997), *Protein, Nucleic Acid and Enzyme*, 41, 603 (1996); *Experimental Medicine*, 11, 2491 (1993); *cDNA Cloning* (Yodo-sha) (1996); *Methods for Preparing Gene Libraries* (Yodo-sha) (1994)] may be used in the following analysis.

[0563] A gene encoding FcγR can be obtained by preparing primers specific for 5'-terminal and 3'-terminal nucleotide sequences based on the nucleotide sequences of various FcγRIIIa, and amplifying DNA by PCR [*PCR Protocols*, Academic Press (1990)] using a prepared cDNA library as the template.

[0564] Whether the thus obtained gene is a DNA encoding FcγRIIIa can be confirmed by analyzing it according to the generally used nucleotide sequence analyzing method such as the dideoxy method of Sanger et al. [*Proc. Natl. Acad. Sci. U.S.A.*, 74, 5463 (1977)] or by using a nucleotide sequence analyzer such as ABI PRISM 377 DNA Sequencer (manufactured by PE Biosystems).

[0565] The nucleotide sequence of a gene encoding FcγRIIIa obtained by the above method includes the nucleotide sequence of FcγRIIIa represented by SEQ ID NO:27.

[0566] The gene encoding FcγRIIIa can also be obtained based on the determined DNA nucleotide sequence by carrying out chemical synthesis by a DNA synthesizer such as DNA Synthesizer Model 392 manufactured by Perkin Elmer using a phosphoramidite method.

[0567] A recombinant vector is prepared by inserting the thus obtained cDNA encoding FcγRIIIa into downstream of the promoter of an appropriate expression vector.

[0568] A transformant which produces an antibody molecule can be obtained by introducing the recombinant vector into a host cell suitable for the expression vector.

[0569] As the host cell, any of yeast, an animal cell, an insect cell, a plant cell or the like can be used, so long as it can express the gene of interest.

[0570] As the expression vector, a vector which is autonomously replicable in the above host cell or can be integrated into the chromosome and comprises a promoter at such a position that the DNA encoding the FcγRIIIa of interest can be transferred is used.

[0571] When a yeast is used as the host cell, the expression vector includes YEP13 (ATCC 37115), YEP24 (ATCC 37051), YCP50 (ATCC 37419) and the like.

[0572] Any promoter can be used, so long as it can function in yeast. Examples include a promoter of a gene of the glycolytic pathway such as a hexose kinase gene, PHOS promoter, PGK promoter, GAP promoter, ADH promoter, gal 1 promoter, gal 10 promoter, heat shock protein promoter, MF α1 promoter, CUP 1 promoter and the like.

[0573] The host cell includes microorganisms belonging to the genus *Saccharomyces*, the genus *Schizosaccharomyces*, the genus *Kluyveromyces*, the genus *Trichosporon*, the genus *Schwanniomyces* and the like, such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Trichosporon pullulans* and *Schwanniomyces alluvius*.

[0574] As the method for introducing the recombinant vector, any method can be used, so long as it can introduce DNA into yeast. Examples include electroporation [*Method in Enzymology*, 194, 182 (1990)], spheroplast method [*Proc. Natl. Acad. Sci. USA*, 84, 1929 (1978)], lithium acetate method [*J. Bacteriol.*, 153, 163 (1983)], a method described in *Proc. Natl. Acad. Sci. USA*, 75, 1929 (1978) and the like.

[0575] When an animal cell is used as the host, the expression vector includes pcDNA1, pcDM8 (available from Funakoshi), pAGE107 Japanese Published Unexamined Patent Application No. 22979/91; *Cytotechnology*, 3, 133 (1990)], pAS3-3, (Japanese Published Unexamined Patent Application No. 227075/90), pCDM8 [*Nature*, 329-840 (1987)], pcDNA1/Amp (manufactured by Invitrogen), pREP4 (manufactured by Invitrogen), pAGE103 [*J. Biochemistry*, 101, 1307 (1987)], pAGE210 and the like.

[0576] 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), an early promoter of SV40, a promoter of retrovirus, a promoter of metallothionein, a heat shock promoter, an SR α promoter and the like. Also, an enhancer of the IE gene of human CMV may be used together with the promoter.

[0577] The host cell includes a human cell such as Namalwa cell, a monkey cell such as COS cell, a Chinese hamster cell such as CHO cell or HBT5637 (Japanese Published Unexamined Patent Application No. 299/88), a rat myeloma cell, a mouse myeloma cell, a cell derived from syrian hamster kidney, an embryonic stem cell, a fertilized egg cell and the like.

[0578] As the method for introducing the recombinant vector, any method can be used, so long as it can introduce DNA into an animal cell. Examples include electroporation [*Cytotechnology*, 3, 133 (1990)], the calcium phosphate method (Japanese Published Unexamined Patent Application No. 227075/90), the lipofection method [*Proc. Natl. Acad. Sci. USA*, 84, 7413 (1987)], the injection method (*Manipulating the Mouse Embryo, A Laboratory Manual*), a method using particle gun (gene gun) (Japanese Patent No. 2606856, Japanese Patent No. 2517813), the DEAE-dextran method [*Biomanual Series 4—Gene Transfer and Expression Analysis* (Yodo-sha), edited by Takashi Yokota and Kenichi Arai (1994)], the virus vector method (*Manipulating Mouse Embryo*, Second Edition) and the like.

[0579] When an insect cell is used as the host, the protein can be expressed by the method described in *Current Protocols in Molecular Biology, Baculovirus Expression Vectors, A Laboratory Manual*, W.H. Freeman and Company, New York (1992), *Bio/Technology*, 6, 47 (1988) or the like.

[0580] That is, the protein can be expressed by co-introducing a recombinant gene-introducing vector and a baculovirus into an insect cell to obtain a recombinant virus in an

insect cell culture supernatant and then infecting the insect cell with the recombinant virus.

[0581] The gene introducing vector used in the method includes pVL1392, pVL1393, pBlueBacIII (all manufactured by Invitrogen) and the like.

[0582] The baculovirus includes *Autographa californica* nuclear polyhedrosis virus which is infected by an insect of the family Barathra.

[0583] The insect cell includes *Spodoptera frugiperda* oocytes Sf9 and Sf1 [*Current Protocols in Molecular Biology, Baculovirus Expression Vectors, A Laboratory Manual*, W.H. Freeman and Company, New York (1992)], a *Trichoplusia ni* oocyte High 5 (manufactured by Invitrogen) and the like.

[0584] The method for co-introducing the recombinant gene-introducing vector and the baculovirus for preparing the recombinant virus includes the calcium phosphate method (Japanese Published Unexamined Patent Application No. 227075/90), the lipofection method [*Proc. Natl. Acad. Sci. USA*, 84, 7413 (1987)] and the like.

[0585] When a plant cell is used as the host, the expression vector includes Ti plasmid, tobacco mosaic virus and the like.

[0586] As the promoter, any promoter can be used, so long as it can function in a plant cell. Examples include cauliflower mosaic virus (CaMV) 35S promoter, rice actin 1 promoter and the like.

[0587] The host cell includes plant cells of tobacco, potato, tomato, carrot, soybean, rape, alfalfa, rice, wheat, barley and the like.

[0588] As the method for introducing the recombinant vector, any method can be used, so long as it can introduce DNA into a plant cell. Examples include a method using *Agrobacterium* (Japanese Published Unexamined Patent Application No. 140885/84, Japanese Published Unexamined Patent Application No. 70080/85, WO94/00977), electroporation (Japanese Published Unexamined Patent Application No. 251887/85), a method using a particle gun (gene gun) (Japanese Patent No. 2606856, Japanese Patent No. 2517813) and the like.

[0589] As the method for expressing a gene, secretion production, expression of a fusion protein with other protein and the like can be carried out in accordance with the method described in *Molecular Cloning*, Second Edition or the like, in addition to the direct expression.

[0590] Fc γ RIIIa can be produced by culturing the thus obtained transformant in a medium to produce and accumulate Fc γ RIIIa in the culture and then recovering it from the resulting culture. The method for culturing the transformant using a medium can be carried out in accordance with a general method which is used for culturing host cells.

[0591] As the medium for culturing a transformant obtained by using yeast as the host, the medium may be either a natural medium or a synthetic medium, so long as efficiently carried out.

[0592] As the carbon source, those which can be assimilated by the yeast can be used. Examples include carbohydrates such as glucose, fructose, sucrose, molasses contain-

ing them, starch and starch hydrolysate; organic acids such as acetic acid and propionic acid; alcohols such as ethanol and propanol; and the like.

[0593] The nitrogen source includes ammonia; ammonium salts of inorganic acid or organic acid such as ammonium chloride, ammonium sulfate, ammonium acetate and ammonium phosphate, other nitrogen-containing compounds; peptone; meat extract; yeast extract; corn steep liquor; casein hydrolysate; soybean meal; soybean meal hydrolysate; various fermented cells and hydrolysates thereof; and the like.

[0594] The inorganic salt includes potassium dihydrogen phosphate, dipotassium hydrogen phosphate, magnesium phosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, copper sulfate, calcium carbonate, and the like.

[0595] The culturing is carried out generally under aerobic conditions such as shaking culture or submerged-aeration stirring culture. The culturing temperature is preferably 15 to 40° C., and the culturing time is generally 16 hours to 7 days. During the culturing, the pH is maintained at 3.0 to 9.0. The pH is adjusted with an inorganic or organic acid, an alkali solution, urea, calcium carbonate, ammonia or the like.

[0596] If necessary, an antibiotic such as ampicillin or tetracycline can be added to the medium during the culturing.

[0597] When yeast transformed with a recombinant vector obtained by using an inducible promoter as the promoter is cultured, an inducer can be added to the medium, if necessary. For example, when yeast transformed with a recombinant vector obtained using lac promoter is cultured, isopropyl- β -D-thiogalactopyranoside can be added to the medium, and when yeast transformed with a recombinant vector obtained using trp promoter is cultured, indoleacrylic acid and the like can be added to the medium.

[0598] When a transformant obtained by using an animal cell as the host cell is cultured, the medium includes generally used RPMI 1640 medium [*The Journal of the American Medical Association*, 199, 519 (1967)], Eagle's MEM medium [*Science*, 122, 501 (1952)], Dulbecco's modified MEM medium [*Virology*, 8, 396 (1959)], 199 medium [*Proceeding of the Society for the Biological Medicine*, 73, 1 (1950)] and Whitten's medium [*Developmental Engineering Experimentation Manual (Hassei Kogaku Jikken Manual)—Preparation of Transgenic Mice* (Kodansha), edited by M. Katsuki (1987)], the media to which fetal calf serum, etc. is added, and the like.

[0599] The culturing is carried out generally at a pH of 6.0 to 8.0 and 30 to 40° C. for 1 to 7 days in the presence of 5% CO₂. If necessary, an antibiotic such as kanamycin or penicillin can be added to the medium during the culturing.

[0600] The medium for the culturing of a transformant obtained by using an insect cell as the host includes generally used TNM-FH medium (manufactured by Pharmingen), Sf-900 II SFM medium (manufactured by Life Technologies), ExCell 400 and ExCell 405 (both manufactured by JRH Biosciences), Grace's Insect Medium [*Nature* 195, 788 (1962)] and the like.

[0601] The culturing is carried out generally at a medium pH of 6.0 to 7.0 and 25 to 30° C. for 1 to 5 days.

[0602] In addition antibiotics such as gentamicin can be added to the medium during the culturing, if necessary.

[0603] A transformant obtained by using a plant cell as the host cell can be cultured as a cell or by differentiating it into a plant cell or organ. The medium for culturing the transformant includes generally used Murashige and Skoog (MS) medium and White medium, the media to which a plant hormone such as auxin or cytokinin is added, and the like.

[0604] The culturing is carried out generally at a pH of 5.0 to 9.0 and 20 to 40° C. for 3 to 60 days.

[0605] If necessary, an antibiotic such as kanamycin or hygromycin can be added to the medium during the culturing.

[0606] As discussed above, Fc γ RIIIa can be produced by culturing a transformant derived from a microorganism, an animal cell, an insect cell or a plant cell which comprises a recombinant vector into which a DNA encoding Fc γ RIIIa is inserted, in accordance with the general culturing method to thereby produce and accumulate Fc γ RIIIa, and then recovering Fc γ RIIIa from the culture.

[0607] As the method for expressing Fc γ RIIIa, secretion production, expression of fusion protein and the like can be carried out in accordance with the method described in *Molecular Cloning*, Second Edition in addition to the direct expression.

[0608] The method for producing Fc γ RIIIa includes a method of intracellular expression in a host cell, a method of extracellular secretion from a host cell, and a method of production on a host cell membrane outer envelope. The method can be selected by changing the host cell used or the structure of Fc γ RIIIa produced.

[0609] When Fc γ RIIIa is produced in a host cell or on a host cell membrane outer envelope, it can be positively secreted extracellularly in accordance with the method of Paulson et al. [*J. Biol. Chem.*, 264, 17619 (1989)], the method of Lowe et al. [*Proc. Natl. Acad. Sci. USA*, 86, 8227 (1989), *Genes Develop.*, 4, 1288 (1990)], the methods described in Japanese Published Unexamined Patent Application No. 336963/93 and Japanese Published Unexamined Patent Application No. 823021/94 and the like.

[0610] That is, Fc γ RIIIa of interest can be positively secreted extracellularly from a host cell by inserting a DNA encoding Fc γ RIIIa and a signal peptide suitable for the expression of Fc γ RIIIa into an expression vector by using a gene recombination technique, and then expressing the vector.

[0611] Also, its production amount can be increased in accordance with the method described in Japanese Published Unexamined Patent Application No. 227075/90 based on a gene amplification system using a dihydrofolate reductase gene.

[0612] In addition, Fc γ R can also be produced by using a gene-introduced animal individual (transgenic non-human animal) or a plant individual (transgenic plant) which is constructed by the redifferentiation of an animal or plant cell into which the gene is introduced.

[0613] When the transformant is an animal individual or a plant individual, Fc γ RIIIa can be produced in accordance with a general method by rearing or cultivating it to thereby

produce and accumulate FcγR and then recovering FcγRIIIa from the animal or plant individual.

[0614] The method for producing FcγRIIIa by using an animal individual includes a method in which FcγRIIIa of interest is produced in an animal constructed by introducing a gene in accordance with a known method [*American Journal of Clinical Nutrition*, 63, 639 (1996); *American Journal of Clinical Nutrition*, 63, 627S (1996); *Bio/Technology*, 9, 830 (1991)].

[0615] In the case of an animal individual, FcγRIIIa can be produced by rearing a transgenic non-human animal into which a DNA encoding FcγRIIIa is introduced to thereby produce and accumulate FcγRIIIa in the animal, and then recovering FcγRIIIa from the animal. The place of the animal where FcγRIIIa is produced and accumulated includes milk (Japanese Published Unexamined Patent Application No. 309192/88) and eggs of the animal. As the promoter used in this case, any promoter can be used, so long as it can function in an animal. Preferred examples include mammary gland cell-specific promoters such as β casein promoter, casein promoter, β lactoglobulin promoter and whey acidic protein promoter.

[0616] The method for producing FcγRIIIa by using a plant individual includes a method in which FcγRIIIa is produced by cultivating a transgenic plant into which a DNA encoding FcγR is introduced by a known method [*Tissue Culture (Soshiki Baiyo)*, 20 (1994); *Tissue Culture (Soshiki Baiyo)*, 21 (1995); *Trends in Biotechnology*, 15, 45 (1997)] to produce and accumulate FcγR in the plant, and then recovering FcγRIIIa from the plant.

[0617] Regarding purification of FcγRIIIa produced by a transformant into which a gene encoding FcγRIIIa is introduced, for example, when FcγRIIIa is intracellularly expressed in a dissolved state, the cells after culturing are recovered by centrifugation, suspended in an aqueous buffer and then disrupted using ultrasonic oscillator, French press, Manton Gaulin homogenizer, dynamill or the like to obtain a cell-free extract. A purified product of FcγRIIIa can be obtained from a supernatant obtained by centrifuging the cell-free extract, by using an ordinary enzyme isolation purification technique such as solvent extraction; salting out and desalting with ammonium sulfate, etc. precipitation with an organic solvent; anion exchange chromatography using a resin such as diethylaminoethyl (DEAE)-Sephacel or DIAION HPA-75 (manufactured by Mitsubishi Chemical); cation exchange chromatography using a resin such as S-Sepharose FF (manufactured by Pharmacia); hydrophobic chromatography using a resin such as butyl-Sepharose or phenyl-Sepharose; gel filtration using a molecular sieve, affinity chromatography; chromatofocusing; electrophoresis such as isoelectric focusing; and the like which may be used alone or in combination.

[0618] Also, when FcγRIIIa is expressed intracellularly by forming an insoluble body, the cells are recovered, disrupted and centrifuged in the same manner, and the insoluble body of FcγRIIIa is recovered as a precipitation fraction. The recovered insoluble body of FcγRIIIa is solubilized by using a protein denaturing agent. FcγRIIIa is made into a normal three-dimensional structure by diluting or dialyzing the solubilized solution, and then a purified product of FcγRIIIa is obtained by the same isolation purification method.

[0619] When FcγRIIIa is secreted extracellularly, FcγRIIIa or derivatives thereof can be recovered from the culture

supernatant. That is, the culture is treated by a similar technique such as centrifugation to obtain a soluble fraction, and a purified preparation of FcγRIIIa can be obtained from the soluble fraction by the same isolation purification method.

[0620] (2) Measurement of Binding Activity to FcγRIIIa

[0621] Binding activity of the antibody composition to FcγRIIIa expressed on the cell membrane can be measured by the immunofluorescent method [*Cancer Immunol. Immunother.*, 36, 373 (1993)] or the like. Also, binding activity to the purified FcγRIIIa prepared by the method described in the item 4(1) can be measured according to the immunological determination method such as Western staining described in literatures [*Monoclonal Antibodies: Principles and Applications*, Wiley-Liss, Inc., (1995); *Enzyme Immunoassay (Koso Men-eki Sokutei Ho)*, 3rd edition, Igaku Shoin (1987), reversed edition; *Enzyme Antibody Method (Koso Kotai Ho)*, Gakusai Kikaku (1985)], RIA (radioimmunoassay), VIA (viroimmunoassay), EIA (enzyme or immunoassay), FIA (fluoroimmunoassay) or MIA (metalloimmunoassay), for example, as follows.

[0622] FcγRIIIa is immobilized on a plastic plate for EIA and is allowed to react with a sample containing an antibody composition. Next, an amount of the bound antibody composition is measured by using an appropriate secondary antibody.

[0623] In addition, binding activity to the purified FcγRIIIa can also be measured by a measuring method using biosensor [e.g., BIAcore (manufactured by BIACORE)][*J. Immunol. Methods*, 200, 121 (1997)], isothermal titration calorimetry [*Proc. Natl. Acad. Sci. U.S.A.*, 97, 9026 (2000)] or the like.

[0624] 5. Activity Evaluation of Antibody Composition

[0625] As the method for measuring the amount of the purified antibody composition, its binding activity to an antigen, its binding activity to FcγRIIIa and its effector function, the known method described in *Monoclonal Antibodies, Antibody Engineering* and the like can be used.

[0626] For example, when the antibody composition is a humanized antibody, the binding activity to an antigen, binding activity to an antigen-positive cultured clone and binding activity to FcγRIIIa can be measured by methods such as ELISA and the immunofluorescent method [*Cancer Immunol. Immunother.*, 36, 373 (1993)], measurement using biosensor [for example, using BIAcore (manufactured by BIACORE)][*J. Immunol. Methods*, 200, 121 (1997)], isothermal titration calorimetry method [*Proc. Natl. Acad. Sci. U.S.A.*, 97, 9026 (2000)] and the like. Among the effector functions, the cytotoxic activity against an antigen-positive cultured clone can be evaluated by measuring CDC activity, ADCC activity [*Cancer Immunol. Immunother.*, 36, 373 (1993)] and the like.

[0627] 6. Analysis of Sugar Chains of Antibody Molecule Expressed in Various Cells

[0628] The sugar chain structure binding to an antibody molecule expressed in various cells can be analyzed in accordance with the general analysis of the sugar chain structure of a glycoprotein. For example, the sugar chain which is bound to IgG molecule comprises a neutral sugar such as galactose, mannose, fucose, an amino sugar such as

N-acetylglucosamine and an acidic sugar such as sialic acid, and can be analyzed by a method such as a sugar chain structure analysis using sugar composition analysis, two dimensional sugar chain mapping or the like.

[0629] (1) Analysis of Neutral Sugar and Amino Sugar Compositions

[0630] The composition analysis of the sugar chain of an antibody molecule can be carried out by acid hydrolysis of sugar chains with trifluoroacetic acid or the like to release a neutral sugar or an amino sugar and measuring the composition ratio.

[0631] Examples include a method using a sugar composition analyzer (BioLC) manufactured by Dionex. The BioLC is an apparatus which analyzes a sugar composition by HPAEC-PAD (high performance anion-exchange chromatography-pulsed amperometric detection) [*J. Liq. Chromatogr.*, 6, 1577 (1983)].

[0632] The composition ratio can also be analyzed by a fluorescence labeling method using 2-aminopyridine. Specifically, the composition ratio can be calculated in accordance with a known method [*Agric. Biol. Chem.*, 55, 283 (1991)] by labeling an acid-hydrolyzed sample with a fluorescence by 2-aminopyridylation and then analyzing the composition by HPLC.

[0633] (2) Analysis of Sugar Chain Structure

[0634] The sugar chain structure binding to an antibody molecule can be analyzed by the two dimensional sugar chain mapping method [*Anal. Biochem.*, 171, 73 (1988), *Biochemical Experimentation Methods 23—Methods for Studying Glycoprotein Sugar Chains* (Japan Scientific Societies Press) edited by Reiko Takahashi (1989)]. The two dimensional sugar chain mapping method is a method for deducing a sugar chain structure by, e.g., plotting the retention time or elution position of a sugar chain by reverse phase chromatography as the X axis and the retention time or elution position of the sugar chain by normal phase chromatography as the Y axis, respectively, and comparing them with those of known sugar chains.

[0635] Specifically, sugar chains are released from an antibody by subjecting the antibody to hydrazinolysis, and the released sugar chains are subjected to fluorescence labeling with 2-aminopyridine (hereinafter referred to as "PA") [*J. Biochem.*, 95, 197 (1984)], and then the sugar chains are separated from an excess PA-treating reagent by gel filtration, and subjected to reverse phase chromatography. Thereafter, each peak of the separated sugar chains are subjected to normal phase chromatography. From these results, the sugar chain structure can be deduced by plotting the results on a two dimensional sugar chain map and comparing them with the spots of a sugar chain standard (manufactured by Takara Shuzo) or a literature [*Anal. Biochem.*, 171, 73 (1988)].

[0636] The structure deduced by the two dimensional sugar chain mapping method can be confirmed by further carrying out mass spectrometry such as MALDI-TOP-MS of each sugar chain.

[0637] 7. Immunological Determination Method for Identifying Sugar Chain Structure of Antibody Molecule

[0638] An antibody composition comprises an antibody molecule in which sugar chains binding to the Fc region of

the antibody are different in structure. The antibody composition comprising a sugar chain in which fucose is not bound to N-acetylglucosamine in the reducing end in the sugar chain among the total complex N-glycoside-linked sugar chains binding to the Fc region in the antibody composition reducing end can be identified by using the method for analyzing the sugar chain structure binding to an antibody molecule described in the item 6. Also, it can also be identified by an immunological determination method using a lectin.

[0639] The sugar chain structure binding to an antibody molecule can be identified by the immunological determination method using a lectin in accordance with the known immunological determination method such as Western staining, IRA (radioimmunoassay), VIA (viroimmunoassay), EIA (enzymimmunoassay), FIA (fluoroimmunoassay) or MIA (metalloimmunoassay) described in literatures [*Monoclonal Antibodies: Principles and Applications*, Wiley-Liss, Inc. (1995); *Immunoassay (Koso Meneki Sokuteiho)*, 3rd Ed., Igakushoin (1987); Revised Edition, *Enzyme Antibody Method (Koso Kotaiho)*, Gakusai Kikaku (11985)] and the like.

[0640] A lectin which recognizes the sugar chain structure binding to an antibody molecule comprised in an antibody composition is labeled, and the labeled lectin is allowed to react with a sample antibody composition. Then, the amount of the complex of the labeled lectin with the antibody molecule is measured.

[0641] The lectin used for identifying the sugar chain structure binding to an antibody molecule includes WGA (wheat-germ agglutinin derived from *T. vulgaris*), ConA (cocoavalin A derived from *C. ensiformis*), RIC (toxin derived from *R. communis*), L-PHA (leucoagglutinin derived from *P. vulgaris*), LCA (lentil agglutinin derived from *L. culinaris*), PSA (pea lectin derived from *P. sativum*), AAL (*Aleuria aurantia* lectin), ACL (*Amaranthus caudatus* lectin), BPL (*Bauhinia purpurea* lectin), DSL (*Datura stramonium* lectin), DBA (*Dolichos biflorus* agglutinin), EBL (elderberry balk lectin), ECL (*Erythrina cristagalli* lectin), EEL (*Euonymus europaeus* lectin), GNL (*Galanthus nivalis* lectin), GSL (*Griffonia simplicifolia* lectin), HPA (*Helix pomatia* agglutinin), HM (*Hippeastrum* hybrid lectin), Jacalin, LTL (*Lotus tetragonolobus* lectin), LEL (*Lycopersicon esculentum* lectin), MAL (*Maackia amurensis* lectin), MPL (*Maclura pomifera* lectin), NPL (*Narcissus pseudonarcissus* lectin), PNA (peanut agglutinin), E-PHA (*Phaseolus vulgaris* erythroagglutinin), PTL (*Psophocarpus tetragonolobus* lectin), RCA (*Ricinus communis* agglutinin), STL (*Solanum tuberosum* lectin), SIA (*Sophora japonica* agglutinin), SBA (soybean agglutinin), UEA (*Ulex europaeus* agglutinin), VVL (*Vicia villosa* lectin) and WFA (*Wisteria floribunda* agglutinin).

[0642] It is preferable to use a lectin which specifically recognizes a sugar chain structure wherein fucose binds to the N-acetylglucosamine in the reducing end in the complex N-glycoside-linked sugar chain. Examples include *Lens culinaris* lectin LCA (lentil agglutinin derived from *Lens culinaris*), pea lectin PSA (pea lectin derived from *Pisum sativum*), broad bean lectin VFA (agglutinin derived from *Vicia faba*) and *Aleuria aurantia* lectin AAL (lectin derived from *Aleuria aurantia*).

[0643] 8. Method for Measuring Binding Activity of Antibody Composition to FcγRIIIa

[0644] The present invention relates to a method for detecting the ratio of a sugar chain in which fucose is not bound to N-acetylglucosamine in the reducing end in an antibody composition by using a measuring method which comprises reacting an antigen with a tested antibody composition to form a complex of the antigen and the antibody composition; contacting the complex with an Fcγ receptor IIIa. Furthermore, the present invention relates to a method for detecting the antibody-dependent cell-mediated cytotoxic activity.

[0645] The measuring method used in the present invention is described below in detail.

[0646] First, an antigen is fixed on a plate, and a sample of antibody composition is allowed to react with it. The resulting complex after the antigen-antibody reaction is allowed to react with human FcγRIIIa.

[0647] The human FcγRIIIa to be allowed to react is labeled with a label such as an enzyme, a radioisotope or an fluorescent, and the binding activity of the antibody bound to the antigen can be measured by an immunological measuring method.

[0648] The immunological measuring method includes any method which uses an antigen-antibody reaction such as an immunoassay, an immunoblotting, a coagulation reaction, a complement binding reaction, a hemolysis reaction, a precipitation reaction, a colloidal gold method, a chromatography or an immune staining method. Among these, the immunoassay is preferred.

[0649] Also, human FcγRIIIa having a tag can be obtained by ligating a nucleotide sequence encoding a short peptide to a gene encoding the human FcγRIIIa, and expressing the product by genetic engineering techniques. The tag includes histidine and the like.

[0650] Accordingly, when the above reaction is carried out by using the human FcγRIIIa having a tag, an immunoassay having high sensitivity can be carried out by applying an antibody against the tag after the reaction and labeling the antibody against the tag as described above, or using a labeled antibody which binds to the antibody against the tag.

[0651] Also, human FcγRIIIa having a tag can be obtained by ligating a nucleotide sequence encoding a short peptide to a gene encoding the human FcγRIIIa, and expressing the product by genetic engineering techniques. The tag includes histidine and the like.

[0652] Accordingly, when the above reaction is carried out by using the human FcγRIIIa having a tag, an immunoassay having high sensitivity can be carried out by applying an antibody against the tag after the reaction and labeling the antibody against the tag as described above, or using a labeled antibody which binds to the antibody against the tag.

[0653] The detection method of the present invention can also be carried out by directly contacting a sample of antibody composition with FcγRIIIa, without reacting an antigen with the sample of antibody component. For example, a labeled antibody which recognizes the human Fc region can be detected by solid-phasing an antibody against

the tag and reacting the antibody with tag-containing FcγRIIIa, followed by reaction with a tested antibody composition.

[0654] The method for detecting the ratio of a sugar chain in which fucose is not bound to N-acetylglucosamine in the reducing end of an antibody composition can be carried out according to the following method.

[0655] First, antibody compositions (standards) necessary for the preparation of a standard curve having a different ratio of a sugar chain in which fucose is not bound to N-acetylglucosamine in the sugar chain reducing end are prepared and the sugar chain analysis of an antibody composition is carried out. In this case, the concentrations of the antibody compositions are adjusted to the same. Each of the binding activities of the prepared antibody composition samples to FcγRIIIa is measured, and a standard curve between the ratio of sugar chain and the binding activity to FcγRIIIa is prepared.

[0656] Based on the above standard curve, the binding activity of the antibody composition sample to be measured to FcγRIIIa is measured while keeping the definite concentration of the antibody composition according to a measuring method similar to the above.

[0657] Based on the above standard curve, the binding activity of the antibody composition to FcγRIIIa is measured with the same concentration of the sample to be measured according to the measuring method similar to the above, so that the ratio of a sugar chain in which fucose is not bound to N-acetylglucosamine in the sugar chain reducing end in the antibody composition sample can be obtained.

[0658] Furthermore, detection of ADCC activity can be carried out by the following method.

[0659] ADCC activities of the standards used for the preparation of the standard curve in the method for detecting the ratio of a sugar chain in which fucose is not bound to N-acetylglucosamine in the sugar chain reducing end of the above antibody composition are measured. The measuring method includes the method for measuring ADCC described below. Each of binding activities of the prepared antibody composition samples is measured by the above measuring method, and a standard curve between the ADCC activity and the binding activity to FcγRIIIa is prepared.

[0660] Based on the above standard curve, the binding activity of the antibody composition sample to be measured to FcγRIIIa is measured while keeping the definite concentration of the antibody composition according to the measuring method used above, so that the ADCC activity can be obtained.

[0661] 8 Screening Method for Antibody Composition

[0662] The present invention relates to a method for screening an antibody composition having a higher binding activity to FcRIIIa, which comprises reacting an antigen with a tested antibody, followed by treatment with FcγRIIIa.

[0663] The screening method of the present invention is described below in detail.

[0664] An antigen is fixed on a plate and allowed to react with an antibody to be tested. Human FcγRIIIa is allowed to react with the complex of the antigen-antibody reaction.

[0665] The human FcγRIIIa to be allowed to react is labeled with a label such as an enzyme, a radioisotope or an fluorescent, and the binding activity of the antibody bound to the antigen can be measured by an immunological measuring method.

[0666] The immunological measuring method includes any method which uses an antigen-antibody reaction such as an immunoassay, an immunoblotting, a coagulation reaction, a complement binding reaction, a hemolysis reaction, a precipitation reaction, a colloidal gold method, a chromatography or an immune staining method. Among these, the immunoassay is preferred.

[0667] Also, human FcγRIIIa having a tag can be obtained by ligating a nucleotide sequence encoding a short peptide to a gene encoding the human FcγRIIIa, and expressing the product by genetic engineering techniques. The tag includes histidine and the like.

[0668] Accordingly, when the above reaction is carried out by using the human FcγRIIIa having a tag, an immunoassay having high sensitivity can be carried out by applying an antibody against the tag after the above reaction and labeling the antibody against the tag as described above, or using a labeled antibody which binds to the antibody against the tag.

[0669] 10. Application of Antibody Composition of the Present Invention

[0670] The antibody composition obtained by the screening method of the present invention has high ADCC activity.

[0671] An antibody having high ADCC activity is useful for preventing and treating various diseases including cancers, inflammatory diseases, immune diseases such as autoimmune diseases and allergies, cardiovascular diseases and viral or bacterial infections.

[0672] In the case of cancers, namely malignant tumors, cancer cells grow. General anti-tumor agents are characterized by inhibiting the growth of cancer cells. In contrast, an antibody having high ADCC activity can treat cancers by injuring cancer cells through its cell killing effect, and therefore, it is more effective as a therapeutic agent than the general anti-tumor agents. At present, in regard to therapeutic agents for cancers, an anti-tumor effect of an antibody medicament alone is insufficient, so that it has been taken to chemotherapy [Science, 280, 1197 (1998)]. If higher anti-tumor effect is found by the antibody composition of the present invention alone, the dependency on chemotherapy will be decreased and side effects will be reduced.

[0673] In immune diseases such as inflammatory diseases, autoimmune diseases and allergies, in vivo reactions of the diseases are induced by the release of a mediator molecule by immunocytes, so that the allergy reaction can be inhibited by eliminating immunocytes using an antibody having high ADCC activity.

[0674] The cardiovascular diseases include arteriosclerosis and the like. The arteriosclerosis is treated by using balloon catheter at present, but cardiovascular diseases can be prevented and treated by using an antibody having high ADCC activity because growth of arterial cells in restructure after above treatment can be inhibited by using the antibody.

[0675] Various diseases including viral and bacterial infections can be prevented and treated by inhibiting pro-

liferation of cells infected with a virus or bacterium using an antibody having high ADCC activity.

[0676] Specific examples of an antibody which recognizes a tumor-related antigen, an antibody which recognizes an allergy- or inflammation-related antigen, an antibody which recognizes cardiovascular disease-related antigen and an antibody which recognizes a viral or bacterial infection-related antigen are described below.

[0677] The antibody which recognizes a tumor-related antigen includes anti-GD2 antibody [Anticancer Res., 13, 331 (1993)], anti-GD3 antibody [Cancer Immunol. Immunother., 36, 260 (1993)], anti-GM2 antibody [Cancer Res., 54, 1511 (1994)], anti-HER2 antibody [Proc. Natl. Acad. Sci. USA, 89, 4285 (1992)], anti-CD52 antibody [Proc. Natl. Acad. Sci. USA, 89, 4285 (1992)], anti-MAGE antibody [British J. Cancer, 83, 493 (2000)], anti-HM1.24 antibody [Molecular Immunol., 36, 387 (1999)], anti-parathyroid hormone-related protein (PTHrP) antibody [Cancer, 88, 2909 (2000)], anti-basic fibroblast growth factor antibody and anti-FGF8 antibody [Proc. Natl. Acad. Sci. USA, 86, 9911 (1989)], anti-basic fibroblast growth factor receptor antibody and anti-FGF8 receptor antibody [J. Biol. Chem., 265, 16455 (1990)], anti-insulin-like growth factor antibody [J. Neurosci. Res., 40, 647 (1995)], anti-insulin-like growth factor receptor antibody [J. Neurosci. Res., 40, 647 (1995)], anti-PMSA antibody [J. Urology, 160, 2396 (1998)], anti-vascular endothelial cell growth factor antibody [Cancer Res., 57, 4593 (1997)], anti-vascular endothelial cell growth factor receptor antibody [Oncogene, 19, 2138 (2000)] and the like.

[0678] The antibody which recognizes an allergy- or inflammation-related antigen includes anti-interleukin 6 antibody [Immunol. Rev., 127, 5 (1992)], anti-interleukin 6 receptor antibody [Molecular Immunol., 31, 371 (1994)], anti-interleukin 5 antibody [Immunol. Rev., 127, 5 (1992)], anti-interleukin 5 receptor antibody and anti-interleukin 4 antibody [Cytokine, 3, 562 (1991)], anti-interleukin 4 receptor antibody [J. Immunol. Methods, 217, 41 (1998)], anti-tumor necrosis factor antibody [Hybridoma, 13, 183 (1994)], anti-tumor necrosis factor receptor antibody [Molecular Pharmacol., 58, 237(2000)], anti-CCR4 antibody [Nature, 400, 776 (1999)], anti-chemokine antibody [J. Immunol. Meth., 174, 249 (1994)], anti-chemokine receptor antibody [J. Exp. Med., 186, 1373 (1997)] and the like. The antibody which recognizes a cardiovascular disease-related antigen includes anti-GpIIb/IIIa antibody [J. Immunol., 152, 2968 (1994)], anti-platelet-derived growth factor antibody [Science, 253, 1129 (1991)], anti-platelet-derived growth factor receptor antibody [J. Biol. Chem., 272, 17400 (1997)] and anti-blood coagulation factor antibody [Circulation, 101, 1158 (2000)] and the like.

[0679] The antibody which recognizes a viral or bacterial infection-related antigen includes anti-gp120 antibody [Structure, 8, 385 (2000)], anti-CD4 antibody [J. Rheumatology, 25, 2065 (1998)], anti-CCR5 antibody and anti-Vero toxin antibody [J. Clin. Microbiol., 37, 396 (1999)] and the like.

[0680] These antibodies can be obtained from public organizations such as ATCC (The American Type Culture Collection), RIKEN Gene Bank at The Institute of Physical and Chemical Research and National Institute of Bioscience and Human Technology, Agency of Industrial Science and Tech-

nology, or private reagent sales companies such as Dainippon Pharmaceutical, R & D SYSTEMS, PharMingen, Cosmo Bio and Funakoshi Co., Ltd.

[0681] The antibody composition obtained by the process of the present invention can be administered as various therapeutic agents alone, but generally, it is preferable to provide it as a pharmaceutical formulation produced by an appropriate method well known in the technical field of pharmaceutical, by mixing it with one or more pharmaceutically acceptable carriers.

[0682] It is preferable to select a route of administration which is most effective in treatment. Examples include oral administration and parenteral administration, such as buccal, tracheal, rectal, subcutaneous, intramuscular and intravenous. In the case of an antibody preparation, intravenous administration is preferred.

[0683] The dosage form includes sprays, capsules, tablets, granules, syrups, emulsions, suppositories, injections, ointments, tapes and the like.

[0684] The pharmaceutical preparation suitable for oral administration include emulsions, syrups, capsules, tablets, powders, granules and the like.

[0685] Liquid preparations such as emulsions and syrups can be produced by using, as additives, water; sugars such as sucrose, sorbitol and fructose, glycols such as polyethylene glycol and propylene glycol, oils such as sesame oil, olive oil and soybean oil; antiseptics such as p-hydroxybenzoic acid esters; flavors such as strawberry flavor and peppermint; and the like.

[0686] Capsules, tablets, powders, granules and the like can be prepared by using, as additives, excipients such as lactose, glucose, sucrose and mannitol; disintegrating agents such as starch and sodium alginate; lubricants such as magnesium stearate and talc; binders such as polyvinyl alcohol, hydroxypropylcellulose and gelatin; surfactants such as fatty acid ester; plasticizers such as glycerine; and the like.

[0687] The pharmaceutical preparation suitable for parenteral administration includes injections, suppositories, sprays and the like.

[0688] Injections may be prepared by using a carrier such as a salt solution, a glucose solution or a mixture thereof. Also, powdered injections can be prepared by freeze-drying the antibody composition in the usual way and adding sodium chloride thereto.

[0689] Suppositories may be prepared by using a carrier such as cacao butter, hydrogenated fat or carboxylic acid.

[0690] Also, sprays may be prepared by using the antibody composition as such or using a carrier, etc. which do not stimulate the buccal or airway mucous membrane of the patient and can facilitate absorption of the antibody composition by dispersing it as fine particles.

[0691] The carrier includes lactose, glycerine and the like. Depending on the properties of the antibody composition and the carrier, it is possible to produce pharmaceutical preparations such as aerosols and dry powders. In addition, the components exemplified as additives for oral preparations can also be added to the parenteral preparations.

[0692] Although the clinical dose or the frequency of administration varies depending on the objective therapeutic effect, administration method, treating period, age, body weight and the like, it is usually 10 μ g/kg to 20 mg/kg per day and per adult.

[0693] Also, as the method for examining antitumor effect of the antibody composition against various tumor cells, in vitro tests include CDC activity measuring method, ADCC activity measuring method and the like, and in vivo tests include antitumor experiments using a tumor system in an experimental animal such as a mouse, and the like.

[0694] CDC activity and ADCC activity measurements and antitumor experiments can be carried out in accordance with the methods described in *Cancer Immunology Immunotherapy*, 36, 373 (1993); *Cancer Research*, 54, 1511 (1994) and the like.

[0695] The present invention will be described below in detail based on Examples; however, Examples are only simple illustrations, and the scope of the present invention is not limited thereto.

BEST MODE FOR CARRYING OUT THE INVENTION

EXAMPLE 1

[0696] Preparation of Anti-Ganglioside GD3 Human Chimeric Antibody:

[0697] 1. Construction of Tandem Expression Vector pChi641LHGM4 for Anti-Ganglioside GD3 Human Chimeric Antibody

[0698] A plasmid pChi641LGM40 was constructed by ligating a fragment of about 4.03 kb containing an L chain cDNA, obtained by digesting an L chain expression vector pChi641LGM4 [*J. Immunol. Methods*, 167, 271 (1994)] for anti-ganglioside GD3 human chimeric antibody (hereinafter referred to as "anti-GD3 chimeric antibody") with restriction enzymes MluI (manufactured by Takara Shuzo) and SalI (manufactured by Takara Shuzo) with a fragment of about 3.40 kb containing a G418-resistant gene and a splicing signal, obtained by digesting an expression vector pAGE107 [*Cytotechnology*, 3, 133 (1990)] for animal cell with restriction enzymes MluI (manufactured by Takara Shuzo) and SalI (manufactured by Takara Shuzo) using DNA Ligation Kit (manufactured by Takara Shuzo), and then transforming *E. coli* HB101 (*Molecular Cloning*, Second Edition) with the ligated product.

[0699] Next, a fragment of about 5.68 kb containing an L chain cDNA, obtained by digesting the constructed plasmid pChi641LGM40 with a restriction enzyme ClaI (manufactured by Takara Shuzo), changing it to blunt-end using DNA Blunting Kit (manufactured by Takara Shuzo) and further digesting it with MluI (manufactured by Takara Shuzo), was ligated with a fragment of about 8.40 kb containing an H chain cDNA, obtained by digesting an anti-GD3 chimeric antibody H chain expression vector pChi641GM4 [*J. Immunol. Methods*, 1, 271 (1994)] with a restriction enzyme (manufactured by Takara Shuzo), changing it to blunt-end using DNA Blunting Kit (manufactured by Takara Shuzo) and further digesting it with MluI (manufactured by Takara Shuzo) using DNA Ligation Kit (manufactured by Takara Shuzo), and then *E. coli* HB101 (*Molecular Cloning*, Sec-

ond Edition) was transformed with the ligated product to thereby construct a tandem expression vector pChi641LHGM4 for anti-GD3 chimeric antibody.

[0700] 2. Preparation of Cell Stably Producing Anti-GD3 Chimeric Antibody

[0701] Cells capable of stably producing an anti-GD3 chimeric antibody were prepared by introducing the tandem expression vector pChi641LHGM4 for anti-GD3 chimeric antibody constructed in the item 1 of Example 1 and selecting suitable clones, as described below.

[0702] (1) Preparation of Producing Cell Using Rat Myeloma YB2/0 Cell

[0703] After introducing 5 μ g of the anti-GD3 chimeric antibody expression vector pChi641LHGM4 into 4×10^6 cells of rat myeloma YB2/0 [ATCC CRL-1662, *J. Cell. Biol.*, 93, 576 (1982)] by electroporation [*Cytotechnology*, 3, 133 (1990)], the cells were suspended in 40 ml of RPMI1640-FBS(10) (RPMI1640 medium comprising 10% (fetal bovine serum (hereinafter referred to as "FBS") (manufactured by GIBCO BRL)) and dispensed at 200 μ l/well into a 96 well culture plate (manufactured by Sumitomo Bakelite). After culturing at 37° C. for 24 hours in a 5% CO₂ incubator, G418 was added to give a concentration of 0.5 mg/ml, followed by culturing for 1 to 2 weeks. The culture supernatant was recovered from wells in which colonies of transformants showing G418 resistance were formed and growth of colonies was observed, and the antigen binding activity of the anti-GD3 chimeric antibody in the supernatant was measured by the ELISA shown in the item 3 of Example 1.

[0704] Regarding the transformants in wells in which production of the anti-GD3 chimeric antibody was observed in culture supernatants, in order to increase the amount of the antibody production using a DHFR gene amplification system, each of them was suspended in the RPMI1640-FBS(10) medium comprising 0.5 mg/ml G418 and 50 nmol/L DHFR inhibitor, methotrexate (hereinafter referred to as "MTX"; manufactured by SIGMA) to give a density of 1 to 2×10^5 cells/ml, and the suspension was dispensed at 2 ml into each well of a 24 well plate (manufactured by Greiner). Transformants showing 50 nmol/L MTX resistance were induced by culturing at 37° C. for 1 to 2 weeks in a 5% CO₂ incubator. The antigen binding activity of the anti-GD3 chimeric antibody in culture supernatants in wells in which growth of transformants was observed was measured by the ELISA shown in the item 3 of Example 1. Regarding the transformants in wells in which production of the anti-GD3 chimeric antibody was observed in culture supernatants, the MTX concentration was increased to 100 nmol/L and then to 200 nmol/L, and transformants capable of growing in the RPMI1640-FBS(10) medium comprising 0.5 mg/ml G418 and 200 nmol/L MTX and capable of producing the anti-GD3 chimeric antibody in a large amount were finally obtained by the same method as described above. Among the obtained transformants, suitable clones were selected and were made into a single cell (cloning) by limiting dilution twice.

[0705] The obtained anti-GD3 chimeric antibody-producing transformed cell clone 7-9-51 has been deposited on Apr. 5, 1999, as FERM BP-6691 in National Institute of Bioscience and Human Technology, Agency of Industrial

Science and Technology (Higashi 1-1-3, Tsukuba, Ibaraki, Japan) (present name: International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology (Tsukuba Central 6, 1, Higashi 1-Chome Tsukuba-shi, Ibaraki-ken, Japan)).

[0706] (2) Preparation of Producing Cell Using CHO/DG44 Cell

[0707] After introducing 4 μ g of the anti-GD3 chimeric antibody expression vector pChi641LHGM4 into 1.6×10^6 cells of CHO/DG44 cell [*Proc. Natl. Acad. Sci. USA*, 77, 4216 (1980)] by electroporation [*Cytotechnology*, 3, 133 (1990)], the cells were suspended in 10 ml of IMDM-FBS(10)-HT(1) [IMDM medium comprising 10% FBS and 1 \times concentration of HT supplement (manufactured by GIBCO BRL)] and dispensed at 200 μ l/well into a 96 well culture plate (manufactured by Iwaki Glass). After culturing at 37° C. for 24 hours in a 5% CO₂ incubator, G418 was added to give a concentration of 0.5 mg/ml, followed by culturing for 1 to 2 weeks. The culture supernatant was recovered from wells in which colonies of transformants showing G418 resistance were formed and growth of colonies was observed, and the antigen binding activity of the anti-GD3 chimeric antibody in the supernatant was measured by the ELISA shown in the item 3 of Example 1.

[0708] Regarding the transformants in wells in which production of the anti-GD3 chimeric antibody was observed in culture supernatants, in order to increase the amount of the antibody production using a DHFR gene amplification system, each of them was suspended in an IMDM-dFBS(10) medium [IMDM medium comprising 10% dialyzed fetal bovine serum (hereinafter referred to as "dFBS"; manufactured by GIBCO BRL)] comprising 0.5 mg/ml G418 and 10 nmol/L MTX to give a density of 1 to 2×10^5 cells/ml, and the suspension was dispensed at 0.5 ml into each well of a 24 well plate (manufactured by Iwaki Glass). Transformants showing 10 nmol/L MTX resistance were induced by culturing at 37° C. for 1 to 2 weeks in a 5% CO₂ incubator. Regarding the transformants in wells in which their growth was observed, the MTX concentration was increased to 100 nmol/L, and transformants capable of growing in the IMDM-dFBS(10) medium comprising 0.5 mg/ml G418 and 100 nmol/L MTX and of producing the anti-GD3 chimeric antibody in a large amount were finally obtained by the same method as described above. Among the obtained transformants, suitable clones were selected and were made into a single cell (cloning) by limiting dilution twice.

[0709] (3) Preparation of Producing Cell Using Mouse Myeloma NS0 Cell

[0710] After introducing 5 μ g of the anti-GD3 chimeric antibody expression vector pChi641LHGM4 into 4×10^6 cells of mouse myeloma NS0 by electroporation [*Cytotechnology*, 3, 133 (1990)], the cells were suspended in 40 ml of EX-CELL302-FBS(10) (EX-CELL302 medium comprising 10% FBS and 2 mmol/L L-glutamine (hereinafter referred to as "L-Gln"; manufactured by GIBCO BPL) and dispensed at 200 μ l/well into a 96 well culture plate (manufactured by Sumitomo Bakelite). After culturing at 37° C. for 24 hours in a 5% CO₂ incubator, G418 was added to give a concentration of 0.5 mg/ml, followed by culturing for 1 to 2 weeks. The culture supernatant was recovered from wells in which colonies of transformants showing G418 resistance were formed and growth of colonies was observed, and the

antigen binding activity of the anti-GD3 chimeric antibody in the supernatant was measured by the ELISA shown in the item 3 of Example 1.

[0711] Regarding the transformants in wells in which production of the anti-GD3 chimeric antibody was observed in culture supernatants, in order to increase the amount of the antibody production using a DHFR gene amplification system, each of them was suspended in an EX-CELL302-dFBS(10) medium (EX-CELL302 medium comprising 10% dFBS and 2 mmol/L L-Gln) comprising 0.5 mg/ml G418 and 50 nmol/L MTX to give a density of 1 to 2×10^5 cells/ml, and the suspension was dispensed at 2 ml into each well of a 24 well plate (manufactured by Greiner). Transformants showing 50 nmol/L MTX resistance were induced by culturing at 37° C. for 1 to 2 weeks in a 5% CO₂ incubator. The antigen binding activity of the anti-GD3 chimeric antibody in culture supernatants in wells in which growth of transformants was observed was measured by the ELISA shown in the item 3 of Example 1. Regarding the transformants in wells in which production of the anti-GD3 chimeric antibody was observed in culture supernatants, the MTX concentration was increased to 100 nmol/L and then to 200 nmol/L, and transformants capable of growing in the EX-CELL302-dFBS(10) medium comprising 0.5 mg/ml G418 and 200 nmol/L MTX and of producing the anti-GD3 chimeric antibody in a large amount was finally obtained by the same method as described above. Among the obtained transformants, suitable clones were selected and were made into a single cell (cloning) by limiting dilution twice. Also, using the method for determining the transcription product of an α 1,6-fucosyltransferase gene shown in Example 9, a clone producing a relatively small amount of the transcription product was selected and used as a suitable clone.

[0712] 3. Measurement of Binding Activity of Antibody to GD3 (ELISA)

[0713] The binding activity of the antibody to GD3 was measured as described below.

[0714] In 2 ml of an ethanol solution containing 10 μ g of dipalmitoylphosphatidylcholine (manufactured by SIGMA) and 5 μ g of cholesterol (manufactured by SIGMA), 4 nmol of GD3 (manufactured by Snow Brand Milk Products) was dissolved. Into each well of a 96 well plate for ELISA (manufactured by Greiner), 20 μ l of the solution (40 pmol/well in final concentration) was dispensed, followed by air-drying, 1% bovine serum albumin (hereinafter referred to as "S" manufactured by SIGMA)-containing PBS (hereinafter referred to as "1% BSA-PBS") was dispensed at 100 μ l/well, and then the reaction was carried out at room temperature for 1 hour to block remaining active groups. After discarding 1% BSA-PBS, a culture supernatant of a transformant or a diluted solution of a human chimeric antibody was dispensed at 50 μ l/well to carry out the reaction at room temperature for 1 hour. After the reaction, each well was washed with 0.05% Tween 20 (manufactured by Wako Pure Chemical Industries)-containing PBS (hereinafter referred to as "Tween-PBS"), a peroxidase-labeled goat anti-human IgG (H & L) antibody solution (manufactured by American Qualex) diluted 3,000 times with 1% BSA-PBS was dispensed at 50 μ l/well as a secondary antibody solution, and then the reaction was carried out at room temperature for 1 hour. After the reaction and subsequent washing with Tween-PBS, ABTS substrate solution

[solution prepared by dissolving 0.55 g of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)ammonium salt in 1 liter of 0.1 mol/L citrate buffer (pH 4.2) and adding 1 μ l/ml of hydrogen peroxide to the solution just before use (hereinafter the same solution was used)] was dispensed at 50 μ l/well for color development, and then absorbance at 415 nm (hereinafter referred to as "OD415") was measured.

[0715] 4. Purification of Anti-GD3 Chimeric Antibody

[0716] (1) Culturing of Producing Cell Derived from YB 2/0 Cell and Purification of Antibody

[0717] The anti-GD3 chimeric antibody-producing transformed cell clone obtained the item 2(1) of Example 1 was suspended in the Hybridoma-SFM medium comprising 0.2% BSA, 200 nmol/L MTX and 100 nmol/L triiodothyronine (hereinafter referred to as "T3"; manufactured by SIGMA) to give a density of 3×10^5 cells/ml and cultured in a 2.0 liter bottle (manufactured by Iwaki Glass) under stirring at a rate of 50 rpm. After culturing at 37° C. for 10 days in a temperature-controlling room, the culture supernatant was recovered. The anti-GD3 chimeric antibody was purified from the culture supernatant using a Prosep-A (manufactured by Bioprocessing) column in accordance with the manufacture's instructions. The purified anti-GD3 chimeric antibody was named YB2/0-GD3 chimeric antibody.

[0718] (2) Culturing of Producing Cell Derived from CHO/DG44 Cell and Purification of Antibody

[0719] The anti-GD3 chimeric antibody-producing transformed cell clone obtained in the item 2(2) of Example 1 was suspended in the EX-CELL302 medium comprising 3 mmol/L L-Gln, 0.5% fatty acid concentrated solution (hereinafter referred to as "CDLC"; manufactured by GIBCO BRL) and 0.3% Pluronic F68 (hereinafter referred to as "PF68"; manufactured by GIBCO BRL) to give a density of 1×10^6 cells/ml, and the suspension was dispensed at 50 ml into 175 mm² flasks (manufactured by Greiner). After culturing at 37° C. for 4 days in a 5% CO₂ incubator, the culture supernatant was recovered. The anti-GD3 chimeric antibody was purified from the culture supernatant using a Prosep-A (manufactured by Bioprocessing) column in accordance with the manufacture's instructions. The purified anti-GD3 chimeric antibody was named CHO/DG44-GD3 chimeric antibody.

[0720] (3) Culturing of Producing Cell Derived from NSO Cell and Purification of Antibody

[0721] The anti-GD3 chimeric antibody-producing transformed cell clone obtained in the item 2(3) of Example 1 was suspended in the EX-CELL302 medium comprising 2 mmol/L L-Gln, 0.5 mg/ml G418, 200 nmol/L MTX and 1% FBS, to give a density of 1×10^6 cells/ml, and the suspension was dispensed at 200 ml into 175 mm² flasks (manufactured by Greiner). After culturing at 37° C. for 4 days in a 5% CO₂ incubator, the culture supernatant was recovered. The anti-GD3 chimeric antibody was purified from the culture supernatant using a Prosep-A (manufactured by Bioprocessing) column in accordance with the manufacture's instructions. The purified anti-GD3 chimeric antibody was named NSO-GD3 chimeric antibody (302).

[0722] Also, the transformed cell clone was suspended in the GIT medium comprising 0.5 mg/ml G418 and 200

nmol/L MTX to give a density of 3×10^5 cells/ml, and the suspension was dispensed at 200 ml into 175 mm² flasks (manufactured by Greiner). After culturing at 37° C. for 10 days in a 5% CO₂ incubator, the culture supernatant was recovered. The anti-GD3 chimeric antibody was purified from the culture supernatant using a Prosep-A (manufactured by Bioprocessing) column in accordance with the manufacture's instructions. The purified anti-GD3 chimeric antibody was named NS0-GD3 chimeric antibody (GIT).

[0723] (4) Culturing of Producing Cell Derived from SP2/0 Cell and Purification of Antibody

[0724] The anti-GD3 chimeric antibody-producing transformed cell clone (KM-871 (FERM BP-3512)) described in Japanese Published Unexamined Patent Application No. 304989/93 (EP 533199) was suspended in the GIT medium comprising 0.5 mg/ml G418 and 200 nmol/L MTX to give a density of 3×10^5 cells/ml, and the suspension was dispensed at 200 ml into 175 mm² flasks (manufactured by Greiner). After culturing at 37° C. for 8 days in a 5% CO₂ incubator, the culture supernatant was recovered. The anti-GD3 chimeric antibody was purified from the culture supernatant using a Prosep-A (manufactured by Bioprocessing) column in accordance with the manufacture's instructions. The purified anti-GD3 chimeric antibody was named SP2/0-GD3 chimeric antibody.

[0725] 5. Analysis of Purified Anti-GD3 Chimeric Antibody

[0726] In accordance with a known method [*Nature*, 227, 680 (1970)], 4 µg of each of the five kinds of the anti-GD3 chimeric antibodies produced by and purified from respective animal cells, obtained in the item 4 of Example 1, was subjected to SDS-PAGE to analyze the molecular weight and purity. The results are shown in **FIG. 1**. As shown in **FIG. 1**, a single band of about 150 kilodaltons (hereinafter referred to as "Kd") in molecular weight was found under non-reducing conditions, and two bands of about 50 Kd and about 25 Kd under reducing conditions, in each of the purified anti-GD3 chimeric antibodies. The molecular weights almost coincided with the molecular weights deduced from the cDNA nucleotide sequences of H chain and L chain of the antibody (H chain: about 49 Kd, L chain: about 23 Kd, whole molecule: about 144 Kd), and also coincided with the reports rating that the IgG antibody has a molecular weight of about 150 Kd under non-reducing conditions and is degraded into H chains having a molecular weight of about 50 Kd and L chains having a molecular weight of about 25 Kd under reducing conditions due to cutting of the disulfide bond (hereinafter referred to as "S—S bond") in the molecule (*Antibodies*, Chapter 14; *Monoclonal Antibodies*), so that it was confirmed that each anti-GD3 chimeric antibody was expressed and purified as an antibody molecule having the true structure.

EXAMPLE 2

[0727] Activity Evaluation of Anti-GD3 Chimeric Antibody:

[0728] 1. Binding Activity of Anti-GD3 Chimeric Antibody to GD3 (ELISA)

[0729] Binding activities of the five kinds of the purified anti-GD3 chimeric antibodies obtained in the item 4 of Example 1 to GD3 were measured by the ELISA shown in

the item 3 of Example 1. **FIG. 2** shows results of the examination of the binding activity measured by changing the concentration of the anti-GD3 chimeric antibody to be added. As shown in **FIG. 2**, the five kinds of the anti-GD3 chimeric antibodies showed almost the same binding activity to GD3. The result shows that antigen binding activities of these antibodies are constant independently of the antibody-producing animal cells and their culturing methods. Also, it was suggested from the comparison of the NS0-GD3 chimeric antibody (302) with the NS0-GD3 chimeric antibody (GIT) that the antigen binding activities are constant independently of the media used in the culturing.

[0730] 2. ADCC Activity of Anti-GD3 Chimeric Antibody

[0731] ADCC activities of the five kinds of the purified anti-GD3 chimeric antibodies obtained in the item 4 of Example 1 were measured in accordance with the following method.

[0732] (1) Preparation of Target Cell Solution

[0733] A human melanoma cell line G-361 (ATCC CRL 1424) was cultured in the RPMI1640-FBS(10) medium to prepare 1×10^6 cells, and the cells were radioisotope-labeled by reacting them with 3.7 MBq equivalents of a radioactive substance Na₂⁵¹CrO₄ at 37° C. for 1 hour. After the reaction, the cells were washed three times through their suspension in the RPMI1640-FBS(10) medium and centrifugation, re-suspended in the medium and then allowed to react at 4° C. for 30 minutes on ice for spontaneous dissolution of the radioactive substance. After centrifugation, the precipitate was adjusted to 2×10^5 cells/ml by adding 5 ml of the RPMI1640-FBS(10) medium and used as the target cell solution.

[0734] (2) Preparation of Effector Cell Solution

[0735] From a healthy donor, 50 ml of venous blood was collected, and gently mixed with 0.5 ml of heparin sodium (manufactured by Takeda Pharmaceutical). The mixture was centrifuged to isolate a mononuclear cell layer using Lymphoprep (manufactured by Nycomed Pharma AS) in accordance with the manufacture's instructions. After washing with the RPMI1640-FBS(10) medium by centrifugation three times, the resulting precipitate was re-suspended to give a density of 2×10^6 cells/ml by using the medium and used as the effector cell solution.

[0736] (3) Measurement of ADCC Activity

[0737] Into each well of a 96 well U-shaped bottom plate (manufactured by Falcon), 50 µl of the target cell solution prepared in the above (1) (1×10^4 cells/well) was dispensed. Next, 100 µl of the effector cell solution prepared in the above (2) was added thereto (2×10^5 cells/well, the ratio of effector cells to target cells becomes 20:1). Subsequently, each of the anti-GD3 chimeric antibodies was added at various concentrations, followed by reaction at 37° C. for 4 hours. After the reaction, the plate was centrifuged, and the amount of ⁵¹Cr in the supernatant was measured with a γ-counter. The amount of spontaneously released ⁵¹Cr was calculated by the same operation using only the medium instead of the effector cell solution and the antibody solution, and measuring the amount of ⁵¹Cr in the supernatant. The amount of total released ⁵¹Cr was calculated by the same operation as above using only the medium instead of the antibody solution and adding 1 N hydrochloric acid

instead of the effector cell solution, and measuring the amount of ^{51}Cr in the supernatant. The ADCC activity was calculated from the following equation (I).

$$\text{ADCC activity (\%)} = \frac{\frac{{}^{51}\text{Cr in sample supernatant} - \text{spontaneously released } {}^{51}\text{Cr}}{\text{total released } {}^{51}\text{Cr} - \text{spontaneously released } {}^{51}\text{Cr}} \times 100}{(1)}$$

[0738] The results are shown in **FIG. 3**. As shown in **FIG. 3**, among the five kinds of the anti-GD3 chimeric antibodies, the YB2/0-GD3 chimeric antibody showed the highest ADCC activity, followed by the SP2/0-GD3 chimeric antibody, NS0-GD3 chimeric antibody and CHO-GD3 chimeric antibody in that order. No difference in the ADCC activity was found between the NS0-GD3 chimeric antibody (302) and NS0-GD3 chimeric antibody (GIT) prepared by using different media in the culturing. The above results show that the ADCC activity of antibodies greatly varies depending on the kind of the animal cells to be used in their production. As its mechanism, since their antigen binding activities were equal, it was considered that ADCC activity depends on a difference in the structure of the Fc region of the antibody.

EXAMPLE 3

[0739] Activity Evaluation of Anti-GD3 Chimeric Antibodies Having a Different Ratio of a Sugar Chain in which 1-Position of Fucose is not Bound to 6-Position of N-acetylglucosamine in the Reducing End:

[0740] 1. Preparation of Anti-GD3 Chimeric Antibodies Having a Different Ratio of a Sugar Chain in which 1-Position of Fucose is not Bound to 6-Position of N-acetylglucosamine in the Reducing End through α -Bond

[0741] In accordance with the method described in the item 2(1) of Example 1, some transformed clones derived from YB2/0 cell capable of producing an anti-GD3 chimeric antibody was obtained. Antibodies were prepared from the transformed clones derived from YB2/0 cell and named lot 1, lot 2 and lot 3. Sugar chain analysis of the anti-GD3 chimeric antibodies of lot 1, lot 2 and lot 3 was carried out by the following method.

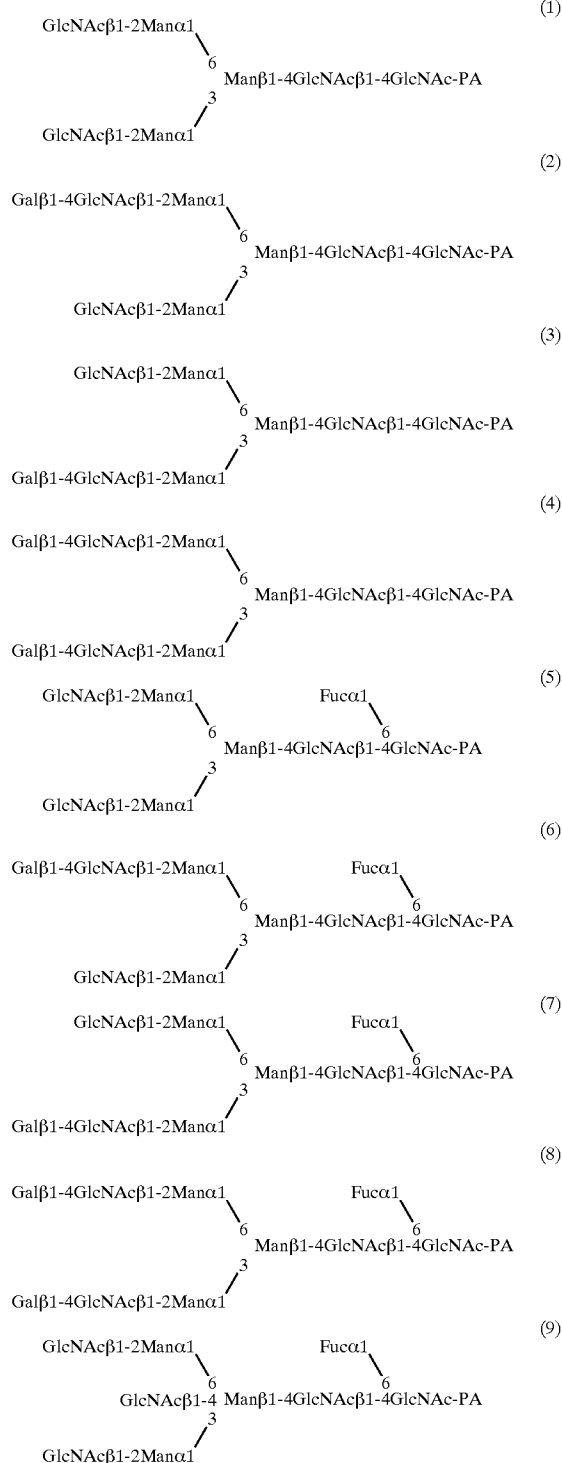
[0742] The solution of each purified antibody was exchanged to 10 mmol/L KH_2PO_4 using Ultra Free 0.5-10K (manufactured by Millipore). The exchange was carried out in such a manner that the exchanging ratio became 80-fold or more.

[0743] Into Hydracub S-204 test tube, 100 μg of each antibody was put and dried with a centrifugal evaporator. The dried sample was subjected to hydrazinolysis using Hydracub manufactured by Hohnen. The sample was allowed to react with hydrazine at 110° C. for 1 hour using a hydrazinolysis reagent manufactured by Hohnen [*Method of Enzymology*, 83, 263 (1982)]. After the reaction, hydrazine was evaporated under a reduced pressure, and the reaction tube was returned to room temperature by allowing it to stand for 30 minutes. Next, 250 of an acetylation reagent manufactured by Hohnen and 25 μl of acetic anhydride were added thereto, followed by thoroughly stirred for reaction at room temperature for 30 minutes. Then, 250 μl of

the acetylation reagent and 25 μl of acetic anhydride were further added thereto, followed by thoroughly stirring for reaction at room temperature for 1 hour. The sample was frozen at -80° C. in a freezer and freeze-dried for about 17 hours. Sugar chains were recovered from the freeze-dried sample by using Cellulose Cartridge Glycan Preparation Kit manufactured by Takara Shuzo. The sample sugar chain solution was dried with a centrifugal evaporator and then subjected to fluorescence labeling with 2-aminopyridine [*J. Biochem.*, 95, 197 (1984)]. The 2-aminopyridine solution was prepared by adding 760 μl of HCl per 1 g of 2-aminopyridine (1 \times PA solution) and diluting the solution 10-fold with reverse osmosis purified water (10-fold diluted PA solution). The sodium cyanoborohydride solution was prepared by adding 20 μl of 1 \times PA solution and 430 μl of reverse osmosis purified water per 10 mg of sodium cyanoborohydride. To the sample, 67 μl of a 10 fold-diluted PA solution was added, followed by reaction at 100° C. for 15 minutes and spontaneously cooled, and 2 μl of sodium cyanoborohydride was further added thereto, followed by reaction at 90° C. for 12 hours for fluorescence labeling of the sample sugar chains. The fluorescence-labeled sugar chain group (PA-treated sugar chain group) was separated from excess reagent by using Superdex Peptide HR 10/30 column (manufactured by Pharmacia). This step was carried out by using 10 mmol/L ammonium bicarbonate as the eluent at a flow rate of 0.5 ml/ml and at a column temperature of room temperature, and using a fluorescence detector of 320 nm excitation wavelength and 400 nm fluorescence wavelength. The eluate was recovered 20 to 30 minutes after addition of the sample and dried with a centrifugal evaporator to be used as purified PA-treated sugar chains.

[0744] Next, reverse phase HPLC analysis of the purified PA-treated sugar chains was carried out by using CLC-ODS column (manufactured by Shimadzu, ϕ 6.0 mm \times 159 mm). The step was carried out at a column temperature of 55° C. and at a flow rate of 1 ml/min and using a fluorescence detector of 320 nm excitation wavelength and 400 nm fluorescence wavelength. The column was equilibrated with a 10 mmol/L sodium phosphate buffer (pH 3.8) and elution was carried out for 80 minutes by a 0.5% 1-butanol linear density gradient. **FIG. 4** shows elution patterns of the purified PA-treated sugar chains of the anti-GD3 antibody of lot 2. Each of the PA-treated sugar chain was identified by post source decay analysis of each peak of the separated PA-treated sugar chains using matrix-assisted laser ionization type of flight mass spectrometry (MALDI-TOF-MS analysis), comparison of elution positions with standards of PA-treated sugar chain manufactured by Takara Shuzo, and reverse phase HPLC analysis after digestion of each PA-treated sugar chain using various enzymes.

[0745] The sugar chain content was calculated from each of the peak area of PA-treated sugar chain by reverse HPLC analysis. A PA-treated sugar chain whose reducing end is not N-acetylglucosamine was excluded from the peak area calculation, because it is an impurity or a by-product during preparation of PA-treated sugar chain. Peaks (i) to (ix) in the figure show the following structures (1) to (9), respectively.



[0746] GlcNAc, Gal, Man, Fuc and PA indicate N-acetylglucosamine, galactose, mannose, fucose and a pyridylamino group, respectively. In FIG. 4, the ratio of a sugar chain in which 1-position of fucose was not bound to 6-position of N-acetylglucosamine in the reducing end

through α -bond was calculated from the area occupied by the peaks (i) to (iv) among (i) to (ix), and the ratio of a sugar chain in which 1-position of fucose was bound to 6-position of N-acetylglucosamine in the reducing end through α -bond was calculated from the area occupied by the peaks (v) to (ix) among (i) to (ix). Each ratio of a sugar chain was shown as an average value of the result of two sugar chain analyses.

[0747] As a result, the ratios of a sugar chain in which 1-position of fucose was not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond were 50%, 45% and 29% in lot 1, lot 2 and lot 3, respectively. Herein, these samples were named anti-GD3 chimeric antibody (50%), anti-GD3 chimeric antibody (45%) and anti-GD3 chimeric antibody (29%).

[0748] Also, sugar chains of the anti-GD3 chimeric antibody derived from the CHO/DG44 cell prepared in the item 2(2) of Example 1 were analyzed in accordance with the above-described method, and it was found that the ratio of a sugar chain in which 1-position of fucose was not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond was 7%. Herein, the sample was named anti-GD3 chimeric antibody (7%).

[0749] Further, the anti-GD3 chimeric antibody (45%) and anti-GD3 chimeric antibody (7%) were mixed at a ratio of anti-GD3 chimeric antibody (45%) anti-GD3 chimeric antibody (7%)=5:3 and 1:7, respectively. Sugar chains of the samples were analyzed in accordance with the above-described method, and the ratios of a sugar chain in which 1-position of fucose was not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond were 24% and 13%. Herein, they were named anti-GD3 chimeric antibody (24%) and anti-GD3 chimeric antibody (13%).

[0750] 2. Evaluation of Binding Activity to GD3 (ELISA)

[0751] The binding activities of the six kinds of the anti-GD3 chimeric antibodies having a different ratio of a sugar chain in which 1-position of fucose was not bound to position of N-acetylglucosamine in the reducing end through α -bond prepared in the item 1 of Example 3 to GD3 were measured by the ELISA shown in the item 3 of Example 1. As a result, all of the six kinds of the anti-GD3 chimeric antibodies showed almost the same GD3-binding activity as shown in FIG. 5, and it was found that the ratio of a sugar chain in which 1-position of fucose was not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond does not have influence on the antigen binding activity of the antibody.

[0752] 3. Evaluation of ADCC Activity on Human Melanoma Cell Line

[0753] ADCC activities of six kinds of the anti-GD3 chimeric antibodies having a different ratio of a sugar chain in which 1-position of fucose was not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond prepared in the item 1 of Example 3 to human melanoma cell line G-361 (ATCC CRL1424) were measured according to the method described in the item 2 of Example 2.

[0754] FIGS. 6 and 7 show results of the measurement of ADCC activity of the six kinds of the anti-GD3 chimeric antibodies having a different ratio of a sugar chain in which

1-position of fucose was bound to 6-position of N-acetylglucosamine in the reducing end through α -bond at various concentrations (0.0005 to 5 $\mu\text{g/ml}$) using effector cells of two healthy donors (A and B). As shown in **FIGS. 6 and 7**, the ADCC activity of the anti-GD3 chimeric antibodies showed a tendency to increase in proportion to the ratio of a sugar chain in which 1-position of fucose was not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond at each antibody concentration. The ADCC activity decreases when the antibody concentration is low.

[0755] At an antibody concentration of 0.05 $\mu\text{g/ml}$, the antibody in which the ratio of a sugar chain in which 1-position of fucose was not bound to 6-position of acetylglucosamine in the reducing end through α -bond was 24%, 29%, 45% or 50% showed almost the same high ADCC activity, but the antibody (13%) or (7%) in which the ratio of a sugar chain in which 1-position of fucose was bound to 6-position of N-acetylglucosamine in the reducing end through (α -bond is less than 20%, 13% or 7% showed the low ADCC activity. These results did not change even if the effector cell having donor was different.

EXAMPLE 4

[0756] Activity Evaluation of Anti-CCR4 Chimeric Antibodies Having a Different Ratio of a Sugar Chain in which 1-Position of Fucose was not Bound to 6-Position of N-acetylglucosamine in the Reducing End through α -Bond.

[0757] 1. Preparation of Cells Stably Producing Anti-CCR4 Chimeric Antibody

[0758] Cells which are capable of stably producing an anti-CCR4 chimeric antibody were prepared as follows by using a tandem type expression vector pKANTEX2160 for an anti-CCR4 chimeric antibody described in WO01/64754.

[0759] (1) Preparation of Producing Cell Using Rat Myeloma YB2/0 Cell

[0760] After introducing 10 μg of the anti-CCR4 chimeric antibody expression vector pKANTEX2160 into 4×10^6 cells of rat myeloma YB2/0 cell (ATCC CRL 1662) by electroporation [*Cytotechnology*, 3, 133 (1990)], the cells were suspended in 40 ml of Hybridoma-SFM-FBS(5) [Hybridoma-SFM medium (manufactured by Invitrogen) comprising 5% FBS (manufactured by PAA Laboratories)] and dispensed at 200 μl /well into a 96 well culture plate (manufactured by Sumitomo Bakelite). After culturing at 37°C for 24 hours in a 5% CO_2 incubator, G418 was added to give a concentration of 1 mg/ml, followed by culturing for 1 to 2 weeks. Culture supernatant was recovered from wells in which growth of transformants showing G418 resistance was observed by the formation of colonies, and antigen binding activity of the anti-CCR4 chimeric antibody in the supernatant was measured by the ELISA described in the item 2 of Example 4.

[0761] Regarding the transformants in wells in which production of the anti-CCR4 chimeric antibody was observed in culture supernatants, in order to increase an amount of the antibody production using a DHFR gene amplification system, each of them was suspended in the Hybridoma-SFM-FBS(5) medium comprising 1 mg/ml G418 and 50 nmol/L DHFR inhibitor MTX (manufactured by SIGMA) to give a density of 1 to 2×10^5 cells/ml, and the suspension was dispensed at 1 ml into each well of a 24 well

plate (manufactured by Greiner). After culturing at 37°C for 1 to 2 weeks in a 5% CO_2 incubator, transformants showing 50 nmol/L MTX resistance were induced. Antigen binding activity of the anti-CCR4 chimeric antibody in culture supernatants in wells in which growth of transformants was observed was measured by the ELISA described in the item 2 of Example 4.

[0762] Regarding the transformants in wells in which production of the anti-CCR4 chimeric antibody was observed in culture supernatants, the MTX concentration was increased by the same method, and a transformant capable of growing in the Hybridoma-SFM-FBS(5) medium comprising 200 nmol/L MTX and of producing the anti-CCR4 chimeric antibody in a large amount was finally obtained. The obtained transformant was made into a single cell (cloning) by limiting dilution twice, and the obtained clone was named KM2760#58-35-16.

[0763] (2) Preparation of Producing Cell Using CHO/DG44 Cell

[0764] After introducing 4 μg of the anti-CCR4 chimeric antibody expression vector pKANTEX2160 into 1.6×10^6 cells of CHO/DG44 cell by electroporation [*Cytotechnology*, 3, 133 (1990)], the cells were suspended in 10 ml of IMDM-dFBS(10)-HT(1) [IMDM medium (manufactured by Invitrogen) comprising 10% dFBS (manufactured by Invitrogen) and 1 \times concentration of MT supplement (manufactured by Invitrogen)] and dispensed at 100 μl /well into a 96 well culture plate (manufactured by Iwaki Glass). After culturing at 37°C for 24 hours in a 5% CO_2 incubator, the medium was changed to MD-dFBS(10) (IMDM medium comprising 10% of dialyzed FBS) followed by culturing for 1 to 2 weeks. Culture supernatant was recovered from wells in which the growth was observed due to formation of a transformant showing HT-independent growth, and an expression amount of the anti-CCR4 chimeric antibody in the supernatant was measured by the ELISA described in the item 2 of Example 4.

[0765] Regarding the transformants in wells in which production of the anti-CCR4-chimeric antibody was observed in culture supernatants, in order to increase an amount of the antibody production using a DHFR gene amplification system, each of them was suspended in the IMDM-dFBS(10) medium comprising 50 nmol/L MTX to give a density of 1 to 2×10^5 cells/ml, and the suspension was dispensed at 0.5 ml into each well of a 24 well plate (manufactured by Iwaki Glass). After culturing at 37°C for 1 to 2 weeks in a 5% CO_2 incubator, transformants showing 50 nmol/L MTX resistance were induced. Regarding the transformants in wells in which the growth was observed, the MTX concentration was increased to 200 nmol/L by the similar method as above, and a transformant capable of growing in the IMDM-dFBS(10) medium comprising 200 nmol/L MTX and of producing the anti-CCR4 chimeric antibody in a large amount was finally obtained. The obtained transformant was named clone 5-03.

[0766] 2. Binding Activity of Antibody to CCR4 Partial Peptide (ELISA)

[0767] Compound 1 (SEQ ID NO:25) was selected as a human CCR4 extracellular region peptide capable of reacting with the anti-CCR4 chimeric antibody. In order to use it in the activity measurement by ELISA, a conjugate with

BSA (manufactured by Nacalai Tesque) was prepared by the following method and used as the antigen. That is, 100 ml of a DMSO solution comprising 25 mg/ml SMCC [4-(N-maleimidomethyl)-cyclohexane-1-carboxylic acid N-hydroxysuccinimide ester] (manufactured by Sigma) was added dropwise to 900 ml of a 10 μ g BSA-containing PBS solution under stirring with a vortex, followed by gently stirring for 30 minutes. To a gel filtration column such as NAP-10 column equilibrated with 25 ml of PBS, 1 ml of the reaction solution was applied and then eluted with 1.5 ml of PBS and the resulting eluate was used as a BSA-SMCC solution (BSA concentration was calculated based on A_{280} measurement). Next, 250 ml of PBS was added to 0.5 mg of Compound 1 and then completely dissolved by adding 250 ml of DMF, and the BSA-SMCC solution was added thereto under vortex, followed by gently stirring for 3 hours. The reaction solution was dialyzed against PBS at 4° C. overnight, sodium azide was added thereto to give a final concentration of 0.05%, and the mixture was filtered through a 0.22 mm filter to be used as a BSA-compound 1 solution.

[0768] The prepared conjugate was dispensed at 0.05 μ g/ml and 50 μ l/well into a 96 well EIA plate (manufactured by Greiner) and incubated for adhesion at 4° C. overnight. After washing each well with PBS, 1% BSA-PBS was added thereto in 100 μ l/well and allowed to react at room temperature to block the remaining active groups. After washing each well with Tween-PBS, a culture supernatant of a transformant was added at 50 μ l/well and allowed to react at room temperature for 1 hour. After the reaction, each well was washed with Tween-PBS, and then a peroxidase-labeled goat anti-human IgG(γ) antibody solution (manufactured by American Qualex) diluted 6000 times with 1% BSA-PBS as the secondary antibody solution was added at 50 μ l/well and allowed to react at room temperature for 1 hour. After the reaction and subsequent washing with Tween-PBS, the ABTS substrate solution was added at 50 μ l/well for color development, and 20 minutes thereafter, the reaction was stopped by adding a 5% SDS solution at 50 μ l/well. Thereafter, the absorbance at OD₄₁₅ was measured. The anti-CCR4 chimeric antibody obtained in the item 1 of Example 4 showed the binding activity to CCR4.

[0769] 3. Purification of Anti-CCR4 Chimeric Antibody

[0770] (1) Culturing of Producing Cell Derived from YB2/0 Cell and Purification of Antibody

[0771] The anti-CCR4 chimeric antibody-expressing transformant cell clone KM2760#58-35-16 obtained in the item 1(1) of Example 4 was suspended in Hybridoma-SFM (manufactured by Invitrogen) medium comprising 200 nmol/L MTX and 5% of Daigo's GF21 (manufactured by Wako Pure Chemical Industries) to give a density of 2×10^5 cells/ml and subjected to fed-batch shaking culturing with a spinner bottle (manufactured by Iwaki Glass) in a constant temperature chamber of 37° C. After culturing for 8 to 10 days and recovering the culture supernatant, the anti-CCR4 chimeric antibody was purified using Prosep-A (manufactured by Millipore) column and gel filtration. The purified anti-CCR4 chimeric antibody was named KM2760-1.

[0772] (2) Culturing of Producing Cell Derived from CHO-DG44 Cell and Purification of Antibody

[0773] The anti-CCR4 chimeric antibody-producing transformant clone 5-03 obtained in the item 1(2) of

Example 4 was cultured at 37° C. in a 5% CO₂ incubator using IMDM-dFBS(10) medium in a 182 cm² flask (manufactured by Greiner). When the cell density reached confluent after several days, the culture supernatant was discarded, and the cells were washed with 25 ml of PBS buffer and then mixed with 35 ml of EXCELL 301 medium (manufactured by JRH). After culturing at 37° C. for 7 days in a 5% CO₂ incubator, the culture supernatant was recovered. The anti-CCR4 chimeric antibody was purified from the culture supernatant using Prosep-A (manufactured by Millipore) column in accordance with the manufacture's instructions. The purified anti-CCR4 chimeric antibody was named KM3060.

[0774] When the binding activity to CCR4 of KM2760-1 and KM3060 was measured by the ELISA described in the item 2 of Example 4, they showed equivalent binding activity.

[0775] 4. Analysis of Purified Anti-CCR4 Chimeric Antibodies

[0776] Each 4 μ g of the two kinds of the anti-CCR4 chimeric antibodies produced by and purified from various animal cells, obtained in the item 3 of Example 4 was subjected to SDS-PAGE in accordance with a known method [*Nature*, 227, 680 (1970)], and the molecular weight and purity were analyzed. In each of the purified anti-CCR4 chimeric antibodies, a single band corresponding to the molecular weight of about 150 Kd was found under non-reducing conditions, and two bands of about 50 Kd and about 25 Kd were found under reducing conditions. The molecular weights almost coincided with the molecular weights deduced from the cDNA nucleotide sequences of antibody H chain and L chain (H chain: about 49 Kd, L chain: about 23 Kd, whole molecule about 144 Kd) and further coincided with reports stating that an IgG type antibody has a molecular weight of about 150 Kd under non-reducing conditions and is degraded into H chain having a molecular weight of about 50 Kd and L chain having a molecular weight of about 25 Kd under reducing conditions caused by cutting an S—S bond in the molecule (*Antibodies*, Chapter 14 (1988), *Monoclonal Antibodies*), thus confirming that the anti-CCR4 chimeric antibody was expressed and purified as an antibody molecule having a correct structure.

[0777] 5. Preparation of Anti-CCR4 Chimeric Antibody Having a Different Ratio of a Sugar Chain in which 1-Position of Fucose was not Bound to 6-Position of N-acetylglucosamine in the Reducing End through α -Bond

[0778] Sugar chains of the anti-CCR4 chimeric antibody KM2760-1 derived from YB2/0 cell and the anti-CCR4 chimeric antibody KM3060 derived as from CHO/DG44 cell prepared in the item 3 of Example 4 were analyzed in accordance with the method in the item 1 of Example 3. The ratios of a sugar chain in which 1-position of fucose was not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond were 87% and 8% in 2760-1 and KM3060, respectively. Herein, the samples were named anti-CCR4 chimeric antibody (87%) and anti-CCR4 chimeric antibody (8%).

[0779] Furthermore, the anti-CCR4 chimeric antibody (87%) and anti-CCR4 chimeric antibody (8%) were mixed at a ratio of anti-CCR4 chimeric antibody (87%): anti-CCR4

chimeric antibody (8%)=1:39, 16:67, 22:57, 32:47 and 42:37, respectively. Sugar chains of these samples were analyzed in accordance with the method of the item 1 of Example 3. The ratios of a sugar chain in which 1-position of fucose was not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond were 9%, 18%, 27%, 39% and 46%, respectively. Herein, these samples were named anti-CCR4 chimeric antibody (9%), anti-CCR4 chimeric antibody (18%), anti-CCR4 chimeric antibody (27%), anti-CCR4 chimeric antibody (39%) and anti-CCR4 chimeric antibody (46%).

[0780] Results of the sugar chain analysis of each of the samples are shown in **FIG. 8**. The ratio of a sugar chain in which 1-position of fucose was not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond was shown as an average value of the result of two sugar chain analyses. Hs

[0781] 6. Evaluation of Binding Activity of Antibody to CCR4 Partial Peptide (ELISA)

[0782] Binding activities of the six kinds of the anti-CCR4 chimeric antibodies having a different ratio of a sugar chain in which 1-position of fucose was not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond prepared in the item 5 of Example 4 to a CCR4 partial peptide were measured in accordance with the method described in the item 2 of Example 4.

[0783] As a result, as shown in **FIG. 9**, the six kinds of the anti-CCR4 chimeric antibodies showed almost the same CCR4-binding activity, and it was found that the ratio of a sugar chain in which 1-position of fucose was not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond does not have influence on the antigen-binding activity of the antibody.

[0784] 7. Evaluation of ADCC Activity on Human CCR4-High Expressing Clone

[0785] The ADCC activity of the anti-CCR4 chimeric antibodies against a human CCR4-high highly expressing cell was measured as follows.

[0786] (1) Preparation of Target Cell Suspension

[0787] Cells (1.5×10^6) of a human CCR4-highly expressing cell, CCR4/EL-4 cell, described in WO01/64754 were prepared and a 5.55 MBq equivalent of a radioactive substance $\text{Na}_2^{51}\text{CrO}_4$ was added thereto, followed by reaction at 37° C. for 1.5 hours to thereby label the cells with a radioisotope. After the reaction, the cells were washed three times by suspension in a medium and subsequent centrifugation, resuspended in the medium and then incubated at 4° C. for 30 minutes on ice for spontaneous dissociation of the radioactive substance. After centrifugation, the cells were adjusted to give a density of 2×10^5 cells/ml by adding 7.5 ml of the medium and used as a target cell suspension.

[0788] (2) Preparation of Human Effector Cell Suspension

[0789] From a healthy donor, 60 ml of peripheral blood was collected, 0.6 ml of heparin sodium (manufactured by Shimizu Pharmaceutical) was added thereto, followed by gently mixing. The mixture was centrifuged (800 g, 20 minutes) to isolate a mononuclear cell layer using Lymphoprep (manufactured by AXIS SHIELD) in accordance with the manufacture's instructions. The cells were washed by

centrifuging (1,400 rpm, 5 minutes) three times in a medium and then re-suspended in the medium to give a density of 5×10^6 cells/ml and used as a human effector cell suspension.

[0790] (3) Measurement of ADCC Activity

[0791] The target cell suspension prepared in the above item (1) was dispensed at 50 μl (1×10^4 cells/well) into each well of a 96 well U-bottom plate (manufactured by Falcon). Next, 100 μl of the effector cell suspension prepared in the above item (2) was added thereto (5×10^5 cells/well, ratio of the human effector cells to the target cells was 50:1). Furthermore, each of the anti-CCR4 chimeric antibodies was added thereto to give a final concentration of 0.0001 to 10 $\mu\text{g}/\text{ml}$ followed by reaction at 37° C. for 4 hours. After the reaction. The plate was centrifuged and the amount of ^{51}Cr in the supernatant was measured with a γ -counter. An amount of the spontaneously dissociated ^{51}Cr was calculated by carrying out the same procedure as above using the medium alone instead of the human effector cell suspension and antibody solution, and measuring the amount of ^{51}Cr in the supernatant. An amount of the total dissociated ^{51}Cr was calculated by carrying out the same procedure as above using 1 mol/L hydrochloric acid solution instead of the antibody solution and human effector cell suspension, and measuring the amount of ^{51}Cr in the supernatant. The ADCC activity (%) was calculated based on the above-mentioned equation (I).

[0792] **FIGS. 10 and 11** show results of the measurement of ADCC activity of the anti-CCR4 chimeric antibodies having a different ratio of a sugar chain in which 1-position of fucose was not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond at various concentrations (0.001 to 10 $\mu\text{g}/\text{ml}$) using effector cells of two healthy donors (A and B), respectively. As shown in **FIGS. 10 and 11**, the ADCC activity of the anti-CCR4 chimeric antibodies showed a tendency to increase in proportion to the ratio of a sugar chain in which 1-position of fucose was not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond at each antibody concentration. The ADCC activity decreases when the antibody concentration is low. At an antibody concentration of 0.01 g/ml, the antibody in which the ratios of a sugar chain in which 1-position of fucose was not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond was 27%, 39% and 46%, respectively, showed almost the same high ADCC activity but the ADCC activity was low in the antibody in which the ratio of a sugar chain in which 1-position of fucose was not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond is less than 20%. These results did not change even if the effector cell having donor was different.

EXAMPLE 5

[0793] Determination of Transcription Product of $\alpha 1,6$ -fucosyltransferase (FUT8) Gene in Host Clone:

[0794] 1. Preparation of Single-Stranded cDNA from Various Clones

[0795] Single-stranded cDNAs were prepared from dhfr-deleted CHO/DG44 cells and rat myeloma YB2/0 cells by the following procedure.

[0796] The CHO/DG44 cells were suspended in IMDM medium (manufactured by Life Technologies) supplemented

with 10% FBS (manufactured by Life Technologies) and 1× concentration HT supplement (manufactured by Life Technologies), and 15 ml of the suspension was inoculated into T75 flask for adhesion cell culture use (manufactured by Greiner) at a density of 2×10^5 cells/ml. Also, the YB2/0 cells were suspended in RPMI 1640 medium (manufactured by Life Technologies) supplemented with 10% FBS (manufactured by Life Technologies) and 4 mmol/l L-GLN (manufactured by Life Technologies), and 15 ml of the suspension was inoculated into T75 flask for suspension cell culture (manufactured by Greiner) at a density of 2×10^5 cells/ml. They were cultured at 37° C. in a 5% CO₂ incubator, and 1 of respective host cells were recovered on the 1st, 2nd, 3rd, 4th and 5th days of the culturing to extract total RNA using RNeasy (manufactured by QIAGEN) in accordance with the manufacture's instructions.

[0797] The total RNA was dissolved in 45 μ l of sterile water, 1 μ l of RQ1 RNase-Free DNase (manufactured by Promega), 5 μ l of the attached 10×DNase buffer and 0.5 μ l of RNasin Ribonuclease Inhibitor (manufactured by Promega) were added thereto, followed by reaction at 37° C. for 30 minutes to degrade genomic DNA contaminated in the sample. After the reaction, the total RNA was purified again using RNeasy (manufactured by QIAGEN) and dissolved in 50 μ l of sterile water.

[0798] In a 20 μ l of the reaction mixture using oligo(dT) as a primer, single-stranded cDNA was synthesized from 3 μ g of each of the obtained total RNA samples by reverse transcription reaction using SUPERSRIPT™ Preamplification System for First Strand cDNA Synthesis (manufactured by Life Technologies) in accordance with the manufacture's instructions. For the cloning of FUT8 and β -actin derived from respective host cells, 1× concentration solution of the reaction solution was used, and for the determination of each gene transcription amount by competitive PCR, 50 fold-diluted aqueous solution of the reaction solution were used. The solutions were stored at -80° C. until use.

[0799] 2. Preparation Method of cDNA Partial Fragments of Chinese Hamster FUT8 and Rat FUT8

[0800] Each cDNA partial fragment of Chinese hamster FUT8 and rat FUT8 was prepared by the following procedure (FIG. 12).

[0801] First, primers (represented by SEQ ID NOs:4 and 5) specific for nucleotide sequences common to human FUT8 cDNA [*J. Biochem.*, 121, 626 (1997)] and swine FUT8 cDNA [*J. Biol. Chem.*, 271, 27810 (1995)] were designed.

[0802] Next, 25 μ l of a reaction solution [ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/l dNTPs and 0.5 μ mol/l of the above gene-specific primers (SEQ ID NOs:4 and 5)] containing 1 μ l of each of the cDNA prepared from CHO cell and cDNA prepared from YB2/0 cell, both obtained in the item 1 of Example 5 on the second day after culturing, and polymerase chain reaction (hereinafter referred to as "PCR") was carried out by using a DNA polymerase ExTaq (manufactured by Takara Shuzo). The PCR was carried out by heating at 94° C. for 1 minute, subsequent 30 cycles of heating at 94° C. for 30 seconds, 55° C. for 30 seconds and 72° C. for 2 minutes as one cycle, and further heating at 72° C. for 10 minutes.

[0803] After the PCR, the reaction solution was subjected to 0.8% agarose gel electrophoresis, and a specific amplified

fragment of 979 bp was purified using GENECLEAN Spin Kit (manufactured by BIO 101) and eluted with 10 μ l of sterile water (hereinafter, the method was used for the purification of DNA fragments from agarose gel). Into a plasmid pCR2.1, 4 μ l of the amplified fragment was employed to insert in accordance with the manufacture's instructions of TOPO TA Cloning Kit (manufactured by Invitrogen), and *E. coli* XL1-Blue was transformed with the reaction solution by the method of Cohen et al. [*Proc. Natl. Acad. Sci. USA*, 69, 2110 (1972)] (hereinafter, the method was used for the transformation of *E. coli*). Plasmid DNA samples were isolated in accordance with a known method [*Nucleic Acids Research*, 7, 1513 (1979)] (hereinafter, the method was used for the isolation of plasmid) from cDNA-inserted six clones among the obtained kanamycin-resistant colonies.

[0804] The nucleotide sequences of cDNAs inserted into the plasmids were determined by using DNA Sequencer 377 (manufactured by Parkin Elmer) and BigDye Terminator Cycle Sequencing FS Ready Reaction kit (manufactured by Parkin Elmer) in accordance with the method of the manufacture's instructions. It was confirmed that all of the inserted cDNAs of which sequences were determined by the method encode the open reading frame (ORF) partial sequences of Chinese hamster FUT8 and rat FUT8 (represented by SEQ ID NOs:6 and 7). Among these, plasmid DNA samples containing absolutely no reading error by the PCR in the sequences were selected. Herein, these plasmids were named CHFT8-pCR2.1 and YBFT8-pCR2.1.

[0805] 3. Preparation of Chinese Hamster β -Actin and Rat β -Actin cDNA

[0806] Chinese hamster β -actin and rat β -actin were prepared by the following procedure (FIG. 13).

[0807] First, a forward primer specific for a common sequence containing translation initiation codon (represented by SEQ ID NO:8) and reverse primers specific for respective sequences containing translation termination codon (represented by SEQ ID NOs:9 and 10) were designed from Chinese hamster β -actin genomic sequence (GenBank, U20114) and rat β -actin genomic sequence [*Nucleic Acids Research*, 11, 1759 (1983)].

[0808] Next, 25 μ l of a reaction solution [1× concentration KOD buffer #1 (manufactured by Toyobo), 0.2 mmol/l dNTPs, 1 mmol/l MgCl₂, 0.4 μ mol/l of the above gene-specific primers (SEQ ID NOs:8 and 9, or SEQ ID NOs:8 and 10) and 5% DMSO] containing 1 μ l of each of the cDNA prepared from CHO cell and cDNA prepared from YB2/0 cell, both obtained in the item 1 of Example 5 on the second day after culturing was prepared, and PCR was carried out by using a DNA polymerase KOD (manufactured by Toyobo). The PCR was carried out by heating at 94° C. for 4 minutes and subsequent 25 cycles of heating at 98° C. for 15 seconds, 65° C. for 2 seconds and 74° C. for 30 seconds as one cycle.

[0809] After the PCR, the reaction solution was subjected to 0.8% agarose gel electrophoresis, and a specific amplified fragment of 1128 bp was purified. The DNA fragment was subjected to 5'-terminal phosphorylation using MEGALABEL (manufactured by Takara Shuzo) in accordance with the manufacture's instructions. The DNA fragment was recovered from the reaction solution using an ethanol precipitation method and dissolved in 10 μ l of sterile water.

[0810] Separately, 3 μ g of a plasmid pBluescript II KS(+) (manufactured by Stratagene) was dissolved in 35 μ l of NEBuffer 2 (manufactured by New England Biolabs), and 16 units of a restriction enzyme EcoRV (manufactured by Takara Shuzo) were added thereto for digestion reaction at 37° C. for 3 hours. To the reaction solution, 35 μ l of 1 mol/l Tris-HCl buffer (pH 8.0) and 3.5 μ l of *E. coli* C15-derived alkaline phosphatase (manufactured by Takara Shuzo) were added thereto, followed by reaction at 65° C. for 30 minutes to thereby dephosphorylate the DNA terminus. The reaction solution was extracted with phenol/chloroform, followed by ethanol precipitation, and the recovered DNA fragment was dissolved in 100 μ l of sterile water.

[0811] Each 4 μ l of the amplified fragment prepared from Chinese hamster cDNA and the amplified fragment (11192 bp) prepared from rat cDNA was mixed with 1 μ l of the EcoRV-EcoRV fragment (about 3.0 Kb) prepared from plasmid pBluescript II KS(+) and 5 μ l of Ligation High (manufactured by Toyobo) for ligation reaction at 16° C. for 30 minutes. Using the reaction solution, *E. coli* XL1-Blue was transformed, and plasmid DNA samples were isolated respectively from the obtained ampicillin-resistant clones in accordance with a known method.

[0812] The nucleotide sequence of each cDNA inserted into the plasmid was determined by using DNA Sequencer 377 (manufactured by Parkin Elmer) and BigDye Terminator Cycle Sequencing FS Ready Reaction kit (manufactured by Parkin Elmer) in accordance with the method of the manufacture's instructions. It was confirmed that all of the inserted cDNAs of which sequences were determined by the method encode the ORF full sequences of Chinese hamster β -actin or rat β -actin. Among these, plasmid DNA samples containing absolutely no reading error of bases by the PCR in the sequences were selected. Herein, the plasmids are called CHAc-pBS and YBAc-pBS.

[0813] 4. Preparation of FUT8 Standard and Internal Control

[0814] In order to measure a transcription amount of mRNA derived from FUT8 gene in each cell, CHFT8-pCR2.1 or YBFT8-pCR2.1, as plasmids in which cDNA partial fragments prepared in the item 2 of Example 5 from Chinese hamster FUT8 or rat FUT8 were inserted into pCR2.1, respectively, were digested with a restriction enzyme EcoRI, and the obtained linear DNAs were used as the standards for the preparation of a calibration curve. CHFT8d-pCR2.1 and YBFT8d-pCR2.1, which were obtained from the CHFT8-pCR2.1 and YBFT8-pCR2.1, by deleting 203 bp between ScaI and HindIII, an inner nucleotide sequence of Chinese hamster FUT8 and rat FUT8, respectively, were digested with a restriction enzyme EcoRI, and the obtained linear DNAs were used as the internal standards for FUT8 amount determination. Details thereof are described below.

[0815] Chinese hamster FUT8 and rat FUT8 standards were prepared as follows. In 40 μ l of NEBuffer 2 (manufactured by New England Biolabs), 2 μ g of the plasmid CHFT8-pCR2.1 was dissolved, 24 units of a restriction enzyme EcoRI (manufactured by Takara Shuzo) were added thereto, followed by digestion reaction at 37° C. for 3 hours. Separately, 2 μ g of the plasmid YBFT8-pCR2.1 was dissolved in 40 μ l of NEBuffer 2 (manufactured by New England Biolabs), and 24 units of a restriction enzyme

EcoRI (manufactured by Takara Shuzo) were added thereto, followed by digestion reaction at 37° C. for 3 hours. By subjecting a part of each of the reaction solutions to 0.8% agarose gel electrophoresis, it was confirmed that an EcoRI-EcoRI fragment (about 1 Kb) containing each of cDNA partial fragments of Chinese hamster FUT8 and rat FUT8 was separated from the plasmids CH8-pCR2.1 and YBFT8-pCR2.1 by the above restriction enzyme digestion reactions. Each of the reaction solutions was diluted with 1 μ g/ml of baker's yeast t-RNA (manufactured by SIGMA) to give a concentration of 0.02 fg/ μ l, 0.2 fg/ μ l, 1 fg/ μ l, 2 fg/ μ l, 10 fg/ μ l, 20 fg/ μ l and 100 fg/ μ l and used as the Chinese hamster FUT8 and rat FUT8 standards.

[0816] Controls of Chinese hamster FUT8 and rat FUT8 were prepared as follows (FIG. 14). By using a DNA polymerase KOD (manufactured by Toyobo), 25 μ l of a reaction solution [1 \times concentration KOD buffer #1 (manufactured by Toyobo), 0.2 mmol/l dNTPs, 1 mmol/l MgCl₂, 0.4 μ mol/l gene-specific primers (SEQ ID NOs:11 and 12) and 5% DMSO] containing 5 ng of CHFT-pCR2.1 or YBFT8-pCR2.1 was prepared, and PCR was carried out. The PCR was carried out by heating at 94° C. for 4 minutes and subsequent 25 cycles of heating at 98° C. for 1 seconds, 65° C. for 2 seconds and 74° C. for 30 seconds as one cycle. After the PCR, the reaction solution was subjected to 0.8% agarose gel electrophoresis, and a specific amplified fragment of about 4.7 Kb was purified. The DNA fragment was subjected to 5'-terminal phosphorylation using MEGALABEL (manufactured by Takara Shuzo) in accordance with the manufacture's instructions, and then the DNA fragment was recovered from the reaction solution by ethanol precipitation and dissolved in 50 μ l of sterile water. The above obtained DNA fragment (5 μ l, about 4.7 kb) and 5 μ l of Ligation High (manufactured by Toyobo) were mixed, followed by self-cyclization reaction at 16° C. for 30 minutes.

[0817] Using the reaction solution, *E. coli* DH5 α was transformed, and plasmid DNA samples were isolated in accordance with a known method from the obtained ampicillin-resistant clones. The nucleotide sequence of each plasmid DNA was determined by using DNA Sequencer 377 (manufactured by Parkin Elmer) and BigDye Terminator Cycle Sequencing FS Ready Reaction kit (manufactured by Parkin Elmer), and it was confirmed that a 203 bp inner nucleotide sequence between ScaI and HindIII of Chinese hamster FUT8 or rat FUT8 was deleted. The obtained plasmids were named CHFT8d-pCR2.1 or YBFT8d-pCR2.1, respectively.

[0818] Next, 2 μ g of the plasmid CHFT8d-pCR2.1 was dissolved in 40 μ l of NEBuffer 2 (manufactured by New England Biolabs), and 24 units of a restriction enzyme EcoRI (manufactured by Takara Shuzo) were added thereto, followed by digestion reaction at 37° C. for 3 hours. Separately, 2 μ g of the plasmid YBFT8d-pCR2.1 was dissolved in 40 μ l of NEBuffer 2 (manufactured by New England Biolabs), and 24 units of a restriction enzyme EcoRI (manufactured by Takara Shuzo) were added thereto, followed by digestion reaction at 37° C. for 3 hours. A part of each of the reaction solutions was subjected to 0.8% agarose gel electrophoresis, and it was confirmed that an EcoRI-EcoRI fragment (about 800 bp) containing a fragment from which 203 bp of the inner nucleotide sequences of Chinese hamster FUT8 or rat FUT8 partial fragments was deleted was separated from the plasmids CHFT8d-pCR2.1

or YBFT8d-pCR2.1 by the above restriction enzyme digestion reactions. Dilutions of 2 fg/ μ l were prepared from the reaction solutions using 1 μ g/ml baker's yeast t-RNA (manufactured by SIGMA) and used as the Chinese hamster FUT8 or rat FUT8 internal controls.

[0819] 5. Preparation of β -Actin Standard and Internal Control

[0820] In order to measure a transcription amount of mRNA derived from β -actin gene in various host cells, CHAc-pBS and YBAC-pBS, as plasmids in which the ORF full length of each cDNA of Chinese hamster β -actin and rat β -actin prepared in the item 3 of Example 5 was inserted into pBluescript II KS(+), respectively, were digested with restriction enzymes HindIII and PstI and restriction enzymes HindIII and KpnI, respectively, and the digested linear DNAs were used as the standards for the preparation of a calibration curve. CHAc-pBS and YBAC-pBS which were obtained from the CHAc-pBS and YBAC-pBS by deleting 180 bp between DraIII and DraIII of an inner nucleotide sequence of Chinese hamster β -actin and rat β -actin were digested with restriction enzymes HindIII and PstI and restriction enzymes HindIII and KpnI, respectively, and the digested linear DNAs were used as the internal controls for β -actin amount determination. Details thereof are described below.

[0821] Chinese hamster β -actin and rat β -actin standards were prepared as follows. In 40 μ l of NEBuffer 2 (manufactured by New England Biolabs), 2 μ g of the plasmid CHAc-pBS was dissolved, and 25 units of a restriction enzyme HindIII (manufactured by Takara Shuzo) and 20 units of PstI (manufactured by Takara Shuzo) were added thereto, followed by digestion reaction at 37° C. for 3 hours. Separately, 2 μ g of the plasmid YBAC-pBS was dissolved in 40 μ l of NEBuffer 2 (manufactured by New England Biolabs), and 25 units of a restriction enzyme HindIII (manufactured by Takara Shuzo) and 24 units of KpnI (manufactured by Takara Shuzo) were added thereto, followed by digestion reaction at 37° C. for 3 hours. A part of each of the reaction solutions was subjected to 0.8% agarose gel electrophoresis, and it was confirmed that a HindIII-PstI fragment and a HindIII-KpnI fragment (about 1.2 Kb) containing the full length ORF of each cDNA of Chinese hamster β -actin and rat β -actin were separated from the plasmids CHAc-pBS and YBAC-pBS by the above restriction enzyme digestion reactions. Each of the reaction solutions was diluted with 1 μ g/ml baker's yeast t-RNA (manufactured by SIGMA) to give a concentration 2 pg/ μ l, 1 pg/ μ l, 200 fg/ μ l, 100 fg/ μ l and 20 fg/ μ l and used as the Chinese hamster β -actin or rat β -actin standards.

[0822] Chinese hamster β -actin and rat β -actin internal controls were prepared as follows (**FIG. 15**). In 100 μ l of NEBuffer 3 (manufactured by New England Biolabs) containing 100 ng/ μ l of BSA (manufactured by New England Biolabs), 2 μ g of CHAc-pBS was dissolved, and 10 units of a restriction enzyme DraIII (manufactured by New England Biolabs) were added thereto, followed by digestion reaction at 37° C. for 3 hours. DNA fragments were recovered from the reaction solution by ethanol precipitation and the DNA termini were changed to blunt-ends using DNA Blunting Kit (manufactured by Takara Shuzo) in accordance with the manufacture's instructions, and then the reaction solution was divided into two equal parts. First, to one part of the

reaction solution, 35 μ l of 1 mol/l Tris-HCl buffer (pH 8.0) and 3.5 μ l of *E. coli* C15-derived alkaline phosphatase (manufactured by Takara Shuzo) were added thereto, followed by reaction at 65° C. for 30 minutes for dephosphorylating the DNA termini. The DNA fragment was recovered by carrying out dephosphorylation treatment, phenol/chloroform extraction treatment and ethanol precipitation treatment and then dissolved in 10 μ l of sterile water. The remaining part of the reaction solution was subjected to 0.8% agarose gel electrophoresis to purify a DNA fragment of about 1.11 Kb containing the ORF partial fragment of Chinese hamster β -actin.

[0823] The dephosphorylated DraIII-DraIII fragment (0.5 μ l), 4.5 μ l of the DraIII-DraIII fragment of about 1.1 Kb and 5 μ l of Ligation High (manufactured by Toyobo) were mixed, followed by ligation reaction at 16° C. for 30 minutes. Using the reaction solution, *E. coli* DH5 α was transformed, and plasmid DNAs were isolated in accordance with a known method from the obtained ampicillin-resistant colonies. The nucleotide sequence of each plasmid DNA was determined using DNA Sequencer 377 (manufactured by Parkin Elmer) and BigDye Terminator Cycle Sequencing FS Ready Reaction kit (manufactured by Parkin Elmer), and it was confirmed that a Chinese hamster β -actin DraIII-DraIII 180 bp inserted into the plasmid was deleted. The plasmid was named CHAc-pBS.

[0824] Also, a plasmid in which rat β -actin DraIII-DraIII 180 bp was deleted was prepared via same steps of CHAc-pBS. The plasmid was named YBAC-pBS.

[0825] Next, 2 μ g of the plasmid CHAc-pBS was dissolved in 40 μ l of NEBuffer 2 (manufactured by New England Biolabs), and 25 units of a restriction enzyme HindIII (manufactured by Takara Shuzo) and 20 units of PstI (manufactured by Takara Shuzo) were added thereto, followed by digestion reaction at 37° C. for 3 hours. Separately, 2 μ g of the plasmid YBAC-pBS was dissolved in 40 μ l of NEBuffer 2 (manufactured by New England Biolabs), and 25 units of a restriction enzyme HindIII (manufactured by Takara Shuzo) and 24 units of KpnI (manufactured by Takara Shuzo) were added thereto, followed by digestion reaction at 37° C. for 3 hours. A part of each of the reaction solutions was subjected to 0.8% agarose gel electrophoresis, and it was confirmed that an HindIII-PstI fragment and HindIII-KpnI fragment (about 1.0 Kb) containing a fragment in which 180 bp of the inner nucleotide sequence of the ORF full length of each cDNA of Chinese hamster β -actin and rat β -actin was deleted were separated from the plasmids CHAc-pBS and YBAC-pBS by the restriction enzyme digestion reactions. Dilutions of 200 fg/ μ l were prepared from the reaction solutions using 1 μ g/ml baker's yeast t-RNA (manufactured by SIGMA) and used as the Chinese hamster β -actin and rat β -actin internal controls.

[0826] 6. Determination of Transcription Amount by Competitive PCR

[0827] Competitive PCR was carried out by using the FUT8 internal control DNA prepared in the item 4 of Example 5 and the host cell-derived cDNA obtained in the item 1 of Example 5 as the templates, and the determined value of the FUT8 transcription product in the host clone was calculated from the relative value of the amount of the amplified product derived from each template. On the other hand, since it is considered that the β -actin gene is tran-

scribed constantly in each cell and its transcription amount is approximately the same between cells, transcription amount of the β -actin gene was determined as an indication of the efficiency of synthesis reaction of cDNA derived from each host clone. That is, the PCR was carried out by using the β -actin internal control DNA prepared in the item 5 of Example 5 and the host cell-derived cDNA obtained in the item 1 of Example 5 as the templates, the determined value of the β -actin transcription product in the host clone was calculated from the relative value of the amount of the amplified product derived from each template. Details thereof are described below.

[0828] The FUT8 transcription product was determined by the following procedure. First, a set of sequence-specific primers (represented by SEQ ID NOs:13 and 14) common to the inner sequences of the ORF partial sequences of Chinese hamster FUT8 and rat FUT8 obtained in the item 2 of Example 5 were designed.

[0832] Next, PCR was carried out by using a DNA polymerase ExTaq (manufactured by Takara Shuzo) in 20 μ l in total volume of a reaction solution [1 \times concentration ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/l dNTPs, 0.5 μ mol/l of the above gene-specific primers (SEQ ID NOs:15 and 16, or SEQ ID NOs:17 and 18) and 5% DMSO] containing 5 μ l of 50 fold-diluted cDNA solution prepared from respective host clone in the item 1 of Example 5 and 5 μ l (1 pg) of the plasmid for internal control. The PCR was carried out by heating at 94° C. for 3 minutes and subsequent 17 cycles of heating at 94° C. for 30 seconds, 65° C. for 1 minute and 72° C. for 2 minutes as one cycle.

[0833] Also, PCR was carried out in a series of reaction in which 5 μ l (10 pg, 5 pg, 1 pg, 500 fg or 100 fg) of the β -actin standard plasmid obtained in the item 5 of Example 5 was added instead of the each host clone-derived cDNA, and used in the preparation of a calibration curve for the β -actin transcription amount.

TABLE 1

Target gene	Primer set*	Size of PCR amplification product (bp)	
		Target	Competitor
FUT8	F: 5'-GTCCATGGTGATCCTGCAGTGTGG-3' R: 5'-CACCAATGATATCTCCAGGTTCC-3'	638	431
β -Actin (Chinese hamster)	F: 5'-GATATCGCTGCGCTCGTTGTCGAC-3' R: 5'-CAGGAAGGAAGGCTGGAAGAGAGC-3'	789	609
β -Actin (Rat)	F: 5'-GATATCGCTGCGCTCGTCGTCGAC-3' R: 5'-CAGGAAGGAAGGCTGGAAGAGAGC-3'	789	609

*F: forward primer, R: reverse primer

[0829] Next, PCR was carried out by using a DNA polymerase ExTaq (manufactured by Takara Shuzo) in 20 μ l in total volume of a reaction solution [1 \times concentration ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/l dNTPs, 0.5 μ mol/l of the above gene-specific primers (SEQ ID NOs:13 and 14) and 5% DMSO] containing 5 μ l of 50 fold-diluted cDNA solution prepared from each of respective host clone in the item 1 of Example 5 and 5 μ l (10 fg) of the plasmid for internal control. The PCR was carried out by heating at 94° C. for 3 minutes and subsequent 32 cycles of heating at 94° C. for 1 minute, 60° C. for 1 minute and 72° C. for 1 minute as one cycle.

[0830] Also, PCR was carried out in a series of reaction in which 5 μ l (0.1 fg, 1 fg, 5 fg, 10 fg, 50 fg, 100 fg, 500 fg or 1 pg) of the FUT8 standard plasmid obtained in the item 4 of Example 5 was added instead of the each host clone-derived cDNA, and used in the preparation of a calibration curve for the FUT8 transcription amount.

[0831] The β -actin transcription product was determined by the following procedure. First, two sets of respective gene-specific primers common to the inner sequences of the ORF full lengths of Chinese hamster β -actin and rat β -actin obtained in the item 3 of Example 5 were designed (the former are represented by SEQ ID NOs:15 and 16, and the latter are represented by SEQ ID NOs:17 and 18).

[0834] By carrying out PCR using the primer set shown in Table 1, a DNA fragment having a size shown in the target column of Table 1 can be amplified from each gene transcription product and each standard, and a DNA fragment having a size shown in the competitor column of Table 1 can be amplified from each internal control.

[0835] After 7 μ l of each of the solutions after PCR was subjected to 1.75% agarose gel electrophoresis, the gel was stained by soaking it for 30 minutes in 1 \times concentration SYBR Green I Nucleic Acid Gel Stain (manufactured by Molecular Probes). The amount of the amplified DNA fragment was measured by calculating luminescence intensity of each amplified DNA using a fluoro-imager (FluorImager SI; manufactured by Molecular Dynamics).

[0836] The amount of an amplified product formed by PCR using a standard plasmid as the template was measured by the above-mentioned method, and a calibration curve was prepared by plotting the measured values against the amounts of the standard plasmid. Using the calibration curve, the amount of cDNA of a gene of interest in each clone was calculated from the amount of the amplified product when each expression clone-derived total cDNA was used as the template, and the amount was defined as the mRNA transcription amount in each clone.

[0837] The amount of the FUT8 transcription product in each host clone when a rat FUT8 sequence was used as the

standard and internal control is shown in **FIG. 16**. Throughout the culturing period, the CHO clone showed a transcription amount 10-fold or higher than that of the YB2/0 clone. The tendency was also found when a Chinese hamster FUT8 sequence was used as the standard and internal control.

[0838] Also, the FUT8 transcription amounts are shown in Table 2 as relative values to the amount of the β -actin transcription product. Throughout the culturing period, the FUT8 transcription amount in the YB 2/0 clone was around 0.1% of β -actin while it was 0.5% to 2% in the CHO/DG44 clone.

[0839] The results shows that the amount of the FUT8 transcription product in YB2/0 clone was significantly smaller than that in the CHO/DG44 clone.

TABLE 2

Clone	Culture days				
	1st	2nd	3rd	4th	5th
CHO	1.95	0.90	0.57	0.52	0.54
YB2/0	0.12	0.11	0.14	0.08	0.07

EXAMPLE 6

[0840] Determination of Transcription Product of FUT8 Gene in Anti-GD3 Chimeric Antibody-Producing Clone:

[0841] 1. Preparation of Single-Stranded cDNA Derived from Various Antibody-Producing Clones

[0842] Single-stranded cDNA was prepared from anti-GD3 chimeric antibody-producing cell clones DCHI01-20 and 61-33 as follows. The clone DCHI01-20 is a transformant clone derived from the CHO/DG4 cell described in item 2(2) of Example 1. Also, the clone 61-33 is a clone obtained by carrying out serum-free adaptation of YB2/0-derived transformant cell clone 7-9-51 (FERM BP-6691, International Patent Organism Depository, National Institute of Advanced Industrial Science and Technology) and then carrying out single cell isolation by two limiting dilution.

[0843] The clone DCHI01-2 were suspended in EXCELL 302 medium (manufactured by JRH BIOSCIENCES) supplemented with 3 mmol/l L-GLN (manufactured by Life Technologies), 0.3% PLURONIC F-68 (manufactured by Life Technologies) and 0.5% fatty acid concentrate (manufactured by Life Technologies), and 15 ml of the suspension was inoculated into T75 flask for suspension cell culture (manufactured by Greiner) at a density of 2×10^5 cells/ml. Also, cells of the clone 61-33 were suspended in Hybridoma-SFM medium (manufactured by Life Technologies) supplemented with 0.2% BSA, and 15 ml of the suspension was inoculated into T75 flask for suspension cell culture (manufactured by Greiner) at a density of 2×10^5 cells/ml. They were cultured at 37° C. in a 5% CO₂ incubator, and 1, 2, 3, 4 and 5 days after culturing 1×10^7 of respective host cells were recovered to extract total RNA using RNeasy (manufactured by QIAGEN) in accordance with the manufacture's instructions.

[0844] The total RNA was dissolved in 45 μ l of sterile water, and 1 μ l of RQ1 RNase-Free DNase (manufactured by Promega), 5 μ l of the attached 10 \times DNase buffer and 0.5 μ l of RNasin Ribonuclease Inhibitor (manufactured by

Promega) were added thereto, followed by reaction at 37° C. for 30 minutes to degrade genomic DNA contaminated in the sample. After the reaction, the total RNA was purified again using RNeasy (manufactured by QIAGEN) and dissolved in 50 μ l of sterile water.

[0845] In a 20 μ l reaction mixture using oligo(dT) as a primer, single-stranded cDNA was synthesized from 3 μ g of each of the obtained total RNA samples by reverse transcription reaction using SUPERScript™ Preamplification System for First Strand cDNA Synthesis (manufactured by Life Technologies) in accordance with the manufacture's instructions. The reaction solution was diluted 50-fold with water and stored at -80° C. until use.

[0846] 2. Determination of Transcription Amounts of Each Gene by Competitive PCR

[0847] The transcription amount of each of the genes of the cDNA derived from the antibody-producing clone obtained in the item 1 of Example 6 was determined by competitive PCR in accordance with the item 6 of Example 5.

[0848] The FUT8 gene-derived mRNA transcription amount in each antibody-producing clone was determined by the following procedure.

[0849] CHFT8-pCR2.1 and YBFT8-pCR2.1, as plasmids in which cDNA partial fragments prepared in item 2 of Example 5 from Chinese hamster FUT8 and rat FUT8, respectively, were inserted into pCR2.1, were digested with a restriction enzyme EcoRI, and the obtained linear DNAs were used as the standards in the preparation of a calibration curve for determining the FUT8 transcription amount.

[0850] CHFT8d-pCR2.1 and YBFT8d-pCR2.1, which were obtained by deleting 203 bp between ScaI and HindIII of an inner nucleotide sequence of Chinese hamster FUT8 and rat FUT8, respectively, in the item 4 of Example 9 were digested with a restriction enzyme EcoRI, and the obtained linear DNAs were used as the internal controls for FUT8 amount determination.

[0851] PCR was carried out by using a DNA polymerase ExTaq (manufactured by Takara Shuzo) in 20 μ l in total volume of a reaction solution [1 \times concentration ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/l dNTPs, 0.5 μ mol/l FUT8 gene-specific primers (SEQ ID NOs:13 and 14) and 5% DMSO] containing 5 μ l of 50 fold-diluted cDNA solution derived from each of the antibody-producing clone in the item 1 of Example 6 and 5 μ l (10 fg) of the plasmid for internal control. The PCR was carried out by heating at 94° C. for 3 minutes and subsequent 32 cycles of heating at 94° C. for 1 minute, 60° C. for 1 minute and 72° C. for 1 minute as one cycle.

[0852] Also, PCR was carried out in a series of reaction in which 5 μ l (0.1 fg, 1 fg, 5 fg, 10 fg, 50 fg, 100 fg, 500 fg or 1 pg) of the FUT8 standard plasmid was added instead of the each antibody-producing clone-derived cDNA, and used in the preparation of a calibration curve for the FUT8 transcription amount. In this case, 1 μ g/ml of a baker's yeast t-RNA (manufactured by SIGMA) was used for the dilution of the standard plasmid.

[0853] On the other hand, since it is considered that the β -actin gene is transcribed constantly in each cell and its transcription amount is approximately the same between

cells, the transcription amount of the β -actin gene was determined as an index of the efficiency of synthesis reaction of cDNA in each antibody-producing clone.

[0854] CHAc-pBS and YBAC-pBS as plasmids in which the OR full length of each cDNA of Chinese hamster β -actin and rat β -actin prepared in the item 3 of Example 5 were inserted into pBluescript II KS(+), respectively, were digested with restriction enzymes HindIII and KpnI, and the obtained linear DNAs were used as the standards in the preparation of a calibration curve for determining the β -actin gene transcription amount.

[0855] CHAc-pBS and YBAC-pBS which were obtained by deleting 180 bp between DraIII and DraIII of an inner nucleotide sequence of Chinese hamster β -actin and rat β -actin, respectively in the item 5 of Example 5, were digested with restriction enzymes HindIII and KpnI, and the obtained linear DNAs were used as the internal controls for β -actin determination.

[0856] PCR was carried out by using a DNA polymerase ExTaq (manufactured by Takara Shuzo) in 20 μ l in total volume of a reaction solution [1 \times concentration ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/l dNTPs, 0.5 μ mol/l β -actin-specific primers (SEQ ID NOs:17 and 118) and 5% DMSO] containing 5 μ l of 50 fold-diluted cDNA solution derived from each of the antibody-producing clones and 5 μ l (1 pg) of the plasmid for internal control. The PCR was carried out by heating at 94° C. for 3 minutes and subsequent 17 cycles of heating at 94° C. for 30 seconds, 65° C. for 1 minute and 72° C. for 2 minutes as one cycle. Also, PCR was carried out in a series of reaction in which 10 pg, 5 pg, 1 pg, 500 fg or 100 fg of the β -actin standard plasmid was added instead of the each antibody-producing clone-derived cDNA, and used in the preparation of a calibration curve for the β -actin transcription amount. In this case, 1 μ g/ml of a baker's yeast t-RNA (manufactured by SIGMA) was used for the dilution of standard plasmid.

[0857] By PCR using the primer set described in Table 1, a DNA fragment having a size shown in the target column of Table 1 can be amplified from each gene transcription product and each standard, and a DNA fragment having a size shown in the competitor column of Table 1 can be amplified from each internal control.

[0858] After 7 μ l of the solutions after PCR was subjected to 1.75% agarose gel electrophoresis, the gel was stained by soaking it for 30 minutes in 1 \times concentration SYBR Green I Nucleic Acid Gel Stain (manufactured by Molecular Probes). The amount of the amplified DNA fragment was measured by calculating luminescence intensity of each amplified DNA using a fluoro-imager (FluorImager SI; manufactured by Molecular Dynamics).

[0859] The amount of the amplified product formed by PCR which used a standard plasmid as the template was measured by the above method, and a calibration curve was prepared by plotting the measured values against the amounts of the standard plasmid. Using the calibration curve, the amount of cDNA of a gene of interest in each clone was calculated from the amount of the amplified product when each antibody-producing clone-derived total cDNA was used as the template, and the value was defined as the mRNA transcription amount in each clone.

[0860] The FUT8 transcription amounts are shown in Table 3 as relative values to the amount of the β -actin

transcription product. Throughout the culturing period, the FUT8 transcription amount in the YB2/0 cell-derived antibody-producing clone 61-33 was 0.3% or less of β -actin while the FUT8 transcription amount in the CHO-derived antibody producing clone DCHI01-20 was 0.7% to 1.5% in the CHO cell-derived antibody-producing cell. The results shows that the amount of the FUT8 transcription product in the YB2/0 cell-derived antibody-producing clone was significantly less than that in the CHO cell-derived antibody-producing clone.

TABLE 3

Clone	Culture days				
	1st	2nd	3rd	4th	5th
DCHI01-20	0.75	0.73	0.99	1.31	1.36
61-33	0.16	0.19	0.24	0.30	<0.10

EXAMPLE 7

[0861] Preparation of Lectin-Resistant CHO/DG44 Cell and Production of Antibody Using the Cell

[0862] 1. Preparation of Lectin-Resistant CHO/DG44

[0863] CHO/DG44 cells were cultured in a 75 cm² flask for adhesion culture (manufactured by Greiner) in IMDM-FBS(10)-HT(1) medium [IMDM medium comprising 10% of FBS and 1 \times concentration of HT supplement (manufactured by GIBCO BRL)] to grow until they reached a stage of just before confluent. After washing the cells with 5 ml of PBS (manufactured by Invitrogen), 1.5 ml of 0.05% trypsin (manufactured by Invitrogen) diluted with Dulbecco PBS was added thereto and cultured at 37° C. for 5 minutes to remove the cells from the flask bottom. The removed cells were recovered by a centrifugation operation generally used in cell culture and suspended in IMDM-FBS(10) medium to give a density of 1 \times 10⁵ cells/ml, and then 0.1 μ g/ml of an alkylating agent N-methyl-N'-nitro-N-nitrosoguanidine (hereinafter referred to as "MNNG", manufactured by Sigma) was added or not added thereto. After culturing at 37° C. for 3 days in a CO₂ incubator (manufactured by TABAI), the culture supernatant was discarded, and the cells were again washed, removed and recovered by the same operations as described above, suspended in IMDM-FBS(10)-HT(1) medium and then inoculated into an adhesion culture 96 well plate (manufactured by IWAKI Glass) to give a density of 1 \times 10³ cells/well. To each well, as the final concentration in medium, 1 mg/ml *Lens culinaris* agglutinin (hereinafter referred to as "LCA", manufactured by Vector), 1 mg/ml *Aleuria aurantia* agglutinin (*Aleuria aurantia* lectin; herein after referred to as "AAL", manufactured by Vector) or 1 mg/ml kidney bean agglutinin (*Phaseolus vulgaris* leucoagglutinin; hereinafter referred to as "L-PHA", manufactured by Vector) was added. After culturing at 37° C. for 2 weeks in a CO₂ incubator, the appeared colonies were obtained as lectin-resistant clone CHO/DG44. Regarding the obtained lectin-resistant clone CHO/DG44, an LCA-resistant clone was named clone CHO-LCA, an AAL-resistant clone was named clone CHO-AAL and an L-PHA-resistant clone was named clone CHO-PHA. When the resistance of these clones to various kinds of lectin was examined, it was found that the clone CHO-LCA was also resistant to AAL and the clone CHO-AAL

was also resistant LCA. In addition, the clone CHO-LCA and the clone CHO-AAL, also showed a resistance to a lectin which recognizes a sugar chain structure identical to the sugar chain structure recognized by LCA and AAL, namely a lectin which recognizes a sugar chain structure in which 6-position of fucose is bound to 1-position of N-acetylglucosamine residue in the reducing end through α -bond in the N-glycoside-linked sugar chain. Specifically, it was found that the clone CHO-LCA and the clone CHO-AAL can show resistance and survive even in a medium supplemented with 1 mg/ml at a final concentration of a pea agglutinin (*Pisum sativum* agglutinin; hereinafter referred to as "PSA", manufactured by Vector). In addition, even when the alkylating agent MNNG was not added, it was able to obtain lectin-resistant clones by increasing the number of cells to be treated. Hereinafter, these clones were used in analyses.

[0864] 2. Preparation of Anti-CCR4 Chimeric Antibody-Producing Cell

[0865] An anti-CCR4 chimeric antibody expression plasmid pKANTEX2160 was introduced into the three kinds of the lectin-resistant clones obtained in the item 1 of Example 7 by the method described in Example 4, and gene amplification by a drug MTX was carried out to prepare an anti-CCR4 human chimeric antibody-producing clone. By measuring an amount of antibody expression by the ELISA described in the item 2 of Example 4, antibody-expressing transformants were obtained from each of the clone CHO-LCA, the clone CHO-AAL and the clone CHO-PHA. Regarding each of the obtained transformants, a transformant derived from the clone CHO-LCA was named clone CHO/CCR4-LCA, a transformant derived from the clone CHO-AAL was named clone CHO/CCR4-AAL and a transformant derived from the clone CHO-PHA was named clone CHO/CCR4-PHA. Further, the clone CHO/CCR4-LCA, as a name of Nega-13, has been deposited on Sep. 26, 2001, as FERM BP-7756 in International Patent Organism Depository, National Institute of Advanced Industrial Science and Technology (Tsukuba Central 6, 1, Higashi 1-Chome Tsukuba-shi, Ibaraki-ken, Japan).

[0866] 3. Production of High ADCC Activity Antibody by Lectin-Resistant CHO Cell

[0867] Using the three kinds of the transformants obtained in the item 2 of Example 7, purified antibodies were obtained by the method described in the item 3 of Example 4. The antigen binding activity of the purified anti-CCR4 chimeric antibodies was evaluated by the ELISA described in the item 2 of Example 4. The antibodies produced by all transformants showed an antigen binding activity similar to that of the antibody produced by a recombinant clone (clone 5-03) prepared in Example 4 using normal CHO/DG44 cell as the host. Using these purified antibodies, ADCC activity of each of the anti-CCR4 chimeric antibodies was evaluated in accordance with the method described in the item 7 of Example 4. The results are shown in **FIG. 17**. In comparison with the antibody produced by the clone 5-03, about 100 fold-increased. ADCC activity was observed in the antibodies produced by the clone CHO/CCR4-LCA and the clone CHO/CCR4-AAL. On the other hand, no significant increase in the ADCC activity was observed in the antibody produced by the clone CHO/CCR4-PHA. Also, when ADCC activities of the antibodies produced by the clone CHO/

CCR4-LCA and the YB2/0 cell-derived clone were compared in accordance with the method described in the item 7 of Example 4, it was found that the antibody produced by the clone CHO/CCR4-LCA shows higher ADCC activity, similar to the case of the antibody KM2760-1 produced by the YB2/0 cell-derived clone prepared in the item 1 of Example 4 (**FIG. 18**).

[0868] 4. Sugar Chain Analysis of Antibody Produced by Lectin-Resistant CHO Cell

[0869] Sugar chains of the anti-CCR4 chimeric antibodies purified in the item 3 of Example 7 were analyzed according to the method described in the item 3 of Example 3. **FIG. 19** shows elution patterns of the purified PA-treated sugar chains of the various anti-CCR4 chimeric antibodies.

[0870] Table 4 shows the result of ratios (%) of a sugar chain in which 1-position of fucose was not bound to 6-position of N-acetylglucosamine in the reducing end obtained by the result of the sugar chain analysis of the anti-CCR4 chimeric antibodies produced by various lectin-resistant clones.

TABLE 4

Antibody producing cell	Ratio of α 1,6-Fucose-free sugar chain (%)
Clone 5-03	9
Clone CHO/CCR4-LCA	48
Clone CHO/CCR4-AAL	27
Clone CHO/CCR4-PHA	8

[0871] In comparison with the antibody produced by the clone 5-03, the ratio of a sugar chain in which 1-position of fucose was not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond was increased from 9% to 48% in the antibody produced by the clone CHO/CCR4-LCA. The ratio of α 1,6-fucose-free sugar chains was increased from 9% to 27% in the antibody produced by the clone CHO/CCR4-AAL. On the other hand, changes in the sugar chain pattern and the ratio of α 1,6-fucose-free sugar chains were hardly found in the clone CHO/CCR4-PHA when compared with the clone 5-03.

EXAMPLE 8

[0872] Analysis of Lectin-Resistant CHO Clone:

[0873] 1. Analysis of Expression Amount of GMD Enzyme in Anti-CCR4 Chimeric Antibody-Producing Clone CHO/CCR4-LCA

[0874] The expression amount of each of the genes of GMD, GFPP and FX, known as fucose biosynthesis enzymes and FUT8 as a fucosyltransferase, in the anti-CCR4 human chimeric antibody-producing clone CHO/CCR4-LCA obtained in Example 7, was analyzed using RT-PCR method.,

[0875] (1) Preparation of RNA from Various Clones

[0876] Each of CHO/DG44 cell, the anti-CCR4 human chimeric antibody-producing clone 5-03 obtained in the item 1(2) of Example 4 and the anti-CCR4 chimeric antibody-producing clone CHO/CCR4-LCA obtained in the item 2 of Example 7 was subcultured at 37° C. in a 5% CO₂ incubator and then cultured for 4 days. After culturing, RNA was

prepared from 1×10^7 cells of each clone using RNeasy Protect Mini Kit (manufactured by QIAGEN) in accordance with the manufacture's instructions. Subsequently, single-stranded cDNA was synthesized from 5 μ g of each RNA in a 20 μ l of a reaction solution using SUPER SCRIPT First-Strand Synthesis System for RT-PCR (manufactured by GIBCO BRL) in accordance with the manufacture's instructions.

[0877] (2) Analysis of Expression Amount of GMD Gene Using RT-PCR

[0878] In order to amplify GMD cDNA by PCR, a 24 mer synthetic DNA primer having the nucleotide sequence shown by SEQ ID NO. 32 and a 26 mer synthetic DNA primer having the nucleotide sequence shown by SEQ ID NO:33 were prepared based on the CHO cell-derived GMD cDNA sequence shown in the item 1 of Reference Example 2.

[0879] Next, 20 μ l of a reaction solution [1 \times concentration Ex Taq buffer (manufactured by Takara Shuzo), 0.2 mmol/L dNTPs, 0.5 unit of Ex Taq polymerase (manufactured by Takara Shuzo) and 0.5 μ l of the synthetic, DNA primers of SEQ ID NOs:32 and 33] containing 0.5 μ l of the single-stranded cDNA derived from each clone in the item 1(1) of Example 8 as the template was prepared, and PCR was carried out by using DNA Thermal Cycler 480 (manufactured by Perkin Elmer) by heating at 94° C. for 5 minutes and subsequent 30 cycles of heating at 94° C. for 1 minute and 68° C. for 2 minutes as one cycle. After subjecting 10 μ l of the PCR reaction solution to agarose electrophoresis, DNA fragments were stained with Cyber Green (manufactured by BMA) and then the amount of the DNA fragment of about 350 bp was measured by using Fluor Imager SI (manufactured by Molecular Dynamics).

[0880] (3) Analysis of Expression Amount of GFPP Gene Using RT-PCR

[0881] In order to amplify GFPP cDNA by PCR, a 27 mer synthetic DNA primer having the nucleotide sequence shown by SEQ ID NO:34 and a 23 mer synthetic DNA primer having the nucleotide sequence shown by SEQ ID NO:35 were prepared based on the CHO cell-derived GFPP cDNA sequence obtained in the item 2 of Reference Example 1.

[0882] Next, 20 μ l of a reaction solution [1 \times Ex Taq buffer (manufactured by Takara Shuzo), 0.2 mmol/L dNTPs, 0.5 unit of Ex Taq polymerase (manufactured by Takara Shuzo) and 0.5 μ l mol/L of the synthetic DNA primers of SEQ ID NOs:34 and 35] containing 0.5 μ l of the single-stranded cDNA prepared from each clone in the item 1(1) of Example 8 as the template was prepared, and PCR was carried out by using DNA Thermal Cycler 480 (manufactured by Perkin Elmer) by heating at 94° C. for 5 minutes and subsequent 24 cycles of heating at 94° C. for 1 minute and 68° C. for 2 minutes as one cycle. After subjecting 10 μ l of the PCR reaction solution to agarose electrophoresis, DNA fragments were stained with Cyber Green (manufactured by BMA) and then the amount of the DNA fragment of about 600 bp was measured by using Fluor Imager SI (manufactured by Molecular Dynamics).

[0883] (4) Analysis of Expression Amount of FX Gene Using RT-PCR

[0884] In order to amplify FX cDNA by PCR, a 28 mer synthetic DNA primer having the nucleotide sequence shown by SEQ ID NO:36 and a 28 mer synthetic DNA primer having the nucleotide sequence shown by SEQ ID NO:37 were prepared based on the CHO cell-derived FX cDNA sequence shown in the item 1 of Reference Example 1.

[0885] Next, 20 μ l of a reaction solution [1 \times concentration Ex Taq buffer (manufactured by Takara Shuzo), 0.2 mmol/L dNTPs, 0.5 unit of Ex Taq polymerase (manufactured by Takara Shuzo) and 0.5 μ mol/L of the synthetic DNA primers of SEQ ID NO:36 and SEQ ID NO:7] containing 0.5 μ l of the single-stranded cDNA derived from each clone in the item 1(1) of Example 8 as the template was prepared, and PCR was carried out by using DNA Thermal Cycler 480 (manufactured by Perkin Elmer) by heating at 94° C. for 5 minutes and subsequent 22 cycles of heating at 94° C. for 1 minute and 68° C. for 2 minutes as one cycle. After subjecting 10 μ l of the PCR reaction solution to agarose electrophoresis, DNA fragments were stained with Cyber Green (manufactured by BMA) and then the amount of the DNA fragment of about 300 bp was measured by using Fluor Imager Si (manufactured by Molecular Dynamics).

[0886] (5) Analysis of Expression Amount of FUT8 Gene Using RT-PCR

[0887] In order to amplify FUT8 cDNA by PCR, 20 μ l of a reaction solution [1 \times Ex Taq buffer (manufactured by Takara Shuzo), 0.2 mmol/L dNTPs, 0.5 unit of Ex Taq polymerase (manufactured by Takara Shuzo) and 0.5 μ mol/L of the synthetic DNA primers of SEQ ID NOs:13 and 14] containing 0.5 μ l of the single-stranded cDNA derived from each clone in the item 1(1) of Example 8 as the template was prepared, and PCR was carried out by using DNA Thermal Cycler 480 (manufactured by Perkin Elmer) by heating at 94° C. for 5 minutes and subsequent 20 cycles of heating at 94° C. for 1 minute and 68° C. for 2 minutes as one cycle. After subjecting 10 μ l of the PCR reaction solution to agarose electrophoresis, DNA fragments were stained with Cyber Green (manufactured by BMA) and then amount of the DNA fragment of about 600 bp was measured using Fluor Imager SI (manufactured by Molecular Dynamics).

[0888] (6) Analysis of Expression Amount of β -Actin Gene Using RT-PCR

[0889] In order to amplify β -actin cDNA by PCR, 20 μ l of a reaction solution [1 \times Ex Taq buffer (manufactured by Takara Shuzo), 0.2 mmol/L dNTPs, 0.5 unit of Ex Taq polymerase (manufactured by Takara Shuzo) and 0.5 μ mol/L of the synthetic DNA primers of SEQ ID NOs:15 and 16] containing 0.5 μ l of the single-stranded cDNA derived from each clone in the item 1(1) of Example 8 as the template was prepared, and PCR was carried out by using DNA Thermal Cycler 480 (manufactured by Perkin Elmer) by heating at 94° C. for 5 minutes and subsequent 14 cycles of heating at 94° C. for 1 minute and 68° C. for 2 minutes as one cycle. After subjecting 10 μ l of the PCR reaction solution to agarose electrophoresis, DNA fragments were stained with Cyber Green (manufactured by BMA) and then the amount of the DNA fragment of about 800 bp was measured using Fluor Imager SI (manufactured by Molecular Dynamics).

[0890] (7) Expression Amount of GMD, GFPP, FX and FUT8 Genes in Each Clone

[0891] The amount of the PCR-amplified fragment of each gene in the clone 5-03 and the clone CHO/CCR4-LCA was calculated by dividing values of the amounts of PCR-amplified fragments derived from GMD, GFPP, FX and FUT cDNA in each clone measured in the items 1(2) to 1(6) of Example 8 by the value of the amount of PCR-amplified fragment derived from β -actin cDNA in each clone, and defining the amount of the PCR-amplified fragments in CHO/DG44 cell as 1. The results are shown in

TABLE 5

	GMD	GEPP	FX	FUT8
Clone CHO/DG44	1	1	1	1
Clone CHO/DG44	1.107	0.793	1.093	0.901
Anti-CCR4 antibody-producing cell				
Clone 5-03				
Derived from clone 5-03	0.160	0.886	0.920	0.875
LCA-resistant cell				
CHO/CCR4-LCA				

[0892] As shown in Table 5, the expression amount of GMD gene in the clone CHO/CCR4-LCA was decreased to about $\frac{1}{10}$ in comparison with other clones. In this case, the test was independently carried out twice, and the average value was used.

[0893] 2. Analysis Using Anti-CCR4 Chimeric Antibody-Producing Clone CHO/CCR4-LCA in which GMD Gene was Forced to Express

[0894] (1) Construction of CHO Cell-Derived GMD Gene Expression Vector pAGE249GMD

[0895] Based on the CHO cell-derived GMD cDNA sequence obtained in the item 1 of Reference Example 2, a 28 mer primer having the nucleotide sequence shown by SEQ ID: NO:38 and a 29 mer primer having the nucleotide sequence shown by SEQ ID NO:39 were prepared. Next, 20 μ l of a reaction solution [1 \times concentration Ex Taq buffer (manufactured by Takara Shuzo), 0.2 mmol/L dNTPs, 0.5 unit of Ex Taq polymerase (manufactured by Takara Shuzo) and 0.5 μ mol/L of the synthetic DNA primers of SEQ ID NOs:38 and 391 containing 0.5 μ l of the CHO cell-derived single-stranded cDNA prepared in the item 1(1) of Example 8 as the template was prepared, and PCR was carried out by using DNA Thermal Cycler 480 (manufactured by Perkin Elmer) by heating at 94° C. for 5 minutes and subsequently 8 cycles of heating at 94° C. for 1 minute, 58° C. for 1 minute and 72° C. for 1 minute as one cycle, and then 22 cycles of heating at 94° C. for 1 minute and 68° C. as one cycle. After completion of the reaction, the PCR reaction solution was fractionated by agarose electrophoresis, and then a DNA fragment of about 600 bp was recovered. The recovered DNA fragment was connected to pT7Blue(R) vector (manufactured by Novagen) by using DNA Ligation Kit (manufactured by Takara Shuzo), and *E. coli* DH5 α (manufactured by Toyobo) was transformed using the obtained recombinant plasmid DNA to obtain a plasmid mt-C (FIG. 20).

[0896] Next, based on the CHO cell-derived GMD cDNA sequence obtained in the item 1 of Reference Example 2, a 45 mer primer having the nucleotide sequence shown by

SEQ ID NO:40 and a 31 mer primer having the nucleotide sequence shown by SEQ ID NO:41 were prepared. Next, 20 μ l of a reaction solution [1 \times Ex Taq buffer (manufactured by Takara Shuzo), 0.2 mmol/L dNTPs, 0.5 unit of Ex Taq polymerase (manufactured by Takara Shuzo) and 0.5 μ mol/L of the synthetic DNA primers of SEQ ID NOs:40 and 41] containing 0.5 μ l of the CHO cell-derived single-stranded cDNA prepared in the item 1(1) of Example 8 as the template was prepared, and PCR was carried out by using DNA Thermal Cycler 480 (manufactured by Perkin Elmer) by heating at 94° C. for 5 minutes and subsequently 8 cycles of heating at 94° C. for 1 minute, 57° C. for 1 minute and 72° C. for 1 minute as one cycle, and then 22 cycles of heating at 94° C. for 1 minute and 68° C. for 2 minutes as one cycle. After completion of the reaction, the PCR reaction solution was fractionated by agarose electrophoresis, and then a DNA fragment of about 150 bp was recovered. The recovered DNA fragment was connected to pT7Blue(R) vector (manufactured by Novagen) by using DNA Ligation Kit (manufactured by Takara Shuzo), and *E. coli* DH5 α (manufactured by Toyobo) was transformed using the obtained recombinant plasmid DNA to obtain a plasmid ATG (FIG. 21).

[0897] Next, 3 μ g of the plasmid CHO-GMD prepared in the item 1 of Reference Example 2 was allowed to react with a restriction enzyme SacI (manufactured by Takara Shuzo) at 37° C. for 16 hours, a DNA was recovered by carrying out phenol/chloroform extraction and ethanol precipitation and allowed to react with a restriction enzyme EcoRI (manufactured by Takara Shuzo) at 37° C. for 16 hours. A digest DNA was fractionated by agarose electrophoresis and then a DNA fragment of about 900 bp was recovered. The plasmid mt-C (1.4 μ g) was allowed to react with a restriction enzyme SacI (manufactured by Takara Shuzo) at 37° C. for 16 hours, DNA was recovered by carrying out phenol/chloroform extraction and ethanol precipitation and allowed to react with a restriction enzyme EcoRI (manufactured by Takara Shuzo) at 37° C. for 16 hours. A digested DNA was fractionated by agarose electrophoresis and, then a DNA fragment of about 3.1 kbp was recovered. The recovered DNA fragments were ligated by using DNA Ligation Kit (manufactured by Takara Shuzo), and *E. coli* DH5 α was transformed using the obtained recombinant plasmid DNA to obtain a plasmid WT-N(-) (FIG. 22).

[0898] Next, 2 μ g of the plasmid WT-N(-) was allowed to react with a restriction enzyme BamHI (manufactured by Takara Shuzo) at 37° C. for 16 hours, DNA was recovered by carrying out phenol/chloroform extraction and ethanol precipitation and allowed to react with a restriction enzyme EcoRI (manufactured by Takara Shuzo) at 37° C. for 16 hours. A digested DNA was fractionated by agarose electrophoresis and then a DNA fragment of about 1 kbp was recovered by using Gene Clean II Kit (manufactured by BIO 101) in accordance with the manufacture's instructions. The plasmid pBluescript SK(-) (3 μ g; manufactured by Stratagene) was allowed to react with a restriction enzyme BamHI (manufactured by Takara Shuzo) at 37° C. for 16 hours, DNA was recovered by carrying out phenol/chloroform extraction and ethanol precipitation and allowed to react with a restriction enzyme EcoRI (manufactured by Takara Shuzo) at 37° C. for 16 hours. A digested DNA was fractionated by agarose electrophoresis and then a DNA fragment of about 3 kbp was recovered. The respective recovered DNA fragments were ligated by using DNA

Ligation Kit (manufactured by Takara Shuzo), and *E. coli* DH5 α was transformed using the obtained recombinant plasmid DNA to obtain a plasmid WT-N(-) in pBS (cf. FIG. 23).

[0899] Next, 2 μ g of the plasmid WT-N(-) in pBS was allowed to react with a restriction enzyme HindIII (manufactured by Takara Shuzo) at 37° C. for 16 hours, DNA was recovered by carrying out phenol/chloroform extraction and ethanol precipitation and allowed to react with a restriction enzyme EcoRI (manufactured by Takara Shuzo) at 37° C. for 16 hours. A digested DNA was fractionated by agarose electrophoresis and then a DNA fragment of about 4 kbp was recovered. After 2 μ g of the plasmid ATG was allowed to react with a restriction enzyme HindIII (manufactured by Takara Shuzo) at 37° C. for 16 hours, DNA was recovered by carrying out phenol/chloroform extraction and ethanol precipitation and allowed to react with a restriction enzyme EcoRI (manufactured by Takara Shuzo) at 37° C. for 16 hours. A digested DNA was fractionated by agarose electrophoresis and then a DNA fragment of about 150 bp was recovered. The respective recovered DNA fragments were ligated by using DNA Ligation Kit (manufactured by Takara Shuzo), and *E. coli* DH5 α was transformed using the obtained recombinant plasmid DNA to obtain a plasmid WT in pBS (FIG. 24).

[0900] Next, 2 μ g of the plasmid pAGE249 was allowed to react with restriction enzymes HindIII and BamHI (both manufactured by Takara Shuzo) at 37° C. for 16 hours. A digested DNA was fractionated by agarose electrophoresis and then a DNA fragment of about 6.5 kbp was recovered. The plasmid WT (2 μ g) in pBS was allowed to react with restriction enzymes HindIII and BamHI (both manufactured by Takara Shuzo) at 37° C. for 16 hours. A digested DNA was fractionated by agarose electrophoresis and then a DNA fragment of about 1.2 kbp was recovered. The respective recovered DNA fragments were ligated by using DNA Ligation Kit (manufactured by Takara Shuzo), and *E. coli* DH5 α was transformed using the obtained recombinant plasmid DNA to obtain a plasmid pAGE249GM (FIG. 25).

[0901] (2) Stable Expression of GMD Gene in Clone CHO/CCR4-LCA

[0902] The CHO cell-derived GMD gene expression vector pAGE249GMD (5 μ g) which was made into linear form by digesting it with a restriction enzyme FspI (manufactured by NEW ENGLAND BIOLABS), was introduced into at 1.6×10^6 cells of the clone CHO/CCR4-LCA by electroporation [Cytotechnology 3, 133 (1990)]. Then, the cells were suspended in 30 ml of IMDM-dFBS(10) medium (M medium (manufactured by GIBCO BRL) supplemented with 10% of dFBS) comprising 200 nmol/L MTX (manufactured by SIGMA), and cultured in a 182 cm² flask (manufactured by Greiner) at 37° C. for 24 hours in a 5% CO₂ incubator. After culturing, the medium was changed to IMDM-dFBS(10) medium containing 0.5 mg/ml hygromycin and 200 nmol/L MTX (manufactured by SIGMA), followed by culturing for 19 days to obtain colonies of hygromycin-resistant transformants.

[0903] In the same manner, the pAGE249 vector was introduced into the clone CHO/CCR4-LCA by the same method as above to obtain colonies of hygromycin-resistant transformants.

[0904] (3) Culturing of GMD Gene-Expressed Clone CHO/CCR4-LCA and Purification of Antibody

[0905] Using IMDM-dFBS(10) medium comprising 200 nmol/L MTX (manufactured by SIGMA) and 0.5 mg/ml hygromycin, the GMD-expressing transformant cells obtained in the item 2(2) of Example 8 were cultured in a 182 cm² flask (manufactured by Greiner) at 37° C. in a 5% CO₂ incubator. Several days thereafter, when the cell density reached confluent, the culture supernatant was discarded and the cells were washed with 25 ml of PBS buffer (manufactured by GIBCO BRL) and mixed with 35 ml of EXCELL301 medium (manufactured by JRH). After culturing at 37° C. in a 5% CO₂ incubator for 7 days, the culture supernatant was recovered. An anti-CCR4 chimeric antibody was purified from the culture supernatant by using Prosep-A column (manufactured by Millipore) in accordance with the manufacture's instructions.

[0906] In the same manner, the pAGE249 vector-introduced transformant cells were cultured by the same method as above and then anti-CCR4 chimeric antibody was recovered and purified from the culture supernatant.

[0907] (4) Measurement of Lectin Resistance in Transformed Cells

[0908] The GMD gene-expressing transformant cells obtained in the item 2(2) of Example 8 were suspended in IMDM-dFBS(10) medium comprising 200 nmol/L MTX (manufactured by SIGMA) and 0.5 mg/ml hygromycin to give a density of 6×10^4 cells/ml, and the suspension was dispensed at 50 l/well into a 96 well culture plate (manufactured by Iwaki Glass). Next, a medium prepared by suspending LCA (*Lens culinaris* agglutinin: manufactured by Vector Laboratories) at concentrations of 0 mg/ml, 0.4 mg/ml, 1.6 mg/ml or 4 mg/ml in IMDM-dFBS(10) medium containing 200 nmol/L MTX (manufactured by SIGMA) and 0.5 mg/ml hygromycin was added to the plate at 50 μ l/well, followed by culturing at 37° C. for 96 hours in a 5% CO₂ incubator. After culturing, WST-1 (manufactured by Boehringer) was added at 10 μ l/well and incubated at 37° C. for 30 minutes in a 5% CO₂ incubator for color development, and then the absorbance at 450 nm and 595 nm (hereinafter referred to as "OD450" and "OD595", respectively) was measured by using Microplate Reader (manufactured by BIO-RAD). In the same manner, the pAGE249 vector-introduced transformant cells were measured by the same method as above. The above-mentioned test was carried out twice independently.

[0909] FIG. 26 shows the number of survived cells in each well by percentage when a value calculated by subtracting OD595 from OD450 measured in the above is used as the survived number of each cell group and the number of survived cells in each of the LCA-free wells is defined as 100%. As shown in FIG. 26, decrease in the LCA-resistance was observed in the GMD-expressed clone CHO/CCR4-LCA, and the survival ratio was about 40% in the presence of 0.2 mg/ml LCA and the survival ratio was about 20% in the presence of 0.8 mg/ml LCA. On the other hand, in the pAGE249 vector-introduced stain CHO/CCR4-LCA, the survival ratio was 100% in the presence of 0.2 mg/ml LCA and the survival ratio was about 80% even in the presence of 0.8 mg/ml LCA. Based on the above results, it was suggested that expression amount of GMD gene in the clone CHO/CCR4-LCA was decreased and, as a result, the resistance against LCA was obtained.

[0910] (5) ADCC Activity of Anti-CCR4 Chimeric Antibody Obtained from GMD-Expressed Clone CHO/CCR4-LCA

[0911] ADCC activity of the purified anti-CCR4 chimeric antibody obtained in the item 2(3) of Example 8 was measured in accordance with the following method.

[0912] i) Preparation of Target Cell Suspension

[0913] CCR4/ELA cell described in WO01/64754 was prepared at 1×10^6 cells and 3.7 MBq equivalent of a radioactive substance $\text{Na}_2^{51}\text{CrO}_4$ was added to thereto, followed by reaction at 37°C . for 90 minutes to thereby label the cells with a radioisotope. After the reaction, the cells were washed three times by suspension in the RPMI1640-FBS(10) medium and subsequent centrifugation, resuspended in the medium and then incubated at 4°C . for 30 minutes on ice for spontaneous dissociation of the radioactive substance. After centrifugation, the cells were adjusted to 2.0×10^5 cells/ml by adding 5 ml of the RPMI1640-FBS(10) medium and used as a target cell suspension.

[0914] ii) Preparation of Effector Cell Suspension

[0915] From a healthy donor, 50 ml of venous blood was collected and gently mixed with 0.5 ml of heparin sodium (manufactured by Takeda Pharmaceutical). Using Lymphoprep (manufactured by Nycomed Pharma AS), the mixture was centrifuged in accordance with the manufacture's instructions to separate a mononuclear cell layer. The cells were washed three times by centrifuging with the RPMI1640-FBS(10) medium and then resuspended in the medium to give a density of 2.5×10^6 cells/ml and used as a effector cell suspension.

[0916] iii) Measurement of ADCC Activity

[0917] The target cell suspension prepared in the above i) was dispensed at $50 \mu\text{l}$ (1×10^4 cells/well) into each well of a 96 well U-bottom plate (manufactured by Falcon). Next, $100 \mu\text{l}$ of the effector cell suspension prepared in the above ii) was added thereto (2×10^5 cells/well, ratio of the effector cells to the target cells was 25:1). Each of various anti-CCR4 chimeric antibodies was further added thereto to give a final concentration of 0.0025 to $2.5 \mu\text{g/ml}$, followed by reaction at 37°C . for 4 hours. After the reaction, the plate was centrifuged and the amount ^{51}Cr in the supernatant was measured with a γ -counter. The amount of the spontaneously dissociated ^{51}Cr was calculated by carrying out the same procedure using the medium alone instead of the effector cell suspension and antibody solution, and measuring the amount ^{51}Cr in the supernatant. The amount of the total dissociated ^{51}Cr was calculated by carrying out the same procedure using the medium alone instead of the antibody solution and adding 1 mol/L hydrochloric acid instead of the effector cell suspension and measuring the amount of ^{51}Cr in the supernatant. The ADCC activity was calculated based on the above equation (1).

[0918] Results of the measurement of ADCC activity are shown in FIG. 27. As shown in FIG. 27, ADCC activity of the purified anti-CCR4 chimeric antibody obtained from the GMD-expressed clone CHO/CCR4-LCA was decreased to a similar degree to that of the KM3060 produced by the normal CHO cell-derived antibody-producing clone obtained in Example 4. On the other hand, ADCC activity of the purified anti-CCR4 chimeric antibody obtained from the

pAGE249 vector-introduced clone CHO/CCR4-LCA showed a similar degree of ADCC activity to that of the purified anti-CCR4 chimeric antibody obtained from the clone CHO/CCR4-LCA. Based on the above results, it was suggested that expression amount of GMD gene in the clone CHO/CCR4-LCA is decreased and, as a result, an antibody having high ADCC activity can be produced.

[0919] (6) Sugar Chain Analysis of Anti-CCR4 Chimeric Antibody Derived from GMD-Expressed Clone CHO/CCR4-LCA

[0920] Sugar chains binding to the purified anti-CCR4 chimeric antibody obtained in the item 2(3) of Example 8 were analyzed in accordance with the method shown in the item 1 or Example 3, and the analyzed results are shown in FIG. 28. In comparison with the purified anti-CCR4 chimeric antibody prepared from the clone CHO/CCR4-LCA in Example 7, the ratio of a sugar chain in which 1-position of fucose was not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond in the purified anti-CCR4 chimeric antibody derived from the GMD gene-expressed clone CHO/CCR4-LCA was decreased to 9% when calculated from the peak area. Thus, it was shown that the ratio of a sugar chain in which 1-position of fucose was not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond in the antibody produced by the cell is decreased to similar level of the antibody produced by the clone 5-03.

EXAMPLE 9

[0921] Preparation of Anti-Fibroblast Growth Factor-8 Human Chimeric Antibody

[0922] 1. Preparation of Cells Stably Producing Anti-Fibroblast Growth Factor-8 Human Chimeric Antibody

[0923] Using a tandem type expression vector pKAN-TEX134 of an anti-fibroblast growth factor-8 (hereinafter referred to as "FGF-8") human chimeric antibody described in Reference Example 3, cells stably producing the anti-FGF-8 human chimeric antibody (hereinafter referred to as "anti-FGF-8 chimeric antibody") was prepared as follows.

[0924] (1) Preparation of Producing Cell Using Rat Myeloma YB2/0 Cell

[0925] After introducing $10 \mu\text{g}$ of the anti-FGF-8 chimeric antibody expression vector pKANTEX1334 into 4×10^6 cells of rat myeloma YB2/0 cell (ATCC CRL 1662) by electroporation [Cytotechnology, 3, 133 (1990)], the cells were suspended in 40 ml of Hybridoma-SFM-FBS(5) [Hybridoma-SFM medium (manufactured by Invitrogen) containing 5% FBS (manufactured by PAA Laboratories)] and dispensed at $200 \mu\text{l/well}$ into a 96 well culture plate (manufactured by Sumitomo Bakelite). After culturing at 37°C . for 24 hours in a 5% CO_2 incubator, G418 was added to give a concentration of 0.5 mg/ml, followed by culturing for 1 to 2 weeks. Culture supernatants were recovered from wells in which colonies of transformants showing G418 resistance were formed and their growth was confirmed, and antigen-binding activity of the anti-FGF-8 chimeric antibody in the supernatants was measured by the ELISA described in the item 2 of Example 9.

[0926] Regarding the transformants in wells in which production of the anti-FGF-8 chimeric antibody was found

in the culture supernatants, in order to increase the antibody production amount by using a dhfr gene amplification system, each of them was suspended to give a density of 1 to 2×10^5 cells/ml in the Hybridoma-SFM-FBS(5) medium containing 0.5 mg/ml G418 and 50 nmol/l DHFR inhibitor MTX (manufactured by SIGMA) and dispensed at 1 ml into each well of a 24 well plate (manufactured by Greiner). After culturing at 37° C. for 1 to 2 weeks in a 5% CO_2 incubator, transformants showing 50 nmol/l MTX resistance were induced. Antigen-binding activity of the anti-FGF-8 chimeric antibody in culture supernatants in wells where growth of transformants was observed was measured by the ELISA described in the item 2 of Example 9.

[0927] Regarding the transformants in wells in which production of the anti-FGF-8 chimeric antibody was found in culture supernatants, the MTX concentration was increased by a method similar to the above to thereby finally obtain a transformant 5-D capable of growing in the Hybridoma-SFM-FBS(5) medium containing 0.5 mg/ml G418 and 200 nmol/l MTX and also highly producing the anti-FGF-8 chimeric antibody. The resulting transformant was subjected to cloning by limiting dilution, and the resulting transformant cell clone was named 5-D-10.

[0928] (2) Preparation of Producing Cell Using CHO/DG44 Cell

[0929] In accordance with the method described in Example 4, the anti-FGF-8 chimeric antibody expression plasmid pKANTEX1334 was introduced into CHO/DG44 cell and gene amplification was carried out by using the drug MTX to obtain a transformant highly producing the anti-FGF-8 chimeric antibody. The antibody expression amount was measured using the ELISA described in the item 2 of Example 9. The resulting transformant was cloned twice by limiting dilution, and the resulting transformant cell clone was named 7-D-1-5.

[0930] 2. Binding Activity of Antibody to FGF-8 Partial Peptide (ELISA)

[0931] Compound 2 (SEQ ID NO:21) was selected as a human FGF-8 peptide with which the anti-FGF-8 chimeric antibody can react. For the activity measurement by the ELISA, a conjugate with BSA (manufactured by Nacalai Tesque) was prepared by the following method and used as the antigen. That is, 100 ml of a 25 mg/ml SMCC [4-(N-maleimidomethyl)cyclohexane-1-carboxylic acid N-hydroxysuccinimide ester] (manufactured by SIGMA)-DMSO solution was added dropwise to 900 ml of a PBS solution containing 10 mg of BSA under stirring, followed by slowly stirred for 30 minutes. To a gel filtration column such as NAP-10 column or the like which had been equilibrated with 25 ml of PBS, 1 ml of the reaction solution was applied, and the eluate eluted with 1.5 ml of PBS was used as a BSA-SMCC solution (BSA concentration was calculated from A_{280} measurement). Next, 250 ml of PBS was added to 0.5 mg of Compound 2, 250 ml of DMF was added thereto and completely dissolved, and then the above BSA-SMCC solution (1.25 mg as BSA) was added thereto under stirring, followed by slow stirring for 3 hours. The reaction solution was dialyzed against PBS at 4° C. overnight, sodium azide was added thereto to give a final concentration of 0.05% and then filtered through a $0.22 \mu\text{m}$ filter and used as a BSA-compound 2 solution.

[0932] The conjugate prepared in the above was dispensed at $1 \mu\text{g}/\text{ml}$ and $50 \mu\text{l}/\text{well}$ into a 96 well plate for ELISA

(manufactured by Greiner) and adhered thereto by allowing it to stand at 4° C. overnight. After washing with PBS, 1% BSA-PBS was added at $100 \mu\text{l}/\text{well}$ and allowed to react at room temperature for 1 hour to block the remaining active groups. After washing each well with Tween-PBS, culture supernatant of a transformant or a purified antibody was added at $50 \mu\text{l}/\text{well}$ and allowed to react at room temperature for 1 hour. After the reaction and subsequent washing of each well with Tween-PBS, a peroxidase-labeled goat anti-human IgG (γ) antibody solution (manufactured by American Qualex) diluted 3,000-fold with 1% BSA-PBS was added as a secondary antibody solution at $50 \mu\text{l}/\text{well}$ and allowed to react at room temperature for 1 hour. After the reaction and subsequent washing with Tween-PBS, the ABTS substrate solution was added at $50 \mu\text{l}/\text{well}$ to develop color, and the reaction was stopped 10 minutes thereafter by adding 5% SDS solution at $50 \mu\text{l}/\text{well}$. Thereafter, OD415 was measured.

[0933] 3. Purification of Anti-FGF-8 Chimeric Antibody

[0934] (1) Culturing of YB2/0 Cell-Derived Producing Cell and Purification of Antibody

[0935] The anti-FGF-8 chimeric antibody-expressing transformant 5-D obtained in the item 1(1) of Example 9 was cultured in Hybridoma-SFM (manufactured by Invitrogen) medium containing 200 nmol/l of MTX and 5% Daigo's GF21 (manufactured by Wako Pure Chemical Industries) in a 182 cm^2 flask (manufactured by Greiner) at 37° C. in a 5% CO_2 incubator. After culturing for 8 to 10 days, the anti-FGF-8 chimeric antibody was purified from the culture supernatant recovered by using Prosep-A (manufactured by Millipore) column and in accordance with the attached manufacture's instructions. The purified anti-FGF-8 chimeric antibody was named YB2/0-FGF8 chimeric antibody.

[0936] (2) Culturing of CHO-DG44 Cell-Derived Antibody-Producing Cells and Purification of Antibody

[0937] The anti-FGF-8 chimeric antibody-producing transformant cell clone 7-D-I-5 obtained in the item 1(2) of Example 9 was cultured in the IMDM-dFBS(10) medium in a 182 cm^2 flask (manufactured by Greiner) at 37° C. in a 5% CO_2 incubator. At the stage where the cell density reached confluent several days thereafter, the culture supernatant was discarded, the cells were washed with 25 ml of PBS buffer and then 35 ml of EXCELL301 medium (manufactured by JRH) was added thereto. After the culturing for 7 days at 37° C. in a 5% CO_2 incubator, the culture supernatant was recovered. The anti-FGF-8 chimeric antibody was purified from the culture supernatant by using Prosep-A (manufactured by Millipore) column and in accordance with the manufacture's instructions. The purified anti-FGF-8 chimeric antibody was named CHO-FGF8 chimeric antibody.

[0938] When the binding activity of the YB2/0-FGF8 chimeric antibody and CHO-FGF8 chimeric antibody to FGF-8 was measured by the ELISA described in the item 2 of Example 9, they showed similar binding activity.

[0939] 4. Analysis of Purified Anti-FGF-8 Chimeric Antibody

[0940] Each $4 \mu\text{g}$ of the two anti-FGF-9 chimeric antibodies produced by respective animal cells and purified in the item 3 of Example 9 was subjected to SDS-PAGE according to a known method [*Nature*, 227, 680 (1970)] and the

molecular weight and purity were analyzed. In each of the purified anti-FGF-8 chimeric antibodies, a single band of about 150 Kd in molecular weight was found under non-reducing conditions and two bands of about 50 Kd and about 25 Kd were found under reducing conditions. These molecular weights almost coincided with the molecular weights deduced from the cDNA nucleotide sequences of the antibody H chain and L chain (H chain: about 49 Kd, L chain: about 23 Kd, whole molecule: about 144 Kd), and also coincided with the reports showing that the IgG type antibody shows a molecular weight of about 150 Kd under non-reducing conditions and is degraded into H chain having a molecular weight of about 50 Kd and L chain having a molecular weight of about 25 Kd under reducing conditions due to cleavage of the intramolecular S—S bond (*Antibodies*, Chapter 14 (1988); *Monoclonal Antibodies*). Thus it was confirmed that the anti-FGF-8 chimeric antibody was purified as antibody molecules having correct structures.

[0941] 5. Sugar Chain Analysis of Purified Anti-FGF-8 Chimeric Antibodies

[0942] Sugar chain analysis of the YB2/0-FGF8 chimeric antibody which is YB2/0 cell-derived anti-FGF-8 chimeric antibody and anti-FGF-8 chimeric antibody CHO-FGF8 chimeric antibody which is CHO/DG44 cell-derived prepared in the item 4 of Example 9 was carried out in accordance with the method described in the item 1 of Example 3. As a result, the ratio of a sugar chain in which 1-position of fucose was not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond were 58% in the YB2/0-FGF8 chimeric antibody and 13% in the CHO-FGF8 chimeric antibody. Hereinafter, these samples are called anti-FGF-8 chimeric antibody (58%) and anti-FGF-8 chimeric antibody (13%).

EXAMPLE 10

[0943] Preparation of Soluble Human Fc γ RIIIa Protein

[0944] 1. Construction of a Soluble Human Fc γ RIIIa Protein Expression Vector

[0945] (1) Preparation of Human Peripheral Blood Monocyte cDNA

[0946] From a healthy donor, 30 ml of vein blood was collected, gently mixed with 0.5 ml of heparin sodium (manufactured by Shimizu Pharmaceutical) and then mixed with 30 ml of physiological saline (manufactured by Otsuka Pharmaceutical). After the mixing, 10 ml of each mixture was gently overlaid on 4 ml of Lymphoprep (manufactured by NYCOMED PHARMA AS) and centrifuged at 2,000 rpm for 30 minutes at room temperature. The separated monocyte fractions in respective centrifugation tubes were combined and suspended in 30 ml of RPMI1640-FBS(10). After centrifugation at room temperature and at 1,200 rpm for 15 minutes, the supernatant was discarded and the cell were suspended in 20 ml of RPMI1640-FBS(10). This washing operation was repeated twice and then 2×10^6 cells/ml of peripheral blood monocyte suspension was prepared using RPMI1640-FBS(10).

[0947] After 5 ml of the resulting peripheral blood monocyte suspension was centrifuged at room temperature and at 800 rpm for 5 minutes in 5 ml of PBS, the Supernatant was discarded and the residue was suspended in 5 ml of PBS. After centrifugation at room temperature and at 800 rpm for

5 minutes, the supernatant was discarded and total RNA was extracted by QIAamp RNA Blood Mini Kit (manufactured by QIAGEN) and in accordance with the manufacture's instructions.

[0948] A single-stranded cDNA was synthesized by reverse transcription reaction to 2 μ g of the resulting total RNA, in a series of 40 μ l containing oligo(dT) as primers using SUPERSCRIPTM Preamplification System for First Strand cDNA Synthesis (manufactured by Life Technologies) according to the manufacture's instructions.

[0949] (2) Preparation Method of cDNA Encoding Human Fc γ RIIIa Protein

[0950] A cDNA encoding a human Fc γ RIIIa protein (hereinafter referred to as "hFc γ RIIIa") was prepared as follows.

[0951] First, a specific forward primer containing a translation initiation codon (represented by SEQ ID NO:22) and a specific reverse primer containing a translation termination codon (represented by SEQ ID NO:26) were designed from the nucleotide sequence of hFc γ RIIIa cDNA [*J. Exp. Med.* 170, 481 (1989)].

[0952] Next, using a DNA polymerase ExTaq (manufactured by Takara Shuzo), 50 μ l of a reaction solution [1 \times concentration ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/l dNTPs, 1 μ mol/l of the above gene-specific primers (SEQ ID NOs:22 and 26)] containing 5 μ l of 20-fold diluted solution of the human peripheral blood monocyte-derived cDNA solution prepared in the item 1(1) of Example 10 was prepared, and PCR was carried out. The PCR was carried out by 35 cycles of a reaction at 94° C. for 30 seconds, at 56° C. for 30 seconds and at 72° C. for 60 seconds as one cycle.

[0953] After the PCR, the reaction solution was purified by using QIAquick PCR Purification Kit (manufactured by QIAGEN) and dissolved in 20 μ l of sterile water. The products were digested with restriction enzymes EcoRI (manufactured by Takara Shuzo) and BamHI (manufactured by Takara Shuzo) and subjected to 0.8% agarose gel electrophoresis to recover about 800 bp of a specific amplification fragment.

[0954] On the other hand, 2.5 μ g of a plasmid pBluescript II SK(−) (manufactured by Stratagene) was digested with restriction enzymes EcoRI (manufactured by Takara Shuzo) and BamHI (manufactured by Takara Shuzo), and digested products were subjected to 0.8% agarose gel electrophoresis to recover a fragment of about 2.9 kbp.

[0955] The human peripheral blood monocyte cDNA-derived amplification fragment and plasmid pBluescript II SK(−)-derived fragment obtained in the above were ligated by using DNA Ligation Kit Ver. 2.0 (manufactured by Takara Shuzo). The strain *Escherichia coli* DH5 α (manufactured by TOYOBO) was transformed by using the reaction solution, and a plasmid DNA was isolated from each of the resulting ampicillin-resistant colonies according to a known method.

[0956] A nucleotide sequence of the cDNA inserted into each plasmid was determined by using DNA Sequencer 377 (manufactured by Parkin Elmer) and BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (manufactured by Parkin Elmer) according to the manufacture's instructions. It was confirmed that all of the inserted cDNAs whose

sequences were determined by this method encodes a complete ORF sequence of the cDNA encoding hFcγRIIIa. A plasmid DNA containing absolutely no reading error of bases in the sequence accompanied by PCR was selected from them. Hereinafter, the plasmid is called pBSFcγRIIIa5-3.

[0957] The thus determined full length cDNA sequence for hFcγRIIIa is represented by SEQ ID NO:27, and its corresponding amino acid sequence is shown in by SEQ ID NO:28.

[0958] (3) Preparation of a cDNA Encoding Soluble hFcγRIIIa

[0959] A cDNA encoding soluble hFcγRIIIa (hereinafter referred to as "shFcγRIIIa") having the extracellular region of hFcγRIIIa (positions 1 to 193 in SEQ ID NO:28) and a His-tag sequence at the C-terminal was constructed as follows.

[0960] First, a primer FcgR3-1 (represented by SEQ ID NO. 29) specific for the extracellular region was designed from the nucleotide sequence of hFcγRIIIa cDNA (represented by SEQ ID NO:27).

[0961] Next, using a DNA polymerase ExTaq (manufactured by Takara Shuzo), 50 μl of a reaction solution [1× concentration ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/l dNTPs, 1 μmol/l of the primer FcgR3-1, 1 μmol/l of the primer M13M4 (manufactured by Takara Shuzo) containing 5 ng of the plasmid pBSFcγRIIIa5-3 prepared in the item 1(2) of Example 10 was prepared, and PCR was carried out. The PCR was carried out by 35 cycles of a reaction at 94° C. for 30 seconds, at 56° C. for 30 seconds and at 72° C. for 60 seconds as one cycle.

[0962] After the PCR, the reaction solution was purified by using QIAquick PCR Purification Kit (manufactured by QIAGEN) and dissolved in 20 μl of sterile water. The products were digested with restriction enzymes PstI (manufactured by Takara Shuzo) and BamHI (manufactured by Takara Shuzo) and subjected to 0.8% agarose gel electrophoresis to recover about 110 bp of a specific amplification fragment.

[0963] On the other hand, 2.5 g of the plasmid pBSFcγRIIIa5-3 was digested with restriction enzymes PstI (manufactured by Takara Shuzo) and BamHI (manufactured by Takara Shuzo), and the digested products were subjected to 0.8% agarose gel electrophoresis to recover a fragment of about 3.5 kbp.

[0964] The hFcγRIIIa cDNA-derived amplification fragment and plasmid pBSFcγRIIIa5-3-derived fragment obtained in the above were ligated by using DNA Ligation Kit Ver. 2.0 (manufactured by Takara Shuzo). The strain *Escherichia coli* DH5α (manufactured by TOYOBO) was transformed by using the reaction solution, and a plasmid DNA was isolated from each of the resulting ampicillin-resistant colonies according to a known method.

[0965] A nucleotide sequence of the cDNA inserted into each plasmid was determined by using DNA Sequencer 377 (manufactured by Parkin Elmer) and BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (manufactured by Parkin Elmer) according to the manufacture's instructions. It was confirmed that all of the inserted cDNAs whose sequences were determined by this method encodes a com-

plete ORF sequence of the cDNA encoding shFcγRIIIa of interest. A plasmid DNA containing absolutely no reading error of bases in the sequence accompanied by PCR was selected from them. Hereinafter, this plasmid is named pBSFcγRIIIa+His3.

[0966] The thus determined full length cDNA sequence for shFcγRIIIa is represented by SEQ ID NO:30, and its corresponding amino acid sequence is represented by SEQ ID NO:31.

[0967] (4) Construction of shFcγRIIIa Expression Vector

[0968] The shFcγRIIIa expression vector was constructed as follows.

[0969] After 3.0 μg of the plasmid pBSFcγRIIIa+His3 obtained in the item 1(3) of Example 10 was digested with restriction enzymes EcoRI (manufactured by Takara Shuzo) and BamHI (manufactured by Takara Shuzo), the digested products were subjected to 0.8% agarose gel electrophoresis to recover a fragment of about 620 bp.

[0970] Separately, 2.0 μg of the plasmid pKANTEX93 described in WO97/10354 was digested with restriction enzymes EcoRI (manufactured by Takara Shuzo) and BamHI (manufactured by Takara Shuzo), and the digested products were subjected to 0.8% agarose gel electrophoresis to recover a fragment of about 10.7 kbp.

[0971] The DNA fragment containing shFcγRIIIa cDNA and the plasmid pKANTEX93-derived fragment obtained in the above were ligated by using DNA Ligation Kit Ver. 2.0 (manufactured by Takara Shuzo). The strain *Escherichia coli* DH5α (manufactured by TOYOBO) was transformed by using the reaction solution, and a plasmid DNA was isolated from each of the resulting ampicillin-resistant colonies according to a known method.

[0972] A nucleotide sequence of the cDNA inserted into each plasmid was determined by using DNA Sequencer 377 (manufactured by Parkin Elmer) and BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (manufactured by Parkin Elmer)+, in accordance with the manual attached thereto. It was confirmed that all of the plasmids whose sequences were determined by this method encodes the cDNA of interest encoding shFcγRIIIa. Hereinafter, the obtained expression vector was named pKANTEXFcγRIIIa-His.

[0973] 2. Preparation of Cell Stably Producing shFcγRIIIa

[0974] Cells stably producing shFcγRIIIa were prepared by introducing the shFcγRIIIa expression vector pKANTEXFcγRIIIa-His constructed in the item 1 of Example 10 into rat myeloma YB2/0 cell (ATCC CRL-1662, *J. Cell. Biol.*, 93, 576 (1982)).

[0975] pKANTEXFcγRIIIa-His was digested with a restriction enzyme AatII to obtain a linear fragment, 10 μg thereof was introduced into 4×10⁶ cells by electroporation [*Cytotechnology*, 1, 133 (1990)], and the resulting cells were suspended in 40 ml of Hybridoma-SFM-FBS(10) [Hybridoma-SFM medium (manufactured by Life Technologie) containing 10% FBS] and dispensed at 200 μl/well into a 96 well culture plate (manufactured by Sumitomo Bakelite). After culturing at 37° C. for 24 hours in a 5% CO₂ incubator, G418 was added to give a concentration of 1.0 mg/ml, followed by culturing for 1 to 2 weeks. Culture supernatants

were recovered from wells in which colonies of transformants showing G418 resistance were formed and their growth was confirmed, and expression amount of shFcγRIIIa in the supernatants was measured by the ELISA described in the item 3 of Example 10.

[0976] Regarding the transformants in wells in which expression of the shFcγRIIIa was confirmed in the culture supernatants, in order to increase the antibody production by using a dhfr gene amplification system, each of them was suspended to give a density of 1 to 2×10 cells/ml in the Hybridoma-SFM-FBS(10) medium containing 1.0 mg/ml G418 and 50 nmol/l DHFR inhibitor MTX (manufactured by SIGMA) and dispensed at 2 ml into each well of a 24 well plate (manufactured by Greiner). After culturing at 37° C. for 1 to 2 weeks in a 5% CO₂ incubator, transformants showing 50 nmol/l MTX resistance were induced. Expression level of shFcγRIIIa in culture supernatants in wells where growth of transformants was observed was measured by the ELISA described in The item 3 of Example 10. Regarding the transformants in wells in which expression of the shFcγRIIIa was found in culture supernatants, the MTX concentration was increased to 100 nmol/l and then to 200 nmol/l sequentially by a method similar to the above to thereby finally obtain a transformant capable of growing in the Hybridoma-SFM-FBS(10) medium containing 1.0 mg/ml G418 and 200 nmol/l MTX and also of highly producing shFcγRIIIa. The resulting transformant was cloned twice by limiting dilution. The obtained transformant was named clone KC1107.

[0977] 3. Detection of shFcγRIIIa (ELISA)

[0978] shFcγRIIIa in culture supernatant or purified shFcγRIIIa was detected or determined by the ELISA shown below.

[0979] A solution of a mouse antibody against His-tag, Tetra-His Antibody (manufactured by QIAGEN), adjusted to 5 μg/ml with PBS was dispensed at 50 μl/well into each well of a 96 well plate for ELISA (manufactured by Greiner) and allowed to react at 4° C. for 12 hours or more. After the reaction, 1% BSA-PBS was added at 100 μl/well and allowed to react at room temperature for 1 hour to block the remaining active groups. After 1% BSA-PBS was discarded, culture supernatant of the transformant or each of various dilution solutions of purified shFcγRI was added at 50 μl/well and allowed to react at room temperature for 1 hour. After the reaction and subsequent washing of each well with Tween-PBS, a biotin-labeled mouse anti-human CD16 antibody solution (manufactured by PharMingen) diluted 50-fold with 1% BSA-PBS was added at 50 μl/well and allowed to react at room temperature for 1 hour. After the reaction and subsequent washing with Tween-PBS, a peroxidase-labeled Avidin D solution (manufactured by Vector) diluted 4,000-fold with 1% BSA-PBS was added at 50 μl/well and allowed to react at room temperature for 1 hour. After the reaction and subsequent washing with Tween-PBS, the ABTS substrate solution was added at 50 μl/well to develop color, and OD415 was measured.

[0980] 4. Purification of shFcγRIIIa

[0981] The shFcγRIIIa-producing transformant cell clone KC1107 obtained in the item 2 of Example 10 was suspended in Hybridoma-SFM-GF(5) [Hybridoma-SFM medium (manufactured by Life Technologic) containing 5%

Daigo's GF21 (manufactured by Wako Pure Chemical Industries)] to give a density of 3×10 cells/ml and dispensed at 50 ml into 182 cm² flasks (manufactured by Greiner). After culturing at 37° C. for 4 days in a 5% CO₂ incubator, the culture supernatants were recovered. shFcγRIIIa was purified from the culture supernatants by using Ni-NTA agarose (manufactured by QIAGEN) column according to the manufacture's instructions.

[0982] 5. Analysis of Purified shFcγRIIIa

[0983] A concentration of purified shFcγRIIIa obtained in the item 4 of Example 10 was calculated by amino acid composition analysis as follows. A part of purified shFcγRIIIa was suspended in 6 mol/l hydrochloric acid-1% phenol solution, and hydrolysed in a gas phase at 110° C. for 20 hours. Work Station manufactured by Waters was used for the hydrolysis. Amino acids after the hydrolysis were converted into PTC-amino acid derivatives in accordance with the method of Bidlingmeyer et al. [*J. Chromatogr.*, 336, 93 (1984)] and analyzed by using PicoTag Amino Acid Analyzer (manufactured by Waters).

[0984] Next, about 0.5 μg of purified shFcγRIIIa was subjected to SDS-PAGE under reducing conditions according to a known method [*Nature* 227, 680 (1970)] to analyze its molecular weight and purity. The results are shown in FIG. 6. As shown in FIG. 29, a broad band of 36 to 38 Kd in molecular weight was detected in purified can be bound are present in the extracellular region of hFcγRIIIa [*J. Exp. Med.*, 170, 481 (1989)], it was considered that the broad molecular weight distribution of purified shFcγRIIIa is based on the irregularity of sugar chain addition. On the other hand, when the N-terminal amino acid sequence of purified shFcγRIIIa was analyzed by automatic Edman degradation using a protein sequencer PPSQ-10 (manufactured by Shimadzu), a sequence expected from the cDNA of shFcγRIIIa was obtained, so that it was confirmed that shFcγRIIIa of interest was purified.

EXAMPLE 11

[0985] Evaluation of Binding Activity of Various Chimeric Antibodies to shFcγRIIIa

[0986] 1. Evaluation of shFcγRIIIa-Binding Activity of Anti-GD3 Chimeric Antibodies Having a Different Ratio of a Sugar Chain in which 1-Position of Fucose was not Bound to 6-Position of N-acetylglucosamine in the Reducing End through α-Bond

[0987] The shFcγRIIIa-binding activity of the anti-GD3 chimeric antibody (45%) and anti-GD3 chimeric antibody (7%) described in the item 1 of Example 3 which are two anti-GD3 chimeric antibodies having a different ratio of a sugar chain in which 1-position of fucose was not bound to 6-position of N-acetylglucosamine in the reducing end through α-bond was measured by ELISA as follows.

[0988] According to the method described in the item 3 of Example 1, GD3 was immobilized at 100 pmol/well on a 96 well plate for ELISA (manufactured by Greiner). The 1% BSA-PBS was added at 100 μl/well and allowed to react at room temperature for 1 hour to block the remaining active groups. After washing each well with Tween-PBS, a solution of each anti-GD3 chimeric antibody diluted with 1% BSA-PBS was added at 50 μl/well and allowed to react at room temperature for 1 hour. After the reaction and subsequent

washing of each well with Tween-PBS, an shFcγRIIIa solution prepared by diluting it to 2.3 μg/ml with 1% BSA-PBS was added at 50 μl/well and allowed to react at room temperature for 1 hour. After the reaction and subsequent washing with Tween-PBS, a solution of a mouse antibody against His-tag, Tetra-His Antibody (manufactured by QIAGEN), adjusted to 1 μg/ml with 1% BSA-PBS was added at 50 μl/well and allowed to react at room temperature for 1 hour. After the reaction and subsequent washing with Tween-PBS, a peroxidase-labeled goat anti-mouse IgG1 antibody solution (manufactured by ZYMED) diluted 200-fold with 1% BSA-PBS was added at 50 μl/well and allowed to react at room temperature for 1 hour. After the reaction and subsequent washing with Tween-PBS, the ABTS substrate solution was added at 50 μl/well to develop color, and OD415 was measured. In addition, it was confirmed that there is no difference in the amount of the anti-GD3 chimeric antibodies bound to the plate by adding each of the anti-GD3 chimeric antibodies to another plate and carrying out the ELISA described in item 3 of Example 1. The results of the measurement of the binding activity of the various anti-GD3 chimeric antibodies for shFcγRIIIa are shown in **FIG. 30**. As shown in **FIG. 30**, regarding the binding activity of the anti-GD3 chimeric antibodies to shFcγRIIIa, the anti-GD3 chimeric antibody (45%) having a high ratio of a sugar chain in which 1-position of fucose was not bound to 6-position of N-acetylglucosamine in the reducing end through α-bond had 10 times or more higher activity.

[0989] 2. Evaluation of shFcγRIIIa-Binding Activity of Anti-FGF-8 Chimeric Antibodies Having a Different Ratio of a Sugar Chain in which 1-Position of Fucose is not Bound to 6-Position of N-acetylglucosamine in the Reducing End through α-Bond

[0990] The shFcγRIIIa-binding activity of the anti-FGF-8 chimeric antibody (58%) and anti-FGF-8 chimeric antibody (13%) described in the item 5 of Example 9 which were as two anti-FGF-8 chimeric antibodies having different ratio of a sugar chain in which 1-position of fucose was not bound to 6-position of N-acetylglucosamine in the reducing end through α-bond was measured by ELISA as follows.

[0991] The human FGF-8 peptide conjugate prepared in the item 2 of Example 9 at a concentration of 1.0 μg/ml was dispensed at 50 μl/well into a 96-well plate for ELISA (manufactured by Greiner) and adhered thereto by allowing it to stand at 4° C. overnight. After washing with PBS, 1% BSA-PBS was added at 100 μl/well and allowed to react at room temperature for 1 hour to block the remaining active groups. After washing each well with Tween-PBS, a solution of each anti-FGF-8 chimeric antibody diluted with 1% BSA-PBS was added at 50 μl/well and allowed to react at room temperature for 1 hour. After the reaction and subsequent washing of each well with Tween-PBS, an shFcγRIIIa solution prepared by diluting it to 3.0 μg/ml with 1% BSA-PBS was added at 50 μl/well and allowed to react at room temperature for 1 hour. After the reaction and subsequent washing with Tween-PBS, a solution of a mouse antibody against His-tag, Tetra-His Antibody (manufactured by QIAGEN), adjusted to 1 μg/ml with 1% BSA-PBS, was added at 50 μl/well and allowed to react at room temperature for 1 hour. After the reaction and subsequent washing with Tween-PBS, a peroxidase-labeled goat anti-mouse IgG1 antibody solution (manufactured by ZYMED) diluted 200-fold with 1% BSA-PBS was added at 50 μl/well and allowed

to react at room temperature for 1 hour. After the reaction and subsequent washing with Tween-PBS, the ABTS substrate solution was added at 50 μl/well to develop color, and OD415 was measured. In addition, by adding each of the anti-FGF-8 chimeric antibodies to another plate and carrying out the ELISA described in the item 2 of Example 9, it was confirmed that there is no difference in the amount of the various anti-FGF-8 chimeric antibodies bound to the plate. The results of the measurement of the binding activity of the various anti-FGF-8 chimeric antibodies for shFcγRIIIa are shown in **FIG. 31**. As shown in **FIG. 31**, regarding the binding activity of the anti-FGF8 chimeric antibodies to shFcγRIIIa the anti-FGF-8 chimeric antibody (58%) having a high ratio of a sugar chain in which 1-position of fucose was not bound to 6-position of N-acetylglucosamine in the reducing end through α-bond had 100 times or more higher activity.

[0992] 3. Evaluation of shFcγRIIIa-Binding Activity of Anti-CCR4 Chimeric Antibodies Having a Different Ratio of a Sugar Chain in which 1-Position of Fucose is not Bound to 6-Position of N-acetylglucosamine in the Reducing End through α-Bond

[0993] The shFcγRIIIa-binding activity of the seven anti-CCR4 chimeric antibodies described in the item 5 of Example 4, having a different ratio of a sugar chain in which 1-position of fucose was not bound to 6-position of N-acetylglucosamine in the reducing end through α-bond, was measured by ELISA as follows.

[0994] The human CCR4 extracellular peptide conjugate prepared in the item 2 of Example 4 at a concentration of 1.0 μg/ml was dispensed at 50 μl/well into a 96 well plate for ELISA (manufactured by Greiner) and adhered thereto by allowing it to stand at 4° C. overnight. After washing with PBS, 1% BSA-PBS was added at 100 μl/well and allowed to react at room temperature for 1 hour to block the remaining active groups. After washing each well with Tween-PBS, a solution of each anti-CCR4 chimeric antibody diluted with 1% BSA-PBS was added at 50 μl/well and allowed to react at room temperature for 1 hour. After the reaction and subsequent washing of each well with Tween-PBS, an shFcγRIIIa solution prepared by diluting it to 3.0 μg/ml with 1% BSA-PBS was added at 50 μl/well and allowed to react at room temperature for 1 hour. After the reaction and subsequent washing with Tween-PBS, a solution of a mouse antibody against His-tag, Tetra-His Antibody (manufactured by QIAGEN), adjusted to 1 μg/ml with 1% BSA-PBS, was added at 50 μl/well and allowed to react at room temperature for 1 hour. After the reaction and subsequent washing with Tween-PBS, a peroxidase-labeled goat anti-mouse IgG1 antibody solution (manufactured by ZYMED) diluted 200-fold with 1% BSA-PBS was added at 50 μl/well and allowed to react at room temperature for 1 hour. After the reaction and subsequent washing with Tween-PBS, the ABTS substrate solution was added at 50 μl/well to develop color, and OD415 was measured. In addition, it was confirmed that there is no difference in the amount of the anti-CCR4 chimeric antibodies bound to the plate by adding each of the anti-CCR4 chimeric antibodies to another plate and carrying out the ELISA described in the item 2 of Example 4. The results of the measurement of the binding activity of the various anti-CCR4 chimeric antibodies to shFcγRIIIa are shown in **FIG. 32A**. As shown in **FIG. 32A**, the binding activity of the anti-CCR4 chimeric antibodies to shFcγRIIIa

increased in proportion to the ratio of a sugar chain in which 1-position of fucose was not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond. **FIG. 32B** shows a plotted graph on a relationship between the ratio of a sugar chain in which 1-position of fucose was not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond, at antibody concentrations of 4 μ g/ml and 40 μ g/ml (the abscissa) and the shFc γ RIIIa-binding activity (the ordinate). As shown in **FIG. 32B**, the shFc γ RIIIa-binding activity was hardly detected in the anti-CCR4chimeric antibody (8%), anti-CCR4 chimeric antibody (9%) and anti-CCR4 chimeric antibody (18%), as antibodies having 20% or less of the ratio of a sugar chain in which 1-position of fucose was not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond.

[0995] The above results clearly show that antibodies having a sugar chain in which 1-position of fucose is not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond have higher shFc γ RIIIa-binding activity than antibodies having a α 1,6-fucose sugar chain. In addition, since antibodies having a sugar chain in which 1-position of fucose is not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond has higher ADCC activity than antibodies having a α 1,6-fucose sugar chain as shown in Examples 3 and 4, it was strongly suggested that the high ADCC activity of antibodies a sugar chain in which 1-position of fucose is not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond is based on the high shFc γ RIIIa-binding activity. As shown in **FIG. 32B**, the proportion between Fc γ RIIIa binding activity and the ratio of a sugar chain in which 1-position of fucose is not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond is found. Therefore, Fc γ RIIIa-binding activity can be measured and the ratio of a sugar chain in which 1-position of fucose is not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond can be determined by constructing such a calibration curve in advance. By using the above method, the cytotoxic activity can be prospected easily without determining the cytotoxic activity of the antibody composition.

EXAMPLE 12

[0996] Evaluation of Antibody Produced by Lectin-Resistant CHO/DG44 Cells to shFc γ RIIIa

[0997] Binding activities of the anti-CCR4 chimeric antibody produced by the lectin-resistant clone CHO/CCR4-LCA purified in the item 3 of Example 7 [hereinafter referred to as anti-CCR4 chimeric antibody (48%)], the anti-CCR4 chimeric antibody KM2760-1 produced by a YB2/0 cell-derived antibody-producing clone purified in the item 3 of Example 4 [anti-CCR4 chimeric antibody (87%)] and the anti-CCR4 chimeric antibody KM3060 produced by a CHO/DG44 cell-derived antibody-producing clone 5-03 (anti-CCR4 chimeric antibody (8%)] to shFc γ RIIIa were measured according to the method described in the item 3 of Example 11. As a result, as shown in **FIG. 33**, the anti-CCR4 chimeric antibody (48%) produced by the lectin-resistant clone CHO/CCR4-LCA showed 100 times or more higher binding activity to shFc γ RIIIa than the anti-CCR4 chimeric antibody (8%) produced by the clone 5-03. Also, this activity was about $\frac{1}{3}$ of the anti-CCR4 chimeric antibody (87%) produced by the YB2/0 cell-derived antibody-producing clone.

[0998] The above results clearly show that an antibody having 100 times or more higher binding activity to shFc γ RIIIa can be prepared by using lectin-resistant CHO/DG44 cell than by using CHO/DG44 cell which is its parent cell.

EXAMPLE 13

[0999] Method for Screening Antibody Composition Having High ADCC Activity Based on Binding Activity to shFc γ RIIIa

[1000] A anti-GD3 chimeric antibody expression plasmid pCHI641LHGM4 was introduced into the LCA-resistant clone CHO-LCA obtained in the item 1 of Example 7 according to the method described in the item 2(2) of Example 1, and gene amplification was carried out by using MTX to produce a transformant producing an anti-GD3 chimeric antibody. Cloning was carried out by limiting dilution method using the obtained transformant to obtain several clones. After each clone was cultured, the culture medium was recovered when it became confluent. A concentration of the anti-GD3 chimeric antibody in the culture supernatant was diluted to 1 μ g/ml, and the shFc γ RIIIa-binding activity was measured using the diluted antibody solution by the ELISA described in the item 1 of Example 11. At the same time, solutions of the anti-GD3 chimeric antibody produced by a YB2/0 cell-derived antibody-producing clone and the anti-GD3 chimeric antibody produced by a CHO/DG44 cell-derived antibody-producing clone, purified in the item 4 of Example 1 were prepared by diluting them to 1 μ g/ml, and their shFc γ RIIIa-binding activities were also measured.

[1001] Based on the measured results, a transformant cell clone capable of producing an antibody showing the activity equal to or higher than the binding activity of the anti-GD3 chimeric antibody produced by the CHO/DG44 cell-derived antibody-producing clone and also equal to or lower than the binding activity of the anti-GD3 chimeric antibody produced by the YB2/0 cell-derived antibody-producing clone was selected.

[1002] A purified antibody was obtained from the culture supernatant by culturing the selected transformant cell clone according to the method described in the item 4(2) of Example 1. When monosaccharide composition analysis of the purified antibody was carried out according to the method described in the item 1 of Example 3, the ratio of a sugar chain in which 1-position of fucose was not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond was 42%. Hereinafter, this sample is called anti-GD3 chimeric antibody (42%). When antigen-binding activity of the purified anti-GD3 chimeric antibody (42%) was evaluated by the ELISA described in the item 3 of Example 11, it was equivalent to those of the anti-GD3 chimeric antibody produced by a YB2/0 cell-derived antibody-producing clone and the anti-GD3 chimeric antibody produced by a CHO/DG44 cell-derived antibody-producing clone purified in the item 4 of Example 1. In addition, the ADCC activity of each anti-GD3 chimeric antibody was evaluated according to the method described in the item 2 of Example 2. For comparison, the ADCC activity of a sample of the anti-GD3 chimeric antibody produced by a CHO/DG44 cell-derived antibody-producing clone, wherein the ratio of a sugar chain in which 1-position of fucose was not

bound to 6-position of acetylglucosamine in the reducing end through α -bond was 12% as a result of its monosaccharide composition analysis [hereinafter named "anti-GD3 chimeric antibody (12%)"] was measured. The results are shown in **FIG. 34**. In comparison with the anti-GD3 chimeric antibody (12%) produced by a CHO/DG44 cell-derived antibody-producing clone, about 30 times increase in the ADCC activity was observed in the anti-GD3 chimeric antibody (42%) produced by an antibody-producing clone selected based on the high binding activity to shFc γ RIIIa.

[1003] Based on the above results, it was found that an antibody composition having high ADCC activity can be screened by screening an antibody composition having high binding activity to shFc γ RIIIa.

EXAMPLE 14

[1004] Preparation of FGF-8b/Fc Fusion Protein.

[1005] 1. Construction of the Expression Vector of FGF-8b/Fc Fusion Protein

[1006] A humanized antibody expression vector pKAN-TEX93 [*Mol. Immunol.*, 37, 1035 (2000)] was digested with restriction enzymes ApaI and BamHI and a fragment containing about 1.0 kbp of human IgG1 subclass CH (hC γ 1) was obtained by using QIAquick Gel Extraction Kit (manufactured by QIAGEN). A plasmid pBluescript II SK(-) (manufactured by STRATAGENE) was also digested with similar restriction enzymes to obtain a fragment of about 2.9 kbp. The fragments were ligated using Solution I of TAKARA DNA Ligation Kit Ver. 2 (manufactured by Takara Shuzo) and *E. coli* DH5 α (manufactured by TOYOBO) was transformed to construct a plasmid pHc γ 1/SK(-).

[1007] In order to ligate hC γ 1 with cDNA of FGF8, a synthetic DNA having the nucleotide sequence represented by SEQ ID NO:86 was designed. The synthetic DNA contains plural restriction enzyme recognition sequences at its 5' terminal for cloning to pBluescript II SK(-), and the synthesis of the DNA was consigned to Prologo. To 50 μ l of a solution containing EX Taq Buffer (Mg²⁺ plus) in TaKaRa Ex Taq (manufactured by Takara Shuzo) at 1 \times concentration, 1 ng of plasmid pHc γ 1/SK(-), 0.25 mmol/L dNTPs, 0.5 μ mol/L of the synthetic DNA having the nucleotide sequence represented by SEQ ID NO:86, 0.5 μ mol/L M13 primer RV and 1.25 unit of TaKaRa Ex Taq were added, and the resulting solution was subjected to 30 cycles of heating at 94° C. for 30 minutes, 56° C. for 30 minutes and 72° C. for 1 minute as one cycle by using DNA thermal cycler GeneAmp PCR System 9700 (manufactured by PERKIN ELMER). A PCR amplified fragment was purified from the total reaction solution by using QIAquick PCR purification Kit (manufactured by QIAGEN). Then, the purified fragment was digested with restriction enzymes KpnI and BamHI to obtain a fragment of about 0.75 kbp. In addition, the pBluescript II SK(-) (manufactured by STRATAGENE) was digested with similar restriction enzymes to obtain a fragment of about 2.9 kbp. These fragments were ligated by using Ligation high (manufactured by TOYOBO) and *E. coli* DH5 α (manufactured by TOYOBO) was transformed to construct a plasmid pAhC γ 1/SK(-).

[1008] Using the FGF-8b gene cloned plasmid pSC17 [*Proc. Natl. Acad. Sci.*, 89, 8928 (1992)] as the template, PCR was carried out as follows to obtain a structure gene

region fragment of FGF-8b. To 50 μ l of a solution containing EX Taq Buffer (Mg²⁺, plus) in TaKaRa Ex Taq (manufactured by Takara Shuzo) at 1 \times concentration, 1 ng of plasmid pSC17, 0.25 mmol/L dNTPs, 10 μ mol/L of synthetic DNAs having the nucleotide sequence represented by SEQ ID NOs:87 and 88 and 2.5 unit of TaKaRa Ex Taq were added, and the resulting solution was subjected to 35 cycles of heating at 94° C. for 1 minute, 55° C. for 1 minute and 72° C. for 2 minutes as one cycle, followed by heating at 72° C. for 10 minutes, by using DNA thermal cycler GeneAmp PCR System 9700 (manufactured by PERKIN ELMER). A PCR amplified fragment was purified from the total reaction solution, and the purified fragment was digested with restriction enzymes EcoRI and BamHI to obtain a fragment of about 0.66 kbp. Also, plasmid pBluescript II SK(-) (manufactured by STRATAGENE) was digested with similar restriction enzymes to obtain a fragment of about 2.9 kbp. Then, the fragments were ligated by using T4 DNA Ligase (manufactured by Takara Shuzo) and *E. coli* DH5 α (manufactured by TOYOBO) was transformed to construct a plasmid pFGF-8b/SK(-).

[1009] Next, the plasmid pAhC γ 1/SK(-) was digested with restriction enzymes ApaI and EcoRI to obtain a fragment of about 3.7 kbp. In addition, the plasmid pFGF-8b/SK(-) was digested with similar restriction enzymes to obtain a fragment of about 0.6 kbp. Then, the fragments were ligated by using Ligation high (manufactured by TOYOBO) and *E. coli* DH5 α (manufactured by TOYOBO) was transformed to construct a plasmid pFGF8b+hIgG/SK(-).

[1010] Next, the thus constructed plasmid pFGF8b+hIgG/SK(-) was digested with restriction enzymes EcoRI and BamHI, a fragment of about 1.34 kbp was obtained. Also, pKANTEX93 was digested with similar restriction enzymes to obtain a fragment of about 8.8 kbp. Then, the fragments were ligated by using Ligation high (manufactured by TOYOBO) and *E. coli* DH5 D (manufactured by TOYOBO) was transformed to construct an expression vector pKANTEX/FGF8Fc for animal cell containing cDNA of FGF8b-Fc fusion protein represented by SEQ ID NO:89.

[1011] 2. Stable Expression Using Animal Cell of FGF-8b/Fc Fusion Protein

[1012] A stable expression clone of FGF-8/Fc fusion protein was prepared by introducing the FGF-8 fusion protein expression vector pKANTEX/FGF8Fc for animal cell which was constructed in the item 1 of Example 14 into various cells, and selecting a suitable clone.

[1013] (1) Preparation of Producing Cell Using Rat Myeloma YB2/0 Cells

[1014] After 10 μ g of the FGF8-Fc fusion protein expression vector pKANTEX/FGF8Fc was introduced into 4 \times 10⁶ cells of rat myeloma YB2/0 cell by electroporation, the resulting cells were suspended in 20 to 40 ml of RPMI1640-FBS(10) and the solution was dispensed at 200 μ l/well into a 96-well culture plate (manufactured by Sumitomo Bakelite). After culturing at 37° C. for 24 hours in a 5% CO₂ incubator, G418 was added thereto to give a concentration of 0.5 ml/ml, followed by culturing for 1 to 2 weeks. The culture supernatant was recovered from wells in which colonies of G418 resistance transformants showing were formed and growth of colonies was observed, and the

binding activity of the FGF8-Fc fusion protein in the supernatant to an anti-FGF-8 antibody was measured by the ELISA described in the item 4 of Example 14. As the anti-FGF-8 antibody, KM1334 (U.S. Pat. No. 5,952,472) was used.

[1015] Regarding the transformants in wells in which production of the FGF-8/Fc protein was observed in culture supernatants, in order to increase the production amount of the fusion protein by using a dhfr gene amplification system, each of them was suspended in the Hybridoma-SFM-FBS(S) medium comprising 0.5 mg/ml G418 and 50 nmol/L DHFR inhibitor, MTX (manufactured by SIGMA), and dispensed into each well of a 24 well plate for expansion culturing. After culturing at 37° C. for 1 to 2 weeks in a 5% CO₂ incubator to induce a transformant showing 50 nmol/l MTX resistance. Binding activity of FGF8b-Fc fusion protein in the supernatant in wells where growth of the transformant was observed, to KM1334 was measured by the ELISA described in the item 4 of Example 14.

[1016] Regarding the transformant in wells where production of FGF-8/Fc fusion protein was observed in the culture supernatant, according to the method similar to the above, a transformant KC1178 was obtained, which could grow in Hybridoma-SFM FBS(5) medium comprising 0.5 mg/ml G418 and 200 nmol/l MTX as final concentrations by increase of the MTX concentration and highly produce FGF8-Fc fusion protein. It was found that KC1178 was a lectin-resistant clone having a relatively low transcript amount obtained by the measuring method of FUT8 gene transcript described in Example 8 of WO00/61739. Also, KC1178 has been deposited on Apr. 1, 2003, as FERM BP-8350 in International Patent Organism Depository, National Institute of Advanced Industrial Science and Technology (AIST Tsukuba Central 6, 1-1, Higashi 1-Chome Tsukuba-shi, Ibaraki-ken, Japan).

[1017] (2) Preparation of Producing Cell Using CHO/DG44 Cells

[1018] After 10 µg of the FGF8b-Fc fusion protein expression vector pKANTEX/FGF8c was introduced into 1.6×10⁶ cells of CHO/DG 44 cell by electroporation, the resulting cells were suspended in 30 ml of IMDM-dFBS(10)-HT(1) and the solution was dispensed at 100 µl/well into a 96-well culture plate (manufactured by Sumitomo Bakelite). After culturing at 37° C. for 24 hours in a 5% CO₂ incubator, the medium was changed to IMDM-dFBE(10), followed by culturing for 1 to 2 weeks. The culture supernatant was recovered from wells in which colonies of transformants showing HT-independent growth were formed and growth of colonies was observed, and the binding activity of the FGF8-Fc fusion protein in the supernatant to KM1334 was measured by the ELISA shown in the item 4 of Example 14.

[1019] Regarding the transformants in wells in which production of the FGF-g/Fc protein was observed in culture supernatants, in order to increase the production amount of the fusion protein by using a dhfr gene amplification system, each of them was suspended in the IMDM-dFBS(10) medium comprising 50 nmol/L of MTX (manufactured by SIGMA), and dispensed into each well of a 24 well plate for expansion culturing. After culturing at 37° C. for 1 to 2 weeks in a 5% CO₂ incubator to induce a transformant showing 50 nmol/l MTX resistance. Binding activity of FGF8b-Fc fusion protein in the supernatant in wells where

growth was observed, to KM1334 was measured by the ELISA described in the item 4 of Example 14.

[1020] Regarding the transformant in wells where production of FGF-8/Fc fusion protein was observed in the culture supernatant, according to the method similar to the above, a transformant KC1179 was obtained, which could grow in IMDM-dFBS(10) medium comprising 500 nmol/l MTX by increase of the MTX concentration and highly produce FGF8-Fc fusion protein. Also, KC1179 has been deposited on Apr. 1, 2003, as FERM BP-8351 in International Patent Organism Depository, National Institute of Advanced Industrial Science and Technology (Tsukuba Central 6, 1, Higashi 1-Chome Tsukuba-shi, Ibaraki-ken, Japan).

[1021] 3. Purification of FGF8-Fc Fusion Protein

[1022] The producing cell of FGF8-Fc fusion protein prepared in the item 2 of Example 14 was cultured in an appropriate culture medium (e.g., H-SFM comprising 5% GF21 (manufactured by Wako Pure Chemical Industries), 0.5 mg/ml of G418 and 200 nmol/l of MTX for YB2/0 cell-derived cells; EXCELL301 (manufactured by JRH) comprising 500 nmol/l MTX for CHO/D644 cell-derived cells) at a scale of 100 to 200 ml. FGF8-Fc fusion protein was purified from the culture supernatant by using Prosep G (manufactured by Millipore) column according to the manufacture's instruction. The deduced amino acid sequence of the purified protein is shown by SEQ ID NO:90.

[1023] 4. Binding Activity to Anti-FGF-9 Antibody

[1024] Binding activities of the FGF-8/Fc fusion protein produced by YB2/0 and the FGF-S/Fc fusion protein produced by CHO described in the item 2 of Example 14, to KM1334 were measured by the ELISA as follows. KM1334 of 1 µg/ml was dispensed at 50 µl/well into a 96-well plate for ELISA (manufactured by Greiner) and allowed to stand at 4° C. overnight for adsorption. After washing with PBS, 1% BSA-PBS was added at 100 µl/well and allowed to react at room temperature for 1 hour to block the remaining active group. After washing each well with Tween-PBS, the culture supernatant of the transformant or a purified protein was added at 50 µl/well, followed reaction at room temperature. After the reaction, each well was washed with Tween-PBS, and peroxidase labeled goat-anti-human IgG(γ) antibody solution (manufactured by American Qualex), which was diluted 3000-fold with 1% BSA-PBS, was added at 50 µl/well as the secondary antibody solution, followed by reaction at room temperature for 1 hour. After the reaction, each well was washed with Tween-PBS, and ABTS substrate solution was added at 50 µl/well, followed by reaction. After the color developed sufficiently, 5% SDS solution was added at 50 µl/well to stop the reaction. Then, the absorbance was measured at a wavelength of 415 nm and a reference wavelength of 490. As shown in FIG. 35, the FGF-8/Fc fusion protein obtained in the item 2 of Example 14 showed binding activity to KM1334.

[1025] 5. Binding Activity to FcγRIIIa

[1026] Binding activities of FGF-8/Fc fusion protein produced by YB2/0 and FGF-8/Fc fusion protein derived from CHO described in the item 2 of Example 14, to FcγRIIIa was measured by ELISA as follows. Tetra-His Antibody, mouse antibody against tag (manufactured by QIAGEN), of 5 µg/ml was dispensed at 50 µl/well into a 96-well plate for ELISA (manufactured by GREINER) and allowed to stand

at 4° C. overnight for adsorption. After washing with PBS, 1% BSA-PBS was added at 100 μ l/well, followed by reaction at room temperature for 1 hour to block the remaining active group. After washing each well with Tween-PBS, shFc γ RIIIa (V) solution diluted to 5 μ g/ml with 1% BSA-PBS was added at 50 μ l/well, followed by reaction at room temperature for 2 hours. After the reaction, each well was washed with Tween-PBS, and a solution prepared by diluting purified FGF-8/Fc fusion protein to different concentrations with 1% BSA-PBS was added at 50 μ l/well, followed by reaction at room temperature for 2 hours. After the reaction, each well was washed with Tween-PBS, and biotinized KM1334 diluted to 1 μ g/ml with 1% BSA-PBS was added at 50 μ l/well, followed by reaction at room temperature for 1 hour. After the reaction, each well was washed with Tween-PBS, and a solution of peroxidase labeled Avidin-D (manufactured by VECTOR) diluted 4000-fold with 1% BSA-PBS was added at 50 μ l/well, followed by reaction at room temperature for 1 hour. After the reaction, each well was washed with Tween-PBS, and ABTS substrate solution was added at 50 μ l/well for color development, and after 15 minutes, 5% SDS solution was added at 50 μ l/well to stop the reaction. The absorbance of the resulting solution was measured at a wavelength of 415 nm and a reference wavelength of 490 nm.

[1027] **FIG. 36** shows the measurement results of the binding activity of various FGF-8/Fc fusion proteins to shFc γ RIIIa (V). As apparent in **FIG. 36**, the FGF-8/Fc fusion protein produced by YB2/0 showed higher binding activity to shFc γ RIIIa (V) than the FGF-8/Fc fusion protein produced by CHO/DG44 cell.

REFERENCE EXAMPLE 1

[1028] Preparation of genes Encoding Various Enzymes Relating to Sugar Chain Synthesis Derived from CHO Cell:

[1029] 1. Determination of FX cDNA Sequence in CHO Cell

[1030] (1) Extraction of Total RNA Derived from CHO/DG44 Cell

[1031] CHO/DG44 cells were suspended in IMDM medium containing 100/a fetal bovine serum (manufactured by Life Technologies) and 1x concentration HT supplement (manufactured by Life Technologies), and 15 ml of the suspension was inoculated into a T75 flask for adhesion cell culture use (manufactured by Greiner) to give a density of 2x10 cells/ml. On the second day after culturing at 37° C. in a 5% CO₂ incubator, 1x10⁷ of the cells were recovered and a total RNA was extracted therefrom by using RNAeasy (manufactured by QIAGEN) in accordance with the manufacture's instructions.

[1032] (2) Preparation of Total Single-Stranded cDNA from CHO/DG44 Cell

[1033] The total RNA prepared in the item 1(1) of Reference Example 1 was dissolved in 45 μ l of sterile water, and 1 μ l of RQ1 RNase-Free DNase (manufactured by Promega), 5 μ l of the attached 10x DNase buffer and 0.5 of RNasin Ribonuclease Inhibitor (manufactured by Promega) were added thereto, followed by reaction at 37° C. for 30 minutes to degrade genomic DNA contaminated in the sample. After the reaction, the total RNA was purified again using RNAeasy (manufactured by QIAGEN) and dissolved in 50 μ l of sterile water.

[1034] In a 20 μ l of reaction mixture using oligo(dT) as a primer, single-stranded cDNA was synthesized from 3 μ g of the obtained total RNA samples by carrying out reverse transcription reaction using SUPERScript™ Preamplification System for First Strand cDNA Synthesis (manufactured by Life Technologies) in accordance with the manufacture's instructions. A 50 fold-diluted aqueous solution of the reaction solution was used in the cloning of GFPP and FX. This was stored at -80° C. until use,

[1035] (3) Preparation Method of cDNA Partial Fragment of Chinese Hamster-Derived FX

[1036] An FX cDNA partial fragment derived from Chinese hamster was prepared by the following procedure.

[1037] First, primers (represented by SEQ ID NOs:42 and 43) specific for common nucleotide sequences registered at a public data base, namely a human FX cDNA (Genebank Accession No. U58766) and a mouse cDNA (Genebank Accession No. M30127), were designed.

[1038] Next, 25 μ l of a reaction solution [1x concentration ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/l dNTPs and 0.5 μ mol/l of the above gene-specific primers (SEQ ID NOs:42 and 43)] containing 1 μ l of the CHO/DG44-derived single-stranded cDNA prepared in the item 1(2) of Reference Example 1 was prepared by using a DNA polymerase ExTaq (manufactured by Takara Shuzo), and PCR was carried out. The PCR was carried out by heating at 94° C. for 5 minutes, subsequent 30 cycles of heating at 94° C. for 1 minute, 58° C. for 2 minutes and 72° C. for 3 minutes as one cycle, and further heating at 72° C. for 10 minutes.

[1039] After the PCR, the reaction solution was subjected to 2% agarose gel electrophoresis, and a specific amplified fragment of 301 bp was purified using QuiaexII Get Extraction Kit (manufactured by QIAGEN) and eluted with 20 μ l of sterile water (hereinafter, the method was used for the purification of DNA fragments from agarose gel). Into a plasmid pCR2.1, 4 μ l of the above amplified fragment was employed to insert in accordance with the instructions attached to TOPO TA Cloning Kit (manufactured by Invitrogen), and *E. coli* DH5 α was transformed with the reaction solution. Each plasmid DNA was isolated in accordance with a known method from the obtained several kanamycin-resistant colonies to obtain 2 clones into which FX cDNA partial fragments were respectively inserted. They were named pCRFX clone 8 and pCRFX clone 12.

[1040] The nucleotide sequence of the cDNA inserted into each of the FX clone 8 and FX clone 12 was determined using DNA Sequencer 377 (manufactured by Parkin Elmer) and BigDye Terminator Cycle Sequencing FS Ready Reaction kit (manufactured by Parkin Elmer) in accordance with the method of the manufacture's instructions. It was confirmed that each of the inserted cDNA whose sequence was determined encodes an ORF partial sequence of the Chinese hamster FX.

[1041] (4) Synthesis of Single-Stranded cDNA for RACE

[1042] Single-stranded cDNA samples for 5' and 3' RACE were prepared from the CHO/DG44 total RNA extracted in the item 1(1) of Reference Example 1 using SMART™ RACE cDNA Amplification Kit (manufactured by CLONTECH) in accordance with the manufacture's instructions.

In the case, PowerScript™ Reverse Transcriptase (manufactured by CLONTECH) was used as the reverse transcriptase. Each single-stranded cDNA after the preparation was diluted 10-fold with the Tricin-EDTA buffer attached to the kit and used as the template of PCR.

[1043] (5) Determination of Chinese Hamster-Derived FX Full Length cDNA by RACE Method

[1044] Based on the FX partial sequence derived from Chinese hamster determined in the item 1(3) of Reference Example 1, primers FXGSP1-1 (SEQ ID NO:44) and FXGSP1-2 (SEQ ID NO:45) for the Chinese hamster FX-specific 5' RACE and primers FXGSP2-1 (SEQ DD NO:46) and FXGSP2-2 (SEQ ED NO:47) for the Chinese hamster FX-specific 3' RACE were designed.

[1045] Next, 50 μ l of a reaction solution [1 \times concentration Advantage2 PCR buffer (manufactured by CLONTECH), 0.2 mmol/L dNTPs, 0.2 μ mol/l Chinese hamster FX-specific primers for RACE and 1 \times concentration of common primers (manufactured by CLONTECH)] containing 1 μ l of the CHO/DG44-derived single-stranded cDNA for RACE prepared in the item 1(4) of Reference Example 1 was prepared by using Advantage2 PCR Kit (manufactured by CLONTECH) and PCR was carried out. The PCR was carried out by 20 cycles of heating at 94° C. for 5 seconds, 68° C. for 10 seconds and 72° C. for 2 minutes as one cycle.

[1046] After completion of the reaction, 1 μ l of the reaction solution was diluted 50-fold with the Tricin-EDTA buffer, and 1 μ l of the diluted solution was used as a template. The reaction solution was again prepared and the PCR was carried out under the same conditions. The templates, the combination of primers used in the first and second PCRs and the length of amplified DNA fragments by the PCRs are shown in Table 6.

TABLE 6

Combination of primers used in Chinese hamster FX cDNA RACE PCR and the size of PCR products			
	FX-specific primers	Common primers	PCR-amplified product size
<u>5' RACE</u>			
First	FXGSP1-1	UPM (Universal primer mix)	300 bp
Second	FXGSP1-2	NUP (Nested Universal primer)	
<u>3' RACE</u>			
First	FXGSP2-1	UPM (Universal primer mix)	1,100 bp
Second	FXGSP2-2	NUP (Nested Universal primer)	

[1047] After the PCR, the reaction solution was subjected to 1% agarose gel electrophoresis, and the specific amplified fragment of interest was recovered and eluted with 20 μ l of sterile water. Into a plasmid pCR2.1, 4 μ l of the amplified fragment was inserted, and *E. coli* DH5 α was transformed by using the reaction solution in accordance with the instructions attached to TOPO TA Cloning Kit (manufactured by Invitrogen). Plasmid DNAs were isolated from the obtained kanamycin-resistant colonies to obtain 5 cDNA clones containing Chinese hamster FX 5' region. They were named FX5' clone 25, FX5' clone 26, FX5' clone 27, FX5' clone 28, FX5' clone 31 and FX5' clone 32.

[1048] In the same manner, 5 cDNA clones containing Chinese hamster FX 3' region were obtained. These FX3' clones were named FX3' clone 1, FX3' clone 3, FX3' clone 6, FX3' clone 8 and FX3' clone 9.

[1049] The nucleotide sequence of the cDNA moiety of each of the clones obtained by the 5' and 3' RACE was determined by using DNA Sequencer 377 (manufactured by Parkin Elmer) in accordance with the method described in the manufacture's instructions. By comparing the cDNA nucleotide sequences determined by the method, reading errors of nucleotide bases due to PCR were excluded and the full length nucleotide sequence of Chinese hamster FX cDNA was determined. The determined sequence is represented by SEQ ID NO:48.

[1050] 2. Determination of CHO Cell-Derived GFPP cDNA Sequence

[1051] (1) Preparation of GFPP cDNA Partial Fragment Derived from Chinese Hamster

[1052] GFPP cDNA partial fragment derived from Chinese hamster was prepared by the following procedure.

[1053] First, nucleotide sequences of a human GFPP cDNA (Genebank Accession No. AF017445), mouse EST sequences having high homology with the sequence (Genebank Accession Nos. AI467195, AA422658, BE304325 and AI466474) and rat EST sequences (Genebank Accession Nos. BF546372, AI058400 and AW144783), registered at public data bases, were compared, and primers GFPP FW9 and GFPP RV9. (SEQ ID NOs:49 and 50) specific for rat GFPP were designed on a highly preserved region among these three species.

[1054] Next, 25 μ l of a reaction solution [1 \times concentration ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/L dNTPs and 0.5 μ mol/l of the above GFPP-specific primers GFPP FW9 and GFPP RV9 (SEQ ID NOs:49 and 50)] containing 1 μ l of the CHO/DG44-derived single-stranded cDNA prepared in the item 1(2) of Reference Example 1 was prepared by using a DNA polymerase ExTaq (manufactured by Takara Shuzo), and PCR was carried out. The PCR was carried out by heating at 94° C. for 5 minutes, subsequent 30 cycles of heating at 94° C. for 1 minute, 58° C. for 2 minutes and 72° C. for 3 minutes as one cycle, and further heating at 72° C. for 10 minutes.

[1055] After the PCR, the reaction solution was subjected to 2% agarose gel electrophoresis, and a specific amplified fragment of 1.4 Kbp was recovered and eluted with 20 μ l of sterile water. Into a plasmid pCR2.1, 4 μ l of the above amplified fragment was inserted in accordance with the instructions attached to TOPO TA Cloning Kit (manufactured by Invitrogen), and *E. coli* DH5 α was transformed by using the reaction solution. Plasmid DNAs were isolated from the obtained kanamycin resistant clones to obtain 3 clones into which GFPP cDNA partial fragments were respectively integrated. They were named GFPP clone 8, GFPP clone 11 and GFPP clone 12.

[1056] The nucleotide sequence of the cDNA inserted into each of the GFPP clone 8, GFPP clone 11 and GFPP clone 12 was determined by using DNA Sequencer 377 (manufactured by Parkin Elmer) and BigDye Terminator Cycle Sequencing FS Ready Reaction kit (manufactured by Parkin Elmer) in accordance with the method described in the

manufacture's instructions. It was confirmed that the inserted cDNA whose sequence was determined according to the present invention encodes an ORF partial sequence of the Chinese hamster GFPP.

[1057] (2) Determination of Chinese Hamster GFPP Full Length cDNA by RACE Method

[1058] Based on the Chinese hamster FX partial sequence determined in the item 2(1) of Reference Example 1, primers GFPP GSP1-1 (SEQ ID NO:52) and GFPP GSP1-2 (SEQ ID NO:53) for the Chinese hamster FX-specific 5' RACE and primers GFPP GSP2-1 (SEQ ID NO:54) and GFPP GSP2-2 (SEQ ID NO:55) for the Chinese hamster GFPP-specific 3' RACE were designed.

[1059] Next, 50 μ l was of a reaction solution [1 \times concentration Advantage2 PCR buffer (manufactured by CLONTECH), 0.2 mmol/L dNTPs, 0.2 μ mol/l Chinese hamster GFPP-specific primers for RACE and 1 \times concentration of common primers (manufactured by CLONTECH)] containing 1 μ l of the CHO/DG44 cell-derived single-stranded cDNA for RACE prepared in the item 1(4) of Reference Example 1 was prepared by using Advantage2 PCR Kit (manufactured by CLONTECH), and PCR was carried out. The PCR was carried out by 20 cycles of heating at 94° C. for 5 seconds, 68° C. for 10 seconds and 72° C. for 2 minutes as one cycle.

[1060] After completion of the reaction, 1 μ l of the reaction solution was diluted 50-fold with the Tricin-EDTA buffer, and 1 μ l of the diluted solution was used as a template. The reaction solution was again prepared and the PCR was carried out under the same conditions. The templates, the combination of primers used in the first and second PCRs and the size of amplified DNA fragments by the PCRs are shown in Table 7.

TABLE 7

Combination of primers used in Chinese hamster GFPP cDNA RACE PCR and the size of PCR products			
	GFPP-specific primers	Common primers	PCR-amplified product size
<u>5' RACE</u>			
First	GFPPGSP1-1	UPM (Universal primer mix)	1,100 bp
Second	GFPPGSP1-2	NUP (Nested Universal primer)	
<u>3' RACE</u>			
First	GFPPGSP2-1	UPM (Universal primer mix)	1,400 bp
Second	GFPPGSP2-2	NUP (Nested Universal primer)	

[1061] After the PCR, the reaction solution was subjected to 1% agarose gel electrophoresis, and the specific amplified fragment of interest was recovered and eluted with 20 μ l of sterile water. Into a plasmid pCR2.1, 4 μ l of the above amplified, fragment was inserted and *E. coli* DH5 α was transformed with the reaction solution in accordance with the instructions attached to TOPO TA Cloning Kit (manufactured by Invitrogen). Plasmid DNAs were isolated from the obtained several kanamycin-resistant clones to obtain 4

cDNA clones containing Chinese hamster GFPP 5' region. They were named GFPP5' clone 1, GFPP5' clone 2, GFPP5' clone 3 and GFPP5' clone 4.

[1062] In the same manner, 5 cDNA clones containing Chinese hamster GFPP 3' region were obtained. They were named GFPP3' clone 10, GFPP3' clone 16 and GFPP3' clone 20.

[1063] The nucleotide sequence of the cDNA protein of each of the clones obtained in the above 5' and 3' RACE was determined by using DNA Sequencer 377 (manufactured by Parkin Elmer) in accordance with the method described in the manufacture's instructions. By comparing the cDNA nucleotide sequences after the nucleotide sequence determination, reading errors of bases due to PCR were excluded and the full length nucleotide sequence of Chinese hamster GFPP cDNA was determined. The determined sequence is represented by SEQ ID NO:5.

REFERENCE EXAMPLE 2

[1064] Preparation of CHO Cell-Derived GMD Gene:

[1065] 1. Determination of CHO Cell-Derived GMD cDNA Sequence

[1066] (1) Preparation of CHO Cell-Derived GMD Gene cDNA (Preparation of Partial cDNA Excluding 5'- and 3'-Terminal Sequences)

[1067] Rodents-derived GMD cDNA was searched in a public data base (BLAST) using a human-derived GMD cDNA sequence (GenBank Accession No. AF042377) registered at GenBank as a query, and three kinds of mouse EST sequences were obtained (GenBank Accession Nos. BE986856, BF158988 and BE284785). By ligating these EST sequences, a deduced mouse GMD cDNA sequence was determined.

[1068] On the basis of the mouse-derived GMD cDNA sequence, a 28 mer primer having the nucleotide sequence represented by SEQ ID NO:56, a 27 mer primer having the nucleotide sequence represented by SEQ ID NO:57, a 25 mer primer having the nucleotide sequence represented by SEQ ID NO:58, a 24 mer primer having the nucleotide sequence represented by SEQ ID NO:59 and a 25 mer primer having the sequence represented by SEQ ID NO:60 were prepared.

[1069] Next, in order to amplify the CHO cell-derived GMD cDNA, PCR was carried out by the following method. A reaction solution at 20 μ l [1 \times concentration Ex Taq buffer (manufactured by Takara Shuzo), 0.2 mmol/L dNTPs, 0.5 unit of Ex Taq polymerase (manufactured by Takara Shuzo) and 0.5 μ mol/L of two synthetic DNA primers] containing 0.5 μ l of the CHO cell-derived single-stranded cDNA prepared in the item 1(1) of Example 8 as the template was prepared. In this case, combinations of SEQ ID NO:56 with SEQ ID NO:57, SEQ ID NO:58 with SEQ ID NO:57, SEQ ID NO:56 with SEQ ID NO:59 and SEQ ID NO:56 with SEQ ID NO:60 were used as the synthetic DNA primers. The reaction was carried out by using DNA Thermal Cycler 480 (manufactured by Perkin Elmer) by heating at 94° C. for 5 minutes and subsequent 30 cycles of heating at 94° C. for 1 minute and 68° C. for 2 minutes as one cycle.

[1070] The PCR reaction solution was fractionated by agarose electrophoresis to find that a DNA fragment of about

1.2 kbp was amplified in the PCR product when synthetic DNA primers of SEQ ID NOs:56 and 57 were used, a fragment of about 1.1 kbp was amplified in the PCR product when synthetic DNA primers of SEQ ID NOs:57 and 59 were used, a fragment of about 350 bp was amplified in the PCR product when synthetic DNA primers of SEQ ID NOs:56 and 59 were used and a fragment of about 1 kbp was amplified in the PCR product when synthetic DNA primers of SEQ ID NOs:56 and 60 were used. The DNA fragments were recovered. The recovered DNA fragments were ligated to a pT7Blue(R) vector (manufactured by Novagen) by using DNA Ligation Kit (manufactured by Takara Shuzo), and *E. coli* DH5 strain (manufactured by Toyobo) was transformed by using the obtained recombinant plasmid DNA samples to thereby obtain plasmids 22-8 (having a DNA fragment of about 1.2 kbp amplified from synthetic DNA primers of SEQ ID NO:56 and SEQ ID NO:57), 23-3 (having a DNA fragment of about 1.1 kbp amplified from synthetic DNA primers of SEQ ID NO:58 and SEQ ID NO:57), 31-5 (a DNA fragment of about 350 bp amplified from synthetic DNA primers of SEQ ID NO:56 and SEQ ID NO:59) and 34-2 (having a DNA fragment of about 1 kbp amplified from synthetic DNA primers of SEQ ID NO:56 and SEQ ID NO:60). The CHO cell-derived GMD cDNA sequence contained in these plasmids was determined in the usual way by using a DNA sequencer ABI PRISM 377 (manufactured by Parkin Elmer) (since a sequence of 28 bases in downstream of the initiation codon methionine in the 5'-terminal side and a sequence of 27 bases in upstream of the termination codon in the 3'-terminal side are originated from synthetic oligo DNA sequences, they are mouse GMD cDNA sequences).

[1071] In addition, the following steps were carried out in order to prepare a plasmid in which the CHO cell-derived GMD cDNAs contained in the plasmids 22-8 and 34-2 are combined. The plasmid 22-8 (1 μ g) was allowed to react with a restriction enzyme EcoRI (manufactured by Takara Shuzo) at 37° C. for 16 hours, the digest was subjected to agarose electrophoresis and then a DNA fragment of about 4 kbp was recovered. The plasmid 34-2 (2 μ g) was allowed to react with a restriction enzyme EcoRI at 37° C. for 16 hours, the digest was subjected to agarose electrophoresis and then a DNA fragment of about 150 bp was recovered. The recovered DNA fragments were respectively subjected to terminal dephosphorylation using Calf Intestine Alkaline Phosphatase (manufactured by Takara Shuzo) and then ligated by using DNA Ligation Kit (manufactured by Takara Shuzo), and *E. coli* DH5 α strain (manufactured by Toyobo) was transformed by using the obtained recombinant plasmid DNA to obtain a plasmid CHO-GMD (cf. FIG. 37).

[1072] (2) Determination of 5'-Terminal Sequence of CHO Cell-Derived GMD cDNA

[1073] A 24 mer primer having the nucleotide sequence represented by SEQ ID NO:61 was prepared from 5'-terminal side non-coding region nucleotide sequences of CHO cell-derived human and mouse GMD cDNA, and a 32 mer primer having the nucleotide sequence represented by SEQ ID NO:62 from CHO cell-derived GMD cDNA sequence were prepared, and PCR was carried out by the following method to amplify cDNA. Then 20 μ l of a reaction solution [1 \times concentration Ex Taq buffer (manufactured by Takara Shuzo), 0.2 mmol/L dNTPs, 0.5 unit of Ex Taq polymerase (manufactured by Takara Shuzo) and 0.5 μ mol/L of the

synthetic DNA primers of SEQ ID NO:61 and SEQ ID NO:62] containing 0.5 μ l of the CHO cell-derived single-stranded cDNA obtained in the item 1(1) of Example 8 was prepared as the template, and the reaction was carried out therein by using DNA Thermal Cycler 480 (manufactured by Perkin Elmer) by heating at 94° C. for 5 minutes, subsequent 20 cycles of heating at 94° C. for 1 minute, 55° C. for 1 minute and 72° C. for 2 minutes as one cycle and further 18 cycles of heating at 94° C. for 1 minute and 68° C. for 2 minutes as one cycle. After fractionation of the PCR reaction solution by agarose electrophoresis, a DNA fragment of about 300 bp was recovered. The recovered DNA fragment was ligated to a pT7Blue(R) vector (manufactured by Novagen) by using DNA Ligation Kit (manufactured by Takara Shuzo), and *E. coli* DH5 α strain (manufactured by Toyobo) was transformed by using the obtained recombinant plasmid DNA samples to thereby obtain a plasmid 5'GMD. Using DNA Sequencer 377 (manufactured by Parkin Elmer), the nucleotide sequence of 28 bases in downstream of the initiation methionine of CHO-derived GMD cDNA contained in the plasmid was determined.

[1074] (3) Determination of 3'-Terminal Sequence of CHO Cell-Derived GMD cDNA

[1075] In order to obtain 3'-terminal cDNA sequence of CHO cell-derived GMD, RACE method was carried out by the following method. A single-stranded cDNA for 3' RACE was prepared from the CHO cell-derived RNA obtained in the item 1(1) of Example 8 by using SMART™ RACE cDNA Amplification Kit (manufactured by CLONTECH) in accordance with the manufacture's instructions. In the case, PowerScript™ Reverse Transcriptase (manufactured by CLONTECH) was used as the reverse transcriptase. The single-stranded cDNA after the preparation was diluted 10-fold with the Tricin-EDTA buffer attached to the kit and used as the template of PCR.

[1076] Next, 20 μ l of a react-on solution [1 \times concentration ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/L dNTPs, 0.5 unit of EX Taq polymerase (manufactured by Takara Shuzo), 0.5 μ mol/L of the 24 mer synthetic DNA primer represented by SEQ ID NO:63 [prepared on the basis of the CHO cell-derived GMD cDNA sequence determined in the item 1(1) of Reference Example 2] and 1 \times concentration of Universal Primer Mix (attached to SMART™ RACE cDNA Amplification Kit; manufactured by CLONTECH)] containing 1 μ l of the above single-stranded cDNA for 3, RACE as the template was prepared, and the reaction was carried out by using DNA Thermal Cycler 480 (manufactured by Perkin Elmer) by heating at 94° C. for 5 minutes and subsequent 30 cycles of heating at 94° C. for 1 minute and 68° C. for 2 minutes as one cycle.

[1077] After completion of the reaction, 1 μ l of the PCR reaction solution was diluted 20-fold with Tricin-EDTA buffer (manufactured by CLONTECH). Then, 20 μ l of a reaction solution [1 \times concentration ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/L dNTPs, 0.5 unit of EX Taq polymerase (manufactured by Takara Shuzo), 0.5 μ mol/L of the 25 mer synthetic DNA primer represented by SEQ ID NO:64 [prepared on the basis of the CHO cell-derived GMD cDNA sequence determined in the item 1(1) of Reference Example 2] and 0.5 μ mol/L of Nested Universal Primer (attached to SMART™ RACE cDNA Amplification Kit; manufactured by CLONTECH)] containing 1 μ l

of the 20 fold-diluted aqueous solution as the template was prepared, and the reaction was carried out by using DNA Thermal Cycler 480 (manufactured by Perkin Elmer) by heating at 94° C. for 5 minutes and subsequent 30 cycles of heating at 94° C. for 1 minute and 68° C. for 2 minutes as one cycle.

[1078] After completion of the reaction, the PCR reaction solution was fractionated by agarose electrophoresis and then a DNA fragment of about 700 bp was recovered. The recovered DNA fragment was ligated to a pT7Blue(R) vector (manufactured by Novagen) by using DNA Ligation Kit (manufactured by Takara Shuzo), and *E. coli* DH5 α strain (manufactured by Toyobo) was transformed by using the obtained recombinant plasmid DNA, thereby obtaining a plasmid 3'GMD. Using DNA Sequencer 377 (manufactured by Parkin Elmer), the nucleotide sequence of 27 bases in upstream of the termination codon of CHO-derived GMD cDNA and 415 bp of 3'-terminal side non-coding region contained in the plasmid was determined.

[1079] The full length cDNA sequence of the CHO-derived GMD gene determined by the items 1(1), 1(2) and 1(3) of Reference Example 2 is represented by SEQ ID NO:65, and the corresponding amino acid sequence is represented by SEQ ID NO:71.

[1080] 2. Determination of Genomic Sequence Containing CHO/DG44-Derived Cell GMD Gene

[1081] A 25 mer primer having the nucleotide sequence represented by SEQ ID NO:66 was prepared from the mouse GMD cDNA sequence determined in the item 1 of Reference Example 2. Next, a CHO cell-derived genomic DNA was obtained by the following method. A CHO/DG44 cell-derived KC861 cell was suspended in IMDM-dFBS(10)-HT(1) medium [IMDM-dFBS(10) medium comprising 1 \times concentration of HT supplement (manufactured by Invitrogen)] to give a density of 3 \times 10 cells/ml, and the suspension was dispensed at 2 ml/well into a 6 well flat bottom plate for adhesion cell use (manufactured by Greiner). After culturing at 37° C. in a 5% CO₂ incubator until the cells became confluent on the plate, genomic DNA was prepared from the cells on the plate by a known method [*Nucleic Acids Research*, 3, 2303 (1976)] and dissolved overnight in 150 μ l of TE-RNase buffer (pH 8.0) (10 mmol/l Tris-HCl, 1 mmol/l EDTA, 200 μ g/ml RNase A).

[1082] A reaction solution (20 μ l) [1 \times concentration Ex Taq buffer (manufactured by Takara Shuzo), 0.2 mmol/L dNTPs, 0.5 unit of EX Taq polymerase (manufactured by Takara Shuzo) and 0.5 μ mol/L of synthetic DNA primers of SEQ ID NO:59 and SEQ ID NO:66] containing 100 ng of the obtained CHO/DG44 cell-derived genomic DNA was prepared, and PCR was carried out by using DNA Thermal Cycler 480 (manufactured by Perkin Elmer) by heating at 94° C. for 5 minutes and subsequent 30 cycles of heating at 94° C. for 1 minute and 68° C. for 2 minutes as one cycle. After completion of the reaction, the PCR reaction solution was fractionated by agarose electrophoresis and then a DNA fragment of about 100 bp was recovered. The recovered DNA fragment was ligated to a pT7Blue(R) vector (manufactured by Novagen) by using DNA Ligation Kit (manufactured by Takara Shuzo), and *E. coli* DH5 α strain (manufactured by Toyobo) was transformed by using the obtained recombinant plasmid DNA, thereby obtaining a plasmid ex3. Using DNA Sequencer 377 (manufactured by Parkin

Elmer), the nucleotide sequence of CHO cell-derived genomic DNA contained in the plasmid was determined. The nucleotide sequence was represented by SEQ ID NO:67.

[1083] Next, a 25 mer primer having the nucleotide sequence represented by SEQ ID NO:68 and a 25 mer primer having the nucleotide sequence represented by SEQ ID NO:69 were prepared on the basis of the CHO cell-derived GMD cDNA sequence determined in the item 1 of Reference Example 2. Next, 20 μ l of a reaction solution [1 \times concentration Ex Taq buffer (manufactured by Takara Shuzo), 0.2 mmol/L dNTPs, 0.5 unit of EX Taq polymerase (manufactured by Takara Shuzo) and 0.5 mmol/L of the synthetic DNA primers of SEQ ID NO:68 and SEQ ID NO:69] containing 100 ng of the CHO/DG44-derived genomic DNA was prepared, and PCR was carried out by using DNA Thermal Cycler 480 (manufactured by Perkin Elmer) by heating at 94° C. for 5 minutes and subsequent 30 cycles of heating at 94° C. for 1 minute and 68° C. for 2 minutes as one cycle.

[1084] After completion of the reaction, the PCR reaction solution was fractionated by agarose electrophoresis and then a DNA fragment of about 200 bp was recovered. The recovered DNA fragment was ligated to a pT7Blue(R) vector (manufactured by Novagen) by using DNA Ligation Kit (manufactured by Takara Shuzo), and *E. coli* DH5 α strain (manufactured by Toyobo) was transformed by using the obtained recombinant plasmid DNA, thereby obtaining a plasmid ex4. Using DNA Sequencer 377 (manufactured by Parkin Elmer), the nucleotide sequence of CHO cell-derived genomic DNA contained in the plasmid was determined. The result nucleotide sequence was represented by SEQ ID NO:70.

REFERENCE EXAMPLE 3

[1085] Preparation of Anti-FGF-8 Chimeric Antibody

[1086] 1. Isolation and Analysis of a cDNA Encoding the V Region of a Mouse Antibody Against FGF-8

[1087] (1) Preparation of mRNA from Hybridoma Cells which Produces a Mouse Antibody Against FGF-8.

[1088] About 8 μ g of mRNA was prepared from 1 \times 10⁷ cells of a hybridoma KM1334 (FERM BP-5451) which produces a mouse antibody against FGF-8 (anti-FGF-8 mouse antibody), using a mRNA preparation kit Fast Track mRNA Isolation Kit (manufactured by Invitrogen) according to the attached manufacture's instructions.

[1089] (2) Production of cDNA Libraries of Anti-FGF-8 Mouse Antibody H Chain and L Chain

[1090] A cDNA having EcoRI-NotI adapters on both termini was synthesized from 5 μ g of the KM1334 mRNA obtained in the item 1(1) of Reference Example 3 by using Time Saver cDNA Synthesis Kit (manufactured by Amersham Pharmacia Biotech) according to the attached manufacture's instructions. A full amount of the prepared cDNA was dissolved in 20 μ l of sterile water and then fractionated by agarose gel electrophoresis, and about 1.5 kb of a cDNA fragment corresponding to the H chain of an IgG class antibody and about 1.0 kb of a cDNA fragment corresponding to the L chain of a κ class were recovered each at about 0.1 μ g. Next, 0.1 μ g of the cDNA fragment of about 1.5 kb

and 0.1 μ g of the cDNA fragment of about 1.0 kb were respectively digested with restriction enzyme EcoRI and then ligated with 1 μ g of λ ZAPII vector whose termini had been dephosphorylated with calf intestine alkaline phosphatase, using λ ZAPII Cloning Kit (manufactured by Stratagene) according to the attached manufacture's instructions.

[1091] Using Gigapack II Packaging Extracts Gold (manufactured by Stratagene), 4 μ l of each reaction solution after ligation was packaged in λ phage according to the manufacture's instructions, and *Escherichia coli* XL1-Blue [Biotechniques, 5, 376 (1987)] was infected with an adequate amount of the package to obtain about 8.1×10^4 and 5.5×10^4 phage clones as H chain cDNA library and L chain cDNA library, respectively, of KM334. Next, respective phages were immobilized on a nylon membrane according to a known method (*Molecular Cloning*, Second Edition).

[1092] (3) Cloning of cDNAs of Anti-FGF-8 Mouse Antibody H Chain and L Chain

[1093] Nylon membranes of the H chain cDNA library and L chain cDNA library of KM1334 prepared in the item 1(2) in Reference Example 3 were detected using a cDNA of the C region of a mouse antibody [H chain is a DNA fragment containing mouse C γ 1 cDNA (*J. Immunol.*, 146, 2010 (1991)), L chain is a DNA fragment containing mouse CK cDNA (*Cell*, 22, 197 (1980))] as a probe, using ECL Direct Nucleic Acid Labeling and Detection Systems (manufactured by Amersham Pharmacia Biotech) according to the attached manufacture's instructions, and phage clones strongly linked to the probe, 10 clones for each of H chain and L chain, were obtained. Next, each phage clone was converted into a plasmid by the in vivo excision method according to the manufacture's instructions attached to λ ZAPII Cloning Kit (manufactured by Stratagene) A nucleotide sequence of a cDNA contained in each of the obtained plasmids was determined by the dideoxy method (*Molecular Cloning*, Second Edition) by using Big Dye Terminator Kit ver. 2 (manufactured by Applied Biosystems). As a result, a plasmid pKM1334H7-1 containing a full length and functional H chain cDNA and a plasmid pKM1334L7-1 containing L chain cDNA, having an ATG sequence considered to be the initiation codon in the 5'-terminal of the cDNA were obtained.

[1094] (4) Analysis of Amino Acid Sequence of V Region of Anti-FGF-8 Mouse Antibody

[1095] A full length nucleotide sequence of VH contained in the plasmid pKM1334H7-1 and a deduced complete length amino acid sequence are represented by SEQ ID NO:72 and SEQ ID NO:73, respectively, and a full length nucleotide sequence of VL contained in the plasmid pKM1334L7-1 and a deduced complete length amino acid sequence are represented by SEQ ID NO:74 and SEQ ID NO:75, respectively. As a result of comparing these sequences to both known sequence data of mouse antibodies (*Sequences of Proteins of Immunological Interest*, U.S. Dept. Health and Human Services, 1991) and the comparison with the results of analysis of N-terminal amino acid sequences of H chain and L chain of the purified anti-FGF-8 mouse antibody KM1334, carried out by their automatic Edman degradation using a protein sequencer PPSQ-10 (manufactured by Shimadzu), it was found that each of the isolated cDNA is a full length cDNA encoding the anti-FGF-8 mouse antibody KM1334 containing a secretory

signal sequence, and positions 1 to 19 in the amino acid sequence represented by SEQ ID NO:73 and positions 1 to 19 in the amino acid sequence described in SEQ ID NO:75 are secretory signal sequences of H chain and L chain, respectively.

[1096] Next, novelty of the amino acid sequences (sequences excluding secretory signal sequence) of VH and VL of the anti-FGF-8 mouse antibody KM1334 was examined. Using GCG Package (version 9.1 manufactured by Genetics Computer Group) as a sequence analyzing system, an amino acid sequence data base of known proteins (PIR-Protein (Release 56.0)) was searched by the BLAST method [*J. Mol. Biol.*, 215, 403 (1990)]. As a result, completely coincided sequences were not found for both of the H chain and L chain, so that it was confirmed that the VH and VL of the anti-FGF-8 mouse antibody KM1334 are novel amino acid sequences.

[1097] Also, the CDR of VH and VL of the anti-FGF-8 mouse antibody KM1334 was identified by comparing with amino acid sequences of known antibodies. Amino acid sequences of CDR 1, 2 and 3 of VH of the anti-FGF-8 mouse antibody KM1334 are represented by SEQ DD NOs:76, 77 and 78, respectively, and amino acid sequences of CDR 1, 2 and 3 of VL in SEQ ID NOs:79, 80 and 81, respectively.

[1098] 2. Stable Expression of Anti-FGF-8 Chimeric Antibody Using Animal Cell

[1099] (1) Construction of Anti-FGF-8 Chimeric Antibody Expression Vector pKANTEX1334

[1100] An anti-FGF-8 chimeric antibody expression vector pKANTEX1334 was constructed as follows using the vector pKANTEX93 for humanized antibody expression described in WO97/10354 and the plasmids pKM1334H7-1 and pKM1334L7-1 obtained in the item 1(3) of Reference Example 3.

[1101] Using 50 ng of the plasmid pKM1334H7-1 obtained in the item 1(3) of Reference Example 3 as the template and by adding synthetic DNAs having the nucleotide sequences described in SEQ ID NOs:24 and 25 (manufactured by GENSET) as primers to give a final concentration 0.3 μ M, PCR were carried out in a system of 50 μ l by first heating at 94° C. for 2 minutes and subsequent 30 cycles of heat at 94° C. for 15 seconds, at 55° C. for 30 seconds and at 68° C. for 1 minute according to the manufacture's instructions attached to KOD plus polymerase (manufactured by TOYOBO). The reaction solution was precipitated with ethanol, dissolved in sterile water and then allowed to react at 37° C. for 1 hour by using 10 units of a restriction enzyme ApaI (manufactured by Takara Shuzo) and 10 units of a restriction enzyme NotI (manufactured by New England Biolabs). About 0.3 μ g of an ApaI-NotI fragment of about 0.47 kb was recovered By fractionating the reaction solution by agarose gel electrophoresis.

[1102] Next, 3 μ g of the vector pKANTEX93 for humanized antibody expression was allowed to react at 37° C. for 1 hour by using 10 units of restriction enzyme ApaI (manufactured by Takara Shuzo) and 10 units of restriction enzyme NotI (manufactured by New England Biolabs). About 2 g of an ApaI-NotI fragment of about 12.75 kb was recovered, by fractionating the reaction solution by an agarose gel electrophoresis.

[1103] Next, 0.1 μ g of the NotI-ApaI fragment derived from the PCR product and 0.1 μ g of the NotI-ApaI fragment derived from the plasmid pKANTEX93, obtained in the above, were added to 10 μ l of sterile water in total amount and ligated by using Ligation High (manufactured by TOYOBO). The plasmid pKANTEX1334H shown in FIG. 38 was obtained by transforming *Escherichia coli* JM109 by using the recombinant plasmid DNA solution obtained in this manner.

[1104] Next, using 50 ng of the plasmid pKM1334L7-1 obtained in the item 1(3) of Reference Example 1 as the template and by adding synthetic DNAs having the nucleotide sequences described in SEQ ID NOS:82 and 83 (manufactured by GENSET) as primers to give a final concentration of 0.3 μ M, PCR was carried out in a system of 50 μ l by first heating at 94° C. for 2 minutes and subsequent 30 cycles of heating at 94° C. for 15 seconds, at 55° C. for 30 seconds and 68° C. for 1 minute according to the manufacturer's instructions attached to KOD plus polymerase (manufactured by TOYOBO). The reaction solution was precipitated with ethanol, dissolved in sterile water and then allowed to react at 37° C. for 1 hour by using 10 units of a restriction enzyme EcoRI (manufactured by Takara Shuzo) and 10 units of a restriction enzyme BsiWI (manufactured by New England Biolabs). About 0.3 μ g of an EcoRI-BsiWI fragment of about 0.44 kb was recovered by fractionating the reaction solution by agarose gel electrophoresis.

[1105] Next, 3 μ g of the plasmid pKANTEX1134H obtained in the above was allowed to react at 37° C. for 1 hour by using 10 units of a restriction enzyme EcoRI

(manufactured by Takara Shuzo) and a restriction enzyme BsiWI (manufactured by New England Biolabs). About 2 μ g of an EcoRI-BsiWI fragment of about 13.20 kb was recovered by fractionating said reaction solution by an agarose gel electrophoresis.

[1106] Next, 0.1 μ g of the EcoRI-BsiWI fragment derived from the PCR product and 0.1 μ g of the EcoRI-BsiWI fragment derived from the plasmid pKANTEX1334H, obtained in the above, were added to 10 μ l of sterile water in total amount and ligated by using Ligation High (manufactured by TOYOBO). The plasmid pKANTEX1334 shown in FIG. 38 was obtained by transforming *Escherichia coli* JM109 using the recombinant plasmid DNA solution obtained in this manner.

[1107] As a result of carrying out analysis of a nucleotide sequence using 400 ng of the obtained plasmid by the dideoxy method (*Molecular Cloning*, Second Edition) using Big Dye Terminator Kit ver. 2 (manufactured by Applied Biosystems), it was confirmed that a plasmid comprising a cloned DNA of interest was obtained.

[1108] While the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skill in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof. All references cited herein are incorporated in their entirety.

[1109] This application is based on Japanese application No. 2002-106950 filed on Apr. 9, 2002, the entire contents of which being incorporated hereinto by reference.

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acagtgcacg aattctcttt aaacctataa tggetccctc tgtggtggta tcccttttct 8160
tgctctcatc cgttctctcc ctgactagat cttctctgct cctcatgtcc tcctctcccc 8220
tccccttctc ccttctcttt tcttctaact cctctctccc tccacccacg atccccatta 8280
gcttatgaga tcttgcctt attttagcaa aacctttttg gctataaaat taattaattt 8340
aatatgctta tatcagggtt attttggtta gtatttgtat gtgtttggtt agtgttttta 8400
accttaattg acatgtatcc ttatatattg acacagattt aaatatattga agtttttttt 8460
tttttttttt ttaaagattt atttattttt tatgtcttct gcctgcatgc cagaagaggg 8520
caccagatct cattcaaggt ggttgtagc caccatgtgg ttgctgggaa ttgaactcag 8580
gacctctgga agaacagtca gtgctcttaa ccgctgagcc atctctccag cccctgaagt 8640
gtttctttta aagaggatag cagtgcacaa tttttccctt tgaccaatga ctctacctt 8700
actgaattgt tttagccatt tatatgtaat gctgttacca ggtttacatt ttcttttatc 8760
ttgctaaatt tcttccctgt ttgtctcatc tcttattttt gtctgttgga ttatataggc 8820
ttttattttt ctgtttttac agtaagttat atcaaattaa aattatttta tggaatgggt 8880
gtgttgacta catgtatgct tgtgcaccat gtgctgacct ggtcttggcc agaagaaggt 8940
gtcatattct ctgaaactgg tattgtggat gttacgaact gccatagggt gctaggaatc 9000
aaaccccagc tcctctggaa aagcagccac tgctctgagc cactgagtcc tctcttcaag 9060
caggtgatgc caacttttaa tggttaccag tggataagag tgcttgatc tctagacccc 9120
atgaaaaatt atgcattgct atatgggctt gtcacttcag cattgtgtga cagagacagg 9180
aggatcccaa gagctc 9196

```

```

<210> SEQ ID NO 4
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
DNA

```

```
<400> SEQUENCE: 4
```

```
actcatcttg gaatctcaga attgg 25
```

```

<210> SEQ ID NO 5
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
DNA

```

```
<400> SEQUENCE: 5
```

```
cttgaccggt tctatcttct ctgc 24
```

```

<210> SEQ ID NO 6
<211> LENGTH: 979
<212> TYPE: DNA

```

-continued

<213> ORGANISM: *Cricetulus griseus*

<400> SEQUENCE: 6

```

actcatcttg gaatctcaga attggcgcta tgctactgga ggatgggaga ctgtgttttag    60
acctgtaagt gagacatgca cagacaggtc tggcctctcc actggacact ggtcagggtga    120
agtgaaggac aaaaatgttc aagtggtcga gctccccatt gtagacagcc tccatcctcg    180
tcctccttac ttacccttgg ctgtaccaga agaccttgca gatcgactcc tgagagtcca    240
tggtgatcct gcagtgtggt ggggtatccca gtttgtcaaa tacttgatcc gtccacaacc    300
ttggctggaa agggaaatag aagaaccac caagaagctt ggcttcaaac atccagttat    360
tggagtccat gtcagacgca ctgacaaagt gggaacagaa gcagccttcc atcccattga    420
ggaatacatg gtacacgttg aagaacattt tcagcttctc gaacgcagaa tgaaagtggga    480
taaaaaaaga gtgtatcttg ccactgatga cccttctttg ttaaaggagg caaagacaaa    540
gtactccaat tatgaattta ttagtgataa ctctatttct tggtcagctg gactacacaa    600
ccgatacaca gaaaattcac ttcggggcgt gatcctggat atacactttc tctcccaggc    660
tgacttcctt gtgtgtactt tttcatccca ggtctgtagg gttgcttatg aaatcatgca    720
aacactgcat cctgatgcct ctgcaaaact ccattcttta gatgacatct actatttttg    780
aggccaaaat gcccacaaac agattgcagt ttatcctcac caacctcgaa ctaaaggagg    840
aatcccatg gaacctggag atatcattgg tgtggctgga aaccattgga atggttactc    900
taaagggtgc aacagaaaac taggaaaaac aggcctgtac ccttcctaca aagtcgaga    960
gaagatagaa acggtcaag                                     979

```

<210> SEQ ID NO. 7

<211> LENGTH: 979

<212> TYPE: DNA

<213> ORGANISM: *Rattus norvegicus*

<400> SEQUENCE: 7

```

actcatcttg gaatctcaga attggcgcta tgctactggt ggatgggaga ctgtgttttag    60
acctgtaagt gagacatgca cagacagatc tggcctctcc actggacact ggtcagggtga    120
agtgaatgac aaaaatattc aagtggtgga gctccccatt gtagacagcc tccatcctcg    180
gcctccttac ttaccactgg ctgttcocaga agaccttgca gatcgactcg taagagtcca    240
tggtgatcct gcagtgtggt ggggtgccca gttcgtcaaa tatttgattc gtccacaacc    300
ttggctagaa aaggaaatag aagaagccac caagaagctt ggcttcaaac atccagtcac    360
tggagtccat gtcagacgca cagacaaagt gggaacagag gcagccttcc atcccatcga    420
agagtacatg gtacatgttg aagaacattt tcagcttctc gcacgcagaa tgcaagtggga    480
taaaaaaaga gtatatcttg ctaccgatga ccctgctttg ttaaaggagg caaagacaaa    540
gtactccaat tatgaattta ttagtgataa ctctatttct tggtcagctg gactacacaa    600
tcggtacaca gaaaattcac ttcggggcgt gatcctggat atacactttc tctctcaggc    660
tgacttccta gtgtgtactt tttcatccca ggtctgtcgg gttgcttatg aaatcatgca    720
aaccctgcat cctgatgcct ctgcaaaact ccactcttta gatgacatct actatttttg    780
aggccaaaat gcccacaaac agattgccgt ttatcctcac aaacctcgaa ctgatgagga    840
aattccaatg gaacctggag atatcattgg tgtggctgga aaccattggg atggttattc    900

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-continued

taaaggtgtc aacagaaaac ttggaaaaac aggcttatat ccctcctaca aagtccgaga 960

gaagatagaa acgggtcaag 979

<210> SEQ ID NO 8

<211> LENGTH: 40

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic DNA

<400> SEQUENCE: 8

aagtataagc ttacatggat gacgatatcg ctgcgctcgt 40

<210> SEQ ID NO 9

<211> LENGTH: 40

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic DNA

<400> SEQUENCE: 9

atttaactgc aggaagcatt tgcggtggac gatggagggg 40

<210> SEQ ID NO 10

<211> LENGTH: 40

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic DNA

<400> SEQUENCE: 10

atttaaggta ccgaagcatt tgcggtgcac gatggagggg 40

<210> SEQ ID NO 11

<211> LENGTH: 23

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic DNA

<400> SEQUENCE: 11

ctccaattat gaatttatta gtg 23

<210> SEQ ID NO 12

<211> LENGTH: 25

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic DNA

<400> SEQUENCE: 12

ggatgtttga agccaagctt cttgg 25

<210> SEQ ID NO 13

<211> LENGTH: 24

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic

-continued

DNA

<400> SEQUENCE: 13

gtccatggtg atcctgcagt gtgg

24

<210> SEQ ID NO 14

<211> LENGTH: 23

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
DNA

<400> SEQUENCE: 14

caccaatgat atctccaggt tcc

23

<210> SEQ ID NO 15

<211> LENGTH: 24

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
DNA

<400> SEQUENCE: 15

gatatcgctg cgctcgttgt cgac

24

<210> SEQ ID NO 16

<211> LENGTH: 24

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
DNA

<400> SEQUENCE: 16

caggaaggaa ggctggaaaa gagc

24

<210> SEQ ID NO 17

<211> LENGTH: 24

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
DNA

<400> SEQUENCE: 17

gatatcgctg cgctcgtcgt cgac

24

<210> SEQ ID NO 18

<211> LENGTH: 24

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
DNA

<400> SEQUENCE: 18

caggaaggaa ggctggaaga gagc

24

<210> SEQ ID NO 19

<211> LENGTH: 321

<212> TYPE: PRT

<213> ORGANISM: Cricetulus griseus

-continued

<400> SEQUENCE: 19

```

Met Gly Glu Pro Gln Gly Ser Arg Arg Ile Leu Val Thr Gly Gly Ser
  1           5           10           15
Gly Leu Val Gly Arg Ala Ile Gln Lys Val Val Ala Asp Gly Ala Gly
  20           25           30
Leu Pro Gly Glu Glu Trp Val Phe Val Ser Ser Lys Asp Ala Asp Leu
  35           40           45
Thr Asp Ala Ala Gln Thr Gln Ala Leu Phe Gln Lys Val Gln Pro Thr
  50           55           60
His Val Ile His Leu Ala Ala Met Val Gly Gly Leu Phe Arg Asn Ile
  65           70           75           80
Lys Tyr Asn Leu Asp Phe Trp Arg Lys Asn Val His Ile Asn Asp Asn
  85           90           95
Val Leu His Ser Ala Phe Glu Val Gly Thr Arg Lys Val Val Ser Cys
  100          105          110
Leu Ser Thr Cys Ile Phe Pro Asp Lys Thr Thr Tyr Pro Ile Asp Glu
  115          120          125
Thr Met Ile His Asn Gly Pro Pro His Ser Ser Asn Phe Gly Tyr Ser
  130          135          140
Tyr Ala Lys Arg Met Ile Asp Val Gln Asn Arg Ala Tyr Phe Gln Gln
  145          150          155          160
His Gly Cys Thr Phe Thr Ala Val Ile Pro Thr Asn Val Phe Gly Pro
  165          170          175
His Asp Asn Phe Asn Ile Glu Asp Gly His Val Leu Pro Gly Leu Ile
  180          185          190
His Lys Val His Leu Ala Lys Ser Asn Gly Ser Ala Leu Thr Val Trp
  195          200          205
Gly Thr Gly Lys Pro Arg Arg Gln Phe Ile Tyr Ser Leu Asp Leu Ala
  210          215          220
Arg Leu Phe Ile Trp Val Leu Arg Glu Tyr Asn Glu Val Glu Pro Ile
  225          230          235          240
Ile Leu Ser Val Gly Glu Glu Asp Glu Val Ser Ile Lys Glu Ala Ala
  245          250          255
Glu Ala Val Val Glu Ala Met Asp Phe Cys Gly Glu Val Thr Phe Asp
  260          265          270
Ser Thr Lys Ser Asp Gly Gln Tyr Lys Lys Thr Ala Ser Asn Gly Lys
  275          280          285
Leu Arg Ala Tyr Leu Pro Asp Phe Arg Phe Thr Pro Phe Lys Gln Ala
  290          295          300
Val Lys Glu Thr Cys Ala Trp Phe Thr Asp Asn Tyr Glu Gln Ala Arg
  305          310          315          320
Lys

```

<210> SEQ ID NO 20

<211> LENGTH: 590

<212> TYPE: PRT

<213> ORGANISM: *Cricetulus griseus*

<400> SEQUENCE: 20

```

Met Ala Ser Leu Arg Glu Ala Ser Leu Arg Lys Leu Arg Arg Phe Ser
  1           5           10           15

```

-continued

Glu	Met	Arg	Gly	Lys	Pro	Val	Ala	Thr	Gly	Lys	Phe	Trp	Asp	Val	Val
			20					25					30		
Val	Ile	Thr	Ala	Ala	Asp	Glu	Lys	Gln	Glu	Leu	Ala	Tyr	Lys	Gln	Gln
		35					40					45			
Leu	Ser	Glu	Lys	Leu	Lys	Arg	Lys	Glu	Leu	Pro	Leu	Gly	Val	Asn	Tyr
	50					55					60				
His	Val	Phe	Thr	Asp	Pro	Pro	Gly	Thr	Lys	Ile	Gly	Asn	Gly	Gly	Ser
65					70					75					80
Thr	Leu	Cys	Ser	Leu	Gln	Cys	Leu	Glu	Ser	Leu	Tyr	Gly	Asp	Lys	Trp
				85					90					95	
Asn	Ser	Phe	Thr	Val	Leu	Leu	Ile	His	Ser	Gly	Gly	Tyr	Ser	Gln	Arg
		100						105					110		
Leu	Pro	Asn	Ala	Ser	Ala	Leu	Gly	Lys	Ile	Phe	Thr	Ala	Leu	Pro	Leu
		115					120					125			
Gly	Glu	Pro	Ile	Tyr	Gln	Met	Leu	Asp	Leu	Lys	Leu	Ala	Met	Tyr	Met
	130					135					140				
Asp	Phe	Pro	Ser	Arg	Met	Lys	Pro	Gly	Val	Leu	Val	Thr	Cys	Ala	Asp
145					150					155					160
Asp	Ile	Glu	Leu	Tyr	Ser	Ile	Gly	Asp	Ser	Glu	Ser	Ile	Ala	Phe	Glu
			165					170					175		
Gln	Pro	Gly	Phe	Thr	Ala	Leu	Ala	His	Pro	Ser	Ser	Leu	Ala	Val	Gly
		180						185					190		
Thr	Thr	His	Gly	Val	Phe	Val	Leu	Asp	Ser	Ala	Gly	Ser	Leu	Gln	His
		195					200					205			
Gly	Asp	Leu	Glu	Tyr	Arg	Gln	Cys	His	Arg	Phe	Leu	His	Lys	Pro	Ser
	210					215				220					
Ile	Glu	Asn	Met	His	His	Phe	Asn	Ala	Val	His	Arg	Leu	Gly	Ser	Phe
225					230					235					240
Gly	Gln	Gln	Asp	Leu	Ser	Gly	Gly	Asp	Thr	Thr	Cys	His	Pro	Leu	His
			245					250					255		
Ser	Glu	Tyr	Val	Tyr	Thr	Asp	Ser	Leu	Phe	Tyr	Met	Asp	His	Lys	Ser
		260					265					270			
Ala	Lys	Lys	Leu	Leu	Asp	Phe	Tyr	Glu	Ser	Val	Gly	Pro	Leu	Asn	Cys
		275				280					285				
Glu	Ile	Asp	Ala	Tyr	Gly	Asp	Phe	Leu	Gln	Ala	Leu	Gly	Pro	Gly	Ala
	290				295					300					
Thr	Ala	Glu	Tyr	Thr	Lys	Asn	Thr	Ser	His	Val	Thr	Lys	Glu	Glu	Ser
305					310					315					320
His	Leu	Leu	Asp	Met	Arg	Gln	Lys	Ile	Phe	His	Leu	Leu	Lys	Gly	Thr
			325						330					335	
Pro	Leu	Asn	Val	Val	Val	Leu	Asn	Asn	Ser	Arg	Phe	Tyr	His	Ile	Gly
		340					345					350			
Thr	Thr	Glu	Glu	Tyr	Leu	Leu	His	Phe	Thr	Ser	Asn	Gly	Ser	Leu	Gln
		355					360					365			
Ala	Glu	Leu	Gly	Leu	Gln	Ser	Ile	Ala	Phe	Ser	Val	Phe	Pro	Asn	Val
	370					375					380				
Pro	Glu	Asp	Ser	His	Glu	Lys	Pro	Cys	Val	Ile	His	Ser	Ile	Leu	Asn
385					390					395					400
Ser	Gly	Cys	Cys	Val	Ala	Pro	Gly	Ser	Val	Val	Glu	Tyr	Ser	Arg	Leu
			405						410					415	
Gly	Pro	Glu	Val	Ser	Ile	Ser	Glu	Asn	Cys	Ile	Ile	Ser	Gly	Ser	Val

-continued

420	425	430
Ile Glu Lys Ala Val Leu Pro	Pro Cys Ser Phe Val	Cys Ser Leu Ser
435	440	445
Val Glu Ile Asn Gly His Leu	Glu Tyr Ser Thr Met Val	Phe Gly Met
450	455	460
Glu Asp Asn Leu Lys Asn Ser	Val Lys Thr Ile Ser	Asp Ile Lys Met
465	470	475
Leu Gln Phe Phe Gly Val Cys	Phe Leu Thr Cys Leu	Asp Ile Trp Asn
485	490	495
Leu Lys Ala Met Glu Glu Leu	Phe Ser Gly Ser Lys Thr	Gln Leu Ser
500	505	510
Leu Trp Thr Ala Arg Ile Phe	Pro Val Cys Ser Ser	Leu Ser Glu Ser
515	520	525
Val Ala Ala Ser Leu Gly Met	Leu Asn Ala Ile Arg	Asn His Ser Pro
530	535	540
Phe Ser Leu Ser Asn Phe Lys	Leu Leu Ser Ile Gln	Glu Met Leu Leu
545	550	555
Cys Lys Asp Val Gly Asp Met	Leu Ala Tyr Arg Glu	Gln Leu Phe Leu
565	570	575
Glu Ile Ser Ser Lys Arg Lys	Gln Ser Asp Ser Glu	Lys Ser
580	585	590

<210> SEQ ID NO 21
 <211> LENGTH: 25
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 21

Gln Val Thr Val Gln Ser Ser	Pro Asn Phe Thr Gln His Val	Arg Glu
1	5	10
Gln Ser Leu Val Thr Asp Gln	Leu Cys	
20	25	

<210> SEQ ID NO 22
 <211> LENGTH: 32
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
 DNA

<400> SEQUENCE: 22

taaatagaat tcggcatcat gtggcagctg ct

32

<210> SEQ ID NO 23
 <211> LENGTH: 575
 <212> TYPE: PRT
 <213> ORGANISM: Cricetulus griseus

<400> SEQUENCE: 23

Met Arg Ala Trp Thr Gly Ser	Trp Arg Trp Ile Met Leu Ile	Leu Phe
1	5	10
Ala Trp Gly Thr Leu Leu Phe	Tyr Ile Gly Gly His Leu Val	Arg Asp
20	25	30
Asn Asp His Pro Asp His Ser	Ser Arg Glu Leu Ser Lys Ile	Leu Ala
35	40	45

-continued

Lys	Leu	Glu	Arg	Leu	Lys	Gln	Gln	Asn	Glu	Asp	Leu	Arg	Arg	Met	Ala
50						55					60				
Glu	Ser	Leu	Arg	Ile	Pro	Glu	Gly	Pro	Ile	Asp	Gln	Gly	Thr	Ala	Thr
65					70					75					80
Gly	Arg	Val	Arg	Val	Leu	Glu	Glu	Gln	Leu	Val	Lys	Ala	Lys	Glu	Gln
				85					90					95	
Ile	Glu	Asn	Tyr	Lys	Lys	Gln	Ala	Arg	Asn	Asp	Leu	Gly	Lys	Asp	His
			100					105					110		
Glu	Ile	Leu	Arg	Arg	Arg	Ile	Glu	Asn	Gly	Ala	Lys	Glu	Leu	Trp	Phe
		115					120					125			
Phe	Leu	Gln	Ser	Glu	Leu	Lys	Lys	Leu	Lys	Lys	Leu	Glu	Gly	Asn	Glu
	130					135					140				
Leu	Gln	Arg	His	Ala	Asp	Glu	Ile	Leu	Leu	Asp	Leu	Gly	His	His	Glu
145					150					155					160
Arg	Ser	Ile	Met	Thr	Asp	Leu	Tyr	Tyr	Leu	Ser	Gln	Thr	Asp	Gly	Ala
				165					170					175	
Gly	Glu	Trp	Arg	Glu	Lys	Glu	Ala	Lys	Asp	Leu	Thr	Glu	Leu	Val	Gln
		180						185					190		
Arg	Arg	Ile	Thr	Tyr	Leu	Gln	Asn	Pro	Lys	Asp	Cys	Ser	Lys	Ala	Arg
		195					200					205			
Lys	Leu	Val	Cys	Asn	Ile	Asn	Lys	Gly	Cys	Gly	Tyr	Gly	Cys	Gln	Leu
	210					215					220				
His	His	Val	Val	Tyr	Cys	Phe	Met	Ile	Ala	Tyr	Gly	Thr	Gln	Arg	Thr
225					230					235					240
Leu	Ile	Leu	Glu	Ser	Gln	Asn	Trp	Arg	Tyr	Ala	Thr	Gly	Gly	Trp	Glu
			245						250					255	
Thr	Val	Phe	Arg	Pro	Val	Ser	Glu	Thr	Cys	Thr	Asp	Arg	Ser	Gly	Leu
		260						265					270		
Ser	Thr	Gly	His	Trp	Ser	Gly	Glu	Val	Lys	Asp	Lys	Asn	Val	Gln	Val
		275					280					285			
Val	Glu	Leu	Pro	Ile	Val	Asp	Ser	Leu	His	Pro	Arg	Pro	Pro	Tyr	Leu
	290					295					300				
Pro	Leu	Ala	Val	Pro	Glu	Asp	Leu	Ala	Asp	Arg	Leu	Leu	Arg	Val	His
305					310					315					320
Gly	Asp	Pro	Ala	Val	Trp	Trp	Val	Ser	Gln	Phe	Val	Lys	Tyr	Leu	Ile
			325						330					335	
Arg	Pro	Gln	Pro	Trp	Leu	Glu	Arg	Glu	Ile	Glu	Glu	Thr	Thr	Lys	Lys
		340						345					350		
Leu	Gly	Phe	Lys	His	Pro	Val	Ile	Gly	Val	His	Val	Arg	Arg	Thr	Asp
		355					360					365			
Lys	Val	Gly	Thr	Glu	Ala	Ala	Phe	His	Pro	Ile	Glu	Glu	Tyr	Met	Val
	370					375					380				
His	Val	Glu	Glu	His	Phe	Gln	Leu	Leu	Glu	Arg	Arg	Met	Lys	Val	Asp
385					390					395					400
Lys	Lys	Arg	Val	Tyr	Leu	Ala	Thr	Asp	Asp	Pro	Ser	Leu	Leu	Lys	Glu
			405						410					415	
Ala	Lys	Thr	Lys	Tyr	Ser	Asn	Tyr	Glu	Phe	Ile	Ser	Asp	Asn	Ser	Ile
		420						425					430		
Ser	Trp	Ser	Ala	Gly	Leu	His	Asn	Arg	Tyr	Thr	Glu	Asn	Ser	Leu	Arg
		435					440					445			
Gly	Val	Ile	Leu	Asp	Ile	His	Phe	Leu	Ser	Gln	Ala	Asp	Phe	Leu	Val

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450					455					460					
Cys	Thr	Phe	Ser	Ser	Gln	Val	Cys	Arg	Val	Ala	Tyr	Glu	Ile	Met	Gln
465					470					475					480
Thr	Leu	His	Pro	Asp	Ala	Ser	Ala	Asn	Phe	His	Ser	Leu	Asp	Asp	Ile
				485					490					495	
Tyr	Tyr	Phe	Gly	Gly	Gln	Asn	Ala	His	Asn	Gln	Ile	Ala	Val	Tyr	Pro
			500					505					510		
His	Gln	Pro	Arg	Thr	Lys	Glu	Glu	Ile	Pro	Met	Glu	Pro	Gly	Asp	Ile
		515					520					525			
Ile	Gly	Val	Ala	Gly	Asn	His	Trp	Asn	Gly	Tyr	Ser	Lys	Gly	Val	Asn
	530					535					540				
Arg	Lys	Leu	Gly	Lys	Thr	Gly	Leu	Tyr	Pro	Ser	Tyr	Lys	Val	Arg	Glu
545					550					555					560
Lys	Ile	Glu	Thr	Val	Lys	Tyr	Pro	Thr	Tyr	Pro	Glu	Ala	Glu	Lys	
				565					570					575	

<210> SEQ ID NO 24

<211> LENGTH: 575

<212> TYPE: PRT

<213> ORGANISM: Mus musculus

<400> SEQUENCE: 24

Met	Arg	Ala	Trp	Thr	Gly	Ser	Trp	Arg	Trp	Ile	Met	Leu	Ile	Leu	Phe
1				5					10					15	
Ala	Trp	Gly	Thr	Leu	Leu	Phe	Tyr	Ile	Gly	Gly	His	Leu	Val	Arg	Asp
			20					25					30		
Asn	Asp	His	Pro	Asp	His	Ser	Ser	Arg	Glu	Leu	Ser	Lys	Ile	Leu	Ala
		35				40						45			
Lys	Leu	Glu	Arg	Leu	Lys	Gln	Gln	Asn	Glu	Asp	Leu	Arg	Arg	Met	Ala
	50					55					60				
Glu	Ser	Leu	Arg	Ile	Pro	Glu	Gly	Pro	Ile	Asp	Gln	Gly	Thr	Ala	Thr
65				70					75						80
Gly	Arg	Val	Arg	Val	Leu	Glu	Glu	Gln	Leu	Val	Lys	Ala	Lys	Glu	Gln
				85				90						95	
Ile	Glu	Asn	Tyr	Lys	Lys	Gln	Ala	Arg	Asn	Gly	Leu	Gly	Lys	Asp	His
		100						105					110		
Glu	Ile	Leu	Arg	Arg	Arg	Ile	Glu	Asn	Gly	Ala	Lys	Glu	Leu	Trp	Phe
	115					120						125			
Phe	Leu	Gln	Ser	Glu	Leu	Lys	Lys	Leu	Lys	His	Leu	Glu	Gly	Asn	Glu
	130					135					140				
Leu	Gln	Arg	His	Ala	Asp	Glu	Ile	Leu	Leu	Asp	Leu	Gly	His	His	Glu
145				150						155					160
Arg	Ser	Ile	Met	Thr	Asp	Leu	Tyr	Tyr	Leu	Ser	Gln	Thr	Asp	Gly	Ala
				165					170					175	
Gly	Asp	Trp	Arg	Glu	Lys	Glu	Ala	Lys	Asp	Leu	Thr	Glu	Leu	Val	Gln
		180						185					190		
Arg	Arg	Ile	Thr	Tyr	Leu	Gln	Asn	Pro	Lys	Asp	Cys	Ser	Lys	Ala	Arg
		195					200					205			
Lys	Leu	Val	Cys	Asn	Ile	Asn	Lys	Gly	Cys	Gly	Tyr	Gly	Cys	Gln	Leu
	210					215					220				
His	His	Val	Val	Tyr	Cys	Phe	Met	Ile	Ala	Tyr	Gly	Thr	Gln	Arg	Thr
225					230					235					240

-continued

```

Leu Ile Leu Glu Ser Gln Asn Trp Arg Tyr Ala Thr Gly Gly Trp Glu
      245                      250                      255
Thr Val Phe Arg Pro Val Ser Glu Thr Cys Thr Asp Arg Ser Gly Leu
      260                      265                      270
Ser Thr Gly His Trp Ser Gly Glu Val Asn Asp Lys Asn Ile Gln Val
      275                      280                      285
Val Glu Leu Pro Ile Val Asp Ser Leu His Pro Arg Pro Pro Tyr Leu
      290                      295                      300
Pro Leu Ala Val Pro Glu Asp Leu Ala Asp Arg Leu Leu Arg Val His
      305                      310                      315                      320
Gly Asp Pro Ala Val Trp Trp Val Ser Gln Phe Val Lys Tyr Leu Ile
      325                      330                      335
Arg Pro Gln Pro Trp Leu Glu Lys Glu Ile Glu Glu Ala Thr Lys Lys
      340                      345                      350
Leu Gly Phe Lys His Pro Val Ile Gly Val His Val Arg Arg Thr Asp
      355                      360                      365
Lys Val Gly Thr Glu Ala Ala Phe His Pro Ile Glu Glu Tyr Met Val
      370                      375                      380
His Val Glu Glu His Phe Gln Leu Leu Ala Arg Arg Met Gln Val Asp
      385                      390                      395                      400
Lys Lys Arg Val Tyr Leu Ala Thr Asp Asp Pro Thr Leu Leu Lys Glu
      405                      410                      415
Ala Lys Thr Lys Tyr Ser Asn Tyr Glu Phe Ile Ser Asp Asn Ser Ile
      420                      425                      430
Ser Trp Ser Ala Gly Leu His Asn Arg Tyr Thr Glu Asn Ser Leu Arg
      435                      440                      445
Gly Val Ile Leu Asp Ile His Phe Leu Ser Gln Ala Asp Phe Leu Val
      450                      455                      460
Cys Thr Phe Ser Ser Gln Val Cys Arg Val Ala Tyr Glu Ile Met Gln
      465                      470                      475                      480
Thr Leu His Pro Asp Ala Ser Ala Asn Phe His Ser Leu Asp Asp Ile
      485                      490                      495
Tyr Tyr Phe Gly Gly Gln Asn Ala His Asn Gln Ile Ala Val Tyr Pro
      500                      505                      510
His Lys Pro Arg Thr Glu Glu Glu Ile Pro Met Glu Pro Gly Asp Ile
      515                      520                      525
Ile Gly Val Ala Gly Asn His Trp Asp Gly Tyr Ser Lys Gly Ile Asn
      530                      535                      540
Arg Lys Leu Gly Lys Thr Gly Leu Tyr Pro Ser Tyr Lys Val Arg Glu
      545                      550                      555                      560
Lys Ile Glu Thr Val Lys Tyr Pro Thr Tyr Pro Glu Ala Glu Lys
      565                      570                      575

```

<210> SEQ ID NO 25

<211> LENGTH: 18

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 25

```

Asp Glu Ser Ile Tyr Ser Asn Tyr Tyr Leu Tyr Glu Ser Ile Pro Lys
  1             5             10             15

```

Pro Cys

-continued

```

<210> SEQ ID NO 26
<211> LENGTH: 34
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA

<400> SEQUENCE: 26

aataaaggat cctgggggtca tttgtcttga ggggt                               34

<210> SEQ ID NO 27
<211> LENGTH: 788
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 27

gaa ttc ggc atc atg tgg cag ctg ctc ctc cca act gct ctg cta ctt         48
      Met Trp Gln Leu Leu Leu Pro Thr Ala Leu Leu Leu
      1             5             10

cta gtt tca gct ggc atg cgg act gaa gat ctc cca aag gct gtg gtg         96
Leu Val Ser Ala Gly Met Arg Thr Glu Asp Leu Pro Lys Ala Val Val
      15             20             25

ttc ctg gag cct caa tgg tac agg gtg ctc gag aag gac agt gtg act        144
Phe Leu Glu Pro Gln Trp Tyr Arg Val Leu Glu Lys Asp Ser Val Thr
      30             35             40

ctg aag tgc cag gga gcc tac tcc cct gag gac aat tcc aca cag tgg        192
Leu Lys Cys Gln Gly Ala Tyr Ser Pro Glu Asp Asn Ser Thr Gln Trp
      45             50             55             60

ttt cac aat gag agc ctc atc tca agc cag gcc tcg agc tac ttc att        240
Phe His Asn Glu Ser Leu Ile Ser Ser Gln Ala Ser Ser Tyr Phe Ile
      65             70             75

gac gct gcc aca gtc gac gac agt gga gag tac agg tgc cag aca aac        288
Asp Ala Ala Thr Val Asp Asp Ser Gly Glu Tyr Arg Cys Gln Thr Asn
      80             85             90

ctc tcc acc ctc agt gac ccg gtg cag cta gaa gtc cat atc ggc tgg        336
Leu Ser Thr Leu Ser Asp Pro Val Gln Leu Glu Val His Ile Gly Trp
      95             100            105

ctg ttg ctc cag gcc cct cgg tgg gtg ttc aag gag gaa gac cct att        384
Leu Leu Leu Gln Ala Pro Arg Trp Val Phe Lys Glu Glu Asp Pro Ile
      110            115            120

cac ctg agg tgt cac agc tgg aag aac act gct ctg cat aag gtc aca        432
His Leu Arg Cys His Ser Trp Lys Asn Thr Ala Leu His Lys Val Thr
      125            130            135            140

tat tta cag aat ggc aaa ggc agg aag tat ttt cat cat aat tct gac        480
Tyr Leu Gln Asn Gly Lys Gly Arg Lys Tyr Phe His His Asn Ser Asp
      145            150            155

ttc tac att cca aaa gcc aca ctc aaa gac agc ggc tcc tac ttc tgc        528
Phe Tyr Ile Pro Lys Ala Thr Leu Lys Asp Ser Gly Ser Tyr Phe Cys
      160            165            170

agg ggg ctt ttt ggg agt aaa aat gtg tct tca gag act gtg aac atc        576
Arg Gly Leu Phe Gly Ser Lys Asn Val Ser Ser Glu Thr Val Asn Ile
      175            180            185

acc atc act caa ggt ttg gca gtg tca acc atc tca tca ttc ttt cca        624
Thr Ile Thr Gln Gly Leu Ala Val Ser Thr Ile Ser Ser Phe Phe Pro
      190            195            200

cct ggg tac caa gtc tct ttc tgc ttg gtg atg gta ctc ctt ttt gca        672
Pro Gly Tyr Gln Val Ser Phe Cys Leu Val Met Val Leu Leu Phe Ala
      205            210            215            220

```

-continued

```
gtg gac aca gga cta tat ttc tct gtg aag aca aac att cga agc tca      720
Val Asp Thr Gly Leu Tyr Phe Ser Val Lys Thr Asn Ile Arg Ser Ser
                225                230                235
```

```
aca aga gac tgg aag gac cat aaa ttt aaa tgg aga aag gac cct caa      768
Thr Arg Asp Trp Lys Asp His Lys Phe Lys Trp Arg Lys Asp Pro Gln
                240                245                250
```

```
gac aaa tga ccc cag gat cc      788
Asp Lys
```

<210> SEQ ID NO 28

<211> LENGTH: 254

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 28

```
Met Trp Gln Leu Leu Leu Pro Thr Ala Leu Leu Leu Val Ser Ala
  1                      5                      10                      15
```

```
Gly Met Arg Thr Glu Asp Leu Pro Lys Ala Val Val Phe Leu Glu Pro
                20                25                30
```

```
Gln Trp Tyr Arg Val Leu Glu Lys Asp Ser Val Thr Leu Lys Cys Gln
                35                40                45
```

```
Gly Ala Tyr Ser Pro Glu Asp Asn Ser Thr Gln Trp Phe His Asn Glu
  50                55                60
```

```
Ser Leu Ile Ser Ser Gln Ala Ser Ser Tyr Phe Ile Asp Ala Ala Thr
  65                70                75                80
```

```
Val Asp Asp Ser Gly Glu Tyr Arg Cys Gln Thr Asn Leu Ser Thr Leu
                85                90                95
```

```
Ser Asp Pro Val Gln Leu Glu Val His Ile Gly Trp Leu Leu Leu Gln
                100                105                110
```

```
Ala Pro Arg Trp Val Phe Lys Glu Glu Asp Pro Ile His Leu Arg Cys
                115                120                125
```

```
His Ser Trp Lys Asn Thr Ala Leu His Lys Val Thr Tyr Leu Gln Asn
                130                135                140
```

```
Gly Lys Gly Arg Lys Tyr Phe His His Asn Ser Asp Phe Tyr Ile Pro
                145                150                155                160
```

```
Lys Ala Thr Leu Lys Asp Ser Gly Ser Tyr Phe Cys Arg Gly Leu Phe
                165                170                175
```

```
Gly Ser Lys Asn Val Ser Ser Glu Thr Val Asn Ile Thr Ile Thr Gln
                180                185                190
```

```
Gly Leu Ala Val Ser Thr Ile Ser Ser Phe Phe Pro Pro Gly Tyr Gln
                195                200                205
```

```
Val Ser Phe Cys Leu Val Met Val Leu Leu Phe Ala Val Asp Thr Gly
                210                215                220
```

```
Leu Tyr Phe Ser Val Lys Thr Asn Ile Arg Ser Ser Thr Arg Asp Trp
                225                230                235                240
```

```
Lys Asp His Lys Phe Lys Trp Arg Lys Asp Pro Gln Asp Lys
                245                250
```

<210> SEQ ID NO 29

<211> LENGTH: 51

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic DNA

-continued

<400> SEQUENCE: 29

tg	ttg	gat	cc	tg	tca	at	gat	gat	gat	at	gac	ctt	ga	tg	gat	ggt	ga	t	51
----	-----	-----	----	----	-----	----	-----	-----	-----	----	-----	-----	----	----	-----	-----	----	---	----

<210> SEQ ID NO 30

<211> LENGTH: 620

<212> TYPE: DNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 30

gaa	ttc	ggc	atc	atg	tgg	cag	ctg	ctc	ctc	cca	act	gct	ctg	cta	ctt	48
				Met	Trp	Gln	Leu	Leu	Leu	Pro	Thr	Ala	Leu	Leu	Leu	
			1					5					10			

cta	gtt	tca	gct	ggc	atg	cgg	act	gaa	gat	ctc	cca	aag	gct	gtg	gtg	96
Leu	Val	Ser	Ala	Gly	Met	Arg	Thr	Glu	Asp	Leu	Pro	Lys	Ala	Val	Val	
		15					20					25				

ttc	ctg	gag	cct	caa	tgg	tac	agg	gtg	ctc	gag	aag	gac	agt	gtg	act	144
Phe	Leu	Glu	Pro	Gln	Trp	Tyr	Arg	Val	Leu	Glu	Lys	Asp	Ser	Val	Thr	
	30					35					40					

ctg	aag	tgc	cag	gga	gcc	tac	tcc	cct	gag	gac	aat	tcc	aca	cag	tgg	192
Leu	Lys	Cys	Gln	Gly	Ala	Tyr	Ser	Pro	Glu	Asp	Asn	Ser	Thr	Gln	Trp	
	45				50					55				60		

ttt	cac	aat	gag	agc	ctc	atc	tca	agc	cag	gcc	tcg	agc	tac	ttc	att	240
Phe	His	Asn	Glu	Ser	Leu	Ile	Ser	Ser	Gln	Ala	Ser	Ser	Tyr	Phe	Ile	
			65						70				75			

gac	gct	gcc	aca	gtc	gac	gac	agt	gga	gag	tac	agg	tgc	cag	aca	aac	288
Asp	Ala	Ala	Thr	Val	Asp	Asp	Ser	Gly	Glu	Tyr	Arg	Cys	Gln	Thr	Asn	
			80					85					90			

ctc	tcc	acc	ctc	agt	gac	ccg	gtg	cag	cta	gaa	gtc	cat	atc	ggc	tgg	336
Leu	Ser	Thr	Leu	Ser	Asp	Pro	Val	Gln	Leu	Glu	Val	His	Ile	Gly	Trp	
		95				100						105				

ctg	ttg	ctc	cag	gcc	cct	cgg	tgg	gtg	ttc	aag	gag	gaa	gac	cct	att	384
Leu	Leu	Leu	Gln	Ala	Pro	Arg	Trp	Val	Phe	Lys	Glu	Glu	Asp	Pro	Ile	
	110					115					120					

cac	ctg	agg	tgt	cac	agc	tgg	aag	aac	act	gct	ctg	cat	aag	gtc	aca	432
His	Leu	Arg	Cys	His	Ser	Trp	Lys	Asn	Thr	Ala	Leu	His	Lys	Val	Thr	
	125				130				135					140		

tat	tta	cag	aat	ggc	aaa	ggc	agg	aag	tat	ttt	cat	cat	aat	tct	gac	480
Tyr	Leu	Gln	Asn	Gly	Lys	Gly	Arg	Lys	Tyr	Phe	His	His	Asn	Ser	Asp	
			145						150				155			

ttc	tac	att	cca	aaa	gcc	aca	ctc	aaa	gac	agc	ggc	tcc	tac	ttc	tgc	528
Phe	Tyr	Ile	Pro	Lys	Ala	Thr	Leu	Lys	Asp	Ser	Gly	Ser	Tyr	Phe	Cys	
		160						165				170				

agg	ggg	ctt	ttt	ggg	agt	aaa	aat	gtg	tct	tca	gag	act	gtg	aac	atc	576
Arg	Gly	Leu	Phe	Gly	Ser	Lys	Asn	Val	Ser	Ser	Glu	Thr	Val	Asn	Ile	
	175					180					185					

acc	atc	act	caa	ggt	cat	cat	cat	cat	cat	cat	tga	cag	gat	cc	620
Thr	Ile	Thr	Gln	Gly	His	His	His	His	His	His					
	190				195										

<210> SEQ ID NO 31

<211> LENGTH: 199

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 31

Met	Trp	Gln	Leu	Leu	Leu	Pro	Thr	Ala	Leu	Leu	Leu	Leu	Val	Ser	Ala
1				5					10					15	

-continued

Gly Met Arg Thr Glu Asp Leu Pro Lys Ala Val Val Phe Leu Glu Pro
 20 25 30
 Gln Trp Tyr Arg Val Leu Glu Lys Asp Ser Val Thr Leu Lys Cys Gln
 35 40 45
 Gly Ala Tyr Ser Pro Glu Asp Asn Ser Thr Gln Trp Phe His Asn Glu
 50 55 60
 Ser Leu Ile Ser Ser Gln Ala Ser Ser Tyr Phe Ile Asp Ala Ala Thr
 65 70 75 80
 Val Asp Asp Ser Gly Glu Tyr Arg Cys Gln Thr Asn Leu Ser Thr Leu
 85 90 95
 Ser Asp Pro Val Gln Leu Glu Val His Ile Gly Trp Leu Leu Leu Gln
 100 105 110
 Ala Pro Arg Trp Val Phe Lys Glu Glu Asp Pro Ile His Leu Arg Cys
 115 120 125
 His Ser Trp Lys Asn Thr Ala Leu His Lys Val Thr Tyr Leu Gln Asn
 130 135 140
 Gly Lys Gly Arg Lys Tyr Phe His His Asn Ser Asp Phe Tyr Ile Pro
 145 150 155 160
 Lys Ala Thr Leu Lys Asp Ser Gly Ser Tyr Phe Cys Arg Gly Leu Phe
 165 170 175
 Gly Ser Lys Asn Val Ser Ser Glu Thr Val Asn Ile Thr Ile Thr Gln
 180 185 190
 Gly His His His His His His
 195

<210> SEQ ID NO 32
 <211> LENGTH: 24
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
 DNA

<400> SEQUENCE: 32

aggaaggtgg cgctcatcac gggc

24

<210> SEQ ID NO 33
 <211> LENGTH: 26
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
 DNA

<400> SEQUENCE: 33

taaggccaca agtcttaatt gcatcc

26

<210> SEQ ID NO 34
 <211> LENGTH: 27
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
 DNA

<400> SEQUENCE: 34

caggggtggt ccottgagga ggtggaa

27

-continued

<210> SEQ ID NO 35
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
DNA

<400> SEQUENCE: 35

cccctcacgc atgaagcctg gag 23

<210> SEQ ID NO 36
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
DNA

<400> SEQUENCE: 36

ggcaggagac caccttgcca gtgcccac 28

<210> SEQ ID NO 37
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
DNA

<400> SEQUENCE: 37

ggcgctggct taccgggaga ggaatggg 28

<210> SEQ ID NO 38
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
DNA

<400> SEQUENCE: 38

aaaaggcctc agttagtga ctgtatgg 28

<210> SEQ ID NO 39
<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
DNA

<400> SEQUENCE: 39

cgcgatcct caagcgttg ggttggtcc 29

<210> SEQ ID NO 40
<211> LENGTH: 45
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
DNA

<400> SEQUENCE: 40

cccaagcttg ccaccatggc tcacgtccc gctagctgcc cgagc 45

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<210> SEQ ID NO 41
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
DNA

<400> SEQUENCE: 41
ccggaattct gccaaagtatg agccatcctg g 31

<210> SEQ ID NO 42
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
DNA

<400> SEQUENCE: 42
gccatccaga aggtggt 17

<210> SEQ ID NO 43
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
DNA

<400> SEQUENCE: 43
gtcttgtcag ggaagat 17

<210> SEQ ID NO 44
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
DNA

<400> SEQUENCE: 44
ggcaggagac caccttgcca gtgcccac 28

<210> SEQ ID NO 45
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
DNA

<400> SEQUENCE: 45
gggtgggctg taccttctgg aacagggc 28

<210> SEQ ID NO 46
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
DNA

<400> SEQUENCE: 46

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ggcgctggct taccggaga ggaatggg 28

<210> SEQ ID NO 47
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
DNA

<400> SEQUENCE: 47

ggaatgggtg tttgtctcct ccaaagatgc 30

<210> SEQ ID NO 48
<211> LENGTH: 1316
<212> TYPE: DNA
<213> ORGANISM: Cricetulus griseus

<400> SEQUENCE: 48

gccccgcccc ctccacctgg accgagagta gctggagaat tgtgcaccgg aagtagctct 60
tggactgggtg gaacctctgc caggtgcagc aacaatgggt gagccccagg gatccaggag 120
gatacctagt acagggggct ctggactggt gggcagagct atccagaagg tggtcgdaga 180
tggcgctggc ttaccggag aggaatgggt gtttgtctcc tccaaagatg cagatctgac 240
ggatgcagca caaacccaag ccctgttcca gaaggtacag cccacccatg tcatccatct 300
tgctgcaatg gtaggaggcc ttttcggaa tatcaaatac aacttgatt tctggaggaa 360
gaatgtgcac atcaatgaca acgtcctgca ctacagcttc gaggtgggca ctgcaagggt 420
ggtctcctgc ctgtccacct gtatcttccc tgacaagacc acctatccta ttgatgaaac 480
aatgatccac aatgtccac cccacagcag caattttggg tactcgtatg ccaagaggat 540
gattgacgtg cagaacagg cctacttcca gcagcatggc tgcacctca ctgctgtcat 600
ccctaccaat gtctttggac ctcatgacaa cttcaacatt gaagatggcc atgtgctgcc 660
tggcctcatc cataaggtgc atctggccaa gagtaatggt tcagccttga ctgtttgggg 720
tacagggaaa ccacggagc agttcatcta ctactggac ctagccggc tcttcatctg 780
ggtcctgcgg gagtacaatg aagttgagcc catcatcctc tcagtgggcg aggaagatga 840
agtctccatt aaggaggcag ctgaggctgt agtggaggcc atggacttct gtggggaagt 900
cacttttgat tcaacaaagt cagatgggca gtataagaag acagccagca atggcaagct 960
tcgggcctac ttgcctgatt tccgtttcac acccttcaag caggctgtga aggagacctg 1020
tgcttggttc accgacaact atgagcagc ccggaagtga agcatgggac aagcgggtgc 1080
tcagctggca atgccagtc agtaggctgc agtctcatca tttgctgtc aagaactgag 1140
gacagtatcc agcaacctga gccacatgct ggtctctctg ccagggggct tcatgcagcc 1200
atccagtagg gccatgttt gtccatcctc gggggaaggc cagaccaaca ccttgtttgt 1260
ctgtctctgc cccaacctca gtgcatccat gctggctctg ctgtcccttg tctaga 1316

<210> SEQ ID NO 49
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
DNA

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<400> SEQUENCE: 49

gatcctgctg ggaccaaagt tgg 23

<210> SEQ ID NO 50

<211> LENGTH: 22

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic DNA

<400> SEQUENCE: 50

cttaacatcc caagggatgc tg 22

<210> SEQ ID NO 51

<211> LENGTH: 1965

<212> TYPE: DNA

<213> ORGANISM: Cricetulus griseus

<400> SEQUENCE: 51

acggggggct cccggaagcg gggaccatgg cgtctctgcg cgaagcgagc ctgcggaagc 60
tgcggcgctt ttccgagatg agaggcaaac ctgtggcaac tgggaaattc tgggatgtag 120
ttgtaataac agcagctgac gaaaagcagg agcttgctta caagcaacag ttgtcggaga 180
agctgaagag aaaggaattg ccccttggag ttaactacca tgttttctact gatcctctctg 240
gaaccaaagt tggaaatgga ggatcaacac tttgttctct tcagtgcctg gaaagcctct 300
atggagacaa gtggaattcc ttcacagtcc tgttaattca ctctggtggc tacagtcaac 360
gacttcccaa tgcaagcgct ttaggaaaaa tcttcacggc tttaccactt ggtgagccca 420
tttatcagat gttggactta aaactagcca tgtacatgga tttccctca cgcagtaagc 480
ctggagtttt ggtcacctgt gcagatgata ttgaactata cagcattggg gactctgagt 540
ccattgcatt tgagcagcct ggctttactg ccctagccca tccatctagt ctggctgtag 600
gcaccacaca tggagttttt gtattggact ctgccggttc tttgcaacat ggtgacctag 660
agtagcagga atgccaccgt ttcctccata agcccagcat tgaaaacatg caccacttta 720
atgccgtgca tagactagga agctttgtgc aacaggactt gagggggggt gacaccacct 780
gtcatccatt gcactctgag tatgtctaca cagatagcct attttacctg gatcataaat 840
cagccaaaaa gctacttgat ttctatgaaa gtgtaggccc actgaactgt gaaatagatg 900
cctatggtga ctttctgcag gcactgggac ctggagcaac tgcagagtac accaagaaca 960
cctcacacgt cactaaagag gaatcacact tgttggacat gaggcagaaa atattccacc 1020
tcctcaaggg aacacccctg aatgtgtgtg tccttaataa ctccaggttt tatcacattg 1080
gaacaacgga ggagtatctg ctacatttca cttccaatgg ttcgttacag gcagagctgg 1140
gcttgcaatc catagctttc agtgtcttcc caaatgtgcc tgaagactcc catgagaaac 1200
cctgtgtcat tcacagcctc ctgaattcag gatgctgtgt gggccctggc tcagtggtag 1260
aatattccag attagacctt gaggtgtcca tctcgaaaaa ctgcattatc agcggttctg 1320
tcataaaaa agctgtttctg ccccatgtt ctttcgtgtg ctctttaagt gtggagataa 1380
atggacactt agaataattc actatggtgt ttggcatgga agacaacttg aagaacagt 1440
ttaaaacctt atcagatata aagatgcttc agttcttttg agtctgtttc ctgacttggt 1500

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tagatatattg gaaccttaaa gctatggaag aactattttc aggaagtaag acgcagctga	1560
gcctgtggac tgctcgaatt ttccctgtct gttcttctct gagtgagtcg gttgcagcat	1620
cccttgggat gttaaatgcc attcgaaacc attcgccatt cagcctgagc aacttcaagc	1680
tgctgtccat ccaggaaatg cttctctgca aagatgtagg agacatgctt gcttacaggg	1740
agcaactctt tctagaaatc agttcaaaga gaaaacagtc tgattcggag aaatcttaaa	1800
tacaatggat ttgcttgga aacaggattg caaatgcagg catattctat agatctctgg	1860
gttcttcttt ctttctcccc tctctccttt cctttccctt tgatgtaatg acaaaggtaa	1920
aaatggccac ttctgatgga aaaaaaaaaa aaaaaaaaaa aaaaaa	1965

<210> SEQ ID NO 52
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
DNA

<400> SEQUENCE: 52

caggggtggt cccttgagga ggtggaa	27
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<210> SEQ ID NO 53
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
DNA

<400> SEQUENCE: 53

cactgagcca ggggccacac agcatcc	27
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<210> SEQ ID NO 54
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
DNA

<400> SEQUENCE: 54

cccctcacgc atgaagcctg gag	23
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<210> SEQ ID NO 55
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
DNA

<400> SEQUENCE: 55

tgccaccgtt tcctccataa gccccagc	27
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<210> SEQ ID NO 56
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
DNA

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<400> SEQUENCE: 56

atgggtcaag ctcccgttaa gtgcccga

28

<210> SEQ ID NO 57

<211> LENGTH: 27

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
DNA

<400> SEQUENCE: 57

tcaagcgttt gggttggtcc tcatgag

27

<210> SEQ ID NO 58

<211> LENGTH: 25

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
DNA

<400> SEQUENCE: 58

tccgggggatg gcgagatggg caagc

25

<210> SEQ ID NO 59

<211> LENGTH: 24

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
DNA

<400> SEQUENCE: 59

cttgacatgg ctctgggctc caag

24

<210> SEQ ID NO 60

<211> LENGTH: 25

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
DNA

<400> SEQUENCE: 60

ccacttcagt cggtcggtag tattt

25

<210> SEQ ID NO 61

<211> LENGTH: 24

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
DNA

<400> SEQUENCE: 61

cgctcaccg cctgaggcga catg

24

<210> SEQ ID NO 62

<211> LENGTH: 32

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

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<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
DNA

<400> SEQUENCE: 62

ggcaggtgct gtcggtgagg tcaccatagt gc 32

<210> SEQ ID NO 63
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
DNA

<400> SEQUENCE: 63

ggggccatgc caaggactat gtcg 24

<210> SEQ ID NO 64
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
DNA

<400> SEQUENCE: 64

atgtggctga tgttacaaaa tgatg 25

<210> SEQ ID NO 65
<211> LENGTH: 1504
<212> TYPE: DNA
<213> ORGANISM: Cricetulus griseus

<400> SEQUENCE: 65

atg gct cac gct ccc gct agc tgc ccg agc tcc agg aac tct ggg gac 48
Met Ala His Ala Pro Ala Ser Cys Pro Ser Ser Arg Asn Ser Gly Asp
1 5 10 15

ggc gat aag ggc aag ccc agg aag gtg gcg ctc atc acg ggc atc acc 96
Gly Asp Lys Gly Lys Pro Arg Lys Val Ala Leu Ile Thr Gly Ile Thr
20 25 30

ggc cag gat ggc tca tac ttg gca gaa ttc ctg ctg gag aaa gga tac 144
Gly Gln Asp Gly Ser Tyr Leu Ala Glu Phe Leu Leu Glu Lys Gly Tyr
35 40 45

gag gtt cat gga att gta cgg cga tcc agt tca ttt aat aca ggt cga 192
Glu Val His Gly Ile Val Arg Arg Ser Ser Ser Phe Asn Thr Gly Arg
50 55 60

att gaa cat tta tat aag aat cca cag gct cat att gaa gga aac atg 240
Ile Glu His Leu Tyr Lys Asn Pro Gln Ala His Ile Glu Gly Asn Met
65 70 75 80

aag ttg cac tat ggt gac ctc acc gac agc acc tgc cta gta aaa atc 288
Lys Leu His Tyr Gly Asp Leu Thr Asp Ser Thr Cys Leu Val Lys Ile
85 90 95

atc aat gaa gtc aaa cct aca gag atc tac aat ctt ggt gcc cag agc 336
Ile Asn Glu Val Lys Pro Thr Glu Ile Tyr Asn Leu Gly Ala Gln Ser
100 105 110

cat gtc aag att tcc ttt gac tta gca gag tac act gca gat gtt gat 384
His Val Lys Ile Ser Phe Asp Leu Ala Glu Tyr Thr Ala Asp Val Asp
115 120 125

gga gtt ggc acc ttg cgg ctt ctg gat gca att aag act tgt ggc ctt 432
Gly Val Gly Thr Leu Arg Leu Leu Asp Ala Ile Lys Thr Cys Gly Leu
130 135 140

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ata aat tct gtg aag ttc tac cag gcc tca act agt gaa ctg tat gga Ile Asn Ser Val Lys Phe Tyr Gln Ala Ser Thr Ser Glu Leu Tyr Gly 145 150 155 160	480
aaa gtg caa gaa ata ccc cag aaa gag acc acc cct ttc tat cca agg Lys Val Gln Glu Ile Pro Gln Lys Glu Thr Thr Pro Phe Tyr Pro Arg 165 170 175	528
tcg ccc tat gga gca gcc aaa ctt tat gcc tat tgg att gta gtg aac Ser Pro Tyr Gly Ala Ala Lys Leu Tyr Ala Tyr Trp Ile Val Val Asn 180 185 190	576
ttt cga gag gct tat aat ctc ttt gcg gtg aac ggc att ctc ttc aat Phe Arg Glu Ala Tyr Asn Leu Phe Ala Val Asn Gly Ile Leu Phe Asn 195 200 205	624
cat gag agt cct aga aga gga gct aat ttt gtt act cga aaa att agc His Glu Ser Pro Arg Arg Gly Ala Asn Phe Val Thr Arg Lys Ile Ser 210 215 220	672
cgg tca gta gct aag att tac ctt gga caa ctg gaa tgt ttc agt ttg Arg Ser Val Ala Lys Ile Tyr Leu Gly Gln Leu Glu Cys Phe Ser Leu 225 230 235 240	720
gga aat ctg gac gcc aaa cga gac tgg ggc cat gcc aag gac tat gtc Gly Asn Leu Asp Ala Lys Arg Asp Trp Gly His Ala Lys Asp Tyr Val 245 250 255	768
gag gct atg tgg ctg atg tta caa aat gat gaa cca gag gac ttt gtc Glu Ala Met Trp Leu Met Leu Gln Asn Asp Glu Pro Glu Asp Phe Val 260 265 270	816
ata gct act ggg gaa gtt cat agt gtc cgt gaa ttt gtt gag aaa tca Ile Ala Thr Gly Glu Val His Ser Val Arg Glu Phe Val Glu Lys Ser 275 280 285	864
ttc atg cac att gga aag acc att gtg tgg gaa gga aag aat gaa aat Phe Met His Ile Gly Lys Thr Ile Val Trp Glu Gly Lys Asn Glu Asn 290 295 300	912
gaa gtg ggc aga tgt aaa gag acc ggc aaa att cat gtg act gtg gat Glu Val Gly Arg Cys Lys Glu Thr Gly Lys Ile His Val Thr Val Asp 305 310 315 320	960
ctg aaa tac tac cga cca act gaa gtg gac ttc ctg cag gga gac tgc Leu Lys Tyr Tyr Arg Pro Thr Glu Val Asp Phe Leu Gln Gly Asp Cys 325 330 335	1008
tcc aag gcg cag cag aaa ctg aac tgg aag ccc cgc gtt gcc ttt gac Ser Lys Ala Gln Gln Lys Leu Asn Trp Lys Pro Arg Val Ala Phe Asp 340 345 350	1056
gag ctg gtg agg gag atg gtg caa gcc gat gtg gag ctc atg aga acc Glu Leu Val Arg Glu Met Val Gln Ala Asp Val Glu Leu Met Arg Thr 355 360 365	1104
aac ccc aac gcc tga gcacctctac aaaaaaattc gcgagacatg gactatggtg Asn Pro Asn Ala 370	1159
cagagccagc caaccagagt ccagccactc ctgagaccat cgaccataaa cctcgcactg	1219
cctgtgtcgt cccacagct aagagctggg ccacaggttt gtgggcacca ggacggggac	1279
actccagagc taaggccact tcgcttttgt caaaggctcc tctcaatgat tttgggaaat	1339
caagaagttt aaaatcacat actcatttta cttgaaatta tgtcactaga caacttaa	1399
ttttgagtct tgagattgtt tttctctttt cttattaaat gatctttcta tgaccagca	1459
aaaaaaaaa aaaaaaggga tataaaaaaa aaaaaaaaaa aaaaa	1504

<210> SEQ ID NO 66

<211> LENGTH: 25

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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA

<400> SEQUENCE: 66

atgaagttgc actatggtga cctca                               25

<210> SEQ ID NO 67
<211> LENGTH: 59
<212> TYPE: DNA
<213> ORGANISM: Cricetulus griseus

<400> SEQUENCE: 67

ccgacagcac ctgcctagta aaaatcatca atgaagtcaa acctacagag atctacaat       59

<210> SEQ ID NO 68
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA

<400> SEQUENCE: 68

gacttagcag agtacactgc agatg                                   25

<210> SEQ ID NO 69
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA

<400> SEQUENCE: 69

accttgata gaaaggggtg gtctc                                   25

<210> SEQ ID NO 70
<211> LENGTH: 125
<212> TYPE: DNA
<213> ORGANISM: Cricetulus griseus

<400> SEQUENCE: 70

ttgatggagt tggcaccttg cggcttctgg atgcaattaa gacttgtggc cttataaatt       60
ctgtgaagtt ctaccaggcc tcaactagtg aactgtatgg aaaagtgcaa gaaatacccc     120
agaaa                                           125

<210> SEQ ID NO 71
<211> LENGTH: 372
<212> TYPE: PRT
<213> ORGANISM: Cricetulus griseus

<400> SEQUENCE: 71

Met Ala His Ala Pro Ala Ser Cys Pro Ser Ser Arg Asn Ser Gly Asp
  1             5             10            15

Gly Asp Lys Gly Lys Pro Arg Lys Val Ala Leu Ile Thr Gly Ile Thr
          20            25            30

Gly Gln Asp Gly Ser Tyr Leu Ala Glu Phe Leu Leu Glu Lys Gly Tyr
          35            40            45

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Glu Val His Gly Ile Val Arg Arg Ser Ser Ser Phe Asn Thr Gly Arg
 50 55 60
 Ile Glu His Leu Tyr Lys Asn Pro Gln Ala His Ile Glu Gly Asn Met
 65 70 75 80
 Lys Leu His Tyr Gly Asp Leu Thr Asp Ser Thr Cys Leu Val Lys Ile
 85 90 95
 Ile Asn Glu Val Lys Pro Thr Glu Ile Tyr Asn Leu Gly Ala Gln Ser
 100 105 110
 His Val Lys Ile Ser Phe Asp Leu Ala Glu Tyr Thr Ala Asp Val Asp
 115 120 125
 Gly Val Gly Thr Leu Arg Leu Leu Asp Ala Ile Lys Thr Cys Gly Leu
 130 135 140
 Ile Asn Ser Val Lys Phe Tyr Gln Ala Ser Thr Ser Glu Leu Tyr Gly
 145 150 155 160
 Lys Val Gln Glu Ile Pro Gln Lys Glu Thr Thr Pro Phe Tyr Pro Arg
 165 170 175
 Ser Pro Tyr Gly Ala Ala Lys Leu Tyr Ala Tyr Trp Ile Val Val Asn
 180 185 190
 Phe Arg Glu Ala Tyr Asn Leu Phe Ala Val Asn Gly Ile Leu Phe Asn
 195 200 205
 His Glu Ser Pro Arg Arg Gly Ala Asn Phe Val Thr Arg Lys Ile Ser
 210 215 220
 Arg Ser Val Ala Lys Ile Tyr Leu Gly Gln Leu Glu Cys Phe Ser Leu
 225 230 235 240
 Gly Asn Leu Asp Ala Lys Arg Asp Trp Gly His Ala Lys Asp Tyr Val
 245 250 255
 Glu Ala Met Trp Leu Met Leu Gln Asn Asp Glu Pro Glu Asp Phe Val
 260 265 270
 Ile Ala Thr Gly Glu Val His Ser Val Arg Glu Phe Val Glu Lys Ser
 275 280 285
 Phe Met His Ile Gly Lys Thr Ile Val Trp Glu Gly Lys Asn Glu Asn
 290 295 300
 Glu Val Gly Arg Cys Lys Glu Thr Gly Lys Ile His Val Thr Val Asp
 305 310 315 320
 Leu Lys Tyr Tyr Arg Pro Thr Glu Val Asp Phe Leu Gln Gly Asp Cys
 325 330 335
 Ser Lys Ala Gln Gln Lys Leu Asn Trp Lys Pro Arg Val Ala Phe Asp
 340 345 350
 Glu Leu Val Arg Glu Met Val Gln Ala Asp Val Glu Leu Met Arg Thr
 355 360 365
 Asn Pro Asn Ala
 370

<210> SEQ ID NO 72
 <211> LENGTH: 420
 <212> TYPE: DNA
 <213> ORGANISM: Mus musculus

<400> SEQUENCE: 72

atg gaa tgg atc tgg atc ttt ctc ttc ttc ctc tca gga act aca ggt
 Met Glu Trp Ile Trp Ile Phe Leu Phe Phe Leu Ser Gly Thr Thr Gly
 1 5 10 15

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gtc tac tcc cag gtt cag ctg cag cag tct gga gct gag gtg gcg agg      96
Val Tyr Ser Gln Val Gln Leu Gln Gln Ser Gly Ala Glu Val Ala Arg
                20                      25                      30

ccc ggg gct tca gtg aaa ctg tcc tgc aag gct tct ggc tac acc ttc      144
Pro Gly Ala Ser Val Lys Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe
                35                      40                      45

act gac tac tat cta aac tgg gtg aag cag agg tct gga cag ggc ctt      192
Thr Asp Tyr Tyr Leu Asn Trp Val Lys Gln Arg Ser Gly Gln Gly Leu
                50                      55                      60

gag tgg att gga gag att gat cct gga agt gat agt ata tat tat aat      240
Glu Trp Ile Gly Glu Ile Asp Pro Gly Ser Asp Ser Ile Tyr Tyr Asn
                65                      70                      75                      80

gaa aac ttg gag ggc agg gcc aca ctg act gca gac aaa tcc tcc agc      288
Glu Asn Leu Glu Gly Arg Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser
                85                      90                      95

aca gcc tac atg cag ctc aac agc ctg aca tct gag gac tct gca gtc      336
Thr Ala Tyr Met Gln Leu Asn Ser Leu Thr Ser Glu Asp Ser Ala Val
                100                      105                      110

tat ttc tgt gca aga tat ggg tat tct aga tac gac gta agg ttt gtc      384
Tyr Phe Cys Ala Arg Tyr Gly Tyr Ser Arg Tyr Asp Val Arg Phe Val
                115                      120                      125

tac tgg ggc caa ggg act ctg gtc act gtc tct aca      420
Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Thr
                130                      135                      140

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<210> SEQ ID NO 73

<211> LENGTH: 140

<212> TYPE: PRT

<213> ORGANISM: Mus musculus

<400> SEQUENCE: 73

```

Met Glu Trp Ile Trp Ile Phe Leu Phe Phe Leu Ser Gly Thr Thr Gly
 1           5           10           15

Val Tyr Ser Gln Val Gln Leu Gln Gln Ser Gly Ala Glu Val Ala Arg
                20                      25                      30

Pro Gly Ala Ser Val Lys Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe
                35                      40                      45

Thr Asp Tyr Tyr Leu Asn Trp Val Lys Gln Arg Ser Gly Gln Gly Leu
                50                      55                      60

Glu Trp Ile Gly Glu Ile Asp Pro Gly Ser Asp Ser Ile Tyr Tyr Asn
                65                      70                      75                      80

Glu Asn Leu Glu Gly Arg Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser
                85                      90                      95

Thr Ala Tyr Met Gln Leu Asn Ser Leu Thr Ser Glu Asp Ser Ala Val
                100                      105                      110

Tyr Phe Cys Ala Arg Tyr Gly Tyr Ser Arg Tyr Asp Val Arg Phe Val
                115                      120                      125

Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Thr
                130                      135                      140

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<210> SEQ ID NO 74

<211> LENGTH: 393

<212> TYPE: DNA

<213> ORGANISM: Mus musculus

<400> SEQUENCE: 74

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atg aag ttg cct gtt agg ctg ttg gtg ctg atg ttc tgg att cct gct      48

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Met	Lys	Leu	Pro	Val	Arg	Leu	Leu	Val	Leu	Met	Phe	Trp	Ile	Pro	Ala		
1				5					10					15			
tcc	agg	agt	gat	gtt	ttg	atg	acc	caa	act	cca	ctc	tcc	ctg	cct	gtc	96	
Ser	Arg	Ser	Asp	Val	Leu	Met	Thr	Gln	Thr	Pro	Leu	Ser	Leu	Pro	Val		
			20					25					30				
agt	ctt	gga	gat	caa	gcc	tcc	atc	tct	tgc	aga	tct	agt	cag	agt	ctt	144	
Ser	Leu	Gly	Asp	Gln	Ala	Ser	Ile	Ser	Cys	Arg	Ser	Ser	Gln	Ser	Leu		
		35					40				45						
gta	cat	agt	aat	gga	aga	acc	tat	tta	gaa	tgg	tac	ctg	cag	aaa	cct	192	
Val	His	Ser	Asn	Gly	Arg	Thr	Tyr	Leu	Glu	Trp	Tyr	Leu	Gln	Lys	Pro		
	50					55				60							
ggc	cag	tca	cca	aag	gtc	ctg	atc	tac	aaa	gtt	tcc	aac	cga	att	tct	240	
Gly	Gln	Ser	Pro	Lys	Val	Leu	Ile	Tyr	Lys	Val	Ser	Asn	Arg	Ile	Ser		
65					70				75					80			
ggg	gtc	cca	gac	agg	ttc	agt	ggc	agt	gga	tca	ggg	aca	gat	ttc	aca	288	
Gly	Val	Pro	Asp	Arg	Phe	Ser	Gly	Ser	Gly	Ser	Gly	Thr	Asp	Phe	Thr		
			85					90					95				
ctc	aaa	atc	agc	aga	gtg	gag	gct	gag	gat	ctg	gga	gtt	tat	ttc	tgc	336	
Leu	Lys	Ile	Ser	Arg	Val	Glu	Ala	Glu	Asp	Leu	Gly	Val	Tyr	Phe	Cys		
			100				105						110				
ttt	cag	ggt	tca	cat	gtt	ccg	tac	acg	ttc	gga	ggg	ggg	acc	aag	ctg	384	
Phe	Gln	Gly	Ser	His	Val	Pro	Tyr	Thr	Phe	Gly	Gly	Gly	Thr	Lys	Leu		
		115				120						125					
gaa	ata	aaa														393	
Glu	Ile	Lys															
		130															

<210> SEQ ID NO 75

<211> LENGTH: 131

<212> TYPE: PRT

<213> ORGANISM: Mus musculus

<400> SEQUENCE: 75

Met	Lys	Leu	Pro	Val	Arg	Leu	Leu	Val	Leu	Met	Phe	Trp	Ile	Pro	Ala		
1				5					10					15			
Ser	Arg	Ser	Asp	Val	Leu	Met	Thr	Gln	Thr	Pro	Leu	Ser	Leu	Pro	Val		
			20					25					30				
Ser	Leu	Gly	Asp	Gln	Ala	Ser	Ile	Ser	Cys	Arg	Ser	Ser	Gln	Ser	Leu		
		35					40				45						
Val	His	Ser	Asn	Gly	Arg	Thr	Tyr	Leu	Glu	Trp	Tyr	Leu	Gln	Lys	Pro		
	50					55				60							
Gly	Gln	Ser	Pro	Lys	Val	Leu	Ile	Tyr	Lys	Val	Ser	Asn	Arg	Ile	Ser		
65					70				75					80			
Gly	Val	Pro	Asp	Arg	Phe	Ser	Gly	Ser	Gly	Ser	Gly	Thr	Asp	Phe	Thr		
			85					90					95				
Leu	Lys	Ile	Ser	Arg	Val	Glu	Ala	Glu	Asp	Leu	Gly	Val	Tyr	Phe	Cys		
			100				105						110				
Phe	Gln	Gly	Ser	His	Val	Pro	Tyr	Thr	Phe	Gly	Gly	Gly	Thr	Lys	Leu		
		115				120						125					
Glu	Ile	Lys															
		130															

<210> SEQ ID NO 76

<211> LENGTH: 5

<212> TYPE: PRT

<213> ORGANISM: Mus musculus

-continued

<400> SEQUENCE: 76

Asp Tyr Tyr Leu Asn
1 5

<210> SEQ ID NO 77

<211> LENGTH: 17

<212> TYPE: PRT

<213> ORGANISM: Mus musculus

<400> SEQUENCE: 77

Glu Ile Asp Pro Gly Ser Asp Ser Ile Tyr Tyr Asn Glu Asn Leu Glu
1 5 10 15

Gly

<210> SEQ ID NO 78

<211> LENGTH: 12

<212> TYPE: PRT

<213> ORGANISM: Mus musculus

<400> SEQUENCE: 78

Tyr Gly Tyr Ser Arg Tyr Asp Val Arg Phe Val Tyr
1 5 10

<210> SEQ ID NO 79

<211> LENGTH: 16

<212> TYPE: PRT

<213> ORGANISM: Mus musculus

<400> SEQUENCE: 79

Arg Ser Ser Gln Ser Leu Val His Ser Asn Gly Arg Thr Tyr Leu Glu
1 5 10 15

<210> SEQ ID NO 80

<211> LENGTH: 7

<212> TYPE: PRT

<213> ORGANISM: Mus musculus

<400> SEQUENCE: 80

Lys Val Ser Asn Arg Ile Ser
1 5

<210> SEQ ID NO 81

<211> LENGTH: 9

<212> TYPE: PRT

<213> ORGANISM: Mus musculus

<400> SEQUENCE: 81

Phe Gln Gly Ser His Val Pro Tyr Thr
1 5

<210> SEQ ID NO 82

<211> LENGTH: 22

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:synthetic
DNA

<400> SEQUENCE: 82

ctgaattcgc ggccgctagt cc

-continued

<210> SEQ ID NO 83
<211> LENGTH: 39
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: synthetic
DNA

<400> SEQUENCE: 83

atggggccctt ggtggaggct gtagagacag tgaccagag 39

<210> SEQ ID NO 84
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:synthetic
DNA

<400> SEQUENCE: 84

ctgaattcgc ggccgctgct gt 22

<210> SEQ ID NO 85
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: synthetic
DNA

<400> SEQUENCE: 85

atcgtacggt ttatttcag ctgtgtcc 28

<210> SEQ ID NO 86
<211> LENGTH: 57
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:synthetic
DNA

<400> SEQUENCE: 86

ttgttggtac cgaattcttt caggggcccg gagccccgag agcccaaadc ttgtgac 57

<210> SEQ ID NO 87
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: synthetic
DNA

<400> SEQUENCE: 87

gcgaattcca ccatgggcag cccccgtcc g 31

<210> SEQ ID NO 88
<211> LENGTH: 32
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:synthetic
DNA

<400> SEQUENCE: 88

cgggatccct atcggggctc cggggccaa gt 32

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<210> SEQ ID NO 89
<211> LENGTH: 1341
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:synthetic
      DNA

<400> SEQUENCE: 89

atg ggc agc ccc cgc tcc gcg ctg agc tgc ctg ctg ttg cac ttg ctg      48
Met Gly Ser Pro Arg Ser Ala Leu Ser Cys Leu Leu Leu His Leu Leu
  1             5             10             15

gtt ctc tgc ctc caa gcc cag gta act gtt cag tcc tca cct aat ttt      96
Val Leu Cys Leu Gln Ala Gln Val Thr Val Gln Ser Ser Pro Asn Phe
             20             25             30

aca cag cat gtg agg gag cag agc ctg gtg acg gat cag ctc agc cgc      144
Thr Gln His Val Arg Glu Gln Ser Leu Val Thr Asp Gln Leu Ser Arg
             35             40             45

cgc ctc atc cgg acc tac cag ctc tac agc cgc acc agc ggg aag cac      192
Arg Leu Ile Arg Thr Tyr Gln Leu Tyr Ser Arg Thr Ser Gly Lys His
  50             55             60

gtg cag gtc ctg gcc aac aag cgc atc aac gcc atg gca gaa gac gga      240
Val Gln Val Leu Ala Asn Lys Arg Ile Asn Ala Met Ala Glu Asp Gly
  65             70             75             80

gac ccc ttc gcg aag ctc att gtg gag acc gat act ttt gga agc aga      288
Asp Pro Phe Ala Lys Leu Ile Val Glu Thr Asp Thr Phe Gly Ser Arg
             85             90             95

gtc cga gtt cgc gcc gca gag aca ggt ctc tac atc tgc atg aac aag      336
Val Arg Val Arg Gly Ala Glu Thr Gly Leu Tyr Ile Cys Met Asn Lys
  100            105            110

aag ggg aag cta att gcc aag agc aac ggc aaa ggc aag gac tgc gta      384
Lys Gly Lys Leu Ile Ala Lys Ser Asn Gly Lys Gly Lys Asp Cys Val
  115            120            125

ttc aca gag atc gtg ctg gag aac aac tac acg gcg ctg cag aac gcc      432
Phe Thr Glu Ile Val Leu Glu Asn Asn Tyr Thr Ala Leu Gln Asn Ala
  130            135            140

aag tac gag gcc tgg tac atg gcc ttt acc cgc aag ggc cgg ccc cgc      480
Lys Tyr Glu Gly Trp Tyr Met Ala Phe Thr Arg Lys Gly Arg Pro Arg
  145            150            155            160

aag ggc tcc aag acg cgc cag cat cag cgc gag gtg cac ttc atg aag      528
Lys Gly Ser Lys Thr Arg Gln His Gln Arg Glu Val His Phe Met Lys
             165            170            175

cgc ctg ccg cgg gcc cac cac acc acc gag cag agc ctg cgc ttc gag      576
Arg Leu Pro Arg Gly His His Thr Thr Glu Gln Ser Leu Arg Phe Glu
             180            185            190

ttc ctc aac tac ccg ccc ttc acg cgc agc ctg cgc ggc agc cag agg      624
Phe Leu Asn Tyr Pro Pro Phe Thr Arg Ser Leu Arg Gly Ser Gln Arg
  195            200            205

act tgg gcc ccg gag ccc cga gag ccc aaa tct tgt gac aaa act cac      672
Thr Trp Ala Pro Glu Pro Arg Glu Pro Lys Ser Cys Asp Lys Thr His
  210            215            220

aca tgc cca ccg tgc cca gca cct gaa ctc ctg ggg gga ccg tca gtc      720
Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val
  225            230            235            240

ttc ctc ttc ccc cca aaa ccc aag gac acc ctc atg atc tcc cgg acc      768
Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr
  245            250            255

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cct gag gtc aca tgc gtg gtg gtg gac gtg agc cac gaa gac cct gag	816
Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu	
260 265 270	
gtc aag ttc aac tgg tac gtg gac ggc gtg gag gtg cat aat gcc aag	864
Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys	
275 280 285	
aca aag ccg cgg gag gag cag tac aac agc acg tac cgt gtg gtc agc	912
Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser	
290 295 300	
gtc ctc acc gtc ctg cac cag gac tgg ctg aat ggc aag gag tac aag	960
Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys	
305 310 315 320	
tgc aag gtc tcc aac aaa gcc ctc cca gcc ccc atc gag aaa acc atc	1008
Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile	
325 330 335	
tcc aaa gcc aaa ggg cag ccc cga gaa cca cag gtg tac acc ctg ccc	1056
Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro	
340 345 350	
cca tcc cgg gat gag ctg acc aag aac cag gtc agc ctg acc tgc ctg	1104
Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu	
355 360 365	
gtc aaa ggc ttc tat ccc agc gac atc gcc gtg gag tgg gag agc aat	1152
Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn	
370 375 380	
ggg cag ccg gag aac aac tac aag acc acg cct ccc gtg ctg gac tcc	1200
Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser	
385 390 395 400	
gac ggc tcc ttc ttc ctc tac agc aag ctc acc gtg gac aag agc agg	1248
Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg	
405 410 415	
tgg cag cag ggg aac gtc ttc tca tgc tcc gtg atg cat gag gct ctg	1296
Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu	
420 425 430	
cac aac cac tac acg cag aag agc ctc tcc ctg tct ccg ggt aaa	1341
His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys	
435 440 445	

<210> SEQ ID NO 90

<211> LENGTH: 447

<212> TYPE: PRT

<213> ORGANISM: homo sapiens

<400> SEQUENCE: 90

Met Gly Ser Pro Arg Ser Ala Leu Ser Cys Leu Leu Leu His Leu Leu	
1 5 10 15	
Val Leu Cys Leu Gln Ala Gln Val Thr Val Gln Ser Ser Pro Asn Phe	
20 25 30	
Thr Gln His Val Arg Glu Gln Ser Leu Val Thr Asp Gln Leu Ser Arg	
35 40 45	
Arg Leu Ile Arg Thr Tyr Gln Leu Tyr Ser Arg Thr Ser Gly Lys His	
50 55 60	
Val Gln Val Leu Ala Asn Lys Arg Ile Asn Ala Met Ala Glu Asp Gly	
65 70 75 80	
Asp Pro Phe Ala Lys Leu Ile Val Glu Thr Asp Thr Phe Gly Ser Arg	
85 90 95	
Val Arg Val Arg Gly Ala Glu Thr Gly Leu Tyr Ile Cys Met Asn Lys	
100 105 110	

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Lys Gly Lys Leu Ile Ala Lys Ser Asn Gly Lys Gly Lys Asp Cys Val
 115 120 125
 Phe Thr Glu Ile Val Leu Glu Asn Asn Tyr Thr Ala Leu Gln Asn Ala
 130 135 140
 Lys Tyr Glu Gly Trp Tyr Met Ala Phe Thr Arg Lys Gly Arg Pro Arg
 145 150 155 160
 Lys Gly Ser Lys Thr Arg Gln His Gln Arg Glu Val His Phe Met Lys
 165 170 175
 Arg Leu Pro Arg Gly His His Thr Thr Glu Gln Ser Leu Arg Phe Glu
 180 185 190
 Phe Leu Asn Tyr Pro Pro Phe Thr Arg Ser Leu Arg Gly Ser Gln Arg
 195 200 205
 Thr Trp Ala Pro Glu Pro Arg Glu Pro Lys Ser Cys Asp Lys Thr His
 210 215 220
 Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val
 225 230 235 240
 Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr
 245 250 255
 Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu
 260 265 270
 Val Lys Phe Asn Trp Tyr Val Asp Gly Val Gln Val His Asn Ala Lys
 275 280 285
 Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser
 290 295 300
 Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys
 305 310 315 320
 Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile
 325 330 335
 Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro
 340 345 350
 Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu
 355 360 365
 Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn
 370 375 380
 Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser
 385 390 395 400
 Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg
 405 410 415
 Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu
 420 425 430
 His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
 435 440 445

<210> SEQ ID NO 91

<211> LENGTH: 1245

<212> TYPE: DNA

<213> ORGANISM: Cricetulus griseus

<400> SEQUENCE: 91

gaacttcacc caagccatgt gacaattgaa ggctgtaccc ccagacccta acatcttgga 60

gccctgtaga ccagggagtg cttctggccg tggggtgacc tagctcttct accaccatga 120

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acagggcccc tctgaagcgg tccaggatcc tgcgcacggc gctgactgga ggctccactg 180
cctctgagga ggcagatgaa gacagcagga acaagccgtt tctgctgcgg gcgctgcaga 240
tcgcgctggt cgtctctctc tactgggtca cctccatctc catgggtattc ctcaacaagt 300
acctgctgga cagccctctc ctgcagctgg atacccttat cttcgtcact ttctaccaat 360
gcctggtgac ctctctgctg tgcaagggcc tcagcactct ggccacctgc tgccctggca 420
ccgttgactt cccaccctg aacctggacc ttaaggtggc ccgcagcgtg ctgccactgt 480
cggtagtctt cattggcatg ataagtttca ataacctctg cctcaagtac gtaggggtgg 540
ccttctacaa cgtggggcgc tcgctcacca ccgtgttcaa tgtgcttctg tcctacctgc 600
tgctcaaaca gaccatttcc ttctatgccc tgctcacatg tggcatcatc attggtgggt 660
tctggtggg tatagaccaa gagggagctg agggcaccct gtccctcata ggcaccatct 720
tcggggtgct ggccagcctc tgcgtctccc tcaatgccat ctataccaag aaggtgctcc 780
cagcagtgga caacagcatc tggcgccctaa ccttctataa caatgtcaat gcctgtgtgc 840
tcttcttgcc cctgatggtt ctgctgggtg agctccgtgc cctccttgac ttgctcatc 900
tgtacagtgc ccacttctgg ctcatgatga cgctgggtgg cctcttcggc ttgcccattg 960
gctatgtgac aggactgcag atcaaattca ccagtccctt gaccacaat gtatcaggca 1020
cagccaaggc ctgtgcgcag acagtgtctg ccgtgctcta ctatgaagag actaagagct 1080
tcctgtggtg gacaagcaac ctgatggtgc tgggtggctc ctcagcctat acctgggtca 1140
ggggctggga gatgcagaag acccaagagg accccagctc caaagagggt gagaagagt 1200
ctattggggt gtgagcttct tcagggaact gggactgaac ccaag 1245

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<210> SEQ ID NO 92

<211> LENGTH: 365

<212> TYPE: PRT

<213> ORGANISM: Cricetulus griseus

<400> SEQUENCE: 92

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Met Asn Arg Ala Pro Leu Lys Arg Ser Arg Ile Leu Arg Met Ala Leu
 1             5             10            15
Thr Gly Gly Ser Thr Ala Ser Glu Glu Ala Asp Glu Asp Ser Arg Asn
      20            25            30
Lys Pro Phe Leu Leu Arg Ala Leu Gln Ile Ala Leu Val Val Ser Leu
      35            40            45
Tyr Trp Val Thr Ser Ile Ser Met Val Phe Leu Asn Lys Tyr Leu Leu
      50            55            60
Asp Ser Pro Ser Leu Gln Leu Asp Thr Pro Ile Phe Val Thr Phe Tyr
      65            70            75            80
Gln Cys Leu Val Thr Ser Leu Leu Cys Lys Gly Leu Ser Thr Leu Ala
      85            90            95
Thr Cys Cys Pro Gly Thr Val Asp Phe Pro Thr Leu Asn Leu Asp Leu
      100           105           110
Lys Val Ala Arg Ser Val Leu Pro Leu Ser Val Val Phe Ile Gly Met
      115           120           125
Ile Ser Phe Asn Asn Leu Cys Leu Lys Tyr Val Gly Val Ala Phe Tyr
      130           135           140
Asn Val Gly Arg Ser Leu Thr Thr Val Phe Asn Val Leu Leu Ser Tyr
      145           150           155           160

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Leu	Leu	Leu	Lys	Gln	Thr	Thr	Ser	Phe	Tyr	Ala	Leu	Leu	Thr	Cys	Gly
			165						170					175	
Ile	Ile	Ile	Gly	Gly	Phe	Trp	Leu	Gly	Ile	Asp	Gln	Glu	Gly	Ala	Glu
			180					185					190		
Gly	Thr	Leu	Ser	Leu	Ile	Gly	Thr	Ile	Phe	Gly	Val	Leu	Ala	Ser	Leu
		195					200					205			
Cys	Val	Ser	Leu	Asn	Ala	Ile	Tyr	Thr	Lys	Lys	Val	Leu	Pro	Ala	Val
	210					215					220				
Asp	Asn	Ser	Ile	Trp	Arg	Leu	Thr	Phe	Tyr	Asn	Asn	Val	Asn	Ala	Cys
225					230					235					240
Val	Leu	Phe	Leu	Pro	Leu	Met	Val	Leu	Leu	Gly	Glu	Leu	Arg	Ala	Leu
			245						250					255	
Leu	Asp	Phe	Ala	His	Leu	Tyr	Ser	Ala	His	Phe	Trp	Leu	Met	Met	Thr
		260						265					270		
Leu	Gly	Gly	Leu	Phe	Gly	Phe	Ala	Ile	Gly	Tyr	Val	Thr	Gly	Leu	Gln
	275						280					285			
Ile	Lys	Phe	Thr	Ser	Pro	Leu	Thr	His	Asn	Val	Ser	Gly	Thr	Ala	Lys
	290					295					300				
Ala	Cys	Ala	Gln	Thr	Val	Leu	Ala	Val	Leu	Tyr	Tyr	Glu	Glu	Thr	Lys
305					310					315					320
Ser	Phe	Leu	Trp	Trp	Thr	Ser	Asn	Leu	Met	Val	Leu	Gly	Gly	Ser	Ser
			325						330					335	
Ala	Tyr	Thr	Trp	Val	Arg	Gly	Trp	Glu	Met	Gln	Lys	Thr	Gln	Glu	Asp
		340					345						350		
Pro	Ser	Ser	Lys	Glu	Gly	Glu	Lys	Ser	Ala	Ile	Gly	Val			
	355						360					365			

<210> SEQ ID NO 93

<211> LENGTH: 1095

<212> TYPE: DNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 93

atgaataggg cccctctgaa gcggtccagg atcctgcaca tggcgctgac cggggcctca	60
gaccctctg cagaggcaga ggccaacggg gagaagccct ttctgctgcg ggcattgcag	120
atcgcgctgg tgggtctcct ctactgggtc acctccatct ccattggtgt ccttaataag	180
tacctgctgg acagcccctc cctgcggctg gacaccccca tcttcgtcac cttctaccag	240
tgcttggtga ccacgtgct gtgcaaaggc ctcagcgctc tggccgcctg ctgccctggt	300
gccgtggact tccccagctt gcgcctggac ctcagggtgg ccgcagcgt cctgccctg	360
tcggtggtct tcatcgcat gatcaccttc aataacctct gcctcaagta cgtcggtgtg	420
gccttttaca atgtgggccc ctcactcacc accgtcttca acgtgctgct ctctacctg	480
ctgtcaagc agaccacctc cttctatgcc ctgtcacct gcggtatcat catcgggggc	540
ttctggcttg gtgtggacca ggagggggca gaaggcacc tgctgtggct gggcaccgtc	600
ttcggcgtgc tggctagcct ctgtgtctcg ctcaacgcc tctacaccac gaagggtgctc	660
ccggcggtgg acggcagcat ctggcgctg actttctaca acaacgtcaa cgcctgcac	720
ctcttctgc ccctgctcct gctgctcggg gagcttcagg ccctgctga ctttgcccag	780
ctgggcagt cccacttctg ggggatgat acgctgggag gcctgtttg ctttgccatc	840
ggctacgtga caggactgca gatcaagttc accagtccgc tgaccacaaa tgtgtcgggc	900

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acggccaagg cctgtgcca gacagtgtg gccgtgtct actacgagga gaccaagagc 960
ttctcttggg ggacgagcaa catgatggg ctgggggggt cctccgccta cacctgggtc 1020
aggggctggg agatgaagaa gactccggag gagcccagcc ccaaagacag cgagaagagc 1080
gccatggggg tgtga 1095

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<210> SEQ ID NO 94
<211> LENGTH: 364
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

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<400> SEQUENCE: 94

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Met Asn Arg Ala Pro Leu Lys Arg Ser Arg Ile Leu His Met Ala Leu
 1             5             10            15

Thr Gly Ala Ser Asp Pro Ser Ala Glu Ala Glu Ala Asn Gly Glu Lys
      20            25            30

Pro Phe Leu Leu Arg Ala Leu Gln Ile Ala Leu Val Val Ser Leu Tyr
      35            40            45

Trp Val Thr Ser Ile Ser Met Val Phe Leu Asn Lys Tyr Leu Leu Asp
      50            55            60

Ser Pro Ser Leu Arg Leu Asp Thr Pro Ile Phe Val Thr Phe Tyr Gln
      65            70            75            80

Cys Leu Val Thr Thr Leu Leu Cys Lys Gly Leu Ser Ala Leu Ala Ala
      85            90            95

Cys Cys Pro Gly Ala Val Asp Phe Pro Ser Leu Arg Leu Asp Leu Arg
      100           105           110

Val Ala Arg Ser Val Leu Pro Leu Ser Val Val Phe Ile Gly Met Ile
      115           120           125

Thr Phe Asn Asn Leu Cys Leu Lys Tyr Val Gly Val Ala Phe Tyr Asn
      130           135           140

Val Gly Arg Ser Leu Thr Thr Val Phe Asn Val Leu Leu Ser Tyr Leu
      145           150           155           160

Leu Leu Lys Gln Thr Thr Ser Phe Tyr Ala Leu Leu Thr Cys Gly Ile
      165           170           175

Ile Ile Gly Gly Phe Trp Leu Gly Val Asp Gln Glu Gly Ala Glu Gly
      180           185           190

Thr Leu Ser Trp Leu Gly Thr Val Phe Gly Val Leu Ala Ser Leu Cys
      195           200           205

Val Ser Leu Asn Ala Ile Tyr Thr Thr Lys Val Leu Pro Ala Val Asp
      210           215           220

Gly Ser Ile Trp Arg Leu Thr Phe Tyr Asn Asn Val Asn Ala Cys Ile
      225           230           235           240

Leu Phe Leu Pro Leu Leu Leu Leu Gly Glu Leu Gln Ala Leu Arg
      245           250           255

Asp Phe Ala Gln Leu Gly Ser Ala His Phe Trp Gly Met Met Thr Leu
      260           265           270

Gly Gly Leu Phe Gly Phe Ala Ile Gly Tyr Val Thr Gly Leu Gln Ile
      275           280           285

Lys Phe Thr Ser Pro Leu Thr His Asn Val Ser Gly Thr Ala Lys Ala
      290           295           300

Cys Ala Gln Thr Val Leu Ala Val Leu Tyr Tyr Glu Glu Thr Lys Ser
      305           310           315           320

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Phe Leu Trp Trp Thr Ser Asn Met Met Val Leu Gly Gly Ser Ser Ala
 325 330 335

Tyr Thr Trp Val Arg Gly Trp Glu Met Lys Lys Thr Pro Glu Glu Pro
 340 345 350

Ser Pro Lys Asp Ser Glu Lys Ser Ala Met Gly Val
 355 360

<210> SEQ ID NO 95

<211> LENGTH: 2609

<212> TYPE: DNA

<213> ORGANISM: Mus musculus

<400> SEQUENCE: 95

```

gagccgaggg tgggtgctgca ggtgcacccg agggcacccg cgagggtgag caccaggtcc      60
ctgcatcagc caggacacca gagcccagtc ggggtggacgg acgggcgcct ctgaagcggc      120
ccaggatcct gcgcattggc ctgactggag tctctgctgt ctccgaggag tcagagagcg      180
ggaacaagcc atttctgtct cggtctctgc agatcgcgct ggtggtctct ctctactggg      240
tcacctccat ttccatggta ttcctcaaca agtacctgct ggacagcccc tccctgcagc      300
tggtatcccc catttttgtc accttctacc aatgcctggt gacctactg ctgtgcaagg      360
gcctcagcac tctggccacc tgetgccccg gcattgtaga cttccccacc ctaaacctgg      420
acctcaaggt ggcccgaagt gtgctgccgc tgtcagtggt ctttatcggc atgataacct      480
tcaataacct ctgcctcaag tacgtagggg tgcccttcta caacgtggga cgctcgtcct      540
ccaccgtgtt caacgttctt ctctctctacc tgetgctcaa acagaccact tccttctatg      600
ccctgctcac ctgcggcgct atcattgggt gtttctggct gggtagtagc caagaaggag      660
ctgagggaac ctgtgccctg acgggcacca tcttcggggg gctggccagc ctctgctgtc      720
ccctcaatgc catctatacc aagaaggctg tccctgcagt agaccacagt atctggcgcc      780
taaccttcta taacaatgtc aatgcctgcg tgctcttctt gccctgatg atagtgtctg      840
gcgagctccg tgccctcctg gccttcactc atctgagcag tgcccacttc tggctcatga      900
tgacgtctgg tggcctgttt ggctttgcca tcggctatgt gacaggactg cagatcaa      960
tcaccagtcc cctgacccat aacgtgtcag gcacggccaa ggcctgtgca cagacagtgc     1020
tgccctgtct ctactacgaa gagattaaga gcttctgtg gtggacaagc aacctgatgg     1080
tgctgggtgg ctctccgccc tacacctggg tcaggggctg ggagatgcag aagaccaggg     1140
aggacccagc ctccaaagat ggtgagaaga gtgctatcag ggtgtgagct ccttcaggga     1200
gccagggctg agctcgggtg gggcctgccc agcacggaag gcttcccata gagcctactg     1260
ggtagtgccc tgagcaataa tgtttacatc cttctcagaa gaccatctaa gaagagccag     1320
gttcttttct gataatgtca gaaagctgcc aaatctcctg cctgccccat cttctagtct     1380
tgggaaagcc ctaccaggag tggcaccctt cctgcctcct cctggggcct gtctacctcc     1440
atatggtctc tgggggttgg gccagctgca ctctttgggc actggactga tgaagtgtatg     1500
tcttactttc tacacaaggg agatgggttg tgaccctact atagctagtt gaaggagagct     1560
gtgtaacccc acatctcttg ggccttgggc aggtagcata atagctaggt gctattaaca     1620
tcaataacac ttcagactac ctttgagggc agttgggagc tgagccgaga gagagagatg     1680
gccattctgc cctcttctgt gtggatgggt atgacagacc aactgtccat ggggtgactg     1740

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acacctccac acttcatatt ttcaacttta gaaaaggggg agccacacgt tttacagatt 1800
aagtggagtg atgaatgcct ctacagcccc taaccccact ttccctgcct ggcttctctt 1860
ggcccagaag ggccaccatc ctgttctcca acacctgacc cagctatctg gctatactct 1920
ctttctgtac tcccctcccc ttcccccccc cattagcctc ctccccaaca cctccatctt 1980
caggcaggaa gtggggtcca ctcagcctct gttcccatct gcttggaccc ctgagcctct 2040
catgaaggta ggcttatgtt ctctgaggct ggggccggag gagcgactg attctcggag 2100
ttatcccatc aggcctctgt cacaaaatag cctaggccgt gtgtctaaga acagtggagg 2160
ttggcttata actgttctgg gggcagcgaa gccacatca aggtactcat agaccagta 2220
ttctgagga aacccctgtc cacatcctca cttggtaaag gcacagataa tctccctcag 2280
gcctcttgta taggagcact agccctggga gggctccgcc ccatgacctg atcaccctaa 2340
agccttcaac agaaggattc caacatgaat ttggggacag aagcactcag accacgatgc 2400
ccagcaccac accctcctat cctcagggta gctgtcactg tcctagtccc ttctgtttgg 2460
cctttgttac cctcatcttc ttggcgctat gtttgatgct tttgtctctt tcgtgagaag 2520
atggggaaac catgtcagcc tctgcttccg acttcccatg ggttctaag aagttgggtg 2580
ggcctgatgc cctgagttgt atgtgattt 2609

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<210> SEQ ID NO 96

<211> LENGTH: 350

<212> TYPE: PRT

<213> ORGANISM: Mus musculus

<400> SEQUENCE: 96

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Met Ala Leu Thr Gly Val Ser Ala Val Ser Glu Glu Ser Glu Ser Gly
  1             5             10            15
Asn Lys Pro Phe Leu Leu Arg Ala Leu Gln Ile Ala Leu Val Val Ser
          20             25             30
Leu Tyr Trp Val Thr Ser Ile Ser Met Val Phe Leu Asn Lys Tyr Leu
          35             40             45
Leu Asp Ser Pro Ser Leu Gln Leu Asp Thr Pro Ile Phe Val Thr Phe
          50             55             60
Tyr Gln Cys Leu Val Thr Ser Leu Leu Cys Lys Gly Leu Ser Thr Leu
          65             70             75             80
Ala Thr Cys Cys Pro Gly Met Val Asp Phe Pro Thr Leu Asn Leu Asp
          85             90             95
Leu Lys Val Ala Arg Ser Val Leu Pro Leu Ser Val Val Phe Ile Gly
          100            105            110
Met Ile Thr Phe Asn Asn Leu Cys Leu Lys Tyr Val Gly Val Pro Phe
          115            120            125
Tyr Asn Val Gly Arg Ser Leu Thr Thr Val Phe Asn Val Leu Leu Ser
          130            135            140
Tyr Leu Leu Leu Lys Gln Thr Thr Ser Phe Tyr Ala Leu Leu Thr Cys
          145            150            155            160
Gly Val Ile Ile Gly Gly Phe Trp Leu Gly Ile Asp Gln Glu Gly Ala
          165            170            175
Glu Gly Thr Leu Ser Leu Thr Gly Thr Ile Phe Gly Val Leu Ala Ser
          180            185            190
Leu Cys Val Ser Leu Asn Ala Ile Tyr Thr Lys Lys Val Leu Pro Ala
          195            200            205

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Val Asp His Ser Ile Trp Arg Leu Thr Phe Tyr Asn Asn Val Asn Ala
 210 215 220

Cys Val Leu Phe Leu Pro Leu Met Ile Val Leu Gly Glu Leu Arg Ala
 225 230 235 240

Leu Leu Ala Phe Thr His Leu Ser Ser Ala His Phe Trp Leu Met Met
 245 250 255

Thr Leu Gly Gly Leu Phe Gly Phe Ala Ile Gly Tyr Val Thr Gly Leu
 260 265 270

Gln Ile Lys Phe Thr Ser Pro Leu Thr His Asn Val Ser Gly Thr Ala
 275 280 285

Lys Ala Cys Ala Gln Thr Val Leu Ala Val Leu Tyr Tyr Glu Glu Ile
 290 295 300

Lys Ser Phe Leu Trp Trp Thr Ser Asn Leu Met Val Leu Gly Gly Ser
 305 310 315 320

Ser Ala Tyr Thr Trp Val Arg Gly Trp Glu Met Gln Lys Thr Gln Glu
 325 330 335

Asp Pro Ser Ser Lys Asp Gly Glu Lys Ser Ala Ile Arg Val
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<210> SEQ ID NO 97

<211> LENGTH: 1053

<212> TYPE: DNA

<213> ORGANISM: Rattus norvegicus

<400> SEQUENCE: 97

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<210> SEQ ID NO 98

<211> LENGTH: 350

<212> TYPE: PRT

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<213> ORGANISM: Rattus norvegicus

<400> SEQUENCE: 98

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Asn Lys Pro Phe Leu Leu Arg Ala Leu Gln Ile Ala Leu Val Val Ser
      20           25           30
Leu Tyr Trp Val Thr Ser Ile Ser Met Val Phe Leu Asn Lys Tyr Leu
      35           40           45
Leu Asp Ser Pro Ser Leu Gln Leu Asp Thr Pro Ile Phe Val Thr Phe
      50           55           60
Tyr Gln Cys Leu Val Thr Ser Leu Leu Cys Lys Gly Leu Ser Thr Leu
      65           70           75           80
Ala Thr Cys Cys Pro Gly Met Val Asp Phe Pro Thr Leu Asn Leu Asp
      85           90           95
Leu Lys Val Ala Arg Ser Val Leu Pro Leu Ser Val Val Phe Ile Gly
      100          105          110
Met Ile Thr Phe Asn Asn Leu Cys Leu Lys Tyr Val Gly Val Ala Phe
      115          120          125
Tyr Asn Val Gly Arg Ser Leu Thr Thr Val Phe Asn Val Leu Leu Ser
      130          135          140
Tyr Leu Leu Leu Lys Gln Thr Thr Ser Phe Tyr Ala Leu Leu Thr Cys
      145          150          155          160
Ala Ile Ile Ile Gly Gly Phe Trp Leu Gly Ile Asp Gln Glu Gly Ala
      165          170          175
Glu Gly Thr Leu Ser Leu Thr Gly Thr Ile Phe Gly Val Leu Ala Ser
      180          185          190
Leu Cys Val Ser Leu Asn Ala Ile Tyr Thr Lys Lys Val Leu Pro Ala
      195          200          205
Val Asp His Ser Ile Trp Arg Leu Thr Phe Tyr Asn Asn Val Asn Ala
      210          215          220
Cys Val Leu Phe Leu Pro Leu Met Val Val Leu Gly Glu Leu His Ala
      225          230          235          240
Leu Leu Ala Phe Ala His Leu Asn Ser Ala His Phe Trp Val Met Met
      245          250          255
Thr Leu Gly Gly Leu Phe Gly Phe Ala Ile Gly Tyr Val Thr Gly Leu
      260          265          270
Gln Ile Lys Phe Thr Ser Pro Leu Thr His Asn Val Ser Gly Thr Ala
      275          280          285
Lys Ala Cys Ala Gln Thr Val Leu Ala Val Leu Tyr Tyr Glu Glu Ile
      290          295          300
Lys Ser Phe Leu Trp Trp Thr Ser Asn Leu Met Val Leu Gly Gly Ser
      305          310          315          320
Ser Ala Tyr Thr Trp Val Arg Gly Trp Glu Met Gln Lys Thr Gln Glu
      325          330          335
Asp Pro Ser Ser Lys Glu Gly Glu Lys Ser Ala Ile Gly Val
      340          345          350

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<210> SEQ ID NO 99

<211> LENGTH: 7752

<212> TYPE: DNA

<213> ORGANISM: Mus musculus

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<400> SEQUENCE: 99

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gttctggaag gaaagggccc ccgggaagg gatacagcat tgagaagctc agaggctttg	180
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gtgctgcttg gatctccggg acttcatgtc ctgactaggt ctggaagcgg tgaataagg	300
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<210> SEQ ID NO 100

<211> LENGTH: 4039

<212> TYPE: DNA

<213> ORGANISM: *Cricetulus griseus*

<400> SEQUENCE: 100

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1. A method for enhancing a binding activity of an antibody composition to Fc γ receptor IIIa, which comprises modifying a complex N-glycoside-linked sugar chain which is bound to the Fc region of an antibody molecule.

2. The method according to claim 1, wherein the modification of a complex N-glycoside-linked sugar chain which is bound to the Fc region of an antibody molecule is to bind a sugar chain in which 1-position of fucose is not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond in the complex N-glycoside-linked sugar chain to the Fc region in the antibody molecule.

3. The method according to claim 1, wherein the sugar chain is synthesized by a cell in which the activity of a protein relating to modification of a sugar chain in which fucose is bound to N-acetylglucosamine in the reducing end in the complex N-glycoside-linked sugar chain is decreased or deleted.

4. The method according to claim 3, wherein the protein relating to

- (a) a protein relating to synthesis of an intracellular sugar nucleotide, GDP-fucose;
- (b) a protein relating to modification of a sugar chain in which 1-position of fucose is bound to 6-position of N-acetylglucosamine in the reducing end through α -bond in a complex N-glycoside-linked sugar chain;
- (c) a protein relating to transport of an intracellular sugar nucleotide, GDP-fucose to the Golgi body.

5. The method according to claim 3, wherein the cell is resistant to a lectin which recognizes a sugar chain structure in which 1-position of fucose is bound to 6-position of N-acetylglucosamine in the reducing end through α -bond in a complex N-glycoside-linked sugar chain.

6. The method according to claim 3, wherein the cell is resistant to at least one lectin selected from the group consisting of the following (a) to (d):

- (a) a *Lens culinaris* lectin;

7. The method according to claim 3, wherein the cell is selected from the group consisting of a yeast, an animal cell, an insect cell and a plant cell.

8. The method according to claim 3, wherein the cell is selected from the group consisting of the following (a) to (i):

- (a) a CHO cell derived from a Chinese hamster ovary tissue;
- (b) a rat myeloma cell line YB2/3HL.P2.G11.16Ag.20 cell;
- (c) a BHK cell derived from a Syrian hamster kidney tissue;
- (d) a mouse myeloma cell line NS0 cell;
- (e) a mouse myeloma cell line SP2/0-Ag14 cell;
- (f) a hybridoma cell;
- (g) a human leukemic cell line Namalwa cell;
- (h) an embryonic stem cell;
- (i) a fertilized egg cell.

9. The method according to claim 1,

- (c) an antibody fragment comprising the Fc region of (a) or (b);
- (d) a fusion protein comprising the Fc region of (a) or (b).

10. The method according to claim 1, wherein the antibody molecule belongs to an IgG class.

11. The method according to claim 1, wherein, in the complex N-glycoside-linked sugar chain which is bound to the Fc region of an antibody molecule, the ratio of a sugar chain in which 1-position of fucose is not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond is 20% or more of total complex N-glycoside-linked sugar chains.

12. A method for enhancing an antibody-dependent cell-mediated cytotoxic activity of an antibody composition, which comprises using the method according to claim 1.

13. A process for producing an antibody composition having an

14. The process according to claim 13, wherein the modification of a complex N-glycoside-linked sugar chain which is bound to the Fc region of an antibody molecule is to bind a sugar chain in which 1-position of fucose is not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond in the complex N-glycoside-linked sugar chain to the Fc region in the antibody molecule.

15. The process according to claim 13, wherein the sugar chain is synthesized by a cell in which the activity of a protein relating to modification of a sugar chain in which fucose is bound to N-acetylglucosamine in the reducing end in the complex N-glycoside-linked sugar chain is decreased or deleted.

16. The process according to claim 15, wherein the protein relating to modification of a sugar chain in which fucose is bound to N-acetylglucosamine in the reducing end in the complex N-glycoside-linked sugar chain is selected from the group consisting of the following (a), (b) and (c):

(a) a protein relating to synthesis of an intracellular sugar nucleotide, GDP-fucose;

(c) a protein relating to transport of an intracellular sugar nucleotide, GDP-fucose to the Golgi body.

17. The process according to claim 15, wherein the cell is resistant to a lectin which recognizes a sugar chain structure in which 1-position of fucose is bound to 6-position of N-acetylglucosamine in the reducing end through α -bond in a complex N-glycoside-linked sugar chain.

18. The process according to claim 15, wherein the cell is resistant to at least one lectin selected from the group consisting of the following (a) to (d):

(a) a *Lens culinaris* lectin;

(b) a *Pisum sativum* lectin;

(c) a *Vicia faba* lectin;

(d) an *Aleuria aurantia* lectin.

19. The process according to claim 15

20. The process according to claim 15, wherein the cell is selected from the group consisting of the following (a) to (i):

(a) a CHO cell derived from a Chinese hamster ovary tissue;

(b) a rat myeloma cell line YB2/3HL.P2.G11.16Ag.20 cell;

(c) a BHK cell derived from a Syrian hamster kidney tissue;

(d) a mouse myeloma cell line NS0 cell;

(e) a mouse myeloma cell line SP2/0-Ag14 cell;

(f) a hybridoma cell;

(g) a human leukemic cell line Namalwa cell;

(h) an embryonic stem cell;

(i) a fertilized egg cell.

21. The process according to claim 13, wherein the antibody molecule is selected from the group consisting of the following (a) to (d):

(a) a human antibody;

(b) a humanized antibody;

22. The process according to claim 13, wherein the antibody molecule belongs to an IgG class.

23. The method according to claim 13, wherein, in the complex N-glycoside-linked sugar chain which is bound to the Fc region of an antibody molecule, the ratio of a sugar chain in which 1-position of fucose is not bound to 6-position of N-acetylglucosamine in the reducing end through α -bond is 20% or more of total complex N-glycoside-linked sugar chains.

24. A process for producing an antibody composition having an increased antibody-dependent cell-mediated cytotoxic activity, which comprises using the process according to claim 12.

25. An antibody composition produced by the process according to claim 13.

26. A method for detecting the ratio of a sugar chain in which with an Fc γ receptor IIIa to measure the binding activity to the Fc γ receptor IIIa; and detecting the ratio of a sugar chain in a standard antibody composition with a standard curve showing the binding activity to the Fc γ receptor IIIa.

27. A method for detecting the antibody-dependent cell-mediated cytotoxic activity in an antibody composition, which comprises: reacting an antigen with a tested antibody composition to form a complex of the antigen and the antibody composition; contacting the complex with an Fc γ receptor IIIa to measure the binding activity to the Fc γ receptor IIIa; and detecting the ratio of a sugar chain in a standard antibody composition with a standard curve showing the binding activity to the Fc γ receptor IIIa.

28. A method for detecting the ratio of a sugar chain in which fucose is not bound to N-acetylglucosamine in the reducing end in the sugar chain among total complex N-glycoside-linked sugar chains bound to the Fc region in an antibody composition, which comprises: contacting a tested antibody composition with a Fc γ receptor IIIa to measure the binding activity of the antibody composition to the Fc γ

29. A method for detecting the antibody-dependent cell-mediated cytotoxic activity in an antibody composition, which comprises: contacting a tested antibody composition with a Fc γ receptor IIIa to measure the binding activity of the antibody composition to the Fc γ receptor IIIa; and detecting the ratio of a sugar chain in a standard antibody composition with a standard curve showing the binding activity to the Fc γ receptor IIIa.

30. An Fc fusion protein composition produced by using a cell resistant to a lectin which recognizes a sugar chain structure in which 1-position of fucose is bound to 6-position of N-acetylglucosamine in the reducing end through α -bond in a complex N-glycoside-linked sugar chain.

31. The Fc fusion protein composition according to claim 30, wherein the cell is selected from the group consisting of the following (a), (b) and (c):

(a) an enzyme protein relating to synthesis of an intracellular sugar nucleotide, GDP-fucose;

(b) an enzyme protein relating to modification of a sugar chain in which 1-

wherein the activity of the protein is decreased or deleted.

32. The Fc fusion protein composition according to claim 30, wherein the cell is resistant to at least one lectin selected from the group consisting of the following (a) to (d):

(a) a *Lens culinaris* lectin;

(b) a *Pisum sativum* lectin;

(c) a *Vicia faba* lectin;

(d) an *Aleuria aurantia* lectin.

33. The Fc fusion protein composition according to claim 30, wherein the cell is a cell into which a gene encoding an Fc fusion protein is introduced.

34. The Fc fusion protein composition according to claim 33, wherein the Fc is derived from an IgG class of an antibody molecule.

36. The Fc fusion protein composition according to claim 30, wherein the cell is a mouse myeloma cell.

37. The Fc fusion protein composition according to claim 36, wherein the mouse myeloma cell is NS0 cell or SP2/0-Ag14 cell.

38. The Fc fusion protein composition according to claim 30, wherein the cell is selected from the group consisting of the following (a) to (g):

(a) a CHO cell derived from a Chinese hamster ovary tissue;

(b) a rat myeloma cell line YB2/3HL.P2.G11.16Ag.20 line;

(c) a BHK cell derived from a Syrian hamster kidney tissue;

(d) hybridoma cell;

(e) a human leukemic cell line Namalwa cell;

(f) an embryonic stem cell;

(g) a fertilized egg cell.

39. An Fc fusion protein composition comprising an Fc fusion protein having an complex N-glycoside-linked sugar chain at the Fc region of an antibody molecule, wherein the ratio of a sugar chain in which fucose is not bonded to

N-acetylglucosamine in the reducing end in the sugar chain is 20% or more of total complex N-glycoside-linked sugar chains which are bound to the Fc region in the composition.

40. The Fc fusion protein composition according to claim 39, wherein the sugar chain in which fucose is not bound is a sugar chain in which 1-position of fucose is not bound to 6-position of N-acetylglucosamine in the reducing end in the complex N-glycoside-linked sugar chain through α -bond.

41. The Fc fusion protein composition according to claim 39, wherein the antibody molecule belongs to an IgG class.

42. The Fc fusion protein composition according to claim 30, wherein the Fc fusion protein composition is Fc-fused fibroblast growth factor-8.

43. A cell which produces the Fc fusion protein composition according to claim 30.

45. The cell according to claim 43, which is a mouse myeloma cell.

46. The cell according to claim 45, wherein the mouse myeloma cell is NS0 cell or SP2/0-Ag14 cell.

47. The cell according to claim 43, which is selected from the group consisting of the following (a) to (g):

(a) a CHO cell derived from a Chinese hamster ovary tissue;

(b) a rat myeloma cell line YB2/3HL.P2.G11.16Ag.20 line;

(c) a BHK cell derived from a Syrian hamster kidney tissue;

(d) an antibody-producing hybridoma cell;

(e) a human leukemic cell line Namalwa cell;

(f) an embryonic stem cell;

(g) a fertilized egg cell.

48. A process for producing an Fc fusion protein

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