(c) a technique for introducing mutation into the enzyme;

(d) a technique for inhibiting transcription and/or translation of a gene encoding the enzyme.

(35) The cell according to any one of (23) to (34), which is resistant to at least 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 the *N*-glycoside-linked sugar chain.

(36) The cell according to any one of (23) to (35), which is a cell 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 mouse myeloma cell line, NSO cell;

(d) a mouse myeloma cell line, SP2/0-Ag14 cell;

(e) a BHK cell derived from a syrian hamster kidney tissue;

(f) an antibody-producing hybridoma cell;

(g) a human leukemia cell line, Namalwa cell;

(h) an embryonic stem cell;

(i) a fertilized egg cell.

(37) The cell according to any one of (23) to (36) into which a gene encoding an antibody molecule is introduced.

(38) The cell according to (37), wherein the antibody molecule belongs to an IgG class.

(39) A method for producing an antibody composition, which comprises culturing the cell according to (37) or (38) in a medium to produce and accumulate the antibody composition in the culture; and recovering the antibody composition from the culture.

(40) The method according to (39), which produces an antibody composition having higher antibody-dependent cell-

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mediated cytotoxic activity than an antibody composition obtained from its parent cell line.

(41) An antibody composition which is produced using the method according to (39) or (40).

(42) A transgenic non-human animal or plant or the progenies thereof, comprising a genome which is modified such that the activity of an enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose and/or the activity of an enzyme relating to the 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 the *N*-glycoside-linked sugar chain is decreased.

(43) The transgenic non-human animal or plant or the progenies thereof according to (42), wherein a gene encoding the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or a gene encoding the enzyme relating to the 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 the *N*-glycoside-linked sugar chain is knocked out.

(44) The transgenic non-human animal or plant or the progenies thereof according to (42) or (43), wherein the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose is an enzyme selected from the group consisting of the following (a), (b) and (c):

(a) GMD (GDP-mannose 4,6-dehydratase);

(b) Fx (GDP-keto-6-deoxymannose 3,5-epimerase, 4-reductase);

(c) GFPP (GDP-beta-L-fucose pyrophosphorylase).

(45) The transgenic non-human animal or plant or the progenies thereof according to (44), wherein the GMD is a protein encoded by a DNA of the following (a) or (b):

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

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(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.

(46) The transgenic non-human animal or plant or the progenies thereof according to (44), wherein the Fx is a protein encoded by a DNA of the following (a) or (b):

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

(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.

(47) The transgenic non-human animal or plant or the progenies thereof according to (44), wherein the GFPP is a protein encoded by a DNA of the following (a) or (b):

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

(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.

(48) The transgenic non-human animal or plant or the progenies thereof according to (42) or (43), wherein the enzyme relating to the 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 the *N*-glycoside-linked sugar chain is α-1,6-fucosyltransferase.

(49) The transgenic non-human animal or plant or the progenies thereof according to (48), wherein the α-1,6-fucosyltransferase is a protein encoded by a DNA selected from the group consisting of the following (a), (b), (c) and (d):

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

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

(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;

(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.

(50) The transgenic non-human animal or plant or the progenies thereof according to any one of (42) to (49), wherein the transgenic non-human animal is an animal selected from the group consisting of cattle, sheep, goat, pig, horse, mouse, rat, fowl, monkey and rabbit.

(51) A method for producing an antibody composition, which comprises introducing a gene encoding an antibody molecule into the transgenic non-human animal or plant or the progenies thereof according to any one of (42) to (50); rearing the animal or plant; isolating tissue or body fluid comprising the introduced antibody from the reared animal or plant; and recovering the antibody composition from the isolated tissue or body fluid.

(52) The method according to (51), wherein the antibody molecule belongs to an IgG class.

(53) The method according to (51) or (52), which produces an antibody composition having higher antibody-dependent cell-mediated cytotoxic activity than an antibody composition obtained from a non-human animal or plant or the progenies thereof whose genome is not modified.

(54) An antibody composition which is produced using the method according to any one of (51) to (53).

(55) A medicament comprising the antibody composition according to any one of (21), (22), (41) and (54) as an active ingredient.

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(56) The medicament according to (55), wherein the medicament is a diagnostic drug, a preventive drug or a therapeutic drug for diseases accompanied by tumors, diseases accompanied by allergies, diseases accompanied by inflammations, autoimmune diseases, circulatory organ diseases, diseases accompanied by viral infections or diseases accompanied by bacterial infections.

(57) A protein selected from the group consisting of the following (a), (b), (c), (d), (e), (f), (g), (h), (i) and (j):

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

(b) 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;

(c) a protein comprising the amino acid sequence represented by SEQ ID NO:72;

(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:72 and has Fx activity;

(e) a protein comprising the amino acid sequence represented by SEQ ID NO:73;

(f) 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:73 and has GFPP activity;

(g) a protein comprising the amino acid sequence represented by SEQ ID NO:23;

(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:23 and has α-1,6-fucosyltransferase activity;

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(i) a protein comprising the amino acid sequence represented by SEQ ID NO:24;

(j) 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 the α-1,6-fucosyltransferase activity.

(58) A DNA which encodes the protein according to (57).

(59) A DNA selected from the group consisting of the following (a), (b), (c), (d) and (e):

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

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

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

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

(e) a DNA comprising the nucleotide sequence represented by SEQ ID NO:51.

(60) A genome DNA selected from the group consisting of the following (a), (b) and (c):

(a) a genome DNA comprising the nucleotide sequence represented by SEQ ID NO:3;

(b) a genome DNA comprising the nucleotide sequence represented by SEQ ID NO:67;

(c) a genome DNA comprising the nucleotide sequence represented by SEQ ID NO:70.

(61) A target vector for homologous recombination, comprising a full length of the DNA according to any one of (58) to (60), or a part thereof.

BRIEF EXPLANATION OF THE DRAWINGS

Fig. 1 shows electrophoresis patterns of SDS-PAGE of five purified anti-GD3 chimeric antibodies (using

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gradient gel from 4 to 15%). Fig. 1A and Fig. 1B show a result of the electrophoresis under non-reducing conditions and that 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.

Fig. 2 shows activities of five purified anti-GD3 chimeric antibodies to bind to GD3, measured by changing the antibody concentration. The ordinate and the abscissa show the binding activity with GD3 and the antibody concentration, respectively. "○", "●", "□", "■" and "△" 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.

Fig. 3 shows ADCC activities of five purified anti-GD3 chimeric antibodies for a human melanoma cell line G-361. The ordinate and abscissa show the cytotoxic activity and the antibody concentration, respectively. "○", "●", "□", "■" and "△" 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.

Fig. 4 shows electrophoresis patterns of SDS-PAGE of three purified anti-hIL-5Rα CDR-grafted antibodies (using gradient gel from 4 to 15%). Fig. 4A and Fig. 4B show results of the electrophoresis carried out under non-reducing conditions and those under reducing conditions, respectively. Lanes 1 to 5 show electrophoresis patterns of high molecular weight markers, YB2/0-hIL-5R CDR antibody, CHO/d-hIL-5R CDR antibody, NS0-hIL-5R CDR antibody and low molecular weight markers, respectively.

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Fig. 5 shows activities of three purified anti-hIL-5Rα CDR-grafted antibodies to bind to hIL-5Rα, measured by changing the antibody concentration. The ordinate and the abscissa show the binding activity with hIL-5Rα and the antibody concentration, respectively. "○", "●" and "□" show the activities of YB2/0-hIL-5R CDR antibody, CHO/d-hIL-5R CDR antibody and NS0-hIL-5R CDR antibody, respectively.

Fig. 6 show ADCC activities of three purified anti-hIL-5Rα CDR-grafted antibodies for an hIL-5R expressing mouse T cell line CTLL-2(h5R). The ordinate and the abscissas show the cytotoxic activity and the antibody concentration, respectively. "○", "●" and "□" show the activities of YB2/0-hIL-5RCDR antibody, CHO/d-hIL-5R CDR antibody and NS0-hIL-5R CDR antibody, respectively.

Fig. 7 shows inhibition activities of three purified anti-hIL-5Rα CDR-grafted antibodies in an hIL-5-induced eosinophil increasing model of *Macaca faseicularis*. The ordinate and the abscissa show the number of eosinophils in peripheral blood and the number of days (the day of the commencement of antibody and hIL-5 administration was defined as 0 day). "101 and 102", "301, 302 and 303", "401, 402 and 403" and "501, 502 and 503" show results in the antibody non-administration group, the YB2/0-hIL-5R CDR antibody administered group, the CHO/d-hIL-5R CDR antibody administered group and the NS0-hIL-5R CDR antibody administered group, respectively.

Fig. 8 shows elution patterns of reverse phase HPLC elution of a PA-treated sugar chain (left side), and an elution pattern obtained by treating the PA-treated sugar chain with α-L-fucosidase and then analyzed by reverse phase HPLC (right side), of the purified anti-hIL-5Rα CDR-grafted antibody produced by YB2/0 (Fig. 8A) and the purified anti-hIL-5Rα CDR-grafted antibody produced by NS0 (Fig. 8B). The ordinates and the abscissas show the

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relative fluorescence intensity and the elution time, respectively.

Fig. 9 shows an elution pattern obtained by preparing a PA-treated sugar chain from the purified anti-hIL-5Rα CDR-grafted antibody produced by CHO/d cell and analyzing it by reverse phase HPLC. The ordinate and the abscissa show the relative fluorescence intensity and the elution time, respectively.

In Fig. 10, Fig. 10A shows the GD3-binding activities of a non-adsorbed fraction and a part of an adsorbed fraction, measured by changing the antibody concentration. The ordinate and the abscissa show the binding activity with GD3 and the antibody concentration, respectively. "●" and "○" show the non-adsorbed fraction and a part of the adsorbed fraction, respectively. Fig. 10B shows the ADCC activities of the non-adsorbed fraction and a part of the adsorbed fraction for a human melanoma line G-361. The ordinate and the abscissa show the cytotoxic activity and the antibody concentration, respectively. "●" and "○" show the non-adsorbed fraction and a part of the adsorbed fraction, respectively.

Fig. 11 shows elution patterns obtained by analyzing PA-treated sugar chains prepared from a non-adsorbed fraction and a part of an adsorbed fraction by a reverse HPLC. Fig. 11A and Fig. 11B show an elution pattern of the non-adsorbed fraction and an elution pattern of a part of the adsorbed fraction, respectively. The ordinates and the abscissas show the relative fluorescence strength and the elution time, respectively.

Fig. 12 shows elution patterns of PA-treated sugar chains prepared from 6 anti-GD3 chimeric antibodies (Fig. 12A to Fig. 12F), obtained by analyzing them by reverse phase HPLC. The ordinates and the abscissas show the relative fluorescence intensity and the elution time, respectively.

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Fig. 13 shows GD3-binding activities of 6 anti-GD3 chimeric antibodies having a different ratio of α-1,6-fucose-free sugar chains measured by changing the antibody concentration. The ordinate and the abscissa show the binding activity with GD3 and the antibody concentration, respectively. "●", "□", "■", "△", "▲" and "×" 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.

Fig. 14 shows ADCC activities of six kinds of anti-GD3 chimeric antibodies having a different ratio of α-1,6-fucose-free sugar chains against a human melanoma cell line G-361, using an effector cell of the donor A. The ordinate and the abscissa show the cytotoxic activity and the antibody concentration, respectively. "●", "□", "■", "△", "." and "×" 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.

Fig. 15 shows ADCC activities of six kinds of anti-GD3 chimeric antibodies having a different ratio of α-1,6-fucose-free sugar chains against a human melanoma cell line G-361, using an effector cell of the donor B. The ordinate and the abscissa show the cytotoxic activity and the antibody concentration, respectively. "●", "□", "■", "△", "." and "×" 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.

Fig. 16 shows elution patterns of PA-treated sugar chains prepared from six kinds of anti-GD3 chimeric

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antibodies, obtained by analyzing them by reverse phase HPLC. The ordinates and the abscissas show the relative fluorescence intensity and the elution time, respectively.

Fig. 17 shows CCR4-binding activities of six kinds of anti-CCR4 chimeric antibodies having a different ratio of α-1,6-fucose-free sugar chains measured by changing the antibody concentration. The ordinate and the abscissa show the binding activity with CCR4 and the antibody concentration, respectively. "■", "□", "▲", "△", "●" 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.

Fig. 18 shows ADCC activities of anti-CCR4 chimeric antibodies having a different ratio of α-1,6-fucose-free sugar chains against CCR4/EL-4 cell, using an effector cell of the donor A. The ordinate and the abscissa show the cytotoxic activity and the antibody concentration, respectively. "■", "□", "▲", "△", "●" 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. Also, Fig. 18A and Fig. 18B show results obtained using effector cells of the donor A and the donor B, respectively.

Fig. 19 shows ADCC activities of anti-CCR4 chimeric antibodies having a different ratio of α-1,6-fucose-free sugar chain against CCR4/EL-4 cell, using an effector cell of the donor B. The ordinate and the abscissa show the cytotoxic activity and the antibody concentration, respectively. "■", "□", "▲", "△", "●" and "○" show the activities of anti-CCR4 chimeric antibody (46%), anti-CCR4 chimeric antibody (39%), anti-CCR4 chimeric antibody (27%),

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anti-CCR4 chimeric antibody (18%), anti-CCR4 chimeric antibody (9%) and anti-CCR4 chimeric antibody (8%), respectively.

Fig. 20 shows construction of plasmids CHFT8-pCR2.1 and YBFT8-pCR2.1.

Fig. 21 shows construction of plasmids CHAc-pBS and YBAc-pBS.

Fig. 22 shows construction of plasmids CHFT8d-pCR2.1 and YBFT8d-pCR2.1.

Fig. 23 shows construction of plasmids CHAcd-pBS and YBAcd-pBS.

Fig. 24 shows results of determination of an 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.

Fig. 25 shows construction of a plasmid mfFUT8-pCR2.1.

Fig. 26 shows construction of a plasmid pBSmfFUT8.

Fig. 27 shows construction of a plasmid pAGEmfFUT8.

Fig. 28 shows results of analysis of expression levels of FUT8 gene by a cell line excessively expressing the gene using a competitive RT-PCR. The ordinate shows relative values of amounts of FUT8 transcription to amounts of β-actin transcription.

Fig. 29 shows ADCC activities of an anti-GD3 chimeric antibody purified from a cell line excessively expressing FUT8 gene against a human melanoma cell line G-361. The ordinate and the abscissa show the cytotoxic activity and the antibody concentration, respectively.

Fig. 30 shows elution patterns of PA-treated sugar chains prepared from antibodies produced by mfFUT8-6 and pAGE249-introduced cell lines, obtained by analyzing them

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by reverse phase HPLC. Fig. 30A and Fig. 30B show elution patterns of PA-treated sugar chains prepared from an antibody produced by mfFUT8-6-introduced cell line and PA-treated sugar chains prepared from an antibody produced by pAGE249-introduced cell line, respectively. The ordinate and the abscissa show the relative fluorescence intensity and the elution time, respectively.

Fig. 31 shows an elution pattern of PA-treated sugar chains prepared from Herceptin, obtained by analyzing them by reverse phase HPLC. The ordinate and the abscissa show the relative fluorescence intensity and the elution time, respectively.

Fig. 32 shows construction of a plasmid CHfFUT8-pCR2.1.

Fig. 33 shows construction of a plasmid ploxPPuro.

Fig. 34 shows construction of a plasmid pKOFUT8gE2-1.

Fig. 35 shows construction of a plasmid pKOFUT8gE2-2.

Fig. 36 shows construction of a plasmid pscFUT8gE2-3.

Fig. 37 shows construction of a plasmid pKOFUT8gE2-3.

Fig. 38 shows construction of a plasmid pKOFUT8gE2-4.

Fig. 39 shows construction of a plasmid pKOFUT8gE2-5.

Fig. 40 shows construction of a plasmid pKOFUT8Puro.

Fig. 41 shows results of genome Southern analyses of 1st.ΔFUT8 2-46-1 and 1st.ΔFUT8 2-46 as α-1,6-fucosyltransferase gene-disrupted CHO cell lines.

Fig. 42 shows ADCC activities of an anti-CCR4 chimeric antibody purified from an FUT8 allele gene-disrupted cell line. The ordinate and the abscissa show the cytotoxic activity and the antibody concentration. "▲"

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and "■" show the activities of a purified antibody derived from an anti-CCR4 chimeric antibody-producing CHO cell 5-03 and a purified antibody derived from 1st.ΔFUT8 2-46-1, respectively.

Fig. 43 shows 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. "□", "■", "◆" and "▲" show the activities of antibodies produced by the strain 5-03, CHO/CCR4-LCA, CHO/CCR4-AAL and CHO/CCR4-PHA, respectively.

Fig. 44 shows 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 activities of antibodies produced by YB2/0 (KM2760 # 58-35-16), 5-03 and CHO/CCR4-LCA, respectively.

Fig. 45 shows elution patterns of PA-treated sugar chains prepared from purified anti-CCR4 human chimeric antibodies, obtained by analyzing them by reverse phase HPLC. The ordinate and the abscissa show the relative fluorescence intensity and the elution time, respectively. Fig. 45A, Fig. 45B, Fig. 45C and Fig. 45D show results of analyses of an antibody produced by the strain 5-03, an antibody produced by CHO/CCR4-LCA, an antibody produced by CHO/CCR4-AAL and an antibody produced by CHO/CCR4-PHA, respectively.

Fig. 46 shows the 1st step of construction of an expression vector of CHO cell-derived GMD (6 steps in total).

Fig. 47 shows the 2nd step of construction of the expression vector of CHO cell-derived GMD (6 steps in total).

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Fig. 48 shows the 3rd step of construction of the expression vector of CHO cell-derived GMD (6 steps in total).

Fig. 49 shows the 4th step of construction of the expression vector of CHO cell-derived GMD (6 steps in total).

Fig. 50 shows the 5th step of construction of the expression vector of CHO cell-derived GMD (6 steps in total).

Fig. 51 shows the 6th step of construction of the expression vector of CHO cell-derived GMD (6 steps in total).

Fig. 52 shows resistance of GMD-expressed 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 CHO/CCR4-LCA introduced with an expression vector pAGE249 for LCA lectin. GMD shows resistance of the CHO/CCR4-LCA introduced with a GMD expression vector pAGE249GMD for LCA lectin.

Fig. 53 shows ADCC activities of an anti-CCR4 chimeric antibody produced by cells of GMD-expressed CHO/CCR4-LCA cell lines. The ordinate and the abscissa show the cytotoxic activity and the antibody concentration, respectively.

Fig. 54 show a production 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.

Fig. 55 shows an elution pattern of PA-treated sugar chains prepared from an anti-CCR4 human chimeric antibody purified from GMD gene-expressed CHO/CCR4-LCA, obtained by analyzing them by reverse phase HPLC. The ordinate and the abscissa show the relative fluorescence intensity and the elution time, respectively.

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DETAILED DESCRIPTION OF THE INVENTION

The Chinese hamster ovary tissue-derived CHO cell into which a gene encoding an antibody molecule is introduced according to the present invention may be any CHO cell, so long as it is a Chinese hamster ovary tissue-derived CHO cell into which a gene encoding an antibody molecule is introduced, which produces an antibody composition comprising complex *N*-glycoside-linked sugar chains bound to the Fc region of an antibody molecule, wherein among the total complex *N*-glycoside-linked sugar chains bound to the Fc region in the composition, 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.

In the present invention, the antibody molecule includes any molecule, so long as it comprises the Fc region of an antibody. Examples include an antibody, an antibody fragment, a fusion protein comprising an Fc region, and the like.

The antibody is a protein which is produced in the living body by immune reaction as a result of exogenous antigen stimulation and has an activity to specifically bind to the antigen. Examples of the antibody include an antibody secreted by a hybridoma cell prepared from a spleen cell of an animal immunized with an antigen; an antibody prepared by a genetic recombination technique, namely an antibody obtained by introducing an antibody gene-inserted antibody expression vector into a host cell; and the like. Specific examples include an antibody produced by a hybridoma, a humanized antibody, a human antibody and the like.

A hybridoma is a cell which is obtained by cell fusion between a B cell obtained by immunizing a mammal other than human with an antigen and a myeloma cell derived

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from mouse or the like and can produce a monoclonal antibody having the desired antigen specificity.

Examples of the humanized antibody include a human chimeric antibody, a human CDR-grafted antibody and the like.

A human chimeric antibody is an antibody which comprises an antibody heavy chain variable region (hereinafter referred to as "HV" or "VH", the heavy chain being "H chain") and an antibody light chain variable region (hereinafter referred to as "LV" or "VL", the light chain being "L chain"), both of an animal other than human, a human antibody heavy chain constant region (hereinafter also referred to as "CH") and a human antibody light chain constant region (hereinafter also referred to as "CL"). As the animal other than human, any animal such as mouse, rat, hamster, rabbit or the like can be used, so long as a hybridoma can be prepared therefrom.

The human chimeric antibody can be produced by obtaining cDNA's 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 human chimeric antibody expression vector, and then introducing the vector into a host cell to express the antibody.

As the CH of human chimeric antibody, any CH can be used, so long as it belongs to human immunoglobulin (hereinafter referred to as "hIg") can be used. But those belonging to the hIgG class are preferable 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.

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A human CDR-grafted antibody is an antibody in which amino acid sequences of CDR's of VH and VL of an antibody derived from an animal other than human are grafted into appropriate positions of VH and VL of a human antibody.

The human CDR-grafted antibody can be produced by constructing cDNA's encoding V regions in which CDR's of VH and VL of an antibody derived from an animal other than human are grafted into CDR's of VH and VL of a 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 human CDR-grafted antibody expression vector, and then introducing the expression vector into a host cell to express the human CDR-grafted antibody.

As the CH of human CDR-grafted antibody, any CH can be used, so long as it belongs to the hIg, but those of the hIgG class are preferable 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.

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 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.

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

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and then cloning it, and the antibody can be purified from the culture.

The human antibody phage library is a library in which antibody fragments such as Fab, single chain antibody and the like 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, using its activity to bind to an antigen-immobilized substrate as the marker. 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.

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 animal 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 chimeric antibody gene into a fertilized egg and developing it, the transgenic animal can be also prepared. Regarding the preparation method of a human antibody from the human antibody-producing transgenic 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 mammals other than human and then culturing it.

Examples of the transgenic non-human animal include cattle, sheep, goat, pig, horse, mouse, rat, fowl, monkey, rabbit and the like.

Also, in the present invention, it is preferable that the antibody is an antibody which recognizes a tumor-related antigen, an antibody which recognizes an allergy- or inflammation-related antigen, an antibody which

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recognizes circulatory organ disease-related antigen, an antibody which recognizes an autoimmune disease-related antigen or an antibody which recognizes a viral or bacterial infection-related antigen, and a human antibody which belongs to the IgG class is preferable.

An antibody fragment is a fragment which comprises the Fc region of an antibody. Examples of the antibody fragment include an H chain monomer, an H chain dimer and the like.

A fusion protein comprising an Fc region is a composition in which an antibody comprising the Fc region of an antibody or the antibody fragment is fused with a protein such as an enzyme, a cytokine or the like.

In the present invention, examples of the sugar chain which binds to the Fc region of an antibody molecule includes an *N*-glycoside-linked sugar chain. Examples of the *N*-glycoside-linked sugar chain include a complex type in which the non-reducing end side of the core structure has one or plural parallel branches of galactose-*N*-acetylglucosamine (hereinafter referred to as "Gal-GlcNAc") and the non-reducing end side of Gal-GlcNAc has a structure such as sialic acid, bisecting *N*-acetylglucosamine or the like.

In one antibody, the Fc region has positions to which an *N*-glycoside-linked sugar chain is bound which will be described later. Accordingly, two sugar chains are bound per one antibody molecule. Since the *N*-glycoside-linked sugar chain which binds to an antibody includes any sugar chain having the core structure represented by the structural formula (I), a number of combinations of sugar chains may possible for the two *N*-glycoside-linked sugar chains which bind to the antibody. Accordingly, identity of substances can be judged from the viewpoint of the sugar structure bound to the Fc region.

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In the present invention, the composition which comprises an antibody molecule having complex *N*-glycoside-linked sugar chains in the Fc region (hereinafter referred to as "the antibody composition of the present invention") may comprise an antibody having the same sugar chain structure or an antibody having different sugar chain structures, so long as the effect of the present invention is obtained from the composition.

In the present invention, 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.

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 sugar chain in which the fucose is not bound to *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain. Examples include a complex *N*-glycoside-linked sugar chain in which 1-position of fucose is not bound to 6-position of *N*-acetylglucosamine through α-bond.

The antibody composition shows high ADCC activity when 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 binding to the Fc region contained in the antibody composition of the present invention is preferably 20% or more, more preferably 25% or more, still more preferably 30% or more, far preferably 40% or more, and most preferably 50% or more. As the antibody concentration

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is decreased, the ADCC activity is decreased, but high ADCC activity can be obtained even when the antibody concentration is low, so long as 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.

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 also be determined by analyzing it with the HPAED-PAD method [*J. Liq. Chromatogr.*, 6, 1557 (1983)].

In the present invention, the Chinese hamster ovary tissue-derived CHO cell includes any cell which is a cell line established from an ovary tissue of Chinese hamster (*Cricetulus griseus*). Examples include CHO cells described in documents 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.*, 60, 1275 (1968); *Cell*, 6, 121 (1975); *Molecular Cell Genetics*, Appendix I, II (pp. 883-900); and the like. In addition, CHO-K1 (ATCC CCL-61), DUXB11 (ATCC CCL-9096) and Pro-5 (ATCC CCL-1781) registered in ATCC (The American Type Culture Collection) and a commercially available CHO-S (Life Technologies, Cat #

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11619) or sub-cell lines obtained by adapting the cell lines using various media can also be exemplified.

In the present invention, the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose 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. The enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose is an enzyme which has influence on the synthesis of the intracellular sugar nucleotide, GDP-fucose.

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 enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose.

Examples of the enzyme relating to the *de novo* synthesis pathway of the intracellular sugar nucleotide, GDP-fucose include GDP-mannose 4,6-dehydratase (hereinafter referred to as "GMD"), GDP-keto-6-deoxymannose 3,5-epimerase 4,6-reductase (hereinafter referred to as "Fx") and the like.

Examples of the enzyme relating to the salvage synthesis pathway of the intracellular sugar nucleotide, GDP-fucose include GDP-beta-L-fucose pyrophosphorylase (hereinafter referred to as "GFPP"), fucokinase and the like.

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 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.

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In the present invention, examples of the GMD include:

a protein encoded by a DNA of the following (a) or (b):

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

(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,

(c) a protein comprising the amino acid sequence represented by SEQ ID NO:71,

(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,

(e) a protein which comprises an amino acid sequence having a homology of at least 80% with the amino acid sequence represented by SEQ ID NO:71 and has GMD activity, and the like.

Also, examples of the DNA encoding the amino acid sequence of GMD include a DNA comprising the nucleotide sequence represented by SEQ ID NO:65 and a DNA which hybridizes with the DNA comprising the nucleotide sequence represented by SEQ ID NO:65 under stringent conditions and encodes an amino acid sequence having GMD activity.

In the present invention, examples of the Fx include:

a protein encoded by a DNA of the following (a) or (b):

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

(b) a DNA which hybridizes with the DNA comprising the nucleotide sequence represented by SEQ ID NO:48 under

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stringent conditions and encodes a protein having Fx activity,

(c) a protein comprising the amino acid sequence represented by SEQ ID NO:72,

(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:72 and has Fx activity,

(e) a protein which comprises an amino acid sequence having a homology of at least 80% with the amino acid sequence represented by SEQ ID NO:72 and has Fx activity, and the like.

Also, examples of the DNA encoding the amino acid sequence of Fx include a DNA comprising the nucleotide sequence represented by SEQ ID NO:48 and a DNA which hybridizes with the DNA comprising the nucleotide sequence represented by SEQ ID NO:48 under stringent conditions and encodes an amino acid sequence having Fx activity.

In the present invention, examples of the GFPP include:

a protein encoded by a DNA of the following (a) or (b):

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

(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,

(c) a protein comprising the amino acid sequence represented by SEQ ID NO:73,

(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:73 and has GFPP activity,

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(e) a protein which comprises an amino acid sequence having a homology of at least 80% with the amino acid sequence represented by SEQ ID NO:73 and has GFPP activity, and the like.

Also, examples of the DNA encoding the amino acid sequence of GFPP include a DNA comprising the nucleotide sequence represented by SEQ ID NO:51 and a DNA which hybridizes with the DNA comprising the nucleotide sequence represented by SEQ ID NO:51 under stringent conditions and encodes an amino acid sequence having Fx activity.

In the present invention, the enzyme relating to the 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 the complex *N*-glycoside-linked sugar chain 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.

Examples of 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 include α-1,6-fucosyltransferase, α-L-fucosidase and the like.

Also, examples include an enzyme which has influence on the activity 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 and an enzyme which has influence on the structure of substances as the substrate of the enzyme.

In the present invention, examples of the α-1,6-fucosyltransferase include:

a protein encoded by a DNA of the following (a), (b), (c) or (d):

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

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

(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;

(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;

(e) a protein comprising the amino acid sequence represented by SEQ ID NO:23,

(f) a protein comprising the amino acid sequence represented by SEQ ID NO:24,

(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,

(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,

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

(j) a protein which comprises an amino acid sequence having a homology of at least 80% with the amino acid sequence represented by SEQ ID NO:24 and has α-1,6-fucosyltransferase activity, and the like.

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Also, examples of the DNA encoding the amino acid sequence of α-1,6-fucosyltransferase include a DNA having the nucleotide sequence represented by SEQ ID NO:1 or 2 and a DNA which hybridizes with the DNA having the nucleotide sequence represented by SEQ ID NO:1 or 2 under stringent conditions and encodes an amino acid sequence having α-1,6-fucosyltransferase activity.

In the present invention, 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 or 65 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 M sodium chloride using a filter to which colony- or plaque-derived DNA fragments are immobilized, and then washing the filter at 65°C using 0.1 to 2 × SSC solution (composition of the 1 × SSC solution comprising 150 mM sodium chloride and 15 mM 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. Examples of the hybridizable DNA include 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 or 65.

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In the present invention, 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:23, 24, 71, 72 or 73 and has α-1,6-fucosyltransferase activity, GMD activity, Fx activity or GFPP 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, 65, 48 or 51, 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 is one or more, 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.

Also, in order to maintain the α-1,6-fucosyltransferase activity, GMD activity, Fx activity or GFPP 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:23, 24, 71, 72 or 73, when calculated using an analyzing soft such as BLAST [*J. Mol. Biol.*, 215, 403 (1990)], FASTA [*Methods in Enzymology*, 183, 63 (1990)] or the like.

Examples of the CHO cell of the present invention include a cell in which the enzyme activity is decreased or deleted.

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The cell in which the enzyme activity is decreased or deleted include cells in which the activity of an enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the activity of an enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain is decreased or deleted. As the method for obtaining such cells, any technique can be used, so long as it can reduce or delete the enzyme activity of interest. Examples of the technique for reducing or deleting the enzyme activity include:

(a) a gene disruption technique targeting a gene encoding the enzyme,

(b) a technique for introducing a dominant negative mutant of a gene encoding the enzyme,

(c) a technique for introducing mutation into the enzyme,

(d) a technique for inhibiting transcription and/or translation of a gene encoding the enzyme,

(e) a technique for selecting a cell line 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 the complex *N*-glycoside-linked sugar chain, and the like.

Herein, the lectin-resistant cell line can be obtained by culturing a cell line in a medium comprising a predetermined concentration of lectin and then by selecting a cell line which acquires such a property that its survival rate is increased at least 2 times, preferably 3 times, and more preferably 5 times or more, than the parent cell line with statistical significance. Also, it can also be obtained by culturing a cell line in a medium comprising lectin and then by selecting a cell line which can be cultured at a certain survival rate, e.g., 80% survival

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rate, at a lectin concentration of at least 2 times, preferably 5 times, more preferably 10 times, and most preferably 20 times or more, than the parent cell line.

As the 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, any lectin which can recognize the sugar chain structure can be used. 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.

The CHO cell of the present invention can produce an antibody composition having higher ADCC activity than that of an antibody composition produced by the parent CHO cell before applying the technique for decreasing or deleting the enzyme activity of interest.

Also, the CHO cell of the present invention can produce an antibody composition having higher ADCC activity than that of an antibody composition in which, among the total complex *N*-glycoside-linked sugar chains bound to the Fc region contained in the antibody composition, the ratio of a sugar chain in which fucose is not bound to *N*-acetylglucosamine in the reducing end in the sugar chain is less than 20%.

An example of the parent cell line to be used in the present invention is a cell in which the activity of an enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the activity of an enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain is not decreased.

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Specifically, a cell which is not treated to decrease or delete the activity of an enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the activity of an enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain is used.

In the present invention, the ADCC activity is a cytotoxic activity in which an antibody bound to a cell surface antigen on a tumor cell in the living body activate an effector cell through an Fc receptor existing on the antibody Fc region and effector cell surface and thereby obstruct the tumor cell and the like [*Monoclonal Antibodies: Principles and Applications*, Wiley-Liss, Inc., Chapter 2.1 (1955)]. Examples of the effector cell include a killer cell, a natural killer cell, an activated macrophage and the like.

The present invention also relates to a cell in which the activity of an enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the activity of an enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain is decreased by a genetic engineering technique (hereinafter referred to as "the host cell of the present invention"). The host cell of the present invention is useful as a host cell for producing an antibody composition having high ADCC activity.

The host cell of the present invention may be any host, so long as it can express an antibody molecule. Examples include a yeast cell, an animal cell, an insect cell, a plant cell and the like. Examples of the cells include those which will be later in the item 3. Among

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animal cells, preferred examples include a CHO cell derived from a Chinese hamster ovary tissue, a rat myeloma cell line YB2/3HL.P2.G11.16Ag.20 cell, a mouse myeloma cell line NSO cell, a mouse myeloma SP2/0-Ag14 cell, a BHK cell derived from a syrian hamster kidney tissue, an antibody producing-hybridoma cell, a human leukemia cell line Namalwa cell, an embryonic stem cell, a fertilized egg cell and the like.

The present invention is described below in detail.

1. Preparation of the host cell of the present invention

The host cell of the present invention can be prepared by the following techniques.

(1) Gene disruption technique targeting at a gene encoding an enzyme

The host cell of the present invention can be prepared using a gene disruption technique by targeting at an enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or an enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain. Examples of the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose include GMD, Fx, GFPP, fucokinase and the like. Examples of the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain include α-1,6-fucosyltransferase, α-L-fucosidase and the like.

The gene as used herein includes DNA and RNA.

The gene disruption method may be any method, so long as it can disrupt the gene of the target enzyme is included. Examples include an antisense method, a ribozyme

method, a homologous recombination method, an RDO method, an RNAi method, a retrovirus-employed method, a transposon-employed method and the like. The methods are specifically described below.

(a) Preparation of the host cell of the present invention by the antisense method or the ribozyme method

The host cell of the present invention can be prepared by the 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 by targeting at an enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or an enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain.

A cDNA or a genome DNA encoding an enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or an enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain is prepared.

The nucleotide sequence of the prepared cDNA or genome DNA is determined.

Based on the determined DNA sequence, an appropriate length of an antisense gene or ribozyme construct comprising a DNA moiety which encodes the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-

linked sugar chain a part of its non-translation region or an intron, is designed.

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.

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

The host cell of the present invention can be obtained by selecting a transformant using, as a marker, the activity of the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain. The host cell of the present invention can also be obtained by selecting a transformant as a measure of the sugar chain structure of a glycoprotein on the cell membrane or the sugar chain structure of the produced antibody molecule.

As the host cell used for the production of the host cell of the present invention, any cell such as yeast, animal cell, insect cell or plant cell can be used, so long as it has a gene encoding the target enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the target enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain. Examples include host cells which will be described later in the item 3.

As the expression vector, a vector which is autonomously replicable in the host cell or can be

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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 which will be described later in the item 3.

Regarding the method for introducing a gene into various host cells, the methods for introducing recombinant vectors suitable for various host cells, which will be described later in the item 3, can be used.

The following method can be exemplified as the method for selecting a transformant as a measure of the activity of an enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the activity of an enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain.

Method for selecting transformant:

Examples of the method for selecting a cell in which the activity of an enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the activity of an enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain is decreased include biochemical methods or genetic engineering techniques described in *New Biochemical Experimentation Series 3-Saccharides I, Glycoprotein* (Tokyo Kagaku Dojin), edited by Japanese Biochemical society (1988); *Cell Engineering, 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;

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Current Protocols in Molecular Biology; and the like. Examples of the biochemical method include a method in which the enzyme activity is evaluated using an enzyme-specific substrate and the like. Examples of 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.

Examples of the method for selecting a transformant using the sugar chain structure of a glycoprotein on the cell membrane as a marker include the methods which will be described later in the item 1(5). Examples of the method for selecting a transformant using the sugar chain structure of a produced antibody molecule as a marker include the methods which will be described later in the items 5 and 6.

As the method for preparing cDNA encoding an enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or an enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain, the following method is exemplified.

Preparation of DNA:

A total RNA or mRNA is prepared from a human or non-human animal tissue or cell.

A cDNA library is prepared from the prepared total RNA or mRNA.

Degenerative primers are produced based on the amino acid sequence of an enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or an enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain, and a gene fragment

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encoding the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain is obtained by PCR using the prepared cDNA library as the template.

A DNA encoding the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain can be obtained by screening the cDNA library using the obtained gene fragment as a probe.

Regarding the mRNA of a human or non-human tissue or cell, a commercially available product (e.g., manufactured by Clontech) may be used or it may be prepared from a human or non-human animal tissue or cell in the following manner. Examples of the method for preparing a total RNA from a human or non-human animal tissue or cell include 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*, 9, 1937 (1991)] and the like.

Also, examples of the method for preparing mRNA from a total RNA as poly(A)$^+$ RNA include an oligo(dT)-immobilized cellulose column method (*Molecular Cloning*, Second Edition) and the like.

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

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A cDNA library is prepared from the prepared mRNA of a human or non-human animal tissue or cell. Examples of the method for preparing cDNA libraries include the methods described in *Molecular Cloning*, Second Edition; *Current Protocols in Molecular Biology*; *A Laboratory Manual*, Second Edition (1989); and the like, or methods using commercially available kits such as SuperScript Plasmid System for cDNA Synthesis and Plasmid Cloning (manufactured by Life Technologies), ZAP-cDNA Synthesis Kit (manufactured by STRATAGENE) and the like.

As the cloning vector for use in the preparation of the cDNA library, any vector such as a phage vector, a plasmid vector or the like 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.

Any microorganism can be used as the host microorganism, but *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

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

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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 Protein*, 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.

Degenerative primers specific for the 5'-terminal and 3'-terminal nucleotide sequences of a nucleotide sequence presumed to encode the amino acid sequence are prepared based on the amino acid sequence of the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain, 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 enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain.

It can be confirmed that the obtained gene fragment is a DNA encoding the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain, by a method usually used for analyzing a nucleotide, such as the dideoxy method of Sanger *et al.* [*Proc. Natl. Acad. Sci. USA*, 74, 5463 (1977)], a nucleotide sequence analyzer such as ABIPRISM

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377 DNA Sequencer (manufactured by PE Biosystems) or the like.

A DNA encoding the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain 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.

Also, a DNA encoding the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain can also be obtained by carrying out screening by PCR using the cDNA or cDNA library synthesized from the mRNA contained in a human or non-human animal tissue or cell as the template and using the primers used for obtaining the gene fragment encoding the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain.

The nucleotide sequence of the obtained DNA encoding the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in

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the complex *N*-glycoside-linked sugar chain is analyzed from its terminus and determined by a method usually used for analyzing a nucleotide, such as the dideoxy method of Sanger *et al.* [*Proc. Natl. Acad. Sci. USA*, 74, 5463 (1977)], a nucleotide sequence analyzer such as ABIPRISM 377 DNA Sequencer (manufactured by PE Biosystems) or the like.

A gene encoding the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain can also be determined from genes in data bases by searching nucleotide sequence data bases such as GenBank, EMBL, DDBJ and the like using a homology retrieving program such as BLAST based on the determined cDNA nucleotide sequence.

Examples of the nucleotide sequence of the gene obtained by the method encoding the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose include the nucleotide sequence represented by SEQ ID NO:48, 51 or 65. Examples of the nucleotide sequence of the gene encoding the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain include the nucleotide sequence represented by SEQ ID NO:1 or 2.

The cDNA encoding the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain can also be obtained by chemically synthesizing it with a DNA synthesizer such as DNA Synthesizer model 392 manufactured by Perkin Elmer

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or the like using the phosphoamidite method, based on the determined DNA nucleotide sequence.

As an example of the method for preparing a genome DNA encoding the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain, the method described below is exemplified.

Preparation of genome DNA:

Examples of the method for preparing genome DNA include known methods described in *Molecular Cloning*, Second Edition; *Current Protocols in Molecular Biology*; and the like. In addition, a genome DNA encoding the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain can also be isolated using a kit such as Genome DNA Library Screening System (manufactured by Genome Systems), Universal GenomeWalker™ Kits (manufactured by CLONTECH) or the like.

Examples of the nucleotide sequence of the genome DNA obtained by the method encoding the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose include the nucleotide sequence represented by SEQ ID NO:67 or 70. Examples of the nucleotide sequence of the genome DNA encoding the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar

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chain include the nucleotide sequence represented by SEQ ID NO:3.

In addition, the host cell of the present invention 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 enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain.

The antisense oligonucleotide or ribozyme can be prepared in the usual method or 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 10 to 40 bases, among nucleotide sequences of a cDNA and a genome DNA encoding the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain, by synthesizing an oligonucleotide which corresponds to a sequence complementary to the oligonucleotide (antisense oligonucleotide) or a ribozyme comprising the oligonucleotide sequence.

Examples of the oligonucleotide include oligo RNA and derivatives of the oligonucleotide (hereinafter referred to as "oligonucleotide derivatives").

Examples of the oligonucleotide derivatives include oligonucleotide derivatives in which a phosphodiester bond in the oligonucleotide is converted into a phosphorothioate bond, an oligonucleotide derivative in which a

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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*, 16, 1463 (1997)].

(b) Preparation of the host cell of the present invention by homologous recombination

The host cell of the present invention can be produced by modifying a target gene on chromosome through a homologous recombiniation technique, using a gene encoding an enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or an enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain as the target gene.

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); *Biomanual Series 8, Gene Targeting, Preparation of Mutant Mice using ES Cells*, Yodo-sha (1995) (hereinafter referred to as "*Preparation of Mutant Mice using ES Cells*"); or the like, for example, as follows.

A genome DNA encoding an enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or an enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain is prepared.

Based on the nucleotide sequence of the genome DNA, a target vector is prepared for homologous recombination of a target gene to be modified (e.g., structural gene of the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain, or a promoter gene).

The host cell 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.

As the host cell, any cell such as yeast, animal cell, insect cell or plant cell can be used, so long as it has a gene encoding the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain. Examples include the host cells which will be described later in the item 3.

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Examples of the method for preparing a genome DNA encoding the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain include the methods described in the preparation of genome DNA in the item 1(1)(a) and the like.

Examples of the nucleotide sequence of genome DNA encoding the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose include the nucleotide sequence represented by SEQ ID NO:67 or 70. Examples of the nucleotide sequence of genome DNA encoding the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain include the nucleotide sequence represented by SEQ ID NO:3.

The target vector for use in 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); *Biomanual Series 8, Gene Targeting, Preparation of Mutant Mice using ES Cells*, Yodo-sha (1995); or the like. The target vector can be used as either a replacement type or an insertion type.

For introducing the target vector into various host cells, the methods for introducing recombinant vectors suited for various host cells, which will be described later in the item 3, can be used.

Examples of the method for efficiently selecting a homologous recombinant include a method such as the positive selection, promoter selection, negative selection or polyA selection described in *Gene Targeting, A Practical*

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Approach, IRL Press at Oxford University Press (1993); *Biomanual Series 8, Gene Targeting, Preparation of Mutant Mice using ES Cells*, Yodo-sha (1995); or the like. Examples of the method for selecting the homologous recombinant of interest from the selected cell lines include the Southern hybridization method for genome DNA (*Molecular Cloning*, Second Edition), PCR [*PCR Protocols*, Academic Press (1990)], and the like.

(c) Preparation of the host cell of the present invention by RDO method

The host cell of the present invention can be prepared by an RDO (RNA-DNA oligonucleotide) method by targeting at a gene encoding an enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or an enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain, for example, as follows

A cDNA or a genome DNA encoding an enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or an enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain is prepared.

The nucleotide sequence of the prepared cDNA or genome DNA is determined.

Based on the determined DNA sequence, an appropriate length of an RDO construct comprising a DNA moiety which encodes the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in

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the complex *N*-glycoside-linked sugar chain or a part of its non-translation region or an intron, is designed and synthesized.

The host cell 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, namely the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain.

As the host cell, any cell such as yeast, animal cell, insect cell or plant cell can be used, so long as it has a gene encoding the target enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or of the target enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain. Examples include the host cells which will be described later in the item 3.

Examples of the method for introducing RDO into various host cells include the methods for introducing recombinant vectors suited for various host cells, which will be described later in the item 3.

Examples of the method for preparing cDNA encoding the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain include the methods described in the preparation of DNA in the item 1(1)(a) and the like.

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Examples of the method for preparing a genome DNA encoding the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain include the methods in preparation of genome DNA described in the item 1(1)(a) and the like.

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) or the like, subjecting the clones to the reaction generally used as a method for analyzing a nucleotide sequence such as the dideoxy method [*Proc. Natl. Acad. Sci. USA*, 74, 5463 (1977)] of Sanger *et al.* or the like, and then analyzing the clones using an automatic nucleotide sequence analyzer such as A.L.F. DNA Sequencer (manufactured by Pharmacia) or the like.

The RDO can be prepared by a usual method or using a DNA synthesizer.

Examples of the method for selecting a cell in which a mutation occurred, by introducing the ROD into the host cell, in the gene encoding the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain include the methods for directly detecting mutations in chromosomal genes described in *Molecular Cloning*, Second Edition, *Current Protocols in Molecular Biology* and the like;

the methods described in the item 1(1)(a) for selecting a transformant through the evaluation of the

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activity of the introduced enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain; the method for selecting a transformant using the sugar structure of a glycoprotein on the cell membrane which will be described later in the item 1(5); and the method for selecting a transformant as a measure of the sugar structure of the produced antibody molecule which will be described later in the item 5 or 6, and the like.

The construct of the ROD 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. Dematol.*, 111, 1172 (1998); *Nature Biotech.*, 16, 1343 (1998); *Nature Biotech.*, 18, 43 (2000); *Nature Biotech.*, 18, 555 (2000); and the like.

(d) Preparation of the host cell of the present invention by RNAi method

The host cell of the present invention can be prepared by the RNAi (RNA interference) method by targeting at a gene of an enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or of an enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain, for example, as follows.

A cDNA encoding an enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or an

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enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain is prepared.

The nucleotide sequence of the prepared cDNA is determined.

Based on the determined DNA sequence, an appropriate length of an RNAi gene construct comprising the DNA coding moiety encoding the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain or a part of its non-translation region, is designed.

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.

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

The host cell of the present invention can be obtained by selecting a transformant as a measure of the activity of the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain, or the sugar chain structure of a glycoprotein on the cell membrane or of the produced antibody molecule.

As the host cell, any cell such as yeast, animal cell, insect cell or plant cell can be used, so long as it has a gene encoding the target enzyme relating to the

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synthesis of an intracellular sugar nucleotide, GDP-fucose or the target enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain. Examples include the host cells which will be described later in the item 3.

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 RNAi gene can be transferred is used. Examples include the expression vectors which will be described later in the item 3.

As the method for introducing a gene into various host cells, the methods for introducing recombinant vectors suitable for various host cells, which will be described later in the item 3, can be used.

Examples of the method for selecting a transformant as a measure of the activity of the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain include the methods described in the item 1(1)(a).

Examples of the method for selecting a transformant as a measure of the sugar chain structure of a glycoprotein on the cell membrane include the methods which will be described later in the item 1(5). Examples of the method for selecting a transformant as a measure of the sugar chain structure of a produced antibody molecule include the methods which will be described later in the item 5 or 6.

Examples of the method for preparing cDNA encoding the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the enzyme relating to the

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modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain include the methods described in preparation of DNA in the item 1(1)(a) and the like.

In addition, the host cell 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 encoding the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain.

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

The RNAi gene construct can be designed in accordance with the methods described in *Nature*, 391, 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.

(e) Preparation of the host cell of the present invention by a method using transposon

The host cell of the present invention can be prepared by inducing mutation using a transposon system described in *Nature Genet.*, 25, 35 (2000) or the like, and then by selecting a mutant as a measure of the activity of the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-

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linked sugar chain, or the sugar chain structure of a glycoprotein of a produced antibody molecule or on the cell membrane.

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.

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

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

As the host cell, any cell such as yeast, animal cell, insect cell or plant cell can be used, so long as it has a gene encoding the target enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or of the target enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain. Examples include the host cells which will be described later in the item 3. For introducing the gene into various host cells, the method for introducing recombinant vectors suitable for various host cells, which will be described later in the item 3, can be used.

Examples of the method for selecting a mutant as a measure of the activity of the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain include the methods described in the item 1(1)(a).

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Examples of the method for selecting a mutant as a measure of the sugar chain structure of a glycoprotein on the cell membrane include the methods which will be described later in the item 1(5). Examples of the method for selecting a mutant as a measure of the sugar chain structure of a produced antibody molecule include the methods which will be described later in the item 5 or 6.

(2) Method for introducing a dominant negative mutant of a gene encoding an enzyme

The host cell of the present invention can be prepared by targeting a gene encoding an enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or an enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain, using a technique for introducing a dominant negative mutant of the enzyme. Examples of the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose include GMD, Fx, GFPP, fucokinase and the like. Examples of the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain include α-1,6-fucosyltransferase, α-L-fucosidase and the like.

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 catalyze 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.

As a result of the analysis of the three-dimensional structure of *E. coli*-derived GMD, it has been

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revealed 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. For example, in GMD (SEQ ID NO:65) derived from CHO cell, a dominant negative mutant can be prepared by substituting threonine position 155, glutamic acid at position 157, tyrosine at position 179 and lysine at position 183 with other amino acids, 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. 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.

The host cell of the present invention can be prepared in accordance with the method described in *Molecular Cloning*, Second Edition, *Current Protocols in Molecular Biology* or the like, using the prepared dominant negative mutant gene of the target enzyme, for example, as follows.

A gene encoding a dominant negative mutant (hereinafter referred to as "dominant negative mutant gene") of the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the enzyme relating to the modification of a sugar chain wherein 1-

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position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain is prepared.

Based on the prepared full length DNA of dominant negative mutant gene, a DNA fragment of an appropriate length containing a moiety encoding the protein is prepared, if necessary.

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

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

The host cell of the present invention can be prepared by selecting a transformant as a measure of the activity of the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the activity of the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain, or the sugar chain structure of a glycoprotein of a produced antibody molecule or on the cell membrane.

As the host cell, any cell such as yeast, animal cell, insect cell or plant cell can be used, so long as it has a gene encoding the target enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or of the target enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain. Examples include the host cells which will be described later in the item 3.

As the expression vector, a vector which is autonomously replicable in the host cell or can be

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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 which will be described later in the item 3.

For introducing the gene into various host cells, the method for introducing recombinant vectors suitable for various host cells, which will be described later in the item 3, can be used.

Examples of the method for selecting a transformant as a measure of the activity of the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain include the methods described in the item 1(1)(a).

Examples of the method for selecting a transformant as a measure of the sugar chain structure of a glycoprotein on the cell membrane include the methods which will be described later in the item 1(5). Examples of the method for selecting a transformant as a measure of the sugar chain structure of a produced antibody molecule include the methods which will be described later in the item 5 or 6.

(3) Method for introducing a mutation into an enzyme

The host cell of the present invention can be prepared by introducing a mutation into a gene encoding an enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or an enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain, and then by selecting a cell line of interest in which the mutation occurred in the enzyme.

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Examples of the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose include GMD, Fx, GFPP, fucokinase and the like. Examples of the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain include α-1,6-fucosyltransferase, α-L-fucosidase and the like.

Examples of the method include 1) a method in which a desired cell line is selected from mutants obtained by a mutation-inducing treatment of a parent cell line with a mutagen or spontaneously generated mutants, as a measure of the activity of an enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the activity of an enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain, 2) a method in which a desired cell line is selected from mutants obtained by a mutation-inducing treatment of a parent cell line with a mutagen or spontaneously generated mutants, as a measure of the sugar chain structure of a produced antibody molecule and 3) a method in which a desired cell line is selected from mutants obtained by a mutation-inducing treatment of a parent cell line with a mutagen or spontaneously generated mutants, as a measure of the sugar chain structure of a glycoprotein on the cell membrane.

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.

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. Examples of the

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method for allowing a mutagen to act upon cells include 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.

Examples of the spontaneously generated mutant include mutants which are spontaneously formed by continuing subculture under general cell culture conditions without applying special mutation-inducing treatment.

Examples of the method for measuring the activity of the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the activity of the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain include the methods described in the item 1(1)(a). Examples of the method for discriminating the sugar chain structure of a prepared antibody molecule include the methods which will be described later in the item 5 or 6. Examples of the method for discriminating the sugar chain structure of a glycoprotein on the cell membrane include the methods which will be described later in the item 1(5).

(4) Method for inhibiting transcription and/or translation of a gene encoding an enzyme

The host cell of the present invention can be prepared by inhibiting transcription and/or translation of a target gene through a method such as 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, using a gene

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encoding an enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose and/or an enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain, as the target.

Examples of the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose include GMD, Fx, GFPP, fucokinase and the like. Examples of the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain include α-1,6-fucosyltransferase, α-L-fucosidase and the like.

(5) Method for selecting a cell line 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

The host cell of the present invention can be prepared by using a method for selecting a cell line 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.

Examples of the method for selecting a cell line 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 include the methods using lectin described in *Somatic Cell Mol. Genet.*, 12, 51 (1986) and the like. 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

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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.

Specifically, the cell line 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 *N*-glycoside-linked sugar chain can be selected by culturing cells for 1 day to 2 weeks, preferably from 1 day to 1 week, using a medium comprising the lectin at a concentration of 1 μg/ml to 1 mg/ml, 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. Examples of the cell line obtained by the method include CHO/CCR4-LCA Nega-13 (FERM BP-7756) obtained in Example 14(2) which will be described later.

2. Preparation of a transgenic non-human animal or plant or the progenies thereof of the present invention

The transgenic non-human animal or plant or the progenies thereof of the present invention is a transgenic non-human animal or plant or the progenies thereof in which a genome gene is modified in such a manner that the activity of an enzyme relating to the modification of a sugar chain of an antibody molecule can be controlled, and it can be prepared according to the method similar to that in the item 1, using a gene encoding an enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or an enzyme relating to the modification of a sugar

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chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain, as the target.

In a transgenic non-human animal, the embryonic stem cell of the present invention in which the activity of the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the activity of the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain is controlled 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, rabbit or the like.

Specifically, a mutant clone is prepared in which a gene encoding the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain is inactivated or substituted with any sequence, by a known homologous recombination technique [e.g., *Nature*, 326, 6110, 295 (1987); *Cell*, 51, 3, 503 (1987); or the like]. 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 enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the activity of the enzyme relating to the modification of a sugar chain

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wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain is decreased or deleted in the whole body cells can be obtained.

Also, a fertilized egg cell of the present invention in which the activity of an enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the activity of an enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain 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.

A transgenic non-human animal in which the activity of an enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the activity of an enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain is decreased 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.

In a transgenic plant, the callus of the present invention in which the activity of an enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the activity of an enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-

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linked 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.

A transgenic plant in which the activity of an enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or the activity of an enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain is decreased can be prepared by culturing the prepared callus using a medium comprising auxin and cytokinin to redifferentite it in accordance with a known method [*Tissue Culture*, 20 (1994); *Tissue Culture*, 21 (1995); *Trends in Biotechnology*, 15, 45 (1997)].

3. Method for producing an antibody composition

The antibody composition can be obtained by expressing it in a host cell using the methods described in *Molecular Cloning*, Second Edition; *Current Protocols in Molecular Biology*; *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988 (hereinafter referred also to as "*Antibodies*"); *Monoclonal Antibodies: Principles and Practice*, Third Edition, Acad. Press, 1993 (hereinafter referred also to as "*Monoclonal Antibodies*"); and *Antibody Engineering, A Practical Approach*, IRL Press at Oxford University Press (hereinafter referred also to as "*Antibody Engineering*"), for example, as follows.

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

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.

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

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

A cell such as yeast, animal cell, insect cell, 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.

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.

The cDNA can be prepared from a human or non-human tissue or cell using, e.g., a probe primer specific for the antibody molecule of interest, in accordance with the methods described in the preparation of DNA in the item 1(1)(a).

When a yeast is used as the host cell, examples of the expression vector include YEP13 (ATCC 37115), YEp24 (ATCC 37051), YCp50 (ATCC 37419) and the like.

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, *etc.*, PH05 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.

Examples of the host cell include microorganisms belonging to the genus *Saccharomyces*, the genus *Schizosaccharomyces*, the genus *Kluyveromyces*, the genus

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Trichosporon, the genus *Schwanniomyces* and the like, such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Trichosporon pullulans* and *Schwanniomyces alluvius*, *etc.*

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.

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

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 SRα promoter and the like. Also, an enhancer of the IE gene of human CMV may be used together with the promoter.

Examples of the host cell include a human cell such as Namalwa cell, a monkey cell such as COS cell, a Chinese humster cell such as CHO cell or HBT5637 (Japanese Published Examined 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.

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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 Examined 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.

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.

That is, the protein can be expressed by simultaneously 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.

Examples of the gene introducing vector used in the method include pVL1392, pVL1393, pBlueBacIII (all manufactured by Invitrogen) and the like.

Examples of the baculovirus include *Autographa californica* nuclear polyhedrosis virus which is infected with an insect of the family *Barathra*.

Examples of the insect cell include *Spodoptera frugiperda* oocytes Sf9 and Sf21 [*Current Protocols in Molecular Biology, Baculovirus Expression Vectors, A Laboratory Manual*, W.H. Freeman and Company, New York

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(1992)], a *Trichoplusia ni* oocyte High 5 (manufactured by Invitrogen) and the like.

Examples of the method for the simultaneously introducing the recombinant gene-introducing vector and the baculovirus for preparing the recombinant virus include the calcium phosphate method (Japanese Published Examined Patent Application No. 227075/90), the lipofection method [*Proc. Natl. Acad. Sci. USA*, 84, 7413 (1987)] and the like.

When a plant cell is used as the host, examples of the expression vector include Ti plasmid, tobacco mosaic virus and the like.

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.

Examples of the host cell include plant cells of tobacco, potato, tomato, carrot, soybean, rape, alfalfa, rice, wheat, barley, *etc.*, and the like.

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 Examined Patent Application No. 140885/84, Japanese Published Examined Patent Application No. 70080/85, WO 94/00977), electroporation (Japanese Published Examined Patent Application No. 251887/85), a method using a particle gun (gene gun) (Japanese Patent No. 2606856, Japanese Patent No. 2517813) and the like.

As the method for expressing a 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.

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When a gene is expressed by a bacterium, a 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.

An antibody composition can be obtained 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 using a medium can be carried out in accordance with a general method which is used for the culturing of host cells.

As the medium for culturing a transformant obtained using a prokaryote such as *Escherichia coli etc.* or a eukaryote such as yeast *etc.* 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, an inorganic salt and the like which can be assimilated by the organism and culturing of the transformant can be efficiently carried out.

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

Examples of the nitrogen source include ammonia; ammonium salts of inorganic acid or organic acid such as ammonium chloride, ammonium sulfate, ammonium acetate, ammonium phosphate, *etc.*; 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.

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Examples of the inorganic material include potassium dihydrogen phosphate, dipotassium hydrogen phosphate, magnesium phosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, copper sulfate, calcium carbonate, and the like

The culturing is carried out generally under aerobic conditions such as a shaking culture, submerged-aeration stirring culture or the like. 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 using an inorganic or organic acid, an alkali solution, urea, calcium carbonate, ammonia or the like.

If necessary, an antibiotic such as ampicillin, tetracycline or the like may be added to the medium during the culturing.

When a microorganism transformed with a recombinant vector obtained using an inducible promoter as the promoter is cultured, an inducer may be added to the medium, if necessary. For example, when a microorganism transformed with a recombinant vector obtained using *lac* promoter is cultured, isopropyl-β-D-thiogalactopyranoside may be added to the medium, and when a microorganism transformed with a recombinant vector obtained using *trp* promoter is cultured, indoleacrylic acid may be added to the medium.

When a transformant obtained using an animal cell as the host cell is cultured, examples of the medium include 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

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by M. Katshuki (1987)], the media to which fetal calf serum, *etc.* is added, and the like.

The culturing is carried out generally at a pH of 6 to 8 and 30 to 40°C for 1 to 7 days in the presence of 5% CO_2. If necessary, an antibiotic such as kanamycin, penicillin or the like may be added to the medium during the culturing.

Examples of the medium for use in the culturing of a transformant obtained using an insect cell as the host include usually 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.

The culturing is carried out generally at a medium pH of 6 to 7 and 25 to 30°C for 1 to 5 days.

In addition, antibiotics such as gentamicin may be added to the medium during the culturing as occasion demands.

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

The culturing is carried out generally at a pH of 5 to 9 and 20 to 40°C for 3 to 60 days.

If necessary, an antibiotic such as kanamycin, hygromycin or the like may be added to the medium during the culturing.

Accordingly, an antibody composition can be produced by culturing a transformant derived from a microorganism, an animal cell or a plant cell, which comprises a recombinant vector into which a DNA encoding an

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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.

As the method for expressing the gene, 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.

Examples of the method for producing an antibody composition include 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.

When the antibody composition of the present invention 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 Examined Patent Application No. 336963/93 and Japanese Published Examined Patent Application No. 823021/94 and the like.

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 using a gene recombination technique, introducing the expression vector into the host cell and then expressing the antibody molecule.

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

In addition, the antibody composition can also be produced 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.

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.

Examples of the method for producing an antibody composition using an animal individual include 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, 627S (1996); *Bio/Technology*, 9, 830 (1991)].

In the case of an animal individual, an antibody composition can be produced 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. Examples of the place of the animal where the composition is produced and accumulated include milk (Japanese Published Examined 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

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as α casein promoter, β casein promoter, β lactoglobulin promoter, whey acidic protein promoter and the like.

Example of the method for producing an antibody composition using a plant individual include 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*, 20 (1994); *Tissue Culture*, 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.

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 using ultrasonic oscillator, French press, Manton Gaulin homogenizer, dynomill 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, by using a general enzyme isolation purification techniques such as solvent extraction; salting out; desalting with ammonium sulfate, *etc.*; precipitation with an organic solvent; anion exchange chromatography using a resin such as diethylaminoethyl (DEAE)-Sepharose, DIAION HPA-75 (manufactured by Mitsubishi Chemical), *etc.*; cation exchange chromatography using a resin such as S-Sepharose FF (manufactured by Pharmacia), *etc.*; hydrophobic chromatography using a resin such as butyl-Sepharose, phenyl-Sepharose, *etc.*; gel filtration using a molecular sieve; affinity chromatography; chromatofocusing; electrophoresis such as isoelectric focusing, *etc.*; and the like which may be used alone or in combination.

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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 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.

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 by a technique such as centrifugation or the like 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.

Examples of the thus obtained antibody composition include an antibody, the fragment of the antibody, a fusion protein comprising the Fc region of the antibody, and the like.

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

(1) Construction of vector for humanized antibody expression

A vector for humanized antibody expression is an expression vector for animal cell into which genes encoding the heavy chain (H chain) and light chain (L chain) C regions of a human antibody are inserted, which can be

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constructed by cloning each of genes encoding the H chain and L chain C regions of a human antibody into an expression vector for animal cell.

The C regions of a human antibody may be the H chain and L chain 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 "hCγ1"), the C region belonging to κ class in the L chain of a human antibody (hereinafter referred to as "hCκ"), and the like.

As the genes encoding the H chain and L chain C regions of a human antibody, a chromosomal DNA comprising an exon and an intron can be used or a cDNA can also be used.

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. Examples of the promoter and enhancer in the expression vector for animal cell include 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.

The humanized antibody expression vector 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 (tandem type). In respect of easiness of construction of a humanized antibody expression vector, easiness of introduction into animal cells, and balance between the expression amounts of the H and L chains of an antibody in animal cells, a tandem

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type of the humanized antibody expression vector is more preferred [*J. Immunol. Methods*, 167, 271 (1994)].

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

(2) Preparation of cDNA encoding V region of antibody derived from animal other than human

cDNAs encoding the H chain and L chain V regions of an antibody derived from an animal other than human, such as a mouse antibody, can be obtained in the following manner.

A cDNA is synthesized by extracting mRNA 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 the H chain V region and a recombinant phage or recombinant plasmid comprising a cDNA encoding the L chain V region is isolated from the library using a C region part or a V region part of an existing mouse antibody as the probe. Full nucleotide sequences of the H chain and L chain V regions of the mouse antibody of interest on the recombinant phage or recombinant plasmid are determined, and full amino acid sequences of the H chain and L chain V regions are deduced from the nucleotide sequences.

As the animal other than human, any animal such as mouse, rat, hamster, rabbit or the like can be used so long as a hybridoma cell can be produced therefrom.

Examples of the method for preparing total RNA from a hybridoma cell include the guanidine thiocyanate-cesium trifluoroacetate method [*Methods in Enzymology*, 154, 3 (1987)] and the like, and examples of the method for preparing mRNA from total RNA, an oligo(dT)-immobilized

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cellulose column method (*Molecular Cloning*, Second Edition) and the like. In addition, examples of a kit for preparing mRNA from a hybridoma cell include Fast Track mRNA Isolation Kit (manufactured by Invitrogen), Quick Prep mRNA Purification Kit (manufactured by Pharmacia) and the like.

Examples of the method for synthesizing cDNA and preparing a cDNA library include 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.

In preparing the cDNA library, the vector into which a cDNA synthesized 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)], λzapII (manufactured by Stratagene), λgt10 and λgt11 [*DNA Cloning, A Practical Approach*, I, 49 (1985)], Lambda BlueMid (manufactured by Clontech), λExCell, pT7T3 18U (manufactured by Pharmacia), pcD2 [*Mol. Cell. Biol.*, 3, 280 (1983)], pUC18 [*Gene*, 33, 103 (1985)] and the like.

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.

As the method for selecting a cDNA clone encoding the H chain and L chain V regions of an antibody derived

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from an animal other than human 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 H chain and L chain V regions 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.

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) or the like, carrying out the reaction of a generally used nucleotide sequence analyzing method such as the dideoxy method [*Proc. Natl. Acad. Sci. USA*, 74, 5463 (1977)] of Sanger *et al.* or the like and then analyzing the clones using an automatic nucleotide sequence analyzer such as A.L.F. DNA Sequencer (manufactured by Pharmacia) or the like. Whether or not the obtained cDNAs are encoding the full amino acid sequences of the H chain and L chain V regions of the antibody containing a secretory signal sequence can be confirmed by deducing the full amino acid sequences of the H chain and L chain V regions from the determined nucleotide sequence and comparing them with the full amino acid sequences of the H chain and L chain V regions of known antibodies [*Sequences of Proteins of Immunological Interest*, US Dep. Health and Human Services (1991)].

(3) Analysis of amino acid sequence of V region of antibody derived from animal other than human

Regarding the full amino acid sequences of the H chain and L chain V regions of the antibody containing a

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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 comparing them with the full amino acid sequences of the H chain and L chain V regions of known antibodies [*Sequences of Proteins of Immunological Interest*, US Dep. Health and Human Services, (1991)]. In addition, the amino acid sequences of the H chain and L chain V regions of each CDR can also be found by comparing them with the amino acid sequences of the H chain and L chain V regions of known antibodies [*Sequences of Proteins of Immunological Interest*, US Dep. Health and Human Services, (1991)].

(4) Construction of human chimeric antibody expression vector

A human chimeric antibody expression vector can be constructed by cloning cDNAs encoding the H chain and L chain V regions of an antibody derived from an animal other than human into upstream of genes encoding the H chain and L chain C regions of a human antibody in the vector for humanized antibody expression constructed in the item 3(1). For example, a human chimeric antibody expression vector can be constructed by linking each of cDNAs encoding the H chain and L chain V regions of an antibody derived from an animal other than human to a synthetic DNA comprising nucleotide sequences at the 3'-terminals of the H chain and L chain V regions of an antibody derived from an animal other than human and nucleotide sequences at the 5'-terminals of the H chain and L chain C regions 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 the H chain and L chain C regions of a human antibody contained in the vector for humanized antibody expression constructed described in the item 3(1).

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(5) Construction of cDNA encoding V region of human CDR-grafted antibody

cDNAs encoding the H chain and L chain V regions of a human CDR-grafted antibody can be obtained as follows. First, amino acid sequences of the frameworks (hereinafter referred to as "FR") of the H chain and L chain V regions of a human antibody for grafting CDR of the H chain and L chain V regions of an antibody derived from an animal other than human is selected. As the amino acid sequences of FRs of the H chain and L chain V regions 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 the H chain and L chain V regions of human antibodies registered at databases such as Protein Data Bank, *etc.*, amino acid sequences common in each subgroup of FRs of the H chain and L chain V regions of human antibodies [*Sequences of Proteins of Immunological Interest*, US Dep. Health and Human Services (1991)] and the like. But in order to produce a human CDR-grafted antibody having potent activity, it is preferable to select an amino acid sequence having a homology as high as possible (at least 60% or more) with amino acid sequences of the H chain and L chain V regions of an antibody of interest derived from an animal other than human.

Next, the amino acid sequences of CDRs of the H chain and L chain V regions of the antibody of interest derived from an animal other than human are grafted to the selected amino acid sequences of FRs of the H chain and L chain V regions of a human antibody to design amino acid sequences of the H chain and L chain V regions 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*

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Interest, US Dep. Health and Human Services (1991)], and the DNA sequences encoding the amino acid sequences of the H chain and L chain V regions of the human CDR-grafted antibody are designed. Based on the designed DNA sequences, several synthetic DNA fragments having a length of about 100 bases are synthesized, and PCR is carried out using them. In this case, it is preferable 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.

Also, they can be easily cloned into the vector for humanized antibody expression constructed in the item 3(1) by introducing recognition sequences of an appropriate restriction enzyme into the 5'-terminals of the synthetic DNA present on both terminals. After the PCR, the amplified product is cloned into a plasmid such as pBluescript SK(-) (manufactured by Stratagene) or the like 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 the H chain and L chain V regions of the desired human CDR-grafted antibody.

(6) Construction of human CDR-grafted antibody expression vector

A human CDR-grafted antibody expression vector can be constructed by cloning the cDNAs encoding the H chain and L chain V regions of the human CDR-grafted antibody constructed in the item 3(5) into upstream of the gene encoding H chain and L chain C regions of a human antibody in the vector for humanized antibody expression described in the item 3(1). For example, the human CDR-grafted antibody expression vector can be constructed by introducing recognizing sequences of an appropriate restriction enzyme into the 5'-terminals of both terminals

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of a synthetic DNA fragment, among the synthetic DNA fragments which are used when PCR is carried out in the item 3(5) for constructing the H chain and L chain V regions of the human CDR-grafted antibody, so that they are cloned into upstream of the genes encoding the H chain and L chain C regions of a human antibody in the vector for humanized antibody expression described in the item 3(1) in such a manner that they can be expressed in a suitable form.

(7) Stable production of humanized antibody

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 humanized antibody expression vectors described in the items 3(4) and (6) into an appropriate animal cell.

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

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.

Examples include mouse myeloma cells such as NSO 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.

After introduction of the humanized antibody expression vector, a transformant capable of stably

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producing the humanized antibody can be selected using a medium for animal cell culture comprising an agent such as G418 sulfate (hereinafter referred to as "G418"; manufactured by SIGMA) and the like in accordance with the method disclosed in Japanese Published Examined Patent Application No. 257891/90. Examples of the medium for animal cell culture include 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 expression level 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: A Laboratory Manual*, Cold Spring Harbor Laboratory, Chapter 14 (1998), *Monoclonal Antibodies: Principles and Practice*, Academic Press Limited (1996)] or the like. Also, the expression level of the humanized antibody by the transformant can be increased using a DHFR gene amplification system in accordance with the method disclosed in Japanese Published Examined Patent Application No. 257891/90.

The humanized antibody can be purified from a culture supernatant of the transformant using a protein A column [*Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Chapter 8 (1988), *Monoclonal Antibodies: Principles and Practice*, Academic Press Limited (1996)]. 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

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of a gel filtration, an ion exchange chromatography and an 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, A Laboratory Manual*, Cold Spring Harbor Laboratory, Chapter 12, (1988), *Monoclonal Antibodies: Principles and Practice*, Academic Press Limited (1996)] or the like.

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

When a host cell has the ability to express an antibody molecule innately, the antibody composition of the present invention can be produced by preparing a cell expressing an antibody molecule using the method described in the item 1, culturing the cell and then purifying the antibody composition of interest from the resulting culture.

4. Activity evaluation of antibody composition

As the method for measuring the amount of the purified antibody composition, the activity to bind to an antibody and the effector function of the purified antibody composition, the known method described in *Monoclonal Antibodies*, *Antibody Engineering* and the like can be used.

As the examples, when the antibody composition is a humanized antibody, the binding activity with an antigen and the binding activity with an antigen-positive cultured cell line can be measured by methods such as ELISA, an immunofluorescent method [*Cancer Immunol. Immunother.*, *36*, 373 (1993)] and the like. The cytotoxic activity against an antigen-positive cultured cell line can be evaluated by

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measuring CDC activity, ADCC activity [*Cancer Immunol. Immunother.*, 36, 373 (1993)] and the like.

Also, safety and therapeutic effect of the antibody composition in human can be evaluated using an appropriate model of animal species relatively close to human, such as *Macaca fascicularis* or the like.

5. Analysis of sugar chains binding to antibody molecules expressed in various cells

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

(1) Analysis of neutral sugar and amino sugar compositions

The sugar chain composition binding to an antibody molecule can be analyzed by carrying out acid hydrolysis of sugar chains with an acid such as trifluoroacetic acid or the like to release a neutral sugar or an amino sugar and measuring the composition ratio.

Example 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)].

The composition ratio can also be analyzed by a fluorescence labeling method using 2-aminopyridine.

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Specifically, the compositional ratio can be calculated in accordance with a known method [*Agric. Biol. Chem.*, 55(1), 283-284 (1991)], by labeling an acid-hydrolyzed sample with a fluorescence with 2-aminopyridylation and then analyzing the composition by HPLC.

(2) Analysis of sugar chain structure

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 such results of known sugar chains.

Specifically, sugar chains are released from an antibody by subjecting the antibody to hydrazinolysis, and the released sugar chain is 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. 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)].

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The structure deduced by the two dimensional sugar chain mapping method can be confirmed by further carrying out mass spectrometry such as MALDI-TOF-MS of each sugar chain or the like.

6. Immunological determination method for discriminating sugar chain structure of antibody molecule

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 in which 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 among the total complex *N*-glycoside-linked sugar chains binding to the Fc region in the antibody composition reducing end has potent ADCC activity. The antibody composition can be identified by using the method for analyzing the sugar chain structure of an antibody molecule described in the item 6. Also, it can also be identified by an Immunological determination method using a lectin.

The sugar chain structure of 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 (enzymoimmunoassay), FIA (fluoroimmunoassay), MIA (metalloimmunoassay) and the like described in *Monoclonal Antibodies: Principles and Applications*, Wiley-Liss, Inc. (1995); Immunoassay, 3rd Ed., Igakushoin (1987); *Enzyme Antibody Method*, Revised Edition, Gakusai Kikaku (1985); and the like.

A lectin which recognizes the sugar chain structure of an antibody molecule comprised in an antibody composition is labeled, and the labeled lectin is allowed to react with an antibody composition which is a sample.

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Then, the amount of the complex of the labeled lectin with the antibody molecule is measured.

Examples of the lectin used for identifying the sugar chain structure of an antibody molecule include WGA (wheat-germ agglutinin derived from *T. vulgaris*), ConA (cocanavalin A derived from *C. ensiformis*), RIC (a 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 eoropaeus* lectin), GNL (*Galanthus nivalis* lectin), GSL (*Griffonia simplicifolia* lectin), HPA (*Helix pomatia* agglutinin), HHL (*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), SJA (*Sophora japonica* agglutinin), SBA (soybean agglutinin), UEA (*Ulex europaeus* agglutinin), VVL (*Vicia villosa* lectin) and WFA (*Wisteria floribunda* agglutinin).

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*).

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7. Application of antibody molecule of the present invention

The antibody composition of the present invention has potent antibody-dependent cell-mediated cytotoxic activity. An antibody having potent antibody-dependent cell-mediated cytotoxic activity is useful for preventing and treating various diseases including cancers, inflammatory diseases, immune diseases such as autoimmune diseases, allergies and the like, circulatory organ diseases and viral or bacterial infections.

In the case of cancers, namely malignant tumors, cancer cells grow. General anti-tumor agents inhibit the growth of cancer cells. In contrast, an antibody having potent antibody-dependent cell-mediated cytotoxic 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 the therapeutic agent for cancers, an anti-tumor effect of an antibody medicament alone is insufficient so that combination therapy with chemotherapy has been carried out [*Science*, 280, 1197 (1998)]. If more potent 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.

In immune diseases such as inflammatory diseases, autoimmune diseases, allergies and the like, *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 potent antibody-dependent cell-mediated cytotoxic activity.

Examples of the circulatory organ diseases include arteriosclerosis and the like. The arteriosclerosis is

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treated using balloon catheter at present, but circulatory organ diseases can be prevented and treated by inhibiting growth of arterial cells in restricture after treatment using an antibody having potent antibody-dependent cell-mediated cytotoxic activity.

Various diseases including viral and bacterial infections can be prevented and treated by inhibiting proliferation of cells infected with a virus or bacterium using an antibody having potent antibody-dependent cell-mediated cytotoxic activity.

Examples of an antibody which recognizes a tumor-related antigen, an antibody which recognizes an allergy- or inflammation-related antigen, an antibody which recognizes circulatory organ disease-related antigen and an antibody which recognizes a viral or bacterial infection-related antigen are described below.

Examples of the antibody which recognizes a tumor-related antigen include anti-GD2 antibody (Ohta *et al.*, *Anticancer Res.*, 13, 331-336, 1993), anti-GD3 antibody (Ohta *et al.*, *Cancer Immunol. Immunother.*, 36, 260-266, 1993), anti-GM2 antibody (Nakamura *et al.*, *Cancer Res.*, 54, 1511-1516, 1994), anti-HER2 antibody (Carter *et al.*, *Proc. Natl. Acad. Sci. USA*, 89, 4285-4289, 1992), anti-CD52 antibody (Carter *et al.*, *Proc. Natl. Acad. Sci. USA*, 89, 4285-4289, 1992), anti-MAGE antibody (Jungbluth *et al.*, *British J. Cancer*, 83, 493-497, 2000), anti-HM1.24 antibody (Ono *et al.*, *Molecular Immunol.*, 36, 387-395, 1999), anti-parathyroid hormone-related protein (PTHrP) antibody (Ogata *et al.*, *Cancer*, 88, 2909-2911, 2000), anti-basic fibroblast growth factor antibody and anti-FGF8 antibody (Matsuzaki *et al.*, *Proc. Natl. Acad. Sci. USA*, 86, 9911-9915, 1989), anti-basic fibroblast growth factor receptor antibody and anti-FGF8 receptor antibody (Kuo *et al.*, *J. Biol. Chem.*, 265, 16455-16463, 1990), anti-insulin-like growth factor antibody (Yao *et al.*, *J. Neurosci. Res.*, 40, 647-659, 1995),

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anti-insulin-like growth factor receptor antibody (Yao *et al.*, *J. Neurosci. Res.*, 40, 647-659, 1995), anti-PMSA antibody (Murphy *et al.*, *J. Urology*, 160, 2396-2401, 1998), anti-vascular endothelial cell growth factor antibody (Presta *et al.*, *Cancer Res.*, 57, 4593-4599, 1997), anti-vascular endothelial cell growth factor receptor antibody (Kanno *et al.*, *Oncogene*, 19, 2138-2146, 2000) and the like.

Examples of the antibody which recognizes an allergy- or inflammation-related antigen include anti-interleukin 6 antibody (Abrams *et al.*, *Immunol. Rev.*, 127, 5-24, 1992), anti-interleukin 6 receptor antibody (Sato *et al.*, *Molecular Immunol.*, 31, 371-381, 1994), anti-interleukin 5 antibody (Abrams *et al.*, *Immunol. Rev.*, 127, 5-24, 1992), anti-interleukin 5 receptor antibody and anti-interleukin 4 antibody (Biord *et al.*, *Cytokine*, 3, 562-567, 1991), anti-tumor necrosis factor antibody (Tempest *et al.*, *Hybridoma*, 13, 183-190, 1994), anti-tumor necrosis factor receptor antibody (Amrani *et al.*, *Molecular Pharmacol.*, 58, 237-245, 2000), anti-CCR4 antibody (Campbell *et al.*, *Nature*, 400, 776-780, 1999), anti-chemokine antibody (Peri *et al.*, *J. Immuno. Meth.*, 174, 249-257, 1994), anti-chemokine receptor antibody (Wu *et al.*, *J. Exp. Med.*, 186, 1373-1381, 1997) and the like. Examples of the antibody which recognizes a circulatory organ disease-related antigen include anti-GpIIb/IIIa antibody (Co *et al.*, *J. Immunol.*, 152, 2968-2976, 1994), anti-platelet-derived growth factor antibody (Ferns *et al.*, *Science*, 253, 1129-1132, 1991), anti-platelet-derived growth factor receptor antibody (Shulman *et al.*, *J. Biol. Chem.*, 272, 17400-17404, 1997) and anti-blood coagulation factor antibody (Peter *et al.*, *Circulation*, 101, 1158-1164, 2000) and the like.

Examples of the antibody which recognizes a viral or bacterial infection-related antigen include anti-gp120 antibody (Tugarinov *et al.*, *Structure*, 8, 385-395, 2000), anti-CD4 antibody (Schulze-Koops *et al.*, *J. Rheumatology*,

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25, 2065-2076, 1998), anti-CCR4 antibody and anti-Vero toxin antibody (Karnali *et al.*, *J. Clin. Microbiol.*, 37, 396-399, 1999) and the like.

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, National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology (present name, International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology) and the like, or private reagent sales companies such as Dainippon Pharmaceutical, R & D SYSTEMS, PharMingen, Cosmo Bio, Funakoshi and the like.

The medicament comprising the antibody composition of the present invention can be administered as a therapeutic agent 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 manufacturing pharmacy, by mixing it with at least one pharmaceutically acceptable carrier.

It is desirable 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, intravenous or the like. In an antibody preparation, intravenous administration is preferable.

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

Examples of the pharmaceutical preparation suitable for oral administration include emulsions, syrups, capsules, tablets, powders, granules and the like.

Liquid preparations, such as emulsions and syrups, can be produced using, as additives, water; saccharides, such as sucrose, sorbitol, fructose, etc.; glycols, such as

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polyethylene glycol, propylene glycol, *etc.*; oils, such as sesame oil, olive oil, soybean oil, *etc.*; antiseptics, such as p-hydroxybenzoic acid esters, *etc.*; flavors, such as strawberry flavor, peppermint, *etc.*; and the like.

Capsules, tablets, powders, granules and the like can be produced using, as additive, fillers, such as lactose, glucose, sucrose, mannitol, *etc.*; disintegrating agents, such as starch, sodium alginate, *etc.*; lubricants, such as magnesium stearate, talc, *etc.*; binders, such as polyvinyl alcohol, hydroxypropylcellulose, gelatin, *etc.*; surfactants, such as fatty acid ester, *etc.*; plasticizers, such as glycerine, *etc.*; and the like.

Examples of the pharmaceutical preparation suitable for parenteral administration include injections, suppositories, sprays and the like.

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

Suppositories may be prepared using a carrier such as cacao butter, hydrogenated fat, carboxylic acid or the like.

Also, sprays may be prepared using the antibody composition as such or using a carrier which does 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.

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

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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 μg/kg to 20 mg/kg per day and per adult.

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, *etc.* and the like.

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.

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.

Example 1

Production of anti-ganglioside GD3 human chimeric antibody:

1. Construction of tandem expression vector pChiLHGM4 for anti-ganglioside GD3 human chimeric antibody

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 *Mlu*I (manufactured by Takara Shuzo) and *Sal*I (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

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pAGE107 [*Cytotechnology*, 3, 133 (1990)] for animal cell with restriction enzymes *Mlu*I (manufactured by Takara Shuzo) and *Sal*I (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.

Next, a fragment of about 5.68 kb containing an L chain cDNA, obtained by digesting the constructed plasmid pChi641LGM40 with a restriction enzyme *Cla*I (manufactured by Takara Shuzo), blunt-terminating it using DNA Blunting Kit (manufactured by Takara Shuzo) and further digesting it with *Mlu*I (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, pChi641HGM4 [*J. Immunol. Methods*, 167, 271 (1994)] with a restriction enzyme, *Xho*I (manufactured by Takara Shuzo), blunt-terminating it using DNA Blunting Kit (manufactured by Takara Shuzo) and further digesting it with *Mlu*I (manufactured by Takara Shuzo), using DNA Ligation Kit (manufactured by Takara Shuzo), and then *E. coli* HB101 (*Molecular Cloning*, Second Edition) was transformed with the ligated product to thereby construct a tandem expression vector pChi641LHGM4 for anti-GD3 chimeric antibody.

2. Preparation of cells stably producing anti-GD3 chimeric antibody

Cells capable of stably producing an anti-GD3 chimeric antibody were prepared using the tandem expression vector pChi641LHGM4 for anti-GD3 chimeric antibody constructed in the item 1 of Example 1, as described below.

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(1) Preparation of antibody-producing cell using rat myeloma YB2/0 cell

After introducing 5 μg of the anti-GD3 chimeric antibody expression vector pChi641LHGM4 into 4×10^6 cells of rat myeloma YB2/0 [ATCC CRL-1662, J.V. Kilmarin *et al.*, *J. Cell. Biol.*, 93, 576-582 (1982)] by electroporation [*Cytotechnology*, 3, 133 (1990)], the cells were suspended in 40 ml of RPMI1640-FBS(10) (RPMI1640 medium comprising 10% FBS (manufactured by GIBCO BRL)) and dispensed in 200 μl/well into a 96 well culture plate (manufactured by Sumitomo Bakelite). After culturing them at 37°C for 24 hours in a 5% CO_2 incubator, G418 was added to 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.

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 nM 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 in 2 ml into wells of a 24 well plate (manufactured by Greiner). Transformants showing 50 nM MTX resistance were induced by culturing at 37°C for 1 to 2 weeks in a 5% CO_2 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

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transformants in wells in which production of the anti-GD3 chimeric antibody was observed in culture supernatants, the MTX concentration was increased to 100 nM and then to 200 nM, and transformants capable of growing in the RPMI1640-FBS(10) medium comprising 0.5 mg/ml G418 and 200 nM 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 cell lines 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 cell line producing a relatively small amount of the transcription product was selected and used as a suitable cell line.

The obtained anti-GD3 chimeric antibody-producing transformed cell clone 7-9-51 has been deposited on April 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 (AIST Tsukuba Central 6, 1-1, Higashi 1-Chome Tsukuba-shi, Ibaraki-ken, Japan)).

(2) Preparation of antibody-producing cell using CHO/DG44 cell

After introducing 4 μg of the anti-GD3 chimeric antibody expression vector, pChi641LHGM4, into 1.6×10^6 cells of CHO/DG44 [G. Urlaub and L.A. Chasin, *Proc. Natl. Acad. Sci. USA*, 77, 4216-4220 (1980)] by electroporation [*Cytotechnology*, 3, 133 (1990)], the cells were suspended in 10 ml of IMDM-FBS(10) [IMDM medium comprising 10% FBS and 1x concentration of HT supplement (manufactured by GIBCO BRL)] and dispensed in 200 μl/well into a 96 well

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culture plate (manufactured by Iwaki Glass). After culturing them 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. 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.

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 nM MTX to give a density of 1 to 2×10^5 cells/ml, and the suspension was dispensed in 0.5 ml into wells of a 24 well plate (manufactured by Iwaki Glass). Transformants showing 10 nM MTX resistance were induced by culturing at 37°C for 1 to 2 weeks in a 5% CO_2 incubator. Regarding the transformants in wells in which their growth was observed, the MTX concentration was increased to 100 nM, and transformants capable of growing in the IMDM-dFBS(10) medium comprising 0.5 mg/ml G418 and 100 nM 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 cell lines 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 cell line producing a relatively

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small amount of the transcription product was selected and used as a suitable cell line.

(3) Preparation of antibody-producing cell using mouse myeloma NS0 cell

After introducing 5 μg 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 mM L-glutamine [hereinafter referred to as "L-Gln"; manufactured by GIBCO BRL)] and dispensed in 200 μl/well into a 96 well culture plate (manufactured by Sumitomo Bakelite). After culturing them 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. 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.

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 mM L-Gln) comprising 0.5 mg/ml G418 and 50 nM MTX to give a density of 1 to 2×10^5 cells/ml, and the suspension was dispensed in 2 ml into wells of a 24 well plate (manufactured by Greiner). Transformants showing 50 nM MTX resistance were induced by culturing at 37°C for 1 to 2 weeks in a 5% CO_2 incubator. The antigen binding activity of the anti-GD3 chimeric antibody in culture supernatants

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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 nM and then to 200 nM, and transformants capable of growing in the EX-CELL302-dFBS(10) medium comprising 0.5 mg/ml G418 and 200 nM 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, elite cell lines 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 cell line producing a relatively small amount of the transcription product was selected and used as a suitable cell line.

3. Measurement of binding activity of antibody to GD3 (ELISA)

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

In 2 ml of ethanol solution containing 10 μg of dipalmitoylphosphatidylcholine (manufactured by SIGMA) and 5 μg of cholesterol (manufactured by SIGMA), 4 nmol of GD3 was dissolved. Into each well of a 96 well plate for ELISA (manufactured by Greiner), 20 μl of the solution (40 pmol/well in final concentration) was dispensed, followed by air-drying, 1% bovine serum albumin (hereinafter referred to as "BSA"; manufactured by SIGMA)-containing PBS (hereinafter referred to as "1% BSA-PBS") was dispensed in 100 μl/well, and then the reaction was carried out at room temperature for 1 hour for blocking remaining active groups. After discarding 1% BSA-PBS, a culture supernatant of a transformant or a diluted solution of a human chimeric

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antibody was dispensed in 50 µl/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 in 50 µl/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 M citrate buffer (pH 4.2) and adding 1 µl/ml of hydrogen peroxide to the solution just before use (hereinafter the same solution was used)] was dispensed in 50 µl/well for color development, and then absorbance at 415 nm (hereinafter referred to as "OD415") was measured.

4. Purification of anti-GD3 chimeric antibody

(1) Culturing of antibody-producing cell derived from YB2/0 cell and purification of antibody

The anti-GD3 chimeric antibody-producing transformed cell clone obtained in the item 2(1) of Example 1 was suspended in the Hybridoma-SFM medium comprising 0.2% BSA, 200 nM MTX and 100 nM triiodothyronine (hereinafter referred to as "T3"; manufactured by SIGMA) to give a density of 3×10^5 cells/ml and cultured using a 2.0 liter capacity spinner bottle (manufactured by Iwaki Glass) under agitating at a rate of 50 rpm. After culturing them 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

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purified anti-GD3 chimeric antibody was named YB2/0-GD3 chimeric antibody.

(2) Culturing of antibody-producing cell derived from CHO/DG44 cell and purification of antibody

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 mM 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 in 50 ml into 175 mm^2 flasks (manufactured by Greiner). After culturing them at 37°C for 4 days in a 5% CO_2 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.

(3) Culturing of antibody-producing cell derived from NS0 cell and purification of antibody

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 mM L-Gln, 0.5 mg/ml G418, 200 nM MTX and 1% FBS, to give a density of 1×10^6 cells/ml, and the suspension was dispensed in 200 ml into 175 mm^2 flasks (manufactured by Greiner). After culturing them at 37°C for 4 days in a 5% CO_2 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

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instructions. The purified anti-GD3 chimeric antibody was named NS0-GD3 chimeric antibody (302).

Also, the transformed cell clone was suspended in the GIT medium comprising 0.5 mg/ml G418 and 200 nM MTX to give a density of 3×10^5 cells/ml, and the suspension was dispensed in 200 ml into 175 mm^2 flasks (manufactured by Greiner). After culturing them at 37°C for 10 days in a 5% CO_2 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).

(4) Culturing of antibody-producing cell derived from SP2/0 cell and purification of antibody

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 nM MTX to give a density of 3×10^5 cells/ml, and the suspension was dispensed in 200 ml into 175 mm^2 flasks (manufactured by Greiner). After culturing them at 37°C for 8 days in a 5% CO_2 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.

5. Analysis of purified anti-GD3 chimeric antibody

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

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respective animal cells, obtained in the item 4 of Example 1, was subjected to SDS-PAGE to analyze the molecular weight and purification degree. 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 stating 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: A Laboratory Manual*, Cold Spring Harbor Laboratory, Chapter 14 (1998); *Monoclonal Antibodies: Principles and Practice*, Academic Press Limited (1996)], 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

Activity evaluation of anti-GD3 chimeric antibody:

1. Binding activity of anti-GD3 chimeric antibody to GD3 (ELISA)

The activity of the five kinds of the purified anti-GD3 chimeric antibodies obtained in the item 4 of Example 1 to bind to GD3 (manufactured by Snow Brand Milk Products) was measured by the ELISA shown in the item 3 of Example 1. Fig. 2 shows a result of the examination of the

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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.

2. *In vitro* cytotoxic activity (ADCC activity) of anti-GD3 chimeric antibody

In order to evaluate *in vitro* cytotoxic activity of the five kinds of the purified anti-GD3 chimeric antibodies obtained in the item 4 of Example 1, the ADCC activity was measured in accordance with the following method.

(1) Preparation of target cell solution

A human melanoma cultured cell line G-361 (ATCC CRL 1424) was cultured using 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 $Na_2{}^{51}CrO_4$ 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 incubated at 4°C for 30 minutes in 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.

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(2) Preparation of effector cell solution

From a healthy person, 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 using the medium and used as the effector cell solution.

(3) Measurement of ADCC activity

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 to give a final concentration from 0.0025 to 2.5 µg/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 using a γ-counter. The amount of spontaneously released ^{51}Cr 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 ^{51}Cr in the supernatant. The amount of total released ^{51}Cr was calculated by the same operation 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 (II):

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

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 most potent 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 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 identical, it was considered that it is caused by a difference in the structure binding to the antibody Fc region.

Example 3

Preparation of anti-human interleukin 5 receptor α chain human CDR-grafted antibody:

1. Preparation of cell stably producing anti-human interleukin 5 receptor α chain human CDR-grafted antibody

(1) Preparation of antibody-producing cell using rat myeloma YB2/0 cell

Using the anti-human interleukin 5 receptor α chain human CDR-grafted antibody (hereinafter referred to as "anti-hIL-5Rα CDR-grafted antibody") expression vector, pKANTEX1259HV3LV0, described in WO 97/10354, cells capable of stably producing anti-hIL-5Rα CDR-grafted antibody were prepared as described below.

After introducing 5 μg of the anti-hIL-5Rα CDR-grafted antibody expression vector pKANTEX1259HV3LV0 into 4×10^6 cells of rat myeloma YB2/0 by electroporation

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[*Cytotechnology*, 3, 133 (1990)], the cells were suspended in 40 ml of RPMI1640-FBS(10) and dispensed in 200 µl/well into a 96 well culture plate (manufactured by Sumitomo Bakelite). After culturing them 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. 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-hIL-5Rα CDR-grafted antibody in the supernatant was measured by the ELISA shown in the item 2 of Example 3.

Regarding the transformants in wells in which production of the anti-hIL-5Rα CDR-grafted antibody was observed in culture supernatants, in order to increase amount of the antibody production using a DHFR gene amplification system, each of the them was suspended in the RPMI1640-FBS(10) medium comprising 0.5 mg/ml G418 and 50 nM MTX to give a density of 1 to 2×10^5 cells/ml, and the suspension was dispensed in 2 ml into wells of a 24 well plate (manufactured by Greiner). Transformants showing 50 nM MTX resistance were induced by culturing at 37°C for 1 to 2 weeks in a 5% CO_2 incubator. The antigen binding activity of the anti-hIL-5Rα CDR-grafted antibody in culture supernatants in wells in which growth of transformants was observed was measured by the ELISA shown in the item 2 of Example 3. Regarding the transformants in wells in which production of the anti-hIL-5Rα CDR-grafted antibody was observed in culture supernatants, the MTX concentration was increased to 100 nM and then to 200 nM, and transformants capable of growing in the RPMI1640-FBS(10) medium comprising 0.5 mg/ml G418 and 200 nM MTX and of producing the anti-hIL-5Rα CDR-grafted antibody in a large amount were finally obtained in the same manner as described above. Among the obtained transformants, elite

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cell lines 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 cell line producing a relatively small amount of the transcription product was selected and used as a suitable cell line. The obtained anti-hIL-5Rα CDR-grafted antibody-producing transformed cell clone No. 3 has been deposited on April 5, 1999, as FERM BP-6690 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 (AIST Tsukuba Central 6, 1-1, Higashi 1-Chome Tsukuba-shi, Ibaraki-ken, Japan)).

(2) Preparation of antibody-producing cell using CHO/dhfr$^-$ cell

After introducing 4 μg of the anti-hIL-5Rα CDR-grafted antibody expression vector pKANTEX1259HV3LV0 described in WO 97/10354 into 1.6×10^6 cells of CHO/dhfr$^-$ by electroporation [*Cytotechnology*, 3, 133 (1990)], the cells were suspended in 10 ml of IMDM-FBS(10) and dispensed in 200 μl/well into a 96 well culture plate (manufactured by Iwaki Glass). After culturing them 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. The culture supernatant was recovered from respective well in which colonies of transformants showing G418 resistance were formed and growth of colonies was observed, and the antigen binding activity of the anti-hIL-5Rα CDR-grafted antibody in the supernatant was measured by the ELISA shown in the item 2 of Example 3.

Regarding the transformants in wells in which production of the anti-hIL-5Rα CDR-grafted antibody was

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observed in culture supernatants, in order to increase amount of the antibody production using a DHFR gene amplification system, each of the transformants was suspended in an IMDM-dFBS(10) medium comprising 0.5 mg/ml G418 and 10 nM MTX to give a density of 1 to 2×10^5 cells/ml, and the suspension was dispensed in 0.5 ml into wells of a 24 well plate (manufactured by Iwaki Glass). Transformants showing 10 nM MTX resistance were induced by culturing at 37°C for 1 to 2 weeks in a 5% CO_2 incubator. Regarding the transformants in wells in which their growth was observed, the MTX concentration was increased to 100 nM and then to 500 nM, and transformants capable of growing in the IMDM-dFBS(10) medium comprising 0.5 mg/ml G418 and 500 nM MTX and of producing the anti-hIL-5Rα CDR-grafted antibody in a large amount were finally obtained in the same manner as described above. Among the obtained transformants, elite cell lines 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 cell line producing a relatively small amount of the transcription product was selected and used as a suitable cell line.

(3) Production of antibody-producing cell using mouse myeloma NS0 cell

An anti-hIL-5Rα CDR-grafted antibody expression vector was prepared in accordance with the method of Yarranton *et al.* [*BIO/TECHNOLOGY*, 10, 169 (1992)] and using the antibody H chain cDNA and L chain cDNA on the anti-hIL-5Rα CDR-grafted antibody expression vector pKANTEX1259HV3LV0 described in WO 97/10354, and NS0 cell was transformed to obtain transformants capable of producing the anti-hIL-5Rα CDR-grafted antibody in a large amount. Among the obtained transformants, elite cell lines

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were selected and were made into a single cell (cloning) by limiting dilution twice. Also, using the method for determining the transcription production of an α-1,6-fucosyltransferase gene shown in Example 9, a cell line producing a relatively small amount of the transcription product was selected and used as a suitable cell line.

2. Measurement of binding activity of antibody to hIL-5Rα (ELISA)

The binding activity of the antibody to hIL-5Rα was measured as described below.

A solution was prepared by diluting the anti-hIL-5Rα mouse antibody KM1257 described in WO 97/10354 with PBS to give a concentration of 10 μg/ml, and 50 μl of the resulting solution was dispensed into each well of a 96 well plate for ELISA (manufactured by Greiner), followed by reaction at 4°C for 20 hours. After the reaction, 1% BSA-PBS was dispensed in 100 μl/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 solution prepared by diluting the soluble hIL-5Rα described in WO 97/10354 with 1% BSA-PBS to give a concentration of 0.5 μg/ml was dispensed in 50 μl/well, followed by reaction at 4°C for 20 hours. After the reaction, each well was washed with Tween-PBS, culture supernatants of transformants or diluted solutions of a purified human CDR-grafted antibodies were dispensed in 50 μg/well to carry out the reaction at room temperature for 2 hours. After the reaction, each well was washed with 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 in 50 μl/well as a secondary antibody solution, followed by reaction at room temperature for 1 hour. After the reaction and subsequent washing with Tween-PBS, the ABTS substrate solution was

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dispensed in 50 µl/well for color development, and then the absorbance at OD415 was measured.

3. Purification of anti-hIL-5Rα CDR-grafted antibody

(1) Culturing of antibody-producing cell derived from YB2/0 cell and purification of antibody

The anti-hIL-5Rα CDR-grafted antibody-producing transformed cell clone obtained in the item 1(1) of Example 3 was suspended in the GIT medium comprising 0.5 mg/ml G418 and 200 nM MTX to give a density of 3×10^5 cells/ml and dispensed in 200 ml into 175 mm^2 flasks (manufactured by Greiner). After culturing them at 37°C for 8 days in a 5% CO_2 incubator, the culture supernatant was recovered. The anti-hIL-5Rα CDR-grafted antibody was purified from the culture supernatant using ion exchange chromatography and a gel filtration method. The purified anti-hIL-5Rα CDR-grafted antibody was named YB2/0-hIL-5R CDR antibody.

(2) Culturing of antibody-producing cell derived from CHO/dhfr$^-$ cell and purification of antibody

The anti-hIL-5Rα CDR-grafted antibody-producing transformed cell clone obtained in the item 1(2) of Example 3 was suspended in the EX-CELL302 medium comprising 3 mM L-Gln, 0.5% CDLC and 0.3% PF68 to give a density of 3×10^5 cells/ml and cultured using a 4.0 liter capacity spinner bottle (manufactured by Iwaki Glass) under agitating at a rate of 100 rpm. After culturing them at 37°C for 10 days in a temperature-controlling room, the culture supernatant was recovered. The anti-hIL-5Rα CDR-grafted antibody was purified from the culture supernatant using ion exchange chromatography and a gel filtration method. The purified anti-hIL-5Rα CDR-grafted antibody was named CHO/d-hIL-5R CDR antibody.

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(3) Culturing of antibody-producing cell derived from NS0 cell and purification of antibody

The anti-hIL-5Rα CDR-grafted antibody-producing transformed cell clone obtained in the item 1(3) of Example 3 was cultured in accordance with the method of Yarranton *et al.* [*BIO/TECHNOLOGY*, 10, 169 (1992)] and then a culture supernatant was recovered. The anti-hIL-5Rα CDR-grafted antibody was purified from the culture supernatant using ion exchange chromatography and the gel filtration method. The purified anti-hIL-5Rα CDR-grafted antibody was named NS0-hIL-5R CDR antibody.

4. Analysis of purified anti-hIL-5Rα CDR-grafted antibodies

In accordance with a known method [*Nature*, 227, 680 (1970)], 4 μg of each of the three kinds of the anti-hIL-5Rα CDR-grafted antibodies produced by and purified from each animal cells, obtained in the item 3 of Example 3, was subjected to SDS-PAGE to analyze the molecular weight and purification degree. The results are shown in Fig. 4. As shown in Fig. 4, 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 under reducing conditions, in each of the purified anti-hIL-5Rα CDR-grafted 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 stating 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 S-S bond in the molecule [*Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory*, Chapter 14 (1998); *Monoclonal Antibodies:*

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Principles and Practice, Academic Press Limited (1996)], so that it was confirmed that each anti-hIL-5Rα CDR-grafted antibody was expressed and purified as an antibody molecule having the true structure.

Example 4

Activity evaluation of anti-hIL-5Rα CDR-grafted antibody:

1. Binding activity of anti-hIL-5Rα CDR-grafted antibody to hIL-5Rα (ELISA)

The activity of the three kinds of the purified anti-hIL-5Rα CDR-grafted antibodies obtained in the item 3 of Example 3 to bind to hIL-5Rα was measured by the ELISA shown in the item 2 of Example 3. Fig. 5 shows a result of the examination of the binding activity measured by changing concentration of the anti-hIL-5Rα CDR-grafted antibody to be added. As shown in Fig. 5, the three kinds of the anti-hIL-5Rα CDR-grafted antibodies showed almost the same binding activity to hIL-5Rα. The result shows that the antigen binding activities of these antibodies are constant independently of the antibody-producing animal cells and their culturing methods, similar to the result of the item 1 of Example 2.

2. *In vitro* cytotoxic activity (ADCC activity) of anti-hIL-5Rα CDR-grafted antibody

In order to evaluate *in vitro* cytotoxic activity of the three kinds of the purified anti-hIL-5Rα CDR-grafted antibodies obtained in the item 3 of Example 3, the ADCC activity was measured in accordance with the following method.

(1) Preparation of target cell solution

A mouse T cell line CTLL-2(h5R) expressing the hIL-5Rα chain and β chain described in WO 97/10354 was cultured using the RPMI1640-FBS(10) medium to give a density of

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1×10^6 cells/0.5 ml, and the cells were radioisotope-labeled by reacting them with 3.7 MBq equivalents of a radioactive substance $Na_2{}^{51}CrO_4$ at 37°C for 1.5 hours. After the reaction, the cells were washed three times through their suspension in the RPMI1640-FBS(10) medium and centrifugation, resuspended in the medium and then incubated at 4°C for 30 minutes in ice for spontaneous dissolution of the radioactive substance. After the centrifugation, the precipitate was adjusted to give a density of 2×10^5 cells/ml by adding 5 ml of the RPMI1640-FBS(10) medium and used as the target cell solution.

(2) Preparation of effector cell solution

From a healthy person, 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 separate a mononuclear cell layer using Polymorphprep (manufactured by Nycomed Pharma AS) and in accordance with the manufacture's instructions. After washing with the RPMI1640-FBS(10) medium by centrifugation three times, the resulting cells were resuspended to give a density of 9×10^6 cells/ml using the medium and used as the effector cell solution.

(3) Measurement of ADCC activity

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 dispensed (9×10^5 cells/well, the ratio of effector cells to target cells becomes 90:1). Subsequently, each of the anti-hIL-5Rα CDR-grafted antibodies was added to give a final concentration of 0.001 to 0.1 µg/ml, followed by reaction at 37°C for 4 hours. After the reaction, the plate was centrifuged, and the

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amount of ^{51}Cr in the supernatant was measured using a γ-counter. The amount of spontaneously released ^{51}Cr 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 ^{51}Cr in the supernatant. The amount of total released ^{51}Cr was calculated by the same operation 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 above equation (II).

The results are shown in Fig. 6. As shown in Fig. 6, among the three kinds of the anti-hIL-5Rα CDR-grafted antibodies, the YB2/0-hIL-5R CDR antibody showed the most potent ADCC activity, followed by the CHO/d-hIL-5R CDR antibody and the NS0-hIL-5R CDR antibody in this order. Similar to the result of the item 2 of Example 2, the above results show that the ADCC activity of antibodies greatly varies depending on the animal cells to be used in their production. In addition, since the antibodies produced by the YB2/0 cell showed the most potent ADCC activity in both cases of the two kinds of the humanized antibodies, it was revealed that an antibody having potent ADCC activity can be produced by using the YB2/0 cell.

3. *In vivo* activity evaluation of anti-hIL-5Rα CDR-grafted antibody

In order to evaluate *in vivo* activity of the three kinds of the purified anti-hIL-5Rα CDR-grafted antibodies obtained in the item 3 of Example 3, the inhibition activity in an hIL-5-induced eosinophilia increasing model of *Macaca faseicularis* was examined in accordance with the following method.

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The hIL-5 (preparation method is described in WO 97/10354) was administered to *Macaca faseicularis* under the dorsal skin at a dose of 1 μg/kg, starting on the first day and once a day for a total of 14 times. Each anti-hIL-5Rα CDR-grafted antibody was intravenously administered at a dose of 0.3 mg/kg one hour before the hIL-5 administration on the day zero. An antibody-non-added group was used as the control. In the antibody-administered groups, three animals of *Macaca faseicularis* were used in each group (No. 301, No. 302, No. 303, No. 401, No. 402, No. 403, No. 501, No. 502 and No. 503), and two animals (No. 101 and No. 102) were used in the antibody-non-added group. Starting 7 days before commencement of the administration and until 42 days after the administration, about 1 ml of blood was periodically collected from a saphena or a femoral vein, and the number of eosinophils in 1 μl of peripheral blood was measured. The results are shown in Fig. 7. As shown in Fig. 7, increase in the blood eosinophil was completely inhibited in the group to which the YB2/0-hIL-5R CDR antibody was administered. On the other hand, complete inhibition activity was found in one animal in the group to which the CHO/d-hIL-5R CDR antibody was administered, but the inhibition activity was not sufficient in two animals. In the group to which NS0-hIL-5R CDR antibody was administered, complete inhibition activity was not found and its effect was not sufficient.

The above results show that the *in vivo* activity of antibodies greatly varies depending on the animal cells to be used in their production. In addition, since a positive correlation was found between the degree of the *in vivo* activity of the anti-hIL-5Rα CDR-grafted antibody and the degree of its ADCC activity described in the item 2 of Example 4, it was indicated that the degree of ADCC activity is remarkably important for its activity expression.

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Based on the above results, it is expected that an antibody having potent ADCC activity is useful also in the clinical field for various diseases in human.

Example 5

Analysis of sugar chain which enhances ADCC activity:

1. Preparation of 2-aminopyridine-labeled sugar chain (PA-treated sugar chain)

The humanized antibody of the present invention was acid-hydrolyzed with hydrochloric acid to remove sialic acid. After hydrochloric acid was completely removed, the sugar chain was cleaved from the protein by hydrazinolysis [*Method of Enzymology*, 83, 263 (1982)]. Hydrazine was removed, and *N*-acetylation was carried out by adding an aqueous ammonium acetate solution and acetic anhydride. After lyophilizing, fluorescence labeling with 2-aminopyridine was carried out [*J. Biochem.*, 95, 197 (1984)]. The fluorescence-labeled sugar chain (PA-treated sugar chain) was separated from impurity using Surperdex Peptide HR 10/30 Column (manufactured by Pharmacia). The sugar chain fraction was dried using a centrifugal concentrator and used as a purified PA-treated sugar chain.

2. Reverse phase HPLC analysis of PA-treated sugar chain of purified anti-hIL-5Rα CDR-grafted antibody

According to the method in the item 1 of Example 5, various anti-hIL-5Rα CDR-grafted antibodies produced in Example 3 were subjected to PA-treated sugar chain treatment, and reverse phase HPLC analysis was carried out by CLC-ODS column (manufactured by Shimadzu). An excess amount of α-L-fucosidase (derived from bovine kidney, manufactured by SIGMA) was added to the PA-treated sugar chain for digestion (37°C, 15 hours), and then the products were analyzed by reverse phase HPLC (Fig. 8). It was confirmed that the asparagine-linked sugar chain is eluted

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for 30 minutes to 80 minute using PA-treated sugar chain standards manufactured by Takara Shuzo. The ratio of sugar chains whose reverse phase HPLC elution positions were shifted (sugar chains eluted for 48 minutes to 78 minutes) by the α-L-fucosidase digestion was calculated. The results are shown in Table 1.

Table 1

Antibody-producing cell	α-1,6-Fucose-linked sugar chain (%)
YB2/0	47
NS0	73

About 47% of the anti-hIL-5R CDR-grafted antibody produced by the YB2/0 cell and about 73% of the anti-hIL-5R CDR-grafted antibody produced by the NS0 cell were sugar chains 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 (hereinafter referred to as "sugar chain having α-1,6-fucose"). Thus, the ratio of sugar chains in which 1-position of fucose is not bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the *N*-glycoside-linked sugar chain (hereinafter referred to as "α-1,6-fucose-free sugar chain") is higher in the antibody produced by the YB2/0 cell than in the antibody produced by the NS0 cell.

3. Analysis of monosaccharide composition of purified anti-hIL-5Rα CDR-grafted antibody

Sugar chains of anti-hIL-5Rα CDR-grafted antibodies produced by the YB2/0 cell, NS0 cell and CHO/d cell were hydrolyzed into monosaccharides by acid hydrolysis with trifluoroacetic acid, and monosaccharide composition analysis was carried out using BioLC (manufactured by Dionex).

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Among *N*-glycoside-linked sugar chains, there are 3 mannose units in one sugar chain in the complex type *N*-glycoside-linked sugar chain. A relative ratio of each monosaccharide obtained by calculating the number of mannose as 3 is shown in Table 2.

Table 2

Antibody-producing cell	Fuc	GlcNAc	Gal	Man	ADCC activity (%)*
YB2/0	0.60	4.98	0.30	3.00	42.27
NS0	1.06	3.94	0.66	3.00	16.22
CHO/dhFr⁻	0.85	3.59	0.49	3.00	25.73
CHO/dhFr⁻	0.91	3.80	0.27	3.00	25.73

*: Antibody concentration: 0.01 μg/ml

Since the relative ratios of fucose were in an order of YB2/0 < CHO/d < NS0, the sugar chain produced in the antibody produced by YB2/0 cell showed the lowest fucose content as also shown in the present results.

4. Sugar chain analysis of antibody produced by CHO/dhfr⁻ cell

PA-treated sugar chains were prepared from purified anti-hIL-5Rα CDR-grafted antibody produced by CHO/dhfr⁻ cell, and reverse phase HPLC analysis was carried out using CLC-ODS column (manufactured by Shimadzu) (Fig. 9). In Fig. 9, an elution time from 35 to 45 minutes corresponded to sugar chains having no fucose and an elution time from 45 to 60 minutes corresponded to sugar chains having fucose. Similar to the case of the antibody produced by mouse myeloma NS0 cell, the anti-hIL-5Rα CDR-grafted antibody produced by CHO/dhfr⁻ cell had less fucose-free sugar chain content than the antibody produced by rat myeloma YB2/0 cell.

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Example 6

Separation of potent ADCC activity antibody:

The anti-hIL-5Rα CDR-grafted antibody produced by rat myeloma YB2/0 cell was separated using a lectin column which binds to sugar chains having fucose. HPLC was carried out using LC-6A manufactured by Shimadzu at a flow rate of 1 ml/min and at room temperature as the column temperature. After equilibration with 50 mM Tris-sulfate buffer (pH 7.3), the purified anti-hIL-5Rα CDR-grafted antibody was injected and then eluted by a linear density gradient (60 minutes) of 0.2 M α-methylmannoside (manufactured by Nakalai Tesque). The anti-hIL-5Rα CDR-grafted antibody was separated into non-adsorbed fraction and adsorbed fraction. When the non-adsorbed fraction and a part of the adsorbed fraction were sampled and their binding activity to hIL-5Rα was measured, they showed similar binding activity (Fig. 10A). When the ADCC activity was measured, the non-adsorbed fraction showed potent ADCC activity (100 to 1000 folds) than that of the part of adsorbed fraction (Fig. 10B). In addition, PA-treated sugar chains were prepared from the non-adsorbed fraction and a part of the adsorbed fraction, and reverse HPLC analysis was carried out using CLC-ODS column (manufactured by Shimadzu) (Fig. 11). In the non-adsorbed fraction, an antibody binding to fucose-free sugar chains was mainly present, and in the part of adsorbed fraction, an antibody binding to sugar chains having fucose was mainly present.

Example 7

Activity evaluation of anti-GD3 chimeric antibody having different ratio of α-1,6-fucose-free sugar chain:

1. Preparation of anti-GD3 chimeric antibodies having different ratio of α-1,6-fucose-free sugar chain

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In accordance with the method described in the item 2(1) of Example 1, 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. Each sugar chain which is bound to the anti-GD3 chimeric antibodies of lot 1, lot 2 and lot 3 was analyzed in accordance with the method of Example 11(6), and it was found that the ratios of α-1,6-fucose-free sugar chains were 50%, 45% and 29%, respectively. Herein, these samples are referred to as anti-GD3 chimeric antibody (50%), anti-GD3 chimeric antibody (45%) and anti-GD3 chimeric antibody (29%).

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 method of Example 11(6), and it was found that the ratio of α-1,6-fucose-free sugar chains was 7%. Herein, the sample is referred to as anti-GD3 chimeric antibody (7%).

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 or 1:7. Sugar chains of the samples were analyzed in accordance with the method of Example 10(6), and it was found that samples having the ratio of α-1,6-fucose-free sugar chains of 24% and 13% (calculated value) were prepared. Herein, they are referred to as anti-GD3 chimeric antibody (24%) and anti-GD3 chimeric antibody (13%).

Results of the sugar chain analysis of each of the samples are shown in Fig. 12. The ratio of α-1,6-fucose-free sugar chains was shown as an average value of the result of two sugar chain analyses.

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2. Evaluation of binding activity to GD3 (ELISA)

The binding activities of the six kinds of the anti-GD3 chimeric antibodies having a different ratio of α-1,6-fucose-free sugar chains prepared in the item 1 of Example 7 against GD3 (manufactured by Snow Brand Milk Products) 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. 13, and it was found that the ratio of the α-1,6-fucose-free sugar chains does not have influence on the antigen binding activity of the antibody.

3. Evaluation of ADCC activity on human melanoma cell line

The ADCC activity of anti-GD3 chimeric antibodies on a human melanoma cell line G-361 (ATCC CRL 1424) was measured as follows.

(1) Preparation of target cell suspension

1×10^6 cells of a human melanoma cell line G-361 were prepared, a 3.7 MBq equivalent of a radioactive substance $Na_2{}^{51}CrO_4$ was added thereto and the mixture was allowed to react at 37°C for 1 hour to label the cells with the radioisotope. After the reaction, the cells were washed three times by a procedure of their suspension in a medium and subsequent centrifugation, re-suspended in the medium and then incubated at 4°C for 30 minutes in ice to effect spontaneous dissociation of the radioactive substance. After centrifugation, the cells were adjusted to 2×10^5 cells/ml by adding 5 ml of the medium and used as a target cell suspension.

(2) Preparation of human effector cell suspension

A 50 ml portion of peripheral blood was collected from a healthy person and gently mixed with 0.5 ml of heparin sodium (manufactured by Shimizu Pharmaceutical).

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Using Lymphoprep (manufactured by AXIS SHIELD), this was centrifuged (800 g, 20 minutes) in accordance with the manufacture's instructions to separate a mononuclear cell layer. The cells were washed by centrifuging (1,200 rpm, 5 minutes) three times using a medium and then re-suspended in the medium to give a density of 2×10^6 cells/ml and used as a human effector cell suspension.

(3) Measurement of ADCC activity

The target cell suspension prepared in the (1) was dispensed in 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 human effector cell suspension prepared in the (2) was added thereto (2×10^5 cells/well, ratio of the human effector cells to the target cells was 20 : 1). Each of the anti-GD3 chimeric antibodies was added thereto to give a final concentration of 0.0005 to 5 µg/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 using a γ-counter. An amount of the spontaneously dissociated ^{51}Cr was calculated by carrying out the same procedure 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 using the medium alone instead of the antibody solution and a 1 mol/l hydrochloric acid solution instead of the human effector cell suspension, and measuring the amount of ^{51}Cr in the supernatant. The cytotoxic activity (%) was calculated using equation (II).

Figs. 14 and 15 show results of the measurement of ADCC activity of the six kinds of the anti-GD3 chimeric antibodies having a different ratio of α-1,6-fucose-free sugar chains at various concentrations (0.0005 to 5 µg/ml)

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using effector cells of two healthy donors (A and B), respectively. As shown in Figs. 14 and 15, ADCC activity of the anti-GD3 chimeric antibodies showed a tendency to increase in proportion to the ratio of α-1,6-fucose-free sugar chains at each antibody concentration. The ADCC activity decreases when the antibody concentration is low. At an antibody concentration of 0.05 μg/ml, the antibody in which the ratio of α-1,6-fucose-free sugar chains is 24%, 29%, 45% or 50% showed almost the same potent ADCC activity but the ADCC activity was low in the antibody (13%) or (7%) in which the ratio of α-1,6-fucose-free sugar chains is less that 20%. These results were the same when the effector cell donor was changed.

Example 8

Activity evaluation of anti-CCR4 chimeric antibody having different ratio of α-1,6-fucose-free sugar chain:

1. Production of cells stably producing anti-CCR4 chimeric antibody

Cells which capable of stably producing an anti-CCR4 chimeric antibody were prepared as follows using a tandem type expression vector pKANTEX2160 for an anti-CCR4 chimeric antibody described in WO 01/64754.

(1) Preparation of antibody-producing cell using rat myeloma YB2/0 cell

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 in 200 μl/well into a 96 well culture plate (manufactured by Sumitomo Bakelite). After culturing them at 37°C for 24

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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 8.

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 nM DHFR inhibitor MTX (manufactured by SIGMA) to give a density of 1 to 2 x 10^5 cells/ml, and the suspension was dispensed in 1 ml portions into wells of a 24 well plate (manufactured by Greiner). After culturing them at 37°C for 1 to 2 weeks in a 5% CO_2 incubator, transformants showing 50 nM 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 8.

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 nM 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 cloned cell line was named KM2760 #58-35-16. In this case, using the method for determining the transcription product of an α-1,6-fucosyltransferase gene shown in Example 9, a cell line

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producing a relatively small amount of the transcription product was selected and used as a suitable cell line.

(2) Preparation of antibody-producing cell using CHO/DG44 cell

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 × concentration of HT supplement (manufactured by Invitrogen)] and dispensed in 100 μl/well into a 96 well culture plate (manufactured by Iwaki Glass). After culturing them at 37°C for 24 hours in a 5% CO_2 incubator, the medium was changed to IMDM-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 level of the anti-CCR4 chimeric antibody in the supernatant was measured by the ELISA described in the item 2 of Example 8.

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 nM MTX to give a density of 1 to 2×10^5 cells/ml, and the suspension was dispensed in 0.5 ml into wells of a 24 well plate (manufactured by Iwaki Glass). After culturing them at 37°C for 1 to 2 weeks in a 5% CO_2 incubator, transformants showing 50 nM MTX resistance were induced. Regarding the transformants in wells in which the growth was observed, the MTX

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concentration was increased to 200 nM by the same method, and a transformant capable of growing in the IMDM-dFBS(10) medium comprising 200 nM MTX and of producing the anti-CCR4 chimeric antibody in a large amount was finally obtained. The obtained transformant was named 5-03. In this case, using the method for determining the transcription product of an α-1,6-fucosyltransferase gene shown in Example 9, a cell line producing a relatively small amount of the transcription product was selected and used as a suitable cell line.

2. Antibody binding activity to CCR4 partial peptide (ELISA)

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 (bovine serum albumin) (manufactured by Nakalai 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 mg BSA-containing PBS solution under stirring using a vortex, followed by gently stirring for 30 minutes. A 1 ml portion of the reaction solution was applied to a gel filtration column such as NAP-10 column or the like equilibrated with 25 ml of PBS, 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

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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.

The prepared conjugate was dispensed at 0.05 μg/ml and 50 μl/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 μl/well and allowed to react at room temperature to block the remaining active groups. After washing each well with PBS containing 0.05% Tween 20 (hereinafter referred to as "Tween-PBS"), a culture supernatant of a transformant was added at 50 μl/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 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 for color development, and 20 minutes thereafter, the reaction was stopped by adding a 5% SDS solution at 50 μl/well. Thereafter, the absorbance at OD_{415} was measured. The anti-CCR4 chimeric antibody obtained in the item 1 of Example 8 showed the binding activity to CCR4.

3. Purification of anti-CCR4 chimeric antibody

(1) Culturing of antibody-producing cell derived from YB2/0 cell and purification of antibody

The anti-CCR4 chimeric antibody-expressing transformant cell clone KM2760 # 58-35-16 obtained in the item 1(1) of Example 8 was suspended in Hybridoma-SFM (manufactured by Invitrogen) medium comprising 200 nM MTX and 5% of Daigo's GF21 (manufactured by Wako Pure Chemical Industries) to give a density of 2×10^5 cells/ml and

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subjected to fed-batch shaking culturing using a spinner bottle (manufactured by Iwaki Glass) in a constant temperature chamber of 37°C. After culturing them for 8 to 10 days, the anti-CCR4 chimeric antibody was purified from the culture supernatant recovered using Prosep-A (manufactured by Millipore) column and gel filtration. The purified anti-CCR4 chimeric antibody was named KM2760-1.

(2) Culturing of antibody-producing cell derived from CHO-DG44 cell and purification of antibody

The anti-CCR4 chimeric antibody-producing transformant cell line 5-03 obtained in the item 1(2) of Example 8 was cultured at 37°C in a 5% CO_2 incubator using IMDM-dFBS(10) medium in a 182 cm^2 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 them at 37°C for 7 days in a 5% CO_2 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.

When the binding activity to CCR4 of KM2760-1 and KM3060 was measured by ELISA, they showed equivalent binding activity.

4. Analysis of purified anti-CCR4 chimeric antibodies

Each (4 μg) of the two kinds of the anti-CCR4 chimeric antibodies produced by and purified from in different animal cells, obtained in the item 1 of this Example was subjected to SDS-PAGE in accordance with a known method [*Nature*, 227, 680 (1970)], and the molecular weight and purification degree were analyzed. In each of

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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 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: A Laboratory Manual*, Cold Spring Harbor Laboratory, Chapter 14 (1988), *Monoclonal Antibodies: Principles and Practice*, Academic Press Limited (1996)], thus confirming that the anti-CCR4 chimeric antibody was expressed and purified as an antibody molecule having a correct structure.

5. Preparation of anti-CCR4 chimeric antibody having different ratio of α-1,6-fucose-free sugar chain

Sugar chains which are bound to anti-CCR4 chimeric antibody KM2760-1 derived from YB2/0 cell prepared in the item 3(1) of Example 8 and the anti-CCR4 chimeric antibody KM3060 derived from CHO/DG44 cell prepared in the item 3(2) of Example 8 were analyzed in accordance with the method of Example 10(6). The ratio of α-1,6-fucose-free sugar chains was 87% and 8% in KM2760 and KM3060, respectively. Herein, the samples are referred to as anti-CCR4 chimeric antibody (87%) and anti-CCR4 chimeric antibody (8%).

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 or 42 : 37. Sugar

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chains of these samples were analyzed in accordance with the method of Example 10(6). The ratio of α-1,6-fucose-free sugar chains was 9%, 18%, 27%, 39% and 46%, respectively. Herein, these samples are referred to as 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%).

Results of the sugar chain analysis of each of the samples are shown in Fig. 16. The ratio of α-1,6-fucose-free sugar chains was shown as an average value of the result of two sugar chain analyses.

6. Evaluation of binding activity to CCR4 partial peptide (ELISA)

Binding activity of the six kinds of the different anti-CCR4 chimeric antibodies having a different α-1,6-fucose-free sugar chain prepared in the item 5 of Example 8 to CCR4 partial peptide was measured in accordance with the method described in the item 2 of Example 8.

As a result, as shown in Fig. 17, the six kinds of the anti-CCR4 chimeric antibodies showed almost the same CCR4-binding activity, it was found that the ratio of α-1,6-fucose-free sugar chains does not have influence on the antigen-binding activity of the antibody.

7. Evaluation of ADCC activity on human CCR4-high expressing cell line

The ADCC activity of the anti-CCR4 chimeric antibodies against a human CCR4-high expressing cell CCR4/EL-4 cell (WO 01/64754) was measured as follows.

(1) Preparation of target cell suspension

Cells (1.5×10^6) of a human CCR4-expressing cell, CCR4/EL-4 cell, described in WO 01/64754 were prepared and

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a 5.55 MBq equivalent of a radioactive substance $Na_2{}^{51}CrO_4$ 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 in 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.

(2) Preparation of human effector cell suspension

From a healthy person, 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 using 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.

(3) Measurement of ADCC activity

The target cell suspension prepared in the (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 human effector cell suspension prepared in the (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 µg/ml, followed by reaction at 37°C for 4 hours. After the

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reaction, the plate was centrifuged and the amount of ^{51}Cr in the supernatant was measured using a γ-counter. An amount of the spontaneously dissociated ^{51}Cr was calculated by carrying out the same procedure 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 using a 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 equation (II).

Figs. 18 and 19 show results of the measurement of ADCC activity of the anti-CCR4 chimeric antibodies having a different ratio of α-1,6-fucose-free sugar chains at various concentrations (0.001 to 10 μg/ml) using effector cells of two healthy donors (A and B), respectively. As shown in Figs. 18 and 19, the ADCC activity of the anti-CCR4 chimeric antibodies showed a tendency to increase in proportion to the ratio of α-1,6-fucose-free sugar chains at each antibody concentration. The ADCC activity decreases when the antibody concentration is low. At an antibody concentration of 0.01 μg/ml, the antibody in which the α-1,6-fucose-free sugar chains is 27%, 39% or 46% showed almost the same potent ADCC activity but the ADCC activity was low in the antibody in which the ratio of α-1,6-fucose-free sugar chains is less than 20%. The results were the same as the case when the effector cell donor was changed.

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Example 9

Determination of transcription product of α-1,6-fucosyltransferase gene in host cell line:

(1) Preparation of single-stranded cDNA from various cell lines

Single-stranded cDNA samples were prepared from dihydrofolate reductase gene (dhfr)-deleted CHO/DG44 cells derived from Chinese hamster ovary and rat myeloma YB2/0 cells by the following procedure.

The CHO/DG44 cells were suspended in IMDM medium (manufactured by Life Technologies) supplemented with 10% fetal bovine serum (manufactured by Life Technologies) and 1 x 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% fetal bovine serum (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% CO_2 incubator, and 1×10^7 of respective host cells were recovered on the 1st, 2nd, 3rd, 4th and 5th days of the culturing to extract total RNA using RNAeasy (manufactured by QIAGEN) in accordance with the manufacture's instructions.

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

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RNAeasy (manufactured by QIAGEN) and dissolved in 50 μl of sterile water.

In a 20 μl of the reaction mixture using oligo(dT) as a primer, single-stranded cDNA was synthesized from 3 μg 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) and in accordance with the manufacture's instructions. A 1 x concentration solution of the reaction solution was used for the cloning of α-1,6-fucosyltransferase (hereinafter referred sometimes to as "FUT8") and β-actin derived from respective host cells, and 50 folds-diluted aqueous solution of the reaction solution for the determination of each gene transcription amount by competitive PCR, and the solutions were stored at -80°C until use.

(2) Preparation of cDNA partial fragments of Chinese hamster FUT8 and rat FUT8

Each cDNA partial fragment of Chinese hamster FUT8 and rat FUT8 was prepared by the following procedure (Fig. 20).

First, primers (shown in 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.

Next, 25 μl of a reaction solution [ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/l dNTPs and 0.5 μmol/l gene-specific primers (SEQ ID NOs:4 and 5)] containing 1 μl of each of the cDNA prepared from CHO cell and cDNA prepared from YB2/0 cell, both obtained in the item (1) 2 days after culturing, and polymerase chain reaction (PCR) was carried out 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

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heating at 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 2 minutes as one cycle, and final heating at 72°C for 10 minutes.

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 µl of sterile water (hereinafter, the method was used for the purification of DNA fragments from agarose gel). Into a plasmid pCR2.1, 4 µl 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 6 clones among the obtained kanamycin-resistant colonies.

The nucleotide sequence of each cDNA inserted into the plasmid 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 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 or rat FUT8 (shown in 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 are referred to as CHFUT8-pCR2.1 and YBFUT8-pCR2.1.

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(3) Preparation of Chinese hamster β-actin and rat β-actin cDNA

Chinese hamster β-actin and rat β-actin cDNA were prepared by the following procedure (Fig. 21).

First, a forward primer specific for a common sequence containing translation initiation codon (shown in SEQ ID NO:8) and reverse primers specific for respective sequences containing translation termination codon (shown in 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).

Next, 25 µl of a reaction solution [KOD buffer #1 (manufactured by Toyobo), 0.2 mmol/l dNTPs, 1 mmol/l $MgCl_2$, 0.4 µmol/l gene-specific primers (SEQ ID NOs:8 and 9, or SEQ ID NOs:8 and 10) and 5% DMSO] containing 1 µl of each of the cDNA prepared from CHO cell and cDNA prepared from YB2/0 cell, both obtained in the item (1) 2 days after culturing was prepared, and PCR was carried out using a DNA polymerase KOD (manufactured by Toyobo). The PCR was carried out by heating at 94°C for 1 minute 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.

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 DNA 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 ethanol precipitation method and dissolved in 10 µl of sterile water.

Separately, 3 µg of a plasmid pBluescript II KS(+) (manufactured by Stratagene) was dissolved in 35 µl of NEBuffer 2 (manufactured by New England Biolabs), and 16 units of a restriction enzyme *Eco*RV (manufactured by Takara

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Shuzo) were added thereto for digestion reaction at 37°C for 3 hours. To the reaction solution, 35 µl of 1 mol/l Tris-HCl buffer (pH 8.0) and 3.5 µl 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 dephophorylate the DNA terminus. The reaction solution was extracted with phenol/chloroform, followed by ethanol precipitation, and the recovered DNA fragment was dissolved in 100 µl of sterile water.

Each 4 µl of the amplified fragment prepared from Chinese hamster cDNA or the amplified fragment (1192 bp) prepared from rat cDNA was mixed with 1 µl of the *Eco*RV-*Eco*RV fragment (about 3.0 Kb) prepared from plasmid pBluescript II KS(+) and 5 µl 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 in accordance with a known method from the obtained ampicillin-resistant clones.

The nucleotide sequence of each cDNA inserted into the plasmid 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 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.

(4) Preparation of FUT8 standard and internal control

In order to measure a transcription level of FUT8 gene mRNA in each cell, CHFT8-pCR2.1 or YBFT8-pCR2.1, as

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plasmids in which cDNA partial fragments prepared in the item (2) from Chinese hamster FUT8 or rat FUT8 were inserted into pCR2.1, respectively, were digested with a restriction enzyme *Eco*RI, 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 *Sca*I and *Hin*dIII, an inner nucleotide sequence of Chinese hamster FUT8 and rat FUT8, respectively, were digested with a restriction enzyme *Eco*RI, and the obtained linear DNAs were used as the internal standards for FUT8 amount determination. Details thereof are described below.

Chinese hamster FUT8 and rat FUT8 standards were prepared as follows. In 40 µl of NEBuffer 2 (manufactured by New England Biolabs), 2 µg of the plasmid CHFT8-pCR2.1 was dissolved, 24 units of a restriction enzyme *Eco*RI (manufactured by Takara Shuzo) were added thereto, followed by digestion reaction at 37°C for 3 hours. Separately, 2 µg of the plasmid YBFT8-pCR2.1 was dissolved in 40 µl of NEBuffer 2 (manufactured by New England Biolabs), and 24 units of a restriction enzyme *Eco*RI (manufactured by Takara Shuzo) were added thereto, followed by digestion reaction at 37°C for 3 hours. By subjecting a portion of each of the reaction solutions to 0.8% agarose gel electrophoresis, it was confirmed that an *Eco*RI-*Eco*RI fragment (about 1 Kb) containing each of cDNA partial fragments of Chinese hamster FUT8 and rat FUT8 was separated from the plasmids CHFT8-pCR2.1 and YBFT8-pCR2.1 by the restriction enzyme digestion reactions. Each of the reaction solutions was diluted with 1 µg/ml of baker's yeast t-RNA (manufactured by SIGMA) to give a concentration of 0.02 fg/µl, 0.2 fg/µl, 1 fg/µl, 2 fg/µl, 10 fg/µl, 20 fg/µl and 100 fg/µl and used as the Chinese hamster FUT8 and rat FUT8 standards.

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Internal standards of Chinese hamster FUT8 and rat FUT8 were prepared as follows (Fig. 22). A reaction solution [KOD buffer #1 (manufactured by Toyobo), 0.2 mmol/l dNTPs, 1 mmol/l $MgCl_2$, 0.4 μmol/l gene-specific primers (SEQ ID NOs:11 and 12) and 5% DMSO] containing 5 ng of CHFT8-pCR2.1 or YBFT8-pCR2.1 was prepared, and PCR was carried out 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. 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 5'-terminal was phosphorylated 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 μl of sterile water. The obtained DNA fragment (5 μl, about 4.7 kb) and 5 μl of Ligation High (manufactured by Toyobo) were mixed, followed by self-cyclization reaction at 16°C for 30 minutes.

Using the reaction solution, *E. coli* DH5α 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 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 *Sca*I and *Hind*III of Chinese hamster FUT8 or rat FUT8 was deleted. The obtained plasmids are referred to as CHFT8d-pCR2.1 or YBFT8d-pCR2.1, respectively.

Next, 2 μg of the plasmid CHFT8d-pCR2.1 was dissolved in 40 μl of NEBuffer 2 (manufactured by New England Biolabs), and 24 units of a restriction enzyme

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*Eco*RI (manufactured by Takara Shuzo) were added thereto, followed by digestion reaction at 37°C for 3 hours. Separately, 2 μg of the plasmid YBFT8d-pCR2.1 was dissolved in 40 μl of NEBuffer 2 (manufactured by New England Biolabs), and 24 units of a restriction enzyme *Eco*RI (manufactured by Takara Shuzo) were added thereto, followed by digestion reaction at 37°C for 3 hours. A portion of each of the reaction solutions was subjected to 0.8% agarose gel electrophoresis, and it was confirmed that an *Eco*RI-*Eco*RI 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 restriction enzyme digestion reactions. Dilutions of 2 fg/μl were prepared from the reaction solutions using 1 μg/ml baker's yeast t-RNA (manufactured by SIGMA) and used as the Chinese hamster FUT8 or rat FUT8 internal controls.

(5) Preparation of β-actin standard and internal control

In order to measure the transcription amount of β-actin gene mRNA 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) was inserted into pBluescript II KS(+), respectively, were digested with restriction enzymes *Hin*dIII and *Pst*I and restriction enzymes *Hin*dIII and *Kpn*I, respectively, and the digested linear DNAs were used as the standards for the preparation of a calibration curve. CHAcd-pBS and YBAcd-pBS which were obtained from the CHAc-pBS and YBAc-pBS by deleting 180 bp between *Dra*III and *Dra*III of an inner nucleotide sequence of Chinese hamster β-actin and rat β-actin were digested with restriction enzymes *Hin*dIII and *Pst*I and restriction enzymes *Hin*dIII and *Kpn*I, respectively, and the digested linear DNAs were

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used as the internal standards for β-actin amount determination. Details thereof are described below.

Chinese hamster β-actin and rat β-actin standards were prepared as follows. In 40 µl of NEBuffer 2 (manufactured by New England Biolabs), 2 µg of the plasmid CHAc-pBS was dissolved, and 25 units of a restriction enzyme *Hin*dIII (manufactured by Takara Shuzo) and 20 units of *Pst*I (manufactured by Takara Shuzo) were added thereto, followed by digestion reaction at 37°C for 3 hours. Separately, 2 µg of the plasmid YBAc-pBS was dissolved in 40 µl of NEBuffer 2 (manufactured by New England Biolabs), and 25 units of a restriction enzyme *Hin*dIII (manufactured by Takara Shuzo) and 25 units of *Kpn*I (manufactured by Takara Shuzo) were added thereto, followed by digestion reaction at 37°C for 3 hours. A portion of each of the reaction solutions was subjected to 0.8% agarose gel electrophoresis, and it was confirmed that a *Hin*dIII-*Pst*I fragment and a *Hin*dIII-*Kpn*I 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 restriction enzyme digestion reactions. Each of the reaction solutions was diluted with 1 µg/ml baker's yeast t-RNA (manufactured by SIGMA) to give a concentration 2 pg/µl, 1 pg/µl, 200 fg/µl, 100 fg/µl and 20 fg/µl and used as the Chinese hamster β-actin and or β-actin standards.

Chinese hamster β-actin and rat β-actin internal standards were prepared as follows (Fig. 23). In 100 µl of NEBuffer 3 (manufactured by New England Biolabs) containing 100 ng/µl of BSA (manufactured by New England Biolabs), 2 µg of CHAc-pBS was dissolved, and 10 units of a restriction enzyme *Dra*III (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

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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 µl of 1 mol/l Tris-HCl buffer (pH 8.0) and 3.5 µl 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 µl 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 1.1 Kb containing the ORF partial fragment of Chinese hamster β-actin.

The dephosphorylated *Dra*III-*Dra*III fragment (4.5 µl), 4.5 µl of the *Dra*III-*Dra*III fragment of about 1.1 Kb and 5 µl of Ligation High (manufactured by Toyobo) were mixed, followed by ligation reaction at 16°C for 30 minutes. Using the reaction solution, *E. coli* DH5α was transformed, and plasmid DNAs were isolated in accordance with a known method from the obtained ampicillin-resistant clones. 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 *Dra*III-*Dra*III 180 bp inserted into the plasmid was deleted. The plasmid is referred to as CHAcd-pBS.

Also, a plasmid in which rat β-actin *Dra*III-*Dra*III 180 bp was deleted was prepared via the same steps of CHAcd-pBS. The plasmid is referred to as YBAcd-pBS.

Next, 2 µg of the plasmid CHAcd-pBS was dissolved in 40 µl of NEBuffer 2 (manufactured by New England

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Biolabs), and 25 units of a restriction enzyme *Hin*dIII (manufactured by Takara Shuzo) and 20 units of *Pst*I (manufactured by Takara Shuzo) were added thereto, followed by digestion reaction at 37°C for 3 hours. Separately, 2 μg of the plasmid YBAcd-pBS was dissolved in 40 μl of NEBuffer 2 (manufactured by New England Biolabs), and 25 units of a restriction enzyme *Hin*dIII (manufactured by Takara Shuzo) and 24 units of *Kpn*I (manufactured by Takara Shuzo) were added thereto, followed by digestion reaction at 37°C for 3 hours. A portion of each of the reaction solutions was subjected to 0.8% agarose gel electrophoresis, and it was confirmed that an *Hin*dIII-*Pst*I fragment and *Hin*dIII-*Kpn*I 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 CHAcd-pBS and YBAcd-pBS by the restriction enzyme digestion reactions. Dilutions of 200 fg/μl were prepared from the reaction solutions using 1 μg/ml baker's yeast t-RNA (manufactured by SIGMA) and used as the Chinese hamster β-actin and rat β-actin internal controls.

(6) Determination of transcription amount by competitive PCR

Competitive PCR was carried out using the FUT8 internal control DNA prepared in the item (4) and the host cell-derived cDNA obtained in the item (1) as the templates, the determined value of the FUT8 transcription product in the host cell line 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 transcribed continuously in each cell and its transcription level is approximately the same between cells, transcription level of the β-actin gene was determined as a measure of the efficiency of synthesis

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reaction of cDNA in each host cell line. That is, the PCR was carried out using the β-actin internal control DNA prepared in the item (5) and the host cell-derived cDNA obtained in the item (1) as the templates, the determined value of the β-actin transcription product in the host cell line was calculated from the relative value of the amount of the amplified product derived from each template. Details thereof are described below.

The FUT8 transcription product was determined by the following procedure. First, a set of sequence-specific primers (shown in 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) were designed.

Next, PCR was carried out using a DNA polymerase ExTaq (manufactured by Takara Shuzo) in 20 μl in total volume of a reaction solution [ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/l dNTPs, 0.5 μmol/l gene-specific primers (SEQ ID NOs:13 and 14) and 5% DMSO] containing 5 μl of 50 folds-diluted cDNA solution prepared from each of respective host cell line in the item (1) and 5 μl (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.

Also, PCR was carried out in a series of reaction in which 5 μl (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) was added instead of the each host cell line-derived cDNA, and used in the preparation of a calibration curve for the FUT8 transcription level.

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) were designed (the former are

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shown in SEQ ID NOs:15 and 16, and the latter are shown in SEQ ID NOs:17 and 18).

Next, PCR was carried out using a DNA polymerase ExTaq (manufactured by Takara Shuzo) in 20 µl in total volume of a reaction solution [ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/l dNTPs, 0.5 µmol/l gene-specific primers (SEQ ID NOs:15 and 16, or SEQ ID NOs:17 and 18) and 5% DMSO] containing 5 µl of 50 folds-diluted cDNA solution prepared from respective host cell line in the item (1) and 5 µl (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 5 µl (10 pg, 5 pg, 1 pg, 500 fg or 100 fg) of the β-actin standard plasmid obtained in the item (5) was added instead of the each host cell line-derived cDNA, and used in the preparation of a calibration curve for the β-actin transcription level.

Table 3

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

* F: forward primer, R: reverse primer

By carrying out PCR using the primer set described in Table 3, a DNA fragment having a size shown in the

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target column of Table 3 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 3 can be amplified from each internal control.

A 7 µl portion of each of the solutions after PCR was subjected to 1.75% agarose gel electrophoresis, and then the gel was stained by soaking it for 30 minutes in 1 × 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).

The amount of an amplified product formed by PCR using a standard plasmid as the template was measured by the 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 cell line was calculated from the amount of the amplified product when each expression cell line-derived cDNA was used as the template, and the amount was defined as the mRNA transcription amount in each cell line.

The amount of the FUT8 transcription product in each host cell line when a rat FUT8 sequence was used in the standard and internal control is shown in Fig. 24. Throughout the culturing period, the CHO cell line showed a transcription amount 10 folds or higher than that of the YB2/0 cell line. The tendency was also found when a Chinese hamster FUT8 sequence was used in the standard and internal control.

Also, the FUT8 transcription amounts are shown in Table 4 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 line was

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around 0.1% of β-actin while it was 0.5% to 2% in the CHO cell line.

The results shows that the amount of the FUT8 transcription product in YB2/0 cell line was significantly smaller than that in the CHO cell line.

Table 4

	Culture days				
Cell line	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 10

Determination of transcription product of α-1,6-fucosyltransferase (FUT8) gene in anti-ganglioside GD3 chimeric antibody-producing cell line:

(1) Preparation of single-stranded cDNA from various antibody-producing cell lines

Single-stranded cDNA was prepared from anti-ganglioside GD3 chimeric antibody-producing cell lines DCHI01-20 and 61-33 as follows. The DCHI01-20 is a transformant clone derived from the CHO/DG44 cell described in item 2(2) of Example 1. Also, the 61-33 is a clone obtained by carrying out serum-free adaptation of YB2/0-derived transformant cell 7-9-51 (FERM BP-6691, International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology (AIST Tsukuba Central 6, 1-1, Higashi 1-Chome Tsukuba-shi, Ibaraki-ken 305-8566 Japan)) and then carrying out single cell isolation by two limiting dilution.

Cells of the DCHI01-20 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),

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and 15 ml of the suspension was inoculated into T75 flask for suspension cell culture use (manufactured by Greiner) at a density of 2×10^5 cells/ml. Also, cells of the 61-33 were suspended in Hybridoma-SFM medium (manufactured by Life Technologies) supplemented with 0.2% bovine serum albumin fraction V (manufactured by Life Technologies) (hereinafter referred to as "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% CO_2 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 RNAeasy (manufactured by QIAGEN) in accordance with the manufacture's instructions.

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

In a 20 µl reaction mixture using oligo(dT) as a primer, single-stranded cDNA was synthesized from 3 µg 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 folds with water and stored at -80°C until use.

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(2) Determination of transcription amounts of each gene by competitive PCR

The transcription amount of each of the genes on the cDNA derived from the antibody-producing cell line obtained in the item (1) was determined by competitive PCR in accordance with Example 9(6).

The FUT8 gene-derived mRNA transcription amount in each of the antibody-producing cell lines was determined by the following procedure.

CHFT8-pCR2.1 and YBFT8-pCR2.1, as plasmids in which cDNA partial fragments prepared in Example 9(2) from Chinese hamster FUT8 and rat FUT8, respectively, were inserted into pCR2.1, were digested with a restriction enzyme *Eco*RI, and the obtained linear DNAs were used as the standards in the preparation of a calibration curve for determining the FUT8 transcription amount.

CHFT8d-pCR2.1 and YBFT8d-pCR2.1, which were obtained by deleting 203 bp between *Sca*I and *Hin*dIII of an inner nucleotide sequence of Chinese hamster FUT8 and rat FUT8, respectively, in Example 9(4) were digested with a restriction enzyme *Eco*RI, and the obtained linear DNAs were used as the internal standards for FUT8 amount determination.

PCR was carried out using a DNA polymerase ExTaq (manufactured by Takara Shuzo) in 20 μl in total volume of a reaction solution [ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/l dNTPs, 0.5 μmol/l FUT8 gene-specific primers (SEQ ID NOs:13 and 14) and 5% DMSO] containing 5 μl of 50 folds-diluted cDNA solution prepared from each of the antibody-producing cell line in the item (1) and 5 μl (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.

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Also, PCR was carried out in a series of reaction in which 5 μl (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 cell line-derived cDNA, and used in the preparation of a calibration curve for the FUT8 transcription amount. In this case, 1 μg/ml of a baker's yeast t-RNA (manufactured by SIGMA) was used for the dilution of the standard plasmid.

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 cell line.

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 Example 9(3) were inserted into pBluescript II KS(+), respectively, were digested with restriction enzymes *Hin*dIII and *Kpn*I, and the obtained linear DNA samples were used as the standards in the preparation of a calibration curve for determining the β-actin transcription amount.

CHAcd-pBS and YBAcd-pBS which were obtained by deleting 180 bp between *Dra*I and *Dra*I of an inner nucleotide sequence of Chinese hamster β-actin and rat β-actin, respectively in Example 9(5), were digested with restriction enzymes *Hin*dIII and *Kpn*I, and the obtained linear DNAs were used as the internal standards for β-actin determination.

PCR was carried out using a DNA polymerase ExTaq (manufactured by Takara Shuzo) in 20 μl in total volume of a reaction solution [ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/l dNTPs, 0.5 μmol/l β-actin-specific primers (SEQ ID NOs:17 and 18) and 5% DMSO] containing 5 μl of 50 folds-diluted cDNA solution prepared from each of the

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antibody-producing cell lines and 5 μl (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 cell line-derived cDNA, and used in the preparation of a calibration curve for the β-actin transcription amount. In this case, 1 μg/ml of a baker's yeast t-RNA (manufactured by SIGMA) was used for the dilution of standard plasmid.

By PCR using the primer set described in Table 3, a DNA fragment having a size shown in the target column of Table 3 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 3 can be amplified from each internal control.

A 7 μl portion of each of the solutions after PCR was subjected to 1.75% agarose gel electrophoresis, and then the gel was stained by soaking it for 30 minutes in 1 × 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).

The amount of the amplified product formed by PCR which used a standard plasmid as the template was measured by the 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 cell line was calculated from the amount of the amplified product when each antibody-producing cell line-derived cDNA was used as

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the template, and the value was defined as the mRNA transcription amount in each cell line.

The FUT8 transcription amounts are shown in Table 5 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 61-33 was 0.3% or less of β-actin while it 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 cell line was significantly less than that in the antibody-producing cell line derived from the CHO cell.

Table 5

	Culture days				
Cell line	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 11

Preparation of mouse α-1,6-fucosyltransferase (FUT8) gene over-expressing cell line:

(1) Construction of mouse α-1,6-fucosyltransferase (FUT8) expression plasmid

Total RNA was extracted from 1×10^7 cells of a mouse myeloma NSO cell (RCB0213, Cell Bank at The Institute of Physical and Chemical Research) subcultured using IMDM medium (manufactured by Life Technologies) containing 10% fetal bovine serum (manufactured by Life Technologies), using RNAeasy (manufactured by QIAGEN) in accordance with the manufacture's instructions. The total RNA was dissolved in 45 μl of sterile water, and 1 μl of RQ1 RNase-Free DNase (manufactured by Promega), 5 μl of the attached 10 x DNase buffer and 0.5 μl of RNasin Ribonuclease

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Inhibitor (manufactured by Promega) were added thereto, followed by reaction at 37°C for 30 minutes to degrade genome DNA contaminated in the sample. After the reaction, the total RNA was purified again using RNAeasy (manufactured by QIAGEN) and dissolved in 50 µl of sterile water. In a 20 µl reaction mixture using oligo(dT) as a primer, single-stranded cDNA was synthesized from 3 µg of the obtained total RNA by reverse transcription reaction using SUPERSCRIPT™ Preamplification System for First Strand cDNA Synthesis (manufactured by Life Technologies) in accordance with the manufacture's instructions.

Mouse FUT8 cDNA was prepared by the following procedure (Fig. 25).

First, a forward primer specific for a sequence containing a translation initiation codon (shown in SEQ ID NO:19) and a reverse primer specific for a sequence containing translation termination codon (shown in SEQ ID NO:20) were designed from a mouse FUT8 cDNA sequence (GenBank, AB025198).

Next, 25 µl of a reaction solution [ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/l dNTPs, 4% DMSO and 0.5 µmol/l specific primers (SEQ ID NO:19 and SEQ ID NO:20)] containing 1 µl of the NSO cell-derived cDNA was prepared, and PCR was carried out 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 final heating at 72°C for 10 minutes.

After the PCR, the reaction solution was subjected to 0.8% agarose gel electrophoresis, and a specific amplified fragment of 1728 bp was purified. Into a plasmid pCR2.1, 4 µl of the DNA fragment was employed to insert in accordance with the manufacturer's instructions attached to TOPO TA Cloning Kit (manufactured by Invitrogen), and

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E. coli DH5α was transformed with the reaction solution. Plasmid DNAs were isolated in accordance with a known method from cDNA-inserted 6 clones among the obtained kanamycin-resistant colonies.

The nucleotide sequence of each cDNA inserted into the plasmid 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 all of the inserted cDNAs of which sequences were determined encode the ORF full sequence of mouse FUT8. Among these, a plasmid DNA containing absolutely no reading error of bases by the PCR in the sequences were selected (its DNA sequence and amino acid sequence are shown in SEQ ID NOs:2 and 24, respectively). Also, inconsistency of 3 bases due to amino acid substitution was found in the sequence when compared with the mouse FUT8 sequence registered on GenBank. Herein, the plasmid is referred to mfFUT8-pCR2.1.

Next, a plasmid pBSmfFUT8 containing the ORF full sequence of mouse FUT8 was constructed as follows (Fig. 26). First, 1 μg of a plasmid pBluescript II KS(+) (manufactured by Stratagene) was dissolved in 35 μl of NEBuffer 2 (manufactured by New England Biolabs), and 20 units of a restriction enzyme *Eco*RI (manufactured by Takara Shuzo) were added thereto, followed by digestion reaction at 37°C for 2 hours. To the reaction solution, 35 μl of 1 mol/l Tris-HCl buffer (pH 8.0) and 3.5 μl of *E. coli* C15-derived alkaline phosphatase (manufactured by Takara Shuzo) were added, followed by reaction at 65°C for 30 minutes for dephosphorylate the DNA termini. The reaction solution was extracted with phenol/chloroform, followed by ethanol precipitated, and the recovered DNA fragment was dissolved in 10 μl of sterile water.

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Separately, 1 μg of the plasmid mfFUT8-pCR2.1 was dissolved in 35 μl of NEBuffer 2 (manufactured by New England Biolabs), and 20 units of a restriction enzyme *Eco*RI (manufactured by Takara Shuzo) were added thereto, followed by digestion reaction at 37°C for 2 hours. The reaction solution was subjected to 0.8% agarose gel electrophoresis to purify a DNA fragment of about 1.7 Kb containing the ORF full sequence of mouse FUT8 cDNA.

The obtained plasmid pBluescript II KS(+)-derived *Eco*RI-*Eco*RI fragment (1 μl, 2.9 Kb), 4 μl of the *Eco*RI-*Eco*RI fragment (1.7 Kb) prepared from the plasmid mfFUT8-pCR2.1 and 5 μl of Ligation High (manufactured by Toyobo) were mixed, followed by ligation reaction at 16°C for 30 minutes. Using the reaction solution, *E. coli* DH5α was transformed, and plasmid DNAs were isolated in accordance with a known method from the obtained ampicillin-resistant clones. Herein, the plasmid is referred to pBSmfFUT8.

Using the pBSmfFUT8 and pAGE249, a mouse FUT8 expression vector pAGEmfFUT8 was constructed by the following procedure (Fig. 27). The pAGE249 is a derivative of pAGE248 [*J. Biol. Chem.*, 269, 14730 (1994)], as a vector in which an *Sph*I-*Sph*I fragment (2.7 Kb) containing a dihydrofolate reductase gene (dhfr) expression unit was removed from the pAGE248.

In 50 μl of Universal Buffer H (manufactured by Takara Shuzo), 1 μg of the pAGE249 was dissolved, and 20 units of a restriction enzyme *Sal*I (manufactured by New England Biolabs) were added thereto, followed by digestion reaction at 37°C for 2 hours. A DNA fragment was recovered from the reaction solution by ethanol precipitation and dissolved in 35 μl of NEBuffer 2 (manufactured by New England Biolabs), and 20 units of a restriction enzyme *Bam*HI (manufactured by New England Biolabs) were added thereto, followed by digestion reaction at 37°C for 2 hours. After the digestion reaction, to the reaction solution, 35

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μl of 1 mol/l Tris-HCl buffer (pH 8.0) and 3.5 μl of *E. coli* C15-derived alkaline phosphatase (manufactured by Takara Shuzo) were added thereto, followed by reaction at 65°C for 30 minutes to dephosphorylate the DNA termini. The reaction solution was extracted with phenol/chloroform extraction, followed by ethanol precipitation, and the recovered DNA fragment was dissolved in 10 μl of sterile water.

Separately, 1 μg of pBSmfFUT8 was dissolved in 50 μl of Universal Buffer H (manufactured by Takara Shuzo), and 20 units of a restriction enzyme *Sal*I (manufactured by New England Biolabs) were added thereto, followed by digestion reaction at 37°C for 2 hours. A DNA fragment was recovered from the reaction solution by ethanol precipitation and dissolved in 35 μl of NEBuffer 2 (manufactured by New England Biolabs), and 20 units of a restriction enzyme *Bam*HI (manufactured by New England Biolabs) were added thereto, followed by digestion reaction at 37°C for 2 hours. After the digestion reaction, the solution was subjected to 0.8% agarose gel electrophoresis to purify a DNA fragment of about 1.7 Kb containing the ORF full sequence of mouse FUT8 cDNA.

The obtained plasmid pAGE249-derived *Bam*HI-*Sal*I fragment (1 μl, 6.5 Kb), 4 μl of the *Bam*HI-*Sal*I fragment (1.7 Kb) prepared from the plasmid pBSmfFUT8 and 5 μl of Ligation High (manufactured by Toyobo) were mixed, followed by ligation reaction at 16°C for 30 minutes. Using the reaction solution, *E. coli* DH5α was transformed, and a plasmid DNA was isolated in accordance with a known method from the obtained ampicillin-resistant clones. Herein, the plasmid is referred to pAGEmfFUT8.

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(2) Preparation of mouse α-1,6-fucosyltransferase (FUT8) gene over-expressing cell line

A stable FUT8 gene-expressing cell line was obtained by introducing the mouse FUT8 expression vector pAGEmfFUT8 constructed in the item (1) into 61-33. The 61-33 is a clone obtained by carrying out serum-free adaptation of a transformant cell 7-9-51 (FERM BP-6691, International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology) derived from a YB2/0 cell highly producing an anti-ganglioside GD3 chimeric antibody, and then carrying out single cell isolation by two limiting dilution.

The plasmid pAGEmfFUT8 was transferred into 61-33 by the following procedure in accordance with the electroporation [*Cytotechnology*, 3, 133 (1990)]. First, 30 μg of the plasmid pAGEmfFUT8 was dissolved in 600 μl of NEBuffer 4 (manufactured by New England Biolabs), and 100 units of a restriction enzyme *Fsp*I (manufactured by New England Biolabs) were added thereto, followed by digestion reaction at 37°C for 2 hours to obtain a linear fragment. The reaction solution was subjected to ethanol precipitation, and the recovered linear plasmid was made into a 1 μg/μl aqueous solution. Next, the 61-33 was suspended in a K-PBS buffer (137 mol/l KCl, 2.7 mol/l NaCl, 8.1 mol/l Na_2HPO_4, 1.5 mol/l KH_2PO_4, 4.0 mol/l $MgCl_2$) to give a density of 2×10^7 cells/ml, and 200 μl of the cell suspension (4×10^6 cells) was mixed with 10 μl (10 μg) of the linear plasmid. The cell-DNA mixture was transferred into Gene Pulser Cuvette (inter-electrode distance, 2 mm) (manufactured by BIO-RAD) and then electroporation was carried out using a cell fusion apparatus Gene Pulser (manufactured by BIO-RAD) at 0.2 KV pulse voltage and 250 μF electric capacity. The cell suspension was mixed with 10 ml of Hybridoma-SFM medium (manufactured by Life Technologies) supplemented with 5% fetal bovine dialyzed

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serum (manufactured by Life Technologies) and 0.2% BSA (manufactured by Life Technologies) and dispensed in 100 µl portions into a 96 well plate for suspension cell use (manufactured by Greiner). After culturing them at 37°C for 24 hours in 5% CO_2, 50 µl of the culture supernatant was removed, and Hybridoma-SFM medium (manufactured by Life Technologies) supplemented with 0.5 mg/ml Hygromycin B (manufactured by Wako Pure Chemical Industries), 5% fetal bovine dialyzed serum (manufactured by Life Technologies) and 0.2% BSA (manufactured by Life Technologies) was dispensed at 100 µl. They were cultured for 3 weeks while repeating the medium exchange step at intervals of 3 to 4 days, and 14 cell lines showing hygromycin resistance were obtained.

On the other hand, a negative control cell line was prepared by introducing the plasmid pAGE249 as a parent vector of the pAGEmfFUT8 into the 61-33. According to the above procedure, 10 µg of the plasmid pAGE249 converted into linear form with a restriction enzyme *Fsp*I was introduced into 4×10^6 cells of the 61-33 using the electroporation. The cells were mixed with 15 ml of Hybridoma-SFM medium (manufactured by Life Technologies) supplemented with 5% fetal bovine dialyzed serum (manufactured by Life Technologies) and 0.2% BSA (manufactured by Life Technologies), transferred into a T75 flask for suspension cell (manufactured by Greiner) and then cultured at 37°C for 24 hours in 5% CO_2. After culturing them, a half of the culture supernatant (7.5 ml) was removed by centrifugation at 800 rpm for 4 minutes, and the cells were suspended in 7.5 ml of Hybridoma-SFM medium (manufactured by Life Technologies) supplemented with 0.5 mg/ml Hygromycin B (manufactured by Wako Pure Chemical Industries), 5% fetal bovine dialyzed serum (manufactured by Life Technologies) and 0.2% BSA (manufactured by Life Technologies) and transferred into the T75 flask for

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suspension cell (manufactured by Greiner). They were cultured for 3 weeks while repeating the medium exchange at intervals of 3 to 4 days, a hygromycin-resistant cell line was obtained.

(3) Analysis of expression level of α-1,6-fucosyltransferase (FUT8) gene in cell lines over-expressing the gene

Using 6 cell lines optionally selected from the 14 mouse FUT8-over expressing cell lines prepared from 61-33 in the item (2) and the negative control cell line, the FUT8 expression levels were compared using competitive RT-PCR.

Each of these over-expression cell lines was suspended in Hybridoma-SFM medium (manufactured by Life Technologies) supplemented with 0.5 mg/ml Hygromycin B (manufactured by Wako Pure Chemical Industries), 5% fetal bovine dialyzed serum (manufactured by Life Technologies) and 0.2% BSA (manufactured by Life Technologies) to give a density of 3×10^5 cells/ml and then transferred into a T75 flask for suspension cell culture use (manufactured by Greiner). After culturing them at 37°C for 24 hours in 5% CO_2, 1×10^7 of intact cells were recovered to extract total RNA using RNAeasy (manufactured by QIAGEN) in accordance with the manufacture's instructions. The total RNA was dissolved in 45 µl of sterile water, and 0.5 U/µl of RQ1 RNase-Free DNase (manufactured by Promega), 5 µl of the attached 10 × DNase buffer and 0.5 µl of RNasin Ribonuclease Inhibitor (manufactured by Promega) were added thereto, followed by reaction at 37°C for 30 minutes to degrade genome DNA contaminated in the sample. After the reaction, the total RNA was purified again using RNAeasy (manufactured by QIAGEN) and dissolved in 50 µl of sterile water.

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In a 20 µl reaction mixture using oligo(dT) as a primer, single-stranded cDNA was synthesized from 2.5 µg of the obtained total RNA 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 folds with water and the transcription amount of each gene was determined by the competitive PCR in accordance with Example 9(6).

The FUT8 gene-derived mRNA transcription amount in each expression cell line was determined by the following procedure.

YBFT8-pCR2.1, as a plasmid in which a cDNA partial fragment prepared in Example 9(2) from rat FUT8 was inserted into pCR2.1, was digested with a restriction enzyme *Eco*RI, and the obtained linear DNA was used as the standard in the preparation of a calibration curve for determining the FUT8 transcription amount.

Among the YBFT8-pCR2.1 prepared in Example 9(4), YBFT8d-pCR2.1 obtained by deleting 203 bp between *Sca*I and *Hind*III of an inner nucleotide sequence of rat FUT8 was digested with a restriction enzyme *Eco*RI, and the obtained linear DNA was used as the internal control for FUT8 determination.

PCR was carried out using a DNA polymerase ExTaq (manufactured by Takara Shuzo) in 20 µl in total volume of a reaction solution [ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/l dNTPs, 0.5 µmol/l FUT8 gene-specific primers (SEQ ID NOs:13 and 14) and 5% DMSO] containing 5 µl of 50 folds-diluted cDNA solution prepared from respective expression cell line in the above and 5 µl (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.

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Also, PCR was carried out in a series of reaction in which 5 μl (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 expression cell line-derived cDNA, and used in the preparation of a calibration curve for the FUT8 transcription amount. In this case, 1 μg/ml baker's yeast t-RNA (manufactured by SIGMA) was used for the dilution of standard plasmid.

On the other hand, since it is considered that the β-actin gene is transcribed constantly in each cell and its transcription level 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 expression cell line.

YBAc-pBS, as a plasmid in which the ORF full sequence of cDNA of rat β-actin was inserted into pBluescript II KS(+) prepared in Example 9(3), was digested with restriction enzymes *Hin*dIII and *Kpn*I, and the obtained linear DNA was used as the standard in the preparation of a calibration curve for determining the β-actin gene transcription amount.

YBAcd-pBS obtained from the YBAc-pBS by deleting 180 bp between *Dra*I and *Dra*I of an inner nucleotide sequence of rat β-actin was digested with restriction enzymes *Hin*dIII and *Kpn*I, and the obtained linear DNA was used as the internal standards for β-actin amount determination.

PCR was carried out using a DNA polymerase ExTaq (manufactured by Takara Shuzo) in 20 μl in total volume of a reaction solution [ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/l dNTPs, 0.5 μmol/l β-actin-specific primers (SEQ ID NOs:17 and 18) and 5% DMSO] containing 5 μl of 50 folds-diluted cDNA solution prepared from each of the expression cell lines and 5 μl (1 pg) of the plasmid for internal control. The PCR was carried out by heating at

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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 expression cell line-derived cDNA, and used in the preparation of a calibration curve for the β-actin transcription amount. In this case, 1 μg/ml baker's yeast t-RNA (manufactured by SIGMA) was used for diluting the standard plasmid.

By carrying out PCR using the primer set described in Table 3, a DNA fragment having a size shown in the target column of Table 3 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 3 can be amplified from each internal control.

Each (7 μl) of the solutions after PCR was subjected to a 1.75% agarose gel electrophoresis, and then the gel was stained by soaking it for 30 minutes in 1 x concentration SYBR Green I Nucleic Acid Gel Stain (manufactured by Molecular Probes). By calculating luminescence intensity of each amplified DNA fragment using a fluoro-imager (FluorImager SI; manufactured by Molecular Dynamics), the amount of the amplified DNA fragment was measured.

The amount of an amplified product formed by PCR using the standard plasmid as the template was measured by the 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 cell line was calculated from the amount of an amplified product when each expression cell line-derived cDNA was used as the template, and the amount was defined as the mRNA transcription amount in each cell line.

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Fig. 28 shows the FUT8 transcription amounts as relative values to the amount of β-actin transcription product. Three cell lines mfFUT8-1, mfFUT8-2 and mfFUT8-4 and the pAGE249-introduced cell line were cell lines having a relatively small FUT8 transcription amount, which was equivalent to 0.3 to 10% of a β-actin transcription amount. On the other hand, other three cell lines mfFUT8-3, mfFUT8-6 and mfFUT8-7 were cell lines having a relatively large FUT8 transcription amount, which was equivalent to 20 to 40% of a β-actin transcription amount.

(4) Purification of antibody produced by α-1,6-fucosyltransferase (FUT8) gene over-expressing cell line

Each of the six FUT8 gene over-expressing cell lines and one negative control cell line obtained in the item (2) was suspended in Hybridoma-SFM medium (manufactured by Life Technologies) supplemented with 200 nmol/l MTX, 0.5 mg/ml Hygromycin B (manufactured by Wako Pure Chemical Industries) and 0.2% BSA (manufactured by Life Technologies) to give a density of 2×10^5 cells/ml, and then 100 ml in total of the suspension was inoculated into three T225 flasks for suspension cell culture use (manufactured by IWAKI). After culturing them at 37°C for 7 to 9 days in a 5% CO_2 incubator, the number of intact cells was counted to confirm that their viability was almost the same (each 30% or less), and then each cell suspension was recovered. Each of the cell suspensions was centrifuged at 3,000 rpm at 4°C for 10 minutes, and the recovered supernatant was centrifuged at 10,000 rpm at 4°C for 1 hour and then filtered using PES Filter Unit (manufactured by NALGENE) having a pore diameter of 0.22 μm with 150 ml capacity.

Prosep-A HighCapacity (manufactured by bioPROCESSING) was packed in a 0.8 cm diameter column to a

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thickness of 2 cm and washed with 10 ml of 0.1 mol/l citrate buffer (pH 3.0) and 10 ml of 1 mol/l glycine/NaOH-0.15 mol/l NaCl buffer (pH 8.6) in that order to effect equilibration the carrier. Next, 100 ml of each of the culture supernatant was passed through the column and washed with 50 ml of 1 mol/l glycine/NaOH-0.15 mol/l NaCl buffer (pH 8.6). After washing them, the antibody absorbed to Prosep-A was eluted using 2.5 ml of a 0.1 mol/l citrate buffer (pH 3.0), the eluate was fractionated at 500 µl and each fraction was neutralized by mixing with 100 µl of 2 mol/l Tris-HCl (pH 8.5). Two fractions containing the antibody at a high concentration (1.2 ml in total) were selected by the BCA method [*Anal. Biochem.*, 150, 76 (1985)], combined and then dialyzed against 10 mol/l citrate buffer (pH 6.0) at 4°C for a whole day and night. After the dialysis, the antibody solution was recovered and subjected to sterile filtration using a 0.22 µm pore size Millex GV (manufactured by MILLIPORE).

(5) *In vitro* cytotoxic activity (ADCC activity) of antibody produced by mouse α-1,6-fucosyltransferase (FUT8) gene over-expressing cell line

In order to evaluate *in vitro* cytotoxic activity of the anti-GD3 antibodies purified in the item (4), ADCC activity was measured using a GD3-positive cell, human melanoma cultured cell line G-361 (RCB0991, Cell Bank at The Institute of Physical and Chemical Research).

The G-361 cells subcultured in RPMI1640 medium (manufactured by Life Technologies) containing 10% fetal bovine serum (manufactured by Life Technologies) (hereinafter referred to as "RPMI1640-FBS(10)") were suspended in 500 µl of RPMI1640-FBS(10) at a density of 1×10^6 cells, and 3.7 MBq of $Na_2{}^{51}CrO_4$ was added thereto, followed by culturing at 37°C for 30 minutes for labeling the cells with a radioisotope. After centrifugation at

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1,200 rpm for 5 minutes, the supernatant was discarded and the target cells were suspended in 5 ml of RPMI1640-FBS(10). The washing step was repeated three times and then the cell suspension was incubated for 30 minutes on ice for spontaneous dissociation of the radioactive substance. The washing step was again repeated twice and then the cells were suspended in 5 ml of RPMI1640-FBS(10) to thereby prepare 2×10^5 cells/ml of a target cell suspension.

On the other hand, 30 ml of peripheral blood was collected from a healthy person and 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 the mixture was gently overlaid on 4 ml of Lymphoprep (manufactured by NYCOMED PHARMA AS) and centrifuged at room temperature at 2,000 rpm for 30 minutes. The separated mononuclear cell fractions were collected from the centrifugation tubes, combined and then suspended in 30 ml of RPMI1640-FBS(10). After centrifugation at room temperature at 1,200 rpm for 5 minutes, the supernatant was discarded and the cells were suspended in 20 ml of RPMI1640-FBS(10). The washing step was repeated twice and then 2×10^6 cells/ml of an effector cell suspension was prepared using RPMI1640-FBS(10).

The target cell suspension was dispensed at 50 µl (1×10^4 cells/well) into each well of a 96 well U-bottom plate (manufactured by Falcon). Subsequently, the effector cell suspension was dispensed at 100 µl (2×10^5 cells/well) into each well to thereby adjust the ratio of the effector cells to the target cells to 20 : 1. Next, using a 10 M citrate buffer (pH 6.0), a series of dilution solution of 0.01 µg/ml, 0.1 µg/ml, 1 µg/ml and 10 µg/ml of each anti-GD3 antibody obtained in the item (4) was prepared, and the diluted solutions were dispensed at 50 µl into the wells to give final concentrations of 0.0025 µg/ml,

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0.025 μg/ml, 0.25 μg/ml and 2.5 μg/ml, respectively. After carrying out the reaction at 37°C for 4 hours in 5% CO_2, the plate was centrifuged at 1,200 rpm for 5 minutes. Into a 12 mm diameter RIA tube (manufactured by IWAKI), 50 μl of the supernatant in each well was transferred and, and the amount of the dissociated ^{51}Cr was measured using MINAX-γ auto-gamma counter 5550 (manufactured by PACKARD).

Also, the amount of the spontaneously dissociated ^{51}Cr was calculated by carrying out the same reaction in a reaction mixture in which 150 μl of RPMI1640-FBS(10) was added instead of the effector cell suspension and antibody solution. The amount of the total dissociated ^{51}Cr was calculated by carrying out the same reaction in a reaction mixture in which 100 μl of 1 N hydrochloric acid and 50 μl of RPMI1640-FBS(10) were added instead of the effector cell suspension and antibody solution. Using these values, the ADCC activity was calculated based on the formula (II) described in the item 2(3) of Example 2.

Fig. 29 shows ADCC activity of each of the anti-GD3 antibodies for G-361 cell. Three cell lines mfFUT8-1, mfFUT8-2 and mfFUT8-4 having a low FUT8 expression level as shown in Fig. 28 showed potent ADCC activity equivalent to that of the negative control pAGE249-introduced cell line. On the other hand, other three cell lines mfFUT8-3, mfFUT8-6 and mfFUT8-7 having a high FUT8 expression level as shown in Fig. 28 showed low ADCC activity equivalent to that of the anti-GD3 antibody produced from CHO cell. Based on these results, it was shown that the ADCC activity of produced antibodies can be controlled by regulating the expression level of FUT8 in host cells.

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(6) Sugar chain analysis of antibody produced by mouse α-1,6-fucosyltransferase (FUT8) gene over-expressing cell line

Sugar chains of the anti-GD3 antibodies purified in the item (4) were analyzed. The sugar chains binding to the antibodies produced by mfFUT8-6 and pAGE249-introduced cell lines were cleaved from proteins by subjecting the antibodies to hydrazinolysis [*Method of Enzymology*, 83, 263 (1982)]. After removing hydrazine by evaporation under a reduced pressure, *N*-acetylation was carried out by adding an aqueous ammonium acetate solution and acetic anhydride. After freeze-drying, fluorescence labeling by 2-aminopyridine [*J. Biochem.*, 95, 197 (1984)] was carrying out. A fluorescence-labeled sugar chain group (PA-treated sugar chain group) was separated from excess reagents using Superdex Peptide HR 10/30 column (manufactured by Pharmacia). The sugar chain fractions were dried using a centrifugation concentrator and used as a purified PA-treated sugar chain group. Next, the purified PA-treated sugar chain group was subjected to reverse phase HPLC analysis using a CLC-ODS column (manufactured by Shimadzu) (Fig. 30). When calculated from the peak area, the content of α-1,6-fucose-free sugar chains in mfFUT8-6 was 10%, and the content of α-1,6-fucose-bound sugar chains was 90%. The content of α-1,6-fucose-free sugar chains in pAGE249 was 20%, and the content of α-1,6-fucose-bound sugar chains was 80%. Based on these results, it was found that the content of α-1,6-fucose-bound sugar chains of a produced antibody is increased by over-expressing the FUT8 gene.

Fig. 30 shows elution patterns obtained by carrying out reverse phase HPLC analysis of each of PA-treated sugar chains prepared from antibodies produced by mfFUT8-6 and pAGE249-introduced cell lines. Figs. 30A and 30B show elution patterns of mfFUT8-6 and pAGE249, respectively. The relative fluorescence intensity and the elution time

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are plotted as the ordinate and the abscissa, respectively. Using a sodium phosphate buffer (pH 3.8) as buffer A and a sodium phosphate buffer (pH 3.8) + 0.5% 1-butanol as buffer B, the analysis was carried out by the following gradient.

Time (minute)	0	80	90	90.1	120
Buffer B (%)	0	60	60	0	0

Peaks (i) to (ix) shown in Fig. 30 and Fig. 31 show the following structures.

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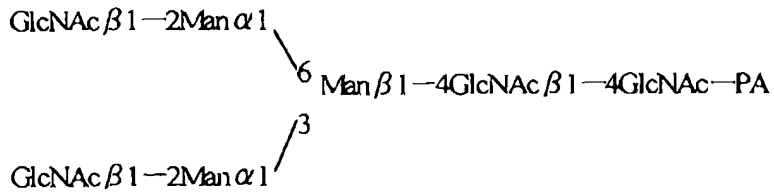
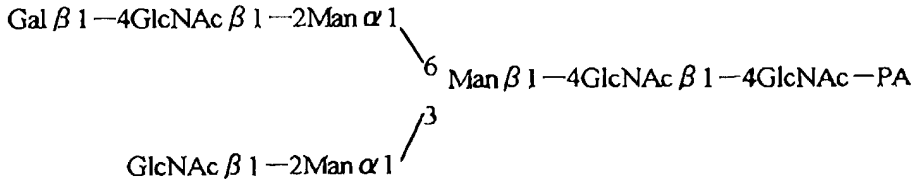
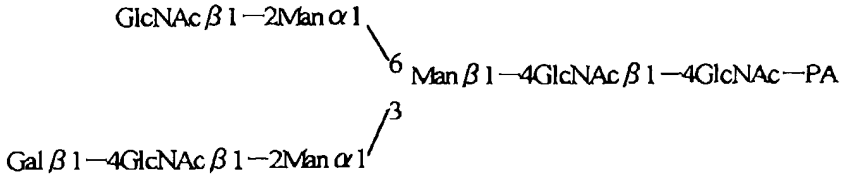
(i)



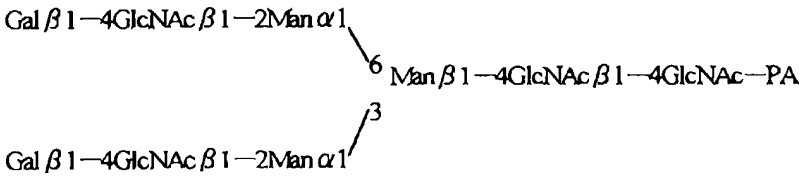
(ii)



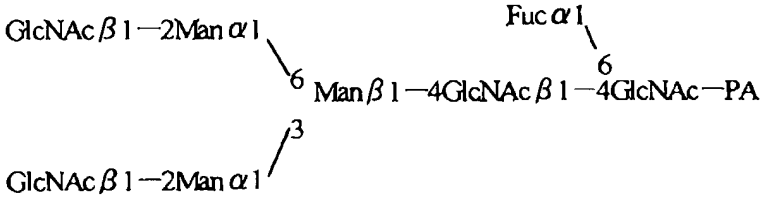
(iii)



(iv)



(v)



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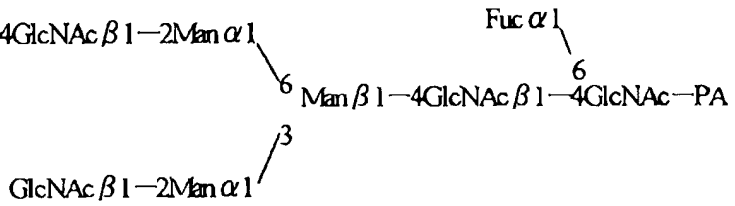
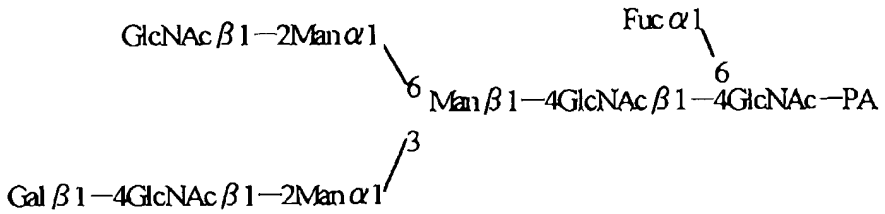
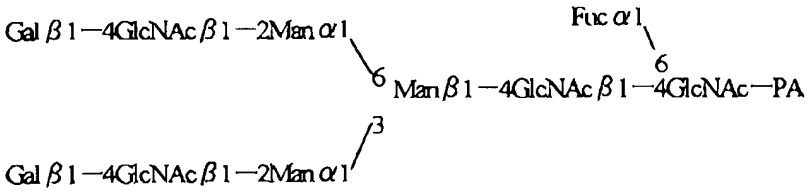
(vi)



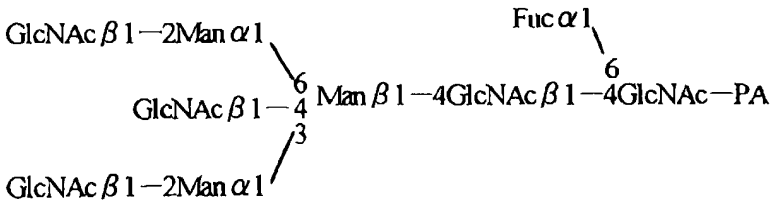
(vii)



(viii)



(ix)



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GlcNAc, Gal, Man, Fuc and PA indicate *N*-acetylglucosamine, galactose, mannose, fucose and a pyridylamino group, respectively. In Figs. 30 and 31, the ratio of the α-1,6-fucose-free sugar chain group was calculated from the area occupied by the peaks (i) to (iv) among (i) to (ix), and the ratio of the α-1,6-fucose-bound sugar chain group from the area occupied by the peaks (v) to (ix) among (i) to (ix).

Example 12

Preparation of CHO cell α-1,6-fucosyltransferase (FUT8) gene:

(1) Preparation of CHO cell α-1,6-fucosyltransferase (FUT8) cDNA sequence

From a single-stranded cDNA prepared from CHO/DG44 cells on the 2nd day of culturing in Example 9(1), Chinese hamster FUT8 cDNA was obtained by the following procedure (Fig. 32).

First, a forward primer specific for a 5'-terminal non-translation region (shown in SEQ ID NO:21) and a reverse primer specific for a 3'-terminal non-translation region (shown in SEQ ID NO:22) were designed from a mouse FUT8 cDNA sequence (GenBank, AB025198).

Next, 25 μl of a reaction solution [ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/l dNTPs, 4% DMSO and 0.5 μmol/l specific primers (SEQ ID NOs:21 and 22)] containing 1 μl of the CHO/DG44 cell-derived cDNA was prepared and PCR was carried out 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 final heating at 72°C for 10 minutes.

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

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amplified fragment of about 2 Kb was purified. Into a plasmid pCR2.1, 4 μl of the DNA fragment was employed to insert in accordance with the instructions attached to TOPO TA Cloning Kit (manufactured by Invitrogen), and *E. coli* DH5α was transformed with the reaction solution. Plasmid DNAs were isolated in accordance with a known method from cDNA-inserted 8 clones among the obtained kanamycin-resistant colonies.

The nucleotide sequence of each cDNA inserted into the plasmid 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 by the method that all of the inserted cDNAs encode a sequence containing the full ORF of CHO cell FUT8. Among these, a plasmid DNA containing absolutely no reading error of bases by the PCR in the sequences was selected. Herein, the plasmid is referred to as CHfFUT8-pCR2.1. The determined nucleotide sequence and the amino acid sequence of the cDNA of CHO FUT8 are shown in SEQ ID NOs:1 and 23, respectively.

(2) Preparation of CHO cell α-1,6-fucosyltransferase (FUT8) genomic sequence

Using the ORF full length cDNA fragment of CHO cell FUT8 obtained in the item (1) as a probe, a CHO cell FUT8 genomic clone was obtained in accordance with a known genome screening method described, e.g., in *Molecular Cloning*, Second Edition, *Current Protocols in Molecular Biology, A Laboratory Manual*, Second Edition (1989). Next, after digesting the obtained genomic clone using various restriction enzymes, the Southern hybridization was carried out using an *Afa*I-*Sau*3AI fragment (about 280 bp) containing initiation codon of the CHO cell FUT8 cDNA as a probe, and then a *Xba*I-*Xba*I fragment (about 2.5 Kb) and a *Sac*I-*Sac*I

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fragment (about 6.5 Kb) were selected from restriction enzyme fragments showing positive reaction, inserted into pBluescript II KS(+) (manufactured by Stratagene), respectively.

The nucleotide sequence of each of the obtained genomic fragments 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. Thereby, it was confirmed that the *XbaI-XbaI* fragment encodes a sequence of an upstream intron of about 2.5 Kb containing exon 2 of the CHO cell FUT8, and the *SacI-SacI* fragment encodes a sequence of a downstream intron of about 6.5 Kb containing exon 2 of the CHO cell FUT8. Herein, the plasmid containing *XbaI-XbaI* fragment is referred to as pFUT8fgE2-2, and the plasmid containing *SacI-SacI* fragment is referred to as pFUT8fgE2-4. The determined nucleotide sequence (about 9.0 Kb) of the genome region containing exon 2 of the CHO cell FUT8 is shown in SEQ ID NO:3.

Example 13

Preparation of CHO cell in which α-1,6-fucose transferase gene is disrupted and production of antibody using the cell:

A CHO cell from which the genome region comprising the CHO cell α-1,6-fucosyltransferase (FUT8) gene exon 2 was deleted was prepared and the ADCC activity of an antibody produced by the cell was evaluated.

1. Construction of Chinese hamster α-1,6-fucosyltransferase (FUT8) gene exon 2 targeting vector plasmid pKOFUT8Puro

(1) Construction of plasmid ploxPPuro

A plasmid ploxPPuro was constructed by the following procedure (Fig. 33).

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In 35 µl of NEBuffer 4 (manufactured by New England Biolabs), 1.0 µg of a plasmid pKOSelectPuro (manufactured by Lexicon) was dissolved, and 20 units of a restriction enzyme *Asc*I (manufactured by New England Biolabs) were added thereto, followed by digestion reaction at 37°C for 2 hours. After the digestion reaction, the solution was subjected to 0.8% (w/v) agarose gel electrophoresis to purify a DNA fragment of about 1.5 Kb containing a puromycin resistance gene expression unit.

On the other hand, 1.0 µg of a plasmid ploxP described in Japanese Published Examined Patent Application No. 314512/99 was dissolved in 35 µl of NEBuffer 4 (manufactured by New England Biolabs), and 20 units of a restriction enzyme *Asc*I (manufactured by New England Biolabs) were added thereto, followed by digestion reaction at 37°C for 2 hours. After the digestion reaction, the solution was subjected to 0.8% (w/v) agarose gel electrophoresis to purify a DNA fragment of about 2.0 Kb.

The obtained *Asc*I-*Asc*I fragment (4.5 µl, about 1.5 Kb) derived from the plasmid pKOSelectPuro, 0.5 µl of the *Asc*I-*Asc*I fragment (about 2.0 Kb) derived from the plasmid ploxP and 5.0 µl of Ligation High (manufactured by Toyobo) were mixed, followed by ligation reaction at 16°C for 30 minutes. *E. coli* DH5α was transformed using the reaction solution, and a plasmid DNA was isolated in accordance with a known method from the obtained ampicillin-resistant clones. Herein, the plasmid is referred to as ploxPPuro.

(2) Construction of plasmid pKOFUT8gE2-1

A plasmid pKOFUT8gE2-1 was constructed by the following procedure (Fig. 34), using the plasmid pFUT8fgE2-2 obtained in Example 12(2) having a genome region comprising exon 2 of Chinese hamster FUT8.

In 35 µl of NEBuffer 1 (manufactured by New England Biolabs) containing 100 µg/ml of BSA (manufactured by New

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England Biolabs), 2.0 μg of the plasmid pFUT8fgE2-2 was dissolved, and 20 units of a restriction enzyme *Sac*I (manufactured by New England Biolabs) were added thereto, followed by digestion reaction at 37°C for 2 hours. A DNA fragment was recovered from the reaction solution by ethanol precipitation and dissolved in 35 μl of NEBuffer 2 (manufactured by New England Biolabs) containing 100 μg/ml of BSA (manufactured by New England Biolabs), and 20 units of a restriction enzyme *Eco*RV (manufactured by New England Biolabs) were added thereto, followed by digestion reaction at 37°C for 2 hours. After the digestion reaction, the solution was subjected to 0.8% (w/v) agarose gel electrophoresis to purify a DNA fragment of about 1.5 Kb.

Separately, 1.0 μg of a plasmid LITMUS28 (manufactured by New England Biolabs) was dissolved in 35 μl of NEBuffer 1 (manufactured by New England Biolabs) containing 100 μg/ml of BSA (manufactured by New England Biolabs), and 20 units of a restriction enzyme *Sac*I (manufactured by New England Biolabs) were added thereto, followed by digestion reaction at 37°C for 2 hours. A DNA fragment was recovered from the reaction solution by ethanol precipitation and dissolved in 35 μl of NEBuffer 2 (manufactured by New England Biolabs) containing 100 μg/ml of BSA (manufactured by New England Biolabs), and 20 units of a restriction enzyme *Eco*RV (manufactured by New England Biolabs) were added thereto, followed by digestion reaction at 37°C for 2 hours. After the digestion reaction, the solution was subjected to 0.8% (w/v) agarose gel electrophoresis to purify a DNA fragment of about 2.8 Kb.

The obtained *Eco*RV-*Sac*I fragment (4.5 μl, about 1.5 Kb) derived from the plasmid pFUT8fgE2-2, 0.5 μl of the *Eco*RV-*Sac*I fragment (about 2.8 Kb) derived from the plasmid LITMUS28 and 5.0 μl of Ligation High (manufactured by Toyobo) were mixed, followed by ligation reaction at 16°C for 30 minutes. *E. coli* DH5α was transformed using the

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reaction solution, and a plasmid DNA was isolated in accordance with a known method from the obtained ampicillin-resistant clones. Herein, the plasmid is referred to as pKOFUT8gE2-1.

(3) Construction of plasmid pKOFUT8gE2-2

A plasmid pKOFUT8gE2-2 was constructed by the following procedure (Fig. 35), using the plasmid pKOFUT8gE2-1 obtained in the item (2).

In 30 µl of NEBuffer 2 (manufactured by New England Biolabs) containing 100 µg/ml of BSA (manufactured by New England Biolabs), 2.0 µg of the plasmid pKOFUT8gE2-1 was dissolved, and 20 units of a restriction enzyme *Eco*RV (manufactured by New England Biolabs) were added thereto, followed by digestion reaction at 37°C for 2 hours. A DNA fragment was recovered from the reaction solution by ethanol precipitation and dissolved in 30 µl of NEBuffer 1 (manufactured by New England Biolabs) containing 100 µg/ml of BSA (manufactured by New England Biolabs), and 20 units of a restriction enzyme *Kpn*I (manufactured by New England Biolabs) were added thereto, followed by digestion reaction at 37°C for 2 hours. After the digestion reaction, the solution was subjected to 0.8% (w/v) agarose gel electrophoresis to purify a DNA fragment of about 1.5 Kb.

Separately, 1.0 µg of the plasmid ploxPPuro was dissolved in 30 µl of NEBuffer 4 (manufactured by New England Biolabs), and 20 units of a restriction enzyme *Hpa*I (manufactured by New England Biolabs) were added thereto, followed by digestion reaction at 37°C for 2 hours. A DNA fragment was recovered from the reaction solution by ethanol precipitation and dissolved in 30 µl of NEBuffer 1 (manufactured by New England Biolabs) containing 100 µg/ml of BSA (manufactured by New England Biolabs), and 20 units of a restriction enzyme *Kpn*I (manufactured by New England Biolabs) were added thereto, followed by digestion reaction

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at 37°C for 2 hours. After the digestion reaction, the solution was subjected to 0.8% (w/v) agarose gel electrophoresis to purify a DNA fragment of about 3.5 Kb.

A 4.0 µl portion of the obtained *Eco*RV-*Kpn*I fragment (about 1.5 Kb) derived from the plasmid pKOFUT8gE2-1, 1.0 µl of the *Hpa*I-*Kpn*I fragment (about 3.5 Kb) derived from the plasmid ploxPPuro and 5.0 µl of Ligation High (manufactured by Toyobo) were mixed and allowed to undergo the ligation reaction at 16°C for 30 minutes. *E. coli* DH5α was transformed using the reaction solution, and a plasmid DNA was isolated in accordance with a known method from the obtained ampicillin-resistant clones. Herein, the plasmid is referred to pKOFUT8gE2-2.

(4) Construction of plasmid pscFUT8gE2-3

A plasmid pscFUT8gE2-3 was constructed by the following procedure (Fig. 36), using the plasmid pFUT8fgE2-4 obtained in Example 12(2) having a genome region comprising exon 2 of Chinese hamster FUT8.

In 35 µl of NEBuffer 1 (manufactured by New England Biolabs), 2.0 µg of the plasmid pFUT8fgE2-4 was dissolved, and 20 units of a restriction enzyme *Hpa*II (manufactured by New England Biolabs) were added thereto, followed by digestion reaction at 37°C for 2 hours. A DNA fragment was recovered from the reaction solution by ethanol precipitation, and then the DNA termini were changed to blunt ends using Blunting High (manufactured by Toyobo) in accordance with the manufacture's instructions. The DNA fragment was recovered by carrying out phenol/chloroform extraction and ethanol precipitation and dissolved in 35 µl of NEBuffer 2 (manufactured by New England Biolabs), and 20 units of a restriction enzyme *Hind*III (manufactured by New England Biolabs) were added thereto, followed by digestion reaction at 37°C for 2 hours. After the digestion reaction,

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the solution was subjected to 0.8% (w/v) agarose gel electrophoresis to purify a DNA fragment of about 3.5 Kb.

On the other hand, 1.0 μg of a plasmid LITMUS39 (manufactured by New England Biolabs) was dissolved in 35 μl of NEBuffer 2 (manufactured by New England Biolabs), and the solution was mixed with 20 units of a restriction enzyme *Eco*RV (manufactured by New England Biolabs) and 20 units of a restriction enzyme *Hind*III (manufactured by New England Biolabs) and subjected to the digestion reaction at 37°C for 2 hours. After the digestion reaction, the solution was subjected to 0.8% (w/v) agarose gel electrophoresis to purify a DNA fragment of about 2.8 Kb.

The obtained *Hpa*II-*Hind*III fragment (4.0 μl, about 3.5 Kb) derived from the plasmid pFUT8fgE2-4, 1.0 μl of the *Eco*RV-*Hind*III fragment (about 2.8 Kb) derived from the plasmid LITMUS39 and 5.0 μl of Ligation High (manufactured by Toyobo) were mixed, followed by ligation reaction at 16°C for 30 minutes. *E. coli* DH5α was transformed using the reaction solution, and a plasmid DNA was isolated in accordance with a known method from the obtained ampicillin-resistant clones. Herein, the plasmid is referred to pscFUT8gE2-3.

(5) Construction of plasmid pKOFUT8gE2-3

A plasmid pKOFUT8gE2-3 was constructed by the following procedure (Fig. 37), using the plasmid pFUT8fgE2-4 obtained in Example 12(2) having a genome region comprising exon 2 of Chinese hamster FUT8.

In 35 μl of NEBuffer for *Eco*RI (manufactured by New England Biolabs), 2.0 μg of the plasmid pFUT8fgE2-4 was dissolved, and 20 units of a restriction enzyme *Eco*RI (manufactured by New England Biolabs) and 20 units of a restriction enzyme *Hind*III (manufactured by New England Biolabs) were added thereto, followed by digestion reaction at 37°C for 2 hours. After the digestion reaction, the

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solution was subjected to 0.8% (w/v) agarose gel electrophoresis to purify a DNA fragment of about 1.8 Kb.

Separately, 1.0 μg of a plasmid pBluescript II KS(+) (manufactured by Stratagene) was dissolved in 35 μl of NEBuffer for *Eco*RI (manufactured by New England Biolabs), and 20 units of a restriction enzyme *Eco*RI (manufactured by New England Biolabs) and 20 units of a restriction enzyme *Hin*dIII (manufactured by New England Biolabs) were added thereto, followed by digestion reaction at 37°C for 2 hours. After the digestion reaction, the solution was subjected to 0.8% (w/v) agarose gel electrophoresis to purify a DNA fragment of about 3.0 Kb.

The obtained *Hin*dIII-*Eco*RI fragment (4.0 μl, about 1.8 Kb) derived from the plasmid pFUT8fgE2-4, 1.0 μl of the *Hin*dIII-*Eco*RI fragment (about 3.0 Kb) derived from the plasmid pBluescript II KS(+) and 5.0 μl of Ligation High (manufactured by Toyobo) were mixed, followed by ligation reaction at 16°C for 30 minutes. *E. coli* DH5α was transformed using the reaction solution, and a plasmid DNA was isolated in accordance with a known method from the obtained ampicillin-resistant clones. Herein, the plasmid is referred to pKOFUT8gE2-3.

(6) Construction of plasmid pKOFUT8gE2-4

A plasmid pKOFUT8gE2-4 was constructed by the following procedure (Fig. 38), using the plasmids pscFUT8fgE2-3 and pKOFUT8gE2-3 obtained in the items (4) and (5).

In 35 μl of NEBuffer for *Sal*I (manufactured by New England Biolabs) containing 100 μg/ml of BSA (manufactured by New England Biolabs), 1.0 μg of the plasmid pscFUT8gE2-3 was dissolved, and 20 units of a restriction enzyme *Sal*I (manufactured by New England Biolabs) were added thereto, followed by digestion reaction at 37°C for 2 hours. A DNA fragment was recovered from the reaction solution by

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ethanol precipitation and dissolved in 30 µl of NEBuffer 2 (manufactured by New England Biolabs) containing 100 µg/ml of BSA (manufactured by New England Biolabs), and 20 units of a restriction enzyme *Hin*dIII (manufactured by New England Biolabs) were added thereto, followed by digestion reaction at 37°C for 2 hours. After the digestion reaction, the solution was subjected to 0.8% (w/v) agarose gel electrophoresis to purify a DNA fragment of about 3.6 Kb.

Separately, 1.0 µg of the plasmid pKOFUT8gE2-3 was dissolved in 35 µl of NEBuffer for *Sal*I (manufactured by New England Biolabs), and 20 units of a restriction enzyme *Sal*I (manufactured by New England Biolabs) were added thereto, followed by digestion reaction at 37°C for 2 hours. A DNA fragment was recovered from the reaction solution by ethanol precipitation and dissolved in 35 µl of NEBuffer 2 (manufactured by New England Biolabs), and 20 units of a restriction enzyme *Hin*dIII (manufactured by New England Biolabs) were added thereto, followed by digestion reaction at 37°C for 2 hours. After the digestion reaction, 35 µl of 1 mol/l Tris-HCl buffer (pH 8.0) and 3.5 µl of *E. coli* C15-derived alkaline phosphatase (manufactured by Takara Shuzo) were added thereto, followed by reaction at 65°C for 30 minutes to dephosphorylate the DNA termini. After the dephosphorylation treatment, a DNA fragment was recovered by carrying out phenol/chloroform extraction and ethanol precipitation and dissolved in 10 µl of sterile water.

The obtained *Sal*I-*Hin*dIII fragment (4.0 µl, about 3.1 Kb) derived from the plasmid pscFUT8gE2-3, 1.0 µl of the *Sal*I-*Hin*dIII fragment (about 4.8 Kb) derived from the plasmid pKOFUT8gE2-3 and 5.0 µl of Ligation High (manufactured by Toyobo) were mixed, followed by ligation reaction at 16°C for 30 minutes. *E. coli* DH5α was transformed using the reaction solution, and a plasmid DNA was isolated in accordance with a known method from the

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obtained ampicillin-resistant clones. Herein, the plasmid is referred to pKOFUT8gE2-4.

(7) Construction of plasmid pKOFUT8gE2-5

A plasmid pKOFUT8gE2-5 was constructed by the following procedure (Fig. 39), using the plasmids pKOFUT8gE2-2 and pKOFUT8gE2-4 obtained in the items (3) and (6).

In 30 µl of NEBuffer 4 (manufactured by New England Biolabs), 1.0 µg of the plasmid pKOFUT8gE2-2 was dissolved, and 20 units of a restriction enzyme *Sma*I (manufactured by New England Biolabs) were added thereto, followed by digestion reaction at 25°C for 2 hours. A DNA fragment was recovered from the reaction solution by ethanol precipitation and dissolved in 30 µl of NEBuffer 2 (manufactured by New England Biolabs), and 20 units of a restriction enzyme *Bam*HI (manufactured by New England Biolabs) were added thereto, followed by digestion reaction at 37°C for 2 hours. After the digestion reaction, 30 µl of 1 mol/l Tris-HCl buffer (pH 8.0) and 3.0 µl of *E. coli* C15-derived alkaline phosphatase (manufactured by Takara Shuzo) were added thereto, followed by reaction at 65°C for 1 hour to dephosphorylate the DNA termini. After the dephosphorylation treatment, the DNA fragment was recovered by carrying out phenol/chloroform extraction and ethanol precipitation and dissolved in 10 µl of sterile water.

Separately, 1.0 µg of the plasmid pKOFUT8gE2-4 was dissolved in 30 µl of NEBuffer 4 (manufactured by New England Biolabs), and 20 units of a restriction enzyme *Sma*I (manufactured by New England Biolabs) were added thereto, followed by digestion reaction at 25°C for 2 hours. A DNA fragment was recovered from the reaction solution by ethanol precipitation and dissolved in 30 µl of NEBuffer 2 (manufactured by New England Biolabs), and 20 units of a restriction enzyme *Bam*HI (manufactured by New England

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Biolabs) were added thereto, followed by digestion reaction at 37°C for 2 hours. After the digestion reaction, the solution was subjected to 0.8% (w/v) agarose gel electrophoresis to purify a DNA fragment of about 5.2 Kb.

The obtained *Sma*I-*Bam*HI fragment (0.5 µl, about 5.0 Kb) derived from the plasmid pKOFUT8gE2-2, 4.5 µl of the *Sma*I-*Bam*HI fragment (about 5.4 Kb) derived from the plasmid pKOFUT8gE2-4 and 5.0 µl of Ligation High (manufactured by Toyobo) were mixed, followed by ligation reaction at 16°C for 15 hours. *E. coli* DH5α was transformed using the reaction solution, and a plasmid DNA was isolated in accordance with a known method from the obtained ampicillin-resistant clones. Herein, the plasmid is referred to pKOFUT8gE2-5.

(8) Construction of plasmid pKOFUT8Puro

A plasmid pKOFUT8Puro was constructed by the following procedure (Fig. 40), using the plasmid pKOFUT8gE2-5 obtained in the item (7).

In 50 µl of NEBuffer 4 (manufactured by New England Biolabs), 1.0 µg of a plasmid pKOSelectDT (manufactured by Lexicon) was dissolved, and 16 units of a restriction enzyme *Rsr*II (manufactured by New England Biolabs) were added thereto, followed by digestion reaction at 37°C for 2 hours. After the digestion reaction, the solution was subjected to 0.8% (w/v) agarose gel electrophoresis to purify a DNA fragment of about 1.2 Kb comprising a diphtheria toxin expression unit.

Separately, 1.0 µg of the plasmid pKOFUT8gE2-5 was dissolved in 50 µl of NEBuffer 4 (manufactured by New England Biolabs), and 16 units of a restriction enzyme *Rsr*II (manufactured by New England Biolabs) were added thereto, followed by digestion reaction at 37°C for 2 hours. After the digestion reaction, 30 µl of 1 mol/l Tris-HCl buffer (pH 8.0) and 3.0 µl of *E. coli* C15-derived alkaline

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phosphatase (manufactured by Takara Shuzo) were added thereto, followed by reaction at 65°C for 1 hour to dephosphorylate the DNA termini. After the dephosphorylation treatment, the DNA fragment was recovered by carrying out phenol/chloroform extraction and ethanol precipitation and dissolved in 10 µl of sterile water.

The obtained *Rsr*II-*Rsr*II fragment (1.0 µl, about 1.2 Kb) derived from the plasmid pKOSelectDT, 1.0 µl of the *Rsr*II-*Rsr*II fragment (about 10.4 Kb) derived from the plasmid pKOFUT8gE2-5, 3.0 µl of sterile water and 5.0 µl of Ligation High (manufactured by Toyobo) were mixed, followed by ligation reaction at 16°C for 30 minutes. *E. coli* DH5α was transformed using the reaction solution, and a plasmid DNA was isolated in accordance with a known method from the obtained ampicillin-resistant clones. Herein, the plasmid is referred to pKOFUT8Puro.

2. Preparation of CHO cell in which one copy of the genome region containing α-1,6-fucosyltransferase (FUT8) gene exon 2 was disrupted

(1) Introduction of targeting vector

A Chinese hamster FUT8 genome region targeting vector pKOFUT8Puro constructed in the item 1 of this Example was introduced into the strain 5-03 prepared in the item 1(2) of Example 8.

A gene of the plasmid pKOFUT8Puro was introduced into the strain 5-03 as described below in accordance with the electroporation method [*Cytotechnology*, 3, 133 (1990)]. First, 150 µg of the plasmid pKOFUT8Puro was dissolved in 1.8 ml of NEBuffer for *Sal*I (manufactured by New England Biolabs), and 600 units of a restriction enzyme *Sal*I (manufactured by New England Biolabs) were added thereto, followed by digestion reaction at 37°C for 5 hours to obtain a linear fragment. The reaction solution was extracted with phenol/chloroform extraction, followed by

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ethanol precipitation, and the recovered linear plasmid was made into a 1 μg/μl aqueous solution. Separately, the strain 5-03 was suspended in a K-PBS buffer (137 mmol/l KCl, 2.7 mmol/l NaCl, 8.1 mmol/l Na_2HPO_4, 1.5 mmol/l KH_2PO_4, 4.0 mmol/l $MgCl_2$) to give a density of 8×10^7 cells/ml. After mixing 200 μl of the cell suspension (1.6×10^6 cells) with 4 μl (4 μg) of the linear plasmid, an entire volume of the cell-DNA mixture was transferred into Gene Pulser Cuvette (inter-electrode distance, 2 mm) (manufactured by BIO-RAD) and then the electroporation was carried out using a cell fusion apparatus Gene Pulser (manufactured by BIO-RAD) at 350 V pulse voltage and 250 μF electric capacity. After carrying out the electroporation using 30 cuvettes in the same manner, the cell suspension was suspended in IMDM medium (manufactured by Life Technologies) supplemented with 10% fetal bovine serum (manufactured by Life Technologies) and 1 × concentration HT supplement (manufactured by Life Technologies) and inoculated into 30 adhesion cell culture dishes of 10 cm in diameter (manufactured by Falcon). After culturing them at 37°C for 24 hours in 5% CO_2, the culture supernatant was removed, and IMDM medium (manufactured by Life Technologies) supplemented with 15 μg/ml puromycin (manufactured by SIGMA) and 10% fetal bovine dialyzed serum (manufactured by Life Technologies) was dispensed in 10 ml portions. After culturing them for 10 days while repeating the medium exchange at intervals of 3 to 4 days, puromycin-resistant cell lines were obtained.

(2) Preparation of targeting vector-introduced cell lines

Arbitrary 900 colonies were obtained as follows from the puromycin-resistant cell lines obtained in the item (1).

First, culture supernatant was removed from the 10 cm dish in which colony of puromycin-resistant cell lines

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were formed and 7 ml of a phosphate buffer was added to the dish which was subsequently set under a stereoscopic microscope. Next, each colony was scratched and sucked up using Pipetteman (manufactured by GILSON) and transferred into a 96 well round-bottom plate (manufactured by Falcon). After a trypsin-treatment, each clone was inoculated into a 96 well flat-bottom plate for adhesion cell culture use (manufactured by Iwaki Glass) and cultured for 1 week using IMDM medium (manufactured by Life Technologies) supplemented with 15 μg/ml puromycin (manufactured by SIGMA) and 10% fetal bovine dialyzed serum (manufactured by Life Technologies).

After culturing them, each clone in the plate was subjected to trypsin treatment and then mixed with two volumes of a freeze drying medium (20% DMSO, 40% fetal bovine serum, 40% IMDM). A half of the mixture was inoculated into a 96 well flat-bottom plate for adhesion cell culture (manufactured by Iwaki Glass) as a replica plate, while the remaining half of the mixture was subjected to cryopreservation as master plates. The replica plate was cultured for 1 week using IMDM medium (manufactured by Life Technologies) supplemented with 15 μg/ml of puromycin (manufactured by SIGMA) and 10% fetal bovine dialyzed serum (manufactured by Life Technologies).

(3) Diagnosis of homologous recombination by genomic PCR

Diagnosis of homologous recombination in the 900 clones obtained in the item (2) was carried out by genomic PCR.

First, genome DNA of each clone was prepared from the replica plate prepared in the item (2) in accordance with a known method [*Analytical Biochemistry*, 201, 331 (1992)] and dissolved overnight in 30 μl of a TE-RNase buffer (pH 8.0) (10 mmol/l Tris-HCl, 1 mmol/l EDTA, 200 μg/ml RNase A). Also, a primer (shown in SEQ ID NO:26)

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which binds to a sequence outside the targeting vector homologous region among the FUT8 genome region obtained in Example 12 and a primer (shown in SEQ ID NO:27) which binds to the loxP sequence in the vector were designed.

Using a DNA polymerase ExTaq (manufactured by Takara Shuzo), 25 μl of a reaction solution [ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/l dNTPs and 0.5 μmol/l gene-specific primers (SEQ ID NO:26 and SEQ ID NO:27)] containing 10 μl of each the above-prepared genome DNA solution were prepared, and polymerase chain reaction (PCR) was carried out. The PCR was carried out by heating at 94°C for 3 minutes and subsequent 38 cycles of heating using the reaction at 94°C for 1 minute, 60°C for 1 minute and 72°C for 2 minutes as one cycle.

After the PCR, the reaction solution was subjected to 0.8% (w/v) agarose gel electrophoresis, and a specifically amplifying fragment of about 1.7 Kb containing a border region between the CHO cell genome region and the targeting vector homologous region was identified as a positive clone. One positive clone was found by the method.

(4) Diagnosis of homologous recombination by genome Southern blotting

Diagnosis of homologous recombination in the 1 clone, whose positive signal was confirmed in the item (3), was carried out by genome Southern blotting.

Among the master plates cryo-preserved in the item (2), a 96 well plate containing the positive clone found in the item (3) was selected and incubated at 37°C for 10 minutes in 5% CO_2. After the incubation, cells were collected from a well corresponding to the positive clone and inoculated into a 24 well flat bottom plate for adhesion cell (manufactured by Greiner). After culturing them for 1 week using IMDM medium (manufactured by Life Technologies) supplemented with 15 μg/ml of puromycin

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(manufactured by SIGMA) and 10% fetal bovine dialyzed serum (manufactured by Life Technologies), the cells were inoculated into a 6 well flat bottom plate for adhesion cell (manufactured by Greiner). Genome DNA was prepared from the clone in the plate in accordance with a known method [*Nucleic Acids Research*, 3, 2303 (1976)] and dissolved overnight in 150 µl of a TE-RNase buffer (pH 8.0) (10 mmol/l Tris-HCl, 1 mmol/l EDTA, 200 µg/ml RNase A).

In 120 µl of NEBuffer 3 (manufactured by New England Biolabs), 12 µg of the obtained genome DNA was dissolved, and 25 unites of a restriction enzyme *Pst*I (manufactured by New England Biolabs) were added thereto, followed by digestion reaction at 37°C overnight. A DNA fragment was recovered from the reaction solution by ethanol precipitation, dissolved in 20 µl of TE buffer (pH 8.0) (10 mmol/l Tris-HCl, 1 mmol/l EDTA) and then subjected to 0.8% (w/v) agarose gel electrophoresis. After the electrophoresis, the genome DNA was transferred onto a nylon membrane in accordance with a known method [*Proc. Natl. Acad. Sci. USA*, 76, 3683 (1979)]. After completion of the transfer, the nylon membrane was heated at 80°C for 2 hours.

Separately, a probe used in the Southern blotting was prepared as follows. First, primers (SEQ ID NOs:28 and 29) which bind to a sequence outside the targeting vector homologous region with the FUT8 genome region obtained in Example 12 were designed. Next, using a DNA polymerase ExTaq (manufactured by Takara Shuzo), 20 µl of a reaction solution [ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/l dNTPs and 0.5 µmol/l gene-specific primers (SEQ ID NOs:28 and 29)] containing 4.0 ng of the plasmid pFUT8fgE2-2 obtained in Example 12(2) was prepared, and polymerase chain reaction (PCR) was carried out. The PCR was carried out by heating at 94°C for 1 minute and subsequent 25 cycles of heating at 94°C for 30 seconds, 55°C for 30

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seconds and 74°C for 1 minute as one cycle. After the PCR, the reaction solution was subjected to 1.75% (w/v) agarose gel electrophoresis to purify a probe DNA fragment of about 230 bp. The obtained probe DNA solution (5 µl) was labeled with a radioisotope using 1.75 MBq of [α-^{32}P]dCTP and Megaprime DNA Labeling System, dCTP (manufactured by Amersham Pharmacia Biotech).

The hybridization was carried out as follows. First, the nylon membrane was sealed in a roller bottle, and pre-hybridization was carried out at 65°C for 3 hours by adding 15 ml of a hybridization solution [5 × SSPE, 50 × Denhaldt's solution, 0.5% (w/v) SDS, 100 µg/ml salmon sperm DNA]. Next, the ^{32}P-labeled probe DNA was heat-denatured and put into the bottle. Then, the nylon membrane was heated at 65°C overnight.

After the hybridization, the nylon membrane was soaked in 50 ml of 2 × SSC-0.1% (w/v) SDS and heated at 65°C for 15 minutes. After repeating the washing step twice, the membrane was soaked in 50 ml of 0.2 × SSC-0.1% (w/v) SDS and heated at 65°C for 15 minutes. After the washing, the nylon membrane was exposed to an X-ray film at -80°C for two nights for development.

By the restriction enzyme *Pst*I treatment, a DNA fragment of about 4.4 Kb is formed from a wild type FUT8 allele. On the other hand, a DNA fragment of about 6.0 Kb is formed from an allele in which homologous recombination with a targeting vector was generated.

By the method, such specific fragments of about 4.4 Kb and about 6.0 Kb were found from the positive clone genome DNA in the item (3). Since the quantitative ratio of both fragments was 1 : 1, it was confirmed that the clone is a clone in which 1 copy of the FUT8 allele was disrupted. Hereinafter, the clone is referred to as the strain 1st.ΔFUT8 2-46.

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3. Deletion of drug resistance gene from CHO cell in which 1 copy of the α-1,6-fucosyltransferase (FUT8) gene was disrupted

(1) Introduction of Cre recombinase expression vector

A Cre recombinase expression vector pBS185 (manufactured by Life Technologies) was introduced into the strain 1st.ΔFUT8 2-46 prepared in the item 2 of this Example.

A gene of the plasmid pBS185 was introduced into the strain 1st.ΔFUT8 2-46 as follows in accordance with the electroporation method [*Cytotechnology*, 3, 133 (1990)]. First, the strain 1st.ΔFUT8 2-46 was suspended in a K-PBS buffer (137 mmol/l KCl, 2.7 mmol/l NaCl, 8.1 mmol/l Na_2HPO_4, 1.5 mmol/l KH_2PO_4, 4.0 mmol/l $MgCl_2$) to give a density of 8×10^7 cells/ml. After mixing 200 µl of the cell suspension (1.6×10^6 cells) with 4 µg of the plasmid pBS185, an entire volume of the cell-DNA mixture was transferred into Gene Pulser Cuvette (inter-electrode distance, 2 mm) (manufactured by BIO-RAD) and then the gene transfer was carried out using a cell fusion apparatus Gene Pulser (manufactured by BIO-RAD) at 350 V pulse voltage and 250 µF electric capacity. After the gene transfer, the cell suspension was suspended in 10 ml of IMDM medium (manufactured by Life Technologies) supplemented with 10% fetal bovine serum (manufactured by Life Technologies) and 1 x concentration HT supplement (manufactured by Life Technologies) and further diluted 20,000 folds using the same medium. The cells were inoculated into 7 adhesion cell culture dishes of 10 cm in diameter (manufactured by Falcon) and then cultured at 37°C for 24 hours in 5% CO_2. After culturing them, the culture supernatant was removed and IMDM medium (manufactured by Life Technologies) supplemented with 10% fetal bovine dialyzed serum (manufactured by Life Technologies) was dispensed at 10 ml.

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Culturing was carried out for 10 days while repeating the medium exchange at intervals of 3 to 4 days.

(2) Preparation of Cre recombinase expression vector-introduced cell lines

Arbitrary 400 colonies were obtained as follows from the cell line obtained in the item (1).

First, culture supernatant was removed from the 10 cm dish and 7 ml of a phosphate buffer was added to the dish which was subsequently set under a stereoscopic microscope. Next, each colony was scratched and sucked up using Pipetteman (manufactured by GILSON) and transferred into a 96 well round-bottom plate (manufactured by Falcon). After a trypsin-treatment, each clone was inoculated into a 96 well flat-bottom plate for adhesion cell culture (manufactured by Iwaki Glass) and cultured for 1 week using IMDM medium (manufactured by Life Technologies) supplemented with 10% fetal bovine dialyzed serum (manufactured by Life Technologies).

After the culturing, each clone in the plate was subjected to trypsin treatment and then mixed with two volumes of a freeze drying medium (20% DMSO, 40% fetal bovine serum, 40% IMDM). A half of the mixture was inoculated into a 96 well flat-bottom plate for adhesion cell culture use (manufactured by Iwaki Glass) to prepare a replica plate, while the remaining half was subjected to cryopreservation as a master plate.

Next, the replica plate was cultured for 6 days using IMDM medium (manufactured by Life Technologies) supplemented with 15 μg/ml of puromycin (manufactured by SIGMA) and 10% fetal bovine dialyzed serum (manufactured by Life Technologies). A positive clone from which the puromycin resistance gene interposed between loxP sequences was eliminated by the expression of Cre recombinase dies

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out in the presence of puromycin. By the selection method, 91 positive clones were found.

(3) Diagnosis of drug resistance gene elimination by genome Southern blotting

Diagnosis of drug resistance gene elimination by the genome Southern blotting was carried out on optional 6 clones among the positive clones found in the item (2).

Among the master plates cryo-preserved in the item (2), 96 well plates containing the 6 positive clones were selected and incubated at 37°C for 10 minutes in 5% CO_2. After the incubation, cells were collected from a well corresponding to each positive clone and inoculated into a 24 well flat bottom plate for adhesion cell use (manufactured by Greiner). After culturing them for 1 week using IMDM medium (manufactured by Life Technologies) supplemented with 10% fetal bovine dialyzed serum (manufactured by Life Technologies), the cells were inoculated into a 6 well flat bottom plate for adhesion cell use (manufactured by Greiner). Genome DNAs were prepared from each clone in the plate in accordance with a known method [*Nucleic Acids Research*, 3, 2303 (1976)] and dissolved overnight in 150 µl of a TE-RNase buffer (pH 8.0) (10 mmol/l Tris-HCl, 1 mmol/l EDTA, 200 µg/ml RNase A).

In 120 µl of NEBuffer for *Bam*HI (manufactured by New England Biolabs), 12 µg of the obtained genome DNA was dissolved, and 20 unites of a restriction enzyme *Bam*HI (manufactured by New England Biolabs) were mixed, followed by digestion reaction at 37°C overnight. A DNA fragment was recovered from the reaction solution by ethanol precipitation, dissolved in 20 µl of TE buffer (pH 8.0) (10 mmol/l Tris-HCl, 1 mmol/l EDTA) and then subjected to 0.4% (w/v) agarose gel electrophoresis. After the electrophoresis, the genome DNA was transferred onto a nylon membrane in accordance with a known method [*Proc.*

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Natl. Acad. Sci. USA, *16*, 3683 (1979)]. After completion of the transfer, the nylon membrane was heated at 80°C for 2 hours.

On the other hand, a probe used in the Southern blotting was prepared as follows. First, primers (SEQ ID NOs:30 and 31) which bind to a sequence outside the targeting vector homologous region among the FUT8 genome region obtained in Example 12 were designed. Next, polymerase chain reaction (PCR) was carried out using a DNA polymerase ExTaq (manufactured by Takara Shuzo), by preparing 20 μl of a reaction solution [ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/l dNTPs and 0.5 μmol/l gene-specific primers (SEQ ID NOs:30 and 31)] containing 4.0 ng of the plasmid pFUT8fgE2-2 obtained in Example 12(2). The PCR was carried out by heating at 94°C for 1 minute and subsequent 25 cycles of heating at 94°C for 30 seconds, 55°C for 30 seconds and 74°C for 1 minute as one cycle. After the PCR, the reaction solution was subjected to 1.75% (w/v) agarose gel electrophoresis to purify a probe DNA fragment of about 230 bp. A 5 μl portion of the obtained probe DNA solution was radioisotope-labeled using 1.75 MBq of [α-^{32}P]dCTP and Megaprime DNA Labeling System, dCTP (manufactured by Amersham Pharmacia Biotech).

The hybridization was carried out as follows. First, the nylon membrane was sealed in a roller bottle, and pre-hybridization was carried out at 65°C for 3 hours by adding 15 ml of a hybridization solution [5 × SSPE, 50 × Denhaldt's solution, 0.5% (w/v) SDS, 100 μg/ml salmon sperm DNA]. Next, the ^{32}P-labeled probe DNA was heat-denatured and put into the bottle and the nylon membrane was heated overnight at 65°C.

After the hybridization, the nylon membrane was soaked in 50 ml of 2 × SSC-0.1% (w/v) SDS and heated at 65°C for 15 minutes. After repeating the washing step

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twice, the membrane was soaked in 50 ml of 0.2 × SSC-0.1% (w/v) SDS and heated at 65°C for 15 minutes. After washing the nylon membrane, it was exposed to an X-ray film two nights at -80°C for development.

By the restriction enzyme *Bam*HI treatment, a DNA fragment of about 19.0 Kb was formed from a wild type FUT8 allele. Also, a DNA fragment of about 12.5 Kb was formed from an allele in which homologous recombination with a targeting vector was generated. In addition, when the puromycin resistance gene (about 1.5 Kb) was deleted from the allele in which homologous recombination was generated, a DNA fragment of about 11.0 Kb was formed by the same treatment.

By the method, such specific fragments of about 19.0 Kb and about 11.0 Kb were found from the genome DNA of 5 clones among the 6 clones. Since the quantitative ratio of both fragments was 1 : 1, it was shown that the puromycin resistance gene was deleted from the cell lines in which 1 copy of the FUT8 genome region was disrupted. Hereinafter, one of the clone is called 1st.ΔFUT8 2-46-1. Also, results of the genome Southern blotting of the strain 1st.ΔFUT8 2-46-1, 1st.ΔFUT8 2-46 and 5-03 are shown in Fig. 41. Also, the strain 1st.ΔFUT8 2-46-1, as a name of 2-46-1, has been deposited on September 26, 2001, as FERM BP-7755 in International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology (AIST Tsukuba Central 6, 1-1, Higashi 1-Chome Tsukuba-shi, Ibaraki-ken 305-8566 Japan)).

4. Purification of antibody produced by α-1,6-fucosyltransferase (FUT8) gene-disrupted cell line

1st.ΔFUT8 2-46-1 obtained in the item 3 of this Example by disrupting one copy of the FUT8 allele was suspended in IMDM medium (manufactured by Life Technologies) supplemented with 15 μg/ml of puromycin

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(manufactured by SIGMA) and 10% fetal bovine dialyzed serum (manufactured by Life Technologies) to give a density of 3×10^5 cells/ml, and then 60 ml in total of the suspension was inoculated into two T182 flasks for adhesion cell culture use (manufactured by Greiner). After culturing them for 3 days, the supernatant was discarded and changed to a total of 60 ml of EXCELL301 medium (manufactured by JRH Biosciences).

After culturing them at 37°C for 7 days in a 5% CO_2 incubator, the number of intact cells was counted to confirm that their viability was almost the same (each 30% or less), and then each cell suspension was recovered. The cell suspension was centrifuged at 3,000 rpm at 4°C for 10 minutes, and the recovered supernatant was centrifuged at 10,000 rpm at 4°C for 1 hour and then filtered using 150 ml capacity PES Filter Unit (manufactured by NALGENE) having a pore diameter of 0.22 μm.

Prosep-A HighCapacity (manufactured by bioPROCESSING) was packed in a 0.8 cm diameter column to a thickness of 2 cm and washed with 10 ml of 0.1 mol/l citrate buffer (pH 3.0) and 10 ml of 1 mol/l glycine/NaOH-0.15 mol/l NaCl buffer (pH 8.6) in this order to effect equilibrate the carrier. Next, 100 ml of each of the culture supernatant was passed through the column and washed with 50 ml of 1 mol/l glycine/NaOH-0.15 mol/l NaCl buffer (pH 8.6). After washing it, the antibody absorbed to Prosep-A was eluted using 2.5 ml of 0.1 mol/l citrate buffer (pH 3.0), the eluate was fractionated in 500 μl portions and each fraction was neutralized by mixing with 100 μl of 2 mol/l Tris-HCl (pH 8.5). Two fractions containing the antibody at a high concentration (1.2 ml in total) were selected by the BCA method [*Anal. Biochem.*, 150, 76 (1985)], combined and then dialyzed against 10 mol/l citrate-0.15 mol/l NaCl buffer (pH 6.0) at 4°C for a whole day and night. After the dialysis, the antibody solution

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was recovered and subjected to sterile filtration using a 0.22 μm pore size Millex GV (manufactured by MILLIPORE).

5. *In vitro* cytotoxic activity (ADCC activity) of antibody produced by α-1,6-fucosyltransferase (FUT8) gene-disrupted cell line

In order to evaluate *in vitro* cytotoxic activity of the anti-CCR4 antibody purified in the item 4 of this Example, ADCC activity was measured using the CCR4-positive cell line CCR4/EL-4 described in Example 8.

The CCR4/EL-4 cells subcultured in RPMI1640 medium (manufactured by Life Technologies) containing 10% fetal bovine serum (manufactured by Life Technologies) (hereinafter referred to as "RPMI1640-FBS(10)") were suspended in 500 μl of RPMI1640-FBS(10) at a density of 1×10^6 cells, and 3.7 MBq of $Na_2{}^{51}CrO_4$ was added thereto, followed by culturing at 37°C for 90 minutes to label the cells with a radioisotope. After centrifugation at 1,200 rpm for 5 minutes, the supernatant was discarded and the target cells were suspended in 5 ml of RPMI1640-FBS(10). The washing step was repeated three times and then the cell suspension was incubated for 30 minutes on ice for spontaneous dissociation of the radioactive substance. The washing step was again repeated twice and then the cells were suspended in 5 ml of RPMI1640-FBS(10) to thereby prepare 2.0×10^5 cells/ml of a target cell suspension.

Separately, 30 ml of venous blood was collected from a healthy person, 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 mixing them, 10 ml of the mixture was gently overlaid on 4 ml of Lymphoprep (manufactured by NYCOMED PHARMA AS) and centrifuged at room temperature at 2,000 rpm for 30 minutes. The separated mononuclear cell fractions were collected from the

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centrifugation tubes, combined and then suspended in 30 ml of RPMI1640-FBS(10). After centrifugation at room temperature at 1,200 rpm for 15 minutes, the supernatant was discarded and the cells were suspended in 20 ml of RPMI1640-FBS(10). The washing step was repeated twice and then 2.5×10^6 cells/ml of an effector cell suspension was prepared using RPMI1640-FBS(10).

The target cell suspension was dispensed at 50 µl (1×10^4 cells/well) into each well of a 96 well U-bottom plate (manufactured by Falcon). Subsequently, the effector cell suspension was dispensed at 100 µl (2.5×10^5 cells/well) into each well to thereby adjust the ratio of the effector cells to the target cells to 25 : 1. Next, using RPMI1640-FBS(10), a series of dilution solution of 0.01 µg/ml, 0.1 µg/ml, 1 µg/ml and 10 µg/ml was prepared from each of the anti-CCR4 antibodies obtained in the item 5 of Example 13, and the diluted solutions were dispensed in 50 µl portions into the wells to give final concentrations of 0.0025 µg/ml, 0.025 µg/ml, 0.25 µg/ml and 2.5 µg/ml, respectively. After the reaction at 37°C for 4 hours in 5% CO_2, the plate was centrifuged at 1,200 rpm for 5 minutes. Into a 12 mm diameter RIA tube (manufactured by IWAKI), 75 µl of the supernatant in each well was batched off and the amount of the dissociated ^{51}Cr was measured using MINAX-γ auto-gamma counter 5550 (manufactured by PACKARD).

Also, the amount of the spontaneously dissociated ^{51}Cr was calculated by carrying out the same reaction in a reaction mixture in which 150 µl of RPMI1640-FBS(10) was added instead of the effector cell suspension and antibody solution. The amount of the total dissociated ^{51}Cr was calculated by carrying out the same reaction in a reaction mixture in which 100 µl of 1 N hydrochloric acid and 50 µl of RPMI1640-FBS(10) were added instead of the effector cell

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suspension and antibody solution. Using these values, the ADCC activity was calculated based on equation (II).

Fig. 42 shows ADCC activity of each of the anti-CCR4 antibodies. The antibody obtained from the strain 1st.ΔFUT8 2-46-1 in which 1 copy of the FUT8 allele was disrupted showed a significantly more potent ADCC activity than the antibody produced by the strain 5-03 which is the CHO cell line before gene disruption. Also, changes in the antigen binding activity of these antibodies were not observed. Based on the results, it was confirmed that the ADCC activity of produced antibodies can be improved by disrupting the FUT8 allele in host cells.

Example 14

Preparation of lectin-resistant CHO/DG44 cell and production of antibody using the cell:

(1) Preparation of lectin-resistant CHO/DG44

CHO/DG44 cells were grown until they reached a stage of just before confluent, by culturing in a 75 cm^2 flask for adhesion culture (manufactured by Greiner) using IMDM-FBS(10) medium [IMDM medium comprising 10% of fetal bovine serum (FBS) and 1 × concentration of HT supplement (manufactured by GIBCO BRL)]. After washing the cells with 5 ml of Dulbecco PBS (manufactured by Invitrogen), 1.5 ml of 0.05% trypsin (manufactured by Invitrogen) diluted with Dulbecco PBS was added thereto and incubated at 37°C for 5 minutes for peel the cells from the flask bottom. The peeled cells were recovered by a centrifugation operation generally used in cell culture and suspended in IMDM-FBS(10) medium to give a density of 1×10^5 cells/ml, and then 0.1 μg/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 incubating them at 37°C for 3 days in a CO_2 incubator (manufactured by TABAI), the culture supernatant was

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discarded, and the cells were again washed, peeled and recovered by the same operations, suspended in IMDM-FBS(10) medium and then inoculated into an adhesion culture 96 well plate (manufactured by IWAKI Glass) to give a density of 1,000 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; hereinafter 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 them at 37°C for 2 weeks in a CO_2 incubator, the appeared colonies were obtained as lectin-resistant CHO/DG44. Regarding the obtained lectin-resistant CHO/DG44, an LCA-resistant cell line was named CHO-LCA, an AAL-resistant cell line was named CHO-AAL and an L-PHA-resistant cell line was named CHO-PHA. When the resistance of these cell lines to various kinds of lectin was examined, it was found that the CHO-LCA was also resistant to AAL and the CHO-AAL was also resistant LCA. In addition, the CHO-LCA and 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 1-position of fucose is bound to 6-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 CHO-LCA and 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 cell lines by increasing the

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number of cells to be treated. Hereinafter, these cell lines were used in analyses.

(2) Preparation of anti-CCR4 human chimeric antibody-producing cell

An anti-CCR4 human chimeric antibody expression plasmid pKANTEX2160 was introduced into the three lectin-resistant cell lines obtained in the (1) by the method described in Example 8, and gene amplification by a drug MTX was carried out to prepare an anti-CCR4 human chimeric antibody-producing cell line. By measuring an amount of antibody expression by the ELISA described in Example 8-2, antibody-expressing transformants were obtained from each of the CHO-LCA, CHO-AAL and CHO-PHA. Regarding each of the obtained transformants, a transformant derived from CHO-LCA was named CHO/CCR4-LCA, a transformant derived from CHO-AAL was named CHO/CCR4-AAL and a transformant derived from CHO-PHA was named CHO/CCR4-PHA. Also, the CHO/CCR4-LCA, as a name of Nega-13, has been deposited on September 26, 2001, as FERM BP-7756 in International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology (AIST Tsukuba Central 6, 1-1, Higashi 1-Chome Tsukuba-shi, Ibaraki-ken 305-8566 Japan)).

(3) Production of potent ADCC activity antibody by lectin-resistant CHO cell

Using the three transformants obtained in the (2), purified antibodies were obtained by the method described in Example 8-3. The antigen binding activity of each of the purified anti-CCR4 human chimeric antibodies was evaluated using the ELISA described in Example 8-2. The antibodies produced by all transformants showed an antigen binding activity similar to that of the antibody produced by a recombinant cell line (strain 5-03) prepared in Example 8 using general CHO/DG44 cell as the host. Using

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these purified antibodies, ADCC activity of each of the purified anti-CCR4 human chimeric antibodies was evaluated in accordance with the method described in Example 8-7. The results are shown in Fig. 43. In comparison with the antibody produced by the strain 5-03, about 100 folds-increased ADCC activity was observed in the antibodies produced by the CHO/CCR4-LCA and CHO/CCR4-AAL. On the other hand, no significant increase in the ADCC activity was observed in the antibody produced by the CHO/CCR4-PHA. Also, when ADCC activities of the antibodies produced by the CHO/CCR4-LCA and YB2/0 were compared in accordance with the method described in Example 8-7, it was found that the antibody produced by the CHO/CCR4-LCA shows more potent ADCC activity than the antibody produced by the strain 5-03, similar to the case of the antibody KM2760-1 produced by the YB2/0 cell line prepared in Example 8-1 (Fig. 44).

(4) Sugar chain analysis of antibodies produced by lectin-resistant CHO cell

Sugar chains of the anti-CCR4 human chimeric antibodies purified in the (3) were analyzed. The solution of each of the purified antibodies was exchanged to 10 mM 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 folds or more. The concentration of the antibodies after the solution exchange was measured using UV-1600 (manufactured by Shimadzu). The molar absorption coefficient was calculated from the amino acid sequence of each antibody based on the following equation (III) [*Advances in Protein Chemistry*, 12, 303 (1962)], and the concentration was determined by defining the absorbance at 280 nm as 1.38 mg/ml.

$$E_{1mol/l} = A \times n1 + B \times n2 + C \times n3 \quad \text{(III)}$$

$$E_{1mol/ml} = E_{1mol/l}/MW$$

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$E_{1mol/l}$: absorption coefficient at 280 nm (mg^{-1} ml cm^{-1})

$E_{1mol/ml}$: molar absorption coefficient at 280 nm (M^{-1} cm^{-1})

A: molar absorption coefficient of tryptophan at 280 nm = 5550 (M^{-1} cm^{-1})

B: molar absorption coefficient of tyrosine at 280 nm = 1340 (M^{-1} cm^{-1})

C: molar absorption coefficient of cystine at 280 nm = 200 (M^{-1} cm^{-1})

n1: the number of tryptophan per 1 antibody molecule

n2: the number of tyrosine per 1 antibody molecule

n3: the number of cystine per 1 antibody molecule

MW: molecular weight of antibody (g/mol)

Into Hydraclub S-204 test tube, 100 µg of each antibody was put and dried using a centrifugal evaporator. The dried sample in the test tube was subjected to hydrazinolysis using Hydraclub manufactured by Hohnen. The sample was allowed to react with hydrazine at 110°C for 1 hour using a hydrazinolysis reagent manufactured by Hohnen hydrazinolysis [*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 µl 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

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frozen at -80°C in a freezer and freeze-dried for about 17 hours. Sugar chains were recovered from the freeze-dried sample using Cellulose Cartridge Glycan Preparation Kit manufactured by Takara Syuzo Co., Ltd. The sample sugar chain solution was dried using 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 × PA solution) and diluting the solution 10 folds with reverse osmosis purified water (10-folds diluted PA solution). The sodium cyanoborohydride solution was prepared by adding 20 µl of 1 × PA solution and 430 µl of reverse osmosis purified water per 10 mg of sodium cyanoborohydride. To the sample, 67 µl of a 10 folds-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 using Superdex Peptide HR 10/30 column (manufactured by Pharmacia). This step was carried out using 10 mM ammonium bicarbonate as the eluent at a flow rate of 0.5 ml/min 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 using a centrifugal evaporator to be used as purified PA-treated sugar chains. Next, reverse phase HPLC analysis of the purified PA-treated sugar chains was carried out using CLC-ODS column (manufactured by Shimadzu, ϕ 6.0 nm × 159 nm). 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

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400 nm fluorescence wavelength. The column was equilibrated with a 10 mM sodium phosphate buffer (pH 3.8) and elution was carried out for 80 minutes by a 0.5% 1-butanol linear density gradient. 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 time of flight mass spectrometry (MALDI-TOF-MS analysis), comparison of elution positions with standards of PA-treated sugar chain manufactured by Takara Syuzo, and reverse phase HPLC analysis after digestion of each PA-treated sugar chain using various enzymes (Fig. 45). Each of 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.

The analysis was carried out in the same manner as in Example 11(6) using a sodium phosphate buffer (pH 3.8) as buffer A and a sodium phosphate buffer (pH 3.8) + 0.5% 1-butanol as buffer B.

In Fig. 45, the ratio of the α-1,6-fucose-free sugar chain group was calculated from the area occupied by the peaks (i) to (iv) among (i) to (viii), and the ratio of the α-1,6-fucose-bound sugar chain group from the area occupied by the peaks (v) to (viii) among (i) to (viii).

Results of the sugar chain structure analysis of the purified anti-CCR4 human chimeric antibodies produced by lectin-resistant cell lines are shown in Table 6. The result shows the analysis of sugar chains of the anti-CCR4 human chimeric antibody produced by lectin-resistant cell lines. The ratio of α-1,6-fucose-free sugar chains (%) calculated from peak areas by analyzing by the method described in Example d(4) is shown in the table.

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Table 6

Antibody producing cells	α-1,6-Fucose-free complex double-chain sugar chain (%)
Strain 5-03	9
Strain CHO/CCR4-LCA	48
Strain CHO/CCR4-AAL	27
Strain CHO/CCR4-PHA	8

In comparison with the antibody produced by the strain 5-03, the ratio of the α-1,6-fucose-free sugar chains was increased from 9% to 48% in the antibody produced by the CHO/CCR4-LCA when calculated from the analyzed peak area. The ratio of α-1,6-fucose-free sugar chains was increased from 9% to 27% in the antibody produced by the CHO/CCR4-AAL. On the other hand, changes in the sugar chain pattern and ratio of the α-1,6-fucose-free sugar chains were hardly found in the PHA-resistant cell line when compared with the strain 5-03.

Example 15

Analysis of lectin-resistant CHO cell line:

1. Analysis of expression level of GMD enzyme in an anti-CCR4 human chimeric antibody-producing cell line CHO/CCR4-LCA

The expression level of each of the genes of GMD (GDP-mannose 4,6-dehydratase), GFPP (GDP-keto-6-deoxymannose 3,5-epimerase, 4-reductase) and FX (GDP-beta-L-fucose pyrophosphorylase) known as fucose biosynthesis enzymes and FUT8 (α-1,6-fucosyltransferase) as a fucose transferase, in the anti-CCR4 human chimeric antibody-producing cell line CHO/CCR4-LCA obtained in Example 14, was analyzed using RT-PCR method.

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(1) Preparation of RNA from various cell lines

Each of the CHO/DG44 cell, the anti-CCR4 human chimeric antibody-producing cell line 5-03 obtained in Example 8-1(2) and the anti-CCR4 human chimeric antibody-producing cell line CHO/CCR4-LCA obtained in Example 14(2) was subcultured at 37°C in a 5% CO_2 incubator and then cultured for 4 days. After culturing them, total RNA was prepared from 1 × 10^7 cells of each cell line using RNeasy Protect Mini Kit (manufactured by QIAGEN) in accordance with the manufacture's instructions. Subsequently, single-stranded cDNA was synthesized from 5 μg of each RNA in a 20 μl 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.

(2) Analysis of expression level of GMD gene using RT-PCR

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 Example 17-1.

Next, 20 μl of a reaction solution [1 × Ex Taq buffer (manufactured by Takara Shuzo), 0.2 mM dNTPs, 0.5 unit of Ex Taq polymerase (manufactured by Takara Shuzo) and 0.5 μM of the synthetic DNA primers of SEQ ID NOs:32 and 33] containing 0.5 μl of the single-stranded cDNA prepared from each cell line in the item (1) as the template was prepared, and PCR was carried out using DNA Thermal Cycler 480 (manufactured by Perkin Elmer) by heating at 94°C for 5 minutes and subsequent 30 cycles of heating of 94°C for 1 minute and 68°C for 2 minutes as one cycle. After subjecting 10 μl of the PCR reaction solution to agarose electrophoresis, DNA fragments were stained using Cyber Green (manufactured by BMA) and then the amount

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of the DNA fragment of about 350 bp was measured using Fluor Imager SI (manufactured by Molecular Dynamics).

(3) Analysis of expression level of GFPP gene using RT-PCR

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 Example 16-2.

Next, 20 μl of a reaction solution [1 × Ex Taq buffer (manufactured by Takara Shuzo), 0.2 mM dNTPs, 0.5 unit of Ex Taq polymerase (manufactured by Takara Shuzo) and 0.5 μM of the synthetic DNA primers of SEQ ID NOs:34 and 35] containing 0.5 μl of the single-stranded cDNA prepared from each cell line in the item (1) as the template was prepared, and PCR was carried out 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 μl of the PCR reaction solution to agarose electrophoresis, DNA fragments were stained using Cyber Green (manufactured by BMA) and then the amount of the DNA fragment of about 600 bp was measured using Fluor Imager SI (manufactured by Molecular Dynamics).

(4) Analysis of expression level of FX gene using RT-PCR

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 Example 16-1.

Next, 20 μl of a reaction solution [1 × Ex Taq buffer (manufactured by Takara Shuzo), 0.2 mM dNTPs, 0.5

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unit of Ex Taq polymerase (manufactured by Takara Shuzo) and 0.5 μM of the synthetic DNA primers of SEQ ID NO:36 and SEQ ID NO:37] containing 0.5 μl of the single-stranded cDNA prepared from each cell line in the item (1) as the template was prepared, and PCR was carried out 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 μl of the PCR reaction solution to agarose electrophoresis, DNA fragments were stained using Cyber Green (manufactured by BMA) and then the amount of the DNA fragment of about 300 bp was measured using Fluor Imager SI (manufactured by Molecular Dynamics).

(5) Analysis of expression level of FUT8 gene using RT-PCR

In order to amplify FUT8 cDNA by PCR, 20 μl of a reaction solution [1 x Ex Taq buffer (manufactured by Takara Shuzo), 0.2 mM dNTPs, 0.5 unit of Ex Taq polymerase (manufactured by Takara Shuzo) and 0.5 μM of the synthetic DNA primers of SEQ ID NOs:13 and 14] containing 0.5 μl of the single-stranded cDNA prepared from each cell line in the item (1) as the template was prepared, and PCR was carried out 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 μl of the PCR reaction solution to agarose electrophoresis, DNA fragments were stained using 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).

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(6) Analysis of expression level of β-actin gene using RT-PCR

In order to amplify β-actin cDNA by PCR, 20 μl of a reaction solution [1 × Ex Taq buffer (manufactured by Takara Shuzo), 0.2 mM dNTPs, 0.5 unit of Ex Taq polymerase (manufactured by Takara Shuzo) and 0.5 μM of the synthetic DNA primers of SEQ ID NOs:15 and 16] containing 0.5 μl of the single-stranded cDNA prepared from each cell line in the item (1) as the template was prepared, and the reaction was carried out 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 μl of the PCR reaction solution to agarose electrophoresis, DNA fragments were stained using 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).

(7) Expression levels of GMD, GFPP, FX and FUT8 genes in each cell line

The amount of the PCR-amplified fragment of each gene in the strain 5-03 and the 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 cell line measured in the items (2) to (6) by the value of the amount of PCR-amplified fragment derived from β-actin cDNA in each cell line, and defining the amount of the PCR-amplified fragments in CHO/DG44 cell as 1. . The results are shown in Table 7.

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Table 7

	GMD	GEPP	FX	FUT8
Strain CHO/DG44	1	1	1	1
Strain 5-03	1.107	0.793	1.093	0.901
Strain 5-03-derived LCA-resistant cell CHO/CCR4-LCA	0.160	0.886	0.920	0.875

As shown in Table 7, the expression level of GMD gene in the CHO/CCR4-LCA was decreased to about 1/10 in comparison with other cell lines. In this case, the test was independently carried out twice, and the average value was used.

2. Analysis using anti-CCR4 human chimeric antibody-producing CHO/CCR4-LCA in which GMD gene was forced to express

(1) Construction of CHO cell-derived GMD gene expression vector pAGE249GMD

Based on the CHO cell-derived GMD cDNA sequence obtained in Example 17-1, 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 µl of a reaction solution [1 × Ex Taq buffer (manufactured by Takara Shuzo), 0.2 mM dNTPs, 0.5 unit of Ex Taq polymerase (manufactured by Takara Shuzo) and 0.5 µM of the synthetic DNA primers of SEQ ID NOs:38 and 39] containing 0.5 µl of the CHO cell-derived GMD single-stranded cDNA prepared in the item 1(1) of this Example as the template was prepared, and PCR was carried out 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

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fractionated by agarose electrophoresis, and then a DNA fragment of about 600 bp was recovered using Gene Clean II Kit (manufactured by BIO 101) in accordance with the manufacture's instructions. The recovered DNA fragment was connected to pT7Blue(R) vector (manufactured by Novagen) using DNA Ligation Kit (manufactured by Takara Shuzo), and *E. coli* DH5α (manufactured by Toyobo) was transformed using the obtained recombinant plasmid DNA to obtain a plasmid mt-C (cf. Fig. 46).

Next, based on the CHO cell-derived GMD cDNA sequence obtained in Example 17-1, 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 μl of a reaction solution [1 × Ex Taq buffer (manufactured by Takara Shuzo), 0.2 mM dNTPs, 0.5 unit of Ex Taq polymerase (manufactured by Takara Shuzo) and 0.5 μM of the synthetic DNA primers of SEQ ID NOs:40 and 41] containing 0.5 μl of the CHO cell-derived GMD single-stranded cDNA prepared in the item 1(1) of this Example as the template was prepared, and PCR was carried out 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 using Gene Clean II Kit (manufactured by BIO 101) in accordance with the manufacture's instructions. The recovered DNA fragment was connected to pT7Blue(R) vector (manufactured by Novagen) using DNA Ligation Kit (manufactured by Takara Shuzo), and *E. coli* DH5α (manufactured by Toyobo) was transformed using the obtained recombinant plasmid DNA to obtain a plasmid ATG (cf. Fig. 47).

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Next, 3 μg of the plasmid CHO-GMD prepared in Example 17-1 was allowed to react with a restriction enzyme *Sac*I (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 *Eco*RI (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 using Gene Clean II Kit (manufactured by BIO 101) in accordance with the manufacture's instructions. The plasmid mt-C (1.4 μg) was allowed to react with a restriction enzyme *Sac*I (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 *Eco*RI (manufactured by Takara Shuzo) at 37°C for 16 hours, the digest was fractionated by agarose electrophoresis and then a DNA fragment of about 3.1 kbp was recovered using Gene Clean II Kit (manufactured by BIO 101) in accordance with the manufacture's instructions. The recovered DNA fragments were ligated using DNA Ligation Kit (manufactured by Takara Shuzo), and *E. coli* DH5α was transformed using the obtained recombinant plasmid DNA to obtain a plasmid WT-N(-) (cf. Fig. 48).

Next, 2 μg of the plasmid WT-N(-) was allowed to react with a restriction enzyme *Bam*HI (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 *Eco*RI (manufactured by Takara Shuzo) at 37°C for 16 hours, the digest was fractionated by agarose electrophoresis and then a DNA fragment of about 1 kbp was recovered using Gene Clean II Kit (manufactured by BIO 101) in accordance with the manufacture's instructions. The plasmid pBluescript SK(-) (3 μg; manufactured by

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Stratagene) was allowed to react with a restriction enzyme *Bam*HI (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 *Eco*RI (manufactured by Takara Shuzo) at 37°C for 16 hours, the digest was fractionated by agarose electrophoresis and then a DNA fragment of about 3 kbp was recovered using Gene Clean II Kit (manufactured by BIO 101) in accordance with the manufacture's instructions. The recovered respective DNA fragments were ligated using DNA Ligation Kit (manufactured by Takara Shuzo), and *E. coli* DH5α was transformed using the obtained recombinant plasmid DNA to obtain a plasmid WT-N(-) in pBS (cf. Fig. 49).

Next, 2 μg of the plasmid WT-N(-) in pBS was allowed to react with a restriction enzyme *Hind*III (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 *Eco*RI (manufactured by Takara Shuzo) at 37°C for 16 hours, the digest was fractionated by agarose electrophoresis and then a DNA fragment of about 4 kbp was recovered using Gene Clean II Kit (manufactured by BIO 101) in accordance with the manufacture's instructions. A 2 μg portion of the plasmid ATG was allowed to react with a restriction enzyme *Hind*III (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 *Eco*RI (manufactured by Takara Shuzo) at 37°C for 16 hours, the digest was fractionated by agarose electrophoresis and then a DNA fragment of about 150 bp was recovered using Gene Clean II Kit (manufactured by BIO 101) in accordance with the manufacture's instructions. The recovered respective DNA fragments were ligated using DNA Ligation Kit

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(manufactured by Takara Shuzo), and *E. coli* DH5α was transformed using the obtained recombinant plasmid DNA to obtain a plasmid WT in pBS (cf. Fig. 50).

Next, 2 μg of the plasmid pAGE249 was allowed to react with restriction enzymes *Hin*dIII and *Bam*HI (both manufactured by Takara Shuzo) at 37°C for 16 hours, the digest was fractionated by agarose electrophoresis and then a DNA fragment of about 6.5 kbp was recovered using Gene Clean II Kit (manufactured by BIO 101) in accordance with the manufacture's instructions. The plasmid WT (2 μg) in pBS was allowed to react with restriction enzymes *Hin*dIII and *Bam*HI (both manufactured by Takara Shuzo) at 37°C for 16 hours, the digest was fractionated by agarose electrophoresis and then a DNA fragment of about 1.2 kbp was recovered using Gene Clean II Kit (manufactured by BIO 101) in accordance with the manufacture's instructions. The recovered respective DNA fragments were ligated using DNA Ligation Kit (manufactured by Takara Shuzo), and *E. coli* DH5α was transformed using the obtained recombinant plasmid DNA to obtain a plasmid pAGE249GMD (cf. Fig. 51).

(2) Stable expression of GMD gene in CHO/CCR4-LCA

The CHO cell-derived GMD gene expression vector pAGE249GMD (5 μg) made into linear form by digesting it with a restriction enzyme *Fsp*I (manufactured by NEW ENGLAND BIOLABS), which was introduced into 1.6×10^6 cells of CHO/CCR4-LCA by electroporation [*Cytotechnology*, 3, 133 (1990)]. Then, the cells were suspended in 30 ml of IMDM-dFBS(10) medium [IMDM medium (manufactured by GIBCO BRL) supplemented with 10% of dFBS] containing 200 nM MTX (manufactured by SIGMA), and cultured using a 182 cm^2 flask (manufactured by Greiner) at 37°C for 24 hours in a 5% CO_2 incubator. After culturing them, the medium was changed to IMDM-dFBS(10) medium containing 0.5 mg/ml hygromycin and 200 nM MTX (manufactured by SIGMA), followed by culturing

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for 19 days to obtain colonies of hygromycin-resistant transformants.

In the same manner, the pAGE249 vector was introduced into the CHO/CCR4-LCA by the same method to obtain colonies of hygromycin-resistant transformants.

(3) Culturing of GMD gene-expressed CHO/CCR4-LCA and purification of antibody

Using IMDM-dFBS(10) medium comprising 200 nM MTX (manufactured by SIGMA) and 0.5 mg/ml hygromycin, the GMD-expressing transformant cells obtained in the item (2) were cultured using a 182 cm^2 flask (manufactured by Greiner) at 37°C in a 5% CO_2 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 them at 37°C in a 5% CO_2 incubator for 7 days, the culture supernatant was recovered. An anti-CCR4 chimeric antibody was purified from the culture supernatant using Prosep-A (manufactured by Millipore) in accordance with the manufacture's instructions.

In the same manner, the pAGE249 vector-introduced transformant cells were cultured by the same method and then anti-CCR4 chimeric antibody was recovered and purified from the culture supernatant.

(4) Measurement of lectin resistance in transformed cells

The GMD-expressing transformant cells obtained in the item (2) were suspended in IMDM-dFBS(10) medium comprising 200 nM 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 in 50 µl/well portions into a 96 well culture plate (manufactured by Iwaki Glass). Next, a medium prepared by suspending at concentrations of 0 mg/ml,

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0.4 mg/ml, 1.6 mg/ml or 4 mg/ml LCA (*Lens culinaris* agglutinin: manufactured by Vector Laboratories) in IMDM-dFBS(10) medium containing 200 nM MTX (manufactured by SIGMA) and 0.5 mg/ml hygromycin was added to the plate at 50 µl/well, followed by culturing at 37°C for 96 hours in a 5% CO_2 incubator. After culturing them, WST-I (manufactured by Boehringer) was added at 10 µl/well and incubated at 37°C for 30 minutes in a 5% CO_2 incubator to effect color development, and then the absorbance at 450 nm and 595 nm (hereinafter referred to as "OD450" and "OD595", respectively) was measured using Microplate Reader (manufactured by BIO-RAD). In the same manner, the pAGE249 vector-introduced transformant cells were measured by the same method. The test was carried out twice independently.

Fig. 52 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. 52, decrease in the LCA-resistance was observed in the GMD-expressed 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 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 these results, it was suggested that expression level of GMD gene in the CHO/CCR4-LCA was decreased and, as a result, the resistance against LCA was obtained.

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(5) *In vitro* cytotoxic activity (ADCC activity) of anti-CCR4 chimeric antibody obtained from GMD-expressed CHO/CCR4-LCA

In order to evaluate *in vitro* cytotoxic activity of the purified anti-CCR4 chimeric antibody obtained in the item (3), the ADCC activity was measured in accordance with the following methods.

i) Preparation of target cell suspension

A 3.7 MBq equivalent of a radioactive substance $Na_2{}^{51}CrO_4$ was added to 1×10^6 cells of the CCR4-EL4 (cf. Example 8-7) cultured using a medium prepared by adding 500 μg/ml G418 sulfate (manufactured by Nakalai Tesque) to the RPMI1640-FBS(10) medium, 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, re-suspended in the medium and then incubated at 4°C for 30 minutes in ice for spontaneous dissociation of the radioactive substance. After centrifugation, the cells were adjusted to 2.5×10^5 cells/ml by adding 5 ml of the RPMI1640-FBS(10) medium and used as a target cell suspension.

ii) Preparation of effector cell suspension

From a healthy person, 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 using the RPMI1640-FBS(10) medium and then resuspended in the medium to give a density of 2×10^6 cells/ml and used as a effector cell suspension.

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iii) Measurement of ADCC activity

The target cell suspension prepared in the 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 2) 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 (the anti-CCR4 chimeric antibody purified in the item (3), and KM2760-1 and KM3060) was further added thereto to give a final concentration of 0.0025 to 2.5 μg/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 using 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 of ^{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 N 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 formula (II).

Results of the measurement of ADCC activity are shown in Fig. 53. As shown in Fig. 53, ADCC activity of the purified anti-CCR4 chimeric antibody obtained from the GMD-expressed CHO/CCR4-LCA was decreased to a similar degree to that of the KM3060 obtained in Example 8. On the other hand, ADCC activity of the purified anti-CCR4 chimeric antibody obtained from the pAGE249 vector-introduced CHO/CCR4-LCA showed a similar degree of ADCC activity to that of the purified anti-CCR4 chimeric antibody obtained from the CHO/CCR4-LCA. Based on the results, it was suggested that expression level of GMD gene

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in the CHO/CCR4-LCA is decreased and, as a result, an antibody having potent ADCC activity can be produced

(6) Sugar chain analysis of anti-CCR4 chimeric antibody derived from GMD-expressed CHO/CCR4-LCA

Sugar chains binding to the purified anti-CCR4 chimeric antibody obtained in the item (3) were analyzed in accordance with the method shown in Example 14(4), with the analyzed results shown in Fig. 55. In comparison with the purified anti-CCR4 chimeric antibody prepared from CHO/CCR4-LCA in Example 14, the ratio of sugar chain having no α-1,6-fucose in the purified anti-CCR4 chimeric antibody derived from GMD-expressed CHO/CCR4-LCA was decreased to 9% when calculated from the peak area. Thus, it was shown that the ratio of sugar chain having no α-1,6-fucose in the antibody produced by the cell is decreased to similar level of the antibody produced by the strain 5-03, by expressing GMD gene in the CHO/CCR4-LCA

Example 16

Preparation of various genes encoding enzymes relating to the sugar chain synthesis in CHO cell:

1. Determination of CHO cell-derived FX cDNA sequence

(1) Extraction of total RNA from CHO/DG44 cell

CHO/DG44 cells were suspended in IMDM medium containing 10% fetal bovine serum (manufactured by Life Technologies) and 1 × 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 2×10^5 cells/ml. On the second day after culturing them at 37°C in a 5% CO_2 incubator, 1×10^7 of the cells were recovered and total RNA was extracted therefrom using RNAeasy (manufactured by QIAGEN) in accordance with the manufacture's instructions.

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(2) Preparation of total single-stranded cDNA from CHO/DG44 cell

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

In a 20 μl of reaction mixture using oligo(dT) as a primer, single-stranded cDNA was synthesized from 3 μg 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 folds-diluted aqueous solution of the reaction solution was used in the cloning of GFPP and FX. This was stored at -80°C until use.

(3) Preparation of cDNA partial fragment of Chinese hamster-derived FX

FX cDNA partial fragment derived from Chinese hamster was prepared by the following procedure.

First, primers (shown in 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.

Next, 25 μl of a reaction solution [ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/l dNTPs and 0.5 μmol/l gene-specific primers (SEQ ID NOs:42 and 43)] containing 1 μl of the CHO/DG44-derived single-stranded

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cDNA prepared in the item (2) was prepared, and polymerase chain reaction (PCR) was carried out using a DNA polymerase ExTaq (manufactured by Takara Shuzo). 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 final heating at 72°C for 10 minutes.

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 Gel Extraction Kit (manufactured by Quiagen) and eluted with 20 µl of sterile water (hereinafter, the method was used for the purification of DNA fragments from agarose gel). Into a plasmid pCR2.1, 4 µl of the amplified fragment was employed to insert in accordance with the instructions attached to TOPO TA Cloning Kit (manufactured by Invitrogen), and *E. coli* DH5α 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 was isolated in accordance with a known method [*Nucleic Acids Research*, 7, 1513 (1979)] (hereinafter, the method was used for the isolation of plasmid) from the obtained several kanamycin-resistant colonies to obtain 2 clones into which FX cDNA partial fragments were respectively inserted. They are referred to as pCRFX clone 8 and pCRFX clone 12.

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

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determined encodes open reading frame (ORF) partial sequence of the Chinese hamster FX.

(4) Synthesis of single-stranded cDNA for RACE

Single-stranded cDNA samples for 5' and 3' RACE were prepared from the CHO/DG44 total RNA extracted in the item (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 folds with the Tricin-EDTA buffer attached to the kit and used as the template of PCR.

(5) Determination of Chinese hamster-derived FX full length cDNA by RACE method

Based on the FX partial sequence derived from Chinese hamster determined in the item (3), 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 ID NO:46) and FXGSP2-2 (SEQ ID NO:47) for the Chinese hamster FX-specific 3' RACE were designed.

Next, polymerase chain reaction (PCR) was carried out using Advantage2 PCR Kit (manufactured by CLONTECH), by preparing 50 μl of a reaction solution [Advantage2 PCR buffer (manufactured by CLONTECH), 0.2 mM dNTPs, 0.2 μmol/l Chinese hamster FX-specific primers for RACE and 1 × concentration of common primers (manufactured by CLONTECH)] containing 1 μl of the CHO/DG44-derived single-stranded cDNA for RACE prepared in the item (4).

The PCR was carried out by repeating 20 cycles of heating at 94°C for 5 seconds, 68°C for 10 seconds and 72°C for 2 minutes as one cycle.

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After completion of the reaction, 1 μl of the reaction solution was diluted 50-folds with the Tricin-EDTA buffer, and 1 μl 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 8.

Table 8

Combination of primers used in Chinese hamster FX cDNA RACE PCR and the size of PCR products

5' RACE	FX-specific primers	Common primers	PCR-amplified product size
First	FXGSP1-1	UPM (Universal primer mix)	
Second	FXGSP1-2	NUP (Nested Universal primer)	300 bp

3' RACE	FX-specific primers	Common primers	PCR-amplified product size
First	FXGSP2-1	UPM (Universal primer mix)	
Second	FXGSP2-2	NUP (Nested Universal primer)	1,100 bp

After the PCR, the reaction solution was subjected to 1% agarose gel electrophoresis, and the specific amplified fragment of interest was purified using QiaexII Gel Extraction Kit (manufactured by Qiagen) and eluted with 20 μl of sterile water. Into a plasmid pCR2.1, 4 μl of the amplified fragment was inserted, and *E. coli* DH5α was transformed using the reaction solution in accordance with the instructions attached to TOPO TA Cloning Kit (manufactured by Invitrogen).

Plasmid DNAs were isolated from the appeared several kanamycin-resistant colonies to obtain 5 cDNA clones containing Chinese hamster FX 5' region. They are referred to as FX5' clone 25, FX5' clone 26, FX5' clone 27, FX5' clone 28, FX5' clone 31 and FX5' clone 32.

In the same manner, 5 cDNA clones containing Chinese hamster FX 3' region were obtained. These FX3'

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clones are referred to as FX3' clone 1, FX3' clone 3, FX3' clone 6, FX3' clone 8 and FX3' clone 9.

The nucleotide sequence of the cDNA moiety of each of the clones obtained by the 5' and 3' RACE was determined 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 shown in SEQ ID NO:48.

2. Determination of CHO cell-derived GFPP cDNA sequence

(1) Preparation of GFPP cDNA partial fragment derived from Chinese hamster

GFPP cDNA partial fragment derived from Chinese hamster was prepared by the following procedure.

First, nucleotide sequences of a human GFPP cDNA (Genebank Accession No. number 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.

Next, polymerase chain reaction (PCR) was carried out using a DNA polymerase ExTaq (manufactured by Takara Shuzo), by preparing 25 μl of a reaction solution [ExTaq buffer (manufactured by Takara Shuzo), 0.2 mM dNTPs and 0.5 μmol/l GFPP-specific primers GFPP FW9 and GFPP RV9 (SEQ ID NOs:49 and 50)] containing 1 μl of the CHO/DG44-derived single-stranded cDNA prepared in the item 1(2). The PCR was carried out by heating at 94°C for 5 minutes,

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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 final heating at 72°C for 10 minutes.

After the PCR, the reaction solution was subjected to 2% agarose gel electrophoresis, and a specific amplified fragment of 1.4 Kbp was purified using QuiaexII Gel Extraction Kit (manufactured by Quiagen) and eluted with 20 μl of sterile water. Into a plasmid pCR2.1, 4 μl of the amplified fragment was employed to insert in accordance with the instructions attached to TOPO TA Cloning Kit (manufactured by Invitrogen), and *E. coli* DH5α was transformed using the reaction solution.

Plasmid DNAs were isolated from the appeared several kanamycin-resistant colonies to obtain 3 clones into which GFPP cDNA partial fragments were respectively integrated. They are referred to as GFPP clone 8, GFPP clone 11 and GFPP clone 12.

The nucleotide sequence of the cDNA inserted into each of the GFPP clone 8, GFPP clone 11 and GFPP 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 described in the manufacture's instructions. It was confirmed that the inserted cDNA whose sequence was determined encodes open reading frame (ORF) partial sequence of the Chinese hamster GFPP.

(2) Determination of Chinese hamster GFPP full length cDNA by RACE method

Based on the Chinese hamster FX partial sequence determined in the item 2(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.

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Next, polymerase chain reaction (PCR) was carried out using Advantage2 PCR Kit (manufactured by CLONTECH), by preparing 50 µl of a reaction solution [Advantage2 PCR buffer (manufactured by CLONTECH), 0.2 mM dNTPs, 0.2 µmol/l Chinese hamster GFPP-specific primers for RACE and 1 × concentration of common primers (manufactured by CLONTECH)] containing 1 µl of the CHO/DG44-derived single-stranded cDNA for RACE prepared in the item (4).

The PCR was carried out by repeating 20 cycles of heating at 94°C for 5 seconds, 68°C for 10 seconds and 72°C for 2 minutes as one cycle.

After completion of the reaction, 1 µl of the reaction solution was diluted 50-folds with the Tricin-EDTA buffer, and 1 µl 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 9.

Table 9

Combination of primers used in Chinese hamster GFPP cDNA RACE PCR and the size of PCR products

5' RACE	GFPP-specific primers	Common primers	PCR-amplified product size
First	GFPPGSP1-1	UPM (Universal primer mix)	
Second	GFPPGSP1-2	NUP (Nested Universal primer)	1,100 bp

3' RACE	GFPP-specific primers	Common primers	PCR-amplified product size
First	GFPPGSP2-1	UPM (Universal primer mix)	
Second	GFPPGSP2-2	NUP (Nested Universal primer)	1,400 bp

After the PCR, the reaction solution was subjected to 1% agarose gel electrophoresis, and the specific amplified fragment of interest was purified using QiaexII Gel Extraction Kit (manufactured by Qiagen) and eluted with 20 µl of sterile water. Into a plasmid pCR2.1, 4 µl of the

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amplified fragment was employed to insert and *E. coli* DH5α 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 colonies to obtain 4 cDNA clones containing Chinese hamster GFPP 5′ region. They are referred to as GFPP5′ clone 1, GFPP5′ clone 2, GFPP5′ clone 3 and GFPP5′ clone 4.

In the same manner, 5 cDNA clones containing Chinese hamster GFPP 3′ region were obtained. They are referred to as GFPP3′ clone 10, GFPP3′ clone 16 and GFPP3′ clone 20.

The nucleotide sequence of the cDNA of each of the clones obtained by the 5′ and 3′ RACE was determined 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 shown in SEQ ID NO:51.

Example 17

Preparation of CHO cell-derived GMD gene:

1. Determination of CHO cell-derived GMD cDNA sequence

(1) Preparation of CHO cell-derived GMD gene cDNA (preparation of partial cDNA excluding 5′- and 3′-terminal sequences)

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).

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By ligating these EST sequences, a deduced mouse GMD cDNA sequence was determined.

On the base of the mouse-derived GMD cDNA sequence, a 28 mer primer having the sequence represented by SEQ ID NO:56, a 27 mer primer having the sequence represented by SEQ ID NO:57, a 25 mer primer having the sequence represented by SEQ ID NO:58, a 24 mer primer having the sequence represented by SEQ ID NO:59 and a 25 mer primer having the sequence represented by SEQ ID NO:60 were generated.

Next, in order to amplify the CHO cell-derived GMD cDNA, PCR was carried out by the following method. A 20 µl portion of a reaction solution [1 × Ex Taq buffer (manufactured by Takara Shuzo), 0.2 mM dNTPs, 0.5 unit of Ex Taq polymerase (manufactured by Takara Shuzo) and 0.5 µM of two synthetic DNA primers] containing 0.5 µl of the CHO cell-derived single-stranded cDNA prepared in Example 15-1(1) 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 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.

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

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the PCR product when synthetic DNA primers of SEQ ID NOs:56 and 60 were used. The DNA fragments were recovered using Gene Clean II Kit (manufactured by BIO 101) in accordance with the manufacture's instructions. The recovered DNA fragments were ligated to a pT7Blue(R) vector (manufactured by Novagen) using DNA Ligation Kit (manufactured by Takara Shuzo), and *E. coli* DH (manufactured by Toyobo) was transformed 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 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).

In addition, the following steps were carried out in order to prepare a plasmid in which the CHO cell-derived GMD cDNA fragments contained in the plasmids 22-8 and 34-2 are combined. The plasmid 22-8 (1 μg) was allowed to react with a restriction enzyme *Eco*RI (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 using Gene Clean II Kit (manufactured by BIO 101) in accordance with the manufacture's instructions. The plasmid 34-2 (2 μg) was allowed to react with a

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restriction enzyme *Eco*RI at 37°C for 16 hours, the digest was subjected to agarose electrophoresis and then a DNA fragment of about 150 bp was recovered using Gene Clean II Kit (manufactured by BIO 101) in accordance with the manufacture's instructions. The recovered DNA fragments were respectively subjected to terminal dephosphorylation using Calf Intestine Alkaline Phosphatase (manufactured by Takara Shuzo) and then ligated using DNA Ligation Kit (manufactured by Takara Shuzo), and *E. coli* DH5α (manufactured by Toyobo) was transformed using the obtained recombinant plasmid DNA to obtain a plasmid CHO-GMD (cf. Fig. 54).

(2) Determination of 5'-terminal sequence of CHO cell-derived GMD cDNA

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 µl of a reaction solution [1 × Ex Taq buffer (manufactured by Takara Shuzo), 0.2 mM dNTPs, 0.5 unit of Ex Taq polymerase (manufactured by Takara Shuzo) and 0.5 µM of the synthetic DNA primers of SEQ ID NO:61 and SEQ ID NO:62] containing 0.5 µl of the single-stranded cDNA prepared in Example 15-1(1) was prepared as the template, and the reaction was carried out therein 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

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electrophoresis, a DNA fragment of about 300 bp was recovered using Gene Clean II Kit (manufactured by BIO 101) in accordance with the manufacture's instructions. The recovered DNA fragment was ligated to a pT7Blue(R) vector (manufactured by Novagen) using DNA Ligation Kit (manufactured by Takara Shuzo), and *E. coli* DH5α (manufactured by Toyobo) was transformed 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 cell-derived GMD cDNA contained in the plasmid was determined.

(3) Determination of 3'-terminal sequence of CHO cell-derived GMD cDNA

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 Example 15-1(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. The single-stranded cDNA after the preparation was diluted 10 folds with the Tricin-EDTA buffer attached to the kit and used as the template of PCR.

Next, 20 μl of a reaction solution [ExTaq buffer (manufactured by Takara Shuzo), 0.2 mM dNTPs, 0.5 unit of EX Taq polymerase (manufactured by Takara Shuzo), 0.5 μM of the 25 mer synthetic DNA primer shown in SEQ ID NO:63 [generated on the base of the CHO cell-derived GMD cDNA sequence determined in the item (1)] and 1 × concentration of Universal Primer Mix (attached to SMART™ RACE cDNA Amplification Kit; manufactured by CLONTECH] containing 1

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μl of the cDNA for 3' RACE as the template was prepared, and PCR was carried out 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, 1 μl of the PCR reaction solution was diluted 20 folds with Tricin-EDTA buffer (manufactured by CLONTECH). Then, 20 μl of a reaction solution [ExTaq buffer (manufactured by Takara Shuzo), 0.2 mM dNTPs, 0.5 unit of EX Taq polymerase (manufactured by Takara Shuzo), 0.5 μM of the 25 mer synthetic DNA primer shown in SEQ ID NO:64 [generated on the base of the CHO cell-derived GMD cDNA sequence determined in the item (1)] and 0.5 μM of Nested Universal Primer (attached to SMART™ RACE cDNA Amplification Kit; manufactured by CLONTECH) containing 1 μl of the 20 folds-diluted aqueous solution as the template] was prepared, and the reaction was carried out using DNA Thermal Cycler 480 (manufactured by Perkin Elmer) by heating at 94°C for 5 minutes and subsequent 30 cycles 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 700 bp was recovered using Gene Clean II Kit (manufactured by BIO 101) in accordance with the manufacture's instructions. The recovered DNA fragment was ligated to a pT7Blue(R) vector (manufactured by Novagen) using DNA Ligation Kit (manufactured by Takara Shuzo), and *E. coli* DH5α (manufactured by Toyobo) was transformed 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 cell-derived GMD cDNA contained in the plasmid was determined.

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The full length cDNA sequence of the CHO-derived GMD gene determined by the items (1), (2) and (3) and the corresponding amino acid sequence are shown in SEQ ID NOs:65 and 71, respectively.

2. Determination of genomic sequence containing CHO/DG44-derived cell GMD gene

A 25 mer primer having the nucleotide sequence represented by SEQ ID NO:66 was prepared from the mouse GMD cDNA sequence determined in Example 17-1. Next, a CHO cell-derived genome DNA was obtained by the following method. A CHO/DG44 cell-derived KC861 was suspended in IMDM-dFBS(10)-HT(1) medium [IMDM-dFBS(10) medium comprising 1 x concentration of HT supplement (manufactured by Invitrogen)] to give a density of 3×10^5 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 them at 37°C in a 5% CO_2 incubator until the cells became confluent on the plate, genome DNA was prepared from the cells on the plate by a known method [*Nucleic Acids Research*, 3, 2303 (1976)] and dissolved overnight in 150 µl of TE-RNase buffer (pH 8.0) (10 mmol/l Tris-HCl, 1 mmol/l EDTA, 200 µg/ml RNase A).

A reaction solution (20 µl) [1 x Ex Taq buffer (manufactured by Takara Shuzo), 0.2 mM dNTPs, 0.5 unit of EX Taq polymerase (manufactured by Takara Shuzo) and 0.5 µM of synthetic DNA primers of SEQ ID NO:59 and SEQ ID NO:66] containing 100 ng of the obtained CHO/DG44 cell-derived genome DNA was prepared, and PCR was carried out 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 using

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Gene Clean II Kit (manufactured by BIO 101) in accordance with the manufacture's instructions. The recovered DNA fragment was ligated to a pT7Blue(R) vector (manufactured by Novagen) using DNA Ligation Kit (manufactured by Takara Shuzo), and *E. coli* DH5α (manufactured by Toyobo) was transformed 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 genome DNA contained in the plasmid was determined. The result is shown in SEQ ID NO:67.

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 generated on the base of the CHO cell-derived GMD cDNA sequence determined in Example 17-1. Next, 20 μl of a reaction solution [1 × Ex Taq buffer (manufactured by Takara Shuzo), 0.2 mM dNTPs, 0.5 unit of EX Taq polymerase (manufactured by Takara Shuzo) and 0.5 μM of the synthetic DNA primers of SEQ ID NO:68 and SEQ ID NO:69] containing 100 ng of the CHO/DG44-derived genome DNA was prepared, and PCR was carried out 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 200 bp was recovered using Gene Clean II Kit (manufactured by BIO 101) in accordance with the manufacture's instructions. The recovered DNA fragment was ligated to a pT7Blue(R) vector (manufactured by Novagen) using DNA Ligation Kit (manufactured by Takara Shuzo), and *E. coli* DH5α (manufactured by Toyobo) was transformed using the obtained recombinant plasmid DNA, thereby obtaining a plasmid ex4. Using DNA Sequencer 377 (manufactured by Parkin Elmer), the nucleotide sequence of

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CHO cell-derived genome DNA contained in the plasmid was determined. The result is shown in SEQ ID NO:70.

Example 18

Sugar chain analysis of conventionally available antibodies:

Sugar chains binding to a conventionally available anti-HER2/neu antibody Herceptin (manufactured by GENENTECH and Roche) produced by CHO cell as the host cell was analyzed in accordance with the method of Example 10(6) (Fig. 31). When calculated from each peak area of elution diagram, the content of α-1,6-fucose-free sugar chains of Herceptin was 16%, and the content of α-1,6-fucose-bound sugar chains was 84%. The same analysis was carried out on other commercially available antibodies, Rituxan (manufactured by GENENTECH, Roche and IDEC) and Zenapax (manufactured by Roche and PDL), and the α-1,6-fucose-free sugar chain content of was less than that in Herceptin.

Fig. 31 is a graph showing elution pattern of PA-treated sugar chains prepared from Herceptin, obtained by analyzing them by reverse phase HPLC. The relative fluorescence intensity and the elution time are plotted as the ordinate and the abscissa, respectively. The reverse phase HPLC analysis conditions, sugar chain structure analysis and calculation of the ratio of sugar chain group containing no α-1,6-fucose sugar chain were carried out by the same methods of Example 11(6).

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.

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WHAT IS CLAIMED IS:

1. A Chinese hamster ovary tissue-derived CHO cell into which a gene encoding an antibody molecule is introduced, which produces an antibody composition comprising an antibody molecule having complex *N*-glycoside-linked sugar chains bound to the Fc region, wherein among the total complex *N*-glycoside-linked sugar chains bound to the Fc region in the composition, 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.

2. The CHO cell according to claim 1, wherein the sugar chain to which fucose is not bound is a complex *N*-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.

3. The CHO cell according to claim 1 or 2, wherein the antibody molecule belongs to an IgG class.

4. The CHO cell according to any one of claims 1 to 3, wherein the activity of an enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose and/or the activity of an enzyme relating to the 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 the complex *N*-glycoside-linked sugar chain is decreased or deleted.

5. The CHO cell according to claim 4, wherein the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose is an enzyme selected from the group consisting of the following (a), (b) and (c):

(a) GMD (GDP-mannose 4,6-dehydratase);

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(b) Fx (GDP-keto-6-deoxymannose 3,5-epimerase, 4-reductase);

(c) GFPP (GDP-beta-L-fucose pyrophosphorylase).

6. The CHO cell according to claim 5, wherein the GMD is a protein encoded by a DNA of the following (a) or (b):

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

(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.

7. The CHO cell according to claim 5, wherein the GMD is a protein selected from the group consisting of the following (a), (b) and (c):

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

(b) 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;

(c) a protein which comprises an amino acid sequence having a homology of at least 80% with the amino acid sequence represented by SEQ ID NO:71 and has GMD activity.

8. The CHO cell according to claim 5, wherein the Fx is a protein encoded by a DNA of the following (a) or (b):

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

(b) a DNA which hybridizes with the DNA comprising the nucleotide sequence represented by SEQ ID NO:48 under

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stringent conditions and encodes a protein having Fx activity.

9. The CHO cell according to claim 5, wherein the Fx is a protein selected from the group consisting of the following (a), (b) and (c):

(a) a protein comprising the amino acid sequence represented by SEQ ID NO:72;

(b) 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:72 and has Fx activity;

(c) a protein which comprises an amino acid sequence having a homology of at least 80% with the amino acid sequence represented by SEQ ID NO:72 and has Fx activity.

10. The CHO cell according to claim 5, wherein the GFPP is a protein encoded by a DNA of the following (a) or (b):

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

(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.

11. The CHO cell according to claim 5, wherein the GFPP is a protein selected from the group consisting of the following (a), (b) and (c):

(a) a protein comprising the amino acid sequence represented by SEQ ID NO:73;

(b) a protein which comprises an amino acid sequence in which at least one amino acid is deleted,

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substituted, inserted and/or added in the amino acid sequence represented by SEQ ID NO:73 and has GFPP activity;

(c) a protein which comprises an amino acid sequence having a homology of at least 80% with the amino acid sequence represented by SEQ ID NO:73 and has GFPP activity.

12. The CHO cell according to claim 4, wherein the enzyme relating to the modification of a sugar chain in which 1-position of fucose is bound to 6-position of the *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain is α-1,6-fucosyltransferase.

13. The CHO cell according to claim 12, wherein the α-1,6-fucosyltransferase is a protein encoded by a DNA of the following (a) or (b):

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

(b) 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.

14. The CHO cell according to claim 12, wherein the α-1,6-fucosyltransferase is a protein selected from the group consisting of the following (a), (b) and (c):

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

(b) 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;

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(c) a protein which comprises an amino acid sequence having a homology of at least 80% with the amino acid sequence represented by SEQ ID NO:23 and has α-1,6-fucosyltransferase activity.

15. The CHO cell according to any one of claims 4 to 14, wherein the enzyme activity is decreased or deleted by a technique selected from the group consisting of the following (a), (b), (c), (d) and (e):

(a) a gene disruption technique targeting a gene encoding the enzyme;

(b) a technique for introducing a dominant negative mutant of a gene encoding the enzyme;

(c) a technique for introducing mutation into the enzyme;

(d) a technique for inhibiting transcription and/or translation of a gene encoding the enzyme;

(e) a technique for selecting a cell line 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 the complex *N*-glycoside-linked sugar chain.

16. The CHO cell according to any one of claims 4 to 15, which is resistant to at least 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 the complex *N*-glycoside-linked sugar chain.

17. The CHO cell according to any one of claims 4 to 16, which produces an antibody composition having higher antibody-dependent cell-mediated cytotoxic activity than an antibody composition produced by its parent CHO cell.

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18. The CHO cell according to claim 17, which produces an antibody composition having higher antibody-dependent cell-mediated cytotoxic activity than an antibody composition in which among the total complex *N*-glycoside-linked sugar chains bound to the Fc region contained in the antibody composition, the ratio of a sugar chain in which fucose is not bound to *N*-acetylglucosamine in the reducing end in the sugar chain is less than 20%.

19. The CHO cell according to claim 18, wherein the sugar chain to which fucose is not bound is a complex *N*-glycoside-linked sugar chain in which 1-position of the fucose is not bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond.

20. A method for producing an antibody composition, which comprises culturing the CHO cell according to any one of claims 1 to 19 in a medium to produce and accumulate an antibody composition in the culture; and recovering the antibody composition from the culture.

21. An antibody composition which is produced using the method according to claim 20.

22. An antibody composition which comprises an antibody molecule having complex *N*-glycoside-linked sugar chains bound to the Fc region which is produced by a CHO cell, wherein among the total complex *N*-glycoside-linked sugar chains bound to the Fc region in the composition, 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.

23. A cell in which the activity of an enzyme relating to the synthesis of an intracellular sugar

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nucleotide, GDP-fucose and/or the activity of an enzyme relating to the modification of a sugar chain wherein 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the complex *N*-glycoside-linked sugar chain is decreased or deleted by a genetic engineering technique.

24. The cell according to claim 23, wherein the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose is an enzyme selected from the group consisting of the following (a), (b) and (c):

(a) GMD (GDP-mannose 4,6-dehydratase);

(b) Fx (GDP-keto-6-deoxymannose 3,5-epimerase, 4-reductase);

(c) GFPP (GDP-beta-L-fucose pyrophosphorylase).

25. The cell according to claim 24, wherein the GMD is a protein encoded by a DNA of the following (a) or (b):

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

(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.

26. The cell according to claim 24, wherein the GMD is a protein selected from the group consisting of the following (a), (b) and (c):

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

(b) 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;

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(c) a protein which comprises an amino acid sequence having a homology of at least 80% with the amino acid sequence represented by SEQ ID NO:71 and has GMD activity.

27. The cell according to claim 24, wherein the Fx is a protein encoded by a DNA of the following (a) or (b):

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

(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.

28. The cell according to claim 24, wherein the Fx is a protein selected from the group consisting of the following (a), (b) and (c):

(a) a protein comprising the amino acid sequence represented by SEQ ID NO:72;

(b) 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:72 and has Fx activity;

(c) a protein which comprises an amino acid sequence having a homology of at least 80% with the amino acid sequence represented by SEQ ID NO:72 and has Fx activity.

29. The cell according to claim 24, wherein the GFPP is a protein encoded by a DNA of the following (a) or (b):

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

(b) a DNA which hybridizes with the DNA comprising the nucleotide sequence represented by SEQ ID NO:51 under

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stringent conditions and encodes a protein having GFPP activity.

30. The cell according to claim 24, wherein the GFPP is a protein selected from the group consisting of the following (a), (b) and (c):

(a) a protein comprising the amino acid sequence represented by SEQ ID NO:73;

(b) 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:73 and has GFPP activity;

(c) a protein which comprises an amino acid sequence having a homology of at least 80% with the amino acid sequence represented by SEQ ID NO:73 and has GFPP activity.

31. The cell according to claim 23, wherein the enzyme relating to the 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 the *N*-glycoside-linked sugar chain is α-1,6-fucosyltransferase.

32. The cell according to claim 31, wherein the α-1,6-fucosyltransferase is a protein encoded by a DNA selected from the group consisting of the following (a), (b), (c) and (d):

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

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

(c) a DNA which hybridizes with the DNA comprising the nucleotide sequence represented by SEQ ID NO:1 under

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stringent conditions and encodes a protein having α-1,6-fucosyltransferase activity,;

(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.

33. The cell according to claim 31, wherein the α-1,6-fucosyltransferase is a protein selected from the group consisting of the following (a), (b), (c), (d), (e) and (f):

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

(b) a protein comprising the amino acid sequence represented by SEQ ID NO:24;

(c) 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;

(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:24 and has α-1,6-fucosyltransferase activity;

(e) a protein which comprises an amino acid sequence having a homology of at least 80% with the amino acid sequence represented by SEQ ID NO:23 and has α-1,6-fucosyltransferase activity;

(f) a protein which comprises an amino acid sequence having a homology of at least 80% with the amino acid sequence represented by SEQ ID NO:24 and has α-1,6-fucosyltransferase activity.

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34. The cell according to any one of claims 23 to 33, wherein the genetic engineering technique is a technique selected from the group consisting of the following (a), (b), (c) and (d):

(a) a gene disruption technique targeting a gene encoding the enzyme;

(b) a technique for introducing a dominant negative mutant of a gene encoding the enzyme;

(c) a technique for introducing mutation into the enzyme;

(d) a technique for inhibiting transcription and/or translation of a gene encoding the enzyme.

35. The cell according to any one of claims 23 to 34, which is resistant to at least 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 the *N*-glycoside-linked sugar chain.

36. The cell according to any one of claims 23 to 35, which is a cell 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 mouse myeloma cell line, NSO cell;

(d) a mouse myeloma cell line, SP2/0-Ag14 cell;

(e) a BHK cell derived from a syrian hamster kidney tissue;

(f) an antibody-producing hybridoma cell;

(g) a human leukemia cell line Namalwa cell;

(h) an embryonic stem cell;

(i) a fertilized egg cell.

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37. The cell according to any one of claims 23 to 36 into which a gene encoding an antibody molecule is introduced.

38. The cell according to claim 37, wherein the antibody molecule belongs to an IgG class.

39. A method for producing an antibody composition, which comprises culturing the cell according to claim 37 or 38 in a medium to produce and accumulate the antibody composition in the culture; and recovering the antibody composition from the culture.

40. The method according to claim 39, which produces an antibody composition having higher antibody-dependent cell-mediated cytotoxic activity than an antibody composition obtained from its parent cell line.

41. An antibody composition which is produced using the method according to claim 39 or 40.

42. A transgenic non-human animal or plant or the progenies thereof, comprising a genome which is modified such that the activity of an enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose and/or the activity of an enzyme relating to the 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 the *N*-glycoside-linked sugar chain is decreased.

43. The transgenic non-human animal or plant or the progenies thereof according to claim 42, wherein a gene encoding the enzyme relating to the synthesis of an

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intracellular sugar nucleotide, GDP-fucose or a gene encoding the enzyme relating to the 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 the *N*-glycoside-linked sugar chain is knocked out.

44. The transgenic non-human animal or plant or the progenies thereof according to claim 42 or 43, wherein the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose is an enzyme selected from the group consisting of the following (a), (b) and (c):

(a) GMD (GDP-mannose 4,6-dehydratase);

(b) Fx (GDP-keto-6-deoxymannose 3,5-epimerase, 4-reductase);

(c) GFPP (GDP-beta-L-fucose pyrophosphorylase).

45. The transgenic non-human animal or plant or the progenies thereof according to claim 44, wherein the GMD is a protein encoded by a DNA of the following (a) or (b):

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

(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.

46. The transgenic non-human animal or plant or the progenies thereof according to claim 44, wherein the Fx is a protein encoded by a DNA of the following (a) or (b):

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

(b) a DNA which hybridizes with the DNA comprising the nucleotide sequence represented by SEQ ID NO:48 under

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stringent conditions and encodes a protein having Fx activity.

47. The transgenic non-human animal or plant or the progenies thereof according to claim 44, wherein the GFPP is a protein encoded by a DNA of the following (a) or (b):

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

(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.

48. The transgenic non-human animal or plant or the progenies thereof according to claim 42 or 43, wherein the enzyme relating to the 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 the *N*-glycoside-linked sugar chain is α-1,6-fucosyltransferase.

49. The transgenic non-human animal or plant or the progenies thereof according to claim 48, wherein the α-1,6-fucosyltransferase is a protein encoded by a DNA selected from the group consisting of the following (a), (b), (c) and (d):

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

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

(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;

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(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.

50. The transgenic non-human animal or plant or the progenies thereof according to any one of claims 42 to 49, wherein the transgenic non-human animal is an animal selected from the group consisting of cattle, sheep, goat, pig, horse, mouse, rat, fowl, monkey and rabbit.

51. A method for producing an antibody composition, which comprises introducing a gene encoding an antibody molecule into the transgenic non-human animal or plant or the progenies thereof according to any one of claims 42 to 50; rearing the animal or plant; isolating tissue or body fluid comprising the introduced antibody from the reared animal or plant; and recovering the antibody composition from the isolated tissue or body fluid.

52. The method according to claim 51, wherein the antibody molecule belongs to an IgG class.

53. The method according to claim 51 or 52, which produces an antibody composition having higher antibody-dependent cell-mediated cytotoxic activity than an antibody composition obtained from a non-human animal or plant or the progenies thereof whose genome is not modified.

54. An antibody composition which is produced using the method according to any one of claims 51 to 53.

55. A medicament comprising the antibody composition according to any one of claims 21, 22, 41 and 54 as an active ingredient.

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56. The medicament according to claim 55, wherein the medicament is a diagnostic drug, a preventive drug or a therapeutic drug for diseases accompanied by tumors, diseases accompanied by allergies, diseases accompanied by inflammations, autoimmune diseases, circulatory organ diseases, diseases accompanied by viral infections or diseases accompanied by bacterial infections.

57. A protein selected from the group consisting of the following (a), (b), (c), (d), (e), (f), (g), (h), (i) and (j):

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

(b) 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;

(c) a protein comprising the amino acid sequence represented by SEQ ID NO:72;

(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:72 and has Fx activity;

(e) a protein comprising the amino acid sequence represented by SEQ ID NO:73;

(f) 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:73 and has GFPP activity;

(g) a protein comprising the amino acid sequence represented by SEQ ID NO:23;

(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

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sequence represented by SEQ ID NO:23 and has α-1,6-fucosyltransferase activity;

(i) a protein comprising the amino acid sequence represented by SEQ ID NO:24;

(j) 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 the α-1,6-fucosyltransferase activity.

58. A DNA which encodes the protein according to claim 57.

59. A DNA selected from the group consisting of the following (a), (b), (c), (d) and (e):

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

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

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

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

(e) a DNA comprising the nucleotide sequence represented by SEQ ID NO:51.

60. A genome DNA selected from the group consisting of the following (a), (b) and (c):

(a) a genome DNA comprising the nucleotide sequence represented by SEQ ID NO:3;

(b) a genome DNA comprising the nucleotide sequence represented by SEQ ID NO:67;

(c) a genome DNA comprising the nucleotide sequence represented by SEQ ID NO:70.

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61. A target vector for homologous recombination, comprising a full length of the DNA according to any one of claims 58 to 60, or a part thereof.

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ABSTRACT OF THE DISCLOSURE

The present invention relates to a cell for the production of an antibody molecule such as an antibody useful for various diseases having high antibody-dependent cell-mediated cytotoxic activity, a fragment of the antibody and a fusion protein having the Fc region of the antibody or the like, a method for producing an antibody composition using the cell, the antibody composition and use thereof.

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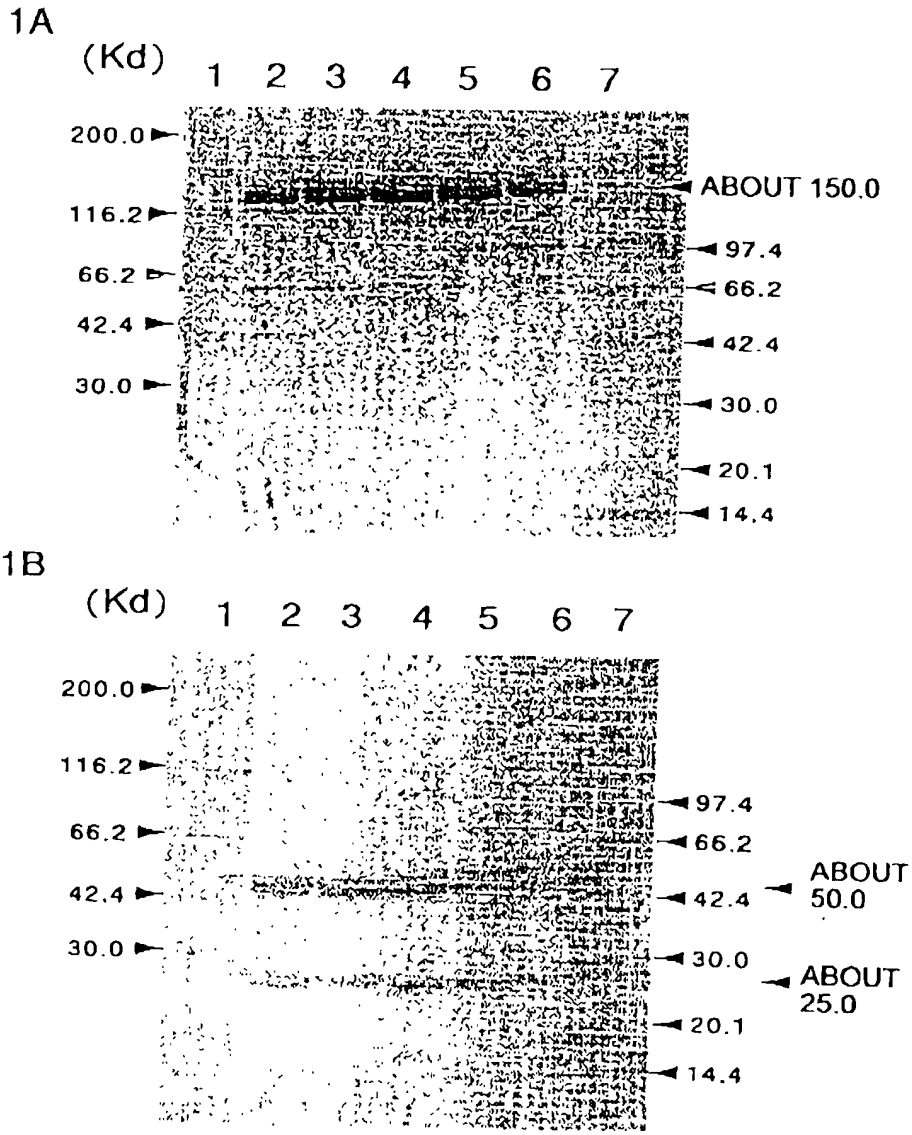
FIG. 1



Aragen/Transposagen Ex. 1036 - Part G

FIG. 2

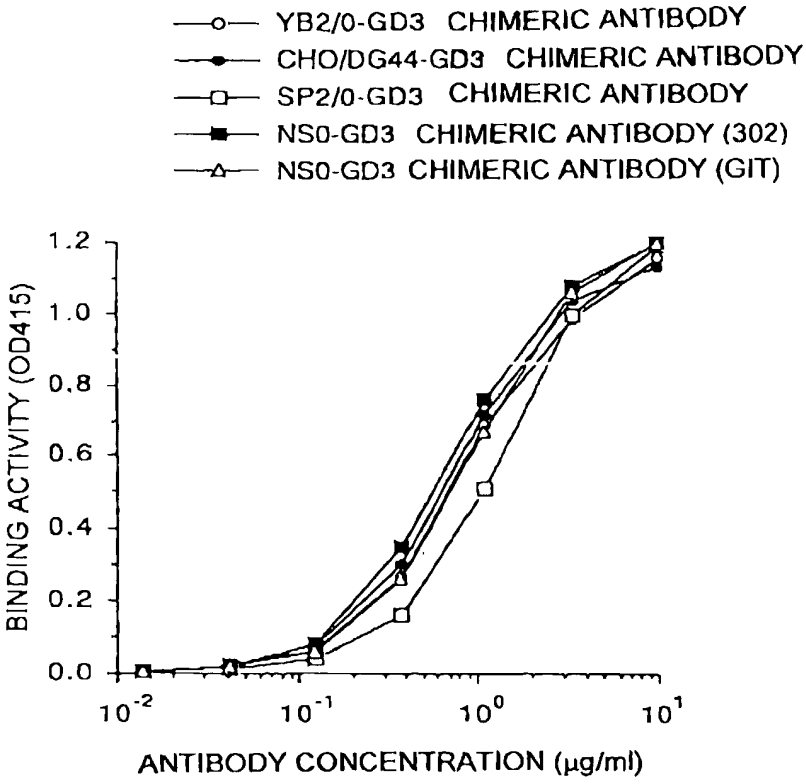

YB2/0-GD3 CHIMERIC ANTIBODY
CHO/DG44-GD3 CHIMERIC ANTIBODY
SP2/0-GD3 CHIMERIC ANTIBODY
NS0-GD3 CHIMERIC ANTIBODY (302)
NS0-GD3 CHIMERIC ANTIBODY (GIT)
BINDING ACTIVITY (OD415)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10^-2
10^-1
10^0
10^1
ANTIBODY CONCENTRATION (μg/ml)

FIG. 3

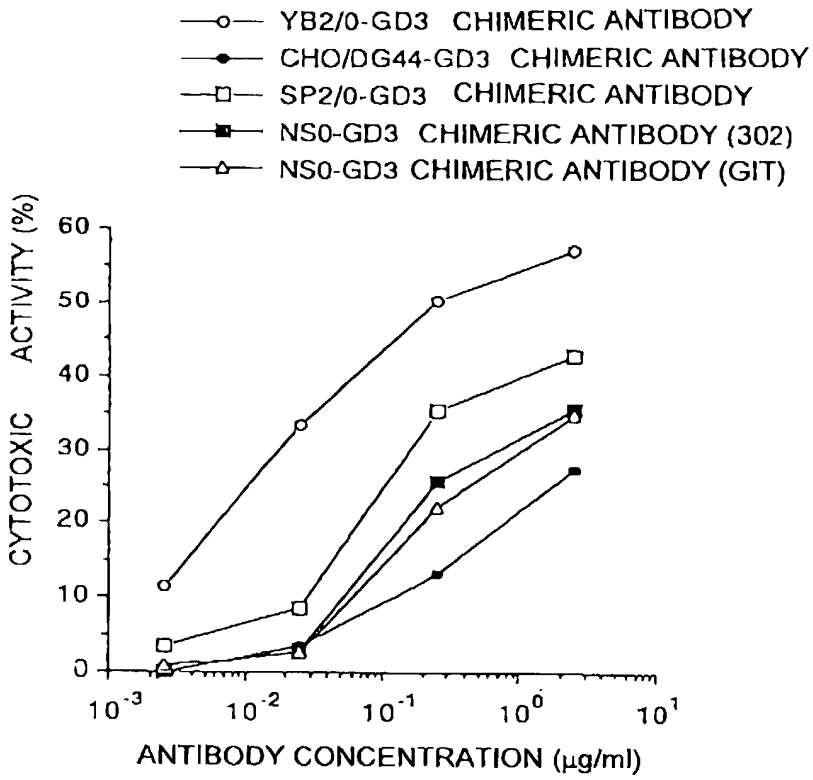


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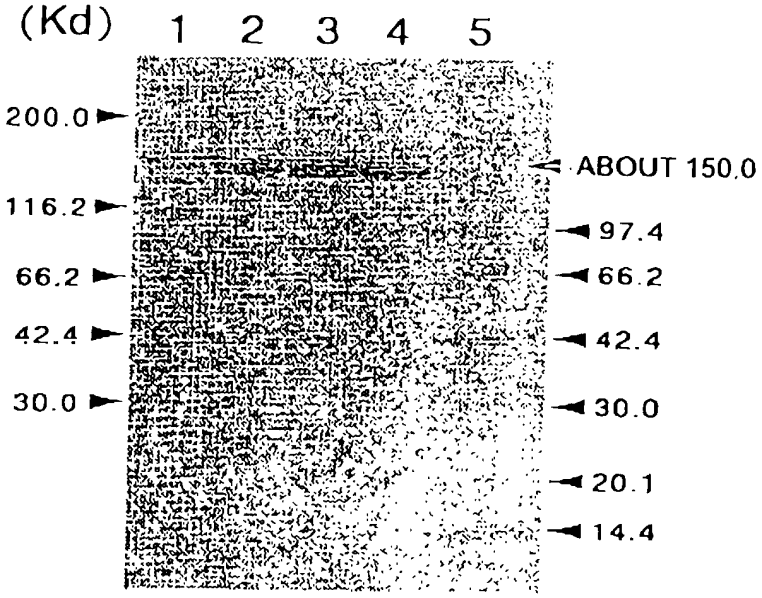
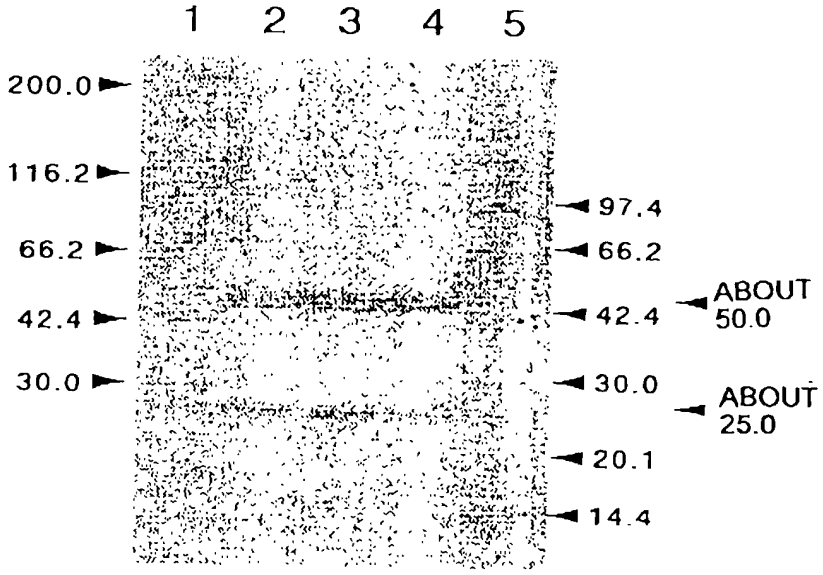
FIG. 4

4A

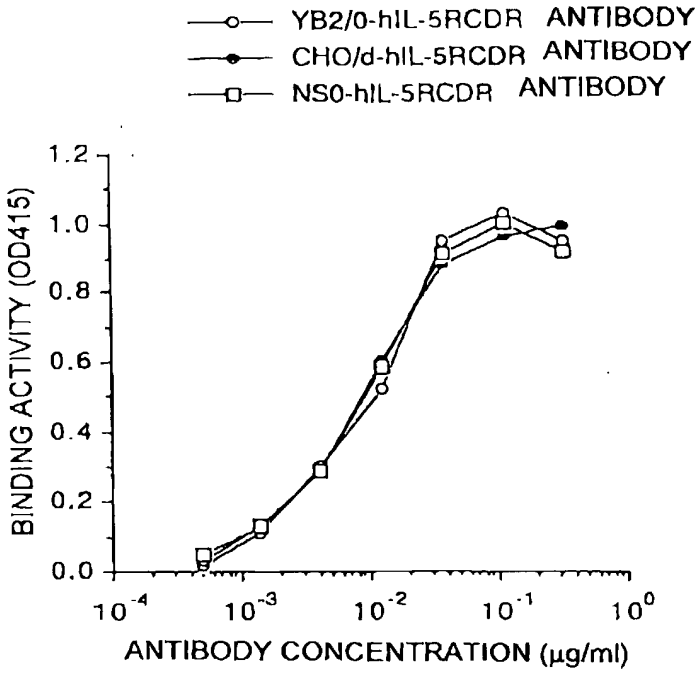


4B



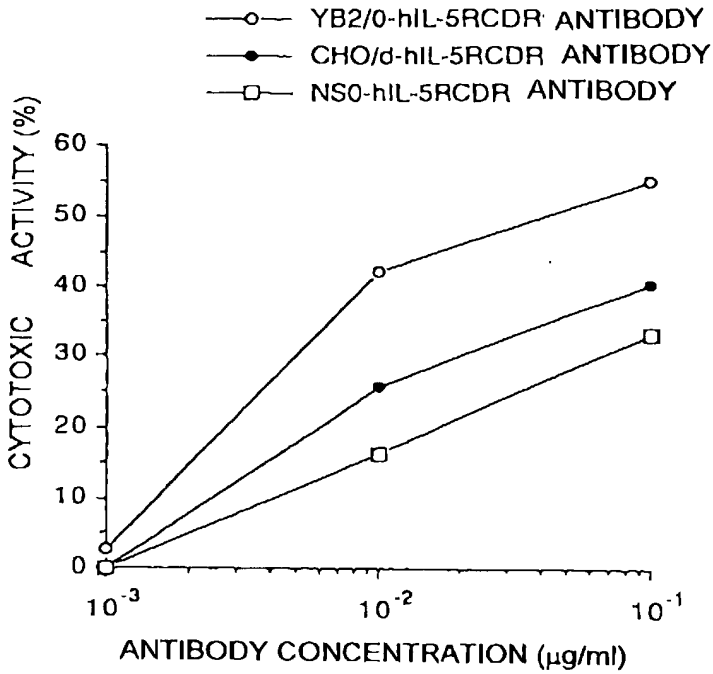
Aragen/Transposagen Ex. 1036 - Part G

FIG. 5



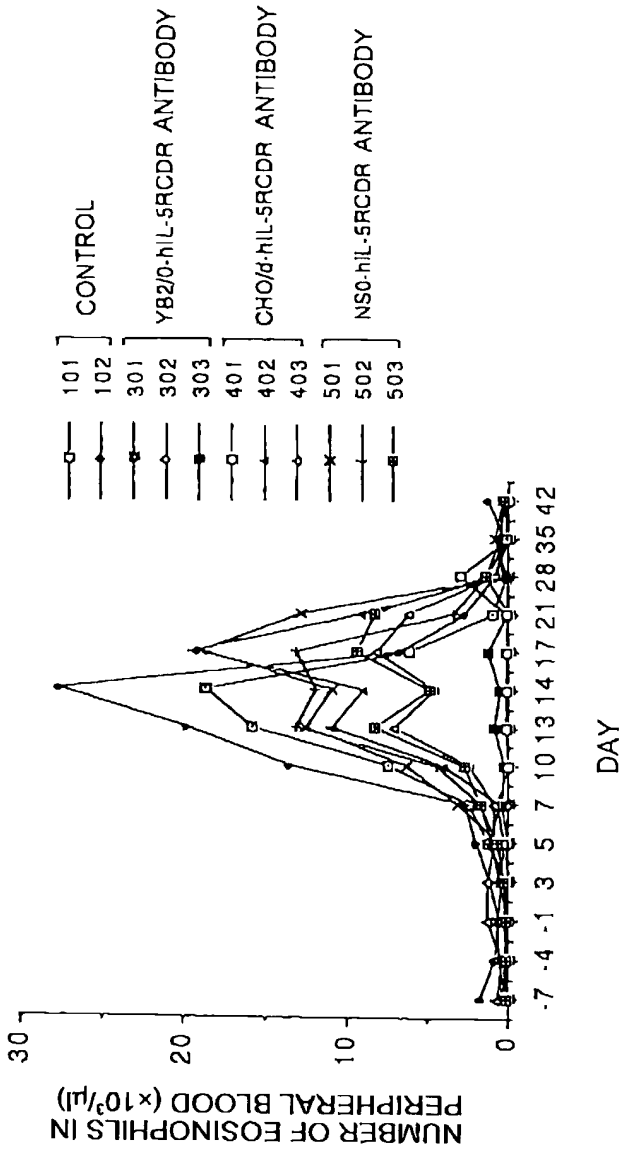
Aragen/Transposagen Ex. 1036 - Part G

FIG. 6



Aragen/Transposagen Ex. 1036 - Part G

FIG. 7

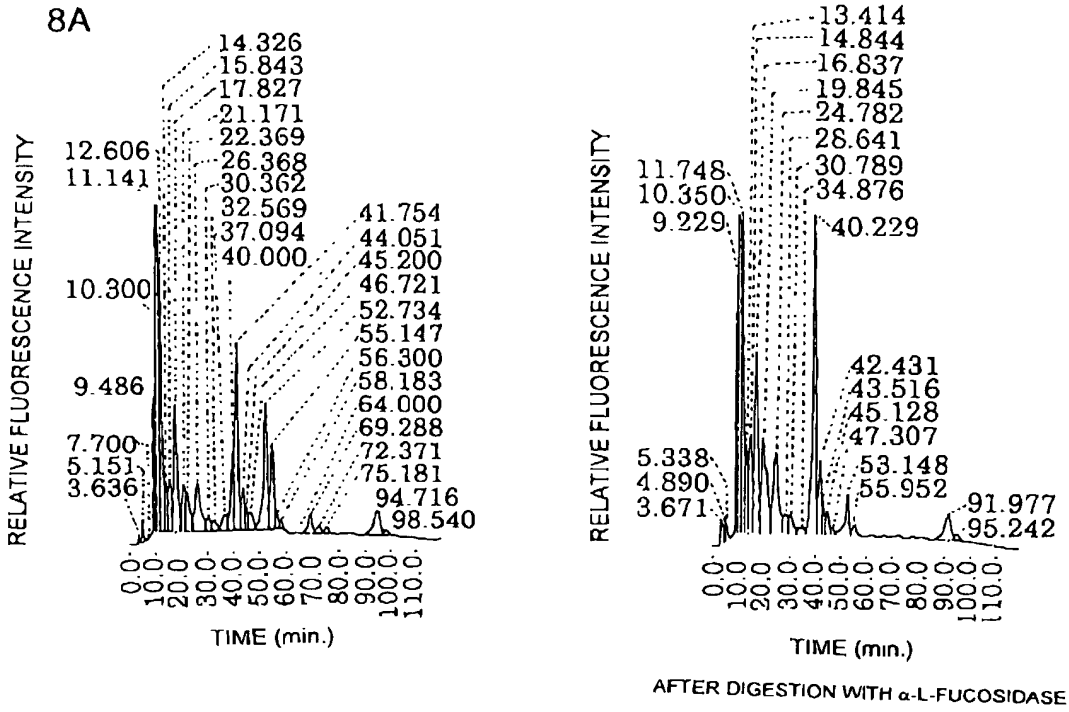


Aragen/Transposagen Ex. 1036 - Part G

FIG. 8

YB2/0-hIL-5RCDR ANTIBODY

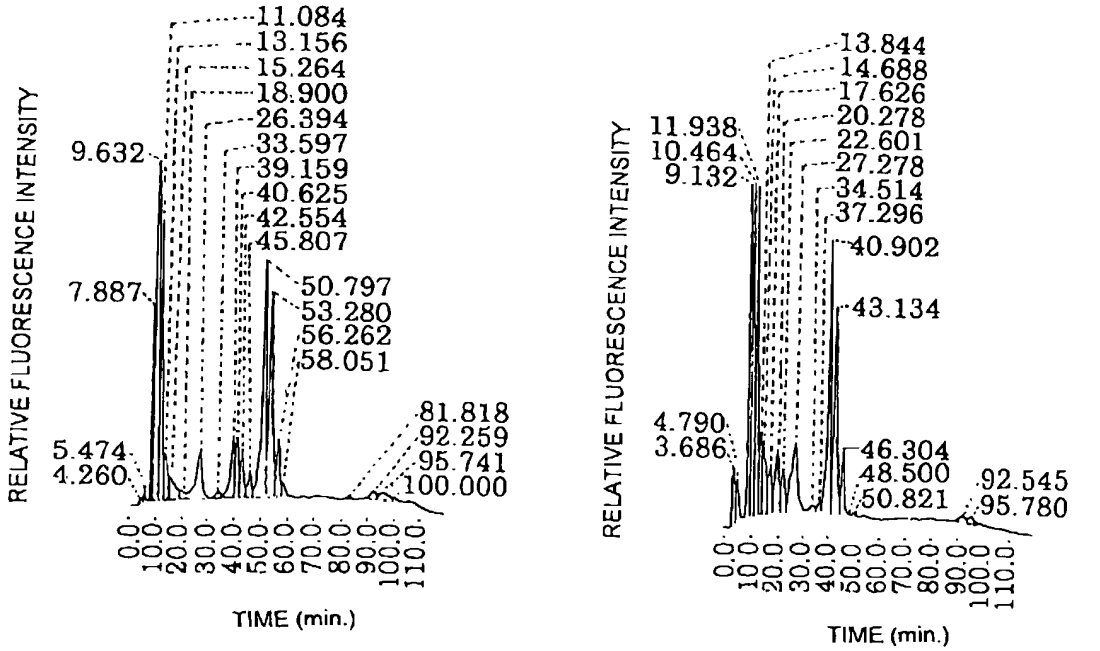
8A



AFTER DIGESTION WITH α-L-FUCOSIDASE

8B

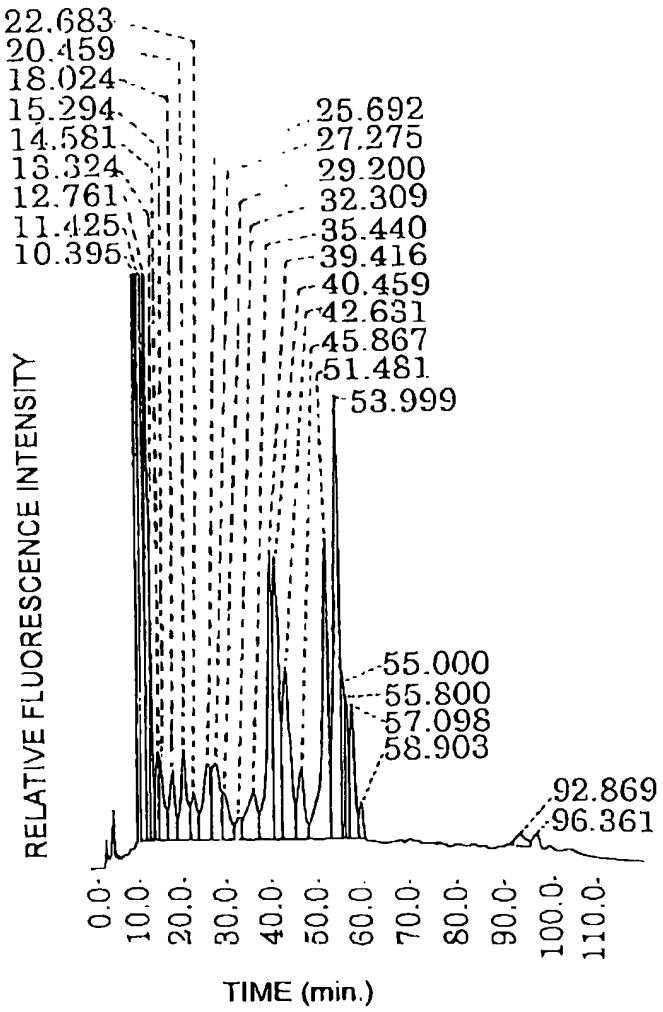
NS0-hIL-5RCDR ANTIBODY



AFTER DIGESTION WITH α-L-FUCOSIDASE

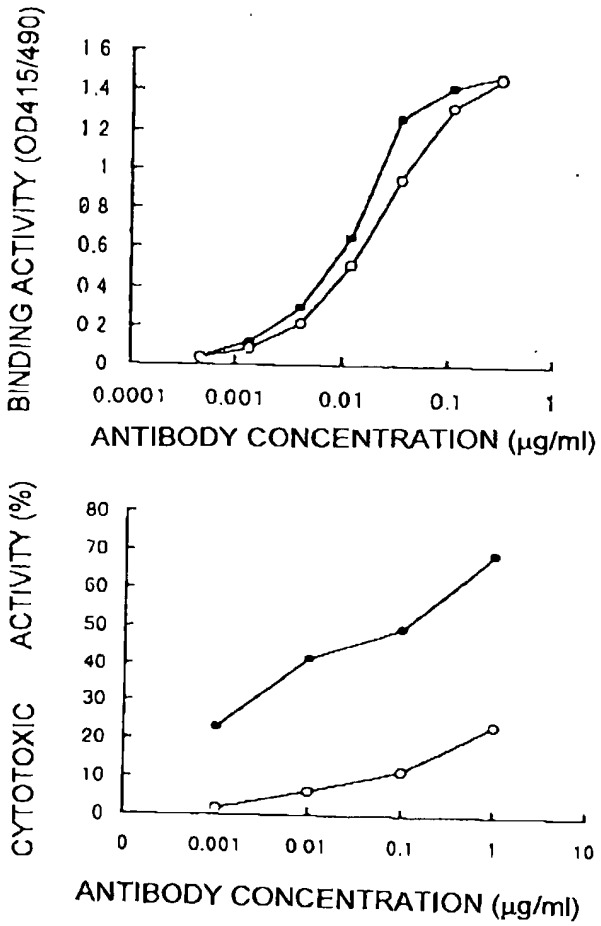
Aragen/Transposagen Ex. 1036 - Part G

FIG. 9



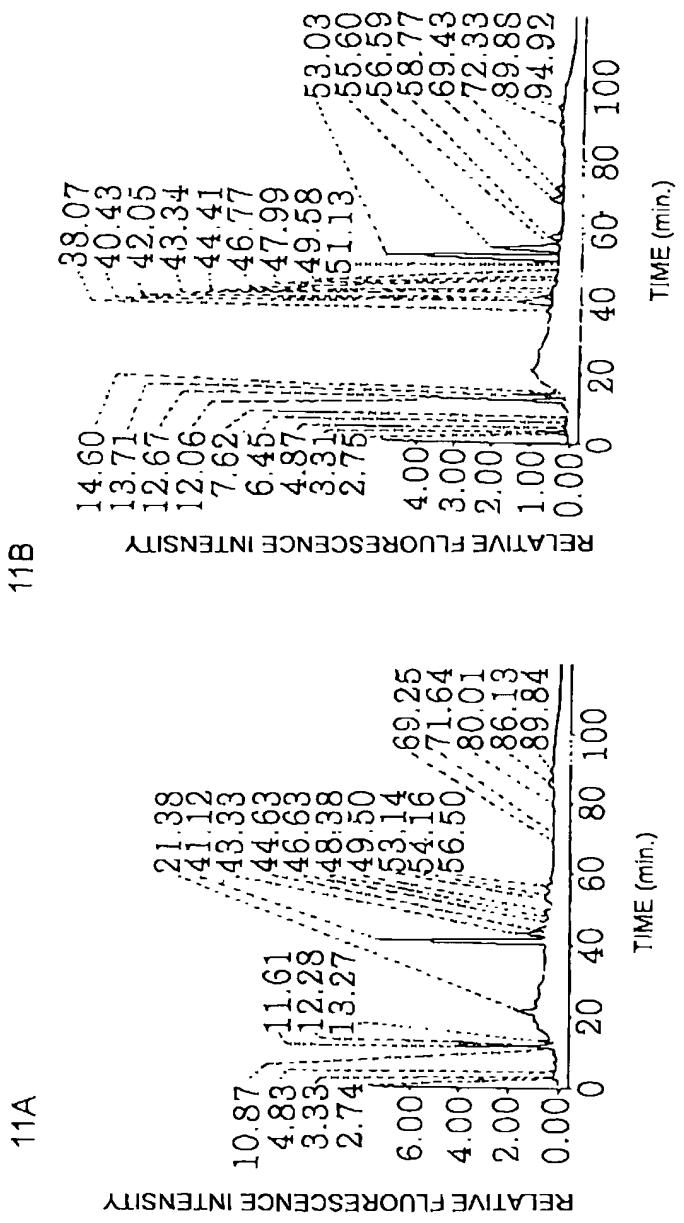
Aragen/Transposagen Ex. 1036 - Part G

FIG. 10



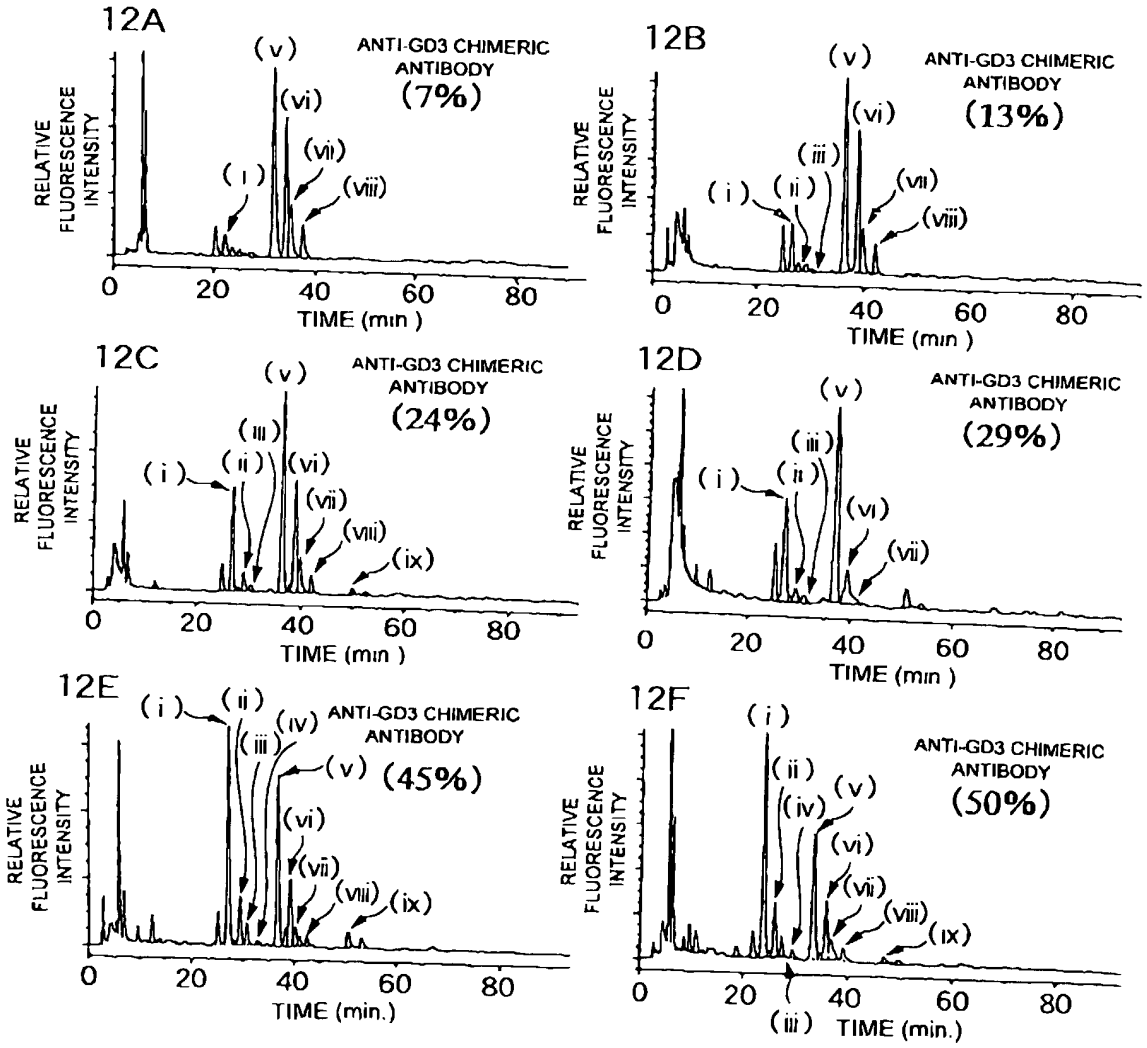
Aragen/Transposagen Ex. 1036 - Part G

FIG. 11



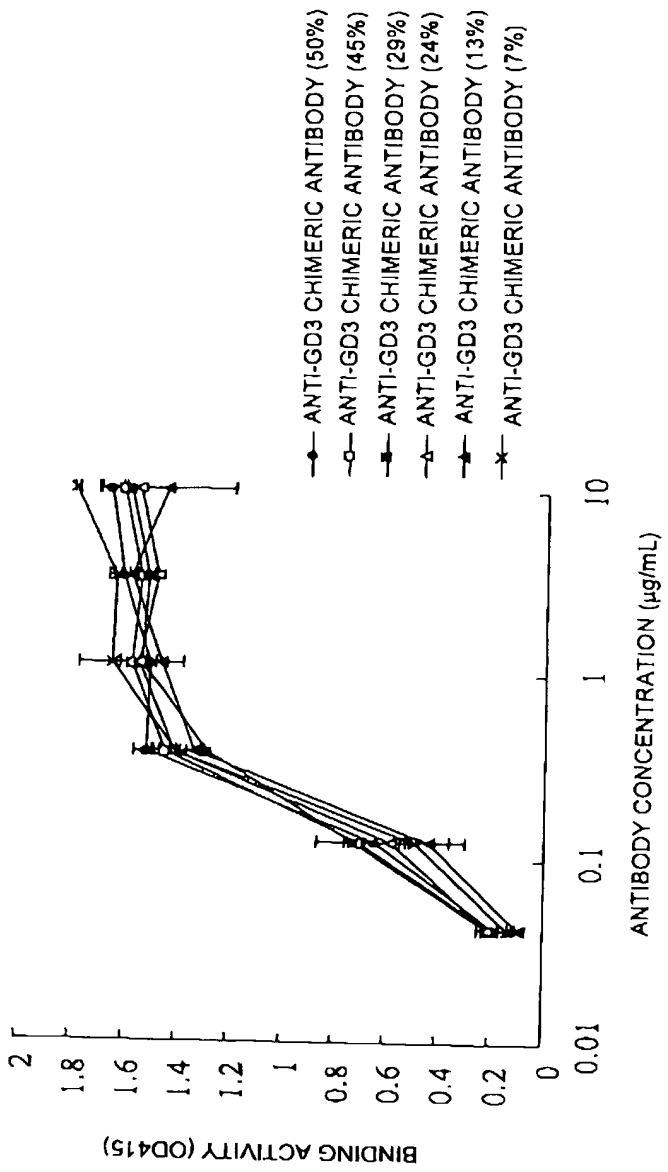
Aragen/Transposagen Ex. 1036 - Part G

FIG. 12



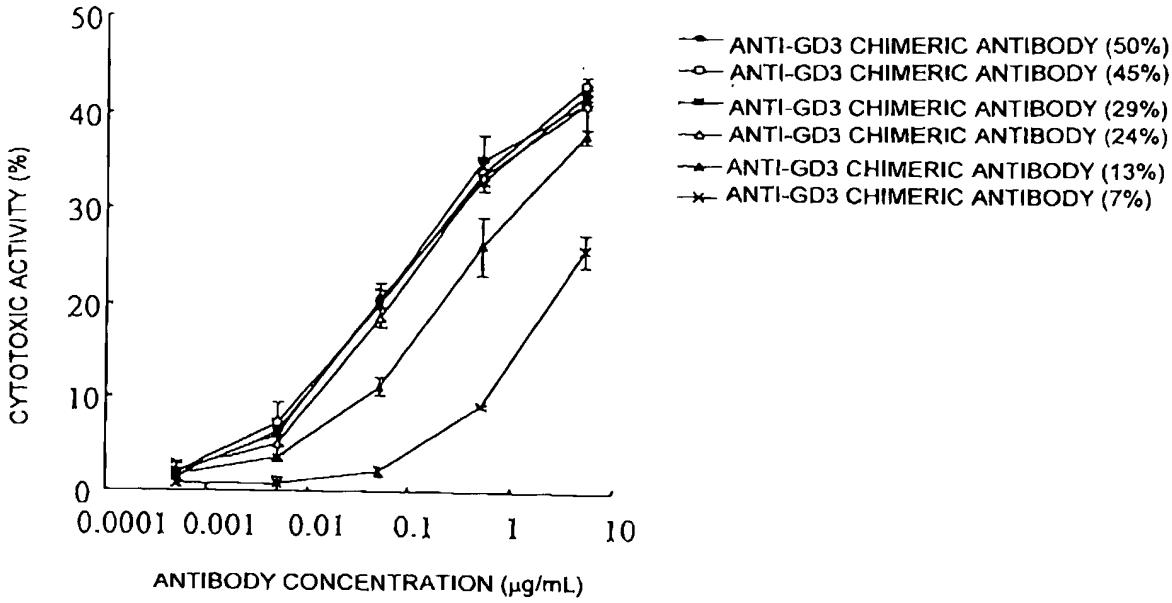
Aragen/Transposagen Ex. 1036 - Part G

FIG. 13



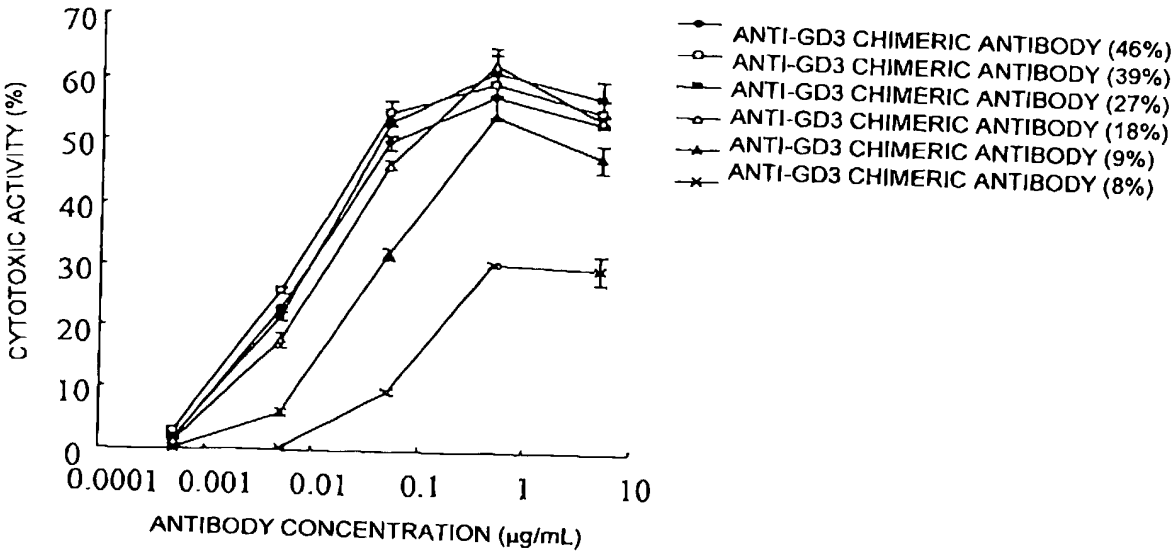
Aragen/Transposagen Ex. 1036 - Part G

FIG. 14



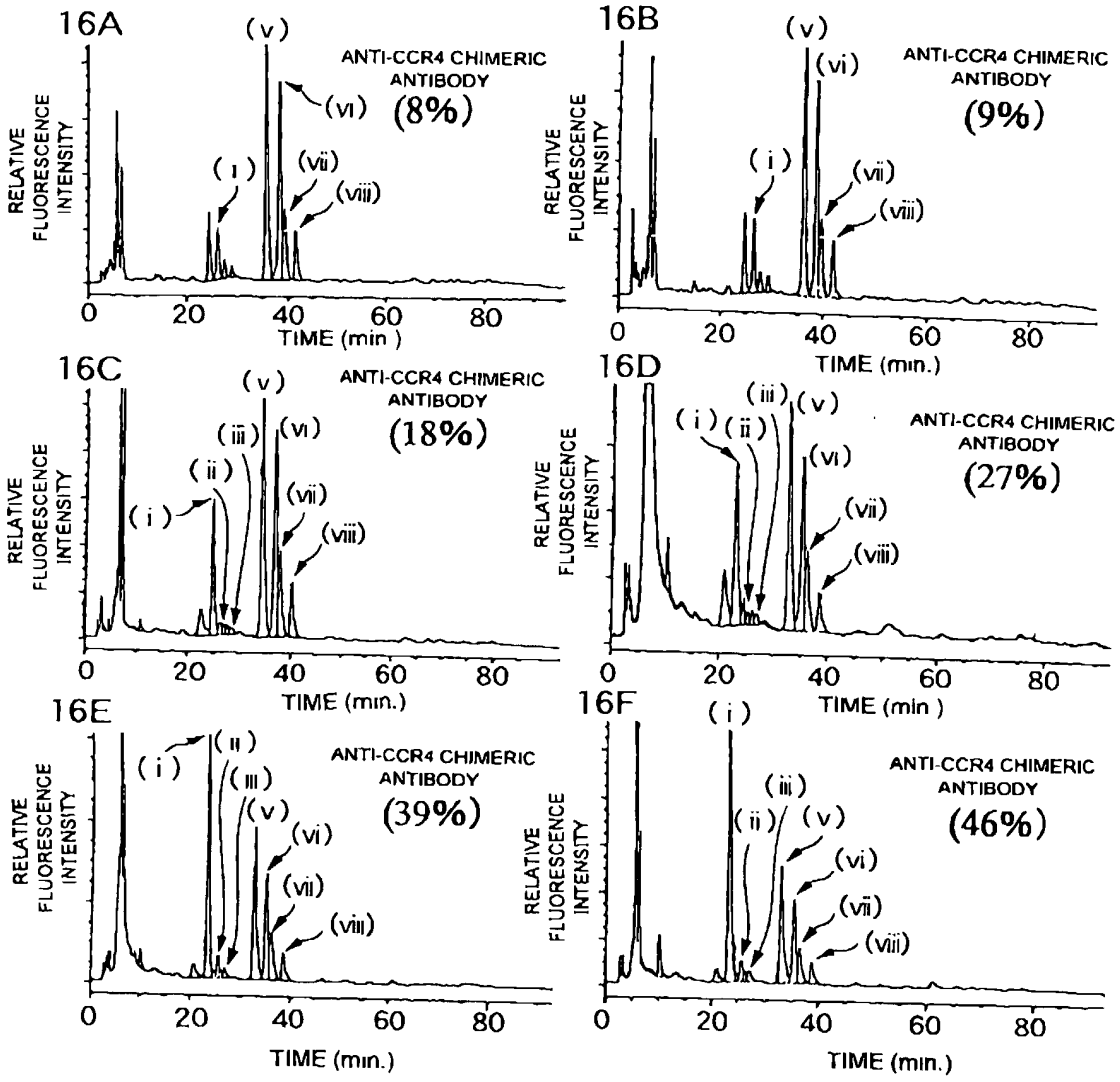
Aragen/Transposagen Ex. 1036 - Part G

FIG. 15



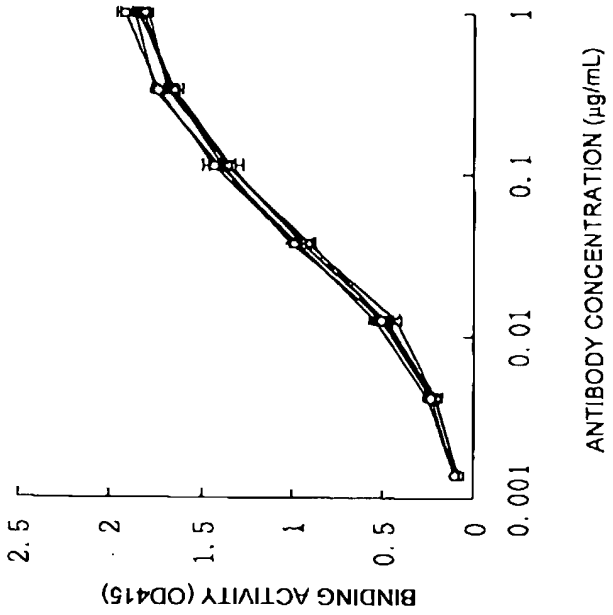
Aragen/Transposagen Ex. 1036 - Part G

FIG. 16



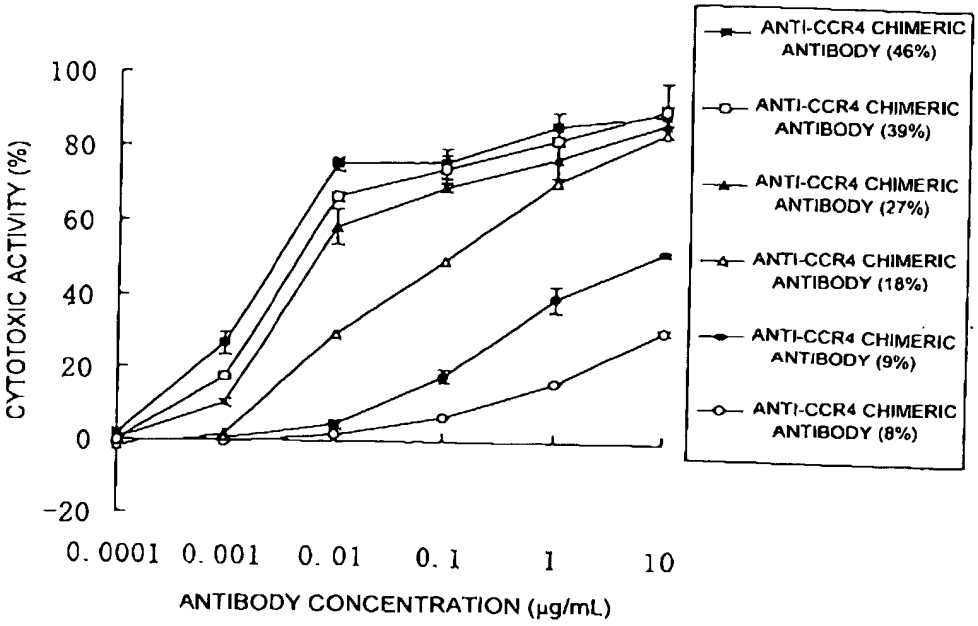
Aragen/Transposagen Ex. 1036 - Part G

FIG. 17



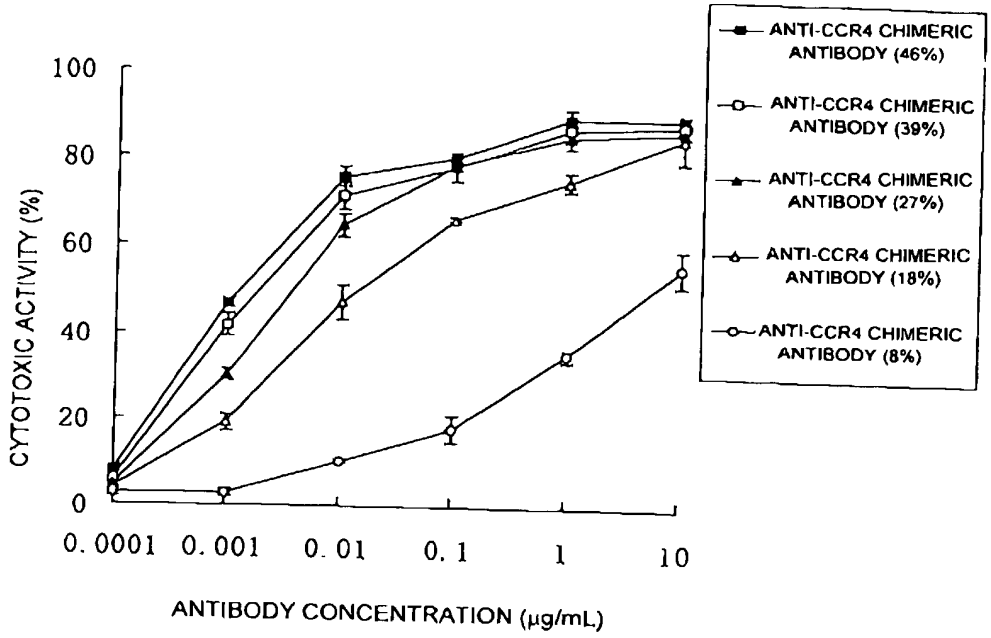
Aragen/Transposagen Ex. 1036 - Part G

FIG. 18



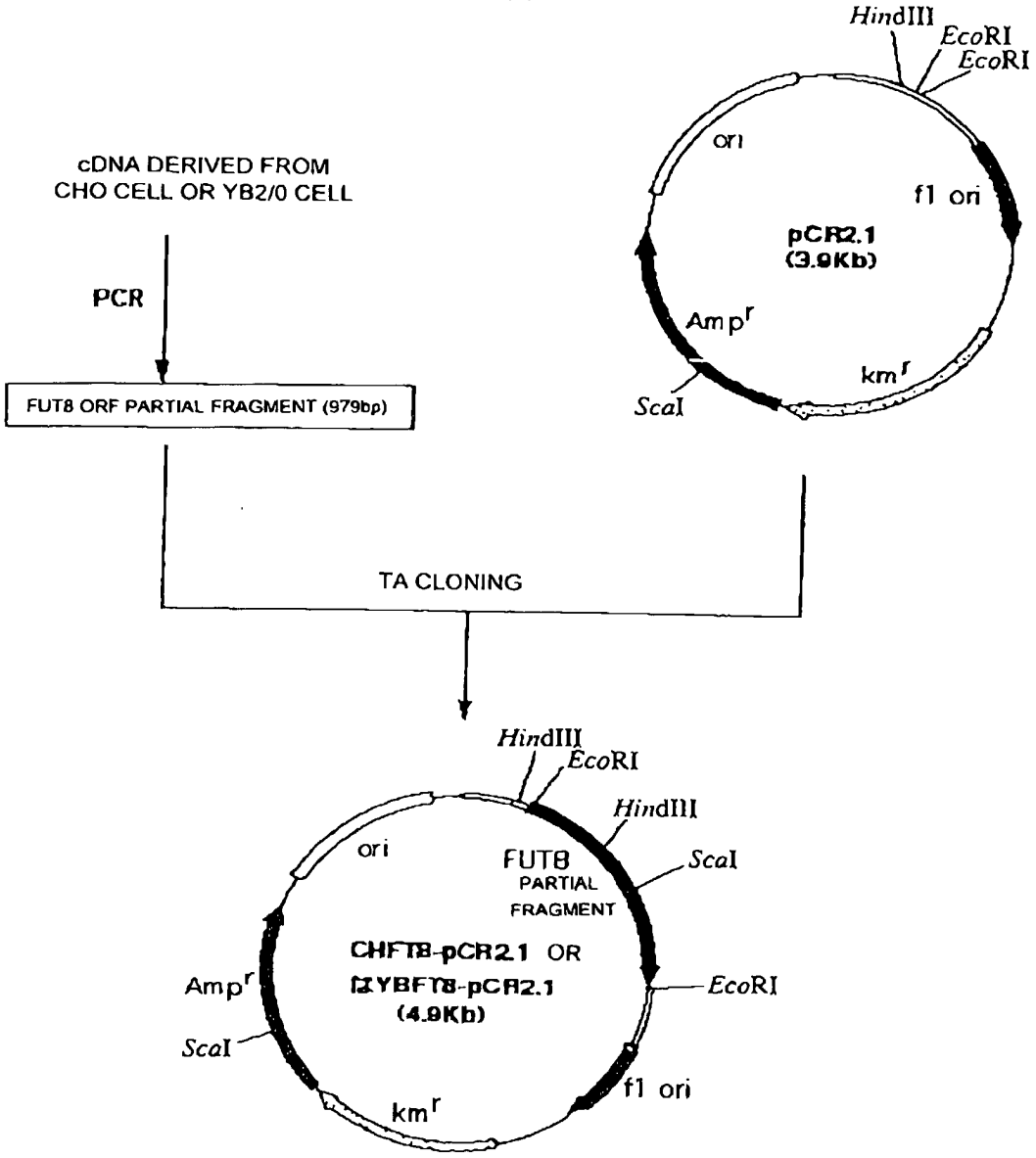
Aragen/Transposagen Ex. 1036 - Part G

FIG. 19



Aragen/Transposagen Ex. 1036 - Part G

FIG. 20



Aragen/Transposagen Ex. 1036 - Part G

FIG. 21

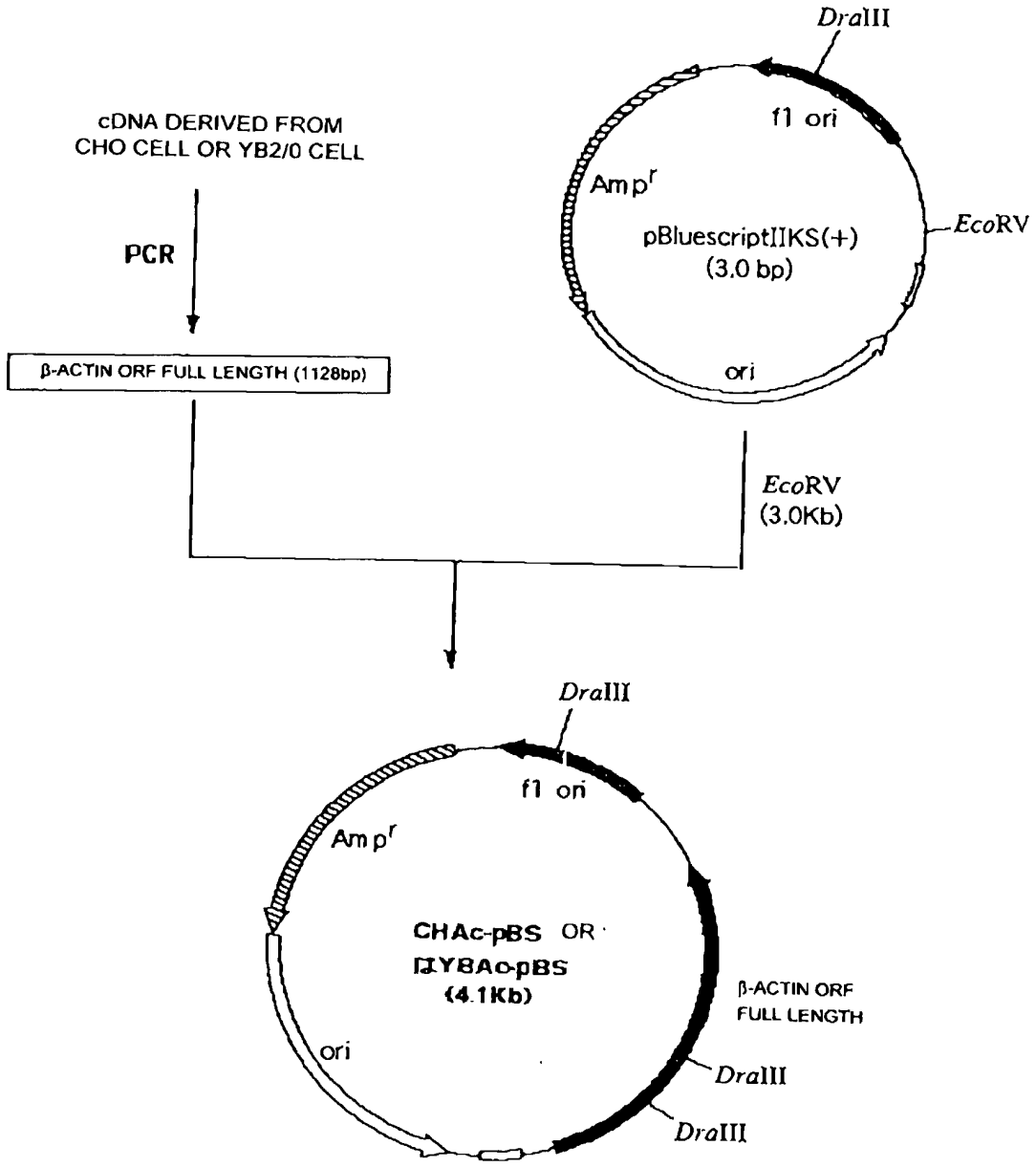
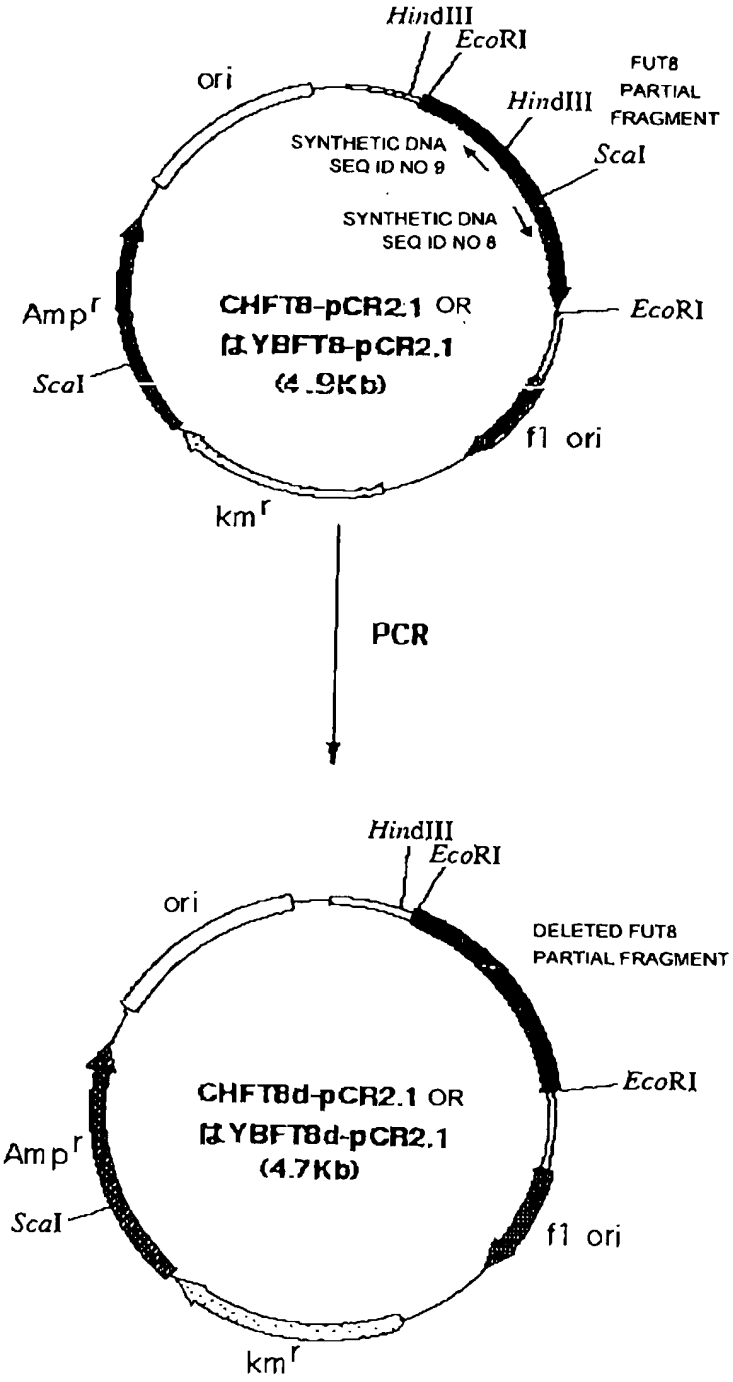
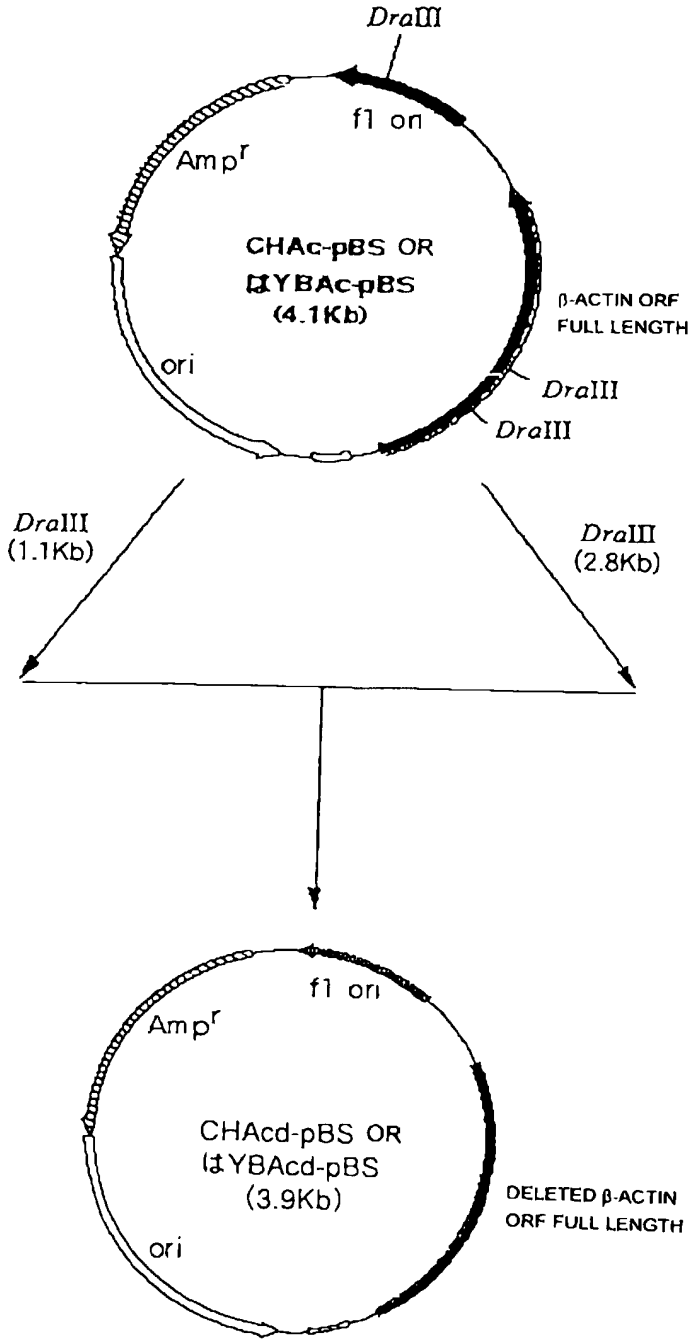

cDNA DERIVED FROM
CHO CELL OR YB2/0 CELL
PCR
β-ACTIN ORF FULL LENGTH (1128bp)
DraIII
f1 ori
Amp^r
pBluescriptIIKS(+)
(3.0 bp)
EcoRV
ori
EcoRV
(3.0Kb)
DraIII
f1 ori
Amp^r
CHAc-pBS OR
YB8Ac-pBS
(4.1Kb)
β-ACTIN ORF
FULL LENGTH
ori
DraIII
DraIII

FIG. 22



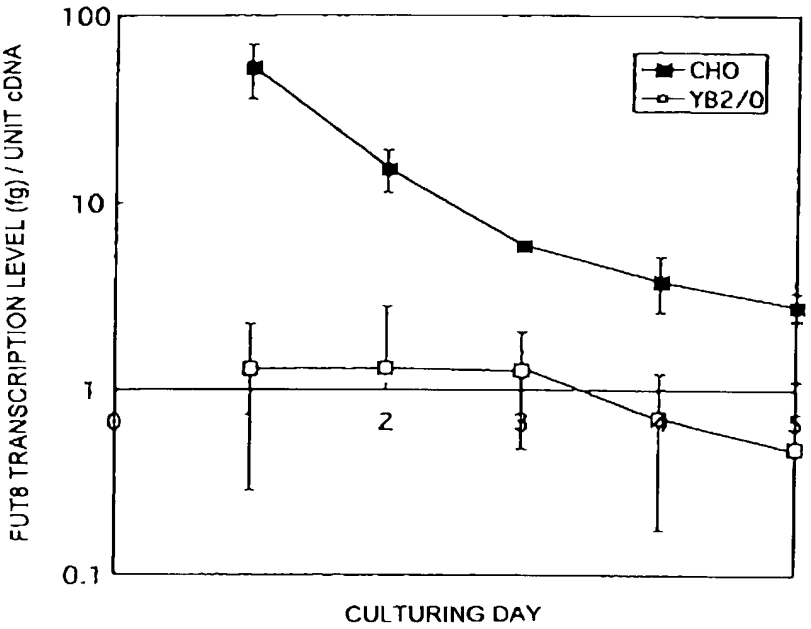
Aragen/Transposagen Ex. 1036 - Part G

FIG. 23



Aragen/Transposagen Ex. 1036 - Part G

FIG. 24



Aragen/Transposagen Ex. 1036 - Part G

FIG. 25

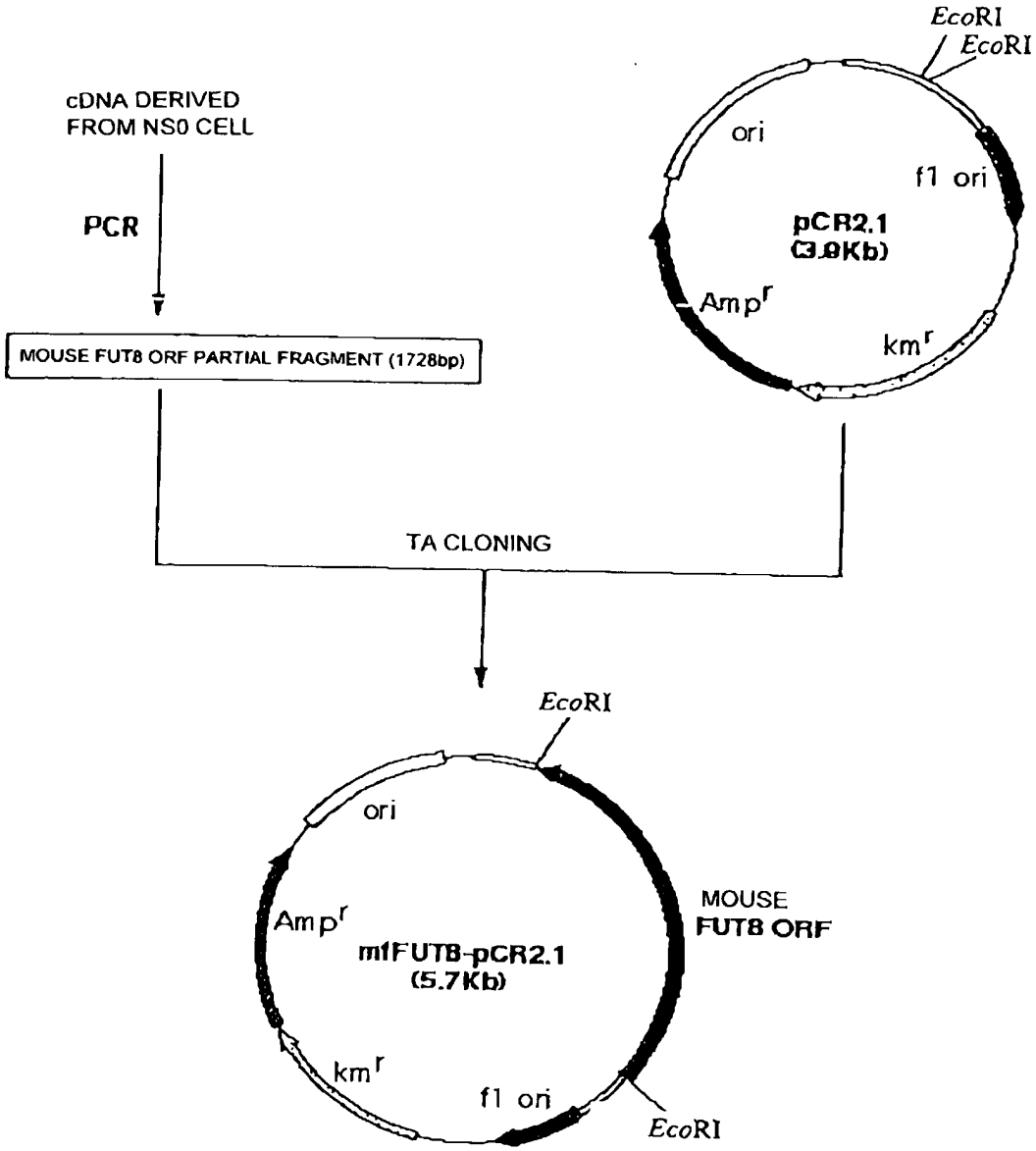
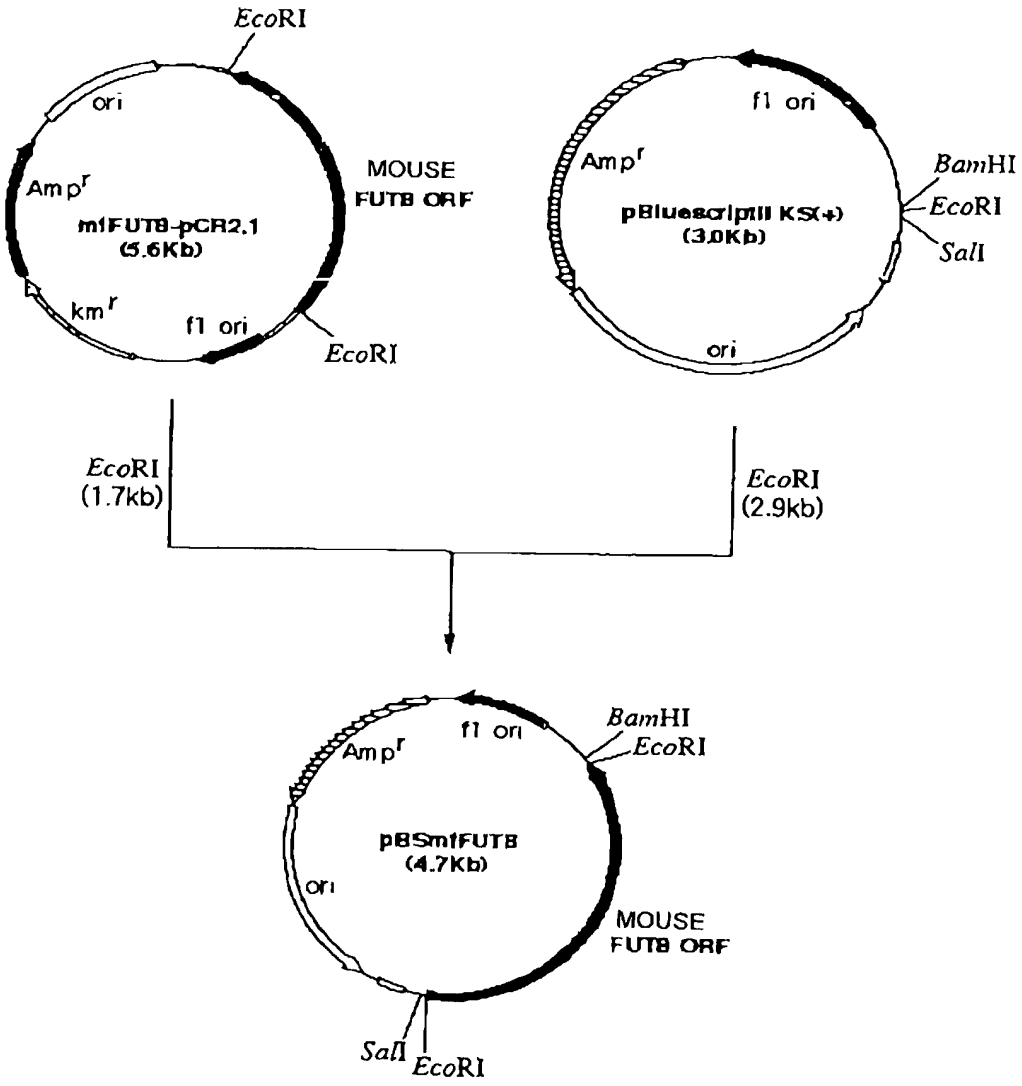
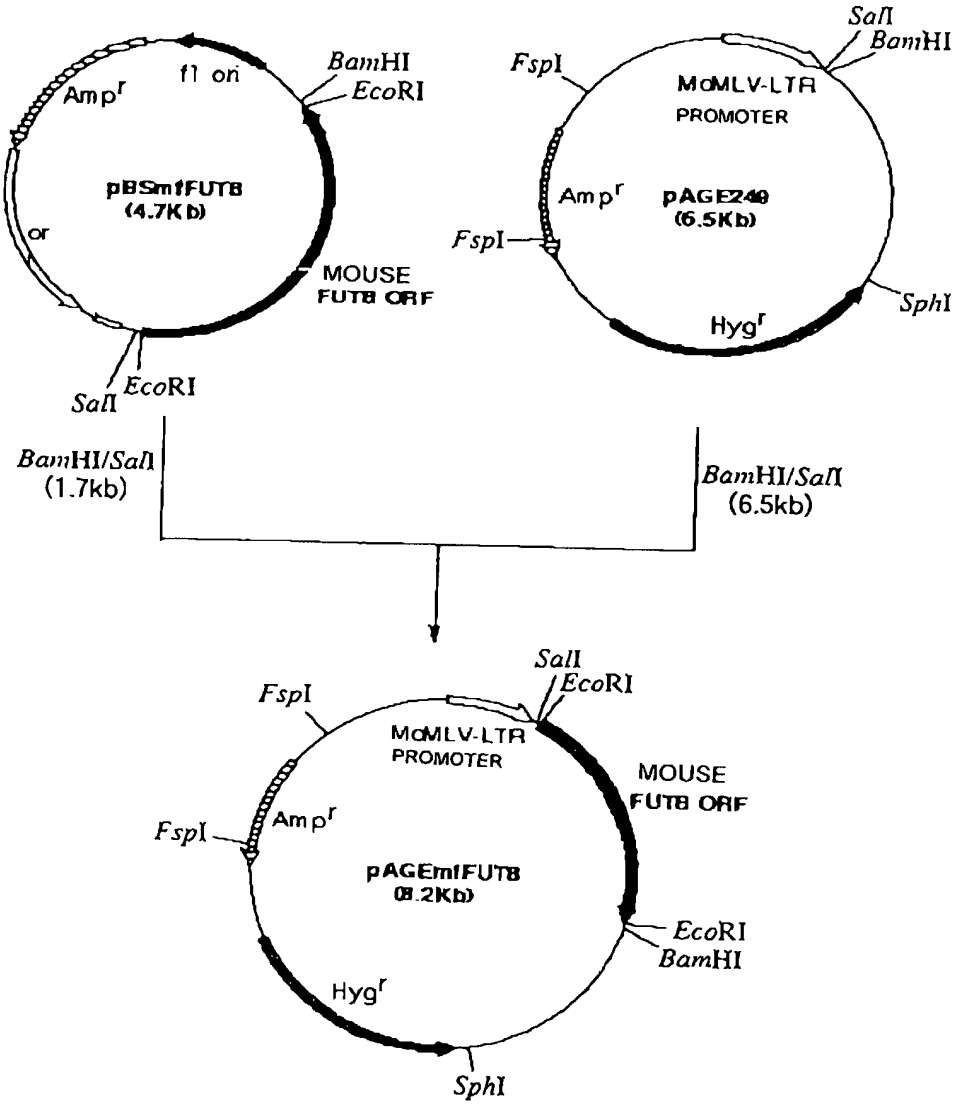

cDNA DERIVED
FROM NS0 CELL
PCR
MOUSE FUT8 ORF PARTIAL FRAGMENT (1728bp)
EcoRI
EcoRI
ori
f1 ori
pCR2.1
(3.9Kb)
Amp^r
km^r
TA CLONING
EcoRI
ori
Amp^r
m1FUT8-pCR2.1
(5.7Kb)
km^r
f1 ori
EcoRI
MOUSE
FUT8 ORF

FIG. 26



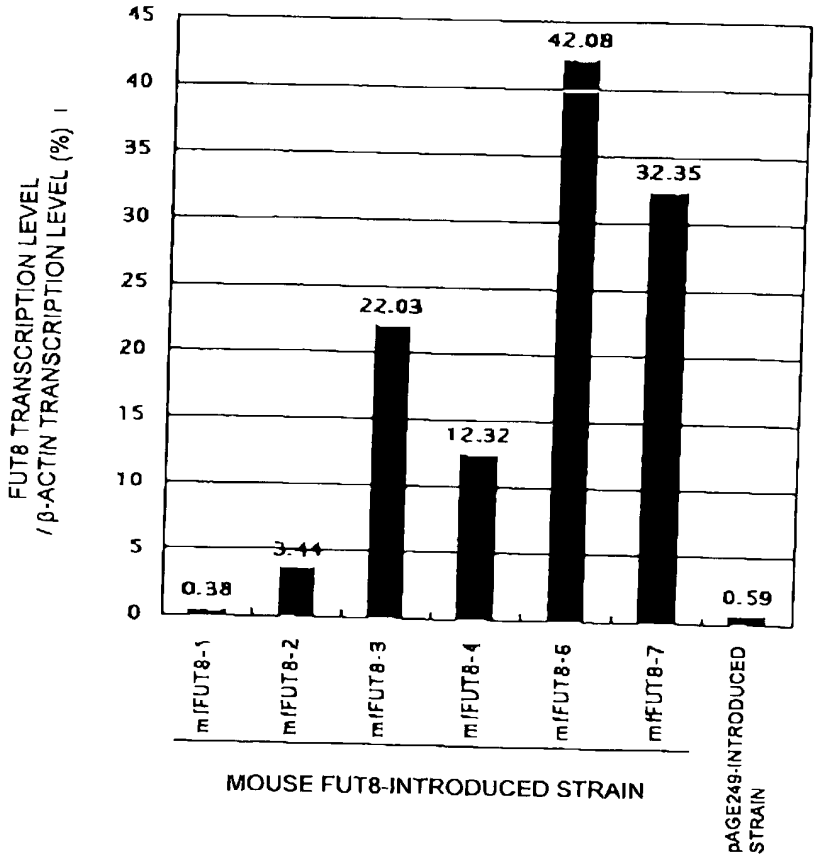
Aragen/Transposagen Ex. 1036 - Part G

FIG. 27



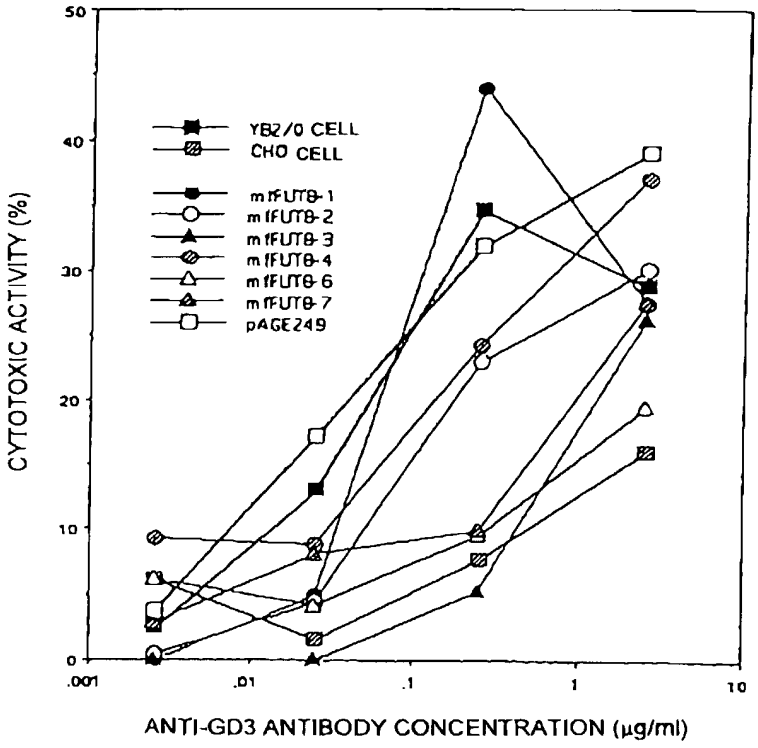
Aragen/Transposagen Ex. 1036 - Part G

FIG. 28



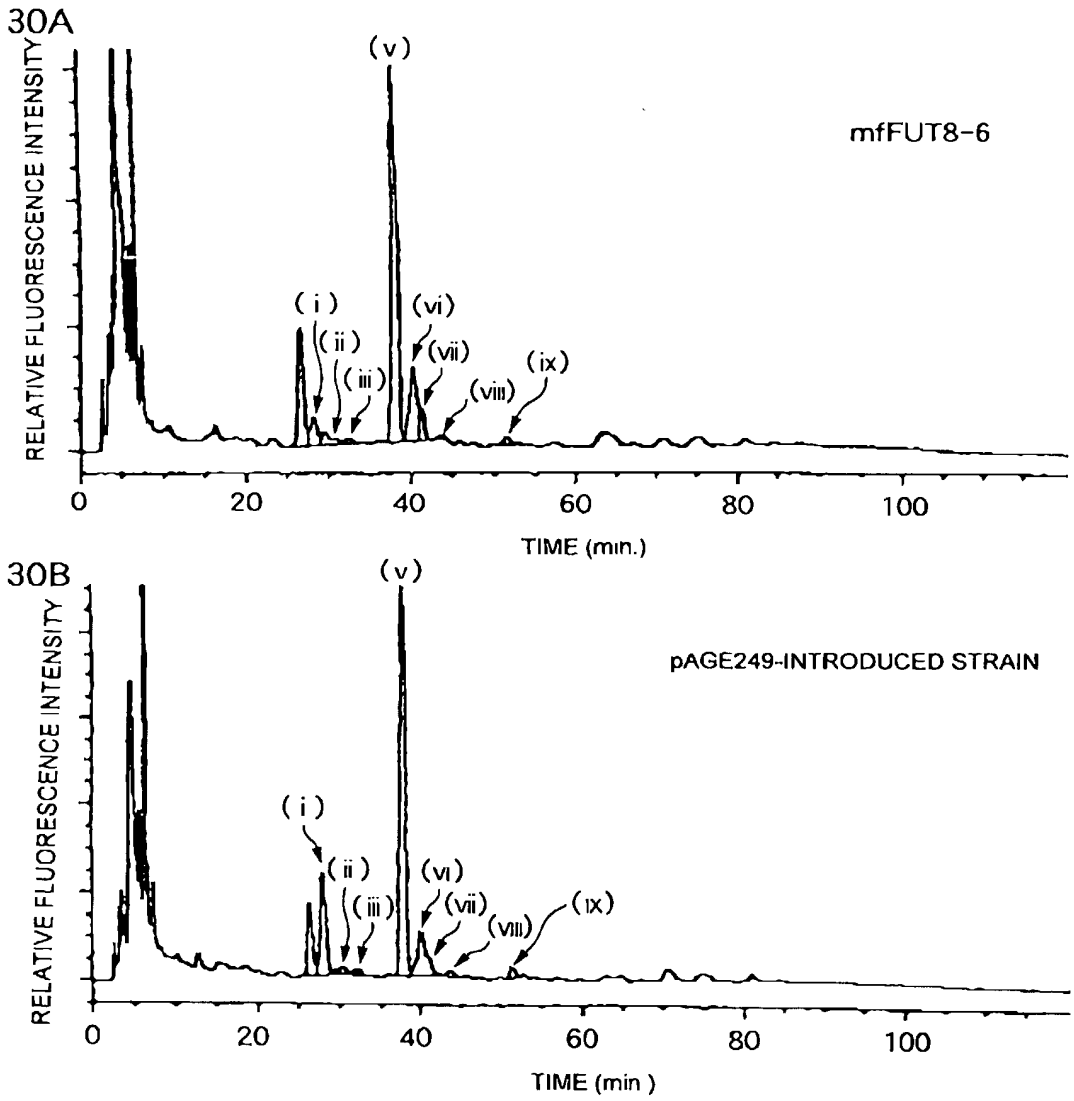
Aragen/Transposagen Ex. 1036 - Part G

FIG. 29



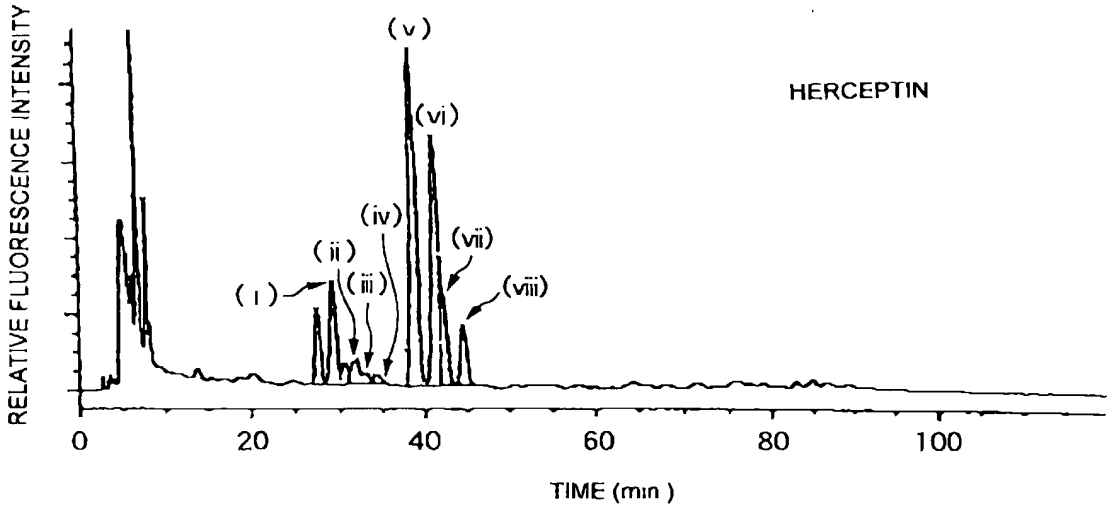
Aragen/Transposagen Ex. 1036 - Part G

FIG. 30



Aragen/Transposagen Ex. 1036 - Part G

FIG. 31



Aragen/Transposagen Ex. 1036 - Part G

FIG. 32

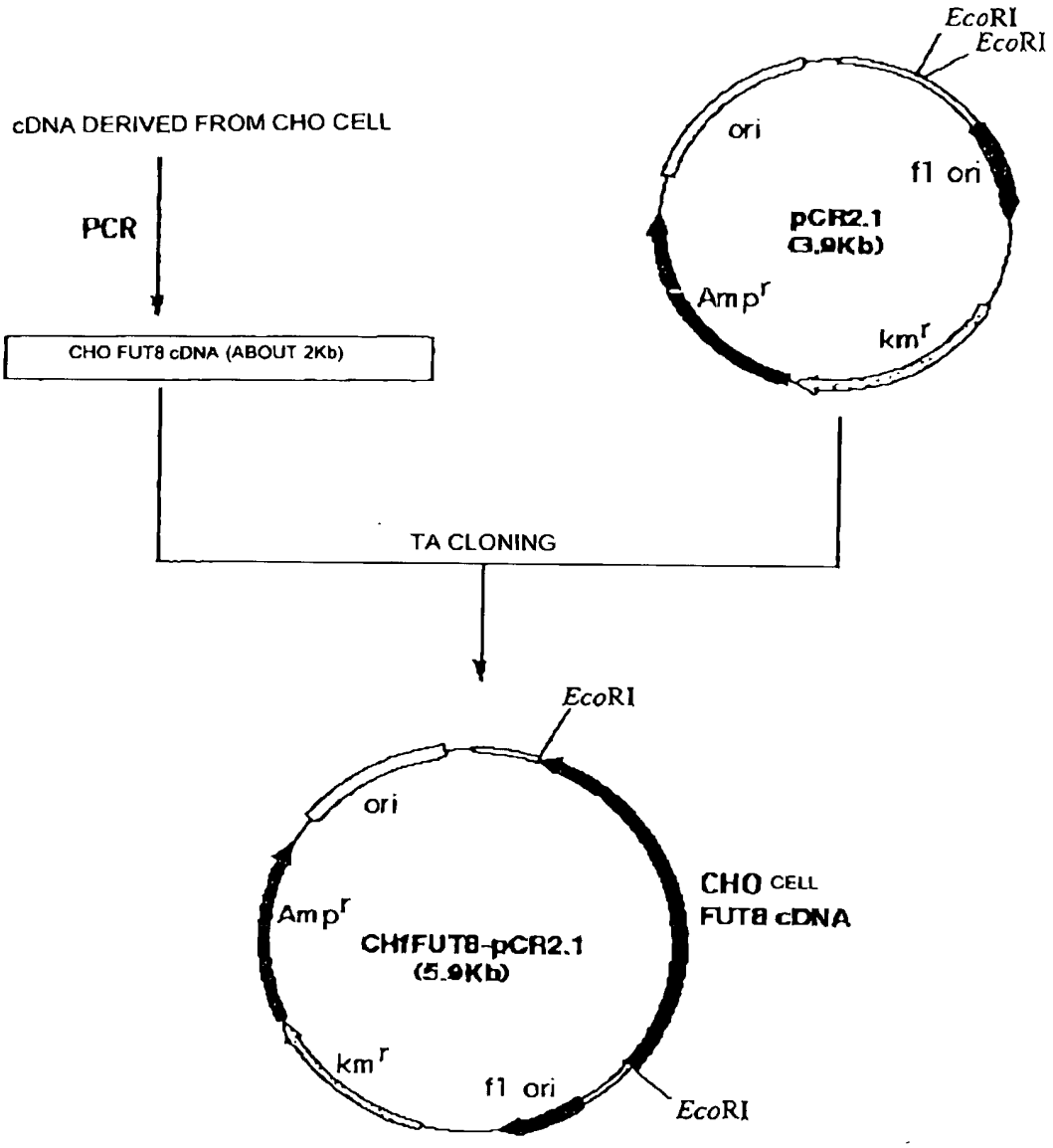

cDNA DERIVED FROM CHO CELL
PCR
CHO FUT8 cDNA (ABOUT 2Kb)
EcoRI
EcoRI
ori
f1 ori
pCR2.1
(3.9Kb)
Amp r
km r
TA CLONING
EcoRI
ori
Amp r
CHFUT8-pCR2.1
(5.9Kb)
km r
f1 ori
EcoRI
CHO CELL
FUT8 cDNA

FIG. 33

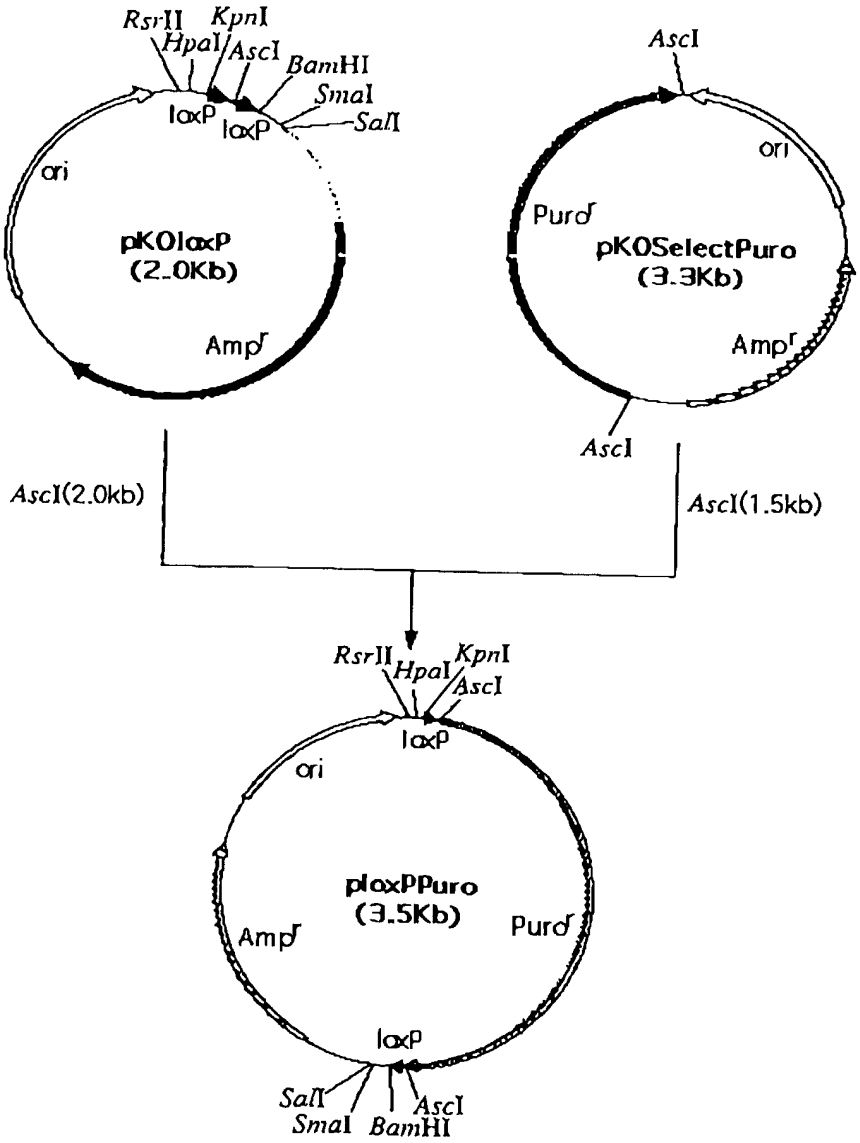
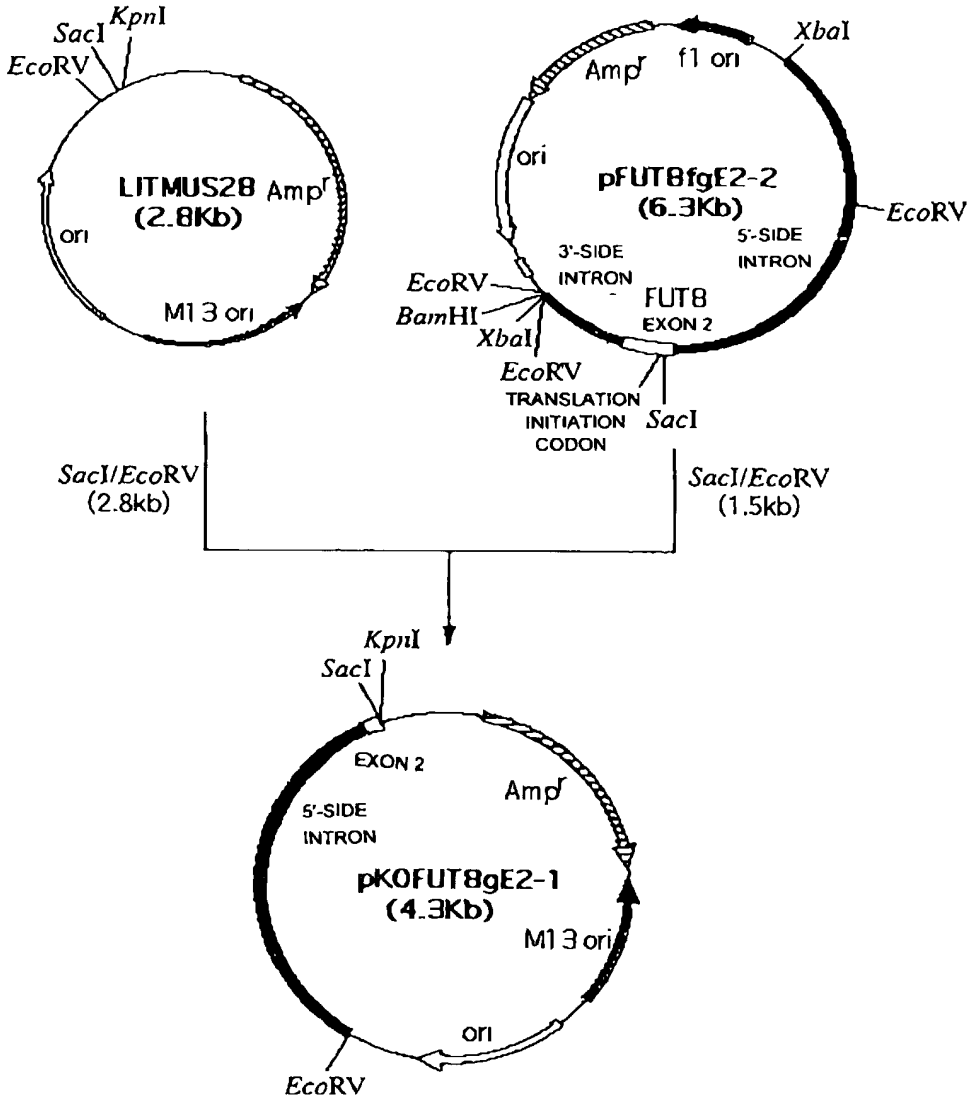
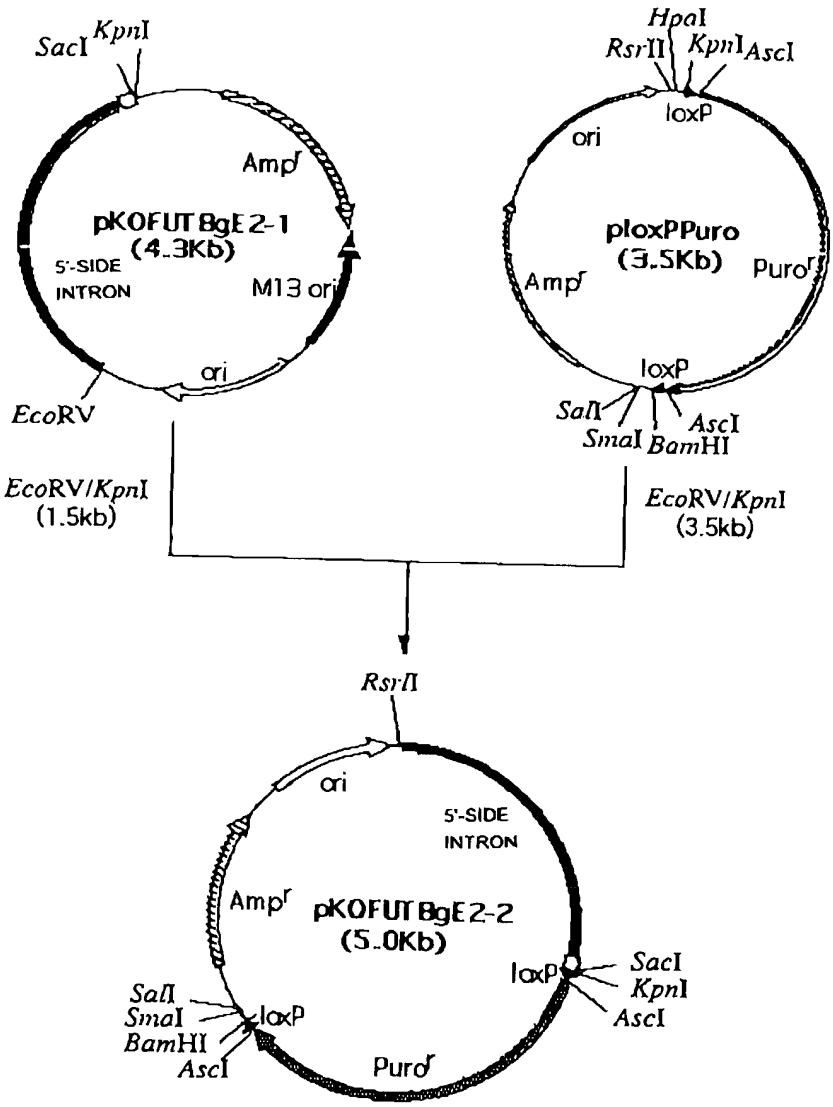

RsrII
KpnI
HpaI
AscI
BamHI
SmaI
SalI
loxP
loxP
ori
pKOloxP
(2.0Kb)
Amp
AscI
ori
Puro
pKOSelectPuro
(3.3Kb)
Amp
AscI
AscI(2.0kb)
AscI(1.5kb)
RsrII
HpaI
KpnI
AscI
loxP
ori
ploxPPuro
(3.5Kb)
Amp
Puro
loxP
SalI
SmaI
BamHI
AscI

FIG. 34



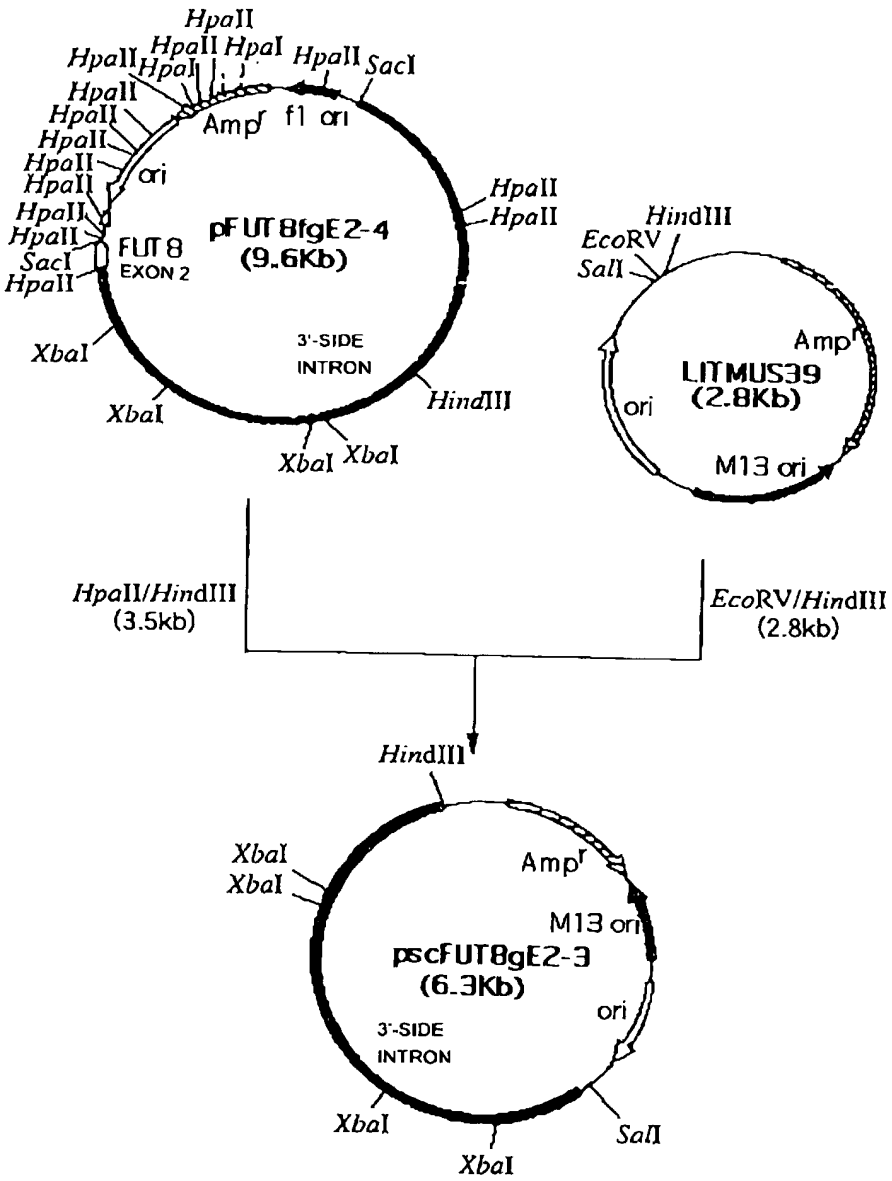
Aragen/Transposagen Ex. 1036 - Part G

FIG. 35



Aragen/Transposagen Ex. 1036 - Part G

FIG. 36



Aragen/Transposagen Ex. 1036 - Part G

FIG. 37

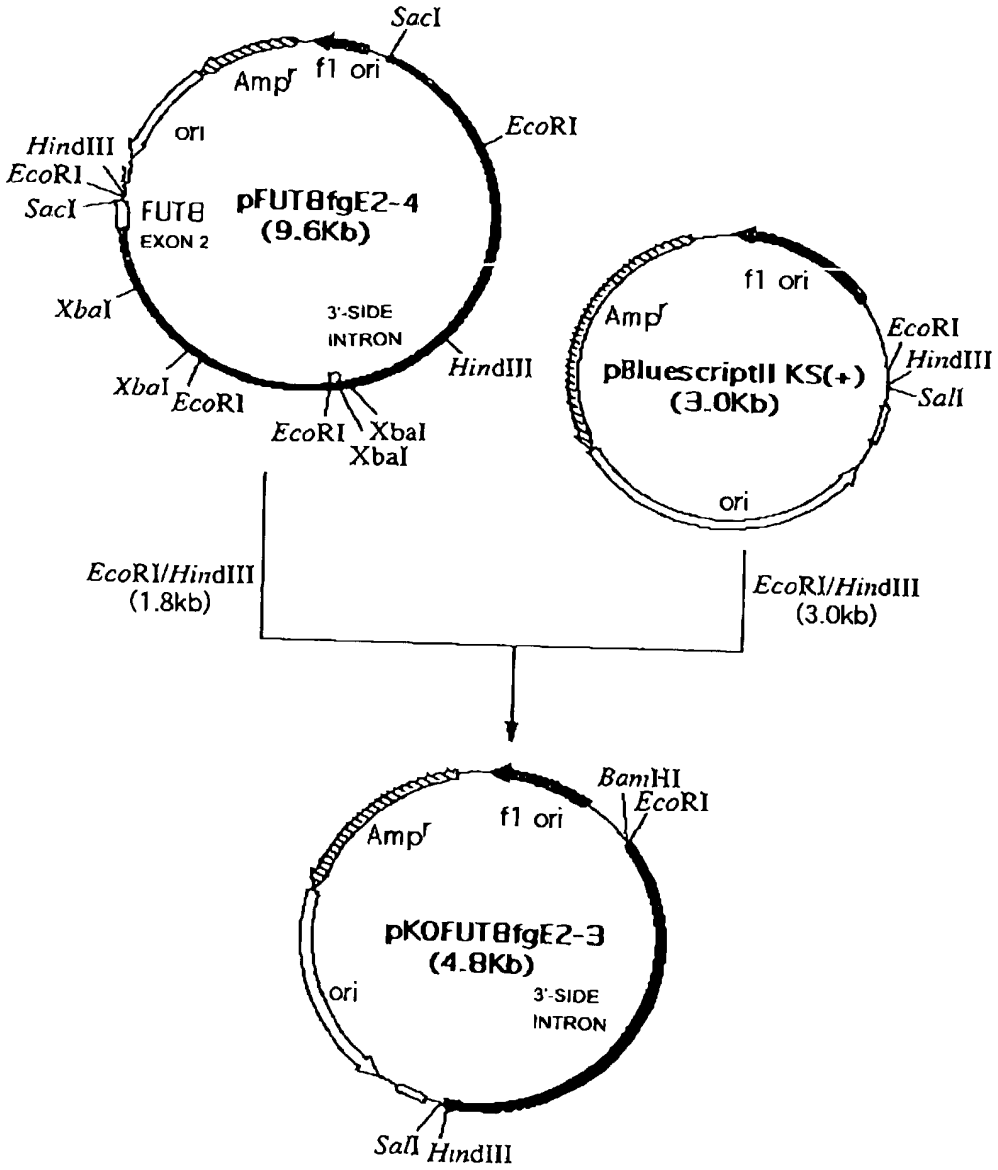
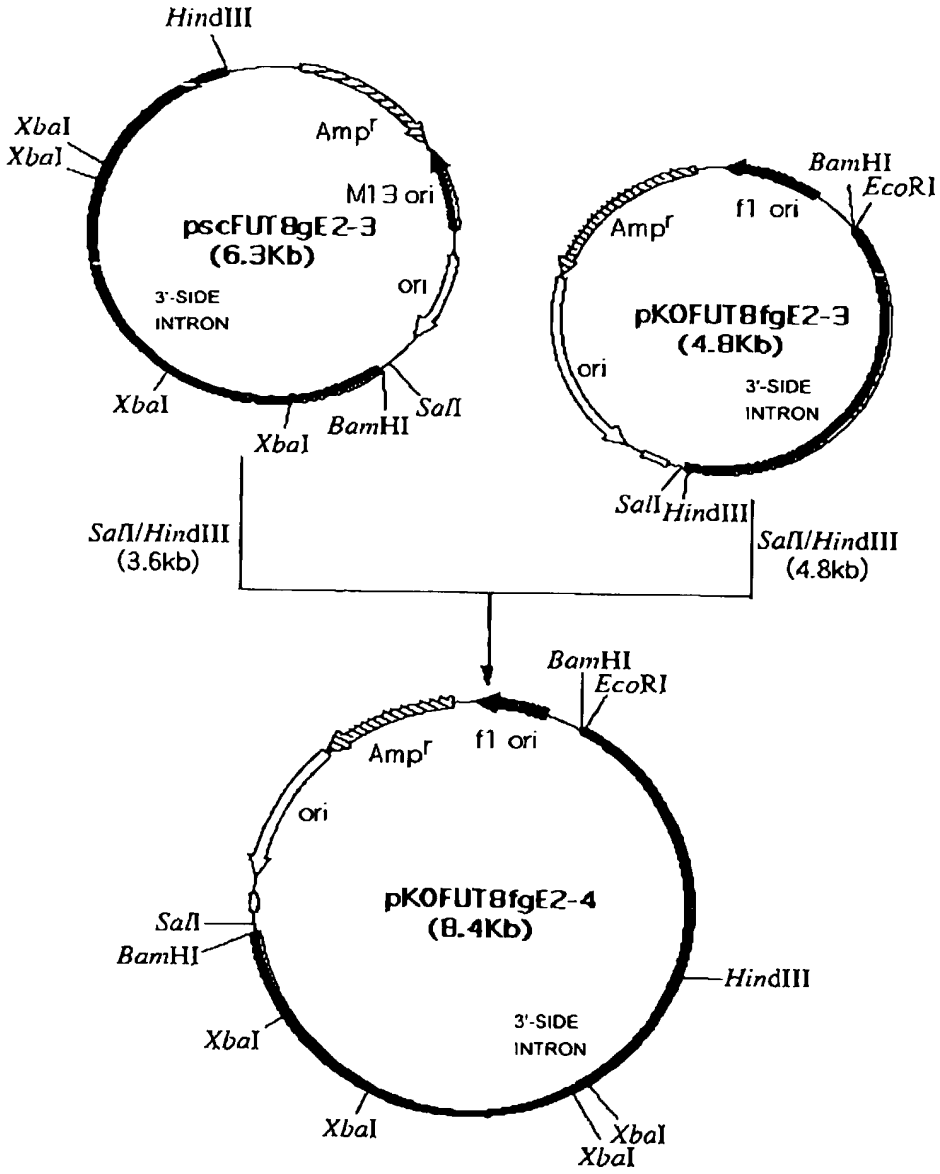
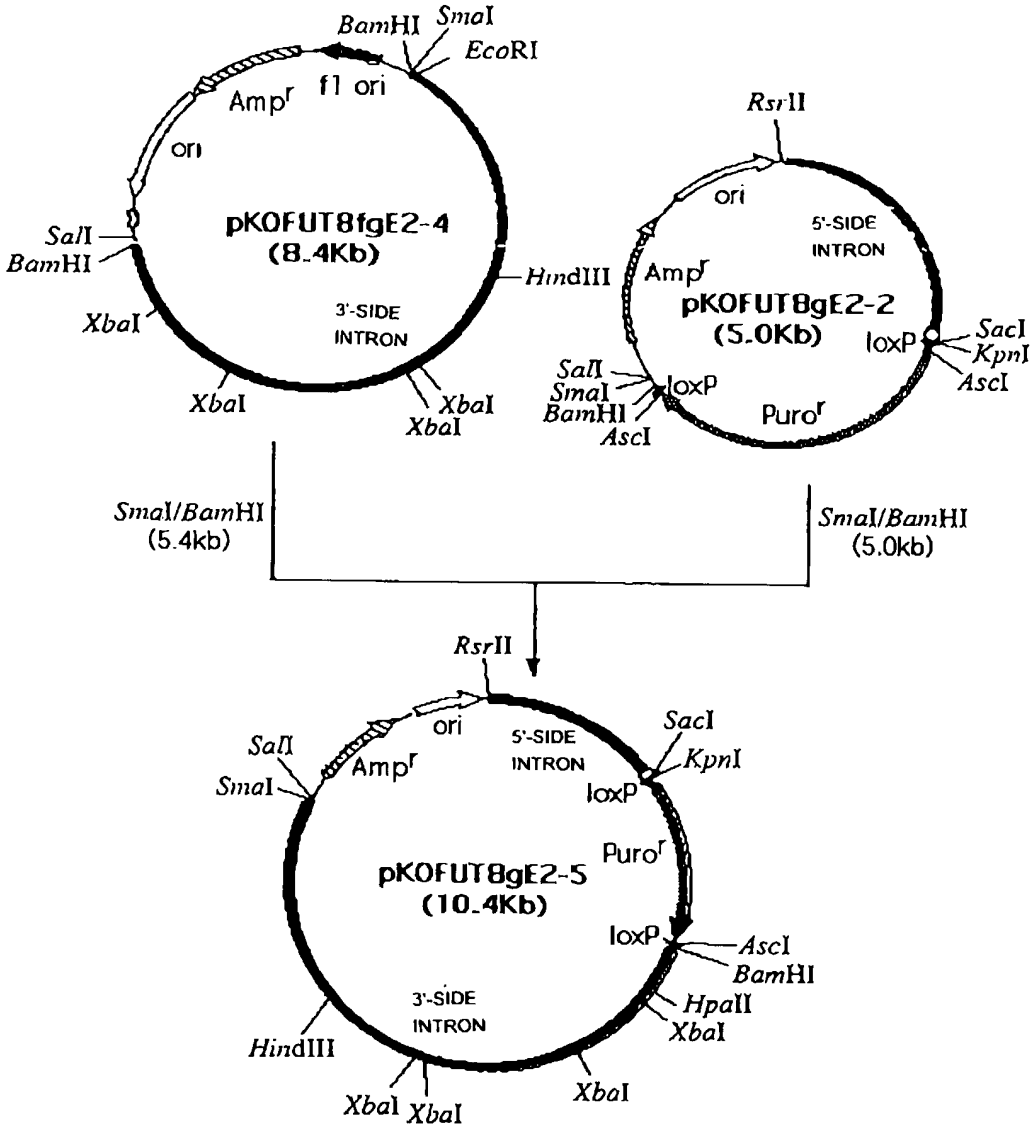

SacI
Ampr
f1 ori
EcoRI
HindIII
EcoRI
SacI
ori
FUT8
EXON 2
pFUT8fgE2-4
(9.6Kb)
XbaI
3'-SIDE
INTRON
HindIII
XbaI
EcoRI
EcoRI
XbaI
XbaI
f1 ori
Ampr
EcoRI
HindIII
SalI
pBluescriptII KS(+)
(3.0Kb)
ori
EcoRI/HindIII
(1.8kb)
EcoRI/HindIII
(3.0kb)
BamHI
EcoRI
f1 ori
Ampr
pKOFUT8fgE2-3
(4.8Kb)
ori
3'-SIDE
INTRON
SalI
HindIII

FIG. 38



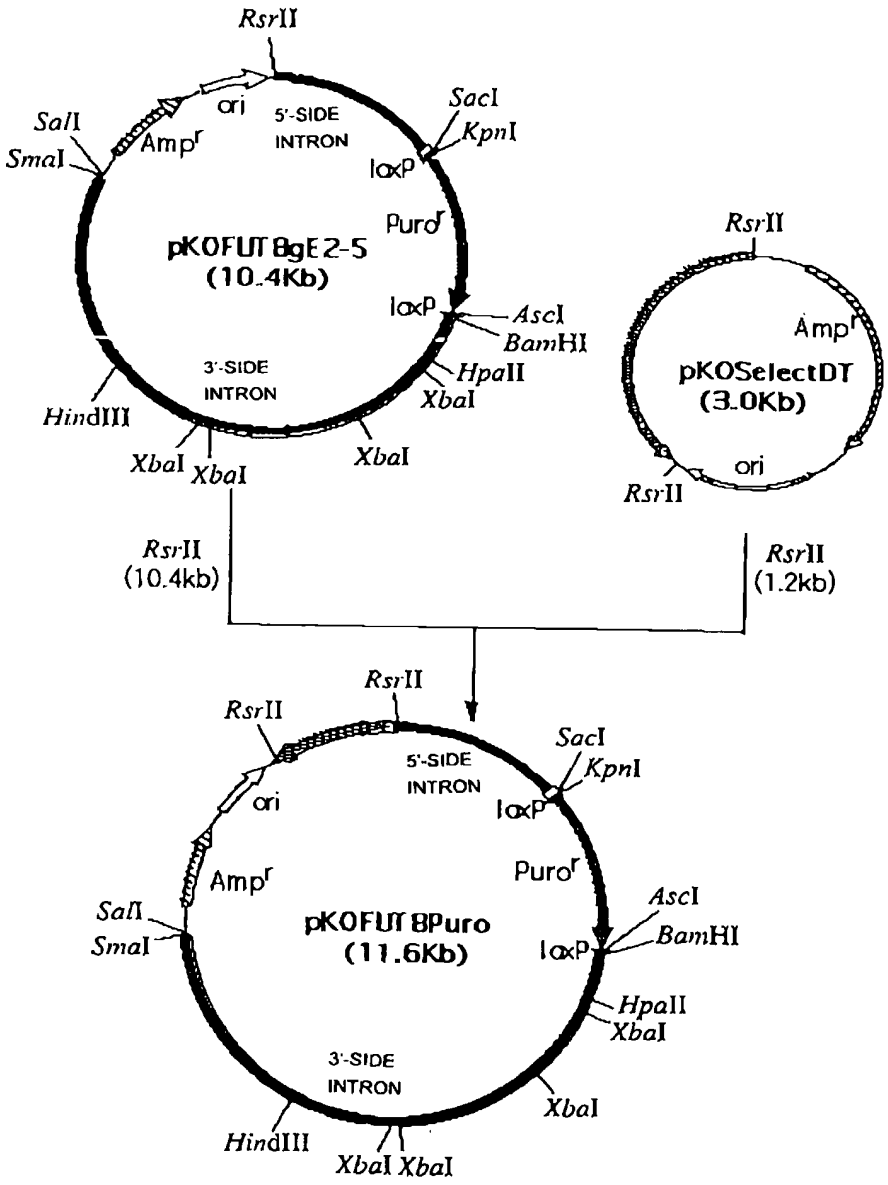
Aragen/Transposagen Ex. 1036 - Part G

FIG. 39



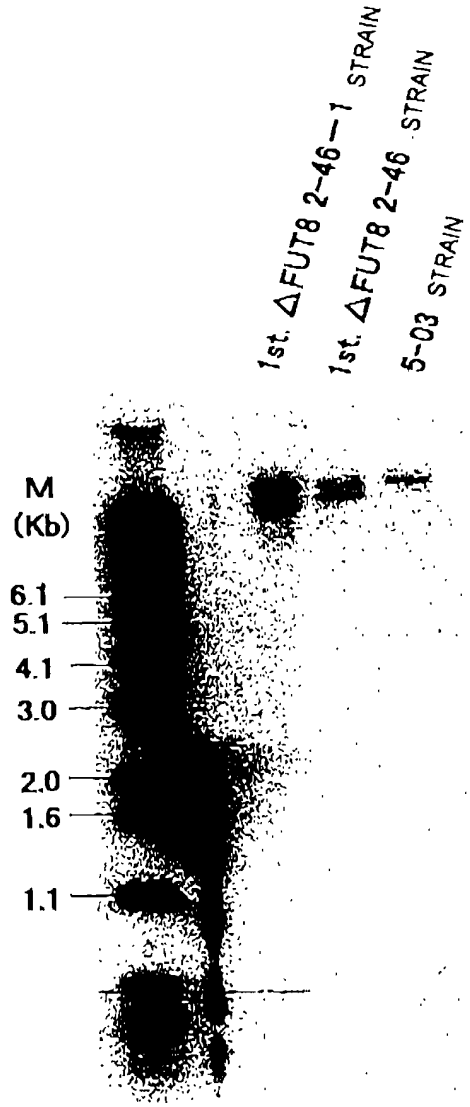
Aragen/Transposagen Ex. 1036 - Part G

FIG. 40



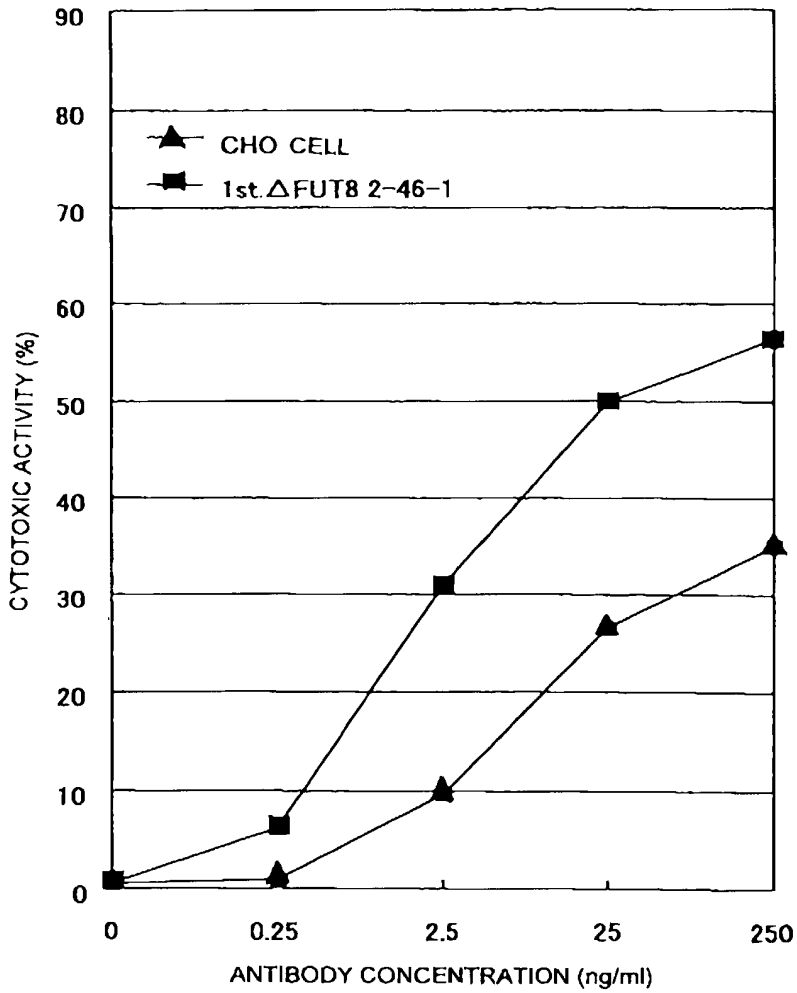
Aragen/Transposagen Ex. 1036 - Part G

FIG. 41



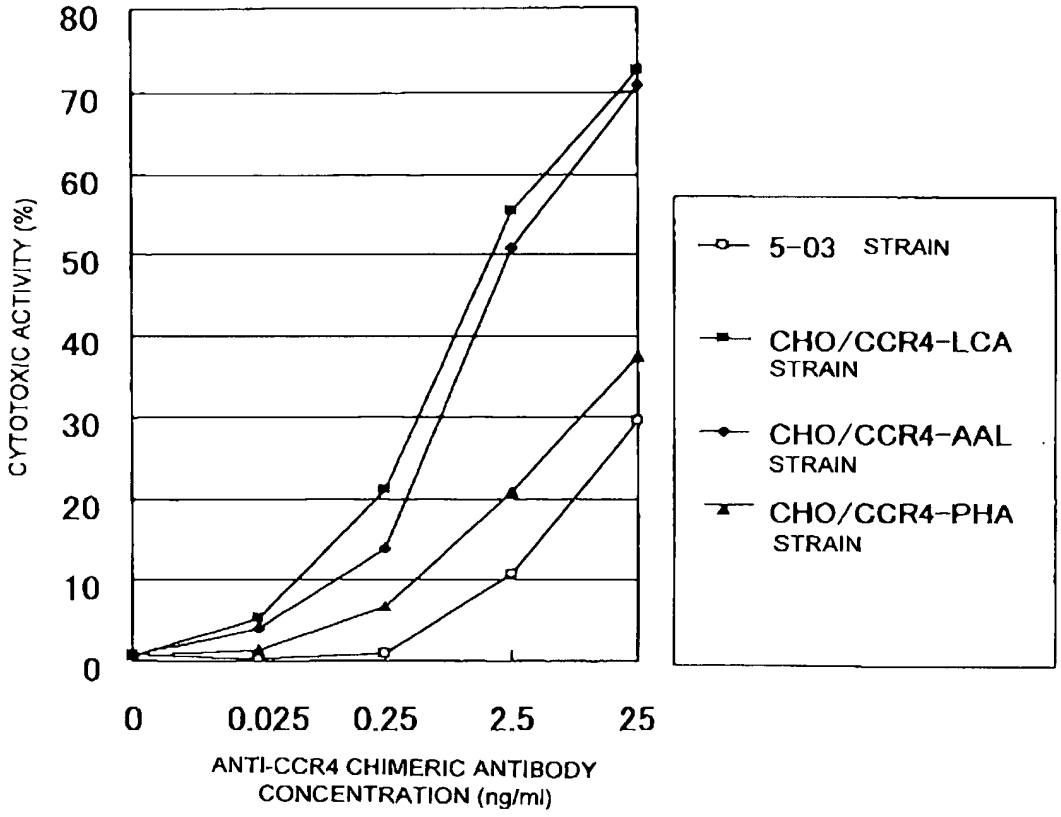
Aragen/Transposagen Ex. 1036 - Part G

FIG. 42



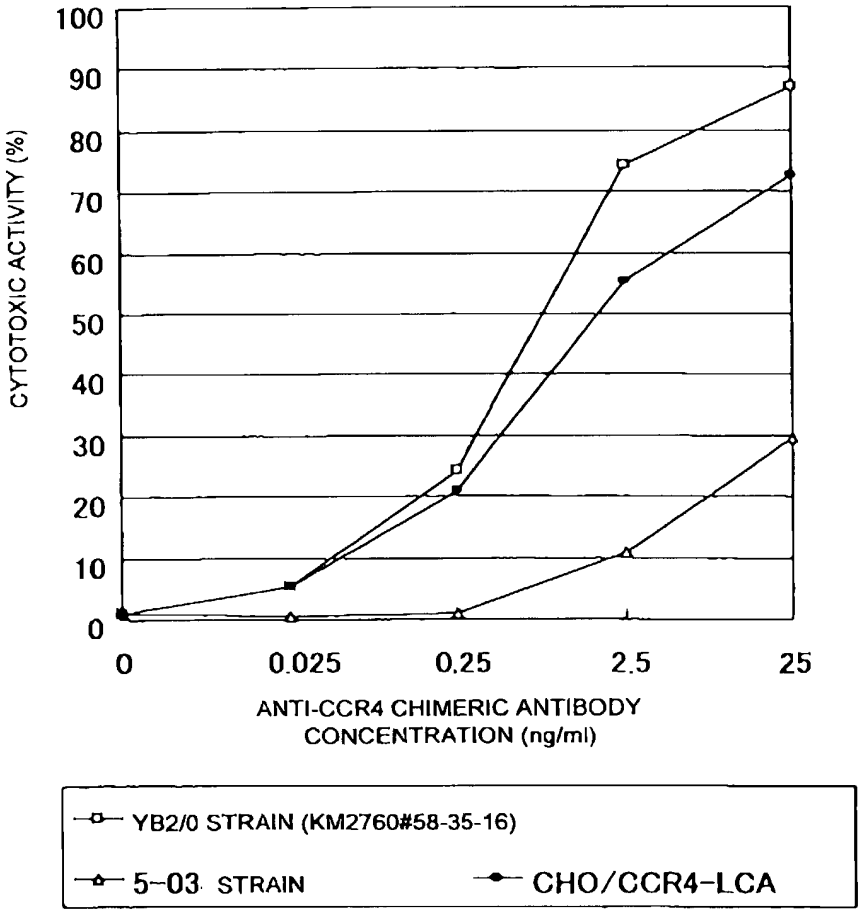
Aragen/Transposagen Ex. 1036 - Part G

FIG. 43



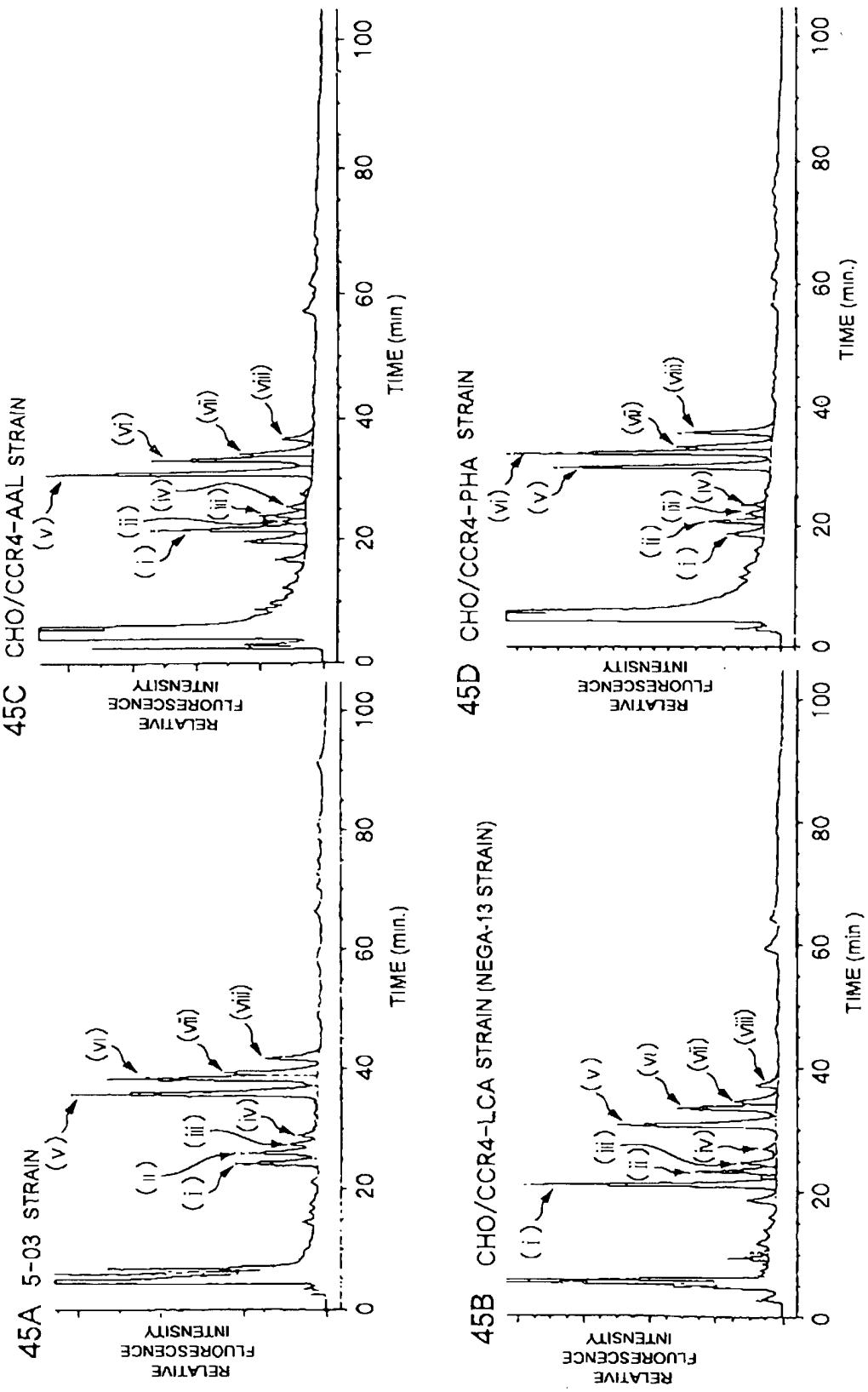
Aragen/Transposagen Ex. 1036 - Part G

FIG. 44



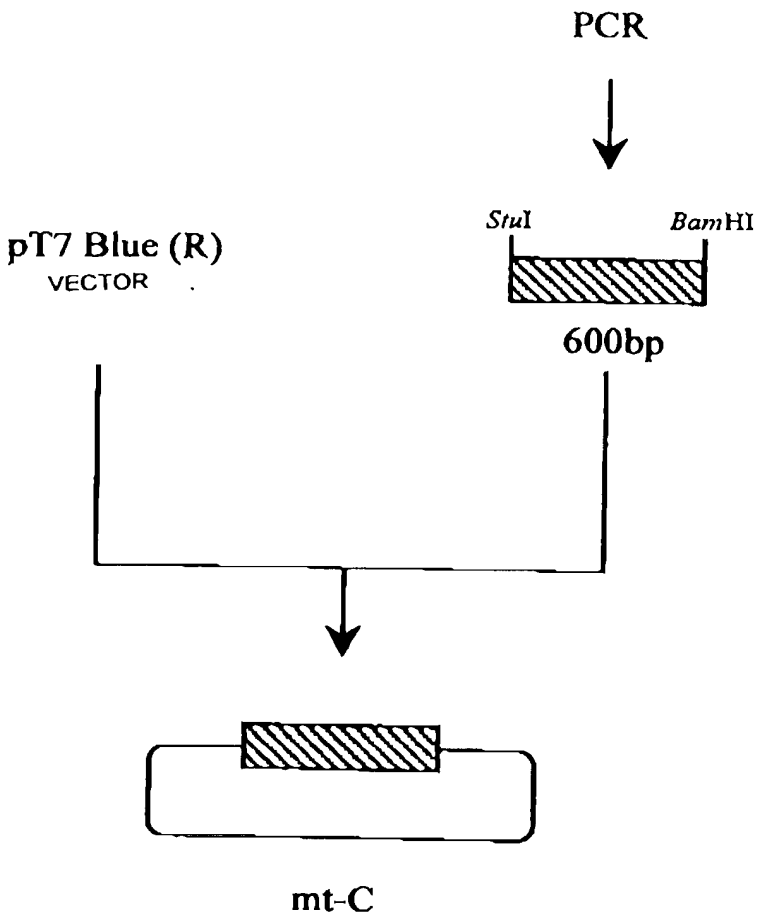
Aragen/Transposagen Ex. 1036 - Part G

FIG. 45



Aragen/Transposagen Ex. 1036 - Part G

FIG. 46



Aragen/Transposagen Ex. 1036 - Part G

FIG. 47

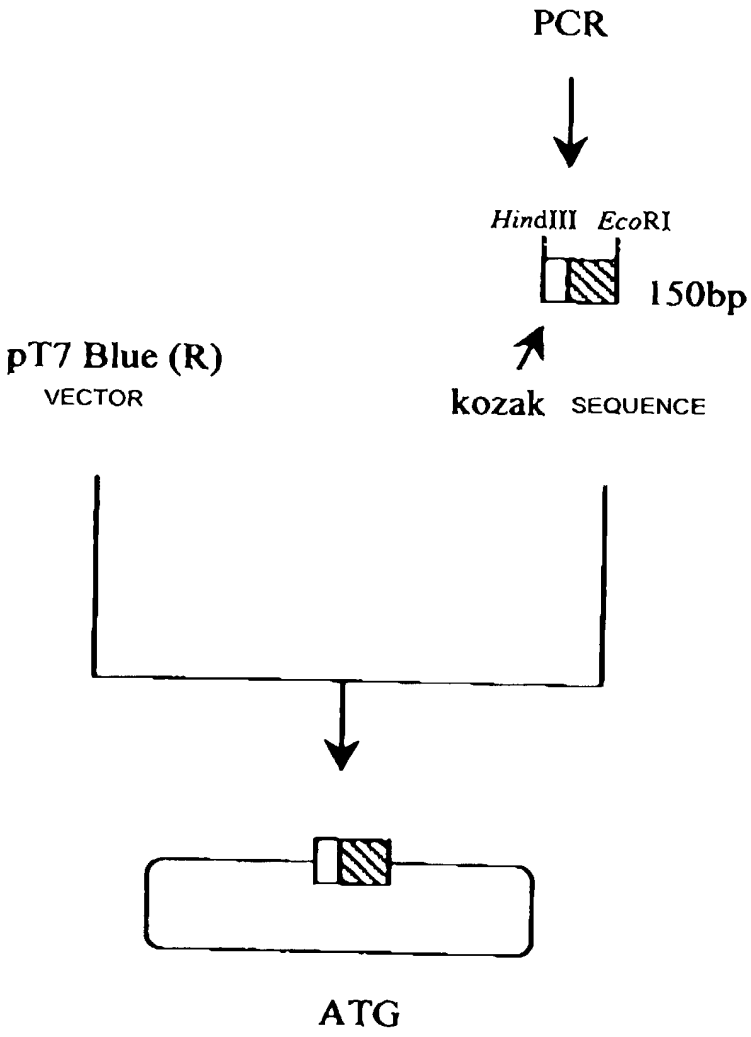
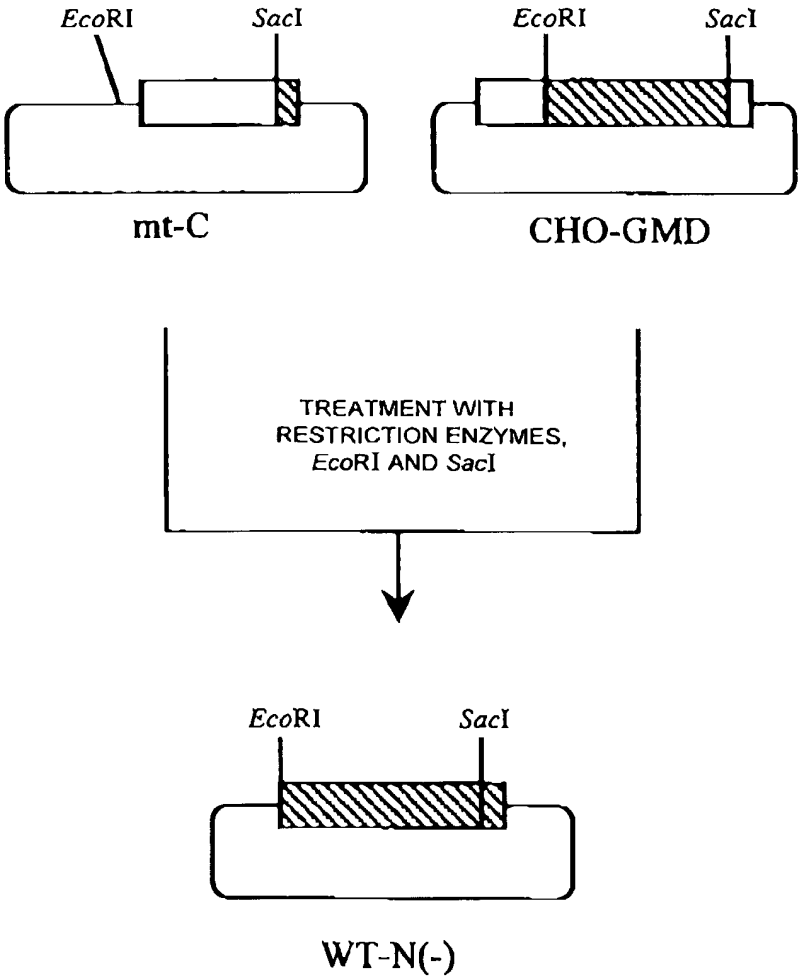
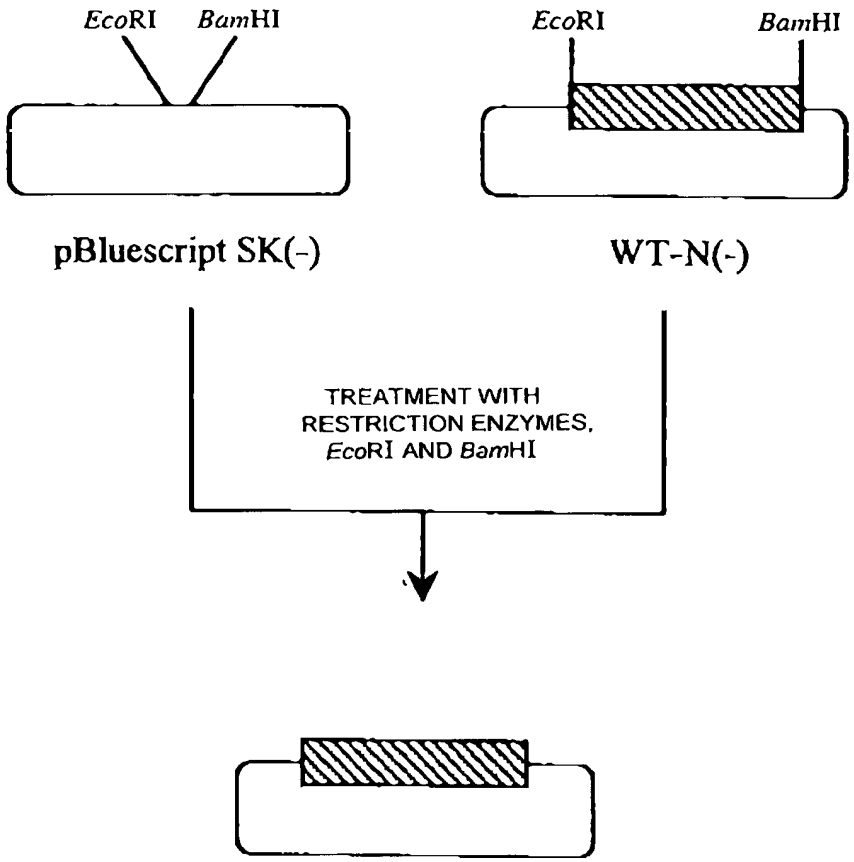

PCR
HindIII EcoRI
150bp
pT7 Blue (R)
VECTOR
kozak SEQUENCE
ATG

FIG. 48



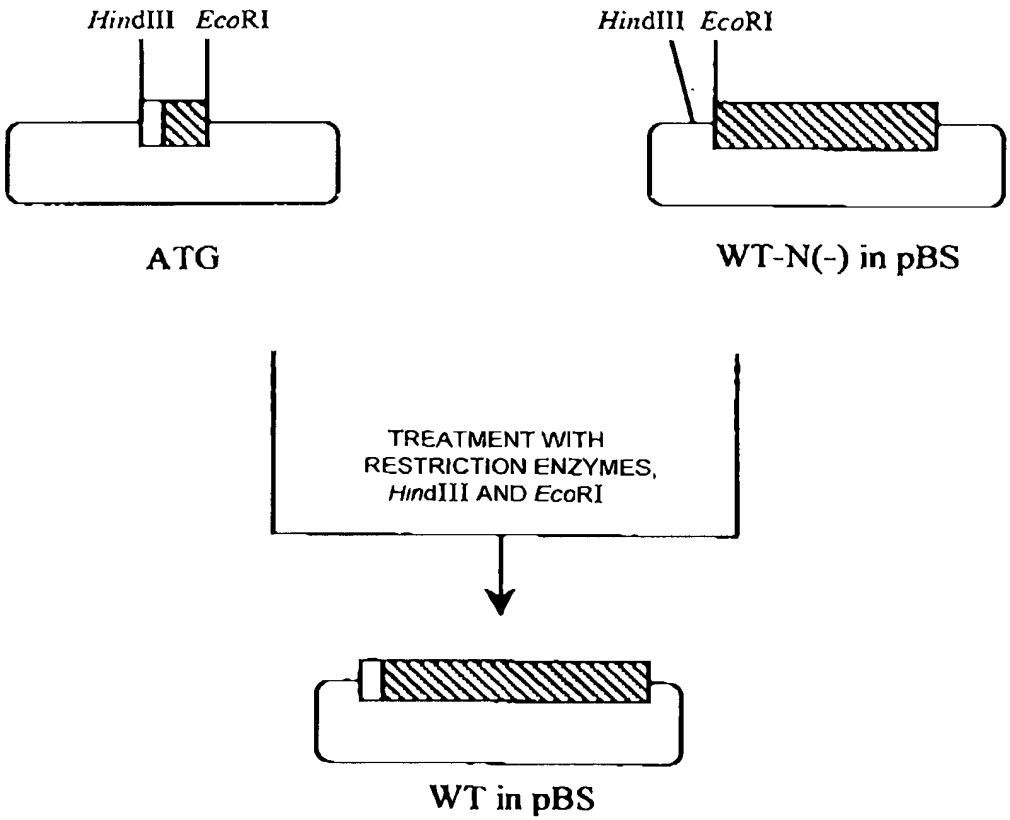
Aragen/Transposagen Ex. 1036 - Part G

FIG. 49



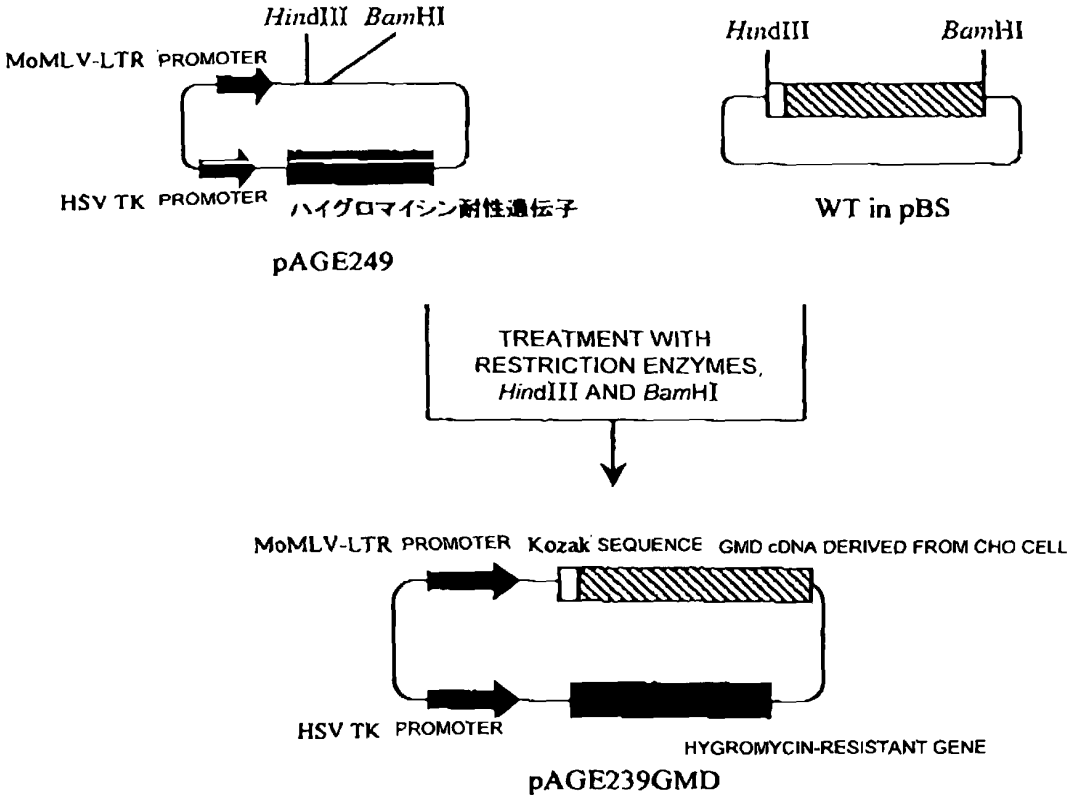
Aragen/Transposagen Ex. 1036 - Part G

FIG. 50



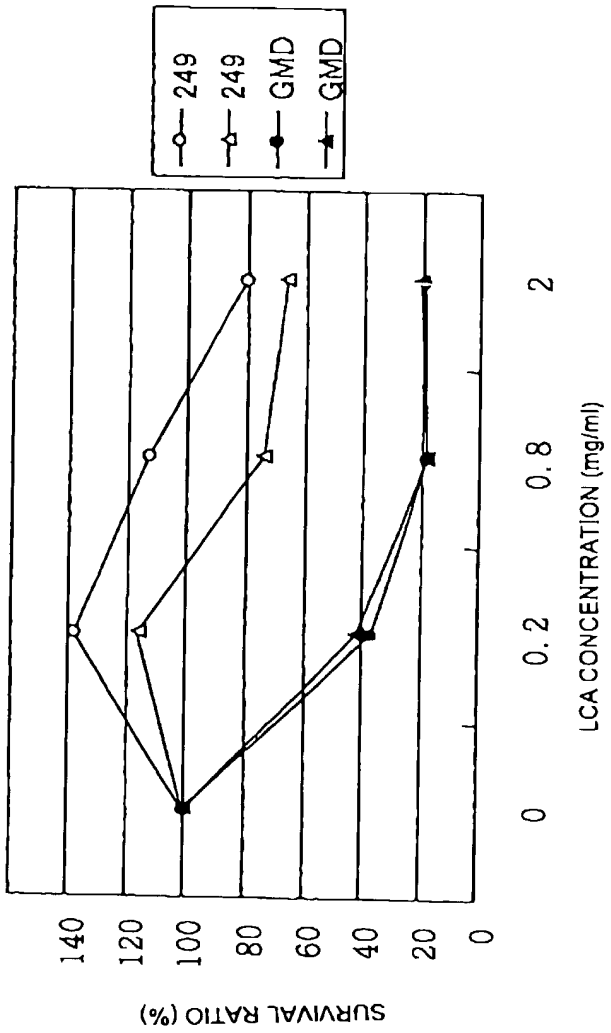
Aragen/Transposagen Ex. 1036 - Part G

FIG. 51



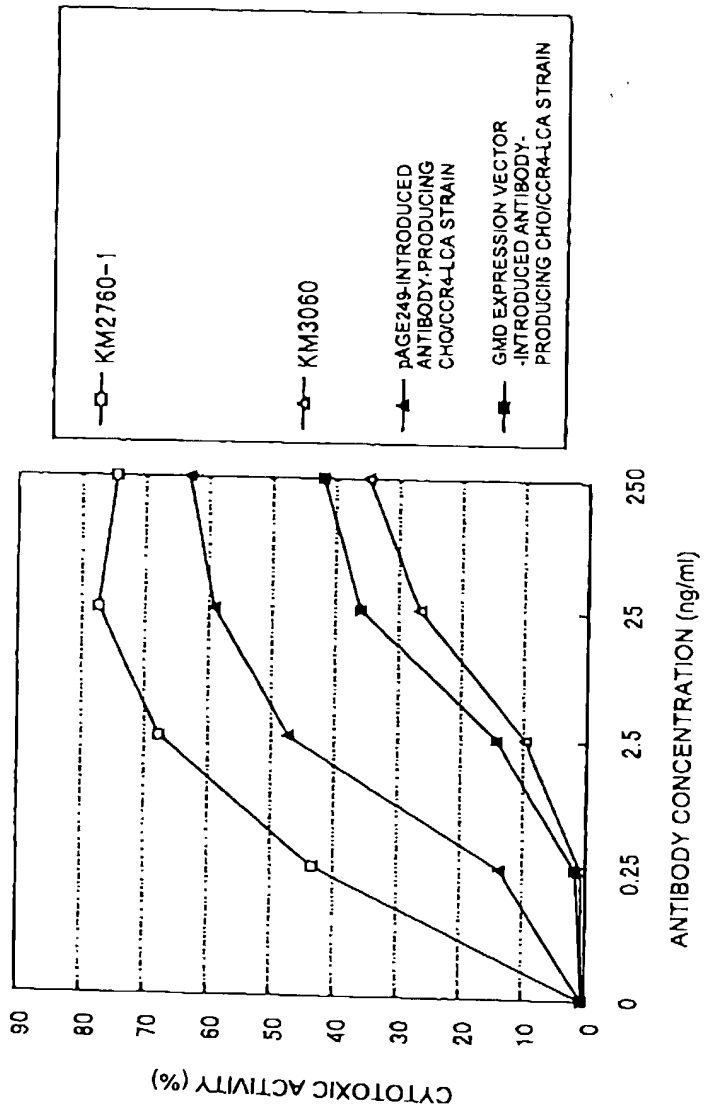
Aragen/Transposagen Ex. 1036 - Part C

FIG. 52



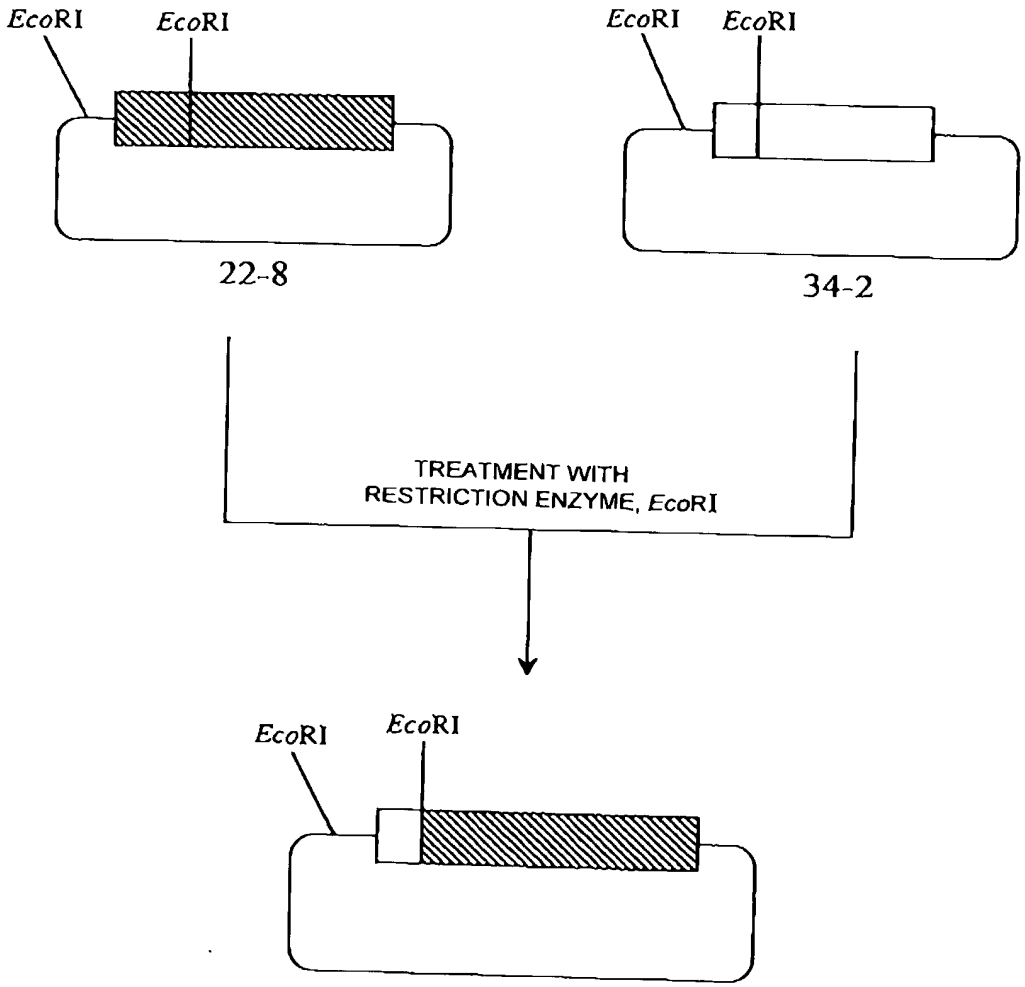
Aragen/Transposagen Ex. 1036 - Part G

FIG. 53



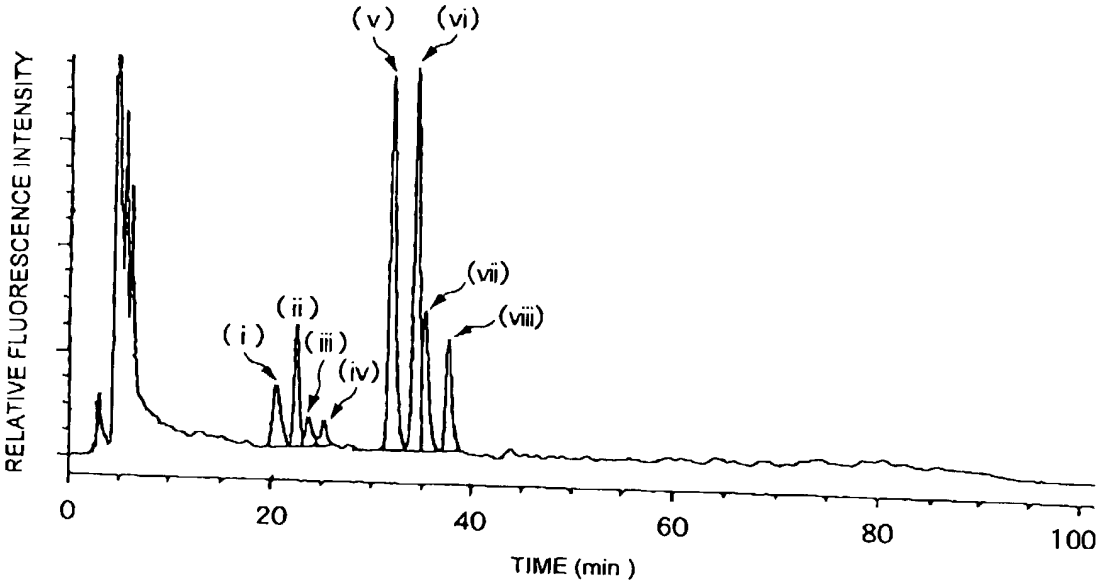
Aragen/Transposagen Ex. 1036 - Part G

FIG. 54



Aragen/Transposagen Ex. 1036 - Part G

FIG. 55



Aragen/Transposagen Ex. 1036 - Part G

JUN 17 2002 PATENT & TRADEMARK OFFICE

#4

Case No. _____

Nixon & Vanderhye P.C (12/97)

RULE 63 (37 C.F.R. 1.63)
DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name and I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

ANTIBODY COMPOSITION-PRODUCING CELL

the specification of which (check applicable box(s)):

☐ is attached hereto
☒ was filed on October 9, 2001 as U S Application Serial No 09/971,773
☐ was filed as PCT International application No _____ on _____
and (if applicable to U S or PCT application) was amended on _____

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with 37 C.F.R. 1.56. I hereby claim foreign priority benefits under 35 U.S.C 119/365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed or, if no priority is claimed, before the filing date of this application:

Priority Foreign Application(s):

Application Number	Country	Day/Month/Year Filed
P. 2000-308526	Japan	06/October/2000

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below

Application Number	Date/Month/Year Filed
60/268,916	16/February/2001

I hereby claim the benefit under 35 U S C 120/365 of all prior United States and PCT International applications listed above or below and, insofar as the subject matter of each of the claims of this application is not disclosed in such prior applications in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose material information as defined in 37 C.F.R. 1.56 which occurred between the filing date of the prior applications and the national or PCT international filing date of this application:

Prior U.S./PCT Application(s): Application Serial No.	Day/Month/Year Filed	Status: patented pending, abandoned
PCT/JP01/08804	05/October/2001	pending

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon. And I hereby appoint **NIXON & VANDERHYE P.C., 1100 North Glebe Rd., 8th Floor, Arlington, VA 22201-4714, telephone number (703) 816-4000 (to whom all communications are to be directed)**, and the following attorneys thereof (of the same address) individually and collectively my attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and with the resulting patent: Arthur R. Crawford, 25327; Larry S. Nixon, 25640, Robert A. Vanderhye, 27076; James T. Hosmer, 30184; Robert W. Fans, 31352; Richard G. Besha, 22770; Mark E. Nusbaum, 32348, Michael J. Keenan, 32106, Bryan H. Davidson, 30251, Stanley C. Spooner, 27393, Leonard C. Mitchard, 29009, Duane M. Byers, 33363; Jeffry H. Nelson, 30481, John R. Lastova, 33149; H. Warren Burnam, Jr. 29366; Thomas E. Byrne, 32205; Mary J. Wilson, 32955; J. Scott Davidson, 33489; Alan M. Kagen, 36178, William J. Griffin, 31260; Robert A. Molan, 29834, B. J. Sadoff, 36663, James D. Berquist, 34776; Updeep S. Gill, 37334

1. Inventor's Signature: Yutaka Kanda Date: December 20, 2001
Inventor: Yutaka (first) MI KANDA (last) Japan (citizenship)
Residence: (city) Machida-shi, Tokyo (state/country) Japan
Post Office Address: c/o Tokyo Research Laboratories, KYOWA HAKKO KOGYO CO., LTD. 6-6, Asahi-machi 3-chome,
(Zip Code) Machida-shi, Tokyo 194-8533 Japan

2. Inventor's Signature: Mitsuo Satoh Date: December 20, 2001
Inventor: Mitsuo (first) MI SATOH (last) Japan (citizenship)
Residence: (city) Machida-shi, Tokyo (state/country) Japan
Post Office Address: c/o Tokyo Research Laboratories, KYOWA HAKKO KOGYO CO., LTD., 6-6 Asahi-machi 3-chome,
(Zip Code) Machida-shi, Tokyo 194-8533 Japan

3. Inventor's Signature: Kazuyasu Nakamura Date: December 20, 2001
Inventor: Kazuyasu (first) MI NAKAMURA (last) Japan (citizenship)
Residence: (city) Machida-shi, Tokyo (state/country) Japan
Post Office Address: c/o Tokyo Research Laboratories, KYOWA HAKKO KOGYO CO., LTD. 6-6, Asahi-machi 3-chome,
(Zip Code) Machida-shi Tokyo 194-8533 Japan

FOR ADDITIONAL INVENTORS, check box ☒ and attach sheet with same information and signature and date for each

268050

NIXON JUN 17 2002 PATENT & TRADEMARK OFFICE JC98

Case No. _____

Nixon & Vanderhye P.C. (12/97)

RULE 63 (37 C.F.R. 1.63)
DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

4. Inventor's Signature Kazuhisa Uchida. Date: December 20, 2001
Inventor: Kazuhisa (first) | MI | UCHIDA (last) | Japan (citizenship)
Residence: (city) Machida-shi, Tokyo (state/country) Japan
Post Office Address: c/o Tokyo Research Laboratories, KYOWA HAKKO KOGYO CO., LTD., 6-6, Asahi-machi 3-chome,
(Zip Code) Machida-shi, Tokyo 194-8533 Japan

5. Inventor's Signature: Toyohide Shinkawa Date: December 20, 2001
Inventor: Toyohide (first) | MI | SHINKAWA (last) | Japan (citizenship)
Residence (city) Machida-shi, Tokyo (state/country) Japan
Post Office Address: c/o Tokyo Research Laboratories, KYOWA HAKKO KOGYO CO., LTD., 6-6, Asahi-machi 3-chome,
(Zip Code) Machida-shi, Tokyo 194-8533 Japan

6. Inventor's Signature: Naoko Yamane Date: December 20, 2001
Inventor: Naoko (first) | MI | YAMANE (last) | Japan (citizenship)
Residence: (city) Machida-shi, Tokyo (state/country) Japan
Post Office Address: c/o Tokyo Research Laboratories, KYOWA HAKKO KOGYO CO., LTD., 6-6, Asahi-machi 3-chome,
(Zip Code) Machida-shi, Tokyo 194-8533 Japan

7. Inventor's Signature: Emi Hosaka Date: December 20, 2001
Inventor: Emi (first) | MI | HOSAKA (last) | Japan (citizenship)
Residence: (city) Machida-shi, Tokyo (state/country) Japan
Post Office Address: c/o Tokyo Research Laboratories, KYOWA HAKKO KOGYO CO., LTD., 6-6, Asahi-machi 3-chome,
(Zip Code) Machida-shi, Tokyo 194-8533 Japan

8. Inventor's Signature: Kazuya Yamano Date: December 20, 2001
Inventor: Kazuya (first) | MI | YAMANO (last) | Japan (citizenship)
Residence: (city) Machida-shi, Tokyo (state/country) Japan
Post Office Address: c/o Tokyo Research Laboratories, KYOWA HAKKO KOGYO CO., LTD., 6-6, Asahi-machi 3-chome,
(Zip Code) Machida-shi, Tokyo 194-8533 Japan

9. Inventor's Signature: Motoo Yamasaki Date: December 20, 2001
Inventor: Motoo (first) | MI | YAMASAKI (last) | Japan (citizenship)
Residence: (city) Machida-shi, Tokyo (state/country) Japan
Post Office Address: c/o Tokyo Research Laboratories, KYOWA HAKKO KOGYO CO., LTD., 6-6, Asahi-machi 3-chome,
(Zip Code) Machida-shi, Tokyo 194-8533 Japan

10. Inventor's Signature: Nobuo Hanai Date: December 20, 2001
Inventor: Nobuo (first) | MI | HANAI (last) | Japan (citizenship)
Residence: (city) Machida-shi, Tokyo (state/country) Japan
Post Office Address: c/o Tokyo Research Laboratories, KYOWA HAKKO KOGYO CO., LTD., 6-6, Asahi-machi 3-chome,
(Zip Code) Machida-shi, Tokyo 194-8533 Japan

11. Inventor's Signature _____ Date. _____
Inventor: _____ (first) | MI | _____ (last) | _____ (citizenship)
Residence (city) _____ (state/country) _____
Post Office Address _____
(Zip Code) _____

12. Inventor's Signature: _____ Date. _____
Inventor: _____ (first) | MI | _____ (last) | _____ (citizenship)
Residence: (city) _____ (state/country) _____
Post Office Address: _____
(Zip Code) _____

268050

NIXON PEABODY
JUN 17 2002
PATENT & TRADEMARK OFFICE
JC98

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of
Yutaka KANDA *et al.*
Appln. No.: 09/971,773 Group Art Unit:
Filed: October 9, 2001 Examiner:
For: ANTIBODY COMPOSITION-PRODUCING CELL

DECLARATION

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir/Madam:

I, Eiichi Kobayashi, do declare and state that:

I graduated from the University of Tokyo, Faculty of Agriculture, Department in Agricultural Chemistry, having received a Master's Degree of Agriculture in March, 1992.

I understand the Japanese and English languages. Attachment is an accurate English translation made by me of U.S. Patent Application No. 09/971,773, filed October 9, 2001 in Japanese language.

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date : June 14, 2002 Name : E Kobayashi
Eiichi Kobayashi

#8

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<223> Description of Artificial Sequense: Synthetic DNA

<400> 5
cttgaccgtt tctatcttct ctcg 24

<210> 6
<211> 979
<212> DNA
<213> Cricetulus griseus

<400> 6
actcatcttg gaatctcaga attggcgcta tgctactgga ggatgggaga ctgtgtttag 60

acctgtaagt gagacatgca cagacaggtc tggcctctcc actggacact ggtcaggtga 120

agtgaaggac aaaaatgttc aagtggtcga gctccccatt gtagacagcc tccatcctcg 180

tcctccttac ttacccttgg ctgtaccaga agaccttgca gatcgactcc tgagagtcca 240

tggtgatcct gcagtgtggt gggtatccca gtttgtcaaa tacttgatcc gtccacaacc 300

ttggctggaa agggaaatag aagaaaccac caagaagctt ggcttcaaac atccagttat 360

tggagtccat gtcagacgca ctgacaaagt gggaacagaa gcagccttcc atcccattga 420

ggaatacatg gtacacgttg aagaacattt tcagcttctc gaacgcagaa tgaaagtgga 480

taaaaaaaga gtgtatctgg 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 ctgcaaactt ccattcttta gatgacatct actattttgg 780

aggccaaaat gcccacaacc agattgcagt ttatcctcac caacctcgaa ctaaagagga 840

aatccccatg gaacctggag atatcattgg tgtggctgga aaccattgga atggttactc 900

taaaggtgtc aacagaaaac taggaaaaac aggcctgtac ccttcctaca aagtccgaga 960

gaagatagaa acggtcaag 979

<210> 7
<211> 979
<212> DNA
<213> Rattus norvegicus

<400> 7
actcatcttg gaatctcaga attggcgcta tgctactggt ggatgggaga ctgtgtttag 60

acctgtaagt gagacatgca cagacagatc tggcctctcc actggacact ggtcaggtga 120

agtgaatgac aaaaatattc aagtggtgga gctccccatt gtagacagcc ttcatcctcg 180

gcctccttac ttaccactgg ctgttccaga agaccttgca gatcgactcg taagagtcca 240

tggtgatcct gcagtgtggt gggtgtccca gttcgtcaaa tatttgattc gtccacaacc 300

ttggctagaa aaggaaatag aagaagccac caagaagctt ggcttcaaac atccagtcat 360

tggagtccat gtcagacgca cagacaaagt gggaacagag gcagccttcc atcccatcga 420

agagtacatg gtacatgttg aagaacattt tcagcttctc gcacgcagaa tgcaagtgga 480

taaaaaaaga gtatatctgg 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 ctgcaaactt ccactcttta gatgacatct actattttgg 780

aggccaaaat gcccacaacc agattgccgt ttatcctcac aaacctcgaa ctgatgagga 840

aattccaatg gaacctggag atatcattgg tgtggctgga aaccattggg atggttattc 900

taaaggtgtc aacagaaaac ttggaaaaac aggcttatat ccctcctaca aagtccgaga 960

gaagatagaa acggtcaag 979

<210> 8
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequense: Synthetic DNA

<400> 8
aagtataagc ttacatggat gacgatatcg ctgcgctcgt 40

<210> 9
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequense: Synthetic DNA

<400> 9
atttaactgc aggaagcatt tgcggtggac gatggagggg 40

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<210> 10
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequense: Synthetic DNA

<400> 10
atttaaggta ccgaagcatt tgcggtgcac gatggagggg 40

<210> 11
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequense: Synthetic DNA

<400> 11
ctccaattat gaatttatta gtg 23

<210> 12
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequense: Synthetic DNA

<400> 12
ggatgtttga agccaagctt cttgg 25

<210> 13
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequense: Synthetic DNA

<400> 13
gtccatggtg atcctgcagt gtgg 24

<210> 14
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequense: Synthetic DNA

<400> 14
caccaatgat atctccaggt tcc 23

<210> 15
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequense: Synthetic DNA

<400> 15
gatatcgctg cgctcgttgt cgac 24

<210> 16
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequense: Synthetic DNA

<400> 16
caggaaggaa ggctggaaaa gagc 24

<210> 17
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequense: Synthetic DNA

<400> 17
gatatcgctg cgctcgtcgt cgac 24

<210> 18
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequense: Synthetic DNA

<400> 18
caggaaggaa ggctggaaga gagc 24

<210> 19
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequense: Synthetic DNA

<400> 19
atgcgggcat ggactggttc ctgg 24

<210> 20
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequense: Synthetic DNA

<400> 20
ctatttttca gcttcaggat atgtggg 27

<210> 21
<211> 24
<212> DNA

<213> Artificial Sequence

<220>
<223> Description of Artificial Sequense: Synthetic DNA

<400> 21
gtctgaagca ttatgtgttg aagc 24

<210> 22
<211> 23
<212> DNA
<213> Artificial Sequence
<220>
<223> Description of Artificial Sequense: Synthetic DNA

<400> 22
gtgagtacat tcattgtact gtg 23

<210> 23
<211> 575
<212> PRT
<213> Cricetulus griseus

<400> 23
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 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

Aragen/Transposagen Ex. 1036 - Part G

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
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

Aragen/Transposagen Ex. 1036 - Part G

<210> 24
<211> 575
<212> PRT
<213> Mus musculus

<400> 24

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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

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
```


Aragen/Transposagen Ex. 1036 - Part G

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> 25
<211> 18
<212> PRT
<213> Homo sapiens

<400> 25
Asp Glu Ser Ile Tyr Ser Asn Tyr Tyr Leu Tyr Glu Ser Ile Pro Lys
1 5 10 15

Pro Cys

<210> 26
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequense: Synthetic DNA

<400> 26

cttgtgtgac tcttaactct cagag 25

<210> 27
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequense: Synthetic DNA

<400> 27
ccctcgagat aacttcgtat agc 23

<210> 28
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequense: Synthetic DNA

<400> 28
ggtaggcctc actaactg 18

<210> 29
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequense: Synthetic DNA

<400> 29
catagaaaca agtaacaaca gccag 25

<210> 30
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequense: Synthetic DNA

<400> 30
gagacttcag cccacttcaa ttattggc 28

<210> 31
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequense: Synthetic DNA

<400> 31
gaggccactt gtgtagcgcc aagtg 25

<210> 32
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic DNA

<400> 32
aggaaggtgg cgctcatcac gggc 24

<210> 33
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic DNA

<400> 33
taaggccaca agtcttaatt gcatcc 26

<210> 34
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic DNA

<400> 34
caggggtgtt cccttgagga ggtggaa 27

<210> 35
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic DNA

<400> 35
cccctcacgc atgaagcctg gag 23

<210> 36
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic DNA

<400> 36
ggcaggagac caccttgcga gtgcccac 28

<210> 37
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic DNA

<400> 37
ggcgctggct tacccggaga ggaatggg 28

<210> 38

<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic DNA

<400> 38
aaaaggcctc agttagtgaa ctgtatgg 28

<210> 39
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic DNA

<400> 39
cgcggatcct caagcgttgg ggttggtcc 29

<210> 40
<211> 45
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic DNA

<400> 40
cccaagcttg ccaccatggc tcacgctccc gctagctgcc cgagc 45

<210> 41
<211> 31
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic DNA

<400> 41
ccggaattct gccaagtatg agccatcctg g 31

<210> 42
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic DNA

<400> 42
gccatccaga aggtggt 17

<210> 43
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic DNA

<400> 43

gtcttgtcag ggaagat 17

<210> 44
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic DNA

<400> 44
ggcaggagac caccttgcga gtgcccac 28

<210> 45
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic DNA

<400> 45
gggtgggctg taccttctgg aacagggc 28

<210> 46
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic DNA

<400> 46
ggcgctggct tacccggaga ggaatggg 28

<210> 47
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic DNA

<400> 47
ggaatgggtg tttgtctcctc caaagatgc 28

<210> 48
<211> 1316
<212> DNA
<213> Cricetulus griseus

<400> 48
gccccgcccc ctccacctgg accgagagta gctggagaat tgtgcaccgg aagtagctct 60

tggactggtg gaaccctgcg caggtgcagc aacaatgggt gagccccagg gatccaggag 120

gatcctagtg acaggggggt ctggactggt gggcagagct atccagaagg tggtcgcaga 180

tggcgctggc ttacccggag aggaatgggt gtttgtctcc tccaaagatg cagatctgac 240

ggatgcagca caaacccaag ccctgttcca gaaggtacag cccacccatg tcatccatct 300

tgctgcaatg gtaggaggcc ttttccggaa tatcaaatac aacttggatt tctggaggaa 360

gaatgtgcac atcaatgaca acgtcctgca ctcagctttc gaggtgggca ctcgcaaggt 420

ggtctcctgc ctgtccacct gtatcttccc tgacaagacc acctatccta ttgatgaaac 480

aatgatccac aatggtccac cccacagcag caattttggg tactcgtatg ccaagaggat 540

gattgacgtg cagaacaggg cctacttcca gcagcatggc tgcaccttca ctgctgtcat 600

ccctaccaat gtctttggac ctcatgacaa cttcaacatt gaagatggcc atgtgctgcc 660

tggcctcatc ataaggtgc atctggccaa gagtaatggt tcagccttga ctgtttgggg 720

tacagggaaa ccacggaggc agttcatcta ctcactggac ctagcccggc 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

tgcctggttc accgacaact atgagcaggc ccggaagtga agcatgggac aagcgggtgc 1080

tcagctggca atgcccagtc agtaggctgc agtctcatca tttgcttgtc aagaactgag 1140

gacagtatcc agcaacctga gccacatgct ggtctctctg ccaggggggct tcatgcagcc 1200

atccagtagg gcccatgttt gtccatcctc gggggaaggc cagaccaaca ccttgtttgt 1260

ctgcttctgc cccaacctca gtgcatccat gctggtcctg ctgtcccttg tctaga 1316

<210> 49
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic DNA

<400> 49
gatcctgctg ggaccaaaat tgg 23

<210> 50
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic DNA

<400> 50
cttaacatcc caagggatgc tg 22

<210> 51
<211> 1965
<212> DNA
<213> Cricetulus griseus

<400> 51
acgggggggct cccggaagcg gggaccatgg cgtctctgcg cgaagcgagc ctgcggaagc 60

tgcggcgctt ttccgagatg agaggcaaac ctgtggcaac tgggaaattc tgggatgtag 120

ttgtaataac agcagctgac gaaaagcagg agcttgctta caagcaacag ttgtcggaga 180

agctgaagag aaaggaattg cccottggag ttaactacca tgttttcact gatcctcctg 240

gaaccaaaat tggaaatgga ggatcaacac tttgttctct tcagtgcctg gaaagcctct 300

atggagacaa gtggaattcc ttcacagtcc tgttaattca ctctggtggc tacagtcaac 360

gacttcccaa tgcaagcgct ttaggaaaaa tcttcacggc tttaccactt ggtgagccca 420

tttatcagat gttggactta aaactagcca tgtacatgga tttcccctca cgcatgaagc 480

ctggagtttt ggtcacctgt gcagatgata ttgaactata cagcattggg gactctgagt 540

ccattgcatt tgagcagcct ggctttactg ccctagccca tccatctagt ctggctgtag 600

gcaccacaca tggagtattt gtattggact ctgccggttc tttgcaacat ggtgacctag 660

agtacaggca atgccaccgt ttcctccata agcccagcat tgaaaacatg caccacttta 720

atgccgtgca tagactagga agctttggtc aacaggactt gagtgggggt gacaccacct 780

gtcatccatt gcactctgag tatgtctaca cagatagcct attttacatg 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 aatgttgttg tccttaataa ctccaggttt tatcacattg 1080

gaacaacgga ggagtatctg ctacatttca cttccaatgg ttcgttacag gcagagctgg 1140

gcttgcaatc catagctttc agtgtctttc caaatgtgcc tgaagactcc catgagaaac 1200

cctgtgtcat tcacagcatc ctgaattcag gatgctgtgt ggcccctggc tcagtggtag 1260

aatattccag attaggacct gaggtgtcca tctcggaaaa ctgcattatc agcggttctg 1320

tcatagaaaa agctgttctg cccccatgtt ctttcgtgtg ctctttaagt gtggagataa 1380

atggacactt agaatattca actatggtgt ttggcatgga agacaacttg aagaacagtg 1440

ttaaaaccat atcagatata aagatgcttc agttctttgg agtctgtttc ctgacttgtt 1500

tagatatttg 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 tttgcctgga aacaggattg caaatgcagg catattctat agatctctgg 1860

gttcttcttt ctttctcccc tctctccttt cctttccctt tgatgtaatg acaaaggtaa 1920

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<210> 53
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<210> 54
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<212> DNA
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<210> 55
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<210> 56
<211> 28
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<210> 57
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<400> 57
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<210> 58

Aragen/Transposagen Ex. 1036 - Part G

<211> 25
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<213> Artificial Sequence

<220>
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<400> 58
tccggggatg gcgagatggg caagc 25

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cgctcacccg cctgaggcga catg 24

<210> 62
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<210> 63
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<212> DNA
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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                 100

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
            105                 110                 115

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
        120                 125                 130

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
    135                 140                 145

ata aat tct gtg aag ttc tac cag gcc tca act agt gaa ctg tat gga  480
Ile Asn Ser Val Lys Phe Tyr Gln Ala Ser Thr Ser Glu Leu Tyr Gly
150                 155                 160

aaa gtg caa gaa ata ccc cag aaa gag acc acc cct ttc tat cca agg  528
Lys Val Gln Glu Ile Pro Gln Lys Glu Thr Thr Pro Phe Tyr Pro Arg
165                 170                 175                 180
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Aragen/Transposagen Ex. 1036 - Part G

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tcg ccc tat gga gca gcc aaa ctt tat gcc tat tgg att gta gtg aac   576
Ser Pro Tyr Gly Ala Ala Lys Leu Tyr Ala Tyr Trp Ile Val Val Asn
                185                 190                 195

ttt cga gag gct tat aat ctc ttt gcg gtg aac ggc att ctc ttc aat   624
Phe Arg Glu Ala Tyr Asn Leu Phe Ala Val Asn Gly Ile Leu Phe Asn
            200                 205                 210

cat gag agt cct aga aga gga gct aat ttt gtt act cga aaa att agc   672
His Glu Ser Pro Arg Arg Gly Ala Asn Phe Val Thr Arg Lys Ile Ser
        215                 220                 225

cgg tca gta gct aag att tac ctt gga caa ctg gaa tgt ttc agt ttg   720
Arg Ser Val Ala Lys Ile Tyr Leu Gly Gln Leu Glu Cys Phe Ser Leu
    230                 235                 240

gga aat ctg gac gcc aaa cga gac tgg ggc cat gcc aag gac tat gtc   768
Gly Asn Leu Asp Ala Lys Arg Asp Trp Gly His Ala Lys Asp Tyr Val
245                 250                 255                 260

gag gct atg tgg ctg atg tta caa aat gat gaa cca gag gac ttt gtc   816
Glu Ala Met Trp Leu Met Leu Gln Asn Asp Glu Pro Glu Asp Phe Val
                265                 270                 275

ata gct act ggg gaa gtt cat agt gtc cgt gaa ttt gtt gag aaa tca   864
Ile Ala Thr Gly Glu Val His Ser Val Arg Glu Phe Val Glu Lys Ser
            280                 285                 290

ttc atg cac att gga aag acc att gtg tgg gaa gga aag aat gaa aat   912
Phe Met His Ile Gly Lys Thr Ile Val Trp Glu Gly Lys Asn Glu Asn
        295                 300                 305

gaa gtg ggc aga tgt aaa gag acc ggc aaa att cat gtg act gtg gat   960
Glu Val Gly Arg Cys Lys Glu Thr Gly Lys Ile His Val Thr Val Asp
    310                 315                 320

ctg aaa tac tac cga cca act gaa gtg gac ttc ctg cag gga gac tgc  1008
Leu Lys Tyr Tyr Arg Pro Thr Glu Val Asp Phe Leu Gln Gly Asp Cys
325                 330                 335                 340

tcc aag gcg cag cag aaa ctg aac tgg aag ccc cgc gtt gcc ttt gac  1056
Ser Lys Ala Gln Gln Lys Leu Asn Trp Lys Pro Arg Val Ala Phe Asp
                345                 350                 355

gag ctg gtg agg gag atg gtg caa gcc gat gtg gag ctc atg aga acc  1104
Glu Leu Val Arg Glu Met Val Gln Ala Asp Val Glu Leu Met Arg Thr
            360                 365                 370

aac ccc aac gcc tga gcacctctac aaaaaaattc gcgagacatg gactatggtg  1159
Asn Pro Asn Ala
        375

cagagccagc caaccagagt ccagccactc ctgagaccat cgaccataaa ccctcgactg 1219
cctgtgtcgt ccccacagct aagagctggg ccacaggttt gtgggcacca ggacggggac 1279
actccagagc taaggccact tcgcttttgt caaaggctcc tctcaatgat tttgggaaat 1339
caagaagttt aaaatcacat actcatttta cttgaaatta tgtcactaga caacttaaat 1399
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<210> 66
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Aragen/Transposagen Ex. 1036 - Part G

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<210> 67
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<212> DNA
<213> Cricetulus griseus

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agaaa 125

<210> 71
<211> 376
<212> PRT
<213> Cricetulus griseus

<400> 71
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Gly Gln Asp Gly Ser Tyr Leu Ala Glu Phe Leu Leu Glu Lys Gly Tyr
35 40 45

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

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65 70 75 80

Lys Leu His Tyr Gly Asp Leu Thr Asp Ser Thr Cys Leu Val Lys Ile
85 90 95 100

Ile Asn Glu Val Lys Pro Thr Glu Ile Tyr Asn Leu Gly Ala Gln Ser
105 110 115

His Val Lys Ile Ser Phe Asp Leu Ala Glu Tyr Thr Ala Asp Val Asp
120 125 130

Gly Val Gly Thr Leu Arg Leu Leu Asp Ala Ile Lys Thr Cys Gly Leu
135 140 145

Ile Asn Ser Val Lys Phe Tyr Gln Ala Ser Thr Ser Glu Leu Tyr Gly
150 155 160

Lys Val Gln Glu Ile Pro Gln Lys Glu Thr Thr Pro Phe Tyr Pro Arg
165 170 175 180

Ser Pro Tyr Gly Ala Ala Lys Leu Tyr Ala Tyr Trp Ile Val Val Asn
185 190 195

Phe Arg Glu Ala Tyr Asn Leu Phe Ala Val Asn Gly Ile Leu Phe Asn
200 205 210

His Glu Ser Pro Arg Arg Gly Ala Asn Phe Val Thr Arg Lys Ile Ser
215 220 225

Arg Ser Val Ala Lys Ile Tyr Leu Gly Gln Leu Glu Cys Phe Ser Leu
230 235 240

Gly Asn Leu Asp Ala Lys Arg Asp Trp Gly His Ala Lys Asp Tyr Val
245 250 255 260

Glu Ala Met Trp Leu Met Leu Gln Asn Asp Glu Pro Glu Asp Phe Val
265 270 275

Ile Ala Thr Gly Glu Val His Ser Val Arg Glu Phe Val Glu Lys Ser
280 285 290

Phe Met His Ile Gly Lys Thr Ile Val Trp Glu Gly Lys Asn Glu Asn
295 300 305

Glu Val Gly Arg Cys Lys Glu Thr Gly Lys Ile His Val Thr Val Asp
310 315 320

Leu Lys Tyr Tyr Arg Pro Thr Glu Val Asp Phe Leu Gln Gly Asp Cys
325 330 335 340

Ser Lys Ala Gln Gln Lys Leu Asn Trp Lys Pro Arg Val Ala Phe Asp
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Glu Leu Val Arg Glu Met Val Gln Ala Asp Val Glu Leu Met Arg Thr
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Asn Pro Asn Ala
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<210> 72
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<213> Cricetulus griseus

<400> 72
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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

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Lys

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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
            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                 480

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                 560

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
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US 0997177306P1

Creation date: 09-26-2003
Indexing Officer: OADAN - ORLANDO ADAN
Team: OIPEBackFileIndexing
Dossier: 09971773

Legal Date: 06-17-2003

No.	Doccode	Number of pages
1	LET.	3
2	DRW	55

Total number of pages: 58

Remarks:

Order of re-scan issued on .....

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

#3

OIPE JC17 NOV 2 3 2001 PATENT & TRADEMARK OFFICE

In re Patent Application of

KANDA et al

Serial No. 09/971,773

Filed: October 9, 2001

Atty. Ref.: 249-202

Group:

Examiner:

For: ANTIBODY COMPOSITION-PRODUCING CELL

* * * * * * * * * * * *

November 23, 2001

Assistant Commissioner for Patents
Office of Initial Patent Examination
Customer Service Center
Washington, DC 20231

Sir:

REQUEST FOR CORRECTION OF FILING RECEIPT

Enclosed is a copy of the Filing Receipt for the above-identified application which incorrectly states the cities and countries of the inventors as Machida-shi or Machidi-shi, Japan. The cities and countries for all of the inventors should read Tokyo, Japan. The corrections are shown in red on the attached copy of the filing receipt. Please note your records accordingly. Correction of same is respectfully requested.

Respectfully submitted,

NIXON & VANDERHYE P.C.

By: _____

B. J. Sadoff
Reg. No. 36,663

BJS:eaw
1100 North Glebe Road, 8th Floor
Arlington, VA 22201-4714
Telephone: (703) 816-4000
Facsimile: (703) 816-4100

BJS

UNITED STATES PATENT AND TRADEMARK OFFICE

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WASHINGTON, D.C. 20231
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APPLICATION NUMBER	FILING DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET NO	DRAWINGS	TOT CLAIMS	IND CLAIMS
09/971,773	10/09/2001		0.00	249-202	55	1	1

CONFIRMATION NO. 2525

FILING RECEIPT

OC000000007076733

NIXON & VANDERHYE P.C.
8th Floor
1100 North Glebe Road
Arlington, VA 22201-4714

OIPE JC17 NOV 23 2001 PATENT & TRADEMARK OFFICE

Date Mailed: 11/15/2001

Receipt is acknowledged of this nonprovisional Patent Application. It will be considered in its order and you will be notified as to the results of the examination. Be sure to provide the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION when inquiring about this application. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please write to the Office of Initial Patent Examination's Customer Service Center. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections (if appropriate).**

Applicant(s)

Yutaka Kanda, ~~Machida-shi~~, JAPAN; Tokyo
Mitsuo Satoh, ~~Machida-shi~~, JAPAN; Tokyo
Kazuyasu Nakamura, ~~Machida-shi~~, JAPAN; Tokyo
Kazuhisa Uchida, ~~Machida-shi~~, JAPAN; Tokyo
Toyohide Shinkawa, ~~Machida-shi~~, JAPAN; Tokyo
Naoko Yamane, ~~Machidi-shi~~, JAPAN; Tokyo
Emi Hosaka, ~~Machida-shi~~, JAPAN; Tokyo
Kazuya Yamano, ~~Machida-shi~~, JAPAN; Tokyo
Motoo Yamasaki, ~~Machida-shi~~, JAPAN; Tokyo
Nobuo Hanai, ~~Machida-shi~~, JAPAN; Tokyo

Domestic Priority data as claimed by applicant

THIS APPLN CLAIMS BENEFIT OF 60/268,916 02/16/2001

Foreign Applications

JAPAN 2000-308526 10/06/2000

If Required, Foreign Filing License Granted 11/15/2001

Pr jected Publication Date: To Be Determined - pending completion of Missing Parts

Non-Publication Request: No

Early Publication Request: No

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WASHINGTON, D.C. 20231
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APPLICATION NUMBER	FILING/RECEIPT DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NUMBER
09/971,773	10/09/2001	Yutaka Kanda	249-202

CONFIRMATION NO. 2525

FORMALITIES LETTER

OC000000007076734

NIXON & VANDERHYE P.C.
8th Floor
1100 North Glebe Road
Arlington, VA 22201-4714

Date Mailed: 11/15/2001

NOTICE TO FILE MISSING PARTS OF NONPROVISIONAL APPLICATION

FILED UNDER 37 CFR 1.53(b)

Filing Date Granted

An application number and filing date have been accorded to this application. The item(s) indicated below, however, are missing. Applicant is given **TWO MONTHS** from the date of this Notice within which to file all required items and pay any fees required below to avoid abandonment. Extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 CFR 1.136(a).

- The statutory basic filing fee is missing.
  Applicant must submit $ **740** *to complete the basic filing fee for a non-small entity. If appropriate, applicant may make a written assertion of entitlement to small entity status and pay the small entity filing fee (37 CFR 1.27).*
- The oath or declaration is missing.
  A properly signed oath or declaration in compliance with 37 CFR 1.63, identifying the application by the above Application Number and Filing Date, is required.
- To avoid abandonment, a late filing fee or oath or declaration surcharge as set forth in 37 CFR 1.16(l) of $130 for a non-small entity, must be submitted with the missing items identified in this letter.
- The application was filed in a language other than English. Applicant is required to provide an English translation of the specification and a statement that the translation is accurate. (See 37 CFR 1.52(d)).
- Applicant must file an English translation of the application, the $ 130 fee set forth in 37 CFR 1.17(i), unless previously submitted, and a statement that the translation is accurate (37 CFR 1.52(d)).
- **The balance due by applicant is $ 1000.**
- Because your specification was filed in a language other than English, the Office was unable to determine the number of claims submitted. Additional claim fees may be due once the number of claims can be determined.

The application is informal since it does not comply with the regulations for the reason(s) indicated below.

The required item(s) identified below must be timely submitted to avoid abandonment:

- Substitute drawings in compliance with 37 CFR 1.84 because:
  - drawing figures contain text that is not in English (including, for example, a flow chart

that was originally not in English that has been marked up to include the English text) see 37 CFR 1.84(p)(2) and 37 CFR 1.52(d)(1);

- This application does not contain a statement that the content of the sequence listing information recorded in computer readable form is identical to the written (on paper or compact disc) sequence listing and, where applicable, includes no new matter, as required by 37 CFR 1.821(e), 1.821(f), 1.821(g), 1.825(b), or 1.825(d). Applicant must provide such statement. If the effective filing date is on or after September 8, 2000, see the final rulemaking notice published in the Federal Register at 65 FR 54604 (September 8, 2000) and 1238 OG 145 (September 19, 2000).
- A copy of the "Sequence Listing" in computer readable form has not been submitted as required by 37 C.F.R. 1.821(e). If the effective filing date is on or after September 8, 2000, see the final rulemaking notice published in the Federal Register at 65 FR 54604 (September 8, 2000) and 1238 OG 145 (September 19, 2000). Applicant must provide an initial computer readable form (CRF) copy of the "Sequence Listing" and a statement that the content of the sequence listing information recorded in computer readable form is identical to the written (on paper or compact disc) sequence listing and, where applicable, includes no new matter, as required by 37 CFR 1.821(e), 1.821(f), 1.821(g), 1.825(b), or 1.825(d). If applicant desires the sequence listing in the instant application to be identical with that of another application on file in the U.S. Patent and Trademark Office, such request in accordance with 37 CFR 1.821(e) may be submitted in lieu of a new CRF.

For questions regarding compliance to these requirements, please contact:

- **For Rules Interpretation, call (703) 308-4216**
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A copy of this notice <u>MUST</u> be returned with the reply.

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Initial Patent Examination Division (703) 308-1202

PART 3 - OFFICE COPY

J1080 U.S. PTO
09/971773
10/09/01

	Subclass	ISSUE CLASSIFICATION
	Class	

PATENT NUMBER

JC 525

U.S. UTILITY Patent Application

O.I.P.E.	PATENT DATE
SCANNED TOI Q.A.	

DS ST 1600

APPLICATION NO.	CONT/PRIOR	CLASS	SUBCLASS	ART UNIT	EXAMINER
09/971773	D F	800	6	1632	LI, J.

Claim 42

APPLICANTS

Yutaka Kanda
Mitsuo Satoh
Kazuyasu Nakamura
Kazuhisa Uchida

TITLE

Antibody composition-producing cell

PTO-2040
12/99

ISSUING CLASSIFICATION

ORIGINAL		CROSS REFERENCE(S)	
CLASS	SUBCLASS	CLASS	SUBCLASS (ONE SUBCLASS PER BLOCK)
INTERNATIONAL CLASSIFICATION			
			☐ Continued on Issue Slip Inside File Jacket

☐ TERMINAL DISCLAIMER	DRAWINGS			CLAIMS ALLOWED	
	Sheets Drwg.	Figs. Drwg.	Print Fig.	Total Claims	Print Claim for O.G.
☐ The term of this patent subsequent to _____ (date) has been disclaimed.	(Assistant Examiner) (Date)			NOTICE OF ALLOWANCE MAILED	
☐ The term of this patent shall not extend beyond the expiration date of U.S Patent. No. _____	(Primary Examiner) (Date)			ISSUE FEE	
				Amount Due	Date Paid
☐ The terminal ____months of this patent have been disclaimed.	(Legal Instruments Examiner) (Date)			ISSUE BATCH NUMBER	

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SEARCHED

Class	Sub.	Date	Exmr.

INTERFERENCE SEARCHED

Class	Sub.	Date	Exmr.

SEARCH NOTES
(INCLUDING SEARCH STRATEGY)

	Date	Exmr.

ISSUE SLIP STAPLE AREA (for additional cross references)

POSITION	INITIALS	ID NO.	DATE
FEE DETERMINATION			
O.I.P.E. CLASSIFIER		10	10-30-01
FORMALITY REVIEW		535	11-15-01
RESPONSE FORMALITY REVIEW		35886	04-01-02
		852	09-27-02

INDEX OF CLAIMS

✔	Rejected	N	Non-elected
=	Allowed	I	Interference
—	(Through numeral)... Canceled	A	Appeal
÷	Restricted	O	Objected

Claim Final	Claim Original	Date 6.16.03
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Claim Final	Claim Original	Date 6.16.03
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If more than 150 claims or 10 actions staple additional sheet here

(LEFT INSIDE)

IN[illegible] UNITED STATES PATENT AND TRADEM[illegible] OFFICE
RE[illegible]T FOR FILING APPLICATION UNDER 37[illegible]. 1.53(b)
WITHOUT FILING FEE *AND/OR* WITHOUT EXECUTED INVENTOR'S DECLARATION

10/09/01 J1155 U.S. PTO

Assistant Commissioner for Patents
Washington, DC 20231

Atty. Dkt. 249-202

Date: October 9, 2001

J1050 U.S. PTO 09/971773 10/09/01

This is a request for filing a new PATENT APPLICATION under Rule 53(b) entitled:

ANTIBODY COMPOSITION-PRODUCING CELL

without a filing fee and/or without an executed inventor's oath/declaration.

This application is made by the below identified inventor(s). Attached hereto are the following papers:

☐ Newly executed Declaration, ☐ Copy of Declaration from prior application, ☒ Abstract
131 pages of specification and claims (including 61 numbered claims), and
55 sheets of accompanying drawing/s.
☐ Record the attached assignment and return to the undersigned.
☐ Attached is a Power of Attorney.
☒ Priority is hereby claimed under 35 U.S.C. § 119 based on the following foreign applications:

Application Number	**Country**	**Day/Month/Year Filed**
2000-308526	Japan	6 October 2000

, respectively, the entire content of which is hereby incorporated by reference in this application..

☐ Certified copy(ies) of foreign application(s) is/are attached.
☐ Certified copy(ies) filed on _____ in prior application no. _____ filed _____
☒ Please amend the specification by inserting the following paragraph before the first line: --This application claims the benefit of Provisional Application No. 60/268,916, filed February 16, 2001, the entire content of which is hereby incorporated by reference in this application.--
☐ Please amend the specification by inserting the following paragraph before the first line: --This application is a ☐ continuation/☐ division/☐ continuation-in-part of Application No. , filed , , the entire content of which is hereby incorporated by reference in this application.--
☐ Petition filed in prior application to extend its life to insure co-pendency.
☐ The prior application is assigned to .
☐ It is hereby requested that the Examiner consider the art cited in the above parent application(s) by applicant and/or the Examiner for the reasons stated therein. A listing of that art is attached, but pursuant to Rule 98(d) copies are not required.
☐ Applicant claims "small entity" status. ☐ "Small entity" statement attached.
☐ Please enter the attached and/or below preliminary amendment **prior** to calculation of filing fee:

☒ Also attached: ☐ **Information Disclosure Statement**; ☐ **Non-Publication Request**; ☒ **Sequence Listing**; ☐ **Statement deleting Inventor(s) named in prior application**; ☐ **Other:**

1. Inventor: Yutaka (first) MI KANDA (last) Japanese (citizenship)
Residence: (city) Machida-shi (state/country) Tokyo, Japan
Mailing Address: c/o Tokyo Research Laboratories, KYOWA HAKKO KOGYO CO., LTD., 6-6, Asahi-machi 3-chome, Machida-shi, Tokyo, Japan
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2. Inventor: Mitsuo (first) MI SATOH (last) Japanese (citizenship)
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(Zip Code) 194-8533

☒ *See attached sheet(s) for additional inventor(s) information!!*

Address all future communications to NIXON & VANDERHYE P.C., 1100 North Glebe Road, 8th Floor, Arlington, VA 22201.

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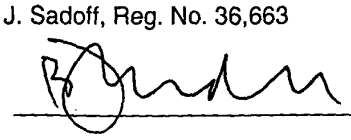
Arlington, Virginia 22201-4714
Telephone: (703) 816-4000
Facsimile: (703) 816-4100
BJS:tat

NIXON & VANDERHYE P.C.
By Atty: B. J. Sadoff, Reg. No. 36,663

Signature: 

3. Inventor: Kazuyasu (first) MI NAKAMURA (last) Japanese (citizenship)
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Mailing Address: c/o Tokyo Research Laboratories, KYOWA HAKKO KOGYO CO., LTD., 6-6, Asahi-machi 3-chome, Machida-shi, Tokyo, Japan
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4. Inventor: Kazuhisa (first) MI UCHIDA (last) Japanese (citizenship)
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5. Inventor: Toyohide (first) MI SHINKAWA (last) Japanese (citizenship)
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(Zip Code) 194-8533

6. Inventor: Naoko (first) MI YAMANE (last) Japanese (citizenship)
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7. Inventor: Emi (first) MI HOSAKA (last) Japanese (citizenship)
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8. Inventor: Kazuya (first) MI YAMANO (last) Japanese (citizenship)
Residence: (city) Machida-shi (state/country) Tokyo, Japan
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(Zip Code) 194-8533

9. Inventor: Motoo (first) MI YAMASAKI (last) Japanese (citizenship)
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(Zip Code) 194-8533

10. Inventor: Nobuo (first) MI HANAI (last) Japanese (citizenship)
Residence: (city) Machida-shi (state/country) Tokyo, Japan
Mailing Address: c/o Tokyo Research Laboratories, KYOWA HAKKO KOGYO CO., LTD., 6-6, Asahi-machi 3-chome, Machida-shi, Tokyo, Japan
(Zip Code) 194-8533

PTO/SB/05 (08-00) Approved for use through 10/31/2002. OMB 0651-0032

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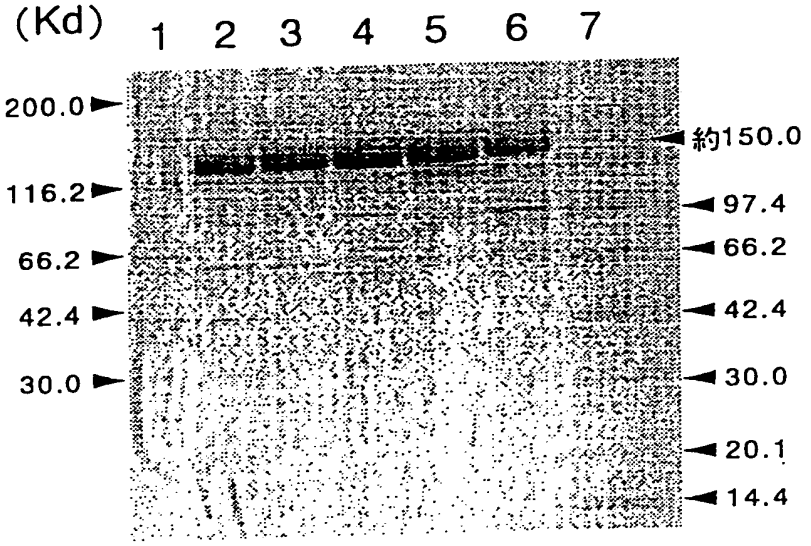

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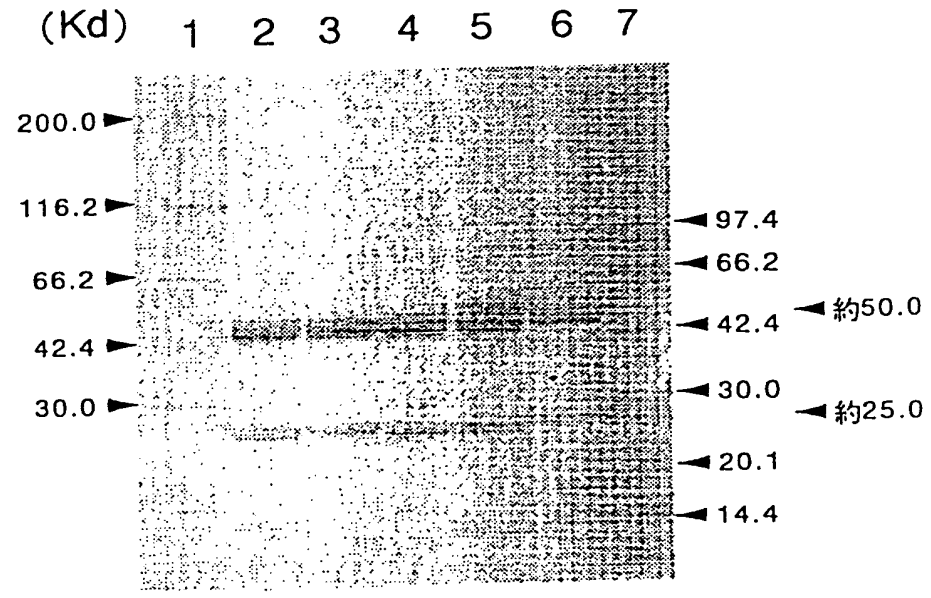

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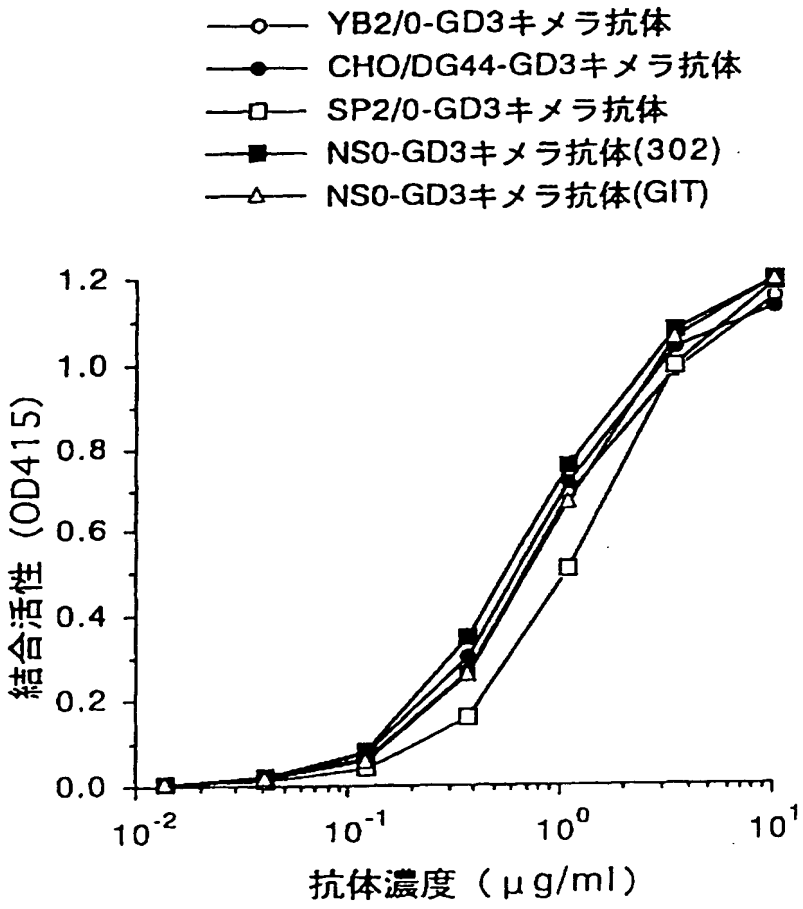

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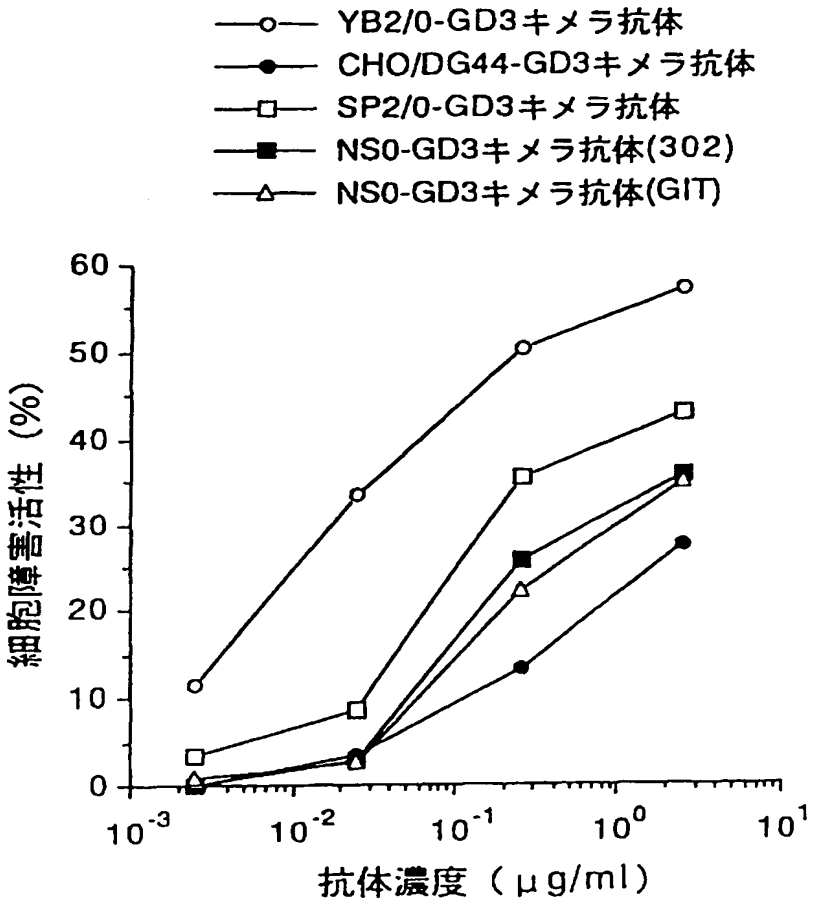

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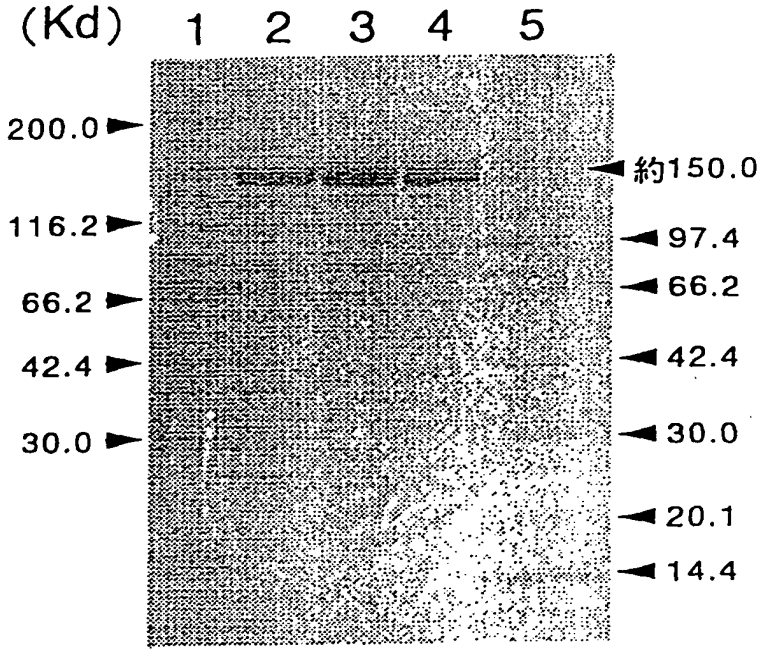

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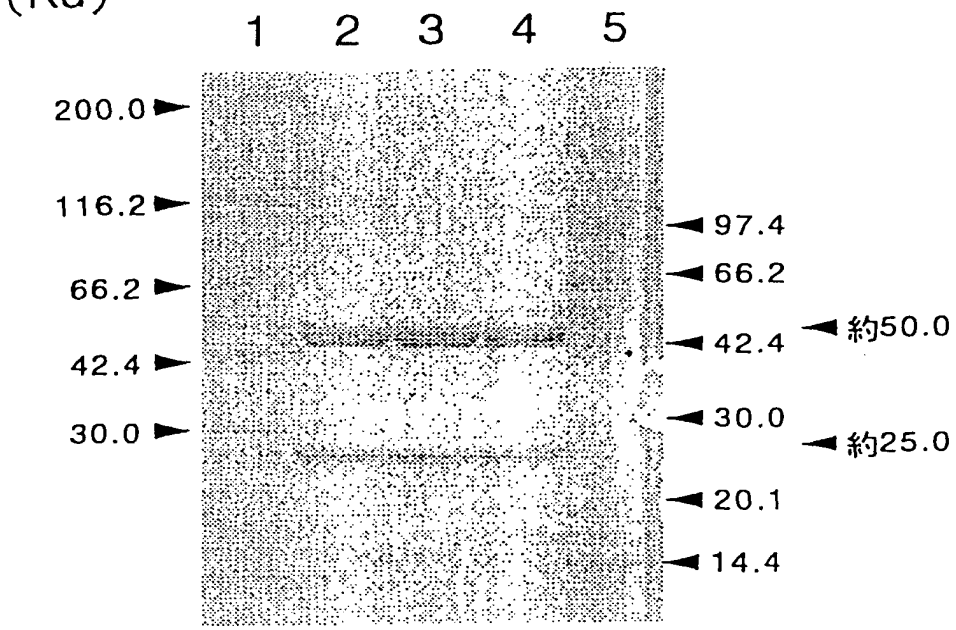

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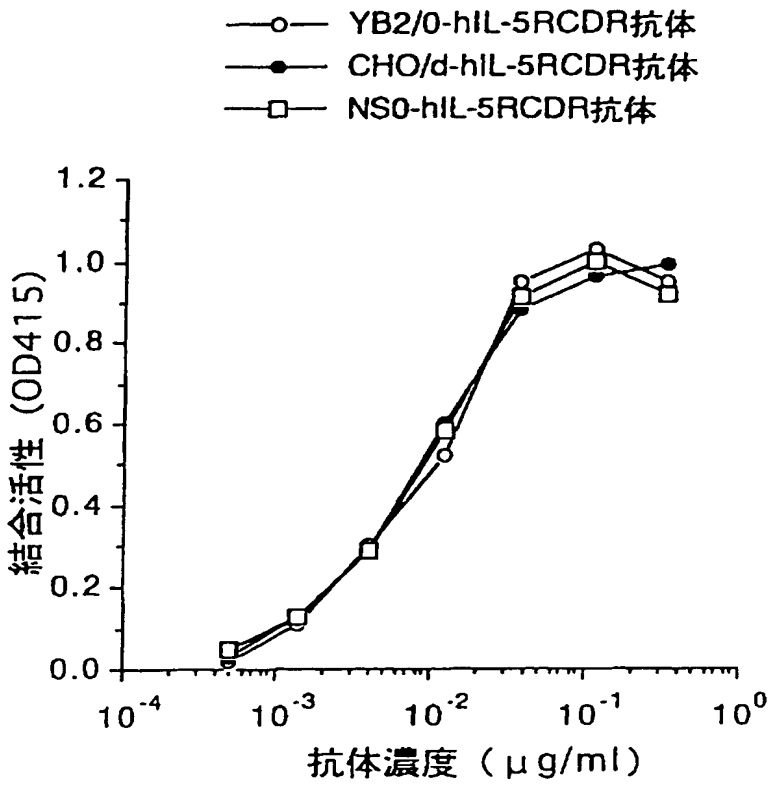

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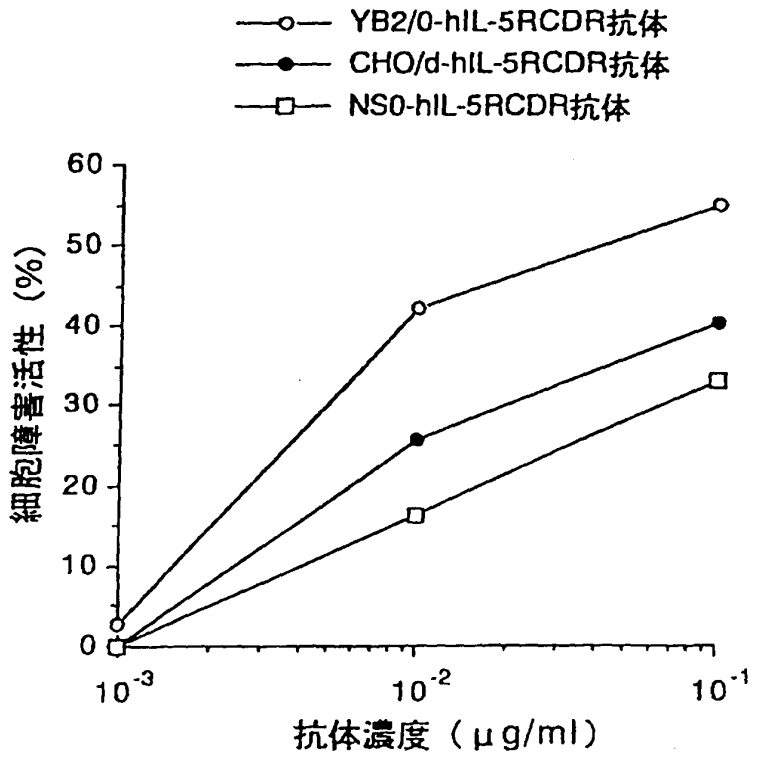

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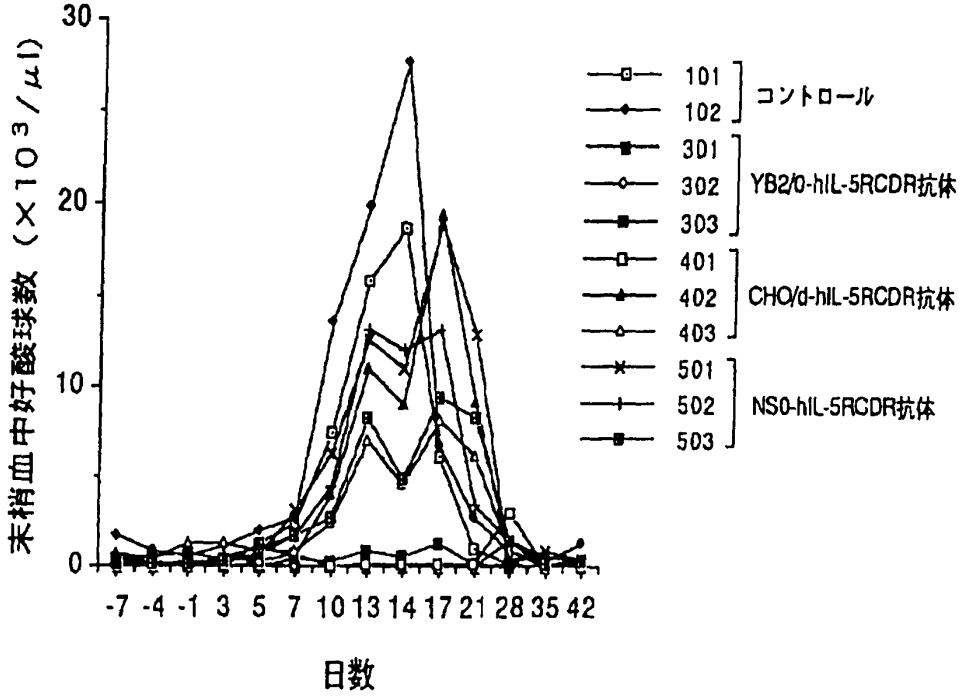

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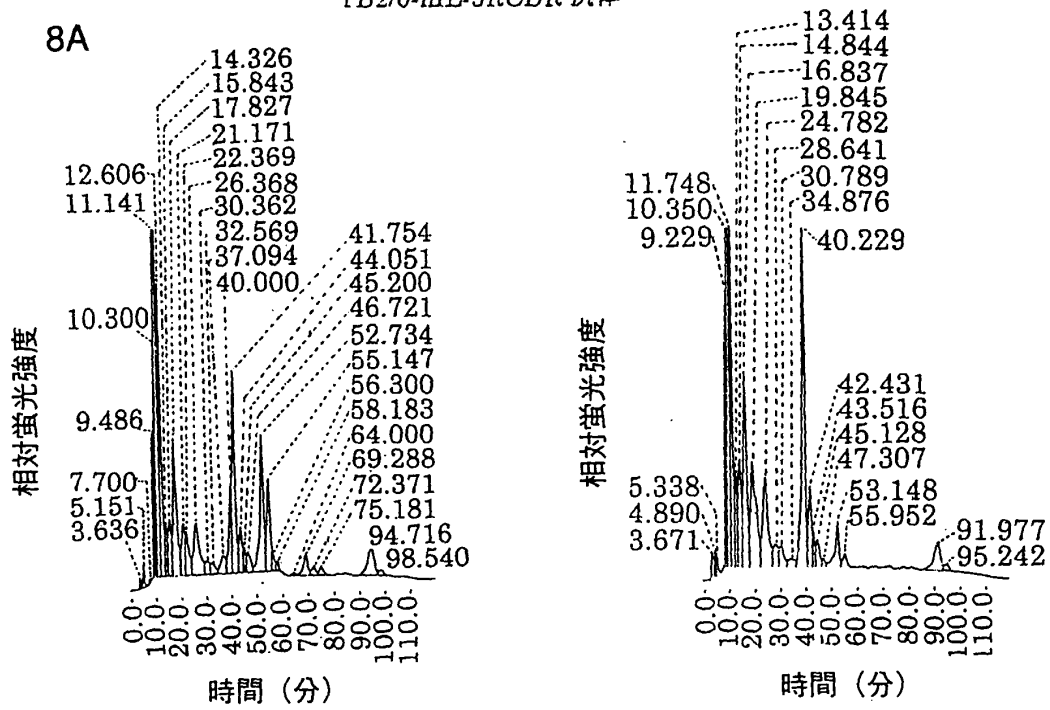

α-L-フコシダーゼ消化後

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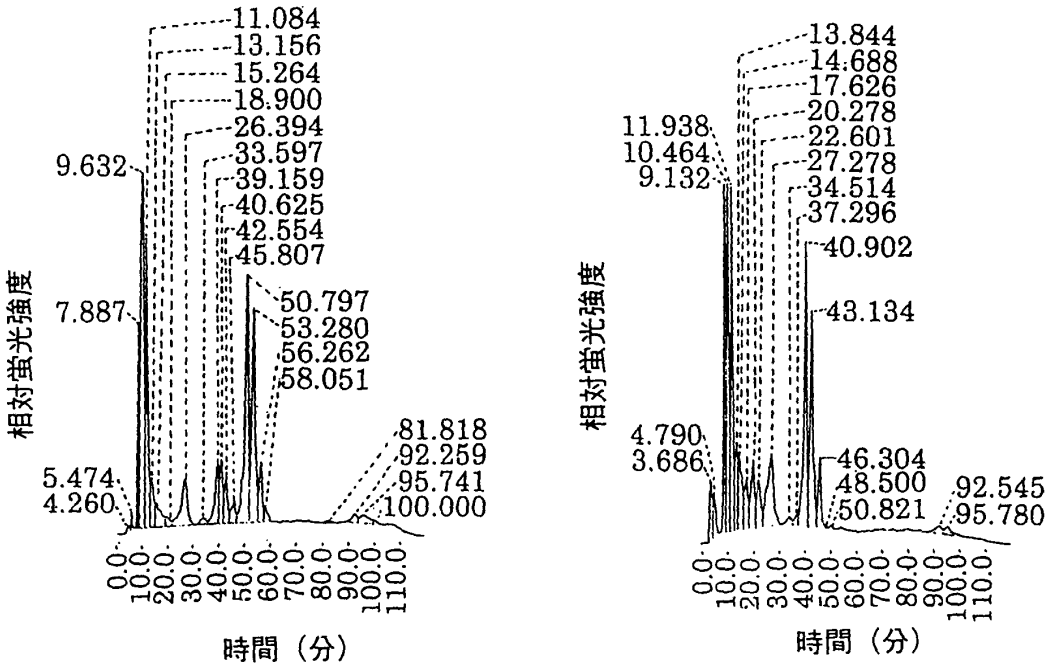

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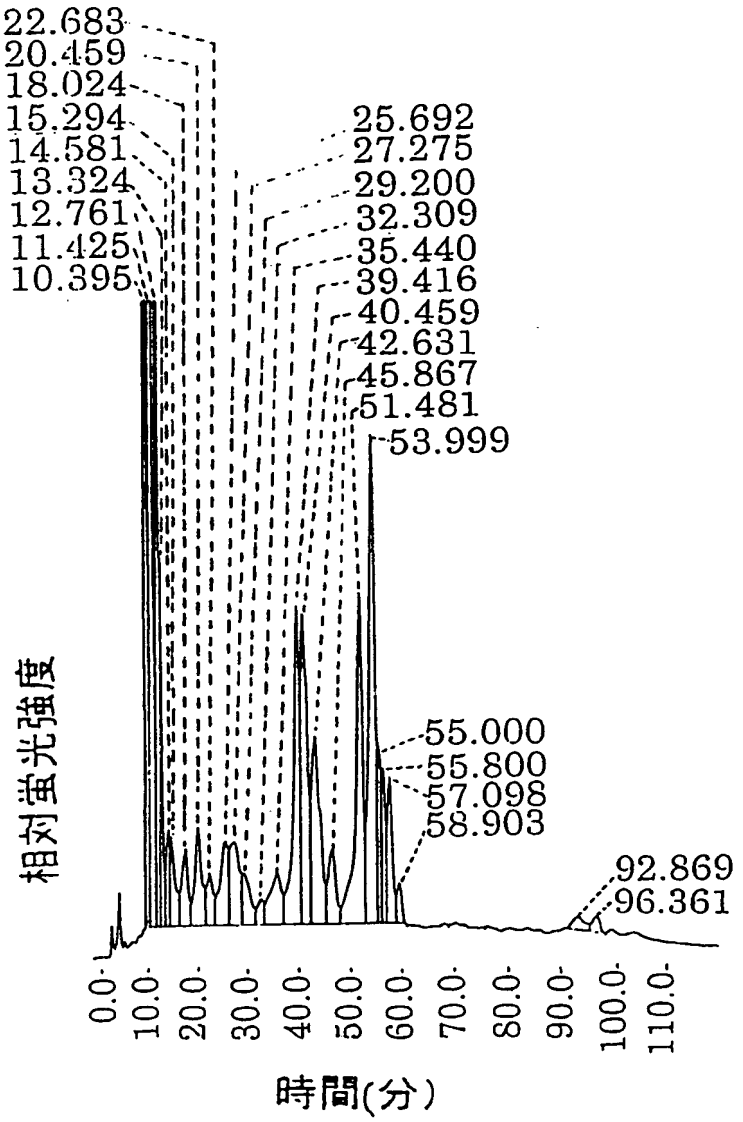

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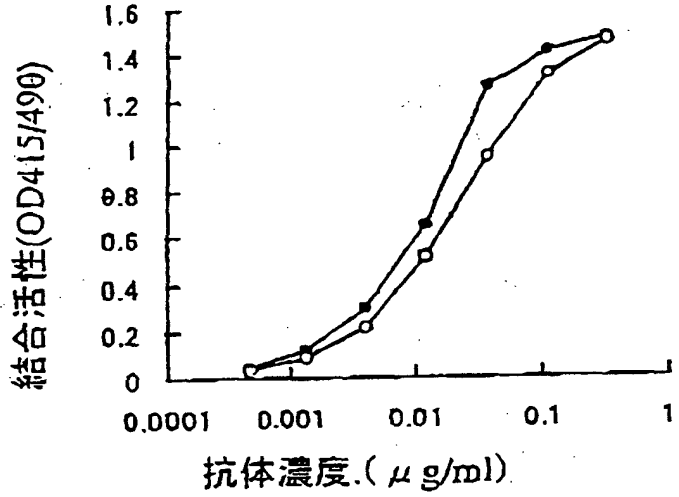

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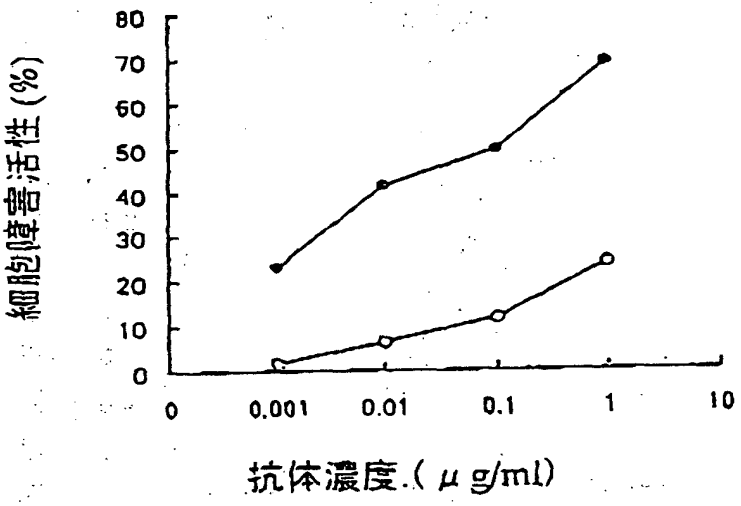

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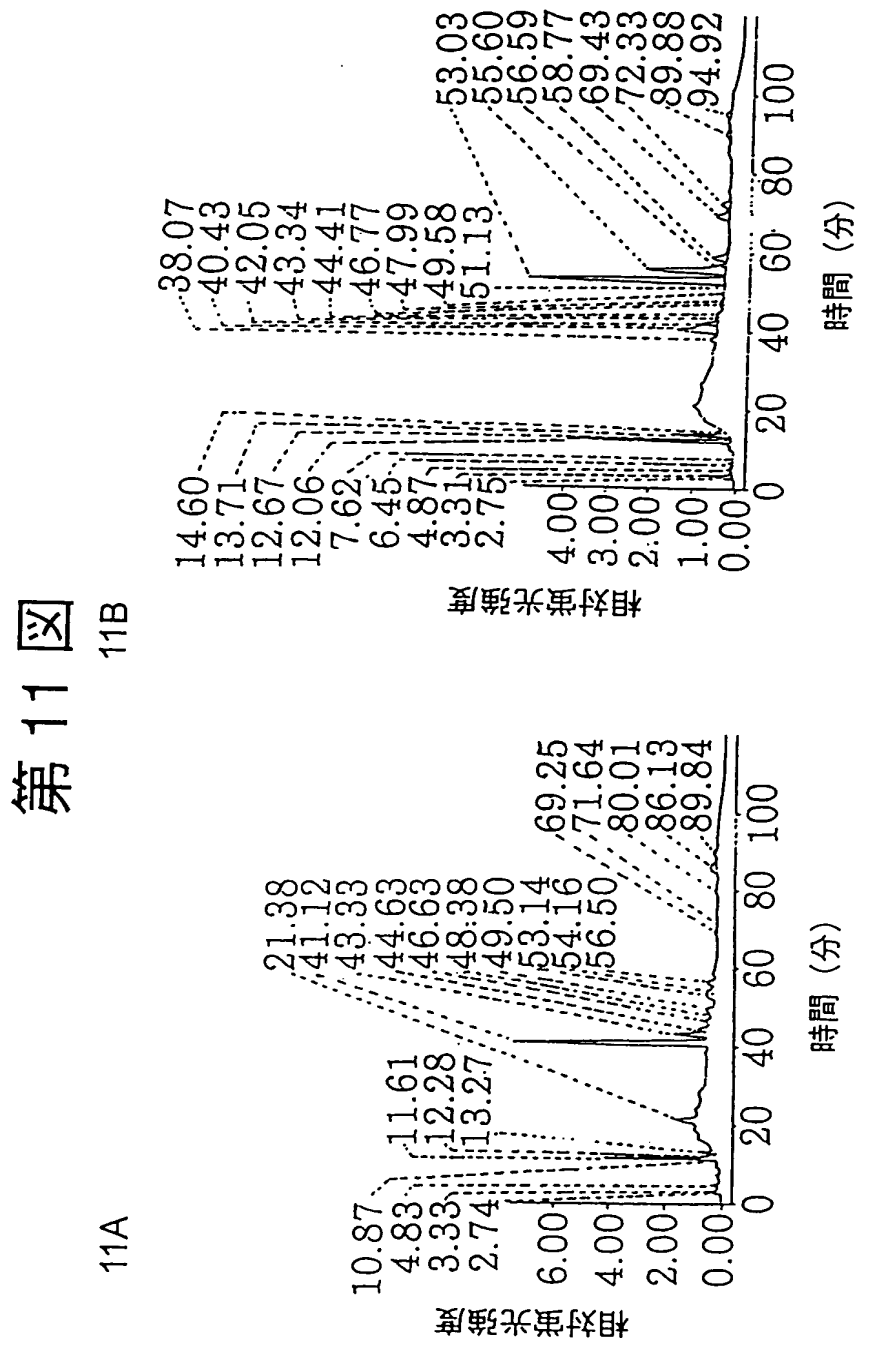

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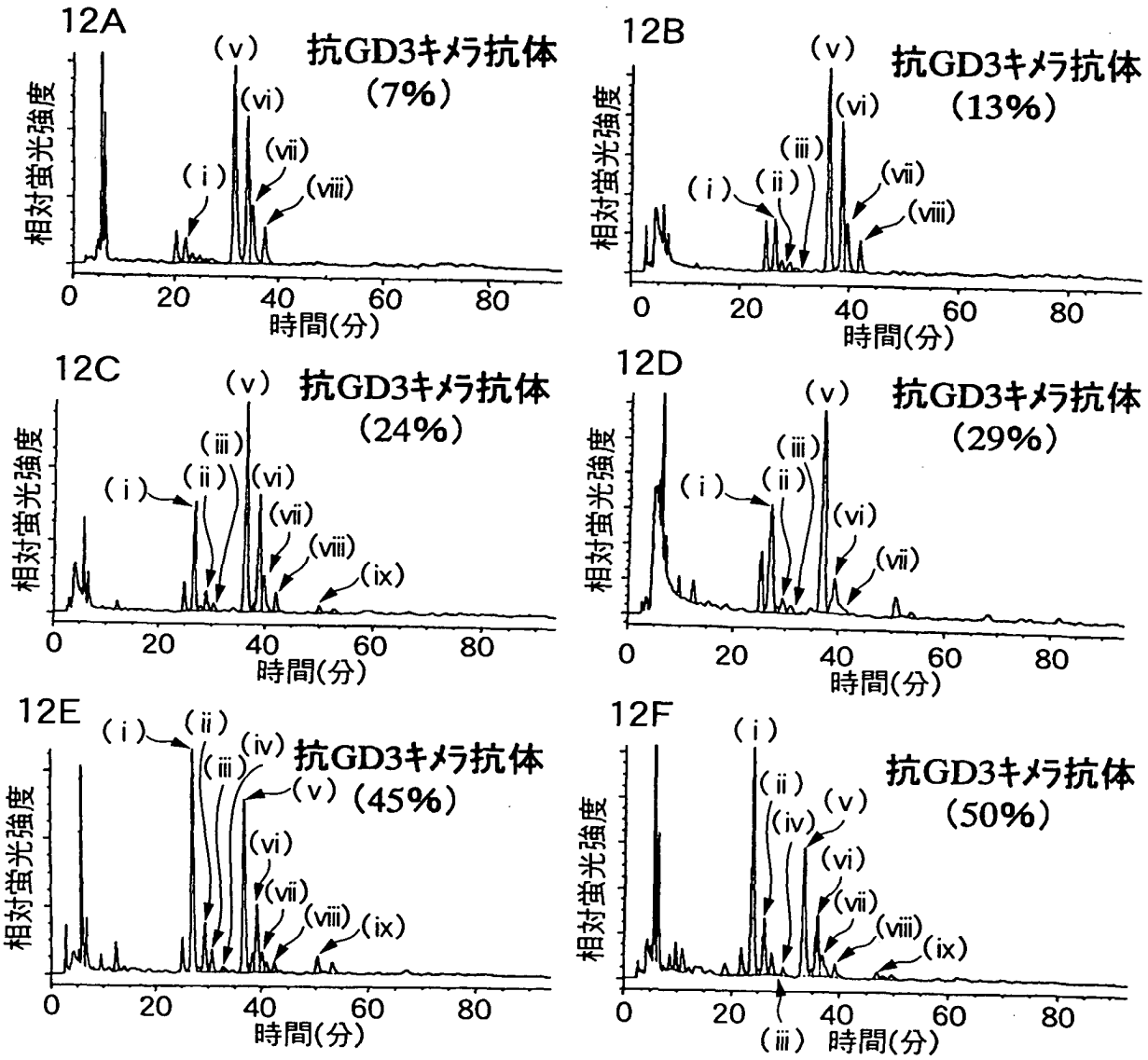

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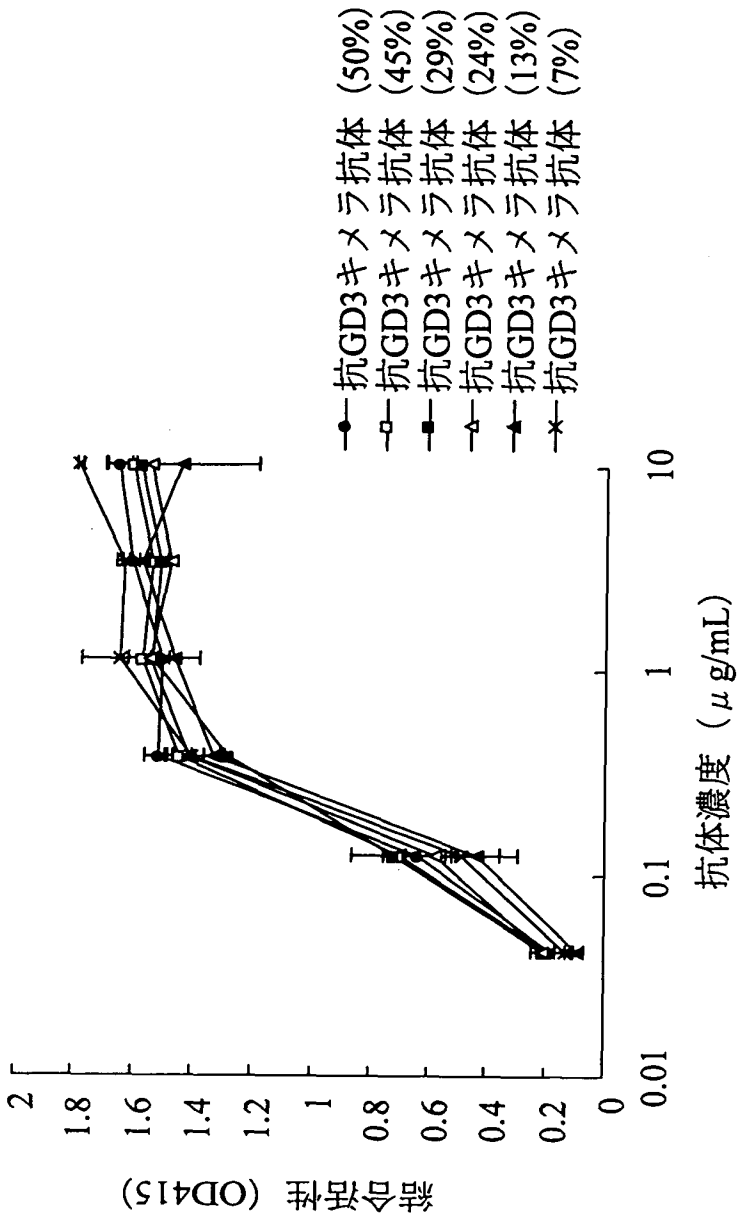

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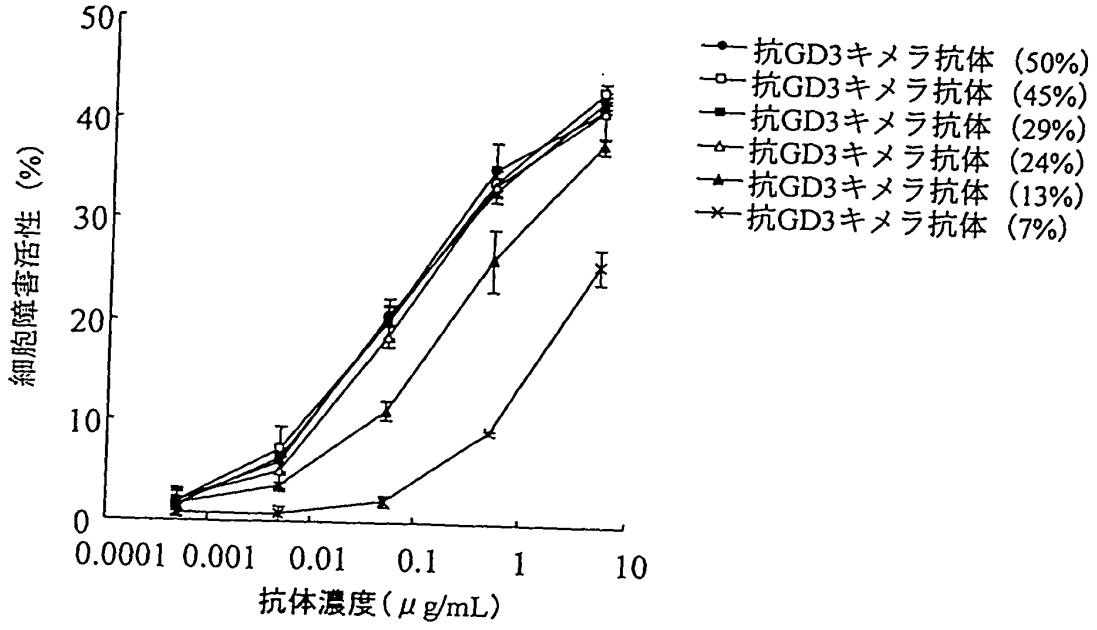

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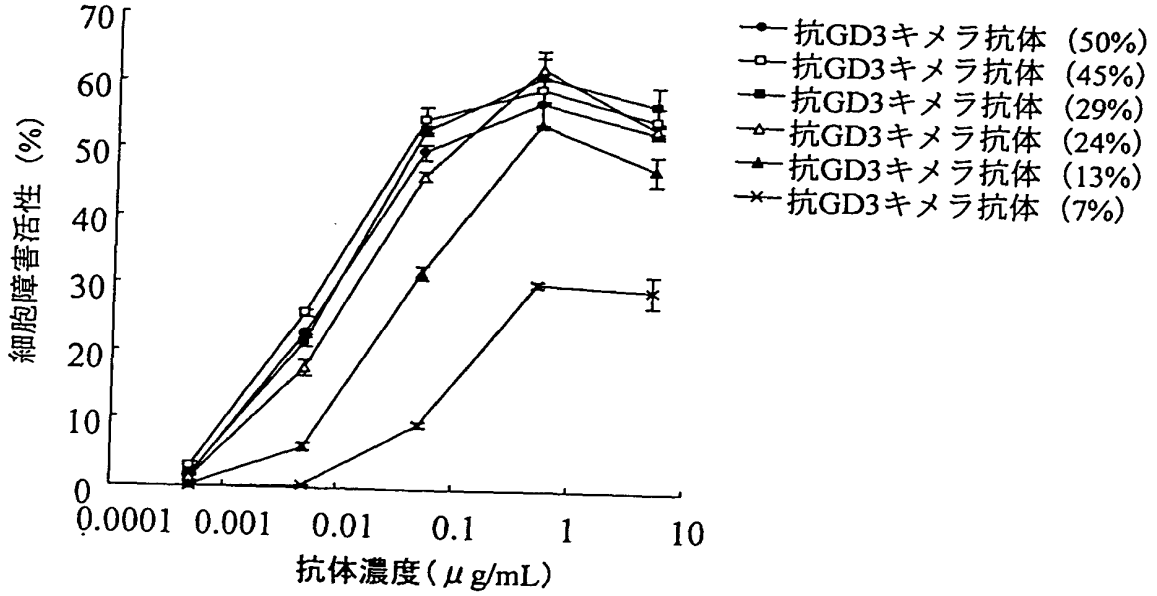

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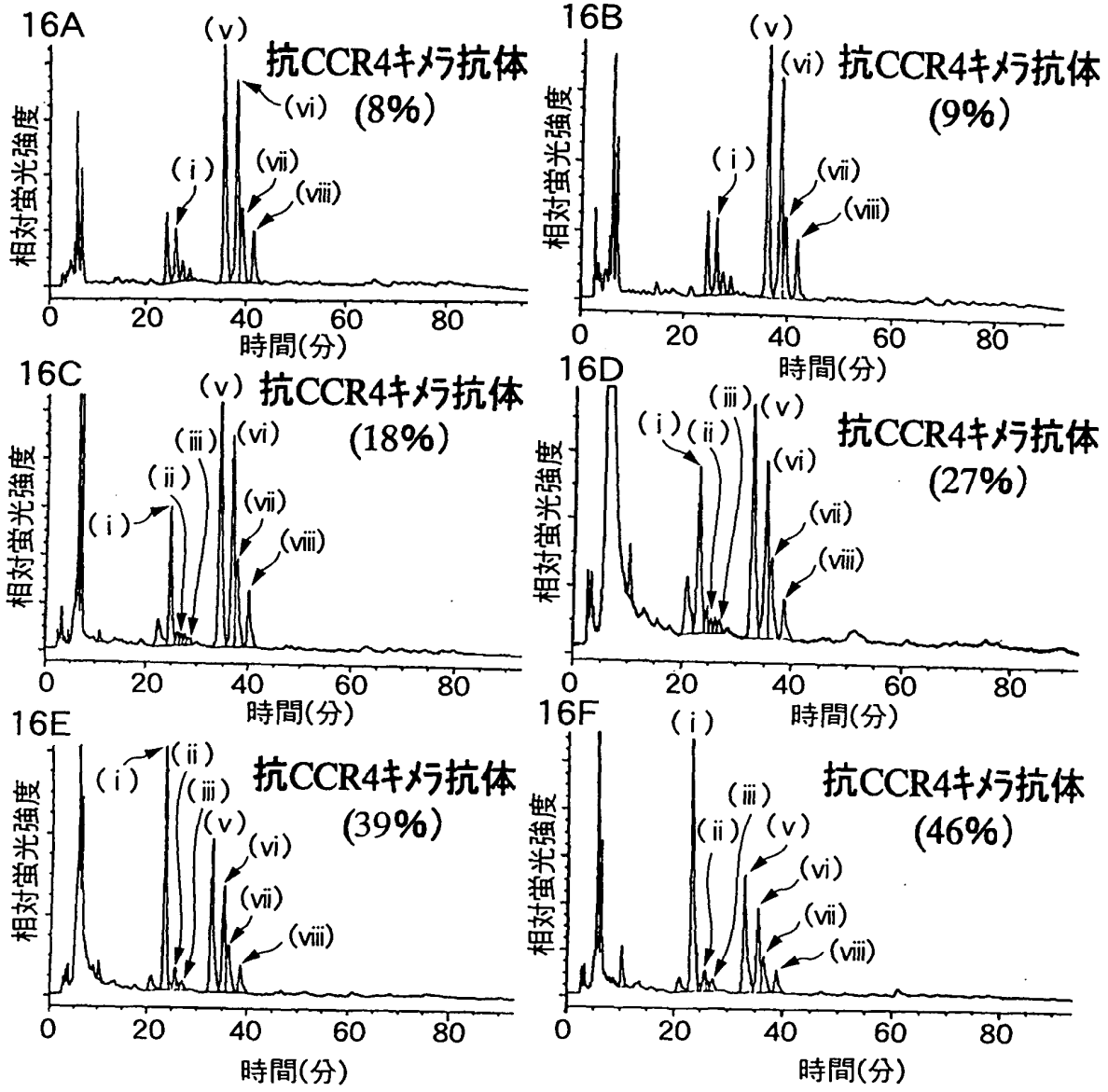

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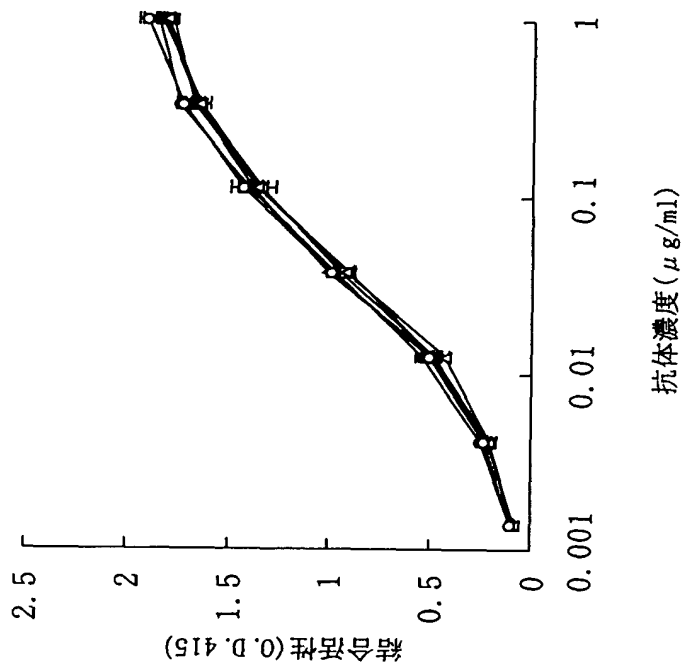

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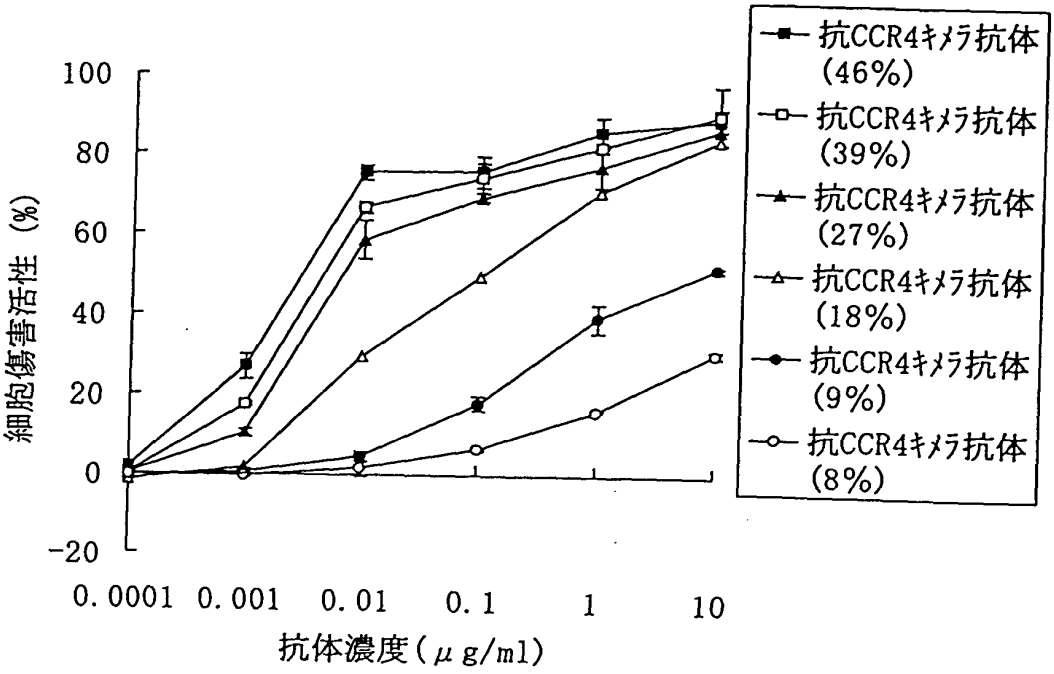

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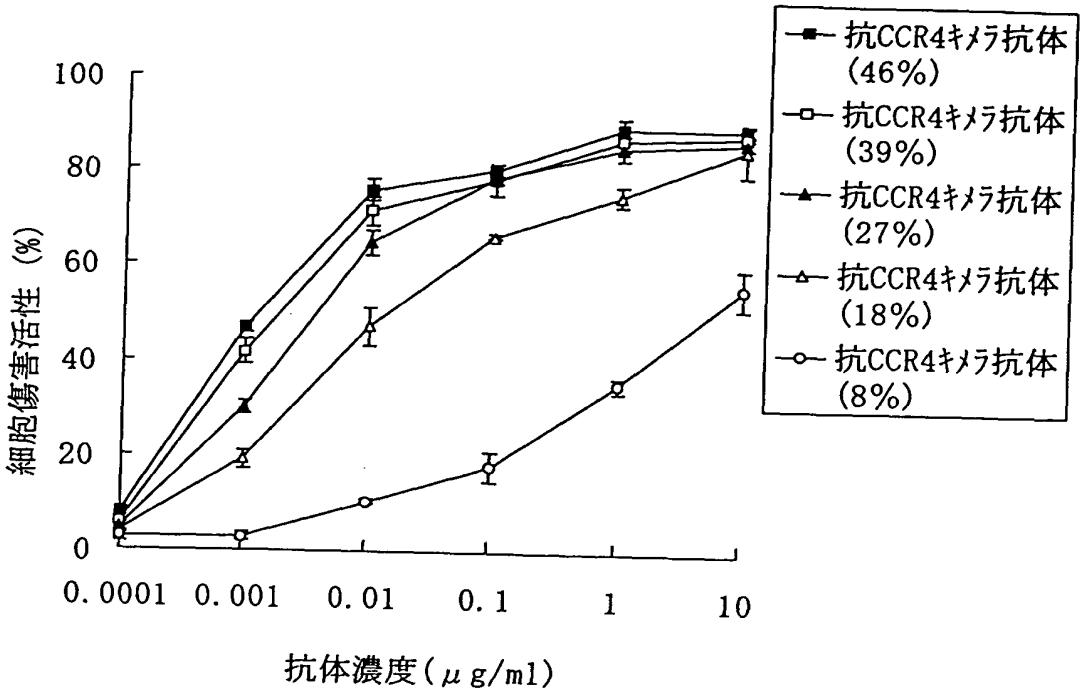

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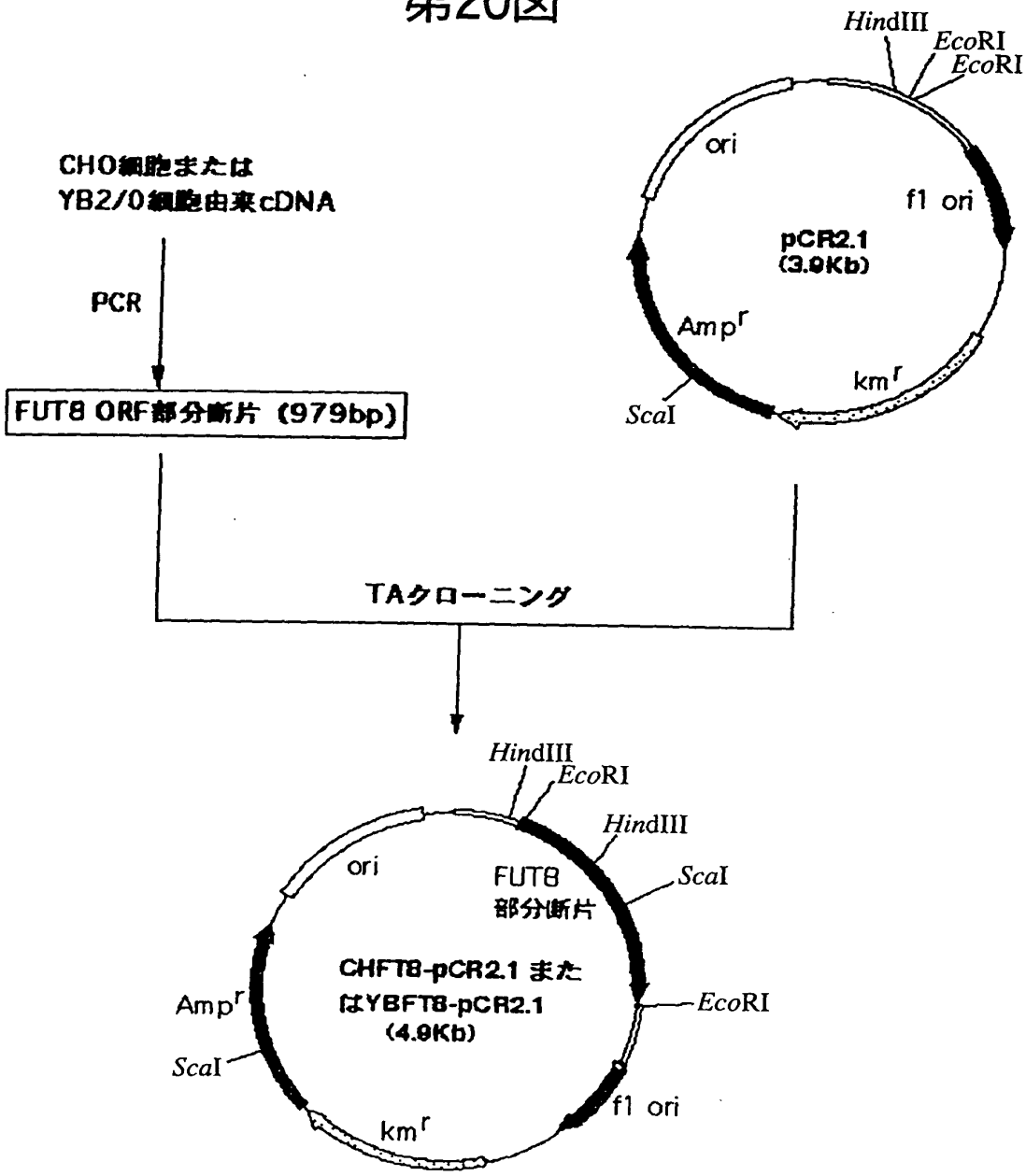

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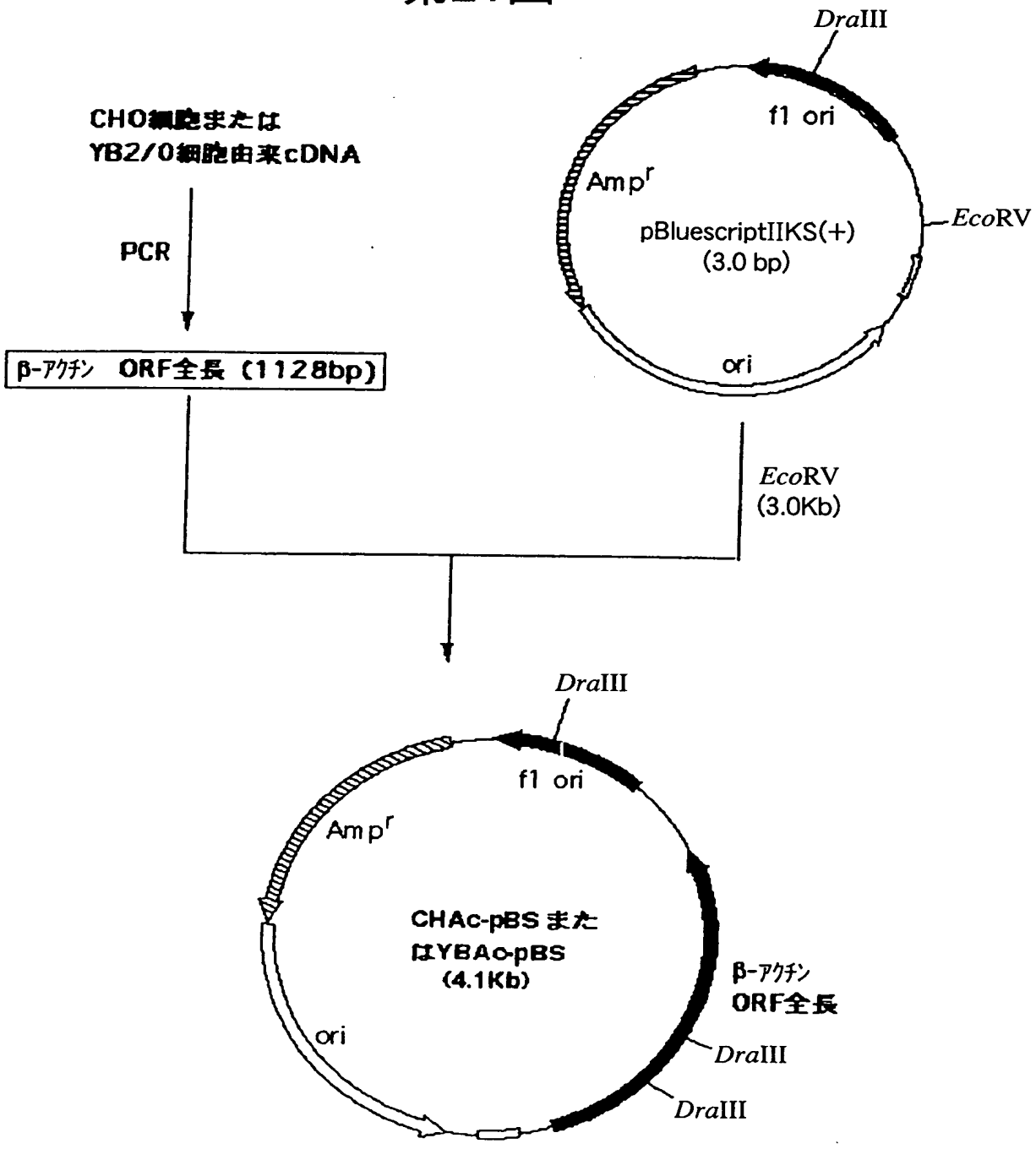

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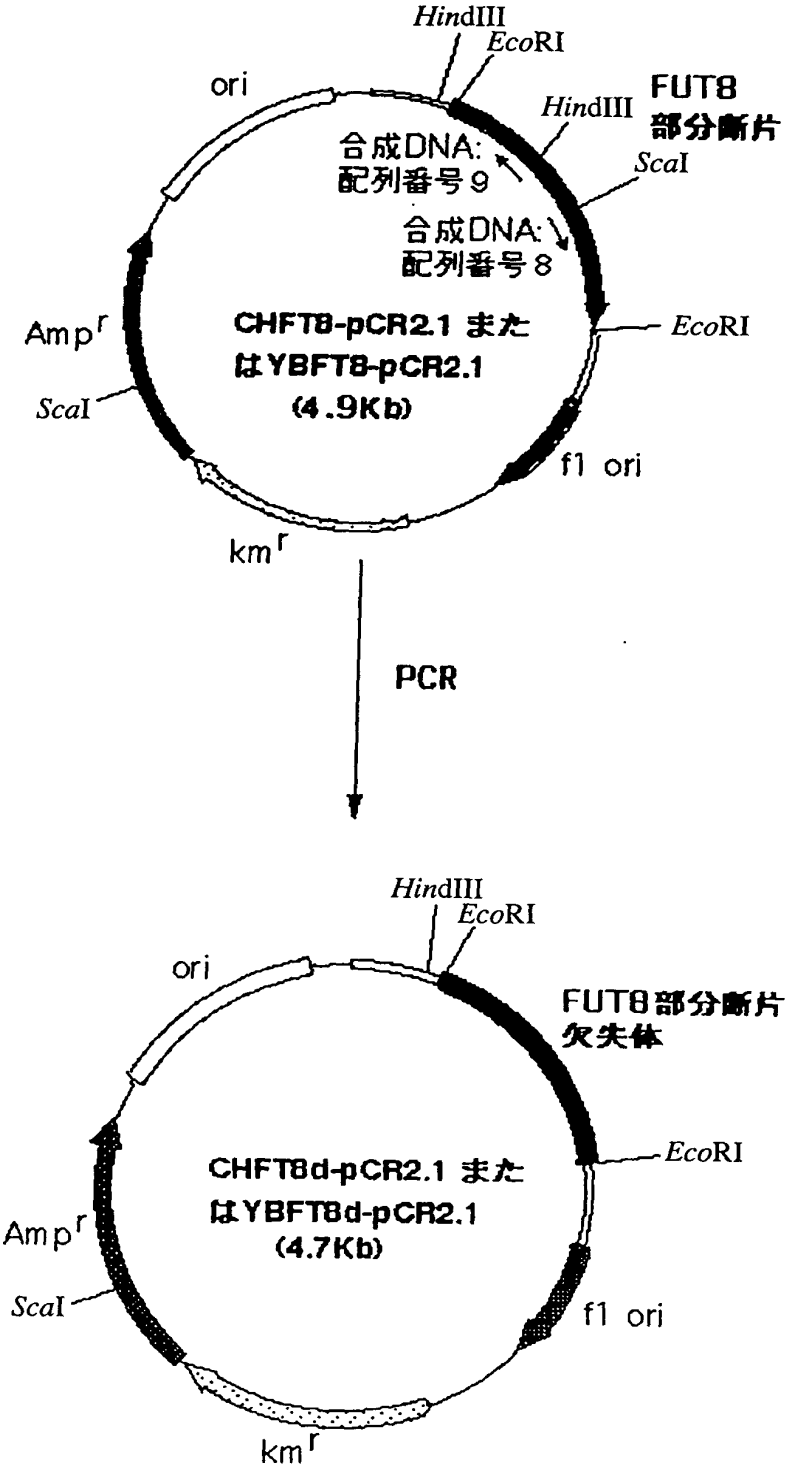

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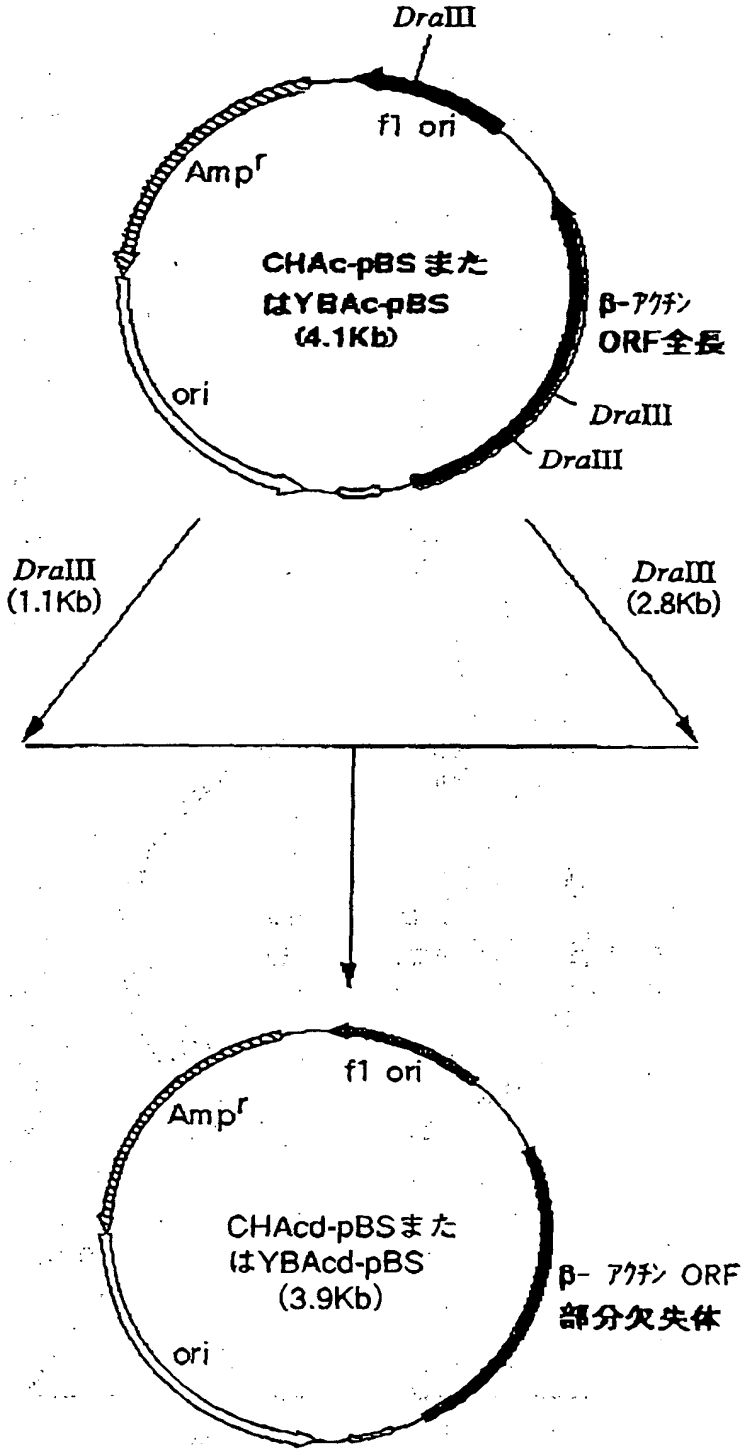

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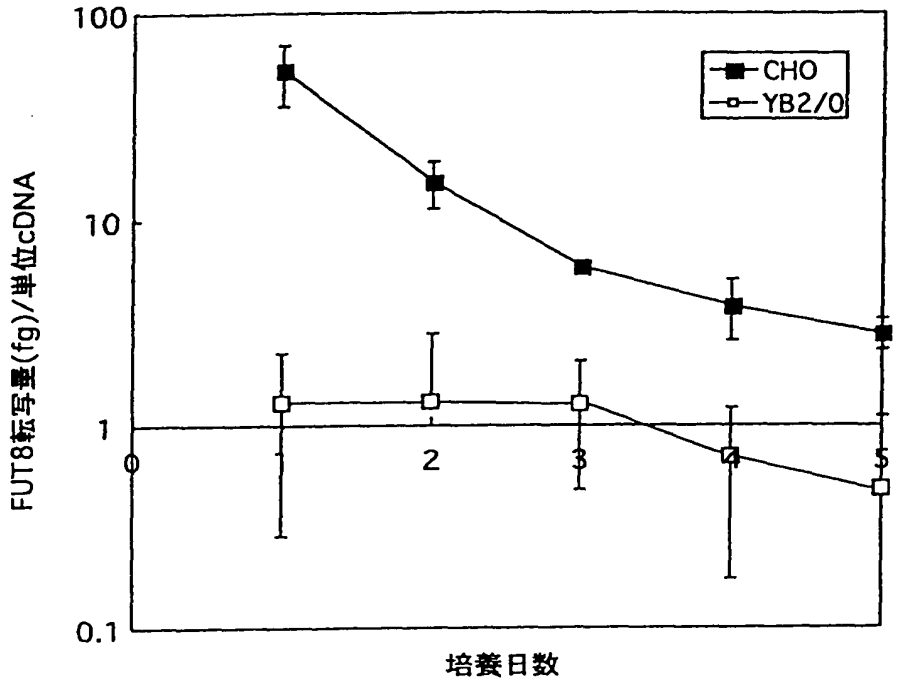

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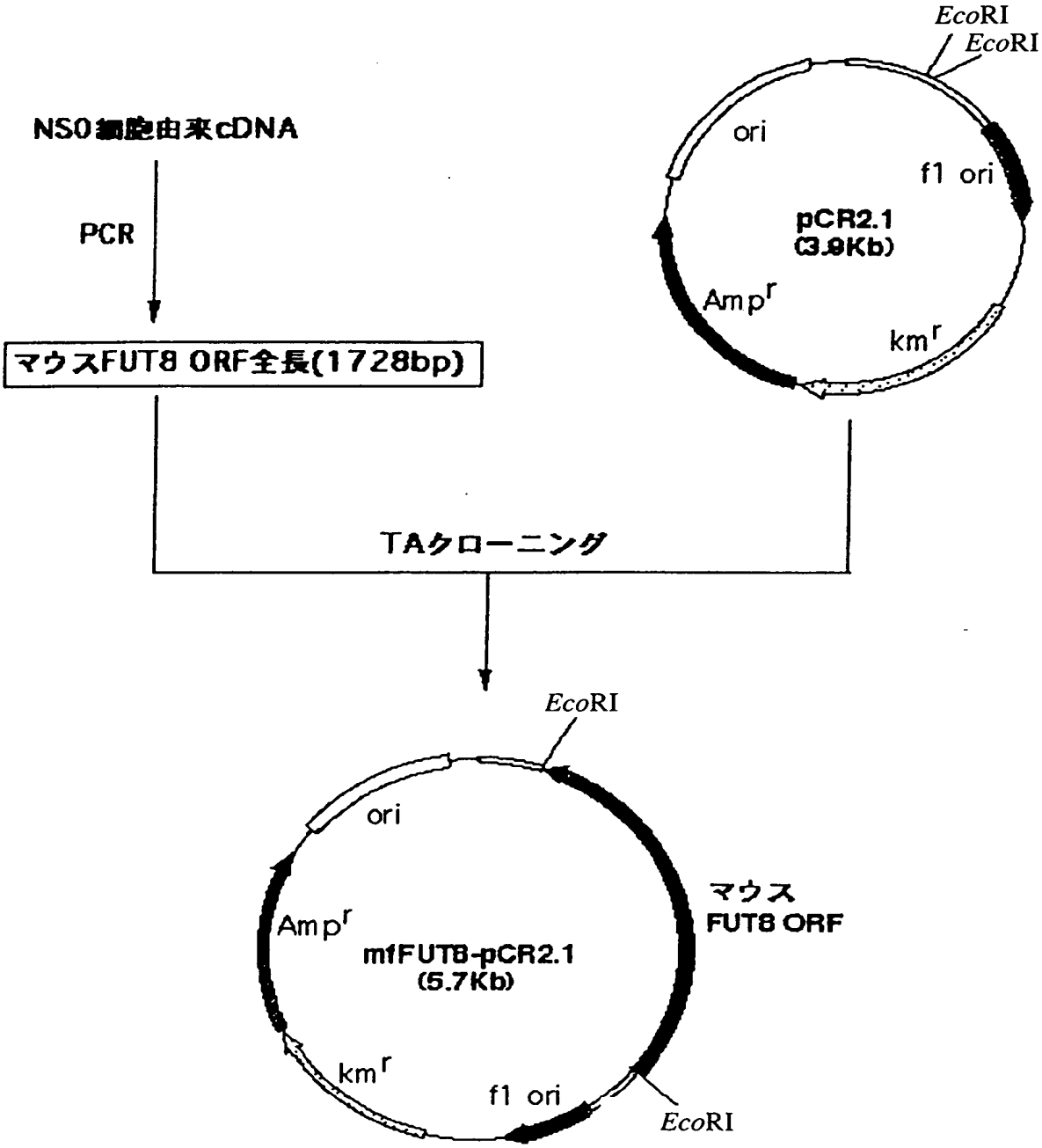

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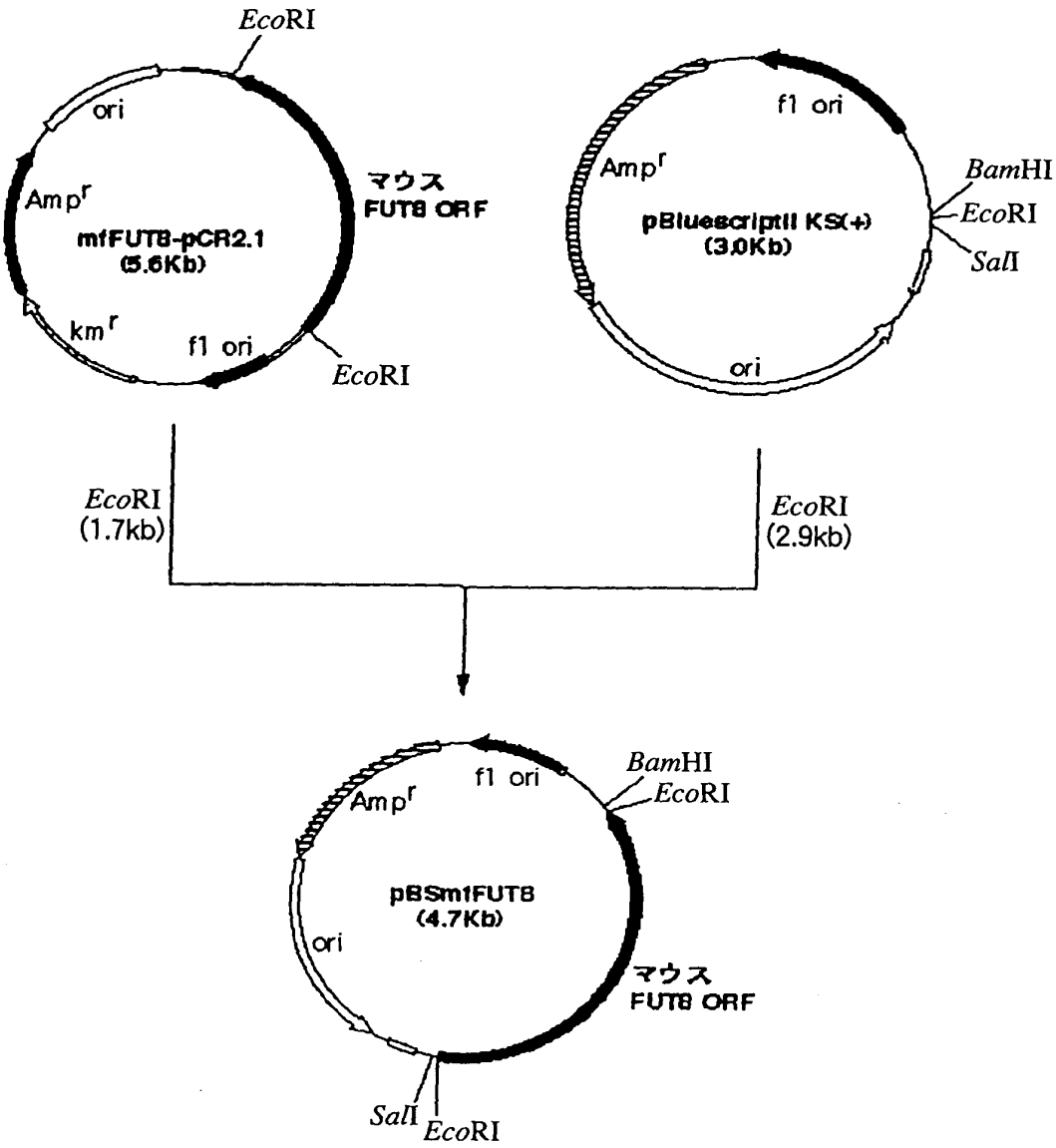

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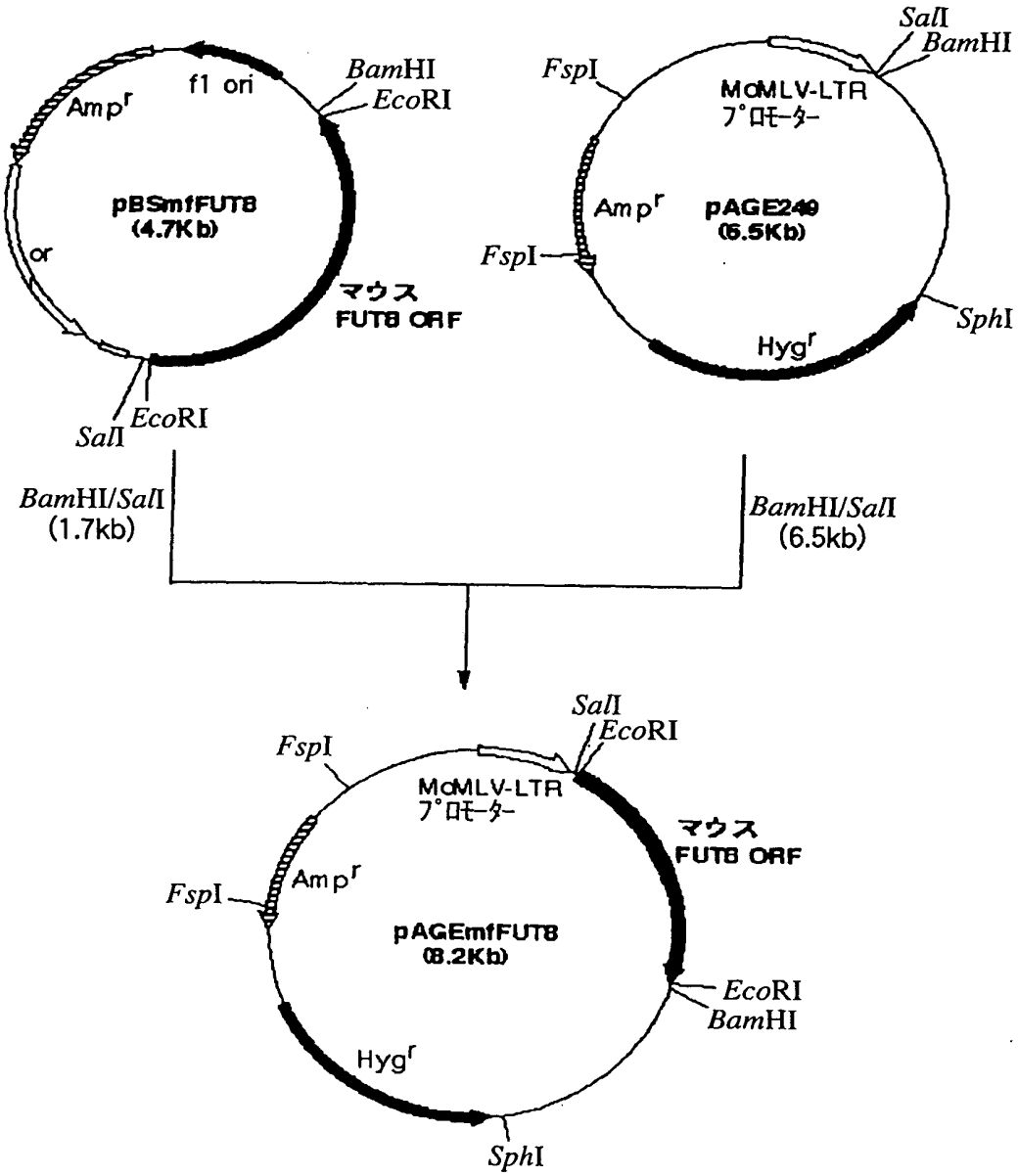

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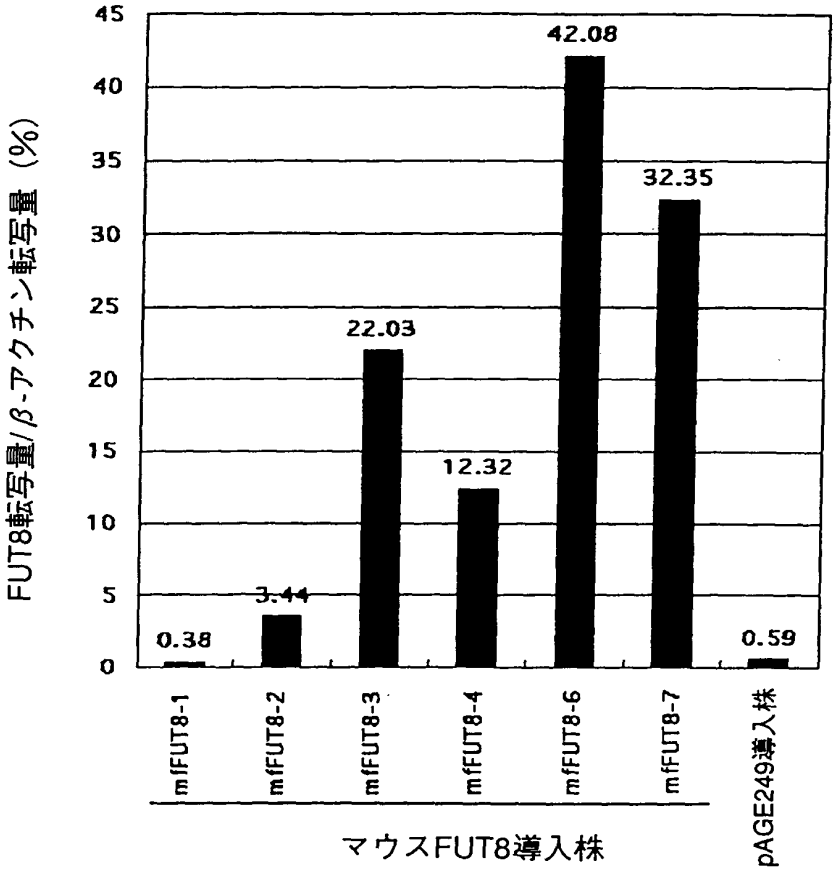
第 28 図



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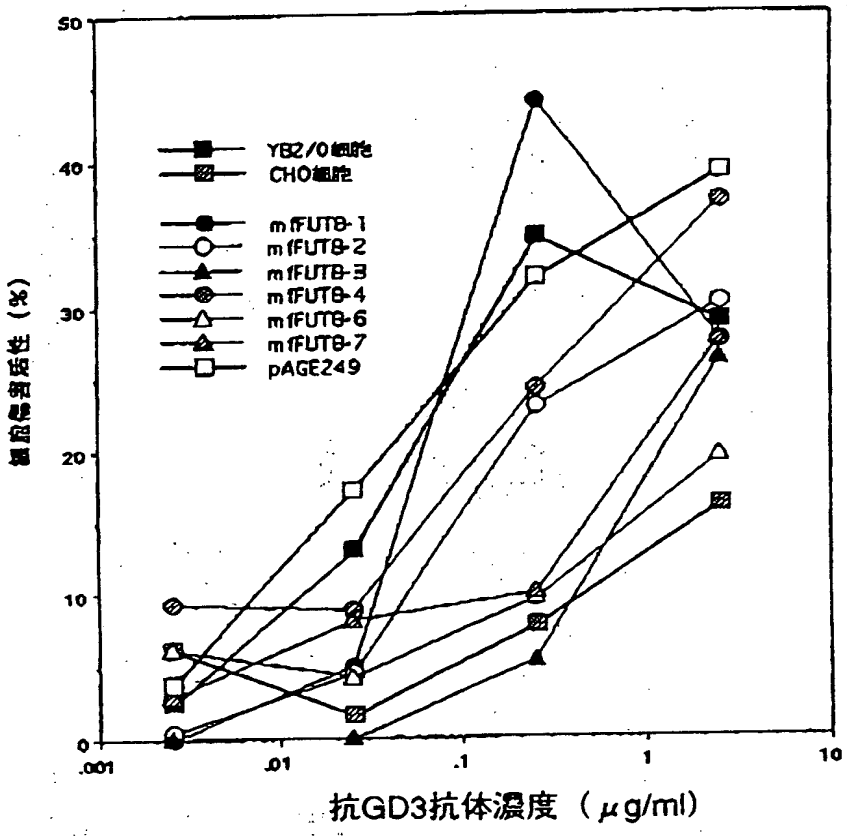
第 29 図



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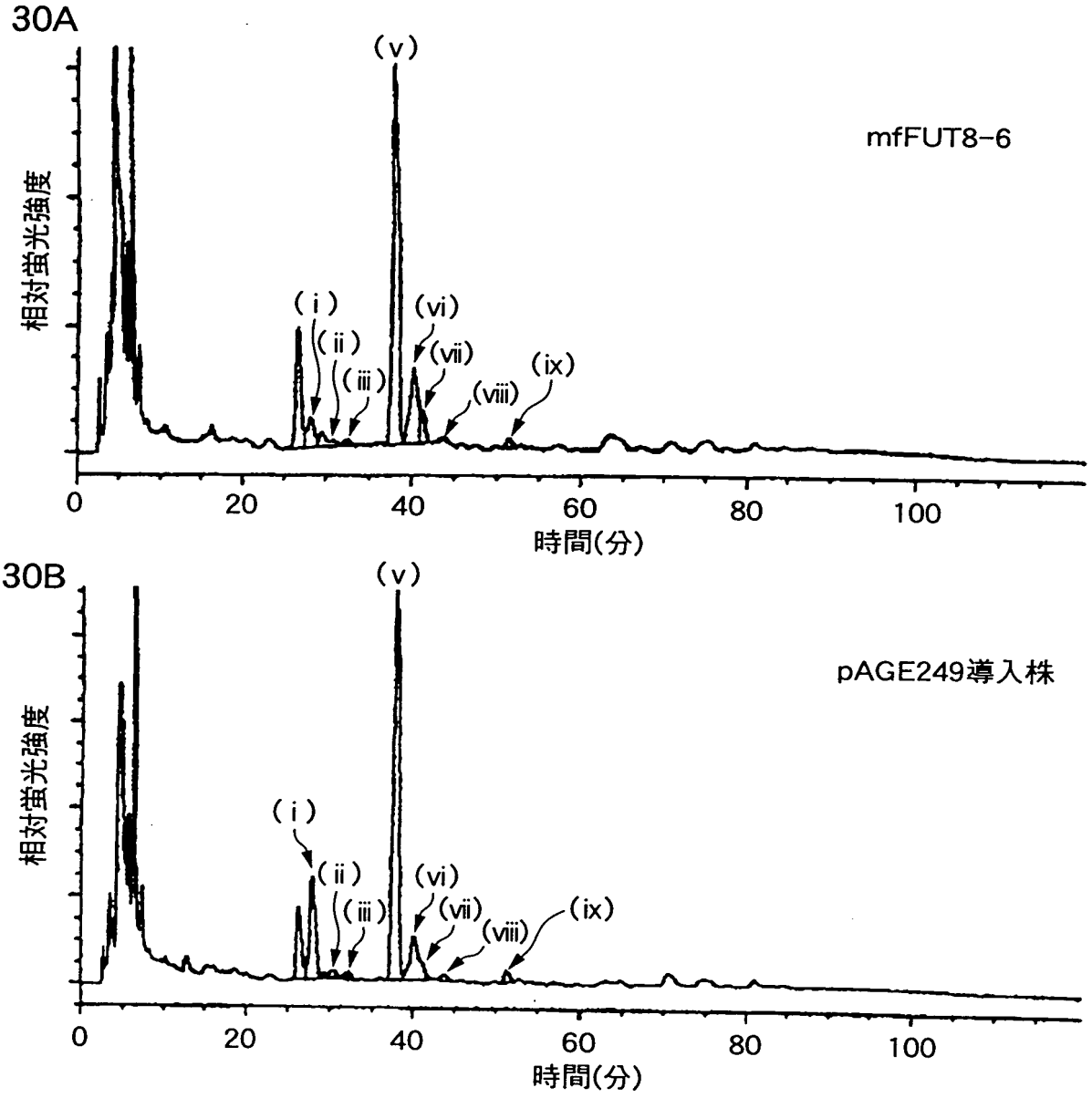

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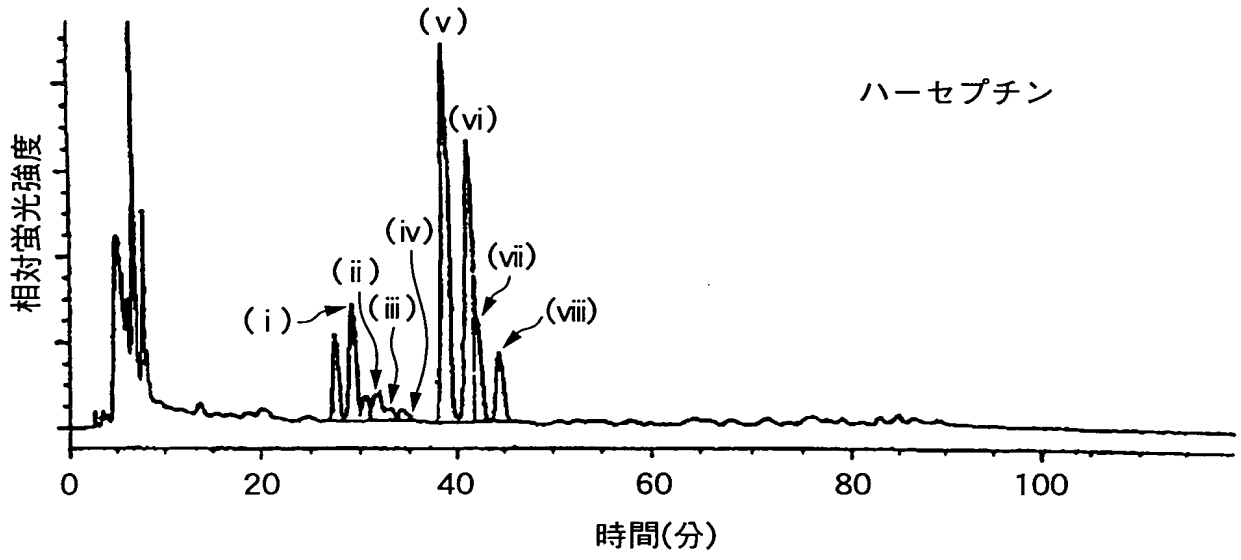

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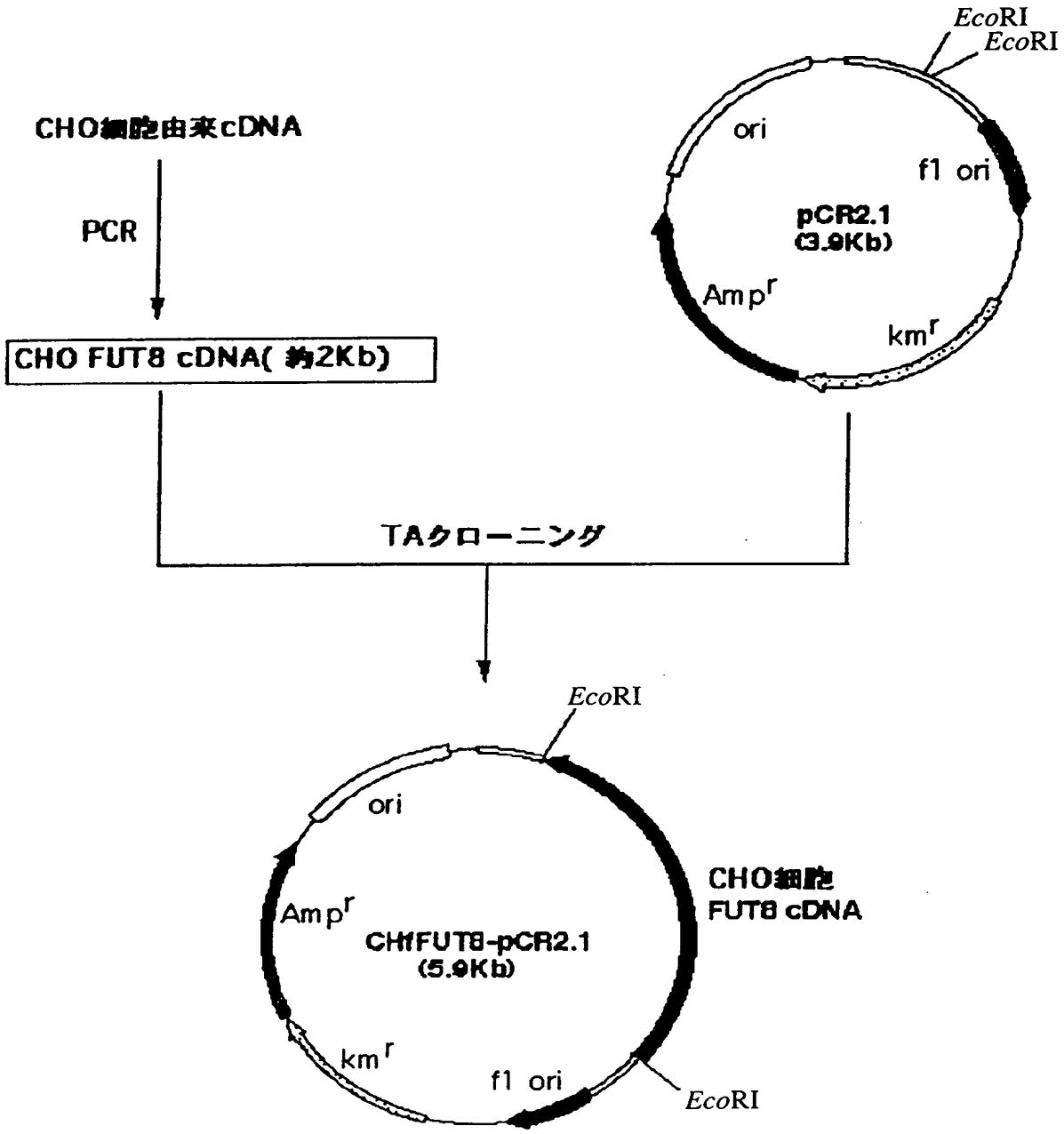

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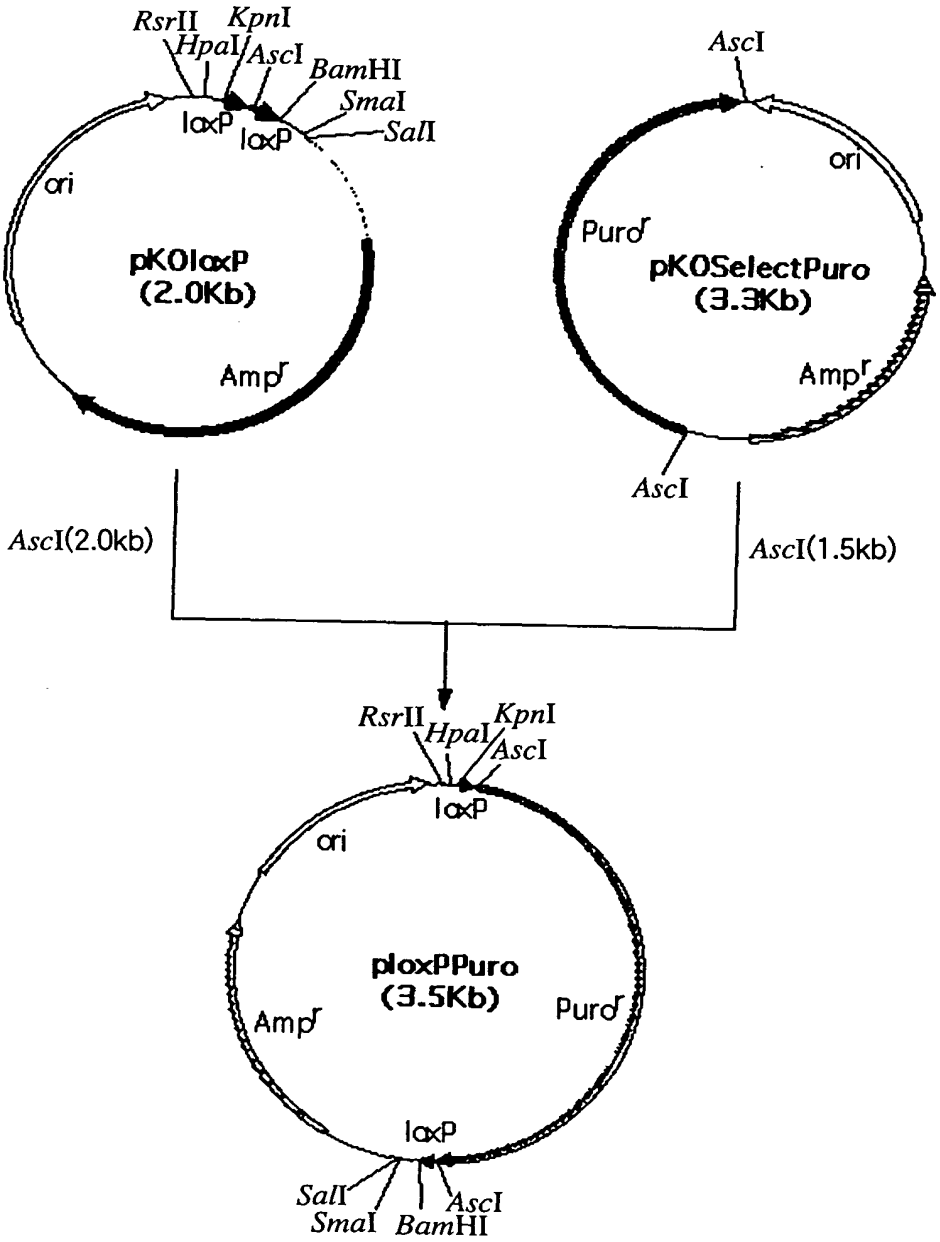

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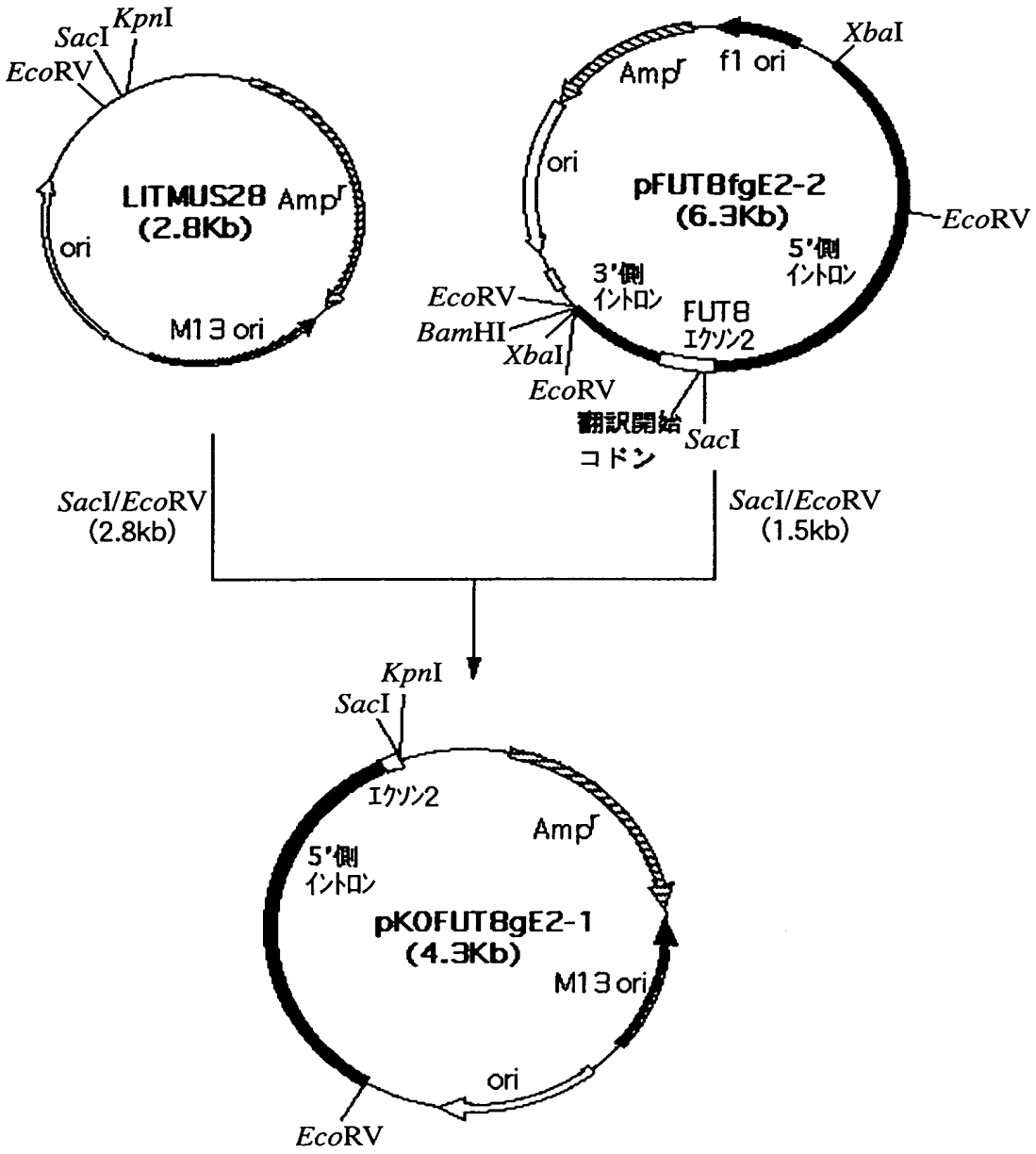

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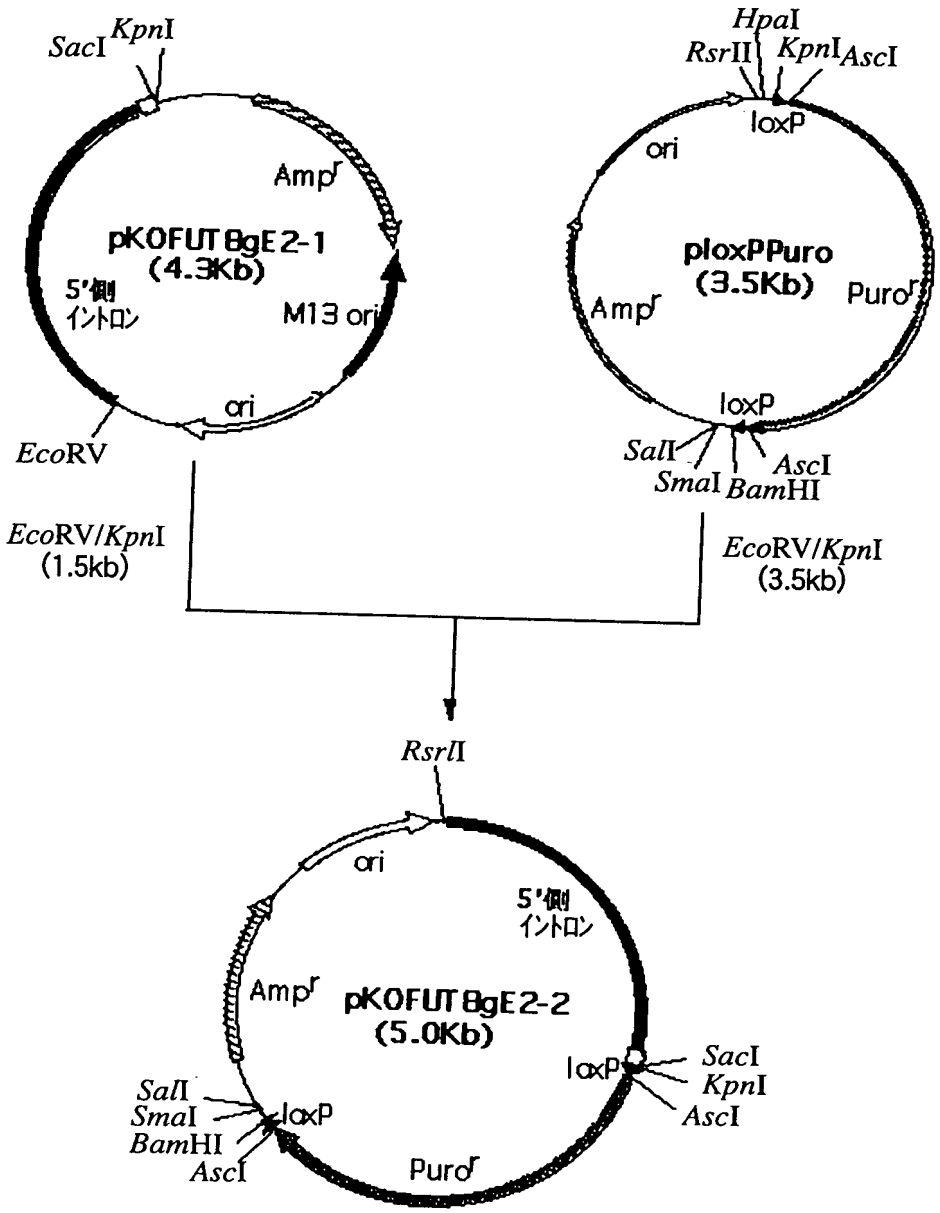

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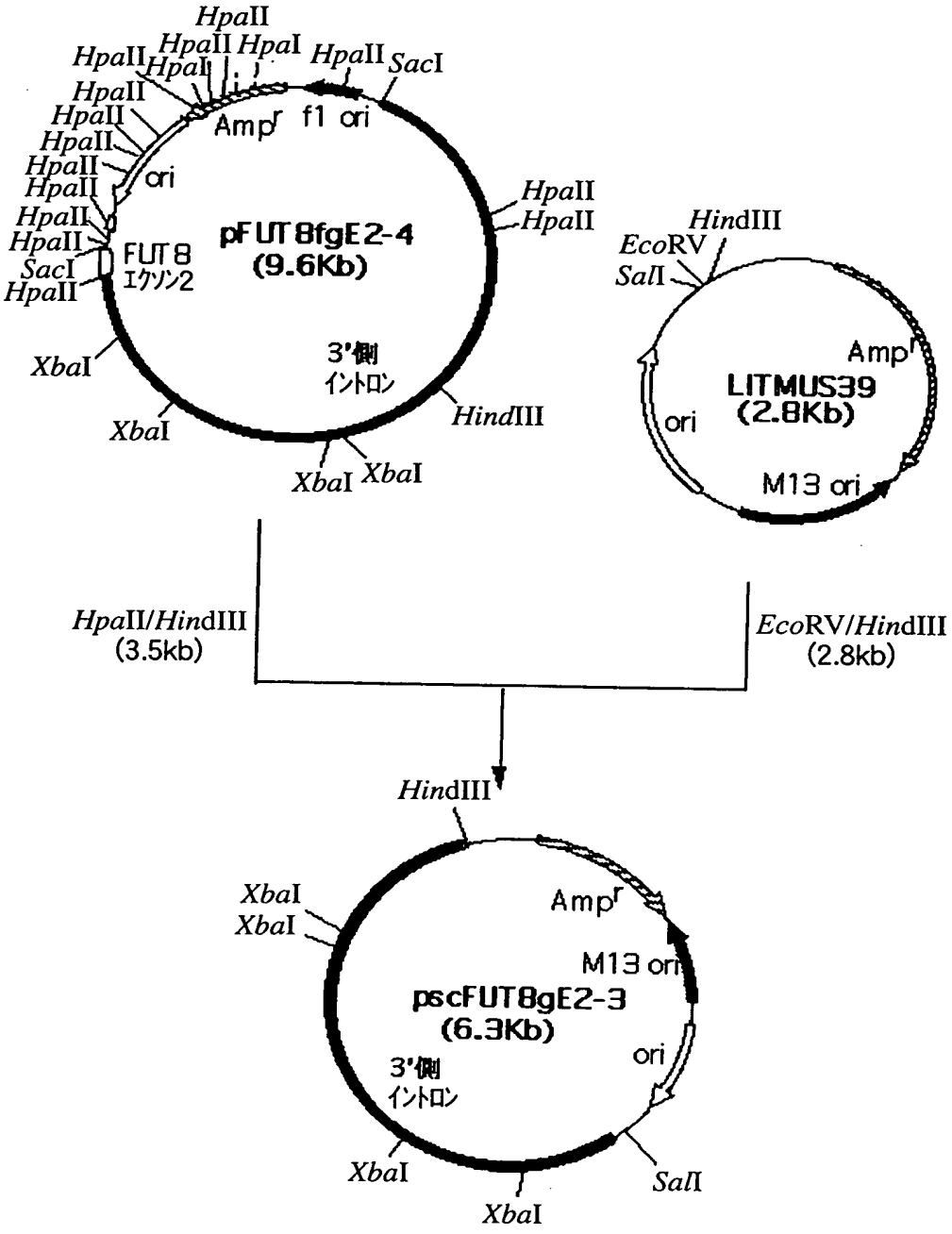
第36図



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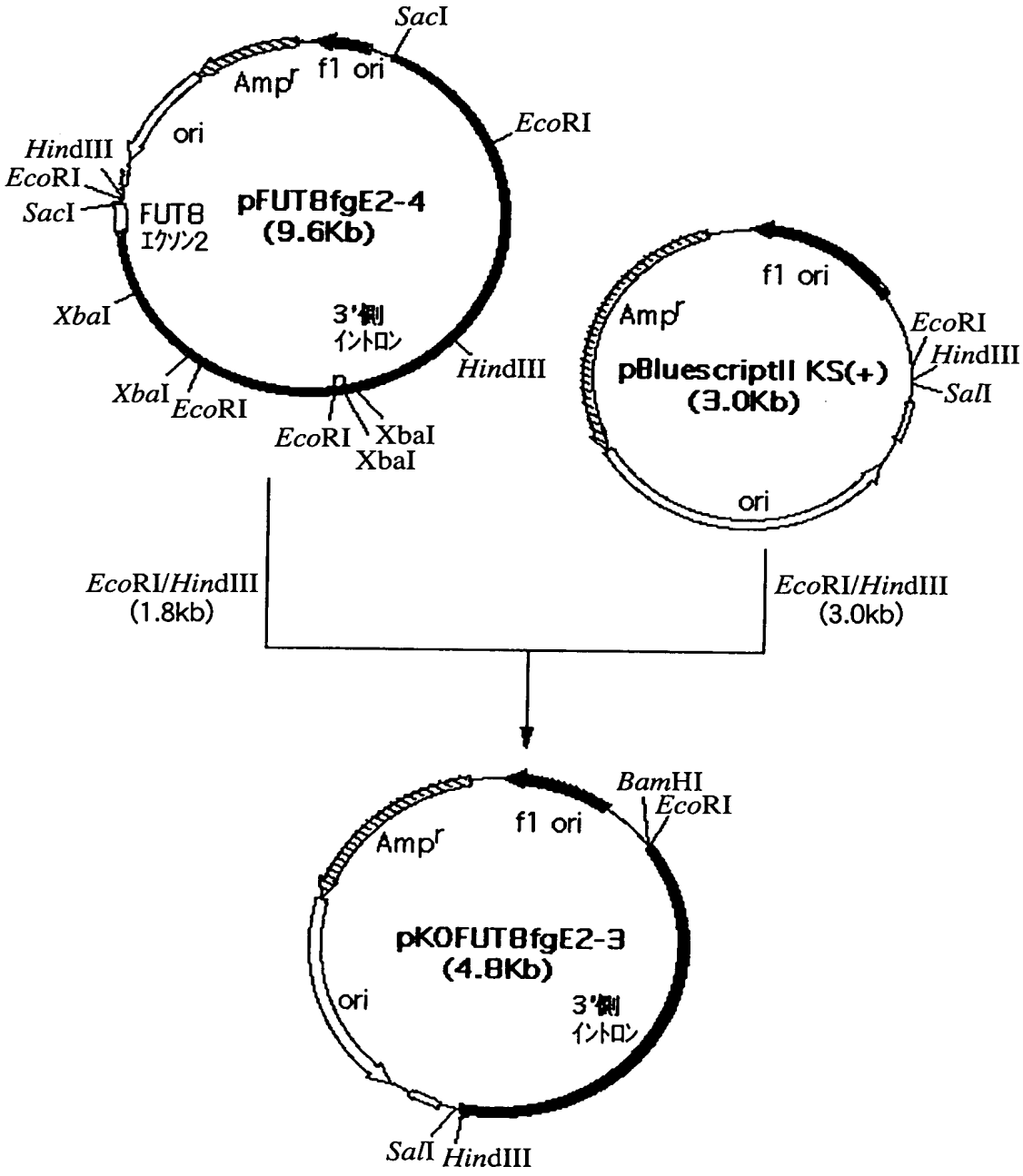

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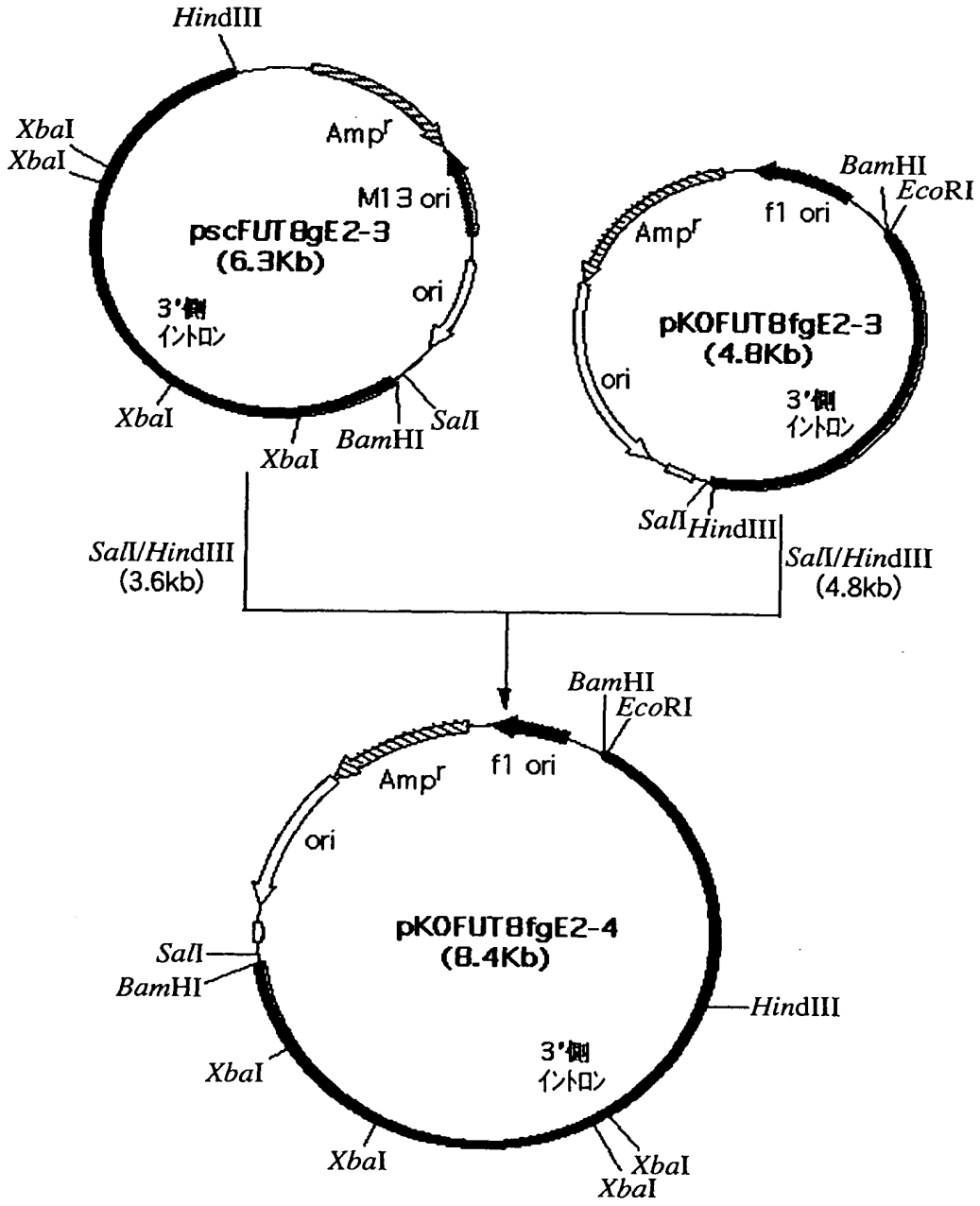

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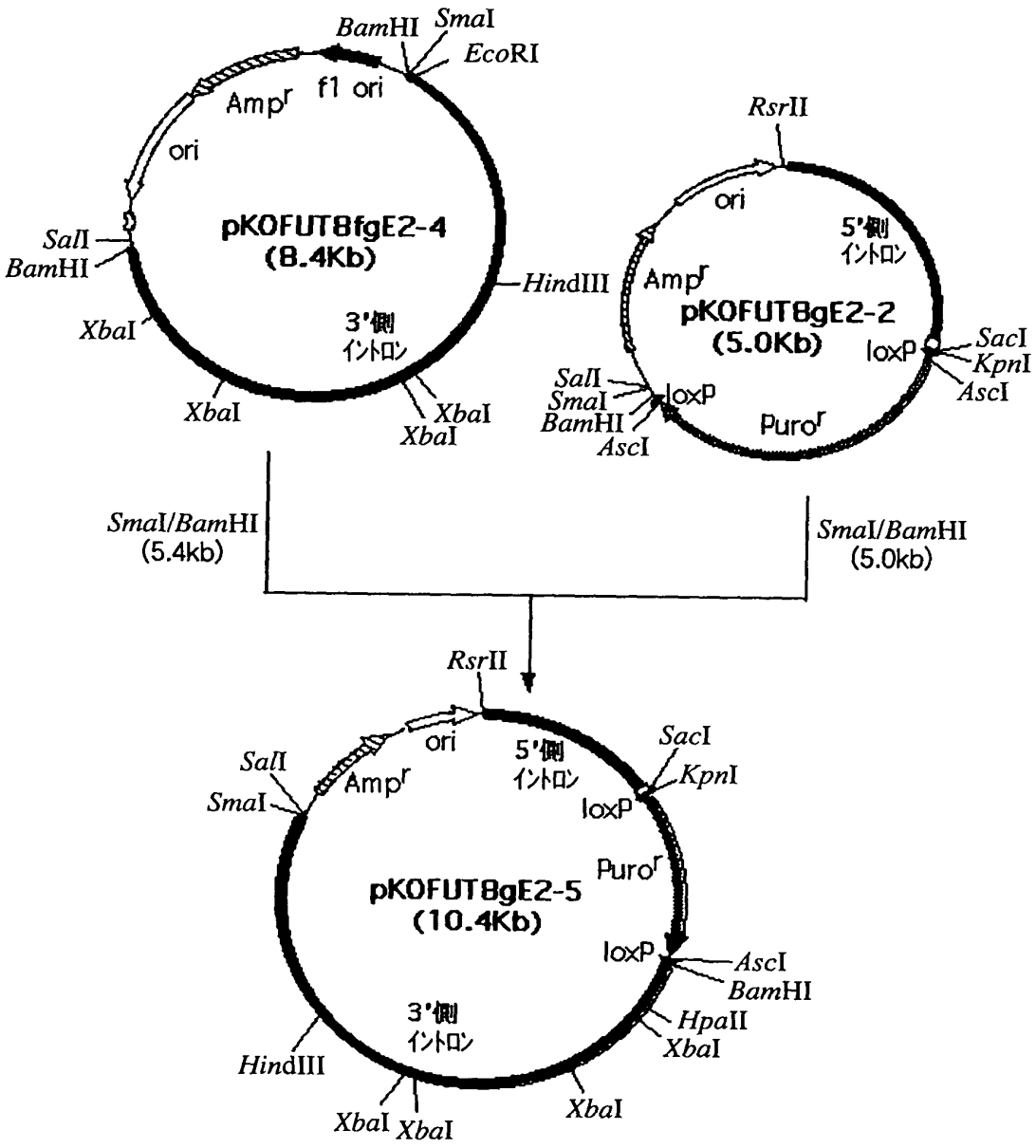

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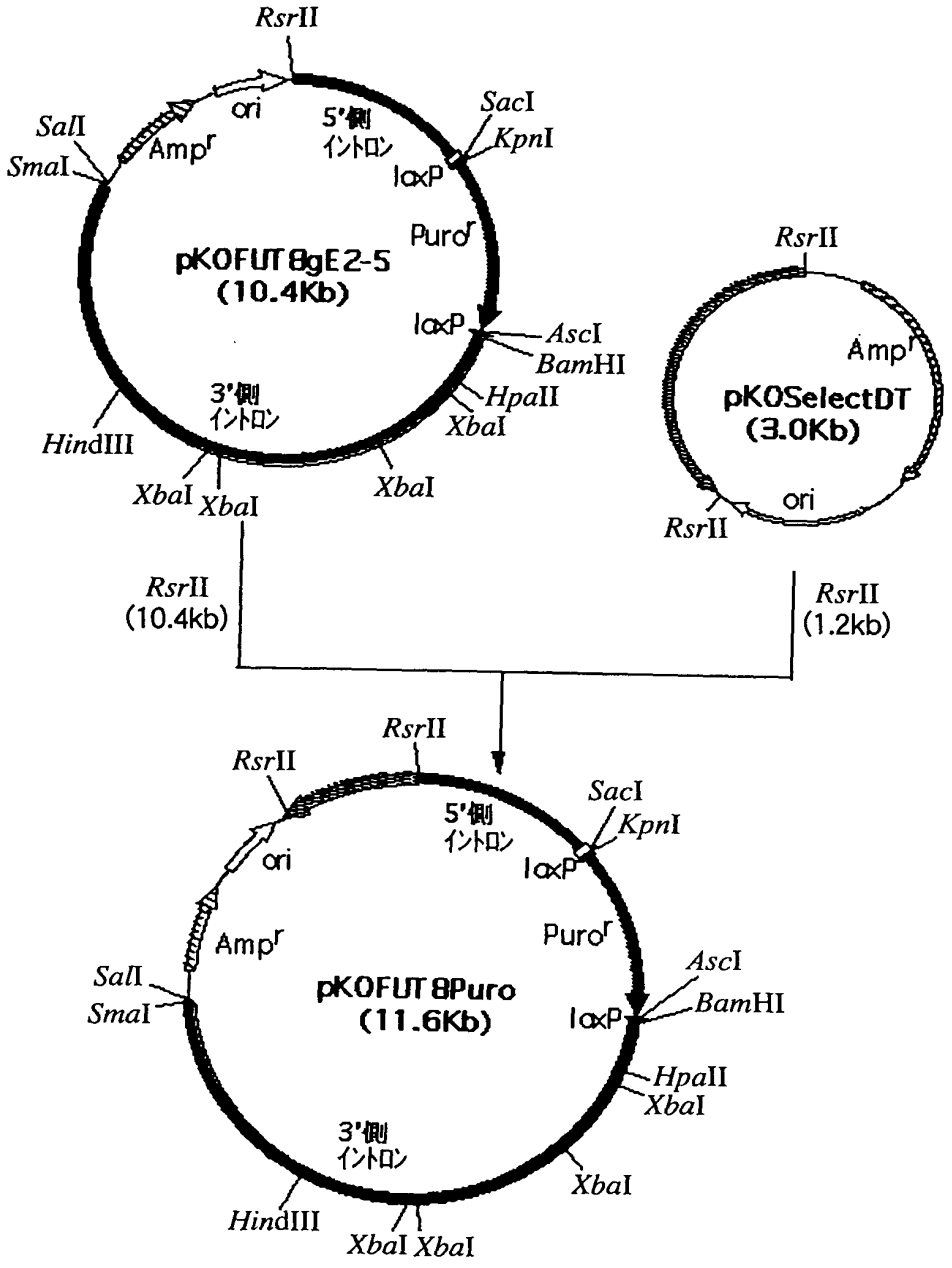

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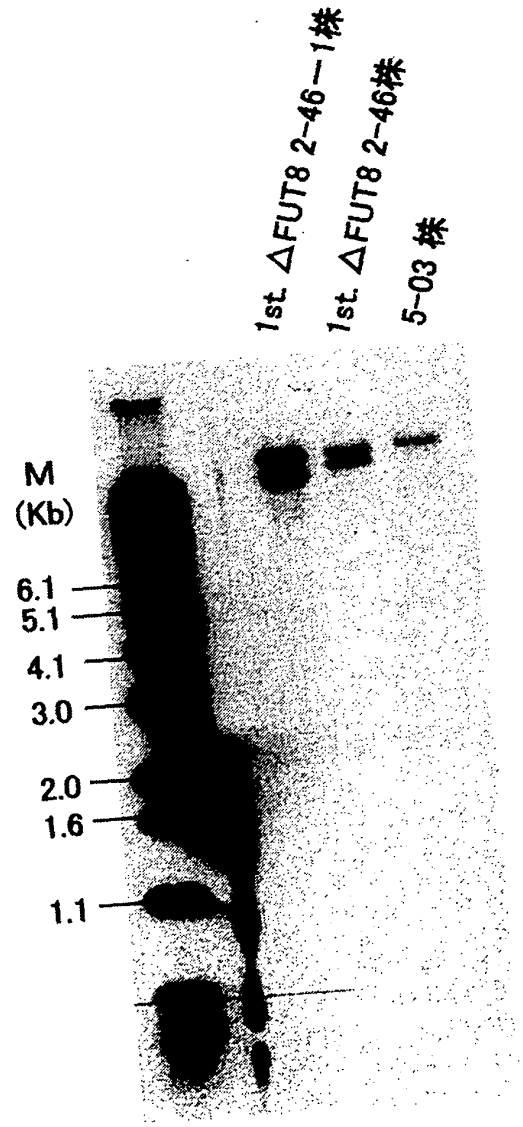

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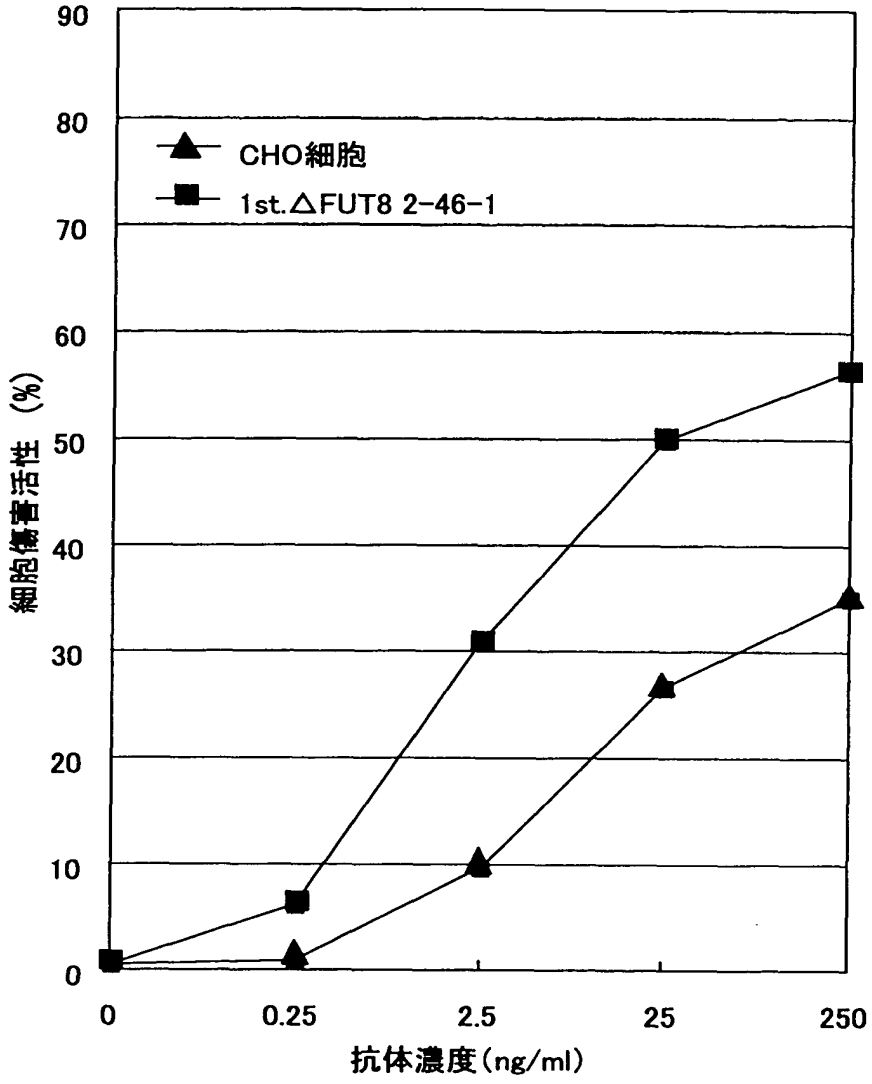

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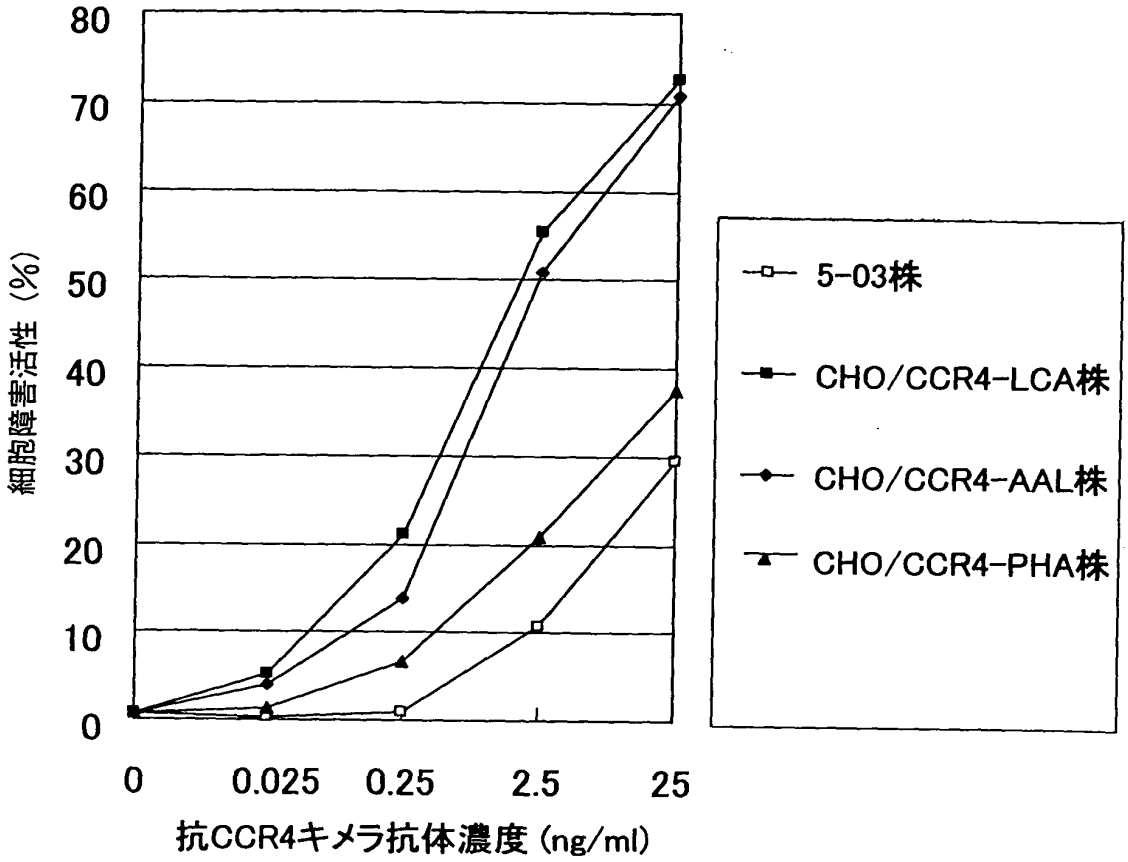

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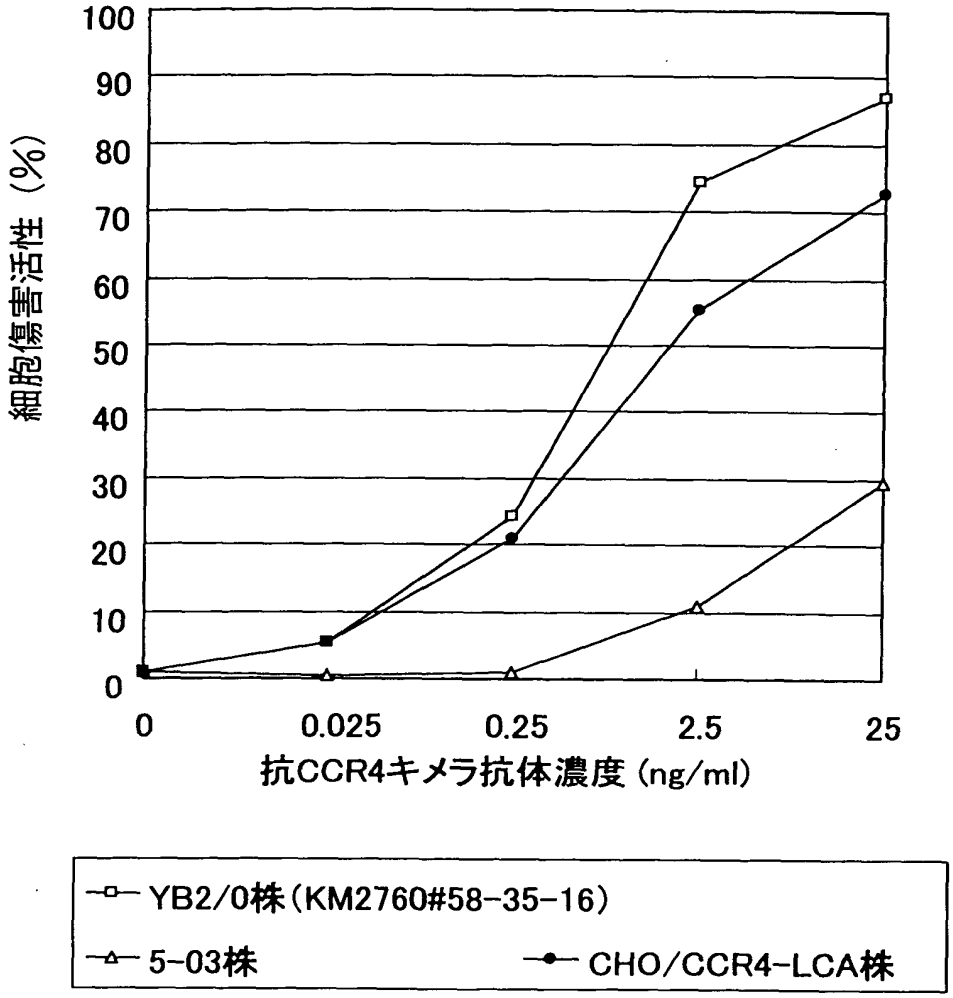

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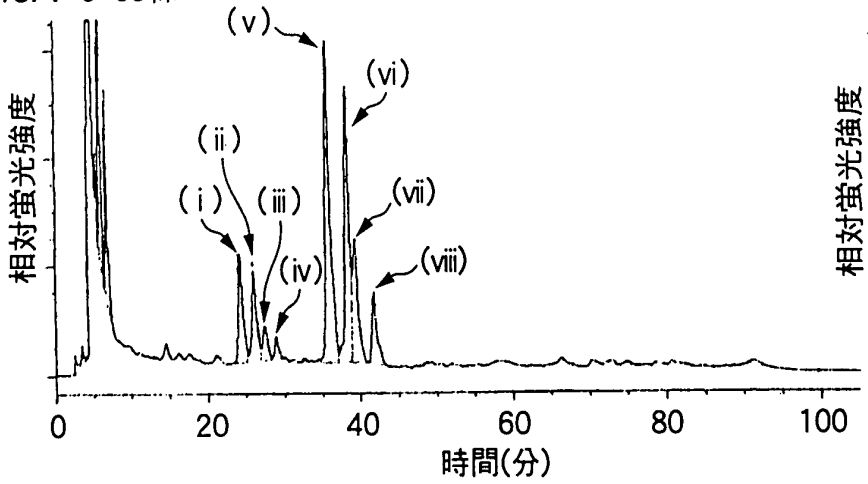
第45図

45A 5-03株



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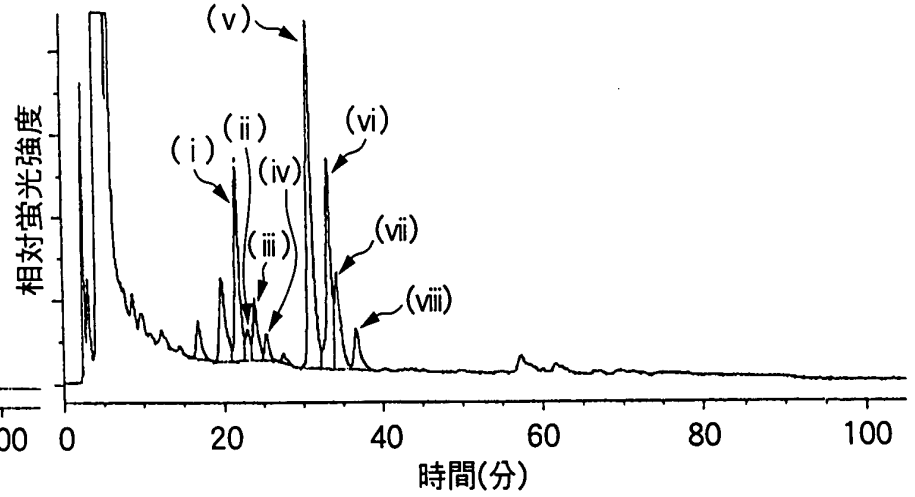

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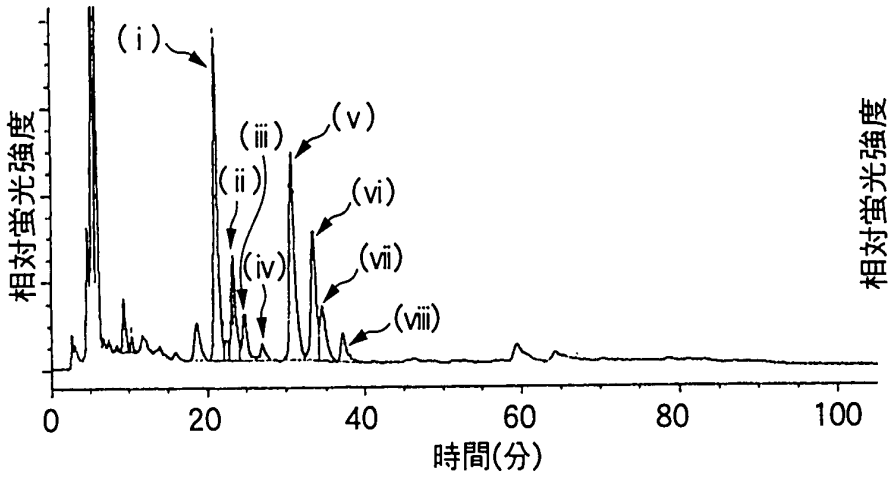

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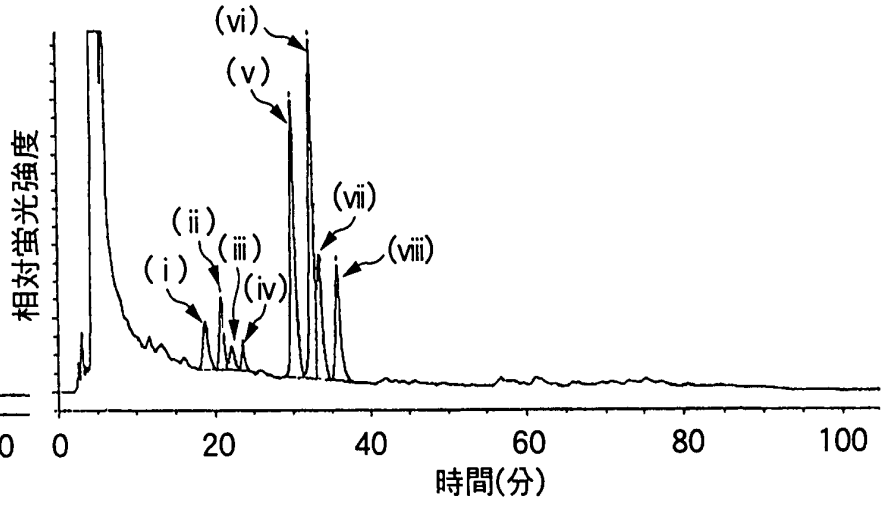

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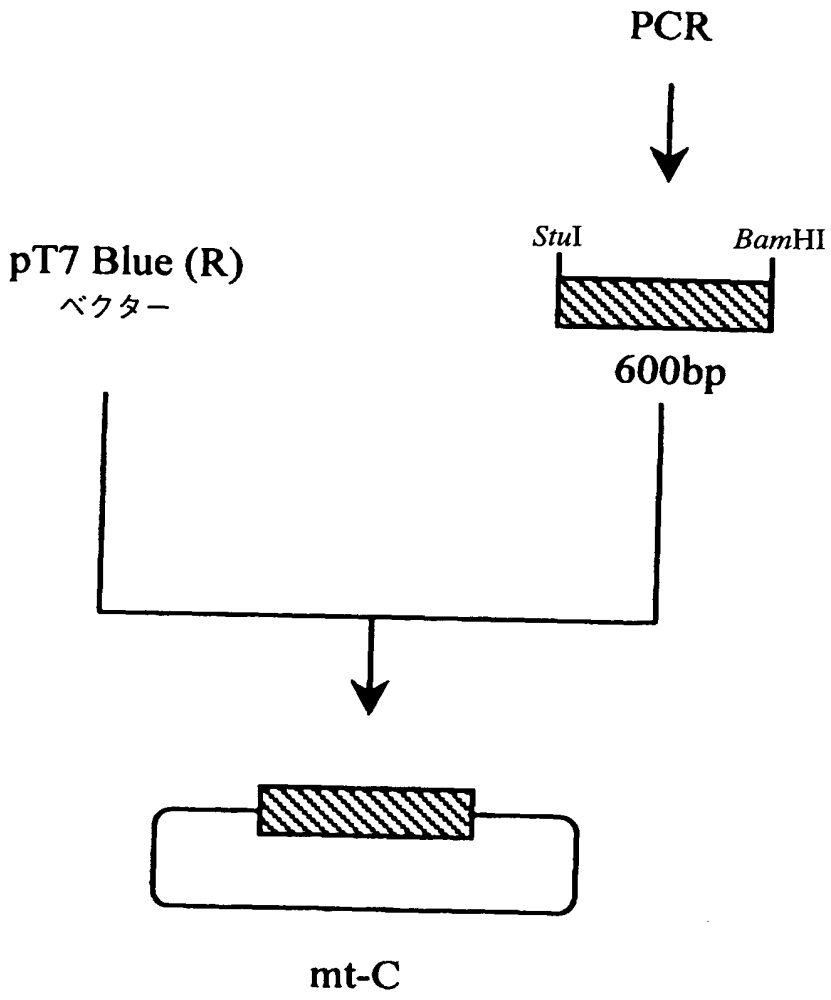

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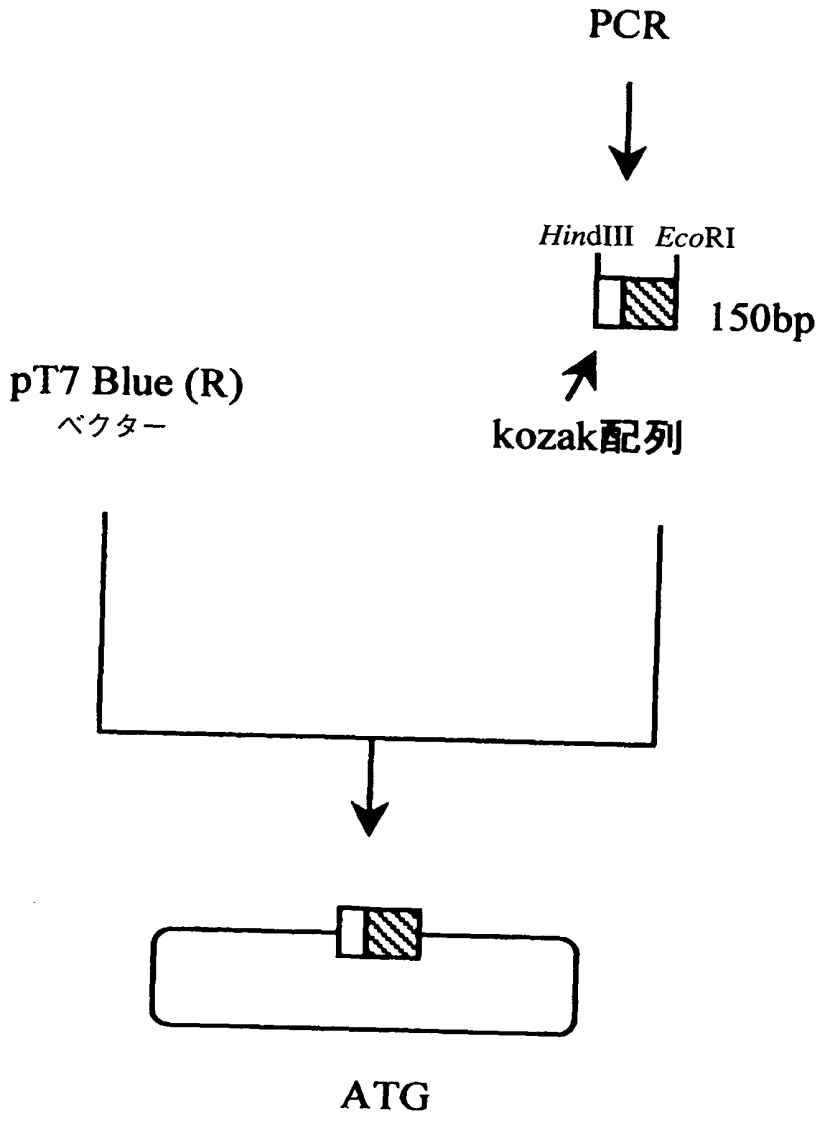

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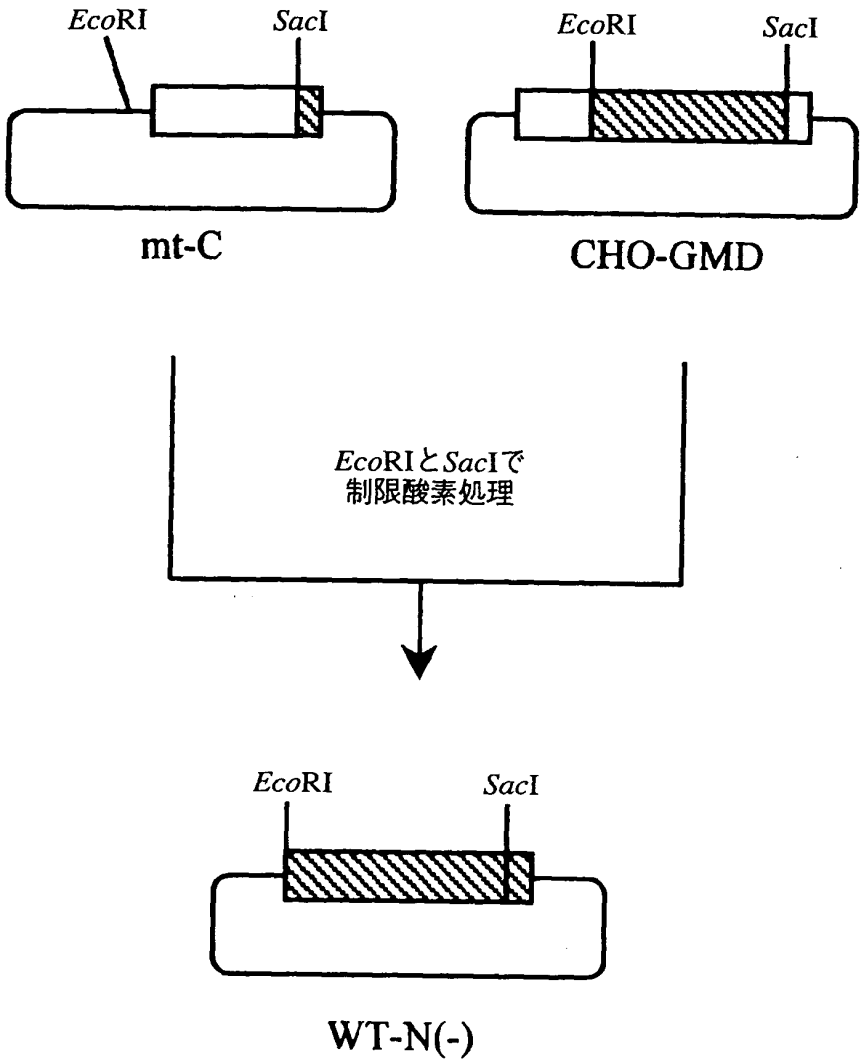

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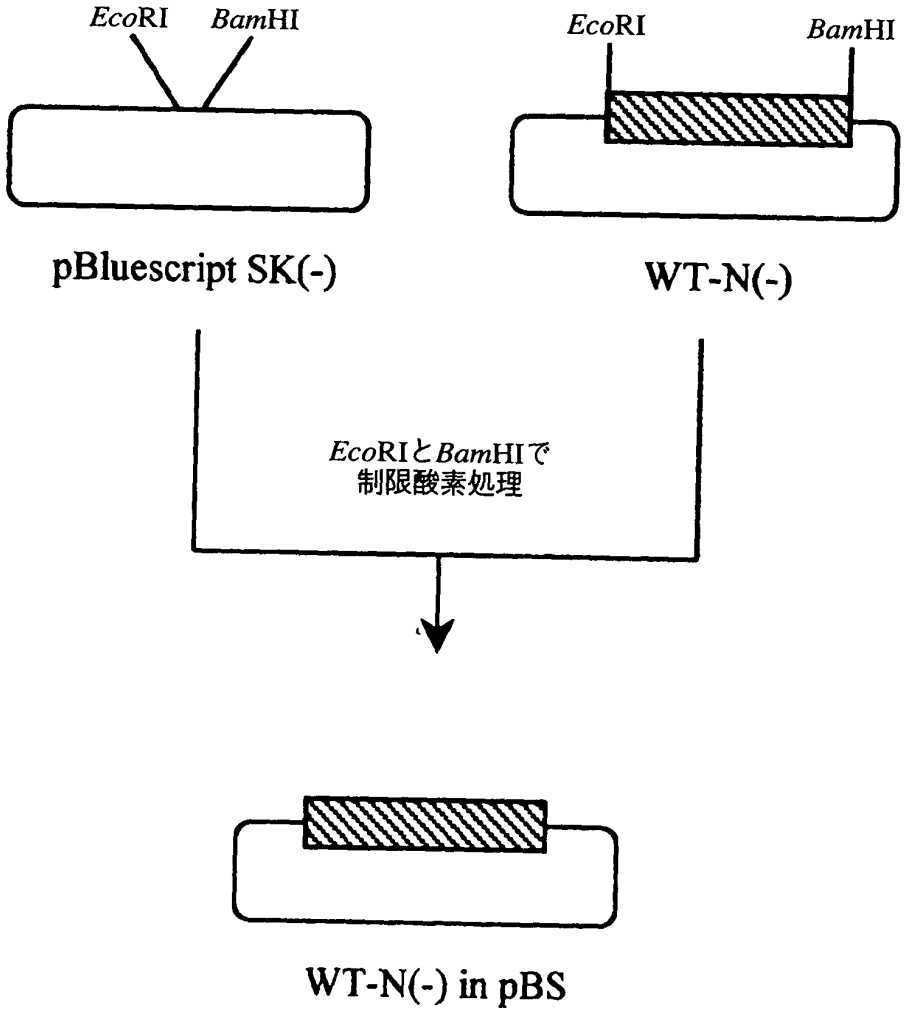

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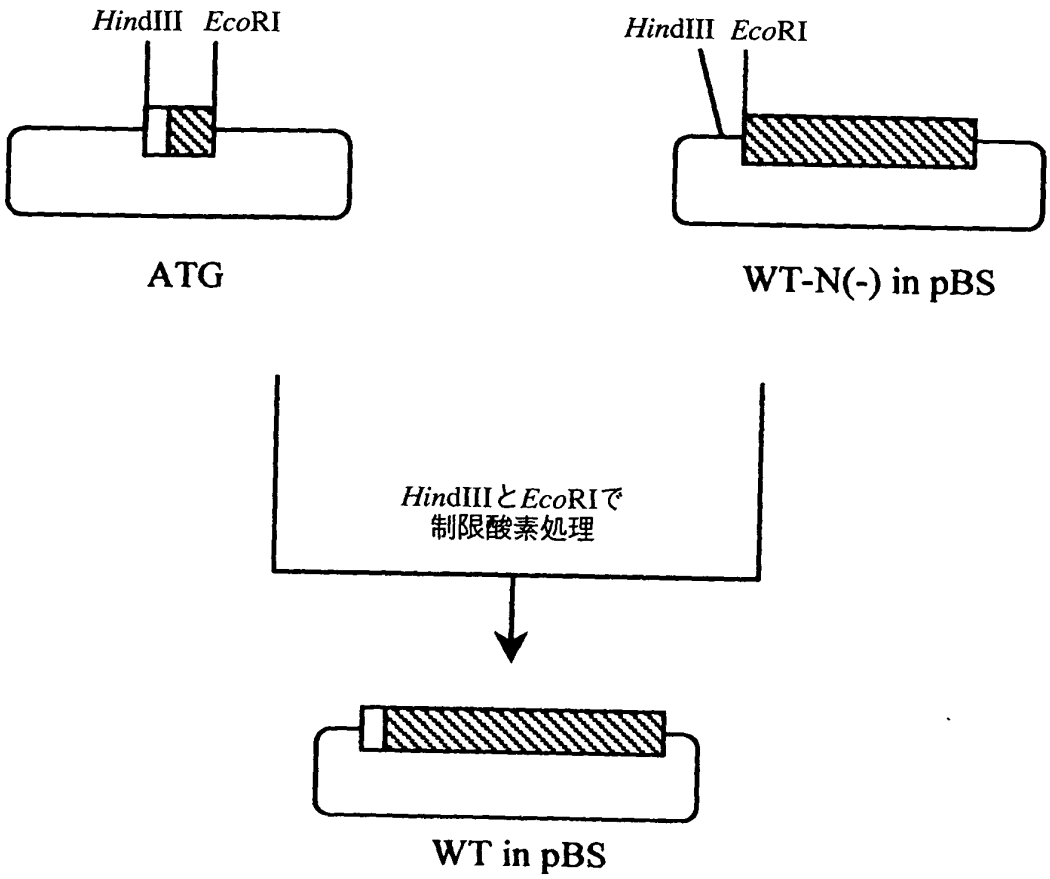

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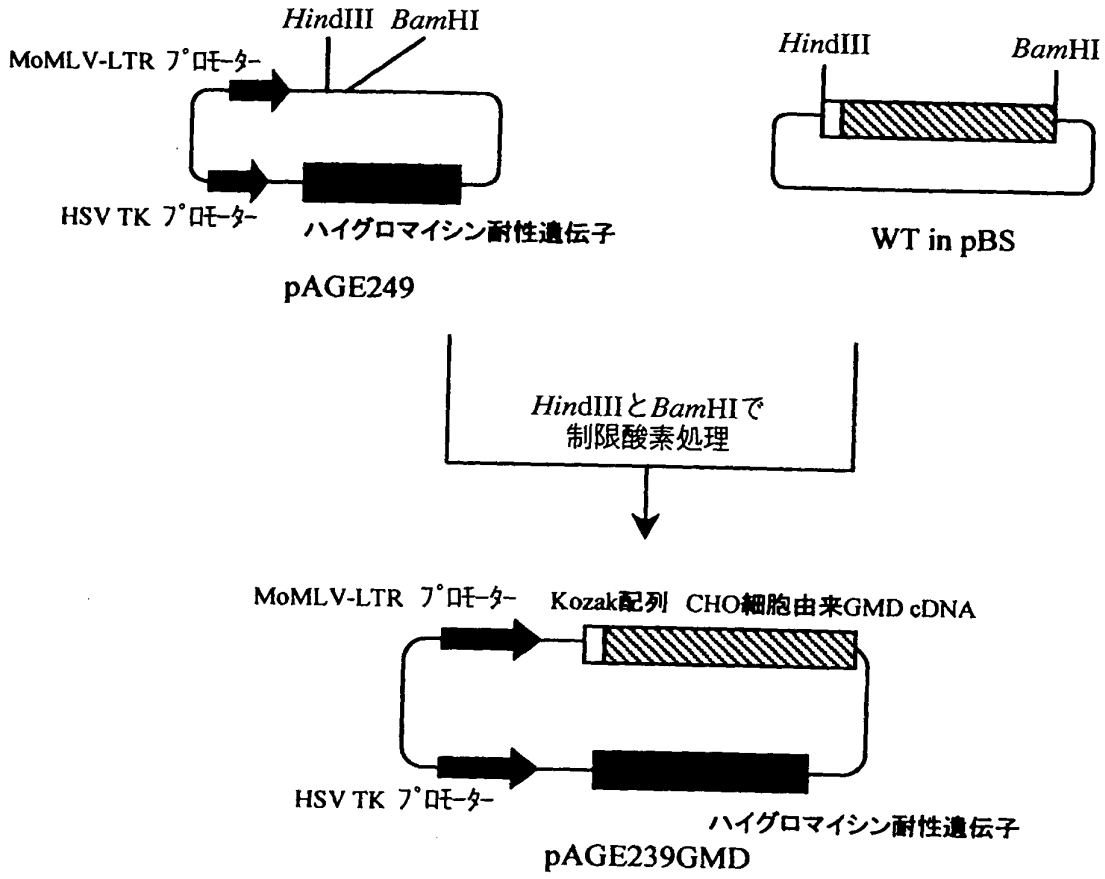

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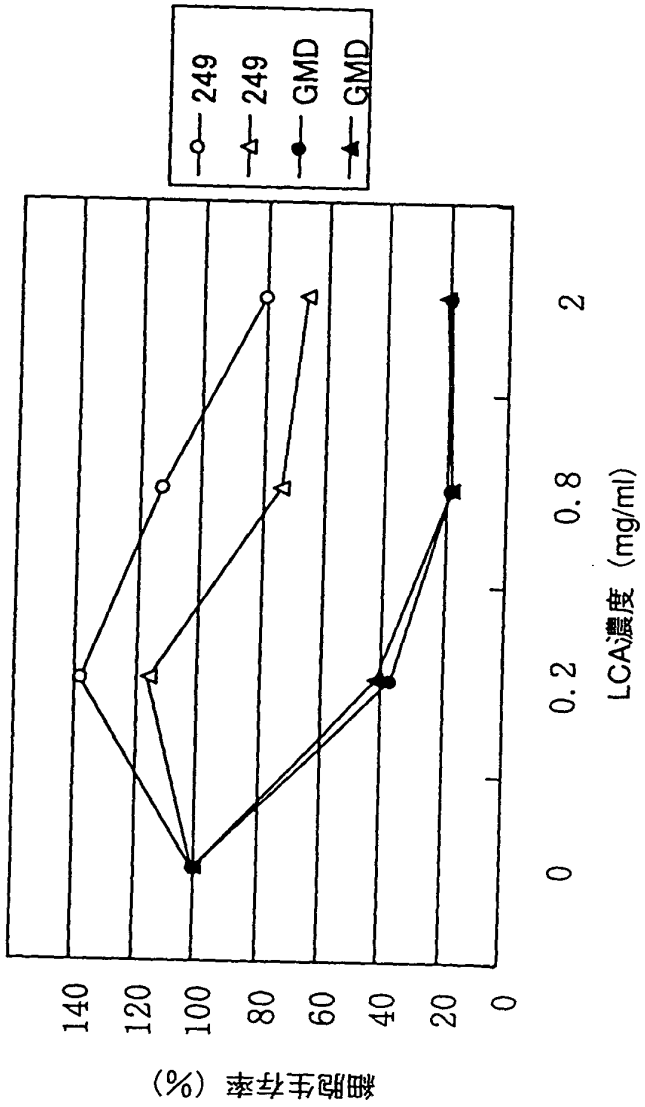

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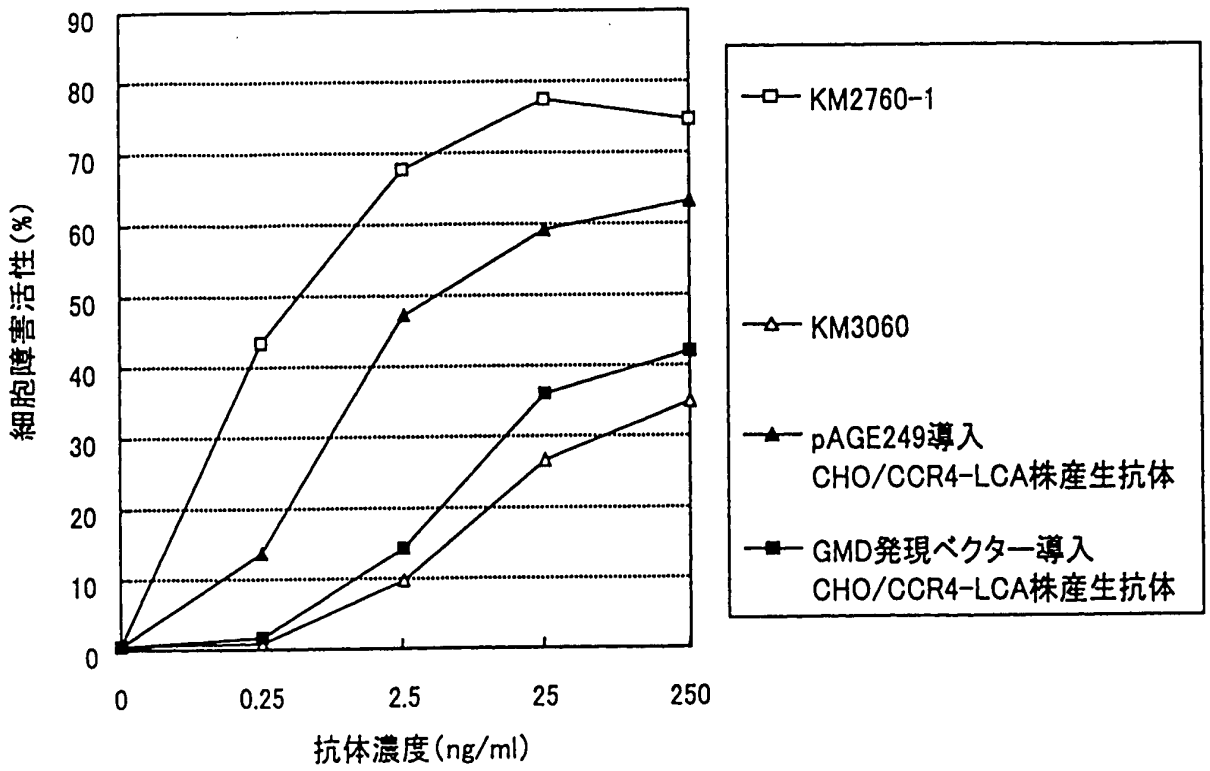
第 53 図



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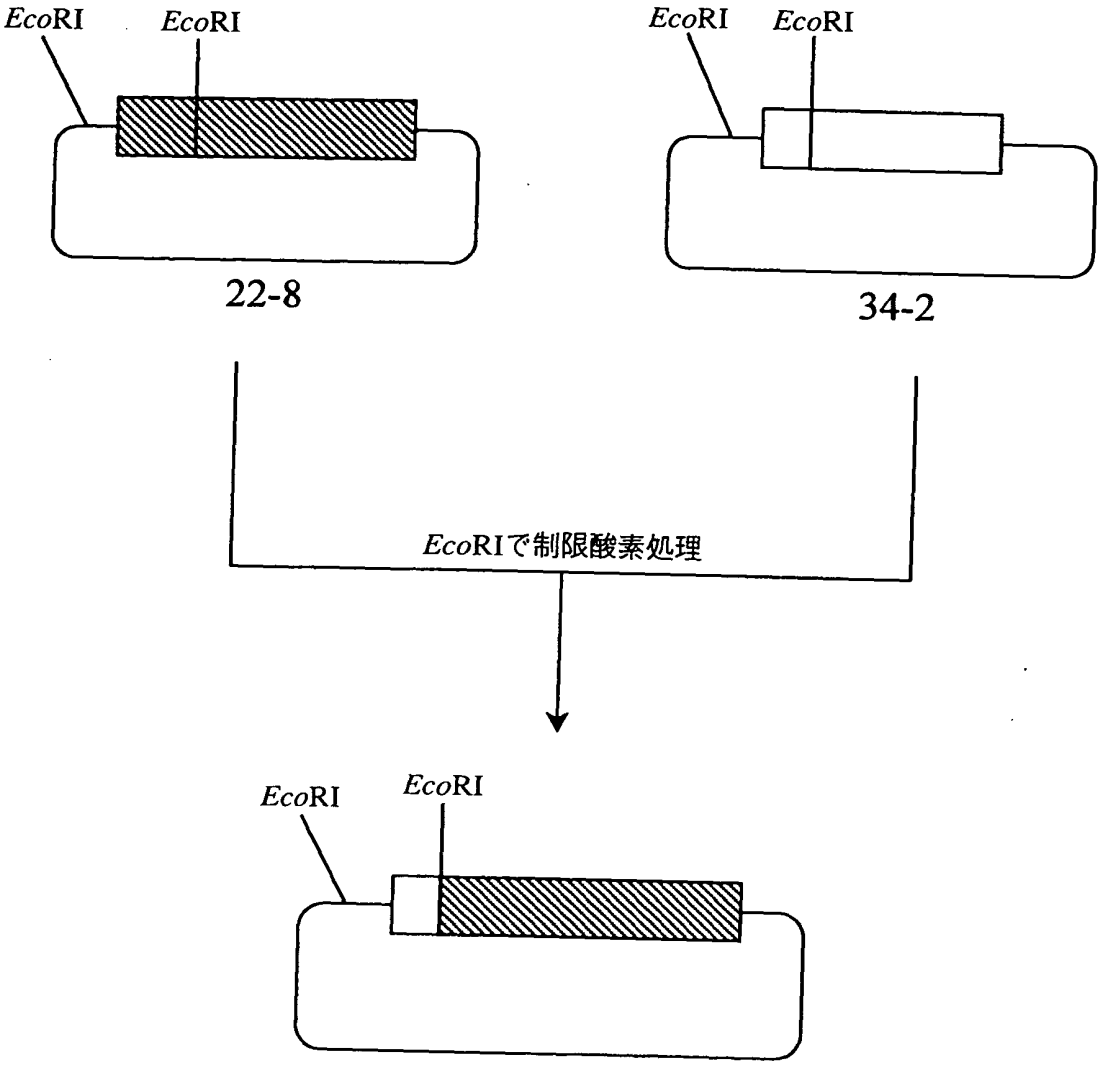

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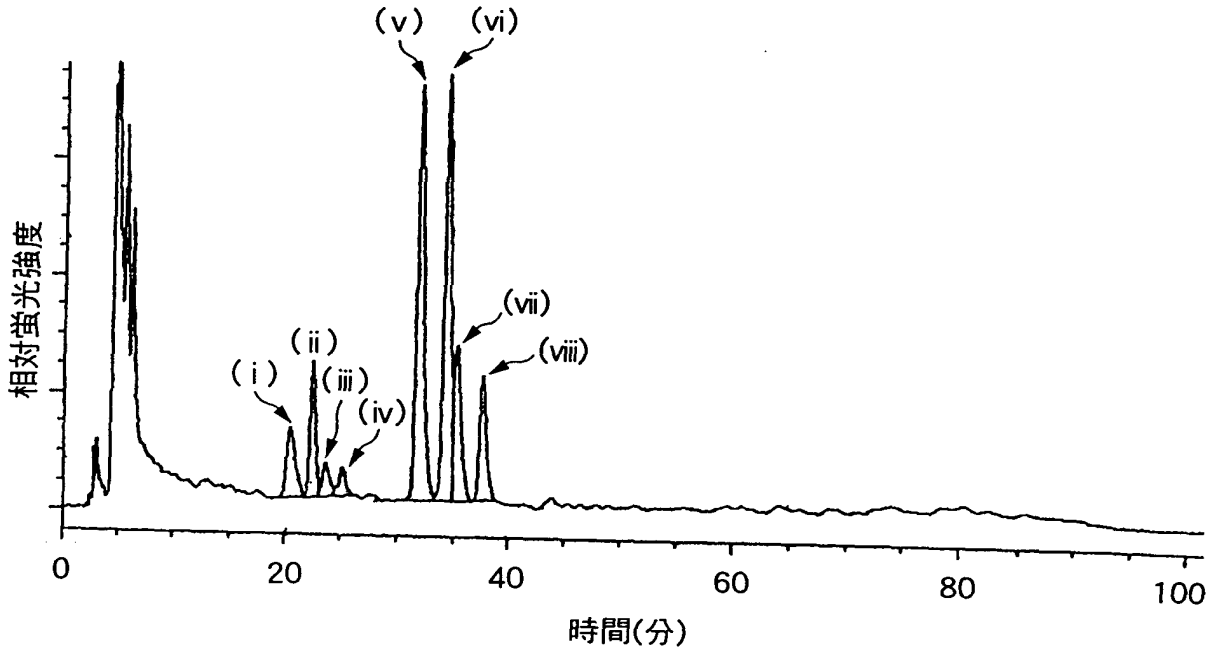

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Our Ref.: 249-202
K46-131990C/EK

U.S. PATENT APPLICATION

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Invention: ANTIBODY COMPOSITION-PRODUCING CELL

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SPECIFICATION

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ANTIBODY COMPOSITION-PRODUCING CELL

BACKGROUND OF THE INVENTION

1. Field of the Invention

本発明は、種々の疾患に有用な抗体、抗体の断片、抗体の Fc 領域を有する融合タンパク質などの抗体分子の製造に用いる細胞、該細胞を用いた抗体組成物の製造方法、抗体組成物、およびその用途に関する。

2. Brief Description of the Background Art

抗体は、高い結合活性、結合特異性及び血中での高い安定性を有することから、ヒトの各種疾患の診断、予防及び治療への応用が試みられてきた [Monoclonal Antibodies: Principles and Applications, Wiley-Liss, Inc., Chapter 2.1 (1995)]。また、遺伝子組換え技術を利用して、ヒト以外の動物の抗体からヒト型キメラ抗体或いはヒト型相補性決定領域（以下、CDR と表記する）移植抗体の様なヒト化抗体を作製することが試みられている。ヒト型キメラ抗体とは、抗体可変領域（以下、V 領域と表記する）がヒト以外の動物の抗体で、定常領域（以下、C 領域と表記する）がヒト抗体である抗体である。ヒト型 CDR 移植抗体とは、ヒト抗体の CDR をヒト以外の動物の抗体の CDR と置換した抗体である。

哺乳類の抗体には、IgM、IgD、IgG、IgA、IgE の 5 種類のクラスが存在することが明らかとなっているが、ヒトの各種疾患の診断、予防及び治療には血中半減期が長く、各種エフェクター機能を有する等の機能特性からヒト IgG クラスの抗体が主として利用されている [Monoclonal Antibodies: Principles and Applications, Wiley-Liss, Inc., Chapter 1 (1995)]。ヒト IgG クラスの抗体は、更に IgG1、IgG2、IgG3、IgG4 の 4 種類のサブクラスに分類されている。IgG クラスの抗体のエフェクター機能である抗体依存性細胞障害活性（以下、ADCC 活性と表記する）や補体依存性細胞障害活性（以下、CDC 活性と表記する）については、これまでに多数の研究が行われ、ヒト IgG クラスでは、IgG1 サブクラスの抗体が最も高い ADCC 活性、CDC 活性を有していることが報告されている [Chemical Immunology, 65, 88 (1997)]。以上の観点から、市販のリツキサン、ハーセプチンを始めとして、その効果発現に高いエフェクター機能を必要とする抗腫瘍ヒト化抗体の殆どはヒト IgG1 サブクラスの抗体である。

ヒト IgG1 サブクラスの抗体の ADCC 活性及び CDC 活性の発現には、抗体 Fc 領域と、キラー細胞、ナチュラルキラー細胞、活性化されたマクロファージ等のエフェクター細胞表面上に存在する抗体レセプター（以下、FcγR と表記する）及び各種補体成分との結合が必要であり、その結合については、抗体のヒンジ領域及び C 領域の第 2 番目のドメイン（以下、Cγ2 ドメインと表記する）内のいくつかのアミノ酸残基の重要性 [Eur. J. Immunol., 23, 1098 (1993)、Immunology, 86, 319 (1995)、Chemical Immunology, 65, 88 (1997)] の他、Cγ2 ドメインに結合している糖鎖の重要性 [Chemical Immunology, 65, 88 (1997)] が示唆されている。

糖鎖に関しては、Boyd らは、チャイニーズハムスター卵巣細胞（CHO 細胞）或いはマウスミエローマ NS0 細胞（NS0 細胞）で生産したヒト型 CDR 移植抗体 CAMPATH-1H（ヒト IgG1 サ

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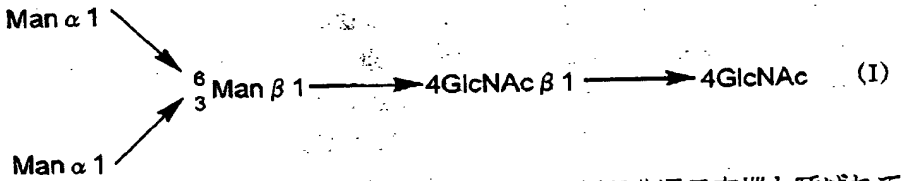
ブクラス）を各種糖分解酵素で処理し、糖鎖の ADCC 活性、CDC 活性に対する影響を検討した結果、非還元末端のシアル酸の除去は、両活性に影響を与えないが、更にガラクトース残基を除去することで CDC 活性のみが影響を受け、約 50%程度活性が低下すること、糖鎖の完全な除去は、両活性を消失させることを報告した [Molecular Immunol., 32, 1311 (1995)]。また、Lifely らは、CHO 細胞、NSO 細胞或いはラットミエローマ YO 細胞で生産したヒト型 CDR 移植抗体 CAMPATH-1H（ヒト IgG1 サブクラス）の糖鎖の分析及び ADCC 活性を測定した結果、YO 細胞由来の CAMPATH-1H が最も高い ADCC 活性を示し、その活性にはバイセクティングに位置する N-アセチルグルコサミン（以下、GlcNAc とも表記する）が重要であることを示唆した [Glycobiology, 5, 813 (1995) : WO99/54342]。これらの報告は、ヒト IgG1 サブクラスの抗体のエフェクター機能に糖鎖の構造が極めて重要な役割を果たしており、糖鎖の構造を変えることでより高いエフェクター機能を有する抗体を作製することが可能であることを示している。しかし、実際には糖鎖の構造は多様かつ複雑であり、エフェクター機能に真に重要な構造を特定できたとは言い難い。

糖タンパク質の糖鎖は、タンパク質部分との結合様式により、アスパラギンと結合する糖鎖（N-グリコシド結合糖鎖）とセリン、スレオニンなどと結合する糖鎖（O-グリコシル結合糖鎖）の２種類に大別される。N-グリコシド結合糖鎖は、様々な構造を有しているが [生物化学実験法 23-糖タンパク質糖鎖研究法(学会出版センター)高橋禮子編（1989 年)]、いずれの場合も以下の構造式（I）に示す基本となる共通のコア構造を有することが知られている。



(I)

アスパラギンと結合する糖鎖の末端が還元末端、反対側が非還元末端と呼ばれている。N-グリコシド結合糖鎖には、コア構造の非還元末端にマンノースのみが結合するハイマンノース型、コア構造の非還元末端側にガラクトース-N-アセチルグルコサミン（以下、Gal-GlcNAc と表記する）の枝を並行して 1 ないしは複数本有し、更に Gal-GlcNAc の非還元末端側にシアル酸、バイセクティングの N-アセチルグルコサミンなどの構造を有するコンプレックス型、コア構造の非還元末端側にハイマンノース型とコンプレックス型の両方の枝を持つハイブリッド型などがあることが知られている。

抗体 IgG 分子の Fc 領域には、２個所の N-グリコシド型の糖鎖結合部位が存在しており、血清中の IgG では、通常、この部位に、シアル酸やバイセクティングの N-アセチルグルコサミンの付加の程度が少ない複数本の枝を持つコンプレックス型糖鎖が結合している。このコンプレックス型糖鎖の非還元末端でのガラクトースの付加および還元末端の N-アセチルグルコサミンへのフコースの付加に関しては多様性があることが知られている [Biochemistry, 36, 130 (1997)]。

このような糖鎖の構造は、糖鎖遺伝子、すなわち、糖鎖を合成する糖転移酵素と糖鎖を分解する糖分解酵素の遺伝子によって規定されていると考えられている。

以下に、N-グリコシド結合糖鎖の生合成に関して述べる。

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糖タンパク質は、小胞体（以下、ＥＲと表記する）内腔で糖鎖の修飾を受ける。N-グリコシド結合糖鎖の生合成過程では、比較的大きな糖鎖が、ＥＲ内腔で伸長しつつあるポリペプチド鎖に転移される。この際、糖鎖はまず、ドリコールリン酸（以下、P-Dol とも表記する）と呼ばれるα-イソプレン単位を２０個程度含む長鎖の脂質担体のリン酸基に順次付加される。すなわち、ドリコールリン酸に N-アセチル-グルコサミンが転移され GlcNAc-P-P-Dol となり、続いてもう１個 GlcNAc が転移され GlcNAc-GlcNAc-P-P-Dol となる。次いで、マンノース（以下、Man とも表記する）が５個転移され $(Man)_5$-$(GlcNAc)_2$-P-P-Dol に、さらに、Man が４個、グルコース（以下、Glc とも表記する）が３個転移される。このようにして、コアオリゴ糖と呼ばれる糖鎖の前駆体 $(Glc)_3$-$(Man)_9$-$(GlcNAc)_2$-P-P-Dol ができる。この１４個の糖からなる糖鎖の前駆体はアスパラギン-X-セリンまたはアスパラギン-X-スレオニン配列を持ったポリペプチドへＥＲ内腔でひとかたまりのまま転移される。この際、コアオリゴ糖に結合していたドリコールピロリン酸（P-P-Dol）は遊離するが、ピロホスファターゼの分解を受けて再びドリコールリン酸となり再利用される。糖鎖のトリミングは、糖鎖がポリペプチドに結合すると直ちに開始される。すなわち、３個の Glc と１ないし２個の Man がＥＲ上で除去され、この除去にはα-1,2-グルコシダーゼ I、α-1,3-グルコシダーゼ II およびα-1,2-マンノシダーゼが関与することが知られている。

ＥＲ上でトリミングを受けた糖タンパク質はゴルジ体へ輸送され様々な修飾を受ける。ゴルジ体シス部には、マンノースリン酸を付加する N-アセチルグルコサミンホスホトランスフェラーゼ、N-アセチルグルコサミン-1-ホスホジエステルα-N-アセチルグルコサミニダーゼおよびα-マンノシダーゼ I が存在し、Man 残基を５個にまで減少させる。ゴルジ体メディア部には、コンプレックス型の N-グリコシド結合糖鎖の最初の外側の GlcNAc を付加する N-アセチルグルコサミン転移酵素 I (GnTI)、２個の Man を除去するα-マンノシダーゼ II、外側から２個目の GlcNAc を付加する N-アセチルグルコサミン転移酵素 II (GnTII)、還元末端の N-アセチルグルコサミンにフコースを付加するα-1,6-フコシルトランスフェラーゼが存在する。ゴルジ体トランス部にはガラクトースを付加するガラクトース転移酵素、N-アセチルノイラミン酸などのシアル酸を付加するシアル酸転移酵素が存在する。このような各種酵素の作用を受けて N-グリコシド結合糖鎖が作られることが知られている。

一般的に、医薬への応用が考えられているヒト化抗体の多くは、遺伝子組換え技術を用いて作製され、チャイニーズハムスター卵巣組織由来 CHO 細胞を宿主細胞として用い製造されているが、上述したように、抗体のエフェクター機能には糖鎖構造が極めて重要な役割を担っていること、宿主細胞によって発現された糖タンパク質の糖鎖構造に違いが観察されることから、より高いエフェクター機能を有する抗体を作製することが可能な宿主細胞の開発が望まれている。

生産される糖タンパク質の糖鎖構造を改変するために、１）糖鎖の修飾に係わる酵素の阻害剤の応用、２）突然変異体の選択、３）糖鎖の修飾に係わる酵素遺伝子の導入などの方法が試みられている。以下に、それら具体的例を述べる。

糖鎖の修飾に係わる酵素の阻害剤としては、N-グリコシド結合糖鎖の前駆体であるコアオリゴ糖形成の最初のステップである GlcNAc-P-P-Dol の形成を選択的に阻害するツニカマイシン、グリコシダーゼ I の阻害剤であるカスタノスペルミンや N-メチル-1-デオキシノジリ

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マイシン、グルコシダーゼ II の阻害剤であるブロモコンズリトール、マンノシダーゼ I の阻害剤である1-デオキシノジリマイシンや 1,4-ジオキシ-1,4-イミノ-D-マンニトール、マンノシダーゼ II の阻害剤であるスワンソニンなどが知られている。糖転移酵素の特異的な阻害剤としては、N-アセチルグルコサミン転移酵素 V (GnTV) などに対する基質のデオキシ誘導体が知られている[グライコバイオロジーシリーズ2—糖鎖の細胞における運命（講談社サンエンティフィック）永井克孝・箱守仙一朗・木幡陽編 (1993)]。また、1-デオキシノジリマイシンはコンプレックス型糖鎖の合成を抑え、ハイマンノース型やハイブリッド型糖鎖の割合を増加させることが知られている。実際に、これら阻害剤を培地に添加することで IgG の糖鎖構造が変化し、抗原結合性などが変化することが報告されている[Molecular. Immunol., 26, 1113 (1989)]。

糖鎖の修飾に係わる酵素の活性に関する突然変異体は、主に、レクチン耐性株として選択され取得されている。例えば、WGA (*T. vulgaris* 由来の wheat-germ agglutinin)、ConA (*C. ensiformis* 由来の concanavalin A)、RIC (*R. communis* 由来の毒素)、L-PHA (*P. vulgaris* 由来の leukoagglutinin)、LCA (*L. culinaris* 由来の lentil agglutinin)、PSA (*P. sativum* 由来の Pea lectin)などのレクチンを用い、様々な糖鎖構造を有する CHO 細胞変異株がレクチン耐性株として取得されている [Somatic Cell Mol. Genet., 12, 51 (1986)]。

糖鎖の修飾に係わる酵素の遺伝子を宿主細胞に導入して生産物の糖鎖構造を改変した例としては、ラットのβ-ガラクトシド-α-2,6-シアリルトランスフェラーゼを CHO 細胞に導入することで糖鎖の非還元末端にシアル酸が多く付加されたタンパク質の製造が可能であることが報告されている [J. Biol. Chem., 261, 13848 (1989)]。

また、ヒトのβ-ガラクトシド-2-α-フコシルトランスフェラーゼをマウスL細胞に導入することで糖鎖の非還元末端にフコース（以下、Fuc とも表記する）が付加されたH抗原 (Fucα1-2Galβ1-) の発現が確認されている [Science, 252, 1668 (1991)]。さらに、Umana らは、N-グリコシド結合糖鎖のバイセクティングに位置する N-アセチルグルコサミンの付加が抗体の ADCC 活性に重要であるとの知見に基づき、β-1,4-N-アセチルグルコサミン転移酵素 III (GnTIII) を発現させた CHO 細胞を作製し親株との比較を行っている。親株の CHO 細胞では GnTIII の発現が観察されておらず[J. Biol. Chem., 261, 13370 (1984)]、作製した GnTIII 発現 CHO 細胞を用いて発現させた抗体は親株で発現させた抗体と比べ16倍高い ADCC 活性を有していることを確認している [Glycobiology, 5, 813 (1995) : WO99/54342]。またこの際、Umana らは、β-1,4-N-アセチルグルコサミン転移酵素 V (GnTV) の遺伝子を導入した CHO 細胞も作製しており、GnTIII または GnTV の過剰発現は CHO 細胞に対して毒性を示すことを報告している。

このように、生産される糖タンパク質の糖鎖構造を改変するために、宿主細胞の糖鎖の修飾に係わる酵素の活性を調節する試みがなされているが、実際には糖鎖の構造は多様かつ複雑であり、かつ糖鎖が持つ生理的な役割の解明も十分とは言い難いため試行錯誤を繰り返しているのが現状である。特に、抗体のエフェクター機能は糖鎖構造により大きな影響を受ける事が明らかになりつつあるが、真に重要な糖鎖構造の特定には至っていない。従って、抗体のエフェクター機能に影響を及ぼす糖鎖構造の同定と、そのような糖鎖構造の付加が可能な宿主細胞の開発が医薬開発の上で求められている。

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SUMMARY OF THE INVENTION

本発明は、抗体分子の糖鎖構造を制御することが可能な、抗体組成物を生産する宿主細胞、ADCC 活性が高い抗体組成物を生産することが可能な細胞、該細胞を用いた抗体組成物の製造方法、該製造方法で製造された抗体組成物を提供することを目的とする。

本発明は、以下の (1)～(61) に関する。

(1) N-グリコシド結合複合型糖鎖を Fc 領域に有する抗体分子からなる組成物であって、該組成物中に含まれる Fc 領域に結合する全 N-グリコシド結合複合型糖鎖のうち、糖鎖還元末端の N-アセチルグルコサミンにフコースが結合していない糖鎖の割合が 20%以上である抗体組成物を生産する、抗体分子をコードする遺伝子を導入したチャイニーズハムスター卵巣組織由来の CHO 細胞。

(2) フコースが結合していない糖鎖が、該フコースの 1 位が N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの 6 位にα結合していない糖鎖である、(1) に記載の CHO 細胞。

(3) 抗体分子のクラスが IgG である、(1) または(2)に記載の CHO 細胞。

(4) 細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素の活性または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの 6 位にフコースの 1 位がα結合する糖鎖修飾に関与する酵素の活性が低下または欠失した(1)～(3)のいずれか 1 項に記載の CHO 細胞。

(5) 細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素が、以下の(a)、(b) 及び(c)からなる群から選ばれる酵素である、(4) に記載の CHO 細胞。

(a) GMD (GDP-mannose 4,6-dehydratase) ;

(b) Fx (GDP-keto-6-deoxymannose 3,5-epimerase, 4-reductase) ;

(c) GFPP (GDP-beta-L-fucose pyrophosphorylase)。

(6) GMD が、以下の (a) または (b) である DNA がコードする蛋白質である、(5) に記載の CHO 細胞。

(a) 配列番号 65 で表される塩基配列からなる DNA ;

(b) 配列番号 65 で表される塩基配列からなる DNA とストリンジェントな条件でハイブリダイズし、かつ GMD 活性を有する蛋白質をコードする DNA。

(7) GMD が、以下の (a)、(b) 及び (c) からなる群から選ばれる蛋白質である、(5) に記載の CHO 細胞。

(a) 配列番号 71 で表されるアミノ酸配列からなる蛋白質 ;

(b) 配列番号 71 で表されるアミノ酸配列において、1 以上のアミノ酸が欠失、置換、挿入および／または付加されたアミノ酸配列からなり、かつ GMD 活性を有する蛋白質。

(c) 配列番号 71 で表されるアミノ酸配列と８０%以上の相同性を有するアミノ酸配列からなり、かつ GMD 活性を有する蛋白質。

(8) Fx が、以下の (a) または (b) である DNA がコードする蛋白質である、(5) に記載の CHO 細胞。

(a) 配列番号 48 で表される塩基配列からなる DNA ;

(b) 配列番号 48 で表される塩基配列からなる DNA とストリンジェントな条件でハイブリダ

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イズし、かつFx活性を有する蛋白質をコードするDNA。

(9) Fxが、以下の(a)、(b)及び(c)からなる群から選ばれる蛋白質である、(5)に記載のCHO細胞。

(a) 配列番号72で表されるアミノ酸配列からなる蛋白質；

(b) 配列番号72で表されるアミノ酸配列において、1以上のアミノ酸が欠失、置換、挿入および／または付加されたアミノ酸配列からなり、かつFx活性を有する蛋白質；

(c) 配列番号72で表されるアミノ酸配列と８０％以上の相同性を有するアミノ酸配列からなり、かつFx活性を有する蛋白質。

(10) GFPPが、以下の(a)または(b)であるDNAがコードする蛋白質である、(5)に記載のCHO細胞。

(a) 配列番号51で表される塩基配列からなるDNA；

(b) 配列番号51で表される塩基配列からなるDNAとストリンジェントな条件でハイブリダイズし、かつGFPP活性を有する蛋白質をコードするDNA。

(11) GFPPが、以下の(a)、(b)及び(c)からなる群から選ばれる蛋白質である、(5)に記載のCHO細胞。

(a) 配列番号73で表されるアミノ酸配列からなる蛋白質；

(b) 配列番号73で表されるアミノ酸配列において、1以上のアミノ酸が欠失、置換、挿入および／または付加されたアミノ酸配列からなり、かつGFPP活性を有する蛋白質；

(c) 配列番号73で表されるアミノ酸配列と８０％以上の相同性を有するアミノ酸配列からなり、かつGFPP活性を有する蛋白質。

(12) N-グリコシド結合複合型糖鎖還元末端のN-アセチルグルコサミンの6位にフコースの1位がα結合する糖鎖修飾に関与する酵素がα-1,6-フコシルトランスフェラーゼである、(4)に記載のCHO細胞。

(13) α-1,6-フコシルトランスフェラーゼが、以下の(a)または(b)であるDNAがコードする蛋白質である、(12)に記載のCHO細胞。

(a) 配列番号1で表される塩基配列からなるDNA；

(b) 配列番号1で表される塩基配列からなるDNAとストリンジェントな条件でハイブリダイズし、かつα-1,6-フコシルトランスフェラーゼ活性を有する蛋白質をコードするDNA。

(14) α-1,6-フコシルトランスフェラーゼが、以下の(a)、(b)及び(c)からなる群から選ばれる蛋白質である、(12)に記載のCHO細胞。

(a) 配列番号23で表されるアミノ酸配列からなる蛋白質；

(b) 配列番号23で表されるアミノ酸配列において、1以上のアミノ酸が欠失、置換、挿入および／または付加されたアミノ酸配列からなり、かつα-1,6-フコシルトランスフェラーゼ活性を有する蛋白質；

(c) 配列番号23で表されるアミノ酸配列と８０％以上の相同性を有するアミノ酸配列からなり、かつα-1,6-フコシルトランスフェラーゼ活性を有する蛋白質。

(15) 酵素の活性が、以下の(a)、(b)、(c)、(d)及び(e)からなる群から選ばれる手法により低下または欠失した、(4)～(14)のいずれか1項に記載のCHO細胞。

(a) 酵素の遺伝子を標的した遺伝子破壊の手法；

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(b) 酵素の遺伝子のドミナントネガティブ体を導入する手法；

(c) 酵素についての突然変異を導入する手法；

(d) 酵素の遺伝子の転写又は翻訳を抑制する手法；

(e) N-グリコシド結合糖鎖還元末端の N-アセチルグルコサミンの 6 位とフコースの 1 位がα結合した糖鎖構造を認識するレクチンに耐性である株を選択する手法。

(16) 少なくとも N-グリコシド結合糖鎖還元末端の N-アセチルグルコサミンの 6 位とフコースの 1 位がα結合した糖鎖構造を認識するレクチンに耐性である、(4) ～(15)のいずれか 1 項に記載の CHO 細胞。

(17) 親株である CHO 細胞が生産する抗体組成物より、抗体依存性細胞障害活性が高い抗体組成物を生産する、(4) ～(16)のいずれか 1 項に記載の CHO 細胞。

(18) 抗体組成物中に含まれる Fc 領域に結合する全 N-グリコシド結合複合型糖鎖のうち、糖鎖還元末端の N-アセチルグルコサミンとフコースが結合していない糖鎖の割合が 20%未満である抗体組成物よりも抗体依存性細胞障害活性が高い抗体組成物を生産する、(17) 記載の CHO 細胞。

(19) フコースが結合していない糖鎖が、該フコースの 1 位が N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの 6 位にα結合していない糖鎖である、(18) 記載の CHO 細胞。

(20) (1) ～(19)のいずれか 1 項に記載の CHO 細胞を培地に培養し、培養物中に抗体組成物を生成蓄積させ、該培養物から抗体組成物を採取する工程を含む、抗体組成物を製造する方法。

(21) (20) に記載の方法を用いて製造される抗体組成物。

(22) CHO 細胞が産生する N-グリコシド結合複合型糖鎖を Fc 領域に有する抗体分子からなる組成物であって、該組成物中に含まれる Fc 領域に結合する全 N-グリコシド結合糖鎖のうち、糖鎖還元末端の N-アセチルグルコサミンにフコースが結合していない糖鎖の割合が 20%以上である抗体組成物。

(23) 細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素の活性または N-グリコシド結合糖鎖還元末端の N-アセチルグルコサミンの 6 位にフコースの 1 位がα結合する糖鎖修飾に関与する酵素の活性が遺伝子工学的な手法により低下または欠失した細胞。

(24) 細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素が、以下の(a)、(b) 及び (c)からなる群から選ばれる酵素である、(23) 記載の細胞。

(a) GMD (GDP-mannose 4,6-dehydratase)；

(b) Fx (GDP-keto-6-deoxymannose 3,5-epimerase, 4-reductase)；

(c) GFPP (GDP-beta-L-fucose pyrophosphorylase)。

(25) GMD が、以下の (a) または (b) である DNA がコードする蛋白質である、(24) に記載の細胞。

(a) 配列番号 65 で表される塩基配列からなる DNA；

(b) 配列番号 65 で表される塩基配列からなる DNA とストリンジェントな条件でハイブリダイズし、かつ GMD 活性を有する蛋白質をコードする DNA。

(26) GMD が、以下の (a)、(b) 及び (c) からなる群から選ばれる蛋白質である、(24)

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に記載の細胞。
(a) 配列番号 71 で表されるアミノ酸配列からなる蛋白質；
(b) 配列番号 71 で表されるアミノ酸配列において、１以上のアミノ酸が欠失、置換、挿入および／または付加されたアミノ酸配列からなり、かつ GMD 活性を有する蛋白質。
(c) 配列番号 71 で表されるアミノ酸配列と８０％以上の相同性を有するアミノ酸配列からなり、かつ GMD 活性を有する蛋白質。
(27) Fx が、以下の (a) または (b) である DNA がコードする蛋白質である、(24) に記載の細胞。
(a) 配列番号 48 で表される塩基配列からなる DNA；
(b) 配列番号 48 で表される塩基配列からなる DNA とストリンジェントな条件でハイブリダイズし、かつ Fx を有する蛋白質をコードする DNA。
(28) Fx が、以下の (a)、(b) 及び (c) からなる群から選ばれる蛋白質である、(24) に記載の細胞。
(a) 配列番号 72 で表されるアミノ酸配列からなる蛋白質；
(b) 配列番号 72 で表されるアミノ酸配列において、１以上のアミノ酸が欠失、置換、挿入および／または付加されたアミノ酸配列からなり、かつ Fx 活性を有する蛋白質；
(c) 配列番号 72 で表されるアミノ酸配列と８０％以上の相同性を有するアミノ酸配列からなり、かつ Fx 活性を有する蛋白質。
(29) GFPP が、以下の (a) または (b) である DNA がコードする蛋白質である、(24) に記載の細胞。
(a) 配列番号 51 で表される塩基配列からなる DNA；
(b) 配列番号 51 で表される塩基配列からなる DNA とストリンジェントな条件でハイブリダイズし、かつ GFPP 活性を有する蛋白質をコードする DNA。
(30) GFPP が、以下の (a)、(b) 及び (c) からなる群から選ばれる蛋白質である、(24) に記載の細胞。
(a) 配列番号 73 で表されるアミノ酸配列からなる蛋白質；
(b) 配列番号 73 で表されるアミノ酸配列において、１以上のアミノ酸が欠失、置換、挿入および／または付加されたアミノ酸配列からなり、かつ GFPP 活性を有する蛋白質；
(c) 配列番号 73 で表されるアミノ酸配列と８０％以上の相同性を有するアミノ酸配列からなり、かつ GFPP 活性を有する蛋白質。
(31) N-グリコシド結合糖鎖還元末端の N-アセチルグルコサミンの 6 位にフコースの 1 位がα結合する糖鎖修飾に関与する酵素がα-1,6-フコシルトランスフェラーゼである、(23) に記載の細胞。
(32) α-1,6-フコシルトランスフェラーゼが、以下の (a)、(b)、(c)及び(d)からなる群から選ばれる DNA がコードする蛋白質である、(31) に記載の細胞。
(a) 配列番号 1 で表される塩基配列からなる DNA；
(b) 配列番号 2 で表される塩基配列からなる DNA；
(c) 配列番号 1 で表される塩基配列からなる DNA とストリンジェントな条件でハイブリダイズし、かつα-1,6-フコシルトランスフェラーゼ活性を有する蛋白質をコードする DNA；

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(d) 配列番号 2 で表される塩基配列からなる DNA とストリンジェントな条件でハイブリダイズし、かつα-1,6-フコシルトランスフェラーゼ活性を有する蛋白質をコードする DNA。

(33) α-1,6-フコシルトランスフェラーゼが、以下の (a)、(b)、(c)、(d)、(e)及び (f) からなる群から選ばれる蛋白質である、(31) に記載の細胞。

(a) 配列番号 23 で表されるアミノ酸配列からなる蛋白質；

(b) 配列番号 24 で表されるアミノ酸配列からなる蛋白質；

(c) 配列番号 23 で表されるアミノ酸配列において、1 以上のアミノ酸が欠失、置換、挿入および／または付加されたアミノ酸配列からなり、かつα-1,6-フコシルトランスフェラーゼ活性を有する蛋白質；

(d) 配列番号 24 で表されるアミノ酸配列において、1 以上のアミノ酸が欠失、置換、挿入および／または付加されたアミノ酸配列からなり、かつα-1,6-フコシルトランスフェラーゼ活性を有する蛋白質；

(e) 配列番号 23 で表されるアミノ酸配列と８０％以上の相同性を有するアミノ酸配列からなり、かつα-1,6-フコシルトランスフェラーゼ活性を有する蛋白質；

(f) 配列番号 24 で表されるアミノ酸配列と８０％以上の相同性を有するアミノ酸配列からなり、かつα-1,6-フコシルトランスフェラーゼ活性を有する蛋白質。

(34) 遺伝子工学的な手法が、以下の (a)、(b)、(c) 及び (d) からなる群から選ばれる手法である、(23) ～(33)のいずれか 1 項に記載の細胞。

(a) 酵素の遺伝子を標的した遺伝子破壊の手法；

(b) 酵素の遺伝子のドミナントネガティブ体を導入する手法；

(c) 酵素についての突然変異を導入する手法；

(d) 酵素の遺伝子の転写又は翻訳を抑制する手法。

(35) 少なくとも N-グリコシド結合糖鎖還元末端の N-アセチルグルコサミンの 6 位とフコースの 1 位がα結合した糖鎖構造を認識するレクチンに耐性である、(23) ～(34)のいずれか 1 項に記載の細胞。

(36) (23) ～(35)のいずれか 1 項に記載の細胞が、下記の (a)～(i) からなる群から選ばれる細胞。

(a) チャイニーズハムスター卵巣組織由来 CHO 細胞；

(b) ラットミエローマ細胞株 YB2/3HL.P2.G11.16Ag.20 細胞；

(c) マウスミエローマ細胞株 NSO 細胞；

(d) マウスミエローマ細胞株 SP2/0-Ag14 細胞；

(e) シリアンハムスター腎臓組織由来 BHK 細胞；

(f) 抗体を産生するハイブリドーマ細胞；

(g) ヒト白血病細胞株ナマルバ細胞；

(h) 胚性幹細胞；

(i) 受精卵細胞。

(37) (23) ～(36)のいずれか 1 項に記載の細胞に、抗体分子をコードする遺伝子を導入した細胞。

(38) 抗体分子のクラスが IgG である、(37) 記載の細胞。

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(39) (37) または(38)項に記載の細胞を培地に培養し、培養物中に抗体組成物を生成蓄積させ、該培養物から抗体組成物を採取する工程を含む、抗体組成物の製造方法。

(40) 親株から得られる抗体組成物よりも、抗体依存性細胞障害活性が高い抗体組成物を生産する、(39) に記載の方法。

(41) (39) または(40)に記載の方法を用いて製造される、抗体組成物。

(42) 細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素の活性または N-グリコシド結合糖鎖還元末端の N-アセチルグルコサミンの 6 位にフコースの 1 位がα結合する糖鎖修飾に関与する酵素の活性が低下するように、ゲノムが改変されたトランスジェニック非ヒト動物あるいは植物、またはその子孫。

(43) 細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素の遺伝子または N-グリコシド結合糖鎖還元末端の N-アセチルグルコサミンの 6 位にフコースの 1 位がα結合する糖鎖修飾に関与する酵素の遺伝子がノックアウトされた、(42) 記載のトランスジェニック非ヒト動物あるいは植物、またはその子孫。

(44) 細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素が、以下の(a)、(b) 及び (c)からなる群から選ばれる酵素である、(42) または(43)に記載のトランスジェニック非ヒト動物あるいは植物、またはその子孫。

(a) GMD (GDP-mannose 4,6-dehydratase) ;

(b) Fx (GDP-keto-6-deoxymannose 3,5-epimerase, 4-reductase) ;

(c) GFPP (GDP-beta-L-fucose pyrophosphorylase)。

(45) GMD が、以下の (a) または (b) である DNA がコードする蛋白質である、(44) に記載のトランスジェニック非ヒト動物あるいは植物、またはその子孫。

(a) 配列番号 65 で表される塩基配列からなる DNA ;

(b) 配列番号 65 で表される塩基配列からなる DNA とストリンジェントな条件でハイブリダイズし、かつ GMD 活性を有する蛋白質をコードする DNA。

(46) Fx が、以下の (a) または (b) である DNA がコードする蛋白質である、(44) に記載のトランスジェニック非ヒト動物あるいは植物、またはその子孫。

(a) 配列番号 48 で表される塩基配列からなる DNA ;

(b) 配列番号 48 で表される塩基配列からなる DNA とストリンジェントな条件でハイブリダイズし、かつ Fx 活性を有する蛋白質をコードする DNA。

(47) GFPP が、以下の (a) または (b) である DNA がコードする蛋白質である、(44) に記載の細胞。

(a) 配列番号 51 で表される塩基配列からなる DNA ;

(b) 配列番号 51 で表される塩基配列からなる DNA とストリンジェントな条件でハイブリダイズし、かつ GFPP 活性を有する蛋白質をコードする DNA。

(48) N-グリコシド結合糖鎖還元末端の N-アセチルグルコサミンの 6 位にフコースの 1 位がα結合する糖鎖修飾に関与する酵素がα-1,6-フコシルトランスフェラーゼである、(42) または(43)に記載のトランスジェニック非ヒト動物あるいは植物、またはその子孫。

(49) α-1,6-フコシルトランスフェラーゼが、以下の (a)、(b)、(c) 及び (d) からなる群から選ばれる DNA がコードする蛋白質である、(48) に記載のトランスジェニック非ヒ

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ト動物あるいは植物、またはその子孫。

(a) 配列番号1で表される塩基配列からなるDNA；

(b) 配列番号2で表される塩基配列からなるDNA；

(c) 配列番号1で表される塩基配列からなるDNAとストリンジェントな条件でハイブリダイズし、かつα-1,6-フコシルトランスフェラーゼ活性を有する蛋白質をコードするDNA；

(d) 配列番号2で表される塩基配列からなるDNAとストリンジェントな条件でハイブリダイズし、かつα-1,6-フコシルトランスフェラーゼ活性を有する蛋白質をコードするDNA。

(50) トランスジェニック非ヒト動物が、ウシ、ヒツジ、ヤギ、ブタ、ウマ、マウス、ラット、ニワトリ、サル及びウサギからなる群から選ばれる動物である、(42)～(49)のいずれか1項に記載のトランスジェニック非ヒト動物、またはその子孫。

(51) (42)～(50)のいずれか1項に記載のトランスジェニック非ヒト動物あるいは植物、またはその子孫に抗体分子をコードする遺伝子を導入し、該動物あるいは植物を飼育し、飼育した動物あるいは植物から導入した抗体を含む組織あるいは体液を取得し、取得した組織あるいは体液から目的とする抗体組成物を採取する工程を含む、抗体組成物を製造する方法。

(52) 抗体分子のクラスがIgGである、(51)に記載の方法。

(53) ゲノムが改変されていない非ヒト動物あるいは植物、またはその子孫から得られる抗体組成物よりも、抗体依存性細胞障害活性が高い抗体組成物を生産する、(51)または(52)に記載の方法。

(54) (51)～(53)のいずれか1項に記載の方法を用いて製造される、抗体組成物。

(55) (21)、(22)、(41)または(54)のいずれか1項に記載の抗体組成物を有効成分として含有する医薬。

(56) 医薬が、腫瘍を伴なう疾患、アレルギーを伴なう疾患、炎症を伴なう疾患、自己免疫疾患、循環器疾患、ウイルス感染を伴なう疾患または細菌感染を伴なう疾患に対する診断薬、予防薬又は治療薬である、(55)に記載の医薬。

(57) 以下の(a)、(b)、(c)、(d)、(e)、(f)、(g)、(h)、(i)及び(j)からなる群から選ばれる蛋白質。

(a) 配列番号71で表されるアミノ酸配列からなる蛋白質；

(b) 配列番号71で表されるアミノ酸配列において、1以上のアミノ酸が欠失、置換、挿入および／または付加されたアミノ酸配列からなり、かつGMD活性を有する蛋白質；

(c) 配列番号72で表されるアミノ酸配列からなる蛋白質；

(d) 配列番号72で表されるアミノ酸配列において、1以上のアミノ酸が欠失、置換、挿入および／または付加されたアミノ酸配列からなり、かつFx活性を有する蛋白質；

(e) 配列番号73で表されるアミノ酸配列からなる蛋白質；

(f) 配列番号73で表されるアミノ酸配列において、1以上のアミノ酸が欠失、置換、挿入および／または付加されたアミノ酸配列からなり、かつGFPP活性を有する蛋白質；

(g) 配列番号23で表されるアミノ酸配列からなる蛋白質；

(h) 配列番号23で表されるアミノ酸配列において、1以上のアミノ酸が欠失、置換、挿入および／または付加されたアミノ酸配列からなり、かつα-1,6-フコシルトランスフェラーゼ活性を有する蛋白質；

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(i) 配列番号 24 で表されるアミノ酸配列からなる蛋白質；

(j) 配列番号 24 で表されるアミノ酸配列において、1以上のアミノ酸が欠失、置換、挿入および／または付加されたアミノ酸配列からなり、かつα-1,6-フコシルトランスフェラーゼ活性を有する蛋白質。

(58) (57) 記載の蛋白質をコードする DNA。

(59) 以下の (a)、(b)、(c)、(d)及び (e) からなる群から選ばれる DNA。

(a) 配列番号 1 で表される塩基配列を含む DNA；

(b) 配列番号 2 で表される塩基配列を含む DNA；

(c) 配列番号 65 で表される塩基配列を含む DNA；

(d) 配列番号 48 で表される塩基配列を含む DNA；

(e) 配列番号 51 で表される塩基配列を含む DNA。

(60) 以下の (a)、(b) 及び (c)からなる群から選ばれるゲノム DNA。

(a) 配列番号 3 で表される塩基配列を含むゲノム DNA；

(b) 配列番号 67 で表される塩基配列を含むゲノム DNA；

(c) 配列番号 70 で表される塩基配列を含むゲノム DNA。

(61) (58) ～(60)のいずれか 1 項に記載の DNA 全長あるいは一部を含む相同組み換えのためのターゲットベクター。

BRIEF EXPLANATION OF THE DRAWINGS

第 1 図は、精製した 5 種類の抗 GD3 キメラ抗体の SDS-PAGE (4～15%グラジエントゲルを使用) の電気泳動パターンを示した図である。1A 図が非還元条件、1B 図が還元条件でそれぞれ電気泳動を行った図である。レーン 1 が高分子量マーカー、2 が YB2/0-GD3 キメラ抗体、3 が CHO/DG44-GD3 キメラ抗体、4 が SP2/0-GD3 キメラ抗体、5 が NSO-GD3 キメラ抗体 (302)、6 が NSO-GD3 キメラ抗体 (GIT)、7 が低分子量マーカーの泳動パターンをそれぞれ示す。

第 2 図は、精製した 5 種類の抗 GD3 キメラ抗体の GD3 との結合活性を抗体濃度を変化させて測定した図である。縦軸は GD3 との結合活性、横軸は抗体濃度をそれぞれ示す。○が YB2/0-GD3 キメラ抗体、●が CHO/DG44-GD3 キメラ抗体、□が SP2/0-GD3 キメラ抗体、■が NSO-GD3 キメラ抗体 (302)、△が NSO-GD3 キメラ抗体 (GIT) の活性をそれぞれ示す。

第 3 図は、精製した 5 種類の抗 GD3 キメラ抗体のヒトメラノーマ細胞株 G-361 に対する ADCC 活性を示した図である。縦軸に細胞障害活性、横軸に抗体濃度をそれぞれ示す。○が YB2/0-GD3 キメラ抗体、●が CHO/DG44-GD3 キメラ抗体、□が SP2/0-GD3 キメラ抗体、■が NSO-GD3 キメラ抗体 (302)、△が NSO-GD3 キメラ抗体 (GIT) の活性をそれぞれ示す。

第 4 図は、精製した 3 種類の抗 hIL-5Rα CDR 移植抗体の SDS-PAGE (4～15%グラジエントゲルを使用) の電気泳動パターンを示した図である。4A 図が非還元条件、4B 図が還元条件でそれぞれ電気泳動を行った図である。レーン 1 が高分子量マーカー、2 が YB2/0-hIL-5RCDR 抗体、3 が CHO/d-hIL-5RCDR 抗体、4 が NSO-hIL-5RCDR 抗体、5 が低分子量マーカーの泳動パターンをそれぞれ示す。

第 5 図は、精製した 3 種類の抗 hIL-5Rα CDR 移植抗体の hIL-5Rα との結合活性を抗体濃度を変化させて測定した結果を示した図である。縦軸は hIL-5Rα との結合活性、横軸は抗体濃

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度をそれぞれ示す。○が YB2/0-hIL-5RCDR 抗体、　が CHO/d-hIL-5RCDR 抗体、□が NS0-hIL-5RCDR 抗体の活性をそれぞれ示す。

第 6 図は、精製した 3 種類の抗 hIL-5Rα CDR 移植抗体の hIL-5R 発現マウス T 細胞株 CTLL-2 (h5R) に対する ADCC 活性を示した図である。縦軸に細胞障害活性、横軸に抗体濃度をそれぞれ示す。○が YB2/0-hIL-5RCDR 抗体、●が CHO/d-hIL-5RCDR 抗体、□が NS0-hIL-5RCDR 抗体の活性をそれぞれ示す。

第 7 図は、精製した 3 種類の抗 hIL-5Rα CDR 移植抗体のカニクイザルの hIL-5 誘発好酸球増加モデルに対する抑制作用を示した図である。縦軸に末梢血中好酸球数、横軸に日数（抗体及び hIL-5 の投与開始日を 0 日とした）をそれぞれ示す。101、102 が抗体非投与群、301、302、303 が YB2/0-hIL-5RCDR 抗体投与群、401、402、403 が CHO/d-hIL-5RCDR 抗体投与群、501、502、503 が NS0-hIL-5RCDR 抗体投与群の結果をそれぞれ示す。

第 8 図は、YB2/0 が生産した精製抗 hIL-5Rα CDR 移植抗体（8A 図）および NS0 が生産した精製抗 hIL-5Rα CDR 移植抗体（8B 図）の PA 化糖鎖の逆相 HPLC 溶離の溶離図（左図）とその PA 化糖鎖をα-L-フコシダーゼ処理した後に逆相 HPLC で分析して得た溶離図（右図）を示したものである。縦軸に相対蛍光強度、横軸に溶出時間をそれぞれ示す。

第 9 図は、CHO/d 細胞が生産した精製抗 hIL-5Rα CDR 移植抗体から PA 化糖鎖を調製し、逆相 HPLC で分析して得た溶離図を示したものである。縦軸に相対蛍光強度、横軸に溶出時間をそれぞれ示す。

第 10 図で、10A 図は、非吸着画分、吸着画分の一部の GD3 との結合活性を、抗体濃度を変化させて測定した図である。縦軸は GD3 との結合活性、横軸は抗体濃度をそれぞれ示す。●が非吸着画分、○が吸着画分の一部をそれぞれ示す。10B 図は非吸着画分、吸着画分の一部のヒトメラノーマ細胞株 G-361 に対する ADCC 活性を示した図である。縦軸に細胞障害活性、横軸に抗体濃度をそれぞれ示す。●が非吸着画分、○が吸着画分の一部をそれぞれ示す。

第 11 図は、非吸着画分、吸着画分の一部から調製した PA 化糖鎖を逆相 HPLC で分析して得た溶離図を示した図である。11A 図に非吸着画分の溶離図、11B 図に吸着画分の一部の溶離図をそれぞれ示す。縦軸に相対蛍光強度、横軸に溶出時間をそれぞれ示す。

第 12 図は、6 種類の抗 GD3 キメラ抗体（12A 図～12F 図）から調製した PA 化糖鎖を、逆相 HPLC で分析して得た溶離図を示した図である。縦軸に相対蛍光強度、横軸に溶出時間をそれぞれ示す。

第 13 図は、α-1,6-フコースを持たない糖鎖の割合が異なる 6 種類の抗 GD3 キメラ抗体の GD3 に対する結合活性を抗体濃度を変化させて測定した図である。縦軸は GD3 との結合活性、横軸は抗体濃度をそれぞれ示す。●が抗 GD3 キメラ抗体（50%）、□が抗 GD3 キメラ抗体（45%）、■が抗 GD3 キメラ抗体（29%）、△が抗 GD3 キメラ抗体（24%）、▲が抗 GD3 キメラ抗体（13%）、×が抗 GD3 キメラ抗体（7%）の活性をそれぞれ示す。

第 14 図は、ドナーA のエフェクター細胞を用いた、α-1,6-フコースを持たない糖鎖の割合が異なる 6 種類の抗 GD3 キメラ抗体のヒトメラノーマ細胞株 G-361 に対する ADCC 活性を示した図である。縦軸に細胞障害活性、横軸に抗体濃度をそれぞれ示す。●が抗 GD3 キメラ抗体（50%）、□が抗 GD3 キメラ抗体（45%）、■が抗 GD3 キメラ抗体（29%）、△が抗 GD3 キメラ抗体（24%）、▲が抗 GD3 キメラ抗体（13%）、×が抗 GD3 キメラ抗体（7%）の活性

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をそれぞれ示す。

第 15 図は、ドナーB のエフェクター細胞を用いた、α-1,6-フコースを持たない糖鎖の割合が異なる 6 種類の抗 GD3 キメラ抗体のヒトメラノーマ細胞株 G-361 に対する ADCC 活性を示した図である。縦軸に細胞障害活性、横軸に抗体濃度をそれぞれ示す。●が抗 GD3 キメラ抗体（50%）、□が抗 GD3 キメラ抗体（45%）、■が抗 GD3 キメラ抗体（29%）、△が抗 GD3 キメラ抗体（24%）、▲が抗 GD3 キメラ抗体（13%）、×が抗 GD3 キメラ抗体（7%）の活性をそれぞれ示す。

第 16 図は、6 種類の抗 CCR4 キメラ抗体から調製した PA 化糖鎖を、逆相 HPLC で分析して得た溶離図を示したものである。縦軸に相対蛍光強度、横軸に溶出時間をそれぞれ示す。

第 17 図は、α-1,6-フコースを持たない糖鎖の割合が異なる 6 種類の抗 CCR4 キメラ抗体の CCR4 に対する結合活性を抗体濃度を変化させて測定した図である。縦軸は CCR4 との結合活性、横軸は抗体濃度をそれぞれ示す。■が抗 CCR4 キメラ抗体（46%）、□が抗 CCR4 キメラ抗体（39%）、▲が抗 CCR4 キメラ抗体（27%）、△が抗 CCR4 キメラ抗体（18%）、●が抗 CCR4 キメラ抗体（9%）、○が抗 CCR4 キメラ抗体（8%）の活性をそれぞれ示す。

第 18 図は、ドナーA のエフェクター細胞を用いた、α-1,6-フコースを持たない糖鎖の割合が異なる抗 CCR4 キメラ抗体の CCR4/EL-4 細胞に対する ADCC 活性を示した図である。縦軸に細胞障害活性、横軸に抗体濃度をそれぞれ示す。■が抗 CCR4 キメラ抗体（46%）、□が抗 CCR4 キメラ抗体（39%）、▲が抗 CCR4 キメラ抗体（27%）、△が抗 CCR4 キメラ抗体（18%）、が抗 CCR4 キメラ抗体（9%）、○が抗 CCR4 キメラ抗体（8%）の活性をそれぞれ示す。また、18A 図はドナーA、18 図はドナーB のエフェクター細胞を用いた結果を示す。

第 19 図は、ドナーB のエフェクター細胞を用いた、α-1,6-フコースを持たない糖鎖の割合が異なる抗 CCR4 キメラ抗体の CCR4/EL-4 細胞に対する ADCC 活性を示した図である。縦軸に細胞障害活性、横軸に抗体濃度をそれぞれ示す。■が抗 CCR4 キメラ抗体（46%）、□が抗 CCR4 キメラ抗体（39%）、▲が抗 CCR4 キメラ抗体（27%）、△が抗 CCR4 キメラ抗体（18%）、が抗 CCR4 キメラ抗体（9%）、○が抗 CCR4 キメラ抗体（8%）の活性をそれぞれ示す。

第 20 図は、プラスミド CHFT8-pCR2.1 および YBFT8-pCR2.1 の構築を示した図である。

第 21 図は、プラスミド CHAc-pBS および YBAc-pBS の構築を示した図である。

第 22 図は、プラスミド CHFT8d-pCR2.1 および YBFT8d-pCR2.1 の構築を示した図である。

第 23 図は、プラスミド CHAcd-pBS および YBAcd-pBS の構築を示した図である。

第 24 図は、競合的 RT-PCR 法を用いた各宿主細胞株における FUT8 転写産物量の定量結果を示した図である。ラット FUT8 配列をスタンダード、内部コントロールに用いた場合の各宿主細胞株における FUT8 転写産物の量を示す。■が CHO 細胞株、□が YB2/0 細胞株を宿主細胞として用いた結果をそれぞれ示す。

第 25 図は、プラスミド mfFUT8-pCR2.1 の構築を示した図である。

第 26 図は、プラスミド pBSmfFUT8 の構築を示した図である。

第 27 図は、プラスミド pAGEmfFUT8 の構築を示した図である。

第 28 図は、競合的 RT-PCR 法を用いた FUT8 遺伝子過剰発現株の該遺伝子発現量解析結果を示した図である。縦軸にβ-アクチン転写量に対する FUT8 転写量の相対値を示す。

第 29 図は、FUT8 遺伝子過剰発現株より精製した抗 GD3 キメラ抗体のヒトメラノーマ細胞

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株 G-361 に対する ADCC 活性を示した図である。縦軸に細胞傷害活性、横軸に抗体濃度をそれぞれ示す。

第 30 図は、mfFUT8-6、pAGE249 導入株によって産生した抗体から調製した PA 化糖鎖を、それぞれ逆相 HPLC で分析して得た溶離図を示したものである。30A 図に mfFUT8-6 株によって産生した抗体から調製した PA 化糖鎖、30B 図に pAGE249 導入株によって産生した抗体から調製した PA 化糖鎖の溶離図をそれぞれ示す。縦軸に相対蛍光強度、横軸に溶出時間をそれぞれ示す。

第 31 図は、Herceptin から調製した PA 化糖鎖を、逆相 HPLC で分析して得た溶離図を示したものである。縦軸に相対蛍光強度、横軸に溶出時間をそれぞれ示す。

第 32 図は、プラスミド CHfFUT8-pCR2.1 の構築を示した図である。

第 33 図は、プラスミド ploxPPuro の構築を示した図である。

第 34 図は、プラスミド pKOFUT8gE2-1 の構築を示した図である。

第 35 図は、プラスミド pKOFUT8gE2-2 の構築を示した図である。

第 36 図は、プラスミド pscFUT8gE2-3 の構築を示した図である。

第 37 図は、プラスミド pKOFUT8gE2-3 の構築を示した図である。

第 38 図は、プラスミド pKOFUT8gE2-4 の構築を示した図である。

第 39 図は、プラスミド pKOFUT8gE2-5 の構築を示した図である。

第 40 図は、プラスミド pKOFUT8Puro の構築を示した図である。

第 41 図は、α-1,6-フコシルトランスフェラーゼ 遺伝子破壊 CHO 細胞株である 1st.△FUT8 2-46-1 株及び 1st.△FUT8 2-46 株のゲノムサザン解析結果を示した図である。

第 42 図は、FUT8 対立遺伝子破壊株より精製した抗 CCR4 キメラ抗体の ADCC 活性を示した図である。縦軸に細胞傷害活性、横軸に抗体濃度をそれぞれ示す。▲、■はそれぞれ、抗 CCR4 キメラ抗体産生 CHO 細胞 5-03 株由来の精製抗体および 1st.△FUT8 2-46-1 株由来の精製抗体の活性をそれぞれ示す。

第 43 図は、レクチン耐性株が生産した抗 CCR4 ヒト型キメラ抗体の ADCC 活性を評価した結果を示した図である。縦軸に細胞傷害活性、横軸に抗体濃度をそれぞれ示す。□は 5-03 株、■は CHO/CCR4-LCA 株、◆は CHO/CCR4-AAL 株、▲は CHO/CCR4-PHA 株が生産した抗体の活性をそれぞれ示す。

第 44 図は、レクチン耐性株が生産した抗 CCR4 ヒト型キメラ抗体の ADCC 活性を評価した結果を示したものである。縦軸に細胞傷害活性、横軸に抗体濃度をそれぞれ示す。□は YB2/0 株 (KM2760#58-35-16)、△は 5-03 株、●は CHO/CCR4-LCA 株が生産した抗体の活性をそれぞれ示す。

第 45 図は、精製した抗 CCR4 ヒト型キメラ抗体から調製した PA 化糖鎖を、逆相 HPLC で分析して得た溶離図を示した図である。縦軸に相対蛍光強度、横軸に溶出時間をそれぞれ示す。45A 図は 5-03 株が生産する抗体、45B 図は CHO/CCR4-LCA 株が生産する抗体、45C 図は CHO/CCR4-AAL 株が生産する抗体、および 45D 図は CHO/CCR4-PHA 株が生産した抗体の分析結果を示す。

第 46 図は、CHO 細胞由来 GMD の発現ベクター構築（全 6 工程）の第 1 の工程を示した図である。

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第 47 図は、CHO 細胞由来 GMD の発現ベクター構築（全 6 工程）の第 2 の工程を示した図である。

第 48 図は、CHO 細胞由来 GMD の発現ベクター構築（全 6 工程）の第 3 の工程を示した図である。

第 49 図は、CHO 細胞由来 GMD の発現ベクター構築（全 6 工程）の第 4 の工程を示した図である。

第 50 図は、CHO 細胞由来 GMD の発現ベクター構築（全 6 工程）の第 5 の工程を示した図である。

第 51 図は、CHO 細胞由来 GMD の発現ベクター構築（全 6 工程）の第 6 の工程を示した図である。

第 52 図は、GMD を発現させた CHO/CCR4-LCA 株の LCA レクチンに対する耐性度を示した図である。LCA レクチンを添加せずに培養した細胞群の生存率を 100%とし、２回測定を行った図である。図中 249 は、発現ベクターpAGE249 を導入した CHO/CCR4-LCA 株の LCA レクチンに対する生存率を示す。GMD は GMD 発現ベクターpAGE249GMD を導入した CHO/CCR4-LCA 株の LCA レクチンに対する耐性度を示す。

第 53 図は、GMD を発現させた CHO/CCR4-LCA 株の細胞群が生産した抗 CCR4 キメラ抗体の ADCC 活性を示した図である。縦軸に細胞傷害活性、横軸に抗体濃度をそれぞれ示す。

第 54 図は、CHO 細胞由来の GMD cDNA クローン 22-8 の 5' 末端にクローン 34-2 の 5' 末端を導入したプラスミド CHO-GMD の作製工程を示した図である。

第 55 図は、GMD 遺伝子を発現させた CHO/CCR4-LCA 株より精製した抗 CCR4 キメラ抗体から調製した PA 化糖鎖を、逆相 HPLC で分析して得た溶離図を示した図である。縦軸に相対蛍光強度、横軸に溶出時間をそれぞれ示す。

DETAILED DESCRIPTION OF THE INVENTION

本発明の抗体分子をコードする遺伝子を導入したチャイニーズハムスター卵巣組織由来 CHO 細胞とは、N-グリコシド結合複合型糖鎖を Fc 領域に有する抗体分子からなる組成物であって、該組成物中に含まれる Fc 領域に結合する全 N-グリコシド結合複合型糖鎖のうち、糖鎖還元末端の N-アセチルグルコサミンにフコースが結合していない糖鎖の割合が２０%以上である抗体組成物を生産する、抗体分子をコードする遺伝子を導入したチャイニーズハムスター卵巣組織由来の CHO 細胞であればいかなる CHO 細胞も包含される。

本発明において、抗体分子とは、抗体の Fc 領域を含む分子であればいかなる分子も包含される。具体的には、抗体、抗体の断片、Fc 領域を含む融合タンパク質などをあげることができる。

抗体とは、外来抗原刺激の結果、免疫反応によって生体内に産生される蛋白質で、抗原と特異的に結合する活性を有するものをいう。抗体としては動物に抗原を免疫し、免疫動物の脾臓細胞より作製したハイブリドーマ細胞が分泌する抗体のほか、遺伝子組換え技術により作製された抗体、すなわち、抗体遺伝子を挿入した抗体発現ベクターを、宿主細胞へ導入することにより取得された抗体などがあげられる。具体的には、ハイブリドーマが生産する抗体、ヒト化抗体、ヒト抗体などをあげることができる。

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ハイブリドーマとは、ヒト以外の哺乳動物に抗原を免疫して取得されたB細胞と、マウス等に由来するミエローマ細胞とを細胞融合させて得られる、所望の抗原特異性を有したモノクローナル抗体を産生する細胞を意味する。

ヒト化抗体としては、ヒト型キメラ抗体、ヒト型CDR移植抗体などがあげられる。

ヒト型キメラ抗体は、ヒト以外の動物の抗体重鎖可変領域（以下、重鎖はH鎖としてHVまたはVHとも称す）および抗体軽鎖可変領域（以下、軽鎖はL鎖としてLVまたはVLとも称す）とヒト抗体の重鎖定常領域（以下、CHとも称す）およびヒト抗体の軽鎖定常領域（以下、CLとも称す）とからなる抗体を意味する。ヒト以外の動物としては、マウス、ラット、ハムスター、ラビット等、ハイブリドーマを作製することが可能であれば、いかなるものも用いることができる。

ヒト型キメラ抗体は、モノクローナル抗体を生産するハイブリドーマより、VHおよびVLをコードするcDNAを取得し、ヒト抗体CHおよびヒト抗体CLをコードする遺伝子を有する宿主細胞用発現ベクターにそれぞれ挿入してヒト型キメラ抗体発現ベクターを構築し、宿主細胞へ導入することにより発現させ、製造することができる。

ヒト型キメラ抗体のCHとしては、ヒトイムノグロブリン（以下、hIgと表記する）に属すればいかなるものでもよいが、hIgGクラスのものが好適であり、更にhIgGクラスに属するhIgG1、hIgG2、hIgG3、hIgG4といったサブクラスのいずれも用いることができる。また、ヒト型キメラ抗体のCLとしては、hIgに属すればいかなるものでもよく、κクラスあるいはλクラスのものを用いることができる。

ヒト型CDR移植抗体は、ヒト以外の動物の抗体のVHおよびVLのCDRのアミノ酸配列をヒト抗体のVHおよびVLの適切な位置に移植した抗体を意味する。

ヒト型CDR移植抗体は、ヒト以外の動物の抗体のVHおよびVLのCDR配列を任意のヒト抗体のVHおよびVLのCDR配列に移植したV領域をコードするcDNAを構築し、ヒト抗体のCHおよびヒト抗体のCLをコードする遺伝子を有する宿主細胞用発現ベクターにそれぞれ挿入してヒト型CDR移植抗体発現ベクターを構築し、該発現ベクターを宿主細胞へ導入することによりヒト型CDR移植抗体を発現させ、製造することができる。

ヒト型CDR移植抗体のCHとしては、hIgに属すればいかなるものでもよいが、hIgGクラスのものが好適であり、更にhIgGクラスに属するhIgG1、hIgG2、hIgG3、hIgG4といったサブクラスのいずれも用いることができる。また、ヒト型CDR移植抗体のCLとしては、hIgに属すればいかなるものでもよく、κクラスあるいはλクラスのものを用いることができる。

ヒト抗体は、元来、ヒト体内に天然に存在する抗体を意味するが、最近の遺伝子工学的、細胞工学的、発生工学的な技術の進歩により作製されたヒト抗体ファージライブラリーならびにヒト抗体産生トランスジェニック動物あるいはヒト抗体産生トランスジェニック植物から得られる抗体等も含まれる。

ヒト体内に存在する抗体は、例えば、ヒト末梢血リンパ球を単離し、EBウイルス等を感染させ不死化、クローニングすることにより、該抗体を産生するリンパ球を培養でき、培養物中より該抗体を精製することができる。

ヒト抗体ファージライブラリーは、ヒトB細胞から調製した抗体遺伝子をファージ遺伝子に挿入することによりFab、一本鎖抗体等の抗体断片をファージ表面に発現させたライブラ

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リーである。該ライブラリーより、抗原を固定化した基質に対する結合活性を指標として所望の抗原結合活性を有する抗体断片を発現しているファージを回収することができる。該抗体断片は、更に遺伝子工学的手法により、2本の完全なH鎖および2本の完全なL鎖からなるヒト抗体分子へも変換することができる。

ヒト抗体産生トランスジェニック非ヒト動物は、ヒト抗体遺伝子が細胞内に組込まれた動物を意味する。具体的には、マウスES細胞へヒト抗体遺伝子を導入し、該ES細胞を他のマウスの初期胚へ移植後、発生させることによりヒト抗体産生トランスジェニック動物を作製することができる。また、動物の受精卵にヒト抗体遺伝子を導入し、該受精卵を発生させることにヒト抗体酸産生トランスジェニック動物を作製することもできる。ヒト抗体産生トランスジェニック動物からのヒト抗体の作製方法は、通常のヒト以外の哺乳動物で行われているハイブリドーマ作製方法によりヒト抗体産生ハイブリドーマを得、培養することで培養物中にヒト抗体を産生蓄積させることができる。

トランスジェニック非ヒト動物は、ウシ、ヒツジ、ヤギ、ブタ、ウマ、マウス、ラット、ニワトリ、サル又はウサギ等があげられる。

また、本発明において、抗体が、腫瘍関連抗原を認識する抗体、アレルギーあるいは炎症に関連する抗原を認識する抗体、循環器疾患に関連する抗原を認識する抗体、自己免疫疾患に関連する抗原を認識する抗体、またはウイルスあるいは細菌感染に関連する抗原を認識する抗体であることが好ましく、抗体のクラスがIgGのヒト抗体が好ましい。

抗体の断片とは、上記抗体のFc領域を含んだ断片を意味する。抗体の断片としては、H鎖の単量体、H鎖の2量体などがあげられる。

Fc領域を含む融合タンパク質とは、抗体のFc領域を含んだ抗体あるいは抗体の断片と、酵素、サイトカインなどのタンパク質とを融合させた物質を意味する。

本発明において、抗体分子のFc領域に結合する糖鎖としては、N-グリコシド結合糖鎖が挙げられ、そのN-グリコシド結合糖鎖としては、コア構造の非還元末端側にガラクトース-N-アセチルグルコサミン（以下、Gal-GlcNAcと表記する）の枝を並行して1ないしは複数本有し、更にGal-GlcNAcの非還元末端側にシアル酸、バイセクティングのN-アセチルグルコサミンなどの構造を有するコンプレックス型（複合型）をあげることができる。

抗体分子のFc領域には、後述するN-グリコシド結合糖鎖がそれぞれ1カ所ずつ結合する領域を有しているので、抗体1分子あたり2本の糖鎖が結合している。抗体に結合するN-グルコシド結合糖鎖としては、前記構造式（I）で示されるコア構造を有するいかなる糖鎖も包含されるので、抗体に結合する2本のN-グルコシド結合糖鎖には多数の糖鎖の組み合わせが存在することになる。したがって、Fc領域に結合した糖鎖構造の観点から物質の同一性を判断することができる。

本発明において、N-グリコシド結合複合型糖鎖をFc領域に有する抗体分子からなる組成物（以下、本発明の抗体組成物と称する）とは、本発明の効果が得られる範囲であれば、単一の糖鎖構造を有する抗体から構成されていてもよいし、複数の異なる糖鎖構造を有する糖鎖から構成されていてもよい。

本発明において、抗体組成物中に含まれるFc領域に結合する全N-グリコシド結合複合型糖鎖のうち、糖鎖還元末端のN-アセチルグルコサミンにフコースが結合していない糖鎖の割

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合とは、該組成物中に含まれる Fc 領域に結合する全ての N-グリコシド結合複合型糖鎖の合計数に対して、糖鎖還元末端の N-アセチルグルコサミンにフコースが結合していない糖鎖の数が占める割合をいう。

本発明において、N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンにフコースが結合していない糖鎖とは、該フコースが、N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンにα結合していない糖鎖を意味する。具体的には、該フコースの１位が N-グリコシド結合複合型糖鎖の N-アセチルグルコサミンの６位にα結合していない糖鎖があげられる。

本発明の抗体組成物中に含まれる Fc 領域に結合する全 N-グリコシド結合複合型糖鎖のうち、糖鎖還元末端の N-アセチルグルコサミンにフコースが結合していない糖鎖の割合が、好ましくは２０％以上、より好ましくは２５％以上、さらに好ましくは３０％以上、特に好ましくは４０％以上、最も好ましくは５０％以上である抗体組成物は、高い ADCC 活性を有する。抗体濃度が低下すれば、それに伴って ADCC 活性が低下するが、糖鎖還元末端の N-アセチルグルコサミンにフコースが結合していない糖鎖の割合が２０％以上の場合、抗体濃度が低くても高い ADCC 活性を獲得することができる。

N-グリコシド結合複合型糖鎖を Fc 領域に有する抗体分子からなる組成物中に含まれる、糖鎖還元末端の N-アセチルグルコサミンにフコースが結合していない糖鎖の割合は、抗体分子からヒドラジン分解や酵素消化などの公知の方法[生物化学実験法 23―糖タンパク質糖鎖研究法（学会出版センター）高橋禮子編（1989）]を用い、糖鎖を遊離させ、遊離させた糖鎖を蛍光標識又は同位元素標識し、標識した糖鎖をクロマトグラフィー法にて分離することによって決定することができる。また、遊離させた糖鎖を HPAED-PAD 法[J. Liq. Chromatogr., 6, 1577 (1983)]によって分析することによっても決定することができる。

本発明において、チャイニーズハムスター卵巣組織由来の CHO 細胞とは、チャイニーズハムスター（Chinese hamster ; *Cricetulus griseus*）の卵巣組織から樹立された株化細胞であればいかなる細胞も包含される。その具体的な例としては、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. 60, 1275 (1968)、Cell, 6, 121 (1975)、Molecular Cell Genetics, Appendix I, II (p883-900)等の文献に記載されている CHO 細胞をあげることができる。また、ATCC (The American Type Culture Collection) に登録されている CHO-K1 株 (ATCC CCL-61)、DUXB11 株 (ATCC CRL-9096)、Pro-5 株 (ATCC CRL-1781) や、市販の CHO-S 株 (Lifetechnologies 社 Cat#11619)、あるいはこれら株を様々な培地に馴化させた亜株なども具体的な例としてあげることができる。

本発明において、細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素とは、細胞内で糖鎖へのフコースの供給源である糖ヌクレオチド GDP-フコースの合成に関与する酵素であればいかなる酵素も包含される。細胞内糖ヌクレオチド GDP-フコースの合成に係わる酵素とは、細胞内糖ヌクレオチド GDP-フコースの合成に影響を与える酵素のことを意味する。

細胞内の糖ヌクレオチド GDP-フコースは、de novo の合成経路あるいは Salvage 合成経路

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により供給されている。したがって、これら合成経路に関与する酵素はすべて細胞内 GDP-フコースの合成に係わる酵素に包含される。

細胞内の糖ヌクレオチド GDP-フコースの de novo の合成経路に関与する酵素としては、具体的には、GDP-mannose 4,6-dehydratase (GDP-マンノース 4,6-デヒドラターゼ；以下、GMD と表記する)、GDP-keto-6-deoxymannose 3,5-epimerase, 4,6-reductase (GDP-ケト-デオキシマンノース 3,5-エピメラーゼ, 4,6-リダクターゼ；以下、Fx と表記する) などがあげられる。

細胞内の糖ヌクレオチド GDP-フコースの Salvage 合成経路に関与する酵素としては、具体的には、GDP-beta-L-fucose pyrophosphorylase (GDP-ベータ-L-フコース-ピロホスフォリラーゼ；以下、GFPP と表記する)、Fucokinase (フコキナーゼ) などがあげられる。

細胞内糖ヌクレオチド GDP-フコースの合成に影響を与える酵素としては、上述の細胞内の糖ヌクレオチド GDP-フコースの合成経路に関与する酵素の活性に影響を与えたり、該酵素の基質となる物質の構造に影響を与える酵素も包含される。

本発明において、GMD としては、

下記(a)または(b)の DNA がコードする蛋白質、

(a) 配列番号 65 で表される塩基配列からなる DNA

(b) 配列番号 65 で表される塩基配列からなる DNA とストリンジェントな条件でハイブリダイズし、かつ GMD 活性を有する蛋白質をコードする DNA

または、

(c) 配列番号 71 で表されるアミノ酸配列からなる蛋白質

(d) 配列番号 71 で表されるアミノ酸配列において、１以上のアミノ酸が欠失、置換、挿入および／または付加されたアミノ酸配列からなり、かつ GMD 活性を有する蛋白質

(e) 配列番号 71 で表されるアミノ酸配列と８０%以上の相同性を有するアミノ酸配列からなり、かつ GMD 活性を有する蛋白質

等があげられる。

また、GMD のアミノ酸配列をコードする DNA としては、配列番号 65 で表される塩基配列を有する DNA、配列番号 65 で表される塩基配列を有する DNA とストリンジェントな条件でハイブリダイズし、かつ GMD 活性を有するアミノ酸配列をコードする DNA などがあげられる。

本発明において、Fx としては、

下記(a)または(b)の DNA がコードする蛋白質、

(a) 配列番号 48 で表される塩基配列からなる DNA

(b) 配列番号 48 で表される塩基配列からなる DNA とストリンジェントな条件でハイブリダイズし、かつ Fx 活性を有する蛋白質をコードする DNA

または、

(c) 配列番号 72 で表されるアミノ酸配列からなる蛋白質

(d) 配列番号 72 で表されるアミノ酸配列において、１以上のアミノ酸が欠失、置換、挿入および／または付加されたアミノ酸配列からなり、かつ Fx 活性を有する蛋白質

(e) 配列番号 72 で表されるアミノ酸配列と８０%以上の相同性を有するアミノ酸配列からなり、かつ Fx 活性を有する蛋白質

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等があげられる。

また、Fx のアミノ酸配列をコードする DNA としては、配列番号 48 で表される塩基配列を有する DNA、配列番号 48 で表される塩基配列を有する DNA とストリンジェントな条件でハイブリダイズし、かつ Fx 活性を有するアミノ酸配列をコードする DNA などがあげられる。

本発明において、GFPP としては、

下記(a)または(b)の DNA がコードする蛋白質、

(a) 配列番号 51 で表される塩基配列からなる DNA

(b) 配列番号 51 で表される塩基配列からなる DNA とストリンジェントな条件でハイブリダイズし、かつ GFPP 活性を有する蛋白質をコードする DNA

または、

(c) 配列番号 73 で表されるアミノ酸配列からなる蛋白質

(d) 配列番号 73 で表されるアミノ酸配列において、1以上のアミノ酸が欠失、置換、挿入および／または付加されたアミノ酸配列からなり、かつ GFPP 活性を有する蛋白質

(e) 配列番号 73 で表されるアミノ酸配列と８０％以上の相同性を有するアミノ酸配列からなり、かつ GFPP 活性を有する蛋白質

等があげられる。

また、GFPP のアミノ酸配列をコードする DNA としては、配列番号 51 で表される塩基配列を有する DNA、配列番号 51 で表される塩基配列を有する DNA とストリンジェントな条件でハイブリダイズし、かつ Fx 活性を有するアミノ酸配列をコードする DNA などがあげられる。

本発明において、N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの 6 位にフコースの 1 位がα結合する糖鎖修飾に関与する酵素とは、N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの 6 位とフコースの 1 位がα結合する反応に関与する酵素であればいかなる酵素も包含される。N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの 6 位とフコースの 1 位がα結合する反応に関与する酵素とは、N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの 6 位とフコースの 1 位がα結合する反応に影響を与える酵素を意味する。

N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの 6 位にフコースの 1 位がα結合する反応に関与する酵素としては、具体的には、α-1,6-フコシルトランスフェラーゼやα-L-フコシダーゼなどがあげられる。

また、上述の N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの 6 位とフコースの 1 位がα結合する反応に関与する酵素の活性に影響を与えたり、該酵素の基質となる物質の構造に影響を与える酵素も包含される。

本発明において、α-1,6-フコシルトランスフェラーゼとしては、

下記(a)、(b)、(c)または(d)の DNA がコードする蛋白質、

(a) 配列番号 1 で表される塩基配列からなる DNA

(b) 配列番号 2 で表される塩基配列からなる DNA

(c) 配列番号 1 で表される塩基配列からなる DNA とストリンジェントな条件でハイブリダイズし、かつα-1,6-フコシルトランスフェラーゼ活性を有する蛋白質をコードする DNA

(d) 配列番号 2 で表される塩基配列からなる DNA とストリンジェントな条件でハイブリダ

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イズし、かつα-1,6-フコシルトランスフェラーゼ活性を有する蛋白質をコードするDNA
または、
(e) 配列番号23で表されるアミノ酸配列からなる蛋白質
(f) 配列番号24で表されるアミノ酸配列からなる蛋白質
(g) 配列番号23で表されるアミノ酸配列において、1以上のアミノ酸が欠失、置換、挿入および／または付加されたアミノ酸配列からなり、かつα-1,6-フコシルトランスフェラーゼ活性を有する蛋白質
(h) 配列番号24で表されるアミノ酸配列において、1以上のアミノ酸が欠失、置換、挿入および／または付加されたアミノ酸配列からなり、かつα-1,6-フコシルトランスフェラーゼ活性を有する蛋白質
(i) 配列番号23で表されるアミノ酸配列と８０％以上の相同性を有するアミノ酸配列からなり、かつα-1,6-フコシルトランスフェラーゼ活性を有する蛋白質
(j) 配列番号24で表されるアミノ酸配列と８０％以上の相同性を有するアミノ酸配列からなり、かつα-1,6-フコシルトランスフェラーゼ活性を有する蛋白質
等があげられる。

また、α-1,6-フコシルトランスフェラーゼのアミノ酸配列をコードするDNAとしては、配列番号1または2で表される塩基配列を有するDNA、配列番号1または2で表される塩基配列を有するDNAとストリンジェントな条件でハイブリダイズし、かつα-1,6-フコシルトランスフェラーゼ活性を有するアミノ酸配列をコードするDNAなどがあげられる。

本発明において、ストリンジェントな条件下でハイブリダイズするDNAとは、例えば配列番号1、2、48、51または65で表される塩基配列を有するDNAなどのDNAまたはその一部の断片をプローブとして、コロニー・ハイブリダイゼーション法、プラーク・ハイブリダイゼーション法あるいはサザンブロットハイブリダイゼーション法等を用いることにより得られるDNAを意味し、具体的には、コロニーあるいはプラーク由来のDNAを固定化したフィルターを用いて、０．７～１．０Mの塩化ナトリウム存在下、６５℃でハイブリダイゼーションを行った後、０．１～２倍濃度のＳＳＣ溶液（１倍濃度のＳＳＣ溶液の組成は、１５０mM塩化ナトリウム、１５mMクエン酸ナトリウムよりなる）を用い、６５℃条件下でフィルターを洗浄することにより同定できるDNAをあげることができる。ハイブリダイゼーションは、Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989)（以下、モレキュラー・クローニング第２版と略す）、Current Protocols in Molecular Biology, John Wiley & Sons, 1987-1997（以下、カレント・プロトコールズ・イン・モレキュラー・バイオロジーと略す）、DNA Cloning 1: Core Techniques, A Practical Approach, Second Edition, Oxford University (1995)等に記載されている方法に準じて行うことができる。ハイブリダイズ可能なDNAとして具体的には、配列番号1、2、48、51または65で表される塩基配列と少なくとも６０％以上の相同性を有するDNA、好ましくは７０％以上、より好ましくは８０％以上、さらに好ましくは９０％以上、特に好ましくは９５％以上、最も好ましくは９８％以上の相同性を有するDNAをあげることができる。

本発明において、配列番号23、24、71、72または73で表されるアミノ酸配列において1以上のアミノ酸が欠失、置換、挿入および／または付加されたアミノ酸配列からなり、かつ

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α-1,6-フコシルトランスフェラーゼ活性、GMD 活性、Fx 活性または GFPP 活性を有する蛋白質は、モレキュラー・クローニング第2版、カレント・プロトコールズ・イン・モレキュラー・バイオロジー、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)等に記載の部位特異的変異導入法を用いて、例えば、配列番号1、2、65、48または51で表されるアミノ酸配列を有する蛋白質をコードする DNA に部位特異的変異を導入することにより取得することができる。欠失、置換、挿入および/または付加されるアミノ酸の数は1個以上でありその数は特に限定されないが、上記の部位特異的変異導入法等の周知の技術により、欠失、置換もしくは付加できる程度の数であり、例えば、1～数十個、好ましくは1～20個、より好ましくは1～10個、さらに好ましくは1～5個である。

また、本発明において、用いられる蛋白質が、α-1,6-フコシルトランスフェラーゼ活性、GMD 活性、Fx 活性または GFPP 活性を有するためには、それぞれ配列番号 23、24、71、72 または 73 で表されるアミノ酸配列とＢＬＡＳＴ[J. Mol. Biol., 215, 403 (1990)]やＦＡＳＴＡ[Methods in Enzymology, 183, 63 (1990)]等の解析ソフトを用いて計算したときに、少なくとも８０％以上、好ましくは８５％以上、より好ましくは９０％以上、さらに好ましくは９５％以上、特に好ましくは９７％以上、最も好ましくは９９％以上の相同性を有する。

本発明の CHO 細胞としては、上述の酵素活性が低下または欠失した細胞があげられる。

上述の酵素活性が低下または欠失した細胞としては、すなわち、細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素の活性または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの6位にフコースの1位がα結合する糖鎖修飾に関与する酵素の活性が親株より低下または欠失した細胞を包含する。このような細胞を取得する方法としては、目的とする酵素活性を低下または欠失させることができる手法であれば、いずれの手法でも用いることができる。上述の酵素活性を低下または欠失させる手法としては、

（a）酵素の遺伝子を標的した遺伝子破壊の手法；

（b）酵素の遺伝子のドミナントネガティブ体を導入する手法；

（c）酵素についての突然変異を導入する手法；

（d）酵素の遺伝子の転写又は翻訳を抑制する手法；

（e）N-グリコシド結合糖鎖還元末端の N-アセチルグルコサミンの6位とフコースの1位がα結合した糖鎖構造を認識するレクチンに耐性である株を選択する手法

などがあげられる。

ここで、レクチンに耐性である株は、ある一定濃度のレクチンを含む培地中で培養した場合に、親株に比べて統計的な有意差を伴って少なくとも2倍、好ましくは3倍、より好ましくは5倍以上生存率に差が生じる性質を獲得する株を選択することで取得することができる。また、レクチンを含む培地中で培養した場合に、ある一定の生存率、例えば８０％の生存率、で培養可能なレクチンの濃度が、親株に比べ少なくとも2倍、好ましくは5倍、より好ましくは10倍、さらに好ましくは20倍以上の濃度となる株を選択することでも取得することができる。

N-グリコシド結合糖鎖還元末端の N-アセチルグルコサミンの6位とフコースの1位がα結

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合した糖鎖構造を認識するレクチンとしては、該糖鎖構造を認識できるレクチンであれば、いずれのレクチンでも用いることができる。その具体的な例としては、レンズマメレクチン LCA (*Lens Culinaris* 由来の Lentil Agglutinin)、エンドウマメレクチン PSA (*Pisum sativum* 由来の Pea Lectin)、ソラマメレクチン VFA (Vicia faba 由来の Agglutinin)、ヒイロチャワンタケレクチン AAL (*Aleuria aurantia* 由来の Lectin) 等をあげることができる。

本発明の CHO 細胞は、上記の目的とする酵素活性を低下または欠失させる手法を施す前の親株である CHO 細胞が生産する抗体組成物より、ADCC 活性が高い抗体組成物を生産することができる。

また、本発明の CHO 細胞は、抗体組成物中に含まれる Fc 領域に結合する全 N-グリコシド結合複合型糖鎖のうち、糖鎖還元末端の N-アセチルグルコサミンとフコースが結合していない糖鎖の割合が２０％未満である抗体組成物よりも ADCC 活性が高い抗体組成物を生産することができる。

本発明において親株としては、例えば、細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素の活性または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの 6 位にフコースの 1 位がα結合する糖鎖修飾に関与する酵素の活性が低下していない細胞があげられる。具体的には、細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素の活性または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素の活性を低下または欠失させるような処理を施していない細胞が用いられる。

本発明において、ADCC 活性とは、生体内で、腫瘍細胞等の細胞表面抗原などに結合した抗体が、抗体 Fc 領域とエフェクター細胞表面上に存在する Fc レセプターとの結合を介してエフェクター細胞を活性化し、腫瘍細胞等を障害する活性を意味する [Monoclonal Antibodies: Principles and Applications, Wiley-Liss, Inc., Capter 2.1 (1995)]。エフェクター細胞としては、キラー細胞、ナチュラルキラー細胞、活性化されたマクロファージ等があげられる。

本発明は、また、細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素の活性または N-グリコシド結合等鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素の活性または N-グリコシド結合糖鎖修飾に関与する酵素の活性が、遺伝子工学的な手法により低下した細胞（以下、本発明の宿主細胞と略記する）に関する。本発明の宿主細胞は、ADCC 活性が高い抗体組成物を生産するための宿主細胞として有用である。

本発明の宿主細胞としては、抗体分子を発現できる宿主細胞であればいかなる細胞も包含する。その例として、酵母、動物細胞、昆虫細胞、植物細胞などがあげられる。これらの細胞の具体的な例としては、後述の３．に記載のものがあげられる。特に、動物細胞の中でも、チャイニーズハムスター卵巣組織由来の CHO 細胞、ラットミエローマ細胞株 YB2/3HL.P2.G11.16Ag.20 細胞、マウスミエローマ細胞株 NS0 細胞、マウスミエローマ細胞株 SP2/0-Ag14 細胞、シリアンハムスター腎臓組織由来 BHK 細胞、抗体を産生するハイブリドーマ細胞、ヒト白血病細胞株ナマルバ細胞、胚性幹細胞、受精卵細胞などが好ましい。

以下、本発明を詳細に説明する。

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1．　本発明の宿主細胞の作製

本発明の宿主細胞は、以下に述べる手法により作製することができる。

（1）酵素の遺伝子を標的とした遺伝子破壊の手法

本発明の宿主細胞は、細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素またはN-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素の遺伝子を標的とし、遺伝子破壊の方法を用いることにより作製することができる。細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素としては、具体的には、GMD、Fx、GFPP、Fucokinase などがあげられる。N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素としては、具体的には、α-1,6-フコシルトランスフェラーゼ、α-L-フコシダーゼなどがあげられる。

ここでいう遺伝子とは、DNA または RNA を含む。

遺伝子破壊の方法としては、標的とする酵素の遺伝子を破壊することができる方法であればいかなる方法も包含される。その例としては、アンチセンス法、リボザイム法、相同組換え法、RDO 法、RNAi 法、レトロウイルスを用いた方法、トランスポゾンを用いた方法等があげられる。以下これらを具体的に説明する。

（a）アンチセンス法又はリボザイム法による本発明の宿主細胞の作製

本発明の宿主細胞は、細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素またはN-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素遺伝子を標的とし、細胞工学，12，239（1993）、BIO/TECHNOLOGY，17，1097（1999）、Hum. Mol. Genet.，5，1083（1995）、細胞工学，13，255（1994）、Proc. Natl. Acad. Sci. USA，96，1886（1999）等に記載されたリボザイム法を用いて、例えば、以下のように作製することができる。

細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素をコードする cDNA あるいはゲノム DNA を調製する。

調製したあるいはゲノム DNA の塩基配列を決定する。

決定した DNA の配列に基づき、細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素をコードする DNA 部分、非翻訳領域の部分あるいはイントロン部分を含む適当な長さのアンチセンス遺伝子またはリボザイムのコンストラクトを設計する。

該アンチセンス遺伝子、またはリボザイムを細胞内で発現させるために、調製した DNA の断片、または全長を適当な発現ベクターのプロモーターの下流に挿入することにより、組換えベクターを作製する。

該組換えベクターを、該発現ベクターに適合した宿主細胞に導入することにより形質転換体を得る。

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細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素の活性または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素の活性を指標として形質転換体を選択することにより、本発明の宿主細胞を得ることができる。また、細胞膜上の糖タンパク質の糖鎖構造または産生抗体分子の糖鎖構造を指標として形質転換体を選択することにより、本発明の宿主細胞を得ることもできる。

本発明の宿主細胞を作製するために用いられる宿主細胞としては、酵母、動物細胞、昆虫細胞、植物細胞など、標的とする細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素の遺伝子を有しているものであればいずれも用いることができる。具体的には、後述の３．に記載の宿主細胞があげられる。

発現ベクターとしては、上記宿主細胞において自立複製可能ないしは染色体中への組み込みが可能で、設計したアンチセンス遺伝子、またはリボザイムを転写できる位置にプロモーターを含有しているものが用いられる。具体的には、後述の３．に記載の発現ベクターがあげられる。

各種宿主細胞への遺伝子の導入方法としては、後述の３．に記載の各種宿主細胞に適した組換えベクターの導入方法を用いることができる。

細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素の活性または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素の活性を指標として形質転換体を選択する方法としては、例えば、以下の方法があげられる。

<u>形質転換体を選択する方法</u>

細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素の活性または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素の活性が低下した細胞を選択する方法としては、文献［新生化学実験講座３―糖質Ⅰ，糖タンパク質(東京化学同人)日本生化学会編(1988)］、文献[細胞工学，別冊，実験プロトコールシリーズ，グライコバイオロジー実験プロトコール，糖タンパク質･糖脂質･プロテオグリカン(秀潤社製)谷口直之・鈴木明美・古川清・菅原一幸監修（1996）]、モレキュラー･クローニング第２版、カレント･プロトコールズ･イン･モレキュラー･バイオロジー等に記載された生化学的な方法あるいは遺伝子工学的な方法などがあげられる。生化学的な方法としては、例えば、酵素特異的な基質を用いて酵素活性を評価する方法があげられる。遺伝子工学的な方法としては、例えば、酵素遺伝子の mRNA 量を測定するノーザン解析やRT-PCR 法等があげられる。

細胞膜上の糖タンパク質の糖鎖構造を指標として形質転換体を選択する方法としては、例えば、後述の１の（５）に記載の方法があげられる。産生抗体分子の糖鎖構造を指標として形質転換体を選択する方法としては、例えば、後述の５または後述の６に記載の方法があげられる。

細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合

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型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素をコードする cDNA を調製する方法としては、例えば、以下に記載の方法があげられる。

DNA の調製方法

ヒト又は非ヒト動物の組織又は細胞から全 RNA 又は mRNA を調製する。

調製した全 RNA 又は mRNA から cDNA ライブラリーを作製する。

細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素のアミノ酸配列に基づいて、デジェネレイティブプライマーを作製し、作製した cDNA ライブラリーを鋳型として PCR 法にて 細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素をコードする遺伝子断片を取得する。

取得した遺伝子断片をプローブとして用い、cDNA ライブラリーをスクリーニングし、細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素をコードする DNA を取得することができる。

ヒト又は非ヒト動物の組織又は細胞の mRNA は市販のもの(例えば Clontech 社)を用いてもよいし、以下のごとくヒト又は非ヒト動物の組織又は細胞から調製してもよい。ヒト又は非ヒト動物の組織又は細胞から全 RNA を調製する方法としては、チオシアン酸グアニジン-トリフルオロ酢酸セシウム法 [Methods in Enzymology, 154, 3 (1987)]、酸性チオシアン酸グアニジン・フェノール・クロロホルム（ＡＧＰＣ）法 [Analytical Biochemistry, 162, 156 (1987); 実験医学、9, 1937 (1991)] などがあげられる。

また、全 RNA からｐｏｌｙ（Ａ）＋RNA として mRNA を調製する方法としては、オリゴ（ｄＴ）固定化セルロースカラム法（モレキュラー・クローニング第２版）等があげられる。

さらに、Fast Track mRNA Isolation Kit（Invitrogen 社）、Quick Prep mRNA Purification Kit (Pharmacia 社) などのキットを用いることにより mRNA を調製することができる。

調製したヒト又は非ヒト動物の組織又は細胞 mRNA から cDNA ライブラリーを作製する。cDNA ライブラリー作製法としては、モレキュラー・クローニング第２版、カレント・プロトコールズ・イン・モレキュラー・バイオロジー、A Laboratory Manual, 2nd Ed. (1989) 等に記載された方法、あるいは市販のキット、例えば SuperScript Plasmid System for cDNA Synthesis and Plasmid Cloning（Life Technologies 社）、ZAP-cDNA Synthesis Kit (STRATAGENE 社) を用いる方法などがあげられる。

cDNA ライブラリーを作製するためのクローニングベクターとしては、大腸菌 K12 株中で自立複製できるものであれば、ファージベクター、プラスミドベクター等いずれでも使用できる。具体的には、ZAP Express [STRATAGENE 社、Strategies, 5, 58 (1992)]、pBluescript II SK(+) [Nucleic Acids Research, 17, 9494 (1989)]、Lambda ZAP II (STRATAGENE 社)、

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λ gt10、λ gt11 [DNA cloning, A Practical Approach, 1, 49 (1985)]、λ TriplEx (Clontech 社)、λ ExCell (Pharmacia 社)、pT7T318U (Pharmacia 社)、pcD2 [Mol. Cell. Biol., 3, 280 (1983)] および pUC18 [Gene, 33, 103 (1985)] 等をあげることができる。

宿主微生物としては、微生物であればいずれでも用いることができるが、好ましくは大腸菌が用いられる。具体的には、*Escherichia coli* XL1-Blue MRF' [STRATAGENE 社、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)] および *Escherichia coli* JM105 [Gene, 38, 275 (1985)] 等が用いられる。

この cDNA ライブラリーを、そのまま以降の解析に用いてもよいが、不完全長 cDNA の割合を下げ、なるべく完全長 cDNA を効率よく取得するために、菅野らが開発したオリゴキャップ法 [Gene, 138, 171 (1994); Gene, 200, 149 (1997); 蛋白質核酸酵素, 41, 603 (1996); 実験医学, 11, 2491 (1993); cDNA クローニング(羊土社)(1996); 遺伝子ライブラリーの作製法(羊土社) (1994)] を用いて調製した cDNA ライブラリーを以下の解析に用いてもよい。

細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素のアミノ酸配列に基づいて、該アミノ酸配列をコードすることが予測される塩基配列の 5' 端および 3' 端の塩基配列に特異的なデジェネレイティブプライマーを作製し、作製した cDNA ライブラリーを鋳型として PCR 法 [PCR Protocols, Academic Press (1990)] を用いて DNA の増幅を行うことにより、細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素をコードする遺伝子断片を取得することができる。

取得した遺伝子断片が細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素をコードする DNA であることは、通常用いられる塩基配列解析方法、例えば Sanger らのジデオキシ法 [Proc. Natl. Acad. Sci. USA, 74, 5463 (1977)] あるいはＡＢＩＰＲＩＳＭ３７７DNA シークエンサー (PE Biosystems 社製) 等の塩基配列分析装置を用いて分析することにより、確認することができる。

該遺伝子断片を DNA をプローブとして、ヒト又は非ヒト動物の組織又は細胞に含まれる mRNA から合成した cDNA あるいは cDNA ライブラリー対してコロニーハイブリダイゼーションやプラークハイブリダイゼーション（モレキュラー・クローニング第２版）を行うことにより、細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素の DNA を取得することができる。

また、細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖

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修飾に関与する酵素をコードする遺伝子断片を取得するために用いたプライマーを用い、ヒト又は非ヒト動物の組織又は細胞に含まれる mRNA から合成した cDNA あるいは cDNA ライブラリーを鋳型として、PCR 法を用いてスクリーニングを行うことにより、細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素の DNA を取得することもできる。

取得した細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素をコードする DNA の塩基配列を末端から、通常用いられる塩基配列解析方法、例えば Sanger らのジデオキシ法［Proc. Natl. Acad. Sci. USA, 74, 5463 (1977)］あるいはＡＢＩＰＲＩＳＭ３７７DNA シークエンサー (PE Biosystems 社製) 等の塩基配列分析装置を用いて分析することにより、該 DNA の塩基配列を決定する。

決定した cDNA の塩基配列をもとに、ＢＬＡＳＴ等の相同性検索プログラムを用いて、ＧｅｎＢａｎｋ、ＥＭＢＬおよびＤＤＢＪなどの塩基配列データベースを検索することにより、データベース中の遺伝子の中で細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素をコードしている遺伝子を決定することもできる。

上記の方法で得られる細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素をコードする遺伝子の塩基配列としては、例えば、配列番号 48、51 または 65 に記載の塩基配列があげられる。N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素をコードする遺伝子の塩基配列としては、例えば、配列番号１または２に記載の塩基配列があげられる。

決定された DNA の塩基配列に基づいて、フォスフォアミダイト法を利用したパーキン・エルマー社の DNA 合成機 model 392 等の DNA 合成機で化学合成することにより、細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素の cDNA を取得することもできる。

細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素のゲノム DNA を調製する方法としては、例えば、以下に記載の方法があげられる。

<u>ゲノム DNA の調製方法</u>

ゲノム DNA を調製する方法としては、モレキュラー・クローニング第２版やカレント・プロトコールズ・イン・モレキュラー・バイオロジー等に記載された公知の方法があげられる。また、ゲノム DNA ライブラリースクリーニングシステム (Genome Systems 社) や Universal GenomeWalker™ Kits (CLONTECH 社) などを用いることにより、細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素のゲノム DNA を単離することもできる。

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上記の方法で得られる細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素のゲノム DNA の塩基配列として、例えば配列番号 67 または 70 に記載の塩基配列があげられる。N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素のゲノム DNA の塩基配列として、例えば配列番号３に記載の塩基配列があげられる。

また、発現ベクターを用いず、細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素の塩基配列に基づいて設計したアンチセンスオリゴヌクレオチドまたはリボザイムを、直接宿主細胞に導入することで、本発明の宿主細胞を得ることもできる。

アンチセンスオリゴヌクレオチドまたはリボザイムは、常法または DNA 合成機を用いることにより調製することができる。具体的には、細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素をコードする cDNA およびゲノム DNA の塩基配列のうち、連続した５～１５０塩基、好ましくは５～６０塩基、より好ましくは１０～４０塩基に相当する配列を有するオリゴヌクレオチドの配列情報に基づき、該オリゴヌクレオチドと相補的な配列に相当するオリゴヌクレオチド（アンチセンスオリゴヌクレオチド）または該オリゴヌクレオチドの配列を含むリボザイムを合成することで調製することができる。

オリゴヌクレオチドとしては、オリゴ RNA および該オリゴヌクレオチドの誘導体（以下、オリゴヌクレオチド誘導体という）等があげられる。

オリゴヌクレオチド誘導体としては、オリゴヌクレオチド中のリン酸ジエステル結合がホスフォロチオエート結合に変換されたオリゴヌクレオチド誘導体、オリゴヌクレオチド中のリン酸ジエステル結合がＮ３'－Ｐ５'ホスフォアミデート結合に変換されたオリゴヌクレオチド誘導体、オリゴヌクレオチド中のリボースとリン酸ジエステル結合がペプチド核酸結合に変換されたオリゴヌクレオチド誘導体、オリゴヌクレオチド中のウラシルがＣ－５プロピニルウラシルで置換されたオリゴヌクレオチド誘導体、オリゴヌクレオチド中のウラシルがＣ－５チアゾールウラシルで置換された誘導体オリゴヌクレオチド、オリゴヌクレオチド中のシトシンがＣ－５プロピニルシトシンで置換されたオリゴヌクレオチド誘導体、オリゴヌクレオチド中のシトシンが phenoxazine-modified cytosine で置換されたオリゴヌクレオチド誘導体、オリゴヌクレオチド中のリボースが２'－Ｏ－プロピルリボースで置換されたオリゴヌクレオチド誘導体、あるいはオリゴヌクレオチド中のリボースが２'－メトキシエトキシリボースで置換されたオリゴヌクレオチド誘導体等があげられる［細胞工学，16，1463 (1997)］。

（ｂ）相同組換え法による本発明の宿主細胞の作製

本発明の宿主細胞は、細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素の遺伝子を標的とし、染色体上の標的遺伝子を相同組換

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え法を用い改変することによって作製することができる。

染色体上の標的遺伝子の改変は、Manipulating the Mouse Embryo A Laboratory Manual, S cond Edition, Cold Spring Harbor Laboratory Press (1994) (以下、「マニピュレイティング・ザ・マウス・エンブリオ・ア・ラボラトリー・マニュアル」と略す)、Gene Targeting, A Practical Approach, IRL Press at Oxford University Press (1993)、バイオマニュアルシリーズ8 ジーンターゲッティング, ＥＳ細胞を用いた変異マウスの作製, 羊土社 (1995) (以下、「ＥＳ細胞を用いた変異マウスの作製」と略す) 等に記載の方法を用い、例えば以下のように行うことができる。

細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素のゲノム DNA を調製する。

ゲノム DNA の塩基配列にも基づき、改変する標的遺伝子 (例えば、細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素の構造遺伝子、あるいはプロモーター遺伝子) を相同組換えするためのターゲットベクターを作製する。

作製したターゲットベクターを宿主細胞に導入し、標的遺伝子とターゲットベクターの間で相同組換えを起こした細胞を選択することにより、本発明の宿主細胞を作製することができる。

宿主細胞としては、酵母、動物細胞、昆虫細胞、植物細胞等、標的とする細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素の遺伝子を有しているものであればいずれも用いることができる。具体的には、後述の３．に記載の宿主細胞があげられる。

細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素のゲノム DNA を調製する方法としては、上記１の (1) の (a) に記載のゲノム DNA の調製方法などがあげられる。

上記の方法で得られる細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素のゲノム DNA の塩基配列として、例えば配列番号 67 または 70 に記載の塩基配列があげられる。N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素のゲノム DNA の塩基配列として、例えば配列番号３に記載の塩基配列があげられる。

標的遺伝子を相同組換えするためのターゲットベクターは、 Gene Targeting, A Practical Approach, IRL Press at Oxford University Press (1993)、バイオマニュアルシリーズ8 ジーンターゲッティング, ＥＳ細胞を用いた変異マウスの作製(羊土社)(1995) 等に記載の方法にしたがって作製することができる。ターゲットベクターは、リプレースメント型、インサーション型いずれでも用いることができる。

各種宿主細胞へのターゲットベクターの導入には、後述の３．に記載の各種宿主細胞に適した組換えベクターの導入方法を用いることができる。

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相同組換え体を効率的に選別する方法として、例えば、Gene Targeting, A Practical Approach, IRL Press at Oxford University Press (1993)、バイオマニュアルシリーズ８ジーンターゲッティング，ＥＳ細胞を用いた変異マウスの作製(羊土社)(1995)等に記載のポジティブ選択、プロモーター選択、ネガティブ選択、ポリＡ選択などの方法を用いることができる。選別した細胞株の中から目的とする相同組換え体を選択する方法としては、ゲノムDNA に対するサザンハイブリダイゼーション法（モレキュラー・クローニング第２版）やPCR 法 [PCR Protocols, Academic Press (1990)] 等があげられる。

（ｃ）RDO 方法による本発明の細胞の作製

本発明の宿主細胞は、細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素またはN-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素の遺伝子を標的とし、RDO (RNA-DNA oligonucleotide)法を用い、例えば、以下のように作製することができる。

細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素の cDNA あるいはゲノム DNA を調製する。

調製した cDNA あるいはゲノム DNA の塩基配列を決定する。

決定した DNA の配列に基づき、細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素をコードする部分、非翻訳領域の部分あるいはイントロン部分を含む適当な長さの RDO のコンストラクトを設計し合成する。

合成した RDO を宿主細胞に導入し、標的とした酵素、すなわち細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素に変異が生じた形質転換体を選択することにより、本発明の宿主細胞を作製することができる。

宿主細胞としては、酵母、動物細胞、昆虫細胞、植物細胞等、標的とする細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合型糖鎖還元末端のN-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素の遺伝子を有しているものであればいずれも用いることができる。具体的には、後述の３．に記載の宿主細胞があげられる。

各種宿主細胞への RDO の導入には、後述の３．に記載の各種宿主細胞に適した組み換えベクターの導入方法を用いることができる。

細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素の cDNA を調製する方法としては、例えば、上記１の（１）の（ａ）に記載の「DNA の調製方法」などがあげられる。

細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素のゲノム DNA を調製する方法としては、例えば、上記１の（１）の（ａ）に記

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載のゲノムDNAの調製方法などがあげられる。

DNA の塩基配列は、適当な制限酵素などで切断後、pBluescript SK(-) (Stratagene 社製) 等のプラスミドにクローニングし、通常用いられる塩基配列解析方法、例えば、サンガー (Sanger) らのジデオキシ法 [Proc. Natl. Acad. Sci. USA, 74, 5463 (1977)] 等の反応を行い、塩基配列自動分析装置、例えば、A. L. F. DNA シークエンサー (Pharmacia 社製) 等を用いて解析することで該DNAの塩基配列を決定することができる。

RDOは、常法またはDNA合成機を用いることにより調製することができる。

RDO を宿主細胞に導入し、標的とした酵素、細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの6位にフコースの1位がα結合する糖鎖修飾に関与する酵素の遺伝子に変異が生じた細胞を選択する方法としては、モレキュラー・クローニング第2版、カレント・プロトコールズ・イン・モレキュラー・バイオロジー等に記載された染色体上の遺伝子の変異を直接検出する方法があげられる。

また、前記1の (1) の (a) に記載の、導入した細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素の活性または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの6位にフコースの1位がα結合する糖鎖修飾に関与する酵素の活性を指標として形質転換体を選択する方法、後述の1の (5) に記載の細胞膜上の糖タンパク質の糖鎖構造を指標として形質転換体を選択する方法、あるいは、後述の5または後述の6に記載の産生抗体分子の糖鎖構造を指標として形質転換体を選択する方法も用いることができる。

RDO のコンストラクトは、Science, 273, 1386 (1996); Nature Medicine, 4, 285 (1998); Hepatology, 25, 1462 (1997); Gene Therapy, 5, 1960 (1999); 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. Dematol., 111, 1172 (1998); Nature Biotech., 16, 1343 (1998); Nature Biotech., 18, 43 (2000); Nature Biotech., 18, 555 (2000) 等の記載に従って設計することができる。

(d) RNAi 方法による本発明の宿主細胞の作製

本発明の宿主細胞は、細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの6位にフコースの1位がα結合する糖鎖修飾に関与する酵素の遺伝子を標的とし、RNAi (RNA interference) 法を用い、例えば、以下のように作製することができる。

細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの6位にフコースの1位がα結合する糖鎖修飾に関与する酵素の cDNA を調製する。

調製した cDNA の塩基配列を決定する。

決定した DNA の配列に基づき、細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの6位にフコースの1位がα結合する糖鎖修飾に関与する酵素をコードする部分あるいは非翻訳領域の部分を

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含む適当な長さのRNAi遺伝子のコンストラクトを設計する。

該RNAi遺伝子を細胞内で発現させるために、調製したDNAの断片、または全長を適当な発現ベクターのプロモーターの下流に挿入することにより、組換えベクターを作製する。

該組換えベクターを、該発現ベクターに適合した宿主細胞に導入することにより形質転換体を得る。

導入した細胞内糖ヌクレオチドGDP-フコースの合成に関与する酵素の活性またはN-グリコシド結合複合型糖鎖還元末端のN-アセチルグルコサミンの6位にフコースの1位がα結合する糖鎖修飾に関与する酵素の活性、あるいは産生抗体分子または細胞表面上の糖タンパク質の糖鎖構造を指標に形質転換体を選択することで、本発明の宿主細胞を得ることができる。

宿主細胞としては、酵母、動物細胞、昆虫細胞、植物細胞等、標的とする細胞内糖ヌクレオチドGDP-フコースの合成に関与する酵素またはN-グリコシド結合複合型糖鎖還元末端のN-アセチルグルコサミンの6位にフコースの1位がα結合する糖鎖修飾に関与する酵素の遺伝子を有しているものであればいずれも用いることができる。具体的には、後述の3．に記載の宿主細胞があげられる。

発現ベクターとしては、上記宿主細胞において自立複製可能ないしは染色体中への組み込みが可能で、設計したRNAi遺伝子を転写できる位置にプロモーターを含有しているものが用いられる。具体的には、後述の3．に記載の発現ベクターがあげられる。

各種宿主細胞への遺伝子の導入には、後述の3．に記載の各種宿主細胞に適した組換えベクターの導入方法を用いることができる。

細胞内糖ヌクレオチドGDP-フコースの合成に関与する酵素の活性またはN-グリコシド結合複合型糖鎖還元末端のN-アセチルグルコサミンの6位にフコースの1位がα結合する糖鎖修飾に関与する酵素の活性を指標として形質転換体を選択する方法としては、例えば、前記1の（1）の（a）に記載の方法があげられる。

細胞膜上の糖タンパク質の糖鎖構造を指標として形質転換体を選択する方法としては、例えば、後述の1の（5）に記載の方法があげられる。産生抗体分子の糖鎖構造を指標として形質転換体を選択する方法としては、例えば、後述の5または後述の6に記載の方法があげられる。

細胞内糖ヌクレオチドGDP-フコースの合成に関与する酵素またはN-グリコシド結合複合型糖鎖還元末端のN-アセチルグルコサミンの6位にフコースの1位がα結合する糖鎖修飾に関与する酵素のcDNAを調製する方法としては、例えば、前記1の（1）の（a）に記載されたDNAの調製方法などがあげられる。

また、発現ベクターを用いず、細胞内糖ヌクレオチドGDP-フコースの合成に関与する酵素またはN-グリコシド結合複合型糖鎖還元末端のN-アセチルグルコサミンの6位にフコースの1位がα結合する糖鎖修飾に関与する酵素の塩基配列に基づいて設計したRNAi遺伝子を、直接宿主細胞に導入することで、本発明の宿主細胞を得ることもできる。

RNAi遺伝子は、常法またはDNA合成機を用いることにより調製することができる。

RNAi遺伝子のコンストラクトは、[Nature, 391, 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);

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Proc. Natl. Acad. Sci. USA, 95, 13959 (1998); Nature Cell Biol., 2, 70 (2000)]等の記載に従って設計することができる。

（e）トランスポゾンを用いた方法による、本発明の宿主細胞の作製

本発明の宿主細胞は、Nature Genet., 25, 35 (2000)等に記載のトランスポゾンのシステムを用い、細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素の活性または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素の活性、あるいは産生抗体分子または細胞膜上の糖タンパク質の糖鎖構造を指標に突然変異体を選択することで、本発明の宿主細胞を作製することができる。

トランスポゾンのシステムとは、外来遺伝子をランダムに染色体上に挿入させることで突然変異を誘発させるシステムであり、通常、トランスポゾンに挿まれた外来遺伝子を突然変異を誘発させるベクターとして用い、この遺伝子を染色体上にランダムに挿入させるためのトランスポゼースの発現ベクターを同時に細胞の中に導入する。

トランスポゼースは、用いるトランスポゾンの配列に適したものであればいかなるものも用いることができる。

外来遺伝子としては、宿主細胞の DNA に変異を誘起するものであればいかなる遺伝子も用いることができる。

宿主細胞としては、酵母、動物細胞、昆虫細胞、植物細胞等、標的とする細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素の遺伝子を有しているものであればいずれも用いることができる。具体的には、後述の３．に記載の宿主細胞があげられる。各種宿主細胞への遺伝子の導入には、後述の３．に記載の各種宿主細胞に適した組み換えベクターの導入方法を用いることができる。

細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素の活性または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素の活性を指標として突然変異体を選択する方法としては、例えば、前記１の（１）の（a）に記載の方法があげられる。

細胞膜上の糖タンパク質の糖鎖構造を指標として突然変異体を選択する方法としては、例えば、後述の１の（５）に記載の方法があげられる。産生抗体分子の糖鎖構造を指標として突然変異体を選択する方法としては、例えば、後述の５または後述の６に記載の方法があげられる。

（２）酵素の遺伝子のドミナントネガティブ体を導入する手法

本発明の宿主細胞は、細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素の遺伝子を標的とし、該酵素のドミナントネガティブ体を導入する手法を用いることにより作製することができる。細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素としては、具体的には、GMD、Fx、GFPP、Fucokinase などがあげ

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られる。N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素としては、具体的には、α-1,6-フコシルトランスフェラーゼ、α-L-フコシダーゼなどがあげられる。

これらの酵素は、基質特異性を有したある特定の反応を触媒する酵素であり、このような基質特異性を有した触媒作用を有する酵素の活性中心を破壊することで、これらの酵素のドミナントネガティブ体を作製することができる。標的とする酵素のうち、GMD を例として、そのドミナントネガティブ体に作製について具体的に以下に述べる。

大腸菌由来の GMD の立体構造を解析した結果、４つのアミノ酸（133 番目のトレオニン、135 番目のグルタミン酸、157 番目のチロシン、161 番目のリシン）が酵素活性に重要な機能を担っていることが明らかにされている（Structure, 8, 2, 2000)。すなわち、立体構造の情報にもとづきこれら４つのアミノ酸を異なる他のアミノ酸に置換した変異体を作製した結果、いずれの変異体においても有意に酵素活性が低下していたことが示されている。一方、GMD の補酵素 NADP や基質である GDP-マンノースとの結合能に関しては、いずれの変異体においてもほとんど変化が観察されていない。従って、GMD の酵素活性を担うこれら４つのアミノ酸を置換することによりドミナントネガティブ体を作製することができる。大腸菌由来の GMD の結果に基づき、アミノ酸配列情報をもとにした相同性比較や立体構造予測を行うことにより、例えば、CHO 細胞由来の GMD（配列番号 65）では、155 番目のトレオニン、157 番目のグルタミン酸、179 番目のチロシン、183 番目のリシンを他のアミノ酸に置換することによりドミナントネガティブ体を作製することができる。このようなアミノ酸置換を導入した遺伝子の作製は、モレキュラー・クローニング第２版、カレント・プロトコールズ・イン・モレキュラー・バイオロジー等に記載された部位特異的変異導入法を用いて行うことができる。

本発明の宿主細胞は、上述のように作製した標的酵素のドミナントネガティブ体遺伝子を用い、モレキュラー・クローニング第２版、カレント・プロトコールズ・イン・モレキュラー・バイオロジー、マニピュレーティング・マウス・エンブリオ第２版等に記載された遺伝子導入の方法に従って、例えば、以下のように作製することができる。

細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素のドミナントネガティブ体をコードする遺伝子（以下、ドミナントネガティブ体遺伝子と略記する）を調製する。

調製したドミナントネガティブ体遺伝子の全長 DNA をもとにして、必要に応じて、該タンパク質をコードする部分を含む適当な長さの DNA 断片を調製する。

該 DNA 断片、または全長 DNA を適当な発現ベクターのプロモーターの下流に挿入することにより、組換えベクターを作製する。

該組換えベクターを、該発現ベクターに適合した宿主細胞に導入することにより、形質転換体を得る。

細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素の活性または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素の活性、あるいは産生抗体分子または細胞膜上の糖タンパク質の糖鎖構

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造を指標に形質転換体を選択することで、本発明の宿主細胞を作製することができる。
宿主細胞としては、酵母、動物細胞、昆虫細胞、植物細胞等、標的とする細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素の遺伝子を有しているものであればいずれも用いることができる。具体的には、後述の３．に記載の宿主細胞があげられる。

発現ベクターとしては、上記宿主細胞において自立複製可能ないしは染色体中への組み込みが可能で、目的とするドミナントネガティブ体をコードする DNA を転写できる位置にプロモーターを含有しているものが用いられる。具体的には、後述の３．に記載の発現ベクターがあげられる。

各種宿主細胞への遺伝子の導入には、後述の３．に記載の各種宿主細胞に適した組み換えベクターの導入方法を用いることができる。

細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素の活性または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素の活性を指標として形質転換体を選択する方法としては、例えば、前記１の（１）の（ａ）に記載の方法があげられる。

細胞膜上の糖タンパク質の糖鎖構造を指標として形質転換体を選択する方法としては、例えば、後述の１の（５）に記載の方法があげられる。産生抗体分子の糖鎖構造を指標として形質転換体を選択する方法としては、例えば、後述の５または後述の６に記載の方法があげられる。

（３）酵素についての突然変異を導入する手法

本発明の宿主細胞は、細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素の遺伝子について突然変異を導入し、該酵素に突然変異を生じた所望の細胞株を選択する手法を用いることにより作製できる。

細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素としては、GMD、Fx、GFPP、Fucokinase などがあげられる。N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素としては、具体的には、α-1,6-フコシルトランスフェラーゼ、α-L-フコシダーゼなどがあげられる。

方法としては、１）突然変異誘発処理で親株を処理した突然変異体あるいは自然発生的に生じた突然変異体から、細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素の活性または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素の活性を指標として所望の細胞株を選択する方法、２）突然変異誘発処理で親株を処理した突然変異体あるいは自然発生的に生じた突然変異体から、生産抗体分子の糖鎖構造を指標として所望の細胞株を選択する方法、３）突然変異誘発処理で親株を処理した突然変異体あるいは自然発生的に生じた突然変異体から、該細胞の細胞膜上の糖タンパク質の糖鎖構造を指標として所望の細胞株を選択する方法などがあげられる。

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突然変異誘発処理としては、親株の細胞の DNA に点突然変異、欠失あるいはフレームシフト突然変異を誘起するものであればいかなる処理も用いることができる。
具体的には、エチルニトロソウレア、ニトロソグアニジン、ベンゾピレン、アクリジン色素による処理、放射線の照射などがあげられる。また、種々のアルキル化剤や発癌物質も突然変異誘発物質として用いることができる。突然変異誘発物質を細胞に作用させる方法としては、例えば、組織培養の技術 第三版（朝倉書店）日本組織培養学会編(1996)、Nature Genet., 24, 314, (2000)等に記載の方法をあげることができる。

自然発生的に生じた突然変異体としては、特別な突然変異誘発処理を施さないで、通常の細胞培養の条件で継代培養を続けることによって自然発生的に生じる突然変異体をあげることができる。

細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素の活性または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素の活性を測定する方法としては、例えば、前記１の（１）の（ａ）に記載の方法があげられる。産生抗体分子の糖鎖構造を識別する方法としては、例えば、後述の５または後述の６に記載の方法があげられる。細胞膜上の糖タンパク質の糖鎖構造を識別する方法としては、例えば、後述の１の（５）に記載の方法があげられる。

（４）酵素の遺伝子の転写又は翻訳を抑制する手法

本発明の宿主細胞は、細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素の遺伝子を標的とし、アンチセンス RNA／DNA 技術［バイオサイエンスとインダストリー, 50, 322 (1992)；化学, 46, 681 (1991)、Biotechnology, 9, 358 (1992)、Trends in Biotechnology, 10, 87 (1992)、Trends in Biotechnology, 10, 152 (1992)、細胞工学, 16, 1463 (1997)］、トリプル・ヘリックス技術［Trends in Biotechnology, 10, 132 (1992)］等を用い、標的とする遺伝子の転写または翻訳を抑制することで作製することができる。

細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素としては、具体的には、GMD、Fx、GFPP、Fucokinase などがあげられる。N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素としては、具体的には、α-1,6-フコシルトランスフェラーゼ、α-L-フコシダーゼなどがあげられる。

（５）N-グリコシド結合糖鎖還元末端の N-アセチルグルコサミンの６位とフコースの１位がα結合した糖鎖構造を認識するレクチンに耐性である株を選択する手法

本発明の宿主細胞は、N-グリコシド結合糖鎖還元末端の N-アセチルグルコサミンの６位とフコースの１位がα結合した糖鎖構造を認識するレクチンに耐性である株を選択する手法を用いることにより作製することができる。

N-グリコシド結合糖鎖還元末端の N-アセチルグルコサミンの６位とフコースの１位がα結合した糖鎖構造を認識するレクチンに耐性である株を選択する手法としては、例えば、Somatic Cell Mol. Genet., 12, 51 (1986)等に記載のレクチンを用いた方法があげられる。

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レクチンとしては、N-グリコシド結合糖鎖還元末端の N-アセチルグルコサミンの６位とフコースの１位がα結合した糖鎖構造を認識するレクチンであればいずれのレクチンでも用いることができるが、その具体的な例としては、レンズマメレクチン LCA (*Lens Culinaris* 由来の Lentil Agglutinin)、エンドウマメレクチン PSA (*Pisum sativum* 由来の Pea Lectin)、ソラマメレクチン VFA (Vicia faba 由来の Agglutinin)、ヒイロチャワンタケレクチン AAL (*Aleuria aurantia* 由来の Lectin) 等をあげることができる。

具体的には、１μg/ml～１mg/ml の濃度の上述のレクチンを含む培地で１日～２週間、好ましくは１日～１週間培養し、生存している細胞を継代培養あるいはコロニーをピックアップし別の培養器に移し、さらに引き続きレクチンを含む培地で培養を続けることによってことで、本発明の N-グリコシド結合糖鎖還元末端の N-アセチルグルコサミンの６位とフコースの１位がα結合した糖鎖構造を認識するレクチンに耐性である株を選択することができる。上記方法で得られる株としては、例えば、後述の実施例 14(2) で取得した CHO/CCR4-LCA 株 Nega-13 (FERM BP-7756) があげられる。

２．本発明の、トランスジェニック非ヒト動物あるいは植物またはそれら子孫の作製

本発明の、トランスジェニック非ヒト動物あるいは植物またはそれら子孫は、抗体分子の糖鎖の修飾に係わる酵素の活性が制御されるようにゲノム遺伝子が改変されたトランスジェニック非ヒト動物あるいは植物またはそれら子孫は、細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素の遺伝子を標的として、１．に記載の手法と同様の手法を用いて作製することができる。

トランスジェニック非ヒト動物の場合、目的とする非ヒト動物、例えばウシ、ヒツジ、ヤギ、ブタ、ウマ、マウス、ラット、ニワトリ、サル、ウサギ等の胚性幹細胞に、１．に記載の手法と同様の手法を用いることにより、細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素の活性または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素の活性が制御された本発明の胚性幹細胞を作製することができる。

具体的には、染色体上の細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素の活性または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素をコードする遺伝子を公知の相同組換えの手法[例えば、Nature, 326, 6110, 295 (1987)、Cell, 51, 3, 503 (1987)等] により不活化または任意の配列と置換した変異クローンを作製する。作製した該変異クローンを用い、動物の受精卵の胚盤胞(blastcyst)への注入キメラ法または集合キメラ法等の手法により、胚性幹細胞クローンと正常細胞からなるキメラ個体を調製することができる。このキメラ個体と正常個体の掛け合わせにより、全身の細胞で細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素の活性または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素の活性が低下または欠失したトランスジェニック非ヒト動物を得ることができる。

また、目的とする非ヒト動物、例えばウシ、ヒツジ、ヤギ、ブタ、ウマ、マウス、ラット、

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ニワトリ、サル、ウサギ等の受精卵細胞に、１．に記載の手法と同様の手法を用いることにより、細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素の活性または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素の活性が低下または欠失した本発明の受精卵細胞を作製することができる。

作製した受精卵細胞を、マニピューレーティング・マウス・エンブリオ第２版等に記載の胚移植の方法を用いて偽妊娠雌の卵管あるいは子宮に移植し出産させることで、細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素の活性または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素の活性が低下したトランスジェニック非ヒト動物を作製することができる。

トランスジェニック植物の場合、目的とする植物体カルス又は細胞に、１．に記載の手法と同様の手法を用いることにより、細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素の活性または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位あるいは３位にフコースの１位がα結合する糖鎖修飾に関与する酵素の活性が低下または欠失した本発明のカルスを作製することができる。

作製したカルスを、公知の方法[組織培養, 20 (1994); 組織培養, 21 (1995); Trends in Biotechnology, 15, 45 (1997)]に準じてオーキシン及びサイトカイニンを含む培地で培養することで再分化させ、細胞内糖ヌクレオチド GDP-フコースの合成に関与する酵素の活性または N-グリコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンの６位あるいは３位にフコースの１位がα結合する糖鎖修飾に関与する酵素の活性が低下したトランスジェニック植物を作製することができる。

３．抗体組成物の製造方法

抗体組成物は、モレキュラー・クローニング第２版、カレント・プロトコールズ・イン・モレキュラー・バイオロジー、Antibodies, A Laboratory manual, Cold Spring Harbor Laboratory, 1988 (以下、アンチボディズとも略す)、Monoclonal Antibodies: principles and practice, Third Edition, Acad. Press, 1993 (以下、モノクローナルアンチボディズとも略す)、Antibody Engineering, A Practical Approach, IRL Press at Oxford University Press, 1996 (以下、アンチボディエンジニアリングとも略す) 等に記載された方法を用い、例えば、以下のように宿主細胞中で発現させて取得することができる。

抗体分子の全長 cDNA を調製し、該抗体分子をコードする部分を含む適当な長さの DNA 断片を調製する。

該 DNA 断片、または全長 cDNA を適当な発現ベクターのプロモーターの下流に挿入することにより、組換えベクターを作製する。

該組換えベクターを、該発現ベクターに適合した宿主細胞に導入することにより、抗体分子を生産する形質転換体を得ることができる。

宿主細胞としては、酵母、動物細胞、昆虫細胞、植物細胞等、目的とする遺伝子を発現できるものであればいずれも用いることができる。

抗体分子の Fc 領域に結合する N-グリコシド結合糖鎖の修飾に係わる酵素を、遺伝子工学的

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な手法を用いて導入した、酵母、動物細胞、昆虫細胞、植物細胞等の細胞を宿主細胞として用いることもできる。

発現ベクターとしては、上記宿主細胞において自立複製可能ないしは染色体中への組込が可能で、目的とする抗体分子をコードする DNA を転写できる位置にプロモーターを含有しているものが用いられる。

cDNA は、上記1の（1）の（a）に記載の DNA の調製方法に従い、ヒト又は非ヒト動物の組織又は細胞より、目的とする抗体分子に特異的なプローブプライマー等を用いて調製することができる。

酵母を宿主細胞として用いる場合には、発現ベクターとして、例えば、YEP13 (ATCC37115)、YEp24 (ATCC37051)、YCp50 (ATCC37419) 等をあげることができる。

プロモーターとしては、酵母菌株中で発現できるものであればいずれのものを用いてもよく、例えば、ヘキソースキナーゼ等の解糖系の遺伝子のプロモーター、ＰＨＯ５プロモーター、ＰＧＫプロモーター、ＧＡＰプロモーター、ＡＤＨプロモーター、gal 1プロモーター、gal 10 プロモーター、ヒートショックタンパク質プロモーター、MFα1 プロモーター、CUP 1プロモーター等をあげることができる。

宿主細胞としては、サッカロミセス属、シゾサッカロミセス属、クリュイベロミセス属、トリコスポロン属、シュワニオミセス属等に属する微生物、例えば、*Saccharomyces cerevisiae*、*Schizosaccharomyces pombe*、*Kluyveromyces lactis*、*Trichosporon pullulans*、*Schwanniomyces alluvius* 等をあげることができる。

組換えベクターの導入方法としては、酵母に DNA を導入する方法であればいずれも用いることができ、例えば、エレクトロポレーション法 [Methods. Enzymol., 194, 182 (1990)]、スフェロプラスト法 [Proc. Natl. Acad. Sci. USA, 84, 1929 (1978)]、酢酸リチウム法 [J. Bacteriology, 153, 163 (1983)]、Proc. Natl. Acad. Sci. USA, 75, 1929 (1978)に記載の方法等をあげることができる。

動物細胞を宿主として用いる場合には、発現ベクターとして、例えば、pcDNAI、pcDM8 (フナコシ社より市販)、pAGE107 [特開平 3-22979 ; Cytotechnology, 3, 133 (1990)]、pAS3-3 [特開平 2-227075]、pCDM8 [Nature, 329, 840 (1987)]、pcDNAI/Amp (Invitrogen 社)、pREP4 (Invitrogen 社)、pAGE103 [J. Biochemistry, 101, 1307 (1987)]、pAGE210 等をあげることができる。

プロモーターとしては、動物細胞中で発現できるものであればいずれも用いることができ、例えば、サイトメガロウイルス (CMV) の IE (immediate early) 遺伝子のプロモーター、SV40 の初期プロモーター、レトロウイルスのプロモーター、メタロチオネインプロモーター、ヒートショックプロモーター、ＳＲαプロモーター等をあげることができる。また、ヒトＣＭＶのＩＥ遺伝子のエンハンサーをプロモーターと共に用いてもよい。

宿主細胞としては、ヒトの細胞である Namalwa 細胞、サルの細胞である COS 細胞、チャイニーズ・ハムスターの細胞である CHO 細胞、HBT5637 (特開昭 63-299)、ラットミエローマ細胞、マウスミエローマ細胞、シリアンハムスター腎臓由来細胞、胚性幹細胞、受精卵細胞等をあげることができる。

組換えベクターの導入方法としては、動物細胞に DNA を導入する方法であればいずれも用

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いることができ、例えば、エレクトロポレーション法 [Cytotechnology, 3, 133 (1990)]、リン酸カルシウム法 [特開平 2-227075]、リポフェクション法 [Proc. Natl. Acad. Sci. USA, 84, 7413 (1987)]、インジェクション法[マニピュレイティング・ザ・マウス・エンブリオ・ア・ラボラトリー・マニュアル]、パーティクルガン（遺伝子銃）を用いる方法 [特許第2606856、特許第 2517813]、ＤＥＡＥ-デキストラン法 [バイオマニュアルシリーズ４—遺伝子導入と発現・解析法（羊土社）横田崇・新井賢一編(1994)]、ウイルスベクター法[マニピュレーティング・マウス・エンブリオ第２版]等をあげることができる。

昆虫細胞を宿主として用いる場合には、例えばカレント・プロトコールズ・イン・モレキュラー・バイオロジー、Baculovirus Expression Vectors, A Laboratory Manual, W. H. Freeman and Company, New York (1992)、Bio/Technology, 6, 47 (1988)等に記載された方法によって、タンパク質を発現することができる。

即ち、組換え遺伝子導入ベクターおよびバキュロウイルスを昆虫細胞に共導入して昆虫細胞培養上清中に組換えウイルスを得た後、さらに組換えウイルスを昆虫細胞に感染させ、タンパク質を発現させることができる。

該方法において用いられる遺伝子導入ベクターとしては、例えば、pVL1392、pVL1393、pBlueBacIII（ともに Invitorogen 社）等をあげることができる。

バキュロウイルスとしては、例えば、夜盗蛾科昆虫に感染するウイルスである Autographa californica nuclear polyhedrosis virus 等を用いることができる。

昆虫細胞としては、*Spodopterafrugiperda* の卵巣細胞である Sf9、Sf21 [カレント・プロトコールズ・イン・モレキュラー・バイオロジー、Baculovirus Expression Vectors, A Laboratory Manual, W. H. Freeman and Company, New York (1992)]、*Trichoplusiani* の卵巣細胞である High 5 (Invitrogen 社) 等を用いることができる。

組換えウイルスを調製するための、昆虫細胞への上記組換え遺伝子導入ベクターと上記バキュロウイルスの共導入方法としては、例えば、リン酸カルシウム法（特開平 2-227075)、リポフェクション法 [Proc. Natl. Acad. Sci. USA, 84, 7413 (1987)] 等をあげることができる。

植物細胞を宿主細胞として用いる場合には、発現ベクターとして、例えば、Ｔｉプラスミド、タバコモザイクウイルスベクター等をあげることができる。

プロモーターとしては、植物細胞中で発現できるものであればいずれのものを用いてもよく、例えば、カリフラワーモザイクウイルス (CaMV) の 35S プロモーター、イネアクチン１プロモーター等をあげることができる。

宿主細胞としては、タバコ、ジャガイモ、トマト、ニンジン、ダイズ、アブラナ、アルファルファ、イネ、コムギ、オオムギ等の植物細胞等をあげることができる。

組換えベクターの導入方法としては、植物細胞に DNA を導入する方法であればいずれも用いることができ、例えば *Agrobacterium* を用いる方法 [特開昭 59-140885、特開昭 60-70080、WO94/00977]、エレクトロポレーション法 [特開昭 60-251887]、パーティクルガン（遺伝子銃）を用いる方法 [日本特許第 2606856、日本特許第 2517813] 等をあげることができる。

遺伝子の発現方法としては、直接発現以外に、モレキュラー・クローニング第２版に記載されている方法等に準じて、分泌生産、Fc 領域と他のタンパク質との融合タンパク質発現等

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を行うことができる。

糖鎖の合成に関与する遺伝子を導入した細菌、酵母、動物細胞、昆虫細胞または植物細胞により発現させた場合には、導入した遺伝子によって糖あるいは糖鎖が付加された抗体分子を得ることができる。

以上のようにして得られる形質転換体を培地に培養し、培養物中に抗体分子を生成蓄積させ、該培養物から採取することにより、抗体組成物を製造することができる。形質転換体を培地に培養する方法は、宿主細胞の培養に用いられる通常の方法に従って行うことができる。

大腸菌等の原核生物あるいは酵母等の真核生物を宿主として得られた形質転換体を培養する培地としては、該生物が資化し得る炭素源、窒素源、無機塩類等を含有し、形質転換体の培養を効率的に行える培地であれば天然培地、合成培地のいずれを用いてもよい。

炭素源としては、該生物が資化し得るものであればよく、グルコース、フラクトース、スクロース、これらを含有する糖蜜、デンプンあるいはデンプン加水分解物等の炭水化物、酢酸、プロピオン酸等の有機酸、エタノール、プロパノールなどのアルコール類等を用いることができる。

窒素源としては、アンモニア、塩化アンモニウム、硫酸アンモニウム、酢酸アンモニウム、リン酸アンモニウム等の無機酸もしくは有機酸のアンモニウム塩、その他の含窒素化合物、ならびに、ペプトン、肉エキス、酵母エキス、コーンスチープリカー、カゼイン加水分解物、大豆粕および大豆粕加水分解物、各種発酵菌体およびその消化物等を用いることができる。

無機塩類としては、リン酸第一カリウム、リン酸第二カリウム、リン酸マグネシウム、硫酸マグネシウム、塩化ナトリウム、硫酸第一鉄、硫酸マン癌、硫酸銅、炭酸カルシウム等を用いることができる。

培養は、通常振盪培養または深部通気攪拌培養などの好気的条件下で行う。培養温度は15～40℃がよく、培養時間は、通常16時間～7日間である。培養中のpHは3．0～9．0に保持する。pHの調製は、無機または有機の酸、アルカリ溶液、尿素、炭酸カルシウム、アンモニアなどを用いて行う。

また、培養中必要に応じて、アンピシリンやテトラサイクリン等の抗生物質を培地に添加してもよい。

プロモーターとして誘導性のプロモーターを用いた組換えベクターで形質転換した微生物を培養するときには、必要に応じてインデューサーを培地に添加してもよい。例えば、lacプロモーターを用いた組換えベクターで形質転換した微生物を培養するときにはイソプロピル-β-D-チオガラクトピラノシド等を、trpプロモーターを用いた組換えベクターで形質転換した微生物を培養するときにはインドールアクリル酸等を培地に添加してもよい。

動物細胞を宿主として得られた形質転換体を培養する培地としては、一般に使用されているRPMI1640培地［The Journal of the American Medical Association, 199, 519 (1967)］、EagleのMEM培地［Science, 122, 501 (1952)］、ダルベッコ改変MEM培地［Virology, 8, 396 (1959)］、199培地［Proceeding of the Society for the Biological Medicine, 73, 1 (1950)］、Whitten培地［発生工学実験マニュアル-トランスジェニック・マウスの作り方（講談社）勝木元也編（1987）］またはこれら培地に牛胎児血清等を添加した培地等を用いることができる。

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培養は、通常ｐＨ６～８、３０～４０℃、５％CO_2存在下等の条件下で１～７日間行う。

また、培養中必要に応じて、カナマイシン、ペニシリン等の抗生物質を培地に添加してもよい。

昆虫細胞を宿主として得られた形質転換体を培養する培地としては、一般に使用されているTNM-FH培地（Pharmingen社）、Sf-900 II SFM培地（Life Technologies社）、ExCell400、ExCell405（いずれもJRH Biosciences社）、Grace's Insect Medium［Nature, 195, 788 (1962)］等を用いることができる。

培養は、通常ｐＨ６～７、２５～３０℃等の条件下で、１～５日間行う。

また、培養中必要に応じて、ゲンタマイシン等の抗生物質を培地に添加してもよい。

植物細胞を宿主として得られた形質転換体は、細胞として、または植物の細胞や器官に分化させて培養することができる。該形質転換体を培養する培地としては、一般に使用されているムラシゲ・アンド・スクーグ(MS)培地、White 培地、またはこれら培地にオーキシン、サイトカイニン等、植物ホルモンを添加した培地等を用いることができる。

培養は、通常ｐＨ５～９、２０～４０℃の条件下で３～６０日間行う。

また、培養中必要に応じて、カナマイシン、ハイグロマイシン等の抗生物質を培地に添加してもよい。

上記のとおり、抗体分子をコードするDNAを組み込んだ組換え体ベクターを保有する微生物、動物細胞、あるいは植物細胞由来の形質転換体を、通常の培養方法に従って培養し、抗体組成物を生成蓄積させ、該培養物より抗体組成物を採取することにより、抗体組成物を製造することができる。

抗体遺伝子の発現方法としては、直接発現以外に、モレキュラー・クローニング第２版に記載されている方法に準じて、分泌生産、融合タンパク質発現等を行うことができる。

抗体組成物の生産方法としては、宿主細胞内に生産させる方法、宿主細胞外に分泌させる方法、あるいは宿主細胞外膜上に生産させる方法があり、使用する宿主細胞や、生産させる抗体分子の構造を変えることにより、該方法を選択することができる。

抗体組成物が宿主細胞内あるいは宿主細胞外膜上に生産される場合、ポールソンらの方法［J. Biol. Chem., 264, 17619 (1989)］、ロウらの方法［Proc. Natl. Acad. Sci. USA, 86, 8227 (1989); Genes Develop., 4, 1288 (1990)］、または特開平 5-336963、特開平 6-823021 等に記載の方法を準用することにより、該抗体組成物を宿主細胞外に積極的に分泌させることができる。

すなわち、遺伝子組換えの手法を用いて、発現ベクターに、抗体分子をコードするDNA、および抗体分子の発現に適切なシグナルペプチドをコードするDNAを挿入し、該発現ベクターを宿主細胞へ導入の後に抗体分子で発現させることにより、目的とする抗体分子を宿主細胞外に積極的に分泌させることができる。

また、特開平 2-227075 に記載されている方法に準じて、ジヒドロ葉酸還元酵素遺伝子等を用いた遺伝子増幅系を利用して生産量を上昇させることもできる。

さらに、遺伝子導入した動物または植物の細胞を再分化させることにより、遺伝子が導入された動物個体（トランスジェニック非ヒト動物）または植物個体（トランスジェニック植物）を造成し、これらの個体を用いて抗体組成物を製造することもできる。

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形質転換体が動物個体または植物個体の場合は、通常の方法に従って、飼育または栽培し、抗体組成物を生成蓄積させ、該動物個体または植物個体より該抗体組成物を採取することにより、該抗体組成物を製造することができる。

動物個体を用いて抗体組成物を製造する方法としては、例えば公知の方法［American Journal of Clinical Nutrition, 63, 639S (1996); American Journal of Clinical Nutrition, 63, 627S (1996); Bio/Technology, 9, 830 (1991)］に準じて遺伝子を導入して造成した動物中に目的とする抗体組成物を生産する方法があげられる。

動物個体の場合は、例えば、抗体分子をコードする DNA を導入したトランスジェニック非ヒト動物を飼育し、抗体組成物を該動物中に生成・蓄積させ、該動物中より抗体組成物を採取することにより、抗体組成物を製造することができる。該動物中の生成・蓄積場所としては、例えば、該動物のミルク（特開昭 63-309192）、卵等をあげることができる。この際に用いられるプロモーターとしては、動物で発現できるものであればいずれも用いることができるが、例えば、乳腺細胞特異的なプロモーターであるαカゼインプロモーター、βカゼインプロモーター、βラクトグロブリンプロモーター、ホエー酸性プロテインプロモーター等が好適に用いられる。

植物個体を用いて抗体組成物を製造する方法としては、例えば抗体分子をコードする DNA を導入したトランスジェニック植物を公知の方法［組織培養, 20 (1994); 組織培養, 21 (1995); Trends in Biotechnology, 15, 45 (1997)］に準じて栽培し、抗体組成物を該植物中に生成・蓄積させ、該植物中より該抗体組成物を採取することにより、抗体組成物を生産する方法があげられる。

抗体分子をコードする遺伝子を導入した形質転換体により製造された抗体組成物は、例えば抗体組成物が、細胞内に溶解状態で発現した場合には、培養終了後、細胞を遠心分離により回収し、水系緩衝液にけん濁後、超音波破砕機、フレンチプレス、マントンガウリンホモゲナイザー、ダイノミル等により細胞を破砕し、無細胞抽出液を得る。該無細胞抽出液を遠心分離することにより得られる上清から、通常の酵素の単離精製法、即ち、溶媒抽出法、硫安等による塩析法、脱塩法、有機溶媒による沈殿法、ジエチルアミノエチル（DEAE）-セファロース、DIAION HPA-75（三菱化学（株）製）等レジンを用いた陰イオン交換クロマトグラフィー法、S-Sepharose FF (Pharmacia 社) 等のレジンを用いた陽イオン交換クロマトグラフィー法、ブチルセファロース、フェニルセファロース等のレジンを用いた疎水性クロマトグラフィー法、分子篩を用いたゲルろ過法、アフィニティークロマトグラフィー法、クロマトフォーカシング法、等電点電気泳動等の電気泳動法等の手法を単独あるいは組み合わせて用い、抗体組成物の精製標品を得ることができる。

また、抗体組成物が細胞内に不溶体を形成して発現した場合は、同様に細胞を回収後破砕し、遠心分離を行うことにより、沈殿画分として抗体組成物の不溶体を回収する。回収した抗体組成物の不溶体をタンパク質変性剤で可溶化する。該可溶化液を希釈または透析することにより、該抗体組成物を正常な立体構造に戻した後、上記と同様の単離精製法により該抗体組成物の精製標品を得ることができる。

抗体組成物が細胞外に分泌された場合には、培養上清に該抗体組成物あるいはその誘導体を回収することができる。即ち、該培養物を上記と同様の遠心分離等の手法により処理する

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ことにより可溶性画分を取得し、該可溶性画分から、上記と同様の単離精製法を用いることにより、抗体組成物の精製標品を得ることができる。

このようにして取得される抗体組成物として、例えば、抗体、抗体の断片、抗体の Fc 領域を有する融合タンパク質などをあげることができる。

以下に、抗体組成物の取得のより具体的な例として、ヒト化抗体の組成物の製造方法について記すが、他の抗体組成物を当該方法と同様にして取得することもできる。

（1）ヒト化抗体発現用ベクターの構築

ヒト化抗体発現用ベクターとは、ヒト抗体の重鎖（H鎖）及び軽鎖（L鎖）C 領域をコードする遺伝子が組み込まれた動物細胞用発現ベクターであり、動物細胞用発現ベクターにヒト抗体の H 鎖及び L 鎖 C 領域をコードする遺伝子をそれぞれクローニングすることにより構築することができる。

ヒト抗体の C 領域としては、任意のヒト抗体の H 鎖及び L 鎖 C 領域であることができ、例えば、ヒト抗体の H 鎖の IgG1 サブクラスの C 領域（以下、hCγ1 と表記する）及びヒト抗体の L 鎖のκクラスの C 領域（以下、hCκと表記する）等があげられる。

ヒト抗体の H 鎖及び L 鎖 C 領域をコードする遺伝子としてはエキソンとイントロンから成る染色体 DNA を用いることができ、また、cDNA を用いることもできる。

動物細胞用発現ベクターとしては、ヒト抗体の C 領域をコードする遺伝子を組込み発現できるものであればいかなるものでも用いることができる。例えば、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 βd2-4 [Cytotechnology, 4, 173 (1990)] 等があげられる。動物細胞用発現ベクターに用いるプロモーターとエンハンサーとしては、SV40 の初期プロモーターとエンハンサー [J. Biochem., 101, 1307 (1987)]、モロニーマウス白血病ウイルスの LTR [Biochem. Biophys. Res. Commun., 149, 960 (1987)]、免疫グロブリンH鎖のプロモーター [Cell, 41, 479 (1985)] とエンハンサー [Cell, 33, 717 (1983)] 等があげられる。

ヒト化抗体発現用ベクターは、抗体 H 鎖及び L 鎖が別々のベクター上に存在するタイプあるいは同一のベクター上に存在するタイプ（以下、タンデム型と表記する）のどちらでも用いることができるが、ヒト化抗体発現ベクターの構築の容易さ、動物細胞への導入の容易さ、動物細胞内での抗体 H 鎖及び L 鎖の発現量のバランスが均衡する等の点からタンデム型のヒト化抗体発現用ベクターの方が好ましい [J. Immunol. Methods, 167, 271 (1994)]。

構築したヒト化抗体発現用ベクターは、ヒト型キメラ抗体及びヒト型 CDR 移植抗体の動物細胞での発現に使用できる。

（2）ヒト以外の動物の抗体の V 領域をコードする cDNA の取得

ヒト以外の動物の抗体、例えば、マウス抗体の H 鎖及び L 鎖 V 領域をコードする cDNA は以下のようにして取得することができる。

目的のマウス抗体を産生するハイブリドーマ細胞より mRNA を抽出し、cDNA を合成する。合成した cDNA をファージ或いはプラスミド等のベクターにクローニングして cDNA ライブラ

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リーを作製する。該ライブラリーより、既存のマウス抗体のC領域部分或いはV領域部分をプローブとして用い、H鎖V領域をコードするcDNAを有する組換えファージ或いは組換えプラスミド及びL鎖V領域をコードするcDNAを有する組換えファージ或いは組換えプラスミドをそれぞれ単離する。組換えファージ或いは組換えプラスミド上の目的のマウス抗体のH鎖及びL鎖V領域の全塩基配列を決定し、塩基配列よりH鎖及びL鎖V領域の全アミノ酸配列を推定する。

ヒト以外の動物としては、マウス、ラット、ハムスター、ウサギ等、ハイブリドーマ細胞を作製することが可能であれば、いかなるものも用いることができる。

ハイブリドーマ細胞から全RNAを調製する方法としては、チオシアン酸グアニジン-トリフルオロ酢酸セシウム法 [Methods in Enzymol., 154, 3 (1987)]、また全RNAからmRNAを調製する方法としては、オリゴ(dT)固定化セルロースカラム法 (モレキュラー・クローニング第2版) 等があげられる。また、ハイブリドーマ細胞からmRNAを調製するキットとしては、Fast Track mRNA Isolation Kit (Invitrogen 社製)、Quick Prep mRNA Purification Kit (Pharmacia 社製) 等があげられる。

cDNAの合成及びcDNAライブラリー作製法としては、常法 (モレキュラー・クローニング第2版；カレント・プロトコールズ・イン・モレキュラー・バイオロジー, Supplement 1-34)、或いは市販のキット、例えば、Super Script™ Plasmid System for cDNA Synthesis and Plasmid Cloning (GIBCO BRL 社製) や ZAP-cDNA Synthesis Kit (Stratagene 社製) を用いる方法などがあげられる。

cDNAライブラリーの作製の際、ハイブリドーマ細胞から抽出したmRNAを鋳型として合成したcDNAを組み込むベクターは、該cDNAを組み込めるベクターであればいかなるものでも用いることができる。例えば、ZAP Express [Strategies, 5, 58 (1992)]、pBluescript II SK(+) [Nucleic Acids Research, 17, 9494 (1989)]、λzap II (Stratagene 社製)、λgt10、λgt11 [DNA Cloning: A Practical Approach, I, 49 (1985)]、Lambda BlueMid (Clontech 社製)、λExCell、pT7T3 18U (Pharmacia 社製)、pcD2 [Mol. Cell. Biol., 3, 280 (1983)] 及びpUC18 [Gene, 33, 103 (1985)] 等が用いられる。

ファージ或いはプラスミドベクターにより構築されるcDNAライブラリーを導入する大腸菌としては該cDNAライブラリーを導入、発現及び維持できるものであればいかなるものでも用いることができる。例えば、XL1-Blue MRF' [Strategies, 5, 81 (1992)]、C600 [Genetics, 39, 440 (1954)]、Y1088、Y1090 [Science, 222, 778 (1983)]、NM522 [J. Mol. Biol., 166, 1 (1983)]、K802 [J. Mol. Biol., 16, 118 (1966)] 及びJM105 [Gene, 38, 275 (1985)] 等が用いられる。

cDNAライブラリーからのヒト以外の動物の抗体のH鎖及びL鎖V領域をコードするcDNAクローンの選択法としては、アイソトープ或いは蛍光標識したプローブを用いたコロニー・ハイブリダイゼーション法或いはプラーク・ハイブリダイゼーション法 (モレキュラー・クローニング第2版) により選択することができる。また、プライマーを調製し、mRNAから合成したcDNA或いはcDNAライブラリーを鋳型として、Polymerase Chain Reaction [以下、PCR法と表記する；モレキュラー・クローニング第2版；カレント・プロトコールズ・イン・モレキュラー・バイオロジー, Supplement 1-34] によりH鎖及びL鎖V領域をコードするcDNA

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を調製することもできる。

上記方法により選択された cDNA を、適当な制限酵素などで切断後、pBluescript SK(-) (Stratagene 社製) 等のプラスミドにクローニングし、通常用いられる塩基配列解析方法、例えば、サンガー (Sanger) らのジデオキシ法 [Proc. Natl. Acad. Sci. USA, 74, 5463 (1977)] 等の反応を行い、塩基配列自動分析装置、例えば、A. L. F. DNA シークエンサー (Pharmacia 社製) 等を用いて解析することで該 cDNA の塩基配列を決定することができる。決定した塩基配列からH鎖及びL鎖 V 領域の全アミノ酸配列を推定し、既知の抗体のH鎖及びL鎖 V 領域の全アミノ酸配列 [Sequences of Proteins of Immunological Interest, US Dept. Health and Human Services (1991)] と比較することにより、取得した cDNA が分泌シグナル配列を含む抗体のH鎖及びL鎖 V 領域の完全なアミノ酸配列をコードしているかを確認することができる。

(3) ヒト以外の動物の抗体の V 領域のアミノ酸配列の解析

分泌シグナル配列を含む抗体のH鎖及びL鎖 V 領域の完全なアミノ酸配列に関しては、既知の抗体のH鎖及びL鎖 V 領域の全アミノ酸配列 [Sequences of Proteins of Immunological Interest, US Dept. Health and Human Services (1991)] と比較することにより、分泌シグナル配列の長さ及び N 末端アミノ酸配列を推定でき、更にはそれらが属するサブグループを知ることができる。また、H鎖及びL鎖 V 領域の各 CDR のアミノ酸配列についても、既知の抗体のH鎖及びL鎖 V 領域のアミノ酸配列 [Sequences of Proteins of Immunological Interest, US Dept. Health and Human Services (1991)] と比較することによって見出すことができる。

(4) ヒト型キメラ抗体発現ベクターの構築

本項3の (1) に記載のヒト化抗体発現用ベクターのヒト抗体の H 鎖及び L 鎖 C 領域をコードする遺伝子の上流に、ヒト以外の動物の抗体の H 鎖及び L 鎖 V 領域をコードする cDNA をクローニングし、ヒト型キメラ抗体発現ベクターを構築することができる。例えば、ヒト以外の動物の抗体の H 鎖及び L 鎖 V 領域をコードする cDNA を、ヒト以外の動物の抗体 H 鎖及び L 鎖 V 領域の 3' 末端側の塩基配列とヒト抗体の H 鎖及び L 鎖 C 領域の 5' 末端側の塩基配列とから成り、かつ適当な制限酵素の認識配列を両端に有する合成 DNA とそれぞれ連結し、それぞれを本項3の (1) に記載のヒト化抗体発現用ベクターのヒト抗体の H 鎖及び L 鎖 C 領域をコードする遺伝子の上流にそれらが適切な形で発現するようにクローニングし、ヒト型キメラ抗体発現ベクターを構築することができる。

(5) ヒト型 CDR 移植抗体の V 領域をコードする cDNA の構築

ヒト型 CDR 移植抗体のH鎖及びL鎖 V 領域をコードする cDNA は、以下のようにして構築することができる。まず、目的のヒト以外の動物の抗体の H 鎖及び L 鎖 V 領域の CDR を移植するヒト抗体の H 鎖及び L 鎖 V 領域のフレームワーク (以下、FR と表記する) のアミノ酸配列を選択する。ヒト抗体の H 鎖及び L 鎖 V 領域の FR のアミノ酸配列としては、ヒト抗体由来のものであれば、いかなるものでも用いることができる。例えば、Protein Data Bank 等

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のデータベースに登録されているヒト抗体のH鎖及びL鎖V領域のFRのアミノ酸配列、ヒト抗体のH鎖及びL鎖のV領域のFRの各サブグループの共通アミノ酸配列［Sequences of Proteins of Immunological Interest, US Dept. Health and Human Services (1991)］等があげられるが、その中でも、十分な活性を有するヒト型CDR移植抗体を作製するためには、目的のヒト以外の動物の抗体のH鎖及びL鎖V領域のFRのアミノ酸配列とできるだけ高い相同性（少なくとも60%以上）を有するアミノ酸配列を選択することが望ましい。

次に、選択したヒト抗体のH鎖及びL鎖V領域のFRのアミノ酸配列に目的のヒト以外の動物の抗体のH鎖及びL鎖V領域のCDRのアミノ酸配列を移植し、ヒト型CDR移植抗体のH鎖及びL鎖V領域のアミノ酸配列を設計する。設計したアミノ酸配列を抗体の遺伝子の塩基配列に見られるコドンの使用頻度［Sequences of Proteins of Immunological Interest, US Dept. Health and Human Services (1991)］を考慮してDNA配列に変換し、ヒト型CDR移植抗体のH鎖及びL鎖V領域のアミノ酸配列をコードするDNA配列を設計する。設計したDNA配列に基づき、100塩基前後の長さから成る数本の合成DNAを合成し、それらを用いてPCR法を行う。この場合、PCRでの反応効率及び合成可能なDNAの長さから、H鎖、L鎖とも6本の合成DNAを設計することが好ましい。

また、両端に位置する合成DNAの5'末端に適当な制限酵素の認識配列を導入することで、本項3の（1）で構築したヒト化抗体発現用ベクターに容易にクローニングすることができる。PCR後、増幅産物をpBluescript SK(-)（Stratagene社製）等のプラスミドにクローニングし、本項3の（2）に記載の方法により、塩基配列を決定し、所望のヒト型CDR移植抗体のH鎖及びL鎖V領域のアミノ酸配列をコードするDNA配列を有するプラスミドを取得する。

（6）ヒト型CDR移植抗体発現ベクターの構築

本項3の（1）に記載のヒト化抗体発現用ベクターのヒト抗体のH鎖及びL鎖C領域をコードする遺伝子の上流に、本項3の（5）で構築したヒト型CDR移植抗体のH鎖及びL鎖V領域をコードするcDNAをクローニングし、ヒト型CDR移植抗体発現ベクターを構築することができる。例えば、本項3の（5）でヒト型CDR移植抗体のH鎖及びL鎖V領域を構築する際に用いる合成DNAのうち、両端に位置する合成DNAの5'末端に適当な制限酵素の認識配列を導入することで、本項3の（1）に記載のヒト化抗体発現用ベクターのヒト抗体のH鎖及びL鎖C領域をコードする遺伝子の上流にそれらが適切な形で発現するようにクローニングし、ヒト型CDR移植抗体発現ベクターを構築することができる。

（7）ヒト化抗体の安定的生産

本項3の（4）及び（6）に記載のヒト化抗体発現ベクターを適当な動物細胞に導入することによりヒト型キメラ抗体及びヒト型CDR移植抗体（以下、併せてヒト化抗体と称す）を安定に生産する形質転換株を得ることができる。

動物細胞へのヒト化抗体発現ベクターの導入法としては、エレクトロポレーション法［特開平2-257891; Cytotechnology, 3, 133 (1990)］等があげられる。

ヒト化抗体発現ベクターを導入する動物細胞としては、ヒト化抗体を生産させることがで

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きる動物細胞であれば、いかなる細胞でも用いることができる。

具体的には、マウスミエローマ細胞である NSO 細胞、SP2/0 細胞、チャイニーズハムスター卵巣細胞 CHO/dhfr-細胞、CHO/DG44 細胞、ラットミエローマ YB2/0 細胞、IR983F 細胞、シリアンハムスター腎臓由来である BHK 細胞、ヒトミエローマ細胞であるナマルバ細胞などがあげられるが、好ましくは、チャイニーズハムスター卵巣細胞である CHO/DG44 細胞、ラットミエローマ YB2/0 細胞、５に記載本発明の宿主細胞等があげられる。

ヒト化抗体発現ベクターの導入後、ヒト化抗体を安定に生産する形質転換株は、特開平 2-257891 に開示されている方法に従い、G418 sulfate（以下、G418 と表記する；SIGMA 社製）等の薬剤を含む動物細胞培養用培地により選択できる。動物細胞培養用培地としては、RPMI1640 培地（日水製薬社製）、GIT 培地（日本製薬社製）、EX-CELL302 培地（JRH 社製）、IMDM 培地（GIBCO BRL 社製）、Hybridoma-SFM 培地（GIBCO BRL 社製）、またはこれら培地に牛胎児血清（以下、FBS と表記する）等の各種添加物を添加した培地等を用いることができる。得られた形質転換株を培地中で培養することで培養上清中にヒト化抗体を生産蓄積させることができる。培養上清中のヒト化抗体の生産量及び抗原結合活性は酵素免疫抗体法［以下、ELISA 法と表記する；Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Chapter 14 (1998)、Monoclonal Antibodies: Principles and Practice, Academic Press Limited (1996)］等により測定できる。また、形質転換株は、特開平 2-257891 に開示されている方法に従い、DHFR 遺伝子増幅系等を利用してヒト化抗体の生産量を上昇させることができる。

ヒト化抗体は、形質転換株の培養上清よりプロテイン A カラムを用いて精製することができる［Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Chapter 8 (1988)、Monoclonal Antibodies: Principles and Practice, Academic Press Limited (1996)］。また、その他に通常、タンパク質の精製で用いられる精製方法を使用することができる。例えば、ゲル濾過、イオン交換クロマトグラフィー及び限外濾過等を組み合わせて行い、精製することができる。精製したヒト化抗体の H 鎖、L 鎖或いは抗体分子全体の分子量は、ポリアクリルアミドゲル電気泳動［以下、SDS-PAGE と表記する；Nature, 227, 680 (1970)］やウエスタンブロッティング法［Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Chapter 12 (1988)、Monoclonal Antibodies: Principles and Practice, Academic Press Limited (1996)］等で測定することができる。

以上、動物細胞を宿主とした抗体組成物の製造方法を示したが、上述したように、酵母、昆虫細胞、植物細胞または動物個体あるいは植物個体においても動物細胞と同様の方法により抗体組成物を製造することができる。

すでに宿主細胞が抗体分子を発現する能力を有する場合には、前記１．に記載した方法を用いて抗体分子を発現させる細胞を調製した後に、該細胞を培養し、該培養物から目的とする抗体組成物を精製することにより、本発明の抗体組成物を製造することができる。

４．抗体組成物の活性評価

精製した抗体組成物の蛋白量、抗原との結合活性あるいはエフェクター機能はを測定する方法としては、モノクローナルアンチボディズ、あるいはアンチボディエンジニアリング等

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に記載の公知の方法を用いることができる。

その具体的な例としては、抗体組成物がヒト化抗体の場合、抗原との結合活性、抗原陽性培養細胞株に対する結合活性は ELISA 法及び蛍光抗体法 [Cancer Immunol. Immunother., 36, 373 (1993)] 等により測定できる。抗原陽性培養細胞株に対する細胞障害活性は、CDC 活性、ADCC 活性等を測定することにより、評価することができる [Cancer Immunol. Immunother., 36, 373 (1993)]。

また、抗体組成物のヒトでの安全性、治療効果は、カニクイザル等のヒトに比較的近い動物種の適当なモデルを用いて評価することができる。

5．各種細胞で発現させた抗体分子の糖鎖の分析

各種細胞で発現させた抗体分子の糖鎖構造は、通常の糖タンパク質の糖鎖構造の解析に準じて行うことができる。例えば、IgG 分子に結合している糖鎖はガラクトース、マンノース、フコースなどの中性糖、N-アセチルグルコサミンなどのアミノ糖、シアル酸などの酸性糖から構成されており、糖組成分析および二次元糖鎖マップ法などを用いた糖鎖構造解析等の手法を用いて行うことができる。

（1）中性糖・アミノ糖組成分析

抗体分子の糖鎖の組成分析は、トリフルオロ酢酸等で、糖鎖の酸加水分解を行うことにより、中性糖またはアミノ糖を遊離し、その組成比を分析することができる。

具体的な方法として、Dionex 社製糖組成分析装置 (BioLC) を用いる方法があげられる。BioLC は HPAEC-PAD (high performance anion-exchange chromatography-pulsed amperometric detection) 法 [J. Liq. Chromatogr., 6, 1577 (1983)] によって糖組成を分析する装置である。

また、2-アミノピリジンによる蛍光標識化法でも組成比を分析することができる。具体的には、公知の方法 [Agric. Biol. Chem., 55(1), 283-284 (1991)] に従って酸加水分解した試料を 2-アミノピリジル化で蛍光ラベル化し、HPLC 分析して組成比を算出することができる。

(2) 糖鎖構造解析

抗体分子の糖鎖の構造解析は、2 次元糖鎖マップ法 [Anal. Biochem., 171, 73 (1988)、生物化学実験法 23-糖タンパク質糖鎖研究法 (学会出版センター) 高橋禮子編 (1989 年)] により行うことができる。2 次元糖鎖マップ法は、例えば、X 軸には逆相クロマトグラフィー糖鎖の保持時間または溶出位置を、Y 軸には順相クロマトグラフィーによる糖鎖の保持時間または溶出位置を、それぞれプロットし、既知糖鎖のそれらの結果と比較することにより、糖鎖構造を推定する方法である。

具体的には、抗体をヒドラジン分解して、抗体から糖鎖を遊離し、2-アミノピリジン（以下、PA と略記する）による糖鎖の蛍光標識 [J. Biochem., 95, 197 (1984)] を行った後、ゲルろ過により糖鎖を過剰の PA 化試薬などと分離し、逆相クロマトグラフィーを行う。次いで、分取した糖鎖の各ピークについて順相クロマトグラフィーを行う。これらの結果をも

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とに、2 次元糖鎖マップ上にプロットし、糖鎖スタンダード（TaKaRa 社製）、文献［Anal. Biochem., 171, 73 (1988)］とのスポットの比較より糖鎖構造を推定することができる。

さらに各糖鎖の MALDI-TOF-MS などの質量分析を行い、2 次元糖鎖マップ法により推定される構造を確認することができる。

6．抗体分子の糖鎖構造を識別する免疫学的定量方法

抗体組成物は、抗体の Fc 領域に結合する糖鎖構造が異なった抗体分子から構成されている。本発明の抗体組成物は、Fc 領域に結合する全 N-グリコシド結合複合型糖鎖のうち、糖鎖還元末端の N-アセチルグルコサミンにフコースが結合していない糖鎖の割合が 20%以上であり、高い ADCC 活性を示す特徴を有している。このような抗体組成物は、上記6．に記載の抗体分子の糖鎖構造の分析法を用いることにより識別できる。また、レクチンを用いた免疫学的定量方法を用いることによっても識別できる。

レクチンを用いた免疫学的定量方法を用いた抗体分子の糖鎖構造の識別は、文献［Monoclonal Antibodies: Principles and Applications, Wiley-Liss, Inc. (1995)；酵素免疫測定法，第 3 版，医学書院 (1987)；改訂版，酵素抗体法，学際企画 (1985)］等に記載のウエスタン染色、RIA（Radioimmunoassay）、VIA（Viroimmunoassay）、EIA (Enzymoimmunoassay)、FIA (Fluoroimmunoassay)、MIA (Metalloimmunoassay) などの免疫学的定量方法に準じて、例えば、以下のように行うことができる。

抗体組成物を構成する抗体分子の糖鎖構造を認識するレクチンを標識し、標識したレクチンと試料である抗体組成物を反応させる。次に、標識したレクチンと抗体分子の複合体の量を測定する。

抗体分子の糖鎖構造を識別に用いられるレクチンとしては、例えば、WGA (*T. vulgaris* 由来の wheat-germ agglutinin)、ConA (*C. ensiformis* 由来の concanavalin A)、RIC (*R. communis* 由来の毒素)、L-PHA (*P. vulgaris* 由来の leukoagglutinin)、LCA (*L. culinaris* 由来の lentil agglutinin)、PSA (*P. sativum* 由来の Pea lectin)、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)、HHL (*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)、SJA (*Sophora japonica* Agglutinin)、SBA (Soybean Agglutinin)、UEA (*Ulex europaeus* Agglutinin)、VVL (*Vicia villosa* Lectin)、WFA (*Wisteria floribunda* Agglutinin)があげられる。

N-グルコシド結合複合型糖鎖還元末端の N-アセチルグルコサミンにフコースが結合している糖鎖構造を特異的に認識するレクチンを用いることが好ましく、その具体的な例としては、

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レンズマメレクチン LCA (*Lens Culinaris* 由来の Lentil Agglutinin) エンドウマメレクチン PSA (*Pisum sativum* 由来の Pea Lectin)、ソラマメレクチン VFA (Vicia faba 由来の Agglutinin)、ヒイロチャワンタケレクチン AAL (*Aleuria aurantia* 由来の Lectin) をあげることができる。

7．本発明の抗体分子の利用

本発明の抗体組成物は高い抗体依存性細胞障害活性を有する。高い抗体依存性細胞障害活性を有する抗体は、癌、、炎症疾患、自己免疫疾患、アレルギーなどの免疫疾患、循環器疾患、またはウィルスあるいは細菌感染をはじめとする各種疾患の予防および治療において有用である。

癌、すなわち悪性腫瘍は癌細胞が増殖する。通常の抗癌剤は癌細胞の増殖を抑制することを特徴とする。しかし、高い抗体依存性細胞障害活性を有する抗体は、殺細胞効果により癌細胞を障害することにより癌を治療することができるため、通常の抗癌剤よりも治療薬として有効である。特に癌の治療薬において、現状では抗体医薬単独の抗腫瘍効果は不充分であり、化学療法との併用療法が行われているが [Science, 280, 1197 (1998)]、本発明の抗体組成物単独でのより強い抗腫瘍効果が認められれば、化学療法に対する依存度が低くなり、副作用の低減にもなる。

炎症疾患、自己免疫疾患、アレルギーなどの免疫疾患において、それらの疾患における生体内反応は、免疫細胞によるメディエータ分子の放出により惹起されるため、高い抗体依存性細胞障害活性を有する抗体を用いて免疫細胞を除去することにより、アレルギー反応を抑えることができる。

循環器疾患としては、動脈硬化などがあげられる。動脈硬化は、現在バルーンカテーテルによる治療を行うが、治療後の再狭窄での動脈細胞の増殖を高い抗体依存性細胞障害活性を有する抗体を用いて抑えることより、循環器疾患を予防および治療することができる。

ウィルスまたは細菌に感染細胞を、高い抗体依存性細胞障害活性を有する抗体を用いてウィルスまたは細菌に感染細胞の増殖を抑えることにより、ウィルスまたは細菌感染をはじめとする各種疾患の予防および治療することができる。

腫瘍関連抗原を認識する抗体、アレルギーあるいは炎症に関連する抗原を認識する抗体、循環器疾患に関連する抗原を認識する抗体、またはウイルスあるいは細菌感染に関連する抗原を認識する抗体の具体例を以下に述べる。。

腫瘍関連抗原を認識する抗体としては、抗 GD2 抗体 (Ohta et al., Anticancer Res., 13, 331-336, 1993)、抗 GD3 抗体 (Ohta et al., Cancer Immunol. Immunother., 36, 260-266, 1993)、抗 GM2 抗体 (Nakamura et al., Cancer Res., 54, 1511-1516, 1994)、抗 HER2 抗体 (Carter et al., Proc. Natl. Acad. Sci. USA, 89, 4285-4289, 1992)、抗 CD52 抗体 (Carter et al., Proc. Natl. Acad. Sci. USA, 89, 4285-4289, 1992)、抗 MAGE 抗体 (Jungbluth et al., British J. Cancer, 83, 493-497, 2000)、抗 HM1.24 抗体 (Ono et al., Molecular Immunol., 36, 387-395, 1999)、抗副甲状腺ホルモン関連蛋白 (PTHrP) 抗体 (Ogata et al., Cancer, 88, 2909-2911, 2000)、抗塩基性繊維芽細胞増殖因子抗体、抗ＦＧＦ８抗体 (Matsuzaki et al., Proc. Natl. Acad. Sci. USA, 86, 9911-9915, 1989)、

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抗塩基性繊維芽細胞増殖因子受容体抗体、抗 FGF8 受容体抗体（Kuo et al., J. Biol. Chem., 265, 16455-16463, 1990）、抗インスリン様増殖因子抗体（Yao et al., J. Neurosci. Res., 40, 647-659, 1995）、抗インスリン様増殖因子受容体抗体（Yao et al., J. Neurosci. Res., 40, 647-659, 1995）、抗PMSA抗体（Murphy et al., J. Urology, 160, 2396-2401, 1998）、抗血管内皮細胞増殖因子抗体（Presta et al., Cancer Res., 57, 4593-4599, 1997）、抗血管内皮細胞増殖因子受容体抗体（Kanno et al., Oncogene, 19, 2138-2146, 2000）などがあげられる。

アレルギーあるいは炎症に関連する抗原を認識する抗体としては、抗インターロイキン６抗体（Abrams et al., Immunol. Rev., 127, 5-24, 1992）、抗インターロイキン６受容体抗体（Sato et al., Molecular Immunol., 31, 371-381, 1994）、抗インターロイキン５抗体（Abrams et al., Immunol. Rev., 127, 5-24, 1992）、抗インターロイキン５受容体抗体、抗インターロイキン４抗体（Bird et al., Cytokine, 3, 562-567, 1991）、抗インターロイキン４受容体抗体（Jung et al., J. Immunol. Meth., 217, 41-50, 1998）、抗腫瘍壊死因子抗体（Tempest et al., Hybridoma, 13, 183-190, 1994）、抗腫瘍壊死因子受容体抗体（Amrani et al., Molecular Pharmacol., 58, 237-245, 2000）、抗 CCR4 抗体（Campbell et al., Nature, 400, 776-780, 1999）、抗ケモカイン抗体（Peri et al., J. Immunol. Meth., 174, 249-257, 1994）または抗ケモカイン受容体抗体（Wu et al., J. Exp. Med., 186, 1373-1381, 1997）であり、循環器疾患に関連する抗原を認識する抗体が抗 GpIIb/IIIa 抗体（Co et al., J. Immunol., 152, 2968-2976, 1994）、抗血小板由来増殖因子抗体（Ferns et al., Science, 253, 1129-1132, 1991）、抗血小板由来増殖因子受容体抗体（Shulman et al., J. Biol. Chem., 272, 17400-17404, 1997）、抗血液凝固因子抗体（Peter et al., Circulation, 101, 1158-1164, 2000）などがあげられる。

ウイルスあるいは細菌感染に関連する抗原を認識する抗体としては、抗ｇｐ１２０抗体（Tugarinov et al., Structure, 8, 385-395, 2000）、抗ＣＤ４抗体（Schulze-Koops et al., J. Rheumatology, 25, 2065-2076, 1998）、抗ＣＣＲ４抗体、抗ベロ毒素抗体（Karmali et al., J. Clin. Microbiol., 37, 396-399, 1999）などがあげられる。

上記抗体は、ATCC（The American Type Culture Collection）、理化学研究所細胞開発銀行、工業技術院生命工業技術研究所（現：独立行政法人産業技術総合研究所 特許生物寄託センター）等の公的な機関、あるいは大日本製薬株式会社、R&D SYSTEMS 社、PharMingen 社、コスモバイオ社、フナコシ株式会社等の民間試薬販売会社からも入手することができる。

本発明の抗体組成物を含有する医薬は、治療薬として単独で投与することも可能ではあるが、通常は薬理学的に許容される一つあるいはそれ以上の担体と一緒に混合し、製剤学の技術分野においてよく知られる任意の方法により製造した医薬製剤として提供するのが望ましい。

投与経路は、治療に際して最も効果的なものを使用するのが望ましく、経口投与、または口腔内、気道内、直腸内、皮下、筋肉内および静脈内等の非経口投与をあげることができ、抗体製剤の場合、望ましくは静脈内投与をあげることができる。

投与形態としては、噴霧剤、カプセル剤、錠剤、顆粒剤、シロップ剤、乳剤、座剤、注射剤、軟膏、テープ剤等があげられる。

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経口投与に適当な製剤としては、乳剤、シロップ剤、カプセル剤、錠剤、散剤、顆粒剤等があげられる。

乳剤およびシロップ剤のような液体調製物は、水、ショ糖、ソルビトール、果糖等の糖類、ポリエチレングリコール、プロピレングリコール等のグリコール類、ごま油、オリーブ油、大豆油等の油類、p-ヒドロキシ安息香酸エステル類等の防腐剤、ストロベリーフレーバー、ペパーミント等のフレーバー類等を添加剤として用いて製造できる。

カプセル剤、錠剤、散剤、顆粒剤等は、乳糖、ブドウ糖、ショ糖、マンニトール等の賦形剤、デンプン、アルギン酸ナトリウム等の崩壊剤、ステアリン酸マグネシウム、タルク等の滑沢剤、ポリビニルアルコール、ヒドロキシプロピルセルロース、ゼラチン等の結合剤、脂肪酸エステル等の界面活性剤、グリセリン等の可塑剤等を添加剤として用いて製造できる。

非経口投与に適当な製剤としては、注射剤、座剤、噴霧剤等があげられる。

注射剤は、塩溶液、ブドウ糖溶液、あるいは両者の混合物からなる担体等を用いて調製される。または、抗体組成物を常法に従って凍結乾燥し、これに塩化ナトリウムを加えることによって粉末注射剤を調製することもできる。

座剤はカカオ脂、水素化脂肪またはカルボン酸等の担体を用いて調製される。

また、噴霧剤は該抗体組成物そのもの、ないしは受容者の口腔および気道粘膜を刺激せず、かつ該抗体組成物を微細な粒子として分散させ吸収を容易にさせる担体等を用いて調製される。

担体として具体的には乳糖、グリセリン等が例示される。該抗体組成物および用いる担体の性質により、エアロゾル、ドライパウダー等の製剤が可能である。また、これらの非経口剤においても経口剤で添加剤として例示した成分を添加することもできる。

投与量または投与回数は、目的とする治療効果、投与方法、治療期間、年齢、体重等により異なるが、有効成分の量として、通常成人1日当たり10μg/kg～20mg/kgである。

また、抗体組成物の各種腫瘍細胞に対する抗腫瘍効果を検討する方法は、インビトロ実験としては、CDC活性測定法、ADCC活性測定法等があげられ、インビボ実験としては、マウス等の実験動物での腫瘍系を用いた抗腫瘍実験等があげられる。

CDC活性、ADCC活性、抗腫瘍実験は、文献[Cancer Immunology Immunotherapy, 36, 373 (1993); Cancer Research, 54, 1511 (1994)]等記載の方法に従って行うことができる。

以下の実施例により本発明をより具体的に説明するが、実施例は本発明の単なる例示を示すものにすぎず、本発明の範囲を限定するものではない。

実施例1. 抗ガングリオシドGD3ヒト型キメラ抗体の作製

1. 抗ガングリオシドGD3ヒト型キメラ抗体のタンデム型発現ベクターpChiLHGM4の構築

抗ガングリオシドGD3ヒト型キメラ抗体（以下、抗GD3キメラ抗体と表記する）のL鎖の発現ベクターpChi641LGM4 [J. Immunol. Methods, 167, 271 (1994)]を制限酵素MluI（宝酒造社製）とSalI（宝酒造社製）で切断して得られるL鎖cDNAを含む約4.03kbの断片と動物細胞用発現ベクターpAGE107 [Cytotechnology, 3, 133 (1990)]を制限酵素MluI（宝酒造社製）とSalI（宝酒造社製）で切断して得られるG418耐性遺伝子及びスプライシングシグナルを含む約3.40kbの断片をDNA Ligation Kit（宝酒造社製）を用いて連結、大腸菌

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HB101 株（モレキュラー・クローニング第 2 版）を形質転換してプラスミド pChi641LGM40 を構築した。

次に、上記で構築したプラスミド pChi641LGM40 を制限酵素 ClaI（宝酒造社製）で切断後、DNA Blunting Kit（宝酒造社製）を用いて平滑末端化し、更に MluI（宝酒造社製）で切断して得られる L 鎖 cDNA を含む約 5.68kb の断片と抗 GD3 キメラ抗体の H 鎖の発現ベクター pChi641HGM4 [J. Immunol. Methods, 167, 271 (1994)] を制限酵素 XhoI（宝酒造社製）で切断後、DNA Blunting Kit（宝酒造社製）を用いて平滑末端化し、更に MluI（宝酒造社製）で切断して得られる H 鎖 cDNA を含む約 8.40kb の断片を DNA Ligation Kit（宝酒造社製）を用いて連結、大腸菌 HB101 株（モレキュラー・クローニング第 2 版）を形質転換して抗 GD3 キメラ抗体のタンデム型発現ベクターpChi641LHGM4 を構築した。

2. 抗 GD3 キメラ抗体の安定生産細胞の作製

上記実施例 1 の 1 項で構築した抗 GD3 キメラ抗体のタンデム型発現ベクターpChi641LHGM4 を各種細胞株に導入し、優良株を選択することで抗 GD3 キメラ抗体の安定生産細胞を以下のようにして作製した。

(1) ラットミエローマ YB2/0 細胞を用いた生産細胞の作製

抗 GD3 キメラ抗体発現ベクターpChi641LHGM4 の 5μg を 4×10⁶ 細胞のラットミエローマ YB2/0 細胞 [ATCC CRL-1662、J.V. Kilmarin et al., J. Cell. Biol., 93, 576-582 (1982)] へエレクトロポレーション法 [Cytotechnology, 3, 133 (1990)] により導入後、40ml の RPMI1640-FBS(10) [FBS (GIBCO BRL 社製)を 10%含む RPMI1640 培地] に懸濁し、96 ウェル培養用プレート（住友ベークライト社製）に 200μl/ウェルずつ分注した。5%CO_2 インキュベーター内で 37℃、24 時間培養した後、G418 を 0.5mg/ml になるように添加して 1～2 週間培養した。G418 耐性を示す形質転換株のコロニーが出現し、増殖の認められたウェルより培養上清を回収し、上清中の抗 GD3 キメラ抗体の抗原結合活性を実施例 1 の 3 項に示す ELISA 法により測定した。

培養上清中に抗 GD3 キメラ抗体の生産が認められたウェルの形質転換株については、DHFR 遺伝子増幅系を利用して抗体生産量を増加させる目的で、G418 を 0.5mg/ml、DHFR の阻害剤であるメソトレキセート（以下、MTX と表記する；SIGMA 社製）を 50nM 含む RPMI1640-FBS(10) 培地に 1～2×10⁶ 細胞/ml になるように懸濁し、24 ウェルプレート（Greiner 社製）に 2ml ずつ分注した。5%CO_2 インキュベーター内で 37℃で 1～2 週間培養して、50nM MTX 耐性を示す形質転換株を誘導した。形質転換株の増殖が認められたウェルの培養上清中の抗 GD3 キメラ抗体の抗原結合活性を実施例 1 の 3 項に示す ELISA 法により測定した。培養上清中に抗 GD3 キメラ抗体の生産が認められたウェルの形質転換株については、上記と同様の方法により、MTX 濃度を 100nM、200nM と順次上昇させ、最終的に G418 を 0.5mg/ml、MTX を 200nM の濃度で含む RPMI1640-FBS(10) 培地で増殖可能かつ、抗 GD3 キメラ抗体を高生産する形質転換株を得た。得られた形質転換株の中から優良株を選択し、2 回の限界希釈法による単一細胞化（クローン化）を行った。尚、実施例 9 に示すα-1,6-フコシルトランスフェラーゼの遺伝子の転写物の定量法を用い、該転写物の量が比較的低い株を優良株として選択

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し用いた。

このようにして得られた抗 GD3 キメラ抗体を生産する形質転換細胞クローン 7-9-51 は平成 11 年 4 月 5 日付で工業技術院生命工学工業技術研究所（日本国茨城県つくば市東 1 丁目 1 番 3 号）（現・独立行政法人産業技術総合研究所 特許生物寄託センター（茨城県つくば市東 1 丁目 1 番地 中央第 6））に FERM BP-6691 として寄託されている。

(2) CHO/DG44 細胞を用いた生産細胞の作製

抗 GD3 キメラ抗体発現ベクター pChi641LHGM4 の 4μg を 1.6×10^6 細胞の CHO/DG44 細胞[G. Urlaub and L.A. Chasin, Proc. Natl. Acad. Sci. USA, 77, 4216-4220 (1980)] へエレクトロポレーション法 [Cytotechnology, 3, 133 (1990)] により導入後、10ml の IMDM-FBS(10) [FBS を 10%、HT supplement (GIBCO BRL 社製) を 1 倍濃度で含む IMDM 培地] に懸濁し、96 ウェル培養用プレート（岩城硝子社製）に 200μl/ウェルずつ分注した。5%CO_2 インキュベーター内で 37℃、24 時間培養した後、G418 を 0.5mg/ml になるように添加して 1～2 週間培養した。G418 耐性を示す形質転換株のコロニーが出現し、増殖の認められたウェルより培養上清を回収し、上清中の抗 GD3 キメラ抗体の抗原結合活性を実施例 1 の 3 項に示す ELISA 法により測定した。

培養上清中に抗 GD3 キメラ抗体の生産が認められたウェルの形質転換株については、DHFR 遺伝子増幅系を利用して抗体生産量を増加させる目的で、G418 を 0.5mg/ml、MTX を 10nM 含む IMDM-dFBS(10) 培地 [透析牛胎児血清（以下、dFBS と表記する；GIBCO BRL 社製）を 10% 含む IMDM 培地] に 1～2×10^5 細胞/ml になるように懸濁し、24 ウェルプレート（岩城硝子社製）に 0.5ml ずつ分注した。5%CO_2 インキュベーター内で 37℃で 1～2 週間培養して、10nM MTX 耐性を示す形質転換株を誘導した。増殖が認められたウェルの形質転換株については、上記と同様の方法により、MTX 濃度を 100nM に上昇させ、最終的に G418 を 0.5mg/ml、MTX を 100nM の濃度で含む IMDM-dFBS(10) 培地で増殖可能かつ、抗 GD3 キメラ抗体を高生産する形質転換株を得た。得られた形質転換株の中から優良株を選択し、2 回の限界希釈法による単一細胞化（クローン化）を行った。尚、実施例 9 に示すα-1,6-フコシルトランスフェラーゼの遺伝子の転写物の定量法を用い、該転写物の量が比較的低い株を優良株として選択し用いた。

(3) マウスミエローマ NS0 細胞を用いた生産細胞の作製

抗 GD3 キメラ抗体発現ベクター pChi641LHGM4 の 5μg を 4×10^6 細胞のマウスミエローマ NS0 細胞へエレクトロポレーション法 [Cytotechnology, 3, 133 (1990)] により導入後、40ml の EX-CELL302-FBS(10) [FBS を 10%、L-グルタミン（以下、L-Gln と表記する；GIBCO BRL 社製）を 2mM 含む EX-CELL302 培地] に懸濁し、96 ウェル培養用プレート（住友ベークライト社製）に 200μl/ウェルずつ分注した。5%CO_2 インキュベーター内で 37℃、24 時間培養した後、G418 を 0.5mg/ml になるように添加して 1～2 週間培養した。G418 耐性を示す形質転換株のコロニーが出現し、増殖の認められたウェルより培養上清を回収し、上清中の抗 GD3 キメラ抗体の抗原結合活性を実施例 1 の 3 項に示す ELISA 法により測定した。

培養上清中に抗 GD3 キメラ抗体の生産が認められたウェルの形質転換株については、DHFR

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遺伝子増幅系を利用して抗体生産量を増加させる目的で、G418 を 0.5mg/ml、MTX を 50nM 含む EX-CELL302-dFBS(10) 培地（dFBS を 10%、L-Gln を 2mM 含む EX-CELL302 培地）に 1～2×10^5 細胞/ml になるように懸濁し、24 ウェルプレート（Greiner 社製）に 2ml ずつ分注した。5%CO_2 インキュベーター内で 37℃で 1～2 週間培養して、50nM MTX 耐性を示す形質転換株を誘導した。形質転換株の増殖が認められたウェルの培養上清中の抗 GD3 キメラ抗体の抗原結合活性を実施例 1 の 3 項に示す ELISA 法により測定した。培養上清中に抗 GD3 キメラ抗体の生産が認められたウェルの形質転換株については、上記と同様の方法により、MTX 濃度を 100nM、200nM と順次上昇させ、最終的に G418 を 0.5mg/ml、MTX を 200nM の濃度で含む EX-CELL302-dFBS(10) 培地で増殖可能かつ、抗 GD3 キメラ抗体を高生産する形質転換株を得た。得られた形質転換株の中から優良株を選択し、2 回の限界希釈法による単一細胞化（クローン化）を行った。尚、実施例 9 に示すα-1,6-フコシルトランスフェラーゼの遺伝子の転写物の定量法を用い、該転写物の量が比較的低い株を優良株として選択し用いた。

3. 抗体の GD3 に対する結合活性の測定（ELISA 法）

抗体の GD3 に対する結合活性は以下のようにして測定した。

4nmol の GD3 を 10μg のジパルミトイルフォスファチジルコリン（SIGMA 社製）と 5μg のコレステロール（SIGMA 社製）とを含む 2ml のエタノール溶液に溶解した。該溶液の 20μl（40pmol/ウェルとなる）を 96 ウェルの ELISA 用のプレート（Greiner 社製）の各ウェルにそれぞれ分注し、風乾後、1%牛血清アルブミン（以下、BSA と表記する；SIGMA 社製）を含む PBS（以下、1%BSA-PBS と表記する）を 100μl/ウェルで加え、室温で 1 時間反応させて残存する活性基をブロックした。1%BSA-PBS を捨て、形質転換株の培養上清或いは精製したヒト型キメラ抗体の各種希釈溶液を 50μl/ウェルで加え、室温で 1 時間反応させた。反応後、各ウェルを 0.05%Tween20（和光純薬社製）を含む PBS（以下、Tween-PBS と表記する）で洗浄後、1%BSA-PBS で 3000 倍に希釈したペルオキシダーゼ標識ヤギ抗ヒト IgG（H&L）抗体溶液（American Qualex 社製）を二次抗体溶液として、50μl/ウェルで加え、室温で 1 時間反応させた。反応後、Tween-PBS で洗浄後、ABTS 基質液 [2,2'-アジノ-ビス(3-エチルベンゾチアゾリン-6-スルホン酸)アンモニウムの 0.55g を 1L の 0.1M クエン酸緩衝液（pH4.2）に溶解し、使用直前に過酸化水素を 1μl/ml で添加した溶液（以下、同様）] を 50μl/ウェルで加えて発色させ、415nm の吸光度（以下、OD415 と表記する）を測定した。

4. 抗 GD3 キメラ抗体の精製

(1) YB2/0 細胞由来の生産細胞の培養及び抗体の精製

上記実施例 1 の 2 項 (1) で得られた抗 GD3 キメラ抗体を生産する形質転換細胞クローンを BSA を 0.2%、MTX を 200nM、トリヨードチロニン（以下、T3 と表記する；SIGMA 社製）を 100nM の濃度で含む Hybridoma-SFM 培地に 3×10^5 細胞/ml となるように懸濁し、2.0L スピナーボトル（岩城硝子社製）を用いて 50rpm の速度で撹拌培養した。37℃の恒温室内で 10 日間培養後、培養上清を回収した。培養上清より Prosep-A (Bioprocessing 社製) カラムを用いて、添付の説明書に従い、抗 GD3 キメラ抗体を精製した。精製した抗 GD3 キメラ抗体は、YB2/0-GD3 キメラ抗体と名付けた。

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(2) CHO/DG44 細胞由来の生産細胞の培養及び抗体の精製

上記実施例 1 の 2 項 (2) で得られた抗 GD3 キメラ抗体を生産する形質転換細胞クローンを L-Gln を 3mM、脂肪酸濃縮液 (以下、CDLC と表記する ; GIBCO BRL 社製) を 0.5%、プルロニック F68 (以下、PF68 と表記する ; GIBCO BRL 社製) を 0.3%の濃度で含む EX-CELL302 培地に 1×10^6 細胞/ml となるように懸濁し、175mm² フラスコ (Greiner 社製) に 50ml ずつ分注した。5%CO_2 インキュベーター内で 37℃で 4 日間培養後、培養上清を回収した。培養上清より Prosep-A (Bioprocessing 社製) カラムを用いて、添付の説明書に従い、抗 GD3 キメラ抗体を精製した。精製した抗 GD3 キメラ抗体は、CHO/DG44-GD3 キメラ抗体と名付けた。

(3) NSO 細胞由来の生産細胞の培養及び抗体の精製

上記実施例 1 の 2 項 (3) で得られた抗 GD3 キメラ抗体を生産する形質転換細胞クローンを L-Gln を 2mM、G418 を 0.5mg/ml、MTX を 200nM、FBS を 1%の濃度で含む EX-CELL302 培地に 1×10^6 細胞/ml となるように懸濁し、175mm² フラスコ (Greiner 社製) に 200ml ずつ分注した。5%CO_2 インキュベーター内で 37℃で 4 日間培養後、培養上清を回収した。培養上清より Prosep-A (Bioprocessing 社製) カラムを用いて、添付の説明書に従い、抗 GD3 キメラ抗体を精製した。精製した抗 GD3 キメラ抗体は、NSO-GD3 キメラ抗体 (302) と名付けた。

また、該形質転換細胞クローンを G418 を 0.5mg/ml、MTX を 200nM の濃度で含む GIT 培地に 3×10^5 細胞/ml となるように懸濁し、175mm² フラスコ (Greiner 社製) に 200ml ずつ分注した。5%CO_2 インキュベーター内で 37℃で 10 日間培養後、培養上清を回収した。培養上清より Prosep-A (Bioprocessing 社製) カラムを用いて、添付の説明書に従い、抗 GD3 キメラ抗体を精製した。精製した抗 GD3 キメラ抗体は、NSO-GD3 キメラ抗体 (GIT) と名付けた。

(4) SP2/0 細胞由来の生産細胞の培養及び抗体の精製

特開平 5-304989 (EP533199) に記載の抗 GD3 キメラ抗体を生産する形質転換細胞クローン (KM-871 (FERM BP-3512)) を G418 を 0.5mg/ml、MTX を 200nM の濃度で含む GIT 培地に 3×10^5 細胞/ml となるように懸濁し、175mm² フラスコ (Greiner 社製) に 200ml ずつ分注した。5%CO_2 インキュベーター内で 37℃で 8 日間培養後、培養上清を回収した。培養上清より Prosep-A (Bioprocessing 社製) カラムを用いて、添付の説明書に従い、抗 GD3 キメラ抗体を精製した。精製した抗 GD3 キメラ抗体は、SP2/0-GD3 キメラ抗体と名付けた。

5. 精製した抗 GD3 キメラ抗体の解析

上記実施例 1 の 4 項で得られた各種動物細胞で生産、精製した 5 種類の抗 GD3 キメラ抗体の各 4μg を公知の方法 [Nature, 227, 680 (1970)] に従って SDS-PAGE し、分子量及び精製度を解析した。その結果を第 1 図に示した。第 1 図に示したように、精製した各抗 GD3 キメラ抗体は、いずれも非還元条件下では分子量が約 150 キロダルトン (以下、Kd と表記する) の単一のバンドが、還元条件下では約 50Kd と約 25Kd の 2 本のバンドが認められた。これらの分子量は、抗体の H 鎖及び L 鎖の cDNA の塩基配列から推定される分子量 (H 鎖 : 約 49Kd、L 鎖 : 約 23Kd、分子全体 : 約 144Kd) とほぼ一致し、更に、IgG 型の抗体は、非還元条件下

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では分子量は約 150Kd であり、還元条件下では分子内のジスルフィド結合（以下、S-S 結合と表記する）が切断され、約 50Kd の分子量を持つ H 鎖と約 25Kd の分子量を持つ L 鎖に分解されるという報告 [Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Chapter 14 (1988)、Monoclonal Antibodies: Principles and Practice, Academic Press Limited (1996)] と一致し、各抗 GD3 キメラ抗体が正しい構造の抗体分子として発現され、かつ精製されたことが確認された。

実施例 2. 抗 GD3 キメラ抗体の活性評価

1. 抗 GD3 キメラ抗体の GD3 に対する結合活性（ELISA 法）

上記実施例 1 の 4 項で得られた 5 種類の精製抗 GD3 キメラ抗体の GD3（雪印乳業社製）に対する結合活性を実施例 1 の 3 項に示す ELISA 法により測定した。第 2 図は、添加する抗 GD3 キメラ抗体の濃度を変化させて結合活性を検討した結果である。第 2 図に示したように、5 種類の抗 GD3 キメラ抗体は、ほぼ同等の GD3 に対する結合活性を示した。この結果は抗体の抗原結合活性は、抗体を生産する動物細胞やその培養方法に関わらず、一定であることを示している。また、NSO-GD3 キメラ抗体（302）と NSO-GD3 キメラ抗体（GIT）の比較から抗原結合活性は、培養に用いる培地にも依らず、一定であることが示唆された。

2. 抗 GD3 キメラ抗体の in vitro 細胞障害活性（ADCC 活性）

上記実施例 1 の 4 項で得られた 5 種類の精製抗 GD3 キメラ抗体の in vitro 細胞障害活性を評価するため、以下に示す方法に従い、ADCC 活性を測定した。

(1) 標的細胞溶液の調製

RPMI1640-FBS(10) 培地で培養したヒトメラノーマ培養細胞株 G-361 (ATCC CRL1424) の 1×10^6 細胞を調製し、放射性物質である $Na_2{}^{51}CrO_4$ を 3.7MBq 当量加えて 37℃で 1 時間反応させ、細胞を放射線標識した。反応後、RPMI1640-FBS(10) 培地で懸濁及び遠心分離操作により 3 回洗浄し、培地に再懸濁し、4℃で 30 分間氷中に放置して放射性物質を自然解離させた。遠心分離後、RPMI1640-FBS(10) 培地を 5ml 加え、2×10^5 細胞/ml に調製し、標的細胞溶液とした。

(2) エフェクター細胞溶液の調製

健常人静脈血 50ml を採取し、ヘパリンナトリウム（武田薬品社製）0.5ml を加え穏やかに混ぜた。これを Lymphoprep (Nycomed Pharma AS 社製) を用いて使用説明書に従い、遠心分離して単核球層を分離した。RPMI1640-FBS(10) 培地で 3 回遠心分離して洗浄後、培地を用いて 2×10^6 細胞/ml の濃度で再懸濁し、エフェクター細胞溶液とした。

(3) ADCC 活性の測定

96 ウェルU字底プレート（Falcon 社製）の各ウェルに上記 (1) で調製した標的細胞溶液の 50μl (1×10^4 細胞/ウェル) を分注した。次いで (2) で調製したエフェクター細胞溶液を 100μl (2×10^5 細胞/ウェル、エフェクター細胞と標的細胞の比は 20:1 となる) 添加した。

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更に、各種抗 GD3 キメラ抗体を各最終濃度 0.0025～2.5μg/ml となるように加え、37℃で 4 時間反応させた。反応後、プレートを遠心分離し、上清の ^{51}Cr 量をγ-カウンターにて測定した。自然解離 ^{51}Cr 量は、エフェクター細胞溶液、抗体溶液の代わりに培地のみを用いて上記と同様の操作を行い、上清の ^{51}Cr 量を測定することにより求めた。全解離 ^{51}Cr 量は、抗体溶液の代わりに培地のみを、エフェクター細胞溶液の代わりに 1 規定塩酸を添加し、上記と同様の操作を行い、上清の ^{51}Cr 量を測定することにより求めた。ADCC 活性は下式 (II) により求めた。

$$\text{ADCC 活性 (\%)} = \frac{\text{検体上清中の } ^{51}Cr \text{ 量} - \text{自然解離 } ^{51}Cr \text{ 量}}{\text{全解離 } ^{51}Cr \text{ 量} - \text{自然解離 } ^{51}Cr \text{ 量}} \times 100 \qquad \text{(II)}$$

その結果を第 3 図に示した。第 3 図に示したように、5 種類の抗 GD3 キメラ抗体のうち、YB2/0-GD3 キメラ抗体が最も高い ADCC 活性を示し、次いで SP2/0-GD3 キメラ抗体、NS0-GD3 キメラ抗体、CHO-GD3 キメラ抗体の順に高い ADCC 活性を示した。培養に用いた培地の異なる NS0-GD3 キメラ抗体 (302) と NS0-GD3 キメラ抗体 (GIT) では、それらの ADCC 活性に差は認められなかった。以上の結果は、抗体の ADCC 活性は、生産に用いる動物細胞によって大きく異なることを示している。その機構としては、抗原結合活性が同等であったことから、抗体の Fc 領域の構造の差に起因していることが推定された。

実施例 3. 抗ヒトインターロイキン 5 レセプターα鎖ヒト型 CDR 移植抗体の作製

1. 抗ヒトインターロイキン 5 レセプターα鎖ヒト型 CDR 移植抗体の安定生産細胞の作製

(1) ラットミエローマ YB2/0 細胞を用いた生産細胞の作製

WO97/10354 に記載の抗ヒトインターロイキン 5 レセプターα鎖ヒト型 CDR 移植抗体 (以下、抗 hIL-5Rα CDR 移植抗体と表記する) の発現ベクターpKANTEX1259HV3LV0 を各種細胞株に導入し、優良株を選択することで抗 hIL-5Rα CDR 移植抗体の安定生産細胞を以下のようにして作製した。

抗 hIL-5Rα CDR 移植抗体発現ベクターpKANTEX1259HV3LV0 の 5μg を 4×10^6 細胞のラットミエローマ YB2/0 細胞へエレクトロポレーション法 [Cytotechnology, 3, 133 (1990)]により導入後、40ml の RPMI1640-FBS(10) に懸濁し、96 ウェル培養用プレート (住友ベークライト社製) に 200μl/ウェルずつ分注した。5%CO_2 インキュベーター内で 37℃、24 時間培養した後、G418 を 0.5mg/ml になるように添加して 1～2 週間培養した。G418 耐性を示す形質転換株のコロニーが出現し、増殖の認められたウェルより培養上清を回収し、上清中の抗 hIL-5Rα CDR 移植抗体の抗原結合活性を実施例 3 の 2 項に示す ELISA 法により測定した。

培養上清中に抗 hIL-5Rα CDR 移植抗体の生産が認められたウェルの形質転換株については、DHFR 遺伝子増幅系を利用して抗体生産量を増加させる目的で、G418 を 0.5mg/ml、MTX を 50nM 含む RPMI1640-FBS(10) 培地に $1\sim2\times10^5$ 細胞/ml になるように懸濁し、24 ウェルプレート (Greiner 社製) に 2ml ずつ分注した。5%CO_2 インキュベーター内で 37℃で 1～2 週間培

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養して、50nM MTX 耐性を示す形質転換株を誘導した。形質転換株の増殖が認められたウェルの培養上清中の抗 hIL-5Rα CDR 移植抗体の抗原結合活性を実施例 3 の 2 項に示す ELISA 法により測定した。培養上清中に抗 hIL-5Rα CDR 移植抗体の生産が認められたウェルの形質転換株については、上記と同様の方法により、MTX 濃度を 100nM、200nM と順次上昇させ、最終的に G418 を 0.5mg/ml、MTX を 200nM の濃度で含む RPMI1640-FBS(10) 培地で増殖可能かつ、抗 hIL-5Rα CDR 移植抗体を高生産する形質転換株を得た。得られた形質転換株の中から優良株を選択し、2 回の限界希釈法による単一細胞化（クローン化）を行った。尚、実施例 9 に示すα-1,6-フコシルトランスフェラーゼの遺伝子の転写物の定量法を用い、該転写物の量が比較的低い株を優良株として選択し用いた。このようにして得られた抗 hIL-5Rα CDR 移植抗体を生産する形質転換細胞クローン No.3 は平成 11 年 4 月 5 日付で工業技術院生命工学工業技術研究所（日本国茨城県つくば市東 1 丁目 1 番 3 号）（現・独立行政法人産業技術総合研究所 特許生物寄託センター（茨城県つくば市東 1 丁目 1 番地 中央第 6)）に FERM BP-6690 として寄託されている。

(2) CHO/dhfr-細胞を用いた生産細胞の作製

WO97/10354 に記載の抗 hIL-5Rα CDR 移植抗体発現ベクターpKANTEX1259HV3LV0 の 4μg を 1.6×10^6 細胞の CHO/dhfr-細胞へエレクトロポレーション法 [Cytotechnology, 3, 133 (1990)] により導入後、10ml の IMDM-FBS(10)に懸濁し、96 ウェル培養用プレート（岩城硝子社製）に 200μl/ウェルずつ分注した。5%CO_2 インキュベーター内で 37℃、24 時間培養した後、G418 を 0.5mg/ml になるように添加して 1～2 週間培養した。G418 耐性を示す形質転換株のコロニーが出現し、増殖の認められたウェルより培養上清を回収し、上清中の抗 hIL-5Rα CDR 移植抗体の抗原結合活性を実施例 3 の 2 項に示す ELISA 法により測定した。

培養上清中に抗 hIL-5Rα CDR 移植抗体の生産が認められたウェルの形質転換株については、DHFR 遺伝子増幅系を利用して抗体生産量を増加させる目的で、G418 を 0.5mg/ml、MTX を 10nM 含む IMDM-dFBS(10) 培地に $1\sim2\times10^5$ 細胞/ml になるように懸濁し、24 ウェルプレート（岩城硝子社製）に 0.5ml ずつ分注した。5%CO_2 インキュベーター内で 37℃で 1～2 週間培養して、10nM MTX 耐性を示す形質転換株を誘導した。増殖が認められたウェルの形質転換株については、上記と同様の方法により、MTX 濃度を 100nM、500nM に上昇させ、最終的に G418 を 0.5mg/ml、MTX を 500nM の濃度で含む IMDM-dFBS(10) 培地で増殖可能かつ、抗 hIL-5Rα CDR 移植抗体を高生産する形質転換株を得た。得られた形質転換株の中から優良株を選択し、2 回の限界希釈法による単一細胞化（クローン化）を行った。尚、実施例 9 に示すα-1,6-フコシルトランスフェラーゼの遺伝子の転写物の定量法を用い、該転写物の量が比較的低い株を優良株として選択し用いた。

(3) マウスミエローマ NS0 細胞を用いた生産細胞の作製

Yarranton らの方法 [BIO/TECHNOLOGY, 10, 169 (1992)] に従い、WO97/10354 に記載の抗 hIL-5Rα CDR 移植抗体発現ベクターpKANTEX1259HV3LV0 上の抗体 H 鎖及び L 鎖 cDNA を用いて抗 hIL-5Rα CDR 移植抗体発現ベクターを作製し、NS0 細胞を形質転換し、抗 hIL-5Rα CDR 移植抗体を高生産する形質転換株を得た。得られた形質転換株の中から優良株を選択し、2 回

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の限界希釈法による単一細胞化（クローン化）を行った。尚、実施例 9 に示すα-1,6-フコシルトランスフェラーゼの遺伝子の転写物の定量法を用い、該転写物の量が比較的低い株を優良株として用いた。

2. 抗体の hIL-5Rα に対する結合活性の測定（ELISA 法）

抗体の hIL-5Rα に対する結合活性は以下のようにして測定した。

WO97/10354 に記載の抗 hIL-5Rα マウス抗体 KM1257 を PBS で 10μg/ml の濃度に希釈した溶液の 50μl を 96 ウェルの ELISA 用のプレート（Greiner 社製）の各ウェルにそれぞれ分注し、4℃で 20 時間反応させた。反応後、1%BSA-PBS を 100μl/ウェルで加え、室温で 1 時間反応させて残存する活性基をブロックした。1%BSA-PBS を捨て、WO97/10354 に記載の可溶性 hIL-5Rα を 1%BSA-PBS で 0.5μg/ml の濃度に希釈した溶液を 50μl/ウェルで加え、4℃で 20 時間反応させた。反応後、各ウェルを Tween-PBS で洗浄後、形質転換株の培養上清或いは精製したヒト型 CDR 移植抗体の各種希釈溶液を 50μl/ウェルで加え、室温で 2 時間反応させた。反応後、各ウェルを Tween-PBS で洗浄後、1%BSA-PBS で 3000 倍に希釈したペルオキシダーゼ標識ヤギ抗ヒト IgG (H&L) 抗体溶液（American Qualex 社製）を二次抗体溶液として、50μl/ウェルで加え、室温で 1 時間反応させた。反応後、Tween-PBS で洗浄後、ABTS 基質液を 50μl/ウェルで加えて発色させ、OD415 を測定した。

3. 抗 hIL-5Rα CDR 移植抗体の精製

(1) YB2/0 細胞由来の生産細胞の培養及び抗体の精製

上記実施例 3 の 1 項 (1) で得られた抗 hIL-5Rα CDR 移植抗体を生産する形質転換細胞クローンを G418 を 0.5mg/ml、MTX を 200nM の濃度で含む GIT 培地に 3×10^5 細胞/ml となるように懸濁し、175mm² フラスコ（Greiner 社製）に 200ml ずつ分注した。5%CO_2 インキュベーター内で 37℃で 8 日間培養後、培養上清を回収した。培養上清よりイオン交換クロマトグラフィー及びゲル濾過法を用いて抗 hIL-5Rα CDR 移植抗体を精製した。精製した抗 hIL-5Rα CDR 移植抗体は、YB2/0-hIL-5RCDR 抗体と名付けた。

(2) CHO/dhfr-細胞由来の生産細胞の培養及び抗体の精製

上記実施例 3 の 1 項 (2) で得られた抗 hIL-5Rα CDR 移植抗体を生産する形質転換細胞クローンを L-Gln を 3mM、CDLC を 0.5%、PF68 を 0.3%の濃度で含む EX-CELL302 培地に 3×10^5 細胞/ml となるように懸濁し、4.0L スピナーボトル（岩城硝子社製）を用いて 100rpm の速度で撹拌培養した。37℃の恒温室内で 10 日間培養後、培養上清を回収した。培養上清よりイオン交換クロマトグラフィー及びゲル濾過法を用いて抗 hIL-5Rα CDR 移植抗体を精製した。精製した抗 hIL-5Rα CDR 移植抗体は、CHO/d-hIL-5RCDR 抗体と名付けた。

(3) NSO 細胞由来の生産細胞の培養及び抗体の精製

上記実施例 3 の 1 項 (3) で得られた抗 hIL-5Rα CDR 移植抗体を生産する形質転換細胞クローンを Yarranton らの方法 [BIO/TECHNOLOGY, 10, 169 (1992)] に従い、培養後、培養上清を回収した。培養上清よりイオン交換クロマトグラフィー及びゲル濾過法を用いて抗 hIL-

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5Rα CDR 移植抗体を精製した。精製した抗 hIL-5Rα CDR 移植抗体は、NSO-hIL-5RCDR 抗体と名付けた。

4. 精製した抗 hIL-5Rα CDR 移植抗体の解析

上記実施例 3 の 3 項で得られた各種動物細胞で生産、精製した 3 種類の抗 hIL-5Rα CDR 移植抗体の各 4μg を公知の方法 [Nature, 227, 680 (1970)] に従って SDS-PAGE し、分子量及び精製度を解析した。その結果を第 4 図に示した。第 4 図に示したように、精製した各抗 hIL-5Rα CDR 移植抗体は、いずれも非還元条件下では分子量が約 150Kd の単一のバンドが、還元条件下では約 50Kd と約 25Kd の 2 本のバンドが認められた。これらの分子量は、抗体の H 鎖及び L 鎖の cDNA の塩基配列から推定される分子量 (H 鎖：約 49Kd、L 鎖：約 23Kd、分子全体：約 144Kd) とほぼ一致し、更に、IgG 型の抗体は、非還元条件下では分子量は約 150Kd であり、還元条件下では分子内の S-S 結合が切断され、約 50Kd の分子量を持つ H 鎖と約 25Kd の分子量を持つ L 鎖に分解されるという報告 [Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Chapter 14 (1988)、Monoclonal Antibodies: Principles and Practice, Academic Press Limited (1996)] と一致し、各抗 hIL-5Rα CDR 移植抗体が正しい構造の抗体分子として発現され、かつ、精製されたことが確認された。

実施例 4. 抗 hIL-5Rα CDR 移植抗体の活性評価

1. 抗 hIL-5Rα CDR 移植抗体の hIL-5Rα に対する結合活性 (ELISA 法)

上記実施例 3 の 3 項で得られた 3 種類の精製抗 hIL-5Rα CDR 移植抗体の hIL-5Rα に対する結合活性を実施例 3 の 2 項に示す ELISA 法により測定した。第 5 図は、添加する抗 hIL-5Rα CDR 移植抗体の濃度を変化させて結合活性を検討した結果である。第 5 図に示したように、3 種類の抗 hIL-5Rα CDR 移植抗体は、ほぼ同等の hIL-5Rα に対する結合活性を示した。この結果は実施例 2 の 1 項の結果と同様に、抗体の抗原結合活性は、抗体を生産する動物細胞やその培養方法に関わらず、一定であることを示している。

2. 抗 hIL-5Rα CDR 移植抗体の in vitro 細胞障害活性 (ADCC 活性)

上記実施例 3 の 3 項で得られた 3 種類の精製抗 hIL-5Rα CDR 移植抗体の in vitro 細胞障害活性を評価するため、以下に示す方法に従い、ADCC 活性を測定した。

(1) 標的細胞溶液の調製

WO97/10354 に記載の hIL-5Rα 鎖及びβ鎖を発現しているマウス T 細胞株 CTLL-2(h5R) を RPMI1640-FBS(10) 培地で培養し、1×10^6 細胞/0.5ml となるように調製し、放射性物質である $Na_2{}^{51}CrO_4$ を 3.7MBq 当量加えて 37℃で 1.5 時間反応させ、細胞を放射線標識した。反応後、RPMI1640-FBS(10) 培地で懸濁及び遠心分離操作により 3 回洗浄し、培地に再懸濁し、4℃で 30 分間氷中に放置して放射性物質を自然解離させた。遠心分離後、RPMI1640-FBS(10) 培地を 5ml 加え、2×10^5 細胞/ml に調製し、標的細胞溶液とした。

(2) エフェクター細胞溶液の調製

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健常人静脈血 50ml を採取し、ヘパリンナトリウム（武田薬品社製）0.5ml を加え穏やかに混ぜた。これを Polymorphprep (Nycomed Pharma AS 社製) を用いて使用説明書に従い、遠心分離して単核球層を分離した。RPMI1640-FBS(10) 培地で 3 回遠心分離して洗浄後、培地を用いて 9×10^6細胞/ml の濃度で再懸濁し、エフェクター細胞溶液とした。

(3) ADCC 活性の測定

96 ウェルU字底プレート (Falcon 社製) の各ウェルに上記 (1) で調製した標的細胞溶液の 50μl (1×10^4 細胞/ウェル) を分注した。次いで (2) で調製したエフェクター細胞溶液を 100μl (9×10^5細胞/ウェル、エフェクター細胞と標的細胞の比は 90:1 となる) 添加した。更に、各種抗 hIL-5Rα CDR 移植抗体を各最終濃度 0.001～0.1μg/ml となるように加え、37℃で 4 時間反応させた。反応後、プレートを遠心分離し、上清の ^{51}Cr 量をγ-カウンターにて測定した。自然解離 ^{51}Cr 量は、エフェクター細胞溶液、抗体溶液の代わりに培地のみを用いて上記と同様の操作を行い、上清の ^{51}Cr 量を測定することにより求めた。全解離 ^{51}Cr 量は、抗体溶液の代わりに培地のみを、エフェクター細胞溶液の代わりに 1 規定塩酸を添加し、上記と同様の操作を行い、上清の ^{51}Cr 量を測定することにより求めた。ADCC 活性は前記式 (II) により求めた。

その結果を第 6 図に示した。第 6 図に示したように、3 種類の抗 hIL-5Rα CDR 移植抗体のうち、YB2/0-hIL-5RCDR 抗体が最も高い ADCC 活性を示し、次いで CHO/d-hIL-5RCDR 抗体、NSO-hIL-5RCDR 抗体の順に高い ADCC 活性を示した。以上の結果は実施例 2 の 2 項の結果と同様に、抗体の ADCC 活性は、生産に用いる動物細胞によって大きく異なることを示している。更に、2 種類のヒト化抗体のいずれの場合も YB2/0 細胞で生産した抗体が最も高い ADCC 活性を示したことから、YB2/0 細胞を用いることにより、ADCC 活性の高い抗体を製造できることが明らかとなった。

3. 抗 hIL-5Rα CDR 移植抗体の in vivo における活性評価

上記実施例 3 の 3 項で得られた 3 種類の精製抗 hIL-5Rα CDR 移植抗体の in vivo における活性を評価するため、以下に示す方法に従い、カニクイザルの hIL-5 誘発好酸球増加モデルに対する抑制作用を検討した。

カニクイザルに初日より hIL-5 (調製方法は WO97/10354 に記載) を 1μg/kg で 1 日 1 回、計 14 回背部皮下より投与した。各種抗 hIL-5Rα CDR 移植抗体を 0 日の hIL-5 の投与 1 時間前に 0.3mg/kg で静脈内に単回投与した。抗体非投与群をコントロールとして用いた。抗体投与群は各群 3 頭 (No.301、No.302、No.303、No.401、No.402、No.403、No.501、No.502、No.503)、抗体非投与群は 2 頭 (No.101、No.102) のカニクイザルを用いた。投与開始の 7 日前より投与後 42 日目まで経時的に約 1ml の血液を伏在静脈または大腿静脈より採取し、1μl の末梢血中の好酸球数を測定した。その結果を第 7 図に示した。第 7 図に示したように、YB2/0-hIL-5RCDR 抗体を投与した群では、血中好酸球の増加が完全に抑制された。一方、CHO/d-hIL-5RCDR 抗体の投与群では、1 頭で完全な抑制作用が認められたものの、2 頭ではその抑制作用は不充分であった。NSO-hIL-5RCDR 抗体の投与群では、完全な抑制作用は認められず、その効果は不充分であった。

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以上の結果は、抗体の in vivo 活性は、生産に用いる動物細胞によって大きく異なることを示している。更に、抗 hIL-5Rα CDR 移植抗体ではその in vivo 活性の高さは、実施例 4 の 2 項で述べた ADCC 活性の高さと正の相関が認められたことから、その活性発現には、ADCC 活性の高さが極めて重要であることが示唆された。

以上の結果から、ADCC 活性の高い抗体は、ヒトの各種疾患の臨床においても有用であることが期待される。

実施例 5. ADCC 活性を高める糖鎖の解析

1. 2-アミノピリジン標識糖鎖（PA 化糖鎖）の調製

本発明のヒト化抗体を塩酸による酸加水分解にてシアル酸を除去した。塩酸を完全に除去した後、ヒドラジン分解により糖鎖をタンパク質から切断した [Method of Enzymology, 83, 263 (1982)]。ヒドラジンを除去した後、酢酸アンモニウム水溶液と無水酢酸加えて N-アセチル化を行った。凍結乾燥後、2-アミノピリジンによる蛍光標識を行った [J. Biochem., 95, 197 (1984)]。蛍光標識した糖鎖（PA 化糖鎖）を、Superdex Peptide HR 10/30 カラム (Pharmacia 社製) を用いて不純物と分離した。糖鎖画分を遠心濃縮機にて乾固させ、精製 PA 化糖鎖とした。

2. 精製抗 hIL-5Rα CDR 移植抗体の PA 化糖鎖の逆相 HPLC 分析

上記実施例 5 の 1 項の方法で実施例 3 で作製された各種抗 hIL-5RCDR 抗体について PA 化糖鎖を行った後、CLC-ODS カラム (Shimadzu 社製) による逆相 HPLC 分析を行った。過剰量のα-L-フコシダーゼ（ウシ腎由来、SIGMA 社製）を PA 化糖鎖に添加して消化を行い（37℃、15 時間）、逆相 HPLC で分析した（第 8 図）。アスパラギン結合糖鎖は 30 分間から 80 分間の範囲に溶出することを TaKaRa 社製 PA 化糖鎖スタンダードを用いて確認した。α-L-フコシダーゼ消化によって、逆相 HPLC の溶出位置が移動する糖鎖（48 分間から 78 分間に溶出される糖鎖）の全体に占める割合を計算した。結果を第 1 表に示す。

第 1 表

抗体の生産細胞	α-1,6-フコース結合糖鎖（%）
YB2/0	47
NS0	73

YB2/0 細胞で生産させた抗 hIL-5RCDR 移植抗体は約 47%、NS0 細胞で生産させた抗 hIL-5RCDR 移植抗体は約 73%が N グリコシド結合糖鎖還元末端の N-アセチルグルコサミンの 6 位にフコースの 1 位がα結合した糖鎖（以下、「α-1,6-フコースを持つ糖鎖」とも表記する）であった。よって、YB2/0 細胞で生産した抗体は、NS0 細胞で生産した抗体と比較して N-グリコシド結合糖鎖還元末端の N-アセチルグルコサミンの 6 位にフコースが結合していない糖鎖(以下、単に、「α-1,6-フコースを持たない糖鎖」と表記する）の割合がα-1,6-フコースを持たない糖鎖が多かった。

3. 精製抗 hIL-5Rα CDR 移植抗体の単糖組成分析

トリフルオロ酢酸による酸加水分解により、YB2/0 細胞、NS0 細胞および CHO/d 細胞で生

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産した抗 hIL-5Rα CDR 移植抗体の糖鎖を単糖に分解し、BioLC (Dionex 社製) を用いて単糖組成分析を行った。

N-グリコシド結合糖鎖のうち、コンプレックス型では、1 本の糖鎖におけるマンノース数は3であるため、マンノースを3として計算した場合の各単糖の相対比を第2表に示す。

第2表

抗体の生産細胞	Fuc	GlcNAc	Gal	Man	ADCC 活性 (%)*
YB2/0	0.60	4.98	0.30	3.00	42.27
NS0	1.06	3.94	0.66	3.00	16.22
CHO/dhfr-	0.85	3.59	0.49	3.00	25.73
CHO/dhfr-	0.91	3.80	0.27	3.00	25.73

*抗体濃度 0.01μg/ml

フコースの相対比は、 YB2/0＜CHO/d＜NS0 であり、本結果でも YB2/0 細胞で生産した抗体の糖鎖はフコース含量が最も低かった。

4. CHO/dhfr-細胞生産抗体の糖鎖解析

CHO/dhfr-細胞で生産した精製抗 hIl-5Rα CDR 移植抗体から PA 化糖鎖を調製し、CLC-ODS カラム (島津社製) を用いて逆相 HPLC 分析を行った(第 9 図)。第 9 図において、溶出時間 35～45 分間がフコースを持たない糖鎖、45～60 分間がフコースを持つ糖鎖であった。CHO/dhfr-細胞で生産した抗 hIl-5Rα CDR 移植抗体は、マウスミエローマ NS0 細胞で生産させた抗体と同様に、ラットミエローマ YB2/0 細胞で生産させた抗体よりもフコースを持たない糖鎖の含量が少なかった。

実施例 6. 高 ADCC 活性抗体の分離

フコースを持つ糖鎖に結合するレクチンカラムを用いて、ラットミエローマ YB2/0 細胞で生産させた抗 hIl-5Rα CDR 移植抗体の分離を行った。HPLC は島津社製 LC-6A を用い、流速は 1ml/分、カラム温度は室温で行った。50mM トリス-硫酸緩衝液 (pH7.3) で平衡化し、精製された抗 hIL-5Rα CDR 移植抗体を注入後、0.2Mα-メチルマンノシド (ナカライテスク社製) の直線濃度勾配 (60 分間) にて溶出した。抗 hIl-5Rα CDR 移植抗体を非吸着画分と吸着画分とに分離した。非吸着画分、吸着画分の一部をとり、hIL-5Rα に対する結合活性を測定すると、同様の結合活性を示した (10A 図)。ADCC 活性を測定すると、非吸着画分の方が吸着画分の一部よりも高い (100～1000 倍) ADCC 活性を示した (10B 図)。さらに、非吸着画分、吸着画分の一部から PA 化糖鎖を調製し、CLC-ODS カラム (島津社製) を用いて逆相 HPLC 分析を行った (第 11 図)。非吸着画分は主としてフコースのない糖鎖をもつ抗体であり、吸着画分の一部は主としてフコースがある糖鎖もつ抗体であった。

実施例 7. α-1,6-フコースを持たない糖鎖の割合の異なる抗 GD3 キメラ抗体の活性評価

1. α-1,6-フコースを持たない糖鎖の割合の異なる抗 GD3 キメラ抗体の調製

実施例 1 の 2 項 (1) に記載した方法に従って、抗 GD3 キメラ抗体を生産する YB2/0 細胞由来の形質転換クローンを得た。それぞれの YB2/0 細胞由来の形質転換クローンより抗体を

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調製し、それぞれをロット1、ロット2、ロット3とした。抗GD3キメラ抗体ロット1、ロット2、ロット3の糖鎖分析を、実施例11の（6）の方法に従って行った結果、α-1,6-フコースを持たない糖鎖の割合は、それぞれ50%、45%、29%であった。以下、これらの試料を、抗GD3キメラ抗体（50%）、抗GD3キメラ抗体（45%）、抗GD3キメラ抗体（29%）と表記する。

また、実施例1の2項（2）で調製したCHO/DG44細胞由来の抗GD3キメラ抗体の糖鎖分析を実施例11の（6）の方法に従って行った結果、α-1,6-フコースを持たない糖鎖の割合は、7%であった。以下、本試料を抗GD3キメラ抗体（7%）と表記する。

さらに、抗GD3キメラ抗体（45%）と抗GD3キメラ抗体（7%）を用い、抗GD3キメラ抗体（45%）:抗GD3キメラ抗体（7%）=5:3および1:7の割合で混合した。これらの試料を、実施例10の（6）の方法に従って糖鎖分析を行った結果、α-1,6-フコースを持たない糖鎖の割合が24%および13%（分析値）の試料を調製した。これらを以下、抗GD3キメラ抗体（24%）、抗GD3キメラ抗体（13%）と表記する。

第12図には、各試料の糖鎖分析の結果を示した。α-1,6-フコースを持たない糖鎖の割合は、2回の糖鎖分析の結果を平均した値を用いた。

2. GD3に対する結合活性の評価（ELISA法）

実施例7の1項で調製したα-1,6-フコースを持たない糖鎖の割合の異なる6種類の抗GD3キメラ抗体のGD3（雪印乳業社製）に対する結合活性は、実施例1の3項に示すELISA法により測定した。その結果、第13図に示したように、6種類の抗GD3キメラ抗体は、いずれも同等のGD3に対する結合活性を示し、α-1,6-フコースを持たない糖鎖の割合は、抗体の抗原結合活性に影響を与えないことが明らかとなった。

3. ヒトメラノーマ細胞株に対するADCC活性の評価

抗GD3キメラ抗体のヒトメラノーマ細胞株G-361（ATCC CRL1424）に対するADCC活性は、以下のようにして測定した。

(1) 標的細胞溶液の調製

ヒトメラノーマ細胞株G-361の1×10^6細胞を調製し、放射性物質である$Na_2{}^{51}CrO_4$を3.7MBq当量加えて37℃で1時間反応させ、細胞を放射線標識した。反応後、培地を用いた懸濁及び遠心分離操作により3回洗浄し、培地に再懸濁し、4℃で30分間氷中に放置して放射性物質を自然解離させた。遠心分離後、培地を5mL加え、2×10^5細胞/mLに調製し、標的細胞溶液とした。

(2) ヒトエフェクター細胞溶液の調製

健常人末梢血50mLを採取し、ヘパリンナトリウム（清水製薬社製）を0.5mLを加え穏やかに混ぜた。これをLymphoprep（AXIS SHIELD社製）を用いて使用説明書に従い、遠心分離（800g、20分間）して単核球層を分離した。培地で3回遠心分離（1200rpm、5分間）して洗浄後、培地を用いて2×10^6細胞/mLの濃度で再懸濁し、ヒトエフェクター細胞溶液とした。

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(3) ADCC 活性の測定

96 ウェルU字底プレート（Falcon 社製）の各ウェルに上記（1）で調製した標的細胞溶液の 50μL（1×10^4 細胞/ウェル）を分注した。次いで上記（2）で調製したヒトエフェクター細胞溶液を 100μL（2×10^5 細胞/ウェル、ヒトエフェクター細胞と標的細胞の比は 20:1 となる）添加した。さらに、抗 GD3 キメラ抗体を各最終濃度 0.0005～5μg/mL となるように加え、37℃で 4 時間反応させた。反応後、プレートを遠心分離し、上清中の ^{51}Cr 量をγ-カウンターにて測定した。自然解離 ^{51}Cr 量は、ヒトエフェクター細胞溶液、抗体溶液の代わりに培地のみを用いて上記と同様の操作を行い、上清中の ^{51}Cr 量を測定することにより求めた。全解離 ^{51}Cr 量は、抗体溶液の代わりに培地のみを、ヒトエフェクター細胞溶液の代わりに 1mol/L の塩酸溶液を添加し、上記と同様の操作を行い、上清中の ^{51}Cr 量を測定することにより求めた。細胞障害活性（%）は前記式（II）により求めた。

第 14 図および第 15 図には、α-1,6-フコースを持たない糖鎖の割合の異なる 6 種類の抗 GD3 キメラ抗体の各種濃度（0.0005～5μg/mL）における ADCC 活性を 2 名の健常人ドナー（A、B)のエフェクター細胞を用いて測定した結果をそれぞれ示した。第 14 図および第 15 図に示したように、抗 GD3 キメラ抗体の ADCC 活性は、いずれの抗体濃度においてもα-1,6-フコースを持たない糖鎖の割合に比例して上昇する傾向を示した。抗体濃度が低ければ、ADCC 活性は低下する。抗体濃度が 0.05μg/ml では、α-1,6-フコースを持たない糖鎖が２４%、２９%、４５%および５０%の ADCC 活性はほぼ同様の高い活性を示したが、α-1,6-フコースを持たない糖鎖が２０%未満の抗体である、１３%および７%では、ADCC 活性は低かった。本結果は、エフェクター細胞のドナーが異なっても同様であった。

実施例 8. α-1,6-フコースを持たない糖鎖の割合の異なる抗 CCR4 キメラ抗体の活性評価

1. 抗 CCR4 キメラ抗体の安定生産細胞の作製

WO01/64754 記載の抗 CCR4 キメラ抗体のタンデム型発現ベクターpKANTEX2160 を用いて抗 CCR4 キメラ抗体の安定生産細胞を以下のようにして作製した。

(1) ラットミエローマ YB2/0 細胞を用いた生産細胞の作製

10μg の抗 CCR4 キメラ抗体発現ベクターpKANTEX2160 を 4×10^6 細胞のラットミエローマ YB2/0 細胞（ATCC CRL1662）へエレクトロポレーション法［Cytotechnology, 3, 133 (1990)］により導入後、40mL の Hybridoma-SFM-FBS(5)［FBS（PAA ラボラトリーズ社製）を 5%含む Hybridoma-SFM 培地（インビトロジェン社製)］に懸濁し、96 ウェル培養用プレート（住友ベークライト社製）に 200μL/ウェルずつ分注した。5%CO_2インキュベーター内で 37℃、24 時間培養した後、G418 を 1mg/mL になるように添加して 1～2 週間培養した。G418 耐性を示す形質転換株のコロニーが出現し、増殖の認められたウェルより培養上清を回収し、上清中の抗 CCR4 キメラ抗体の抗原結合活性を実施例 8 の 2 項記載の ELISA 法により測定した。

培養上清中に抗 CCR4 キメラ抗体の生産が認められたウェルの形質転換株については、DHFR 遺伝子増幅系を利用して抗体生産量を増加させる目的で、G418 を 1mg/mL、DHFR の阻害剤である MTX（SIGMA 社製）を 50nM 含む Hybridoma-SFM-FBS(5) 培地に $1\sim2\times10^5$ 細胞/ml に

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なるように懸濁し、24 ウェルプレート（Greiner 社製）に 1ml ずつ分注した。5%CO_2 インキュベーター内で 37℃で 1～2 週間培養して、50nM MTX 耐性を示す形質転換株を誘導した。形質転換株の増殖が認められたウェルの培養上清中の抗 CCR4 キメラ抗体の抗原結合活性を実施例 8 の 2 項記載の ELISA 法により測定した。

培養上清中に抗 CCR4 キメラ抗体の生産が認められたウェルの形質転換株については、上記と同様の方法により、MTX 濃度を上昇させ、最終的に MTX を 200nM の濃度で含む Hybridoma-SFM-FBS(5) 培地で増殖可能かつ、抗 CCR4 キメラ抗体を高生産する形質転換株を得た。得られた形質転換株について、2 回の限界希釈法による単一細胞化（クローン化）を行い、得られたクローン化株を KM2760#58-35-16 と名付けた。尚、実施例 9 に示すα-1,6-フコシルトランスフェラーゼの遺伝子の転写物の定量法を用い、該転写物の量が比較的低い株を優良株として選択し用いた。

(2) CHO/DG44 細胞を用いた生産細胞の作製

抗 CCR4 キメラ抗体発現ベクターpKANTEX2160 の 4μg を 1.6×10^6 細胞の CHO/DG44 細胞へエレクトロポレーション法 [Cytotechnology, 3, 133 (1990)] により導入後、10ml の IMDM-dFBS(10)-HT(1) [dFBS（インビトロジェン社製）を 10%、HT supplement（インビトロジェン社製）を 1 倍濃度で含む IMDM 培地（インビトロジェン社製)] に懸濁し、96 ウェル培養用プレート（岩城硝子社製）に 100μl/ウェルずつ分注した。5%CO_2 インキュベーター内で 37℃、24 時間培養した後、IMDM-dFBS(10)（透析 FBS を 10%で含む IMDM 培地）に培地交換し、1～2 週間培養した。HT 非依存的な増殖を示す形質転換株のコロニーが出現したため、増殖の認められたウェルより培養上清を回収し、上清中の抗 CCR4 キメラ抗体の発現量を実施例 8 の 2 項記載の ELISA 法により測定した。

培養上清中に抗 CCR4 キメラ抗体の生産が認められたウェルの形質転換株については、DHFR 遺伝子増幅系を利用して抗体生産量を増加させる目的で、MTX を 50nM 含む IMDM-dFBS(10) 培地に 1～2×10^5 細胞/ml になるように懸濁し、24 ウェルプレート（岩城硝子社製）に 0.5ml ずつ分注した。5%CO_2 インキュベーター内で 37℃で 1～2 週間培養して、50nM MTX 耐性を示す形質転換株を誘導した。増殖が認められたウェルの形質転換株については、上記と同様の方法により、MTX 濃度を 200nM に上昇させ、最終的に MTX を 200nM の濃度で含む IMDM-dFBS(10) 培地で増殖可能かつ、抗 CCR4 キメラ抗体を高生産する形質転換株を得た。得られた形質転換株は 5-03 株と名付けた。尚、実施例 9 に示すα-1,6-フコシルトランスフェラーゼの遺伝子の転写物の定量法を用い、該転写物の量が比較的低い株を優良株として選択し用いた。

2. 抗体 CCR4 部分ペプチドに対する結合活性（ELISA 法）

抗 CCR4 キメラ抗体が反応し得るヒト CCR4 細胞外領域ペプチドとして化合物 1（配列番号 25）を選択した。ELISA 法による活性測定に用いるため、以下の方法で BSA (Bovine Serum Albumin)（ナカライテスク社製）とのコンジュゲートを作製し、抗原として用いた。すなわち、10 mg の BSA を含む PBS 溶液 900 mL に、100ml の 25mg/mL SMCC [4-(N-マレイミドメチル) シクロヘキサン-1-カルボキシリックアシッド N-ヒドロキシサクシンイミドエステル]

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(シグマ社製)-DMSO 溶液を vortex しながら滴下し、30 分間ゆっくりと撹拌した。25 mL PBS で平衡化した NAP-10 カラムなどのゲルろ過カラムに反応液 1mL をアプライし、1.5mL の PBS で溶出させた溶出液を BSA-SMCC 溶液とした（A_{280} 測定から BSA 濃度を算出)。次に、0.5 mg の化合物 1 に 250mL PBS を加え、次いで 250mL DMF を加えて完全に溶解させた後、前述の BSA-SMCC 溶液（BSA 換算 1.25mg）を vortex 下で添加して 3 時間ゆっくり撹拌した。反応液を PBS に対して 4℃、一晩透析し、最終濃度 0.05%となるようにアジ化ナトリウムを添加して、0.22 mm フィルターでろ過した後 BSA-化合物 1 溶液とした。

96 穴の EIA 用プレート（グライナー社）に、上述のように調製したコンジュゲートを 0.05μg/mL、50μl/ウェルで分注し、4℃で一晩放置して吸着させた。PBS で洗浄後、1%BSA-PBS を 100μL/ウェルで加え、室温で 1 時間反応させて残存する活性基をブロックした。各ウェルを 0.05%Tween20 を含む PBS（以下、Tween-PBS と表記する）で洗浄後、形質転換株の培養上清を 50μL/ウェルで加え、室温で 1 時間反応させた。反応後、各ウェルを Tween-PBS で洗浄後、1%BSA-PBS で 6000 倍に希釈したペルオキシダーゼ標識ヤギ抗ヒト IgG(γ) 抗体溶液（American Qualex 社製） を二次抗体溶液として、それぞれ 50μL/ウェルで加え、室温で 1 時間反応させた。反応後、Tween-PBS で洗浄後、ABTS 基質液を 50μL/ウェルで加えて発色させ、20 分後に 5%SDS 溶液を 50μL/ウェル加えて反応を停止した。その後 OD415 を測定した。実施例 8 の 1 項で得られた抗 CCR4 キメラ抗体は、CCR4 に対する結合活性を示した。

3. 抗 CCR4 キメラ抗体の精製

(1) YB2/0 細胞由来の生産細胞の培養及び抗体の精製

実施例 8 の 1 項 (1) で得られた抗 CCR4 キメラ抗体を発現する形質転換細胞クローン KM2760#58-35-16 を 200nM MTX、Daigo's GF21（和光純薬製）を 5％の濃度で含む Hybridoma-SFM（インビトロジェン社製）培地に 2×10^5 細胞/ml となる様に懸濁し、スピナーボトル（岩城硝子社製）を用いて 37℃の恒温室内で Fed-Batch 撹拌培養した。8-10 日間培養して回収した培養上清より、Prosep-A（ミリポア社製）カラム及びゲルろ過法を用いて、抗 CCR4 キメラ抗体を精製した。精製した抗 CCR4 キメラ抗体を KM2760-1 と名づけた。

(2) CHO/DG44 細胞由来の生産細胞の培養及び抗体の精製

実施例 8 の 1 項 (2) で得られた抗 CCR4 キメラ抗体を生産する形質転換細胞株 5-03 株を IMDM-dFBS(10) 培地中で、182cm^2 フラスコ（Greiner 社製）にて 5%CO_2 インキュベーター内で 37℃にて培養した。数日後、細胞密度がコンフルエントに達した時点で培養上清を除去し、25ml の PBS バッファーにて細胞を洗浄後、EXCELL301 培地（JRH 社製）を 35ml 注入した。5%CO_2 インキュベーター内で 37℃にて 7 日間培養後、培養上清を回収した。培養上清より Prosep-A（ミリポア社製）カラムを用いて、添付の説明書に従い、抗 CCR4 キメラ抗体を精製した。精製した抗 CCR4 キメラ抗体は KM3060 と名付けた。

KM2760-1 および KM3060 の CCR4 に対する結合活性を ELISA により測定した結果、同等の結合活性を示した。

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4. 精製した抗 CCR4 キメラ抗体の解析

本実施例 1 項で得られた各種動物細胞で生産、精製した 2 種類の抗 CCR4 キメラ抗体の各 4 μg を公知の方法 [Nature, 227, 680 (1970)] に従って SDS-PAGE し、分子量及び製精度を解析した。精製した各抗 GD3 キメラ抗体は、いずれも非還元条件下では分子量が約 150Kd の単一のバンドが、還元条件下では約 50Kd と約 25Kd の 2 本のバンドが認められた。これらの分子量は、抗体の H 鎖及び L 鎖の cDNA の塩基配列から推定される分子量 (H 鎖：約 49Kd、L 鎖：約 23Kd、分子全体：約 144Kd) とほぼ一致し、更に、IgG 型の抗体は、非還元条件下では分子量は約 150Kd であり、還元条件下では分子内の S-S 結合が切断され、約 50Kd の分子量を持つ H 鎖と約 25Kd の分子量を持つ L 鎖に分解されるという報告 [Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Chapter 14 (1988)、Monoclonal Antibodies: Principles and Practice, Academic Press Limited (1996)] と一致し、抗 CCR4 キメラ抗体が正しい構造の抗体分子として発現され、かつ精製されたことが確認された。

5. α-1,6-フコースを持たない糖鎖の割合の異なる抗 CCR4 キメラ抗体の調製

実施例 8 の 3 項 (1) で調製した、YB2/0 細胞由来の抗 CCR4 キメラ抗体 KM2760-1 と、実施例 8 の 3 項 (2) で調製した、CHO/DG44 細胞由来の抗 CCR4 キメラ抗体 KM3060 の糖鎖分析を、実施例 10 の (6) の方法に従って行った。α-1,6-フコースを持たない糖鎖の割合は、KM2760 は 87%、KM3060 は 8%であった。以下、これらの試料を、抗 CCR4 キメラ抗体 (87%)、抗 CCR4 キメラ抗体 (8%) と表記する。

さらに、抗 CCR4 キメラ抗体 (87%) と抗 CCR4 キメラ抗体 (8%) を用い、抗 CCR4 キメラ抗体 (87%):抗 CCR4 キメラ抗体 (8%)＝1:39、16:67、22:57、32:47、42:37 の割合で混合した。これらの試料を実施例 10 の (6) の方法にしたがって糖鎖分析を行なった。α-1,6-フコースを持たない糖鎖の割合は、それぞれ 9%、18%、27%、39%、46%であった。以下、これらの試料を抗 CCR4 キメラ抗体 (9%)、抗 CCR4 キメラ抗体 (18%)、抗 CCR4 キメラ抗体 (27%)、抗 CCR4 キメラ抗体 (39%)、抗 CCR4 キメラ抗体 (46%) と表記する。

第 16 図には、各試料の糖鎖分析の結果を示した。α-1,6-フコースを持たない糖鎖の割合は、2 回の結果を平均した値を用いた。

6. CCR4 部分ペプチドに対する結合活性の評価 (ELISA 法)

実施例 8 の 5 項で調製したα-1,6-フコースを持たない糖鎖の割合の異なる 6 種類の抗 CCR4 キメラ抗体の CCR4 部分ペプチドに対する結合活性は実施例 8 の 2 に記載の方法に従って測定した。

その結果、第 17 図に示したように、6 種類の抗 CCR4 キメラ抗体は、いずれも同等の CCR4 に対する結合活性を示し、α-1,6-フコースを持たない糖鎖の割合は、抗体の抗原結合活性に影響を与えないことが明らかとなった。

7. ヒト CCR4 高発現細胞株に対する ADCC 活性の評価

抗 CCR4 キメラ抗体のヒト CCR4 高発現細胞である CCR4/EL-4 細胞 (WO01/64754) に対する ADCC 活性は、以下のようにして測定した。

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(1) 標的細胞溶液の調製

WO01/64754 に記載のヒト CCR4 を発現している CCR4/EL-4 細胞の 1.5×10^6 細胞を調製し、放射性物質である $Na_2{}^{51}CrO_4$ を 5.55MBq 当量加えて 37℃で 1 時間 30 分間反応させ、細胞を放射線標識した。反応後、培地を用いた懸濁及び遠心分離操作により 3 回洗浄し、培地に再懸濁し、4℃で 30 分間氷中に放置して放射性物質を自然解離させた。遠心分離後、培地を 7.5mL 加え、2×10^5 細胞/mL に調製し、標的細胞溶液とした。

(2) ヒトエフェクター細胞溶液の調製

健常人末梢血 60mL を採取し、ヘパリンナトリウム（清水製薬社製）を 0.6mL を加え穏やかに混ぜた。これを Lymphoprep（AXIS SHIELD 社製）を用いて使用説明書に従い、遠心分離（800g、20 分間）して単核球層を分離した。培地で 3 回遠心分離（1400rpm、5 分間）して洗浄後、培地を用いて 5×10^6 細胞/mL の濃度で再懸濁し、ヒトエフェクター細胞溶液とした。

(3) ADCC 活性の測定

96 ウェルU字底プレート（Falcon 社製）の各ウェルに上記（1）で調製した標的細胞溶液の 50μL（1×10^4 細胞/ウェル）を分注した。次いで上記（2）で調製したヒトエフェクター細胞溶液を 100μL（5×10^6 細胞/ウェル、ヒトエフェクター細胞と標的細胞の比は 50:1 となる）添加した。さらに、抗 CCR4 キメラ抗体を各最終濃度 0.0001～10μg/mL となるように加え、37℃で 4 時間反応させた。反応後、プレートを遠心分離し、上清中の ^{51}Cr 量をγ-カウンターにて測定した。自然解離 ^{51}Cr 量は、ヒトエフェクター細胞溶液、抗体溶液の代わりに培地のみを用いて上記と同様の操作を行い、上清中の ^{51}Cr 量を測定することにより求めた。全解離 ^{51}Cr 量は、抗体溶液とヒトエフェクター細胞溶液の代わりに 1mol/L の塩酸溶液を添加し、上記と同様の操作を行い、上清中の ^{51}Cr 量を測定することにより求めた。ADCC 活性（%）は前記式（II）により求めた。

第 18 図および第 19 図には、α-1,6-フコースを持たない糖鎖の割合の異なる抗 CCR4 キメラ抗体の各種濃度（0.001～10μg/mL）における ADCC 活性を 2 名の健常人ドナー（A, B）のエフェクター細胞を用いて測定した結果をそれぞれ示した。第 18 図および第 19 図に示したように、抗 CCR4 キメラ抗体の ADCC 活性はいずれの抗体濃度においてもα-1,6-フコースを持たない糖鎖の割合に比例して上昇する傾向を示した。抗体濃度が低ければ、ADCC 活性は低下する。抗体濃度が 0.01μg/ml では、α-1,6-フコースを持たない糖鎖が２７%、３９%および４６%の ADCC 活性はほぼ同様の高い活性を示したが、α-1,6-フコースを持たない糖鎖が２０%未満の抗体では、ADCC 活性は低かった。本結果は、エフェクター細胞のドナーが異なっても同様であった。

実施例 9. 宿主細胞株におけるα-1,6-フコシルトランスフェラーゼ遺伝子の転写物の定量

(1) 各種細胞株由来一本鎖 cDNA の調製

ジヒドロ葉酸還元酵素遺伝子（dhfr）を欠損したチャイニーズハムスター卵巣由来 CHO/DG44 細胞およびラットミエローマ YB2/0 細胞より、以下の手順で一本鎖 cDNA を調製し

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た。

CHO/DG44 細胞を 10% ウシ胎児血清 (Life Technologies 社製) および 1 倍濃度の HT supplement (Life Technologies 社製) を添加した IMDM 培地 (Life Technologies 社製) に懸濁し、2×10^5 個/ml の密度で接着細胞培養用 T75 フラスコ (Greiner 社製) に 15ml 播種した。また、YB2/0 細胞を 10% ウシ胎児血清 (Life Technologies 社製)、4mmol/l L-GLN (Life Technologies 社製) を添加した RPMI1640 培地 (Life Technologies 社製) に懸濁し、2×10^5 個/ml の密度で浮遊細胞培養用 T75 フラスコ (Greiner 社製) に 15ml 播種した。これらを 37℃の 5%CO_2 インキュベーター内で培養し、培養 1 日目、2 日目、3 日目、4 日目および 5 日目に各宿主細胞 1×10^7 個を回収後、RNAeasy (QIAGEN 社製) により添付の説明書に従って全 RNA を抽出した。

全 RNA を 45μl の滅菌水に溶解し、RQ1 RNase-Free DNase (Promega 社製) 1μl、付属の 10×DNase buffer 5μl、RNasin Ribonuclease inhibitor (Promega 社製) 0.5μl をそれぞれに添加して、37℃で 30 分間反応させることにより、試料中に混入したゲノム DNA を分解した。反応後、RNAeasy (QIAGEN 社製) により全 RNA を再精製し、50μl の滅菌水に溶解した。

得られた各々の全 RNA3μg に対し、SUPERSCRIPT™ Preamplification System for First Strand cDNA Synthesis (Life Technologies 社製) を用いて添付の説明書に従い、オリゴ (dT) をプライマーとした 20μl の系で逆転写反応を行うことにより、一本鎖 cDNA を合成した。各宿主細胞由来α-1,6-フコシルトランスフェラーゼ (以下、FUT8 ともいう)、β-アクチンのクローニングには該反応液の 1 倍濃度液を、競合的 PCR による各遺伝子転写量の定量には該反応液の 50 倍希釈水溶液を用い、各々使用するまで-80℃で保管した。

(2) チャイニーズハムスターFUT8 およびラット FUT8 の各 cDNA 部分断片の取得

チャイニーズハムスターFUT8 およびラット FUT8 の各 cDNA 部分断片の取得は、以下の手順で行った (第 20 図)。

まず、ヒト FUT8 の cDNA [J. Biochem., 121, 626 (1997)] およびブタ FUT8 の cDNA [J. Biol. Chem., 271, 27810 (1996)] に共通の塩基配列に対して特異的なプライマー (配列番号 4 および配列番号 5 に示す) を設計した。

次に DNA ポリメラーゼ ExTaq (宝酒造社製) を用いて、本項 (1) で調製した培養 2 日目の CHO 細胞由来 cDNA および YB2/0 細胞由来 cDNA を各々1μl を含む 25μl の反応液 [ExTaq buffer (宝酒造社製)、0.2mmol/l dNTPs、0.5μmol/l 上記遺伝子特異的プライマー (配列番号 4 および配列番号 5)] を調製し、ポリメラーゼ連鎖反応 (PCR) を行った。PCR は、94℃で 1 分間の加熱の後、94℃で 30 秒間、55℃で 30 秒間、72℃で 2 分間からなる反応を 1 サイクルとして 30 サイクルの後、さらに 72℃で 10 分間加熱する条件で行った。

PCR 後、反応液を 0.8%アガロースゲル電気泳動に供し、特異的増幅断片 979bp を GENECLEAN Spin Kit (BIO 101 社製) を用いて精製し、滅菌水 10μl で溶出した (以下、アガロースゲルからの DNA 断片の精製にはこの方法を用いた)。上記増幅断片 4μl を、TOPO TA cloning Kit (Invitrogen 社製) の説明書に従って、プラスミド pCR2.1 へ挿入し、該反応液を用いて大腸菌 XL1-Blue 株をコーエンらの方法 [Proc. Natl. Acad. Sci. USA, 69,

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