

AUSTRALIA

PATENTS ACT 1990

PATENT REQUEST: STANDARD PATENT

I/We, the Applicant(s)/Nominated Person(s) specified below, request I/We be granted a patent for the invention disclosed in the accompanying standard complete specification.

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[54] Invention Title:

Process for Producing Humanized Chimera Antibody

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	Details of Basic Application(s):	
[31] Appl'n No(s):	[33] Country:	[32] Application Date:
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In accordance with Regulation 3.25, samples of materials deposited in accordance with the Budapest Treaty in relation to this Patent Request are only to be provided before: the patent is granted on the application; or the application has lapsed or been withdrawn or refused; to a person who is: a skilled addressee without an interest in the invention; and nominated by a person who makes a request for the furnishing of those samples.

DATED this SEVENTEENTH day of SEPTEMBER 1992

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By:



Registered Patent Attorney

IRN: 221055

INSTR CODE: 59080

Australia*Patents Act 1990***NOTICE OF ENTITLEMENT**

I, John David O'Connor, of 31 Market Street, Sydney, New South Wales, 2000, Australia, patent attorney for the Applicant/Nominated Person in respect of Application No. 24581/92 state the following:-

The Applicant/Nominated Person has entitlement from the actual inventors as follows:-

The Applicant/Nominated Person is the assignee of the actual inventors.

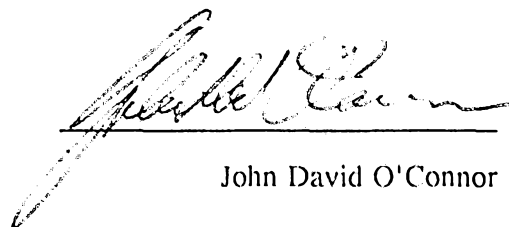
The Applicant/Nominated Person is the applicant of the basic application listed on the Patent Request.

The basic application listed on the Patent Request is the application first made in a Convention Country in respect of the invention.

The Applicant/Nominated Person is the depositor of the following deposits with the Fermentation Research Institute Agency of Industrial Science and Technology:

Organism	Deposit Date	Accession No.
KM-641	27 September 1990	FERM BP-3116
KM-871	13 August 1991	FERM BP-3512

Dated 3 November 1992



John David O'Connor



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PROCESS FOR PRODUCING HUMANIZED CHIMERA ANTIBODY
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- (56) Prior Art Documents
BARKER ET AL: CANCER RESEARCH, 51, 144-149, 1 JAN 1991
MUELLER ET AL: J OF IMMUN, 144, 4, 1386, 15 FEB 1990
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- (57)

An object of the present invention is to provide a process for the production of humanized chimera antibody by which the chimera antibody is produced easily without changing any of the amino acids of its mouse antibody variable region. Another object of the present invention is to provide a humanized chimera antibody to ganglioside GD₃ and a process for the production of such antibody.

CLAIM

1. A process for producing humanized chimera antibody without changing any of the amino acids of its non-human antibody variable region which comprises the steps of:
- (1) constructing a cassette vector by inserting a cDNA coding for human antibody C_H into an expression vector for animal cell use and establishing a cloning site in the upstream region C_H of said cassette vector for inserting a cDNA which encodes nonhuman animal V_H;
 - (2) digesting a cDNA coding for nonhuman animal antibody V_H with restriction enzyme;
 - (3) inserting said cDNA coding for nonhuman animal antibody V_H into the cassette vector, using a synthetic DNA which comprises a base sequence corresponding to the 5'-end side of said human antibody C_H and a base sequence corresponding to the 3'-end side

of said nonhuman animal V_H and is possessed of the restriction enzyme recognition sites on both of its ends, thereby constructing a humanized chimera antibody heavy chain expression vector in which said cDNA coding for the heavy chain variable region of human antibody are linked together through said synthetic DNA;

(4) constructing a cassette vector by inserting a cDNA coding for a light chain constant region of human antibody into an expression vector for animal cell use and establishing a cloning site in the upstream region of the light chain constant region of said cassette vector for inserting a cDNA which encodes a light chain variable region of nonhuman animal antibody;

(5) digesting a cDNA coding for the light chain variable region of nonhuman animal antibody with restriction enzymes;

(6) inserting said cDNA coding for the light chain variable region of nonhuman animal antibody into the cassette vector using a synthetic DNA which comprises a base sequence corresponding to the 5'-end side of said light chain constant region of human antibody and a base sequence corresponding to the 3'-end side of said light chain variable region of nonhuman animal antibody and is possessed of the restriction enzyme recognition sites on both of its ends, thereby constructing a humanized chimera antibody light chain expression vector in which said cDNA coding for the light chain constant region of human antibody and said cDNA coding for the light chain variable region of nonhuman animal antibody are linked together through said synthetic DNA;

(7) introducing these expression vectors into host cells to obtain a transformant; and

(8) culturing said transformant in an appropriate

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culture medium, thereby allowing the transformant to produce and accumulate a humanized chimera antibody, and collecting said humanized chimera antibody from the resulting culture broth.

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COMPLETE SPECIFICATION

FOR A STANDARD PATENT

ORIGINAL

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Invention Title:

Process for Producing Humanized Chimera Antibody

The following statement is a full description of this invention, including the
best method of performing it known to me/us:-

Process for Producing Humanized Chimera Antibody

FIELD OF THE INVENTION

This invention relates to a process for the production of humanized chimera antibody. In contrast to mouse monoclonal
5 antibody, humanized chimera antibody does not cause formation of anti-mouse immunoglobulin antibody in the body of a patient. Thus, side effects are reduced or eliminated and half life in blood increases when the chimera antibody is used. Therapeutic effects which are superior to those obtained in the case of
10 using mouse monoclonal antibody can be obtained in the treatment of human cancers and the like.

BACKGROUND OF THE INVENTION

It is known that, when mouse antibodies are administered to humans, they are recognized as foreign
15 substances and cause formation of anti-mouse immunoglobulin antibodies in the human body, and the thus formed antibodies react with the administered mouse antibodies. As a result, side effects occur (J. Clin. Oncol., 2, 881 (1984); Blood, 65, 1349-1363 (1985); J. Natl. Cancer Inst., 80, 932 (1988); Proc.
20 Natl. Acad. Sci. U.S.A., 82, 1242 (1985)), the antibodies are cleared away quickly (J. Nucl. Med., 26, 1011 (1985); Blood, 65, 1349-1363 (1985); J. Natl. Cancer Inst., 80, 937 (1988)) and effects of the antibodies are reduced (J. Immunol., 135, 1530 (1985); Cancer Res., 46, 6489 (1986)). When mouse
25 monoclonal antibody is converted into humanized chimera antibody, human anti-mouse immunoglobulin antibody form in

minimal amounts if at all, and the half life of the chimera antibody in human blood is six times as long as that of mouse monoclonal antibody (Proc. Natl. Acad. Sci. U.S.A., 86, 4220 (1989)). In addition, it is probable that the Fc region of
5 mouse antibody does not fully activate human complement and human effector cells, in comparison with the Fc region of human antibody. For example, the antitumor activity of mouse monoclonal antibody to ganglioside GD₂, which is effected via human effector cells, is improved when the monoclonal antibody
10 is converted into chimera antibody that has the human antibody Fc region (J. Immunol., 144, 1382-1386 (1990)).

Ganglioside is one of the animal cell membrane-constituting glycolipids and is composed of a sugar chain as a hydrophilic side chain, sphingosine as a hydrophobic side chain
15 and fatty acids. It is known that expression of ganglioside varies depending on the type of cells, organs and animal species. In addition, it has been revealed that quantity and quality of the expressed ganglioside change during the canceration process of cells (Cancer Res., 45, 2405 (1985)).

20 For example, it has been reported that gangliosides GD₂, GD₃, GM₂ and the like which hardly exist in normal cells were found in the cells of neuroblastoma, lung small cell carcinoma and melanoma belonging to neuroectodermal-origin tumor which is
said to be highly malignant (J. Exp. Med., 155, 1133 (1982); J. Biol. Chem., 257, 12752 (1982); Cancer Res., 47, 225 (1987);
25 ibid., 47, 1098 (1987); ibid., 45, 2642 (1985); Proc. Natl.

Acad. Sci. U.S.A., 80, 5392 (1983)).

Ganglioside GD₃ has been found most frequently in melanoma cells among the neuroectodermal-origin tumors, and anti-ganglioside GD₃ monoclonal antibodies (to be referred to
5 as "anti-GD₃ monoclonal antibody" hereinafter) belonging to the mouse IgM class and IgG class have been reported (Int. J. Cancer, 29, 269 (1982); J. Biol. Chem., 257, 12752 (1982); Cancer Res., 47, 225 (1987); Acta Neuropathol., 79, 317 (1989); Proc. Natl. Acad. Sci. U.S.A., 77, 6114 (1980); J. Exp. Med.,
10 155, 1133 (1982); Proc. Natl. Acad. Sci. U.S.A., 81, 5767 (1984)).

KM-641 (FERM BP-3116) disclosed in EP-A-0 493 686 is an anti-GD₃ monoclonal antibody belonging to the mouse IgG3 class, which reacts not only with ganglioside GD₃ but also with
15 ganglioside 3',8'-LD1 and is possessed of a broad range of antitumor spectrum. In addition, KM-641 has stronger binding activities to antigens than anti-GD₃ monoclonal antibody R24 which has been disclosed in J. Exp. Med., 155, 1133 (1982) and it shows strong antitumor activities.

20 The mouse monoclonal antibody R24 to the ganglioside GD₃ was once used for the treatment of melanoma, but the administered mouse monoclonal antibody R24 did not fully exert its effect due to the formation of anti-mouse immunoglobulin
antibody in the patient's body (Eur. J. Cancer Clin. Oncol.,
25 24, suppl 2, s 65 (1988)).

Consequently, the use of chimera antibody for anti-GD₃

monoclonal antibody would be advantageous in that anti-mouse immunoglobulin antibody does not form in the body, side effects are reduced or eliminated, its half life in blood is prolonged and its antitumor effector effect increases, and thus
5 therapeutic effects of the chimera antibody which are superior to those of mouse monoclonal antibody can be obtained in the treatment of human cancers and the like.

Several processes for the production of humanized chimera antibodies are known. Humanized chimera antibody, in
10 which constant regions of the heavy chain (to be referred to as "H chain" hereinafter) and the light chain (to be referred to as "L chain" hereinafter) of mouse monoclonal antibody are converted into human constant regions, is produced in animal cells making use of recombinant DNA techniques. Examples of
15 such processes include a process in which humanized chimera antibody is produced using chromosomal DNA as a gene which encodes mouse H chain variable region (to be referred to as "V_H" hereinafter) and L chain variable region (to be referred to as "V_L" hereinafter) (Morrison et al., Proc. Natl. Acad.
Sci. U.S.A., 81, 6851 (1984); Neuberger et al., Nature, 314,
268 (1985); Nishimura et al., Cancer Res., 47, 999 (1987);
Dorai et al., J. Immunol., 139, 4232 (1987); Kameyama et al.,
FEBS letter, 244, 301 (1989)) and another process in which
humanized chimera antibody is produced using cDNA (Gillies et
25 al., J. Immunol. Methods, 125, 191 (1989); Liu et al., published International Application in Japan No. 2-501886).

Cloning and base sequence determination of hybridoma cell chromosomal DNA which encodes mouse V_H and V_L require much time and labor in comparison with those of cDNA that encodes mouse V_H and V_L . Consequently, the process in which cDNA is used for
5 the production of humanized chimera antibody is more desirable than the chromosomal DNA process.

Gillies et al. have succeeded in expressing humanized chimera antibody in animal cells, making use of an expression vector for animal cells having inserted therein a humanized
10 chimera H chain gene obtained by linking mouse V_H -encoding cDNA with human C_H -encoding chromosomal DNA, and a humanized chimera L chain gene obtained by linking mouse V_L -encoding cDNA with human C_L -encoding chromosomal DNA (J. Immunol. Methods, 125, 191 (1989)). However, when an attempt was made to prepare
15 chimera antibodies from several types of antibodies, a problem was found that there were certain chimera antibodies whose L chains could not be expressed without converting leader sequences. In addition, humanized chimera antibody can be produced more simply when cDNA which encodes human C_H and C_L is
20 used instead of the human C_H - and C_L -encoding chromosomal DNA.

In published International Application in Japan No. 2-501886, Liu et al. discloses a process for the expression of humanized chimera antibody in animal cells, which comprises
25 using an expression vector for animal cells having inserted therein a chimera H chain cDNA obtained by linking mouse V_H -encoding cDNA with human C_H -encoding cDNA and a chimera L chain

cDNA obtained by linking mouse V_L -encoding cDNA with human C_L -encoding cDNA. According to this process, however, it is necessary to alter the J_H portion of the V_H -encoding cDNA and the J_L portion of the V_L -encoding cDNA by means of mutation, because the cDNA which encodes mouse V_H or V_L is linked with the human C_H - or C_L -encoding cDNA at the J region in the mouse variable region. In addition, with regard to the chimera L chain prepared using mouse Jk5, leucine which is one of the amino acids of the framework 4 is changed to isoleucine when made into humanized chimera antibody. Although amino acid sequence of complementarity-determining region (to be referred to as "CDR" hereinafter) is especially important for antigen-antibody binding, the amino acid sequence of the framework is also an important factor. For example, Riechmann et al. have prepared CDR graft antibody by grafting a rat antibody CDR into a human antibody framework and reported that binding activity of the antibody was reduced by the framework conversion and the antibody activity increased when amino acid sequence of the framework was partially changed (Nature, 332, 323 (1988)). Consequently, there is a possibility that the binding activity of humanized chimera antibody is undesirably reduced when the antibody is produced by the mouse Jk5-aided process disclosed by Liu et al.

In view of the above, when any mouse antibody is converted into humanized chimera antibody, it has been desired to simply and easily produce humanized chimera antibody in

which amino acids of the mouse antibody variable region remain completely unchanged.

Objects of the Invention

An object of the present invention is to provide a process for the production of humanized chimera antibody by which the chimera antibody is produced easily without
5 changing any of the amino acids of its mouse antibody variable region. Another object of the present invention is to provide a humanized chimera antibody to ganglioside GD₃ and a process for the production of such antibody.

Summary of the Invention

The present invention relates to a process for producing humanized chimera
10 antibody without changing any of the amino acids of its non-human antibody variable region which comprises the steps of:

- (1) constructing a cassette vector by inserting a cDNA coding for human antibody C_H into an expression vector for animal cell use and establishing a cloning site in the upstream region C_H of said cassette vector for inserting a cDNA which encodes
15 nonhuman animal V_H;
- (2) digesting a cDNA coding for nonhuman animal antibody V_H with restriction enzyme;
- (3) inserting said cDNA coding for nonhuman animal antibody V_H into the cassette vector, using a synthetic DNA which comprises a base sequence corresponding to the 5'-
20 end side of said human antibody C_H and a base sequence corresponding to the 3'-end side of said nonhuman animal V_H and is possessed of

restriction enzyme recognition sites on both of its ends,
thereby constructing a humanized chimera antibody H chain
expression vector in which said cDNA coding for human antibody
C_H and said cDNA coding for nonhuman animal antibody V_H are
5 linked together through said synthetic DNA;

(4) constructing a cassette vector by inserting a cDNA coding
for human antibody C_L into an expression vector for animal cell
use and establishing a cloning site in the upstream region of
the C_L of said cassette vector for inserting a cDNA which
10 encodes nonhuman animal antibody V_L;

(5) digesting a cDNA coding for nonhuman animal antibody V_L
with restriction enzymes;

(6) inserting said cDNA coding for nonhuman animal antibody V_L
into the cassette vector, using a synthetic DNA which comprises
15 a base sequence corresponding to the 5'-end side of said human
antibody C_L and a base sequence corresponding to the 3'-end
side of said nonhuman animal antibody V_L and is possessed of
restriction enzyme recognition sites on both of its ends,

thereby constructing a humanized chimera antibody L chain
expression vector in which said cDNA coding for human antibody
C_L and said cDNA coding for nonhuman animal antibody V_L are
20 linked together through said synthetic DNA;

(7) introducing these expression vectors into host cells to
obtain a transformant; and

25 (8) culturing said transformant in an appropriate culture
medium, thereby allowing the transformant to produce and

accumulate a humanized chimera antibody, and collecting said humanized chimera antibody from the resulting culture broth.

The cassette vector to be used in the present invention is a vector which is obtained by inserting a cDNA that encodes a constant region of human antibody into an expression vector for animal cell use, in which a cloning site is located in the upstream region of the constant region for inserting a cDNA that encodes a variable region of nonhuman animal antibody. An expression vector for humanized chimera antibody can be constructed easily by inserting a variable region of nonhuman animal antibody into the cloning site of the cassette vector, using a synthetic DNA which comprises a base sequence corresponding to the 5'-end side of a constant region of human antibody and a base sequence corresponding to the 3'-end side of a variable region of nonhuman animal antibody and is possessed of restriction enzyme recognition sites on its both ends.

The present invention also relates to a humanized chimera antibody obtainable by the above-described process, a pharmaceutical composition comprising the humanized chimera antibody and a pharmaceutically acceptable carrier, and use of said humanized chimera antibody for the manufacture of the medicament for treating cancer.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows a restriction enzyme cleavage map of a 9.3 kb XbaI fragment of KM50 cell chromosomal DNA.

Fig. 2 shows a construction scheme for plasmid pKMB11.
 Fig. 3 shows a construction scheme for plasmid pKMD6.
 Fig. 4 shows a construction scheme for plasmid pEPKMA1.
 Fig. 5 shows a construction scheme for plasmid pEPKMB1.
 5 Fig. 6 shows a construction scheme for plasmid pAGE501.
 Fig. 7 shows a construction scheme for plasmid pAGE109.
 Fig. 8 shows a construction scheme for plasmid pAGE502.
 Fig. 9 shows a construction scheme for plasmid pAGE503.
 Fig. 10 shows a construction scheme for plasmid pSEd1.
 10 Fig. 11 shows a construction scheme for plasmid pSE1D2.
 Fig. 12 shows a construction scheme for plasmid
 pIG1SE1d2.
 Fig. 13 shows a construction scheme for plasmid
 pIG1SE1d3.
 15 Fig. 14 shows a construction scheme for plasmid
 pIG1SE1d4.
 Fig. 15 shows a construction scheme for plasmid pPMOL2.
 Fig. 16 shows a construction scheme for plasmid pPMOL3.
 Fig. 17 shows a construction scheme for plasmid
 20 pchCKA7.
 Fig. 18 shows a construction scheme for plasmid
 pchCKB1.
 Fig. 19 shows a construction scheme for plasmid
 pckCKC1.
 25 Fig. 20 shows a construction scheme for plasmid
 pChiIgHB2.

Fig. 21 shows a construction scheme for plasmid pChiIgLA1.

Fig. 22 shows plasmids pKM641HA3 and pKM641LA2.

Fig. 23 shows plasmid pChi641HA1.

5 Fig. 24 shows a construction scheme for plasmid pKM641HE1.

Fig. 25 shows a construction scheme for plasmid pKM641HF1.

10 Fig. 26 shows a construction scheme for plasmid pChi641HA1.

Fig. 27 shows a construction scheme for plasmid pChi641HAM1.

Fig. 28 shows plasmid pChi641LG11.

15 Fig. 29 shows a construction scheme for plasmid pChi641LG11.

Fig. 30 shows a construction scheme for plasmid pChi641LGM11.

20 Fig. 31 shows a pattern of SDS-PAGE (4 to 15% gradient gel) of purified anti-GD₃ chimera antibody KM-871 (about 5 µg/lane) carried out under reductive condition (A) or non-reductive condition (B), where the lanes starting from the left respectively indicate electrophoretic patterns of molecular weight markers, human IgG standard, mouse anti-GD₃ antibody KM-641 and anti-GD₃ chimera antibody KM-871.

25 Fig. 32 is a graph showing reactivity of anti-GD₃ chimera antibody KM-871 with ganglioside GD₃-positive G361 and

SK-MEL-28 cells measured by fluorescent antibody technique with the cell number on the ordinate and the fluorescence intensity on the abscissa. A dotted line shows reactivity in the absence of the antibody, while a solid line shows reactivity in the presence of KM-871.

Fig. 33 is a graph showing complement-dependent cytotoxicity (CDC) of anti-GD₃ chimera antibody KM-871 and anti-GD₃ mouse antibody KM-641 against ganglioside GD₃-positive G361 and SK-MEL-28 cells with cytotoxicity on the ordinate and an antibody concentration on the abscissa. A blackened bar shows CDC activity of KM-871, while a striped bar shows that of KM-641.

Fig. 34 is a graph showing antibody-dependent cell-mediated cytotoxicity (ADCC) of KM-871 and KM-641 against ganglioside GD₃-positive cell G361 with a ratio of effector cells to target cells on the ordinate and ADCC activity on the abscissa. A blackened bar shows ADCC activity of KM-871, a dotted bar shows ADCC activity of KM-641 and a striped bar shows control (in the absence of the antibody). PMN means polymorphonuclear leukocyte.

Fig. 35 is a graph showing therapeutic effect of KM-871 on transplanted tumors with the tumor size on the ordinate and days after transplantation of tumors on the abscissa, in which closed circle shows effect of anti-Sialyl Le^a monoclonal antibody AMC-462, open square shows that of KM-641 and open triangle shows that of KM-871.

DETAILED DESCRIPTION OF THE INVENTION

1. Construction of cassette vector

The cassette vector to be used in the present invention is constructed by inserting a cDNA which encodes a human antibody constant region into an expression vector for animal cell use. Essential components in the expression vector for animal cell use include promoter, enhancer, polyA signal, splicing signal, drug resistance gene as a selection marker (e.g., ampicillin resistance gene, etc.) and the like. Any expression vector for animal cell use may be used for this purpose, as long as it can contain and express the cDNA molecule which encodes a human antibody constant region. For example, pAGE107 (Cytotechnology, 3, 133 (1990)) is useful as such an expression vector. Examples of the promoter and enhancer for use in the expression vector for animal cell use include: SV40 early promoter and enhancer (J. Biochem., 101, 1307 (1987)); LTR promoter and enhancer of Moloney mouse leukemia virus (Biochem. Biophys. Res. Commun., 149, 960 (1987)); and immunoglobulin H chain promoter (Cell, 41, 479 (1985)) and enhancer (Cell, 33, 717 (1983)). The immunoglobulin H chain promoter and enhancer can be prepared using appropriate antibody-producing hybridoma cells, such as rat hybridoma KM50 cells which produce anti-human serum albumin antibody as disclosed in JP-A-60-258128 (the term "JP-A" as used herein means an "unexamined published Japanese patent application"). The following describes processes for the

preparation of the immunoglobulin H chain promoter and enhancer making use of KM50 cells.

Each chromosomal DNA is obtained from cultured KM50 cells, and P3X63Ag8U.1 (to be referred to as "P3U1" hereinafter) cells (ATCC CRL1597) which are to be fused with KM50 and rat kidney cells in accordance with the procedure disclosed in Molecular Cloning (2nd. ed., Cold Spring Harbor Laboratory Press, 1989, p9.14). Next, a DNA fragment containing immunoglobulin promoter and enhancer and a gene of the variable region of activated immunoglobulin H chain, in which DNA rearrangement has been induced, is isolated from the chromosomal DNA extracted from KM50 cells, in accordance with the procedure disclosed in FEBS letter 244, 301 (1989). The immunoglobulin promoter and enhancer are cut out from the thus isolated DNA fragment and inserted into the aforementioned expression vector for animal cell use. Plasmid pIg1SE1d4 is an illustrative example of the animal cell expression vector which contains the immunoglobulin H chain promoter and enhancer.

Next, a cloning site is established in the upstream region of a human constant region of a cassette vector, for inserting a cDNA which encodes a variable region of nonhuman animal antibody. Into the thus established cloning site is inserted a cDNA which encodes a variable region of nonhuman animal antibody, using a synthetic DNA which comprises a base sequence corresponding to the 5'-end side of a constant region of human antibody and a base sequence corresponding to the 3'-

end side of a variable region of nonhuman animal antibody and is possessed of restriction enzyme recognition sites on both of its ends. In this way, a humanized chimera antibody expression vector is constructed in which the cDNA coding for human antibody constant region and the cDNA coding for the variable region of nonhuman animal antibody are linked together through the synthetic DNA. The synthetic DNA to be used may be prepared using a DNA synthesizer, based on the base sequence which corresponds to the 5'-end side of a constant region of human antibody and the base sequence that corresponds to the 3'-end side of a variable region of nonhuman animal antibody. Illustrative examples of cloning site-containing cassette vectors include a cassette vector pChiIgHB2 which is used for the construction of an expression vector for the expression of humanized chimera antibody H chain and a cassette vector pChiIgLA2 which is used for the construction of an expression vector for the expression of humanized chimera antibody L chain.

A cassette vector for use in the construction of an expression vector for the expression of humanized chimera antibody H chain is constructed, for example, by cutting out a human C_H-encoding cDNA-containing fragment, from an ApaI site in the vicinity of the 5'-end of the cDNA to its 3'-end, and inserting the fragment into an appropriate expression vector for animal cell use such as plasmid pIg1SEld4 or the like. Then, a cloning site is established in the thus constructed

cassette vector for inserting a cDNA which encodes a V_H of nonhuman animal antibody. Into the thus established cloning site is then inserted a cDNA fragment encoding a nonhuman animal antibody V_H , which is obtained by digesting a V_H -encoding cDNA with an appropriate restriction enzyme, using a synthetic DNA molecule which comprises a base sequence corresponding to the 5'-end side (5'-end to ApaI site) of a human antibody C_H and a base sequence corresponding to the 3'-end side of a nonhuman animal antibody V_H and is possessed of restriction enzyme recognition sites on both of its ends. In this way, an expression vector for use in the expression of humanized chimera antibody H chain is easily obtained without altering amino acid sequence of the expressed V_H .

A cassette vector for constructing of an expression vector for the expression of humanized chimera antibody L chain may be constructed for example by introducing an EcoRV site into the vicinity of 5'-end side of a human C_L -encoding cDNA by means of mutation, cutting out a fragment from the resulting human cDNA from the EcoRV site to the 3'-end and inserting the fragment into an appropriate expression vector such as plasmid pIg1SEld4 or the like. Then, a cloning site is established in the thus constructed cassette vector for inserting a cDNA which encodes a nonhuman animal antibody V_L . Into the thus established cloning site is then inserted a cDNA fragment encoding a nonhuman animal antibody V_L , which is obtained by digesting a V_L -encoding cDNA with an appropriate restriction

enzyme, using a synthetic DNA which comprises a base sequence corresponding to the 5'-end side (5'-end to EcoRV site) of a human antibody C_L and a base sequence corresponding to the 3'-end side of a nonhuman animal antibody V_L and is possessed of restriction enzyme recognition sites on both of its ends. In this way, an expression vector for use in the expression of humanized chimera antibody L chain is easily obtained without altering amino acid sequence of the expressed V_L.

Examples of the cDNAs which encode the human C_H and human C_L described above are disclosed, for instance, in Cell 22, 197 (1982). Such cDNAs can be prepared from human antibody-producing myeloma cells, humanized monoclonal antibody-producing hybridoma cells, humanized chimera antibody-producing cells (SP2-PC chimera; FEBS Letters, 244, 301 (1989)) and the like, in accordance with known procedures disclosed for instance in Proc. Natl. Acad. Sci. U.S.A. 82, 7025 (1985) and ibid., 79 7025 (1985). That is, cDNA is synthesized using mRNA extracted from the above-described cells, in accordance with the procedure disclosed in Molecular Cloning 2nd. ed.; 1989, p8.1. A library is prepared from the thus synthesized cDNA using a phage vector or a plasmid vector, in accordance with the procedure disclosed in Molecular Cloning 2nd. ed.; 1989, p8.1, 1.53. Next, a recombinant phage or a recombinant plasmid which contains human C_H-encoding cDNA or human C_L-encoding cDNA is obtained from the thus prepared library using a human antibody constant region or a human antibody variable

region as a probe, in accordance with the procedure disclosed in Molecular Cloning 2nd. ed.; 1989, p8.1, 1.53. Base sequences of the human C_H-encoding cDNA and the human C_L-encoding cDNA are determined in accordance with the procedure
5 disclosed in Molecular Cloning, 2nd. ed.; 1989, p13.1. Introduction of an appropriate restriction enzyme recognition site into the human C_L-encoding cDNA, for example insertion of an EcoRV recognition site into a region in the vicinity of the 5'-end of the cDNA, may be effected in accordance with the
10 procedure disclosed in Molecular Cloning, 2nd. ed.; 1989, p15.1.

2. Production of humanized chimera antibody

Firstly, cDNAs which encode V_H and V_L of nonhuman animal antibody, such as mouse anti-GD₃ monoclonal antibody, are
15 prepared in the following manner.

That is, cDNA is synthesized using mRNA extracted from appropriate hybridoma cells which produce mouse anti-GD₃ monoclonal antibody, such as mouse anti-GD₃ monoclonal antibody KM-641 (FERM BP-3116). A library is prepared from the thus
20 synthesized cDNA using a phage vector or a plasmid vector. Next, a recombinant phage or a recombinant plasmid which contains V_H-encoding cDNA or V_L-encoding cDNA is obtained from the thus prepared library using a constant region or a variable
25 region of nonhuman antibody, such as mouse antibody, as a probe. Base sequences of the V_H-encoding cDNA and the V_L-encoding cDNA are determined in accordance with the

aforementioned procedure.

A fragment of the V_H -encoding cDNA, ranging from the 5'-end to an appropriate restriction enzyme site near the 3'-end (to be referred to as "site A" hereinafter), is cut out and
5 inserted into the cloning site of the aforementioned cassette vector, using a synthetic DNA which comprises a base sequence corresponding to the 5'-end side of a human antibody C_H and a base sequence corresponding to the 3'-end side (from 3'-end to site A) of a nonhuman animal antibody V_H and is possessed of
10 restriction enzyme recognition sites on both of its ends. In this way, an expression vector for use in the expression of humanized chimera antibody H chain is constructed by linking the human antibody C_H -encoding cDNA with the nonhuman antibody V_H -encoding cDNA through the synthetic DNA. In the same way,
15 a fragment of the V_L -encoding cDNA, ranging from the 5'-end to an appropriate restriction enzyme site near the 3'-end (to be referred to as "site B" hereinafter), is cut out and inserted into the cloning site of the aforementioned cassette vector, using a synthetic DNA molecule which comprises a base sequence
20 corresponding to the 5'-end side of a human antibody C_L and a base sequence corresponding to the 3'-end side (from 3'-end to site B) of a nonhuman animal antibody V_L and is possessed of restriction enzyme recognition sites on both of its ends. In
25 this way, an expression vector for use in the expression of humanized chimera antibody L chain is constructed by linking the human antibody C_L -encoding cDNA with the nonhuman antibody

V_L-encoding cDNA through the synthetic DNA.

A transformant which is capable of producing humanized chimera antibody is obtained by transforming appropriate host cells with the thus prepared expression vectors for use in the
5 expression of the H chain and L chain of humanized chimera antibody.

Any type of cells may be used as host cells for use in the introduction of the humanized chimera antibody expression vectors, as long as these cells are capable of expressing the
10 humanized chimera antibody. Illustrative examples of such host cells include mouse SP2/0-Ag14 cells (ATCC CRL1581; to be referred to as "SP2/0 cells" hereinafter), mouse P3X63-Ag8.653 (ATCC CRL1580) and CHO cells which are deficient in dihydrofolate reductase gene (to be referred to as "dhfr"
15 hereinafter) (Urlaub et al., Proc. Natl. Acad. Sci. U.S.A., 77, 4216 (1980)).

Introduction of the expression vectors for use in the expression of the H chain and L chain of humanized chimera antibody into host cells may be effected for example by the
20 electroporation technique disclosed in JP-A-2-257891. A transformant capable of producing the humanized chimera antibody may be selected using RPMI1640 medium supplemented with G418 and fetal calf serum, in accordance with the procedure disclosed in JP-A-2-257891. A transformant, KM-871,
25 which produces humanized chimera antibody that reacts with ganglioside GD₃ is an illustrative example of the transformant

capable of producing humanized chimera antibody. KM-871 has been deposited on August 13, 1991, with Fermentation Research Institute, Agency of Industrial Science and Technology of 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki, Japan under the Budapest
5 Treaty, and has been assigned the accession number FERM BP-3512.

For the cultivation of the thus-obtained transformants, any medium can be used as long as the desired antibody can be produced and accumulated in the medium. An example of such
10 medium is RPMI1640 medium supplemented with G418 and fetal calf serum. The transformants may be inoculated into 200 μ l to 100 ml of the above-mentioned medium to give a cell concentration of 1×10^5 to 1×10^7 cells/ml and cultivated at 37°C in a 5% CO₂ incubator for 1 to 7 days. The desired chimera antibody is
15 produced and accumulated in the culture medium.

Activity of the humanized chimera antibody in the culture broth is measured by enzyme-linked immunosorbent assay (ELISA method; E. Harlow et al., Manual of Antibody Experiments, Cold Spring Harbor Laboratory Press, 1988).
..... 20 Productivity of the humanized chimera antibody in the transformant can be improved making use of a dhfr amplification
..... system in accordance with the procedure disclosed in JP-A-2-
..... 257891.

The humanized chimera antibody thus produced can be
25 purified from supernatant fluid of the aforementioned cultured mixture making use of a protein A column (E. Harlow et al.,

Manual of Antibody Experiments, Cold Spring Harbor Laboratory Press, 1988). Illustrative examples of humanized chimera antibodies obtained in this way include those which react with ganglioside GD₃, such as humanized chimera antibody KM-871 and
5 the like.

Reactivity of humanized chimera antibody is measured by ELISA method. The molecular weight of the H chain, the L chain or the entire molecule of purified humanized chimera antibody is measured by means of polyacrylamide gel electrophoresis
10 (SDS-PAGE), Western blotting method (E. Harlow et al., Manual of Antibody Experiments, Cold Spring Harbor Laboratory Press, 1988) or the like.

Binding activity, or avidity, of the humanized chimera antibody to ganglioside GD₃ to a cultured cancer cell line is
15 measured by means of the fluorescent antibody technique, the ELISA method or the like. Complement-dependent cytotoxicity (CDC activity) and antibody-dependent cell-mediated cytotoxicity (ADCC activity) of humanized chimera antibody to a cultured cancer cell line are measured in accordance with the
20 procedures disclosed in Menekigaku Jikken Nyumon, (Manual of Immunological Experiments) Matsushashi et al., Gakkai Shuppan Center, Japan, 1981).

The humanized chimera antibodies according to the present invention can be used alone as an anticancer agent.
25 They may be formulated into an anticancer composition together with at least one pharmaceutically acceptable carrier. For

instance, the humanized chimera antibodies are dissolved in physiological saline, an aqueous solution of glucose, lactose or mannitol and the like. The powder of the humanized chimera antibodies for injection can be prepared by lyophilizing the
5 humanized chimera antibodies in accordance with the conventional method and mixing the lyophilized products with sodium chloride. The anticancer composition may further contain additives conventionally used well known in the art of medical preparation, for example, pharmaceutically acceptable
10 salts.

The humanized chimera antibodies according to the present invention can be administered in the form of the above-described anticancer composition to mammals including human in a dose of 0.2 to 20 mg/kg/day. The dose may vary depending on
15 the age, condition, etc. of patients. The administration of the anticancer composition can be effected by intravenous injection once a day (single administration or consecutive administration) or intermittently one to three times a week or once every two to three weeks.

20 The anticancer composition is expected to be useful for treating cancer such as melanoma, neuroblastoma and glioma.

25 The following examples and reference examples are provided to further illustrate the present invention. It is to be understood, however, that the examples are for purpose of illustration only and are not to be construed to limit the invention.

Example 1

Construction of cassette vector

1. Isolation of promoter and enhancer genes of KM50 cell-derived immunoglobulin H chain

5 (1) Preparation of chromosomal DNA from KM50 cells, P3U1 cells and rat kidney

Chromosomal DNA was prepared in the following manner in accordance with the procedure disclosed in Molecular Cloning, Maniatis et al., 1989, p9.16.

1.2 x 10⁸ KM50 cells, 2 x 10⁸ P3U1 cells (ATCC CRL1597)
10 and 1.6 g of rat kidney (a kidney sample frozen at -80°C was smashed thoroughly using a mallet) were each suspended in 2 ml of a buffer solution (pH 7.5) containing 10 mM Tris-HCl, 150 mM sodium chloride and 10 mM sodium ethylenediaminetetraacetate (to be referred to as "EDTA" hereinafter). To this suspension
15 were added 0.8 mg of Proteinase K (Sigma Chemical Co.) and 10 mg of sodium lauryl sulfate (to be referred to as "SDS" hereinafter). After incubation at 37°C for 10 hours, the resulting mixture was extracted with the same volume of phenol (once), chloroform (twice) and ether (once) in this order, and
20 the extract was dialyzed for 10 hours against a buffer solution (pH 7.5) containing 10 mM Tris-HCl and 1 mM EDTA. A DNA solution was recovered from the dialysis tube and Ribonuclease A (Sigma Chemical Co.) was added thereto to give a final concentration of 20 µg/ml. After incubating at 37°C for 6
25 hours to decompose RNA completely, the resulting solution was

mixed with 15 mg of SDS and 1 mg of Proteinase K and incubated at 37°C for 10 hours. The thus treated solution was extracted with the same volume of phenol, chloroform and ether (twice for each) in this order, and the extract was dialyzed for 10 hours
5 against a buffer solution (pH 7.5) containing of 10 mM Tris-HCl and 1 mM EDTA. The DNA solution was recovered from the dialysis tube and used as a chromosomal DNA sample. A DNA concentration of each sample was determined by measuring the absorbance at 260 nm and, as a result, it was found that 1.6
10 mg, 1.5 mg and 1.9 mg of chromosomal DNA was obtained from 1.2×10^8 KM50 cells, 2×10^8 P3U1 cells and 1.6 g of rat kidney, respectively.

(2) Identification of activated immunoglobulin H chain gene in KM50 cells by Southern blotting

15 A 3 µg portion of each of the chromosomal DNA samples obtained in the above step (1) from KM50 cells, P3U1 cells and rat kidney was dissolved in 25 µl of a buffer solution containing 10 mM Tris-HCl (pH 7.5), 6 mM magnesium chloride and 100 mM sodium chloride. Each of the thus prepared solution was
20 mixed with 15 units of XbaI (Takara Shuzo Co., Ltd.; all restriction enzymes used in the following experiments were purchased from the same company) and incubated at 37°C for 2
25 hours to cleave the chromosomal DNA at the XbaI site. The reaction mixture was subjected to agarose gel electrophoresis, resulting DNA fragments were transferred to a nitrocellulose filter in accordance with the method of Southern et al. (J.

Mol. Biol., 98, 503, (1975)) and then subjected to hybridization in the known method (Kameyama et al., FEBS Letters, 244, 301-306 (1989)) using a mouse JH probe which is disclosed in the FEBS Letters article. A band equivalent to
5 about 9.3 kb was observed only in the DNA sample of KM50 cells. In consequence, it was considered that the XbaI fragment of immunoglobulin DNA found in this band contained the activated immunoglobulin H chain gene derived from KM50 cells.

(3) Preparation of KM50 cell chromosomal DNA library

10 A 60 µg portion of the KM50 cell chromosomal DNA obtained in the above step (2) was dissolved in 250 µl of a buffer solution containing 10 mM Tris-HCl (pH 7.5), 6 mM magnesium chloride and 100 mM sodium chloride. The thus prepared solution was mixed with 150 units of XbaI and
15 incubated at 37°C for 2 hours to cleave the chromosomal DNA at the XbaI site. The reaction mixture was subjected to agarose gel electrophoresis and a 9.3 kb-equivalent fraction was recovered as about 2 µg of 9.3 kb DNA sample of KM50 cells, making use of the DEAE paper method (Maniatis et al., Molecular
20 Cloning, 1989, p6.24). Separately, a 3 µg portion of lambda-ZAP (Stratagene Cloning Systems) to be used as a vector was dissolved in 200 µl of a buffer solution containing 10 mM Tris-HCl (pH 7.5), 6 mM magnesium chloride and 100 mM sodium chloride. The thus prepared solution was mixed with 50 units
25 of XbaI and incubated at 37°C for 2 hours to cleave the DNA at the XbaI site. The resulting reaction mixture was extracted

with phenol-chloroform and then treated with ethanol to precipitate and recover about 3 μ g of DNA. The thus recovered DNA sample was dissolved in a 100 μ l of 100 mM Tris-HCl buffer (pH 7.5), and the resulting solution was mixed with 1 unit of
5 alkaline phosphatase (Takara Shuzo Co., Ltd.) to effect dephosphorylation of restriction enzyme cleavage ends of the vector DNA. The resulting reaction mixture was extracted with phenol-chloroform and then treated with ethanol to precipitate and recover 2 μ g of DNA. The thus recovered DNA sample was
10 dissolved in 10 μ l of a buffer solution containing 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA to serve as a vector DNA sample. Next, 0.2 μ g of the thus prepared vector DNA sample and 0.2 μ g of the KM50 cell-derived 9.3 kb DNA sample were dissolved in 5 μ l of a buffer solution containing 66 mM Tris-HCl (pH 7.5), 6.6
15 mM magnesium chloride, 10 mM dithiothreitol (to be referred to as "DTT" hereinafter) and 0.1 mM adenosine triphosphate (to be referred to as "ATP" hereinafter) (to be referred to as "T4 ligase buffer" hereinafter). The resulting solution was mixed with 175 units of T4 DNA ligase (Takara Shuzo Co., Ltd.) and
20 incubated at 4°C for 3 days. A 2 μ l portion of the resulting reaction mixture was subjected to lambda phage packaging in the known method (Maniatis et al., Molecular Cloning, 1989, p2.95) using GigaPack Gold purchased from Stratagene Cloning Systems.
25 E. coli BB4 cells were infected with this phage to obtain 200,000 phage clones. 100,000 out of these phage clones were fixed on nitrocellulose filters in the known method (Maniatis

et al., Molecular Cloning, 1989, p2.112).

(4) Selection of recombinant DNA containing a gene of the activated (anti-human serum albumin) immunoglobulin H chain variable region in KM50 cells

5 Two clones showing strong reaction with the ^{32}P -labeled mouse JH probe at 65°C were isolated from the 100,000 phage clones prepared in the above step (3) in accordance with the procedure of Kameyama et al. (FEBS Letters, 44, 301-306, 1989). When the phage DNA was recovered in the conventional manner
10 (Maniatis et al., Molecular Cloning, 1989, p2.118-2.169), it was found that the 9.3 kb XbaI fragment of the KM50 cell-derived chromosomal DNA was incorporated into the phage DNA.

(5) Base sequence of the gene of the activated (anti-human serum albumin) immunoglobulin H chain variable region in KM50
15 cells

Restriction enzyme cleavage maps of the two clones obtained in the above step (4) was prepared by digesting them with various restriction enzymes and it was found that completely the same DNA fragment (9.3 kb) has been inserted
20 into these clones (Fig. 1). Next, base sequence of a part of the 9.3 kb DNA fragment, which was considered to contain the
25 promoter and variable regions of the rat immunoglobulin H chain, was determined in accordance with the Sanger method (Sanger et al., Proc. Natl. Acad. Sci. U.S.A., 74, 5463 (1977); M13 Cloning and Sequencing Handbook, Amersham). In SEQ ID NO: 1, a region containing octamer sequences such as ATGCAAAT and

TATA box sequences such as TTGAAAA and the like can be regarded as the immunoglobulin promoter region.

2. Construction of heterologous protein expression vector using promoter and enhancer of the activated (anti-human serum albumin) immunoglobulin H chain variable region in KM50 cells

(1) Construction of pKMB11

A 1 µg portion of the 9.3 kb fragment of the immunoglobulin H chain variable region gene obtained in 1-(5) was dissolved in 30 µl of a buffer solution containing 10 mM Tris-HCl (pH 7.5), 6 mM magnesium chloride and 100 mM sodium chloride. The thus prepared solution was mixed with 10 units of BglII and 10 units of HindIII and incubated at 37°C for 2 hours to cleave the DNA fragment at the BglII and HindIII sites. The resulting reaction mixture was subjected to agarose gel electrophoresis and 0.01 µg of a DNA fragment containing 0.8 kb immunoglobulin promoter was recovered. Separately, a 1 µg portion of a plasmid pBR322-BglII (Kuwana et al., FEBS Letters, 219, 360 (1987)) was dissolved in 30 µl of a buffer solution containing 10 mM Tris-HCl (pH 7.5), 6 mM magnesium chloride and 100 mM sodium chloride. The thus prepared solution was mixed with 10 units of BglII and 10 units of HindIII and incubated at 37°C for 2 hours to cleave the plasmid at the BglII and HindIII sites. The resulting reaction mixture was subjected to agarose gel electrophoresis, a DNA fragment of about 4.2 kb was recovered. A 0.1 µg portion of the thus obtained pBR322-BglII derived DNA fragment of about 4.2 kb and

0.01 µg of the immunoglobulin promoter-containing DNA fragment were dissolved in 20 µl of a T4 ligase buffer, and the resulting solution was mixed with 175 units of T4 DNA ligase (Takara Shuzo Co., Ltd.) and incubated at 4°C for 24 hours.

5 Using the resulting reaction mixture, transformation of *E. coli* HB101 (J. Mol. Biol., 41, 459 (1969)) was carried out in accordance with the method of Scott et al. (M. Shigesada, Saibo Kogaku, 2, 616 (1983)) to isolate a colony having ampicillin resistance (to be referred to as "ApR" hereinafter). Plasmid
10 DNA was recovered from the colony to obtain pKMB11 as shown in Fig. 2.

(2) Construction of pKMD6

In order to establish an appropriate restriction enzyme recognition site in downstream region of the immunoglobulin promoter, the plasmid pKMB11 constructed in the above step (1)
15 was digested with nuclease BAL31 from the NcoI site. A 10 µg portion of the plasmid pKMB11 was dissolved in 100 µl of a buffer solution containing 10 mM Tris-HCl (pH 7.5), 6 mM magnesium chloride and 50 mM potassium chloride. The thus
20 prepared solution was mixed with 30 units of NcoI and incubated at 37°C for 2 hours to cleave the plasmid at the NcoI site. The resulting reaction mixture was extracted with phenol and
chloroform and treated with ethanol. The thus precipitated DNA
fragments were dissolved in 100 µl of BAL31 buffer which
25 contained 20 mM Tris-HCl (pH 8.0), 600 mM sodium chloride, 12 mM calcium chloride, 12 mM magnesium chloride and 1 mM EDTA,

and the resulting solution was mixed with 0.25 unit of BAL31 (Bethesda Research Laboratories, Inc. (BRL)) and incubated at 37°C for 5 seconds. The reaction was stopped by extracting the reaction mixture with phenol. After extraction with chloroform and precipitation with ethanol, 1 µg of DNA was recovered. A 0.1 µg portion of the thus obtained DNA sample and 0.01 µg of a synthetic DNA linker SalI were dissolved in 20 µl of the T4 ligase buffer, and the resulting solution was mixed with 175 units of T4 DNA ligase and incubated at 4°C for 24 hours. Using the resulting reaction mixture, transformation of E. coli HB101 was carried out in accordance with the method of Scott et al. to isolate an ApR colony. Plasmid DNA was recovered from the colony to obtain pKMD6 as shown in Fig. 3. The base sequence of the BAL31-digested portion of this plasmid was determined in accordance with the Sanger method and it was found that bases up to the third base (the 303 position base in the SEQ ID NO: 1) upstream from the initiation codon ATG of the immunoglobulin gene.

(3) Construction of pEPKMA1, pEPKMB1 and pAGE501

Since original promoter and enhancer of the immunoglobulin gene are separated from each other, it is necessary to construct a vector in which the promoter and enhancer are connected together so that it can be used as a vector for the expression of a heterologous protein. The following manipulation was carried out to construct such vectors.

A 1 µg portion of the 9.3 kb fragment of the immunoglobulin H chain variable region gene obtained in 1-(5) was dissolved in 30 µl of a buffer solution containing 10 mM Tris-HCl (pH 7.5), 6 mM magnesium chloride and 100 mM sodium chloride. The thus prepared solution was mixed with 10 units of EcoRV and 10 units of XbaI and incubated at 37°C for 2 hours to cleave the DNA fragment at the EcoRV and XbaI sites. The resulting reaction mixture was subjected to agarose gel electrophoresis and 0.1 µg of a DNA fragment of about 1 kb containing the immunoglobulin enhancer region was recovered. Separately, a 1 µg portion of the plasmid pKMD6 obtained in the above step (2) was dissolved in 100 µl of a buffer solution containing 10 mM Tris-HCl (pH 7.5), 6 mM magnesium chloride and 100 mM sodium chloride. The thus prepared solution was mixed with 10 units of BglII and incubated at 37°C for 2 hours to cleave the plasmid at the BglII site. The resulting reaction mixture was extracted with phenol and chloroform and precipitated with ethanol. The thus precipitated DNA fragments were dissolved in 40 µl of DNA polymerase I buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM magnesium chloride, 0.1 mM dATP (deoxyadenosine triphosphate), 0.1 mM dCTP (deoxycytidine triphosphate), 0.1 mM dGTP (deoxyguanosine triphosphate) and 0.1 mM dTTP (deoxythymidine triphosphate). The resulting solution was mixed with 6 units of E. coli DNA polymerase I Klenow fragment and incubated at 16°C for 90 minutes to convert the cohesive 5'-end formed by the BglII digestion into blunt

end. The reaction was stopped by extracting the reaction mixture with phenol. After extraction with chloroform and precipitation with ethanol, the resulting DNA fragments were dissolved in 30 µl of a buffer solution containing 10 mM Tris-HCl (pH 7.5), 6 mM magnesium chloride and 50 mM sodium chloride. The thus prepared solution was mixed with 10 units of HindIII and incubated at 37°C for 2 hours to cleave the DNA fragment at the HindIII site. The resulting reaction mixture was subjected to agarose gel electrophoresis, 0.1 µg of a DNA fragment of about 0.8 kb containing the immunoglobulin promoter region was recovered. Next, a 0.2 µg portion of plasmid pUC18 (Messing, Methods in Enzymology, 101, 20 (1983)) was dissolved in 30 µl of a buffer solution containing 10 mM Tris-HCl (pH 7.5), 6 mM magnesium chloride and 100 mM sodium chloride. The thus prepared solution was mixed with 10 units of HindIII and 10 units of XbaI and incubated at 37°C for 2 hours to cleave the plasmid at the HindIII and XbaI sites. The resulting reaction mixture was subjected to agarose gel electrophoresis, 0.1 µg of a DNA fragment of about 2.7 kb was recovered. A 0.1 µg portion of the thus obtained pKMD6-derived 0.8 kb DNA fragment, 0.02 µg of the DNA fragment containing the immunoglobulin enhancer region and 0.1 µg of the pUC18 fragment were dissolved in 20 µl of the T4 ligase buffer, and the resulting solution was mixed with 175 units of T4 DNA ligase and incubated at 4°C for 24 hours. Using the resulting reaction mixture, transformation of E. coli HB101 was carried

out to isolate an ApR colony. Plasmid DNA was recovered from the colony to obtain pEPKMA1 as shown in Fig. 4.

Next, a 1 µg portion of the plasmid pEPKMA1 was dissolved in 100 µl of a buffer solution containing 10 mM Tris-HCl (pH 7.5), 6 mM magnesium chloride and 100 mM sodium chloride. The thus prepared solution was mixed with 10 units of XbaI and incubated at 37°C for 2 hours to cleave the plasmid at the XbaI site. The resulting reaction mixture was extracted with phenol and chloroform and precipitated with ethanol. The thus precipitated DNA fragments were dissolved in 40 µl of the aforementioned DNA polymerase I buffer solution, and the resulting solution was mixed with 6 units of E. coli DNA polymerase I Klenow fragment and incubated at 16°C for 90 minutes to convert the cohesive 5'-end formed by the XbaI digestion into blunt end. The reaction was stopped by extracting the reaction mixture with phenol. After extraction with chloroform and precipitation with ethanol, DNA fragments was recovered. The thus obtained DNA sample and 0.01 µg of a synthetic DNA XhoI linker (Takara Shuzo Co., Ltd.) were dissolved in 20 µl of the T4 ligase buffer, and the resulting solution was mixed with 175 units of T4 DNA ligase and incubated at 4°C for 24 hours. Using the resulting reaction mixture, transformation of E. coli HB101 was carried out to isolate an ApR colony. Plasmid DNA was recovered from the colony to obtain pEPKMB1 as shown in Fig. 5.

Next, SV40 early gene promoter and enhancer regions (to

be referred to as "P_{SE}" hereinafter) of an expression vector pAGE107 for use in the expression of heterologous genes in animal cells (Miyaji et al., Cytotechnology, 3, 133 - 140 (1990)) were converted into KM50-derived immunoglobulin H chain promoter and enhancer (to be referred to as "P_{IH}" hereinafter) of pEPKMB1 in the following manner.

A 1 µg portion of the plasmid pAGE107 was dissolved in 30 µl of a buffer solution containing 10 mM Tris-HCl (pH 7.5), 6 mM magnesium chloride and 150 mM sodium chloride. The thus prepared solution was mixed with 10 units of SalI and 10 units of XhoI and incubated at 37°C for 2 hours to cleave the plasmid at the SalI and XhoI sites. The resulting reaction mixture was subjected to agarose gel electrophoresis and 0.5 µg of a DNA fragment of about 5.95 kb containing G418 resistance gene was recovered. Next, a 1 µg portion of the plasmid pEPKMB1 was dissolved in 30 µl of a buffer solution containing 10 mM Tris-HCl (pH 7.5), 6 mM magnesium chloride and 150 mM sodium chloride. The thus prepared solution was mixed with 10 units of SalI and 10 units of XhoI and incubated at 37°C for 2 hours to cleave the plasmid at the SalI and XhoI sites. The resulting reaction mixture was subjected to agarose gel electrophoresis and 0.1 µg of a DNA fragment of about 1.7 kb containing immunoglobulin promoter and enhancer regions was recovered. A 0.1 µg portion of the thus obtained pAGE107-derived 5.95 kb DNA fragment and 0.02 µg of the DNA fragment containing immunoglobulin promoter and enhancer regions were

dissolved in 20 μ L of the T4 ligase buffer, and the resulting solution was mixed with 175 units of T4 DNA ligase and incubated at 4°C for 24 hours. Using the resulting reaction mixture, transformation of *E. coli* HB101 was carried out to isolate an ApR colony. Plasmid DNA was recovered from the colony to obtain pAGE501 as shown in Fig. 6.

5 (4) **Construction of pAGE109**

In order to construct pAGE109, it is first necessary to prepare plasmid pAGE106 in the following manner:

(a) **Plasmid pAGE106**

Construction of the Recombinant Plasmid pAGE105M:

10 An *E. coli* HB101 transformant harbouring the plasmid pAGE28 constructed by the present inventors [Mizukami *et al.*, J. Biochem., 101, 1307-1310 (1987)] was cultured and the pAGE28 DNA was prepared from cultured cells by a conventional method. About 2 μ g of the pAGE28 DNA obtained was dissolved in 30 μ L of Y-100 buffer, 8 units of *Xho*I and 12 units of *Sca*I were added, and the digestion reaction was conducted
15 at 37°C for 2 hours. After 10 minutes of heat treatment at 65°C, a DNA fragment about 2.8 kb was purified using the AFT method. Separately, an *Escherichia coli* HB101 transformant harbouring the plasmid pAGE103 constructed by the present inventors [Mizukami *et al.*, Biochem., 101, 1307-1310 (1987)] was cultured and the pAGE103 DNA was prepared from cultured cells in a conventional manner. About 3 μ g of the
20 pAGE103 DNA obtained was dissolved in 30 μ L of Y-100 buffer, 10 units of *Eco*RI was added, and the digestion reaction was conducted at 37°C for 2 hours. After phenol extraction and chloroform extraction, a DNA fragment was recovered by precipitation with ethanol. This DNA fragment was dissolved in a total of 40 μ L of polymerase buffer, 6 units of Klenow fragment were added, and the reaction was conducted at 15°C
25 for 1 hour to thereby convert the *Eco*RI protruding ends to blunt ends. The reaction was terminated by phenol extraction and, after chloroform extraction, a DNA fragment was recovered by precipitation with ethanol. This DNA fragment was dissolved in 30 μ L of Y-100 buffer, 12 units of *Xho*I was added, and the digestion reaction was conducted at 37°C for 2 hours. After 10 minutes of heat treatment at 65°C, a DNA fragment about 0.4
30 kb was purified using the AFT method. Separately, an *Escherichia coli* transformant harbouring the plasmid pKCR constructed by O'Hara *et al.* [Proc. Natl. Acad. Sci. USA, 78, 1527 (1981)] was cultured and the pKCR DNA was prepared from cultured cells by the conventional method. About 2 μ g of the pKCR DNA obtained was dissolved in 30 μ L of Y-150 buffer, 12 units of *Bam*HI and 16 units of *Sal*I were added, and the digestion
35 reaction was conducted at 37°C for 2 hours. After phenol extraction and chloroform extraction, a DNA fragment was recovered by precipitation with ethanol. This DNA fragment was dissolved in a total of 40 μ L of polymerase buffer, 6 units of Klenow fragment were added, and the reaction was conducted at 15°C for 1 hour for the conversion of the *Bam* HI and *Sal*I protruding ends to blunt ends.



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[N:Abuu]00407:KEH

After 10 minutes of heat treatment at 65°C, a DNA fragment about 1.85 kb was purified using the AFT method.

The thus-obtained pAGE-28-derived DNA fragment (about 2.8 kb, about 0.05 µg), pAGE-103-derived DNA fragment (about 0.4 kb, about 0.03 µg) and pKCR-derived DNA fragment (about 1.85 kb, about 0.2 µg) were dissolved in 20 µL of T4 ligase buffer, 300 units of T4 DNA ligase was added, and the ligation reaction was conducted at 4°C for 18 hours.

The thus-obtained reaction mixture was used to transform *Escherichia coli* MM294 to give kanamycin (hereinafter referred to as "Km")-resistant transformants. The plasmid pAGE-105M isolated from one of these transformants was found to have the desired structure upon structural analysis comprising digestion with restriction enzymes (cf. Fig. 28).

Construction of the recombinant plasmid pAGE106:

About 2 µg of the pAGE105M DNA obtained as described above was dissolved in 30 µL of Y-100 buffer, 12 units of *ScaI* was added, and the digestion reaction was conducted at 37°C for 2 hours. After 10 minutes of heat treatment at 65°C, a DNA fragment about 5.0 kb was purified using the AFT method. Separately, 20 picomoles of the *EcoRI* linker GGAATTCC (Collaborative Research) was 5'-phosphorylated in the same manner as mentioned hereinbefore.

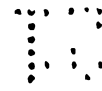
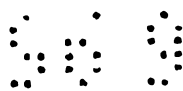
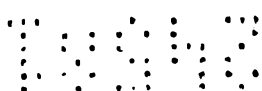
The thus-obtained pAGE105M-derived DNA fragment (about 5.0 kb, about 0.1 µg), 5'-phosphorylated *EcoRI* linker (1 picomole) were dissolved in 20 µL T4 ligase buffer, 100 units of T4 DNA ligase was added, and the ligation reaction was conducted at 4°C for 18 hours.

The thus-obtained reaction mixture was used to transform *Escherichia coli* MM294 to give Km-resistant transformants. The plasmid DNA pAGE106 isolated from one of these transformants was found to have the desired structure upon structural analysis comprising digestion with restriction enzymes (cf. Fig. 29).

Plasmid pAGE109

One of the two *EcoRI* cleavage sites in plasmid pAGE106 was deleted in the following manner to construct pAGE109.

A 2 µg portion of the expression vector pAGE106 for use in the expression of heterologous genes in animal cells was dissolved in 100 µL of a buffer solution containing 10 mM Tris-HCl (pH 7.5), 6 mM magnesium chloride and 50 mM sodium chloride. The thus prepared solution was mixed with 10 units of *EcoRI* and 10 units of *SacI* and incubated at 37°C for 4 hours. The resulting reaction mixture was subjected to agarose gel electrophoresis and about 1.5 µg of a pAGE106 DNA fragment (4.3 kb) was recovered which contained the SV40 early gene promoter and G418 resistance gene cleaved with *EcoRI* and *SacI*. The thus recovered DNA fragment was dissolved in 40 µL of the DNA polymerase I buffer solution, and the resulting solution was mixed with 5



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units of *E. coli* DNA polymerase I large fragment and incubated at 16°C for 2 hours to convert the cohesive 3'-end formed by the *Sac*I digestion and the cohesive 5'-end formed by the *Eco*RI digestion into blunt ends. The resulting reaction mixture was extracted



1.1.1.1

2.2.2.2

3.3.3.3

[N:\libuu]00407:KEH

with phenol and chloroform and then treated with ethanol. The thus precipitated sample was dissolved in 20 μ l of the T4 ligase buffer, and the resulting solution was mixed with 350 units of T4 DNA ligase and incubated at 4°C for 4 hours. Using
5 the thus obtained recombinant plasmid DNA, transformation of E. coli HB101 was carried out to obtain plasmid pAGE109 as shown in Fig. 7.

(5) Construction of pAGE502

Plasmid pAGE502 was constructed in the following manner
10 in order to convert the SV40 promoter and enhancer of pAGE107 into immunoglobulin H chain promoter and enhancer.

A 2 μ g portion of the plasmid pAGE107 disclosed in JP-A-3-22979 or EP-A-0 405 285 was dissolved in 100 μ l of a buffer solution containing 10 mM Tris-HCl (pH 7.5), 6 mM magnesium
15 chloride and 50 mM sodium chloride. The thus prepared solution was mixed with 10 units of HindIII and incubated at 37°C for 4 hours. The resulting reaction mixture was subjected to phenol-chloroform extraction and ethanol precipitation and the thus recovered sample was dissolved in 40 μ l of the DNA polymerase
20 I buffer solution. The resulting solution was mixed with 5 units of E. coli DNA polymerase I Klenow fragment and incubated at 16°C for 2 hours to convert the cohesive 5'-end formed by the HindIII digestion into blunt end. The resulting reaction mixture was extracted with phenol and chloroform and then
25 treated with ethanol. The thus precipitated sample was dissolved in 30 μ l of a buffer solution containing 10 mM Tris-

HCl (pH 7.5), 6 mM magnesium chloride and 100 mM sodium chloride. The thus prepared solution was mixed with 10 units of XhoI and incubated at 37°C for 4 hours. The resulting reaction mixture was subjected to agarose gel electrophoresis and about 1.5 µg of a pAGE107 DNA fragment of about 5.95 kb was obtained which contained G418 resistance gene and ApR gene cleaved with XhoI and HindIII.

Next, a 2 µg portion of the plasmid pAGE501 obtained in the above step (3) was dissolved in 100 µl of a buffer solution containing 10 mM Tris-HCl (pH 7.5), 6 mM magnesium chloride and 175 mM sodium chloride. The thus prepared solution was mixed with 10 units of SalI and incubated at 37°C for 4 hours. After subjecting the resulting reaction mixture to phenol-chloroform extraction and ethanol precipitation, the thus recovered sample was dissolved in 40 µl of the DNA polymerase I buffer solution. The resulting solution was mixed with 5 units of E. coli DNA polymerase I Klenow fragment and incubated at 16°C for 2 hours to convert the cohesive 5'-end formed by the SalI digestion into blunt end. The resulting reaction mixture was extracted with phenol and chloroform and then treated with ethanol. The thus precipitated sample was dissolved in 30 µl of a buffer solution containing 10 mM Tris-HCl (pH 7.5), 6 mM magnesium chloride and 100 mM sodium chloride. The thus prepared solution was mixed with 10 units of XhoI and incubated at 37°C for 4 hours. The resulting reaction mixture was subjected to agarose gel electrophoresis and about 0.2 µg of a pAGE501 DNA

fragment of about 1.8 kb was obtained which contained KM50 immunoglobulin H chain promoter and enhancer genes cleaved with XhoI and SalI.

Next, 0.1 µg of the thus obtained pAGE107 HindIII-XhoI fragment (about 5.95 kb) and 0.1 µg of the pAGE501 SalI-XhoI fragment (about 1.8 kb) were dissolved in 20 µl of the T4 ligase buffer, and the resulting solution was mixed with 350 units of T4 DNA ligase and incubated at 4°C for 24 hours. Using the thus obtained recombinant plasmid DNA, transformation of E. coli HB101 was carried out to obtain plasmid pAGE502 as shown in Fig. 8.

(6) Construction of pAGE503

One of the two EcoRI cleavage sites in plasmid pAGE502 was deleted in the following manner to construct pAGE503.

A 2 µg portion of the plasmid pAGE109 obtained in the above step (4) was dissolved in 30 µl of a buffer solution containing 10 mM Tris-HCl (pH 7.5), 6 mM magnesium chloride and 50 mM sodium chloride. The thus prepared solution was mixed with 10 units of HindIII and 10 units of ClaI and incubated at 37°C for 4 hours. The resulting reaction mixture was subjected to agarose gel electrophoresis and about 0.2 µg of a pAGE109 DNA fragment of about 1 kb was recovered which contained the poly(A) signal gene of beta-globin and SV40 early genes cleaved with ClaI and HindIII.

Next, a 2 µg portion of the plasmid pAGE502 obtained in the above step (5) was dissolved in 30 µl of a buffer solution

containing 10 mM Tris-HCl (pH 7.5), 6 mM magnesium chloride and 50 mM sodium chloride. The thus prepared solution was mixed with 10 units of HindIII and 10 units of ClaI and incubated at 37°C for 4 hours. The resulting reaction mixture was subjected
5 to agarose gel electrophoresis and then to the aforementioned DEAE paper method to recover about 1 µg of a pAGE502 DNA fragment of about 6.1 kb which contained KM50 immunoglobulin H chain promoter and enhancer genes, ApR gene and G418 resistance gene cleaved with HindIII and ClaI. Next, 0.1 µg of the thus
10 obtained pAGE109 HindIII-ClaI fragment (about 1 kb) and 0.1 µg of the pAGE502 HindIII-ClaI fragment (about 6.1 kb) were dissolved in 20 µl of the T4 ligase buffer, and the resulting solution was mixed with 350 units of T4 DNA ligase and incubated at 4°C for 24 hours. Using the thus obtained
15 recombinant plasmid DNA, transformation of E. coli HB101 was carried out to obtain plasmid pAGE503 as shown in Fig. 9.

(7) Construction of pSEld1

A dhfr gene was introduced into plasmid pAGE107 in the following manner to construct plasmid pSEld1.

20 A 2 µg portion of the plasmid pAGE107 disclosed in JP-A 3-22979 or EP-A-0 405 285 was dissolved in 100 µl of a buffer solution containing 100 mM Tris-HCl (pH 7.5), 6 mM magnesium chloride and 50 mM sodium chloride. The thus prepared solution was mixed with 10 units of EcoRI and incubated at 37°C for 4
25 hours. After subjecting the resulting reaction mixture to phenol-chloroform extraction and ethanol precipitation, the

thus recovered sample was dissolved in 40 μ l of the DNA polymerase I buffer solution. The resulting solution was mixed with 5 units of E. coli DNA polymerase I Klenow fragment and incubated at 16°C for 2 hours to convert the cohesive 5'-end
5 formed by the EcoRI digestion into blunt end. The resulting reaction mixture was extracted with phenol and chloroform and then treated with ethanol. The thus precipitated sample was dissolved in 30 μ l of a buffer solution which was composed of 10 mM Tris-HCl (pH 7.5), 6 mM magnesium chloride and 50 mM
10 sodium chloride. The thus prepared solution was mixed with 10 units of HindIII and incubated at 37°C for 4 hours. The resulting reaction mixture was subjected to agarose gel electrophoresis and about 1.5 μ g of a pAGE107 DNA fragment of about 5.6 kb was recovered which contained G418 resistance gene
15 and ApR gene cleaved with EcoRI and HindIII.

Next, a 2 μ g portion of a plasmid pSV2-dhfr (Subramani et al., Mol. Cell. Biology, 1, 854 (1981)) was dissolved in 100 μ l of a buffer solution containing 10 mM Tris-HCl (pH 7.5), 6 mM magnesium chloride and 100 mM sodium chloride. The thus
20 prepared solution was mixed with 10 units of BglII and incubated at 37°C for 4 hours. After subjecting the resulting reaction mixture to phenol-chloroform extraction and ethanol precipitation, the thus recovered sample was dissolved in 40 μ l
25 of the DNA polymerase I buffer solution. The resulting solution was mixed with 5 units of E. coli DNA polymerase I Klenow fragment and incubated at 16°C for 2 hours to convert

the cohesive 5'-end formed by the BglII digestion into blunt end. The resulting reaction mixture was extracted with phenol and chloroform and then treated with ethanol. The thus precipitated sample was dissolved in 30 µl of a buffer solution
5 containing 10 mM Tris-HCl (pH 7.5), 6 mM magnesium chloride and 100 mM sodium chloride. The thus prepared solution was mixed with 10 units of HindIII and incubated at 37°C for 4 hours. The resulting reaction mixture was subjected to agarose gel electrophoresis, about 0.2 µg of a pSV2-dhfr DNA fragment of
10 about 0.76 kb was recovered which contained dhfr gene cleaved with BglII and HindIII.

Next, 0.1 µg of the thus obtained pAGE107 HindIII-EcoRI fragment (about 5.6 kb) and 0.1 µg of the pSV2-dhfr BglII-HindIII fragment (about 0.76 kb) were dissolved in 20 µl of the
15 T4 ligase buffer, and the resulting solution was mixed with 350 units of T4 DNA ligase and incubated at 4°C for 24 hours. Using the thus obtained recombinant plasmid DNA, transformation of E. coli HB101 was carried out to obtain plasmid pSEld1 as shown in Fig. 10.

20 (8) Construction of pSEld2

25 The HindIII cleavage site was removed from the plasmid pSEld1 in the following manner to construct plasmid pSEld2.

30 A 2 µg portion of the plasmid pSEld1 obtained in the above step (7) was dissolved in 100 µl of a buffer solution containing 10 mM Tris-HCl (pH 7.5), 6 mM magnesium chloride and
35 50 mM sodium chloride. The thus prepared solution was mixed

with 10 units of HindIII and incubated at 37°C for 4 hours. After subjecting the resulting reaction mixture to phenol-chloroform extraction and ethanol precipitation, the thus recovered sample was dissolved in 40 µl of the DNA polymerase I buffer solution. The resulting solution was mixed with 5 units of E. coli DNA polymerase I Klenow fragment and incubated at 16°C for 2 hours to convert the cohesive 5'-end formed by the HindIII digestion into blunt end. The resulting reaction mixture was extracted with phenol and chloroform and then treated with ethanol. The thus precipitated sample was dissolved in 20 µl of the T4 ligase buffer, and the resulting solution was mixed with 350 units of T4 DNA ligase and incubated at 4°C for 24 hours. Using the thus obtained recombinant plasmid DNA, transformation of E. coli HB101 was carried out to obtain plasmid pSEld2 as shown in Fig. 11.

(9) Construction of pIg1SEld2

The dhfr gene was introduced into plasmid pAGE503 in the following manner to construct plasmid pIg1SEld2.

A 2 µg portion of the plasmid pAGE503 obtained in the above step (6) was dissolved in 100 µl of a buffer solution containing 100 mM Tris-HCl (pH 7.5), 6 mM magnesium chloride and 50 mM sodium chloride. The thus prepared solution was mixed with 10 units of ClaI and incubated at 37°C for 4 hours. After subjecting the resulting reaction mixture to phenol-chloroform extraction and ethanol precipitation, the thus recovered sample was dissolved in 40 µl of the DNA polymerase

I buffer solution. The resulting solution was mixed with 5 units of E. coli DNA polymerase I Klenow fragment and incubated at 16°C for 2 hours to convert the cohesive 5'-end formed by the ClaI digestion into blunt end. The resulting reaction mixture was extracted with phenol and chloroform and then treated with ethanol. The thus precipitated sample was dissolved in 30 µl of a buffer solution containing 10 mM Tris-HCl (pH 7.5), 6 mM magnesium chloride and 50 mM sodium chloride. The thus prepared solution was mixed with 10 units of MluI and incubated at 37°C for 4 hours. The resulting reaction mixture was subjected to agarose gel electrophoresis and about 1 µg of a pAGE503 DNA fragment of about 5.4 kb was recovered which contained the KM50 immunoglobulin H chain promoter and enhancer genes cleaved with ClaI and MluI.

Next, a 2 µg portion of the plasmid pSEld2 obtained in the above step (8) was dissolved in 100 µl of a buffer solution containing 10 mM Tris-HCl (pH 7.5), 6 mM magnesium chloride and 100 mM sodium chloride. The thus prepared solution was mixed with 10 units of XhoI and incubated at 37°C for 4 hours. The resulting reaction mixture was subjected to phenol-chloroform extraction and ethanol precipitation and the thus recovered sample was dissolved in 40 µl of the DNA polymerase I buffer solution. The resulting solution was mixed with 5 units of E. coli DNA polymerase I Klenow fragment and incubated at 16°C for 2 hours to convert the cohesive 5'-end formed by the XhoI digestion into blunt end. The resulting reaction mixture was

extracted with phenol and chloroform and then treated with ethanol. The thus precipitated sample was dissolved in 30 µl of a buffer solution containing 10 mM Tris-HCl (pH 7.5), 6 mM magnesium chloride and 100 mM sodium chloride. The thus
5 prepared solution was mixed with 10 units of MluI and incubated at 37°C for 4 hours. The resulting reaction mixture was subjected to agarose gel electrophoresis and about 1 µg of a pSEld2 DNA fragment of about 3.8 kb was recovered which contained dhfr gene cleaved with XhoI and MluI.

10 Next, 1 µg of the thus obtained pAGE503 ClaI-MluI fragment (about 5.4 kb) and 1 µg of the pSEld2 XhoI-MluI fragment (about 3.8 kb) were dissolved in 20 µl of the T4 ligase buffer, and the resulting solution was mixed with 350 units of T4 DNA ligase and incubated at 4°C for 24 hours.
15 Using the thus obtained recombinant plasmid DNA, transformation of E. coli HB101 was carried out to obtain plasmid pIg1SEld2 as shown in Fig. 12.

(10) Construction of pIg1SEld3

20 The ApaI cleavage site was removed from the plasmid pIg1SEld2 in the following manner to construct plasmid pIg1SEld3.

25 A 2 µg portion of the plasmid pIg1SEld2 obtained in the above step (9) was dissolved in 100 µl of a buffer solution containing 10 mM Tris-HCl (pH 7.5) and 6 mM magnesium chloride.

30 The thus prepared solution was mixed with 10 units of ApaI and incubated at 37°C for 4 hours. After subjecting the resulting

reaction mixture to phenol-chloroform extraction and ethanol precipitation, the thus recovered sample was dissolved in 40 μ l of the DNA polymerase I buffer solution. The resulting solution was mixed with 5 units of E. coli DNA polymerase I Klenow fragment and incubated at 16°C for 2 hours to convert the cohesive 3'-end formed by the ApaI digestion into blunt end. The resulting reaction mixture was extracted with phenol and chloroform and then treated with ethanol. The thus precipitated sample was dissolved in 20 μ l of the T4 ligase buffer, and the resulting solution was mixed with 350 units of T4 DNA ligase and incubated at 4°C for 24 hours. Using the thus obtained recombinant plasmid DNA, transformation of E. coli HB101 was carried out to obtain plasmid pIg1SEld3 as shown in Fig. 13.

(11) Construction of pIg1SEld4

In order to establish a cloning site between HindIII cleavage site and EcoRI cleavage site of the plasmid pIg1SEld3, plasmid pIg1SEld4 was constructed by inserting the synthetic DNA shown in SEQ ID NO:2 into the plasmid pIg1SEld3 in the following manner.

A 2 μ g portion of the plasmid pIg1SEld3 obtained in the above step (10) was dissolved in 30 μ l of a buffer solution containing 10 mM Tris-HCl (pH 7.5), 6 mM magnesium chloride and 50 mM sodium chloride. The thus prepared solution was mixed with 10 units of HindIII and 10 units of EcoRI and incubated at 37°C for 4 hours. The resulting reaction mixture was subjected

to agarose gel electrophoresis and about 1 µg of a pIg1SE1d3 DNA fragment of about 9.2 kb was recovered which contained the KM50 immunoglobulin H chain promoter and enhancer genes, ApR gene, G418 resistance gene and dhfr gene cleaved with *HindIII* and *EcoRI*.

5 Next, 0.1 µg of the thus obtained pIg1SE1d3 *HindIII-EcoRI* fragment (about 9.2 kb) and 10 ng of the synthetic DNA (SEQ ID NO:2) were dissolved in 2 µL of the T4 ligase buffer, and the resulting solution was mixed with 350 units of T4 DNA ligase and incubated at 4°C for 24 hours. Using the thus obtained recombinant plasmid DNA, transformation of *E. coli* HB101 was carried out to obtain plasmid pIg1SE1d4 as shown
10 in Fig. 14.

3. Preparation of Moloney mouse leukemia virus long terminal repeat (to be referred to as "MoLTR" hereinafter)

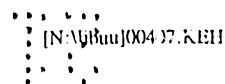
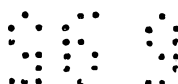
Since MoLTR is known to have promoter and enhancer activities (Kuwana *et al.*, Biochem. Biophys. Res. Commun., **149**, 960 (1987)), a plasmid pPMOL3 containing
15 MoLTR was prepared in the following manner in order to use MoLTR as cassette vector promoter and enhancer.

Five µg of plasmid pMolp-1 [Virology, **141**, 30-42 (1985)] in which MMuLV-LTR DNA had been introduced were suspended in 30 µL of a solution containing 10 mM Tris-HCl (pH 7.5), 7 mM MgCl₂ and 50 mM NaCl (hereinafter referred to as Y-50 buffer)
20 and 20 units each of *KpnI* and *ClaI* (Takara Shuzo Co., Ltd.; unless otherwise specified, all the restriction enzymes described below are manufactured by the same company.) to conduct the digestion reaction at 37°C for 2 hours. In accordance with the method described in Nature **293**, 543-548 (1981), about 0.3 µg of a DNA fragment of about 0.62 Kb containing MMuLV-LTR in pMOLp-1 was obtained from the reaction mixture by the
25 LGT method.

Separately, 3 µg of pBR322 was suspended in 30 µL of a solution containing 10 mM Tris-HCl (pH 7.5), 7 mM MgCl₂ and 100 mM NaCl (hereinafter referred to as Y-100 buffer) and 10 units of *PstI* and 10 units of *ClaI* were added thereto to conduct the digestion reaction at 37°C for 2 hours. About 0.3 µg of a DNA fragment of about 0.77
30 Kb was obtained from the reaction mixture by the LGT method.

Separately, 2 µg of plasmid pKYP26 (JP-A-62-48699) were suspended in a solution containing 10 mM Tris-HCl (pH 7.5), 7 mM MgCl₂ and 50 mM NaCl and 10 units of *PstI* and 10 units of *KpnI* were added thereto followed by the digestion reaction at 37°C for 2 hours. A DNA fragment of about 1.7 Kb containing lpp terminator was obtained
35 from the reaction mixture by the LGT method.

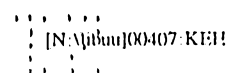
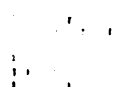
Then, 0.04 µg of pMO1p-1-derived *ClaI-KpnI* fragment (0.062 kb), 0.04 µg of pBR322-derived *ClaI-PstI* fragment (about 0.77 Kb) and 0.05 µg of pKYP26 derived *PstI-KpnI* fragment (about 1.7 kb) were suspended in 20 µL of a solution containing 50



mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 10 mM DTT and 1 mM ATP. Six units of T4 DNA ligase were added thereto to conduct the ligation reaction at 40°C for 18 hours.

The resulting mixture was used to transform *Escherichia coli* HB101 to obtain Ap² colony. The plasmid DNA was recovered from the colony and pPMOL1 shown in Fig. 5
5 was obtained.

A 3 µg portion of the plasmid pPMOL1 was dissolved in 30 µL of a buffer solution containing 10 mM Tris-HCl (pH 7.5), 7 mM magnesium chloride and 6 mM 2-mercapto-ethanol. The thus prepared solution was mixed with 10 units of *Cla*I and incubated at 37°C for 4 hours. After subjecting the resulting reaction mixture to phenol-chloroform



extraction and ethanol precipitation, the thus recovered sample
 was dissolved in 40 μ l of the DNA polymerase I buffer solution.
 The resulting solution was mixed with 5 units of E. coli DNA
 polymerase I Klenow fragment and incubated at 16°C for 2 hours
 5 to convert the cohesive 5'-end formed by the ClaI digestion
 into blunt end. The reaction was stopped by phenol extraction,
 followed by chloroform extraction and ethanol precipitation to
 recover 2 μ g of DNA fragments. The thus precipitated DNA
 sample and 0.01 μ g of a synthetic DNA XhoI linker (Takara Shuzo
 10 Co., Ltd.) were dissolved in 20 μ l of the T4 ligase buffer, and
 the resulting solution was mixed with 175 units of T4 DNA
 ligase and incubated at 4°C for 24 hours. Using the resulting
 reaction mixture, transformation of E. coli HB101 was carried
 out to obtain plasmid pPMOL2 as shown in Fig. 15. Next, a 3 μ g
 15 portion of the thus obtained plasmid pPMOL2 was dissolved in 30
 μ l of a buffer solution containing 10 mM Tris-HCl (pH 7.5), 7
 mM magnesium chloride, 10 mM sodium chloride and 6 mM 2-
 mercaptoethanol. The thus prepared solution was mixed with 10
 units of SmaI and incubated at 37°C for 4 hours. The resulting
 20 reaction mixture was subjected to phenol-chloroform extraction
 and ethanol precipitation and 2 μ g of DNA fragments were
 recovered. The thus recovered DNA sample and 0.01 μ g of a
 synthetic DNA EcoRI linker (Takara Shuzo Co., Ltd.) were
 dissolved in 20 μ l of the T4 ligase buffer, and the resulting
 25 solution was mixed with 175 units of T4 DNA ligase and
 incubated at 4°C for 24 hours. Using the resulting reaction

mixture, transformation of E. coli HB101 was carried out to obtain plasmid pPMOL3 as shown in Fig. 16.

4. Cloning of H chain constant region (Cg1) cDNA and L chain constant region (Ck) cDNA of human immunoglobulin IgG

5 (1) Preparation of mRNA from chimera antibody-producing SP2-PC Chimera-1 cells

Using a mRNA extraction kit, Fast Track (No. K1593-02, available from Invitrogen), 6.2 µg of mRNA was obtained from 1×10^8 cells of chimera antibody-producing SP2-PC Chimera-1 which has anti-phosphorylcholine activity and is disclosed in
10 FEBS Letters (244, 301 - 306 (1989)).

(2) Preparation of SP2-PC Chimera-1 cDNA library and cloning of human immunoglobulin H chain constant region (Cg1) cDNA and L chain constant region (Ck) cDNA

15 A 2 µg portion of the mRNA obtained in the above step (1) was subjected to EcoRI adaptor addition using cDNA Synthesis Kit (No. 27-9260-01, available from Pharmacia) followed by kination. The resulting cDNA solution was subjected to phenol-chloroform extraction and ethanol
20 precipitation to recover 4 µg of cDNA. The thus recovered cDNA was dissolved in 20 µl of sterile water, and the resulting solution was subjected to agarose gel electrophoresis to
25 recover about 0.3 µg of a DNA fragment of about 1.8 kb and about 0.3 µg of a DNA fragment of about 1.0 kb.

26 Next, a 5 µg portion of the vector pUC18 was dissolved in 100 µl of a buffer solution containing 100 mM Tris-HCl (pH

7.5), 6 mM magnesium chloride and 100 mM sodium chloride. The thus prepared solution was mixed with 50 units of EcoRI and incubated at 37°C for 4 hours to cleave the pUC18 DNA at its EcoRI cleavage site. The resulting reaction mixture was
5 subjected to phenol-chloroform extraction and ethanol precipitation to recover about 3 µg of a pUC18 DNA fragment cleaved with EcoRI.

Next, 0.1 µg of the thus obtained pUC18 EcoRI fragment (about 2.7 kb) and the 1.8 kb and 1.0 kb cDNA fragments (0.1 µg
10 for each) prepared from the SP2-PC Chimera-1 cells were dissolved in 20 µl of the T4 ligase buffer, and the resulting solution was mixed with 350 units of T4 DNA ligase and incubated at 4°C for 24 hours.

Using the thus obtained recombinant plasmid DNA,
15 transformation of E. coli LE392 was carried out. About 3,000 colonies thus obtained were fixed on nitrocellulose filters. Two ³²P-labeled probes were prepared from human immunoglobulin constant region chromosomal genes (Cg1 as an IgG1 H chain constant region and Ck as an IgG1 L chain constant region)
20 which have been isolated by Kameyama et al. (FEBS Letters, 244, 301 (1989)). From colonies which showed strong reactions at 65°C with these probes, one showing strong reaction with Cg1 (pPCVHhCGI1) and the other showing strong reaction with Ck (pPCVLhCK1) were obtained.

25 (3) Introduction of EcoRV site into human Ig k chain constant region

An EcoRV site was introduced into 5'-end side of the human Ig k chain constant region by means of site-specific mutagenesis using a kit purchased from Promega (Catalogue No. Q6210). A 2 µg portion of the plasmid pPCVLhCK1 was dissolved
5 in 30 µl of a buffer solution containing 10 mM Tris-HCl (pH 7.5), 6 mM magnesium chloride and 50 mM sodium chloride. The thus prepared solution was mixed with 10 units of EcoRI and 10 units of KpnI and incubated at 37°C for 4 hours. The resulting reaction mixture was subjected to agarose gel electrophoresis
10 and about 0.2 µg of a pPCVLhCK1 DNA fragment of about 0.8 kb was recovered which contained the human immunoglobulin L chain constant region cleaved with EcoRI and KpnI.

Next, a 2 µg portion of pSELECT1 (a kit available from Promega, Catalogue No. Q6210) was dissolved in 30 µl of a
15 buffer solution containing 10 mM Tris-HCl (pH 7.5), 6 mM magnesium chloride and 50 mM sodium chloride. The thus prepared solution was mixed with 10 units of EcoRI and 10 units of KpnI and incubated at 37°C for 4 hours. The resulting reaction mixture was subjected to agarose gel electrophoresis
20 and about 1 µg of a pSELECT1 DNA fragment of about 5.7 kb cleaved with EcoRI and KpnI was recovered.

Next, 0.1 µg of the pPCVLhCK1 EcoRI-KpnI fragment (about 0.8 kb) and 0.1 µg of the pSELECT1 EcoRI-KpnI fragment (about 5.7 kb) obtained above were dissolved in 20 µl of the T⁴
25 ligase buffer, and the resulting solution was mixed with 350 units of T4 DNA ligase and incubated at 4°C for 24 hours.

Using the thus obtained recombinant plasmid DNA, transformation of E. coli JM109 was carried out to obtain plasmid pchCKA7 as shown in Fig. 17.

Next, using the plasmid pchCKA7 thus obtained and the
5 synthetic DNA of SEQ ID NO:3 as mutagenesis primer, the ACC sequence of the human immunoglobulin L chain constant region (12 to 14 position bases from the N-terminal) was converted into GAT in order to construct a plasmid pchCKB1 (Fig. 18) in which an EcoRV site was introduced into the converted site.

10 Next, the EcoRV site of the plasmid pchCKB1 was converted into HindIII cleavage site in the following manner. A 2 µg portion of the plasmid pchCKB1 obtained above was dissolved in 10 µl of a buffer solution containing 100 mM Tris-HCl (pH 7.5), 6 mM magnesium chloride and 100 mM sodium
15 chloride. The thus prepared solution was mixed with 10 units of EcoRI and incubated at 37°C for 4 hours. After subjecting the resulting reaction mixture to phenol-chloroform extraction and ethanol precipitation, the thus recovered sample was dissolved in 40 µl of the DNA polymerase I buffer solution.
20 The resulting solution was mixed with 5 units of E. coli DNA polymerase I Klenow fragment and incubated at 37°C for 30 minutes to convert the cohesive 5'-end formed by the EcoRI
25 digestion into blunt end. The resulting reaction mixture was extracted with phenol and chloroform and then treated with ethanol. The thus precipitated sample was dissolved in 20 µl of the T4 ligase buffer containing 0.1 µg of HindIII linker

(Takara Shuzo Co., Ltd.), and the resulting solution was mixed with 350 units of T4 DNA ligase and incubated at 4°C for 24 hours. Using the thus obtained recombinant plasmid DNA, transformation of E. coli HB101 was carried out to obtain
5 plasmid pchCKC1 as shown in Fig. 19.

5. Construction of cassette vector

(1) Construction of a cassette vector for use in the construction of humanized chimera antibody H chain expression vector

10 A 2 µg portion of the plasmid pIg1SEId4 obtained in the aforementioned step 2-(11) was dissolved in 30 µl of a buffer solution containing 10 mM Tris-HCl (pH 7.5), 6 mM magnesium chloride and 100 mM sodium chloride. The thus prepared solution was mixed with 10 units of EcoRV and 10 units of ApaI
15 and incubated at 37°C for 4 hours. The resulting reaction mixture was subjected to agarose gel electrophoresis and about 1.5 µg of a pIg1SEId4 DNA fragment of about 9.2 kb cleaved with EcoRV and ApaI was recovered.

20 Next, a 2 µg portion of the plasmid pPCVHhCGI1 obtained in the aforementioned step 4-(2) was dissolved in 30 µl of a buffer solution containing 10 mM Tris-HCl (pH 7.5) and 6 mM magnesium chloride. The thus prepared solution was mixed with
25 10 units of ApaI and 10 units of SmaI and incubated at 37°C for 1 hour. The resulting reaction mixture was subjected to agarose gel electrophoresis and about 0.2 µg of a pPCVHhCGI1 DNA fragment of about 1 kb was recovered which contained the human

immunoglobulin H chain constant region gene cleaved with ApaI and SmaI.

Next, 0.1 µg of the pIg1SEId4 EcoRV-ApaI fragment (about 9.2 kb) and 0.1 µg of the pPCVHhCGI1 ApaI-SmaI fragment (about 1 kb) prepared above were dissolved in 20 µl of the T4 ligase buffer. The resulting solution was mixed with 350 units of T4 DNA ligase and incubated at 4°C for 24 hours. Using the thus obtained recombinant plasmid DNA, transformation of E. coli HB101 was carried out to obtain a plasmid pChiIgHB2 (Fig. 20) as a cassette vector for use in the construction of a humanized chimera antibody H chain expression vector.

(2) Construction of a cassette vector for use in the construction of humanized chimera antibody L chain expression vector

A 2 µg portion of the plasmid pIg1SEId4 obtained in the aforementioned step 2-(11) was dissolved in 30 µl of a buffer solution containing 10 mM Tris-HCl (pH 7.5), 6 mM magnesium chloride and 100 mM sodium chloride. The thus prepared solution was mixed with 10 units of EcoRV and 10 units of HindIII and incubated at 37°C for 4 hours. The resulting reaction mixture was subjected to agarose gel electrophoresis and about 1.5 µg of a pIg1SEId4 DNA fragment of about 9.2 kb cleaved with EcoRV and HindIII was recovered.

Next, a 2 µg portion of the plasmid pchCKC1 obtained in the aforementioned step 4-(3) was dissolved in 30 µl of a buffer solution containing 10 mM Tris-HCl (pH 7.5), 6 mM

magnesium chloride and 100 mM sodium chloride. The thus prepared solution was mixed with 10 units of EcoRV and 10 units of HindIII and incubated at 37°C for 1 hour. The resulting reaction mixture was subjected to agarose gel electrophoresis and about 0.2 µg of a pPCVLhCK1 DNA fragment of about 0.6 kb was recovered which contained the human immunoglobulin L chain constant region gene cleaved with EcoRV and HindIII.

Next, 0.1 µg of the pIg1SEId4 EcoRV-HindIII fragment (about 9.2 kb) and 0.1 µg of the pchCKC1 EcoRV-HindIII fragment (about 0.6 kb) prepared above were dissolved in 20 µl of the T4 ligase buffer. The resulting solution was mixed with 350 units of T4 DNA ligase and incubated at 4°C for 24 hours. Using the thus obtained recombinant plasmid DNA, transformation of E. coli HB101 was carried out to obtain a plasmid pChiIgLA1 (Fig. 21) as a cassette vector for use in the construction of a humanized chimera antibody L chain expression vector.

Example 2

Anti-GD₃ chimera antibody

1. Preparation of mRNA from a hybridoma cell line capable of producing mouse anti-GD₃ monoclonal antibody KM-641

Using a mRNA extraction kit, Fast Track (No. K1593-02, available from Invitrogen), 34 µg of mRNA was prepared from 1 x 10⁸ cells of a hybridoma cell line which is capable of producing mouse anti-GD₃ monoclonal antibody KM-641 prepared by the method described later in Reference Example shown below.

2. Preparation of cDNA libraries of KM-641 H chain and L chain

genes

Using ZAP-cDNA Synthesis Kit (No. sc200400), a cDNA synthesis kit available from Stratagene Cloning Systems, cDNA having EcoRI adaptor on its 5'-end and XhoI adaptor on its 3'-
5 end was prepared from 3 µg of the mRNA obtained in the above procedure 1. About 6 µg of the cDNA was dissolved in 10 µl of sterile water and subjected to agarose gel electrophoresis to recover 0.1 µg of an H chain-corresponding cDNA fragment of about 1.8 kb and 0.1 µg of an L chain-corresponding cDNA
10 fragment of about 1.0 kb. Next, 0.1 µg of the 1.8 kb cDNA fragment, 0.1 µg of the 1.0 kb cDNA fragment and 1 µg of Uni-ZAP XR (available from Stratagene Cloning Systems; a preparation obtained by digesting Lambda ZAPII vector with EcoRI and XhoI, followed by treatment with calf intestine
15 alkaline phosphatase) to be used as a vector were dissolved in 11.5 µl of the T4 ligase buffer, and the resulting solution was mixed with 175 units of T4 DNA ligase and incubated at 12°C for 10 hours and then at room temperature for 2 hours. A 4 µl portion of the resulting reaction mixture was subjected to
20 lambda phage packaging using Giga Pack Gold (Stratagene Cloning Systems) in accordance with the conventional method (Maniatis et al., Molecular Cloning, 1989, p2.95). An E. coli strain PLK-F was infected with the thus packaged product in accordance
25 with the conventional method (Maniatis et al., Molecular Cloning, 1989, p2.95-107) to obtain an H chain cDNA library and an L chain cDNA library, each containing about 10,000 phage

clones. Next, these phage particles were fixed on nitrocellulose filters in accordance with the conventional method (Maniatis et al., Molecular Cloning, 1989, p2.112).

3. Cloning of monoclonal antibody KM-641 H chain and L chain cDNA

Two ^{32}P -labeled probes were prepared from an EcoRI fragment of about 6.8 kb containing a mouse immunoglobulin constant region chromosomal gene Cg1 (Roeder et al., Proc. Natl. Acad. Sci. U.S.A., 78, 474 (1981)) and a mouse Ck gene-
10 containing HindIII-BamHI fragment of about 3 kb (Sakano et al., Nature, 280, 288 (1979)). A phage clone which showed strong reaction at 65°C with one of these two probes were obtained from each of the H chain cDNA library and the L chain cDNA library prepared in the above procedure 2 in accordance with
15 the conventional method (Maniatis et al., Molecular Cloning, 1989, p2.108). Next, using ZAP-cDNA Synthesis Kit (No. sc200400), a cDNA synthesis kit of Stratagene Cloning Systems each of the thus obtained phage clones was introduced into plasmid pBluescript to isolate a recombinant plasmid pKM641HA3
20 containing the KM-641 H chain cDNA and a recombinant plasmid pKM641LA2 containing the KM-641 L chain cDNA. When each of the plasmids pKM641HA3 and pKM641LA2 was digested with EcoRI and
25 XhoI, it was found that cDNA of about 1.6 kb had been inserted into the former plasmid, and cDNA of about 0.9 kb into the latter (Fig. 22).

4. Immunoglobulin variable region base sequences of KM-641 H

chain cDNA (pKM641HA3) and KM-641 L chain cDNA (pKM641LA2).

Immunoglobulin variable region base sequences of the plasmids pKM641HA3 and pKM641LA2 obtained in the above procedure 3 were determined by the dideoxy method (Maniatis *et al.*, Molecular Cloning, 1989, p13.42) using Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical Corporation). The results are shown in SEQ ID NO:4 and SEQ ID NO:5. The amino acid sequence as defined by from the first residue to the 120th residue in SEQ ID NO:4 corresponds to the heavy chain variable region of KM-641 and the amino acid sequence as defined by from the first residue to the 108th residue in SEQ ID NO:5 corresponds to the light chain variable region of KM-641.

10 The plasmid pKM641LA2 was a complete cDNA containing a leader sequence and having a methionine-corresponding sequence which was assumed to be the initiation codon ATC located close to the 5'-end. The plasmid pKM641HA3, on the other hand, did not have such a methionine-corresponding initiation codon-like sequence on its 5'-end side, and its leader sequence was partially deficient.

15 5. Construction of KM-641 chimera H chain expression vector

H chain variable region gene obtained by cleaving the plasmid pKM641HA3 variable region at the 5'-end *AluI* site and 3'-end *SlyI* site was ligated with the cassette vector for use in the construction of the humanised chimera antibody H chain obtained in Example 1 using the synthetic DNA sequences shown in SEQ ID NO:6 and SEQ ID NO:7, thereby constructing a humanised chimera antibody H chain vector pchi641HA1 (Fig. 23).

20

Firstly, the DNA shown in SEQ ID NO:7 (see Fig. 23) was synthesised using a DNA synthesizer. This synthetic DNA comprises a base sequence derived from plasmid pKM641HA3



10043

36 9

[N-Mou]00407:KEI

ranging from the 3'-end of its immunoglobulin H chain variable region to a StyI cleavage site in the vicinity of the 3'-end and a base sequence derived from plasmid pAGE28 ranging from the 5'-end of its immunoglobulin H chain constant region to an
5 ApaI cleavage site in the vicinity of the 5'-end. Thus, the synthetic DNA has a StyI cleavage site and an ApaI cleavage site on both of its end. Next, the thus synthesized DNA was introduced into the plasmid pKM641HA3 in the following manner.

A 3 µg portion of the plasmid pKM641HA3 was dissolved
10 in 30 µl of a buffer solution containing 50 mM Tris-HCl (pH 7.5), 10 mM magnesium chloride, 50 mM sodium chloride and 1 mM DTT. The thus prepared solution was mixed with 10 units of EcoRI and 10 units of StyI and incubated at 37°C for 4 hours. The resulting reaction mixture was subjected to agarose gel
15 electrophoresis and about 0.3 µg of a DNA fragment of about 0.41 kb was recovered. Next, a 3 µg portion of pAGE28 (Mizukami et al., J. Biochem., 101, 1307-1310 (1987)) was dissolved in 30 µl of a buffer solution containing 10 mM Tris-HCl (pH 7.5), 7 mM magnesium chloride and 6 mM 2-
20 mercaptoethanol. The thus prepared solution was mixed with 10 units of EcoRI and 10 units of ApaI and incubated at 37°C for 4 hours. The resulting reaction mixture was subjected to agarose gel electrophoresis and about 2 µg of a DNA fragment of
25 about 2.45 kb was recovered. Next, 0.1 µg of the pKM641HA3 EcoRI-StyI fragment (about 0.41 kb) and 0.1 µg of the pAGE28 EcoRI-ApaI fragment (about 2.45 kb) prepared above and 0.3 µg

of the synthetic DNA of SEQ ID NO:7 were dissolved in 20 µl of the T4 ligase buffer solution. The resulting solution was mixed with 350 units of T4 DNA ligase and incubated at 4°C for 24 hours. Using the thus obtained recombinant plasmid DNA, transformation of E. coli HB101 was carried out to obtain a plasmid pKM641HE1 as shown in Fig. 24.

Since the thus constructed plasmid pKM641HE1 lacks a leader sequence, the following attempt was made to supplement the plasmid with the leader sequence using the synthetic DNA of SEQ ID NO: 6.

A 3 µg portion of the plasmid pKM641HE1 was dissolved in 30 µl of a buffer solution containing 10 mM Tris-HCl (pH 7.5), 7 mM magnesium chloride and 6 mM 2-mercaptoethanol. The thus prepared solution was mixed with 10 units of EcoRI and 10 units of ApaI and incubated at 37°C for 4 hours. The resulting reaction mixture was subjected to agarose gel electrophoresis and about 0.4 µg of a DNA fragment of about 0.42 kb was recovered. Next, a 0.4 µg portion of the thus prepared pKM641HE1 EcoRI-ApaI fragment (about 0.42 kb) was dissolved in 30 µl of a buffer solution containing 10 mM Tris-HCl (pH 7.5), 7 mM magnesium chloride, 50 mM sodium chloride and 6 mM 2-mercaptoethanol. The thus prepared solution was mixed with 10 units of AluI and incubated at 37°C for 4 hours. The resulting reaction mixture was subjected to phenol-chloroform extraction and ethanol precipitation and about 0.3 µg of a DNA fragment of about 0.4 kb was recovered.

Next, 0.1 µg of the pKM641HE1 AluI-ApaI fragment (about 0.4 kb) and 0.1 µg of the pAGE28 EcoRI-ApaI fragment (about 2.45 kb) prepared above and 0.3 µg of the synthetic DNA of SEQ ID NO:6 were dissolved in 20 µl of the T4 ligase buffer solution. The resulting solution was mixed with 350 units of T4 DNA ligase and incubated at 4°C for 24 hours. Using the thus obtained recombinant plasmid DNA, transformation of E. coli HB101 was carried out to obtain a plasmid pKM641HF1 as shown in Fig. 25.

Next, immunoglobulin H chain variable region of the thus obtained plasmid pKM641HF1 was introduced into the aforementioned cassette vector pChiIgHB2 in the following manner.

A 3 µg portion of the plasmid pKM641HF1 was dissolved in 30 µl of a buffer solution containing 10 mM Tris-HCl (pH 7.5), 7 mM magnesium chloride and 6 mM 2-mercaptoethanol. The thus prepared solution was mixed with 10 units of EcoRI and 10 units of ApaI and incubated at 37°C for 4 hours. The resulting reaction mixture was subjected to agarose gel electrophoresis and about 0.5 µg of a DNA fragment of about 0.44 kb was recovered. Next, a 3 µg portion of the pChiIgHB2 was dissolved in 30 µl of a buffer solution containing 10 mM Tris-HCl (pH 7.5), 7 mM magnesium chloride and 6 mM 2-mercaptoethanol. The thus prepared solution was mixed with 10 units of EcoRI and 10 units of ApaI and incubated at 37°C for 4 hours. The resulting reaction mixture was subjected to phenol-chloroform extraction

and ethanol precipitation and about 3 µg of DNA was recovered. Next, 0.1 µg of the pKM641HF1 EcoRI-ApaI fragment (about 0.44 kb) and 0.1 µg of the pChiIgHB2 EcoRI-ApaI fragment (about 10.1 kb) prepared above were dissolved in 20 µl of the T4 ligase buffer. The resulting solution was mixed with 350 units of T4 DNA ligase and incubated at 4°C for 24 hours. Using the thus obtained recombinant plasmid DNA, transformation of E. coli HB101 was carried out to obtain a plasmid pChi641HA1 as shown in Fig. 26.

Next, KM50-derived immunoglobulin H chain promoter and enhancer regions of the thus obtained plasmid pChi641HA1 were converted into MoLTR in the following manner.

A 3 µg portion of the plasmid pChi641HA1 was dissolved in 30 µl of a buffer solution containing 50 mM Tris-HCl (pH 7.5), 10 mM magnesium chloride, 50 mM sodium chloride and 1 mM DTT. The thus prepared solution was mixed with 10 units of EcoRI and 10 units of XhoI and incubated at 37°C for 4 hours. The resulting reaction mixture was subjected to agarose gel electrophoresis and about 0.2 µg of a DNA fragment of about 8.8 kb was recovered. Next, a 3 µg portion of the pPMOL3 prepared in procedure 2 of Example 1 was dissolved in 30 µl of a buffer solution containing 50 mM Tris-HCl (pH 7.5), 10 mM magnesium chloride, 50 mM sodium chloride and 1 mM DTT. The thus prepared solution was mixed with 10 units of EcoRI and 10 units of XhoI and incubated at 37°C for 4 hours. The resulting reaction mixture was subjected to agarose gel electrophoresis

and about 0.3 µg of a DNA fragment of about 0.63 kb containing MoLTR was recovered. Next, 0.1 µg of the pChi641HA1 EcoRI-XhoI fragment and 0.1 µg of the pPMOL3 EcoRI-XhoI fragment prepared above were dissolved in 20 µl of the T4 ligase buffer solution.

5 The resulting solution was mixed with 100 units of T4 DNA ligase and incubated at 4°C for 24 hours. Using the resulting reaction mixture, transformation of E. coli HB101 was carried out to obtain a plasmid pChi641HAM1 (Fig. 27) as a KM-641 chimera H chain expression vector.

10 6. Construction of KM-641 chimera L chain expression vector

L chain variable region gene obtained by cleaving the plasmid pKM641LA2 variable region gene at its 5'-end EcoRI site and 3'-end HindIII site was ligated with the cassette vector for the expression of chimera L chain, using the synthetic DNA
15 shown in SEQ ID NO: 8, thereby constructing an L chain expression vector pchi641LG11 (Fig. 28).

Firstly, the DNA of SEQ ID NO: 8 (see Fig. 29) was synthesized using a DNA synthesizer. This synthetic DNA comprises a base sequence corresponding to a region of the
20 plasmid pKM641LA2 ranging from the 3'-end of the immunoglobulin L chain variable region to a HindIII cleavage site in the vicinity of the 3'-end and a base sequence corresponding to a
region of the plasmid pChiIgLA1 ranging from the 5'-end to an
30 EcoRV cleavage site in the vicinity of the 5'-end. Thus, it has a HindIII cleavage site and an EcoRV cleavage site on both
ends. Next, the thus synthesized DNA was introduced into the

plasmid pKM641LA2 in the following manner.

A 3 µg portion of the plasmid pKM641LA2 was dissolved in 30 µl of a buffer solution containing 10 mM Tris-HCl (pH 7.5), 7 mM magnesium chloride, 50 mM sodium chloride and 6 mM 2-mercaptoethanol. The thus prepared solution was mixed with 10 units of EcoRI and 10 units of HindIII and incubated at 37°C for 4 hours. The resulting reaction mixture was subjected to agarose gel electrophoresis and about 0.3 µg of a DNA fragment of about 0.35 kb was recovered. Next, a 3 µg portion of pChiIgLA1 was dissolved in 30 µl of a buffer solution containing 50 mM Tris-HCl (pH 7.5), 10 mM magnesium chloride, 50 mM sodium chloride and 1 mM DTT. The thus prepared solution was mixed with 10 units of EcoRI and 10 units of EcoRV and incubated at 37°C for 4 hours. The resulting reaction mixture was subjected to phenol-chloroform extraction and ethanol precipitation and about 3 µg of DNA was recovered and dissolved in 10 µl of the TE solution (a buffer solution containing 10 mM Tris-HCl and 1 mM EDTA (pH 7.5)). Next, 0.1 µg of the pKM641LA2 EcoRI-HindIII fragment (about 0.35 kb) and 0.1 µg of the pChiIgLA1 EcoRI-EcoRV fragment (about 9.7 kb) prepared above and 0.3 µg of the synthetic DNA of SEQ ID NO:8 were dissolved in 20 µl of the T4 ligase buffer solution. The resulting solution was mixed with 350 units of T4 DNA ligase and incubated at 4°C for 24 hours. Using the thus obtained recombinant plasmid DNA, transformation of E. coli HB101 was carried out to obtain a plasmid pChi641LG11 as shown in Fig. 29.

Next, KM50-derived immunoglobulin H chain promoter and enhancer regions of the thus obtained plasmid pChi641LG11 were converted into MoLTR in the following manner.

A 3 µg portion of the plasmid pChi641LG11 was dissolved
5 in 30 µl of a buffer solution containing 50 mM Tris-HCl (pH 7.5), 10 mM magnesium chloride, 50 mM sodium chloride and 1 mM DTT. The thus prepared solution was mixed with 10 units of EcoRI and 10 units of XhoI and incubated at 37°C for 4 hours. The resulting reaction mixture was subjected to agarose gel
10 electrophoresis and about 0.2 µg of a DNA fragment of about 8.3 kb was recovered. Next, 0.1 µg of the pChi641LG11 EcoRI-XhoI fragment and 0.1 µg of the pPMOL3 EcoRI-XhoI fragment prepared above were dissolved in 20 µl of the T4 ligase buffer. The resulting solution was mixed with 175 units of T4 DNA ligase
15 and incubated at 4°C for 24 hours. Using the resulting reaction mixture, transformation of E. coli HB101 was carried out to obtain a plasmid pChi641LGM11 (Fig. 30) as a KM-641 chimera L chain expression vector.

7. Expression of anti-GD₃ chimera antibody in SP2/0 cells

20 Introduction of plasmid into SP2/0 cells was carried out making use of the electroporation technique in accordance with the method of Miyaji et al. (Cytotechnology, 3, 133 - 140 (1990)).

25 The plasmids pChi641LG11 and pChi641HA1 (2 µg for each), or the plasmids pChi641LGM11 and pChi641HAM1 (2 µg for each), were simultaneously introduced into 2 x 10⁶ of SP2/0

cells, and the resulting cells were suspended in 40 ml of RPMI1640-FCS(10) which has been prepared by supplementing RPMI1640 medium (Nissui Pharmaceutical Co., Ltd.) with 10% of FCS, 1/40 volume of 7.5% NaHCO_3 , 3% of 200 mM L-glutamine solution (available from GIBCO) and 0.5% of a penicillin-streptomycin solution (GIBCO, a solution containing 5,000 units/ml of penicillin and 5,000 units/ml of streptomycin). The thus prepared cell suspension was distributed in 200 μl -portions into wells of a 96-well microtiter plate (Flow Laboratories), and the cells were cultured at 37°C in a CO_2 incubator. After 24 hours of the culturing, G418 (GIBCO) was added to the cell suspension to a final concentration of 0.5 mg/ml, and the culturing was continued for 1 to 2 weeks. When transformant colonies were developed and grown into confluent stages, culture broths were recovered from the wells to measure anti-GD₃ chimera antibody activities by ELISA method in the following manner.

<Enzyme immunoassay (ELISA)>

A 2 ng portion of GD₃ (available from Iatron) or other type of ganglioside was dissolved in 2 μl of ethanol solution containing 5 ng of phosphatidylcholine (Sigma Chemical Co.) and 2.5 ng of cholesterol (Sigma Chemical Co.). A 20 μl portion of the thus prepared solution or the same volume of its dilution solution was distributed into each well of a 96-well microtiter plate (available from Greiner). After air-drying, blocking was effected with PBS containing 1% BSA. To each well was added 50

to 100 μ l of a culture supernatant of a transformant, a purified mouse monoclonal antibody solution or a purified chimera antibody solution. After reaction at 4°C for 10 hours, each well was washed with PBS and charged with 50 to 100 μ l of
5 peroxidase-labeled protein A (Funakoshi Pharmaceutical Co., Ltd.), followed by 1 to 2 hours of reaction at room temperature. After washing with PBS, 50 to 100 μ l of ABTS substrate solution prepared by dissolving 550 mg of diammonium 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) in 0.1 M
10 citrate buffer (pH 4.2) and adding 1 μ l/ml of hydrogen peroxide to the resulting solution just before its use was added to each well to develop color, and OD₄₁₅ of the reaction mixture was measured.

Among clones thus obtained, culture broth of a clone
15 having the highest activity measured by ELISA method contained anti-GD₃ chimera antibody in an amount of about 0.1 μ g/ml.

The clone having anti-GD₃ chimera antibody activity was suspended in the aforementioned RPMI1640-FCS(10) medium supplemented with 0.5 mg/ml of G418 and 50 nM of methotrexate
20 (to be referred to as "MTX" hereinafter) to a final cell density of 1-2 x 10⁵ cells /ml. The thus prepared cell suspension was distributed in 2-ml portions into wells of a 24 well plate, and the cells were cultured at 37°C for 2 to 3 weeks in a CO₂ incubator to induce clones resistant to 50 nM
25 MTX. When the thus induced clones were grown into confluent stages, anti-GD₃ chimera antibody activities in the culture

broths were measured by the ELISA method. Among clones thus obtained, culture broth of a 50 nM MTX-resistant clone having the highest activity measured by ELISA method contained anti-GD₃ chimera antibody in an amount of about 0.3 µg/ml.

5 The 50 nM MTX-resistant clone was suspended in the RPMI1640-FCS(10) medium supplemented with 0.5 mg/ml of G418 and 200 nM of MTX to a final cell density of $1-2 \times 10^5$ cells /ml. The thus prepared cell suspension was distributed in 2-ml portions into wells of a 24 well plate, and the cells were
10 cultured at 37°C for 2 to 3 weeks in a CO₂ incubator to induce clones resistant to 200 nM MTX. When the thus induced clones were grown into confluent stages, anti-GD₃ chimera antibody activities in the culture broths were measured by the ELISA method. Among clones thus obtained, culture broth of a 200 nM
15 MTX-resistant clone having the highest activity measured by ELISA method contained anti-GD₃ chimera antibody in an amount of about 2 µg/ml. This 200 nM MTX-resistant clone was named transformant KM-871.

20 Expression of anti-GD₃ chimera antibody protein in the transformant KM-871 was confirmed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in the following manner.

25 The transformant KM-871 was suspended in GIT medium (Nihon Seiyaku Co., Ltd.) supplemented with 0.5 mg/ml of G418 and 200 nM of MTX to a final cell density of $1-2 \times 10^5$ cells /ml. The thus prepared cell suspension was distributed in 100-ml portions in 175 cm² flasks (available from Greiner), and the

cells were cultured at 37°C for 3 to 5 days in a CO₂ incubator. When the cells were grown into confluent stage, the resulting culture broth (about 900 ml) was recovered and subjected to salting-out with 50% ammonium sulfate. Using Affigel Protein
5 A MAPS-II Kit (Bio-Rad Laboratories), about 100 µg of purified anti-GD₃ chimera antibody KM-871 was obtained. About 5 µg of the thus purified anti-GD₃ chimera antibody KM-871 was subjected to electrophoresis in accordance with the conventional method (Laemmli, Nature, 227, 680 (1970)) to check
10 its molecular weight. The results are shown in Fig. 31. As shown in the figure, under reductive conditions, molecular weights of the chimera H chain and the chimera L chain were found to be about 50 kilodaltons and about 25 kilodaltons, respectively, thus confirming expression of the H and L chains
15 having correct molecular weights. Under non-reductive conditions, molecular weight of the chimera antibody was found to be about 150 kilodaltons which confirmed expression of the correct size antibody consisting of two H chains and two L chains.

20 8. Reaction specificity of anti-GD₃ chimera antibody KM-871

Reactivities of the anti-GD₃ chimera antibody with ganglioside GM₁, N-acetyl GM₂ (Boehringer-Mannheim Corp.), N-glycolyl GM₂, N-acetyl GM₃, N-glycolyl GM₃, GD_{1a}, GD_{1b} (Iatron),
GD₂, GD₃ (Iatron), GT_{1b} (Funakoshi Pharmaceutical Co., Ltd.) and
25 GQ_{1b} (Iatron) were measured by the ELISA method. In this instance, GM₁ and GD_{1a} were purified from bovine brain, N-

glycolyl GM₂ and N-glycolyl GM₃ from mouse liver, N-acetyl GM₃ from dog erythrocytes and GD₂ from a cultured cell line IMR32 (ATCC CCL127), in accordance with the conventional method (J. Biol. Chem., 263, 10915 (1988)). The results are shown in
5 Table 1.

Table 1

	<u>Ganglioside</u>	<u>Binding activity of antibody (OD₄₁₅)</u>	
		<u>Anti-GD₃ chimera antibody (0.3 µg/ml)</u>	<u>Mouse anti-GD₃ antibody (0.4 µg/ml)</u>
10	N-acetyl GM ₃	0.007	0.006
	N-glycolyl GM ₃	0	0
	N-acetyl GM ₂	0	0
	N-glycolyl GM ₂	0	0
	GM ₁	0	0
15	GD ₂	0	0
	GD ₃	0.717	1.33
	GD _{1a}	0	0
	GD _{1b}	0	0
	GT _{1b}	0	0
20	GQ _{1b}	0	0.16

As shown in Table 1, anti-GD₃ chimera antibody KM-871 and mouse anti-GD₃ antibody KM-641 reacted only with GD₃, thus showing no changes in the reaction specificity by the chimera formation.

25 9. Reactivity of anti-GD₃ chimera antibody KM-871 by

fluorescent antibody technique

The cultured human malignant melanoma SK-MEL-28 (ATCC HTB72) and G361 cells (JCRB) both of which produced ganglioside GD₃ were placed in a microtube (Treff) to give a cell number of 1 x 10⁶ cells per tube and washed by centrifugation (1,200 rpm, 5 minutes) with PBS. 50 µl of anti-GD₃ chimera antibody KM-871 (10 µg/ml) was added to the microtube and the mixture was allowed to react at 4°C for 30 minutes. Thereafter, the cells were washed three times by centrifugation (1,200 rpm, 5 minutes) with PBS, then 20 µl of fluorescein isocyanate-labeled Protein A (Boehringer Mannheim-Yamanouchi, 30-fold diluted) was added and, after stirring, the mixture was allowed to react at 4°C for 30 minutes. Thereafter, the cells were washed three times by centrifugation (1,200 rpm, 5 minutes) with PBS, further suspended in PBS, and submitted for analysis using FCS-1 flow cell sorter (Nippon Bunko).

Control tests without the addition of KM-871 were performed by the same analytical procedure as mentioned above.

The results are shown in Fig. 32. The fluorescence intensity peak for KM-871 showed shifting to the right (increased fluorescence intensity) as compared with the control, indicating that this antibody had reacted directly with ganglioside GD₃ on the surface of the SK-MEL-28 and G361 cells.

10. In vitro antitumor effect of anti-GD₃ chimera antibody KM-871 (complement-dependent cytotoxicity: CDC)

(a) Preparation of target cells

Suspensions of the target cells, namely SK-MEL-28 cells and G361 cells, in RPMI-1640 medium supplemented with 10% FCS were respectively adjusted to a cell concentration of 1×10^7 cells/ml, $\text{Na}_2^{51}\text{CrO}_4$ was added to a concentration of 100 $\mu\text{Ci}/1 \times 10^7$ cells, reaction was performed at 37°C for 1 hour and, thereafter, the cells were washed three times with the medium. The cells were allowed to stand in the medium at 4°C for 30 minutes for spontaneous dissociation and then centrifuged (1,200 rpm, 5 minutes), and the medium was added to adjust the cell concentration to 4×10^6 cells/ml.

(b) Preparation of complement

Serum from three healthy subjects were mixed to serve as a source of human complement.

(c) CDC activity measurement

To U-bottomed 96-well plates was added anti-GD₃ chimera antibody KM-871 or anti-GD₃ mouse antibody KM-641 to final concentrations within the range of 0.05 $\mu\text{g}/\text{ml}$ to 50 $\mu\text{g}/\text{ml}$. To each well were added 2×10^5 target cells. Reaction was performed at room temperature for 1 hour. The supernatants were removed by centrifugation (1,200 rpm, 5 minutes), the complement solution prepared as described above under (b) was added in 150- μl portions (final concentration of 15 v/v%), and reaction was performed at 37°C for 1 hour. After centrifugation (1,200 rpm, 5 minutes), the amount of ^{51}Cr in each supernatant was determined using a γ -counter. The amount

of ^{51}Cr resulting from spontaneous dissociation was determined by adding to target cells the medium alone in place of the antibody and complement solution and determining the amount of ^{51}Cr in the supernatant in the same manner as described above.

5 The total amount of free ^{51}Cr was determined by adding 5 N sodium hydroxide in place of the antibody and complement solution, proceeding as described above, and determining the amount of ^{51}Cr in the supernatant.

The CDC activity was calculated as follows:

10 CDC activity (%) =

$$\frac{\text{Amount of } ^{51}\text{Cr in sample supernatant} - \text{Amount of } ^{51}\text{Cr resulting from spontaneous dissociation}}{\text{Total amount of free } ^{51}\text{Cr} - \text{Amount of } ^{51}\text{Cr resulting from spontaneous dissociation}} \times 100$$

15

The results are shown in Fig.33. It was found from the results that chimera antibody KM-871 showed strong cytotoxicity against the SK-MEL-28 and G361 cells as compared to mouse antibody KM-641, which indicates that chimera antibody KM-871 would be clinically more useful than mouse antibody KM-641.

11. In vitro antitumor effect of anti-GD₃ chimera antibody KM-871 (antibody-dependent cell-mediated cytotoxicity:ADCC)

(a) Preparation of target cells

25 The target SK-MEL-28 and G361 cells were prepared in the same manner as described above under 10 (a).

(b) Preparation of effector cells

50 ml of human venous blood was collected, 0.5 ml of heparin sodium (Takeda Chemical Industries, 1,000 units/ml) was added, and the mixture was stirred gently and then centrifuged (1,500 to 1,800 g, 15 minutes) using Polymorphprep (Nycomed
5 Pharma AS). The layer of lymphocytes and polymorphonuclear leukocytes was separated, and the cells were washed three times by centrifugation (1,500 to 1,800 g, 15 minutes) with RPMI-1640 medium and suspended in RPMI-1640 medium supplemented with 10% FCS (5×10^6 cells/ml) for use as effector cells.

10 (c) ADCC activity measurement

To U-bottomed 96-well plates was added anti-GD₃ chimera antibody KM-871 or anti-GD₃ mouse antibody KM-641 in 50- μ l portions to final concentrations of 10 μ g/ml. To each well were added 100 μ l of target cells (2×10^5 cells) and 50 μ l of
15 effector cells (5×10^5 cells) so that the ratio of effector cells to target cells should be 50:1 or 100:1. Reaction was performed at 37°C for 4 hours, followed by centrifugation (1,200 rpm, 5 minutes). The amount of ^{51}Cr in each supernatant was determined using a γ -counter. The amount of ^{51}Cr resulting
20 from spontaneous dissociation was determined by adding to target cells the medium alone in place of the antibody and effector cells and measuring the amount of ^{51}Cr in the supernatant in the same manner as described above. The total
amount of free ^{51}Cr was determined by adding 5 N sodium
25 hydroxide in place of the antibody and effector cells, proceeding as described above, and determining the amount of

^{51}Cr in the supernatant.

The ADCC activity was calculated as follows:

ADCC activity (%) =

$$\frac{\text{Amount of } ^{51}\text{Cr in sample supernatant} - \text{Amount of } ^{51}\text{Cr resulting from spontaneous dissociation}}{\text{Total amount of free } ^{51}\text{Cr} - \text{Amount of } ^{51}\text{Cr resulting from spontaneous dissociation}} \times 100$$

As a control, the medium was added in place of the antibodies, the procedure mentioned above was followed, and the amount of ^{51}Cr in the control supernatant was determined for ADCC activity calculation.

The results are shown in Fig. 34. In both cases of using lymphocytes and polymorphonuclear leukocytes as effector cells, chimera antibody KM-871 showed strong antibody-dependent cell-mediated cytotoxicity against the G361 cells as compared to mouse antibody KM-641, which indicates that chimera antibody KM-871 would be clinically more useful than mouse antibody KM-641.

12. In vivo therapeutic effect of anti-GD₃ chimera antibody KM-871 (therapeutic effect on transplanted tumors)

Human malignant melanoma G361 cells (1×10^7 cells) were intracutaneously transplanted to abdominal parts of Balb/c nu/nu mice (5 to 7 animals/group). Anti-GD₃ chimera antibody KM-871 (100 $\mu\text{g}/\text{animal}$) was intravenously administered into mice

four times starting from the next day of the transplantation of the tumor cells. To the mice of the control group, 100 µg of anti-GD₃ mouse antibody KM-641 or anti-Sialyl Le^a monoclonal antibody AMC-462 (ECACC 86050801) was intravenously administered five times starting from the day of the transplantation. The therapeutic effect on transplanted tumor cells was determined in terms of tumor size (volume) calculated by the following equation.

$$\text{Tumor size (mm}^3\text{)} = 0.4 \times a \times b^2$$

a: major axis

b: minor axis

The results are shown in Fig. 35. As shown in Fig. 35, remarkable growth of tumors was observed in the control group to which AMC-462 was administered, while the growth of tumors was significantly suppressed in the group to which KM-641 was administered. KM-871 showed further stronger therapeutic effect so that the establishment of tumors was completely inhibited 65 days after the transplantation.

Reference Example 1

(1) Preparation of antigen

In 30 ml of chloroform/methanol (2/1) solution were dissolved 5 µg of ganglioside GD₃ having NeuAcα2→8NeuAcα2→3Gal sugar chain on its non-reducing end (Iatron), 0.5 µmol of dipalmitoylphosphatidylcholine (Sigma Chemical Co.), 0.5 µmol

of cholesterol (Nakalai Tesque), 0.05 μ mol of dipalmitoylphosphatidylc acid (Sigma Chemical Co.) and 2.5 μ g of Lipid A (Funakoshi Pharmaceutical Co., Ltd.). The thus prepared solution was warmed at 45°C to remove solvents, 5 thereby obtaining a uniform lipid thin film. After completely removing solvents by sucking the film for 1 hour using a vacuum pump, the resulting film was mixed with 0.5 ml of PBS solution and stirred at 45°C to obtain an antigen solution.

(2) Preparation of antibody-producing cells

10 Mice was immunized by administering 0.5 ml of the antigen solution obtained in the above step (1) into the caudal vein once every week for 7 weeks. For further immunization, ganglioside GD₃-positive SK-MEL-28 (ATCC HTB 72) cells (1×10^7 cells) were intraperitoneally administered once every week for 15 three weeks. On the third day after the last administration, spleen cells were prepared from each mouse for use in the following cell fusion.

(3) Preparation of mouse myeloma cells

20 A mouse myeloma cell line P3-U1 having 8-azaguanine resistance was cultured in normal medium (RPMI1640 medium containing 10% fetal calf serum (FCS)) to obtain 2×10^7 or more cells for use in the following cell fusion as parent cells.

.... (4) Preparation of hybridoma

25 The spleen cells and myeloma cells obtained in the above steps (2) and (3), respectively, were used in ratio of

10:1 and subjected to cell fusion in accordance with the
aforementioned procedure. After culturing at 37°C for 14 days
in HAT medium (prepared by supplementing normal medium with
hypoxanthin (10^{-4} M), thymidine (1.5×10^{-5} M) and aminopterin
5 (4×10^{-7} M)) under an atmosphere of 5% CO₂, fused cells were
selected and cultured in HT medium (HAT medium minus
aminopterin). Then, active wells were selected by assaying
the antibody titers against ganglioside GD₃, and after changing
to normal medium, cloning was repeated twice. Thereafter,
10 hybridomas which showed specific reaction with ganglioside GD₃
were selected by enzyme immunoassay or immunohistological
evaluation (ABC method). That is, 2 ng of ganglioside GM₃
(purified from dog erythrocytes in accordance with the method
of Nores et al., J. Immunol., 139, 3171 (1987)) and 2 ng of
15 ganglioside GD₃ (Iatron) were dissolved in 2 ml of ethanol
solution containing 5 ng of phosphatidylcholine (Sigma Chemical
Co.) and 2.5 ng of cholesterol (Sigma Chemical Co.). The thus
prepared solution was distributed in 20-μl portions into wells
of a 96 well microtiter plate (Flow Laboratories), air-dried
20 and then subjected to blocking using 1% BSA-PBS solution. Each
of the resulting hybridoma culture supernatant was distributed
in 50-μl portions into the plate wells carrying a ganglioside
GD₃ adsorbed and the plate carrying ganglioside GM₃ adsorbed
thereon, and the reaction was allowed to proceed at 4°C for 18
25 hours.

After the reaction, a hybridoma strain capable of

producing mouse monoclonal antibody specifically reactive with ganglioside GD₃ but not with ganglioside GM₃ were selected in accordance with the known method (Cancer Res., 46, 4438 (1986)). This mouse monoclonal antibody was named "mouse
5 monoclonal antibody KM-641", and the hybridoma which produces this antibody was named "hybridoma KM-641". The hybridoma KM-641 has been deposited on September 27, 1990, with Fermentation Research Institute, Agency of Industrial Science and Technology, under the Budapest Treaty and has been assigned the
10 designation as FERM BP-3116.

The present invention provides a process for the production of humanized chimera antibody wherein the chimera antibody can be produced easily without changing any one of amino acids of its mouse antibody variable region, as well as
15 a humanized chimera antibody specific for ganglioside GD₃.

While the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the
20 spirit and scope of the invention.

SEQUENCE LISTING

SEQ ID NO:1:

SEQUENCE TYPE: nucleic acid
SEQUENCE LENGTH: 812 base pairs

STRANDEDNESS: double
TOPOLOGY: linear
MOLECULE TYPE: DNA (genomic)

ORIGINAL SOURCE:
IMMEDIATE EXPERIMENTAL SOURCE
NAME OF CELL LINE: hybridoma KM50

FEATURES:
from 256 to 262 bp TATA signal

AAAGTCAGAC AACTTTGTAG AGTAGGTTCT ATCAATCCTA CTGCAATCCA ACATCACTGA	60
GGACAAATGT TTATACTGAG GAACCTGGTC TTGTGTGATA CGTACTTTCT GTGGGAAGCA	120
GATACGCACT CTCATGTGGC TCCTGAATTT CCCATCAGAG AATGATACAT CTTGAGTCCT	180
AAAATTTAAG TACACCATCA GTGTCAGCAC CTGGTGAGGA AATGCAAATC TCTCCTGGAT	240
CCACCCAACC TTGGGTTGAA AAGCCAAAGC TGGGCCTGGG TACTCACTGG TGTGCAGCC	299
ATG GAC AGG CTT ACT TCC TCA TTC CTA CTG CTG ATG GTC CCT GCA T	345
Met Asp Arg Leu Thr Ser Ser Phe Leu Leu Leu Met Val Pro Ala	
1 5 10 15	
GTGAGTACCA AAGCTTCCTA AGTGATGAAC TGTTCTATCC TCACCTGTTC AAACCTGACC	405
TCCTCCCCTT TGATTTCTCC ACAG AT GTC CTG TCT CAG GTT ACT CTG AAA	455
Tyr Val Leu Ser Gln Val Thr Leu Lys	
-4 1 5	
... GAA TCT GGC CCT GGG ATA TTG CAG CCC TCC CAG ACC CTC AGT CTG ACT	503
Glu Ser Gly Pro Gly Ile Leu Gln Pro Ser Gln Thr Leu Ser Leu Thr	
10 15 20	
... TGC TCT TTC TCT GGG TTT TCA CTG AGC ACT TAT GGT ATG TGT GTG GGC	551
Cys Ser Phe Ser Gly Phe Ser Leu Ser Thr Tyr Gly Met Cys Val Gly	
25 30 35	
TGG ATT CGT CAG TCT TCA GGG AAG GGT CTG GAG TGG CTG GCA AAC GTT	599
Trp Ile Arg Gln Ser Ser Gly Lys Gly Leu Glu Trp Leu Ala Asn Val	
40 45 50	
TGG TGG AGT GAT GCT AAG TAC TAC AAT CCA TCT CTG AAA AAC CGG CTC	647

Trp	Trp	Ser	Asp	Ala	Lys	Tyr	Tyr	Asn	Pro	Ser	Leu	Lys	Asn	Arg	Leu	
55						60					65					
ACA	ATC	TCC	AAG	GAC	ACC	TCC	AAC	AAC	CAA	GCA	TTC	CTC	AAG	ATC	ACC	695
Thr	Ile	Ser	Lys	Asp	Thr	Ser	Asn	Asn	Gln	Ala	Phe	Leu	Lys	Ile	Thr	
70					75					80					85	
AAT	ATG	GAC	ACT	GCA	GAT	ACT	GCC	ATA	TAC	TAC	TGT	GCT	GGG	AGA	GGG	743
Asn	Met	Asp	Thr	Ala	Asp	Thr	Ala	Ile	Tyr	Tyr	Cys	Ala	Gly	Arg	Gly	
				90					95					100		
GCT	ACG	GAG	GGT	ATA	GTG	AGC	TTT	GAT	TAC	TGG	GGC	CAC	GGA	GTC	ATG	791
Ala	Thr	Glu	Gly	Ile	Val	Ser	Phe	Asp	Tyr	Trp	Gly	His	Gly	Val	Met	
			105					110					115			
GTC	ACA	GTC	TCC	TCA	GGTAAG											812
Val	Thr	Val	Ser	Ser												
			120													

SEQ ID NO:2:

SEQUENCE TYPE: 46 base pairs
SEQUENCE LENGTH: nucleic acid

STRANDEDNESS: double
TOPOLOGY: linear
MOLECULE TYPE: other nucleic acid, synthetic DNA

AGCTGAATTC GGGCCCGATA TCAAGCTTGT CGACTCTAGA GGTACC 46

SEQ ID NO:3:

SEQUENCE TYPE: nucleic acid
SEQUENCE LENGTH: 29 base pairs

STRANDEDNESS: double
TOPOLOGY: linear
MOLECULE TYPE: other nucleic acid, synthetic DNA

...
GATGAAGACA GATATCGCAG CCACAGTTC 29

SEQ ID NO:4:

SEQUENCE TYPE: nucleic acid
SEQUENCE LENGTH: 403 base pairs

STRANDEDNESS: double
TOPOLOGY: linear
MOLECULE TYPE: cDNA to mRNA

ORIGINAL SOURCE:

IMMEDIATE EXPERIMENTAL SOURCE
NAME OF CELL LINE: hybridoma KM-641

FEATURES:
from 14 to 43 sig peptide

IDENTIFICATION METHOD: S

AATTCGGCAC	GAG	CTT	GTC	CTT	GTT	TTC	AAA	GGT	GTT	CAG	TGT	GAA	GTG		49	
		Leu	Val	Leu	Val	Phe	Lys	Gly	Val	Gln	Cys	Glu	Val			
		-10					-5					1				
ACG	CTG	GTG	GAG	TCT	GGG	GGA	GAC	TTT	GTG	AAA	CCT	GGA	GGG	TCC	CTG	97
Thr	Leu	Val	Glu	Ser	Gly	Gly	Asp	Phe	Val	Lys	Pro	Gly	Gly	Ser	Leu	
		5					10					15				
AAA	GTC	TCC	TGT	GCA	GCC	TCT	GGA	TTC	GCT	TTC	AGT	CAT	TAT	GCC	ATG	145
Lys	Val	Ser	Cys	Ala	Ala	Ser	Gly	Phe	Ala	Phe	Ser	His	Tyr	Ala	Met	
	20					25					30					
TCT	TGG	GTT	CGC	CAG	ACT	CCG	GCG	AAG	AGG	CTG	GAA	TGG	GTC	GCA	TAT	193
Ser	Trp	Val	Arg	Gln	Thr	Pro	Ala	Lys	Arg	Leu	Glu	Trp	Val	Ala	Tyr	
	35				40					45					50	
ATT	AGT	AGT	GGT	GGT	AGT	GGC	ACC	TAC	TAT	TCA	GAC	AGT	GTA	AAG	GGC	241
Ile	Ser	Ser	Gly	Gly	Ser	Gly	Thr	Tyr	Tyr	Ser	Asp	Ser	Val	Lys	Gly	
			55					60						65		
CGA	TTC	ACC	ATT	TCC	AGA	GAC	AAT	GCC	AAG	AAC	ACC	CTG	TAC	CTG	CAA	289
Arg	Phe	Thr	Ile	Ser	Arg	Asp	Asn	Ala	Lys	Asn	Thr	Leu	Tyr	Leu	Gln	
			70					75					80			
ATG	CGC	AGT	CTG	AGG	TCT	GAG	GAC	TCG	GCC	ATG	TAT	TTC	TGT	ACA	AGA	337
Met	Arg	Ser	Leu	Arg	Ser	Glu	Asp	Ser	Ala	Met	Tyr	Phe	Cys	Thr	Arg	
		85					90					95				
GTT	AAA	CTG	GGA	ACC	TAC	TAC	TTT	GAC	TCC	TGG	GGC	CAA	GGC	ACC	ACT	385
Val	Lys	Leu	Gly	Thr	Tyr	Tyr	Phe	Asp	Ser	Trp	Gly	Gln	Gly	Thr	Thr	
	100					105					110					

CTC ACT GTC TCC TCA GCT
 Leu Thr Val Ser Ser Ala
 115 120

403

SEQ ID NO:5:

SEQUENCE TYPE: nucleic acid
 SEQUENCE LENGTH: 408 base pairs

STRANDEDNESS: double
 TOPOLOGY: linear
 MOLECULE TYPE: cDNA to mRNA

ORIGINAL SOURCE:
 IMMEDIATE EXPERIMENTAL SOURCE
 NAME OF CELL LINE: hybridoma KM-641

FEATURES:
 from 25 to 84 sig peptide

IDENTIFICATION METHOD: S

AATTCGGCAC GAGTCAGCCT GGAC ATG ATG TCC TCT GCT CAG TTC CTT GGT	51
Met Met Ser Ser Ala Gln Phe Leu Gly	
-20 -15	
CTC CTG TTG CTC TGT TTT CAA GGT ACC AGA TGT GAT ATC CAG ATG ACA	99
Leu Leu Leu Leu Cys Phe Gln Gly Thr Arg Cys Asp Ile Gln Met Thr	
-10 -5 1 5	
CAG ACT GCA TCC TCC CTG CCT GCC TCT CTG GGA GAC AGA GTC ACC ATC	147
Gln Thr Ala Ser Ser Leu Pro Ala Ser Leu Gly Asp Arg Val Thr Ile	
10 15 20	
AGT TGC AGT GCA AGT CAG GAC ATT AGT AAT TAT TTA AAC TGG TAT CAA	195
Ser Cys Ser Ala Ser Gln Asp Ile Ser Asn Tyr Leu Asn Trp Tyr Gln	
25 30 35	
... CAG AAA CCA GAT GGA ACT GTT AAA CTC CTG ATC TTT TAC TCA TCA AAT	243
Gln Lys Pro Asp Gly Thr Val Lys Leu Leu Ile Phe Tyr Ser Ser Asn	
40 45 50	
... TTA CAC TCG GGA GTC CCA TCA AGG TTC AGT GGC GGT GGG TCC GGG ACA	291
Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly Gly Gly Ser Gly Thr	
55 60 65	
... GAT TAT TCT CTC ACC ATC AGC AAC CTG GAG CCT GAA GAT ATT GCC ACT	339
Asp Tyr Ser Leu Thr Ile Ser Asn Leu Glu Pro Glu Asp Ile Ala Thr	
70 75 80 85	
TAC TTT TGT CAT CAG TAT AGT AAG CTT CCG TGG ACG TTC GGT GGA GGC	387

Tyr Phe Cys His Gln Tyr Ser Lys Leu Pro Trp Thr Phe Gly Gly Gly
90 95 100

ACC AAG CTG GAA ATC AAA CGG
Thr Lys Leu Glu Ile Lys Arg
105

408

SEQ ID NO:6:

SEQUENCE TYPE: nucleic acid
SEQUENCE LENGTH: 35 base pairs

STRANDEDNESS: double
TOPOLOGY: linear
MOLECULE TYPE: other nucleic acid, synthetic DNA

AATTCACC ATG GAG TTT GGG CTC AGC TGG CTT TTT
Met Glu Phe Gly Leu Ser Trp Leu Phe
1 5

35

SEQ ID NO:7:

SEQUENCE TYPE: nucleic acid
SEQUENCE LENGTH: 43 base pairs

STRANDEDNESS: double
TOPOLOGY: linear
MOLECULE TYPE: other nucleic acid, synthetic DNA

CAA GGT ACC ACG TTA ACT GTC TCC TCA GCC TCC ACC AAG GGC
Cln Gly Thr Thr Leu Thr Val Ser Ser Ala Ser Thr Lys Gly
1 5 10

42

C

43

SEQ ID NO:8:

SEQUENCE TYPE: nucleic acid
SEQUENCE LENGTH: 61 base pairs

STRANDEDNESS: double
TOPOLOGY: linear
MOLECULE TYPE: other nucleic acid, synthetic DNA

AG CTT CCA TGG ACG TTC GGT GGA GGC ACC AAG CTG GAA ATC AAA CGA
Leu Pro Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Arg
1 5 10 15

47

ACT GTG GCT GCA CC
Thr Val Ala Ala

61

The claims defining the invention are as follows:-

1. A process for producing humanized chimera antibody without changing any of the amino acids of its non-human antibody variable region which comprises the steps of:
 - (1) constructing a cassette vector by inserting a cDNA coding for human antibody C_H into an expression vector for animal cell use and establishing a cloning site in the upstream region C_H of said cassette vector for inserting a cDNA which encodes nonhuman animal V_H;
 - (2) digesting a cDNA coding for nonhuman animal antibody V_H with restriction enzyme;
 - 10 (3) inserting said cDNA coding for nonhuman animal antibody V_H into the cassette vector, using a synthetic DNA which comprises a base sequence corresponding to the 5'-end side of said human antibody C_H and a base sequence corresponding to the 3'-end side of said nonhuman animal V_H and is possessed of the restriction enzyme recognition sites on both of its ends, thereby constructing a humanized chimera antibody heavy chain
15 expression vector in which said cDNA coding for the heavy chain variable region of human antibody are linked together through said synthetic DNA;



(4) constructing a cassette vector by inserting a cDNA coding for a light chain constant region of human antibody into an expression vector for animal cell use and establishing a cloning site in the upstream region of the light chain constant region of said cassette vector for inserting a cDNA which encodes a light chain variable region of nonhuman animal antibody;

(5) digesting a cDNA coding for the light chain variable region of nonhuman animal antibody with restriction enzymes;

(6) inserting said cDNA coding for the light chain variable region of nonhuman animal antibody into the cassette vector using a synthetic DNA which comprises a base sequence corresponding to the 5'-end side of said light chain constant region of human antibody and a base sequence corresponding to the 3'-end side of said light chain variable region of nonhuman animal antibody and is possessed of the restriction enzyme recognition sites on both of its ends, thereby constructing a humanized chimera antibody light chain expression vector in which said cDNA coding for the light chain constant region of human antibody and said cDNA coding for the light chain variable region of nonhuman animal antibody are linked together through said synthetic DNA;

(7) introducing these expression vectors into host cells to obtain a transformant; and

(8) culturing said transformant in an appropriate

culture medium, thereby allowing the transformant to produce and accumulate a humanized chimera antibody, and collecting said humanized chimera antibody from the resulting culture broth.

2. The process according to claim 1, wherein said nonhuman animal is mouse.

3. The process according to claim 1, wherein said nonhuman animal antibody is reactive with ganglioside.

4. The process according to claim 1, wherein said nonhuman animal antibody is reactive with ganglioside GD₃.

5. The process according to claim 1, wherein said nonhuman animal antibody is reactive with mouse ganglioside GD₃.

6. The process according to claim 1, wherein said heavy chain variable region of antibody comprises the polypeptide having the amino acid sequence as defined by from the first residue to the 120th residue in SEQ ID NO: 4 and said light chain variable region of antibody comprises the polypeptide having the amino acid sequence as defined by from the first residue to the 108th residue in SEQ ID NO: 5.

7. The process according to claim 1, wherein said humanized chimera antibody is reactive with ganglioside GD₃.

8. The process according to claim 1, wherein said humanized chimera antibody is an antibody KM-871 reactive with ganglioside GD₃.

9. A humanized chimera antibody which comprises a

variable region of a mouse monoclonal antibody KM-641 (FERM BP-3116) which is reactive with ganglioside GD_3 and 3',8'-LD1 and a human antibody constant region.

10. A humanised chimera antibody which is reactive with ganglioside GD_3 .

11. A humanised chimera antibody KM-871 which is reactive with ganglioside
5 GD_3 .

12. A transformant capable of producing a humanised chimera antibody, wherein said antibody comprises a variable region of a mouse monoclonal antibody KM-641 (FERM BP-3116) which is reactive with ganglioside GD_3 and 3',8'-LD1 and a human antibody constant region.

10 13. A transformant capable of producing a humanised chimera antibody, wherein said antibody is reactive with ganglioside GD_3 .

14. A transformant KM-871 (FERM BP-3512) capable of producing humanized chimera antibody KM-871, wherein said antibody is reactive with ganglioside GD_3 .

15 15. A pharmaceutical composition comprising a humanized chimera antibody which is reactive with ganglioside GD_3 .

16. Use of a humanized chimera antibody which is reactive with ganglioside GD_3 for the manufacture of the medicament for treating cancer.

17. A process for producing humanized chimera antibody, substantially as hereinbefore described with reference to any one of the Examples.

20 18. A humanized chimera antibody, substantially as hereinbefore described with reference to any one of the Examples.

19. A transformant capable of producing a humanized chimera antibody, substantially as hereinbefore described with reference to any one of the Examples.

25 20. A humanized chimera antibody whenever prepared by the process according to any one of claims 1 to 8.

Dated 19 June, 1995

Kyowa Hakko Kogyo Co., Ltd.

Patent Attorneys for the Applicant/Nominated Person

SPRUSON & FERGUSON



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ABSTRACT OF THE DISCLOSURE

A process for the production of humanized chimera antibody, wherein the chimera antibody is produced easily without changing any of the amino acids of its mouse antibody variable region, which comprises the steps of: (1) constructing
5 a cassette vector by inserting a cDNA coding for a heavy chain constant region of human antibody into an expression vector for animal cell use and establishing a cloning site in the upstream region of the heavy chain constant region of said cassette vector for inserting a cDNA which encodes a heavy chain
10 variable region of nonhuman animal antibody; (2) digesting a cDNA coding for the heavy chain variable region of nonhuman animal antibody with restriction enzymes; (3) inserting said cDNA coding for the heavy chain variable region of nonhuman animal antibody into the cassette vector, using a synthetic DNA
15 which comprises a base sequence corresponding to the 5'-end side of said heavy chain constant region of human antibody and a base sequence corresponding to the 3'-end side of said heavy chain variable region of nonhuman animal antibody and is
....
:....: possessed of the restriction enzyme recognition sites on both
20 of its ends, thereby constructing a humanized chimera antibody heavy chain expression vector in which said cDNA coding for the heavy chain constant region of human antibody and said cDNA coding for the heavy chain variable region of nonhuman animal antibody are linked together through said synthetic DNA; (4)
25 constructing a cassette vector by inserting a cDNA coding for

a light chain constant region of human antibody into an expression vector for animal cell use and establishing a cloning site in the upstream region of the light chain constant region of said cassette vector for inserting a cDNA which
5 encodes a light chain variable region of nonhuman animal antibody; (5) digesting a cDNA coding for the light chain variable region of nonhuman animal antibody with restriction enzymes; (6) inserting said cDNA coding for a light chain variable region of nonhuman animal antibody into the cassette
10 vector using a synthetic DNA which comprises a base sequence corresponding to the 5'-end side of said light chain constant region of human antibody and a base sequence corresponding to the 3'-end side of said light chain variable region of nonhuman animal antibody and is possessed of the restriction enzyme
15 recognition sites on both of its ends, thereby constructing a humanized chimera antibody light chain expression vector in which said cDNA coding for the light chain constant region of human antibody and said cDNA coding for the light chain variable region of nonhuman animal antibody are linked together
....
:....: 20 through said synthetic DNA; (7) introducing these expression vectors into host cells to obtain a transformant; and (8) culturing said transformant in an appropriate culture medium, thereby allowing the transformant to produce and accumulate a humanized chimera antibody, and collecting said humanized
25 chimera antibody from the resulting culture broth.

Fig. 1

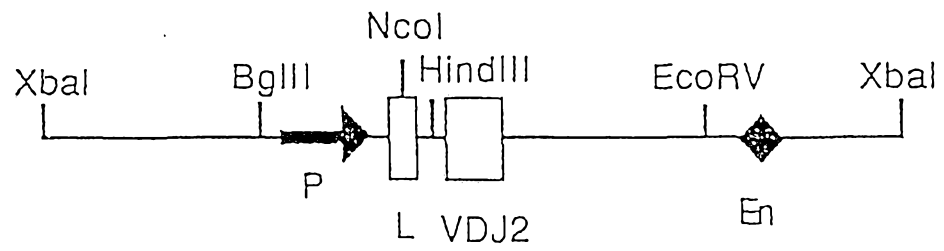


Fig. 2

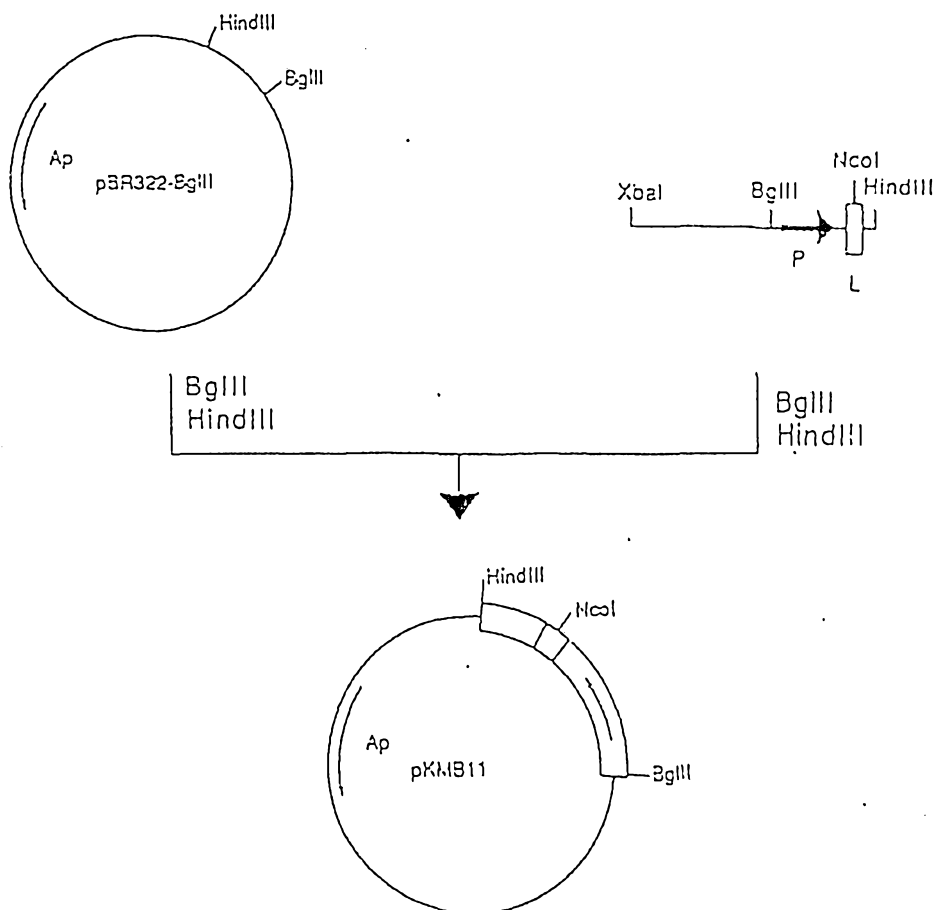


Fig. 3

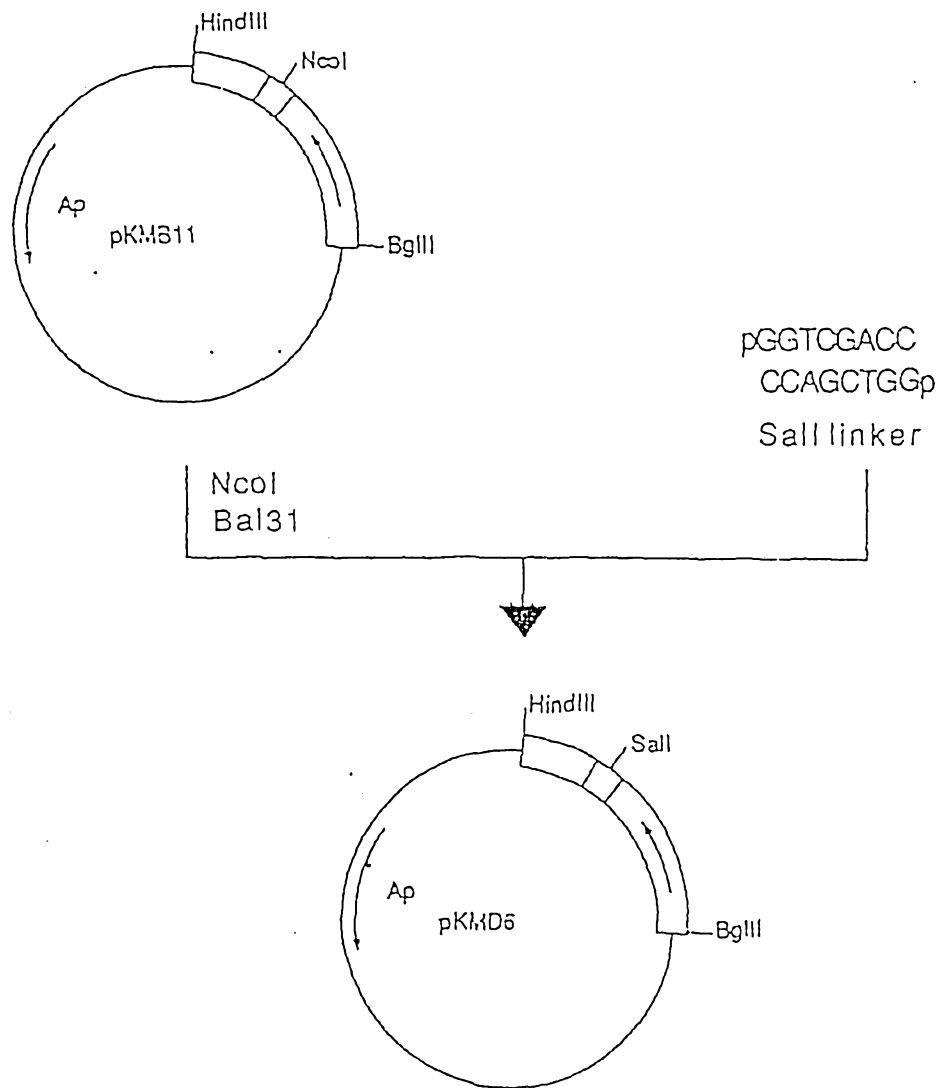


Fig. 4

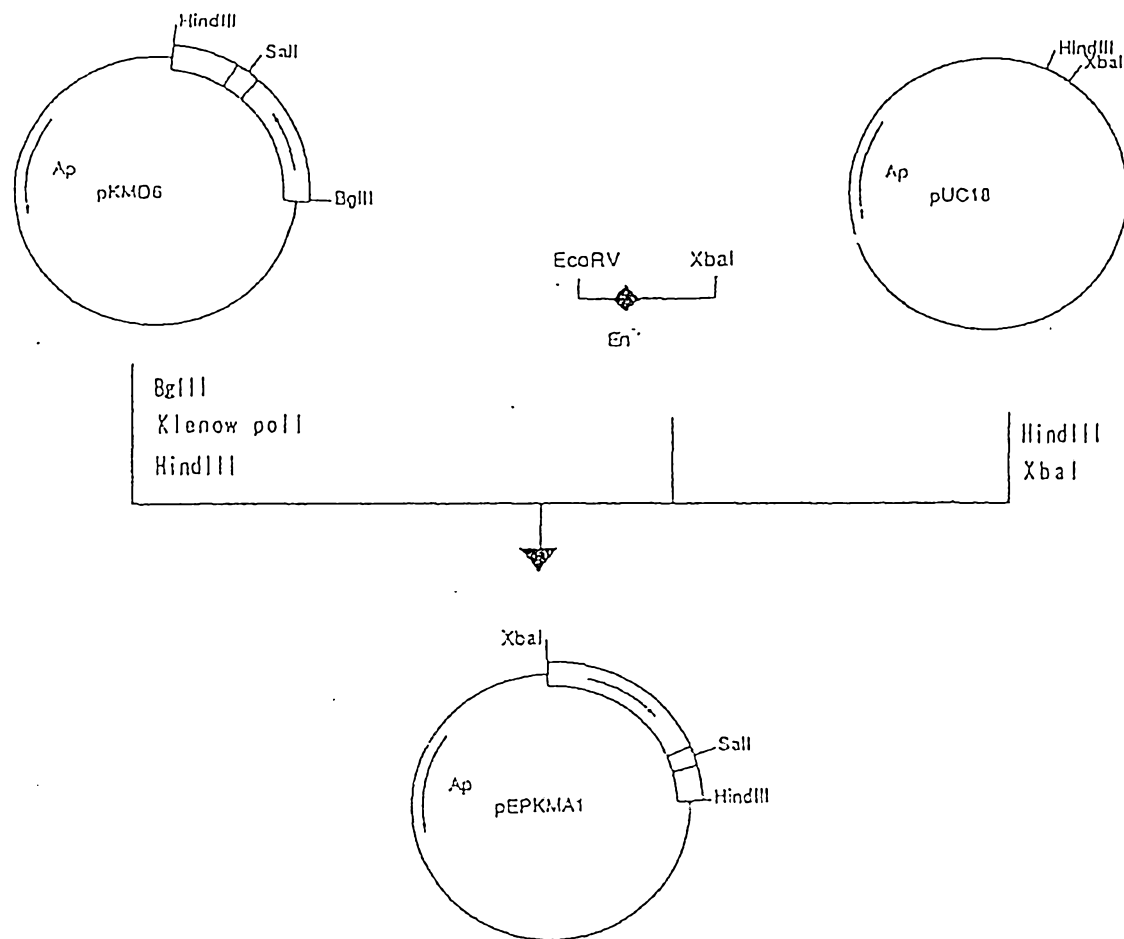


Fig. 5

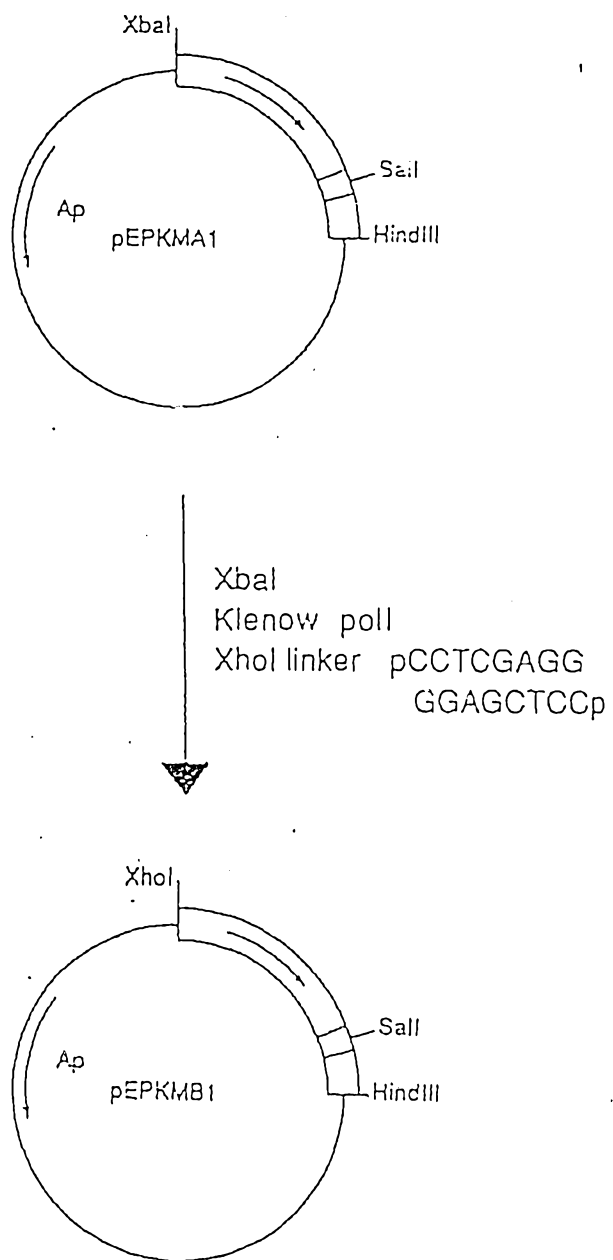


Fig. 6

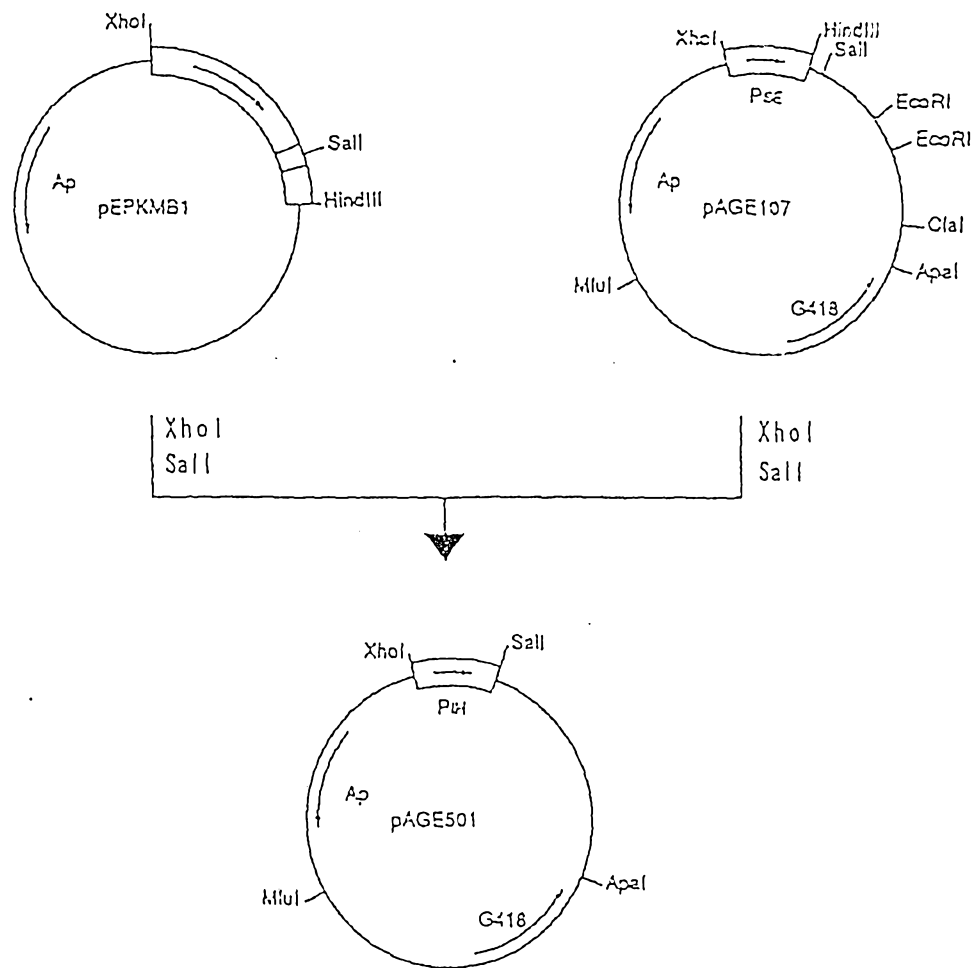


Fig. 7

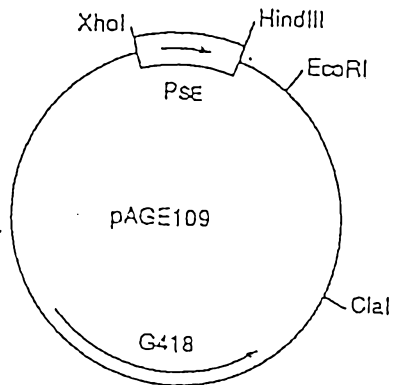
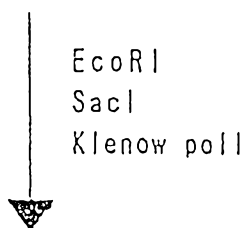
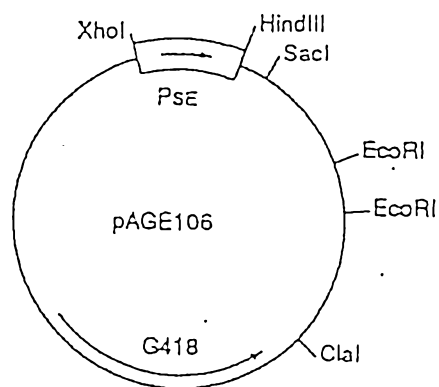


Fig. 8

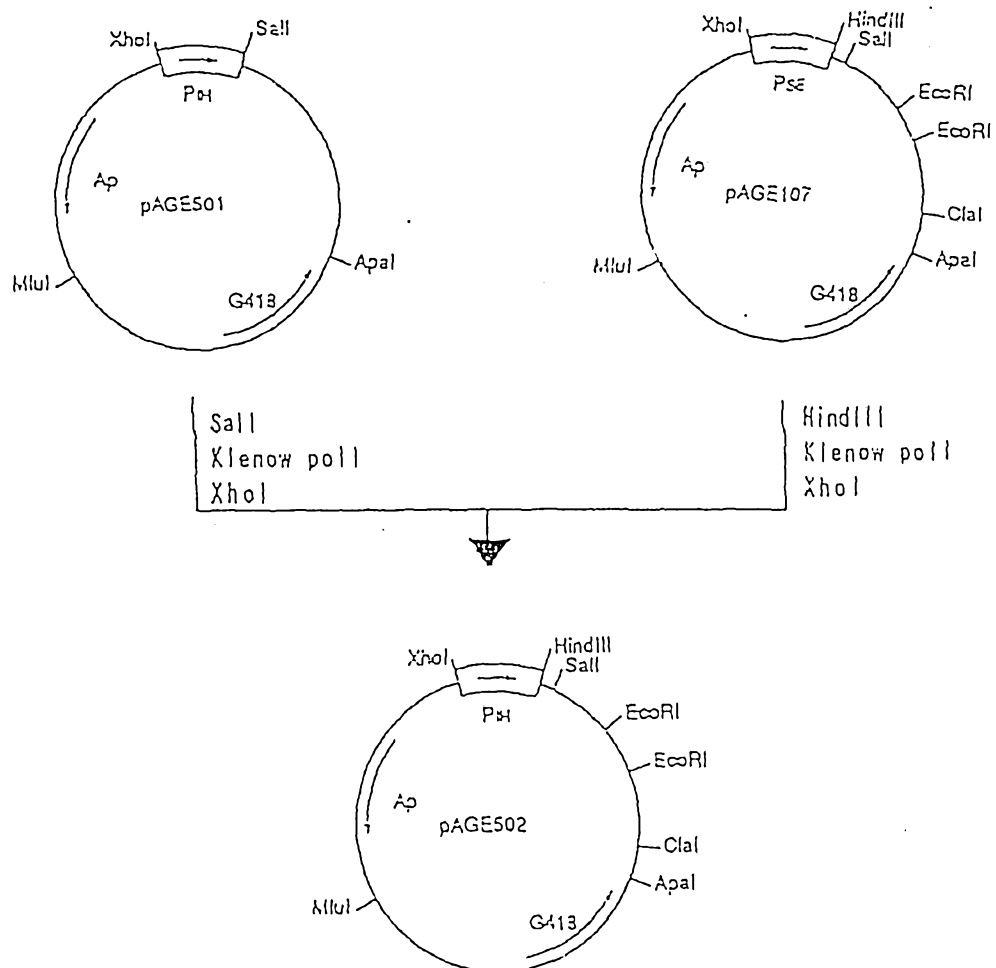


Fig. 9

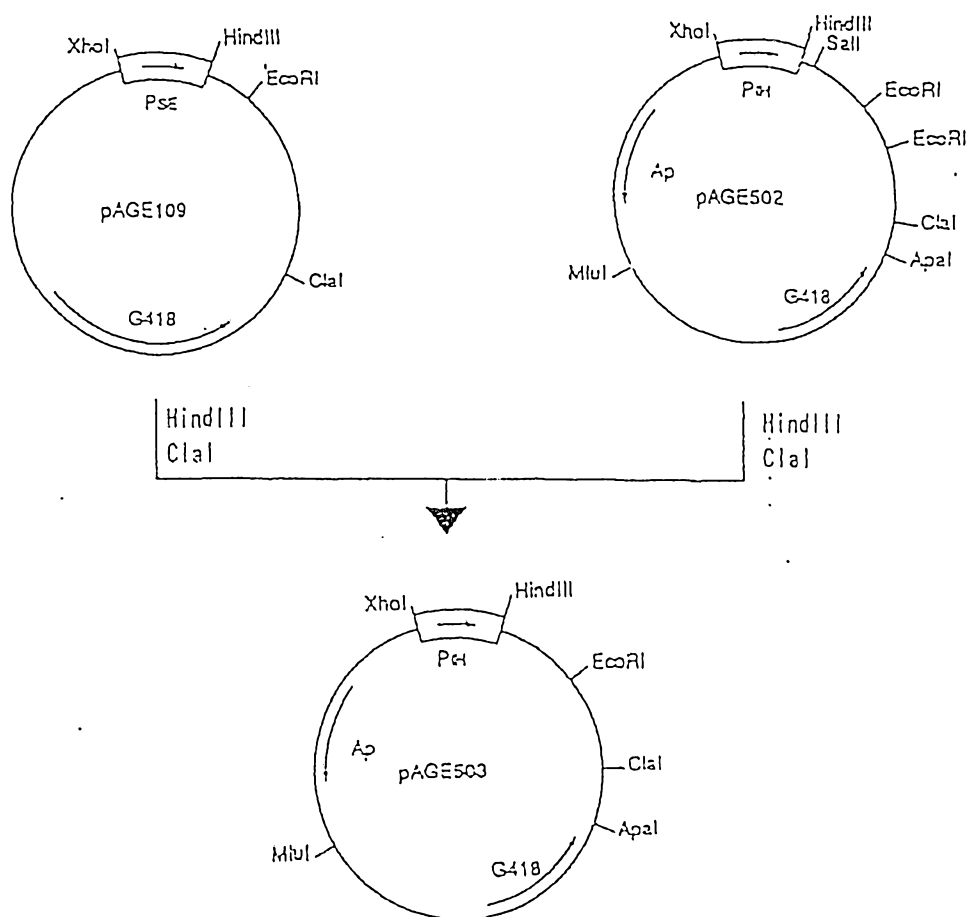


Fig. 10

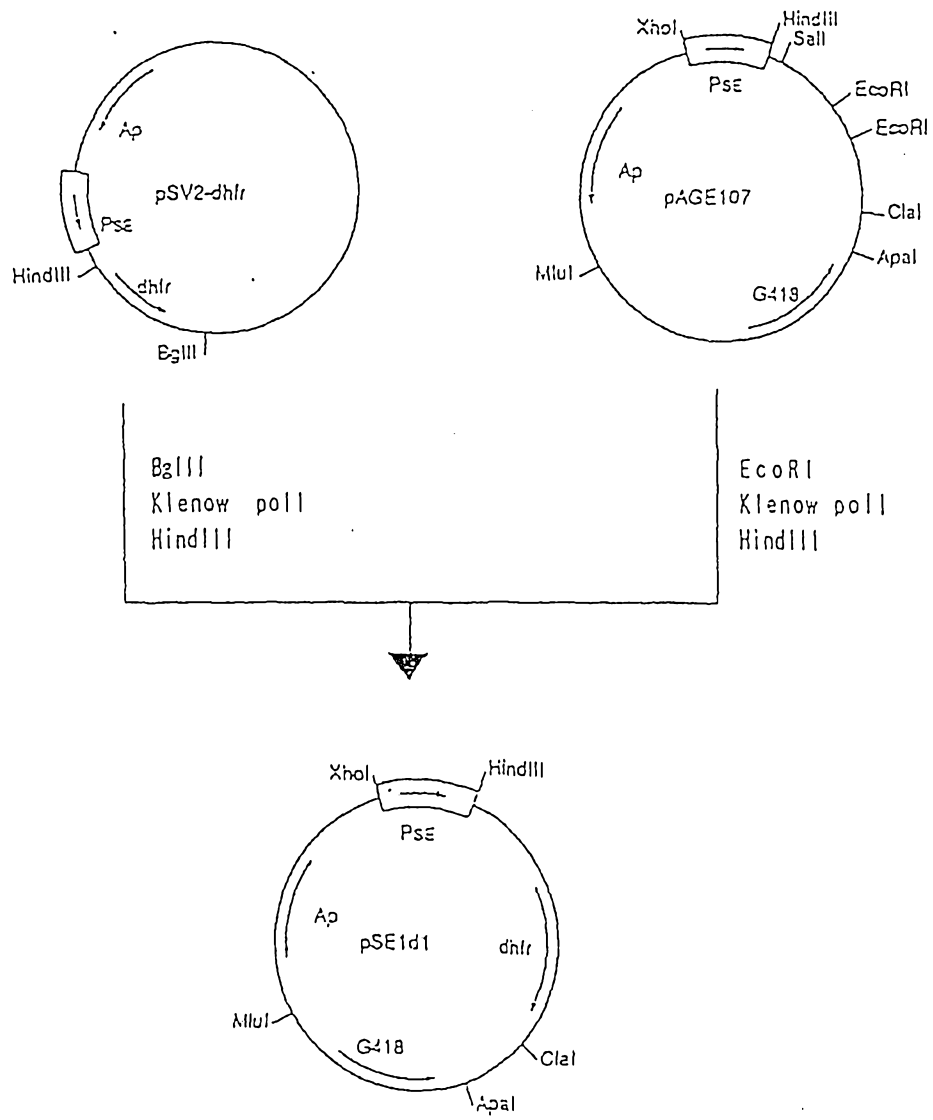
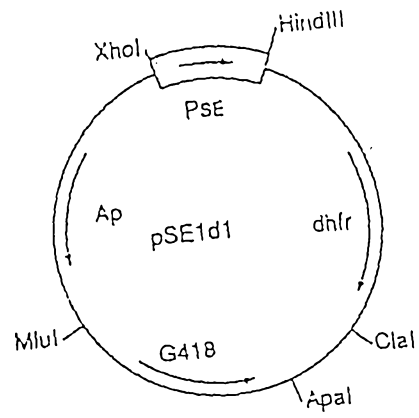


Fig. 11



HindIII
Klenow pol

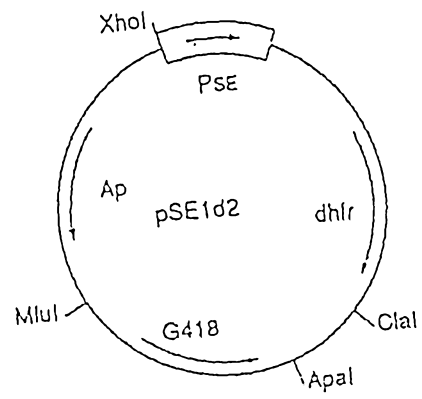


Fig. 12

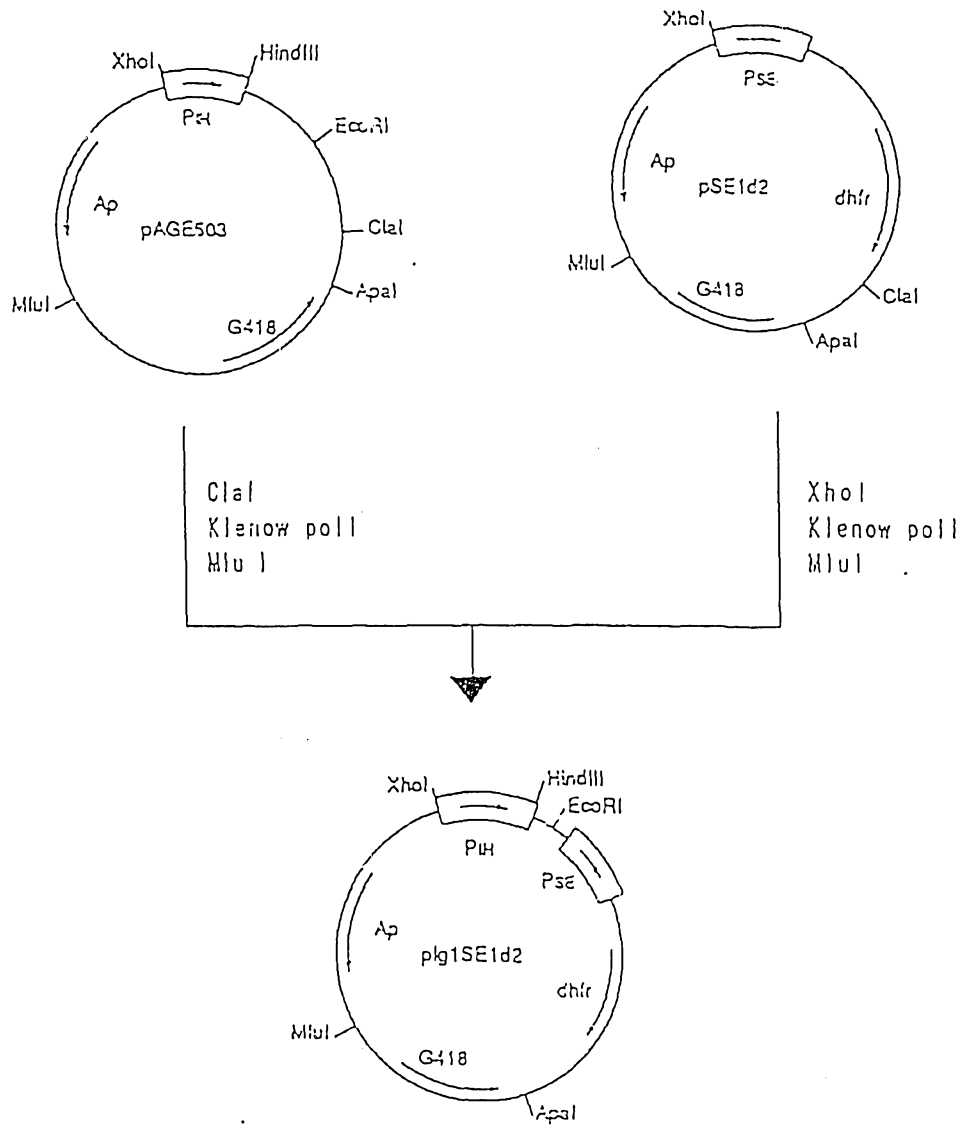


Fig. 13

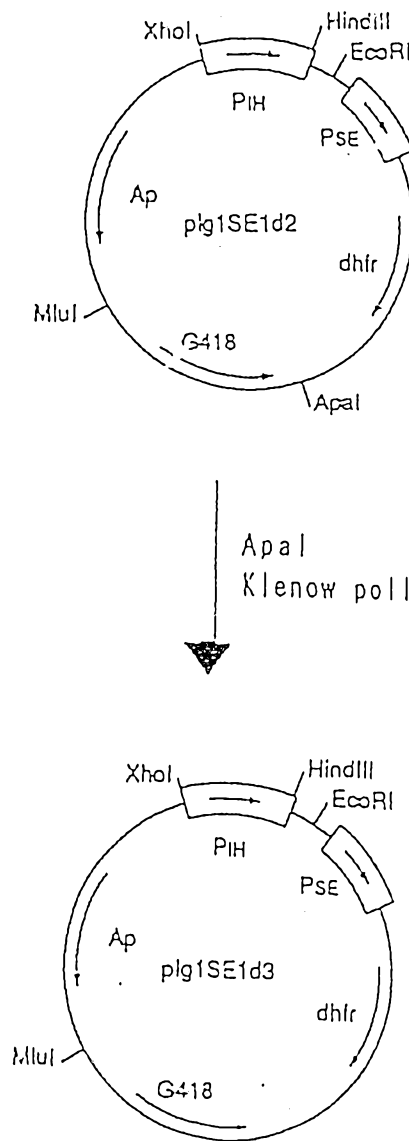


Fig. 14

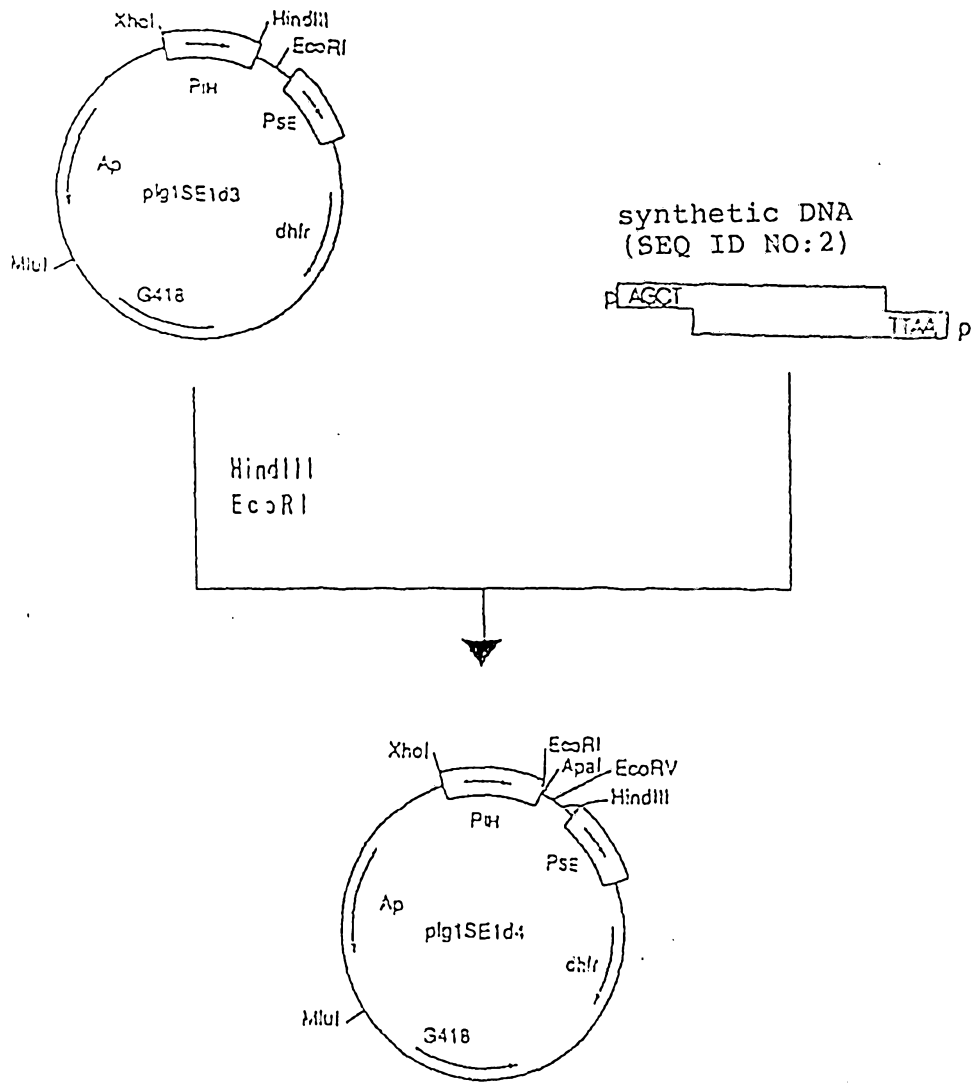


Fig. 15

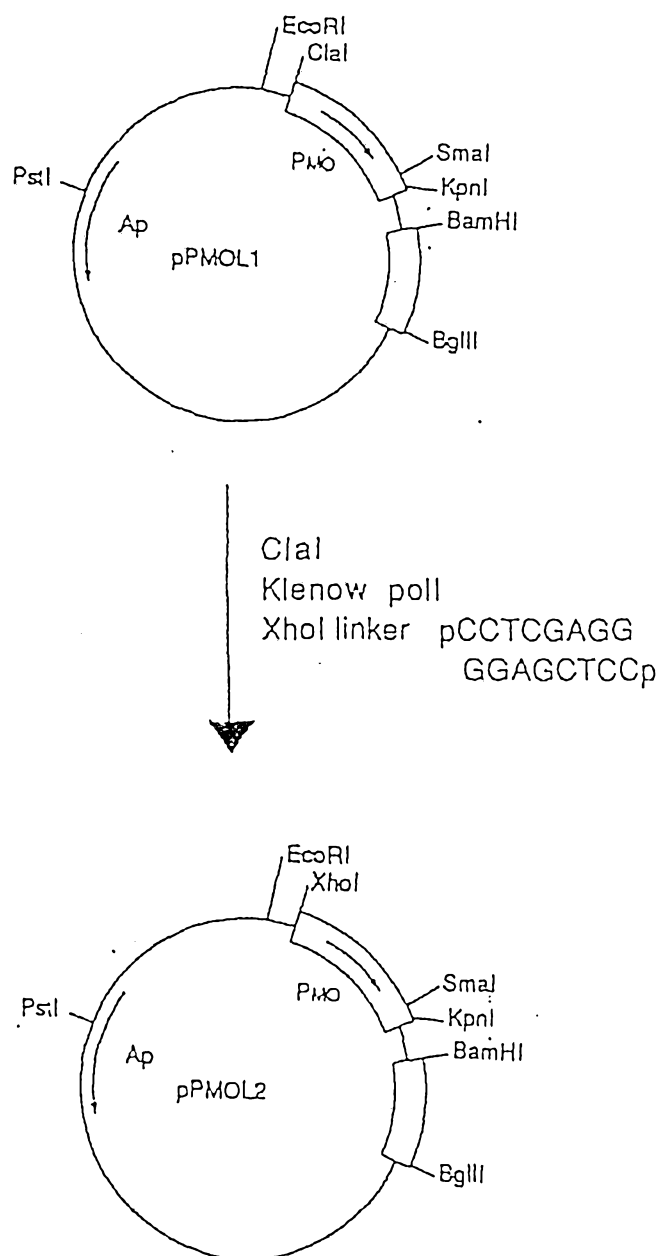


Fig. 16

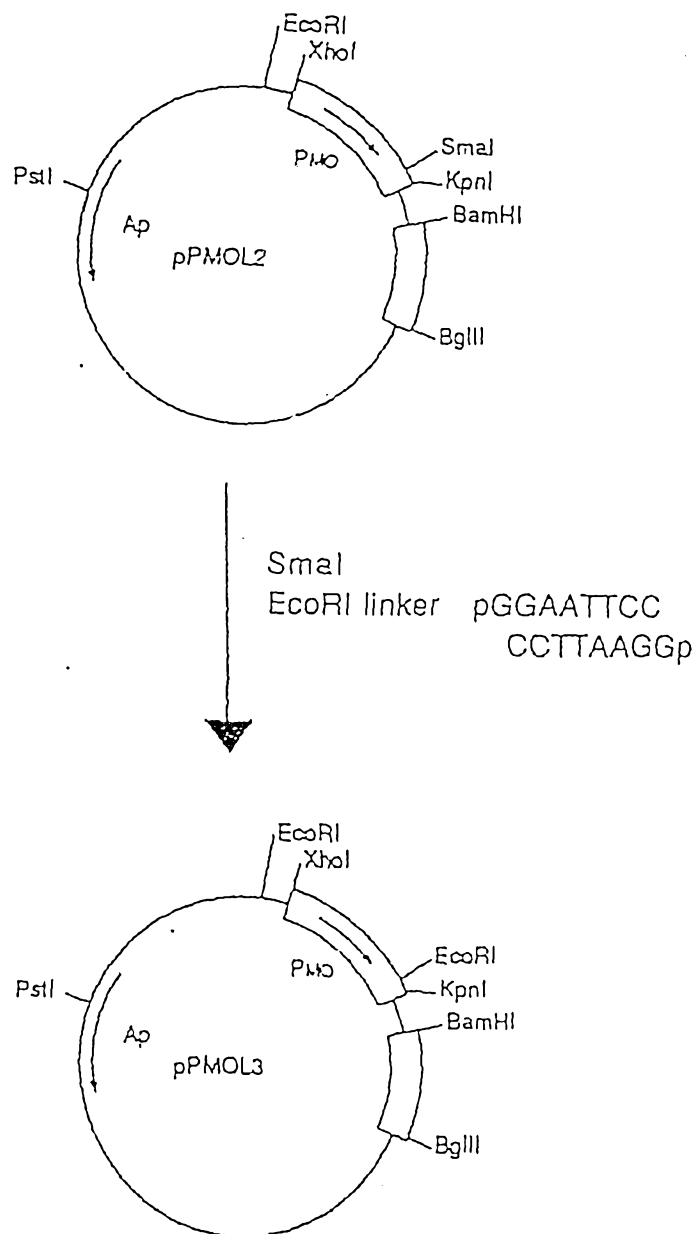


Fig. 17

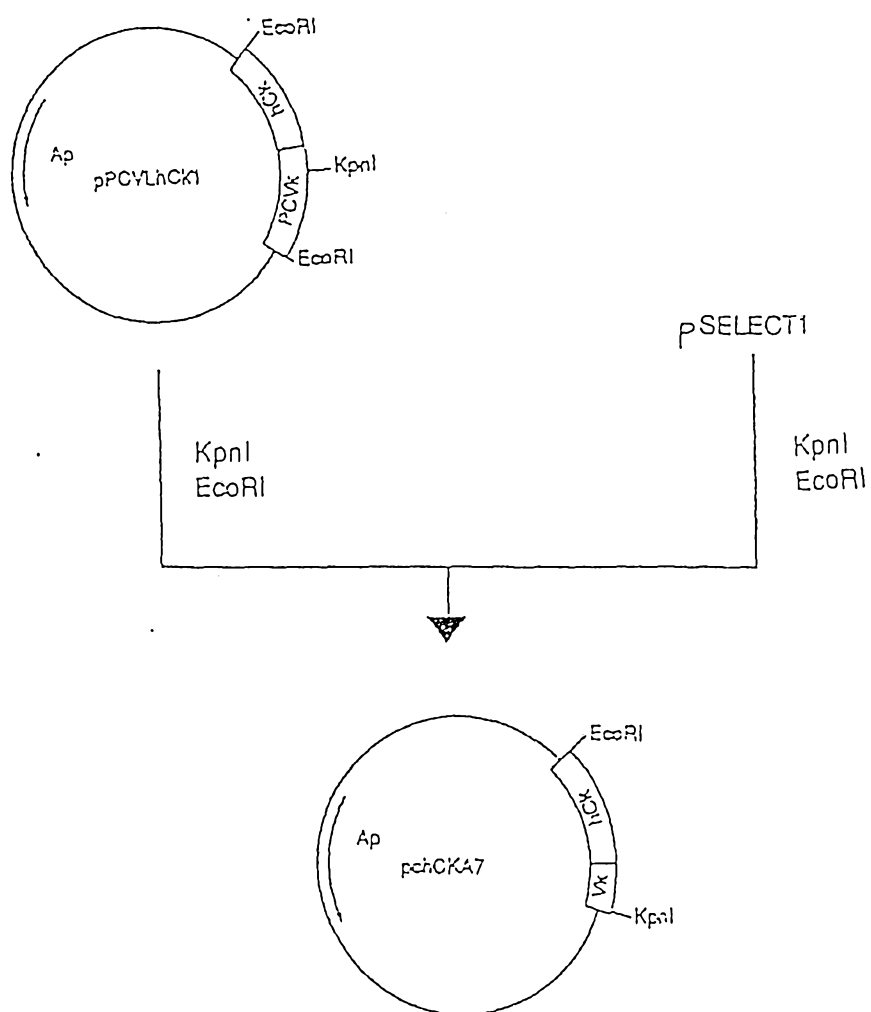
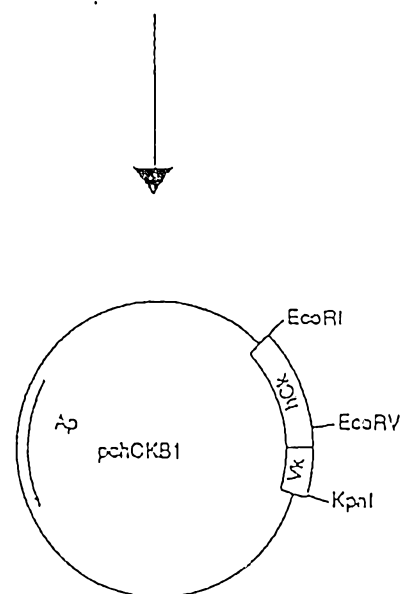
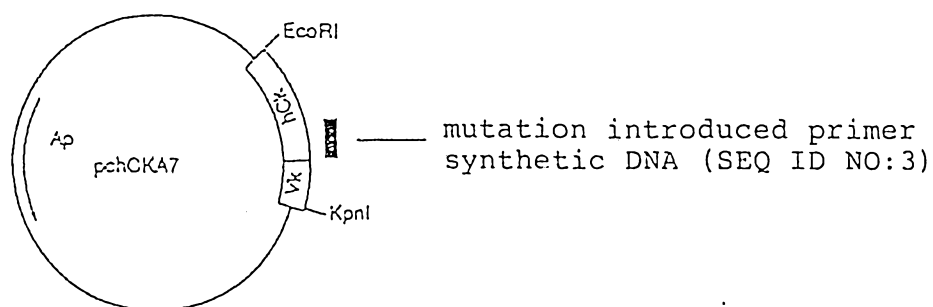


Fig. 18



PCVL	Human Ck	
AAAC	GAACTGTGGCTGCACCATCTGTC	pchCKA7
	GAT	pchCKB1

Fig. 19

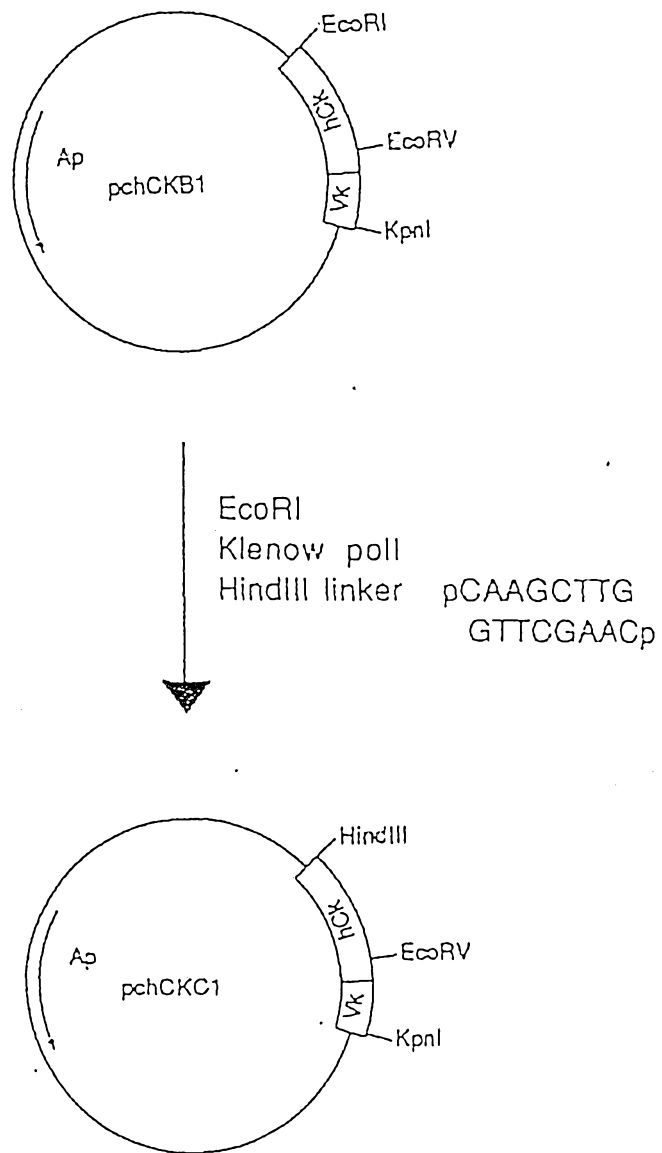


Fig. 20

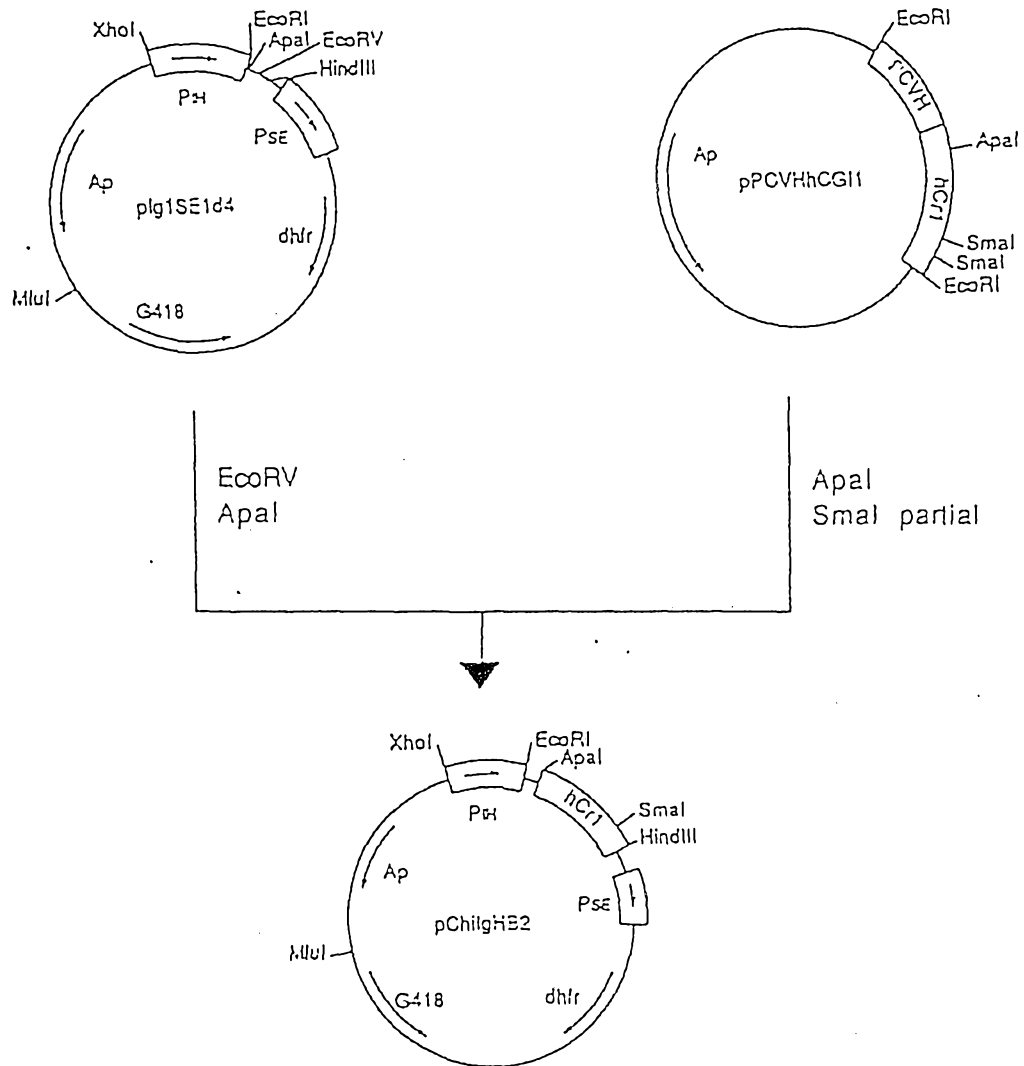


Fig. 21

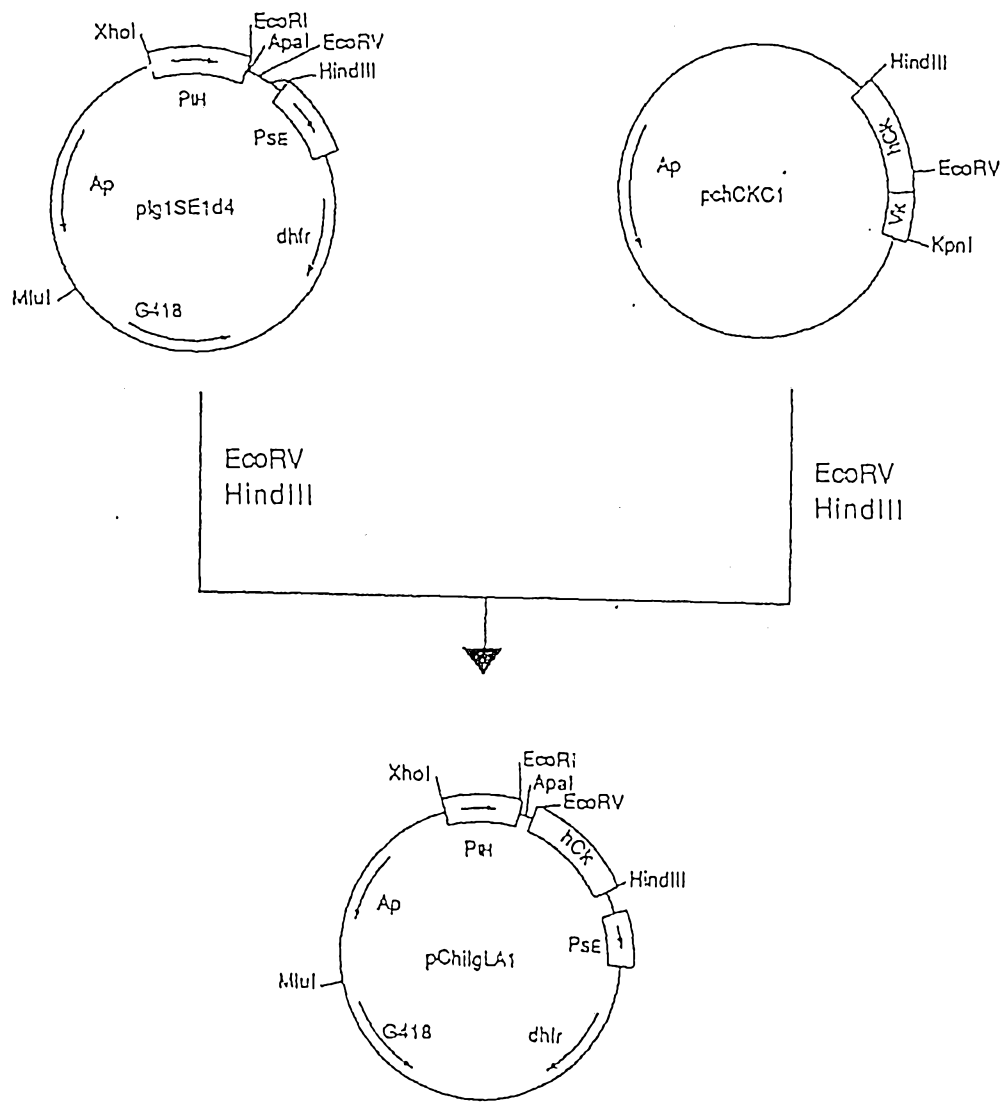


Fig. 22

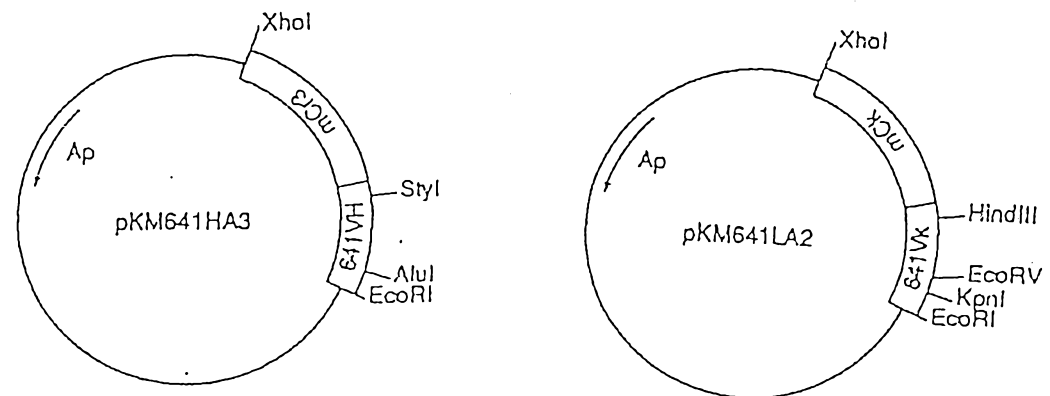
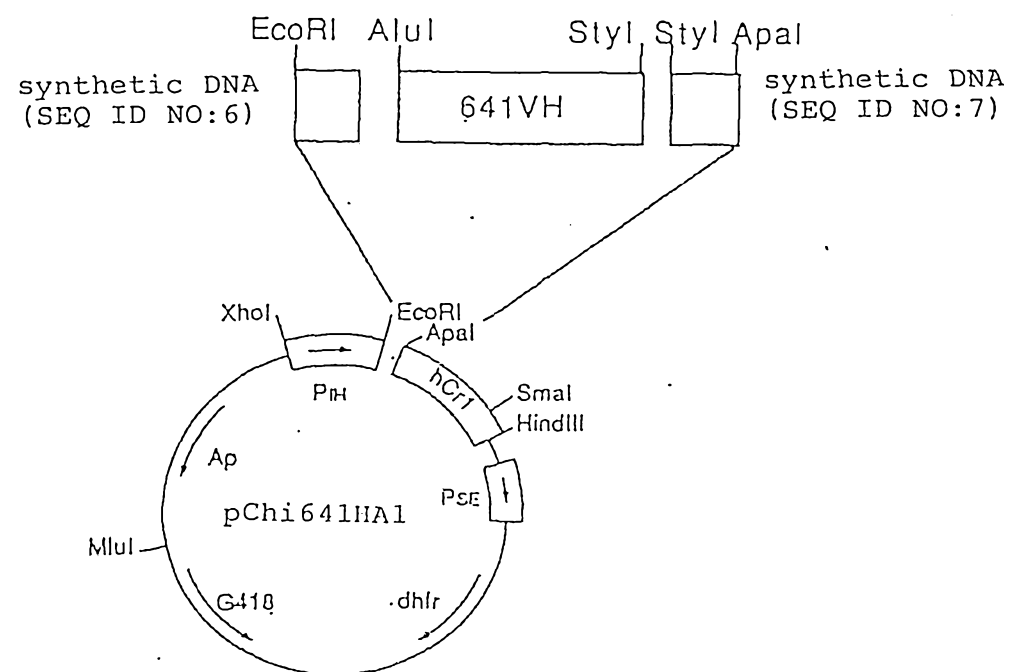


Fig. 23



11

Fig. 24

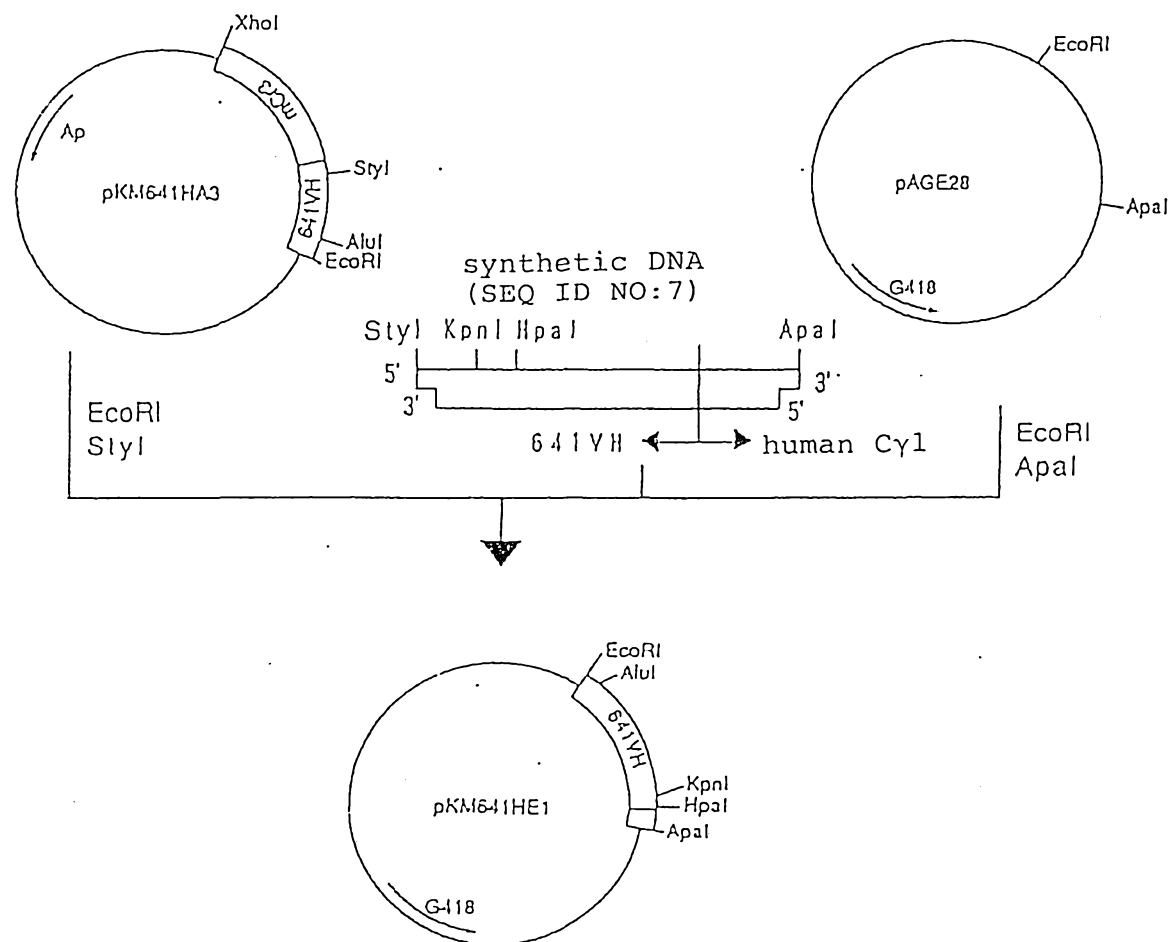


Fig. 25

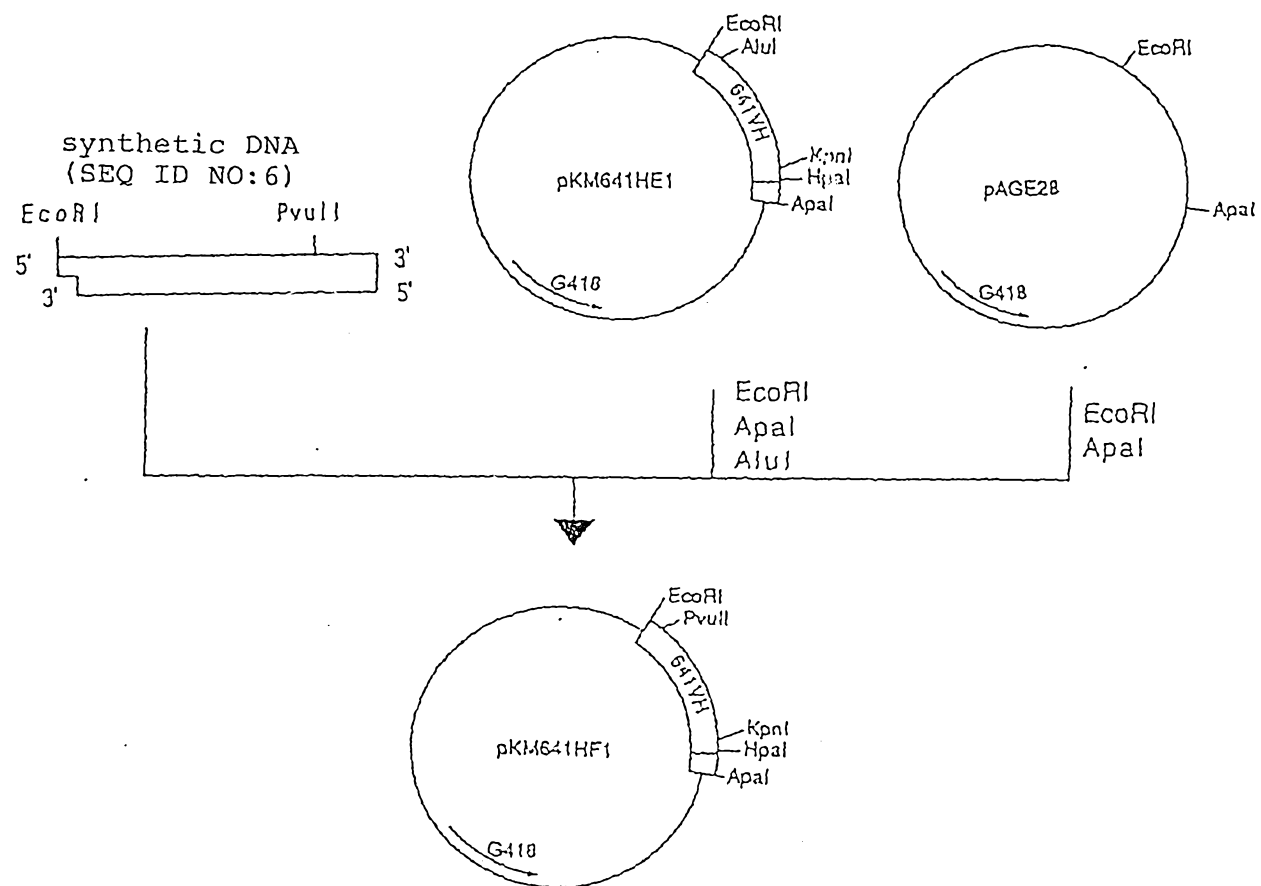


Fig. 26

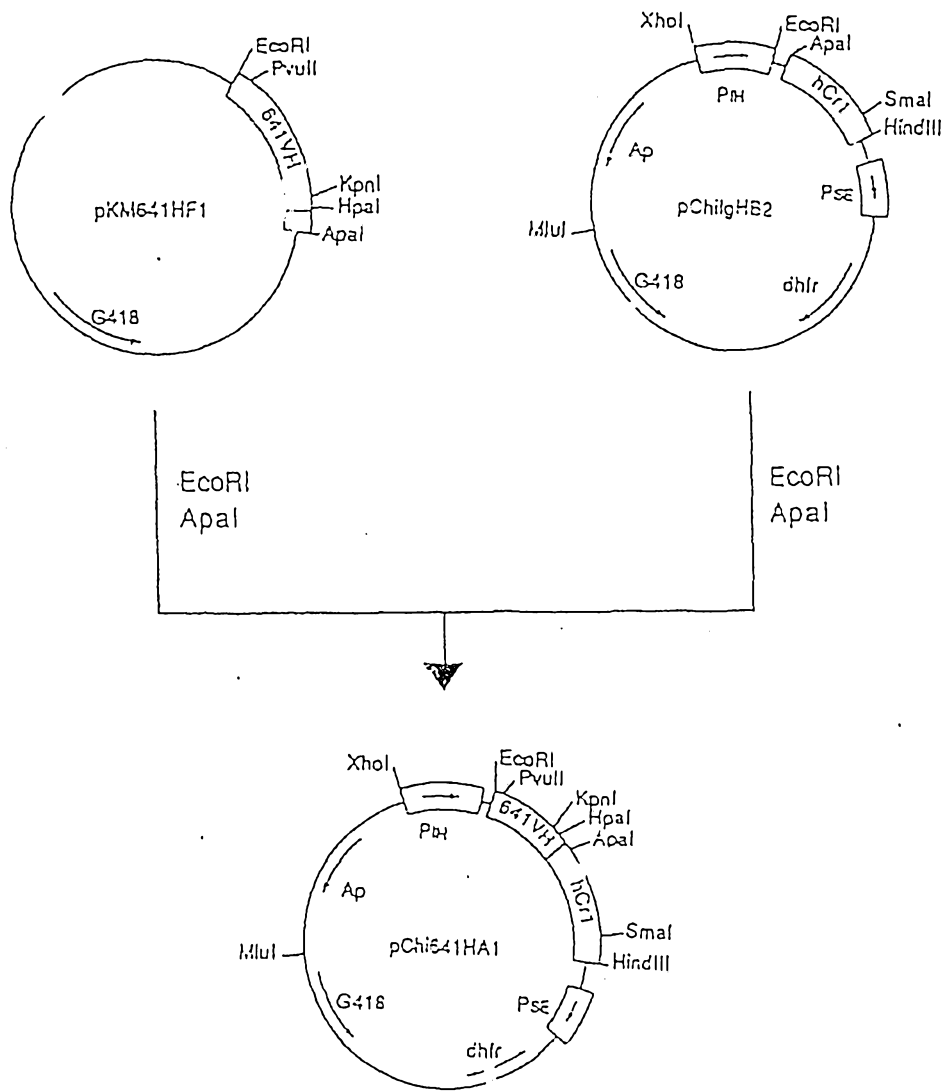


Fig. 27

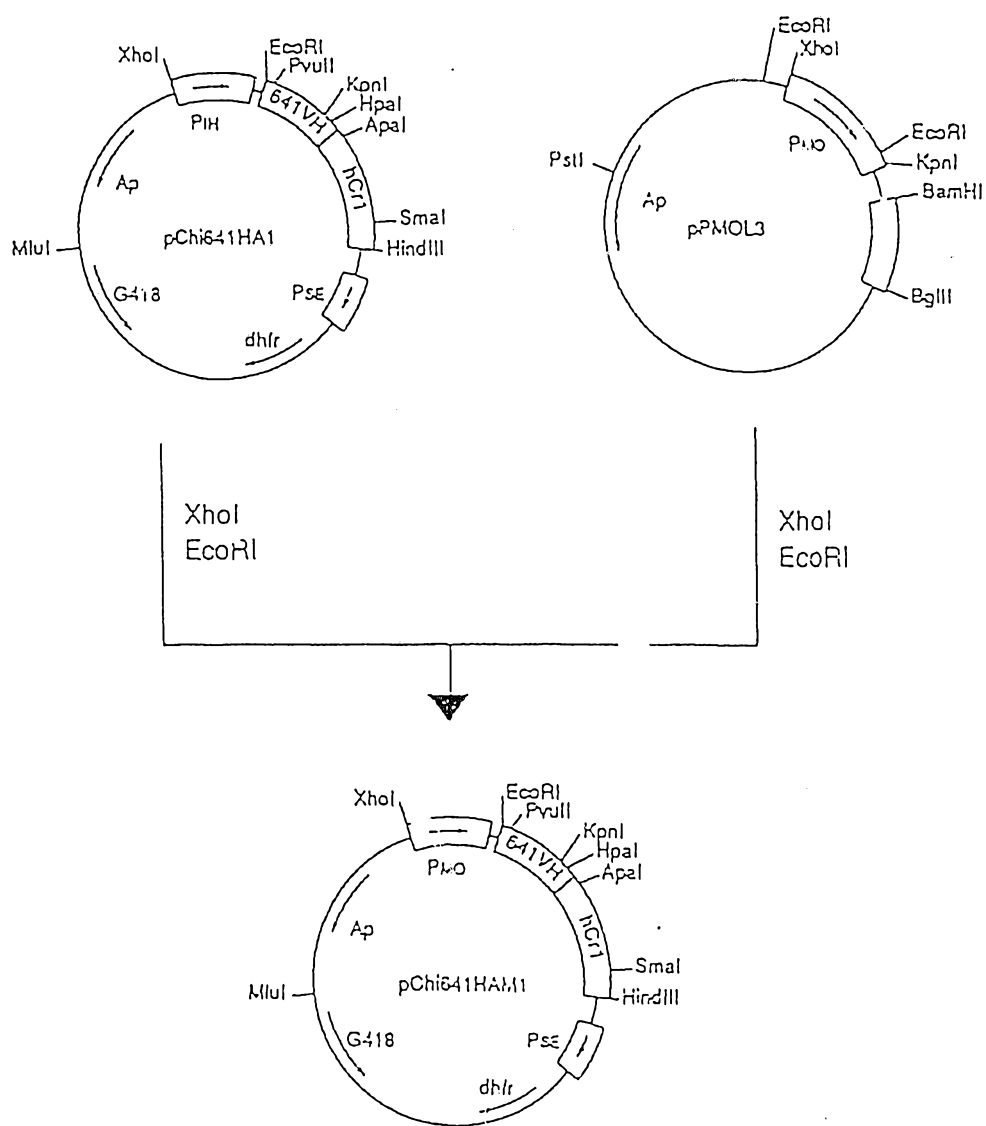


Fig. 28

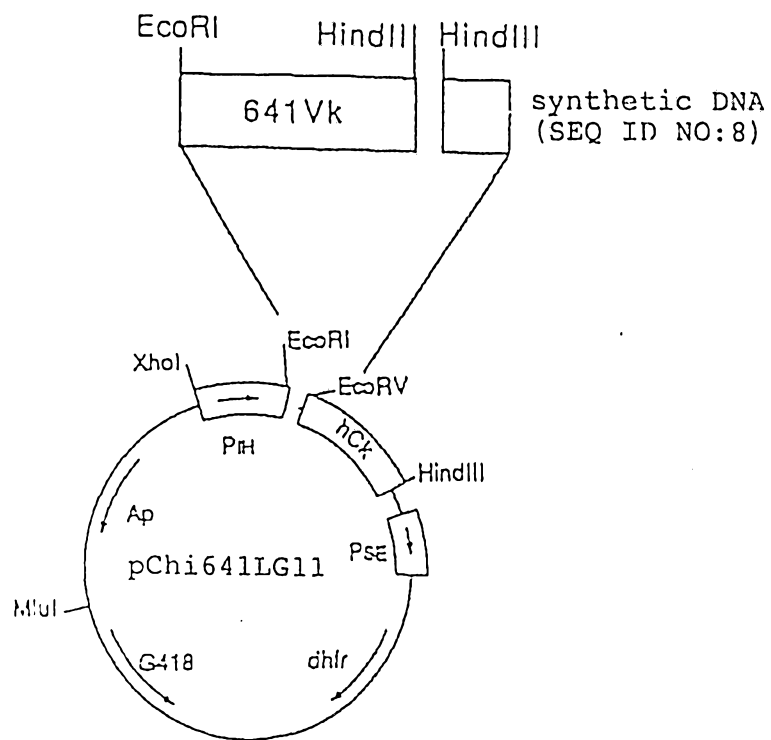


Fig. 29

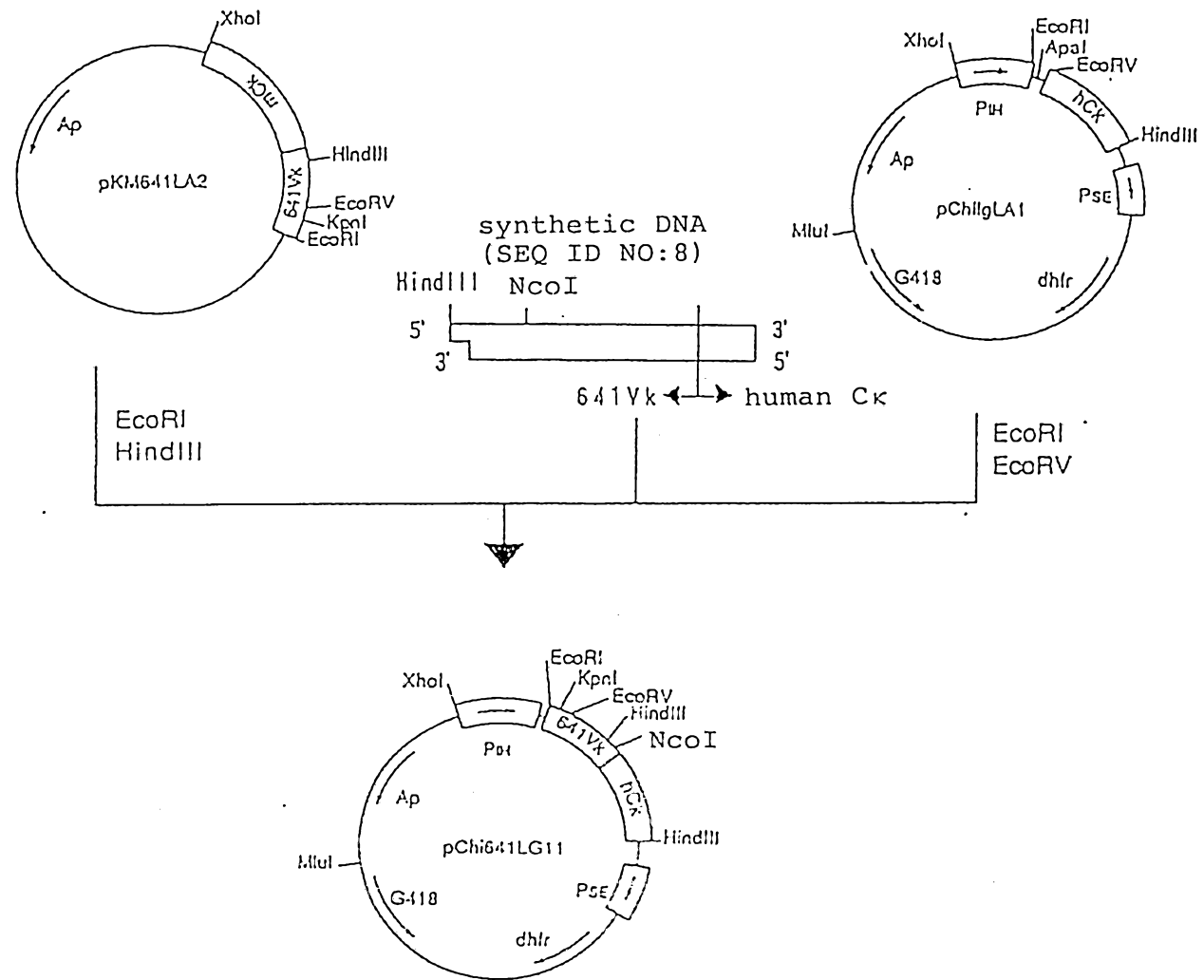


Fig. 30

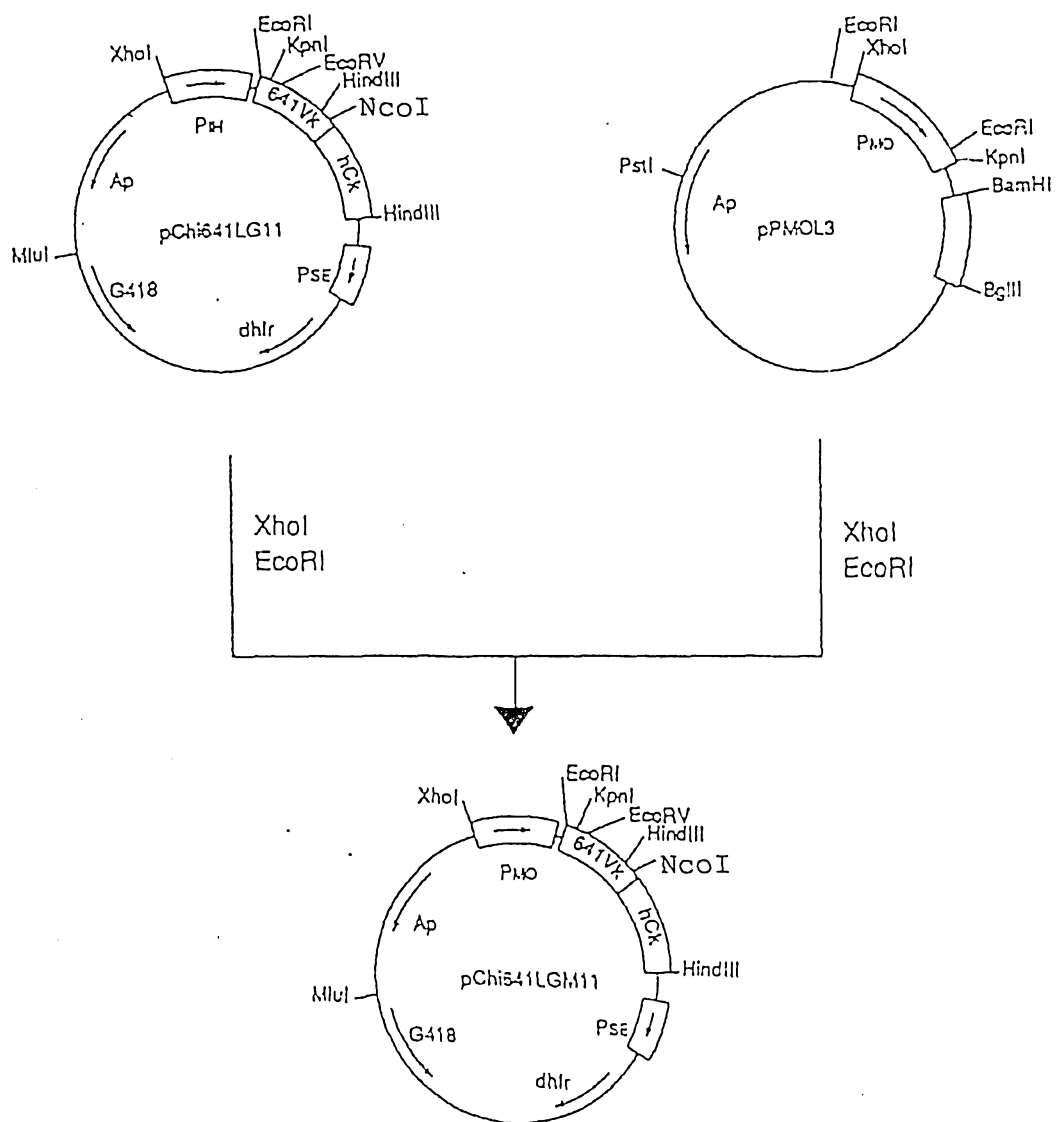


Fig. 31

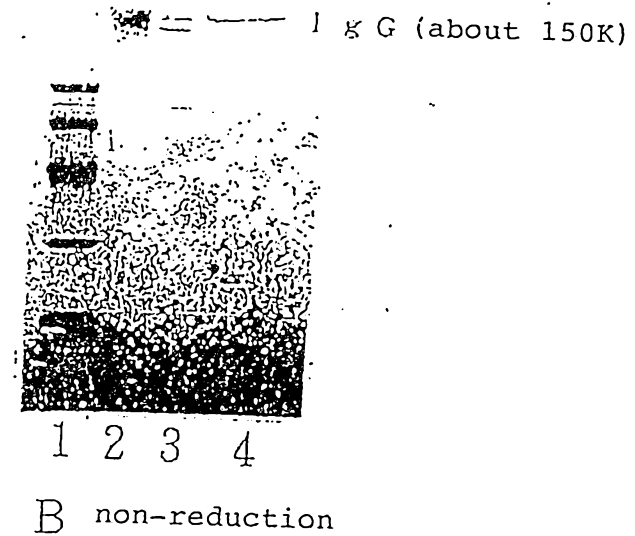
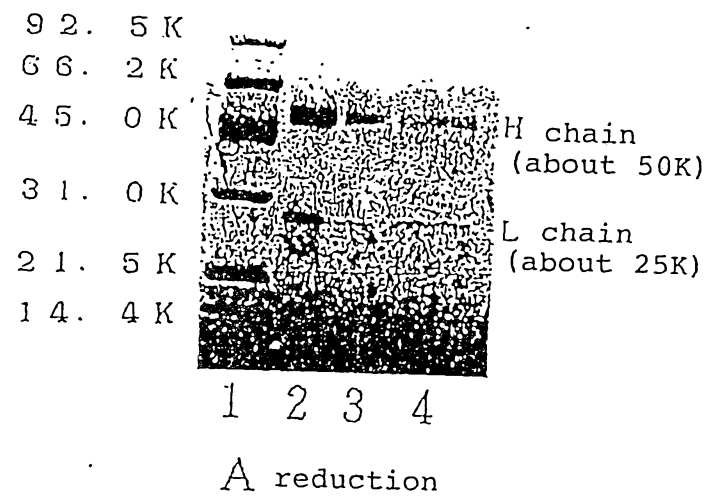
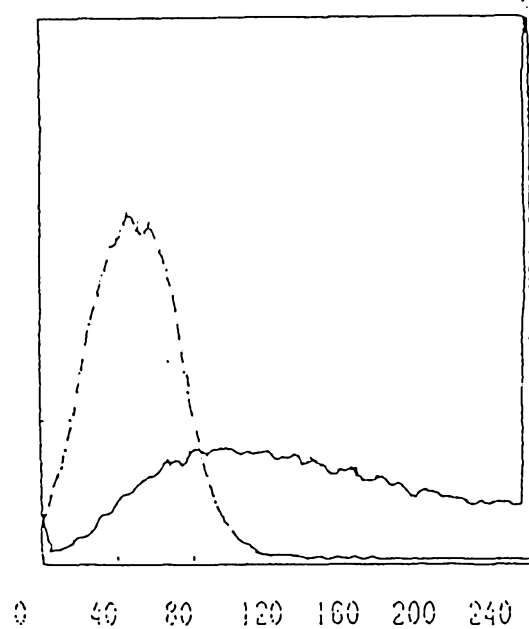


Fig. 32

Melanoma
SK-MEL-28



Melanoma
G361

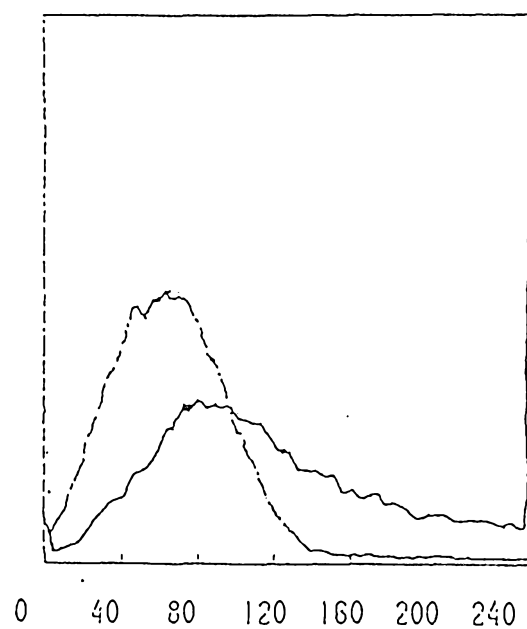


Fig. 33

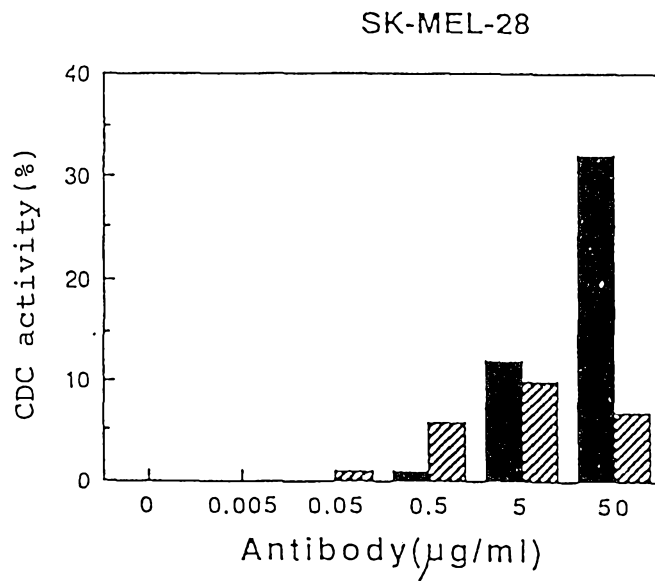
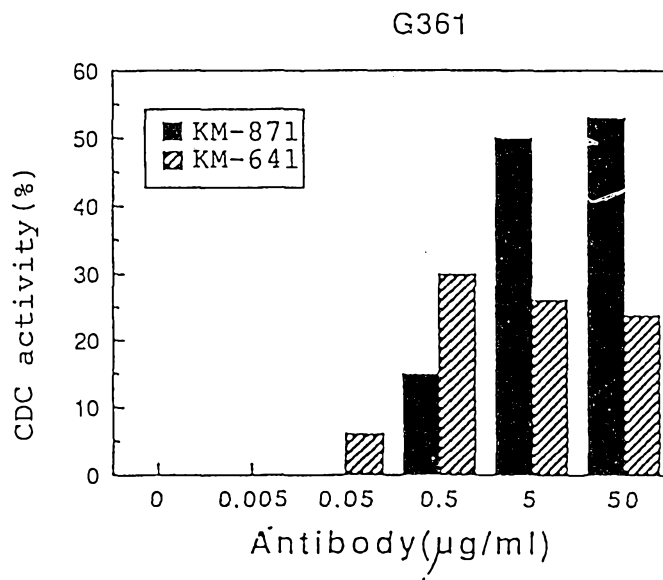


Fig. 34

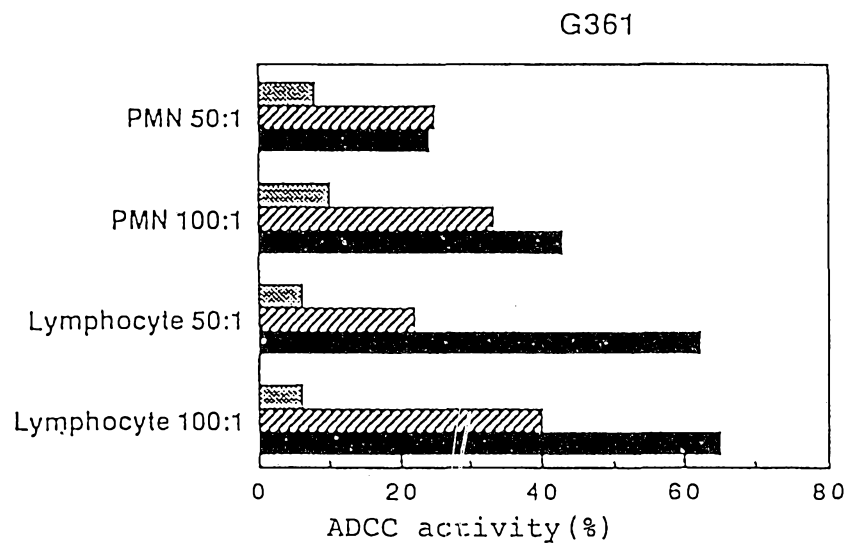


Fig. 35

