627432

COMMONWEALTH of AUSTRALIA

**PATENTS ACT 1952**

APPLICATION FOR A STANDARD PATENT

~~I~~ We JURIDICAL FOUNDATION THE CHEMO-SERO-THERAPEUTIC RESEARCH INSTITUTE of 668, Okubo, Shimizu-machi, Kumamoto-shi, Kumamoto-ken, Japan and TEIJIN LIMITED of No. 11, Minami-hommachi 1-chome, Higashi-ku, Osaka-shi, Osaka-fu, Japan

hereby apply for the grant of a Standard Patent for an invention entitled:

PROCESS FOR PRODUCING RECOMBINANT HUMAN FACTOR VIII:C AND TRANSFORMANT TO BE USED THEREFOR

which is described in the accompanying ~~provisional~~ complete specification.

Details of basic application(s):—

| Number | Convention Country | Date |
|---|---|---|
| 225147/1987 | JAPAN | September 10, 1987 |
| 85454/1988 | JAPAN | April 8, 1988 |

The address for service is care of DAVIES & COLLISON, Patent Attorneys, of 1 Little Collins Street, Melbourne, in the State of Victoria, Commonwealth of Australia.

Dated this 8th day of September 19 88.

Keith Walsh

.....
(a member of the firm of DAVIES & COLLISON for and on behalf of the Applicant).

To: THE COMMISSIONER OF PATENTS

COMMONWEALTH OF AUSTRALIA

PATENTS ACT 1952

# DECLARATION IN SUPPORT OF CONVENTION OR NON-CONVENTION APPLICATION FOR A PATENT

In support of the Application made for a patent for an invention

Insert title of invention.

entitled : PROCESS FOR PRODUCING RECOMBINANT HUMAN FACTOR VIII:C AND TRANSFORMANT TO BE USED THEREFOR

Insert full name(s) and address(es) of declarant(s) being the applicant(s) or person(s) authorized to sign on behalf of an applicant company.

We SANEO NONAKA of Juridical Foundation The Chemo-Sero-Therapeutic Research Institute, of No. 668, Okubo, Shimizu-machi, Kumamoto-shi, Kumamoto-ken, Japan and KAZUO OGATA of Teijin Limited, of No. 11, Minami-hommachi 1-chome, Higashi-ku, Osaka-shi, Osaka-fu, Japan

do solemnly and sincerely declare as follows :-

Cross out whichever of paragraphs 1(a) or 1(b) does not apply

1(a) relates to application made by individual(s)

1(b) relates to application made by company; insert name of applicant company.

1. (a) We are authorized by JURIDICAL FOUNDATION THE CHEMO-SERO-THERAPEUTIC RESEARCH INSTITUTE and TEIJIN LIMITED, respectively,

the applicant...S..... for the patent to make this declaration on their behalf.

Cross out whichever of paragraphs 2(a) or 2(b) does not apply

2(a) relates to application made by inventor(s)

2(b) relates to application made by company(s) or person(s) who are not inventor(s); insert full name(s) and address(es) of inventors.

2. (a) ~~I am / We are the actual inventor..... of the invention~~

or (b) Takashi SUGIYAMA, of No. 3-18-4, Tamadaira, Hino-shi, Tokyo-to; Kenichi MASUDA, of No. 2-23-8, Myojin-cho, Hachioji-shi, Tokyo-to; Yoshitaka TAJIMA, of No. 27-62, Hakenomiya 1-chome, Kumamoto-shi, Kumamoto-ken; Hiroshi YONEMURA, of No. 35-37, Ikeda 1-chome, Kumamoto-shi, Kumamoto-ken; all of JAPAN

are the actual inventor....S..... of the invention and the facts upon which the applicant..S..... are entitled to make the application are as follows :-

State manner in which applicant(s) derive title from inventor(s)

The actual inventors have assigned the invention to the said applicants.

Cross out paragraphs 3 and 4 for non-convention applications. For convention applications, insert basic country(s) followed by date(s) and basic applicant(s).

3. The basic application..S..... as defined by Section 141 of the Act was/were made

in JAPAN on the September 10, 1987

by JURIDICAL FOUNDATION THE CHEMO-SERO-THERAPEUTIC RESEARCH INSTITUTE and TEIJIN LIMITED

in JAPAN on the April 8, 1988

by JURIDICAL FOUNDATION THE CHEMO-SERO-THERAPEUTIC RESEARCH INSTITUTE and TEIJIN LIMITED

4. The basic application..S..... referred to in paragraph 3 of this Declaration was/were the first application..S..... made in a Convention country in respect of the invention the subject of the application.

Insert place and date of signature.

Declared at Kumamoto and Osaka, JAPAN this 22nd & 23rd day of August 1988, respectively

Juridical Foundation The Chemo-Sero-Therapeutic Research Institute

Teijin Limited

Signature of declarant(s) (no attestation required)

Saneo Nonaka
President

Kazuo Ogata
Vice President

Note: Initial all alterations.

DAVIES & COLLISON, MELBOURNE and CANBERRA.

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**PROCESS FOR PRODUCING RECOMBINANT HUMAN FACTOR VIII:C AND TRANSFORMANT TO BE USED THEREFOR**

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(57) In the present invention, the natural type active human Factor VIII:C means a protein which lacks a part or all of the peptide of B domain ranging from Ser-741 to Arg-1648 of natural human Factor VIII:C, wherein the amino terminus of the light chain is covalently bonded through a peptide bond to the carboxy terminus of the heavy chain.

## CLAIM

1. An animal cell line having been transformed with an expression vector containing a gene encoding natural type active human Factor VIII:C as hereinbefore defined in which Arg-740 of the carboxyl terminus of the H chain is directly bonded through a peptide bond with 1649 glutamic acid of the amino terminus of the L chain.

22. A method for producing recombinant human Factor VIII:C as hereinbefore defined which comprises cultivating an animal cell line transformed with an expression vector containing a gene encoding natural type active human Factor VIII:C in which Arg-740 of the carboxyl terminus of the H chain is directly bonded through a peptide bond with 1649 glutamic acid of the amino terminus of the L chain.

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COMPLETE SPECIFICATION

(ORIGINAL)

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Published:

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Related Art:

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NAME OF APPLICANT: JURIDICAL FOUNDATION THE CHEMO-SERO-THERAPEUTIC RESEARCH INSTITUTE and TEIJIN LIMITED

ADDRESS OF APPLICANT: 668, Okubo, Shimizu-machi, Kumamoto-shi, Kumamoto-ken, Japan and

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**1 Little Collins Street, Melbourne, 3000.**

**COMPLETE SPECIFICATION FOR THE INVENTION ENTITLED:**

PROCESS FOR PRODUCING RECOMBINANT HUMAN FACTOR VIII:C AND TRANSFORMANT TO BE USED THEREFOR

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

# PROCESS FOR PRODUCING RECOMBINANT HUMAN FACTOR VIII:C AND TRANSFORMANT TO BE USED THEREFOR

This invention relates to a novel transformant and a process for producing recombinant human Factor VIII:C by using said transformant. More particularly, it relates to a transformant of animal cells carring a novel expression vector which can produce recombinant human Factor VIII:C using genetic engineering technology and relates also to a process for producing the recombinant human Factor VIII:C by using said transformant.

## Prior Art

Human Factor VIII:C is a blood plasma glycoprotein which is involved in the intrinsic pathway of blood coagulation and functions as a cofactor for Factor IXa, serine protease being capable of activating Factor X. In patients of hemophilia A which is a hereditary X chromosome-linked recessive bleeding disorder, Factor VIII:C is defective or absent in the blood. For the treatment of bleeding in hemophilia A patients caused by blood coagulation disorder, there has clinically been used so-called Factor VIII:C replacement therapy, i.e. a method for stopping the bleeding by supplement of the Factor VIII:C, which is known as the most reasonable and reliable therapy.

For the supplement of Factor VIII:C deficient in the hemophilia A patients, there have usually been used

blood plasma or cryoprecipitate obtained by freezing the blood plasma, followed by thawing of the plasma. Nowadays Factor VIII concentrate antibodies have been used for said purpose.

The source of human Factor VIII:C is human blood plasma. Commercially available Factor VIII concentrates have nowadays a content of Factor VIII:C of about 0.04 % by weight. Thus, the conventional Factor VIII concentrates usually contain various types of impurities derived from human blood plasma. While attemps have been made in the donor selection and development of technique for inactivation of viruses, there may be a danger of contamination of viruses such as those causing in hepatitis or Acquired Immune Deficiency Syndorome (AIDS).

Under the circumstances, in order to obtain more reliable Factor VIII preparations at lower cost without relying on the quantitatively limited human blood plasma source and also without anxiety of the undesirable contamination of viruses contained in pooled human blood plasma, it has currently been attempted to produce Factor VIII:C using recombinant DNA technology, i.e. by cloning genes encoding human Factor VIII:C and expressing the genes in animal cells and microorganism cells.

Many attempts have been reported for the production of human Factor VIII:C by gene recombinant technology, after human Factor VIII:C was cloned, for the first time, by J.

Gitschier et al (cf. Nature 312, 326 - 330, 1984), and then expressed in baby hamster kidney cells by W.I. Wood et al (cf. Nature 312, 330 - 337, 1984). The attempts are exemplified by cloning of cDNA of human Factor VIII:C and expression in mammalian cells by J.J. Toole et al (cf. Nature 312, 342 - 347, 1984), by M.A. Truett et al (cf. DNA 4 333-349, 1985), by N. Sarver et al (cf. European patent application No. 265,778), and by A. Pavirani et al (cf. Biotechnology 5, 389 - 392, 1987), respectively. In addition, the cloning of cDNA of human Factor VIII:C and expression in microorganisms by V. Ooyen et al (cf. European patent application No. 253,455) is also exemplified. However, according these methods, the expression of gene is transient or the gene expression levels are very low partly because the human Factor VIII:C is a glycoprotein with a very large molecular weight. Accordingly, these disclosed methods are not satisfactory from the points of an industrial production of human Factor VIII:C.

It is believed that the natural human Factor VIII:C has a domain structure of A1-A2-B-A3-C1-C2 in which A1, A2 and A3 have homologous amino acid sequences, and C1 and C2 have also homologous amino acid sequences (G.A. Vehar et al, Nature 312, 337 - 342, 1984). It is supposed that a part or all of the B domain is not necessary for the essential cofactor activity of human factor VIII:C. Thus, in order to increase the gene expression level in producing Factor

VIII:C by recombinant mode, it has been attempted to create a molecular species which exhibits a biological activity while being modified to be as small as possible by deleting a part or all of the B domain.

(1) Definition of natural type active human Factor VIII:C and abbreviation thereof:

Natural human Factor VIII:C is a polymorphic glycoprotein which is constituted by 2332 amino acid residues and has a molecular weight of about 300 KDa. in which the sections covering from the amino acid at the position 1 (Ala-1) up to the amimo acid at the position of 1648 (Arg-1648) are generally referred to as heavy chains while the sections from Glu-1649 up to Tyr-2332 are called as light chains. Natural human Factor VIII:C is subjected to proteolytic cleavage by enzymes such as thrombin, Factor Xa and activated protein C into lower molecular fragments and the Factor VIII:C is activated or inactivated in this way.

Biologically active Factor VIII:C in plasma contains a heterogenous mixture of heavy chains ranging in length from 1648 amino acids down to 740 amino acids (cf. L.-O. Andersson et al, Proc. Natl. Acad. Sci. USA 83, 2979-2983, 1986). Accordingly to the Andersson's observation, the smallest biologically active Factor VIII:C consists of a heavy chain from Ala-1 to Arg-740 and a light chain from 1649-Glu to Try-2332, wherein the heavy and light chains are linked via a calcium-ion bridge.

In the present invention, the natural type active human Factor VIII:C means a protein which lacks a part or all of the peptide of B domain ranging from Ser-741 to Arg-1648 of natural human Factor VIII:C, wherein the amino terminus of the light chain is covalently bonded through a peptide bond to the carboxy terminus of the heavy chain.

The natural type active human Factor VIII:C is abbreviated, for example, as follows. The natural type active human Factor VIII:C where a polypeptide fragment (H chain) ranging from Ala-1 to Arg-740 is linked covalently to the amino acid of a polypeptide (L chain) ranging from Glu-1649 to Try-2332 is abbreviated as "740 Arg-1649 Glu Factor VIII:C". "740 Arg-1649 Glu Factor VIII:C" may be hereinafter referred to as RE.

(2) Preparation of natural type active human Factor VIII:C molecule:

It has been succeeded by J.J. Toole et al in the expression of 981 Pro-1563 Asp Factor VIII:C and 759 Thr-1640 Pro Factor VIII:C in COS cells (cf. Proc. Natl. Acad. Sci. USA 83, 5939 - 5942, 1986) and in Chinese Hamster Ovary cells (CHO cells) (cf. PCT application No. WO 86/06101). It has also been succeeded by R. Kaufman in the expression of 740 Arg-1647 Gln Factor VIII:C in Chinese Hamster Ovary cells (cf. PCT application No. WO 87/04187). It has been succeeded by D.L. Eaton et al in the expression of 796 Gln-1563 Asp Factor VIII:C in mammalian cells (cf. Biochemistry 25, 8343 - 8347, 1986), and by C. Gorman (cf. European

patent application No. 260148) and by A. Pavirani et al in the expression of 116 Asp-1455 Asp Factor VIII:C in BHK-21 cells (cf. Japanese patent first publication (Kokai) No. 22195/1988), and by N. Sarver et al in the expression of 746 Ser-1561 Leu Factor VIII:C in Mouse C127 cells (cf. European patent application No. 265,778), and further, it has been succeeded by M. Pasek et al in construction of cDNA encoding 740 Arg-1649 Glu Factor VIII:C and 744 Gln-1563 Asp Factor VIII:C, etc. and expression thereof in BMT-10 cells (R.D. Gerard et al, Mol. Cell. Biol 5, 3231 - 3240, 1985) (cf. PCT application No. WO 88/831). In microorganisms, 866 Thr-1663 Asp Factor VIII:C gene is expressed by V. Ooyen et al (cf. European patent application No. 253,455).

It appears that the expression levels achieved by the methods described in the above literatures are not satisfactory.

It has been attempted to express an exogenous gene by a genetic engineering technology using eukaryotic cells such as animal cells as host cells, for example, by a method employing Chinese Hamster Ovary cells deficient in dihydrofolate reductase (dhfr): dhfr(-) CHO cells (cf. G. Urlaub et al, Prot. Natl. Acad. Sci. USA 77, 4216 - 4220, 1980). The cells are eukaryotic cells which can constantly express the exogenous genes. Although there is disclosed in PCT applications WO 86/06101 and WO 87/04187 CHO cells which can produce a specific natural type active human Factor VIII:C, no report is seen as to establishment of a cell line which

can constantly produce 740 Arg-1649 Glu Factor VIII:C at a high production efficiency.

Brief Description of the Invention

The present invention provides an animal cell line having been transformed with an expression vector containing a gene encoding natural type active human Factor VIII:C in which Arg-740 of the carboxyl terminus of the H chain is directly bonded through a peptide bond with 1649 glutamic acid of the amino terminus of the L chain.

The present invention also contemplates a method for producing recombinant human Factor VIII:C as hereinbefore defined which comprises cultivating an animal cell line transformed with an expression vector containing a gene encoding natural type active human Factor VIII:C in which Arg-740 of the carboxyl terminus of the H chain is directly bonded through a peptide bond with 1649 glutamic acid of the amino terminus of the L chain.

Brief Description of the Drawings

Fig. 1 shows DNA and amino acid sequences of RE (which codes for 740 Arg-1649 Glu Factor VIII:C) in plasmid Ad.RE.neo. Fig. 2 shows restriction enzyme maps of plasmid Ad.RE.neo(a) and pSV2-dhfr(b) which are used in Example 1.

Fig. 3 graphically shows Factor VIII:C activity in the supernatant of culture obtained by cultivating the transformant in Example 3. Fig. 4 shows a schematic representation of plasmid Ad.RE.dhfr used in Example 5. Fig. 5 shows Factor VIII:C activity in the supernatant of culture obtained by cultivating the transformant in Example 7. Fig. 6 shows an amount of Factor VIII:C antigen in the culture medium obtained by cultivating the transformant in Example 7. Fig. 7 shows the antigen level of Factor VIII:C - von Willebrand factor complex in the culture supernant in Example 7.

Detailed Description of the Invention

The transformant of the invention are generated by transforming animal cells with natural type active human Factor VIII:C expression vectors.

The natural type active human Factor VIII:C expression vectors carrying genes encoding natural type active human Factor VIII:C and at least one promoter upstream thereof and optionally carrying at least one enhancer, said promoter and enhancer being originated from animal genes and animal viruses (e.g. monkey virus, adenovirus, etc.) and further carries a selective gene in animal cells (e.g. neomycin resistant gene: transposon Tn5, dihydrofolate reductase gene (dhfr), etc.) and an amplifiable gene (e.g. dihydrofolate reductase gene (dhfr), adenosine deaminase gene, etc.).

In preferred embodiment, the natural type active human Factor VIII:C expression vector constitutes of a gene coding for natural type active human Factor VIII:C having a signal peptide (necessary for secretion) gene at the upstream thereof, and a DNA sequence of a poly rA addition signal downstream thereof, under the control of an enhancer and a promoter which function within the eukaryotic cells, and a neomycin-resistant gene (transposon Tn5), a replication origin in Escherichia coli and an antibiotics-resistant gene. Such an expression vector is, for example, plasmid Ad.RE.neo which is disclosed in PCT application No. WO 88/831 wherein "RE" represents 740 Arg-1649 Glu Factor VIII:C.

RE (740 Arg-1649 Glu Factor VIII:C) has an amino acid and DNA sequences as shown in the accompanying Fig. 1.

The signal peptide DNA sequence and an amino acid sequence in the present invention is preferably of natural human Factor VIII:C (cf. Nature 312, 330 - 337, 1984) but includes any ones which can precisely be cleaved between the signal peptide region and the mature protein region in secretion process.

Any promoter which can function in eukaryotic cells can be used in the expression vector according to the present invention. Such promoters include promoters of animal virus genes and promoters of animal genes which are effective for transcription of natural type active human Factor VIII:C genes in animal cells. Suitable examples of

the animal virus promoters include SV40 early region promoter, SV40 late region promoter, adenovirus-2 major late promoter, HB virus gene promoter, retrovirus gene promoter, and the like. Suitable examples of the animal cell gene promoter are a promoter of a thymidine kinase gene, a promoter of metarothioneine gene, a promoter of interferon gene, and the like. Among them, preferred promoters are SV40 eary region promoter, SV40 late region promoter, and adenovirus-2 major late promoter. The promoters may be inducible or constitutive.

The natural type active human Factor VIII:C expression vector carries a gene resistant to antibiotics or derivatives thereof (e.g. Geneticin, G418, etc.), for example, neomycin-resistant gene, in order to make easier the selection of the transformed animal cells. Besides, linkage with a replication origin of E. coli and an antibiotics-resistant gene is useful in order to allow easy preparation of a pure vector DNA in a large amount. The replication origin is preferably a plasmid derived from colicine E1 plasmid, for example pBR322 and its analogous plasmids, but is not limited thereto. The antibiotics-resistant genes include ampicillin-resistant gene, tetracycline-resistant gene, kanamycin-resistant gene, streptomycin-resistant gene, neomycin-resistant gene, and the like.

The eukaryotic host cells for the natural type active human Factor VIII:C gene expression in this invention include any animal cells which can express natural type

active human Factor VIII:C gene. While a wide variety of animal cell lines are useful in expressing the genes according to the present invention, hamster cells are preferable and Chinese hamster ovary cells are more preferable. Most preferable cells are Chinese hamster ovary cells which are deficient in dihydrofolate reductase activity (DHFR), since such cells are suitable for selection and isolation of the desired transformants as well as for effective gene amplification. Particularly preferred cell lines are DHFR(-) CHO cells, DXB11, and DHFR(-) CHO cells, DG44 (cf. G. Urlaub et al., Proc. Natl. Acad. Sci. USA 77, 4216-4220, 1980), of which DG44 is more prefereble in terms of stable and easy culturing.

Introduction of DNA into eukaryotic cells can be carried out by known methods, for example, by a method of F.L. Graham et al (cf. Virology 54, 536 - 539, 1973), or a method of H. Potter et al (cf. Proc. Natl. Acad. Sci. USA 81, 7161 - 7165, 1984). When animal cells deficient in a gene, e.g. CHO cells deficient in dihydrofolate reductase (dhfr) gene, are subjected to co-transfection (cf. Cell 16, 777 - 785, 1979) with a dhfr gene-carrying plasmid (e.g. pSV2-dhfr, cf. Mol. Cell Biol. 1, 854 - 864, 1981) and natural type active human Factor VIII:C expression vector, followed by selection in a selection medium which lacks hypoxanthine, glycine and thymidine, there can be obtained CHO cells in which dhfr genes are introduced and expressed,

and natural type active human Factor VIII:C gene is possibly introduced.

When the transformation is carried out by employing eukaryotic cells by the above method, it is preferable to use a linearized plasmid transfered into the cells which is prepared by cleaving previously with appropriate restriction enzymes which do not affect on the expression of the desired product, by which the desired exogenous gene can be integrated into the chromosomes of the eukaryotic cells in high efficiency.

Moreover, the animal cells integrated with a natural type active human Factor VIII:C gene can express the inserted neomycin-resistant gene (transposon Tn5) together with said gene, and hence, the animal cells can easily be selected and isolated by growing the cells grown in a medium added with a neomycin derivative (e.g. Geneticin®, G418, etc.).

The natural type active human Factor VIII:C-producing transformants can also be selected and isolated by the above-mentioned methods in combination.

Alternatively a dhfr gene is inserted in advance into above-mentioned natural type human Factor VIII:C expression vector and this composite vector is inserted into the host cells. One can effectively integrate both of the natural type active human Factor VIII:C gene and the dhfr gene into adjacent positions in the chromosomes of the host cells.

In the transformant thus prepared, amplification of the natural type active Factor VIII:C gene in the chromosomes proceeds effectively. Thus, there can be obtained a transformant which can express the natural type human Factor VIII:C gene in higher yields.

When the transformants of the invention containing an amplifiable genes (e.g. dhfr gene, adenosine deaminase gene, etc.) are cultured under an appropriate condition suitable for amplification of gene (in case of dhfr gene, the amplification of gene is carried out in the presence of methotrexate in a concentration of 1 - 10,000 nM), the natural type active human Factor VIII:C gene carried in the transformants as well as the dhfr gene can be amplified. As a result, the transformant produces a large amount of desired protein due to the increased gene dosage, and hence, there can be produced the desired recombinant human Factor VIII:C in the culture medium in a large amount.

The transformants expressing the natural type active human Factor VIII:C gene can also be cultivated in a non-serum medium like in the conventional serum-containing medium to give the desired recombinant human Factor VIII:C. In addition, the transformants can also be cultivated in a suspension with a carrier or without a carrier.

The cultivation of the transformants may preferably be carried out by adding a substance for increasing the

amount of the desired recombinant human Factor VIII:C accumulated in the medium. Such substances include preferably various types of proteins, such as albumin, von Willebrand factor and further include polyethylene glycol, sodium selenite, protease inhibitors such as ε-aminocaproic acid, PMSF (phenylmethanesulfonyl fluoride), and aprotinin, and cyclodextrins, and the like.

The recombinant human Factor VIII:C according to the present invention, i.e., the expression product of natural type active human Factor VIII:C gene, is synthesized as a single chain in the transformant. Some population of such expression products may be secreted into the culture medium in the form of a single chain. Other population of the products may be secreted in the form of plural-chains resulting from proteolytic cleavage in the transformant. In the case of 740 Arg-1649 Glu Factor VIII:C, the proteolytic cleavage may predominantly occur at the peptide bond between Arg-740 and Glu-1649, in which the two chains are linked to each other via a calcium-ion bridge. The resulting two chains-form active Factor VIII:C is considered to have the same structure as active Factor VIII:C molecular species in the human blood plasma as discovered by L.-O. Andersson et al (Proc. Natl. Acad. Sci. USA 83, 2979-2983, 1986).

The degree of the proteolytic processing in the transformant can be controlled through optimizing the conditions for the cultivation.

The recombinant Factor VIII:C protein produced and accumulated in the culture medium can be purified by removing the cells from the culture medium, concentrating the medium and subjecting the supernatant thereof to a combination of a variety of conventional purification methods and strategies.

The conventional purification methods include a method utilizing a defference in solubility (e.g. salting-out, precipitation in solvent, etc.), a method utilizing a difference in molecular weight (e.g. dialysis, ultrafiltration, gel filtration, SDS-polyacrylamide gel electrophoresis, etc.), a method utilizing a difference in electric charge (e.g. ion exchange chromatography, etc.), a method utilizing specific affinity (e.g. affinity chromatography, etc.), a method utilizing a difference in hydrophobicity (e.g. reverse phase high performance liquid chromatography, etc.), a method utilizing a difference in isoelectric point (e.g. isoelectric electrophoresis, etc.), and the like.

While the recombinant Factor VIII:C of this invention may be administered in the form either of a single-chain form or of a two-chains form as they are produced, it should also be understood that it is within the scope of this invention to administer such recombinant Factor VIII:C after subjecting it to proteolytic cleavage <u>in vitro</u>. For example, the single-chain recombinant human

Factor VIII:C as produced can be cleaved *in vitro* into the appropriate two-chains form followed by linking the chains via a calcium or another alkaline earth metal bridge, before, during or after purification. The recombinant human Factor VIII:C of this invention may be formulated, using coventional methods, into pharmaceutically acceptable compositions.

Recombinant human Factor VIII:C-containing solution obtained by the present invention may optionally be lyophilized to give a powdery product. The lyophilization may be carried out in the presence of a stabilizer such as sorbitol, mannitol, dextrose, maltose, glycerol, human serum albumin (HSA), and the like, or a combination thereof.

The transformants of animal cells of the present invention can constantly and continuously produce the desired recombinant human Factor VIII:C in high yield and can grow rapidly in a cheap medium, and which can propagate substantially in a permanent manner. Thus, according to the present invention, the desired recombinant human Factor VIII:C can be produced on an industrial scale. Particularly, the present invention has made it possible on a commercial scale to produce a recombinant Factor VIII:C which is considered to correspond to the smallest species of active and intact Factor VIII:C molecules in the human blood plasma. The recombinant human Factor VIII:C produced by the present invention is useful for the treatment of bleeding in

hemophilia A patients who are deficient in Factor VIII:C.

The present invention is illustrated by the following Examples but should not be construed to be limited thereto.

The transformant E. coli HB101(RE) prepared by transforming with an expression vector plasmid Ad.RE.neo used in Examples has been deposited as ATCC 53517 by Biogen Inc.

Example 1

In a FALCON® bottle (culture flask) (cultivation surface area, 150 $cm^2$), DHFR(-) CHO cells DG44 (cf. G. Urlaub et al, Proc. Natl. Acad. Sci. USA 77, 4216 - 4220, 1980, and others) were cultivated in Ham's F12 medium containing 10 % fetal bovine serum at 37°C overnight. After the cultivation, the cells were floated with trypsin and thereto were introduced plasmid Ad.RE.neo which is a natural type active human Factor VIII:C expression vector (20 µg per 3 x $10^6$ cells) and plasmid pSV2-dhfr (cf. Mol. Cell Biol. 1, 854 - 864, 1981) (10 µg per 3 x $10^6$ cells) by an electrophoration method by H. Potter et al (cf. Proc. Natl. Acad. Sci. USA 81, 7161 - 7165, 1984). The mixture was allowed to stand at room temperature for 5 minutes and thereafter was cultivated in Ham's F12 medium containing 10 % fetal bovine serum for 2 days. After replacing the medium by Ham's F12 medium for selection of DHFR(+) cells containing 10 % dialyzed fetal bovine serum but lacking

hypoxanthine, glycine and thymidine, the cultivation was continued at 37°C, during which the culture medium was changed at intervals of 3 to 4 days. After cultivating for about two weeks, the cells becoming DHFR(+) were grown to form colonies.

The DHFR(+) transformed cells thus obtained was further cultivated in the second selection medium, i.e. Ham's F12 medium containing 10 % fetal bovine serum and G418 (Geneticin®, manufactured by GIBCO, 400 μg/ml). After cultivating for 1 to 2 weeks, the G418-resistant cells, neo(+) transformed cells were grown to give colonies.

Cloning of the neo(+), DHFR(+) transformed cells was carried out by a known method (e.g. limited dilution method). After the cloning, the cloned cells were cultivated in Ham's F12 medium containing 10 % dialyzed fetal bovine serum and G418 (400 μg/ml) (lacking hypoxanthine, glycine and thymidine). The cloned cells were separated and cultivated to about 80 % confluence. After changing with a fresh medium, the cultivation was continued for 48 hours, and the activity of Factor VIII:C in the culture medium was measured by a synthetic substrate method (using Coatest® Factor VIII:C, manufactured by Kabi Vitrum) and activated partial thromboplastin coagulation time (APTT). The results are shown in Table 1.

The transformant RE.1A-3 shown in Table 1 has been deposited at Fermentation Research Institute, Agency of

Industrial Science and Technology, Japan, in deposit No. FERM P-9873 (as CHO-TK-0001) on February 17, 1988, which was re-deposited at the same depositary under Budapest Treaty as FERM BP-2014 on August 24, 1988.

Table 1

| Transformants | Activity of Factor VIII:C (mU/ml) | |
|---|---|---|
| | Synthetic substrate method | APTT |
| RE.1A-3 | 10.4 | 9.2 |
| RE.1A-5 | 8.8 | 8.4 |
| RE.1B-1 | 7.7 | 7.9 |
| RE.1B-3 | 10.0 | 9.9 |
| RE.1C-2 | 9.7 | 9.8 |
| RE.1C-3 | 12.5 | - |
| RE.1C-5 | 9.6 | - |
| RE.2A-3 | 8.4 | - |
| RE.2A-4 | 7.3 | - |
| RE.2A-6 | 10.8 | - |
| RE.2C-3 | 7.2 | - |
| RE.2D-6 | 9.5 | - |
| RE.3A-2 | 7.3 | - |
| RE.3C-6 | 7.7 | - |
| RE.3D-5 | 6.1 | - |

Example 2

The CHO cell line RE.1C-3 being capable of producing natural type human Factor VIII:C (740 Arg-1649 Glu Factor VIII:C) prepared in Example 1 was cultivated in Ham's F12 medium containing 20 nM methotrexate (MTX), 10 % dialyzed fetal bovine serum and G418 400 µg/ml but lacking

hypoxanthine, glycine and thymidine. The grown cells were subcultured in sequentially increasing concentrations of MTX in the medium starting 100 nM with step to 500 nM. The MTX-resistant clones were cultivated under the same conditions as used in Example 1, and the activity of Factor VIII:C in the culture medium was measured likewise. The results are shown in Table 2.

Table 2

| Trans-formants | Activity of Factor VIII:C (mU/ml) (measured by synthetic substrate method) | | | |
|---|---|---|---|---|
| | Original cells (RE.1C-3) | MTX 20 nM-resist. cells | MTX 100 nM-resist. cells | MTX 500 nM-resist. cells |
| RE.1C-3 | 12.5 | 20.7 | 26.4 | 42.0 |

Example 3

The CHO transformed cell line, RE.1C-3 prepared in Example 1 and clone RE.1C-3(500) derived from the MTX (500 nM)-resistant cells in Example 2, the activity of Factor VIII:C in the culture medium was measured with a lapse of time.

In a culture flask (cultivation surface area: 150 $cm^2$), CHO cells RE.1C-3 and CHO cells RE.1C-3(500) (each $10^5$ cells) were cultivated in Ham's F12 medium supplemented with 10 % fetal bovine serum (30 ml) at 37°C in a carbon dioxide incubator. From two days after the initiation of cultivation, the count of cells and the activity of Factor

VIII:C in the culture medium were examined everyday. The results are shown in the accompanying Fig. 3. As is shown in Fig. 3, Factor VIII:C was accumulated in the culture medium with growth of the cells, and even after growing of cells was stopped, Factor VIII:C was continuously produced. The maximum activity was 12.3 mU/ml in CHO cells RE.1C-3 and 42 mU/ml in CHO cells RE.1C-3(500).

Example 4

In a culture flask (cultivation surface area: 25 $cm^2$), DHFR(-) CHO cells DG44 and DHFR(-) CHO cells DXB11 were cultivated in MEM alpha medium containing the nucleosides (manufactured by GIBCO) supplemented with 10 % fetal bovine serum at 37°C overnight. After the cultivation, into the resulting cells were introduced a linearized Ad.RE.neo (10 μg) by digestion with Pvu I and a linearized pSV2-dhfr (0.5 μg) by digestion with Pvu I using the DNA-calcium phosphate co-precipitation method of F.L. Graham et al (cf. Virology, 52, 456, 1973).

After the gene introduction, the medium was changed with a fresh one, and the cells were fed at 37°C overnight. Thereafter, the medium was changed with a medium for selecting DHFR(+) neo(+) cell line, i.e. MEM alpha medium supplemented with 10 % dialized fetal bovine serum and G418 (400 μg/ml) but lacking nucleosides (manufactured by GIBCO), and the cultivation was continued at 37°C. After cultivation for about 2 weeks, DHFR(+) neo(+) cells were grown to form colonies.

The DHFR(+), neo(+) transformed CHO cells DG44 and CHO cells DXB11 thus isolated were cultivated in MEM alpha medium supplemented with 20 nM MTX, 10 % dialyzed fetal bovine serum and G418 (400 µg/ml) but lacking the nucleosides. The grown cells were continuously subjected to subculturing in stepwise increasing concentrations of MTX in the medium 100 nM and 500 nM. The cells were grown to about 80 % confluence. The medium was changed with a fresh one, and the cultivation was continued for 3 days, and thereafter, the activity of Factor VIII:C in the culture medium was measured by a synthetic substrate method. The results are shown in Table 3.

Table 3

| Host cells | Activity of Factor VIII:C (mU/ml) (measured by synthetic substrate method) | | | |
|---|---|---|---|---|
| | Original transformant | MTX 20 nM-resist. cells | MTX 100 nM-resist. cells | MTX 500 nM-resist. cells |
| CHO cells DG44 | 5.2 | 28 | 97 | 213 |
| CHO cells DXB11 | 0.4 | 1.9 | 30 | 105 |

The DHFR(+), neo(+) CHO cells DG44 (MTX 500 nM-resistant) was cloned to give CHO cells clones as shown in Table 4 which can produce recombinant Factor VIII:C (740 Arg-1649 Gln Factor VIII:C) in high levels.

Table 4

| Clones | Activity of Factor VIII:C (U/ml) (measured by synthetic substrate method) |
|---|---|
| RE-KSG-1H | 0.64 |
| RE-KSG-2D | 1.06 |
| RE-KSG-3C | 0.96 |
| RE-KSG-4C | 0.94 |
| RE-KSG-7A | 0.78 |

Example 5

We isolated a fragment of dihydrofolate reductase gene from dhfr-expressing plasmid pSV2-dhfr as used in Example 1 by digesting restriction enzymes Pvu II and EcoR I and then filled out 5'AATT overhang with $T_4$ DNA polymerase to make blunt ended DNA. A fragment carrying dhfr gene was isolated after 0.7 % agarose gel electrophoresis.

Separately, a natural type active human Factor VIII:C-expressing plasmid Ad.RE.neo was digested with a restriction enzyme Sma I and then we inserted the above dhfr gene fragment into it with $T_4$ DNA ligase by which there was constructed plasmid Ad.RE.dhfr which is a vector being capable of expressing both natural type human Factor VIII:C gene and dihydrofolate reductase gene.

The plasmid Ad.RE.dhfr was made linearized by digesting with a restriction enzyme Pvu I, and the resulting linear DNA (10 µg) was mixed with a carrier DNA (calf thymus DNA, 10 µg) in 0.25 M $CaCl_2$ (100 µl), and thereto was added dropwise HEPES buffered saline (100 µl). The DNA-calcium

phosphate complex precipitate thus prepared was applied to DHFR(-) CHO cells DG44 that were previously subcultured in the same manner as described in Example 1.

The cells were cultivated for 15 hours, and after replacing the medium with MEM alpha medium containing no ribonuleosides and deoxyribonucleosides (manufactured by GIBCO) supplemented with 10 % dialyzed fetal bovine serum (hereinafter, referred to as "a medium for selecting dhfr-expressing cells"), the cultivation was continued to give DHFR(+)-transformed CHO cells RED44. After cultivating for 12 days, the activity of Factor VIII:C in the culture medium was measured by a synthetic substrate method. As a result, it was expressed in an amount of 10 mU/ml.

Subsequently, the RED44 cells were cloned by a limited dilution method.

After the cloning, the cells were plated in 24 wells culture plate (manufactured by Nunk) and were cultivated in a medium for selecting dhfr-expressing cells (1 ml). We grew each cloned cells to confluent, and the activity of Factor VIII:C in the culture medium was measured by a synthetic substrate method. The results are shown in Table 5.

Table 5

| Clones | Activity of Factor VIII:C (U/ml) (measured by synthetic substrate method) |
|---|---|
| A7 | 126 |
| D2 | 117 |
| D7 | 123 |
| D12 | 98 |
| 3C8 | 85 |
| 2E8 | 58 |

Example 6

Four clones of CHO cells RED44 being capable of producing recombinant Factor VIII:C isolated in Example 5 (shown in Table 5) were added to FALCON® 6 wells culture plate and cultivated in the medium for selecting DHFR-expressing cells which contained 10 nM methotrexate (MTX). The cells were cultured in sequentially increasing concentrations of MTX in the medium starting 50 nM with step to 100 nM. We grew cells to 95% confluence, three or four days after changing the medium, the activity of Factor VIII:C in the culture medium was measured likewise. The results are shown in Table 6.

Table 6

| Clones | Activity of Factor VIII:C (mU/ml) (measured by synthetic substrate method) | | |
|---|---|---|---|
| | MTX concentration: 10 nM | 50 nM | 100 nM |
| A7 | 490 | 800 | 1080 |
| D2 | 120 | 670 | 880 |
| D7 | 180 | 690 | 842 |
| D12 | 420 | 704 | 1100 |

Among the CHO cells being capable of producing natural type human Factor VIII:C, the CHO cells RED44. A7 shown in Table 6 was deposited at Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, in deposit No. FERM P-9935 (as CHO RED44.A7) on March 11, 1988, which was re-deposited at the same depositary as FERM BP-2016 on August 25, 1988.

Example 7

The MTX-resistant CHO cells RED44.A7 being capable of producing recombinant Factor VIII:C cloned in Example 5 were added to FALCON® 6 wells culture plate (#3046) and cultivated in the medium for selecting DHFR-expressing cells (4 ml) in the presence of 50 nM methotrexate, to which purified human von Willebrand factor was added in a concentration of 0, 1, 3, and 10 U/ml, respectively. While continuing the cultivation, the culture medium was intermittently taken out as a sample for test.

As to the conditioned medium samples thus taken

out, the activity of Factor VIII:C was measured by a synthetic substrate method, and further, an amount of antigen of Factor VIII:C (Factor VIII:C Ag) was measured by ELISA using an anti-Factor VIII:C monoclonal antibodies. Moreover, antigen levels of the Factor VIII:C - von Willebrand factor complex were measured by ELISA using a monoclonal anti-human Factor VIII:C as the first antibody and a rabbit polyclonal anti-human von Willebrand factor antibody (cf. Acta Heamatol. Jpn. 50, 1681 - 1688, 1987). The results of activity of Factor VIII:C measured by a synthetic substrate method are shown in the accompanying Fig. 5. The results of the amount of Factor VIII:C antigen measured by ELISA and the antigen level of Factor VIII:C - von Willebrand factor complex measured by ELISA are shown in Fig. 6 and Fig. 7, respectively.

Example 8

The MTX-resistant CHO cells RED44.A7 being capable of producing natural type human Factor VIII:C prepared in Example 5 were added to FALCON® 6 wells tissue culture plate and cultivated in the medium for selecting DHFR-expressing cells (3 ml) which contained 50 nM methotrexate for 24 hours. The medium was then removed and the cells were fed with commercially available non-serum medium (ASF medium 104, manufactured by Ajinomoto Co., Inc., 3 ml). While continuing the cultivation, the culture medium was intermittently taken out as a sample for test. As to the conditioned medium samples thus taken out, the activity of

Factor VIII:C was measured by a synthetic substrate method. As a control experiment, we used a conventional serum medium (MEM alpha medium containing 10 % fetal calf serum (FCS). The above assays were made at 3, 5 and 7 days after changing the medium. The results are shown in Table 7.

Table 7

| Medium | Activity of Factor VIII:C (mU/ml) (synthetic substrate method) | | |
|---|---|---|---|
| | After 3 days | 5 days | 7 days |
| ASF Medium 104 | 550 | 820 | 570 |
| 10 % FCS-containing MEM alpha medium | 50 | 240 | 270 |

In addition, in the same manner as described above except that to the non-serum medium was added various additives such as polyethylene glycol (PEG) 20,000, sodium selenite, ε-aminocaproic acid, phenylmethanesulfonyl fluoride (PMSF), human von Willebrand factor (h-vWF), bovine serum albumin (BSA), aprotinin or α-cyclodextrin and that the concentration of methotrexate was 100 nM, the MTX-resistant CHO cells RED44.A7 was cultivated, and the activity of Factor VIII:C in the culture medium was measured by a synthetic substrate method with lapse of time. The results are shown in Table 8 to Table 11.

Factor VIII:C antigen was examined in a similar experiment using aprotinin with the result that the expression levels when added with 100 to 10000 KIU/ml is two or three times as high as the control without addition of aprotinin.

Table 8 (Effects of each additive to non-serum medium)

| Additives | Activity of Factor VIII:C (mU/ml) (synthetic substrate method) | | |
|---|---|---|---|
| | After 2 days | 4 days | 9 days |
| PEG 20,000 (0.1 %) | 9 | 136 | 705 |
| Sodium selenite (13 μg/l) | 18 | 95 | 664 |
| PEG + sodium selenite | 33 | 123 | 830 |
| α-Cyclodextrin (0.1 %) | 10 | 150 | 953 |
| Non-additive | 15 | 104 | 498 |

Table 9 (Effects of addition of ε-aminocaproic acid to non-serum medium)

| Additives | Activity of Factor VIII:C (mU/ml) (synthetic substrate method) | | |
|---|---|---|---|
| | After 5 days | 7 days | 10 days |
| 1 mM ε-amino-caproic acid | 338 | 484 | 620 |
| Non-additive | 230 | 351 | 125 |

Table 10 (Effects of addition of PMSF, h-vWF to non-serum medium)

| Additives | Activity of Factor VIII:C (mU/ml) (synthetic substrate method) | | |
|---|---|---|---|
| | After 2 days | 4 days | 7 days |
| 0.1 mM PMSF | 103 | 381 | 784 |
| 2U h-vWF | 332 | 913 | 2910 |
| Non-additive | 148 | 290 | 747 |

Table 11 (Effects of addition of BSA to non-serum medium, cultivation for 2 days)

| Concentration of BSA (µg/ml) | Activity of Factor VIII:C (mU/ml) (synthetic substrate method) |
|---|---|
| 0 | 62 |
| 10 | 50 |
| 50 | 83 |
| 100 | 54 |
| 1000 | 249 |
| 10000 | 540 |

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. An animal cell line having been transformed with an expression vector containing a gene encoding natural type active human Factor VIII:C as hereinbefore defined in which Arg-740 of the carboxyl terminus of the H chain is directly bonded through a peptide bond with 1649 glutamic acid of the amino terminus of the L chain.

2. The cell line according to claim 1, wherein the gene encoding natural type active human Factor VIII:C has a signal peptide gene bonded thereto.

3. The cell line according to claim 1, wherein the expression vector contains further at least one enhancer.

4. The cell line according to claim 3, wherein the enhancer is an enhancer originated from an animal virus.

5. The cell line according to claim 1, wherein the promoter is a promoter originated from an animal virus.

6. The cell line according to claim 4 or 5, wherein the animal virus is a simian virus.

7. The cell line according to claim 5, wherein the animal virus is adenovirus.

8. The cell line according to claim 3, wherein the enhancer is an enhancer derived from SV40 promoter region.

9. The cell line according to claim 1, wherein the promoter is SV40 promoter.

10. The cell line according to claim 1, wherein the promoter is a hybrid promoter derived from adenovirus promoter region and SV40 promoter region.

11. The cell line according to claim 1, wherein the expression vector carries a selective marker gene in animal cells.

12. The cell line according to claim 11, wherein the selective marker gene in animal cells is a neomycin-resistant gene.

13. The cell line according to claim 11, wherein the selective marker gene is dihydrofolate reductase gene (dhfr).

14. The cell line according to claim 11, wherein the selective marker gene is neomycin-resistant gene and dihydrofolate reductase gene (dhfr).

15. The cell line according to claim 1, wherein the expression vector contains a gene-amplifiable gene.

16. The cell line according to claim 15, wherein the gene-amplifiable gene is dihydrofolate reductase gene (dhfr).

17. The cell line according to claim 1, wherein the animal cells are Chinese hamster cells.

18. The cell line according to claim 17, wherein the Chinese hamster cells are Chinese hamster ovary (CHO) cells.

19. The cell line according to claim 18, wherein the Chinese hamster ovary cells are deficient in dihydrofolate reductase activity (DHFR).

20. The cell line according to claim 19, wherein DHFR(-) CHO cells are DXB11 or DG44.

21. The cell line according to claim 1, wherein the transformant is a cotransformant.

22. A method for producing recombinant human Factor VIII:C as hereinbefore defined which comprises cultivating an animal cell line transformed with an expression vector containing a gene encoding natural type active human Factor VIII:C in which Arg-740 of the carboxyl terminus of the H chain is directly bonded through a peptide bond with 1649 glutamic acid of the amino terminus of the L chain.

23. The method according to claim 22, wherein the cultivation of the cell line is carried out under the conditions for amplificating the gene.

24. The method according to claim 22, wherein the cultivation of the cell line is carried out in a serum free medium.

25. The method according to claim 21, wherein the cultivation of the cell line is carried out by adding to the medium a substance selected from the group consisting of von Willbrand factor, albumin, polyethylene glycol, sodium selenite, ε-aminocapropic acid, phenylmethanesulfonyl fluoride (PMSF), aprotinin, and cyclodextrins.

26. A recombinant Factor VIII:C produced by the method as claimed in any one of claims 22 to 25.

27. A cell line according to claim 1, or a method according to claim 22, substantially as hereinbefore described with reference to the drawings and/or examples.

DATED this 11th day of February, 1992

JURIDICAL FOUNDATION THE CHEMO-SERO THERAPEUTIC RESEARCH INSTITUTE
and
TEIJIN LIMITED
By Their Patent Attorneys
DAVIES COLLISON CAVE

Fig. 1 (1)

```
  1                                          10
ala thr arg arg tyr tyr leu gly ala val glu leu ser
GCC ACC AGA AGA TAC TAC CTG GGT GCA GTG GAA CTG TCA

                           20
trp asp tyr met gln ser asp leu gly glu leu pro val
TGG GAC TAT ATG CAA AGT GAT CTC GGT GAG CTG CCT GTG

          30
asp ala arg phe pro pro arg val pro lys ser phe pro
GAC GCA AGA TTT CCT CCT AGA GTG CCA AAA TCT TTT CCA

 40                                             50
phe asn thr ser val val tyr lys lys thr leu phe val
TTC AAC ACC TCA GTC GTG TAC AAA AAG ACT CTG TTT GTA

                                  60
glu phe thr asp his leu phe asn ile ala lys pro arg
GAA TTC ACG GAT CAC CTT TTC AAC ATC GCT AAG CCA AGG

                     70
pro pro trp met gly leu leu gly pro thr ile gln ala
CCA CCC TGG ATG GGT CTG CTA GGT CCT ACC ATC CAG GCT

     80                                          90
glu val tyr asp thr val val ile thr leu lys asn met
GAG GTT TAT GAT ACA GTG GTC ATT ACA CTT AAG AAC ATG

                                    100
ala ser his pro val ser leu his ala val gly val ser
GCT TCC CAT CCT GTC AGT CTT CAT GCT GTT GGT GTA TCC
```

Fig. 1 (2)

```
                        110
tyr trp lys ala ser glu gly ala glu tyr asp asp gln
TAC TGG AAA GCT TCT GAG GGA GCT GAA TAT GAT GAT CAG

        120                                     130
thr ser gln arg glu lys glu asp asp lys val phe pro
ACC AGT CAA AGG GAG AAA GAA GAT GAT AAA GTC TTC CCT

                                    140
gly gly ser his thr tyr val trp gln val leu lys glu
GGT GGA AGC CAT ACA TAT GTC TGG CAG GTC CTG AAA GAG

                        150
asn gly pro met ala ser asp pro leu cys leu thr tyr
AAT GGT CCA ATG GCC TCT GAC CCA CTG TGC CTT ACC TAC

            160
ser tyr leu ser his val asp leu val lys asp leu asn
TCA TAT CTT TCT CAT GTG GAC CTG GTA AAA GAC TTG AAT

170                                     180
ser gly leu ile gly ala leu leu val cys arg glu gly
TCA GGC CTC ATT GGA GCC CTA CTA GTA TGT AGA GAA GGG

                            190
ser leu ala lys glu lys thr gln thr leu his lys phe
AGT CTG GCC AAG GAA AAG ACA CAC ACC TTG CAC AAA TTT

                200
ile leu leu phe ala val phe asp glu gly lys ser trp
ATA CTA CTT TTT GCT GTA TTT GAT GAA GGG AAA AGT TGG

    210                                     220
his ser glu thr lys asn ser leu met gln asp arg asp
CAC TCA GAA ACA AAG AAC TCC TTG ATG CAG GAT AGG GAT
```

Fig. 1 (3)

```
                                        230
ala ala ser ala arg ala trp pro lys met his thr val
GCT GCA TCT GCT CGG GCC TGG CCT AAA ATG CAC ACA GTC

                    240
asn gly tyr val asn arg ser leu pro gly leu ile gly
AAT GGT TAT GTA AAC AGG TCT CTA CCA GGT CTG ATT GGA

        250                                         260
cys his arg lys ser val tyr trp his val ile gly met
TGC CAC AGG AAA TCA GTC TAT TGG CAT GTG ATT GGA ATG

                                    270
gly thr thr pro glu val his ser ile phe leu glu gly
GGC ACC ACT CCT GAA GTG CAC TCA ATA TTC CTC GAA GGT

                    280
his thr phe leu val arg asn his arg gln ala ser leu
CAC ACA TTT CTT GTG AGG AAC CAT CGC CAG GCG TCC TTG

            290
glu ile ser pro ile thr phe leu thr ala gln thr leu
GAA ATC TCG CCA ATA ACT TTC CTT ACT GCT CAA ACA CTC

300                                         310
leu met asp leu gly gln phe leu leu phe cys his ile
TTG ATG GAC CTT GGA CAG TTT CTA CTG TTT TGT CAT ATC

                                320
ser ser his gln his asp gly met glu ala tyr val lys
TCT TCC CAC CAA CAT GAT GGC ATG GAA GCT TAT GTC AAA

                330
val asp ser cys pro glu glu pro gln leu arg met lys
GTA GAC AGC TGT CCA GAG GAA CCC CAA CTA CGA ATG AAA
```

Fig. 1 (4)

```
    340                                                 350
asn asn glu glu ala glu asp tyr asp asp asp leu thr
AAT AAT GAA GAA GCG GAA GAC TAT GAT GAT GAT CTT ACT

                                    360
asp ser glu met asp val val arg phe asp asp asp asn
GAT TCT GAA ATG GAT GTG GTC AGG TTT GAT GAT GAC AAC

                        370
ser pro ser phe ile gln ile arg ser val ala lys lys
TCT CCT TCC TTT ATC CAA ATT CGC TCA GTT GCC AAG AAG

        380                                             390
his pro lys thr trp val his tyr ile ala ala glu glu
CAT CCT AAA ACT TGG GTA CAT TAC ATT GCT GCT GAA GAG

                                        400
glu asn trp asp tyr ala pro leu val leu ala pro asp
GAG GAC TGG GAC TAT GCT CCC TTA GTC CTC GCC CCC CAT

                            410
asp arg ser tyr lys ser gln tyr leu asn asn gly pro
GAC AGA AGT TAT AAA AGT CAA TAT TTG AAC AAT GGC CCT

                420
gln arg ile gly arg lys tyr lys lys val arg phe met
CAG CGG ATT GGT AGG AAG TAC AAA AAA GTC CGA TTT ATG

430                                             440
ala try thr asp glu thr phe lys thr arg glu ala ile
GCA TAC ACA GAT GAA ACC TTT AAG ACT CGT GAA GCT ATT

                                    450
gln his glu ser gly ile leu gly pro leu leu tyr gly
CAG CAT GAA TCA GGA ATC TTG GGA CCT TTA CTT TAT GGG
```

Fig. 1 (5)

```
                    460
glu val gly asp thr leu leu ile ile phe lys asn gln
GAA GTT GGA GAC ACA CTG TTG ATT ATA TTT AAG AAT CAA

    470                                         480
ala ser arg pro tyr asn ile tyr pro his gly ile thr
GCA AGC AGA CCA TAT AAC ATC TAC CCT CAC GGA ATC ACT

                                    490
asp val arg pro leu tyr ser arg arg leu pro lys gly
GAT GTC CGT CCT TTG TAT TCA AGG AGA TTA CCA AAA GGT

                        500
val lys his leu lys asp phe pro ile leu pro gly glu
GTA AAA CAT TTG AAG GAT TTT CCA ATT CTG CCA GGA GAA

        510                                     520
ile phe lys tyr lys trp thr val thr val glu asp gly
ATA TTC AAA TAT AAA TGG ACA GTG ACT GTA GAA GAT GGG

                                        530
pro thr lys ser asp pro arg cys leu thr arg tyr tyr
CCA ACT AAA TCA GAT CCT CGG TGC CTG ACC CGC TAT TAC

                            540
ser ser phe val asn met glu arg asp leu ala ser gly
TCT AGT TTC GTT AAT ATG GAG AGA GAT CTA GCT TCA GGA

                550
leu ile gly pro leu leu ile cys tyr lys glu ser val
CTC ATT GGC CCT CTC CTC ATC TGC TAC AAA GAA TCT GTA

560                                         570
asp gln arg gly asn gln ile met ser asp lys arg asn
GAT CAA AGA GGA AAC CAG ATA ATG TCA GAC AAG AGG AAT
```

Fig. 1 (6)

```
                                580
val ile leu phe ser val phe asp glu asn arg ser trp
GTC ATC CTG TTT TCT GTA TTT GAT GAG AAC CGA AGC TGG

                590
tyr leu thr glu asn ile gln arg phe leu pro asn pro
TAC CTC ACA GAG AAT ATA CAA CGC TTT CTC CCC AAT CCA

    600                                         610
ala gly val gln leu glu asp pro glu phe gln ala ser
GCT GGA GTG CAG CTT GAG GAT CCA GAG TTC CAA GCC TCC

                                    620
asn ile met his ser ile asn gly tyr val phe asp ser
AAC ATC ATG CAC AGC ATC AAT GGC TAT GTT TTT GAT AGT

                        630
leu gln leu ser val cys leu his glu val ala tyr trp
TTG CAG TTG TCA GTT TGT TTG CAT GAG GTG GCA TAC TGG

        640                                         650
tyr ile leu ser ile gly ala gln thr asp phe leu ser
TAC ATT CTA AGC ATT GGA GCA CAG ACT GAC TTC CTT TCT

                                        660
val phe phe ser gly tyr thr phe lys his lys met val
GTC TTC TTC TCT GGA TAT ACC TTC AAA CAC AAA ATG GTC

                            670
tyr glu asp thr leu thr leu phe pro phe ser gly glu
TAT GAA GAC ACA CTC ACC CTA TTC CCA TTC TCA GGA GAA

            680
thr val phe met ser met glu asn pro gly leu trp ile
ACT GTC TTC ATG TCG ATG GAA AAC CCA GGT CTA TGG ATT
```

Fig. 1 (7)

690 700
leu gly cys his asn ser asp phe arg asn arg gly met
CTG GGG TGC CAC AAC TCA GAC TTT CGG AAC AGA GGC ATG

710
thr ala leu leu lys val ser ser cys asp lys asn thr
ACC GCC TTA CTG AAG GTT TCT AGT TGT GAC AAG AAC ACT

720
gly asp tyr tyr glu asp ser tyr glu asp ile ser ala
GGT GAT TAT TAC GAG GAC AGT TAT GAA GAT ATT TCA GCA

730 740 1649
tyr leu leu ser lys asn asn ala ile glu pro arg glu
TAC TTG CTG AGT AAA AAC AAT GCC ATT GAA CCA AGA GAA

1650 1660
ile thr arg thr thr leu gln ser asp gln glu glu ile
ATA ACT CGT ACT ACT CTT CAG TCA GAT CAA GAG GAA ATT

1670
asp tyr asp asp thr ile ser val glu met lys lys glu
GAC TAT GAT GAT ACC ATA TCA GTT GAA ATG AAG AAG GAA

1680
asp phe asp ile tyr asp glu asp glu asn gln ser pro
GAT TTT GAC ATT TAT GAT GAG GAT GAA AAT CAG AGC CCC

1690 1700
arg ser phe gln lys lys thr arg his tyr phe ile ala
CGC AGC TTT CAA AAG AAA ACA CGA CAC TAT TTT ATT GCT

1710
ala val glu arg leu trp asp tyr gly met ser ser ser
GCA GTG GAG AGG CTC TGG GAT TAT GGG ATG AGT AGC TCC

Fig. 1 (8)

```
                    1720
pro his val leu arg asn arg ala gln ser gly ser val
CCA CAT GTT CTA AGA AAC AGG GCT CAG AGT GGC AGT GTC

        1730                                    1740
pro gln phe lys lys val val phe gln glu phe thr asp
CCT CAG TTC AAG AAA GTT GTT TTC CAG GAA TTT ACT GAT

                                        1750
gly ser phe thr gln pro leu tyr arg gly glu leu asn
GGC TCC TTT ACT CAG CCC TTA TAC CGT GGA GAA CTA AAT

                        1760
glu his leu gly leu leu gly pro tyr ile arg ala glu
GAA CAT TTG GGA CTC CTG GGG CCA TAT ATA AGA GCA GAA

            1770
val glu asp asn ile met val thr phe arg asn gln ala
GTT GAA GAT AAT ATC ATG GTA ACT TTC AGA AAT CAG GCC

1780                                        1790
ser arg pro tyr ser phe tyr ser ser leu ile ser tyr
TCT CGT CCC TAT TCC TTC TAT TCT AGC CTT ATT TCT TAT

                                1800
glu glu asp gln arg gln gly ala glu pro arg lys asn
GAG GAA GAT CAG AGG CAA GGA GCA GAA CCT AGA AAA AAC

                    1810
phe val lys pro asn glu thr lys thr tyr phe trp lys
TTT GTC AAG CCT AAT GAA ACC AAA ACT TAC TTT TGG AAA

    1820                                        1830
val gln his his met ala pro thr lys asp glu phe asp
GTG CAA CAT CAT ATG GCA CCC ACT AAA GAT GAG TTT GAC
```

Fig. 1 (9)

```
                                        1840
cys lys ala trp ala tyr phe ser asp val asp leu glu
TGC AAA GCC TGG GCT TAT TTC TCT GAT GTT GAC CTG GAA

                    1850
lys asp val his ser gly leu ile gly pro leu leu val
AAA GAT GTG CAC TCA GGC CTG ATT GGA CCC CTT CTG GTC

        1860                                        1870
cys his thr asn thr leu asn pro ala his gly arg gln
TGC CAC ACT AAC ACA CTG AAC CCT GCT CAT GGG AGA CAA

                                            1880
val thr val gln glu phe ala leu phe leu thr ile phe
GTG ACA GTA CAG GAA TTT GCT CTG TTT CTC ACC ATC TTT

                        1890
asp glu thr lys ser trp tyr phe thr glu asn met glu
GAT GAG ACC AAA AGC TGG TAC TTC ACT GAA AAT ATG GAA

            1900
arg asn cys arg ala pro cys asn ile gln met glu asp
AGA AAC TGC AGG GCT CCC TGC AAT ATC CAG ATG GAA GAT

1910                                        1920
pro thr phe lys glu asn tyr arg phe his ala ile asn
CCC ACT TTT AAA GAG AAT TAT CGC TTC CAT GCA ATC AAT

                                1930
gly tyr ile met asp thr leu pro gly leu val met ala
GGC TAC ATA ATG GAT ACA CTA CCT GGC TTA GTA ATG GCT

                    1940
gln asp gln arg ile arg trp tyr leu leu ser met gly
CAG GAT CAA AGG ATT CGA TGG TAT CTG CTC AGC ATG GGC
```

Fig. 1 (10)

```
    1950                                                1960
ser asn glu asn ile his ser ile his phe ser gly his
AGC AAT GAA AAC ATC CAT TCT ATT CAT TTC AGT GGA CAT

                                    1970
val phe thr val arg lys lys glu glu tyr lys met ala
GTG TTC ACT GTA CGA AAA AAA GAG GAG TAT AAA ATG GCA

                        1980
leu tyr asn leu tyr pro gly val phe glu thr val glu
CTG TAC AAT CTC TAT CCA GGT GTT TTT GAG ACA GTG GAA

                                                        2000
met leu pro ser lys ala gly ile trp arg val glu cys
ATG TTA CCA TCC AAA GCT GGA ATT TGG CGG GTG GAA TGC

                                        2010
leu ile gly glu his leu his ala gly met ser thr leu
CTT ATT GGC GAG CAT CTA CAT GCT GGG ATG AGC ACA CTT

                            2020
phe leu val tyr ser asn lys cys gln thr pro leu gly
TTT CTG GTG TAC AGC AAT AAG TGT CAG ACT CCC CTG GGA

                2030
met ala ser gly his ile arg asp phe gln ile thr ala
ATG GCT TCT GGA CAC ATT AGA GAT TTT CAG ATT ACA GCT

2040                                                2050
ser gly gln tyr gly gln trp ala pro lys leu ala arg
TCA GGA CAA TAT GGA CAG TGG GCC CCA AAG CTG GCC AGA

                                    2060
leu his tyr ser gly ser ile asn ala trp ser thr lys
CTT CAT TAT TCC GGA TCA ATC AAT GCC TGG AGC ACC AAG
```

Fig. 1 (11)

```
                2070
glu pro phe ser trp ile lys val asp leu leu ala pro
GAG CCC TTT TCT TGG ATC AAG GTG GAT CTG TTG GCA CCA

    2080                                        2090
met ile ile his gly ile lys thr gln gly ala arg gln
ATG ATT ATT CAC GGC ATC AAG ACC CAG GGT GCC CGT CAG

                                    2100
lys phe ser ser leu tyr ile ser gln phe ile ile met
AAG TTC TCC AGC CTC TAC ATC TCT CAG TTT ATC ATC ATG

                        2110
tyr ser leu asp gly lys lys trp gln thr tyr arg gly
TAT AGT CTT GAT GGG AAG AAG TGG CAG ACT TAT CGA GGA

        2120                                        2130
asn ser thr gly thr leu met val phe phe gly asn val
AAT TCC ACT GGA ACC TTA ATG GTC TTC TTT GGC AAT GTG

                                        2140
asp ser ser gly ile lys his asn ile phe asn pro pro
GAT TCA TCT GGG ATA AAA CAC AAT ATT TTT AAC CCT CCA

                            2150
ile ile ala arg tyr ile arg leu his pro thr his tyr
ATT ATT GCT CGA TAC ATC CGT TTG CAC CCA ACT CAT TAT

            2160
ser ile arg ser thr leu arg met glu leu met gly cys
AGC ATT CGC AGC ACT CTT CGC ATG GAG TTG ATG GGC TGT

2170                                        2180
asp leu asn ser cys ser met pro leu gly met glu ser
GAT TTA AAT AGT TGC AGC ATG CCA TTG GGA ATG GAG AGT
```

Fig. 1 (12)

```
                                   2190
lys ala ile ser asp ala gln ile thr ala ser ser tyr
AAA GCA ATA TCA GAT GCA CAG ATT ACT GCT TCA TCC TAC

                  2200
phe thr asn met phe ala thr trp ser pro ser lys ala
TTT ACC AAT ATG TTT GCC ACC TGG TCT CCT TCA AAA GCT

    2210                                        2220
arg leu his leu gln gly arg ser asn ala trp arg pro
CGA CTT CAC CTC CAA GGG AGG AGT AAT GCC TGG AGA CCT

                                       2230
gln val asn asn pro lys glu trp leu gln val asp phe
CAG GTG AAT AAT CCA AAA GAG TGG CTG CAA GTG GAC TTC

                       2240
gln lys thr met lys val thr gly val thr thr gln gly
CAG AAG ACA ATG AAA GTC ACA GGA GTA ACT ACT CAG GGA

        2250                                        2260
val lys ser leu leu thr ser met tyr val lys glu phe
GTA AAA TCT CTG CTT ACC AGC ATG TAT GTG AAG GAG TTC

                                          2270
leu ile ser ser ser gln asp gly his gln trp thr leu
CTC ATC TCC AGC AGT CAA GAT GGC CAT CAG TGG ACT CTC

                          2280
phe phe gln asn gly lys val lys val phe gln gly asn
TTT TTT CAG AAT GGC AAA GTA AAG GTT TTT CAG GGA AAT

              2290
gln asp ser phe thr pro val val asn ser leu asp pro
CAA GAC TCC TTC ACA CCT GTG GTG AAC TCT CTA GAC CCA
```

Fig. 1 (13)

```
2300                                ecoPI      2310
pro leu leu thr arg tyr leu arg ile his pro gln ser
CCG TTA CTG ACT CGC TAC CTT CGA ATT CAC CCC CAG AGT

                                    2320
trp val his gln ile ala leu arg met glu val leu gly
TGG GTG CAC CAG ATT GCC CTG AGG ATG GAG GTT CTG GGC

                    2330    2332
cys glu ala gln asp leu tyr end
TGC GAG GCA CAG GAC CTC TAC TGA
```

Fig. 2(a)

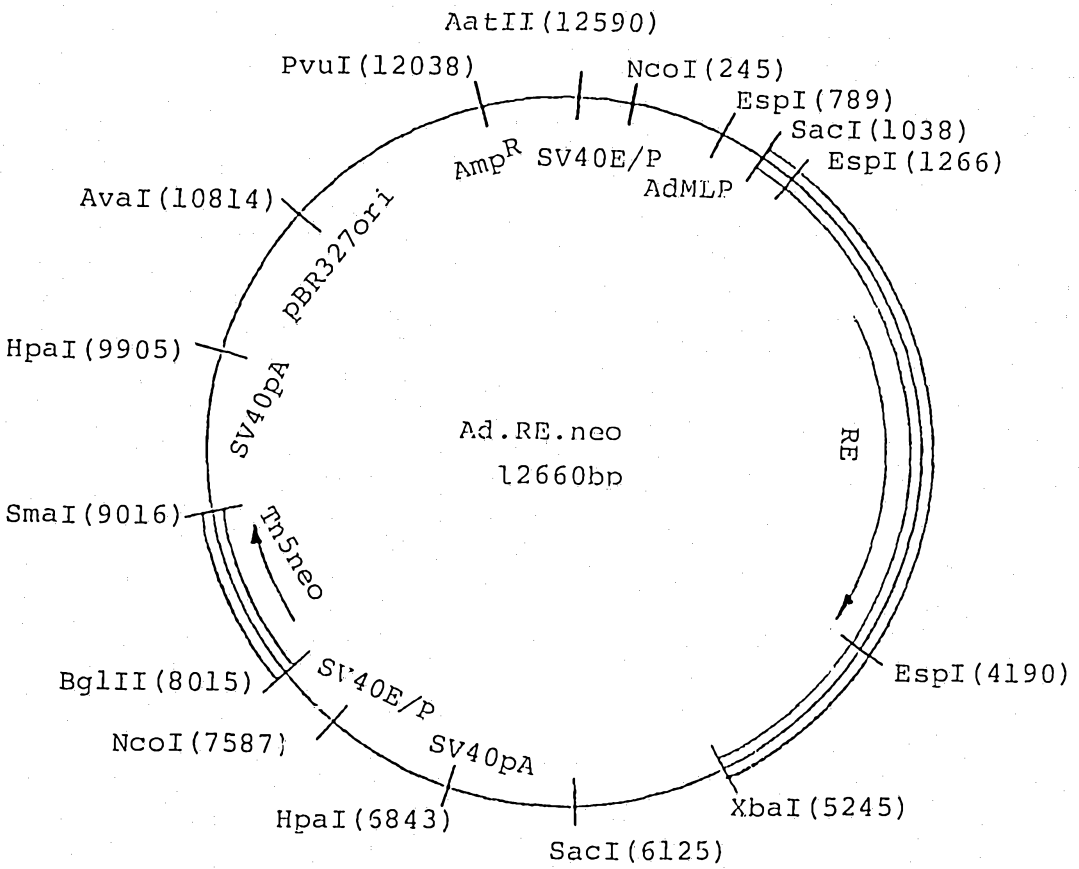

AatII(12590)
PvuI(12038)
NcoI(245)
EspI(789)
SacI(1038)
EspI(1266)
AmpR
SV40E/P
AdMLP
AvaI(10814)
pBR327ori
HpaI(9905)
SV40pA
Ad.RE.neo
12660bp
RE
SmaI(9016)
Tn5neo
BglII(8015)
SV40E/P
NcoI(7587)
SV40pA
EspI(4190)
HpaI(6843)
XbaI(5245)
SacI(6125)

Fig. 2(b)

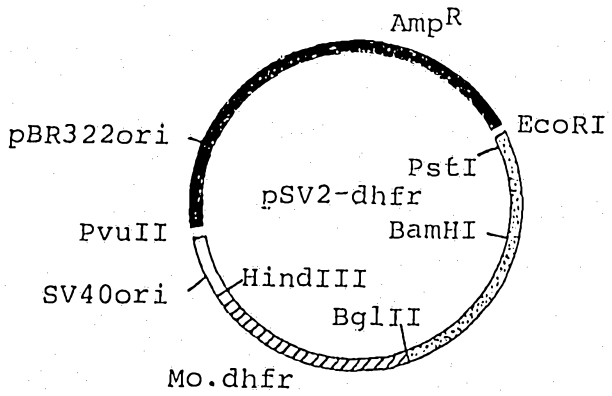

AmpR
EcoRI
pBR322ori
PstI
pSV2-dhfr
BamHI
PvuII
HindIII
SV40ori
BglII
Mo.dhfr

Fig. 3

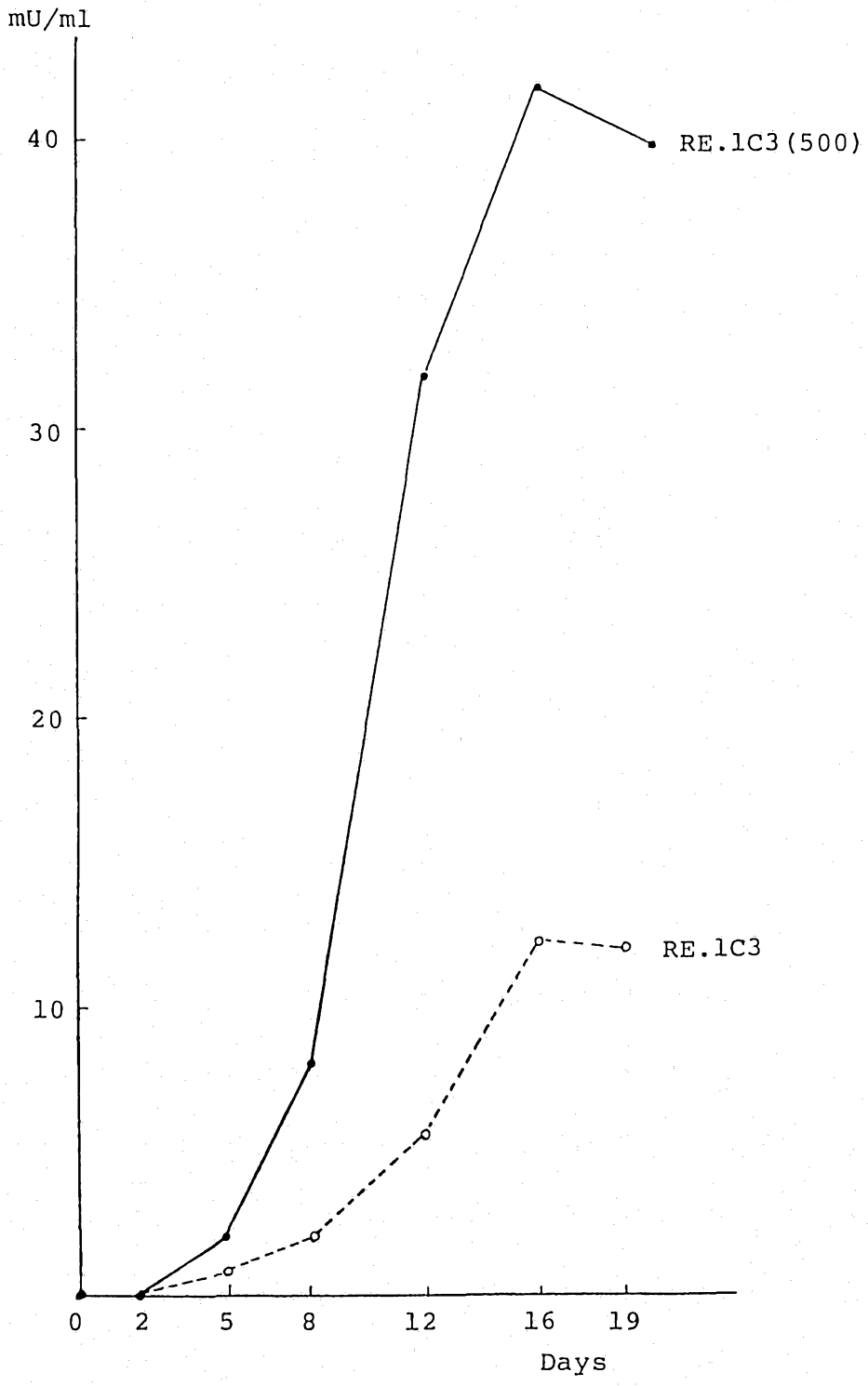


Fig. 4

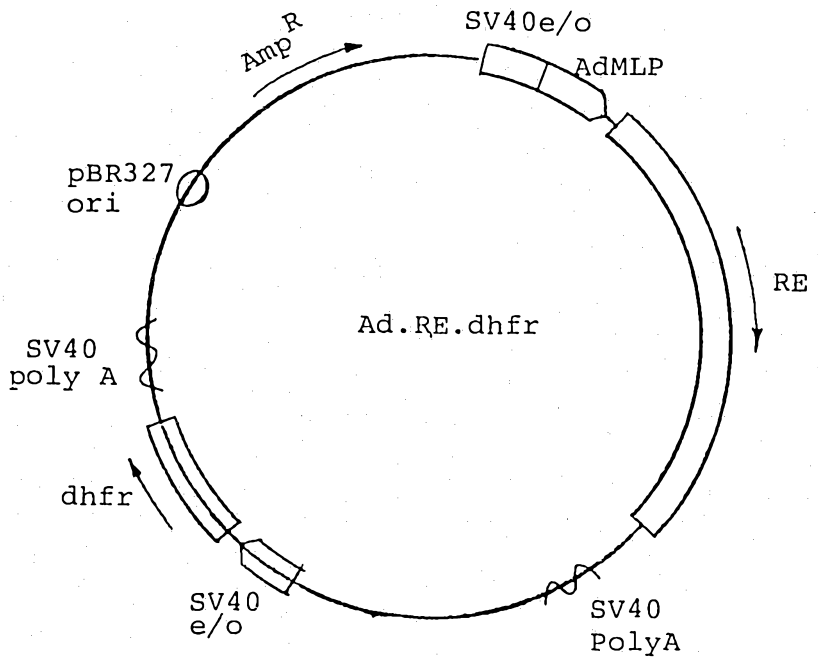

Amp R
SV40e/o
AdMLP
pBR327
ori
RE
Ad.RE.dhfr
SV40
poly A
dhfr
SV40
e/o
SV40
PolyA

Fig. 5

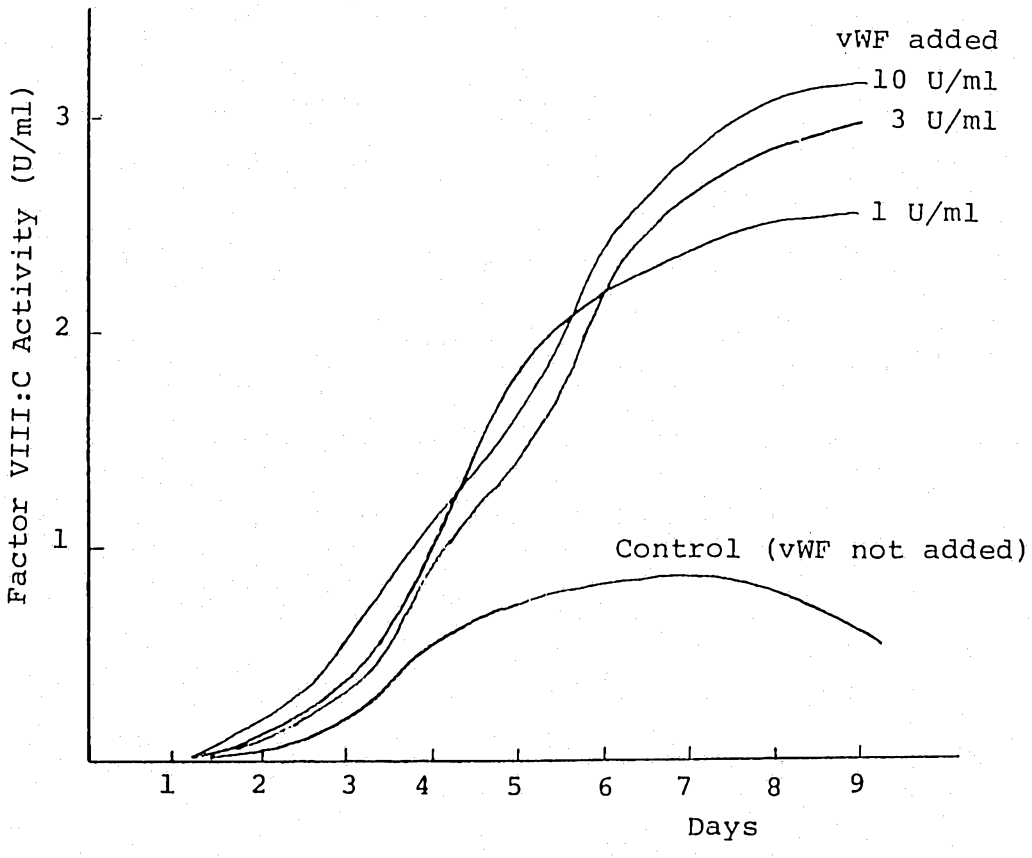

vWF added
10 U/ml
3 U/ml
1 U/ml
Control (vWF not added)
Factor VIII:C Activity (U/ml)
1
2
3
1
2
3
4
5
6
7
8
9
Days

Fig. 6

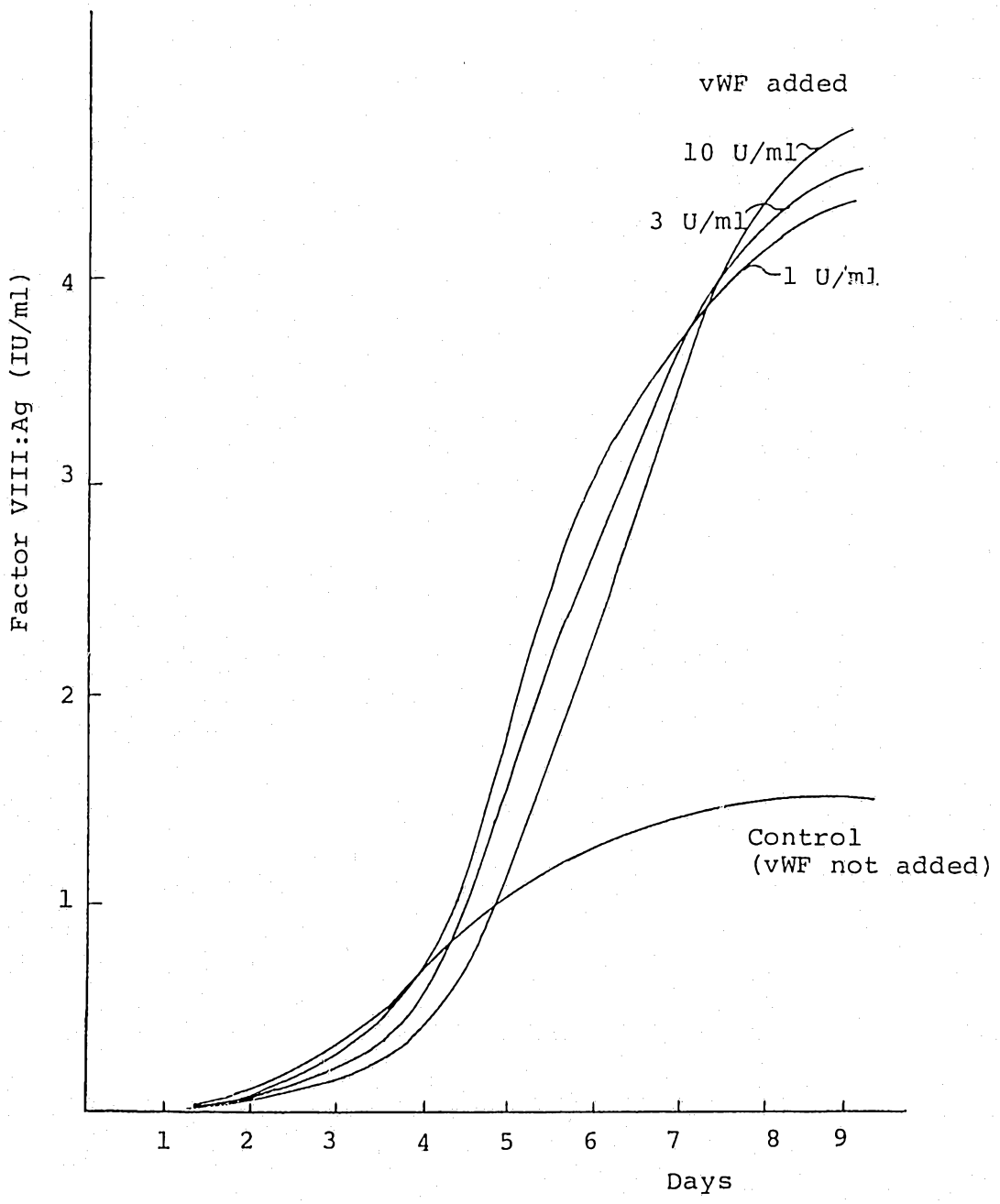
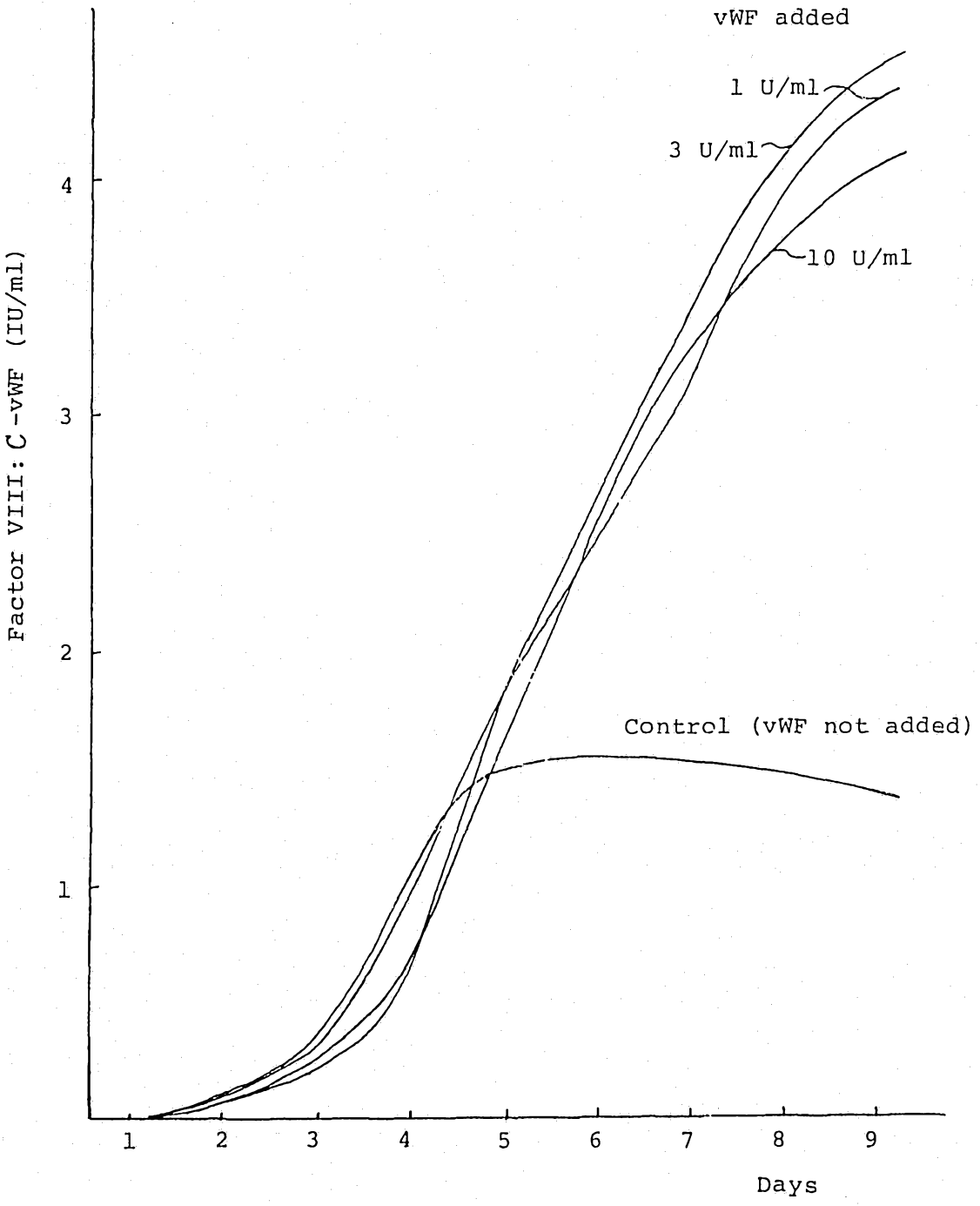

vWF added
10 U/ml
3 U/ml
1 U/ml
Control
(vWF not added)
Factor VIII:Ag (IU/ml)
1
2
3
4
1
2
3
4
5
6
7
8
9
Days

Fig. 7


vWF added
1 U/ml
3 U/ml
10 U/ml
Control (vWF not added)
Factor VIII: C -vWF (IU/ml)
1
2
3
4
1
2
3
4
5
6
7
8
9
Days