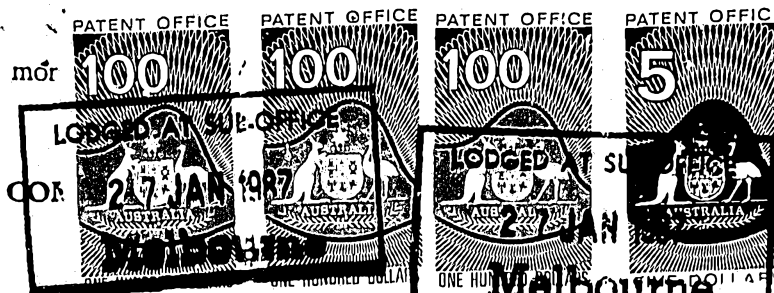


(CONVENTION. By one or mor

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CONVENTION APPLICATION FOR A PATENT

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27 JAN 1987
Melbourne

(1) Here insert (in full) Name of Applicant or Applicants, followed by Address (es).

R
We (1) CHIRON CORPORATION and NORDISK GENTOFTE,
of 4560 Horton Street, Emeryville, California 94608
United States of America and Niels Steensensvej
1, DK-2820 Gentofte, Denmark, respectively.

(2) Here insert Title of Invention.

hereby apply for the grant of a Patent for an invention entitled: (2)
METHOD OF PRODUCING A RECOMBINANT PROTEIN COMPLEX HAVING
HUMAN FACTOR VIII:C ACTIVITY

(3) Here insert number(s) of basic application(s)

which is described in the accompanying complete specification. This application is a
Convention application and is based on the application numbered (3)
822,989

(4) Here insert Name of basic Country or Countries, and basic date or dates

for a patent or similar protection made in (4) United States of America
on 27th January 1986

APPLICATION ACCEPTED AND AMENDMENTS

ALLOWED 20-12-90

My
Our address for service is Messrs. Edwd. Waters & Sons, Patent Attorneys,
50 Queen Street, Melbourne, Victoria, Australia.

DATED this 23rd day of January 1987

(5) Signature (s) of Applicant (s) or Seal of Company and Signatures of its Officers as prescribed by its Articles of Association.

(5)

CHIRON CORPORATION and NORDISK GENTOFTE

by

Louis C. Gebhardt

Registered Patent Attorney

To:

THE COMMISSIONER OF PATENTS

COMMONWEALTH OF AUSTRALIA

THE PATENTS ACT 1952

DECLARATION IN SUPPORT OF A
CONVENTION APPLICATION FOR A PATENTAUSTRALIA
CONVENTION
STANDARD
& PETTY PATENT
DECLARATION
SFP 4

In support of the Convention Application made for a
patent for an invention entitled: METHOD OF PRODUCING A RECOMBINANT PROTEIN
COMPLEX HAVING HUMAN FACTOR VIII:C ACTIVITY

Title of Invention

I, HENRY BRENNUM, President of 1
~~xxx~~ NORDISK GENTOFTE

Full name(s) and
address(es) of
Declarant(s)

Of Niels Steensensvej 1, DK-2820 Gentofte, Denmark

do solemnly and sincerely declare as follows:-

Full name(s) of
Applicant(s)

1. ~~xxxxxx the applicant(s) for the patent~~

(or, in the case of an application by a body corporate)

1. ~~xxxx~~ We are authorised by NORDISK GENTOFTE

CO-

the applicant(s) for the patent to make this declaration on
its/~~their~~ behalf.

2. The basic application(s) as defined by Section 141 of the
Act was/~~were~~ made

Basic Country(ies)

in the United States of America

Priority Date(s)

on 27 January 1986

Basic Applicant(s)

by RAE LYN BURKE and MIRELLA EZBAN RASMUSSEN

Full name(s) and
address(es) of
inventor(s)

~~xxxxxx We are the actual inventor(s) of the invention referred
to in the basic application(s)~~

~~(xxxxxx person(s) who the applicant)~~

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~~is~~ are the actual inventor(s) of the invention and the facts upon
which the/~~CO-~~applicant(s) is/~~are~~ entitled to make the application are
as follows:

The applicant is the assignee of the inventors.

Set out how Applicant(s)
derive title from actual
inventor(s) e.g. The
Applicant(s) is/are the
assignee(s) of the
invention from the
inventor(s)

4. The basic application(s) referred to in paragraph 2 of this
Declaration was/were the first application(s) made in a Convention
country in respect of the invention(s) the subject of the application.

✓ Declared at Torshavn this 12 day of March 19 87

To: The Commissioner of Patents

Signature of Declarant(s)

11/81

(12) PATENT ABRIDGMENT (11) Document No. AU-B-68014/87
(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 610059

(54) Title
HUMAN FACTOR VIII C

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(56) Prior Art Documents
AU 75754/87 C12N 15/00 C12P 21/02
AU 50253/85 C07K 15/12
EP 150735

(57) Factor VIII:C is a very large glycoprotein (native M_r 330-360 kiloDaltons (kD)), which is present in plasma at extremely low concentrations. The protein is very susceptible to cleavage by thrombin, plasmin, protease C, and other serine proteases. It is generally isolated from plasma or plasma products as a series of related polypeptides ranging from 160-40kD with predominant species of 92kD and 80-77kD.

CLAIM

1. A method of producing a recombinant protein complex having human Factor VIII:C activity but lacking all or part of the B domain of human Factor VIII:C comprising co-expressing in trans in a eukaryotic transformant host cell.

(a) a first gene encoding a signal sequence and a polypeptide having substantially the same amino acid sequence as the A domain of human Factor VIII:C and

optionally including an N-terminal portion of the B domain up to amino acid 1405 of human Factor VIII:C; and

(b) a second gene encoding a signal sequence and a polypeptide having substantially the same amino acid sequence as the C domain of human Factor VIII:C; and ^{isolating the secreted} ~~secreting the recombinant~~ protein complex.

2. A method according to claim 1, wherein not more than about 5 number% of the amino acids of said amino acid sequences have been substituted with natural amino acids other than the amino acids specified by the naturally occurring amino acid sequences of the Factor VIII:C A and C domains at the positions of substitution.

3. The method of claim 1 wherein the amino acid sequence of the polypeptide encoded by the second gene is the same as the amino acid sequence of amino acids 1649-2322 of human Factor VIII:C.

4. The method of claim 3 wherein the amino acid sequence of the polypeptide encoded by the first gene is the same as the amino acid sequence of amino acids 1-740 of human Factor VIII:C.

11. The method of any of claims 1-10 wherein the first gene and second gene are in separate expression plasmids.

12. A method for treating an individual requiring Factor VIII:C activity comprising administering to the individual a sufficient amount of a protein complex having human Factor VIII:C activity but lacking all or part of the B domain of human Factor VIII:C prepared by the method of any one of claims 1 to 11 to enhance blood clotting activity in the individual.

13. A DNA composition comprising first and second expression cassettes, said first express cassette comprising a gene encoding a signal sequence and a polypeptide comprising substantially the same sequence as the A-domain of human Factor VIII:C and optionally an N-terminal portion

(11) AU-B-68014/87

-3-

(10) 610059

of the B-domain of human Factor VIII:C under transcriptional and translational regulatory signals functional in a mammalian host cell, and said second expression cassette comprising a gene encoding a signal sequence and a polypeptide comprising substantially the same sequence as the C-domain of human Factor VIII:C and not including the B-domain under transcriptional and translational regulatory signals functional in said mammalian host cell.

COMMONWEALTH OF AUSTRALIA

PATENTS ACT 1952-69

610059

Form 10

COMPLETE SPECIFICATION

(ORIGINAL)

Class

Int. Class

Application Number: 68014/87.
Lodged:

Complete Specification Lodged:

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Published:

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Related Art :

This document contains the
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Complete Specification for the invention entitled:

METHOD OF PRODUCING A RECOMBINANT PROTEIN COMPLEX HAVING
HUMAN FACTOR VIII:C ACTIVITY

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

5

METHOD OF PRODUCING A RECOMBINANT PROTEIN COMPLEX
HAVING HUMAN FACTOR VIII:C ACTIVITY

10

Description

Background

Hemophilia A is an X-chromosome-linked
inherited disease which afflicts 1-2 males per 10,000.
15 The defect is manifest as a bleeding disorder due to the
absence of clot formation arising from a lack of Factor
VIII:C. Factor VIII:C has historically been isolated
from blood in a concentrated form for therapeutic
treatment of hemophilia. However, concerns about
20 transmission of hepatitis and now AIDS have galvanized
activity to provide alternative supplies of Factor
VIII:C. It is of substantial interest to be able to
supply compositions having Factor VIII:C activity
without concerns as to the transmission viral diseases
25 associated with the native Factor VIII:C.

Factor VIII:C is a very large glycoprotein
(native M_r 330-360 kiloDaltons (kD)), which is present
in plasma at extremely low concentrations. The protein
is very susceptible to cleavage by thrombin, plasmin,
30 protease C, and other serine proteases. It is generally
isolated from plasma or plasma products as a series of
related polypeptides ranging from 160-40kD with
predominant species of 92kD and 80-77kD. This

complex pattern has made the analysis of the structure of active Factor VIII:C very difficult.

Rotblat et al., Biochemistry (1985)

24:4294-4300; Vehar et al., Nature (1984) 312:337-342;

5 Toole et al., Nature (1984) 312:342-347; and Truett et al., DNA (1985) 4:333-349 describe Factor VIII:C and the related polypeptides. Orr et al., Molecular Genetics of Clotting Factors, p. 54, s321, reports a "spacer"

10 function for the heavily glycosylated region of Factor VIII:C. Toole et al., supra; Wood et al., Nature (1984) 312:330-336; and Truett et al., supra, report sequencing of Factor VIII:C. Fulcher et al., Blood (1983)

15 61:807-811, report that the peak of maximum Factor VIII:C activity correlates with the presence of 90kD and 70kD fragments. Fulcher et al., J. Clin. Invest. (1985) 76:117-124, suggest that based on antibody-epitope data with Factor VIII:C, both the 92kD and the 80 kD polypeptides are necessary for Factor VIII:C function.

20 Although full-length recombinant human Factor VIII:C has been produced, it is difficult to purify and characterize, and it is unstable due to proteolysis. The practicality of being able to successfully produce and use the full-length molecule clinically is, therefore, doubtful at this time. Prior attempts to
25 combine the 92kD and 80kD polypeptides in vitro did not produce an active composition. The present invention provides a means of making an active complex of the 92kD and 80kD chains.

30

Disclosure of the Invention

One aspect of the present invention is to provide a method of producing a recombinant protein complex having human Factor VIII:C activity but lacking all or part of the B domain of human Factor VIII:C

comprising co-expressing in trans in a eukaryotic transformant host cell

(a) a first gene encoding a signal sequence and a polypeptide having substantially the same amino acid sequence as the A domain of human Factor VIII:C and optionally including an N-terminal portion of the B domain ^{up to amino acid 1405} of human Factor VIII:C; and

(b) a second gene encoding a signal sequence and a polypeptide having substantially the same amino acid sequence as the C domain of human Factor VIII:C; - - - and ^{isolating the secreted} ~~secreting the recombinant~~ protein complex. As used herein the C domain includes A3 as described in Wood, et al., supra.

Another aspect of the invention is the protein complex made by the above-described method.

A further aspect of the invention is to provide a pharmaceutical composition comprising the above-described protein complex, and a physiologically acceptable carrier.

A further aspect of the invention is to provide a method for treating an individual requiring Factor VIII:C activity comprising administering to the individual a sufficient amount of the above-described protein complex to enhance blood clotting activity in the individual.

A further aspect of the invention is to provide a DNA composition comprising first and second expression cassettes, said first expression cassette comprising a gene encoding a signal sequence and a ^{polypeptide} ~~peptide~~ comprising substantially the same sequence as the A-domain of human Factor VIII:C and optionally an N-terminal portion of the B-domain of human Factor VIII:C under transcriptional and translational regulatory signals functional in a host cell, and said second expression



cassette comprising a gene encoding a signal sequence
and a ^{polypeptide} ~~peptide~~ comprising substantially the same sequence
as the C-domain of human Factor VIII:C and not including
the B-domain under transcriptional and translational
5 regulatory signals functional in said host cell.

Another aspect of the invention is to provide a
host mammalian cell containing the above-described DNA
composition.

10 Brief Description of the Drawings

Fig. 1A depicts the location of the proteolytic
cleavages responsible for the generation of the 92kD and
80kD chains.

Figs. 1B-E indicate selected restriction maps
15 for the plasmids pSV7d, pSVF8-92, pSVF8-80 and
pSVF8-200, the source or function of fragments and the
number of nucleotides starting from the point indicated
O. The selected restriction sites are: B, BamHI; Bc,
BclI; Bg, BglII; E, EcoRI; H, HindIII; Hp, HpaI; K,
20 KpnI; N, NdeI; Nr, NruI; P, PstI; PvuI, PvuI; PvuII,
PvuII; S, SalI; Sc, SacI; Sm, SmaI; St, StuI; X, XbaI.
Those sites enclosed within parentheses were used in the
vector construction, but were not regenerated.

25 Description of the Specific Embodiments

Novel protein compositions are provided and
methods for their preparation where the compositions
have Factor VIII:C activity. The compositions comprise
two chains, one chain having substantially the same
30 amino acid sequence as the N-terminal portion (A domain)
of Factor VIII:C and the other chain having
substantially the same amino acid sequence as the
C-terminus (C domain) of the Factor VIII:C protein. The
two chains are about 92kD for the A domain (except when



extended into the B domain) and 80kD for the C domain, and are sometimes referred to, respectively, as the "heavy" chain and the "light" chain. Nucleic acid constructs are provided comprising individual expression cassettes having transcriptional and translational regulatory regions functional in a mammalian cell, which control the transcription and the translation of the structural genes, where each structural gene has a sequence encoding for the mature peptide chain joined at its N-terminus to a signal sequence. The expression cassettes are introduced into a mammalian host, whereby a protein complex is produced having Factor VIII:C activity. (In referring to the sequences and domains of Factor VIII:C, the sequences and domains referred to are found on p. 333 of Wood et al. cited above.)

The N-terminal polypeptide will extend from amino acid 10, usually amino acid 1, to at least about amino acid 620, usually at least about amino acid 675, more usually at least about amino acid 740. The polypeptide will include at least about 85% of the A domain (Wood et al., supra), more usually at least about 90% and may optionally include a portion of the N-terminus of the B domain, typically not exceeding about amino acid 1405. Of particular interest is an N-terminal chain having the entire sequence to the thrombolytic cleavage site at Arg₇₄₀-Ser₇₄₁.

The light chain will have an amino acid sequence substantially the same as the amino acid sequence of the C-terminus of a Factor VIII:C polypeptide, usually at least about 80%, more usually at least about 90% of the Factor VIII:C 80kD chain, particularly beginning with amino acid 1570, usually amino acid 1600, particularly amino acid 1625, more particularly amino acid 1640, preferably at about amino

acid 1649, ± 10 amino acids, more particularly ± 1 amino acid, and continuing to at least about amino acid 2300, usually 2310, ± 10 amino acids, preferably 2325, ± 5 amino acids, more preferably to the terminal amino acid (2332). Usually, the light chain will have at least about 85%, more usually at least 95%, of the C1-C2 domains, desirably the A3-C1-C2 domains.

Usually not more than 10, more usually not more than 5 number%, preferably not more than about 1 number% of the amino acids in the chains will differ from the amino acids naturally present in the Factor VIII:C A and C domains. Particularly, not more than about 5%, more usually not more than about 1% will be nonconservative substitutions. Conservative substitutions include: G,A; V,I,L; D,E; K,R; N,Q; F,W,Y, where the letters between the semicolons are conservative substitutions. (The letters are one-letter code for amino acids.) Where an amino acid in one grouping is substituted by an amino acid in another grouping or an amino acid which is not indicated above (C,M,H,P) such change will be considered nonconservative.

DNA constructs will be employed for expression of the indicated polypeptides. Each of the constructs will have in the 5'-3'-direction of transcription a transcriptional initiation and translational initiation region, a structural gene coding region comprising a sequence coding for the peptide signal sequence, including the processing signal, and a sequence coding for the Factor VIII:C heavy or light chains, followed by the translational and transcriptional termination region.

The initiation region may be comprised of a number of different sequences involved with the initiation of transcription and translation. These sequences include enhancer sequences, RNA polymerase binding site,

capping site, ribosomal binding and translational initiation sites, and the like. The transcriptional initiation region may be the natural or wild type region associated with Factor VIII:C or may be alternative
5 region to provide for high transcriptional efficiency. The region may be obtained from viruses having mammalian host ranges or the genes of the host cell or genes from a different mammalian host compatible with the host cell. Numerous transcriptional initiation regions have
10 been isolated and demonstrated to be operative in mammalian hosts. These regions include the SV-40 early promoter and late promoter regions, the adenovirus major late promoter region, β -actin promoter region, the cytomegalovirus 72kD early protein promoter region, the
15 metallothionein promoter, and the like.

The termination region may include the polyadenylation signal sequence, the transcriptional termination sequence, and the like. The termination region may be obtained from the 3'-region of the Factor
20 VIII:C natural or wild type region, or may be from the same structural gene or different structural gene from which the 5'-initiation region was obtained. The 3'-region is not as essential to the level of transcription as the initiation region, so that its
25 choice would be more of a matter of convenience than specific selection.

The structural genes will consist of a signal sequence which codes for the N-terminal amino acid sequence, and which directs the polypeptide into the
30 lumen of the endoplasmic reticulum for processing and maturation. Also included in the signal sequence is the processing signal which is recognized by an endopeptidase, where the endopeptidase cleaves a peptide bond, removing the signal sequence to provide the mature

polypeptide. The signal sequence may be the naturally occurring signal sequence, particularly for the N-terminal peptide or may be any signal sequence which is operative with the peptides to provide for the processing and maturation of the polypeptides.

Various signal sequences have been reported in the literature and include such signal sequences as tissue plasminogen activator, immunoglobulin heavy and light chains, viral membrane glycoproteins such as Herpes Simplex virus glycoproteins gB and gD, and the like.

When necessary, the sequence encoding the mature protein and signal sequence will be joined so as to be in reading frame. Where convenient restriction sites are available, the cohesive or blunt ends may be properly joined. However, for the most part, adaptors will be employed where portions of the coding sequence will be recreated in the synthetic adaptor so that the truncated structural gene and/or truncated signal sequence will be linked through the adaptor, so as to be in proper reading frame. The signal sequence and structural gene may be partially restriction mapped, so as to identify restriction sites, particularly unique restriction sites, which may be employed to link the two sequences together in proper reading frame by means of an appropriate adaptor. Alternatively unique restriction sites may be inserted at the signal sequence recognition site by in vitro mutagenesis.

The translational start and stop signals will normally be part of the structural gene, providing for the appropriate initiation codon at the beginning of translation and one or more stop codons at the termination of translation. These codons will frequently be present in the sequences as employed.

particularly the signal sequences providing the initiation codon. The stop codons may be added as appropriate as part of the termination region or be added to the coding region to provide for convenient
5 3'-terminus for linkage to the transcriptional termination region to provide for a complete termination region.

The various regions of the expression cassette, (the transcriptional and translational initiation region
10 nucleic acid sequence, structural gene nucleic acid sequence encoding one of the polypeptides and under the transcriptional and translational control of the initiation region, and a transcriptional and translational termination region, controlling the
15 processing of the mRNA and the translational termination) which identify the particular nucleotide sequences may be joined using conventional methods. Usually, the sequences obtained will contain, or be modified to contain restriction sites, which may then be
20 annealed, where complementary overhangs or cohesive ends are present. Modification frequently will be in noncoding regions by the introduction of linkers to provide for the desired cohesive ends. The ends will: usually be ligated prior to introduction into the host
25 cell, although the host cell may be allowed to provide the necessary ligation.

The expression cassettes may be joined to a wide variety of other sequences for particular purposes. Where amplification is desired, the
30 expression cassettes may be joined in tandem to a gene which is amplified when induced by an appropriate stimulus. Such genes as the metallothionein genes, e.g., human metallothionein gene, dihydrofolate reductase, and mouse mammary tumor virus LTR may be

joined to the peptide cassettes having their own transcriptional and translational regulatory sequences. By employing heavy metal ions, e.g., copper or cadmium, methotrexate, or glucocorticoids, amplification of the
5 amplifying gene and the gene of the interest (the expression cassette) may be achieved in the host cell. The structural genes will have the appropriate sequences for expression as described for the expression cassette.

The subject expression cassettes may be joined
10 to a vector, which will comprise a replication system functional in the host cell, which replication system may provide for stable episomal maintenance or integration of the expression cassette into the host genome. The vector will also comprise a marker for
15 selection, for selecting host cells containing the DNA construct and the vector from those host cells which lack the DNA construct and vector.

A wide variety of replication systems are available, normally from viruses that infect mammalian
20 host cells. Illustrative replication systems include the replication systems from Simian virus 40, adenovirus, bovine papilloma virus, polyoma virus, Epstein-Barr virus, and the like.

Markers may include resistance to a biocide,
25 particularly an antibiotic, or complementation of auxotrophy to provide a prototrophic host. Particular genes of interest as markers include kanamycin resistance gene (NPTII), chloramphenicol resistance gene (CAT), penicillinase, or the like.

Usually, the vector will be circular, and will
30 have one or more restriction sites which allow for the insertion of the expression cassette, stepwise or as a completed entity, into the vector. Frequently, the vector will also include a bacterial replication and

selection system, which allows for cloning after each of the manipulative steps. In this way, relatively large amounts of the construction at each of the stages may be prepared, isolated, purified, checked to see that the proper joining has occurred, and then used for the next stage.

Various mammalian host cells may be employed in which the regulatory sequences and replication system are functional. Such cells include COS cells, Chinese hamster ovary (CHO) cells, mouse kidney cells, hamster kidney cells, HeLa cells, HepG2 cells, or the like.

The expression cassettes of the desired polypeptides may be linked together in one nucleic acid chain or may be present in separate nucleic acid molecules. Conveniently, the expression cassettes may be parts of different vectors or of the same vector. This is primarily a matter of convenience, although in some situations with particular vectors, one or the other manner of construction may be desirable.

The manner in which the expression cassettes are introduced into the host cell will be conventional. Conveniently, calcium phosphate precipitated DNA or DNA in the presence of DEAE-dextran may be employed for transformation. Where viruses are involved, transfection or transduction may be employed. The particular manner in which the host cell is transformed is not critical to this invention, depending substantially upon whether the expression cassettes are joined to a replication system and the nature of the replication system and associated genes.

The transformed cells may then be grown in an appropriate nutrient medium. The product is obtained as a complex of the two chains, so that the media or cell lysate may be isolated and the Factor VIII:C active complex extracted and purified. Various means are available for extraction and purification, such as

affinity chromatography, ion exchange chromatography, hydrophobic chromatography, electrophoresis, solvent-solvent extraction, selective precipitation, and the like. The particular manner in which the product is isolated is not critical to this invention and will be chosen to minimize denaturation or inactivation and maximize the isolation of a high purity active product.

Compositions are provided where the composition in the Coatest will have at least 0.02U/ml of activity, usually at least about 0.2, more usually at least about 0.5U/ml of activity. The subject product can be purified by affinity chromatography using antibodies, particularly monoclonal antibodies directed against the C-terminus subunit of the Factor VIII:C activity, electrophoresis, extraction, HPLC, etc.

The subject method provides for production of a complex of the heavy and light chains which has Factor VIII:C activity. Production is evidenced by conditioned media as described in the experimental section, which will have at least about 50, usually at least about 70mU/ml, more usually at least about 200mU/ml of Factor VIII:C activity in the Coatest assay.

The complexes having Factor VIII:C activity produced according to the invention have a variety of uses as immunogens for the production of antibodies, for isolation of von Willebrand factor by affinity chromatography, in diagnostic assays for Factor VIII:C and for treatment of hemophiliacs and other hosts having blood clotting disorders. The subject protein complexes may be administered in a physiologically acceptable carrier, such as water, saline, phosphate buffered saline, and citrate buffered saline, at concentrations in the range of about 10-200U/ml. See U.S. Patent Nos. 3,631,018; 3,652,530, and 4,069,216 for methods of

administration and amounts. Other conventional additives may also be included.

The following examples are offered by way of illustration and not by way of limitation.

5

Experimental

Example 1

10 A. Preparation of Expression Plasmids

A.1 pSV7d: A mammalian cell expression vector

The expression cassettes were prepared using the mammalian cell expression vector pSV7d (2423bp).

15

The plasmid pSV7d (see Truett et al., supra) was constructed as follows: The 400bp BamHI/HindIII fragment containing the SV40 origin of replication and early promoter was excised from pSVgtI (Gruss, P., and Khoury, G., Proc. Natl. Acad. Sci. USA (1981) 78:133-137) and purified. The 240bp SV40 BclI/BamHI fragment containing the SV40 polyA addition site was excised from pSV2/DHFR (Subramani et al., Molec. and Cell Biol. (1981) 1:854-864) and purified. The fragments were fused through the following linker:

25

Stop Codons

1 2 3

5'-AGCTAGATCTCCCGGGTCTAGATAAGTAAT-3'
TCTAGAGGGCCCAGATCTATTCATTACTAG

HindIII BglII SmaI XbaI BclI overhang.

30

This linker contains five restriction sites, as well as stop codons in all three reading frames. The resulting 670bp fragment containing the SV40 origin of replication, the SV40 early promoter, the polylinker with stop

codons and the SV40 polyadenylation site was cloned into the BamHI site of pML, a pBR322 derivative with about 1.5kb deletion (Lusky and Botchan, Cell (1984) 36:391), to yield pSV6. The EcoRI and EcoRV sites in the pML sequences of pSV6 were eliminated by digestion with EcoRI and EcoRV, treated with Bal31 nuclease to remove about 200bp on each end, and finally religated to yield pSV7a. The Bal31 resection also eliminated one BamHI restriction site flanking the SV40 region, approximately 200bp away from the EcoRV site. To eliminate the second BamHI site flanking the SV40 region, pSV7a was digested with NruI, which cuts in the pML sequence upstream from the origin of replication. This was recircularized by blunt end ligation to yield pSV7b.

pSV7c and pSV7d represent successive polylinker replacements. First, pSV7b was digested with StuI and XbaI. Then, the following linker was ligated into the vector to yield pSV7c:

BglII EcoRI SmaI KpnI XbaI
5'-AGATCTCGAATTCCCCGGGGTACCT
TCTAGAGCTTAAGGGGCCCCCATGGAGATC

Thereafter, pSV7c was digested with BglII and XbaI, and then ligated with the following linker to yield pSV7d:

BglII EcoRI SmaI XbaI BamHI SalI
5'-GATCTCGAATTCCCCGGGTCTAGAGGATCCGTCGAC
AGCTTAAGGGGCCAGATCTCCTAGGCACGTGGATC

A.2 pSVF8-92: An expression plasmid for the 92kD chain
Starting from the BamHI site in the polylinker pSV7d, pSVF8-92 consists of a 49bp synthetic linker-adaptor molecule from BamHI to SacI encoding

nucleotides -30 to +14 of the Factor VIII:C protein, (numbering from the first A of the translational start site; the sequence is shown below in A.4) a 2267bp SacI to HindIII fragment from the Factor VIII:C DNA contained
5 in pSVF8-200 described below (up to nucleotide +2281), and pSV7d from HindIII to BamHI.

A.3 pSVF8-80: An expression plasmid for the 80kD chain

Starting from the SalI site in the polylinker
10 pSV7d, pSVF8-80 consists of a 201bp fragment of a tissue plasminogen activator cDNA from nucleotides -98 to +103 (relative to the start codon) terminating at a BglII site (tPA sequences given in Degan, S.J.F., et al., J. Biol. Chem. (1986) 261:6972-6985), a 29bp synthetic
15 BglII to BclI linker-adaptor encoding nucleotides +5002 to +5031 of Factor VIII:C ligated to a 2464bp BclI fragment of factor VIII:C spanning from a BclI site created at nucleotide 5028 of the factor VIII:C cDNA through in vitro mutagenesis (Zoller and Smith, Methods
20 in Enzymology (1983) 100:468), to a BclI site in the 3' untranslated region, at nucleotide 7492, and a 400bp fragment of tPA 3' untranslated sequence spanning from a BglII site to a synthetic PstI site generated from the cDNA cloning, followed by the polylinker from the vector
25 M13mp9 (Vieira and Messing, Gene (1982) 19:259) and then pSV7d.

A.4 pSVF8-200: An expression plasmid for the full length Factor VIII:C cDNA

30 The plasmid pSVF8-200 (described in Truett et al.), which contains the entire Factor VIII:C cDNA coding and 3' untranslated sequences, with the 5' untranslated sequences the same as described above for pSVF8-92, was prepared as follows.

Plasmid pSV7d was digested with BamHI to cut in the polylinker region downstream of the SV40 early promoter. The following 49bp BamHI-SacI linker adaptor, which codes for the last 30bp of the 5' untranslated region and the first 15bp of the human Factor VIII:C coding sequence, was chemically synthesized and ligated to pSV7d:

10 -35 -30 -25 -20 -15 -10 -5 met gln ile glu
5' GATCC TCTCC AGTTG AACAT TTGTA GCAAT AAGTC ATG CAA ATA GAG CT 3'
3' BamHI G AGAGG TCAAC TTGTA AACAT CGTTA TTCAG TAC GTT TAT CSacI 5'

This ligated plasmid was subsequently digested with SacI to remove excess linkers and with SalI to provide a SalI overhang.

15 Fragment 1, the 2.9kb SacI fragment from pF8-102 containing the 5' coding region of human Factor VIII:C, and Fragment 2, the 6.5kb SacI-SalI fragment from pF8-6.5 which contains the 3' coding region of the factor, and pSV7d modified vector containing the linker adaptor were ligated together (see Truett et al., supra). This ligation mix was then used to transform E. coli HB101, and colonies were selected by resistance to ampicillin.

25 Three hundred transformants were screened by colony filter hybridization using the BamHI-SacI 5' adaptor or the 2.9kb SacI fragment as probes. Those colonies positive with both probes were then analyzed by restriction mapping. Plasmid pSVF8-200, which contains the entire coding region for the human Factor VIII:C gene and a 5' untranslated region properly fused in transcriptional orientation to the SV40 early promoter, was obtained.

B. Transfection and Culture of COS7 Cells

The plasmids described above were transfected into COS7 cells (Guzman, Cell (1981) 23:175) using the calcium phosphate coprecipitation method (van der Eb and Graham, Meth. Enzymology (1980) 65:826-839) coupled with treatment with chloroquine diphosphate (Luthman and Magnusson, Nucl. Acids Res. (1983) 11:1295-1308) using 50µg of plasmid DNA per 5×10^5 cells for 14hr. Cells may also be transfected by the DEAE-dextran method of Sompayrac and Danna P.N.A.S. (1981) 78:7575-7578.

The COS7 cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, 100U/ml penicillin, 100µg/ml streptomycin, 292µg/ml glutamine, and 110µg/ml sodium pyruvate. Samples were obtained from a 48-hour collection of serum containing medium at 88 hours post transfection.

C. Assays

At specific intervals post transfection, media was removed from the cells, and aliquots were stored at -70°C. Samples were tested for their ability to decrease the prolonged partial thromboplastin time of Factor VIII:C deficient plasma in a standard coagulation assay (Hardisty et al., Thrombosis et Diathesis Haemologica (1962) 72:215). The more specific Coatest assay (Rosen et al. in Thromb. and Haemostasis (1985) 54:818-823), which measures the generation of activated Factor X (Xa) as a linear function of the concentration of exogenously supplied Factor VIII:C, was used to verify the results of the coagulation assay. The concentration of immunologically reactive Factor VIII:C protein in the medium was determined by the application of a radioimmunoassay (RIA) developed to detect the 92kD polypeptide and by an enzyme-linked immunosorbant assay

(ELISA) specific for the 80kD polypeptide (Nordfang et al., Thromb. and Haemostasis (1985) 53:346).

As shown in Table 1, expression of the 92kD polypeptide or of the 80kD polypeptide alone produced no detectable activity even though high levels of each of the individual proteins were present in the conditioned media. When cells were cotransfected with pSVF8-92 and pSVF8-80 plasmids, the media contained about 20mU/ml of coagulation activity. The same relative level of the coagulation activity was secreted by cells transfected with the plasmid pSVF8-200 encoding the complete Factor VIII:C protein.

When conditioned media from the pSVF8-92 and the pSVF8-80 single transfectants were mixed together (using several different conditions as outlined in Table 1) no activity was measurable.

These results indicate that a complex of the amino and carboxyl terminal domains of Factor VIII:C retains intrinsic coagulation activity and that the interior β domain is not essential for activity nor for the assembly of an active complex from separate chains.

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TABLE 1: Assay of Recombinant Factor VIII:C Activity in Conditioned COS Cell Media

ELISA ^d Assay	Coagulation	Activity ^a	Coatest ^b	RIA ^c	
			Activity	Assay	
Plasmid U/ml	Coagulation Time (sec)	mU/ml ^e	mU/ml	92kD U/ml	80kD
pSVF8-92 <.0002	95.7	< .9	< .1	0.15	
pSVF8-80 1.36	97.2	< .9	< .1	<.01	
pSVF8-92 +pSVF8-80 1.13	56.1	22.5	20.4	0.05	
pSVF8-200 0.28	47.7	70.0	43.2	0.12	
none <.0002	94.6	< .9	< .1	<.01	
pSVF8-92} mixed <u>in vitro</u> ^f pSVF8-80	95.7	< .9	< .1	--	

^a Aliquots of 75µl of media conditioned by the growth of COS cells transfected with the indicated plasmids or mock transfected were assayed for their ability to decrease the prolonged partial thromboplastin time of Factor VIII:C deficient plasma in the one-stage assay. Briefly, 75µl of Platelin (General Diagnostics) was incubated for 3min at 37°C followed by the addition of 75µl of Factor VIII:C deficient plasma plus 75µl of the test sample for an additional 5min incubation at 37°C. A 75µl aliquot of prewarmed 0.025M CaCl₂ was added and the clotting time measured with a Becton-Dickinson fibrometer. Normal human plasma diluted in COS cell medium was used as a standard.

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TABLE 1 (continued) . . .

- b The Coatest assay (Kabi) measures the generation of activated Factor X (Xa) as a linear function of the concentration of Factor VIII:C. The concentration of Factor Xa is measured by the proteolytic cleavage of the chromogen para-nitroaniline from a synthetic peptide substrate for Xa. Normal human plasma diluted in 50mM Tris-HCl, pH 7.3, 0.2% BSA was used as the standard.
- c For the RIA assay purified dog factor VIII:C inhibitory IgG was coated onto the wells of a 96-well polystyrene microtiter plate at a concentration of 3.5 µg/ml in 0.1 M sodium carbonate buffer, pH 9.8, by overnight incubation at 37°C. The plates were washed 3 times with 0.1 M NaCl, 0.05% Tween 20 followed by an incubation with a mixture of test medium samples and iodinated VIII:C 92-kDa protein, both diluted in 0.05 M imidazole, 0.1 M NaCl, 1% bovine serum albumin, 0.05% Tween 20, pH 7.3. The VIII:C 92-kDa protein was isolated from plasma and was greater than 50 percent homogenous as estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and silver staining. After incubation for 16 hr at room temperature, the plates were washed, and the amount of ¹²⁵I in the individual wells was measured in a γ counter. An intermediate purified commercial factor VIII:C preparation (factor VIII, NORDISK) with a specific activity of 0.5 unit of coagulation activity per mg was used as the standard. This standard was calibrated against the World Health Organization Third International factor VIII:C standard. We defined our intermediate purified standard to contain a 92-kDa RIA activity/factor VIII:C coagulation activity ratio of 1.
- d For the ELISA assay, purified human Factor VIII:C inhibitory IgG was coated onto the wells of a 96-well polyvinylchloride microtiter plate at a concentration 4.5µg/ml in 0.1M sodium carbonate, pH 9.8, by overnight incubation at 37°C. The wells were washed as above and peroxidase-conjugated F(ab)₂ fragments of the human inhibitory IgG diluted in 0.1M imidazole, 0.15M NaCl, 1% BSA, 0.05% Tween 20, pH 7.3, were added for a final incubation of 16hr at room temperature. The color was developed with o-phenylenediamine solution. Normal human serum was used as a standard.
- e One mU of activity is assumed to correspond to approximately 100pg of Factor VIII:C protein (Fay et al., Proc. Natl. Acad. Sci. USA (1982) 79:7200).
- f A variety of mixing conditions were tested including preincubation for various times up to 2hr at 37°C, 20°C, or 4°C in the presence or absence of 10mM CaCl₂. The value reported in this table is representative of the data obtained.

TABLE 2: Coagulation Inhibition Assay

<u>Experiment I:</u>		
Plasmid	IgG ^a	Coagulation Time (secs)
pSVF8-80 + pSVF8-92	N	51.9
	I	74.5
	B	54.4
pSVF8-200	N	46.4
	I	69.4
	B	46.8

For Experiment I, 160 μ l of the indicated COS cell conditioned media was incubated with 20 μ l of a 100-fold dilution of human Factor VIII:C inhibitory serum (Bethesda titer 1500 units) or a similar dilution of pooled normal human serum, or buffer alone (50mM imidazole, 0.1M NaCl, 100 μ g/ml BSA pH 7.3) for 2hr at 37°C. These samples were then assayed for residual coagulation activity as outline in the legend to Table 1.

<u>Experiment II:</u>		
Plasmid	IgG ^a	Coagulation Time (secs)
pSVF8-92 + pSVF8-80	I	72.9
	B	48.0
pSVF8-200	I	60.9
	B	44.9

For Experiment II, 100 μ l of conditioned media was incubated for 2hr at 37°C with either 10 μ l of a 1 μ g/ μ l solution of anti-Factor VIII:C monoclonal antibody from Hybritech (Bethesda titer 14,000 units) or buffer and then assayed as above.

^a

Normal serum = N; inhibitory serum = I; buffer = B.

To verify that the observed coagulation activity was due to Factor VIII:C, the sensitivity of the coagulation to inhibition by an antibody specific for Factor VIII:C was determined. Prior to assay, 5 aliquots of conditioned media were preincubated for 2hr at 37°C in the presence of dilutions of normal human serum or of serum from a hemophiliac who had developed a high titer of inhibitory antibodies to Factor VIII:C. As shown in Table 2, the activity of the complete 10 molecule, as well as that of the 92kD-80kD complex was reduced specifically by the inhibitory serum. The same results were obtained using three different inhibitory monoclonal antibodies which bind to the 80kD species.

To demonstrate more clearly the existence of a 15 two chain complex, the active species was partially purified from the COS cell media by passage over a monoclonal antibody column directed against the 80kD portion as shown in Table 3. Approximately 65% of the applied activity was retained by the column and 50% of 20 this bound material was eluted in an active form and at a fivefold greater concentration than the initial media. Thus an active complex can be isolated by affinity chromatography using an antibody specific for only the 80kD species.

25 TABLE 3: Partial Purification of 92kD-80kD Coagulation Active Complex

Fraction	Coatest U/ml	80kD ELISA U/ml
Media Flowthrough Eluate	.0044 .0017 .0200	0.175 0.13 0.76

30 TABLE 3: Cont'd

100µg of an anti-80kD monoclonal antibody (56 IgG) (Nordfang *et al.*, *Thromb. Haemostasis* (1985) 53:346) coupled to Sepharose CL4B was incubated overnight at 20°C with 1.4ml of media containing a total of 6.2mU of activity (measured by the Coatest Assay obtained from COS cells cotransfected with pSVF8-92 and pSVF8-80 plasmids). After incubation, the slurry was loaded into a column and the flowthrough fraction was collected. The column was washed with 300µl of Buffer A (50mM imidazole, .1M NaCl, 0.1% sodium insulin, 0.2% NaN₃, pH 7.3) and then eluted with 300µl of Buffer B (2.5M NaCl, 50% ethylene glycol, 0.5M imidazole, 0.1M CaCl₂, 0.1% sodium insulin, 0.2% NaN₃, pH 7.3).

Results reported here demonstrate that expression of the β linker region, containing 918 amino acids or about 40% of the total for the intact protein, is not required for Factor VIII:C activity. Co-expression of individual 92kD and 80kD regions results in a level of Factor VIII:C activity comparable to that obtained from the expression of the whole Factor VIII:C coding region. These proteins assemble *in vivo* to form an active complex linked by a calcium bridge. The assembly does not require the presence of the β region and occurs efficiently for the two chains expressed in *trans*.

It is evident from the above results that Factor VIII:C activity can be achieved by directly producing an N-terminal fragment and a C-terminal fragment which are independently expressed, each having its own signal sequence. Thus, Factor VIII:C can be obtained more efficiently, since the large precursor need not be cloned and used as the coding sequence for the Factor VIII:C activity. Thus, cells may be employed for expression of Factor VIII:C which may be deficient in the capability of the proper maturation of the Factor VIII:C protein.

Example 2

Expression of the 92 kD protein in COS cells using the pSVF8-92 construction was low compared to the amount of 80kD protein produced. Accordingly, the construction was modified in an attempt to increase the level of 92kD protein. Modifications of the following types were made: Changes in the 5' untranslated sequence of the Factor VIII:C gene; inclusion of heterologous 5' untranslated and leader sequences; and changes in the 3' untranslated sequences. These constructs are summarized below.

A. Expression PlasmidsA.1 5' Untranslated Region Modifications

Plasmid pSVF8-92B. This plasmid is a derivative of pSVF8-92 in which the 30bp of 5' untranslated sequence of pSVF8-92 is replaced with the entire 5' untranslated region of human Factor VIII:C cDNA (nucleotides 1 to 171; see Fig. 8 of Truett *et al.*, supra), with a deletion of the G-C tails (by in vitro site-specific mutagenesis), and the three base changes shown below at the starting ATG (at position +172, Fig. 8, Truett, *et al.*, supra) to conform to Kozak's preferred sequences for efficient message translation in eukaryotic cells:

Factor VIII:C:	GTCATG CAA
Kozak consensus:	ACCATG G

This change alters the second amino acid of the signal peptide to Glu from Gln.

Plasmid pSVF8-92E. This plasmid is a derivative of pSVF8-92B in which the polylinker derived from pSV7u 5' to the Factor VIII:C sequences is removed with the exception of the SalI site, and the ATG codon
5 in the 5' untranslated region (at 41 according to Truett et al., supra) is altered to ATT, by in vitro mutagenesis.

10 A.2 Addition of Heterologous 5' Untranslated Regions and Leader Sequences

Plasmids pSVF8-92G, H, and I. These plasmids are derivatives of pSVF8-92B in which the 5' untranslated region as well as the natural Factor VIII:C
15 signal sequences are replaced with the analogous region from the human tissue plasminogen activator (tPA) cDNA gene. In pSVF8-92G the first 35 amino acids (signal sequence) of the tPA 5' region are joined to mature Factor VIII:C 92 kD with a serine substituted for the
20 first amino acid (alanine) of the 92 kD protein. In pSVF8-92H the first 32 amino acids of the tPA 5' region are joined to mature Factor VIII:C 92 kD protein. In pSVF8-92I, the first 23 amino acids of the tPA 5' region are joined to mature Factor VIII:C 92 kD protein. The
25 tPA sequences are the same as those described for pSVF8-80.

Plasmid pSVF8-92J. This plasmid is a derivative of pSVF8-92G in which the tPA 5' region is
30 replaced with 75bp of herpes simplex virus-1 (HSV-1) gD 5' untranslated sequences and 75bp of HSV-1 gD signal sequence. pSVF8-92J also lacks the Ala → Ser substitution (Watson, R.J., et al., Science (1982) 218:381-384).

A.3 3' Untranslated Region Changes

Plasmid pSVF8-92C. This plasmid is a variation of pSVF8-92B in which the 92kD coding region is fused directly to the translational stop codon and natural 3' untranslated sequences of human Factor VIII:C cDNA. This plasmid is a derivative of pSVF8-200.

Plasmid pSVF8-92L. This plasmid is a derivative of pSVF8-92C in which the 3' untranslated region of pSVF8-92C is replaced with the 3' untranslated region of pSVF8-80.

B. Results

Each of the plasmids of part A was transfected into COS7 cells along with pSVF8-80 as described in Example 1 and the media tested for Factor VIII:C activity as in Example 1.

pSVF8-92B, the first tested, showed activity levels ranging from 2-to-8-fold better than pSVF8-92. Of the remaining plasmids pSVF8-92E appeared to be the best, being 1.65-fold better than pSVF8-92B. pSVF8-92J and I also produced substantially higher expression levels than pSVF8-92, being close to that of pSVF8-92E. The expression level of pSVF8-92G approximated that of pSVF8-92, whereas that of pSVF8-92H was substantially less than pSVF8-92. The expression levels of both pSVF8-92C and pSVF8-92L appear to be equivalent to that of pSVF8-92E.

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Example 3

This example describes the preparation of constructs for producing polypeptides that consist of the 92 kD chain and a portion of the B domain. These

derivatives were made in an attempt to develop a heavy chain that is more stable and/or assembles more efficiently into an active complex with the light chain. The derivatives were chosen to mimic molecular species that have been observed in plasma-derived preparations of Factor VIII:C and in cell lysates and conditioned media from cells expressing recombinant full-length Factor VIII:C. These could possibly arise by thrombin cleavages of full-length Factor VIII:C.

A. Preparation of Expression Plasmids

A.1 pSVF8-92S

This plasmid encodes a 982 amino acid heavy chain and was prepared from a full-length cDNA plasmid pSVF8-302 by partial cleavage at the first SacI site of the B-domain coding region. An oligonucleotide adaptor was used to install a translational stop codon and fuse the coding sequence to the natural human Factor VIII:C 3' untranslated sequence beginning at the first BalI site. This plasmid encodes the first 978 amino acids of native human Factor VIII:C and 4 substituted amino acid residues at the carboxy terminus.

A.2 pSVF8-160

This plasmid provides a 1323 amino acid heavy chain and was prepared from a full-length clone (designated pSVF8-303) similar to pSVF8-200, but having the 5' untranslated region of pSVF8-92E. pSVF8-303 was cleaved with EcoRV and SmaI, and the blunt ends were ligated together to form pSVF8-160. This plasmid encodes the first 1315 amino acids of Factor VIII:C. Eight nonsense amino acids are added at the carboxyl terminus as a result of the fusion of the polylinkers of the vector pSV7d.

A.3 pSVF8-170

This plasmid provides a 1416 amino acid heavy chain and was also prepared from pSVF8-303. pSVF8-303 was partially digested with BglII, and the resulting
5 6811 bp fragment was gel isolated and the ends ligated together to form pSVF8-170. This plasmid encodes the first 1405 amino acids of Factor VIII:C and has a carboxyl extension of 11 nonsense amino acids due to fusion of the polylinker of the vector pSV7d.

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A.4 pSVF8-120

This plasmid provides a 1107 amino acid heavy chain and was prepared from pSVF8-303. The plasmid pSVF8-303 was digested with ApaI and the cohesive ends
15 were filled in with T4 polymerase. The resulting molecule was further digested with SmaI, the DNA self-ligated and propagated in E. coli HB101. This plasmid encodes 1102 amino acids from the amino terminus of Factor VIII:C plus an additional 5 nonsense amino
20 acids at the carboxyl terminus.

B. Results

Each of the plasmids of part A was transfected into COS7 cells along with pSVF8-80 as described in
25 Example 1 and the media tested for Factor VIII:C activity as in Example 1.

All of these plasmids showed substantially reduced expression levels compared to that of pSVF8-92E. Interestingly, though, the ratio of RIA to
30 Coatest activity for pSVF8-160 and pSVF8-170 is about 1.8, compared to 7.2 for pSVF8-92E. This result suggests that these longer heavy chain derivatives have a higher specific activity, that is, they are more efficiently assembled into active subunit complexes than

the 92kD molecule itself. Also, the ratio of coagulation activity to Coatest activity is lower for the longer heavy chains at ~1.7 compared to 2.3 for 92kD and 1.35 for the complete molecule, suggesting that these longer polypeptides form complexes which are not as activated as that of the 92kD + 80kD complex.

Example 4

10 This example describes the preparation of stable CHO cell lines that produce the Factor VIII:C 92kD-80kD chain complex.

 CHO-DUKX-B1 cells (Urlaub and Chasin, P.N.A.S. (1980) 77:4216-4220) were cotransfected with pSVF8-92C or pSVF8-92E, pSVF8-80 and pAd-DHFR using the calcium phosphate precipitation method of Graham and Van der Eb (op. cit.) and modifications described by Wigler et al. Cell (1978) 14:725-731) and Lewis et al. Somatic Cell Genetics (1980) 6:333-347. The plasmid pAd-DHFR, bearing the dhfr gene, was constructed by fusing the major late promoter from adenovirus-2 (Ad-MLP, map units 16-17.3) to the mouse dhfr cDNA at the 5' end. DNA coding for the intron for SV40 small t antigen and the SV40 early region polyadenylation site was obtained from pSV2-neo (Southern and Berg, J. Mol. Appl. Genet. (1982) 1:327-341) and fused to the 3' end of the dhfr cDNA. These three segments were subcloned into pBR322 to obtain plasmid pAd-DHFR. The weight ratio of plasmids were, respectively, 10:1:5 in the pSVF8-92C co-transfection and 1:1:1 or 10:10:1 in the pSVF8-92E co-transfections. In one of the pSVF8-92C transfections, the plasmids were digested with PvuI, which cuts uniquely in the beta-lactamase gene of pBR322. In that instance, linear DNA molecules were

introduced instead of supercoiled molecules. Media from the resulting DHFR positive clones were screened by ELISA and Coatest, as described above. The stability of positive clones was evaluated by repeated passage in T-75 flasks and assay of the media. Table 4 below reports the expression levels for the positive, stable clones from the linear DNA.

Table 4

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<u>Clone</u>	<u>mU Coatest</u>
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pSVF8-92C

11-D6	43
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11-D5	30
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pSVF8-92E

8-C1	18.2
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10-C2	70.0
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Primary clones from the pSVF8-92C group were carried in T-75 flasks. Clone 11-D5 proved to be the highest expressing clone, producing >90 mU/ml Coatest activity in T-75 flask cultures. After six weeks passage, this clone was put under selection in 0.025 μ M, 0.05 μ M, 0.1 μ M, and 0.2 μ M methotrexate. Clones appeared on the 0.025 μ M plates, indicating that the DHFR genes have amplified at a low level of methotrexate.

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Lines 8-C1 and 10-C2 of the pSVF8-92E group were selected in methotrexate (0.025 μ M to 0.2 μ M) and resistant clones were assayed for Factor VIII:C activity by Coatest, RIA and ELISA. Twenty-two methotrexate-resistant 8-C1 clones were examined, the

data for 10 of which are reported in Table 5. The amount of Factor VIII:C amplification varies among clones, suggesting that either one of the subunit genes may have been co-amplified with the DHFR cassette, or both of them, or neither one. Note clones 8C1-A2, 8C1-A2, 8C1-C2, and 8C1-C5 as examples of these four possibilities. Similarly, 30 methotrexate-selected derivatives of 10-C2 were evaluated, the data for 20 of which are also represented in Table 5. These also contain a spectrum of activity. Note clones 10C2-A2, 10C2-D2, 10C2-B5, and 10C2-C6 as examples of the four different co-amplification possibilities.

Table 5

	Clone	conc. MTX <u>μU</u>	Coatest <u>μU/ml</u>	ELISA <u>μU/ml</u>	RIA <u>μU/ml</u>
15	8-C1	0	18	1275	n.d.
	8C1-A1	0.1	<50	1750	80
	8C1-A2	0.1	60	1950	>1000
	8C1-A5	0.05	2	100	10
	8C1-B3	0.025	33	1950	1000
20	8C1-B4	0.025	50	3550	820
	8C1-B5	0.025	35	1950	>1000
	8C1-C2	0.025	130	13,100	>>1000
	8C1-C3	0.025	165	3900	>>>1000
	8C1-C5	0.025	30	1750	760
	10-C2	0	200	1400	700
	10C2-A1	0.05	61	1600	400
	10C2-A2	0.1	67	6700	700
25	10C2-A4	0.05	63	2250	1200
	10C2-A5	0.05	183	9450	2660
	10C2-A6	0.05	320	8600	7400
	10C2-B1	0.05	408	8100	4300
	10C2-B3	0.05	134	800	9800
	10C2-B4	0.05	394	18,000	7800
	10C2-B5	0.05	461	15,000	8400
30	10C2-B6	0.05	247	2200	9800
	10C2-C1	0.1	160	8100	7600
	10C2-C2	0.05	228	6000	5600
	10C2-C3	0.05	294	14,850	2650
	10C2-C5	0.05	294	12,400	5400
	10C2-C6	0.05	100	1350	520
	10C2-D2	0.05	496	1560	16,400
	10C2-D3	0.05	242	10,200	2260
	10C2-D4	0.05	165	14,100	3500
	10C2-D5	0.05	316	7800	5200
	10C2-D6	0.05	141	1600	6400

Plasmids pSVF8-92 and pSVF8-80 were deposited at the American Type Culture Collection (ATCC) on 24 January 1986 and given ATCC Accession Nos. 40222 and 40223, respectively. Plasmid pSVF8-200 was deposited at the ATCC on 17 July 1985 and was given ATCC Accession No. 40190.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method of producing a recombinant protein complex having human Factor VIII:C activity but lacking all or part of the B domain of human Factor VIII:C comprising co-expressing in trans in a eukaryotic transformant host cell

(a) a first gene encoding a signal sequence and a polypeptide having substantially the same amino acid sequence as the A domain of human Factor VIII:C and optionally including an N-terminal portion of the B domain up to amino acid 1405 of human Factor VIII:C; and

(b) a second gene encoding a signal sequence and a polypeptide having substantially the same amino acid sequence as the C domain of human Factor VIII:C; and ^{isolating the secreted} ~~secreting the recombinant~~ protein complex.

2. A method according to claim 1, wherein not more than about 5 number% of the amino acids of said amino acid sequences have been substituted with natural amino acids other than the amino acids specified by the naturally occurring amino acid sequences of the Factor VIII:C A and C domains at the positions of substitution.

3. The method of claim 1 wherein the amino acid sequence of the polypeptide encoded by the second gene is the same as the amino acid sequence of amino acids 1649-2322 of human Factor VIII:C.

4. The method of claim 3 wherein the amino acid sequence of the polypeptide encoded by the first gene is the same as the amino acid sequence of amino acids 1-740 of human Factor VIII:C.



5. The method of claim 3 wherein the amino acid sequence of the polypeptide encoded by the first gene is the same as the amino acid sequence of amino acids 1-1102 of human Factor VIII:C.

6. The method of claim 3 wherein the amino acid sequence of the polypeptide encoded by the first gene is the same as the amino acid sequence of amino acids 1-1315 of human Factor VIII:C.

7. The method of claim 3 wherein the amino acid sequence of the polypeptide encoded by the first gene is the same as the amino acid sequence of amino acids 1-1405 of human Factor VIII:C.

8. The method of any of claims 1-7 wherein the eukaryotic transformant host cell is a mammalian cell.

9. The method of any of claims 1-8 wherein the first gene includes a 5' untranslated DNA sequence of human Factor VIII:C that enhances the expression of the polypeptide encoded by the first gene.

10. The method of any of claims 1-8 wherein the first gene includes a 3' untranslated DNA sequence of human Factor VIII:C that enhances the expression of the polypeptide encoded by the first gene.

11. The method of any of claims 1-10 wherein the first gene and second gene are in separate expression plasmids.



12. A method for treating an individual requiring Factor VIII:C activity comprising administering to the individual a sufficient amount of a protein complex having human Factor VIII:C activity but lacking all or part of the B domain of human Factor VIII:C prepared by the method of any one of claims 1 to 11 to enhance blood clotting activity in the individual.

13. A DNA composition comprising first and second expression cassettes, said first express cassette comprising a gene encoding a signal sequence and a polypeptide comprising substantially the same sequence as the A-domain of human Factor VIII:C and optionally an N-terminal portion of the B-domain of human Factor VIII:C under transcriptional and translational regulatory signals functional in a mammalian host cell, and said second expression cassette comprising a gene encoding a signal sequence and a polypeptide comprising substantially the same sequence as the C-domain of human Factor VIII:C and not including the B-domain under transcriptional and translational regulatory signals functional in said mammalian host cell.

14. A DNA composition according to claim 13 wherein said first cassette gene encodes a polypeptide comprising at least about 90% of the amino acid sequence of human Factor VIII:C amino acids 1-740 and said second cassette gene encodes a polypeptide comprising at least about 90% of the amino acid sequence of human Factor VIII:C amino acids 1649-2332.



15. A host mammalian cell containing the DNA composition of claim 13 or 14.

DATED this 11th day of December 1990.

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FIG. 1A

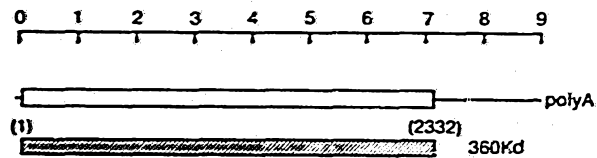


FIG. 1B

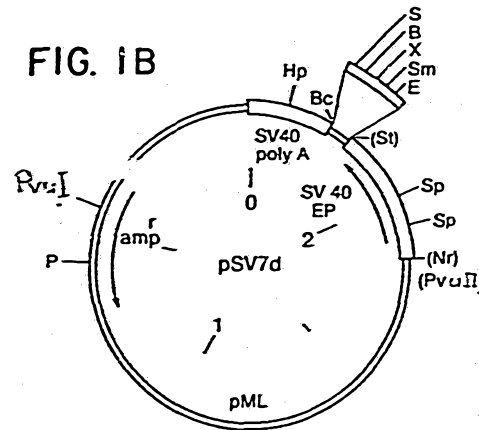


FIG. 1C

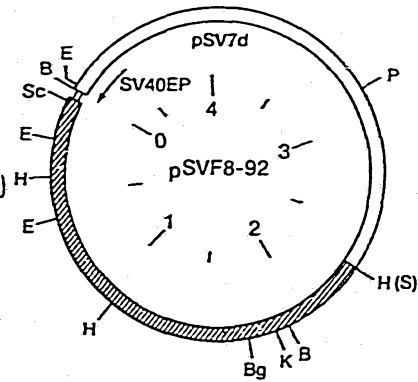


FIG. 1D

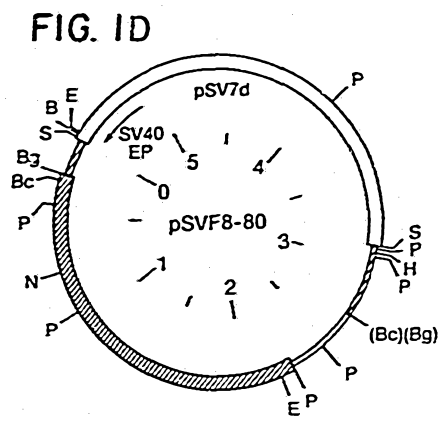


FIG. 1E

