Expression System for Factor VIII

Related applications: The application to Cho designated MSB-7241 (U.S. Ser. No. 09/209,920), "Human Hybrid Host Cell for Mammalian Gene Expression," and the application to Cho and Chan designated MSB-7254 (U.S. Ser. No. 09/209,915), "Vectors Having Terminal Repeat Sequence of Epstein-Barr Virus," contain related subject matter. Both applications were filed on December 10, 1998.

BACKGROUND OF THE INVENTION

Field: The present invention relates to an improved production method for factor VIII and its derivatives. The method relates generally to vector construction, transfection, and selection of cell lines with enhanced productivity under protein-free conditions. In particular, this invention relates to a process for preparing a protein with factor VIII procoagulant activity on an industrial scale.

Background: Human factor VIII is a trace plasma glycoprotein involved as a cofactor in the activation of factor X and factor IXa. Inherited deficiency of factor VIII results in the X-linked bleeding disorder hemophilia A which can be treated successfully with purified factor VIII. The replacement therapy of hemophilia A has evolved from the use of plasma-derived factor VIII to the use of recombinant factor VIII obtained by cloning and expressing the factor VIII cDNA in mammalian cells. (Wood et al., 1984, Nature 312: 330).

Factor VIII has a domain organization of A1-A2-B-A3-C1-C2 and is synthesized as a single chain polypeptide of 2351 amino acids, from which a 19-amino acid signal peptide is cleaved upon translocation into the lumen of the endoplasmic reticulum. Due to the fact that factor VIII is heavily glycosylated, high-level expression (>0.2 pg/c/d) of factor VIII has been difficult to achieve (Lind et al., 1995, Eur J Biochem. **232**: 19-27; Kaufman et al., 1989, Mol Cell Biol. **9**: 1233-1242). Expression of factor VIII in mammalian cells is typically 2-3 orders of magnitude lower than that observed with other genes using similar vectors and approaches. The productivity of production cell lines for factor VIII has been in the range of 0.5 -1 μU/c/d (0.1 - 0.2 pg/c/d).

It has been demonstrated that the B-domain of factor VIII is dispensable for procoagulant activity. Using truncated variants of factor VIII, improved expression of factor VIII in mammalian cells has been reported by various groups (Lind et al., 1995, Eur J Biochem **232**: 19-27; Tajima et al., 1990, Proc 6th Int Symp H.T. p.51-63; US Patent 5,661,008 to Almstedt, 1997). However, the expression level of the factor VIII variants remained below 1 pg/c/d from a stable cell clone.

SUMMARY OF THE INVENTION

We have now discovered (i) a method which derives cell lines with extremely high productivity of proteins having factor VIII procoagulant activity, and (ii) a plasma protein-free production process for proteins having factor VIII procoagulant activity.

A process for the production of proteins having factor VIII procoagulant activity at the industrial scale. Using a newly created cell host, cell clones with specific productivities in the range of 2-4 pg/cell/day (10 - 20 μU/c/d) were derived. Under serum-free conditions, one clone has

sustained a daily productivity of 2 – 4 pg/c/d. Clones with this high level of productivity are able to produce 3 - 4 million units per day in a 15-liter perfusion fermenter. One unit of factor VIII activity is by definition the activity present in one milliliter of plasma. One pg of factor VIII is generally equivalent to about 5 μU of FVIII activity.

As used herein, a protein having factor VIII procoagulant activity is a protein which causes the activation of Factor X in an in vitro or in vivo model system. As non-limiting examples, this definition includes full length recombinant human factor VIII and the B domain deleted factor VIII whose sequence is described in figure 1.

A high level of expression of a protein having factor VIII procoagulant activity means at least about 2 μU/c/d, or more preferably at least about 4 μU/c/d, or most preferably at least about 5 μU/c/d, of factor VIII activity if grown in plasma derived protein-free medium, or at least about 4 μU/c/d, or more preferably at least about 8 μU/c/d, or most preferably at least about 10 μU/c/d, of factor VIII activity if grown in medium supplemented with plasma derived protein. When the protein expressed is BDD-FVIII, cell lines having specific productivities up to about 15 μU/c/d, more preferably up to about 20 μU/c/d may be obtained by the method described herein.

As used herein to describe the origin of cell lines, "derived from" is intended to include, but not be limited to, normal mitotic cell division and processes such as transfections, cell fusions, or other genetic engineering techniques used to alter cells or produce cells with new properties.

BRIEF DESCRIPTION OFTHE FIGURES

Figure 1. Amino Acid Sequence of BDD-FVIII (SEQ ID NO:1).

Figure 2. Sequence of terminal repeat (TR) sequence isolated from Epstein-Barr virus (SEQ ID NO:2).

Figure 3. Plasmid map of pCIS25DTR.

Figure 4(a). Derivation of clone 20B8.

Figure 4(b). Comparison of productivities of several clones in various media. Three data points are presented from a two month stability test of each clone.

Figure 5. Volumetric productivity of clone 20B8.

SPECIFIC EMBODIMENTS

FVIII Assay

The activity of factor VIII derivatives obtained from recombinant gene expression in methotrexate (MTX)-resistant cell populations was measured by a chromogenic assay. Activity was quantitated using Coatest® factor VIII:C/4 kit (Cromogenix, Molndal, Sweden) according to manufacturer's instructions. A U.S. standard anti-hemophilic factor (factor VIII) known as MEGA 1 (Office of Biologics Research and Review, Bethesda, MD) was used as the standard of measurement in this assay. See Barrowcliffe, 1993, Thromb Haem 70: 876.

Construction of expression vectors for B-domain deleted FVIII

The sequence of the B-domain deleted (BDD) FVIII is shown in Figure 1. The 90-kD and 80-kD chains were linked by a linker consisting of 14 amino acids. See Chan, S.-Y., "Production of Recombinant Factor VIII in the Presence of Liposome-like Substances of Mixed Composition," U.S. Patent Application No. 08/634,001, filed April 16, 1996. The expression vector for BDD-FVIII was made using standard recombinant DNA techniques. The structure of the expression vector (pCIS25DTR) is shown in Figure 3. The vector includes a transcriptional unit for BDD-FVIII and a selectable marker, dihydrofolate reductase (dhfr). In addition a terminal repeat sequence from Epstein-Barr virus, which shows enhanced drug selection ratio, (Figure 2) was inserted into the vector to increase the integration efficiency. The vector is essentially a construct of a vector (deposited ATCC 98879) which has been engineered to include a transcriptional unit corresponding to the sequence shown in figure 1. Further information about the terminal repeat sequence can be found in the related patent application, incorporated herein by reference, to Cho and Chan designated MSB-7254, "Terminal repeat sequence of Epstein-Barr virus enhances drug selection ratio," filed on the same day as the current application.

Similar vectors can be constructed and used by those having skill in the art to obtain cells expressing proteins having factor VIII procoagulant activity. For example, coding sequences coding for known variants of factor VIII which retain procoagulant activity can be substituted for the BDD-FVIII coding sequence. Also, instead of dhfr, other selectable markers can be used, such as glutamine synthetase (gs) or multidrug-resistance gene (mdr). The choice of a selection agent must be made accordingly, as is known in the art, i.e. for dhfr, the preferred slection agent is methotrexate, for gs the preferred selection agent is methionine sulfoximine, and for mdr the preferred selection agent is colchicine.

WORKING EXAMPLES

Derivation of cell lines expressing BDD-FVIII: Transfection, drug selection and gene amplification

Thirty micrograms of pCIS25DTR DNA was transferred into HKB11 (ATCC deposit no. CRL 12568 - a hybrid of 293S cells and human Burkitt's lymphoma cells, see U.S. Patent application to Cho et al. filed on the same day as the current application and designated MSB-7241, incorporated herein by reference) cells by electroporation set at 300 volts and 300 micro farads (BTX Electro cell Manipulator 600) using a 2mm cuvette (BTX part #620). In comparative experiments done to parallel work with the HKB11 cells, CHO (Chinese hamster ovary) and 293S (human embryonic kidney) cells were transfected using a cationic lipid reagent DMRIE-C (Life Technologies, Gaithersburg, MD) according to a protocol provided by the Life Technologies. Amplification of transfected cells was done with increasing methotrexate (MTX) concentrations (100nM, 200nM, 400nM, and 800nM) at 1 x 10^6 cells per 96 well plate in a MTX-selection medium lacking hypoxanthine and thymidine (DME/F12 media without hypoxanthine and thymidine plus 5% dialyzed fetal bovine serum from Hyclone, Logan, UT). MTX resistant cells were scored for growth, and secretion of the BDD-FVIII was screened using a Coatest® factor VIII kit about 2 - 3 weeks post-transfection. The cultivation of cells were done at 37°C in a humidified 5% CO_2 incubator.

Limiting Dilution Cloning

Single cell clones (SCC) were derived by limiting dilution cloning (LDC) of high producing populations in 96 well plates under serum-free conditions. Cells were seeded at 1 - 10 cells per well in DME/F12 media supplemented with Humulin® recombinant insulin (Lilly, Indianapolis, IN) at 10 µg/ml, 10X essential amino acids (Life Technology, Gaithersburg, MD), and Plasmanate®

human plasma protein fraction (Bayer, Clayton, NC). Plasmanate® human plasma protein (HPP) fraction contains human albumin (88%) and various globulins (12%). The clones were screened for BDD-FVIII productivity using the Coatest® factor VIII kits. The highest producing clones were selected for stability evaluation in shake flasks. For HKB cells, the first round LDC was performed using selection medium supplemented with 5% dialyzed FBS. The second round LDC was done in serum-free but Plasmanate® HPP fraction-containing medium using the first SCC adapted in serum-free medium supplemented with Plasmanate® HPP fraction.

Derivation of HKB clone 20B8

As summarized in Figure 4(a), the initial population 1C10 was derived from the HKB cells transfected with pCIS25DTR after amplification with 400 nM MTX in the selection medium with 5% FBS. One of the first single cell clones (SCCs), 10A8, derived from 1C10 by a LDC using a selection medium supplemented with 5% FBS was adapted in serum-free medium supplemented with Plasmanate® HPP fraction. Unexpectedly, 10A8 showed extremely increased levels of rFVIII production at this stage (Figure 4b). Therefore, we did a second LDC using the medium supplemented with Plasmanate® HPP fraction. The productivity of SCCs (e.g. 20B8) derived from the second LDC was similar with Plasmanate® HPP fraction-adapted 10A8. 20B8 showed higher levels of BDD-FVIII than original 10A8 derived from the first LDC in serum-containing medium. Finally, 20B8 was adapted to growth in plasma protein-free (PPF) medium. Samples of 20B8 were deposited at the American Type Culture Collection (Manassas, VA) (ATCC deposit no. CRL-12582).

As shown in Table 1, HKB clones exhibit superior productivity for BDD-FVIII. A 10 - 20 fold increase in productivity was observed in HKB cells when compared to clones derived from transfected CHO and 293S cells.

HKB cells, which do not form large aggregates of cells when grown in suspension culture, are preferred cells for the expression of proteins having factor VIII procoagulant activity.

Table 1. Expression of FVIII and BDD-FVIII in human and rodent cell lines

	Specific Productivity (μU/c/d)*			
FVIII Derivatives	BHK	293s	CHO	HKB
Full length FVIII	0.45	1.2	0.5	1.0
BDD-FVIII	ND	2.5	1.0	20

* Average of 5 high producing clones (in serum-free media)
ND = Not done

Plasma-Protein-free adaptation of clones

HKB clones that have been adapted to grow as serum-free suspension cultures were further weaned of plasma protein supplements. The weaning was done in sterile polycarbonate shake flasks (Corning, Corning, NY) at a cell density of about 0.5 x 10^6 cells/ml using plasma derived protein free medium. The plasma protein free (PPF) medium was DME/F12 medium supplemented with pluronic F68 (0.1%), $CuSO_4$ (50 nM), and $FeSO_4$/EDTA (50 μM). Complete medium exchange was done every 48 hours and the shake flasks were re-seeded at 0.5 x 10^6 cells/ml.

Fermentation of clone 20B8

The productivity of clone 20B8 was evaluated in a 15-liter perfusion fermenter. The fermenter was seeded with clone 20B8 cells at a density of about 3 x 10^6 cells/ml. The fermenter was perfused at a rate of 4 volumes per day with the serum-free production medium as described in the preceding paragraph. A final cell density of 2 x 10^7 cells/ml was sustained throughout the evaluation period (45 days). As shown in Figure 5, during the first 4 weeks of fermentation, clone 20B8 was perfused with the serumfree production medium supplemented with Plasmanate® HPP fraction and was

able to sustain high productivity. From day 28 to the end of the fermentation run, the cells were perfused with the same serumfree production medium but without Plasmanate® HPP fraction. As shown in Figure 5, the cells continued to produce high levels of FVIII in a plasma derived protein-free environment. "Plasma derived protein-free" means that essentially no proteins isolated from plasma have been added to the medium.

DISCUSSION

The derivation of HKB cells provides a protein-free production system to produce not only BDD-FVIII but other therapeutic proteins as well. Proteins produced from HKB cells have human glycosylation patterns which may improve the half-life of certain glycoproteins in vivo. These cells should also be useful for the production of adenovirus and adeno-associated virus strains that have been designed for gene therapy purposes.

The above examples are intended to illustrate the invention and it is thought variations will occur to those skilled in the art. Accordingly, it is intended that the scope of the invention should be limited only by the claims below.

* C L M S *

CLAIMS

We claim:

1. A method of producing cells which express a protein having factor VIII procoagulant activity comprising the sequential steps of:
   a) obtaining cells which are solely of human origin,
   b) contacting the cells of step a) with a vector under conditions sufficient to allow the vector to enter the cells, wherein the vector comprises a selectable marker and a sequence coding for the protein having factor VIII procoagulant activity operably linked to a promoter,
   c) selecting the cells from step b) with a selection agent, and
   d) isolating individual clones which express high levels of the protein having factor VIII activity from the cells obtained from step c).
2. The method of claim 1, further comprising the step
   e) adapting the clones of step d) to growth in a plasma derived protein-free medium.
3. The method of claim 1, wherein the cells of step a) are hybrids of human lymphoma cells and 293S cells.
4. The method of claim 1, wherein the cells of step a) are hybrids of 2B8 cells (ATCC CRL-12569) and 293S cells.
5. The method of claim 1, wherein the cells of step a) are HKB11 cells (ATCC CRL-12568).
6. The method of claim 1, wherein the steps c) and d) are performed more than once.
7. The method of claim 1, wherein the sequence of step b) codes for the sequence shown in figure 1 (SEQ ID NO:1).

8. The method of claim 1, wherein the sequence of step b) codes for human factor VIII.

9. The method of claim 1, wherein the selectable marker of step b) is dhfr and the selection agent of step c) is methotrexate.

10. The method of claim 1, wherein the selectable marker of step b) is gs and the selection agent of step c) is methionine sulfoximine.

11. The method of claim 1, wherein the selectable marker of step b) is mdr and the selection agent of step c) is colchicine.

12. A method of producing a protein having factor VIII activity comprising growing the cells produced by the method of claim 1 in a growth medium and then isolating the protein having factor VIII activity from the medium.

13. The method of claim 11 wherein the protein is human factor VIII.

14. The method of claim 11 wherein the protein has the amino acid sequence shown in figure 1 (SEQ ID NO:1]).

15. A human cell line derived from human lymphoma cells and 293S cells which expresses high levels of a protein having factor VIII activity.

16. The human cell line of claim 14, wherein the human cell line is derived from HKB11 cells (ATCC CRL-12568).

17. A human cell line derived from human lymphoma cells and 293S cells which expresses high levels of a protein having factor VIII activity when grown in plasma derived protein-free medium.

18. The cell line of claim 16, wherein the cell line is derived from HKB11 cells (ATCC CRL-12568).

19. A cell line designated 20B8 (ATCC CRL-12582).