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(54) Titre : UTILISATION DE CHAPERONS MOLECULAIRES POUR AUGMENTER LA FABRICATION DE PROTEINES SECRETEES RECOMBINANTES DANS LES CELLULES MAMMALIENNES

(54) Title: USE OF MOLECULAR CHAPERONES FOR THE ENHANCED PRODUCTION OF SECRETED, RECOMBINANT PROTEINS IN MAMMALIAN CELLS

(57) Abrégé/Abstract:
The present invention relates to a method for increased production of a secreted, recombinant protein product through the introduction of molecular chaperones in a mammalian host cell. The present invention also relates to a mammalian host cell with enhanced expression of a secreted recombinant protein product by coexpressing at least one chaperone protein.

ABSTRACT

The present invention relates to a method for increased production of a secreted, recombinant protein product through the introduction of molecular chaperones in a mammalian host cell. The present invention also relates to a mammalian host cell with enhanced expression of a secreted recombinant protein product by coexpressing at least one chaperone protein.

Use of Molecular Chaperones for the Enhanced Production of Secreted, Recombinant Proteins in Mammalian Cells

This application has been divided out of Canadian Patent Application No. 2,850,891 which is itself divided out of Canadian Patent Application No. 2,529,369, which is the national phase application derived from International Application PCT/US2004/007993 filed internationally on March 16, 2004 and published internationally as WO 2005/010046 on February 3, 2005.

Field of the Invention

The present invention relates to the general field of recombinant protein production in a mammalian host cell. Specifically, the present invention relates to enhanced production of a secreted recombinant protein product by coexpressing at least one chaperone protein in the mammalian host cell.

Background of the Invention

In both procaryotic and eucaryotic cells, molecular chaperone proteins catalyze disulfide bond exchange and assist in the proper folding of newly synthesized proteins. This observation has led to a large number of studies and proposed uses for these quality control proteins. For example, increasing pDI (protein disulfide isomerase) activity in bacterial, yeast and insect cell expression systems can have beneficial effects on protein solubility and folding and, in some cases, can lead to an increase in the secretion of heterologous proteins (1-7). In addition, other studies have shown that the molecular chaperones immunoglobulin heavy chain binding protein (BiP, also referred to as glucose regulated protein) and human heat shock protein 70 (Hsp 70) have a beneficial effect on recombinant protein expression in insect cell systems (5, 8-12).

Molecular chaperones have not had the same level of success on recombinant protein expression and secretion in mammalian cell systems. For example, overexpression of the pDI chaperone in Chinese hamster ovary (CHO) cells not only had no effect on the secretion levels of IL-15, but also caused a decrease in secretion, and an increase in cellular retention of a tumor necrosis factor receptor-Fc fusion protein (TNFR:Fc) (13). Other studies have shown that overexpression of the BiP chaperone in mammalian cells

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can lead to increased cellular retention and decreased secretion of recombinant proteins (14-15 and U.S. Patent No. 4,912,040). The regulatory mechanisms involved in protein processing within the mammalian cell are complex, and probably involve the cooperation of many of these chaperone proteins. Therefore, one cannot predict whether a particular chaperone will lead to an increase in the production of a certain recombinant protein.

Because of the contradictory teaching in the field, the effect of chaperone proteins on the production of a secreted recombinant protein product is not understood and appreciated. U.S. Patent No. 6,451,597 (the '597 patent) describes a method for enhanced production of viral particles, and speculates on the effect of chaperones on improving yield of a recombinant protein in eukaryotic cells. However, no actual expression of a recombinant protein is disclosed. However, other studies had found that over-expression of chaperones in eukaryotic cell lines either had no effect on product yields or had reduced secretion of recombinant proteins (14, 15). See also U.S. Patent No. 4,912,040. In light of the contradictory teaching in the field, the '597 patent does not enable one of skill in the art to use chaperones to improve the production and secretion of a recombinant protein in eukaryotic cells. The state of art does not teach one to predict what effect a particular chaperone will have in the production and secretion of a given recombinant protein in cell culture models such as those described herein. The applicants were therefore surprised to find that when the chaperones described in this study were transfected into mammalian cell lines expressing a secreted, recombinant protein, the resultant effect was an overall increase in the production of the secreted protein.

Summary

The present invention relates to mammalian cells, methods and reagents therefor, for enhanced expression of a secreted recombinant protein product in a mammalian host cell.

Certain exemplary embodiments provide a mammalian CHO host cell for enhanced expression of a recombinant bikunin protein or fragment thereof, said mammalian CHO cell having genetic material coding for expression of said recombinant bikunin protein or fragment thereof and transformed with at least one expression vector comprising DNA encoding chaperone protein Erp57.

Other exemplary embodiments provide a mammalian BHK host cell for enhanced expression of a recombinant Factor VIII protein or fragment thereof, said mammalian BHK cell having genetic material coding for expression of said recombinant Factor VIII protein or fragment thereof and transformed with at least one expression vector comprising DNA encoding chaperone protein Erp57 and at least one expression vector comprising calreticulin.

Yet other exemplary embodiments provide a method for enhancing recombinant bikunin protein or fragment thereof yield, wherein genetic material coding for expression of said recombinant protein has been previously introduced into the cell line to form the first cell line, said method comprising the steps of: inserting at least one chaperone protein expression vector comprising DNA encoding Erp57 chaperone protein into said first cell line so as to form a modified cell line; and selecting from said modified cell line at least one second cell line exhibiting enhanced yield of the recombinant protein.

Still yet other exemplary embodiments provide a method for enhancing recombinant Factor VIII protein or fragment thereof yield, wherein genetic material coding for expression of said recombinant protein has been previously introduced into a BHK cell line to form a first cell line, said method comprising the steps of: inserting at least one chaperone protein expression vector comprising DNA encoding Erp57 chaperone protein and DNA encoding calreticulin (CRT) chaperone protein into said first cell line so as to form a modified cell line; and selecting from said modified cell line at least one second cell line exhibiting enhanced yield of the recombinant protein.

In one embodiment of the first aspect of the invention, the recombinant protein product is secreted.

In another embodiment of the invention, the genetic material coding for expression of said recombinant protein product is integrated into host cell DNA.

In another embodiment of the invention, the mammalian host cell is further transformed with an expression vector comprising DNA encoding a glutamine synthetase protein.

In another embodiment of the invention, the recombinant protein product comprises bikunin, Factor VIII, IL2SA, or fragment thereof.

In another embodiment of the invention, the transformation occurs with an expression vector comprising DNA encoding calnexin, calreticulin, Erp57, Hsp40, and Hsp70.

In another embodiment of the invention, the transformation occurs with a first expression vector comprising DNA encoding calreticulin and a second expression vector.

In a second aspect of the invention, a method for producing a mammalian host cell for enhanced expression of a target recombinant protein or fragment thereof is provided, wherein the method comprises providing a mammalian cell having genetic material coding for expression of a target recombinant protein or fragment thereof; and

transforming the mammalian cell with at least one expression vector comprising DNA encoding a chaperone protein selected from the group consisting of calnexin, calreticulin, Erp57, Hsp40, and Hsp70.

In one embodiment of the second aspect of the invention, the recombinant protein product is secreted.

In another embodiment of the invention, the genetic material coding for expression of said recombinant protein product is integrated into host cell DNA.

In another embodiment of the invention, the mammalian host cell is further transformed with an expression vector comprising DNA encoding a glutamine synthetase protein.

In another embodiment of the invention, the recombinant protein product comprises bikunin, Factor VIII, IL2SA, or fragment thereof.

In another embodiment of the invention, the transformation occurs with an expression vector comprising DNA encoding calnexin, calreticulin, Erp57, Hsp40, or Hsp70.

In another embodiment of the invention, the transformation occurs with a first expression vector comprising DNA encoding calreticulin and a second expression vector comprising DNA encoding Erp57.

In a third aspect of the invention, a method for producing a secreted recombinant protein product is provided, the method comprising the steps of: culturing a mammalian host cell, said mammalian host cell having genetic material coding for expression of said recombinant protein product and transformed with at least one expression vector comprising DNA encoding a chaperone protein selected from the group consisting of calnexin, calreticulin, Erp57, hsp40, and Hsp70; and recovering from the culture medium the recombinant protein product so produced and secreted.

In one embodiment of the third aspect of the invention, the recombinant protein product is secreted.

In another embodiment of the invention, the genetic material coding for expression of said recombinant protein product is integrated into host cell DNA.

In another embodiment of the invention, the mammalian host cell is further transformed with an expression vector comprising DNA encoding a glutamine synthetase protein.

In another embodiment of the invention, the recombinant protein product comprises bikunin, Factor VIII, IL2SA, or fragment thereof.

In another embodiment of the invention, the transformation occurs with an expression vector comprising DNA encoding calnexin, calreticulin, Erp57, Hsp40, or Hsp70.

In another embodiment of the invention, the transformation occurs with a first expression vector comprising DNA encoding calreticulin and a second expression vector comprising DNA encoding Erp57.

In a fourth aspect of the invention, a method for enhancing yield of a recombinant protein or fragment thereof in a mammalian cell is provided, the method comprising providing a first cell line having genetic material coding for expression of said recombinant protein product or fragment thereof and introducing at least one chaperone protein expression vector into said first cell line so as to form a modified cell line; and selecting from said modified cell line at least one second cell line exhibiting enhanced yield of the recombinant protein or fragment thereof.

In one embodiment of the fourth aspect of the invention, the recombinant protein product is secreted.

In another embodiment of the invention, the genetic material coding for expression of said recombinant protein product is integrated into host cell DNA.

In another embodiment of the invention, the mammalian host cell is further transformed with an expression vector comprising DNA encoding a glutamine synthetase protein.

In another embodiment of the invention, the recombinant protein product comprises bikunin, Factor VIII, IL2SA, or fragment thereof.

In another embodiment of the invention, the chaperone expression vector comprises DNA encoding calnexin, calreticulin, Erp57, Hsp40, or Hsp70.

In another embodiment of the invention, said introducing occurs with a first chaperone expression vector comprising DNA encoding calreticulin and a second chaperone expression vector comprising DNA encoding Erp57.

In another embodiment of the invention, at least one second cell line is produced from said first cell line by selecting a portion of said first cell line exhibiting integration of the chaperone protein expression vector into host DNA.

In a fifth aspect of the invention, a method for enhancing yield of a recombinant protein or fragment thereof in a mammalian cell is provided, the method comprises introducing genetic material coding for a recombinant protein or fragment thereof into a cell line exhibiting enhanced chaperone protein expression.

In one embodiment of this aspect of the invention, the recombinant protein product is secreted.

In another embodiment of the invention, the genetic material coding for expression of said recombinant protein product is integrated into host cell DNA.

In another embodiment of the invention, the cell is further transformed with an expression vector comprising DNA encoding a glutamine synthetase protein.

In another embodiment of the invention, the recombinant protein product comprises bikunin, Factor VIII, IL2SA, or fragment thereof.

In another embodiment of the invention, the chaperone protein comprises calnexin, calreticulin, Erp57, Hsp40, or Hsp70.

In another embodiment of the invention, the chaperone protein comprises calreticulin and Erp57.

Brief Description of the Drawings

The invention will be better understood from a consideration of the following detailed description and claims, taken in conjunction with the drawings, in which:

Figure **1** depicts the sequences of RT-PCR primers used to amplify cDNA of ER chaperones from a human cDNA library. Underlined indicates a built in EcoRI (5' primer) or XbaI (3' primer) restriction site. CNX: calnexin; CRT: calreticulin;

Figure **2A** depicts the complete nucleotide and amino acid sequences of calnexin cloned by RT-PCR. The 5' EcoRI and 3' XbaI sites within the primers are underlined. The start codon and stop codon are shown in bold text;

Figure **2B** depicts the complete nucleotide and amino acid sequences of calreticulin cloned by RT-PCR. The 5' EcoRI and 3' XbaI sites are underlined. The start codon and stop codon are shown in bold text;

Figure **2C** depicts the complete nucleotide and amino acid sequences of Erp57 cloned by RT-PCR. The 5' EcoRI and 3' XbaI sites are underlined. The start codon and stop codon are shown in bold text;

Figure **2D** depicts the complete nucleotide and amino acid sequences of the coding region of the human Hsp70 gene;

Figure **2E** depicts the complete nucleotide and amino acid sequences of the coding region of the human Hsp40 gene. The start codon is shown in bold and underlined text;

Figure **2F** depicts the complete nucleotide and amino acid sequences of the coding region of the glutamine synthetase gene. The start codon is shown in bold and underlined text;

Figure **3** is an illustration of overexpression of bikunin in clones super transfected with calnexin (X4.14:5, X4/14:30), Hsp70 (7-3) or Erp57(X4/19:62). The specific Bikunin

production rate for all cell lines is expressed as pg Bikunin/cell/day (SPR). Each day cells were harvested and transferred into fresh media and incubated for 24 hours at 37°C in shaking flasks. The following day, cells were harvested again, counted and re-suspended into fresh media of the same volume and incubated similarly for another 24 hours. Bikunin activity measurements (pg/cell/day) were conducted on samples of the spent media. The same procedure was repeated every day until the cell number and viability started to decrease. The control cell line (CF 9-20) expresses bikunin but does not express any of chaperone proteins;

Figure **4** is an illustration of overexpression of bikunin in clones super transfected with Hsp70. All clones except CF9-20 (control cells) are super transfected with Hsp70. The experiment procedure is the same as that described in Figure 3; and

Figure **5** depicts the amino acid sequence of bikunin.

Detailed Description of the Invention

The present invention relates to a method and reagents therefor, for enhanced expression of a secreted recombinant protein product in a mammalian host cell.

In one embodiment of the invention, a mammalian host cell for enhanced expression of a recombinant protein product is provided, wherein said mammalian cell comprises genetic material coding for expression of said recombinant protein product and is further transformed with at least one expression vector comprising DNA encoding a chaperone protein selected from the group consisting of calnexin, calreticulin, Erp57, Hsp40, and Hsp70.

In another embodiment of the invention, the mammalian host cell is stably transformed with the genetic material coding for expression of said recombinant protein product.

The term "mammalian host cell" is used to refer to a mammalian cell which has been transfected, or is capable of being transfected with a nucleic acid sequence and then of expressing a selected gene of interest. The term includes the progeny of the parent cell, whether or not the progeny is identical in morphology or in genetic make-up to the original parent, so long as the selected gene is present.

Suitable mammalian cells for use in the present invention include, but are not limited to Chinese hamster ovary (CHO) cells, baby hamster kidney (BHK) cells, human HeLa cells, monkey COS-1 cell, human embryonic kidney 293 cells, mouse myeloma NSO and human HKB cells (US Patent No. 6,136,599). The other cell lines are readily available from the ATCC.

The term "transfection" is used to refer to the uptake of foreign or exogenous DNA by a cell, and a cell has been "transfected" when the exogenous DNA has been introduced inside the cell membrane. A number of transfection techniques are well known in the art and are disclosed herein. *See, e.g.*, Graham *et al.*, 1973, *Virology* 52:456; Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual* (Cold Spring Harbor Laboratories, 1989); Davis *et al.*, *Basic Methods in Molecular Biology* (Elsevier, 1986); and Chu *et al.*, 1981, *Gene* 13:197. Such techniques can be used to introduce one or more exogenous DNA moieties into suitable host cells.

Suitable techniques of transfection for use in the present invention include, but are not limited to calcium phosphate-mediated transfection, DEAE-dextran mediated transfection, and electroporation. Cationic lipid transfection using commercially available reagents including the Boehringer Mannheim Transfection Reagent (N->1-(2,3-Dioleoyloxy)propyl-N,N,N-trimethyl ammoniummethylsulfate, Boehringer Mannheim, Indianapolis, Ind.) or LIPOFECTIN or LIPOFECTAMIN or DMRIE reagent (GIBCO-BRL, Gaithersburg, MD) may also be used.

As used herein the term "super transfection" refers to transfecting more than one expression vectors to a host cell already expressing a recombinant gene.

The term "transformation" as used herein refers to a change in a cell's genetic characteristics, and a cell has been transformed when it has been modified to contain a new DNA. For example, a cell is transformed where it is genetically modified from its native state. Following transfection, the transforming DNA may recombine with that of the cell by physically integrating into a chromosome of the cell, may be maintained transiently as an episomal element without being replicated, or may replicate independently as a plasmid. A cell is considered to have been stably transformed when the DNA is replicated with the division of the cell.

As used herein the term "modified cell line" refers to a cell line either transiently or stably transformed with one or more DNA constructs.

Polynucleotides, genetic material, recombinant DNA molecules, expression vectors, and such, used in the practice of the present invention may be isolated using standard cloning methods such as those described by Sambrook et al. (Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, N.Y., 1989).

Alternatively, the polynucleotides coding for a recombinant protein product of the present invention may be synthesized using standard techniques that are well known in the art, such as by synthesis on an automated DNA synthesizer. For example, in one embodiment of the invention, DNA sequences encoding the calnexin protein are synthesized by RT-PCR using primers depicted in Figure 1.

As used herein an "expression vector" refers to a DNA molecule, or a clone of such a molecule, which has been modified through human intervention to contain segments of DNA combined and juxtaposed in a manner that would not otherwise exist in nature. DNA constructs may be engineered to include a first DNA segment encoding a polypeptide of the present invention operably linked to additional DNA segments required for the expression of the first DNA segment. Within the context of the present invention additional DNA segments will generally include promoters and transcription terminators and may further include enhancers and other elements. One or more selectable markers may also be included. DNA constructs useful for expressing cloned DNA segments in a variety of prokaryotic and eukaryotic host cells can be prepared from readily available components or purchased from commercial suppliers.

DNA constructs may also contain DNA segments necessary to direct the secretion of a polypeptide or protein of interest. Such DNA segments may include at least one secretory signal sequence. Secretory signal sequences, also called leader sequences, prepro sequences and/or pre sequences, are amino acid sequences that act to direct the secretion of mature polypeptides or proteins from a cell. Such sequences are characterized by a core of hydrophobic amino acids and are typically (but not exclusively) found at the amino termini of newly synthesized proteins. Very often the secretory peptide is cleaved from the mature protein during secretion. Such secretory peptides contain processing sites that allow cleavage of the secretory peptide from the mature protein as it passes through the secretory pathway. A recombinant protein may contain a secretory signal sequence in its original amino acid sequence, or may be engineered to become a secreted protein by

inserting an engineered secretory signal sequence into its original amino acid sequence. The choice of suitable promoters, terminators and secretory signals is well within the level of ordinary skill in the art. Expression of cloned genes in cultured mammalian cells and in E. coli, for example, is discussed in detail in Sambrook et al. (Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, N.Y., 1989).

As used herein, the term "recombinant protein product" refers to a recombinant protein or fragment thereof expressed from the genetic material introduced into the host mammalian cell.

After transfection, the cell may be maintained either transiently transformed or stably transformed with said DNA construct. Introduction of multiple DNA constructs, and selection of cells containing the multiple DNA constructs can be done either simultaneously or, more preferably, sequentially. The technique of establishing a cell line stably transformed with a genetic material or expression vector is well known in the art (Current Protocols in Molecular Biology). In general, after transfection, the growth medium will select for cells containing the DNA construct by, for example, drug selection or deficiency in an essential nutrient, which is complemented by a selectable marker on the DNA construct or co-transfected with the DNA construct. Cultured mammalian cells are generally cultured in commercially available serum-containing or serum-free medium. Selection of a medium appropriate for the particular host cell used is within the level of ordinary skill in the art.

Suitable selectable markers for drug selection used in this invention include, but are not limited to, neomycin (G418), hygromycin, puromycin, zeocin, colchine, methotrexate, and methionine sulfoximine.

Once a drug resistant cell population is established, individual clones may be selected and screened for high expressing clones. Methods of establishing cloned cell line are well known in the art, including, but not limited to, using a cloning cylinder, or by limiting dilution. Expression of the recombinant product of interest from each clone can be measured by methods such as, but not limited to, immunoassay, enzymatic assay, or chromogenic assay.

Cell line stably transformed with a first DNA construct may be then used as host cell for transfection with a second or more DNA constructs, and subjected to different drug selections.

In one particular embodiment there is provided a mammalian host cell for enhanced expression of a recombinant protein product, said mammalian cell having genetic material coding for expression of said recombinant protein product and transformed with at least one expression vector comprising DNA encoding chaperone protein Hsp70.

In another particular embodiment there is provided a mammalian host cell for enhanced expression of bikunin or a fragment thereof, said mammalian cell having genetic material coding for expression of bikunin or a fragment thereof and transformed with at least one expression vector comprising DNA encoding chaperone protein Hsp70.

In yet another particular embodiment there is provided a mammalian host cell for enhanced expression of Factor VIII or a fragment thereof, said mammalian cell having genetic material coding for expression of Factor VIII or a fragment thereof and transformed with at least one expression vector comprising DNA encoding chaperone protein Hsp70.

In still yet another particular embodiment there is provided a mammalian host cell for enhanced expression of IL2SA or a fragment thereof, said mammalian cell having genetic material coding for expression of IL2SA or a fragment thereof and transformed with at least one expression vector comprising DNA encoding chaperone protein Hsp70.

In still yet another particular embodiment there is provided a method for producing a mammalian host cell for enhanced expression of a target recombinant protein or a fragment thereof comprising: providing a mammalian cell having genetic material coding for expression of a target recombinant protein or a fragment thereof; and transforming the mammalian cell with at least one expression vector comprising DNA encoding chaperone protein Hsp70.

In still yet another particular embodiment there is provided a method for producing a secreted recombinant protein product comprising the steps of: culturing a mammalian host cell, said mammalian host cell having genetic material coding for

expression of said recombinant protein product and transformed with at least one expression vector comprising DNA encoding chaperone protein Hsp70; and recovering from the culture medium the recombinant protein product so produced and secreted.

In still yet another particular embodiment there is provided a method for enhancing recombinant bikunin protein yield in a Chinese hamster ovary (CHO) cell line, wherein genetic material coding for expression of said recombinant bikunin has been previously introduced into a first CHO cell line, said method comprising the steps of: inserting at least one chaperone protein expression vector encoding Hsp70 into said first CHO cell line so as to form a modified CHO cell line; and selecting from said modified CHO cell line at least one second cell line exhibiting enhanced yield of the recombinant bikunin protein.

In still yet another particular embodiment there is provided a method for enhancing recombinant Factor VIII yield in a baby hamster kidney (BHK) cell line, wherein genetic material coding for expression of said recombinant Factor VIII has been previously introduced into a first BHK cell line, said method comprising the steps of: inserting at least one chaperone protein expression vector encoding Hsp70 into said first BHK cell line so as to form a modified BHK cell line; and selecting from said modified BHK cell line at least one second cell line exhibiting enhanced yield of the recombinant Factor VIII product.

In still yet another particular embodiment there is provided a method for enhancing recombinant IL2SA protein yield in a CHO cell line, wherein genetic material coding for expression of said recombinant IL2SA has been previously introduced into a first CHO cell line, said method comprising the steps of: inserting at least one chaperone protein expression vector encoding Hsp70 into said first CHO cell line so as to form a modified CHO cell line; and selecting from said modified CHO cell line at least one second cell line exhibiting enhanced yield of the recombinant IL2SA protein.

In still yet another particular embodiment there is provided a method for enhancing yield of a recombinant Factor VIII or a fragment thereof in a BHK cell line comprising introducing genetic material coding for such Factor VIII or a fragment thereof into a BHK cell line exhibiting enhanced chaperone protein expression, wherein the chaperone protein is Hsp70.

In still yet another particular embodiment there is provided a method for enhancing yield of a recombinant IL2SA or a fragment thereof in a CHO cell line comprising introducing genetic material coding for such IL2SA into a CHO cell line exhibiting enhanced chaperone protein Hsp70 expression.

In still yet another particular embodiment there is provided a method for enhancing recombinant Factor VIII protein production comprising the steps of: culturing a mammalian host cell, said mammalian host cell having genetic material coding for expression of the recombinant Factor VIII protein and transformed with at least one expression vector comprising DNA encoding chaperone protein Hsp70; and recovering from the culture medium the recombinant protein product so produced and secreted.

In still yet another particular embodiment there is provided a method for enhancing recombinant IL2SA protein production comprising the steps of: culturing a mammalian host cell, said mammalian host cell having genetic material coding for expression of the recombinant IL2SA protein and transformed with at least one expression vector comprising DNA encoding chaperone protein Hsp70; and recovering from the culture medium the recombinant protein product so produced and secreted.

In one embodiment of the invention, a mammalian host cell with enhanced expression and secretion of bikunin protein or fragment thereof is provided, wherein the

mammalian host cell is further transformed with at least one expression vector comprising DNA encoding a chaperone protein selected from the group consisting of calnexin, calreticulin, Erp57, Hsp40, and Hsp70.

In a preferred embodiment of the invention, the mammalian host cell with enhanced expression and secretion of bikunin is a CHO cell.

As used herein the term "bikunin" refers to any protein, which has at least one Kunitz domain. Kunitz-type domains have been described in references such as Laskowski et al., 1980, Ann Rev Biochem. 49:593-626; and U.S. Patent No. 5,914,315 (June 22, 1999). In one preferred embodiment, the term bikunin used herein refers to the amino acid sequence shown in Figure 5. Other bikunin proteins and fragments thereof are described in U.S. Patent No. 6,583,208, U.S. Patent Publication No. 2003-0194398 A1, and PCT Application serial numbers US97/03894, published as WO 97/33996, and US99/04381, published as WO 00/37099).

In another embodiment of the invention, the invention provides a mammalian host cell with enhanced expression and secretion of Factor VIII protein or fragment thereof, and the mammalian host cell is further transformed with at least one expression vector comprising DNA encoding a chaperone protein selected from the group consisting of calnexin, calreticulin, Erp57, Hsp40, and Hsp70.

In one preferred embodiment, the Factor VIII protein has the sequence depicted in U.S. Patent No. 4,965,199.

In yet another preferred embodiment, the mammalian host cell with enhanced expression and secretion of Factor VIII is a BHK cell.

In another embodiment of the invention, the invention provides a mammalian host cell with enhanced expression and secretion of IL2SA protein or fragment thereof, and the mammalian host cell is further transformed with at least one expression vector comprising DNA encoding a chaperone protein selected from the group consisting of calnexin, calreticulin, Erp57, Hsp40, and Hsp70.

In one preferred embodiment, the IL2SA protein has the sequence depicted in US patent No. 6,348,192.

In yet another preferred embodiment, the mammalian host cell with enhanced expression and secretion of IL2SA is a CHO cell.

In still another embodiment of the invention, the mammalian host cell is further transformed with an expression vector encoding a glutamine synthetase protein.

The present invention also provides a method for producing a mammalian host cell for enhanced expression of a target recombinant protein or fragment thereof comprising: providing a mammalian cell having genetic material coding for expression of a target recombinant protein or fragment thereof; and transforming the mammalian cell with at least one expression vector comprising DNA encoding a chaperone protein selected from the group consisting of calnexin, calreticulin, Erp57, Hsp40, and Hsp70.

In one embodiment of the invention, the genetic material coding for expression of said recombinant protein product is integrated into host cell DNA.

In another embodiment of the invention, the mammalian host cell is further transformed with an expression vector comprising DNA encoding a glutamine synthetase protein.

In one preferred embodiment of the invention, the recombinant protein product is bikunin or fragment thereof and the transformation occurs with an expression vector comprising DNA encoding calnexin, Erp57, calreticulin, or Hsp70.

In another preferred embodiment of the invention, the recombinant protein product is Factor VIII or fragment thereof and the transformation occurs with a first expression vector comprising DNA encoding calreticulin and a second expression vector comprising DNA encoding Erp57.

In another preferred embodiment of the invention, the recombinant protein product is Factor VIII or fragment thereof and the transformation occurs with an expression vector comprising DNA encoding calnexin or Hsp70.

In another preferred embodiment of the invention, the recombinant protein product is IL2SA or fragment thereof and the transformation occurs with an expression vector comprising DNA encoding Hsp70.

The present invention also provides a method for producing a secreted recombinant protein product comprising culturing a mammalian host cell, said mammalian host cell having a genetic material coding for expression of said recombinant product and further transformed with at least one expression vector comprising DNA encoding a chaperone protein elected from the group consisting of calnexin, calreticulin,

Erp57, Hsp40, and Hsp70; and recovering from the culture medium the bikunin protein or fragment thereof so produced and secreted.

In one embodiment of the invention, the method for producing a secreted recombinant protein product comprising culturing a mammalian host cell, wherein the mammalian host cell is stably transformed with a genetic material coding for the expression of said recombinant product.

In another embodiment of the invention, the method for producing a secreted recombinant protein product further comprises transfecting the mammalian host cell with an expression vector encoding a glutamine synthetase protein.

One embodiment of the invention provides a method of producing a bikunin protein or fragment thereof, comprising culturing a mammalian host cell expressing bikunin or fragment thereof, and at least one of the chaperone proteins selected from the group consisting of calnexin, calreticulin, Erp57, Hsp40, and Hsp70; and recovering from the culture medium the bikunin protein or fragment thereof so produced and secreted.

In one embodiment of the invention, a method for enhanced production of a recombinant bikunin protein in a CHO cell is provided, wherein a genetic material coding for expression of said recombinant bikunin has been previously introduced into a first CHO cell line (as described in U.S. Patent Publication No. 2004-0235715 to Chan published November 25, 2004), comprising the steps of: inserting at least one chaperone protein expression vector into said first CHO cell line so as to form a modified CHO cell line; and selecting from said modified CHO cell line at least one second cell exhibiting enhanced yield of the recombinant bikunin protein.

In another embodiment of the invention, the method for enhancing recombinant bikunin yield in a CHO cell line comprises introducing a genetic material for such bikunin into a CHO cell line, wherein the CHO cell line exhibits enhanced chaperone protein expression.

In yet another embodiment of the invention, a method for enhanced production of a recombinant Factor VIII protein in a BHK cells is provided, wherein a genetic material coding for expression of said recombinant Factor VIII has been previously introduced into a first BHK cell line, comprising the steps of: inserting at least one chaperone protein expression vector into said first BHK cell line so as to form a modified BHK cell line; and selecting from said modified BHK cell line at least one second cell exhibiting enhanced yield of the recombinant Factor VIII protein.

In still another embodiment of the invention, the method for enhancing recombinant Factor VIII yield in a BHK cell line comprises introducing a genetic material for such Factor VIII into a BHK cell line, wherein the BHK cell line exhibits enhanced chaperone protein expression.

The present invention also provides a method for enhanced production of a recombinant IL2SA protein into a CHO cell, wherein a genetic material coding for expression of said recombinant IL2SA has been previously introduced into a first CHO cell line, comprising the steps of: inserting at least one chaperone protein expression vector into said first CHO cell line so as to form a modified CHO cell line; and selecting from said modified CHO cell line at least one second cell exhibiting enhanced yield of the recombinant IL2SA protein.

In another embodiment of the invention, the method for enhancing recombinant IL2SA yield in a CHO cell line comprises introducing a genetic material for such IL2SA into a CHO cell line, wherein the CHO cell line exhibits enhanced chaperone protein expression.

The following examples are intended for illustration purposes only, and should not be construed as limiting the scope of the invention in any way.

Examples

Example 1. Cloning of chaperone cDNA.

All chaperone sequences were cloned from human cDNA libraries followed by verification of the nucleotide sequences. DNA sequences representing the three ER chaperones were cloned by RT-PCR from a human cDNA library. The RT-PCR primers used in these reactions were designed to amplify the entire coding region using the appropriate sequences obtained from Genbank. Each pair of 5' and 3' primers include either an EcoRI (5' primer) or XbaI (3' primer) restriction site (Figure 1) to facilitate cloning of the PCR product into the expression vector, pCI-neo (Promega).

The PCR reactions were performed using high fidelity PFU enzyme (Stratagene). Bands of the expected size were purified, digested with EcoR I and Xba I and cloned into the similarly digested pCI-neo vector. Recombinant vectors from this step were propagated in E. Coli followed by isolation and purification of the vector sequences. The

sequence inserts representing the chaperones were sequenced using primers binding just outside the multiple cloning sites of the vector as well as within the chaperone sequence. Sequencing was done using the Big Dye™ terminator method on MJ Research's thermal cycler and analyzed using an ABI 310 Genetic Analyzer. The cDNA sequences of human calnexin, clareticulin and Erp57 are shown in Figures 2A-2C.

The full-length human Hsp70 cDNA fragment was obtained by RT-PCR using human brain polyA$^+$ RNA (CLONTECH Cat: 6516-1) and two primers designated F-Hsp70 = 5'AGG GAA CCG CAT GGC CAA AG and R-Hsp70 = 5' GAA AGG CCCCTA ATC TAC CTC CTC A. The primer sequences of Hsp 70 were derived from the previously published sequence for the human heat shock protein (Hsp70) gene [9]. The F-Hsp70 and R-Hsp70 primers included either an EcoRI or XbaI sequence respectively. The desired PCR fragment was purified by agarose gel electrophoresis and confirmed by nucleotide sequencing. The full-length human Hsp70 cDNA fragment was then inserted into the EcoRI and XbaI cloning sites of the pCI-neo vector to form the pCI-neo-Hsp70 vector. The pCI-neo-Hsp70 vector was propagated in E. Coli followed by isolation and purification of the vector sequences. pCI-neo-Hsp70 plasmid DNA was sequenced by ABI PRISM™ 310 Genetic Analyzer. The sequence of human Hsp70 is shown in Figure 2D.

Example 2. Bikunin production is increased in CHO cells after transfection of an ER chaperone such as calnexin, calreticulin, Erp57 or Hsp70.

A CHO cell line secreting the Bikunin recombinant protein (U.S. Patent Publication No. 2004-0235715) was super transfected with various combinations of the ER chaperones, calnexin (CNX), calreticulin (CRT), ERp57 or Hsp70 followed by selection with G418. Populations were obtained and screened by kallikrein assay (U.S. Publication No. 2004-0235715). Briefly, bikunin standarts or culture fluid was serially diluted and incubated with an equal volume of kallikrein at 37°C for 30 minutes, after which a chromogenic substrate, N-benzoyl-Pro-Phe-Arg-pNA, was added. The reaction was incubated for 15 minutes before the addition of 50% acetic acid. The amount of p-nitroanilide released was measured at 405 nM. Populations showing the highest Bikunin

titers were then single cell cloned and growth expanded over a period of several weeks. Clones showing consistently higher Bikunin titers (2-4x) relative to the control CF9-20 cells were retained and expanded into shake flasks for further analysis. These clones were further narrowed based on Bikunin titers and growth characteristics demonstrated while growing in the shake flask environment. Final candidate clones were selected after several rounds and extensive analyses at the shake flask stage.

The specific Bikunin production rate for all cell lines is expressed as pg Bikunin/cell/day (SPR). Each day cells were harvested and transferred into fresh media and incubated for 24 hours at 37°C in shaking flasks. The following day, cells were harvested again, counted and re-suspended into fresh media of the same volume and incubated similarly for another 24 hours. Bikunin activity measurements (pg/cell/day) were conducted on samples of the spent media. The same procedure was repeated every day until the cell number and viability started to decrease.

The effect of chaperone proteins on bikunin expression is shown in Figures 3 and 4. The control cell line (CF9-20) expresses Bikunin but does not express any of chaperone proteins. The effect of calnexin, calreticulin, and Erp57 on bikunin expression is further summarized in Table 1.

Table 1. Overall Bikunin production levels are 2-4 fold higher in clones that have been super transfected with a chaperone

Clone	Bikunin Increase Relative to Control	Chaperone
X4/14:5	2-4	CNX
X4/14:30	2-4	CNX
X4/19:62	2-4	ERp57
T4/13:22	1.5-2	CRT

Fold activity measurements are relative to a control cell line that expresses Bikunin but does not express any of the chaperone proteins. Cells were grown in serum free media in shake flask cultures.

Example 3. Recombinant Factor VIII production is increased in BHK cells after transfection with ER chaperones.

Stable Factor VIII producing cells (MWCB1) (U.S. Patent No. 4,965,199; ATCC No. CRL 8544) were transfected with chaperone expression vectors in addition to pPUR, a vector containing puromycin-resistant gene, in a 10 : 1 ratio. Approximately 4 x 10^6 MWCB1 cells were transfected with a total of 5 μg of DNA using the DMRIE-C reagent and OPTI-MEM medium (Life Technology, MD) in 6-well plates. Three days post transfection, 100,000 cells were seeded in 6-well plates and then selected in the presence of 1 – 2 μg/ ml puromycin with OPTI-MEM medium containing 2% FBS for 2 weeks. Puromycin resistant colonies were manually picked and seeded into 96 well plates and expanded without the presence of drug. Individual clonal populations were screened for Factor VIII production using a COATEST kit (Chromogenix, Italy) according to manufacturer's instructions. The high producing clones were sequentially expanded from the 6 well dish, to T75 flask, followed by shake flask stage for stability and productivity tests. The Calnexin (CNX), Calreticulin (CRT), Erp57, Hsp40 and Hsp70 chaperones were then transfected into cells individually or in combinations of two. A significant 2 to 3 fold increase of productivity of Factor VIII was observed in clones transfected with CNX, CRT and Erp57, Hsp70, and Hsp40 while the empty vector control (PCI-Neo) showed no difference compared to the parent MWCB1 cells (Table 2).

Table 2. Recombinant Factor VIII productivity in clones

	Factor VIII (U/ml)	Fold of Inc (SPR)
MWCB1(27000JC)	0.11	1.00
PCI-Neo + pPUR	0.09	1.00
CNX + pPUR	0.31	2.88
CRT + pPUR	0.13	1.25
Erp57 + pPUR	0.05	0.91
CRT, Erp57 + pPUR	0.29	2.50
Hsp70 + pPUR	0.37	2.50
Hsp40 + pPUR	0.11	1.00
Hsp70, 40 + pPUR	0.28	1.66

Cells were seeded at 1 x 10^6 per ml, total 15 ml in shake flask 2-day

Example 4. Co-expression of BiP and PDI does not enhance the expression of Factor VIII and anti-TNF antibody in BHK and CHO cells.

Recombinant CHO cells (as described in Example 2) expressing high levels of bikunin, and recombinant BHK cells (as described in Example 3) expressing high levels of recombinant Factor VIII (rFVIII) were super-transfected with pHyg (plasmid conferring hygromycin resistance) and pBiP. The transfection conditions and selection conditions were same as in Example 2. After selection in hygromycin and limiting dilution cloning, clones were evaluated for productivity for bikunin and rFVIII activity. No significant difference in the specific productivity of clones derived from cells transfected only with the control vector (pHyg) and clones derived from cells transfected with pBiP.

Example 5. Transfection of IL2SA-producing clone with Glutamine Synthetase (GS) and Hsp70.

IL2SA (IL2 selective agonist; U.S. Patent No. 6,348,192) producing CHO cell line, 49-19-H42 (a clonal variant of ATCC deposit PTA-8), was co-transfected with PCI-GS and PCI-neo-Hsp70. 4 x 10^6 cells were transfected with 2.5 μg of plasmid DNA using DMRIE-C reagents and OPTI-MEM medium (Life Technology, MD) in 6-well plates according to manufacturer's instructions. Three days after transfection, cells were seeded in 150-mm and 96 well plates and then selected in the presence of 10 μM MSX (methionine sulfoxinmine) and 250 μg/ml G418 with DME:F12 (1:1) medium deficient in glutamine containing 2% dialyzed FBS for 2 weeks. Single cell colonies were picked and re-seeded in 96 wells. The clones were selected for another week with increased concentrations of MSX (20 μM) and G418 (400 μg/ml). A pool is generated from a 150-mm plate after 3 weeks' selection. The pool and clones were gradually expanded to shake flasks and screened for IL2 productivity using ELISA. The expression of GS and Hsp70 proteins were confirmed by FACS™ analysis using a flow cytometer. The "GS positive" cells were cultured in a glutamine-free medium supplement with 5.6 mM glutamate and 4 g/L glucose. The doubling time of these clones varied from 24 to 48 hr. A comparison of the productivity of the parent and clones is shown in Table 3. A 2- 4 fold increase in overall titer and a 2 -3 fold increase in specific productivity was observed in all the single cell clones when compared against either the pool or the parental line.

Table 3. Productivity of IL2SA producing cells

	Titer (μg/ml)	Cell density (10^6/ml)	SPR (pg/c/d)	GS	Hsp70
49-19H42 parent line	18.78	3.51	2.67	(-)	(-)
49-19H42 GShsp70-SC#12	33.87	2.63	6.44	+++	+++
49-19H42 GShsp70-SC#14	22.08	1.83	6.03	+++	+++
49-19H42 GShsp70-SC#17	64.00	3.05	10.50	+++	+++
49-19H42 GShsp70-pool	10.59	1.74	3.04	+++	+

Cells were seeded at 1 million per ml at day 0 in 15 ml of complete (for the parental line) or glutamine-free medium. Samples were taken at 2 day after seeding and analyzed using ELISA. For GS and Hsp70 expression, cells were fixed with 70% EtOH, labeled with proper antibodies, and analyzed by FACS.

+++ = all cells expressed GS or Hsp70; + = 30% of cells expressed GS or Hsp70; (-) = no expression.

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WHAT IS CLAIMED:

1. A mammalian host cell for enhanced expression of a recombinant protein product, said mammalian cell having genetic material coding for expression of said recombinant protein product and transformed with at least one expression vector comprising DNA encoding a chaperone protein selected from the group consisting of calnexin, calreticulin, Erp57, Hsp40, and Hsp70.

2. The mammalian host cell according to claim 1, wherein the chaperone protein is selected from the group consisting of calnexin, calreticulin, and Erp57.

3. The mammalian host cell according to claim 1 or claim 2, wherein the recombinant protein product is secreted.

4. The mammalian host cell according to claim 3, wherein the genetic material coding for expression of said recombinant protein product is integrated into host cell DNA.

5. The mammalian host cell according to claim 4, further transformed with an expression vector comprising DNA encoding a glutamine synthetase protein.

6. The mammalian host cell according to claim 3, wherein the recombinant protein product is bikunin or fragment thereof.

7. The mammalian host cell according to claim 6, wherein the transformation occurs with an expression vector comprising DNA encoding calnexin.

8. The mammalian host cell according to claim 6, wherein the transformation occurs with an expression vector comprising DNA encoding Erp57.

9. The mammalian host cell according to claim 6, wherein the transformation occurs with an expression vector comprising DNA encoding calreticulin.

10. The mammalian host cell according to claim 6, wherein the transformation occurs with an expression vector comprising DNA encoding Hsp70.

11. The mammalian host cell according to claim 3 wherein the recombinant protein product is Factor VIII or fragment thereof.

12. The mammalian host cell according to claim 11wherein said transformation occurs with a first expression vector comprising DNA encoding calreticulin and a second expression vector comprising DNA encoding Erp57.

13. The mammalian host cell according to claim 11 wherein said transformation occurs with an expression vector comprising DNA encoding calnexin.

14. The mammalian host cell according to claim 11, wherein said transformation occurs with an expression vector comprising DNA encoding Hsp70.

15. The mammalian host cell according to claim 3 wherein the recombinant protein product is IL2SA or fragment thereof.

16. The mammalian host cell according to claim 15, wherein said transformation occurs with an expression vector comprising DNA encoding Hsp70.

17. A mammalian host cell for enhanced expression of bikunin or fragment thereof, said mammalian cell having genetic material coding for expression of bikunin or fragment thereof and transformed with at least one expression vector comprising DNA encoding a chaperone protein selected from the group consisting of calnexin, calreticulin, Erp57, Hsp40, and Hsp70.

18. The mammalian host cell according to claim 16, wherein the chaperone protein is selected from the group consisting of calnexin, calreticulin, and Erp57.

[17]19. The mammalian host cell according to claim [16]17 or claim 18, wherein the genetic material coding for expression of bikunin or fragment thereof is integrated into the host cell DNA.

[18]20. The mammalian host cell according to claim [16]17 or claim 18, further transformed with an expression vector comprising DNA encoding a glutamine synthetase protein.

[19]21.A mammalian host cell for enhanced expression of Factor VIII or fragment thereof, said mammalian cell having genetic material coding for expression of Factor VIII or fragment thereof and transformed with at least one expression vector comprising DNA encoding a chaperone protein selected from the group consisting of calnexin, calreticulin, Erp57, hsp40, and Hsp70.

22. The mammalian host cell according to claim 21, wherein the chaperone protein is selected from the group consisting of calnexin, calreticulin, and Erp57.

23. The mammalian host cell according to claim 21 or claim 22, wherein the genetic material coding for expression of Factor VIII or fragment thereof is integrated into the host cell DNA.

24 The mammalian host cell according to claim 23, further transformed with an expression vector comprising DNA encoding a glutamine synthetase protein.

25. A mammalian host cell for enhanced expression of IL2SA or fragment thereof, said mammalian cell having genetic material coding for expression of IL2SA or fragment thereof and transformed with at least one expression vector comprising DNA

encoding a chaperone protein selected from the group consisting of calnexin, calreticulin, Erp57, hsp40, and Hsp70.

26. The mammalian host cell according to claim 25, wherein the chaperone protein is selected from the group consisting of calnexin, calreticulin, and Erp57.

27. The mammalian host cell according to claim 25 or claim 26, wherein the genetic material coding for expression of IL2SA or fragment thereof is integrated into the host cell DNA.

28. The mammalian host cell according to claim 27, further transformed with an expression vector comprising DNA encoding a glutamine synthetase protein.

29. A method for producing a mammalian host cell for enhanced expression of a target recombinant protein or fragment thereof comprising

providing a mammalian cell having genetic material coding for expression of a target recombinant protein or fragment thereof; and

transforming the mammalian cell with at least one expression vector comprising DNA encoding a chaperone protein selected from the group consisting of calnexin, calreticulin, Erp57, Hsp40, and Hsp70.

30. The method according to claim 29, wherein the chaperone protein is selected from the group consisting of calnexin, calreticulin, and Erp57.

31. The method according to claim 29 or claim 30 wherein the recombinant protein product is secreted.

32. The method according to claim 31, wherein the genetic material coding for expression of said recombinant protein product is integrated into host cell DNA.

33. The method according to claim 32, further transformed with an expression vector comprising DNA encoding a glutamine synthetase protein.

34. The method according to claim 31, wherein the recombinant protein product is bikunin or fragment thereof.

35. The method according to claim 34, wherein the transforming occurs with an expression vector comprising DNA encoding calnexin.

36. The method according to claim 34, wherein the transforming occurs with an expression vector comprising DNA encoding Erp57.

37. The method according to claim 34, wherein the transforming occurs with an expression vector comprising DNA encoding calreticulin.

38. The method according to claim 34, wherein the transforming occurs with an expression vector comprising DNA encoding Hsp70.

39. The method according to claim 31, wherein the recombinant protein product is Factor VIII or fragment thereof.

40. The method according to claim 39, wherein said transforming occurs with a first expression vector comprising DNA encoding calreticulin and a second expression vector comprising DNA encoding Erp57.

41. The method according to claim 39, wherein said transforming occurs with an expression vector comprising DNA encoding calnexin.

42. The method according to claim 39, wherein said transforming occurs with an expression vector comprising DNA encoding Hsp70.

43. The method according to claim 39, wherein said transforming occurs with an expression vector comprising DNA encoding calreticulin.

44. The method according to claim 31, wherein the recombinant protein product is IL2SA or fragment thereof.

45. The method according to claim 44, wherein said transforming occurs with an expression vector comprising DNA encoding Hsp70.

46. A method for producing a secreted recombinant protein product comprising the steps of:

culturing a mammalian host cell, said mammalian host cell having genetic material coding for expression of said recombinant protein product and transformed with at least one expression vector comprising DNA encoding a chaperone protein selected from the group consisting of calnexin, calreticulin, Erp57, hsp40, and Hsp70; and

recovering from the culture medium the recombinant protein product so produced and secreted.

47. The method according to claim 46, wherein the chaperone protein is selected from the group consisting of calnexin, calreticulin, Erp57.

48. The method according to claim 46 or claim 47, wherein the genetic material coding for expression of said recombinant protein product is integrated into the host cell DNA.

49. The method according to claim 48, wherein said mammalian host cell is further transformed with an expression vector comprising DNA encoding a glutamine synthetase protein.

50. A method for producing a bikunin protein or fragment thereof which comprises culturing the mammalian host cell according to claim 6 and recovering from the culture medium the bikunin protein or fragment thereof so produced and secreted.

51. The method according to claim 50, wherein the chaperone protein is selected from the grouped consisting of calnexin, calreticulin, and Erp57.

52.. The method according to claim 50, wherein said mammalian host cell is further transformed with an expression vector comprising DNA encoding a glutamine synthetase protein.

53. A method for producing a Factor VIII protein or fragment thereof which comprises culturing the mammalian host cell according to claim 11 and recovering from the culture medium the Factor VIII protein or fragment thereof so produced and secreted.

54. The method according to claim 53, wherein the chaperone protein is selected from the grouped consisting of calnexin, calreticulin, and Erp57.

55. The method according to claim 53, wherein said mammalian host cell is further transformed with an expression vector comprising DNA encoding a glutamine synthetase protein.

56. A method for producing an IL2SA protein or fragment thereof which comprises culturing the mammalian host cell according to claim 15 and recovering from the culture medium the IL2SA protein or fragment thereof so produced and secreted.

57. The method according to claim 56, wherein the chaperone protein is selected from the grouped consisting of calnexin, calreticulin, and Erp57.

58. The method according to claim 56, wherein said mammalian host cell is further transformed with an expression vector comprising DNA encoding a glutamine synthetase protein.

59. A method for enhancing recombinant bikunin protein yield in a Chinese hamster ovary (CHO) cell line, wherein genetic material coding for expression of said recombinant bikunin has been previously introduced into a first CHO cell line, said method comprising the steps of:

inserting at least one chaperone protein expression vector into said first CHO cell line so as to form a modified CHO cell line; and

selecting from said modified CHO cell line at least one second cell line exhibiting enhanced yield of the recombinant bikunin protein.

60. The method according to claim 59, wherein the genetic material coding for expression of said recombinant bikunin is integrated into the first CHO cell DNA.

61. The method according to claim 60, wherein the second cell line is further transformed with an expression vector comprising DNA encoding a glutamine synthetase protein.

62. The method according to claim 59, wherein at least one second cell line is produced from said first cell line by selecting a portion of said first cell line exhibiting integration of the chaperone protein expression vector into host DNA.

63. The method according to claim 59, wherein said chaperone protein expression vector comprises DNA encoding a chaperone protein selected from the group consisting of calnexin, calreticulin, Erp57, Hsp40, and Hsp70.

64. The method according to claim 63, wherein the chaperone protein is selected from the group consisting of calnexin, calreticulin, and Erp57.

65. The method according to claim 59, wherein said selection occurs in the presence of G418.

66. A method for enhancing recombinant Factor VIII yield in a baby hamster kidney (BHK) cell line, wherein genetic material coding for expression of said recombinant Factor VIII has been previously introduced into a first BHK cell line, said method comprising the steps of:

inserting at least one chaperone protein expression vector into said first BHK cell line so as to form a modified BHK cell line; and

selecting from said modified BHK cell line at least one second cell line exhibiting enhanced yield of the recombinant Factor VIII product.

67. The method according to claim 66, wherein the genetic material coding for expression of said recombinant Factor VIII is integrated into the first BHK cell DNA.

68. The method according to claim 66, wherein the second cell line is further transformed with an expression vector comprising DNA encoding a glutamine synthetase protein.

69. The method according to claim 66, wherein at least one second cell line is produced from said first cell line by selecting a portion of said first cell line exhibiting integration of the chaperone protein expression vector into host DNA.

70. The method according to claim 66, wherein said chaperone protein expression vector comprises DNA encoding a chaperone protein selected from the group consisting of calnexin, calreticulin, Erp57, Hsp40 or Hsp70.

71. The method according to claim 70, wherein the chaperone protein is selected from the group consisting of calnexin, calreticulin, and Erp57.

72. The method according to claim 66, wherein said BHK cell is further transfected with a vector including a puromycin-resistant gene.

73. The method according to claim 66, wherein the selection occurs in the presence of puromycin.

74. A method for enhancing recombinant IL2SA protein yield in a CHO cell line, wherein genetic material coding for expression of said recombinant IL2SA has been previously introduced into a first CHO cell line, said method comprising the steps of:

inserting at least one chaperone protein expression vector into said first CHO cell line so as to form a modified CHO cell line; and

selecting from said modified CHO cell line at least one second cell line exhibiting enhanced yield of the recombinant IL2SA protein.

75. The method according to claim 74, wherein the genetic material coding for expression of said recombinant IL2SA is integrated into the first CHO cell DNA.

76. The method according to claim 75, wherein the second cell line is further transformed with an expression vector comprising DNA encoding a glutamine synthetase protein.

77. The method according to claim 74, wherein said chaperone protein expression vector comprises DNA encoding a chaperone protein selected from the group consisting of calnexin, calreticulin, Erp57, Hsp40, and Hsp70.

78. The method according to claim 77, wherein the chaperone protein is selected from the group consisting of calnexin, calreticulin, and Erp57.

79. A method for enhancing yield of a recombinant bikunin or fragment in a CHO cell line comprising introducing genetic material coding for bikunin or fragment thereof into a CHO cell line exhibiting enhanced chaperone protein expression, wherein

the chaperone protein is selected from the group consisting of calnexin, calreticulin, and Erp57.

80. The method according to claim 79, wherein the CHO cell line is further transformed with an expression vector comprising DNA encoding a glutamine synthetase protein.

81. A method for enhancing yield of a recombinant Factor VIII or fragment thereof in a BHK cell line comprising introducing genetic material coding for such Factor VIII or fragment thereof into a BHK cell line exhibiting enhanced chaperone protein expression, wherein the chaperone protein is selected from the group consisting of calnexin, calreticulin, Erp57, and Hsp70.

82. The method according to claim 81, wherein the BHK cell line is further transformed with an expression vector comprising DNA encoding a glutamine synthetase protein.

83. A method for enhancing yield of a recombinant IL2SA or fragment thereof in a CHO cell line comprising introducing genetic material coding for such IL2SA into a CHO cell line exhibiting enhanced chaperone protein Hsp70 expression.

84. The method according to claim 83, wherein the CHO cell line is further transformed with an expression vector comprising DNA encoding a glutamine synthetase protein.

85. A method for enhancing recombinant bikunin protein production comprising the steps of:

culturing a mammalian host cell, said mammalian host cell having genetic material coding for expression of the recombinant bikunin protein and transformed with at least one expression vector comprising DNA encoding a chaperone protein selected from the group consisting of calnexin, calreticulin, and Erp57; and

recovering from the culture medium the recombinant protein product so produced and secreted.

86. The method according to claim 85, wherein said mammalian host cell is a CHO cell.

87. The method according to claim 85, wherein the genetic material coding for expression of said recombinant protein product is integrated into the host cell DNA.

88. The method according to claim 85, wherein said mammalian host cell is further transformed with an expression vector comprising DNA encoding a glutamine synthetase protein.

89. A method for enhancing recombinant Factor VIII protein production comprising the steps of:

culturing a mammalian host cell, said mammalian host cell having genetic material coding for expression of the recombinant Factor VIII protein and transformed with at least one expression vector comprising DNA encoding a chaperone protein selected from the group consisting of calnexin, calreticulin, Erp57, and Hsp70; and

recovering from the culture medium the recombinant protein product so produced and secreted.

90. The method according to claim 89, wherein said mammalian host cell is a BHK cell.

91. The method according to claim 89, wherein the genetic material coding for expression of said recombinant protein product is integrated into the host cell DNA.

92. The method according to claim 89, wherein said mammalian host cell is further transformed with an expression vector comprising DNA encoding a glutamine

synthetase protein.

93. A method for enhancing recombinant IL2SA protein production comprising the steps of:

culturing a mammalian host cell, said mammalian host cell having genetic material coding for expression of the recombinant IL2SA protein and transformed with at least one expression vector comprising DNA encoding chaperone protein Hsp70; and

recovering from the culture medium the recombinant protein product so produced and secreted.

94. The method according to claim 93, wherein said mammalian host cell is a CHO cell.

95. The method according to claim 93, wherein the genetic material coding for expression of said recombinant protein product is integrated into the host cell DNA.

96. The method according to claim 93, wherein said mammalian host cell is further transformed with an expression vector comprising DNA encoding a glutamine synthetase protein.

97. A CHO cell line with enhanced expression of bikunin protein generated according to claim 59 or 79.

98. A BHK cell line with enhanced expression of Factor VIII generated according to claim 66 or 81.

99. A CHO cell line with enhanced expression of IL2SA generated according to claim 74 or 83.

CNX: 5' primer: ATGAATTCCGGGAGGCTAGAGATCATGG
3' primer: ATTCTAGATGCAGGGGAGGAGGGAGAAG
CRT: 5' primer: ATGAATTCCCGCCATGCTGCTATCCGTG
3' primer: ATTCTAGACTGGAGGCAGGCCTCTCTAC
Erp57: 5' primer: ATGAATTCCTCCGCAGTCCCAGCCGAGC
3' primer: ATTCTAGACTCTCGGCCCTGAGAGGTAA

FIG. 1

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```
                                          M  E  G     K  W  L
  1 GAATTCCGGG AGGCTAGAGA TCATGGAAGG GAAGTGGTTG
     L  C  M  L     L  V  L     G  T  A     I  V  E  A ·
 41 CTGTGTATGT TACTGGTGCT TGGAACTGCT ATTGTTGAGG
    · H  D  G     H  D  D     D  V  I  D     I  E  D ·
 81 CTCATGATGG ACATGATGAT GATGTGATTG ATATTGAGGA
    · D  L  D     D  V  I  E     E  V  E     D  S  K
121 TGACCTTGAC GATGTCATTG AAGAGGTAGA AGACTCAAAA
     P  D  T  T     A  P  P     S  S  P     K  V  T  Y ·
161 CCAGATACCA CTGCTCCTCC TTCATCTCCC AAGGTTACTT
    · K  A  P     V  P  T     G  E  V  Y     F  A  D ·
201 ACAAAGCTCC AGTTCCAACA GGGGAAGTAT ATTTTGCTGA
    · S  F  D     R  G  T  L     S  G  W     I  L  S
241 TTCTTTTGAC AGAGGAACTC TGTCAGGGTG GATTTTATCC
     K  A  K  K     D  D  T     D  D  E     I  A  K  Y ·
281 AAAGCCAAGA AAGACGATAC CGATGATGAA ATTGCCAAAT
    · D  G  K     W  E  V     E  E  M  K     E  S  K ·
321 ATGATGGAAA GTGGGAGGTA GAGGAAATGA AGGAGTCAAA
    · L  P  G     D  K  G  L     V  L  M     S  R  A
361 GCTTCCAGGT GATAAAGGAC TTGTGTTGAT GTCTCGGGCC
     K  H  H  A     I  S  A     K  L  N     K  P  F  L ·
401 AAGCATCATG CCATCTCTGC TAAACTGAAC AAGCCCTTCC
    · F  D  T     K  P  L     I  V  Q  Y     E  V  N ·
441 TGTTTGACAC CAAGCCTCTC ATTGTTCAGT ATGAGGTTAA
    · F  Q  N     G  I  E  C     G  G  A     Y  V  K
481 TTTCCAAAAT GGAATAGAAT GTGGTGGTGC CTATGTGAAA
     L  L  S  K     T  P  E     L  N  L     D  Q  F  H ·
521 CTGCTTTCTA AAACACCAGA ACTCAACCTG GATCAGTTCC
    · D  K  T     P  Y  T     I  M  F  G     P  D  K ·
561 ATGACAAGAC CCCTTATACG ATTATGTTTG GTCCAGATAA
    · C  G  E     D  Y  K  L     H  F  I     F  R  H
601 ATGTGGAGAG GACTATAAAC TGCACTTCAT CTTCCGACAC
     K  N  P  K     T  G  I     Y  E  E     K  H  A  K ·
641 AAAAACCCCA AAACGGGTAT CTATGAAGAA AAACATGCTA
    · R  P  D     A  D  L     K  T  Y  F     T  D  K ·
681 AGAGGCCAGA TGCAGATCTG AAGACCTATT TTACTGATAA
    · K  T  H     L  Y  T  L     I  L  N     P  D  N
```

FIG. 2A

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```
721 GAAAACACAT CTTTACACAC TAATCTTGAA TCCAGATAAT
      S   F   E   I     L   V   D     Q   S   V     V   N   S   G  ·
761 AGTTTTGAAA TACTGGTTGA CCAATCTGTG GTGAATAGTG
    ·   N   L   L     N   D   M     T   P   P   V     N   P   S  ·
801 GAAATCTGCT CAATGACATG ACTCCTCCTG TAAATCCTTC
    ·   R   E   I     E   D   P     E   D   R   K     P   E   D
841 ACGTGAAATT GAGGACCCAG AAGACCGGAA GCCCGAGGAT
      W   D   E   R     P   K   I     P   D   P     E   A   V   K  ·
881 TGGGATGAAA GACCAAAAAT CCCAGATCCA GAAGCTGTCA
    ·   P   D   D     W   D   E     D   A   P   A     K   I   P  ·
921 AGCCAGATGA CTGGGATGAA GATGCCCCTG CTAAGATTCC
    ·   D   E   E     A   T   K     P   E   G   W     L   D   D
961 AGATGAAGAG GCCACAAAAC CCGAAGGCTG GTTAGATGAT
      E   P   E   Y     V   P   D     P   D   A     E   K   P   E  ·
1001 GAGCCTGAGT ACGTACCTGA TCCAGACGCA GAGAAACCTG
     ·   D   W   D     E   D   M     D   G   E   W     E   A   P  ·
1041 AGGATTGGGA TGAAGACATG GATGGAGAAT GGGAGGCTCC
     ·   Q   I   A     N   P   R   C     E   S   A     P   G   C
1081 TCAGATTGCC AACCCTAGAT GTGAGTCAGC TCCTGGATGT
       G   V   W   Q     R   P   V     I   D   N     P   N   Y   K  ·
1121 GGTGTCTGGC AGCGACCTGT GATTGACAAC CCCAATTATA
     ·   G   K   W     K   P   P     M   I   D   N     P   S   Y  ·
1161 AAGGCAAATG GAAGCCTCCT ATGATTGACA ATCCCAGTTA
     ·   Q   G   I     W   K   P   R     K   I   P     N   P   D
1201 CCAGGGAATC TGGAAACCCA GGAAAATACC AAATCCAGAT
       F   F   E   D     L   E   P     F   R   M     T   P   F   S  ·
1241 TTCTTTGAAG ATCTGGAACC TTTCAGAATG ACTCCTTTTA
     ·   A   I   G     L   E   L     W   S   M   T     S   D   I  ·
1281 GTGCTATTGG TTTGGAGCTG TGGTCCATGA CCTCTGACAT
     ·   F   F   D     N   F   I   I     C   A   D     R   R   I
1321 TTTTTTTGAC AACTTTATCA TTTGTGCTGA TCGAAGAATA
       V   D   D   W     A   N   D     G   W   G     L   K   K   A  ·
1361 GTTGATGATT GGGCCAATGA TGGATGGGGC CTGAAGAAAG
     ·   A   D   G     A   A   E     P   G   V   V     G   Q   M  ·
1401 CTGCTGATGG GGCTGCTGAG CCAGGCGTTG TGGGGCAGAT
       ·   I   E   A     A   E   E   R     P   W   L     W   V   V
1441 GATCGAGGCA GCTGAAGAGC GCCCGTGGCT GTGGGTAGTC
       Y   I   L   T     V   A   L     P   V   F     L   V   I   L  ·
1481 TATATTCTAA CTGTAGCCCT TCCTGTGTTC CTGGTTATCC
     ·   F   C   C     S   G   K     K   Q   T   S     G   M   E  ·
```

FIG. 2A (Continued)

```
1521 TCTTCTGCTG TTCTGGAAAG AAACAGACCA GTGGTATGGA
      · Y  K  K   T  D  A  P   Q  P  D   V  K  E
1561 GTATAAGAAA ACTGATGCAC CTCAACCGGA TGTGAAGGAA
       E  E  E  E   K  E  E   E  K  D   K  G  D  E ·
1601 GAGGAAGAAG AGAAGGAAGA GGAAAAGGAC AAGGGAGATG
      · E  E  E   G  E  E   K  L  E  E   K  Q  K ·
1641 AGGAGGAGGA AGGAGAAGAG AAACTTGAAG AGAAACAGAA
      · S  D  A   E  E  D  G   G  T  V   S  Q  E
1681 AAGTGATGCT GAAGAAGATG GTGGCACTGT CAGTCAAGAG
       E  E  D  R   K  P  K   A  E  E   D  E  I  L ·
1721 GAGGAAGACA GAAAACCTAA AGCAGAGGAG GATGAAATTT
      · N  R  S   P  R  N   R  K  P  R   R  E  * ·
1761 TGAACAGATC ACCAAGAAAC AGAAAGCCAC GAAGAGAGTG
      . *
1801 AAACAATCTT AAGAGCTTGA TCTGTGATTT CTTCTCCCTC
1841 CTCCCCTGCA TCTAGA
```

FIG. 2A(Continued)

```
                    M  L  L   S  V  P  L   L  L  G ·
  1 GAATTCCCGC CATGCTGCTA TCCGTGCCGC TGCTGCTCGG
    · L  L  G   L  A  V  A   E  P  A   V  Y  F
 41 CCTCCTCGGC CTGGCCGTCG CCGAGCCTGC CGTCTACTTC
     K  E  Q  F   L  D  G   D  G  W   T  S  R  W ·
 81 AAGGAGCAGT TTCTGGACGG AGACGGGTGG ACTTCCCGCT
    · I  E  S   K  H  K   S  D  F  G   K  F  V ·
121 GGATCGAATC CAAACACAAG TCAGATTTTG GCAAATTCGT
    · L  S  S   G  K  F  Y   G  D  E   E  K  D
161 TCTCAGTTCC GGCAAGTTCT ACGGTGACGA GGAGAAAGAT
     K  G  L  Q   T  S  Q   D  A  R   F  Y  A  L ·
201 AAAGGTTTGC AGACAAGCCA GGATGCACGC TTTTATGCTC
    · S  A  S   F  E  P   F  S  N  K   G  Q  T ·
241 TGTCGGCCAG TTTCGAGCCT TCAGCAACA AAGGCCAGAC
    · L  V  V   Q  F  T  V   K  H  E   Q  N  I
281 GCTGGTGGTG CAGTTCACGG TGAAACATGA GCAGAACATC
     D  C  G  G   G  Y  V   K  L  F   P  N  S  L ·
321 GACTGTGGGG GCGGCTATGT GAAGCTGTTT CCTAATAGTT
    · D  Q  T   D  M  H   G  D  S  E   Y  N  I ·
361 TGGACCAGAC AGACATGCAC GGAGACTCAG AATACAACAT
    · M  F  G   P  D  I   C  G  P  G   T  K  K
401 CATGTTTGGT CCCGACATCT GTGGCCCTGG CACCAAGAAG
     V  H  V  I   F  N  Y   K  G  K   N  V  L  I ·
441 GTTCATGTCA TCTTCAACTA CAAGGGCAAG AACGTGCTGA
    · N  K  D   I  R  C   K  D  D  E   F  T  H ·
481 TCAACAAGGA CATCCGTTGC AAGGATGATG AGTTTACACA
    · L  Y  T   L  I  V  R   P  D  N   T  Y  E
521 CCTGTACACA CTGATTGTGC GGCCAGACAA CACCTATGAG
     V  K  I  D   N  S  Q   V  E  S   G  S  L  E ·
561 GTGAAGATTG ACAACAGCCA GGTGGAGTCC GGCTCCTTGG
    · D  D  W   D  F  L   P  P  K  K   I  K  D ·
601 AAGACGATTG GGACTTCCTG CCACCCAAGA AGATAAAGGA
    · P  D  A   S  K  P  E   D  W  D   E  R  A
641 TCCTGATGCT TCAAAACCGG AAGACTGGGA TGAGCGGGCC
     K  I  D  D   P  T  D   S  K  P   E  D  W  D ·
681 AAGATCGATG ATCCCACAGA CTCCAAGCCT GAGGACTGGG
    · K  P  E   H  I  P   D  P  D  A   K  K  P ·
721 ACAAGCCCGA GCATATCCCT GACCCTGATG CTAAGAAGCC
    · E  D  W   D  E  E  M   D  G  E   W  E  P
```

FIG. 2B

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```
761  CGAGGACTGG GATGAAGAGA TGGACGGAGA GTGGGAACCC
       P  V  I  Q   N  P  E   Y  K  G   E  W  K  P ·
801  CCAGTGATTC AGAACCCTGA GTACAAGGGT GAGTGGAAGC
     · R  Q  I   D  N  P   D  Y  K  G   T  W  I ·
841  CCCGGCAGAT CGACAACCCA GATTACAAGG GCACTTGGAT
     · H  P  E   I  D  N  P   E  Y  S   P  D  P
881  CCACCCAGAA ATTGACAACC CCGAGTATTC TCCCGATCCC
       S  I  Y   A  Y  D  N   F  G  V   L  G  L  D ·
921  AGTATCTATG CCTATGATAA CTTTGGCGTG CTGGGCCTGG
     · L  W  Q   V  K  S  G   T  I  F   D  N  F ·
961  ACCTCTGGCA GGTCAAGTCT GGCACCATCT TTGACAACTT
     · L  I  T   N  D  E  A   Y  A  E   E  F  G
1001 CCTCATCACC AACGATGAGG CATACGCTGA GGAGTTTGGC
       N  E  T   W  G  V  T   K  A  A   E  K  Q  M ·
1041 AACGAGACGT GGGGCGTAAC AAAGGCAGCA GAGAAACAAA
     · K  D  K   Q  D  E  E   Q  R  L   K  E  E ·
1081 TGAAGGACAA ACAGGACGAG GAGCAGAGGC TTAAGGAGGA
     · E  E  D   K  K  R  K   E  E  E   E  A  E
1121 GGAAGAAGAC AAGAAACGCA AAGAGGAGGA GGAGGCAGAG
       D  K  E   D  D  E  D   K  D  E   D  E  E  D ·
1161 GACAAGGAGG ATGATGAGGA CAAAGATGAG GATGAGGAGG
     · E  E  D   K  E  E  D   E  E  E   D  V  P ·
1201 ATGAGGAGGA CAAGGAGGAA GATGAGGAGG AAGATGTCCC
     · G  Q  A   K  D  E  L   *
1241 CGGCCAGGCC AAGGACGAGC TGTAGAGAGG CCTGCCTCCA
1281 GTCTAGA
```

FIG. 2B(Continued)

```
1       GAATTCCTCC GCAGTCCCAG CCGAGCCGCG ACCCTTCCGG
                                    M  R  L  R  R  L ·
41  CCGTCCCCAC CCCACCTCGC CGCCATGCGC CTCCGCCGCC
    · A  L  F  P  G  V  A  L  L  L  A  A  A ·
81  TAGCGCTGTT CCCGGGTGTG GCGCTGCTTC TTGCCGCGGC
    ·R  L  A  A  A  S  D  V  L  E  L  T  D
121 CCGCCTCGCC GCTGCCTCCG ACGTGCTAGA ACTCACGGAC
     D  N  F  E  S  R  I  S  D  T  G  S  A  G ·
161 GACAACTTCG AGAGTCGCAT CTCCGACACG GGCTCTGCGG
    · L  M  L  V  E  F  F  A  P  W  C  G  H ·
201 GCCTCATGCT CGTCGAGTTC TTCGCTCCCT GGTGTGGACA
    ·C  K  R  L  A  P  E  Y  E  A  A  A  T
241 CTGCAAGAGA CTTGCACCTG AGTATGAAGC TGCAGCTACC
     R  L  K  G  I  V  P  L  A  K  V  D  C  T ·
281 AGATTAAAAG GAATAGTCCC ATTAGCAAAG GTTGATTGCA
    · A  N  T  N  T  C  N  K  Y  G  V  S  G ·
321 CTGCCAACAC TAACACCTGT AATAAATATG GAGTCAGTGG
    ·Y  P  T  L  K  I  F  R  D  G  E  E  A
361 ATATCCAACC CTGAAGATAT TTAGAGATGG TGAAGAAGCA
     G  A  Y  D  G  P  R  T  A  D  G  I  V  S ·
401 GGTGCTTATG ATGGACCTAG GACTGCTGAT GGAATTGTCA
    · H  L  K  K  Q  A  G  P  A  S  V  P  L ·
441 GCCACTTGAA GAAGCAGGCA GGACCAGCTT CAGTGCCTCT
    ·R  T  E  E  E  F  K  K  F  I  S  D  K
481 CAGGACTGAG GAAGAATTTA AGAAATTCAT TAGTGATAAA
     D  A  S  I  V  G  F  F  D  D  S  F  S  E ·
521 GATGCCTCTA TAGTAGGTTT TTTCGATGAT TCATTCAGTG
    · A  H  S  E  F  L  K  A  A  S  N  L  R ·
561 AGGCTCACTC CGAGTTCCTA AAAGCAGCCA GCAACTTGAG
    ·D  N  Y  R  F  A  H  T  N  V  E  S  L
601 GGATAACTAC CGATTTGCAC ATACGAATGT TGAGTCTCTG
     V  N  E  Y  D  D  N  G  E  G  I  I  L  F ·
641 GTGAACGAGT ATGATGATAA TGGAGAGGGT ATCATCTTAT
    · R  P  S  H  L  T  N  K  F  E  D  K  T ·
681 TTCGTCCTTC ACATCTCACT AACAAGTTTG AGGACAAGAC
    ·V  A  Y  T  E  Q  K  M  T  S  G  K  I
721 TGTGGCATAT ACAGAGCAAA AAATGACCAG TGGCAAAATT
      K  K  F  I  Q  E  N  I  F  G  I  C  P  H ·
761 AAAAAGTTTA TCCAGGAAAA CATTTTTGGT ATCTGCCCTC
    · M  T  E  D  N  K  D  L  I  Q  G  K  D ·
```

FIG. 2C

```
801 ACATGACAGA AGACAATAAA GATTTGATAC AGGGCAAGGA
    · L  L  I   A  Y  Y  D   V  D  Y   E  K  N
841 CTTACTTATT GCTTACTATG ATGTGGACTA TGAAAAGAAC
     A  K  G  S   N  Y  W   R  N  R   V  M  M  V ·
881 GCTAAAGGTT CCAACTACTG GAGAAACAGG GTAATGATGG
    · A  K  K   F  L  D   A  G  H  K   L  N  F ·
921 TGGCAAAGAA ATTCCTGGAT GCTGGGCACA AACTCAACTT
    · A  V  A   S  R  K  T   F  S  H   E  L  S
961 TGCTGTAGCT AGCCGCAAAA CCTTTAGCCA TGAACTTTCT
     D  F  G  L   E  S  T   A  G  E   I  P  V  V ·
1001 GATTTTGGCT TGGAGAGCAC TGCTGGAGAG ATTCCTGTTG
    · A  I  R   T  A  K   G  E  K  F   V  M  Q ·
1041 TTGCTATCAG AACTGCTAAA GGAGAGAAGT TTGTCATGCA
    · E  E  F   S  R  D  G   K  A  L   E  R  F
1081 GGAGGAGTTC TCGCGTGATG GGAAGGCTCT GGAGAGGTTC
     L  Q  D  Y   F  D  G   N  L  K   R  Y  L  K ·
1121 CTGCAGGATT ACTTTGATGG CAATCTGAAG AGATACCTGA
    · S  E  P   I  P  E   S  N  D  G   P  V  K ·
1161 AGTCTGAACC TATCCCAGAG AGCAATGATG GGCCTGTGAA
    · V  V  V   A  E  N  F   D  E  I   V  N  N
1201 GGTAGTGGTA GCAGAGAATT TTGATGAAAT AGTGAATAAT
     E  N  K  D   V  L  I   E  F  Y   A  P  W  C ·
1241 GAAAATAAAG ATGTGCTGAT TGAATTTTAT GCCCCTTGGT
    · G  H  C   K  N  L   E  P  K  Y   K  E  L ·
1281 GTGGTCATTG TAAGAACCTG GAGCCCAAGT ATAAAGAACT
    · G  E  K   L  S  K  D   P  N  I   V  I  A
1321 TGGCGAGAAG CTCAGCAAAG ACCCAAATAT CGTCATAGCC
     K  M  D  A   T  A  N   D  V  P   S  P  Y  E ·
1361 AAGATGGATG CCACAGCCAA TGATGTGCCT TCTCCATATG
    · V  R  G   F  P  T   I  Y  F  S   P  A  N ·
1401 AAGTCAGAGG TTTTCCTACC ATATACTTCT CTCCAGCCAA
    · K  K  L   N  P  K  K   Y  E  G   G  R  E
1441 CAAGAAGCTA AATCCAAAGA AATATGAAGG TGGCCGTGAA
     L  S  D  F   I  S  Y   L  Q  R   E  A  T  N ·
1481 TTAAGTGATT TTATTAGCTA TCTACAAAGA GAAGCTACAA
    · P  P  V   I  Q  E   E  K  P  K   K  K  K ·
```

FIG. 2C (Continued)

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```
1521 ACCCCCCTGT AATTCAAGAA GAAAAACCCA AGAAGAAGAA
     · K  A  Q   E  D  L  *
1561 GAAGGCACAG GAGGATCTCT AAAGCAGTAG CCAAACACCA
1601 CTTTGTAAAA GGACTCTTCC ATCAGAGATG GGAAAACCAT
1641 TGGGGAGGAC TAGGACCCAT ATGGGAATTA TTACCTCTCA
1681 GGGCCGAGAG TCTAGA
```

FIG. 2C (Continued)

```
      M  A  K  A  A  A  I  G  I  D  L  G  T  T  Y  S  C ·
1     ATGGCCAAAG CCGCGGCGAT CGGCATCGAC CTGGGCACCA CCTACTCCTG
      · V  G  V  F  Q  H  G  K  V  E  I  I  A  N  D  Q  G ·
51    CGTGGGGGTG TTCCAACACG GCAAGGTGGA GATCATCGCC AACGACCAGG
      · N  R  T  T  P  S  Y  V  A  F  T  D  T  E  R  L
101   GCAACCGCAC CACCCCCAGC TACGTGGCCT TCACGGACAC CGAGCGGCTC
       I  G  D  A  A  K  N  Q  V  A  L  N  P  Q  N  T  V ·
151   ATCGGGGATG CGGCCAAGAA CCAGGTGGCG CTGAACCCGC AGAACACCGT
      · F  D  A  K  R  L  I  G  R  K  F  G  D  P  V  V  Q ·
201   GTTTGACGCG AAGCGGCTGA TCGGCCGCAA GTTCGGCGAC CCGGTGGTGC
      · S  D  M  K  H  W  P  F  Q  V  I  N  D  G  D  K
251   AGTCGGACAT GAAGCACTGG CCTTTCCAGG TGATCAACGA CGGAGACAAG
       P  K  V  Q  V  S  Y  K  G  E  T  K  A  F  Y  P  E ·
301   CCCAAGGTGC AGGTGAGCTA CAAGGGGGAG ACCAAGGCAT TCTACCCCGA
      · E  I  S  S  M  V  L  T  K  M  K  E  I  A  E  A  Y ·
351   GGAGATCTCG TCCATGGTGC TGACCAAGAT GAAGGAGATC GCCGAGGCGT
      · L  G  Y  P  V  T  N  A  V  I  T  V  P  A  Y  F
401   ACCTGGGCTA CCCGGTGACC AACGCGGTGA TCACCGTGCC GGCCTACTTC
       N  D  S  Q  R  Q  A  T  K  D  A  G  V  I  A  G  L ·
451   AACGACTCGC AGCGCCAGGC CACCAAGGAT GCGGGTGTGA TCGCGGGGCT
      · N  V  L  R  I  I  N  E  P  T  A  A  A  I  A  Y  G ·
501   CAACGTGCTG CGGATCATCA ACGAGCCCAC GGCCGCCGCC ATCGCCTACG
      · L  D  R  T  G  K  G  E  R  N  V  L  I  F  D  L
551   GCCTGGACAG AACGGGCAAG GGGGAGCGCA ACGTGCTCAT CTTTGACCTG
       G  G  G  T  F  D  V  S  I  L  T  I  D  D  G  I  F ·
601   GGCGGGGGCA CCTTCGACGT GTCCATCCTG ACGATCGACG ACGGCATCTT
      · E  V  K  A  T  A  G  D  T  H  L  G  G  E  D  F  D ·
651   CGAGGTGAAG GCCACGGCCG GGGACACCCA CCTGGGTGGG GAGGACTTTG
      · N  R  L  V  N  H  F  V  E  E  F  K  R  K  H  K
701   ACAACAGGCT GGTGAACCAC TTCGTGGAGG AGTTCAAGAG AAAACACAAG
       K  D  I  S  Q  N  K  R  A  V  R  R  L  R  T  A  C ·
751   AAGGACATCA GCCAGAACAA GCGAGCCGTG AGGCGGCTGC GCACCGCCTG
      · E  R  A  K  R  T  L  S  S  S  T  Q  A  S  L  E  I ·
801   CGAGAGGGCC AAGAGGACCC TGTCGTCCAG CACCCAGGCC AGCCTGGAGA
      · D  S  L  F  E  G  I  D  F  Y  T  S  I  T  R  A
851   TCGACTCCCT GTTTGAGGGC ATCGACTTCT ACACGTCCAT CACCAGGGCG
       R  F  E  E  L  C  S  D  L  F  R  S  T  L  E  P  V ·
901   AGGTTCGAGG AGCTGTGCTC CGACCTGTTC CGAAGCACCC TGGAGCCCGT
      · E  K  A  L  R  D  A  K  L  D  K  A  Q  I  H  D  L ·
951   GGAGAAGGCT CTGCGCGACG CCAAGCTGGA CAAGGCCCAG ATTCACGACC
      · V  L  V  G  G  S  T  R  I  P  K  V  Q  K  L  L
1001  TGGTCCTGGT CGGGGGCTCC ACCCGCATCC CCAAGGTGCA GAAGCTGCTG
       Q  D  F  F  N  G  R  D  L  N  K  S  I  N  P  D  E ·
1051  CAGGACTTCT TCAACGGGCG CGACCTGAAC AAGAGCATCA ACCCCGACGA
      · A  V  A  Y  G  A  A  V  Q  A  A  I  L  M  G  D  K ·
1101  GGCTGTGGCC TACGGGGCGG CGGTGCAGGC GGCCATCCTG ATGGGGGACA
      · S  E  N  V  Q  D  L  L  L  L  D  V  A  P  L  S
1151  AGTCCGAGAA CGTGCAGGAC CTGCTGCTGC TGGACGTGGC TCCCCTGTCG
```

FIG. 2D

```
          L  G  L  E   T  A  G   G  V  M   T  A  L  I   K  R  N ·
1201  CTGGGGCTGG AGACGGCCGG AGGCGTGATG ACTGCCCTGA TCAAGCGCAA
      · S  T  I   P  T  K  Q   T  Q  I   F  T  T   Y  S  D  N ·
1251  CTCCACCATC CCCACCAAGC AGACGCAGAT CTTCACCACC TACTCCGACA
      · Q  P  G   V  L  I   Q  V  Y  E   G  E  R   A  M  T
1301  ACCAACCCGG GGTGCTGATC CAGGTGTACG AGGGCGAGAG GGCCATGACG
       K  D  N  N  L  L  G   R  F  E   L  S  G  I   P  P  A ·
1351  AAAGACAACA ATCTGTTGGG GCGCTTCGAG CTGAGCGGCA TCCCTCCGGC
      · P  R  G   V  P  Q  I   E  V  T   F  D  I   D  A  N  G ·
1401  CCCCAGGGGC GTGCCCCAGA TCGAGGTGAC CTTCGACATC GATGCCAACG
      · I  L  N   V  T  A   T  D  K  S   T  G  K   A  N  K
1451  GCATCCTGAA CGTCACGGCC ACGGACAAGA GCACCGGCAA GGCCAACAAG
       I  T  I  T   N  D  K   G  R  L   S  K  E  E   I  E  R ·
1501  ATCACCATCA CCAACGACAA GGGCCGCCTG AGCAAGGAGG AGATCGAGCG
      · M  V  Q   E  A  E  K   Y  K  A   E  D  E   V  Q  R  E ·
1551  CATGGTGCAG GAGGCGGAGA AGTACAAAGC GGAGGACGAG GTGCAGCGCG
      · R  V  S   A  K  N   A  L  E  S   Y  A  F   N  M  K
1601  AGAGGGTGTC AGCCAAGAAC GCCCTGGAGT CCTACGCCTT CAACATGAAG
       S  A  V  E   D  E  G   L  K  G   K  I  S  E   A  D  K ·
1651  AGCGCCGTGG AGGATGAGGG GCTCAAGGGC AAGATCAGCG AGGCCGACAA
      · K  K  V   L  D  K  C   Q  E  V   I  S  W   L  D  A  N ·
1701  GAAGAAGGTG CTGGACAAGT GTCAAGAGGT CATCTCGTGG CTGGACGCCA
      · T  L  A   E  K  D   E  F  E  H   K  R  K   E  L  E
1751  ACACCTTGGC CGAGAAGGAC GAGTTTGAGC ACAAGAGGAA GGAGCTGGAG
       Q  V  C  N   P  I  I   S  G  L   Y  Q  G  A   G  G  P ·
1801  CAGGTGTGTA ACCCCATCAT CAGCGGACTG TACCAGGGTG CCGGTGGTCC
      · G  P  G   G  F  G  A   Q  G  P   K  G  G   S  G  S  G ·
1851  CGGGCCTGGG GGCTTCGGGG CTCAGGGTCC CAAGGGAGGG TCTGGGTCAG
      · P  T  I   E  E  V  D  *
1901  GCCCCACCAT TGAGGAGGTA GATTAG
```

FIG. 2D (Continued)

```
    M  G  K  D   Y  Y  Q   T  L  G   L  A  R  G   A  S  D ·
1   ATGGGTAAAG ACTACTACCA GACGTTGGGC CTGGCCCGCG GCGCGTCGGA
    · E  E  I   K  R  A  Y   R  R  Q   A  L  R   Y  H  P  D ·
51  CGAGGAGATC AAGCGGGCCT ACCGCCGCCA GGCGCTGCGC TACCACCCGG
    · K  N  K   E  P  G  A   E  E  K   F  K  E   I  A  E
101 ACAAGAACAA GGAGCCCGGC GCCGAGGAGA AGTTCAAGGA GATCGCTGAG
     A  Y  D  V   L  S  D   P  R  K   R  E  I  F   D  R  Y ·
151 GCCTACGACG TGCTCAGCGA CCCGCGCAAG CGCGAGATCT TCGACCGCTA
    · G  E  E   G  L  K  G   S  G  P   S  G  G   S  G  G  G ·
201 CGGGGAGGAA GGCCTAAAGG GGAGTGGCCC CAGTGGCGGT AGCGGCGGTG
    · A  N  G   T  S  F   S  Y  T  F   H  G  D   P  H  A
251 GTGCCAATGG TACCTCTTTC AGCTACACAT TCCATGGAGA CCCTCATGCC
     M  F  A  E   F  F  G   G  R  N   P  F  D  T   F  F  G ·
301 ATGTTTGCTG AGTTCTTCGG TGGCAGAAAT CCCTTTGACA CCTTTTTTGG
    · Q  R  N   G  E  E  G   M  D  I   D  D  P   F  S  G  F ·
351 GCAGCGGAAC GGGGAGGAAG GCATGGACAT TGATGACCCA TTCTCTGGCT
    · P  M  G   M  G  G   F  T  N  V   N  F  G   R  S  R
401 TCCCTATGGG CATGGGTGGC TTCACCAACG TGAACTTTGG CCGCTCCCGC
     S  A  Q  E   P  A  R   K  K  Q   D  P  P  V   T  H  D ·
451 TCTGCCCAAG AGCCCGCCCG AAAGAAGCAA GATCCCCCAG TCACCCACGA
    · L  R  V   S  L  E  E   I  Y  S   G  C  T   K  K  M  K ·
501 CCTTCGAGTC TCCCTTGAAG AGATCTACAG CGGCTGTACC AAGAAGATGA
    · I  S  H   K  R  L  N   P  D  G   K  S  I   R  N  E
551 AAATCTCCCA CAAGCGGCTA AACCCCGACG GAAAGAGCAT TCGAAACGAA
     D  K  I  L   T  I  E   V  K  K   G  W  K  E   G  T  K ·
601 GACAAAATAT TGACCATCGA AGTGAAGAAG GGGTGGAAAG AAGGAACCAA
    · I  T  F   P  K  E  G   D  Q  T   S  N  N   I  P  A  D ·
651 AATCACTTTC CCCAAGGAAG GAGACCAGAC CTCCAACAAC ATTCCAGCTG
    · I  V  F   V  L  K   D  K  P  H   N  I  F   K  R  D
701 ATATCGTCTT TGTTTTAAAG GACAAGCCCC ACAATATCTT TAAGAGAGAT
```

FIG. 2E

```
          G  S  D  V  I  Y  P  A  R  I  S  L  R  E  A  L  C ·
751  GGCTCTGATG TCATTTATCC TGCCAGGATC AGCCTCCGGG AGGCTCTGTG
     · G  C  T  V  N  V  P  T  L  D  G  R  T  I  P  V  V ·
801  TGGCTGCACA GTGAACGTCC CCACTCTGGA CGGCAGGACG ATACCCGTCG
     · F  K  D  V  I  R  P  G  M  R  R  K  V  P  G  E
851  TATTCAAAGA TGTTATCAGG CCTGGCATGC GGCGAAAAGT TCCTGGAGAA
      G  L  P  L  P  K  T  P  E  K  R  G  D  L  I  I  E ·
901  GGCCTCCCCC TCCCCAAAAC ACCCGAGAAA CGTGGGGACC TCATTATTGA
     · F  E  V  I  F  P  E  R  I  P  Q  T  S  R  T  V  L ·
951  GTTTGAAGTG ATCTTCCCCG AAAGGATTCC CCAGACATCA AGAACCGTAC
     · E  Q  V  L  P  I  *
1001      TTGAGCAGGT TCTTCCAATA TAG
```

FIG. 2E (Continued)

```
      M  T  T  S  A  S  S  H  L  N  K  G  I  K  Q  V  Y·
1  ATGACCACCT CAGCAAGTTC CCACTTAAAT AAAGGCATCA AGCAGGTGTA
   ·M  S  L  P  Q  G  E  K  V  Q  A  M  Y  I  W  I  D·
51 CATGTCCCTG CCTCAGGGTG AGAAAGTCCA GGCCATGTAT ATCTGGATCG
   ·G  T  G  E  G  L  R  C  K  T  R  T  L  D  S  E
101 ATGGTACTGG AGAAGGACTG CGCTGCAAGA CCCGGACCCT GGACAGTGAG
    P  K  C  V  E  E  L  P  E  W  N  F  D  G  S  S  T·
151 CCCAAGTGTG TGGAAGAGTT GCCTGAGTGG AATTTCGATG GCTCCAGTAC
   ·L  Q  S  E  G  S  N  S  D  M  Y  L  V  P  A  A  M·
201 TTTACAGTCT GAGGGTTCCA ACAGTGACAT GTATCTCGTG CCTGCTGCCA
   ·F  R  D  P  F  R  K  D  P  N  K  L  V  L  C  E
251 TGTTTCGGGA CCCCTTCCGT AAGGACCCTA ACAAGCTGGT GTTATGTGAA
    V  F  K  Y  N  R  R  P  A  E  T  N  L  R  H  T  C·
301 GTTTTCAAGT ACAATCGAAG GCCTGCAGAG ACCAATTTGA GGCACACCTG
   ·K  R  I  M  D  M  V  S  N  Q  H  P  W  F  G  M  E·
351 TAAACGGATA ATGGACATGG TGAGCAACCA GCACCCCTGG TTTGGCATGG
   ·Q  E  Y  T  L  M  G  T  D  G  H  P  F  G  W  P
401 AGCAGGAGTA TACCCTCATG GGGACAGATG GGCACCCCTT TGGTTGGCCT
    S  N  G  F  P  G  P  Q  G  P  Y  Y  C  G  V  G  A·
451 TCCAACGGCT TCCCAGGGCC CCAGGGTCCA TATTACTGTG GTGTGGGAGC
   ·D  R  A  Y  G  R  D  I  V  E  A  H  Y  R  A  C  L·
501 AGACAGAGCC TATGGCAGGG ACATCGTGGA GGCCCATTAC CGGGCCTGCT
   ·Y  A  G  V  K  I  A  G  T  N  A  E  V  M  P  A
551 TGTATGCTGG AGTCAAGATT GCGGGGACTA ATGCCGAGGT CATGCCTGCC
    Q  W  E  F  Q  I  G  P  C  E  G  I  S  M  G  D  H·
601 CAGTGGGAAT TTCAGATTGG ACCTTGTGAA GGAATCAGCA TGGGAGATCA
   ·L  W  V  A  R  F  I  L  H  R  V  C  E  D  F  G  V·
651 TCTCTGGGTG GCCCGTTTCA TCTTGCATCG TGTGTGTGAA GACTTTGGAG
   ·I  A  T  F  D  P  K  P  I  P  G  N  W  N  G  A
701 TGATAGCAAC CTTTGATCCT AAGCCCATTC CTGGGAACTG GAATGGTGCA
```

FIG. 2F

```
                G   C   H   T   N   F   S   T   K   A   M   R   E   E   N   G   L ·
751  GGCTGCCATA CCAACTTCAG CACCAAGGCC ATGCGGGAGG AGAATGGTCT
     · K   Y   I   E   E   A   I   E   K   L   S   K   R   H   Q   Y   H ·
801  GAAGTACATC GAGGAGGCCA TTGAGAAACT AAGCAAGCGG CACCAGTACC
     · I   R   A   Y   D   P   K   G   G   L   D   N   A   R   R   L
851  ACATCCGTGC CTATGATCCC AAGGGAGGCC TGGACAATGC CCGACGTCTA
      T   G   F   H   E   T   S   N   I   N   D   F   S   A   G   V   A ·
901  ACTGGATTCC ATGAAACCTC CAACATCAAC GACTTTTCTG CTGGTGTAGC
     · N   R   S   A   S   I   R   I   P   R   T   V   G   Q   E   K   K ·
951  CAATCGTAGC GCCAGCATAC GCATTCCCCG GACTGTTGGC CAGGAGAAGA
     · G   Y   F   E   D   R   R   P   S   A   N   C   D   P   F   S
1001 AGGGTTACTT TGAAGATCGT CGCCCCTCTG CCAACTGCGA CCCCTTTTCG
      V   T   E   A   L   I   R   T   C   L   L   N   E   T   G   D   E ·
1051 GTGACAGAAG CCCTCATCCG CACGTGTCTT CTCAATGAAA CCGGCGATGA
     · P   F   Q   Y   K   N   *
1101 GCCCTTCCAG TACAAAAATT AA
```

FIG. 2F (Continued)

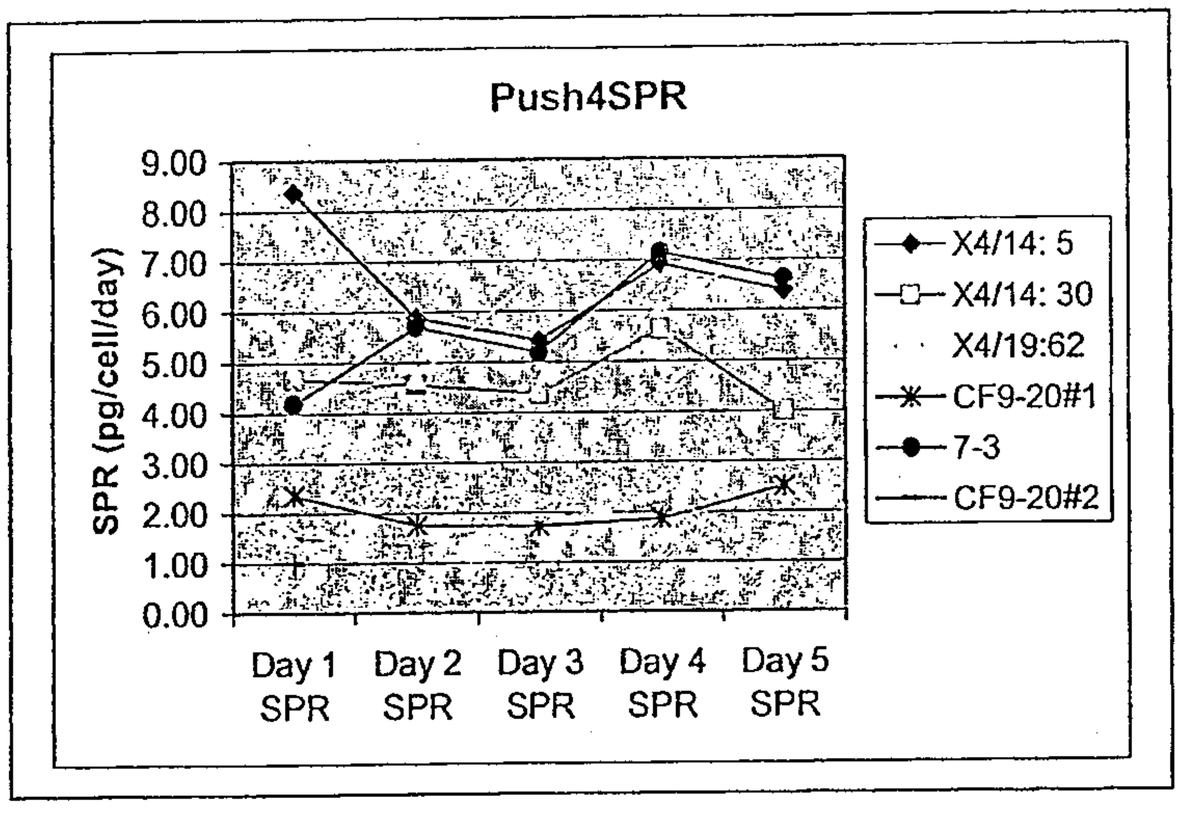
Push4SPR
SPR (pg/cell/day)
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Day 1 SPR
Day 2 SPR
Day 3 SPR
Day 4 SPR
Day 5 SPR
X4/14: 5
X4/14: 30
X4/19:62
CF9-20#1
7-3
CF9-20#2

FIG. 3

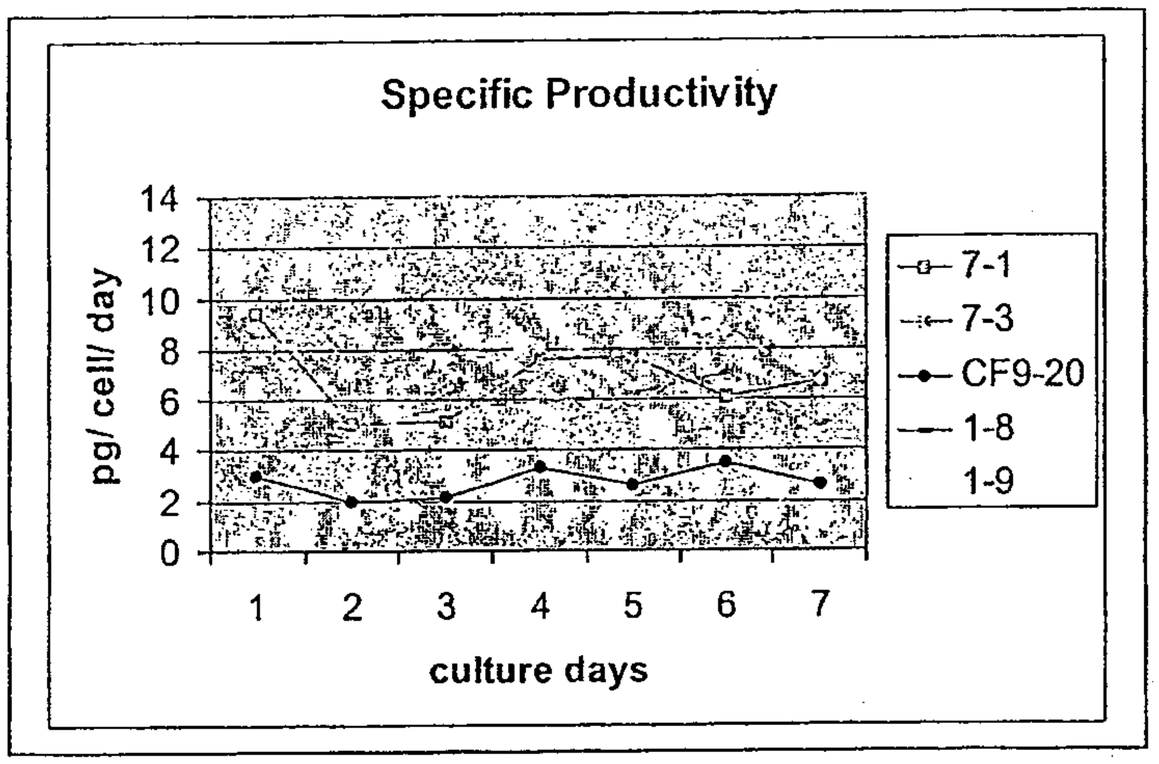
Specific Productivity
pg/ cell/ day
14
12
10
8
6
4
2
0
1
2
3
4
5
6
7
culture days
7-1
7-3
CF9-20
1-8
1-9

FIG. 4

ADRERSIHDF	CLVSKVVGRC	RASMPRWWYN	30
VTDGSCQLFV	YGGCDGNSNN	YLTKEECLKK	60
CATVTENATG	DLATSRNAAD	SSVPSAPRRQ	90
DSEDHSSDMF	NYEEYCTANA	VTGPCRASFP	120
RWYFDVERNS	CNNFIYGGCR	GNKNSYRSEE	150
ACMLRCFRQQ	ENPPLPLGSK		170

FIG. 5