

Methods for Obtaining High Viable Cell Density in Mammalian Cell Culture

Field of the Invention

The present invention relates to methods for achieving high viable cell density and extended culture longevity in fed batch cell culture by using a high glucose feed. The methods are useful for increasing production of secreted proteins of interest.

Background of the Invention

Mammalian cell culture is the system of choice for many recombinant protein production processes due to its ability to produce proteins with proper post-translational modifications. With increasing manufacturing demand, there is a strong motivation to improve process efficiency by increasing product yield. Attaining grams per liter production levels of biotherapeutics or other proteins in commercial production processes relies upon the optimization of both mammalian cell culture and engineering methods. Inherent in current high density, protein-free mammalian cell cultures is the problem of cell death of which apoptosis can account for up to 80% in a typical fed-batch bioreactor, induced in response to conditions such as nutrient and growth factor deprivation, oxygen depletion, toxin accumulation, and shear stress (Goswami et al., *Biotechnol Bioeng* 62:632-640 (1999)). Apoptosis limits the maximum viable cell density, accelerates the onset of the death phase and potentially decreases heterologous protein yield (Chiang and Sisk, *Biotechnol Bioeng* 91:779-792 (2005); Figueroa et al., *Biotechnol Bioeng* 73:211-222 (2001), *Metab Eng* 5:230-245 (2003), *Biotechnol Bioeng* 85:589-600 (2004); Mercille and Massie, *Biotechnol Bioeng* 44:1140-1154 (1994)).

Apoptosis is a result of a complex network of signaling pathways initiating from both inside and outside the cell, culminating in the activation of cysteine aspartate proteases (caspases) that mediate the final stages of cell death. See Fig. 1. Various methods of apoptosis prevention have been

used to maintain cell viability during extended production runs in mammalian cell culture (Arden and Betenbaugh, Trends Biotechnol 22:174-180 (2004); Vives et al., Metab Eng 5:124-132 (2003)). Altering the extracellular environment through media supplementation of growth factors, hydrolysates, and limiting nutrients has led to increased protein production and decreased apoptosis (Burteau et al., In Vitro Cell Dev Biol Anim. 39:291-296 (2003); Zhang and Robinson, Cytotechnology 48: 59-74 (2005)). Other researchers have turned to chemical and genetic strategies to inhibit the apoptotic signaling cascade from within the cell (Sauerwald et al., Biotechnol Bioeng 77:704-716 (2002), Biotechnol Bioeng 81:329-340 (2003)).

Researchers have found that over-expression of genes found upregulated in cancer cells can prolong viability in cells grown in bioreactors by preventing apoptosis upstream of caspase activation (Goswami et al., supra; Mastrangelo et al., Trends Biotechnol 16:88-95 (1998); Meents et al., Biotechnol Bioeng 80:706-716 (2002); Tey et al., J Biotechnol 79:147-159 (2000) and Biotechnol Bioeng 68:31-43 (2000). Upregulation of these proteins in production cell lines effectively suppressed apoptotic signaling within the cell, thereby limiting cell death in order to maintain viability and increase biotherapeutic production in some cases.

Also inherent in high density, protein-free mammalian cell cultures is the problem of waste accumulation and its detrimental effect on cell growth. The two most common cell culture waste products are lactate and ammonia. Numerous strategies have been devised to address the accumulation of excessive lactate build-up including 1) maintaining low medium glucose concentrations (Kurokawa et al., Biotechnol Bioeng 44:95-103 (1994); Xie and Wang, Biotechnol Bioeng 43:1175-1189 (1993); Zhang et al., J Chem Technol Biotechnol 79:171-181 (2004); Zhou et al., Biotechnol Bioeng 46:579-587 (1995)); 2) feeding alternative sugars, including fructose (Martinelle et al., Biotechnol Bioeng 60:508-517 (1998), Altamirano et al., J Biotechnol 110:171-179 (2004) and J Biotechnol 125:547-556 (2006), Walschin and Wu, J

Biotechnol 131:168-176 (2007); 3) partially knocking out lactate dehydrogenase (LDH) expression by homologous recombination or siRNA technology; 4) over-expression of pyruvate carboxylase; 5) use of dichloroacetate (DCA), a pyruvate dehydrogenase (PDH) activator (via PDH kinase inhibition); 6) oxamic acid, an LDH competitive inhibitor; and 7) removal through perfusion (US Pat. Appl. Publ. No. 2009/0042253 A1).

Originally, the exclusive function of many of the apoptotic pathway proteins was believed to be binding at the mitochondrial membrane and regulating apoptosis through modulation of mitochondria permeability. Recent findings have shown that key proteins involved in apoptotic signaling interact with and have effects on proteins that control metabolism and energy homeostasis in the cell. See Majors et al., *Metab Eng* 9:317-326 (2007) for a review; and White et al., *Nat Cell Biol* 7:1021-1028 (2005). In a recent study, microarray analysis of CHO cells over-expressing Bcl-XL show that lactate dehydrogenase, a key enzyme in gluconeogenesis, is up-regulated.

Certain cells and viruses produce anti-apoptotic genes that function in the mitochondrial apoptotic pathway. These can be divided into three groups, namely 1) those that act early in the pathway, e.g., members of the Bcl-2 family of proteins; 2) those that act mid-pathway to disrupt or inhibit the apoptosome complex, e.g., Aven and 3) those that act late in the pathway, e.g., caspase inhibitors such as XIAP. The functionality of the majority of these genes have been studied by over-expressing them in mammalian expression systems, and in some cases the effect of combined over-expression of two or more genes, each derived from a different part of the pathway has been determined. Examples include 1) the additive effect of Bcl-XL and a deletion mutant of XIAP (XIAPΔ) in CHO cells (Figuerola et al., *Metab. Eng.* 5:230-245 (2003)); 2) E1B-19K and Aven in BHK cells (Nivitchanyong et al., *Biotechnol Bioeng* 98:825-841 (2007)) and 3) Bcl-XL, Aven and XIAPΔ (Sauerwald et al., *supra*, (2003); Sauerwald et al., *Biotechnol Bioeng* 94:362-369

(2006)). However, in these studies, the effect of the anti-apoptotic genes on the cellular metabolic state of the cell was not examined. Accordingly, a need exists in mammalian cell culture systems to optimize nutrient consumption and metabolite accumulation conditions to achieve increased viable cell density, longevity and productivity.

Brief Description of the Drawings

Fig. 1 shows mitochondrial apoptosis pathway components.

Figs. 2A-2C show the characterization of apoptotic^R cell lines.

Fig. 3 shows confirmation of apoptotic^R in EA167 and EAX197 by analysis by flow cytometry.

Figs. 4A-4C show growth profiles of apoptotic^R cell lines generated from transfection of E1B-19K, E1B-19K+Aven and E1B-19K + Aven + XIAPΔ. Shake flask batch cultures of control (●), E64 (◆), EA167 (■) and EAX197 (▼) were monitored for viable cell density and viability.

Figs. 5A-5C show metabolite profiles of apoptotic^R cell lines generated from transfection of E1B-19K + AVEN ± XIAPΔ. Shake flask batch cultures of control (●), EA167 (■) and EAX197 (▼) cell lines were monitored for various metabolites.

Figs. 6A and 6B show daily lactate replenishment of apoptotic^R cell lines. A) (●), control and EA167: □, un-fed and ■, lactate-fed) and B) EAX197 (▲, un-fed and ▼, lactate-fed).

Figs. 7A-7G show growth and metabolic profiles of control and apoptotic^R cell lines EA167 and EAX197 in the presence or absence of lactate feeding. Control (●), EA167 (□, un-fed and ■, lactate-fed), EAX197 (▲, un-fed and ▼, lactate-fed).

Figs. 8A-8G show profiles of amino acids depleted by apoptotic^R cell lines upon lactate feeding. Control (●), EA167 (□, un-fed and ■, lactate-fed), EAX197 (▲, un-fed and ▼, lactate-fed).

Figs. 9A-9D show growth profiles and lactate accumulation profiles of control and apoptotic^R cell lines EAX

197 cultured in high concentrations of glucose in a custom medium formulation. Apoptotic^R cell line EAX197 (■); control cell line (♦).

Figs. 10A and 10B show antibody titers of cell lines generated from EAX197 and control host cell lines cultured in custom media formulation containing 30mM or 60mM glucose. Apoptotic^R cell line EAX197 (■); control cell lines (□).

Figs. 11A and 11B show VCD and titer of a cell line containing the apoptotic^R gene Bcl-2 with high and low cell seeding densities and media containing 30mM or 60mM glucose.

Summary of the Invention

One aspect of the invention is a method for obtaining high viable cell density in a fed batch eukaryotic cell culture comprising the steps of:

a) culturing a eukaryotic cell line expressing one or more heterologous apoptotic-resistant (apoptotic^R) genes and one or more genes of interest; and

b) maintaining a high glucose media feed during the exponential and stationary phases of the cell culture.

Another aspect of the invention is a method of increasing production of secreted proteins in a fed batch eukaryotic cell culture comprising the steps of:

a) culturing a eukaryotic cell line expressing one or more heterologous apoptotic-resistant (apoptotic^R) genes and one or more genes of interest; and

b) maintaining a high glucose media feed during the exponential and stationary phases of the cell culture.

Detailed Description of the Invention

All publications, including but not limited to patents and patent applications, cited in this specification are herein incorporated by reference as though fully set forth.

As used herein, the term "fed batch cell culture" means a cell culture process which is based on feeding of a growth limiting nutrient substrate to the culture. The fed-batch strategy is typically used in bio-industrial processes to

reach a high cell density in a bioreactor. However, the addition of a nutrient such as glucose results in formation of metabolic waste products such as lactate and ammonia. Concentrations of 18mM lactate (Kurano et al., 1990) and 8mM ammonia (Hansen and Emborg, 1994) have been reported as inhibitory for eukaryotic cell growth.

Numerous strategies have been devised to address the accumulation of excessive lactate build-up in batch-fed cell culture and are discussed above. In the present invention, an alternative approach to reducing lactate concentration and increasing viable cell density and viability is presented. The method involves over-expressing one or more anti-apoptotic genes in a host cell line. The resultant apoptotic-resistant cell lines stimulate mitochondrial respiration and accumulate less lactate. Therefore, these cell lines can thrive when fed with high concentrations of glucose. Consequently, cultures of these apoptotic-resistant cell lines can reach high viable cell density. These cell lines also possess extended longevity due to their apoptotic-resistant nature.

The methods of the invention are useful for increasing viable cell density and viability in fed batch cell culture, such as Chinese Hamster Ovary (CHO), myeloma or hybridoma cell cultures. In particular, the methods of the invention are useful for increasing the integrated viable cell count (IVCC) of CHO cell cultures. The method of the invention comprises the steps of culturing a cell line expressing one or more heterologous apoptotic-resistant (apoptotic^R) genes and one or more genes of interest; and maintaining a high glucose media feed during the exponential and stationary phases of the cell culture. These lines are superior hosts for the development of production cell lines expressing proteins of interest such as peptides, peptide fusions, growth factors, hormones, antibodies, designed ankyrin repeat proteins (DARPs) and other polypeptides useful for therapeutic, diagnostic or research purposes. CHO cell lines useful in the method of the invention include CHO-K1 (Invitrogen, Carlsbad, CA) and CHOK1SV (Lonza Biologics,

Slough, UK). Myeloma lines useful in the method of the invention include NS0 and Sp2/0.

The cell lines useful in the method of the invention express one or more heterologous anti-apoptosis genes. In particular, the genes encoding ElB19K (SEQ ID NOs: 1 and 2) and Aven (SEQ ID NOs: 3 and 4) are useful. The gene encoding XIAPA (SEQ ID NOs: 5 and 6) can also be used. These anti-apoptotic genes are representatives of the early, mid- and late stages of the apoptotic signaling pathway, respectively. Further, the gene encoding Bcl-2 Δ can also be used (SEQ ID NOs: 7 and 8). Expression of the anti-apoptosis genes can be achieved by transfection techniques known to those skilled in the art. It is contemplated that other anti-apoptosis genes from these stages of the apoptotic signaling pathway, such as MDM2 (SEQ ID NOs: 9 and 10) and Bcl-XL (SEQ ID NOs: 11 and 12), would also be useful in the methods of the invention.

In the present invention, the use of cell lines expressing heterologous anti-apoptotic genes allows these cell lines to secrete less lactate or alternatively, to consume accumulated lactate, to reach integrated viable cell count (IVCC) values about two-fold higher than control cell lines while a high glucose feed is maintained in the culture. By increasing the viable cell densities of production cell lines in culture, the yields of products obtained from a bioreactor run are increased. Such enhanced productivities can result in lower production costs for complex biologics and at the same time, generate product of superior quality due to the absence of cell lysis of the non-viable cells, as lysed cells release proteases that degrade product. Accordingly, these lines are superior hosts for the development of production cell lines expressing a protein or proteins of interest.

In the method of the invention, the ability of the apoptotic^R cell lines to consume or produce less lactate that would otherwise accumulate as waste provides for culture strategies to allow growth under conditions of high glucose as well as following glucose depletion. In different embodiments, the methods of the invention provide for

increased peak viable cell density, increased longevity, , increased secreted protein titer, increased integrated viable cell count, reduced cellular calcium flux and increased mitochondrial membrane potential in batch fed cell culture. Furthermore, strategies of fed-batch cultivation that have been used to limit high levels of toxic lactate and ammonia are unlikely to be necessary for high productivity from these cell lines.

Following transfection, the site of chromosomal integration of an anti-apoptotic transgene can dictate the level of its expression. Moreover, when multiple anti-apoptotic genes are transfected, the level of expression of a particular anti-apoptotic transgene can impact the activity of other anti-apoptotic proteins the cell possesses since many of these proteins interact directly or indirectly to bring about physiological changes in the cell. Thus the quantitative contribution of each anti-apoptotic gene towards the overall apoptotic^R characteristics of a cell line with multiple genes can be difficult to determine due to synergistic effects of the apoptotic^R genes. Additionally, significant clone-to-clone variations exist with respect to apoptotic^R characteristics even though all of them have been transfected with an identical set of transgenes. What is clear from the results presented in the Examples below is that each anti-apoptotic gene tested offered an incremental positive value towards improving the apoptosis^R characteristics of the host cell line into which it was transfected. Within the above constraints, triple transfectants were generally superior to double transfectants, which in turn were superior to transfectants over-expressing only one anti-apoptotic transgene.

Consequently, following transfection with appropriate expression vectors, clones were obtained with variable levels of expression of ElB-19K, Aven, and XIAP Δ . The expression level of ElB-19K was relatively low and rare clones that had high expression level of ElB-19K were either unstable or had poor growth properties (data not shown). In the Examples

below, only limited protection against cell death was observed in CHO cells expressing E1B-19K alone (Fig. 4).

Contrary to results obtained with cell lines expressing E1B-19K alone, data presented in the Examples below demonstrate that CHO K1 cell lines co-expressing Aven (EA167) or Aven and XIAPA (EAX197) exhibited improvements in viability, cell density and IVCC. The results presented in the Examples below further demonstrate that the expression of two anti-apoptosis genes in CHO K1 leads to a significant reduction in caspase activity and improved mitochondrial membrane potential in the presence of insults including staurosporine and extended batch cultures.

In the Examples below, the expression of a combination of E1B-19K, which is a functional homolog of Bcl-XL, plus Aven, and XIAPA was examined for effects on delaying cell death. The level of XIAPA expression in these transfected cells was not high relative to the endogenous wild type XIAP protein already present in the cell, perhaps due to the requirement for transfecting two plasmids in this system. Nonetheless, the addition of XIAPA in EAX197 (in addition to Aven and E1B-19K) provided a consistent enhancement in the maximum viable cell density in both unsupplemented (Figs. 5 and 7) and lactate supplemented cultures (Fig. 7). Indeed, the EAX197 cell line fed with lactate was able to sustain high viabilities at or above nearly 60% for 14 days, which was two days longer than the EA167 cells and four days longer than the control culture. The inclusion of XIAPA likely increases the cell's resistance to caspase activation and this is verified in Fig. 3 by the very low caspase activity of EAX197 even after treatment with staurosporine. Furthermore, the use of a mutant of XIAP (XIAPA) provides enhanced apoptosis resistance relative to the wild type XIAP protein. Thus, the protective capacity of the deletion mutant of XIAP (XIAPA) may be significant even if the expression of the protein is not high in these CHO lines.

In the Examples below, the role of one or more anti-apoptotic gene(s) in cell metabolism was investigated. Nutrient consumption and metabolite production were

determined in the apoptotic^R cell lines expressing ElB-19K in conjunction with Aven and/or XIAP Δ . In particular, the accumulation of the two most common cell culture waste products (i.e., lactate and ammonia) were compared in apoptotic^R cell lines to that of the control cell line. In the data presented below, it was found that in shake-flask batch cultures in CD-CHO medium, on day 6-day 7 post-seeding, the control cell line accumulated 2.8g/L (31mM) lactate and 12mM ammonia (Fig. 5), which are well above the concentrations of 18mM of lactate (Kurano et al., supra) and 8mM of ammonia (Hansen and Emborg, supra) reported as inhibitory for cell growth. These levels may be part of the reason for loss in viability seen by day-8 post-inoculation in CHO control cell line (Fig. 4).

While the data presented below show that apoptotic^R cell lines accumulate some lactate early, these cell lines began to consume lactate during the exponential phase and continued to increase in VCD well beyond that of the control cultures. Either the ability to consume lactate immediately following glucose exhaustion or an increase in apoptosis inhibition or both factors contributed to the continued cell growth. Furthermore, the apoptotic^R cells only entered the stationary phase when the endogenous lactate was exhausted. In order to determine if the consumption of lactate was a general characteristic of these engineered cell lines, exogenous lactate was added to the apoptosis^R cultures to replenish the depleted lactate (Fig. 6). The results presented in the Examples below show that the apoptotic^R cultures consumed the added lactate and maintained high VCD and viabilities for four to seven additional days as compared to control cultures (for un-fed and lactate-fed cultures, respectively; Fig. 7A and B). An added process benefit is that lower levels of lactate in cultures of apoptotic^R cell lines will result in lower osmolarity and higher pH, both of which are highly desirable for superior product quality.

Previous researchers have detected lactate consumption in hybridoma, NS0 myeloma and CHO cultures (deZengotita et al., 2000; Zhou et al., 1995 and 1997; Burky et al., 2007;

Pascoe et al., 2007). However, these cells typically exhibit lactate production in the exponential phase and transition to consumption in the stationary phases to suggest that lactate can represent both an intermediate by-product and a carbohydrate fuel source (Burky et al., 2007; Brooks et al., 1985). In the results presented below, the control CHO cultures appeared to consume some lactate in the stationary phase, particularly in the 'high' glucose medium (Fig. 9), as seen in the previous studies. In contrast, the apoptotic^R cells exhibited a dramatically different profile in which lactate was consumed during the exponential phase and the cells only entered the stationary phase when the exogenous lactate was depleted.

The conversion of pyruvate to lactate, catalyzed by lactate dehydrogenase (LDH), is reversible but strongly favors lactate formation with an equilibrium constant of $3.6 \times 10^4/M$. However, when glycolysis is unable to keep up with tricarboxylic acid (TCA) demand, lactate can be converted back to pyruvate by one of the several isozymes of LDH favoring that reaction. The lactate consumed by apoptotic^R cell lines likely feeds into the TCA cycle for cellular energetics and amino acid production. Interestingly, the ammonia profiles of the apoptotic^R cultures differed from that of control cultures, with a repeated transitory drop in ammonia production for both the un-fed and lactate-fed cell lines as well as an overall decrease in ammonia production. The utilization of amino acids as an energy source in the TCA cycle is likely to lead to the accumulation of ammonia. Consequently, a reduction in ammonia production suggests that the apoptotic^R cell lines may be obtaining a lower relative fraction of their TCA energy requirements from amino acids compared to the control cells, which is reasonable when considering that the apoptotic^R cells are also consuming more lactate as a carbon source. It should be pointed out however that the drop in ammonia is transitory and the apoptotic^R cells may still utilize amino acids as an energy source. Indeed, three amino acids, isoleucine, leucine and valine, all branched-chain amino acids were consumed more rapidly by

apoptotic^R cell lines and this consumption was exacerbated in lactate-fed cultures (Fig. 8A-C). Since the apoptotic^R cell lines experienced continued growth relative to the control cell lines, these three amino acids may form building blocks for fatty acids and other proteins which are needed by the apoptotic^R cells and also can be catabolized as a source of energy for the TCA cycle. A somewhat similar consumption profile is also observed for methionine (Fig. 8D).

In contrast to the amino acids which are observed to decline, a few amino acids were secreted including alanine, transiently and aspartate, transiently in the control cultures. A portion of the pyruvate can be converted to alanine by alanine amino-transferase. This pathway may be more active in apoptotic^R cells leading to a larger rise in alanine concentration relative to the control cell lines (Fig. 8B). Aspartate, on the other hand, is the first member of the aspartate amino acid family and is formed along with alpha-ketoglutarate by the conversion of TCA cycle intermediate oxaloacetate and glutamate using aspartate transaminase. Its level increases only in the control cells for the first six days while being continuously depleted in the apoptotic^R cell lines. Since this reaction is reversible, the depletion of aspartate in the apoptotic^R cell lines early in the growth phase indicates a possible limitation in the reversible oxaloacetate product in the TCA cycle for apoptotic^R cell lines. Given that oxaloacetate also reacts with Acetyl-CoA during the initial step of the TCA cycle, an increased flux of pyruvate into Acetyl-CoA from lactate in the apoptotic^R cell lines may enhance the demand for oxaloacetate. In contrast, there may be less of a demand for Acetyl CoA in the control cell lines since they are not consuming lactate and thus they generate aspartate from oxaloacetate. Indeed, this lack of demand for Acetyl CoA could be driving the build-up of lactate as an alternative by-product to Acetyl CoA in the control cells over the first seven days of the cell culture. Alternatively, a lack of lactate consumption in the control cells could lead to a limitation in the supply of Acetyl-CoA and a concomitant increase in the production of aspartate.

These results are indicative of heightened TCA cycle energetics in cell lines expressing anti-apoptotic genes although an exact interpretation of the cellular reactions awaits more detailed metabolic flux analysis. Irrespective of the fate of the consumed lactate, in the present invention it has been demonstrated that apoptotic^R cell lines consume accumulated lactate, which in turn, aids in their increased longevity and IVCC.

If apoptotic^R cell lines are unique in that they can extend their longevity by consuming the accumulated lactate, then there is a possibility that these lines can be cultured in medium containing lactate or medium containing higher than standard concentrations of glucose. While growth and viability could not be supported in either type of cell line when lactate was provided as the sole carbon source, lactate consumption was observed in apoptotic^R cell lines when it was provided as a component, in addition to glucose (data not shown) or when lactate accumulated as a waste product. This suggests that the apoptotic^R cells cannot perform gluconeogenesis from pyruvate or obtain enough energy from lactate, but if glucose is provided in conjunction to lactate the cells can consume both the glucose as well as lactate accumulated or supplemented to the culture. Moreover, in medium containing high glucose (60mM), the IVCC (and VCD and viability, Figs. 9A-C) of apoptotic^R cell lines was significantly higher than that of control cell lines. This is due to the fact that, in the high glucose medium, the control cell lines amassed more than 20mM lactate, which may have been toxic. The apoptotic^R cell lines on the other hand, were consuming the lactate early in the growth phase and thus able to maintain higher numbers of viable cells that translated to an increase in IVCC of 170% over control (Fig. 9C).

Since the apoptotic^R cell lines consumed lactate and achieved higher IVCC, production cell lines expressing proteins of interest such as therapeutic antibodies derived from apoptotic^R host cell lines achieved significantly higher titers compared to production cell lines derived from control

cell lines. Such higher titers illustrate the potential commercial benefits of using the anti-apoptosis genes in mammalian cell lines producing therapeutic or other proteins of interest.

The present invention will now be described with reference to the following specific, non-limiting examples.

Examples

In the following Examples, CHO cell lines over-expressing anti-apoptosis genes were analyzed in shake flask cultures for peak viable cell density, longevity, caspase-3 activation and mitochondrial membrane potential (MMP). Additionally, nutrient consumption and metabolite production in these cell lines were compared to that of control cell lines to determine if the expression of anti-apoptosis genes had any effect on the nutrient consumption and metabolite accumulation in mammalian cell culture systems.

Materials and Methods:

Cell culture:

CHOK1SV cell line (Lonza Biologics, Slough, UK), designated as the Control cell line C1013A, was cultured in CD-CHO medium (Cat. No. 10743-011, Invitrogen, Carlsbad, CA), containing 30 mM Glucose and supplemented with 6mM L-Glutamine (Invitrogen Cat. No. 10313-021). In some instances, another animal protein-free medium containing various concentrations including 60mM glucose (defined as the high glucose medium) was used. Fetal Bovine serum was purchased from Hyclone Labs, Logan, UT (Cat. No. SH30071.03). Cell cultures were monitored by a Cedex automated cell counting instrument (Innovatis, Germany). Integrated viable cell count (IVCC, cell-day/ml) was calculated using the following formula:

$$IVCC (d1) = [VCD (d0) + VCD (d1)]/2 + VCD (d0) ,$$

where VCD = viable cell density

Plasmid construction:

pBUDCE4.1 vector designed to constitutively express E1B-19K (EF-1a promoter), either alone or in conjunction with Aven (CMV promoter) and has been described (Nivitchanyong et. al., 2007). The vector expressing XIAP Δ (CMV promoter) was as described in Sauerwald et al., 2002. The blank vector refers to the original pBUDCE4.1 vector.

A model antibody (Ab #1) expression vector was constructed by cloning a Heavy and a Light chain cDNA into the Glutamine Synthase (GS) expression vector (obtained from Lonza Biologics, Slough, UK, under a research license).

Generation of apoptotic^R cell lines:

An exponential culture of CHOK1SV cell line was transfected with 1) pBUDCE4.1; 2) pBUDCE4.1-E1B-19K; 3) pBUDCE4.1-E1B-19K-Aven; and 4) pBUDCE4.1-E1B-19K-Aven and pCMV-XIAP Δ . Transfections were performed using Eugene (Roche, Cat. #1815075, Basel, Switzerland) according to the manufacturer's recommendation. Two days post-transfection, cells were plated in 96-well plates in growth medium containing 300 μ g/ml Zeocin (for transfections 1, 2 and 3 above); 300 μ g/ml Zeocin and 400 μ g/ml Hygromycin (for transfection #4), see Table 1. About 200 antibiotic resistant clones from each transfection were expanded and analyzed for caspase3/7 activity as described below. Promising clones underwent stability testing in absence of the respective antibiotics for ten passages.

Table 1

<u>Cell Line</u>	<u>Vector(s) transfected</u>	<u>Genes over-expressed</u>	<u>Clones selected by</u>
Control	None	None	None
Blank	pBUDCE4.1	None	Zeo
E64	pBUDCE4.1-E1B-19K	E1B19K	Zeo
EA63 EA112 EA167 EA190	pBUDCE4.1-E1B-19K-Aven	E1B-19K, Aven	Zeo
EAX64 EAX99	pBUDCE4.1-E1B-19K-Aven and pCMV-XIAP Δ	E1B-19K, Aven, XIAP Δ	Zeo, Hygro

EAX148
EAX197

Initially, a control cell line was developed by transfecting the blank vector into CHOK1SV cell line and selecting zeocin-resistant colonies. These cell lines had on average about 20% lower IVCC compared to the untransfected control CHOK1SV, hence CHOK1SV was subsequently used as the control cell line in all experiments. The most promising apoptotic^R cell line, EAX197 as well as the control cell line were then used to develop production cell lines expressing a model antibody using the GS expression vector according to the manufacturer's recommendation. Antibody in cell culture was measured by Nephelometry (Beckman Array System).

Shake-flask cultures of apoptotic^R cell lines:

Selected apoptotic^R cell lines were cultured in un-fed batch mode in CD-CHO medium supplemented with 6mM Glutamine and the requisite antibiotic selection agent. In some experiments, the batch mode cultures of apoptotic^R cell lines were fed with lactate to replenish the lactate they had depleted. Additionally, select Ab-expressing cell lines were cultured in a custom formulated animal protein-free medium supplemented with 6mM glutamine and 60mM glucose.

Caspase 3/7 activity Assay:

About 3×10^5 cells of each clone were seeded in one ml of growth medium in a 24-well plate. On day 4 (d4) post seeding, about 1×10^5 cells were transferred in triplicate to a 96well plate. Staurosporine (2 μ M fc) was added and the cells were incubated for 16h before assaying for caspase3/7 activity by APO-ONE kit (BD Labs). The procedure was repeated on d10, except that Staurosporine was omitted. The clones that had significantly lower caspase3/7 activity on both days were expanded into SF. The apoptotic^R nature of the selected clones was confirmed by flow cytometry analysis (see below) and in some instances, by measuring the mitochondrial membrane potential.

Analysis of apoptotic^R clones by flow cytometry:

About 1×10^6 cells from exponential cultures were withdrawn from each shake flask into 24well plates, incubated with Staurosporine (2 μ M fc) for 16h, harvested and washed once in PBS. The cells were then incubated with CytoPerm (Cat. No. 2075KK, BD BioScience) to fix and permeablized them. Following a PBS wash, cells were incubated with FITC-labeled anti-caspase3 (Cat. No. 68654, BD BioScience) antibody before subjecting them to analysis by flow cytometry.

Mitochondrial membrane potential (MMP) assay:

Cells from shake flask cultures were withdrawn on d6 post-seeding and incubated with Staurosporine (5 μ M fc) for 2 hours. They were subsequently washed and processed with lipophilic cationic dye, JC-1 (Cayman Labs, Ann Arbor, MI) as per manufacturer's recommendation. Plates were read at FL535 and FL595 and the ratio was calculated.

Western blotting:

About 1×10^7 cells from indicated cell lines were harvested, washed in PBS and lysed in RIPA buffer containing 1% NP-40, 120mM Tris-HCl, 150mM NaCl, 0.2mM PMSF and 1mM EDTA. Fifty micrograms of cytosolic protein from each sample was loaded on a 4-12% NuPAGE gradient gel and separated by SDS-PAGE in a Novex system (Invitrogen). Separated protein bands were transferred onto nitrocellulose and analyzed by using: 1) an anti-mouse antibody to human ElB-19K, 1:40 dilution, (Cat No. DP-17, CalBiochem, Gibbstown, MD); 2) an anti-Rabbit antibody to human Aven, 1:1,000 dilution, (Cat No. 612521, BD BioScience, San Jose, CA); and 3) an anti-mouse antibody to human XIAP (Cat No. 616713, BD BioScience). The above protocol, including the antibodies used, was optimized and standardized using known quantities (~10ng each) of purified ElB-19K, Aven and XIAP, purchased from commercial sources. The protein bands were visualized using ECL detection kit (GE Healthcare, Piscataway, NJ).

Determination of metabolite concentrations in culture medium:

Concentration of all key metabolites and waste products were determined using an YSI 2700 automated analyzer. Ammonium ion concentration was determined by flow injection analysis (Campmajo et al, 1994). Amino acids were measured by HPLC (Waters, Milford, MA), using a reversed-phase column (Waters).

Example 1**Generation and characterization of apoptotic-resistant cell lines**

A list of cell lines used in this study, the expression plasmids that were transfected in each case to generate the cell lines and the selection agent(s) used for isolating the transfectomas is shown in Table 1 above. The cell lines listed are representative of multiple clones that were generated from each transfection. The name of each cell line was derived from the anti-apoptotic genes that were transfected into the host cell line. For example, EAX197 is a

cell line that was transfected with ElB-19K (E), Aven (A) and XIAPA (X). The notation, apoptotic^R, will be used to refer to a cell line that has been transfected by one or more of these anti-apoptotic genes.

All apoptotic^R cell lines used in this study were characterized by Western blotting, caspase 3/7 activity and mitochondrial membrane potential (data not shown). The single transfectant E64, or double transfectants EA63, EA112, EA167 and EA190, all expressed levels of ElB-91K that were above background levels observed in the control, untransfected cell line, C1013A. Of the four double transfectants, cell lines EA112 and EA167 expressed both ElB-19K (~19KDa) and Aven (~55 KDa). Cell lines EA63 and EA190 expressed significant levels of ElB-19K but only very low levels of Aven. Of the four cell lines that were generated by transfecting the dual vector pBUDCE4.1-ElB-19K-Aven as well as pCMV-XIAPA, namely, EAX64, EAX99, EAX148 and EAX197, only EAX197 expressed detectable levels of all three proteins.

An ~55kDa band was observed in all lanes including in the untransfected control lane, C1013A, above the recombinant Aven band was believed to be the endogenous Aven protein as it was visualized with post-immune but not with pre-immune sera (Sauerwald et al., 2002, Chau et al., 2000). The transfected Aven gene lacks the first six amino acids, which have been shown not to impair the biological activity of the protein. Consequently, transfected Aven is slightly smaller than its endogenous counterpart. A 40KDa band, which may represent a degradation product of Aven, was observed in all cell lines that over-expressed this protein in conjunction with ElB-19K. Degradative products of similar sizes were observed previously when Aven was over-expressed with Bcl-XL (Sauerwald et al., 2005) or Bcl-2 (Figuerola, unpublished observations). Additionally, the expression of Aven was significantly lower in triple transfectants, including EAX197 over-expressing ElB-19K, Aven and XIAPA.

Interestingly, the level of expression of transfected XIAPA was significantly lower compared to the endogenous levels of the intact XIAP protein. Note that the apparent difference

in expression level between XIAP and XIAPA could be explained in part by the difference in antibody binding efficiency of these two forms of the anti-apoptotic protein. The apparent expression level difference between the endogenous XIAP and the transgenic XIAPA questions the contribution of this transfected protein in preventing apoptosis. Indeed this study suggests that there is not always a direct correlation between the expression level of a given anti-apoptotic gene and the level of apoptosis inhibition the protein can confer on a cell line.

Each apoptotic^R cell line was examined for caspase 3/7 activity by APO-ONE as this activity provides an indication of the degree of resistance of a cell line to apoptosis. Briefly, in this assay, the profluorescent substrate Z-DEVD-R110 is cleaved by caspase 3/7 in a dose-dependent manner. Exponential cultures of EA167 and EAX197 as well as the control cell line were treated with staurosporine for 16 hours to induce apoptosis, following which caspase 3/7 was measured. The caspase 3/7 activity in staurosporine treated control cells was arbitrarily set at 100%. Under these conditions, the caspase activities of the EA167 and EAX197 cells were 30% and 35%, respectively, of the control, suggesting that these cell lines were at least partially resistant to apoptosis induction (Fig. 2A). As further evidence of the apoptotic^R nature of EA167 and EAX197, staurosporine-treated cultures of these two cell lines and the control cell line, were labeled with FITC-conjugated anti-caspase 3 antibody followed by analysis by flow cytometry. As can be seen from Fig. 3, all three cell lines, whether untreated or vehicle (DMSO)-treated, had 1% or less caspase 3-positive population. However, 91% of the control cell population was positive for caspase 3 following staurosporine treatment. In contrast, only 9% of the population derived from cell line EAX197 and 30% of that derived from cell line EA167 were positive for caspase 3, confirming the apoptotic^R phenotype of these two cell lines.

To examine if the resistance to apoptosis leads to increased longevity, multiple EA cell lines were grown in shake-flasks (batch mode) and both viable cell density (VCD) and integrated viable cell count (IVCC) were monitored.

Increases in the integrated viable cell count improve the volumetric productivity of a culture, assuming that the specific productivity of the cell remains unaltered. Therefore IVCC can be used as a parameter for screening superior apoptotic^R cell lines that can serve as hosts for the development of production cell lines. As shown in Fig. 2B, the cell lines with lower relative caspase 3/7 activity tended to generate cell lines with higher IVCC. Although each of the EA cell lines showed reduced caspase 3/7 activity relative to the control, there was no strict correlation between IVCC and the level of caspase 3/7 that each cell line possessed. Moreover, some apoptotic^R cell lines were more stable than their sister clones with respect to the degree of anti-apoptotic phenotype. EA167 was chosen for further study because it possessed both potent anti-apoptotic activity and robust growth in shake flask cultures. Additionally, EA167 was the most stable cell line in this panel, expressing Aven at high levels as detected by Western blot.

Many of the signaling molecules in the apoptosis pathway interact with or reside within the mitochondria. There these proteins interact with the mitochondrial membrane and regulate apoptosis through modulation of the mitochondria permeability. Therefore, mitochondrial membrane potential (MMP) serves as another monitor of cell physiology in which to compare the characteristics of the apoptotic^R nature of EA167 and EAX197 versus control. As seen in the assay results of Fig. 2C, both EA167 and EAX197 had higher MMP than the control cell line.

Example 2

Effect of E1B-19K on viable cell density of CHOK1SV

The growth profiles of double transfectants over-expressing E1B-19K in conjunction with Aven (EA167), triple transfectants over-expressing E1B-19K in conjunction with Aven and XIAPA (EAX197), as well as the transfectant over-expressing E1B-19K only (E64) are compared to the control (host) cell line in Fig. 4. While the peak VCD of the control cell line reached 7.7×10^6 cells/ml and the peak VCD of E64 (expressing E1B-19K

only) was 8.9×10^6 cells/ml, that of EA167 was 1.4×10^7 cells/ml and that of EAX197 was more than 1.5×10^7 cells/ml, an increase of over 190% above the control cell line. The viability of EA167 and EAX197 showed a sharp decline by d8 or d9 post-seeding, presumably because of nutrient depletion, while the control and the E64 cell lines viability declined more slowly (Fig. 4B). The net effect on IVCC is shown in Fig. 4C. The IVCC of the control cell line was 4.9×10^7 cell-day/ml, followed by that of E64 (5.2×10^7 cell-day/ml). Clearly, the IVCC of the two apoptotic^R cell lines, EA167 (6.3×10^7 cell-day/ml) and EAX197 (6.6×10^7 cell-day/ml) were significantly higher (136% and 140% for EA167 and EAX197, respectively) than that of control cell lines.

In addition to EA167 and EAX197, several other apoptotic^R lines exhibited similarly low lactate production phenotype (data not shown). Additionally, wild-type cell lines with low lactate production characteristics, though rare, have been reported (Pascoe et al, 2007).

Example 3

Metabolic profile of apoptotic^R cell lines

In order to compare the physiology of the apoptotic^R cell lines EA167 and EAX197 which could protect against apoptosis to the control cell line in batch cultures, the levels of key nutrients and amino acids that were provided at the beginning of the batch culture, namely, glucose, glutamate and glutamine, were monitored. Additionally, the accumulation of the waste products ammonia and lactate were also monitored. Shown in Fig. 5 are the levels of these metabolites when EAX197 and EA167 as well as control cell line were cultured in batch mode in shake-flasks in commercial CD-CHO medium. As shown in Fig. 5A, glucose is rapidly depleted from the medium of all three cell lines by day-6 or day-7 post-seeding. No significant differences in glutamine depletion were discernable between apoptotic^R and control cell lines and glutamate levels did not vary greatly for the three cell lines across all days (data not shown). All three cell lines showed an accumulation of lactate during early cell growth phase up to day-4 (Fig.

5B). In the control cell line, lactate continued to increase up until day-6 (at which time it reached 2.8g/L) and then a slight decrease was observed with the lactate concentration eventually reaching 1.8g/L. This amount of lactate is expected to lower the pH of the culture and in bioreactors, where pH is controlled by the addition of bicarbonate, the osmolarity would be elevated. All these characteristics, in addition to other stress factors, could lead to a toxic environment for the animal cells. In contrast, the lactate levels in the apoptotic^R cell lines increased for a shorter period and then dropped rapidly from day 5 onward with the lactate being completely eliminated by day 7 (EA167) or day 8 (EAX197). Interestingly, the time point at which the lactate dropped to zero immediately preceded the time at which the VCD dropped precipitously in Fig. 5A for these two cell lines, suggesting that the loss in cell viability was due to the absence of any available glucose or lactate in the medium.

Another difference observed between apoptotic^R and control cell lines is the ammonia profile (Fig. 5C). The ammonia levels in EA167 and EAX197 declined slightly at day 6, at which time the decline in lactate had begun, and then ultimately increased again. However, the overall ammonia levels of the apoptotic^R lines were lower than that of control lines, which was surprising since the apoptotic^R lines reached higher IVCC and thus attained more overall cell growth compared to the control cell line (Fig. 4C).

From the above data, it is obvious that the characteristics of the apoptotic^R cell lines are dramatically different from that of the control cell lines. However, when multiple clones are examined under somewhat different experimental conditions, a wide spectrum of phenotypes is observed among the apoptosis^R transfectants. Since phenotypic variability exists in clonal isolates of the control cell line as well, it is possible to select rare apoptotic^R clones from control cell line by utilizing a suitable screening regimen. In this respect, Mattanovich and Borth (2006) have reviewed the application of cell sorting to isolate rare variants with desirable properties from wild-type (control) cell line.

Example 4

Effect of lactate feeding

The amount of lactate present in a cell culture at any point is dependent on the amount of lactate secreted by that cell line less the amount consumed by it. In order to determine if the apoptotic^R cell lines were actually consuming lactate or if the net lactate production was lower in these cell lines, cultures of all three cell lines were monitored daily and any major difference in lactate concentration between apoptotic^R and control cell line was eliminated by the addition of exogenous lactate. As observed previously, the lactate level in the un-fed apoptotic^R EA167 and EAX197 began to drop at day-5 (Figs. 6A and 6B) with exhaustion of lactate by day-7 (EA67, un-fed) or day-8 (EAX197, un-fed). The lactate profile of the control cell line continued to increase until d6 at which time the level declined slightly for the next three days but never dropped to zero. Upon daily monitoring of the apoptosis^R culture, lactate was added to separate fed-batch cultures up to 2.5g/L at day-7 (for EA167, Fig. 6A) and 2g/L at day-8 (for EAX197, Fig. 6B) in order for levels to be similar to that of the control cell line. Even with the addition of supplemental lactate, the lactate level dropped much more rapidly in the apoptotic^R cultures compared than the controls from day-6 to day-12. Indeed, the lactate level dropped from 2.5 to 0.2g/L in the EA167 fed culture from day-6 to day-7 while the lactate decline was only from 2.7 to 2.4g/L in the control culture over the same time period. Thus, the lactate appears to be consumed in the apoptotic^R cell lines at an accelerated rate compared to the control cell lines for at least 7 days following addition of supplemental lactate.

The effect of lactate supplementation on VCD and cell viability is shown in Figs. 7A and 7B, respectively. As previously observed (Fig. 5A), the VCD of the un-fed apoptotic^R cell lines declined rapidly starting at day-8 (EA167) and day-9 (EAX197). This is presumably because one or more essential media components, including glucose and/or lactate, have been exhausted in these cultures. The control cell line exhibited a

slow decline starting at day-7. However the lactate-supplemented cultures (EAX167, lactate-fed and EAX197, lactate-fed) exhibited a much slower decline in VCD, and in one cell line (EAX197, lactate-fed), the VCD stabilized at between 8×10^6 and 10×10^6 cells/ml until d14 before this culture eventually succumbed. The cell viabilities shown in Fig. 7B exhibited a similar more gradual decline for the two apoptotic^R cultures with supplemented lactate in the media. These results suggest that the apoptotic^R cell lines are capable of utilizing both endogenous and supplemental lactate efficiently to retain their viable cell density and viability while the control cell line is unable to implement a similar efficient lactate consumption strategy. This difference is reflected in the IVCC of the lactate-fed apoptotic^R cell lines, which were nearly 180-220% higher than the un-fed apoptotic^R cell lines and about 235%-250% higher than the control cultures.

Most of the other nutrients and metabolites shown in Figs. 7D-F exhibited profiles similar to that observed previously in the un-fed cultures of Fig. 5. Glucose and glutamine were rapidly consumed at early time points in the culture. The ammonia profile of the apoptotic^R cell lines once again exhibited a slight decline at the time at which lactate consumption began in both the unsupplemented and lactate-supplemented cultures. As in the previous experiments, the final ammonia level of the apoptotic^R cell lines was below control levels even though the IVCC was considerably higher in all four apoptotic^R cultures. The glutamate levels differed between lactate-fed and un-fed cultures with glutamate depletion being significantly increased in the fed culture as compared to the un-fed and control cell lines.

Example 5

Amino acid profile of apoptotic^R and control cell lines

The concentrations of each of the twenty amino acids were monitored periodically in control cell lines as well as in un-fed and lactate-fed apoptotic^R cell line cultures (Table II and Fig. 8). With respect to the consumption or production of amino acids, other than glutamine and glutamate, it should be first stated that none of the nine essential amino acids (phenylalanine, threonine, methionine, lysine, tryptophan, leucine, isoleucine, valine and histidine) were exhausted, as indicated in Table II. In apoptotic^R (un-fed) and control cell line cultures, asparagine, serine, tryptophan and cysteine (highlighted in italics) were almost completely exhausted. Interestingly, all three branched-chain amino acids, namely, isoleucine, leucine and valine (shaded light grey in Table II) were more rapidly depleted from the culture medium by apoptotic^R cell lines compared to the control, and this depletion is more pronounced in lactate-fed cultures (Figs. 8A-D). In addition to glutamate, the final levels of these three amino acids were much lower from the medium in lactate-fed cultures. Other amino acids with a high percentage consumed (final concentration below 50% of their initial concentration in the medium) included tyrosine, phenylalanine, methionine (Figure 8D) and aspartic acid (Fig. 8E).

A comparison of the amino acids that were excreted into the culture medium between the control and the apoptotic^R cell lines is also of interest to consider. As shown in Fig. 8E, the control cell lines accumulated aspartic acid in the culture medium over the first six days while there was a decline in the aspartic acid levels over that same time period for all four apoptotic^R cultures. Additionally, the apoptotic^R cell lines secrete significantly more alanine (Fig. 8F) over the first 6 days as compared to the control cell line, while all the cell lines consume it at later times. Also, the apoptotic^R cell lines produce more homocysteine over the entire cell culture in comparison to the control cell lines (Fig. 8G).

Example 6

Batch culture in the presence of high glucose

If the apoptotic^R cell lines can utilize lactate, then there is a possibility that these lines may potentially thrive at glucose concentrations that could be inhibitory for control cells due to accumulation of toxic levels of lactate. Additionally, it is possible that growth of these lines could be supported by culture medium containing lactate as the sole carbon source. It was not possible to use the CD-CHO medium used in the previous experiments because such as high glucose would lead to unsustainable osmolarity and thus a new medium had to be utilized for these experiments. Therefore, we compared the growth kinetics of EAX197, which exhibited the highest sustained growth in previous experiments, to that of control cell lines in a custom-formulated animal protein-free medium containing 60mM glucose (high glucose) or 30mM lactate. Neither the apoptotic^R nor the control cell lines exhibited sustained growth in the complete absence of glucose, i.e., in the presence of lactate only (data not shown). When both cell lines were cultured in the same custom-formulated medium containing 'high' (60mM) concentrations of glucose, EAX197 exhibited a higher VCD, viability and IVCC (Figs. 9A-C) than the control cell line especially at later times in the cell culture. EAX197 cell line exhibited a smaller overall increase in IVCC (130%) over control than in the previous CD-CHO medium but the difference was still significant due to increased viability at later times. The lactate profiles of these two cell lines cultivated in 'high' glucose are shown in Fig. 9D. As in the case with IVCC, the lactate profiles of both lines differed somewhat from that observed in CD-CHO medium (Fig. 5D). Consistent with previous results with the CD-CHO medium, the apoptotic^R cell line in the custom formulation medium initiated net lactate consumption earlier (by day 2, as shown in Fig. 9D) than the control and the maximum lactate level was much lower (1.2g/L compared to 2.4g/L for the control cell line). The control cell exhibited a net production of lactate until day 5 at which point the control cells also began to consume the lactate in the medium. Since the CD-CHO medium is a commercial formulation with a propriety composition, it was

not possible to compare exactly the medium component(s) in the two formulations in order to understand the cause of the different lactate consumption profiles in the different formulations.

Example 7

Productivity of an apoptotic^R cell line in high glucose medium

The apoptotic^R cell line, EAX197 along with the control cell line were subsequently used as hosts for the development of production cell lines expressing a model antibody. An equal number of clones were surveyed for each cell line and the best clone generated from each host was then compared in shake-flask-batch mode for Ab productivity. Custom formulated medium containing 60mM ('high') glucose as a carbon source was used in order to compare their performance in a high glucose environment. In the standard medium (Fig. 10A), the production cell line derived from EAX197 had titers of 489mg/L, which is 145% increase over the control cell line, which had a titer of 337mg/L. The same cell line in high glucose medium (Fig. 10B) had titers of 687mg/L, which is 178% increase over that of the control cell line (384mg/L). The enhanced production was due at least in part to a 188% increase in IVCC for EAX197 versus the control cell line (data not shown).

Example 8

Effect of over-expression of ElB19K+AVEN +/- XIAPΔ in blocking apoptosis

The major activators of the apoptosis cascade in mitochondria are shown in Fig. 1. In our attempt to develop an apoptotic-resistant CHO cell line, we selected three anti-apoptotic genes that act at early, mid- and late stage of apoptosis. These genes were ElB19K (E), AVEN (A) and XIAPΔ (X). CHOK1SV, the widely used transfection host cell line was transfected with ElB19K+AVEN with/without XIAPΔ. A large number of transfectomas were screened and a few clones from each transfection were selected for further study. Growth profile study of these cell lines show that they reach very high VCD and in general, their IVCC was about two-fold higher

than that of control. The apoptotic^R cell lines lost viability by day-8 post-seeding, but regained it day-10 onwards by consuming lactate. The culture fluid of apoptotic^R lines expressing E1B19K+AVEN±XIAPA had reduced or undetectable levels of ammonia and lactate and depleted glutamate from the culture media.

Example 9

Use of Bcl-2d to increase antibody titer

The results above demonstrate that apoptotic^R cell lines accumulate less lactate compared to control cells and viability of apoptotic^R cultures can be maintained in high glucose medium. In the following shake-flask batch experiment, an apoptotic^R cell line expressing Bcl-2d and antibody heavy and light chains (C2088B) was seeded at various seeding densities in standard (4.8g/L) and high concentration of glucose (9.5g/L). The results show that the apoptotic^R cell line, when seeded at 1e6cells/ml in medium containing 9.5g/L glucose, reached peak VCD of 14e6cells/ml on day-7 post-seeding (Fig. 11A), and antibody titer of 2g/L. In comparison, C2088B cultured under the standard conditions (0.3e5cells/ml inoculation and 4.8g/L) had peak VCD of 7e6cells/ml and antibody titer of 1g/L (Fig. 11B). This translates to a 200% increase in antibody titers for high glucose condition as compared to standard conditions (i.e., seeding density of 0.3e6c/ml in medium containing 4.8g/L glucose).

The present invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

Claims

1. A method of increasing viable cell density in a fed batch eukaryotic cell culture comprising the steps of:
 - a) culturing a eukaryotic cell line expressing one or more heterologous apoptotic-resistant (apoptotic^R) genes and one or more genes of interest; and
 - b) maintaining a high glucose media feed containing about 60 mM glucose during the exponential and stationary phases of the cell culture, wherein the apoptotic^R genes comprise ElB19K and AVEN and the lactate concentration in the cell culture is about 1 g/L or less after 5 days of culture.
2. The method of claim 1 wherein the eukaryotic cell line is a Chinese Hamster Ovary (CHO) cell line.
3. The method of claim 2 wherein the CHO cell line is CHO-K1.
4. The method of claim 2 wherein the CHO cell line is CHO-K1SV.
5. The method of claim 1 wherein the eukaryotic cell line is a myeloma cell line.
6. The method of claim 5 wherein the myeloma cell line is NS0.
7. The method of claim 5 wherein the myeloma cell line is Sp2/0.
8. The method of claim 1 wherein the eukaryotic cell line is a hybridoma.
9. The method of claim 1 wherein the apoptotic^R genes further comprise XIAPΔ.
10. The method of claim 1 wherein the peak viable cell density (VCD) is increased.
11. The method of claim 1 wherein the longevity of the cell culture is extended.
12. The method of claim 1 wherein the titer of the cell culture is increased.

13. The method of claim 1 wherein the integrated viable cell count (IVCC) of the cell culture is increased.

14. The method of claim 1 wherein the cellular calcium flux is reduced.

15. The method of claim 1 wherein the mitochondrial membrane potential is increased.

16. The method of claim 1 wherein the genes of interest encode an antibody heavy chain and an antibody light chain.

17. The method of claim 1 wherein the high glucose media feed contains 60 mM glucose and the lactate concentration in the cell culture is about 0.1 g/L or less after 7 days of culture.

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Cys	Leu	Arg	Leu	Asn	Val	Ala	Ala	Glu	Leu	Val	Gln	Gly	Thr	Val	Pro
		195					200					205			
Leu	Glu	Val	Pro	Gln	Val	Lys	Pro	Lys	Arg	Thr	Asp	Asp	Gly	Lys	Gly
	210					215					220				

SEQLIST

Leu Gly Met Gln Leu Lys Gly Pro Leu Gly Pro Gly Gly Arg Gly Pro
 225 230 235 240
 Ile Phe Glu Leu Lys Ser Val Ala Ala Gly Cys Pro Val Leu Leu Gly
 245 250 255
 Lys Asp Asn Pro Ser Pro Gly Pro Ser Arg Asp Ser Gln Lys Pro Thr
 260 265 270
 Ser Pro Leu Gln Ser Ala Gly Asp His Leu Glu Glu Glu Leu Asp Leu
 275 280 285
 Leu Leu Asn Leu Asp Ala Pro Ile Lys Glu Gly Asp Asn Ile Leu Pro
 290 295 300
 Asp Gln Thr Ser Gln Asp Leu Lys Ser Lys Glu Asp Gly Glu Val Val
 305 310 315 320
 Gln Glu Glu Glu Val Cys Ala Lys Pro Ser Val Thr Glu Glu Lys Asn
 325 330 335
 Met Glu Pro Glu Gln Pro Ser Thr Ser Lys Asn Val Thr Glu Glu
 340 345 350
 Leu Glu Asp Trp Leu Asp Ser Met Ile Ser
 355 360

<210> 5
 <211> 1356
 <212> DNA
 <213> Homo sapiens

<400> 5
 atgacttttta acagtttttga aggatctaaa acttgtgttac ctgcagacat caataaggaa 60
 gaagaattttg tagaagagtt taatagatta aaaactttttg ctaattttcc aagtggtagt 120
 cctgttttcag catcaacact ggcacgagca gggttttcttt atactgggtga aggagatacc 180
 gtgcgggtgct ttagttgtca tgcagctgta gatagatggc aatatggaga ctccagcagtt 240
 ggaagacaca ggaaagtatc cccaaattgc agattttatca acggcttttta tcttgaaaaat 300
 agtgccacgc agtctacaaa ttctgggtatc cagaatggtc agtacaaaagt tgaaaactat 360
 ctgggaagca gagatcattt tgccttagac aggccatctg agacacatgc agactatctt 420
 ttgagaactg ggcaggttgt agatatatca gacaccatat acccgaggaa ccctgccatg 480
 tatagtgaag aagctagatt aaagtccttt cagaactggc cagactatgc tcacctaacc 540
 ccaagagagt tagcaagtgc tggactctac tacacaggta ttggtgacca agtgcagtgc 600
 tttgtttgtg gtggaaaact gaaaaatttg gaacctttgt atcgtgcctg gtcagaacac 660
 aggcgacact ttcctaattg cttctttgtt ttggggccgga atcttaatat tcgaagttaa 720
 tctgatgctg tgagtcttga taggaatttc ccaaattcaa caaatcttcc aagaaatcca 780
 tccatggcag attatgaagc acggatcttt acttttggga catggatata ctcatgtaac 840
 aaggagcagc ttgcaagagc tggattttat gcttttaggtg aagggtgataa agtaaaagtgc 900
 tttcactgtg gaggagggct aactgatttg aagcccagtg aagacccttg ggaacaacat 960
 gctaaatggt atccaggggt caaatatctg ttagaacaga agggacaaga atatataaac 1020
 aatattcatt taactcattc acttgaggag tgtctggtaa gaactactga gaaaacacca 1080
 tcactaacta gaagaattga tgataccatc ttccaaaatc ctatggtaca agaagctata 1140
 cgaatggggt tcagttttcaa ggacattaa aaaataatgg agggaaaaat tcagatatct 1200
 gggagcaact ataaatcact tgaggttctg gttgcagatc tagtgaatgc tcagaaagac 1260
 agtatgccag atgagtcaag tcagacttca ttacagaaag agattagtac tgaagagcag 1320
 ctaaggcgcc tgcaagagga gaagcttatc gattga 1356

<210> 6
 <211> 451
 <212> PRT
 <213> Homo sapiens

<400> 6
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 Ile Asn Lys Glu Glu Glu Phe Val Glu Glu Phe Asn Arg Leu Lys Thr
 20 25 30
 Phe Ala Asn Phe Pro Ser Gly Ser Pro Val Ser Ala Ser Thr Leu Ala
 35 40 45
 Arg Ala Gly Phe Leu Tyr Thr Gly Glu Gly Asp Thr Val Arg Cys Phe
 50 55 60
 Ser Cys His Ala Ala Val Asp Arg Trp Gln Tyr Gly Asp Ser Ala Val
 65 70 75 80
 Gly Arg His Arg Lys Val Ser Pro Asn Cys Arg Phe Ile Asn Gly Phe
 85 90 95
 Tyr Leu Glu Asn Ser Ala Thr Gln Ser Thr Asn Ser Gly Ile Gln Asn

SEQLIST

SEQUENCE															
			100						105			110			
Gly	Gln	Tyr	Lys	Val	Glu	Asn	Tyr	Leu	Gly	Ser	Arg	Asp	His	Phe	Ala
		115					120					125			
Leu	Asp	Arg	Pro	Ser	Glu	Thr	His	Ala	Asp	Tyr	Leu	Leu	Arg	Thr	Gly
	130					135					140				
Gln	Val	Val	Asp	Ile	Ser	Asp	Thr	Ile	Tyr	Pro	Arg	Asn	Pro	Ala	Met
	145				150					155					160
Tyr	Ser	Glu	Glu	Ala	Arg	Leu	Lys	Ser	Phe	Gln	Asn	Trp	Pro	Asp	Tyr
				165					170					175	
Ala	His	Leu	Thr	Pro	Arg	Glu	Leu	Ala	Ser	Ala	Gly	Leu	Tyr	Tyr	Thr
			180					185					190		
Gly	Ile	Gly	Asp	Gln	Val	Gln	Cys	Phe	Cys	Cys	Gly	Gly	Lys	Leu	Lys
		195					200					205			
Asn	Trp	Glu	Pro	Cys	Asp	Arg	Ala	Trp	Ser	Glu	His	Arg	Arg	His	Phe
	210					215					220				
Pro	Asn	Cys	Phe	Phe	Val	Leu	Gly	Arg	Asn	Leu	Asn	Ile	Arg	Ser	Glu
	225				230					235					240
Ser	Asp	Ala	Val	Ser	Ser	Asp	Arg	Asn	Phe	Pro	Asn	Ser	Thr	Asn	Leu
				245					250					255	
Pro	Arg	Asn	Pro	Ser	Met	Ala	Asp	Tyr	Glu	Ala	Arg	Ile	Phe	Thr	Phe
			260					265					270		
Gly	Thr	Trp	Ile	Tyr	Ser	Val	Asn	Lys	Glu	Gln	Leu	Ala	Arg	Ala	Gly
		275					280					285			
Phe	Tyr	Ala	Leu	Gly	Glu	Gly	Asp	Lys	Val	Lys	Cys	Phe	His	Cys	Gly
	290					295					300				
Gly	Gly	Leu	Thr	Asp	Trp	Lys	Pro	Ser	Glu	Asp	Pro	Trp	Glu	Gln	His
	305				310					315					320
Ala	Lys	Trp	Tyr	Pro	Gly	Cys	Lys	Tyr	Leu	Leu	Glu	Gln	Lys	Gly	Gln
				325					330					335	
Glu	Tyr	Ile	Asn	Asn	Ile	His	Leu	Thr	His	Ser	Leu	Glu	Glu	Cys	Leu
			340					345					350		
Val	Arg	Thr	Thr	Glu	Lys	Thr	Pro	Ser	Leu	Thr	Arg	Arg	Ile	Asp	Asp
		355					360					365			
Thr	Ile	Phe	Gln	Asn	Pro	Met	Val	Gln	Glu	Ala	Ile	Arg	Met	Gly	Phe
	370					375					380				
Ser	Phe	Lys	Asp	Ile	Lys	Lys	Ile	Met	Glu	Glu	Lys	Ile	Gln	Ile	Ser
	385				390					395					400
Gly	Ser	Asn	Tyr	Lys	Ser	Leu	Glu	Val	Leu	Val	Ala	Asp	Leu	Val	Asn
				405					410					415	
Ala	Gln	Lys	Asp	Ser	Met	Pro	Asp	Glu	Ser	Ser	Gln	Thr	Ser	Leu	Gln
			420					425					430		
Lys	Glu	Ile	Ser	Thr	Glu	Glu	Gln	Leu	Arg	Arg	Leu	Gln	Glu	Glu	Lys
		435				</									

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<210> 7
<211> 573
<212> DNA
<213> Homo sapiens
```

<400> 7							
atggcgcacg	ctgggagaac	agggtagcat	aaccgggaga	tagtgatgaa	gtacatccat	60	
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ccacctgtgg	tccacctgac	cctccgccag	gccggcgacg	acttctcccg	ccgctaccgc	180	
cgcgacttcg	ccgagatgtc	cagccagctg	cacctgacgc	ccttcaccgc	gcggggacgc	240	
tttgccacgg	tgggtggagga	gctcttcagg	gacgggggtga	actgggggag	gattgtggcc	300	
ttctttgagt	tcgggtgggg	catgtgtgtg	gagagcgtca	accgggagat	gtcgcacctg	360	
gtggacaaca	tcgccctgtg	gatgacttag	tacctgaacc	ggcaccgtca	cacctggatc	420	
caggataaac	gaggctggga	tgcctttgtg	gaactgtacg	gccccagcat	gcggcctctg	480	
tttgatttct	cctggctgtc	tctgaagact	ctgctcagtt	tggccctggg	gggagcttgc	540	
atcacccctgg	qtgcctatct	qagccacaag	tga			573	

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<210> 8
<211> 190
<212> PRT
<213> Homo sapiens
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SEQLIST

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<400> 8
Met Ala His Ala Gly Arg Thr Gly Tyr Asp Asn Arg Glu Ile Val Met
1      5      10      15
Lys Tyr Ile His Tyr Lys Leu Ser Gln Arg Gly Tyr Glu Trp Asp Ala
20      25      30
Ala Gly Pro Ala Leu Ser Pro Val Pro Pro Val Val His Leu Thr Leu
35      40      45
Arg Gln Ala Gly Asp Asp Phe Ser Arg Arg Tyr Arg Arg Asp Phe Ala
50      55      60
Glu Met Ser Ser Gln Leu His Leu Thr Pro Phe Thr Ala Arg Gly Arg
65      70      75      80
Phe Ala Thr Val Val Glu Glu Leu Phe Arg Asp Gly Val Asn Trp Gly
85      90      95
Arg Ile Val Ala Phe Phe Glu Phe Gly Gly Val Met Cys Val Glu Ser
100     105     110
Val Asn Arg Glu Met Ser Pro Leu Val Asp Asn Ile Ala Leu Trp Met
115     120     125
Thr Glu Tyr Leu Asn Arg His Leu His Thr Trp Ile Gln Asp Asn Gly
130     135     140
Gly Trp Asp Ala Phe Val Glu Leu Tyr Gly Pro Ser Met Arg Pro Leu
145     150     155     160
Phe Asp Phe Ser Trp Leu Ser Leu Lys Thr Leu Leu Ser Leu Ala Leu
165     170     175
Val Gly Ala Cys Ile Thr Leu Gly Ala Tyr Leu Ser His Lys
180     185     190

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<210> 9
<211> 1476
<212> DNA
<213> Homo sapiens

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<400> 9
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gcttcggaac aagagaccct ggtagacca aagccattgc ttttgaagtt attaaagtct 120
gttggtgcac aaaaagacac ttatactatg aaagagggtc ttttttatct tggccagtat 180
attatgacta aacgattata tgatgagaag caacaacata ttgtatattg ttcaaagtat 240
cttctaggag atttgtttgg cgtgccaaagc ttctctgtga aagagcacag gaaaatatat 300
accatgatct acaggaactt ggtagtagtc aatcagcagg aatcatcgga cttaggtaca 360
tctgtgagtg agaacagggtg tcaccttgaa ggtgggagtg atcaaaaagga ccttgtacaa 420
gagcttcagg aagagaaacc ttcatcttca catttggttt ctagaccatc tacctcatct 480
agaaggagag caattagtga gacagaagaa aattcagatg aattatctgg tgaacgacaa 540
agaaaaacgcc acaaactctga tagtatttcc ctttcctttg atgaaagcct ggctctgtgt 600
gtaataaggg agatatgttg tgaaagaagc agtagcagtg aatctacagg gacgccatcg 660
aatccggatc ttgatgtcgg tgtaagtga cttcaggtg attggttgga tcaggattca 720
gtttcagatc agtttagtgt agaatttgaa gttgaatctc tcgactcaga agattatagc 780
cttagtgaag aaggacaaga actctcagat gaagatgatg aggtatatca agttactgtg 840
tatcaggcag gggagagtga tacagattca tttgaagaag atcctgaaat ttccttagct 900
gactattgga aatgcacttc atgcaatgaa atgaatcccc cccttccatc acattgcaac 960
agatgttggg cccttcgtga gaattggctt cctgaagata aagggaaaga taaaggggaa 1020
atctctgaga aagccaaact ggaaaactca acacaagctg aagagggtt tgatgttcct 1080
gattgtaaaa aaactatagt gaatgattcc agagagtcac gtgttgagga aaatgatgat 1140
aaaattacac aagcttcaca atcacaagaa agtgaagact attctcagcc atcaacttct 1200
agtagcatta ttatagcag ccaagaagat gtgaaagagt ttgaaagggg agaaacccaa 1260
gacaaagaag agagtgtgga atctagtttg ccccttaatg ccattgaacc ttgtgtgatt 1320
tgtcaaggtc gacctaaaaa tggttgcatt gtccatggca aaacaggaca tcttatggcc 1380
tgctttacat gtgcaaagaa gctaaagaaa aggaataagc cctgcccagt atgtagacaa 1440
ccaattcaaa tgattgtgct aacttatttc ccctag
1476

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<210> 10
<211> 491
<212> PRT
<213> Mus musculus

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<400> 2
Met Cys Asn Thr Asn Met Ser Val Pro Thr Asp Gly Ala Val Thr Thr
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Ser Gln Ile Pro Ala Ser Glu Gln Glu Thr Leu Val Arg Pro Lys Pro
20      25      30

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SEQLIST

Leu Leu Leu Lys Leu Leu Lys Ser Val Gly Ala Gln Lys Asp Thr Tyr
 35 40 45
 Thr Met Lys Glu Val Leu Phe Tyr Leu Gly Gln Tyr Ile Met Thr Lys
 50 55 60
 Arg Leu Tyr Asp Glu Lys Gln Gln His Ile Val Tyr Cys Ser Asn Asp
 65 70 75 80
 Leu Leu Gly Asp Leu Phe Gly Val Pro Ser Phe Ser Val Lys Glu His
 85 90 95
 Arg Lys Ile Tyr Thr Met Ile Tyr Arg Asn Leu Val Val Val Asn Gln
 100 105 110
 Gln Glu Ser Ser Asp Ser Gly Thr Ser Val Ser Glu Asn Arg Cys His
 115 120 125
 Leu Glu Gly Gly Ser Asp Gln Lys Asp Leu Val Gln Glu Leu Gln Glu
 130 135 140
 Glu Lys Pro Ser Ser Ser His Leu Val Ser Arg Pro Ser Thr Ser Ser
 145 150 155 160
 Arg Arg Arg Ala Ile Ser Glu Thr Glu Glu Asn Ser Asp Glu Leu Ser
 165 170 175
 Gly Glu Arg Gln Arg Lys Arg His Lys Ser Asp Ser Ile Ser Leu Ser
 180 185 190
 Phe Asp Glu Ser Leu Ala Leu Cys Val Ile Arg Glu Ile Cys Cys Glu
 195 200 205
 Arg Ser Ser Ser Ser Glu Ser Thr Gly Thr Pro Ser Asn Pro Asp Leu
 210 215 220
 Asp Ala Gly Val Ser Glu His Ser Gly Asp Trp Leu Asp Gln Asp Ser
 225 230 235 240
 Val Ser Asp Gln Phe Ser Val Glu Phe Glu Val Glu Ser Leu Asp Ser
 245 250 255
 Glu Asp Tyr Ser Leu Ser Glu Glu Gly Gln Glu Leu Ser Asp Glu Asp
 260 265 270
 Asp Glu Val Tyr Gln Val Thr Val Tyr Gln Ala Gly Glu Ser Asp Thr
 275 280 285
 Asp Ser Phe Glu Glu Asp Pro Glu Ile Ser Leu Ala Asp Tyr Trp Lys
 290 295 300
 Cys Thr Ser Cys Asn Glu Met Asn Pro Pro Leu Pro Ser His Cys Asn
 305 310 315 320
 Arg Cys Trp Ala Leu Arg Glu Asn Trp Leu Pro Glu Asp Lys Gly Lys
 325 330 335
 Asp Lys Gly Glu Ile Ser Glu Lys Ala Lys Leu Glu Asn Ser Thr Gln
 340 345 350
 Ala Glu Glu Gly Phe Asp Val Pro Asp Cys Lys Lys Thr Ile Val Asn
 355 360 365
 Asp Ser Arg Glu Ser Cys Val Glu Glu Asn Asp Asp Lys Ile Thr Gln
 370 375 380
 Ala Ser Gln Ser Gln Glu Ser Glu Asp Tyr Ser Gln Pro Ser Thr Ser
 385 390 395 400
 Ser Ser Ile Ile Tyr Ser Ser Gln Glu Asp Val Lys Glu Phe Glu Arg
 405 410 415
 Glu Glu Thr Gln Asp Lys Glu Glu Ser Val Glu Ser Ser Leu Pro Leu
 420 425 430
 Asn Ala Ile Glu Pro Cys Val Ile Cys Gln Gly Arg Pro Lys Asn Gly
 435 440 445
 Cys Ile Val His Gly Lys Thr Gly His Leu Met Ala Cys Phe Thr Cys
 450 455 460
 Ala Lys Lys Leu Lys Lys Arg Asn Lys Pro Cys Pro Val Cys Arg Gln
 465 470 475 480
 Pro Ile Gln Met Ile Val Leu Thr Tyr Phe Pro
 485 490

<210> 11
 <211> 513
 <212> DNA
 <213> Homo sapiens

<400> 11
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 ggatacagct ggagtcagtt tagtgatgtg gaagagaaca ggactgaggc cccagaaggg 120
 actgaatcgg agatggagac cccagtgcc atcaatggca acccatcctg gcacctggca 180

SEQLIST

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gacagcccccg cggatgaatgg agccactggc cacagcagca gtttggatgc ccgggaggtg 240
atcccccattg cagcagtaaa gcaagcgctg agggaggcag gcgacgagtt tgaactgcgg 300
taccggcggg cattcagtga cctgacatcc cagctccaca tcaccccagg gacagcatat 360
cagagctttt aacaggatac ttttgtggaa ctctatggga acaatgcagc agccgagagc 420
cgaaagggcc aggaacgctt caaccgctgg ttcttgacgg gcatgactgt ggccggcgtg 480
gttctgctgg gctcactctt cagtcggaat tga
513

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<210> 12
 <211> 233
 <212> PRT
 <213> Homo sapiens

<400> 12

Met	Ser	Gln	Ser	Asn	Arg	Glu	Leu	Val	Val	Asp	Phe	Leu	Ser	Tyr	Lys
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Leu	Ser	Gln	Lys	Gly	Tyr	Ser	Trp	Ser	Gln	Phe	Ser	Asp	Val	Glu	Glu
		20					25					30			
Asn	Arg	Thr	Glu	Ala	Pro	Glu	Gly	Thr	Glu	Ser	Glu	Met	Glu	Thr	Pro
		35					40					45			
Ser	Ala	Ile	Asn	Gly	Asn	Pro	Ser	Trp	His	Leu	Ala	Asp	Ser	Pro	Ala
	50					55					60				
Val	Asn	Gly	Ala	Thr	Gly	His	Ser	Ser	Ser	Leu	Asp	Ala	Arg	Glu	Val
65					70					75				80	
Ile	Pro	Met	Ala	Ala	Val	Lys	Gln	Ala	Leu	Arg	Glu	Ala	Gly	Asp	Glu
			85						90					95	
Phe	Glu	Leu	Arg	Tyr	Arg	Arg	Ala	Phe	Ser	Asp	Leu	Thr	Ser	Gln	Leu
			100					105					110		
His	Ile	Thr	Pro	Gly	Thr	Ala	Tyr	Gln	Ser	Phe	Glu	Gln	Val	Val	Asn
		115					120					125			
Glu	Leu	Phe	Arg	Asp	Gly	Val	Asn	Trp	Gly	Arg	Ile	Val	Ala	Phe	Phe
	130					135					140				
Ser	Phe	Gly	Gly	Ala	Leu	Cys	Val	Glu	Ser	Val	Asp	Lys	Glu	Met	Gln
145					150					155				160	
Val	Leu	Val	Ser	Arg	Ile	Ala	Ala	Trp	Met	Ala	Thr	Tyr	Leu	Asn	Asp
				165					170					175	
His	Leu	Glu	Pro	Trp	Ile	Gln	Glu	Asn	Gly	Gly	Trp	Asp	Thr	Phe	Val
			180					185					190		
Glu	Leu	Tyr	Gly	Asn	Asn	Ala	Ala	Ala	Glu	Ser	Arg	Lys	Gly	Gln	Glu
		195				200						205			
Arg	Phe	Asn	Arg	Trp	Phe	Leu	Thr	Gly	Met	Thr	Val	Ala	Gly	Val	Val
	210					215					220				
Leu	Leu	Gly	Ser	Leu	Phe	Ser	Arg	Lys							
225					230										