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(54) Title: HOST CELLS HAVING IMPROVED CELL SURVIVAL PROPERTIES AND METHODS TO GENERATE SUCH CELLS

(57) Abstract: The present invention relates to genetically engineered mammalian host cells comprising an enhanced level of active anti-apoptosis genes and methods to generate such host cells. More particularly, the invention pertains to methods which modulate the level of anti-apoptosis active genes within host cells and to host cells showing an enhanced cell viability by delaying/inhibiting programmed cell death naturally occurring in such cells. The present invention also provides new anti-apoptosis genes suitable fro the preparing host cells showing an enhanced cell viability by delaying/inhibiting programmed cell death naturally occurring in such cells.

HOST CELLS HAVING IMPROVED CELL SURVIVAL PROPERTIES AND METHODS TO GENERATE SUCH CELLS

FIELD OF THE INVENTION

The present invention relates to genetically engineered mammalian host cells comprising an enhanced level of active anti-apoptosis genes and methods to generate such host cells. More particularly, the invention pertains to methods which modulate the level of anti-apoptosis active genes within host cells and to host cells showing an enhanced cell viability by delaying/ inhibiting programmed cell death naturally occurring in such cells.

BACKGROUND OF THE INVENTION

Mammalian cells are the preferred host for the production of most complex protein therapeutics, as functionally and pharmacokinetically relevant post-translational modifications are highly human-compatible. Commercially relevant cell types include hybridomas, myelomas, chinese hamster ovary (CHO) cells and baby hamster kidney (BHK) cells. CHO derivatives are being increasingly used in industry due to their straightforward adaptation for growth in serum-/ protein-free media and their consideration as safe production hosts by key regulatory agencies.

During the standard biopharmaceutically production processes - bioreactor operations - a substantial percentage of the production cell population die following a genetically determined program known as apoptosis (Al-Rubeai et al., 1998; Goswami et al., 1999; Lakenet al., 2001). Apoptosis is known as an active cellular suicide program activated as a result of either extrinsic or intrinsic signals, such as serum deprivation, nutrient limitation, oxygen limitation and mechanical stress. Apoptosis is characterized by plasma membrane blebbing, cell volume loss, nuclear condensation, and endonuleolytic degradation of DNA at nucleosomal intervals (Wyllie et al., 1980).

Serum components have been identified as major effective apoptosis-protective agents. However, there is a strong drive within the biotechnology industry, also pushed by the key regulatory agencies for drug registration, towards the development of serum-free and protein-free manufacturing processes. The main reasons are reduction of costs, less interference during

protein purification, and reduction of the potential for introduction of pathogens, such as prions or viruses. However, omission of serum often leads to an increased sensitivity of production cell lines to programmed cell death, making cells more vulnerable to cultural insults (Franek et al., 1991; Goswami et al., 1999; Moore et al., 1995; Zanghi et al., 1999). Premature cell death, e.g. by apoptosis, represents a high loss of valuable resources, because protein production is primarily a function of the viable producing cell population. The longer a high density of viable and productive cells in culture can be maintained, the more effective and profitable is the production process due to higher product yields per production run. In the absence of serum the cells die more rapidly at reaching the stationary growth phase at maximal cell density, thus cutting the production phase short. Yields are even further reduced by product degradation by proteases released from apoptotic cells and interference during the harvest caused by high numbers of cell debris.

A variety of physiological engineering strategies have been initiated to solve the dilemma of the increased apoptosis sensitivity of production cells lines, particularly of those cell lines being adapted and completely cultivated under serum-free conditions. Some of those strategies are focused at the reduction/elimination of the induction of endogenous apoptosis-response programs under production-mimicking cell culture conditions. One approach is directed on alleviating nutrient deprivation by feeding (deZengotita et al., 2000; Franek et al., 1996; Mercille et al., 1994; Sanfeliu et al., 1999). Another method is focussed on the use of apoptosis-suppressing chemical additives for blocking key effectors of apoptosis response mechanisms (Mastrangelo et al., 1999; Sanfeliu et al., 2000; Simpson et al., 1998; Zanghi et al., 2000). An alternative strategy represents a genetic approach. It includes the manipulation of the cell itself using anti-apoptotic survival genes or engineered anti-apoptosis determinants derived from survival-maintaining regulatory networks or viruses (Cotter et al., 1995; Chung et al., 1998; Fussenegger et al., 2000; Mastrangelo et al., 1998, 2000a and 2000b; Mercille et al., 1999a and 1999b; Pan et al., 1998; Simpson et al., 1999; Terada et al., 1997; Tey et al., 2000a and 2000b).

Of the latter strategy bcl-2 and bcl-xL, two apoptosis suppressors and key members of a family of highly conserved pro- (for example bad, bak, bax, bok, bcl-xS, bik, bid, hrk, bim, blk, bcl-y) and anti-apoptotic (for example bcl-2, bcl-xL, blc-w, bfl-1, a1, mcl-1, boo, brag-1, nr-13, bhrf-1, ced 9, cdn-1, cdn-2, cdn-3) response regulator genes, have been postulated to be potent candidates for anti-apoptosis engineering in the biotech community.

Bcl-2 has been shown to modulate induction of caspase-9-dependent apoptosis pathway at the outer membrane of mitochondria in response to a molecular rheostat localized at the outer mitochondrial membrane consisting of homo- and heterodimerized pro- and anti-apoptotic BCL-2-type proteins. Biased expression towards a pro-apoptotic subfamily member by culture constraints and endogenous signals results in release of Apaf-1-caspase-9 complex and auto-activation of this cysteine-containing aspartate-specific protease correlating with dramatic cytochrome c efflux from mitochondria into the cytosol. Assembly of major parts of the apoptosis-controlling machinery in the outer mitochondrial membrane bring these cellular power stations, acting as stress sensors and executioners for maintenance of the apoptosis process, in the focus of anti-apoptosis engineering (Follstad et al., 2000; Green et al., 1998; Tsujimoto, 1998).

There are some reports on the positive effect of expression of a heterologous anti-apoptosis bcl-2 gene on survival/viability and robustness of engineered cell lines, adapted on and cultivated in presence of calf serum. Those cells include myelomas, hybridomas, baby hamster kidney cells (BHK), and chinese hamster ovary cells (CHO). The positive effect of BCL-2 expression has been described in connection with various environmental insults like serum and glucose deprivation, growth factor withdrawal or viral infections (Fassnacht et al., 1998; Figueroa et al., 2001; Fussenegger et al., 2000; Itoh et al., 1995; Mastrangelo et al., 2000a and 2000b; Reynolds et al., 1996; Simpson et al., 1997, 1998 and 1999; Tey et al., 2000a and 2000b)

BCL-xL-based metabolic engineering has been described for human T cells and $CD34^+$ hematopoietic cells, respectively (US patent No. 6,143,291, WO 00/75291). Fussenegger et al. al., 1998 and also Mastrangelo et al., 2000a and 2000b have described a positive effect of BCL-xL expression on survival of hamster cells.

However, in all those reports either non-production cell lines such as human T cells and $CD34^+$ hematopoietic cells were used, or if production cell lines, e.g. hamster or mice cell lines, were used, they were adapted and routinely grown in the presence of serum and in most cases even as adherent cells. Studies on the effect of serum withdrawal on the survival of recombinant cells expressing heterologous BCL-2 or BCL-xL did not include cultivation for several passages in serum-free medium. Rather, serum-cultivated cells were either seeded into completely serum-

free medium or medium with reduced serum content and the effect of this cultural insult on cell survival in batch culture was followed for several days. This way the actual onset of cultural insult caused by serum withdrawal might also be delayed due to still ongoing intracellular reactions triggered by apoptosis-protective agents within the serum.

Only few experiments have been performed on production relevant recombinant cell lines adapted on and constantly grown in suspension in serum-free medium. These conditions are most favored in biotechnology industry nowadays, but at the same time cells are also more fragile and less robust. In the publication of Goswami et al., 1999, experiments are described in which CHO cells expressing γ-interferon were cultivated under those conditions. Survival of cultures expressing in addition heterologous BCL-2 were improved but limited to about 40% viable cells after day 7 in a batch culture. Nothing is known about the effect of BCL-xL expression on viability/survival of host cells, adapted and permanently grown under serum-free conditions.

From the above discussion, it is apparent that there is a need in the biotech community for further increasing cell viability, especially of cell lines adapted to serum free growth and qualified for serum free production of biopharmaceuticals for therapeutic and diagnostic use. The extension of cell survival at high viabilities as the cells reach the stationary phase in batch cultures would significantly influence productivity and cost-effectiveness, whereby every additional day gained during the production phase would make a great contribution.

In the present invention strategies are provided to further improve the survival of production cell lines grown constantly in suspension in serum-free medium. The strategies are based on engineering host cells in order to improve the intracellular-level of anti-apoptotic acting polypeptides. It has been surprisingly found, that the level of intracellular anti-apoptotic acting genes can substantially improve cell viability without showing any negative effect on cell productivity.

SUMMARY OF THE INVENTION

It has been demonstrated, that the expression of a non-amplified heterologous introduced anti-apoptosis gene, e.g. BCL-2 or BCL-xL has only a minor effect on the viability/survival of cells

adapted and generally grown under serum- and/or protein-free condition (see Figure 5). This finding is supported by the publication of Goswami et al., 1999. The present invention describes/provides now a new approach to dramatically improve the viability/survival of host cells, particularly when established and cultivated under serum-free conditions. Apoptosis delay/inhibition is achieved by improving the intracellular level of anti-apoptotic acting proteins, e.g. BCL-xL or BCL-2, using amplification-mediated over-expression. It has been surprisingly found by the present invention, that an enormous over-expression of an anti-apoptosis protein such as BCL-xL is a more than suitable tool to further improve cell viability/survival, without negatively affecting the expression level of a desired protein. On the contrary, cell productivity in BCL-xL over-expressing cells is enhanced (for example see Figure 2 and 3). Therefore, the present invention provides a person skilled in the art with new genetically modified host cells, in particular hamster or murine production cell lines, and also with methods to generate such inventive cell lines. This finding is highly advantageous for the production of biopharmaceutical peptides/proteins as now the economic viability of production processes can be improved in a very sufficient manner. A great advantage of the present invention over the art consists in that methods are provided which connect improvement of cell viability, a limiting factor in protein production processes, with the high yield protein production.

In a first aspect of the present invention, there is provided a hamster host cell genetically modified by introducing nucleic acid sequences that encode for an anti-apoptosis gene, a selectable amplifiable marker gene, and at least one gene of interest.

In a second aspect of the present invention, there is provided a murine myeloma cell genetically modified by introducing nucleic acid sequences that encode for an anti-apoptosis gene, a selectable amplifiable marker gene, and at least one gene of interest.

In a third aspect of the present invention, there is provided a host cell as defined in the second aspect, wherein the host cell is a NS0 or SP2/0-Ag14 cell, or the progeny of any of such cell line.

An embodiment may be genetically engineered host cells having improved survival properties and being highly suitable for the production of biopharmaceutical proteins. The improved survival properties are based on an enhanced level of active anti-apoptosis genes within the cells. Preferred are host cells of mammalian origin, more preferred murine hybridoma/myelomas or hamster cells, especially if adapted and constantly grown in serum-free and/or protein-free media.

In a fourth aspect of the present invention there is provided a method of generating mammalian host cells showing such an enhanced expression level of an anti-apoptosis gene, comprising (*i.*) introducing into a mammalian cell population nucleic acid sequences that encode for an anti-apoptosis gene, a selectable amplifiable marker gene, and optionally at least one gene of interest, wherein said genes are operatively linked to at least one regulatory sequence allowing for expression of said genes, (*ii.*) cultivating said cell population under conditions where at least said selectable amplifiable marker gene and said anti-apoptosis gene are expressed, and which are favourable

for obtaining multiple copies at least of the anti-apoptosis gene, and (*iii.*) selecting cells from the cell population that incorporate multiple copies at least of the anti-apoptosis gene.

In a fifth aspect of the present invention, there is provided a method of expressing an anti-apoptosis gene, an selectable amplifiable marker gene and at least one gene of interest in a mammalian host cell, comprising (*a.*) introducing into a hamster host cell population the nucleic acid sequences that encode for an anti-apoptosis gene, a selectable amplifiable marker gene, and a gene (s) of interest, wherein said genes are operatively linked to at least one regulatory sequence allowing for expression of said genes, and (*b.*) cultivating said host cell population under conditions wherein said genes are expressed.

In a sixth aspect of the present invention, there is provided a method of generating a mammalian host cell comprising: (a) introducing into a mammalian host cell population an anti-apoptosis gene and the DHFR gene; (b) amplifying the anti-apoptosis gene in the presence of methotrexate.

In a seventh aspect of the present invention, there is provided host cells obtainable by a method as defined above.

In an eighth aspect of the present invention, there is provided a method for inhibiting or delaying cell death in a host cell, comprising cultivating host cells as defined above under conditions where at least the anti-apoptosis gene is expressed in such that cell death is inhibited or delayed in said host cells.
Manipulation of host cells by any of these methods provides host cells comprising at least a high copy number of an anti-apoptosis gene.

In a ninth aspect of the present invention there is provided a process for producing a protein of interest in a host cell comprising (*a.*) cultivating cells as defined above under conditions which are favourable for the expression of the anti-apoptosis gene and the gene(s) of interest and (*b.*) isolating the protein of interest from the cells and/or the cell culture supernatant.

In a tenth aspect of the present invention, there is provided a use of a cell as defined above for production of at least one protein encoded by a gene of interest.
In an eleventh aspect of the present invention, there is provided a host cell comprising at least 5 copies of a heterologous anti-apoptosis gene.

In a twelfth aspect of the present invention, there is provided a host cell comprising at least 10 copies of a heterologous anti-apoptosis gene.

In a thirteenth aspect of the present invention, there is provided a host cell comprising at least 20 copies of a heterologous anti-apoptosis gene.

In a fourteenth aspect of the present invention, there is provided a host cell comprising at least 50 copies of a heterologous anti-apoptosis gene.

In a fifteenth aspect of the present invention, there is provided a host cell comprising at least 100 copies of a heterologous anti-apoptosis gene.

In a sixteenth aspect of the present invention, there is provided a DNA comprising a nucleic acid sequence encoding a biologically active bcl-xL gene, wherein the nucleic acid is: (a) a nucleic acid having the sequence of SEQ ID NO:3, or the complementary strand thereof; (b) functional fragments, variants or mutants (degenerative and non degenerative) of the nucleic acid sequence defined in (a); (c) a nucleic acid having at least 95% homology to the nucleic acid sequence defined in (a); or (d) a nucleic acid which hybridizes to any of the nucleic acid sequences defined in (a), (b), or (c) under stringent conditions.

In a seventeenth aspect of the present invention, there is provided a DNA comprising a nucleic acid sequence encoding a biologically active bcl-xL gene, wherein the nucleic acid is: (a) a nucleic acid having the sequence of SEQ ID NO:5, SEQID NO:7, SEQ ID NO:9, SEQ ID NO:11, or the complementary strand of any of those; (b) functional variants or mutants (degenerative and non degenerative) of any of the nucleic acid sequences defined in (a); (c) a nucleic acid having at least 95% homology to any of the nucleic acid sequences defined in (a); or (d) a nucleic acid which hybridizes to any of the nucleic acid sequences defined in (a), (b), or (c) under stringent conditions.

In an eighteenth aspect of the present invention, there is provided a DNA comprising a nucleic acid sequence encoding a biologically active bcl-xL gene having the sequence of SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9 or SEQ ID NO:11.

In a nineteenth aspect of the present invention, there is provided a polypeptide encoded by a DNA as defined in the sixteenth to eighteenth aspects.

In a twentieth aspect of the present invention, there is provided a host cell comprising a DNA as defined in the sixteenth to eighteenth aspects.

Finally, the present invention also provides a person skilled in the art with a new anti-apoptosis gene originally isolated from hamster (*Cricetulus griseus*), and also with several mutants of that gene. Both, wild type and modified gene(s) can be used in any of the inventive processes, described herein, to improve cell viability and cell productivity. Furthermore, the present invention is also directed to the proteins, which are encoded by any of these new anti-apoptosis genes.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 schematically shows the expression vector designs used for the transfection of CHO-DG44 cells. P/E means a composite unit that contains both enhancer and promoter element, P a promoter element and T a transcription termination site required for polyadenylation of transcribed messenger RNA. sICAM refers to the gene of interest and bcl-2 or bcl-xl to the anti-

apoptosis genes. dhfr refers to the amplifiable selectable marker dihydrofolate reductase. An arrow indicates the site of transcription initiation within a transcription unit.

Figure 2 compares the expression profiles of transiently transfected CHO-DG44 cells 48 hours post transfection. In Figure 2A pBID-sICAM, in Figure 2B pSEAP2-Control (Clontech, Palo Alto, CA) and in Figure 2C pGL2-Control (Promega, Madison, WI) was cotransfected with either pBID-bcl-2, pBID-bcl-xL or the control pBID.

Figure 3 shows sICAM expression profiles of stable mixed populations of CHO-DG44 cells cotransfected with pBID-sICAM and either pBID, pBID-bcl-2 or pBID-bcl-xL, selected and cultivated in hypoxanthine/thymidine free CHO-S-SFMII medium (Invitrogen, Carlsbad, CA). Each value resulted from an average sICAM production of six mixed populations during a 3 day culture period over 5 passages.

Figure 4 assesses the viability of clonal cell lines HMNI-1/2 (control cell lines cotransfected with pBID-sICAM and pBID), HMNIBC-1/2 (cotransfected with pBID-sICAM and pBID-bcl-2) and HMNIBX-1/2 (cotransfected with pBID-sICAM and pBID-bcl-xL) during a 7-day batch cultivation period using trypan blue dye exclusion. The recombinant CHO-DG44 cells were selected and cultivated in hypoxanthine/thymidine free CHO-S-SFMII medium (Invitrogen, Carlsbad, CA).

Figure 5 shows the percent viability profiles, using trypan blue dye exclusion, of methotrexate-amplified cell clones HMNI-3/4 (control cell lines cotransfected with pBID-sICAM and pBID), HMNIBC-3/4 (cotransfected with pBID-sICAM and pBID-bcl-2) and HMNIBX-3/4 (cotransfected with pBID-sICAM and pBID-bcl-xL) during a 9-day batch cultivation in hypoxanthine/thymidine free CHO-S-SFMII medium (Invitrogen, Carlsbad, CA) and the presence of 20 nM MTX.

Figure 6 shows the apoptosis characteristics of methotrexate-amplified CHO-DG44 cell clones expressing BCL-2 or BCL-xL. Percentage of apoptotic cells among HMNI-3/4 (control cell lines cotransfected with pBID-sICAM and pBID), HMNIBC-3/4 (cotransfected with pBID-sICAM and pBID-bcl-2) and HMNIBX-3/4 (cotransfected with pBID-sICAM and pBID-bcl-xL) batch cultures in hypoxanthine/thymidine free CHO-S-SFMII medium (Invitrogen, Carlsbad, CA) plus

20 nM MTX were assessed at days 2, 4 and 6 using a fluorescence-based TUNEL assay (BD Biosciences PharMingen, San Diego,CA).

Figure 7 shows the Western blot analysis of BCL-2 and BCL-xL in different recombinant CHO-DG44 cell clones. It compares BCL-2 (Fig. 7A) and BCL-xL (Fig. 7B) expression in either unamplified (BCL-2: HMNIBC-1/2; BCL-xL: HMNIBX-1/2) or amplified (BCL-2: HMNIBC-3/4; BCL-xL: HMNIBX-3/4) cell clones expressing the anti-apoptosis genes in monocistronic configuration. Anti-apoptosis gene expression was compared to the parental (CHO-DG44) and sICAM-only producing (HMNI-1) control cell lines. Proteins were detected using mouse monoclonal antibodies from Santa Cruz Biotechnology (Santa Cruz, CA) specific for BCL-2 or BCL-xL and an anti-mouse peroxidase-coupled secondary antibody (Dianova GmbH, Hamburg, Germany).

Figure 8 shows the MitoTracker-based quantification (Molecular Probes, Eugene, OR) of the mitochondria content in CHO-DG44 cells grown in the presence and absence of serum. FACS-mediated mitochondria counts were performed on CHO-DG44 cells cultivated in the presence (grey line) of 10% serum and in the absence (black line) of serum.

Figure 9 shows the nucleotide sequence and predicted open reading frame of new hamster bcl-xL gene (SEQ ID NOS: 3 and 4). The nucleotide sequence represents a composite sequence whereby the 5′ and 3′ untranslated region have been obtained from genomic clones and the coding region from a cDNA clone.

Figure 10 schematically shows the eukaryotic expression vector design. P/E means a composite unit that contains both enhancer and promoter element, P a promoter element and T a transcription termination site required for polyadenylation of transcribed messenger RNA. bcl-xL refers to the hamster anti-apoptosis bcl-xL cDNA and dhfr to the amplifiable selectable marker dihydrofolate reductase. An arrow indicates the site of transcription initiation within a transcription unit.

Figure 11 shows schematically the general cloning strategy used for the generation of the hamster bcl-xL deletion mutants. The expression vector pBID/bcl-xL, encoding the hamster bcl-xL wildtype cDNA, served as template in the PCRs. Using a combination of vector specific

primer 1 (VSP1) and gene specific primer del26, del46 or del66 the various 5'ends of the deletion mutants were generated. For subcloning the PCR products (black arrows) were digested with NotI, for which a restriction enzyme site was newly introduced by the gene specific primers, and SnaBI, located within the promoter/enhancer (P/E) region. For the generation of the various 3'ends of the deletion mutants a combination of vector specific primer 2 (VSP2) and gene specific primers del63 or del83 were used and the resulting PCR products (arrows with broken lines) were digested with NotI, located within the sequence of the gene specific primer, and EcoRI, located upstream of the terminator region (T). For the construction of the expression vectors containing the deletion mutants of the hamster bcl-xL cDNA (pBID/bcl-xL del) a 5' and a 3' cDNA fragment were cloned directionally into the SnaBI/EcoRI digested vector pBID.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides host cells which are genetically modified by introducing nucleic acid sequences that encode for an anti-apoptotic gene, a selectable amplifiable marker gene, and at least one gene of interest. In a preferred embodiment the anti-apoptosis gene, the selectable amplifiable marker gene and the gene(s) of interest are operatively linked to at least one regulatory sequence allowing for expression of said genes. In a more preferred embodiment the anti-apoptosis, the selectable amplifiable marker gene and the gene(s) of interest are operatively linked to only one promotor sequence allowing for co-expression of said genes. In another preferred embodiment the anti-apoptosis, and the selectable amplifiable marker gene are operatively linked to only one promotor sequence allowing for co-expression of said genes. In a further embodiment, the genetic modification of host cells includes the introduction of more than one anti-apoptosis gene.

Host cells modified in this manner allow co-expression of the anti-apoptosis gene(s) together with an selectable amplifiable marker, and optionally with a gene of interest. The selectable amplifiable marker not only enables selection of stable transfected host cell clones but also amplification of the anti-apoptosis gene(s). It is shown by the current invention that amplification of an anti-apoptosis gene provides a more efficient method for achieving intracellular levels of anti-apoptotic acting proteins, which are sufficient to improve survival of the host cells compared to host cells without any amplified anti-apoptosis protein encoding

sequence.

Genetically modified host cells according to this invention are characterized by an enhanced cell survival attributed to an delayed or inhibited programmed cell death, e.g. apoptosis, within the host cells. It has been surprisingly found that a population of host cells modified by any method of this invention are being cultivable for at least 9 days, wherein the total viability of cells is at least about 50% (see also Figure 5). After a 8-day batch cultivation viability of at least about 60% is achievable by host cells generated according to this invention. In a further embodiment of this invention, cell viability of at least about 75% is obtainable for a 7-day batch cultivation. In another embodiment, after a 6-day cultivation period cell viability of at least about 85% can be achieved. Equivalent levels of viability are not known in the art, at least not for serum free cultivation, especially for cells which are permentely cultivated under serum free conditions, and/or in connection with serum free production of biopharmaceuticals. Viability described in the art is limited to 40% of cells after a 7-day batch cultivation (Goswami et al., 1999). Therefore, host cells characterized accordingly are within the meaning of the present invention.

"Cell viability" or "cell survival" is the ability of a target cell to continue to remain alive and functional, and include the protection of the cell from cell death due to inhibition or delay of apoptosis or natural cell death. Ways of measuring cell viability or survivability are well known in the art. For example, cell viability can be determined by phase contrast microscopy, fluorescence microscopy or flow cytometry using non-cell permeable dyes such as trypan blue, propidium iodide or a combination of propidium iodide/acridine orange. Those methods are exemplary described in Current Protocols in Cytometry, John Wiley & Sons, Inc., updated, incorporated by reference.

The term „permentely cultivated under serum free conditions" means that not only cell cultivation is performed under serum free conditions, but also cell transformation, cell expansion by multiplying the number of cells and also cell storage, e.g. in fluid nitrogen.

Therefore, the present invention also concerns methods for inhibiting or delaying cell death in a host cell, comprising cultivating host cells of this invention, e.g. host cells as described above, under condition where at least the anti-apoptosis gene is expressed in such that cell death is

inhibited or delayed in said host cells. In a preferred embodiment, cell death of said cells is caused by programmed cell death, more preferably by apoptosis.

The present invention also provides methods of generating mammalian host cells showing an enhanced expression level of an anti-apoptosis gene comprising, (*i.*) introducing into a mammalian cell population nucleic acid sequences that encode for an anti-apoptosis gene, a selectable amplifiable marker gene, and optionally at least one gene of interest, wherein said genes are operatively linked to at least one regulatory sequence allowing for expression of said genes, (*ii.*) cultivating said cell population under conditions where at least said selectable amplifiable marker gene and said anti-apoptosis gene are expressed, and which are favorable for obtaining multiple copies at least of the anti-apoptosis gene, and (*iii.*) selecting cells from the cell population that incorporate multiple copies at least of the anti-apoptosis gene. It is preferred to place at least the anti-apoptosis and the selectable amplifiable marker gene in close spatial proximity to allow for a more effective amplification of the anti-apoptosis gene. Therefore in preferred embodiment of method described above, at least the anti-apoptosis and the selectable amplifiable are encoded by the same DNA molecule ,e.g. if both genes are placed on the same expression vector, and will therefore be commonly introduced into the host cell. In a more preferred embodiment of this method the expression of the anti-apoptosis gene and of the selectable amplifiable marker gene are operatively linked to each other, e.g. by using a common promotor to allow for co-expression of said genes.

In a further preferred embodiment the anti-apoptosis gene, the selectable amplifiable marker gene and the gene(s) of interest are encoded by only one DNA molecule, e.g. if all of these genes are placed on the same expression vector, and will be commonly introduced in said host cell. In a more preferred embodiment, the anti-apoptosis, the selectable amplifiable marker gene and the gene(s) of interest are not only encoded by one DNA-molecule but also are operatively linked to only one promotor sequence to allow for co-expression of said genes.

Host cells genetically modified by any method according to this invention show an increased expression of an anti-apoptosis protein compared to non-transfected as well as non-amplified parental cells. A "parental cell" means a cell that does not show enhanced expression of the anti-apoptosis gene, and is generally grown under the same or substantially the same conditions as the genetically modified cell according to the present invention, e.g. modified by introducing and

amplifying the nucleic acid sequences that encode for the anti-apoptosis gene(s), a selectable amplifiable marker gene, and optionally at least one gene of interest. "Increased expression" of the anti-apoptosis protein means for example an over-expression, that is at least 30-fold increased, preferably at least 50-fold increased, even more preferably at least 100-fold increased compared to the expression-level of that protein in host cells which are not modified according to any method of this invention, e.g modified by introducing and amplifying the nucleic acid sequences that encode for the anti-apoptosis gene(s), a selectable amplifiable marker gene, and optionally at least one gene of interest.

Therefore, this invention also provides host cells, that are genetically modified by any method described herein and showing an over-expression of an anti-apoptosis gene that is at least about 30-fold increased compared to the expression-level of said gene in host cells which are not modified according to any method of this invention, are provided by this invention. In a further embodiment, the present invention also concerns to host cells showing a level of over-expression which is at least 50-fold increased, compared to cells not modified in the meaning of this invention. In another embodiment host cells are provided, showing a level of over-expression that is at least about 100-fold increased compared to the expression-level of cells which are not modified in any way disclosed by this invention. Expression-level achievable by host cells generated by a method of the present invention are described examplary in Figure 7. Expression levels of an anti-apoptosis gene can be further increased by applying the principles of this invention on the modification procedure of host cells, e.g. by further increasing the copy number of the anti-apoptosis gene. Also conceivable is the introduction of multiple copies of one or more anti-apoptosis genes into a host cell.

"Host cells" or "target cells" in the meaning of the present invention are hamster cells, preferably BHK21, BHK TK$^-$, CHO, CHO-K1, CHO-DUKX, CHO-DUKX B1, and CHO-DG44 cells or the derivatives/progenies of any of such cell line. Particularly preferred are CHO-DG44, CHO-DUKX, CHO-K1 and BHK21, and even more preferred CHO-DG44 and CHO-DUKX cells. In a further embodiment of the present invention host cells also mean murine myeloma cells, preferably NS0 and Sp2/0 cells or the derivatives/progenies of any of such cell line. Examples of murine and hamster cells which can be used in the meaning of this invention are also summarized in Table 1. However, derivatives/progenies of those cells, other mammalian cells, including but not limited to human, mice, rat, monkey, and rodent cell lines, or eukaryotic cells,

including but not limited to yeast, insect and plant cells, can also be used in the meaning of this invention, particularly for the production of biopharmaceutical proteins.

TABLE 1: Hamster and murine production cell lines

Cell line	Order Number
NS0	ECACC No. 85110503
Sp2/0-Ag14	ATCC CRL-1581
BHK21	ATCC CCL-10
BHK TK$^-$	ECACC No. 85011423
HaK	ATCC CCL-15
2254-62.2 (BHK-21 derivative)	ATCC CRL-8544
CHO	ECACC No. 8505302
CHO-K1	ATCC CCL-61
CHO-DUKX (= CHO duk$^-$, CHO/dhfr$^-$)	ATCC CRL-9096
CHO-DUKX B1	ATCC CRL-9010
CHO-DG44	Urlaub et al., Cell 33[2], 405-412, 1983
CHO Pro-5	ATCC CRL-1781
V79	ATCC CCC-93
B14AF28-G3	ATCC CCL-14
CHL	ECACC No. 87111906

Host cells are most preferred, when being established, adapted, and completely cultivated under serum free conditions, and optionally in media which are free of any protein/peptide of animal origin. Commercially available media such as Ham's F12 (Sigma, Deisenhofen, Germany), RPMI-1640 (Sigma), Dulbecco's Modified Eagle's Medium (DMEM; Sigma), Minimal Essential Medium (MEM; Sigma), Iscove's Modified Dulbecco's Medium (IMDM; Sigma), CD-CHO (Invitrogen, Carlsbad, CA), CHO-S-Invtirogen), serum-free CHO Medium (Sigma), and protein-free CHO Medium (Sigma) are exemplary appropriate nutrient solutions. Any of the media may be supplemented as necessary with a variety of compounds examples of which are hormones and/or other growth factors (such as insulin, transferrin, epidermal growth factor, insulin like growth factor), salts (such as sodium chloride, calcium, magnesium, phosphate), buffers (such as HEPES), nucleosides (such as adenosine, thymidine), glutamine, glucose or other equivalent energy sources, antibiotics, trace elements. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. In the present invention the use of serum-free medium is preferred, but media supplemented with a suitable amount of serum can also be used for the cultivation of host cells. For the growth

and selection of genetically modified cells expressing the selectable gene a suitable selection agent is added to the culture medium.

The present invention also concerns high-level expression of an anti-apoptosis gene of the bcl-2 superfamily in order to improve the viability of the host cells, preferably of production cells as described above. Genes encoding for the bcl-2 superfamily are therefore suitable to generate host cells according to this invention. Thus host cells genetically modified by introducing nucleic acid sequences that encode for a gene of the bcl-2 superfamily, a selectable amplifiable marker gene, and at least one gene of interest are within the meaning of the present invention. In Table 2 examples of members belonging to the bcl-2 superfamily, that inhibit or delay programmed cell death or apoptosis, are listed. Most proteins of this family of mammalian, C. elegans or viral origin have two to four regions with extensive amino acid sequence similarity with BCL-2 (BCL-2 homology regions BH1-BH4), the prototypical inhibitor of apoptosis. All these are suitable candidates of anti-apoptosis genes/proteins to carry out the present invention.

In a further embodiment of this invention cells are preferred, wherein survival is prolonged by introducing nucleic acid sequences encoding for BCL-xL, BCL-2, BCL-w, BFL-1, A1, MCL-1, BOO, BRAG-1, NR-13, CDN-1, CDN-2, CDN-3, BHRF-1, LMW5-HL or CED-9. In a more preferred embodiment, the nucleic acid sequence encoding the anti-apoptosis gene of the present invention is a DNA molecule from a vertebrate species. A preferred vertebrate is a mammal. More preferably, a nucleic acid of the present invention encodes polypeptides designated BCL-xL or BCL-2. The BCL-xL encoding gene is the most preferred. In a further preferred embodiment, the nucleic acid sequence encoding for BCL-xL is isolated from hamster, preferably from *Cricetulus griseus*. Also preferred is a nucleic acid having the sequence of SEQ ID NO:1 (identical with GenBank Accession Number Z23115),SEQ ID NO:2 (identical with GenBank Accession Number M13995), SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9 or SEQ ID NO:11, wherein SEQ ID NO:3 is originally isolated from hamster and SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, and SEQ ID NO:11 are modified variants of the nucleic acid sequence of SEQ ID NO: 3. Most preferred is a sequence of SEQ ID NO:1 or SEQ ID NO:3. Any homologs of any of these genes encoded from other vertebrate species are also suitable to carry out the present invention. Moreover, also preferred are nucleotide sequences encoding for any of the anti-apoptosis genes of Table 2, particularly having the sequence given by any of the Genbank Accession Numbers cited in Table 2. Examples for anti-apoptosis acting

gene are also described in US patents No. 6,303,331, No. 5,834,309 and No. 5,646,008 as well as in WO 95/006342, which are herein incorporated by reference. Reference is also made to Boise et al., Cell 74, 597 – 608, 1993. In WO 95/006342 isolation of the BCL-xL encoding sequence is exemplary described.

A nucleic acid sequence encoding for an anti-apoptosis protein is intended to include any nucleic acid sequence that will be transcribed and translated into an anti-apoptosis protein either *in vitro* or upon introduction of the encoding sequence into a target cell. The anti-apoptosis protein encoding sequences can be native (wild-type) genes as well as naturally occurring (by spontaneous mutation), or recombinantly engineered mutants and variants, truncated versions and fragments, functional equivalents, derivatives, homologs and fusions of the naturally occurring or wild-type proteins as long as the biological functional activity, meaning the anti-apoptotic function, of the encoded polypeptide is maintained and not substantially altered. A preferred encoded polypeptide has at least 50%, more preferred at least 80% and even more preferred at least 100% or more of functional biological activity compared to the corresponding wild-type anti-apoptosis protein. "Wild-type protein" means, a complete, non truncated, non modified, naturally occurring allele of the encoding polypeptide.

The "functional biological activity" of an anti-apoptosis polypeptide, for example of BCL-xL, can be determined by quantitative apoptosis assays on transfected cells expressing the particular polypeptide such as, for example, TUNEL assay, Annexin V assay, acridine orange/ethidum bromide staining or propidium iodide/acridine orange staining using fluorescence microscopy or flow cytometry analysis or other assays. Those assays are well known in the art and for example described in Current Protocols in Cytometry, John Wiley & Sons, Inc., updated).

The terms "functional biologically active polypeptide(s)" or "functional biologically active fragment(s)" refers to polypeptide(s) or fragment(s) having the functional biological activity of anti-apoptosis peptide. This means, that a functional biologically active polypeptide or a functional biologically active fragment has at least 50%, more preferred at least 80% and even more preferred at least 100% or more of the functional biological activity compared to the corresponding wild-type anti-apoptosis protein.

The modified nucleic acid sequences can be prepared using standard techniques well known to one of skill in the art, e.g. site-specific mutagenesis or polymerase chain reaction mediated mutagenesis. In the present invention, a particularly preferred bcl-xL nucleic acid sequence is the isolated polynucleotide of SEQ ID NO:1 (identical with GenBank Accession Number Z23115), SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9 or SEQ ID NO:11. The invention also includes functionally equivalent nucleic acid sequences to those sequences and to any other BCL-xL encoding sequences, including recombinantly engineered mutants and variants, truncated versions and fragments, functional equivalents, derivatives, homologs and fusions of those nucleic acids. In one preferred embodiment, the nucleic acid sequence encoding a BCL-xL protein is of human origin, in a more preferred embodiment it is of hamster origin.

In this context, a nucleic acid coding for a Bcl-xL homologous gene of hamster has been identified for the first time and is also provided by the present invention. The isolated hamster bcl-xl gene is a matter of nucleic acid having SEQ ID NO:3 which has been isolated and cloned from *Cricetulus griseus*. The use of an anti-apoptosis protein encoded by this sequence or any functional fragments, variants or mutants (degenerative and non degenerative) thereof, in a process prolonging cell survival by inhibition or delaying apoptosis is a preferred embodiment of the present invention. However, nucleic acid sequences encoding BCL-xL from other species are also encompassed in the meaning of this invention.

BCL-xL protein is encoded by a bcl-x gene. It is known from the human bcl-xL gene, that two different RNA molecules are produced, one of which codes for BCL-xL (long form) and one of which codes for BCL-xS (short form). The BCL-xS lacks a section of 63 amino acids found in the BCL-xL. BCL-xS has been shown to favor apoptosis, and therefore it is preferable to use a cDNA for expression of the BCL-xL rather than a genomic fragment. In another preferred embodiment a BCL-xL mutant, or a BCL-2 mutant, with improved anti-apoptosis properties is encompassed, e.g. by deleting a non-conserved region between the BH3 and BH4 conserved regions and thus increasing the protein stability of the mutant protein variants (Chang et al., 1997; Figueroa et al., 2001).

In a further preferred embodiment the present invention provides, modified variants of the hamster bcl-xL gene, especially of the hamster bcl-xL gene encoded by SEQ ID NO:3 as mentioned above. Such modified variants include but are not limited to nucleic acids molecules

having the sequence of SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9 or SEQ ID NO:11, or any functional variants or mutants (degenerative and non degenerative) thereof.

A "functional mutant" includes but is not limited to a DNA molecule having at least 95%, preferably 96%, more preferably 97%, even more preferably 98% and most preferably 99% homology to anyone of the nucleic acid sequences desribed above, preferably to anyone of the nucleic acids having the sequence of SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9 or SEQ ID NO:11. The mutation may be caused by insertions, substitutions and/or deletions of one or more nucleotides of the original nucleic acid sequence. The term "functional mutant" also includes any functional biologically active fragment, deletion or insertion mutant of any of the above mentioned nucleic acid molecules, which have a homology of at least 95%, preferably of at least 96%, more preferably of at least 97%, even more preferably of at least 98% and most preferably of at least 99% to anyone of these molecules, or at least to functional fragments of these molecules. The term "functional mutant" also includes any functional biological active fragment, deletion or insertion mutant of any of the above mentioned nucleic acid molecules, wherein biological active part of those fragments shows at least 95%, preferably of at least 96%, more preferably of at least 97%, even more preferably of at least 98%, and most preferably of at least 99% homology to the biological active part of the bcl-xL gene of hamster.

A "functional fragment" also means a DNA molecule, which encodes for a functionally active portion of the hamster bcl-xL gene, especially of the nucleic acid having the sequence of SEQ ID NO:3. Examples of preferred functional fragments of a hamster bcl-xL gene are the nucleic acid molecules having the sequence of SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9 or SEQ ID NO:11.

"Functional variants" include nucleic acid molecules which hybridize under stringent conditions to a nucleic acid having any of the sequences defined above, e.g. having the sequence of SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9 or SEQ ID NO:11 and encoding for a functional biologically active bcl-xL gene of hamster. The term "variants" refers in general to an alternate form of a polynucleotide, which may have a substitution, deletion or addition of one or more nucleotides which does not substantially alter the function of the encoded polypeptide.

"Degenerative mutants" refer in general to DNA molecules having different nucleic acid sequences but encoding for polypeptides having the identical amino acid sequence. This means, that any nucleic acid which encodes for a polypeptide having the amino acid sequence which is encoded by any of the sequences of SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9 or SEQ ID NO:11 is a degenerative mutant thereof.

According to the above-described aspect, the present invention also provides a DNA comprising a nucleic acid sequence encoding a biologically active bcl-xL gene, wherein the nucleic acid is selected from: (a) nucleic acid having the sequence of SEQ ID NO:3, or the complementary strand thereof; (b) a functional fragment, variant or mutant (degenerative and non degenerative) of the nucleic acid sequence defined in (a); (c) a nucleic acid having at least 95% homology to the nucleic acid sequence defined in (a); and (d) a nucleic acid which hybridize to any of the nucleic acid sequences defined in (a), (b), or (c) under stringent conditions. The present invention further provides a DNA comprising a nucleic acid sequence encoding a biologically active bcl-xL gene, wherein the nucleic acid is selected from (a) a nucleic acid having the sequence of SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, or the complementary strand of any of those; (b) functional variants or mutants (degenerative and non degenerative) of any of the nucleic acid sequnces defined in (a); (c) a nucleic acid having at least 95% homology to any of the nucleic acid sequences defined in (a); and (d) a nucleic acid which hybridizes to any of the nucleic acid sequences defined in (a), (b), or (c) under stringent conditions. In a more preferred embodiment the present invention is also directed to a DNA comprising a nucleic acid sequence encoding a biologically active bcl-xL gene having the sequence of SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9 or SEQ ID NO:11.

The proteins encoded by anyone of those DNA sequences, mentioned above, result in a more or less stabilized form of hamster BCl-xL protein. The use of one or more of these hamster BCL-xL mutants in any inventive process which prolongs cell survival by inhibiting or delaying apoptosis, as described herein, is a highly preferred embodiment of the present invention. The present invention is therefore also directed to a polypeptide, which is encoded by any of the nucleic acids sequences described above which encodes for a functional biologically active bcl-xL gene.

TABLE 2: Anti-apoptosis genes of the bcl-2 superfamily

Polynucleotide	GenBank Accession No.	Polynucleotide	GenBank Accession No.
bcl-xL (human) bcl-xL (mouse) bcl-xL (rat) bcl-xL (chicken)	Z23115 L35049 U34963 Z23110	nr-13 (chicken)	AF120211
bcl-2 (human) bcl-2 (mouse) bcl-2 (rat) bcl-2 (chicken) bcl-2 (hamster)	M13995 M16506 L14680 Z11961 AJ271720	bhfr-1 (herpesvirus) bhfr-1 (Epstein-Barr virus)	AF120456 A22899
bcl-w (human) bcl-w (mouse) bcl-w (rat)	U59747 U59746 AF096291	lmw5-HL (African swine fever virus)	L09548
bfl-1 (human) A1 (mouse)	U27467 U23774	cdn-1 (human)	AAQ95492 (NAGENESEQ)
mcl-1 (human) mcl-1 (mouse) mcl-1 (rat) mcl-1 (chicken)	AF147742 U35623 AF115380 AF120210	cdn-2 (human)	AAQ95493 (NAGENESEQ)
boo (mouse)	AF102501	cdn-3 (human)	AAQ95494 (NAGENESEQ)
brag-1 (human)	S82185	ced-9 (C. elegans)	L26545

A "nucleic acid sequence" as used herein refers to an oligonucleotide, nucleotide or polynucleotide and fragments and portions thereof and to DNA or RNA of genomic or synthetic origin, which may be single or double stranded and represent the sense or antisense strand. The polynucleotides of the invention include nucleic acid regions wherein one or more codons have been replaced by their synonyms. Additionally included are polynucleotides which have a coding sequence which is a naturally occurring allelic variant of a coding sequence, for example, a variant of the coding sequence of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9 or SEQ ID NO:11. As known in the art, an allelic variant is an alternate form of a polynucleotide, which may have a substitution, deletion or addition of one or more nucleotides which does not substantially alter the function of the encoded polypeptide. The function of a polypeptide is in general determined by the functional biological activity of said encoding polypeptide. Therefore, not substantially alter the function of the encoded polypeptides means, that the level of functional biological activity of the encoded polypeptide is at least 50%, more preferred at least 80% and even more preferred at least 100% or more compared to the corresponding wild-type encoded polypeptide.

The term "encoding" refers to the inherent property of specific sequences of nucleotides in a nucleic acid, such as a gene in chromosome or a mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having a defined sequence of nucleotides (i.e. rRNA, tRNA, other RNA molecules) or amino acids and the biological properties resulting therefrom. Thus a gene encodes a protein, if transcription and translation of mRNA produced by that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence and is usually provided in sequence listings, and non-coding strand, used as the template for the transcription, of a gene or cDNA can be referred to as encoding the protein or other product of that gene or cDNA. A nucleic acid that encodes a protein includes any nucleic acids that have different nucleotide sequences but encode the same amino acid sequence of the protein due to the degeneracy of the genetic code. Nucleic acids and nucleotide sequences that encode proteins may include introns.

The term "cDNA" in the context of this invention refers to deoxyribonucleic acids produced by reverse transcription and typically second-strand synthesis of mRNA or other RNA produced by a gene. If double-stranded, a cDNA molecule has both a coding or sense and a non-coding or antisense strand.

In general as used herein upper case letters (e.g. BCL-xL) indicate polypeptides (e.g. products of gene expression) and lower case letters (e.g. bcl-xL) indicates polynucleotides (e.g. genes). Additionally, throughout this specification, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise.

The term "polypeptide" is used interchangeably with amino acid residue sequences or protein and refers to polymers of amino acids of any length. These terms also include proteins that are post-translationally modified through reactions that include, but are not limited to, glycosylation, acetylation, phosphorylation or protein processing. Modifications and changes, for example fusions to other proteins, amino acid sequence substitutions, deletions or insertions, can be made in the structure of a polypeptide while the molecule maintains its biological functional activity. For example certain amino acid sequence substitutions can be made in a polypeptide or its underlying nucleic acid coding sequence and a protein can be obtained with like properties. As

mentioned above a preferred protein is the BCL-xL protein sequence encoded by nucleic acid sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9 or SEQ ID NO:11 , or any functional fragment, variant or mutant (degenerative and non degenerative) thereof Amino acid substitutions that provide functionally equivalent BCL-xL polypeptides by use of the hydrophathic index of amino acids (Kyte et al., J. Mol. Biol. 157: 105-132, 1982) can be prepared by performing site-specific mutagenesis or polymerase chain reaction mediated mutagenesis on its underlying nucleic acid sequence.

As mentioned above, the present invention concerns genetically modified host cells and also methods for generating such host cells. In one embodiment, the host cells comprise heterologous introduced polynucleotide sequences that encode an anti-apoptosis gene, a selectable amplifiable marker gene and optionally at least one gene of interest. The "selectable amplifiable marker gene" has the properties of a selectable marker gene, but additionally allows amplification (i.e., generating additional copies of the gene which survive in intra-chromosomal or extra-chromosomal form) of the gene itself and also of genes adjacent to that selectable amplifiable gene when cells are cultured under appropriate conditions. The present invention particularly provides host cells, comprising not only one copy of an introduced anti-apoptosis gene but comprising multiple copies of said anti-apoptosis gene, e.g. more than 5, 10, 20, 50 or 100 copies of the introduced anti-apoptosis gene.

Such host cells are obtainable, for example, by the method comprising (*i.*) introducing into a mammalian cell population nucleic acid sequences that encode for an anti-apoptosis gene, a selectable amplifiable marker gene, and optionally at least one gene of interest, wherein said genes are operatively linked to at least one regulatory sequence allowing for expression of said genes, (*ii.*) cultivating said cell population under conditions where at least said selectable amplifiable marker gene and said anti-apoptosis gene are expressed, and which are favorable for obtaining multiple copies at least of the anti-apoptosis gene, and (*iii.*) selecting cells from the cell population that incorporate multiple copies at least of the anti-apoptotic gene. As mentioned above, it is preferred to place at least the anti-apoptosis and the selectable amplifiable marker gene in close spatial proximity to allow a more effective amplification of the anti-apoptosis gene. Therefore in preferred embodiment of method described herein, at least the anti-apoptosis and the selectable amplifiable are encoded by the same DNA molecule ,e.g. both genes are placed on the same expression vector, and will therefore be commonly introduced into the host cell. In a

more preferred embodiment of this method the expression of the anti-apoptosis gene and of the selectable amplifiable marker gene are operatively linked to each other, e.g. by using a common promotor allowing for co-expression of said genes.

Host cells generated in such a manner comprise for example at least 5 copies of the incorporated heterologous anti-apoptosis gene. In a further embodiment host cells comprise for example at least 10 copies of the incorporated heterologous anti-apoptosis gene. In another embodiment host cells are obtainable comprising at least 50 copies of the introduced heterologous anti-apoptosis gene. In further embodiment host cells comprise for example at least 100 copies of a incorporated heterologous anti-apoptotic gene. Appropriate anti-apoptosis genes are those mentioned above, in particular those listed in Table 2, e.g. those encoding for any of the BCL-xL polypeptides described herein.

In general, host cells are preferred by the invention comprising the anti-apoptosis gene in copy numbers allowing its expression at levels which are sufficiently high to enhance survival of the cell population by inhibiting or delaying programmed cell death. More preferred are host cells not only comprising the anti-apoptosis gene in copy numbers which allow to improve cell survival but also showing high level of expression of the gene of interest, also encoded by the genetically modified host cells. Methods to find out the highest-tolerable expression level for an anti-apoptosis gene having the maximum effect on reduction of apoptosis rate without leading to dramatically decreased cell growth rates or genetic instabilities and without a negative impact on the productivity with regard to the protein of interest are well known for a skilled person in the art. They include but are not limited to, for example, generating growth curves, monitoring the viability of cells by measuring exclusion of non-permeable dyes by microscopy or flow cytometry, quantifying apoptosis by TUNEL or Annexin V assays, determination of product titers by ELISA and evaluation of the genetic stability of the transfected genes over several passages by Southern and dot blot analysis or quantitative PCR.

In this invention, for example, a sufficiently enhanced cell survival is given, if the expression level of the anti-apopotsis gene, e.g. the bcl-xL gene, reaches intracellular survival-effective concentrations of the encoding polypeptide compared to the parental, non-amplified cell. A sufficient enhanced cell survival is provided for example, when the level for bcl-xL gene expression, or for any other anti-apoptosis gene results in a cell population having at least an

increase in survival of at least about 20% after a 6-, 7-, 8-, or 9-day cultivation compared to the parental cell population. More preferred is an increase in survival of at least about 30% and even more preferred an increase in survival of at least about 50% after a 6-, 7-, 8-, or 9-day cultivation compared to the parental cell population. Sufficient cell survival is also given, when at least about 50% of the cultivated cells are viable after a cultivation period of 9 days. If the anti-apoptosis gene encodes for BCl-xL, sufficient cell survival is also given, when at least about 60% of the cell are viable after 9-day cultivation. In another embodiment a sufficient enhanced cell survival is reached, when at least about 60% of the cell population is viable after 8 days. In a further embodiment, a sufficient enhanced cell survival is achieved, when at least about 75% of the cell population is viable after 7 days, furthermore if at least about 85% of the cells are viable after 6 days.

The "selectable amplifiable marker gene" usually encodes an enzyme which is required for growth of eukaryotic cells under those conditions. For example, the selectable amplifiable marker gene may encode DHFR which gene is amplified when a host cell transfected therewith is grown in the presence of the selective agent, methotrexate (MTX). The non-limited exemplary selectable genes in Table 3 are also amplifiable marker genes, which can be used to carry out the present invention. For a review of the selectable amplifiable marker genes listed in Table 3, see Kaufman, Methods in Enzymology, 185:537-566 (1990), incorporated by reference. Accordingly, host cells genetically modified according to any method described herein are encompassed by this invention, wherein the selectable amplifiable marker gene encodes for a polypeptide having the function of dihydrofolate reductase (DHFR), glutamine synthetase, CAD, adenosine deaminase, adenylate deaminase, UMP synthetase, IMP 5'-dehydrogenase, xanthine guanine phosphoribosyl transferase, HGPRTase, thymidine kinase, thymidylate synthetase, P glycoprotein 170, ribonucleotide reductase, asparagine synthetase, arginosuccinate synthetase, ornithine decarboxylase, HMG CoA reductase, acetylglucosaminyl transferase, threonyl-tRNA synthetase or Na^+K^+-ATPase.

A preferred selectable amplifiable marker gene is the gene encoding dihydrofolate reductase (DHFR) which is necessary for the biosynthesis of purines. Cells lacking the DHFR gene will not grow on medium lacking purines. The DHFR gene is therefore useful as a dominant selectable marker to select and amplify genes in such cells growing in medium lacking purines. The selection agent used in conjunction with a DHFR gene is methotrexate (MTX). The present

invention therefore includes a method of generating a mammalian host cell comprising (*i.*) introducing into a mammalian host cell population an anti-apoptosis gene, a DHFR encoding gene, and (*ii.*) amplifying the anti-apoptosis gene in the presence of methotrexate. In a more preferred embodiment of the present invention, the anti-apoptosis gene and the DHFR encoding gene are placed on the same DNA-molecule when introduced in the host cell. In a more preferred embodiment both genes are oberatively linked to each other and transcribed by only one common promotor. Accordingly, transfected cells are cultivable in a hypoxanthine/thymidine-free medium in the absence of serum and increasing amounts of MTX, starting with no MTX in the medium to MTX concentrations between 2 nM and 2000 nM, more typically between 2 nM and 1000 nM. The concentration of MTX is increased gradually by a factor between 2 and 10. In a preferred process, the anti-apoptosis gene additionally encodes for any one of the genes listed in Table 2. In a more preferred method the anti-apoptosis gene encodes for BCL-2 or BCL-xL, and in a even more preferred method for BCL-xL.

TABLE 3: Selectable amplifiable marker genes

Selectable Amplifiable Marker Gene	Accession Number	Selection Agent
Dihydrofolate reductase	M19869 (hamster) E00236 (mouse)	Methotrexate (MTX)
Metallothionein	D10551 (hamster) M13003 (human) M11794 (rat)	Cadmium
CAD (Carbamoyl-phosphate synthetase:Aspartate transcarbamylase: Dihydroorotase)	M23652 (hamster) D78586 (human)	N-Phosphoacetyl-L-aspartate
Adenosine deaminase	K02567 (human) M10319 (mouse)	Xyl-A- or adenosine, 2′deoxycoformycin
AMP (adenylate) deaminase	D12775 (human) J02811 (rat)	Adenine, azaserine, coformycin
UMP synthase	J03626 (human)	6-Azauridine, pyrazofuran
IMP 5′dehydrogenase	J04209 (hamster) J04208 (human) M33934 (mouse)	Mycophenolic acid
Xanthine-guanine phosphoribosyltransferase	X00221 (E. coli)	Mycophenolic acid with limiting xanthine
Mutant HGPRTase or mutant thymidine kinase	J00060 (hamster) M13542, K02581 (human) J00423, M68489(mouse) M63983 (rat)	Hypoxanthine, aminopterin, and thymidine (HAT)

	M36160 (herpesvirus)	
Thymidylate synthetase	D00596 (human) M13019 (mouse) L12138 (rat)	5-Fluorodeoxyuridine
P-glycoprotein 170 (MDR1)	AF016535 (human) J03398 (mouse)	Multiple drugs, e.g. adriamycin, vincristine, colchicine
Ribonucleotide reductase	M124223, K02927 (mouse)	Aphidicolin
Glutamine synthetase	AF150961 (hamster) U09114, M60803 (mouse) M29579 (rat)	Methionine sulfoximine (MSX)
Asparagine synthetase	M27838 (hamster) M27396 (human) U38940 (mouse) U07202 (rat)	β-Aspartyl hydroxamate, Albizziin, 5′Azacytidine
Argininosuccinate synthetase	X01630 (human) M31690 (mouse) M26198 (bovine)	Canavanine
Ornithine decarboxylase	M34158 (human) J03733 (mouse) M16982 (rat)	α-Difluoromethylornithine
HMG-CoA reductase	L00183, M12705 (hamster) M11058 (human)	Compactin
N-Acetylglucosaminyl transferase	M55621 (human)	Tunicamycin
Threonyl-tRNA synthetase	M63180 (human)	Borrelidin
Na^+K^+-ATPase	J05096 (human) M14511 (rat)	Ouabain

Suitable host cells for using a DHFR encoding gene as a selectable amplifiable marker are mammalian cells, preferably murine myeloma or hamster cells. More preferred are CHO-DUKX (ATCC CRL-9096) and CHO-DG44 (Urlaub et al., Cell 33[2], 405 – 412, 1983) cells which are deficient in DHFR activity. To extend the DHFR amplification method to other cell types, a mutant DHFR gene that encodes a protein with reduced sensitivity to methotrexate may be used in conjunction with host cells that contain normal numbers of an endogenous wild-type DHFR gene (Simonsonet al., 1983; Wigler et al., 1980; Haberet al., 1982).

Another embodiment of this invention also provides host cells which are genetically modified by introducing nucleic acid sequences that encode for an anti-apoptosis gene, a DHFR gene, and at least one gene of interest. In a further embodiment, the anti-apoptosis gene of said host cells encodes for any of the anti-apoptosis gene listed in Table 2, preferably for the bcl-xL gene, more preferably for the bcl-xL gene of human or hamster origin, even more preferably having the

sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9 or SEQ ID NO:11, or any functional fragment, variant or mutant (degenerative and non degenerative) thereof.

Host cells genetically modified by introducing nucleic acid sequences that encode for bcl-xL, the DHFR gene, and at least one gene of interest are most preferred by this invention and therefore subject of the present invention. However, in general host cells are within the meaning of the present invention comprising at least a heterologous anti-apoptosis gene, a selectable amplifiable marker gene and one gene of interest, wherein the anti-apoptosis gene is any gene of Table 2,or any other described herein and the selectable amplifiable marker gene is any of the genes listed in Table 3.

The term "selection agent" refers to a substance that interferes with the growth or survival of a host cell that is deficient in a particular selectable gene. For example, to select for the presence of an antibiotic resistance gene like APH (aminoglycoside phosphotransferase) in a transfected cell the antibiotic Geneticin (G418) is used. The selection agent can also comprise an "amplifying agent" which is defined for purposes herein as an agent for amplifying copies of the amplifiable gene if the selectable marker gene relied on is an amplifiable selectable marker. For example, MTX is a selection agent useful for the amplification of the DHFR gene. Examplary amplifying selection agents are but not limited to those listed in Table 3.

The present invention is suitable to generate host cells for the production of biopharmaceutical polypeptides/proteins. The invention is particularly suitable for the high-yield expression of a large number of different genes of interest by cells showing an enhanced cell viability and productivity. "Gene of interest", "selected sequence", or "product gene" have the same meaning herein and refer to a polynucleotide sequence of any length that encodes a product of interest, also mentioned by the term "desired product". The selected sequence can be full length or a truncated gene, a fusion or tagged gene, and can be a cDNA, a genomic DNA, or a DNA fragment, preferably, a cDNA. It can be the native sequence, i.e. naturally occurring form(s), or can be mutated or otherwise modified as desired. These modifications include codon optimizations to optimize codon usage in the selected host cell, humanization or tagging. The selected sequence can encode a secreted, cytoplasmic, nuclear, membrane bound or cell surface polypeptide. The "desired product" includes proteins, polypeptides, fragments thereof, peptides,

antisense RNA all of which can be expressed in the selected host cell. Desired proteins can be for example antibodies, enzymes, cytokines, lymphokines, adhesion molecules, receptors and derivatives or fragments thereof, and any other polypeptides that can serve as agonists or antagonists and/or have therapeutic or diagnostic use. Examples for a desired protein/polypeptide are also given below.

By definition any sequences or genes introduced into a host cell are called "heterologous sequences" or "heterologous genes" with respect to the host cell, even if the introduced sequence or gene is identical to an endogenous sequence or gene in the host cell. For example, a hamster bcl-xL gene, introduced into a hamster host cell, is by definition a heterologous gene.

Heterologous gene sequences can be introduced into a target cell, for example a nucleic acid sequence encoding BCL-xL, by using an "expression vector", preferably an eukaryotic, and even more preferably a mammalian expression vector. Methods used to construct vectors are well known to a person skilled in the art and described in various publications. In particular techniques for constructing suitable vectors, including a description of the functional components such as promoters, enhancers, termination and polyadenylation signals, selection markers, origins of replication, and splicing signals, are reviewed in considerable details in Sambrook et al., 1989 and references cited therein. Vectors may include but are not limited to plasmid vectors, phagemids, cosmids, articificial/mini-chromosomes, or viral vectors such as baculovirus, retrovirus, adenovirus, adeno-associated virus, herpes simplex virus, retroviruses, bacteriophages. The eukaryotic expression vectors will typically contain also prokaryotic sequences that facilitate the propagation of the vector in bacteria such as an origin of replication and antibiotic resistance genes for selection in bacteria. A variety of eukaryotic expression vectors, containing a cloning site into which a polynucleotide can be operatively linked, are well known in the art and some are commercially available from companies such as Stratagene, La Jolla, CA; Invitrogen, Carlsbad, CA; Promega, Madison, WI or BD Biosciences Clontech, Palo Alto, CA.

In a preferred embodiment the expression vector comprises at least one nucleic acid sequence which is a regulatory sequence necessary for transcription and translation of nucleotide sequences that encode for a peptide/polypeptide. In a more specific embodiment, the expression vector comprises at least one regulatory sequence allowing the transcription and translation

(expression) of the nucleotide sequences that encode for the anti-apoptosis gene, the selectable amplifiable maker gene, and a gene of interest.

"Regulatory sequences" include promoters, enhancers, termination and polyadenylation signals, and other expression control elements. Both inducible and constitutive regulatory sequences are known in the art to function in various cell types. Transcriptionally regulatory elements normally comprise a promoter upstream of the gene sequence to be expressed, transcriptional initiation and termination sites, and a polyadenylation signal sequence. The term transcriptional initiation site refers to the nucleic acid in the construct corresponding to the first nucleic acid incorporated into the primary transcript, i.e., the mRNA precursor; the transcriptional initiation site may overlap with the promoter sequences. The term transcriptional termination site refers to a nucleotide sequence normally represented at the 3′end of a gene of interest or the stretch of sequences to be transcribed, that causes RNA polymerase to terminate transcription. The polyadenylation signal sequence or poly-A addition signal provides the signal for the cleavage at a specific site at the 3′end of eukaryotic mRNA and the post-transcriptional addition in the nucleus of a sequence of about 100 – 200 adenine nucleotides (polyA tail) to the cleaved 3′end. The polyadenylation signal sequence includes the sequence AATAAA located at about 10 – 30 nucleotides upstream from the site of cleavage, plus a downstream sequence. Various polyadenylation elements are known, e.g. SV40 late and early polyA, or BGH polyA. Translational regulatory elements include a translational initiation site (AUG), stop codon and poly A signal for each individual polypeptide to be expressed. An internal ribosome entry site (IRES) is included in some constructs. IRES is defined below. In order to optimize expression it may be necessary to remove, add or alter 5′ and/or 3′untranslated portions of the nucleic acid sequence to be expressed to eliminate potentially extra inappropriate alternative translation initiation codons or other sequences that may interfere with or reduce expression, either at the level of transcription or translation. Alternatively consensus ribosome binding sites can be inserted immediately 5′of the start codon to enhance expression. To produce a secreted polypeptide, the selected sequence will generally include a signal sequence encoding a leader peptide that directs the newly synthesized polypeptide to and through the ER membrane where the polypeptide can be routed for secretion. The leader peptide is often but not universally at the amino terminus of a secreted protein and is cleaved off by signal peptidases after the protein crosses the ER membrane. The selected sequence will generally, but not necessarily, include its own signal sequence. Where the native signal sequence is absent, a heterologous signal sequence

can be fused to the selected sequence. Numerous signal sequences are known in the art and available from sequence databases such as GenBank and EMBL.

The expression vector can contain a single transcription unit for expression of the anti-apoptosis gene, the selectable amplifiable marker gene and optionally the gene(s) of interest, making for example use of IRES elements or intron positioning of some of the genes to operatively link all elements to the same promoter or promoter/enhancer. Alternatively, the expression vector can have one or more transcription units and the aforementioned elements can be expressed from separate transcription units with each transcription unit containing the same or different regulatory sequences. In another alternative more than one expression vector comprising one or more transcription units, each of which can be used for the expression of one or more of the elements mentioned above, can be used and introduced into a host cell by co-transfection or in sequential rounds in any order of the introduced transcription units. Any combination of elements on each vector can be chosen as long as the transcription units are sufficiently expressed. Further elements as deemed necessary may be positioned on an expression vector such as additional anti-apoptosis gene(s), gene(s) of interest or selection markers. The prerequisite for the present invention is the co-introduction of the anti-apoptosis gene and the selectable amplifiable maker gene at the same time, either in separate transcription units on one or two vectors or both in one transcription unit in a single vector, to allow for the co-amplification of the anti-apoptosis gene along with the selectable amplifiable marker gene. In a further embodiment, the anti-apoptosis gene, the selectable amplifiable maker gene and also the gene(s) of interest are being incorporated at the same time, either in separate transcription units on one, two or more vectors or in one or more transcription unit in a single vector, to allow for the co-amplification of the anti-apoptosis gene along with the selectable amplifiable marker and the gene(s) of interest. It is preferred to place at least the anti-apoptosis and the selectable amplifiable marker gene in close spatial proximity to allow a more effective amplification of the anti-apoptosis gene. Therefore it is preferred that at least the anti-apoptosis and the selectable amplifiable are encoded by the same DNA molecule, e.g. if both genes are placed on the same expression vector, and will therefore be commonly introduced into the host cell. It is further more preferred to place both genes in one transcription unit, e.g. by using a common promotor element, to allow for co-expression and amplification of said genes.

A "promoter" refers to a polynucleotide sequence that controls transcription of a gene or sequence to which it is operatively linked. A promoter includes signals for RNA polymerase binding and transcription initiation. A promoter used will be functional in the cell type of the host cell in which expression of the selected sequence is contemplated. A large number of promoters, including constitutive, inducible and repressible promoters from a variety of different sources, are well known in the art (and identified in databases such as GenBank) and are available as or within cloned polynucleotides (from, e.g. depositories such as ATCC as well as other commercial or individual sources). With inducible promoters, the activity of the promoter increases or decreases in response to a signal. For example, the tetracycline (tet) promoter containing the tetracycline operator sequence (tetO) can be induced by a tetracycline-regulated transactivator protein (tTA). Binding of the tTA to the tetO is inhibited in the presence of tet. For other inducible promoters including jun, fos, metallothionein and heat shock promoters, see, e.g., Sambrook et al., 1989 and Gossen et al., 1994. Among the eukaryotic promoters that have been identified as strong promoters for high-level expression are the hamster promoter of the Ubiquitin S27a gene and functional fragments thereof as described in WO97/15664, the SV40 early promoter, adenovirus major late promoter, mouse methallothionein-I promoter, Rous sarcoma virus long terminal repeat and human cytomegalovirus immediate early promoter (CMV). Other heterologous mammalian promoters include, e.g., actin promoter, immunoglobulin promoter, heat-shock promoters. The aforementioned promoters are well known in the art. Any of the above mentioned promoter is highly suitable to controll and initiate the expression of at least the anti-apoptosis gene in all of the inventive host cells described herein. For example, a sutiable expression vector for introducing the anti-apoptosis gene and the selectable amplifiable marker gene in a mammalian host cell comprisses an anti-apoptosis gene, a selectable amplifiable marker gene, and the hamster promoter of the Ubiquitin S27a gene or a functional fragments thereof as described in WO97/15664. Preferably at least the anti-apopotsis gene is under control of said hamster promoter of the Ubiquitin S27a gene.

An "enhancer", as used herein, refers to a polynucleotide sequence that acts on a promoter to enhance transcription of a gene or coding sequence to which it is operatively linked. Unlike promoters, enhancers are relatively orientation and position independent and have been found 5'or 3'to the transcription unit, within an intron as well as within the coding sequence itself. Therefore, enhancers may be placed upstream or downstream form the transcription initiation site or at considerable distances from the promoter, although in practice enhancers may overlap

physically and functionally with promoters. A large number of enhancers from a variety of different sources are well known in the art (and identified in databases such as GenBank, e.g. SV40 enhancer, CMV enhancer, polyoma enhancer, adenovirus enhancer) and available as or within cloned polynucleotide sequences (from, e.g., depositories such as the ATCC as well as other commercial or individual sources). A number of polynucleotides comprising promoter sequences (such as the commonly used CMV promoter) also comprise enhancer sequences. For example, all of the strong promoters listed above also contain strong enhancers.

A "transcription unit" defines a region within a construct that contains one or more genes to be transcribed, wherein the genes contained within the segment are operatively linked to each other and transcribed from a single promoter, and as result, the different genes are at least transcriptionally linked. More than one protein or product can be transcribed and expressed from each transcription unit. Each transcription unit will comprise the regulatory elements necessary for the transcription and translation of any of the selected sequence that are contained within the unit.

"Operatively linked" means that two or more nucleic acid sequences or sequence elements are positioned in a way that permits them to function in their intended manner. For example, a promoter and/or enhancer is operatively linked to a coding sequence if it acts in cis to control or modulate the transcription of the linked sequence. Generally, but not necessarily, the DNA sequences that are operatively linked are contiguous and, where necessary to join two protein coding regions or in the case of a secretory leader, contiguous and in reading frame. However, although an operatively linked promoter is generally located upstream of the coding sequence, it is not necessarily contiguous with it. Enhancers do not have to be contiguous as long as they increase the transcription of the coding sequence. For this they can be located upstream or downstream of the coding sequence and even at some distance. A polyadenylation site is operatively linked to a coding sequence if it is located at the 3'end of the coding sequence in a way that transcription proceeds through the coding sequence into the polyadenylation signal. Linking is accomplished by recombinant methods known in the art, e.g. using PCR methodology, by ligation at suitable restrictions sites or by annealing. Synthetic oligonucleotide linkers or adaptors can be used in accord with conventional practice if suitable restriction sites are not present.

The term "expression" as used herein refers to transcription and/or translation of a heterologous nucleic acid sequence within a host cell. The level of expression of a desired product in a host cell may be determined on the basis of either the amount of corresponding mRNA that is present in the cell, or the amount of the desired polypeptide encoded by the selected sequence. For example, mRNA transcribed from a selected sequence can be quantitated by Northern blot hybridization, ribonuclease RNA protection, in situ hybridization to cellular RNA or by PCR (see Sambrook et al., 1989; Ausubel et al., 1987 updated). Proteins encoded by a selected sequence can be quantitated by various methods, e.g. by ELISA, by Western blotting, by radioimmunoassays, by immunoprecipitation, by assaying for the biological activity of the protein, or by immunostaining of the protein followed by FACS analysis (see Sambrook et al., 1989; Ausubel et al., 1987 updated).

As used herein the terms "enhanced expression", "over-expression" or "high-level expression" refer to sustained and sufficiently high expression of a heterologous selected sequence, in the present invention preferentially an anti-apoptosis gene, e.g. bcl-xL or bcl-2, and/or a gene of interest, introduced into a host cell. Enhanced expression can be achieved by different means. In the present invention expression of an anti-apoptosis gene such as bcl-xL or any other gene listed in Table 2, together with a selectable amplifiable marker gene provides a more efficient method of selecting for and identifying host cells expressing a heterologous gene at high levels. The selectable amplifiable marker not only allows selection of stable transfected host cell lines but allows gene amplification of the heterologous gene of interest. The additional copies of the nucleic acid sequences may be integrated into the cell's genome or an extra artificial chromosome/mini-chromosome or may be located episomally. This approach can be combined with a fluorescence activated cell sorting (FACS)-supported selection for recombinant host cells which have co-amplified the anti-apoptosis gene by using as additional selection marker for example a fluorescent protein (e.g. GFP) or a cell surface marker. Other methods for achieving enhanced expression (either on its own or in combination with the possibilities mentioned above) may include but are not limited to use of (artificial) transcription factors or treatment of cells with natural or synthetic agents to up-regulate endogenous or heterologous gene expression, improvement of either the stability of the mRNA encoding for BCL-xL or of the protein itself (e.g. deletion of protease-susceptible regions not relevant for the biological function of the protein), increasing the translation of the mRNA, or increasing gene dosage by use of episomal plasmids (based on viral sequences for replication origins, such as SV40, polyoma, adenovirus,

EBV or BPV), amplification-promoting sequences (Hemann et al., 1994), or in vitro amplification systems based on DNA-concatemers (Monaco et al., 1996). A person skilled in the art will be able to identify and quantify increased copy numbers of, for example, the amplified heterologous anti-apoptosis gene by methods of the art, e.g., by Southern blot analysis or quantitative PCR methods. It is within the knowledge of a person skilled in the art how to choose the most suitable controls and to perform such assays. One example of such a method is given by the Examples described herein.

By the methods described above, host cells can be generated in such that they comprise multiple copies at least of the introduced anti-apoptosis gene, e.g. at least 5, 10, 20, 50, or 100 copies of the introduced anti-apoptosis gene.

An "internal ribosome entry site" or "IRES" describes a sequence which functionally promotes translation initiation independent from the gene 5'of the IRES and allows two cistrons (open reading frames) to be translated from a single transcript in an animal cell. The IRES provides an independent ribosome entry site for translation of the open reading frame immediately downstream of it. Unlike bacterial mRNA which can be polycistronic, i.e., encode several different polypeptides that are translated sequentially from the mRNAs, most mRNAs of animal cells are monocistronic and code for the synthesis of only one protein. With a polycistronic transcript in a eukaryotic cell, translation would initiate from the 5'most translation initiation site, terminate at the first stop codon, and the transcript would be released from the ribosome, resulting in the translation of only the first encoded polypeptide in the mRNA. In a eukaryotic cell, a polycistronic transcript having an IRES operably linked to the second or subsequent open reading frame in the transcript allows the sequential translation of that downstream open reading frame to produce the two or more polypeptides encoded by the same transcript. The IRES can be of varying length and from various sources, e.g. encephalomyocarditis virus (EMCV) or picornavirus. Various IRES sequences and their use in vector construction has been previously described (Pelletier et la., 1988; Jang et al., 1989; Davies et al., 1992; Adam et al., 1991; Morgan et al., 1992; Sugimoto et al., 1994; Ramesh et al., 1996; Mosser et al., 1997). The downstream coding sequence isoperatively linked to the 3'end of the IRES at any distance that will not negatively affect the expression of the downstream gene. The optimum or permissible distance between the IRES and the start of the downstream gene can be readily determined by varying the distance and measuring expression as a function of the distance.

The term "intron" as used herein, refers to a non-coding nucleic acid sequence of varying length, normally present within many eukaryotic genes, which is removed from a newly transcribed mRNA precursor by the process of splicing for which highly conserved sequences at or near either end of the intron are necessary. In general, the process of splicing requires that the 5'and 3'ends of the intron be correctly cleaved and the resulting ends of the mRNA be accurately joined, such that a mature mRNA having the proper reading frame for protein synthesis is produced. Many splice donor and splice acceptors sites, meaning the sequences immediately surrounding the exon-intron- and intron-exon-boundaries, have been characterized and Ohshima et al., 1987 provides a review of those.

"Transfection" of eukaryotic host cells with a polynucleotide or expression vector, resulting in genetically modified cells or transgenic cells, can be performed by any method well known in the art and described, e.g., in Sambrook et al., 1989 or Ausubel et al., 1987 (updated). Transfection methods inlcude but are not limited to liposome-mediated transfection, calcium phosphate co-precipitation, electroporation, polycation (such as DEAE-dextran)-mediated transfection, protoplast fusion, viral infections and microinjection. Preferably, the transfection is a stable transfection. The transfection method that provides optimal transfection frequency and expression of the heterologous genes in the particular host cell line and type is favored. Suitable methods can be determined by routine procedures. For stable transfectants the constructs are either integrated into the host cell's genome or an artificial chromosome/mini-chromosome or located episomally so as to be stably maintained within the host cell.

A "selectable marker gene" is a gene that only allows cells carrying the gene to be specifically selected for or against in the presence of a corresponding selection agent. By way of illustration, an antibiotic resistance gene can be used as a positive selectable marker gene that allows the host cell transformed with the gene to be positively selected for in the presence of the corresponding antibiotic; a non-transformed host cell would not be capable of growth or survival under the selection culture conditions. Selectable markers can be positive, negative or bifunctional. Positive selectable markers allow selection for cells carrying the marker by conferring resistance to a drug or compensate for a metabolic or catabolic defect in the host cell. In contrast, negative selection markers allow cells carrying the marker to be selectively eliminated. For example, using the HSV-tk gene as a marker will make the cells sensitive to agents such as acyclovir and

gancyclovir. The selectable marker genes used herein, including the amplifiable selectable genes, will include recombinantly engineered mutants and variants, fragments, functional equivalents, derivatives, homologs and fusions of the native selectable marker gene so long as the encoded product retains the selectable property. Useful derivatives generally have substantial sequence similarity (at the amino acid level) in regions or domains of the selectable marker associated with the selectable property. A variety of marker genes have been described, including bifunctional (i.e. positive/negative) markers (see e.g. WO 92/08796 and WO 94/28143), incorporated by reference herein. For example, selectable genes commonly used with eukaryotic cells include the genes for aminoglycoside phosphotransferase (APH), hygromycin phosphotransferase (HYG), dihydrofolate reductase (DHFR), thymidine kinase (TK), glutamine synthetase, asparagine synthetase, and genes encoding resistance to neomycin (G418), puromycin, histidinol D, bleomycin and phleomycin.

Selection may also be made by fluorescence activated cell sorting (FACS) using for example a cell surface marker, bacterial β-galactosidase or fluorescent proteins (e.g. green fluorescent proteins (GFP) and their variants from Aequorea victoria and Renilla reniformis or other species; red fluorescent proteins, fluorescent proteins and their variants from Discosoma or other species) to select for recombinant cells.

In accordance with the teachings herein, the present invention provides also a method for the expression of an anti-apoptosis gene, a selectable amplifiable marker gene and at least one gene of interest in a host cell. Said method comprising the steps: (*i.*) introducing into a host cell population, preferably into a hamster host cell population, the nucleic acid sequences that encode for an anti-apoptosis gene, a selectable amplifiable marker gene, and at least one gene of interest, wherein said genes are operatively linked to at least one regulatory sequence allowing for expression of said genes, and (*ii.*) cultivating said host cell population under conditions wherein said genes are expressed. Methods, teaching to a skilled person in the art how to introduce one or more polynucleotides into a host cell are exemplary described above. Moreover, examples are also given by the invention how one or more genes can be linked with at least one regulatory sequence allowing for expression of said genes. Moreover, it is also described that is preferred to to place at least the anti-apoptosis and the selectable amplifiable marker gene in close spatial proximity to allow for a more effective amplification of the anti-apoptosis gene. Host cells, suitable for expressing said genes are those mentioned by the invention. Suitable candidates of

an anti-apoptosis gene are listed under Table 2. Preferred is the expression of any of the sequences encoding BCL-xL or BCL-2, more preferred any sequence encoding BCL-xL, even more preferred any BCL-xL encoding sequence of human or hamster origin. In a further preferred embodiment of that method, co-expressing host cells include any of the sequences encoding for any one of the selectable amplifiable marker listed in Table 3. Even more preferred is a method, wherein the host cells comprise a sequence encoding for a heterologous BCL-xL and also sequences encoding for any one of the selectable amplifiable marker listed in Table 3. Most preferred is a method, where the host cell comprises the sequences encoding for a heterologous BCL-xL and DHFR, especially if the BcL-xL encoding sequence is of human or hamster origin.

Methods provided by the present invention allows a person skilled in the art to generate new host cells, particularly for production purposes, showing enhanced cell survival attributed to an delayed or inhibited programmed cell death. Beneficial strategies have been found that dramatically increase viability and productivity of cells, constantly grown in suspension and especially in serum free-medium. Cells according to this invention are highly suitable for the production of several desired polypeptides. The genetically modified host cells described herein not only encode for the genes that are responsible for the enhanced cell survival, e.g. an anti-apoptosis gene and an amplifiable selectable marker gene, but optionally for any gene of interest encoding for a desired peptide. Accordingly, the present invention also provides to persons skilled in the art a process for producing a protein of interest in a host cell, comprising (*i.*) cultivating any host cells of this invention under conditions which are favorable for the expression of the anti-apoptosis gene and the gene(s) of interest, and (*ii.*) isolating the protein of interest from the cells and/or the cell culture supernatant. Also provided is the use of said cells for the production of at least one desired protein encoded by a gene of interest.

Desired proteins are those mentioned above. Especially, desired proteins/polypeptides are for example, but not limited to insulin, insulin-like growth factor, hGH, tPA, cytokines, such as interleukines (IL), e.g. IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, interferon (IFN) alpha, IFN beta, IFN gamma, IFN omega or IFN tau, tumor necrosisfactor (TNF), such as TNF alpha and TNF beta, TNF gamma, TRAIL; G-CSF, GM-CSF, M-CSF, MCP-1 and VEGF. Also included is the production of erythropoietin or any other hormone growth factors. The method according to the invention can

also be advantageously used for production of antibodies or fragments thereof. Such fragments include e.g. Fab fragments (Fragment antigen-binding = Fab). Fab fragments consist of the variable regions of both chains which are held together by the adjacent constant region. These may be formed by protease digestion, e.g. with papain, from conventional antibodies, but similar Fab fragments may also be produced in the mean time by genetic engineering. Further antibody fragments include F(ab')2 fragments, which may be prepared by proteolytic cleaving with pepsin.

Using genetic engineering methods it is possible to produce shortened antibody fragments which consist only of the variable regions of the heavy (VH) and of the light chain (VL). These are referred to as Fv fragments (Fragment variable = fragment of the variable part). Since these Fv-fragments lack the covalent bonding of the two chains by the cysteines of the constant chains, the Fv fragments are often stabilised. It is advantageous to link the variable regions of the heavy and of the light chain by a short peptide fragment, e.g. of 10 to 30 amino acids, preferably 15 amino acids. In this way a single peptide strand is obtained consisting of VH and VL, linked by a peptide linker. An antibody protein of this kind is known as a single-chain-Fv (scFv). Examples of scFv-antibody proteins of this kind known from the prior art are described in Huston et al. (1988, PNAS 16: 5879-5883).

In recent years, various strategies have been developed for preparing scFv as a multimeric derivative. This is intended to lead, in particular, to recombinant antibodies with improved pharmacokinetic and biodistribution properties as well as with increased binding avidity. In order to achieve multimerisation of the scFv, scFv were prepared as fusion proteins with multimerisation domains. The multimerisation domains may be, e.g. the CH3 region of an IgG or *coiled coil* structure (helix structures) such as *Leucin-zipper* domains. However, there are also strategies in which the interaction between the VH/VL regions of the scFv are used for the multimerisation (e.g. dia-, tri- and pentabodies). By diabody the skilled person means a bivalent homodimeric scFv derivative. The shortening of the *Linker* in an scFv molecule to 5- 10 amino acids leads to the formation of homodimers in which an inter-chain VH/VL-superimposition takes place. Diabodies may additionally be stabilised by the incorporation of disulphide bridges. Examples of diabody-antibody proteins from the prior art can be found in Perisic et al. (1994, Structure 2: 1217-1226).

By minibody the skilled person means a bivalent, homodimeric scFv derivative. It consists of a fusion protein which contains the CH3 region of an immunoglobulin, preferably IgG, most preferably IgG1 as the dimerisation region which is connected to the scFv via a *Hinge region* (e.g. also from IgG1) and a *Linker* region. Examples of minibody-antibody proteins from the prior art can be found in Hu et al. (1996, Cancer Res. 56: 3055-61).

By triabody the skilled person means a: trivalent homotrimeric scFv derivative (Kortt et al. 1997 Protein Engineering 10: 423-433). ScFv derivatives wherein VH-VL are fused directly without a linker sequence lead to the formation of trimers.

The skilled person will also be familiar with so-called miniantibodies which have a bi-, tri- or tetravalent structure and are derived from scFv. The multimerisation is carried out by di-, tri- or tetrameric coiled coil structures (Pack et al., 1993 Biotechnology 11:, 1271-1277; Lovejoy et al. 1993 Science 259: 1288-1293; Pack et al., 1995 J. Mol. Biol. 246: 28-34).

Preferred host cells of the present invention are those described herein and which are being characterized by an enhanced cell survival. In particular, preferred are host cells genetically modified by any method according to this invention, comprising the nucleic acid sequences that encode for an anti-apoptosis gene, a selectable amplifiable marker gene and at least one gene of interest. More preferred are host cells comprising multiple copies of those genes, at least of the anti-apoptosis gene and the selectable amplifiable marker gene. Multiple copies of those genes are obtainable by methods also described herein, e.g by gene-amplification processes, and/or by providing host cells with multiple gene copies in any other way, known to person skilled in the art (e.g. introducing concatemers of the gene(s)).

Suitable anti-apoptosis genes are but not limited to those listed in Table 2. Preferred are host cells comprising multiple copies of a BCL-xL encoding gene and multiple copies of any one of the selectable amplifiable marker genes listed in Table 3. The most preferred selectable amplifiable marker gene is a DHFR encoding gene.

Host cells suitable for protein production are showing over-expression of the anti-apoptosis protein that is at least about 30-fold increased, preferably at least about 50-fold, even more

preferably at least about 100-fold increased compared to the expression-level of said protein in host cells which are not modified according to any method of this invention.

Furthermore, suitable is a population of host cells showing a viability of at least about 50% when cultivated for 9 days. Furthermore, host cells are suitable and preferred by the present invention that show a viability of at least about 60% after 8 days of cultivation. Moreover, host cells are suitable in the meaning of this invention, when at least about 75% of the cell population are viable for at least 7 days. In another embodiment, a host cell population is suitable and preferred, that show a cell viability of at least about 85% after a 6-day cultivation. In the case that the anti-apoptosis encodes for BCl-xL cell viability of about 60% after 9 day cultivation, 75% after 8-day cultivation, and about 85% after 7 day cultivation are obtainable (e.g. see Figure 5). Also, host cells are particularly suitable, having at least an increase in survival of at least about 20% after a 6-, 7-, 8-, or 9-day cultivation compared to the parental cell population, which is not modified by gene-amplification methods. More preferred are those host cell populations having an increase in survival of at least about 30%, and even more preferred are host cell populations having an increase in survival of about at least about 50% after a 6-, 7-, 8-, or 9-day cultivation compared to the parental cell population.

Suitable host cells or host cell population in the meaning of the present invention includes hamster cells, preferably BHK21, BHK TK$^-$, CHO, CHO-K1, CHO-DUKX, CHO-DUKX B1, and CHO-DG44 cells or the derivatives/progenies of any of such cell line. Particularly preferred are CHO-DG44, CHO-DUKX, CHO-K1 and BHK21, and even more particularly CHO-DG44 and CHO-DUKX cells. In a further embodiment of the present invention host cells also mean murine myeloma cells, preferably NS0 and Sp2/0 or the derivatives/progenies of any of such cell line. However, derivatives/progenies of those cells, other mammalian cells, including but not limited to human, mice, rat, monkey, and rodent cell lines, or eukaryotic cells, including but not limited to yeast, insect and plant cells, can also be used in the meaning of this invention for production purposes.

The selection of recombinant host cells showing increased viability and expressing high levels of a desired protein generally is a multi-step process. Selection strategy depends on whether cells are co-transfected at least by all three relevant genes (anti-apoptosis gene, selectable amplifiable marker gene(s), gene(s) of interest, and optionally additional selection marker gene(s)) in

parallel, whether the gene(s) of interest will be introduced into cells already showing increased cell viability as described herein or whether the anti-apoptosis gene(s) along with a selectable amplifiable marker will be introduced into host cells expressing already a gene(s) of interest.

In the first case, transfected cells are screened or selected for the expression of any selection marker(s) co-transfected with the gene of interest, to identify cells that have incorporated the gene(s) of interest. Typically, the transfected host cells are subjected to selection for expression of the selectable marker(s) by culturing in selection medium, e.g. for about 2 weeks. Following that, cells are exposed stepwise to successively increasing amounts of the amplifying selection agent(s) to co-amplify the nucleic acid sequences encoding for at least the anti-apoptosis gene(s),the selectable amplifiable marker gene(s) and the gene(s) of interest until sufficient expression of the heterologous desired product(s) and also of the anti-apoptosis gene product(s) is obtained. Each selection step can be performed on cell pools or it can be combined with clone selection by, e.g., limited dilution. Selection can be supported by fluorescence activated cell sorting (FACS) if an appropriate marker such as GFP is used for selection.

In the second case, suitable host cells are transfected by at least the gene(s) of interest and the selectable marker gene(s), optionally a selectable amplifiable gene, which differs from the selectable amplifiable marker gene used before to generate the host cells of enhanced viability. Afterwards transfected cells are screened or selected at least for the expression of the selection marker co-transfected with the gene of interest, to identify cells that have incorporated the gene of interest. Typically, the transfected host cells are subjected to selection for expression of the selectable marker(s) by culturing in selection medium. Optionally, the selection medium also contains the selective agent which is specific for the selectable amplifiable marker used before to generate a host cell of enhanced viability. The selection step can be performed on cell pools or it can be combined with clone selection by, e.g., limited dilution. Selection can be supported by fluorescence activated cell sorting (FACS) if an appropriate marker such as GFP is used for selection. Optionally, cells can undergo further amplification steps to increase the copy number at least of the gene of interest, if another selectable amplifiable marker was used for transfection.

In the third case, recombinant host cells or production cell lines already expressing the gene(s) of interest are co-transfected with the anti-apoptosis gene(s), the selectable amplifiable marker gene(s) and optionally with additional selection marker gene(s) and/or gene(s) of interest,

whereby markers different from the ones used for establishing the recombinant production cell line are used for selection. The transfected host cells are subjected to selection for expression of the newly introduced marker gene(s) by culturing in selection medium for about 2 weeks. Optionally, the selection medium contains also the selective agent which is specific for the selectable marker(s) used before to generate the recombinant production cell line. Following that, the cells are exposed stepwise to successively increasing amounts of the amplifying selection agents to co-amplify at least the anti-apoptosis gene(s) and the selectable amplifiable marker gene and optionally co-transfected gene(s) of interest until sufficient expression of at least the anti-apoptosis gene product is obtained. Each selection step can be performed on cell pools or it can be combined with clone selection by, e.g., limited dilution. Selection can be supported by fluorescence activated cell sorting (FACS), if an appropriate marker gene such as GFP is used for selection.

The protein of interest is preferably recovered from the culture medium as a secreted polypeptide, or it can be recovered from host cell lysates if expressed without a secretory signal. It is necessary to purify the protein of interest from other recombinant proteins and host cell proteins in a way that substantially homogenous preparations of the protein of interest are obtained. As a first step, cells and/or particulate cell debris are removed from the culture medium or lysate. The product of interest thereafter is purified from contaminant soluble proteins, polypeptides and nucleic acids, for example, by fractionation on immunoaffinity or ion-exchange columns, ethanol precipitation, reverse phase HPLC, Sephadex chromatography, chromatography on silica or on a cation exchange resin such as DEAE. In general, methods teaching a skilled person how to purify a protein heterologous expressed by host cells, are well known in the art. Such methods are for example described by Harris and Angal, Protein Purification Methods, in Rickwood and Hames eds., The Practical Approach Series, IRL Press (1995) or Robert Scopes, Protein Purification, Springer-Verlag (1988), both incorporated by reference.

One general subject of the present invention is to provide a person skilled in the art with host cells showing an increased cell survival. Cell survival can be increased by inhibiting or delaying programmed cell death, e.g, apoptosis, by any methods as described herein. The most sufficient way to enhance cell survival in the meaning of this invention is to provide a host cell with multiple copies of at least a heterologous introduced anti-apoptosis gene and culturing the host

cells under conditions allowing high expression of those anti-apoptosis gene copies. The present invention also provides host cells, comprising at least 5 copies of a heterologous anti-apoptosis gene. Also provided are host cells, comprising at least 10 copies of a heterologous anti-apoptosis gene. Moreover, the present invention also provides host cells, comprising at least 20 copies, in another embodiment at least 50 copies, and in a further embodiment at least 100 copies of the heterologous anti-apoptosis gene(s). The present invention provides several methods to a skilled person in the art how to generate such host cells, comprising multiple copies of an heterologous anti-apoptosis gene. Those methods include but are not limited to those described above: e.g. (*i.*) stepwise amplification of at least one heterologous introduced anti-apoptosis gene driven by an selective agent, (*ii.*) increasing gene dosage of at least one heterologous introduced anti-apoptosis gene by use and introduction of episomal plasmids as described above, (*iii.*) or using in vitro amplification systems based on DNA-concatemers as described for example by Monaco et al., 1996, incorporated herein by reference.

Suitable anti-apoptosis genes are any of those listed in Table 2 or described herein in general, particularly encoded by any of the nucleic acid sequences referred to in Table 2 or by any of the sequences encoded by SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9 or SEQ ID NO:11, or any functional variants or mutants (degenerative and non degenerative) thereof.. Preferred is a host comprising multiple copies of a gene encoding for BCL-xL or BCL-2, more preferred for BCL-xL, and even more preferred for BCL-xL of human or hamster origin. Even more preferred is a host cell comprising multiple copies of a nucleic acid having the sequence of any one of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9 or SEQ ID NO:11, or any functional variants or mutants (degenerative and non degenerative) thereof. In a further embodiment the anti-apoptosis gene encoding BCL-2 or having the sequence of SEQ ID NO:2 is preferred. Also preferred are host cells comprising different anti-apoptosis genes, e.g. selected from those listed in Table 2 or any of the others described herein. In a more preferred embodiment of this invention, at least one copy of the mixture encodes for a heterologous BCL-xL.

Suitable host cells are any of those described in the present invention, e.g. those mentioned in Table 1. More preferred are host cells of hamster or murine origin, e.g. murine myeloma cells. Even more preferred are CHO or BHK cells, particularly CHO-DG44, BHK21, BHK TK$^-$, CHO, CHO-K1, CHO-DUKX, CHO-DUKX B1, and CHO-DG44 cells or the derivatives/progenies of

any of such cell line. Most preferred are CHO-DG44, CHO-DUKX, CHO-K1 and BHK21, particularly CHO-DG44 and CHO-DUKX cells.

Host cells described above can be additionally modified by introducing at least one heterologous gene of interest such that the present invention also concerns host cells comprising multiple copies of an anti-apoptosis gene as described above, and further comprising at least one gene of interest.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, molecular biology, cell culture, immunology and the like which are in the skill of one in the art. These techniques are fully disclosed in the current literature. See e.g. Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989); Ausubel et al., Current Protocols in Molecular Biology (1987, updated); Brown ed., Essential Molecular Biology, IRL Press (1991); Goeddel ed., Gene Expression Technology, Academic Press (1991); Bothwell et al. eds., Methods for Cloning and Analysis of Eukaryotic Genes, Bartlett Publ. (1990); Wu et al., eds., Recombinant DNA Methodology, Academic Press (1989); Kriegler, Gene Transfer and Expression, Stockton Press (1990); McPherson et al., PCR: A Practical Approach, IRL Press at Oxford University Press (1991); Gait ed., Oligonucleotide Synthesis (1984); Miller & Calos eds., Gene Transfer Vectors for Mammalian Cells (1987); Butler ed., Mammalian Cell Biotechnology (1991); Pollard et al., eds., Animal Cell Culture, Humana Press (1990); Freshney et al., eds., Culture of Animal Cells, Alan R. Liss (1987); Studzinski, ed., Cell Growth and Apoptosis, A Practical Approach, IRL Press at Oxford University Presss (1995); Melamed et al., eds., Flow Cytometry and Sorting, Wiley-Liss (1990); Current Protocols in Cytometry, John Wiley & Sons, Inc. (updated); Wirth & Hauser, Genetic Engineering of Animals Cells, in: Biotechnology Vol. 2, Pühler ed., VCH, Weinheim 663-744; the series Methods of Enzymology (Academic Press, Inc.), and Harlow et al., eds., Antibodies: A Laboratory Manual (1987).

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications cited herein are hereby incorporated by reference in their entirety in order to more fully describe the state of the art to which this invention pertains. The invention generally described above will be more readily understood by reference to the following examples, which

are hereby included merely for the purpose of illustration of certain embodiments of the present invention and are not intended to limit the invention in any way.

EXAMPLES

Abbreviations

CHO:	chinese hamster ovary
DHFR:	dihydrofolate reductase
ELISA:	enzyme-linked immunosorbant assay
FACS:	fluorescence activated cell sorter
FITC:	fluorescein isothiocyanate
HRPO:	horseradish peroxidase
HT:	hypoxanthine and thymidine
IRES:	internal ribosome entry site
MTX:	methotrexate
PAGE:	polyacrylamide gel electrophoresis
PBS:	phosphate buffered saline
PCR:	polymerase chain reaction
PI:	propidium iodide
RT-PCR:	reverse transcriptase – polymerase chain reaction
SEAP:	secreted alkaline phosphatase
SDS:	sodium dodecyl sulfate
sICAM:	soluble intracellular adhesion molecule

Methods

1. Cell culture

CHO-DG44/dhfr$^{-/-}$ (Urlaub et al., 1983), grown permanently in suspension in the serum-free medium CHO-S-SFMII (Invitrogen, Carlsbad, CA) supplemented with hypoxanthine and thymidine (Invitrogen, Carlsbad, CA), were incubated in cell culture flasks at 37°C in a humidified atmosphere containing 5% CO_2. Cells were seeded at a concentration of $2x10^5$ cells/ml in fresh medium every two to three days and cell suspensions (500 µl) were analyzed for cell number and viability using a CASY1 cell counter (Schaerfe System; Germany). Viability was also confirmed by trypan blue dye exclusion. Single cell cloning was done by standard dilution technology in 96-well chambers. For the DHFR-based selection of stable transfected CHO-DG44 cells CHO-S-SFMII medium without hypoxanthine and thymidine was used. DHFR-based gene amplification was achieved by adding 20 nM MTX (Sigma, Germany) as amplifying selection agent to the medium. For batch cultivation clones were seeded in duplicate into cell culture flasks (T25) containing 12 ml of the appropriate medium and kept for 7 or 9

days in the incubator without adding any fresh medium. When CHO-DG44 cells were cultivated in the presence of serum CHO-S-SFMII medium was supplemented with 10% fetal calf serum (Sigma, Germany). For passaging cells were trypsinized (in the presence of serum CHO-DG44 became adherent) and seeded at a concentration of $2x10^5$ cells/ml in fresh medium every two to three days.

2. Expression vectors

The basic vector pBID, based on the pAD-CMV vector (Werner et al., 1998), mediates constitutive expression of the heterologous genes driven by the CMV promoter/enhancer. In addition, pBID encodes the dhfr mini gene as amplifiable selectable marker (see for example EP 0 393 438). sICAM was isolated as HindIII/SalI fragment from pAD-sICAM (Werner et al., 1998) and cloned into the corresponding sites (HindIII and SalI) of pBID, resulting in pBID-sICAM. The human homologous of bcl-xL (SEQ ID NO:1, GenBank Accession Number Z23115) and bcl-2 (SEQ ID NO:3, GenBank Accession Number M13995) were cloned into the expression vectors as follows. For the construction of pBID-bcl-2, bcl-2 was at first PCR-amplified from human total RNA and subcloned into the pCR2.1-TOPO cloning vector (Invitrogen, Carlsbad, CA). From there it was excised using EcoRI and ligated into the corresponding EcoRI site of pBID. Using Klenow-DNA polymerase the filled in PmeI-excised IRES-bcl-xL fragment of pDD6 (Fussenegger et al., 1998) was cloned into the filled in XbaI site of pBID-sICAM resulting in the bicistronic expression vector pBID-sICAM-bcl-xL. This vector was the basis for the construction of pBID-bcl-xL by eliminating the sICAM-IRES element from pBID-sICAM-bcl-xL by SalI/XhoI digestion and religation. pSEAP2-Control harbors the human secreted alkaline phosphatase under control of the SV40 promoter (Clontech, Palo Alto, CA). pGL2-Control contains the firefly luciferase driven by the SV40 promoter (Promega, Madison, USA).

3. Transient and stable transfections

Transfections were conducted using Fugene6 reagent (Roche Diagnostics GmbH, Mannheim, Germany). Per transfection $0,6x10^6$ exponentially growing CHO-DG44 cells in 2ml HT-supplemented CHO-S-SFMII medium were seeded in a well of a 6-well chamber. A total of 4 µg plasmid-DNA and 10 µl Fugene6 reagent were used for each transfection, following the protocol of the manufacturer. Transient transfections were performed in triplicate and supernatant and cells were harvested 48 hours post transfection. For stable transfections the medium was

replaced with HT-free CHO-S-SFMII medium 72 hours post transfection and the mixed cell populations were selected for two weeks prior to analysis or cloning with medium changes every 3 to 4 days. Single cell cloning was done by standard dilution technology. For amplification of the heterologous genes, integrated into the chromosomes of the host cells, single cells derived from a mixed genetically modified host cell population were seeded into 96-well chambers that contained 200 µl HT-free CHO-S-SFMII medium supplemented with 20 nM MTX. Amplified single cell clones were selected during 3 weeks and expanded into cell culture flasks.

4. sICAM ELISA

sICAM titers in supernatants were quantified by ELISA with standard protocols (Ausubel et al., 1994, updated) using two in house developed sICAM specific monoclonal antibodies (as described for example in US patents No. 5,284,931, 5,475,091), whereby one of the antibodies is a HRPO-conjugated antibody. Purified sICAM protein was used as a standard. Samples were analyzed using a Spectra Fluor Plus reader (TECAN, Crailsheim, Germany).

5. Reporter enzyme assays

The firefly luciferase reporter enzyme activity was determined with the Luciferase Assay System from Promega (Madison, WI) according to protocol. SEAP activity was measured with the Great EscAPe SEAP kit from Clontech (Palo Alto, CA) according to protocol. Samples were analyzed using a Spectra Fluor Plus reader (TECAN, Crailsheim, Germany).

6. Western blot analysis

The BCL-2 and BCL-xL expression of the parental host cells CHO-DG44 and the genetically modified host cells was confirmed by Western Blot analysis. About $1x10^6$ cells were extracted in 500 µl lysis buffer (1% Triton X-100, 15 mM NaCl, 10 mM Tris-HCl pH 7.5 with 50 µg/ml phenyl methanesulfonyl fluoride and 200 µl of the protease inhibitor cocktail Complete from Roche Diagnostics). The lysates were clarified by centrifugation at 13.000 xg for 10 min. Protein concentrations were determined by Bradford assay according to the manufacturer's protocol (Bio-Rad Laboratories GmbH, Munich, Germany). The extracts were then stored at –80°C until needed. Equal amounts of protein (20 µg) were subjected to 10% SDS-PAGE (Novex; Invitrogen, Carlsbad, CA) and subsequently electroblotted onto nitrocellulose membranes (Invitrogen, Carlsbad, CA). After blocking with 5% skim milk, proteins were detected using mouse monoclonal antibodies from Santa Cruz Biotechnology (Santa Cruz, CA) specific for

BCL-2 or BCL-xL. Proteins were visualized with an anti-mouse peroxidase-coupled secondary antibody (Dianova GmbH, Hamburg, Germany) using ECL detection system (Amersham Biosciences, Freiburg, Germany).

7. *Quantification of apoptosis*

Fragmented DNA levels were quantified using a fluorescence-based TUNEL-assay (BD Biosciences PharMingen, San Diego, CA). $1x10^6$ cells were harvested, washed with PBS and treated with 1% paraformaldehyde solution in PBS for 15 min at 4°C. Following an additional washing step using PBS the cells were fixed in 70% ethanol and analyzed according to the manufacturer's protocol. FITC and PI emission profiles of 10.000 cells per sample were analyzed using a FACSCalibur (Becton-Dickinson).

8. *Flow cytometry of labeled mitochondria*

500.000 cells per sample were harvested, washed with PBS, and treated with 1% paraformaldehyde in PBS for 15 min at 4°C. After a second PBS wahsing step the cells were ready for the fluorescence-based staining of their mitochondria. A PBS solution containing 500 pM of the mitochondrion-selective MitoTracker Green FM dye (Molecular Probes, Eugene, OR) was prepared from a 1 mM stock solution. Cells were incubated in 200 µl staining solution for 15 min at 37°C. Subsequently, the cells were washed twice with PBS, resuspended in 400 µl PBS and subjected to flow cytometric analysis. The green fluorescence emission of 10.000 cells per sample was determined.

9. *Hybridization assay*

Hybridization analysis of DNA or RNA blots using either DNA or RNA probes (>100 bp) is carried out according to standard protocols described in Ausubel et al., 1994, updated. Specificity of hybridization is achieved in the post-hybridization washes, whereby the critical parameters are the ionic strength of the final wash solution and the temperature at which the wash is carried out. Stringent conditions are achieved by performing the wash in 0.2xSSC/0.1% SDS at 65°C. For highly stringent conditions the wash is performed in 0.1xSSC/0.1% SDS at 65°C.

Example 1: Overexpression of survival and product genes in transient and stable mode

The common cold therapeutic sICAM (soluble intercellular adhesion molecule 1) competes with the ICAM receptor for rhinovirus binding and prevents interaction of this common cold etiological agent with the ICAM receptor which is required for cell entry and subsequent infection (Bella et al., 1999; Marlin et al., 1990). For detailed assessment of transient expression of anti-apoptosis genes bcl-2 or bcl-xL on the production of sICAM equal amounts of the sICAM encoding plasmid pBID-sICAM were co-transfected in triplicate with either the pBID-bcl-2, pBID-bcl-xL or the isogenic control pBID (Figure 1) into dhfr-deficient CHO-DG44. The cells were grown permanently in suspension in the serum-free medium CHO-S-SFMII (Invitrogen, Carlsbad, CA) supplemented with hyopxanthine and thymidine. sICAM titers in supernatants were quantified 48 hours post transfection by ELISA using in house produced sICAM specific antibodies. Whereas BCL-2 expression dramatically reduced sICAM production to 10% of the control pBID, ectopic BCL-xL expression increased the yield of sICAM by 60% (Figure 2A). These data were confirmed by SEAP expression profiles (exchanging pBID-sICAM by pSEAP2-Control) demonstrating that observed production characteristics of BCL-2 and BCL-xL were of general rather than a combinatorial effect of sICAM/BCL-2 or sICAM/BCL-xL expression (Figure 2B). Also, in order to assess whether the impact of BCL-2 and BCL-xL expression on cellular production parameters is linked or limited to secreted product proteins, identical production profiling was performed using the intracellular luciferase reporter (exchanging pBID-sICAM by pGL2-Control). Luciferase production characteristics correlated with aforementioned SEAP and sICAM expression profiles confirming the positive (bcl-xL) and negative (bcl-2) impact of these apoptosis-suppression genes on the overall productivity of CHO-DG44 cells (Figure 2C). Reduced production of desired proteins following expression of BCL-2 has also been observed in COS-7 cells (ATCC CRL-1651), anchorage-dependent CHO-K1 (ATCC CCL-61) and CHO-DUKX cells (ATCC CRL-9096) adapted for growth in suspension.

Although transient transfection experiments enable highly reliable information on the metabolic engineering capacity of heterologous genes over a wide range of cell types and copy numbers, only stable mixed populations can confirm beyond doubt the observed phenotype. Therefore sICAM titer profiles of 6 mixed populations for each pBID-sICAM/pBID-bcl-2, pBID-sICAM/pBID-bcl-xL, pBID-sICAM/pBID configuration were generated. Transfected cell populations were selected for the expression of the heterologous genes by DHFR-based selection

in HT-free CHO-S-SFMII medium. Figure 3 shows the absolute sICAM yield produced by those stable transfected mixed populations during a 3 day cultivation period. Each value represents an average sICAM production of all populations over five passages, whereby at each passage a sample was taken. As seen with transient transfections, BCL-2 expression has a negative impact on sICAM production with titers reduced by 50%, whereas BCL-xL expression increases the overall yield of this common cold therapeutic by 30% compared to the control populations engineered for sICAM-only expression. These correlating results obtained in transient and stable expression configurations suggest that design of anti-apoptosis engineering strategies should only be considered following careful analysis of available survival determinants.

Example 2: Effect of dhfr-amplified gene expression of bcl-2 and bcl-xL on viability and apoptosis of stable sICAM producing cell clones

For the evaluation of the effect of anti-apoptosis engineering on the viability of transgenic CHO-DG44 cell lines in serum-free batch cultivation, the highest sICAM-producing cell populations out of 6 mixed cell populations each, generated by stable co-transfection of CHO-DG44 with either pBID-sICAM/pBID-bcl-2, pBID-sICAM/pBID-bcl-xL or pBID-sICAM/pBID vector combinations, were selected for further analysis. Two parallel single cell cloning procedures by limited cell dilution in 96-well chambers were conducted, one in HT-free CHO-S-SFMII medium (non-amplified cell clones) and one including a single round of MTX-induced DHFR-based amplification by supplementing the HT-free CHO-S-SFMII medium with 20 nM MTX (amplified cell clones). The cell clones were screened for the highest sICAM producers by ELISA and for each gene configuration the best two cell clones were selected for further analysis. In addition to pBID-sICAM these cell clones also contain one of the following constructs: (*i.*) pBID (vector-only control) in HMNI-1, HMNI-2 (not amplified) and HMNI-3, HMNI-4 (amplified), (*ii.*) pBID-bcl-2 in HMNIBC-1, HMNIBC-2 (not amplified) and HMNIBC-3, HMNIBC-4 (amplified), (*iii.*) pBID-bcl-xL in HMNIBX-1, HMNIBX-2 (not amplified) and HMNIBX-3, HMNIBX-4 (amplified).

Viability of all cell clones in batch cultivation in cell culture flasks was monitored daily for a period of one week. As seen in Figure 4 all non-amplified cell clones, including the vector-only control, exhibited similar viability profiles clearly showing the negligible effect of BCL-2 and BCL-xL on viability. At day 7 viability was down to approximately 30 – 40% in all cases.

However, significant increases in cell viability could be observed during the decline phase of clones containing amplified heterologous genes. BCL-xL was clearly outperforming BCL-2 with respect to cell death protection (Figure 5). In BCL-xL expressing cell lines (HMNIBX-3/4) there were still 70% viable cells in the culture at day 9, compared to about 60% in the BCL-2 expressing cell lines (HMNIBC-3/4) and no viable cells in the transgenic control cell lines (HMNI-3/4). Correlating with an increase in viability amplified cell clones also showed a dramatic reduction in the percentage of cells which initiate apoptosis programs, assessed at days 2, 4 and 6 using a fluorescence-based TUNEL assay (Figure 6). Whereas at day 2 almost no apoptotic cells were found in all transgene configurations, a significant increase in apoptotic cell numbers was seen at day 4. The least increase up to 5% was found in BCL-xL expressing cells, followed by BCL-2 expressing cells with 10% and the transgenic control cells already with 30% apoptotic cells. At day 6 the difference between the various transfected cell lines was even more pronounced. The apoptosis-suppressing potential of BCL-xL is at least three-fold higher compared to BCL-2, where 30% of apoptotic cells compared to 10% in BCL-xL expressing cells were found at this stage. In the transgenic control cells, not expressing any heterologous anti-apoptosis gene, the number of apoptotic cells amounted already to 60%.

In order to confirm the amplification status of the anti-apoptosis genes BCL-2- and BCL-xL-directed Western blot analysis was performed. Figure 7 demonstrates only basal BCL-2 (Figure 7A) and BCL-xL (Figure 7B) expression in the transgenic control cell line HMNI-1 at levels near the detection limit and comparable to the parental CHO-DG44 cells. This indicates survival gene expression is not upregulated by DHFR-based selection as was shown to occur in an earlier report following G418-mediated selection which biased results towards enhanced survival (Tey et al., 2000a). BCL-2 and BCL-xL expression profiles could be increased at least 80- to 100-fold and 30- to 40-fold, respectively, by DHFR-based amplification compared to the transgenic control HMNI-1 and the parental CHO-DG44 (HMNIBC-3/4 and HMNIBX-3/4; Figure 12A and 12B). In the non-amplified transgenic cell lines BCL-2 and BCL-xL expression levels were increased less than 10-fold. This protein level was obviously to low for a sufficient protective anti-apoptosis effect (Figure 5). Comparable results were obtained with cells transfected with pBID-sICAM-bcl-xL, an expression vector with a dicistronic configuration. In this set-up translation of sICAM in the first cistron of the transcription unit was initiated in a classical cap-dependent manner and of bcl-xL in the second cistron in cap-independent manner driven by an IRES element derived from the encephalomyocarditis virus. The selectable amplifiable marker dhfr was contained in a separate transcription unit on the same vector.

The detailed analysis of BCL-2 and BCL-xL-mediated anti-apoptosis engineering exemplifies that high level expression of these survival determinants is required in order to exhibit any significant protective effects in CHO-DG44 cells cultivated permanently under serum-free medium conditions.

Example 3: Determination of gene copy numbers by dot blot analysis

Copy numbers of heterologous anti-apoptosis genes bcl-2 or bcl-xL integrated in the genome of the transfected host cells CHO-DG44 are determined by dot blot analysis. For this purpose genomic DNA is isolated from the transgenic and the parental CHO-DG44 cell lines by using the commercially availabe Genomic Blood & Tissue Kit from Qiagen (Hilden, Germany) according to the protocol of the manufacturer. Various amounts of the genomic DNA, usually between 0,5 µg and 15 µg, are applied in duplicate in alkaline buffer to positively charged Hybond N+ nylon membrane (Amersham Biosciences, Freiburg, Germany) using a manifold attached to a suction device (for detailed protocol see Ausubel et al., 1994, updated). The amount of genomic DNA that should be blotted will depend on the relative abundance of the target sequence that will be subsequently sought by hybridization probing. Hybridization analysis is then carried out to determine the abundance of the bcl-2 or bcl-xL sequences in the blotted DNA preparations. Following the protocol of the Gene Images random prime labelling module (Amersham Biosciences, Freiburg, Germany) random-primed FITC-dUTP labelled bcl-2 and bcl-xL specific probes are generated, using either the bcl-2 specific 640 bp fragment obtained by EcoRI digestion of pBID-bcl-2 or the bcl-xL specific 730 bp PvuII/BclI fragment generated from digestion of pBID-bcl-xL as template in the labelling reaction. Hybridisation is performed overnight at 65°C in a hybridization oven according to the Gene Images random prime labelling module protocol. After hybridisation the filter is washed twice with 0.2x SSC/0.1% SDS at 65°C for 20 min before proceeding with the antibody development and the detection according to the Gene Images CDP-Star detection module (Amersham Biosciences). Quantification of the signals is performed using the VDS-CL Imager System (Amersham Biosciences). Signal intensities of bcl-2 or bcl-xL specific signals from the genomic DNA, isolated from the transgenic cells, are compared to a standard curve of defined molecule numbers of either the pBID-bcl-2 or the pBID-bcl-xL expression plasmid (numbers calculated on the basis of the molecular weight of the plasmids), respectively, and are used to estimate the absolute amount of target DNA within the

genomic DNA samples. Hybridization signals from the genomic DNA of the parental cell line CHO-DG44, due to the endogenous bcl-2 and bcl-xL genes, are substracted from the transgenic signals. The copy numbers of the transgenic samples are then divided by the DNA content of each sample to obtain the gene copy number per cell and multiplied by 5 pg, based on 5 pg DNA content per cell. For normalization of DNA contents among samples that are being compared, part of the hamster mbh-1 gene (myc-basic motif homolog 1) is used as an internal control gene probe in a second hybridization reaction on the same filter after stripping the filter of the first probes.

By this method genetically modified host cells generated by introducing and amplifying the bcl-2 or bcl-xL gene (e.g. as described herein) can be determined, comprising at least 5, 10, 20, 50 or 100 copies of a heterologous bcl-2 or bcl-xL gene.

Example 4: Modulation of mitochondria number by serum additives

The obvious requirement for a high BCL-2 or BCL-xL dosage in order to achieve significant survival impact in CHO-DG44 grown under serum-free conditions initiated speculations to whether the mitochondria content and therefore sensitivity to programmed cell death is dependent on serum. This hypothesis is even more convincing as serum has been found to be a key factor for apoptosis protection and because mitochondria are a major platform for cell death modulators (like BCL-2 and BCL-xL).

With a focus on determining the serum-dependence of the specific mitochondria content, CHO-DG44 cells were cultivated either in suspension in serum-free CHO-S-SFMII medium or as adherent cells in CHO-S-SFMII medium supplemented with 10% FCS. Following cultivation for two weeks under stated conditions, the cells of both cultures were profiled for their specific mitochondria content using MitoTracker Green FM, a mitochondrion-specific fluorescent dye. As MitoTracker Green FM accumulates in mitochondria in a membrane potential-independent manner it is a well-established tool for the quantification of these organelles (Metivier et al., 1998). Mitochondria-specific staining was significantly increased in cells cultivated in the absence of serum compared to cells cultivated in the presence of serum. FACS-mediated analysis quantified an up to 3-fold boost in specific mitochondria content (Figure 8). Given the clear amplification of mitochondria under serum-free conditions it is straightforward to suggest that successful anti-apoptosis engineering of serum-independent cell lines requires increased BCL-2

or BCL-xL expression levels to compensate for specific increases in apoptosis machineries associated with these organelles.

Example 5: Cloning of hamster bcl-xL cDNA

The following 5′end primer (Bcl for1: 5′-GCCACCATGT CTCAGAGCAAACCGGGAG-3′; SEQ ID NO: 13) and 3′end primer (Bcl rev1: 5′-TCAYTTCCGACTGAAGAGYGARCC-3′; SEQ ID NO: 14) were designed. These primers were used in a One-Step RT-PCR according to protocol (Invitrogen, Carlsbad, CA) on 1 µg total RNA isolated from the hamster cell line CHO-DG44 (Urlaub et al., 1983) to obtain the hamster homologue of bcl-xL cDNA. The resulting 700 bp cDNA product was subcloned into a TA-type cloning vector (Invitrogen) and the nucleotide sequence of both cDNA strands was determined on an ABI 373A automatic sequencer (Applied Biosystems, Weiterstadt, Germany) using vector specific (M13 reverse, T7) and gene specific primers (Bcl for1 and Bcl rev1) to initiate the extension reaction in the Big Dye Terminator Cycle Sequencing reaction according to the manufacturer's protocol (Applied Biosystems). Homology search against GenBank and EMBL databanks confirmed the identity of the isolated cDNA and that it was comprising the entire coding region of the hamster bcl-xL gene.

In order to determine the true 5′ and 3′ end sequence of the hamster bcl-xL coding region a genomic walking approach was used. For the isolation of the genomic region covering the very 5′ end and 3′end of the coding region, respectively, the following primers, based on the hamster bcl-xL sequence, were designed:

(i) overlapping primers Bcl rev5 (5′-CATCACTAAACTGACTCCAGCTG-3′; SEQ ID NO: 15) and Bcl rev6 (5′-TGACTCCAGCTGTATCCTTTCTG-3′; SEQ ID NO: 16) located downstream of the 5′end of the coding region and with complementarity to the coding strand for the isolation of the 5′end

(ii) overlapping primers Bcl for2 (5′-GACGGGCATGACTGTGGCTG-3′; SEQ ID NO: 17) and Bcl for3 (5′-TGACTGTGGCTGGTGTGGTTCT-3′; SEQ ID NO: 18) located upstream of the 3′end of the coding region with complementarity to the non-coding strand for the isolation of the 3′end.

Adaptor-ligated genomic CHO-DG44 DNA served as template in a nested PCR. The primary PCR was conducted with a combination of primers complementary to the adaptor and a bcl-xL

specific primer (Bcl rev5 or Bcl for2, respectively). A secondary PCR was performed on the primary PCR products with a combination of an inner adaptor primer and a nested bcl-xL specific primer (Bcl rev6 or Bcl for3, respectively). The resulting DNA fragments with sizes between 0.5 kb and 1.8 kb, starting with a known sequence at the bcl-xl primer end and extended into unknown adjacent genomic DNA of various length, were cloned into a TA-type cloning vector (Invitrogen) and further analyzed by sequence analysis. All the overlapping DNA fragments contained either the 5'end or the 3'end of the hamster bcl-xL coding region and in addition sequences of either the 5' or 3' untranslated region. Based on this sequence information the One-Step RT-PCR was repeated this time using in the reaction the hamster specific 5'end primer Eco-Bcl for (5'-TCCGGAATTCGCCACCATGTCTCAGAGCAACC GGGAG-3'; SEQ ID NO: 19) and 3'end primer Xho-Bcl rev (5'-TCCGCTCGAGTCACTTCCGACTGAA GAGAGAGCC-3'; SEQ ID NO: 20). By this way a complete bcl-xL coding region of hamster origin was obtained (Figure 9). The primers Eco-Bcl for and Xho-Bcl rev include in addition to the bcl-xL sequence a restriction enzyme site for EcoRI or XhoI, respectively, for subcloning purposes into the eukaryotic expression vector pBID, resulting in pBID/bcl-xL (Figure 10). This vector, based on the pAD-CMV vector (Werner et al., 1998), mediates constitutive expression of the heterologous genes driven by the CMV promoter/enhancer. In addition, pBID encodes the dhfr mini gene as amplifiable selectable marker (see for example EP 0 393 438).

Example 6: Generation of hamster bcl-xL deletion mutants

Several hamster bcl-xL mutants with different deletions of the non-conserved unstructured loop region were cloned as follows. Briefly, the 5' end and the 3' end of each deletion mutant were generated separately by PCR, joined together via a newly introduced NotI restriction site, cloned directly into the eukaryotic expression vector pBID and confirmed by sequencing analysis. Deleted amino acid residues were thereby uniformely replaced by a sequence of 4 alanines which serve as a linker to bridge the adjacent domains. The expression vector pBID/bcl-xL, containing the hamster bcl-xL wildtype cDNA, was used as template in the PCRs with various primer combinations to generate the required components for the 5' or 3' part of the coding region upstream or downstream of the deleted DNA sequence (Figure 11):

(i) 5' part:

a) vector-specific upstream primer combined with del26 (5′-ATAGTTATGCTGCG GCCGC ACTCCAGCTGTATCCTTTCTGG-3′) (SEQ ID NO: 19)

b) vector-specific upstream primer combined with del46 (5′-ATAGTTATGCTGC GGCCGCCCTCTCTGATTCAGTTCCTTCTG-3′) (SEQ ID NO: 20)

c) vector-specific upstream primer combined with del66 (5′-ATAGTTATGCTGCG GCCGCTACCGCGGGGCTGTCCGCC-3′) (SEQ ID NO: 21)

(ii) 3′ part

a) vector-specific downstream primer combined with del63 (5′-AAGTAAGAAGCG GCCGCAGCAGCGGTAAATGGAGCCACTGGC-3′) (SEQ ID NO: 22)

b) vector-specific downstream primer combined with del83 (5′-AAGTAAGAAGC GGCCGCAGCAGCAGCCGTAAAGCAAGCGCTG-3′) (SEQ ID NO: 23)

For the generation of the deletion mutants pBID/bcl-xLdel26-83 having the nt and aa sequences of SEQ ID NOs: 5 and 6, respectively, pBID/bcl-xLdel46-83 having the nt and aa sequences of SEQ ID NOs: 7 and 8, respectively, pBID/bcl-xLdel66-83 having the nt and aa sequences of SEQ ID Nos: 9 and 10, respectively, and pBID/bcl-xLdel46-63 having the nt and aa sequences of SEQ ID NOs: 11 and 12, respectively, the PCR fragments del26 and del83, del46 and del83, del66 and del83 or del46 and del63, respectively, were joined *via* the introduced NotI restriction site and cloned into the expression vector pBID as EcoRI/SnaBI restriction fragment. Numbers are indicative of the deleted amino acids, e.g. del26-83 means, that in this mutant amino acids 26 to 83 of the hamster wildtype BCL-xL sequence were deleted.

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180 185 190

Gly Ser Leu Phe Ser Arg Lys
195

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actgaatcag agagggagac ccccagtgcc atcaatggca acccatcctg gcacctggcg 180
gacagccccg cggtagcggc cgcagcagca gccgtaaagc aagcgctgag agaggccggc 240
gatgagtttg agctgcggta ccggcgggcg ttcagtgatc taacatccca gcttcatata 300
accccaggga ctgcatatca aagctttgaa caggtagtga atgaactctt ccgggatggg 360
gtaaactggg gtcgcattgt ggcctttttc tccttcggtg gagccctctg tgtggaaagc 420
gtagacaagg agatgcaggt attggtgagt cggatcgcaa gttggatggc cacctacctg 480
aatgaccacc tagagccttg gatccaggac aacggcggct gggacacttt cgtggaactc 540
tacggaaaca atgcagcagc tgagagccgg aaaggccagg agcgcttcaa ccgctggttc 600
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<210> 10
<211> 219
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1 5 10 15

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Leu Ser Gln Lys Gly Tyr Ser Trp Ser Gln Phe Ser Asp Val Glu Glu
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Asn Arg Thr Glu Ala Pro Glu Gly Thr Glu Ser Glu Arg 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 Ala Ala Ala Ala Ala Ala Val Lys Gln Ala Leu Arg Glu Ala Gly
65                  70                  75                  80

Asp Glu Phe Glu Leu Arg Tyr Arg Arg Ala Phe Ser Asp Leu Thr Ser
                85                  90                  95

Gln Leu His Ile Thr Pro Gly Thr Ala Tyr Gln Ser Phe Glu Gln Val
            100                 105                 110

Val Asn Glu Leu Phe Arg Asp Gly Val Asn Trp Gly Arg Ile Val Ala
        115                 120                 125

Phe Phe Ser Phe Gly Gly Ala Leu Cys Val Glu Ser Val Asp Lys Glu
    130                 135                 140

Met Gln Val Leu Val Ser Arg Ile Ala Ser Trp Met Ala Thr Tyr Leu
145                 150                 155                 160

Asn Asp His Leu Glu Pro Trp Ile Gln Asp Asn Gly Gly Trp Asp Thr
                165                 170                 175

Phe Val Glu Leu Tyr Gly Asn Asn Ala Ala Ala Glu Ser Arg Lys Gly
            180                 185                 190

Gln Glu Arg Phe Asn Arg Trp Phe Leu Thr Gly Met Thr Val Ala Gly
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Val Val Leu Leu Gly Ser Leu Phe Ser Arg Lys
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<210> 11
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<220>
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actgaatcag agagggcggc cgcagcagcg gtaaatggag ccactggcca cagcagcagt 180
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gatgagtttg agctgcggta ccggcgggcg ttcagtgatc taacatccca gcttcatata 300
accccaggga ctgcatatca aagctttgaa caggtagtga atgaactctt ccgggatggg 360
gtaaactggg gtcgcattgt ggcctttttc tccttcggtg gagccctctg tgtggaaagc 420
gtagacaagg agatgcaggt attggtgagt cggatcgcaa gttggatggc cacctacctg 480
aatgaccacc tagagccttg gatccaggac aacggcggct gggacacttt cgtggaactc 540
tacggaaaca atgcagcagc tgagagccgg aaaggccagg agcgcttcaa ccgctggttc 600
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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	Arg	Ala	Ala	Ala
		35					40					45			

Ala	Ala	Val	Asn	Gly	Ala	Thr	Gly	His	Ser	Ser	Ser	Leu	Asp	Ala	Arg
	50					55					60				

Glu	Val	Ile	Pro	Met	Ala	Ala	Val	Lys	Gln	Ala	Leu	Arg	Glu	Ala	Gly
65					70					75					80

Asp	Glu	Phe	Glu	Leu	Arg	Tyr	Arg	Arg	Ala	Phe	Ser	Asp	Leu	Thr	Ser
				85					90					95	

Gln	Leu	His	Ile	Thr	Pro	Gly	Thr	Ala	Tyr	Gln	Ser	Phe	Glu	Gln	Val
			100					105					110		

Val	Asn	Glu	Leu	Phe	Arg	Asp	Gly	Val	Asn	Trp	Gly	Arg	Ile	Val	Ala
		115					120					125			

Phe	Phe	Ser	Phe	Gly	Gly	Ala	Leu	Cys	Val	Glu	Ser	Val	Asp	Lys	Glu
	130					135					140				

Met	Gln	Val	Leu	Val	Ser	Arg	Ile	Ala	Ser	Trp	Met	Ala	Thr	Tyr	Leu
145					150					155					160

Asn	Asp	His	Leu	Glu	Pro	Trp	Ile	Gln	Asp	Asn	Gly	Gly	Trp	Asp	Thr
				165					170					175	

Phe Val Glu Leu Tyr Gly Asn Asn Ala Ala Ala Glu Ser Arg Lys Gly
180 185 190

Gln Glu Arg Phe Asn Arg Trp Phe Leu Thr Gly Met Thr Val Ala Gly
195 200 205

Val Val Leu Leu Gly Ser Leu Phe Ser Arg Lys
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<210> 17
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<210> 19
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<210> 20
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<210> 22
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<210> 24
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<210> 25
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<220>
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<400> 25
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CLAIMS

1. A hamster host cell genetically modified by introducing nucleic acid sequences that encode for an anti-apoptosis gene, a selectable amplifiable marker gene, and at least one gene of interest.

2. A host cell of claim 1, wherein the host cell is a Chinese Hamster Ovary (CHO) cell or a Baby Hamster Kidney (BHK) cell.

3. A host cell according to claim 1 or 2, wherein the host cell is a CHO-DG44, CHO-K1, CHO-DUKX, CHO-DUKX B1, CHO Pro-5, V79, B14AF28-G3, BHK-21, BHK TK$^-$, HaK, or BHK-21(2254-62.2) cell, or the progeny of any of such cell line.

4. A murine myeloma cell genetically modified by introducing nucleic acid sequences that encode for an anti-apoptosis gene, a selectable amplifiable marker gene, and at least one gene of interest.

5. A host cell of claim 4, wherein the host cell is a NS0 or SP2/0-Ag14 cell, or the progeny of any of such cell line.

6. A host cell according to any of claims 1 to 5, wherein the anti-apoptosis gene encodes for a member of the Bcl-2 superfamily that can act as cell death repressor.

7. A host cell according to any one of claims 1 to 6, wherein the anti-apoptosis gene encodes for BCL-xL, BCL-2, BCL-w, BFL-1, A1, MCL-1, BOO, BRAG-1, NR-13, CDN-1, CDN-2, CDN-3, BHRF-1, LMW5-HL or CED-9.

8. A host cell according to any one of claims 1 to 6, wherein the anti-apoptosis gene encodes for BCL-xL or BCL-2.

9. A host cell according to any one of claims 1 to 7, wherein the anti-apoptosis gene has the sequence of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9 or SEQ ID NO:11, or any biologically active fragment, variant, or derivative thereof.

10. A host cell according to any one of claim 1 to 7, encoding for BCL-xL.

11. A host cell according to any one of claims 1 to 7, wherein the anti-apoptosis gene has the sequence of SEQ ID NO:1, , SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9 or SEQ ID NO:11, or any biologically active fragment, variant, or derivative thereof.

12. A host cell according to any one of claims 1 to 9, wherein the selectable amplifiable marker gene encodes for dihydrofolate reductase (DHFR), glutamine synthetase, CAD, adenosine

deaminase, adenylate deaminase, UMP synthetase, IMP 5'-dehydrogenase, xanthine guanine phosphoribosyl transferase, HGPRTase, thymidine kinase, thymidylate synthetase, P glycoprotein 170, ribonucleotide reductase, asparagine synthetase, arginosuccinate synthetase, ornithine decarboxylase, HMG CoA reductase, acetylglucosaminyl transferase, threonyl-tRNA synthetase or Na^+K^+-ATPase.

13. A host cell according to any one of claims 1 to 12, wherein the anti-apoptosis gene encodes for BCL-xL and the selectable amplifiable marker gene for DHFR, glutamine synthetase, CAD, adenosine deaminase, adenylate deaminase, UMP synthetase, IMP 5'-dehydrogenase, xanthine guanine phosphoribosyl transferase, HGPRTase, thymidine kinase, thymidylate synthetase, P glycoprotein 170, ribonucleotide reductase, asparagine synthetase, arginosuccinate synthetase, ornithine decarboxylase, HMG CoA reductase, acetylglucosaminyl transferase, threonyl-tRNA synthetase or Na^+K^+-ATPase

14. A host cell according to any of claims 1 to 12, wherein the anti-apoptosis gene encodes for BCL-xL and the selectable amplifiable marker gene for DHFR.

15. A host cell according to any of claims 1 to 14, wherein the anti-apoptosis gene, the selectable amplifiable marker gene and the gene(s) of interest are operatively linked to at least one regulatory sequence allowing for expression of said genes.

16. A method of expressing an anti-apoptosis gene, an selectable amplifiable marker gene and at least one gene of interest in a mammalian host cell comprising:

(a) introducing into a hamster host cell population the nucleic acid sequences that encode for an anti-apoptosis gene, a selectable amplifiable marker gene, and a gene(s) of interest, wherein said genes are operatively linked to at least one regulatory sequence allowing for expression of said genes;

(b) cultivating said host cell population under conditions where said genes are expressed.

17. A method of generating mammalian host cells showing an enhanced expression level of an anti-apoptosis gene comprising:

(a) introducing into a mammalian host cell population nucleic acid sequences that encode for an anti-apoptosis gene, a selectable amplifiable marker gene, and optionally at least one gene of interest, wherein said genes are operatively linked to at least one regulatory sequence allowing for expression of said genes;

(b) cultivating said cell population under conditions where at least said selectable amplifiable marker gene and said anti-apoptosis gene are expressed, and which are favourable for obtaining multiple copies at least of the anti-apoptosis gene;

(c) selecting cells from the cell population that incorporate multiple copies at least of the anti-apoptosis gene.

18. The method of claim 17, wherein the gene(s) of interest is introduced into the mammalian host cell population.

19. The method of any one of claims 16 to 18, wherein the mammalian host cells are murine myeloma or hamster cells.

20. The method of claim 19, wherein the hamster cells are Chinese Hamster Ovary (CHO) cells or Baby Hamster Kidney (BHK) cells.

21. The method of any one of claims 16 to 18, wherein the mammalian host cell is NS0 or SP2/0-Ag14 cell.

22. The method of any one of claims 15 to 19, wherein the anti-apoptosis gene encodes for BCL-xL, BCL-2, BCL-w, BFL-1, A1, MCL-1, BOO, BRAG-1, NR-13, CDN-1, CDN-2, CDN-3, BHRF-1, LMW5-HL or CED-9.

23. The method of any one of claims 16 to 22, wherein the anti-apoptosis gene encodes for BCL-xL or BCL-2.

24. The method of any one of claims 16 to 22, wherein the anti-apoptosis gene has the sequence of SEQ ID NO:1 or SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9 or SEQ ID NO:11, or any functional fragments, variants or mutants (degenerative and non degenerative) thereof.

25. The method of any one of claims 16 to 22, wherein the anti-apoptosis gene encodes for BCL-xL.

26. The method of any one of claims 16 to 22, wherein the anti-apoptosis gene has the sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9 or SEQ ID NO:11, or any functional fragments, variants or mutants (degenerative and non degenerative) thereof.

27. The method of any one of claims 16 to 26, wherein the selectable amplifiable marker gene encodes for the dihydrofolate reductase (DHFR), glutamine synthetase, CAD, adenosine deaminase, adenylate deaminase, UMP synthetase, IMP 5'-dehydrogenase, xanthine guanine phosphoribosyl transferase, HGPRTase, thymidine kinase, thymidylate synthetase, P glycoprotein 170, ribonucleotide reductase, asparagine synthetase, arginosuccinate synthetase, ornithine decarboxylase, HMG CoA reductase, acetylglucosaminyl transferase, threonyl-tRNA synthetase or Na^+K^+-ATPase.

28. The method of any one of claims 16 to 21, wherein the anti-apoptosis gene encodes for BCL-xL and the selectable amplifiable marker gene for DHFR.

29. A method of generating a mammalian host cell comprising:

(a) introducing into a mammalian host cell population an anti-apoptosis gene and the DHFR gene;

(b) amplifying the anti-apoptosis gene in the presence of methotrexate.

30. The method of claim 29, wherein the anti-apoptosis gene encodes for BCl-2 or BCl-xL.

31. The method of claim 29, wherein the anti-apoptosis gene encodes for BCl-xL.

32. The method of any of claims 29 to 31, wherein the host cells are murine myeloma or hamster cells.

33. The method of any of claims 29 to 31, wherein the hamster cells are Chinese Hamster Ovary (CHO) cells or Baby Hamster Kidney (BHK) cells.

34. Host cells obtainable by a method of any one of claims 16 to 33.

35. A method for inhibiting or delaying cell death in a host cell, comprising cultivating host cells according to any of claims 1 to 15 or 34 under conditions where at least the anti-apoptosis gene is expressed in such that cell death is inhibited or delayed in said host cells.

36. The method according to claim 35, wherein the cell death is caused by programmed cell death.

37. The method of claim 35, wherein the cell death is caused by apoptosis.

38. The method according to any one of claims 16 to 33 or 35 to 37, wherein the cells are cultivated in a serum and/or protein free culture medium.

39. A process for producing a protein of interest in a host cell, comprising:

(a) cultivating cells according to any of claims 1 to 15 or 34 under conditions which are favourable for the expression of the anti-apoptosis gene and the gene(s) of interest;

(b) isolating the protein of interest from the cells and/or the cell culture supernatant.

40. Use of a cell according to any of claims 1 to 15 or 34 for production of at least one protein encoded by a gene of interest.

41. A host cell comprising at least 5 copies of a heterologous anti-apoptosis gene.

42. A host cell comprising at least 10 copies of a heterologous anti-apoptosis gene.

43. A host cell comprising at least 20 copies of a heterologous anti-apoptosis gene.

44. A host cell comprising at least 50 copies of a heterologous anti-apoptosis gene.

45. A host cell comprising at least 100 copies of a heterologous anti-apoptosis gene.

46. A host cell according to any of claims 37 to 41, wherein the anti-apoptosis gene encodes for BCL-xL, BCL-2, BCL-w, BFL-1, A1, MCL-1, BOO, BRAG-1, NR-13, CDN-1, CDN-2, CDN-3, BHRF-1, LMW5-HL or CED-9.

47. A host cell according to any of claims 41 to 45, wherein the anti-apoptosis gene is BCL-xL or BCL-2.

48. A host cell according to any of claims 41 to 45, wherein the anti-apoptosis gene has the sequence of of SEQ ID NO:1 or SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9 or SEQ ID NO:11, or any functional fragments, variants or mutants (degenerative and non degenerative) thereof.

49. A host cell according to any of claims 41 to 45, wherein the anti-apoptosis gene is BCL-xL.

50. A host cell according to any of claims 41 to 49, wherein the host cell is a murine hybridoma or hamster cell.

51. A host cell according to any of claims 41 to 49, wherein the host cell is a Chinese Hamster Ovary (CHO) cell or a Baby Hamster Kidney (BHK) cell.

52. A host cell according to claim 50, wherein the murine myeloma cell is a NS0 or SP2/0-Ag14 cell, or the progeny of any of such cell line.

53. A host cell according to any of claims 41 to 49, wherein the host cell is a CHO-DG44, CHO-K1, CHO-DUKX, CHO-DUKX B1, CHO Pro-5, V79, B14AF28-G3, BHK-21, BHK TK$^-$, HaK, BHK-21(2254-62.2), or the progeny of any of such cell line.

54. A host cell according to any of claims 41 to 53, further comprising at least one heterologous gene of interest.

55. A host cell according to any of claims 1 to 15, or 34 comprising at least 5 copies of the heterologous anti-apoptosis gene.

56. A host cell according to any of claims 1 to 15, or 34 comprising at least 10 copies of the heterologous anti-apoptosis gene.

57. A host cell according to any of claims 1 to 15, or 34 comprising at least 20 copies of the heterologous anti-apoptosis gene.

58. A host cell according to any of claims 1 to 15, or 33 comprising at least 50 copies of the heterologous anti-apoptosis gene.

59. A host cell according to any of claims 1 to 15, or 34 comprising at least 100 copies of the heterologous anti-apoptosis gene.

60. A DNA comprising a nucleic acid sequence encoding a biologically active bcl-xL gene, wherein the nucleic acid is:

(a) a nucleic acid having the sequence of SEQ ID NO:3, or the complementary strand thereof;

(b) functional fragments, variants or mutants (degenerative and non degenerative) of the nucleic acid sequence defined in (a);

(c) a nucleic acid having at least 95% homology to the nucleic acid sequence defined in (a); or

(d) a nucleic acid which hybridizes to any of the nucleic acid sequences defined in (a), (b), or (c) under stringent conditions.

61. A DNA comprising a nucleic acid sequence encoding a biologically active bcl-xL gene, wherein the nucleic acid is:

(a) a nucleic acid having the sequence of SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, or the complementary strand of any of those;

(b) functional variants or mutants (degenerative and non degenerative) of any of the nucleic acid sequences defined in (a);

(c) a nucleic acid having at least 95% homology to any of the nucleic acid sequences defined in (a); or

(d) a nucleic acid which hybridizes to any of the nucleic acid sequences defined in (a), (b), or (c) under stringent conditions.

62. A DNA comprising a nucleic acid sequence encoding a biologically active bcl-xL gene having the sequence of SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9 or SEQ ID NO:11.

63. A polypeptide encoded by a DNA according to any of claims 60 to 62.

64. A host cell comprising a DNA according to claims 60 to 62.