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Regulated overexpression of the survival factor *bcl-2* in CHO cells increases viable cell density in batch culture and decreases DNA release in extended fixed-bed cultivation

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Abstract

Using multicistronic expression technology we generated a stable Chinese hamster ovary (CHO) cell line (MG12) expressing a model secreted heterologous glycoprotein, the secreted form of the human placental alkaline phosphatase (SEAP), and *bcl-2*, best known as an apoptosis inhibitor, in a tetracycline-repressible dicistronic configuration. In batch cultivations in serum-containing medium, MG12 cells reached twice the final viable cell density when Bcl-2 was overexpressed (in the absence of tetracycline) compared to MG12 populations cultured under tetracycline-containing conditions (*bcl-2* repressed). However, *bcl-2*-expressing MG12 cells showed no significant retardation of the decline phase compared to batch cultures in which the dicistronic expression unit was repressed. Genetic linkage of *bcl-2* expression with the reporter protein SEAP in our multicistronic construct allowed online monitoring of Bcl-2 expression over an extended, multistage fixed-bed bioreactor cultivation. The cloned multicistronic expression unit proved to be stable over a 100 day bioreactor run. CHO MG12 cells in the fixed-bed reactor showed a drastic decrease in the release of DNA into the culture supernatant under conditions of reduced tetracycline (and hence derepressed SEAP and *bcl-2* overexpression). This observation indicated enhanced robustness associated with *bcl-2* overexpression, similar to recent findings for constitutive Bcl-2-overexpressing hybridoma cells under the same bioprocess conditions. These findings indicate, in these serum-containing CHO cell cultures, that overexpression of Bcl-2 results in desirable modifications in culture physiology.

Nomenclature

c_i = substrate or product concentration in the reactor (mmol l^{-1})
c_{i0} = substrate or product concentration in the feed (mmol l^{-1})
q_i^* = substrate uptake or metabolite production rate per volume conditioning vessel (mmol l^{-1} h^{-1})
$q_{i,FB}^*$ = substrate uptake or metabolite production rate per volume fixed-bed (mmol l_{FB}^{-1} h^{-1})
V_{FB} = volume of the fixed-bed (l)
V_C = volume of the conditioning vessel (l)
D_{FB} = dilution rate related to the fixed-bed volume (d^{-1})
D = dilution rate related to the conditioning vessel (d^{-1})
t = time (d)

Introduction

Chinese Hamster ovary cells are the most important animal cell hosts for industrial manufacturing of protein pharmaceuticals. Understanding of the mechanism and kinetics for both cell growth and cell death

in various culture systems is of central importance for the optimization of recombinant protein production. While the parameters for cell growth are well established for many production cell lines and bioreactor conditions, prevention of cell death has only recently attracted much interest in the bioengineering community (de la Broise et al., 1991; Duval et al., 1991; Al-Rubeai et al., 1995; Cotter and Al-Rubeai, 1995; Mastrangelo and Betenbaugh, 1998; Dickson, 1998).

Contemporary basic research has revealed an intrinsic, genetically determined program which commits a cell to die in response to physiological constraints such as serum, nutrient and oxygen depletion. Programmed cell death, or apoptosis, is a complex and active energy-dependent biochemical network controlled by an enzymatic cascade and intracellular signal transduction circuits (Hale et al., 1996; Cohen, 1997; Newton and Strasser, 1998; Fussenegger and Bailey, 1998; Fussenegger et al., 1998c). Cell death by apoptosis differs from 'classical' cell death or necrosis, whereby the cells die by total disintegration and release the cellular content into the surrounding area. Apoptosis instead involves distinct morphological characteristics such as volume loss by condensation of chromatin (accompanied by DNA breakdown) and cytosplasmatic organelles, disintegration of the nucleus, and the bulging out of membrane blebs (apoptotic bodies) containing cytoplasmatic components. *In vivo* these apoptotic bodies are phagocytosed by the surrounding tissue, but under cell culture conditions they eventually lyse by a process called secondary necrosis.

Hybridoma cells have been reported to be very sensitive to apoptosis, particularly when they have reached their final cell density and enter the decline phase (de la Broise et al., 1991; Mercille and Massie, 1994). Additionally, cell culture additives which arrest cell growth were shown to enhance antibody production of hybridoma cells but also to induce apoptosis (Suzuki and Ollis, 1990; Franek and Dolnikova 1991; Al-Rubeai et al., 1992; Terada et al., 1997; Franek et al., 1998). Given the negative effect of programmed cell death for hybridoma culture productivity these cell types became the paradigm for apoptosis suppression by metabolic engineering.

Bcl-2 is the first key negative regulator of apoptosis which has been described (Tsujimoto and Croce, 1986). Since its discovery, many homologous Bcl-2-like family members have been identified, some of which inhibit (Bcl-2, Bcl-x_L, Bcl-w, A1, Mcl-1) or promote programmed cell death (Bax, Bak, Bad, Bcl-x_S) (Adams and Cory, 1998; Fussenegger and Bailey, 1998; Newton and Strasser, 1998). All of these *bcl-2*-like proteins form a complex rheostat-like intrafamily network in which the sum of the positive and negative interactions determines the apoptotic response to physiological stresses. Bcl-2 is intimately linked with the cell cycle since the central cell-cycle regulator and tumor suppressor gene p53 acts as higher order regulator of apoptosis by repressing *bcl-2* expression while inducing *bax* (Ko and Prives, 1996). Although the role of Bcl-2 in the signalling network seems to be well established at the molecular level, the target genes and mechanisms by which Bcl-2 effects apoptosis suppression remain somewhat obscure. Linked with the remaining secrets of Bcl-2 is its subcellular localization to nuclear membrane as well as to the membranes of the endoplasmatic reticulum and the mitochondria, a feature which *bcl-2* shares with *bcl-x_L* (Chen-Levy and Cleary, 1990; De Jong et al., 1992; Adams and Cory, 1998; Thornberry and Lazebnik, 1998). Although the significance of the membrane localization to cell death has been unclear, there is now good evidence that the mitochondria may play a pivotal role in a cell death pathway (Green and Reed, 1998). The connection between the anti-apoptotic function of Bcl-2 and Bcl-x_L and their localization at the mitochondrial membrane was recently suggested to reside in a block of cytochrome C release from mitochondria (Kluck et al., 1997; Yang et al., 1997; Kharbanda et al., 1997; Adams and Cory, 1998; Green and Reed, 1998). The release of cytochrome C has recently been shown to be sufficient for the activation of initiating procaspases such as caspase-9 or caspase-3 which in turn activate a nuclear endonuclease responsible for DNA fragmentation as well as PAK2, a kinase which appears to be required for plasma membrane blebbing and the formation of apoptotic bodies (Liu et al., 1997; Rudel and Bokoch, 1997).

Bcl-2-based metabolic engineering has been successfully used to suppress apoptosis in many cell lines in response to various chemical, genetic, biochemical and viral apoptosis-inducing conditions (Balajthy et al., 1997; Camilleri-Broet et al., 1998; Chung et al., 1997; He et al., 1996; Mastrangelo and Betenbaugh, 1998; Meikrantz et al., 1998; Reynolds et al., 1996a, b; Zhang et al., 1995). Also, high level expression of human *bcl-2* resulted in better survival of mouse myeloid cells in the absence of growth factors (Tsujimoto, 1989; Vaux et al., 1988). Deregulated *bcl-2* expression prolonged survival of growth factor-deprived hemopoietic cell lines (Nunez et al., 1990)

and protects human B cells from a variety of physiological stress conditions (Tsujimoto, 1989; Miyashita and Reed, 1992). Furthermore, Bcl-2 was extremely successful in suppressing apoptosis in hybridoma cell cultures, leading sometimes to enhanced specific antibody production (Itoh et al., 1995; Fassnacht et al., 1998a) or enabling production technology by the addition of growth-inhibiting cell culture additives (Terada et al., 1997). However, *bcl-2* may not be a universal apoptosis inhibitor, and its potential and function may vary among different cell lines. For example, Bcl-2 showed no apoptosis-suppressing activity in NSO myeloma cells (Murray et al., 1996).

In contrast to hybridoma cells, cytostatic culture technology implemented in CHO cells by the conditional overexpression of p27 or p21 lead to G1-phase-specific growth arrest and increased heterologous protein production, but the induction of apoptosis was not observed in the arrested CHO cell cultures (Fussenegger et al., 1997; Fussenegger et al., 1998b; Mazur et al., 1998). However, CHO cells in batch culture have been reported to die almost exclusively via apoptosis (Moore et al., 1995). In this study, we combine regulated gene expression and multicistronic expression technology to assess the effect of Bcl-2 in CHO cells under batch culture conditions and in fixed-bed reactors in an absolutely isogenic background and in the absence of chemical, genetic and biochemical apoptosis inducers against most of which Bcl-2 has previously been reported to provide some protection (Balajthy et al., 1997; Camilleri-Broet et al., 1998; Chung et al., 1997; He et al., 1996; Meikrantz et al., 1988; Reynolds et al., 1996a, b; Zhang et al., 1995).

Regulated expression technology eliminates secondary effects of clonal variation commonly encountered by classical polyclonal or single-clone analyses. Furthermore, isogenic metabolic engineering assessments achieved by regulated gene expression reduces genetic drift in long-term, reactor cultivations.

Materials and methods

Cell culture and transfection

For the generation of stable cell lines Chinese hamster ovary cells (CHO-K1, ATCC: CCL 61) were cultured in FMX-8 medium (Dr. F. Messi Cell Culture Systems, Switzerland) supplemented with 10% fetal calf serum (FCS, Boehringer Mannheim, Lot Nr. 14713602). The cell line XMK1-9 is a derivative of CHO-K1 which constitutively expresses the tetracycline-responsive transactivator tTA (Fussenegger et al., 1997; Fussenegger et al., 1998b; Mazur et al., 1998). XMK1-9 was cultured in FMX-8 medium that also contained 400 μg/ml of G418 (GIBCO BRL, Life Technologies). For repression of the tetracycline regulatable promoter $P_{hCMV*-1}$ (Gossen and Bujard, 1992), 2 μg/ml of tetracycline (Sigma) was used. For high efficiency transfection, a modified $CaPO_4$ protocol was used (P. Umaña et al., in preparation). For batch cultures and cultivation in the fixed-bed reactor a 1:1 mixture of Iscove's MDM and Ham's F12 (GIBCO BRL, Life Technologies) supplemented with 2 mmol l^{-1} L-glutamine (GIBCO BRL, Life Technologies), 2 g l^{-1} $NaHCO_3$, 0.01% PEG, 50 mmol l^{-1} ethanolamine, 6 mg l^{-1} puromycin, 400 mg l^{-1} G418 (Calbiochem) and 3% (v/w) horse serum (GIBCO BRL, Life Technologies; Lot Nr. 30A1045D) was used. The trypan blue dye exclusion method was used to distinguish viable from dead cells on the basis of membrane integrity. Cells that exclude trypan blue have an intact membrane and are viable (MI) whereas cells which take this dye up show a disrupted membrane which is indicative of dead cells. The membrane intact index was calculated as the ratio of cells with an intact membrane to total cell number (Simpson et al., 1997; Fassnacht et al., 1998a).

The eukaryotic expression vector pSFFVNeo-bcl2 encoding the human anti-apoptosis factor Bcl-2 has been reported by Tsujimoto and Croce (1986). *bcl-2* was released by restriction of pSFFVNeo-bcl2 with *Eco*RI and was subsequently cloned into the *Eco*RI site of pBluescript^{circR} II SK$^-$ placing this gene under the control of the *lacZ* promoter to give plasmid pMF137. *bcl-2* was excised from pMF137 as a *Not*I/*Sal*I fragment and ligated to the corresponding sites of pSBC2 resulting in pMF147 (Dirks et al., 1993).

Enzymatic assays

The SEAP concentration in the culture supernatant was determined by measuring the kinetics of the hydrolysis of p-nitrophenolphosphate (Sigma) for 10 min at 30 °C and 405 nm in a 96-well multiplate reader (Spectra Thermo, SLT Laborinstrumente)(adapted from Berger et al., 1988). 80 μl of sample were mixed with 100 μl of SEAP buffer (20 mM L-homoarginine, 2 M diethanolamine, 1 mM $MgCl_2$, pH 9.8). 20 μl of substrate was added immediatelly prior to measurement. SEAP activity was measured in units per liter. One unit is defined as the amount of SEAP which

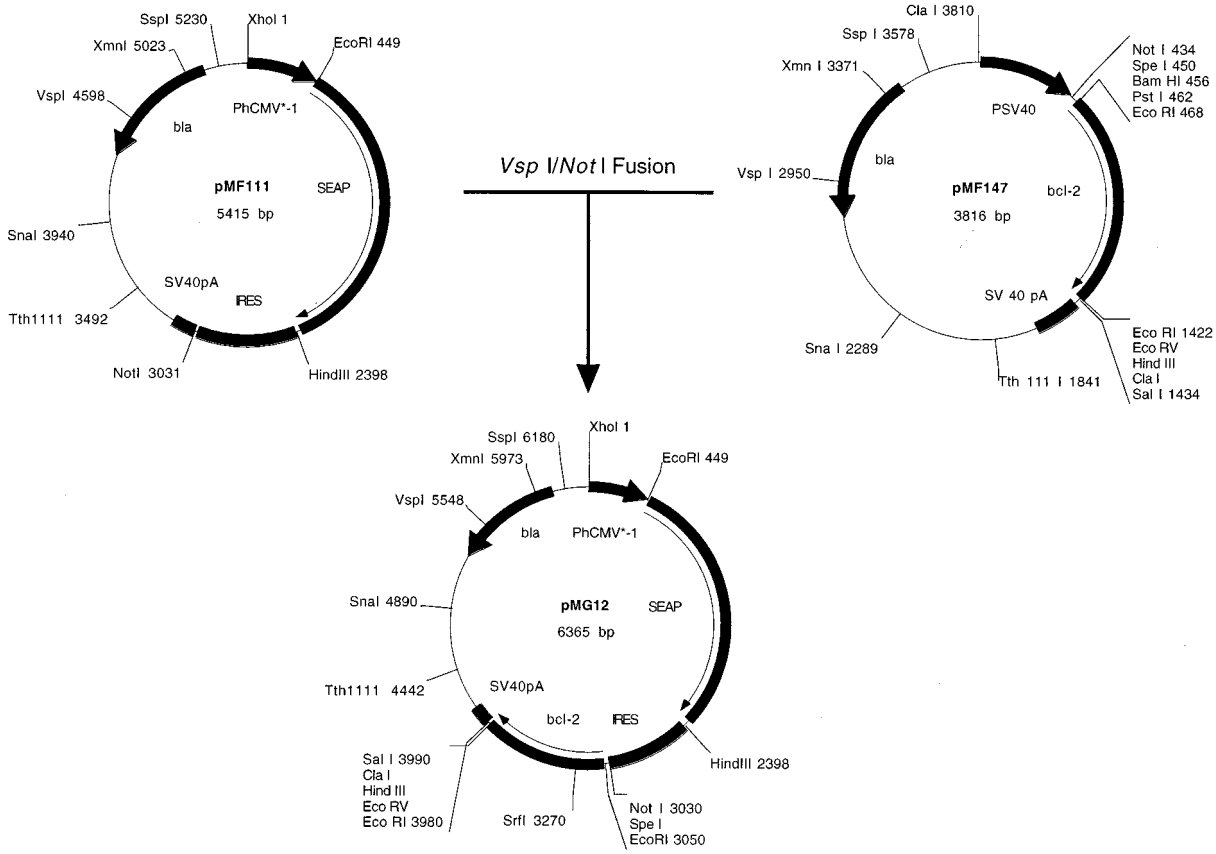

Figure 1. Construction of the dicistronic expression vector pMG12. pMG12 was fused from two monocistronic expression vectors pMF111 and pMF147 and contains a dicistronic expression unit under control of the tetracycline-repressible promoter $P_{hCMV^{*}-1}$. The dicistronic expression unit encodes the model product gene *seap* and the survival gene *bcl-2* which are transcribed into a single mRNA. Both genes are separated by the internal ribosomal entry site (*IRES*) which mediates cap-independent translation initiation of the second, *bcl-2* encoding cistron and enables simultaneous and coordinated expression of *seap* and *bcl-2*.

hydrolyze 1 nmol of p-nitrophenylphosphate per min. A concentration of 1 mU per liter corresponds to an increase of 0.028 ΔE per minute given a liquid height of 0.7 cm (Berger et al., 1988).

Glucose and lactate were determined with a YSI-Analyzer 2700 (YSI, U.S.A.). Glutamine and ammonia were analyzed enzymatically according to the manufacturer's protocols (Boehringer Mannheim, Germany).

Western blot analysis

Tetracycline-responsive expression of *bcl-2* coordinated with SEAP expression in MG12 cells was confirmed by Western Blot analysis. 10^6 cells were detached from the culture vessel, washed twice with PBS and resuspended in lysis buffer (1% IGEPAL (Sigma, CA-630), 150 mM NaCl, 50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM PMSF, 21 μg/ml aprotinin, 10 μg/ml leupeptin) for 30 min on ice (adapted from Behrens et al, 1994). The cells were then sonified twice for 1 min on ice before the cell lysate was centrifuged at 14 000 $\times$ g for 10 min at 4 °C. The protein concentration of the supernatant was determined using a BCA* protein assay (Pierce) according to the manufacturer's protocol. Equal protein concentrations were applied on a 12.5% polyacrylamide-SDS gel and the proteins were subsequently blotted onto nitrocellulose membranes using a Bio-Rad electroblotter (Sambrook et al., 1989). The blot was developed using the primary monoclonal antibody against human Bcl-2 (purified mouse monoclonal anti-bcl-2 IgG lot# 9612009; Ancell Corp. (Bayport, MN, U.S.A.) and the ECL Western Blot kit (Amersham) using the manufacturer's suggested protocol.

Table 1. Plasmids used in this study

Plasmid	Description	Reference or source
pTBC1	Cloning vector containing the tetracycline-regulatable promoter $P_{hCMV*-1}$	Dirks et al., 1994
pSBC2	Cloning vector	Dirks et al., 1993
pSFFVNeo-bcl2	pSFFV-Neo expression vector containing the human *bcl-2* gene as an *Eco*RI fragment	Tsujomoto and Croce, 1986
pMF111	pTBC1containing *seap* as *Eco*RI/*Hind*III fragment of pSBC2-SEAP	Fussenegger et al., 1997a
pMF137	pSSFNeo-bcl2 was restricted with *Eco*RI and the *bcl-2* containing fragment was cloned into pBluescript® II SK$^-$(*Eco*RI)	Fussenegger et al., 1998
pMF147	pSBC2 containing the *bcl-2* gene as *Not*I/*Sal*I fragment of pMF137	This work
pMG12	Dicistronic tet-regulatable SEAP-Bcl-2 expression vector; *Not*I/*Vsp*I fusion of pMF137 and pMF147	This work

Measurement of the DNA concentration in the culture supernatant

Since dead and damaged cells release their chromosomal DNA, the time course of DNA concentration in the supernatant is indicative of the extent of cell death (Sramkova et al., 1997). The DNA concentration in the cell culture supernatant was determined by Hoechst-33258 (Sigma) staining. 100 μg of Hoechst-33258 were dissolved in Ca^{2+}- and Mg^{2+}-free PBS. 13 μl of this stock solution was added to 1050 μl Tris-buffer (250 mmol l^{-1}, pH 8) and 250 μl of culture supernatant. The fluorescence of the DNA-fluorochrome complex, excited at 353 nm, was measured at 460 nm on a spectrofluorometer (Shimadzu, RF-540). The DNA concentration was determined using a calibration curve.

Apoptosis assays

The terminal stage of apoptosis includes degradation of chromosomal DNA at nucleosomal intervals ('DNA ladder'. Therefore, chromosomal DNA of CHO cells was isolated as follows: 3.5×10^5 cells were incubated in 100 μl PBS containing 100 μg DNAse-free RNAse A (Boehringer Mannheim) for 1 h at room temperature. The DNA was extracted twice using first 50 μl phenol and 50 μl of a chloroform/iso-amylic alcohol mixture (24:1) and subsequently 100 μl of the chloroform/iso-amylic alcohol mixture (24:1). The DNA in the aqueous supernatant was then precipitated with 10 volumes isopropanol and 1 volume sodium acetate (3 M, pH 5.2) for 10 min at –20 °C. The precipitated DNA was collected by centrifugation at 10 000 × g for 15 min, the pellet was washed twice with ice-cold 70% ethanol and resuspended in 20 μl water. In order to monitor the ladder-like degradation of the DNA following induction of the cellular apoptosis program, the DNA was visualized by agarose gel electrophoresis as described before (Sambrook et al., 1989). Apoptosis was also quantified using an ApoAlert^{TM} CPP32/Caspase-3 assay kit (Clontech) according to the manufacturer's protocol.

Cultivations in the fixed-bed reactor

For extended cultivation of the CHO cell line MG12, a fixed-bed reactor (Meredos, Germany) was used. The fixed-bed reactor setup consists of a 1 liter conditioning vessel and a 50 ml fixed-bed in which the cells are immobilized on porous glass beads (3 to 5 mm in diameter (Siran, Schott, Germany)), a carrier which proved to be particularly suitable for cultivation of suspendable cells such as transfectoma or hybridoma as well as for adherent cells including CHO-K1 (Sràmkovà et al., 1995; Lüdemann et al., 1996; Pörtner et al., 1997). The fixed-bed is integrated in the conditioning vessel which allows simultaneous temperature control and maintenance of both subsystems at 37 °C by electrical heating. Between the fixed-bed and the conditioning vessel, a closed medium circuit is established with medium being pumped from the conditioning vessel through the fixed-bed (axially) and back into the conditioning vessel. The maximum superficial flow velocity in the fixed-bed was constantly kept below 0.9 mm s^{-1} to minimize cell washout from the fixed-bed. This superficial flow velocity was determined by dividing the volumetric flow rate by the cross sectional area of the fixed-bed (20 cm^2). The dissolved oxygen in the conditioning vessel (equipment by Ingold, Germany) was maintained between 75 and 85% of air saturation by regulating the gassing rate. pH was measured and automatically controlled by the addition of CO_2 to the air used for bubble aeration

(electrode CPS11-1DA4GSA, Endress + Hauser, Germany). Sampling and medium exchange during continuous operation were carried out in the conditioning vessel.

For inoculation, the circulation between the conditioning vessel and the fixed-bed was closed. The cells were filled into a bottle which was connected to the fixed-bed by a separate circulation loop. The cells were then slowly pumped through the fixed-bed for approximately 2 h until the inoculation suspension in the bottle became clear. The inoculation circuit was then closed and the circulation between the conditioning vessel and the fixed-bed was re-opened. The fixed-bed was inoculated with 1×10^6 cells per ml fixed-bed which corresponds to a total cell number of 5×10^7 cells.

The following equations were applied for the fixed-bed reactor:

$$\frac{dc_i}{dt} = D\cdot(c_{i0} - c_i) + q_i^* \qquad (1)$$

D is the dilution rate of medium in the conditioning vessel, c_i the substrate or product concentration, c_{i0} the substrate or product feed concentration and q_i^* indicates the production rate of component i per volume conditioning vessel.

The dilution rate and the substrate consumption or metabolite production rates per volume fixed-bed are defined in Equations (2) and (3). V_C is the volume of the conditiong vessel and V_{FB} the volume of the fixed-bed.

$$D_{\mathrm{FB}} = \frac{V_C}{V_{\mathrm{FB}}}\cdot D \qquad (2)$$

$$q^*_{i,\mathrm{FB}} = \frac{V_C}{V_{\mathrm{FB}}}\cdot q_i^* \qquad (3)$$

Using Equations (1), (2) and (3), the following Equations (4) are obtained in case of a steady-state assuming ideal mixing in the conditioning vessel.

$$\frac{dc_i}{dt} \stackrel{!}{=} 0 \Rightarrow \begin{array}{l} D\cdot(c_i - c_{i0}) + q_i^* \\ D_{\mathrm{FB}}\cdot(c_i - c_{i0}) + q^*_{i,\mathrm{FB}} \end{array} \qquad (4)$$

Results

Construction of a dicistronic, tetracycline-regulatable expression unit encoding the model product protein SEAP and the survival factor Bcl-2

Dicistronic expression systems enable simultaneous and coordinated expression of two independent genes from a single promoter. While the translation of the first cistron is mediated by classical cap-dependent translation, translation-initation of the second cistron relies on a genetic element of polioviral origin the internal ribosomal entry sites (*IRES*). By *Vsp*I/*Not*I-mediated fusion of the two independent expression vectors pMF111, encoding the model product gene the human placental alkaline phosphatase (SEAP; Fussenegger et al., 1997) and pMF147, encoding the Bcl-2, pMG12 was generated which harbors SEAP-bcl-2 as a single dicistronic expression unit under control of the tetracycline-repressible promoter $\mathrm{P_{hCMV^*-1}}$ (Figure 1). Activity as well as tetracycline-repressible expression of pMG12 was tested by transient transfection of this dicistronic expression vector into the CHO-K1 derivative XMK1-9 which was previously engineered to constitutively express the tetracycline-responsive transactivator (tTA; Fussenegger et al., 1997). In all cases the expression of SEAP and *bcl-2* in transfected CHO populations was strictly correlated and tightly repressed by the addition of the external stimulus tetracycline ($\mathrm{Tet_{off}}$) as confirmed by enzymatic SEAP assays and *bcl-2*-directed immunofluorescence (data not shown).

Construction of a CHO cell line which stably expresses the dicistronic, tetracycline-regulatable SEAP-bcl-2 expression unit

Plasmid pMG12 was cotransfected with pPur, an expression vector encoding resistance against the antibiotic puromycin, into XMK1-9 overexpressing the tetracycline-responsive transactivator. A mixed population was selected in 13 mg $\mathrm{l^{-1}}$ puromycin and single clones were then generated by two subsequent rounds of limiting dilution. Since constitutive expression of both genes was shown to have no deleterious effects in various cells (Huang et al., 1997; Terada et al., 1997; Fussenegger et al., 1998a), the selection procedure was carried out in the absence of tetracycline in the culture medium, and thus under full expression of the dicistronic expression unit. Expression of the dicistronic expression unit enabled easy,

SEAP-based detection of cells harboring pMG12 during the entire selection procedure. Several individual pMG12-containing clones were generated and tested for tetracycline-mediated repression of the dicistronic expression unit. As the tightness of repression of the dicistronic expression unit minimizes or largely excludes the influence of host promoter and/or enhancer elements on the dicistronic expression unit, the CHO cell clone MG12, which showed a slope of $<10^{-4}$ in phosphatase kinetics, was selected for further investigations. Due to the dicistronic nature of the SEAP-bcl-2-encoding expression unit, *bcl-2* expression was also highly tetracycline-regulatable as shown by Western blot analysis of MG (Figure 2) which was strictly coordinated with SEAP expression (data not shown).

Batch cultivations of MG12

For investigation of the influence of Bcl-2 expression on CHO cell culture processes, MG12 was cultivated in the presence ($MG12_{+tet}$; *bcl-2* repressed) and absence of tetracycline ($MG12_{-tet}$; *bcl-2* overexpression). Since MG12 cells were typically propagated in the absence of tetracycline $MG12_{+tet}$ populations were precultured for 5 days in tetracycline-containing medium to avoid the adaptation to the repressed state (SEAP-Bcl-2 off) to take place during the batch experiment. This batch cultivation was extended to 18 days to include exponential growth (ideal growth conditions) as well as the death phase (sub-optimal growth conditions). During the first three days of cultivation, both cultures $MG12_{+tet}$ and $MG12_{-tet}$ showed a similar specific growth rate to a viable (membrane intact) cell density of 3.5×10^5 cells ml^{-1} (Figure 3A). However, whereas $MG12_{+tet}$ ceased to grow exponentially and reached a final viable cell density of 5.5×10^5 cells ml^{-1}, the *bcl-2*-expressing $MG12_{-tet}$ culture continued to grow exponentially until day 6, finally reaching a viable cell density of 1.2×10^6, more than twice as much as $MG12_{+tet}$ populations (Figure 3A). In order to exclude a potential negative effect of tetracycline on the growth behavior, CHO-K1 as well as its tTA-expressing derivative XMK1-9 were grown in the presence and absence of tetracycline with no difference in the growth behavior of both cell lines (data not shown). Furthermore, regulation effective concentrations of tetracycline in the cell culture medium are generally accepted not to influence cell physiology (Gossen and Bujard, 1992).

After both cell populations ($MG12_{+tet}$ and $MG12_{-tet}$) had reached their final cell densities, they started to die (Figure 3B). A significant difference in the decline phases of $MG12_{+tet}$ and $MG12_{-tet}$ could not be detected (Figure 3B). Electrophoresis of chromosomal DNA isolated from $MG12_{+tet}$ and $MG12_{-tet}$ cells at day 14 showed discrete bands of 180 bp multimers which is a consequence of the chromosomal breakup at nucleosomal intervals by a specific endonuclease typically active in the apoptotic cell death program (Figure 4; Adams and Cory, 1998; Newton and Strasser, 1998). Furthermore, the ApoAlert™ assay (Clontech) failed to detect significant differences in the levels of the initiator caspase-3 in $MG12_{+tet}$ and $MG12_{-tet}$ cultures suggesting that *bcl-2*-mediated apoptosis protection is not detectable under conditions studied here.

Due to the dicistronic nature of the SEAP-bcl-2 expression construct, the coordinated expression of both genes allows *bcl-2* expression to be monitored via concomitant SEAP expression and secretion. The SEAP activity measured in the culture supernatant confirms expression of the dicistronic expression unit in the absence of tetracycline and repression in the presence of tetracycline over the entire cultivation period, respectively (Figure 3C).

Figure 5 shows the glutamine/ammonia as well as the glucose/lactate time courses of $MG12_{+tet}$ and $MG12_{-tet}$ populations over the entire cultivation period. Whereas *bcl-2*-expressing populations had already metabolized the glutamine pool at day 6 $MG12_{+tet}$ cells were running out of glutamine only at day ten (Figure 5). In spite of the more rapid glutamine consumption, Bcl-2-expressing cultures had lower ammonia levels during most of the first 8 days of culture. Glucose utilization profiles were very similar for both cultures. Lactate was first produced and then consumed. Lactate was produced more rapidly by $MG12_{+tet}$ cultures, attaining a maximal lactate concentration almost twice as high compared to the $MG12_{-tet}$ cultures. The reason for the lower maximal lactate concentration of the Bcl-2-expressing cell line is that glutamine limitation and consumption of lactate started on day 5. For the other cell state (*bcl-2* repressed) glutamine became limited only beyond day 10 and more lactate could therefore accumulate.

Fixed-bed cultivations of MG12

In order to assess the effect of *bcl-2* on CHO cells under conditions of a high cell density, perfused culture system, and to study the long-term stability of multicistronic expression technology, MG12 was cul-

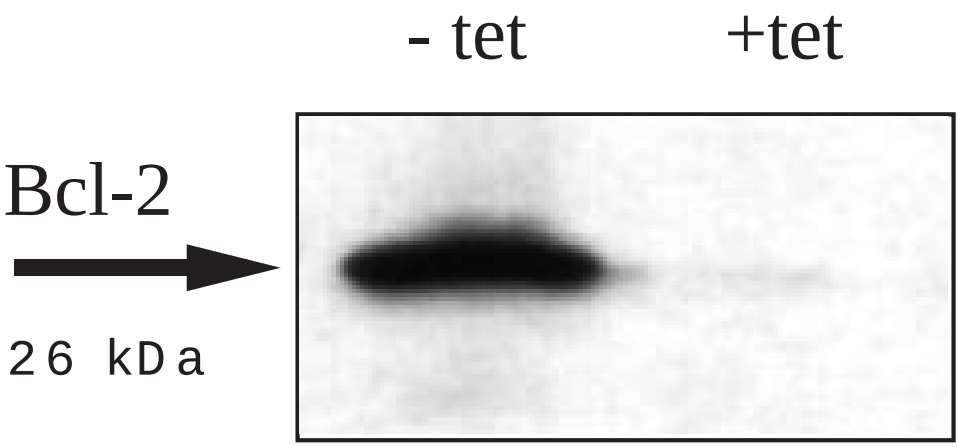

Figure 2. Western blot analysis of Bcl-2 expression of MG12 grown in the absence (–tet) and the presence (+tet) of tetracycline. Expression of Bcl-2 (26 kDa) is undetectable in the presence of tetracycline in the cell culture medium and Bcl-2 protein is overexpressed in its absence.

tivated in a fixed-bed reactor. Following inoculation into tetracycline-free culture medium the fixed-bed reactor was run in batch mode for 4 days until glucose was nearly consumed. Thereafter, continuous operation of the fixed-bed reactor was initiated. In order to impose further stress on the cells, the cultivation was performed at a low dilution rate (D_{FB}) of 3 d^{-1}. In the tetracycline-free medium SEAP and *bcl-2* are fully expressed and the increase in SEAP activity during the first 40 days may indicate increase in cell density which correlates with decreasing glutamine and glucose concentrations (data not shown) (Figure 6). A first steady-state appeared after 45 days of continuous operation. On day 61, the culture feed medium was changed to contain 2 mg l^{-1} tetracycline which induced a decrease in SEAP activity, and a new steady-state for all parameters was reached on day 86 (Figure 6). The gradual decrease in SEAP activity during the culture period between day 60 and day 80 is due to the low wash-out of this protein from the reactor given the fixed-bed reactor configuration and setup.

The steady-state concentrations and the specific substrate uptake or metabolite production rates per volume fixed-bed of glucose, lactate, glutamine, ammonia, the SEAP activity and the DNA content are shown in Table 2 for tetracycline-free (day 45 to 60) and tetracycline-containing cultivation periods (day 86–100). In addition, DNA release kinetics is shown in Figure 6. Apart from the DNA content (and of course the SEAP activity), there is no significant difference in key culture parameters between both cultivation conditions which suggest a high stability of the cell line MG12 and the fixed-bed reactor set-up over long cultivation periods. Due to the different conditions of batch and fixed-bed cultivations the relative metabolic production and consumption rates of $MG12_{+tet}$ and $MG12_{-tet}$ populations can not be directly compared. For example, in batch cultivation lactate was not the final metabolite, but can be consumed under unfavorable condition such as glutamine limitation.

Since dead and damaged cells release their DNA, the DNA concentration in the culture supernatant is indicative of cell death, comparable to the release of the lactate dehydrogenase (LDH) (Sramkova et al., 1997). Therefore, the rate of DNA accumulation in the culture supernatant correlates with the death rate of cultured cells. $MG12_{+tet}$ with repressed *bcl-2* expression show 80±10% higher DNA accumulation rate compared to $MG12_{-tet}$ cells, suggesting a protective effect of *bcl-2* on the death rate under unfavorable conditions in the fixed-bed ractor. Similar results have been found for a *bcl-2* expressing hybridoma cell line, where a decrease in the cell turn-over led to higher antibody yields in the fixed-bed system (Fassnacht et al., 1998b).

Discussion

Conditionally-regulated expression of endogenous genes is a desirable goal in stable cell line and transgenic animal systems, as well as in clinical gene therapy. Although regulated gene expression technology is a well established tool in contemporary basic research since it simplifies and greatly speeds up functional genomic research by enabling the assessment of gene function in a strictly isogenic background,

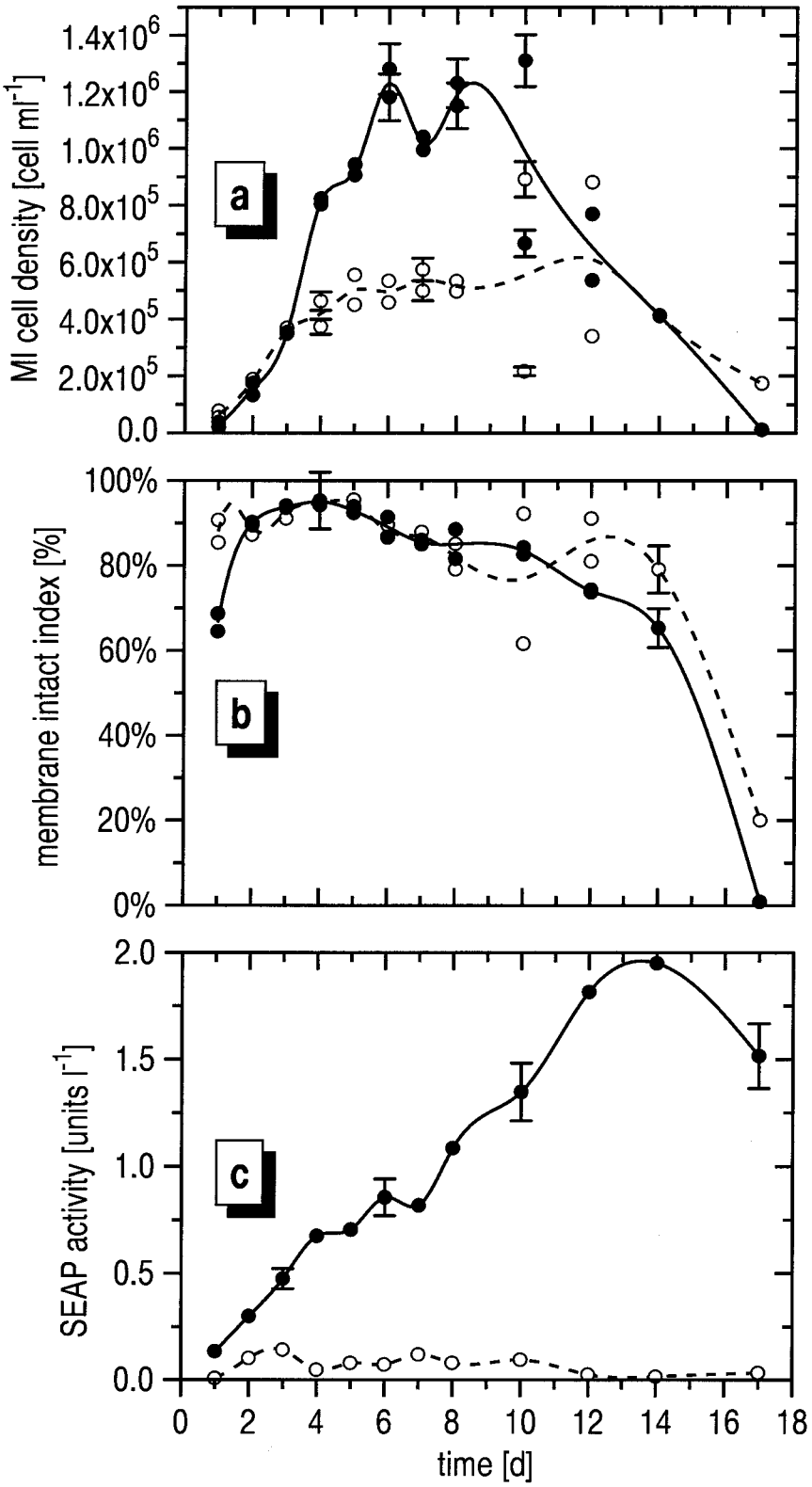

Figure 3. Batch cultivation of MG12 in tetracycline-free (●) and tetracycline-containing (○) medium. Viable cell density (cells with intact membrane, MI) (A), membrane intact index (B) and SEAP production (C) was assessed during a cultivation priod of 18 days. Bcl-2-expressing MG12 cells grow to double the final cell density as isogenic MG12 populations with repressed dicistronic expression unit (A). However, Bcl-2 overexpression provides little or no protective effects during the decline phase of both cultures starting at day 14 (B). The expression of the dicistronic expression unit can be monitored online by measuring the activity of the secreted reporter protein SEAP which correlates with expression of Bcl-2 (C).

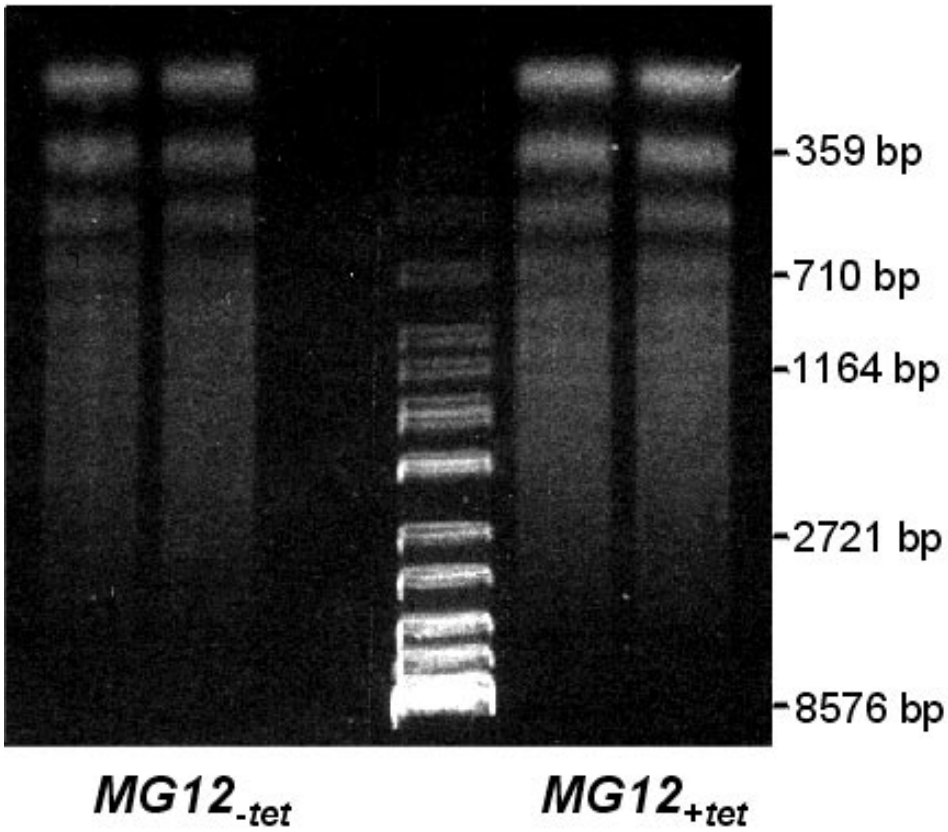

Figure 4. Electrophoretical analysis of chromosomal DNA isolated from MGg12 cells grown in batch cultures in the presence ($MG12_{+tet}$) and in the absence of tetracycline ($MG12_{-tet}$). Both cell populations show the DNA laddering characteristic of apoptosis caused by intranucleosomal strand cleavage by an apoptosis-specific nuclease. M represents a DNA size marker.

Table 2. Culture supernatant concentrations and consumption or production rates per volume fixed-bed of the steady-state fixed-bed data (calculated by using Equations (1)–(3))

	induced Bcl-2 and SEAP (45–60 days)	repressed Bcl-2 and SEAP (86–100 days)	
$c_{glucose}$	[mmol l^{-1}]	0.35	0.3
$c_{lactate}$	[mmol l^{-1}]	20	15
$c_{glutamine}$	[mmol l^{-1}]	0.15	0.15
$c_{ammonia}$	[mmol l^{-1}]	3.1	3.3
c_{SEAP}	[units l^{-1}]	0.69	0.19
c_{DNA}	[mg l^{-1}]	0.75	1.4
$q^*_{FB,glucose}$	[mmol $l_{FB}{}^{-1}$ h^{-1}]	–2.2	–2.2
$q^*_{FB,lactate}$	[mmol $l_{FB}{}^{-1}$ h^{-1}]	2.5	1.9
$q^*_{FB,glutamine}$	[mmol $l_{FB}{}^{-1}$ h^{-1}]	–0.42	–0.42
$q^*_{FB,ammonia}$	[mmol $l_{FB}{}^{-1}$ h^{-1}]	0.39	0.41
$q^*_{FB,SEAP}$	[units $l_{FB}{}^{-1}$ h^{-1}]	0.086	0.024
$q^*_{FB,DNA}$	[mg $l_{FB}{}^{-1}$ h^{-1}]	0.094	0.17

bioengineers discovered only recently the potential of regulated gene expression for the expression of toxic transgenes (Cook et al., 1997) or the development of novel cytostatic bioprocesses for enhanced production of heterologous proteins (Fussenegger et al., 1997; Fussenegger et al., 1998b; Fussenegger et al., 1998c; Mazur et al., 1998).

Classical metabolic engineering of industrially relevant cell lines often involves selection of cell clones which stably and constitutively express the transgenes. However, as the transfected transgenic information is integrated into the host chromosome by illegitimate recombination at random sites, the outcome of classical metabolic engineering can depend significantly on high clonal variations between generated cell lines which complicates interpretation of specific gene functions and engineering effects: (i) random integration of the transgene may result in disruption of host chromosomal loci which could lead to secondary effects unrelated to the metabolic engineering intent, (ii) the chromosomal context at the integration site may influence transgene expression, and (iii) multiple integration of transgenes at different locations may cause dosage effects, function as targets for secondary recombinations or simply potentiate the problematic genetic configurations stated above.

Several strategies have been developed to alleviate clonal variation following metabolic engineering including characterization of multiple clones, transient transfections, stable polyclonal populations and targeted integration. Apart from targeted integration, which requires complex pre-engineering and screening for appropriate integration sites (Karremann et al., 1996), the transient transfection and polyclonal population strategies certainly reduce unrelated effects resulting from clonal variations, but they do not allow long-term cultivations as the transgene is lost in a few generations in transient transfection experiments and mixed populations may change due to different growth behavior of individual cell clones.

Regulated gene expression has the potential to alleviate most critical issues of clonal variations and enables the metabolic engineering effector, here the survival factor *bcl-2*, to be studied in a completely isogenic background:

(i) complete repression of the transgene guarantees the absence of host promoter/enhancer elements influencing the heterologous expression unit;
(ii) assessment of two different cell states (expression and repression of the gene of interest) in a strictly isogenic background;
(iii) disruption of chromosomal target genes by integration of the gene of interest is normalized by repressed configurations;
(iv) multiple integrations are still possible but regulated expression system enforces coordinated behavior of different loci. Therefore, regulated expression of metabolic transgenes offers a straightforward strategy to assess the potential of a candidate gene for metabolic engineering.

Although apoptosis is a vital mechanism for multicellular life (Raff, 1992; Von Boehmer, 1994) it is an undesired phenomenon in biotechnological production processes since premature cell death eliminates viable cell population and hence production potential (Franek and Dolnikova, 1991; Duval et al., 1991). Furthermore, because of the complex signalling pathways involved in the induction of apoptosis, cell death signals could be expected to rapidly spread within a culture and affect the remaining healthy cell population.

One of the most important and intrinsic causes of apoptosis in mammalian cell culture is the deprivation of nutrients and special survival factors during the course of cultivation. Recent studies suggest that depletion of any amino acid can induce apoptosis and the effect is greatest when there is a lack in essential amino acids (Duval et al., 1991; Simpson et al., 1998). The discovery of nutrient depletion-induced programmed cell death stimulated sophisticated feeding strategies as well as research on chemical cell culture additives suppressing apoptosis. It has been demonstrated that apoptotic hybridoma cultures could be rescued by the addition of a single amino acid (Franek and Sramkova, 1996). However, although feeding strategies may successfully retard the onset of apoptosis in hybridoma and myeloma cell lines they do not necessarily enhance cellular productivity (Franek and Dolnikova, 1991). In fact heterologous protein productivity was greatest in growth-arrested or slow growing cells and growth-inhibitory strategies such as nutrient deprivation and the addition of specific chemicals (thymidine or hydroxyurea, TGF-β; Suzuki and Ollis, 1990; Al-Rubeai et al., 1992) have long ago been elaborated to increase productivity of these cell lines.

However, not all cell lines respond by apoptosis to nutrient deprivation. Some cell lines, including HeLa and HL60 cells have been suggested to block cellular proliferation following nutrient deprivation to repair defects or await improvement of physiological conditions rather than initiating programmed cell death

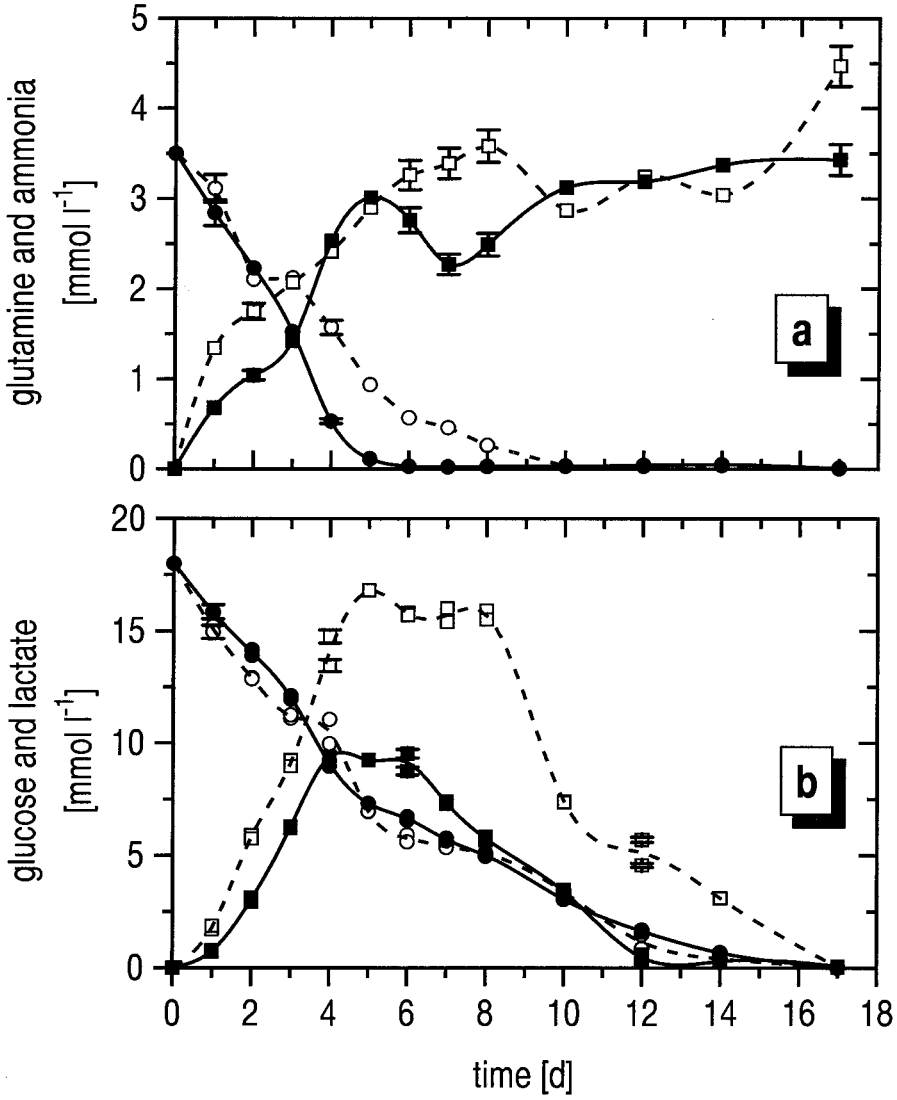

Figure 5. Physiological parameters: glutamine (○/●) and ammonia (□/■) (A) and glucose (○/●) and lactate (□/■) (B) concentrations. MG12 cells were grown under conditions repressing (○/□, presence of tetracycline) or inducing (●/■, absence of tetracycline) coordinated *bcl-2* and SEAP expression. The *bcl-2*-expressing MG12 culture consumes glutamine faster and exhibits lower lactate concentrations.

(Perreaut and Lemmieux, 1993). The phenomenon of G1-phase accumulation in response to apoptosis induction in the cultured population has also been observed in CHO cells (Moore et al., 1995). However, we recently showed that CHO cell engineered for tetracycline-regulated expression of tumor suppressor genes showed greatly enhanced heterologous protein productivity under G1-arrested conditions without any signs of apoptosis (Fussenegger et al., 1998b; Mazur et al., 1998). It seems that in some cell lines apoptosis may be repressed in the G1-phase or at least in some stages of G1-phase, a cell-cycle phase which often functions as recovery phase from a wide variety of physiological insults before entering the cell division program (Fussenegger et al., 1997; Fussenegger et al., 1998b; Mazur et al., 1998).

Besides special feeding strategies and the addition of anti-apoptotic medium additives, metabolic engineering of key regulators of cellular survival pathways has been used to prevent the induction of apoptosis by physiological stresses typically encountered during biopharmaceutical manufacturing (Singh et al., 1994; Al-Rubeai et al., 1995). Bcl-2-mediated anti-apoptosis engineering was found to be particularly successful

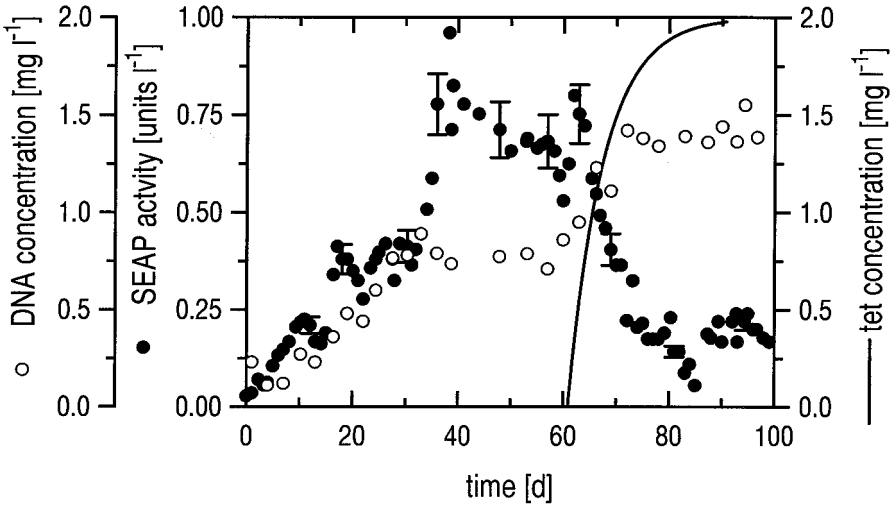

Figure 6. Fixed-bed reactor cultivation of MG12 cells for 100 days. SEAP production (●), DNA concentration (○) as well as the modeled tetraycycline concentration (−−) is shown. The reactor consisted of a 50 ml fixed-bed and a 1000 ml conditioning vessel and was operated at a dilution rate of 3 ml medium per ml fixed bed per day. This reactor configuration shows the regulatability and the genetic stability of multicistronic expression unit over an extended cultivation period of 100 days.

and relevant in hybridoma cells since this cell line is highly sensitive to apoptosis and since strategies to arrest cell growth of hybridoma cells lead to increased antibody titers but also to enhanced apoptosis (Suzuki and Ollis, 1990; Al-Rubeai et al., 1992; Mercille and Massie, 1994; Takahashi et al., 1994; Itoh et al., 1995; Singh et al., 1996; Huang et al., 1997; Simpson et al., 1997; Terada et al., 1997; Fassnacht et al., 1998a, b; Chung et al., 1998; Mastrangelo and Betenbaugh, 1998; Huang et al., 1997; Terada et al., 1997). Although the overexpression of anti-apoptosis effectors could neither prevent nor completely block the onset of apoptosis in hybridoma cell cultures a consensus finding was that apoptosis was significantly delayed in *bcl-2*-overexpressing cultures during the decline phase (Singh et al., 1996; Chung et al., 1997; Fassnacht et al., 1998a, b).

However, reports on the effect of Bcl-2-mediated protection under suboptimal nutrient conditions were more controversal, reaching from only marginal effects (Chung et al., 1998) to growth under complete exhaustion of glutamine (Singh et al., 1996). Recently, Simpson et al. (1998) showed that the protective effect of Bcl-2 varied in response to medium deprivation for different essential amino acids, suggesting that apoptosis suppression could be involved in the down-regulation of non-essential cellular functions (Simpson et al., 1998). The variety of effects with respect to protection against apoptosis as well as the recent controversy whether Bcl-2 expression increases antibody productivity of hybridoma cells (Itoh et al., 1995) or not (Fassnacht et al., 1998a) may reside either in the various cell lines and cell systems used or are a consequence of clonal variation, effects of integration sites and expression levels.

Therefore, we regulated gene expression to assess Bcl-2 function in a complete isogenic cellular background in CHO cell lines. Although high level expression of Bcl-2 as determined by Western blot analysis and immunofluorescence microscopy using human-specific antibodies could be observed in the absence of the external regulatory stimulus tetracycline, no difference in the decline phase of MG12 populations with respect of the state of Bcl-2 expression could be detected. Apoptosis assays such as DNA fragmentation determination and quantification of casepase 3 using Clontech's ApoAlert technology failed to detect apoptosis characteristics in batch culture before day 14 when the cells enter the decline phase and where the membrane intact index dramatically decreases and the classical DNA laddering becomes apparent. This result does not necessarily suggest that Bcl-2 is not protective in CHO cells since Bcl-2-expressing cells seem to be more robust and grow to a double final cell density as respective control populations with repressed Bcl-2 transgene. However, *bcl-2* has previously been shown to be little or not protective against programmed cell death of NSO cells (Murray et al., 1996). NSO cells naturally express high levels of the Bcl-2 homologue Bcl-x_L which may

render Bcl-2 overexpression redundant and therefore ineffective in the NSO cell background (Murray et al., 1996). Relatively high Bcl-2 and Bcl-x_L expression levels have also been found in CHO cells (Cotter et al., unpublished observation) which may explain why Bcl-2-based metabolic engineering is little protective against apoptosis of CHO cells grown in batch culture.

Certainly, various reports show that Bcl-2 is able to delay the onset of apoptosis in CHO cells but induction of programmed cell death has only been achieved by severe chemical, genetic, biochemical or viral insults (Balajthy et al., 1997; Chung et al., 1997; He et al., 1996; Meikrantz et al., 1998; Reynolds et al., 1996a, b; Zhang et al., 1995). However, such severe chemical, genetic, biochemical or viral insults are known to trigger specific cellular repair and survival programs which may particularly interfere with Bcl-2 protective pathways and could lead to overestimation of the survival potential of Bcl-2 in batch culture or normal bioreactor operation. However, we could show that overexpression of *bcl-2* reproducibly allows CHO cells to grow to double the final cell density in batch cultures compared to the same cells grown with repressed *bcl-2*-encoding expression unit. Despite this phenomenon which suggest a higher robustness of CHO cells under *bcl-2*-expressing conditions, no significant difference of apoptosis characteristics could be detected between the two isogenic cell culture states. However, Bcl-2-mediated increase of final cell density of CHO cultures could have positive impact on industrially relevant production processes since heterologous protein production is primarily a function of the viable cell population. Whether higher overall antibody production of Bcl-2-overexpressing hybridoma cell lines reported by Itoh et al. (1995) is based on the same phenomenon remains to be investigated.

We recently reported a novel metabolic engineering strategy to arrest CHO cells in G1-phase of the cell cycle by the overexpression of critical tumor suppressor genes. Despite the complete growth block arrested CHO populations showed no apoptotic behavior except for overexpression of p53 (Mazur et al., 1998). Following this observation we included the survival gene *bcl-x_L* (a homologue of *bcl-2*) also in a p27-based tricistronic cytostatic expression unit. Interestingly, Bcl-x_L-overexpressing CHO cells showed no enhanced protective properties against apoptosis but an enhancement in the final heterologous protein production by a factor up to 3 compared to arrested CHO populations lacking this additional survival factor (Fussenegger et al., 1998b). Although these phenomena cannot yet be explained on a mechanistic and molecular level, these findings support the idea that *bcl-2* and *bcl-x_L* are not exclusively associated with the well-established apoptosis pathway. For example, Minn et al. (1996) demonstrated that overexpression of the Bcl-2 related protein Bcl-x_L induces tetraploidy, a phenomenon which may correlate with higher higher heterologous protein production due to amplification of genetic information.

Although the effects of Bcl-2 overexpression in the CHO batch cultures reported here are different from those reported for hybridoma cells, similar protective effects are found for both cell types in fixed-bed reactors as judged by the DNA released into the culture supernatant. These findings indicate that the effect of Bcl-2 overexpression depends not only on the cell line but also on the cultivation conditions used. This latter notion is particularly supported by direct comparison of relative Bcl-2-mediated effects on CHO populations grown in batch culture and in the fixed-bed reactor. Whereas in the batch cultures Bcl-2 overexpression resulted in higher final cell densities and almost no apoptosis-protective effects, lower DNA release indicated lower death rate in fixed-bed reactor configurations. Since the glucose and glutamine consumption rates were identical for Bcl-2 expressing (-tet) and Bcl-2 repressing steady-states (+tet) in the fixed-bed reactor, it can be assumed that the cell density within the fixed-bed remained largely unchanged. However, the molecular basis for such diverse Bcl-2-mediated effects under different culture conditions remains to be investigated.

Although fixed-bed reactors are the reactor type of choice for extended high density cell cultures, the genetic characterization of the cell line is impaired by the limited access to the cells for off-line analysis. Multicistronic expression technology, which allows strict linkage of the metabolic engineering effector to a secreted reporter gene proved extremely useful to monitor expression of the effector transgene *bcl-2*. Also, multicistronic expression technology could be shown to be very stable over extended culture periods and still retain full regulatability by tetracycline. This high genetic stability would allow the use of regulated multigene metabolic engineering for extended industrially relevant production processes as has recently been developed, for example, for cytostatic production technology (Fussenegger et al., 1997, 1998b; Mazur et al., 1998).

Regulated multigene metabolic engineering showed

the potential to assess genetic targets for metabolic engineering in a pure isogenic background. Anti-apoptosis engineering is not only important for maintaining high viability when grown in reactors but is gaining increasing importance for *in vivo* cultures of primary cell lines for use in tissue culture and gene therapy. A combined effect of *bcl-2* to suppress apoptosis and to increase the final cell density would therefore greatly stimulate the use of *bcl-2* for various applied bioprocesses.

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