Doctoral Thesis

Overcoming production bottlenecks of chinese hamster ovary cells by multilevel genetic engineering

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OVERCOMING PRODUCTION BOTTLENECKS OF CHINESE HAMSTER OVARY CELLS BY MULTI-LEVEL GENETIC ENGINEERING

A dissertation submitted to the
SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH
for the degree of
Doctor of Natural Sciences

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Zurich 2002
To my family

Fredi, Annemarie, Janine, Vasco & Sabina

for the love, support, encouragement and confidence

you offered throughout my education
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CURRICULUM VITAE
SUMMARY

Mammalian cells are successfully used for the production of numerous therapeutic proteins, but still there are many bottlenecks to overcome to improve specific productivity. The aim of this thesis was to develop new concepts for improving the overall production capacity.

The first concept to improve the cellular protein productivity was based on controlled growth arrest at high cell density to enable an extended period of high production. Chinese hamster ovary (CHO) cells, engineered to express the cyclin-dependent kinase inhibitor (CKI) p21 in combination with the p21-stabilizing differentiation factor CCAAT/enhancer-binding protein α (C/EBPα) in a tetracycline-responsive manner, showed a sustained, G1-phase-specific cell-cycle arrest. The cellular productivity of non-dividing cells, as judged by quantification of the model protein, namely secreted alkaline phosphatase (SEAP), was increased 10-15 times relative to an isogenic growth-competent control cell line. A similar approach using the CKI p27 and the apoptosis-blocker protein bcl-xL also found to enable cell-cycle arrest predominantly in G1-phase in CHO cells and resulted in up to 30-fold higher specific SEAP productivity.

As a consequence of genetic drift and strong counterselection, rare growth-competent mutants emerged during the growth-arrested production period. Therefore, in a second approach, a novel strategy was developed to maintain the producer cells in culture, while selecting against the outgrowth of mutants. Concomitant expression of a cell-surface displayed single chain antibody enabled selective isolation of recombinant producer cells based on hapten-antibody interactions. CHO cells displaying the surface antibody could be selectively enriched from mixed populations either mediated by fluorescence-based immunolabeling and FACS technology or by magnetic selection based on interaction of the surface antibody with its hapten immobilized on metal particles.

Quantitative assessment of cellular protein production is an important issue in biotechnology to design metabolic engineering strategies. Standard practice to quantify the cellular production capacity is based on expression profiling of a reporter enzyme. In a third project we adapted the Bacillus stearothermophilus α-amylase gene for use as an intracellular and secreted, eukaryotic reporter protein. The genetically modified secreted alkaline phosphatase (SAMY) was efficiently secreted from a variety of mammalian and human cell lines and could be quantified from the corresponding cell culture supernatants by a spectrophotometric assay.
A prerequisite for any genetic engineering approach is the ability to control gene expression locally and in time. Several groups have addressed this issue by specific promoter/transactivator systems that enabled drug-regulatable gene transcription. In the final part of the thesis two novel production control strategies were explored which rely on translation control rather than transcriptional regulation. The first translation control system (TCS) is based on the specific interaction of the cellular nucleic acid binding protein (CNBP) or La autoantigen (La) with a terminal oligopyrimidine (TOP) sequence. Translation blockage of a SEAP-mRNA exerted by this TOP sequence in the 5’ UTR could be released depending on the presence of either CNBP or La protein enabling conditional translational regulation. The levels of CNBP or La were thereby adjusted with the tetracycline regulation system. Alternatively, to regulate CNBP and La titers, TOP-mediated translation control could also be adjusted by artificial phosphorothioate anti-TOP oligodeoxynucleotides leading to repression of the system.

The second TCS mimics the events following polioviral infection that result in shutoff of host mRNA translation. The change from host- to virus-protein synthesis is mediated by clipping of host initiation factor 4G (eIF4G) to abolish its ability to promote host-mRNA translation. Separate fusion of the eIF4G protein domains to rapamycin-binding proteins and subsequent expression in CHO cells enabled controlled assembly of the two eIF4G fragments in a rapamycin-inducible manner, resulting in adjustable expression of SEAP driven by cap-dependent, as well as cap-independent translation initiation.
ZUSAMMENFASSUNG

Säugetierzellen werden erfolgreich zur Produktion von vielen Protein-Therapeutika eingesetzt, obschon die Produktionsverfahren noch wesentliches Potential zur Verbesserung aufweisen. Das Thema dieser Arbeit beinhaltet die Entwicklung neuer Konzepte zur Optimierung der Kultivierung von Säugetierzellen für biotechnologische Prozessverfahren und der Generierung von künstlichen Geweben.


Das Detektieren und Quantifizieren der Syntheseleistungen von Säugetierzellen ist ein wichtiges Mittel der Biotechnologie um die Effektivität von neu entwickelten Strategien zur Verbesserung von Stoffwechselwegen zu erfassen. Gebräuchliche Verfahren zur Erfassungzellulärer Syntheseleistungen, bedienen sich eines quantifizierbaren Reporterenzymes. In einem dritten Projekt wurde das α-Amylase Gen von Bacillus stearothermophilus zur
Verwendung als intra-, sowie extrazelluläres Reportergen adaptiert. Die genetisch modifizierte, sekretierte Amylase (SAMY) wurde effizient aus mehreren Säugetier- und Humanzelllinien ausgeschleust und konnte mittels spektrophotometrischem Nachweisverfahren aus dem Kulturüberstand bestimmt werden.


CHAPTER 1

Introduction
THE EUKARYOTIC CELL CYCLE

The ultimate destiny of a cell to undergo division, differentiation, survival, and death results from an intricate balance between multiple regulators governed by the cell cycle, being the fundamental means by which all living things are propagated.

The cell cycle is an ordered set of events, culminating in cell growth and division into two daughter cells. The eukaryotic cell cycle is divided into four stages: G1, S, G2 and M. G1 is the gap-phase during which cells prepare for the process of DNA replication. S-phase is defined as the stage in which DNA synthesis occurs. G2 is the second gap-phase during which the cell prepares for the process of division and M stands for mitosis, the phase in which the replicated chromosomes are segregated into separate nuclei and cytokinesis occurs to form two daughter cells. In addition to G1, S, G2, and M, the term G0 is used to describe cells that have exited the cell cycle and become quiescent (Johnson and Walker, 1999).

Most of the regulatory events that affect proliferation occur in the G1-phase of the cell cycle. Growth and proliferation of normal cells are regulated by complex interactions between growth factors, cell density and attachment to substrate (Baserga, 1994). In contrast to extracellular cues, the internal integrity of a cell is examined by distinct checkpoints at work

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Fig. 1: The eukaryotic cell cycle. Adapted from (Weinert, 1998).
throughout the entire cell cycle. Each of these processes controls the successful completion of a previous phase and is defined as “checkpoint control” (Weinert, 1998). An important checkpoint in G₁ has been identified in both yeast and mammalian cells. Referred to as START in yeast and restriction point in mammalian cells, this is the stage at which the cell becomes committed to DNA replication and completing a cell cycle (Hartwell et al., 1974; Pardee, 1974). Arrest in G₁ prevents aberrant replication of damaged DNA and arrest in G₂ (before mitosis) allows the cells to avoid segregation of defective chromosomes (Agami and Bernards, 2002) (Fig. 1).

**The Building Blocks**

* Cyclins and cyclin-dependent protein kinases (CDKs)

Cyclin-dependent protein kinases (CDKs) are the engines that drive the events of the eukaryotic cell cycle and the clock that times them. In complex cell cycles, CDKs are also the information processors that integrate extracellular and intracellular signals to ensure the smooth coordination of cell-cycle events in the face of environmental change or mechanical failure (Levine and Cross, 1995). CDK catalytic subunits do not act alone; their ability to

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*Table 1: Mammalian cyclins. Adapted from (Johnson and Walker, 1999).*
trigger cell-cycle events is completely dependent on associated cyclin subunits, whose oscillating concentrations underlie the stage-specific timing of CDK activity (De Bondt et al., 1993; Jeffrey et al., 1995). In contrast to only the one or two CDK-like gene products found in lower eukaryotes, nine CDKs, in addition to at least 16 cyclins have been identified in mammalian cells. All cyclins contain a common region of homology known as the cyclin box, which is a domain for binding and activating of CDKs (Johnson and Walker, 1999) (Table 1).

Control of cyclin gene transcription and ubiquitin-dependent cyclin proteolysis mediated by a small sequence motif (the destruction box) near the N terminus of mitotic cyclins governs cell-cycle progression in many contexts in all eukaryotes and gives rise to successive waves of cell-cycle regulated cyclin expression. The irreversibility of proteolysis provides a strong directionality to the cell cycle, forcing it to go forward at several critical steps (King et al., 1996; Pagano, 1997).

Although cyclin binding is the primary determinant of CDK function, layers of additional regulatory subunits and protein kinases also modulate CDK activity, substrate recognition, and subcellular location. This fine-tuned regulatory network ensures the precise timing and coordination of the mechanical events that duplicate and divide the cell (Morgan, 1997).

In eukaryotes, distinct CDKs regulate different cell-cycle processes. For instance, CDK4 and CDK6 regulate cell-cycle progression through mid-G1, CDK2 activation is associated with entry into S-phase, whereas CDK1 primarily regulates mitosis. Distinct cyclins associate and activate different CDKs throughout the cell cycle. D-cyclins (cyclin-: D1, D2, and D3) bind CDK4/6 to wire external signals to the cell cycle and regulate progression through mid-G1. CyclinE binds CDK2 in late G1 and its activity is rate-limiting for progression from G1 to S phase. The function of each of these complexes is quantitatively regulated throughout the cell cycle and qualitatively targets a unique set of substrates (Baldin et al., 1993; Sherr, 1996).

Not all cyclins and CDKs function in regulating the cell cycle. Other functions identified for cyclins and CDKs include controlling of transcription, DNA repair, differentiation, and apoptosis (Roy et al., 1994; Rickert et al., 1996; Peng et al., 1998).

**Cyclin-dependent kinase inhibitors (CKIs)**

The activity of cyclin-CDK complexes is modulated by both activating and inhibiting phosphorylation of the CDKs, and by binding to cyclin-dependent kinases inhibitors (CKIs) (Sherr and Roberts, 1999) (Fig. 2).

In mammalian cells, two classes of CKIs, the Cip/Kip and Ink4 families, provide a tissue-specific mechanism by which cell-cycle progression can be restrained in response to
extracellular and intracellular signals (Harper and Elledge, 1994). The INK4 family contains
four members (p15, p16, p18, and p19) that interact only with CDK4/6 and inhibit their
association with and activation by the D-type cyclins (Carnero and Hannon, 1998). The
Cip/Kip family includes p21, p27, and p57, which inhibit CDK2- and CDK4/6-cyclin
complexes involved in G1 and G1/S control.

p21 was identified as a gene induced by the p53 tumor suppressor protein (El-Deiry et al.,
1993; Harper and Elledge, 1994). In response to DNA damage, the p53 protein is stabilized
and activated as a transcription factor. The p21 gene promoter contains a p53-binding site that
allows p53 to transcriptionally activate the p21 gene. Induction of p21 inhibits cell-cycle
progression in two ways: (a) by inhibiting a variety of cyclin-CDK complexes and (b) by
inhibiting DNA synthesis (Deng et al., 1995). Thus p21 appears to be the critical mediator of
p53's response to DNA damage through its ability to inhibit cell proliferation but allow DNA
repair. p21 was also isolated as a gene that accumulated as aged cells approached senescence,
suggesting that p21 may play a role in this cellular process as well (Noda et al., 1994).

The other two members of the Cip/Kip family are p27 and p57. Like p21, p27 and p57
bind to a variety of cyclin-CDK complexes through a conserved amino-terminal domain
(Polyak et al., 1994; Matsuoka et al., 1995). p27 has been implicated in mediating several
growth inhibitory signals including transforming growth factor-β (TGF-β) and contact

**Fig. 2:** Regulation of cyclin, CDK and CKI activities. Adapted from (Morgan, 1997).
inhibition (Lee et al., 1995).

**Progression from $G_0$ through the Cell Cycle**

The D-type cyclins are the first cyclins to be induced as $G_0$ cells are stimulated to enter the cell cycle (Sherr, 1994). Unlike many other cyclins, D-type cyclins do not oscillate during the cell cycle, but rather their levels are controlled by the presence of growth factors. The cyclinD/E-CDK complexes synergize to cause phosphorylation of the retinoblastoma family of tumor suppressor proteins (pRb, p107 and p130) in $G_1$ and abrogate their growth suppressive activity (Lipinski and Jacks, 1999).

pRb plays a critical role in regulating $G_1$-progression and is likely a key component of the molecular network controlling the restriction point. pRb has been shown to bind and regulate a large number of cellular proteins, including members of the E2F family of transcription factors (Johnson and Schneider-Broussard, 1998). E2F factors regulate the expression of many genes that encode proteins involved in cell-cycle progression and DNA synthesis, including cyclinE, cyclinA and CDK1. Binding of pRb to E2F inhibits E2F's transcriptional activation capacity and, in at least some cases, converts E2F factors from transcriptional activators to transcriptional repressors. Phosphorylation of pRb by D-type cyclin kinases results in the dissociation of pRb from E2F and the expression of the above mentioned E2F-regulated genes. Through the activation of E2F, cyclinE is the next cyclin to be induced during the progression of cells through $G_1$ (Ohtani et al., 1995; Geng et al., 1996). CyclinE

![Fig. 3: Regulation of E2F transcriptional activity through the cell cycle. Adapted from (Johnson and Walker, 1999).](image-url)
associates with CDK2, and this kinase complex is required for cells to make the transition from G1 into S phase (Ohtsubo et al., 1995). CyclinE-CDK2 participates in maintaining pRb in the hyperphosphorylated state and thus participates in a positive feedback loop for the accumulation of active E2F (Hinds et al., 1992) (Fig. 3).

CyclinA, which is also regulated in part by E2F, accumulates at the G1/S phase transition and persists through S phase (Schulze et al., 1995). CyclinA initially associates with CDK2 and then, in late S phase, associates with CDK1. CyclinA-associated kinase activity is required for entry into S phase, completion of S phase, and entry into M phase (Lehner and O'Farrell, 1989; Walker and Maller, 1991). E2F binds cyclinA, allowing cyclinA-associated kinases to phosphorylate the E2F heterodimerization partner DP1, resulting in an inhibition of E2F DNA-binding activity. Thus, whereas cyclinE positively regulates E2F activity, cyclinA participates in a negative feedback loop for E2F regulation (Johnson and Walker, 1999) (Fig. 3).

The G2 phase also contains a checkpoint that responds to DNA damage and causes a delay to allow DNA repair before the cell enters into mitosis. Mitosis is regulated by CDK1 in association with cyclinA, cyclinB1, and cyclinB2 (King et al., 1994; Arellano and Moreno, 1997). The proteins phosphorylated by these cyclin-CDK1 complexes include cytoskeleton proteins such as lamins, histone H1, and possibly components of the mitotic spindle. For cells to exit mitosis, cyclinA and cyclinB must be degraded. After mitosis, cells again enter G1 and, at the restriction point, must decide whether to proceed into another cell cycle.

**Mechanisms Restricting Progression through G1**

Acute damage to DNA, such as double stranded breaks or bulky adducts caused by ionizing radiation or cis-platin treatments, activate a checkpoint that arrests the cells in G1. This arrest requires both functional p53 and pRB proteins and is built up through two distinct and independent mechanisms, referred to as “initiation” and “maintenance” (Harrington et al., 1998; Agami and Bernards, 2000). The initiation phase of the cell-cycle arrest is rapid, and depends uniquely on cyclinD1 destruction. p53 contributes very little to this initial response. Destruction of cyclinD1 releases a wave of p21 molecules from CDK4 complexes to arrest cell-cycle progression in G1, at least in part by inhibiting CDK2 activity. Thus, the main feature of this initial arrest is that it does not require protein synthesis, but rather relies on rapid destruction and redistribution of pre-existing cellular components. However, this initial arrest is transient as the pool of p21, bound by cyclinD1-CDK4, is rapidly exhausted and
newly synthesized CDK2-cyclinE complexes are active to push the cells back into the cycle in the presence of damaged DNA. In contrast, the classical p53 response does require protein synthesis and is therefore inherently slower. The p53-dependent maintenance phase of cell-cycle arrest follows the initiation phase, in which accumulation of p21 by DNA-damage activated p53 arrests the cells in G1 as long as the damage is present (Deng et al., 1995; Agami and Bernards, 2000). p21 and p27 bind both CDK2 and CDK4/6 complexes, but play a distinct role in each case (Blain et al., 1997; LaBaer et al., 1997). They inhibit cyclinE-CDK2 activity but are essential for complex formation and activity of cyclinD-CDK4/6 (LaBaer et al., 1997; Cheng et al., 1999). The association of D-type cyclins with CDK4/6 sequesters p21 and p27 away from cyclinE-CDK2 complexes, thereby releasing them from their inhibitory effect on cyclinE-CDK2 (Cheng et al., 1999) (Fig. 4).

When cells are deprived of mitogens, accumulation of p27 is required for efficient exit from the cell cycle and entry into a quiescent state (Coats et al., 1996; Rivard et al., 1996). During cell-cycle progression p27 is a very labile protein and its destruction relies primarily on the E3 ligase complex SCF$^{SKP2}$ (Carrano et al., 1999). Accumulation of p27 by mitogen

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**Fig. 4:** Mechanisms used for withdrawal from the cell cycle in response to DNA damage and mitogen deprivation. Adapted from (Agami and Bernards, 2002).
deprivation depends on inhibition of its proteolysis (Pagano et al., 1995; Hengst and Reed, 1996). Mitogenic signals activate a pathway that inhibits GSK3-β and thereby stabilizes D-type cyclins. As a consequence, the level of D-type cyclins in resting cell is low due to the combined effects of reduced promoter activity and decreased protein stability. CyclinD down-regulation leads to a rapid loss of cyclinD-CDK4-p27 complexes, which in turn results in a release and redistribution of p27 to bind and inhibit CDK2 kinase activity. Thus, in most cases it appears that mitogen deprivation causes activation of the restriction point and exit from G\textsubscript{1} to G\textsubscript{0} by combined effects of reduction in cyclinD levels and activity, and elevation of the amount of p27 (Agami and Bernards, 2002) (Fig. 4).

**MAMMALIAN CELLS AS PROTEIN PRODUCTION DEVICES**

**Host and Cultivation Systems**

The advent of recombinant DNA technology enabled heterologous gene expression in living cells and thereby production of protein pharmaceuticals in large quantities. Often heterologous proteins for therapeutic use are produced from bacterial and yeast cells because of their rapid growth and higher expression levels. However, many of these pharmaceuticals require posttranslational modifications such as glycosylation for full therapeutic efficacy. In these cases, mammalian cells such as Chinese hamster ovary (CHO) are the preferred hosts to obtain protein products with minimal alterations in posttranslational modifications compared to their physiological forms in humans (Zang et al., 1995; Kaufmann et al., 2001).

Batch cultivation is often the method of choice for large-scale protein production, because it provides a relatively simple yet reliable and controllable process (Leelavatcharamas et al., 1994; Seewoster et al., 1997). However, one of the major limitations of batch culture processes to date is limitation of the period of highest protein production to a short interval of the bioprocess either during exponential growth or when cells have reached their maximal cell density (Pendse et al., 1992; Tonouchi et al., 1992).

In view of the relatively high costs and low yields of mammalian cell culture processes compared with bacterial fermentations, there is a strong demand for optimization in terms of cellular productivity and product quality.
Gene Shuttles

The genetic modification of cultured cells or whole animals by various gene transfer methods has allowed the production of proteins intended for both basic research and for large-scale biotechnological production of therapeutic proteins. Gene delivery technology presents the major obstacle to the success of this field, and a consensus has emerged that the development of vectors that can deliver and appropriately express relevant gene products is much needed (Verma and Somia, 1997; Nabel, 1999).

Many approaches focusing on the expression of one or more proteins in transgenic cell cultures or organisms require a coexpression of heterologous gene products. Standard methods used to generate stable cell lines require transfection of a host cell line with two expression cassettes, one expressing the protein of interest and the other an antibiotic resistance marker for selection. This is usually achieved either by the cotransfer of two independent vector systems or by placing both expression cassettes, provided with two heterologous sets of regulatory elements, on the same vector (Mielke et al., 2000).

Internal initiation sites

A valuable tool to physically link the expression of a selection marker with another gene of interest was provided by the discovery of the function of the 5' untranslated regions of transcripts made by polio- and encephalomyocarditis viruses. These sequences mediate an efficient translational initiation of the uncapped viral transcript by directing initiating ribosomes to a defined start codon about 600-1200 nucleotides downstream, and were hence called internal ribosome entry sites (IRES) and cap-independent translation enhancers (CITE) (Jang et al., 1988; Molla et al., 1992). Whereas initiation of the overwhelming majority of eukaryotic mRNAs proceeds by a cap-dependent mechanism, IRES elements function in artificial bicistronic mRNAs when placed between the two reading frames, mediating a cap-independent translational initiation of the second cistron (Kozak, 1989b; Mountford and Smith, 1995). Such internal entry sites led to the development of multicistronic expression vectors allowing coordinated protein production from a single mRNA transcript (Dirks et al., 1993; Fussenegger et al., 1998) (Fig. 5).

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**Fig. 5:** Tricistronic, eukaryotic expression unit. Adapted from (Fussenegger et al., 1998).
Controlling transcription by a regulatable promoter; the tetracycline system

An essential requirement for establishing and controlling biotechnological production processes is restriction of product synthesis to a desired time period of the cultivation. An elegant approach to solve this problem is to express the product genes under the control of a tetracycline- (tet) regulatable promoter (P_{hCMV*-1}). Employing the tetracycline gene regulation system provides the means to tightly regulate the expression level of a specific eukaryotic gene. By fusing the tet repressor (tetR) with the transcription activation domain of virion protein 16 of herpes simplex virus (VP16), a tetracycline-controlled transactivator (tTA) was generated that stimulates the transcription from a minimal promoter sequence. This minimal promoter sequence consists of the human cytomegalovirus promoter IE fused to seven tet operator sequences (tetO). Depending on the concentration of tetracycline in the culture medium, expression can be regulated over 5 orders of magnitude (Gossen and Bujard, 1992; Furth et al., 1994) (Fig. 6).

Controlling transcription by dimerizing technology; the rapamycin system

A completely different approach for transcriptional control is based on the principle of

![Diagram](image-url)

Fig. 6: The tetracycline-regulatable promoter system. Adapted from (Fussenegger, 2001)
chemically induced dimerization (CID), a technique first applied to the activation of cell-surface receptors (Spencer et al., 1993). Several dimerizer systems have been developed since then based on both natural metabolites and completely synthetic molecules (Klemm et al., 1998).

Rivera and coworkers demonstrated rapamycin-dependent dimerization of FK506 binding-protein (FKBP) and FRB (FKBP-rapamycin binding domain of FKBP-rapamycin associated protein). By fusing each of the DNA-binding (tetR) and transactivation domains (VP16) to FKBP or FRB, transcriptional activation can be rendered dependent on the presence of the bivalent drug rapamycin that can simultaneously bind the two fusion proteins through their drug-binding domains (Magari et al., 1997; Rollins et al., 2000). The optimal response in the rapamycin-dependent induction of reporter gene transcription was obtained when three FKBP domains were fused tandemly to a DNA binding domain and an activation domain fused to one FRB (Rivera, 1998; Pollock et al., 2000) (Fig. 7).

![Transcriptional control system based on rapamycin-dependent dimerization](image)

Fig. 7: Transcriptional control system based on rapamycin-dependent dimerization. Adapted from (Fussenegger, 2001).
**Biphasic Production Processes**

In standard cultivation processes cells proliferate at high rates until cell death due to nutrient depletion causes a decline in the number of viable cells. However, several findings pointed to a beneficial effect of reduced growth rates on product synthesis rates per cell. The phenomenon that reduced specific growth rates may increase productivity of the cells was first observed with hybridoma cells producing monoclonal antibodies (Al-Rubeai et al., 1992; Bibila et al., 1994).

Recent research in the field of cell-cycle control demonstrated the potential of growth-arrested cells to produce a multiple amount of protein with a given volume and cell density and an additional improvement in the efficiency of medium usage due to the maintenance of the cells in a non-dividing yet product-synthesizing state (Al-Rubeai et al., 1992; Singh et al., 1994). The first relevant biotechnological contribution to protein production in proliferation-inhibited cells came from the isolation of a temperature-sensitive CHO cell line. These mutants showed 3-4 times higher production of a tissue inhibitor of metalloproteinases when growth arrest was induced by a temperature shift to 39°C (Jenkins and Hovey, 1993).

The first attempt to control proliferation by genetic engineering of the biotechnologically relevant baby hamster kidney (BHK) cell line was based on the estrogen-regulated overexpression of the interferon-responsive factor, IRF-1, a DNA-binding transcription activator, which accumulates in response to interferons and leads to the general antiviral state.
Introduction

of the cells (Koester et al., 1995). This technology gives rise to an ideal production process where the cells will grow to the desired density in a first stage, followed by a second production stage where the engineered genes are turned on resulting in cell-cycle arrest and overproduction of the desired protein. Besides the better cost-effectiveness this technology allows for the production of cell-toxic compounds not currently practicable (Fussenegger and Bailey, 1998) (Fig. 8).

As outlined in the first part of the introduction, over the past years a great body of research revealed how regulatory protein networks function to control the cell cycle in eukaryotes. The identification of cyclin-dependent kinase inhibitors and other proteins that inhibit cell growth at specific points in the cell cycle enabled the development of novel strategies to control proliferation of mammalian cells by engineering cells to overexpress one such inhibitor (Mazur et al., 1998). The productivity enhancement achieved through saving of metabolic growth energy is not the only advantage of using non-proliferating cells for the winning of heterologous proteins, but also proved the fact, that all cells remain in the same cell-cycle state promising with regard to the homology of the manufactured products (Kaufmann et al., 2001).

Maintaining the Producer Cells in Culture

One major problem relying on biotechnological applications is genetic drift of the producer cells, a mechanism that is often observed in heterologous protein production processes or metabolic engineering (Morrison et al., 1997; Mazur et al., 1999).

The necessity to discriminate different clonal variants of a cell lineage is not only a prerequisite for biotechnological applications but is also of fundamental interest for corrective gene transfer for therapeutic intervention in metabolic and hematopoietic disorders. Hematopoietic cell cultures, or ex-vivo expansion of blood cells, is an enabling technology with many potential applications in bone-marrow transplantation, immunotherapy, gene therapy and the production of blood products. The culturing of hematopoietic cells is hampered by their inherent complexity due to the heterogeneous cell types at different stages of development present as well as their strong interaction with each other and the environment through cytokines and adhesion molecules (Hayworth et al., 1990; Nielsen, 1999).

Surface selection technology

Increasing knowledge of surface antigen expression specific for developmental stages or the course of an illness offers new possibilities towards cellular discrimination by flow cytometry.
based on panels of surface antigens (Lai et al., 2000; Porwit-MacDonald et al., 2000). According to this technology there have also been attempts to overcome the still inadequate gene transfer efficiency which hampers a long-term correction for gene therapy of most disorders. Transfection of the therapeutic gene together with a cell-surface antigen enabled the selective enrichment of successfully transfected cells by flow cytometry based on cell-surface fluorescence intensity derived from the engineered antigen (Migita et al., 1995; Medin et al., 1996). Even though this technology can speed up establishment of stable cell lines or enable the selective analysis of cells with a specific surface pattern out of cell mixtures it is not suitable as selection system for larger scale. On one side the processing of large cell numbers and volumes is too labor-intensive and on the other side too time-consuming.

Different groups reported on magnetically-activated cell sorting as an approach enabling a more careful treatment and the processing of a larger number of cells in a reasonable amount of time thus increasing the yield of cells per round of selection (Geiselhart et al., 1996; Siegel et al., 1997). Such systems utilizes recombinant antibody technology to produce a “molecular Hook” by displaying a hapten-binding antibody on the cell surface enabling selective magnetic isolation by virtue of the ability to bind to hapten-coated metal particles (Chesnut et al., 1996) (Fig. 9).

**Monitoring of Expression Levels**

A major area of current research in eukaryotic molecular biology involves the elucidation of factors which control the level of gene expression. Reporter gene assays are widely used for studying gene regulation and function in cell biology since they enable mapping of promoter and enhancer regions, and identification of factors, mechanisms, or compounds that alter gene

![Fig. 9: Magnetic surface selection of cells presenting a recombinant antibody. Adapted from (Chesnut et al., 1996).](image-url)

Reporter genes code for proteins that possess a unique enzymatic activity or are otherwise easily distinguishable from the mixture of intra- or extracellular proteins. The effectiveness and sensitivity of a reporter system depends upon a number of factors including level of expression in a cell, stability of the expressed protein in the intracellular milieu and sensitivity of detection of the expressed protein (Bronstein et al., 1994; Olesen et al., 2000).

Several genes have been adapted as reporters of transcriptional activity, including chloramphenicol acetyltransferase, β-galactosidase (β-Gal), β-glucuronidase (GUS), placental alkaline phosphatase (PLAP), human growth hormone (hGH), plasminogen activator (PA), firefly luciferase (luc), green fluorescent protein (GFP) and others (Gorman et al., 1982; Selden et al., 1986; Berger et al., 1988; Jefferson, 1989; Williams et al., 1989; Jain and Magrath, 1991; Chalfie et al., 1994; Langer et al., 1995).

**Human secreted placental alkaline phosphatase**

A gene construct bearing a mutation in the membrane localization domain of human placental alkaline phosphatase (PLAP) causes the normally membrane-bound protein to be secreted from the cell (SEAP) (Berger et al., 1988). Thus, SEAP detection is performed with a sample of cell culture medium while the cell population remains intact. SEAP is able to dephosphorylate a long list of natural and synthetic substrates. Accordingly, a number of different assays with varying degrees of sensitivity are available for measuring SEAP activity. The standard colorimetric assay, which is based on the hydrolysis of p-nitrophenylphosphate to p-nitrophenol, is rapid, simple, and inexpensive (Alam and Cook, 1990). The chemiluminescent 1,2-dioxetane substrate (CSPD: disodium 3-(4-methoxyspiro [1,2-dioxetane-3,2'(5'-chloro)-tricyclo-[3.3.1.13,7]decan]-4-yl)phenyl phosphate), can be used for highly sensitive detection of either SEAP or non-secreted placental alkaline phosphatase.

![Fig. 10: Light emission mechanism of the 1-2-dioxetan substrate CSPD. Adapted from (Olesen et al., 2000).](image-url)
Hydrolytic cleavage of CSPD by alkaline phosphatase results in the formation of a metastable dioxetane phenolate anion, which initiates a decomposition mechanism called chemically initiated electron exchange luminescence (CIEEL), in part influenced by the remote chlorine substituent on the adamantine group (Koo et al., 1978). Charge transfer from the phenolate to the dioxetane ring promotes a concerted cleavage of two bonds of the cyclic peroxide, releasing about 100 kcal to chemiexcite one of the resulting carbonyl fragments to a singlet electronic state. The excited species emits light at approximately 480 nm as it converts back to the ground state (Olesen et al., 2000) (Fig. 10).

**Human low molecular weight urokinase-type plasminogen activator**

Plasminogen activation is a cascade-like process and represents a major extracellular proteolytic mechanism. Plasminogen activators (PAs) are highly specific serine proteases, named for their ability to cleave the inactive zymogen plasminogen to the active general protease plasmin. PAs play an essential role in the process of fibrinolysis and a variety of other biological processes (Saksela, 1985; Collen et al., 1988). There are two types of PAs, urokinase-type (u-PA) and the tissue-type (t-PA). u-PA operates as a fibrin-independent, largely receptor-bound, plasminogen activator, whereas t-PA acts as a fibrin-dependent and primarily intravascular activation enzyme (Chapman, 1997). u-PA has been purified as a single-chain molecule consisting of 411 amino acids from several natural sources including

![Fig. 11: u-PA_{LMW}-dependent plasminogen cleavage and proteolytic cleavage of the plasmin substate S-2251.](image-url)
urine, plasma, or conditioned cell culture media. In contrast to u-PA, its low molecular-weight derivative (u-PA\textsubscript{LMW}) consisting of only 267 residues, does not interact with cell membrane anchored u-PA receptors and is secreted as an inactive proform. Upon activation by traces of plasmin, u-PA\textsubscript{LMW} displays an enzymatic activity comparable to that of the wild-type protein (Lijnen et al., 1988). The activity of u-PA\textsubscript{LMW} can be assessed by measuring u-PA\textsubscript{LMW}-dependent conversion of plasminogen to plasmin. Subsequent plasmin-mediated cleavage of the tripeptide substrate, S-2251 (H-D-Val-Leu-Lys-p-nitroanilin) results in the chromophore p-nitroanilide, which can be quantified by measuring the increase in light absorbance at 405 nm (Langer et al., 1995) (Fig. 11).

*Photinus pyralis* firefly luciferase

The luciferase isolated from the common North American firefly *Photinus pyralis* is one of the most extensively studied of the enzymes that catalyse light production in bioluminescent organisms (DeLuca and McElroy, 1978). Firefly luciferase (luc) is a 62 kDa monomeric protein with no prosthetic group. Its cDNA and that of several other beetle luciferases have been cloned and expressed in *Escherichia coli* and many eukaryotes (Wood et al., 1989; Devine et al., 1993). Luciferase first catalyses the condensation of luciferin, benzothiazoylthiazole with ATP in the presence of Mg\textsuperscript{2+}, followed by the reaction of the adenylate with oxygen and cyclization of the peroxide; ATP provides the good leaving group AMP. The breakdown of the dioxetanone releases the energy, 50 kcal/mol, necessary to generate the excited state of oxyluciferin and CO\textsubscript{2} with an overall efficiency reportedly close to 1 photon per oxidized luciferin (McCapra and Perring, 1985; McElroy and DeLuca, 1985). Even

![Firefly luciferase reaction](image-url)

**Fig. 12:** The hypothetic bioluminescence reaction of firefly luciferin. Adapted from (Wilson and Hastings, 1998).
though the luciferin is the same in all beetles, their emission span a wide-wavelength range, from green to red. Emission originates from the enzyme-bound mono-anion of oxyluciferin in its keto form, and the energy of its excited state, hence the color of the emission, probably depends on the tertiary structure of the catalytic site (Wilson and Hastings, 1998) (Fig. 12).

CONTROL OF TRANSLATION IN EUKARYOTES

The importance of the translation machinery and its fidelity have been apparent since the discovery of the genetic code. However, the broad impact of translational regulation in eukaryotic cells has emerged explosively only in the last few years.

Translational regulation plays critical roles in cell growth, proliferation, and development. Advantages of translational control include rapid response and a means to affect the level of a gene product in the absence of transcription. Translation rates can be controlled at each of the three steps of translation: initiation, elongation, and termination. However, regulation occurs predominantly at initiation, when the ribosome is recruited to an mRNA and positioned at the initiation codon (Morley, 2001).

There are two general forms of translational control: (i) A specific mRNA or subset of mRNAs is regulated. Such regulation can be quantitative, determining the amount of protein produced; this may be all-or-none, or graded. Specific regulation can also be qualitative, enabling a single mRNA to produce several different proteins. (ii) Regulation is global and modulates rates and patterns of protein synthesis, thereby contributing to the overall regulation of cell growth and metabolism. These two forms of regulation are not mutually exclusive (Mathews et al., 1996).

Mechanisms of Translation-Initiation

Initiation is a complex multi-step process involving a large number of protein factors and multi-protein complexes, in addition to ribosomes. At least 25 proteins are involved in the

![Fig. 13: Cap-dependent translation initiation pathway.](image)
initiation process per se, excluding ribosomal proteins and tRNA synthetases (Merrick and Hershey, 1996). Translation initiation consists of several steps and is catalysed by proteins referred to as eukaryotic initiation factors (eIFs) (Dever, 2002).

Eukaryotic mRNAs receive a 5’ cap structure (m7G) and a 3’ polyadenylated tail (AAA) as
a nuclear dowry before their export into the cytoplasm as mature mRNAs. Both these moieties activate translation initiation in concert with cytoplasmic binding proteins, the cap-binding protein eIF4E, and the polyA binding protein (Pab1p). However, some picornaviral RNAs and a few cellular mRNAs have specialized sequences within their 5' untranslated regions that directly promote ribosome binding independent of a cap structure. These are the internal ribosome entry sequences (IRES) (Oh and Sarnow, 1993) (Fig. 14).

**Cap-dependent**

A single, cap-dependent mechanism accounts for the translation of the vast majority of cellular mRNAs. The minimal features of an mRNA essential for cap-dependent initiation are deceptively simple: a cap structure and an AUG in a favorable context (Kozak, 1989a).

Initiation commences with dissociation of a preexisting 80 S ribosome, which is assisted by a number of initiation factors (eIFs), including eIF-3. Joining of an eIF2-GTP-Met-tRNA\textsubscript{i} ternary complex to the small ribosomal subunit (40 S) forms a 43 S pre-initiation complex. This 43 S complex is then recruited, with the aid of the eIF4 group of initiation factors, to the 5' end of the mRNA. Mammalian eIF4F is composed of three subunits: eIF4E (which binds to the m7G cap, an interaction crucial to recruiting eIF4F), eIF4A (which has ATPase-dependent RNA helicase activity), and eIF4G (which through its interaction with eIF3 aids the binding of the 43 S pre-initiation complex). It is generally accepted that the helicase activity of eIF4A unwinds secondary structures from the 5' untranslated region (5'UTR), which would otherwise impede the initiation apparatus. This helicase activity is stimulated by eIF4B, which binds simultaneously with, or very closely after, eIF4F. The 40 S subunit, with its associated cohort of initiation factors (43 S complex), traverses the mRNA 5' UTR in an ATP-dependent process known as scanning, until it encounters an initiation codon (AUG) in a favorable sequence context (Jackson, 1996). Following AUG recognition, the associated initiation factors are released in a process mediated by a GTPase-activating protein (GAP), eIF5, which promotes GTP hydrolysis by eIF2 in the ternary complex. Dissociation of the initiation factors allows for joining of the 60S subunit and the beginning of polypeptide elongation (Gale et al., 2000; Pestova et al., 2001) (Figs. 13, 14).

**IRES-mediated internal (cap-independent)**

Internal initiation, sometimes referred to as cap-independent initiation, is mediated by a secondary structure within the 5'UTR known as internal ribosome entry site (IRES) and was first discovered in picornaviruses (Jackson and Kaminski, 1995). IRES-mediated translation
does not require a free 5' end, as demonstrated by the translation of circular IRES-containing

Fig. 14: Several ways of engaging the small ribosomal subunit (40 S) with mRNAs mediated by the adapter protein eIF4G. Adapted from (Hentze, 1997).
RNAs (Chen and Sarnow, 1995). IRESes can be functionally discriminated from other 5’ UTR secondary structures by their ability to mediate translation of the downstream ORF of a bicistronic reporter mRNA, independent of the translational status of the first ORF (Pelletier and Sonenberg, 1988; Gingras et al., 2001).

Picornaviruses such as poliovirus or foot-and-mouth-disease virus encode proteases that specifically clip the N-terminal third off eIF4G (eIF4G\(^{\Delta}\)). This separates the cap-binding function of eIF4G from its RNA-helicase and ribosome-binding activities and thus inactivates translation of most cellular mRNAs (Etchison et al., 1982). However, \(^{\Delta}\)eIF4G can substitute for intact eIF4G in IRES-mediated translation and in fact may be more efficient (Ohlmann et al., 1996; Pestova et al., 1996). The cap-binding protein, eIF4E, can directly plug into the eIF4G\(^{\Delta}\), while \(^{\Delta}\)eIF4G associates with eIF3, a multimeric complex that is bound to the 40 S subunit (Mader et al., 1995). Therefore, eIF4G forms a bridge between the cap structure (via eIF4E) and the 40 S subunit (involving eIF3) that is broken by the picornaviral proteases. The proteolytic clip kills two birds with one stone, because it not only uncouples the cap from eIF4G, but the liberated \(^{\Delta}\)eIF4G actively supports IRES-mediated translation (Lamphear et al., 1995). Similar to the cap in conjunction with eIF4E, \(^{\Delta}\)eIF4G binds strongly and specifically to a structural element within the encephalomyocarditis virus IRES upstream of the initiation codon. By means of IRES-binding proteins (X) such as polypyrimidine tract-binding protein (PTB) and the La autoantigen. Thus, eIF4G can also build a molecular bridge between this IRES and the ribosome (Svitkin et al., 1994; Hellen and Wimmer, 1995) (Fig. 14).

**An alternative route: Paip1**

Studies in yeast indicated that the polyA tail stimulates 40 S subunit recruitment to the mRNA (Tarun and Sachs, 1995). This recruitment is mediated by polyA-binding protein (Pab1p), which acts synergistically with the cap structure, but can function independently as well. Once more, eIF4G is the responsible molecular adapter. Interestingly, Pab1p and eIF4G interact only in the presence of RNA. eIF4G thus integrates the functions of the cap and the polyA tail in translation. A simultaneous interaction of eIF4G with the cap-binding protein eIF4E and the polyA binding protein Pab1p circularize the mRNA and was shown to enhance translational efficiency (Wells et al., 1998) (Fig. 14).
REFERENCES


Chapter 1


CHAPTER 2

CONTROLLED PROLIFERATION BY MULTIGENE METABOLIC ENGINEERING ENHANCES THE PRODUCTIVITY OF CHINESE HAMSTER OVARY CELLS

ABSTRACT

The eukaryotic cell cycle is regulated by a complex network of many proteins. Effective reprogramming of this complex regulatory apparatus to achieve bioprocess goals, such as cessation of proliferation at high cell density to allow an extended period of high production, can require coordinated manipulation of multiple genes. Previous efforts to establish inducible cell-cycle arrest of Chinese hamster ovary (CHO) cells by regulated expression of the cyclin-dependent kinase inhibitor (CDI) p21 failed. Here, by tetracycline-regulated coexpression of p21 and the differentiation factor CCAAT/enhancer-binding protein α (C/EBPα) (which both stabilizes and induces p21), effective cell-cycle arrest has been achieved, and per cell production of a model heterologous protein (secreted alkaline phosphatase; SEAP) has been increased 10-15 times relative to an isogenic control cell line. Because activation of apoptosis response is a possible complication in a proliferation-arrested culture, the survival gene bcl-xL was coexpressed with another CDI, p27, found to enable CHO cell-cycle arrest predominantly in G1-phase. CHO cells stably transfected with a tricistronic construct containing the genes for these proteins and for SEAP showed 30-fold higher SEAP expression than controls, a factor of three increase relative to CHO cells engineered to overexpress only p27. Multigene metabolic engineering of the cell cycle has thus proven effective in achieving a difficult-to-attain cell culture state and surprisingly large enhancements in expression of a heterologous secreted protein.

INTRODUCTION

Classical metabolic engineering most often involves modulating the expression of a single key gene of a critical pathway (for example, overexpression of Vitreoscilla hemoglobin enhances several aerobic bioprocesses (Holmberg et al., 1997), or overexpression of cyclinE stimulates mammalian proliferation in protein-free culture (Renner et al., 1995)).

However, complex biological functions involve multiple gene products, presumably because this affords greater efficiency or higher quality control, or simply because a single protein cannot, alone, provide the required function. Therefore, metabolic engineering strategies which coordinately modify multigene expression have the potential to achieve previously inaccessible metabolic states and thereby to empower qualitative changes in useful cell characteristics. One striking example is synthesis of many variant and novel non-natural polyketides by recombinant Streptomyces expressing new combinations of polyketide synthases (Alvarez et al., 1996; McDaniel et al., 1993; McDaniel et al., 1995).
Some of the most complex and important regulatory mechanisms of eukaryotic cells are those that govern cell division. The decision for a cell to divide is a tightly regulated process that integrates signals of several types such as presence of growth and differentiation factors, DNA damage and availability of nutrients. At the centre of these signalling pathways, the cyclin-dependent kinases (CDK) coordinate different cell-cycle events in space and time (Graña and Reddy, 1995). Whereas positive growth control relies on complexes of CDKs and cyclins (a family of proteins synthesised and degraded in a cell-cycle-dependent manner and involves multiple phosphorylation and dephosphorylation events), negative proliferation control is regulated by cyclin-dependent kinase inhibitors (CDIs) which bind to and inactivate cyclin-CDK complexes (Graña and Reddy, 1995). The two most prominent members of the CDI family are p21 and p27 (El-Diery et al., 1993; Polyak et al., 1994a). These are highly homologous but differ in their affinity to cyclin-CDK complexes, and their regulation (Polyak et al., 1994a; Toyoshima and Hunter, 1994). Only at elevated intracellular concentrations p21 binds to cyclin-CDK complexes and inhibits the progression of cell-cycle particularly at G1-phase (Harper et al., 1993; Xiong et al., 1993). Besides p53-mediated transcriptional activation to counteract DNA damage, p21 can also be induced by the CCAAT/enhancer-binding protein α (C/EBPα) as part of the cell-cycle inhibition program following terminal differentiation (Gartel et al., 1996; Timchenko et al., 1996). C/EBPα also stabilises p21 at the protein level, increasing its half-life (Timchenko et al., 1996). Like many cell-cycle regulatory proteins, turnover of this CDI is very high (Graña and Reddy, 1995).

Whereas the overexpression of the CDIs p21 or p27 have already proven to be effective in cancer therapy (Bertelsen et al., 1995; Chen et al., 1996; Clayman et al., 1996; Hartwell and Kastan, 1994; Pennisi, 1996; Yang et al., 1995), we recently showed that their transient overexpression controls proliferation of CHO cells and leads to a 4-fold increase in productivity of the model product protein SEAP in the G1-arrested state (Fussenegger et al., 1997). However, in a stable genetic configuration, only regulated overexpression of p27 was successful in inducing a sustained CHO cell growth arrest in G1-phase, which also resulted in a 10-fold increase in per cell SEAP productivity (Mazur et al., 1998). Stable overexpression of p21 did not result in growth arrest (Mazur et al., 1998). Here we use multigene metabolic engineering to enable a cytostatic production process with the CDI p21, and to increase greatly the productivity of CHO cells arrested by the overexpression of p27.
MATERIALS AND METHODS

Cell Culture, Transfection, SEAP Activity Test, Flow Cytometry, Western Blot Analysis and Apoptosis Assay

The cell line CHO-K1 (CHO-K1, ATCC: CCL 61), its tTA-expressing derivative CHO-XMK1\textsubscript{9}, the SEAP producing cell line CHO-XMK11\textsubscript{10} as well as the basic cell culture technologies including medium composition, transfection and selection procedures, tetracycline-responsive gene expression, flow cytometric analysis and immunofluorescence microscopy have been described before (Fussenegger et al., 1997, Mazur et al., 1998; Fussenegger et al., 1998; Gossen and Bujard, 1992; Zang et al., 1995). Stable growth-regulatable CHO cell lines were generated by transfection of plasmids pDD6 or pSS5 into CHO-XMK1\textsubscript{9} under selection (6 µg/ml) of the cotransfected puromycin resistance plasmid (pPur; Clontech). For permanent repression of the cytostatic expression unit during selection and the two subsequent rounds of cloning, 2 µg/ml tetracycline were added to the cell culture medium with medium exchanges every other day.

The SEAP activity test measuring the change in absorbance of a chromogenic substrate was adapted from Berger (Berger et al., 1988). The slope of such an absorbance time course is directly proportional to the SEAP production of the population and was divided by the number of cells to generate SEAP production data for arrested (P) and proliferation-competent (P*) cell population on a per cell basis. Thus, the factor P/P* is a quantitative measure indicating productivity of growth arrested cells relative to proliferating cells (Fussenegger et al., 1997; Mazur et al., 1998).

Protein isolations were adapted from Behrens (Behrens et al., 1994). Western Blot analysis was performed with the ECL Western blot kit (Amersham) according to the manufacturer’s protocol using the same primary antibodies as for immunofluorescence: bcl-2 (rabbit polyclonal (ΔC21) cat# sc-783, lot# A137; mouse monoclonal bcl-2 (100), cat# sc-509, lot# C187); bcl-x\textsubscript{S/L} (rabbit polyclonal (S-18), cat# sc-634, lot# L106); C/EBP\textgreek{a} (rabbit polyclonal (14AA), cat# sc-061, lot# L046) (all from Santa Cruz Biotechnology Inc.), p21- and p27-specific antibodies were described before (Fussenegger et al., 1997; Mazur et al., 1998; Fussenegger et al., 1998). Apoptosis was measured without or with induction of programmed cell death by incubation with various actinomycin D concentrations for 6 h using the ApoAlertTM apoptosis assay (Clontech).
**Plasmid Constructions**

Various dicistronic expression vectors expressing CDIs concomitantly with the model product gene SEAP, the isogenic monocistronic SEAP expression vector pMF111 as well as the family of tricistronic expression vectors including pTRIDENT1 and pTRIDENT4 have been described before and contain the tetracycline-repressible promoter P_{hCMV\textsuperscript{-}1} (Fussenegger et al., 1997; Mazur et al., 1998; Fussenegger et al., 1998; Gossen and Bujard, 1992). We constructed pTRIDENT1 and pTRIDENT4-derived tricistronic expression vectors which are isogenic to their dicistronic counterparts for the expression of SEAP and p21 or p27 from the first and second cistron, respectively, but which contain an additional third cistron which encodes either of the survival factors bcl-2 (Tsujimoto and Croce, 1986), bcl-x\textsubscript{L} (Boise et al., 1993) or the p21 stabilising differentiation factor C/EBP\textalpha (Timchenko et al., 1996).

SEAP was cloned into the first cistron of pTRIDENT1 and pTRIDENT4 by cutting the monocistronic, SEAP production vector pMF111 (Fussenegger et al., 1997; Mazur et al., 1998) with EcoRI/HindIII and ligating the SEAP gene to the corresponding sites of the pTRIDENT vectors to give pMF127 (pTRIDENT1) and pMF124 (pTRIDENT4), respectively. Subsequently, pMF124 was cut with NotI and BclI and p27 amplified with primers OMF46: GATCACTAGTGATATCGCGGCCGCGGTCGTGCAGACCCGG and OMF47: GATCATCGATGGATGGATCCGCCCGGGCTTACGT TTGACGTCTTCTTCTTG from pMF113 and p21 amplified with primers OMF44: GATCACTAGTGATATCGCGGCCCTTCGCCGAGGCACCGAGG and OMF45: GATCATCGATGGATGGATCCGCCCGGGCTTAGGGCTTCCTTGGG from pMF112 (Fussenegger et al., 1997) were cloned as NotI/BamHI fragments to give plasmids pMF128 (SEAP-p27) and pMF130 (SEAP-p21), respectively (Fussenegger et al., 1998). Similarly, pMF127 was cut with NotI/BclI and PCR-amplified p21 (OMF44 and OMF45) was cloned as NotI/BamHI fragment to give pMF129 (SEAP-p21).

The survival factors bcl-2 and bcl-x\textsubscript{L} were cut out of pSFFNeo-bcl2 (Behrens et al., 1994) and pSFFNeo-bclx\textsubscript{L} (Boise et al., 1993) by EcoRI and cloned into pBluescriptII SK\textsuperscript{-} placing these genes under the control of the lacZ promoter to give plasmids pMF137 (bcl-2) and pMF138 (bcl-x\textsubscript{L}), respectively. Similarly, the gene coding for the human differentiation factor C/EBP\textalpha contained in pCMV\textalpha (Timchenko et al., 1996) was restricted with BamHI and ligated to the corresponding BamHI site of pBluescriptII SK\textsuperscript{-} (pSS2), thus placing this gene under the control of the lacZ promoter. pMF137 and pMF138 were restricted with SpeI/EcoRV and the corresponding genes, bcl-2 and bcl-x\textsubscript{L} were either ligated to the SpeI/SwaI sites of pMF128 to
give plasmids pDD4 (SEAP-p27-bcl-2) and pDD6 (SEAP-p27-bcl-xL) or to pMF130 to give pDD1 (SEAP-p21-bcl-2) and pDD3 (SEAP-p21-bcl-xL). In the same way, pSS2 was restricted by SpeI/EcoRV and ligated to pMF129 (SpeI/SwaI) to produce plasmid pSS5 (SEAP-p21-C/EBPα).

RESULTS

Tetracycline-Responsive Proliferation Control of CHO Cell Lines Stably Expressing the Model Product Protein SEAP, the Cell-Cycle Inhibitor p27, and either of the Two Survival Genes bcl-2 or bcl-xL from a Tricistronic Expression Unit

In order to reduce the potential of apoptosis in cytostatic cell cultures as observed with ongoing expression of p53 even in its mutated, apoptosis-deficient form (p53175P) (Mazur et al., 1998; Rowan et al., 1996), we engineered CHO cells with either of the two survival factors bcl-2 or bcl-xL which were previously shown to minimize the induction of programmed cell death in proliferation-competent cell cultures (Huang et al., 1997; Itoh et al., 1995; Singh et al., 1996). The recently reported pTRIDENT family of expression vectors allowed the construction of multicistronic, operon-like genetic configurations (Fussenegger et al., 1998) containing the model product protein SEAP as well as the cytostatic gene p27 and one of the survival genes bcl-2 or bcl-xL. This tricistronic expression unit is driven by the tetracycline-repressible promoter P_{hCMV*-1} so that growth arrest, heterologous protein production and cell death protection can be simultaneously induced upon withdrawal of tetracycline from the cell culture medium.

Two tricistronic expression vectors pDD4 (SEAP-p27-bcl-2) and pDD6 (SEAP-p27-bcl-xL) were generated and stably transfected into the tTA-expressing CHO cell line CHO-XMK19 (Fussenegger et al., 1997). Surprisingly, no stable clones could be generated harboring pDD4. However, 20 stable pDD6-containing clones were produced, and three (CHO-DD6X1, CHO-DD6X2, CHO-DD6X3) were randomly chosen for further analysis. Western Blot analysis of CHO-DD6X1 and CHO-DD6X2 showed not only simultaneous, coordinated and differential expression of p27 and bcl-xL in the absence of tetracycline but also a strict linkage of their expression levels (Fig. 1). Strict tetracycline-mediated regulatability of the multicistronic expression unit of individual cells was demonstrated by p27-targeted immunofluorescence (Fig.2). Individual clones differed in the overall expression levels of their multicistronic expression units: CHO-DD6X1 shows a slightly higher expression of the tricistronic
expression unit (Fig. 1). Whereas the growth behaviour of CHO-DD6\(_{X1}\), CHO-DD6\(_{X2}\) and CHO-DD6\(_{X3}\) is identical to a wild-type or the control cell line CHO-XMK111\(_{10}\).

**Fig. 1.** Western blot analysis of CHO-XMK1\(_{10}\) cells stably transfected with the tricistronic expression vector pDD6 (SEAP-p27-bcl-x\(_L\)). Two independent cell lines, CHO-DD6\(_{X1}\) and CHO-DD6\(_{X2}\), were analysed for the production of the survival gene bcl-x\(_L\) encoded by the third cistron of pDD6 (A). Both cell lines were grown in the presence and the absence of tetracycline (+ tet; - tet) in the culture medium resulting in the expression of the tricistronic message and consequently in growth arrest (due to the concomitant expression of p27 (B), see also Fig. 2E) and coordinated expression of bcl-x\(_L\) in the absence of tetracycline (CHO-DD6\(_{X1}\) -tet; CHO-DD6\(_{X2}\) -tet). The size of bcl-x\(_L\) corresponds to its deduced amino acid sequence of 26 kDa (see ->). The membrane was reprobed with anti-p27 antibody (B).
(Mazur et al., 1998) in the presence of tetracycline, all pDD6-derived cell lines show an immediate and strong growth arrest, predominantly at G₁-phase (CHO-DD6ₓ₁: 60% compared to 45% of proliferating cells), when tricistronic expression is activated in the absence of tetracycline (Fig. 3). Interestingly, the sustenance of the cell-cycle arrest seems to correlate with the expression level of the multicistronic expression unit, since CHO-DD6ₓ₁ shows a more restricted proliferation control than CHO-DD6ₓ₂ (Figs. 1 and 3). Under cytostatic conditions both cell clones (CHO-DD6ₓ₁; CHO-DD6ₓ₂) remain cell-cycle arrested.

Fig. 2. Immunofluorescence analysis of the stable cell line CHO-SS5₁₅₃ harboring the tricistronic SEAP-p21-C/EBPα-encoding expression unit under growth-arrested (A, C) and proliferation-competent conditions (B, D). A and B show the expression of p21 and B and C the expression of the differentiation factor C/EBPα always under full induction (cytostatic condition: absence of tetracycline; A and C) or repression (proliferation-competent condition: presence of tetracycline; B and D) of the multicistronic expression unit. Similarly, p27-targeted immunofluorescence was analyzed in growth-arrested (E) and proliferating CHO-DD6ₓ₁ (F).
over extended periods (up to or exceeding two weeks) and probably beyond this time. However, after two weeks of sustained cytostatic culture, mutant clones begin to become apparent which escape the engineered proliferation control, most probably as a result of a damaged multicistronic expression unit (data not shown). During growth-arrested periods, the cells remain viable, show no particular morphological changes which are suggestive for apoptosis, and can resume the cell-cycle upon addition of tetracycline which shows that tetracycline-responsive proliferation control is completely reversible (data not shown).

**Proliferation Control of CHO Cells Using the CDI p21 in a Tricistronic Genetic Configuration**

Analogous to p27-encoding plasmids we constructed and transfected p21-based tricistronic proliferation control vectors pDD1 (SEAP-p21-bcl-2) and pDD3 (SEAP-p21-bcl-xL) into CHO cells. As with pDD4 (SEAP-p27-bcl-2), no stable clones could be found following transfection of pDD1. All of the CHO clones that were generated by the transfection of pDD3 showed high as well as tetracycline-regulatable expression of all three cistrons, but these clones displayed no growth arrest in tetracycline-free medium. Several other efforts to generate growth controlled pDD3-containing stable CHO cells were also unsuccessful, as were prior attempts to establish p21-based proliferation control in a dicistronic, PhCMV*-1-driven configuration (Mazur et al., 1998). However, p21-based proliferation control in CHO was found to be possible when p21 was expressed either transiently or from a very strong constitutive promoter (Fussenegger et al., 1997; Mazur et al., 1998). Both experiments indicated that, similar to the wild-type configuration (Harper et al., 1993; Xiong et al., 1993), p21-engineering can inhibit cyclin-CDK complexes and promote cell-cycle arrest in CHO cells only at high intracellular levels which may not be attained in stable clones expressing cloned p21 under control of PhCMV*-1.

Therefore, as a further fundamental example of multigene metabolic engineering, we coexpressed the differentiation factor C/EBPα together with SEAP and p21 in a tricistronic configuration (pSS5) in order to coordinately stabilize p21 and also possibly induce expression of endogenous p21 in a p53-independent manner (Timchenko et al., 1996). Simultaneous, coordinated, as well as tetracycline-repressible expression of p21 and C/EBPα was shown by immunofluorescence (Fig. 2). Using this bipartite p21-C/EBPα-based engineering strategy we could generate stable pSS5-containing cell lines (CHO-SS5153; CHO-SS5127; CHO-SS5156; CHO-SS5135) in the same fashion as described above for p27-
expressing CHO derivatives. Full induction of $p21$ and $C/EBP\alpha$ in the absence of

![Graph A](image1)

![Graph B](image2)

**Fig. 3.** Growth behavior of arrested cell lines. The growth potential of an initial population of $2 \times 10^5$ cells of several pDD6- (A; SEAP-$p27$-bcl-$xL$) and pSS5-harboring (B; SEAP-$p21$-C/EBP$\alpha$) proliferation-inhibited cell lines were compared to the isogenic proliferation-competent control cell line CHO-XMK111$_{10}$. Whereas cell lines CHO-DD6$_{X1}$ and CHO-SS5$_{153}$ show the most restricted growth behavior in the absence of tetracycline, other cell lines are less restricted but show significant slower growth than the control cell line CHO-XMK111$_{10}$. 
tetracycline results in a growth arrest which varies in strength and sustenance among different clones (Fig. 3). Characterization of CHO-SS5$_{153}$; CHO-SS5$_{127}$; CHO-SS5$_{156}$; CHO-SS5$_{135}$ with respect to cell viability, absence of apoptotic morphologies, sustenance of growth arrest and outgrowth of mutant clones revealed no significant difference between CHO-SS5 (SEAP-\textit{p21-C/EBP}$_\alpha$) and CHO-DD6 (SEAP-\textit{p27-bcl-xL}) derivatives.

**Productivity of Proliferation-Inhibited Cell Lines**

As the model secreted heterologous product gene \textit{SEAP} is coordinately expressed from the same tricistronic expression unit as the CDIs, the survival gene or the differentiation factor, growth arrest is coupled with the production of SEAP. Concomitant with studies on the growth behaviour of the cell lines, SEAP activity was measured every day in their culture supernatants. As control we used the proliferation-competent cell line CHO-XMK11110 which represents the highest SEAP-producing, isogenic, continuously proliferating cell line out of 30 individual clones generated by transfection of the tetracycline-responsive transactivator (\textit{tTA}) overexpressing CHO derivative CHO-XMK1$_9$ with pMF111, a monocistronic expression vector harbouring \textit{SEAP} under control of the \textit{tTA}-responsive promoter P$_{hCMV^*-1}$ (Fussenegger et al., 1997; Mazur et al., 1998). The SEAP productivity (SEAP activity on a per cell basis) of both growth-arrested (P) and the proliferation-competent control cell line CHO-XMK11110 (P*) were separately measured under tetracycline-free conditions and plotted as the relative productivity P/P* (Table 1). Thus, the factor P/P* is a quantitative measure indicating productivity of growth-arrested cells relative to proliferating cells.

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<td>CHO-DD6$_{X1}$</td>
<td>32.5 ± 5.9</td>
<td>26.1 ± 2.3</td>
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<td>CHO-DD6$_{X2}$</td>
<td>15.3 ± 4.6</td>
<td>18.8 ± 5.4</td>
<td>11.8 ± 1.5</td>
<td>14.5 ± 0.6</td>
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<td>3.54 ± 0.3</td>
<td>4.80 ± 0.1</td>
<td>1.30 ± 0.5</td>
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<td>\textit{p21+ C/EBP}$_\alpha$</td>
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<td>CHO-SS5$_{153}$</td>
<td>2.16 ± 0.6</td>
<td>13.4 ± 0.8</td>
<td>15.9 ± 1.1</td>
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<td>CHO-SS5$_{127}$</td>
<td>2.06 ± 0.1</td>
<td>8.66 ± 0.5</td>
<td>10.6 ± 1.0</td>
<td>18.3 ± 3.6</td>
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<td>CHO-SS5$_{156}$</td>
<td>1.36 ± 0.6</td>
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<td>CHO-SS5$_{135}$</td>
<td>0.92 ± 0.1</td>
<td>1.98 ± 0.01</td>
<td>1.7 ± 0.1</td>
<td>2.49 ± 0.3</td>
<td>1.47 ± 0.1</td>
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**Table 1:** SEAP activity (P) of growth-arrested CHO cell populations is scaled, on a per cell basis, by the SEAP activity (P*) of the most productive, proliferation competent clone (CHO-XMK11110). Errors indicate standard deviations of three replicate experiments.
contrast to the control cells which were selected for high SEAP production, cells transfected with tricistronic expression vectors were isolated, as described above, without any selection or screening for SEAP.

All growth-arrested cultures show a higher overall per cell productivity compared to the proliferation competent control cell line CHO-XMK11110 (Table 1). Differences in SEAP productivity of growth-arrested cell lines correlate with the extent of proliferation-inhibition. For example, CHO-SS5153 and CHO-DD6X1 which show a more sustained growth arrest and express higher levels of the CDIs produce also more SEAP compared to the other cell clones (Figs. 1 and 3). Whereas the 10- to 15-fold increases in per cell SEAP productivity of CHO-SS5 (SEAP-p21-C/EBP\(\alpha\)) derivatives are comparable to increases reached by dicistronic, SEAP-p27-encoding stable cell lines (Mazur et al., 1998), the per cell SEAP productivity of SEAP-p27-bcl-x\(L\) (pDD6) harboring CHO cell derivatives is increased by an additional factor of 3 compared to the dicistronic counterparts (Mazur et al., 1998), reaching values of 30 times greater than the per cell SEAP productivity observed with the proliferating control cell line CHO-XMK11110 (Table 1). However, the exact role of bcl-x\(L\) in this observed increase in productivity remains to be elucidated. This effect does not appear related to the anti-apoptotic function of bcl-x\(L\), since growth-arrested CHO-DD6 cells respond similarly to apoptosis-inducing agents as do their proliferation-competent counterparts (data not shown). However, observed tetraploidy and consequent amplification of genetic information following bcl-x\(L\) overexpression (Minn et al., 1996) could account for the additional increase in per cell SEAP productivity of proliferation-inhibited pDD6-harboring (SEAP-p27-bcl-x\(L\)) cell lines compared to their dicistronic counterparts. The reason why no stable bcl-2-expressing CHO clones could be generated remains unclear since anti-apoptosis engineering using this type of survival factor has already been established for several other cell types (Huang et al., 1997; Itoh et al., 1995; Singh et al., 1996) and has been achieved in CHO cells carrying dicistronic SEAP-bcl-2 vectors without any cloned CDI coexpression (data not shown).

**DISCUSSION**

In contrast to the use of p27, p21-mediated inducible proliferation control was not successful using one-gene metabolic engineering. Shortcomings in one-gene metabolic engineering are not surprising since many key regulatory circuits as i.e. found in the cell-cycle machinery are highly redundant. Such redundant pathways likely evolved for robust maintenance of critical regulation even in the presence of one or more mutations affecting some component(s) of the
control system. One-step multigene metabolic engineering technology has superior potential to cope with such situations since it enables simultaneous reprogramming of several cellular processes, greatly increases the potential to gain control over the desired cellular pathway or process. \(p21\)-based proliferation control therefore represents a fundamental example for multigene metabolic engineering. Two independent signalling pathways converge at \(p21\) to mediate cell-cycle arrest: (i) \(p21\) induction by the \(p53\)-dependent pathway in response to DNA damage (El-Diery et al., 1994; Polyak et al., 1994b); and (ii) induction and stabilization of \(p21\) by C/EBP\(\alpha\) to maintain proliferation arrest in terminally differentiated cells (Timchenko et al., 1996). Stimulation of \(p21\) expression by overexpression of \(p53\) is not a promising strategy in view of the apoptosis-inducing role of \(p53\) (Mazur et al., 1998; Ko and Prives, 1996; Yonish-Rouach et al., 1991). On the other hand overexpression of C/EBP\(\alpha\) was not reported to be problematic in several transformed human cell lines (Timchenko et al., 1996). Therefore, multigene metabolic engineering using a combination of coordinately expressed \(p21\) and C/EBP\(\alpha\) on regulated tricistronic expression vectors enabled \(p21\)-based proliferation control. Productivity assays of these constructs confirmed, in a genetic configuration totally different from \(p27\)-mediated proliferation control, that an individual cell is more productive in a cell-cycle-arrested state.

**ACKNOWLEDGEMENTS**

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CHAPTER 3

NOVEL SURFACE TAGGING TECHNOLOGY FOR SELECTION OF COMPLEX PROLIFERATION-CONTROLLED MAMMALIAN CELL PHENOTYPES

ABSTRACT
Regulated overexpression of the cyclin-dependent kinase inhibitor p27 enables biphasic production processes which consist of a non-producing expansion phase followed by an extended proliferation-arrested production phase. During the growth-arrested production phase proliferation-competent mutants emerge as a consequence of genetic drift and strong counterselection. Here we evaluate the use of cell surface markers for ex vivo selection of growth-arrested phenotypes by magnetic or FACS-mediated cell sorting. Multigene metabolic engineering resulted in a Chinese hamster ovary- (CHO) derived cell line CHO-SS1015, which expresses the model product protein SEAP (secreted alkaline phosphatase), the human cyclin-dependent kinase inhibitor p27 and a membrane-anchored multi-domain surface marker Hook in a tricistronic tetracycline-repressible manner. In the absence of tetracycline in the cell culture medium, p27 mediated a G1-phase-specific cell-cycle arrest of CHO-SS1015 and resulted in a 5-fold increase in SEAP production compared to proliferation-competent control cells. Concomitant expression of Hook enabled FACS- or magnetic-based selection of CHO-SS1015 cells from various mixed populations. Sophisticated surface selection of engineered cells will likely become important for biopharmaceutical manufacturing and for in vivo maintenance of treated cells in gene therapy and tissue engineering.

INTRODUCTION
Mammalian cell culture technology is becoming increasingly important for industrial production of complex human therapeutics ranging from glycoproteins, viral vectors for gene therapy to activated human cells (Fussenegger et al., 1999a; for a review; Fussenegger et al., 1999b; Cotter and Al-Rubeai, 1995). The variety of therapeutic products produced by mammalian cells represents a constant challenge for bioengineers to achieve an optimal balance between product quality, safety and cost-performance.

Biphasic production concepts meet with most of the requirements of modern biopharmaceutical manufacturing (Fussenegger et al., 1998b; Geserick et al., 2000). In a biphasic production process, cells are first grown to a desired cell density (non-producing proliferation phase) and then growth-arrested to enable maximum production (proliferation-controlled production phase) (Fussenegger et al., 1998b; Geserick et al., 2000). The switch between both phases is mediated by induction of growth-suppressing genes such as the cyclin-dependent kinase inhibitors p21 (Gartel et al., 1996; Michieli et al., 1994; Zeng and El-Deiry, 1996, for a review,) and p27 (Mazur et al., 1998; Toyoshima and Hunter, 1994), the
differentiation gene C/EBPα (Timchenko et al., 1995; Timchenko et al., 1996), the tumor suppressor gene p53 (Agarwal et al., 1995; Del Santo et al., 1996, for a review, Fussenegger et al., 1997a; Fussenegger and Bailey, 1998; Ko and Prives, 1996), antisense c-jun (Kim et al., 1998) or the interferon-responsive factor 1 (IRF-1) (Geserick et al., 2000; Mueller et al., 1999). In an optimal set-up, the product gene is induced concomitantly (preferably in a multicistronic configuration) with the growth-arresting gene to enable product production in a defined cell-cycle phase. Induction of product and growth-controlling genes is mediated by heterologous gene regulation technologies (Gossen and Bujard, 1992; Fussenegger and Bailey, 1998; Fussenegger et al., 2000; Mueller et al., 1999). The tetracycline-repressible expression concept is ideally used in combination with controlled proliferation technology since the natural breakdown of this antibiotic under culture conditions enables automatic well-defined transition from expansion to production phase (TETSWITCH; Mazur et al., 1999).

Several reports have shown that p27- or IRF-1-based proliferation control technology significantly increases specific productivity compared to proliferation-competent production processes (Fussenegger et al., 1997a; Fussenegger et al., 1998b; Mazur et al., 1998; Geserick et al., 2000), while maintaining identical product quality (Kaufmann et al., 2000; Mueller et al., 1999).

A major concern of controlled proliferation technology is the strong counterselection of arrested populations which results in increased genetic drift and outgrowth of proliferation-competent mutant cells (Mazur et al., 1999). These mutants were shown to have deleted the multicistronic expression cassette encoding the product gene and the growth-suppressing determinant (Mazur et al., 1999). Elimination of heterologous genes is often observed with production of difficult-to-express proteins or strong counterselection following metabolic engineering of mammalian cells (Cooke et al., 1997; Couture and Heath, 1995; Morrison et al., 1997).

In order to eliminate proliferation-competent mutant cells which overgrow arrested populations and to minimize genetic drift in production cultures, sophisticated selection technology will be required to continuously remove undesired cell mutants. The scenario of removing mutant cells from production processes is very similar to elimination of neoplastic cells from the hematopoietic system. Human-compatible in vivo selection technologies are of fundamental interest for corrective therapeutic interventions in metabolic and hematopoietic disorders. A further key to successful hematopoietic cell-based therapies is in vitro cultivation of (autologous) blood stem cells which are genetically engineered and reimplanted into the patient’s body. In vitro cultivation of hematopoietic cells is very difficult due to the potential
of these cells to differentiate into a variety of cell lineages (Hayworth et al., 1990, for a review, Nielsen, 1999). *Ex vivo* selection of treated cells prior to reimplantation is a prerequisite for successful therapy (Medin et al., 1996; Migita et al., 1995). Also, monitoring of blood counts following reimplantation of engineered cells and elimination of leukemic cells requires a sophisticated selection technology.

In principle, selection procedures may be based on antibiotic selection. Although antibiotic-based selection is routinely used for biotechnological applications, it is not compatible with *in vivo* selection of desired cell phenotypes in humans. The increasing knowledge of surface antigens expressed during different developmental stages or on diseased cells enables new opportunities for sophisticated surface-based selection technologies (de Wynter et al., 1995, de Winter et al., 1999; Kraguljac et al., 2000; Lai et al., 2000; Porwit-MacDonald et al., 2000).

Here we evaluate a model surface selection system (“the Hook system”) initially developed for rapid selection of transiently transfected cells (Chalmers et al., 1998; Lea et al., 1986; Manyonda et al., 1992; Tibbe et al., 1999) for use in controlled proliferation technology and future gene therapy applications.

**MATERIALS AND METHODS**

**Plasmid Construction, Cell Culture, Transfection and Production of Stable Cell Lines**

The gene encoding the single-chain variable fragment antibody (sFv) *Hook* contained in pHook™-2 (Invitrogen) was restricted by *XhoI*/EcoRV, polished with Pfu polymerase (Stratagene) and ligated to the *SmaI* site of pBluescriptII KS™ (Stratagene) to give plasmid pSS100.

CHO-XMK1₀ are Chinese hamster ovary cells (CHO-K₁, ATCC: CCL 61) stably transfected with the *tTA* (tetracycline-dependent transactivator) encoding plasmid pUHD15-1 (Gossen and Bujard, 1992) and pSV2neo (Clontech, CA) harboring the G418 resistance gene (Mazur et al., 1998).

CHO-SS101 cells are CHO-XMK1₀ cells stably transfected with pSS101 encoding the human secreted alkaline phosphatase (*SEAP*), the human cyclin-dependent kinase inhibitor *p27* and the surface-selection marker *Hook* in a tricistronic *PᵦCMV*-1-*SEAP*-IRES-*p27*-CITE*-Hook*-pA) and *Pₚur* conferring resistance to puromycin (Clontech, CA). CHO cells and their derivatives (30%–
50% confluent) were transfected using an optimized protocol typically yielding transfection rates of over 30% (Fussenegger et al., 1997a). Transfected populations were grown in the presence of FMX-8 medium (Dr. F. Messi Cell Culture Systems, Switzerland) and 400 µg/ml G418 (Alexis Biochemicals) to ensure tTA expression, 6 µg/ml puromycin (Alexis Biochemicals) to select for integration of pSS101, and 2.5 µg/ml tetracycline (Sigma) to repress the tricistronic SEAP-p27-Hook-encoding expression unit. Mixed populations were cultivated to a total cell number of 3x10^6 and subsequently cloned by FACS-mediated single cell sorting using a FACStarPlus and Cell Quest™ software (Becton-Dickinson, San Jose, CA).

All cell lines were cultivated in FMX-8 medium supplemented with 10% fetal calf serum (PAA Laboratories GmbH, Austria, cat# A15-022, Lot# A01129-242) and the appropriate antibiotics at 37°C in a humidified 5% CO₂ atmosphere.

**SEAP Assay**

Culture supernatants were centrifuged for 2 min at 14000 x g. 25 µl of the supernatant were removed and prepared for chemiluminescent SEAP quantification according to the manufacturer’s protocol (Great EscAPE™ SEAP reporter system 2, chemiluminescent, Roche Biochemicals). The SEAP activity was measured during 10 s using a Turner Designs Luminometer (TD20-20). The readout in relative light units per second (RLUs⁻¹) is directly proportional to the amount of SEAP in the culture supernatant.

SEAP production of stable cell lines was determined in triplicate over a period of 5 days. An initial cell population of 2.5x10^4 cells was seeded into 6-well plates containing selection medium (FMX-8, 10% FCS, 400 µg/ml neomycin, 6 µg/ml puromycin) and were grown in the presence (+tet; 2.5 µg/ml) or absence (-tet) of tetracycline. SEAP production was quantified by chemiluminescence (RLUs⁻¹; relative light units) on a per cell basis (RLUs⁻¹Cell⁻¹).

**Characterization of the Growth Behavior of Stable Cell Lines**

Parallel to quantification of SEAP production (see above), the total cell number of all cell cultures (+tet/-tet) was measured in triplicate. Cells were detached using dissolvation solution (Sigma) and resuspended in filter-purified casyton buffer (pH 7.3; 7.93 g/l NaCl; 0.38 g/l Na₂EDTA; 0.4 g/l KCl; 0.19 g/l NaHPO₄; 1.95 g/l Na₂H₂(PO₄)₂; 0.3 g/l NaF). The cell number (CellsWell⁻¹10⁻⁵) was determined as the average of three independent readings taken by a Casy1® cell counter (Schärfe System, Germany).
Immunofluorescence Analysis

Cells were transfected as described above and grown in the presence or absence of tetracycline (repression or induction of the multicistronic expression unit) on chamber slides (Lab-Tek, Nunc, Inc.) for 48 h. Alternatively, stable cell lines were seeded in chamber slides at low densities. The cell monolayer was washed once with phosphate-buffered saline (PBS, Sigma) and submerged with 3% paraformaldehyde solution in PBS for 15 min. Cells were then washed again with PBS and submerged with 0.2% Triton X-100 for 10 min. After another washing step, the cells were immersed in 2% BSA in PBS for 15 min and subsequently incubated for 1 h with the antibodies specific for p27 (mouse monoclonal p27, (F-8), cat# sc-1641, Santa Cruz) and for the HA epitope present in the Hook determinant (rabbit polyclonal HA-Probe, (Y-11), cat# sc-805, Santa Cruz). Thereafter, the cells were washed three times for 5 min with PBS before the secondary antibodies (anti-mouse or anti-rabbit, Capple or Pierce) conjugated to FITC or Texas Red (Jackson ImmunoResearch Laboratories) were applied for 1 h. Alternatively, to determine Hook-specific hapten-binding capacity, secondary antibodies were applied together with hapten-coated metal particles (Capture-Tec™ Beads, Invitrogen). Cells were then washed again three times for 5 min with PBS before they were fixed with embedding medium (70% (v/v) glycerol, 30 mM Tris-HCl (pH 9.5); 240 mM n-propyl gallate (Sigma)) and examined under a fluorescence microscope (Leica, Leitz DMRB).

SDS PAGE and Western Blot Analysis

For preparation of whole cell extracts, cells were solubilized in NP-40 extraction buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 25 mM β-glycerophosphate, 25 mM NaF, 5 mM EGTA, 1 mM EDTA, 1% NP-40, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM PMSF, 1 mM sodium orthovanadate) for 10 min on ice. The lysates were clarified by centrifugation at 14000 x g for 15 min. Protein concentrations were determined using a Bradford assay (Biorad). Equal amounts of protein were subjected to 12.5% SDS-PAGE and subsequently electroblotted onto polyvinylidene difluoride membranes. After blocking with 20% horse serum (Gibco BRL) in TTBS (50 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween-20), filters were probed with antibodies specific to p27 (mouse monoclonal Kip1, cat# K25020, Transduction Laboratories) and the HA epitope of Hook (rabbit polyclonal HA-Probe (Y-11), cat# sc-805, Santa Cruz). Proteins were visualized by peroxidase-coupled secondary antibodies using the ECL detection system (Amersham).
**FACS Analysis**

Cells were washed twice with PBS prior to detachment using dissolvation solution (Sigma) and centrifugation at 1500 x g. The supernatant was removed and the cells were resuspended in PBSAz (0.1% NaAz and 2% fetal calf serum in PBS) and centrifuged again. After a second washing step, 10^6 cells were dissolved in 750 µl of ice-cold PBSAz. While vortexing, 250 µl of 1% PFA in PBS were added and the mixture was incubated at 4°C for 1 h. Following centrifugation the cells were dissolved in 1 ml of 0.2% Tween-20 in PBS and incubated for 15 min at 37°C. Cells were then washed twice with PBSAz and incubated with antibodies specific for the HA epitope (rabbit polyclonal HA-Probe (Y-11), cat# sc-805, Santa Cruz) for 1 h at 37°C. Thereafter, cells were washed three times with PBSAz before the secondary antibody (anti-rabbit) conjugated to FITC (Jackson ImmunoResearch Laboratories, Inc.) was applied for 1 h at 37°C in the dark. Cells were then washed again three times with PBSAz before they where incubated in 500 µl propidium iodide staining medium (10 mg/l propidium iodide and 1 mg/l RNAse A in PBSAz) for 30 min at 37°C in the dark. Analysis was performed on a FACStarPlus using the Cell Quest™ software (Becton-Dickinson, San Jose, CA).

**Magnetic Cell Sorting**

CHO-XMK19 and CHO-SS1015 cells were grown for 2 days without tetracycline in T150-flasks before they were washed twice with PBS and incubated with hapten- (phOx) coated metal particles in PBS for 30 min at 37°C in a humidified 5% CO2 atmosphere. Cells were then washed twice with PBS, detached with dissolvation solution, counted and mixed at a ratio of 10% CHO-SS1015 and 90% CHO-XMK19 cells in PBS. A 5 ml pipette (length 24 cm; diameter 0.6 cm) was filled with PBS and subjected to a strong magnetic field (permanent magnet). To control gravity flow through the pipette, the top of the pipette was connected via a 0.5 mm tube to the falcon tube containing the cell mixture. While CHO-XMK19 cells passed through the pipette, CHO-SS1015 cells bound to the metal particles via the Hook-phOx interaction were retained in the magnetic field. The CHO-SS1015 cells were eluted by gravity flow following removal of the magnetic field. In order to disrupt the interaction between the metal particles and the cells, the cell suspension was incubated in phOx solution (10 mM phOx (Sigma) dissolved in 10% EtOH in PBS) for 10 min at 37°C in a humidified 5% CO2 atmosphere. Release of phOx-conjugated metal particles from CHO-SS1015 by excess phOx hapten was monitored by light microscopy.
RESULTS

Construction of the Multicistronic Expression Vector pSS101

In order to test controlled proliferation technology (Fussenegger et al., 1997a; Fussenegger et al., 1998b; Mazur et al., 1998) in combination with surface selection we constructed pSS101 which encodes the gene for the human glycoprotein SEAP (secreted alkaline phosphatase; Berger et al., 1988), a model product protein, the human cyclin-dependent kinase inhibitor p27 (Toyoshima and Hunter, 1994; Mazur et al., 1998), an inhibitor of G1-S transition, and for the surface selection marker Hook (Chestnut et al., 1996) in a tricistronic PhCMV*-1-driven configuration (Gossen and Bujard, 1992) (PhCMV*-1-SEAP-IRES-p27-CITE*-Hook-pA; Figure 1). Hook is an artificial multi-domain protein which contains a membrane localization signal.

Fig. 1: Plasmid map of pSS101. pSS101 enables tetracycline-responsive PhCMV*-1 (tetracycline-responsive promoter) driven expression of the tricistronic expression unit encoding the model product gene SEAP (human secreted alkaline phosphatase), the growth-controlling cyclin-dependent kinase inhibitor p27, and the multi-domain surface marker Hook. The tetracycline-responsive promoter (PhCMV*-1) produces a tricistronic message encoding SEAP, p27 and Hook. Whereas translation of SEAP is mediated in a classical cap-dependent manner, translation-initiation of p27 and Hook are cap-independent and driven by internal ribosomal entry sites of polioviral origin (IRES) or of encephalomyocarditis virus (CITE*). The polyadenylation site (pASV40) is derived from Simian virus.
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derived from murine Ig κ-chain V-J2-C region, a hemagglutinin A epitope tag (HA), the sFv region specific for phOx (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one), two c-myc epitope tags and a membrane anchor from the platelet derived growth factor receptor (Chesnut et al., 1996).

For construction of pSS101, the Hook-encoding fragment was excised from pSS100 by SpeI/EcoRV and inserted SpeI/SwaI into the third cistron of pMF128 (PrCMV*-1-SEAP-IRES-p27-MCS-pA; Fussenegger et al., 1997b; Fussenegger et al., 1998a; Fussenegger et al., 1998b) (Figure 1). In order to demonstrate tetracycline-responsive expression of all three transgenes, pSS101 was transiently transfected into the tTA-expressing CHO-XMK1 cell line (Gossen and Bujard, 1992; Mazur et al., 1998). While expression of SEAP was determined by chemiluminiscence, p27 and Hook expression was assessed by immunofluorescence. Expression of all three cistrons was coordinated and responsive to tetracycline antibiotics (data not shown; see Fig. 4 for stable expression).

**Construction of the CHO-SS101 Cell Line Stably Expressing pSS101**

For construction of CHO-SS101 cell lines pSS101 was cotransfected with plasmid pPur (conferring puromycin resistance) into the tTA-expressing CHO-K1-derived CHO-XMK1 cell line and selected in the presence of puromycin. In order to prevent expression of the growth-arresting p27 gene, the tricistronic expression unit was repressed during the selection procedure by the addition of tetracycline. Complete repression of the tricistronic expression unit was monitored by SEAP assays performed daily (data not shown). The mixed population was cloned by FACS-mediated cell sorting and 6 SEAP-expressing clones were randomly chosen for further analysis. Concomitant with SEAP expression, p27-induced G1-specific growth arrest and tetracycline-responsive expression profiles of p27 and Hook (via HA or c-myc epitopes) were determined by Western blot analysis. CHO-SS1012 and CHO-SS1015 cell lines showed the tightest expression of all three transgenes and were therefore chosen for further analysis (see below; Fig. 3).

**Characterization of the Growth Behavior and SEAP Production of CHO-SS101**

Controlled proliferation by regulated overexpression of tumor-suppressor genes has previously been shown to result in higher specific productivity of CHO cells compared to isogenic proliferation-competent control cell lines (CHO-XMK11110, Mazur et al., 1998). In order to assess the growth and SEAP production characteristics of CHO-XMK11110 and
CHO-SS1015 we cultivated these cell lines for 5 days in the presence and absence of tetracycline. SEAP productivity (SEAP activity on a per cell basis) of both, growth-arrested CHO-SS1015 cells in the presence or absence of tetracycline (+tet; -tet) over a period of 5 days. The SEAP productivity of growth-arrested CHO-SS1015 (P) relative to the SEAP productivity of the proliferation-competent control cell line CHO-XMK11110 (P*) is shown as a line.

Fig. 2A: Columns correspond to the average per cell SEAP productivity of CHO-SS1015 and CHO-XMK11110 cells in the presence or absence of tetracycline (+tet; -tet) over a period of 5 days. The SEAP productivity of growth-arrested CHO-SS1015 (P) relative to the SEAP productivity of the proliferation-competent control cell line CHO-XMK11110 (P*) is shown as a line.

Fig. 2B: Growth characteristics of CHO-SS1015 and CHO-XMK11110 in the presence or absence of tetracycline (+tet; -tet) over a period of 5 days. Whereas tetracycline has no significant influence on the growth behavior of CHO-XMK1110 cells, CHO-SS1015 become growth-arrested upon removal of tetracycline, due to the overexpression of p27.
CHO-SS101$_5$ (P) and the proliferation competent cell line CHO-XMK111$_{10}$ (P*) were independently measured under tetracycline-free conditions and plotted as the relative productivity (P/P*; RLUs$^{-1}$Cell$^{-1}$; RLU, relative light units).

In accordance with previous experiments, arrested CHO-SS101$_5$ produced up to 5-fold more SEAP compared to the proliferation-competent control cell line CHO-XMK111$_{10}$ (Figure 2A). Interestingly, the relative productivity decreased after 3 days correlating with the emergence of proliferation-competent mutants which lost the multicistronic expression unit (Mazur et al., 1999) (Figure 2A). Increased SEAP expression in CHO-SS101$_5$ correlated with a p27-induced G$_1$-phase-specific growth arrest (over 90% reduction of average doubling time within 5 days) (Figure 2B).

**Western Blot Analysis of Tetracycline-Responsive p27 and Hook Expression**

For western blot analysis of tetracycline-responsive expression of p27 and Hook in CHO-SS101$_2$ and CHO-SS101$_5$, these cell clones were grown for 3 days either in the presence or absence of tetracycline before they were lysed and equal amounts of protein extracts were loaded on a 12.5% SDS-PAGE gel. Protein extract of CHO-XMK1$_9$ cells was loaded as a negative control for Hook as well as for an estimation of endogenous p27 levels. Extracts of

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![Western Blot Analysis](Fig. 3.png)

**Fig. 3:** Western Blot analysis of cell clones CHO-SS101$_2$ and CHO-SS101$_5$. Tetracycline-responsive expression of the cyclin-dependent kinase inhibitor p27 and the multi-domain surface marker Hook of cell clones CHO-SS101$_2$ and CHO-SS101$_5$ grown in the presence or absence of tetracycline (+tet; -tet). The parent CHO-XMK1$_9$ cell line transiently transfected with control plasmids pHook$^{TM}$-2 and pSS101 is used as a control.
CHO-XMK19 cells transiently transfected with either the original plasmid $p^{\text{Hook}}_{\text{TM-2}}$ or pSS101 served as positive controls for the HA epitope. In addition, pSS101 is a reference for $p27$ expression. Western blot analysis showed high-level expression of $p27$ and $\text{Hook}$ in a tetracycline-responsive manner (Figure 3).

**Immunofluorescence-Based Detection of the Hook-Hapten Interaction**

CHO-SS101$_5$ cells were cultivated for 3 days in the presence (multicistronic expression unit

![Images of immunofluorescence and light microscopy](image)

**Fig. 4:** Immunofluorescence and light micrographs of CHO-SS101$_5$. Immunofluorescence-based characterization of the cyclin-dependent kinase inhibitor $p27$ (nuclear staining) and the surface marker Hook (surface staining) in the presence or absence of tetracycline (+tet; -tet). Light microscopy pictures of CHO-SS101$_5$ grown in the presence or absence of tetracycline (+tet; -tet), incubated with phOx-coated metal particles (orange staining). Metal particles interact with Hook-expressing CHO-SS101$_5$ cells (-tet).
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repressed) or absence (multicistronic expression unit induced) of tetracycline, immunostained for p27 (FITC) and HA (Texas Red) or incubated with phOx-coated metal particles. Following several washing steps labeled CHO-SS1015 cells were examined by fluorescence microscopy. As shown in Figure 4, nuclear green fluorescence, and surface-stained red fluorescence confirmed simultaneous expression of p27 and Hook in the absence of tetracycline, respectively. Binding of phOx to Hook is visualized by light-interference which identifies the metal particles as orange spheres. In addition, CHO-SS1015 cells grown in the absence of tetracycline, show a fried-egg-like shape typically associated with overexpression of cyclin-dependent kinase inhibitors and G1-phase specific growth arrest (Fussenegger et al., 1997a; Fussenegger et al., 1998b; Mazur et al., 1998).

FACS-Mediated Sorting of Surface-Tagged Cells

For characterization of FACS-mediated sorting of recombinant cells expressing the Hook surface marker, CHO-SS1015 cells were grown in the absence of tetracycline, stained with propidium iodide and immuno-labeled with FITC-coupled and HA-specific antibodies. CHO-SS1015 were mixed with CHO-XMK19 cells at a ratio of 1:9 and analyzed by flow cytometry (Figure 5). Cell-cycle distribution of the mixed population was assessed by propidium iodide

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**Fig. 5A/B:** FACS-mediated cell-cycle analysis of proliferation-competent CHO-XMK19 and growth-arrested CHO-SS1015 cells in the absence of tetracycline. Both cell lines were incubated with the DNA-staining chemical propidium iodide for DNA content-based assessment of cell-cycle distribution. CHO-SS1015 cells were also FITC-stained for Hook expression. Whereas growth competent CHO-XMK19 cells display a low G1-phase content (A), growth-arrested CHO-SS1015 cells reside predominantly in the G1-phase of the cell cycle due to overexpression of p27 (B).
fluorescence which is proportional to the DNA content of individual cells. Whereas analysis of pure CHO-SS1015 and CHO-XMK19 populations showed a typical cell-cycle distribution of either cell type the cell-cycle profile of the mixed population correlated with the mix factor. Also, gating for FITC-fluorescence (expression of HA surface marker) revealed the identical DNA content and cell-cycle distribution as pure CHO-SS1015 populations grown in the absence of tetracycline. These experiments demonstrate FACS-mediated selection of cells expressing recombinant surface markers in the terminal cistron of a multicitronic expression configuration.

**Magnetic Cell Sorting**

In order to demonstrate the principle of magnetic field-mediated cell sorting CHO-XMK19 and CHO-SS1015 were grown for two days in the absence of tetracycline prior to incubation with phOx-coated metal particles. Both cell populations were mixed at a 1:9 ratio (CHO-SS1015 to CHO-XMK19). The mixed population was applied onto a column and eluted by gravity flow (Fig. 6A). A permanent magnet was used to maintain a magnetic field along the column axis. Cells expressing the Hook surface marker were retained in the magnetic field when bound to metal particles via phOx and separated from CHO-XMK19 cells devoid of surface markers specific for phOx. Magnetically separated cell populations were replated to compare their SEAP expression profiles to the original CHO-SS1015 for three days. Based on SEAP production we deduced the percentage of CHO-SS1015 cells present in the fraction

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**Fig. 5C/D:** Cell-cycle distribution of a 9:1 mixture of CHO-XMK19 and CHO-SS1015 cells either gated for propidium iodide (PI) alone (C) or in combination with FITC (D).
retained by the magnetic field. Magnetic one-step sorting resulted in 8-fold enrichment of CHO-SS1015 from the original mixed population (Fig. 6B). SEAP expression-based determination of the sorting efficiency was complemented by cell counting and Hook-targeted immunofluorescence (data not shown).

For some therapeutic applications of magnetic field-based cell sorting it will be desirable

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**Fig. 6A:** Scheme of magnetic cell sorting. The individual cell lines were incubated with hapten-coated metal particles, mixed at a ratio of 9 (CHO-XMK1,9) to 1 (CHO-SS1015, parental) and applied to a pipette containing a magnet along its vertical axis. Unlike CHO-XMK1,9 cells which pass the pipette by gravity flow, CHO-SS1015 cells bound to hapten-coated metal particles are retained by the magnetic field.
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to eliminate phOx-coated metal particles from cell suspensions. We therefore incubated cell populations with surface-bound metal particles with excess free phOx (not linked to metal particles) and analyzed the flow-through of a “magnetic column” by light microscopy. About 90% (± 5%) of the eluted cells were completely released from the metal particles by competing free phOx.

**DISCUSSION**

Controlled proliferation technology, the concept of arresting cell growth in the production phase, has had a long tradition in biopharmaceutical manufacturing. Strategies to block proliferation include addition of growth-inhibiting chemicals or consist of growing cells at low temperatures (Fussenegger et al., 1999a and Fussenegger et al., 1999b, for a review; Kaufmann et al., 1999; Kaufmann et al., 2001). Recent developments were based on metabolic engineering strategies to control proliferation during the production phase by regulated overexpression of growth-inhibiting genes (Fussenegger et al., 1998b; Mueller et al., 1999; Geserick et al., 2000). The success of controlled proliferation technology is based

Fig. 6B: SEAP expression profiles of the parental CHO-SS1015 culture and the subpopulation retained in the magnetic field. Both cell populations were seeded at identical densities in 6-well plates. The values indicate average SEAP production of both CHO-SS1015 cell populations (parental and retained) over a period of five days determined by three independent experiments.
on the observation that arrested cell populations show increased specific productivity compared to proliferation-competent control cells.

A general problem associated with controlled proliferation technology, no matter how growth inhibition is achieved, is the outgrowth of proliferation-competent mutants which eventually overgrow arrested populations (Mazur et al., 1999). Similar to the situation in a proliferation-controlled production process, cells of most mammalian tissues remain quiescent in the G₁- or G₀-phase unless mutations lead to neoplastic outgrowth of malignant phenotypes. Therefore, maintaining mammalian cells in a growth-arrested state is a current priority in cancer therapies and gene therapy scenarios particularly those which are designed to correct hematopoietic disorders.

In order to evaluate a surface selection technology for on-line selection of desired phenotypes we have constructed the stable model cell line CHO-SS101 which expressed the product gene (SEAP), the growth-inhibiting gene (p27) and a cell surface marker (Hook) in a tricistronic pTRIDENT-derived configuration. In principle, SEAP could be exchanged by any product gene (for proliferation-controlled production processes) or disease-correcting gene (for gene therapy concepts) and Hook could be replaced by other endogenous or heterologous surface markers. FACS-mediated cell sorting based on expression of endogenous surface antigens is a standard technology for enrichment of cell populations (de Wynter et al., 1995; de Wynter et al., 1999; Kraguljac et al., 2000; Lai et al., 2000; Porwitt-MacDonald et al., 2000). For example, CD34⁺ hematopoietic progenitor cells of Gaucher patients were enriched by FACS and transduced using a dicistronic retroviral vector encoding the wildtype glucocerebrosidase gene for correction of Gaucher disease and a CD24 or HSA (heat-stable antigen) surface marker. FACS-mediated enrichment of CD24- or HSA-positive cells resulted in cell populations showing increased expression of heterologous glucocerebrosidase (Migita et al., 1995; Medin et al., 1996). Further developments in surface selection technology may therefore be promising for in vivo and ex vivo gene therapy.

FACS-mediated sorting of engineered cells is well suited for isolation of a desired subpopulation from a complex cell mixture. However, such technology is tedious and requires expensive equipment. Recently, magnetically-activated cell sorting of transiently transfected cells has been reported to have significant advantages in speed and scale-up compared to FACS-mediated cell sorting and may likely have great impact on in vivo cell sorting in the not-too-distant future (Chesnut et al., 1996; Geiselhart et al., 1996; Siegel et al., 1997).

We have assessed magnetic cell sorting for selection of the growth-arrested surface antigen-expressing cell line CHO-SS1015 from mixed populations. Magnetic sorting of CHO-
SS1015 cells, via binding of hapten-coated metal particles to single chain antibodies exposed on the surface of CHO-SS1015 cells enabled over 8-fold enrichment in a single selection procedure. Prototype equipment for on-line selection of desired phenotypes by magnetic forces in perfusion reactor configurations is currently under development. On-line selection may limit the genetic drift of cell populations and will likely enable production of difficult-to-express proteins for extended cultivation periods.

Technology for enrichment of cells by selecting for a lineage-specific surface antibody or hapten will have significant impact on ex vivo gene therapy. Furthermore, the simple hardware requirements facilitate adaptation of surface-based selection technologies for example to select treated cells in the hematopoietic system. A fundamental characteristic of in vivo selection concepts will require the successful removal of hapten-coated beads from the blood stream. We have demonstrated in vitro that free hapten is competing with particle-bound hapten which is then released from the cell surface. In order to minimize host immune responses, human compatibility of surface antigens, haptens and hapten-coated particles is critical for in vivo applications of surface selection technologies. Future in vivo systems will therefore require significant refinements in human compatibility. Biodegradable beads facilitating clearance from the hematopoietic system are currently under investigation as matrix for different haptens.

Our model surface selection technology presented here proved to be a versatile tool for selecting highly engineered cells with complex and multiple phenotypes in a single step. Although characterization focused primarily on biotechnological applications, novel surface selection technologies will likely become increasingly important for corrective gene therapies as basic research identifies more disease-specific surface antigens.

ACKNOWLEDGEMENTS

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REFERENCES


CHAPTER 4

**SAMY, A NOVEL MAMMALIAN REPORTER GENE DERIVED FROM BACILLUS STEAROTHERMOPHILUS α-AMYLASE**

ABSTRACT

The *Bacillus stearothermophilus* α-amylase (*amyS*) is a heat-stable monomeric exoenzyme which catalyzes random hydrolysis of 1,4-α-glucosidic linkages in polyglucosans. The *Bacillus* α-amylase was engineered for use as an intracellular (AmyS\textsubscript{AS}) as well as a secreted reporter protein (SAMY; secreted α-amylase) in mammalian cells. The 5’ end of *amyS* containing the prokaryotic secretion signal was either deleted (*amyS\textsubscript{AS}) or replaced by a murine immunoglobulin secretion signal. SAMY was cloned under control of the cytomegalovirus promoter (P\textsubscript{CMV}) in a mammalian expression vector or the promoter of the human elongation factor 1α (P\textsubscript{EF1-α}) in a lentiviral expression context. A variety of mammalian and human cell lines growing as monolayers, in suspension or as three-dimensional spheroids were transfected/transduced with SAMY- or *amyS\textsubscript{AS}-encoding expression/lentiviral vectors and α-amylase activity was measured in cell lysates and culture supernatants. These experiments showed that SAMY and AmyS\textsubscript{AS} were either secreted or remained intracellular as highly sensitive reporter enzymes. SAMY expression and detection was fully compatible with established SEAP (human secreted alkaline phosphatase) and u-PA\textsubscript{LMW} (low molecular weight urokinase-type plasminogen activator) reporter systems and could be used to quantify expression of up to three independent genes in one culture supernatant.

INTRODUCTION

Reporter gene assays have become essential for the study of gene regulatory elements (Fussenegger et al., 2000), as biotechnological production markers (Schlatter et al., 2001), expression tracers (Schlatter et al., 2001) and for high-throughput antibiotic screening programs (Aubel et al., 2001).

Several genes have been adapted as indicators of transcriptional activities in mammalian cells including the *Escherichia coli* (*E. coli*) galactokinase, *E. coli* xanthine-guanine phosphoribosyl transferase, luciferase, thymidine kinase, interleukin-2 (*IL-2*), the *E. coli* chloramphenicol acetyltransferase (*CAT*), β-galactosidase, β-glucuronidase, and fluorescent proteins (Alam and Cook, 1990; Berger et al., 1988; Kricka et al., 2000; Ormö et al., 1996; Prasher et al., 1992; Thompson et al., 1990 and references therein). Analytical techniques ranged from cumbersome extractions followed by thin-layer chromatography and radioisotope detection for CAT quantification to highly sensitive colorimetric, fluorescent
(including FACS), immunological and chemiluminescent assays (Kricka et al., 2000). However, since all of these reporter proteins accumulate intracellularly their quantification requires tedious, time-consuming and hardware-intensive protocols which may be incompatible with complex kinetic studies, robotic high-throughput analysis/screening, long-term expression in stable mammalian cells and in vivo applications (Ormö et al., 1996).

Therefore, secreted proteins are becoming increasingly important as mammalian reporter systems and start to replace classical intracellular markers of transcriptional activities whenever simple, sensitive and non-invasive long-term monitoring of gene expression is required in cell culture, animal models and high throughput screening (Aubel et al., 2001; Cullen and Mallim, 1992; Fussenegger et al., 2000; Schlatter et al., 2001; Thompson et al., 1990). Among the most prominent secreted reporter proteins are the human growth hormone (hGH; Selden et al., 1986), the tissue plasminogen activator (tPA; Cheng et al., 1987), the human secreted alkaline phosphatase (SEAP; Berger et al., 1988), the Vargula hilgendorfii luciferase (Thompson et al., 1990), apoaequorin (Inouye and Tsuji, 1992), GFP from Aequoria victoria (Laukkanen et al., 1996), the low molecular weight urokinase-type plasminogen activator (u-PA<sub>LMW</sub>; Langer et al., 1995), and the Renilla reniformis luciferase (LUC; Liu and Escher, 1999).

Of these reporter proteins, only hGH, tPA and the Vargula luciferase are naturally secreted. All others have been engineered for secretion either by deletion of retention signals (SEAP; u-PA<sub>LMW</sub>), or addition of heterologous mammalian secretion signals to intracellular proteins (secretion signal of the human follistatin to apoaequorin, chromogranin B secretion signal to GFP, signal peptide of human IL-2 to Renilla reniformis luciferase). hGH, tPA and u-PA<sub>LMW</sub> have the potential disadvantage of inducing pleiotropic effects or being biased by similar endogenous activities in mammalian cells. For Vargula luciferase there is no commercially available substrate which restricts its use as a reporter protein.

Due to its sensitivity and the availability of easy-to-handle and low-cost colorimetric, fluorescence and chemiluminescent assay technology SEAP is one of the most widely used secreted mammalian reporter genes which has also been established as human model product glycoprotein for biopharmaceutical manufacturing (Schlatter et al., 2001) and for antibiotic screening programmes (Aubel et al., 2001).

All known species of the genus Bacillus produce α-amylase exoenzymes which are capable of random hydrolysis of 1,4-α-glucosidic linkages in polyglucosans (amylose, amylopectin, glycogen, and dextrins) degrading them into maltose and larger
oligosaccharides. α-amylases are important for the liquefaction of starch required for brewing and sweetener industries. α-amylases are also produced by plants for degradation of endosperm starch to initiate germination (Kumangai et al., 1990). Based on the coloration of starch by iodide solutions, the starch-degrading activity of α-amylases is often used in bacterial and yeast reporter systems to quantify protein production and gene expression in liquid and solid media (Natarajan et al., 2000). For example, α-amylase producing bacteria spotted on starch-containing agar plates reveal cleared zones following flooding of the plates with an iodide-containing solution (Natarajan et al., 2000). In the clinics, α-amylases serve as indicator enzymes for acute pancreatitis, mumps, hepatic disorders, impaired renal function and peritonitis. Due to its widespread use a variety of quantitative assays have been developed for α-amylases (Bernfeld, 1955; Muscholl-Silberhorn, 2000).

Recently, a thermostable α-amylase has been cloned from *Bacillus stearothermophilus* (*amyS*; Suominen et al., 1987a and 1987b). *amyS* is 1650 bp (coding for a 63 kDa preprotein) in length including a typical bacterial signal sequence for protein secretion (bp 1-102; Suominen et al., 1987a and 1987b). Taking advantage of the thermostability of *Bacillus stearothermophilus* α-amylase which resists boiling for over 15 min. and has an optimal assay temperature of 70°C, we have adapted *amyS* for use as a mammalian reporter gene and expression tracer in a variety of mammalian and human cell lines.

**MATERIALS AND METHODS**

**Vector Constructions**

To enable efficient secretion of the *Bacillus stearothermophilus* ATCC 12980 α-amylase (AmyS; Suominen et al., 1987b) in mammalian cells the bacterial secretion signal of *amyS* (bp 1-102 according to Suominen et al., 1987b) was replaced by the signal peptide (METDTLLLWVLWLVPGSTGD) derived from the murine Ig κ-chain V-J2-C region (Coloma et al., 1992) using PCR technology. *amyS* was amplified from pCSS1 (Suominen et al., 1987a and 1987b) using primers OSS1: gatcgccgccgactagtccaccatggagacagacacactctgetatggtacactgetcttgtcagttcaggtctctca and OSS2: gatcagatcaagctgTCAAGGCACACCATGCA (containing NotI and SpeI sites (underlined), a Kozak sequence (italics), the secretion signal (bold) and the annealing sequence (capital letters, starting at position 103)).
Fig. 1A: Maps of plasmids constructed. All eukaryotic expression plasmids are derived from pcDNA3.1/V5-HisTOPO and contain a cytomegalovirus promoter (P\text{CMV}) and a polyadenylation site derived from the bovine growth hormone (pA\text{BGH}). All vectors are isogenic and contain the secreted mammalian α-amylase (\text{SAMY}) , the full-length \textit{Bacillus stearothermophilus} α-amylase (\text{amyS}), an \text{amyS} derivate without signal sequence (\text{amyS}\_\Delta\text{S}) in some cases fused to the hemagglutinin A epitope tag (HA) and/or the signal sequence derived from the murine Ig κ-chain V-12-C region (S\text{Igκ}), the human secreted alkaline phosphatase (SEAP) or the low molecular weight urokinase-type plasminogen activator (u-\text{PA}\_\text{LMW}). A, Apal; B, BamHI; B\_2, Bgl\_II; B\_3, BstXI; C, Clal, D, DraI; E, EagI; E\_2, EcoRI; E\_3, EcoRV; H, HindIII; K, KpnI; M, MluI; N, NorI; N\_2, NruI; S, SacII; S\_2, SbfI; S\_3, SfiI; S\_d, SpeI; S\_s, SphI; S\_t, SrfI; X, XbaI; X\_2, XhoI.
(containing HindIII and BglII sites (underlined); annealing sequence in capital letters) and the 1611 bp secreted mammalian α-amylase (SAMY) fragment was cloned in sense orientation into pcDNA3.1/V5/His-TOPO (Invitrogen, San Diego, CA) to result in plasmid pSS158 (P_{CMV-SAMY-pA}) (Fig. 1A).

The isogenic control vector pSS186 was constructed by amplifying full length amyS from pCSS1 using oligonucleotides OSS3: gatcaactagtgccgccaagcttccaccATGCTAACGTTTCACCGCATCATTCG (containing SpeI, NotI, HindIII sites (underlined), the Kozak (italics) and annealing sequences (starting at bp 2) and a G --> A (bold) transition to generate an ATG start codon required for expression in mammalian cells) and OSS2 followed by cloning in sense orientation into pcDNA3.1/V5/His-TOPO (Invitrogen, San Diego, CA) (pSS186: P_{CMV-amyS-pA}).

An amyS derivative (amyS_{AS}) lacking the bacterial signal sequence (ΔS; bp 1-102) was constructed by amplification of amyS (bp 103-1611; Suominen et al., 1987b) using OSS4: gatcaactagtgccgccaagcttccaccATGGCCGCACCGTTTAACGGCA (containing SpeI, NotI and HindIII sites (underlined), Kozak (italics) and annealing sequences (capital letters; starting at bp 103) as well as an ATG start codon (bold)) and OSS2 and cloning in sense orientation into pcDNA3.1/V5/His-TOPO (Invitrogen, San Diego, CA) to give plasmid pSS188 (P_{CMV-amyS_{AS}-pA}).

Plasmids pSS185, pSS187 and pSS189 are pcDNA3.1/V5/His-TOPO derivatives (isogenic to pSS158, pSS185 and pSS188, respectively) containing C-terminally HA-tagged (YPYDVPDYA) SAMY, amyS and amyS_{AS}. These vectors were constructed by amplifying SAMY, amyS and amyS_{AS} with the respective 5' primers OSS1, OSS3 and OSS4 and the 3' primer OSS5: gatcaacgctgcccagctcatactggaacatcatatggataAGGCCATGCCACAACCG (containing MluI and NotI sites (underlined), the HA-tag (bold) and the annealing sequence)

Fig. 1B: pMF364 is a third-generation replication-incompetent self-inactivating HIV-1-based lentiviral vector encoding SAMY under control of the human elongation factor 1α promoter (P_{EF1α}). 5’LTR, 5’ Long terminal repeat of HIV-1; 3’LTR_{ΔU3}; 3’ Long terminal repeat of HIV-1 containing a U3 deletion; ψ’, packaging signal; PPT, polyuridine track; RRE, nuclear export signal for HIV-1 RNA; ori_{SV40} large T-dependent origin of replication derived from simian virus 40 (SV40).
(capital letters)) followed by cloning into pcDNA3.1/V5/His-TOPO in sense orientation (pSS185: P<sub>CMV</sub>-SAMY-HA-pA; pSS187: P<sub>CMV</sub>-amyS-HA-pA; pSS189: P<sub>CMV</sub>-amyS<sub>∆S</sub>-HA-pA; Fig. 1A).

pSS173 (P<sub>CMV</sub>-SEAP-pA) and pSS179 (P<sub>CMV</sub>-u-PA<sub>LM</sub>-pA) are isogenic to pSS158. pSS173 was constructed by a two-step cloning procedure. (i) pSEAP2-control- (Clontech, Palo Alto, CA) derived SEAP was excised from pSAM237 (Moser et al., 2001) by NotI and cloned in sense orientation into the NotI site (second cistron) of pTRIDENT4 (Fussenegger et al., 1998) to result in plasmid pSS139. (ii) SEAP was excised from pSS139 by NotI/HindIII and ligated to the corresponding sites (NotI/HindIII) of pcDNA3.1/V5-TOPO/lacZ (Clontech, Palo Alto, CA) thereby replacing lacZ and resulting in pSS173 (P<sub>CMV</sub>-SEAP-pA). pSS179 is a pcDNA3.1/V5-TOPO derivative containing the low molecular weight urokinase-type plasminogen activator (u-PA<sub>LM</sub>) amplified from pDL205 (Langer et al., 1995) with OSS8: gatctattaacttgtgatedccggccgcaagcttgctgcaGTCTAGCGCCCCGACCT (containing PacI, SpeI, EcoRV and NotI sites (underlined) and the annealing sequence (capital letters)) and OSS9: gatcagatctagctgatedccggccgcaagcttgctgcaGTCTAGCGCCCCGACCT (containing BglII, MluI, EcoRV, NotI and HindIII (underlined) and the annealing sequence (capital letters)) in sense orientation.

The SAMY-encoding HIV type 1-based lentiviral expression vector pMF364 was constructed by cloning SAMY, excised from pSS158 by SpeI/EcoRV into the SpeI/PmeI sites of pMF359 (5’LTR-ψ<sup>+</sup>-RRE-P<sub>EF1α</sub>-SAMY-3’LTR<sub>AU3</sub>; ψ<sup>+</sup>, packaging signal; RRE, nuclear RNA export signal; P<sub>EF1α</sub>, human EF1α promoter; 3’LTR<sub>AU3</sub>, 3’LTR containing a deletion in the U3 region (self-inactivating phenotype)) (Mochizuki et al., 1998).

**Cell Culture and Transfections**

Chinese hamster ovary cells (CHO; ATCC CCL-61), NIH/3T3 (ATCC CRL-1658), baby hamster kidney cells BHK-21 (ATCC CCL-10), human fibrosarcoma cells HT-1080 (ATCC CCL-121), human cervical adenocarcinoma cells HeLa (ATCC CCL-2), human hepatocellular carcinoma cells HepG2 (ATCC HB-8065) and human chronic myelogenous leukemia cell line K-562 (ATCC-243) were cultivated in FMX-8 (CHO-K1; Cell Culture Technologies GmbH, Switzerland), Dulbecco’s modified eagle medium (NIH/3T3, BHK-21, HT-1080, HeLa, HepG2; cat# 52100-039; Life Technologies AG, Basel, Switzerland) or Iscove’s modified Dulbecco’s medium (K-562, cat# 4220-022; Life Technologies AG, Basel,
Switzerland) supplemented with either 10% calf serum (NIH/3T3; cat# 16170-078; lot# 1022174) or 10% fetal calf serum (all other cell lines; PAA Vienna, Austria; Lot Nr. A01129-242). Spheroids of HT-1080 and HepG2 cells were produced using common hanging drop technology. All cell lines were transfected using a modified CaPO₄-based transfection protocol. In brief, 100’000 cells per 6-well were transfected with a CaPO₄-DNA complex produced by drop-wise addition of 100 µl of 1.2 µg DNA-containing 0.25 M CaCl₂ solution to 100 µl 2x HBS solution (280 mM NaCl; 100 mM HEPES; 1.5 mM Na₂HPO₄; pH 7.1). After 16 hours the CaPO₄-DNA complex was removed by medium exchange and reporter gene expression was assayed after 48 hours.

For counting, cells were detached using dissociation solution (Sigma Chemie, Buchs, Switzerland) and resuspended in filter-purified casyton buffer (pH 7.3; 7.93 g/l NaCl; 0.38 g/l Na₂EDTA; 0.4 g/l KCl; 0.19 g/l NaHPO₄; 1.95 g/l Na₂H₂(PO₄)₂; 0.3 g/l NaF). The viable cell number (CellsWell⁻¹ 10⁻⁵) was determined as the average of three independent readings taken by a Casy1® cell counter (Schärfe System, Germany).

**Western Blot and Glycosylation Analysis**

For preparation of whole cell extracts, cells were solubilized in NP-40 extraction buffer (50 mM Heps, pH 7.4, 150 mM NaCl, 25 mM β-glycerophosphate, 25 mM NaF, 5 mM EGTA, 1 mM EDTA, 1% NP-40, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM PMSF, 1 mM sodium orthovanadate) for 10 min on ice. The lysates were clarified by centrifugation at 14000 x g for 15 min. For detection of secreted proteins, 10 ml cell culture supernatant were concentrated to 1 ml using a Centiprep-Concentrator 10 kDa (Amicon Inc., Beverly, MA). For glycosylation analysis, 25 µg (approximately 150 µl) of concentrated supernatant were diluted in 350 µl PNGase F buffer (20 mM sodium phosphate, 0.02% NaN₃, pH 7.5) and concentrated to 4 µl using a Centiprep-Concentrator 5 kDa (Amicon Inc., Beverly, MA). The sample was then mixed with 1 µl denaturing buffer (2.5% SDS, 125 mM DTT) and boiled for 2 min, before 2.5 µl 5% Triton X-100, 12.5 µl PNGase F buffer and 5 µl PNGase F (0.5 U/µl; OxfordGlycoScience) were added. Deglycosylation was performed at 37°C for 20 h. Protein concentrations were determined using a Bradford assay (Bio-Rad Laboratories AG, Glattbrugg, Switzerland). Equal amounts of protein were subjected to 7.5% SDS-PAGE and subsequently electroblotted onto polyvinylidene difluoride membranes. After blocking with 20% horse serum (Life Technologies AG, Basel, Switzerland) in TTBS (50 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween-20), filters were probed with antibodies specific to the HA
epitope (rabbit polyclonal HA-Probe (Y-11), cat# sc-805, Santa Cruz Biotechnology Inc., Santa Cruz, CA). Proteins were visualized by peroxidase-coupled secondary antibodies using the ECL detection system (Amersham Pharmacia Biotech, Duebendorf, Switzerland).

**Lentivirus Technology**

For production of replication-incompetent, self-inactivating lentiviruses a mixture containing 94 µl DMEM, 6 µl FUGENE (Roche Diagnostics AG, Rotkreuz, Switzerland), 25 mM chloroquine, 1.5 µg pLTR-G (encoding the pseudotyping envelope protein VSV-G of the vesicular stomatitis virus; Mochizuki et al., 1998), 1.5 µg of the helper construct pCD/NL-BH* (Mochizucki et al., 1998) and 1.5 µl of the SAMY-encoding lentiviral expression vector pMF364 was transfected into human embryonic kidney cells (HEK293-T). The medium was replaced after 24 hours and virus particles were produced for another 48 h. Viral particles were collected from the HEK293-T supernatant by filtration through a 0.45 mm filter (Schleicher & Schuell GmbH, Dassel, Germany: FP 030/2) yielding typical titers of 2 x 10^7 viral particles per ml. 100'000 target cells per 6-well were infected with 200 µl viral supernatant also containing 8 µg/ml polybrene (hexadimethrine bromide; Sigma Chemie, Buchs, Switzerland).

**Colorimetric 96-Well Plate SEAP Assay (Adapted from Berger et al., 1988)**

100 µl cell culture supernatant were heat-inactivated for 30 min at 65°C and centrifuged at 14’000 x g for 2 min. to remove cell debris. 50 µl were subsequently transferred into a chamber of a 96-well plate and adjusted to 37°C. 45 µl 2x SEAP assay buffer (20 mM homoarginine, 1 mM MgCl₂, 21% diethanolamin, pH 9.8) are mixed with 5 µl substrate solution (120 mM p-nitrophenylphosphate (pNPP; Sigma Chemie, Buchs, Switzerland) in 2x SEAP assay buffer), adjusted to 37°C and added to the cell culture supernatant. Absorbance readings are taken at 405 nm (p-Nitrophenol; pNP) for 15 min. from which SEAP activity can be calculated as follows:

\[
\text{Lambert-Beer's law: } A = \varepsilon * c * d \quad \rightarrow \quad \frac{\Delta c}{\text{min}} = \frac{\Delta A}{\text{min} * \varepsilon * d}
\]

where: Extinction coefficient [M⁻¹cm⁻¹]: \( \varepsilon_{pNP} = 18600 \)
Lightpath [cm]: \( d = \frac{V_{\text{Measurement}}}{\pi \cdot r_{\text{well}}^2} \)

Slope \([\text{min}^{-1}]\): \( S = \frac{\Delta A}{\text{min}} \)

Dilution factor \([1]\): \( v = \frac{V_{\text{Measurement}}}{V_{\text{Sample}}} \)

Enzymatic Activity \([\text{Ul}^{-1}]\): \( EA = \frac{\Delta c}{\text{min}} = \frac{S \cdot v}{\varepsilon_{\text{pNA}} \cdot d} \)

Colorimetric Assay for the Low Molecular Weight Urokinase-Type Plasminogen Activator (u-PA\textsubscript{LMW}) (Adapted from Langer et al., 1995)

100 \(\mu\)l cell culture supernatant were centrifuged at 14’000 x g for 2 min. 50 \(\mu\)l were subsequently transferred into a chamber of a 96-well plate and adjusted to 37\(^\circ\)C. 25 \(\mu\)l plasminogen-specific substrate (1.6 mM S-2251: H-D-Val-Leu-Lys-pNA\#2HCl (cat# 820332; Chromogenix, Milano, Italy) in u-PA\textsubscript{LMW} assay buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 1mM EDTA, 0.01% Tween 80) were mixed with 25 \(\mu\)l plasminogen solution (1 mg/ml human plasminogen in H\(_2\)O (cat# 820332; Chromogenix, Milano, Italy) adjusted to 37\(^\circ\)C and added to the cell culture supernatant. The absorbance timecourse is measured at 405 nm (p-nitroanillin; pNA) for 15 min and u-PA\textsubscript{LMW} levels are determined as follows (see section 2.5):

\[
EA = \frac{\Delta c}{\text{min}} = \frac{S \cdot v}{\varepsilon_{\text{pNA}} \cdot d}
\]

where: Extinction coefficient \([\text{M}^{-1}\text{cm}^{-1}]\): \(\varepsilon_{\text{pNA}} = 9600\)

Dinitrosalicylic Acid (DNS)-Based SAMY Assay (Adapted from \(\alpha\)-Amylase Assay by Bernfeld, 1955)

100 \(\mu\)l cell culture supernatant were centrifuged at 14’000 x g for 2 min. 50 \(\mu\)l were mixed with 50 \(\mu\)l substrate solution (100 mM NaOAc, 5 mM CaCl\(_2\), 1% soluble starch, pH 5.5). The reaction is incubated for 15 min. at 70\(^\circ\)C and then stopped by addition of 100 \(\mu\)l DNS solution
(dissolve 1 g 3,5-dinitrosalicylic acid (Sigma Chemie, Buchs, Switzerland) and 30 g sodium potassium tartrate in 20 ml 2N NaOH and adjust to 100 ml using dH2O). The mixture is then boiled for 5 min. and 800 µl dH2O were added. The absorbance is measured at 540 nm and compared to a glucose calibration curve produced with glucose solution (100 mM NaOAc, 5 mM CaCl₂, 1% glucose, pH 5.5). α-amylase units are calculated according to the following equation:

\[
\text{Enzymatic Activity [Ul}^{-1}\text{]}: \ EA = \mu\text{molmin}^{-1}\text{l}^{-1} \text{glucose produced}
\]

**Assaying SAMY Using the Phadebas® Method (Adapted from α-Amylase Assay by Muscholl-Silberhorn, 2000)**

100 µl cell culture supernatant were centrifuged at 14’000 x g for 2 min. 50 µl are transferred into an Eppendorf cup also containing 1 ml substrate solution (45 mg blue starch (1 Phadebas® tablet, cat# 10-5380-32, Pharmacia & Upjohn, Uppsala, Sweden), 4 ml dH₂O) and incubated at 70°C for 15 min. The reaction is stopped by addition of 250 µl 0.5 M NaOH, centrifuged for 5 min. at 14’000 x g and the supernatant is transferred into a 1 ml cuvette. Alternatively, for quantification of intracellular AmyS₃S levels the cell monolayer was washed twice with PBS. Cells were incubated with 500 µl/6-well lysis buffer (reporter gene assay lysis buffer, Roche Diagnostics AG, Rotkreuz, Switzerland) for 30 min at 37°C. The cell extracts were then centrifuged for 2 min. at 14’000 x g and 50 µl of the supernatant were assayed as described above. α-amylase levels are measured at 620 nm against dH₂O using the following equation:

\[
\text{Enzymatic Activity (Ul}^{-1}\text{)}: \ EA = e^N
\]

where: \[ N = -B + \frac{1}{2}(C + D \cdot \ln(Abs)) \]

\(B, C, D:\) Lot-specific parameters indicated by the manufacturer

**Preparation of Human Serum**

5 ml blood were taken using a BD vacutainer SST (BD Vacutainer Systems, Plymouth, UK). The sample was incubated in the dark at 37°C for 20 min. After centrifugation at 1800 x g for
10 min., the serum in the upper phase was separated from clotted blood and mixed 1:1 with FMX-8 medium.

RESULTS

Construction of the Mammalian Reporter Gene SAMY

Since prokaryotic signal sequences are typically incompatible with mammalian protein secretion machineries the signal sequence of *Bacillus stearothermophilus* α-amylase (AmyS) comprising bp 1-102 (GTG...GCT, Suominen et al., 1987; *amyS*ΔS) was replaced by a mammalian secretion signal encoding the signal peptide of the murine Ig κ-chain V-J2-C region previously reported to promote efficient secretion of heterologous fusion proteins in mammalian cells (Coloma et al., 1992; Schlatter et al., 2001). The resulting Ig κ-chain V-J2-C-AmySΔ fusion protein was designated SAMY (secreted α-amylase) and cloned under transcriptional control of the CMV immediate-early promoter (pSS158: P<sub>CMV</sub>-SAMY-pA; Fig. 1A). pSS158 was introduced into Chinese hamster ovary cells (CHO-K1) by using a

![Fig. 2A: Immunodetection of α-amylase derivatives in CHO-K1 cells. Culture supernatants and whole cell extracts of CHO-K1 cells transiently transfected with pSS185 (P<sub>CMV</sub>-SAMY-HA-pA), pSS187 (P<sub>CMV</sub>-amyS-HA-pA) and pSS189 (P<sub>CMV</sub>-amySΔS-pA) were used for HA-directed Western blot analysis of α-amylase expression.](image-url)
calcium phosphate transfection protocol. No differences in cell morphology or cell growth were observed between mock-transfected cells and CHO-K1 cells expressing SAMY (Fig. 6B).

Using dinitrosalicylic- (Bernfeld, 1955) acid or Phadebas®-based colorimetric detection assays, SAMY can easily be detected in the supernatant of CHO-K1 cells transfected with SAMY (pSS158), AmyS (pSS186) and AmyS_{AS} (pSS188) in CHO-K1 cells.

Fig. 2B: Quantification of α-amylase derivatives in CHO-K1 cells. Colorimetric Phadebas®-based quantification of SAMY (pSS158), AmyS (pSS186) and AmyS_{AS} (pSS188) in CHO-K1 cells.

Fig. 2C: PNGase F-digested or untreated supernatant of CHO-K1 cells transiently transfected with pSS185 (PCMV-SAMY-HA-pA) were used for HA-directed Western blot analysis of the SAMY glycosylation status.
pSS158 (450 mU/ml) while α-amylase activity was undetectable in the supernatant of untransfected control cells (1.2 mU/ml; Fig. 2B). Using the colorimetric Phadebas® assay as described in materials and methods SAMY activity as low as 70 µU per 100 µl sample could be detected. The assay sensitivity could be further increased by reducing the sample dilution and extending the reaction time.

**Transient Expression of SAMY in CHO-K1 Cells**

In order to localize expression of α-amylases in CHO-K1 cells several C-terminally HA-tagged derivatives were constructed: (i) full length SAMY (SAMY-HA; pSS185; Fig. 1A); (ii) *Bacillus stearothermophilus* full length amyS (amyS-HA; pSS186; Fig. 1A), and (iii) amyS lacking any signal sequence (bp 1-102; amySΔS-HA; pSS189; Fig. 1A). Western blot analysis of cell extracts and supernatants as well as quantitative α-amylase assays (see below) of CHO-K1 cells transfected with pSS185/158 demonstrate efficient secretion of SAMY into the culture supernatant (Fig. 2). Interestingly, the prokaryotic signal sequence of *B. stearothermophilus* promotes secretion of AmyS even in CHO-K1 cells although to a lesser extent than the Ig κ-chain V-J2-C signal sequence of SAMY (Fig. 2). AmySΔS could only be detected in cell extracts but not in the supernatant which demonstrates that SAMY and AmyS were truly secreted and not simply released from dead cells (Fig. 2). Quantitative expression analysis of SAMY (pSS158), amyS (pSS186) and amySΔS (pSS188) confirm the Western blot results of HA-tagged α-amylase derivatives (Fig. 2A) and validate SAMY as efficiently secreted mammalian reporter protein (Fig. 2B).

PNGase F-based deglycosylation of SAMY resulted in a faster migrating SAMY isoform as shown in HA-targeted Western blot analysis indicating N-linked glycosylation at the 4 putative glycosylation sites Asn26, Asn53, Asn302 and Asn476 (Asn6, Asn33, Asn282 and Asn456 for AmySΔS) (Fig. 2C).

**Using AmySΔS as Intracellular Reporter Protein in Mammalian Cells**

The finding that *B. stearothermophilus* AmyS devoid of the bacterial signal (AmySΔS, pSS188 and pSS189) sequence remains predominantly in the cytosol of mammalian cells would validate amySΔS as an intracellular reporter gene variant isogenic to its secreted homologue SAMY. AmySΔS is extracted from CHO-K1 cells transiently transfected with pSS188 (PCMV-amySΔS-pA; Fig. 1A) technology and assayed using the Phadebas® technology (see section 3.4.). Intracellular amySΔS expression could clearly be monitored compared to mock-
transfected CHO-K1 cells (Fig. 2B). Therefore, \textit{amyS} and \textit{SAMY} represent a unique set of reporter genes for quantifying either intracellular or secreted expression using a single quantitative detection technology.

**Quantitative SAMY Analysis Using Colorimetric Assays**

Four commonly used techniques for the determination of \(\alpha\)-amylase activity have been developed in the past: The Nelson colorimetric copper method, the iodine-starch method, the dinitrosalicylic (DNS) method and the Remazol Brilliant Blue (RBB) dye Phadebas® method (Muscholl-Silberhorn et al., 2000). We focused on the DNS and the Phadebas® technology for their simplicity, speed and low cost. A detailed analysis of key parameters including absorbance, temperature and pH for DNS- and Phadebas®-based SAMY quantification is indicated in Fig. 3. Like \textit{B. stearothermophilus} AmyS, SAMY is extremely heat-stable

![Fig. 3: Temperature and pH profiles of SAMY. SAMY activity was measured in the culture supernatant of CHO-K1 cells transfected with pSS158 (P\textsubscript{CMV-}\textit{SAMY}-pA) at different reaction temperatures and pH using dinitrosalicylic acid (DNS)- and Phadebas®-based \(\alpha\)-amylase assays. SAMY retains the heat stability of \textit{Bacillus stearothermophilus} AmyS and shows optimal enzymatic properties at 70°C (Phadebas® (T); DNS (T)) and pH 5.5 (DNS (pH); the pH of Phadebas® is set by the assay kit), conditions which typically inactivate endogenous mammalian \(\alpha\)-amylases. Phadebas® and DNS assays are of comparable sensitivity.](image-url)
resisting temperatures of 100°C for several minutes and shows an optimal activity at 70°C and pH 5.5 (Fig. 3). Endogenous mammalian α-amylases have typical reaction optima at pH 7 and are inactivated at temperatures above 40°C (Bernfeld, 1955). The heat-resistance of SAMY enables effective elimination of endogenous α-amylases a prerequisite for its use as an ideal reporter gene in mammalian cells and possibly in vivo. All of the mammalian and human cell lines tested display like CHO-K1 no significant endogenous α-amylase activity (Fig. 2B and data not shown). The DNS and Phadebas® assays show comparable sensitivity (Fig. 3).

**SAMY Expression Analysis in a Variety of Cell Lines Using Lentiviral Transduction Technology**

In order to establish *SAMY* as an universal reporter gene for mammalian cells, we tested its expression in a variety of mammalian cell lines (BHK-21; CHO-K1; NIH/3T3) including human ones (HeLa; HepG2; HT-1080; K-562) growing as monolayers (BHK-21; CHO-K1; HeLa; HepG2; HT-1080; NIH/3T3), in suspension (K-562) or as three-dimensional spheroids (HepG2S; HT-1080S) (Fig. 4). In order to ensure high transfection rates we constructed a

![Fig. 4: Expression of *SAMY* in different mammalian cell lines. BHK-21; CHO-K1; HeLa, NIH/3T3, K-562, HepG2, and HT-1080 were grown as monolayers, in suspension and as three-dimensional spheroids ("s"; 300 μm diameter on average) and infected with a *SAMY*-encoding pMF364-based lentivirus (Fig. 1B). Culture supernatants were analysed in triplicate after 72 hours using the Phadebas® α-amylase quantification technology.](image)
SAMY-encoding HIV-1-based, self-inactivating and replication-incompetent lentiviral expression vector pMF364 which contains SAMY under control of the ubiquitously active human elongation factor 1α promoter (\(P_{\text{EF1α}}\); Fig. 1B). In all cell lines tested SAMY is highly expressed and could be quantified using the Phadebas® detection technology (Fig. 4).

![Graph](image)

**Fig. 5:** Stability of SAMY, human α-amylase, AmyS\(_{AS}\), SEAP and u-PA\(_{LMW}\) under cell culture conditions. Three days post transfection, SAMY, SEAP and u-PA\(_{LMW}\) produced by CHO-K1 cells transfected with pSS158, pSS173 or pSS179 were transferred to untransfected CHO-K1 cultures. Stability of the intracellular AmyS\(_{AS}\) was directly measured in cell extracts from CHO-K1 transiently transfected with pSS188. Human α-amylase contained in serum was mixed 1:1 with FMX-8 medium and transferred to CHO-K1 cells. Reporter protein activities were measured daily and plotted as log % activity vs. time. Linear regression of decreasing enzymatic activities was used to determine the half-life of the reporter proteins.
Therefore, SAMY is a promising reporter set-up for a variety of mammalian cell lines.

**Stability Profiles of SAMY, Human α-Amylase, AmySΔS, SEAP and u-PA_LMW**

High stability is an important characteristic for reporter proteins since it enables more reliable quantitative correlation of transcription with a specific assay without the need to consider protein degradation profiles under different assay conditions. Short half-lives of reporter proteins is often mentioned as a requirement for time-course studies, however, previously secreted protein can be removed prior to time-dependent analysis of gene expression profiles.

In order to assess the stability of SAMY, human α-amylase, AmySΔS, SEAP and u-PA_LMW, culture supernatants of CHO-K1 transiently transfected with pSS158, pSS188, pSS173 or pSS179 were transferred 3 days post transfection to CHO-K1 cultures grown at 37°C. For stability assessment of human α-amylase, human serum was mixed 1:1 with FMX-8 medium prior to addition to the CHO-K1 cells. Daily reporter protein quantification revealed high stability for SAMY (half-life (t½) = 1505 h), correlating with previous reports on *B. subtilis* α-amylase stability (t½ = 120 h at 70°C; Vihinen and Mantsala, 1990). SEAP displayed a t½ of 502 h, u-PA_LMW of 61 h, AmySΔS of 58 h and human α-amylase of 21 h (Fig. 5).

**Simultaneous and Comparative Analysis of SAMY, SEAP and u-PA_LMW Expression in CHO-K1 Cells**

Analysis of combinatorial gene expression is of major interest for elucidating the genetic interplay of key regulatory mammalian networks in the post-genome era. The precise and simultaneous expression profiling of several independent genes requires compatible reporter systems which enable independent readings of different reporter genes from a single culture supernatant. We have therefore assessed the compatibility of SAMY, SEAP and u-PA_LMW by cotransfecting 0.4 µg of the isogenic expression vectors pSS158 (P_CMV-SAMY-pA), pSS173 (P_CMV-SEAP-pA) and pSS179 (P_CMV-u-PA_LMW-pA) into CHO-K1 cells. α-amylase,

**Fig. 6:** Compatibility, expression kinetics, production levels and impact on cell growth characteristics of SAMY, SEAP and u-PA_LMW. (A) For assessment of SAMY, SEAP and u-PA_LMW compatibility 0.4 µg of pSS158, pSS173 and pSS179 were cotransfected into CHO-K1 cells. Black bars indicate independent quantification of all three reporter enzymes from the same culture supernatant. Individual transfection of 0.4 µg of SAMY, SEAP and u-PA_LMW expression constructs revealing the expression kinetics and production levels are shown as grey bars. (B) Growth profiles of CHO-K1 cells transiently expressing SAMY, SEAP and u-PA_LMW compared to mock-transfected control populations.
phosphatase and peptide-cleaving activities could be independently quantified from a single
CHO-K1 supernatant without any interference between SAMY, SEAP and u-PA activity tests (Fig. 6A).

Simultaneous quantification of all three reporter genes in a completely isogenic background also confirmed that the SAMY reporter system is up to 90-fold more sensitive than SEAP (Berger et al., 1988) and up to $4 \times 10^5$-fold more sensitive compared to $u$-PA assays (Lijnen et al., 1988; Langer et al., 1995). The SAMY, SEAP and $u$-PA reporter are completely compatible with each other and could be used together to quantify expression of up to three genes independently. The combination of SAMY and SEAP has recently been used to assess the regulation performance of dual-regulated expression technology designed for gene therapy and tissue engineering applications (Fussenegger, 2001; Moser et al., 2001).

For analysis of production kinetics, maximum expression levels and impact on cell growth characteristics, CHO-K1 were separately transfected with 0.4 $\mu$g of pSS158 (PCMV-SAMY-pA), pSS173 (PCMV-SEAP-pA) or pSS179 (PCMV-$u$-PA-pA). SAMY expression is detectable about two hours post transfection and shows steady daily increments for several days. Due to its higher activity SAMY expression is detectable significantly earlier than previous SEAP and $u$-PA reporter systems (Fig. 6A).

In this comparative setup SAMY production (147 mU/ml; 146 ng/ml) compares favourably with SEAP (2.3 mU/ml; 1.2 ng/ml) and $u$-PA (0.14 mU/ml; 1.2 ng/ml) considering values for specific activity of $\alpha$-amylase (1010 U/mg; Vihinen and Mantsala 1990), SEAP (2000 U/mg; Ezra et al., 1983) and $u$-PA (120 U/mg; Lijnen et al., 1988). These data qualify SAMY as an alternative model product glycoprotein to SEAP in pilot biopharmaceutical manufacturing studies.

As shown in Fig. 6B, transfection of any reporter gene expression construct had no significant influence on the growth characteristics of transfected CHO-K1 cells.

**DISCUSSION**

Secreted enzymatic reporter systems including the human secreted alkaline phosphatase (SEAP; Berger et al., 1988), *Vargula hilgendorfii* luciferase (Thompson et al., 1990), apoaequorin (Inouye and Tsuji, 1992), GFP from *Aequoria victoria* (Laukkanen et al., 1996), tissue plasminogen activator (tPA; Chen et al., 1987), the low molecular weight urokinase-type plasminogen activator ($u$-PA; Langer et al., 1995), the secreted soft coral *Renilla reniformis* luciferase (Liu and Escher, 1999) and the secreted $\alpha$-amylase derived from *Bacillus stearothermophilus* presented here provide several advantages for the analysis of
transcription control elements (Fussenegger et al., 2000), gene expression studies (Moser et al., 2001), enhancer traps, biotechnological production markers (Schlatter et al., 2001) and non-selective gene expression tracers (Schlatter et al., 2001) compared to intracellular reporter concepts (CAT, β-Gal, GUS, LUC). Key characteristics of an ideal mammalian reporter system are: (i) high affinity for a range of substrates, (ii) high substrate turnover rate, (iii) good enzymatic stability (e.g. resistance to high temperatures), (iv) avoidance of cell extraction procedures, (v) compatible with sequential sampling for kinetic studies, (vi) efficient secretion in a wide variety of mammalian cells, (vi) no requirement of expensive hardware for quantification, (vii) availability of low-cost assays, (viii) high sensitivity and wide linear dynamic range, (x) detection in small samples (e.g. 96-well plate format), (x) fast time course of appearance, (xi) low background from endogenous activities, (xii) the reporter gene product must not alter the physiology of mammalian cells, (xiii) non-invasive assay to leave cells intact for further investigation, (xiv) sample collection and assay are compatible with robotic high-throughput multi-well technology, and (xv) compatibility with other reporter systems.

Although none of the reporter systems developed thus far is considered universal in its application, secreted mammalian reporter technology based on SEAP, u-PA LMW and Renilla luciferase are gathering momentum as they meet with most of the criteria for an ideal reporter system. One of the most widely used secreted mammalian reporter systems is SEAP which has found applications beyond characterization of promoter and enhancer elements as human model glycoprotein for biopharmaceutical manufacturing (Schlatter et al., 2001), as expression tracer for on-line monitoring of difficult-to-quantify genes (Schlatter et al., 2001) and as readout system for high throughput antibiotic screening programs (Aubel et al., 2001). Disadvantages of the SEAP reporter concepts include the requirement for dimerization which may limit the sensitivity, possible in vivo interference of heterologous phosphatases with proper functioning of endogenous phosphoproteins and endogenous expression of placental alkaline phosphatase (PLAP) in many transformed cell lines (Cullen and Malim, 1992). Similar in vivo incompatibilities are also expected for u-PA LMW as this protein is believed to be involved in tissue remodelling and invasive growth in its wildtype configuration. Furthermore, the u-PA LMW assay is of limited sensitivity and requires expensive human plasminogen (Langer et al., 1995).

SAMY has been shown to be secreted by a wide variety of mammalian cell lines showing higher sensitivity compared to SEAP and clearly outperforms u-PA LMW using colorimetric assays. However, neither the DNS- nor Phadebas®-based α-amylase detection technology can
keep up with the sensitivity of the latest-generation chemiluminescence assays designed for SEAP (Kricka et al., 2000). In principle, substrates for chemiluminescent detection of SAMY may be similar to the substrate Galacton Plus used for β-Gal reporter gene assays but should contain dioxetane in α-1,4 instead of β-1,4 linkage to a sugar residue (Martin et al., 1996).

Like SEAP, SAMY is N-terminally glycosylated which enables its use as a model product protein for biopharmaceutical manufacturing or basic glycosylation studies. Despite glycosylation, SAMY fully retains its heat stability associated with AmyS of Bacillus stearothermophilus. This stability is exemplified by the resistance to boiling and the high temperatures (70°C) required for optimal activity thereby eliminating background activity of endogenous α-amylases which are inactivated at temperatures above 40°C. A variety of different tests for quantification of α-amylase have been developed and are routinely used in the brewing and sweetener industries as well as in the clinics since α-amylase is an important diagnosis factor for various diseases (Muscholl-Silberhorn et al., 2000). Because of their low-cost set-up, their easy handling and high sensitivity we have optimized the DNS- and Phadebas®-based α-amylase detection technology as standard SAMY assay. These assays are completely compatible with colorimetric SEAP and u-PA.LMW assays and could be used for independent quantification of up to three expression units.

Surprisingly, B. stearothermophilus AmyS is secreted by mammalian cells (although to a lesser extent than SAMY) and remains in the cytosol only when the 102 bp prokaryotic signal sequence is removed (amyS∆S). It remains to be elucidated why and on what basis the B. stearothermophilus α-amylase signal sequence is compatible with the mammalian protein secretion machinery. AmyS∆S shows similar heat stability as SAMY and can be quantified using identical assays. Therefore, AmyS∆S represents a highly sensitive isogenic intracellular reporter alternative to SAMY similar to Renilla reniformis luciferase which has been engineered for use as intracellular and secreted reporter gene (Liu and Escher, 1999).

Due to the ubiquitous distribution of α-amylases in nature (mammals, plants, insects, yeast and bacteria), we believe that following the example of SAMY design, Bacillus stearothermophilus α-amylase has all assets for easy adaptation as reporter gene in distantly related species. For mammalian cells, SAMY already represents a welcome expansion of the existing secreted reporter gene portfolio and will, alone or in combination with similar systems, be a powerful technology in basic and applied research areas to monitor combinatorial gene expression profiles in complex gene-function analysis, trace complex
(multiregulated) multigene molecular interventions and speed up development of multifactorial screening systems.

ACKNOWLEDGEMENTS

This work was supported by the Swiss Priority Program in Biotechnology (SPP BioTech outphasing), by the Swiss Bundesamt für Bildung und Wissenschaft (BBW) within EC Framework 5 (QLRT-2000-00721) and Cistronics Cell Technology GmbH, Einsteinstrasse 1-5, CH-8093 Zurich, Switzerland. We thank Wolfgang Minas for providing pCSS1, Jacob Reiser for 3rd generation lentiviral expression technology, Gernod Langer for pDL205, Andreas Zisch for HEK293-T cells, Sabina Hug for medical assistance and blood serum, Claudia Ferrara for help with the deglycosylation assay and Barbara Lennon and Wilfried Weber for critical comments on the manuscript.

REFERENCES


Chapter 5

NOVEL CNBP- AND LA-BASED TRANSLATION CONTROL SYSTEMS FOR MAMMALIAN CELLS

ABSTRACT

Throughout the development of *Xenopus*, production of ribosomal proteins (rp) is regulated at the translational level. Translation control is mediated by a terminal oligopyrimidine element (TOP) present in the 5’ untranslated region (UTR) of *rp*-encoding mRNAs. TOP elements adopt a specific secondary structure which prevents ribosome-binding and translation-initiation of *rp*-encoding mRNAs. However, binding of CNBP (cellular nucleic acid binding protein) or La proteins to the TOP hairpin structure abolishes the TOP-mediated transcription block and induces rp production. Based on the specific CNBP-TOP/La-TOP interactions we have designed a translation control system (TCS) for conditional as well as adjustable translation of desired transgene mRNAs in mammalian cells. The generic TCS configuration consists of a plasmid encoding CNBP or La under control of the tetracycline-responsive expression system (TET\textsubscript{OFF}) and a target expression vector containing a TOP module between a constitutive P\textsubscript{SV40} promoter and the human model product gene SEAP (human secreted alkaline phosphatase) (P\textsubscript{SV40}-TOP-SEAP-pA). The TCS technology showed excellent SEAP regulation profiles in transgenic Chinese hamster ovary (CHO) cells. Alternatively to CNBP and La, TOP-mediated translation control can also be adjusted by artificial phosphorothioate anti-TOP oligodeoxynucleotides. Confocal Laser-scanning microscopy demonstrated cellular uptake of FITC-labelled oligodeoxynucleotides and their localization in perinuclear organelles within 24 hours. Besides their TOP-based translation-controlling capacity, CNBP and La were also shown to increase cap-independent translation from polioviral internal ribosomal entry sites (IRES) and La alone to boost cap-dependent translation initiation. CNBP and La exemplify for the first time the potential of RNA-binding proteins to exert translation control of desired transgenes and to increase heterologous protein production in mammalian cells. We expect both of these assets to advance current gene therapy and biopharmaceutical manufacturing strategies.

INTRODUCTION

In mammalian cells, regulation of protein production occurs at several levels including transcription, translation (mRNA concentration, stability and processing), and post-translational modification. Although significant progress has been achieved in the discovery and design of natural as well as heterologous mammalian transcription control systems in past decades, mechanisms underlying translation control remained largely obscure (Harvey and Caskey, 1998; Weber and Fussenegger, 2002; Weber et al., 2002). Systems which modulate
translation were only recently found to play a pivotal role in key developmental and cell-cycle regulatory networks (Pyronnet and Sonenberg, 2001).

Since the discovery of small non-coding RNA controlling copy number of ColE1-derived plasmids a variety of artificial RNA types have been designed to modulate gene expression in mammalian cells which include (i) antisense RNA, (ii) small interference RNA, (iii) ribozymes and (iv) aptamers (Stougaard et al., 1981; Tomizawa et al., 1981; Famulok and Mayer, 1999; Wilson and Szostak, 1999; Levy and Ellington, 2001; Braasch and Corey, 2002; Brantl, 2002). Besides antisense RNA and RNA interference, several different mechanisms have evolved to control translation in nature including (Szymanski and Barciszewski, 2002): (i) secondary structures in the 5’ and 3’ untranslated regions (UTR) as well as the polyadenylation tail of mRNAs (Jackson and Wickens, 1997; Wickens et al., 1997; Conne et al., 2000), (ii) availability of translation-initiation factors (Gray and Wickens, 1998), (iii) cell-cycle-dependent rate of ribosomal recruitment to mRNA (Pyronnet and Sonenberg, 2001), (iv) intracellular localization of mRNA (Bassell and Singer, 1997), and (v) mRNA stability in general (Peng et al., 1998; Nocker et al., 2001).

Key components of the eukaryotic translation machinery, including eER-1α and eEF2, are regulated at the translational level mediated by terminal oligopyrimidinidine (TOP) motifs in the 5’ UTR of their mRNAs (Meyuhas, 2000). These TOP elements adopt a particular secondary structure which impedes translation-initiation at specific developmental stages and growth phases (Pierandrei-Amaldi et al., 1982; Polymenis and Schmidt, 1997). Control of translation-initiation of TOP-harboring mRNAs is mediated by TOP-binding proteins recently identified in Xenopus as cellular nucleic acid binding protein (CNBP) and La autoantigen (Pellizzoni et al., 1997; Perrillou et al., 1998).

CNBP is a small protein containing seven zinc-finger domains of the CCHC type which is highly conserved among human, mouse, rat, chicken and Xenopus species and expressed in a variety of tissues (Warden et al., 1994). CNBP has been reported to be a single-stranded nucleic acid-binding protein involved in the production of the low-density lipoprotein (Rajavashisth et al., 1989), regulation of the c-myc oncogene (Michelotti et al., 1995) and the expression of the human β-myosin heavy chain (Flink and Morkin, 1995a). The predominant localization of CNBP in the cytoplasm or the endoplasmic reticulum is consistent with its preferential binding to specific RNA sequences (Warden et al., 1994). Recently, Pierandrei-Amaldi and co-workers have identified CNBP in vitro as a key translation regulator of TOP-containing ribosomal protein-encoding mRNAs in Xenopus (De Dominicis et al., 2000).
La (also referred to as SS-B; (Wolin and Cedervall, 2002) is a multifunctional phosphoprotein which has first been identified as an autoantigen in patients suffering from rheumatic diseases known as systemic \textit{lupus erythematosus} or Sjogren’s syndrome (van Venrooij et al., 1993). La homologues have since been identified in a variety of insect, yeast and mammalian species (Chan et al., 1989; Scherly et al., 1993; Bai et al., 1994; Pardigon and Strauss, 1996). Although its physiological role has not yet been fully established La has been implied in a variety of functions including (i) initiation and termination of RNA polymerase III-mediated transcription (Fan et al., 1997), (ii) nuclear RNA export (Simons et al., 1996), (iii) interaction with tRNAs, 5S rRNA, U6 small nuclear RNA, 7SL RNAs, cytoplasmic Y RNAs, 4.5S\textsubscript{i} and 4.5S\textsubscript{ii} RNAs and Alu transcripts (Hendrick et al., 1981; Lerner et al., 1981; Rinke and Steitz, 1982; Chambers et al., 1983; Rinke and Steitz, 1985; Fabini et al., 2000), (iv) chaperoning of small RNAs (Kufel et al., 2000), (v) interaction with 5’ non-coding regions of viruses (Kurilla and Keene, 1983; Svitkin et al., 1994; Park and Katze, 1995; Pardigon and Strauss, 1996; Duncan and Nakhasi, 1997; Goodier et al., 1997), and (vi) translation enhancement of endogenous and viral mRNAs (Kim and Jang, 1999; Ali et al., 2000; Carter and Sarnow, 2000; Crosio et al., 2000; Kim et al., 2001; Waysbort et al., 2001; Zhu et al., 2001).

We designed a heterologous mammalian translation control system based on the specific interactions of CNBP and La with TOP-containing and transgene-encoding mRNAs. Translation of a TOP-engineered \textit{SEAP}-encoding reporter-mRNA could be reversibly adjusted by binding of human CNBP or La to the TOP sequence in the 5’ UTR of the target mRNA or by a specific anti-TOP oligodeoxynucleotide. To our knowledge the translation control system (TCS) described here represents the first example of metabolic engineering of translation in mammalian cells. We expect translation engineering to significantly increase the technology portfolio for improving biopharmaceutical manufacturing, gene therapy and tissue engineering.

**MATERIALS AND METHODS**

**Plasmid Constructions (see Table 1)**

\textbf{pSS106}: The gene conferring puromycin resistance (\textit{pur}) was excised from \textit{pPur} (Clontech, Palo Alto, CA) by \textit{HindIII}/\textit{XbaI}, polished with Pfu polymerase (Stratagene, La Jolla, CA) and ligated into the \textit{SmaI} site of \textit{pBluescriptII KS’} (Stratagene).
**pSS107:** The human cellular nucleic acid binding protein (CNBP) was excised from pSG5-CNBP (Flink and Morkin, 1995b; Flink and Morkin, 1995a) by StuI/AflIII, polished with Pfu polymerase and ligated into the SmaI site of pBluescriptII KS⁺.

**pSS108:** The human La gene was excised from pGEM-3Zf(-)-hLa (Pruijn et al., 1991) with EcoRI and ligated into the corresponding site (EcoRI) of pBluescriptII KS⁺.

**pSS110:** The gene conferring zeocin resistance (zeo) was amplified by PCR from pVgRXR (Invitrogen, Carlsbad, CA) using primers OSS20: gatccctcgaactagGGGCCACCATGGCCAAGT (XhoI and SpeI sites underlined, Kozak sequence in italics, annealing sequence in capital letters) and OSS21: gategctatttaaatCGGTCAGTCCTGCTCCTC (BglII and SwaI sites underlined, annealing sequence in capital letters), and the 420 bp zeo fragment was cloned in sense orientation into pCR2.1-TOPO (Invitrogen).

**pSS113:** SEAP was amplified by PCR from pSEAP2-Basic (Clontech) using primers OSS23: gatcgcggcCGCACCATGC TGCTGC (NotI site underlined, Kozak sequence in italics, annealing sequence in capital letters) and OSS24: gategtcagTTATCATGTCTGCTCGAAGCG (PstI site underlined, annealing sequence in capital letters) and cloned in sense orientation into pCR2.1-TOPO.

**pSS115:** SEAP was amplified by PCR from pSEAP2-Basic using primers OSS25: gatcgaattcccttttctcttcgtggccgctgtggagaagcagcgaggagCCACCATGCTGCTGCTGCT (EcoRI site underlined, terminal oligopyrimidine (TOP) adapted from the Xenopus L4 5' untranslated region (UTR) (Pellizzoni et al., 1998) in bold letters, Kozak sequence in italics, annealing sequence in capital letters) and OSS24 and cloned in sense orientation into pCR2.1-TOPO.

**pSS123:** The gene encoding the low molecular weight urokinase-type plasminogen activator (u-PALMW).

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**Table 1:** Plasmids used in this study: CITE* (optimized, eukaryotic cap-independent translation enhancer element from encephalomyocarditis virus), CNBP (human cellular nucleic acid binding protein gene), IRESI and IRESII (eukaryotic internal ribosomal entry site from poliovirus), La (human La autoantigen gene), neo (neomycin resistance conferring gene), pA SV40 (eukaryotic polyadenylation sequence from simian virus 40), Pbcmv (constitutive, eukaryotic promoter from cytomegalovirus), Pbcmv*-1 (tetracycline-regulatable, eukaryotic promoter from cytomegalovirus), PlacZ (constitutive, prokaryotic promoter from the lacZ gene), Pmpsv (constitutive, eukaryotic promoter from myeloproliferative sarcoma virus), PSV40 (constitutive, eukaryotic promoter from simian virus 40), pur (puromycin resistance conferring gene), SEAP (human secreted alkaline phosphatase gene), TOP (eukaryotic terminal oligopyrimidine sequence), tTA (tetracycline-dependent transactivator gene), n-PAlaw (human low molecular weight urokinase-type plasminogen activator gene), zeo (zeocin resistance conferring gene).
The activator (u-PALMW) was excised from pDL205 (Langer et al., 1995) by HindIII and ligated into the corresponding site (HindIII) of the first cistron of pTRIDENT1 (Fussenegger et al., 1998).

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<th>Plasmid</th>
<th>Description</th>
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Cell Culture, Transfection and Production of Stable Cell Lines

All cell lines were cultivated in FMX-8 medium (Cell Culture Technologies, Zurich, Switzerland) supplemented with 10% fetal calf serum (PAA Laboratories GmbH, Austria, cat# A15-022, Lot# A01129-242) and the appropriate antibiotics at 37°C in a humidified 5% CO₂ atmosphere. CHO-XMK1₉ are Chinese hamster ovary cells (CHO-K1, ATCC: CCL 61) stably expressing the tetracycline-dependent transactivator (tTA) (Gossen and Bujard, 1992; Mazur et al., 1998). CHO-XMK111₁₀ is a CHO-XMK1₉ derivative also producing SEAP under control of the tetracycline-dependent promoter (P_hCMV*-1; P_hCMV*-1-SEAP-pASV₄₀⁻) (Mazur et al., 1998).

CHO-K1 cells and their derivatives (30%-50% confluent) were transfected using an optimized CaPO₄-based transfection protocol typically yielding transfection rates of over 30% (Fussenegger et al., 1997). In brief, 24h before transfection 10⁵ cells were seeded in 6 well plates. Thereafter, 6 µg DNA were incubated as CaPO₄-precipitates for 5 h with the cells before a glycerol shock (15% glycerol in culture medium) was applied for 30s. The cells were then washed three times with PBS and cultivated with fresh culture medium.

CHO-SS1924 and CHO-SS1925 are CHO-XMK1₉-derived cell lines stably transfected with pSS119 and either pSS124 (CHO-SS1924) or pSS125 (CHO-SS1925). pSS119 harbors a dicistronic P_SV₄₀-driven expression unit encoding a TOP-SEAP cassette in the first and the puromycin resistance gene (pur) in the second cistron (P_SV₄₀-TOP-SEAP-RES-pur-pA; pA, polyadenylation site) (Fig. 1B). pSS124 encodes a tricistronic expression unit encoding u-PALMW, human CNBP and zeo under control of the tetracycline-responsive promoter (P_hCMV*-1) (P_hCMV*-1-u-PALMW-RES-CNBP-CITE*-zeo-pA). pSS125 is isogenic to pSS124 but contains human La instead of CNBP in the second cistron (P_hCMV*-1-u-PALMW-RES-La-CITE*-zeo-pA) (Fig. 1B).

Transfected populations were grown in culture medium supplemented with 400 mg/l G418 (Alexis Biochemicals, Laefelfingen, Switzerland) to maintain tTA expression, 5 mg/l puromycin (Alexis Biochemicals) to select for integration of pSS119 and 2.5 mg/l tetracycline (Sigma, Buchs, Switzerland) to repress the tricistronic expression unit. Mixed populations were cultivated to a total cell number of 3*10⁶ and subsequently cloned by FACS-mediated single cell sorting using a FACStar⁺ and Cell Quest™ software (Becton-Dickinson, San Jose, CA).

Individual CHO-SS1924 and CHO-SS1925 clones were numbered by subscripts (for example CHO-SS1924₆).
Chemiluminescence-Based SEAP Assay and Modulation of SEAP Production

Cell culture supernatants were centrifuged for 2 min at 14,000 g. 25 µl of the supernatant was removed and prepared for chemiluminescence-based quantification of SEAP according to the manufacturer’s protocol (Great EscAPE™ SEAP reporter system 2, chemiluminescent, Roche Biochemicals, Basel, Switzerland). SEAP activity was measured during 10 s using a Turner Designs Luminometer (TD20-20, Turner Designs, Sunnyvale, CA). The readout in relative light units per second (RLUs⁻¹) is directly proportional to the amount of SEAP in the culture supernatant. SEAP was quantified by chemiluminescence per volume (RLUs⁻¹l⁻¹10⁻⁹).

Alternatively, for assessment of SEAP production of transiently transfected cells, SEAP measurement was started 24h post-transfection (RLUs⁻¹l⁻¹10⁻⁸). Average SEAP productions (including standard deviations) of stable cell lines were determined in triplicate over a period of 4 days. An initial cell population of 2.5*10⁴ cells was seeded into 6-well plates containing selection medium (FMX-8, 10% FCS, 400 mg/l neomycin, 5 mg/l puromycin) and grown in the presence (+tet; 2.5 µg/l) or absence (–tet) of tetracycline. For assessment of CNBP- or La-induced SEAP production expression levels of these TOP-binding proteins were adjusted by incubation of the respective transgenic cell lines with varying concentrations of tetracycline (0, 1, 5, 20, 100 and 5000 µg/l). Effects of the anti-TOP phosphorothioate oligo (gaagagaaaaaggaattcgc-FITC) on TOP-dependent SEAP-mRNA translation were evaluated by incubation of CHO-SS1925₂ cells in tetracycline-free medium (induction of CNBP- / La-expression) supplemented with different amounts of the oligodeoxynucleotide (gaagagaaaaaggaattcgc-FITC; 0, 0.1, 0.2, 0.5, 1 or 2 µM).

Colorimetric Assay for Quantification of Low Molecular Weight Urokinase-Type Plasminogen Activator (u-PA_LMW)

100 µl cell culture supernatant were centrifuged at 14’000 x g for 2 min. Subsequently, 50 µl were transferred into a chamber of a 96-well plate and adjusted to 37°C. 25 µl plasminogen-specific substrate (1.6 mM S-2251: H-D-Val-Leu-Lys-pNA₂HCl (cat# 820332; Chromogenix, Milano, Italy) in u-PA_LMW assay buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.01% Tween 80) were mixed with 25 µl plasminogen solution (1 g/l human plasminogen in H₂O (cat# 820332; Chromogenix, Milano, Italy) adjusted to 37°C and added to the cell culture supernatant. The absorbance time-course was measured at 405 nm (p-nitroanillin; pNA) for 15 min and u-PA_LMW levels (U⁻¹) are calculated as described before (Schlatter et al., 2002).
Parallel to quantification of SEAP and u-PA<sub>LMW</sub> production, the average viable cell number of all cell cultures was measured in triplicate. Cells were detached using dissociation solution (Sigma) and resuspended in filter-purified casein buffer (pH 7.3; 7.93 g/l NaCl; 0.38 g/l Na<sub>2</sub>EDTA; 0.4 g/l KCl; 0.19 g/l NaH<sub>2</sub>PO<sub>4</sub>; 1.95 g/l Na<sub>2</sub>H<sub>2</sub>(PO<sub>4</sub>)<sub>2</sub>; 0.3 g/l NaF). The viable cell number (Cells l<sup>-1</sup> 10<sup>-8</sup>) was determined as the average of three independent readings taken by a Casyl® cell counter (Schärfe System, Reutlingen, Germany).

Confocal Laser-Scanning Microscopy of CHO Cell Derivatives Grown in the Presence of FITC-Labelled Anti-TOP Oligodeoxynucleotides

2.5*10<sup>4</sup> CHO-SS1924<sub>6</sub> (CHO-SS1925<sub>2</sub>) or CHO-XMK111<sub>10</sub> cells were seeded in chamber slides (Lab-Tek, Nunc, Inc., IL, USA) and grown for 24 h in tetracycline-free selection medium (induction of CNBP or La expression). After cultivation for another 24 h in the presence of 2 µM anti-TOP oligodeoxynucleotides the cell monolayer was washed once with phosphate-buffered saline (PBS, Dulbecco’s phosphate-buffered saline, Sigma, cat.# D5773) and submerged with 4% para-formaldehyde (PFA) solution in PBS for 30 min. Cells were then washed three times with PBS before they were fixed with embedding medium (70% (v/v) glycerol, 30 mM Tris-HCl (pH 9.5); 240 mM n-propyl gallate (Sigma)). The imaging system consisted of an inverted fluorescence microscope (Leica DMIRB/E, Heerbrugg, Switzerland), a confocal scanner (Leica TCS NT) and an argon/krypton mixed gas laser. Image processing was performed on a Silicon Graphics workstation using Imaris software (Bitplane AG, Zurich, Switzerland) (Messerli et al., 1993).

RESULTS

Construction and Assembly of the Translation Control Systems (Table 1)

The terminal oligopyrimidine (TOP) sequence in the 5’ untranslated region (UTR) of ribosomal mRNAs has been identified as binding target for the cellular nucleic acid binding protein (CNBP) and the La protein in <i>Xenopus</i> and was ascribed to confer translational regulation of these mRNAs (Pellizzoni et al., 1997).

Based on this protein-RNA interactions we constructed binary translation control systems (TCS) consisting of CNBP/La expression vectors and a transgene-encoding plasmid harboring
the TOP module between a constitutive promoter and the transgene of interest. When placed in the 5’ UTR of the constitutive SEAP expression unit (P_{SV40}-TOP-SEAP-pA; pSS119, see below), TOP adopts a particular secondary structure which abolishes translation of this human model glycoprotein (TOP-SEAP-mRNA) (Fig. 1A; Fig. 2 and 3 for stable expression; Fig. 5 for transient expression). Binding of human CNBP or human La to TOP resolves its translation-inhibiting secondary structure and SEAP production is initiated (Figure 1A). CNBP and La are cloned under control of the tetracycline-responsive promoter (P_{hCMV*-1}) to adjust expression of these TOP-modulating proteins by tetracycline antibiotics. Consequently, the presence of tetracycline in the culture medium prevents CNBP/La expression and therefore suppresses SEAP-mRNA translation whereas cells grown in the absence of this antibiotic produce the TOP-binding proteins which induce maximum SEAP production (Fig. 1A).

The design of the TCS systems included construction of the SEAP reporter plasmid pSS119 containing a dicistronic P_{SV40}-driven SEAP-IRES-pur expression unit with a TOP sequence in its 5’ UTR. The internal ribosome entry site (IRES) mediates coordinated cap-independent translation-initiation of the terminal pur gene conferring resistance to the antibiotic puromycin (P_{SV40}-TOP-SEAP-IRES-pur-pA). pSS119 was assembled by ligating

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**Fig. 1A:** Schematic representation of the translation control system (TCS) and its plasmids. The TOP-SEAP-mRNA encoded on pSS119 is continuously transcribed under the control of the constitutive P_{SV40} promoter. SEAP-mRNA translation is blocked due to the secondary structure of the terminal oligopyrimidine element (TOP). In the absence of tetracycline pSS124- and pSS125-encoded RNA-binding protein CNBP and La are expressed by the tetracycline-responsive promoter (P_{hCMV*-1}), bind to the TOP structure and enable translation of the reporter gene encoding the human secreted alkaline phosphatase (SEAP).
the \textit{pur}-encoding \textit{NotI/EcoRV} fragment of pSS106 into the compatible \textit{NotI/SwaI} sites of pSS118 (Fig. 1B).

The vectors for expression of the TOP binding proteins \textit{CNBP} (Flink and Morkin, 1995b; Flink and Morkin, 1995a) and \textit{La} (van Venrooij et al., 1993) are isogenic and contain a tricistronic \textit{PhCMV*-1}-driven expression cassette encoding in the first cistron the single-chain

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Fig. 1B: Key plasmids of the translation control systems as well as relevant control vectors. Expression units encoding \textit{SEAP}, the human cellular nucleic acid binding protein (\textit{CNBP}) or human \textit{La} autoantigen are either driven by the constitutive \textit{PSV40} or the tetracycline-adjustable promoter (\textit{PhCMV*-1}) and cloned in a monocistronic or multicistronic expression configuration also encoding reporter (low molecular weight urokinase-type plasminogen activator; \textit{u-PALMW}), or selection markers (\textit{pur}, puromycin resistance gene; \textit{zeo}, zeocin resistance gene). Genes encoded in the second cistron are translated by an internal ribosome entry site of polioviral origin (IRES) while third cistrons rely on optimized encephalomyocarditis-derived cap-independent translation enhancers (CITE*) for translation-initiation. Restriction sites: \textit{AscI} (A), \textit{BclI} (B), \textit{BglII} (B2), \textit{BssHII} (B3), \textit{ClaI} (C), \textit{EcoRI} (E), \textit{EcoRV} (E2), \textit{HindIII} (H), \textit{MluI} (M), \textit{NotI} (N), \textit{PacI} (P), \textit{PmeI} (P2), \textit{PstI} (P3), \textit{SalI} (S), \textit{SbfI} (S2), \textit{SgfI} (S3), \textit{SpeI} (S4), \textit{SrfI} (S5), \textit{SwaI} (S6), \textit{XbaI} (X) and \textit{XhoI} (X2).
\end{verbatim}
urokinase-type plasminogen activator (u-PALMW, Langer et al., 1995), either CNBP (pSS124) or La (pSS125) in the second and the zeocin resistance marker in the terminal cistron (pSS124: PhCMV*-1-u-PALMW-IRESCNBP-CITE*-zeo-pA; pSS125: PhCMV*-1-u-PALMW-IRESLa-CITE*-zeo-pA) (Fig. 1B). pSS124 was cloned following a four-step procedure: (i) zeo was excised from pSS110 by SpeI/SwaI and cloned into the corresponding sites (SpeI/SwaI) of TRIDENT3’s third cistron (Fussenegger et al., 1998) to give pSS121 (pSS121, PhCMV*-1-MCSI-IRES-MCSII-CITE*-zeo-pA). (ii) human CNBP was excised from pSS107 by NotI/ClaI and cloned into the corresponding sites (NotI/ClaI) of the second cistron of pSS121 to result in pSS122 (PhCMV*-1-MCSI-IRES-CNBP-CITE*-zeo-pA). (iii) u-PALMW was excised from pSS123 by SspI/NotI and cloned into the first cistron of pSS122 to result in pSS124 (PhCMV*-1-u-PALMW-IRESCNBP-CITE*-zeo-pA). (iv) pSS125 was constructed by replacing CNBP of pSS124 by NotI/ClaI with the corresponding La-encoding fragment excised from pSS108 (NotI/ClaI) (PhCMV*-1-u-PALMW-IRESLa-CITE*-zeo-pA) (Fig. 1B).

All plasmids were transiently transfected into the tTA-expressing CHO-K1-derived CHO-XMK19 cell line (Mazur et al., 1998) to demonstrate functionality. In particular, to monitor CNBP- and La-mediated induction of SEAP-mRNA translation, pSS119 was cotransfected with pSS124 or pSS125 (see below and Fig. 5). While SEAP expression was determined by chemiluminescence, u-PA LMW levels were assessed using a colorimetric assay. u-PALMW expression of pSS124 and pSS125 was responsive to tetracycline, whereas SEAP expression from pSS119 was not altered by the antibiotic, unless cotransfected with either pSS124 or pSS125 (see below for stable expression, Fig. 2).

Construction of the CHO-SS1924 (CHO-SS1925) Cell Line Stably Expressing pSS119 and pSS124 (pSS125)

For construction of the CHO-SS1924 and CHO-SS1925 cell lines pSS119 was cotransfected with either pSS124 or pSS125 into the tTA-expressing CHO-K1-derived CHO-XMK19 cell line and selected in the presence of puromycin. In order to prevent expression of CNBP and La during the selection procedure, the tricistronic expression units contained on pSS124 and pSS125 were repressed by addition of tetracycline. Complete repression of the tricistronic expression unit during selection of mixed populations was monitored by parallel u-PA LMW assays (data not shown). The mixed populations were cloned by FACS-mediated cell sorting and 6 clones co-expressing SEAP and u-PALMW in the absence of tetracycline were randomly chosen for further analysis (CHO-SS1924, pSS119 + pSS124; CHO-SS1925, pSS119 +
pSS125). Since cell clones CHO-SS1924\textsubscript{6} and CHO-SS1925\textsubscript{2} showed the optimal combination of tightest tetracycline-dependent \textit{u-PA}_{LMW} expression and maximum \textit{SEAP} expression in the absence of tetracycline they were chosen for further analysis (see below and Figs. 2, 3 and 4).

**Characterization of the Growth Behavior and SEAP and \textit{u-PA}_{LMW} Production of CHO-SS1924\textsubscript{6} (CHO-SS1925\textsubscript{2}) Cells**

![Graph showing SEAP and \textit{u-PA}_{LMW} production over time](image)

**Fig. 2A:** SEAP and \textit{u-PA}_{LMW} production of cell lines CHO-SS1924\textsubscript{6} and CHO-SS1925\textsubscript{2}. Readings of SEAP (RLUs\textsuperscript{10\textsuperscript{-9}}) and \textit{u-PA}_{LMW} (UI\textsuperscript{-1}) production of CHO-SS1924\textsubscript{6} and CHO-SS1925\textsubscript{2} were taken in the presence or absence of tetracycline (+tet; -tet) over a period of 4 days and represent the average of three independent experiments.
For characterization of the growth behavior, SEAP production and u-PA\textsubscript{LMW} expression profiles of CHO-SS1924\textsubscript{6} and CHO-SS1925\textsubscript{2} we cultivated these cell lines for four days in the presence (+ tet) or absence (–tet) of tetracycline. SEAP and u-PA\textsubscript{LMW} production profiles were independently measured and plotted as reporter protein activities (SEAP [RLUs\textsuperscript{-1}l\textsuperscript{-1}10\textsuperscript{-9}] and u-PA\textsubscript{LMW} [Ul\textsuperscript{-1}]; RLU, relative light units and U, international units) (Fig. 2). Concomitantly with the daily determination of reporter protein levels the cells were detached and counted. The average cell number of three independent experiments is shown in Fig. 2 (Cells l\textsuperscript{-1}10\textsuperscript{-8}).

By comparing SEAP expression levels of CHO-SS1924\textsubscript{6} or CHO-SS1925\textsubscript{2} cells grown in tetracycline-free media (CNBP and La expression induced) with the same cell lines cultivated in the tetracycline-containing control configuration (CNBP and La expression repressed) the CNBP-TOP and La-TOP-mediated translation control capacity could be assessed. Overall, the translation of the TOP-SEAP-mRNA as evidenced by the SEAP titer increased 45.81±1.93- (7.91±0.52-) fold following induction of CNBP (La) (Fig. 2; SEAP). Tetracycline-dependent control of CNBP and La expression as represented by u-PA\textsubscript{LMW} titers reached ratios of 23.18±6.56 and 60.07±2.52, respectively under identical conditions (Fig. 2; u-PA\textsubscript{LMW}). The 2-fold increase in maximum SEAP expression of CHO-SS1925\textsubscript{2} (19.62±1.33 RLUs\textsuperscript{-1}l\textsuperscript{-1}10\textsuperscript{-9}) compared to CHO-SS1924\textsubscript{6} (9.22±0.38 RLUs\textsuperscript{-1}l\textsuperscript{-1}10\textsuperscript{-9}) correlated with 2-fold higher La levels.

![Graph](image_url)

**Fig. 2B:** Growth characteristics cell lines CHO-SS1924\textsubscript{6} and CHO-SS1925\textsubscript{2}. Cell number (Cells l\textsuperscript{-1}10\textsuperscript{-8}) of CHO-SS1924\textsubscript{6} and CHO-SS1925\textsubscript{2} in the presence or absence of tetracycline (+tet; -tet) over a period of four days.
compared to CNBP as deduced from u-PA\textsubscript{LMW}-based expression linkage (1.06±0.05 vs. 0.46±0.01 Ul\textsuperscript{-1}). Neither the presence of tetracycline nor increased expression of $u$-$PA_{LMW}$, CNBP, La or zeo affected growth characteristics of CHO-SS1924\textsubscript{6} or CHO-SS1925\textsubscript{2} (4.94±0.22 and 4.37±0.36 Cells l\textsuperscript{-1} 10\textsuperscript{-8}) (Fig. 2).

**Adjustable Translation Control in Response to Tetracycline**

**Fig. 3**: Tetracycline-adjustable SEAP production profiles of CHO-SS1924\textsubscript{6} and CHO-SS1925\textsubscript{2}. CHO-SS1924\textsubscript{6} and CHO-SS1925\textsubscript{2} cells were grown in the presence of different tetracycline concentrations (0, 1, 5, 20, 100 and 5000 μg/l). Over a time period of four days, SEAP production was assessed daily using a chemiluminescence assay. Columns represent average SEAP values (RLUs^\textsuperscript{-1} l\textsuperscript{-1} 10\textsuperscript{-9}) of three independent experiments.
In the previous section we have demonstrated that CNBP-TOP- as well as La-TOP-mediated translation control can be switched “ON” and “OFF”. However, to generate high impact among bioengineering and gene therapy communities, TCS technology has to be adjustable so that translation of transgenes can be fine-tuned to any desired level. Therefore, we have quantitatively assessed the fine-tuning capacity of TCS by cultivating CHO-SS1924 and CHO-SS1925 in the presence of increasing concentrations of tetracycline and analyzing the SEAP titer in the cell culture medium during four days. SEAP expression could be adjusted by CNBP to 100 ± 4.19, 59.43 ± 6.63, 31.29 ± 3.04, 17.89 ± 1.5, 12.67 ± 1.2 or 7.41 ± 1.11 % and by La to 100 ± 8.83, 53.54 ± 5.98, 21.37 ± 2.08, 4.33 ± 0.36, 3.44 ± 0.33 or 3.21 ± 0.48 % of maximal SEAP expression by addition of 1, 5, 20, 100 or 5000 µg/l of tetracycline (Fig. 3).

Translation Control Using Anti-TOP Phosphorothioate Oligodeoxynucleotides

Pierandrei-Amaldi and coworkers repeatedly demonstrated in vitro that binding of CNBP or La to their cognate TOP RNA structure mediated translation of downstream messages (Crosio et al., 2000). Although the precise mechanisms by which the CNBP-TOP or La-TOP interactions abolish the TOP-mediated translation block remains unknown, it is commonly

![Fig. 4A: Anti-TOP oligodeoxynucleotide-based regulation of TOP-SEAP-mRNA translation: Secondary structure of the TOP-sequence (adapted from the Xenopus L4 rp-mRNA (Pellizzoni et al., 1998)) as engineered into the 5' UTR of the reporter gene SEAP. Hydrogen bonds are depicted as dotted lines, Kozak sequence and start codon are shown in italics. The binding site for CNBP (La) (Pellizzoni et al., 1997) is represented by grey (black) circles. The 3'-FITC-labelled phosphorothioate anti-TOP oligo directed against the La-binding site is shown as solid black line.](image-url)
accepted that the disruption of the TOP-adopted hairpin structure induced by the two RNA-binding proteins may result in unencumbered ribosome scanning and translation of the TOP-containing mRNA. In case such a scenario would hold true it can be expected that a complementary TOP oligodeoxynucleotide may compete for CNBP and La binding to the TOP-hairpin structure thereby blocking translation.

In order to test whether artificial TOP-specific nucleic acids may indeed modulate TOP’s translation-suppressing activity we have designed a 3’-FITC-labelled anti-TOP phosphorothioate oligodeoxynucleotide complementary to the La binding site which is expected to compete with TOP’s secondary structure and abolish it (Fig. 4A). Initial experiments revealed that lipofection was equally efficient in modulating target mRNA translation compared to simple addition of the anti-TOP oligo to the culture medium. Furthermore, concentrations up to 5 µM anti-TOP exerted no deleterious effects on cell growth and viability of the target cells (data not shown). The influence of the anti-TOP oligo on the translation of TOP-tagged SEAP-mRNA was analyzed by incubating CHO-SS1925_2 in tetracycline-free medium (full induction of CNBP- / La- expression) enriched with various concentrations of anti-TOP oligo followed by daily assessment of SEAP production over a period of four days. Increasing concentrations of the anti-TOP oligo (0, 0.1, 0.2, 0.5, 1

![Graph showing SEAP expression over time with different concentrations of anti-TOP oligo.](image)

**Fig. 4B:** Anti-TOP-responsive SEAP expression of cell clone CHO-SS1925_2 grown in the presence of varying concentrations of this oligo (0, 0.1, 0.2, 0.5, 1 or 2 µM) but in the absence of tetracycline (full induction of CNBP / La expression) during four days. Average daily SEAP-levels (RLUs⁻¹l⁻¹10⁹) from three independent experiments are indicated.
or 2 µM) resulted in a concentration-dependent shut down of SEAP expression (100±1.98, 89.99±2.15, 76.95±3.06, 45.58±3.66, 30.19±3.13 or 10.15±1.41(2-1) %) (Fig. 4B). As shown in Fig. 4B, cells rapidly take up the anti-TOP oligo as they mediate translation-induction of SEAP-mRNA already after 24 h (Fig. 4B). In order to demonstrate that anti-TOP-mediated translation control is implemented via binding of these oligos to their complementary TOP-containing mRNA we grew CHO-SS1924₆, CHO-SS1925₂ and the control cell line CHO-XMK11₁₀ expressing TOP-less SEAP-mRNA (Mazur et al., 1998) for 24 h on cover slides in the presence of 2 µM anti-TOP oligos followed by confocal microscopy of intracellular FITC signals. Whereas the FITC-tagged anti-TOP oligo was randomly distributed in the CHO-XMK11₁₀ cytoplasm (no anti-TOP oligo target present), it accumulated in the perinuclear organelles of CHO-SS192₄₆ and CHO-SS192₅₂ which coincide with increased transcriptional activities including the TOP-tagged SEAP-mRNA (Fig. 4C).

**Fig. 4C:** Phase contrast, FITC fluorescence or superimposed confocal micrographs of the cell clones CHO-SS192₄₆, CHO-SS192₅₂ and the control cell line CHO-XMK11₁₀ (Mazur et al., 1998) grown in the presence of 2 µM anti-TOP oligo.
Impact of CNBP and La on TOP-, Cap-, IRES- or CITE*-Mediated Translation

Based on the fact that CNBP and La may have activities unrelated to TOP binding we investigated whether these RNA-binding proteins could boost production of proteins translated in a cap-, IRES- or CITE*-dependent manner. A variety of isogenic reporter plasmids pSS118, pSS143, pSS139 and pSS138 were constructed which encode SEAP preceeded by the four different translation-modulating elements TOP, 5'-Cap, the polioviral internal ribosome entry site (IRES) and the optimised cap-independent translation enhancer (CITE*) derived from the encephalomyocarditis virus (pSS118, PSV40-TOP-SEAP-IRES-CITE*-pA; pSS143, PSV40-SEAP-IRES-CITE*-pA; pSS139, PSV40-IRES-SEAP-CITE*-pA; pSS138, PSV40-IRES-CITE*-SEAP-pA). pSS118 was constructed by cloning the TOP-SEAP-encoding EcoRI fragment of pSS115 into the corresponding site (EcoRI) of pTRIDENT4’s first cistron (Fussenegger et al., 1998). Excision of SEAP from plasmid pSS113 by EcoRI, NotI/EcoRV or SpeI/EcoRV followed by cloning into the EcoRI, NotI/Srfl sites of the first,
The second or third cistron of pTRIDENT4 resulted in plasmids pSS143, pSS139 and pSS138, respectively (Fig. 1B).

CHO-XMK19 cells were transiently transfected with one of the four different reporter plasmids pSS118 (TOP-SEAP), pSS143 (5'-Cap-SEAP), pSS139 (IRES-SEAP) or pSS138 (CITE*-SEAP) alone or together either with the CNBP-encoding pSS124 or the La expression construct pSS125. Confirming the results shown in Fig. 3 with CHO-SS1924 and CHO-SS1925, CNBP as well as La induced TOP-dependent SEAP mRNA translation 5.23±0.72- and 4.84±0.49-fold in this transient expression configuration, respectively. Furthermore, CNBP- / La-dependent translation of TOP-tagged SEAP mRNA compares with translation efficiency of isogenic cap-dependent SEAP transcripts driven by the strong viral P_{SV40} promoter (Fig. 5). In contrast, cap-dependent SEAP mRNA translation was not increased following coexpression of CNBP, whereas La protein doubled production of SEAP translated in a classical cap-dependent manner. Furthermore, cap-independent translation mediated by the IRES element was increased by the presence of CNBP (1.49±0.31-fold) or La (2.71±0.61-fold). CITE*-mediated translation was not affected neither by CNBP nor by La (Fig. 5).

**DISCUSSION**

Advances in biomedicine have accentuated the use of gene therapy as an attractive platform for the delivery of a therapeutic protein. Human-compatible gene regulation technology will become an increasingly important option for pharmacological control of in vivo-delivered therapeutic transgenes since it (i) enables titration of the protein therapeutic into the desired range, (ii) facilitates adaptation of a therapy to fluctuating daily dosing regimes, and (iii) allows reversibility of the therapy as needed. Compact genetic design and low immunogenicity are particularly important for therapeutic applications, including somatic gene therapy and tissue engineering (Clackson, 2000; Fussenegger, 2001).

Most of the heterologous gene regulation systems available to date are based on chimeric heterologous transcription control mechanisms. The generic concept for transgene transcription control includes an artificial transcription factor, typically a fusion protein of a DNA-binding motif and a potent transactivation domain, and a heterologous promoter assembled by placing a cognate transactivator-specific operator adjacent of a minimal eukaryotic promoter (Fussenegger, 2001). Transcription of desired genes is implemented by conditional binding of the transactivator to its target promoter in response to small-molecule
drugs. Although controlling transcription is an obvious target for titration desired protein products, other steps including translation of the transgene message as well as protein secretion may be considered highly valuable alternatives for fine-tuning protein levels at different sites in the body or to study intracellular protein trafficking as well as mRNA stability. Owing to their ambition to increase product titers in biopharmaceutical manufacturing, bioengineers have long focused on comprehensive aspects of gene-product transformations. However, since the gene-to-product balance with its specific parameters (transient) transfection, transcription, translation, glycosylation, and secretion together with attendant permutations of such factors and their stability contains too many variables for a generic solution further studies on particular constants will be required. Although bioengineers are highly proficient in producing enough message of desired product genes, knowledge on efficient processing of gene transcripts, in particular translation and protein secretion, remains rather rudimentary. The design of translation and secretion control systems is essential for providing the molecular assets to study these bottlenecks in biopharmaceutical manufacturing and eventually improve product titers beyond the common levels.

Prototype technologies for controlled protein secretion and mRNA translation have been reported (Werstuck and Green, 1998; Rivera et al., 2000). However, these systems have been designed for therapeutic use to modulate insulin secretion of transgenic cell lines and to block translation of viral transcripts by rather toxic molecules without the scope of improving biopharmaceutical manufacturing. We have adapted naturally evolved human protein-RNA interactions for use as an artificial translation control system which can either be adjusted by antibiotics of the tetracycline class or by synthetic oligodeoxynucleotides. Key components of these novel translation control systems (TCS) are the human CNBP and La proteins which bind to a terminal oligopyrimidine module (TOP). CNBP or La binding enables efficient translation of TOP-containing mRNAs which is otherwise blocked by the particular secondary structure adopted by unbound TOP. Translation of desired target genes could be adjusted either indirectly by fine-tuning transcription of CNBP or La or directly by designed anti-TOP oligos which interfere with the CNBP / La interaction in a dose-dependent manner. Both systems are human-compatible and have proven to provide unprecedented precision in controlling translation in stable transgenic mammalian cell lines. Under fully induced conditions CNBP / La completely neutralizes TOP’s translation-blocking activity which results in maximum product titers comparable to isogenic TOP-less expression units.

Although the tightness of TCS is remarkable two major factors may contribute to leaky translation: (i) the well-known leakiness of the tetracycline-responsive gene regulation system
controlling heterologous CNBP / La expression (Freundlieb et al., 1997) and (ii) endogenous CNBP / La titers. While repression of CNBP / La transcription may be improved by using tighter transcription control modalities (Fussenegger et al., 2000; Weber et al., 2002), endogenous CNBP / La levels remain an inherent characteristic associated with the host cell line.

The basic TCS system only requires expression of *CNBP* or *La* and engineering of the TOP module 5’ of the Kozak sequence of the target gene. Since the TOP-modified transgene can in principle be driven by any mammalian promoter, we expect the TCS technology to be amenable to tissue-specific translation control using tissue-specific promoters. In addition to its translation-controlling capacity, La has been found to boost titers of proteins which are either encoded in a classical cap-dependent configuration or translated via a polioviral IRES element. With the assets of controlling and boosting translation of desired transgenes, *CNBP*- and *La*-based metabolic engineering may become of central interest for a variety of applications, including functional genomics, biopharmaceutical manufacturing, gene therapy, and tissue engineering.

ACKNOWLEDGEMENTS

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REFERENCES


CHAPTER 6

MODULATION OF TRANSLATION-INITIATION IN CHO-K1 CELLS BY RAPAMYCIN-INDUCED HETERODIMERIZATION OF ENGINEERED EIF4G FUSION PROTEINS

**ABSTRACT**

Translation-initiation is a predominant checkpoint in mammalian cells which controls protein synthesis and fine-tunes the flow of information from gene to protein. In eukaryotes, translation-initiation is typically initiated at a 7-methyl-guanylic acid cap posttranscriptionally linked to the 5’ end of mRNAs. Alternative cap-independent translation-initiation involves 5’ untranslated regions (UTR) known as internal ribosome entry sites, which adopt a particular secondary structure. Translation-initiating ribosome assembly at cap or IRES elements is mediated by a multi-protein complex of which the initiation factor 4F (eIF4F) consisting of eIF4A (helicase), eIF4E (cap-binding protein) and eIF4G is a major constituent. eIF4G is a key target of picornaviral protease 2A, which cleaves this initiation factor into eIF4G∆ and ∆eIF4G to redirect the cellular translation machinery exclusively to its own IRES-containing transcripts. We have designed a novel translation control system (TCS) for conditional as well as adjustable translation of cap- and IRES-dependent transgene mRNAs in mammalian cells. eIF4G∆ and ∆eIF4G were fused C- and N-terminally to the FK506-binding protein (FKBP) and the FKBP-rapamycin-binding domain (FRB) of the human FKBP-rapamycin-associated protein (FRAP), respectively. Rapamycin-induced heterodimerization of eIF4G∆-FKBP and FRB-∆eIF4G fusion proteins reconstituted a functional chimeric elongation factor 4G in a dose-dependent manner. Rigorous quantitative expression analysis of cap- and IRES-dependent SEAP- (human placental secreted alkaline phosphatase) and luc- (Photinus pyralis luciferase) encoding reporter constructs confirmed adjustable translation control and revealed increased production of desired proteins in response to dimerization-induced heterologous eIF4G in Chinese hamster ovary (CHO-K1) cells.

**INTRODUCTION**

Translation is the final step in the conversion of genetic information to protein and a key checkpoint for controlling protein synthesis. Modulation of mRNA translation enables mammalian cells to rapidly adjust protein production in the absence of mRNA synthesis, processing or export (Kaufman, 1994). Translation-initiation is the rate-limiting step on which internal and external signals impinge to control assembly of a translation-competent initiation factor-containing multi-protein complex at the 5’ end of target mRNAs (Gray and Wickens, 1998; Kozak, 2001; Morley, 2001).
Initiation of translation and protein synthesis in mammalian cells is a complex multi-step process involving eukaryotic initiation (eIF) and elongation factors (EF) most of which are oligoprotein complexes. To date, more than eleven initiation factors orchestrate the first steps in protein synthesis (Hershey, 1991; Gingras et al., 2001; Dever, 2002): (i) assembly of a ternary complex between initiation factor eIF2, GTP and initiator methionyl tRNA (Met-tRNAi); (ii) eIF2-directed binding of the ternary complex to the 40 S ribosomal subunit to form the 43 S complex; (iii) eIF3 contained in this 43 S pre-initiation complex mediates its binding to the 5’ end of mRNA via interaction with the cap-binding initiation factor 4F (eIF4F); (iv) AUG scanning followed by (v) joining of the large 60 S ribosomal subunit, which contains the peptidyl-transferase active site required for a translation-competent 80 S ribosome (Kaufman, 1994; Morley, 2001; Pestova et al., 2001).

Mammalian eIF4F consists of three subunits: eIF4A, eIF4E and eIF4G (Gray and Wickens, 1998; Gingras et al., 2001). eIF4A-associated helicase activity resolves secondary mRNA structures in an ATP-dependent manner (Hershey, 1991; Gale et al., 2000) and the cap-binding protein eIF4E harbors the binding site for the cap structure (Zhang et al., 1994; Rau et al., 1996). Two eIF4G isoforms (previously referred to as eIF-4γ or p220), eIF4GI (Yan et al., 1992) and eIF4GII (Gradi et al., 1998a), an eIF4GI homologue harboring a 158 amino acids extension at its N-terminus, function as bridge proteins and tether 40 S-bound eIF3 to the cap-binding protein eIF4E (Imataka and Sonenberg, 1997). Lamphear and coworkers have shown that eIF4E interacts with the N-terminal domain of eIF4GI, whereas eIF4A and eIF3 bind to its C-terminus (Lamphear et al., 1995). In addition, eIF4GII has the exclusive capacity to interact with poly(A)-bound PABP (poly(A)-binding protein) and stimulate, following a supposed closed-loop translation model, translation-initiation by facilitating 5’- 3’ interactions of target mRNAs (Imataka et al., 1998; Joachims et al., 1999; Wakiyama et al., 2000).

Devoid of their own translation machinery, some viruses have evolved so-called cap-independent translation-initiation strategies to ensure efficient synthesis of viral proteins by the translation machinery of the infected host cell (Jackson and Wickens, 1997; Aranda and Maule, 1998; Gale et al., 2000). In many viruses cap-independent translation-initiation is mediated by internal ribosome entry sites (IRES) contained in the 5’ untranslated regions (UTR) of viral transcripts, which form ribosome assembling complexes with specific host proteins (Johannes and Sarnow, 1998; Pestova et al., 2001). Such IRES-mediated translation-initiation does not require a free 5’ end and was first discovered in picornaviruses (Jackson and Kaminski, 1995). Most of the cellular initiation factors, including eIF4F, are essential for
IRES-mediated translation-initiation (Jackson, 1996). A key event of picornaviral take-over of target cells is the complete shut-down of cellular protein synthesis accompanied by exclusive production of viral proteins six to twelve hours post infection (Belsham and Sonenberg, 1996). This major shift from cap-dependent to cap-independent translation-initiation is mediated by the viral protease 2A, which cleaves the eIF4G (eIF4G\textsuperscript{I} and eIF4G\textsuperscript{II}) subunit of the eIF4F holoenzyme complex thereby preventing cap-dependent translation of host mRNAs and redirecting the remaining cellular translation machinery entirely to IRES-containing transcripts and production of viral proteins (Borman et al., 1997; Bovee et al., 1998). Protease 2A-mediated cleavage of eIF4G\textsuperscript{I} (TLSTR\textsuperscript{↓}GP) and eIF4G\textsuperscript{II} (TPGGR\textsuperscript{↓}GV) produces N-terminal fragments eIF4G\textsuperscript{ΔI} and eIF4G\textsuperscript{ΔII}, which remain bound to the eIF4E-cap complex, and the C-terminal fragments eIF4G\textsuperscript{ΔI} and eIF4G\textsuperscript{ΔII}, which continue to interact with eIF3 and the helicase eIF4A (Jackson and Wickens, 1997). Ohlmann and coworkers have reported that eIF4A-bound eIF4G\textsuperscript{ΔI} or eIF4G\textsuperscript{ΔII} stimulates IRES-mediated translation of viral transcripts. Thus, protease 2A-mediated cleavage of eIF4G represents the central irreversible molecular switch in the transition from cellular to viral protein production (Ohlmann et al., 1996; Gradi et al., 1998a; Kolupaeva et al., 1998; Novoa and Carrasco, 1999; Svitkin et al., 1999).

Based on molecular events mimicking picornaviral infection, we have designed a novel heterologous mammalian translation control system (TCS). This system capitalizes on tetracycline-adjustable expression of the two cleavage products eIF4G\textsuperscript{ΔI} / eIF4G\textsuperscript{ΔII} and eIF4G\textsuperscript{ΔI} / eIF4G\textsuperscript{ΔII} fused to triplicate FKBP (FK506-binding protein; eIF4G\textsuperscript{ΔI}-FKBP\textsubscript{3} / eIF4G\textsuperscript{ΔII}-FKBP\textsubscript{3}) and to FRB (FKBP-rapamycin binding domain; FRB; FRB\textsuperscript{ΔI} / FRB\textsuperscript{ΔII}-eIF4G), respectively (Bierer et al., 1990; Brown et al., 1994; Chen et al., 1995; Choi et al., 1996). Addition of rapamycin results in chemically induced heterodimerization of FKBP\textsubscript{3} / FRB'-containing fusion proteins and reconstitution of functional chimeric eIF4G in a dose-dependent and reversible manner (Rivera et al., 1996; Amara et al., 1997; Clackson, 1997; Magari et al., 1997; Harvey and Caskey, 1998; Rollins et al., 2000; Pollock et al., 2002). Tetracycline-adjustable overexpression of eIF4G-derived fusion proteins combined with rapamycin-induced heterodimerization of functional chimeric eIF4G enabled unmatched translation control of cap- and IRES-driven target mRNAs as well as increased production of encoded product proteins.

We expect translation engineering to generate therapeutic impact by adjusting cellular translation bottlenecks towards optimized production of desired proteins in current biopharmaceutical manufacturing, gene therapy and tissue engineering scenarios.
MATERIALS AND METHODS

mRNA Isolation and RT-PCR Technology

4 \times 10^6 HeLa cells were lysed in 1 ml denaturing solution (4 M guanidine thiocyanate, 25 mM sodium citrate, pH 7, 0.5% N-lauroylsarcosine, 0.1 M 2-mercaptoethanol). After addition of 50 µl acetate solution (2 M sodium acetate, pH 4), 500 µl phenol solution (75% phenol, 25% H₂O), 100 µl chloroform-isoamyl alcohol (98% chloroform, 2% isoamyl alcohol) the sample was mixed and incubated on ice for 15 min prior to centrifugation for 20 min at 10’000*g and 4ºC. Incubation of the aqueous phase with 1 ml isopropanol at -20ºC for 1 h was followed by additional centrifugation for 20 min at 10’000*g and 4ºC. The pellet was then dissolved in 300 µl denaturing solution and again precipitated by addition of 300 µl isopropanol and incubation at -20ºC for 1 h. After centrifugation for 20 min at 4ºC, the pellet was resuspended in 500 µl ice-cold 75% ethanol and centrifuged again for 5 min at 4ºC. This washing step was repeated twice before the pellet was finally dissolved in 50 µl RNase-free water.

The reverse transcription polymerase chain reaction (RT-PCR) was performed by using the OneStep RT-PCR kit according to the manufacturer’s protocol (cat# 210210, QIAGEN, Valencia, CA).

Vector Constructions (see Table 1)

pCS4, pCS5, pCS9, pCS21 and pCS43: RT-PCR-based amplification of the coding sequences of ‘FKBP’ (‘: missing start (ATG), stop (TGA) codon), ‘FKBP, FRB’, eIF4G-II (nt 1-2076, Met¹-Arg⁶⁹² (Gradi et al., 1998a)) and eIF4G-II-HpaI (nt 1-2775, Met¹-Leu⁹²⁵) from HeLa total RNA using oligonucleotides OCS7 (gatcctagaAGTGCAGGTGGAAACCAT, BlnI site in bold, annealing sequence in capital letters) and OCS8 (gategetagTTTAGAAGCTCCACATCGAAGACG, NheI site in bold, annealing sequence in capital letters) (‘FKBP’) or OSC9 (gatcctagaCTCCACATCGAAGACG; MluI, NotI and EcoRI sites in bold, stop codon underlined, annealing sequence in capital letters) (‘FKBP’), OCS10 (gategetagTTTAGAAGCTCCACATCGAAGGCCT; PacI, NotI, EcoRI sites in bold, Kozak sequence underlined, annealing sequence in capital letters) and OCS11 (gategetagTTTAGAAGCTCCACATCGAAGACAT; NheI site in bold, annealing sequence in capital letters) (FRB), as well as OCS14
Plasmids used in this study: CITE* (optimized, eukaryotic cap-independent translation enhancer element from encephalomyocarditis virus), ΔΙ elF4G / ΔΙΙ elF4G / ΔΙΙΙ elF4G; human elongation initiation factor 4G, nt 1-1455 / 1-2076 / 1-2775, Met 1-Arg485 / Met 1-Arg692 / Met 1-Leu925), ΔΙ elF4G / ΔΙ II elF4G / ΔΙΙ elF4G-HpaI (nt 1-1455 / 1-2076 / 1-2775, Met 1-Arg485 / Met 1-Arg692 / Met 1-Leu925), ΔΙ elF4G / ΔΙ II elF4G (nt 1-4215 / 1-4758, Met 1-Asn1404 / Met 1-Asn1585), 'FKBP' (‘ ‘; missing start (ATG), stop (TGA) codon), 'FKBP', 'FKBP'; ('FKBP'-'FKBP'), 'FKBP', ('FKBP'- 'FKBP'- 'FKBP') (FKBP; human FK506-binding protein), 'FRB' (FRB; FKBP-rapamycin binding domain of human FRAP (FKBP-rapamycin-associated protein), HA (hemagglutinin A epitope tag), IRESI and IRESII (eukaryotic internal ribosomal entry site from poliovirus), luc (Photinus pyralis firefly luciferase), myc (c-myc oncoprotein epithope tag), pABGH (eukaryotic polyadenylation sequence from bovine growth hormone), pA SV40 (eukaryotic polyadenylation sequence from simian virus 40), P CMV (constitutive, eukaryotic promoter from cytomegalovirus), P hCMV*-1 (tetracycline-regulatable, eukaryotic promoter derived from cytomegalovirus), P lacZ (constitutive, prokaryotic promoter derived from the lacZ gene), P MPSV (constitutive, eukaryotic promoter derived from myeloproliferative sarcoma virus), P SV40 (constitutive, eukaryotic promoter from simian virus 40), pur (puromycin resistance conferring gene), SEAP (human secreted alkaline phosphatase gene), u-PA LMG (human low molecular weight urokinase-type plasminogen activator gene).
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<td>Fussenegger et al., 1998a</td>
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Note: EcoRI sites in bold, stop codon underlined, annealing sequence in capital letters.
(\textsuperscript{ΔII}eIF4G) followed by cloning into pcDNA3.1/V5/His-TOPO resulted in plasmids pCS3 (PCMV-\textsuperscript{ΔII}eIF4G-pABGH), pCS10 (PCMV-\textsuperscript{ΔII}eIF4G-pABGH) and pCS22 (PCMV-\textsuperscript{ΔII}eIF4G-pABGH).

**pCS6 and pCS7:** pCS7 (PCMV-‘FKBP3-pABGH) was cloned in a two step procedure: (i) pCS4 (PCMV-‘FKBP-pABGH) was restricted with BlnI-DraIII and the ‘FKBP’ fragment was fused to the 3’ end of ‘FKBP’, contained in the Nhel-DraIII-restricted pCS4 to result in pCS6 (PCMV-‘FKBP’-pABGH). (ii) The ‘FKBP-containing BlnI-DraIII fragment of pCS5 (PCMV-‘FKBP-pABGH) was ligated 3’ to the ‘FKBP’-encoding Nhel-DraIII fragment of pCS6.

**pCS8 and pCS23:** Plasmids pCS8 (PCMV-eIF4G\textsuperscript{ΔΙ}-‘FKBP3-pABGH) and pCS23 (PCMV-eIF4G\textsuperscript{ΔΙΙ}-‘FKBP3-pABGH) encoding the eIF4G-derived fusion proteins were assembled by ligation of the BlnI-PmeI fragment of pCS7 (‘FKBP3) with the Nhel-PmeI fragment of pCS3 (eIF4G\textsuperscript{ΔΙ}) or pCS21 (eIF4G\textsuperscript{ΔΙΙ}).

**pCS35 and pCS37:** In order to enable immunodetection of eIF4G\textsuperscript{ΔΙ}-‘FKBP3 (pCS8) and eIF4G\textsuperscript{ΔΙΙ}-‘FKBP3 (pCS23) they were fused by PCR to a Hemagglutinin A epitope tag (YPYDVPDYA (Kolodziej and Young, 1991)) using oligonucleotides OCS16 (gateATTAACGCCGCGCtATTCCA; NotI site in bold, beginning of Kozak sequence underlined, annealing sequence in capital letters) or OCS20 (gategcgggcgcCCACCATGAACACGCTTCT; NotI site in bold, Kozak sequence underlined, annealing sequence in capital letters) and OCS17 (gategcgcgcgcgtagcataatctggaactataggatatAGCTTTAGAAGCTCCACATCGAA; NotI site in bold, stop codon underlined, HA epitope sequence in italics, annealing sequence in capital letters). The resulting fragments were cloned into pcDNA3.1/V5/His-TOPO vectors to give plasmids pCS35 (PCMV-eIF4G\textsuperscript{ΔΙ}-‘FKBP3-HA-pABGH) and pCS37 (PCMV-eIF4G\textsuperscript{ΔΙΙ}-‘FKBP3-HA-pABGH).

**pCS11 and pCS24:** Similarly, pCS11 (PCMV-FRB\textsuperscript{ΔΙ}eIF4G-pABGH) and pCS24 (PCMV-FRB\textsuperscript{ΔΙΙ}eIF4G-pABGH) were constructed by fusion of the Nhel-DraIII fragment of pCS9 (FRB) with the BlnI-DraIII fragment of pCS10 (\textsuperscript{ΔΙ}eIF4G) or pCS22 (\textsuperscript{ΔΙΙ}eIF4G).

**pCS36 and pCS38:** PCR-mediated fusion of the human c-myc epitope tag (EQKLISEEDL (Kolodziej and Young, 1991)) to FRB\textsuperscript{ΔΙ}eIF4G or FRB\textsuperscript{ΔΙΙ}eIF4G was performed by using oligonucleotides OCS18 (gatecctaattaaGAATTCCACCATGGAGATGTGG; PacI, EcoRI sites in bold, Kozak sequence underlined, annealing sequence in capital letters) and OCS19 (gategcgggtgatatcagacatatctactggagatatAGCTTGGAGCTCCACATCGAA; MluI, EcoRI sites in bold, stop codon underlined, c-myc epitope sequence in italics, annealing sequence in capital letters) or OCS21...
**Chapter 6**

(gatcagcgtgaattctcacagatcttcttgagatgagttttgttcGTTGTGGTCAGACTCCTCCT; MluI, EcoRI sites in bold, stop codon underlined, c-myc epitope sequence in italics, annealing sequence in capital letters). The corresponding pcDNA3.1/V5/His-TOPO-derived plasmids were designated pCS36 (P<sup>CMV</sup>-FRB<sup>ΔΙ</sup>eIF4G-myc-p<sub>ABGH</sub>) and pCS38 (P<sup>CMV</sup>-FRB<sup>ΔIΙ</sup>eIF4G-myc-p<sub>ABGH</sub>).

**pCS40:** The coding sequence of full-length eIF4GI (nt 1-4215, Met<sup>1</sup>-Asn<sup>1404</sup>) contained in pSK(-)-HFC1 was fused to a c-myc epitope tag by PCR using oligos OCS5 (gcttaattaaggccgeggaattctcacCATGTCTGGGGCCGC; PacI, NotI, EcoRI sites in bold, Kozak sequence underlined, annealing sequence in capital letters) and OCS19 (gatcagcgtgaattctcacagatcttcttgagatgagttttgttcGACTCCTCCTCTGACTTCAC; MluI, EcoRI sites in bold, stop codon underlined, c-myc epitope sequence in italics, annealing sequence in capital letters). Insertion of the aforementioned PCR fragment into pcDNA3.1/V5/His-TOPO resulted in plasmid pCS40 (P<sup>CMV</sup>-eIF4GI-myc-p<sub>ABGH</sub>).

**pCS47:** pCS47 (P<sup>CMV</sup>-eIF4GII-myc-p<sub>ABGH</sub>) encoding myc-tagged full-length eIF4GII (nt 1-4758, Met<sup>1</sup>-Asn<sup>1585</sup>) was assembled by ligation of the HpaI-IIeIF4G-encoding SspI/HpaI fragment of pCS38 with the corresponding SspI/HpaI fragment (eIF4GII-HpaI) of pCS43.

**pSS106, pSS113 and pSS123:** Construction of the prokaryotic cloning vectors pSS106, encoding the puromycin resistance conferring gene (<i>pur</i>), pSS113 harboring the SEAP gene as well as the eukaryotic expression vector pSS123 containing the low molecular weight urokinase-type plasminogen activator (<i>u-PALMW</i>) have been described before (Schlatter and Fussenegger, 2002; Schlatter et al., 2002).

**pSS157:** <i>luc</i> (<i>Photinus pyralis</i> firefly luciferase) was excised from pGL3-Basic (cat# E1751, Promega, Madison, WI) by <i>NheI</i>/<i>XbaI</i> and cloned in antisense orientation into the <i>XbaI</i> site of pBluescriptII KS<sup>+</sup> (cat# 212208, Stratagene, La Jolla, CA).

RT-PCR and PCR products, as well as all gene fusions were sequenced to confirm correct junctions and open reading frames (ORFs).

**Cell Culture and Transfections**

CHO-XMK1<sub>9</sub> are Chinese hamster ovary cells (CHO-K1, ATCC: CCL 61) stably transfected with the tTA- (tetracycline-dependent transactivator) encoding plasmid pUHD15-1 (Gossen and Bujard, 1992) and pSV2neo (Clontech, Palo Alto, CA; no longer available) harboring the G418 resistance gene (Mazur et al., 1998).
CHO-XMK1₀ cells (30%-50% confluent) were transfected using an optimized protocol typically yielding transfection rates of over 30%. In brief, 10⁵ cells were transfected with a CaPO₄-DNA complex produced by drop-wise addition of 100 µl DNA solution (1.2 µg DNA, 0.25 M CaCl₂) to 100 µl 2* HBS solution (280 mM NaCl, 100 mM HEPES, 1.5 mM Na₂HPO₄, pH 7.1). After 16 hours the CaPO₄-DNA complex was removed and the cell monolayer washed with PBS prior to medium exchange and analysis at 72h transfection.

CHO-XMK1₀ and human cervical adenocarcinoma cells (HeLa; ATCC CCL-2) were cultivated in FMX-8 (Cell Culture Technologies, Zurich, Switzerland) or Dulbecco’s modified eagle medium (cat# 52100-039, Life Technologies AG, Basel, Switzerland) supplemented with 10% fetal calf serum (cat# A15-022, Lot# A01129-242, PAA Laboratories GmbH, Linz, Austria) and the appropriate antibiotics at 37°C in a humidified 5% CO₂ atmosphere.

**Western Blot and Immunoprecipitation Analysis**

Preparation of whole cell extracts was initiated by cell lysis in NP-40 extraction buffer (50 mM Heps, pH 7.4, 150 mM NaCl, 25 mM β-glycerophosphate, 25 mM NaF, 5 mM EGTA, 1 mM EDTA, 1% NP-40, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM PMSF, 1 mM sodium orthovanadate) for 10 min on ice. The lysates were clarified by centrifugation at 10000*g for 15 min.

Alternatively, for immunoprecipitations (cat# 17-6002-35, Immunoprecipitation Starter Pack, Amersham Pharmacia Biotech, Piscataway, NJ), equal amounts of total protein were incubated with myc-specific antibodies (cat# sc-40, mouse monoclonal (9E10), Santa Cruz Biotechnology Inc., Santa Cruz, CA) for 2 h on ice. Immune complexes were collected with 50 µl protein A- or protein G-Sepharose and washed three times with 1 ml of NP-40 extraction buffer and once with washing buffer (50 mM Tris, 140 mM NaCl, and 5 mM EDTA). Precipitated proteins were released from antibodies by boiling in 30 µl of sample buffer, subjected to SDS-PAGE and blotted onto a PVDF membrane.

Following blocking with 5% blocking reagent (cat# RNP 2108, ECL Western blotting analysis system, Amersham Pharmacia Biotech, Dubendorf, Switzerland) in TTBS (50 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween-20), filters were probed with antibodies specific for myc or HA (cat# sc-805, rabbit polyclonal HA-probe (Y-11), Santa Cruz Biotechnology Inc.) epitopes. Proteins were visualized by peroxidase-coupled secondary antibodies using the ECL detection system.
Immuno-fluorescence Microscopy

5*10^4 cells were seeded into chamber slides (Lab-Tek, Nunc, Inc., Naperville, IL) and grown for 24 h prior to transfection. 16 h post transfection cells were washed with PBS and cultivated for another 72 h in FMX-8 medium containing 400 µg/ml G418 and either 2.5 µg/ml tetracycline (+tet; repression of eIF4G variants encoded on the tricistronic units) or no tetracycline (-tet; induction of P_hCMV*-1-driven expression of eIF4G variants encoded on the tricistronic units). The cell monolayer was washed once with phosphate-buffered saline (PBS, Sigma) and submerged with 3% paraformaldehyde (PFA) solution in PBS for 15 min. Cells were washed again with PBS and submerged with 0.2% Triton X-100 for 10 min. After another washing step, the cells were immersed in 2% BSA in PBS for 15 min and subsequently incubated for 1 h with the antibodies specific for myc and HA epitopes. Thereafter, the cells were washed three times for 5 min with PBS before the secondary antibodies (cat# 115-075-146, goat anti-mouse IgG conjugated to TR (Texas Red) or cat# 111-095-144, goat anti-rabbit IgG conjugated to FITC (Fluorescin Isothiocyanate), Jackson ImmunoResearch Laboratories, West Grove, PA) were applied for 1 h. Cells were then washed again three times for 5 min with PBS before they were fixed with embedding medium (70% (v/v) glycerol, 30 mM Tris-HCl, pH 9.5, 240 mM n-propyl gallate) and examined under a fluorescence microscope (Leitz DMRB, Leica, Bannockburn, IL).

Colorimetric Assay for Quantification of Low Molecular Weight Urokinase-Type Plasminogen Activator (u-PALMW)

72 h post transfection cell culture supernatants of cells grown in repression (+tet; repression of P_hCMV*-1-responsive transcription) or induction (-tet; induction of P_hCMV*-1-responsive transcription) medium were assayed for u-PA_LMW activity using a previously described chromogenic assay (Schlatter et al., 2002).

Chemiluminescence-Based SEAP Quantification

For SEAP quantification cell culture supernatant was centrifuged for 2 min at 10000*g. 25 µl of the supernatant were prepared for chemiluminescence-based quantification of SEAP according to the manufacturer’s protocol (Great EscAPe™ SEAP reporter system 2, chemiluminescent, Roche Biochemicals. Basel, Switzerland). SEAP activity was measured during 10 s using a Turner Designs Luminometer (TD20-20, Turner Designs, Sunnyvale, CA). The readout in relative light units per second (RLUs^-1) is directly proportional to the
amount of SEAP in the culture supernatant. Average SEAP productions (including standard deviations) were determined in triplicate (RLUs$^{-1}l^{-1}10^8$).

**Quantitative Chemiluminescence Assay for Luciferase (luc)**

Concomitant with SEAP, the luciferase activity of whole cell extracts was quantified using a modified chemiluminescent assay (cat# BC100L, Luciferase Assay Kit, Tropix, Bedford, MA). Cells were harvested and washed two times in PBS and lysed by addition of 500 µl lysis buffer (100 mM K$_2$PO$_4$, 0.2% Triton X-100, 1 mM DTT). The lysates were centrifuged for 2 min at 10000*g and 100 µl of the cell free lysates were added to 100 µl of substrate A. Within 10 min 100 µl of substrate B were injected. The enzyme activity was immediately measured during 10 s using a Turner Designs Luminometer. Average luc productions (including standard deviations) were determined in triplicate (RLUs$^{-1}l^{-1}10^6$).

**Characterization of the Growth Behavior of Transiently Transfected Cells**

Parallel to quantification of SEAP and luc production, the average viable cell number of all cell cultures was measured in triplicate. Cells were detached using dissociation solution (cat# C-5914, Cell Dissociation Solution, Sigma Chemical Co., St. Louis, MO) and resuspended in filter-purified casyton buffer (pH 7.3; 7.93 g/l NaCl; 0.38 g/l Na$_2$EDTA; 0.4 g/l KCl; 0.19 g/l NaHPO$_4$; 1.95 g/l Na$_2$H$_2$(PO$_4$)$_2$; 0.3 g/l NaF). The viable cell number (Cell$^{-1}$l$^{-1}10^9$) was determined as the average of three independent readings taken by a cell counter (Casy1®, Scharfe System, Reutlingen, Germany).

**RESULTS**

**Construction of Eukaryotic Expression Vectors**

**pCS50, pCS53, pCS55 and pCS57:** The tricistronic expression vectors pCS50, pCS53, pCS55 and pCS57, encoding either of the fusion proteins $FRB^-\Delta I eIF4G$-myc (FRB; FKBP-rapamycin binding domain of human FRAP (FKBP-rapamycin-associated protein) (Rivera et al., 1996), $\Delta I eIF4G$; human elongation initiation factor 4G, nt 1456-4215, Gly$^{486}$-Asn$^{1404}$ (Yan et al., 1992), $myc$; c-myc epitope tag (Kolodziej and Young, 1991)) or $FRB^-\Delta II eIF4G$-myc ($\Delta II eIF4G$; nt 2077-4758, Gly$^{643}$-Asn$^{1585}$ (Gradi et al., 1998a)), $eIF4G^{\Delta II} FKBP3$-HA ($eIF4G^{\Delta II}$, nt 1-1455, Met$^1$-Arg$^{485}$, FKBP; human FK506-binding protein (Rivera et al., 1996), $HA$;
hemagglutinin A epitope tag (Kolodziej and Young, 1991) or eIF4G\(^{AI\text{-}}\)‘FKBP\(_3\)-HA (eIF4G\(^{AI\text{ll}}\), nt 1-2076, Met\(^1\)-Arg\(^{692}\)) and the reporter gene low molecular weight urokinase-type plasminogen activator (\(u-PALMW\) (Langer et al., 1995)) in different configurations, were assembled following multi-step cloning procedures.

pCS50 and pCS53: (i) pCS48 / pCS51 was constructed by ligation of the FRB\(^{\text{-}AI}\)eIF4G-myc- / FRB\(^{\text{-}AII}\)eIF4G-myc-encoding EcoRI fragment of pCS36 / pCS38 into the first cistron of pTRIDENT3 (EcoRI; (Fussenegger et al., 1998a) (P\(_{\text{BCMV+1}}\)FRB\(^{\text{-}AI}\)eIF4G-myc-IRES-CITE*-pASV40 / P\(_{\text{BCMV+1}}\)FRB\(^{\text{-}AII}\)eIF4G-myc-IRES-CITE*-pASV40). (ii) Subsequently, the eIF4G\(^{AI\text{-}}\)‘FKBP\(_3\)-HA- / eIF4G\(^{AII\text{-}}\)‘FKBP\(_3\)-HA-containing Not\(_I\) fragment of pCS35 / pCS37 was cloned into the second cistron (Not\(_I\)) of pCS48 / pCS51 to give pCS49 / pCS52 (P\(_{\text{BCMV+1}}\)FRB\(^{\text{-}AI\text{ll}}\)eIF4G-myc-IRES-eIF4G\(^{AI\text{-}}\)‘FKBP\(_3\)-HA-CITE*-pASV40 / P\(_{\text{BCMV+1}}\)FRB\(^{\text{-}AII\text{ll}}\)eIF4G-myc-IRES-eIF4G\(^{AII\text{-}}\)‘FKBP\(_3\)-HA-CITE*-pASV40). (iii) Finally, the reporter gene \(u-PALMW\) was excised from pSS179 (Schlatter et al., 2002) as EcoRV fragment and inserted into the SwaI site of the third cistron of pCS49 / pCS52 thereby resulting in the final tetracycline-regulatable, tricistronic, eukaryotic expression vector pCS50 / pCS53 (P\(_{\text{BCMV+1}}\)FRB\(^{\text{-}AI\text{ll}}\)eIF4G-myc-IRES-eIF4G\(^{AII\text{-}}\)‘FKBP\(_3\)-HA-CITE*-u-\(PA_{LMW}\)-pASV40) (Table 1 and Fig. 1).

pCS55 and pCS57: (i) Ligation of the FRB\(^{\text{-}AII}\)eIF4G-myc- / FRB\(^{\text{-}AI\text{ll}}\)eIF4G-myc-encoding Pac\(_I\)/MluI fragment of pCS36 / pCS38 into the corresponding sites (Pac\(_I\)/MluI) of the third

Fig. 1: Schematic representation of key expression units. All eukaryotic expression plasmids are derived from pTRIDENT4, pTRIDENT3, pTRIDENT1 (Fussenegger et al., 1998a) or pcDNA3.1/V5/His-TOPO (Invitrogen) and contain a tetracycline-responsive promoter (P\(_{\text{BCMV+1}}\)) (Gossen and Bujard, 1992)) or a constitutive simian virus 40 promoter (P\(_{\text{ASV40}}\)), a polyadenylation site derived from simian virus 40 (P\(_{\text{ASV40}}\)) or bovine growth hormone (P\(_{\text{BGH}}\)) and either eukaryotic internal ribosomal entry sites of poliovirus (IRES) or optimized eukaryotic cap-independent enhancer elements of encephalomyocarditis virus (CITE*). The tricistronic vectors contain reporter genes encoding the intracellular firefly luciferase from *Photinus pyralis* (luc (Gould and Subramani, 1988)), the human placental secreted alkaline phosphatase (SEAP (Berger et al., 1988)), human low molecular weight urokinase-type plasminogen activator (\(u-PALMW\) (Langer et al., 1995)) and a selection marker conferring puromycin resistance (\(pur\) (de la Luna et al., 1988)). The fusion proteins consist of domains derived from human elongation initiation factor 4G (eIF4G\(^{\text{G}}\) / eIF4G\(^{\text{H}}\)), human FK506-binding protein (FKBP (Rivera et al., 1996), FKBp-rapamycin binding domain of human FRAP (FKBP-rapamycin-associated protein) (FRB (Rivera et al., 1996)), and epitope tags derived from Hemagglutinin A (HA (Kolodziej and Young, 1991)) or c-myc oncogene (myc (Kolodziej and Young, 1991)). Sites specific for restriction endonucleases: A, AscI; B, BclI; B\(_2\), BglII; B\(_3\), BsoHI; C, ClaI; E, EcoRI; E\(_2\), EcoRV; F, FseI; H, HindIII; M, MluI; N, NaeI; N\(_2\), NotI; P, PacI; P\(_2\), PmeI; S, Sall; S\(_2\), SbfI; S\(_3\), SgfI; S\(_4\), SpeI; S\(_5\), SrfI; X, XbaI; X\(_2\), XhoI; X\(_3\), XmnI.
cistron of pSS123 (Schlatter and Fussenegger, 2002) resulted in plasmid pCS56 / pCS54 (P_{hCMV*-1-u-PA_{LAMW}}-IRESI-IRESI-FRB^-\Delta eIF4G-myc-pASV40 / P_{hCMV*-1-u-PA_{LAMW}}-IRESI-IRESI-FRB^-\Delta eIF4G-myc-pASV40). (ii) Subsequently, the eIF4G^-\Delta FKBP3-HA- / eIF4G^-\Delta FKBP3-HA-containing NotI fragment of pCS35 / pCS37 was ligated into the NotI site of the second cistron of pCS56 / pCS54 thereby resulting in the final tetracycline-regulatable, tricistronic, eukaryotic expression vector pCS57 / pCS55 (P_{hCMV*-1-u-PA_{LAMW}}-IRESI-eIF4G^-\Delta FKBP3-HA-IRESI-FRB^-\Delta eIF4G-myc-pASV40 / P_{hCMV*-1-u-PA_{LAMW}}-IRESI-eIF4G^-\Delta FKBP3-HA-IRESI-FRB^-\Delta eIF4G-myc-pASV40) (Table 1 and Fig. 1).
pCS60 and pCS62: The monocistronic, tetracycline-regulatable expression plasmids pCS60 (P*hCMV*-1-\(eIF4G^I\)-myc-pABGH) and pCS62 (P*hCMV*-1-\(eIF4G^II\)-myc-pABGH), harboring myc-tagged full-length \(eIF4G^I\) / \(eIF4G^II\) (nt 1-4215 / 1-4758, Met^1-Asn\(^{1404}\) / Met^1-Asn\(^{1585}\)) were constructed by promoter swapping. The P_CMV-promoter encoded on the SspI/HindIII fragment of pCS40 (P_CMV-\(eIF4G^I\)-myc-pABGH) and pCS47 (P_CMV-\(eIF4G^II\)-myc-pABGH) was exchanged by the SspI/HindIII fragment of pTRIDENT3 harboring the tetracycline-responsive PhCMV*-1 promoter (Table 1 and Fig. 1).

pCS20: The constitutive, tricistronic, eukaryotic reporter and selection vector pCS20, encoding the genes for human placental secreted alkaline phosphatase (SEAP (Berger et al., 1988)), the Photinus pyralis firefly luciferase (\(luc\) (Gould and Subramani, 1988)) and the puromycin resistance-conferring gene (\(pur\) (de la Luna et al., 1988)) was assembled following a two-step cloning procedure. (i) Ligation of the \(pur\)-containing SpeI/EcoRV fragment of pSS106 (Schlatter and Fussenegger, 2002) into the SpeI/SwaI sites of the third cistron of pSS143 (Schlatter and Fussenegger, 2002) resulted in pCS14 (P_SV40-SEAP-IRES-CITE*-\(pur\)-pASV40). (ii) The SrfI/EcoRV fragment of pSS157 encoding the luciferase gene was ligated into the SrfI site of the second cistron of pCS14 thereby resulting in pCS20 (P_SV40-SEAP-IRES-\(luc\)-CITE*-\(pur\)-pASV40) (Table 1 and Fig. 1).

Western Blot Analysis of CHO Cells Transiently Expressing pCS50, pCS53, pCS55 or pCS57

In order to demonstrate functionality of designed expression vectors, the CHO-K1-derived cell line CHO-XMK1\(^9\) (Mazur et al., 1998) stably expressing the tetracycline-dependent transactivator (\(tTA\) (Gossen and Bujard, 1992)) required for tetracycline-adjustable expression of transgenes driven by the tetracycline-responsive promoter (P*hCMV*-1 (Gossen and Bujard, 1992)) was transiently transfected with each of the four plasmids pCS50, pCS53, pCS55 or pCS57, encoding different \(eIF4G\) domains. The cells were grown for 3 days in the absence of tetracycline (-tet; induction of P*hCMV*-1-responsive expression) prior to lysis and Western blot-mediated analysis of the respective protein extracts. myc- or HA-specific immunodetection of the four different fusion proteins FRB\(^+\)-\(\Delta^I\)\(eIF4G\)-myc, FRB\(^+\)-\(\Delta^II\)\(eIF4G\)-myc, \(\text{eIF4G}^\text{Al}\)-\(\text{FKBP}^\text{3-HA}\) and \(\text{eIF4G}^\text{Al}\)-\(\text{FKBP}^\text{3-HA}\) confirmed the integrity of the gene sequences as well as their functional expression (Figs. 2A and 2B).
Rapamycin-Induced Heterodimerization of Chimeric eIF4G

Functional rapamycin (rap)-induced heterodimerization of eIF4G-containing fusion proteins (eIF4GΔL-'FKBP3-HA-rap-FRB'-ΔelF4G-myc / eIF4GΔL-'FKBP3-HA-rap-FRB'-ΔelF4G-myc) was investigated by transient transfection of CHO-XMK19 with pCS50, pCS53, pCS55 or pCS57 followed by cultivation for 72 h in rapamycin-free (-rap; repression of dimerization) or -supplemented medium (+rap; 10 nM, induction of rapamycin-dependent FRB'-'FKBP3 dimerization). Whole cell extracts of transfected CHO-XMK19 were incubated with anti-myc antibodies resulting in immunoprecipitation of myc-tagged protein complexes.

Fig. 2: Western blot analysis of eIF4G-derived fusion proteins. The CHO-K1-derived cell line CHO-XMK19 (Mazur et al., 1998), stably expressing the tetracycline-dependent transactivator (tTA (Gossen and Bujard, 1992)) was transiently transfected with the tetracycline-responsive, tricistronic expression vectors pCS50, pCS53, pCS55 or pCS57. Whole cell extracts were used for myc- (Fig. 2A) or HA- (Fig. 2B) directed Western blot analysis. The size of FRB'-ΔelF4G-myc, FRB'-ΔelF4G-myc, eIF4GΔL-'FKBP3-HA and eIF4GΔL-'FKBP3-HA correspond to their deduced amino acid sequence.
Immunocomplexes were purified using Protein G Sepharose, separated by SDS-PAGE and further analyzed by HA tag-specific Western blotting. HA-specific immunodetection for all transfectants grown in the presence of rapamycin demonstrates functional rapamycin-responsive heterodimerization of the heterologous chimeric translation-initiation factor eIF4G (Fig. 3).

Tetracycline-Responsive Expression of Engineered eIF4G Derivatives

In order to ensure simultaneous, coordinated as well as tetracycline-responsive expression, all eIF4G-encoding tricistronic expression vectors (pCS50, pCS53, pCS55 or pCS57) were transfected into CHO-XMK19 cells and positive u-PA Lưu expression analyzed 72 h post transfection (data not shown). Tetracycline-responsive expression of eIF4G-encoding domains from pCS50, pCS53, pCS55 or pCS57 was confirmed by myc- (TR, texas red) and eIF4G^ΔΩ-FKBP3-HA and eIF4G^ΔΩ-FKBP3-HA correspond to their deduced amino acid sequence.

Fig. 3: Co-immunoprecipitation of eIF4GΔΙ^- FKBP3-HA / eIF4GΔΩ^- FKBP3-HA and FRB^-ΔΙ elf4G-myc / FRB^-ΔΩ elf4G-myc. CHO-XMK19 transiently transfected with plasmids pCS50, pCS53, pCS55 or pCS57 were grown in the absence (-rap; repression of rapamycin-responsive dimerization) or presence (+rap; induction of rapamycin-responsive dimerization) of 10 nM rapamycin. Cell extracts immunoprecipitated with anti-myc antibodies were visualized by HA-dependent Western blot analysis. The size of eIF4GΔΙ^- FKBP3-HA and eIF4GΔΩ^- FKBP3-HA correspond to their deduced amino acid sequence.

Fig. 4: Immunofluorescence-based characterization of CHO-XMK19 cells transiently expressing the fusion proteins FRB^-ΔΙ elf4G-myc / FRB^-ΔΩ elf4G-myc (red fluorescence; TR (Texas Red)), eIF4G^ΔΩ^- FKBP3-HA / eIF4G^ΔΩ^- FKBP3-HA (green fluorescence; FITC (Fluorescin Isothiocyanate)) or full-length eIF4GΔ^- myc / eIF4GΔ^- myc (red fluorescence; TR) encoded on plasmids pCS50, pCS53, pCS55, pCS57, pCS60 or pCS62. Cells were grown in the presence (+tet) or absence (-tet) of tetracycline, corresponding to repression or induction of the PmCMV^-I-driven expression units.
HA- (FITC, fluorescin isothiocyanate) specific immunofluorescence of transfected cells grown for 3 days in the presence (multicistronic expression unit repressed) or absence (multicistronic expression unit induced) of tetracycline. We have also analyzed in the same
way myc-tagged full-length eIF4G\textsubscript{I} / eIF4G\textsubscript{II} (pCS60 / pCS62) to provide isogenic immunofluorescence-based wildtype eIF4G expression controls. Cell-specific red / green staining of fluorescence micrographs shown in Fig. 4 confirmed simultaneous as well as tetracycline-responsive expression of FRB\textsuperscript{-ΔΙ}eIF4G-myc / FRB\textsuperscript{-ΔΙΙ}eIF4G-myc and eIF4G\textsuperscript{ΔΙ}-'FKBP\textsubscript{3}-HA / eIF4G\textsuperscript{ΔΙΙ}-'FKBP\textsubscript{3}-HA or eIF4G\textsuperscript{I}-myc / eIF4G\textsuperscript{II}-myc.

**Effect of Full-Length eIF4G Expression on Transgene Translation and Growth Characteristics of CHO Cells**

In order to determine whether ectopic expression of heterologous eIF4G\textsubscript{I} / eIF4G\textsubscript{II} increases cap-dependent and/or cap-independent translation-initiation, plasmid pCS20 (P\textsubscript{SV40}-SEAP-IRES-luc-CITE\textsuperscript{*}-pur-pA\textsubscript{SV40}) was either transfected alone as control or cotransfected with plasmids pCS60 (PhCMV*-1-eIF4G\textsubscript{I}-myc-pABGH) and pCS62 (PhCMV*-1-eIF4G\textsubscript{II}-myc-pABGH) into CHO-XMK1\textsubscript{9} cells. The reporter vector pCS20 harbors the tricistronic SEAP-luc-pur-encoding cassette in a constitutive, P\textsubscript{SV40}-driven expression configuration, whereas expression from plasmids pCS60 and pCS62 is controlled by the tetracycline-responsive promoter PhCMV*-1. In order to assess the effects of eIF4G\textsubscript{I}-myc / eIF4G\textsubscript{II}-myc expression on translation, the transfected cells were grown for 72 h in the presence (+tet; eIF4G\textsubscript{I}-myc / eIF4G\textsubscript{II}-myc expression repressed) or absence (-tet; eIF4G\textsubscript{I}-myc / eIF4G\textsubscript{II}-myc expression induced) of tetracycline. SEAP and luc titers, reflecting the efficiency of cap-dependent and -independent translation-initiation, were increased 6.2- / 5.3- and 5- / 7- fold upon removal of tetracycline (induction of eIF4G\textsubscript{I}-myc / eIF4G\textsubscript{II}-myc expression), respectively. By contrast, SEAP and luc titers of pCS20-only transfected control cells remained unchanged following removal of tetracycline from the cell culture medium. Similarly, neither the presence of tetracycline nor expression of eIF4G\textsubscript{I}-myc / eIF4G\textsubscript{II}-myc affected cell growth characteristics. These findings demonstrate the potential of full-length eIF4G to dramatically increase translation and

**Fig. 5:** Influence of eIF4G\textsubscript{I} and eIF4G\textsubscript{II} expression on cap-dependent SEAP and cap-independent luc expression as well as on the growth characteristics of CHO cell derivatives. Average SEAP (RLUs\textsuperscript{-1}\textsuperscript{-10\textsuperscript{-8}}) and luc (RLUs\textsuperscript{-1}\textsuperscript{-10\textsuperscript{-6}}) productivities, as well as cell numbers (Cells\textsuperscript{-1}\textsuperscript{-10\textsuperscript{-9}}) of CHO-XMK1\textsubscript{9} cells transiently expressing the constitutive reporter vector pCS20 and either of the tetracycline-responsive eIF4G expression plasmids pCS60 or pCS62. After cultivation of transfected cells for 72 h in the presence (+tet; repression of PhCMV*-1-responsive expression) or absence (-tet; induction of PhCMV*-1-responsive expression) of tetracycline, cell culture supernatants, cell extracts and dissociated cells were assayed in triplicate for SEAP and luc activity as well as for cell numbers. The solid lines indicate the relative production and growth (induction / repression).
attendant protein titers (Fig. 5).

<table>
<thead>
<tr>
<th>Plasmids Transfected</th>
<th>luc [RLUs·10⁻¹⁰]</th>
<th>SEAP [RLUs·10⁻¹⁰]</th>
<th>Cells [10⁹]</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCS20</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0.5</td>
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<tr>
<td>pCS60</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>pCS62</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

- **Repression**: +tet
- **Induction**: -tet
- **Induction / Repression**
Rapamycin-Induced Heterodimerization of Engineered eIF4G Domains and the Influence of Reconstituted eIF4G on Transgene Translation and Cell Growth

In order to evaluate the impact of different combinations of $\Delta I$eIF4G / $\Delta II$eIF4G domains on cap-dependent as well as cap-independent translation-initiation (Ohlmann et al., 1996) CHO-XMK19 cells were either transfected with pCS20 alone as control or together with plasmids pCS50, pCS53, pCS55 or pCS57. In this configuration, expression of the fusion proteins FRB′-$\Delta I$eIF4G-myc / FRB′-$\Delta II$eIF4G-myc and eIF4G$^{\Delta I}$-‘FKBP3-HA / eIF4G$^{\Delta II}$-‘FKBP3-HA can be adjusted in response to tetracycline. Furthermore, the aforementioned fusion proteins allow for rapamycin-inducible heterodimerization via ‘FKBP3 and FRB′ which results in reconstitution of heterologous translation-initiation factors eIF4G$^{\Delta I}$-‘FKBP3-HA-rap-FRB′-$\Delta I$eIF4G-myc and eIF4G$^{\Delta II}$-‘FKBP3-HA-rap-FRB′-$\Delta II$eIF4G-myc. In contrast to P$_{hCMV*1}$-driven transcription of eIF4G-derived domains from pCS50, pCS53, pCS55 and pCS57, expression of SEAP, luc and pur encoded on pCS20 is mediated by a constitutive P$_{SV40}$ promoter. Whereas translation of SEAP encoded in the first cistron of pCS20 is exclusively cap-dependent, translation-initiation of luc (second cistron) occurs in a cap-independent manner driven by the polioviral IRES element.

16 h post transfection the cells were cultivated in repression (+tet / -rap; repression of tetracycline-responsive transcription and rapamycin-dependent dimerization), induction (-tet / -rap; induction of tetracycline-responsive transcription and repression of rapamycin-dependent dimerization) or dimerization medium (-tet / +rap; induction of tetracycline-responsive transcription and rapamycin-dependent dimerization) for 72 h. The impact of
individual, dimerized eIF4G or full-length eIF4G on cap-dependent as well as cap-independent translation-initiation was determined by chemiluminescence-based SEAP and luc
Expression of the individual eIF4G domains upon removal of tetracycline (induction medium) resulted in 4.6- / 2.3- / 2- or 1.9- fold increased SEAP (cap-dependent translation) and 7.3- / 2.1- / 2.8- or 8.2- fold increased luc (cap-independent translation) protein titers for cells harboring pCS20 and either pCS50 / pCS53 / pCS55 or pCS57 compared to the same populations grown in repression medium. SEAP and luc production relying on cap-dependent and cap-independent translation, respectively, could be further increased following heterodimerization (dimerization medium) of individual eIF4G domains by a factor of 6.7 / 6 / 6 / 8.7 (SEAP: cap-dependent for pCS50 / pCS53 / pCS55 or pCS57) and 15.4 / 4.6 / 10.9 / 41.9 (luc: cap-independent for pCS50 / pCS53 / pCS55 or pCS57) compared to repressed conditions (growth in repression medium) (Fig. 6). For cotransfectants expressing pCS20 and either of the tricistronic expression vectors pCS50, pCS53, pCS55 or pCS57, the results show that withdrawal of tetracycline as well as addition of rapamycin increase SEAP as well as luciferase levels (Fig. 6). Control configurations based on pCS20-only transfection demonstrated that neither tetracycline withdrawal nor addition of rapamycin alone have any influence on SEAP or luc expression profiles (Fig. 6).

DISCUSSION

In recent years advances in disease-related gene-function analysis, therapeutic molecular interventions as well as biopharmaceutical manufacturing have in many cases been based on technology which enables stringent and adjustable transcription control of desired transgenes (Rivera et al., 1996; Wang et al., 1997; Fussenegger et al., 1998b; Harvey and Caskey, 1998; Liu et al., 1998; Umana et al., 1999; Clackson, 2000; Fussenegger, 2001). Yet controlling transcription of particular target genes is only one intervention mode on the way to eventually modulate protein titers in a desired way. The overall cellular production of a product protein results from integration of a sequence of events including (i) transcription, (ii) mRNA processing, (iii) mRNA stability, (iv) translation, (v) secretion, (vi) posttranslational modifications of proteins as well as (vii) their stability. Although overall protein production may be modulated in principle at any of these events, bioengineers have long been focussing on optimizing transcription while leaving other potential production bottlenecks largely unconsidered. However, due to its importance in viral infection, maternal effects and epigenetic phenomena, translation control of mRNA transcripts may be considered, besides transcription, a highly important checkpoint in the flow of genetic information from genes to
proteins, which integrates complex regulatory networks to provide a cell with desired proteins at the right time and concentration (Pyronnet and Sonenberg, 2001; Klahre et al., 2002). Also, translation control enables a cell to rapidly modulate protein production in the absence of transcription (Kaufman, 1994; Gray and Wickens, 1998; Kozak, 2001; Morley, 2001). Exploitation of these complex cellular transcription control scenarios to increase overall product titers, so-called translation engineering, is therefore in the immediate interest of the bioengineering community.

Due to its central role in linking the mammalian translation machinery to capped mRNA transcripts the eukaryotic translation-initiation factor eIF4G seems to be an obvious target for translation engineering. Two homologous mammalian eIF4G isoforms are known to date, eIF4GI and eIF4GII, which differ in a 158 amino acid extension at the N-terminus of eIF4GII (Yan et al., 1992; Gradi et al., 1998a). The longer eIF4GII isoform has been associated with binding to the poly(A)-binding protein (PABP) and was hypothesized to mediate translation in a poly(A)-dependent manner (Imataka et al., 1998). However, in our experimental settings eIF4GI and eIF4GII showed similar translation-initiation capacity suggesting their functional equivalence for translation engineering in CHO-K1-derived cell lines.

We have designed a novel translation control system (TCS) which is based on rapamycin-induced heterodimerization of two eIF4G domains transcribed in a tetracycline-responsive manner. The two eIF4G domains, eIF4GAI and eIF4GII (eIF4GAI and eIF4GII), typically occur in mammalian cells following protease 2A-mediated cleavage of this translation-initiation factor in the course of picornaviral infection (Gradi et al., 1998b). Whereas full length eIF4G is essential for cap-dependent as well as cap-independent translation-initiation the C-terminal cleavage product (eIF4GAI or eIF4GII) has been reported to be sufficient for increased cap-independent translation of IRES-containing transcripts; a fact that could be confirmed in our experimental set-up by an up to 42-fold increase of IRES-driven translation-initiation (see below) (Ohlmann et al., 1996; Pestova et al., 1996; Kolupaeva et al., 1998).

In order to reconstitute heterologous chimeric eIF4G from engineered protease 2A cleavage domains we have implemented the rapamycin-induced dimerization technology which heterodimerizes proteins fused to FKBP and FRB in a rapamycin-dependent manner (Bierer et al., 1990; Spencer et al., 1993; Brown et al., 1994; Chen et al., 1995; Choi et al., 1996; Rivera, 1998). Rapamycin is a clinically licensed small-molecule drug routinely used to prevent transplant rejection (Luengo et al., 1995; Liberles et al., 1997; Gonzalez et al., 2001).
Isogenic configurations used to evaluate eIF4G-based translation engineering included multicistronic expression vectors for tetracycline-responsive expression of eIF4G-derived fusion proteins as well as a tricistronic SEAP-luc-reporter construct for simultaneous and quantitative assessment of cap-dependent (SEAP) and cap-independent (luc) translation-initiation. Interestingly, expression of individual non-dimerized eIF4GΔI and ΔΙeIF4G or eIF4GΔII and ΔΙΙeIF4G increased cap-dependent (SEAP; 5- or 2-fold) as well as IRES-dependent (luc; 7- or 8-fold) translation upon coexpression of these eIF4G domains in the absence of tetracycline in the culture medium. Increased cap-dependent SEAP production by overexpression of non-dimerized eIF4GΔI and ΔΙeIF4G or eIF4GΔII and ΔΙΙeIF4G is at first sight surprising as the N-terminal eIF4G domain (eIF4GΔI / eIF4GΔII) has no documented function and the C-terminal eIF4G domain (ΔΙeIF4G / ΔΙΙeIF4G) no longer able to participate in the assembly of the translation-initiation complex at the cap. However, the transgenic C-terminal domains ΔΙeIF4G and ΔΙΙeIF4G have been shown to target an IRES element contained in the 5’ UTR of wildtype eIF4GΙ and may thus induce endogenous initiation factor 4GΙ which in turn increases cap-dependent SEAP translation of pCS20-based reporter transcripts (Johannes and Sarnow, 1998).

SEAP and luc titers could be further increased by rapamycin-mediated heterodimerization of eIF4GΔI and ΔΙeIF4G or eIF4GΔII and ΔΙΙeIF4G (SEAP; 7- or 9-fold and luc; 15- or 42-fold). Similarly, the titers of both model product proteins increased 6- or 7-fold upon expression of full-length eIF4GΙ or eIF4GΙΙ. However, in control configurations devoid of heterologous eIF4GΔI, ΔΙeIF4G, eIF4GΔII, ΔΙΙeIF4G, eIF4GΙ and eIF4GΙΙ neither SEAP / luc production nor cell growth was affected by tetracycline or rapamycin demonstrating that increased translation-initiation associated with ectopic expression of eIF4G derivatives was not related to the addition of these small molecule drugs.

Most notably, reconstituted chimeric eIF4G was not only more potent in promoting cap-dependent as well as cap-independent translation-initiation compared to monomeric eIF4G derivatives but also superior to heterologous wildtype eIF4G. Therefore, in addition to providing adjustable translation control in response to rapamycin (and tetracycline via transcription of eIF4G-derived fusion proteins) the engineered eIF4G derivatives pioneers a novel metabolic engineering strategy to significantly increase production of desired protein therapeutics.

With these combined assets of controlling as well as boosting translation of desired transgenes, eIF4G-based translation engineering may become a valuable tool for a variety of
applications, including functional genomics, biopharmaceutical manufacturing, gene therapy, and tissue engineering.

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CHAPTER 7

CONCLUSION
CONTROLLED PROLIFERATION

Reprogramming of the mammalian cell cycle to achieve bioprocess goals, such as cessation of proliferation at a high cell density to enable an extended production period, requires coordinated expression modulation of multiple genes.

In chapter 2, inducible cell-cycle arrest of Chinese hamster ovary (CHO) cells was achieved by tetracycline-responsive expression of either the cyclin-dependent kinase inhibitors (CKI) \( p21 \) or \( p27 \) in combination with the CCAAT-enhancer binding (CEBP/\( \alpha \)) or the apoptosis-suppressing gene \( bcl-x_L \). One-gene genetic engineering of CHO cells with \( p21 \) in previous experiments were insufficient to promote proliferation control (Fussenegger et al., 1997). A stable CHO cell line was therefore established that additionally harbored the CEBP/\( \alpha \) gene sequence, reported to induce transcription of \( p21 \) and to stabilize it at the protein level (Timchenko et al., 1996). Productivity and growth assays of CEBP/\( \alpha \)- and \( p21 \)-expressing cell lines confirmed the beneficial effect of CEBP/\( \alpha \) on \( p21 \) levels and proliferation control. The increased capacity of non-dividing cells to produce proteins was impressively shown by 10-15-fold elevated secreted alkaline phosphatase (SEAP) reporter protein levels in the cell culture supernatant.

An even more pronounced growth arrest with up to 30-fold higher productivity was achieved by coexpression of \( p27 \) and \( bcl-x_L \). In contrast to \( p21 \), \( p27 \) overexpression has already proven to abolish cell growth and increase productivity 10-fold, even in a one-gene configuration (Fussenegger et al., 1997). To minimize the induction of programmed cell death in proliferation-competent cell cultures, CHO cells were engineered to express \( p27 \) together with \( bcl-x_L \) (Itoh et al., 1995; Singh et al., 1996; Huang et al., 1997). Coexpression of \( bcl-x_L \) in proliferation-arrested configurations resulted in up to 30-fold higher specific productivity of transgenic CHO cell lines.

SURFACE SELECTION TECHNOLOGY

The ability to control cell proliferation enables the development of biphasic fermentation processes, where a growth phase up to the desired cell density is proceeded by an extended, growth-arrested production phase. Such technology is hampered by recent findings, where outgrowth of mutants lacking heterologous \( p27 \) or \( p21 \) prevented extended growth-arrested production phases (Mazur et al., 1999). Since strong counterselection of arrested populations causing mutant outgrowth is an intrinsic characteristic of biphasic fermentation processes,
there is no possibility to omit mutant appearance. Rather a strategy enabling continuous separation of the producer cells from revertants keeps promising. One possible attempt to deal with this situation is described in chapter 3. Chesnut and coworkers developed a cell-surface-displayed antibody that specifically binds to the commercially available hapten phOx immobilized on metal particles. Incubation of transiently transfected populations with hapten-coated metal particles enabled subsequent magnetic enrichment of recombinant cells bound to metal particles (Chesnut et al., 1996). Adaptation of this technology for continuous enrichment of recombinant producer cells in biphasic production processes offers opportunities to cope with mutant outgrowth. To prevent loss of the selection marker, the reporter gene \textit{SEAP}, the cytostatic gene \textit{p27} and the \textit{Hook} gene, encoding for the phOx-specific surface antibody, were genetically linked on a tetracycline-regulatable tricistronic expression unit (Fussenegger et al., 1998). Upon removal of tetracycline, cells stably harboring the genetic cassette showed sustained growth arrest in G1 phase accompanied by 5-fold increased productivity compared to isogenic growing cells. Recombinant cells could be selectively enriched from mixed populations by FACS-mediated or magnetic techniques. Such technique enables development of on-line cultivation systems where desired cell phenotypes are continuously selected for transgene expression throughout the production process by magnetic forces. In addition to biopharmaceutical manufacturing scenarios, surface-based selection technologies may also be a promising tool for \textit{ex vivo} gene therapy, by enabling enrichment of genetically corrected cells prior to reimplantation (Migita et al., 1995; Medin et al., 1996; Siegel et al., 1997).

Summarized the combination of growth-arrest and surface selection technology represents a versatile tool for high-yield, next-generation fermentation processes.

\textbf{ENZYMES AS REPORTERS OF CELLULAR PROTEIN SYNTHESIS CAPACITY}

Reporter systems are the biotechnological tool to quantify the protein production capacity of mammalian cell lines. A reporter enzyme represents the final product of gene expression, depending on transcription, mRNA processing and export, translation, posttranslational modification and targeting. Accordingly, the final enzymatic activity is the result of a sequence of events integrating information of all stages to the final protein. Comparison of different promoters, the adjustability of transcriptional regulation systems, translational processing efficiency or the potential of protein targeting sequences (e.g. secretion signal) are
just a few possible applications. Available reporter systems range from galactokinase, xanthine-guanine phosphoribosyl transferase, luciferase, alkaline phosphatase, plasminogen activator, thymidine kinase, interleukin-2 chloramphenicol acetyltransferase, β-galactosidase, β-glucuronidase to fluorescent proteins (Berger et al., 1988; Alam and Cook, 1990; Thompson et al., 1990; Prasher et al., 1992; Langer et al., 1995; Ormo et al., 1996; Kricka et al., 2000). Accordingly, the corresponding chemical assays are very diverse (Kricka et al., 2000).

In chapter 4 a novel reporter enzyme, derived from Bacillus stearothermophilus α-amylase, was adapted to eukaryotes (Suominen et al., 1987a; Suominen et al., 1987b). The thermostable α-amylase enzyme from Bacillus stearothermophilus is capable of randomly hydrolyzing 1,4-α-glucosidic linkages in polyglucosans (amylose, amylpectin, glycogen, and dextrins) degrading them into maltose and larger oligosaccharides. The bacterial gene (amyS) encodes for a 63 kDa preprotein and contains a typical bacterial signal sequence for protein secretion. Replacement of the 5' bacterial signal sequence (S) with a start codon (ATG) resulted in an intracellular mammalian reporter gene (amySΔS), that could be quantified by adaptation of the commercially available Phadebas amylase assay. Alternatively, the signal sequence was replaced by a human immunoglobulin secretion signal to create an eukaryotic secreted α-amylase derivative (SAMY). SAMY was secreted from a variety of mammalian and human cell lines, growing as monolayers, in suspension or as three-dimensional spheroids. Optimal reaction conditions of pH 5.5 and 70°C prevented any interfering endogenous enzyme activities. SAMY was fully compatible with SEAP and low molecular weight urokinase-type plasminogen activator (u-PA<sub>LMW</sub>) reporter systems enabling its combination with these assays (Berger et al., 1988; Langer et al., 1995).

Taking advantage of the thermostability, the broad-range applicability and combination with pre-existing reporter assays, SAMY is a powerful technology to monitor gene expression in eukaryotic cells.

**TRANSLATIONAL CONTROL OF PROTEIN EXPRESSION**

The technology of controlling protein synthesis represents a prerequisite for future gene therapy and tissue engineering applications. Most of the gene regulation systems available to date are based on transcriptional control mechanisms, enabling titration of the target proteins into the therapeutic range (Fussenegger, 2001). Controlling translation of heterologous mRNA or targeting of the recombinant protein represent valuable alternatives for regulating
protein levels at different sites in the body or studying intracellular protein trafficking or mRNA stability (Werstuck and Green, 1998; Rivera et al., 2000).

**CNBP- / La-Mediated Translational Control Systems**

In chapter 5, naturally evolved RNA-binding proteins together with their cognate RNA were adapted for use as artificial translation control systems (TCS). The protein-RNA interactions under investigation were developed by Pierandrei-Amaldi and coworkers, who identified the cytoplasmic cellular nucleic acid binding (CNBP) and La autoantigen proteins which specifically bind to the 5’ untranslated region (UTR) of terminal oligopyrimidine- (TOP) containing mRNAs in *Xenopus* (Pellizzoni et al., 1996; Pellizzoni et al., 1997). The interaction was found to coordinate the synthesis of the various ribosomal proteins (rp) through redistribution of the rp-mRNAs between active polysomes and untranslated messenger ribonucleoprotein particles (mRNPs) in response to cellular need for ribosomes. Regulation is exerted by binding of CNBP or La to the TOP sequence, found in the 5’ leader of rp-mRNAs, and subsequent translational activation of the downstream message (Crosio et al., 2000).

Establishment of a CHO cell line transgenic for a *SEAP* reporter-mRNA with the TOP sequence in the 5’ UTR (TOP-*SEAP*-mRNA) and for human *CNBP* or *La* autoantigen enabled investigation of the TCS. Since expression of *SEAP* is driven by a constitutive promoter element leading to a constant TOP-*SEAP*-mRNA level, any changes in final SEAP protein titers were the result of altered posttranscriptional events. Regulation of CNBP or La protein levels by a tetracycline-regulatable promoter enabled the assessment of translational activation exerted by these proteins as evidenced by measurement of SEAP protein titers. CNBP and La proved very efficient in promoting translation of the TOP-*SEAP*-mRNA by a factor of 46 and 8, respectively. Alternative to addition of tetracycline antibiotics to reduce CNBP or La titers, translation of the TOP-*SEAP*-mRNA could also be silenced down to 10% of the maximum by addition of anti-TOP phosphorothioate oligodeoxynucleotides. CNBP and La protein were also competent of promoting cap-independent *SEAP* translation from the polioviral internal ribosomal entry site (IRES; IRES-*SEAP*) by a factor of 1.5 and 2.7, respectively.

Summarized, the CNBP- or La-based TCS represent a welcome expansion of the current gene regulation portfolio. Boosting translation of desired transgenes with the additional
possibility for combination with pre-existing transcription regulation systems suits the TCS for a variety of applications in basic and applied research.

**eIF4G-Based Translational Control System**

An alternative approach for translational regulation of protein expression is described in chapter 6. The TCS is designed to mimic the events following polioviral infection. In contrast to eukaryotes, virus mRNAs possess specialized secondary structures in their 5' UTRs, referred to as internal ribosomal entry sites (IRES). Whereas cellular translation follows a cap-dependent mechanism relying on a methyl guanylic acid structure (cap) common for most cellular mRNAs, the viral IRES elements mediate an alternative translation mode, referred to as cap-independent translation (Jackson and Kaminski, 1995; Gale et al., 2000). The difference is defined by the requirement for cellular initiation factors to assemble the translational machinery on the mRNA prior to translation. For both translation modes, most of the cellular initiation factors are essential, but IRES-mediated translation does not require the cap-binding protein eIF4E (Jackson, 1996). Polioviruses encode a protease that clips the eIF4E-binding protein eIF4G, thereby separating the N-terminal eIF4E-binding domain (eIF4G∆) from the C-terminal part (∆eIF4G) needed for translational activation. Accordingly, the cleaved protein is still able to promote cap-independent translation but lost its ability for cap-binding via eIF4E, resulting in a complete shutdown of host mRNA translation (Borman et al., 1997; Bovee et al., 1998).

Separate expression of the cleaved eIF4G domains fused to rapamycin-binding proteins in CHO cells enabled mimicking of the events that follow polioviral infection. By fusion of the rapamycin binding proteins FKBP (FK506-binding protein) and FRB (FKBP-binding domain of human FKBP-rapamycin associated protein (FRAP)) to eIF4G∆ (eIF4G∆-FKBP) and ∆eIF4G (FRB-∆eIF4G) the separately encoded eIF4G fragments become combined upon addition of rapamycin, by virtue of the drug to bind FKBP and FRB simultaneously (Rivera et al., 1996; Clackson, 1997; Pollock et al., 2002). The impact of separated or dimerized eIF4G fragments on translation was assessed by measuring SEAP and Photinus pyralis luciferase (luc) reporter enzyme levels, encoded on a dicistronic reporter unit (cap-SEAP-IRES-luc) (Gould and Subramani, 1988).

Expression of the separated eIF4G fragments in CHO cells revealed a 5-fold increase in cap-dependent (SEAP) and 7-fold increase in cap-independent (luc) translation. Upon addition
of rapamycin, the heterodimerized proteins further increased SEAP and luc protein titers 9- and 42-fold.

Although the TCS is not suited for complete on/off regulation, eIF4G-based controlling and boosting of target gene expression in cap-dependent and -independent configurations represents a effective technology for various applications.

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