Regulated multicistronic expression technology for mammalian metabolic engineering

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Abstract

Contemporary basic research is rapidly revealing increasingly complex molecular regulatory networks which are often interconnected via key signal integrators. These connections among regulatory and catalytic networks often frustrate bioengineers as promising metabolic engineering strategies are bypassed by compensatory metabolic responses or cause unexpected, undesired outcomes such as apoptosis, product protein degradation or inappropriate post-translational modification. Therefore, for metabolic engineering to achieve greater success in mammalian cell culture processes and to become important for future applications such as gene therapy and tissue engineering, this technology must be enhanced to allow simultaneous, in cases conditional, reshaping of metabolic pathways to access difficult-to-attain cell states. Recent advances in this new territory of multigene metabolic engineering are intimately linked to the development of multicistronic expression technology which allows the simultaneous, and in some cases, regulated expression of several genes in mammalian cells. Here we review recent achievements in multicistronic expression technology in view of multigene metabolic engineering.

Introduction

There are two general levels of genetic engineering in which a suitable production cell line is generated; (i) stable introduction of the genetic information for the product protein and (ii) an optional metabolic engineering step to improve cellular activities by the manipulation of enzymatic, transport, and regulatory functions of the cell (Bailey, 1991). Metabolic engineering of animal cells has already been proven useful for improving diverse key characteristics of cultured cells including cell viability (apoptosis engineering: Cotter and Al-Rubeai, 1995; Mastrangelo and Betenbaugh, 1998), product quality (glycosylation engineering: Bailey et al., 1998; Jenkins et al., 1996), product yield (controlled proliferation technology: Fussenegger et al., 1997a; Fussenegger et al., 1998a; Fussenegger et al., 1998b; Papoutsakis, 1998) and growth in protein-free medium (cell-cycle engi-

neering: Renner et al., 1995; Lee et al., 1996; Rivard et al., 1996; Greulich and Erikson, 1998). Most of these successes have been realised by the addition of a single gene to the host cell's genome. However, just as single-gene interpretations of human disease have limited scope (Lander and Schork, 1994), one-gene metabolic engineering cultured cells cannot access anything approaching the full potential set of useful engineered phenotypes (Papoutsakis, 1998).

Owing to the genetic complexity of higher eukaryotic cells and the absence of sophisticated genetic tools (compared to those for several microbial hosts), introduction of heterologous genetic information into mammalian hosts is usually achieved by cotransfection of a selection marker and the gene of interest with subsequent selection for clones containing the marker, and as empirical experience has shown, often also include the cotransfected gene (Kaufman and Sharp, 1982). Many undesired phenomena accompany this haphazard genetic engineering of mammalian cells

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because of the undefined, mechanistically obscure se-lection of random integration sites in different stable clones, giving rise to variability in product expression levels, genetic stability, and second order effects on growth, viability, and productivity resulting from disruption of host genes (or regulatory loci) at the integration site. Recently, chromosomal locations of some industrially relevant mammalian cells lines have been found which show high transcription and stability for integration of transgenes (Karreman et al., 1996). Screening for such sites is a time-consuming process that involves establishment of a genetic platform for subsequent targeted integration. However, unlike the situation in mouse ES stem cells (Hicks et al., 1997) gene targeting is difficult to achieve in most indus-trially relevant cell lines because they seem to lack necessary basic recombination machinery, and there-fore they require installation of complex heterologous site-specific recombination systems (Fukushige and Sauer, 1992; Karreman et al., 1996).

Regardless of the method of integration and the chosen combination of product and metabolic engi-neering genes, it is desirable to manipulate the cell in a minimal number of steps. This goal is addressed by technology for simultaneous cloning and subse-quent expression of multiple genes in a desired host. Besides providing a platform for future metabolic engineering breakthroughs, multicistronic expression technology should speed basic functional genomic re-search and new applications in tissue engineering and gene therapy.

Here we review recent developments in multi-cistronic expression technology and their use to enable one-step multigene metabolic engineering, positive feedback regulation circuits and auto-selective expres-sion systems.

Internal translation initiation, the key to multicistronic expression technology

Bacteria have evolved expression units called oper-ons which unite functionally related genes under the control of a single promoter, thus enabling coordinated, simultaneous and rapid expression of metabolically coordinated genes in response to spe-cific environmental signals or physiological con-straints (the classic example is the lactose operon; Dickson et al., 1975). Individual genes in a bacterial operon are preceded by characteristic sequences, so-called ribosomal binding sites (*RBS*), for translation-initiation at appropriate points within a single mRNA molecule. In contrast to bacterial multigene tran-scripts, most eukaryotic mRNAs are monocistronic, and optimal translation of the encoded gene relies on a post-transcriptional 5′ modification (capping) for ribosome binding and subsequent AUG-scanning (Shatkin, 1985; Kozak, 1989). However, other cap-independent modes of translation-initiation such as leaky scanning, termination-reinitiation, and internal initiation are used in rare cases (Jackson et al., 1995; Table 1; Figure 1).

As part of their pathogenic life cycle, picor-naviruses have evolved specific genetic elements (in-ternal ribosomal entry sites; *IRES*) in their 5′ non-translated leader regions (ntr) which adopt a par-ticular secondary structure able to attract eukaryotic ribosomes and to allow internal translation-initiation (Belsham and Sonenberg, 1996; Table 1). The pivotal role of *IRES* in picornaviral pathogenesis is based on the expression of a viral protease which cleaves the host cap-binding translation-initiation factor eIF4G and allows redirection of host translation machinery for exclusive translation-initiation of *IRES*-containing viral mRNAs (Etchison et al., 1982; Pelletier and So-nenberg, 1988; Jackson et al., 1990; Belsham and Sonenberg, 1996; Rueckert, 1996).

IRES-like elements are present in other viral sys-tems and were recently discovered in eukaryotic cells which give certain mRNA molecules cap-independent translation ability in response to viral infection or stress conditions, as was shown for immunoglobulin heavy chain binding protein (Bip) and the cap-binding protein eIF4G (Macejak and Sarnow, 1991; Gan and Rhoads, 1996) (Table 1). Cap-independent transla-tion can also enforce an alternative translation start site, resulting in translation of different proteins from the same mRNA, such as that mediated by the hu-man fibroblast growth factor 2 (FGF2) (Vagner et al., 1995). Recently, *IRES* elements were also iden-tified in the translation regulation of developmentally regulated genes such as the homeotic gene *Anten-napedia* or *Ultrabithorax* of Drosophila (Oh et al., 1992; Ye et al., 1997), the genes for human insulin-like growth factor (IGF-II) (Teerink et al., 1995), and the platelet-derived growth factor B (developmental *IRES* or D-*IRES*; Bernstein et al., 1997). The poten-tial for cap-independent translation-initiation has also been found in yeast and Xenopus oocytes (Iizuka et al., 1994; Kneiper and Rhoads, 1997). Furthermore, the finding of an internal ribosomal entry site in the 5′ untranslated region of c-myc suggests that *IRES*-

a) Leaky scanning
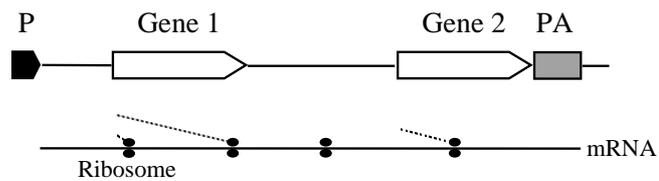
P Gene 1 Gene 2 PA

Ribosome

mRNA

b) Splicing

P SD Gene 1 SA Gene 2 PA

c) Dicistronic expression

P Gene 1 IRES Gene 2 PA

d) Tricistronic operon

P Gene 1 IRES Gene 2 IRES Gene 3 PA

e) Autoregulation

$P_{hCMV*-1}$ tTA IRES Gene 2 IRES Gene 3 PA

tTA
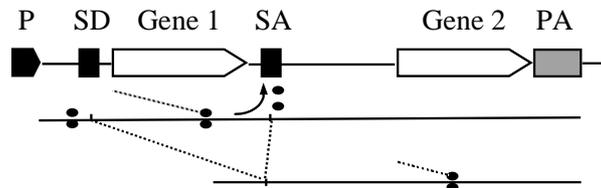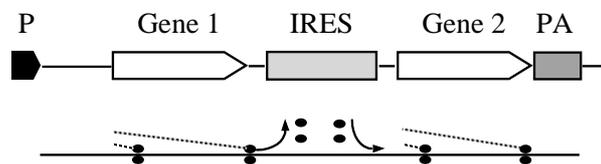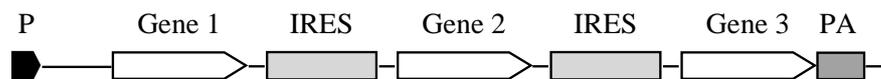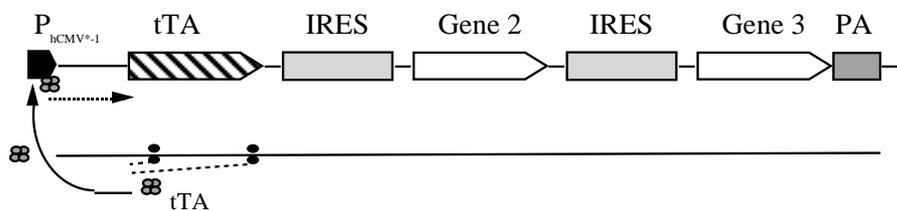
f) One-step auto-regulation and autoselection

$P_{hCMV*-1}$ tTA IRES Gene 2 IRES Gene 3 CITE Resistance PA
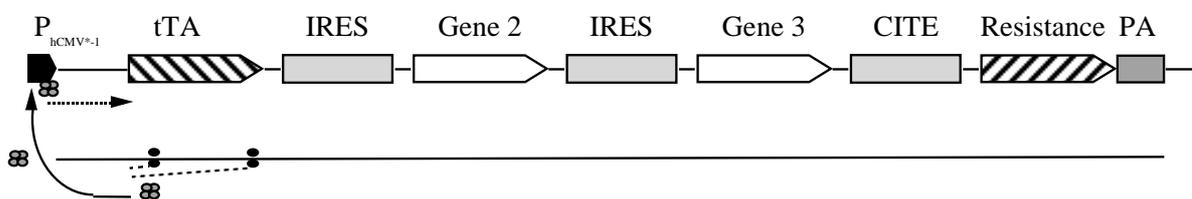
Figure 1. Strategies for simultaneous and in some cases regulated expression of more than one gene in mammalian cells. Key genetic elements for expression in mammalian cells such as the promoter (P), internal ribosomal entry sites of polioviralorigin (*IRES*) or derived from the encephalomyocarditis virus (CITE), the splice donor (SD) and acceptor (SA) and the polyadenylation site (PA) as well as for regulated gene expression including the tetracycline-responsive transactivator (tTA) and the tet-responsive promoter ($P_{hCMV*-1}$) are indicated. In some cases translation is shown below the genetic configuration (mRNA, ribosome, proteins).

Table 1. Origins of different internal ribosomal entry sites (IRES)

Class	Origin	Natural mechanism	Used for dicistronic expression vectors	Reference
Viral	Encephalomyocarditis virus	Viral replication strategy	yes	Jang et al., 1988
	Poliovirus	Viral replication strategy	yes	Pelletier and Sonenberg, 1988
	Foot-and-mouth disease virus	Viral replication strategy	yes	Belsham and Brangwyn, 1990
	Coronavirus infectious Bronchitis virus	Translation of protein 3c	no	Liu and Inglis, 1992
	Hepatitis A virus	Viral replication strategy	yes	Glass et al., 1993
	Hepatitis C virus	Viral replication strategy	yes	Wang et al., 1993
	Leishmania RNA virus 1	Viral replication strategy	no	Maga et al., 1995
	Human rhinovirus 2	Viral replication strategy	yes	Rojas-Eisenring et al., 1995
	Harvey murine sarcoma virus	Translation of VL30 protein	no	Berrlioz et al., 1995
	Mengo virus	Viral replication strategy	yes	Hofmann et al., 1996
	Crucifer infecting Tobamovirus	Translation of CP protein	no	Ivanov et al., 1997
	Reticuloendotheliosis virus type A	Translation of Gag protein	no	Lopez-Lastra et al., 1997
	Classical swine fever virus	Viral replication strategy	yes	Rijnbrand et al., 1997
	Bovine viral diarrhoea virus	Viral replication strategy	yes	Schumacher and Wirth, 1998
Mammalian	Human	Translation of the immunoglobulin heavy-chain binding protein	yes	Macejak and Sarnow, 1990
	Human	Translation of the insulin growth factor	no	Teerink et al., 1995
	Human	Translation of the fibroblast growth factor 2	no	Vagner et al., 1995
	Human	Translation of elongation factor 4G	no	Gan and Rhoads, 1996
	Human	Developmental regulation of the plate-let-derived growth factor B translation	no	Bernstein et al., 1997
	Human	Translation of c-myc proto-oncogene	no	Stoneley et al., 1998
Amphibian	Xenopus	Cap-independent translation in oocytes	no	Kneiper and Rhoads, 1997
Insect	Drosophila	Developmental regulation of the Ultra-bitorax and Antennapedia translation	no	Ye et al., 1997
Fungi	Saccaromyces cerevisiae	Translation of HAP4 and TFIID protein	no	Iizuka et al., 1994

mediated translational control may be vital for higher organisms as aberrant translational regulation of c-myc is likely to play a role in tumorigenesis (Stoneley et al., 1998).

Despite the potential of *IRES* as key elements of operon-like multicistronic expression units in mammalian genomes, such genetic configurations seem to have rarely evolved in a natural context, perhaps because most complex and fine-tuned regulatory circuits in mammalian cells are best configured with independent regulation of individual genes.

Dicistronic expression, a first step towards multicistronic expression systems

Since the transcription and translation of separate co-transfected genes is not strictly correlated, the reliability of product expression based on selection of the cotransfected marker gene can be very low. Further-

Table 2. IRES-based expression technology enables various genetic applications

IRES	Genetic determinants	Application	Reference
PV	luc–seap	Two monocistronic vectors that can be fused to a dicistronic expression system	Dirks et al., 1993
PV	MCS–seap	Cloning vector containing secreted alkaline phosphatase (seap)	Kirchhoff et al., 1995
EMCV	Hepatitis B surface antigen – B7-1 molecule	Adenoviral and retroviral vectors for dicistronic expression	He et al., 1996
EMCV	MCS–hygromycin	Cloning vector	Gurtu et al., 1996
EMCV	MCS-βgal; MCS–neo	Cloning vector	Kobayashi et al., 1996
EMCV	MCS–gfp	Cloning vector	Mosser et al., 1997
EMCV	env–v-fos-CD3	Replication-competent retrovirus for high-level expression of an exogenous gene	Murakami, 1997
HCV	luc–blasticidin S-resistance	Use of the smallest IRES element of HCV to maximize insert size of retroviral vector	Urabe et al., 1997
Mengo virus	lacZ–tTA	Retroviral vector tetracycline autoregulatory cassette	Hofmann et al., 1996
EMCV	tTA–neo	Autoregulated system	Hoshimaru et al., 1996
EMCV	MCS–tTA	Autoregulated system, cloning vector	Zhang et al., 1997
PV	gfp–tTA	pSAM202, autoregulated expression vector	Fussenegger et al., 1997b
PV	MCSI–MCSII–MCSIII	pTRIDENT1, PhCMV*-1-containing tricistronic expression vector	Fussenegger et al., 1998a
PV/EMCV	MCSI–MCSII–MCSIII	pTRIDENT3, PhCMV*-1-containing tricistronic expression vector	Fussenegger et al., 1998a
PV	MCSI–MCSII–MCSIII	pTRIDENT2, PSV40-containing tricistronic expression vector	Fussenegger et al., 1998a
PV/EMCV	MCSI–MCSII–MCSIII	pTRIDENT4, PSV40-containing tricistronic expression vector	Fussenegger et al., 1998a
PV	MCSI–MCSII–MCSIII	pTRIDENT7, ecdysone-resp. promoter-containing tricistronic expression vector	Fussenegger et al., 1998a
PV/EMCV	MCSI–MCSII–MCSIII	pTRIDENT8, ecdysone-resp. promoter-containing tricistronic expression vector	Fussenegger et al., 1998a
PV	tTA–MCS–MCS	pTRIDENT-tTA, autoregulated tricistronic expression vector	Fussenegger et al., 1997b
PV/EMCV	tTA–MCS–MCS–neo	pQuattro-tTA, autoregulated quattrocistronic expression vector	Fussenegger et al., 1997b
EMCV	hyg–neo	Retroviral vector to test dicistronic expression	Koo et al., 1992
EMCV	neo–cat	Retroviral vector to test dicistronic expression	Morgan et al., 1992
EMCV, PV	luc, cat, HIV-1 gp120	Retroviral vector with different IRES for test purposes	Alexander et al., 1994
EMCV	MCS–dhfr	Testvector for dhfr-based amplification procedures	Kaufman et al., 1991
EMCV, PV	Poliovirus proteins	Poliovirus with different IRES elements	Schmid et al., 1994
BIP	GP2,HGP2–NA	Retroviral vector for dicistronic expression of an effector and a marker gene	Garcia-Sastre et al. 1994
FMDV	neo, EP, G-CSF, ADA–βgal	Retroviral vector for dicistronic expression of an effector and a marker gene	Ramesh et al., 1996
MLV-VL30	alkaline phosphatase–neo	Retroviral vector for dicistronic expression	Torrent et al., 1996
EMCV	neo–lacZ	Testvector for dicistronic expression in ES cells and mice	Kim et al., 1992
EMCV	MCS–CD4	Surface marker expression	Smarda and Lipsic, 1994
EMCV	FIX–neo	Factor IX production	Chen et al., 1997
EMCV	Light chain–heavy chain	Recombinant antibody production	Kolb and Siddell, 1997
EMCV	G/CSF–tymidine kinase	Granulocyte colony-stimulating factor overexpression in mice	Veelken et al., 1996

Table 2. *(continued)*

IRES	Genetic determinants	Application	Reference
EMCV	Ebna-1–neo	Human shuttle vector; enforced selection	Ramage et al., 1997
EMCV	NTP–HSVtk	Surface marker in T lymphocytes	Gallardo et al., 1997
EMCV	CFTR–βGeo (βGal:neo)	Detection of CFTR expression	Vasseaux and Huxley, 1997
EMCV	Stem cell-specific transcription factor Oct-4–βgal	Investigation of mammalian emryogenesis	Mountford et al., 1994
EMCV	ADA–βGeo	Dicistronic vetors for knock out constructs due to homologous recombination	Vaulont et al., 1995
EMCV	p53 antisense–resistance	Antisense technology	Yamauchi et al., 1996
EMCV	p16–IAP (phosphatase)	Cytostatic technology	Poulos et al., 1996
PV	seap–p27	pMF113, cytostatic expression vector	Fussenegger et al., 1997a
PV	seap–p53	pMF114, cytostatic expression vector	Fussenegger et al., 1997a
PV	seap–p21	pMF112, cytostatic expression vector	Fussenegger et al., 1997a
PV/EMCV	seap–p27–bcl-xL	pDD6, cytostatic expression vector	Fussenegger et al., 1998c
PV	seap–p21–c/ebpα	pSS5, cytostatic expression vector	Fussenegger et al., 1998c
PV	tTA–p21–gfp	pSAM204, autoregulated expression vector	Fussenegger et al., 1997b
EMCV	Ion channel–gfp	Ion channel characterization	Trouet et al., 1997
EMCV	neo–lacZ	Gene trap vector	Kang et al., 1997
EMCV	MCS-βgal; MCS–lacZ	Genome sequencing/mapping; gene targeting	Yang et al., 1997
EMCV	HSVtk–cytokine	Cancer gene therapy	Castleden et al., 1995
EMCV	dystrophin–βgal	Gene therapy for muscle dystrophy	Fassati et al., 1996
EMCV	MDR–p67	Gene therapy for cronic granulomatous disease	Nunoi et al., 1995
EMCV	Tk1–Na	Cancer gene therapy	Hwang et al., 1996
EMCV	HSVtk–IL2	Gene therapy	Okada et al., 1996
PV	neo–VZVtk	Breast cancer therapy	Grignet and Calberg 1995
EMCV	MCS–gfp	Investigation of micrometastases	Chishima et al., 1997
EMCV	Rev–Lyt2	Inhibition of human immunodeficiency virus replication	Bonyhadi et al., 1997

more, even under high selection pressure, the genetic stability of the expressed product can not be assured in long term cultivations. The combination of product and marker genes on the same vector does not completely alleviate these complications. For these reasons, dicistronic genetic configurations were developed. The first dicistronic constructs used *IRES* elements of picornaviral origin or from encephalomyocarditis virus (EMCV) for cap-independent translation of the second cistron while the first cistron relied on classical cap-dependent translation-initiation (Pelletier and Sonnenberg, 1988; Kaufman et al., 1991). Although the two *IRES* elements differ completely at the sequence level, their secondary structure is very similar and typical for such internal translation initiators. A large number of dicistronic product-marker configurations have since been developed for many different applications. Table 2 gives an overview of recent dicistronic expression vectors. Although genetic combinations used for dicistronic expression vary among different applications, EMCV and picornaviral *IRES* remained the most popular cap-independent translation-initiators for dicistronic configurations because these elements function in a wide variety of cell lines including the industrially relevant CHO and BHK cell lines (Borman et al., 1997). However, recent reports of varying translation-initiation capabilities of *IRES* in different host cell environments and discovery of new *IRES* elements is stimulating new development to apply these *IRES* elements for multicistronic expression (Bernstein et al., 1997; Schumacher and Wirth, 1997).

Dicistronic genetic configurations which contain the marker gene in the second cistron enable autoselective expression in addition to simultaneous and coordinated gene expression. Resistance to the marker gene or expression of the reporter gene is only possible if all 5′-encoded genetic elements are intact. This intrinsic self-selective program was found to be very reliable, with nearly all of the resistant cells

also expressing the desired product gene (Gurtu et al., 1996; Rees et al., 1996). Furthermore, product-marker configurations can also be used for efficient generation and screening of high producing cell clones: *IRES*-based translation-initiation of the second cistron is usually less efficient compared to cap-dependent translation, and can be decreased further by loss-in-function mutations of the *IRES* or the marker gene itself. The overall lower translation efficiency or activity is then compensated under high selective pressure by integration of the dicistronic expression unit into chromosomal sites with high transcriptional activity (Kaufman et al., 1991; Gurtu et al., 1996; Rees et al., 1996). Certainly, simultaneous expression of two genetic traits can also be achieved by gene fusions (Krömer et al., 1997; pTracer plasmids of Clontech) or recently developed splicing expression technology (Lucas et al., 1996; Figure 1), but gene fusion strategies are limited in functional applications or may lead to fusion products with altered physiologic specificities, and splicing-based two-gene expression leads to unequal expression levels of both proteins. Only *IRES*-based dicistronic expression guarantees simultaneous and coordinated expression of both transgenes at comparable levels for multi-subunit proteins (for example antibodies) which enables genetic configurations for a wide variety of contemporary research and development applications which are also listed in Table 2 (Dirks et al., 1993; Dirks et al., 1994; Fussenegger et al., 1997a). Furthermore, *IRES*-mediated expression systems can be extended beyond the dicistronic level to tri- or even quattrocistronic artificial eukaryotic operons (Fussenegger et al., 1997b; Fussenegger et al., 1998c).

pTRIDENT, tricistronic artificial mammalian operons

Despite the numerous expression vectors available containing dicistronic expression units (Table 2), most of these expression systems express a marker or reporter gene in a fixed configuration, leaving only one cistron free for heterologous gene expression. However, for one-step transfection of a product protein, metabolic engineering, and a selection marker in a single expression unit, multicistronic artificial mammalian operons with 3 or even 4 cistrons are desirable. We recently reported the construction of a novel vector family, pTRIDENT, for tricistronic gene expression in mammalian cells (Fussenegger et al., 1998c; Fig-

ure 2). A single promoter allows high level expression and, in some vectors, adjustable transcription of all three genes. Whereas the first cistron is translated in a classical cap-dependent manner, translation-initiation of the subsequent two cistrons rely on *IRES* elements of picornaviral (*IRES*; pTRIDENT1) and EMCV origin (denoted here *CITE*, cap-independent translation enhancer; third cistron; pTRIDENT3; Fussenegger et al., 1998c).

Tricistronic pTRIDENT1- and pTRIDENT3-derived test vectors encoding the model product gene SEAP (secreted alkaline phosphatase; first cistron), a metabolic engineering determinant (the cyclin-dependent kinase inhibitor p21 (CDI) second cistron), and the reporter gene GFP (green fluorescent protein; third cistron) were transfected into a CHO cell derivative which allows tetracycline-responsive gene expression (Fussenegger et al., 1998c). Both tricistronic configurations were stable in CHO cells and showed strict simultaneous, coordinated as well as regulated expression of all three cistrons. The expression levels of individual cistrons were assessed by comparison to respective values of isogenic mono-cistronic expression vectors. Although expression levels of genes encoded on different cistrons are largely dependent on the overall stability of the polycistronic mRNA and therefore a direct function of the genetic configuration of encoded genes, our test vectors showed similar expression levels to those provided by the monocistronic vector on the first two cistrons and approximately 35% (*CITE*) to 50% (*IRES II*) lower expression levels on the third cistron. For enhanced translation-initiation of the third cistron, *CITE* was specially mutated (*CITE∗*) to avoid erroneous translation-initiation at upstream ATG start codons (Jackson et al., 1990; Kaufman et al., 1991; Davies and Kaufman, 1992; Rees et al., 1996; Fussenegger et al., 1998c). Initially, the use of pTRIDENT3 derivatives (*IRES-CITE*) was preferred over double *IRES*-containing counterparts because pTRIDENT3-based vectors show a slightly higher translation efficiency of the third cistron, and they contain no duplicated sequence elements (*IRESI-IRESII*; pTRIDENT1) which bear the risk of recombination-mediated deletion of the second cistron. However, genetic rearrangements or deletions in double *IRES*-containing pTRIDENT1 derivatives were never observed during cloning steps in *recA⁻ E.coli* nor in mammalian cells (Fussenegger et al., 1998a and 1998c).

pTRIDENT vector backbones encode a bacterial ampicillin resistance and origin of replication (*ori*)

Figure 2. Examples for multicistronic expression in mammalian cells: pTRIDENT1 and pQuattro-tTA. Both vectors allow tetracycline-regulated expression of all transgenes. Since pQuattro-tTA encodes all genetic elements for regulated gene expression in its multicistronic expression unit, pQuattro-tTA allows autoregulation and can be used to achieve one-step regulated expression the genes of interest in any cell line where the internal ribosomal entry sites of polioviral origin function (*IRES, CITE*).

for high copy number amplification of these plasmids (Figure 2). High copy number amplifications in bacterial hosts is a prerequisite for large-scale transient transfection protocols which are becoming increasingly popular for industrial R&D applications (Fussenegger et al., 1997a). The tricistronic expression unit contains three multiple cloning sites (MCS) with up to 18 unique restriction sites, many for 8 bp-targeting, rare-cutting enzymes to allow sequential, complication-free cloning of all three transgenes into pTRIDENT. The general modular set-up of the key genetic elements in the pTRIDENT series, including, promoter, *IRES* elements, polyadenylation site, and vector backbone with their well selected flanking (or sometimes internal) restriction sites or MCS allows straightforward elimination or exchange of cistrons between existing conventional monocistronic or pTRIDENT expression vectors. Also, the modular set-up enables rapid adaptation of the pTRIDENT vector concept for special applications and stimulates future developments in expression vector design. Based on the compatibility of pTRIDENT to existing vector families, for example the one presented by Dirks et al. (1993 and 1994), recent developments of the growing pTRIDENT family resulted in tricistronic vector derivatives with various constitutive (P_{SV40}, P_{MPSV}), tetracycline- and ecdysone- regulated promoters ($P_{hCMV*-1}$; P_{EC}) and in construction of auto-

regulated, self-selective, one-step transfection systems described below.

pTRIDENT-based multigene metabolic engineering greatly improves cytostatic production technology

Pioneering reports by Suzuki and Ollis (1990) and Al-Rubeai et al. (1992) showing increased specific productivity of growth-inhibited hybridoma cells stimulated research on chemical culture additives to arrest cell growth and initiated efforts to control cell growth by controlled overexpression or inhibition of selected genes. Three successful one-gene metabolic engineering strategies have been developed to reversibly control mammalian cell growth: (i) estrogen-regulated overexpression of the interferon-responsive factor (IRF-1), a transcription factor which is upregulated by interferons as response to viral cell invasion, in BHK cells (Koester et al., 1995); (ii) dexamethasone-inducible suppression of the key transcription factor c-jun by antisense technology in Friend murine erythroleukemia cells (F-MEL) (Kim et al., 1998); and (iii) tetracycline-regulated overexpression of negative key regulators of the cell-cycle including the tumor suppressor p53 and the CDIs p21 and p27 in CHO cells (Fussenegger et al., 1997a; Fussenegger et al., 1998a and 1998b). Overexpression of IRF-1

resulted in cell-cycle-independent growth arrest, but heterologous gene expression was not enhanced unless the exogenous genes were placed under control of IRF1-responsive promoters. Furthermore, IRF-1-overexpressing BHK cells rapidly die, probably by an apoptosis-independent pathway (Koester et al., 1995; Müller et al., 1998). On the contrary, c-jun suppression leads to sustained G0-phase arrest of F-MEL cells for over two weeks and protects these cells against apoptosis (Kim et al., 1998). Unfortunately, this promising antisense technology remains to be assessed in an industrially relevant cell line and in connection with cloned protein production. However, G0-arrested cells have previously shown to produce exogenous protein at a lower rate (Kim et al., 1998).

In another strategy, transient tetracycline-responsive overexpression of p53, p21 or p27 in a dicistronic configuration (SEAP-p53; SEAP-p21; SEAP-p27) led to G1-specific cell-cycle arrest, and in each case was accompanied by an up to 4-fold increase in SEAP production compared to proliferation-competent control cells (Fussenegger et al., 1997a). These results compare favourably with those from G1-arrested, temperature-sensitive CHO cells generated by random mutagenesis, which also showed a 3-4-fold higher heterologous protein production upon growth arrest but retained low cell viability at elevated permissive temperatures (Jenkins and Hovey, 1993).

However, in a stable genetic configuration in CHO cells, only SEAP-p27 overexpression lead to a significant increase in productivity, with specific SEAP productivity increasing by 15-fold compared to control cells (Fussenegger et al., 1998b). Intracellular p21 levels were probably insufficiently high to cause significant growth inhibition, and p53-based cell-cycle arrest led to rapid decrease in cell viability accompanied by cell morphologies indicative of apoptosis, even when achieved by overexpression of the apoptosis-deficient mutant p53175P (Rowan et al., 1996), a phenomenon which could not be observed with p27-induced G1-arrest (Fussenegger et al., 1998b).

The failure to produce stable growth-controllable CHO cells by p21-mediated overexpression exemplifies current limitations of one-gene metabolic engineering strategies. Although global regulatory proteins certainly exist, such key metabolic effectors are rare, difficult to find and their overexpression may imbalance fine-tuned interconnected cellular circuits, as seems the case with overexpression of p53.

Using tricistronic expression technology we extended the SEAP-p21-encoding dicistronic configuration by an additional cistron harbouring the differentiation factor, CCAAT/enhancer binding protein α (C/EBPα) (pSS5; Figure 3). C/EBPα has been shown to stabilise p21 at the protein level and also to induce endogenous p21 alleles (Timchenko et al., 1996). Using this tricistronic set-up for metabolic engineering, the induction of conditional growth arrest of CHO cells was successful, and the sustained cell-cycle arrest achieved was accompanied by an up to 15-fold higher specific SEAP productivity compared to proliferation-competent control cells, similar to that achieved by p27-based one-gene metabolic engineering (Fussenegger et al., 1998a and 1998c).

In a further preventive measure against possible apoptosis, which was strongly suggested by morphologies of p53 overexpressing cells, we linked SEAP-p27 expression with the expression of the survival gene bcl-x_L in a tricistronic configuration (pDD6; Figure 3). bcl-x_L belongs to the family of bcl-2 anti-apoptosis genes which have been successfully used to suppress apoptosis in production cell lines (Cotter and Al-Rubeai, 1995; Mastrangelo and Betenbaugh, 1998). Although overexpression of SEAP-p27-bcl-x_L induced sustained growth arrest in CHO cells like its dicistronic counterpart, the specific SEAP productivity of arrested cells was increased by an additional factor of three, which corresponds to 30-times higher specific SEAP productivity than respective proliferation-competent control cell lines (Fussenegger et al., 1998a). This unexpected effect of bcl-x_L expression cannot be explained based on the current knowledge of cell-cycle and apoptosis regulatory pathways, and further investigations are needed to reveal the mechanism of this new, apparently apoptosis-unrelated effect of bcl-x_L.

Thus, using controlled proliferation technology as an example, multigene metabolic engineering has proven to be useful for achieving difficult-to-attain cell culture states, and the combinatorial expression of an intuitively unrelated gene revealed previously unknown functions and potential molecular links of complex cellular pathways.

Mammalian expression systems for one-step cloning of autoregulated product gene expression

There is much current interest in the development of regulatable expression systems in basic functional genomic research, since externally regulated transcription enables the effects of a particular gene product
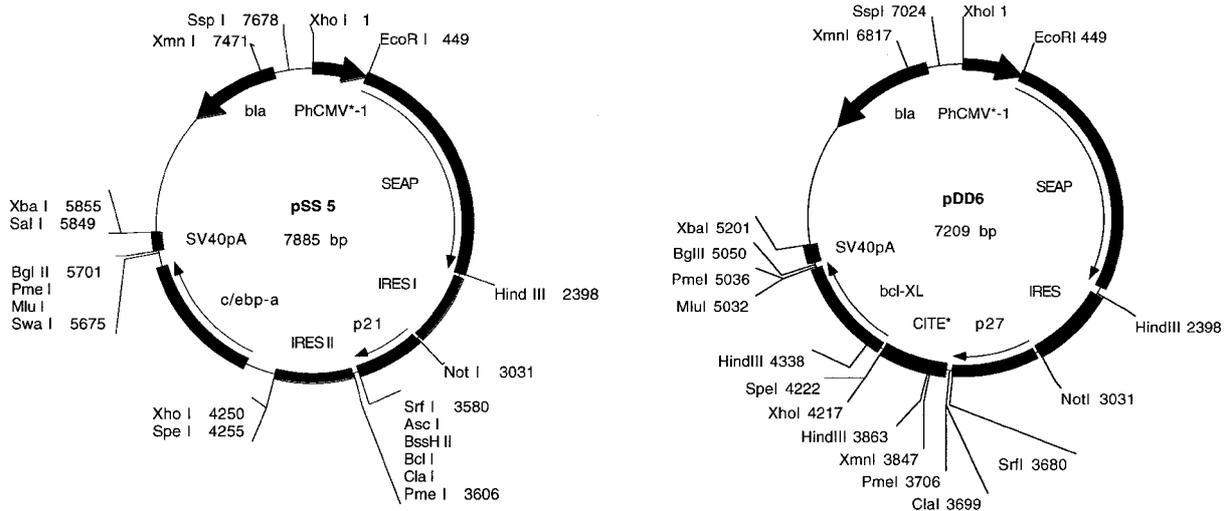
Figure 3. Tricistronic expression vectors enabling multigene metabolic engineering. Both vectors express the model product gene, the secreted alkaline phosphatase (SEAP) and one of the cell-cycle inhibitors p21 (pSS5) and p27 (pDD6). While the expression of p27 is sufficient to cause cell-cycle arrest and result in enhanced specific SEAP productivity which is additionally increased by coexpression of the anti-apoptosis gene bcl-x_L, cell-cycle arrest using p21 is only effective when the differentiation factor $c/ebp\alpha$ is coexpressed and stabilizes p21.

to be assessed in an identical genetic background. Regulated gene expression is also gaining increasing importance for biotechnological applications since it allows conditional metabolic engineering and achievement of specific cell culture states in a timely manner (Fussenegger et al., 1997a; Fussenegger et al., 1998a). For example, regulated metabolic engineering in a multicistronic configuration allows differentiation of a cell culture process into two stages: a non-productive growth phase in which the cells are rapidly expanded to the desired cell density, and a subsequent non-proliferating production phase where the cells can devote all of their metabolic capabilities to the production of protein instead of biomass.

Several *in vivo* regulated eukaryotic promoters have been described (Schweinfest et al., 1988; Israel and Kaufman, 1989; Ko et al., 1989; Hu and Davidson; 1990; Mattioni et al., 1994) and used for regulated gene expression. However, as most of these regulated promoters are derived from regulatory circuits which mediate metabolic responses, the corresponding regulating external stimuli may lead to undesired pleiotropic effects. More successful transcriptional regulation circuits rely on basic regulatory machineries of heterologous origin which are genetically adapted for use in mammalian cells. Besides the lac switch (Fieck et al., 1992) and the ecdysone-responsive system (No et al., 1996), the tetracycline-regulatable expression system (tet system; Gossen and

Bujard, 1992) is by far the most popular. The tet system consists of two separate genetic entities, the tet-responsive transactivator (tTA) and the tTA responsive promoter, $P_{hCMV*-1}$ each of which represents a chimeric genetic configuration composed, respectively, of a protein fusion between the bacterial tet repressor and the VP16 domain of the herpes simplex virus (tTA) and a genetic fusion which places a tet operator adjacent to a minimal cmV promoter ($P_{hCMV*-1}$). While the bacterial parts are responsible for promoter recognition and integrate responsiveness to the external stimulus tetracycline, the viral parts initiate transcription (Gossen and Bujard, 1992).

Despite its success story, the tet system has two major limitations. First, prior to introduction of the regulated transgene, each host cell line must be engineered to express tTA in a fashion which affords efficient tetracycline-mediated control of $P_{hCMV*-1}$-initiated transcription. Screening for this phenotype is tedious and time-consuming. Cotransfection of the tTA-expression plasmid and the vector encoding the gene of interest or the transfection of a single vector with a combination of both genes, are not recommended since, in either case, the close proximity of the two genes in the host chromosome may cause the enhancer of tTA-driving promoter expression to interfere with $P_{hCMV*-1}$ thus leading to hardly regulatable configurations (Gossen and Bujard, 1992). Second, a transcriptional 'squelching' effect by the

VP16 transactivator domain may be lethal for the host cell, even at moderate expression levels (Gill and Ptashne, 1988). Consequently, since the activity of $P_{hCMV*-1}$ is proportional to the intracellular tTA levels, moderate tTA expression may lead to apparently low expression levels of regulated transgenes (Furth et al., 1994). Several improvements have been made to alleviate these complications including (i) fusion of the tTA to the ligand-binding domain of the estrogen receptor to control the transfer of tTA into the nucleus (Iida et al., 1996), (ii) fusion of tTA to a nuclear localisation signal enabling tight regulation and high-level induction (Yoshida and Hamada, 1997), (iii) construction of a regulatory cascade by controlling tTA expression by another higher-order control system, for example, by the lac switch (Aubrecht et al., 1996), (iv) construction of $P_{hCMV*-1}$ derivatives harbouring minimal promoters of various sources which show altered regulatory features, promoter strength and tTA responsiveness (Hoffmann et al., 1997), and (v) placing tTA expression itself under control of $P_{hCMV*-1}$ to prevent accumulation of toxic tTA levels prior to induction (Shocket et al., 1995). However, all of these improvements still require two rounds of transfection for their implementation.

We recently reported one-step, auto-regulated and auto-selective multicistronic mammalian expression systems which included the tTA in a multicistronic, pTRIDENT-based or quattrocistronic configuration (pQuattro-tTA; Fussenegger et al., 1997b; Figure 2). Since the tTA gene is encoded on the multicistronic expression unit itself, little or no tTA is expressed under repressive conditions. This genetic configuration alleviates intracellular accumulation of toxic tTA levels. However, when the auto-regulated system is induced, the few tTA molecules originating from the leakiness of $P_{hCMV*-1}$ activate this tet-responsive promoter (Figure 1). Since tTA is itself encoded on the artificial operon, every round of transcription generates also an additional tTA message resulting in a positive feedback regulation system with high tTA levels and consequently high level expression of all cocistronically expressed transgenes. Since all genetic elements essential for regulated gene expression are united in a single vector, these autoregulated pTRIDENT derivatives mediate one-step regulated gene expression in various cell lines including CHO, BHK and HeLa cells (Fussenegger et al., 1997b). Previously, HeLa cells have been reported to be very sensitive to squelching, and prolonged screening procedures are usually necessary to select HeLa clones with moderate tTA

expression to avoid this problem (Gossen and Bujard, 1992). To our knowledge, no convincing tet-regulation gene expression has previously been established in BHK cells apart from a recent report by Sekigushi and Hunter (1998) which shows very high background under repressed conditions and only 10-fold induction. On the contrary, our positive feedback regulation system showed both tight regulation as well as high levels of tet-responsive gene expression in all these cell lines with no signs of squelching. The lack of squelching is rather surprising, considering that the positive feedback circuit is expected to produce high intracellular levels of tTA. However, recent experiments with a monocistronic positive feedback configuration in transgenic animals also showed no detrimental effects (Shocket et al., 1995).

Positive feedback configurations with tTA in the last cistron consist of both essential and interdependent elements for regulated expression, with $P_{hCMV*-1}$ and tTA at the perimeters of the multicistronic expression unit. This set-up harbours an intrinsic, auto-selective program which guarantees full length transcripts and maintains the functional integrity of all genetic elements encoded on this autoregulatory operon. Recently, a similar autoregulated dicistronic expression system was reported (Shocket et al., 1995; Hofmann et al., 1996; Zhang et al., 1997), but only pTRIDENT-based or pQuattro-tTA systems allow one-step, autoregulated and auto-selective multigene metabolic engineering in industrially-relevant cell lines (Fussenegger et al., 1997b).

Conclusions

Since one-gene metabolic engineering will necessarily reach its limits when coping with today's increasingly complex challenges, the recent development of artificial eukaryotic operons enables effective multigene metabolic engineering of mammalian cells. This greatly expands possibilities to reprogram interconnected cellular networks in desired ways to improve key characteristics of mammalian cells. Besides use in next-generation multigene metabolic engineering, multicistronic expression units are expected to have great impact on very specific applications including (i) straightforward combinatorial evaluation of gene functions and metabolic networks, (ii) one-step transfection, selection and maintenance of difficult-to-express (multi-subunit) proteins, (iii) selection of high

producer cell lines, and (iv) genetic immunisation and gene therapy in combination using sense, antisense or ribozyme technology.

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