

Transient Recombinant Protein Expression in Mammalian Cells: the Role of mRNA Level and Stability

THÈSE N° 4393 (2009)

PRÉSENTÉE LE 15 MAI 2009

À LA FACULTÉ SCIENCES DE LA VIE

LABORATOIRE DE BIOTECHNOLOGIE CELLULAIRE (SV/STI/SB)

PROGRAMME DOCTORAL EN BIOTECHNOLOGIE ET GÉNIE BIOLOGIQUE

ÉCOLE POLYTECHNIQUE FÉDÉRALE DE LAUSANNE

POUR L'OBTENTION DU GRADE DE DOCTEUR ÈS SCIENCES

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Suisse
2009

To my family

Acknowledgements

The completion of this thesis would not have been possible without the support of a lot of people. In particular I would like to thank:

Prof. Florian Wurm for welcoming me into his laboratory and accepting me as a PhD student. When I started my thesis at the LBTC I knew cell culture only by theory, but however he believed in me and gave me a great chance of growing as scientist!

Dr. David Hacker for being always there to listen and discuss! I will never forget all the support and help he gave in correcting my thesis, manuscripts and publications!

Dr. Lucia Baldi and Dr. Mattia Matasci for the useful discussions, the help, the ideas and the support during these years!

All the people with whom I spent great times: Agata, Andrea, Céline, Delphine, Fanny, Gilles, Jean, Matthieu, Sophie N., Tatiana, Virginie, Vincent, Zuzana ... thank you for creating a nice ambience in the lab! All the LBTC members from the past and the present thank you for your support and help in the lab,: Anne-Charlotte, Alex, Balaji, Daniel, Fabienne, Gaurav, Gnagna, Hajer, Harriett, Ivan, Ione, Marie-France, Markus, Martin, Nan, Nicolas, Sagar, Sandrine, Sebastien, Yashas and Xiaowei.

All the interns, trainees and master students that helped me: Sophie B., Elodie, Stéphanie and Christophe! You did a great job! I wish you all the best for your future!

Carine, Divor, Evi and Myriam ... thank you for everything! Your friendship is precious for me! We spent great moments together and, wherever the future will bring us, I will do all my best in order not to lose you!

All my Italian friends: Luca, Andrea, Stefania, Stefano, Cristina, Mary, Fede, Valeria & Beppe, Anna & Beppe. We are far, but you were always there, bringing me up when I was down or distracting me from stressful situations with crazy weekends! Thank you all!

My cousin, bridesmaid and “sister” Daniela: thank you for the encouragement and the “virtual” psychological support!

Mum, Dad and Grandma: I would never have crossed this final lane without you. You always believed in me. I know that today you are happy and proud: I hope these feelings will partially payback all the sacrifices and efforts you made during these 29 years!

Filippo, loved husband, soul mate, Ohana and best friend: there are no words for all you made for me. Thank you for your love, trust, faith and support. With you I always feel safe and as long as we will stay together we can overcome any difficulty!

Abstract

Transient gene expression (TGE) is a rapid method for generating recombinant proteins in mammalian cells, but the volumetric productivities for secreted proteins in transiently transfected CHO DG44 cells are typically more than an order of magnitude lower than the yields achieved with recombinant CHO-derived cell lines. The goals of the thesis are to identify the limitations to higher TGE yields in CHO DG44 cells and to find possible solutions to overcome the problems. Initially an attempt was made to enhance TGE production by increasing the amount of transfected plasmid DNA. However, this approach did not result in increased recombinant protein levels; on the contrary, transfection with an excess of plasmid DNA ($> 1.25 \mu\text{g/ml}$) had a negative impact on transgene mRNA levels and protein production. Moreover, it was also observed that recombinant protein yield was strongly dependent on the mRNA level. Therefore, three strategies aimed at increasing the amount of transgene mRNA were investigated. For the first approach, transfected cells were exposed to hypothermic conditions during the production phase. It was already known that lower temperatures increase protein production several fold in recombinant CHO DG44-derived cell lines. The second strategy involved the treatment of transfected cells with valproic acid, a histone deacetylase inhibitor that reduces the effects of gene silencing. The third approach aimed to increase transgene mRNA levels by overexpressing transcription factors and growth factors. With the first two strategies recombinant antibody yields of 60-80 mg/L were achieved whereas the untreated control transfections produced only 5-10 mg/L. Combination of the two strategies led to the production of 90 mg/L of antibody. Moreover, in the treated cultures, the steady-state level of transgene mRNA was 3-5 times higher than in the untreated cultures and remained stable up to 6 days post-transfection. Using the third approach, the increase in recombinant protein production was moderate and transgene mRNA amounts were only 2-fold higher in treated samples compared to the control. When specific proteins such as c-fos, c-jun, NF-kB, and acidic fibroblast growth factor (aFGF) were overexpressed the recombinant antibody production was 20 mg/L compared to 5 mg/L for the control transfection. Overexpression of either a transcription factor or a growth factor in combination with treatment with valproic acid allowed the recombinant protein yield to reach 90 mg/L. However, the benefit of the overexpressed factors was minimal compared to the effect of valproic acid alone. In conclusion, it was demonstrated that the level and stability of transgene mRNA are important factors for increasing volumetric yields in transiently transfected CHO DG44 cells. Furthermore, three approaches aimed to increase mRNA amounts were tested. Exposure to hypothermic conditions and treatment with valproic acid were the two best strategies tested. Both are simple, cost-effective, and scalable making transient gene expression in CHO DG44 cells a feasible alternative for rapid production of gram amounts of recombinant protein.

Key words: Mammalian cell culture, recombinant protein, transient gene expression, monoclonal antibody, hypothermia, histone deacetylase, transcription factor, growth factor

Riassunto

L'espressione genica transiente è un metodo rapido e semplice per generare, in cellule di mammifero, ogni tipo di proteina ricombinante. Tuttavia la produttività specifica e volumetrica in cellule CHO trasfettate transientemente è inferiore di almeno un ordine di grandezza rispetto a quella normalmente ottenuta con linee cellulari stabili. L'obiettivo della tesi è di identificare le limitazioni nella produzione transiente in cellule CHO DG44 e trovare possibili soluzioni per risolvere il problema. Inizialmente si è provato ad aumentare la produzione transiente aumentando la quantità di DNA usata per la trasfezione. Tuttavia, questo approccio non ha portato ad un aumento dei livelli di proteina prodotta; al contrario, trasfettare le cellule con un eccesso di DNA plasmidico ($> 1.25 \mu\text{g}/\text{mL}$) ha avuto un impatto negativo sui livelli di mRNA transgenico e sulla produzione di proteina. Inoltre si è anche osservato che la quantità di proteina prodotta era fortemente dipendente dai livelli di mRNA; perciò, sono state studiate tre diverse strategie per aumentare la quantità di RNA messaggero. Il primo approccio è consistito nell'espore le cellule ad ipotermia durante la fase produttiva. E' infatti noto che, in linee cellulari derivanti da cellule CHO, le basse temperature aumentano i livelli di proteina prodotta. La seconda strategia ha implicato il trattamento, post-trasfezione, delle cellule con acido valproico, un inibitore delle istone-deacetilasi in grado di ridurre gli effetti del silenziamento genico. Il terzo approccio ha previsto di aumentare i livelli di mRNA sovraesprimendo fattori di trascrizione e di crescita. Utilizzando le prime due strategie elencate si sono potuti raggiungere dei titoli di anticorpo ricombinante pari a 60-80 mg/L, mentre la coltura di controllo ha prodotto solo 5-10 mg/L; la combinazione delle due strategie ha permesso la produzione di 90 mg/L di proteina. Inoltre, nelle colture trattate, il livello di mRNA messaggero era 3-5 volte più alto che in quelle non trattate ed è rimasto stabile per oltre 6 giorni dopo trasfezione. Con il terzo approccio, si è riscontrato un aumento della produzione di proteina ricombinante e di mRNA transgenico alquanto moderato se paragonato a quanto ottenuto con le prime due strategie. Infatti nel caso di sovraespressione di proteine specifiche quali c-fos, c-jun, NF-kB e aFGF sono stati prodotti solo 20 mg/L di anticorpo. La combinazione della sovraespressione di questi fattori e il trattamento con acido valproico ha permesso di raggiungere nuovamente la produzione di 90 mg/L di proteina ricombinante. Tuttavia, il beneficio tratto dalla presenza dei fattori è stato minimo paragonato all'effetto dell'acido valproico in sé. Concludendo, si è dimostrato che il livello e la stabilità dell'mRNA transgenico sono fattori importanti per aumentare la produttività volumetrica in cellule CHO DG44 trasfettate transientemente; in aggiunta, sono stati testati tre approcci per aumentare la quantità di mRNA. L'esposizione delle cellule a condizioni ipotermiche o a acido valproico si sono rivelate essere le migliori strategie. Entrambe sono semplici da attuare, a basso costo, e scalabili: pertanto la loro utlizzazione potrebbe rendere l'espressione genica transiente una possibile alternativa per la rapida produzione di grammi di proteine ricombinanti.

Parole chiave: colture di cellule di mammifero, proteina ricombinante, espressione genica transiente, anticorpo monoclonale, ipotermia, istone-deacetilasi, fattore di trascrizione, fattore di crescita.

Résumé

L'expression génique transitoire est une méthode rapide et simple pour générer des protéines recombinantes dans des cellules de mammifères. Cependant la productivité spécifique et volumétrique des cellules transfectées transitoirement est inférieure d'au moins un ordre de grandeur à celle normalement obtenue en utilisant des lignées cellulaires stables. L'objectif de cette thèse est d'identifier les limitations de la production transitoire en cellules CHO DG44 et de trouver des solutions possibles pour résoudre ce problème. Dans un premier temps, la quantité d'ADN utilisé pour la transfection a été augmentée afin d'améliorer la production de protéine. Cependant, cette approche n'a pas permis d'augmenter la quantité de produit ; au contraire, transfecter les cellules avec un excès d'ADN ($> 1.25 \mu\text{g/mL}$) a eu un impact négatif sur la quantité d'ARN messager et sur la production de protéine. En outre il a aussi été observé que la quantité de protéine produite était dépendante de la quantité d'ARN transgénique. Par conséquent trois stratégies ont été formulées et étudiées pour augmenter la quantité d'ARN. Dans une première approche, les cellules en phase de production ont été exposées à des conditions d'hypothermie. Il est en effet connu, que lignées cellulaires dérivant des CHO, produisent plus de protéines recombinantes si elles sont cultivées à basse température. La deuxième stratégie a impliqué le traitement après-transfection des cellules avec de l'acide valproïque, un inhibiteur des histones deacetylases capable de réduire les effets du *gene silencing*. La troisième approche s'est basée sur la surexpression de facteurs de transcription et de croissance. En utilisant les deux premières stratégies indiquées, 60-80 mg/L d'anticorps recombinant ont été produits, tandis que le contrôle n'a produit que 5-10 mg/L. La combinaison de ces deux stratégies a permis la production de 90 mg/L de protéine. De plus, dans les cultures traitées, la quantité d'ARN messager était 3 à 5 fois plus élevée que dans le contrôle et elle est restée stable pendant au moins 6 jours après la transfection. Avec la troisième stratégie, une faible augmentation de la production de protéine et de la quantité d'ARN a été détectée. En effet, quand c-fos, c-jun, NFkB ou aFGF ont été surexprimés, la production d'anticorps n'a été que de 20 mg/L seulement. La combinaison de la surexpression de ces facteurs avec le traitement à l'acide valproïque a permis de produire de nouveau 90 mg/L de protéine. Cependant, le bénéfice de la présence des facteurs a été minimal comparé à l'effet de l'acide valproïque seul. En conclusion, il a été démontré que la quantité et la stabilité de l'ARN messager sont des facteurs importants pour augmenter la productivité volumétrique et spécifique des cellules CHO DG44 transfectées transitoirement. En outre, trois approches pour augmenter la quantité d'ARN transgénique ont été testées. Le traitement des cellules avec des conditions d'hypothermie ou avec de l'acide valproïque se sont révélées être les meilleures stratégies. Toutes les deux sont simples, peu chères et peuvent être amplifiées à large échelle : c'est pourquoi leur utilisation permettrait à l'expression génique transitoire d'être une bonne alternative pour la production rapide de plusieurs grammes de protéines recombinantes.

Mots clés: culture de cellules de mammifère, protéine recombinante, expression génique transitoire, anticorps monoclonal, hypothermie, histone-deacetylase, facteur de transcription, facteur de croissance.

Zusammenfassung

Transiente Genexpression (TGE) erlaubt die schnelle Produktion von rekombinanten Proteinen in Säugetierzellen, wobei die volumetrische Produktivität im Vergleich zu genetisch modifizierten CHO-Zelllinien um einige Größenordnungen geringer ausfällt. Das Ziel dieser Arbeit ist es die einschränkenden Faktoren für die rekombinanten Proteinträge in TGE in CHO-Zellen zu identifizieren und Lösungsmöglichkeiten hierzu zu finden. Zunächst wurde versucht die Produktivität in TGE durch eine erhöhte Menge an transfizierter DNA zu steigern. Dieser Ansatz war allerdings nicht erfolgreich und führte im Gegensatz zu einer Verringerung der transgenen mRNA Levels und der rekombinanten Proteinproduktion bei Transfektionen mit $> 1.25 \mu\text{g/ml}$ Plasmid DNA. Überdies wurde beobachtet, dass der Ertrag an rekombinantem Protein stark vom Niveau der entsprechenden mRNA abhing. Deswegen wurden drei Strategien getestet, um die Menge an transgener mRNA in den Zellen und damit die Produktivität zu erhöhen. Im ersten Ansatz wurden transfizierte Zellen hypothermischen Bedingungen während der Produktionsphase ausgesetzt. Es war bereits bekannt, dass niedrigere Temperaturen die Erträge von genetisch modifizierten CHO-Zelllinien um einiges erhöhen. Die zweite Strategie betraf das Hinzufügen von Valproinsäure in transfizierten Zellkulturen. Valproinsäure hemmt die Histonacetylase und verringert dadurch den Effekt der Genstilllegung. Das dritte Vorgehen zielte darauf ab, die transgenen mRNA Mengen durch die Überexpression von Transkriptions- und Wachstumsfaktoren zu erreichen. Mit den zwei erst genannten Methoden konnten Erträge von 60-80 mg/L rekombinanten Antikörpern erreicht werden, im Vergleich zu nur 5-10 mg/L in den unbehandelten Kontroll-Transfektionen. Die Kombination von Hypothermia und Valproinsäure steigerte die Erträge bis zu 90 mg/L Antikörper. Hinzu kam, dass im Durchschnitt 3-5 mal mehr transgene mRNA bis zu 6 Tagen nach der Transfektion in den behandelten Zellkulturen vorhanden war im Vergleich zu den Kontrollkulturen. Mit dem dritten Ansatz wurde nur eine geringe Verbesserung der rekombinanten Erträge erzielt und behandelte Zellkulturen zeigten nur 2 mal soviel transgene mRNA wie die Kontrollkulturen. Wenn spezifische Faktoren wie c-fos, c-jun, NF- κ B und aFGF (acidic fibroblast growth factor) überexprimiert wurden, wurden rekombinante Antikörpererträge von 20 mg/L erreicht, im Vergleich zu 5 mg/L in den Kontroll-Transfektionen. Die Überexpression eines Wachstumsfaktors oder eines Transkriptionsfaktors in Kombination mit Valproinsäure ergab Erträge bis zu 90 mg/L. Jedoch war der Nutzen der überexprimierten Faktoren gering im Gegensatz zu der Wirkung von Valproinsäure. Zusammenfassend kann gesagt werden, dass die Menge und Stabilität der transgenen mRNA ein bestimmender Parameter für die volumetrische Produktivität von TGE in CHO-Zellen ist. Darüberhinaus wurden drei Methoden getestet um die Menge an transgener mRNA in der Zelle und damit die Produktivität zu erhöhen. Die Kultivierung der Zellen unter Hypothermia und die Behandlung mit Valproinsäure waren die zwei erfolgreichsten Strategien. Diese beiden Methoden sind einfach, kostengünstig und skalierbar wodurch die TGE in CHO-Zellen eine attraktive Alternative, wird um rekombinante Proteine im Gramm-Masstab zu produzieren.

Stichwörter: Säugetierzellkulturen, rekombinante Proteine, transiente Genexpression, monoklonale Antikörper, Hypothermia, Histoneacetylase, Transkriptionsfaktoren, Wachstumsfaktoren

List of abbreviations

CHO	Chinese hamster ovary (cells)
CV	column volume
CMV	citomegalovirus
DHFR	dihydrofolate reductase
GFP	green fluorescent protein
ELISA	enzyme linked immunosorbent assay
EF-1 α	human elongation factor 1 α
HEK293	human embryonic kidney 293
IgG	immunoglobulin G
iHDAC	inhibitor of histone deacetylase
NaBut	sodium butyrate
PCV	packed cell volume
pDNA	plasmid DNA
PEI	polyethylenimine
qRT-PCR	quantitative real-time polymer chain reaction
SGE	stable gene expression
TGE	transient gene expression
VPA	valproic acid
VPD	valpromide
WPRE	Woodchuck hepatitis virus posttranscriptional element

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Curriculum Vitae

Chapter 1

Introduction

1.1 Introduction to recombinant proteins

Proteins are macromolecules located everywhere in cells: they are involved in cell growth, metabolism, organization, transport, motility (1). In multicellular organisms, proteins control the immune system, the neuron signalling, the growth and the differentiation of the whole organism (1). Mutation or the abnormal expression of proteins could be the origin of several diseases. Considering that the human genome contains more than 30,000 genes and that the number of proteins produced is even higher (2;3), the opportunities for pharmaceutical companies to develop new drugs are tremendously high (4). Therefore during the last decades, proteins became targets or candidates for development of new treatments.

When proteins were first used as therapeutics, they were purified from animal or human sources. Examples include human growth hormone from human cadavers (5); insulin from pigs or cows (6); and human follicle stimulating hormone from the urine of females (7). However, the use of these sources has many concerns about the safety (5;6), the amount of product harvested and also the patients compliance.

Fortunately, nowadays, products from animal origins are less in use. In fact since the late 1970's, progress in biotechnology has allowed the synthesis of proteins from recombinant DNA using either prokaryotic or eukaryotic (yeast or mammalian) host systems. These proteins are termed "recombinant" (rec- or r-proteins) in order to distinguish them from endogenous proteins and to signify that they are produced from the expression of recombinant genes. The first rec-protein approved for human use was insulin produced in *E. coli* by Eli Lilly (Indianapolis, USA) in 1984 (8). The first rec-protein

produced in a eukaryotic host was tissue-type plasminogen activator (tPA) from Chinese hamster ovary (CHO) cells by Genentech in 1986 (9;10). By 2007 there were more than 170 biopharmaceutical products approved by the Food and Drug Administration (FDA) in the USA, but this number is expected to increase in the coming years, considering that in 2006 about 5,000 potential protein products were reportedly in discovery, preclinical studies and clinical trials (11;12).

Among the host expression systems, the most widely used are *E. coli* and cultivated mammalian cells. About half the therapeutic protein products on the market are made in mammalian cells (9;11;12). Even if mammalian cell culture is more complicated and expensive than microbial cultivation, the former is often preferred because mammalian cells are capable of performing post-translation modifications (PTMs) of proteins, especially glycosylation (12;13). Other hosts capable of glycosylating proteins include yeast and cultivated insect and plant cells. The glycosylation in these hosts differs from that observed in mammalian cells (12); however, efforts are underway to “humanize” the glycosylation in these non-mammalian hosts (14-16). In 2007 the FDA approved the first biopharmaceutical produced in insect cells for use in humans (11). Alternative hosts include transgenic animals and plants, but these are still be in the development phase (12).

1.2 Recombinant protein production in mammalian cells

Most of the mammalian cell-derived rec-proteins for clinical applications are produced in immortalized CHO cells, but other cell lines such as the mouse myelomas NS0 and Sp2/0 and human embryo kidney (HEK293) and baby hamster kidney (BHK-21) cells are also approved for production (9). The recombinant DNA is delivered to cells either by mechanical, chemical, or biological methods. Once it reaches the nucleus it can either be integrated into the genome or remain as an episomal DNA element until it is degraded. Alternatively, RNA virus vectors that replicate in the cytoplasm such as Semliki forest virus and vaccinia virus have been used for transient rec-protein production (17-19).

1.2.1 Stable gene expression (SGE)

The production of rec-proteins for the pharmaceutical market is based on the constitutive expression of the recombinant gene following the gene's integration along with a selectable gene into the cell's genome. Cells having undergone a gene integration event are genetically selected for the selection marker from the transfected cell population to generate clonal recombinant cell lines. The clonal cell lines with the desired phenotype for protein expression and cell growth are used for protein production.

Advantages of this process are:

- I. all cells in the culture are expressing the transgene;
- II. the recombinant DNA is replicated along with the cellular genome and is therefore not lost during cell division;
- III. yields of rec-protein can be as high as 5 g/L in fed-batch cultures have been achieved.

The main disadvantage of this approach is the time (6-12 months) and the cost required for the generation and characterization of the cell lines.

1.2.2 Transient gene expression (TGE)

As an alternative to SGE, rec-proteins can also be produced by TGE. Compared to SGE, it is simpler and faster.

The advantages of TGE are:

- I. there is no need to select for the cells with integrated recombinant DNA;
- II. the time frame from transfection to product purification is short (usually less than 3 weeks);
- III. the simplicity and the versatility of the technique.

TGE provides flexibility in construction of expression vectors; it also permits to change between a wide variety of host cell lines. Therefore, TGE is ideal for the rapid generation of multiple proteins or variants of a single protein (20) and for the production of quantities of rec-protein suitable for preclinical studies.

The differences between TGE and SGE are summarized in Table 1.1. One of the main differences is that genetic selection is not necessary for TGE. The recombinant DNA that reaches the nucleus following DNA delivery can either integrate or not into the host genome; in both cases it will be transcribed. The transfected cells are maintained as a batch or fed-batch culture for a defined period (up to two weeks) and then discarded. During the cultivation period the recombinant DNA can be degraded, transcriptionally inactivated (silenced) via epigenetic pathways (21-24), or “diluted” at each cellular division so that the copy number of plasmid per cell decreases.

Table 1.1. Comparison of SGE and TGE.

	SGE	TGE
Genetic selection	Yes	No
Time from DNA to product	6-12 months	up to 3 weeks
Specific productivity (pg/cell/day)	up to 50	below 10
Volumetric productivity (g/L)	up to 5	0.02 - 0.08
Scalability	up to 20,000 L	up to 100 L
Application	Large scale production of therapeutic rec-protein	Small-scale production of protein for research

The preferred hosts for TGE are CHO or HEK cells (25). These cell lines are approved for therapeutic protein production and can grow in suspension under serum-free conditions (26-28). It is also possible to transiently produce rec-proteins in COS (African green monkey kidney) (29) and BHK cells (30).

Secreted, intracellular, and membrane have been produced by TGE (20;25;31). For the production of intracellular proteins TGE is more suitable than SGE. In fact stable cell lines usually do not express intracellular recombinant proteins to high levels, unless controlled by an inducible promoter (32;33).

TGE has been shown to be scalable up to 100 L (27;28;34), however since the average amount of DNA per one million transfected cells is $\sim 1 \mu\text{g}$, the DNA cost is a factor that should be taken into consideration when choosing TGE for a large scale process.

Another major disadvantage of this technique compared to SGE is the moderate yield of rec-protein. The average yield for either secreted or non-secreted proteins is usually in the range of 10-80 mg/L depending on the cell line used and on the protein itself (25-28). Therefore, the main challenge of TGE is to close up the gap with SGE with regard to volumetric yield. The productivity of TGE depends on several factors including the gene delivery method, the medium, the culture environment, the host cell line, and the expression vector. Comparing HEK293 and CHO cell lines it appears that HEK293 cells produce higher amounts of protein than CHO cells (25). HEK293 cells are also transfected more efficiently than CHO cells when using chemical DNA delivery agents. Recently, a fed-batch TGE process in HEK 293 cells yielded a volumetric productivity of 1 g/L of a monoclonal antibody (35). Thus, for this cell it is possible to achieve by TGE the same volumetric yields obtained by SGE. For TGE in CHO cells, the upper limit for the volumetric yield has probably not been achieved and further developments will be necessary to increase transient rec-protein transient production from this host. Possibilities for further enhancements of TGE include the improvement of low-cost transfection methods and the use of high cell densities processes combined with feeding strategies: these approaches have been shown to be successful for TGE in HEK293 cells (36-38). Additional strategies include genetic modification of the host (39;40); decreasing of the epigenetic regulation of the host cell (41-43) and cell cycle arrest during the production phase (44;45). Some of the characteristics of TGE are summarized in Table 1.2 and explained in more detail in this chapter.

Table 1.2. Summary of TGE characteristics.

Host cells used	CHO, HEK, COS, BHK
Categories of proteins produced	Secreted, intracellular, membrane
Amount of DNA per million of cells	About 1 μg
Vectors used for TGE	Both non-viral and viral
DNA delivery methods for TGE	Chemical delivery (usually)
Advantages	Fast, simple, versatile
Disadvantages	Low yields and high cost of DNA
Key factors for TGE	Host cell, gene delivery method, medium, vectors,
Highest titer	1 g/L in HEK293 cells
Possible improvements	High cell densities transfection, feeding strategies, genetic modification of the host, enhanced transcription, cell cycle arrest during production

Currently, there are no therapeutic rec-proteins on the biopharmaceutical market that are produced by TGE. This is mainly due to the low yields from TGE process and the high cost of plasmid DNA, limiting the production of enough protein to meet market needs. There are also concerns about the heterogeneity of the product from batch to batch. However, it should be mentioned that some PTMs, like glycosylation, are dependent on the cell culture process (46;47), therefore it is also possible to find variability from batch to batch and within batches in SGE-derived products.

1.2.3 Vectors used for TGE

Non-viral vectors are the most commonly used ones in TGE. These plasmid vectors usually contain a strong, constitutive promoter, optimized mRNA processing and translational signals (i.e. Kozak sequence, mRNA cleavage and polyadenylation signals), and prokaryotic elements for plasmid amplification and selection in bacteria (48). The transcriptional promoters used for TGE are mainly from viral sources but cellular promoters are also employed. The immediate early cytomegalovirus (CMV) promoter/enhancer from mouse (mCMV) or human CMV (hCMV) and the simian virus 40 (SV40) early promoter/enhancer are the most frequently used elements (25;48), but the adenovirus major late promoter and the Rous sarcoma virus promoter are alternatives (48). Among the cellular promoters, that from the human elongation factor 1- α (EF-1 α) gene has been shown to be as active as the viral promoters listed above in terms of recombinant gene expression (25;49). Other elements such as an intron can be added. The presence of an intron in the 5' untranslated region of the transgene has been shown to increase recombinant protein production (50).

Plasmid DNA for TGE is usually produced by amplification in bacteria and purified using ion exchange chromatography. To achieve a high transfection efficiency, purified pDNA should not contain contaminants traces of bacterial RNA or protein, since these components may interfere with DNA delivery (51).

1.2.4 Chemical gene delivery

Gene delivery can be performed using mechanical, chemical, or biological methods. The mechanical methods include microinjection (52) and electroporation (53). The chemical transfection methods depend on complex formation between positively charged

molecules such as calcium phosphate (CaPi) (54-57), lipids (58;59), or cationic polymers (60) and negatively charged DNA. Biological gene delivery is usually performed with viruses, especially retroviruses (61;62). Chemical delivery is the most widely chosen method for TGE (25), especially with low cost compounds such as calcium phosphate (34;54;56;57) and polyethylenimine (PEI) (26;27;60;63).

CaPi transfections have been performed with different cell lines at volumetric scales up to 100 L (34). The DNA/CaPi co-precipitate is produced by mixing CaCl_2 and DNA and then adding phosphate. Thus the DNA is entrapped in the CaPi precipitate (57;64;65). After addition to the culture and binding to the cell surface, the complexes enter cells by phagocytosis (66). After disruption of precipitate-containing vesicles, the DNA is released in the cytoplasm and probably enters the nucleus when the nuclear membrane is disrupted during mitosis (57;67;68). The major disadvantage of this method, however, is its reliance on serum in the culture medium at the time of transfection (69).

Cationic lipids have been shown to complex with DNA and enter cells, therefore they are widely used both *in vivo* and *in vitro* to deliver drugs or macromolecules (59;61;70). It was a common belief that liposomes were able to deliver their “cargo” by fusing with phospholipids within cell membrane. Now, it has been shown that only a small percentage of liposomes enter cells in this way. Most of them are internalized through endocytosis (61). However, the main disadvantage of these lipid-based chemicals is the high cost; therefore they are not a suitable strategy for large scale transfection.

Polycations such as PEI (60;71), DEAE-dextran (72), or polylysine (73;74) are widely used for efficient gene delivery to mammalian cells. Among them, PEI is the most commonly employed in TGE due to its low cost and high efficiency for DNA delivery (25-27;36;39;63;75). Due to its positive charge, PEI is able to interact with DNA to form positively charged complexes that bind to the negatively charged proteoglycans present on the surface of the cell membrane and enter the cell by endocytosis (61;76). Many theories have been proposed about the escape of the complexes from the endosome (60;61;77), however the “proton sponge” mechanism (60;78) is still the most accepted one. PEI is a polycation containing amino groups with buffering capacity. The degree of protonation of the amine increases from 20 to 45% as pH decreases from 7 to 5 (78). Endosomes are usually acidified by the cellular ATPases present on the membrane of these organelles. The influx of protons is accompanied by an influx of Cl^- ions to neutralize the positive charge. This ion influx increases the osmolarity in the endosomes. Subsequently, a water influx causes swelling and disruption of the endosomes and the release of the PEI-DNA

complexes. Once in the cytosol, the fate of PEI-DNA complexes, especially their delivery to the nucleus, is not fully understood. However, DNA can be released from the complexes in the presence of a competing polyanion, such as RNA or DNA, present in the cytoplasm or nucleus (51). The main advantage of PEI-mediated transfection is that it is efficient in serum-free medium (27). In fact, the presence of serum may have deleterious effect on transfection efficiency. There is evidence that protein in the medium disrupts PEI-DNA complexes and decreases transfection efficiency (*Duhr and Wurm, unpublished data*).

1.3 Research objectives

Prior to the performance of the research described here, the highest yield of rec-protein produced in CHO cells by TGE was 10-20 mg/L (26;27). At the onset, the aim of this thesis was to determine some of the limiting factors in TGE in CHO cells and to propose strategies to solve the problem.

Experiments showing that the transgene mRNA level and not the level of pDNA uptake is a limiting factor for TGE are described in Chapter 3. The amount of pDNA transfected did not correlate with transgene mRNA and rec-protein levels. However, transgene mRNA levels and rec-protein levels were shown to correlate. In the subsequent chapters experiments to develop three different strategies for enhancing transgene mRNA levels and TGE are described.

TGE under hypothermic conditions is described in Chapter 4. It was shown that hypothermia is able to modify cell metabolism and also to increase transgene mRNA amounts relative to the control transfections at 37°C. The system is easily scalable and rec-protein yields obtained were in the range of 60-80 mg/L.

The effect of valproic acid (VPA), a histone deacetylase inhibitor, on TGE is described in Chapter 5. As in the previous chapter, the transgene mRNA levels were higher and more stable over time as compared to transfections at 37°C. The transient rec-protein yield reached 90 mg/L when VPA was combined with hypothermic treatment.

In Chapter 6 the possibility of enhancing transcription by overexpression of either transcription or growth factors was explored. Some factors increased rec-protein production by ~3-fold and mRNA levels by 2-fold relative to the control transfection, however IgG titers were lower compared to the ones obtained under hypothermic conditions or in the presence of VPA.

Additional experiments describing the establishment of an inducible system for the overexpression of transcription factors in CHO cells are reported in the Appendix 1. Finally, the purification and partial characterization of the recombinant monoclonal antibody used as reporter protein in all the experiments is reported in Appendix 2.

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Chapter 2

Materials and methods

2.1 Cell culture

Suspension-adapted CHO DG44 cells (*dhfr*^{-/-}) were routinely cultivated in ProCHO5 medium (Lonza, Verviers, Belgium) supplemented with 0.68 mg/L hypoxanthine, 0.194 mg/L thymidine, and 4 mM glutamine (SAFC Biosciences, St. Louis, MO) in orbitally 250-mL square shaped bottles shaken at 110 rpm in a ISF-4-X incubator (Adolf Kühner AG, Birsfelden, Switzerland) as previously described (1). Temperature and humidity of the incubator were kept constantly at 37°C and 20%, respectively. Manual counting was performed with a Neubauer hemocytometer. Cell viability was assessed using the Trypan blue exclusion method. Biomass was determined by the packed cell volume (PCV) method using ValuPac tubes (Sartorius AG, Göttingen, Germany): cells were loaded in the tube and centrifuged at 2500 g for 1 minute as previously described (2). Under standard cultivation conditions at 37 °C, a cell density of 1 x 10⁶ cells/mL was equivalent to a PCV of 0.175% for CHO DG44 cells (2).

2.2 Transfection

One day prior to transfection, cells were seeded at a PCV of 0.35% in the appropriate volume of fresh ProCHO5 in a square-shaped glass bottle. On the day of transfection, the cells were centrifuged at 800 rpm for 5 min and resuspended in fresh, supplemented ProCHO5 (if not otherwise specified) at 0.70% PCV. Transfections (if not otherwise specified) were performed in CultiFlask 50 tubes (Sartorius AG) (3). Each transfection was

performed in 5 mL of culture using 12.5 µg of DNA and 50 µg of linear 25 kDa PEI (4). The DNA and PEI were each diluted in 250 µL of 150 mM NaCl, mixed, and then allowed to stand for 10 min at room temperature prior to addition to the cells. The transfected cultures were incubated in a ISF-4-X incubator (Adolf Kühner AG) at 37 °C in 5% CO₂ and 85% humidity with agitation at 180 rpm (4). After 4 h, the cultures were diluted with 5 mL of ProCHO5 with supplements (if not otherwise specified). For temperatures other than 37 °C, the cultures were maintained in an ISF-1-W incubator-shaker (Adolf Kühner AG) in the presence of 5% CO₂ and 85% humidity.

2.3 Plasmids

2.3.1 pEGFP-N1

pEGFPN1 expressing the enhanced green fluorescent protein (GFP) gene was purchased from ClonTech (Palo Alto, CA, USA) (Figure 2.1).

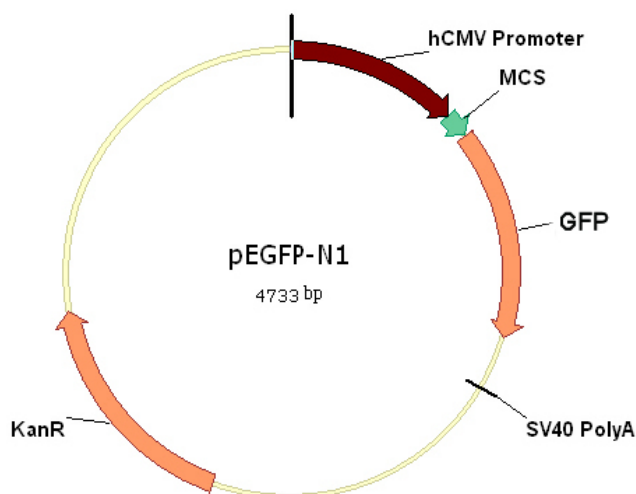


Figure 2.1. Schematic diagram of pEGFPN1. The plasmid contains the human cytomegalovirus promoter (hCMV promoter), a multiple cloning site (MCS), the eGFP, the polyadenylation sequence from SV40 (SV40 PolyA) and the resistance to Kanamycin (KanR) for bacterial selection.

2.3.2 pEAK8-LH39, pEAK8-LH41, pKML and pKMH

The expression vectors for the human IgG light (pEAK8-LH39 and pKML) and heavy chain (pEAK8-LH41 and pKMH) genes have been described previously (5;6) (Figure 2.2).

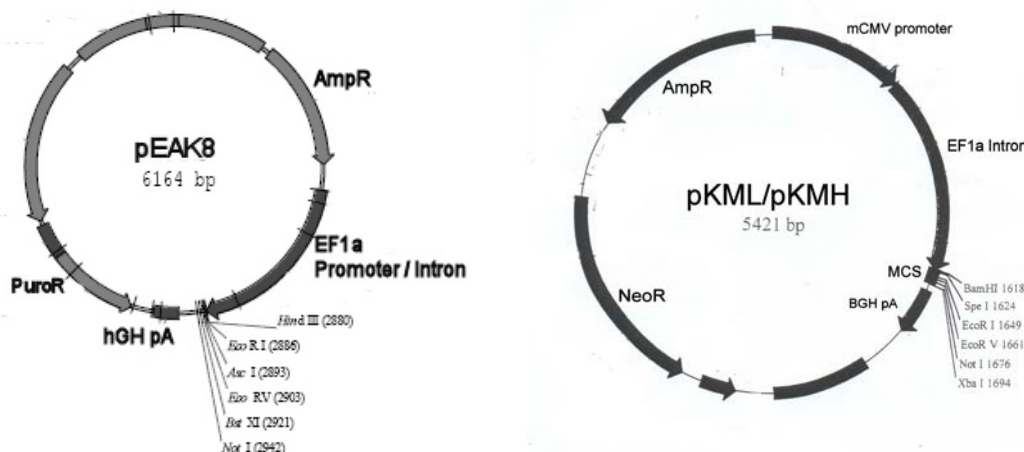


Figure 2.2. Schematic diagrams of pEAK8 and pKMH/pKML. pEAK8 contains the elongation factor 1- α (EF1 α) promoter fused with the EF1 α intron, human growth hormone (hGH) polyA, Puromycin resistance (PuroR) for mammalian selection and Ampicillin resistance (AmpR) for bacterial selection. pKML/H contains the mouse cytomegalovirus promoter (mCMV promoter), EF1 α intron, bovine growth hormone (BGH) polyA, Neomycin resistance (NeoR) for mammalian selection and AmpR for bacterial selection.

2.3.3 pXLG^{HEK}

The IgG light and heavy chain cDNAs from pKML and pKMH and the GFP gene from pEGFP-N1 were individually subcloned into pXLG^{HEK} to produce pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP, respectively, as described (7;8) (Figure 2.3).

pXLG^{HEK}- Δ WPRES-RhLC and pXLG^{HEK}- Δ WPRES-RhHC were generated through removal of the WPRES by restriction digestion of pXLG^{HEK}-RhLC and pXLG^{HEK}-RhHC, respectively, with BamHI and BglIII followed by religation.

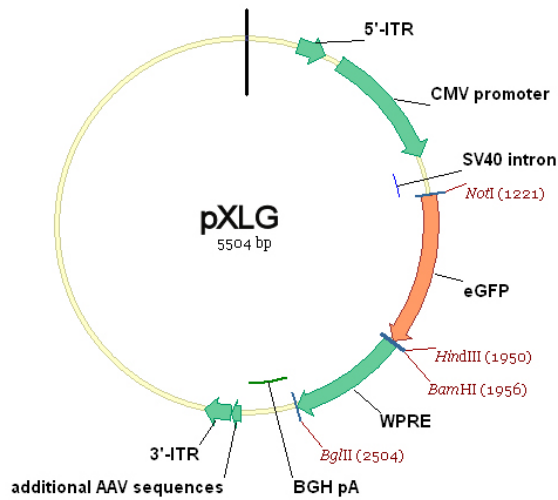


Figure 2.3. Schematic diagram of pXLG^{HEK}-EGFP. The plasmid contains inverted terminal repeats (ITR) before and after the mammalian expression cassette, the hCMV promoter, the intron from SV40, the eGFP, the Woodchuck hepatitis post-transcriptional regulatory element (WPRE), BGH polyA and additional sequences from adeno-associate viruses (AAV).

2.3.4 pMYKEF1-puro

pMYKEF1-puro and pMYKEF1-EGFP-puro have been described previously (9) (Figure 2.4).

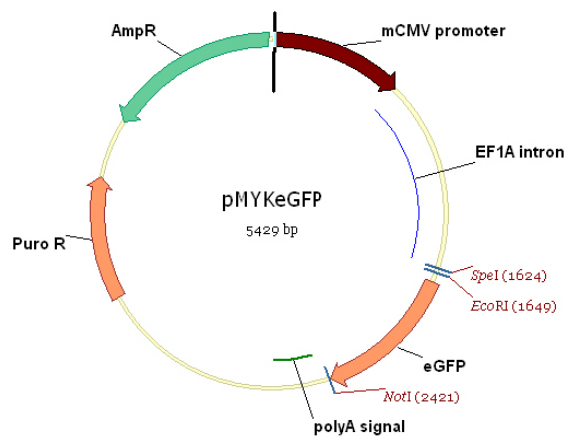


Figure 2.4. Schematic diagram of pMYKEF1-EGFP-puro. The plasmid contains the same elements described for pKML/pKMH except for the Neomycin resistance that was substituted with Puromycin resistance (PuroR).

2.3.5 Cold induced proteins

The CIRBP and Rbm3 cDNAs were purchased from ImaGenes (ImaGenes GmbH, Berlin, Germany) and amplified by PCR with specific primers (see Table 2.1) to create a SpeI restriction site at the 5' end and an EcoRI site at the 3' end of the coding region. The amplified sequences were then ligated into pMYKEF1 vector, previously digested with SpeI and EcoRI in order to generate pMYK-CIRBP and pMYK-Rbm3, respectively.

2.3.6 Transcription factors

All cDNAs coding for transcription factors were purchased from ImaGenes GmbH, amplified by PCR with specific primers (see Table 2.1) to create a NotI restriction site at the 5' end and an HindIII site at the 3' end of the coding region, and cloned into pXLG^{HEK}. For the NF- κ B cDNA, a two-step cloning scheme was necessary due to the length of the gene.

Table 2.1. Primers used for PCR amplifications of transcription factor cDNAs.

Gene	5' - Primer ^a	3' - Primer ^a
CIRBP	ACTAGTATGGCATCAGATGAAGGC	GAATTCCTACTCGTTGTGTGTAGC
Rbm3	GAATTCTCAGTTGTCATAATTGTC	ACTAGTATGTCCTCTGAAGATGGA
c-fos	GCGGCCGCCATGATGTTCTCGGGCTTCA	AAGCTTATCACAGGGCCAGCAGCGTGGG
c-fos^b	AGGCGAATTCATGATGTTCTCGGG	ACCGGGACACTTCGAACCTAGGTT
c-jun	GCGGCCGCCATGACTGCAAAGATGGAAA	AAGCTTATCAAAATGTTTGCAACTGCTG
NF-κB	GCGGCCGCCATGGCAGAAGATGATCCAT CTGACTTGAACTAGTGAACCAAAC	GTTTTGGTTCACTAGTTTCAAAGTCAG AAGCTTACTAAATTTTGCCTTCTAGAGG
CREB	GCGGCCGCCATGACCATGGAATCTGGAG	AAGCTTATTAATCTGATTTGTGGCAGTA
Sp1	GCGGCCGCCATGAGCGACCAAGATCAC	AAGCTTATCAGAAGCCATTGCCACTGAT
Elk1	GCGGCCGCCATGGACCCATCTGTGACGC	AAGCTTATCATGGCTTCTGGGGCCCTGG

^a The sequences are reported in the 5' – 3' sense.

^b The sequences reported are the primers used for cloning of c-Fos in the gas inducible plasmid, pWW192 (see Section 2.3.7)

2.3.7 Gas-inducible vectors

pWW195 and pWW192 (Figure 2.6) were kindly provided by Dr. Wilfred Weber (ETHZ, Zürich, Switzerland) and they were previously described in literature (10). pWW192GFP was generated through removal of the SEAP cDNA and the internal ribosome entry site (IRES) from pWW192 by restriction digestion with EcoRI and NotI and cloning of the GFP gene after its recovery by restriction digestion from pMYKEF1-EGFP-puro.

The c-fos cDNA was amplified by PCR from pXLG^{HEK}-c-fos. Specific primers (reported as c-fos^b in Table 2.1) were used to create a EcoRI site at the 5' and a HindIII site at the 3' of the PCR product. The amplified cDNA was then ligated into pWW192 after digestion with EcoRI and HindIII.

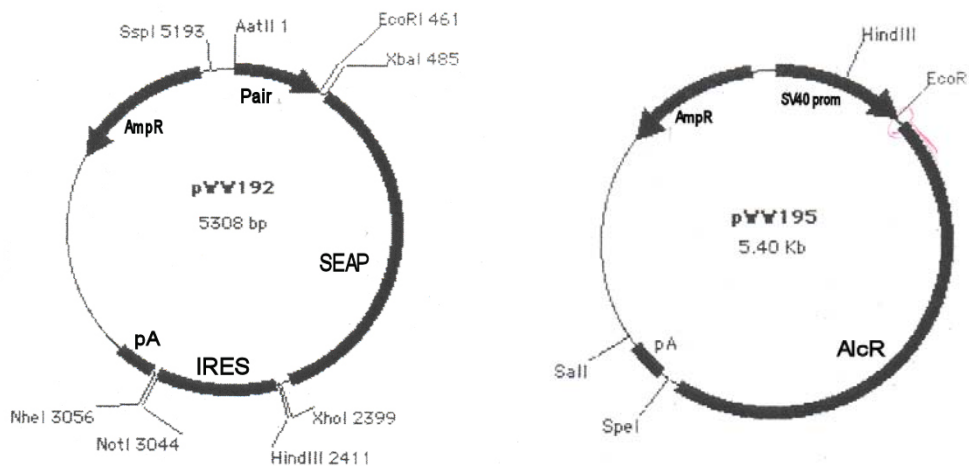


Figure 2.6. Schematic diagrams of pWW192 and pWW195. pWW192 contains a promoter (P_{air}) composed by the *A. Nidulans* operon responsive to acetaldehyde and a minimal hCMV promoter, the sequence codifying for the secreted alkaline phosphatase (SEAP), an internal ribosome entry site (IRES), a poly adenylation site (pA) and AmpR for bacterial selection. pWW195 expresses a constitutive SV40 promoter (SV40 prom), the sequence of the acetaldehyde responsive element (AlcR), a polyA site.

2.3.8 Summary of plasmid elements

Table 2.2 reports the primary elements (promoters, introns, mammalian resistance and other sequences) present on the vectors used in this work.

Table 2.2. Primary transcriptional elements of the plasmids used in this study.

Plasmid	Promoter	Intron	Selection marker	Other elements
pEGFPN1	hCMV	-	-	-
pEAK8	EF-1 α	EF-1 α	Puromycin	-
pMYKEF1	mCMV	EF-1 α	Puromycin	-
pKMH/pKML	mCMV	EF-1 α	Neomycin	-
pXLG ^{HEK}	hCMV	SV40	-	WPRE and AAV ITRs
pWW195	SV40	-	-	-
pWW192	hCMV _{min} + OAlcR	-	-	IRES

2.4 Plasmid purification and quantification

Plasmid DNA for transfection was purified using a NucleobondAX anion exchange column (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol and stored in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4). The purified plasmid was quantified by UV absorbance at 260 nm.

2.5 Nutrient and metabolite quantification

After transfection, 500 μ L aliquots of culture were centrifuged, and the supernatant was used to quantify the levels of glucose, glutamine, ammonium, and lactate using the RX Daytona chemistry analyzer (Radox Laboratories Ltd., Crumlin, UK).

2.6 Cell size analysis

After transfection, 20 μ L aliquots of culture were diluted with 10 mL of CasyTon solution (Innovatis AG, Durmersheim, Switzerland), and the mean cell volume and cell diameter were quantified with a Casy Counter (Innovatis AG).

2.7 mRNA extraction and quantification

At different times after transfections, 1×10^6 cells were collected by centrifugation and washed twice with sterile PBS. Total RNA was extracted using the GenElute mRNA kit (Sigma-Aldrich GbmH, Buchs, Switzerland) according to the manufacturer's protocol. Samples were treated with 1 U of DNase I (Invitrogen AG, Basel, Switzerland) for 15' at room temperature, the enzyme was then inhibited by addition of EDTA and by heating. First-strand cDNA synthesis was performed from 1 μ g of RNA with the M-MLV reverse transcriptase (Invitrogen AG) using oligo dT (New England Biolabs, Ipswich, MA) as the primer. Each reaction was diluted 10-fold in RNase-free water for quantitative PCR. The oligonucleotide primers for the amplification (Table 2.3) of cDNAs were purchased from SAFC Biosciences (St. Louis, MO, USA).

Table 2.3. Primers used for qPCR in this study.

Gene	5'- Primer ^a	3' - Primer ^a
β-Actin	GCTCTTTTCCAGCCTTCCTT	GAGCCAGAGCAGTGATCTCC
LC	TGTCTTCATCTTCCCGCCA	GCGTTATCCACCTTCCACTGT
HC	AAGGCTTCTATCCCAGCGACA	GCATCACGGAGCATGAGAAG

^a The sequences are reported in the 5' – 3' sense.

The samples from the first-strand cDNA synthesis were mixed with the appropriate oligonucleotide primer pair and the reaction mix from the Absolute qPCR SYBR Green ROX mix (Axon Lab AG, Baden-Dättwil, Switzerland) and amplified according to the manufacturer's protocol. All experiments were performed on an ABI 7700 Real Time PCR instrument (Applied Biosystems, Foster City, CA, USA). All samples were analyzed in triplicate. Collected data were processed using SDS software (Applied Biosystems) to obtain Ct values. The comparative Ct method was used to calculate the relative quantity of

IgG light and heavy chain mRNAs as normalized to the quantity of β -actin mRNA (Equations 2.1, 2.2, and 2.3) (11). Non-transfected cells were used as calibrator.

$$\Delta C_T = C_{t \text{ sample}} - C_{t \text{ HKG}} \quad (\text{eq. 2.1})$$

$$\Delta\Delta C_t = \Delta C_{t \text{ sample}} - \Delta C_{t \text{ calibrator}} \quad (\text{eq. 2.2})$$

$$\text{Normalized mRNA levels} = 2^{-\Delta\Delta C_t} \quad (\text{eq. 2.3})$$

Equations used for normalize mRNA levels. The delta Ct (ΔC_T) was calculated by subtracting the value of Ct obtained for the housekeeping gene to the value of Ct of the sample (eq. 2.1). To obtain the delta delta Ct ($\Delta\Delta C_t$) the ΔC_t of the calibrator was subtracted to the ΔC_t of the sample (eq. 2.2). The normalized mRNA levels were obtained by elevating 2 to the $-\Delta\Delta C_t$.

2.8 Total DNA extraction from mammalian cells

At different times after transfection, 1×10^6 cells were harvested and treated with 100 U of DNase I (Roche SA, Rotkreuz, Switzerland) for 15 min at 37°C. The enzyme was inhibited by addition of 25 mM of EDTA followed by heating at 65°C for 10 min. Pellets were frozen at -20°C and then thawed or directly treated with 500 μ L of TENS buffer for 3 h at 50 °C as described (12). Total DNA was then extracted with 500 μ L of phenol/chloroform/isoamyl alcohol (IAA) (24:25:1) (Sigma-Aldrich GmbH). The aqueous phase was transferred to an Eppendorf tube and treated with 200 μ g/mL of RNase at 37 °C for 1 h. A second step of phenol/chloroform/IAA extraction was performed in order to remove the RNase. Two volumes of cold 100% ethanol and 0.5 volumes of cold 7.5 M $\text{NH}_4\text{Acetate}$ were then added. The DNA was allowed to precipitate overnight at -20 °C. After centrifugation, the DNA pellet was washed with 70% ethanol and resuspended in TE buffer. Total DNA was quantified by UV absorbance at 260 nm.

2.9 Quantification of plasmid copy number per cell

DNA purified from mammalian cells was diluted with ultrapure milliQ water, mixed with Absolute qPCR SYBR Green ROX mix (Axon Lab AG) and amplified according to the manufacturer's protocol. Primers used for amplification of the polyA region of pXLG^{HEK} vector were reported in Table 2.4. A known amount of pXLG^{HEK}EGFP plasmid quantified by UV absorbance was used to create a copy number standard curve.

Table 2.4. Primers used for qPCR of the BGH polyA element in pXLG^{HEK}.

Target	5' - Primer ^a	3' - Primer ^a
BGH pA	CTTCTAGTTGCCAGCCATCTGTT	AGAATGACACCTACTCAGACAATGC

^a The sequences are reported in the 5' – 3' sense.

2.10 Transfection efficiency analysis

Transfection efficiency based on exogenous GFP expression was analyzed using a GuavaEasyCyte flow cytometer (Guava Technologies, Hayward, USA) with the Guava Express Plus assay. Data were processed with the Guava Cyto SoftTM software (Guava Technologies).

2.11 GFP fluorescence quantification

For GFP analysis, 100 μ L of culture was lysed by addition of 1 volume of 1% Triton X-100 in PBS. After incubation at 37 °C for 1 h with agitation, the fluorescence was measured using a TECAN Sapphire II plate-reading fluorometer (TECAN, Männedorf, Switzerland). GFP was excited at 485 nm with a bandwidth of 10 nm, and the emission fluorescence was measured at 515 nm with a bandwidth of 10 nm.

2.12 Recombinant IgG quantification

IgG concentration was determined by sandwich ELISA using a goat anti-human kappa light chain antibody (BioSource, Lucerne, Switzerland) for capture and alkaline phosphatase-conjugated goat anti-human IgG (BioSource) for detection (13).

2.13 Recombinant IgG purification

Recombinant antibody was partially purified by affinity chromatography using Streamline Protein A (GE Healthcare, Uppsala, Sweden). A PolyPrep column (BioRad, Hercules, CA) containing Streamline Protein A was equilibrated with 5 column volumes (CVs) of 50 mM sodium phosphate (pH 7.0), and then 10 mL of the clarified culture medium was loaded. The column was washed with 10 CVs of 50 mM sodium phosphate (pH 7.0), and IgG was eluted with 2 CVs of 0.1 M sodium citrate (pH 3.0) and immediately buffered with 10% (v/v) 1 M Tris-HCl (pH 8.0). The neutralized eluate was desalted and concentrated, and the buffer was replaced with deionized water or 10 mM sodium phosphate pH 7.2 using a Microcon or Centricon centrifuge filter with 10 KDa cut off (Millipore, Bedford, MA, USA).

2.14 Recombinant IgG deglycosylation

The antibody was treated with PNGase F (QAbio, Palm Desert, CA) for 12 hours at 37°C, according to the manufacturer's protocol and analyzed on a 4-12% polyacrylamide gradient gel (Invitrogen) in reducing conditions. Proteins were visualized by Coomassie Blue staining.

2.15 Intracellular proteins extraction

Three days after transfection, five millions of cells were harvested and centrifuged at 13000 rpm for 5 min. Supernatant was discharged and pellets were washed three times with cold PBS 1x. Samples were then treated with M-PER Mammalian Protein Extraction Reagent (Pierce – Thermo Scientific, Rockford, IL) according to the manufacturer's protocol and either immediately loaded on an SDS-PAGE gel or stored at -20°C.

2.16 SDS-PAGE and Western Blot analyses

TRIS-Glycine SDS-PAGE gels with different acrylamide concentrations were prepared and run using a Mini Protean 3 Cell system (BioRAD). Samples were mixed with 2x sample buffer, boiled for 5 min and then electrophoresed at 100 V for the stacking gel and at 150 V for the separating gel buffer.

After electrophoresis the gels were either stained with Coomassie Blue or transferred onto a PVDF membrane (Millipore) for immunoblotting. The transfer was done at 60 mA for 1 h at 4 °C. The membrane was then blocked with 10 mL of blocking solution for 1 h at room temperature (or overnight at 4 °C). The solution was discarded and the membrane was incubated for 1 h at room temperature (or overnight at 4 °C) with 10 mL of blocking solution containing the primary antibody (Table 2.6). The membrane was then washed three times with washing solution and incubated with the secondary antibody-HRP conjugated (Table 2.6) diluted in blocking solution. After two washes in washing solution, the membrane was treated with ECL reagent (GE Healthcare) and developed on Hyperfilm (GE Healthcare).

Table 2.6. Primary and secondary antibodies used for Immunoblotting.

Target Protein	Primary Antibody			Secondary Antibody	
	Host	Supplier	Dilution	Supplier	Dilution
CIRBP	Rabbit – polyclonal	ProteinTech, IL, USA	1:200	Sigma, MO, USA	1:10000
Rbm3	Rabbit – polyclonal	Scripps Research Institute, CA, USA (14)	1:2000	Sigma, MO, USA	1:10000
c-FOS	Rabbit – polyclonal	Calbiochem, CA, USA	1:1000	Sigma, MO, USA	1:10000
aFGF	Mouse – monoclonal	Sigma, MO, USA	1:1000	Sigma, MO, USA	1:50000

2.17 Buffers

TENSK: 25 mM Tris pH 8.0
 10 mM EDTA
 200 mM NaCl
 1% SDS
 500 µg/ml Proteinase K

TE: 10 mM Tris-HCl pH 7.4
 1 mM EDTA

PBS 10x: 30 mM NaH₂PO₄•H₂O
 70 mM Na₂HPO₄•2H₂O
 1.5 M NaCl

Coomassie Blue Stain: 0.25% (w/v) Coomassie Blue R-250
 20% (v/v) isopropanol

Coomassie Blue Destain: 7% (v/v) acetic acid

4x Separating Buffer for SDS-PAGE: 1 M Tris-HCl pH 8.8

4x Stacking buffer for SDS-PAGE: 1.5 M Tris- HCl pH 6.8

10x Running buffer for SDS-PAGE: 0.25 M Tris
1.9 M glycine
1% (w/v) SDS

2x Loading buffer: 25% (V/V) 4x stacking buffer
20% (v/v) glycerol
0.5% (w/v) Bromophenol Blue
4% (w/v) SDS
10% (v/v) 2-mercaptoethanol

1x Transfer Buffer: 25 mM Tris
190 mM glycine
20% (v/v) methanol

Blocking solution for western blot: PBS 1x
0.1% (v/v) Tween 20
3% (w/v) dry milk

Washing solution for western blot: PBS 1x
0.1% (v/v) Tween 20

2.18 References

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Chapter 3

Relevance of mRNA level in transient gene expression

3.1 Introduction

TGE in CHO cells is a well-established and scalable technique (1-4). However, the highest reported yields of secreted recombinant proteins in this host have been about 10-20 mg/L after six days of production (1-3;5). The main cause(s) of low TGE yields in CHO cells have not been yet determined. However, the exposure of transfected CHO cells to hypothermic conditions (30-33 °C) (6;7) or histone deacetylase inhibitors (8-11) enhanced TGE yields significantly, meaning that improvements are possible for this host system.

This chapter focuses on the role of pDNA amounts and mRNA level in TGE in CHO cells under standard transfection conditions at 37°C in order to begin to understand the molecular limitations of this host. First, it was observed that the pDNA copy number per cell did not correlate with the level of transgene mRNA. Instead, there appeared to be an upper limit to the amount of transgene mRNA produced by transfected cells. It was also observed that recombinant protein yield was strongly dependent on the mRNA level. Moreover, it was found that the amount of transgene mRNA decreased rapidly after reaching an optimum after transfection. Furthermore, protein expression from three different vectors was compared. In all cases, the transgene mRNA level correlated with the recombinant protein yield. Thus, methods to increase the transgene mRNA level appeared to be critical for enhancing TGE yields in CHO cells; therefore, the remaining chapters of this thesis describe experiments to address this problem in more detail.

3.2 Results

3.2.1 DNA amount used for transfection

The amount of DNA needed for optimal TGE yield was investigated by transfecting CHO DG44 cells with various amounts of a mix of three plasmids (pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP at a mass ratio of 49:49:2). The amount of PEI was also varied to keep the PEI:DNA ratio constant (4:1). Steady-state plasmid DNA and transgene mRNA levels were determined by qRT-PCR and the yield of IgG was measured by ELISA. The plasmid copy number per cell increased as the pDNA amount transfected increased (Figure 3.1).

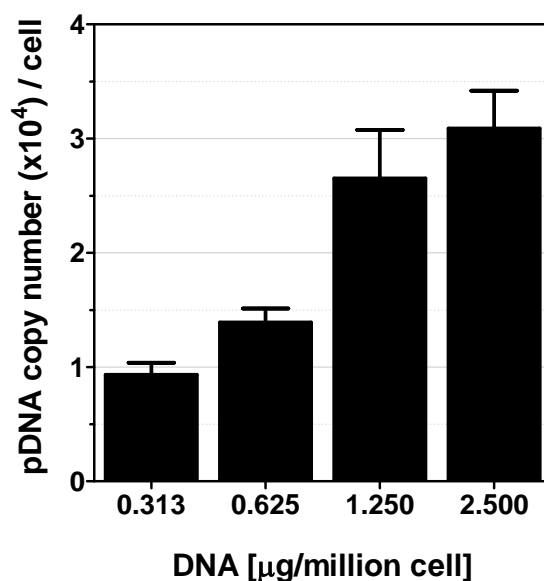


Figure 3.1. Analysis of plasmid copy number after PEI-mediated transfection with increasing amounts of DNA. CHO DG44 cells were transfected with pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP at a 49:49:2 ratio (w/w/w) with increasing amounts of DNA as indicated. Four hours post-transfection the pDNA was extracted and quantified by qRT-PCR. Errors bars represent the standard deviation from two parallel experiments

The increasing amount of pDNA copy number per cell described above did not correlate with the steady-state level of transgene mRNA or the IgG yield in these transfections. The maximal transgene mRNA level and IgG yield were reached when cells were transfected with 0.625 μg of DNA/million cells (Figure 3.2). The levels of transgene mRNA and IgG then decreased as the amount of pDNA per cell increased (Figure 3.2). The stability of pDNA over time was also analyzed by qPCR. The maximum pDNA copy number per cell was found at 4 h post-transfection, then the pDNA copy number declined with time (Figure 3.3).

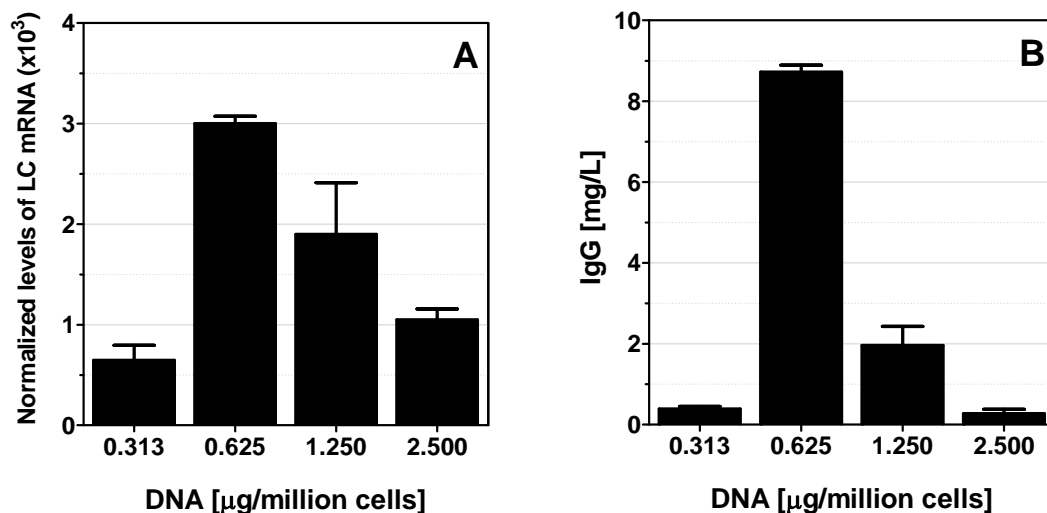


Figure 3.2. Effect of pDNA amount on transgene mRNA levels and recombinant protein production after PEI-mediated transfection. CHO DG44 cells were transfected with pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP at a 49:49:2 ratio (w/w/w) with increasing amounts of DNA as indicated. (A) At day 3 post-transfection total mRNA was extracted and quantified by qRT-PCR. The delta-delta-Ct method was used to calculate the relative quantity of light chain mRNA using β -actin mRNA and mRNA from non-transfected cells for normalization. (B) At the same time IgG yield were measured by ELISA. Errors bars represent the standard deviation from two parallel experiments.

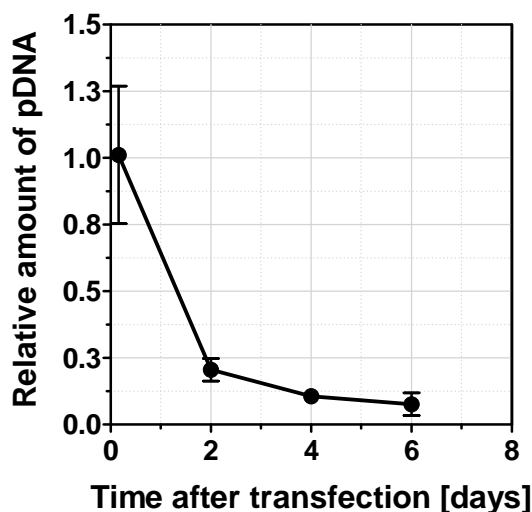


Figure 3.3. pDNA stability over time in transiently transfected CHO cells. CHO DG44 cells were transfected with pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP at a 49:49:2 ratio (w/w/w). At the times indicated, one million cells were harvested, and the pDNA was extracted and analyzed by qRT-PCR. A known amount of pDNA was used to create the standard curve. Error bars represent the standard deviation from two parallel experiments.

3.2.2 Influence on TGE of vectors used for transfection

To determine the variation in transient recombinant protein yield with different expression vectors, CHO DG44 cells were transfected with three different combinations of plasmids: (1) pEAK8-LH39, pEAK8-LH41, and pEGFP-N1 at a ratio of 29:69:2 (w/w/w); (2) pKML, pKMH, and pMYKpuro-EGFP at a ratio of 49:49:2 (w/w/w); and (3) pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP at a ratio of 49:49:2 (w/w/w). The ratios for the IgG light and heavy chain plasmids for the different vector sets were individually optimized previously for transient expression at 37 °C (4;12;13). Total GFP-specific fluorescence was measured 3 days post-transfection, whereas IgG yields were quantified over a time period of 6 days after transfection. The highest GFP-specific fluorescence was observed after transfection with pMYKpuro-EGFP, whereas GFP expression was lower for transfections with pXLG^{HEK}-EGFP and pEGFPN1 (Figure 3.4).

Antibody expression over time also varied with the three sets of plasmids. With pKML/pKMH and the pXLG^{HEK} vectors, the IgG production peaked at 3 days post-transfection, whereas using the pEAK8 vectors the maximal IgG level was found at day 5 post-transfection (Figure 3.5).

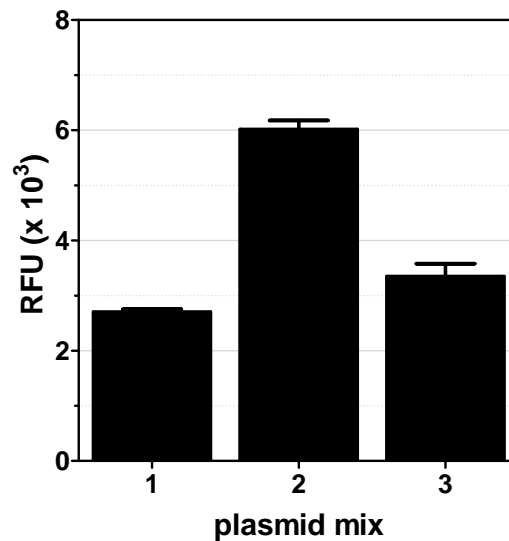


Figure 3.4. Transient GFP-specific fluorescence using different vectors for transfection. Cells were transfected with (1) pEAK8-LH39, pEAK8-LH41, and pEGFP-N1 at a ratio of 29:69:2 (w/w/w); (2) pKML, pKMH, and pMYKpuro-EGFP at a ratio of 49:49:2 (w/w/w); and (3) pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP, at a ratio of 49:49:2 (w/w/w). At day 3 post-transfection the GFP-specific fluorescence was measured by fluorometry. Errors bars represent the standard deviation from three parallel experiments.

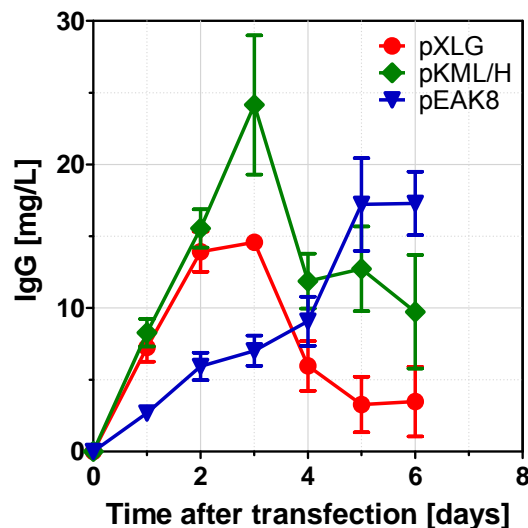


Figure 3.5. Transient IgG production over time after transfection with different vectors. Cells were transfected with (pXLG) pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP, at a ratio of 49:49:2 (w/w/w); (pKML/H) pKML, pKMH, and pMYKpuro-EGFP at a ratio of 49:49:2 (w/w/w); and (pEAK8) pEAK8-LH39, pEAK8-LH41, and pEGFP-N1 at a ratio of 29:69:2 (w/w/w). At the times indicated the IgG concentration was quantified by ELISA. Errors bars represent the standard deviation from three parallel experiments.

3.2.3 Correlations between mRNA and protein levels

To determine if the level of transgene mRNA correlates with recombinant protein yield by TGE, CHO cells were transfected with pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP at a ratio of 49:49:2 (w/w/w), and the IgG light chain mRNA level and IgG yield were determined at various times post-transfection. The highest steady-state level of IgG light chain mRNA was detected at 1 day post-transfection, whereas the highest concentration of IgG was found at day 3 post-transfection (Figure 3.6).

In addition, the IgG light chain mRNA levels after transfection with different plasmids were quantified. The highest transgene mRNA level was found in cells transfected with pKML/pKMH followed by the pXLG^{HEK} vectors and finally the pEAK8 vectors (Figure 3.7).

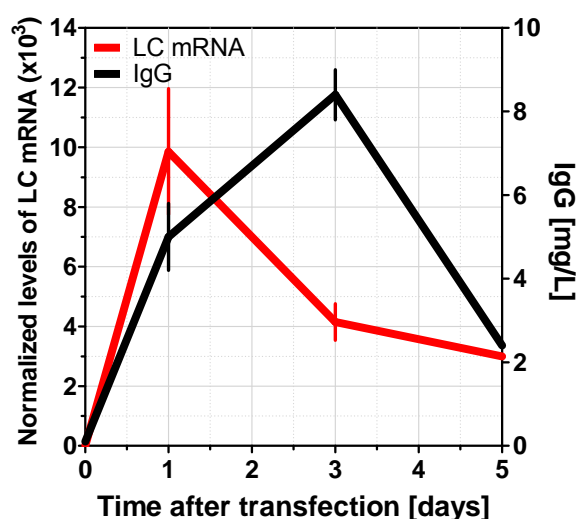


Figure 3.6. IgG light chain mRNA levels and IgG production over time. CHO DG44 cells were transfected with pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP at a 49:49:2 ratio (w/w/w). At the times indicated, one million of cells were harvested. Total mRNA was extracted and light chain mRNA was quantified by qRT-PCR. The delta-delta-Ct method was used to calculate the relative quantity of light chain mRNA using β -actin mRNA and mRNA from non-transfected cells for normalization. IgG titres were measured by ELISA at the times indicated. Error bars represent the standard deviation from two parallel experiments.

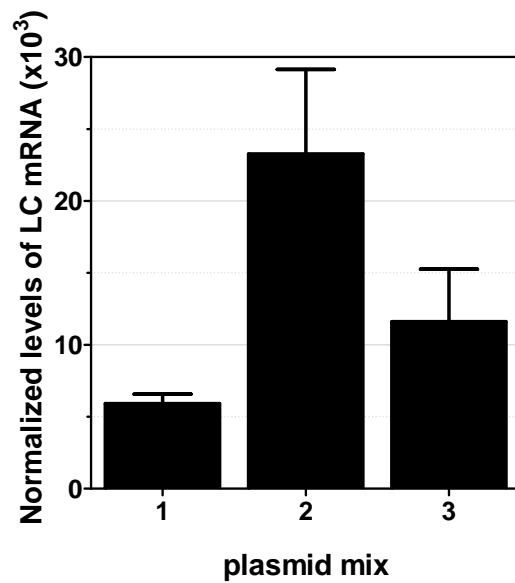


Figure 3.7. Transient light chain mRNA levels following transfection with different vectors. Cells were transfected with (1) pEAK8-LH39, pEAK8-LH41, and pEGFP-N1 at a ratio of 29:69:2 (w/w/w); (2) pKML, pKMH, and pMYKpuro-EGFP at a ratio of 29:69:2 (w/w/w); and (3) pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP at a ratio of 49:49:2 (w/w/w). One day post-transfection, one million cells were harvested, and total mRNA was extracted and light chain mRNA was quantified by qRT-PCR. The delta-delta-Ct method was used to calculate the relative quantity of light chain mRNA using the β -actin mRNA and mRNA from non-transfected cells for normalization. Error bars represent the standard deviation from three parallel experiments.

3.3 Discussion and conclusions

Performing efficient PEI-mediated transfections to get high transient production yields is not an easy task. The plasmid DNA needs to enter the cells, reach the nucleus, and be transcribed. This work showed the importance of the transgene mRNA level in transient gene expression in CHO cells. The pDNA level in transfected cells, in fact, did not correlate with the levels of transgene mRNA level and recombinant protein. These levels were also dependent on the expression vector used for transfection.

The amount of pDNA in cells after transfection was found to be in the range of 10,000-40,000 copies per cell, depending on the amount of DNA used for transfection; these values were similar to those previously published for CaPi transfection in CHO cells (14). However, the pDNA level in cells decreased rapidly. At day 2 post-transfection the amount of pDNA found in cells was decreased by 70% relative to the maximum level soon after transfection. Six days post-transfection, the pDNA quantity found was only 10% of the maximum level. Moreover, increasing the DNA amount used for transfection did not lead to higher protein yields. A similar phenomenon has previously been reported for PEI-mediated transfections in CHO cells (1). Here it was shown that the maximal amount of pDNA that can be delivered to cells to obtain good recombinant protein yields was 0.625 µg/million cell (~15,000 copies/cell). Transfection with higher amounts of pDNA resulted in more pDNA uptake into cells but less transgene mRNA accumulation and recombinant protein production. In the presence of 30,000 copies of pDNA per cell, the transgene mRNA level was 3 times lower than in presence of only 15,000 plasmid copies per cell. The lack of correlation between pDNA copy number and the transgene mRNA level may have been due to a limitation in transcription.

Different expression vectors were compared in terms of recombinant protein production in CHO cells. Two plasmids, both carrying CMV promoters (4;13), gave the highest yields during the first three days of production, but from day 4 IgG levels decreased dramatically. In contrast, the pEAK8 plasmids (12), carrying the EF-1 α promoter, showed a more constant production over six days after transfection. A possible explanation is that the viral promoters may be more prone to gene silencing than the promoter from a cellular housekeeping gene (15-17). No clear explanation was found for the decrease observed in recombinant IgG yields over time for the transfections with the vectors with a cytomegalovirus promoter. It is possible that proteases secreted by CHO cells degraded the recombinant IgG in the medium (18;19).

In conclusion, the data presented in this chapter demonstrated that the level of transgene mRNA appeared to be one of the main bottlenecks in transient gene expression in CHO cells. In the remaining chapters of this thesis, different strategies to increase the level and stability of transgene mRNA in transiently transfected CHO cells are described.

3.4 References

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Chapter 4

Mild hypothermia improves transient gene expression yields in CHO cells

4.1 Introduction

In standard bioprocesses, mammalian cells are cultivated at 37 °C, but it is well-known that mild hypothermic conditions (27-34 °C) can induce an increase in protein production in some recombinant cell lines (1-6). More recently, hypothermic conditions have been shown to enhance TGE in CHO cells (7;8). The reason(s) for the low-temperature enhancement of protein production from mammalian cells are not yet fully understood. Mild hypothermia causes an accumulation of cells in the G1 phase of the cell cycle and an increase in mRNA stability (9;10). Changes in cell metabolism and gene expression also occur at low temperatures (4;11;12). For example, the consumption of glucose and glutamine is reduced, leading to a lower production of cytotoxic metabolites such as lactate and ammonium; proteins able to stabilize mRNA such as cold-induced RNA binding protein (CIRBP) and Rbm3 are expressed; and the overall activity of the transcriptional-translational machinery is reduced (6;13).

By performing TGE under mild hypothermic conditions, a substantial increase in volumetric productivity of recombinant anti-Rhesus D antibody was achieved in CHO cells. The increase in production correlated with accumulation of the transfected cells in G1, reduced cellular metabolism, increased steady-state levels of transgene mRNAs, increased cell size, and increased cell viability. The effect of hypothermia on TGE was time dependent, with the highest yields obtained when cells were transferred to low

temperature at 4 h after DNA addition. Part of the enhancement in antibody production resulted from the inclusion of the woodchuck hepatitis virus posttranscriptional element (WPRE) in the 3' untranslated region of the transgene mRNA. Furthermore, it was shown that plasmid DNA stability was not enhanced by hypothermic conditions. Instead, increased transgene mRNA stability was found to be a key factor for the higher recombinant protein production. The results contribute to the understanding of low-temperature enhancement of recombinant gene expression and demonstrate the feasibility of producing high quantities of secreted recombinant proteins in CHO cells by TGE.

4.2 Results

4.2.1 Transient gene expression under hypothermic conditions

To determine if low-temperature exposure enhanced TGE, CHO cells were transfected with a mixture of pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP at a mass ratio of 49:49:2 for the expression of recombinant antibody and GFP. At different times after DNA addition, the cultures were transferred to 31 °C or maintained at 37 °C as a control. The effect of the temperature shift on recombinant protein yield was most pronounced when the cells were transferred to 31 °C at 4 h after DNA addition (Figure 4.1). Transfer of the cultures to 31 °C after maintenance at 37 °C for more than 2 days had little effect on transient recombinant protein yield (Figure 4.1). For the culture shifted to 31 °C at 4 h post-transfection, the antibody yield was about 80 mg/L after 6 days. Similar results were obtained for transfections with the same three plasmids in a 20 L disposable reactor with a culture volumes of 10 L with a temperature shift to 31 °C at 4 h post-transfection (Figure 4.2).

The same three expression vectors were then used to determine the effect of temperatures other than 31 °C on TGE. Cells were transfected as described above and shifted to 29, 31, or 33 °C at 4 h post-transfection. At all three temperatures tested there was enhancement of antibody and GFP production relative to the control culture at 37 °C, with the highest yields of GFP (Figure 4.3A) and antibody (Figure 4.3B) at 31 and 33 °C, respectively. Overall, recombinant antibody yield was enhanced 12- to 18-fold and GFP yield was enhanced 2- to 4-fold by exposure of cells to mild hypothermia after transfection as compared to protein yields at 37 °C (Figure 4.3).

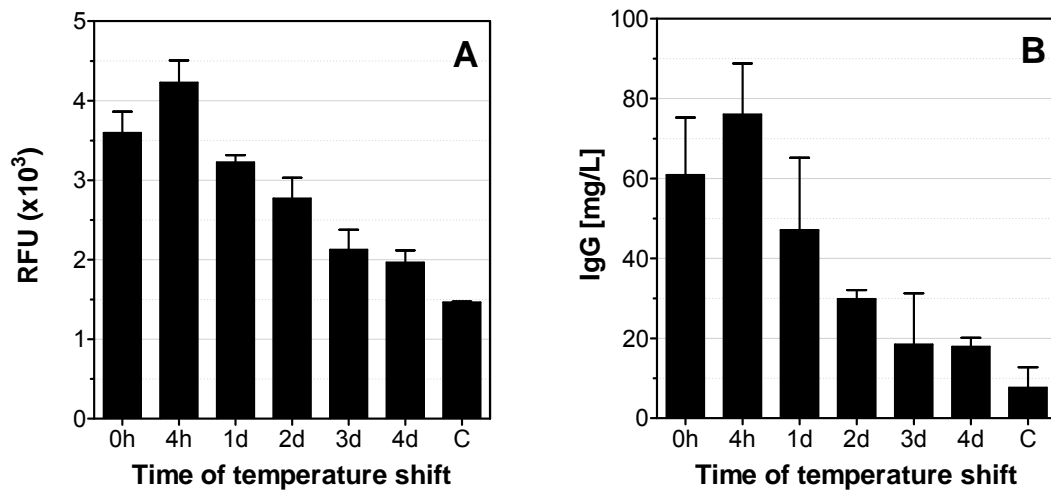


Figure 4.1. Effect of mild hypothermia on TGE. Cells were transfected with pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP at a 49:49:2 ratio (w/w/w). The transfected cells were shifted to 31 °C at the times indicated. The control transfection [C] was maintained at 37 °C. GFP-specific fluorescence (A) and antibody concentration in the medium (B) were measured at 6 days post-transfection. The error bars represent the standard deviation from three parallel experiments.

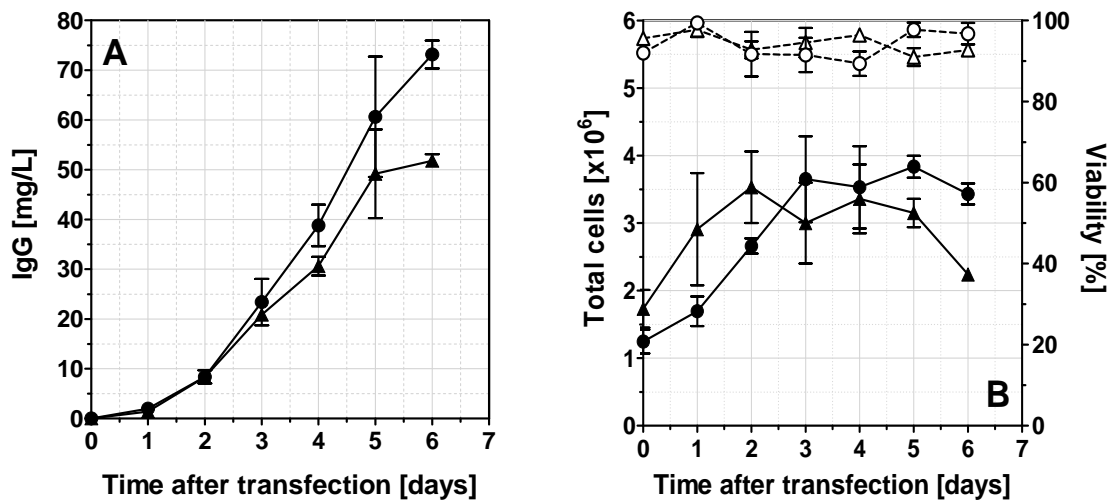


Figure 4.2. Scale-up of TGE under hypothermic conditions. CHO DG44 cells were transfected with pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-GFP at a 49:49:2 ratio (w/w/w) at the 10 mL scale [●] and the 10 L scale [▲]. (A) Recombinant IgG titres were determined at the times indicated. (B) Cell growth [closed symbols] and viability [open symbols] were measured at the times indicated. Error bars represent the standard deviation from three parallel experiments for the 10 mL scale and from three samples of a single culture for the 10 L scale.

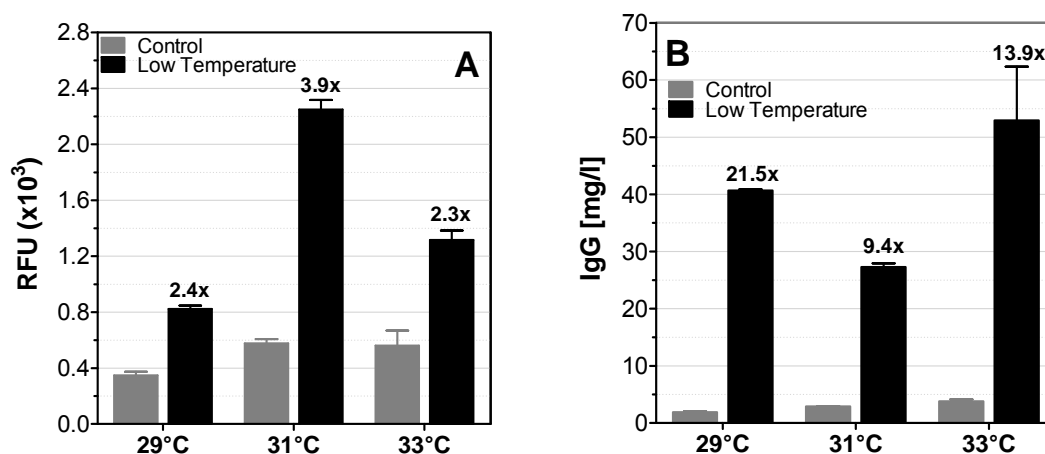


Figure 4.3. Enhancement of TGE at different temperatures. Cells were transfected with pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP at a 49:49:2 ratio (w/w/w) and either shifted to low temperature (29, 31, or 33 °C) at 4 h post-transfection or maintained at 37 °C (control) as indicated. GFP-specific fluorescence (A) and antibody concentration in the medium (B) were measured at 6 days post-transfection. The fold increase over the control is indicated above the bar representing the reporter protein yield at low temperature. The error bars represent the standard deviation from three parallel experiments.

4.2.2 Vector influence on the low-temperature enhancement of TGE

To determine if the low-temperature enhancement of TGE was dependent on the expression vector, cells were then transfected with three different combinations of plasmids: (1) pEAK8-LH39, pEAK8-LH41, and pEGFP-N1 at a ratio of 29:69:2 (w/w/w); (2) pKML, pKMH, and pMYKpuro-EGFP at a ratio of 49:49:2 (w/w/w); (3) pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP at a ratio of 49:49:2 (w/w/w). The ratios for the IgG light and heavy chain plasmids for the different vector sets were individually optimized previously for transient expression at 37 °C (14-16). The cells were shifted to 31 °C at 4 h post-transfection while the control transfections were maintained at 37 °C. For all three sets of vectors, enhanced antibody and GFP production at 31 °C relative to that of the control transfection was observed (Figure 4.4). Among the three GFP vectors, the GFP yield was clearly highest for pEGFP-N1 (Figure 4.4A), while the highest antibody yield was seen with pXLG^{HEK}-RhLC and pXLG^{HEK}-RhHC (Figure 4.4B).

To further investigate the low-temperature enhancement of antibody production from cells transfected with pXLG^{HEK}-RhHC and pXLG^{HEK}-RhLC, the two vectors were modified to eliminate the post-transcriptional regulatory element WPRE to create

pXLG^{HEK}- Δ WPRES-RhLC and pXLG^{HEK}- Δ WPRES-RhHC. Cells were transfected with the two sets of light and heavy chain vectors at a 1:1 (w/w) ratio. At 4 h post-transfection the cultures were shifted to 31 °C, while the control transfections were maintained at 37 °C. The low-temperature enhancement of antibody yield was about 18-fold in the presence of the WPRES but only about 6-fold in its absence (Figure 4.5A). For each transfection, the steady-state levels of the IgG light and heavy chain mRNAs were determined by qRT-PCR. Mild hypothermic conditions enhanced the levels of both the heavy and light chain mRNAs at 3 days post-transfection regardless of the presence or absence of the WPRES (data not shown). However, the light to heavy chain mRNA ratio at both 31 and 37 °C was higher in the presence of the WPRES than in its absence (Figure 4.5B).

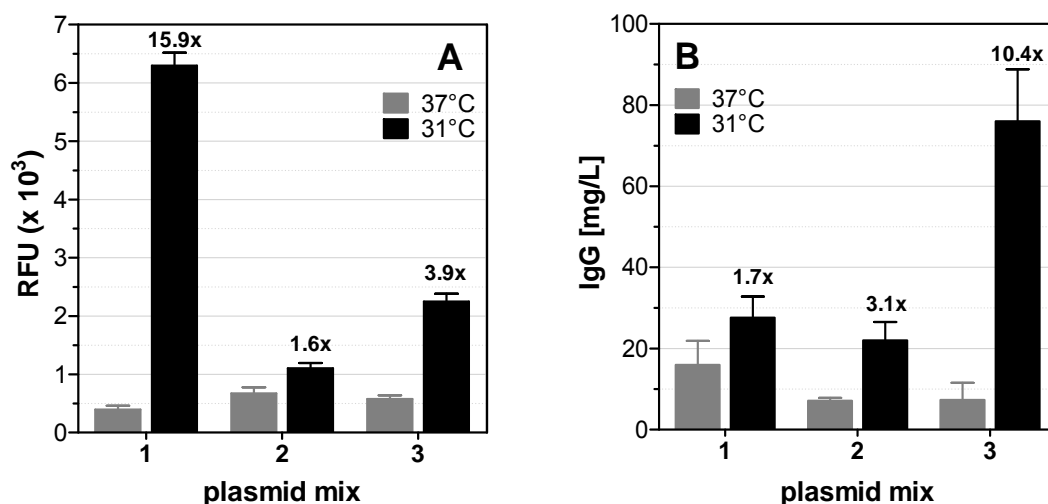


Figure 4.4. Vector influence on low-temperature enhancement of TGE. Cells were transfected with (1) pEAK8-LH39, pEAK8-LH41, and pEGFP-N1 at a ratio of 29:69:2 (w/w/w); (2) pKML, pKMH, and pMYKpuro-EGFP at a ratio of 49:49:2 (w/w/w); and (3) pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP at a ratio of 49:49:2 (w/w/w). The cells were either shifted to 31 °C at 4 h post-transfection or maintained at 37 °C. GFP-specific fluorescence (A) and antibody concentration in the medium (B) were measured at 6 days post-transfection. The fold increase over the control is indicated above the bar representing the reporter protein yield at low temperature. The error bars represent the standard deviation from three parallel experiments.

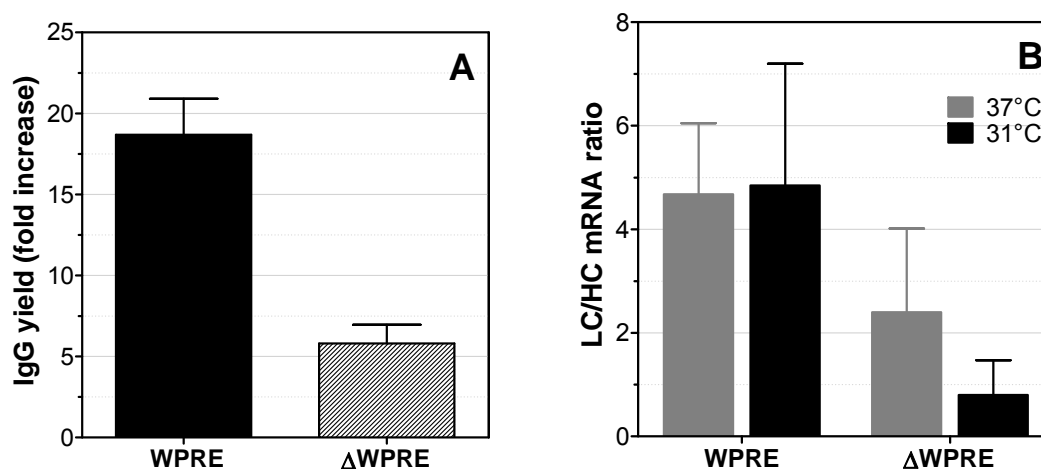


Figure 4.5. Effect of the WPRE on low-temperature enhancement of TGE. Cells were transfected with a 1:1 ratio (w/w) of pXLG^{HEK}-RhLC and pXLG^{HEK}-RhHC (designated WPRE) or pXLG^{HEK}-ΔWPRE-RhLC and pXLG^{HEK}-ΔWPRE-RhHC (designated ΔWPRE). (A) Antibody concentration in the medium was measured by ELISA at 6 days post-transfection. Antibody levels from temperature-shifted cultures were normalized to those of the control cultures at 37 °C. qRT-PCR was used to determine the steady-state levels of the IgG heavy (HC) and light chain (LC) mRNAs at 3 days post-transfection. The levels of these two mRNAs were normalized to that of β-actin mRNA with the delta Ct method. (B) The ratios of the relative levels of LC and HC mRNA for each transfection were determined. The error bars represent the standard deviation from three parallel experiments.

4.2.3 Effect of hypothermia on GFP expression level over time

To investigate other possible causes of the enhanced recombinant protein production under hypothermia, CHO cells were transfected with pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP at a 49:49:2 ratio (w/w/w), and at 4 h after transfection half of the samples were shifted to 31°C. The percentage of GFP-positive cells was determined by flow cytometry each day. The percentage of GFP-positive cells was slightly higher for the cultures maintained at 37 °C as compared to those at 31 °C at most of the time points (Figure 4.6). However, the percentage of GFP-positive cells remained stable for 6 days for the cultures at 31 °C whereas for those at 37°C this number decreased over time from a maximum at day 3 post-transfection (Figure 4.6). This decline may have been caused by the decrease of cell viability at 37°C after day 3.

Furthermore, in transfected cells maintained at 31°C, the GFP expression level increased over time (Figure 4.7). For the cultures at 37 °C, the percentage of GFP-positive cells expressing high (++) levels of GFP decreased after day 3 post-transfection (Figure

4.8A). In contrast, for the cells at 31°C the percentage of GFP-positive cells with high (++) or very high (+++) levels of GFP expression increased over time (Figure 4.8B). This phenomenon explains why the fluorescence measured at 31°C was higher than that measured at 37°C, even if the overall percentage of GFP-positive cells at 31°C was a lower than the one reported for the cells at 37°C.

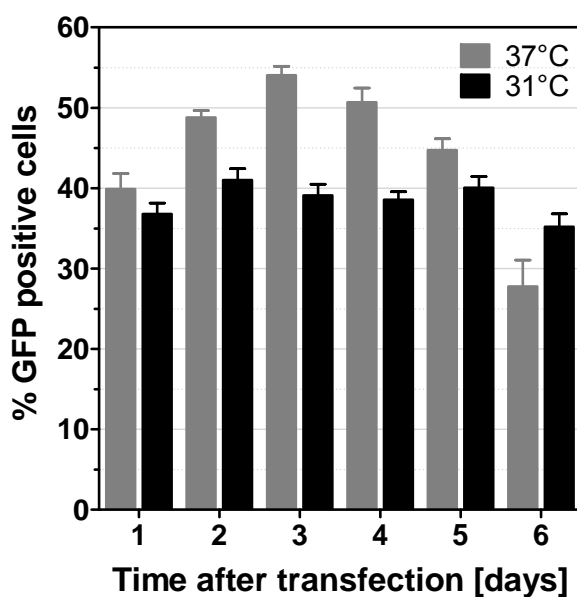


Figure 4.6. Overall percentage of GFP-positive cells at 37 °C and 31 °C. Cells were transfected with pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP at a 49:49:2 ratio (w/w/w). At 4 h after transfection, cells were diluted and half of the samples were shifted to 31 °C. Every day after transfection a sample was recovered and analyzed by flow cytometry. The error bars represent the standard deviation from three parallel experiments.

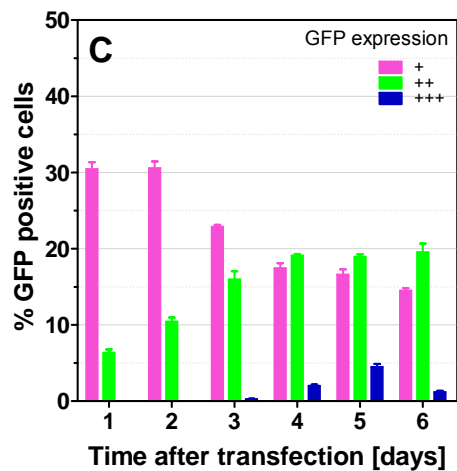
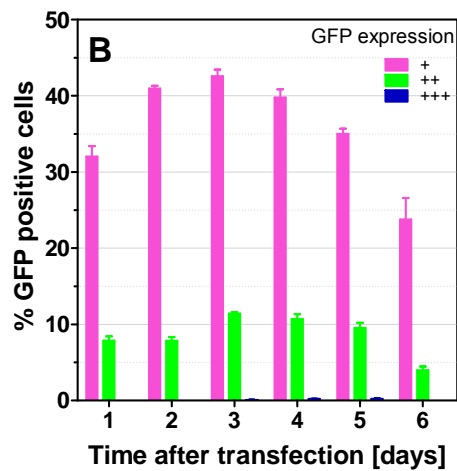
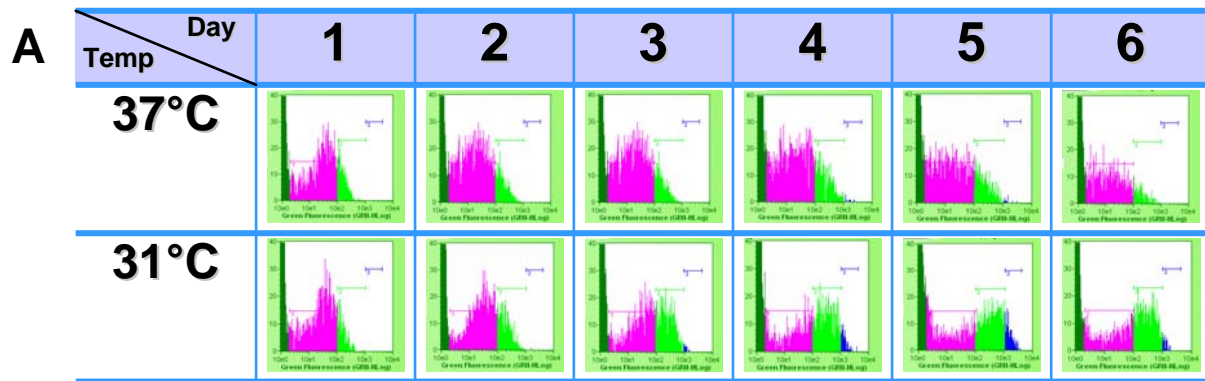


Figure 4.7. Distribution of population of GFP positive cells at 37 °C and 31 °C. Cells were transfected with pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP at a 49:49:2 ratio (w/w/w). At 4 h after transfection, cells were diluted and half of the samples were shifted to 31 °C. Every day after transfection a sample was recovered and analyzed by flow cytometry (A). The lower panels represent the analyzed data for cultures at 37 °C (B) and 31 °C (C). The error bars represent the standard deviation from two parallel experiments.

4.2.4 Effects of hypothermia on transgene mRNA and plasmid DNA stability

Cells were transfected with pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP at a 49:49:2 ratio (w/w/w). The cultures were maintained at either 31 °C or 37 °C, and the steady-state level of IgG light chain mRNA was quantified daily. By 24 h post-transfection the cells kept at 31 °C contained more light chain mRNA than those at 37 °C (Figure 4.8). This difference became greater with time as the steady-state transgene mRNA level decreased with time in the cells at 37 °C (see Chapter 3) but not in those at 31 °C (Figure 4.8). Since it has previously been shown that the level of the IgG light chain polypeptide is limiting for antibody assembly and production, only the steady-state level of this mRNA was determined (17;18).

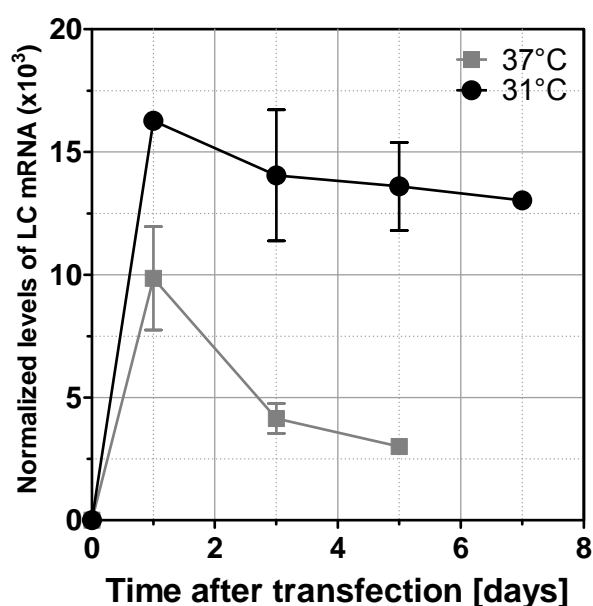


Figure 4.8. Stability of IgG light chain mRNA over time. CHO DG44 cells were transfected with pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP at a 49:49:2 ratio (w/w/w). At 0, 1, 3, 5 and 7 days post-transfection, one million of cells were harvested, and total mRNA was extracted and retro-transcribed into cDNA. Samples were then analyzed by qRT-PCR. The delta-delta-Ct method was used to calculate the relative quantity of light chain mRNA using the β -actin mRNA and not transfected mRNA for normalization. Error bars represent the standard deviation from two parallel experiments.

One explanation for the increase of light chain mRNA stability under hypothermic conditions is that the temperature may have increased the stability of the plasmid DNA. To investigate this possibility, pDNA was extracted from transfected CHO DG44 cells maintained at either 31 °C or 37 °C at different times post-transfection and then quantified using qRT-PCR. For both cultures, the level of pDNA decreased dramatically during the first 48 h after transfection relative to the level at 4 h post-transfection (Figure 4.9). The pDNA level then remained the same until the end of the two cultures. The similarity of the results from cultures at 37 °C and 31 °C implied that the hypothermic conditions did not alter the stability of transfected pDNA.

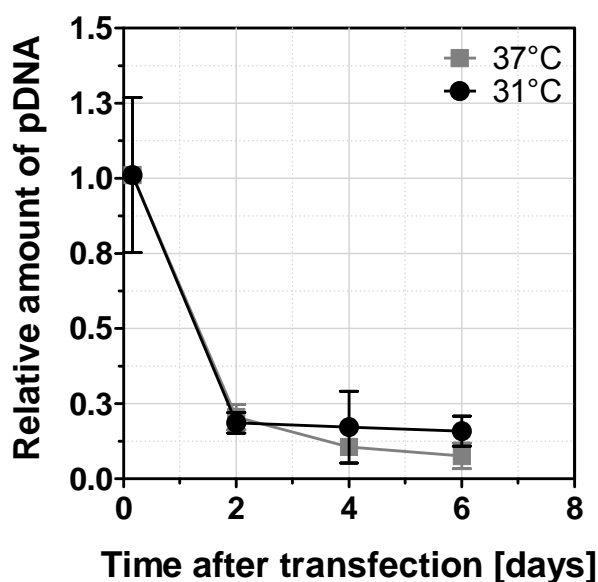


Figure 4.9. Stability of transfected pDNA over time. CHO DG44 cells were transfected with pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP at a 49:49:2 ratio (w/w/w). At 0, 2, 4, 6 days after transfection one million of cells were harvested, and the pDNA was extracted with Phenol/Chlorophorm. Samples were then analyzed by qRT-PCR. A known amount of pDNA was used to create the standard curve. Error bars represent the standard deviation from two parallel experiments.

4.2.5 Effects of overexpression of cold shock proteins

It is known that the cultivation of cells under hypothermic conditions induces the expression of several endogenous genes including those for the RNA binding proteins CIRBP and Rbm3 (13). These two proteins are thought to bind and stabilize mRNA under hypothermic stress (6). To determine if these proteins play a role in the low-temperature enhancement of TGE, CHO cells were co-transfected with a mixture of pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP, and either pMYK-CIRBP, pMYK-Rbm3, or pXLG^{HEK} at a mass ratio of 44:44:2:10. The cells were maintained at 37°C throughout the experiment. The presence of the cold-shock proteins, however, had almost no effect on both GFP and antibody production under the conditions tested (Fig. 4.10). Similar results were observed when transfecting higher or lower amounts of the plasmids encoding the two cold-shock proteins (data not shown) or even after shifting cells to 31 °C. The overexpression of recombinant CIRBP and Rbm3 was confirmed by western blot analysis (Figure 4.11). The polyclonal antibody used for the detection of Rbm3 recognized also other unidentified cellular proteins (bands at ~40 KDa). Furthermore, from these results it appeared that the endogenous Rbm3 was expressed at low levels by CHO cells at 37 °C (Figure 4.11B, Control lane).

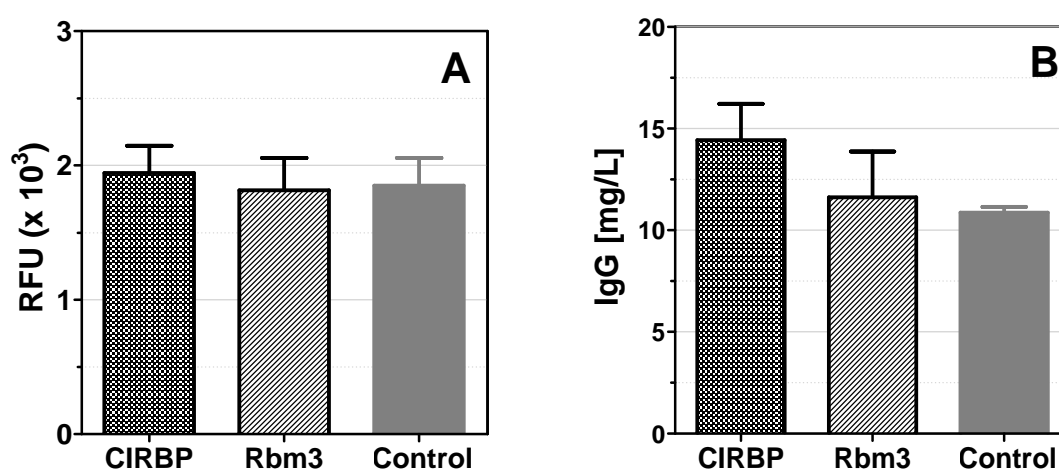


Figure 4.10. Effect of overexpression of cold-shock proteins. Cells were transfected with pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP, and either pMYK-CIRBP (CIRBP), pMYK-Rbm3 (Rbm3), or pXLG^{HEK} (Control) at a ratio of 44:44:2:10 (w/w/w/w) and maintained at 37°C. GFP-specific fluorescence (A) and antibody concentration in the medium (B) were measured 3 days after transfection. Error bars represent the standard deviation from three parallel experiments.

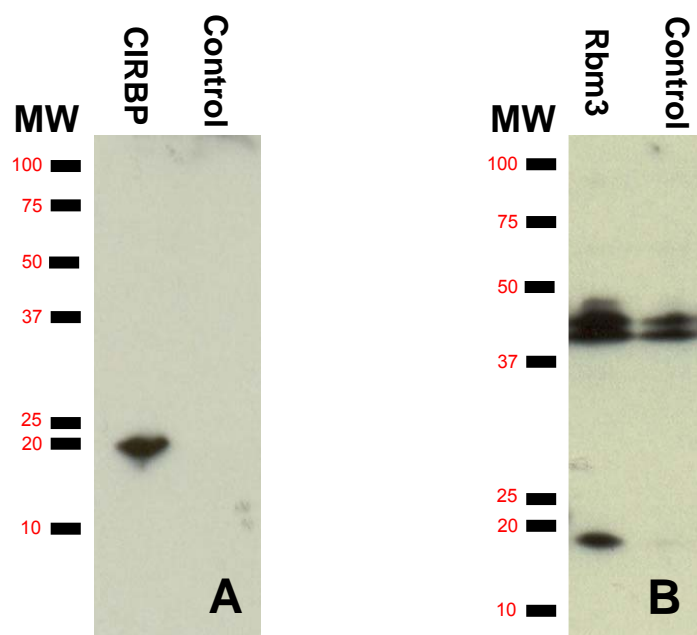


Figure 4.11. CIRBP (A) and Rbm3 (B) detection by Western Blot. CHO DG44 cells were transfected with pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP, and either pMYK-CIRBP (CIRBP), pMYK-Rbm3 (Rbm3) or herring sperm DNA (Control) at a mass ratio of 44:44:2:10. Three days after transfection five millions of cells were harvested, intracellular proteins were extracted, separated by SDS-PAGE, and immunoblotted with a monoclonal anti-CIRBP (A) or with a polyclonal anti-Rbm3 (B) antibody. Molecular weights [MW] are expressed in kDa.

4.2.6 Characterization of hypothermic effects on transfected cells

To better understand the mechanism of low temperature enhancement of TGE, CHO cells transfected with pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP were partially characterized by periodically analyzing biomass, cell viability, glucose and glutamine consumption, lactate and ammonium production, and cell size. The cells transferred to 31 °C at 4 h post-transfection were compared to those maintained at 37 °C. To compare the cell cycle phase distribution of cells maintained at 31 °C and 37 °C, CHO cells were transfected with herring sperm DNA and either shifted to 31 °C at 4 h post-transfection or maintained at 37 °C. At different times the cells were stained with propidium iodide and analyzed by flow cytometry. By 3 days post-transfection, the cell cycle distribution at 31 °C was distinctly different from that at 37 °C, with a higher percentage of cells in the G1 phase for the 31 °C culture (Figure 4.12). By 6 days post-transfection, almost all of the cells at 31 °C were in G1, but this was not the case for the culture maintained at 37 °C (Figure 4.12).

Surprisingly, the biomass accumulation for the two cultures was similar at times up to 5 days post-transfection (Figure 4.13A). This could be explained in part by lower cell viability for the cultures at 37 °C (Figure 4.13B) and an increase in the average cell size at 31 °C as compared to cells at 37 °C. For the cells at 31 °C, both the cell diameter (Figure 4.13C) and the cell volume (Figure 4.13D) were greater as compared to cells at 37 °C. At 31 °C, cell viability was >95% throughout the cultivation period, while cell viability at 37 °C decreased to 40% by 6 days post-transfection (Figure 4.13B). In addition, the levels of glucose and glutamine consumption were lower at 31 °C than at 37 °C (Figure 4.13E, F), and the level of ammonium production was less at 31 °C than at 37 °C (Figure 4.13G). Lactate production was also lower at 31 °C than at 37 °C, but only up to 3 days post-transfection (Figure 4.13H).

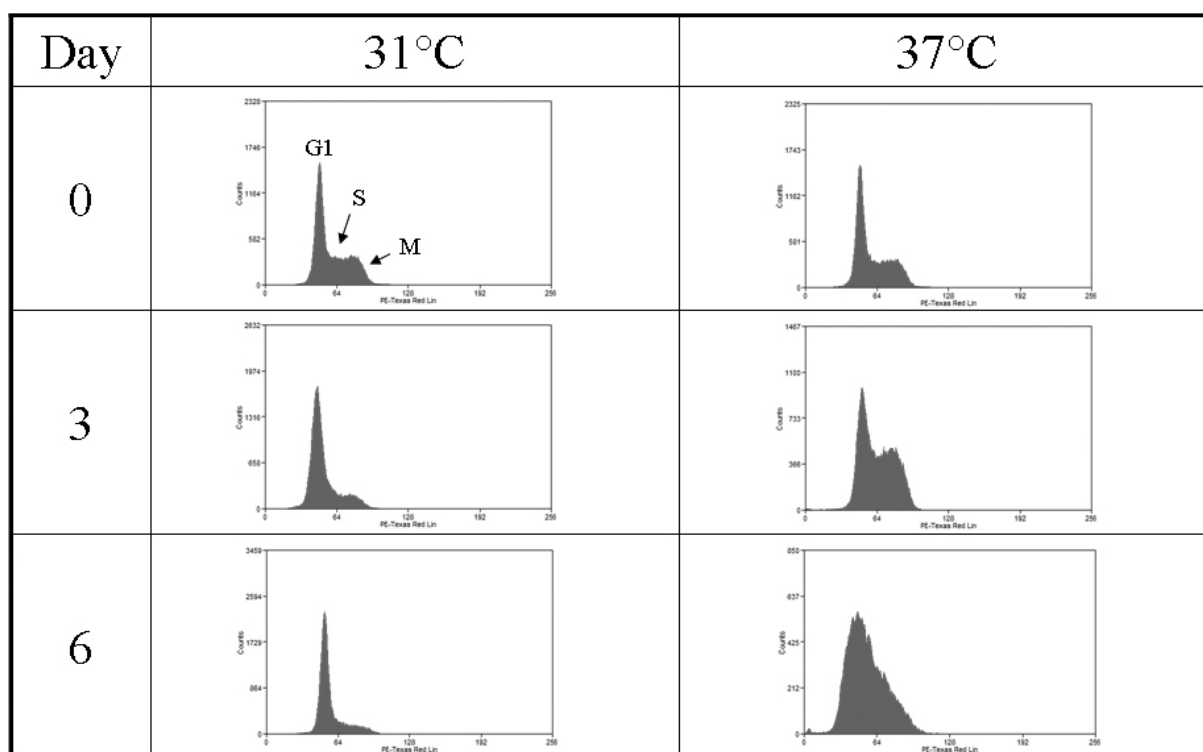


Figure 4.12. Effects of mild hypothermia on cell cycle phase distribution. Cells were transfected with herring sperm DNA and either shifted to 31 °C at 4 h post-transfection (left panels) or maintained at 37 °C (right panels). Flow cytometry analysis was performed at the times indicated. The positions of cells in G1, S, and G2 plus M (M) are indicated in the upper left-hand panel.

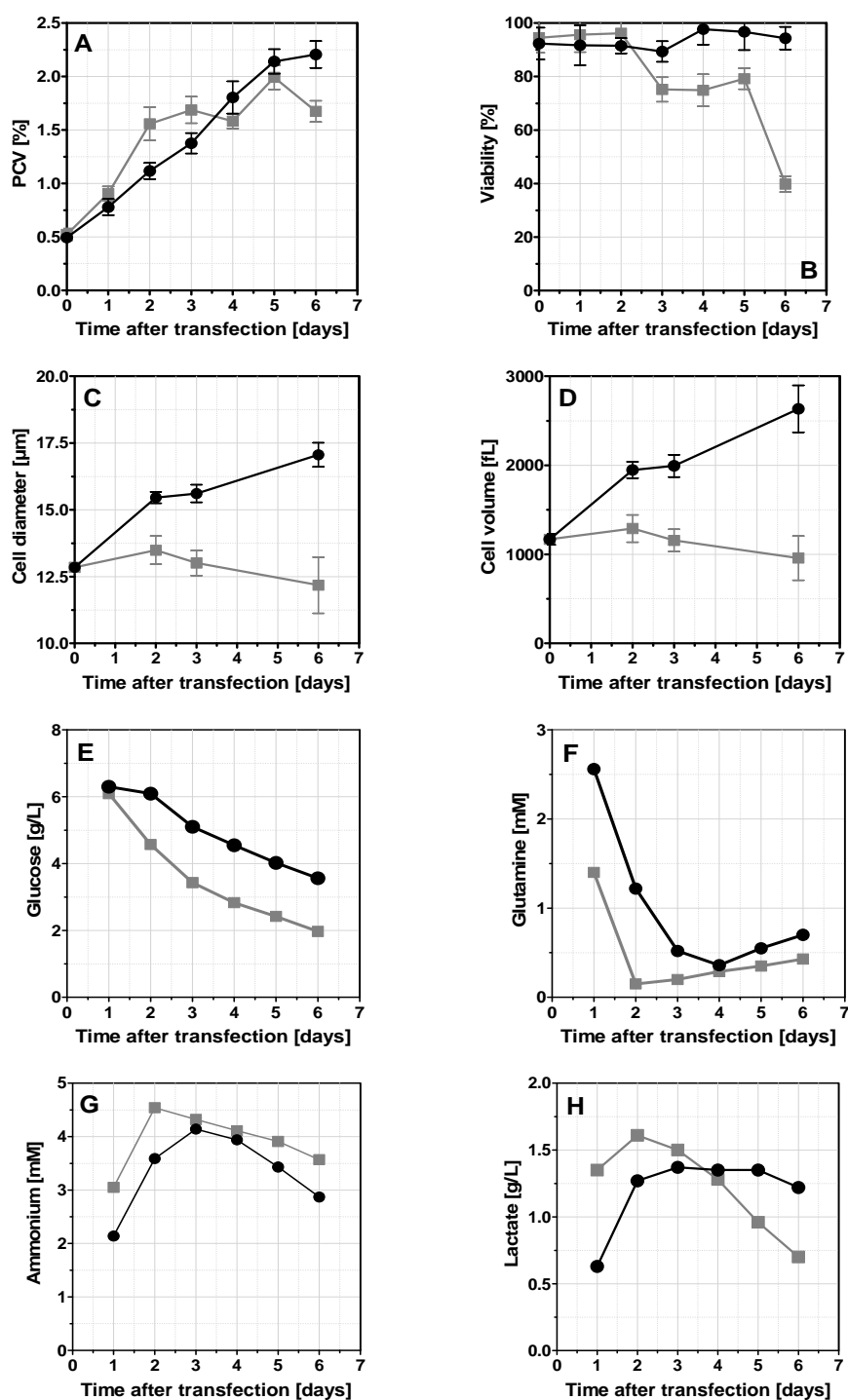


Figure 4.13. Effects of mild hypothermia on cell growth and metabolism. Cells were transfected with pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP at a ratio of 49:49:2 (w/w/w) and shifted to 31 °C at 4 h post-transfection (black ●) or maintained at 37 °C (grey ■). At the times indicated, biomass (A), cell viability (B), cell diameter (C), cell volume (D) glucose (E), glutamine (F), ammonium (G), and lactate (H) were measured as described in Materials and Methods. Each point represents the average of three independent experiments. Error bars, when present, represent the standard deviation from two parallel experiments.

4.3 Discussion and conclusions

This study demonstrated that a shift in cultivation temperature from 37 to 31 °C at 4 h after the transfection of CHO cells with PEI increased transient recombinant antibody yield up to 60-80 mg/L by 6 days post-transfection. This represents the highest level of transient antibody production in mammalian cells reported to date for a batch culture (19). These results have been extended by the demonstration that the low-temperature cultivation of transfected CHO cells yielded 50-60 mg/L of antibody within 10 days in orbitally shaken bottles of 10 or 20 L (14). The enhancement of TGE at 31 °C correlated with an accumulation of cells in the G1 phase of the cell cycle, reduced cellular metabolism, greater cell viability, increased cell size, shift of cell population towards higher recombinant protein production, and increased steady-state levels over time of transgene mRNA as compared to cells kept at 37 °C. The extent of the increase in protein yield was partly dependent on the expression vector used for transfection. More specifically, WPRE, which has a post-transcriptional effect on heterologous gene expression, was particularly effective in increasing transient recombinant protein yield at low temperatures.

The enhancement of TGE by mild hypothermia was found to be time-dependent, with the greatest effect observed when cells were shifted to 31 °C 4 h after transfection. In contrast, little effect on protein yield was observed when the cells were shifted at 3 or more days after transfection. In a separate study on PEI-mediated transfection kinetics, it was shown for CHO cells in suspension that most of the DNA uptake occurs within 60 min of DNA-PEI addition (20). Thus, exposure of cells to low-temperature probably did not have a significant effect on DNA uptake since the protein yields following temperature shifts at the time of DNA addition or 4 h later were about the same.

Furthermore, hypothermia did not have an influence on plasmid DNA stability. The level of pDNA decreased dramatically during the first days after transfection both at 37 and at 31 °C. However, the transgene mRNA level in cells under hypothermic conditions was not only higher than at 37 °C, as previously reported (2;6), but the steady-state level of the mRNA was more stable over time. Thus, the temperature shift may have affected one or more steps in the life cycle of the IgG light chain mRNA such as mRNA transcription, processing, transport, and degradation.

Experiments addressing the importance of the WPRE indicated that post-transcriptional events play a significant role in the low-temperature enhancement of TGE in CHO cells. This RNA element is known to have effects on polyadenylation, mRNA

transport, and translation (21). Here we showed that the ratio of the IgG light to heavy chain mRNA was at least 2-fold higher in the presence of the WPRE than in its absence at both 31 and 37 °C. In the absence of the WPRE, the light to heavy chain mRNA ratio was nearly 1:1 at 31 °C, while this ratio in the presence of the WPRE was about 5:1. It has previously been shown that the level of the light chain polypeptide is limiting for antibody assembly (17;18). Thus, the effect of the WPRE may be to increase the steady-state level of the light chain mRNA, resulting in higher light chain protein levels and higher antibody secretion.

Interestingly, the overexpression in CHO cells of two cold-shock proteins (CIRBP and Rbm3) that were expected to stabilize mRNA under hypothermic conditions (13) failed to enhance transient recombinant protein production. Other studies have shown an increase in recombinant protein yield when either one of these proteins was stably expressed in a recombinant CHO-derived cell line (22;23).

We also observed that mild hypothermia had an impact on the distribution of the GFP-positive cell population; on the viability, size, and metabolism of cells; and on the cell cycle distribution. The accumulation of cells in the G1 phase of the cell cycle as the result of mild hypothermia has previously been described for recombinant CHO cell lines (3;11;24). Even though most of the cells shifted to 31 °C were no longer dividing by 3 days post-transfection, the average cell volume increased about 2-fold relative to cells at 37 °C. Thus, the biomass levels of the transfected cultures at 31 and 37 °C were approximately the same up to 5 days post-transfection. After this time point, cell viability at 37 °C decreased sharply. A similar increase in cell size and recombinant protein production was reported for transfected CHO cells blocked in the G2/M phase of the cell cycle following treatment with nocodazole (25). The difference in viability described here between the cultures at 31 and 37 °C was already evident by 3 days post-transfection, and this difference was increased by 6 days post-transfection. Cells at 31 °C maintained a high viability (>90%) for a longer time than at 37 °C. This may have been due to the reduced consumption of glucose and glutamine and the consequent reduction of lactate and ammonium production. A similar reduction in cellular metabolism has been observed for recombinant CHO cells lines maintained under mild hypothermic conditions (11;12). Subsequent transfections of CHO at scales up to 50 L demonstrated that the viability of CHO cells can remain above 90% for up to 11 days post-transfection at 31 °C (14). Transfected cells under hypothermic conditions had about the same percentage of GFP-positive cells than the transfected cells cultured at 37°C. However, at 31°C the GFP-positive population was shifted towards high-producing cells, leading to a higher GFP

level. These changes in cell growth and physiology due to mild hypothermia are likely to be important for enhancing transient recombinant protein yield, but additional studies are necessary to determine the precise pathways involved.

Although the different mammalian expression vectors described in this study responded differently to low-temperature conditions, in all cases recombinant protein production was increased under mild hypothermia. Only the total protein yields and the extent of the enhancement of recombinant protein production varied. pEGFPN1 and pXLG^{HEK}-RhLC/pXLG^{HEK}-RhHC provided the highest absolute levels of GFP and antibody, respectively. The level of enhancement over the control transfection was 10-fold for pEGFP-N1 and 10-18-fold for pXLG^{HEK}-RhLC/pXLG^{HEK}-RhHC. Regardless of the fact that for all three of these plasmids the recombinant gene was under the control of the hCMV immediate early promoter, we cannot conclude that it is superior to the other promoters used in the study since the various plasmids included other elements which may have affected transgene expression at low temperatures. For example, the pXLG^{HEK}-based plasmids included the adeno-associated virus inverted terminal repeats which have intrinsic promoter activity and the wild-type WPRE which encodes a truncated form of the woodchuck hepatitis virus X protein (26-28). Furthermore, the two reporter proteins also responded differently to hypothermic conditions which may signify differential low-temperature effects on secreted and intracellular proteins.

In conclusion, it was demonstrated that hypothermic conditions help to increase and stabilize transgene mRNA levels in transiently transfected CHO cells. Furthermore, the recombinant antibody levels observed here were the highest ever reported for transiently transfected CHO cells, and the present studies have been extended by performing transfections at scales up to 50 L in disposable containers agitated by orbital shaking (14). Thus, low-temperature TGE is a very promising technology for the rapid production of gram quantities of secreted recombinant proteins from CHO cells.

4.4 References

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Chapter 5

Valproic acid improves mRNA levels and transient gene expression yields in CHO cells

5.1 Introduction

Valproic acid (VPA or 2-propylpentanoic acid) is a carboxylic acid widely used in medicine for the treatment of epilepsy, depression, and other psychiatric disorders (1;2). Recently VPA has been identified as a histone deacetylase inhibitor (iHDAC) (1;3;4) and, like other iHDACs (i.e. sodium butyrate and Tricostatin A) (5-8), it has been used in cell cultures to increase recombinant protein yields (9). Its activity is not only selective to class I HDAC, but it has been reported to inhibit class II HDACs via proteasome-mediated degradation (10). Like other iHDACs, VPA has been shown to alter the cell cycle distribution and block cell growth *in vivo* and *in vitro* (4;11;12). As already reported in Chapter 4, those effects may be positive for recombinant protein production using mammalian cells as the host system. In fact, under hypothermic conditions we observed a lower cell metabolism level and a block in the G1 phase of the cell cycle in addition to higher recombinant protein titers.

In this chapter, the treatment of transiently transfected CHO DG44 cells with VPA is reported. A substantial increase in the volumetric productivity of GFP and IgG was achieved. With increasing VPA concentrations, cell viability and growth were negatively influenced but the IgG mRNA and protein levels increased with increasing VPA concentrations. As already reported in Chapter 4, increased transgene mRNA stability was found to be a key factor for enhanced transient recombinant protein production in this cell

line. Moreover, the enhancement on recombinant protein production in the presence of VPA was dependent on the expression vector used for transfection and on the expressed protein. A synergistic effect of VPA and hypothermia was also observed but only for low concentrations of VPA. The cause of the improved recombinant protein yield described here was mainly due to the iHDAC activity of VPA; however, changes in cell growth and metabolism also had an impact as shown with treatment of transfected cells with valpromide (VPD), a VPA analogue without iHDAC activity (1). Even if many questions about VPA activity in transiently transfected cells are still open, the results presented here contribute to the understanding of the mechanism of the enhancement of transient gene expression after VPA treatment.

5.2 Results

5.2.1 Effect of VPA on transient protein production

To determine if VPA-enhanced TGE, CHO cells were transfected with a plasmid mix containing pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP at a mass ratio of 49:49:2. Four hours after transfection, cells were diluted according to the standard protocol, and VPA was added to different final concentrations from 0 - 5 mM. Cells were cultured at 37 °C and GFP and IgG expression were measured at various times after transfection.

The presence of VPA significantly enhanced the expression of GFP and IgG. Antibody production was enhanced to the same level relative to the untreated control for all VPA concentrations tested (Figure 5.1). The transient IgG levels in the VPA-treated transfections were about 4-fold higher at day 3 post-transfection and almost 23-fold higher at day 6 post-transfection (Figure 5.1). Furthermore, IgG levels in the presence of VPA increased over-time and reached a plateau at day 7. This was different from the untreated control transfection in which the highest level of recombinant IgG was observed at day 3 (Figure 5.1; see also Chapter 3, Section 3.2.2). GFP fluorescence was almost 2-fold higher in the presence of 1.25 mM VPA as compared to the untreated control (Figure 5.2). Increased GFP levels were also observed at high VPA amounts were added to the culture (Figure 5.2), but if VPA concentrations exceeded 2.5 mM the total fluorescence was only 1.5-fold higher than the control. These results were confirmed by flow cytometry analyses: the percentage of GFP positive cells was slightly higher for the cultures treated with 1.25 mM of VPA, but then decreased with increasing VPA concentration (Figure 5.3A). However, for the cells treated with VPA, the percentage of GFP-positive cells with high (++) or very high (+++) levels of GFP expression was higher than for the untreated control transfection (Figures 5.3B and 5.3C). This phenomenon explains why the total fluorescence measured in the presence of VPA was higher than that measured for the control, even if the overall percentage of GFP-positive cells treated with VPA was almost the same in all the transfections.

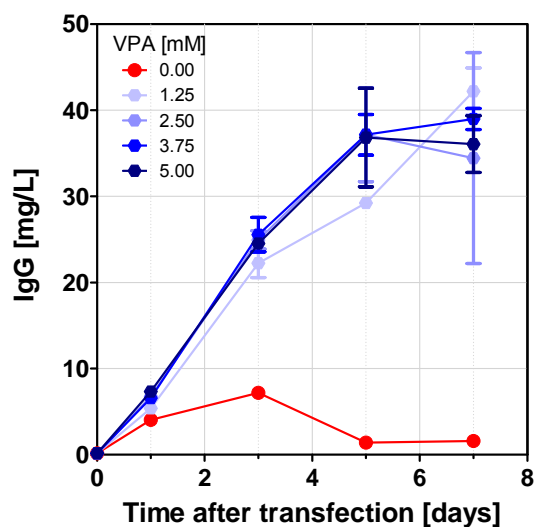


Figure 5.1. Effect VPA treatment on transient IgG production. CHO DG44 cells were transfected with pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-GFP at a 49:49:2 ratio (w/w/w). Four hours after transfection, VPA was added to different concentrations. The concentration of recombinant IgG was measured by ELISA at the times indicated. Error bars represent the standard deviation from two parallel experiments.

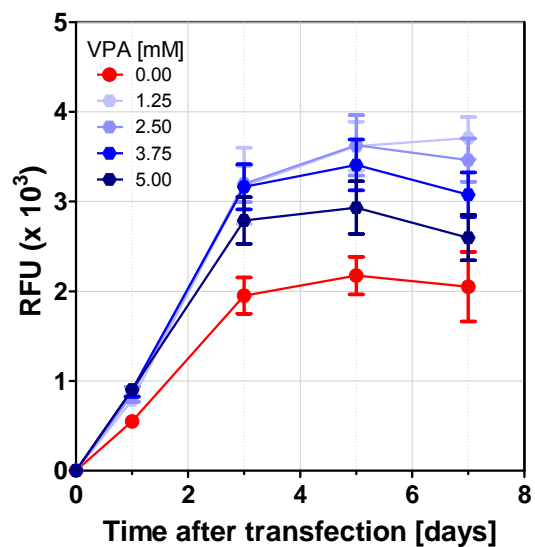


Figure 5.2. Effect of VPA on transient GFP expression. CHO DG44 cells were transfected with pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-GFP at a 49:49:2 ratio (w/w/w). Four hours after transfection, VPA was added to different concentrations. GFP expression was measured at the times indicated. Error bars represent the standard deviation from two parallel experiments.

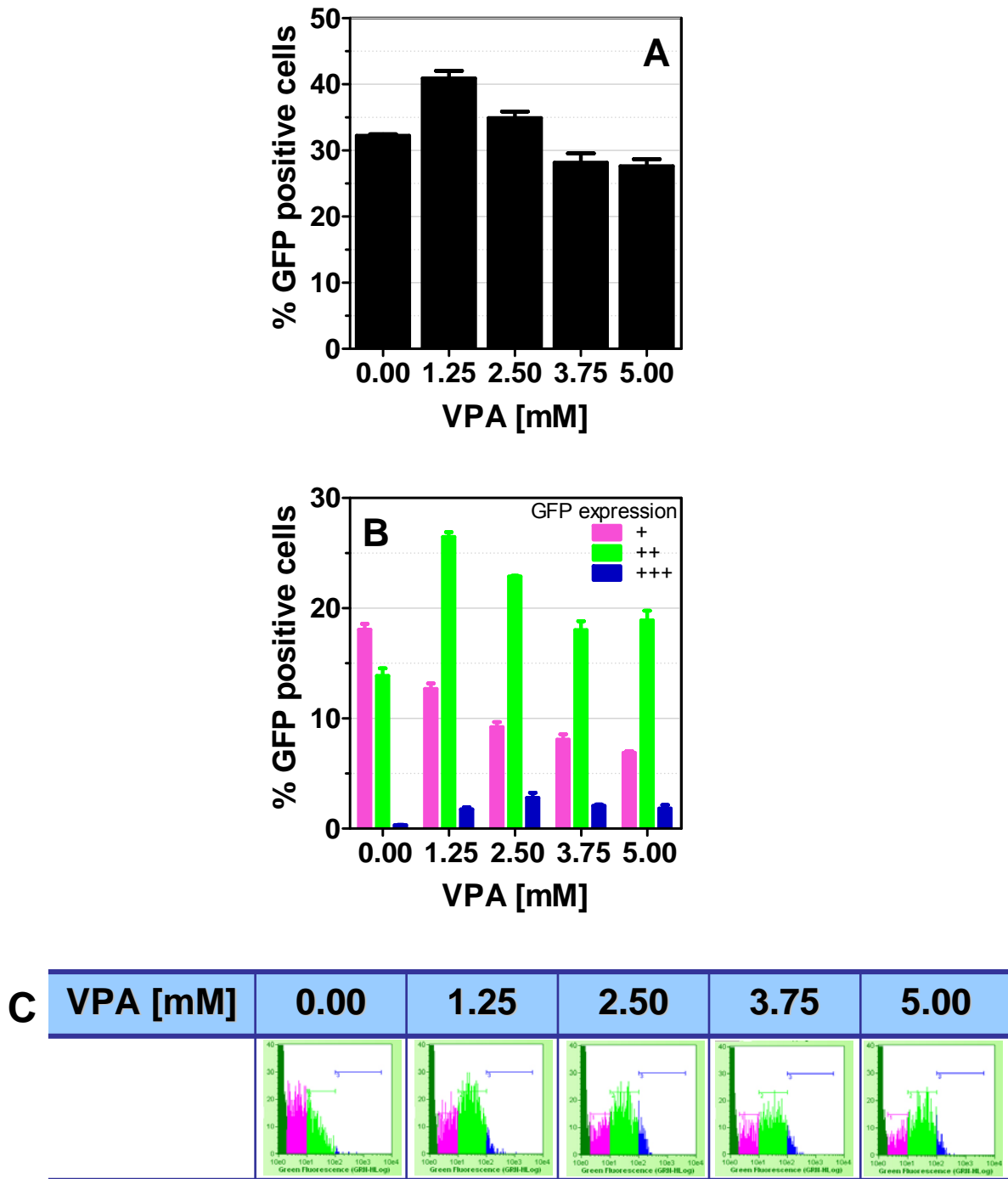


Figure 5.3. Effect of VPA on transient GFP expression. CHO DG44 cells were transfected with pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-GFP at a 49:49:2 ratio (w/w/w). Four hours after transfection, VPA at different concentrations was added to different concentrations. The cultures were sampled at day 3 post-transfection. The percentage (A) and distribution of GFP-positive cells (B,C) were determined by flow cytometry at each VPA concentration. Error bars represent the standard deviation from two parallel experiments.

5.2.2 Growth and viability of cells treated with VPA

CHO cells were transfected as described in Section 5.2.1 and 4 hours after transfection different concentrations of VPA were added. At various times after transfection, the cell density and viability were determined by manual cell counting and the biomass was measured using mini-PCV tubes. VPA significantly influenced the cell density (Figure 5.4A). Cultures treated with VPA at a concentration of 2.5 mM or higher did not reach a cell concentration greater than 4×10^6 cells/mL whereas the cell density of the untreated culture reached 7×10^6 cells/mL (Figure 5.4A). The impact of VPA on cell viability was even more pronounced. The viability decreased dramatically with increasing VPA concentration (Figure 5.4B). At day 7 post-transfection, all the cells in the presence of 3.75 and 5 mM VPA were dead. It was observed by light microscopy that cells treated with VPA were smaller than the untreated control cells. This phenomenon could correlate to the huge influence that VPA had on total biomass. The biomass of cultures treated with VPA was almost 3.5-fold lower that of the untreated control transfection (Figure 5.4C).

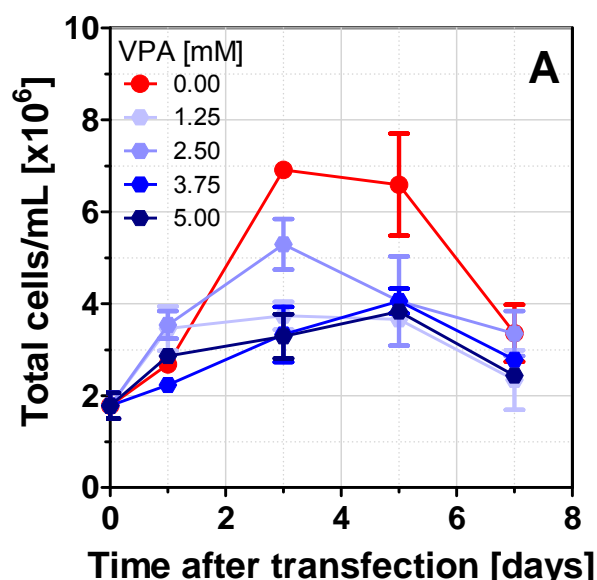


Figure 5.4A. Effect of VPA on cell growth. CHO DG44 cells were transfected with pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-GFP at a 49:49:2 ratio (w/w/w). Four hours after transfection, VPA was added to different concentrations. Cell growth was measured at various times after transfection. Error bars represent the standard deviation from two parallel experiments.

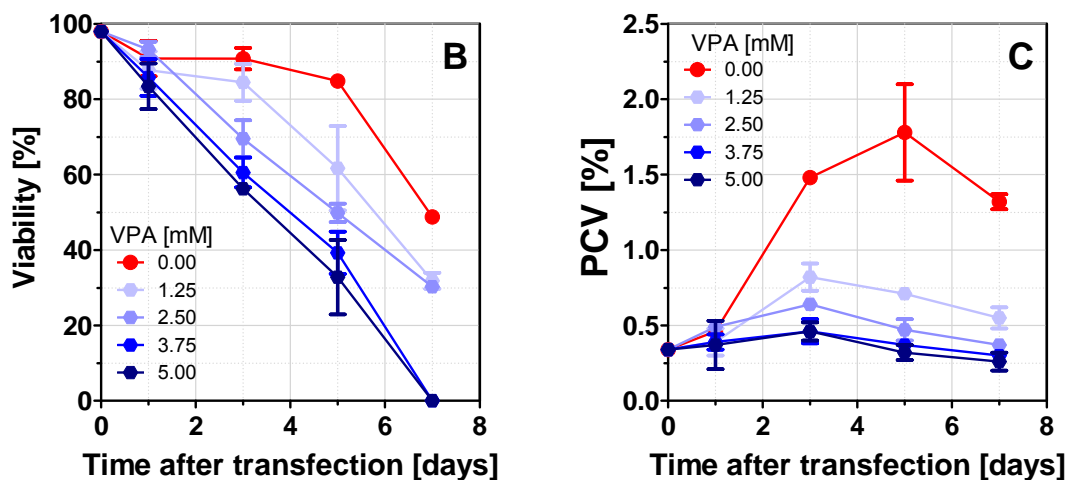


Figure 5.4B and 5.4C. Effect of VPA on viability and biomass. CHO DG44 cells were transfected with pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-GFP at a 49:49:2 ratio (w/w/w). Four hours after transfection, VPA was added to different concentrations. Viability (B), and PCV (C) were measured at various times after transfection. Error bars represent the standard deviation from two parallel experiments.

5.2.3 Effect of VPA on mRNA level and stability

To better characterize the intracellular effects of VPA on CHO cells, the steady-state level of IgG light chain mRNA was quantified 3 days after transfection. CHO cells were transfected with pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP at a 49:49:2 ratio (w/w/w) and 4 h after transfection VPA was added to various concentrations. The IgG light chain mRNA level, as determined by qRT-PCR, increased as the VPA concentration increased and was 13-fold higher than in the untreated control in the presence of 5 mM of VPA (Figure 5.5A). However, the recombinant IgG level did not follow the same pattern as the IgG light chain mRNA level. The highest levels of IgG were reached in the presence of 2.5-3.75 mM VPA and did not increase with further addition of VPA (Figure 5.5B). The plateau of IgG production with increasing concentrations of VPA may have been due to the low viability associated with the VPA treatment.

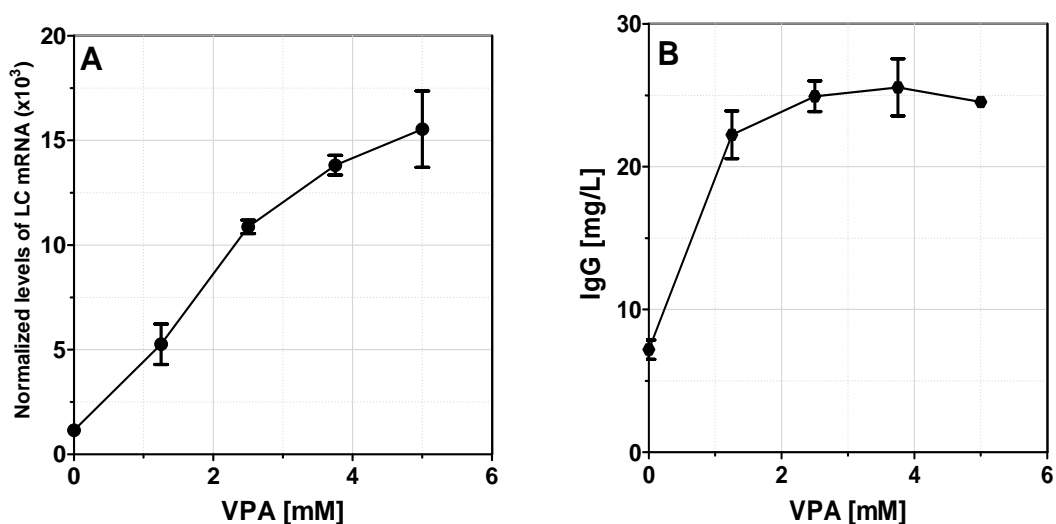


Figure 5.5. Effect of VPA on the steady-state IgG light chain mRNA level and on rhIgG production. CHO DG44 cells were transfected with pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-GFP at a 49:49:2 ratio (w/w/w). Four hours after transfection, VPA was added to different concentrations. mRNA (A) and IgG levels (B) were measured at day 3 post-transfection. The delta-delta-Ct method was used to calculate the relative quantity of light chain mRNA using the β -actin mRNA and mRNA from non-transfected cells for normalization. Error bars represent the standard deviation from two parallel experiments.

To further characterize the effect of VPA on transfected cells, the steady-state IgG light chain mRNA level was measured in the presence of 3.75 mM VPA at different times after transfection. Cells were transfected as described in the previous experiment, and the relative steady-state level of IgG light chain mRNA was determined by qRT-PCR. Levels of mRNA were significantly higher in the presence of VPA as compared to the untreated control culture, and this difference increased with time (Figure 5.6A). Transient recombinant IgG production reflected the steady-state IgG light chain mRNA level (Figure 5.6B). Similar results were observed for the quantification of the IgG heavy chain mRNA (data not shown).

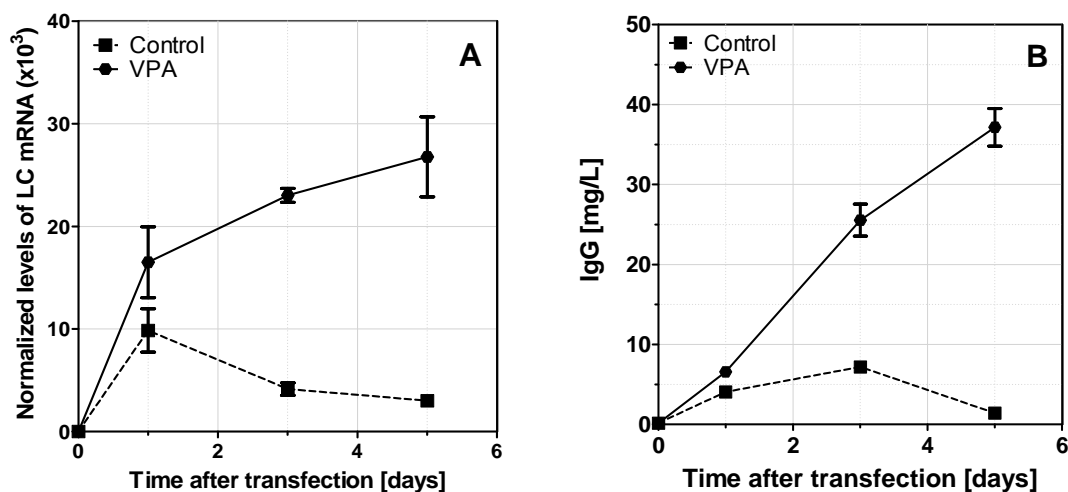


Figure 5.6. Effect of VPA on the steady-state IgG light chain mRNA level and on IgG production. CHO DG44 cells were transfected with pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-GFP at a 49:49:2 ratio (w/w/w). Four hours after transfection, VPA was added to 3.75 mM. The steady-state IgG light chain mRNA level (A) and recombinant IgG (B) were measured at the times indicated. The delta-delta-Ct method was used to calculate the relative quantity of light chain mRNA using the β -actin mRNA and mRNA from non-transfected cells for normalization. Error bars represent the standard deviation from two independent experiments.

5.2.4 pDNA stability after VPA treatment

The IgG light chain mRNA levels following transfection in the presence of VPA may have enhanced due to higher pDNA stability. To test this possibility, CHO cells were transfected with pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP at a ratio of 49:49:2 (w/w/w), and at 4 h after transfection, 3.75 mM VPA was added. pDNA was extracted at various times after transfection. As already reported for hypothermic conditions (see Chapter 4), the steady-state level of pDNA was decreased by about 50% within 24 h post-transfection and continued to decrease over time in both the VPA-treated and the untreated culture as determined by qRT-PCR (Figure 5.7). Thus, VPA did not help to stabilize pDNA in cells after transfection.

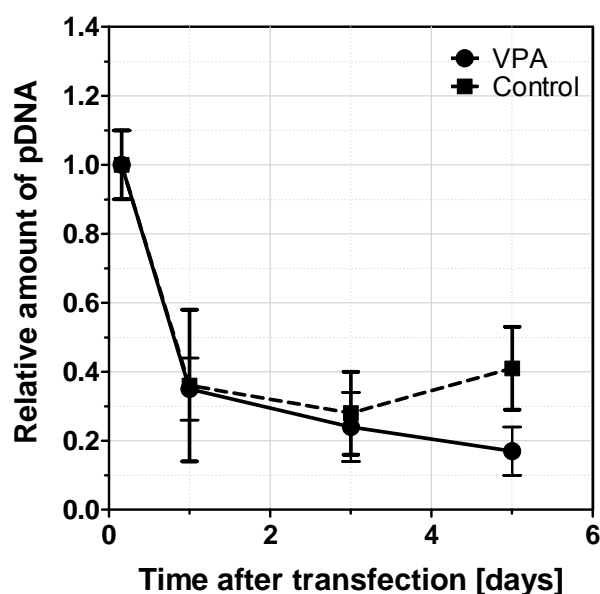


Figure 5.7. Effect of VPA on pDNA stability in CHO cells. CHO DG44 cells were transfected with pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP at a 49:49:2 ratio (w/w/w). Four hours after transfection VPA was added to 3.75 mM. Transfected cells not treated with VPA were used as the control. At the times indicated, one million cells were harvested, and the pDNA was extracted. Samples were then analyzed by qRT-PCR. A known amount of pDNA was used to create the standard curve. Error bars represent the standard deviation from two separate experiments

5.2.5 Influence of expression vector on the VPA enhancement of TGE

CHO cells were transfected with three different combinations of plasmids: (1) pEAK8-LH39, pEAK8-LH41, and pEGFP-N1 at a ratio of 29:69:2 (w/w/w); (2) pKML, pKMH, and pMYKpuro-EGFP at a ratio of 49:49:2 (w/w/w); (3) pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP at a ratio of 49:49:2 (w/w/w). The ratios for the IgG light and heavy chain plasmids for the different vector sets were individually optimized previously for transient expression at 37 °C (13-15). The cells were treated with 3.75 mM VPA at 4 h post-transfection, and at 3 days post-transfection IgG titers were quantified by ELISA. The greatest effect on IgG yield- almost 8-fold increase over the untreated control- was observed using the pXLG^{HEK} vectors (Figure 5.8). By comparison, VPA increased IgG production 3-fold following transfection with pKML and pKMH, while IgG production was decreased 0.5-fold when using the pEAK8 vectors (Figure 5.8).

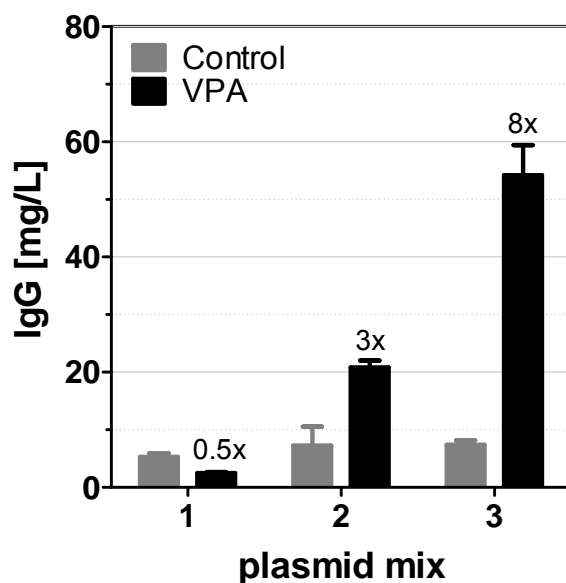


Figure 5.8. Vector influence on VPA enhancement of TGE. Cells were transfected with (1) pEAK8-LH39, pEAK8-LH41, and pEGFP-N1 at a ratio of 29:69:2 (w/w/w); (2) pKML, pKMH, and pMYKpuro-EGFP at a ratio of 29:69:2 (w/w/w); and (3) pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP at a ratio of 49:49:2 (w/w/w). The cells were either treated with 3.75 mM VPA at 4 h post-transfection (VPA) or not treated (control). The recombinant IgG concentration in the medium was measured at 3 days post-transfection. The fold increase over the control is indicated above the bar representing the reporter protein yield after treatment with VPA. The error bars represent the standard deviation from two independent experiments.

5.2.6 Use of VPA under hypothermic conditions

To determine if VPA treatment in combination with a hypothermic shift could have a synergistic effect on TGE, CHO cells were transfected with pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP at a 49:49:2 ratio (w/w/w). Four hours after transfection VPA was added to different concentrations and the cultures were shifted to 31 °C. In contrast to the enhancement of IgG production in the presence of VPA at 37 °C (see Figures 5.1 and 5.5B), at 31 °C the addition of 1.25 mM VPA only increased IgG yields about 30% relative to the untreated control transfection at 31 °C (Figure 5.9). At higher VPA concentrations a negative impact on recombinant protein production was observed (Figure 5.9). By analyzing the distribution of GFP-positive cells after transfection, it was found that the level of high-producing GFP-positive cells (++++) was 2-fold higher in the presence of VPA than in its absence at 31 °C (Figure 5.10). Finally, the steady-state IgG light chain mRNA

level in the presence of 1.25 mM VPA with incubation at 31 °C did not differ from the control transfection as determined by qRT-PCR (Figure 5.11).

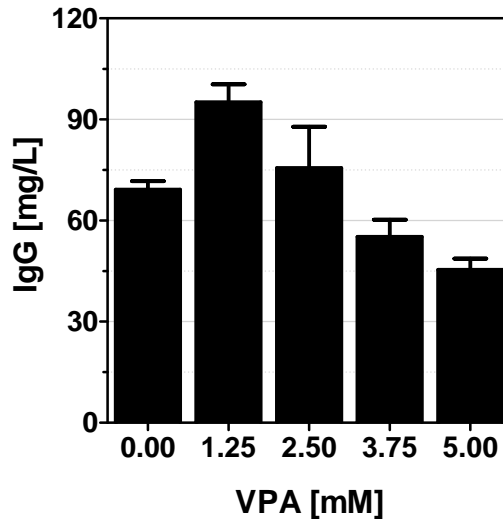


Figure 5.9. Effect of VPA on IgG production under hypothermic conditions. CHO DG44 cells were transfected with pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-GFP at a 49:49:2 ratio (w/w/w). Four hours after transfection, VPA was added to 1.25 mM and the cells were shifted to 31 °C. IgG titers were measured at day 6 post-transfection. Error bars represent the standard deviation from two independent experiments.

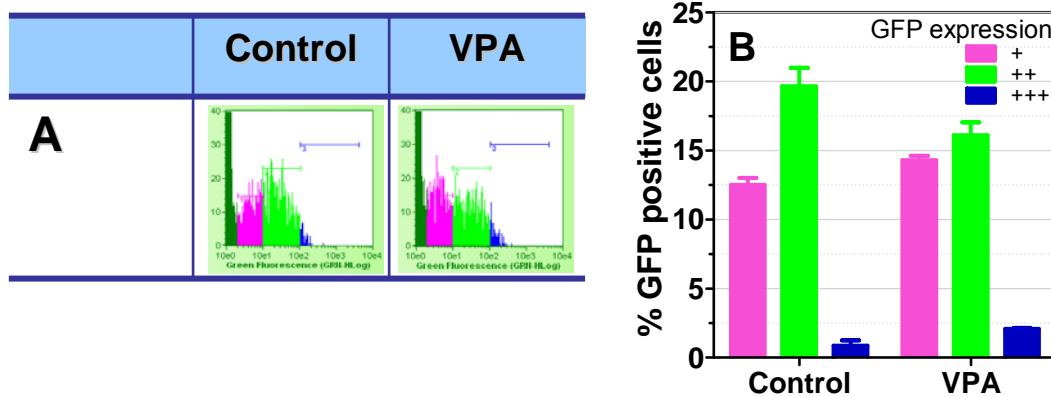


Figure 5.10. Effect of VPA and hypothermic treatment on GFP expression. CHO DG44 cells were transfected with pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-GFP at a 49:49:2 ratio (w/w/w). Four hours after transfection, 1.25 mM VPA was added and cells were shifted to 31 °C. The control culture was not treated with VPA but was only cultivated at 31 °C after transfection. At day 3 post-transfection the cells were analyzed by flow cytometry. Error bars represent the standard deviation from two independent experiments. The histograms from the flow cytometer (A) and the distribution of GFP-positive cells into different arbitrary categories (B) are shown.

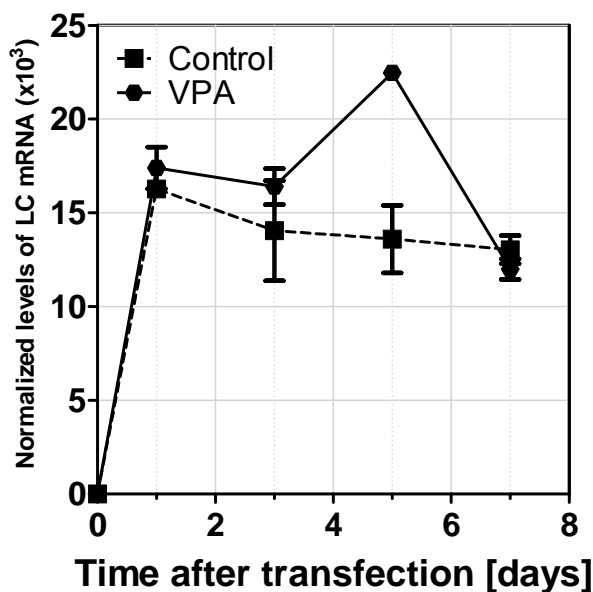


Figure 5.11. Effect of VPA on the steady-state IgG light chain mRNA level under hypothermic conditions. CHO DG44 cells were transfected with pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-GFP at a 49:49:2 ratio (w/w/w). Four hours after transfection, VPA was added to 1.25 mM and the cells were shifted to 31 °C. Steady-state IgG light chain mRNA levels were measured at day 3 post-transfection. The delta-delta-Ct method was used to calculate the relative quantity of light chain mRNA using the beta-actin mRNA and the mRNA from non-transfected cells for normalization. Error bars represent the standard deviation from two independent experiments

5.2.7 The impact of VPA's iHDAC activity on TGE

To better understand the possible influence of the iHDAC activity of VPA, transiently transfected CHO cells were treated either with VPA or with VDP, an analogue of VPA that does not inhibit HDAC activity (Figure 5.12) (1).

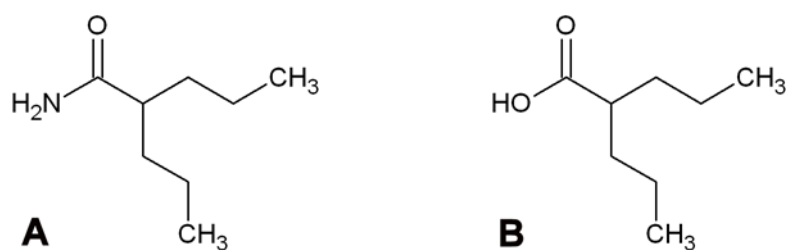


Figure 5.12. Schematic diagrams of the chemical structures of VPD (A) and VPA (B).

CHO cells were transfected with pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP at a 49:49:2 ratio (w/w/w). Four hours after transfection, VPA or VPD was added to various concentrations. The recombinant IgG yield of each transfection was determined at 6 days post-transfection. Although both VPA and VPD had positive effects on recombinant IgG yield, but the highest titers achieved with VPD were only half of those reached with VPA (Figure 5.13).

The effect of VPD on TGE was also analyzed with other IgG expression vector combinations. CHO cells were transfected with: (1) pEAK8-LH39, pEAK8-LH41, and pEGFP-N1 at a ratio of 29:69:2 (w/w/w); (2) pKML, pKMH, and pMYKpuro-EGFP at a ratio of 49:49:2 (w/w/w); and (3) pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP at a ratio of 49:49:2 (w/w/w). Four hours after transfection, 2.5 mM VPA or VPD were added to the cultures. The recombinant IgG yields were quantified 3 days post-transfection. The IgG yields for each set of transfections varied (Figure 5.14). With the set of pXLG^{HEK} vectors, VPA had a greater effect than VPD on recombinant IgG production. In contrast, IgG production was about the same from pKML and pKMH and from the pEAK8 vector set in the presence of either VPA or VPD (Figure 5.14).

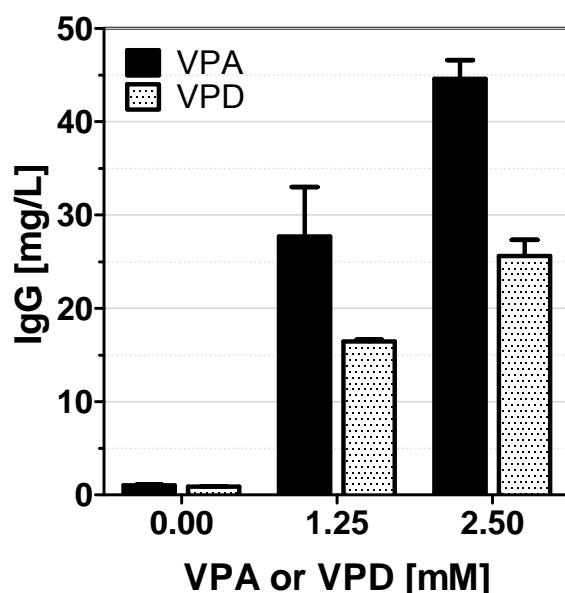


Figure 5.13. Effect of VPA and VPD on transient IgG production. CHO DG44 cells were transfected with pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-GFP at a 49:49:2 ratio (w/w/w). Four hours after transfection, VPA or VPD was added to various concentrations. Recombinant IgG titers were measured by ELISA at day 6 post-transfection. Error bars represent the standard deviation from two independent experiments.

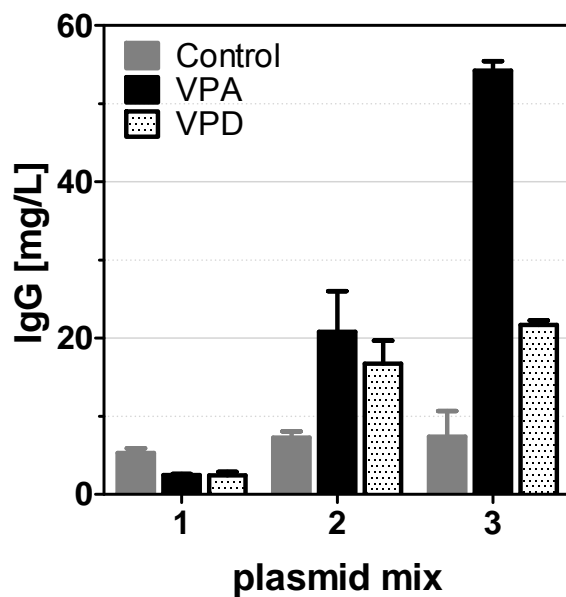


Figure 5.14. Effect of VPA and VPD on transient rhIgG production from different vectors. CHO DG44 cells were transfected with (1) pEAK8-LH39, pEAK8-LH41, and pEGFP-N1 at a ratio of 29:69:2 (w/w/w); (2) pKML, pKMH, and pMYKpuro-EGFP at a ratio of 29:69:2 (w/w/w); and (3) pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP at a ratio of 49:49:2 (w/w/w). The cells were treated with 2.5 mM VPA or VPD at 4 h post-transfection. Recombinant IgG concentration in the medium was measured at 3 days post-transfection by ELISA. The error bars represent the standard deviation from two independent experiments.

5.3 Discussion and conclusions

The data reported here provide a better understanding of the effects of VPA on TGE in CHO cells. VPA increased recombinant protein yields up to 20-fold at 37 °C. The enhancement was dependent on the VPA concentration, the recombinant protein being expressed, and the expression vector itself. The increased protein production was related to the increase in the steady-state level and the stability of the transgene mRNA, at least for the IgG light and heavy chain mRNAs. This was due in part to the ability of VPA to inhibit HDAC activity (3;4). However, it was also observed that VPD, a VPA analogue without iHDAC activity, also enhanced transient recombinant protein production. Thus, the ability of VPA and VPD to arrest cell growth was also important for the enhancement of TGE.

With VPA treatment, the transient production of both reporter proteins, GFP and IgG, increased. Cells treated with VPA had a 4-fold increase of IgG production at day 3 post-transfection. Moreover, the IgG yields from cultures treated with VPA increased up to day 7 post-transfection as did the IgG light chain mRNA level. Here it was shown that steady-state transgene mRNA levels were higher in the presence of VPA than in its absence. Similar results on transgene mRNA levels have previously been reported for the effects of sodium butyrate, another histone deacetylase inhibitor (5). Furthermore, in the presence of VPA, the transgene mRNA level increased over time and in a dose-dependent manner. In contrast, the level of pDNA was not stabilized after treatment with VPA. The pDNA copy number per cell decreased dramatically from the first hours after transfection either in the presence or in absence of VPA.

The enhancement of the recombinant IgG yield was not dose-dependent whereas that of GFP was. Since GFP is an intracellular protein, its expression and accumulation is strongly correlated to cell viability. Samples treated with high concentrations of VPA (> 2.5 mM) had low cell viability, confirming what has already been reported in literature (1;11;12). However, GFP expression in the presence of VPA was always higher than in the untreated control. After VPA treatment, a greater percentage of the GFP-positive cells were present as high-producing cells, leading to a higher total fluorescence.

It has been reported that VPA has an impact on cell growth (1;11;12). Recently, VPA treatment of HeLa cells was shown to up-regulate p21/WAF-1, a cyclin-dependent kinase inhibitor, and blocked the cell cycle in the G1/G0 phase (16). Here it was shown that the cell growth arrest induced by VPA was dose-dependent. Moreover, cell morphology was altered in the presence of VPA. Cell size was reduced as the concentration of VPA

increased, leading to a lower biomass accumulation in the transfected cultures. By treating transfected cells with VPD, an analogue of VPA that does not inhibit HDACs (1), it was shown that the iHDAC activity of VPA was not the only factor in the enhancement of TGE. Like VPA, VPD also arrested CHO growth and reduced total biomass, but it only enhanced transient IgG production 4-10-fold as compared to 8-20-fold for VPA.

Lowered cell metabolism, increased steady-state transgene mRNA levels, and increased recombinant protein production are phenomena that have also been observed in transfected CHO DG44 cells cultured under hypothermic conditions (17;18)(see also Chapter 4). For this reason, the effect of VPA at 31 °C was investigated. The combination of hypothermia and VPA led to a increase in transient recombinant protein yield compared to either treatment alone but only when low VPA concentrations were used. This improvement in yield appeared to be related to an increase in the number of high-producing cells in the transfected population. However, the synergistic effect of VPA combined with low temperature was not accompanied by an additional increase in the steady-state level of transgene mRNA as compared to the cells maintained at 31 °C in the absence of VPA.

Until now, there has been no report concerning the effect of VPA on transient protein production with different expression vectors. Here it was shown that the enhancement of protein yield in the presence of VPA was vector-dependent. Three days post-transfection, the enhancement of transient gene expression from pXLG^{HEK} and pKML/pKMH vectors was up to 8-fold following VPA treatment, whereas VPA had a negative effect on transgene expression when using the EF-1 α promoter. The vectors that supported the greatest enhancement of TGE in the presence of VPA (pXLG^{HEK} and pKML/pKMH) were the ones in which the transgene was under the control of the human or mouse CMV promoter/enhancer. It has been reported that viral promoters are prone to epigenetic silencing (5;19;20). Thus a possible explanation for the differential effects of VPA on the various promoters tested here is that the mCMV and the hCMV were more subject to negative epigenetic effects than was the cellular EF-1 α promoter. However, other hypotheses could explain the different behaviour of the three plasmids. For example, each vector was different with regard to other sequence elements besides the promoter that may have been prone to epigenetic silencing (21;22). The negative effect of VPA treatment on the pEAK8 vectors appeared to be linked to cell growth arrest since VPD treatment also had a negative effect on recombinant protein production when these vectors were used.

In conclusion, this study demonstrated that VPA increases recombinant protein production in transiently transfected CHO cells. Furthermore, it was shown that the steady-state level and stability of the transgene mRNA were increased in the presence of VPA. Due to its low cost and ease of use, the treatment of transfected cells with VPA is a promising strategy to improve TGE at small or large scale. However, further studies will be necessary to better understand the mechanism of VPA on transient recombinant protein productivity.

5.4 References

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Chapter 6

Overexpression of selected transcription factors and growth factors enhances TGE yields in CHO cells

6.1 Introduction

Gene expression in mammalian cells is a complex phenomenon regulated by specific transcription factors. To accomplish their function these factors are able to bind specific DNA sequences within the enhancer and/or promoter of a gene and to recruit transcriptional activators and the RNA Polymerase II holoenzyme (1;2). The expression of the transcription factors themselves or their activation from an inactive form can be induced by external stimuli such as exposure to growth factors that function through phosphorylation cascades (1). The human cytomegalovirus early promoter/enhancer has several binding sites for transcription factors, particularly NF-kB, c-jun, c-fos, CREB, Elk1, and Sp1 (3-7). Therefore, the transcription of a transgene under its control may be expected to be stimulated by overexpression of these proteins.

In this chapter two different approaches aimed at increasing TGE yields following transfection with plasmids containing the hCMV promoter/enhancer are reported. The first strategy involved the overexpression of different transcription factors together with the reporter gene(s) in CHO cells. The second strategy was aimed at increasing TGE yields through external stimulation with growth factors (8-11). This work leads to possible approaches to increase transgene mRNA levels and thus recombinant protein expression in CHO cells.

6.2 Results

6.2.1 Effect of overexpression of transcription factors on TGE

CHO DG44 cells were transfected with a mixture containing 30% of the transcription factor cDNA cloned in pXLG^{HEK} and 70% of pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP [49:49:2 (w/w/w)]. IgG and GFP yields were analyzed at day 3 post-transfection. For both proteins a 2- to 3-fold increase in yield was observed when the transcription factors c-jun, c-fos, NF-kB, or Sp1 were overexpressed (Figure 6.1). In contrast, overexpression of CREB and Elk1 did not contribute to the enhancement of recombinant protein expression.

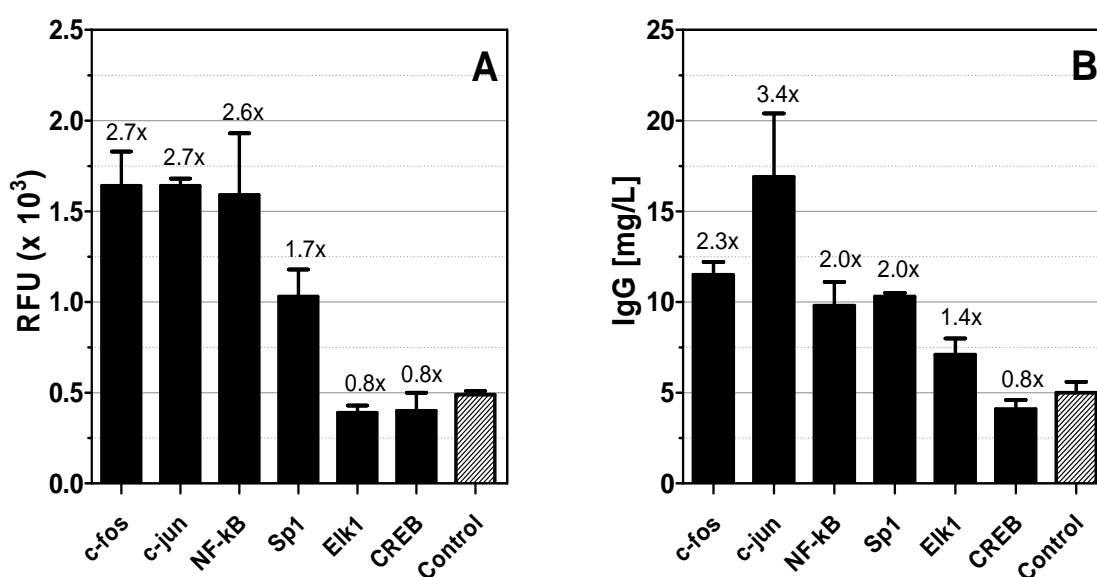


Figure 6.1. Effect of overexpression of transcription factors on TGE. Cells were transfected with a mixture containing 30% of pXLG^{HEK}-transcription factor and 70% of pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP (mass ratio 49:49:2). For the control transfections, pXLG^{HEK} replaced pXLG^{HEK}-transcription factor. GFP-specific fluorescence (A) and antibody concentration in the medium (B) were measured at 3 days post-transfection. The fold increase over the control is indicated above the bar representing each overexpressed transcription factor. The error bars represent the standard deviation from two parallel experiments.

For each transfection described above, the IgG light chain mRNA level was determined by qRT-PCR (12;13). The relative level of IgG light chain mRNA was 2.5-fold higher in the presence of NF- κ B and 2-fold higher when c-fos, c-jun or Sp1 were overexpressed (Figure 6.2). The overexpression of CREB led to a relative decrease in the amount of the light chain mRNA, whereas the presence of Elk1 increased the light chain mRNA level 1.5-fold relative to the control.

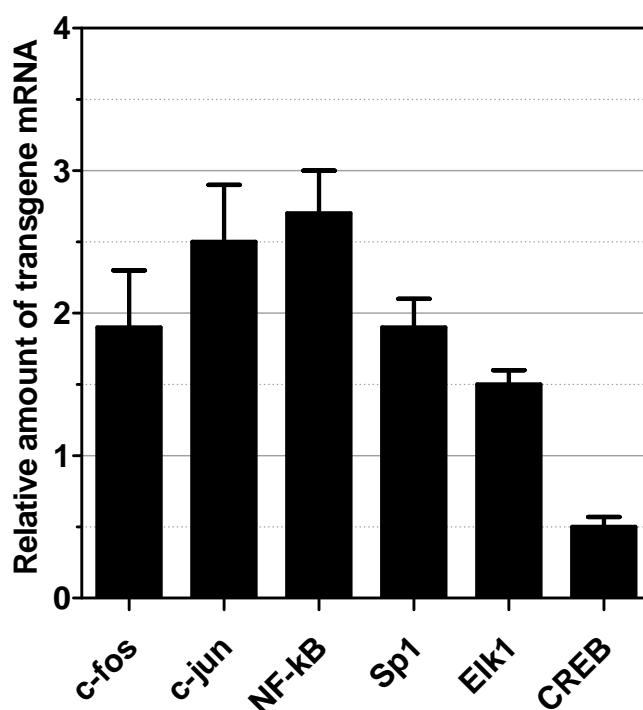


Figure 6.2. Effect of transcription factor overexpression on relative IgG light chain mRNA accumulation. Cells were transfected with a mixture containing 30% of pXLG^{HEK}-transcription factor and 70% of pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP (mass ratio of 49:49:2). Control transfections were performed by replacing pXLG^{HEK}-transcription factor with pXLG^{HEK}. At day 3 post-transfection, one million cells were harvested, and total mRNA was extracted and retrotranscribed into cDNA. Samples were then analyzed by qRT-PCR. The delta-delta-Ct method was used to calculate the relative quantity of IgG light chain mRNA using β -actin mRNA and mRNA from non-transfected cells for normalization. Error bars represent the standard deviation from two parallel experiments.

To determine the optimal amount of transcription factor plasmid per transfection, CHO DG44 cells were transfected as described above except that pXLG^{HEK}-transcription factor was varied from 0 to 40%. For the c-fos, c-jun, NF-kB, and Sp1 vectors, the maximum effect on reporter protein yield was observed when these plasmids represented 20-30% of the transfected DNA (Table 6.1). The presence of CREB and Elk1 did not enhance transient recombinant protein yields at any conditions tested (Table 6.1). Moreover combinations of different transcription factor vectors were tested but there were no differences compared to the use of single vectors in terms of IgG yields (data not shown).

Table 6.1. Optimal amounts of transcription factor vectors for TGE.

Transcription Factor	Optimal amount of plasmid DNA
c-fos	30 %
c-jun	20 %
NF-kB	30 %
Sp1	30 %
Elk1	0 %
CREB	0 %

6.2.2 Effect of VPA in combination with exogenous transcription factors on TGE yields

The influence of VPA on TGE yields in CHO cells has been observed (see Chapter 5). CHO cells were transfected with a mixture of plasmids containing 30% pXLG^{HEK}-transcription factor and a 1:1 (w/w) mix of pXLG^{HEK}-RhHC and pXLG^{HEK}-RhLC (70% of the total DNA). At 4 h post-transfection, VPA was added to a final concentration of 2.5 mM (Chapter 5). Antibody production was quantified 3 and 6 days post-transfection. Overall, the recombinant IgG yields were higher with VPA (Figure 6.3), but the strongest effect was noticed six days post-transfection (Figure 6.3B). The highest IgG yields were observed when the cells treated with VPA or NaBut had been co-transfected with vectors for c-fos, c-jun, or NF-kB overexpression (Figure 6.3B).

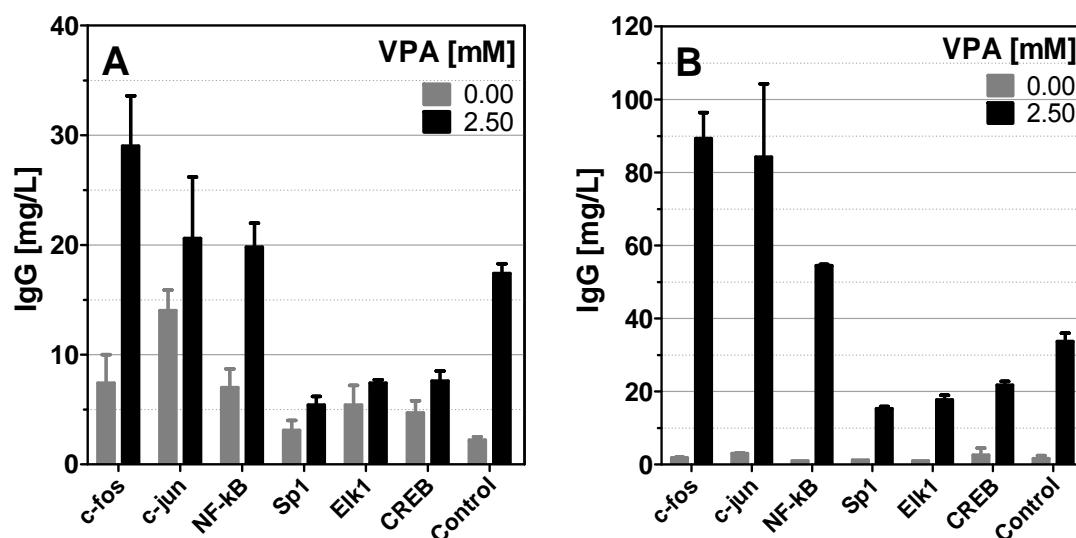


Figure 6.3. Effect of VPA and overexpression of transcription factors on transient IgG production. Cells were transfected with a mixture containing 30% of pXLG^{HEK}-transcription factor and 70% of pXLG^{HEK}-RhLC and pXLG^{HEK}-RhHC (mass ratio of 50:50). The control transfections were performed by replacing a transcription factor vector with pXLG^{HEK}. At 4 h post-transfection VPA to 2.5 mM was added to the culture. At 3 (A) and 6 (B) days post-transfection, the recombinant IgG concentration was determined by ELISA. Error bars represent the standard deviation from three parallel experiments.

6.2.3 TGE yields in stable cells overexpressing c-fos

Pools of CHO cells stably overexpressing c-fos were selected in the presence of puromycin after co-transfection of pMYKEF1-puro-EGFP and pXLG^{HEK}-c-fos. Control cell lines were selected after transfection with pMYKEF1-puro-EGFP alone. Pools of puromycin-resistant cells (\pm c-fos overexpression) were transfected with pXLG^{HEK}-RhLC and pXLG^{HEK}-RhHC (mass ratio of 50:50) and IgG yields were quantified 6 days post-transfection. In the presence of c-fos the antibody production was 1.5-fold higher than in the control cell pool (Figure 6.4A). Immunoblot with a polyclonal antibody against c-fos confirmed its overexpression in the pool of cells transfected with pXLG^{HEK}-c-fos (Figure 6.4B). To better control exogenous c-fos expression, the gene was cloned under the control of an acetaldehyde inducible promoter¹. However, also in that case it was observed a minimal benefit of c-fos (see Appendix 1, Section A1.2.5).

¹ More details about the system are reported in the Appendix 1

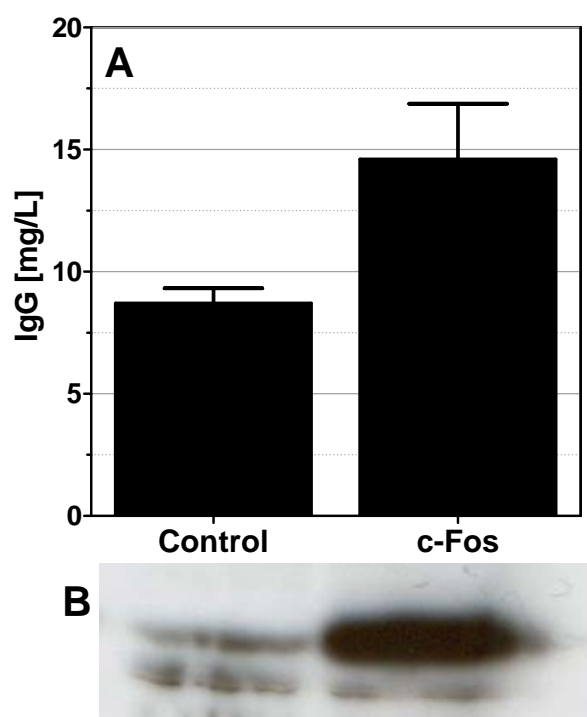


Figure 6.4. Effect of stable overexpression of c-fos on TGE. Pools of CHO cells stably expressing c-fos (c-fos) or GFP (Control) were transfected with pXLG^{HEK}-RhLC and pXLG^{HEK}-RhHC (mass ratio of 50:50). (A) At 3 days post-transfection IgG yields were quantified by ELISA. Error bars represent the standard deviation from two parallel experiments. (B) At 3 days post-transfection, 5 million cells were harvested from each transfection and lysed. Each lysate was analyzed by immunoblot for the expression of c-fos using a polyclonal antibody anti human c-fos (Chapter 2, section 2.16).

6.2.4 Effect of overexpression of growth factors on TGE yields

To determine the effect of growth factors on TGE, vectors constructed with the cDNAs of different growth factors² (10% of the total DNA) were co-transfected in CHO cells with a mix (90% of the total DNA) of pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP (at a mass ratio of 49:49:2). The control transfections were performed by replacing the growth factor vectors with pXLG^{HEK}. IgG expression was quantified by ELISA at 3 and 6 days post-transfection. By 3 days post-transfection, none of the exogenous growth factors demonstrated an enhancement of IgG yield (Figure 6.5). By 6

² The vectors were kindly provided by Dr. Gaurav Backliwal (14;15)

days post-transfection, the culture transfected with pMYKEF1-aFGF yielded ~17 mg/L of IgG while the yields of the other transfections were less than 3 mg/L (Figure 6.5).

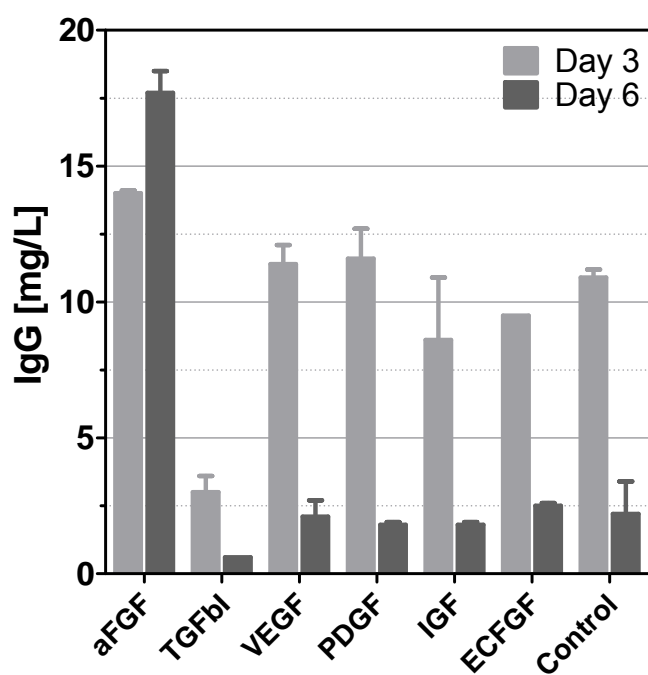


Figure 6.5. Effect of overexpression of growth factors on TGE yield. Cells were transfected with a mixture containing 10% of plasmid carrying a growth factor gene and 90% of a mix of pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP (at a mass ratio of 49:49:2). For the control transfection, pXLG^{HEK} was substituted for the growth factor vector. Antibody concentration in the medium was measured 3 and 6 days post-transfection. The error bars represent the standard deviation from two parallel experiments.

To find the optimal amount of aFGF plasmid for transfection, CHO cells were transfected with varying amounts of the vector (0 to 30% of total DNA) and a mix of pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP (mass ratio of 49:49:2) (70% of the total DNA). If necessary, the DNA was increased to 100% by addition of pXLG^{HEK}. At 3 days post-transfection, the transient IgG yields were quantified by ELISA. The highest concentration of IgG was observed when pMYKEF1-aFGF amount of 5% of the total DNA. Further increases in the amount of the aFGF vector resulted in decreased recombinant protein production (Figure 6.6A). The presence of aFGF in the supernatant was confirmed by immunoblot with a monoclonal antibody against aFGF (Figure 6.6B).

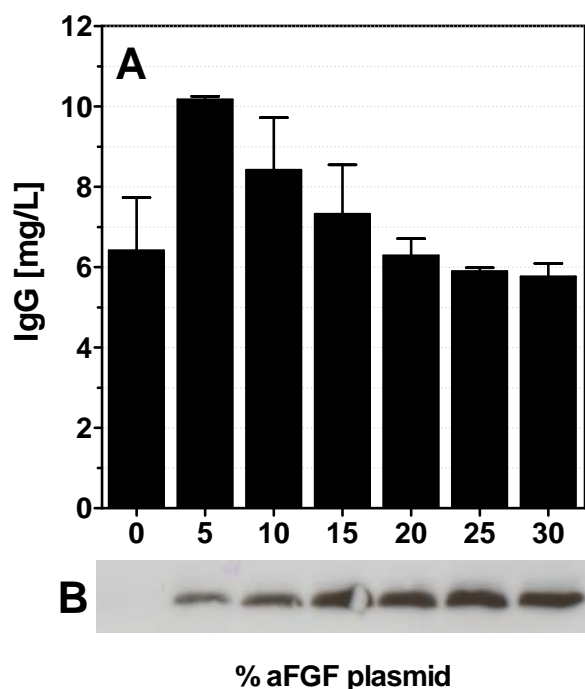


Figure 6.6. Optimization of pXLG^{HEK}-aFGF amount for transfection. Cells were transfected with a mixture containing 0-30% of pMYKEF1-aFGF and 70% of a mix of pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP (mass ratio of 49:49:2). The total DNA was made 100% by addition of pXLG^{HEK}, as needed. (A) Antibody concentration in the medium was measured 3 days post-transfection by ELISA. The error bars represent the standard deviation from two parallel experiments. (B) The presence of aFGF in the supernatant at 3 days post-transfection by immunoblot using an anti-human aFGF monoclonal antibody (Chapter 2, Section 2.16).

6.2.5 Co-cultivation of cells transiently expressing IgG and cells transiently expressing aFGF

As another approach to increase the TGE yield by aFGF treatment, CHO cells were separately transfected with pMYKEF1-aFGF and added to a culture of cells transiently transfected with a 1:1 mix (w/w) of pXLG^{HEK}-RhLC and pXLG^{HEK}-RhHC. In this way, the amounts of IgG light and heavy chain vectors in the transfection were increased as compared to the transfections reported in the previous section. Since aFGF can function in a paracrine manner, it was expected to enhance the TGE yield even if not expressed from the same cells as the recombinant IgG (Figure 6.7) (8).

CHO cells were transiently transfected with either pMYKEF1-aFGF or a mix of pXLG^{HEK}-RhLC and pXLG^{HEK}-RhHC as described above. At 4 h post-transfection

different amounts of CHO cells transiently expressing aFGF were mixed with CHO cells expressing IgG. Antibody yields were quantified at 6 days post-transfection and aFGF expression was confirmed by immunoblot. The highest IgG yields were observed in cultures in which 20% of the cells expressed aFGF (Figure 6.8).

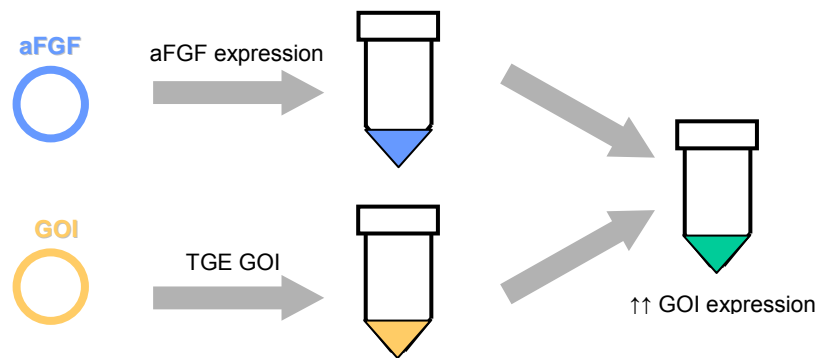


Figure 6.7. Schematic diagram of the co-cultivation strategy. Cells are transfected with the gene of interest (GOI) vector and mixed with cells transiently transfected with pMYKEF1-aFGF.

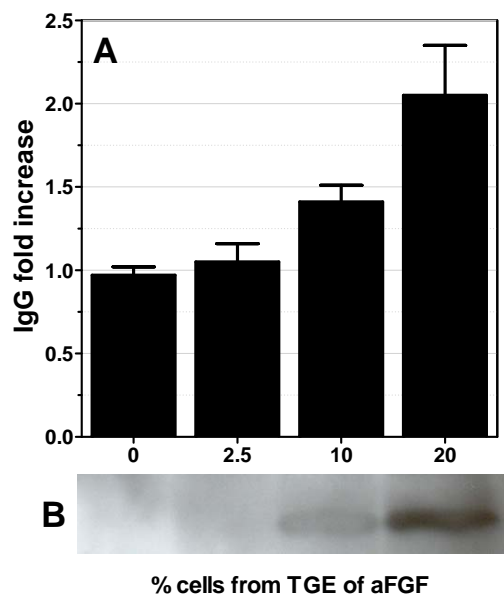


Figure 6.8. Effect of co-cultivation of cells transiently expressing aFGF on TGE yields. Cells were transfected either with pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC (mass ratio of 50:50) or with pMYKEF1-aFGF. Four hours after transfection, different amounts of cells expressing aFGF were added to cells transiently producing IgG. (A) Antibody yields were quantified 6 days after transfection and reported as fold increase compared to the control (0% of cells transiently producing aFGF). The error bars represent the standard deviation from two parallel experiments. (B) aFGF expression was confirmed by immunoblot as described in Figure 6.6.

6.2.6 Co-cultivation of cells transiently expressing IgG and cells stably expressing aFGF

To extend the strategy described in the previous section, pooled CHO cells stably expressing aFGF were co-cultivated with cells transiently transfected with pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC vectors (mass ratio of 50:50). Cell pools stably expressing GFP were used as a control. IgG yields increased as the percentage of aFGF-expressing cells increased (Figure 6.9). As expected, the transient IgG yields decreased as the percentage of cells stably expressing GFP increased (Figure 6.9).

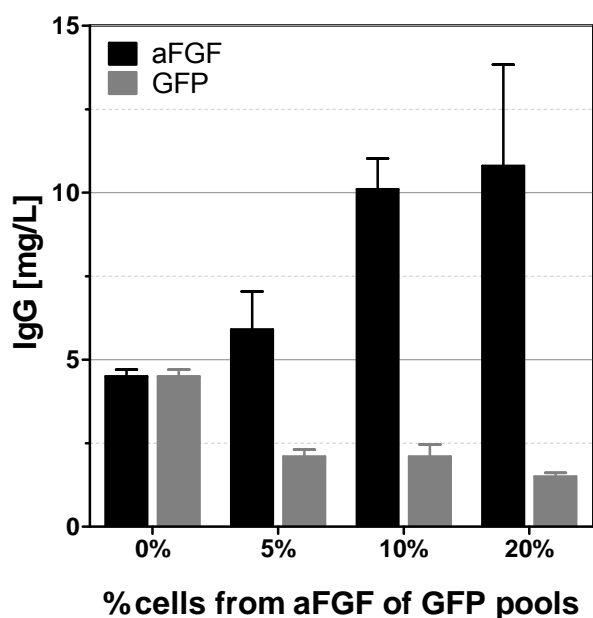


Figure 6.9. Transient IgG production from CHO cells co-cultured with stable pools expressing aFGF or GFP. Cells were transfected with pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC (mass ratio of 50:50). At 4 h post-transfection different amounts of cells stably expressing either aFGF or GFP were added to the transfected cell population. The IgG concentration was determined at 6 days post-transfection by ELISA. The error bars represent the standard deviation from two parallel experiments.

The experiment was then repeated with several clonal cell lines stably expressing aFGF. Each cell line was co-cultured with CHO cells transfected with pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC (mass ratio of 50:50). The final percentage of the aFGF expressing clones in the culture was 20%. At 6 days post-transfection, the transient IgG concentration was quantified by ELISA. Most of the the samples treated with the clones showed an

improvement on IgG titers (Figure 6.12A) and the enhancement seemed to correlate with the aFGF amount in the culture (Figure 6.10).

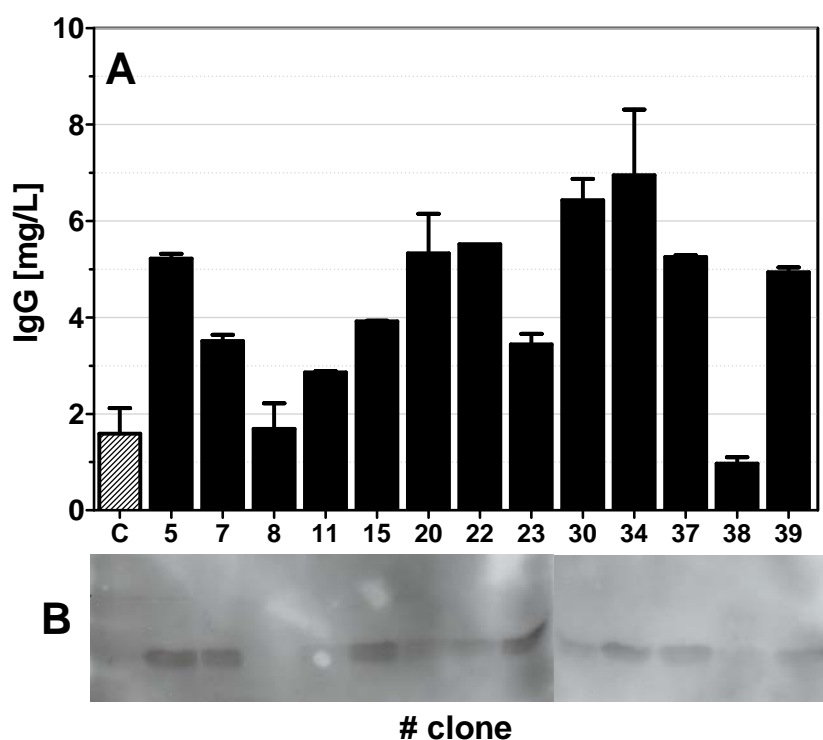


Figure 6.10. Transient IgG production from CHO cells co-cultured with clonal cell lines expressing aFGF. Cells were transfected with pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC (mass ratio of 50:50). At 4 h post-transfection the transfected cells were mixed with different CHO-derived cell lines expressing aFGF. The final percentage of the aFGF expressing clones was 20%. The control sample was a stable clone over-expressing GFP. (A) The recombinant IgG concentration was determined at 6 days post-transfection by ELISA. The error bars represent the standard deviation from two parallel experiments. (B) aFGF expression from each cell line was analyzed by immunoblot after 3 days of culture as described in Figure 6.6.

6.2.7 Effect of VPA or hypothermia on TGE in the presence of cells stably expressing aFGF

The influence of VPA and hypothermia on TGE yields in CHO cells has been described (Chapters 4 and 5). Here the possible synergistic effect on TGE yields of each of these treatments in the presence of aFGF was investigated. CHO cells were transfected

with a mix of DNAs containing pXLG^{HEK}-RhLC and pXLG^{HEK}-RhHC at a mass ratio of 50:50. At 4 h post-transfection, cells stably expressing aFGF as described above were added to a final cell concentration of 20% of the total. Cells were then treated with 2.5 mM VPA or shifted to 31°C. The control transfection was treated with VPA or incubated at 31°C without the addition of stable aFGF-expressing cells. IgG yields were quantified by ELISA at 6 days post-transfection. The presence of aFGF in the culture had the greatest effect when the cells were not treated with VPA or exposed to hypothermic conditions (Figure 6.11). Under hypothermic conditions and in the presence of VPA, the presence of aFGF did not have a significant impact on the recombinant IgG yield (Figure 6.11).

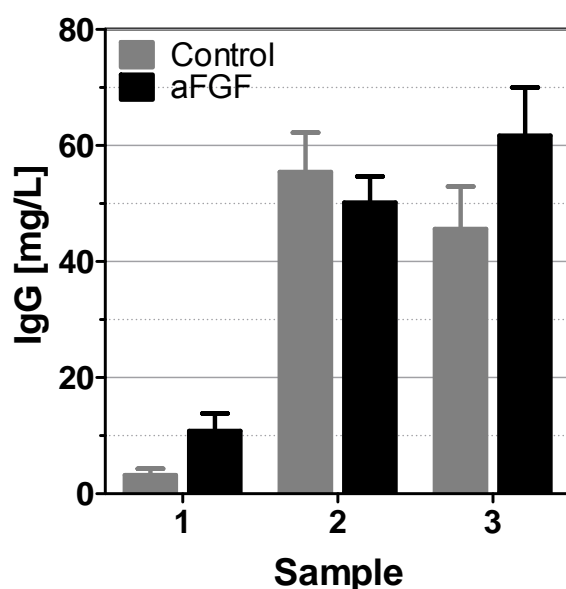


Figure 6.11. Transient IgG production from CHO cells co-cultured with pools expressing aFGF either under hypothermic conditions or after VPA treatment. Cells were transfected with pXLG^{HEK}-RhLC and pXLG^{HEK}-RhHC (mass ratio of 50:50). At 4 h post-transfection the transfected cells were mixed with cells stably expressing aFGF to a final concentration of 20% of the total cells. The control samples were not mixed with cells expressing aFGF. The recombinant IgG production was quantified by ELISA at 6 days post-transfection. Transfected cells were maintained at 37°C (1), maintained at 31°C (2), or maintained at 37°C and treated with 2.5 mM VPA. The error bars represent the standard deviation from two parallel experiments.

6.3 Discussion and conclusions

In this chapter two strategies for increasing TGE in CHO cells by enhancing transgene transcription were presented. Transcription factors and growth factors able to activate transcription of transgenes controlled by the immediate early cytomegalovirus promoter/enhancer were screened for their ability to increase transgene transient recombinant IgG production (3-7). Among the transcription factors tested, c-fos, c-jun, and NF- κ B were the ones that increased TGE yields to the greatest extent. Among the growth factors tested, only aFGF was able to improve TGE yields.

Transient overexpression of the three transcription factors mentioned above in addition to Sp1 increased transient IgG and GFP mRNA and protein levels. In contrast, the transient overexpression of CREB or Elk1 did not increase TGE yields. It was recently reported that the induction of c-fos, c-jun or NF κ B expression by doxorubicin increased the production of transgenes under the control of the immediate early CMV promoter/enhancer (4;5). The addition of VPA to the cells co-transfected with transcription factor genes had a synergistic effect on TGE yields. Similar effects of VPA and NaBut on recombinant protein production in CHO cells have previously been reported (16). In the presence of VPA cells overexpressing c-fos or c-jun produced ~90 mg/L of recombinant antibody by 6 days post-transfection.

Based on the results from transient expression of transcription factors, it was decided to try to develop an engineered CHO line that stably overexpressed c-fos from either a constitutive promoter. However, the benefit for TGE yields from constitutive c-fos expression was not as great as that observed in cells transiently producing c-fos. It is possible that the constitutive expression of c-fos had a negative impact on the cells. For this reason, c-fos was also transiently expressed from a gas inducible promoter (17;18). However, the enhancement of TGE yields after the induced expression of c-fos was not as high as in the cells co-transfected with a vector that constitutively expressed c-fos.

The second strategy to increase TGE yields involved the co-expression of growth factors. Those chosen were ones that were expected to activate the transcription of NF κ B, c-fos and c-jun (9-11). aFGF showed the highest improvement on protein transient protein production as previously reported (14). To eliminate the need to co-transfection with the aFGF vector, stable cell lines overexpressing aFGF were generated and co-cultured with transiently transfected CHO cells. In this experiment, transient IgG production was

enhanced by the presence of aFGF and the transient recombinant protein yield correlated with the amount of aFGF in the medium.

Both the approaches proposed in this chapter were found to increase transient recombinant protein production in CHO cells, but the yields were significantly lower compared to the ones reported after hypothermic shift (Chapter 4) or VPA treatment (Chapter 5). For the transcription factors tested, only the combination of VPA addition in presence of transient c-fos or c-jun expression resulted in comparable IgG yields to those reported in Chapters 4 and 5. However, similar yields were also reached with the combination of VPA and hypothermia, a simpler and more reproducible strategy compared to overexpression of a transcription factor either transiently or in an engineered cell line. VPA was also found to increase the TGE yields from cultures treated with aFGF; however, the benefit of the presence of aFGF was minimal compared to the effect of VPA alone. Since recombinant aFGF can be purified and used as media additive, further developments may be possible by adding it to transiently transfected CHO cells treated with VPA.

6.4 References

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Chapter 7

Conclusions and perspectives

7.1 Conclusions

Transient gene expression is a fast and easy method to produce recombinant proteins. Until now, however, the average yields produced by TGE have been modest compared to those obtained by SGE. The highest reported yields achieved in CHO cells were ~10-20 mg/L (1-4). Improvements in protein production by TGE have recently been reported, and it was possible to achieve almost 1 g/L of recombinant antibody using HEK293 cells culture in high cell and treated with VPA and cell cycle arrest genes (5).

The work described here was aimed at finding methods to enhance the productivity of TGE processes in CHO cells. The major bottleneck for recombinant protein expression was identified as the transgene mRNA level. Increasing the quantity of DNA used for transfection did not increase mRNA levels or recombinant protein yields. Instead, the amount of protein produced correlated with the amount of the transgene mRNA. It was also reported here that the recombinant protein yield was vector dependent and that the steady-state transgene mRNA level decreased within 3 days post-transfection. Therefore, three different strategies based on increasing and stabilizing transgene mRNA levels were proposed and analyzed.

- (I) Hypothermia was shown to modify cell metabolism, block cell cycle and increase transgene mRNA levels. It is not clear if all those phenomena act together in a synergistic way, however, significant improvements on recombinant protein yields were achieved after hypothermic shift of transiently transfected CHO cells to 31 °C. Moreover, for the first time it was reported that the steady-state transgene mRNA level under hypothermic conditions was

stabilized over time. The extent of the increase in recombinant protein yield was also partly dependent on the expression vector used for transfection and on the protein expressed.

- (II) Similar results were obtained with the treatment of transiently transfected CHO cells with VPA, a histone deacetylase inhibitor. In the presence of VPA, cell growth was slowed and the steady-state transgene mRNA level was higher and more stable over time as compared to the control transfection, leading to an increase in protein production up to 60 mg/L. Again, the extent of the yield increase was dependent on the plasmid used; the expression vectors with a viral promoter were more sensitive to VPA treatment.
- (III) The third strategy proposed for increasing the transgene mRNA level was the over expression of transcription factors and growth factors. Among the factors tested, only c-fos, c-jun, NF-kB and aFGF were able to increase recombinant protein expression. However, the amount of recombinant protein produced was lower compared to the yields obtained with the other two strategies.

By combining different strategies the recombinant protein production achieved the highest yield ever reported for a TGE process in CHO cells - 90 mg/L. This result was obtained in the presence of VPA in combination with hypothermia or by overexpressing c-fos in the presence of VPA. However, the first approach appeared to be a better approach to increase recombinant protein production in CHO cells because it is a simpler, more reproducible and less expensive method compared to the overexpression of a transcription factor.

7.2 Perspectives

The strategies investigated here improved TGE in CHO cells. However there are still some key areas that need to be addressed:

- It is essential to have a better understanding of the effects of VPA and/or hypothermia on the transfected cells. It appeared that they both act at the level of transcription or mRNA stability but the exact mechanism(s) is not known.
 - It has been reported that under hypothermic conditions, the cell's siRNA and miRNA machinery is modified (6). Therefore, the siRNAs and miRNAs that may affect transgene expression in CHO cells should be quantified. Small RNAs are not only directly responsible for transcript cleavage (7) but they are also involved in epigenetic regulation of the gene (8).
 - Another strategy to understand the molecular mechanism(s) behind hypothermia and VPA treatment are DNA microarrays. The comparison of the transcriptomes of untreated cells and cells cultured either under hypothermic conditions or with VPA may provide information about the genetic regulation in the treated samples. Unfortunately, cDNA microarrays specific for CHO cells are still under development. qRT-PCR targeting specific genes (i.e. those involved in cell cycle or in DNA silencing) could be used as alternative strategy.
 - Epigenetic silencing of the transfected pDNA should be analyzed. It was reported that the extent of the response to hypothermia or to VPA was vector dependent. If epigenetic regulatory mechanisms are involved in TGE, then the extent of plasmid DNA methylation and histone modification, especially acetylation, should be determined on the promoter/enhancer elements of different expression vectors used for transfection.
- The possibility to increase TGE in CHO cells to the g/L level as has been achieved in HEK 293 cells should also be investigated. Performing the transfections at higher cell densities combined with hypothermia and VPA in fed-batch processes is expected to further improve on the yields reported here.
- Engineering a host cell line overexpressing a transcription factor or a growth factor may not be the best strategy to increase TGE yields. An alternative strategy is the induction of endogenous transcription factors by addition of small molecules such

as doxorubicin to the culture medium (9;10). However, the benefit/cost issue should be taken in account when using this approach.

- It has been presented that the level and stability of transgene mRNA is one of the main bottlenecks for transient recombinant protein production in CHO cells. It would be interesting to investigate if this phenomenon also occurs in transiently transfected HEK 293 cells. If VPA has the same effect both in CHO and in HEK 293 cells, then a comparative study may yield clues as to how VPA transient recombinant protein production.

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Appendix 1

Set up of a gas inducible system for transient gene expression in CHO cells

A1.1 Introduction

The acetaldehyde inducible expression system developed by the Weber et al. (1-3) takes advantage of the natural ability of the fungus *Aspergillus Nidulans* of surviving in the presence of ethanol as its sole carbon source (Figure A1.1). Ethanol is oxidized into acetaldehyde by alcohol dehydrogenase (ADH), and the acetaldehyde is converted into acetate by aldehyde dehydrogenase (ALDH). Acetate is then converted into acetylCoA and can enter the Krebs' cycle. In the presence of acetaldehyde, the AlcR (Acetaldehyde receptor) transactivator binds specific operators to induce the expression of its own cistron as well as the transcription of the two enzymes (ADH and ALDH) necessary for survival (4). The ADH promoter (AlcP) harbours several elements (O_{AlcA}) with high affinity for AlcR (1;4). Based on these observations, two vectors were created to create a gas inducible system for regulated gene expression in mammalian cells (1). pWW195 (see Fig. 2.8) has the AlcR gene under the control of the SV40 early promoter. pWW192 (Fig. 2.8) allows the cloning of the gene of interest under the control of the P_{AIR} promoter. P_{AIR} is composed of eight *A. Nidulans* P_{AlcA} -derived modules and a minimal version of the human CMV promoter ($hCMV_{min}$). Thus, transfection of cells with both plasmids followed by induction with acetaldehyde results in induction of GOI expression. Without induction, GOI expression is minimal (Figure A1.2).

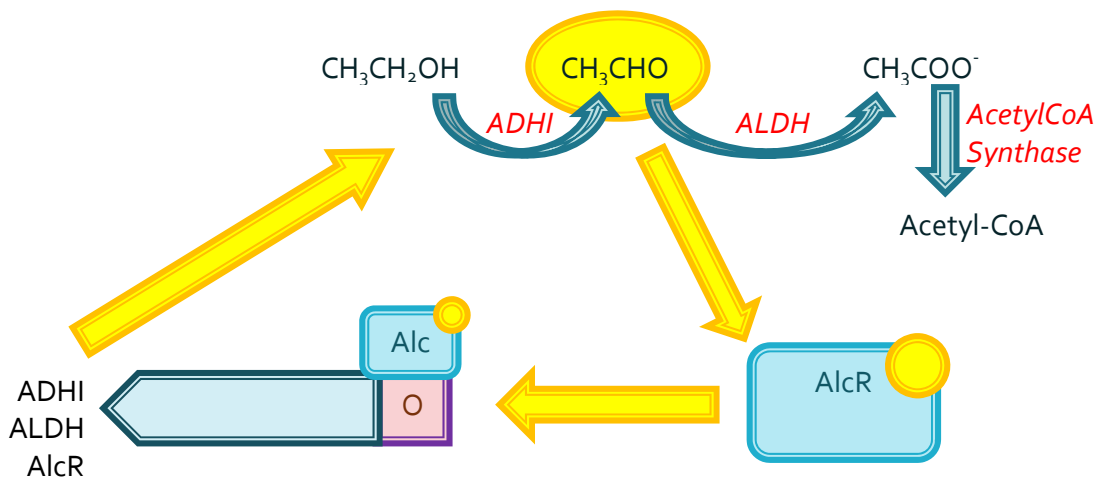


Figure A1.2. Schematic diagram of acetaldehyde inducible gene expression in *Aspergillus nidulans*.

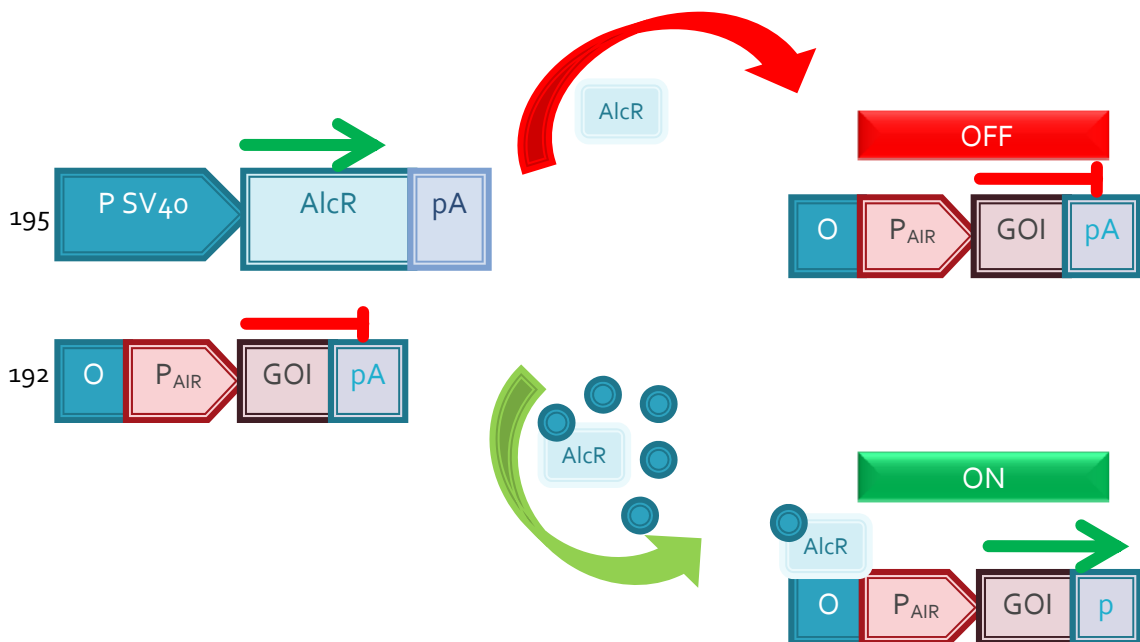


Figure A1.2. Schematic diagram of acetaldehyde inducible gene expression in a mammalian cell.

A1.2 Results

A1.2.1 Optimization of pWW195:pWW192 ratio

Initial tests to apply the acetaldehyde inducible system to TGE in CHO DG44 cells employed GFP as a reporter protein. CHO DG44 cells were transfected with pWW192eGFP and pWW195 at different ratios. The cultures were treated with 20 mg/mL of acetaldehyde and GFP expression was measured two days after induction. The highest level of GFP-specific fluorescence was recorded when cells were transfected with 60% of pWW195 and 40% of pWW192eGFP (Figure A1.3).

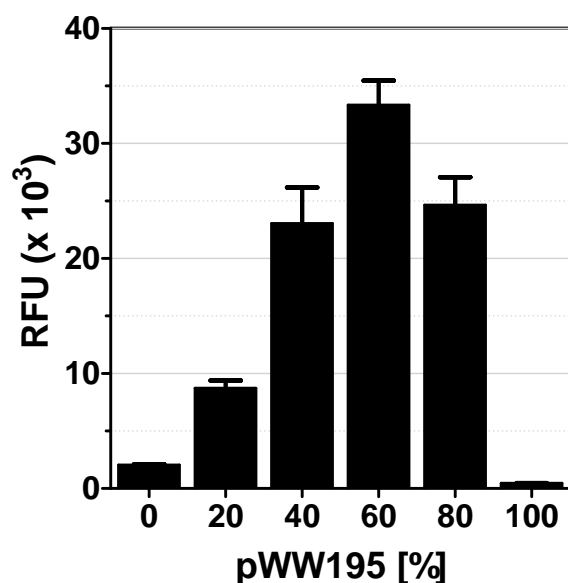


Figure A1.3. Optimization of pWW195-pWW192eGFP ratio for TGE in CHO DG44 cells. Cells were transfected with pWW195 and pWW192eGFP at different ratios as indicated. Cells were induced with acetaldehyde and GFP-specific fluorescence was measured at 2 days post-induction. The error bars represent the standard deviation from two parallel experiments.

A1.2.2 Optimization of Acetaldehyde concentration

To quantify the optimal acetaldehyde concentration for the induction, CHO DG44 cells were transfected with pWW195 and pWW192eGFP at a 60:40 ratio and induced with increasing amount of acetaldehyde. GFP-specific fluorescence was measured 2 days after induction. The acetaldehyde concentration from 20 to 100 mg/mL increased GFP expression of 6-7 fold over the uninduced control (Figure A1.4 - left and right panels). High levels of acetaldehyde (> 100 mg/mL) were toxic for the cells. In fact, GFP expression and cell viability decreased proportionally with the increase of the acetaldehyde concentration (Figure A1.4 - right panel). GFP-specific fluorescence and cell viability levels reached zero for acetaldehyde concentrations of 250 mg/mL and higher.

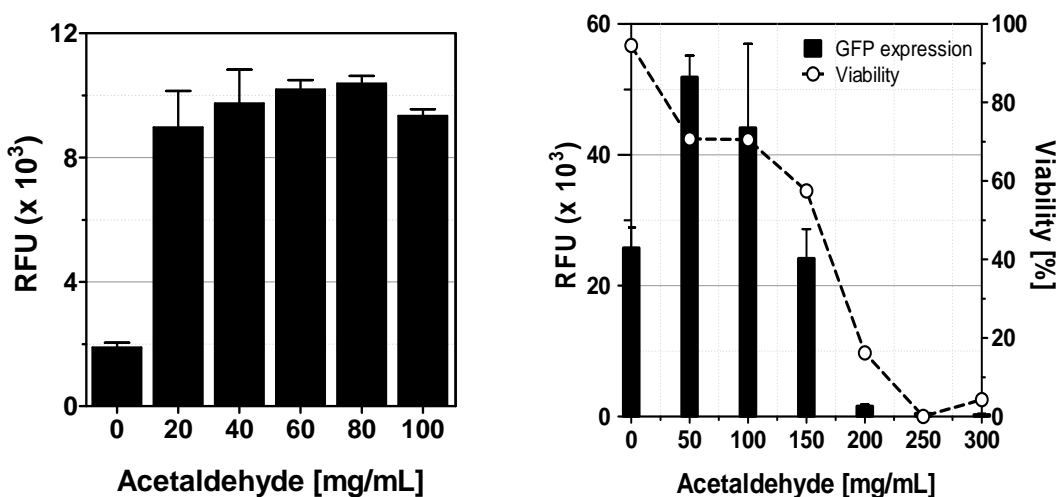


Figure A1.4. Effect of acetaldehyde on gene induction (left and right panels) and cell viability (right panel). CHO DG44 cells were transfected with pWW195:pWW192GFP at a ratio of 60:40 (w/w). Liquid acetaldehyde was added to each culture to reach the concentrations indicated. Two days after induction cells were harvested, and GFP expression and cell viability were quantified. Error bars represent the standard deviation from two different experiments. Differences between the two expression levels were due to two different batches of medium used for transfection.

A1.2.3 Optimization of the time of induction

In transient gene expression timing can have a big impact on protein expression. Therefore it was important to set up when the transfected cells can be induced. We excluded the addition of Acetaldehyde during transfection, in order to avoid any influence on transfection efficiency. CHO DG44 cells were transfected with pWW195 and pWW192eGFP as in the previous section. Acetaldehyde was added either at the time of dilution (4 h post-transfection) or at 24 h after transfection. Based on the level of GFP-specific fluorescence at 2 days post-induction, the better time for induction was 4 h post-transfection (Figure A1.5).

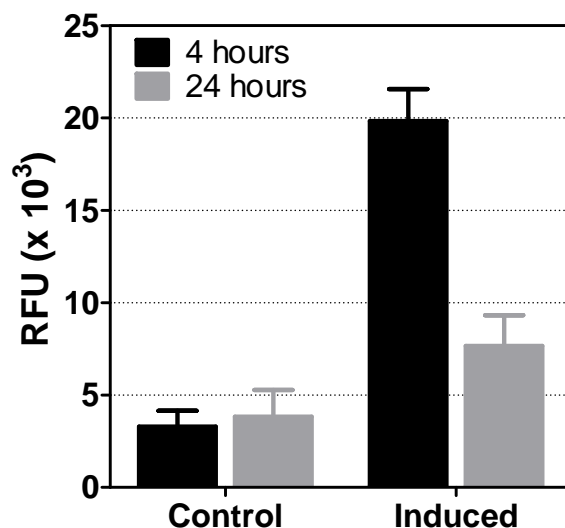


Figure A1.5. Effect of time of acetaldehyde addition on level of GFP expression. CHO DG44 cells were transfected with pWW195:pWW192GFP at a ratio 60:40 (w/w). Acetaldehyde was added to a final concentration of 20 mg/mL at either 4 h (black bars) or 24 h (gray bars) post-transfection. Two days after induction, the level of GFP-specific fluorescence was quantified. Error bars represent the standard deviation from two parallel experiments.

A1.2.4 Kinetics of GFP expression after induction

Finally, GFP expression was analyzed at various times after induction to determine both the time of its highest level of accumulation and the reversibility of the inducible system. CHO DG44 cells were transfected with pWW195 and pWW192eGFP at a 60:40 ratio (w/w) and induced with 20 mg/mL acetaldehyde. Each day after induction, GFP-specific fluorescence was measured. The highest level of GFP expression was reached at 48 h after induction and then decreased over time, demonstrating the reversibility of the inducible system (Figure A1.6).

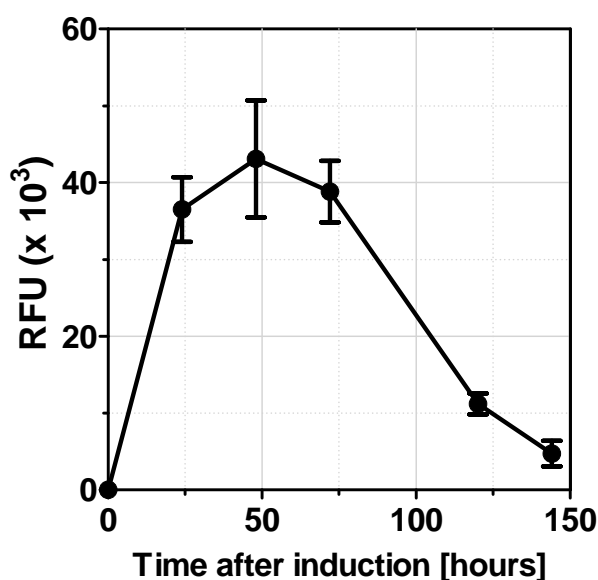


Figure A1.6. Kinetics of transient GFP expression after induction with acetaldehyde. CHO DG44 cells were transfected with pWW195:pWW192GFP at a ratio of 60:40 (w/w). At 4 h after transfection, 20 mg/mL of acetaldehyde were added. GFP-specific fluorescence was quantified at the times indicated. Error bars represent the standard deviation from two parallel experiments.

A1.2.5 Overexpression of c-fos from a gas inducible promoter

CHO cells were transfected with pWW192c-fos (Chapter 2, section 2.3.7) and either induced or not with 20 mg/mL of acetaldehyde. Cell lysates were analyzed by immunoblot to determine the level of c-fos expression with a polyclonal antibody against c-fos. Cells transfected with pXLG^{HEK}-c-fos were used as positive control. As expected, the sample from the acetaldehyde-induced cells had a higher level of c-fos expression than the non-induced one (Figure A1.7).

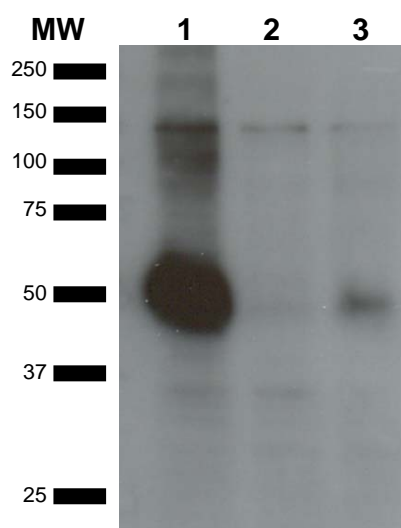


Figure A1.7. Inducible transient expression of c-fos. CHO cells were transfected with pXLG^{HEK}-c-fos (1) or pWW192c-fos (2,3). The cultures transfected with the latter were left untreated (2) or induced with 20 mg/mL acetaldehyde (3). Three days after transfection, 5 million cells were harvested, and total intracellular proteins were extracted and analyzed by immunoblot to detect c-fos using a polyclonal antibody anti human c-fos (Chapter 2, section 2.16).

CHO cells were transfected with pWW192c-fos, pXLG^{HEK}-c-fos, or pXLG^{HEK}. At 4 h post-transfection 20 mg/mL of acetaldehyde were added to each culture. At 2 days post-induction, the cells were transfected with pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP (mass ratio of 49:49:2), and 6 days after the second transfection, the recombinant IgG concentration was quantified by ELISA. Despite the very low IgG yields in this experiment, the cells transfected with pWW192c-fos expressed almost 2-fold more antibody than the control which lacked exogenous c-fos overexpression (Figure 6.6). The highest IgG yield, however, was observed in the culture transfected with pXLG^{HEK}-c-fos

(Figure A1.8). In this experiment double-transfections were used in order to transfect cells with the c-fos vector and then induce them prior to the second transfection with the IgG vectors. The double transfection may result in cell stress that prevented higher TGE yields. Therefore, it may be suitable to try establishing a stable cell line that can be induced to express c-fos.

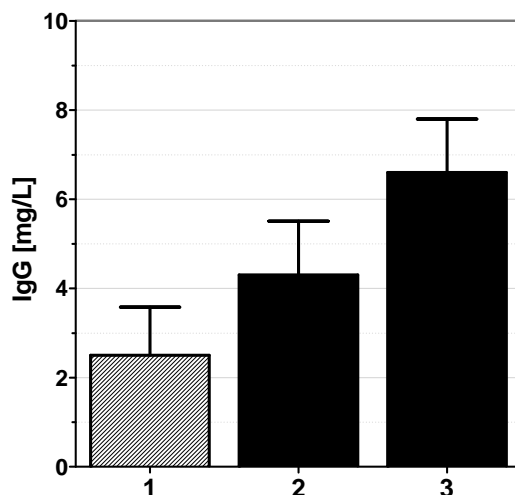


Figure A1.8. Effect of c-fos overexpression on TGE. CHO cells were transfected with pXLG^{HEK} (1), pWW192c-fos (2), or pXLG^{HEK}-c-fos (3). Four hours after transfection all samples were induced with 20 mg/mL of acetaldehyde. Two days after transfection, cells were transfected with pXLG^{HEK}-RhLC, pXLG^{HEK}-RhHC, and pXLG^{HEK}-EGFP (mass ratio of 49:49:2). Six days after the second transfection, IgG yields were quantified by ELISA. Error bars represent the standard deviation from two parallel experiments,

A1.3 References

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Appendix 2

Partial characterization of a recombinant antibody

A2.1 Introduction

The reporter protein used in all the experiments reported in this thesis was the anti-Rhesus D recombinant IgG antibody. Antibodies are globular proteins, with a molecular weight of ~160 KDa. They are composed by two heavy chains of ~55 KDa and two light chains of ~25 KDa. The light and heavy chains are bound together by disulphide bridges. The heavy chains present an N-glycosylation site on Asn297. A complete characterization of the recombinant product should include the use of several preparative and analytical methods summarized below:

- (I) purification by affinity, ion exchange and size exclusion chromatography
- (II) SDS-PAGE gel and isoelectrofocusing (IEF)
- (III) analytical chromatography (size exclusion/reverse phase/ion exchange) to determine the purity of the sample
- (IV) N-terminal sequencing (Edman degradation) to identify possible N-terminal truncations
- (V) Elman assay to check the thiols content
- (VI) Amino acid analysis as an additional check for purity
- (VII) peptide mapping on the intact protein and on impurities present in the reverse phase or ion exchange chromatograms
- (VIII) glycosylation profile analysis by HPLC/capillary electrophoresis/HPLC-MS

Since our laboratory is not equipped with all of these analytical tools, only a partial characterization of the recombinant antibody was performed and reported.

A2.2 Results

A2.2.1 UV spectrum

The IgG was purified by affinity chromatography and desalted as reported in Chapter 2, Section 2.13. To determine the concentration of the purified protein and to evaluate the purity, the sample was analyzed by UV spectroscopy from 220 to 350 nm (Figure A2.1) using a Nanodrop ND-1000 spectrophotometer (Witeg, Littau, Switzerland). The ratio between the absorbance at 260 nm and 280 nm was ~ 0.5 , meaning that the sample was not contaminated by DNA. The total amount of protein contained in the sample was determined to be 0.53 mg/mL using the Lambert Beer equation and considering the extinction coefficient (ϵ , $\text{L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$) equal to 146,400 (*I*).

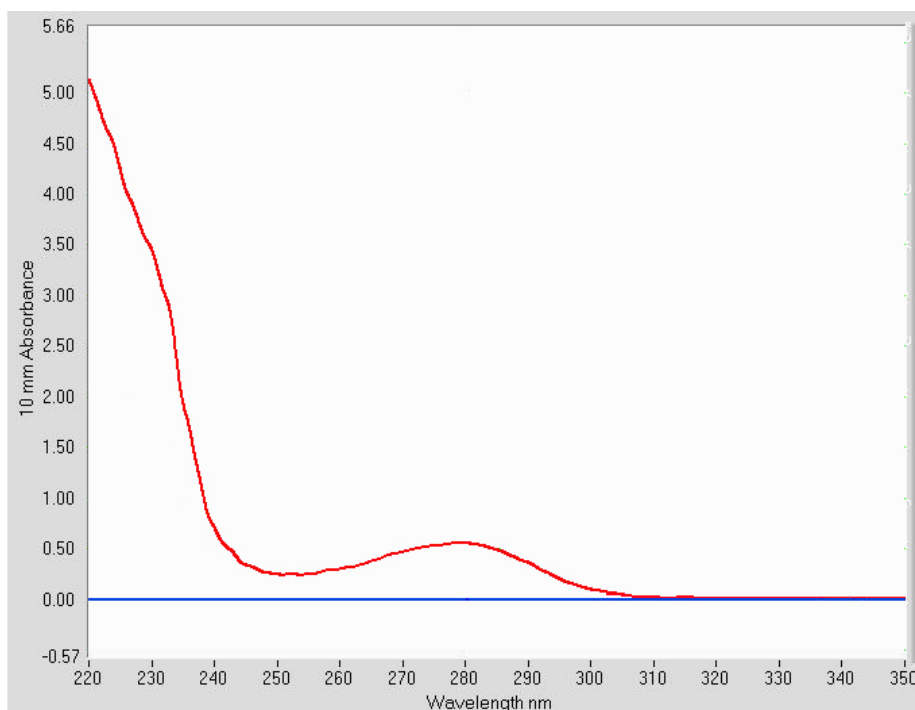


Figure A2.1. UV spectrum of the purified IgG. Recombinant human IgG was purified by affinity chromatography as described in Chapter 2, Section 2.13. After desalting, the sample was analyzed by UV spectroscopy in the range of 220 and 350 nm using a Nanodrop ND-1000 spectrophotometer.

A2.2.2 Size exclusion chromatography

To check for the presence of IgG aggregates or other impurities, the sample was analyzed by size exclusion chromatography using a Superose 12-HR column (GE Healthcare, Otelfingen, Switzerland) and an ÄKTA-FPLC system with a 280 nm UV detector (GE Healthcare). The sample was eluted isocratically at 1 mL/min using a mobile phase composed of 50 mM sodium phosphate and 50 mM NaCl at pH 7.20.

Retention times of proteins with known molecular weight were calculated using the Unicorn Software v.4.0 (GE Healthcare) and then used to create a standard curve. The retention time of the sample was calculated after integration of the chromatogram with the Unicorn Software v.4.0 (Figure A2.2) and fitted to the standard curve previously created in order to obtain the molecular weight of the protein. The molecular weight of ~164 KDa from the standard curve correlated with the predicted molecular weight.

From the chromatogram it was also observed that the peak was wide and tailed, suggesting that the sample contained different molecular weight species (Figure A2.2).

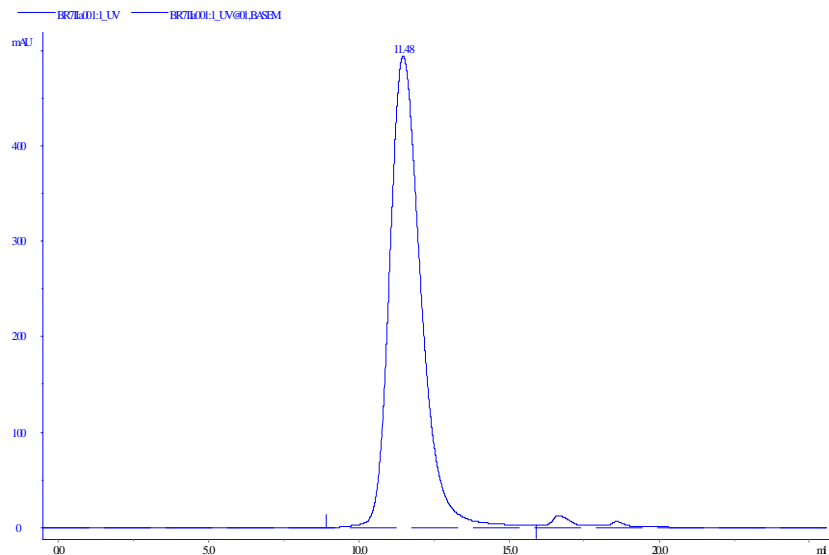


Figure A2.2. Size exclusion chromatography of the purified recombinant IgG. Recombinant human IgG was purified by affinity chromatography as described in Chapter 2, Section 2.13. After desalting, 250 μ L of the sample were injected in a Superose 12-HR column and eluted isocratically as described in the text. The retention time was calculated after integration of the chromatogram with the Unicorn Software and fitted in a standard curve in order to obtain the molecular weight of the protein.

A2.2.3 SDS-PAGE

As a second measure of sample purity and also for molecular weight analysis, the purified protein was analyzed by SDS-PAGE using 4-12% gradient gels (Invitrogen). The sample was treated at 95°C for 5 min in either non-reducing or reducing sample buffer (Chapter 2, Section 2.16) and loaded in the gel. After electrophoresis, the gel was stained with Coomassie Blue.

The sample electrophoresed under reducing conditions migrated as two bands at 55 KDa and 27 KDa corresponding to the molecular weights of IgG heavy and light chains, respectively (Figure A2.3A). The sample electrophoresed under non-reduced conditions showed a major species that migrated with an apparent molecular weight of ~160 KDa, correlating with the IgG molecular weight (Figure A2.3B). On the same gel, two or more minor species migrating at lower molecular weights were visible. It was not possible to clearly identify these minor species. However, western blot analyses using either an anti-Fc or an anti-light chain antibody identified these fragment as IgG (data not shown). Since under reducing conditions only two protein species were observed it is possible that the antibody encountered reducing degradation either in cells or in the cell culture medium after secretion.

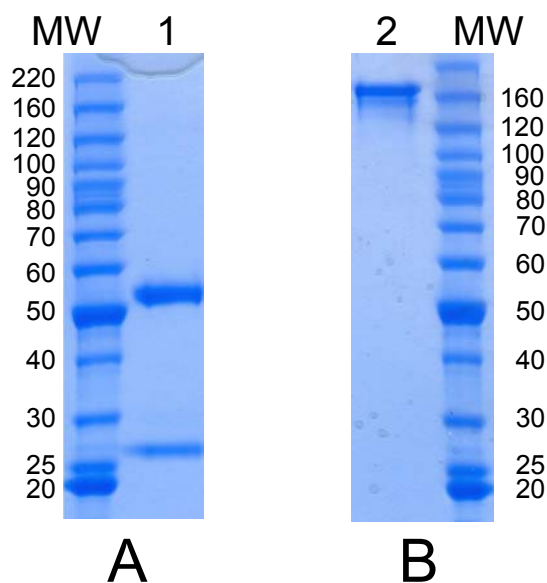


Figure A2.3. SDS-PAGE of the purified recombinant IgG. Recombinant human IgG was purified as described in Chapter 2, Section 2.13. After desalting, samples were treated at 95°C for 5' with reducing (A) or non-reducing (B) sample buffer and electrophoresed in a 4-12% gradient gel. The gel was stained with Coomassie Blue.

A2.2.4 Deglycosylation with PNGase F

The extent of the heavy chain glycosylation was estimated by SDS-PAGE with 4-12% gradient gels (Invitrogen). Recombinant antibody transiently produced at 31 °C or 37 °C was partially purified by affinity chromatography and then treated for 12 hours at 37 °C with peptide-N-glycosidase F (PNGase F). PNGase F is a deamidase and it is one of the most widely used enzymes for the deglycosylation of glycoproteins. The enzyme releases asparagine-linked (N-linked) oligosaccharides from glycoproteins and glycopeptides (2).

Samples were treated at 95°C for 5 min in reducing sample buffer (Chapter 2, Section 2.16) and electrophoresed. The gel was stained with Coomassie Blue. The IgG heavy chain had about 3 kDa of glycosylation judging from the band shift after PNGase F treatment (Figure A2.4). The difference in band intensity is due to the higher amount of purified antibody recovered from samples cultured at 31°C (Figure A2.4, lanes 3 and 4).

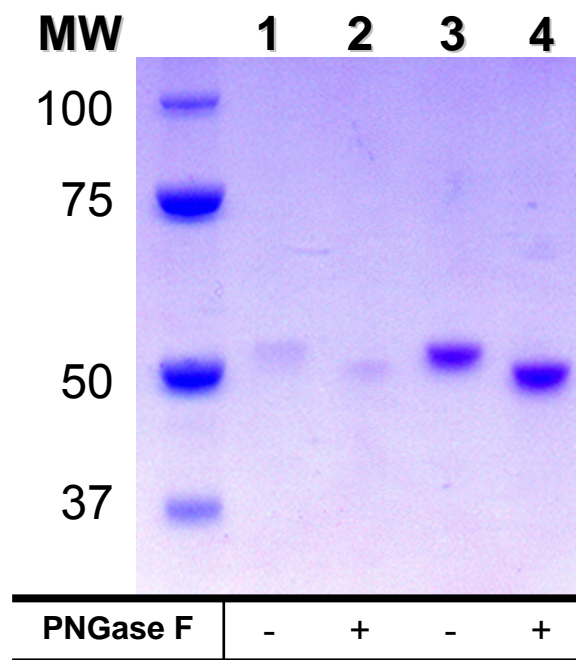


Figure A2.4. SDS-PAGE analysis of antibody glycosylation. Affinity purified antibody produced by TGE at 37°C (lanes 1-2) and 31°C (lanes 3-4) were deglycosylated with PNGase F. Protein samples before (lanes 1 and 3) and after (lanes 2 and 4) treatment with PNGase F were electrophoresed on a 4-12% polyacrylamide gradient gel.

A2.3 Conclusions

Even if the recombinant product was already identified as anti-Rhesus D antibodies from the ELISA analyses performed for quantification in the supernatant, from the analyses reported after purification it was possible to conclude that:

- (I) in the purified sample are present species of low molecular weight (less than 160 kDa) that were also determined to be the recombinant antibody;
- (II) the recombinant antibody had 3 kDa of N-linked glycosylation on the heavy chain.

A2.4 References

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Education

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1998-2003 MSc Degree in Industrial Biotechnology - University of Turin (Italy)
Diploma work title: "*Development of analytical techniques in capillary electrophoresis with laser induced fluorescence for the analysis of proteins. Focusing on transgenic proteins applications*". Department of Bioanalytical Chemistry of the University of Turin (Italy). Grade: 110/110 *cum Laude*

1993-1998 Classical Studies at the "C. Cavour" High School in Turin. Grade: 60/60

Job Experiences

July 2005 - Present Teaching assistant:
- design and supervision of laboratory courses and lectures
- supervision of students, trainees and technicians

2003-2004 One year student in Serono SA (Drug Delivery System, Colletterto Giacosa – Turin, Italy). Main topic: "*Development of new analytical methods for the analysis of protein aggregation state by Capillary Electrophoresis*".

Skills

Languages:

- Italian: mother tongue
- English (spoken and written): good knowledge (First Certificate – 2002)
- French (spoken and written): fluent (Alliance Française – 1994)

Use of personal computer:

Very good use of Microsoft Office Suite and Windows 9x, 2000, XP, Vista.

Technical skills:

- Good expertise in mammalian cell culture: adherent and in suspension cultures, shaking technology, culture in disposable devices or in stirred bioreactor (laboratory scale)
- Good expertise in protein production in mammalian cells: transfection, transient gene expression and establishment of stable cell lines
- Good knowledge of molecular biology techniques: gene cloning and amplification, RT-PCR, qRT-PCR
- Good know-how in protein analyses techniques: SDS-PAGE, IEF, Immunoblot, ELISA, Capillary Electrophoresis, Chromatography (SE, Affinity) - Basic expertise in LC-MS

Professional training

- Aug 2006** “*An advanced course in Cellular Bioprocess Techonology*” – University of Minneapolis – Minnesota (USA)
- Jun 2004** “*10th Thermoweeek: Course of Chromatography – Mass Spectrometry*” – Acitrezza (CT) – Italy
- Jun 2004** Course of “*Design of Experiments*” – Sero DS – Collettero Giacosa (Italy)

Additional activities

- 2004** Volunteer at the Dog Pound of Turin
- 2002-2003** Projectionist at the Greenwich Village Cinema in Turin
- 1994-2002** Active member of a hiking group; during the last years responsible for organization of trekking activities and summer camps

Interests

Mountain sports, running, photography, animals.

Scientific contributions

Publications

- 2008 **Mild Hypothermia Improves Transient Gene Expression Yields Several Fold in Chinese Hamster Ovary Cells.**, Wulhfard S., Tissot S, Bouchet S, Cevey J, Jesus MD, Hacker DL, Wurm FM., *Biotechnol Prog.* 2008 Jan 26
- 2008 **Rational vector design and multi-pathway modulation of HEK 293E cells yield recombinant antibody titers exceeding 1 g/l by transient transfection under serum-free conditions.**, Backliwal G, Hildinger M, Chenuet S, Wulhfard S., De Jesus M, Wurm FM., *Nucleic Acids Res.* 2008 Sep;36(15):e96
- 2007 **Scalable transient gene expression in Chinese hamster ovary cells in instrumented and non-instrumented cultivation systems**, Muller N, Derouazi M, Van Tilborgh F, Wulhfard S., Hacker DL, Jordan M, Wurm FM., *Biotechnol Lett.* 2007 May; 29(5):703-11
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Oral presentations

- 2008 Wulhfard S., Burki C, Engelhardt EE, De Sanctis D, Perrone M, Stettler M, Tissot S, Zhang X, Muller N, Anderlei T, Hacker D, Vario G, Parolini N, Adam M, Baldi L, De Jesus M, Wurm FM - **Proof of principle for recombinant protein production: a disposable 1000 liter orbital shaken bioreactor system for mammalian cell culture – Cell Culture Engineering XI – Queensland (Australia)**
- 2004 Wulhfard S., Giovannoli C, Giraudi G – **Development of analytical techniques in capillary electrophoresis for the analysis of transgenic proteins - 4° Sigma Aldrich Young Chemists Symposium – Riccione (Italy) - 2004**
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Posters

- 2008 Wulhfard S., Degen C, Hildinger M, Hacker D, Wurm FM - **Overexpression of transcription factors increases transient recombinant protein production in mammalian cells - Cell Culture Engineering XI – Queensland (Australia)**
- 2008 Wulhfard S., Tissot S, Bouchet S, Hacker D, Wurm FM - **Mild hypothermia improves transient gene expression yields several fold in Chinese Hamster Ovary cells – Union of the Swiss Societies for Experimental Biology (USGEB) – Lausanne (Switzerland)**
- 2007 Wulhfard S., Tissot S, Bouchet S, Hacker D, Wurm FM - **Transient gene expression in Chinese hamster ovary cells at low temperature: the effects of cold-induced proteins and an mRNA regulatory element – ESACT 2007 – Dresden (Germany)**
- 2006 Wulhfard S., Hildinger M, Hacker D, Wurm FM - **Large scale transient gene expression in agitated bottles: over 100 mg/l of rhIgG in 10 liters - Forth European BioTechnology Workshop – Ittingen (Switzerland)**
- 2004 Wulhfard S., Giovannoli C, Giraudi G – **Development of analytical techniques in capillary electrophoresis for the analysis of transgenic proteins - 4° Sigma Aldrich Young Chemists Symposium – Riccione (Italy) - 2004**
-

Patents

- 2006 Hildinger,M., Wulhfard,S., Backliwal,G., Hacker,D., De Jesus M., and Wurm,F.M. **Producing recombinant protein in a mammalian cell at high specific productivity, high batch yield, and high volumetric yield, by introducing nucleic acid molecules encoding recombinant protein of interest by transient transfection.** EXCELLEGENE,SA ed. [US2008145893-A1]
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