Transient Gene Expression in Mammalian Cells for the Production of Recombinant Protein Therapeutics

Laurens Moore van Tienen
MSc Chemistry, track Biomolecular Sciences
Student ID: 5659191

University of Amsterdam, SILS
Primary supervisor: Dr. J. A. Verhees
Co-supervisor: Prof. M. J. Teixeira de Mattos
Credits: 12 ECTS

June 2012, Amsterdam, the Netherlands
Abstract

Protein-based therapeutics are becoming increasingly important in the pharmaceutical market. Mammalian cells are currently the standard host for the production of therapeutic proteins, as they carry out the proper post-translational modifications and folding. Industrial laboratories typically develop stable cell lines that have the gene coding for the recombinant protein (r-protein) integrated into their genome. At present, a major bottleneck in the development process is the extensive time required to isolate high-producing stable cell lines. A solution to this bottleneck would be to enable the rapid assessment of candidate r-proteins in pre-clinical studies prior to the development of stable cell lines. Transient gene expression (TGE) is well suited for this purpose, since it has the potential to generate therapeutic r-proteins within a few weeks time instead of the more than 6 months when using stable cell lines. However, TGE currently has shortcomings, in part concerning the moderate r-protein yields that it can generate. The volumetric yield of a TGE system is dependent on the following four components: (1) the expression vector, (2) the transfection method, (3) the growth medium and bioreactor conditions, and (4) the nature and state of the host cell line and r-protein. This article aims to provide an overview of the advancements that have been made in each of these components in the past decade, and to address the current limitations that prevent TGE from being commonly applied in clinical settings.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TITLE PAGE</td>
<td>1</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>2</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>3</td>
</tr>
<tr>
<td>GENERAL INTRODUCTION</td>
<td>4</td>
</tr>
<tr>
<td>CHAPTER 1: TRANSIENT GENE EXPRESSION SYSTEMS</td>
<td>7</td>
</tr>
<tr>
<td>Introduction</td>
<td>8</td>
</tr>
<tr>
<td>Expression Vectors</td>
<td>11</td>
</tr>
<tr>
<td>Transfection Methods</td>
<td>15</td>
</tr>
<tr>
<td>Growth Medium Composition and Bioreactor Conditions</td>
<td>20</td>
</tr>
<tr>
<td>Cell Lines</td>
<td>24</td>
</tr>
<tr>
<td>CHAPTER 2: STRATEGIES TO OVERCOME THE CELLULAR CONSTRAINTS IN TRANSIENT GENE EXPRESSION</td>
<td>27</td>
</tr>
<tr>
<td>Introduction</td>
<td>28</td>
</tr>
<tr>
<td>mRNA Stability</td>
<td>31</td>
</tr>
<tr>
<td>Translation</td>
<td>34</td>
</tr>
<tr>
<td>Post-translational Events</td>
<td>36</td>
</tr>
<tr>
<td>CONCLUSIONS AND FUTURE PERSPECTIVES</td>
<td>39</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>42</td>
</tr>
</tbody>
</table>
General Introduction

Protein-based therapeutics, such as monoclonal antibodies, have been in high demand ever since they have been introduced to the pharmaceutical market. Therapeutic proteins often require extensive post-translational modifications to be fully bioactive. Unlike bacteria and yeast, mammalian cells have the proper complement of enzymes and organelles to perform these modifications, and to permit correct physiological protein folding. As such, cultivated mammalian cells have become the primary host for the manufacturing of complex therapeutic proteins.

Industrial laboratories generally make use of stable transfected cell lines that have the gene coding for the recombinant protein (r-protein) integrated into their genome\(^1\). Consequently, stable expression of the transgene results in the constitutive production of the r-protein during prolonged cultivation times. To generate stable cell lines, the gene coding for the r-protein and a selection marker such as dihydrofolate reductase (\(dhfr\)) are co-transfected. A selection process is instigated in which the cells that initially survive have successfully integrated the transfected DNA into a random genomic location and subsequently expressed it. To achieve maximum expression of the r-protein, the selective pressure is gradually increased during subsequent rounds of selection (for example in the presence of increasingly higher concentrations of the DHFR inhibitor methotrexate) to isolate high-producing clones that have amplified the selection marker together with the gene coding for the r-protein. Approximately six months of selection are usually required to obtain clones that provide sufficient yield for their use in the large-scale production of the r-protein. This time-consuming and costly phase, wherein high-producing
clones are isolated, currently represents a major bottleneck in the development of r-proteins. Using this approach, the screening and further development of r-proteins can be initiated only after large amounts of time and money have been invested. As a result, many desire an alternative system that can generate large amounts of r-proteins quickly, which will subsequently enable r-proteins to enter the pre-clinical screening phase faster and at lower costs.

Transient gene expression (TGE) systems could meet this need, as they have the potential to generate enough r-protein suitable for initial characterization and clinical trials within three weeks at relatively low-cost\(^2,3\). In contrast to stable gene expression (SGE), TGE does not require the use of mutant cell lines (\(dhfr^{-}\)) and subsequent rounds of sub-cloning to isolate a high-producing clone with the requisite production and growth characteristics. Instead, cells are transiently transfected with plasmid DNA containing the gene coding for the r-protein, after which the transfected cells are cultured for a period of one or two weeks. It is during this period that the transfected cells can produce the r-protein.

However, TGE currently has several limitations that prevent it from being commonly applied on a large scale (> 1 l cell cultures). Firstly, TGE requires large amounts of purified plasmid DNA, which is costly. Secondly, standard transfection procedures for TGE systems are difficult to scale up. Lastly, the volumetric yields that can be obtained from TGE are still relatively low. The latter constraint has been the main focus of TGE research in the past decade, and considerable progress in volumetric yield has been made. The differences between SGE and TGE are outlined in Table 1. This review will discuss the recent advancements that have been made in TGE, as well as address specific aspects of TGE that could be improved with particular emphasis on increasing r-protein volumetric yield. In the first chapter we will discuss the general aspects involving TGE. The second chapter will specifically address constraints in cellular
mechanisms of mammalian cells that may limit the production of therapeutic proteins by means of TGE.

Table 1. Differences between SGE and TGE in the production of r-proteins.

<table>
<thead>
<tr>
<th></th>
<th>SGE</th>
<th>TGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genetic selection</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>Time from initial transfection to product</td>
<td>≥ 6 months</td>
<td>1-2 weeks</td>
</tr>
<tr>
<td>Amount of DNA required for transfection</td>
<td>1-3 μg/ml</td>
<td>1-50 μg/ml</td>
</tr>
<tr>
<td>Specific productivity</td>
<td>10-90 pg/cell/day</td>
<td>0.5-15 pg/cell/day</td>
</tr>
<tr>
<td>Volumetric yield</td>
<td>1-5 g/l</td>
<td>0.02-1 g/l</td>
</tr>
</tbody>
</table>
Chapter 1

Transient Gene Expression Systems

L. M. van Tienen and J. A. Verhees
Introduction

The transfection procedure for TGE is essentially comparable to the procedure for SGE, and many transient transfection protocols have been published to date\(^4-7\). Linearized plasmid DNA is preferred in stable transfections, as it enhances the probability of genomic integration. In contrast, transient transfections generally make use of circular plasmid DNA, since in TGE the DNA is not meant to be integrated into the genome, but rather functions as an autonomous carrier of genetic information. Nevertheless, it has been shown that supercoiled plasmid DNA may also integrate into the genome due to the presence of endogenous nucleases and DNA recombination enzymes, albeit at low frequency\(^1\).

Although plasmid DNA can be encountered in cells within several hours post-transfection, the cell specific productivity in most cell lines seems to peak between 1 and 3 days post-transfection\(^8,9\). The production phase can last up to 14 days following transfection, depending on the specific composition of the TGE system\(^10\). In clinical production settings, the extract is finally subjected to downstream purification procedures to isolate the r-protein.

Disregarding the downstream purification process, TGE systems generally consist of four components that could affect the costs and the volumetric yield: (1) the expression vector, (2) the transfection method, (3) the growth medium and bioreactor conditions, and (4) the nature and state of the host cell line and the complexity of the r-protein. The volumetric yield of a TGE system can be improved in three ways: by increasing the number of cells that successfully express the transgene, by increasing the average viable cell density and the duration of the production phase, and by increasing the cell specific productivity.
The number of cells that successfully express the transgene is mainly dependent on the transfection efficiency, which can be defined as the ratio of the number of cells that contain plasmid DNA after transfection to the total number of cells, and is generally expressed as a percentage. The number of cells that internalized the plasmid DNA can be determined by counting the number of fluorescent cells one day post-transfection by using plasmid DNA that is labeled with a fluorescent dye such as YOYO-1 (Invitrogen, Carlsbad CA, USA)\(^8\). It is important to note that the number of successfully transfected cells is generally higher than the number of cells that successfully express the transgene, since successful uptake of plasmid DNA does not always lead to expression of the transgene.

The integral of viable cell density (IVCD), also called the integral cumulative cell time, is the most frequently used method to compare the average viable cell density among different TGE systems. The IVCD is represented by the area under the curve when the concentration of viable cells is plotted against the culture time. Thus, when two cell cultures have the same average cell specific productivities, the one with the greater IVCD will generate a greater volumetric yield.

The cell specific productivity, generally expressed in pg/cell/day, is usually calculated during exponential cellular growth phase based on the increase of r-protein in the culture medium divided by the increase in the number of viable cells\(^1\). Different methods have been employed to determine antibody titers directly, including enzyme-linked immune sorbent assays (ELISA) and high-performance liquid chromatography (HPLC). In addition, secreted alkaline phosphatase (SEAP) and green fluorescent protein (GFP) are two widely used reporter genes to study gene expression. However, no single method to measure the volumetric yield has been
universally adopted to date. Unfortunately, these experimental heterogeneities often make it difficult to compare volumetric or specific productivities among different publications.
Expression Vectors

Both viral and non-viral expression vectors have been used in TGE experiments. Viruses that are used to deliver DNA into mammalian cells include adenoviruses, adeno-associated viruses (AAV), lentiviruses, and retroviruses. An advantage of viral DNA delivery compared to existing transfection methods is that, depending on the virus and the host cell line, transduction efficiencies of up to 100% can be easily reached. However, several downsides to the use of viruses have favored the use of non-viral means of DNA delivery in TGE systems. Firstly, the infectious nature of viruses requires that their handling is restricted to special designated laboratories. Secondly, the isolation of recombinant viruses is labor intensive and costly. Thirdly, viral DNA delivery is currently incompatible with large-scale transient transfections. Finally, many viruses have a relatively low DNA carrying capacity; for instance, the maximal carrying capacity of AAV vectors is merely 5.2 kb\(^{12}\).

Plasmids used for TGE generally contain the following elements: a prokaryotic cassette (required for the amplification of the plasmid DNA in bacteria) containing an origin of replication in addition to a bacterial selection marker, and a eukaryotic cassette containing a strong constitutive or inducible promoter upstream of the gene coding for the r-protein, as well as a transcription terminator in combination with a polyadenylation signal sequence downstream of the gene coding for the r-protein. The nature of the promoter has a large effect on the transgene expression level. Promoter sequences that are often used for TGE include the human and mouse cytomegalovirus (hCMV/mCMV) early promoters, the simian virus 40 (SV40) early promoter, and the non-viral elongation factor 1α (EF1α) promoter. The hCMV promoter results in a 3-fold
higher volumetric yield than the EF1α promoter, and a 50% higher volumetric yield than the mCMV promoter under identical TGE conditions in human embryonic kidney (HEK) 293E cells\textsuperscript{13}. Thus, the use of different promoters is another factor that makes it difficult to compare volumetric yields among TGE systems.

In many plasmids, a multi cloning site is located downstream of the promoter to facilitate the cloning of the gene coding for the r-protein behind the promoter sequence. The ideal procedure for the isolation and cloning of the transgene depends on its nature. Repertoire cloning technologies such as phage display are commonly employed in the isolation genes coding for Fab fragments of monoclonal antibodies\textsuperscript{14}. It is often most straightforward to clone the mRNA-derived cDNA of a gene in the plasmid. However, this cDNA lacks introns which have the ability to enhance the nuclear export rate and the stability of the mRNA\textsuperscript{15}. For example, the presence of an intron between the promoter and the 5'-end of the gene has been shown to increase transgene expression levels\textsuperscript{16}. A polyadenylation signal sequence is present downstream of the transgene. Polyadenylation signal sequences that are often used include that from the SV40 (SV40pA), and that from the bovine growth hormone gene (BGHpA). Finally, strong terminator sequences downstream of the polyadenylation signal sequence ensure that the transcript is properly terminated, although the exact mechanisms of the termination process are not yet fully understood\textsuperscript{17}.

In SGE systems the transgene is inherited by the daughter cells upon mitosis. Consequently, the transgene copy number remains rather constant in cells of succeeding generations. In contrast, in TGE systems the transgene copy number per cell is reduced by 50 percent during every cell division, since each daughter cell receives on average half of the total amount of plasmid DNA. It is therefore inevitable that the expression level of the transgene per
cell, and thereby cell specific productivity, will reduce with every generation. To solve this issue, several mammalian cell lines including HEK293 cells and Chinese hamster ovary (CHO) cells have been engineered to express the SV40 large T-antigen or the Epstein-Barr virus (EBV) nuclear antigen-1 (EBNA1). The large T-antigen and EBNA1 protein support the episomal amplification of plasmids containing the SV40 and EBV origin of replication sequences, respectively. Episomal amplification of the plasmids is independent of mitosis of the host cell, resulting in the continuous amplification of the transgene, and potentially higher transgene expression levels.

In addition to mitosis, nucleases also contribute to the reduction of intracellular plasmid DNA concentrations. Besides the degradation of intracellular plasmid DNA, the transgene transcription level may even further decrease due to DNA methylation or the association of the plasmid DNA with nuclear deacetylated histone proteins. To reduce epigenetic transcriptional suppression, DNA methyltransferase (DNMT) and histone deacetylase (HDAC) inhibitors can be added to the cell culture. Examples of DNMT inhibitors are azacytidine, procainamide and hydralazine, and examples of HDAC inhibitors are byturate, Trichostatin A, and valproic acid. Valproic acid has been shown to be particularly useful for TGE, since it is relatively cheap and can increase IgG antibody titers 2 to 5-fold depending on the cell-line.

Since large quantities of plasmid DNA are required for transient transfections, it is important for TGE to apply an inexpensive and efficient method to purify the plasmid DNA. The fact that TGE does not require a eukaryotic selection marker to be present provides that all the required genetic information for TGE can generally be stored on a single plasmid, thereby saving costs in purification and transfection. Typical transient transfection protocols use 1-50 μg of plasmid DNA per ml culture, depending on the cell density at the time of transfection.
Therefore, for large-scale TGE, in the order of grams of plasmid DNA are often required. Commercially available plasmid purification kits, such as the Qiagen Plasmid Giga Kit (Qiagen, Venlo, the Netherlands), allow for the purification of such amounts. Recent efforts have been made to make plasmid purification for TGE more cost-effective by making the isolation of large quantities of high-grade plasmid material easier. In most purification procedures, plasmids are grown in competent bacterial cells such as *Escherichia coli* strain DH5α and subsequently extracted by alkaline lysis, followed by ion-exchange or size-exclusion chromatography. As is true for any purification, there is often a trade-off between the plasmid yield and the purity of the extract. High amounts of contaminants, such as bacterial endotoxins, can interfere with the transfection procedure, with the cell viability, or with the downstream purification of the r-protein. However, it has been shown that endotoxin concentrations of up to 10,000 U/mg do not interfere with PEI-mediated transfection of HEK293E cells. This may render costly endotoxin-free purification procedures for TGE expendable.
Transfection Methods

There are principally three existing methods to deliver DNA into cells: using viral vehicles, using physical gene transfer methods, and using chemical agents. For the transgene to be transcribed, plasmid DNA first has to be delivered into the cytoplasm and from there into the nuclei of cells. Viruses have naturally evolved to be extremely efficient DNA delivery vehicles, but as previously mentioned, pose several disadvantages. The main advantage of physical gene transfer methods, such as microinjection and nucleofection, is that they can deliver naked DNA directly to the nucleus. However, these methods have not yet been successfully applied in large-scale TGE. Chemical agents, on the other hand, have been successful in large-scale TGE, but are required to move the DNA past physical barriers such as the cell membrane and the nuclear envelope before it is delivered into the nucleus. It is possible to distinguish three different types of chemical agents: inorganic compounds, cationic lipids, and cationic polymers. Many extensive reviews have been published on these chemical agents, and they will only briefly be discussed here.

Calcium phosphate (CaPi) is the most renowned and widely used inorganic compound for the transfection of mammalian cells. The CaPi method involves mixing the DNA with a calcium chloride solution, followed by the addition of a phosphate solution to precipitate CaPi-DNA complexes. These complexes are then added to the cell culture, after which they are probably taken up in endosomes through phagocytosis, and released in the cytoplasm. It has been suggested that the DNA subsequently enters the nucleus during mitosis when the nuclear envelope is temporarily disassembled. CaPi is inexpensive and can be readily applied in
transient transfections, as it does not interfere with the downstream purification of r-proteins. In addition, it has been successfully applied in large-scale transient transfections of cell cultures up to a 100 L\textsuperscript{33}. There are, however, several drawbacks to the use of CaPi, of which perhaps the most notable is the requirement for serum in the growth medium. The presence of serum in the medium limits the aggregation of the CaPi-DNA precipitate into cytotoxic complexes, thereby enhancing the transfection efficiency compared to cells that are transfected under serum-free conditions\textsuperscript{34}. In commercial settings the use of serum-free media is currently strongly preferred.

Cationic lipids are a group of amphipathic molecules that are positively charged at physiological pH, and interact with the negatively charged DNA through electrostatic interactions. The lipid-DNA complexes, also called lipoplexes, are internalized through endocytosis and subsequently release the DNA in the cytoplasm\textsuperscript{35}. Commercially available cationic lipids such as Lipofectamine (Invitrogen) and Fugene (Roche, Basel, Switzerland) can result in very high transfection efficiencies of near 100%, depending in the cell density and conditions of the medium at the time of transfection\textsuperscript{36}. However, their relatively high costs and relative low transfection efficiency in suspension cells currently make them impractical candidates for large-scale transient transfections.

Examples of cationic polymers that are used for the transfection of mammalian cells are: DEAE-dextran, polyllysine and polyethyleneimine (PEI). PEI is the most widely used polymer for TGE, and much is known about it\textsuperscript{37}. Branched and linear PEIs with a wide range of molecular weights are synthesized from azridine or 2-ethyl-2-oxazoline monomers, respectively\textsuperscript{38,39}. PEI forms complexes with DNA, called polyplexes, which have a net positive charge. The polyplexes are thought to interact with negatively charged proteoglycans on the cell surface, thereby promoting endocytosis\textsuperscript{40}. Several mechanisms have been suggested as to how
the polyplexes subsequently escape the endosome before lysosomal degradation. One such mechanism, called the proton-sponge mechanism, suggests that PEI can delay the acidification of the endosome due to the buffering capacity of its amine groups. The influx of ions into the endosomes is accompanied with an increase in the endosomal osmolarity. As a result, the endosomes might swell and burst before they become part of a lysosome, releasing the polyplexes into the cytoplasm. It is not fully understood how the DNA is subsequently delivered into the nucleus. The fact that PEI is found in the nucleus after it has been microinjected into the cytosol, and that microinjection of polyplexes instead of naked DNA results in the increased expression of the transgene, implies that PEI promotes nuclear translocation of DNA. The transfection efficiency of PEI has been shown to be cell-type dependent, and is caused by different rates at which polyplexes escape from endosomes and enter the cytoplasm. In addition, it has been suggested that the transfection mechanisms of branched and linear PEI are different, since branched PEI forms large aggregates associated with the cell membrane or nucleus whereas linear PEI remain distinct particles in the cytoplasm. To date, the most successful transient transfections have been performed with linear 25 kDa PEI.

Both CaPi and PEI are commonly applied in large-scale transient transfections, as they are inexpensive and efficient for the transfection of suspension adapted mammalian cells. However, there are several practical advantages to the use of PEI. Firstly, PEI is compatible with serum-free media, whereas CaPi is not. Secondly, CaPi requires a complete change of growth medium prior to tranfection. Finally, many factors in the cell culture can influence the stability of CaPi-DNA complexes, since the formation of these complexes is a reversible process. This makes it difficult to obtain reproducible transfections with CaPi.
Since no selective phase is applied after transfection, it can be postulated that the cells in TGE systems die and proliferate regardless of the amount of plasmid copies. Therefore, in order to maximize the volumetric yield, it is not only important to maximize the transfection efficiency, but also the number of plasmid copies per cell. Typical transient transfection protocols use 1 - 2.5 μg of plasmid DNA per ml culture for standard cell densities of 2·10^6 cells/ml, and 50 μg of plasmid DNA per ml culture for high cell densities of up to 20·10^6 cells/ml. Assuming an average molecular weight of 660 per base pair, and a plasmid size between 3 kb and 10 kb, these plasmid DNA quantities correspond to 10^5 - 10^6 plasmid copies per cell for both normal and high cell density transfections. Not all of this plasmid DNA reaches the interior of the cells; following PEI-mediated transfection of HEK293E cells, the cellular plasmid content seems to peak around 26 hours post-transfection, during which the cells have been shown to contain a maximum of 5.0·10^4 - 6.5·10^4 plasmid copies per cell. Therefore, it can be concluded that much of the transfected plasmid does not get internalized into cells, possibly due to degradation, binding to the bioreactor, or instable complex formation with the tranfection agent.

The aforementioned plasmid DNA quantities and cell densities result in transfection efficiencies of nearly 100% for PEI-mediated transfections, and slightly lower efficiencies for CaPi-mediated transfections under similar conditions in CHO DG44 cells. However, merely 1% of the transfected DNA reaches the nucleus with PEI-mediated transient transfections, making the nuclear plasmid copy number more than one order of magnitude lower than the cytoplasmic plasmid copy number. This illustrates that nuclear translocation of foreign DNA is an extremely inefficient process, and why it may be one of the reasons that only a small fraction of the cellular population successfully expresses the transgene after transient transfection.
Surprisingly, the cells that successfully express the transgene contain on average only a 3-fold higher plasmid copy number in the nucleus than the non-expressing cells. Thus, a significant amount of plasmid DNA is present in the nucleus of non-expressing cells. It is therefore likely that other factors, such as constraints in intracellular conditions, may also contribute to the low numbers of cells that express the transgene following transient transfection\textsuperscript{51}.

To increase the plasmid copy number per cell, it is possible to perform transient transfections with larger plasmid DNA quantities. The amount of plasmid DNA that is taken up by cells generally correlates with the amount of plasmid DNA that is transfected. However, an increase in cellular plasmid copy number does not ineluctably correspond to an increase in the cellular transgene mRNA and r-protein levels\textsuperscript{9}. Up until the transfection range of $\sim 10^5$ - $10^6$ plasmid copies per cell, the amount of transfected plasmid DNA correlates hyperbolic-like with the transgene mRNA and r-protein levels, where the r-proteins levels plateau at the upper end of this range (Figure 1a)\textsuperscript{8}. Then, at higher plasmid DNA transfection quantities, both the mRNA and r-proteins levels decline\textsuperscript{9}. In addition, the IVCD of the cell culture also decreases at these plasmid DNA concentrations, which suggests that the drop in productivity is partly caused by stress-induced apoptosis due to the cytotoxicity of the transfection complexes at high concentrations and possibly by the interference with the expression of endogenous genes of the host cell line.
Growth Medium Composition and Bioreactor Conditions

The choice of the culture medium and the conditions in the bioreactor can have a large effect on the volumetric yield. For mammalian cells to survive and proliferate in vitro, they require basic nutrients including salts, vitamins, sugars, amino acids, and nutritional and growth factors that are naturally found in serum. In the past decade, chemically defined serum-free growth media have become the standard in commercial settings. There are four main reasons for this trend: there is a potential variation between different serum batches, serum is expensive, serum-free media facilitate the downstream purification of r-proteins, and serum-free media evade potential biosafety issues that are associated with animal-derived material. The main biosafety concerns are in regards to the theoretically possible presence of infectious viruses or prions in the serum. Both CHO and HEK293 cell lines are readily adaptable to serum-free suspension conditions. To adapt the cells they are gradually deprived of serum through serial dilution steps. The cells can simultaneously be adapted to suspension growth by transferring them to low attachment shake flasks, spinner flasks, or roller bottles.

Initial attempts to transfect mammalian cells in serum-free media were inefficient. Polyanionic molecules that are present in the medium such as heparin and dextran-sulfate may neutralize polyplexes, thereby preventing them from interacting with proteoglycans on the surface of cells. This issue was later overcome by optimizing the transfection protocol such as the PEI-to-DNA ratio and the cell density during transfection. Higher PEI-to-DNA ratios may provide excess PEI available to neutralize the polyanionic molecules in the medium, so that the
interaction of these molecules with polyplexes is reduced. A disadvantage of high PEI concentrations is that more stress is induced to cells, as PEI is mildly cytotoxic to the cells.

Apart from the basic nutrients, growth media are sometimes supplemented with additional elements. Serum-free media are often fortified with peptones as an alternative to proteins that are naturally found in serum. The addition of peptones to the medium alone may be able to increase volumetric yields by 2-fold\textsuperscript{53}. In addition, small molecule drugs can also be supplemented to the medium to increase the volumetric yield. Examples include DNMT and HDAC inhibitors, or anti-apoptotic agents such as caspase inhibitors\textsuperscript{54}. However, not much research has been conducted to date on the use of small molecules to increase the productivity in SGE and TGE systems.

It is possible to maintain cells in the initial culture medium throughout the entire production phase. The disadvantage of using such batch culture protocols is that the production phase is typically short, since the production of the r-protein decelerates rapidly as soon as the nutrients run out and the cell viability decreases. Alternatively, concentrated nutrients and other supplements may be periodically added to the culture medium in fed-batch processes to prolong the production phase. Recent feeding strategies have shown promising results for TGE systems with both HEK293E and CHO DG44 cells. In preliminary studies with HEK293E cells, volumetric yields of over 2 g/l were obtained by providing the cells with a combination of glucose and peptones throughout the production phase\textsuperscript{55}. Due to these feeding adjustments, the culture attained a considerably higher IVCD. Very similar adjustments have also been made to a TGE system with CHO DG44 cell, where a volumetric yield of 0.5 g/l has been reported using a fed-batch protocol\textsuperscript{56}. Another type of feeding strategy is the so-called exponential batch culture, in which the growth medium is exponentially diluted during the first few days of the production
phase. Even though high absolute yields can be obtained with this strategy, the volumetric yield is low due to continuous dilution, resulting in high expenses during downstream purification of the r-protein.

Large-scale TGE systems make use of suspension adapted cell lines that can grow at relatively high densities. These cell cultures are mainly cultured in stirred tank bioreactors (STB), in which the nutrients are distributed through stirring. Modern advances in the development of bioreactors include the Wave™ bioreactor (Wave Biotech, Tagelswangen, Switzerland), in which cells are maintained in a plastic bag. An external apparatus applies undulation to the bag, causing the nutrients to be distributed uniformly. A major advantage of the Wave™ bioreactor is the fact that the bags are cheap, easy to use, and disposable. This makes them particularly well suited for TGE, as the initial investment is low, and the operation and maintenance of the bioreactor under different conditions requires relatively little calibration.

Although it is customary to incubate mammalian cell cultures at 37°C during the production phase, it has been shown that mild hypothermic conditions (~32°C) at the onset of the production phase can induce an increase in the volumetric yield in TGE systems. Hypothermia increases the overall steady-state levels of mRNA, promotes cell cycle arrest, and leads to lower production of cytotoxic metabolites. Therefore, it is possible that multiple factors could contribute to the observed increase in volumetric yields under these conditions. Interestingly, combining mild hypothermia with the addition of insulin-like growth factor (IGF) to a CHO cell culture resulted in approximately 4-fold higher volumetric yields than each component alone, indicating that they act synergistically (compared to CHO cells that were cultured at 37°C, the combination of mild hypothermia and IGF, mild hypothermia alone, and IGF alone resulted in the 11-fold, 2-fold, and 3-fold increase in volumetric yield, respectively). Further studies on the
effects of hypothermia on cellular metabolism could elucidate the specific role of individual factors on the production or r-proteins.
Cell Lines

The ideal mammalian cell line for the transient production of r-proteins is highly susceptible to transfection, naturally inclined to have a high cell specific productivity, and easy to maintain on large-scale. The cell lines that are most commonly used by the industry today are: CHO cells, mouse myeloma (NS0) cells, baby hamster kidney (BHK) cells, and HEK293 cells. The former three cell lines are rodent derived, and are often employed in SGE systems. In particular the CHO cell strains K1 and DG44 have been used in industrial laboratories for decades, and are therefore well characterized for the different stages of production.

It is important to note that many industrial laboratories are likely to prefer using the same host cell line both in the development of a high-producing SGE system, as well as in the development of a TGE system to transiently produce r-proteins for pre-clinical trials. This has two main reasons: firstly, different cell lines often show minor differences in post-translational glycosylation patterns that could potentially affect the therapeutic efficacy of the r-protein\textsuperscript{61}. Secondly, industrial laboratories are often attached to using one particular cell line and the corresponding standard operating procedure, and somewhat reluctant to adjust to a new cell line.

An example of a cell line that is commonly used for both SGE and TGE is the CHO cell line DG44, in which the $dhfr$ gene has been knocked out\textsuperscript{62}. The $dhfr^{−/−}$ cell lines allows the use of the $dhfr$ gene for genetic selection, since the cells will only survive after the $dhfr$ gene is successfully restored by a functional copy of the gene on the expression vector and subsequently expressed. The $dhfr$ selection system does not serve a purpose in TGE systems, as no selective pressure is exerted in these systems. Furthermore, CHO DG44 cells require a source of purines
and thymidine in the growth medium additional to the standard nutrient requirements, which further increases the operative costs. Still, much TGE research has been focused on this cell line, and this has contributed considerably to the progress in TGE volumetric yields within the past decade. In spite of the longstanding prominence of CHO cells in SGE, human derived HEK293 cells are currently the most commonly used cell line for transient production of r-proteins. These cells constitutively express the adenoviral genes E1A and E1B, resulting from the transformation of HEK cells with fragments of adenoviral DNA. Since then several subclones have been isolated, including the suspension adapted 293N3S cells, the serum-free medium adapted 293S cells, the high density adapted 293SF-3F6 cells, 293T cells expressing the SV40 large T-antigen, and 293E cells expressing the EBV protein EBNA1. HEK293E cells have consistently generated the highest volumetric yields in TGE systems to date, and are therefore the most widely used.

In addition to host cell engineering strategies that directly improve the cell specific productivity, engineering efforts have also been made to reduce the cytotoxic stress in mammalian cell lines during the production phase. The production of r-protein can be an extremely energy demanding process for mammalian cells. Assuming that the average mass of a mammalian cell is 400 pg, a high-producing cell with a specific productivity of 40 pg/cell/day will therefore produce the equivalent of 10% of its mass to the production of r-protein per day. To achieve this, cells have to metabolize large amounts of nutrients, and do so mainly anaerobically. As a result, metabolic byproducts accumulate in the growth medium, which inhibits the growth and productivity of cells.
The accumulations of lactate and ammonia have been long thought to reduce the IVCD (and thereby volumetric yields) of mammalian cell cultures. To reduce the production of lactate, attempts have been made to inhibit the flux of pyruvate to lactate by knocking down lactate dehydrogenase\textsuperscript{68}, and to increase the flux of pyruvate into the TCA cycle by overexpressing pyruvate carboxylase\textsuperscript{69}. Both approaches have shown to be able to increase the volumetric yield by 3-fold. As catabolism of amino acids partly goes through the intermediate conversion to pyruvate, overexpression of pyruvate carboxylase also reduces the amount of ammonia waste produced during the metabolism of glutamine. The accumulation of ammonia has further been reduced more than 25% in CHO DG44 cells by the expression of the urea cycle enzymes carbomyl phosphatase synthetase I and ornithine transcarbamoylase, which increased the cellular growth rate by 15-30\%\textsuperscript{70}.

Both the transfection procedure and culture conditions during the production phase of TGE inflict considerable stress on mammalian cells, which frequently leads cells to initiate apoptosis. Stress-induced apoptosis is mainly caused by a high cytoplasmic osmolarity, and limitations in nutrient and growth factors\textsuperscript{71,72}. Excessive cell death can have a significant impact on the IVCD, as well as the downstream purification of r-proteins\textsuperscript{73}. Consequently, several genetic modifications have been executed to limit early apoptosis during the production phase. Overexpression of Bcl-2 in CHO 22H11 cells resulted in 80% cell viabilities after 5 days, compared to 40% in the control cells, but did not increase the volumetric yield\textsuperscript{74}. On the other hand, overexpression of the anti-apoptotic protein Bcl-xL in CHO DG44 cells resulted in 90% cell viabilities after 14 days, and an up to 4-fold increase in volumetric yield\textsuperscript{75}.
Chapter 2

Strategies to Overcome the Cellular Constraints in Transient Gene Expression

L. M. van Tienen and J. A. Verhees
Introduction

The production of r-proteins by mammalian cells can be organized from transcription, post-transcriptional processing, translation, post-translational processing, to secretion. The productivity of a cell can potentially be regulated at all of these stages. In addition, the cellular machinery that is involved with each one of these stages can reach a point of saturation and thereby hamper the productivity.

In TGE plasmid titration experiments in HEK293E cells, Dorucher and colleagues found that the intracellular transgene copy number does not linearly correlate with the mRNA and r-protein levels (Figure 1a). The fact that the SEAP reporter protein levels plateau at high plasmid DNA quantities indicates that saturation occurs somewhere in the process leading from transcription to secretion. To further investigate at what stage saturation may occur, the mRNA levels were also measured at different plasmid concentrations (Figure 1a). A sharp decrease in both mRNA and SEAP accumulation rates was observed after plasmid DNA concentrations of about 100 ng/ml. This suggests that above these plasmid concentrations there is a reduction in the number of mRNA transcripts that are produced per plasmid (i.e. a reduction in the transcription efficiency), resulting in lower r-protein expression levels. Furthermore, it has been shown that the mRNA levels do not always correlate with the levels of secreted r-protein. This can be observed from the non-linear relationship between SEAP activity and mRNA levels at different plasmid DNA quantities (Figure 1b), and suggests that translational and post-translational events may also limit the cell specific productivity.
Figure 1. Plasmid DNA titration experiment in HEK293E cells. This figure was taken from Carpentier et al.\(^8\). HEK293E cells were transiently transfected with different quantities of supercoiled plasmid DNA containing the SEAP reporter gene. The plasmid DNA quantities ranged from 1% to 100%, corresponding to 10 ng/ml and 1 μg/ml, respectively. Identical amount of DNA were added to the cells by using non-coding stuffer DNA in order to keep to DNA-to-PEI ratios constant. Subsequently, the SEAP activities and mRNA levels were measured three times. The SEAP activities were determined with spectrophotometry from hydrolysis rates of the SEAP substrate para-nitrophenyl phosphate, and mRNA levels were obtained from densitometric analysis of northern blot experiments. (A) Relative SEAP (circles) and mRNA (squares) measurements at different plasmid DNA quantities. (B) The SEAP activity plotted against the mRNA levels, which are both normalized against 100% plasmid DNA quantities.

In SGE systems, the gene coding for the r-protein is generally integrated randomly in the genome of the host cell. The context flanking the transgene can affect the expression level of the transgene due to local differences in the arrangement of the chromatin and/or the presence of regulatory elements, an effect referred to as the “position effect”\(^7\). As a result, expression levels can vary vastly among individual cells. In contrast to SGE, the position effect does not contribute to variation in the expression levels in TGE, implying that transcription may be more limiting in SGE than TGE. Indeed, it has been suggested that while the amount of available transgenic
mRNA is limiting in SGE, translational and post-translational events are mainly limiting in TGE. Moreover, it has been shown that mRNA stability is very important for the transgene expression levels in TGE. Therefore, improving TGE systems at the levels of mRNA stability, translation, and post-translational events could be key factors in enhancing volumetric yields.
mRNA Stability

The steady-state transgenic mRNA levels in a cell are the sum of the transcription levels and the stability of the mRNA molecules. Key features that determine the stability of an mRNA molecule include the 7-methylguanosine cap at the 5’-end, and the polyadenylate tail at the 3’-end. mRNA molecules without 5’-caps are at least 4-fold less stable in cell-free mRNA decay reactions, and deadenylation is the first step necessary for the mRNA to be degraded. It has been shown that homopurine-rich regions in polyadenylation signal sequences, which are prone to form hairpin loop secondary structures, could play an important role in determining mRNA stability. The SV40 and BGH polyadenylation signal sequences contain 3 and 6 purine-rich regions, respectively. Consequently, use of the BGHpA results in higher steady-state mRNA levels than the SV40pA, which has been supported by previous studies. However, the purine-rich sequences in the plasmid DNA make the plasmids more prone to nuclease attack, indicating that a trade off exist between plasmid DNA stability and mRNA stability. Thus, the characteristics of the polyadenylation signal sequence can affect the expression of the transgene, and should be taken into consideration in the design of the expression vector.

All mRNA molecules have a secondary structure which greatly influences stability. The secondary structure determines how the molecule associates with the cellular machinery that is responsible for translation, and also with the machinery that is responsible for the degradation of the mRNA. Certain motifs within the mRNA transcript may lead to structural instabilities that can inhibit the association of a translation initiation complex, or cause the complex to be abortive. Such motives can exist naturally or can be unintentionally created when chimeric r-
proteins are constructed, for instance when antibody sequences are humanized. More knowledge on the sequences that generate such pernicious motives in the mRNA molecule could help improve mRNA stability as well as the translation.

In most genes, the number of introns is positively correlated with mRNA stability. mRNA-binding proteins that define exon-exon junctions during splicing reactions can be retained on the mRNA after it has been transported into the cytoplasm. It has been suggested that these proteins can act as insulators to prevent intra- and inter-molecular mRNA basepairing. Intramolecular basepairing can give rise to stem loop structures that induce endonucleases to cleave the mRNA, subsequently leading to mRNA degradation. Furthermore, RNA interference can result from intermolecular basepairing between the mRNA and other RNAs in the cytoplasm. Thus, the presence of introns may indirectly slow down mRNA degradation and gene silencing. However, the presence of introns might also result in the expression of multiple isoforms of an r-protein when the pre-mRNA is alternatively spliced. This is highly undesirable as usually only one isoform has the correct therapeutic activity. It is therefore important to make use of strongly defined exon-intron junctions, and to eliminate cryptic splice sites from the transgene sequence. Computational tools have been developed that can predict potential cryptic splice sites and poorly defined exon and intron definitions.

The 5′-UTR and especially the 3′-UTR are known to contain important sequences that determine mRNA stability. Most notably, mRNA molecules that contain AU-rich sequences in the 3′-UTR tend to be unstable, as they can accelerate the shortening of the polyadenylate tail or directly promote degradation of the mRNA body. It has been suggested that the AU-rich sequences have different functions depending on the mRNA, the cell type, and even the growth conditions. This makes it difficult to determine the consensus sequences for AU-rich elements,
and how they affect the cell specific productivity. However, it could be valuable to consider the 
effects of these sequences when generating constructs for TGE.

It was previously mentioned that mild hypothermia increases the steady-state mRNA 
levels, although the mechanisms behind this are not yet fully understood. It is possible that the 
increased steady-state mRNA levels are the result of an overall reduction in enzyme activities 
(including ribonucleases) at lower temperatures. A rule of thumb is that most enzyme activities 
are reduced by 50% for a decrease of 10°C. In stable transfected CHO DG44 cells, the steady-
state levels of interferon-γ (IFN-γ) mRNA increased 7 to 8-fold in cells that were cultured at 
32°C compared to cells that were cultured at 37°C. Since a decrease of 5°C resulted in a 7 to 8-
fold increase in mRNA levels, and because hypothermia is known to reduce transcription 
levels, it is unlikely that the increase in mRNA stability is solely a function of the thermal 
effect on enzyme kinetics. It is known that eukaryotic cells exhibit a cold shock response upon a 
sudden temperature downshift. The cold shock response might lead to the inhibition of 
ribonucleases, or the activation of mRNA stabilizing proteins. This idea is supported by the fact 
that several cold shock response proteins are known to be able to bind to RNA.
Translation

The translational efficiency in mammalian cells is dependent on several properties of the mRNA transcript. Besides influencing the stability of the mRNA, the 5’-UTR and 3’-UTR also have an important role during translation. The 5’-UTR contains many sequences that can affect translation such as, binding sites for regulatory proteins that are involved in translation, internal ribosomal entry sites (IRES), upstream start codons, upstream open reading frames (ORF), and the Kozak sequence. The 3’-UTR also often contains binding sites for regulatory factors, including both proteins and trans-acting RNAs. Most of these factors help regulate translation at the level of initiation.

The notion that sequences in the 5’ and 3’-UTR can influence the translation efficiency has encouraged the search for translation enhancing elements (TEE). Different TEEs have been isolated from the 5’ and 3’-UTRs of viral mRNAs, of which one of the most studied is the 68bp omega element from tobacco mosaic virus. Although the omega element is predicted to have little secondary structure, it is highly organized as it contains several sequence repeats. The omega element has been shown to promote translation through the enhanced recruitment of eukaryotic initiation factor (eIF) 4F. A recent study in Drosophila, in which several viral derived TEEs were included in a tropomyosin transgene, resulted in more than 20-fold higher expression levels than transgenes without the TEEs. The use of TEEs in r-protein transgenes may also benefit r-protein expression levels in mammalian cell lines.

The ORF codon composition can greatly influence the translation efficiency. Different organisms favor the use of different codons over synonymous codons (coding for the same
amino acid) in their endogenous mRNA transcripts. The natural enrichment of certain codons often correlates with composition of the tRNA pool, which can differ between organisms. The use of these optimal codons in the ORF of the r-protein mRNA, instead of other synonymous codons, results in higher translational rates and accuracy, thereby enhancing the volumetric yield. For example, codon optimization of human erythropoietin (EPO) has increased the volumetric yield of this r-protein by more than an order of magnitude in CHO K1 cells\textsuperscript{96}. Several codon optimization algorithms have presently been developed that can help compose the most translation efficient host-specific ORFs\textsuperscript{97,98}. The optimized cDNA can be created either by replacing suboptimal codons in the target cDNA through targeted mutagenesis, or through \textit{de novo} synthesis.

[35]
**Post-translational Events**

Proteins that are destined for secretion are generally processed through the ER. Following translation initiation, the premature protein is recognized by the signal recognition particle (SRP), which docks the protein to a translocation channel in the ER membrane. The folding and modification of the protein starts immediately upon translocation into the ER lumen, and this process depends on the availability of molecular chaperones and other enzymes. Since the ER has a finite capacity to process proteins, two coordinated stress responses, the ER-associated degradation (ERAD) and the unfolded protein response (UPR), are initiated whenever the ER is overloaded with misfolded proteins. In the ERAD response, misfolded proteins are eliminated through the ubiquitin-mediated proteolytic pathway in the cytosol. The UPR response is induced by three different ER-transmembrane proteins: IRE1, PERK and ATF6\(^9\). When misfolded proteins accumulate in the ER, these three sensing proteins are activated resulting in the release of transcription factors XBP-1, ATF4 and ATF6 that enhance the expression of genes involved in post-translational modification, protein folding, protein synthesis and degradation, amino acid transport, and ER function. If cells fail to reestablish homeostasis and continue to endure prolonged ER stress, the UPR finally results in apoptosis.

In TGE systems, the UPR may result from the sudden accumulation of misfolded r-proteins present in the ER due to high transgene expression levels, and can limit both the IVCD and the cell specific productivity. Engineering strategies have been applied to mammalian cells in an attempt to reduce the stress of r-protein production on the ER. Overexpression of the HSP70 chaperone in NS0 cells improved the cell viability, but not the cell specific
productivity\textsuperscript{100}. In CHO cells, overexpression of the protein disulfide isomerase (PDI) chaperone did result in a moderate increase of 40\% in volumetric yield, whereas overexpression of the binding protein (BiP) chaperone resulted in a decreased volumetric yield\textsuperscript{101}. Thus, overexpressing different chaperone enzymes can result in very different effects.

Additional engineering strategies have focused on reducing the UPR at higher regulatory levels. Overexpression of the transcription factor XBP-1, a key regulator of the UPR across the entire secretory pathway, increased the volumetric yield up to 6-fold in stably transfected CHO cells\textsuperscript{102}. However, overexpression of XBP-1 did not result in increased volumetric yields in HEK293 cells. It is not clear what may have caused this large discrepancy between the different cell lines.

In a TGE study, CHO cells were engineered to express a mutant of eIF2\textalpha, in which a serine residue at position 51 was replaced with alanine, rendering the protein unsusceptible to phosphorylation at this position\textsuperscript{103}. The eIF2\textalpha protein is a subunit of eIF2 complex, which regulates mRNA translation initiation. When wild type CHO cells initiate the UPR, eIF2\textalpha is phosphorylated by PERK, and subsequently inhibits translation initiation. CHO cells expressing the mutant eIF2\textalpha had at least 3-fold higher r-protein expression levels than the wild type, likely because the translation rate did not reduce as much when the UPR was initiated.

The protein folding machinery may have a large effect on r-protein productivities of mammalian cells. This idea comes forth from the fact that the nature and the complex tertiary structure of the r-protein can have large effects on the volumetric yield. For instance, different combinations of antibody heavy and light chains have been shown to result in varying expression levels of up to 200-fold\textsuperscript{104}. Moreover, a mutation of Ala to Gly at position 49 of an IgG V\textsubscript{H} domain resulted in a more than twofold reduction in the volumetric yield of a TGE system with
CHO cells. It has been suggested that these differences arise from the requirements of molecular chaperones to properly assemble antibody elements in the ER, and could be dependent on the compatibility of the heavy and light chain, as well as proper CDR/framework interactions, since single amino acid changes have been shown to be able to dramatically affect the association of different antibody components.

Finally, glycosylation affects protein folding in the ER as well as the therapeutic quality of the r-protein. Glycoengineering strategies have been applied to eliminate glycosylation patterns that could evoke undesired immunogenic responses in patients. Alternatively, glycans can also be added to r-proteins in order to increase their therapeutic activity. In particular, the glycan linked to the Asn at position 297 in the Fc region of IgGs plays a key role in the therapeutic efficacy of antibodies. In humans, an N-acetylglucosamine residue is added to this glycan at the trimannosyl core through a β(1,4)-linkage by the enzyme GnT-III. However, GnT-III is not naturally present in CHO cells. CHO cells that have been engineered to express GnT-III produce antibodies with higher therapeutic quality, and these antibodies have been approved for clinical use.
Conclusions and Future Perspectives

Large-scale TGE has the potential to generate large amounts of r-protein in a short time and at low cost. This could enable the rapid and high-throughput screening of r-proteins in during pre-clinical stages of drug development, thereby increasing the success rates in the development of therapeutic proteins. However, despite all the progress made in TGE, the volumetric yields that can be obtained are still relatively low. Typical TGE cell specific productivities are around 10 pg/cell/day, whereas SGE cell specific productivities are usually around 40 pg/cell/day. In addition, the cell viability in TGE systems is generally lower throughout the production phase than in SGE systems because the culture conditions in TGE elicit more stress on the cells. This indicates that there is room for improvement in the volumetric yields that can be obtained with TGE.

Several improvements could come from future developments in the transient transfection protocols. The chemical agents CaPi and PEI are currently the most widely used for transient transfection, though they convey only 1% of the total transfected plasmid DNA into the nucleus and they may exert cytotoxic effects under certain conditions. Overcoming gene delivery barriers (such as the escape from the endosome and nuclear translocation) by delivering naked DNA directly into the nucleus would be very useful in large-scale TGE. Electroporation on large-scale cell cultures has already been examined\textsuperscript{108}. The further development of newly explored chemical transfection agents such as dendrimers and chitosan may also improve transient transfection efficiencies in the future\textsuperscript{109,110}. Besides affecting TGE volumetric yields, the currently low transient transfection efficiencies require that large amounts of excess plasmid DNA needs to be
purified. Therefore, new cheaper ways to obtain high-grade plasmid DNA for transient transfection should be explored.

Advancements that have been made in the growth conditions of the cell culture and the composition of the growth medium can also increase the volumetric yields. A major issue in large-scale TGE cell cultures is the high rate of stress-induced apoptosis. Feeding strategies that use peptone and glucose supplements throughout the production phase have been very successful in increasing TGE volumetric yields to date. Additional small molecule supplements such as HDAC inhibitors and caspase inhibitors have also been very beneficial in increasing the cell specific productivity.

Finally, TGE volumetric yields can be increased by directly expanding the processing capacity of the protein production machinery, or by making more efficient use of the existing machinery. For TGE, specifically mRNA stability, translation, and post-translational events may be key modules at which cellular limitations may hinder high specific productivities. Codon optimization and elimination of pernicious motives in the mRNA can increase the mRNA stability and the translation efficiency. Furthermore, mammalian cells can be engineered to reduce negative feedback responses that are associated with high r-protein expression levels.

For TGE to be commonly implemented in industrial laboratories, several requirements will have to be met in the future. Industrial laboratories tend to be rather conservative about making changes in the system that they use to produce r-proteins. For example, the use of a certain production cell line has often led to long-lasting commitments and a production and quality control chain that is specified for the use of a specific cell line. This has implications for TGE, since most of the TGE advances have been made in HEK293 cells, whereas industrial laboratories generally make use of rodent derived cell lines such as CHO cells. In addition, it is
easier for industrial laboratories to experiment with different plasmid purification methods, tranfection methods, and culture conditions, instead of cell lines. In order to pave the way for TGE into commercial settings, futures objectives should be aimed more towards establishing a well-rounded TGE system that is easy to adapt and customize in industrial laboratories, has a low investment risk, and has a high reward in terms of yield.
References


