

## Chapter 2

# Transient Recombinant Protein Expression in Mammalian Cells

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**Abstract** Transient gene expression has evolved into an attractive technology for the rapid production of milligram to gram amounts of recombinant proteins. This review describes the different methods for introducing foreign DNA into suitable mammalian cells with either viral or non-viral vectors. Particular emphasis is given to non-viral transient transfection which represents meanwhile the most prominent variant due to recent progress in the resulting protein productivity. Non-viral transient transfection protocols are always based on the use of specific transfection reagents or the application of an electroporation device. The corresponding methods are compared with regard to their scale-up potential, also in consideration of potential production costs. The underlying cellular pathways of plasmid DNA incorporation, cytoplasmic release and translocation into the nucleus are important details to understand the transfection principle and further improve the technology. Problems associated with the application of transient gene expression at a larger scale are also addressed. In particular, the requirement of different cell culture media conditions for plasmid DNA complex preparation (if necessary), the transfection process itself and a high titer recombinant production need to be harmonized. Strategies to improve recombinant protein productivity by increasing the cell-specific output and/or sustaining the production phase are itemized as well. This can be accomplished by enabling cells to perform episomal plasmid replication, co-transfection with other plasmids, altering the cellular metabolism, temperature reduction, supplementation of specific production enhancers or combinations thereof. A number of examples for successful applications at pilot scale are also provided.

**Keywords** Adenovirus • Alphavirus • BacMam • Baculovirus • Butyrate • Calcium phosphate coprecipitation • Calfection • CHO • EBNA1 • Electroporation • Flavivirus • HEK 293 • Polyethylenimine • Polyplex • Semliki Forest Virus • Sindbis

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## 2.1 Introduction

Transient transfection of animal cells for both recombinant protein production and gene therapy has become more and more important with widespread applicability. Substantial progress was achieved during the 1990s which brought these technologies into the limelight. Accordingly, volume 2 of *Cell Engineering*, published in 2000, was completely dedicated to the topic of transient expression, with particular emphasis on the extremely successful baculovirus expression vector system (Al-Rubeai 2000). Meanwhile, the unsurpassed potential of mammalian cells for posttranslational modifications has further driven the development of mammalian cell-based transient gene expression systems with a remarkable increase in protein yields when compared to the low titers initially obtained. A first increase in recombinant protein productivity was achieved by introducing viral vectors. Later, non-viral systems with improved transfection efficiency and sustained productivity became available. This chapter is addressing the most recent advances in both the fundamental understanding of the transfection technology and methodological tools to further improve transfection protocols with regard to an increased recombinant protein titer and reduction of costs.

## 2.2 Viral Transient Transfection

Besides the baculovirus expression vector system used in combination with lepidopteran cell lines, viral transfection was initially also the method of choice for transient gene expression in mammalian cells due to the very low expression levels in non-viral transfection systems. Meanwhile, non-viral transfection strategies have been improved significantly, thus pushing aside virus-based technologies, which were always hampered by the additional requirement of virus expansion steps, as well as efforts for virus storage and particularly biosafety issues. Viruses used for this purpose are predominantly either single stranded RNA-viruses of the genus alphavirus (Semliki Forest Virus, Sindbis Virus, Venezuelan Equine Encephalitis Virus) or double stranded DNA viruses such as the Vaccinia virus, Adenovirus and Baculovirus. More recently, several flaviviruses have been used as well (Table 2.1). However, all of these expression systems have in common that the virus has to be modified to prevent uncontrolled replication as a result of an accidental release. In addition, viruses had to be either attenuated with regard to their pathogenic properties, or, as a further step, unburdened from most or all of the genes for their structural proteins to become simply replicons which require additional defective helper constructs to generate infective particles (Polo et al. 1999). Some of these

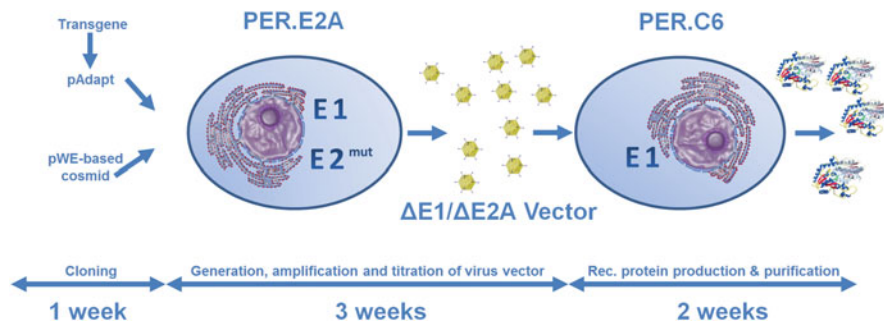
**Table 2.1** Viral vectors used for transient transfection of mammalian cells

Virus vector	Virus type	Family – Genus	Vector	References
Semliki Forest Virus (SFV)	(+) ssRNA	Togaviridae – Alphavirus	Specifically modified virus	Lundstrom (2010), Fernández Núñez et al. (2013)
Sindbis Virus (SIN)	(+) ssRNA	Togaviridae – Alphavirus	Specifically modified virus	Nivitchanyong et al. (2009)
Venezuelan Equine Encephalitis Virus (VEEV)	(+) ssRNA	Togaviridae – Alphavirus	Replicon	Balasuriya et al. (2000)
Tick-borne Encephalitis Virus (TBEV)	(+) ssRNA	Flaviviridae – Flavivirus	Replicon	Gehrke et al. (2005)
Japanese Encephalitis Virus (JEV)	(+) ssRNA	Flaviviridae – Flavivirus	Full length cDNA	Yun et al. (2003)
Vaccinia Virus (VACV)	ds DNA	Poxviridae – Orthopoxvirus	Attenuated Strain	Pradeau-Aubretton et al. (2010)
$\Delta$ E1/ $\Delta$ E2A Ad5 vector (Adenovirus)	ds DNA	Adenoviridae – Mastadenovirus	Specifically modified virus	Havenga et al. (2008)
Baculovirus	ds DNA	Baculoviridae – Alphabaculovirus	Specifically modified virus	Condreay et al. (1999), Ames et al. (2007)

replicons have been further modified to chimera of two related viruses. Most details of the corresponding transfection systems have already been reviewed in detail previously (e.g. Smerdou and Liljeström 2000). Because of their specific properties, replicon-based gene expression is now mostly used for experimental gene vaccine approaches rather than recombinant protein production *in vitro*.

Besides gene therapy applications, adenoviruses were also investigated with regard to their potential as vector for transient protein production. Due to accompanying cytopathic effects virus-susceptible cell lines such as HEK 293 are less suitable, particularly with regard to recombinant protein quality. Therefore, an adenovirus-permissive CHO cell line was generated by repeated virus infection which showed a better performance and was successfully used for production of several secreted proteins (Condon et al. 2003). Another promising approach was accomplished by deleting the viral E1 and E2A in an adenovirus vector which could be easily amplified in a PER.E2A cell line constitutively overexpressing a temperature sensitive, less cytotoxic mutant of E2A. The generated high-titer vector was subsequently used to transduce PER.C6 cells for recombinant protein production (Fig. 2.1). The efficient virus amplification process suggested a high potential for scaling-up this technology (Havenga et al. 2008).

Baculoviruses have also been studied for their ability to transfect mammalian cell lines. The inability of mammalian cells to replicate the baculovirus and to express virus-derived or recombinant DNA was originally considered as a good



**Fig. 2.1** Schematic diagram showing the use of the  $\Delta E1/\Delta E2A$  adenoviral vector-based transient protein production technology in PER.C6 cells including a feasible timeline (Based on Havenga et al. 2008)

sign of biosafety (Carbonell and Miller 1987). Research on protein expression in mammalian cell lines was further driven by the potential of baculoviruses as a safe gene therapy tool. Using the Rous sarcoma virus (RSV) promoter baculovirus was successfully applied to transiently transfect HepG2 cells with moderate expression levels and the requirement of a relatively high multiplicity of infection (Boyce and Bucher 1996). Since baculoviruses allow the insertion of very large genes and their in vitro propagation in lepidopteran cell lines is well-established, a continuing interest lingers on to make them also a tool for transient recombinant protein expression in mammalian cell lines. This led to the development of a substantially improved baculovirus bearing the Vesicular Stomatitis Virus glycoprotein G in its envelope, while keeping the RSV promoter (Barsoum et al. 1997). This virus was able to transfect already a larger number of mammalian cell lines. Subsequently, the technology was developing more rapidly. Another milestone was achieved using expression cassettes under control of the CMV immediate early promoter and the SV40 early promoter. This enabled the transduction of a larger number of mammalian cells including the common production cell lines HEK293, CHO, BHK-21, and HeLa (Condreay et al. 1999). Subsequently, this system was commercialized and named BacMam. An excellent in-depth overview about this expression system is provided by Ames et al. (2007).

## 2.3 Non-viral Transient Transfection

Despite good recombinant protein yields using virus-based transient transfection systems, they were gradually outshone by non-viral transient transfection. A large number of substantial technological improvements during the past decade meanwhile enables recombinant protein production at much higher titers and in a more sustained way. Most prominently, the use of the plasmid replication system of Epstein-Barr virus (EBV) overcame the major problem of low product titers and has

led to a more widespread application of this technology. EBV nuclear antigen 1 in combination with the viral origin of replication *oriP* allows episomal replication of plasmid DNA (Länge-Rouault et al. 1998).

The demand for applying transient transfection at a larger scale is accompanied with requirement of increasing amounts of plasmid DNA. Similar to the necessity to include an additional production step in virus-based systems in order to generate sufficient amounts of virus, substantial amounts of plasmid DNA have to be produced in suitable strains of *E. coli* bacteria and subsequently prepared at a quality compatible to mammalian cell culture applications (e.g. free of endotoxins). At small and medium scale, shaker cultures in combination with subsequent plasmid DNA purification by means of commercially available kits are the method of choice for plasmid preparation. However, with the increasing plasmid demand for transient gene expression in pilot-scale cell culture bioreactors, it became also necessary to scale-up the plasmid preparation accordingly. Thus, plasmid production in bioreactors and purification at a corresponding scale became essential prerequisites (Rozkov et al. 2008; Sun et al. 2008; Tuvesson et al. 2008; Cheng et al. 2011).

## 2.4 Plasmid Vector Design

Suitable plasmid vectors for transient transfections have to meet several requirements. Important criteria for plasmid vectors are:

- Yield of plasmid DNA production in *E. coli*
- Efficiency of cellular delivery upon transfection of host cells
- Strength of recombinant overexpression.

Table 2.2 provides an overview on commonly used vectors for transient transfection.

### 2.4.1 Plasmid Backbone and Size

Transient transcription of mammalian cells on preparative scale requires large amounts of plasmid DNA. High-copy plasmid vectors that can be prepared in high yield from *E. coli* culture are thus preferred. The pUC19 vector was derived from the pBR322 cloning vector, which was cloned from the natural ColE1-class plasmid pMB1 (Yanisch-Perron et al. 1985). Mutation of the pBR322-derived part of pUC19 (GenBank L09137.2) resulted in an ultra-high-copy number of several hundred molecules per *E. coli* cell. The pUC19 origin of replication therefore facilitates the production of large amounts of plasmid DNA in *E. coli* for transient transfection. Most vectors used for cloning and transfection have the ultra-high-copy origin of pUC19, which is characterized by the sequence *ctagaagAac*,

**Table 2.2** Selected plasmid vectors for transient transfection

Vector	Elements	Size (bp)	Source	Reference
pTT	P(CMV), <i>oriP</i> , pUC ori, bla	5,925	NRC-BRI, Montreal, Canada	Durocher et al. (2002)
pTT5	P(CMV), <i>oriP</i> , pUC ori, bla	4,401	NRC-BRI, Montreal, Canada	Zhang et al. (2009)
pCSE2.5-hIgG1Fc-XP	P(CMV), BGH-pA, SV40ori, <i>oriP</i> , pUC ori, bla	5,038	TU Braunschweig, Germany	Jäger et al. (2013)
pCEP4	P(CMV), SV40-pA, <i>oriP</i> , EBNA1, hyg, pUC ori, bla	10,186	Life Technologies, Carlsbad, CA	Parham et al. (2001)
pcDNA3.1	P(CMV), BGH-pA, fl ori, neo, pUC ori, bla	5,428	Life Technologies, Carlsbad, CA	
pSS185	EBNA1, <i>oriP</i> , SV40-pA, TAR, P (CMV), TK pA, hph, Tat, pUC ori, bla	11,952	Bayer Healthcare	Cho et al. (2001)
pCI-neo	P(CMV), intron, SV40-pA, neo, pUC ori, bla	5,472	Promega	GenBank U47120

(CMV) CMV promoter, *oriP* EBV origin of replication, *hyg* hygromycin resistance gene, *neo* neomycin phosphotransferase gene, *pA* polyadenylation signal, *bla*  $\beta$  lactamase gene, *EBNA1* EBV nuclear antigen 1, *PyOri* Polyoma virus origin

comprising a G > A mutation in comparison to pBR322 (Table 2.2). The pUC19 origin is often labelled ‘ColE1 ori’ or ‘pMB1 ori’ in vector maps.

Smaller plasmids are transferred to the nucleus more efficiently during transfections (Yin et al. 2005; Mairhofer and Grabherr 2008). Unnecessary parts of the vector should therefore be removed. Eukaryotic selection markers such as the neomycin phosphotransferase gene are usually not required during transient transfection. Co-expression of selection marker or reporter genes (e.g. GFP) under control of a strong promoter causes an additional metabolic burden for transfected cells, which can result in lower yield of the target protein. Antibiotic selection or cell sorting is also possible by co-transfection of separate plasmids containing these markers.

Vector stability is mainly determined by sequence and size (Oliveira et al. 2009). Larger vectors may contain a larger number of unstable regions and quality control by electrophoresis or chromatography is more difficult. Especially repetitive sequences can reduce plasmid stability, leading to mutated plasmid molecules, or may also induce plasmid degradation by nucleases in the host cell cytoplasm (Oliveira et al. 2009).

Minicircles and other minimal plasmid formats have been developed by reducing or eliminating the bacterial parts of vectors for transfections (Marie et al. 2013; Oliveira and Mairhofer 2013). The development of these systems is driven by gene therapy and DNA vaccination, which require vectors without bacterial antibiotic

resistance markers. For transient transfections, minimal plasmid systems offer improved cellular and nuclear uptake due to minimal plasmid size.

Minicircles are generated by separating the expression module from the bacterial backbone (Broll et al. 2010; Nehlsen et al. 2013). This can be achieved by site-specific recombination. Minicircles are efficiently replicated in mammalian host cells by including copies of scaffold/matrix attachment regions (S/MARs) (Nehlsen et al. 2013).

The bacterial plasmid backbone has alternatively been minimized by replacing the antibiotic selection markers. Ampicillin or Kanamycin resistance markers (830 or 890 bp) can be replaced by the smaller zeocine marker (440 bp). Plasmid propagation can be enforced without antibiotics in specially engineered bacterial strains (Oliveira and Mairhofer 2013). Plasmid propagation has been accomplished with small vector elements such as the amber suppressor tRNAs (~100 bp, pFAR system), the lacO operator (pORT system) (Williams et al. 1998) or antisense RNA (RNA-OUT system) (Luke et al. 2009). The murselect system allows propagation of completely marker-free plasmids. It relies on a bacterial strain overexpressing a regulatory RNA that recognizes the plasmid's origin of replication (Mairhofer et al. 2008).

Despite these interesting developments with focus on gene therapy, most plasmids of choice for transient transfection currently still contain bacterial antibiotic resistance markers and replication origins (Table 2.2). The design of minimal plasmids and their size reduction requires great care. Arrangement and orientation of vector elements in context to others as well as spacer sequences between these elements can dramatically effect plasmid replication and transgene expression. Particularly, DNA elements containing secondary structure information can be strongly influenced by other sequence motifs in their vicinity. Strand separation induced by negative superhelical stress (Stress-induced DNA duplex destabilization, SIDD) and B-form to Z-form (SIBZ) transition may result from a new vector design (Bi and Benham 2004; Zhabinskaya and Benham 2011). We observed that cloning of the highly repetitive EBV origin of plasmid replication (oriP) in direct neighborhood of the bacterial pUC origin of replication reduced the plasmid copy number in *E. coli*. After introduction of a 250-bp spacer between these elements, replication in *E. coli* returned to normal high levels as expected for a high copy plasmid (Jäger et al. 2013).

## 2.4.2 Vector Elements

Vectors for transient transfection require elements that are necessary or support recombinant protein production. Necessary elements are the promoter, the recombinant protein's coding sequence and a signal for transcription stop and polyadenylation. Supporting elements increase product yield, e.g. by stabilizing recombinant transcripts.

Antibody gene sequences were evolved for maximum expression. The coding sequence and untranslated transcript regions of other genes often benefit from optimization (Fath et al. 2011). In certain cases, codon optimization can lead to high expression of a previously undetectable gene product. Fusion partners such as antibody crystallizable fragment (Fc) or serum albumin can also strongly increase expression of difficult target proteins (Carter et al. 2010).

The human Cytomegalovirus (CMV) major immediate early promoter represents the most commonly used promoter for transient transfection. Commonly used CMV promoters comprise the region of ~610 bp upstream of the transcription start of the CMV major immediate-early gene (GenBank M60321), including the promoter's CAAT and TATA box. Mariati et al. have created vectors with additional CMV sequences upstream and downstream of the promoter, including the first exon and intron of the CMV major immediate-early gene (Mariati et al. 2010). Vectors were used for transient and stable transfections of CHO-K1 and HEK293 cells. Luciferase or EPO were produced. A beneficial effect by including the exon and the intron, but not the upstream elements, was observed.

Intron splicing enhances and stabilizes transgene expression by more efficient packaging of the transcript into ribonucleoprotein complexes and accelerates transport to the cytosol. Previous vectors used genomic immunoglobulin intron sequences. They have been replaced by plasmids containing optimized synthetic introns in the 5' UTR (e.g. pCI-neo, Promega) or the translated secretory leader sequence (e.g. pCMV/pCMX vectors, Jäger et al. 2013).

Backliwal et al. optimized IgG production by transient transfection of HEK293 cells (Backliwal et al. 2008d). They observed a twofold increase of production when an intron was placed downstream of the human CMV promoter. The mouse CMV promoter was less efficient. A further increase was obtained by including the post-transcriptional regulatory element WPRE of woodchuck hepatitis virus. WPRE is an RNA element of ~600 bp that acts on different levels of RNA processing, transport, and translation and that can compensate for lack of an intron (Schambach et al. 2000). 5.5-fold increased IgG production was observed in HEK293E cells by including WPREs in the EBV-based episomal vector pCEP4 (Kim et al. 2009). Likewise, a threefold increase of WPRE-mediated antibody production was reported in CHO DG44 cells (Wulhfard et al. 2008).

Xia et al. expressed four genes of interest in HEK293EBNA and CHO-K1 cells using different promoters (Xia et al. 2006). The human CMV promoter with the downstream exon 1 and intron 1 of the CMV major immediate-early gene was the strongest, especially in HEK293EBNA cells, in comparison to a standard human CMV promoter or mouse or rat CMV promoters.

Promoters with synthetic enhancers represent a promising alternative to the CMV promoter. Libraries of promoters are generated by random combinations of transcription factor binding site or random sequences, followed by screening for maximal production (Ogawa et al. 2007; Schlabach et al. 2010). Synthetic promoters twice as active as CMV in transient transfections of CHO-S cells for SEAP production have been reported (Brown et al. 2014).



Transcription factor binding sites not only drive gene expression, but can also mediate the transfer of plasmid DNA from the cytoplasm to the nucleus (Gill et al. 2009). Transfection of HeLa cells using PEI lead to NF- $\kappa$ B-driven nuclear import of plasmids containing an NF- $\kappa$ B binding site (Breuzard et al. 2008). The presence of the NF- $\kappa$ B motif led to a sixfold increase of nuclear plasmid DNA.

### 2.4.3 Episomal Replication

The Epstein-Barr Virus (EBV) maintains its genomic plasmid as an extrachromosomal replicon (Lindner and Sugden 2007). The plasmid's origin of replication, *oriP*, is recognized by the viral protein Epstein-Barr Nuclear Antigen 1 (EBNA1). EBNA1 together with host cell replication factors mediate exactly one round of DNA replication per cell cycle. The viral plasmids are partitioned faithfully to each daughter cell during cell division. EBNA1 has a nuclear localization signal and contributes to nuclear retention of *oriP* containing plasmids.

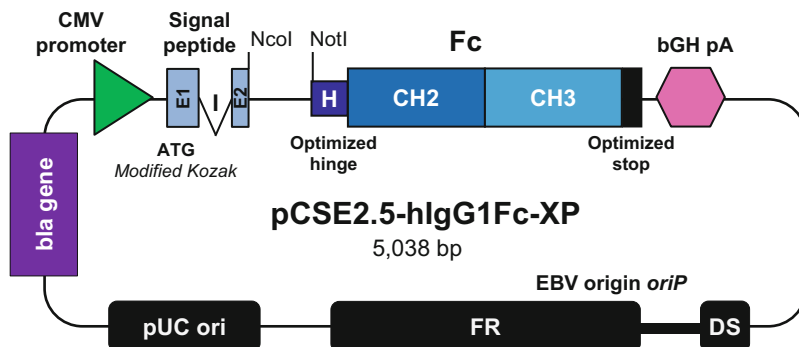
*oriP* is located in a large region of ~2,000 bp of the viral plasmid (GenBank NC\_007605, nt 7315–9312). The Family of Repeats (FR) of *oriP* is composed of 21 imperfect copies of a 30-bp EBNA1 binding site. In combination with EBNA1, FR acts as an enhancer for promoters on the same plasmid. The Dyad Symmetry (DS) element of *oriP* is the site of DNA synthesis initiation.

Transient transfection of HEK293 and CHO cells benefits from the EBV plasmid replication system (Pham et al. 2006). This requires presence of *oriP* in the plasmid vector and overexpression of EBNA1 in the host cell. It should be noted that transient transgene expression benefits from episomal replication only if production time will be longer than 2 or 3 days. Only then the effect becomes significant because non-replicating plasmids are lost during cell divisions.

HKB11 is an EBNA1-expressing human hybrid cell line derived from the fusion of HEK293S and a Burkitt's lymphoma cell line (Table 2.4). Transient transfection of HKB11 with an P(CMV) vector for overexpression of an interleukin was increased threefold by including *oriP* (Cho et al. 2001). The increase was even 18-fold when *oriP* was combined with the Tat/TAR transactivation axis from human immunodeficiency virus type 1 (HIV-1) in the vector **pSS185** (Cho et al. 2001).

Epi-CHO is a transient transfection system based on the plasmid replication system of Polyoma virus (Py). The Py origin of replication (PyOri) and the Py large T antigen (PyLT) are sufficient to launch strong episomal replication in CHO cells (Kunaparaju et al. 2005). The Py and the EBV replication systems were combined. hGH product yield was increased from 10 to 75 mg · L<sup>-1</sup> upon transfecting PyLT-positive CHO (CHO-T) cells by including PyOri, *oriP* and EBNA1 into the vector.

The **pCEP4** vector (Life Technologies, Carlsbad, CA) contains a full-length *oriP* and the EBNA1 gene and replicates autonomously in mammalian cells (Yates et al. 1985). The *oriP*/EBNA1 combination increases protein production upon transient transfection of HEK293 cells (Parham et al. 2001). The **pTT** vector



**Fig. 2.2** Illustration of the optimized transient expression vector pCSE2.5-hIgG1Fc-XP (Jäger et al. 2013). The vector backbone consists of the bacterial high-copy pUC ori and the  $\beta$  lactamase selection marker gene. The expression cassette is under control of the immediate early CMV enhancer/promoter and the short bovine growth hormone (*bGH*) poly A (*pA*) transcription termination signal. The expression cassette is initiated by a modified untranslated 5' region, a strong ribosomal binding site (modified Kozak sequence), and a mouse heavy chain signal peptide with two exons (E1 and 2, *light blue*) interrupted by an intron (*I*). One step cloning of single chain Fv (*scFv*) genes is possible via *NcoI* and *NotI* from HAL antibody gene libraries (Hust et al. 2011). For production of Fc fusion proteins, the vector contains a constant hinge (*H*), a CH<sub>2</sub> and a CH<sub>3</sub> immunoglobulin region derived from human IgG1. A strong stop codon with an optimal fourth nucleotide for efficient translation termination was introduced. A modified EBV oriP for episomal replication in mammalian EBNA1-positive host cells was introduced. The spacer between the highly repetitive framework region (*FR*) and the dyad symmetry (*DS*) element was reduced

contains an *oriP* but lacks EBNA1. It is used with HEK293 cell lines stably expressing EBNA1 (Durocher et al. 2002) or a truncated form (Durocher 2006). **pTT5** was derived from **pTT** and contains just the FR of *oriP* (Zhang et al. 2009).

The **pCSE** vectors were created by us for overexpression of antibody and antigen molecules as Fc fusion proteins (Fig. 2.2) (Jäger et al. 2013). HEK293-6E cells were transiently transfected with plasmids using PEI (Schirrmann and Büsow 2010). The original vector, **pCMV-hIgG1Fc-XP** was optimized in several steps to the final vector **pCSE2.5-hIg1Fc-XP**. Vector size was reduced by removal of a neomycin resistance gene. A minimized *oriP* sequence, shortened by 830 bp, was inserted. These modifications increased production 3.5-fold (Jäger et al. 2013).

## 2.5 Transfection Principles

### 2.5.1 Calcium Phosphate Coprecipitation and Calfection

Calcium phosphate coprecipitation condensing DNA into particles represented one of the first non-viral techniques for introduction of plasmid DNA into mammalian cells. Initially developed by Graham and van der Eb back in 1973 it was optimized for transient transfection of suspension cells at both spinner flask and benchtop

bioreactor scale (Jordan et al. 1998). A further scale-up to 100-L pilot scale was also successfully demonstrated using HEK 293 EBNA1 cells (Girard et al. 2002). However, it's more widespread application was always hindered by the necessity to add serum to the transfection medium, mostly to alleviate toxic side effects of the particles, and by the fact that a number of parameters have to be well adjusted and thoroughly followed to achieve good transfection and expression results. Furthermore, some of the ongoing effects were initially not completely clarified leaving plenty of optimization potential. Diminishing the amount of calcium, adjusting the amount of phosphate and using albumin as a serum replacement for protecting the transfected cells allowed also serum-free transfections (Girard et al. 2001). For CHO cells it was demonstrated that the phase of the cell cycle has a significant influence on the transfection efficiency. When arrested between G0/G1- and S-phase by mimosine and thus synchronized these CHO cells showed a better productivity of the GFP reporter gene (Grosjean et al. 2002), particularly when this was combined with a number of additional process steps such as (repetitive) glycerol shocks or other osmotic shocks momentarily increasing osmolality to approx. 2,000 m Osmol/kg (Grosjean et al. 2006). Obviously, only a proportion of cells, those which are in a specific phase of the cell cycle, are susceptible to successful transfection as shown by repeated transfections which targeted different subpopulations in the same cell culture. Despite of being quite laborious and difficult to perform at larger scale, these techniques provided principally deeper insights about intracellular plasmid DNA processing and transport following endocytosis. Further adjustments were also made with regard to a more feasible incubation period after precipitate formation as a function of temperature, pH and the calcium concentration (Chowdhury et al. 2003). The technology was further improved by omitting the extra precipitation step. This method of forming non- or micro-precipitated calcium-DNA complexes was named *calfection* (Lindell et al. 2004). Again, a successful scale-up to the 30-L scale was demonstrated, although several principal disadvantages of the technology remained. This includes the requirement of a proper adjustment of the pH to slightly alkaline conditions, a narrow range for optimum levels of calcium concentration with additional constraints due to limited reproducibility, a relatively high amount of plasmid DNA required for successful transfection (optimum expression levels at 5 mg L<sup>-1</sup>) and the necessity to add serum at the time of transfection which led to additional burden during downstream processing and, together with the calcium, to an undesired increase of cell aggregation.

### **2.5.2 DNA Lipoplex Formation and Lipid-Based Transfection**

Since the first report describing the use of lipofection with cationic lipids as a tool for DNA-transfection (Felgner et al. 1987), lipids in various forms have been

**Table 2.3** Lipid-based reagents applied for transient transfection of suspension cultures

Name	Manufacturer/ distributor	Type	Specific properties, reference
Lipofectamine®2000 (LF2000)	Life Technologies	Cationic lipid	Sometimes incom- patible for trans- fection in serum- free media
293fectin	Life Technologies	Cationic lipid	Chiou et al. (2014)
DMRIE-C	Life Technologies	Liposome formulation of 1,2-dimyristyloxy-propyl-3- dimethyl-hydroxy ethyl ammonium bromide and cholesterol	
FuGENE 6	Roche, Promega	Non-liposomal lipid-based	
Ro-1539	Roche	Modified dioleoylmelittin combined with a cationic polymer	Legendre et al. (1997), Schlaeger and Christensen (1999)
FreeStyle™ MAX	Life Technologies	Cationic lipid	
ExpiFectamine™293	Life Technologies	Cationic lipid	Chiou et al. (2014)

identified as being particularly well suited for gene delivery into mammalian cells (Table 2.3). In combination with plasmid DNA they form liposomes which are actively incorporated into cells by endocytosis (Zabner et al. 1995). This was confirmed by electron microscopy observations which also revealed significant differences in plasmid uptake between adherently growing cells and suspension cells (Labat-Moleur et al. 1996). Furthermore, the major bottleneck was identified in intracellular translocation of the plasmids into the nucleus, which resulted in the accumulation of substantial amounts of particles in the cytoplasm and was supposed to be responsible for cytotoxicity. Nonetheless, this highly efficient technology provides usually high transfection rates and is one of the methods of choice for both stable and transient transfection at small scale. This is also reflected by a wide range of commercial reagents available for this application.

Different lipid-based transfection reagents have been studied in comparative studies (also with reagents from the group of cationic polymers). The performance was shown to depend highly on the cell line used for transfection as well as other process parameters (Schlaeger et al. 2003; Rosser et al. 2005; Haldankar et al. 2006; Yamano et al. 2010). All lipid-based transfection reagents have in common that they come along with very high costs which are usually precluding applications at a larger (bioreactor-)scale as it is considered necessary for many transient recombinant protein production processes (Haldankar et al. 2006; Liu et al. 2008).

### ***2.5.3 Cationic Polymers and Transient Transfection with DNA Polyplexes***

Polyethylenimines (PEI), a family of cationic polymers were first introduced as a reagent for gene transfer by Boussif et al. (1995). Both a 50 kDa and an 800 kDa PEI not further specified (but presumably branched) were found to be suitable for transfection of embryonic chicken neurons as well as HeLa cells at an optimum N/P ratio of 9 (mole to mole ratio of the amine groups of cationic polymers to the phosphate groups of DNA). Subsequently, linear PEIs were identified to provide also good transfection results at a typically lower toxicity. However, the size of the formed PEI/DNA polyplexes was found to be highly dependent on the solution used (either 5 % glucose or 150 mM NaCl) with different optima of the polyplex size as well as the N/P ratios for either gene therapy applications in vivo or cell transfection in vitro (Goula et al. 1998a, b). Godbey et al. (1999a) investigated also smaller PEI variants down to a molecular weight of 600 but obtained best transfection results with the highest molecular weight variant tested (70 kDa). In another study a linear 22 kDa PEI provided better transfection efficiency than branched PEIs of 25 or 50 kDa (Wiseman et al. 2003). In contrast to the well-understood virus-based transfection mechanisms the principles of the PEI-based non-viral gene transfer had to be elucidated step by step (Kichler et al. 2001). A detailed study about optimum PEI / plasmid DNA ratios (based on suitable N/P ratios), cytotoxicity, stability against DNase digestion, and intracellular translocation of plasmids was performed by Oh et al. (2002). They showed that PEI polyplexes increase the cellular uptake of plasmid DNA, efficiently protect against intracellular enzymatic DNA digestion, and prolong the subcellular DNA retention, whereas a contribution of PEI alone for an improved translocation into the nucleus as suggested previously (Godbey et al. 1999b) could be excluded. Linear PEI of 25 kDa was confirmed as the most suitable variant of PEI when compared to linear PEI of both higher and lower molecular weight as well as branched PEI for either CHO DG44 cells (Derouazi et al. 2004) or HeLa and HEK293 cells (Reed et al. 2006). Moreover, linear PEI with a certain degree of polydispersity was shown to bind DNA less efficiently but provide better recombinant protein yields than more uniform fractions or fully hydrolyzed PEI (PEI “Max”) (Kadlecova et al. 2012). Being currently the most preferable cationic polymer for gene transfer into cells with regard to transfection efficiency and cytotoxic side effects, PEI nevertheless may induce necrosis and apoptosis to some extent (Hunter 2006). In an approach to better understand the transfection principle, Carpentier et al. (2007) investigated the kinetics of plasmid uptake, subsequent translocation into the nucleus, transcription and protein expression. Up to 65,000 plasmid copies in both GFP-expressing and non-expressing cells were detected intracellularly 1 day post transfection, confirming previous results of Kichler et al. (2001) who found 50,000 plasmid copies per cell 7 h post transfection. However, just 1,850 copies were found in the nucleus of GFP-positive cells and 550 copies in GFP-negative cells. Variations in the total amount of plasmid DNA during transfection did not significantly change the ratio of producing and non-producing cell, thus indicating that a cellular state no further specified is of major importance for a successful transient

transfection. This is in accordance with earlier results demonstrating that recombinant protein productivity is dependent on the phase of the cell cycle during transfection (Grosjean et al. 2002).

### **2.5.3.1 Polyplex Uptake, Plasmid Release and Translocation into the Nucleus**

The biochemical principles responsible for the successful use of polythyleneimine as a transfection reagent were initially unknown and it took more than a decade to investigate and understand the most important steps of the underlying pathways. The internalization of the polyplexed plasmid DNA, its intracellular trafficking and release from the polyplex as well as its positioning into the cellular nucleus are based on a series of rather complex mechanisms. PEI:DNA polyplexes are first specifically bound to (heparin sulfate) proteoglycans, in particular syndecans, before being internalized and transported to late endosomes by two constitutive endocytic pathways. Polyplex internalization by both the clathrin- and a caveolae-dependent pathway were observed. Both the transfected cell line and the type of the polyplex appear to determine which of the pathways is used most efficiently (von Gersdorff et al. 2006). For instance, both pathways were observed in COS-7 cells but only the latter was demonstrated to result in a successful gene expression (van der Aa et al. 2007). The mechanism of a successful internalization was shown to be different to and independent of both the clathrin- and the ‘standard’ caveolin-mediated endocytosis by possibly bypassing early endosomes (Payne et al. 2007). Different syndecans are reported to have quite opposing properties with regard to their potential to incorporate the polyplexes. Only syndecan-1 promotes subsequent trafficking of the PEI:DNA complexes (Paris et al. 2007). Using HeLa cells, this slightly different caveolar pathway was compared to the clathrin pathway and confirmed to be the predominant uptake mechanism (Gabrielson and Pack 2009). The initial steps are based on a capture of the polyplexes by filopodia with locally clustering syndecans. By either directional surfing along these filopodia or by an actin driven filopodia retraction the polyplexes are first guided to the cell surface and then specifically internalized via caveolae. This process is far beyond a random attachment of polyplexes by simple electrostatic interactions and contributes significantly to the success of a transient transfection (ur Rehman et al. 2012).

Once in the endosome, polyplexes are proposed to be subjected to the so-called proton sponge effect with an osmotic swelling and eventual vesicle rupture as a result of the ongoing acidification of the maturing vesicle (Nel et al. 2009). Different to that, more recent findings revealed that the corresponding endosome is not disrupted and the release of the plasmid DNA from the polyplex is a more subtle process (ur Rehman et al. 2013). This is in accordance with findings that additional, free PEI molecules might play a pivotal role in the process of DNA release from endosomes (Yue et al. 2011). An early intracellular dissociation of radiolabelled PEI and DNA was also confirmed by subcellular fractionation methods (Shi et al. 2013). Upon dissociation, the relatively large plasmid DNA shows a low mobility (Lukacs et al. 2000) and, at the same time, is very susceptible to

inactivation and degradation by cytosolic nucleases resulting in an apparent half-life of just 50–90 min (Lechardeur et al. 1999). Thus, rapid translocation of naked plasmid DNA into the nucleus is paramount for a successful transient transfection process. The nuclear pore complex may play an important role in non-dividing cells targeted for gene therapy (van der Aa et al. 2006). However, nuclear transport in cultured, proliferating cells is presumably accomplished most efficiently during the temporary nuclear membrane breakdown in mitosis (Grosse et al. 2006). DNA nuclear targeting sequences of the plasmid, originally derived from SV40, might further enhance plasmid uptake into the nucleus (Dean et al. 2005), even if the cells are non-dividing.

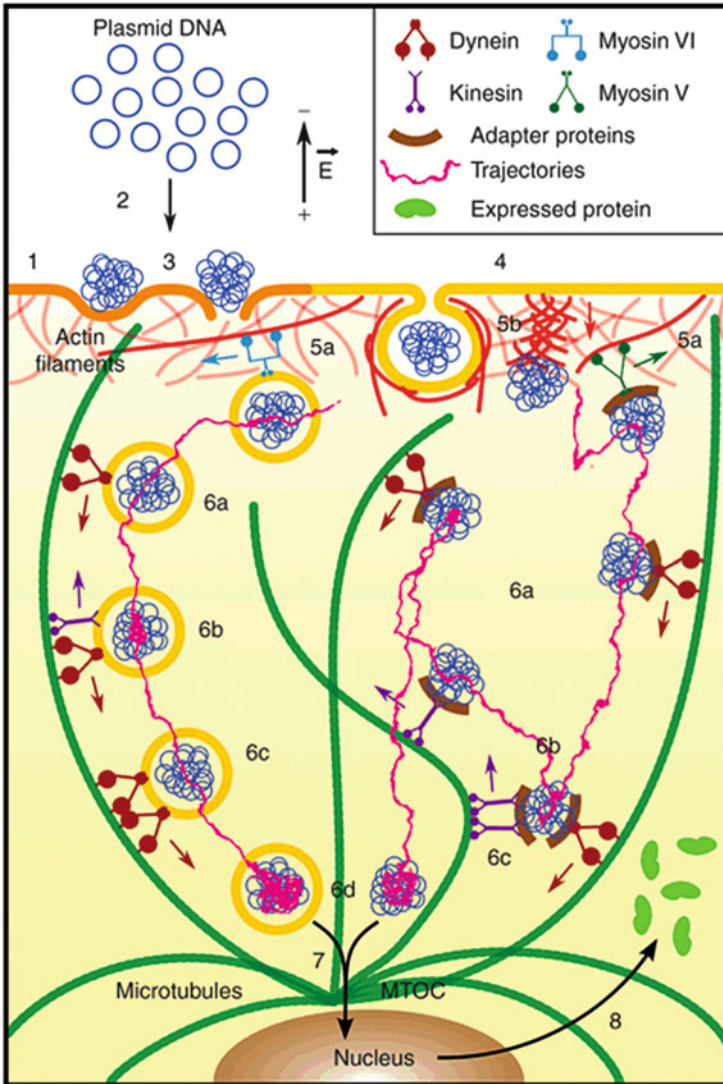
### **2.5.3.2 PEI Derivatives and Other Cationic Polymers as Alternatives to PEI**

Other cationic polymers such as poly(-L-)lysine (PLL), chitosan (Corsi et al. 2003) or linear  $\beta$ -cyclodextrin-containing polymers (Gonzalez et al. 1999) have been also used with some success and usually acceptable cytotoxic effects, but never received widespread application. Irrespective of the successful and meanwhile widespread use of PEI for transient gene expression there are continuing efforts to further improve these cationic polymers by reducing undesired cytotoxic and/or cytostatic effects and by increasing the transfection efficiency with better reproducibility. In addition patent issues regarding the use of PEI for commercial applications and a rapidly growing market for transfection reagents are further stimulating these endeavors. To name a few, this includes the use of PEI/chitosan complexes (Zhao et al. 2009), different PEI-cystamine derivatives (Wang et al. 2011), a family of biodegradable poly(amine-*co*-esters), particularly poly(*N*-methyldiethyleneamine sebacate) (PMSC) (Liu et al. 2011), functional block copolymers with PEG (Hu et al. 2013) or low molecular weight PEI600 cross-linked by  $\beta$ -cyclodextrin (Hu et al. 2012).

### **2.5.4 Transient Transfection by Electroporation**

Being a well-established and reliable technology at small scale (Wong and Neumann 1982), electroporation was initially impossible to perform at larger scale as it is required for recombinant protein production by transiently transfected mammalian cells. First successful attempts on a larger scale were performed by Blasey et al. (1996) with COS cells and by Parham et al. (1998) using several suitable cell lines including HEK293 ( $\pm$ stable EBNA1 expression) and CHO ( $\pm$ stable SV40 large T antigen expression). Different to the principle of DNA internalization by means of transfection reagents, electroporation is based on the process of local membrane destabilizations resulting in an association of membrane and DNA (Phez et al. 2005). Once the plasmid DNA is internalized, the electroporation process is





**Fig. 2.3** Mechanisms of DNA electrotransfer in mammalian cells. During the application of the electric field, (1) the plasma membrane is permeabilized (orange); (2) the DNA is electrophoretically pushed onto the membrane side facing the cathode; therefore, (3) DNA/membrane interactions occur. DNA aggregates are inserted into the membrane and remain there for 10 s to minutes. After the application of the electric field and resealing of the membrane (yellow), (4) the DNA can be internalized through endocytosis (DNA in vesicles) and/or through electropores (free DNA). For gene expression to occur, (5, 6) DNA has to cross the cytoplasm and move toward the nucleus. Rosazza et al. found (5) actin-related motion that can be (5a) transport using myosins (in both directions) and/or (5b) burst of actin polymerization (actin rocketing). Further, they observed (6) microtubule-related motion: (6a) by transport through kinesin and dynein, (6b) by DNA interaction with oppositely directed motors, or by using (6c) several motors of the same type. (6d) Once in the perinuclear region, (7) DNA has to cross the nuclear envelope, after endosomal escape in case of DNA in vesicles. (8) Finally, DNA is expressed in proteins found in the cytoplasm. MTOC microtubule-organizing center (Taken from Rosazza et al. (2013), rights permitted)



additionally important for cytosolic trafficking, since it causes disassembly of the actin cytoskeleton network (Rols and Teissié 1992), which would otherwise significantly slow down the motility of molecules of the size of plasmid DNA (Lechardeur et al. 1999; Dauty and Verkman 2005). Comparable to other transfection methods, trafficking into the cellular nucleus is predominantly associated with nuclear membrane breakdown during mitosis. Correspondingly, cell synchronization in the G2/M phase was shown to increase the efficiency of transfection using cell electroporation (Golzio et al. 2002). A complete scheme describing the mechanisms of plasmid DNA electrotransfer in mammalian cells was provided by Rosazza et al. (2013) (Fig. 2.3). Meanwhile, equipment for transient transfection of up to 200 billion cells has become more generally available. Suitable devices now facilitate routine electroporation with very good transfection rates and preservation of high cell viability (Johansson et al. 2013).

## 2.6 Cell Lines for Non-viral Transient Transfection

In viral expression systems, cells have to be susceptible to the infecting virus for subsequent gene expression. Similarly, the mammalian cell lines used in non-viral approaches have to be compatible to the plasmid DNA and the transfection technology. Initially, COS-cells (in particular COS-7) were frequently used for transient gene expression. However, their relatively low protein expression rates did always encourage the search for better alternatives such like HEK 293 cells (Berntzen et al. 2005). HEK 293 cell lines were found to be particularly well suited as they exhibit relatively low resistance against transient transfection. Nonetheless, tremendous efforts were made to use CHO cell lines as an alternative. Despite the fact that CHO cells proved to be considerably more difficult to transfect transiently, their widespread use in the production of biopharmaceuticals and the corresponding know-how in process technology as well as a posttranslational modification of recombinant proteins similar or identical to stably transfected CHO cells was deemed to justify these additional efforts. Table 2.4 summarizes some commonly used HEK293 and CHO cell lines as well as some other alternatives. Particularly CAP-T, a stably transfected human amniocyte cell line, is showing very good transfectability and high production titers. This versatile line was demonstrated to be an excellent alternative or complement to HEK293-EBNA cell lines (Fischer et al. 2012).

Unfortunately, the nomenclature for the most frequently used HEK 293 cells is considerably diverse. For instance, those cell lines which were initially established at Stanford University by stable transfection to constitutively express EBNA1 and which were subsequently both licensed to Invitrogen (originally named 293-EBNA) and deposited at ATCC as CRL-10852 (originally named 293 c18) are meanwhile described under a plethora of different names (e.g. HEK 293-EBNA, 293E, HEK293E, HEK/EBNA, HEK.EBNA, HE; Geisse 2009). It remains unclear whether these cell lines are originally derived from the same subclone or represent

**Table 2.4** Mammalian cell lines commonly used for transient expression of recombinant proteins

Name	Characteristic features	Suitable plasmid vectors	Culture conditions	Culture media	References
<i>HEK 293 cell lines</i> XDC293	Clonal derivative of HEK 293 cells w. improved transfectability		Not adapted to serum-free suspension culture	DMEM w. 5 % BCS	Reed et al. (2006)
HEK 293SF-3F6	Clonal derivative of HEK 293S cells adapted to both suspension and serum-free culture	E.g. lentiviral vectors	Serum-free suspension culture adapted	Custom made low calcium SFM or HyQSF4TransFx293	Côté et al. (1998), Swiech et al. (2011)
“HEK 293 EBNA1” (ATCC CRL-10852) or 293-EBNA from Invitrogen	Stably transfected cell line expressing EBNA1	pCEP4, pREP4, pcDNA series, pTT series	Adherent culture in serum-supplemented medium or serum-free suspension culture adapted	ExCell 293 293 SFM II or FreeStyle™ 293	Numerous applications; e.g.: Durocher et al. (2002), Geisse and Henke (2005)
HEK/EBNA-SF	EBNA1-expressing 293 c18 (ATCC CRL-10852) adapted to serum-free medium	Same as above	Serum-free adherent culture (microcarriers)	DMEM:Ham's F12-based serum-free medium or EpiSerf	Fliedl and Kaisermayer (2011)
HEK 293T/17 (ATCC CRL-11268)	Stably transfected expressing SV40 large T-antigen		Not adapted to serum-free suspension culture	DMEM w. 10 % FBS	Pear et al. (1993), Numerous applications, e.g.: Chang et al. (2007), Li et al. (2007)
FreeStyle™ 293-F	Derivative of non-transfected HEK 293 cells		Serum-free suspension culture adapted	FreeStyle™ 293 Expression Medium	Liu et al. (2008)
Expi293F™	Derivative of FreeStyle 293-F	E.g. pcDNA3.3	High density serum-free suspension culture adapted	Expi293™ Medium	Chiou et al. (2014), Vasu et al. (2013)

HEK 293SFE (HEK 293SFE41)	Stably transfected HEK 293SF-3F6 expressing EBNA1	pTT series	Serum-free suspension culture adapted	E.g. LC-SFMLB	Pham et al. (2003)
HEK 293-6E	Stably transfected expressing a truncated version of EBNA1	pTT series	Serum-free suspension culture adapted	FreeStyle™ F17	Durocher (2006), Loignon et al. (2008), Carter et al. (2010), Raymond et al. (2011)
293EBNAL T 75	Stably transfected expressing murine polyoma virus large T-antigen (PyLT) + EBNA1	pQCMF plasmids (Icosagen)	Serum-free suspension culture adapted	1:1 mixture of Pro293s-CDM and 293 SFMII	Silla et al. (2011)
HEK 293S GnT <sup>−</sup> (ATCC CRL-3022)	Lacks N-acetylglucosaminyltransferase I (GnTI) activity for less heterogeneous N-glycosylation	Various	Roller bottle cultures w. attached cells or serum-free suspension culture	DMEM w. 1.6–10 % FBS or FreeStyle™ F17 <sup>1</sup>	Reeves et al. (2002), Zhao et al. (2011), <sup>1</sup> Jäger, unpublished results
HKB-11	Somatic hybrid cell line of 293S and Burkitt's lymphoma line 2B8 expressing EBNA1	Tat/TAR-oriP related expression vectors	Serum-free suspension culture adapted	E.g. MICT7.0	Cho et al. (2001), (2002), (2003)
CHO cell lines					
CHO-K1-S	Non-transfected		Serum-free suspension culture adapted	CHO-S-SFM II	Rosser et al. (2005)
FreeStyle™ CHO-S		various (e.g. pCEP4, pcDNA series)	Serum-free suspension culture adapted	FreeStyle™ CHO Expression Medium	Liu et al. (2008)
CHO-T	Stably transfected expressing murine polyoma virus large T-antigen (PyLT)	Epi-CHO system, i.e. pPyEBV plasmids (Pyori, oriP, EBNA-1)	Serum-free suspension culture adapted	Ex-Cell 302 CHO-S-SFM II	Kunaparaju et al. (2005), Codamo et al. (2011)

(continued)

**Table 2.4** (continued)

Name	Characteristic features	Suitable plasmid vectors	Culture conditions	Culture media	References
CHOEBNALT 85	Stably transfected expressing murine polyoma virus large T-antigen (PyLT) + EBNA1	pQCMF plasmids (Icosagen)	Serum-free suspension culture adapted	1:1 mixture of CD CHO and 293 SFMII	Silla et al. (2007), Silla et al. (2011)
Potelligent® CHOK1SV	Glutamine synthetase CHO K-1 cells w. Fucosyltransferase 8 knocked out		Serum-free suspension culture adapted	UltraCHO	Ye et al. (2009)
CHO-3E7	Stably transfected expressing a fusion protein composed of HSV VP16 and a truncated version of EBNA1	pTT series	Serum-free suspension culture adapted	FreeStyle™ CHO Expression Medium	Durocher and Loignon (2009), Exmpl. application: Stoops et al. (2012)
<i>Other cell lines</i>					
CAP-T	Stably transfected expressing SV40 large T-antigen	Plasmids containing SV40ori	Serum-free suspension culture adapted	Protein Expression Medium (PEM)	Wölfel et al. (2011), Fischer et al. (2012)
VERO (ATCC CCL-81)	Non-transfected	E.g. pCEP4	Serum-free adherent culture (microcarriers)	DMEM:Ham's F12-based serum-free formulation	Fliedl and Kaisermayer (2011)

two different clones generated in parallel. In addition, long-term cultivation under various culture conditions (e.g. serum-free suspension culture vs. adherent culture in serum-supplemented medium or combinations thereof) and subsequent recloning might have resulted in substantial genetic and phenotypic diversity.

With the development of stably engineered production cell lines, recombinant protein productivity upon transient transfection was dramatically increased. This was accomplished by both better gene transcription in the nucleus and a more sustained productivity due to the episomal replication of plasmids in the proliferating cell population. However, there are also some reports recently which indicate that high level protein expression is also possible with non-engineered HEK 293 cells (e.g. Expi293F<sup>TM</sup>) when thoroughly following a production protocol based on a proprietary combination of cell line, transfection reagent, culture medium and non-disclosed productivity enhancers (Chiou et al. 2014).

Besides productivity issues, specific applications of the expressed recombinant protein are also influencing the selection of the most suitable cell line. Crystallization of proteins for structural biology research for example requires very high protein homogeneity for crystal formation. This requirement is in contrast to biopharmaceutical applications where the naturally occurring microheterogeneity of glycoproteins is usually acceptable as long as e.g. immunogenicity and pharmacokinetics are not affected. Particularly, when proteins are bearing several *N*-glycosylation sites microheterogeneity might reach an extent which has to be reduced by supplementing specific inhibitors of the *N*-glycan trimming pathway such like kifunensine or swainsonine to the transiently transfected cell culture (Chang et al. 2007). A different approach is followed by cleaving the *N*-glycans from the peptide backbone with specific glycosidases (i.e. PNGaseF or Endo F series) (Lee et al. 2009). Alternatively, the selection of a cell line with altered glycosylation properties such like HEK 293S GnTI<sup>-</sup> which is lacking *N*-acetylglucosaminyltransferase I and thus predominantly expresses glycoproteins with Man<sub>5</sub>GlcNAc<sub>2</sub> *N*-glycans (Reeves et al. 2002) might overcome this problem. Meanwhile, corresponding automated production processes have been established which implement these strategies (Lee et al. 2009; Zhao et al. 2011).

To overcome limitations of CHO cells with regard to their lower recombinant protein yields, a CHO-DG44 cell line was developed stably overexpressing the anti-apoptotic protein bcl-x<sub>L</sub>. PEI-mediated transfection of these cells resulted in a two to threefold increase of productivity and less apoptosis (Majors et al. 2008).

## 2.7 Cell Culture Media Conditions

The selection of suitable cell culture conditions for transient transfection processes is more challenging than for stable cell lines, because the specific requirements of the transfection step itself are also of major importance. Cell culture conditions have to be compatible to all phases of a transient transfection process:

- Complexation of plasmid DNA in a form suitable for later incorporation by target cells
- Transfection of target cells
- High titer production of recombinant proteins

Although the first step is still performed without cells, it has to be considered that the solution with the newly formed complex will be added to the cell culture afterwards and therefore, will influence also all subsequent steps. This is the reason that, instead of simple buffer solutions, cell culture media are frequently applied for this purpose since they are non-cytotoxic and contain also various nutrients and thus diminish the ‘diluting’ effect during transfection with plasmid DNA. As a promising alternative, transfection is also possible without a priori DNA complex formation directly in the cell suspension. This is described in more detail below.

The subsequent steps of transfection and protein production can be easily accomplished at small scale by applying first culture conditions optimized for cell transfection and separating these from a second phase optimized for recombinant protein production by inserting a simple media replacement step of either decantation (for adherently growing cells) or centrifugation (for suspension cells) (e.g. Baldi et al. 2005). However, with increasing scale a complete medium exchange is getting more and more difficult and is accompanied with an increasing expenditure of work and costs. Therefore, it is highly desirable for a reliable scaling-up of the technology that both transfection and recombinant protein production may be performed in the same culture medium thus avoiding a complete medium exchange. Serum usually does not interfere with the transfection process itself but substantially contributes to the burden of contaminating proteins in the supernatant, thus hampering protein purification and – at the same time – does not permit an animal component free process if it is required. Hence, the replacement of serum-supplemented cell culture media by suitable serum-free media is highly desirable. Both, calcium phosphate coprecipitation and calfection represent attractive methods with regard to the overall production costs and their scale-up potential was successfully demonstrated. However, for both methods serum-supplemented media (or the use of albumin as a serum replacement; Girard et al. 2001) are still an essential prerequisite. This significant drawback did finally redirect attention to other transfection technologies, as soon as inexpensive reagents such as PEI and appropriate protocols became available (Baldi et al. 2005).

Apart from serum as an undesirable cell culture supplement, there is a number of other media components which might also significantly interfere with the efficiency of several transient transfection protocols. It is obvious that ingredients such as calcium and phosphate might directly interact with the condensation and complexing of plasmid DNA. There is also evidence about other supplements which disturb transfection in a currently unknown manner. This comprises peptones which are reported to significantly increase recombinant protein productivity (Pham et al. 2003) with major peptone-specific differences in their performance (Pham et al. 2005). Unfortunately, it was also observed that they might interfere with the transfection process, thus suggesting a later supplementation to achieve

maximum transfection rates (i.e. 24–48 h.p.t.) (Pham et al. 2005). Supplementation of different peptones was also successfully applied by Kiseljak et al. (2012) with a remarkable increase in recombinant antibody production. These peptones were preferably added several days post transfection. Again, hydrolysates as well as several other common cell culture media ingredients, namely phosphate and dextran sulfate were reported to inhibit the transient transfection process (Geng et al. 2007). The negative effects on transfection efficiency were circumvented by using different, deficient media during transfection whereas detrimental effects on cell growth and viability were avoided by subsequent complementation with the complete medium.

Iron chelators also represent common ingredients of serum-free media and usually substitute transferrin (either as a natural component of serum or as a defined supplement in serum-free media) as a source of ferric ions. Iron (III) citrate, but neither ferric ions nor citrate, was identified to severely diminish transfection efficiency (Eberhardy et al. 2009). This might also occur as a result of spontaneous iron (III) citrate formation from other media components (i.e. other ferric salts and citrates).

Conditioned media in general were also shown to severely reduce transfection efficiency and recombinant protein titers when compared to transfections in fresh media (Schlaeger and Christensen 1999). This effect was also reported more recently for CHO DG44 cells (Pereira et al. 2011) and also confirmed – although to a much lesser extent – by our own unpublished results when using HEK 293-6E cells. In all these studies cationic polymers (i.e. PEI) were used as transfection reagent. By any means, the detrimental effects of these unidentified cell metabolites on transient gene expression efficiency represent a severe obstacle for the applicability of transient gene expression at a larger scale since a complete media replacement is difficult if not impossible to perform at a multi-liter scale.

On the contrary, Long R3-IGF, well known as a potent growth factor for many cell types, provides enhanced recombinant protein productivity by a factor of 2 and works synergistically with mild hypothermia for CHO cells (Galbraith et al. 2006). However, if Long R3-IGF is already a constituent of the non-disclosed basic formulation of commercially available serum-free media no further increase of productivity is expected upon supplementation.

The supplementation of compounds with no or low cytotoxicity and the potential to increase membrane permeability might also assist the incorporation of plasmid DNA. Both, lithium acetate (3 mM) and dimethyl sulfoxide (DMSO) (1.25 %) are reported to improve PEI-mediated transient transfection in Potelligent® CHOK1SV cells (Ye et al. 2009). Earlier, DMSO was already found to support transfection mediated by electroporation (Melkonyan et al. 1996).

The PEI-mediated transient gene expression in CHO-S cells was shown to be improved by supplementing prior to transfection microtubule disrupting anti-mitotic reagents, namely nocodazole which arrests cells in the G<sub>2</sub>/M phase and hydroxyurea arresting cells in the G<sub>1</sub> phase (Tait et al. 2004). This synchronization technique increases recombinant protein productivity when compared to

unsynchronized cells but requires substantial lab work as the reagents have to be removed afterwards.

### ***2.7.1 Use of Productivity Enhancers***

Gene expression is based on the normal regulation of differential acetylation of nucleosomal histones resulting in either transcriptional activation (hyperacetylation) or repression (hypoacetylation). Different histone deacetylase inhibitors (HDACi) were identified to efficiently increase gene expression because of their interfering effect on this regulation (Hebbar and Archer 2003). Since its first use for enhanced protein expression (Gorman and Howard 1983) sodium butyrate is been in use for a vast number of protein production processes. Parham et al. (1998) were supplementing 2 mM of sodium butyrate to several transiently transfected cell cultures and obtained significantly increased recombinant protein yields. A significant expression-enhancing effect of sodium butyrate was also observed in BacMam transduced cells (Condreay et al. 1999). Whereas most HDACi such as trichostatin A reveal high cytotoxic secondary effects, an inexpensive alternative with a relatively low cytotoxicity was identified more recently in valproic acid or its water soluble sodium salt (Fan et al. 2005). Initially tested for viral gene transfer approaches, valproic acid was subsequently successfully tested for HEK 293 EBNA1 as well as CHO DG44 (Backliwal et al. 2008b) and has meanwhile become a standard supplement in many transient gene expression processes. Less publicized and well-known is the potential of other carboxylic acids to act as productivity enhancers. This includes pentanoic acid (Liu et al. 2001) and sodium propionate (Chun et al. 2003) which are also reported to be less cytotoxic or apoptosis-inducing.

## **2.8 Process Strategies for Improved Recombinant Protein Production**

Numerous transfection and cultivation parameters have been varied to improve the yield of the corresponding recombinant protein production. In the following paragraphs some of the most important steps are summarized.

### ***2.8.1 Mild Hypothermia***

Mild hypothermic conditions represent a well known process variable to increase recombinant protein productivity of stably transfected cell lines (e.g. Weidemann



et al. 1994; Kaufmann et al. 1999). This is usually accomplished in a separate phase initiated by a temperature shift followed by decelerated cell proliferation and an increased protein production in a prolonged cultivation process. Thus, it was obvious to investigate mild hypothermia also in transient gene expression processes. Using the Semliki Forest Virus expression system, some mammalian cell lines (i.e. BHK-21 C13 and CHO/dhfr<sup>-</sup>) showed much better productivity at 33 °C than at the standard temperature of 37 °C (Schlaeger and Lundstrom 1998). In the same study, HEK 293 and 293-EBNA cells were found to be less susceptible to hypothermia. These cells did not grow well at 33 °C and exhibited just slight (HEK 293) or no (293-EBNA) signs of an increased protein expression at the lowered temperature. The good response of different CHO cell lines to temperature reduction was confirmed also in a number of approaches with non-viral transient gene expression. A reduction to 32 °C increased product titers of PEI-transfected CHOK1SV cultures by a factor of 3 with an additional, synergistic effect of supplemented Long R3-IGF (Galbraith et al. 2006). Likewise, antibody expression was also increased several fold by lowering temperature to 31 °C in PEI-transfected CHO DG44 cultures (Wulhfard et al. 2008). The temperature shift was performed just 4 h post transfection, but the uptake of plasmid polyplexes was considered to be already completed. Compared to cells growing at 37 °C more cells were accumulated in the G1 phase of the cell cycle. The productivity enhancing effect was not observed when lowering the temperature 3 days post transfection or later. More recently, this process was further adjusted and optimized by performing also the PEI-mediated transfection itself at a lowered temperature of 31 °C and lessen cytotoxic effects by reducing the amount of polyplexes for transfection (Rajendra et al. 2011).

By contrast, HEK 293 cell lines are reported to be much more sensible to the stress attributed to hypothermic culture conditions (Schlaeger and Lundstrom 1998). Correspondingly, there are few reports on beneficial effects of hypothermic culture conditions using these cell lines. Productivity (and product quality) of recombinant human FVIII expressed in transiently transfected HEK293SF-3F6 cells was improved when cells were transfected repeatedly at lower DNA doses and temperature was simultaneously decreased to 34 °C (Swiech et al. 2011). Our own, unpublished results revealed that, when performing transient gene expression with HEK 293-6E cells, temperature shifts to either 32 °C or 34.5 °C did not provide higher protein expression levels but increased the cytotoxic effects of the transfection process.

### ***2.8.2 High-Density Transfection and ‘Direct’ Transfection***

Transfection protocols with a separate step of polyplex formation a priori are increasingly replaced by the alternative principle of generating PEI:DNA polyplexes directly in the cell culture. This strategy is providing advantages by reducing the variations associated with the polyplex formation step and simplifies

attempts to establish automated processes. This so-called *direct* transfection was accomplished with both HEK 293EBNA (Backliwal et al. 2008a) and CHO DG44 cells (Rajendra et al. 2011) and usually comes along with a transfection at high cell density. However, to achieve this high density prior to transfection these protocols stipulate a centrifugation step which complicates scaling-up. To circumvent this problem, a direct PEI-mediated transfection without centrifugation was established in combination with HEK 293-6E cells (Raymond et al. 2011). In a different approach, high cell densities of HEK293 EBNA cells were obtained using a perfusion bioreactor system (Sun et al. 2008). Several hours after transfection, which was performed using the standard method of separated a priori polyplex formation, surplus polyplexes were rapidly removed by adjusting a high perfusion rate thus lowering possible cytotoxic effects. Subsequently, cells were cultivated either in a fed-batch mode or by perfusion with an enriched medium resulting in significantly improved recombinant protein productivity when compared to previous results (Sun et al 2006).

### 2.8.3 Co-transfection Strategies

Co-transfection with more than one species of plasmid DNA has become a common strategy in transient gene expression approaches. First of all, this is necessary when the gene of interest is composed of more than one peptide chain and multi-cistronic vector plasmids (e.g. Li et al. 2007) are not readily available or applicable. Most commonly, suitable reporter genes for rapid and easy monitoring of the success of the transfection process such like eGFP are applied (e.g. Pick et al. 2002; Galbraith et al. 2006). Apart from these process monitoring aspects the process performance itself can be influenced by transient introduction of additional genes. The co-expression of the growth factor FGF-1 (acidic fibroblast growth factor) already increased recombinant protein titers and cell-specific productivity substantially in both HEK 293 EBNA and CHO DG44 cell cultures (Backliwal et al. 2008c). FGF-1-encoding plasmids were also used in a multi-pathway modulation in combination with additional plasmids for expression of the cell cycle regulatory proteins p18 (cyclin-dependent kinase inhibitor 2C) and p21 (cyclin-dependent kinase inhibitor 1A, WAF1), for suppression of cellular growth by arresting them in the G<sub>1</sub> phase. Both, the overall antibody yield as well as the cell-specific productivity of HEK 293 EBNA cells were increased several fold, reaching accumulated recombinant antibody titers in excess of 1 g L<sup>-1</sup> (Backliwal et al. 2008d). Related to this strategy, Galectin-3, a lectin upregulating p21<sup>WAF1</sup>, is prolonging the viability of cultured HEK 293 EBNA cells at a lowered biomass accumulation and an increased expression of co-transfected GOIs (Delegrange et al. 2012). Transiently transfected CHO DG44 cells behaved differently and just showed a better productivity but no extended survival.

Co-transfection with FGF-2 might also reveal expression-enhancing activity as it was shown for CHO-3E7 cells but not for HEK 293-6E (Durocher and Loignon

2009). The co-expression of an AKT mutant in combination with the supplementation of valproic acid decreases the undesired apoptosis-inducing effects of valproic acid and thus further increases recombinant protein production.

As already described, scaling-up of transient protein production requires also large-scale production and purification of plasmid DNA. If this is not possible to the desired scale, inexpensive “stuffer” or “filler” DNA might replace a certain amount of plasmid DNA without altering the transfection efficiency in PEI-mediated transfections. This allows for maintaining a minimum concentration of transfection reagent (e.g. PEI) required for successful transfection without increasing the amount of precious plasmid DNA accordingly. DNA used for this purpose is either from salmon sperm as successfully tested with HepG2, HEK 293, and A549 cells (Kichler et al. 2005) or from herring sperm as tested more recently with CHO DG44 and HEK 293 EBNA cells (Kiseljak et al. 2011; Rajendra et al. 2012). Recombinant protein production is reduced when adding this non-specific DNA but not to the same extent as the reduction of plasmid DNA would suggest. The addition of this noncoding DNA provides substantially better protein expression levels compared to a lessened amount of encoding plasmid DNA alone.

#### ***2.8.4 Large-Scale and Automated High-Throughput Applications***

Scaling-up of transient transfection processes was usually started almost immediately after the corresponding cell transfection principle was developed. Initially, this was an essential part of various transient protein production projects because of the very low product titers expected. Because of the much better productivity of current transient transfection systems there was no need to further scale-up the production beyond the 100-L scale. Not surprisingly, the type of bioreactors used for transient gene expression purpose are not different to bioreactors used for other mammalian cell cultivation processes with predominantly stirred tank bioreactors operated in batch, fed-batch or perfusion mode or mixtures thereof (Table 2.5). WAVE bioreactors were also used frequently because of their ease of handling and the general tendency to implement single-use bioreactor technology, in particular when setting up new production facilities.

As mentioned earlier however, scale-up of transient transfection processes is associated with a number of individual steps which are more difficult to perform at a larger scale. Besides the preparation of bulk quantities of plasmid DNA, a media replacement prior to transfection to increase transfection efficiency and recombinant protein yield requires additional compatible equipment. Centrifugation of cells at this scale is possible but labor intensive and always associated with a certain risk of contamination. As an alternative, continuous centrifugation was introduced as an intermediate step between a 20-L preculture bioreactor and the 50-L or 100-L production bioreactors (Tuvešson et al. 2008). The use of perfusion technology

**Table 2.5** Examples of large-scale transient transfections

Cell line(s)	Transient transfection system	Bioreactor system	Reference
BHK-21	Semliki Forest Virus expression system	MBR Vibromix™ bioreactor w. 11.5 L working volume	Blasey et al. (2000)
HEK 293/T17	Calcium phosphate co-precipitation	2-L stirred tank bioreactor w. 1.2 L working volume	Jordan et al. (1998)
HEK 293SF-3F6	Ad5-mediated viral transfection system	3-L (helical ribbon) stirred tank bioreactor	Côté et al. (1998)
HEK 293 HEK 293 EBNA	PEI-mediated	12- and 23-L stirred tank bioreactors	Schlaeger and Christensen (1999)
HEK 203 EBNA	Calcium phosphate co-precipitation	3-L stirred tank bioreactor w. 1 L working volume	Meissner et al. (2001)
HEK 293 EBNA SF	Calcium phosphate co-precipitation	150-L stirred tank w. 67 or 110 L working volume	Girard et al., 2002
HEK 293 EBNA	PEI-mediated	3.5-L (helical ribbon) and 14-L stirred tank bioreactors	Durocher et al. (2002)
HEK 293SFE	PEI-mediated	1-, 10- and 14-L stirred tank (pitched-blade) bioreactors	Pham et al. (2003)
CHO DG44	PEI-mediated	3- and 20-L stirred tank bioreactor w. 1.2 or 13 L working volume	Derouazi et al. (2004)
HEK 293 EBNA	PEI-mediated	50-L WAVE bioreactor w. 10 L working volume	Geisse and Henke (2005)
CHO-S	PEI-mediated	50-L WAVE bioreactor w. 10 L working volume	Haldankar et al. (2006)
CHOK1SV	PEI-mediated	20-L WAVE bioreactor w. 8 L working volume	Ye et al. (2009)
HEK 293-6E	PEI-mediated	10-L WAVE bioreactor w. 5 L working volume	Raymond et al. (2011)
HEK 293 EBNA HEK 293-T	PEI-mediated	WAVE bioreactor w. 10 L working volume or stirred tank bioreactor w. 50 or 100 L working volume	Turesson et al. (2008)
CHO DG44	PEI-mediated	50-L orbital shake bioreactor w. 30 L working volume	Stettler et al. (2007)
CHO DG44	PEI-mediated	50-L orbital shake bioreactor w. 30 L working volume	Wulhfard et al. (2008)
HEK 293 EBNA	PEI-mediated	2-L stirred tank bioreactor w. spin filter perfusion system	Sun et al. (2008)
HEK 293-6E	PEI-mediated	Stirred tank bioreactor w. 10 L working volume	Carter et al. (2010)
HEK/EBNA-SF	PEI-mediated	Stirred tank bioreactor w. 1.5 L working volume; Microcarrier culture	Fliedl and Kaisermayer (2011)

represents another approach to remove undesired media ingredients and establish suitable conditions for a high performance transient transfection process (Sun et al. 2008). The perfusion mode was also shown to be usable in combination with a fed-batch strategy.

The development of transient gene expression technologies with high productivity and corresponding recombinant protein yields has enabled the establishment of (semi)-automated high-throughput production facilities. With these facilities it is now possible to manufacture large numbers of smaller quantities of different proteins simultaneously for individual applications (e.g. for high-throughput screening or structural biology research). The principles of the underlying technology and the workflow of these facilities have been described (Davies et al. 2005; Lee et al. 2009; Zhao et al. 2011).

Irrespective of the targeted scale, fine tuning of transient gene expression technology with its multiple parameters affecting each other is becoming more and more important. Design of Experiments (DoE) is a common approach to address this problem and has meanwhile been successfully applied also in this context. In one study, DoE was used to optimize transient transfection of CHO-S and HEK293 EBNA cells with PEI (Bollin et al. 2011). The passage number of transfected CHO-S cells was shown to have an impact on recombinant protein production. Similar, modified PEI:DNA ratios and concentrations were influencing the maximum recombinant protein productivity. A DoE approach was also followed to further increase media performance and the corresponding cell density of HEK 293SF-3F6 resulting in a 2.4 fold increase of the protein productivity (Cervera et al. 2013).

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