Transient gene expression in mammalian cells grown in serum-free suspension culture

Ernst-Jürgen Schlaeger & Klaus Christensen

F. Hoffmann La Roche Ltd., Research Laboratories, CH-4070 Basel, Switzerland

Received 4 May 1998; accepted 22 August 1998

Key words: gene expression, HEK293(EBNA) cells, serum-free, transient transfection

Abstract

In order to establish a simple and scaleable transfection system we have used the cationic polymer polyethylenimine (PEI) to study transient transfection in HEK293 and 293(EBNA) cells grown in serum-free suspension culture. The transfection complexes were made directly within the cell culture by consecutively adding plasmid and PEI (direct method). Alternatively, the DNA-PEI transfection complexes were prepared in fresh medium (1/10 culture volume) and then added to the cells (indirect method). The results of this study clearly show that the ratio of PEI nitrogen to DNA phosphate is very important for high expression levels. The precise ratio is dependent on the DNA concentration. For example, using 1 $\mu\text{g/ml}$ DNA by the indirect method, the ratio of optimal PEI:DNA was about 10–13:1. However, the ratio increases to 33:1 for 0.1–0.2 $\mu\text{g/ml}$ DNA. By testing several different molecular weights of the polycationic polymer we could show that the highest transfection efficiency was obtained with the PEI 25 kDa. Using PEI 25 kDa the indirect method is superior to the direct addition because significantly lower DNA concentrations are needed. The expression levels of the soluble human TNF receptor p55 are even higher at low DNA compared to 1 $\mu\text{g/ml}$ plasmid. The EBV-based pREP vectors gave better transient gene expression when used in 293(EBNA) cells compared to HEK293 cells in suspension culture. No differences in expression levels in the two cell lines were observed when the pC1 (CMV)-TNFR was used. In conclusion, PEI is a low-toxic transfection agent which provides high levels of transient gene expression in 293(EBNA) cells grown in serum-free suspension culture. This system allows highly reproducible, cost-effective production of milligram amounts of recombinant proteins in 2–5 l spinner culture scale within 3–5 days. Fermentor scale experiments, however, are less efficient because the PEI-mediated transient transfection is inhibited by conditioned medium.

Introduction

Efficient gene delivery in gene therapy by non-viral vectors has recently become of considerable interest recently (Ledley, 1995). Substantial work has been done to improve DNA uptake, in which the plasmid is typically complexed with cationic lipids or polycationic polymers or sometimes conjugated with a peptide in order to target a specific cell type (Barthel et al., 1993; Remy et al., 1994; Gao and Huang, 1995; Boussif et al., 1995; Wagner et al., 1992; Legendre et al., 1996; Gottschalk et al., 1996; Mechler and Wagner, 1997; Kircheis et al., 1997). The electrostatic interactions between DNA and the cationic reagent lead to a condensed structure with positive charges

which are important for binding to the cells and protecting the DNA within the cells (Yang and Huang, 1997).

In addition to gene therapy protocols, improved non-viral gene delivery techniques are also of significant interest in transient gene expression in cell culture, especially in suspension culture (Legendre and Szoka, 1993; Jordan et al., 1996). The advantages of such a transient transfection system are numerous. 1) It would speed up the process of expressing milligram amounts of recombinant proteins. 2) It could be used to transfect different cell lines simultaneously to obtain sufficient amounts of 'test' protein in order to decide which cells are most suitable with respect to function and quality. 3) It could be used to produce

Figure 1. Transient gene expression in 293(EBNA) cells. Cells in serum-free HL medium from a spinner flask were centrifuged, washed, resuspended in fresh HL medium and 1 ml cells transferred into 12 well plates. The transfection complexes were formed immediately by adding plasmid and PEI 800 kDa into the suspension culture (direct method) and incubated for 3 days. A: 1 μ g plasmid (pREP7-TNFRp55) was added to 1 ml culture adjusted to 3×10^5 cells and after 1 min different amounts of PEI 800 kDa stock solution were added. B: Increasing amounts of DNA were added to 1 ml culture (3×10^5 c/ml) followed by PEI at a ratio of N:P of 26:1. C: 1 μ g plasmid was added to 1 ml culture with different cell concentrations followed by PEI at a ratio of N:P of 26:1.

recombinant proteins which are poorly expressed in stable cell lines. 4) It could be used for cell-based, high-throughput screening assays.

In order to establish basic data on transient gene expression in mammalian cells grown in suspension culture we decided to start with HEK293 cells (Graham et al., 1977) and with the subclone 293(EBNA). These human cells are becoming increasingly important for production of viral vectors for gene therapy and also for expression of recombinant proteins, e.g. using extrachromosomal EBV-based vectors (Shen et al., 1995). The 293(EBNA) expression system exhibits many advantages compared to CHO or BHK systems. For example, it is of human origin, it exhibits high transfection efficiency, there are autonomously replicating vectors available, it is useful for expressing human receptor proteins and, finally, the cells are relatively easy to grow in serum-free suspension culture (Graham, 1987; Garnier et al., 1994).

Recently, Boussif et al. (1995, 1996) described polyethylenimine (PEI 800 kDa), a highly branched organic macromolecule with a high cationic-charged-density potential, as a very efficient reagent for DNA uptake in various cell lines. It is assumed that PEI behaves as a cationic sponge with a high DNA binding and condensing activity and has a high pH-buffering capacity (Boussif et al., 1995). This latter property is very important for protection of DNA from in-

tracellular degradation and for the release of DNA from endosomal and lysosomal vesicles. Kircheis et al. (1997) used PEI successfully for coupling with a cell-binding ligand for targeting gene delivery. Furthermore, to deliver large DNA molecules into cells, Baker et al. formed PEI-DNA complexes which bind to adenovirus particles by electrostatic interaction. The resulting transfection complexes were delivered to the cells by the adenovirus infection route with a high efficiency (Baker et al., 1997).

PEI exhibits several properties which are extremely important for highly efficient gene delivery (Boussif et al., 1995). For example, PEI-mediated transfection complexes are highly efficient in a broad range of different cell lines and the poly cationic agent shows a low level of cell toxicity. Finally, PEI is cost-effective, an important consideration for scale up experiments.

We were interested in whether the positive gene delivery properties of PEI observed in adherent cells could be applied to serum-free suspension culture. The main goal of our work was to establish an inexpensive, reproducible and scaleable transient gene expression system with mammalian cells.

Figure 2. Transient gene expression in spinner culture. A: Kinetics of gene expression of 293(EBNA) cells in spinner flasks (25ml). Cells were transferred into 20 ml fresh HL medium, adjusted to a cell concentration of 5×10^5 cells/ml and transfected with pREP7-TNFRp55 ($1 \mu\text{g/ml}$) complexed with PEI 800 kDa ($3.6 \mu\text{g/ml}$) giving a ratio of N:P of 26:1 (direct method). 4 days post-transfection the culture was diluted 1:10 with fresh medium and incubated further for 4 days. B: Scale up of culture volumes in transient transfection in spinner flasks. Different culture volumes of EBNA cells in fresh medium (5×10^5 cells/ml) were transferred into spinner flasks of different sizes and transfected with $1 \mu\text{g/ml}$ DNA and $3.6 \mu\text{g/ml}$ PEI 800 kDa using the indirect method and incubated for 3 days.

Materials and Methods

Chemicals and expression vectors

Polyethylenimine (PEI) 800 kDa was obtained as a 50% wt/vol solution from Fluka (Buchs, Switzerland). PEI 60 kDa was from Sigma Chemie (Buchs, Switzerland), PEI 25 and 2 kDa were from Aldrich (Buchs, Switzerland) and PEI 22 kDa (ExGen 500) was kindly given by Euromedex (Souffelweyersheim, France). A 10 mM monomer aqueous stock solution was prepared by mixing 9 mg PEI (50% wt/vol in case of 800, 60, 2 kDa and 100% of 25 kDa) with 10 ml water, neutralized with HCl and sterile filtered (Boussif et al., 1995). The stock solutions were stored at room temperature for two months.

The extracellular domain of the human TNF receptor p55 (Loetscher et al., 1990) was used as a reporter gene throughout this study. The TNFRp55 gene (600 bp) was inserted into the EBV-based pREP expression vectors by standard methods (Invitrogen, Leek, The Netherlands). The vector pREP4 contains the entire EBNA-1 gene whereas pREP7 has a deletion in the EBNA-1 gene. Therefore pREP4 can replicate

extrachromosomally in many cells whereas pREP7 depends on the constitutive expression of EBNA-1 in the host cells. Both plasmids exhibit a very low frequency of chromosomal integration. The TNFRp55 gene was also cloned into the pCI vector and is driven by the strong CMV enhancer/promotor (Promega, Zürich, Switzerland). In addition to the soluble receptor, we also used the firefly luciferase and the green fluorescent protein (GFP) from the jellyfish, which have both emerged as important reporter proteins (Brasier et al., 1989; Chalfie, 1994). The pGL3(CMV) plasmid encoding the luciferase was constructed as described recently (Legendre et al., 1996) and the insertion of the GFP gene into the pC1(CMV) vector was cloned according to standard methods.

Plasmid preparations were done using commercially available miniprep kits (BIO-Rad Laboratories, CA, U.S.A., Quiagen, Hilden Germany). Maxi preparations were made according to a conventional method using alkaline SDS-lysozyme extraction followed by LiCl precipitation and the remaining supernatant was precipitated with isopropanol (Birnbom, 1983). Finally, the dissolved DNA was subjected to RNase

Figure 3. Determination of the optimal N:P ratio using the direct and indirect method. 293(EBNA) cells grown in spinner culture were transferred into 15 ml fresh medium, adjusted to 5×10^5 cells/ml (25 ml spinner flask) and transfected with DNA-PEI complexes which were formed using the direct or indirect method. The incubation time was 3 days. A: pREP7-TNFRp55 ($1 \mu\text{g/ml}$) was complexed with increasing amounts of PEI 800 kDa. B: DNA dose response curve using different amounts of plasmid at a N:P ratio of 13:1 for the indirect and 26:1 for the direct method.

treatment. 1.5 l overnight culture of *E. coli* DH5 α in terrific broth gave 14–20 mg plasmid, sufficient for about 100 l of transfection volume. The plasmid concentrations were determined spectrophotometrically and estimated by (1%) agarose gel electrophoresis ‘calibrated’ with pUC18 DNA (Pharmacia Biotech, Zürich, Switzerland).

Cell culture

The human embryonic kidney cells HEK293 (ATCC CRL 1573 and the subclone 293(EBNA) (Invitrogen) were adapted to serum-free growth in a fortified HL medium (Schumpp and Schlaeger, 1990). The low calcium HL medium is a mixture of fortified DHI (calcium-free) and RPMI 1640 (calcium-free) medium (2:1 by weight) as described recently (Schlaeger, 1996) and contained glutamine, 5 mM, glucose, 3 g/l, fructose and maltose 1 g/l of each, insulin, 5 mg/l (Sigma Chemie), human transferrin, 15 mg/l (Boehringer Mannheim, Rotkreuz Switzerland), Primatone RL, 0.3% (v/v from stock solution 10%, Cheffield Products/Quest International, The

Netherlands), Pluronic F-68, 0.01% (from stock solution 10%), and heparin, 30 $\mu\text{g/ml}$ (Sigma Chemie). Primatone RL contains low amounts of calcium (28 mg/l in a 10% stock solution). The powder media, calcium-free DHI and calcium-free RPMI 1640, were purchased from Life Technologies (Basel, Switzerland). The feed solution was prepared as described recently (Schlaeger, 1996).

The cells were routinely cultured in spinner flasks with 95–105 rpm using 70% of the recommended working volume (Bellco, Inotech AG, Dottikon, Switzerland).

Transfection procedure

HEK293 and 293(EBNA) cells were routinely grown in the presence of heparin to avoid cell aggregation. For transfection experiments cells were cultured to a density of $6\text{--}10 \times 10^5$ cells/ml, centrifuged for 3 min at 600 rpm (Hermle Z364, Germany), washed once with heparin-free HL medium and resuspended in heparin-free HL medium. The cell concentration was adjusted to 5×10^5 cells/ml and the culture was in-

Figure 4. Transient gene expression using low DNA concentration. 293(EBNA) cells were transferred into fresh medium (5×10^5 cells/ml, 25 ml spinner flask) and transfected with DNA-PEI 800 kDa complexes formed with 1 μ g/ml (open bars) and 0.2 μ g/ml DNA (hatched bars) respectively and increasing amounts of PEI 25 kDa using the indirect method and incubated for 3 days.

cubated for at least 3 h before transfection took place. After addition of the transfection complexes, the cells were incubated for 1–5 days without medium replacement. Two days post-transfection, the cultures were generally fed once a day with a nutrient mixture and in some experiments heparin was added 4–6 h after DNA addition in order to monitor the cell density during the expression time.

Preparation of transfection complexes

Two standard methods of forming DNA-PEI complexes have been developed. In the direct method the DNA (1 μ g corresponds to 3 nmol phosphate) and PEI (1 ml stock solution with 0.45 mg/ml corresponds to 10 mmol amino nitrogen) were added directly to the spinner culture. In the indirect method the complexes were formed separately in 1/10 volume of fresh medium.

The direct method was as follows. 1 μ g DNA was added to 1 ml cells and after gentle shaking, 7.8 μ l per milliliter PEI 800 kDa (stock solution 0.45 mg/ml, corresponds to the ratio of N:P of 26:1) were added two minutes later to the culture. The culture was then immediately placed on the magnetic stirrer and incubated further.

Using the indirect method, 1 μ g DNA was added to 0.1 ml fresh medium (1/10 culture volume) and mixed gently. After two minutes, 3.8 μ l PEI 800 kDa (0.45 mg/ml, corresponds to the ratio of N:P of 13:1) were added and mixed. After 10–12 min incubation at room temperature the 0.1 ml transfection complexes was transferred to 1 ml prepared cells, gently shaken and incubated further.

Under optimized gene delivery conditions using the indirect method (0.1) 0.2 μ g DNA was added to 0.1 ml fresh medium and after two minutes 1 μ l PEI

Figure 5. Transient gene expression using different molecular weights of PEI. 15 ml 293(EBNA) cells adjusted to 5×10^5 cells/ml in fresh medium (25 ml spinner flasks) were transfected with transfection complexes using low DNA concentration ($0.2 \mu\text{g/ml}$) and PEI of various molecular weights from stock solutions at a ratio of N:P of 33:1 (indirect method). The culture was incubated for 3 days.

25 kDa (stock solution 0.9 mg/ml, corresponds to the ratio of N:P of 33:1) was added and mixed. After 10–12 min at room temperature the DNA-PEI complexes were transferred to 1 ml cells and culture at 37°C .

Product analysis

The soluble human TNFRp55 in the culture medium was analyzed by ELISA as described earlier (Godfried et al., 1994). The luciferase activity was measured generally 24 h post transfection as follows. At each time point, 2×1 ml samples were taken and centrifuged for 5 min at 2000 rpm at 4°C , the supernatants decanted and the remaining liquid carefully removed. The cell pellets were resuspended mechanically and 1 ml ice cold lysis buffer (Boehringer Mannheim) was added. After mixing the samples were left on ice for 13 min and centrifuged at 12 000 rpm for 10 min to remove cell debris. The supernatants were frozen at -20°C until measurement. The samples were thawed, diluted in lysis buffer, and analyzed using the Lumat LB9501 (Berthold AG, Regensburg, Switzerland) as described (Legendre et al., 1996).

The protein content of the soluble cell extracts was analyzed using the BCA protein assay reagent (Pierce,

Rockford, IL, U.S.A.). Transfected cells expressing GFP were identified using fluorescence microscopy.

Results

The adherent HEK293 and 293(EBNA) cells were adapted to serum-free growth in suspension culture using HL medium. The strong tendency of these cells to form large viable cell aggregates in suspension, especially in the absence of serum, could be significantly reduced either by using calcium-free basic HL medium and particularly by adding heparin (30 u/ml) to the culture medium. Finally, the cell counts could be easily measured and high cell densities of $3\text{--}5 \times 10^6$ cells/ml were achieved if the spinner cultures were fed daily with a mixture of sugar, amino acids and vitamins (data not shown). In large scale cultures the addition of heparin was omitted. The observed growth rate and maximal cell density of 293(EBNA) cells were even better compared to HEK293 cells, probably as a result of the constitutively expressed EBNA-1 gene.

In order to quickly generate a large set of data on transient gene expression, experiments were performed in 12 well plates by transferring 1 ml cell suspension into each well. The transfection complexes were then formed by adding first the plasmid, encoding the soluble human TNF receptor p55 gene, followed by the cationic polymer PEI directly to the culture (direct method). No medium exchange was performed after transfection. First the effect of the PEI:DNA ratio on the expression levels of the receptor was measured. The optimal ratio of PEI nitrogen to DNA phosphate (expressed as PEI/DNA equivalents), which is approximately 26:1 (Figure 1A), differed from the most efficient transfection conditions of around 10–13.5:1 for adherent growing cells described by Boussif et al. (1995), probably due to the different methods of forming transfection complexes. Using the N:P ratio of 26:1 different DNA concentrations and cell concentrations were tested (Figure 1B, C). Figure 1B shows a requirement for at least $1 \mu\text{g/well}$ DNA to sustain reasonable expression levels while the cell densities display a broad maximum (Figure 1C). For subsequent experiments 2×10^5 cells/well were used. However, it should be pointed out that the overall expression in the 12 well plate experiments was very low.

Next we studied transient gene expression in spinner flasks using $1 \mu\text{g/ml}$ plasmid and the PEI:DNA ra-

tio of 26:1. We found that a starting cell concentration of 5×10^5 cells/ml exhibited the highest expression levels (data not shown). For subsequent experiments, 5×10^5 cells/ml were taken. Figure 2A shows the time course of TNFRp55 accumulation. 4 days post-transfection the culture was diluted 1:10 with fresh medium and cultured further. An expression level of 550 ng/ml receptor protein was achieved within 4 days. This is a significantly higher yield compared to the non-agitated culture in 12 well plates. Dilution of the transfected culture followed by incubation for another 3 days led to about a 5-fold decrease of the product titer due to a growth advantage for the non-transfected cells. Therefore, although the EBV-based REP plasmid has the ability to replicate autonomously in the host cells, the whole system remains transient without selection pressure. Demonstration of the scalability of the spinner culture system is shown in Figure 2B. No differences in expression levels were obtained when cell culture volume was 0.2–2 l in corresponding spinner flasks.

We next compared the direct method of forming transfection complexes with the indirect method by studying the different PEI:DNA ratios in detail. The results are shown in Figure 3A. The expression levels were similar for both methods, but the optimal PEI:DNA ratios were quite different. Using the direct method, the spinner culture displayed the highest expression at 20–26 (PEI) nitrogen per (DNA) phosphate, which confirmed our initial finding in 12 well plates. Low ratios of N:P (10–13:1) were optimal for the indirect method. At these ratios, both methods of forming transfection complexes revealed nearly identical DNA dose response curves (Figure 3B). Again, DNA concentrations of at least 1 $\mu\text{g/ml}$ DNA had to be used in spinner cultures to achieve reasonable expression levels.

From PEI-mediated transient gene expression using 1 $\mu\text{g/ml}$ vector DNA encoding the GFP reporter gene we know that about 40–60% of the cells were transfected in 293(EBNA) suspension culture. This means that about 10^5 plasmid molecules are necessary for a single expressing cell. In order to reduce this enormous amount of DNA in transient gene expression, we reinvestigated the ratio of PEI:DNA at lower DNA concentrations using the indirect method of complex formation. To do this, we used a different PEI preparation at lower DNA concentration which had an average molecular weight of 25 kDa. From earlier studies with 1 $\mu\text{g/ml}$ DNA, we know that PEI 25 kDa displays a slight increase in expres-

sion level compared to the PEI 800 kDa. Figure 4 shows the unexpected results. The control DNA concentration of 1 $\mu\text{g/ml}$ again shows the PEI:DNA ratio of 13:1 (PEI 800 kDa) whereas at 0.2 $\mu\text{g/ml}$ DNA a significantly higher ratio of 33:1 is found if PEI 25 kDa was used. More remarkably, the expression levels were not reduced despite the different amount of plasmid added. The experiment was repeated several times with similar results. Additional experiments with low DNA concentration using the direct method did not show measureable transient gene expression (data not shown). This finding clearly demonstrates that the indirect method is superior to the direct one at low DNA concentration if PEI 25 kDa was used and that it is possible to reduce the amount of plasmid by a factor of 5 (–10) without a decrease in the expression level. This has the additional advantage that there is an overall reduction of the cationic polymer concentration in the transfection experiment. Therefore, subsequent experiments were done using only the indirect method of DNA-PEI complex formation at low DNA concentration.

Gene transfer efficiency was next studied as a function of different molecular weights of PEI. Commercially available PEI's with molecular weights of 800, 60, 25, 22 and 2 kDa respectively, were tested. The results of this comparison is shown in Figure 5. Highest expression levels were seen if the transfection complexes were performed with PEI 25 kDa. The 4–5-fold increase of TNFRp55, if PEI 25 kDa was used as transfection agent rather than the 800 kDa polymer, is not well understood. It should be mentioned that the difference in expression levels was significantly smaller when 1 $\mu\text{g/ml}$ plasmid was used (not shown). Almost no activity was seen with the low molecular weight (2 kDa) polymer. Subsequent experiments were performed with PEI 25 kDa.

During the course of the PEI-mediated transfection study, we optimized transient gene expression by changing several parameters like the DNA concentration, PEI:DNA ratio and the MW of the cationic polymer PEI. In Figure 6A the time course of transient gene expression under new optimized conditions is shown. The accumulation of truncated TNF receptor in the culture medium increases until day 5–6. At this time point the high cell viability starts to decline (not shown). Many experiments which we have performed showed average expression levels of the extracellular domain of TNFRp55 ranging from 1.1–1.5 $\mu\text{g/ml}$, i.e. about 50–70% of the level obtained with stable cell lines (not shown).

Figure 6. Kinetic studies of expression levels in transient transfection. A: Time course of TNFRp55 expression using low DNA concentration. 30 ml 293(EBNA) cells (5×10^5 /ml) in fresh medium (50 ml spinner flask) were transfected with 3 ml DNA-PEI complexes ($0.2 \mu\text{g/ml}$ DNA + $0.9 \mu\text{g/ml}$ PEI 25 kDa). B: DNA dose response curve using different amounts of plasmid. 15 ml washed cells (25 ml spinner flasks) were transfected with increasing concentrations of DNA and complexed with PEI 25 kDa at a ratio of N:P of 33:1 and incubated for 3 days. C: SDS-PAGE of purified TNFRp55 from transiently transfected 293(EBNA) cells, stable CHO cells, baculovirus infected Sf9 and from transiently transfected Drosophila SL3 cells.

Figure 7. The influences of various factors on transient gene expression. 15 ml EBNA cells (5×10^5 cells/ml) in fresh and conditioned medium were transfected with DNA-PEI complexes (0.2 – $0.9 \mu\text{g/ml}$ PEI 25 kDa) and incubated for 3 days. Lane 1: complex formation in HL (control), 2: complex formation in HL without Primatone RL, 3: complex formation in NaCl (150 mM), 4: addition of heparin ($30 \mu\text{g/ml}$) to the cells, 5: complex formation in the presence of serum but no serum added to the cells, 6–8: complex formation without serum but addition of serum to the cells: 0, 1 and 4 h post-transfection, 9–11: complexes were formed in fresh medium and added to conditioned cultures 24, 48 and 48 h diluted 1:1 with fresh medium.

Expression experiments under the same conditions using DNA encoding the GFP gene showed transfection efficiency of 50–65% (data not shown).

In order to further reduce the DNA required in transient gene expression, we measured the expression levels using different plasmid concentrations with a high PEI:DNA ratio. DNA concentrations lower than $0.1 \mu\text{g/ml}$ led to almost no expression of TNFR, whereas concentrations higher than $0.2 \mu\text{g/ml}$ did not increase the expression level further (Figure 6B). Obviously, $0.1 \mu\text{g/ml}$ of plasmid gives sufficient amount of recombinant protein and $0.2 \mu\text{g/ml}$ could increase the expression level only about 20–25%. In large scale experiments, it would be an enormous advantage to use only 0.1–0.2 mg/l DNA.

TNF receptors purified from the culture supernatant 4 days post-transfection by affinity chromatography (Loetscher et al., 1990) are shown by SDS gel electrophoresis under non-reducing conditions together with receptors expressed in stable CHO cells,

baculovirus infected Sf9 cells and transiently transfected Drosophila SL3 cells (Figure 6C). The receptor protein expressed in 293(EBNA) cells is heavily glycosylated and exhibits two distinct bands similar to the position of those bands found in CHO cells.

The influence of some medium components and other parameters on the transient gene expression are described in Figure 7. Heparin, which was used to reduce cell aggregation in suspension culture, totally inhibits the PEI-mediated DNA uptake. Therefore, before each transfection experiment, 293(EBNA) cells were transferred into fresh medium without heparin. Because many cell lines were grown in serum containing medium we tested also the influence of serum in PEI-mediated transient transfection in suspension culture. The presence of FCS only during DNA-PEI complex formation reduces the transfection efficiency dramatically as described earlier for cationic liposomes (Yang and Huang, 1997). Addition of FCS to the cells at various time points shows that the ex-

Figure 8. Transient transfection in 293 and 293(EBNA) cells. 15 ml cells were transfected with different plasmids using 0.2 μ g/ml DNA and 0.9 μ g/ml PEI 25 kDa at N:P of 33:1 (indirect method) and incubated for 3 days. Samples were taken 1 and 3 days post-transfection. A: Gene expression using pREP4-, pREP7- and pC1-TNFRp55 plasmids. B: Luciferase gene expression was measured using pGL3-CMV(luc).

pression rate is significantly reduced (27%) at the beginning but after 4 h incubation control values were achieved.

In contrast to this finding, the absence of the protein hydrolysate Primatone RL, another complex nutrient, only in the 1/10 volume complex formation medium decreases the transfection efficiency by about 30–40% compared to the control sample which contains 0.3% hydrolysate. Increasing the Primatone concentration up to 0.8% did not lead to higher expression levels (not shown). Primatone clearly enhances the biological activity of the transfection complexes. The reason for this observation has not yet been determined. It should be mentioned that HEK293 cells grown in serum-free HL medium have to be cultured in the presence of Primatone (or other protein hydrolysates) to obtain a reasonable growth rate and final cell density.

Experiments using a 1/10 volume of NaCl (150 mM) instead of culture medium for the DNA-PEI complex formation as proposed by others (Boussif et al., 1995) led to a significant reduction in productivity (similar to water) under our transfection conditions. This finding disagrees with results obtained by oth-

ers who prepared the transfection complexes in NaCl (150 mM) in order to exclude influences (electrostatic interaction) of medium components on DNA-PEI complexes (Boussif et al., 1996).

The influence of the conditioned medium on the transient gene expression was also investigated. For this purpose, EBNA cells were transferred into heparin-free culture medium and incubated for 48 h. After 24 and 48 h, cells were adjusted to 5×10^5 c/ml with conditioned medium from this time point and transfected as described before. Fresh medium was used for the DNA-PEI complex formation. The results of this experiment clearly show a strong inhibitory effect by the conditioned medium which is significantly linked to the age of the culture.

The inhibition of transient gene expression by conditioned medium, which was also observed in other mammalian cells (not shown), presents a serious problem for large scale transfection experiments. It should be noted that the typical increase in cell aggregation in the absence of heparin, as observed in the conditioned culture, has no significant effect on cell growth and reduces the efficiency of transfection only slightly (data not shown).

The transfection efficiency depends on various different factors, e.g. the strength of the promoter/enhancer, the transfectability (DNA uptake) of a given cell line (which can be altered to some extent if individual cell clones are used) and the vector itself (size, replication). To analyze some of these aspects, two REP vectors (both containing the Rous Sarcoma virus promoter), pREP4 (intact EBNA-1 gene, 293 cells) and pREP7 (deletion in EBNA gene, 293(EBNA) cells) and the pC1 vector which contains the strong CMV promoter were used to measure transient gene expression in HEK293 cells as well as in 293(EBNA) cells. All these vectors express the human soluble TNFRp55.

The two EBV-based vectors of about 10 kb in size can replicate extrachromosomally. In contrast, the smaller pC1 plasmid (4.5 kb) can only survive in transfected cells after integration into the host DNA.

In Figure 8A the expression levels of day 1 and day 3 post-transfection are shown. First, it is evident, that using the REP vectors, the reporter gene is expressed significantly better (6–8-fold) in EBNA cells than in 293 cells, probably due to the constitutively expressed EBV nuclear antigen 1 (EBNA). Although the REP4 vector encodes the complete EBNA, the expression level of this protein is significantly lower in 293 cells. On the other hand, increasing levels of EBNA in 293(EBNA) cells by using the REP4 vector encoding the intact EBNA gene may not be very useful for foreign gene expression. As can be seen, the use of REP7 always yields a slightly higher TNFR titer compared to the REP4 vector.

Compared to the REP vectors, the overall expression levels of receptor protein using the pC1 plasmid were lower. This could be explained by the inability of pC1 to replicate in the host cells. The initial expression levels after day 1 in relation to day 3, however, appear to be higher, probably due to enhanced uptake of the smaller pC1 plasmid. Interestingly, no difference in transient gene expression between 293 and EBNA cells was observed if the pC1 vector was used. Similar luciferase activity was measured in the two cell lines (Figure 8B). The luciferase gene is inserted into the pGL3 vector and is also driven by the strong CMV promoter.

Discussion

In this study we worked out the conditions for transient gene expression in mammalian cells in serum-free sus-

pension culture. All experimental modifications took into consideration the scalability of this system. We realized early in this study that 293(EBNA) cells were superior to HEK293 cells with respect to expression levels (if EBV-based vectors were used) and growth properties. EBNA cells also exhibit a strong tendency to form large viable cell aggregates during serum-free growth in suspension culture. Therefore, we added heparin to the growth medium to reduce cell-cell interactions. On the other hand, however, heparin inhibits DNA uptake in transfection experiments completely. In large scale, however, medium replacement is more difficult and may influence the physiological state of the cells to a certain extent. Surprisingly, the transfection efficiency in cultures containing viable cell aggregates, which are formed in the absence of heparin, is only slightly lower compared to single cells as measured by green fluorescence protein expression (data not shown).

Much more pronounced in this context, however, is the observed inhibitory effect on gene delivery of conditioned medium depending on the age of the culture, see Figure 7. How components (multiple metabolites) in the conditioned medium can influence the gene delivery is not yet well understood, though it appears to be a more general phenomena. It was found not only with other cell lines but also with different cationic transfection agents (data not shown).

We developed strategies to overcome these serious difficulties in scaling up transient gene expression. For example, as a practical solution, we started large scale experiments in fermentors (12 and 23 l) with a freshly centrifuged inoculation culture and transfected the cells after 24 h fermentation in the bioreactor when the cell density of 5×10^5 cells per ml was achieved, accepting lower expression levels.

Much more interesting and efficient is the use of the novel transfection agent dioleoylmelittin (DOM) together with PEI. The synergistic enhancement of the transient gene expression by both agents not only increases the expression levels but also decreases the inhibitory effect of conditioned medium (data not shown).

Obviously, the interaction of DOM and PEI with DNA results in more stable transfection complexes. Work is ongoing to clarify the mechanism of the transfection inhibition by conditioned medium.

During our initial work we found that with increasing DNA concentration, the separate transfection complexes formation with PEI became very turbid and the expression levels decreased significantly. Imple-

menting the direct method by adding first the DNA and later PEI directly into the culture gave more reasonable and reproducible yields. The ratio of PEI:DNA increases to 26:1 if the direct addition was compared to the indirect method (13:1). The reason for this is yet not understood. Interestingly in this context, however, is the observation that at low DNA concentration (0.1–0.2 $\mu\text{g/ml}$) the ratio of N:P increases as well if the indirect method was used.

The ratio of (PEI) nitrogen to (DNA) phosphate is very important for high expression levels in transient gene expression using 293(EBNA) cells. The condensation of sufficient amounts of DNA (1 $\mu\text{g/ml}$) by PEI requires relatively low amounts of cationic polymer (1.9 $\mu\text{g/ml}$). More PEI (0.9 $\mu\text{g/ml}$) is necessary, however, if the plasmid concentration is limited (0.1–0.2 $\mu\text{g/ml}$). The use of low DNA concentrations in transient transfection without reducing the expression levels clearly has advantages in large scale experiments. For example, 2–4 mg plasmid is sufficient to transfect a 23 l fermentor, yielding 10–100 mg recombinant product (depending on the expressed protein). In addition, less transfection agent can be used in the culture, reducing the influence on cell physiology, and therefore the growth properties will be less affected.

Regarding the plasmid preparation, we used a conventional inexpensive method originally described by (Birnboim, 1983). The quality of the DNA in regard to transfection efficiency was sufficient. No difference in expression levels were measured if compared to plasmid preparations isolated with commercially available kits (Quiagen, BIO-RAD). Meanwhile, a large scale plasmid preparation method has been established.

In conclusion PEI-mediated transient gene expression in 293EBNA cells can be used as a low-toxic, scaleable method to express milligram amounts of recombinant protein in suspension culture within a few days. The benefits of this method are its robustness and reproducibility, the high transfection efficiency (about 40–60% of the cells are generally transfected) and the cost effectiveness. A critical aspect in large scale experiments, however, is the observed reduction of transient gene expression in conditioned cultures. Strategies to reduce this inhibitory effect are currently being developed.

Acknowledgements

We are grateful to Drs. F. Grüninger-Leitch and K. Lundström for critical reading of the manuscript. We

thank Dr. U. Deuschle for providing the pGL3-luc and the pC1-GFP plasmid.

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Address for correspondence: Dr Ernst-Jürgen Schlaeger, F. Hoffmann-La Roche Ltd., Research Laboratories, Bldg. 66/108, CH-4070 Basel, Switzerland.

Phone: +61-688-5465;

Fax: +61-688-1448; E-mail: ernst-j.schlaeger@roche.com.