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ORIGINAL RESEARCH

Optimizing the transient transfection process of HEK-293 suspension cells for protein production by nucleotide ratio monitoring

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Abstract Large scale, transient gene expression (TGE) is highly dependent of the physiological status of a cell line. Therefore, intracellular nucleotide pools and ratios were used for identifying and monitoring the optimal status of a suspension cell line used for TGE. The transfection efficiency upon polyethyleneimine (PEI)-mediated transient gene delivery into HEK-293 cells cultured in suspension was investigated to understand the effect of different culture and transfection conditions as well as the significance of the culture age and the quality of the cell line used. Based on two different bicistronic model plasmids expressing the human erythropoietin gene (rHuEPO) in the first position and green fluorescent protein as reporter gene in the second position and vice versa, a completely serum-free transient transfection process was established. The process makes use of a 1:1 mixture of a special calcium-free DMEM and the FreeStyle^{TM} 293 Expression Medium. Maximum transfectability was achieved by adjusting the ratio for complex formation to one mass part of DNA and three parts of PEI corresponding to an N/P (nitrogen residues/DNA phosphates) ratio of 23 representing a minimum amount of DNA for the polycation-mediated gene delivery. Applying this method, maximum transfectabilities between 70 and 96 % and a rHuEPO concentration of 1.6 μg mL^{-1} 72 h post transfection were reached, when rHuEPO gene was expressed from the first position of the bicistronic mRNA. This corresponded to 10 % of the total protein concentration in the cell-free supernatant of the cultures in protein-free medium. Up to 30 % higher transfectabilities were found for cells of early passages compared to those from late passages under protein-free culture conditions. In contrast, when the same cells were propagated in serum-containing medium, higher transfectabilities were found for late-passage cells, while up to 40 % lower transfectabilities were observed for early-passage cells. Nucleotide pools were measured during all cell cultivations and the nucleoside triphosphate/

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uridine ratios were calculated. These 'nucleotide ratios' changed in an age-dependent manner and could be used to distinguish early- from late-passage cells. The observed effects were also dependent on the presence of serum in the culture. Nucleotide ratios were shown being applied to investigate the optimal passage number of cultured cell lines for achieving a maximum productivity in cultures used for transient gene expression. Furthermore, these nucleotide ratios proved to be different for transfected and untransfected cells, providing a high potential tool to monitor the status of transfection under various culture conditions.

Keywords Mammalian biotechnology · Cell culture · Gene expression · Transient transfection

Introduction

Transient gene expression is widely used for producing quantities of recombinant proteins suitable for biochemical and preclinical studies (Wurm and Bernard 1999; Pham et al. 2006; Baldi et al. 2007). Several efficient transient expression systems were successfully applied for the synthesis of a number of different recombinant proteins. These technologies have been used with different cell lines, e.g., COS cells (Blasey et al. 1996), HEK-293 (Jordan et al. 1998; Schlaeger and Christensen 1999; Meissner et al. 2001; Durocher et al. 2002; Geisse and Henke 2005; Pham et al. 2005; Tuvesson et al. 2008), CHO (Derouazi et al. 2004; Muller 2005; Galbraith et al. 2006; Haldankar et al. 2006) and BHK cells (Wurm and Bernard 1999) and even scale-up to larger volumes has been reported for the calcium phosphate co-precipitation method using up to 100-L stirred tank bioreactors (Girard et al. 2002; Lindell et al. 2004) and for the polyethylenimine-mediated gene delivery using 20-L (Derouazi et al. 2004), 100-L (Tuvesson et al. 2008) and 150-L (Muller et al. 2007) stirred tank bioreactors and the 50-L WAVE^{TM} system at 10 L working volume (Geisse et al. 2005; Haldankar et al. 2006) as well as the same volume with DMRIE-C reagent (Invitrogen, Carlsbad, CA, USA) and a 293s cell fused to a human 2B8 suspension cell derived from a Burkitt's lymphoma (HKB11) (Cho et al. 2002, 2003). In addition, transient transfection has been performed using disposable shake tubes (De Jesus et al. 2004) and also larger orbital shake bioreactors up to 50 L scale (Stettler et al. 2007).

Transient gene expression systems provide a rapid method for producing small batches of recombinant protein without the need to isolate high-level expressing clones. Generally these systems yield protein in the microgram quantity range) whereby the genetic construct does not integrate into the genome in contrast to stable expression (Gulick 2001). The productivity of transiently transfected cultures depends on multiple factors. These include the type of the host cell, the transfection efficiency, the rate of gene transcription and translation in the host cell, the extent of post-translational modifications necessary for optimal protein function, the survival rate of the transiently transfected cells, and the availability of culture methods that can handle large volumes of transiently transfected cells (Chen and Okayama 1988; Blasey et al. 1996; Lazar et al. 1993; Curiel 1994; Griffiths and Racher 1994; Hofmann et al. 1995; Baker et al. 1997). Although considerably high expression rates can be achieved with transiently transfected COS cells a routine use was prevented due to difficulties in adaptation to serum-free culture conditions (Ridder et al. 1995; Blasey et al. 1996). Extensive efforts have gone into optimizing gene delivery and insertion methods, developing efficient viral and plasmid vectors. In some cases host cells were genetically modified for optimal transient gene expression, but optimizing culture conditions had to be performed in most cases for gaining high-level protein synthesis. However, the development of technologies that prolong the productive life of transiently transfected cells and facilitate their handling in vitro still lags behind the mentioned efforts. Suspension culture is a proven technology for the industrial-scale growth and manipulation of mammalian cells but the most efficient systems for transient transfections works with anchorage-dependent host cells such as the COS-7 cell line (Lazar et al. 1993; Blasey et al. 1996). Adaptation of anchorage-dependent cells for growth in suspension frequently reduces protein production (Lazar et al. 1993; Griffiths and Racher 1994). In contrast, these cells showed a high level of recombinant protein production in a trimethylamine-coated microcarrier-based suspension culture system used for transient transfection.

Nonviral methods of gene delivery have been used in cell transfection for years, with cationic polymers and liposomes (and combinations of the two) showing

the highest transfection efficiencies of chemical-based delivery systems. One such system, which uses polyethyleneimine (PEI) for gene delivery, has been applied with increasing popularity since its description as a transfection agent in 1995 (Boussif et al. 1995). Since that time, PEI has been tested on a variety of cell types and living animals to establish its efficacy (Abdallah et al. 1996; Boussif et al. 1996; Lambert et al. 1996; Boletta et al. 1997; Ogris et al. 1998; Godbey et al. 1999; Schlaeger and Christensen 1999; Durocher et al. 2002; Geisse and Henke 2005; Pham et al. 2005; Tuvesson et al. 2008; Muller et al. 2007).

Despite all these efforts and for economic reasons, there remains a high demand to simplify or diminish handling steps and to lower the amount of DNA required for transfection. Moreover, for manufacturing purposes the transfection process requires a cell line with reliable and predictive properties as well as complete elimination of serum and undefined components in order to guarantee product safety for biopharmaceutical applications.

In this context, a better understanding of the host cell line and a reliable monitoring and control of its cellular physiology are great challenges in bioprocess engineering of transient gene expression. Unexpected variations in cell's property often accompany cell culture processes and affect their efficiency, as cells in a poor physiological state may respond unfavourably to the transfection stress. Moreover, the expression vectors, the transfection, cloning, and selection steps, and the recombinant product of interest itself may lead to different phenotypes. Therefore, the producer cell line as the "cell factory" is always in the focus of optimisation strategies. Accordingly, parameters that determine the physiological state of the cells are always valuable tools for monitoring in bioprocess optimisation. The relative concentrations of intracellular nucleotides were found to present one of the most reliable parameters to monitor the physiological state of a cell (Ryll and Wagner 1992). Three variables that characterize cell physiology have been described: the nucleoside triphosphate ratio (NTP), the uridine ratio (U) and the combination of both parameters expressed as NTP/U ratio.

Here, we report on the application of nucleotide ratios for the characterization of HEK-293 cells to achieve optimal transfection efficiencies and to monitor the cultivation, transfection and protein production process in transiently transfected HEK-293 cultures.

Materials and methods

Chemicals

All standard chemicals used were of reagent grade, and purchased from Sigma-Aldrich (Carlsbad, CA, USA), Difco Laboratories (Detroit, MI, USA), Merck (Darmstadt, Germany), Serva (Heidelberg, Germany), Riedel-de-Haen (Seelze, Germany), Fluka (Buchs, Switzerland), or J.T. Baker (Griesheim, Germany) if not specified otherwise.

All solutions were prepared in ultra pure water obtained from the water system Super-Q^{TM} (Millipore GmbH, Eschborn, Germany).

Cell line

All experiments were performed using early and late passages of the human embryonic kidney cell line 293s which was adapted to growth in suspension (Coté et al. 1998) and kindly provided by Dr. Amine Kamen (Animal Cell Technology and Downstream Processing Group, Biotechnology Research Institute, National Research Council Canada, Montreal, Canada).

Culture media

Powdered media were dissolved in water and 0.1 μm filtered. A proprietary calcium-free DMEM-based serum-free formulation (DMEM-Ca) was used as basic medium. It contained the following ingredients: [mg L^{-1}] $Fe(NO_3)_3 \times 9\ H_2O$ (0.1), $MgSO_4 \times 7\ H_2O$ (200.0), KCl (400.0), $NaHCO_3$ (3700.0), NaCl (6400.0), NaH_2PO_4 (109.0), L-arginine × HCl (168.6), L-cystine × 2 HCl (62.6), L-glutamine (731.0), glycine (30.0), L-histidine × HCl × H_2O (42.0), L-isoleucine (131.0), L-leucine (131.0), L-lysine × HCl (219.0), L-methionine (60.0), L-phenylalanine (66.0), L-serine (84.0), L-threonine (95.0), L-tryptophane (16.0), L-tyrosine (71.4), L-valine (94.0), D-glucose × H_2O (4950.0); phenol red (1.5×10^{-3}), [% (V V^{-1})] MEM Vitamin Solution 100× (M6895; 4.0, Vit), Pluronic F68 Solution 10 % (0.1). If specified, this medium was supplemented with 5 % fetal calf serum (FCS, Biochrom AG, Berlin, Germany) heated for 1 h at 56 °C for complement inactivation when serum-containing conditions were used (DMEM + FCS). Serum-free cultivation and transfection was performed in DMEM-FS medium, which was a 1:1 mixture of DMEM-Ca-Vit and

FreeStyle™ 293 Expression Medium (Invitrogen) supplemented with 600 µmol L^{-1} aspartate and 100 µmol L^{-1} glutamate (DE). For adaptation of 293s cells to serum-free medium conditions the following media were used: 293 SFM II (Invitrogen) as well as Ex-Cell® 293™ and Ex-Cell 293® modified media. When these media and FreeStyle™ 293 Expression Medium were used for transfection, 1 % fetal calf serum (FCS) was added as recommended by Durocher et al. (2002) and in the FreeStyle™ 293 Expression Manual to improve transient transfection.

Bacteria were cultivated in SOC medium (pH 7.0) consisting of SOB medium formulated by 20 g L^{-1} bacto-tryptone, 5 g L^{-1} bacto-yeast extract, 0.5 g L^{-1} NaCl dissolved in water, autoclaved for 20 min at 121 °C and finally supplemented with 5 mL L^{-1} 2 mmol L^{-1} glucose (0.1 µm filtrated) and 50 mL L^{-1} of 1 mmol L^{-1} $MgSO_4$.

Cell culture

293s cells from stocks stored in serum-free medium in the gas phase of liquid nitrogen (2-mL cryogenic vial filled with 1.8 mL volume containing 1.0×10^7 cells in 10 % DMSO + 10 % methylcellulose + 80 % fresh medium) were revitalized and cultivated in suspension using 150-mL and 1,000-mL spinner flasks (Techne, Cambridge, UK) at 70 and 400 mL working volume, respectively, and incubated at 37 °C, 12 % CO_2 and 90 % humidity at a stirrer rate of 40–80 rpm starting at an initial cell concentration between 2.0×10^5 and 5.0×10^5 mL^{-1} in batch mode.

E. coli bacteria were thawed from −70 °C glycerol stocks and cultivated in Luria–Bertani (LB) medium consisting of 10 g L^{-1} bacto-tryptone, 5 g L^{-1} bacto-yeast extract and 10 g L^{-1} NaCl diluted in water, adjusted to pH 7.0 and autoclaved for 20 min at 121 °C. This medium was supplemented with 50 mg L^{-1} either ampicillin or kanamycin for selection and 15 g L^{-1} bacto-agar if solid media were used.

Cell analysis

Total cell number was either estimated by nuclei fixation and staining using a hemocytometer (Sandford et al. 1951) or by an automatic cell counter (CASY® 1, Innovatis AG, Reutlingen, Germany) according to manufacturer's protocol. Viable cells were counted using the trypan blue exclusion method (Strober 1993). Glucose and lactate concentration were determined daily in duplicates by a glucose/lactate analyzer (YSI 2700 SELECT, YSI Life Sciences, Yellow Springs, OH, USA). The method is based on enzymatic oxidation (immobilized glucose oxidase and L-lactate oxidase, respectively). The formed hydrogen peroxide is oxidized at a platinum electrode and the respective electricity is measured.

Lactate dehydrogenase (LDH) activity was measured spectrophotometrically at 340 nm as described earlier (Wroblewski and LaDue 1955; Ryll et al. 1990; Kratje and Wagner 1992). Free amino acids were quantified using a reversed phase high performance liquid chromatography (RP-HPLC) and an internal norvalin or citrullin standard (Larsen and West 1981) after precipitation of proteins by perchloric acid and transformation of the amino acids with *ortho*-phthaldialdehyde to fluorescent isoindole derivates. Intracellular nucleotides were analyzed using an ion pair reversed-phase HPLC method after an acid cell extraction with perchloric acid as previously reported (Ryll and Wagner 1991, 1992). Nucleotide pools were calculated as following: The adenylate energy charge (AEC; Atkinson 1969), the relative amount of one nucleotide to the total nucleotide pool (NT), the nucleoside triphosphate ratio (NTP), the uridine ratio (U) and the combined NTP–U ratio according to the following equations (Ryll and Wagner 1992):

$$\mathrm{NT} = \frac{\mathrm{NT}}{\sum \mathrm{Nucleotides}} \cdot 100 \tag{1}$$

$$\mathrm{AEC} = \frac{(\mathrm{ATP} + 0.5\mathrm{ADP})}{(\mathrm{ATP} + \mathrm{ADP} + \mathrm{AMP})} \tag{2}$$

$$\mathrm{NTP} = \frac{(\mathrm{ATP} + \mathrm{GTP})}{(\mathrm{UTP} + \mathrm{CTP})} \tag{3}$$

$$\mathrm{U} = \frac{\mathrm{UTP}}{\mathrm{UDP\ -\ GNAc}} \tag{4}$$

$$\frac{\mathrm{NTP}}{\mathrm{U}} = \frac{(\mathrm{ATP} + \mathrm{GTP}) \cdot \mathrm{UDP} - \mathrm{GNAc}}{(\mathrm{UTP} + \mathrm{CTP}) \cdot \mathrm{UTP}} \tag{5}$$

$$\mathrm{UDP} - \mathrm{GNAc} = \mathrm{UDP} - \mathrm{GalNAc} + \mathrm{UDP} - \mathrm{GlcNAc} \tag{6}$$

$$\begin{aligned} \sum \mathrm{Nucleotides} = {} & \mathrm{ATP} + \mathrm{GTP} + \mathrm{UTP} + \mathrm{CTP} \\ & + \mathrm{UDP} - \mathrm{GNAc} + \mathrm{ADP} + \mathrm{GDP} \\ & + \mathrm{AMP} + \mathrm{NAD} + \mathrm{NADP} \end{aligned} \tag{7}$$

Protein analysis

Protein concentration was measured spectrophotometrically at 595 nm according to the Bradford method (Bradford 1976) using the Coomassie Plus™ Protein Assay Reagent (Pierce, Rockford, IL, USA) and bovine serum albumin (BSA, Invitrogen) as reference for the micro and the macro test (Stoscheck 1990).

Expression vectors

For evaluation of the transfection efficiency and the productivity of transiently transfected cells the following bicistronic vectors were used expressing the enhanced green fluorescent protein (EGFP) as reporter and the recombinant human erythropoietin (rHuEPO) as glycoprotein product: (1) pCMV-EGFP-rHuEPO (5,388 bp) bearing as the first cistron EGFP and as second cistron rHuEPO as well as an internal ribosome entry site (IRES) under the control of the cytomegalovirus (CMV) promoter (Fig. 1a). This plasmid was constructed from pSBC-EGFP-rHuEPO expressing both proteins under control of the siman virus (SV) 40 promoter/enhancer (Irani et al. 2002). This promoter was replaced by the CMV promoter applying *Xmn*I and *Xho*I digestion and cloning of the CMV promoter from the plasmid pTracer™-CMV2 (Invitrogen). (2) pEPO-IRES2-EGFP (6,014 bp) bearing first the rHuEPO and second the EGFP as well as an IRES under control of the CMV promoter (Fig. 1b). This plasmid was constructed from pIRES2-EGFP (BD Biosciences, Franklin Lakes, NJ, USA) and pCR-3-EPO (5,715 bp) kindly provided by Dr. Eckart Grabenhorst (Proteinglycosylation Dept., Helmholtz Centre for Infection Research, Braunschweig, Germany) by *Nde*I and *Xho*I restriction and ligation of the respective fragments. pCR3-EPO resulted from plasmid pCR™ 3 (Invitrogen) and the cloned human rHuEPO gene (Powell et al. 1986).

Plasmid production

E. coli strains were transformed by two methods according to the manufacturers' protocols: (1) XL1-Blue competent cells (Stratagene, La Jolla, CA, USA) were transformed by electroporation using an electroporator (Gene Pulser II, Biorad, Hercules, CA, USA) with the following settings: capacitance extender (500 μF), capacitance (25 μF), pulse controller (200 Ω) gene pulser (volt-set, 2.5 kW, max.). (2) JM109 competent cells (Promega) were transformed by the heat shock method for 45–50 s in a water bath at 42 °C. Successful transformations were checked by means of plasmid mini-preparations using the QIAprep® Spin Miniprep Kit (Qiagen, Hilden, Germany). Plasmids were controlled by restriction endonuclease digestion using *EcoR*I, *Nde*I, *Xho*I, *Kpn*I, and *Xmn*I (New England Biolabs Inc., Hitchin, Hertfordshire, UK) and 1 % agarose NA gels (GE Healthcare Biosciences AB, Uppsala, Sweden). Purification of plasmids was performed by using the Plasmid Giga Kit (Qiagen). DNA concentration and quality were determined spectrophotometrically at wavelengths of 260 and 280 nm. DNA concentration was calculated by the following equation:

$$c_{DNA} = OD_{260} \times 50 \times \text{dilution factor} \left[\mu g\, mL^{-1}\right] \quad (8)$$

$OD_{260} = 1$ corresponds to a dsDNA concentration of 50 μg mL^{-1}. DNA quality was estimated by the OD_{260}/OD_{280} ratio. Appropriate preparations should have an OD_{260}/OD_{280} ratio between 1.75 and 2.00.

Transient transfection

293 s cells from the mid-exponential growth phase cultured as single cells or small clusters at a concentration between 5.0×10^5 and 1.0×10^6 mL^{-1} and a viability above 90 % were transfected using the polyethylenimine (PEI)-mediated gene delivery method. Plasmids at a concentration between 0.02 and 2.0 μg μL^{-1} with a OD_{260}/OD_{280} ratio between 1.75 and 2.00 were mixed with 1 mg mL^{-1} 0.22 μm filtrated and HCl-neutralized 25 kDa linear PEI (Polysciences, Warrington, PA, USA) in different ratios as indicated for the respective experiments. For complex formation, DNA was diluted in fresh serum-free medium using a volume equivalent to one-tenth of the culture volume to be used in the transfection system. Two minutes later, PEI was added, and the mixture was immediately homogenized (Vortex Genie® 2, Scientific Industries, Inc., Bohemia, NY, USA) and incubated for 10 min at room temperature (RT) prior to the addition to the cells. Transfected cells were further incubated between 48 and 72 h until being analyzed by flow cytometry. Supernatants were stored at −20 °C for EPO analysis. For transient transfection, either 12-well plates (small-scale) filled with 900 μL per well or 1,000-mL spinner flasks

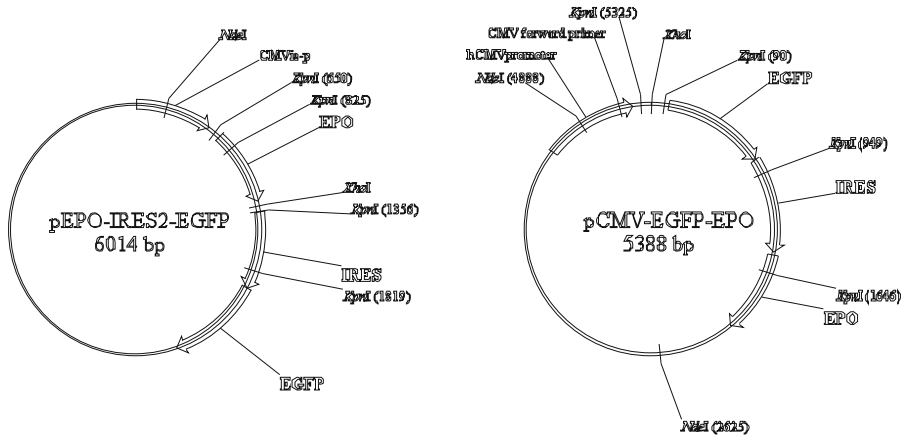

Fig. 1 Schematic drawing of the two bicistronic plasmids used in the experiments. Both genes are under control of the CMV promoter. Vector pCMV-EGFP-EPO carried the EGFP gene in the first position and the human EPO gene in the second (**a**). In contrast, vector pEPO-IRES2-EGFP harbors EPO in the first position and the reporter EGFP in the second. Both vectors were used to characterize the expression efficiencies and the productivities in the different culture conditions and transient transfection processes of 293s cells from different ages

(Techne, Cambridge, UK) at 200 mL working volume (large-scale) were used for the addition of 100 µL or 20 mL DNA, respectively, which was diluted in serum-free medium and complexed with the respective amount of PEI prior to transfection.

Product analysis

Transfection efficiency was evaluated by expression of EGFP and rHuEPO genes. GFP was measured by flow cytometry using a FACSCalibur^{TM} (BD Biosciences) equipped with the CellQuest Pro^{TM} software. R-huEPO was quantified by a sandwich Enzyme-Linked Immunosorbent Assay (ELISA) using 400 ng of the specific monoclonal antibody mAB 2B2 (kindly provided by Dr. Ricardo Kratje, Laboratorio de Cultivos Celulares, Santa Fe, Argentina) diluted in coating buffer (50 mmol L^{-1} Na_2CO_3, pH 9.6) and incubated either 1 h at 37 °C or overnight at 4 °C (Amadeo et al. 2003). The plates were washed with T-PBS (0.05 % Tween 20 in PBS) and then blocked with PBS containing 1 % bovine serum albumin (BSA) to prevent non-specific binding (1 h at 37 °C). After washing with T-PBS, the plates were loaded with the samples. Standard curves (in triplicate) were achieved by 1:2 serial dilutions of purified EPO (Laboratorio de Cultivos Celulares) from 4 µg mL^{-1} to 7.8 ng mL^{-1} dissolved in cell culture medium. Samples were diluted 1:2 in serial dilutions as well. Plates were incubated for 1 h at 37 °C, washed with T-PBS and incubated with rabbit anti-huEPO polyclonal antibodies (pAb, Laboratorio de Cultivos Celulares), diluted 1:1,000 with BSA-T-PBS (0.1 % BSA in T-PBS). After 1 h incubation at 37 °C, the plates were washed and incubated with peroxidase-labeled goat anti-rabbit antibody (DAKO, Glostrup, Denmark) diluted 1:1,000 in BSA-T-PBS. After the final wash, the plates were incubated in the dark with 50 mmol L^{-1} citric acid-phosphate buffer, pH 3.0, containing 3.0 mg mL^{-1} *o*-phenylenediamine and 0.12 % (V V^{-1}) hydrogen peroxide. The absorbance was measured at 450 nm using a Titertek Multiskan Automatic ELISA microplate reader (MCC/340, Huntsville, AL, USA).

Results

Optimization of serum-free medium for transient transfection

For developing a serum-free medium optimized for small-scale transient transfections, different serum-free media were investigated. 293s cells could be fully adapted to 293 SFM II + DE, ExCell 293^{TM} and ExCell 293^{TM} modified media. Furthermore, to improve transient transfection FCS was added to the serum-free media to a final concentration of 1 % in parallel experiments as recommended by Durocher et al. (2002) and as described in the FreeStyle^{TM} 293

Expression Manual. The following media were used: Ex-Cell 293™ medium (1), Ex-Cell 293™ medium + 1 % FCS (2), Ex-Cell 293™ modified medium (3) Ex-Cell 293™ modified medium + 1 % FCS (4), 293 SFM II + DE + 1 % FCS (5).

293s cells cultured in media 1 and 3 were seeded at a concentration of 5.0×10^5 mL^{-1} per well (900 µL) 3 h before transfection. For media 2, 4 and 5, cells growing in the respective medium without serum were washed one time with the corresponding medium containing 1 % FCS and seeded in the plate at the same cell concentration as for medium 1 and 3. All cultures were incubated for 3 h until the transfection procedure was performed. Transfection in DMEM + 5 % FCS (DMEM + FCS) was performed in parallel as a positive control. 293s cells cultured in all different media without transfection were used as negative controls. DNA and PEI were complexed at a DNA:PEI ratio of 1.0:1.5 (µg:µg) in DMEM-Ca for all transfections using the pEPO-IRES2-EGFP vector. The number of GFP-positive cells was measured 48 h post transfection (hpt).

Table 1 shows the transfection efficiency achieved for each medium. Surprisingly, for none of the media tested a substantial gene expression could be observed. A similar result was observed when the transfection complex was prepared in the serum-free test media used in the previous experiment instead of DMEM-Ca medium (48 hpt, transfection efficiency <0.5 %, data not shown).

In order to investigate whether the presence of serum would improve TGF, transfections were done using Ex-Cell 293™ modified medium and the 293 SFM II + DE medium supplemented with 1 % serum. These experiments (see Table 1) and also the addition of 2.5 and 5.0 % of FCS, respectively, did not substantially increase transfectability (data not shown). In these experiments a maximum of 10.0 % GFP-positive cells were obtained after transfection in FreeStyle™ 293 expression medium and complex formation in serum-free DMEM-Ca medium, while maximally 3.0 % GFP-positive cells were found using the Ex-Cell 293™ modified medium. As it was not possible to reach the transfection efficiencies of the positive control (DMEM + FCS) even when supplementing the media with 5.0 % FCS, it was hypothesized that inhibitory components may be present in the serum-free media used.

Table 1 Small-scale transient transfection of 293s cells in different serum-free media

Medium	GFP^+ cells [%]
DMEM + FCS (positive control)	74.6
Ex-Cell 293™	0.2
Ex-Cell 293™ + 1 % FCS	0.1
Ex-Cell 293™ modified	3.0
Ex-Cell 293™ modified + 1 % FCS	1.5
293 SFM II + DE + 1 % FCS	0.1

Three hours before transfection, cells were washed with the respective medium and seeded in the plate for transfection. The DNA(pEPO-IRES2-EGFP):PEI = 1.0:1.5 complex was prepared in DMEM-Ca for all cases, incubated at RT for 10 min and added to the cells. The percentage of GFP-positive cell was determined by flow cytometry 48 hpt. Transfection in DMEM–FCS was performed as positive control. The data represents the average from two experiments

In order to identify a possible inhibition from medium components, the two media Ex-Cell 293™ modified and the 293 SFM II + DE were diluted with DMEM-Ca and the percentage of GFP-positive cells was determined after PEI transfection. The two medium compositions were prepared as follows: Ex-Cell 293™ modified: DMEM-Ca = 1:1 (I) and 293 FreeStyle™ expression medium : DMEM-Ca = 1:1 (II). Cells already adapted to Ex-Cell 293™ modified serum-free medium and taken from the mid-exponential growth phase were washed one time with the respective test medium (I and II) and cultured in these media using an initial cell concentration of 5.0×10^5 mL^{-1}. The two cultures were continuously passaged every second or third day to maintain the cell concentration between 1.5×10^6 and 2.0×10^6 mL^{-1}. After the third passage, cells were prepared for small-scale transient transfection without cell wash. The complex (DNA:PEI = 1.0:1.5) using pEPO-IRES2-EGFP was prepared in DMEM-Ca medium. GFP-positive cells were quantified by FACS analysis 48 hpt. Transfection in DMEM + FCS was used as a positive control. Table 2 shows that the medium composition II consisting of a 1:1 mixture of 293 FreeStyle™ Expression Medium and DMEM-Ca-DE (DMEM-FS, see under Materials and Methods) revealed a transfection efficiency of over 70 % as obtained for the control whereas only 2 % GFP-positive cells were found for cells grown in medium composition I.

Table 2 Optimization of serum-free transfections using modified growth media

Medium	DMEM + FCS = positive control	Ex-Cell 293™: DMEM-Ca = 1:1	293 FreeStyle™ expr. medium: DMEM-Ca = 1:1
GFP^+ cells/%	74.59	2.02	71.32

293s cells growing in DMEM + FCS (positive control), in Ex-Cell 293™ modified: DMEM-Ca = 1:1 (I) and in 293 FreeStyle™ expression medium:DMEM-Ca = 1:1 (II) were transfected with DNA:PEI = 1.0:1.5 prepared in DMEM-Ca. The ratio of GFP expressing cells was measured by flow cytometry (FACS) 48 hpt. Experiments were done in duplicate

Optimization of the DNA–PEI ratio

Different DNA–PEI ratios were evaluated in order to achieve the highest transfection efficiency at a minimum DNA dosage. 293s cells cultured in DMEM-FS serum-free medium were transfected in 12-well plates and the cellular productivity was analyzed 48 hpt by determining the percentage of EGFP-positive cells (FACS analysis) and measuring the rHuEPO concentration in the supernatant using a specific ELISA. Figure 2 shows that the highest transfectability of more than 70 % was obtained using 0.50 µg DNA and 1.50 µg PEI (1:3), corresponding to a N/P ratio of 23. The N/P ratio represents the quotient of the nitrogen residues to DNA phosphates. 1 µg of DNA contains 3 nmol phosphate, and 1 µg of PEI aqueous stock solution carries 23 nmol amine nitrogen based on $NHCH_2CH_2$-monomers; Boussif et al. 1995). As expected for the bicistronic plasmid, both proteins, GFP (Fig. 2a) as well as rHuEPO (Fig. 2b), showed similar relative expression rates for individual DNA–PEI ratios. A further decrease in the amount of DNA using ratios of 0.25:1.00 (1:4) and 0.25:1.50 (1:6) resulted in a lower portion of cells showing EGFP gene expression which was generally below 30 % (data not shown). These results were confirmed by a rHuEPO production of up to 0.8 µg mL^{-1} when a DNA:PEI ratio of 0.50:1.50 (1:3) was used.

Course of transient transfection

For studying the course of transient transfection the process was scaled-up to spinner flasks using 200 mL working volume. Cell growth started at a cell concentration of 3.0×10^5 to 5.0×10^5 mL^{-1} in DMEM-FS

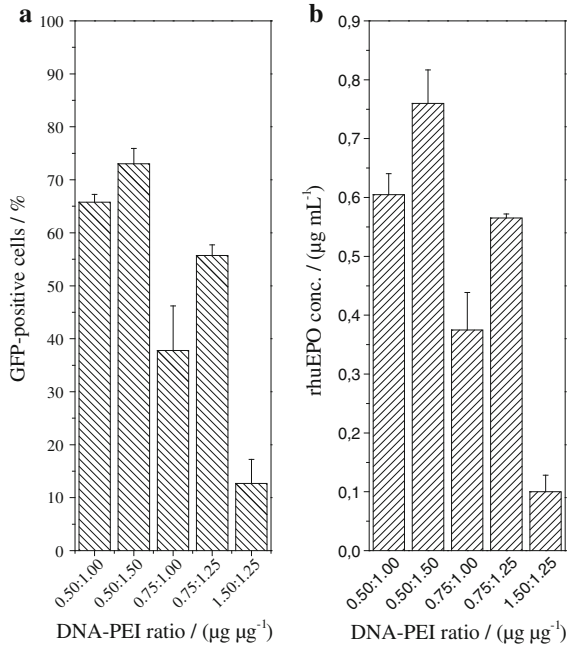

Fig. 2 Transfection efficiency using different DNA–PEI ratios. 293s cells cultured in serum-free DMEM-FS medium were transfected using pEPO-IRES2-EGFP plasmid DNA and PEI. Repeated small-scale transient transfection was performed in 12-well plates. GFP expression (**a**) and EPO production (**b**) were measured 48 hpt by flow cytometry and ELISA, respectively. *Error bars* represent the standard deviation (SD) of two independent experiments measured in triplicate

medium. 293 s cells were transfected when their concentration reached a value between 6.0×10^5 and 1.0×10^6 mL^{-1} and their viability remained above 98 % for 24 or 48 h post seeding (hps). The plasmid pEPO-IRES2-EGFP and the optimal ratio of 0.5 µg DNA and 1.5 µg PEI were used. Samples were taken every 12 hpt for GFP and rHuEPO analysis. Figure 3 shows that the maximum percentage of transfected cells, expressed as GFP-positive cells, was reached between 60 and 72 hpt with a value of 70 %. The highest rHuEPO productivity was reached 72 hpt with a concentration of 1.58 µg mL^{-1}. After a cultivation time of about 120 h, the rHuEPO production ceased and the percentage of GFP-positive cells dropped to about 60 %.

Comparison of 293s cells from early and late passages

For investigating a possible effect of the passage number on gene delivery, transfection experiments

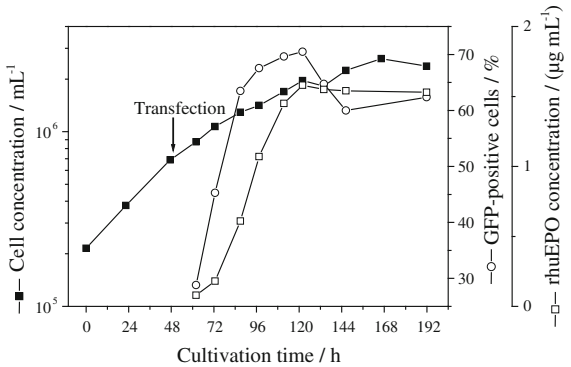

Fig. 3 Growth and productivity of transiently transfected 293s cells growing in serum-free DMEM-FS medium using 1-L spinner flasks filled with 200 mL culture volume and a DNA–PEI ratio of 1:3. Cells were directly transfected without a wash step. GFP-positive cells were quantified by FACS and EPO was measured by ELISA every 12 hpt

were performed using cells from early (between 40 and 77) and late (between 89 and 150) passages. In these experiments the process parameters cell concentration, viability, substrate and metabolite concentrations were determined as well as the concentrations of intracellular nucleotides.

Table 3 summarizes different transient transfections performed under serum-free medium conditions with 293s cells from an early and a late passage using both plasmids, pEPO-IRES2-EGFP and pCMV-EGFP-EPO. GFP-positive cells were identified between 60 and 72 hpt and the rHuEPO produced as well as the total protein concentration was measured. The results showed, that the cells from the early passage showed higher transfection efficiencies compared to 293s cells from a late passage. This result was observed with both plasmids, showing that the effect is not due to the specific nature of the vector. The expression of the rHuEPO also showed the same tendency as the transfection efficiency expressed by the GFP-positive cells. Due to the use of completely serum-free culture media rHuEPO represented between 2.5 and 10 % of the total protein concentration of the culture supernatants.

For comparison, similar experiments were performed with serum-supplemented medium. In this case, higher transfection efficiencies of up to 86 % were reached when late passage cells were transfected either with pEPO-IRES2-EGFP or pCMV-EGFP-EPO, while lower efficiencies ranging below 70 % were obtained for early passage cells. However, this effect was less pronounced than under serum-free conditions and was clearly not reflected by the expression observed for rHuEPO, which tended to be lower for cells from the late passages.

Intracellular nucleotide pools

Intracellular nucleotide pools have been reported to reflect the physiological state of BHK, hybridoma and

Table 3 Transfection of 293s cells in serum-free and serum-containing medium (DNA:PEI = 0.5 µg:1.5 µg), n.d. = not determined

Culture conditions	Cell passage number	Plasmid	GFP-positive cells [%] (60–72 hpt)	rHuEPO conc. [µg mL^{-1}] (60–72 hpt)	Total protein conc. [µg mL^{-1}] (60–72 hpt)
Serum-free	70 (early)	pEPO-IRES2-EGFP	73.22	1.54	14.80
	76 (early)	pEPO-IRES2-EGFP	70.49	1.58	17.12
	89 (late)	pEPO-IRES2-EGFP	51.02	1.17	34.51
	101 (late)	pEPO-IRES2-EGFP	55.62	1.29	51.18
	70 (early)	pCMV-EGFP-EPO	95.97	1.59	19.94
	101 (late)	pCMV-EGFP-EPO	69.48	n.d.	57.08
Serum-containing	40 (early)	pEPO-IRES2-EGFP	71.30	2.60	1,381.96
	42 (early)	pEPO-IRES2-EGFP	43.97	2.07	1,364.24
	116 (late)	pEPO-IRES2-EGFP	80.15	2.34	1,369.50
	42 (early)	pCMV-EGFP-EPO	58.37	n.d.	1,367.46
	150 (late)	pCMV-EGFP-EPO	86.39	1.41	1,380.88

A 15–25 % higher transfectability was found in cells of early passages cultured in serum-free medium and for the same cells of late passages cultured in serum-containing medium compared to late and early passage, respectively

CHO cell cultures (Ryll and Wagner 1992; Ryll et al. 1994; Grammatikos et al. 1999), since they represent a major driving force for cell growth and metabolism. In order to characterize the metabolic energy and the growth potential of transiently transfected 293s cells for optimizing the production process, the influence of different culture conditions on the intracellular nucleotide pattern of these cells was analyzed. 293s cells from early and late passages were cultivated in 200 mL spinner flasks in DMEM + FCS containing 5 % fetal calf serum and in serum-free DMEM-FS medium. In parallel, transfected and non-transfected batch cultures were compared to characterize the cells. Cell concentration, viability, substrate and metabolite concentrations as well as the concentration of intracellular nucleotides were determined daily.

Early versus late passage cultivation

Serum-supplemented growth medium

First, early passage 293s cells (passage 77) and late passage cells (101) growing under serum-containing (DMEM + FCS) conditions were evaluated. Figure 4a shows that cells from the exponential growth phase reached a concentration of about 3.5×10^6 mL^{-1} within 72 hps before they entered the stationary phase. During the first hours of cultivation (cultivation time: 0 and 24 h) the NTP/U ratio maintained at a very low level (below 3.3, horizontal dash line). In the initial growth phase of a culture, this indicates optimal physiological conditions. Subsequently, the ratio increased more than two-fold in cultures with cells from the early passages (from 3.3 to 9.8) and from 3.1 to 4.9 in cultures with cells from the late passages. This increase indicated the initiation of the phase of reduced exponential growth and a worsening of the physiological conditions, which can already be monitored at a very early time point by the determination of the nucleotide ratios (Ryll and Wagner 1992). This change in the state of the culture was confirmed after 72 h of cultivation by other cultivation parameters such as the cell concentration in both cultivations (vertical dash line). Usually, a 50–100 % increase of the NTP/U ratio from the basal level is sufficient to consider this phase as a sub-optimal cell state (Grammatikos et al. 1999). It is remarkable, that the increase of the NTP/U ratio drastically differed between cells from early and late passages. The NTP/U ratio reached a maximum of 128.6 for cells from the early passage 96 hps, whereas cells from the late passage reached a maximum of 67.9 48 h later (dotted lines).

The AEC remained high and revealed values of about 0.96 in both cell clones throughout batch culture (data not shown). This is a characteristic property of all continuous cell lines investigated so far (Ryll et al. 1991). Viability (Fig. 4b), glucose and lactate concentrations (Fig. 4c) as well as the LDH activity (Fig. 4d) were compared between both batch cultures. In each case the vertical dash line in the figures indicate the end of the exponential phase. The behavior of the cells from different passages was the same concerning nearly all cultivation parameters, except for the lactate concentration, which reached a 35 % higher value in cultures with cells from the early passage (Fig. 4c), indicating a lower cell specific lactate production rate in cells from the late passage. No significant differences were found in the LDH activity of both cultures (Fig. 4d).

Serum-free medium

Identical culture parameters were applied to cells growing under serum-free conditions (Fig. 5). 293s cells from the early passage 70 and from the late passage 101 were cultivated in DMEM-FS medium. Figure 5a shows that cells growth was comparable for cells from the early and the late passage. Both cell lines grew exponentially to a concentration of about 4.0×10^6 mL^{-1} within 72–96 hps before entering the stationary growth phase.

Although there were no significant differences observed concerning the growth behavior, the courses of the NTP/U ratio of the two cell lines showed remarkable differences. At the beginning of the cultivation, NTP/U ratios were very low (<2.5) for both cell lines, indicating an optimal physiological state of the cells. During the first 72 h, the NTP/U ratio raised steadily but very slowly (values <5) for both cultures. However, then a strong increase in the NTP/U ratio was observed for the cells of the late passage, reaching a value of about 200 at a cultivation time of 120 h, while the NTP/U ratio was still very low (<5) for the cells of the early passage at this time point. Nevertheless, it also increased for the cells from the early passage afterwards. For both cell lines, maximum NTP/U ratios of about 250–270 were reached during the stationary phase of cultivation, although at different time points.

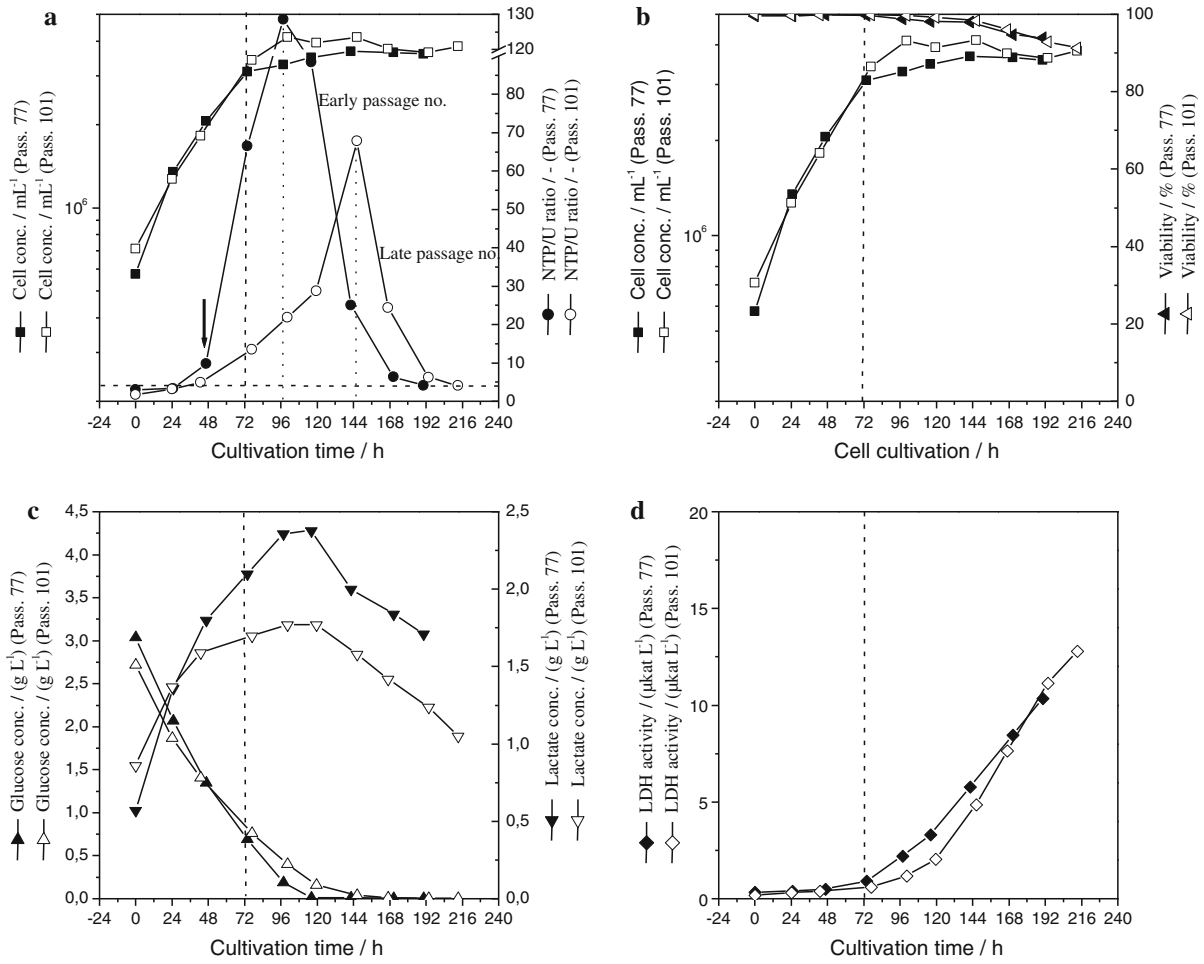

Fig. 4 Progress of 293s cell cultivation from an early (*filled symbols*) and a late (*open symbols*) passage cultured under serum-containing conditions (DMEM + FCS). Cell concentration (*square*) and NTP/U ratio (*circle*) (**a**): The *horizontal dash line* marks the bottom limit for low values of the NTP/U ratio, indicating an optimum physiological state. The *arrow* indicates the point at which an increase in the NTP/U ratio was observed indicating a worsening of the cell conditions. The *vertical dot lines* indicate the maximum increase of the NTP/U ratio for cultures with cells from the early and the late passage, respectively. The *vertical dash line* indicates the end of the exponential growth phase. Viability and total cell concentration (**b**), glucose and lactate concentrations (**c**) and the LDH activity (**d**) are compared between both cells ages. The *vertical black dash line* indicates the end of the exponential phase

The dramatic increase of the NTP/U ratio indicated the onset of a drop in the viability for both cell types (Fig. 5b). Therefore, the increase of the NTP/U ratios also correlated with the increase in LDH activity (Fig. 5d).

Cells grown under serum-free conditions reached the maximum value of their NTP/U ratio 42 h later compared to the corresponding culture grown in serum-supplemented medium (Figs. 4a, 5a). Remarkably, cells grown under serum-free conditions exhibited opposite NTP/U ratios. While the early passage cells showed a fast increase of the NTP/U ratio (onset at 48 h) compared to the late passage cells (onset at 72 h) under serum-containing conditions (Fig. 4a), in these cells the NTP/U ratio increased much slower (onset at 120–144 h) under serum-free conditions. In the late passage cells this increase occurred earlier (onset at 96 h) under these conditions. An AEC of 0.98–0.95 throughout the batch culture was found for low passage cells and of 0.97–0.93 for the respective cells from the late passage as being characteristic for continuous mammalian cell lines independent on the culture conditions selected (Ryll et al. 1991).

Although the growth behavior of both cell types was nearly identical (Fig. 5b), the course of glucose

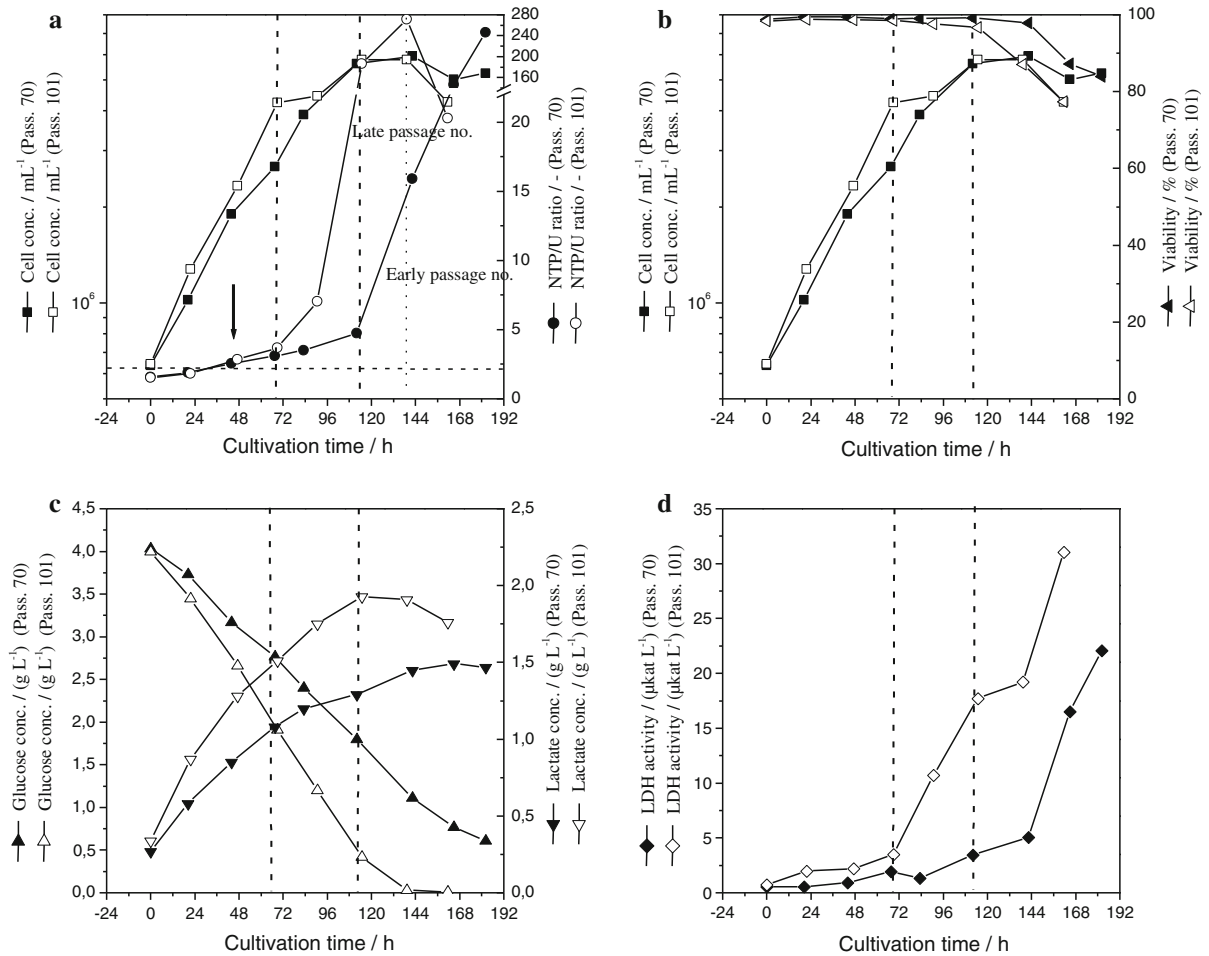

Fig. 5 Progress of 293s cells from an early (*filled symbols*) and a late (*open symbols*) passage cultured under serum-free conditions (DMEM-FS). **a** Cell concentration and NTP/U ratio were evaluated from two batch cultures of 293s from an early (*filled symbol*) and a late (*open symbol*) passage. The *horizontal dash line* showed low values of the NTP/U ratio indicating the optimal physiological state. The *arrow* indicates the point at which an increase in the NTP/U ratio was observed. The *vertical dot line* indicates the maximum increase of the NTP/U ratio for cells from the late passage. The *vertical dash lines* indicate the end of the exponential growth phase for cells from the late and the early passage, respectively. Viability and total cell concentration (**b**), glucose and lactate concentrations (**c**) and the LDH activity (**d**) are compared between cells from both passages. The *vertical dash lines* indicate the end of the exponential phase for early passage and the late passage, respectively

and lactate concentrations distinctly differed in both cultures (Fig. 5c). In cultures with late passage cells, glucose was already depleted 114 hps reaching a concentration of 0.42 g L^{-1} accompanied by a maximum lactate concentration of nearly 2.0 g L^{-1}. In contrast, at the same time, glucose was still present at a concentration of 1.8 g L^{-1} in cultures with cells from the early passage and the lactate concentration only reached 1.3 g L^{-1} upon serum-free growth. As a result of a higher glucose uptake rate, probably due to a faster metabolism, nutrient depletion occurred in the cultures with cells from the late passage at about 144 h, whereas no depletion was observed in the cultures with cells from the early passage.

Early versus late passage—transfection

Serum-containing medium

293 s cells from an early (40) and a late (116) passage were cultivated in serum-containing DMEM + FCS medium in spinner flasks (200 mL working volume). They were transfected following the standard protocol using an N/P ratio of 23 (0.5 µg pEPO-IRES2-EGFP

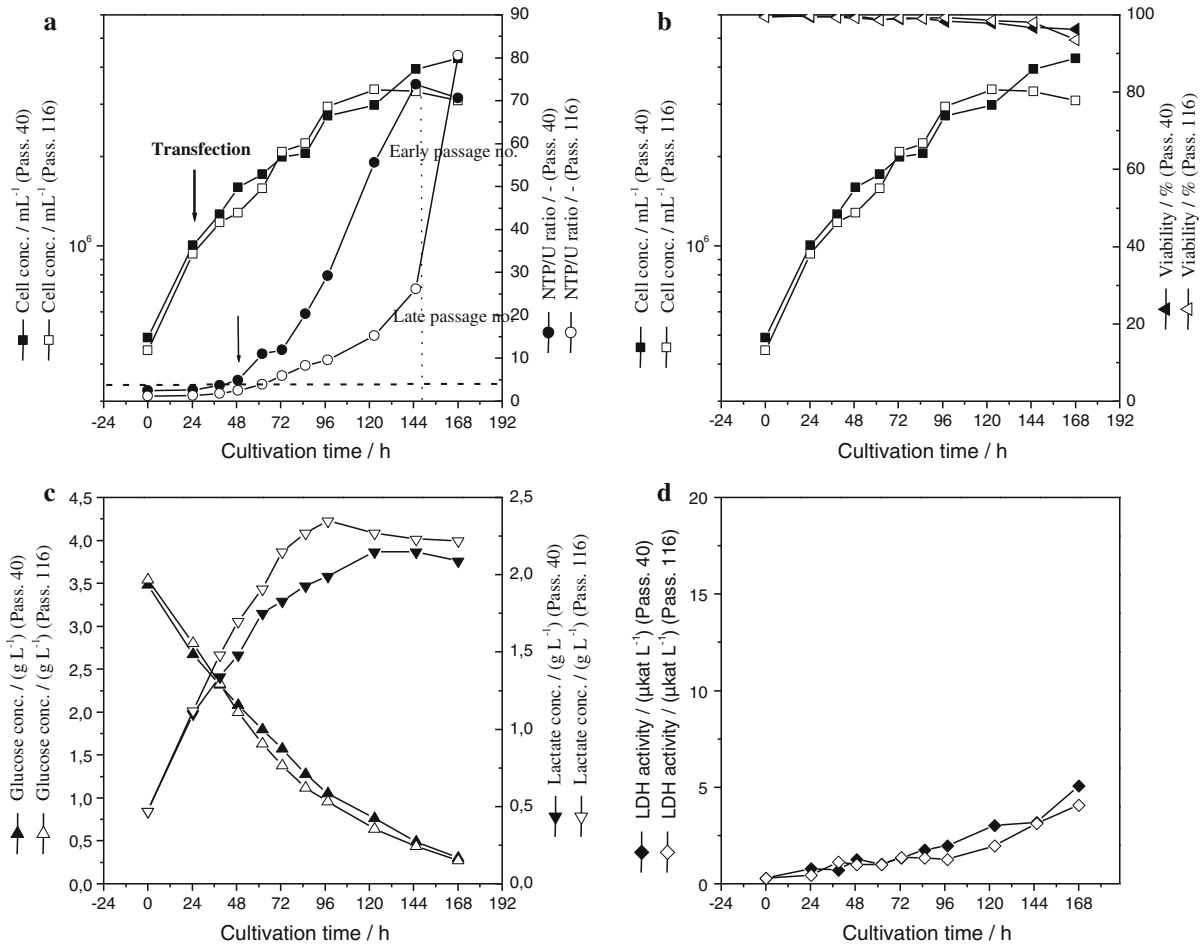

Fig. 6 293s cells from an early (*filled symbols*) and a late (*open symbols*) passage cultured under serum-containing conditions (DMEM + FCS) were transfected using 0.5 µg pEPO-IRES2-EGFP and 1.5 µg PEI. Cell concentration (*square symbol*) and NTP/U ratio (*circle symbol*) (**a**): The *horizontal dash line* shows the low values of the NTP/U ratio, indicating the optimal physiological state. The *arrow* indicates the point at which a slow increase in the NTP/U ratio was observed indicating a worsening of the cell conditions and the initiation of the reduced exponential growth. The *vertical dotted line* indicates the maximum increase of the NTP/U ratio of cells from the early passage. Viability and total cell concentration (**b**), glucose and lactate concentrations (**c**) and the LDH activity (**d**) during progress of cultivation and after transfection of the cells

plasmid and 1.5 µg PEI, 2×10^8 cells). GFP expression was measured by flow cytometry 60 hpt. The transfectability expressed as GFP-positive cells was determined to 71.3 % for cells from the early passage compared to 82.1 % in cultures with cells from the late passage. Figure 6a shows the course of 293 s cell cultivation and transfection, which was performed 24 hps at a cell concentration of 1.0×10^6 mL^{-1} (indicated by an arrow in Fig. 6a). Subsequently, the exponential cell growth continued, but at a reduced rate. During the first hours before transfection this ratio remained low (<5) but started to increase slowly for about 48–60 h (horizontal dash line). After that (denoted by the second arrow in Fig. 6a), the NTP/U ratio increased quickly, indicating a worsening of the cell state and the initiation of reduced exponential growth. Peak values about 70–80 were reached for both cell types at the end of cultivation. Interestingly, although both cell types showed an identical growth behavior, cultures of the low passage cells showed an earlier increase of the NTP/U ratio, which started already 36 hpt in contrast to the cells from a late passage, which showed a delayed onset of the increase at 60 hpt. Although the cells from the late passage reached the stationary phase at the same time as the corresponding cells from the early passage, a

characteristic decline in the NTP/U ratio could not be determined indicating a different behavior of these cells as already shown for the respective untransfected cells under serum-containing conditions (Fig. 4a). For both cell types the course of the NTP/U ratio showed a reduced slope after transfection (Fig. 6a) compared to the respective untransfected cells (Fig. 4a) under serum-containing conditions. In addition, untransfected cells from the late passage showed a lower maximum NTP/U value of 67.9 compared to the early passage (128.61), when cultured in serum-containing medium. In contrast, the respective transfected cells revealed an opposite property concerning their NTP/U maxima which reached 73.7 for the early passage and 80.5 for the late passage, which, however, did not yet represent the peak value (Fig. 6a). The viability remained at a high level for the entire cultivation time for both cultures (Fig. 6b). This result indicated a different impact of the transfection procedure on cells of early and late passages. The AEC showed steady values of approximately 0.97 during the process time for both batch cultures (data not shown), which is characteristic for the continuous cell lines investigated so far (Ryll et al. 1991). In contrast to untransfected cells under serum-containing and serum-free conditions (Figs. 4c, 5c) the course of the glucose and lactate concentrations of transfected cells from the early and late passages in serum-containing medium did not differ significantly (Fig. 6c). The LDH activity showed comparably low maximum values of 5.07 and 4.07 μkat L^{-1} for cells from the early and the late passage, respectively, at the end of cultivation at 168 hps, indicating a high cell integrity in both cases (Fig. 6d).

Serum-free growth medium

293s cells from the early passage 70 and from the late passage 101 were cultivated in serum-free DMEM-FS medium in 200 mL spinner flasks. They were transfected following the standard protocol using 0.5 μg pEPO-IRES2-EGFP together with 1.5 μg of PEI for complex formation as explained above. GFP expression was evaluated using flow cytometry 60 hpt. A substantially higher transfection efficiency was reached in cultures with cells from the early passage (73.2 %) compared to cultures with cells from the late passage (55.6 %). The transfection procedure was performed at a cell concentration of 1.0×10^6 mL^{-1} as shown in Fig. 7a (indicated by an arrow). Subsequently, the growth rate of both cultures slightly decreased, as already shown for transfected cells under serum-containing conditions (Fig. 6a). However, under serum-free conditions, cells from the early passage showed a significantly reduced growth compared to the cells from the late passage. This might be due to the observed difference in transfection efficiency, leading to a higher percentage of transfected cells in the culture with cells from the early passage, which may show reduced growth compared to non-transfected cells. The initial NTP/U ratio was below 2.3, indicating the optimal physiological state, and remained more or less constant during the first hours of cultivation before transfection for both cell cultures (horizontal dash line). Then, the NTP/U ratio slowly increased up to 5 during the following 24 hpt (Fig. 7a). The NTP/U ratio of cells from the late passage dramatically increased under serum-free conditions after 90 hpt and reached a maximum of more than 250 at 144 hps (dotted line). In contrast, the NTP/U ratio of the respective cells from the early passage did not reach a maximum and increased slowly and steadily to a comparatively low value about 10. Consequently, the maximum value of the NTP/U ratio for the cells from the late passage was more than 20-fold higher compared to the respective value obtained from cells of the early passage indicating a drastic difference in cell metabolism. The AEC maintained at 0.98 during both batch cultures and decreased to 0.90 at the end of the culture (161.50 h) for transfected cells from the late passage indicating the beginning of apoptotic conditions. The viability maintained above 90 % during the complete course of cultivation with a decrease to 87 % at the end of the batch culture of transfected cells from the late passage (Fig. 7b). This reduced viability at 161.5 hps correlated to the drastic increase of the LDH activity to 15.14 μkat L^{-1} (Fig. 7d). As already observed for cultures with cells from the late passage under the same medium conditions but without transfection (Fig. 5c), glucose was completely depleted after 140 hps and the lactate concentration reached a maximum of 2.18 g L^{-1} at this time point (Fig. 7c). In the respective culture with cells from the early passage, however, glucose was still present at a concentration above 1 g L^{-1} at the end of cultivation and the lactate concentration reached only a maximum level of 1.6 g L^{-1}. As observed before (Fig. 5), the cells from the late passage reached the stationary phase earlier than the cells from the early passage.

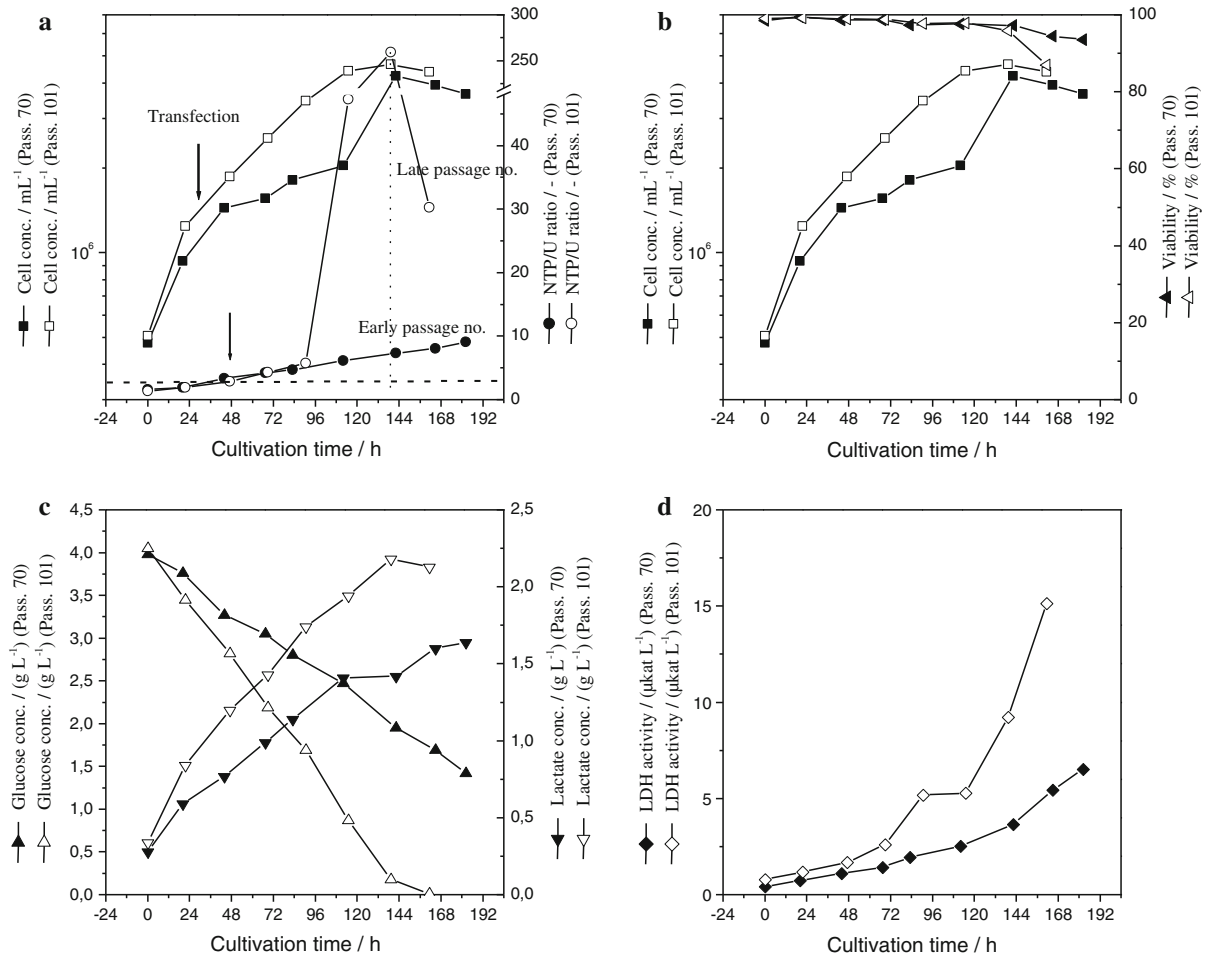

Fig. 7 293 s cells from an early (*filled symbols*) and a late (*open symbols*) passage cultured under serum-free conditions (DMEM-FS) were transfected using 0.5 µg pEPO-IRES2-EGFP and 1.5 µg PEI. Cell concentration (*square symbol*) and NTP/U ratio (*circle symbol*) (**a**): The *horizontal dash line* shows the low values of the NTP/U ratio indicating the optimal physiological state. The *arrow* indicates the point at which a slow increase in NTP/U ratio was observed indicating a worsening of the cell conditions and the initiation of the reduced exponential growth. The *vertical dotted line* indicates the maximum increase of the NTP/U ratio of cells from a late passage. Viability and total cell concentration (**b**), glucose and lactate concentrations (**c**) and the LDH activity (**d**) during progress of cultivation and after transfection of the cells

Discussion

The importance of transient transfection for the manufacturing of recombinant biopharmaceuticals is increasing steadily, especially for early upstream process development. Recently, Backliwal et al. (2008) reported a completely serum-free process. The process involved a suspension-adapted HEK293E cell line (Durocher et al. 2002), which was multi-pathway modulated, and a PEI-based transfection protocol, that yielded 1.1 g L^{-1} of an IgG antibody within 14 days of culture in an orbital shake system. However, optimal transient transfection and recombinant protein expression is only achieved when appropriate host cell lines, transfection vehicle, expression vectors and culture media are used at optimized conditions and concentrations. Therefore, we performed a series of experiments to identify important parameters which influence transient transfection of HEK 293 cells. The knowledge of key factors then can

be used to improve transient transfection protocols. In particular, the role of intracellular nucleotide pools and their ratios was investigated in this context.

The culture medium

The use of serum as a medium supplement is critical for the manufacturing of recombinant biopharmaceuticals due to the associated contamination risk and the respective safety considerations. Therefore, serum-free culture media should be used even in plasmid transfections for generation of recombinant cell lines which are subsequently used in manufacturing processes.

Different research groups reported a serum-free formulation to support growth of HEK-293 cells (Berg et al. 1993; Coté et al. 1998; Schlaeger and Christensen 1999; Durocher et al. 2002). In addition, several commercial, serum-free media were developed. However, these media did not satisfy all the prerequisites for a large-scale transient transfection process. Schlaeger and Christensen (1999) described a serum-free medium, which was supplemented with heparin to avoid cell aggregation. Since heparin inhibits the DNA uptake during the transfection procedure, it has to be removed from the medium before transfection. Consequently, this would require a washing step with a heparin-free medium. For the commercial FreeStyle™ 293 Expression Medium the use of a special transfection medium (Opti-MEM®), which contains about 1 % of FCS is recommended by the manufacturer. Therefore, this system when applied to a transient transfection process for protein production would re-introduce serum into a process that preferentially should be serum-free. Modifications of this medium were also applied to HEK-293 cells, but also required 1 % FCS to improve PEI-mediated gene delivery (Durocher et al. 2002; Pham et al. 2005). The authors suggested that serum supplementation might increase the transcriptional activity of the promoter as the CMV immediate early enhancer contains multiple binding sites for serum-activated transcription factors (Brightwell et al. 1997). However, they obtained only a partial recovery of transgene expression when serum was added to the cells 3 h after transfection in serum-free medium, suggesting that, in addition to the potentially serum-mediated transcriptional activation of the CMV promoter, other serum components might increase the transfection efficacy of DNA-PEI complexes (Durocher et al. 2002).

In a first set of experiments, we tried to establish transient transfection of HEK 293 cells using commercially available, serum-free media. However, we obtained only very low transfection efficiencies of 0.1–3.0 %. In order to investigate whether the presence of serum improves the portion of expressing cells, 1 % FCS was added to the different cultures grown in the absence of serum. Interestingly, expression values declined twofold and transfection efficiencies lower than 1.5 % were obtained (Table 1). These results suggested that the low transfectability might be due to an inhibition by components of the serum-free medium rather than the lack of unknown essential components provided by the addition of FCS. This hint was verified when 60 % higher transfection efficiencies compared to commercial media were obtained using the 293s cells cultivated in a 1:1 mixture of FreeStyle™ 293 Expression Medium with DMEM-Ca medium compared to FreeStyle™ 293 Expression Medium alone (see Table 2). It will be important in the future to identify the unknown inhibitory component(s) in order to better understand the interaction of serum in PEI-mediated transfection processes.

The transfection reagent

The cationic polymer PEI exhibits several properties facilitating gene delivery (Boussif et al. 1995, 1996). PEI-mediated transfection methods are highly efficient in a broad range of different cell lines. The cationic agent shows a low level of cell toxicity, is simple to use and is effective in suspension cultures. Moreover, it is inexpensive and therefore useful for scale-up experiments (Schlaeger and Christensen 1999; Wurm and Bernard 1999; Durocher et al. 2002; Pham et al. 2005, 2006). The polymer is available in both, linear and branched isoforms with a wide range of molecular weights and polydispersities (Godbey et al. 1999). The transfection efficiency and cytotoxicity (Moghimi et al. 2005) of PEI-based transfection systems depends on the molecular weight (MW), the degree of branching, and the cationic charge density and buffer capacity of the polymer (Godbey et al. 1999; von Harpe et al. 2000; Kunath et al. 2003). High MW branched polyethylenimine (BPEI) has been shown to have a superior transfection efficiency compared to BPEIs with lower MW (Godbey et al. 1999), but unfortunately, the higher transfection efficiency was accompanied by a decrease in cell viability. Therefore, among branched PEIs, a MW of 25 kDa is

commonly regarded as most suitable for gene transfer. Polyplexes containing linear polyethylenimines (LPEIs) have recently been shown to have an improved transfection efficiency and cell viability compared to BPEI-based transfection systems (Ferrari et al. 1997; Bragonzi et al. 1999; Goula et al. 1998, 2000). Considering these findings, we used a 25 kDa linear PEI and investigated the effect of different DNA–PEI ratios on the transfection efficiency. The highest transfection efficiency (>70 %) was obtained with a DNA–PEI ratio of 0.5:1.5 (μg:μg), corresponding to an N/P ratio of 23, with 293s cells cultured in a serum-free 1:1 mixture of calcium–free DMEM and FreeStyle™ 293 Expression Medium. DNA–PEI ratios of 1:3 have been reported to give highest expression rates using suspension-adapted HEK-293EBNA cells (Muller 2005), which confirms the findings achieved with the 293s cell line

The impact of passage number on transfectability

Several transfections using cells from an early and a late passage and the two bicistronic plasmids bearing the gene coding for rHuEPO and for EGFP in the first and the second position of the transcribed mRNA and vice versa revealed, that cells from an early passage showed a higher transfection efficiency under serum-free conditions, compared to their counterparts from the late passage (Table 3). This tendency was shown for the expression of both, the reporter GFP and the model gene rHuEPO. Under serum-containing conditions, no clear effect of the passage number on transfection efficiency could be observed. While the percentage of GFP-positive cells surprisingly was higher in transfections with cells from the late passage in the presence of serum, no significant differences in the rHuEPO concentrations obtained with cells from early and late passage could be observed. However, it must be mentioned that the expression of rHuEPO was not analysed on the cellular level. Therefore, it cannot be ruled out that the transfection efficiency for the rHuEPO gene is higher in the advanced cell culture as it could be possible that those cells have a less efficient secretory machinery leading to a rather low cell specific productivity. This would not concern GFP production as this protein is not processed via the secretory pathway.

In any case, the results clearly demonstrate an impact of the passage number on the transfectability of HEK 293 cells. It became also evident that the passage-dependence of the transfection procedure can be influenced by the cultivation conditions such as medium composition.

The rHuEPO concentration achieved under serum-containing culture conditions was higher than in serum-free medium. One reason for this difference could be the release of proteases into the medium (Kratje et al. 1991). This process may occur during cell proliferation as well as after cell death and following lysis. Medium supplemented with serum inhibits proteases, as inhibitors make up 10 % of the total protein content of serum. Therefore, cultures lacking serum, proteases may exert a negative effect upon cell proliferation as well as on the integrity and yield of the desired protein product (Murakami 1998).

The nucleotide ratios

Ryll and Wagner (1992; Ryll et al. 1991) demonstrated the application and usefulness of the intracellular analysis of nucleotides for the cultivation of animal cells and the control of respective processes. Especially the specific combination of these parameters as expressed in particular functions, such as the NTP, U, and NTP/U ratio which are characteristic for the metabolic state of the cell. Several studies (Ryll and Wagner 1991, 1992; Ryll et al. 1994; Moore et al. 1997; Grammatikos et al. 1998, 1999; Schoenherr et al. 2000) verified the applicability of these ratios, and extended their application for monitoring, control, and regulation of bioprocesses. In particular, the sensitivity of the NTP/U ratio and its ability to report on the physiologic status and growth potential of the cells generated a tool to predict the growth behavior of a culture, which was shown for BHK, hybridoma, and CHO cells. The determination of nucleotide ratio NTP/U has been used to monitor cell growth during the exponential phase and represents a predictive value for the onset of the stationary phase in cell culture. In the present work, these results were further extended to suspension cultured HEK-293 cells (293s). Especially, the NTP/U ratio amplifies the growth-dependent NTP (Eq. 3) and U (Eq. 4) ratios by their combination (Eq. 5). This parameter allowed the prediction of the behavior of cells in culture up to 2 days before any changes were noted by classic cell number and viability measurements (see Figs. 4a, 5a, 6a, 7a) (Ryll and Wagner 1992; Grammatikos et al. 1999; Schoenherr et al. 2000).

Nucleotide ratios, passage number and glucose metabolism

HEK-293 suspension cells cultivated either in serum-containing or in serum-free media showed a different property depending whether they were taken from cells of an early (40–77) or a late (89–150) passage. During the course of all batch cultures performed in this work an elevated NTP/U ratio could be calculated (Figs. 4a, 5a, 6a, 7a), however, the increase in the NTP/U ratio occurred at different period. Under serum-free conditions, the maximum NTP/U ratio was reached later for cells from an early passage compared to cells from a late passage (Fig. 5a). In contrast, cells from an early passage growing under serum-containing conditions reached the maximum NTP/U ratio faster than cells from a late passage (Fig. 4a). The maximum NTP/U values were the result of the opposing values of the NTP and the U ratio, which have reached their maximum and minimum at that phase, respectively (see Fig. 8). As was demonstrated earlier (Ryll and Wagner 1992; Grammatikos et al. 1999) and now also confirmed for HEK-293 cells in our experiments (Fig. 8), the NTP ratio increased during progress of the culture, as a result of an appropriate decrease in the cellular UTP and the CTP pools. In parallel, the U ratio decreased which was mainly due to a decrease of the UTP pool and an increase of the respective UDP-GNAc pool as in all cases analyzed so far for continuous animal cell lines (Ryll and Wagner 1992). Surprisingly, this effect did not correlate with the growth behavior of the cells as cells from early and late passages showed comparable growth characteristics.

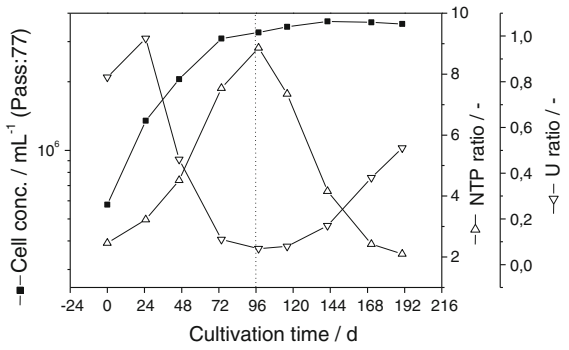

Fig. 8 Progress of 293s cells from the early passage (see Fig. 4) cultured under serum-containing conditions (DMEM + FCS). Cell concentration, NTP, and U ratio. The *vertical dot line* indicates the maximum of the NTP ratio and the minimum of the U ratio. At this point of cultivation time 96 hps, which is exactly identified by the NTP ratio, the starvation phase starts

Another difference between cells from different passage numbers was the glucose utilization. Cells taken from a later passage and cultured under serum-containing conditions utilized glucose more efficient compared to those from the earlier passage. Although glucose was nearly identically consumed in this case, independent of the passage number, lactate production was 35 % lower and glucose limitation occurred about 1 day later in cells from the late passage 101 (Fig. 4c). This behavior changed when the same cells were cultured under serum-free conditions. Particularly, cells from the early passage (passage number 70) consumed glucose and produced lactate at lower rates (Fig. 5c), although the cell growth was comparable for cells from both passages under both medium conditions (Figs. 4a, 5a). The cells from the late passage 101, however, showed nearly identical glucose-lactate rates under serum-containing and serum-free medium conditions. While the passage number of the cells had an impact, the transfection procedure did not influence glucose metabolism as the course of glucose utilization and lactate secretion was comparable for transfected and non-transfected cells (Figs. 6, 7).

When comparing the course of the NTP/U ratios with the course of the glucose concentrations, it became obvious that the pronounced decrease in the NTP/U ratio during the later phase of the batch cultures always occurred just after glucose was depleted, indicating a substrate limitation on the metabolic level (Figs. 4a, c, 5a, c, 6a, c, 7a, c). Actually, glucose depletion (glucose concentration <0.5 g L^{-1}) is known to release the starvation phase, which is exactly indicated by the NTP/U ratio. If no glucose depletion occurred the NTP/U ratio increased steadily during the course of cultivation. Also a correlation between the level of the NTP/U ratio and the glucose concentration could be found as high values for the NTP/U ratio (>10) were only observed when the glucose concentration dropped below 1.5 g L^{-1} (Figs. 4, 5, 6, 7). These observations indicated a close link between glucose and nucleotide metabolism in HEK-293 cells, leading to a regulation of nucleotide pools by the glucose concentration. Obviously, the cells reacted to a decreasing glucose concentration in the cell culture medium with a pronounced rearrangement of the nucleotide pools, after a certain threshold

level was reached. The drop in the values of the NTP/U ratios after glucose depletion could possibly be explained with the decrease in the UDP-GNAc pool (data not shown, see also Ryll and Wagner 1992) as glucose is needed to maintain the UDP-GNAc levels.

The application of nucleotide ratios for the evaluation of cell's susceptibility to high-level transient gene expression

When comparing HEK-293 cells from early to those of late passages up to 25 % higher transfectabilities could be observed for the cells from the early passages, however, only when serum-free conditions were used. In contrast, when FCS was present in the medium, a higher transfectability was found for cells from late passages (Table 3). Furthermore, for transfected cells of the late passage in serum-containing medium (Fig. 6a) and for the respective cells of the early passage under serum-free conditions (Fig. 7a), the NTP-U maximum was reached substantially later compared to the same cells of the respective other passage. The delayed maximum NTP-U value which was observed revealed higher transfection efficiencies compared to their respective counterpart (82.1 vs. 71.3 % in serum-containing medium and 73.2 vs. 55.6 % in serum-free medium). Highest transfection efficiencies were obtained when a low cellular UDP-GNAc content (low NTP/U ratio) was measured during the complete batch culture (data not shown). In serum-free conditions, transfected cells from an early passage showed a low percentage of UDP-GNAc (9–11 %) during the entire culture, whereas cells from a late passage offered values from 10 % at the beginning of the culture up to 35 % at the end at 168 h. In contrast, cells cultured with serum-containing medium showed the lowest percentage of UDP-activated sugars for cells from a late passage (from 11 to 40 %, at the end of the batch culture), whilst transfected cells from an early passage showed values from 14 to 55 % at the same time. Previous work considered the intracellular UDP-GNAc content as a key regulator of cell viability and productivity (Ryll et al. 1994; Barnabé and Butler 1998). In this context, Ryll et al. (1994) suggested that an elevated intracellular UDP-GNAc pool is an indicator of the loss of cell viability induced in cultures by the accumulation or addition of ammonia. Furthermore, it was proposed that if the UDP-GNAc pool is kept at low levels during a production process, it is conceivable that cell growth is improved and uniform product quality is maintained (Grammatikos et al. 1998). In addition, it was shown here, that cultures under the influence of a low percentage of UDP-GNAc also responded with higher transfection efficiencies (Table 4).

Table 4 Transfectability of cells from an early and a late passage cultured under serum-containing and serum-free medium conditions

Culture conditions	Transfection of 293s cells (DNA:PEI= 0.50 μg:1.50 μg)	Transfectability expressed in GFP-positive cells (%) (60 hpt)
Serum-containing	Early passage (40)	71.30
	Late passage (116)	82.07
Serum-free	Early passage (70)	73.22
	Late passage (101)	55.62

Conclusions

Our results present a new approach for the transient transfection of HEK-293 cells in a medium totally free of proteins or animal-derived components, taking into consideration the possible impact of the cell passage number on the transfectability. To our knowledge this is the first report about a passage number effect on the expression efficiency of transiently transfected HEK-293 cells in suspension culture. Furthermore, nucleotide pools and ratios proved to be very sensitive parameters providing valuable information about the transfectability of a cell line. An application of these parameters may therefore help to improve transient transfection protocols in the future.

With our experiments, we also demonstrated that nucleotide ratios can be used to distinguish between early and late passage HEK-293 cells indicating a change in the cell's character during cultivation. The later and the lower increase in the NTP/U ratio of cells from late passages in comparison to those of early passages elucidated a change in the cell physiological character during lifetime of 293s cells. It was shown before that the absolute value of the NTP/U ratio for cells in good physiological state varies with the cell type, medium and culture mode (Grammatikos et al. 1998). Now, we could demonstrate that also the time period a cell line is in culture can have an effect on the

NTP/U ratio. We also could show that the changes in the NTP/U ratio seemed to be closely linked to the glucose metabolism of the cells. All these results could also have an impact on the generation of recombinant cell lines choosing the favorable time in the lifetime of a cell for an efficient and stable transfection.

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