ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Serum-Free Transient Protein Production System Based on Adenoviral Vector and PER.C6 Technology: High Yield and Preserved Bioactivity

M.J.E. Havenga, L. Holterman, I. Melis, S. Smits, J. Kaspers, E. Heemskerk, R. van der Vlugt, M. Koldijk, G.J. Schouten, G. Hateboer, K. Brouwer, R. Vogels, J. Goudsmit

Crucell Holland BV, PO Box 2048, 2301CA Leiden, The Netherlands;
telephone: +31-71-5248736; fax: +31-71-5248702; e-mail: m.havenga@crucell.com

Received 2 August 2007; revision received: 2 November 2007; accepted 14 November 2007
Published online 13 December 2007 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/bit.21757

ABSTRACT: Stable E1 transformed cells, like PER.C6, are able to grow at scale and to high cell densities. E1-deleted adenoviruses replicate to high titer in PER.C6 cells whereas subsequent deletion of E2A from the vector results in absence of replication in PER.C6 cells and drastically lowers the expression of adenovirus proteins in such cells. We therefore considered the use of an ΔE1/ΔE2 type 5 vector (Ad5) to deliver genes to PER.C6 cells growing in suspension with the aim to achieve high protein yield. To evaluate the utility of this system we constructed ΔE1/ΔE2 vector carrying different classes of protein, that is, the gene coding for spike protein derived from the Coronavirus causing severe acute respiratory syndrome (SARS-CoV), a gene coding for the SARS-CoV receptor or the genes coding for an antibody shown to bind and neutralize SARS-CoV (SARS-AB). The ΔE1/ΔE2A-vector backbones were rescued on a PER.C6 cell line engineered to constitutively over express the Ad5 E2A protein. Exposure of PER.C6 cells to low amounts (30 vp/cell) of ΔE1/ΔE2 vectors resulted in highly efficient (>80%) transduction of PER.C6 cells growing in suspension. The efficient cell transduction resulted in high protein yield (up to 60 picogram/cell/day) in a 4 day batch production protocol. FACS and ELISA assays demonstrated the biological activity of the transiently produced proteins. We therefore conclude that ΔE1/ΔE2 vectors in combination with the PER.C6 technology may provide a viable answer to the increasing demand for high quality, high yield recombinant protein.

Biotechnol. Bioeng. 2008;100: 273–283.

KEYWORDS: protein production; human PER.C6® cells; recombinant adenovirus; serum-free medium

Introduction

To date different platforms are used for production of recombinant proteins ranging from bacteria, yeast and fungi to insect-, plant-, and mammalian cells (Andersson et al., 2001; Bao and Fukuhara, 2001; Boedeker, 2001; Cai, 2001; Chen et al., 2001; Friehs and Reardon, 1993; James and Lee, 2001; Jønson and Johnsen, 2001; Konishi et al., 2002; Lazaris et al., 2002; Levy et al., 2001; Mattes, 2001; Massaer et al., 2001; McQuillan et al., 2001; Menassa et al., 2004; Sarkar and Sen, 1998). Given the importance of human type post-translational and peri-translational processing for protein half-life, biological activity, secretion, and protein yields (Berg et al., 1993; Breitbach and Jarvis, 2001; Fussenegger et al., 1999; Jenkins et al., 1998; Kawashima et al., 1993; Lawrence et al., 2001; Lee et al., 1989; Marchal et al., 2001; Mosser et al., 1994; Seo et al., 2001), either human derived cell lines or non-human systems adapted to express human enzymes involved in post-transcriptional modification of proteins, are preferred (Côté et al., 1998; Lawrence et al., 2001; Marchal et al., 2001; Seo et al., 2001). However, mammalian cell-based protein production is time-consuming since it requires stable cell line generation, sub-cloning, screening, and cell clone-selection. Moreover, this process is costly due to the required safety testing of both candidate products as well as cell banks. As such this process does not allow for the rapid production of high quality recombinant protein batches required for candidate protein selection or rapid evaluation of a candidate protein in a clinical setting. Because of these limitations, research attention focuses on novel systems and technology for transient production of high quality protein. Although predominantly large-scale DNA transfection is being investigated (Baldi et al., 2007; Derouazi et al., 2004; Tait et al., 2004) adenoviral vectors are being considered as well (Andersen and Krummen, 2002; Côté et al., 1998; Garnier et al., 1994; Gossen et al., 1994; Massie et al., 1995; Sarkar et al., 2001; Tom et al., 1995; Wu et al., 1996). Attractive features of the adenoviral vector approach for transient protein production include (i) the ability of adenovirus to actively bind to cells growing in suspension, (ii) the high efficiency of vector internalization, and (iii) the high efficiency of nuclear transport of the

M.J.E. Havenga and L. Holterman contributed equally to this work.
Correspondence to: M.J.E. Havenga

adenovirus genome coding for a gene of interest. Because of these features it can be envisioned that the use of adenoviral vectors results in higher protein yields derived from mammalian cells cultured in suspension as compared to current DNA transfection protocols for which processes at scale like cell uptake and nuclear localization, present major technical hurdles.

However, the use of an adenoviral vector based transient protein production system raises several safety issues, which need to be addressed thoroughly. Most importantly, absence of replication competent adenovirus (RCA) as well as replication deficient (ΔE1) virus needs to be demonstrated. Besides these critical parameters, production of proteins derived from the viral backbone is undesired as it may interfere with cell proliferation and viability and could impact on protein purification protocols (Ladisch et al., 1979). Finally, the transient production of protein should be scalable and ideally be performed in absence of any serum components to circumvent transmissible spongiform encephalopathies (TSE) and further facilitate protein purification.

Here we show that ΔE1/ΔE2A Ad5 vectors, produced on PER.E2A cells, are unable to replicate on PER.C6 cells and do not express adenoviral capsid proteins in human indicator cells. We further demonstrate that exposure of PER.C6 cells to a ΔE1/ΔE2A vector carrying the gene coding for SARS-CoV spike protein, the cellular SARS receptor (ACE2) or a SARS-AB results in high yield protein (up to 600 mg/L). Finally, by performing FACS and ELISA analyses we demonstrate that proteins produced using this transient production platform are biologically active. We thus present a new platform that ensures rapid and high yield production of different classes of proteins. Production can be performed in a virus- and serum-free environment, is suitable for scaling to large bioreactors, and thus provides a promising alternative for established protein production systems.

Materials and Methods

Generation of PER.E2A Cell Line and Cell Culture

The complete ts125E2A-coding region was PCR-amplified from DNA isolated from the temperature sensitive adenovirus mutant H5ts125 (Ensinger et al., 1972; Vliet and Sussenbach, 1975; Vliet et al., 1975) with primers DBPpcr1 and DBPpcr2 using the Expand™ Long Template PCR system according to the standard protocol of the supplier (Boeringer, Mannheim, Germany). The primer DBPpcr1: CGG GAT CC*G CCA CC* **A TG**G CCA GTC GGG AAG AGG AG (5′–3′) contained a unique *Bam*HI restriction site (underlined) 5′ of the Kozak sequence (italic) and start codon of the E2A coding sequence (bold). The primer DBPpcr2: CGG AAT TC**T TA**A AAA TCA AAG GGG TTC TGC CGC (5′–3′) contained a unique *Eco*RI restriction site (underlined) 3′ and the stop codon of the E2A coding sequence (bold). The PCR fragment was digested with *Bam*HI/*Eco*RI and cloned into plasmid pcDNA3 (Invitrogen, Breda, The Netherlands) and sequenced giving rise to pcDNA3tsE2A. For transfer of the E2A region, PER.C6 cells were transfected with 5 μg pcDNA3 plasmid containing no insert, wild type E2A, or ts125E2A using the lipofectAMINE Plus reagent kit according to the protocol supplied (Invitrogen). Seventy-two hours after transfection, cells were seeded in DMEM/10% FBS/10 mM $MgCl_2$, supplemented with 0.25 mg/mL G418 to select for transfectants. Stable transfected clones were routinely maintained in the same medium, however the G418 selection was withdrawn from the cultures at the moment ΔE1/ΔE2 viruses were produced. The E2A expression levels in different cell clones were determined by Western blotting using monoclonal antibody B6 (Reich et al., 1983) and horseradish-peroxidase-conjugated-goat anti mouse antibody (BioRad, Hercules, CA) as first and second antibody, respectively. Western blotting procedure and incubations were performed according to the protocol provided by Millipore (Billerica, MA). The complexes were visualized with the ECL detection system according to the manufacturer's protocol (Amersham, Piscataway, NJ). In addition, the selected cell lines were tested for their ability to complement the deleted E2A gene function of adenovirus Ad5.d1802 (Rice and Klessig, 1985) by demonstrating the appearance of cytopathic effect (CPE) upon infection at 32°C. To obtain cells growing in suspension, one selected PER.E2A cell line was cultured at 39°C and 10% CO_2 in a 175 cm^2 tissue culture flask in DMEM, supplemented with 10% FBS and 10 mM $MgCl_2$. At 70–80% confluency cells were washed with PBS (NPBI) and the medium was replaced by 25 mL serum-free suspension medium SFM consisting of Ex-cell™ 525 (JRH) supplemented with 1× L-glutamine (Gibco BRL). Two days later, cells were detached from the flask by flicking and the cells were centrifuged at 1,000 rpm for 5 min. The cell pellet was re-suspended in 5 mL SFM and 0.5 mL cell suspension was transferred to an 80 cm^2 tissue culture flask, together with 12 mL fresh SFM. After 2 days, cells were harvested. Next cells were seeded in a 125 mL tissue culture Erlenmeyer (Corning, Corning, NY) at a seeding density of 3×10^5 cells/mL in a total volume of 20 mL SFM. Cells were further cultured at 125 rpm on an orbital shaker at 39°C, 10% CO_2. Cells were counted at days 1–6 in a Burker cell counter. Human PER.C6® cells are routinely maintained in a humidified 37°C environment in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10 mM $MgCl_2$, either in the presence or absence of fetal bovine serum (FBS). Human A549-cells were obtained from the American Type Culture Collection (ATCC, Mannasas, VA) and were maintained in DMEM/10% FBS. For infection, PER.C6 cells were seeded in roller bottles (100 mL volume) at 0.3×10^6 cells/mL in AEM medium (Invitrogen) supplemented with L-glutamine. Cells were cultured in roller bottles (2 rpm) in a 37°C incubator at 10% CO_2. When cell concentrations reached 2×10^6 cells/mL, cultures were centrifuged for 10 min at 500*g* and pellets were re-suspended in 100 mL fresh AEM/L-glutamine at a final cell concentration of 2.5×10^6 cells/mL. The next day cells were counted using a Casey counter (Schärfe system)

and virus was added to the roller bottles corresponding to the desired MOI. For infection of PER.C6 cells in shaker flasks (25 mL culture volume) cells cultured in roller bottles were counted, centrifuged and cell pellets were re-suspended in fresh AEM/L-glutamine at 2.5×10^6 cells/mL after which virus was added at the desired MOI. Cells were cultured in shaker flasks at 75 rpm, 37°C, 10% CO_2. Harvest of medium was done between day 3 and 14, depending on the type of experiment and the protein expressed.

Construction of ΔE1/ΔE2 Plasmid System and Vector Generation

Recombinant ΔE1/ΔE2 viruses were generated by a homologous recombination procedure using an identical plasmid-based system as previously described for the production of E1-deleted vectors (Havenga et al., 2001) with the exception that a modified cosmid (pWE/Ad.AflII-rITR.ΔE2A) lacking the E2A gene was used. To remove the E2A from the adenoviral backbone assembly PCR amplification and standard molecular cloning techniques were used. Co-transfection into mammalian cells of this cosmid with an expression plasmid encoding the left 5 kb of the adenoviral genome (pAdapt), results in homologous recombination leading to a complete Ad5 genome. The cDNA (3,767 bp) coding for full-length SARS-CoV S1-spike (Brink et al., 2005) was cloned into the expression cassette of pAdapt whereas also the 1,695 bp fragment of S1-spike obtained through restriction analyses was cloned into the expression cassette of pAdapt in order to obtain a 'secreted' or 'soluble' form of S1-spike (pAdapt.S565). Likewise, the 2417 bp fragment coding for complete ACE2 (Genbank sequence AF291820) and a truncated version (sACE2) lacking the trans-membrane region (aa residues 741–805 of ACE2) were cloned into pAdapt. Both soluble proteins (S565 and sACE2) were fused in frame and upstream of a Myc-His tag to enable simple purification and detection of these expressed proteins. To create a ΔE1/ΔE2 vector carrying the light chain (LC) and heavy chain (HC) coding for a SARS-CoV neutralizing antibody (Meulen et al., 2004) the LC and HC genes were subcloned into plasmids that already contained appropriate leader sequences and either the constant CH1, CH2, and CH3 regions of the HC or C kappa, the constant region of the LC. To enable translation of HC and LC from one transcript, a Internal Ribosome Entry Site sequence derived from the EncephaloMyocarditis Virus (Jackson et al., 1990) was inserted in between HC and LC and cloned in the expression cassette of pAdapt. Generation of recombinant ΔE1/ΔE2 adenovirus batches was accomplished at 32°C on adherent PER.E2A cells using Lipofectamine2000 transfection of pAdapt carrying various transgenes and cosmid (pWE/AflII-rITRdE2A) according to instructions provided by the manufacturer (Invitrogen). Homologous recombination between sequences shared between pAdapt and pWE/AflII-rITRdE2A resulted in formation of ΔE1/ΔE2 vector visible by the onset and progression of CPE. Purified virus stocks were obtained using standard two-step CsCl-gradient banding and the isolated virus was dialyzed in three steps to a final formulation in PBS/5% (w/v) sucrose. Virus concentrations were determined based on optical density method (Maizel et al., 1968) in the presence of 1% (w/v) SDS. Infectivity was measured by $TCID_{50}$ assay on PER.E2A cells (3 weeks post-infection) as described previously (Fallaux et al., 1996).

Protein and DNA analyses

Detection of adenoviral proteins in infected cells and DBP protein in PER.E2A cells was performed by Western blot analysis. Hereto, 20 μg of total cellular protein was loaded on a polyacrylamide gel. To visualize DBP, fiber, penton, the pTP/TP proteins, or the E4-*orf6* protein, antibodies B6-mouse (A kind gift from N. Reich, New York, USA; Reich et al., 1983), E641/3-rabbit (a kind gift from R. Gerard, Leuven, Belgium), Pb571-rabbit (a kind gift from P. Boulanger, Montpellier, France), pTP-396-rabbit (a kind gift from P. van Vliet, Utrecht, the Netherlands), RSA-3-mouse (a kind gift from P. Branton, Montreal, Canada), were used at dilutions of 1/1,000, 1/1,000, 1/1,000, 1/750, and 1/377, respectively. Primary antibody binding was visualized utilizing a second antibody (ECL detection kit), that is Goat-anti-mouse, IgG, H + L, (BioRad) or Goat-anti-rabbit, IgG H + L, both at a dilution of 1/7,500. For Southern blot analyses, viral DNA was isolated from infected cells as described previously (Zhang et al., 1995). Viral DNA was digested with *Eco*RI, liberating the LacZ coding domain from the adenoviral backbone. Blots were probed with a ^{32}P-labeled *Eco*RI fragment of the LacZ cDNA. Purified S565; sACE2 and SARS-AB were fractionated by SDS–PAGE on 10% Bis-Tris gels and ran in MOPS running buffer (Invitrogen) and stained using Collodial blue staining (Invitrogen) according to the supplier protocol. For Western blot analysis, fractionated proteins were transferred onto Immobilon-P membranes (Millipore) and incubated with (1:10,000) HRP-conjugated donkey-a-human Mab (Jackson ImmunoResearch, Suffolk, UK) in case of SARS-AB and with anti-c-Myc-HRP (1:10,000) for both S565 and sACE2. Efficiency of gene transfer was typically assessed 48 h after virus addition by determination of LacZ transgene expression either by staining the intact cells as described previously (Havenga et al., 2001) or by preparing a cell lysate of the cells and measuring β-galactosidase activity in the total cell lysate using a β-galactosidase activity kit (Promega, Madsion WI). Transfection experiments were performed with 5 μg of plasmid DNA purified via Qiagen columns (Westburg, The Netherlands). Both soluble (Myc-His tagged) proteins S565 and sACE2 as well as the SARS-AB were produced in 100 mL roller bottles, medium was collected and centrifuged at 500*g* and filtered using a 0.22 μm bottle top filter. S565 and sACE2 proteins were purified on an AKTA explorer (GE Healthcare, Brussels, Belgium) using HisTrap HP (1 mL) affinity and HiTrap desalting (5 mL) columns (GE Healthcare) using the

appropriate buffers as recommended by the supplier. Total protein concentration in culture harvests was determined using the Micro BCA™ protein assay (Pierce; The Netherlands) according to instructions provided by the manufacturer. Samples of the harvests were also serial diluted and separated on SDS–PAGE gels, after colloid blue staining the gel was optically scanned and all single products (in the linear sample range) present in the individual lanes were digitally integrated and converted to integrated optical density (IOD) values using Gel-Pro analyzer developed by Media Cybernetics, Inc. (Bethesda, MD). This enabled the expression of the individual proteins as percentage of total protein per lane and is defined as protein purity. Subsequently, the amount of produced S565 and sACE2 was calculated by multiplication of the purity by the total amount of protein. The SARS virus neutralizing antibody, present in culture medium was purified by HPLC using HiTrap rProtein A protA and HiPrep 26/10 affinity and desalting columns (GE Healthcare) and quantified by optical density measurements at 280 nm. Detection of ACE2 on PER.C6 cells was performed by exposure of PER.C6 cells to a ΔE1/ΔE2 vector carrying the ACE2 coding domain. After 48 h, transiently expressed ACE2 on PER.C6 cell membranes was detected by FACS analysis after cell staining with goat-anti human ACE2 ectodomain polyclonal (0.4 μg/mL; R&D Systems, Oxford, UK) and PE-conjugated (1:100) Donkey anti-goat IgG (Jackson ImmunoResearch). For the detection of proper binding of sACE2 to S1-spike, PER.C6 cells were infected with a ΔE1/ΔE2 vector carrying the S1-spike and after 48 h cells were incubated with transiently produced and purified sACE2 (40 μg/mL) and subsequently with mouse-anti- c-Myc (9E10) FITC (6 μg/mL; Santa Cruz Biotechnologies, Inc., Santa Cruz, Calif.). All incubations and washes were performed at 4°C in PBS supplemented with 1% BSA. Cells were analyzed on a FACSCalibur with CELLQuest Pro software (Becton Dickinson, Franklin Lakes, NJ). To determine functionality of the SARS virus neutralizing antibody, microtiter plates (Nunc, Roskilde, Denmark) were coated overnight with anti-Myc antibody (5 μg/mL PBS, pH 7.4) followed by incubation with purified Myc-tagged S565 (2 μg/well). Concentrations of a transiently or stably produced SARS virus neutralizing antibody range (0.18–370 ng/mL) were added and incubated with (2,000 times diluted) PE-conjugated donkey anti-Human IgG (H + L; Jackson ImmunoResearch). The assay was further developed using *O*-phenylenediamine substrate and stopped by the addition of 1 M H_2SO_4. The absorbance was measured at 492 nm.

Results

Generation of the PER.E2A Cell Line and Production of ΔE1/ΔE2A Vector

Transfection in PER.C6 cells of a plasmid containing a Neomycine resistance ($G418^R$) gene and the E2A coding domain (Vliet and Sussenbach, 1975) under the regulation of the CMV-promoter, resulted in >100 $G418^R$ colonies from which 45 clones were further tested resulting in 37 established cell lines. Each cell line produced high levels of DNA binding protein (DBP) driven from the E2A gene as determined by Western analysis (data not shown). From the 37 cell lines the PER.E2A cell line was selected based on the capability of the cells to stably express the E2A gene in the absence of G418 and the ability to grow in suspension in the absence of animal serum. The PER.E2A cell line was compared to PER.C6 cells showing that during a 10 days culture period at 39°C the proliferation kinetics of the two cell lines were identical (data not shown). At 32°C, both cell lines behaved identical until after approximately 5 days in culture when the cell viability of the PER.E2A cell line started to decrease as a result of E2A toxicity (data not shown) thus providing a 5-day time frame for virus replication and progeny formation of ΔE1/ΔE2A adenoviral vectors. Next, the efficiency of ΔE1/ΔE2A recombinant vector generation on PER.E2A cells in relation to cell culture temperature was established. Hereto, an ΔE1/ΔE2A vector and as control an ΔE1 virus, both carrying the LacZ reporter gene, were generated on PER.E2A and PER.C6 respectively and purified. These vector stocks were used to transduce both PER.C6 and PER.E2A cells at a concentration of 100 virus particles per cell after which cells were cultured at different temperatures. Forty-eight hours later, the amount of infectious virus particles formed was determined by exposing human A549 indicator cells to 100 μL of cleared supernatant and subsequently counting the number of LacZ positive cells obtained. As shown in Figure 1A, an ΔE1 vector can be produced both on PER.C6 as well as PER.E2A cells whereas an ΔE1/ΔE2A vector can be produced only on PER.E2A cells. Moreover, a temperature of 34°C proved optimal for production of an ΔE1/ΔE2A vector on PER.E2A. A subsequent time-course experiment whereby supernatant was harvested at 24, 48, 72, or 120 h after virus exposure demonstrated that the highest titer of ΔE1/ΔE2A vector could be obtained at 72 h post-virus exposure (data not shown).

Using these parameters, more than 25 batches of recombinant ΔE1/ΔE2A viruses carrying different transgenes have since been produced and purified on PER.E2A cells with averaged yield of 4.7×10^{11} virus articles per milliliter and a virus particle per infectious unit ratio (VP/IU) of 22.4 ± 17.9 (average ± SD). These production data are similar to data as established for ΔE1 adenoviral vectors production on PER.C6 cells. A sensitive PCR analyses, discriminating between $E2A^+$ and $E2A^-$ virus, performed on purified virus batches indicated that reversion, that is homologous recombination between E2A DNA in the cell and the viral genome, possibly leading to $E2A^+$ virus does not occur (data not shown).

Replication Specificity and Viral Gene Expression of an ΔE1/ΔE2A Vector

To investigate the replication potential of an ΔE1/ΔE2A and as control ΔE1 recombinant vector in PER.C6 and PER.E2A

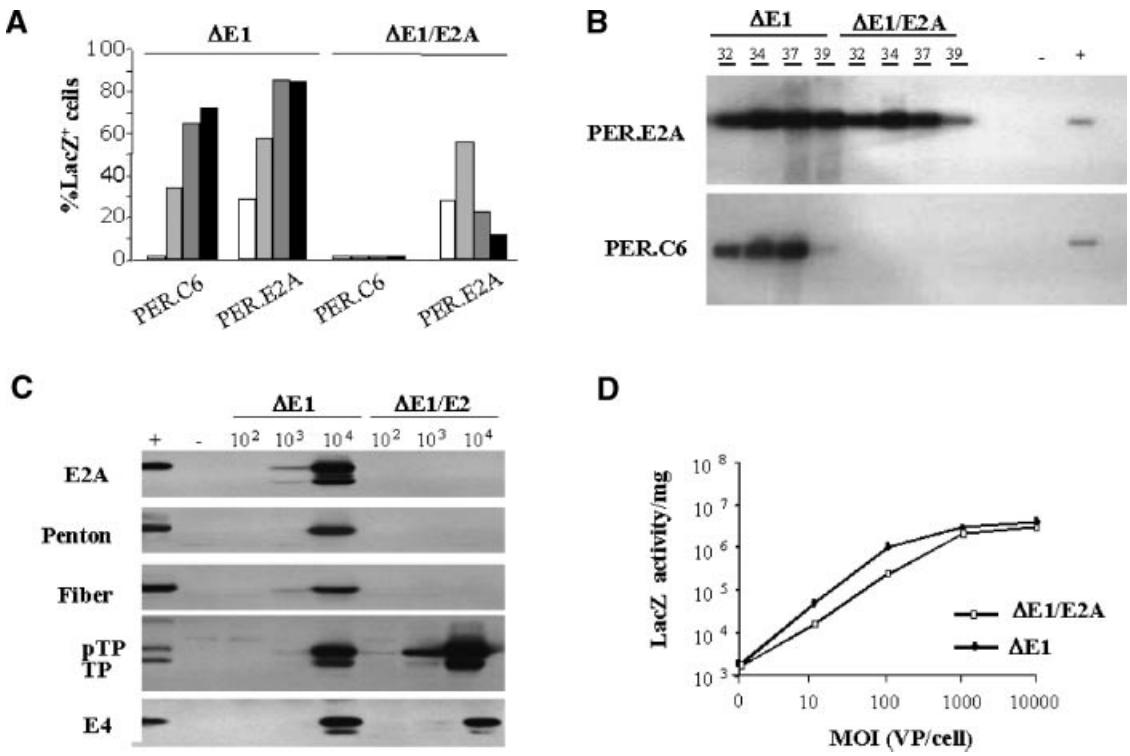

Figure 1. **A**: ΔE1 or ΔE1/E2 virus carrying LacZ (100 vp) was added to PER.C6 or PER.E2A cells cultured at 32°C (white bar), 34°C (light gray bar), 36°C (dark gray bar), or 37°C (black bar). Forty-eight hours later cleared supernatant (100 μL) was added to human A549 cells and 48 h post-inoculation the number of LacZ positive cells was determined. **B**: PER.C6 and PER.E2A cells were infected with 10 virus particles per cell of an ΔE1 or ΔE1/E2A virus carrying the LacZ transgene. Cells were subsequently cultured at 32, 34, 37, and 39°C for 48 h prior to cell harvest and viral DNA isolation. Southern analysis using a ^{32}P labeled probe (708 bp of LacZ gene) was performed with as a positive control, 2×10^8 virus genomes derived from a purified virus batch (+) and as a negative control, genomic DNA of the cell line (−). **C**: Human A549 cells were exposed to 100, 1000, or 10,000 vp/cell of either an ΔE1 or an ΔE1/E2A vector. Forty-eight hours later cells were harvested and protein lysate prepared. Twenty microgram of total cellular protein was loaded for Western analysis and probed with antibodies against DBP (E2A), late genes encoding for fiber or penton, and pTP and TP, or the *orf6* protein derived from the E4 region. As a positive control (+), 20 μg of total cellular protein was loaded derived from PER.C6 cells infected with an ΔE1-LacZ virus. As negative control (−), A549 cell lysate was used. **D**: Human A549 cells were exposed to 0, 100, 1,000, or 10,000 virus particles per cell of an ΔE1 (triangle) or an ΔE1/E2 (square) vector carrying LacZ. Forty-eight hours after infection cells were analyzed for the amount of LacZ activity, expressed in relative LacZ activity per milligram protein.

in relation to cell culture temperature, both cell types were cultured at different temperatures and subsequently transduced with either a ΔE1 or an ΔE1/ΔE2A vector. Forty-eight hours after vector exposure, viral DNA was isolated and Southern analysis was performed. From the results, shown in Figure 1B, the following conclusions were drawn: (i) at 32, 34, and 37°C both an ΔE1 and ΔE1/ΔE2A vector can efficiently replicate in PER.E2A cells resulting in the clear detection of viral DNA copies at 48 post-virus exposure, (ii) at 39°C the replication efficiency of an ΔE1/ΔE2A vector is significantly reduced due to the absence of functional E2A protein, (iii) an ΔE1/ΔE2A vector exposure to PER.C6 cells did not result in detection of viral copies indicative for the absence of viral replication. Since E2A is indirectly involved in activation of the viral major late promoter (MLP), responsible for driving late gene expression (structural proteins), deletion of this region from the adenoviral genome should result in absence of viral capsid proteins in transduced cells. To investigate capsid protein expression, human A549 indicator cells were exposed to an increasing concentration of ΔE1 or ΔE1/ΔE2A vector and Western analyses on protein lysates was performed. As shown in Figure 1C, even at 10,000 virus particles per cell no expression could be observed from either penton or fiber capsid at 48 h after ΔE1/ΔE2A vector exposure. In contrast, expression of both penton and fiber capsid proteins could be detected after exposure of 1,000 virus particles per cell using a ΔE1 vector. Expression of pre-terminal protein (pTP) and terminal protein (TP) derived from the viral E2B region, involved exclusively in promoting viral DNA replication in the PER.E2A cell line, as well as E4 derived *orf-6* protein (E4) involved in trans-activation of the CMV promoter driving expression of the gene encoding for the protein of interest (Gorziglia et al., 1999; Lusky et al., 1998) could still be detected. Finally, we determined whether equal levels of expression from an inserted transgene could be obtained using an ΔE1/ΔE2A as compared to a ΔE1 recombinant virus. Hereto, human A549 indicator cells were transduced with equal doses of vector carrying the LacZ marker gene. Since levels of reporter gene expression proved not

significantly different using either LacZ (Fig. 1D) it was concluded that the vectors are comparable with regard to transduction capacity, capsid stability, and CMV-mediated protein expression. Based on the data obtained thus far it was concluded that an ΔE1/ΔE2A adenoviral vector can be efficiently produced at 34°C on PER.E2A cells and can subsequently serve as a tranducing agent for PER.C6 cells without inducing viral backbone replication or high level viral capsid protein expression in human A549 indicator cells.

ΔE1/ΔE2A Vector Mediated Protein Production in PER.C6

Taking advantage of the observation that ΔE1/ΔE2 vectors do not replicate on PER.C6 cells we were interested to investigate if recombinant protein could be efficiently expressed in PER.C6 cells using this vector. To investigate yield, five ΔE1/ΔE2 vectors were generated and purified, one carrying the SARS-CoV soluble spike protein (S565), one carrying the complete SARS-CoA Spike protein (S1-spike), one carrying the soluble SARS virus receptor (sACE2), one carrying the complete ACE2 cellular receptor, and one carrying the heavy and LC coding for an SARS virus neutralizing antibody (ter Meulen et al., 2004). These proteins were chosen since they represent different classes of proteins and to determine bioactivity of the proteins in binding assays (SARS-AB binds S1-spike and both S1-spike and S565 bind to the ACE2 receptor). The ΔE1/ΔE2 vector coding for complete ACE2 protein was subsequently used to transduce PER.C6 suspension cells cultured in 100 mL roller bottles at increasing concentrations of virus particles per cell. Staining of the cells, 48 h after vector exposure, with a commercially available anti-ACE2 antibody demonstrated that approximately 90% of cells growing in suspension clearly produced the ACE2 protein using as little as 30 virus particles per cell as inoculation dose (Fig. 2A). In a subsequent experiment, shaker flasks (25 mL, 2.5×10^6 cells/mL) were inoculated with 10, 30, or 100 virus particles per cell using the ΔE1/ΔE2 vector expressing the SARS-AB. As shown in Figure 2B the antibody yield increased with increasing inoculation dose up to 60 μg/mL (at 100 VP/cell) over an 8-day batch production protocol. Although the highest antibody concentration was obtained with the highest virus particle per cell concentration, 30 virus particles per cell was chosen as inoculation dose for the remainder of the experiments. Experiments performed thus far used caesium chloride purified ΔE1/ΔE2 vectors (Fang et al., 1996; Goldman et al., 1995; O'Neal et al., 1998). Clearly, timelines required to produce protein could be significantly reduced if PER.C6 cells could be inoculated with non-purified vector stocks produced on the PER.E2A cell line. Therefore a head-to-head comparison was performed whereby four infectious units per cell of either non-purified (cleared vector stock) or purified vector stock (caesium chloride gradient purified) was used to inoculate PER.C6 cells. As shown in Figure 2C the yield of SARS antibody produced did not significantly differ demonstrating that cleared vector stock directly harvested from he PER.E2A cell line can be used for production of recombinant protein without compromising yield.

Next, roller bottles containing 2.5×10^6 PER.C6 cells/mL (100 mL) growing in suspension in absence of serum were inoculated with 30 virus particles per cell using the ΔE1/ΔE2 vector carrying the sACE2 receptor. Four days post-inoculation cells were harvested demonstrating that the total cell count had not increased nor decreased over the 4-day vector exposure period. A volumetric productivity of 611 μg/mL and specific productivity of 61 pg/cell/day (611/4 days/2.5 million cells) was obtained using this production protocol. Inoculation of roller bottles with 30 virus particles per cell of the ΔE1/ΔE2 vector coding for S565 resulted in a volumetric productivity of 370 μg/mL or specific productivity of 37 pg/cell/day. The SARS antibody yielded 140 μg/mL or specific productivity of 7 pg/cell/day during a 8 day batch production protocol.

Since the sACE2 and S565 were generated carrying a Myc-His tag they could be easily purified using a commercially available (Nickel-based) HisTrap HP column that has affinity for the included histidine residues, whereas the SARS antibody was purified using HPLC as described in the Material and Method Section. Purified proteins were run on SDS–PAGE gel and visualized either by whole protein staining (Collodial blue staining) or Western Blot analyses (Fig. 3 panels A and B, respectively) demonstrating protein integrity of transiently produced proteins without apparent aberrant protein being formed.

To assess the bioactivity of the produced proteins two sets of experiments were performed. In the first set of experiments PER.C6 cells transiently expressing complete S1-spike protein were incubated with purified sACE2 protein and subsequently stained with anti-c-Myc (9E10) FITC labeled antibody. As shown in Figure 4A almost all cells expressed on their membrane the S1-spike protein resulting in clear binding of the sACE2 protein as witnessed by high mean fluorescence upon staining with the anti-c-Myc antibody. In the second set of experiments, plates were coated with anti-Myc to capture S565 protein, which in turn was used to capture the SARS-AB. The ELISA was performed using an antibody dose titration and head-to-head comparison of the transiently produced antibody versus the SARS-AB produced and purified from PER.C6 cells that stably expresses this antibody (specific activity 14 pg/cell/day). As shown in Figure 4B no significant difference in binding affinity of the antibody could be observed using transient produced antibody as compared to the antibody produced from a stable cell line.

Collectively the data obtained demonstrate that (i) ΔE1/ΔE2 vectors are suitable for the production of different classes of proteins, that is, viral protein, cellular protein or antibody, (ii) a inoculation dose of 30 virus particles per cell suffices for significant protein yield without the need to purify vector inoculation stocks and is eligible for further optimization, and (iii) proteins produced and purified

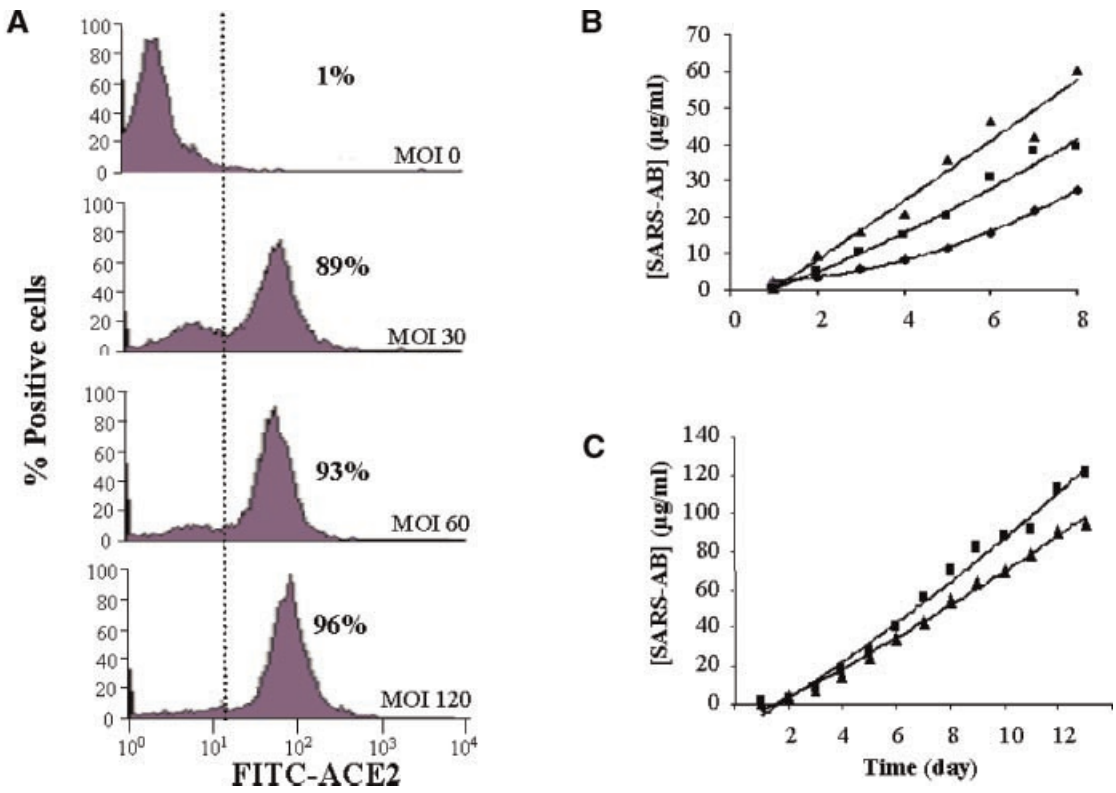

Figure 2. **A**: Shaker flasks were inoculated in parallel with MOI 0, 30, 60 and 120 vp/cell of a ΔE1/ΔE2 vector carrying the complete ACE2 coding region. Forty-eight hours later the percentage of cells positive for ACE2 protein was determined with FACS by staining cells with goat anti-ACE2 and PE-conjugated Donkey anti-human IgG (100-fold dilution). Parental PER.C6 stained with goat anti-ACE2 and PE-conjugated Donkey anti-human IgG served as negative control. **B**: Shaker flasks were inoculated in parallel with 30 (diamond), 60 (square) and 100 (triangle) vp/cell of a ΔE1/ΔE2 vector carrying the SARS-AB coding regions. The concentration of the antibody produced was determined in medium samples using HPLC-protA column chromatography standardized for IgG quantity measurement. **C**: Shaker flasks were inoculated in parallel with 1×10^9 infectious virus particles (4 IU/cell) of either CsCL-purified vector (triangles) or with cleared crude lysate (squares). The concentration of the antibody produced was determined in medium samples using HPLC-protA column chromatography standardized for IgG quantity measurement. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

demonstrate proper integrity and bioactivity as witnessed in binding assays and compared to protein produced in stable cell lines. As such, this platform could deliver high yield recombinant protein batches within approximately 30 working days (see projection in Fig. 5) although clearly further optimization is currently required to achieve vector generation and titration within approximately 4 weeks in order to perform large scale protein production.

Discussion

Several reports have previously described the use of a replication deficient adenovirus in combination with adenovirus production cells (HEK293) with the aim to produce recombinant protein (Andersen and Krummen, 2002; Côté et al., 1998; Garnier et al., 1994; Lamarche et al., 1990; Massie et al., 1995, 1998). In these studies, the E1-deleted vector was allowed to replicate in HEK293 cells, in the process yielding high amounts (4–20% total cell protein) of the protein of interest. However, besides the formation of replication incompetent vectors during production it has been demonstrated that homologous recombination can occur in HEK293 cells between vector and cell line sequences resulting in the formation of RCA (Imler et al., 1995; Lochmüller et al., 1994). Another disadvantage of the reported systems is the relative short time frame allowed for protein production given the fact that the adenovirus lyses the cells that produce the protein of interest within days.

To improve upon the existing systems, PER.C6 cells were chosen as a starting point for the generation of a transient protein production platform (Fallaux et al., 1998). This cell line has been extensively documented, cell banks meet all pertinent US and EEC regulatory requirements, cells can be cultured in suspension without the aid of micro-carriers to high cell densities ($>10^7$ cells/mL), cells can be cultured in suspension in the absence of any serum components, and cultures can be scaled to large bioreactors. In addition, the formation of RCA vectors has never been reported. To meet other criteria, that is absence of replication deficient virus particles and de novo synthesis of viral capsid proteins that could negatively influence cell viability and proliferation, an adenovirus deleted in its genome for both the E1/E2A regions was generated. Deletion of these regions has been previously shown to result in absence of almost all viral gene expression (Gorziglia et al., 1999; Lusky et al., 1998; O'Neal

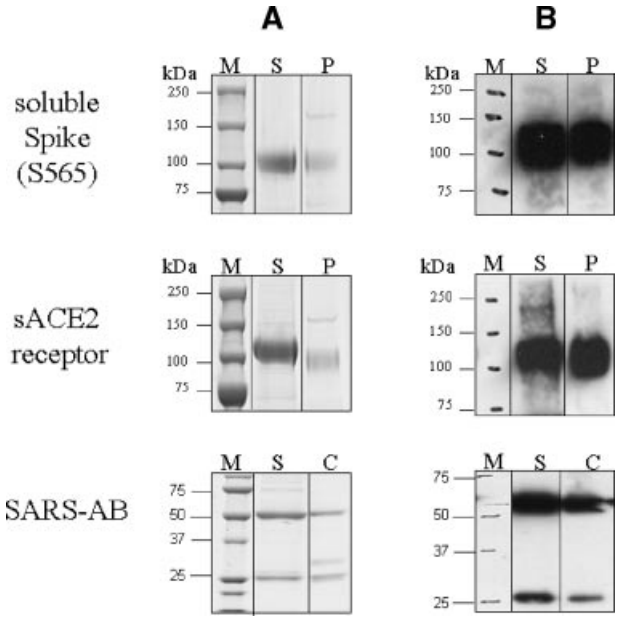

Figure 3. PER.C6 cells cultured in suspension in absence of serum in roller-bottles were inoculated with 30 vp/cell of a ΔE1/ΔE2 vector carrying the S565 coding domain, the ACE2 coding domain or the SARS-AB coding domain. Transiently produced and purified proteins (S) were separated on SDS–PAGE gels (**panel A**) and analyzed via Western blot analyses (**panel B**). S565 and sACE2 were visualized with mouse-anti-c.myc (HRP-conjugated) monoclonal antibody. SARS-AB was visualized with donkey-anti-human IgG (HRP-conjugated) monoclonal antibody. The molecular weight marker is represented (M). Purified S565 transiently produced in HEK293 cells (P), and stably produced CR3014 on PER.C6 cells (C) were included as positive controls. The contents of various lanes are not standardized for equal protein amounts.

et al., 1998) without compromising CMV promoter activity driving foreign gene expression (Gorziglia et al., 1999; Lusky et al., 1998). This report demonstrates that a ΔE1/ΔE2A vector is unable to replicate in PER.C6 cells and does not express viral capsid proteins even after high dose inoculation of susceptible human A549 cells.

We therefore postulated that this vector could provide a safe reagent to introduce DNA into PER.C6 cells growing in suspension for scalable transient protein production purposes. In order to produce high-titer ΔE1/ΔE2A vector we established the PER.E2A cell line. Hereto, a temperature sensitive mutant E2A gene (Vliet and Sussenbach, 1975) was employed since wild type E2A protein expression is known to be toxic to cells (Klessig et al., 1984). The temperature sensitive mutant carries a mutation in codon 413 of the DNA binding protein transcribed from the E2A region of adenovirus serotype 5 (Kruijer et al., 1981), which results in functional E2A protein at 32°C but not at 39°C. This allowed the establishment of a stable E2A expressing cell line that could be cultured without apparent growth deficit or viability loss at 39°C as compared to parental PER.C6 cells. For production of the ΔE1/ΔE2 vector the PER.E2A cells were switched to a temperature of 32°C, which allowed a 5 day time window for vector production before cell viability started to decrease due to E2A expression. To ensure scalability of the platform the PER.E2A cells were adapted to grow in suspension, in the absence of serum components.

This report thus describes for the first time a transient protein production system that can be as safe as the use of naked DNA for large scale transient protein production but solves the current technological bottlenecks associated with

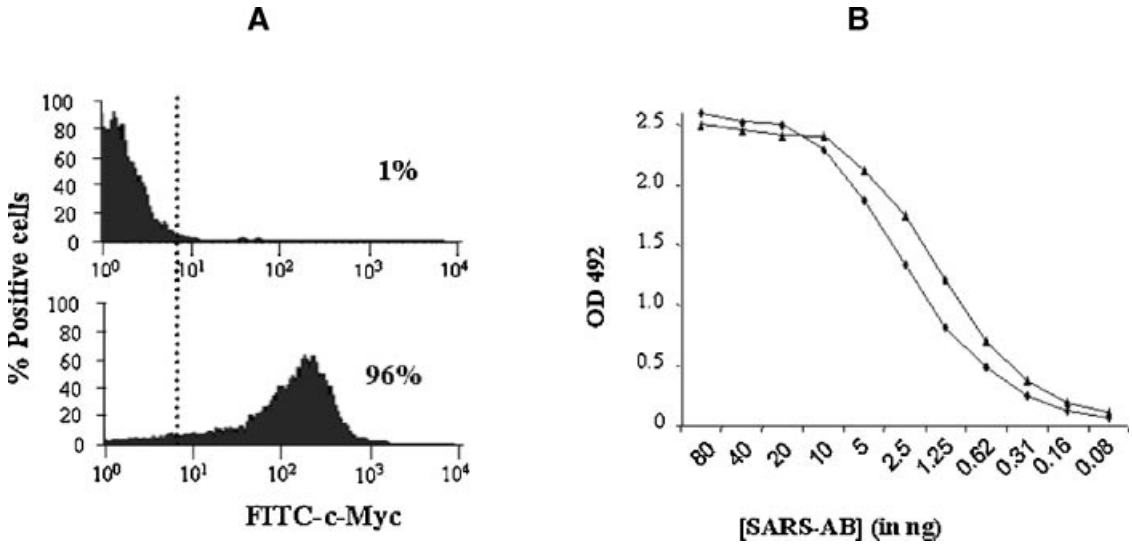

Figure 4. **A**: PER.C6 cells were exposed to 30 vp/cell of an ΔE1/ΔE2 vector carrying the S1-spike coding domain. Forty-eight hours post-exposure cells were harvested and stained with 40 μg/mL transiently produced sACE2. To visualize sACE2 binding to S1-spike cells were further incubated with mouse-anti-c-myc-FITC. Unstained infected PER.C6 cells were used to set back ground cell staining at 1%. **B**: The SARS virus neutralizing antibody produced transiently using a ΔE1/ΔE2 vector (triangles) was compared to the identical antibody produced on PER.C6 cells stably expressing this antibody (diamonds) via ELISA. Binding of the SARS-AB to S565 in a dose dependent manner was detected using peroxidase-conjugated AffiniPure donkey anti-human IgG (Jackson Immuno Research).

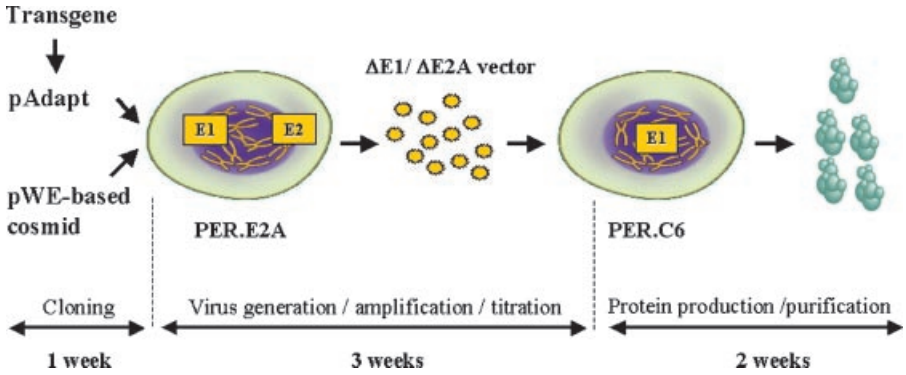

Figure 5. Schematic representation of perceived timelines associated with the use of the novel ΔE1/ΔE2 vector-based transient protein production technology in PER.C6 cells.

the use of naked DNA transfection, that is, limited internalization of plasmid DNA into mammalian cells and limited transport of plasmid DNA to the nucleus. Several improvements to this system that will be investigated next include the set-up of a fast and reliable RT-PCR or FACS-based virus titration assay, needed to replace the laborious $TCID_{50}$ assay. Also, analyses need to be performed regarding the quality of protein produced using this novel system to investigate proper post-translational modification. Regardless of these points of consideration, the data obtained thus far warrant further investigations into the robustness and safety aspects of this transient protein production system given that there is an increasing demand for high quality, high yield recombinant protein to facilitate pre-clinical and clinical pilot studies.

Essentially the process described in Figure 5 is not limited by scale given the calculations demonstrating that at 1,000 liter scale (PER.C6 cell density at 2×10^6 cells/mL $= 2 \times 10^{12}$ cells) a vector inoculation dose (50 IU/cell) of 1×10^{14} IU would be required. This amount of virus can easily be generated due to the efficient virus amplification using the suspension PER.E2A cell line cultured at 2×10^6 cells per mL in a 10 liter bioreactor within the timeframe of 4 weeks.

The authors thank N. Reich (New York), R. Gerard (Leuven, Belgium), P. Boulanger (Montpellier, France), P. van der Vliet (Utrecht, the Netherlands), and P. Branton (Montreal, Canada) for providing antibodies directed against adenoviral proteins. The authors also like to thank K. Verhulst, AGCM Uytdehaag, F. Fallaux, I. van der Velde, and AJ van der Eb for technical and scientific assistance.

References

Andersen DC, Krummen L. 2002. Recombinant protein expression for therapeutic applications. Curr Opin Biotechnol 13(2):117–123.

Andersson C, Hansson M, Power U, Nygren Pk, Ståhl S. 2001. Mammalian cell production of a respiratory syncytial virus (RSV) candidate vaccine recovered using a product-specific affinity column. Biotechnol Appl Biochem 34(Pt 1):25–32.

Baldi L, Hacker DL, Adam M, Wurm FM. 2007. Recombinant protein production by large-scale transient gene expression in mammalian cells: state of the art and future perspectives. Biotechnol Lett 29(5):677–684.

Bao WG, Fukuhara H. 2001. Secretion of human proteins from yeast: Stimulation by duplication of polyubiquitin and protein disulfide isomerase genes in Kluyveromyces lactis. Gene 272(1–2):103–110.

Berg H, Walter M, Mauch L, Seissler J, Northemann W. 1993. Recombinant human preproinsulin. Expression, purification and reaction with insulin autoantibodies in sera from patients with insulin-dependent diabetes mellitus. J Immunol Methods 164(2):221–231.

Boedeker BG. 2001. Production processes of licensed recombinant factor VIII preparations. Semin Thromb Hemost 27(4):385–394.

Breitbach K, Jarvis DL. 2001. Improved glycosylation of a foreign protein by Tn-5B 1-4cells engineered to express mammalian glycosyltransferases. Biotechnol Bioeng 74(3):230–239.

Brink EN van den, Meulen J ter, Cox F, Jongeneelen MA, Thijsse A, Throsby M, Marissen WE, Rood PM, Bakker AB, Gelderblom HR, Martina BE, Osterhaus ADME, Preiser W, Doerr HW, Kruif J de, Goudsmit J. 2005. Molecular and biological characterization of human monoclonal antibodies binding to the spike and nucleocaspid proteins of severe acute respiratory syndrome coronavirus. J Virol 79(3):1635–1644.

Cai X. 2001. Melanin properties studies of the recombinant of Pseudomonas pseudoalcaligenes containing tyrosinase gene. Wei Sheng Wu Xue Bao 41(6):699–703.

Chen L, Colman PM, Cosgrove LJ, Lawrence MC, Lawrence LJ, Tulloch PA, Gorman JJ. 2001. Cloning, expression, and crystallization of the fusion protein of Newcastle disease virus. Virology 290(2):290–299.

Côté J, Garnier A, Massie B, Kamen A. 1998. Serum-free production of recombinant proteins and adenoviral vectors by 293SF-3F6 cells. Biotechnol Bioeng 59(5):567–575.

Derouazi M, Girard P, Van Tilborgh F, Iglesias K, Muller N, Bertschinger M, Wurm FM. 2004. Serum-free large-scale transient transfection of CHO cells. Biotechnol Bioeng 87(4):537–545.

Ensinger MJ, Ginsberg HS. 1972. Selection and preliminary characterization of temperature-sensitive mutants of type 5 adenovirus. J Virol 10(3):328–329.

Fallaux FJ, Kranenburg O, Cramer SJ, Houweling A, Van Ormondt H, Hoeben RC, Van Der Eb AJ. 1996. Characterization of 911: A new helper cell line for the titration and propagation of early region 1-deleted adenoviral vectors. Hum Gene Ther 7(2):215–222.

Fallaux FJ, Bout A, van der Velde I, van den Wollenberg DJ, Hehir KM, Keegan J, Auger C, Cramer SJ, van Ormondt H, van der Eb AJ, Valerio D, Hoeben RC. 1998. New helper cells and matched early

region 1-deleted adenovirus vectors prevent generation of replication-competent adenoviruses. Hum Gene Ther 9(13):1909–1917.
Fang B, Wang H, Gordon G, Bellinger DA, Read MS, Brinkhous KM, Woo SL, Eisensmith RC. 1996. Lack of persistence of E1—Recombinant adenoviral vectors containing a temperature-sensitive E2A mutation in immunocompetent mice and hemophilia B dogs. Gene Ther 3(3): 217–222.
Friehs K, Reardon KF. 1993. Parameters influencing the productivity of recombinant *E. coli* cultivations. Adv Biochem Eng Biotechnol 48:53–77.
Fussenegger M, Bailey JE, Hauser H, Mueller PP. 1999. Genetic optimization of recombinant glycoprotein production by mammalian cells. Trends Biotechnol 17(1):35–42.
Garnier A, Côté J, Nadeau I, Kamen A, Massie B. 1994. Scale-up of the adenovirus expression system for the production of recombinant protein in human 293S cells. Cytotechnology 15(1–3):145–155.
Goldman MJ, Litzky LA, Engelhardt JF, Wilson JM. 1995. Transfer of the CFTR gene to the lung of nonhuman primates with E1-deleted, E2a-defective recombinant adenoviruses: A preclinical toxicology study. Hum Gene Ther 6(7):839–851.
Gorziglia MI, Lapcevich C, Roy S, Kang Q, Kadan M, Wu V, Pechan P, Kaleko M. 1999. Generation of an adenovirus vector lacking E1, e2a, E3, and all of E4 except open reading frame 3. J Virol 73(7):6048–6055.
Gossen M, Bonin AL, Freundlich S, Bujard H. 1994. Inducible gene expression systems for higher eukaryotic cells. Curr Opin Biotechnol 5(5):516–520.
Havenga MJ, Lemckert AA, Grimbergen JM, Vogels R, Huisman LG, Valerio D, Bout A, Quax PH. 2001. Improved adenovirus vectors for infection of cardiovascular tissues. J Virol 75(7):3335–3342.
Imler JL, Bout A, Dreyer D, Dieterlé A, Schultz H, Valerio D, Mehtali M, Pavirani A. 1995. Trans-complementation of E1-deleted adenovirus: A new vector to reduce the possibility of co dissemination of wild-type and recombinant adenoviruses. Hum Gene Ther 6(6):711–721.
Jackson RJ, Howell MT, Kaminski A. 1990. The novel mechanism of initiation of picornavirus RNA translation. Trends Biochem Sci 15(12):477–483.
James E, Lee JM. 2001. The production of foreign proteins from genetically modified plant cells. Adv Biochem Eng Biotechnol 72:127–156.
Jenkins N, Buckberry L, Marc A, Monaco L. 1998. Genetic engineering of alpha 2,6-sialyltransferase in recombinant CHO cells. Biochem Soc Trans 26(2):S115.
Jønson L, Johnsen AH. 2001. Post-translational modifications of heterologously expressed cholecystokinin in *Saccharomyces cerevisiae*. Scand J Clin Lab Invest Suppl 234:87–92.
Kawashima Y, Ogawa T, Kato M, Nakazato A, Tsuchida K, Hatayama K, Hirono S, Moriguchi I. 1993. Structure-activity study of antihypertensive 1,4-dihydropyridine derivatives having nitrooxyalkyl moieties at the 3 and 5 positions. Chem Pharm Bull (Tokyo) 41(6):1060–1065.
Klessig DF, Brough DE, Cleghon V. 1984. Introduction, stable integration, and controlled expression of a chimeric adenovirus gene whose product is toxic to the recipient human cell. Mol Cell Biol 4(7):1354–1362.
Konishi J, Ishii Y, Onaka T, Maruhashi K. 2002. Purification and characterization of the monooxygenase catalyzing sulfur-atom specific oxidation of dibenzothiophene and benzothiophene from the thermophilic bacterium *Paenibacillus* sp. strain A11-2. Appl Microbiol Biotechnol 60(1–2):128–133. Epub 2002 August 24.
Kruijer W, Schaik FM van, Sussenbach JS. 1981. Structure and organization of the gene coding for the DNA binding protein of adenovirus type 5. Nucleic Acid Res 9(18):4439–4457.
Ladisch S, Lovejoy FH, Hierholzer JC, Oxman MN, Strieder D, Vawter GF, Finer N, Moore M. 1979. Extrapulmonary manifestations of adenovirus type 7 pneumonia simulating Reye syndrome and the possible role of an adenovirus toxin. J Pediatr 95(3):348–355.
Lamarche N, Massie B, Richer M, Paradis H, Langelier Y. 1990. High level expression in 293 cells of the herpes simplex virus type 2 ribonucleotide reductase subunit 2 using an adenovirus vector. J Gen Virol 71(Pt 8):1785–1792.
Lawrence SM, Huddleston KA, Tomiya N, Nguyen N, Lee YC, Vann WF, Coleman TA, Betenbaugh MJ. 2001. Cloning and expression of human sialic acid pathway genes to generate CMP-sialic acids in insect cells. Glycoconj J 18(3):205–213.
Lazaris A, Arcidiacono S, Huang Y, Zhou JF, Duguay F, Chretien N, Welsh EA, Soares JW, Karatzas CN. 2002. Spider silk fibers spun from soluble recombinant silk produced in mammalian cells. Science 295(5554): 472–476.
Lee FS, Vallee BL. 1989. Expression of human placental ribonuclease inhibitor in Escherichia coli. Biochem Biophys Res Commun 160(1):115–120.
Levy R, Weiss R, Chen G, Iverson BL, Georgiou G. 2001. Production of correctly folded Fab antibody fragment in the cytoplasm of Escherichia coli trxB gor mutants via the coexpression of molecular chaperones. Protein Expr Purif 23(2):338–347.
Lochmüller H, Jani A, Huard J, Prescott S, Simoneau M, Massie B, Karpati G, Acsadi G. 1994. Emergence of early region 1-containing replication-competent adenovirus in stocks of replication-defective adenovirus recombinants (delta E1 + delta E3) during multiple passages in 293 cells. Hum Gene Ther 5(12):1485–1491.
Lusky M, Christ M, Rittner K, Dieterle A, Dreyer D, Mourot B, Schultz H, Stoeckel F, Pavirani A, Mehtali M. 1998. In vitro and in vivo biology of recombinant adenovirus vectors with E1, E1/E2A, or E1/E4 deleted. J Virol 72(3):2022–2032.
Maizel JV, White DO, Scharff MD. 1968. The polypeptides of adenovirus. I Evidence for multiple protein components in viron and a comparison of types 2,7A and 12. Virology 36:115–125.
Marchal I, Jarvis DL, Cacan R, Verbert A. 2001. Glycoproteins from insect cells: Sialylated or not? Biol Chem 382(2):151–159.
Massaer M, Mazzu P, Haumont M, Magi M, Daminet V, Bollen A, Jacquet A. 2001. High-level expression in mammalian cells of recombinant house dust mite allergen ProDer p 1 with optimized codon usage. Int Arch Allergy Immunol 125(1):32–43.
Massie B, Dionne J, Lamarche N, Fleurent J, Langelier Y. 1995. Improved adenovirus vector provides herpes simplex virus ribonucleotide reductase R1 and R2 subunits very efficiently. Biotechnology (NY) 13(6): 602–608.
Massie B, Couture F, Lamoureux L, Mosser DD, Guilbault C, Jolicoeur P, Bélanger F, Langelier Y. 1998. Inducible overexpression of a toxic protein by an adenovirus vector with a tetracycline-regulatable expression cassette. J Virol 72(3):2289–2296.
Mattes R. 2001. The production of improved tissue-type plasminogen activator in *Escherichia coli*. Semin Thromb Hemost 27(4):325–336.
McQuillan DJ, Seo NS, Hocking AM, McQuillan CI. 2001. Recombinant expression of proteoglycans in mammalian cells. Utility and advantages of the vaccinia virus/T7 bacteriophage hybrid expression system. Methods Mol Biol 171:201–219.
Menassa R, Zhu H, Karatzas CN, Lazaris A, Richman A, Brandle J. 2004. Spider dragline silk proteins in transgenic tobacco leaves: Accumulation and field production. Plant Biotechnol J 2(5):431–438.
Meulen J ter, Bakker AB, Brink EN van den, Weverling GJ, Martina BE, Haagmans BL, Kuiken T, Kruif J de, Preiser W, Spaan W, Gelderblom HR, Goudsmit J, Osterhaus AD. 2004. Human monoclonal antibody as prophylaxis for SARS coronavirus infection in ferrets. Lancet 363 (9427):2139–2141.
Mosser DD, Massie B. 1994. Genetically engineering mammalian cell lines for increased viability and productivity. Biotechnol Adv 12(2): 253–277.
O'Neal WK, Zhou H, Morral N, Aguilar-Cordova E, Pestaner J, Langston C, Mull B, Wang Y, Beaudet AL, Lee B. 1998. Toxicological comparison of E2a-deleted and first-generation adenoviral vectors expressing alpha 1-antitrypsin after systemic delivery. Hum Gene Ther 9(11):1587–1598.
Reich NC, Sarnow P, Duprey E, Levine AJ. 1983. Monoclonal antibodies which recognize native and denatured forms of the adenovirus DNA-binding protein. Virology 128(2):480–484.
Rice SA, Klessig DF. 1985. Isolation and analysis of adenovirus type 5 mutants containing deletions in the gene encoding the DNA-binding protein. J Virol 56(3):767–778.

Sarkar SN, Sen GC. 1998. Production, purification, and characterization of recombinant 2′,5′-oligoadenylate synthetases. Methods 15(3):233–242.
Sarkar S, Miwa N, Kominami H, Igarashi N, Hayashi S, Okada T, Jahangeer S, Nakamura S. 2001. Regulation of mammalian phospholipase D2:Interaction with and stimulation by G(M2) activator. Biochem J 359(Pt 3):599–604.
Seo NS, Hollister JR, Jarvis DL. 2001. Mammalian glycosyltransferase expression allows sialoglycoprotein production by baculovirus-infected insect cells. Protein Expr Purif 22(2):234–241.
Tait AS, Brown CJ, Galbraith DJ, Hines MJ, Hoare M, Birch JR, James DC. 2004. Transient production of recombinant proteins by Chinese hamster ovary cells using polyethyleneimine/DNA complexes in combination with microtubule disrupting anti-mitotic agents. Biotechnol Bioeng 88(6):707–721.
Tom RL, Caron AW, Massie B, Kamen AA. 1995. Scale-up of recombinant virus and protein production in stirred-tank reactors. Methods Mol Biol 39:203–224.
Vliet P van der, Levine AJ, Ensinger MJ, Ginsberg HS. 1975. Thermolabile DNA Binding proteins from cells infected with a temperature-sensitive mutant of adenovirus defective in viral DNA synthesis. J Virol 15(2):348–354.
Vliet P van der, Sussenbach JS. 1975. An Adenovirus type 5 gene function required for initiation of viral DNA replication. J Virol 67(2):415–426.
Wu L, Berk AJ. 1996. Modification of adenovirus as gene transfer vector. Journal of Investigative Medicine 44(1):A190.
Zhang WW, Koch PE, Roth JA. 1995. Detection of wild-type contamination in a recombinant adenoviral preparation by PCR. Biotechniques 18(3):444–447.