Increasing lentiviral vector titer using inhibitors of protein kinase R

Gilles Pernod^{1,2}, Richard Fish¹, Jia Wei Liu¹, and Egbert K.O. Kruithof¹

¹University Hospital of Geneva, Geneva, Switzerland and ²CHU Grenoble, Grenoble, France

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Lentiviral vectors are increasingly used as vehicles for stable gene transfer *ex vivo* and *in vivo* (1). These gene transfer vectors have a large cloning capacity, stably integrate the transgene into the host cell genome, and can transduce nondividing cells such as hematopoietic progenitor cells (2). However, in comparison with the production of retroviral or adenoviral vectors, current methods for producing lentiviral vectors are inefficient, labor-intensive, and time- and reagent-consuming. The mechanisms that limit lentiviral vector production are poorly understood. One limiting factor may be the presence of double-stranded RNA (dsRNA) generated during lentiviral vector production or after transduction of producer cells by released lentiviral vector particles. Indeed, infection of cells by viruses often results in the accumulation of dsRNA in the cytoplasm, which then activates interferon-induced antiviral defense pathways (3). Upon binding to dsRNA, the interferon-inducible protein kinase (PKR) becomes activated. Activated PKR phosphorylates the eukaryotic initiation factor-2 α (eIF-2 α), resulting in a block in protein synthesis initiation. This leads to the inhibition of viral RNA translation, which affects the stability and processing of lentivirus-specific proteins (4–6). A second group of interferon-inducible proteins, the oligoadenylate synthetases (OAS), bind dsRNA and activate RNase L, which degrades both endogenous and viral RNA. Viruses have developed a variety of approaches to repress the innate mammalian antiviral cellular responses. One example is the adenovirus-encoded virus-associated RNA I (VAI RNA), which is transcribed by RNA polymerase III after virus infec-

tion. VAI RNA binds to PKR and prevents its activation by dsRNA (7).

In the present study, we investigated whether lentiviral vector production could be increased by the inhibition of PKR activity. Human immunodeficiency virus (HIV)-based lentiviral vectors were produced by the cotransfection of human embryonic kidney (HEK) 293T cells with three plasmids, using the calcium-phosphate precipitation method (2). pCMVR8.91 (15 μ g) encodes the necessary HIV structural genes, pMD.G (5 μ g) bears the vesicular stomatitis virus glycoprotein G (VSV-G) envelope cDNA, and pLoxCWGFP (20 μ g) is the transfer vector carrying the enhanced green fluorescent protein (EGFP) gene under the control of the cytomegalovirus (CMV) immediate early enhancer/promoter region between modified HIV long terminal repeats (LTRs) (the three plasmids were generously provided by Dr. D. Trono, University Medical Center of Geneva, Geneva, Switzerland). Sixteen hours after transfection, the cell medium was replaced with 15 mL of fresh medium and incubated for an additional 24 h. The conditioned medium was collected and stored at -20°C. Lentiviral vector titers were determined by flow cytometry analysis of EGFP expression in transduced HeLa cells. Vector titers are expressed as transduction units per milliliter (TU/mL).

We employed two approaches to study whether inhibitors of PKR increase lentiviral vector yields. In the first approach, we cotransfected the three lentiviral vector production plasmids with different amounts of the pAdVantageTM plasmid (Promega, Madison, WI, USA) that encodes VAI RNA, an inhibitor of PKR activation, as well as VAI RNA. In the second ap-

proach, we added different concentrations of 2-aminopurine (2-AP) (Sigma, St. Louis, MO, USA), an inhibitor of PKR phosphorylation (8). This inhibitor has been previously shown to increase the yield of different viruses grown on interferon-treated cells (9).

We observed that cotransfection with the pAdVantage plasmid increased lentiviral vector titers in a dose-dependent manner (Figure 1, A and B). A maximal, two-fold increase was obtained when 10 μ g of pAdVantage plasmid were used. Mean fluorescence intensities in transduced cells were not modified by cotransfection with the pAdVantage plasmid. This indicates that the presence of pAdVantage increased the number of lentiviral vector particles but had no effect on the transgene expression level in the transduced cells (Figure 1B). Using 2-AP, we obtained a dose-dependent increase in lentiviral vector production. Compared to controls, almost 4-fold higher titers were obtained using 5 mM 2-AP (Figure 1, C and D). To determine the effect of PKR inhibitors on PKR activity under our experimental conditions, we analyzed the phosphorylation state of eIF-2 α . We observed that cotransfection of 293T cells with the pAdVantage plasmid or incubation with 2-AP markedly decreased the levels of phosphorylated eIF-2 α (Figure 1E). To assess activation of the OAS-mediated interferon response pathway, which ultimately results in RNase L activation, we attempted to compare the levels of OAS1 mRNA in lentiviral vector producing 293T cells with and without pAdVantage and 2-AP. We were unable to detect OAS1 mRNA in any lentiviral vector-producing 293T cells, even after 50 PCR cycles. In other cell types, OAS1 was readily detectable at PCR cycle 25 (data not shown). Therefore, limitations on lentiviral vector production are not linked to the induction of OAS1 in 293T cells. Our results show that inhibitors of PKR activity can be used to increase lentiviral vector yields in HEK 293T producer cells.

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Figure 1. Protein kinase (PKR) inhibitors increase lentiviral vector titers. Lentiviral vectors were produced by cotransfection of three plasmids, as described in the text, with or without pAdVantage or 2-aminopurine (2-AP). Briefly, 5 million human embryonic kidney (HEK) 293T cells were seeded into 100 mm cell culture dishes 1 day prior to transfection in 10 mL of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). The medium was changed at least 1 h prior to transfection. After transfection (24 h), the medium was replaced (15 mL), and vector-containing conditioned medium was collected after an additional 24 h. Vector titers were determined on HeLa cells previously seeded in 6-well plates at 50,000 cells/well 1 day prior to transduction. The vectors were incubated with HeLa cells in medium containing 8 μ g/mL hexadimethrine bromide (Sigma). Flow cytometry was performed 4 days after transduction. (A and B) Effect of pAdVantage, a plasmid that encodes the adenovirus virus-associated RNA I (VAI RNA), an inhibitor of PKR activation. (A) Increasing amounts of pAdVantage were included in transfection mixtures, and resulting vector titers were determined from the percentage of enhanced green fluorescent protein (EGFP⁺) transduced HeLa cells. The figure shows the mean ($\pm$ SEM) of four independent experiments. (B) Representative flow cytometry analysis of HeLa cells transduced with 5 μ L lentiviral vectors produced without (Ctrl) or with 10 μ g of pAdVantage (VAI). Percentage of EGFP⁺ cells was determined from the M1 gating shown. (C and D) Effect of 2-AP. (C) 2-AP was included in the 293T cell medium during lentiviral vector production at the concentrations shown. Vector titers were determined as described above. The figure shows the mean ($\pm$ SEM) of four independent experiments. (D) Representative flow cytometric analysis of HeLa cells transduced with 5 μ L lentiviral vectors made without (Ctrl) or with 0.05, 0.5, or 5 mM 2-AP. Percentage of EGFP⁺ cells was determined from the M1 gating shown. (E) PKR inhibitors reduce eukaryotic initiation factor-2 α (eIF-2 α) phosphorylation in lentiviral vector-producing 293T cells. Vector production was performed in control cells, in cells incubated with 5 mM 2-AP, 10 μ g of pAdVantage, or 5 mM 2-AP and 10 μ g pAdVantage. After vector collection, 48 h posttransfection, 293T vector-producing cells were washed in phosphate-buffered saline (PBS) and lysed in phosphorylation lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM EDTA, 1 mM Zn(C₂H₃O₂)₂, 1 mM NaF, 1 mM Na₃VO₄) supplemented with Complete Mini Protease Inhibitor Cocktail (Roche, Basel, Switzerland). Cell lysates were cleared by centrifugation at 16,000 $\times$ g for 5 min, and protein content was measured using the Bio-Rad[®] Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA), with bovine serum albumin (BSA) as a standard. Twenty-eight micrograms of total protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes for immunoblotting with either anti-eIF-2 α or antiphosphorylated eIF-2 α antibodies (both from Cell Signaling Technologies, Beverly, MA, USA). Specific bands were detected using goat anti-rabbit-horseradish peroxidase (HRP; Bio-Rad Laboratories) secondary antibodies and ECL[®] Western Blotting Detection Reagents (Amersham Biosciences, Uppsala, Sweden). TU/mL, transduction unit per milliliter.

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Address correspondence to Egbert K.O. Kruithof, Division of Angiology and Hemostasis, Department of Internal Medicine, University Hospital of Geneva, CH-1211 Geneva, Switzerland. e-mail: egbert.kruithof@hcuge.ch

Monitoring aerobic *Escherichia coli* growth in shaken microplates by measurement of culture fluorescence

Hartmut F. Zimmermann, Harald Trauthwein, Uwe Dingerdissen, Mechthild Rieping, and Klaus Huthmacher
Degussa AG, Frankfurt am Main, Germany

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Combining the advantages of both the microplate (high-throughput, ease of automation) and the classic shaken Erlenmeyer flask (adequate mixing and oxygen supply), shaken 48-well microplates are an excellent small-scale fermentation system for microbial cultivation (1). Most physiological studies are at least semiquantitative (2), and real-time biomass estimation during a bioprocess is an important goal (3). Therefore, a simple method for the monitoring of microbial growth in these 48-well plates is necessary.

Intracellular NADH and NADPH in living microorganisms can be excited to fluorescence (460 nm) by irritating the cells with UV light (366 nm). This fact is used as the basis of a noninvasive online method for fermentation process monitoring in stirred tank bioreactors (3-5). Since fluorescence readers for microplates are common available analytical instruments, the adaptation of this technique to the microplate should be accomplishable. Researchers, however, have been using either turbidity measurements (6,7) or specially designed microplates with integrated fluorophores (8-10) for growth monitoring in multiwell plates during high-throughput screening (HTS).

The optical density or absorbance (A) of a suspension diluted in such a way that light scattering centers are essentially independent is directly proportional to the biomass concentration (C) (2):

$$A = k \times C \quad [\text{Eq. 1}]$$

where k is the proportionality constant. Biomass C can be calculated from NAD(P)H-dependent fluorescence (F) by (4,5):

$$C = [\exp(-b) \times F]^{1/a} \quad [\text{Eq. 2}]$$

where a and b are constants. Equation 1 may then be written similarly to Equation 2 as:

$$A = [\exp(-b^*) \times F]^{1/a} \quad [\text{Eq. 3}]$$

where $b^* = b - (a \times \ln k)$.

To verify that Equation 3 is likewise true in microscale, both the absorbance and the fluorescence of serial dilutions of two JM101 cultures grown in two different media were determined.

Terrific broth (TB) and Luria Bertani broth (LB) (both from Sigma, St. Louis, MO, USA) were used as media and inoculated with 1% (v/v) of a JM101 glycerol stock with 5×10^8 viable cells/mL. The reaction vessels were glass Erlenmeyer flasks (100 mL, four baffles) and flat-bottom transparent polystyrene arrays, namely nontreated 48-well cell culture plates (BD Biosciences Discovery Labware, Bedford, MA, USA) and 96-well microplates (Greiner, Frickenhausen, Germany). Two flasks were filled with 10 mL inoculated TB and LB, respectively, closed with a cotton plug, and incubated for 18 h at 37°C on a rotary shaker with 25 mm diameter at 180 rpm. Then three serial dilutions of both cultures in respective media were pipetted, each in both a 48-well plate (400 µL/well) and a 96-well plate (200 µL/well) using a Beckman 2000 liquid handler (Beckman, Fullerton, CA, USA). Absorbance was determined by reading the 96-well plate in a SpectraMax® reader (Molecular Devices, Sunnyvale, CA, USA) at 620 nm. Pure media were used as blanks, and data was converted to 1 cm path length. A linear correlation is only true if $A < 0.5$ (7). Higher A values, however, may be calculated from more diluted samples. The means of each 3-fold measurement, as well as the results from calculations, are shown in Figure 1A. F was determined by reading the 48-well plate in a FLUOstar reader (BMG, Offenburg, Germany) using the following crucial settings: no. of flash-