Biochemical and Biophysical Research Communications 417 (2012) 945–950

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Copy number of adenoviral vector genome transduced into target cells can be measured using quantitative PCR: Application to vector titration

Zheng Pei, Saki Kondo, Yumi Kanegae, Izumu Saito *

Laboratory of Molecular Genetics, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan

ARTICLE INFO

Article history:
Received 25 November 2011
Available online 19 December 2011

Keywords:
Adenovirus vector
Virus titer
Quantitative PCR

ABSTRACT

Both transfection and adenovirus vectors are commonly used in studies measuring gene expression. However, the real DNA copy number that is actually transduced into target cells cannot be measured using quantitative PCR because attached DNA present on the cell surface is difficult to distinguish from successfully transduced DNA. Here, we used Cre/*lox*P system to show that most of the transfected DNA was in fact attached to the cell surface; in contrast, most of the viral vector DNA used to infect the target cells was present inside the cells after the cells were washed according to the conventional infection protocol. We applied this characteristic to adenoviral vector titration. Current methods of vector titration using the growth of 293 cells are influenced by the effect of the expressed gene product as well as the cell conditions and culture techniques. The titration method proposed here indicates the copy numbers introduced to the target cells using a control vector that is infected in parallel (relative vector titer: rVT). Moreover, the new titration method is simple and reliable and may replace the current titration methods of viral vectors.

1. Introduction

Transfection is the most commonly used method of choice for examining the nature and function of a gene *in vivo* because this technique is very simple to perform and easy to manipulate. However, the copy numbers of DNA that successfully reach the inside of the target cells cannot be measured using quantitative PCR (qPCR), since experiments using qPCR cannot effectively distinguish DNA present inside the cells from DNA attached to and present on the cell surface. The first-generation adenovirus vector (FG AdV) is now commonly used for gene expression experiments, mainly because the resulting expression level is much higher than that achieved using transfection. Another reason is that the data offered by this vector is quantitative for a linearity range that is about 20-fold wider [1]. However, the vector system is also thought to be unsuitable for qPCR for the same reason mentioned above.

There are several methods for using FG AdV. The most popular titration methods are bioassays of plaque-forming unit (PFU) [2,3] and end-point cytopathic effect (CPE) assay or 50% tissue-culture infectious dose ($TCID_{50}$) assay [3,4]. These methods were actually developed for the titration of wild-type adenoviruses, and not for the titration of FG AdV. At least 4 days or up to 2 weeks are required to obtain the endpoint, and the results often vary depending on the conditions of the 293 cell lines, researchers and laboratories. The immunofluorescent focus assay using a florescent microscope [5,6] and the immunospot assay using 3,3′-diaminobenzidine staining [7] (TaKaRa Bio kit), count the foci of infected 293 cells expressing viral hexon protein. Although the titration can be completed in 2 days, these methods also rely on viral replication in 293 cells. The amount of AdV particles has been measured based on the optical density at 260 nm (OD_{260}) [8], although this method can only be used for purified virus stock. Because the AdVs replicate rapidly in growing 293 cells in all these methods, the titration results are sometimes influenced by the expressed product of an inserted gene if it disturbs viral replication or the growth of 293 cells. Consequently, sometimes the results do not reflect the actual copy number that was transferred to the target cells, which is undoubtedly the most important ability of a "vector".

qPCR has been used to calculate the copy numbers of AdV in viral stocks [9]. In the preparation of helper-dependent AdV (HD AdV), the contaminated helper virus (an FG AdV) in the viral stock has been measured using qPCR [10,11]. Another category of the qPCR method obtains the viral titer not by measuring AdV DNA in the viral stock, but by quantifying the copy numbers of *transduced viral genomes in the target cells* (genomic infectious titer,

Abbreviations: FG, first-generation; AdV, adenoviral vector; PFU, plaque-forming unit; $TCID_{50}$, 50% tissue-culture infectious dose; CPE, cytopathic effect; qPCR, quantitative real-time PCR; HD-AdV, helper-dependent AdV; GIT, genomic infectious titer; rVT, relative vector titer; MOI, multiplicity of infection; NLS, nuclear localization signal; OTC, Ornithine transcarbamylase.

* Corresponding author. Fax: +81 3 5449 5432.
E-mail address: isaito@ims.u-tokyo.ac.jp (I. Saito).

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.12.016

GIT); the total DNA of the infected cells were extracted, and the viral DNA were detected using slot-blot hybridization [12] or qPCR [10,13]. Although all these methods are intended to measure the copy number of the viral genome, there are two problems with using them for the titration of FG AdV. One reason is similar to that described above for transfection but is more crucial: the obtained copy numbers include not only the viral genome of the internally transduced viral particles, but also the DNA of *non-infectious particles* and unpackaged naked DNA that is present either freely in the viral stock or attached to the surface of the target cells. The other problem is that the GIT fluctuates markedly depending on the target cell concentration and conditions; hence, GITs obtained at different times and places are difficult to compare. Therefore, both of these problems must be solved to establish a reliable GIT method. In this paper, we propose a new titration method that solves these problems.

2. Materials and methods

2.1. Cell lines and recombinant adenovirus

The human embryo kidney cell line 293 [14] constitutively expresses adenoviral E1 genes. The cell line CV-1 is derived from African green monkey kidney. HeLa cells are derived from human cervical cancer. The cell line NIH-3T3 was established from an NIH Swiss mouse embryo. AxCANCre, a Cre-expressing AdV tagged with a nuclear localization signal [15], and AxEFdsR, a dsRed-expressing AdV [16], have been described previously. The GFP-expressing AdV AxCAGFP was generated using the COS-TPC method [17]. The AdV AxEFLNLdsRed is identical to AxCALNLZ [15] except that the CAG promoter and the LacZ gene were replaced by the EF1α promoter, and the dsRed gene, respectively. The $TCID_{50}$ was measured according to the protocol described by Kanegae et al. [4]. The plasmid pA14cw contains the AdV genomic DNA of pAdex1w [17] from map units 0 to 14.

2.2. Southern blotting analysis

CV-1 cells in a 6-cm dish were infected with AxCANCre. After 24 h, the cells were infected with AxEFLNLdsRed or transfected with 1 μg of the plasmid pxEFLNLdsRed per 6-cm dish using Transfast (Promega). The total DNA was prepared from the dish [18]. Before alkaline treatment, the agarose gel was exposed to 0.1-N HCl for partial depurination causing DNA fragmentation to several hundred base pairs (bp) to obtain the complete transfer to the membrane [19]; the DNA was then transferred to the nylon membrane Hybond-N (Amersham GE) using the capillary-transfer method [20]. Specific DNA was detected using a DIGDNA Labeling and Detection Kit (Roche Diagnostics). The 0.6-kb *Xmn*I fragment derived from the EF1α promoter region was labeled with digoxigenin-UTP, and specific DNA was detected using the chemiluminescence of CDP-Star (Roche Diagnostics). The bands were visualized using LAS-4000 (Fuji Film) and the densitometry was performed using an image analysis program (Multi Gauge version 3X, Fuji Film). The linear correlation between the DNA amounts and the intensity of the bands was confirmed (Fig. S1 of Supplementary Data), showing that the Southern analysis was quantitative.

2.3. qPCR

The infected total cell DNA was prepared from cells, as described previously [18,21]. Alternatively, we confirmed the total cell DNA prepared using a DNA preparation kit (Macherey–Nagel through TaKaRa Bio). qPCR was performed to detect the AdV genome using a probe for the pIX gene [16] (Fig. 2A). The amount of chromosomal DNA was simultaneously measured to correct the Ct values of the viral genome per cell, and the corrected Ct was shown throughout. The probes were derived from the sequence of the human β-actin gene for HeLa, the human OTC gene for CV-1 [16], and the mouse GAPDH gene for NIH-3T3 (Applied Biosystems, catalog number 7000-1). The qPCR reaction was performed according to the manufacturer's protocol: 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min (Applied BioSystems).

2.4. Generation of a standard curve using qPCR

The copy numbers of the plasmid pA14cw containing the pIX sequences and the cosmid pAxcwit2 [22] containing the full-genome of the FG AdV [22] were calculated according to Puntel et al. [11]. The plasmid and cosmid were serially diluted (Fig. 2B and C). The equivalency between the molecular weight of the plasmid pA14cw and the number of copies was calculated by considering the pA14cw molecular weight and the equivalency between base pairs (pA14cw [4, 247 bp]) and Daltons (Da) ($1bp_{Ad5}$ = 678 Da). $Mass_{pA14cw}$ (Da) = 4, 247 bp/molecule × 678 Da/bp = 2.88×10^6 Da/molecule. We obtained the equivalency of mass 2.88×10^6 Da/molecule × 1.66×10^{-18} μg/Da = 4.78×10^{-12} μg/molecule. The copy numbers of the cosmid pAxcwit2 (42, 698 bp) were similarly calculated.

3. Results and discussion

3.1. Quantification of internally transduced viral copies in target cells

To establish a reliable GIT method, determining the ratio of successfully internalized viral DNA to DNA that has physically attached to the cell surface (that is, naked viral DNA or DNA in inactivated viral particles in AdV-infected target cells) is essential. To estimate the amounts of the former and the latter, we utilized the Cre/*lox*P system. CV-1 cells were infected with the AdV AxCANCre expressing Cre at an MOI of 5. Then, 24 h later, the cells were infected with the target AdV AxEFLNLdsRed at an MOI of 7.5 or were transfected with 1 μg of the target plasmid pxEFLNLdsRed as a control. The target unit in the AdV and the plasmid contains the same sequences of the EF1α promoter and the dsRed gene flanked by two *lox*Ps (Fig. 1A). The total cell DNA was extracted after the indicated number of days and digested with *Bgl*II; the DNA of the target unit was detected using a Southern technique (Fig. 1B and C). The 2.8-kb band (S2.8) indicates the substrate originally present before Cre-mediated recombination, i.e., the unprocessed substrate, while the 1.5-kb band (R1.5) shows the presence of the recombined product. We considered that the viral DNA and the transfected DNA that are physically attached to the cell surface cannot be processed by Cre and remain as unprocessed substrate.

When the cells were transfected with the target plasmid, the majority of the target DNA remained unprocessed even after 72 h (Fig. 1C, column 6). A densitometry analysis showed that only 9 ± 2% (n = 7) of the DNA was processed substrate. Also, a preliminary experiment showed that when using 3 μg of plasmid DNA, the percentage was 12% (data not shown). These results suggested that most of the transfected DNA was possibly present on the cell surface and that the DNA copy number after transfection did not reflect that of the internalized DNA molecules. In contrast, most of the target viral DNA was processed using Cre-mediated recombination by 2 or 3 days after infection (Fig. 1B, columns 5 and 6); the recombination efficiency was 92 ± 3% (n = 4). Considering that the recombination efficiency must not be 100%, the result suggested that at least 92% of the target viral DNA was present inside the infected cells. Similar results were obtained when using