Research Report

Quantitation of Retroviral-Mediated Transfer Using Luciferase in Living and Lysed Cells

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ABSTRACT

We have developed a murine retroviral vector containing an improved luciferase gene for the study of retroviral gene transfer and expression in living or lysed cells. We used a cytosolic form of luciferase gene (*luc+*) with transcriptional enhancements that yielded greater expression levels. The *luc+* gene was subcloned into the retroviral plasmids pDON-AI, in which almost the entire U3 region has been replaced with the heterologous human cytomegalovirus immediate-early promoter. A stable ecotropic and amphotropic retrovirus-producing cell line was generated with a titer 1×10^6 cfu/mL. NIH/3T3(tk-) cells transduced with ecotropic luciferase retrovirus demonstrated a high level of luminescence on the third day. Lysed NIH/3T3(tk-) cells demonstrated a 10-fold increase in activity as compared to living cultures. The creation of a new retroviral system allowed a substantial decrease to 5 days from the 10–14 days previously needed to evaluate viral transfer using the standard neomycin method. Our assay also provides a quantitative assessment in contrast to the β -galactosidase detection method, which also takes 5–6 days but lacks quantitative evaluation. Thus, the expression of an integrated *luc+* gene in eukaryotic cells provides a powerful tool for the study of retroviral gene transfer and will greatly facilitate functional studies in both living and lysed cells.

INTRODUCTION

Human gene therapy involves the transfer of therapeutic genes into living human cells. Murine replication-defective retroviral vectors are widely used as gene transfer vehicles (10), yet much remains to be studied and improved in retroviral vectors. To increase the utility of the retroviral gene delivery system, we developed a retroviral vector with *luc+* gene to study the rate of gene transfer and expression in both living and lysed cells.

The firefly (*Photinus pyralis*) luciferase gene is widely used as a reporter gene because of its sensitivity and ease of detection (2), and it was used to monitor viral dissemination in live infected mammalian cells (9,13). The firefly luciferase gene under the control of the human immunodeficiency virus long terminal repeat has been used to study the effects of drugs on the retroviral replication in vitro (14,17) and in vivo (4,18). Proviral stability has also been assessed by introducing the firefly luciferase gene into Moloney murine leukemia virus-based vectors (19).

Luciferase expression in mammalian cells is typically measured by luminometer analysis of cell lysates (6). This method has the major disadvantage of destroying the biological sample, thus preventing further analysis of luciferase-expressing cells. It was shown that luciferase activity can be measured directly in living cells upon addition of luciferin solution (15). However, the luciferase-directed light output from intact cells has been shown to be decreased when compared with that from cell lysates (6).

Since the ultimate efficiency of

retroviral gene transfer depends on a sufficient viral titer, many applications of retroviruses for both research and gene therapy require titer evaluations. Procedures for screening clones for virus production routinely involve virus quantitation on a target cell line. A common method is the 10- to 14-day drug-resistant colony formation assay using a selectable marker such as a neomycin phosphotransferase (*neo*). More rapid methods involve the use of PCR amplification to quantitate levels of proviral DNA (16) or retroviral RNA (12). Another common method for titer determination is an assay for retrovirally encoded products such as β -galactosidase (3) or green fluorescent protein (GFP) (11,21). For either the β -galactosidase or GFP assay, the scoring can be performed by direct counting 5–6 days after retroviral transduction. However, β -galactosidase and neomycin assays require a time-consuming colonies counting, while the GFP quantitation is limited to using a fluorescence-activated cell sorting (FACS) analysis (unpublished data). Assays for the encoded gene product are important to ensure the integrity and high-efficiency expression of the proviral genome as exemplars of therapeutic gene. When screening individual supernatants from a large number of prospective clones, titer determinations can be time consuming, expensive, and labor intensive. The new assay described here allows rapid retroviral detection and thus permits a number of potentially useful applications. A major advantage of our assay resides in the ability to quantitate retroviral transfer in living cells with improved sensitivity. To monitor retroviral infection based on a more sensi-

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tive and simple assay in living or lysed cells, we constructed a retroviral vector with a modified luciferase gene. While there are many assays for reporter genes (1), this assay is unique in that it requires neither tissue or cell fixation nor lysis. Thus, the sample can be monitored continuously for retroviral gene expression in living cells.

MATERIALS AND METHODS

Cell Line and Cell Culture

The following cell lines were used: NIH/3T3(*tk*-), a murine cell line deficient in thymidine kinase and a ψ -2, a murine ecotropic retrovirus-packaging cell line (8). In addition, the following cell lines were obtained from ATCC (Manassas, VA, USA): PA317, a murine amphotropic retrovirus-packaging cell line (CRL-9078); B16-F1, a murine melanoma cell line (CRL-6323); A375, human malignant melanoma (CRL-1619); and 293, human embryonic kidney (CRL-1573). All cell lines were grown in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT, USA) at 37°C and in the presence of 5% CO₂.

Construction of the Luciferase Retroviral Vector

Plasmid pSP-*luc*+ was obtained from Promega (Madison, WI, USA). Plasmid pDON-AI was obtained from Takara Shuzo (PanVera, Madison, WI, USA). In this retroviral vector, the U3 region was replaced by a heterologous human cytomegalovirus immediately-early promoter (7). All restriction en-

donucleases were obtained from Promega. Plasmid pSP-*luc*+ was digested with *Bgl*II and *Xho*I to obtain the 1.7-kb *luc*+ fragment, which was then gel-isolated using a QIAquick™ Gel Extraction Kit (Qiagen, Valencia, CA, USA). Plasmid pDON-AI was digested with *Bam*HI and *Sal*I. The *luc*+ fragment was then ligated into linearized pDON-AI to produce the plasmid pDONL (Figure 1).

Construction of the Luciferase Vector-Producer Cell Lines

PA317 cells were plated at a density of 2×10^5 cells/well in 6-well plates 24 h before transfection. Plasmid DNA for transfection was isolated and purified using the QIAGEN® Plasmid Maxi Kit (Qiagen). Cells were transfected with 2 μ g pDONL plasmid DNA using SuperFect™ Transfection Reagent (Qiagen) according to the manufacturer's protocol. Forty-eight hours later, the supernatant was collected, filtered (0.45 μ m; Millipore, Bedford, MA, USA), and supplemented with 8 μ g/mL polybrene (Sigma, St. Louis, MO, USA). ψ -2 cells were plated at a density of 2.5×10^4 cells in a 35-mm dish the day before the transduction. One milliliter of supernatant produced as described above from pDONL-transfected PA317 cells was added. The ψ -2 cells were incubated for 2 h at 37°C, and an additional 1 mL medium was added. After 24 h, the ψ -2 cells were trypsin-digested and transferred to a 100-mm tissue culture dish. PA317 and ψ -2 cells were selected in G-418 (Geneticin®; Invitrogen) for 10–14 days with G-418 concentration of 0.4 mg/mL (PA317) and 0.6 mg/mL (ψ -2). Amphotropic retroviral-producing cell line (PA317/pDONL) and ecotropic retroviral-pro-

ducing cell line (ψ -2/pDONL) were obtained. For the generation of clones, the transduced PA317 cells were selected in the presence of G-418, and the surviving clones were isolated by ring cloning, transferred to a 24-well plate, and allowed to grow to confluence for 2 days. Each clone was then replated in a 35-mm tissue culture dish. The clones were then incubated in a CO₂-incubator at 37°C for 24 h. After that, the culture supernatant was used for transduction of the NIH/3T3(*tk*-) cells. The PA317/pDONL clone with the highest luminescent activity was used for transduction of ψ -2 cells as described above.

Cloning of the Stably Transfected Cell Lines

A stable producer cell line was created to demonstrate the utility of the luciferase method for retroviral titer evaluation. The retroviral vector pDONL (Figure 1) was transfected by the SuperFect transfection reagent into the PA317 cell line. The cells were then selected in the presence of G-418 (0.4 mg/mL) for 10–14 days. Surviving clones were isolated by ring cloning, transferred to a 24-well plate, and allowed to grow to confluence. The medium was changed, and the supernatant was used for transduction of NIH/3T3(*tk*-) cells. The luminescence was assayed 24 h later. The clone that showed the highest luminescent activity was next assayed by the standard G-418 resistance viral titer assay. The supernatant from PA317/pDONL cells was also used to obtain a ψ -2/pDONL producer cell line.

Transduction of NIH/3T3(*tk*-) Cells and Neomycin Titer Determination

Vector was obtained from producer cells by growing them at 37°C to 80%–90% confluence, adding 10 mL fresh medium and incubating at 32°C for 48 h. The supernatant was collected, filtered (0.45 μ m), and supplemented with 8 μ g/mL polybrene. One milliliter of retroviral supernatant was added per well of NIH/3T3(*tk*-) cells plated at a density of 2.5×10^4 cells in 6-well plates the day before the experiment. The supernatant was removed 12 h later, replaced with fresh medium containing G-418 (0.8 mg/mL), and selected

Figure 1. Retroviral vector pDONL. LTR, long terminal repeat; *luc*+, cytosolic form of firefly luciferase gene; *neo*, neomycin phosphotransferase; SV40, simian virus 40; HCMV IE promoter, human cytomegalovirus immediate-early promoter; ψ + indicates the presence of the viral packaging sequence (not to scale).

for 10–14 days. After the selection, the titer was determined by counting the number of colonies.

Transduction of NIH/3T3(*tk*-) Cells with Measurement of Luminescent Activity in Living Cells and in Cell Lysates

NIH/3T3(*tk*-) cells were plated at a density of 2.5×10^4 cells in 35-mm tissue culture dishes the day before the ex-

periment. One milliliter of retroviral supernatant was added to the culture dishes. The supernatant was removed 12 h later and replaced with 2 mL fresh medium. After transduction (24, 48, or 72 h), 0.5 mL 0.5 mM luciferin potassium salt solution (Biosynth International, Naperville, IL, USA) was added. The dish was immediately placed in the TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA), and the luminescence signal was measured for 120 s, with a delay pe-

Figure 2. Luminescent activity of NIH/3T3(*tk*-) cells transduced with amphotropic (A) and ecotropic (B) retroviruses expressing the luciferase gene. After the indicated periods of time, each dish of infected NIH/3T3(*tk*-) cells was washed once with PBS, and 0.5 mM solution of luciferin potassium salt in PBS was added. Then, the dish was immediately placed in the TD-20/20 luminometer, and the luminescence signal was measured for 120 s, with the delay period of 3 s. The luciferin solution was then aspirated. The cells were washed once with the PBS, and the fresh medium was added. Measurements were carried out at room temperature. All determinations of luminescent activity were done in triplicate. PBS, Dulbecco's PBS solution.

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Figure 3. Luminescent activity of NIH/3T3(*tk*-) cell lysates 72 h after transduction with ecotropic retrovirus expressing luciferase gene. NIH/3T3(*tk*-) cells were washed once with PBS and lysed with 200 μ L Cell Culture Lysis Reagent, and luminescence signals were measured using a Luciferase Assay System in the TD-20/20 luminometer. Cell lysate (20 μ L) was placed into a test tube, and 100 μ L Luciferase Assay Reagent were used for injection. The luminescent output was read for 15 s, with a delay period of 3 s. Measurements were carried out at room temperature. All determinations of luminescent activity were done in triplicate. PBS, Dulbecco's PBS solution.

Figure 4. Luminescent activity of cell lysates 72 h after transduction with amphotropic retrovirus expressing the luciferase gene. Human melanoma A375, human kidney embryonic 293, murine melanoma B16-F1, and NIH/3T3(*tk*-) cells were washed once with PBS and lysed with 200 μ L Cell Culture Lysis Reagent, and luminescence signals were measured using a Luciferase Assay System in the TD-20/20 luminometer. Cell lysate (20 μ L) was placed into a test tube, and 100 μ L Luciferase Assay Reagent were used for injection. The luminescent output was read for 15 s, with a delay period of 3 s. Measurements were carried out at room temperature. All determinations of luminescent activity were done in triplicate. PBS, Dulbecco's PBS solution.

riod of 3 s. After the measurement the luciferin solution was aspirated, the cells were washed once with the PBS (Invitrogen), and fresh medium was added.

Cells also were lysed with 200 μ L Cell Culture Lysis Reagent (Promega) 72 h after transduction, and luminescence signals were measured using a Luciferase Assay System from Promega according to the manufacturer's protocol in the TD-20/20 luminometer.

RESULTS

Luciferase Expression in Transduced Cells

The NIH/3T3(*tk*-) cells transduced with both PA317/pDONL and ψ -2/pDONL demonstrated titer of 1×10^6 cfu/mL. The NIH/3T3(*tk*-) cells transduced with PA317/pDONL supernatant examined 24, 48, and 72 h after transduction demonstrated luminescence of 0.25 ± 0.03 , 7.8 ± 0.21 , and 38 ± 1.61 relative light units (RLU), respectively. The NIH/3T3(*tk*-) cells transduced with ψ -2/pDONL supernatant examined 24, 48, and 72 h after transduction demonstrated luminescence of 38.6 ± 3.6 , 166.8 ± 3.9 , and 456.9 ± 40.9 RLU, respectively. A 1:10 dilution of the retrovirus resulted in a nearly 10-fold reduction in luminescent activity (Figure 2, A and B). No significant background luminescence was detected in non-transduced cells (Figure 2, A and B). The lysates of NIH/3T3(*tk*-) cells transduced with ψ -2/pDONL supernatant examined 72 h later demonstrated luminescence of 32753 ± 1083 RLU (Figure 3). The luminescent signal in cell lysates was approximately 1 order of magnitude higher than that in living cells. We were able to detect a luminescent signal from a 10^{-5} dilution of a supernatant, which has a 10^6 cfu/mL neomycin titer (Figure 3). We have also measured luminescence in selected human and murine cell lines after transduction with luciferase retroviruses (Figure 4). Surprisingly, the murine melanoma cell line B16-F1 did not show any luminescence after transduction with ecotropic retrovirus but did have a light output after transduction with amphotropic retrovirus. A375, B16-F1, and NIH/3T3(*tk*-) cells trans-

duced with amphotropic virus demonstrated a linear dependence of the signal versus dilution with high reproducibility starting from dilution 10^{-1} , while for 293 cells it began at 10^{-2} dilution (Figure 4). NIH/3T3(*tk*-) cells transduced with ecotropic virus demonstrated linear dependence of the signal versus dilution with high reproducibility starting from non-diluted virus (Figure 3).

DISCUSSION

In the present study, we have demonstrated that luciferase-containing retroviral vectors can be used for the quantitation of retroviral transfer in living cultured mammalian cells. Previously, retroviral studies have been hampered by the time-consuming neomycin titer test. The luciferase-containing retrovirus will allow researchers to track real-time events in living cells, tissues, and animals. Our analysis is real-time and affords quantitative monitoring of luciferase gene activity in live cells. The ability to use this system in living mammalian cells is a powerful tool for retroviral biology. The dynamic range of the assay is over 4 orders of magnitude for both live cells and lysates (Figures 2 and 3).

Using a luciferase gene greatly simplified the measurement of retroviral titer. In living cells, the luminescent reaction is started simply by the addition of luciferin because ATP, magnesium ions, and oxygen are provided by the intracellular pool. Cells that are transfected with luciferase reporter plasmids exhibit luminescence after being incubated in the presence of luciferin solution (15). Instead of native luciferin, an ester of luciferin (5) or a photolyzable "caged" luciferin can also be used (20).

The method we outline also eliminates the need to perform a time-consuming "kill curve" for drug resistance. After establishing the linear range in the cell system being used, a quantitative titer can be obtained with non-diluted or a single dilution of the sample. Thus, the time-consuming process of setting up a serial dilution, as when performing neomycin or β -galactosidase titer assay, is eliminated.

In summary, we have developed a simple and rapid method for retroviral

transfer determination. With this method, the whole procedure from transduction to determination of viral transfer can be shortened to 5 days with no loss of sensitivity.

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