Tracking and Quantitation of Retroviral-Mediated Transfer Using a Completely Humanized, Red-Shifted Green Fluorescent Protein Gene

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ABSTRACT

We have developed murine retroviral vectors (RVs) containing an optimized green fluorescent protein (GFP) gene to study retroviral gene transfer and expression in living cells. We used the codon "humanized", "red-shifted" GFP gene, hGFP-S65T, a gain of function variant of the wild-type GFP from the jellyfish *Aequorea victoria*. We cloned the hGFP-S65T gene into the RV plasmid pLNCX (pLNChG65T). A stable amphotropic RV-producer cell line (VPC), designated LNChG65T VPC, was generated that exhibited bright fluorescence in greater than 95% of the cells. Human A375 melanoma cells and IGROV ovarian carcinoma cells transduced from LNChG65T VPC demonstrated high levels of fluorescence. The expression of a single integrated hGFP-S65T gene in eukaryotic cells provides a powerful tool to study gene transfer, expression and functional studies *in vitro* and *in vivo*.

INTRODUCTION

Human gene therapy involves the transfer of therapeutic genes into living human cells (4,12,15). Murine replication-defective retroviral vectors (RVs) are used as the gene transfer vehicle in two-thirds of the current gene therapy clinical protocols (11). To study gene transfer and expression *in vivo* and *in vitro*, we previously developed an RV using a markedly improved green fluorescent protein (GFP) gene containing a red-shifted, partially humanized GFP (10). We now report using a red-shifted, completely humanized GFP gene, the hGFP-S65T gene, which has 169 codon changes compared to 88 in our prior report. Efficient fluorescence of this GFP protein allowed direct detection of gene transfer and activity in living mammalian cells.

GFP is a 730-bp gene cloned from the jellyfish *Aequorea victoria* that encodes a 27-kDa protein (14). Upon exposure to blue light (450–490 nm), the protein emits a vibrant green fluorescence. Wild-type GFP expression has

been applied in a number of eukaryotic and prokaryotic systems (1,2,9,13,16). Heim and colleagues developed an improved GFP gene variant (S65T) with a "red-shifted" excitation maximum, which has a fluorescent intensity approximately five times stronger than wild-type GFP and fourfold faster fluorophore oxidation (8). Zolotukhin et al. partially "humanized" the "red-shifted" mutant by introducing 92 silent mutations that alter the codons to those more commonly used in human genes (17). The new variant hGFP-S65T gene has even greater modifications (7). Our data with this novel GFP variant gene in human cells demonstrate improved fluorescence compared to previously available GFP variants.

MATERIALS AND METHODS

Cell Culture

The following cell lines were used: A375, a human melanoma cell line (ATCC, Rockville, MD, USA); IGROV,

Figure 1. Retroviral construct containing red-shifted, humanized green fluorescent protein. The hGFP-S65T gene from phGFP-S65T was cloned into the retroviral vector LNCX to produce pLNChG65T. LTR, long terminal repeat; pA, polyadenylation signal; arrows indicate transcriptional start sites; Ψ⁺ indicates the presence of viral packaging sequence; hGFP-S65T, humanized, red-shifted GFP.

Figure 2. Detection of hGFP-S65T activity in transduced cell lines. A) PA317-LNChG65T cells (40 $\times$ magnification). B) A375-LNChG65T cells 72 h after transduction with background light (10 $\times$ magnification). C) IGROV-LNChG65T cells 5 days after transduction and G-418 selection for 4 days (40 $\times$ magnification).

an ovarian carcinoma cell line (kindly provided by Patrick Hwu, National Cancer Institute); PA317, a murine amphotropic, RV-packaging cell line (kindly provided by A.D. Miller, University of Washington); and GPE86, a murine ecotropic, RV-packaging cell line (kindly provided by Arthur Banks, Columbia University). Cells were grown in RPMI supplemented with 10% fetal calf serum (FCS) (both obtained from Life Technologies, Gaithersburg, MD, USA) in monolayers at 37°C and 5% CO₂. All cells were passaged and harvested by standard trypsin (Life Technologies) digestion at 37°C. Cells were routinely passaged at 80%–90% confluence.

Construction of GFP Retroviral Vector

Plasmid pHGFP-S65T was obtained from CLONTECH Laboratories (Palo Alto, CA, USA). Cloned construct plasmid DNA was transformed into MAX EFFICIENCY DH5 α TM Competent Cells (Life Technologies), and colonies were grown on L-broth supplemented with ampicillin (50 μ g/mL) plates (LB/AMP). Positive colonies were grown in LB/AMP broth, and plasmid DNA was isolated using the plasmid kit from Qiagen (Chatsworth, CA, USA). Plasmid pHGFP-S65T was restriction-digested with *Hind*III and *Not*I to obtain the 750-bp hGFP-S65T open reading fragment, which was then gel-isolated (Jet-sorb[®]; Genomed, Raleigh, NC, USA). Plasmid pLNCX was restriction-digested with *Hind*III and *Hpa*I. The hGFP-S65T fragment was then ligated into linearized pLNCX, followed by treatment with a Klenow enzyme to remove the *Not*I overhang. This 3' end was then ligated to the *Hpa*I site to produce plasmid pLNChG65T.

Construction of the LNChG65T Vector-Producer Cell Line

GPE86 cells were plated in a 6-well dish (Falcon[®]) 24 h before transfection. Cells that were at 30%–50% confluence were transfected with 5 μ g of pLNChG65T plasmid DNA and 15 μ L of DOTAP reagent (Boehringer Mannheim, Indianapolis, IN, USA) according to the manufacturer's protocol. After 12 h, the cells were rinsed, and 1 mL

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of fresh medium was placed in each well. RV supernatant was collected 24 h later and filtered (0.45 μ m; Corning Costar, Cambridge, MA, USA); then 10 μ g/mL of protamine sulfate solution were added. This supernatant was transferred to a 6-well dish containing

PA317 cells that were at 30%–50% confluence. After 24 h, both cell lines were trypsin-digested and transferred to a 10-cm tissue culture dish (Falcon). Cells were selected in 1 mg/mL G-418 (Genteticin®; Life Technologies) for 10–14 days.

Transduction of A375 and IGROV Human Tumor Cell Lines

LNChG65T VPC cells were grown to 80%–90% confluence in Nunclon™ T-175 flasks (Allegiance Health Care, McGaw Park, IL, USA). The medium

Figure 3. FACS analysis of hGFP-S65T-transduced PA317 vector-producer cells and human melanoma cells. A) PA317 packaging cells are 98.4% negative (bar 2). B) PA317-LNChG65T cells are 95.8% positive (bar 1). C) A375 cells are 99.5% negative (bar 1). D) A375-LNChG65T cells are 98.7% positive (bar 2). E) IGROV cells are 98.9% negative (bar 1). F) IGROV-LNChG65T cells are 89% positive (bar 2). All FACS analyses used the FL1 emission channel used to monitor green fluorescence. Count: Cell number counted at given fluorescence intensity; log scale represents the mean intensity of green fluorescence detected at 525 nm.

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was replaced with 20 mL of fresh medium, and 24 h later the RV supernatant was collected, filtered and supplemented with 10 μ g/mL protamine sulfate. A375 and IGROV cells were plated onto 10-cm tissue culture dishes and in 6-well dishes containing sterile coverslips. Ten milliliters of the supernatant were added to the culture dishes, and 2 mL were added to each well of the 6-well plates. The supernatant was removed 24 h later and replaced with fresh medium containing G-418 (1 mg/mL) and selected for 7–14 days.

Fluorescent Detection of GFP-Expressing Cells

The hGFP-S65T-expressing cells were visualized with a Nikon Labophot-2TM fluorescent microscope (Melville, NY, USA). The filter cube used in the microscope was the FITC dichroic filter set (excitation at 450–490 nm and

emission at 520 nm). The coverslips from the 6-well dishes were inverted and placed on a glass slide for viewing. Photographs were taken using the MicroflexTM AFX-DX systems (Nikon).

Fluorescence-Activated Cell Sorter (FACS) Analysis of LNChG65T-Transduced Mammalian Cells

Cytometry of transduced cells was performed on an EPICS[®] Profile II Analyzer (Coulter, Miami, FL, USA) with an excitation source of 488 nm. Cells were analyzed using a 525-nm band-pass filter set (Part No. 814036; Coulter). Cultures of cells that were 80%–90% confluent were trypsin-digested, washed with RPMI with 10% FCS and resuspended at a concentration of approximately 1×10^6 cells/mL. All FACS analyses used the FL1 emission channel to monitor green fluorescence (normally an FITC monitor).

RESULTS

Transfected Cell Lines

GPE86 RV-packaging cells were transfected with the construct pLN-ChG65T (Figure 1). Once selected, nearly 100% of the cells emitted green fluorescence after excitation (data not shown). No cytopathic or growth-inhibiting effects due to the expression of pLNChG65T in transfected cells were observed.

Transduced Expression of GFP

The LNChG65T vector-producer cell line (VPC) exhibited intense fluorescence (Figure 2A). A375-LNChG65T-transduced cells (Figure 2B), examined 72 h after transduction, demonstrated fluorescence in approximately 50% of the cells. After 7 days of G-418 selection, approximately 90% of

the A375 (data not shown) and 70% of the IGROV cells demonstrated fluorescent activity (Figure 2C). By 14 days after selection, both of the transduced cell lines demonstrated nearly 100% fluorescence. No significant background fluorescence was detected in nontransduced cells (10).

FACS Analysis of GFP-Transduced Cell Lines

PA317, A375 and IGROV cells transduced by the LNChG65T RV vector and selected in G-418 were analyzed by FACS. The PA317- and A375-transduced cells (Figure 3, B and D) were distinguished by up to a three log shift in mean fluorescent intensity in comparison to nontransduced control cells (Figure 3, A and C). Transduced IGROV cells (Figure 3F) also showed a significant shift in fluorescence intensity (Figure 3E).

DISCUSSION

The ability to observe gene activity in living cells is now optimized with the hGFP-S65T fluorescent gene marker system. Expression studies using the wild-type GFP gene in many eukaryotic cells and organisms have been hampered by the relatively low levels of fluorescence detected. We were not able to detect wild-type GFP gene product by either fluorescence microscopy or FACS in wild-type GFP RV-transduced and selected mammalian cells (10). Several modifications have been applied to improve the expression and fluorescence of GFP (5–8,17). We have investigated several of these variants. In stable retroviral transduction with the red-shift mutation only, fluorescence has been reported (3), but it was at least one log less intense than pLNChRG-expressing cells (10). The “humanization” of 88 codons, without the red-shift mutation, in the GFP open reading frame and subsequent expression in pLNCX showed only moderate levels of fluorescence by both microscopic and FACS analysis (unpublished observation). When we combined 88 codon modifications with the red-shift mutation, a substantial increase in fluores-

cence was observed (10). In this report, we demonstrate a greater improvement using a more extensively modified GFP gene in the pLNChG65T vector. This *hGFP-S65T* gene contains 81 more codon modifications compared to the *hRGFP* gene (10). This improvement is demonstrated by comparing the 79.9% shift in fluorescence in the hRGFP VPC (10), to the 95.8% with the current version (Figure 3B). Furthermore, Southern analysis of an individually selected, highly fluorescent clonal population of the A375-hGFPS65T cells revealed the integration of one copy of the *hGFP-S65T* gene (data not shown), establishing the remarkable sensitivity of this system. Undoubtedly, further modifications will lead to continued development of GFP transgene fluorescence, such as that reported recently by Cormack et al. (5). It is possible that specific GFP variants will be most useful in certain mammalian cell lines or with specific gene transfer vectors. Direct comparison of each available GFP is currently in progress to assess the contribution of each improvement in a retroviral system. The *hGFP-S65T* GFP gene will allow researchers to see real-time events in living cells, tissues and animals and will likely revolutionize the study of gene expression.

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