EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 33, No. 3, 174-178, September 2001

Monitoring transfected cells without selection agents by using the dual-cassette expression EGFP vectors

Sang-Gu Kang[1,4], Eunkyung Lee[2], Scott Schaus[3] and Eric Henderson[3]

[1] Institute of Biotechnology
[2] Department of Pharmacology, Yeungnam University, Kyongsan 712-749, Korea
[3] Department of Zoology and Genetics, Iowa State University, Ames, Iowa 50011, USA
[4] Corresponding Author: Tel, +82-53-810-2986; Fax, +82-53-816-8498; E-mail: kangsg@yu.ac.kr

Accepted 14 September, 2001

Abbreviations: GFP, green fluorescent protein; PBS, phosphate-buffered saline; EM, electron microscopy

Abstract

Conventional methods of selecting gene transfected cells by toxic agents may yield ambiguous results. It is difficult to determine whether cell death is due to selection agents or gene transfection, owing to the substantial overlap of the time-courses for both effects. Therefore, to determine transfection-induced cell toxicity, the mammalian expression vector pEGFP-N1 (CLONTECH Lab., Palo Alto, CA, USA) has been modified to the dual-cassette expression vectors named pEGFP-Ks by the relocation of its EGFP expression cassette. We have precisely monitored the cells transfected with this vector on our custom culture dishes, thereby bypassing the need for selection agent or fluorescent cell sorting. This is a useful method to screen genes encoding potential toxic or useful proteins without performing undesirable selection agent and also can be used to monitor the transfected cells for various purposes, either the inhibition or proliferation of mammalian cells for applications in biotechnology.

Keywords: green fluorescent protein, EGFP, cancer cell proliferation, transfection, dual-cassette expression vector

Introduction

The green fluorescent protein (GFP) gene of *Aequorea victoria* (Prasher *et al*., 1992) has been widely adapted for use as a molecular marker for gene expression in living cells and whole organisms because no substrate or cofactor is required for detection (Chalfie *et al*., 1994). Many different versions of the GFP vectors have been developed and extensively used for promoter analysis, monitoring gene expression, and protein localization analysis (Chalfie *et al*., 1994; Flach *et al*., 1994; Wang and Hazelrigg, 1994; Kain *et al*., 1995; Li *et al*., 1998). The EGFP-N1 vector (CLONTECH Lab.) has been designed to monitor gene expression and protein localization of the gene of interest by fusion to the N-terminus of the EGFP coding sequences as a transcription reporter (Kain *et al*., 1995; Stauber *et al*., 1998). This fusion protein vector system may be directly applied to study the toxicity caused by the transfected cDNA, but is not applicable for non-protein-encoding genes such as tRNA genes, rRNA genes, and snRNA genes. An additional technical difficulty lies in the monitoring method used to identify the cells suppressed by toxicity. For instance, observing sequential changes in transfected target cells on a conventional culture plate is not easily accomplished. Therefore, to facilitate continuous monitoring of cell toxicity resulting from gene transfection, we describe a simple modification of the GFP vector and a custom designed culture dish that have wide applications for biotechnology experiments.

Materials and Methods

Plasmids

To construct a dual-cassette expression vector, the pEGFP-N1 vector (Figure 1A) was digested by *Age* I and *Not* I to delete the EGFP coding sequence, blunted with the Klenow fragment of DNA polymerase (Promega, Madison, WI, USA), then re-circularized with T4 DNA ligase (Epicentre, Madison, WI, USA). This plasmid was named pB1. The CMV early promoter was PCR amplified with Taq polymerase (Sigma, St. Louis, MO, USA) using the primers: a forward primer UCMV1 (5'-TCAGGCCCTCACATGTTCTTTCCTGCG: *Eco* 0109 I underlined) and a reverse primer LCMV1 (5'-AGCTCGAGATCTGAGTCCGGTAGCGC: *Xho* I underlined). The amplified fragment was then cloned into pGEM-T Easy Vector (Promega) and named pCMV. A sequence of the EGFP gene and of SV40 poly A was amplified by using the primers: a forward primer UEGFP (5'-AGCTCGAGCGCCACCATGGTGAGCAAG: *Xho* I underlined) and a reverse primer LSV40 (5'-GCAGGCCCTGATACATTGATGAGTTTG: *Eco* 0109 I underlined). The PCR-generated fragment was then cloned into pGEM-T Easy

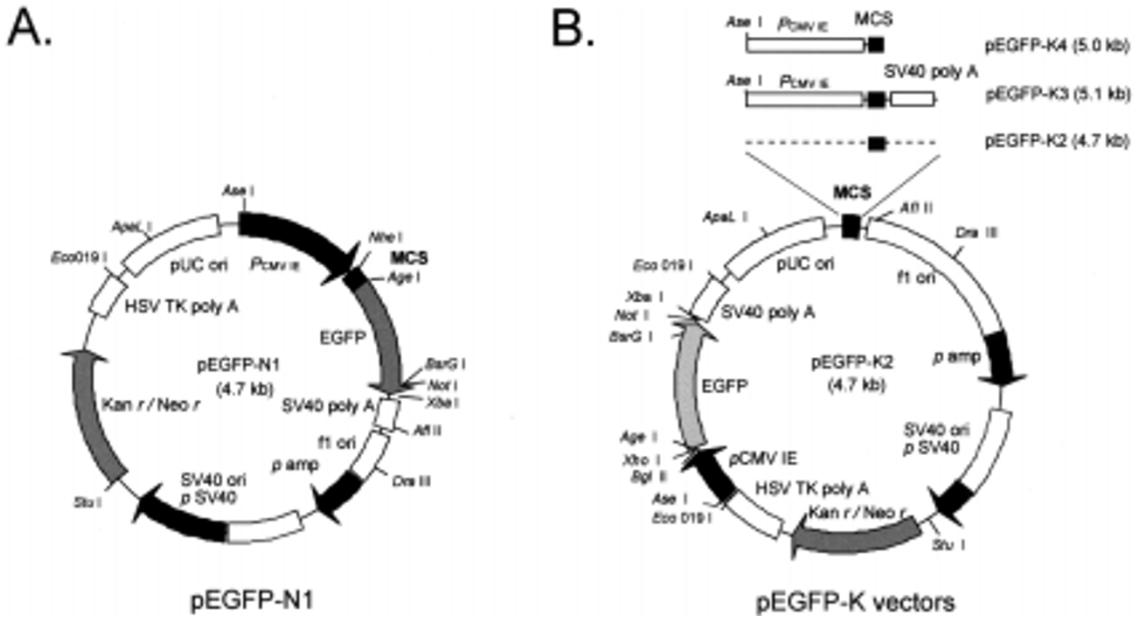

Figure 1. Plasmid maps. Panel A, PEGFP-N1 (GenBank Accession: U55762; CLONTECH Lab.). The EGFP expression unit consists of the immediate early promoter of CMV ($P_{CMV\ IE}$), the multiple cloning site (MCS), the EGFP coding sequences (EGFP), and SV40 early polyadenylation signal (SV40 poly A). Panel B, pEGFP-K vectors. The EGFP expression unit is relocated in between the restriction sites *Eco*0109 I. The MCS in the pEGFP-K vectors is the same as the MCS in the pEGFP-N1. The pEGFP-K2 vector contains only the MCS. The pEGFP-K3 vector contains the EGFP expression cassette, the $P_{CMV\ IE}$, the MCS, and the SV40 poly A. The pEGFP-K4 vector contains the $P_{CMV\ IE}$ and the MCS.

vector (Promega), and named pEGFP/SV40. To create an EGFP expression cassette in a plasmid, the CMV early promoter in the pCMV plasmid was excised using *Sph* I and *Xho* I, and then cloned into the corresponding restriction enzyme sites of the pEGFP/SV40 plasmid. This plasmid, named pCES, contains the complete EGFP expression cassette. Finally, the EGFP expression cassette in the pCES plasmid was excised by *Eco* 0109 I and cloned into the pB1 plasmid to construct the pEGFP-K3 vector (Figure 1B).

The pEGFP-K4 vector that does not contain the SV40 poly A sequence was constructed by modification of the pEGFP-K3 vector (Figure 1B). Briefly, the SV40 poly A sequence was removed from the pEGFP-K3 vector by digestion with *Afl* II and *Bam* HI. This linear vector was then blunted with Klenow fragment and ligated at both ends. The pEGFP-K2 vector contains only a Multiple Cloning Site (MCS) (Figure 1B). To construct the pEGFP-K2, the DNA sequence of the EGFP and SV40 in pEGFP-1 (CLONTECH Lab.) was excised by *Sma* I and *Bstg*98 I, blunted with Klenow fragment, and then ligated at both ends. Next, the *Eco* 0109 I digested EGFP expression cassette (P_{CMV}/EGFP/SV40) from pCES plasmid was directly cloned into the *Eco* 0109 I site of the pEGFP-1 vector.

Cell culture

Custom culture dishes were prepared to facilitate continual fluorescence microscopy monitoring of a single targeted cell. Briefly, a centered, 25-mm hole was cut into the bottom half of a standard 60-mm polystyrene culture dish (Fisher Scientific, Pittsburgh, PA, USA), then a detergent-washed Dasag glass coverslip (#1.5, 30-mm round, Fisher Scientific) was bonded to the underside of the dish with a continuous bead of Sylgard 182 (Dow-Corning, Auburn, MI, USA) deposited around the perimeter of the hole. The Sylgard was cured for 24 h at room temperature, followed by 4-6 h at 60°C, yielding a watertight seal between the glass coverslip and the culture dish. Three 50-mesh EM locator grids were bonded in an asymmetric pattern to the external surface of the coverslip with 0.4% (w/v) Formvar in 1,2-dichloroethane (Sigma) and dried at room temperature. The culture dishes were sterilized by UV exposure prior to use. Cells were cultured as previously described (Rhew *et al.*, 1999). Approximately 1×10^4 human melanoma A375 cells (ATCC, Rockville, MD, USA) were seeded on the 25-mm glass coverslip of the custom culture dish and grown in 0.8-1.0 ml of RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Life Technologies, Gaithersburg, MD, USA). The confluent cells in each dish were transfected with 5.0 μg of plasmid using CLONfectin (CLONTECH Lab.) at 37°C in a 5% CO_2 incubator for 40 min. Transfection was performed as previously described (Cha *et al.*, 2001). Cells were washed twice with 1x PBS and grown by feeding with fresh culture medium every 48 h.

Northern blot analysis

Human telomerase RNA (hTR) cDNA was cloned into

MCS of the pEGFP-K3 with either antisense orientation or sense orientation of hTR. About 450 base pairs of hTR were amplified from A375 genomic DNA using primers: a forward primer U1 (5'- GGGTTGCGGAGG-GTGGGCCTG) and a reverse primer L450 (5'-GAACT-GCATGTGTGAGCCGAG). A restriction enzyme site, either *Hind* III or *Bam* HI was attached to each primer to amplify fragments for either anti-sense hTR or sense hTR. The amplified fragment was then cloned into pGEM-T Easy Vector (Promega). To construct either anti-sense hTR or sense hTR in pEGFP-K3 vector, restriction enzyme sites (*Hind* III and *Bam* HI) in MCS were used. Blank pEGFP-K3 plasmids were used for controls for transfection and Northern analysis. Seven days after transfection, only GFP positive cells were sorted and harvested using Multi-laser FAC system (Becton Dickson Immunocytometry system, San Jose, CA, USA). The harvested GFP cells were cultured again to obtain large number of cells to isolate total RNA.

Total RNA was isolated using Trisol Reagent (Sigma) and evaluated by Northern analysis. Briefly, twenty micrograms of total RNA were electrophoretically separated on 1.4% denaturing agarose gel using 1× MOPS [3-(N-morpholino)-propanesulfonic acid] buffer and transferred to membranes (Hybond N^+, Amersham) in 25 mM phosphate buffer, pH 7.0 for 12 h. The membrane was crosslinked under an UV lamp. RNA probes, sense hTR and antisense hTR from pGEM-T Easy Vector (Promega) were synthesized and labeled with ^{32}P-UTP as described by the procedures of the Riboprobe® System (Promega Co., Madison, WI, USA). Hybridization was performed with ^{32}P-UTP labeled probes for 14 h at 62°C in hybridization buffer (50% formamide, 1.0 M NaCl, 10% (w/v) dextran sulfate, 1.0% SDS, and 0.1 mg/ml denatured salmon sperm DNA). Hybridized membranes were washed twice in 2X SSC, 0.1% SDS, for 5 min at room temperature, once in 1X SSC, 0.1% SDS, for 10 min at room temperature, once in 0.1X SSC, 0.1% SDS, for 20 min at room temperature, and once in 0.1X SSC, 0.1% SDS, for 5 min at 65°C. Washed membranes were exposed for 24 h to X-ray film for autoradiography. EGFP RNA probe was used for a loading and vector control.

Microscopy

Microscopy was performed with a Zeiss Axiovert 135 (Carl Zeiss, Thornwood, NY, USA). A standard fluorescein isothiocyanate (FITC) filter set (Chroma Technology, Brattleboro, VT, USA) was used to excite at 460-510 nm and to detect emission at 515-565 nm wavelength. Images of EGFP cells were taken using Kodak Ektachrome 400 ASA film (Eastman Kodak, Rochester, NY, USA). EGFP expression was analyzed by flow cytometry. Briefly, cells were harvested 48 h after transfection by treatment with 0.25% trypsin (Sigma) at 37°C for 5 min. Flow cytometric analysis was carried out in a flow cytometer (Coulter Co., Miami, FL, USA) using an excitation wavelength of 488 nm in the Cytometry Facility at Iowa State University, Ames, IA, USA.

Results and Discussion

To test the EGFP expression, we transfected A375 cells with either pEGFP-K3 or pEGFP-N1 vectors. The transfected cells were replated at approximately 500 cells per dish and visualized with bright-field light microscopy (Figure 2A, panel 1) and epifluorescence microscopy (Figure 2A, panel 2). Cells expressing EGFP were readily distinguished from non-transfected cells thus facilitating continual monitoring of cell proliferation without resorting to drug selection. Currently, the typical cell culture method for fluorescence microscopy involves growing cells on loose coverslips in a dish, following which the coverslips are inverted and mounted on a

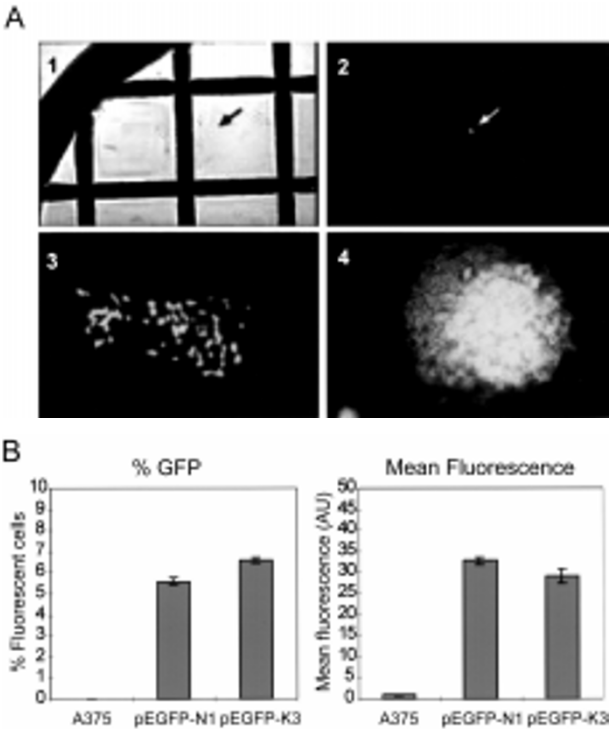

Figure 2. Transient expression of pEGFP-K3 vector in human A375 cells observed by fluorescence microscopy (A) and flow cytometry analysis (B). (A) A375 cells. Cells were transfected with pEGFP-K3 vector and cultured for 24 h. Transfected cells were re-plated at low numbers (approximately 500 cells) per custom culture dish and visualized by bright field (panel 1) or epifluorescence (488 nm excitation) (panel 2) microscopy. The arrows indicate the same EGFP positive cell within one of the EM locator grids. A colony of cells that proliferated from the single cell is shown in panel 1 after 3 days (panel 3). A typical colony was grown from a single transfected cell for 10 days (panel 4). (B) Flow cytometric analysis: the percent of total cells transfected (left panel) and the mean fluorescence of EGFP (right panel) are depicted. The cells were subjected to FACS analysis 3 days after transfection. Total cell count for each FACS analysis was 20,000. The mean ± S.D. of triplicate of independent experiments is indicated.

glass slide for microscopy. However, this method is not suitable for monitoring the growth patterns of a single cell over time. In this report, we successfully monitored a single cell expressing EGFP over time and easily distinguished the target cell from non-transfected cells by using custom culture dishes with the EM locator grids (Figure 2A). In addition, the relatively thin glass coverslip mounted in the custom culture dish permitted the examination of cells by transmitted light and epifluorescence microscopy with high-magnification and short working-distance objectives. Furthermore, since the colony of cells that originated from the single cell was located within a single EM locator grid, we were able to calculate the exact proliferation rate of individual target cells. When a colony grew large enough to preclude visual counting under fluorescence microscopy, we performed the flow cytometry analysis of EGFP expression to count the numbers of cells in each colony.

We compared the levels of pEGFP-K3 expression to that of pEFGP-N1 in transfected cells by calculating 1) the percentage of fluorescence-positive cells, and 2) the mean fluorescence levels of GFP-expressing cells. EGFP-positive cells averaged 5.7% of total cells for EGFP-N1 and 6.7% for EGFP-K3 (Figure 2B). The mean fluorescence of EGFP-N1 was 32.8 Arbitrary Units (AU) and that of EGFP-K3 was 29.1 AU. The levels of EGFP expression of both vectors from transfected cells were nearly identical in Northern blot analysis (Figure 3, EGFP). These FACS analyses and Northern analyses showed that there were no significant differences in EGFP-expression between the two vectors, indicating that pEGFP-K3 expresses EGFP protein effectively as does pEGFP-N1.

To test the expression efficiency of the pCMV IE promoter in pEGFP-K3, we constructed two vectors, which were an antisense orientation of human telomerase component RNA (hTR) (Feng *et al.*, 1995) and a sense orientation of hTR (Figure 3). After transfection, we selected GFP positive cells using Multi-laser FAC system and grew again to get enough cells to perform Northern blot analysis. The ^{32}P-labeled antisense-hTR probe detected both the genomic and vector delivered hTR transcripts (Figure 3A, A1 and A2), indicating that the sense-hTR vector in the transfected cells expressed the hTR RNA. The ^{32}P-labeled sense-hTR probe detected the high level of the antisense-hTR transcripts in the transfected cells with antisense-hTR vector (Figure 3A, B3). Cells transfected with only pEGFP-K3, however, did not show any signal from the ^{32}P-labeled sense-hTR probe (Figure 3A, B1). High levels of EGFP transcripts were detected by ^{32}P-labled antisense-EGFP probe in the cells transfected with pEGFP-K3 vectors (Figure 3B, EGFP). The results of Northern blot experiments indicated that genes constructed in MCS of pEGFP-K3 were successfully transcribed with pCMV IE promoter.

If the expression of introduced genes occurs in nearly the same time frame as cell toxicity due to antibiotic selection, standard drug selection methods may not be suitable. To overcome this limitation, a simple relocation of the EGFP expression cassette in the EGFP reporter vectors combined with culturing cells in modified culture dishes permits direct visual monitoring of transfected cells. By using these methods we were able to accurately count the cell proliferation rates and monitor cell viability without resorting to drug selection procedures. Finally, this protocol is ideal for rapid testing of potentially toxic genes and biologically active chemicals and can be used to monitor transfected cells for manifold purposes in biotechnology.

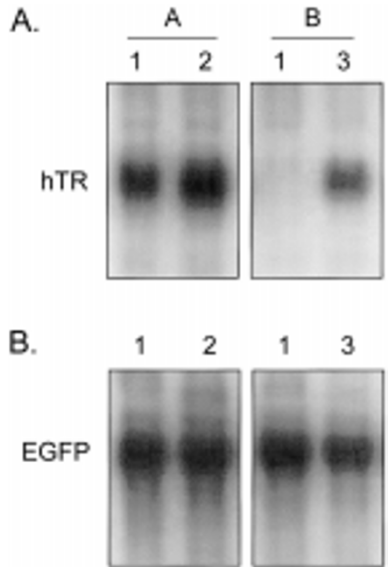

Figure 3. Northern blot analysis of A375 cells transfected with pEGFP-K3 vectors cloned with either sense or antisense hTR. (A) Total RNAs were isolated and loaded from the cells transfected with pEGFP-K3 vector having sense-hTR (2) or antisense-hTR (3). Total RNAs from the cells transfected with the pEGFP-K3 vector without insert are indicated as 1. The hTR transcripts of cells transfected by either pEGFP-K3 vector only (1) or pEGFP-K3 vector with sense-hTR (2) were detected by the ^{32}P-labeled antisense-hTR probe (A). The ^{32}P-labeled sense-hTR probe (B) detected only the transcripts of the vector delivered antisense-hTR (3) in the cells transfected by pEGFP-K3 with antisense-hTR. (B) High levels of EGFP transcripts were detected all of the cells transfected with pEGFP-K3 vectors. Twenty micrograms of total RNA were loaded in each lane.

Acknowledgment

We thank Drs. Donghee Liu and Christi Harkins in the Cytometry Facility; Mike Lynch and Dr. Charles J. Link Jr. in the Department of Zoology and Genetics, Iowa State University, Ames, IA, for technical assistance.

References

Cha JY, Kim HI, Im SS, Li TZ, Ahn YH, FNF1 and/or HNF3 may contribute to the tissue specific expression of glucokinase

gene. Exp Mol Med 2001;33:59-63

Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC. Green fluorescent protein as a marker for gene expression. Science 1994;263:802-5

Feng J, Funk WD, Wang SS, Weinrich SL, Avilion AA, Chiu CP, Adams RR, Chang E, Allsopp RC, Yu J, Le S, West MD, Harley CB, Andrews WH, Greider CW, Villeponteau B. The RNA component of human telomerase. Science 1995;269: 1236-41

Flach J, Bossie M., Vogel J, Corbett A, Jinks T, Willins DA, Silver PA. A yeast RNA-binding protein shuttles between the nucleus and the cytoplasm. Mol Cell Biol 1994;14:8399-407

Kain SR, Adams M, Kondepudi A, Yang TT, Ward WW, Kitts P. Green fluorescent protein as a reporter gene expression and protein localization. BioTechniques 1995;19:650-55

Li X, Zhao X, Fang Y, Jiang X, Duong T, Fan C, Huang CC, Kain SR. Generation of destabilized green fluorescent protein as a transcription reporter. J Biol Chem 1998;273: 34970-75

Prasher DC, Eckenrode VK, Ward WW, Prendergast FG, Cormier MJ. Primary structure of the *Aequorea victoria* green-fluorescent protein. Gene 1992;15:229-33

Rhew JH, Shin YA, Lee BH, Park RW, Kim IS. Induction of fibronectin gene expression by inhibitors of protein phosphatase type 2B in normal and transformed fibroblasts. Exp Mol Med 1999;31:71-75

Stauber RH, Horie K, Carney P, Hudson EA, Tarasova NI, Gaitanaris GA, Pavlakis GN. Development and applications of enhanced green fluorescent protein mutants. BioTecniques 1998;24:462-71

Wang S, Hazelrigg T. Implications for *bcd* mRNA localization from spatial distribution of *exu* protein in *Drosophila* oogenesis. Nature 1994;369:400-3