EGFP-Containing Vector System that Facilitates Stable and Transient Expression Assays

Current methods for the creation of cell lines that express a gene of interest generally involve co-selection for a resistance marker. This process usually requires 5–6 weeks to complete, during which time the culture must be maintained in selection media. Transient assays for gene expression often use co-transfection with another plasmid to identify transfected cells and/or to control for variation in transfection efficiency. We describe an expression system in which the gene of interest is cloned into a vector that contains a gene for enhanced green fluorescent protein (EGFP; CLONTECH Laboratories, Palo Alto, CA, USA). The system is convenient because expressing cells can be directly detected and/or separated within one day of the introduction of the genetic material. It is also flexible because it allows for transient or stable expression using transfection, electroporation or retroviral-mediated gene transfer. We describe the use of this vector system to monitor the down-regulation of CD4 on T cells following expression of the nef gene of simian immunodeficiency virus (SIVmac239).

We replaced the neomycin phosphotransferase gene of the retroviral vector pLXSN with the EGFP gene to create the vector pLXSG (Figure 1). Expression of EGFP in this vector is driven by the simian virus 40 (SV40) early promoter. To ensure high EGFP expression from this plasmid, the consensus eukaryotic translation initiation sequence (GGCCACCATGG) (4) was engineered immediately surrounding its start codon. An additional plasmid (pLnefSG, Figure 1) was created by inserting the nef gene of SIVmac239 into the multiple cloning site of pLXSG that is located immediately downstream of the Moloney murine leukemia virus (MMLV) 5′ long terminal repeat.

pLXSG and pLnefSG were independently introduced into Jurkat T cells by electroporation. Exponentially growing cells were incubated with 50 µg of cesium chloride-purified plasmid in a 0.4-cm gap cuvette and given a 200-V and 960-µF charge from a Gene Pulser® (Bio-Rad, Hercules, CA, USA). By 5 h after electroporation, green fluorescent cells were apparent in the culture (data...
not shown). Fluorescence-activated cell-sorting (FACS) analyses determined that 69.7% of target cells electroporated with pLnefSG (Figure 2A) and 68.5% of target cells electroporated with pLXSG (Figure 2B) expressed EGFP.

It has been well-documented that the nef proteins of SIV and human immunodeficiency virus (HIV) are involved in the down-regulation of the surface expression of CD4 (1–3,5), the receptor common to all SIV and HIV strains. This process takes place within 24 h of initial nef expression in cell culture systems. Jurkat T cells that had been electroporated with either pLXSG or pLnefSG were incubated for 24 h to allow for CD4 down-regulation by nef. At that time, the cells were incubated with an anti-CD4 phycoerythrin (PE)-conjugated monoclonal antibody (Leu-3A; Becton Dickinson Immunocytometry, San Jose, CA, USA). Green fluorescent cells were analyzed using a FACSscan™ (Becton Dickinson Immunocytometry), which measured the surface expression of CD4 in transfected cells. Some of the cells (37.3%) that were transfected with pLnefSG expressed high levels of EGFP and presumably nef (Figure 2A, gate R4). We normalized for the effect of high expression of EGFP on CD4 cell surface expression by comparing pLnefSG- and pLXSG-transfected cells that expressed high levels of EGFP (Figure 2, A and B, gate R4). Cells transfected with pLnefSG (Figure 2A, gate R4) had approximately tenfold less surface expression of CD4 than did analogous cells that were transfected with pLXSG (Figure 2B, gate R4). The mean fluorescence for CD4 was 7.3 and 76.7 (Figure 2, C and D, respectively). We have shown that the EGFP vector can be used as a transient assay system for the expression of foreign genes such as SIV nef. This system is flexible enough to allow for stable gene expression, making it a convenient alternative to co-selection methods.

REFERENCES

We thank Maryann DeMaria for technical assistance. This work was supported by PHS Grants Nos. A125328, A138559 and RR00168. Address correspondence to Ronald C. Desrosiers, New England Regional Primate Research Center, Harvard Medical School, Division of Microbiology, 1 Pine Hill Drive, Southborough, MA 01772-8102, USA.

Received 30 September 1996; accepted 23 December 1996.

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Reliable Method of Isolating Transfected Clones from the LNCaP Human Prostatic Cell Line

BioTechniques 23:66-70 (July 1997)

The LNCaP human prostatic cell line is the only human prostatic cell line available that is both androgen-sensitive and nonmetastatic when implanted into BALB/c/nu/nu mice (4). These properties make it a frequently used model for prostate cancer research. The generation of transfected LNCaP cell lines and the subsequent isolation of transfected clones has been reported by several researchers (3,6,7). Two cloning procedures often used to clone LNCaPs are the cloning ring method (2) and the limit dilution method (5). However, these methods are difficult and tedious and only produce low clone numbers.

Two properties of LNCaP cells make them a particularly difficult cell line to clone. First, LNCaP cells grow in loosely adherent colonies (4) and therefore can easily break away from the main colony. This can make cloning rings unreliable. Second, LNCaP cells have a poor survival rate when plated at very low concentrations in tissue culture flask (unpublished observation), which can affect cloning capacity and efficiency when attempting to produce clonal lines using the limit dilution technique.

To overcome these difficulties in cloning LNCaP cells, we have developed a simple method for isolating clonal LNCaP cells that have been transfected using cationic liposome-mediated transfection (1) with a stable antibiotic resistance marker. We used LNCaP cells (ATCC, Rockville, MD, USA) transfected using LIPOFECTIN® Reagent (Life Technologies, Gaithersburg, MD, USA) with the eukaryotic expression vector pRC/CMV (Invitrogen, San Diego, CA, USA), which contains the neomycin-resistance gene for selection. Transfected cells were then selected in 100 µg/mL of antibiotic G-418 (GENETICIN®; Life Technologies) over 10 days. Because the presence of G-418 was observed to slow down LNCaP cell growth, it was removed, and the heterogeneous population of transfected cells expanded in its absence. These cells have remained stably transfected for at least 60 passages and retained G-418 resistance when reexposed to this antibiotic.

Since G-418 resistance was maintained over the period of study, clones could be established from any passage. Transfected LNCaPs at 60%–70% confluence in a 75-cm² tissue culture flask (Corning Costar, Cambridge, MA, USA) were rinsed with phosphate-buffered saline (PBS). The PBS was removed, and 3 mL 0.2 M EDTA, pH 7.5 were added for 30 s. Following removal of EDTA, cells were incubated in 0.5% trypsin and 5.3 mM EDTA for 15 s, and the excess trypsin was removed. The flask was then placed at 37°C for 5 min; then 3 mL RPMI 1640 medium from the MultiCel™ Kit (with L-glutamine, without sodium bicarbonate) and 10% fetal calf serum (FCS) from the MultiSer™ Kit (both kits from Trace Biosciences, Castle Hill, Australia) were added.

The LNCaPs were then further diluted in RPMI 1640, 10% FCS to a concentration of 25 cells/mL. Forty microliters of this cell suspension were added to each well in a Linbro® 96-well plate (ICN Biomedicals, Costa Mesa, CA, USA) so that, theoretically, each well should only contain one cell. The cells were then left overnight to adhere. The next day, the cell number per well was assessed using a phase-contrast microscope. Two hundred microliters of untransfected LNCaP cells at a concentration of 1 × 10⁴ cells/mL were added to wells that were confirmed to contain one cell. The addition of these untransfected cells acts as a feeder layer and removes the need for addition of any exogenous cytokines or hormones.

Cellular confluence was monitored daily, and cells were passaged when

Figure 1. Phase-contrast photomicrographs showing the establishment of clonal LNCaP cell lines by differential G-418 selection. One transfected LNCaP cell was seeded with two thousand untransfected LNCaPs as described in the text. Cells were then left and passaged until they reached confluence in a 25-cm² flask. (A) Mixed LNCaP cultures before the addition of 500 µg/mL G-418. (B) Mixed LNCaP cultures 6 days after the addition of G-418. (C) An LNCaP clone after 12 days of culture in the presence of G-418. Magnification is 40x.