Versatile Epitope Tagging Vector for Gene Expression in Mammalian Cells

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ABSTRACT

We have constructed an epitope-tagging vector, pCMV-Tag 1, for gene expression in mammalian cells. This vector, which allows for N-terminal, C-terminal and internal tagging of the gene product of interest with the FLAG® and/or c-myc epitopes, enables researchers to rapidly and efficiently characterize gene products in vivo.

INTRODUCTION

Epitope tagging is a convenient technique in which a known peptide epitope is fused to a target protein of interest, allowing expression of the fusion protein to be monitored using a tag-specific antibody. Thus, a new protein can be easily studied without having to generate new, specific antibodies to that protein. Epitope tagging can be used to localize gene products in living cells, identify associated proteins, track the movement of fusion proteins within the cell or characterize new proteins by immunoprecipitation (3,5,7–9). Epitope tags are generally added at the N (amino) or C (carboxy) terminus, as the ends of proteins are more likely to be accessible to the antibody, and the addition is less likely to affect the normal function of proteins (1). However, if the functional portions of the protein are located at either terminus, an internal tagging option is also available using the FLAG® epitope (Scientific Imaging Systems [Eastman Kodak], New Haven, CT, USA). In past experiments, the addition of an epitope coding sequence has normally been performed by individually adding the tag sequence to each construct. More recently, the preferred way to obtain an epitope-tagged protein is to fuse the coding sequence for the target protein to a vector that contains an epitope tag. To date, most epitope-tagging vectors constructed have only one peptide tag and only allowed for tagging at one terminus, which limited the general utility of the vector (3). In this report, we describe a new epitope-tagging vector, pCMV-Tag 1, which contains sequences for both the FLAG and the c-myc epitopes. In addition, pCMV-Tag 1 was specifically designed for versatile cloning purposes, allowing for both internal and terminal cloning with a choice of either epitope tag. Advantageous properties of the FLAG and c-myc epitopes include possessing a strong, specific detection signal with limited background and being innocuous to transfected cells so that they do not interfere with the function of the tagged protein (5,8,10,11). The versatility of pCMV-Tag 1 enables researchers to find a suitable tagging strategy that will greatly assist in biochemical studies of their protein of interest.

MATERIALS AND METHODS

Construction of pCMV-Tag 1

pCMV-Tag 1 is derived from pCMV-Script™ (Stratagene, La Jolla, CA, USA). Briefly, 5 µg of pCMV-Script were digested with *Eco*RV/*Bam*HI and then ligated with an oligonucleotide fragment coding for the sequence of the FLAG epitope, DYKDDDDK (4). This construct was given the name pCMV-Tag 1F and was subsequently digested with *Xho*I/*Kpn*I and then ligated with another oligonucleotide fragment coding for the c-myc epitope, EQKLISEEDL (2), resulting in pCMV-Tag 1. All cloning junctions were verified by DNA sequencing. pCMV-Tag 1 is 4319 bp in size, and the compiled nucleotide sequence is available from GenBank® (Accession No. AF 025668).

Cloning of the Luciferase Gene into the pCMV-Tag 1 Vector

The expression level of the pCMV-Tag 1 vector was tested by cloning the reporter gene luciferase into the construct and

then assaying for function of the gene. To do so, pCMV-Tag 1 was digested with *Bgl*II/*Xho*I, treated with shrimp alkaline phosphatase (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and then ligated with a *Bam*HI/*Xho*I fragment containing the firefly luciferase gene produced by polymerase chain reaction (PCR). The ligated DNA was transformed into XL10-Gold cells (Stratagene), and the correct clones were identified by restriction analysis.

Cell Culture Transfection Assays of pCMV-Tag 1

Chinese hamster ovary (CHO) cells were grown from a stock culture in Dulbecco's modified Eagle medium (DMEM) complete media (Life Technologies, Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS) and 1% glutamine. Transfection of the "pCMV-Tag 1 plus luciferase" construct into CHO cells was performed using the LIPOFECTAMINE™ method (Life Technologies). Two micrograms of DNA were used to transfect approximately 5.0×10^5 cells. The cells were harvested approximately 48 h after the initial transfection.

Stable Line Generation

Stable CHO cell lines containing pCMV-Tag 1 and derived constructs were generated using the $CaPO_4$ Mammalian Transfection Kit (Stratagene). A kill curve experiment was performed before the transfection to optimize the selection concentration of the G418 sulfate (Stratagene). This demonstrated that the optimal amount of G418 was 600 µg/mL for 14 days. Twelve micrograms of DNA were transfected into approximately 5.0×10^5 cells on 100-mm tissue culture plates. The stable line was generated approximately two weeks after the initial transfection.

Western Analysis of Epitope-Tagged Luciferase

Western blots were performed using Xcell II products, reagents and protocols (Novex, San Diego, CA, USA). Approximately 20 µL of cell lysate containing 20 µg of protein were electrophoresed on a 4%–20% Tris-glycine acrylamide gel at 120 V for 1.5 h. Protein was transferred from the gel onto nitrocellulose at 30 V for 1 h, probed with the appropri-

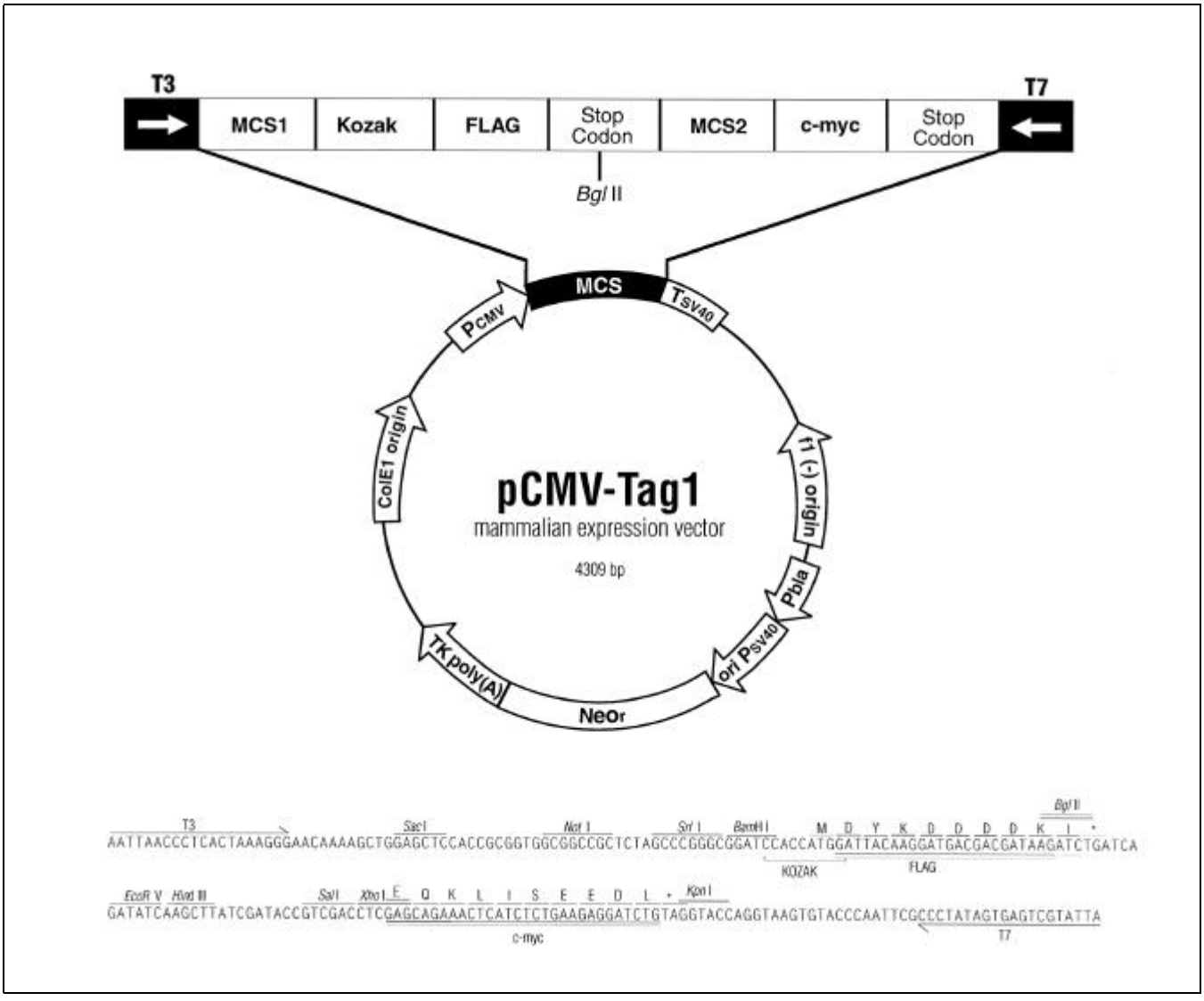

Figure 1. Plasmid map of pCMV-Tag 1. The nucleotide sequence around the MCS with the corresponding amino acid sequences for the epitope tags are shown as well as the unique restriction sites in the MCS region and the T3 and T7 promoters.

ate monoclonal or polyclonal antibodies and detected with a horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit, secondary antibody. The signal was detected by chemiluminescence (SuperSignal™; Pierce Chemical, Rockford, IL, USA), using an exposure time of 1 min. Primary antibodies used were as follows: rabbit polyclonal anti-luciferase (Promega, Madison, WI, USA), mouse monoclonal anti-FLAG® M2 (Stratagene) and mouse monoclonal anti-c-myc (Boehringer Mannheim, Indianapolis, IN, USA). The secondary antibody used was anti-mouse/rabbit conjugated HRP (Amersham Pharmacia Biotech).

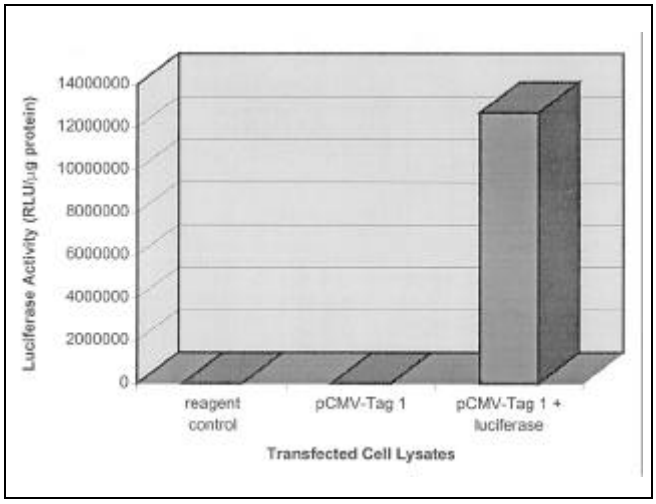

Figure 2. Luciferase assay of epitope-tagged luciferase. CHO cells were transfected with the pCMV-Tag 1 plus luciferase construct and the pCMV-Tag 1 vector alone. One microliter (1 µg protein) of cell lysate was mixed with 100 µL of luciferase assay reagent (Stratagene), and detection was made using a luminometer. The results were normalized using protein concentration. Luciferase activity was measured in relative light units (RLU) per µg of protein.

Enzyme Assays

Luciferase expression levels in CHO cells were determined using a Luciferase Assay Kit (Stratagene). Approximately 1 µg of total cell protein in a volume of 1 µL was added to 100 µL of luciferase assay reagent, and the resulting light was monitored by a luminometer. Protein concentration was determined using a standard Bradford assay (Pierce Chemical).

RESULTS AND DISCUSSION

The pCMV-Tag 1 Epitope Tagging Vector

The pCMV-Tag 1 vector (Figure 1), derived from pCMV-Script, contains sequences for the FLAG and the c-myc epitopes. These specific epitope tags were chosen for their high immuno-reactivity, their small size and their low likelihood to interfere with the function of a protein of interest. The FLAG epitope is a synthetic epitope that consists of 8 amino acid (aa) residues, DYKDDDDK (4), while the c-myc epitope, found in the human c-*myc* gene, is 10-aa-residues long, EQKLISEEDL (4). In addition to the epitope tag sequences, pCMV-Tag 1 contains additional features that enable it to propagate and express fusion proteins in eukaryotic cells. The human cytomegalovirus (CMV) promoter allows for constitutive expression of the cloned gene in a wide variety of mammalian cell lines. Stable selection of clones in eukaryotic cells

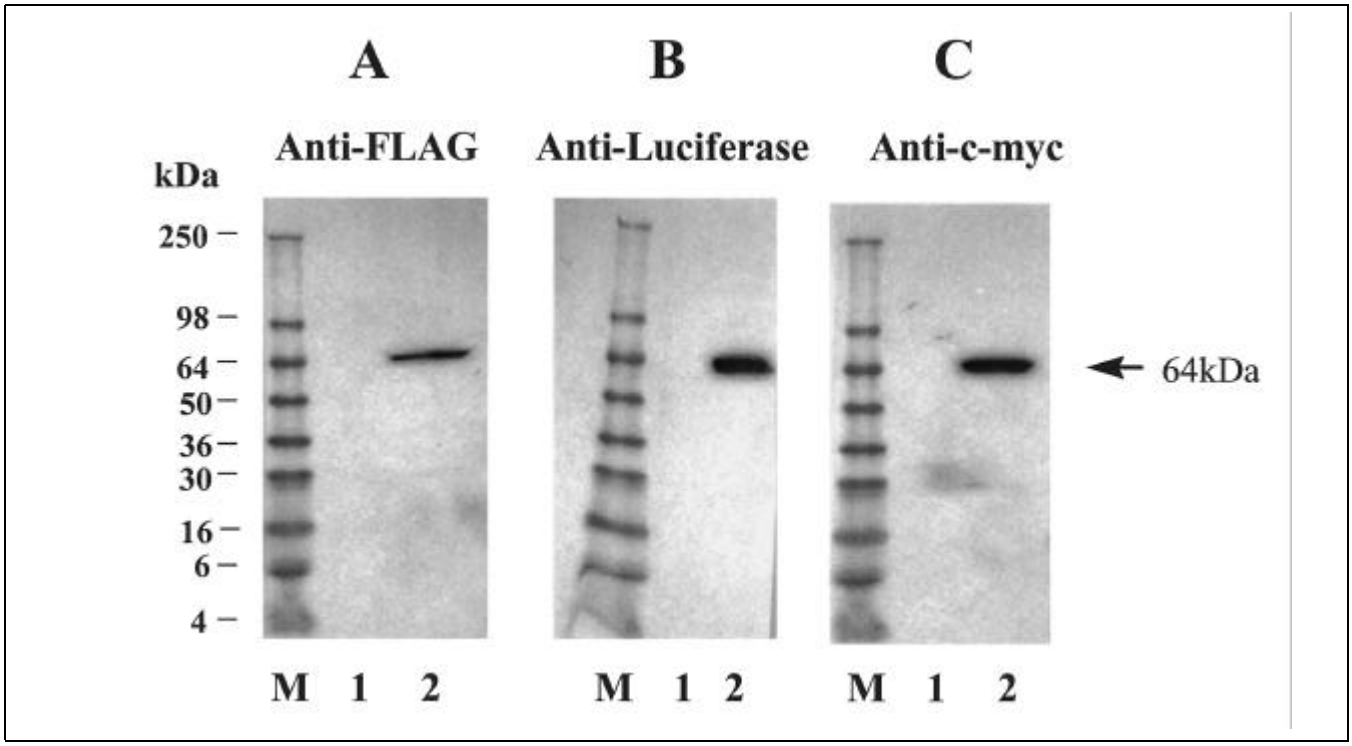

Figure 3. Western analysis of the FLAG-luciferase-c-myc fusion protein. Lane 1 is the pCMV-Tag 1 vector alone, and lane 2 is pCMV-Tag 1 plus luciferase. Twenty microliters of CHO cell lysate (containing 20 µg protein) were electrophoresed on each 4%–20% Tris-glycine gel for 1.5 h at 120 V, transferred to a nitrocellulose membrane for 1 h at 30 V and probed with anti-FLAG (A), anti-luciferase (B) and anti-c-myc (C) antibodies. The signal was detected by chemiluminescence.

Table 1. Cloning Strategies for Epitope Tagging in the pCMV-Tag 1 Vector

Epitope Tag	Tag Location	Cloning Site	Kozak Sequence
FLAG	C terminus	MCS 1	Insert supplied
FLAG	N terminus	*Bgl*II	Vector supplied
FLAG and c-myc	N and C termini	*Bgl*II and MCS 2	Vector supplied
c-myc	C terminus	MCS1 and 2	Insert supplied
FLAG	Internal	MCS1 and *Bgl*II	Insert supplied
No tag	N/A	MCS 1 or MCS 1 and 2	Insert supplied

N/A = not applicable

is made possible by the presence of the neomycin-resistance gene, which is driven by the simian virus 40 (SV40) early promoter and terminated by the thymidine kinase (TK) transcription stop signal. The neomycin-resistance gene is also driven by the β-lactamase promoter to provide selection of the plasmid in *E. coli* cells using kanamycin. The multiple cloning site (MCS) of pCMV-Tag 1 is specifically designed to allow a variety of cloning strategies to be used, resulting in C-terminal, N-terminal and internal tagging of a protein of interest. A KOZAK consensus sequence of GCC(A or G)CCATGG (6) is provided for N-terminal FLAG epitope tagging.

Table 1 summarizes the cloning strategies for various tagging choices. A gene of interest can be efficiently cloned to achieve a terminal tag by using the *Bgl*II, MCS 1 or MCS 2 sites. For internal tagging, initially the 5′ portion of the gene should be cloned within the MCS 1, and then the 3′ portion within the *Bgl*II site. The variety of tagging locations enables researchers to optimize the tagging choices for their proteins.

Expression Analysis of pCMV-Tag 1

We have tested the pCMV-Tag 1 vector by using the firefly luciferase gene as a reporter. The luciferase gene was chosen because it can be assayed both enzymatically and immunologically. The firefly luciferase gene was cloned into the *Bgl*II/*Xho*I site of pCMV-Tag 1 such that the luciferase protein was tagged with the FLAG epitope at the N terminus and with the c-myc epitope at the C terminus. This construct was transiently transfected into CHO cells, and the cell lysates were assayed for luciferase activity. Figure 2 shows the results of transiently expressed luciferase within the pCMV-Tag 1 vector. The data demonstrate that the doubly tagged luciferase is biologically active. The activity of the tagged luciferase is over 6000-fold higher than that of the reagent control and the pCMV-Tag 1 vector alone. This activity is comparable to the activity of untagged firefly luciferase (data not shown).

To demonstrate the ease of detection of the epitope tags, Western analyses were performed. Figure 3 shows the Western analysis of cell lysates derived from CHO cells transfected with the pCMV-Tag 1 vector alone and the vector containing the doubly tagged luciferase. The samples were simultaneously loaded, electrophoresed in triplicate and probed with their respective antibodies. The results clearly indicate that the fusion protein, composed of FLAG-luciferase-c-myc, can be easily detected by antibodies against FLAG, luciferase and c-myc, respectively. Stable CHO cell lines were generated with pCMV-Tag 1 and pCMV-Tag 1 plus luciferase, using the $CaPO_4$ Mammalian Transfection Kit (Stratagene).

CONCLUSIONS

The pCMV-Tag 1 expression vector contains the small and highly immunoreactive FLAG and c-myc epitopes for both N-terminal, C-terminal and internal tagging. These tags eliminate the need for raising specific antisera to study a target gene and both can be easily detected in transfected cells using well-characterized commercially available antibodies. The pCMV-Tag 1 vector offers a fast, versatile and reliable method for analyzing the function of gene products in vivo.

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