Benchmarks

Modified Semliki Forest Virus Expression Vector that Facilitates Cloning

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Many commercially available systems provide a variety of strategies for high-level expression of recombinant proteins. While the prokaryotic and yeast expression systems are highly efficient and easy to use, expression systems for use in higher eukaryotic cells are still hampered by difficulties such as inefficient posttranslational processing, insufficient levels of protein production, restriction in host range and/or complexity of the system (4,8,9). Two mammalian expression systems have been reported to overcome the problems of these other expression systems, one based on Semliki forest virus (SFV) (6) and the other based on Sindbis virus (11). Liljestrom and Garoff (6) reported the development of animal cell expression vectors based on the SFV replicon. This system was reported to be highly efficient and easy to use with a wide range of mammalian cells. The system functions in an unorthodox fashion, consisting of a self-replicating and self-transcribing RNA molecule that is independent of nuclear transcriptional machinery for function. This RNA can be used both for direct transfection of cells and for production of recombinant virus that only expresses the heterologous portion of a recombinant protein (1). The system was commercialized by Life Technologies (Gaithersburg, MD, USA) and released in 1994.

The SFV expression system consists of two plasmids, pSFV1 and pSFV3, based on the full-length cDNA clone of SFV, SFV4 (6,7). These vectors have the coding region of the 26S structural genes deleted from SFV cDNA clone (the 26S promoter is retained) to make way for heterologous inserts, but have preserved the nonstructural coding region that is required for the production of the nsP1-4 replicase complex. The system can be used to express recombinant protein on its own or complemented by the in vivo packaging of recombinant RNAs into infectious SFV particles using a helper construct, pSFV-Helper 2 (1,5).

Both plasmid vectors, pSFV1 and pSFV3, have the same basic polylinker (*Bam*HI, *Sma*I and *Xma*I) downstream from the 26S promoter, followed by translational stop codons in all three reading frames. The vectors differ as to the position where the polylinker cassette has been inserted. In pSFV1, the polylinker is situated 31 bases downstream of the 26S transcription initiation site, whereas in pSFV3, it is placed immediately after the initiation codon (AUG) of the capsid gene.

Our laboratory needed a reliable supply of recombinant respiratory syncytial virus (RSV) G glycoprotein and mutants thereof. The SFV replicon system was selected because of its ability to produce normally glycosylated proteins through expression in a variety of mammalian cells and aberrantly glycosylated protein in insect and other cell types (11). In addition, it has been reported that high-level expression of recombinant protein can be achieved by packaging the expression system (1).

During our attempt to use the vector pSFV1, we have had a number of difficulties with subcloning the G glycoprotein gene. We describe how these problems were overcome by modifying the pSFV1 vector.

The glycoprotein G was reverse-transcribed from mRNA extracted from RSV, A2 strain-infected HEp-2 cells (2). The reverse transcription polymerase chain reaction (RT-PCR) product of 1170 bp was gel-purified and blunt-end cloned within the *Srf*I site of the pCR-Script™ SK(+) phagemid (Stratagene, La Jolla, CA, USA).

The pSFV1 plasmid vector polylinker cassette has sites for only three restriction enzymes (*Bam*HI, *Sma*I and *Xma*I) (Figure 1), and because *Xma*I is a neoschizomer of *Sma*I, there are really only two sites available for cloning. Because of the limited number of sites for cloning, our initial attempts at subcloning the glycoprotein G gene used blunt-end cloning into the *Sma*I site of the pSFV1 vector. The pSFV1 plasmid vector was linearized by digestion with *Sma*I and then dephosphorylated using calf intestinal phosphatase (Promega, Madison, WI, USA). The entire G gene was isolated by double digesting the pCR-Script phagemid clone with *Sac*I and *Eco*RV and ends polished using a standard procedure (10). The prepared fragment was ligated to 50 ng of *Sma*I-cut vector, and the complete ligation mixture was used to transform the *E. coli* ABLE™ K strain cells (Stratagene). Despite repeated attempts, we were unsuccessful at subcloning the G gene into the *Sma*I site of the pSFV1 vector. We hypothesized that our blunt-ending procedure did not make the DNA fragment compatible for ligation. To overcome this problem, we decided to treat the isolated fragment with kinase before performing a fill-in reaction as described by Kanungo and Pandey (3). We also prepared the fragment by double digesting with *Eco*ICRI (an isoschizomer of *Sac*I) and *Eco*RV. Because both these restriction enzymes produce a blunt-ended product, postdigestion manipulations were not required, thus limiting aberrant effect on the fragment. During transformation of the ligated mixture into the *E. coli* ABLE K strain cells, four clones were retrieved using the second procedure. Of these clones, two were correctly oriented and two incorrectly oriented.

Our next attempt involved cloning

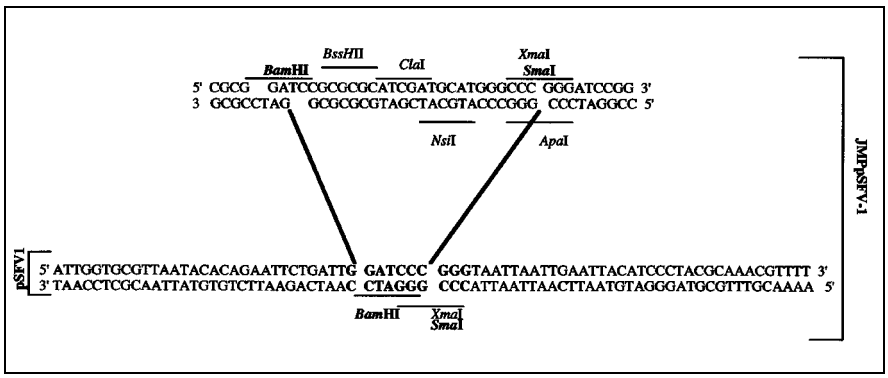

Figure 1. Original (pSFV1) and modified (JMPpSFV1) polylinker regions of SFV vector plasmid.

into the *Bam*HI site of the pSFV1 plasmid vector (Figure 1). The vector was prepared as before. The G gene fragments were prepared using the two methods described above. Isolated fragments from both the preparations were treated with kinase, then ligated to phosphorylated *Bam*HI linkers (CGGATCCG; Progen, Brisbane, Australia) by the routine procedure (3,10). The prepared fragment was digested with *Bam*HI, cleaned with the Prep-A-Gene® Purification Kit (Bio-Rad, Hercules, CA, USA) and ligated to *Bam*HI-cut pSFV1 vector. Following transformation into *E. coli* ABLE K strain cells, no clones were obtained.

Because we planned to use this system for expression of several mutants of glycoprotein G, we needed to create a vector that would guarantee cloning at every attempt and in the correct orientation. We considered modifying the vector by expanding the multiple cloning site and, to do this, analyzed the pSFV1 vector for the absence of restriction sites. Sites for enzymes not found in the vector could then be incorporated into a polylinker, thereby generating unique sites for digestion. Several of these enzymes were rare cutters and are often found within the polylinkers of commonly used cloning vectors. To add the new polylinker, we designed and synthesized a pair of oligomers consisting of 40 nucleotides (sense: 5′-CGCGGATCCGCGCGCATCGATGCATGGGCCCGGGATCCGG-3′ and antisense: 5′-CCGGATCCCGGGCCCATGCATCGATGCGCGCGGATCCGCG-3′). Equimolar amounts of each oligonucleotide were mixed in a 50-mL volume and boiled for 5 min, then allowed to cool slowly at room temperature. When fused together, the sites generated include *Bam*HI, *Bss*HII, *Cla*I, *Nsi*I, *Apa*I, *Sma*I and *Bam*HI (Figure 1). The fused oligomer was double-digested with *Bam*HI and *Sma*I restriction enzymes, purified by chloroform extraction and then ethanol-precipitated. This polylinker sequence was inserted between the *Bam*HI and *Sma*I sites of the pSFV1 vector, and the resulting vector was designated JMPpSFV1. Using this modified vector, we have directionally cloned many G glycoprotein mutants, M protein gene of RSV and capsid gene of hepatitis E virus.

To test whether the modified vector were still functional, capped transcripts were synthesized in vitro from linearized full-length G glycoprotein JMPpSFV1 constructs with SP6 RNA Polymerase (Life Technologies). BHK-21 cells (Life Technologies) were prepared according to the manufacturer's guidelines, and 5 µg of freshly prepared RNA were electroporated according to the protocol optimized by the manufacturer for BHK-21 cells. Following electroporation, transfected cells were

diluted 4-fold in modified Eagle medium (Life Technologies) supplemented with 10% fetal calf serum, seeded on coverslips in 35-mm culture dishes and incubated at 37°C in 5% CO_2. Twenty-four hours later, cells were fixed with chilled 100% acetone for 60 s, probed with a glycoprotein G-specific monoclonal antibody (1:300 dilution) and then labeled with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (Dako, Carpenteria, CA, USA). Slides were examined for immunofluorescence with an Axiovert 100 microscope (Carl Zeiss, Thornwood, NY, USA). Eighty percent of the cells expressed the glycoprotein G as compared to the negative controls.

Modifying the pSFV1 plasmid vector by inserting a synthetic polylinker has enhanced the process of cloning while maintaining the high level of expression of recombinant protein that can be achieved with this system.

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Set of Optimized Luciferase Reporter Gene Plasmids Compatible with Widely Used CAT Vectors

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The expression of reporter genes as a measure for transcriptional activity is widely used for the identification and characterization of regulatory DNA elements in transfection experiments. Over the years, many different reporter gene systems have been described, the products of which are easily detected by their enzymatic activity, reactivity towards specific antibodies or other properties inherent to the protein (1,4).

One of the first and most widespread such reporter genes used in higher eukaryotes is the one coding for the bacterial enzyme chloramphenicol acetyltransferase (CAT), because there is no comparable catalytic activity in eukaryotic cells producing a background signal in the measurements. Consequently, there are several plasmids available containing this gene under the control of different promoters and within various plasmid backbones. A set of two high-copy-number plasmids is widely used throughout the scientific community. One of them contains a promoterless *cat* gene and the other one the same gene under the control of the herpes simplex virus thymidine kinase (*tk*) gene promoter (6), which is ubiquitously active. These plasmids have been modified to eliminate problems resulting from cryptic regulatory elements and transcriptional start sites within the vector backbone (2). However, the determination of CAT protein amounts or enzymatic activity with various types of assays is surpassed in sensitivity by luminescence-based detection sytems. This aspect becomes critical when transfection efficiencies are low, and thus optimal detection of the reporter gene product is required.

One reporter gene, the product of which can be detected by bioluminescence, is the luciferase (*luc*) gene from the firefly *Photinus pyralis*. However, plasmids containing this gene also showed some of the disadvantages of the early constructs containing the *cat* gene. To increase the yield of recover-