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# Rapid, High Level Protein Production Using DNA-based Semliki Forest Virus Vectors*

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**Semliki Forest virus (SFV) vectors can be produced faster, and have a wider host range, than baculovirus vectors. However, the original SFV system requires *in vitro* manipulation of RNA. We have generated a system that is wholly DNA-based. Both the replicon vector, encoding SFV polymerase and the protein of interest, and the helper vector, encoding viral structural proteins, were modified so that expression was RNA polymerase II-dependent. Transfection of the modified replicon plasmid alone generated 20–30-fold more protein than obtained from a simple expression vector. Expression required the SFV replicase, which amplifies replicon RNA. The SFV-based vector generated 10–20-fold more protein than a plasmid based on Sindbis virus. Cotransfection of SFV replicon and helper vectors generated viral titers of around $10^6$ infectious particles/ml. A single electroporation, plated on one 10-cm plate, generated enough virus ($10^7$ particles) to produce >500 μg of protein. Wild type, replication proficient virus was not detected in three tests utilizing almost $10^8$ viral particles, a distinct advantage over a DNA Sindbis-based system in which over half the virus particles generated are fully infectious. The new SFV vectors significantly enhance the utility of this expression system.**

A fundamental technique in both basic and applied molecular biology is the production of heterologous proteins. Bacterial systems are widely utilized, but are hampered by the inability to modify eukaryotic proteins appropriately. In addition, eukaryotic cells have evolved specific mechanisms, which are lacking in bacteria, to fold multidomain proteins (1). Bacterial hosts, therefore, are considerably less efficient at producing well folded, soluble, biochemically active protein than mammalian cells.

Gene expression in mammalian culture is impeded by poor transfection efficiency, limited host cell range, and the complexity of the expression system. Recombinant viruses are the most efficient tools for protein production in higher eukaryotic cells. Baculovirus vectors can produce large amounts of heterologous protein, but utilize insect host cells, which have been shown to glycosylate proteins differently than mammalian cells. The baculovirus and vaccinia systems require screening of viruses generated by homologous recombination (2, 3), which can be time-consuming, especially if large numbers of genetic mutants are required.

To overcome these obstacles, alphavirus vectors have emerged as useful tools in heterologous protein production. The Sindbis virus and Semliki Forest virus (SFV),[1] members of the Togaviridae family, are the best studied alphaviruses (4). Both have been harnessed into gene expression systems because of their self-amplifying genomes that require only the host translational machinery to replicate (5, 6).

In its normal replication cycle, SFV infects the cell and replication commences with translation of the 5′ two-thirds of the positive-sense genomic RNA into a polyprotein, with subsequent autoproteolytic cleavage into four non-structural proteins (nsPs), nsP1 to nsP4, forming an RNA replicase (Fig. 1*A*). This complex targets a replication sequence at the 3′ end of the RNA genome, and replicates full-length (−)-strand RNA from (+)-strand genomic RNA and *vice versa*. The structural proteins (sPs) required for a mature virion are encoded by the last one-third of the SFV genome (Fig. 1*A*). This region is transcribed into a sub-genomic message by the SFV replicase, and translated into a polyprotein that is autoproteolytically cleaved, producing the capsid protein and two envelope glycoproteins (Fig. 1*A*). The capsid protein recognizes a packaging signal buried in the coding region for nsP2 and, together with the full-length RNA, forms a nucleocapsid (7–9). This structure interacts with the envelope proteins' cytoplasmic domains and buds from the plasma membrane to form a mature, infectious virus.

The original SFV expression system employs a plasmid in which the SP6 RNA polymerase promoter lies upstream of the cDNA version of the SFV genome, modified such that the sP coding region has been replaced by the gene of interest (*lacZ* in Fig. 1, *B* and *C*). "Replicon" RNA (re-RNA) is transcribed and capped *in vitro*, and transfected into mammalian cells where it is amplified. Large amounts of target protein are generated from the subgenomic message. Infectious particles carrying re-RNA can be generated *in vivo* by cotransfection of "helper RNA" (5, 10). Helper RNA encodes the viral structural proteins found in the wild type SFV, but lacks the packaging signal (Ψ) found in the nsP2 open reading frame. As a result, only re-RNA is packaged within a wild type viral coat, generating a recom-

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[1] The abbreviations used are: SFV, Semliki Forest virus; nsP, non-structural protein; sP, structural protein; re-RNA, replicon RNA; RPV, replication proficient virus; CMV IE, cytomegalovirus immediate early; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside; PAM, plaque assay medium; PFU, plaque-forming unit; RSV LTR, Rous sarcoma virus long terminal repeat; m.o.i., multiplicity of infection; BHK, baby hamster kidney; PBS, phosphate-buffered saline; FBS, fetal bovine serum; F, farad(s); kb, kilobase pair(s); IMDM, Iscove's modified Dulbecco's medium; ONPG, *O*-nitrophenyl-β-D-galactopyranoside.

binant virus that is capable of one round of infection.

Several features of the SFV have made it a useful vector for the expression of foreign genes. First, and in contrast to baculovirus, SFV will infect almost any mammalian cell. Second, it is possible to generate reasonable amounts of protein simply by transfecting re-RNA (10). This approach avoids generating any virus; however, it is limited by transfection efficiency and requires large amounts of RNA. Third, virus can be produced quickly and efficiently in 2 days (10), which makes it the fastest system available. Fourth, amplification by SFV replicase is so efficient that the level of target protein can reach up to 25% of total cellular protein (10). Finally, mutations within the glycoprotein gene p62 (Fig. 1*A*) prevent infection unless the virus is proteolytically treated with α-chymotrypsin (11). This safeguard feature significantly decreases the likelihood of generating replication proficient virus (RPV) since two events would be required; recombination between re-RNA and helper RNA, and reversion or suppression of the conditional mutation. SFV vectors have been used to generate several recombinant proteins (see, for example, Refs. 10, 12, and 13; reviewed in Ref. 14), to produce hybrid viruses (15, 16), and to study aspects of the SFV life cycle.

The original SFV expression system (Fig. 1*B*) is hampered by the necessity to generate capped RNA transcripts *in vitro* and requires specialized conditions for RNA handling. To overcome these obstacles, we constructed a DNA-based self-amplifying SFV vector by replacing the SP6 promoter used in the original system with the RNA polymerase II-dependent cytomegalovirus immediate early (CMV IE) enhancer/promoter, which drives transcription *in vivo* (Fig. 1*B*). Transfection of this vector into BHK cells generated high levels of protein (20–30 pg/cell), production of which was dependent on a functional SFV polymerase. To complete the DNA-based expression system, a helper plasmid was constructed that encodes SFV sPs. Cotransfection of replicon and helper plasmids generated conditionally infectious virus capable of producing protein at the same high yield as virus generated by the RNA approach. No RPV was detected in several recombinant SFV virus preparations, a distinct advantage over a DNA-based Sindbis system reported previously (17).

## EXPERIMENTAL PROCEDURES

*Plasmids*—pSCAβ was built in three stages. (i) The SV40 polyadenylation signal was amplified by polymerase chain reaction (PCR) using SVhRB (18) as a template, Vent DNA polymerase, and the following primers: O-SV1 (*Spe*I): 5′-gccgactagtgatcataatcagccata-3′ and O-SV2 (*Xba*I): 5′-gccgtctagatccagacatgata-3′. The 0.25-kb product was cut with *Spe*I and *Xba*I, and ligated into *Spe*I-cut pSFV1 (10). The resulting plasmid, pSFVpoly(A), contains a poly(A) signal downstream of the multiple cloning site. (ii) The SP6 promoter and the first 513 base pairs of the 5′ end of the SFV genome were removed from pSFVpoly(A) by digestion with *Sph*I and B*siW*I (Fig. 1*C*). The 10.3-kb fragment was ligated with a 0.7-kb *Sph*I-*Eco*RI CMV-T7 fragment (generated by amplification and digestion of base pairs 1570–2186 of pcDNA/NEO (Invitrogen) with the primers O-CMV1 (*Sph*I): 5′-ccgccggcatgcgtaatcaattacggggtc-3′ and O-CMV2 (*Eco*RI): 5′-gccggaattcaagcttccggtctccctа-3′), and a 0.57-kb *Eco*RI-*Bsi*WI fragment containing the first part of the SFV genome (generated by amplification and digestion of base pairs 1–517 in pSFV1 with the primers O-SFV3(*Eco*RI): 5′-gccggaattcatggcggatgtgtgacat-3′ and reverse primer, O-SFV2: 5′-gtacagcgatgttggtgc-3′). The resulting plasmid, pSCA1, contains the CMV IE/T7 promoters upstream of the SFV replicon and poly(A) region. (iii) pSCAβ was built by ligating a 4.6-kb *Bgl*II-*Spe*I fragment from pSFV3-lacZ (5, 10) to a 9.9-kb *Bgl*II-*Spe*I pSCA1 fragment. This places the *lacZ* gene immediately downstream of the nsP4 coding region, under the control of the SFV subgenomic promoter (Fig. 1*C*). ΔpSCAβ was built by deleting a 2.7-kb *Sac*II fragment from pSCAβ. pSCAHelper (Fig. 6*A*), was constructed by inserting the 3.6-kb *Spe*I/*Acc*I fragment from pSCA1 into the 5.1-kb *Acc*I/*Spe*I fragment from pSFVHelper2 (5, 11).

*Cell Culture Materials*—BHK-21 baby hamster kidney cells and COS-1 cells (SV40 transformed Green monkey kidney) were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum (FBS), 25 mM HEPES buffer, and L-glutamine. Cells were incubated in a humidified atmosphere of 5% $CO_2$ in air.

*Electroporation*—BHK-21 and COS-1 cells were grown to 50% confluence, trypsinized, washed once with IMDM plus FBS, once with PBS and resuspended at $10^7$ cells/ml in a sterile medium comprising IMDM with 10 mM glucose and 0.1 mM β-mercaptoethanol. At room temperature, cells (0.8 ml) were transferred to a 0.4-cm cuvette (Bio-Rad), DNA added, and electroporation carried out using the Bio-Rad Gene Pulser. Cells from one cuvette were plated on five 6-cm plates. To optimize the transfection efficiency, variations in capacitance (25–960 mF), voltage (0.1–0.4 kV), number of pulses (1 or 2), and delay between pulses (0 or 30 s) were carried out in 80 combinations. In initial experiments, two 30-s delayed pulses at 0.4 kV and 960 mF yielded the maximum *lacZ* expression. However, re-optimization was required, and in more recent experiments cells received one pulse at 0.4 kV and 960 μF. Each data point represents the average of three electroporations.

*β-Galactosidase Assay*—β-Galactosidase activity was measured by an ONPG (*O*-nitrophenyl-β-D-galactopyranoside, Ampresco) assay as described previously (19). β-Galactosidase standards (Boehringer Mannheim) were used to convert the readings of $A_{420\ nm}$ for the ONPG assay results into nanograms of protein.

*X-Gal Assay*—Transfected BHK-21 and COS-1 cells were stained by the X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside, Chemica Alta Ltd.) method (20).

*Southern Blot Analysis*—BHK-21 cells ($8 \times 10^6$), electroporated with 2 μg of plasmid, were split into five 6-cm dishes (*i.e.* 0.4 μg of DNA/plate). Total DNA from one plate was isolated by phenol-chloroform extraction (19), and 15% of the sample was digested and run on a 0.8% agarose gel, and transferred to a Gene Screen Plus membrane (NEN Life Science Products) as described previously (21). The 3.1-kb *Bam*HI fragment of pSCAβ, corresponding to the *lacZ* gene, was random-prime labeled (22) with [α-$^{32}$P]dCTP and used as a probe for hybridization. Hybridization was performed as described (21). Blots were washed twice in 2× SSC and 1% SDS, then twice again in 0.1× SSC and 0.1% SDS at 65 °C. Signal was visualized and quantitated using a Molecular Diagnostics PhosphorImager.

*Generation of Infectious SFV Particles*—The transfected amount of pSCAβ and pSCAHelper DNA was optimized to generate the highest viral titer. In duplicate experiments, pSCAβ (2 μg) and pSCAHelper were added in molar ratios of 1:0.5, 1:1, 1:2, and 1:4, respectively, to electroporation media containing $8 \times 10^6$ BHK-21 cells. Cells were electroporated and the contents of each cuvette plated on a 10-cm tissue culture plate. After 4 h, the medium was aspirated, cells washed with PBS, and 8 ml of IMDM (plus 10% FBS) was added. Medium containing the SFV particles was collected 24 and 36 h after electroporation and clarified by centrifugation at 2000 rpm (Sorvall RT 6000 D) for 15 min at 4 °C. Virus was activated with 0.5 mg/ml α-chymotrypsin at room temperature for 45 min, and aprotinin (0.38 mg/ml) added to stop protease activity (11). 0.2 ml of activated virus stock was added to $10^6$ BHK-21 cells on 6-cm tissue culture plates, and incubated for 45 min at 37 °C in a humidified atmosphere of 5% $CO_2$ in air. The infectious medium was aspirated, cells washed with PBS, and IMDM/10% FBS was added. ONPG and X-gal assays were performed 18 h after infection. X-gal-positive and -negative cells were counted in 10 fields viewed under 400× magnification. Total number of cells on the dish was obtained by trypsinizing a duplicate plate of infected cells. Viral titer was estimated by multiplying the total number of cells by both the fraction of X-gal-positive cells and a factor to correct for the volume of virus-containing supernatant used in the assay.

*Determination of Replication Proficient Virus Particles*—A total of $8 \times 10^6$ BHK-21 cells were transfected with a 1:1 molar ratio of pSCAβ (2 μg) and pSCAHelper (1.2 μg) in 10 separate reactions. At 24 h after transfection, 0.5 ml of the growth medium from each 10-cm tissue culture plate was collected and viral titer determined (see above). Without α-chymotrypsin treatment, the remaining 6 ml were divided among six tissue culture plates (6 cm) containing $1 \times 10^6$ BHK cells and incubated for 45 min. The virus-containing medium was aspirated, and the cells were washed in PBS and then overlaid with a plaque assay medium (PAM) containing 0.6% low melt agarose (Bio Shop, Canada) in IMDM (plus 2% FBS, 10% tryptose phosphate (Life Technologies, Inc.), and 20 mM $MgCl_2$). The cells were incubated for 5 days and checked for plaques daily.

Cells infected with activated recombinant virus were tested for production of RPVs. A total of $3 \times 10^7$ α-chymotrypsin activated infectious units, derived from three independent cotransfections of pSCAβ and pSCAHelper DNA, were used to infect $2 \times 10^7$ BHK cells. After 45 min

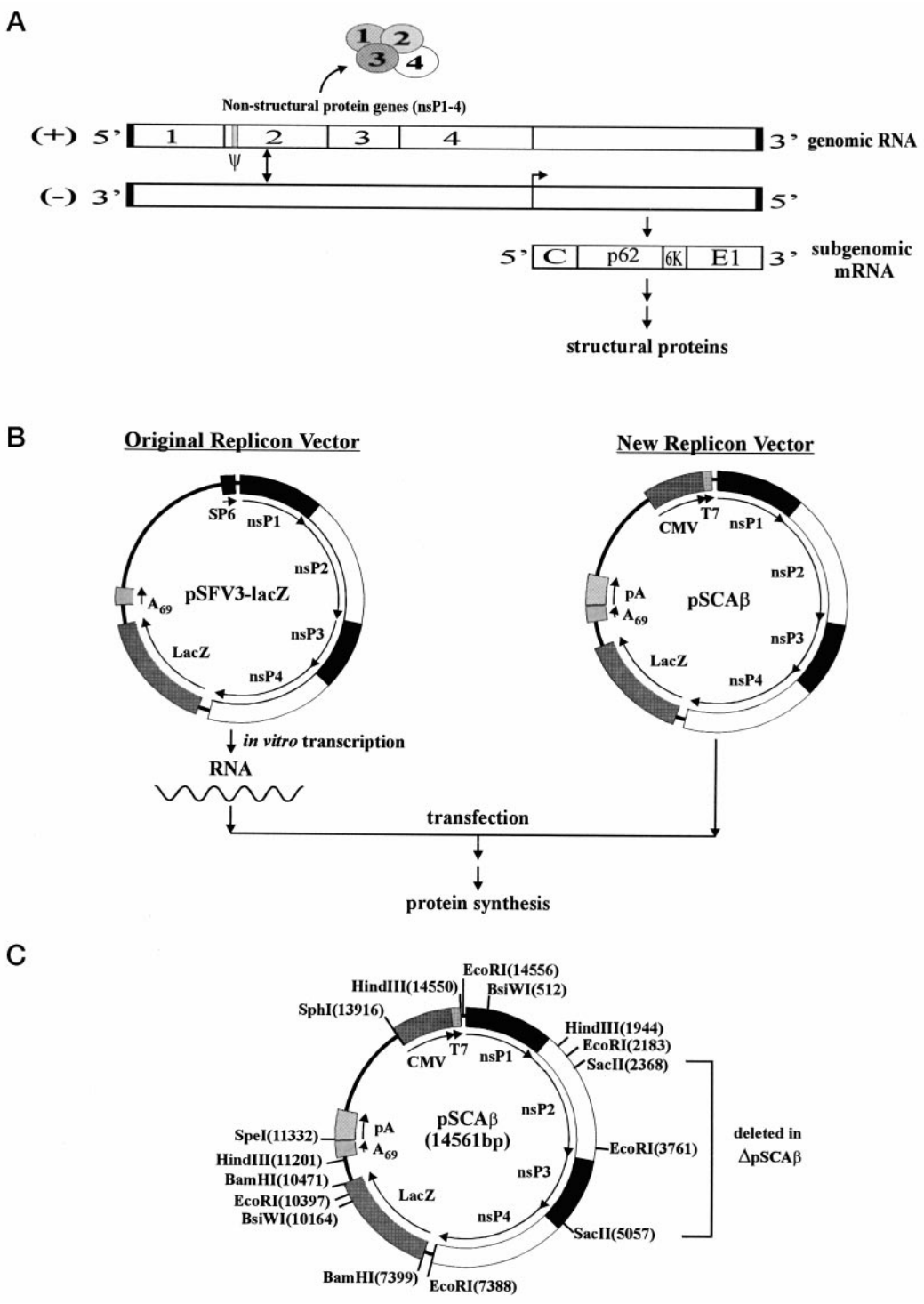


FIG. 1. **The SFV expression system.** *A*, replication of wild type SFV genome. *Filled boxes* represent amplification sequences at either end of the genome. The packaging signal within nsP2 (ψ) is represented by a *stippled box*. The subgenomic promoter is shown as an *arrow*. *B*, comparison of RNA- and DNA-based SFV replicon vectors. pSFV3-lacZ is described in Ref. 10. This scheme shows the steps necessary to obtain protein from transfection of re-RNA or plasmid replicon. Infectious virus can be obtained by co-transfecting pSCAβ with pSCAHelper (Fig. 6*A*). *C*, detailed map of pSCAβ. *Numbers* indicate the start of each restriction site.

of infection, cells were washed with PBS, overlaid with PAM, and checked for plaque-forming units (PFUs) daily over 5 days.

To determine the incidence of RPV in growth media of cells infected with activated virus, $1 \times 10^7$ BHK cells were divided among 10 6-cm plates and infected, in total, with $4.4 \times 10^6$ infectious units of α-chymotrypsin activated recombinant virus. After 18 h, the growth medium was clarified, divided into 10 samples, and applied to 10 tissue culture plates (6 cm) containing a total of $2 \times 10^7$ BHK cells. After 45 min of incubation, PAM was overlaid onto cells and examined for PFUs daily for 5 days.

## RESULTS

*Reporter Gene Expression in Vitro*—The current expression system based on SFV requires transfection of RNA that contains the viral replicase genes and the inserted gene of interest (Fig. 1*B*). RNA is obtained by SP6-driven *in vitro* transcription of an SFV cDNA-geneX cassette. Our goal was to simplify this system by circumventing the need for *in vitro* transcription and RNA handling. Thus, two major modifications were made to

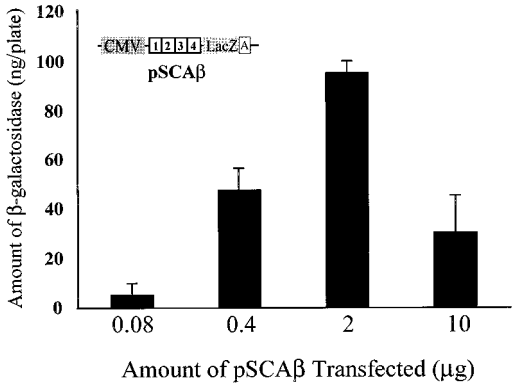


FIG. 2. **Production of β-galactosidase by pSCAβ.** BHK-21 cells were electroporated with the indicated amounts of pSCAβ (shown schematically at the *top left*) and β-galactosidase expression determined 48 h later. Time 0, in this and subsequent graphs, is 4 h after electroporation, when cells had reattached and the medium was replaced. Error bars in this, and subsequent graphs, indicate standard deviation. Note that the amount of DNA per plate is 1/5th of the total electroporated, because transfected cells were split onto five 6-cm dishes. In this experiment, therefore, 2 μg of pSCAβ generated a total of 500 ng β-galactosidase.

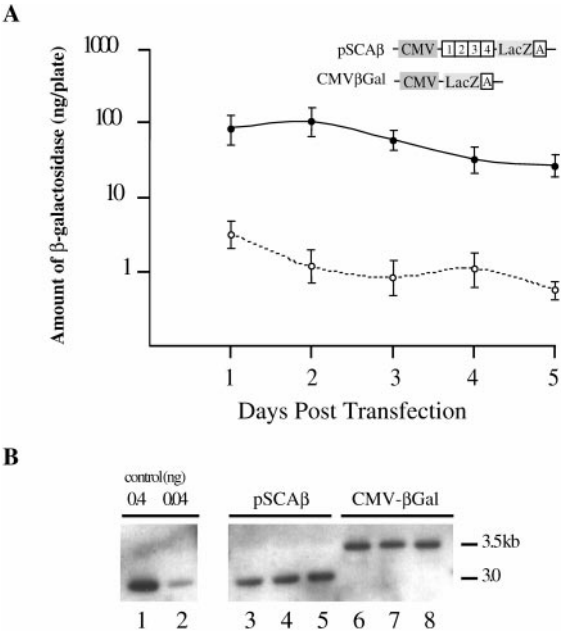


FIG. 3. **Comparison of pSCAβ and a simple, CMV IE promoter-driven vector.** *A*, BHK-21 cells were transfected with 2 μg of pSCAβ (●) or 1 μg of CMVβGal (○) (an equimolar amount), and 1/5th of the cells plated on a 6 cm dish. β-galactosidase levels were measured at the indicated time points after electroporation. The vectors are shown schematically at the *top* of the graph. *B*, Southern analysis of DNA isolated from BHK-21 cells 1 day after transfection with pSCAβ (*lanes 3–5*) or CMVβGal (*lanes 6–8*). Analysis of three separate transfections for each plasmid are shown. pSCAβ and CMVβGal samples were digested with *Bam*HI. The control (*lanes 1* and *2*) is *Bam*HI-digested pSCAβ. Blots were probed with the *Bam*HI *lacZ* fragment from pSCAβ.

the parent vector pSFV1 (5, 10). First, the SV40 termination/polyadenylation signal was added immediately downstream of the 69 A residues at the 3′ end of the SFV cDNA (23). The message expressed from the final vector is expected to have two poly(A) stretches separated by a short SV40 sequence. Second, the SP6 promoter in pSFV1 was replaced by a hybrid CMV IE/T7 promoter derived from the pcDNA vector (Invitrogen). The final vector, pSCAβ, was completed by insertion of the reporter gene, *lacZ*, into the multiple cloning site (Fig. 1). This bifunctional vector can be transcribed *in vivo* or *in vitro* using either the CMV IE or T7 promoter, respectively.

To determine if pSCAβ was functional, the plasmid was transfected into BHK-21 cells and β-galactosidase activity measured in cell lysates. To optimize the amount of β-galactosidase produced, pSCAβ was transfected in 5-fold increments, from 0.08 μg up to 10 μg. β-galactosidase was detected in every case (Fig. 2), indicating that a DNA-based SFV expression system is viable. Maximum expression was obtained following transfection of 2 μg of DNA (Fig. 2), and this amount was used in all subsequent transfection experiments. The amount of β-galactosidase obtained per plate ranged from 100 ng (Fig. 2) to 900 ng in more recent experiments (data not shown), depending on transfection efficiency. The number of transfected cells was determined in several experiments by X-gal staining, and, coupled with determination of enzyme activity, consistently showed that pSCAβ generates around 20–30 pg of β-galactosidase/cell, which is similar to the 80 pg/cell obtained following transfection of *in vitro* generated re-RNA (10).

*pSCAβ Expresses More β-Galactosidase than CMVβGal*—The levels of β-galactosidase obtained with the pSCAβ vector should be much higher than those obtained from a comparable vector that lacks the SFV nsPs. Message levels from CMVβGal (CLONTECH) are dependent on CMV IE promoter activity alone. Thus, BHK-21 cells were transfected with pSCAβ and CMVβGal and β-galactosidase activity measured over 5 days after transfection (Fig. 3*A*). The amount of β-galactosidase produced by pSCAβ reached a maximum on day 2, and on day 1 for the CMVβGal vector. In several replicate experiments, the expression levels obtained with pSCAβ were, on average, 20–30-fold greater than CMVβGal. Similar data were obtained using COS-1 cells (data not shown).

The elevated levels of expression observed with pSCAβ *versus* CMVβGal plasmid is most likely due to the amplification of the message encoded by the SFV-based vector. However, it was also possible that this plasmid-based SFV vector transfected more efficiently. Against this possibility, we found that similar numbers of cells were stained blue in an X-gal assay whether pSCAβ or CMVβGal was transfected (data not shown). As a more quantitative measure of transfection efficiency, DNA was extracted from the same lysates that were used to measure the day 1 β-galactosidase activities shown in Fig. 3*A*. DNA was digested, and transfected plasmid detected on a Southern blot by use of a *lacZ* probe, and quantified on a PhosphorImager. Equal amounts of pSCAβ and CMVβGal were transfected (Fig. 2*B*). Therefore, the higher expression observed with pSCAβ was due to the effects of the SFV polymerase (Fig. 1*A*).

*Reporter Gene Expression from pSCAβ Is Dependent on Functional Replicase Proteins*—To confirm that the SFV nsPs were indeed essential to the activity of pSCAβ, we tested whether a derivative with a large deletion in nsP2–3 could direct β-galactosidase expression. This plasmid (ΔpSCAβ, Fig. 1*C*) was transfected into BHK-21 cells and β-galactosidase activity measured over a 5-day period. pSCAβ expressed an average of 220 ng of β-galactosidase/6-cm plate, but there was no β-galactosidase activity in cells transfected with ΔpSCAβ (Fig. 4*A*). Similarly, when BHK-21 cells were stained for β-galactosidase using X-gal, only pSCAβ-transfected cells were positive (Fig. 4*B*). Southern analysis performed on cells taken from 2, 3, and 4 days after transfection confirmed that both pSCAβ and ΔpSCAβ plasmids had been taken up (Fig. 4*C*). The DNA from each transfection was quantitated on a PhosphorImager and the data used to base-line correct the β-galactosidase levels (Fig. 4*A*). Thus, β-galactosidase expression from pSCAβ is not due to read-through translation of the full-length message, but is dependent on the SFV polymerase, which carries out both amplification and synthesis of the subgenomic *lacZ* transcript (Fig. 1*A*).

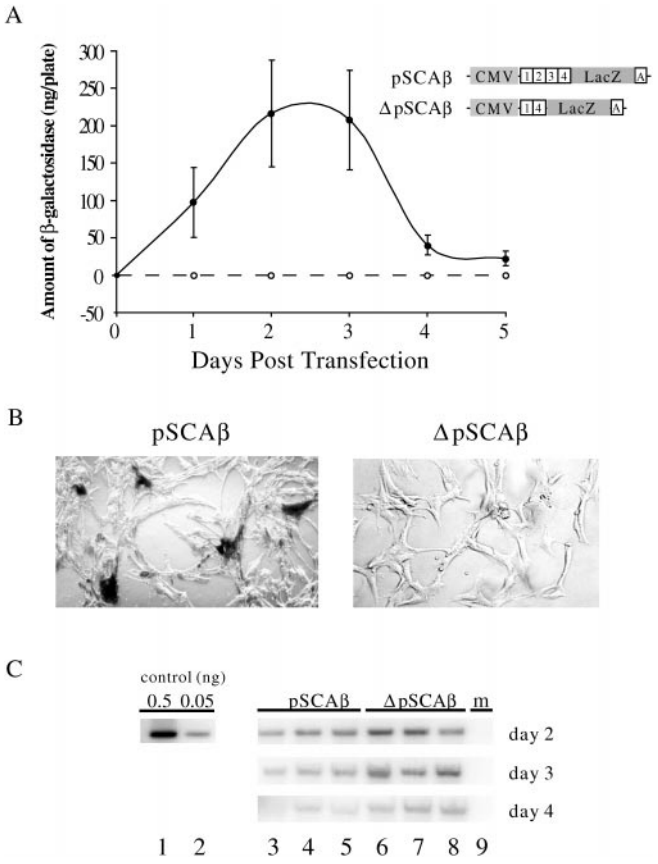


FIG. 4. **pSCA*β* function requires the nsPs.** *A*, BHK-21 cells were transfected with 2 μg of pSCA*β* (●) or ΔpSCA*β* (○) (drawn schematically at the *top right*) and 1/5th of the cells plated on a 6-cm dish. *β*-Galactosidase activity was measured at the indicated time points after electroporation. *B*, cells transfected with pSCA*β* or ΔpSCA*β* were stained with X-gal. *C*, Southern analysis of *Bam*HI-digested pSCA*β* (*lanes 3–5*) or ΔpSCA*β* (*lanes 6–8*) isolated 2–4 days after transfection as indicated. Procedures were as described in the legend to Fig. 3 and under "Materials and Methods." Control DNA (*lanes 1* and *2*) was *Bam*HI-digested pSCA*β*. *M* (*lane 9*) represents a sample from mock-transfected cells.

*pSCA*β *Expresses More Protein than a DNA-based Sindbis Vector*—Sindbis, another alphavirus, was engineered by Wolff and co-workers (24) into a plasmid-based vector. In this system, *in vivo* transcription of the Sindbis RNA was achieved by placing viral cDNA under transcriptional control of the Rous sarcoma virus long terminal repeat (RSV LTR). The Sindbis vector, pSin-nlLacZ, also utilized *lacZ* as a reporter gene. To compare expression from the SFV (pSCA*β*) and Sindbis (pSin-nlLacZ) DNA-based vectors, pSCA*β* and pSin-nlLacZ were transfected into BHK-21 cells and *β*-galactosidase activity was measured over a 5-day period (Fig. 5*A*). *β*-Galactosidase expression peaked on day 2 for both pSCA*β* and pSin-nlLacZ. In several replicate experiments, *β*-galactosidase expression in BHK-21 cells was 10–20-fold higher for pSCA*β* than for pSin-nlLacZ. Transfection efficiency of both plasmids, as assessed by Southern analysis, was similar (data not shown). Thus, higher levels of *β*-galactosidase were obtained using the SFV vector.

*Generation of Infectious Recombinant SFV Using DNA-based Vectors*—To generate infectious virus particles using DNA-based SFV vectors, we constructed pSCAHelper, in which expression of helper RNA is driven by the CMV IE promoter (Fig. 6*A*). This RNA contains the sequences at each end of the SFV genome that mediate amplification by the nsPs, but lacks the packaging signal located in the nsP2 gene (Fig. 1*A*). Thus, cotransfection with the replicon vector will result in amplification of the helper message, transcription of the subgenomic

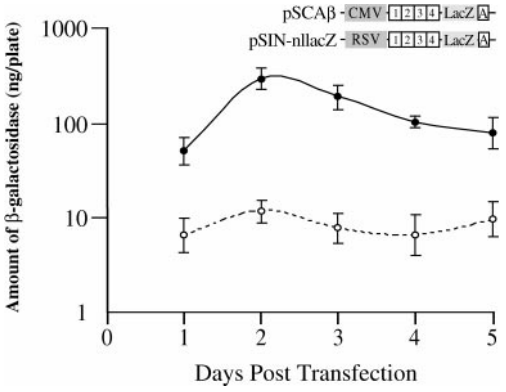


FIG. 5. **Comparison of pSCA*β* and the Sindbis vector, pSin-nlLacZ.** BHK-21 cells were transfected with 2 μg of pSCA*β* (●) or pSin-nlLacZ (○) (drawn schematically at the *top* of the graph), and one-fifth of the cells plated on a 6-cm dish. *β*-Galactosidase levels were measured at the indicated time points after electroporation.

message, and translation of the sPs. The sPs will package re-RNA, but not helper RNA. pSCAHelper carries mutations in the p62 sP gene that alter three amino acids so that generation of E2 and E3 glycoproteins from the p62 precursor and activation of the virus requires *α*-chymotrypsin treatment (11). BHK-21 cells were transfected with pSCA*β* and pSCA-Helper plasmids in four molar ratios. After 24 h, 0.2 ml of the growth medium from the transfected cells was activated with *α*-chymotrypsin, applied to fresh BHK-21 cells, and titer determined. The highest viral yield was obtained with a 1:1 molar ratio, followed by 1:2, 1:0.5, and 1:4 (Fig. 6*B*).

To optimize the postinfection recovery time, BHK-21 cells were transfected with equimolar amounts of pSCA*β* and pSCA-Helper. Medium harvested 24 h after electroporation contained $2.05 \times 10^6$ infectious particles/ml, while medium harvested at 36 h after electroporation contained $0.80 \times 10^6$ infectious particles/ml (Fig. 6*B*). The amount of *β*-galactosidase generated after infection was similar to the amounts reported previously (10). From a single transfection, plated on one 10-cm plate, approximately $10^7$ infectious particles of virus was obtained. Infection of $1.1 \times 10^6$ BHK-21 cells with $3.2 \times 10^5$ particles generated 16 μg of *β*-galactosidase, so the total virus produced from this transfection was enough to generate >500 μg of *β*-galactosidase.

*Absence of Replication Proficient Viruses*—One of the problems associated with any viral expression system is the generation of wild type virus through recombination between the separated components of the viral genome. In alphavirus systems, replication proficient virus (RPV) particles have been detected following cotransfection of replicon and helper RNAs (11, 25). Recombination occurs when the polymerase switches templates during amplification (26). In a DNA-based system, homologous recombination between plasmids provides another level at which a complete virus genome could be generated. However, with the SFV system described here, recombination alone would not generate fully infectious virus since reversion or suppression of the conditional mutation that inhibits p62 cleavage would also be required (11). We performed three assays to estimate the frequency of RPV generation with the DNA-based SFV system (Fig. 7).

To determine whether the growth media from BHK-21 cells transfected with pSCA*β* and pSCAHelper DNA contained any RPV, BHK-21 cells were cotransfected with pSCA*β* (2 μg) and pSCAHelper (1.2 μg) in 10 separate experiments. After 24 h, 0.5 ml of growth medium was removed from each transfection and used to determine viral titer. The remaining growth medium, which contained over $5 \times 10^7$ recombinant virus parti-

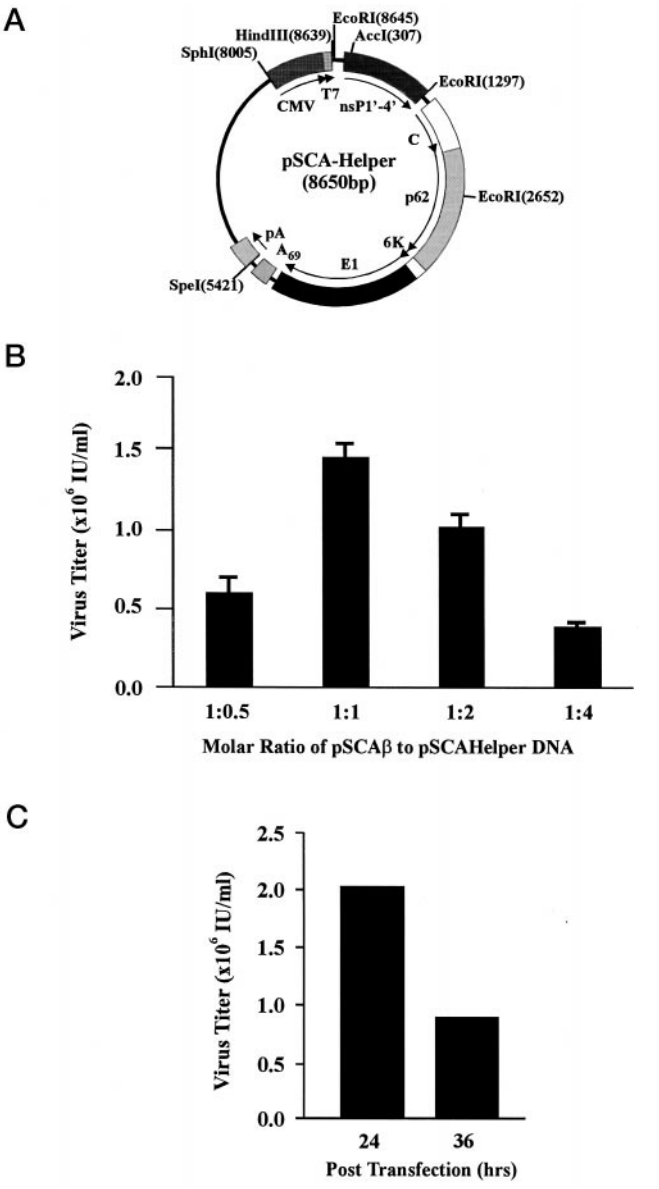


FIG. 6. **Production of infectious recombinant virus.** *A*, diagram of pSCAHelper. Numbers represent the start of the indicated restriction sites. *B*, $8 \times 10^6$ BHK-21 cells were transfected with pSCA$\beta$ (2 $\mu$g) and pSCAHelper in the indicated molar amounts, and all the cells from each transfection plated on one 10-cm dish. Virus was harvested 24 h after transfection and titer assessed on fresh BHK-21 cells 18 h after infection. *C*, BHK-21 cells were transfected with 2 $\mu$g of pSCA$\beta$ and 1.2 $\mu$g of pSCAHelper (an equimolar amount), and all the cells plated on one 10-cm dish. Viral stocks were harvested at the indicated time points and titer assessed on fresh BHK-21 cells.

cles, was added to $6 \times 10^7$ fresh BHK-21 cells without $\alpha$-chymotrypsin treatment. The cells were overlaid with agarose and examined for the appearance of PFUs over 5 days (Fig. 7*A*). No PFUs were detected.

Additionally, no RPV particles were found when plaque assays were performed on $2 \times 10^7$ BHK cells infected with $3 \times 10^7$ $\alpha$-chymotrypsin-activated recombinant viral particles (Fig. 7*B*).

Further experiments were performed to analyze if the culture medium from cells infected with activated recombinant virus contained any RPV. BHK-21 cells were infected with activated recombinant virus over various multiplicities of infection (m.o.i.). A total of $4.4 \times 10^6$ particles were used in these experiments. The medium was collected 18 h after infection, applied to new BHK-21 cells, and tested for the presence of

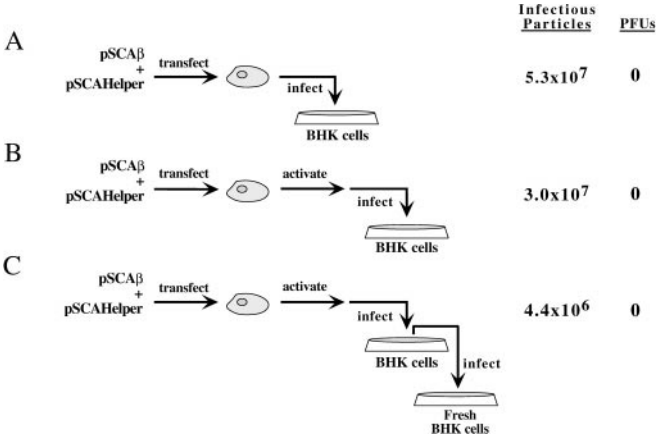


FIG. 7. **Incidence of plaque-forming units (PFUs) due to replication proficient SFV virus.** *A*, 10 individual pSCA$\beta$ and pSCAHelper cotransfections generated $5.3 \times 10^7$ infectious virus units, which, without $\alpha$-chymotrypsin treatment, were added to $6 \times 10^7$ fresh BHK-21 cells. *B*, three individual pSCA$\beta$ and pSCAHelper cotransfections generated $3 \times 10^7$ infectious virus units, which were activated with $\alpha$-chymotrypsin and added to $2 \times 10^7$ fresh BHK-21 cells. *C*, 10 individual pSCA$\beta$ and pSCAHelper cotransfections generated $4.4 \times 10^6$ infectious virus units, which, once activated with $\alpha$-chymotrypsin, were added to $1 \times 10^7$ fresh BHK-21 cells. After 18 h, the growth medium from infected cells was applied to fresh BHK-21 cells. The infected BHK-21 cells in all three experiments (*A–C*) were overlaid with agarose and examined for PFUs over 5 days. The indicated viral titers were determined by infecting $1 \times 10^6$ BHK-21 cells for 18 h with $\alpha$-chymotrypsin-activated virus obtained from the initial transfection and staining fixed cells with X-gal.

RPV (Fig. 7*C*). No PFUs were detected.

In summary, a total of $8.7 \times 10^7$ virus particles obtained by transfection of pSCA$\beta$ and pSCAHelper were negative in three tests for RPVs, indicating that generation of wild type virus is extremely rare.

## DISCUSSION

Alphavirus expression systems have been used to express a wide range of heterologous proteins (reviewed in Refs. 14 and 27). Although the approach is simpler and much faster than vectors such as baculovirus, neither SFV nor the related Sindbis vectors have superceded baculovirus as the expression system of choice. One technical drawback of the original alphavirus systems is that they require production and manipulation of recombinant RNA *in vitro*. Therefore, the design of DNA-based alphavirus vectors, both for SFV (this work) and Sindbis (17, 24), significantly improves the utility of these expression systems. These developments also simplify the use of alphaviruses as *in vivo* gene delivery systems, for example, in the delivery of vaccines (28).

Our observations, and those of others using DNA-based Sindbis vectors (17, 24), show that at least a portion of full-length re-RNA generated in the nucleus must reach the cytoplasm. Indeed, the amount of protein produced per cell with the DNA-based SFV vector was similar to that obtained previously following infection of cells with a recombinant virus (10). Expression was optimal with 2 $\mu$g of pSCA$\beta$ and peaked 48 h after transfection. With these parameters, approximately 20–30 pg of $\beta$-galactosidase was obtained per cell. Protein expression with the DNA-based SFV vector was 20–30-fold higher than with a simple CMV IE-based vector. This difference can be attributed to the effect of the SFV polymerase, since a large deletion in the nsP gene region completely abolished protein expression.

Two groups have developed DNA-based Sindbis expression vectors (17, 24). We compared the performance of the Sindbis vector pSin-nlLacZ (24) against the SFV vector, pSCA$\beta$. pSCA$\beta$

generated 10–20-fold more β-galactosidase than the Sindbis-derived vector. This result is not due to a difference in the efficiency of SFV and Sindbis polymerases, since transfection of SFV or Sindbis re-RNAs yields similar levels of protein (10, 27). Thus, other components of pSCAβ and pSin-nlLacZ must be responsible. pSin-nlLacZ encodes β-galactosidase that is targeted to the nucleus, whereas the protein encoded by pSCAβ lacks a nuclear localization signal, but it seems unlikely that this would explain the large difference in expression levels. An important element required for maximal expression is the viral poly(A) tract placed downstream of the reporter gene (17). However, both pSCAβ and pSin-nlLacZ contain such a motif. pSCAβ contains an additional termination/polyadenylation signal downstream of this tract, whereas pSin-nlLacZ lacks this element. However, this motif inhibited expression of another DNA-based Sindbis vector (24). Other factors that could influence protein expression include the promoters (CMV IE or RSV LTR in pSCAβ and pSin-nlLacZ, respectively), the positioning of *lacZ* relative to the subgenomic promoter, and the efficiency of translation. We suspect that promoter-strength is the major factor, but additional experiments are required to test this idea. Studies with other DNA-based Sindbis vectors also suggest that promoter-strength influences vector efficiency (17).

We also utilized the CMV IE promoter to generate a helper plasmid (pSCAHelper) in which expression of viral sPs is RNA polymerase II-dependent. Cotransfection of pSCAβ and pSCA-Helper generated virus at a titer of around $10^6$ infectious particles/ml (which could be artificially low, since X-gal staining was used to determine titer, and this method has recently been shown to underestimate the number of β-galactosidase-positive cells (20)). Virus generated using the new DNA vectors gives the same high yield of protein as virus generated by the original RNA-based approach (50 μg of β-galactosidase/$10^6$ particles of recombinant virus).

Virus stocks generated using pSCAHelper are conditionally infectious due to amino acid changes within sP p62 at the junction between the E2 and E3 spike proteins (11). This modification reduces the chance of obtaining wild-type virus, which would require recombination between re-RNA and helper RNA, and reversion or suppression of the conditional mutation. Empirical measurements show that the chance of generating RPV, following transfection of re-RNA and helper RNA that encodes α-chymotrypsin-activated p62, is less than $10^{-10}$, and has never been observed (11). A disadvantage of DNA vectors is that they provide another opportunity for recombination. The level of RPVs obtained following transfection of Sindbis DNA-based replicon and helper vectors was 1000-fold higher (~$10^7$ PFU/ml) than that obtained after transfection of *in vitro* generated re-RNA and helper RNA (~$10^4$ PFU/ml) (17). Indeed, a similar number of RPVs and recombinant particles were generated using the DNA-based system Sindbis vectors. In contrast, no RPVs were detected in a population of nearly $10^8$ recombinant particles generated using the DNA-based SFV system described here. These statistics highlight an important advantage of the SFV vectors.

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