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 10⁶ infectious particles/ml. A single electroporation, plated on one 10-cm plate, generated enough virus (10⁷ particles) to produce >500 µg of protein. Wild type, replication proficient virus was not detected in three tests utilizing almost 10⁸ 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), 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 polypeptide, with subsequent autoproteolytic cleavage into four non-structural proteins (nsPs), nsP1 to nsP4, forming an RNA replicase (Fig. 1A). 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. 1A). This region is transcribed into a sub-genomic message by the SFV replicase, and translated into a polypeptide that is autoproteolytically cleaved, producing the capsid protein and two envelope glycoproteins (Fig. 1A). 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 SP6 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-
bicent 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. 1A) 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. 1B) 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. 1B). 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 SVβRR (18) as a template, Vent DNA polymerase, and the following primers: O-SV1 (5′-gccaagcttagtaactagcatga-3′) and O-SV2 (XhoI): 5′-gctctagatccagacatgata-3′. The 0.25-kb product was cut with SpeI and XhoI, and ligated into SpeI-cut pSFV1 (10). The resulting plasmid, pSFpoy(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 pSFpoy(A) by digestion with SplI and BstXI (Fig. 1C). The 10.3-kb fragment was cut with a 0.7-kb SplhI-EcoRI CMV-T7 fragment (generated by amplification and digestion of base pairs 1570–2186 of pCDNA/NEO (Invitrogen) with the primers O-CMV1 (SplI): 5′-gccgcatgtgtaactagcatga-3′ and O-CMV2 (EcoRI): 5′-gctctagatccagacatgata-3′), and a 0.57-kb EcoRI-BstXI 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/EcoRI: 5′-gccaagcttagtaactagcatga-3′) and a reverse primer, O-SFV2: 5′-gtacaagcttagttcgcgctg-3′. The resulting plasmid, pSCA1, contains the CMV IE77 promoters upstream of the SFV replicon and poly(A) region. (iii) pSCAβ was built by ligating a 4.6-kb BglII-SpeI fragment from pSFV3-lucZ (5, 10) to a 9.9-kb BglII-SpeI pSCA1 fragment. This places the luc gene immediately downstream of the promoter under the control of the SFV subgenomic promoter (Fig. 1C). pSCAβ was built by deleting a 2.7-kb SacI-II fragment from pSCAβ, pSCAHelper (Fig. 6A), was constructed by inserting the 3.6-kb SpeI-AccI fragment from pSCA1 into the 5.1-kb AccI-SpeI 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% CO2 in air.

**Electroporation**—BHK-21 and COS-1 cells were grown to confluence, trypsinized, washed once with IMDM plus FBS, and resuspended at 107 cells/ml in 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 Pulsor. Cells from one cuvette were plated on five 6-cm plates. To optimize the transfection efficiency, variations in capacitance (25–960 μF), voltage (0.1–0.4 kV), number of pulses (1 or 2), and delay between pulses (0 or 100 μs) were carried out in 80 combinations. In initial experiments, two 30-s delayed pulses at 0.4 kV and 960 mF yielded the maximum luc 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, Amresco) assay as previously reported (19). The ONPG assay measures the production of O-nitrophenol, which is quantitated by monitoring absorbance at 420 nm (20).

**Southern Blot Analysis**—BHK-21 cells (8 × 106), electroporated with 2 μg of plasmid, were split into five 6-cm dishes (i.e. 0.4 μg DNA per 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 BamHI fragment of pSCAβ, corresponding to the luc gene, was random-prime labeled (22) with α-32PdCTP and used as a probe for hybridization. Hybridization conditions were as described (21). After hybridized filters 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 PhosphoImager.

**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 1.05, 1.1, 1.2, and 1.4, respectively, to electroporated medium containing 8 × 106 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 trypsin, washed once with IMDM plus 10% FBS, and 8 ml of IMDM (plus 10% FBS) was added, and electroporation carried out using the Bio-Rad Gene Pulsor. 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 × 106 BHK-21 cells were transfected with a 1.1 molar ratio of pSCAβ (2 μg) and pSCAHelper (1.1 μg) in 10 replicate 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 × 106 BHK cells and incubated for 45 min. The virus-containing medium was aspirated, and the cells were washed with PBS and then overlaid with a plaque assay medium (3% agarose in IMDM containing 0.1 mg/ml amphotericin B and 20 mM MgCl2). 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 × 106 α-chymotrypsin activated infectious units, derived from three independent cotransfections of pSCAβ and pSCAHelper DNA, were used to infect 2 × 107 BHK cells. After 45 min
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 a-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. 1B). 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...
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. 3A). 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. 3A. 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. 2B). Therefore, the higher expression observed with pSCAβ was due to the effects of the SFV polymerase (Fig. 1A).

Report gene expression from pSCAβ is dependent on functional Replicative proteins—To confirm that the SFV nSpS 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. 1C) 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 dish, but there was no β-galactosidase activity in cells transfected with ΔpSCAβ (Fig. 4A). Similarly, when BHK-21 cells were transfected for β-galactosidase using X-gal, only pSCAβ-transfected cells were positive (Fig. 4B). 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. 4C). The DNA from each transfection was quantitated on a PhosphorImager and the data used to base-line correct the β-galactosidase levels (Fig. 4A). 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. 1A).
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 pSCAβ 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. 6B).

To optimize the postinfection recovery time, BHK-21 cells were transfected with equimolar amounts of pSCAβ 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. 6B). 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 pSCAβ and pSCAHelper DNA contained any RPV, BHK-21 cells were cotransfected with pSCAβ (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-
cles, was added to $6 \times 10^7$ fresh BHK-21 cells without α-chymotrypsin treatment. The cells were overlaid with agarose and examined for the appearance of PFUs over 5 days (Fig. 7A). No PFUs were detected.

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

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

In summary, a total of $8.7 \times 10^7$ virus particles obtained by transfection of pSCΑβ 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 pSCΑβ and peaked 48 h after transfection. With these parameters, approximately 20–30 pg of β-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, pSCΑβ, pSCΑβ...
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-nLacZ must be responsible. pSin-nLacZ encodes polymerase II-dependent. Cotransfection of pSCA plasmid (pSCAHelper) in which expression of viral sPs is RNA ciency (17).

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 10^9 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/10^9 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^-11, 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 observed 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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