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SHORT COMMUNICATION

Expression of Additional Genes in a Vector Derived from a Minimal RNA Virus

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Previous studies have shown that expression of the vesicular stomatitis virus (VSV) glycoprotein (G) from a Semliki Forest virus (SFV) RNA replicon results in the production of propagating infectious particles that we call minimal viruses. These minimal viruses consist of vesicles containing VSV G protein that bud from the plasma membrane and trap the infectious SFV G RNA, but they do not contain other viral structural proteins. The cell binding and membrane fusion activity of the VSV G protein allow minimal viruses to propagate in tissue culture cells. To determine if these minimal viruses could be used to express foreign genes, we added a second SFV promoter and a multiple cloning site downstream of the VSV G gene. We report here expression of three different proteins from this modified, minimal virus vector. Although expression of each foreign, unselected gene was lost rapidly from the vector upon passaging, it was possible after the initial transfection to derive stocks of infectious particles that could be used to infect multiple additional cultures and transfer protein expression efficiently. When cells were infected with these minimal viruses, host protein synthesis was shut off and the foreign protein and VSV G proteins were the major proteins expressed in the infected cells. Both were expressed at similar levels and accumulated to about 1–2% of total cell protein. © 1996 Academic Press, Inc.

High-level expression of proteins in animal cells has been very informative in studies of protein and cellular function, and in many cases has relied on virus-derived expression vectors. Many viruses have evolved to maximize expression of their proteins in host cells and are therefore a good starting point for efficient expression vectors. Vectors based on alphaviruses have proven to be very useful systems for protein expression because they replicate to high levels in the cytoplasm and generate high-level protein expression in mammalian cells (*2, 3*).

The Semliki Forest virus (SFV) vector system (*3, 4*) yields high levels of protein expression in transfected cells. The vector was derived from a cDNA copy of SFV, a positive-strand RNA virus, by replacing the coding region of the structural proteins with a cloning site into which foreign genes are inserted. Cells are transfected with RNA made *in vitro* from this DNA copy, the RNA replicates to high copy number in transfected cells, and the foreign gene is expressed from the subgenomic mRNA. Although this system is very useful, it requires an RNA transfection for each experiment. To circumvent this problem, a helper SFV RNA that encodes the structural proteins but does not contain the RNA packaging signal was developed (*3, 5*). Cotransfection of this RNA into cells with the vector RNA results in the production of infectious SFV particles capable of a single round of infection, replication, and expression of the foreign gene. One difficulty with this system is achieving high-level RNA cotransfection so that adequate titers of infectious particles are produced.

Previously we described insertion of the gene for a single protein, the vesicular stomatitis virus glycoprotein (VSV G), into the SFV vector (*1*). The VSV G protein forms the glycoprotein spikes on the surface of the VSV particles and contains the binding and membrane fusion activities required for VSV entry into cells (*6, 7*). RNA generated from this vector (SFV-G RNA) was transfected into BHK cells where it replicated, expressed VSV G protein, and generated infectious particles that could be propagated indefinitely. We call these infectious particles minimal viruses because they contain VSV G protein as their only viral structural protein and lack any nucleocapsid.

The SFV-G infectious particles can be used to infect additional cells and generate cultures in which all the cells produce VSV G protein while host protein synthesis is suppressed by the SFV replicon (*3*). We reasoned that if we could express proteins in addition to VSV G from the SFV-G vector, the vector might propagate like SFV-G but also express high levels of the additional protein.

Duplication of the subgenomic promoter in Sindbis

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virus, an alphavirus related to SFV, has been used to generate viruses that direct the expression of additional genes (see ref. *8* for review). The subgenomic promoter of the SFV vector has also been duplicated so that an SFV vector expressed two proteins simultaneously (*9*). Here we describe insertion of an additional subgenomic promoter and multiple cloning site into the SFV-G minimal virus vector and we demonstrate the utility of this system as a simple self-propagating animal cell expression vector.

In order to generate a simple propagating vector based on the SFV-G minimal virus we inserted an additional SFV subgenomic promoter immediately downstream from the VSV G gene in pSFV1-VSVG (Fig. 1). Although a core subgenomic promoter region of only 21 nucleotides was identified based on conserved regions of three alphaviruses (*10*), efficient use of the Sindbis promoter was shown to require about 100 base pairs (*11*). We used PCR to amplify 150 nucleotides from the promoter region of pSFV3 (*3*) and inserted these into pSFV1-VSVG. In addition a multiple cloning site (MCS) containing four unique restriction sites was placed downstream from the promoter (Figs. 1A and 1B). Any coding sequence can be inserted into the MCS; a small subgenomic RNA containing the sequence should be made, and the protein should be translated from this RNA. The coding sequence should also be present in the larger subgenomic RNA from which VSV G protein is translated, but would not be translated from this RNA (Fig. 1C).

To determine whether additional proteins could be expressed and propagated from the minimal virus vector pSFVdpG-X, we inserted the coding regions of several proteins into the MCS. We tested two cytoplasmic proteins, bacterial chloramphenicol acetyl transferase (CAT) and the VSV nucleocapsid (VSV N), as well as a cell-surface membrane protein, CD4. To make the pSFVdpG-CAT construct the coding sequence of CAT was amplified by PCR with Vent polymerase (New England Biolabs) from the pSV2-CAT vector (*12*) with primers that included 20 nucleotides homologous to CAT and *Apa*I restriction sites: upstream primer 5′ATCTGGCTACGGGCCCA-AAATGGAGAAAAAAATCAC and downstream primer 5′GTCAGATTGCGGGCCCAAAATTACGCCCCGCCC-TGC. The PCR product was digested with *Apa*I and inserted into the *Apa*I site of pSFVdpG-X. pSFVdpG-N was made similarly by amplifying the VSV N gene from pBS-N (*13*) with primers 5′CGTACGGGCCCAACAGTAATCAA-AATGTCTG and 5′GGATAGGGCCCCATATGTAGCATAA-TATATA. pSFVdpG-CD4 was also constructed in this manner with pBS-CD4 (*14*) as the PCR template and primers 5′GCATCCGAGTGGGCCCGCCACAATGAACCG GGGAGT and 5′CTACCGAGTCGGGCCCTCAAATGGGG-CTACATGTCT.

BHK-21 (American Type Culture Collection CCL 10) cells were transfected using TransfectACE (Life Technol-

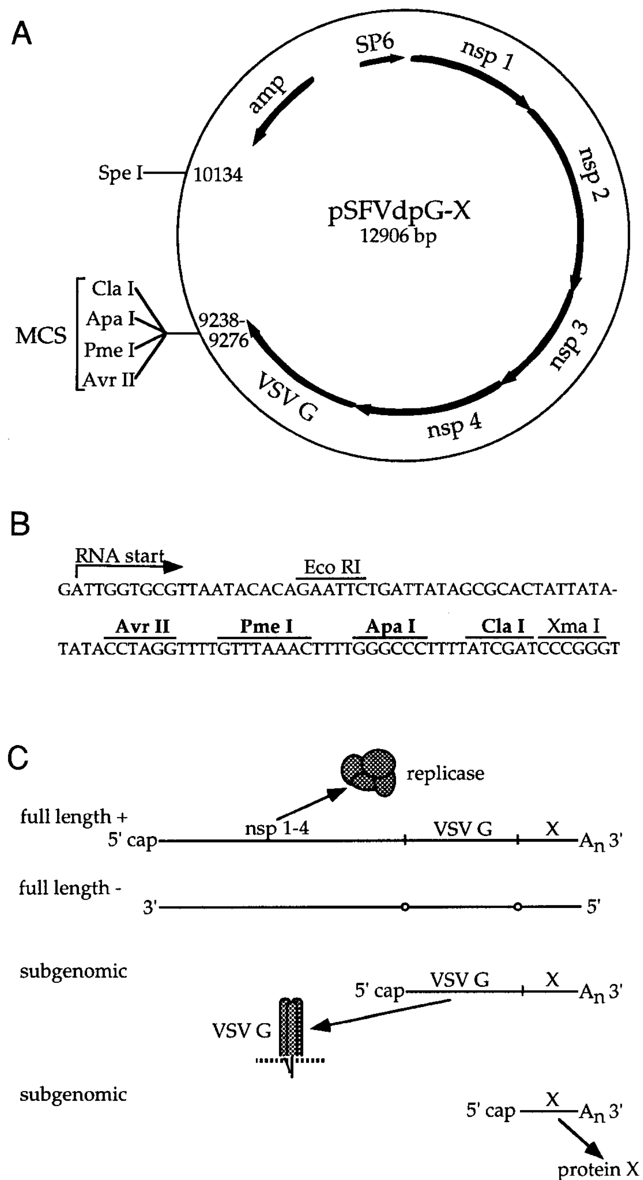

FIG. 1. Diagrams of the SFVdpG-X vector system. (A) The vector pSFVdpG-X contains the VSV G gene followed by an additional SFV subgenomic promoter and a multiple cloning site (MCS) in the background of the pSFV-1 vector. The vector was constructed by amplifying the region containing the subgenomic promoter from the pSFV-3 vector using *Taq* polymerase (Promega). The upstream primer (5′AGCTGCCC-GGGGCTGCAAAAGTATCCTCATA) included 20 bases of homology to the promoter as well as an *Xma*I site (restriction site is underlined) at the 5′ end. The downstream primer (5′AGCTCCCCGGGATCGATAAAAG-GGCCCAAAAGTTTAAACAAAACCTAGGTATATAATAGTGCGCTATA-ATC) also included an *Xma*I site at its 5′ end which was followed by the restriction sites for *Cla*I, *Apa*I, *Pme*I, and *Avr*II and then 20 bases of homology to the promoter. The amplified fragment was cut with *Xma*I and cloned into the *Xma*I site of pSFV1-VSVG (*1*). (B) The MCS of pSFVdpG-X. (C) Replication and transcription events occurring in vector-transfected cells. RNA transcribed from the *Spe*I linearized plasmid with the SP6 polymerase is transfected into tissue culture cells where the replicase is translated from it. The replicase makes a full-length negative-strand copy of the RNA. The negative strand contains two promoters for the subgenomic RNAs, indicated by (°).

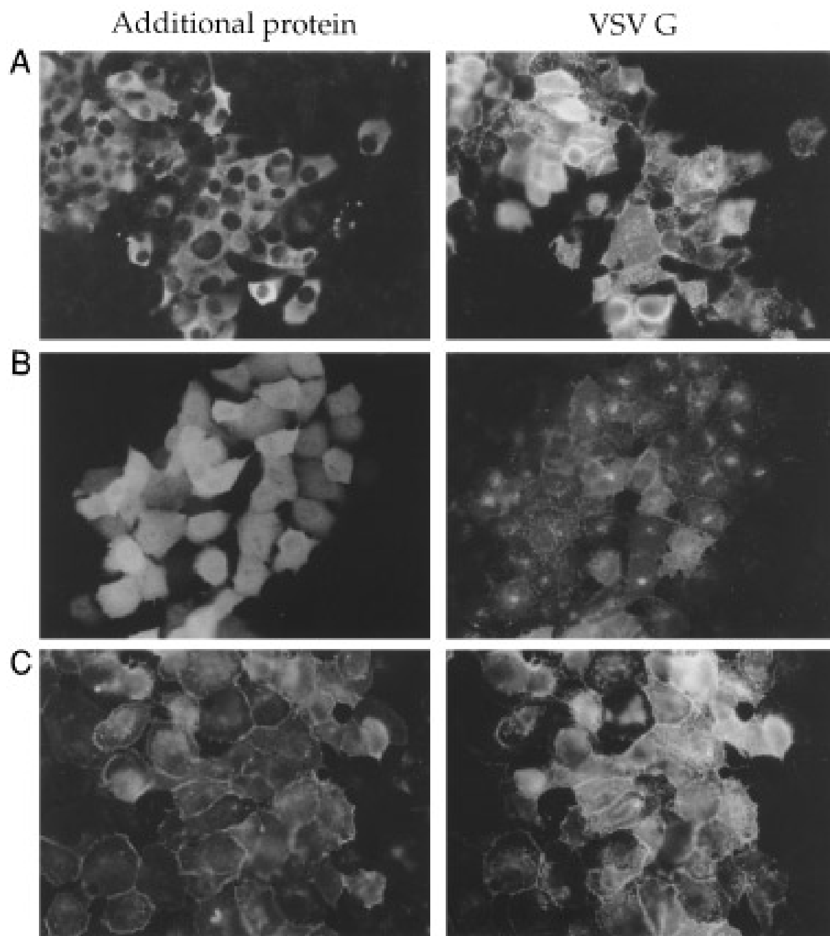

FIG. 2. Expression of additional proteins from the SFVdpG-X vector detected by indirect immunofluorescence. BHK cells transfected with SFVdpG-N, SFVdpG-CAT, and SFVdpG-CD4 RNA are shown in rows A, B, and C, respectively. Protein expression was visualized approximately 48 hr after transfection using indirect immunofluorescence as described (*1*) with antibodies directed against G (right column) and the additional proteins (left column; A, anti-VSV N; B, anti-CAT; C, anti-CD4). Large areas of cells expressing both VSV G and the additional proteins were present in confluent monolayers of cells. The primary antibodies used were monoclonal I14 or a polyclonal rabbit anti-VSV serum to detect VSV G, monoclonal 10G42A9 (kindly provided by L. Lefrancois and D. Lyles) to detect VSV N, monoclonal OKT4 (Becton–Dickinson) to detect CD4, and a polyclonal rabbit anti-CAT serum (5′–3′, Inc.). The secondary antibodies used were rhodamine anti-mouse and FITC-conjugated anti-rabbit (Sigma). Double labeling for CAT and VSV G protein was performed by incubating permeabilized cells with the monoclonal anti-G antibody and the anti-CAT antibody simultaneously, followed by incubation with both secondary antibodies. When G and N proteins were labeled in the same cells, the anti-VSV serum was added before permeabilization so that only G protein at the cell surface would be stained and the N antibody was added after permeabilization, followed by incubation with both secondary antibodies. Cells were photographed with a 40X objective on a Nikon Microphot-FX microscope equipped for epifluorescence.

ogies) with RNAs generated from these vectors *in vitro* as described (*1*) and the protein expression was analyzed by indirect immunofluorescence (Fig. 2). The cells which contained these proteins were usually found in clusters, suggesting propagation of minimal viruses as observed previously (*1*).

To determine whether infectious particles that could transfer expression of the additional proteins were released to the supernatant, we collected supernatants from transfected cells and added a fraction (approximately 5% of the medium) to uninfected cells. Examination of these cells by indirect immunofluorescence 2 days after the transfer indicated that expression of the additional protein could be passaged in the same manner as VSV G. When further passages were done, however, the frequency of coexpression of the additional unselected gene dropped (Fig. 3). Even in the first passage occasional single cells and islands of infected cells could be found that expressed VSV G protein without the additional protein, and upon further passaging expression of the additional gene was lost. By the fifth passage of SFVdpG-N, the double promoter vector expressing VSV N, all the cells expressed VSV G, but none expressed VSV N. Several mechanisms could account for the loss of expression of the additional proteins. Mutations could occur within the gene itself, within the promoter, or the

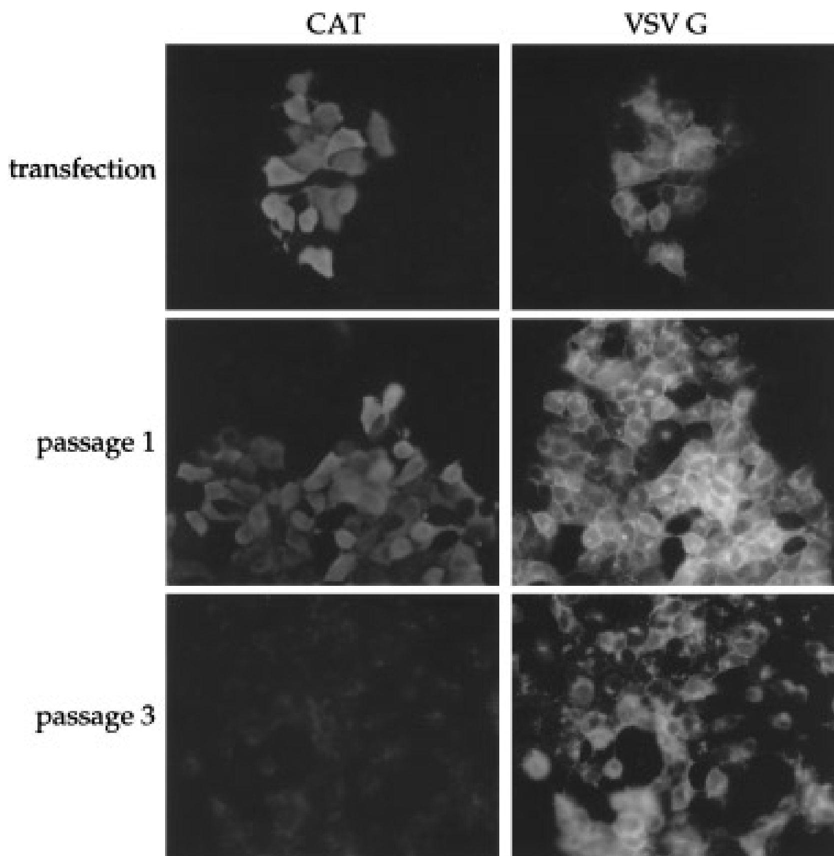

FIG. 3. Indirect immunofluorescence of cells infected with different passages of SFVdpG-CAT. Cells were transfected with SFVdpG-CAT RNA. Supernatants were collected and used to inoculate new cells after 2 days (passage 1). Supernatants from these cells were again collected and passaged after 2 days (passage 2); subsequent passages were performed in the same manner. At the time supernatants were collected the cells were fixed and stained by indirect immunofluorescence as described in Fig. 2. Representative fields are shown stained for VSV G protein and CAT from the transfection, passage 1, and passage 3.

entire gene could be deleted. We also noted that when the expression of the second gene was lost the level of VSV G protein expression was increased. This increased G expression may increase the rate of propagation and thus confer a selective advantage.

We examined RNA isolated from cells infected with different passages of SFVdpG-N to determine if expression of the subgenomic RNA encoding the N protein changed as a function of passaging. The same Northern blot of RNA collected from transfected cells and cells infected with passages 1–4 SFVdpG-N is shown probed for VSV G and VSV N sequences in Fig. 4. The G probe bound to two major bands in the RNA from transfected cells (Fig. 4A). These correspond to the full-length and larger subgenomic species (see Fig. 1C). The N probe bound to these two bands as well as an additional one with higher mobility (Fig. 4B) that corresponds to the smaller subgenomic RNA from which VSV N is translated (Fig. 1C). This profile confirms that the predicted RNA species are made. However, by the second passage, when VSV N expression was reduced, very little N mRNA could be seen on the blot (Fig. 4B) and additional small RNAs containing the N sequence appeared at later passages. The smaller RNAs in the blot probed for VSV G probably represent subgenomic RNAs from which regions in N and the second subgenomic promoter have been deleted. Although we have not analyzed in detail the sequence changes that result in loss of VSV N protein expression, they probably involve mutation in the N mRNA promoter and at least partial deletion of N sequences in many of the replicating genomes in subsequent passages.

The rapid loss of expression of the additional protein as the vector replicates means that large numbers of infectious particles which confer expression of the protein to new cultures cannot be produced by passaging the infectivity. To circumvent this problem we developed a method of producing large numbers of infectious particles from the transfected cells. These infectious particles retain expression of both proteins. We found previously that sonication of cells infected with the SFV-G minimal virus generated as much as 100-fold increases of minimal virus titers compared to those normally released by cells to the supernatant (*1*). Sonication vesiculates the

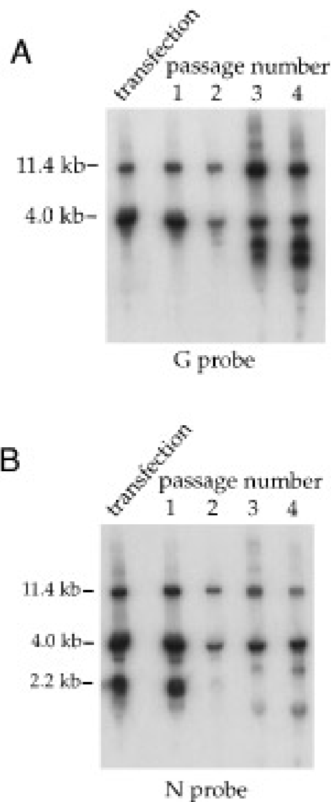

FIG. 4. Northern blot of SFVdpG-N RNAs after minimal virus passaging. BHK cells were transfected with SFVdpG-N RNA and the infectivity was serially passaged four times. At each passage RNA was collected from cells using the Trizol reagent (Life Technologies) 2 days after infection and approximately 4 μg total RNA was analyzed by Northern blotting. The blot was probed sequentially with probes made from the coding regions of VSV G (A) and VSV N (B). Probes for VSV N and VSV G RNAs were prepared from the N and G coding regions using a random primer DNA-labeling kit (Boehringer Mannheim). The nitrocellulose membrane was stripped between incubations using the mild treatment described in (*16*).

plasma membrane which contains VSV G protein and presumably results in particles that nonspecifically entrap the SFV-G RNA in the same manner as the vesicles that bud from infected cells. To test this method for generating infectious particles capable of conferring expression of additional proteins to uninfected cells, approximately 1×10^7 cells in 800 μl Dulbecco's modified Eagle medium were transfected by electroporation with SFVdpG-CAT RNA using a Cell-Porator Electroporation system I (Life Technologies) set at 250 V, 800 μF, low Ω. Most of the cells (500 μl) were plated on a 10-cm dish, and some (60 μl) were plated on a 35-mm dish for parallel immunofluorescence analysis. After 40 hr the cells on the 10-cm dish were scraped into the medium and sonicated in a water bath (Branson 2200) for 3 min in a 15-ml conical polystyrene tube (Corning). A total of 10 ml of infectious material was generated from one transfection. After debris was pelleted by centrifugation in a clinical centrifuge at highest speed for approximately 30 min, aliquots were frozen at −80°. Approximately 1×10^5 BHK cells on 35-mm dishes were infected with different amounts of the stock and with SFV-G particles. At 40 hr postinfection protein expression was examined by radiolabeling the cells for 2 hr with 50 μCi of protein labeling mix (NEN) in methionine-free medium. A fraction (2.5%) of the material was examined by SDS–PAGE (Fig. 5). In SFVdpG-CAT-infected cells, VSV G and CAT were the major proteins synthesized at this time (lanes 2–4), and in SFV-G-infected cells VSV G predominated (lane 5). In addition to giving high levels of specific protein expression, infection with 300 μl of sonicated material (lane 3) was sufficient to infect all of the cells in a culture, 80–90% of which expressed the CAT protein (determined by immunofluorescence staining, not shown). Titers of frozen sonicated stocks were approximately 1×10^5 infectious units/ml as determined by end point dilution.

Approximately equal amounts of VSV G protein and CAT were made from the propagating vector, indicating that the second promoter was used efficiently. Quantitation of the amount of protein expression was performed by determining the total amount of protein in a cell lysate (BCA assay; Pierce) and the amount of VSV G protein. VSV G was quantitated by comparing the amount of VSV G in a portion of the SFVdpG-CAT lysate to that in VSV standards on a Western blot (not shown). Forty hours after 10^5 cells were infected with 100 μl of supernatant from sonicated cells, VSV G comprised 1–2% of total

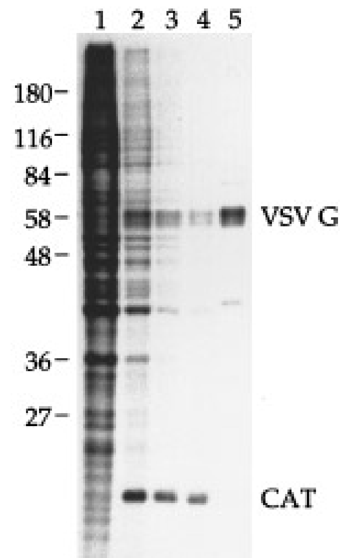

FIG. 5. Identification of CAT and VSV G proteins as the predominant proteins synthesized in cultures of cells infected with SFVdpG-CAT. BHK cells were mock-infected (lane 1), infected with 100 (lane 2), 300 (lane 3), or 500 (lane 4) μl of supernatant from sonicated SFVdpG-CAT transfected cells or 300 μl (lane 5) of supernatant from SFV-G-infected cells. Two days after infection cells were radiolabeled for 2 hr and analyzed by SDS–PAGE.

cellular protein. Since radiolabeling indicated that VSV G protein and CAT were synthesized in approximately equal amounts (Fig. 5; VSV G and CAT contain an equal number of methionines), CAT must also have made up 1–2% of cellular protein.

The SFVdpG-X vector system described here encodes VSV G protein and contains an additional promoter and cloning site for foreign gene expression. Several features make this expression system useful. First, cells transfected with RNA derived from this vector produce infectious, minimal virus particles that propagate the vector, and protein expression, throughout a culture. Thus, the initial transfection frequency does not need to be high to obtain expression of the foreign gene in all of the cells. Second, infectious virus stocks can be derived by sonicating the transfected cells instead of by using cotransfection with an SFV helper virus (*3*). Because the SFVdpG-X vector system does not rely on a helper RNA to be packaged into infectious particles, the possibility of recombination to produce wild-type SFV is also eliminated in our system, although the titers that we have obtained with the minimal virus vector are considerably lower than those that can be obtained with the SFV helper system. Like the original SFV vectors, the SFVdpG-X vector can be manipulated in its DNA form, avoiding the need to produce recombinant virus vectors by homologous recombination. It also expresses the protein of interest very efficiently, and problems with RNA splicing and transport from the nucleus are avoided because SFV replication and transcription occur exclusively in the cytoplasm.

One drawback of the system is the rapid loss of expression of the foreign gene when vector stocks are propagated. Loss of expression of the additional gene was not unexpected because recombinant Sindbis viruses rapidly lose genes encoding unselected proteins on passaging (*15*). Although the nature of the mutations eliminating expression of the unselected gene have not been examined in detail, analysis of the RNA by Northern blots showed rapid loss of the second RNA, suggesting mutations in or deletions of the second promoter. There may in fact be a strong selection favoring high-level expression of G protein since this would be expected to enhance propagation. Elimination of competition between the G promoter and second promoter for SFV transcriptase and competition between the two RNAs for ribosomes may thus be selected for. Consistent with such a model, we have noted that the G protein expression is increased once expression of the second gene is lost.

The host range of the modified SFV vector is probably the same as SFV-G which includes the commonly used cell lines BHK and CHO. However, not all cell lines tested were readily susceptible to SFV-G infection or supported propagation of the minimal virus (*1*).

The cell surface protein CD4 is expressed efficiently from this vector, indicating that the system (including high-level expression of VSV G) does not interfere with the exocytic pathway. Exogenous proteins are expressed within a few hours after the vector reaches the cell cytoplasm, but cells retain relatively normal morphology for up to 48 hr.

Because of the infectious nature of this minimal virus expression system, it is conceivable that it might be useful for gene expression in animals. All enveloped RNA viruses identified so far have their RNA genomes packaged within nucleocapsids. These minimal viruses lack nucleocapsids and use an inefficient, nonspecific RNA packaging mechanism. In addition, they are attenuated in titer by at least 10^5-fold compared to the parent viruses. It is therefore very unlikely that they will be pathogenic in any animal system. However, they might be capable of expressing foreign proteins for a short time in animals and thus might have applications in vaccine development.

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REFERENCES

1. Rolls, M. M., Webster, P., Balba, N. H., and Rose, J. K., *Cell* **79**, 497–506 (1994).
2. Xiong, C., Levis, R., Shen, P., Schlesinger, S., Rice, C. M., and Huang, H. V., *Science* **243**, 1188–1191 (1989).
3. Liljeström, P., and Garoff, H., *Bio/Technology* **9**, 1356–1361 (1991).
4. Liljeström, P., and Garoff, H., *In* "Current Protocols in Molecular Biology" (P. A. Ausubel, Ed.). Greene Publishing and Wiley–Interscience, New York, 1994.
5. Berglund, P., Sjöberg, M., Garoff, H., Atkins, G. J., Sheahan, B. J., and Liljeström, P., *Bio/Technology* **11**, 916–920 (1993).
6. Florkiewicz, R. Z., and Rose, J. K., *Science* **225**, 721–723 (1984).
7. Riedel, H., Kondor-Koch, C., and Garoff, H., *EMBO J.* **3**, 1477–1483 (1984).
8. Piper, R. C., Slot, J. W., Li, G., Stahl, P. D., and James, D. E., *Methods Cell Biol.* **43**, 55–78 (1994).
9. Suomalainen, M., and Garoff, H., *J. Virol.* **68**, 4879–4889 (1994).
10. Ou, J.-H., Rice, C. M., Dalgarno, L., Strauss, E. G., and Strauss, J. H., *Proc. Natl. Acad. Sci. USA* **79**, 5235–5239 (1982).
11. Raju, R., and Huang, H. V., *J. Virol.* **65**, 2501–2510 (1991).
12. Gorman, C. M., Moffat, L. F., and Howard, B. H., *Mol. Cell. Biol.* **2**, 1044–1051 (1982).
13. Stillman, E. A., Rose, J. K., and Whitt, M. A., *J. Virol.* **69**, 2946–2953 (1995).
14. Shaw, A. S., Amrein, K. S., Hammond, C., Stern, D. F., Sefton, B. M., and Rose, J. K., *Cell* **59**, 627–636 (1989).
15. Pugachev, K. V., Mason, P. W., Shope, R. E., and Frey, T. K., *Virology* **212**, 587–594 (1995).
16. Brown, T., *In* "Current Protocols in Molecular Biology" (F. A. Ausubel, Ed.). Greene Publishing and Wiley–Interscience, New York, 1994.