In Vitro Mutagenesis of a Full-Length cDNA Clone of Semliki Forest Virus: the Small 6,000-Molecular-Weight Membrane Protein Modulates Virus Release

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We report on the construction of a full-length cDNA clone of Semliki Forest virus (SFV). By placing the cDNA under the SP6 promoter, infectious RNA can be produced in vitro and used to transfect cells to initiate virus infection. To achieve efficient transfections, a new protocol for electroporation of RNA was developed. This method gave up to 500-fold improvement over the traditional DEAE-dextran transfection procedure. Since virtually 100% of the cells can be transfected by electroporation, this method is a useful tool for detailed biochemical studies of null mutations of SFV that abolish production of infectious virus particles. We used the cDNA clone of SFV to study what effects a deletion of the 6,000-molecular-weight membrane protein (6K membrane protein) had on virus replication. The small 6K protein is part of the structural precursor molecule (C-p62-6K-E1) of the virus. Our results conclusively show that the 6K protein is not needed for the heterodimerization of the p62 and E1 spike membrane proteins in the endoplasmic reticulum, nor is it needed for their transport out to the cell surface. The absence of the 6K protein did, however, result in a dramatic reduction in virus release, suggesting that the protein exerts its function late in the assembly pathway, possibly during virus budding.

Enveloped viruses provide useful model systems with which to study membrane assembly, structure, and function. Recent studies with various retroviruses have demonstrated that the capsid protein alone can direct membrane budding without the involvement of spike glycoproteins (14, 23, 44, 45). However, in the case of alphaviruses, it appears to be the virus transmembrane spike proteins which, through their interaction with the nucleocapsid, form the membrane around the particle (48, 50). Our studies focus on the assembly mechanism of the Semliki Forest virus (SFV). This alphavirus has a single-stranded RNA genome of positive polarity, which therefore functions directly as an mRNA. Replication of this 11,442-nucleotide-long capped and polyadenylated RNA starts soon after infection upon the initial translation of the 5' two-thirds of the genomic RNA, producing a polyprotein which is posttranslationally cleaved into four nonstructural proteins (nsP1 to nsP4). These proteins are responsible for the replication of the plus-strand genome into full-length minus strands, which later in infection are copied into new capped plus-strand genomes. The minus strands also serve as templates for the synthesis of a 4,073-nucleotide long subgenomic RNA species which is colinear with the last one-third of the plus-strand genome. All structural proteins of the virus are encoded by this subgenomic RNA (53).

The structural proteins are synthesized as a polyprotein precursor in the order C (capsid)-E3-E2-6K (6,000-molecular-weight protein)-E1 (20, 49). Once the C protein has been synthesized, it folds to act as a protease, cleaving itself off the nascent chain (28, 40). The cleavage reveals an N-terminal signal sequence in the nascent chain which is recognized by the signal recognition particle, targeting the nascent chain-ribosome complex to the endoplasmic reticulum (ER)

membrane (5, 22), where it is cotranslationally translocated and cleaved by signal peptidase to the three membrane proteins p62 (precursor form of E3/E2), 6K, and E1 (19, 34, 39). Via its E3 domain, the p62 protein forms a heterodimer with E1 (37). The p62-E1 oligomers are transported out to the plasma membrane, where via the cytoplasmic domain of p62, they initiate virus budding (21, 41, 56). At a very late (post-Golgi) stage of transport, the p62 protein is cleaved to E3 and E2 (13), the forms that are found in mature virions. This cleavage exposes the entry functions of the spike and allows the membrane fusion potential of E1 to be activated during virus entry into a new cell (30, 35, 36, 43, 57, 61).

While the major role of p62 appears to be in assembly and that of E1 in entry, the function of the 6K protein is still much of an enigma. 6K was originally found associated with microsomal fractions of infected cells (58, 59), and we have recently shown that 6K is anchored in the membrane as a type I membrane protein. Its signal sequence is located in the cytoplasmic C-terminal region of the p62 polypeptide (34). One function for this small membrane protein is to provide the signal sequence for E1 translocation. However, recent results suggest additional roles for 6K. First, although the E1 signal sequence is located within the C-terminal residues of the 6K, this function can also be provided by the 6K signal sequence at the C terminus of the p62 protein as well (34). Therefore, evolution has had no reason to maintain the 6K gene on the sole basis of E1 signal sequence requirement. Second, the 6K protein is palmitylated, and mutation leading to its underacylation modulate virus budding, leading to the formation of multinucleated virus particles (16).

In this work we have studied the role of 6K. We first describe the construction of a full-length cDNA clone of SFV, which can be used as a template for the production of infectious runoff transcripts, and present a method for an extremely efficient RNA transfection system based on electroporation. Using this system, we show that removal of 6K

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from the SFV genome gene region does not interfere with the synthesis or cell surface transport of the other structural proteins but does severely reduce the release of new virus particles on the infected cell surface.

MATERIALS AND METHODS

Virus growth and purification. BHK-21 cells were grown in BHK medium (GIBCO) supplemented with 5% fetal calf serum, 10% tryptose phosphate broth, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and 2 mM glutamine. Ninety percent confluent monolayers were washed once with phosphate-buffered saline (PBS) and infected with SFV in minimal essential medium (MEM) containing 0.2% bovine serum albumin (BSA), 10 mM HEPES, and 2 mM glutamine at a multiplicity of 0.1. Twenty-four hours postinfection, the medium was collected and cell debris was removed by centrifugation at $8,000 \times g$ for 20 min at 4°C. The virus was pelleted from the medium by centrifugation at 26,000 rpm for 1.5 h in an SW28 rotor at 4°C. The virus was resuspended in Tris-HCl (pH 7.4)–100 mM NaCl–0.5 mM EDTA.

Metabolic labeling and immunoprecipitation. Confluent monolayers of BHK cells grown in MEM supplemented with 10 mM HEPES, 2 mM glutamine, 0.2% BSA, penicillin (100 IU/ml), and streptomycin (100 µg/ml) were infected at a multiplicity of 50 at 37°C. At 1 h postinfection, the medium was replaced with fresh medium and growth continued for 3.5 h. The medium was removed, and the cells were washed once with PBS and overlaid with methionine-free MEM containing 10 mM HEPES and 2 mM glutamine. After 30 min at 37°C, the medium was replaced with the same medium containing [³⁵S]methionine (100 µCi/ml; Amersham), and the plates were incubated for 10 min at 37°C. The cells were washed twice with labeling medium containing a 10-fold excess of methionine and then incubated in same medium for various times. The plates were put on ice, the cells were washed once with ice-cold PBS, and finally lysis buffer (1% Nonidet P-40, 50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 2 mM EDTA, 10 µg of phenylmethylsulfonyl fluoride per ml) was added. Cells were scraped off the plates, and nuclei were removed by centrifugation at 6,000 rpm for 5 min at 4°C in an Eppendorf centrifuge. Immunoprecipitation of proteins with monoclonal antibodies (4) was performed as described previously (57) except that Pansorbin (Calbiochem, La Jolla, Calif.) was used instead of protein A-Sepharose.

Surface labeling of protein. Infected cells were pulse-labeled and chased for the time periods indicated. Cell monolayers were then washed with ice-cold PBS and subsequently incubated with PBS containing sulfo succinimidyl 6-(biotinamido)hexonate (0.5 mg/ml; Pierce-Tecator Inc., Sollentuna, Sweden) for 30 min at 4°C (6). The reaction was stopped by a chase-wash with PBS containing 50 mM lysine. Cells were collected in lysis buffer, and the biotinylated molecules were precipitated by streptavidin agarose (Sigma Chemical Co., St. Louis, Mo.) by incubation overnight at 4°C. The agarose pellet was first washed as normally was done for immunoprecipitates and then once with 1% sodium dodecyl sulfate (SDS). Finally, the samples were prepared for SDS-polyacrylamide gel electrophoresis (PAGE) by heating in SDS sample buffer to 95°C for 10 min.

Electron microscopy. For negative staining of virus particles, virus was collected from the growth medium by sedimentation through a 3-ml 20% sucrose cushion in TNE (50 mM Tris-HCl [pH 7.4], 100 mM NaCl, 1 mM EDTA) in an SW28 rotor at 26,000 rpm for 90 min at 4°C. The supernatant

was removed, and the virus was resuspended in a small volume of TNE overnight on ice. The virus suspension was stained on carbon-coated grids for 10 s with 1% uranyl acetate before analysis by electron microscopy.

General DNA procedures. All basic DNA procedures were done essentially as described previously (47). DNA fragments were isolated from agarose gels by the freeze-thaw method (3), with inclusion of 3 volumes of phenol during the freezing step to increase yield and purity. Fragments were purified by benzoylnaphthoyl-DEAE-cellulose chromatography (52). Plasmids used for production of infectious RNA were purified by sedimentation through 1 M NaCl followed by banding in CsCl (47).

Construction of a full-length SFV cDNA clone. Our strategy for constructing the SFV clone was to prime cDNA synthesis on several positions along the template RNA downstream of suitable restriction endonuclease sites defined by the known nucleotide sequence (18, 55). Virus RNA was isolated by phenol-chloroform extraction from purified virus and used as a template for cDNA synthesis essentially as previously described (27). A full-length cDNA clone of the SFV genome was then constructed by combining independent overlapping subclones, using these sites. After completion of second-strand synthesis, the cDNA was trimmed, the double-stranded adaptor 5'-sticky-*EcoRI*-*HindIII*-*NotI*-*XmaIII*-*SpeI*-blunt-3' was added, and the cDNA was cloned into *EcoRI*-cleaved pTZ18R (Pharmacia, Uppsala, Sweden) as described earlier (29). The 5'-end region was cloned by inserting the cDNA into *SmaI*-*HindIII*-cut pGEM1 (Promega Biotec, Madison, Wis.).

To drive in vitro synthesis of SFV RNA, we used the SP6 promoter carried on a derivative of pGEM1. The first full-length clone candidate, pSP6-SFV3, contained the proper SFV sequence 5'-ATGG, with two additional C residues in front. For the 3'-end of the clone, the cDNA fragment containing 69 A residues was selected. By inclusion of a unique *SpeI* site at the 3' end of the cDNA, the plasmid can be linearized to allow for runoff transcription in vitro.

Site-directed oligonucleotide mutagenesis. For oligonucleotide mutagenesis, relevant fragments of the SFV cDNA clone were subcloned into M13mp18 or M13mp19 (60) and transformed (10) into DH5αF'IQ (GIBCO BRL, Eggenstein, Germany). Replicative-form DNA from these constructs was transformed into RZ1032, and virus was grown in the presence of uridine to incorporate uracil residues into the viral genome. Single-stranded DNA was isolated by phenol extraction from polyethylene glycol-precipitated phage. Phosphorylated oligonucleotides were used in mutagenesis with Sequenase (United States Biochemical Corp., Cleveland, Ohio) as described earlier (32, 54). In vitro-made replicative forms were transformed into DH5αF'IQ, and the resulting phage isolates were analyzed for the presence of correct mutations by dideoxy sequencing according to the U.S. Biochemical protocol for using Sequenase. Deletion of the 6K region has been described elsewhere (34).

In vitro transcription. *SpeI*-linearized plasmid DNA was used as a template for in vitro transcription. RNA was synthesized at 37°C for 1 h in 10- to 50-µl reaction mixtures containing 40 mM Tris-HCl (pH 7.6), 6 mM MgCl₂, 2 mM spermidine-HCl, 5 mM dithiothreitol, 100 µg of nuclease-free BSA per ml, 1 mM each ATP, CTP, and UTP, 500 µM GTP, 1 U of RNasin per µl, and 100 to 500 U of SP6 RNA polymerase (Promega) per ml. For production of capped transcripts (31), the analog m⁷G(5')ppp(5')G or m⁷G(5')ppp(5')A was included in the reaction mixture at 1 mM.

For quantitation of RNA production, trace amounts of [α - 32 P]UTP (Amersham) were included in the reaction mixtures, and incorporation was measured from trichloroacetic acid precipitates.

Typically, about 50 μ g of RNA per μ g of template was obtained with use of 20 U of polymerase, but the yield could be increased considerably by the use of more enzyme. The conditions differ from those reported earlier for the production of infectious transcripts of alphaviruses (12, 46). We found that maximum production of RNA was obtained with ribonucleoside triphosphate concentrations at 1 mM. However, since infectivity also is dependent on the presence of a 5' cap structure (see Table 1), optimal infectivity was obtained when the GTP concentration in the transcription reaction was halved. This drop had only a marginal effect on the amounts of RNA produced but raised the specific infectivity by a factor of 3 (data not shown). We also tested whether an increase in the concentration of cap analog in the transcription reaction (up to 10 mM) would give better specific infectivity, but this was not the case.

RNA transfection. Transfection of BHK monolayer cells by the DEAE-dextran method was done as described previously (46). For transfection by electroporation, RNA was either added directly from the *in vitro* transcription reaction mixture or diluted with transcription buffer containing 5 mM dithiothreitol and 1 Unit of RNasin per ml. Cells were trypsinized, washed once with complete BHK cell medium and once with ice-cold PBS (without $MgCl_2$ and $CaCl_2$), and finally resuspended in PBS to give 10^7 cells per ml. Cells were either used directly or stored (in BHK medium) on ice overnight. For electroporation, 0.5 ml of cells was transferred to a 0.2-cm cuvette (Bio-Rad, Richmond, Calif.), and 10 to 50 μ l of RNA solution was added. Electroporation was performed at room temperature by two consecutive pulses at 1.5 kV and 25 μ F, using a Bio-Rad Gene Pulser apparatus with the pulse controller unit set at maximum resistance. The cells were diluted 1:20 in complete BHK cell medium and transferred onto tissue culture plates. For plaque assays, the electroporated cells were plated together with about 3×10^5 fresh cells per ml, incubated at 37°C for 1.5 h, and then overlaid with 1.8% low-melting-point agarose in complete BHK cell medium. After incubation at 37°C for 48 h, plaques were visualized by staining with neutral red.

Gel electrophoresis. Samples for SDS-PAGE were prepared and run as previously described (9, 11, 34).

RESULTS

In vitro production of infectious RNA. In our first test of pSP6-SFV3 infectivity, *in vitro*-made RNA (cRNA) was transfected into BHK cells by the DEAE-dextran method. However, although the pSP6-SFV3 RNA was of apparent full length and produced virus (data not shown), it had much lower infectivity than did RNA isolated from purified wild-type virus (vRNA) (Table 1). Because of this poor efficiency, we considered the possibility that the three additional non-viral nucleotides GCC, expected to be incorporated into the 5' end of the RNA during transcription, could have a deleterious effect (the G comes from initiation of transcription on the vector part). Therefore, we used oligonucleotide-directed mutagenesis to remove either both C residues or all three GCC residues. The changed fragments were subsequently recloned into pSP6-SFV3, giving pSP6-SFV4 and pSP6-SFV5, respectively.

The three clone derivatives were compared for their efficiencies of promoting RNA production *in vitro*. Since

TABLE 1. Infectivity of SFV RNA

Sample	PFU/ μ g of RNA ^a	
	DEAE-dextran	Electroporation
Virion RNA	1.5×10^4	6.8×10^6
pSP6-SFV3, complete ^b	2	1.0×10^3
pSP6-SFV4, complete	2.8×10^3	1.5×10^6
pSP6-SFV5, complete	ND	1.6×10^6
pSP6-SFV4, no cap analog	ND	2.0×10^4
pSP6-SFV4, DNase I before transcription	ND	0
pSP6-SFV4, DNase I after transcription	ND	1.2×10^6
pSP6-SFV4, RNase A after transcription	ND	0

^a Linearly extrapolated from transfections using 10 to 100 ng of RNA. ND, not determined.

^b Complete, conditions of transcription as outlined in Materials and Methods.

pSP6-SFV5 lacked the G residue at the position of SP6 transcription initiation, transcription reactions with this derivative included the cap analog m⁷G(5')ppp(5')A. The RNA produced was analyzed by agarose gel electrophoresis and was full length in all cases (data not shown). However, transcription of pSP6-SFV4 produced much more RNA than did that of the two other clone derivatives, and quantitation by performing similar transcription reactions in the presence of labeled UTP showed that the transcription levels of pSP6-SFV3 and pSP6-SFV5 were only 16 and 2%, respectively, that of pSP6-SFV4. Thus, the presence of the two C residues immediately downstream of the starting G inhibited efficient transcription in pSP6-SFV3.

Optimization of transfection efficiency. Since it is of utmost importance to be able to perform detailed biochemical analysis of null mutations (i.e., mutations that have abolished virus production), it was crucial to develop a technique that would allow as high RNA transfection efficiencies as possible. If all cells could be transfected, then the viral replication machinery would ensure the same expression level for null mutation as for wild-type infection. Clearly, the use of DEAE-dextran transfection would not be appropriate for such experiments, since optimally only 0.2% of the cells are transfected, which would make analysis difficult because of high background levels from untransfected cells. Therefore, we decided to use the method of electroporation for cell transfection, and experiments were carried out to determine the optimal conditions for RNA transfection in BHK cells by performing plaque titration assays using vRNA as described in Materials and Methods. Optimal transfection close to 100% efficiency was obtained by two consecutive pulses of 1.5 kV at 25 μ F, under which negligible amounts of cells were killed. We found that it was better to keep the cells at room temperature than at 0°C during the procedure. Transfection by electroporation was also measured as a function of input RNA, and about 2 μ g of cRNA was needed to transfect all cells of a sample containing 5×10^6 cells. In comparison, RNA isolated from purified virus was more efficient, needing only 0.5 μ g to reach 100% transfection. Since the 3' poly(A) tail of the cRNA carried four nonviral nucleotides (from the *Spe*I sticky end), the difference could be due to this fact.

Transfection as measured by plaque assays showed that there were qualitative differences in the specific infectivities of the different cRNAs produced. pSP6-SFV4 and pSP6-SFV5 were about equally infectious, whereas the infectivity of pSP6-SFV3 was 1,000-fold lower (Table 1). Since the

FIG. 1. Proteins from SFV-infected cells analyzed by 12% SDS-PAGE. Wild-type genomic RNA (V) or in vitro-made RNA of the wild-type clone (C) or its 6K deletion derivative (Δ) was electroporated into BHK cells and pulse-labeled for 10 min 7 h postelectroporation. Lanes: 1 to 3, lysates after a 10-min chase period; 4 to 6, lysates after a 2-h chase period; 7 to 9, immunoprecipitation with an anti-E1 monoclonal antibody of 10-min-chase lysates; 10 to 12, immunoprecipitation with an anti-E2 monoclonal antibody of 10-min-chase lysates; 13 to 15, immunoprecipitation with anti-E1 of 2-h-chase lysates; 16 to 18, immunoprecipitation with anti-E2 of 2-h-chase lysates; 19 and 20, virus particles pelleted from growth medium after a 2-h chase (note that these lanes have been exposed for a longer period of time to better show the production of the 6K deletion virus). Positions of the 107-kDa, p62, E2, E1, and C proteins are indicated on the left.

infectivities of pSP6-SFV4 and pSP6-SFV5 RNAs were equal but the former allowed much more RNA to be produced in vitro, pSP6-SFV4 was chosen for all subsequent experiments.

Synthesis and assembly of viral proteins when expressed from pSP6-SFV4. To analyze the structural proteins produced, cRNA was electroporated into cells and pulse-chase analysis was performed. vRNA was used as a control. In both cases, transfection resulted in the production of identical structural protein species that had similar transport and assembly properties. After a short pulse and chase, the p62, E1, C, and 6K proteins as well as the 107-kDa species (which represents an uncleaved and unglycosylated protein of p62 plus 6K plus E1) were produced (Fig. 1, lanes 1 and 2; Fig. 2, lanes 1 and 2), a pattern which typically can be seen in SFV-infected cells. Upon a prolonged chase period (120 min), these heterodimers had been transported out of the ER through the trans-Golgi compartment, as visualized by the cleavage of p62 to E2 (and E3, which is not visible on the gel) and by the slight increase in the size of E1 as a result of its sialylation (26) (Fig. 1, lanes 4 and 5). Immunoprecipitation using monoclonal antibodies directed against E1 (Fig. 1, lanes 7, 8, 13, and 14) and E2 (lanes 10, 11, 16, and 17) showed that the p62-E2 and E1 membrane proteins formed heterodimeric complexes. Biotinylation of cell surface proteins showed that pulse-labeled p62 and E1 had reached the surface after a 60-min chase (Fig. 3, lanes 7, 8, 10, and 11) but not after a 10-min chase (lanes 1, 2, 4, and 5). Analysis of the growth medium after a 2-h chase showed that efficient virus formation was taking place with use of cRNA (Fig. 1, lane 29). Thus, the cDNA clone of SFV appeared to function exactly like the vRNA.

6K is not needed for correct production or heterodimerization of p62 and E1. Using a 6K deletion mutation of the 26S cDNA, we have earlier shown that 6K is not needed for in vitro production or membrane translocation of p62 and E1 (34). To determine how the deletion might affect virus assembly events, it was transferred into pSP6-SFV4, and RNA was produced in vitro and electroporated into BHK

cells. We first analyzed the protein synthesis and heterodimerization of p62 and E1 in the ER. Gel analysis of pulse-labeled proteins showed that p62 and E1 were produced, although signal peptidase cleavage between them was not quite as efficient as for the wild type, resulting in a slight accumulation of the 107-kDa species (Fig. 1, lane 3). A portion of this protein had slower mobility, and the larger

FIG. 2. Ten to twenty percent gradient gel analysis of proteins encoded by wild-type (V), clone (C), or deletion 6K (Δ) RNA upon transfection into BHK cells. Cells were pulse-labeled for 10 min and then chased for 2 h. Total lysates are shown.

FIG. 3. Twelve percent SDS-PAGE analysis of surface-labeled SFV membrane proteins. Cells were electroporated with genomic RNA from purified virus (V), clone RNA (C), or deletion 6K RNA (Δ), pulse-labeled with [35 S]methionine for 10 min, and chased for 10 (lanes 1 to 6) or 60 (lanes 7 to 12) min before biotinylation. Lanes: 1 to 3 and 7 to 9, total cell lysates; 4 to 6 and 10 to 12, streptavidin precipitates of biotinylated proteins.

band probably represented molecules that had been partially translocated and thus glycosylated (34). 6K was missing, as expected (Fig. 2, lane 3). Immunoprecipitation of either E1 (Fig. 1, lane 9) or p62 (lane 12) with monoclonal antibodies showed that the 6K deletion had no effect on heterodimerization, since almost quantitative coprecipitation was obtained.

6K is not needed for transport of p62-E1 heterodimers to the cell surface. When the cells expressing the 6K deletion were pulse-labeled and chased for 2 h, p62 was completely cleaved to E2 (and E3) and the apparent size of E1 increased (Fig. 1, lane 6), suggesting that both proteins had been transported from the ER through the Golgi compartment. Coprecipitation of E1 or E2 showed that the complexes were intact and stable even after cleavage of p62 to E2 (Fig. 1, lanes 15 and 18). To prove that the heterodimer had reached the cell surface, we performed cell surface labeling with biotin. Cells were pulse-labeled and then chased for either 10 or 60 min before biotinylation. After the short chase period, no labeled membrane proteins had reached the cell surface and therefore could not react with biotin (Fig. 3, lanes 3 and 6). After the 60-min incubation, when most of the p62 species had been processed (lane 9), both E1 and E2 were efficiently labeled with biotin, showing that both proteins had reached the cell surface (lane 12).

6K is not essential for virus production. To determine whether the 6K deletion construct would allow virus production, we analyzed media from transfected and pulse-labeled cells. After 2 h of chase, virus particles could be detected in the growth medium, but only to about 2% (as quantified from the gel) of the wild-type amount (Fig. 1, lanes 19 and 20). When electroporated cells were assayed for plaque formation, we found no decrease in PFU per microgram of RNA in comparison with the wild type. However, the plaques were clearly smaller, and subsequent virus growth experiments showed that the 6K deletion variant resulted in a marked reduction in virus growth (data not

FIG. 4. Electron micrographs of negatively stained wild-type SFV derived from the cDNA clone (A) and 6K deletion virus (B).

shown). Figure 4 shows an electron microscopic analysis of negatively stained virions. The virus particles derived from the 6K deletion clone derivative had a wild-type appearance.

DISCUSSION

We constructed a cDNA clone of SFV that can be used to produce infectious RNA transcripts *in vitro* by using the SP6 RNA polymerase. The structures of the 5' and 3' ends of the cDNA insert were crucial for both efficient transcription and expression. Transcripts with three extra nonviral nucleotides at the 5' end were substantially lower in specific infectivity than those with only an extra G residue or those without any additional 5' nucleotide. In the case of the closely related Sindbis virus, the presence of eight extra G residues at the 5' end totally abolished infectivity, whereas the presence of only one extra nucleotide at the end had no apparent effect (46). The SFV cRNA was only about 20% as infectious as RNA, probably because of the four nonviral nucleotides present at the 3' end. This finding is consistent with the result with Sindbis virus showing that the 3' end contained nine extra residues, resulting in only 10% infectivity. These were apparently not replicated by the virus replicase and were lost during infection (46).

The most commonly used method of transfecting mammalian cells with nucleic acids has traditionally been by calcium phosphate precipitation (24) or treatment with DEAE-dextran (38). However, these methods suffer from low efficiencies, and therefore new transfection procedures such as the use of liposomes (42) or cationic lipids (2, 15) have been developed. Since most of these techniques are able to transfect only a minor fraction of the cells in a sample, we turned our focus to electroporation, a method that has gained increased popularity for the transfection of DNA, RNA, and proteins into both eukaryotic and prokaryotic cells (references 1, 7, 8, and 51 and references therein). By optimizing the technique, we were able to transfect virtually 100% of the cells, an increase in transfection frequency which in our hands was 500-fold higher than with the DEAE-dextran procedure, which has been used for the transfection of other infectious RNAs of alphaviruses (33, 46). Most important, this method now allows us to perform biochemical experiments with null mutants of the virus (i.e., variants that are unable to produce infectious particles because of a block in the assembly or entry pathways), using the same high expression level as that of wild-type virus. This high expression level and concomitant host shut-off obviate the need for antibody-mediated concentration of viral proteins before SDS-gel analysis.

Also, our expression system is very beneficial for studying viable mutants of SFV since it allows phenotypic analysis after one round of replication. Being an RNA virus, SFV has a high frequency of mutation, and as pointed out by others (25), it is crucial that analysis of mutated genomes be performed very early after transfection without extensive passaging of the virus; otherwise, revertants or second-site mutations may arise, thus interfering with the interpretation of the alleged genotype under study.

As a first application of the cDNA clone to study the assembly of SFV, we introduced a 6K deletion mutation into plasmid pSP6-SFV4. The 6K deletion construction allows the membrane translocation of E1 and internal cleavage from p62 via the remaining signal sequence which resides in the cytoplasmic domain of p62 (34). Our results show that p62 and E1 are synthesized normally and that they complex efficiently with each other as well as become transported to the cell surface. However, a major reduction could be seen in the release of new virus particles, and thus we assume that 6K plays a role at a very late stage of assembly, possibly during the budding process. Recently, Gaedigk-Nitschko and coworkers (16, 17) showed that point mutations of 6K leading to its under palmitoylation also resulted in a substantial reduction in virus release. In contrast to our results, they also showed that the released viruses consisted mainly of aberrantly enveloped particles containing multiple nucleocapsids. One explanation for this phenotype could be that the mutated 6K not only reduced efficiency of budding but also caused interference with the normal envelopment of the nucleocapsid. The same group also showed that small amounts of 6K (8 to 10%) are incorporated into the virus particle. However, the data presented here indicate that 6K is essential for neither the structure nor the infectivity of the virion, since the specific infectivity of the 6K deletion virus equals that of the wild-type virus (data not shown). Work is now directed toward understanding the mechanism by which 6K exerts its function on virus release. We plan to determine the possible associations of 6K with the other virus subcomponents during the maturation process.

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