

Expression of Semliki Forest Virus Proteins from Cloned Complementary DNA. I. The Fusion Activity of the Spike Glycoprotein

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ABSTRACT A complementary (cDNA) molecule encoding the structural proteins of Semliki Forest virus (SFV) has been inserted into a Simian virus 40-derived eucaryotic expression vector lacking introns. Introduction of the recombinant DNA into nuclei of baby hamster kidney cells results in the synthesis of authentic SFV membrane glycoproteins E1 and E2. The glycoproteins are both transported to the cell surface and induce cell-cell fusion after a brief treatment of the cells with low pH medium. The pH dependence of the fusion reaction was the same as that induced by virus particles (White, J., J. Kartenbeck, and A. Helenius, 1980, *J. Cell Biol.*, 89:674–679). Transfection of cells with another recombinant DNA molecule in which the SFV cDNA is engineered into the same expression vector including an intron has been shown before to result in the expression of only the E2 protein on the cell surface, whereas the E1 protein is trapped in the rough endoplasmic reticulum (Kondor-Koch, C., H. Riedel, K. Söderberg, and H. Garoff, 1982, *Proc. Natl. Acad. Sci. USA*, 79:4525–4529). Such cells do not exhibit pH-dependent polykaryon formation, suggesting that the E1 protein is necessary for fusion activity. Immunoblotting experiments show that the RER-trapped E1 protein expressed from the DNA construction with an intron has a smaller apparent molecular weight than authentic E1, and that it has lost its amphipathic characteristics.

The maturation of simple enveloped viruses such as myxo, paramyxo, rhabdo, and alpha viruses involves a series of assembly events through which the viral nucleic acid becomes packaged into a protective coat of protein and lipid (30). In the cytoplasm of the infected cell, the viral nucleic acid is complexed with one or several proteins to form a nucleocapsid. The spike glycoproteins of these viruses are spanning membrane proteins which are made in the rough endoplasmic reticulum (RER) of the host cell and routed to the plasma membrane (PM) via the Golgi complex.¹ At the cell surface the nucleocapsid interacts specifically with the viral spike glycoproteins and is released into the extracellular fluid wrapped in a piece of the host cell PM modified to contain only viral membrane proteins. Clearly, the infection of a cell by an enveloped virus requires that this package of protein and lipid is opened and the viral nucleic acid released into

the cell cytoplasm. The key-role in this process is played by the viral glycoproteins in response to the local pH. Many of the simple enveloped viruses have been shown to enter the cell by receptor-mediated endocytosis from the cell surface into acidic vesicles in the cell cytoplasm (24). The acid milieu triggers a conformational change in the viral glycoproteins such that these mediate fusion between the membrane of the virus and that of the host vesicle (29, 35). This event introduces the nucleocapsid with the viral genome into the cytoplasm of the cell where the replication of the virus can commence.

We are studying the biosynthesis, structure, and function of the Semliki Forest virus (SFV) membrane proteins. The spike glycoproteins of this alpha virus represent a useful model system for eucaryotic plasma membrane proteins (12). Our present approach is to use cloned complementary (cDNA) for expression of the SFV proteins in animal cells. In combination with *in vitro* mutagenesis of the viral protein genes this system offers a unique way of analyzing the assembly and the functions of the virus membrane. As reported in this and the following paper, we investigated two aspects of the SFV

¹ *Abbreviations used in this paper:* HA, hemagglutinin; PM, plasma membrane; SFV, Semliki Forest virus; SV, Simian virus; cDNA, complementary DNA.

membrane proteins: the fusion activity of the spike glycoprotein and, as reported in the accompanying paper, the surface transport of the SFV membrane proteins.

The spike glycoprotein of SFV contains three subunits E1 (438 amino acid residues), E2 (422 amino acid residues), and E3 (66 amino acid residues) (12). E1 and E2 are integral membrane proteins that span the lipid bilayer while E3 is a peripheral membrane protein. In infected cells all the membrane proteins of SFV are translated, together with the nucleocapsid protein, from a 4.1 kilobase (kb) mRNA using a single initiation site. The E3 and the E2 proteins are synthesized as a single precursor protein, p62. This becomes associated in the RER with the E1 glycoprotein, forming an oligomeric complex that is subsequently transported to the cell surface. During this transport the sugar units of the p62-E1 complex are processed to yield their final forms and at a late stage the p62 protein is cleaved to form E2 and E3. The mature E3-E2-E1 spike glycoprotein complex is finally incorporated into the virus envelope during budding at the PM.

The pH-dependent fusion activity of SFV has been demonstrated using several different systems. Below pH 6.0 the virus fuses readily with erythrocytes, with the cell surface of cultured cells, and with liposomes (33, 35–37). However, little is known about how the SFV spike glycoprotein mediates membrane fusion. The fusion activity is inhibited by treatment of the virus with antibodies directed against the E1 protein, but not with anti-E2 or anti-E3 antibodies, suggesting that the E1 subunit plays a crucial role (7, 34). Moreover, the E1 polypeptide contains a hydrophobic region, 16 residues long, situated 80 residues away from its N-terminus (11). This sequence shows a high degree of homology with the corresponding gene region of the Sindbis virus, another alpha virus (27). Such conservation suggests an important function for the encoded peptide, a candidate for which is membrane fusion.

Here we report a system with which to study the structural requirements of the fusion activity of SFV by using expression of the viral proteins in eucaryotic cells from cloned cDNA. A similar approach has been used to study the fusion activity of influenza virus (38). A cloned cDNA molecule, containing the complete coding sequence for all structural proteins of SFV, has been engineered into a Simian virus (SV) 40-derived expression vector lacking introns (pSV S-SFV) and introduced into the nucleus of baby hamster kidney (BHK) cells. This results in the synthesis of authentic E1 and E2 glycoproteins. The glycoproteins are both transported to the cell surface and induce cell-cell fusion after brief treatment of the cells with acidic medium. Previous studies using a vector construction, which contains an intron (pSV 2 SFV), resulted in surface expression of only the E2 protein, the E1 protein remaining associated with the RER (22, 25). We now show that cells transfected with pSV 2-SFV DNA do not exhibit pH-dependent polykaryon formation, indicating that the E1 glycoprotein is indeed necessary for fusion activity. Immunoblotting experiments show that the RER-trapped E1 protein, expressed from the construction with intron, has a smaller apparent molecular weight than authentic E1 and that it has lost its amphipathic characteristics.

MATERIALS AND METHODS

DNA Constructions: Restriction endonucleases were bought from New England Biolabs (Beverly, MA) and Boehringer Mannheim (Mannheim, W. Germany). The *EcoRI*, *HindIII*, and *BamHI* endonucleases were gifts from

V. Pirrotta (European Molecular Biology Laboratory). T4 DNA ligase was a gift from F. Winkler (European Molecular Biology Laboratory). Low gelling temperature agarose was purchased from Bio-Rad Laboratories (Richmond, CA).

The constructions of both pSV S-SFV d-1 and pSV S-SFV involved the ligation of three DNA fragments. The fragments were obtained by restriction endonuclease digestions of various plasmids at conditions recommended by the manufacturers. The digests were run on a 0.8% low gelling temperature agarose gel containing 40 mM Tris, pH 7.8, 5 mM sodium acetate, 1 mM EDTA, and ethidium bromide 0.5 µg/ml. Appropriate bands were identified and cut out under UV light. The agarose was melted by adding 4 vol of water, heating to 68°C for 5 min, and vortexing in between. DNA fragments (~0.2–0.5 µg) to be joined were mixed together and the buffer was adjusted to 50 mM Tris, pH 7.6, 10 mM MgCl₂, and 10 mM dithiothreitol. 1 U of T4 DNA ligase was added per microgram of DNA and the mixture was incubated for the first 2 h at 37°C and then for 10 h at 25°C. The DNA was precipitated together with the agarose in ethanol and taken up in 50 µl of a buffer containing 10 mM Tris, pH 8.0, and 1 mM EDTA. Heating at 68°C for 2 min with intermittent vortexing was necessary to dissolve the agarose. CaCl₂ was added to 100 mM and the mixture was used to transform *Escherichia coli* strain DH1 following standard procedures (32). Extraction of plasmid DNA from ampicillin-resistant bacteria was carried out as described before (2, 16).

Introduction of DNA into Cell Nuclei: Circular forms of plasmid DNA were introduced into the nuclei of cultured BHK cells using microneedle injection or the calcium phosphate co-precipitation technique. In all experiments we analyzed a transient expression of SFV proteins.

Microneedle injection of DNA was used for the cell-cell fusion studies and for the analysis of the cellular location of SFV membrane proteins. The microinjection technique has been described in detail elsewhere (31). Individual BHK cells in a subconfluent monolayer on a coverslip were injected with a glass needle in a paraffin oil chamber using a de Fonbrune micromanipulation set-up. 50–100 cells were injected with DNA at a concentration of 1 µg/µl in each experiment. The coverslip was incubated at 37°C for 1–6 h and then processed for either immunofluorescence or fusion assay.

The precipitation technique was used to obtain a transient expression in a large number of cells, thus yielding enough viral proteins to be detected on a SDS polyacrylamide gel. We followed essentially the protocol of Graham and van der Eb (14). 24 h before transfection, BHK cells were split 1:20 in medium containing 5% fetal calf serum and plated on 10 cm plastic dishes. 4 h before transfection the medium was changed to a fresh one containing 10% fetal calf serum. 10 µg of plasmid DNA were used to transfect the cells in one dish (~1 × 10⁶ cells). The DNA was sterilized by ethanol precipitation, dried in a laminar flow hood, and taken up in 500 µl of a buffer, pH 7.05, containing HEPES 5 g/liter, NaCl 8 g/liter, KCl 0.37 g/liter, Na₂HPO₄·H₂O 0.125 g/liter, and dextrose 1 g/liter. 31 µl of 2 M CaCl₂ were added slowly, under constant agitation, and the mixture was then incubated at room temperature for 45 min for the CaPO₄-DNA precipitate to form. The medium of a dish with cells was replaced with the solution containing the DNA precipitate and left for 20 min at room temperature. Care was taken to distribute the small volume over the whole dish. 10 ml of medium containing 10% FCS were added and the cells incubated at 37°C, first for 8 h and, then, after washing with the medium, for a further 22 h.

In preliminary experiments we followed the time course of the transient expression through immunofluorescence analyses of cells at different times after transfection. SFV proteins were seen 24 h after transfection, reached the highest level of expression at 30 h (~5% of the cells were positive in the immunofluorescence analyses), and were barely detectable at 40 h. We therefore chose a total incubation time of 30 h before processing the transfected cells for the SDS gel analysis. We tried to enhance the efficiencies of transfection through a glycerol or dimethylsulfoxide shock of the transfected cells (26), but without success. On the other hand it proved to be crucial for an efficient transient expression to work with subconfluent cells. The serum proteins were found to make the SDS gel analysis of the cell medium difficult. To circumvent this problem we incubated the transfected cells for the last 6 h in serum-free medium and analysed this for secreted forms of the SFV membrane proteins.

SDS PAGE and Immunoblotting: Altogether, three samples were analysed for each dish of transfected cells: (a) the detergent phase of the cell lysate, (b) the aqueous phase of the lysate, and (c) the serum-free culture medium.

The culture medium was spun for 30 min in a bench-top centrifuge at 0°C to remove cell debris. Proteins were then precipitated with 10% trichloroacetic acid (TCA) and taken up in the sample buffer for the SDS gel electrophoresis (23).

The cells were lysed at 0°C in phosphate-buffered saline containing 0.5% Triton X-114 plus phenylmethylsulphonyl fluoride (Sigma Chemical Co., St. Louis, MO) 40 µg/ml, Trasylol (Bayer, 1:1,000 dilution) and 10 mM iodoacetamide. Nuclei that remained in the cell lysate were removed by a 3-min spin

in an Eppendorf centrifuge at 0°C. The detergent lysate was separated into a detergent phase and an aqueous phase by warming it to 30°C for 3 min (4). Each phase was processed separately for SDS PAGE (23). The detergent droplet was taken up into the SDS gel sample buffer directly, and the proteins in the aqueous phase were first precipitated with TCA.

All samples were run in duplicate on two 10% polyacrylamide gels (23). The separated proteins were blotted onto nitrocellulose filter paper by the procedure of Burnette (6) and viral proteins were detected using rabbit anti-E1 and anti-E2 antibodies and peroxidase-conjugated sheep anti-rabbit IgG as described by Burke et al. (5).

Other Methods: Indirect immunofluorescence analyses of intact and Triton X-100-lysed cells were carried out as described in Ash et al. (1). Cell-cell fusion was induced by treating cells with pH 5.5 medium for 60 s. After an incubation of 1 h in normal medium, the cells were fixed and stained with Giemsa's (37).

RESULTS

Construction of pSV S-SFV d-1 and pSV S-SFV

We previously used the plasmid pSV 2-SFV to express the structural proteins of SFV (22). In this plasmid the SFV cDNA molecule is inserted downstream from the early promoter of SV 40 and upstream from a composite SV 40 fragment encoding the small t intron and the polyadenylation signal for the early transcripts of SV 40. Microinjection of this DNA into BHK cell nuclei resulted in the synthesis of all the structural proteins of SFV. The capsid protein was found in the cell cytoplasm and the E2 protein became transported from the RER to the cell surface as during SFV infection. However, the E1 protein did not appear on the PM, its correct destination, but was seen associated with the RER. One possible explanation for this aberrant behavior is that it resulted from a structural alteration of the E1 polypeptide caused by mis-splicing of its encoding RNA. This could occur if the E1 gene contains a donor site for splicing that is used together with the acceptor site of the small t intron in the vector portion. Regions encoding fortuitous RNA splice signals might occur in the SFV cDNA as it has been reverse transcribed from the 4.1 kb mRNA of SFV that is synthesized in the cell cytoplasm by a viral polymerase. We therefore constructed an expression plasmid that is lacking the region encoding the small t intron of SV 40. The new construct also contains, downstream from the cDNA sequence, an oligonucleotide supplying stop codons in all three reading frames (8). It can therefore be used for the expression of cDNA molecules lacking their own translation terminators.

The new expression plasmid (pSV S-SFV d-1) was constructed by joining together the following three DNA fragments (Fig. 1): (I) the 5,014 bp fragment obtained from a partial *EcoRI* and complete *ClaI* digestion of the plasmid pSV 2-SFV d-1 (see following paper); (II) the smaller *ClaI*-*BamHI* fragment (26 bp) of the plasmid pBR TGA; and (III) the smaller *EcoRI*-*BclI* fragment (988 bp) of SV 40 DNA. Fragment I contains pBR DNA, including the gene for ampicillin resistance and the origin of plasmid replication, the SV 40 early promoter and the 5' portion of the SFV cDNA encompassing the capsid protein gene and a deleted form of the p62 protein gene (d-1 is a designation for the deletion). Fragment II codes for stop codons in all three reading frames in the transcribed RNA molecule. The RNA polyadenylation signal is encoded by fragment III.

Plasmid pSV S-SFV d-1 was used for the construction of pSV S-SFV that contains the coding sequences for all the structural proteins of SFV. A three fragment-ligation was performed (Fig. 1) involving (I), the smaller *PvuI*-*XhoI* fragment (2,954 bp) of pSV S-SFV d-1 containing most of the

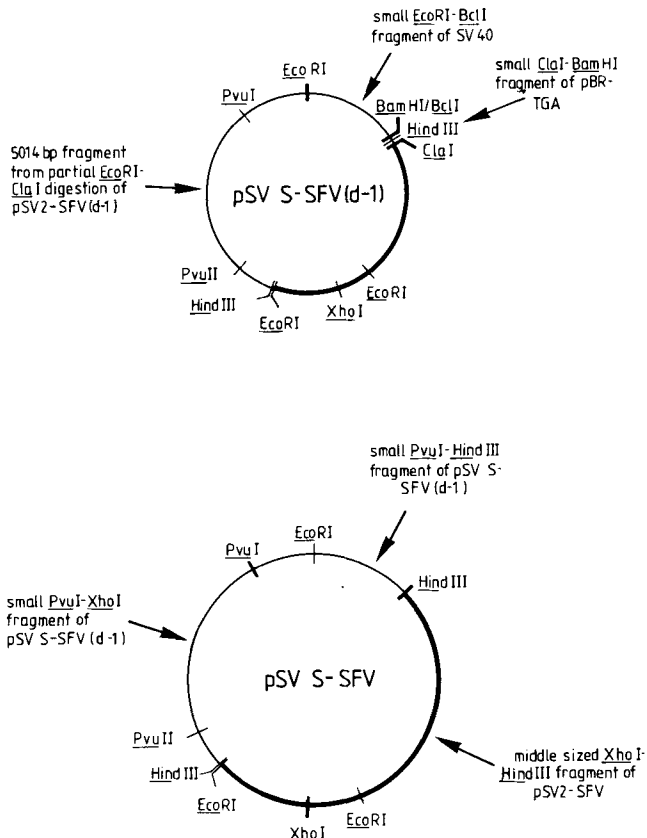


FIGURE 1 Construction scheme for the hybrid plasmids pSV S-SFV d-1 and pSV S-SFV. Both plasmids were engineered from three DNA fragments as indicated by the arrows. The thick bars on the DNA circles represent such restriction endonuclease cleavage sites that have been used in the construction of the hybrid plasmids. The thick parts of the circles indicate sequences encoding SFV proteins (anticlock-wise). Transcription of the SFV sequences is programmed by the SV40 early promoter (between the *PvuII* and the *HindIII* sites). Downstream of the SFV sequences there are the stop translation oligonucleotide and an additional SV 40 fragment encoding a signal for polyadenylation of the mRNA molecule (between the sites for *HindIII* and *EcoRI*).

pBR 322 sequences, the SV 40 early promoter, and the 5' cDNA sequences; (II), the middle-sized *XhoI*-*HindIII* fragment (3,036 bp) of pSV2-SFV encompassing the rest of the SFV cDNA; and (III), the smaller *HindIII*-*PvuI* fragment (1,613 bp) of pSV S-SFV d-1 carrying the oligonucleotide with stop codons, the SV 40 fragment encoding the polyadenylation signal and pBR sequences.

Cellular Location of the SFV Membrane Proteins When Expressed from Plasmid pSV S-SFV

The cellular location of the p62/E2 and E1 proteins expressed from pSV S-SFV was studied by indirect immunofluorescence microscopy. We also analyzed in parallel the cellular location of membrane proteins expressed from pSV 2-SFV. (In many of our analyses we cannot distinguish between E2 and its precursor form p62. In these cases we will use the designation p62/E2 in the text. On the cell surface we assume only E2 protein to be present.) Two pairs of glass slides with subconfluent BHK cells were used for microinjection of both plasmid constructions. After a 6-h incubation at 37°C, one pair of slides was processed for internal and the other pair for

surface immunofluorescence staining of E1 and E2 proteins. Cells which had received pSV 2-SFV DNA expressed the E2 protein at the cell surface as well as in intracellular membranes. The E1 protein as described previously was seen only inside the cell in association with what appeared to be the RER (22). Using pSV S-SFV both E1 and E2 proteins were seen at the cell surface (Fig. 2). The interior of the cell displayed a reticular network within the cytoplasm, a strong perinuclear fluorescence and labeling of the cell margins for both the E1 protein and the E2 protein. These results suggest that both glycoproteins are routed from the RER to the PM via the Golgi complex as during viral infection (15).

Induction of Cell-Cell Fusion by Membrane Glycoproteins Expressed from pSV S-SFV

The fusogenic activity of the viral glycoproteins expressed from our plasmids was tested by observing cell-cell fusion after a brief low pH treatment of microinjected cells. Adjacent BHK cells in a subconfluent monolayer on a glass slide were injected in one (marked) area with the plasmid pSV S-SFV and in another one with pSV 2-SFV. The slide was incubated for 6 h at 37°C to allow expression of viral glycoproteins on the surface of the microinjected cells. After a short (60-s) exposure to medium at pH 5.5, the cells were further incu-

bated for 1 h in normal medium and then analyzed for cell-cell fusion. As shown in Fig. 3, cells injected with the pSV S-SFV DNA and expressing both the E1 and the E2 proteins on their surface fused into a large polykaryon. Those cells microinjected with pSV 2-SFV DNA displaying on their surface only E2 did not fuse with each other. This result shows that cell surface expression of the E1 protein is required for the fusion activity. The pH dependence of the fusion reaction induced by the viral glycoproteins on the cell surface was found to be the same as that induced by virus particles (37). There was a threshold value of pH 6, below which fusion occurred avidly (Table I).

To determine whether the cell-cell fusion could be used as a more sensitive assay for the cell surface expression of the glycoproteins than immunofluorescence, we injected different dilutions of the pSV S-SFV DNA (1, 0.5, 0.1, 0.05, and 0.01 $\mu\text{g}/\mu\text{l}$) into BHK cells on two slides. One of these was processed for surface immunofluorescence, and the other for cell-cell fusion. Clearly, the immunofluorescence assay was more sensitive in detecting viral glycoproteins on the cell surface. Cells injected with DNA at a concentration of 0.01 $\mu\text{g}/\mu\text{l}$ were still weakly positive in the immunofluorescence analyses, whereas cell-cell fusion was observed only in cells injected with the highest three concentrations of DNA. Apparently, a substantial amount of the viral spike glycoproteins is necessary for the cell-cell fusion.

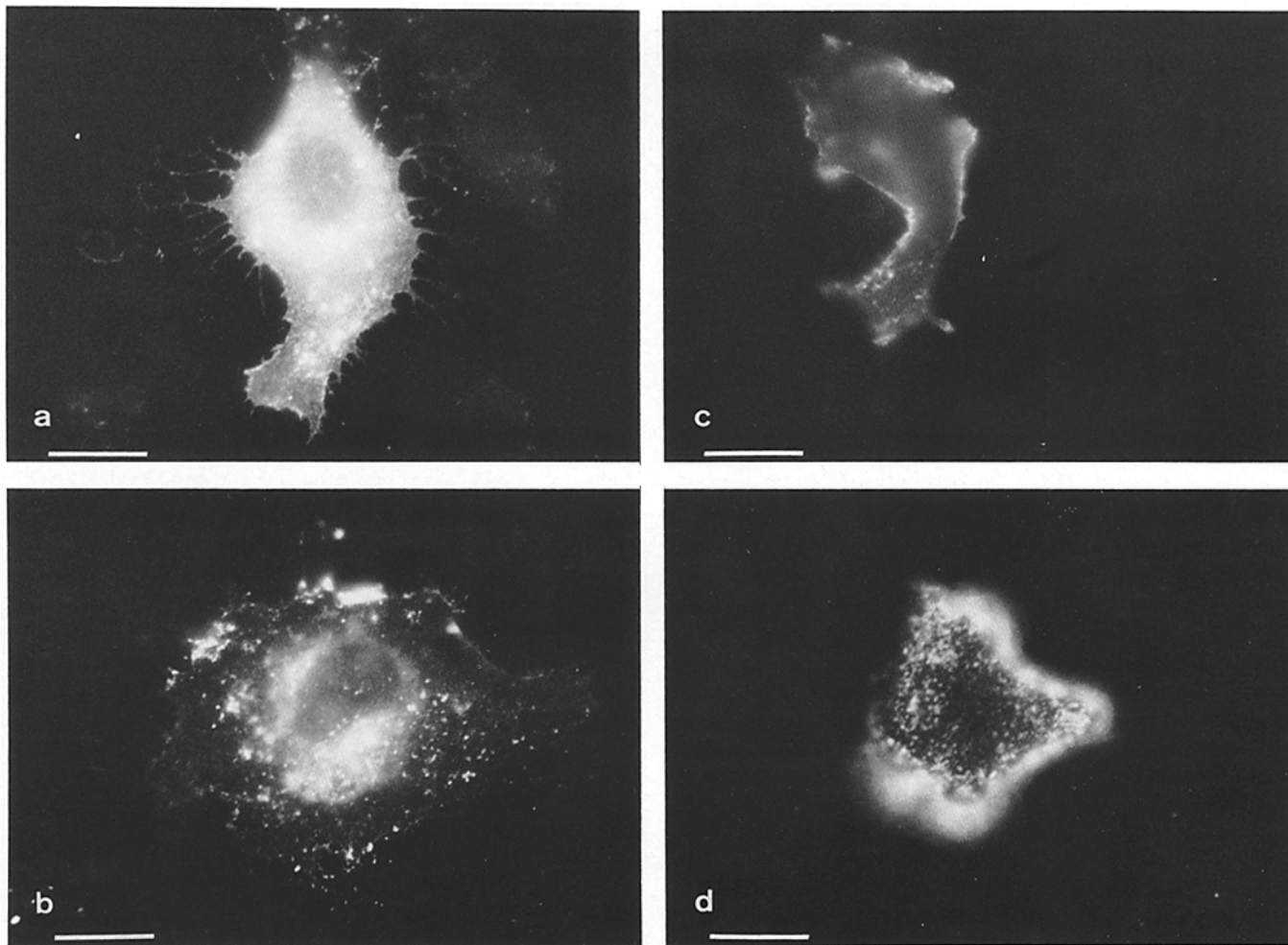


FIGURE 2 Indirect immunofluorescence analysis of the E1 (a and c) and the p62/E2 (b and d) proteins in BHK cells which have been microinjected with pSV S-SFV DNA and incubated at 37°C for 6 h. a and b show internal and c and d surface staining. Bar, 20 μm . $\times 675$.

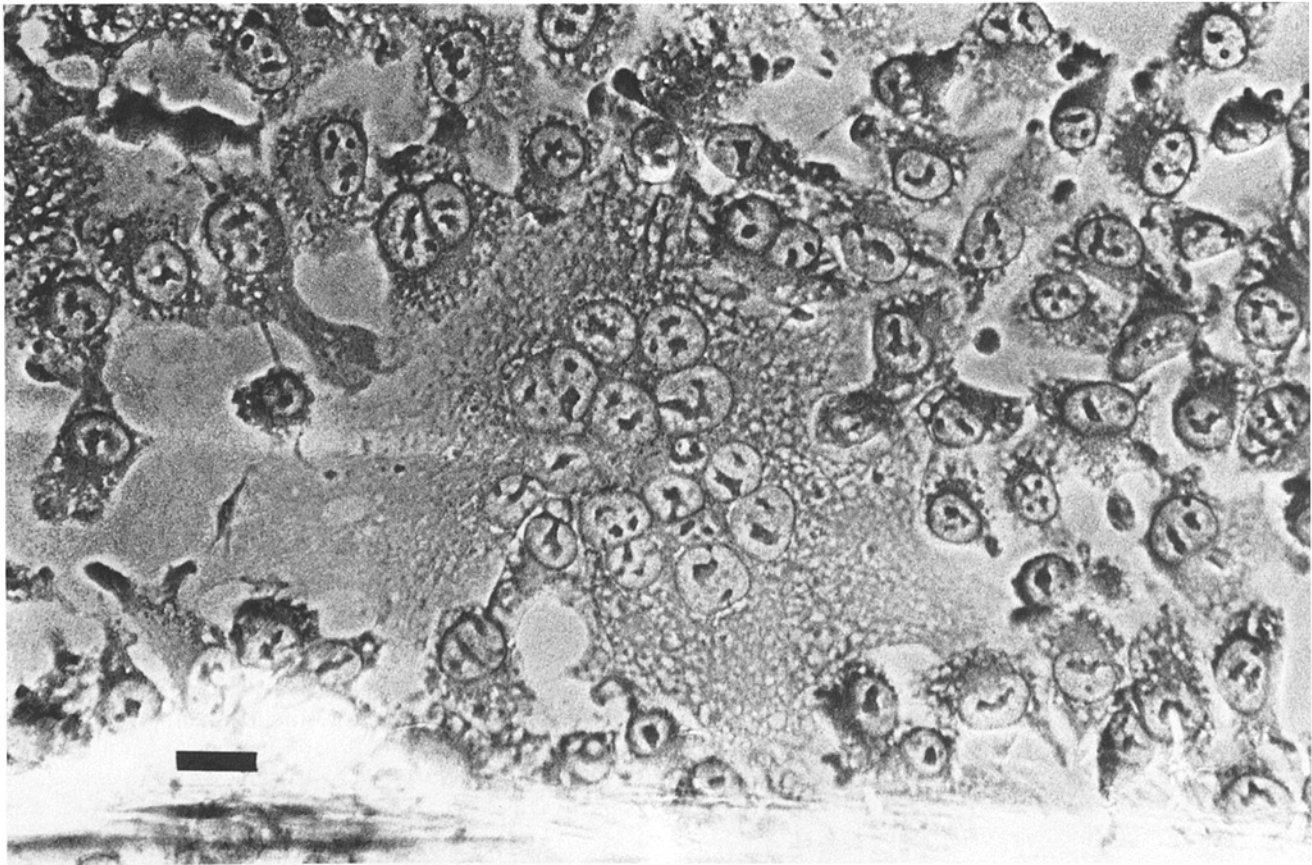


FIGURE 3 Fusion of BHK 21 cells which have been microinjected with pSV S-SFV DNA and express the E1 and E2 proteins on the cell surface. About 50 adjacent cells were microinjected with the DNA, incubated for 6 h, and then treated for 60 s with pH 5.5 medium to induce cell-cell fusion. After further incubation for 60 min the cells were stained with Giemsa's and inspected under the light microscope. The figure shows a polykaryon with 16 nuclei that is located in the microinjected area. Cells outside this area did not show polykaryon formation after low pH treatment. Neither did cells which were injected with the pSV 2-SFV DNA, which results in the expression of only the E2 on the cell surface. Bar, 20 μ m. \times 530.

Physical Characterization of the SFV Membrane Proteins Expressed from Plasmids pSV S-SFV and pSV 2-SFV

So far, our results have shown that both integral membrane proteins of SFV are expressed on the surface of cells that received pSV S-SFV DNA, whereas only E2 can be detected on the PM if pSV 2-SFV DNA is used. Cell-cell fusion can be induced in the first case but not in the latter one. To see whether the membrane proteins expressed from the two vectors were synthesized and processed correctly, we analyzed their apparent molecular weights by SDS PAGE. We were especially interested in the properties of the E1 protein that was expressed from the pSV 2-SFV plasmid and that did not reach the cell surface. We reasoned that this protein could have lost its membrane-spanning segment and therefore included a Triton X-114 extraction step prior to the gel analysis of this and the other samples. It has previously been shown by Bordier (4) that integral membrane proteins, solubilized in solutions of Triton X-114, partition preferentially into the detergent phase rather than the aqueous phase. We also examined the medium of transfected cells for the presence of soluble forms of each of the viral membrane proteins. To maximize both the amounts of expressed proteins and the sensitivity of detection, we used the calcium phosphate precipitation technique for introduction of DNA into cells and

an immunoblotting procedure for detection of the viral membrane proteins.

Two dishes with subconfluent BHK cells were transfected with pSV S-SFV or pSV 2-SFV, respectively. After 24-h incubation at 37°C the medium was changed to one lacking serum and the incubation was continued for a further 6 h. Cells and media were then collected and processed for SDS gel electrophoresis. The media were precipitated with TCA, and the insoluble material was dissolved in SDS gel sample buffer. The cells, on the other hand, were extracted at 0°C in a 0.5% solution of Triton X-114 that was allowed to phase separate at 30°C, thus yielding two samples; a detergent phase and an aqueous phase. For electrophoresis each sample was run in duplicate on separate SDS gels which were subsequently blotted onto nitrocellulose filters. Each filter was then stained either with an E1 specific antiserum (Fig. 4a) or with an E2 antiserum (Fig. 4b). On each gel a Triton X-114 extract from BHK cells infected with SFV was included as a control.

The E1 blot shows single and sharp bands that are easy to interpret; the E2 blot, however, is more problematic. The E2 protein moves as a broad band in SDS gels and is also found in its precursor form p62 (see the control sample in Fig. 4b), which in general appears as a group of closely migrating bands rather than a single one (9, 10). The problematic migration of the p62 and E2 proteins in SDS gels could be caused by this cysteine-rich protein assuming various conformations

TABLE I
Comparison of the pH-dependence for Fusion of BHK-21 Cells Mediated by SFV Particles and Membrane Proteins Expressed from cDNA

pH	Cell fusion mediated		
	By membrane proteins expressed from pSV 2-SFV	By membrane proteins expressed from pSV S-SFV	By SFV particles (36)
7.0	-	-	-
6.5	-	-	-
6.0	-	+	+
5.5	-	+	+
5.0	-	+	+

50 adjacent BHK-21 cells in a subconfluent monolayer on five coverslips were injected with pSV 2-SFV and cells on five others with pSV S-SFV DNA. After incubating for 6 h at 37°C, coverslips with cells from each group were treated with media at pHs ranging from 7.0 to 5.0 for 60 s and then reincubated in normal medium for 1 h. Cell-cell fusion was analyzed in the light microscope after fixation and Giemsa's staining of the cells.

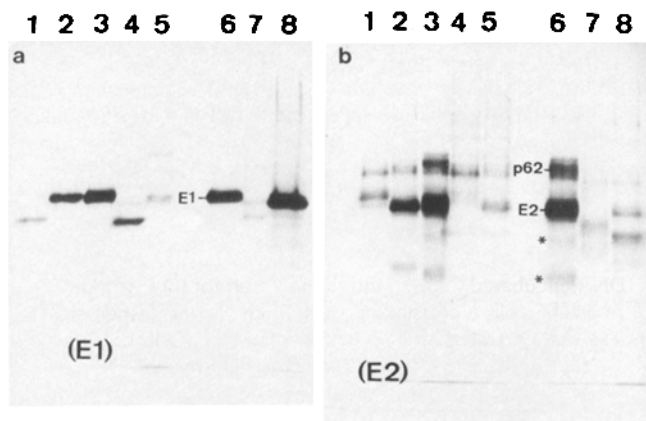


FIGURE 4 Analysis of membrane proteins expressed from pSV S-SFV and pSV 2-SFV by SDS gel electrophoresis. Cells in 10-cm dishes were transfected with pSV 2-SFV and pSV S-SFV DNA. After 30-h incubation at 37°C, samples for SDS gels were prepared as follows: The cells were lysed with Triton X-114 as described in Materials and Methods to yield two samples, a detergent phase and an aqueous phase. TCA-precipitable proteins from the media were analysed directly. 10% of the cell lysate samples and 50% of the medium samples were run in duplicates on two SDS gels. The proteins in the SDS gels were blotted onto nitrocellulose filters, and one of these (a, labeled E1) was stained for E1 proteins using anti E1 antibodies and the other (b, labeled E2) stained for p62/E2 protein using anti E2 antibodies. In each panel, lanes 3 and 6 show the SFV membrane proteins in the lysate of infected cells, lanes 1, 4, and 7 show SFV membrane proteins present in the detergent phase, the aqueous phase and the medium, respectively, of pSV 2-SFV-transfected cells, and lanes 2, 5, and 8 show SFV membrane proteins present in the corresponding three samples of pSV S-SFV-transfected cells. The normal E1 protein is indicated in lane 6 of the (E1) panel and the p62/E2 proteins in lane 6 of the (E2) panel. The stars indicate p62/E2 degradation products. These products increased in quantity with repeated freeze-thawing of the samples.

and/or various degrees of glycosylation in individual molecules (12). N- and C-terminal amino acid analyses have been made of the p62 and E2 proteins, and these indicate homogeneity of the polypeptide chains (3, 18–20).

The E1 membrane protein expressed from the pSV S-SFV

plasmid DNA (Fig. 4a, lane 2) corresponded in apparent molecular weight to that made in the infected cell (Fig. 4a, lane 3), thus suggesting that normal synthesis and processing of E1 glycoprotein occur using pSV S-SFV. This appears to be true for the E2 protein also (Fig. 4b, lane 2). As in the control sample (Fig. 4b, lane 3), most of the p62 protein had been cleaved into E2 and E3 (the latter protein is not shown in the immunoblot). The remaining p62 protein co-migrated with the faster forms of the p62 found in the infected cells. All membrane proteins expressed from pSV S-SFV were predominantly present in the detergent phase of the cell lysate, as is expected for membrane-bound proteins. However, to our surprise a substantial amount of E1 protein was found in the medium of cells transformed with pSV S-SFV (Fig. 4a, lane 8). This E1 protein appeared slightly smaller than the cell-associated E1 molecule (compare lanes 6 and 8 in Fig. 4a). Presumably, it is lost from the cell surface by proteolysis.

The immunoblots of the proteins expressed from pSV 2-SFV showed a pattern of bands quite different from those of the pSV S-SFV and the infected cells. The E1 protein was considerably smaller in size. Furthermore, it appeared predominantly in the aqueous rather than in the detergent phase of the cell lysate (Fig. 4a, lanes 1 and 4). These findings suggest that the E1 protein expressed from pSV 2-SFV has indeed lost its membrane-binding segment. Although this protein appears to be segregated into the lumen of the RER as a hydrophilic product, it is apparently not secreted because we were unable to detect it in the medium (Fig. 4a, lane 7). Three bands were seen using the E2 antiserum (Fig. 4b, lane 1). One of these co-migrated with the faster forms of the p62 protein, while the second migrated with authentic E2. The third and the most heavily labeled band migrated slightly more slowly than authentic E2. Apparently, very little of the p62 protein becomes correctly cleaved into E2, most of it being incorrectly processed into the products migrating slightly more slowly than authentic E2. Most of the anti-E2 reactive material was found in the detergent phase. Altogether, less viral membrane protein was present in cells transfected with pSV 2-SFV than in those transfected with pSV S-SFV.

DISCUSSION

Using the plasmid pSV S-SFV, we expressed the SFV membrane proteins on the surface of BHK cells. These membrane glycoproteins appeared to be synthesized correctly as judged by their apparent molecular weights determined by SDS gel electrophoresis. When treated with low pH medium, the SFV surface glycoproteins induced cell-cell fusion, thus demonstrating that the expressed membrane proteins are also functionally intact. Using this system, it should be possible in future to characterize the structural requirements for the fusion activity of this virus in detail. Earlier studies on the fusion activity of SFV have involved complete virus particles (33, 35–37). Our experiments show that the fusogenic activity can be expressed from glycoproteins that are inserted in the PM of a cell, in the absence of a viral envelope or virus particle. Furthermore, the results obtained with the plasmid pSV 2-SFV that failed to express E1 on the cell surface show unequivocally that this protein is required for fusion activity. This is in agreement with the finding that anti-E1 antibodies inhibit fusion activity of the virus particle (7, 34). However, before this activity can conclusively be allocated to the E1 protein, additional experiments are needed, e.g., expression

of a fusogenic E1 protein on the cell surface in the absence of the E2 protein. Further localisation of this function, e.g., to the putative fusion peptide of E1 (valine 80-cysteine 96), should be possible using *in vitro* mutagenesis of this region and assaying the mutant protein for fusion activity after expressing it on the cell surface. Once the activity is localized to a defined region of a glycopolyptide chain, the fusion activity itself and its low pH requirement can be probed through defined amino acid changes in that region.

The molecular mechanism by which a viral glycoprotein actually promotes membrane fusion has been studied in some detail with the hemagglutinin (HA) molecule of influenza virus. This molecule is made as an HA precursor in the RER of infected cells (21). During its transport to the cell surface, where virus budding takes place, it is cleaved into the subunits HA₁ and HA₂. There are several lines of evidence that suggest that the aminoterminal peptide of the membrane-bound HA₂ subunit has a crucial role in fusion, possibly interacting directly with the target membrane. Firstly, the HA molecule has to be cleaved into its subunits HA₁ and HA₂ to be fusogenic (37, 38). Secondly, the peptide is remarkably hydrophobic and its amino acid sequence shows homology with the corresponding sequence of the membrane-bound F₁ subunit of the Sendai virus fusion protein (13, 28). Thirdly, the cleaved HA molecule undergoes a conformational change at the pH which induces membrane fusion such that it exposes the previously buried amino terminal peptide of HA₂ (29). Further studies of fusion in the HA system will be facilitated by the recent expression of a fusogenic haemagglutinin in an eukaryotic cell from cloned cDNA (38).

Considering the mechanism by which the SFV glycoproteins induce fusion it would be important to find out whether a conformational change similar to that of the HA molecule takes place when the SFV spike protein is treated with low pH medium. Such studies have been hampered by the difficulties of obtaining a soluble spike preparation from SFV. The studies on the low pH-induced changes of the HA molecule were made possible by using the soluble, bromelain released fragment of the HA molecule. This hydrophilic fragment acquired amphipathic properties typical of a membrane-bound protein after acid treatment (29). In this respect it is most interesting that we found large amounts of a soluble form of the E1 protein in the medium of pSV S-SFV-transfected cells. This protein is presumably released from the cell surface by a proteolytic enzyme since we have been unable to detect it within the cytoplasm of the cells. We also found a similar E1 protein in the medium surrounding infected cells (unpublished results). If the E1 protein turns out to be the fusion protein of SFV, then this soluble E1 protein may represent an excellent material for conformational studies.

The pSV S-SFV expression system should also provide the necessary basis for further studies of the synthesis, processing and intracellular transport of the viral membrane proteins as well as their assembly into virus particles. Some investigators have favored the view that the cleavage of p62 into E2 and E3 is closely linked to the budding process (17). Clearly, this cleavage can occur effectively in our expression system where no virus maturation is possible. On the other hand, the pattern of p62 and E2 proteins expressed from the pSV 2-SFV plasmid suggests that p62 has to be complexed with E1 to undergo efficient and correct proteolytic processing.

The use of both the pSV S-SFV and the pSV 2-SFV plasmids for expression of the SFV structural proteins has

shown that one should be cautious in including sequences encoding an intron in the vector molecule. This may be especially true when they are in combination with cDNA sequences obtained from RNA molecules which are replicated in the cell cytoplasm. This is the case for most simple enveloped RNA viruses. Such sequences might contain additional splice signals that, in combination with those in the vector, might lead to RNA splicing within the coding region. Although we have not shown that this is the case for pSV 2-SFV, it does seem to be the most likely explanation for the generation of the hydrophilic and intracellular E1 protein.

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Note Added in Proof: M. Marsh, E. Bolzau, J. White, and A. Helenius (1983, *J. Cell Biol.* 96:455-461) have recently shown that liposomes with SFV spike glycoproteins can fuse with the PM of baby hamster kidney cells at low pH. This provides independent evidence that the fusion activity of SFV is a function of its spike glycoproteins.

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