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SHORT COMMUNICATION

Simian varicella virus open reading frame 63/70 expression is required for efficient virus replication in culture

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Abstract Simian varicella virus (SVV) open reading frame (ORF) 63, duplicated in the virus genome as ORF 70, is homologous to varicella zoster virus ORF 63/70. Transfection of bacterial artificial chromosome clones containing the wild-type SVV genome and mutants with stop codons in ORF 70, in both ORFs 63 and 70 and the repaired virus DNA sequences into Vero cells produced a cytopathic effect (CPE). The onset of CPE was much slower with the double-mutant transfectants (10 days vs. 3 days) and plaques were smaller. While SVV ORF 63 is not required for replication in culture, its expression leads to robust virus replication.

Keywords SVV · BAC · Replication · ORF 63/70

Introduction

Varicella zoster virus (VZV) causes chickenpox (varicella) in humans, becomes latent in sensory ganglia and reactivates decades later to produce shingles (zoster). Reactivation of latent VZV is associated with numerous serious neurological complications (Gilden et al. 2010). VZV ORF 63, which is present as a duplicate copy as ORF 70 on the VZV genome, is the most abundant transcript in latently infected ganglia, and the protein is present in cytoplasm of infected neurons (Cohrs and Gilden 2007; Mahalingam et al. 1996). A recent study of human ganglia latently infected with VZV has shown that the number of neurons expressing VZV ORF 63/70 during latency is much less than previously reported (Zerboni et al. 2010).

Although the exact role of VZV ORF 63/70 protein in the pathogenesis of disease is unclear, it inhibits an α-interferon-induced antiviral response in non-neuronal cells in culture (Ambagala and Cohen 2007), alters the ability of human anti-silencing function 1 protein to bind histones (Ambagala et al. 2009) and inhibits neuronal apoptosis in cultured human ganglia (Hood et al. 2006).

Although VZV does not produce disease in experimentally infected animals, simian varicella virus (SVV) infection of monkeys has served as a useful animal

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model of VZV infection in humans. SVV open reading frame (ORF) 63 is duplicated in the terminal repeat region as ORF 70 and shares 52% amino acid identity with VZV ORF 63/70 (Gray et al. 2001). Like VZV, SVV ORF 63/70 is also transcribed and translated in latently infected ganglia (Mahalingam et al. 1996; Lungu et al. 1998; Kennedy et al. 2000; Messaoudi et al. 2009). Reports regarding the requirement for VZV ORF 63/70 expression in virus replication in culture are conflicting. Sommer et al. (2001) showed a requirement for VZV ORF 63/70 in melanoma cells whereas Cohen et al. (2004) did not; furthermore, replication of the mutant virus was extremely slow. VZV mutants lacking partial or complete segments of ORF 63/70 have also been used to show that the 108 amino acids at the carboxy terminus are important for replication and establishment of ganglionic infection in cotton rats (Cohen et al. 2004, 2005).

The two studies that examined the requirement of VZV ORF 63 for virus replication in culture used overlapping mutant cosmids that spanned the virus genome (Sommer et al. 2001; Cohen et al. 2004). Because varicella ORF 63 is important in latency, we used an SVV BAC (Gray et al. 2011) to construct virus mutants to resolve the important issue of whether varicella ORF 63 expression is required for virus replication.

Results and discussion

Construction of SVV BAC with mutations in ORF 70

Mutant clones containing stop codons in ORF 63, 70, or both coding regions were prepared using a two-step red-mediated mutagenesis to introduce point mutations into the SVV genome (Tischer et al. 2007). We introduced stop codons in SVV ORFs 63 and 70 instead of completely deleting the ORFs because of possible transcription on the opposite strand.

Wild-type SVV BAC containing sequences encoding green-fluorescent protein (GFP) driven by the cytomegalovirus (CMV) immediate-early promoter on the vector was prepared as described (Gray et al. 2011) and electroporated into *Escherichia coli* GS1783. Chloramphenicol-resistant colonies were used for large-scale BAC DNA preparation with the Qiagen DNA midiprep kit (Qiagen, Valencia, CA, USA).

Three in-frame stop codons (TAG) were introduced within SVV ORF 63/70 sequences at amino acid positions 1, 2, and 8 (Table 1) using two-step red-mediated mutagenesis (Tischer et al. 2006) and oligonucleotide primers containing SVV ORF 63/70-specific sequences at the 5′-end and kanamycin-specific sequences at the 3′-end. PCR products generated using primers BK222 and BK223 and the recombinant plasmid pEP-kanS were used to

Table 1 Oligonucleotide primers used for mutagenesis, PCR, and sequencing

Name	Sequence 5′ to 3′[a]
Mutagenesis	
BK222	CCATCTGAATGTTACGTACATAAATAAAACGCTTCTCA**TAGT**AGGCGCCCCGAGATGAA**TA**GACGCCACATGTAGGGATAACAGGGTAATCGATTT
BK223	CCAACCCCAAGCTGTACACATCGATGTGGCGTC**TA**TTCATCTCGGGGCGCCTA**CTA**TGAGAGCCAGTGTTACAACCAATTAACC
BK310	CCATCTGAATGTTACGTACATAAATAAAACGCTTCTCAA**TG**CAGGCGCCCCGAGATGAAA**TG**ACGCTAGGGATAACAGGGTAATCGATTT
BK311	CCAACCCCAAGCTGTACACATCGATGTGGCGTC**AT**TTCATCTCGGGGCGCCT**GCAT**TGAGAAGCCAGTGTTACAACCAATTAACC
PCR[b]	
P1	TGGTCCCCTAACCCCATAAATT
P2	ATAGTGAGGCCGTAGCGTTG
P3	CCATCATCGCCCAATGTTAC
Sequencing	
P4	GGTCCATGTTCCAGGTCATT

[a] Mutations in the SVV ORF 63/70 coding region are bolded; kanamycin-specific sequences are underlined

[b] Primers P1, P2, P3, and P4 are located between nucleotides 110,355 and 110,376, 119,194 and 119,173, 117,082, and 117,101, and 110,600 and 110,618 on the SVV genome (GenBank Accession Number NC 002686)

transform *E. coli* GS1783 containing wild-type SVV BAC. Kanamycin-resistant colonies were selected, and recombinant BAC DNA was extracted and analyzed by *Hind* III digestion and agarose gel electrophoresis. Because the kanamycin cassette insert contains a unique *Hind* III site and because SVV ORF 70 is located within the 17.1-kb *Hind* III fragment, the 17.1-kb fragment (Fig. 1, open triangle) is absent, and additional 13.5 and 4.5-kbp fragments are seen after recombination (Fig. 1, hatched triangles), confirming insertion of the kanamycin cassette at the correct location. After elimination of the kanamycin cassette, the *Hind* III-digestion pattern of the resulting recombinant BAC DNA was identical to that of wild-type SVV BAC (Fig. 1b, lanes 1 and 2). Sequence analysis of PCR fragments generated using the mutant SVV BAC (d70) with primers P1 and P2 (Table 1) for SVV ORF 63, and with primers P1 and P3 for SVV ORF 70 showed that the ORF 70 coding segment contained the three stop codons, while SVV ORF 63 contained wild-type sequences. Thus SVV ORFs 63 and 70- specific sequences were separately PCR-amplified and sequenced.

Construction of SVV BAC with mutations in ORFs 63 and 70

To introduce the stop codon in SVV ORF 63, a single colony of *E. coli* GS1783 containing the SVV mutant d70 BAC, confirmed by sequencing, was transformed again with the same PCR fragment described above. Kanamycin-resistant colonies were selected and recombinant BAC DNA was analyzed by digestion with *Hind* III. Insertion of the kanamycin cassette into SVV ORF 63 was confirmed by the appearance of additional bands at 8.8 and 4.2 kb (closed triangles in Fig. 1c, lane 2). After elimination of the kanamycin cassette, the *Hind* III-digestion pattern of the resulting recombinant BAC DNA was identical to that of wild-type SVV BAC (Fig. 1c, lanes 3 and 4). Sequence analysis of SVV ORF 63- and 70-specific sequences in the

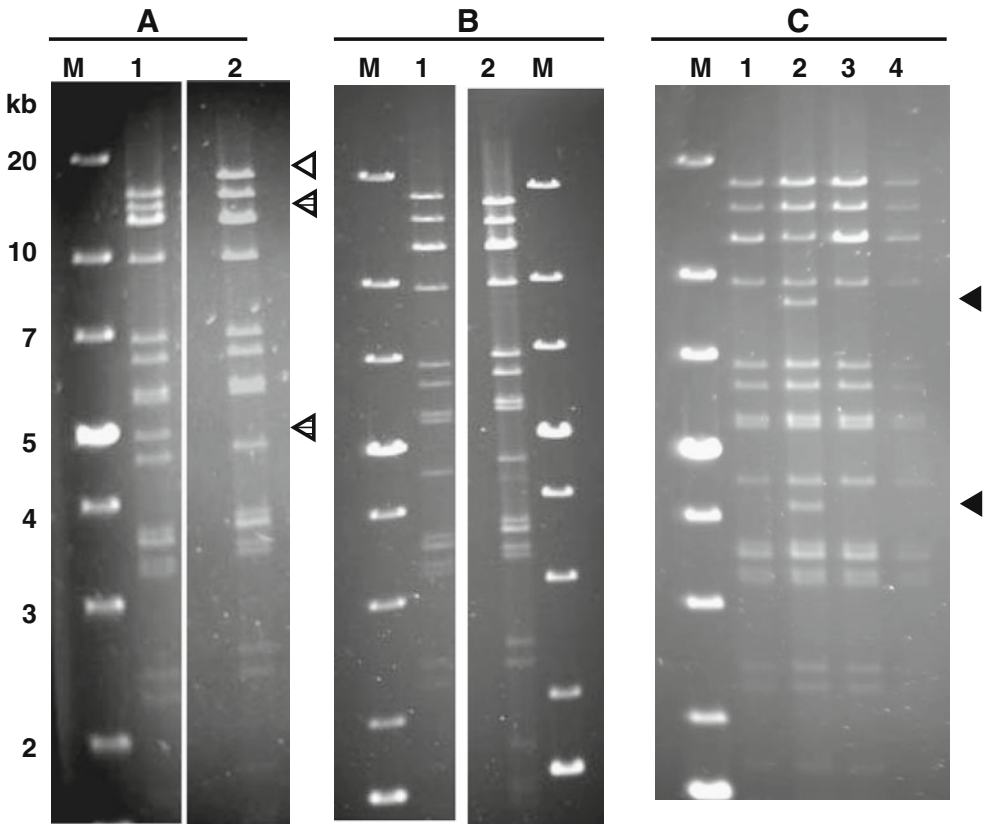

Fig. 1 Sequential insertion of stop codons in SVV ORFs 70 and 63. Agarose gel electrophoresis of *Hind* III-digested kanamycin-resistant (lanes **a***1* and **c***2*), kanamycin-sensitive (lanes **b***1* and **c***3*) and wild-type SVV (lanes **a***2*, **b***2*, and **c***4*) BACs. The disappearance of the 17.1-kbp band in lane **A***1* (*open triangle*) and appearance of 13.5 and 4.5-kbp bands (*hatched triangles*) confirmed insertion of the kanamycin-resistance gene into SVV ORF 70. The hypermolar bands seen around 12 kbp in both lanes **a***1* and **a***2* is due to the presence of two comigrating HindIII fragments that are 12.1 and 12.0 kbp in size. Hind III-digested clones of SVV BACs with mutated ORF 70 before (lane **c***1*) and after (lane **c***2*) insertion of kanamycin cassette into ORF 63 and after removal of the kanamycin cassette (lane **c***3*) are shown. Closed triangles indicate the appearance of bands at 8.8 and 4.2 kb after insertion of the kanamycin cassette. Lanes "*M*" indicate molecular size markers

BAC after PCR amplification showed that both ORFs 63 and 70 contained at least one in-frame stop codon, confirming the generation of an SVV double-mutant BAC (d63/70).

Construction of revertant bacmid

To additionally confirm the absence of additional mutations inadvertently introduced in another region of the SVV genome, a single colony of *E. coli* GS1783 containing the SVV mutant d63/70 BAC confirmed by sequencing was transformed using PCR fragments generated with primers BK310 and BK311 (containing wild-type ORF 63/70 sequences linked to the Kanamycin sequence; Table 1). After electroporation and selection of Kanamycin-resistant colonies, the recombinant DNA was analyzed by digestion with *Hind* III. As described above, the appearance of additional bands at 8.8 and 4.2 kb (closed triangles in Fig. 2a, lane 2) confirmed insertion of the kanamycin cassette into SVV ORF 63. After elimination of the kanamycin cassette, the *Hind* III-digestion pattern of the resulting recombinant BAC DNA was identical to that of wild-type SVV BAC (Fig. 2b, lanes 1 and 2). Thus, the mutation in ORF 63 in d63/70 BAC was repaired to generate wild-type sequences. Both SVV ORFs 63 and 70 sequences were analyzed as described above to confirm that the mutation in SVV ORF 63 (d63 rev) was repaired.

Production of virus CPE in Vero cells by transfection of wild-type and mutant SVV bacmids

Vero cells were transfected with the wild-type BAC, the d70 mutant, the d63/70 mutant or the d63 rev mutant along with or without a recombinant clone containing SVV ORF 62 (a promiscuous transactivator) driven by the strong CMV promoter (Mahalingam et al. 2006) to increase the efficiency of virus reconstitution. A virus-specific CPE was seen 3 days after transfection using wild-type SVV BAC (with or without SVV ORF 62; Fig. 3c and d) or d70 (data not shown) or d63 rev mutants (with or without SVV ORF 62; Fig. 3g and h), whereas the d63/70 mutant BAC with or without SVV ORF 62, did not produce a CPE until 10 days after transfection (Fig. 3a, b, e, and f). Co-transfection of the d63/70 mutant BAC with a CMV-driven SVV ORF 63 plasmid (Mahalingam et al. 2006) produced a CPE in 3 days (data not shown). The size of virus plaques produced by the d63/70 mutant and wild-type bacmids were 49–141 μm and 271–360 μm at 10 and 3 days after transfection, respectively.

Confirmation of replication of mutant SVV in Vero cells

To confirm that the CPE seen in Vero cells transfected with the d63/70 mutant BAC was due to replicating SVV, the

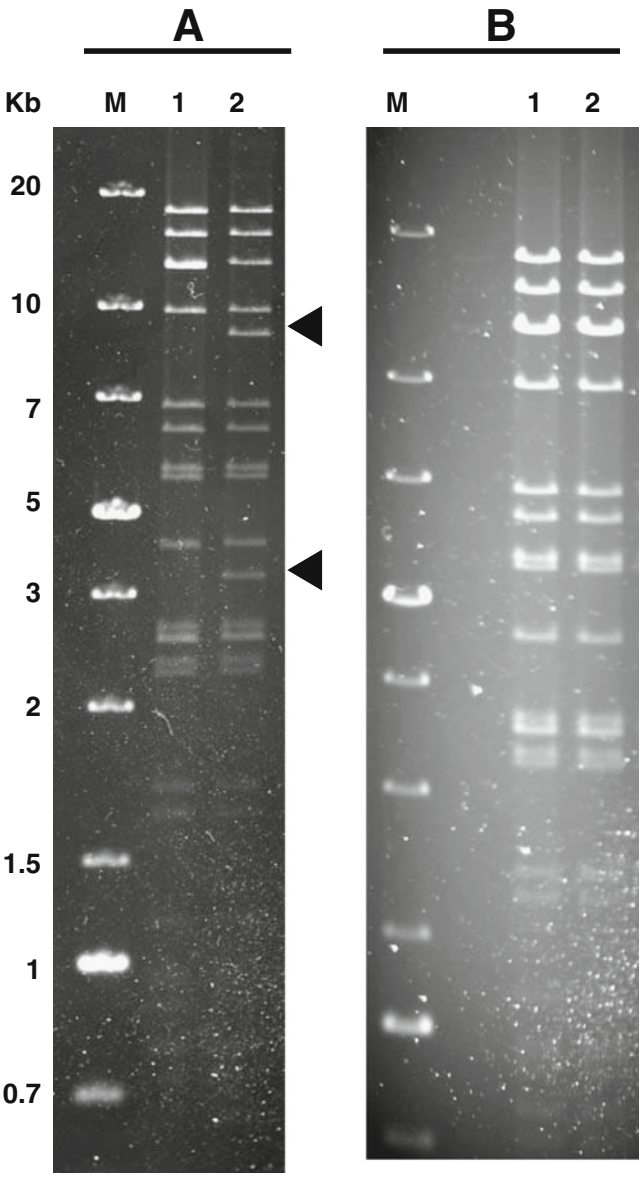

Fig. 2 Removal of stop codons from d63/70 mutant bacmid to generate the revertant bacmid. Agarose gel electrophoresis of HindIII-digested clones of mutant SVV bacmids with double deletion ORFs 63/70 (**a***1*) into which kanamycin-resistant segment along with the wild-type ORF 63 sequences was added (**a***2*). Kanamycin was then deleted as described before and the reverted bacmid was digested with HindIII (**b***1*). The appearance and the disappearance of bands at 8.8 and 4.2 kb SVV ORF63-specific band are indicated by *closed triangles*. Digestion pattern of the wild-type SVV bacmid is used a control (**b***2*). Lanes "*M*" indicate molecular size markers

GFP-positive virus CPE was successively subcultivated three times on Vero cells. DNA and RNA were extracted from cells undergoing a CPE, as described (Owens et al. 2003). PCR amplification of the DNA using SVV-specific primers followed by agarose gel electrophoresis revealed bands of the expected size (data not shown). SVV-specific sequences were readily amplified by one round of PCR using DNA extracted from Vero cells 3 days after transfection with

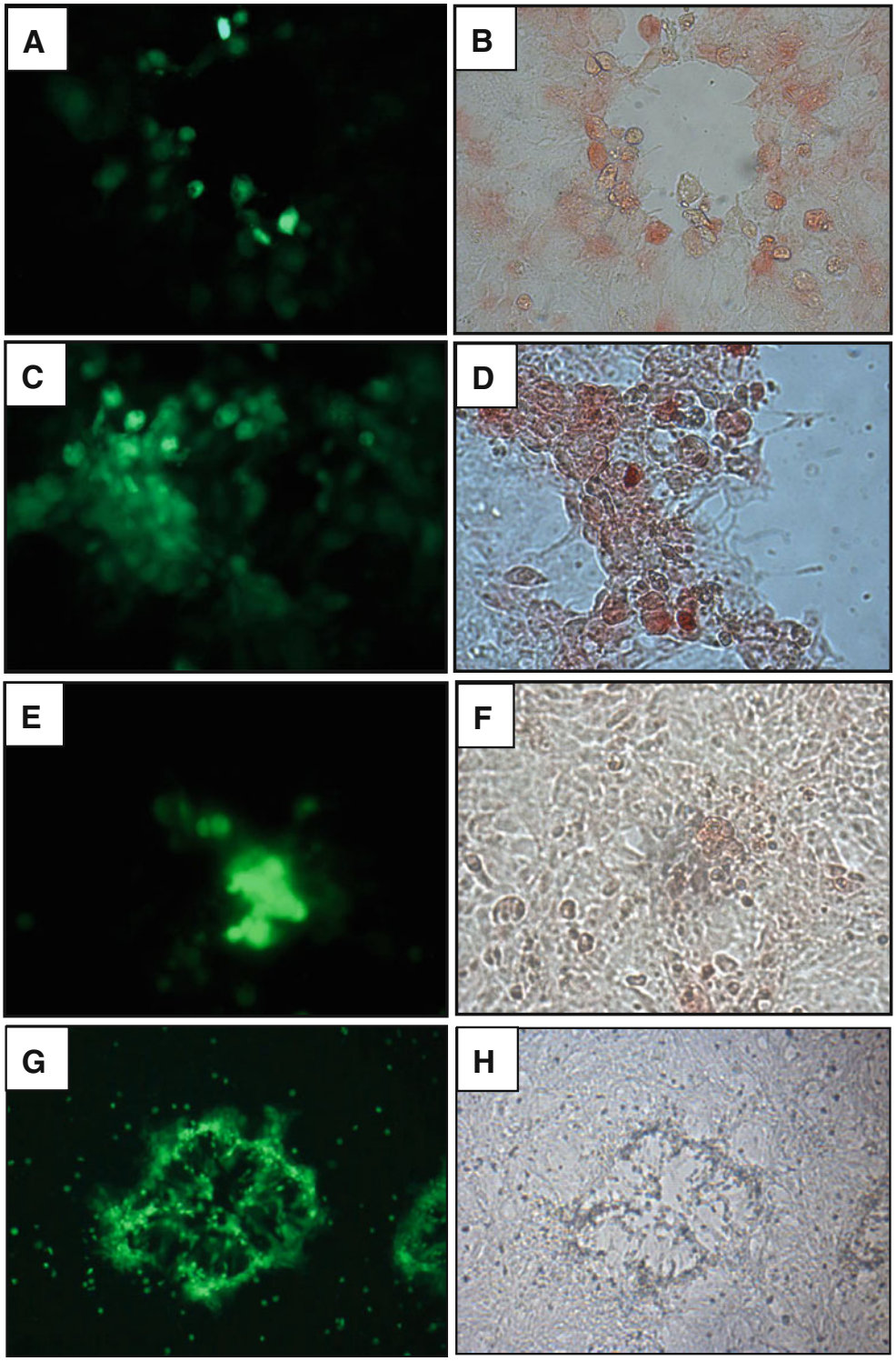

Fig. 3 Analysis of SVV ORF 63/70 expression in cells transfected with d63/70 mutant virus and generation of revertant virus. GFP-associated CPE was visualized in Vero cells transfected with the double-mutant d63/70 BAC (**a** and **e**) or wild-type SVV BAC (**c**) or revertant BAC (**g**). Virus-infected cells were subsequently analyzed by immunohistochemistry using a mixture of rabbit polyclonal antibodies directed against SVV glycoproteins H and L (**b**) or rabbit polyclonal antibodies directed against VZV ORF 63 (**d** and **f**). The detection of SVV glycoproteins in cells infected with the d63/70 mutant confirmed virus replication (**b**). Pronounced expression of SVV ORF 63 was detected in cells transfected with wild-type BAC (**d**) compared to d63/70 mutant transfectants (**f**). A virus CPE detected in Vero cells transfected with the mutant SVV BAC in which ORF 63 mutation was repaired to the wild-type sequence (**h**). Examination of cells by fluorescence (**a**–**g**) and light microscopy (**b**–**h**), respectively, showed that transfection of wild-type and revertant BACs produced an extensive CPE in 3 days (**c**, **d**, **g**, and **h**), while transfection of d63/70 mutant produced rare CPE after 10 days (**a**, **b**, **e** and **f**). Magnification ×400 (**a**–**f**) and ×100 (**g** and **h**)

the wild-type BAC. Whereas DNA extracted from Vero cells 10 days after transfection with the d63/70 mutant BAC required a primary and a nested PCR to be able to detect the same SVV-specific sequences thus confirming the slower replication of the d63/70 mutant virus.

Sequence analysis of PCR fragments specific for SVV ORFs 63 and 70 showed that both ORFs contained stop codons. Real-time RT PCR revealed 93 copies of SVV ORFs 63/70 and 6 copies of SVV ORF 40-specific transcripts per nanogram of GAPdH; no amplification was detected when reverse transcriptase was omitted from the cDNA reaction. The detection of RNA specific for SVV ORF*s* 63/70 indicated that transcription from this region of the virus genome was not affected, and detection

of transcripts specific for SVV ORF 40 (capsid protein) confirmed the presence of replicating SVV.

Vero cells undergoing CPE after transfection with the d63/70 mutant (Fig. 3a and e), with the wild-type BAC (Fig. 3c) or revertant BAC (Fig. 3g) revealed CPE-associated GFP-expression. Subsequent immunohistochemical analysis confirmed the expression of SVV glycoproteins in d63/70 virus infection (Fig. 3b). Analysis of SVV ORF 63 expression indicated an abundant signal with virus generated using wild-type SVV BAC (Fig. 3d) while a negligible amount, if any, of SVV 63 expression was seen with the double deletion mutant 63/70 (Fig. 3f). Even if a small amount of possibly truncated SVV 63 (due to initiation of translation at an internal ATG or due to suppression of stop codon) was present, it was still not sufficient for robust virus replication.

Our results indicate that while SVV ORF 63/70 is not essential for the development of CPE, growth of SVV lacking ORF 63/70 is impaired in Vero cells. These results, obtained using BAC constructs for the first time to examine this requirement question, are consistent with those of Cohen et al. (2004). The d63/70 mutant virus-associated CPE was rare and slow in its rate of growth. Further studies of the role of SVV ORF 63/70 mutants in monkeys await propagation of the mutants to high virus titers in cell lines expressing ORF 63/70.

Materials and methods

Transfection of wild-type SVV BAC into *E. coli* GS1783

Wild-type SVV BAC (100 ng) containing sequences encoding GFP driven by the CMV immediate-early promoter on the vector was prepared as described (Mahalingam et al. 2006) and electroporated into *E. coli* GS1783 (a generous gift from Dr. Gregory Smith). Chloramphenicol-resistant colonies were used for large-scale BAC DNA preparation with the Qiagen DNA midiprep kit (Qiagen).

Viruses and cells

Viruses generated through SVV BACs were propagated by co-cultivation of Vero (African green monkey kidney) cells as described (Gray et al. 2001). Wild-type or mutant viruses were prepared by transfecting Vero cells with SVV bacmids. SVV DNA was extracted from virus-infected cells using the Qiagen DNA extraction kit as per the manufacturer's instructions (Qiagen).

Generation of SVV ORF 63/70 mutants by en passant mutagenesis

Methods described by Tischer et al. (2006) were used to prepare SVV BAC with mutations in ORF*s* 63 and 70. Oligonucleotide primers BK222 and BK223 (Table 1) were designed to mutate wild-type ORF 63/70 ATG, CAG, and ATG to stop codons (TAG). Primers obtained from Integrated DNA Technologies (Coralville, IA, USA) were used in PCR with the template plasmid pEP-kanS. PCR conditions included denaturation at 95°C, annealing at 55–65°C and polymerization at 72°C using Thermo-Start Taq polymerase (Thermo Scientific, Rockford, IL, USA). The amplification product (1,126 bp) was purified by agarose gel electrophoresis and elution using the QIAquick gel extraction kit (Qiagen), electroporated into *E. coli* GS1783 containing wild-type SVV BAC, and kanamycin/chloramphenicol-double-positive colonies were selected. Selected clones were amplified, DNA extracted, digested with *Hind* III and analyzed by (0.7%) agarose gel electrophoresis. The kanamycin cassette was eliminated from the recombinant BAC by red recombination. DNA extracted from kanamycin-sensitive clones was used in PCR with primers P1 and P2 (ORF 63) or P1 and P3 (ORF 70) to generate 2,222- and 2112-base pair (bp) products, respectively, which were gel-purified and sequenced (using primer P4) at the University of Colorado School of Medicine Cancer Center. SVV BAC containing mutated SVV ORF 70 and wild-type ORF 63 (d70) was isolated and processed again as described above to isolate SVV BAC containing mutations in both ORF*s* 63 and 70 (d63/70 mutant).

Generation of SVV ORF 63 revertant BAC

Primers with sequences identical to those of BK222 and BK223 except for the three stop codons that were converted to wild-type codons (primers BK310 and BK311 in Table 1) were used for PCR amplification with pEP-kanS plasmid (Tischer et al. 2006). Amplification products were used in Red recombination with E. coli GS1783 containing the mutant SVV BAC d63/70 as described above to generate an SVV BAC in which the ORF 63 mutation was reverted to wild-type sequence. Sequence analysis confirmed wild-type SVV ORF 63 and mutated ORF 70 sequences in the final SVV ORF 63 revertant BAC DNA. All SVV ORF 63 and 70 mutant and revertant SVV bacmids were confirmed by RFLP and sequence analysis. Table 1 lists the primer sequences.

Reconstitution of SVV from BAC DNA

Vero cells in 60-mm petri dishes (50% to 60% confluent thin monolayers) were transfected with 8 μg of wild-type or mutant SVV BAC DNA, with or without 200 ng of a recombinant DNA clone containing an expression cassette of either SVV ORF 62 or SVV ORF 63 (Mahalingam et al. 2006) using Superfect transfection reagent (Qiagen) as described (Gray and Mahalingam 2005). A recombinant plasmid containing GFP driven by the CMV immediate-early promoter was used as a positive control. A virus-specific cytopathic effect (CPE) was identified by dark-field and fluorescence microscopy. GFP-positive cells associated with virus CPE were carefully transferred to a fresh monolayer of Vero cells, a process repeated three times before DNA extraction using the Qiagen DNeasy kit (Qiagen) or RNA extraction as described (Owens et al. 2003). DNA and reverse transcription-PCR were performed as described (Messaoudi et al. 2009) using primers specific for SVV ORFs 40, 63/70, or *GAPdH*.

Immunohistochemistry

Petri dishes containing GFP-positive virus from the CPE were fixed in 4% paraformaldehyde and analyzed by immunohistochemistry using polyclonal rabbit anti-VZV ORF 63 as described (Mahalingam et al. 1996).

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