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Requirement of Varicella-Zoster Virus Immediate-Early 4 Protein for Viral Replication

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Varicella-zoster virus (VZV) is an alphaherpesvirus that causes two diseases, chickenpox and zoster. VZV open reading frame 4 (ORF4) encodes the immediate-early 4 (IE4) protein, which is conserved among alphaherpesvirus and has transactivation activity in transient transfections. To determine whether the ORF4 gene product is essential for viral replication, we used VZV cosmids to remove ORF4 from the VZV genome. Deleting ORF4 was incompatible with recovery of infectious virus, whereas transfections done by using repaired cosmids with ORF4 inserted at a nonnative site yielded virus. To analyze the functional domain of IE4, we introduced a mutation altering the C-terminal amino acids, KYFKC (K443S), which was designed to disrupt the dimerization of IE4 protein. Transfections with these mutant cosmids yielded no virus, indicating that this KYFKC motif was essential for IE4 function.

Varicella zoster virus (VZV) is a ubiquitous human alphaherpesvirus which causes varicella (chickenpox) during primary infection and herpes zoster (shingles) after its reactivation from sites of latency in sensory ganglia (1, 3). Both varicella and zoster continue to be serious public health problems (1, 11). The expression of VZV genes is regulated in a temporal fashion known as the lytic cascade. The product of open reading frame 4 (ORF4) has been shown to have immediate-early regulatory functions, like immediate-early 62 (IE62) and IE63 (4). The IE4 tegument protein is a 51-kDa phosphoprotein that transactivates genes of all three kinetic classes in transient expression systems and enhances IE62 transactivation (4). In turn, IE62, along with the cellular transcription factor USF, activates the ORF4 promoter (7). IE4 protein resembles its herpes simplex virus (HSV) homolog, ICP27, in its C-terminal residues (38% in amino acids 235 to 449) and complements ICP27 functions in this region, but it does not repair full deletions of the ICP27 gene (8, 9). The equine herpesvirus homolog is UL3 (12). Baudoux et al. have recently mapped IE4 domains in transient expression systems (2), demonstrating that the IE4 transactivating function depends on dimerization mediated by a KYFKC peptide in the C-terminal cysteine-rich region, amino acids 443 to 447. Other mutations in an arginine-rich region of the amino terminus, designated Rb, also reduced transactivation independently of dimerization and may be required for IE4 interactions with cellular proteins, including TATA-binding protein, transcription factor IIB, and the p50 and p65 subunits of NF-κB (5). IE4 also has KH-like motifs that are involved in RNA binding which are found in conserved forms in alphaherpesvirus homologs and are required for HSV replication (13).

The aim of this report was to examine the role of IE4 protein in viral replication by deleting or mutating ORF4 in the context of the viral genome. We showed that ORF4 is an essential gene and that the sequence encoding the C-terminal KYFKC motif in IE4 protein, which mediates dimerization, is required for VZV replication.

Effect of ORF4 deletion on the replication of VZV in melanoma cells. To delete ORF4 from the viral genome, we used a new VZV cosmid system derived from the parent Oka (pOka) virus (10). The complete genome of pOka is contained in four overlapping SuperCos 1 cosmid vectors (Stratagene, Inc.), designated pvFsp73(1-33128), pvSpe14(21796-61868), pvPme2 (53756-96035), and pvSpe23(94055-125123) (Fig. 1, line 2). ORF4 extends from nucleotides 2782 to 4140, located in the cosmid pvFsp73 (Fig. 1, line 3). An 11-kb *Xho*I-*Sac*I DNA fragment from nucleotides 23 to 11436 that contained ORF4 was subcloned into the plasmid vector pLITMUS28 (Invitrogen, Inc.) to generate pLITMUS-Fsp11kb. The 3′-flanking region of the ORF4 gene was amplified from pLITMUS-11kb by using primers 1 and 2 with the introduction of an *Nco*I site (Table 1). The PCR product was ligated into the pCR4-TOPO cloning vector, yielding the plasmid pCR-ORF4C. The sequence of the amplified fragment was confirmed. The plasmids, pLITMUS-Fsp11kb and pCR-ORF4C, were digested with *Pac*I and *Nco*I and were ligated to generate pLITMUS-ΔIE4. Then pLITMUS-ΔIE4 was digested with *Sac*I and *Xho*I, and the resulting 10-kb fragment was ligated with pvFsp73 previously digested with *Sac*I and *Xho*I. The resulting cosmid, pvFspΔ4 (Fig. 1, line 4), was used for transfection. Cotransfection of cosmid clones with full deletions in ORF4 (pvFspΔ4) with pvSpe14, pvPme2, and pvSpe23 yielded no detectable viral plaques. Transfections done with two independently derived pvFspΔ4 cosmids (Table 2) were repeated three times with the same negative result. As a positive control, intact cosmids pvFsp73, pvSpe14, pvPme2, and pvSpe23 were cotransfected in parallel experiments and yielded infectious virus consistently, with plaques visible by 5 to 10 days after transfection (Table 2).

Repair of ORF4 deletion with insertion of ORF4 at a non-

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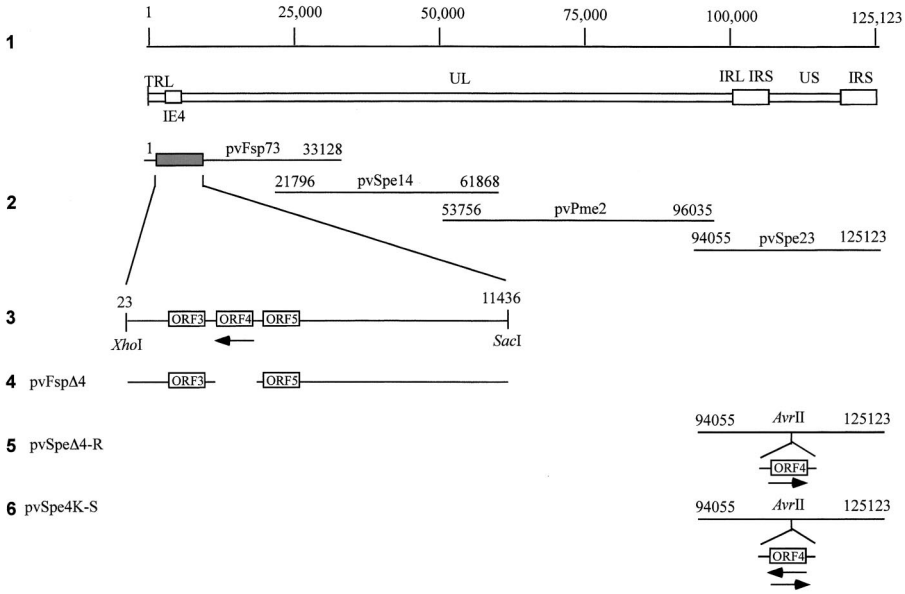

FIG. 1. Schema of cosmid mutagenesis. This diagram illustrates the construction of cosmid vectors with deletion or mutation of VZV ORF4. Line 1, schematic diagram of the VZV genome with the location of the ORF4 gene; line 2, overlapping segments of the VZV genome used to construct the VZV cosmids; line 3, subcloned *Xho*I-*Sac*I fragment from pvFsp73 containing ORF4; line 4, deletion mutant; line 5, insertion of the ORF4 gene and 600 bp of noncoding, putative promoter sequence at the unique *Avr*II site in pvSpe23; line 6, insertion of the ORF4 gene coding for the K443S mutation into pvSpe23.

native *Avr*II site. For the repair of the ORF4 gene, a 1.9-kb fragment containing ORF4 as well as 0.6 kb of noncoding sequence (390 bp upstream) was amplified from pLITMUS-Fsp11kb by using primers 3 and 4 (Table 1) with introduction of an *Avr*II site. The amplified fragment was cloned into the pCR4-TOPO cloning vector, yielding the plasmid pCR-ORF4Avr. The amplified fragment was confirmed by sequencing. Then pCR-ORF4Avr was digested with *Avr*II, and the 1.9-kb fragment was ligated with pvSpe23 previously digested with *Avr*II to generate pvSpeΔ4-R, with the ORF4 gene in the positive orientation (Fig. 1, line 5). Cotransfection using pvSpeΔ4-R, pvSpe14, pvPme2, and pvFspΔ4(1) or pvFspΔ4(2) yielded infectious viruses, which were designated rOkaΔ4-R(1) and rOkaΔ4-R(2) (Table 2). Total DNA from cells infected with these viruses were prepared by using a DNeasy Tissue kit (QIAGEN). As shown in Fig. 2, PCR analysis using primers 7 and 8 (Table 1) of the repaired viruses and cosmids used for the transfection showed the expected 1.4-kb deletion at the endogenous IE4 site (PCR fragment size decreased from 2.5 to 1.1 kb) in rOkaΔ4-R(1) and rOkaΔ4-R(2) viruses and pvFspΔ4(1) and pvFspΔ4(2) cosmids. Another PCR analysis showed the expected 1.9-kb insertion at the *Avr*II site (PCR fragment size increased from 1.1 to 3.0 kb) in rOkaΔ4-R(1) and rOkaΔ4-R(2) and pvSpeΔ4-R cosmid by using primers 9 and 10 (Table 1). These experiments confirm that failure to generate infectious virus by using the pvFspΔ4 cosmids was due specifically to the absence of ORF4 and not to other undetected mutations in the cosmids. The growth kinetics of rOkaΔ4-R(1) and rOkaΔ4-R(2) were determined as described previously (6); this experiment showed that their growth patterns were indistinguishable from intact pOka virus, demonstrating that the location of ORF4 and its putative promoter sequence within the VZV genome did not alter its function (Fig. 3). Plaque morphology of the repaired viruses was also indistinguishable from that of pOka (data not shown).

TABLE 1. Primers used for ORF4 deletion and mutation[a]

Primer	Sequence	Position (nucleotide range)
1	5′-GCGGTATAACTTGTGTAAAC-3′	2356–2375
2	5′-ACCTTTTcCATGgTTGTCAA-3′	2752–2771
3	5′-TCTGGCCTaGGTTGTTGTAT-3′	4502–4521
4	5′-CCCATATATCCCtAggGTCG-3′	2538–2557
5	5′-CGCCCGTCCATACGGTtcATATT TTAAGTG-3′	2801–2830
6	5′-CACTTAAAATATgaACCGTATGG ACGGGCG-3′	2801–2830
7	5′-GACGTCCAAGTCCAATCA-3′	2206–2223
8	5′-CATGCGACGGGATGGTATGA-3′	4691–4710
9	5′-CCACACAAACATCACCTG-3′	116194–116213
10	5′-TTACCACCGCTTCCATCA-3′	117317–117336

[a] Nucleotide numbers refer to those in the nucleotide sequence of the parent Oka strain. Sequence in lowercase indicates introduced mutation.

TABLE 2. Results of transfections

ORF4 mutation in cosmid	No. of transfections	No. positive for infectious virus/no. of transfections	Designation of virus
Intact ORF4	6	6/6	rOka
Δ4(1)	4	0/4	NA[a]
Δ4(2)	4	0/4	NA
Δ4-R(1)	2	1/2	rOkaΔ4-R(1)
Δ4-R(2)	5	4/5	rOkaΔ4-R(2)
4K-S(1)	3	0/3	NA
4K-S(2)	3	0/3	NA

[a] NA, not applicable.

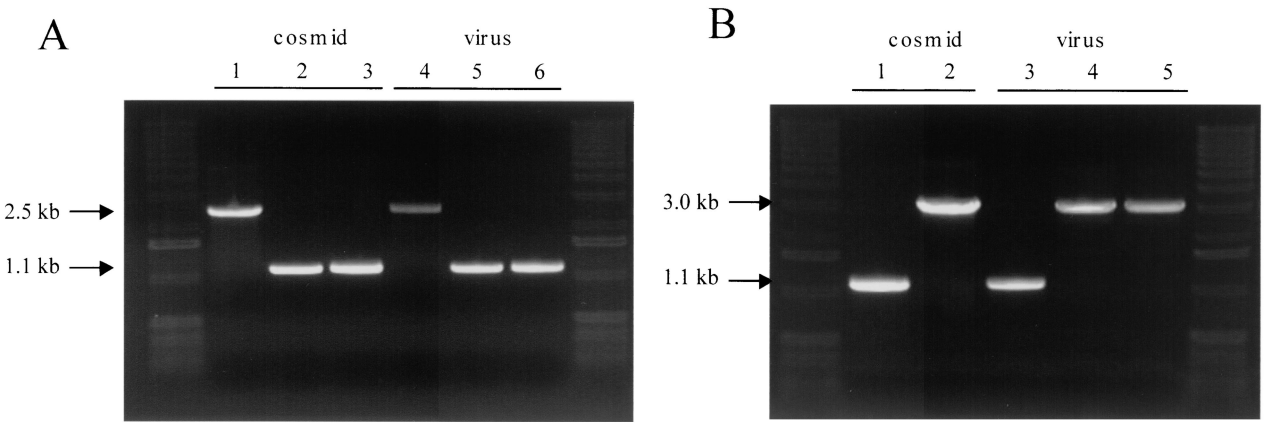

FIG. 2. PCR analysis of VZV DNA from cells infected with ORF4 mutants. (A) PCR products obtained by using primers for deletion of endogenous ORF4. The specimens tested are as follows. Lane 1, pvFsp73; lane 2, pvFspΔ4(1); lane 3, pvFspΔ4(2); lane 4, rOka; lane 5, rOkaΔ4-R(1); lane 6, rOkaΔ4-R(2). (B) PCR products obtained by using primers for insertion of the ORF4 gene at the *Avr*II site. The specimens tested are as follows. Lane 1, pvSpe23; lane 2, pvSpeΔ4-R; lane 3, rOka; lane 4, rOkaΔ4-R(1); lane 5, rOkaΔ4-R(2).

Analysis of the effect of the mutation of the IE4 dimerization site. In transient transfection experiments, mutation of the C-terminal region of IE4 protein was previously shown to disrupt its dimerization and also to disrupt transactivation activity of IE4 protein. To assess the role of dimerization of IE4 protein in the context of the viral genome, the C-terminal KYFKC motif was replaced with SYFKC, which was shown to inhibit dimerization almost completely (2). The point mutation was introduced in the 1.9-kb ORF4 gene fragment in pCR-ORF4Avr by using primers 5 and 6 (Stratagene, Inc.) (Table 1). After the mutation was confirmed by sequencing, the 1.9-kb fragment was inserted at the *Avr*II site in pvSpe23, and the resulting pvSpe4K-S cosmid was used for cotransfection. Cotransfection of cosmid pvSpe4K-S with pvFspΔ4, pvSpe14, and pvPme2 yielded no detectable viral plaques. Transfections done with two independently derived pvSpe4K-S cosmids, which have the mutated ORF4 gene inserted in each orientation at the *Avr*II site (Fig. 1, line 6), were repeated three times with the same negative result, while control rOka virus was consistently generated. This result supported the evidence that the ICP27 of HSV-1 functions as a multimer (14).

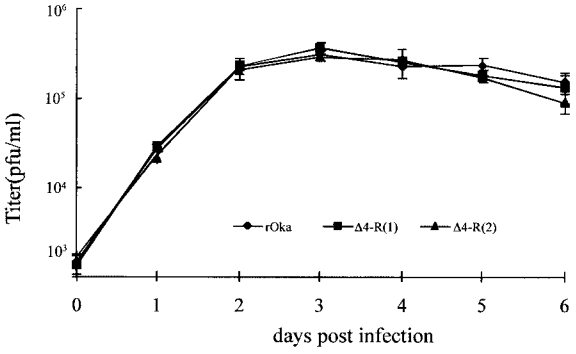

FIG. 3. Replication kinetics of rOka and ORF4 mutant viruses. Virus-infected melanoma cells were seeded onto fresh monolayers of melanoma cells. At days 1 through 6 after infection the infected monolayer was harvested, and the infected cells were serially diluted and used to infect monolayers of melanoma cells in triplicate. At 6 days after infection the melanoma cell monolayers were stained with crystal violet and the number of plaques was counted. The error bars indicate standard deviation.

Summary. This is the first report showing that IE4 protein is essential for VZV replication and demonstrating that the KYFKC motif in the C-terminal region is a critical functional domain in VZV IE4 protein.

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