Mutational Analysis of Open Reading Frames 62 and 71, Encoding the Varicella-Zoster Virus Immediate-Early Transactivating Protein, IE62, and Effects on Replication In Vitro and in Skin Xenografts in the SCID-hu Mouse In Vivo

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The varicella-zoster virus (VZV) genome has unique long (UL) and unique short (US) segments which are flanked by internal repeat (IR) and terminal repeat (TR) sequences. The immediate-early 62 (IE62) protein, encoded by open reading frame 62 (ORF62) and ORF71 in these repeats, is the major VZV transactivating protein. Mutational analyses were done with VZV cosmids generated from parent Oka (pOka), a low-passage clinical isolate, and repair experiments were done with ORF62 from pOka and vaccine Oka (vOka), which is derived from pOka. Transfections using VZV cosmids from which ORF62, ORF71, or the ORF62/71 gene pair was deleted showed that VZV replication required at least one copy of ORF62. The insertion of ORF62 from pOka or vOka into a nonnative site in US allowed VZV replication in cell culture in vitro, although the plaque size and yields of infectious virus were decreased. Targeted mutations in binding sites reported to affect interaction with IE4 protein and a putative ORF9 protein binding site were not lethal. Single deletions of ORF62 or ORF71 from cosmids permitted recovery of infectious virus, but recombination events repaired the defective repeat region in some progeny viruses, as verified by PCR and Southern hybridization. VZV infectivity in skin xenografts in the SCID-hu model required ORF62 expression; mixtures of single-copy recombinant OkaΔ62 (rOkaΔ62) or rOkaΔ71 and repaired rOka generated by recombination of the single-copy deletion mutants were detected in some skin implants. Although insertion of ORF62 into the nonnative site permitted replication in cell culture, ORF62 expression from its native site was necessary for cell-cell spread in differentiated human skin tissues in vivo.

Varicella-zoster virus (VZV) belongs to the alphaherpesvirus subfamily of the Herpesviridae. VZV is the causative agent of varicella, which is characterized by cell-associated viremia and a cutaneous vesicular rash (4). VZV establishes latency in cells within sensory ganglia during primary infection. VZV reactivation from latency results in herpes zoster, a localized skin rash in the distribution of nerves from the affected ganglion. VZV is the first human herpesvirus for which a vaccine has been developed to prevent primary infection (58). This live attenuated varicella vaccine virus (vOka) is the varicella-zoster vaccine virus (vOka).

The VZV genome consists of approximately 125 kb and has at least 70 unique open reading frames (ORFs) (52). As is characteristic of herpesviruses, the double-stranded DNA genome has unique long (UL) and unique short (US) segments which are flanked by internal repeat (IR) and terminal repeat (TR) sequences. Three duplicated genes, ORF62/71, ORF63/69, and ORF64/69, are located in repeats at each end of the US segment. The two VZV origins of replication, designated OriS, are also located in these repeat regions. The immediate-early 62 (IE62) protein, encoded by ORF62 and ORF71, is the major VZV transactivating protein and is the homolog of herpes simplex virus type 1 (HSV-1) ICP4. The IE62 protein induces transcription of all viral genes that have thus far been evaluated (19, 25, 27, 40, 48). The IE63 protein, encoded by ORF63 and ORF70, is also an important VZV regulatory protein which modulates transcription from several genes and has some homology to HSV-1 ICP22 (25).

We have used overlapping VZV cosmids to introduce mutations into the viral genome in order to examine the roles of particular ORFs or nucleotide sequences within VZV genes or their promoters in replication in cell culture in vitro (20, 30, 33, 34, 54). In analyses of ORF63/70 and ORF64/69 gene pairs, one copy of ORF63 was necessary and sufficient for VZV replication, whether it was located at the native site, or introduced into a nonnative site between ORF65 and ORF66 in the US region (54). In contrast, deleting both copies of the ORF64/69 gene pair had no effect on production of infectious virus (54).

When genetic changes were not lethal in vitro, as in the case of the single-copy ORF63 and the dual ORF64/69 deletion mutants, we have evaluated VZV recombinants for infectivity in our SCID-hu mouse model of VZV pathogenesis in vivo (20, 36, 37, 38, 53). Infecting human skin or thymus-liver xenografts in SCID mice permits an assessment of VZV interactions with differentiated human cells located within the intact tissue mi-
croenvironment. VZV replication is not influenced by the host immune response, because the animals are immunodeficient. We have found that experiments in the SCID-hu model are necessary to define the contributions of VZV gene products to pathogenesis, because some VZV proteins, such as glycoprotein I and the ORF47 protein kinase, are dispensable in cell culture but are essential for VZV replication in skin and T cells in vivo (37, 38). Evaluation of the effects of mutating the ORF63/70 and ORF64/69 gene pairs showed that the single-copy ORF63 and the dual ORF64/69 deletion mutants retained typical VZV infectivity in skin xenografts (54).

The third repeated gene pair, ORF62/71, is of particular interest for mutational analysis because it encodes the IE62 protein, which is the primary viral transactivating protein and a component of the VZV virion tegument. The IE62 protein exhibits functional characteristics resembling those of HSV ICP4, which it complements, and has some similarities to HSV VP16 (19, 27, 28, 40, 44, 49, 59). IE62 protein substitutes for ICP4, which it complements, and has some similarities to HSV protein, which is the primary viral transactivating protein and is of interest for mutational analysis because it encodes the IE62 protein. The VZV ORF62 ORF63 and the dual ORF64/69 deletion mutants retained the contributions of VZV gene products to pathogenesis, because some VZV proteins, such as glycoprotein I and the ORF47 protein kinase, are dispensable in cell culture, human skin tissues in vivo.

MATERIALS AND METHODS

Cosmids and plasmids. Four overlapping fragments of genomic DNA from pOka were introduced into SuperCos1 cosmids vectors (Strategene, La Jolla, Calif.) by using methods reported for vOka (22, 30). Deletion of an AvrII site at SuperCos1 nucleotide (nt) 3359 produced a unique AvrII site at VZV nt 112956 (Fig. 1).

Deletions of ORF62, ORF71, and ORF62/71. ORF62 is carried on VZV nt 105164 to 105096, with the gene being in the complementary orientation, located in pSpvec23. Primers were designed to amplify VZV genomic DNA flanking ORF62 by using Elongase enzyme mix (Invitrogen, Inc., Carlsbad, Calif.). These PCR products were then ligated to delete ORF62 before being reintroduced into the cosmids. The 5′ primer 62N was designed to bind to nt 100583 to 100602 and thus to contain an AvrII restriction site (nt 112956) unique in pSpvec23. PCR with these two primers resulted in a 3,869-bp product. This product was digested with Nhel and EcorI and ligated into the pIRE2-EGFP vector (Clontech, Palo Alto, Calif.), which had been digested with the same enzymes. The 5′ primer 71SA was designed to bind to nt 109107 to 109132, with the incorporation of an EcoRI site. The vOka ORF62/63 ORF62/64 ORF62 was designed to delete ORF62 and ORF71 and thus to contain an AvrII restriction site (nt 112956) unique in pSpvec23. PCR with these two primers resulted in a 4,584-bp product. This product was digested with Nhel and EcorI and ligated into the pIRE2-EGFP vector (Clontech, Palo Alto, Calif.), which had been digested with the same enzymes. The ORF71 ORF62 ORF63 ORF71 was digested with Nhel and EcorI and ligated into the pIRE2-EGFP vector (Clontech, Palo Alto, Calif.), which had been digested with the same enzymes. The ORF71 ORF62 ORF63 ORF71 was digested with Nhel and EcorI and ligated into the pIRE2-EGFP vector (Clontech, Palo Alto, Calif.), which had been digested with the same enzymes. The ORF71 ORF62 ORF63 ORF71 was digested with Nhel and EcorI and ligated into the pIRE2-EGFP vector (Clontech, Palo Alto, Calif.), which had been digested with the same enzymes. The ORF71 ORF62 ORF63 ORF71 was digested with Nhel and EcorI and ligated into the pIRE2-EGFP vector (Clontech, Palo Alto, Calif.), which had been digested with the same enzymes.

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FIG. 1. Schema of cosmid mutagenesis. The upper section depicts the VZV IR S-TR S region of the genome containing the coding regions of ORF62 to ORF71, and the unique AvrII site in the US region is indicated. The arrows indicate the orientations of the genes. The nucleotide numbers of the start and stop sites of the ORFs that were deleted (ORF62 and ORF71), as well as the nucleotide numbers of the relevant adjacent ORFs, are given. The designations of the cosmids that are generated are given on the left. Hatched boxes indicate the ORFs that were deleted from the cosmids. The insertions and orientations of vOka ORF62 and pOka ORF62 at the nonnative AvrII site are shown.
proteins were detected with each primary rabbit antibody and secondary goat anti-rabbit immunoglobulin G–horseradish peroxidase conjugate (36).

Infection of SCID-hu skin xenografts. Skin implants were made in homozygous C.B-17 scid/scid mice (35), using human fetal tissues obtained with informed consent according to federal and state regulations. Animal use was in accordance with the Animal Welfare Act and approved by the Stanford University Administrative Panel on Laboratory Animal Care. Eight weeks after implantation, mice were anesthetized and bilateral skin implants were exposed for infection of SCID-hu skin xenografts. Skin implants were made in homozygous C.B-17 scid/scid mice (35), using human fetal tissues obtained with informed consent according to federal and state regulations. Animal use was in accordance with the Animal Welfare Act and approved by the Stanford University Administrative Panel on Laboratory Animal Care. Eight weeks after implantation, mice were anesthetized and bilateral skin implants were exposed for

### RESULTS

Effects of ORF62, ORF71, and dual ORF62/71 deletions. Transfection of the three intact cosmids, pvFsp73, pvSpe14, and pvPme2, and intact pvSpe23 resulted in recovery of recombinant Oka (rOka), with 75% of melanoma cells exhibiting altered morphology. The plaque morphology of rOkaΔORF71 was indistinguishable from that of rOka; rOkaΔORF71 exhibited a smaller plaque phenotype after the initial transfection, but the plaque size became equivalent to that of rOka after passage. In contrast, transfection of pvSpe23ΔORF71 and the intact cosmids yielded no infectious virus in three separate experiments. The presence of the expected mutation in independently derived pvSpe23ΔORF71/71 cosmids was confirmed by PCR, as described below (Fig. 2A and B, lanes 5). Cells were passed for 28 to 30 days to ensure that a virus with a slow-growth phenotype was not missed. These observations suggest that one copy of ORF62 or ORF71 is required for VZV replication in cell culture.

### PCR analysis of mutant cosmids and rOkaΔORF62- and rOkaΔORF71-infected cell DNA. As expected, PCR with primers 1 and 2 (Table 1) to amplify ORF71 generated a 7,468-bp product from pvSpe23 and 3,515-bp products from pvSpe23ΔORF71 and pvSpe23ΔORF62/71 (Fig. 2A). However, PCR of pvSpe23ΔORF62 resulted in a 3,515-bp product as well as the 7,468-bp product. A minor, nonspecific 4,200-bp

### RESULTS

#### Effects of ORF62, ORF71, and dual ORF62/71 deletions.

Transfection of the three intact cosmids, pvFsp73, pvSpe14, and pvPme2, and intact pvSpe23 resulted in recovery of recombinant Oka (rOka), with 75% of melanoma cells exhibiting altered morphology (CPE) within 10 days (Table 2). Parallel transfections in which pvSpe23ΔORF62 or pvSpe23ΔORF71 was substituted for pvSpe23 showed 75% CPE at 17 or 10 days, respectively. Two independently derived VZV recombinants were generated, using separately constructed pvSpe23ΔORF62 or pvSpe23ΔORF71 mutant cosmids, and were designated rOkaΔORF62 and rOkaΔORF71. The plaque morphology of rOkaΔORF71 was indistinguishable from that of rOka; rOkaΔORF71 exhibited a smaller plaque phenotype after the initial transfection, but the plaque size became equivalent to that of rOka after passage. In contrast, transfection of pvSpe23ΔORF71/71 and the intact cosmids yielded no infectious virus in three separate experiments. The presence of the expected mutation in independently derived pvSpe23ΔORF71/71 cosmids was confirmed by PCR, as described below (Fig. 2A and B, lanes 5). Cells were passed for 28 to 30 days to ensure that a virus with a slow-growth phenotype was not missed. These observations suggest that one copy of ORF62 or ORF71 is required for VZV replication in cell culture.

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### TABLE 1. Summary of primers used for cloning and PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>nt in VZV&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>62N</td>
<td>AACGCCTAGCCCCCATGTCATG</td>
<td>100583–100602</td>
</tr>
<tr>
<td>71SA</td>
<td>TGCAGGAAATCCCTTCTTTCTC&lt;sup&gt;c&lt;/sup&gt;</td>
<td>105144–105166, 124937–124959</td>
</tr>
<tr>
<td>71S7</td>
<td>TGAATTGCCAGCAGGACCCGATTCC&lt;sup&gt;c&lt;/sup&gt;</td>
<td>109107–109132, 120971–120996</td>
</tr>
<tr>
<td>62A</td>
<td>TGGATTTGATTGTTCCTAGG</td>
<td>112956–112975</td>
</tr>
<tr>
<td>MARV-L</td>
<td>GAAATTTCTCCAGGTTAAAGGCTTTTC&lt;sup&gt;c&lt;/sup&gt;</td>
<td>NA&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>MARV-U</td>
<td>TTTGCGTTTGCGATGTAGGA</td>
<td>113454–113473</td>
</tr>
<tr>
<td>S245AF</td>
<td>CCGGTCTAGAGGAAAGGGCCCAAGAAAAG</td>
<td>108350–108379</td>
</tr>
<tr>
<td>S245AR</td>
<td>CTTTTTTCCTCGGCGCTCCTTCTTGAGGCGG</td>
<td>108350–108379</td>
</tr>
<tr>
<td>T250AF</td>
<td>GCCGCAAAGAAAAGGGCTTTGAGGTTAAGG</td>
<td>108334–108363</td>
</tr>
<tr>
<td>T250AR</td>
<td>CCTAAACCTTACCACTTTTTCCTCGGCGG</td>
<td>108334–108363</td>
</tr>
<tr>
<td>A28PF</td>
<td>ATGACCTGTGGACCTCGGCCGCGCGGACC</td>
<td>109001–109030</td>
</tr>
<tr>
<td>A28PR</td>
<td>GGCGCGGCGCGCCGCGCTGCAACAGGTTCCAT</td>
<td>109001–109030</td>
</tr>
<tr>
<td>Primer 1</td>
<td>CCCTACGAGAAAGGAAGGGG</td>
<td>105137–105156, 124947–124966</td>
</tr>
<tr>
<td>Primer 2</td>
<td>CCGGAACGTCACCACTTCTA</td>
<td>117499–117518</td>
</tr>
<tr>
<td>Primer 3</td>
<td>CGCGAGCGCAACCAAAATAA</td>
<td>112661–112680</td>
</tr>
<tr>
<td>Primer 4</td>
<td>AATGACGGCTCAGAAAAACCATCG</td>
<td>104171–104194</td>
</tr>
<tr>
<td>Primer 5</td>
<td>CGTCCGGTGCCGCTGATG</td>
<td>124466–124484</td>
</tr>
<tr>
<td>Primer 6</td>
<td>GCCGGGCGACATTTCAACTC</td>
<td>113255–113274</td>
</tr>
</tbody>
</table>

<sup>a</sup> Underlined nucleotides are mutated sequences.

<sup>b</sup> Nucleotide number in parent Oka strain.

<sup>c</sup> NA, not applicable because it anneals to vector sequence.

### TABLE 2. Recovery of infections virus after transfections of cosmids with deletions of ORF62, ORF71, or ORF62/71 and with insertion of ORF62 into a nonnative site in the U5 region

<table>
<thead>
<tr>
<th>Cosmid cotransfected with pvFsp73, pvSpe14, and pvPme2</th>
<th>CPE&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Days after transfection</th>
<th>Designation of recovered virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>pvSpe23</td>
<td>+++</td>
<td>6–10</td>
<td>rOka</td>
</tr>
<tr>
<td>pvSpe23ΔORF62</td>
<td>+++</td>
<td>13–17</td>
<td>rOkaΔORF62</td>
</tr>
<tr>
<td>pvSpe23ΔORF71</td>
<td>+++</td>
<td>6–10</td>
<td>rOkaΔORF71</td>
</tr>
<tr>
<td>pvSpe23ΔORF62/71</td>
<td>28–30&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NA&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>pvSpe23ΔORF62/71(rORF62@Avr)</td>
<td>++</td>
<td>9–13</td>
<td>rOkaΔORF62/71(rORF62-R)</td>
</tr>
<tr>
<td>pvSpe23ΔORF62/71(rORF71@Avr)</td>
<td>++</td>
<td>9–13</td>
<td>rOkaΔORF62/71(rORF62-R)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Proportions of cells exhibiting altered morphology: +, 25 to 50%; ++, 50 to 75%; +++ 75 to 90%; −, no CPE.

<sup>b</sup> Cells were split every 3 to 4 days to ensure that a slow-growth phenotype would not be missed.

<sup>c</sup> NA, not applicable (no virus was recovered from multiple transfections).
FIG. 2. PCR analysis of rOkaΔORF62, rOkaΔORF71, rOkaΔORF62/71v(ORF62-R), and rOkaΔORF62/71p(ORF62-R). PCR analysis was done with cosmid DNA (lanes 2 to 7) or DNA isolated from infected cells (lanes 8 to 12) as described in Materials and Methods. (A to C) PCR products obtained with the ORF71 primers (A), the ORF62 primers (B), or primers for insertion of the ORF62 gene at the AvrII site (C). The specimens tested are as follows: lanes 1 and 13, 1-kb DNA ladder; lane 2, pvSpe23; lane 3, pvSpe23 Δ ORF71; lane 4, pvSpe23ΔORF62; lane 5, pvSpe23ΔORF62/71; lane 6, pvSpe23ΔORF62/71(ORF62ΔAvr); lane 7, pvSpe23ΔORF62/71(ORF62ΔAvr); lane 8, rOka; lane 9, rOka ΔORF71; lane 10, rOkaΔORF62; lane 11, rOkaΔORF62/71(ORF62-R); lane 12, rOkaΔORF62/71(ORF62-R). (D) Expected PCR products. Lines 1 to 3, predicted primer annealing sites of primers 1, 2, 3, and 4 with the intact and deleted cosmids. The predicted fragment sizes for ORF71 PCR are also indicated. Lines 4 to 8, possible mechanism by which the 3,515-bp product in PCR with ORF71 primers and pvSpe23ΔORF62 was generated.
band was observed in all lanes. The 3,515-bp band produced by PCR of pvSpe23ΔORF62 can be explained as illustrated in Fig. 2D. Primers 1 and 2 were designed to amplify regions containing ORF71, independent of the two possible orientations of the inverted repeats. Therefore, primer 1 anneals not only at nt 124974 to 124966 but also at nt 105137 to 105156. In the first few PCR cycles, annealing of primer 1 at nt 105137 to 105156 generates a short fragment containing ORF63 (and ORF64) with ORF62 deleted. After the denaturation step, this fragment could anneal to the fragment generated from primer 2. After these two fragments anneal, in the following elongation step, the short 3,515-bp product is generated. Once this short fragment is generated in the early PCR cycles, it becomes the predominant product. A control experiment using mixtures of pvSpe23 and pvSpe23ΔORF62 in different ratios showed that this 3,515-bp band was 100- to 1,000-fold more likely to be generated than the 7,468-bp band (data not shown). This result indicates that the 3,515-bp band in the PCR of pvSpe23Δ ORF62 (Fig. 2A, lane 4) was a result of the PCR conditions. A similar observation was made when primers 1 and 3 were used to amplify ORF62 (data not shown). PCR with primer 3 and primer 4, which anneal within the U leader region, yielded the expected bands. Taken together, the PCR analyses indicated that the mutant cosmids, pvSpe23ΔORF62 and pvSpe23ΔORF71, were constructed as designed.

In contrast, PCR using DNA from cells infected with rOka ΔORF62, and rOkaΔORF71 yielded some unexpected results. PCR of rOka yielded single products of the expected sizes with the ORF71 and ORF62 primers (Fig. 2A and B, lanes 8). However, PCR of rOkaΔORF71 resulted in two products with each primer set (Fig. 2A and B, lanes 9). The 3,515-bp product generated with the ORF71 primers was expected for ORF71 deletion, but the 7,468-bp product was as expected for intact rOka (Fig. 2A, lane 9). With the ORF62 primers, the expected 8,510-bp product was observed, but a 4,557-bp band was also observed, suggesting recombination during transfection with repair of the deletion. PCR of rOkaΔORF62 with ORF71 primers also yielded both the faint 7,468-bp band and a 3,515-bp band, which is consistent with recombination; the 3,515-bp product could be due in part to PCR conditions, as was observed with pvSpe23ΔORF62 (Fig. 2A, lane 10). The data indicate that recombination occurred during the generation of rOkaΔORF71, and probably of rOkaΔORF62, from cosmids, as we reported with rOkaΔORF63 and rOkaΔORF64 mutants (54).

**Southern blot analysis of rOkaΔORF71 and rOkaΔORF62.**

Southern hybridization of cosmid DNA gave the expected single band (Fig. 3A, lanes 1 to 3 and 7 to 9), confirming the PCR data. Since PCR analyses suggested recombination, the same infected-cell DNA samples were digested with EcoRI and analyzed by Southern hybridization. Two bands were observed when rOka DNA was hybridized with the ORF71 and the ORF62 probes (Fig. 3A, lanes 4 and 10). This pattern is expected in VZV-infected cells because the VZV genome is a mixture of two isoforms with inversion of IR$_S$ - U$_S$ - TR$_S$ (Fig. 3B, lines 1 and 2) (12, 13, 57) and shows that generating VZV genomes from cosmids results in creation of both isoforms, even though the pvSpe23 cosmid has the IR$_S$ - U$_S$ - TR$_S$ sequence in a single orientation. The ORF71 probe should hybridize to a 3.8-kb band in rOkaΔORF71 DNA (Fig. 3B, lines 3 and 4), but hybridization results were the same as with rOka DNA. No 3.8-kb band was detected (Fig. 3A, lane 5), suggesting that intact rOka virus rather than rOkaΔORF71 was the primary recombinant virus generated. PCR showed that some DNA from which ORF71 had been deleted was also present in rOkaΔORF71-infected cells.

The analysis of rOkaΔORF62 DNA also showed that intact rOka was present (Fig. 3A, lane 6). The ORF71 probe should hybridize to 13.0- and 8.0-kb bands in rOkaΔORF62, as in rOka DNA (Fig. 3B, lines 5 and 6). However, a third 3.8-kb band was detected which should only appear in hybridizations with rOkaΔORF71. The ORF62 probe should hybridize only to the 8.0-kb band in rOkaΔORF62, because of the EcoRI site introduced in deleting ORF62 (Fig. 3B, lines 5 and 6). However, hybridization detected not only the 8.0-kb band but also 16.8- and 13.0-kb bands, as with rOka (Fig. 3A, lane 12). The analysis of rOkaΔORF62 suggested recombination, accounting for the 3.8-kb band, yielding a mixture of intact rOka and rOkaΔORF62 (Fig. 3B, lanes 7 and 8). Southern blot analysis of recombinant viruses that had been generated in two independent transfections with separately derived cosmids gave the same patterns (data not shown), indicating that the result was not due to contamination during transfection or cosmid preparation.

**Replication of rOkaΔORF71 and rOkaΔORF62 in vitro.**

When growth kinetics were evaluated over 6 days, no significant difference in viral titers was observed for rOka, rOkaΔ ORF71, or rOkaΔORF62 (see Fig. 5A). However, the kinetic analysis must be interpreted in the context of the PCR and Southern blot evidence of recombination in vitro, resulting in mixtures of intact rOka and single-deletion ORF62 and ORF71 mutants.

**Construction and characterization of rOkaΔORF62/71(pORF62-R) and rOkaΔORF62/71(vORF62-R).**

To confirm that failure to recover VZV from transfections done with pvSpe23ΔORF62/71 was due to absence of the ORF62/71 diploid gene, the pvSpe23ΔORF62/71 cosmid was further modified to insert the pOka ORF62 or the vOka ORF62 sequence into the AvrII site between ORF65 and ORF66 (Fig. 1). The inserted sequence contained ORF62 along with the full intragenic region between ORF62 and ORF63 and 70 bp of ORF63, which was predicted to contain the complete or essential components of the ORF62 promoter. The requirement for at least one copy of the ORF62 or ORF71 gene and its promoter for VZV replication was demonstrated by recovery of infectious viruses, designated rOkaΔORF62/71(pORF62-R) and rOkaΔORF62/71(vORF62-R), in all transfections done with pvSpe23ΔORF62/71(pORF62@Avr) or pvSpe23ΔORF62/71(vORF62@Avr) and the three intact cosmids in two separate experiments (Table 2). However, initial plaque sizes were smaller than those of rOka, and repaired viruses produced only 50% CPE in melanoma cells at 13 days after transfection, suggesting attenuated growth.

Sequencing of ORF62 and ORF71 in the respective pOka and vOka cosmids revealed that these two duplicated ORFs were identical in pOka and also in vOka. When the ORF62/71 sequences from the pOka and vOka cosmids were compared, only two differences were identified, both of which resulted in an amino acid change. An A-to-G transition at position 2872 (A2872G) resulted in an arginine-to-glycine substitution at position 958 (R958G), and a T-to-C substitution at position 3172.
(T3172C) produced a tyrosine-to-histidine substitution at position 1058 (Y1058H). Sequencing of ORF62 in plasmid clones made from cells infected with vaccine Oka virus has shown considerable heterogeneity, as confirmed in two independent reports (3, 17). The A2872G substitution present in the vOka cosmid was one of the numerous mutations that have been identified in ORF62 from vaccine Oka virus stocks, but the T3172C mutation has not been reported previously.

PCR analysis of mutant cosmids and rOkaΔORF62/71(pORF62-R)- and rOkaΔORF62/71(vORF62-R)-infected cell DNA. PCR with the AvrII site primers 5 and 6 (Table 1) showed that the ORF62 gene was inserted into cosmids at the AvrII site in pvSpe23ΔORF62/71(vORF62@Avr) and pvSpe23ΔORF62/71(pORF62@Avr), oriented towards ORF66 (Fig. 2C). PCR with primers 1 and 2 to amplify ORF71 generated a 3,515-bp product from pvSpe23ΔORF62/71(vORF62@Avr) and pvSpe23ΔORF62/71(pORF62@Avr).
and pvSpe23ΔORF62/71(pORF62@Avr) (Fig. 2A, lanes 6 and 7). PCR of rOka and repaired viruses rOkaΔORF62/71 (pORF62-R) and rOkaΔORF62/71(vORF62-R) yielded a single product of the expected size with either the ORF62 or ORF71 primers (Fig. 2A and B, lanes 8, 11, and 12). PCR with the AvrI site primers further confirmed that rOkaΔORF62/71 (pORF62-R) and rOkaΔORF62/71(vORF62-R) had a single copy of ORF62 at the AvrI site, as expected (Fig. 2C, lanes 11 and 12). In contrast to the case for single deletions, no evidence of recombination in vitro was observed when the pvSpe23ΔORF62/71(vORF62@Avr) or pvSpe23ΔORF62/71(pORF62@Avr) cosmids were used to generate these repaired viruses.

Effects of targeted mutations in ORF62. The IE62 protein has been shown previously to bind to the IE4 protein (55) and to the whole ORF9 protein (56). The Ruyechan and Hay laboratories have mapped the region important for IE4 binding to aa 161 to 299 in the IE62 sequence by deletion analysis in vitro expression experiments. Mutations in ORF62 were designed from their evidence that IE4 binding to IE62 protein was eliminated by phosphorylation of IE62 at or near threonine 250, which contains a common phosphorylation site for protein kinases C and A, and a second protein kinase C site at serine 245 (55, 56). To further explore these interactions, the point mutations S245A and T250A were introduced into ORF62 in the repaired single-copy pOka ORF62 gene. In addition, A28P, which was designed to disrupt the predicted alpha-helical structure of the putative ORF9 binding site (Ruyechan and Hay, unpublished observations), was transferred into ORF62. Infectious virus was recovered from all transfections and was further evidence for attenuation as suggested by the small-plaque phenotype of the repaired viruses in vitro, and was further evidence for attenuation associated with insertion of the single copy of ORF62 into a nonnative site in the VZV genome. Viral titers of ORF62 point mutants showed smaller plaques with rOkaΔORF62/71(pORF62-R(A28P)), in which the putative ORF9 binding site was disrupted, compared to rOkaΔORF62/71(pORF62-R) (0.52 ± 0.09 mm versus 1.14 ± 0.12 mm; P < 0.0001). A second experiment showed no difference between the two repaired viruses (data not shown), whereas the slower growth and lower peak titers of rOkaΔORF62/71 (pORF62-R) and rOkaΔORF62/71 (vORF62-R) compared to rOka was confirmed. This viral titration data were consistent with less efficient cell-cell spread, as suggested by the small-plaque phenotype of the repaired viruses in vitro, and was further evidence for attenuation associated with insertion of the single copy of ORF62 into a nonnative site in the VZV genome. Viral titers of ORF62 point mutants were also compared with those of rOkaΔORF62/71 (pORF62-R) and rOka (Fig. 5B). Only the mutant rOkaΔORF62/71 (pORF62-R(A28P)) exhibited slower growth kinetics than rOkaΔORF62/71 (pORF62-R), which was consistent with the plaque size analysis. This observation suggested that IE62 protein interactions with ORF9 protein, predicted to involve this region of the IE62 protein (Ruyechan and Hay, unpublished observations), were important for replication and warrants further investigation. However, the N-terminal region of the IE62 protein also has an acidic activation domain (49); the possibility that the A28P mutation could affect the trans-
activating activity of the IE62 protein, as well as interfering with ORF9 binding, must be examined (29, 56).

Expression of VZV proteins by rOkaΔORF62/71 (pORF62-R) and rOkaΔORF62/71(vORF62-R). To investigate the attenuated growth of the repaired viruses in vitro, VZV protein synthesis was examined in melanoma cells infected with rOka, rOkaΔORF62/71(pORF62-R), and rOkaΔ ORF62/71(vORF62-R) and evaluated by Western blotting at days 1 and 4 (Fig. 6). In addition to IE62 protein, expression of IE4 protein, ORF47 protein kinase (an early gene product), and gE (a late gene product) were analyzed. As shown in Fig. 6, a reduced amount of IE62 protein was detected in cells infected with repaired viruses on days 1 and 4. The limited amount of IE62 protein produced by the repaired viruses was associated with expression of IE4 and ORF47 proteins at levels equivalent to those observed in rOka-infected cells on both days 1 and 4. Little or no gE was detected on day 1 in any specimens, which is consistent with its classification as a late gene product.

However, gE remained markedly reduced in cells infected with rOkaΔORF62/71(pORF62-R) or rOkaΔ ORF62/71(vORF62-R) on day 4, which is consistent with a requirement for IE62 protein in inducing gE expression. Since the VZV gE-gI protein complex is required for cell-cell spread (1, 45, 46), reduced gE expression, secondary to limited IE62 protein expression, can account for the smaller plaque sizes and slower growth kinetics observed in melanoma cells infected with rOkaΔ ORF62/71(pORF62-R) or rOkaΔORF62/71(vORF62-R).

Restoration of plaque size by infection of gE-expressing melanoma cells with rOkaΔORF62/71(pORF62-R) and rOkaΔORF62/71(vORF62-R). To further examine the relationship between reduced gE expression and the attenuated phenotypes of the repaired viruses, Met-gE cells, which are a melanoma cell line with tetracycline-inducible gE expression (33), were inoculated with rOkaΔORF62/71(pORF62-R) or rOkaΔ ORF62/71(vORF62-R). In the absence of doxycycline, the mean plaque size for rOkaΔ62/71(pORF62-R) in Met-gE cells was less than that for rOka (0.75 ± 0.14 mm versus 1.26 ± 0.07 mm; \( P < 0.0001 \)); the rOkaΔORF62/71(vORF62-R) plaque size was 0.75 ± 0.10 mm, which was also reduced compared to that of rOka (\( P < 0.0001 \)) (Fig. 4B). However, in the presence of doxycycline (1.0 \( \mu \)g/ml), the mean plaque sizes of rOkaΔORF62/71(pORF62-R) and rOkaΔORF62/71(vORF62-R) were 1.21 ± 0.10 mm and 1.23 ± 0.09 mm, respectively, and were not significantly different from that of rOka, which was 1.25 ± 0.09 mm (Fig. 4B). The rOka plaque size was not affected by doxycycline.

Infectivity of rOkaΔORF71, rOkaΔORF62, rOkaΔORF62/71(vORF62-R), and rOkaΔORF62/71(pORF62-R) in SCID-hu skin xenografts. Skin xenografts were inoculated with equivalent titers of rOka, rOkaΔORF71, rOkaΔORF62, rOkaΔORF62/71(vORF62-R), rOkaΔORF62/71(pORF62-R), and implants were harvested at days 12, 21, and 28 (Fig. 7). No difference in infectivity was observed between rOka, rOkaΔ71, and rOkaΔ62. This observation was not unexpected, since intact rOka was generated in transfections of the ORF62 and ORF71 single-deletion cosmids, as shown by PCR and Southern hybridization (Fig. 2 and 3). When skin xenograft tissue was analyzed by PCR using primers for amplifying ORF71, ORF62, and ORF4, 2 of 10 implants inoculated with rOkaΔ71 had mixed single-copy rOkaΔ71 and rOka viruses, and intact rOka virus was predominant in 3 implants (Fig. 8A, lanes 6 and 7), 5 had mixed single-copy rOkaΔ71 and rOka viruses, and intact rOka virus was predominant in 3 implants (Fig. 8A, lanes 12, 14, and 15). Seven of nine implants inoculated with rOkaΔ62 had both rOkaΔ62 and rOka viruses, and two implants yielded predominantly intact rOka (Fig. 8A, lanes 17 and 21).

Infectious virus was recovered at low titers from only a single skin xenograft that was tested at day 12 after inoculation with rOkaΔORF62/71(vORF62-R) and from none of those inoculated with rOkaΔORF62/71(pORF62-R). Further, no rOkaΔORF62/71(vORF62-R) or rOkaΔORF62/71(pORF62-R) was recovered from 10 SCID-hu skin implants tested at 21 days after infection. By PCR, two of five xenografts inoculated with rOkaΔORF62/71(vORF62-R) and tested at day 12 had detectable ORF71 DNA. At day 21, all xenografts inoculated with rOkaΔORF62/71(pORF62-R) had detectable ORF71 DNA (Fig. 8B). Western blot analysis for IE4 protein expression was negative in rOkaΔORF62/71(vORF62-R) and rOkaΔORF62/71(pORF62-R)-infected implants evaluated at day 21 (Fig. 8B). Despite failure to recover infectious virus, detection of
VZV DNA in some implants by PCR suggested that rOka/H9004 ORF62/71(pORF62-R) might replicate at a very low rate in vivo. Therefore, a second cohort of mice with skin xenografts were inoculated with rOka/H9004 ORF62/71(vORF62-R) or rOka/H9004 ORF62/71(pORF62-R) and evaluated after 28 days. PCR, virus titrations, and Western blot analysis for VZV protein expression of all of these skin implants were negative for both of the repaired viruses (data not shown). Thus, rOkaΔ ORF62/71(vORF62-R) and rOkaΔ ORF62/71(pORF62-R) were not infectious in vivo or replicated to a very limited extent in only one implant of all of the skin xenografts that were inoculated with these viruses. This defect in replication in differentiated cells in vivo was observed even though insertion of a single copy of vORF62 or pORF62 into the nonnative AvrII site was associated with restoration of the capacity to replicate in vitro, albeit with an attenuated phenotype.

DISCUSSION

The mutational analysis of ORF62 and ORF71, encoding the IE62 protein, demonstrated that one copy of the duplicated gene is required for VZV replication in vitro, either at one of the native locations in the repeat regions flanking the US region of the genome or in the nonnative insertion site in the US region. The requirement for IE62 protein fits the model in which IE62 protein must localize to the nucleus of the infected cell, along with uncoated viral DNA, as an essential first step in VZV replication. These experiments using VZV recombinant viruses support experiments with transient-expression systems showing that no other VZV regulatory protein can substitute for the transactivating effects of IE62 protein on viral gene promoters (19, 40). IE62 transactivation of promoters representative of IE, early, and late kinetic classes of VZV genes has been demonstrated in expression systems (25). The IE62 protein binds to the IE4 and IE63 proteins and is required to recruit other viral regulatory proteins to sites of transcription initiation, such as IE63 to the gI promoter (29, 55, 56). In addition, IE62 protein interacts with cellular proteins involved in viral gene transcription (32). Introducing pOka or vOka ORF62 into the nonnative US site was associated with the recovery of infectious virus. These experiments define ORF62 as an essential gene by showing that failure to generate infectious virus from pvSpe23/H9004 ORF62/71 was due to the simultaneous deletion of ORF62 and ORF71 and not to disruption of promoter sequences or other regulatory regions affecting adjacent genes in the repeat segment or to random, unidentified mutations elsewhere in the VZV genome. The conclusion that the IE62 protein is an essential gene product parallels observations that deleting the duplicate ORFs encoding HSV ICP4 from the repeat regions flanking the US segment is lethal, except in complementing cell lines (47), and that mutating the ICP4 DNA binding domain is also incompatible with HSV replication (2).
When ORF62 or ORF71 was removed from the IR<sub>S</sub> or TR<sub>S</sub> region, respectively, transfections of the mutated cosmid with the single copy of ORF62 or ORF71, along with the other three VZV cosmids, often resulted in the recovery of intact rOka as well as rOka<sup>ΔORF62</sup> or rOka<sup>ΔORF71</sup> mutants. While experimental information is quite limited, VZV is presumed to undergo rolling-circle DNA replication, with theta intermediates, as described for HSV (8, 51, 52). Segment inversion, occurring by intramolecular recombination between the inverted repeats during replication, generates the variant isoforms (7, 8, 52). When the four fragments of VZV DNA are introduced by cosmid transfection, assembly of initial full-length genomes results from recombination due to the overlap between fragments. As shown in our Southern hybridization experiments, segment inversion occurred even though the pvSpe23 cosmid contains the IR<sub>S</sub>-U<sub>S</sub>-TR<sub>S</sub> in one orientation only, presumably during early replication of the initial genomes. In transfections with the single deletion cosmids, pvSpe23ΔORF62 and pvSpe23ΔORF71, replication was associated with repair of the ORF62 or ORF71 deletion, creating VZV DNA with full-length IRs and TRs. It is of interest that experiments with HSV-1 IRS deletion mutants did not identify similar recombinant events (50). In VZV, recombination is likely to be facilitated by the presence of the two R4 repeat element in the
IRs and TRs sequence, which are located in an intragenic region adjacent to each of the two VZV origins of replication (OriS); the R4 repeats are between ORF62 and ORF63 or between ORF71 and ORF70, respectively. R4 is one of the five such elements in the VZV genome, and this R4 repeat is made up of variable numbers of a 27-bp sequence (5, 8). As noted in our previous report about single-copy ORF63 and ORF70 deletions, this sequence is predicted to be single stranded during early replication and could provide the site for a specific crossover event (54). Thus, the intact IRs, or the intact TRs, could be introduced in place of the sequence from which ORF62 or ORF71 had been deleted. Our experiments with the single ORF62 and ORF71 deletion mutations in cell culture and in skin xenografts in vivo indicate that conditions of VZV DNA replication, and presumably cleavage and packaging, favor the restoration of full-length IRs and TRs sequences in the context of the viral genome.

Introducing a single copy of ORF62 into the nonnative Uc site permitted recovery of infectious viruses, rOka/ORF62/71 (vORF62-R) and rOkaΔORF62/71 (pORF62-R), in cell culture, but changing the genomic location of ORF62 had an attenuating effect on VZV replication in vitro and was essentially lethal for infection of skin xenografts in vivo. These experiments, which were done repeatedly with independently derived cosmids, pvSpe23ΔORF62/71 (pORF62@Avr) or pvSpe23ΔORF62/71 (vORF62@Avr), indicate that the ORF62/71 sequence cannot be restored from this foreign location to its native sites during replication. The attenuation phenotypes of rOkaΔORF62/71 (vORF62-R) and rOkaΔ ORF62/71 (pORF62-R) were characterized by small plaques and delayed and diminished production of infectious virus compared to rOka. Introducing point mutations, S245A and T250A, to disrupt the IE4 binding site of the IE62 protein, as mapped by in vitro expression experiments, was not lethal and did not further diminish replication in vitro. However, altering the putative ORF9 binding site with the substitution of A28P was associated with a log decrease in virus yields at each time point in the kinetic analysis; this mutation may also affect the IE62 N-terminal acidic activation domain and warrant further analysis because of the attenuating effect. Mutations interfering with IE62 binding to IE4 and potentially with ORF9 protein are predicted to affect VZV infectivity in vivo, but failure of rOkaΔ ORF62/71 (pORF62-R) to infect skin xenografts prevented an assessment of the consequences of these mutations in vivo. Experiments introducing these targeted mutations into both copies of ORF62/71 in their native locations will be necessary to address this question.

Analysis of the kinetics of viral protein synthesis by rOkaΔ ORF62/71 (vORF62-R) and rOkaΔ ORF62/71 (pORF62-R) in vitro showed that IE62 protein was reduced at day 1, while IE4 and ORF47 protein were not affected, and that delayed IE62 production was associated with reduced synthesis of the essential late VZV gE protein at day 4. Thus, the primary effect of relocating ORF62 and providing only one copy of ORF62 in the VZV genome was to decrease and delay its transcription, which is mediated by the transactivating function of its own gene product, IE62 protein, and ORF10 protein (39), and to reduce late ORF68 (gE) transcription. These observations are of interest because of the evidence that transcription of ORF4 and ORF47 did not require the usual concentrations of IE62 protein present at day 1 after VZV infection of cultured cells. Of note is that eliminating HSV ICP4, the IE62 homolog, resulted in high levels of expression of all IE genes, attributed to diminished repressor activity (9). The promoters of ORF4 and ORF47 have not been mapped, but both contain predicted binding sites for cellular transactivators based on computer sequence analysis; cellular proteins may function in combination with low levels of IE62 protein to mediate their transcription. With regard to glycoproteins, recent analyses have demonstrated that HSV ICP4 contributed to TFIIID binding to a minimal gC promoter, consisting of the TATA box and a G start site sequence that resembled the eukaryotic initiator (Inr) element, thereby facilitating formation of preinitiation complexes (18). Subsequent experiments showed that ICP4 was required for high levels of late HSV glycoprotein gene expression and that this function was mediated by ICP4 interaction with Inr start site regions (24). Since the ORF68 sequence, encoding VZV gE, has this conserved Inr element, IE62 protein may play a similar role in enhancing late gE expression. Characterizing the effects of the ecotopic insertion of ORF62 on the kinetics of VZV protein expression in vivo was not possible because of the failure of these attenuated viruses to replicate in differentiated skin xenografts.

In contrast to the case for IE62, we have not seen any effects on expression in vitro when other essential VZV genes and their promoters, including ORF63, gE (ORF68), and gK (ORF5), were introduced at the unique Avr II site in the Uc (33, 34, 54). When ORF62 was transferred to the Avr II site, 1,658 bp of upstream sequence was included, containing the complete ORF62-ORF63 intragenic region and 70 bp of ORF63. ORF62 and ORF63 are transcribed in opposite directions, and this region is presumed to contain promoter sequences for both genes. The IE62 promoter has three TAAT GARAT motifs and other sites that are known to bind cellular and viral protein complexes, which were preserved in the inserted sequence (11, 31, 41, 42). We cannot exclude the possibility that reduced early IE62 protein synthesis was due to absence of promoter elements located farther upstream within the ORF63 coding sequence. In addition, the lack of recombination events, which occurred with the single ORF62 or ORF71 deletions, meant that only one copy of ORF62 was present in all progeny viruses generated by using the pvSpe23ΔORF62/71 (pORF62@Avr) cosmid, which could affect total IE62 protein synthesis. It is also possible that the insertion of the additional OriS altered IE62 expression from the ORF62 gene at its nonnative site.

The rOkaΔORF62/71 (vORF62-R) and rOkaΔORF62/71 (pORF62-R) mutants had a small-plaque phenotype in vitro, and infectious virus was recovered from only one skin xenograft inoculated with these mutants in vivo. In addition to its association with limited gE production, the small-plaque phenotype was corrected when melanoma cells with inducible gE expression (Met-gE cells) were induced and infected with rOkaΔORF62/71 (vORF62-R) or rOkaΔORF62/71 (pORF62-R). These experiments suggest that the attenuation phenotype was related directly to gE concentrations in infected cells. While dispensable in other alphaherpesviruses, VZV gE is essential for viral replication in vitro, probably because it is required for cell-cell fusion (23, 33). VZV infection of cultured cells is known to be highly cell associated and is characterized by...
extensive formation of multinucleated syncytia (4). VZV gE and gI, encoded by ORF67, form heterodimer complexes that are required for normal gE trafficking within infected cells and to the plasma membrane (1, 20, 30, 45, 46, 61). In our experience, mutations that eliminated gE expression by ORF67 deletion or significantly reduced gE expression by altering binding sites for the cellular transactivating proteins, Sp1 and USF, in the gI promoter have also resulted in recombinants with a small-plaque phenotype; in these experiments, plaque morphology was corrected by insertion of the intact gene and its promoter into the nonnative AIV II site (20, 30). Like for the ROKaΔORF62/71 (vORF62-R) and ROKaΔORF62/71 (pORF62-R) recombinants, the small-plaque phenotype of ROKaΔgI and the dual Sp1/USF gI promoter mutants in vitro was associated with no infectivity or much decreased replication in differentiated human skin cells in SCID-hu xenografts (20, 38). Other mutations that have no effects on plaque size or infectious virus yields in vitro can also be lethal for skin infection in vivo, notably deletion of the ORF47 protein kinase or elimination of its kinase function, by other mechanisms (4a, 37). However, our interpretation of experiments with the small-plaque ROKaΔORF62/71 (vORF62-R) and ROKaΔORF62/71 (pORF62-R) recombinants, in which gE expression was downregulated, as well as with the small-plaque gI deletion and gI promoter mutants, is that the pathogenic effects of VZV in skin in vivo are critically dependent upon intact mechanisms of cell fusion mediated by gE-gI complex formation.

In summary, these experiments constitute the first analysis of the ORF62/71 gene pair in the context of the VZV genome and demonstrate the essential role of the IE62 protein in VZV replication in vitro and for the pathogenesis of VZV infection in skin in vivo.

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