Loss of the Vaccinia Virus 35-Amino-Acid Hydrophobic O3 Protein Is Partially Compensated by Mutations in the Transmembrane Domains of Other Entry Proteins

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ABSTRACT Eleven highly conserved proteins comprise the poxvirus entry-fusion complex (EFC). We focused on vaccinia virus (VACV) O3, a 35-amino-acid, largely hydrophobic component of unknown specific function. Experimental evolution was carried out by blindly passaging a virus that was severely impaired in entry due to deletion of the gene encoding O3. Large plaque variants that arose spontaneously were discerned by round four, and their numbers increased thereafter. Genome sequencing of individual cloned viruses revealed mutations in predicted transmembrane domains of three open reading frames encoding proteins with roles in entry. There were frameshift mutations in consecutive T's in open reading frames F9L and D8L and a nonsynonymous base substitution in L5R. F9 and L5 are EFC proteins, and D8 is involved in VACV cell attachment. The F9L mutation occurred by round four in each of three independent passages, whereas the L5R and D8L mutations were detected only after nearly all of the genomes already had the F9L mutation. Viruses with deletions of O3L and single or double F9L, L5R, and D8L mutations were constructed by homologous recombination. In a single round of infection, viruses with adaptive mutations including F9L alone or in combination exhibited statistically significant higher virus titers than the parental O3L deletion mutant or the L5R or D8L mutants, consistent with the order of selection during the passages. Further analyses indicated that the adaptive F9L mutants also had higher infectivities, entered cells more rapidly, and increased EFC assembly, which partially compensated for the loss of O3.

IMPORTANCE Entry into cells is an essential first step in virus replication and an important target of vaccine-elicited immunity. For enveloped viruses, this step involves the fusion of viral and host membranes to form a pore allowing entry of the genome and associated proteins. Poxviruses are unique in that this function is mediated by an entry-fusion complex (EFC) of 11 transmembrane proteins rather than by one or a few. The large number of proteins has hindered investigation of their individual roles. We focused on O3, a predominantly hydrophobic 35-amino-acid component of the vaccinia virus EFC, and found that spontaneous mutations in the transmembrane domains of certain other entry proteins can partially compensate for the absence of O3. The mutants exhibited increased infectivity, entry, and assembly or stability of the EFC.

KEYWORDS adaptive mutations, experimental evolution, membrane fusion, poxvirus, vaccinia virus, virus entry

Entry of enveloped viruses depends on the fusion of viral and cellular membranes (1). In the best characterized models, transmembrane proteins embedded in the viral envelope undergo conformational changes upon contact with cellular receptors,

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resulting in close apposition and fusion of the viral and host membranes. This step may involve one or two viral fusion proteins that are or become trimeric upon activation. The situation for poxviruses, however, is more complex and not yet well understood. Studies with vaccinia virus (VACV), the prototypical member of the poxvirus family, identified 11 conserved proteins that form the entry-fusion complex (EFC) (2–9). Inactivation of individual protein components prevents the formation of the complex or destabilizes it and abolishes or drastically inhibits entry, despite the formation of normal-looking virus particles containing the other EFC proteins. The EFC proteins have near N- or C-terminal hydrophobic domains and are associated with the membrane of mature virions (MVs). However, they lack signal peptides and are nonglycosylated, raising questions regarding their mode of trafficking to the viral membrane. The structure of the EFC has not yet been determined, though some subunit interactions have been identified (10–13) and the crystal structures of the ectodomains of two have been reported (14, 15).

The VACV O3 protein, the smallest component of the EFC, consists of only 35 amino acids, and the homologs of some other poxviruses are even shorter (9). VACV O3L (gene names are distinguished from proteins by having L or R indicating the direction of transcription) deletion mutants form tiny plaques and have a 3-log-unit reduction in infectivity (9). This reduction is associated with a defect in assembly of the EFC and entry of virus particles into cells. Nevertheless, the absence of O3 does not promote degradation of other EFC proteins or interfere with their trafficking to viral membranes and inclusion in virus particles. Although the O3L homologs have relatively low sequence identity, they are each capable of complementing the VACV O3L deletion mutant (16). Mutagenesis studies revealed that the N-terminal hydrophobic domain, which comprises two-thirds of O3 is sufficient to functionally replace the full-length O3 protein for VACV entry and assembly of the EFC (17). However, the sequence requirements are not stringent, as single amino acid substitutions in O3 do not have a discernible impact (17).

The presence of O3L homologs detected by iterative BLAST searches in all poxviruses except Crocodile poxvirus, Salmon gill poxvirus, and entomopoxviruses testify to the early acquisition of this gene during chordopoxvirus evolution. However, studies with VACV indicate that unlike the other EFC proteins, O3 is not absolutely essential for infection as tiny plaques form when the gene encoding O3 is deleted. On the basis of the latter property, we carried out experimental evolution experiments to determine whether adaptive mutants with increased plaque size would be selected upon blind passaging of a VACV with a deleted O3L gene. In such studies, small improvements are amplified by selection over multiple rounds of infection. The identification and location of compensatory mutations could provide information regarding the role of O3 in the virus life cycle. We report that viruses with increased plaque size were detected and found to have mutations in the hydrophobic domains of two EFC proteins (F9 and L5) and a cell attachment protein (D8). Further studies demonstrated that the mutations partially compensate for the loss of O3L by improving infectivity, cell entry, and assembly or stability of the EFC.

RESULTS

Adaptive mutants arise upon serial passages of an O3L deletion mutant. Three replicate cultures of BS-C-1 cells were infected with 0.01 PFU per cell of vO3Δ, a previously isolated recombinant VACV in which the entire O3L open reading frame (ORF) was replaced with the ORF encoding green fluorescent protein (GFP) regulated by a VACV promoter (9). After 48 h to allow multiple rounds of infection and virus spread, the cells were harvested, and virus titers were determined by plaque assay. This protocol was repeated for a total of 10 rounds, each time infecting with 0.01 PFU/cell of the harvested virus. By round four, we noted plaques with increased size in all three parallel passage series (Fig. 1A). This impression was corroborated by measuring the areas of individual plaques. The increase in mean areas of plaques in successive rounds of

FIG 1 Increase in plaque size during serial passages of a VACV O3L deletion mutant. (A) Three independent passages were carried out by infecting BS-C-1 cells with 0.01 PFU/cell of vO3Δ and harvesting after 48 h. After titrating the amount of virus, the infections were repeated nine more times for each passage series. Virus samples were frozen and stored after each round for further analysis. BS-C-1 monolayers were infected with thawed samples and stained with crystal violet after 48 h. Contrast-enhanced plaques of WT virus and from rounds 1, 5, and 10 (R1, R5, and R10) of the three independent passages (P1 to P3) imaged on an EVOS are shown. (B) To determine plaque sizes, each sample was diluted and used to inoculate three wells of BS-C-1 cells. After 48 h, the areas of individual plaques in each well were determined using ImageJ, and the sums were divided by the number of plaques to determine the mean areas. The error bars are the standard errors of the means (SEM) from triplicate wells of each sample. Samples are labeled according to the passage and round number (e.g., 1.1 indicating passage 1 and round 1).

infection (Fig. 1B) reflect the steady increase in the proportion of large plaques, suggesting the selection of adaptive mutants.

Identification of mutations. Virus was isolated from individual plaques after the initial and final rounds of each parallel passage. Following three successive plaque isolations to achieve clonal purity, the viruses were expanded in BS-C-1 cells and DNA extracted for whole-genome Illumina sequencing. Each of the analyzed clones from round 10 had mutations in two or three ORFs identified as F9L, L5R, and D8L and in one case an additional mutation in an intergenic region (Table 1). In contrast, none of the clones from round one had mutations in these ORFs. The mutation in F9L was caused by insertion of a T residue within a run of five T's, and the mutation in D8L was caused by a deletion of a T within a run of six T's (Fig. 2). *In silico* translation of the F9L and D8L DNA sequences indicated frameshifts followed by premature stop codons (Fig. 3). The mutation in L5R was the substitution of A for G at nucleotide 94 leading to the replacement of a valine with an isoleucine. Each of the three mutations occurred within the transmembrane (TM) domain predicted by the TMPred program and underlined in Fig. 3. In F9 and D8, amino acids distal to TM domains were deleted placing the TM at the C terminus in each case. The change in L5 appeared less dramatic, as the original amino acid, valine, and the substituted amino acid, isoleucine, have nonpolar

TABLE 1 Mutations in cloned virus isolates

Clone ^a	F9L mutation (%) ^c	L5R mutation (%) ^c	D8L mutation (%) ^c
P1R1.1	0	0	0
P1R10.1 ^b	0	100	99.9
P1R10.2	100	0	99.7
P1R10.3	100	0	100
P2R1.1	0	0	0
P2R10.1	99.8	100	0
P2R10.2	100	0	100
P2R10.3	99.6	0	100
P3R1.1	0	0	0
P3R10.1	100	0	100
P3R10.2	99.8	100	0
P3R10.3	99.8	100	100

^aP1, P2, and P3 refer to passages 1, 2, and 3, respectively. R1 and R10 refer to round 1 and round 10, respectively. The numbers after the decimal point refer to individual clones.

^bAn additional G₅₀₃₃₂T mutation was found between the E5R and E6R ORFs.

^c% read counts of specific mutated sequence divided by read counts of mutated plus wild-type sequences $\times$ 100 in DNA from cells infected with cloned viruses.

side chains (Fig. 3). Both F9L and D8L have additional runs of 5 T's or A's preceding and following the one mutated, suggesting that the mutations selected during passaging were specific for the TM domain. Similarly, L5R had unaltered runs of both A's and T's that were not mutated.

Evolutionary dynamics. The finding of mutations in two or three genes of each cloned virus raised the question of the order of their occurrence. Illumina sequencing of DNA extracted from cells at rounds 1, 2, 4, 6, 8, and 10 of each passage series was carried out in order to answer this query. The mutation in the F9L ORF was detected by round four in all three passages (Table 2), the same time that a significant increase in plaque sizes occurred (Fig. 1B). Mutations in the L5R and D8L ORFs were detected by round eight, when the F9 mutation was present in 94% to 99% of the genomes, indicating that the L5R and D8L mutations occurred secondarily. At round 10, more than 50% of the genomes had F9L plus L5R or D8L mutations, suggesting that the double mutations had a further selective advantage. The absence of a significant number of D8L mutations in round 10 of passage series 2 and 3 was surprising, since they were found in viruses cloned from those series. Either rare plaques with these viruses were picked because of their large size or the mutations occurred and were selected during the three repeat plaque purifications and expansion prior to DNA sequencing.

Construction of recombinant viruses with point mutations in F9L, L5R, or D8L.

Because the cloned viruses had more than one mutation, we constructed viruses with single and double mutations to compare their individual and combined impacts. O3L deletion viruses that contained single nucleotide changes in the F9L (vO3ΔF9m), L5R (vO3ΔL5m), and D8L (vO3ΔD8m) ORFs were constructed using a high-efficiency recombination protocol (18). In the procedure outlined in Fig. 4, cells were infected with vO3Δ and transfected with an ~1,000-bp PCR product containing the single nucleotide that differs from the nucleotide sequence of the wild type (WT) near the midpoint. The cells were harvested at 18 h after infection, and the lysates were plated on BS-C-1 cells. DNA samples from individual plaques were analyzed by PCR and Sanger sequencing as depicted for the F9L mutant (Fig. 4). Of 22 plaques analyzed for mutation in F9L, 16 were wild type with five consecutive A's in the noncoding strand and 6 were mutant with six consecutive A's. Similar procedures were used to construct L5R and D8L recombinant viruses. Positive isolates with the expected mutation were then clonally purified and expanded. Double mutants were isolated by repeating the recombination step with the single mutants as the parent viruses using a second PCR product. We succeeded in obtaining O3 deletion mutants with mutations in both F9L and D8L (vO3ΔF9/D8m) and with both F9L and L5R (vO3ΔF9/L5m) mutations but for unknown reasons failed to

F9L

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ATGGCGGAAA CTAAAGAGTT TAAACCTTTG TATAATCTTT TTATAGATAG 50
TTATTTACAA AAATTAGCTC AACATTCTAT CCCTACTAAT GTCACCTGTG 100
CTATTCATAT AGGAGAGGTT ATAGGACAGT TTAAAAATTG CGCGCTCCGA 150
ATAACTAACA AATGCATGAG TAATTCTCGA CTTAGTTTCA CACTCATGGT 200
TGAATCATTT ATTGAAGTGA TTTCATTGCT TCCGGAAAAG GATAGAAGAG 250
CTATCGCTGA AGAAATAGGA ATAGATCTAG ACGATGTACC TAGTGCGGTA 300
TCCAAGCTAG AAAAGAACTG TAATGCGTAT GCGGAGGTTA ATAATATTAT 350
AGATATACAG AAATTAGATA TCGGAGAATG TTCGGCTCCG CCCGGTCAAC 400
ATATGCTTTT ACAGATAGTT AATACAGGAT CCGCGGAAGC AAATTGTGGT 450
TTACAGACAA TTGTTAAGTC CTTAAATAAA ATATACGTTT CACCTATTAT 500
CGAAAACCGA TTGCCGTATT ACGATCCGTG GTTTCTAGTG GGTGTAGCAA 550
TTATTCTAGT TATTTTTTAC TG TAGCTATT TGTTCTATTA GACGAAATCT 600
GGCTCTTAAA TACAGATACG GAACGTTTTT ATACGTTTAA 640

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L5R

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ATGGAGAATG TTCCTAATGT ATACTTTAAT CCTGTGTTTA TAGAGCCAC 50
GTTTAAACAT TCTTTATTAA GTGTTTATAA ACACAGATTA ATAATTTTAT 100
TTGAAGTATT CGTTGTATTC ATTCTAATAT ATGTATTTTT TAGATCTGAA 150
TTAAATATGT TCTTCATGCC TAAACGAAAA ATACCCGATC CTATTGATAG 200
ATTACGACGT GCTAATCTAG CGTGTGAAGA CGATAAATTA ATGATCTATG 250
GATTACCATG GATGACAAC TAAACATCTG CGTTATCAAT AAATAGTAAA 300
CCGATAGTGT ATAAAGATTG TGCAAAGCTT TTGCGATCAA TAAATGGATC 350
ACAACCAGTA TCTCTTAACG ATGTTCTTCG CAGATGA 387

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D8L

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ATGCCGCAAC AACTATCTCC TATTAATATA GAAACTAAAA AAGCAATTTT 50
TAACGCGCGA TTGAAGCCGT TAGACATACA TTATAATGAG TCGAAACCAA 100
CCACTATCCA GAACACTGGA AAAC TAGTAA GGATTAATTT TAAAGGAGGA 150
TATATAAGTG GAGGGTTTCT CCCCAATGAA TATGTGTTAT CATCACTACA 200
TATATATTGG GGAAAGGAAG ACGATTATGG ATCCAATCAC TTGATAGATG 250
TGTACAAATA CTCTGGAGAG ATTAATCTTG TTCATTGGAA TAAGAAAAAA 300
TATAGTTCTT ATGAAGAGGC AAAAAACAC GATGATGGAC TTATCATTAT 350
TTCTATATTC TTACAAGTAT TGGATCATAA AAATGTATAT TTTCAAAGA 400
TAGTTAATCA ATTGGATTCC ATTAGATCCG CCAATACGTC TGCACCGTTT 450
GATTCAAGTAT TTTATCTAGA CAATTTGCTG CCTAGTAAGT TGGATTATTT 500
TACATATCTA GGAACAAC TAACCACTC TGCAGACGCT GTATGGATAA 550
TTTTTCCAAC GCCAATAAAC ATTCATTCTG ATCAACTATC TAAATTCAGA 600
ACACTATTGT CGTCGTCTAA TCATGATGGA AAACCGCATT ATATAACAGA 650
GAACTATAGA AATCCGTATA AATTGAACGA CGACACGCAA GTATATTATT 700
CTGGGGAGAT TATACGAGCA GCAACTACCT CTCCAGCGCG CGAGAACTAT 750
TTTATGAGAT GGTGTGTCGA TTTGAGAGAG ACATGTTTTT CATATTATCA 800
AAAATATATC GAAGAGAATA AAACATTCGC AATTATTGCC ATAGTATTCG 850
TGTTTATACT TACCGCTATT CTCTTTT-A TGAGTCGACG ATATTCGCGA 899
GAAAAACAAA ACTAG 914

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FIG 2 Nucleotide sequences of F9L, L5R, and D8L ORFs of cloned adapted viruses. DNA was extracted from cells infected with cloned viruses and sequenced with an Illumina MySeq-2. Shown in red are the addition of a T nucleotide at the end of a run of T's in F9L, substitution of an A-for-a-G nucleotide in L5R, and deletion (indicated by a red dash) of a T nucleotide at the end of a run of T's in D8L. The remainder of the sequences are identical to the WT WR ORFs.

isolate viruses with both D8L and L5R mutations even though the double mutants arose spontaneously during the passages. The absence of unintended mutations was verified in each case by whole-genome Illumina sequencing.

Plaque formation and replication of recombinant viruses with single or double mutations. The plaques formed by the cloned viruses (Fig. 5A) were compared to those engineered by recombination (Fig. 5B). The plaque sizes of the recombinant viruses

F9 WT	1	MAETKEFKTLYNLFIDSYLQKLAQHSIPTNVTCAIHIGEVIQGFKNALRITNKCMSNSR	60
F9 mut	1	MAETKEFKTLYNLFIDSYLQKLAQHSIPTNVTCAIHIGEVIQGFKNALRITNKCMSNSR	60
F9 WT	61	LSFTLMVESFIEVISLLPEKDRRAIAEEIGIDLDDVPSAVSKLEKNCNAYAEVNNIIDIQ	120
F9 mut	61	LSFTLMVESFIEVISLLPEKDRRAIAEEIGIDLDDVPSAVSKLEKNCNAYAEVNNIIDIQ	120
F9 WT	121	KLDIGECsAPPgQHMLLQIVNTGSAEANCGLQTIVKSLNKIYVPPPIENRLPYYPDPWFLV	180
F9 mut	121	KLDIGECsAPPgQHMLLQIVNTGSAEANCGLQTIVKSLNKIYVPPPIENRLPYYPDPWFLV	180
F9 WT	181	GVAILLVIF <u>TVAICSI</u> RRNLALKYRYGTFLYV	212
F9 mut	181	GVAILLVIF <u>YCSYLFY</u> -----	196
L5WT	1	MENVPNVYFNPVFIETPKHSLLSVYKHRLI <u>VLFEV</u> FVVFILIYVFFRSELNMFMPKRR	60
L5mut	1	MENVPNVYFNPVFIETPKHSLLSVYKHRLI <u>ILFEV</u> FVVFILIYVFFRSELNMFMPKRR	60
L5WT	61	IPDPIDRLRRANLACEDDKLMIYGLPWTQTTSALSINSKPIVYKDCAKLLRSINGSQPV	120
L5mut	61	IPDPIDRLRRANLACEDDKLMIYGLPWTQTTSALSINSKPIVYKDCAKLLRSINGSQPV	120
L5WT	121	SLNDVLR	128
L5mut	121	SLNDVLR	128
D8WT	1	MPQQLSPINIETKKAISNARLKPLDIHYNESKPTTIQNTGKLVRIKGGYISGGFLPNE	60
D8mut	1	MPQQLSPINIETKKAISNARLKPLDIHYNESKPTTIQNTGKLVRIKGGYISGGFLPNE	60
D8WT	61	YVLSSLHIYWGKEDDYGSNHLIDVYKSGEINLVHWNKKYSSYEEAKKHDDGLIIISIF	120
D8mut	61	YVLSSLHIYWGKEDDYGSNHLIDVYKSGEINLVHWNKKYSSYEEAKKHDDGLIIISIF	120
D8WT	121	LQVLDHKNVYFQKIVNQDLSIRSANTSAPFDSVFYLDNLLPSKLDYFTYLGTINHSADA	180
D8mut	121	LQVLDHKNVYFQKIVNQDLSIRSANTSAPFDSVFYLDNLLPSKLDYFTYLGTINHSADA	180
D8WT	181	VWIIFFTPPINIHSQDLSKFRLLSSSNHDKPHYITENYRNPYKLNDTQVYYSGEIIRA	240
D8mut	181	VWIIFFTPPINIHSQDLSKFRLLSSSNHDKPHYITENYRNPYKLNDTQVYYSGEIIRA	240
D8WT	241	ATTSPARENIFYMRWLSDLRETCFSYYQKYIEENKTF FAII IAIVFVFILTAILF <u>FMSRRYSR</u>	300
D8mut	241	ATTSPARENIFYMRWLSDLRETCFSYYQKYIEENKTF FAII IAIVFVFILTAILF <u>L</u> -----	293
D8WT	301	EQQN	304
D8mut		----	

FIG 3 Amino acid sequence alignments of F9L, L5R, and D8L ORFs of WT and cloned adapted viruses. The mutant (mut) DNA sequences shown in Fig. 2 were translated *in silico* and aligned with WT sequences. Predicted TM sequences are underlined, and sequence differences resulting from frameshifts or substitution are shown by red lettering.

were larger than the original O3L deletion virus in order $vO3\Delta F9/L5m > vO3\Delta F9m \sim vO3\Delta F9/D8m \sim vO3\Delta L5m > vO3\Delta D8m$. The plaques made by the recombinant viruses were similar in size to the passaged clones with the same two mutations except for $vO3\Delta F9/D8m$, which were smaller for an unknown reason.

In the initial passaging experiment, BS-C-1 cells were infected with 0.01 PFU/cell at the first and each subsequent round and harvested at 48 h. To mimic the impact of the mutations on a single passage round, the same protocol was used to compare the replication of $vO3\Delta$ and the recombinant viruses. Each of the viruses, with the exception of $vO3\Delta D8m$, achieved twofold- to fourfold-higher titers than $vO3\Delta$ (Fig. 6A), with high significance ($P < 0.0001$) for $vO3\Delta F9m$, $vO3\Delta F9/D8m$, and $vO3\Delta F9/L5m$ (Fig. 6A). The highest titer was attained for $vO3\Delta F9/L5m$, although this was still considerably less than wild-type VACV. The small enhancement of $vO3\Delta D8m$ and $vO3\Delta L5m$ is con-

TABLE 2 Evolutionary dynamics^a

Round	Passage series 1			Passage series 2			Passage series 3		
	F9L	L5R	D8L	F9L	L5R	D8L	F9L	L5R	D8L
1	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0
4	76.0	0	0	85.0	0	0	68.9	0	0
6	90.3	0	0	96.6	0	0	90.5	0	0
8	94.0	0	52.9	98.9	56.6	0	98.8	54.2	0
10	96.2	0	60.2	99.1	57.5	0	99.3	66.5	0

^aPercentages of F9L, L5R, and D8L mutants determined from read counts of mutated sequence divided by read counts of mutated plus wild-type sequence $\times 100$ in cytoplasmic DNA at indicated round of three parallel passage series.

FIG 4 Construction of recombinant VACV with mutated F9L, L5R, or D8L and deleted O3L. Cells infected with vO3Δ were transfected with DNA containing point mutations in F9L, L5R, or D8L. The cells were harvested, and lysates were diluted and applied to fresh BS-C-1 cells. PCR was performed directly from virus obtained from large plaques and used for Sanger sequencing. Sequence data for WT and mutated F9L are shown. Note that a run of A's in the noncoding strand correspond to T's in Fig. 2.

sistent with the late appearance of these mutations after the F9L mutation was present in nearly all viruses.

Genome particle/PFU ratios. The infectivity of O3L null mutant virions, produced by an O3L deletion mutant or by an inducible O3L virus in the absence of inducer, were previously shown to be extremely low due to the defect in their ability to enter cells (9). We considered that if the adaptive mutations in the F9L, L5R, and D8L ORFs increase entry, then the infectivity of the O3 deletion mutant virions should be enhanced. The number of virus genome-containing particles was determined by drop-let digital PCR (ddPCR) following treatment of purified virions with Benzonase nuclease to remove any adventitious DNA and compared to the infectious virus titer determined by plaque assay (19). The lower the particle/PFU ratio, the higher the specific infectivity. The lowest particle/PFU ratio of 13 was found for WT virus, whereas the highest of 538 was found for vO3Δ (Fig. 6B). The viruses with adaptive mutations all had lower particle/PFU ratios than vO3Δ ($P < 0.0001$), indicating increased infectivity. In addition, the viruses with F9L mutations all had significantly lower ratios than D8m ($P \leq 0.005$) and L5m ($P \leq 0.01$). The recombinant virus vO3HAi, which requires the inducer isopropyl-β-D-1-thiogalactopyranoside (IPTG) for expression of a hemagglutinin (HA)-tagged O3, served as another control. The particle/PFU ratio of vO3HAi grown in the absence of IPTG was approximately 25-fold higher than vO3HAi grown in the presence of the inducer (Fig. 6B).

In the previous section, replication of the viruses was compared by infecting cells

FIG 5 Comparison of plaques formed by viruses cloned after passage or constructed by recombination. (A) BS-C-1 cells were infected with WT virus or viruses cloned after passage 1 or 10. After 48 h, the cell monolayers were stained with crystal violet. P1, P2, and P3 refer to passage series. R1 and R10 refer to round 1 and round 10. The numbers after the decimal refer to individual clones. (B) BS-C-1 cells were infected with recombinant viruses vO3ΔF9m, vO3ΔL5m, vO3ΔD8m, vO3ΔF9/L5m, or vO3ΔF9/D8m. After 48 h, the cell monolayers were stained with crystal violet. The letter “m” is used to designate specific mutations made by recombination.

with the same number of PFU (Fig. 6A). Since the particle/PFU ratio of vO3Δ is higher than for the adaptive mutants, more virus particles were used for infection, possibly minimizing the differences in virus titers.

Effects of adaptive mutations on cell entry. The decrease in genome particle/PFU ratios of the adaptive mutants compared to vO3Δ suggested enhanced entry of virus into cells. We used two methods to gauge the effect of the adaptive mutations on virus entry. The first was expression of an early mRNA as a sign of entry, since transcription occurs within cores released into the cytoplasm and can be detected within 20 min (20). In the experiment depicted in Fig. 7A, BS-C-1 cells were infected with 200 particles of vO3Δ, mutant viruses, or WT virus in the presence of the protein synthesis inhibitor cycloheximide. Cycloheximide prevents virus uncoating, thereby prolonging the synthesis of mRNA from virus cores (21). In addition, the protein synthesis inhibitor reduces the cytopathic effect of virus infection and prevents some host responses. The quantity of mRNA transcribed from the early E3 gene was determined by ddPCR. The largest amount of RNA was found after infection with WT virus and the lowest after infection with vO3Δ (Fig. 7A). The adaptive mutants with the F9 mutation expressed two to three times more E3 mRNA than vO3Δ did ($P < 0.0001$). In contrast, the amounts of E3 mRNA in cells infected with the D8 and L5 mutants were only slightly higher than for vO3Δ and were not statistically significant. A time course experiment, carried out with vO3Δ and vO3ΔF9/L5m, showed a similar twofold-greater amount of RNA at all times up to 4 h (Fig. 7B).

For the second method, we also infected BS-C-1 cells with 200 virus particles but used flow cytometry to analyze the percentage of cells that expressed the early E3 protein. At 4 h, ~85% of the cells infected with WT virus stained positively with an E3 monoclonal antibody (MAb) compared to less than 30% infected with vO3Δ (Fig. 8A and B). The percentage of positive cells was between 1.6- and 2.0-fold higher for the viruses with adaptive mutations. Thus, both methods showed similar enhancements in virus entry for the recombinant viruses.

Adaptive mutations enhance assembly/stability of the EFC in the absence of O3. One way that the adaptive mutants could increase virus infectivity is by enhancing the assembly or stability of the EFC. Satheshkumar and Moss (9) showed that O3 expression is required for assembly of the EFC. For the present study, a MAb to the L1

FIG 6 Replication and infectivity of recombinant viruses. (A) BS-C-1 cells were infected with 0.01 PFU/cell of vO3Δ, vO3ΔD8m, vO3ΔL5m, vO3ΔF9m, vO3ΔF9/D8m, vO3ΔF9/L5m, or WT virus. After 48 h, the cells were harvested, and virus titers were determined by plaque assay. Titers obtained with recombinant viruses were compared to vO3Δ for statistical significance. ns, not significant; Symbols: ***, $P < 0.001$; ****, $P < 0.0001$. (B) Genome particle/PFU ratios of purified virus particles. Mutant viruses and vO3HAI, which was grown in the presence (+) or absence (−) of IPTG, were purified by sedimentation through a sucrose cushion and titered by plaque assay. After treatment with Benzonase to remove adventitious DNA, the number of genome copies was determined by ddPCR and the ratio to PFU was determined. The particle/PFU ratios of recombinant viruses were compared to vO3Δ for statistical significance. ****, $P < 0.0001$.

protein component of the EFC was used to capture the complex. Cells were infected with the O3-inducible virus vO3HAI in the absence and presence of IPTG, WT VACV, and the recombinant O3L deletions mutants with mutations in F9L, L5R, and D8L. The lysates were incubated with anti-L1 MAb beads, which were then washed and incubated with lithium dodecyl sulfate to elute bound proteins. The proteins of the total lysates and those bound and eluted from L1 MAb beads were analyzed by sodium dodecyl sulfate (SDS) gel electrophoresis and Western blotting using antibodies that

FIG 7 Copies of viral early mRNA in cells infected with recombinant viruses. (A) BS-C-1 cells treated with 150 μ g/ml of cycloheximide were mock infected or infected with 200 virus particles per cell of WT, vO3 Δ , vO3 Δ D8m, vO3 Δ L5m, vO3 Δ F9m, vO3 Δ F9/D8m, or vO3 Δ F9/L5m. At 4 h after infection, RNA was extracted, and copies of E3 mRNA were determined by ddPCR. The number of copies of E3 mRNA for cells infected with mutant viruses or mock infected is indicated on the left; the number of copies of E3 mRNA for WT virus is shown on the right in blue. Fold values for each recombinant relative to vO3 Δ are indicated. The ratios of RNA copies for recombinant viruses were compared to vO3 Δ for statistical significance. ****, $P < 0.0001$. (B) BS-C-1 cells treated with 150 μ g/ml of cycloheximide were infected with vO3 Δ or vO3 Δ F9m. At 1, 2, 3, and 4 h, RNA was isolated, and copies of E3 mRNA were determined by ddPCR. Fold values and statistical significance were determined as in panel A. ns, not significant; ***, $P < 0.001$; ****, $P < 0.0001$.

reacted specifically with several representative EFC proteins. L1, H2, A21, and A28 were detected by chemiluminescence at similar intensities in the total lysates of each sample (Fig. 9, left panel). In addition, similar amounts of L1 were captured by the MAbs beads (Fig. 9, right panel). The dependence on O3 for capture of other EFC proteins was demonstrated by comparing the lanes of vO3HAi in the absence and presence of IPTG and with WT VACV Western Reserve (WR) (Fig. 9, right panel). Importantly, the H2, A21, L5, and A28 bands were each more intense in the samples from cells infected with the O3 deletion mutants that had adaptive mutations in F9L, L5R, and D8L than the null mutant, indicating that they increased the amount of EFC that could be captured.

FIG 8 Quantitation of cells expressing early E3 protein. BS-C-1 cells were mock infected or infected in duplicate with 200 virus particles per cell of WT, vO3Δ, vO3ΔD8m, vO3ΔL5m, vO3ΔF9m, vO3ΔF9/D8m, or vO3ΔF9/L5m. At 4 h after infection, the cells were fixed, permeabilized, stained with a MAb to the E3 protein followed by a fluorescent secondary antibody, and analyzed by flow cytometry. (A) Histograms indicate the numbers of cells and fluorescence intensities. The bars with numbers above the bars show the gate and percentage of positive cells. (B) Bar graphs show the percentage of total cells expressing E3 (E3+ Cells) and fold differences relative to vO3Δ. Similar results were obtained with the duplicate samples.

DISCUSSION

Experimental evolution provides an unbiased approach to determine genomic alterations that can compensate for modification or loss of genes that are important for virus replication. An important advantage of this method is that a spontaneous mutant with just a slight enhancement in replication can outcompete the original defective virus over multiple rounds of infection. Such small enhancements, however, provide a challenge for follow-up one-step infection studies to elucidate the molecular

FIG 9 Association of representative EFC proteins with L1 protein. BS-C-1 cells were infected with 1.5 PFU per cell of WT WR strain of VACV, vO3HAI grown in the absence (–) or presence (+) of IPTG, vO3ΔD8m, vO3ΔL5m, vO3ΔF9m, vO3ΔF9/D8m, or vO3ΔF9/L5m. After 24 h, the cells were lysed, aliquots were removed (input), and the remainder was incubated with anti-L1 MAb bound to beads. After washing, the proteins were eluted. Input and bound proteins were resolved by SDS-polyacrylamide gel electrophoresis. After transfer, membranes were incubated with polyclonal rabbit antibodies to L1, H2, A21, and A28 proteins and detected by chemiluminescence.

basis for the adaptation. We considered that an experimental evolution strategy to find compensatory mutations for an O3 deletion mutant would be possible because it has a low level of replication and forms tiny plaques on cell monolayers. Although the inability to isolate deletion mutants of other EFC proteins precludes the same strategy, an alternative to consider would involve the passaging of inducible mutants without inducer or only a small amount.

Large plaques were observed by the fourth round of passage of an O3 deletion mutant, and their numbers increased through round eight. Since infectious VACV progeny can be detected as early as 8 h, each 48-h passage round involved multiple opportunities for selection. Whole-genome sequencing of cloned viruses revealed mutations in three genes, the products of which are involved in virus entry, suggesting a relationship to O3. The changes in F9L and D8L resulted from a single nucleotide insertion or deletion in a run of T's, respectively. This type of mutation has been reported previously in experimental evolution studies (22, 23) and is due to slippage of the VACV DNA polymerase resulting in frameshifts. In contrast, the change in L5R was a base substitution leading to a missense mutation, which is relatively infrequent due to the proofreading exonuclease activity of the VACV DNA polymerase. The frameshift mutation in F9L was detected at round four in each of the three passages. Although not noted in DNA from round one or two, the mutation might have been present below the threshold for detection in the starting inoculum. The missense mutation in L5R was first detected in two of the passages at round eight, which was also the round that the frameshift mutation in D8L was found. The latter two mutations were detected only after the F9L mutation was present in ~95% of the viral genomes, indicating that they occurred secondarily. When nine individual viruses were cloned after the 10th passage, none had a single mutation, eight had two mutations, and one had three.

Homologous recombination was used to construct O3L deletion viruses with specific mutations in F9L, L5R, or D8L in order to confirm and investigate their adaptive roles individually as well as together. The F9L and L5R mutations had a greater effect on plaque size than the D8L mutation, and there appeared to be an additive effect particularly with the F9L/L5R double mutant. In order to mimic the replication advantage of the mutants during a single round of the blind passage, we infected BS-C-1 cells with 0.01 PFU/cell of vO3Δ or the recombinant viruses and determined the virus titers after 48 h. Increases in virus production of high statistical significance, though only a

few fold, were found for the same mutations that appeared during the natural selection, i.e., F9L alone and together with L5R or D8L, whereas the single L5R and D8L mutations had a lesser effect on virus yield, consistent with their secondary selection.

Our previous studies with the O3 deletion mutant provided clues regarding possible enhancing effects of the adaptive mutations. The absence of O3 does not promote the degradation of other EFC proteins or interfere with their incorporation into virus particles but instead prevents the formation of the complex of EFC proteins and entry of O3-deficient particles into cells (9). Therefore, it seemed most likely that the adaptive mutants would partially overcome the latter defects. We used two approaches to investigate the effects of the adaptive mutants on cell entry. First, we measured expression of an early mRNA in the presence of the protein synthesis inhibitor cycloheximide. Although early RNA synthesis is a postentry step, the presence of cores in the cytoplasm is sufficient for early transcription as the enzymatic machinery is packaged in virus particles. The viruses with an F9 mutation expressed two to three times more early mRNA than the parental O3 deletion mutant did ($P < 0.0001$), whereas the amounts of mRNA in cells infected with the single D8L and L5R mutants were only slightly higher than the amount for the parental virus and not statistically significant. The second approach involved flow cytometry to determine the percentage of cells that expressed an early protein, which requires an additional translation step following core entry. Here too, the adaptive mutants had an advantage in entry of about twofold compared to the O3 deletion mutant. In addition, we found that the virus particles formed by the adaptive mutants had a greater infectivity (lower particle/PFU ratio) than the parental O3 deletion virus.

The EFC can be captured with affinity beads when an epitope tag is attached to any component, and nine of the proteins including O3 (9) are considered to be central because the absence of any one of these prevents formation of the complex or destabilizes it. Our data indicated that the adaptive mutations all increased the amount of the complex compared to an O3 null mutant, implying that this provides a mechanism for enhanced cell entry. Since the hydrophobic domain of the O3 protein is sufficient for function, O3 likely interacts with the transmembrane domains of other EFC proteins. The location of the F9 and L5 mutations in their hydrophobic domains suggests that these mutations partially overcome their need for interaction with O3 or replace O3 for interaction with other EFC proteins. Since D8 is a cell attachment protein (24), it may have an ancillary effect. However, a more direct effect is not excluded, as chemical cross-linking provided evidence for the close proximity of D8 and the A21 EFC protein (13).

Mutations in additional EFC proteins might further compensate for loss of O3 but did not arise during the 10 passage rounds. Whether the failure to isolate compensatory mutations in other EFC genes is due to a lower probability of their spontaneous formation or to roles independent of O3 cannot be determined from this study. Elsewhere we will show by proximity analysis that O3 is closely associated with numerous EFC proteins.

MATERIALS AND METHODS

Virus and cells. BS-C-1 and HeLa cells were maintained in minimum essential medium with Earle's salts (EMEM) supplemented with 10% fetal bovine serum (FBS), 100 U of penicillin, and 100 μ g of streptomycin per ml (Quality Biologicals, Gaithersburg, MD). The O3 deletion and inducible mutants were derived from the Western Reserve (WR) strain of VACV (ATCC VR1354) and described previously (9).

Sources of antibodies. Mouse MAb 7D11 to the L1 protein (25) was a gift of Alan Schmaljohn (U.S. Army Medical Research Institute of Infectious Diseases [USAMRIID]) and MAb TW2.3 to the E3 protein was described previously (26). Rabbit polyclonal antibodies to A21 (27), A28 (2), H2 (28), and L5 (29) were previously described.

Passaging of vO3 Δ . BS-C-1 cells (ATCC CCL-26) in six-well plates were infected with vO3 Δ at a multiplicity of 0.01 PFU per cell. After 48 h, the cells were lysed, and the virus titers were determined by plaque assays. The infection was repeated by adjusting the input to 0.01 PFU per cell.

Plaque size determination. Virus samples from serial passages 1 to 10 were diluted 10^{-4} and used to infect BS-C-1 monolayers in triplicate in six-well plates. After 48 h, the plates were scanned using an EVOS imaging system (Thermo Fisher Scientific, Waltham, MA), and green fluorescent plaques were

analyzed with ImageJ Fiji. The size limits for plaques were set from 25,000 nm² to infinity and circularity from 0.001 to 1.00.

Sequencing DNA from infected-cell lysates. DNA was extracted from 5×10^6 infected BS-C-1 cells. Briefly, the cells were harvested by scraping, and cell pellets were washed with phosphate-buffered saline (PBS) and resuspended in 20 mM Tris (pH 8.0), 5 mM EDTA, and 1% Triton X-100. After incubation at room temperature for 10 min, NaCl was added to a final concentration of 0.2 M, and the cell extract was centrifuged at $800 \times g$ for 3 min. The clarified supernatant was transferred to a fresh tube and treated with proteinase K and 0.5% SDS for 2 h at 50°C. An equal volume of buffer-saturated phenol was added to the DNA sample, and the aqueous phase was removed and mixed with 2 volumes of cold 95% ethanol. After centrifugation, the DNA pellet was washed twice with cold 80% ethanol. After the ethanol was removed, the pellet was allowed to air dry for 15 min and then dissolved in 100 μ l of nuclease-free water. The concentration of DNA was determined with a DS-11 FX spectrophotometer (Denovix, Wilmington, DE). Sequencing was performed with a MySeq-2 (Illumina, San Diego, CA) with a 150-bp configuration by the Integrated Research Facility at Rocky Mountain Laboratory, NIAID.

Construction of recombinant VACV. BS-C-1 cells were infected with 3 PFU/cell of VACV and after 1.5 h were transfected with ~ 500 ng of a PCR product of approximately 1,000 bp with the desired nucleotide change in the middle using Lipofectamine 3000 (Thermo Fisher). At 18 to 20 h after infection, the cells were lysed, and diluted virus was used to infect BS-C-1 cells in a six-well plate. After 48 h, infected cells from individual plaques were placed in 250 μ l of medium and subjected to three freeze-thaw cycles, and 4 μ l of the lysate was used for PCR (Phusion High-Fidelity PCR Mix; New England BioLabs, Ipswich, MA) with the original primers in a 50- μ l reaction mixture. PCR products were purified with a PCR purification kit (Qiagen, Germantown, MD), and clones with mutations were identified by Sanger sequencing. The virus clones with the desired mutations were plaque purified two additional times, and the virus obtained from the third round of plaque purification was used to infect wells of a 24-well plate of BS-C-1 cells. After 2 or 3 days, when the cytopathic effect was complete, the cells were harvested, and the virus in the lysates was expanded for further experiments.

Genome particle/PFU ratios. Ratios were determined as previously described (19) for virus particles that had been purified by sedimentation through a cushion of sucrose. Approximately 1×10^4 PFU of virus was treated with 18 U of Benzonase nuclease (Sigma, St. Louis, MO) for 1 h at 37°C to remove any adventitious DNA. Benzonase was then inactivated by the addition of EDTA (5 mM final concentration) for 20 min at room temperature. The QiaAMP Minelute Virus Spin kit (Qiagen, Valencia, CA) was used to isolate DNA, and viral genome copies were determined by ddPCR using primers specific for the VACV WR E11L gene. Genome particle/PFU ratios were calculated by dividing the number of genome copies by the amount of infectious virus determined by plaque assay.

Quantitation of transcription of the VACV E3R gene. BS-C-1 cells were infected in duplicate with 200 virus particles per cell at 4°C in the presence of 150 μ g/ml of cycloheximide for 1 h at 4°C. The infected cells were then washed twice with EMEM containing 2% FBS and incubated in EMEM with 2% FBS and 150 μ g/ml of cycloheximide at 37°C for an additional 4 h. For the time course experiment, the incubation period was 1 to 4 h. At specific times, total RNA was extracted from infected cells using the TRIzol Plus RNA purification kit with Phasemaker tubes (Thermo Fisher Scientific). Contaminating DNA was removed from each RNA preparation using the Turbo DNA-free kit (Thermo Fisher Scientific). The RNA was reverse transcribed using the iScript cDNA synthesis kit containing a mixture of oligo(dT) and random primers (Bio-Rad, Hercules, CA). The E3R transcript and 18s rRNA were quantified by ddPCR with specific primers using an automated droplet generator and QX200 droplet reader (Bio-Rad) (30). The values for E3R transcripts were normalized using the 18s RNA for each sample.

Flow cytometry. To examine early expression of E3 protein as a surrogate for core entry, BS-C-1 cells grown in 6-well tissue culture plates were infected in duplicate with 200 virus particles per cell of virus for 1 h at 37°C. After washing the cells, fresh EMEM with 2% FBS was added and the infection continued for an additional 3 to 4 h. Infected cells were washed once with Dulbecco's PBS, trypsinized and resuspended in PBS with 0.5% bovine serum albumin. To detect E3, the cells were fixed and permeabilized with Cytofix/Cytoperm solution for 30 min at 4°C and subsequently washed with $1 \times$ Perm/Wash Buffer (BD Biosciences, San Jose, CA). Cells were then incubated with anti-E3 MAb TW2.3 (26) for 1 h at 4°C. Bound E3 antibody was detected by incubation with anti-mouse-allophycocyanin (Biolegend, San Diego, CA) for 30 min at 4°C. After staining, cells were washed in PBS and resuspended in 2% paraformaldehyde. A FACSCalibur cytometer with Cell Quest software (BD Biosciences) was used to acquire approximately 40,000 events and data were analyzed using FlowJo software (BD Biosciences).

Copurification of proteins using anti-L1 MAb. Adherent HeLa cells grown in 60-mm dishes were infected with 1.5 PFU/cell of VACV WR, O3 deletion viruses, and an O3-inducible mutant (in the presence or absence of IPTG). After 24 h, the infected cells were lysed in cold 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, $1 \times$ Haltprotease, and phosphatase inhibitor cocktail (Thermo Fisher Scientific) for 30 min with rotation at 4°C. Lysates were clarified by centrifugation at $20,000 \times g$ for 15 min at 4°C, and the supernatants were collected and precleared with control Dynabeads (Thermo Fisher Scientific) for 2 h with rotation at 4°C. Precleared extracts were incubated with protein G Dynabeads labeled with 20 μ g of anti-L1 MAb 7D11 for 16 to 18 h with rotation at 4°C. The Dynabeads were washed extensively with cold lysis buffer, and bound proteins were eluted by resuspension of the beads in NuPage lithium dodecyl sulfate sample buffer (Thermo Fisher Scientific) and heating at 98°C for 15 min. Input, unbound, and bound proteins were resolved by SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and probed with polyclonal rabbit antisera specific for individual EFC proteins followed by horseradish peroxidase-conjugated secondary antibodies (Jackson

ImmunoResearch Laboratories, West Grove, PA) and analyzed with the SuperSignal West Dura chemiluminescence reagent (Pierce, Rockford, IL) and a chemiluminescence imaging system (SynGene, Frederick, MD).

Statistical analysis. Analyses were performed using Prism 8 (GraphPad, San Diego, CA). The Shapiro-Wilk test was done to ensure normal distribution of values. One-way analysis of variance (ANOVA) was performed on all data sets, and Tukey's test was used to correct for multiple comparisons using statistical hypothesis testing.

Data availability. Data will be made available without restriction.

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