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An Attenuated LC16m8 Smallpox Vaccine: Analysis of Full-Genome Sequence and Induction of Immune Protection§

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The potential threat of smallpox bioterrorism has made urgent the development of lower-virulence vaccinia virus vaccines. An attenuated LC16m8 (m8) vaccine was developed in 1975 from the Lister strain used in the World Health Organization smallpox eradication program but was not used against endemic smallpox. Today, no vaccines can be tested with variola virus for efficacy in humans, and the mechanisms of immune protection against the major intracellular mature virion (IMV) and minor extracellular enveloped virion (EEV) populations of poxviruses are poorly understood. Here, we determined the full-genome sequences of the m8, parental LC16mO (mO), and grandparental Lister (LO) strains and analyzed their evolutionary relationships. Sequence data and PCR analysis indicated that m8 was a progeny of LO and that m8 preserved almost all of the open reading frames of vaccinia virus except for the disrupted EEV envelope gene B5R. In accordance with this genomic background, m8 induced 100% protection against a highly pathogenic vaccinia WR virus in mice by a single vaccination, despite the lack of anti-B5R and anti-EEV antibodies. The immunogenicity and priming efficacy with the m8 vaccine consisting mainly of IMV were as high as those with the intact-EEV parental mO and grandparental LO vaccines. Thus, mice vaccinated with 10^7 PFU of m8 produced low levels of anti-B5R antibodies after WR challenge, probably because of quick clearance of B5R-expressing WR EEV by strong immunity induced by the vaccination. These results suggest that priming with m8 IMV provides efficient protection despite undetectable levels of immunity against EEV.

Variola virus (VAR), a member of the orthopoxvirus (OPV) family, is the causative agent of smallpox and caused millions of deaths before its eradication. Today, smallpox is again becoming a potential threat to humans, with abuse of VAR as a bioterrorist weapon (10, 15, 20, 26, 30, 37, 40). The World Health Organization (WHO) program for smallpox eradication indicated that vaccinia virus (VV) vaccination is the most effective preventive measure against the disease. However, WHO recommended discontinuing the vaccination in 1980 (55) due to rare (around 20 cases/10^6 vaccinees) but severe complications, such as postvaccinial encephalitis, progressive vaccinia, and eczema vaccinatum with the primary vaccination (4, 17, 34, 57). Thus, after a lag time of more than 20 years, serious attempts have been urged to restart the development of lower-virulence vaccine strains (2, 3, 9, 43, 45, 50). A vaccinia ACAM1000 clone has recently been established using cell cultures from the Dryvax (NYBH strain) vaccine (50), but it may induce myocarditis (4, 11). Modified vaccinia virus Ankara (MVA) and NYVAC (modified Copenhagen strain) replication-incompetent viruses are certainly safer but may require high vaccine doses or boosting with replication-competent vaccines (2, 9).

One of the safest replication-competent vaccines, a vaccinia virus LC16m8 strain (m8), was developed and established in the early 1970s with cell culture systems (24, 25) through a temperature-sensitive and low-virulence LC16mO intermediate clone (mO) from the Lister (Elstree) original strain (LO) that was used worldwide in the WHO program. The m8 virus exhibited the lowest levels of neurovirulence and the mildest adverse events among several vaccine strains, such as NYBH, CV1, and EM63, in monkeys, rabbits, and cortisone-induced immunocompromised mice (24, 38, 39). Its antigenicity was as high as that of the LO vaccine, not only in animals, but also in approximately 50,000 Japanese children vaccinated from 1973 to 1974 (over 90,000 doses in 1974 and 1975) with no reports of severe complications (24, 57). Based on these studies, cell culture-derived m8 was licensed in 1975 in Japan as a second-generation smallpox vaccine, but it has never been confronted with VAR.

Recent progress in molecular genetics has demonstrated that m8 has a single-nucleotide deletion creating a termination codon at amino acid (aa) position 93 in the B5R envelope (*env*) gene (47). Several papers have indicated that the destruction of B5R contributes to attenuation of poxviruses (12, 36, 44, 46, 47, 54). In turn, the B5R Env protein was suggested to function as an antigen that induces neutralizing antibodies (NAbs) to the extracellular enveloped virion (EEV) form of poxviruses (12, 19, 44). EEVs are free virions released from infected cells and may cause long-range dissemination of infection, although

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they comprise less than 1% of the virus population, the majority being the intracellular mature virion (IMV) form (12, 41, 44). In addition, B5R is also a component of viral particles on the cell surface termed cell-associated enveloped virions, which are more abundant than EEV and are important for cell-to-cell spread (44). Consequently, the spread of these VVs seems to be prevented by anti-B5R NAbs.

However, little is as yet understood regarding the mechanisms of immune protection against EEVs, cell-associated enveloped virions, and IMVs of poxviruses. Thus, a concern has arisen that the B5R truncation and other possible mutations introduced into m8 during processes of attenuation of the LO vaccine reduce the generation of the enveloped virions and therefore might make the attenuated m8 vaccine less protective or nonprotective against VAR (5, 44, 45). No vaccines, however, can be tested for efficacy against VAR in humans. Alternatively, intranasal infection with a mouse-adapted and highly pathogenic vaccinia virus Western Reserve (WR) strain provides a mouse model well suited for evaluating protective efficacy (2, 32, 50, 51).

Here, we determined and compared the full-genome sequences of the licensed m8, parental mO, and grandparental LO strains to examine whether m8 has inherited the intact genome of LO or acquired other alterations in the EEV-related genes. We also examined antibody responses to B5R, EEV, and IMV in mice after a single vaccination with m8, mO, and LO and evaluated the protective efficacy against intranasal WR challenge in vaccinated mice. The results suggest that the genes, except for B5R, of m8 are similar to those of LO and that consequently, the immunogenicity and protective efficacy of m8 are similar to those of LO.

MATERIALS AND METHODS

Cells and viruses. RK13 cells were grown in Eagle's minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). HeLa cells were cultured in Dulbecco's modified MEM containing 5% FBS. High five (Tn*5*) insect cells were cultured at 26°C in TC100 medium (JRH Bioscience, Inc.) supplemented with 10% FBS. LO, mO, m8, and WR strains of VV (kind gifts from S. Hashizume) were propagated and titrated on RK13 cell monolayers (58). The WR virus used was selected by sensitivity to 5-bromo-2-deoxyuridine before propagation. When a VV IHD-J strain was used as a high producer of EEV, the virus was freshly prepared, titrated, and inoculated into cells (41).

Purification of viral DNA. RK13 cells infected with m8, mO, or LO virus were harvested and disrupted by sonication in 10 mM Tris (pH 8.0)-1 mM EDTA buffer. Cell debris and nuclei were removed from cell lysates by low-speed centrifugation, and viruses were recovered by centrifugation at 15,000 × *g* for 40 min. Virions suspended in 0.1× Tris-EDTA were purified by centrifugation on 36% sucrose cushions and then on 20 to 40% linear sucrose density gradients, as described previously (29). After each centrifugation step, virion precipitates were resuspended by sonication to avoid virion aggregate formation. Genomic virus DNA was extracted from purified virions with sodium dodecyl sulfate-proteinase K and then with phenol-chloroform as described previously (42).

Sequence analysis of the complete viral DNA genomes. Purified viral DNA was fragmented with a HydroShear recirculating point-sink flow system (GeneMachines). DNA fragments of 1.5 to 2.5 kbp were recovered by 0.8% agarose gel electrophoresis, blunt ended, and cloned into pUC18. The inserts of the shotgun clones were amplified by PCR with primers (5′-CAGTCACGACGTTGTAAA ACGAC-3′ and 5′-GTGTGGAATTGTGAGCGGATAAC-3′) and Ex Taq polymerase (TaKaRa Bio. Inc.). The amplified DNAs were sequenced with a BigDye Terminator v3.1 Cycle Sequencing Kit on PRISM 3700 automated DNA sequencers (Applied Biosystems). The net virus nucleotide sequences were collected with PHRED/PHRAP software and then assembled and edited with Sequencher 4.0 software (GeneCodes Corp.) (13, 14). Primer walking was done for filling gaps and for confirming the order and lengths of the preassembled contigs, as well as the approximately 6-kbp inverted terminal repeats (ITRs) of both genome ends. As the terminal hairpin loops were not sequenced, the leftmost nucleotide of the assembled sequences was arbitrarily designated base number 1. The final DNA sequences of m8, mO, and LO were represented at more than 9.2-, 7.8-, and 8.9-fold redundancy, respectively, at each base position. Open reading frames (ORFs) were identified using National Center for Biotechnology Information BLAST and compared to the GenBank files of the nonredundant protein sequence database, including OPVs and the vaccinia Copenhagen (CPN) strain (21). When there was a large gap between ORFs, mini-ORFs (more than 33 aa) were tentatively predicted for m8 and mO. Noncoding regions were examined for putative early, intermediate, and late promoters with MEME version 3.0 and MAST version 3.0.

PCR analysis. DNAs from LO and mO viruses were analyzed by PCR at six randomly selected loci of LO diversity, numbers L0202, L0403, L0638, L0640, L1000, and L1100, using combinations of the LO- or mO-specific forward primers and the common reverse primers. PCR mixtures were heat denatured at 95°C for 3 min and subjected to 30 cycles of 94°C for 20 s, 63°C for 40 s, and 72°C for 1 min. When the loci L0403 and L1000 were amplified, annealing was done at 61°C. The primers used were as follows: LO-0202 (5′-AGCTATTCTACCATA GCAAAT-3′), mO-0202 (5′-AGCTATTCTACCATAGCAGAA-3′), and R-0202 (5′-CTTGGTTGGTAGAAATGCGG-3′); LO-0403 (5′-TCTAGATAA AATCACTGACTTTC-3′), mO-0403 (5′-TCTAGATAAAATCACTGACTTT T-3′), and R-0403 (5′-AGGAATATGTATAAATGCGGG-3′); LO-0638 (5′-C ATATTAGTAGTTCTGCGCAAT-3′), mO-0638 (5′-CATATTAGTAGTTCT GCGTAAG-3′), and R-0638 (5′-CATTATGGTGGCTAGTGATG-3′); LO-0640 (5′-CACCTCTACCGAATAGAGTA-3′), mO-0640 (5′-CACCTCTA CCGAATAAAGTT-3′), and R-0630 (5′-GATCTAAATAGAATGCCGACC-3′); LO-1000 (5′-TTAATAGTTGATAGATACGCATTT-3′), mO-1000 (5′-AA TAGTTGATAGATACGCGTTC-3′), and R-1000 (5′-CATTTATAACACTGT ACTAAC-3′); and LO-1100 (5′-GAACTTCAGGCTGGTGAATC-3′), mO-1100 (5′-AGAACTTCAGGCTGGTAAATT-3′), and R-1100 (5′-CCATTA GTATCCATATACCATG-3′).

Comparison of EEV *env*-related genes. The B5R gene and other EEV *env*-related genes, A33R, A34R, A36R, A56R, and F13L, of a calf lymph Lister vaccine (ListerVAX), mO, and IHD-J were amplified by PCR, sequenced, and compared in amino acid alignment with the VV CPN (GenBank M35027), WR (GenBank AY243312), and MVA (GenBank, U94848) strains and also with other OPVs: VAR (strain Bangladesh-1975; GenBank L22579), monkeypox virus (MPV) (strain Zaire-96-I-16; GenBank AF380138), and cowpox virus (CPV) (strain GRI-90; GenBank X94355).

Preparation of B5R and vaccinia virus antigens. The ectodomain of B5R was amplified from ListerVAX DNA by PCR using primers B5R-Hisf-Bgl (5′-AGA TCTACATGTACTGTACCCAC-3′) and B5R-ECTr-Bgl (5′-AGATCTATTCT AACGATTCTATTTCTTG-3′) and cloned into pGEM-Teasy (Promega). The B5R-ect insert was excised from the resultant pTe-Lis-B5R-ect and ligated into a pAcYM1 baculovirus transfer plasmid, pAcMel-His, modified with the melitin signal sequence and a six-His tag. A recombinant AcHis-Lister-B5R-ect baculovirus was constructed as described previously (33). Lysates of Tn*5* insect cells were prepared with 1% NP-40 4 days after AcHis-Lister-B5R-ect infection. The lysates were clarified by centrifugation, and the recombinant B5R protein was purified by Ni column (Invitrogen) chromatography. For VV antigens, HeLa cells were infected with LO, harvested 4 days after infection, and lysed with 1% NP-40. The lysates were clarified by centrifugation.

Tests for immunogenicity and protective efficacy. All animal experiments were approved by the Institutional Animal Care and Use Committee of the National Institute of Infectious Diseases. Groups of 15 6-week-old female BALB/c mice were vaccinated with 10^5 or 10^7 PFU of m8, mO, or LO or with PBS. On day 21, five mice from each group were sacrificed to test for prechallenge antibody responses, and the other mice were challenged intranasally with 10^6 PFU of WR in 20 μl PBS (51). The mice were observed for clinical signs, examined for bodyweight, and sacrificed 14 days after WR challenge to test for postchallenge antibody responses. The immunogenicity of the recombinant B5R protein was confirmed by subcutaneous injection of BALB/c mice three times each with mixed-in aluminum adjuvant and with the B5R antigen adsorbed to Ni-agarose beads. The immunized mice were challenged with WR as described above 12 days after the last booster injection.

Anti-B5R and anti-vaccinia virus antibody ELISA. Enzyme-linked immunosorbent assay (ELISA) plates were coated with B5R or VV antigen and blocked with 5% skim milk. Dilutions of serum samples were reacted to the plates, and bound antibodies were detected with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (Zymed Laboratory), followed by a substrate (ABTS; Roche Diagnostics). The cutoff optical density at 405 nm

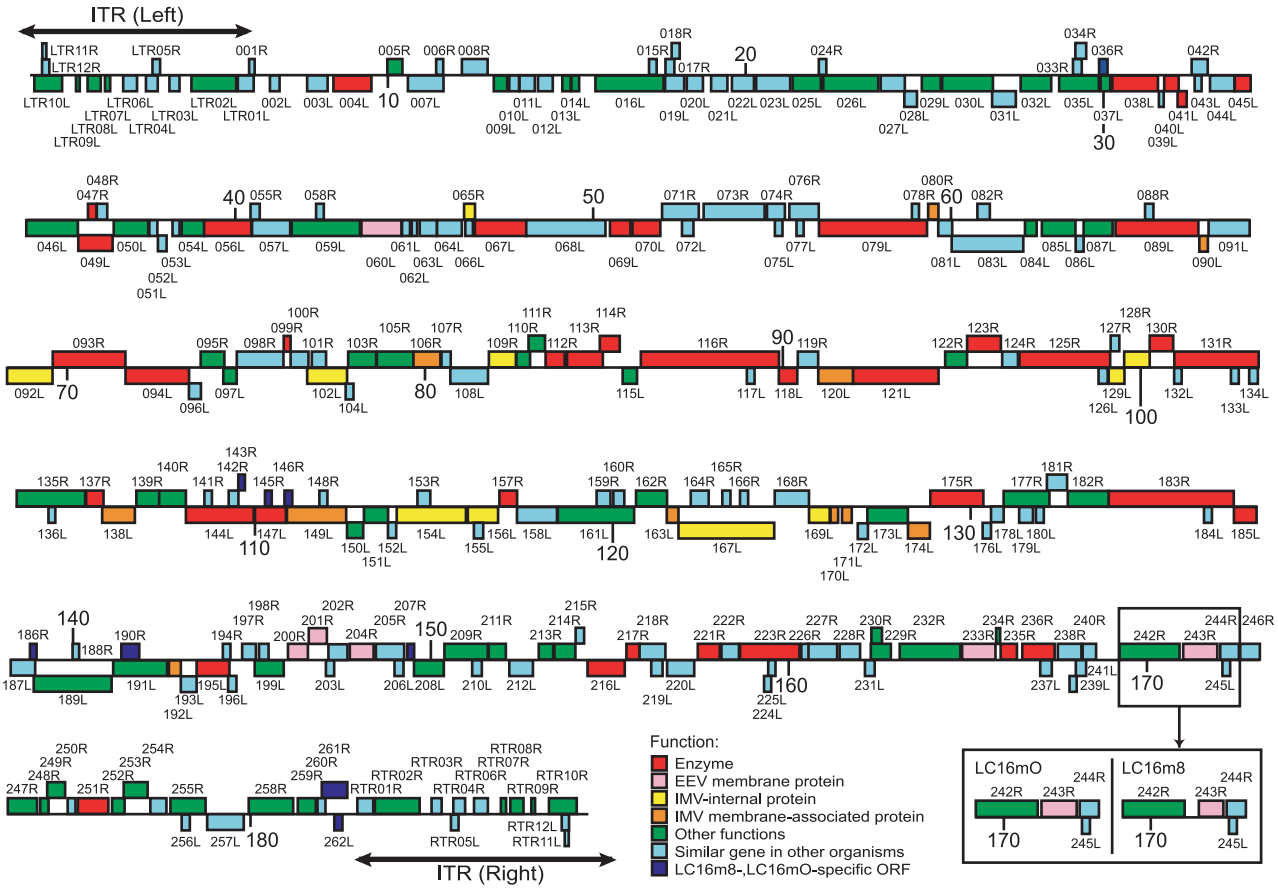

FIG. 1. ORF map of the LC16m8 and LC16mO strains. The ORFs transcribed rightward and leftward are presented above and below the horizontal centerlines, respectively. The major difference between the two strains is boxed. Putative functions of ORFs were evaluated or predicted by a BLAST search of the GenBank database and are expressed in different colors. The double-headed arrows indicate the regions of the ITRs of the left and right ends.

(OD_{405}) value of 0.2 was calculated from the average OD, plus three times the standard deviation, for five mock-immunized mouse sera.

Virus neutralization and comet inhibition assays. LO virus (100 PFU/100 μl determined on HeLa cells) was mixed with serially diluted mouse serum at 37°C for 1 h and then overnight at 4°C. HeLa cells in 24-well plates were infected with the serum-treated virus, cultured for 4 days, and stained with 0.1% crystal violet. The serum dilutions yielding a 50% plaque reduction were defined as IMV-neutralizing antibody titers. Comet-inhibiting activity in serum was examined as an indication of anti-EEV antibody responses (1). RK13 cells in 12-well plates were infected with IHD-J virus (100 PFU/well), incubated for 2 days in 2% FBS-Dulbecco's modified MEM containing mouse serum dilutions, and stained with crystal violet. The lengths of comets formed from primary plaques were measured under a microscope.

Histopathology and immunohistochemistry (IHC). The mouse nasal tissues were fixed in 10% buffered formalin and embedded in paraffin. Paraffin block sections were stained with hematoxylin and eosin (HE). VV antigens were immunohistochemically detected with a labeled-streptavidin-biotin complex staining system (DAKO). Rabbit polyclonal antibodies raised by LO infection were used as a primary antibody. A catalyzed signal amplification method (DAKO) was also used to detect VV antigens with enhanced sensitivity.

Nucleotide sequence accession numbers. The complete sequences of the vaccinia virus m8, mO, and LO strains have been deposited in GenBank under accession numbers AY678275, AY678277, and AY678276, respectively. The *env* gene sequences of IHD-J were deposited in DDBJ: A33R-A34R (accession no. AB191187), A36R (accession no. AB191188), A56R (accession no. AB191189), B5R (accession no. AB191190), and F13L (accession no. AB191191). As there were slight differences between the ListerVAX and compiled shotgun LO sequences, ListerVAX virus sequences were deposited in DDBJ as follows: B5R (accession no. AB191251), A56R (clone 1) (accession no. AB191252), and A56R (clone 3) (accession no. AB191253).

RESULTS

Complete genome sequences of m8, mO, and LO. Genomic DNA was prepared from purified m8, mO, and LO virions, shotgun sequenced, and confirmed by primer walking. As m8 and mO are clonal viruses, their genome sequences were easily assembled to 189,158 and 189,157 bp, respectively, and were analyzed with reference to the GenBank files, including the vaccinia virus CPN strain (21). Comparison of the m8 and mO genomes indicated that their gene structures and organizations were almost the same (Fig. 1 and Table 1). Notably, there were only six point mutations between m8 and mO (Fig. 2A). Three of them were in noncoding regions, probably in promoter regions. A single-amino-acid substitution was found in 4 ORFs out of 286 putative major, minor, and mini-ORFs: a T-to-G mutation caused the change from Ile to Leu in the LC16M098L (F12L for CPN) gene, and an A-to-T mutation caused the replacements of Thr with Ser in the LC16M105R (A ORF T for CPN) gene and Ser with Arg in the LC16M012L (A54L for CPN) gene. The most remarkable change was a deletion of G in the LC16M243R (B5R for CPN)

TABLE 1. ORF locations and features of the LC16m8 and LC16mO genomes

ORF	Position in LC16m8 (aa length)	Position in LC16m0	Promoter type[a]	Putative function	Category	Best-matching ORF[b]			ORF corresponding to CPN
						Name	BLASTP Score	Source	
LC16MLTR12R	300–503 (67)	–[c]		Hypothetical protein	Similar gene in other organisms	C ORF H	2e-36	CPN	C ORF H (2e-36)
LC16MLTR11R	307–420 (37)	–		Hypothetical protein	Similar gene in other organisms	C ORF G	4e-09	CPN	C ORF G (4e-09)
LC16MLTR10L	860–84 (258)	–		Major secreted protein	Other functions	VACWR001	e-113	WR	B29R (e-112)
LC16MLTR09L	1353–1249 (34)	–		Tumor necrosis factor receptor II fragment	Other functions	PredictadbyGaneMark	3e-17	CPN	PredictedbyGeneMark11 (3e-17)
LC16MLTR08L	1940–1572 (122)	–	L?	Tumor necrosis factor receptor II homologue	Other functions	VACWR004	4e-73	WR	C22L (3e-72)
LC16MLTR07L	2204–2058 (48)	–		K1R protein fragment	Other functions	VACWR005	4e-24	WR	PredictedbyGeneMark02 (5e-24)
LC16MLTR06L	2954–2568 (128)	–		Hypothetical protein	Similar gene in other organisms	VACWR007	4e-59	WR	C20L (1e-55)
LC16MLTR05R	3387–3599 (70)	–	L?	Hypothetical protein	Similar gene in other organisms	C ORF F	1e-29	CPN	C ORF F (1e-29)
LC16MLTR04L	3533–3204 (109)	–	L?,E	Hypothetical protein	Similar gene in other organisms	VACWR008	1e-62	WR	C19L (5e-57)
LC16MLTR03L	4141–3860 (93)	–		Hypothetical protein	Similar gene in other organisms	D4L	3e-41	Cowpox	PredictedbyGeneMark09 (3e-18)
LC16MLTR02L	5725–4475 (416)	–	L?	Host range protein	Other functions	C17L	0.0	CPN	C17L (0.0)
LC16M001R	6087–6242 (51)	–		Hypothetical protein	Similar gene in other organisms	TC18R	3e-65	Tian Tan	
LC16MLTR01L	6215–5772 (147)	–		Hypothetical protein	Similar gene in other organisms	C16L	4e-85	CPN	C16L (4e-85)
LC16M002L	6938–6669 (89)	–	L?	Hypothetical protein	Similar gene in other organisms	C15L	1e-35	CPN	C15L (1e-35)
LC16M003L	8281–7709 (190)	–		Hypothetical protein	Similar gene in other organisms	VACWR206	e-108	WR	C14L (3e-37)
LC16M004L	9505–8444 (353)	–	L?	Serine protease	Enzyme	C12L	0.0	CPN	C12L (0.0)
LC16M005R	9950–10372 (140)	–	L?	Growth factor	Other functions	MVA005R	3e-72	MVA	C11R (8e-69)
LC16M006R	11315–11512 (65)	–		Hypothetical protein	Similar gene in other organisms	C ORF E	e-14	CPN	C ORF E (e-14)
LC16M007L	11520–10525 (331)	–	L?	Hypothetical protein	Similar gene in other organisms	C10L	0.0	CPN	C10L (0.0)
LC16M008R	12034–12753 (239)	–	L?	Hypothetical protein	Similar gene in other organisms	C7R	e-105	Cowpox	
LC16M009L	13300–12826 (124)	–	L?	Interleukin 18 binding protein	Other functions	MVA008L	5e-64	MVA	
LC16M010L	13631–13359 (90)	–	E	Hypothetical protein	Similar gene in other organisms	ACAM3000_MVA_009	5e-50	ACAM3000	
LC16M011L	14072–13644 (142)	–	L?	Hypothetical protein	Similar gene in other organisms	ACAM3000_MVA_010	9e-80	ACAM3000	
LC16M012L	14574–14161 (137)	–	L?	Hypothetical protein	Similar gene in other organisms	VACWR015	5e-71	WR	
LC16M013L	15074–14841 (77)	–		Host range protein	Other functions	VACWR016	6e-41	WR	
LC16M014L	15311–15096 (11)	–	L?	Host range protein	Other functions	ACAM3000_MVA_013	9e-41	ACAM3000	
LC16M015R	17265–17477 (70)	–		Hypothetical protein	Similar gene in other organisms	C ORF D	8e-23	CPN	C ORF D (8e-23)
LC16M016L	17671–15767 (634)	–	L?,E	Host range protein	Other functions	C9L	0.0	CPN	C9L (0.0)
LC16M017R	17724–17972 (82)	–	L?	Hypothetical protein	Similar gene in other organisms	C ORF C	7e-33	CPN	C ORF C (7e-33)
LC16M018R	17697–18121 (74)	–		Hypothetical protein	Similar gene in other organisms	C ORF B	2e-37	CPN	C ORF B (2e-37)
LC16M019L	18247–17714 (177)	–	L?	Hypothetical protein	Similar gene in other organisms	VACWR020	e-102	WR	C8L (6e-99)
LC16M020L	18771–18319 (150)	–	L?	Hypothetical protein	Similar gene in other organisms	MVA018L	1e-88	MVA	C7L (2e-88)
LC16M021L	19455–19000 (151)	–	L?	Hypothetical protein	Similar gene in other organisms	MVA019L	6e-85	MVA	C6L (7e-85)
LC16M022L	20196–19582 (204)	–		Hypothetical protein	Similar gene in other organisms	C5L	e-120	CPN	C5L (e-120)
LC16M023L	21209–20259 (316)	–	L?,E	Hypothetical protein	Similar gene in other organisms	C4L	0.0	CPN	C4L (0.0)
LC16M024R	22010–22219 (69)	–	L?	Hypothetical protein	Similar gene in other organisms	C ORF A	2e-36	CPN	C ORF A (2e-36)
LC16M025L	22067–21276 (263)	–	L?	Complement regulatory protein	Other functions	C3L	e-159	CPN	C3L (e-159)
LC16M026L	23672–22134 (512)	–		Kelch-like protein	Other functions	C2L	0.0	CPN	C2L (0.0)
LC16M027L	24413–23739 (224)	–		Hypothetical protein	Similar gene in other organisms	C1L	e-120	CPN	C1L (e-120)

LC16M028L	24753–24400 (117)	–	L?	Hypothetical protein	Similar gene in other organisms	N1L	5e-66	CPN	N1L (5e-66)
LC16M029L	25416–24889 (175)	–		Putative alpha amanitin-sensitive protein	Other functions	N2L	e-100	CPN	N2L (e-100)
LC16M030L	26876–25458 (472)	–		Putative ankyrin isoform	Other functions	M1L	0.0	CPN	M1L (0.0)
LC16M031L	27516–26854 (220)	–	L?	Hypothetical protein	Similar gene in other organisms	M2L	e-132	CPN	M2L (e-132)
LC16M032L	28505–27651 (284)	–	E	Host range protein	Other functions	VACWR032	e-155	WR	K1L (e-153)
LC16M033R	29114–29359 (81)	–		Hypothetical protein	Similar gene in other organisms	K ORF A	4e-45	CPN	K ORF A (4e-45)
LC16M034R	29181–29483 (100)	–		Hypothetical protein	Similar gene in other organisms	K ORF B	1e-40	CPN	K ORF B (1e-40)
LC16M035L	29836–28727 (369)	–	L?,E	Serine protease inhibitor 3	Other functions	K2L	0.0	CPN	K2L (0.0)
LC16M036R	29843–30079 (78)	–	L?	Hypothetical protein	LC16m8, LC16mO specific gene				
LC16M037L	30154–29888 (88)	–	L?,E	eIF-2 alpha protein	Other functions	MVA024L	2e-50	MVA	K3L (1e-49)
LC16M038L	31488–30214 (424)	–	L?,E	Phospholipase D-like protein	Enzyme	K4L	0.0	CPN	K4L (0.0)
LC16M039L	31649–31515 (44)	–		Hypothetical protein	Similar gene in other organisms	ACAM3000_MVA_026	9e-24	ACAM3000	
LC16M040L	32068–31664 (134)	–		Putative monoglyceride lipase	Enzyme	VACWR037	1e-72	WR	K5L (2e-60)
LC16M041L	32291–32037 (84)	–		Lysophospholipase-like protein	Enzyme	K6L	1e-45	CPN	K6L (1e-45)
LC16M042R	32430–32879 (149)	–	L?	Hypothetical protein	Similar gene in other organisms	K7R	2e-86	CPN	K7R (2e-86)
LC16M043L	32708–32514 (64)	–		Hypothetical protein	Similar gene in other organisms	K8	2e-21	WR	
LC16M044L	33624–32944 (226)	–		Hypothetical protein	Similar gene in other organisms	F1L	e-122	CPN	F1L (e-122)
LC16M045L	34079–33638 (147)	–	L?	dUTP pyrophosphatase	Enzyme	MVA030L	3e-76	MVA	F2L (4e-76)
LC16M046L	35545–34103 (480)	–	L	Kalch-like protein	Other functions	F3L	0.0	CPN	F3L (0.0)
LC16M047R	35827–36063 (78)	–		Ribonucleoside-diphosphate reductase	Enzyme	F ORF B	3e-40	CPN	F ORF B (3e-40)
LC16M048R	36075–36365 (96)	–		Hypothetical protein	Similar gene in other organisms	F ORF C	3e-55	CPN	F ORF C (3e-55)
LC16M049L	36515–35556 (318)	–	E	Ribonucleoside-diphosphate reductase	Enzyme	F4L	0.0	CPN	F4L (0.0)
LC16M050L	37512–36547 (321)	–	L?,E	Major membrane protein	Other functions	F5L	e-168	CPN	F5L (e-168)
LC16M051L	37766–37542 (74)	–	L?	Hypothetical protein	Similar gene in other organisms	MVA035L	5e-40	MVA	F6L (7e-40)
LC16M052L	38024–37782 (80)	–	E	Hypothetical protein	Similar gene in other organisms	MVA036L	3e-46	MVA	F7L (6e-43)
LC16M053L	38387–38190 (65)	–	L?	Hypothetical protein	Similar gene in other organisms	ACAM3000_MVA_037	9e-25	ACAM3000	F8L (3e-24)
LC16M054L	39085–38447 (212)	–	L	Putative membrane protein	Other functions	F9L	e-121	CPN	F9L (e-121)
LC16M055R	40370–40627 (85)	–	L?	Hypothetical protein	Similar gene in other organisms	F ORF D	1e-44	CPN	F ORF D (1e-44)
LC16M056L	40391–39072 (439)	–	L	Putative ser/thr protein kinase	Enzyme	F10L	0.0	CPN	F10L (0.0)
LC16M057L	41478–40414 (354)	–	L?,E	Hypothetical protein	Similar gene in other organisms	F11L	0.0	CPN	F11L (0.0)
LC16M058R	42203–42418 (71)	–	L?	Hypothetical protein	Similar gene in other organisms	F ORF E	2e-37	CPN	F ORF E (2e-37)
LC16M059L	43428–41521 (635)	–	L?	Putative EEV maturation protein	Other functions	F12L	0.0	CPN	F12L (0.0)
LC16M060L	44588–43470 (372)	–	L	Major envelope protein	EEV membrane protein	F13L	0.0	CPN	F13L (0.0)
LC16M061L	44827–44606 (73)	–	L?,E	Hypothetical protein	Similar gene in other organisms	MVA044L	3e-28	MVA	F14L (2e-27)
LC16M062L	45026–44877 (49)	–	L	Hypothetical protein	Similar gene in other organisms	PredictedbyGeneMark	7e-22	CPN	PredictedbyGeneMark04 (7e-22)

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TABLE 1—*Continued*

ORF	Position in LC16m8 (aa length)	Position in LC16m0	Promoter type[a]	Putative function	Category	Best-matching ORF[b]			ORF corresponding to CPN
						Name	BLASTP Score	Source	
LC16M063L	45575–45099 (158)	–	L?,E	Hypothetical protein	Similar gene in other organisms	MVA045L	1e-78	MVA	F15L (6e-79)
LC16M064L	46277–45582 (231)	–	L?,E	Hypothetical protein	Similar gene in other organisms	MVA046L	e-122	MVA	F16L (e-121)
LC16M065R	46339–46644 (101)	–	L	Putative DNA-binding virion core protein	IMV internal protein	ACAM3000_MVA_047	8e-44	ACAM3000	F17R (2e-43)
LC16M066L	48586–46374 (70)	–		Hypothetical protein	Similar gene in other organisms	E ORF A	2e-27	CPN	E ORF A (2e-27)
LC16M067L	48080–46641 (479)	–	L?	Poly(A) polymerase large subunit	Enzyme	E1L	0.0	CPN	E1L (0.0)
LC16M068L	50290–48077 (737)	–		Hypothetical protein	Similar gene in other organisms	E2L	0.0	CPN	E2L (0.0)
LC16M069L	50989–50417 (190)	–		Double-stranded RNA-specific adenosine	Enzyme	MVA050L	2e-99	MVA	E3L (3e-99)
LC16M070L	51824–51045 (259)	–	L,E	DNA-directed RNA polymerese	Enzyme	E4L	e-139	CPN	E4L (e-139)
LC16M071R	51873–52898 (341)	–		Hypothetical protein	Similar gene in other organisms	E5R	0.0	CPN	E5R (0.0)
LC16M072L	52750–52430 (106)	–		Hypothetical protein	Similar gene in other organisms	E ORF B	4e-43	CPN	E ORF B (4e-43)
LC16M073R	53035–54738 (567)	–	L?	Hypothetical protein	Similar gene in other organisms	E6R	0.0	CPN	E6R (0.0)
LC16M074R	54805–55305 (166)	–	L	Hypothetical protein	Similar gene in other organisms	MVA054R	6e-89	MVA	E7R (7e-89)
LC16M075L	55236–55026 (70)	–		Hypothetical protein	Similar gene in other organisms	E ORF C	3e-38	CPN	E ORF C (3e-38)
LC16M076R	55430–56251 (273)	–	L?	Hypothetical protein	Similar gene in other organisms	MVA055R	e-161	MVA	E8R (e-160)
LC16M077L	55830–55630 (66)	–		Hypothetical protein	Similar gene in other organisms	E ORF D	5e-36	CPN	E ORF D (5e-36)
LC16M078R	58856–59053 (65)	–		Hypothetical protein	Similar gene in other organisms	E ORF E	2e-36	CPN	E ORF E (2e-36)
LC16M079L	59278–56258 (1006)	–	L,E	DNA-directed DNA polymerase	Enzyme	E9L	0.0	CPN	E9L (0.0)
LC16M080R	59310–59597 (95)	–	L	Putative redox protein	IMV membrane associated protein	MVA057R	2e-54	MVA	E10R (3e-53)
LC16M081L	59981–59592 (129)	–	L	Hypothetical protein	Similar gene in other organisms	MVA058L	3e-73	MVA	E11L (4e-73)
LC16M082R	60686–61033 (115)	–		Hypothetical protein	Similar gene in other organisms	E ORF F	3e-59	CPN	E ORF F (3e-59)
LC16M083L	61968–59968 (655)	–	E	Hypothetical protein	Similar gene in other organisms	O1L	0.0	CPN	O1L (0.0)
LC16M084L	62342–62016 (108)	–	L?	Glutaredoxin	Other functions	ACAM3000_MVA_061	8e-61	ACAM3000	O2L (1e-60)
LC16M085L	63426–62488 (312)	–	L,E	Putative DNA-binding virion care protein	Other functions	I1L	e-147	CPN	I1L (e-147)
LC16M086L	63654–63433 (73)	–	L	Hypothetical protein	Similar gene in other organisms	MVA063L	3e-28	MVA	I2L (4e-28)
LC16M087L	64464–63655 (269)	–	I	DNA binding phosphoprotein	Other functions	MVA064L	e-139	MVA	I3L (e-138)
LC16M088R	65372–65605 (77)	–		Hypothetical protein	Similar gene in other organisms	I ORF A	9e-34	CPN	I ORF A (9e-34)
LC15M089L	66862–64547 (771)	–	L?,E	Ribonucleoside-diphosphate reductase large subunit	Enzyme	I4L	0.0	CPN	I4L (0.0)
LC16M090L	67128–66889 (79)	–	L	Hypothetical protein	IMV membrane associated protein	I5L	3e-40	CPN	I5L (3e-40)
LC16M091L	68295–67147 (382)	–	L?	Hypothetical protein	Similar gene in other organisms	I6L	0.0	CPN	I6L (0.0)
LC16M092L	69559–68288 (423)	–	L	Hypothetical protein	IMV internal protein	I7L	0.0	CPN	I7L (0.0)
LC16M093R	69565–71595 (676)	–	I,L?	RNA helicase/NPH-I/NTPase II	Enzyme	I8R	0.0	CPN	I8R (0.0)
LC16M094L	73374–71599 (591)	–	L	Metalloprotease	Enzyme	G1L	0.0	CPN	G1L (0.0)
LC16M095R	73700–74362 (220)	–	L?	Putative transcriptional elongation factor	Other functions	G2R	e-127	CPN	G2R (e-127)
LC16M096L	73706–73371 (111)	–	L	Hypothetical protein	Similar gene in other organisms	G3L	2e-54	CPN	G3L (2e-54)
LC16M097L	74706–74332 (124)	–	L	Putative glutaredoxin	Other functions	MVA073L	3e-68	MVA	G4L (9e-69)
LC16M098R	74709–76013 (434)	–		Hypothetical protein	Similar gene in other organisms	G5R	0.0	CPN	G5R (0.0)

LC16M099R	76021–76212 (63)	–	L?,E	RNA polymerase	Enzyme	MVA075R	3e-26	MVA	Predicted by Gene Mark05 (5e-26)
LC16M100R	76214–76711 (165)	–	I,L?	Hypothetical protein	Similar gene in other organisms	VACWR084	2e-96	WR	G6R (3e-95)
LC16M101R	76806–77204 (132)	–	L?,E	Hypothetical protein	Similar gene in other organisms	G ORF A	1e-60	CPN	G ORF A (1e-60)
LC16M102L	77791–76676 (371)	–	L	Putative virion core protein	IMV internal	G7L	0.0	CPN	G7L (0.0)
LC16M103R	77822–78604 (250)	–	I,L?	Late transcription factor	Other functions	G8R	e-151	CPN	G8R (e-151)
LC16M104L	77970–77752 (72)	–		Hypothetical protein	Similar gene in other organisms	G ORF B	3e-38	CPN	G ORF B (3e-38)
LC16M105R	78624–79646 (340)	–	L?	Myristytprotein	Other functions	G9R	0.0	CPN	G9R (0.0)
LC16M106R	79647–80399 (250)	–	L	Myristytated membrane protein	IMV membrane associated protein	L1R	e-142	CPN	L1R (e-142)
LC16M107R	80431–80688 (85)	–	E	Hypothetical protein	Similar gene in other organisms	MVA081R	2e-29	MVA	L2R (3e-29)
LC16M108L	81730–80678 (350)	–	L	Hypothetical protein	Similar gene in other organisms	L3L	0.0	CPN	L3L (0.0)
LC16M109R	81755–82510 (251)	–	L	Putative DNA-binding virion core protein	IMV internal protein	MVA083R	e-143	MVA	L4R (e-142)
LC16M110R	82520–82906 (128)	–	L	Putative membrane protein	Other functions	MVA084R	1e-60	MVA	L5R (2e-60)
LC16M111R	82863–83324 (153)	–	L	Dimeric Virion protein	Other functions	MVA085R	3e-82	MVA	J1R (9e-83)
LC16M112R	83340–83873 (177)	–	E	Thymidine kinase	Enzyme	J2R	2e-95	CPN	J2R (2e-95)
LC16M113R	83939–84940 (333)	–	L?,E	Poly(A) polymerase subunit	Enzyme	MVA087R	e-172	MVA	J3R (e-171)
LC16M114R	84855–85412 (185)	–	L?,E	DNA-directed RNA polymerase	Enzyme	J4R	e-104	CPN	J4R (e-104)
LC16M115L	85895–85494 (133)	–	L?	Membrane protein	Other functions	J5L	4e-69	CPN	J5L (4e-69)
LC16M116R	86002–89862 (1286)	–	L?,E	DNA-directed RNA polymerase subunit	Enzyme	J6R	0.0	CPN	J6R (0.0)
LC16M117L	89180–88965 (71)	–	L?	Hypothetical protein	Similar gene in other organisms	H ORF A	8e-36	CPN	H ORF A (8e-36)
LC16M118L	90374–89859 (171)	–	L	Tyrosine phosphatase	Enzyme	MVA091L	1e-91	MVA	H1L (6e-91)
LC16M119R	90388–90957 (189)	–		Hypothetical protein	Similar gene in other organisms	H2R	e-109	CPN	H2R (e-109)
LC16M120L	91934–90960 (324)	–	L	IMV membrane associated protein	IMV membrane associated protein	MVA093L	e-172	MVA	H3L (e-171)
LC16M121L	94322–91935 (795)	–	L	RNA polymerase-associated protein	Enzyme	H4L	0.0	CPN	H4L (0.0)
LC16M122R	94508–95119 (203)	–	L?	Late transcription factor	Other functions	MVA095R	1e-83	MVA	H5R (4e-83)
LC16M123R	95120–96064 (314)	–	L	DNA topoisomerase	Enzyme	H6R	0.0	CPN	H6R (0.0)
LC16M124R	96101–96541 (146)	–	L	Hypothetical protein	Similar gene in other organisms	MVA097R	6e-82	MVA	H7R (7e-82)
LC16M125R	96585–99119 (844)	–	L?,E	mRNA capping enzyme, large subunit	Enzyme	D1R	0.0	CPN	D1R (0.0)
LC16M126L	99049–98795 (84)	–		Hypothetical protein	Similar gene in other organisms	D ORF A	7e-43	CPN	D ORF A (7e-43)
LC16M127R	99133–99375 (80)	–	L?	Hypothetical protein	Similar gene in other organisms	D ORF B	1e-24	CPN	D ORF B (1e-24)
LC16M128R	99511–100224 (237)	–	L?	Structural protein	IMV Internal protein	VACWR108	e-141	WR	D3R (e-140)
LC16M129L	89518–99078 (146)	–	L?	Putative Virion protein	IMV internal protein	MVA099L	1e-81	MVA	D2L (2e-81)
LC16M130R	100224–100850 (218)	–	E	Uracil DNA glycosytase	Enzyme	MVA101R	e-124	MVA	D4R (e-123)
LC16M131R	100912–103269 (785)	–	L,E	Putative NTPase	Enzyme	D5R	0.0	CPN	D5R (0.0)
LC16M132L	101117–100908 (69)	–	L?	Hypothetical protein	Similar gene in other organisms	D ORF C	8e-26	CPN	D ORF C (8e-26)
LC16M133L	102713–102495 (72)	–		Hypothetical protein	Similar gene in other organisms	D ORF D	7e-38	CPN	D ORF D (7e-38)
LC16M134L	103247–103005 (80)	–	L?	Hypothetical protein	Similar gene in other organisms	D ORF E	3e-45	CPN	D ORF E (3e-45)
LC16M135R	103310–105223 (637)	–	L	Early transcription factor	Other functions	D6R	0.0	CPN	D6R (0.0)
LC16M136L	104388–104197 (63)	–		Hypothetical protein	Similar gene in other organisms	F-53	2e-21	WR	
LC16M137R	105250–105735 (161)	–	L	DNA-directed RNA polymerase subunit	Enzyme	MVA104R	2e-90	MVA	D7R (6e-91)
LC16M138L	106612–105698 (304)	–		Cell surface-binding protein	IMV membrane associated protein	VACWR113	e-161	WR	D8L (e-158)

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TABLE 1—*Continued*

ORF	Position in LC16m8 (aa length)	Position in LC16m0	Promoter type[a]	Putative function	Category	Best-matching ORF[b]			ORF corresponding to CPN
						Name	BLASTP Score	Source	
LC16M139R	106654–107295 (213)	–	E	MutT-like protein	Other functions	D9R	e-121	CPN	D9R (e-121)
LC16M140R	107292–108038 (248)	–	L	MutT-like protein	Other functions	VACWR115	e-144	WR	D10R (e-142)
LC16M141R	108556–108765 (69)	–	L?	Hypothetical protein	Similar gene in other organisms	D ORF F	4e-36	CPN	D ORF F (4e-36)
LC16M142R	109234–109506 (90)	–		Hypothetical protein	Similar gene in other organisms	D ORF G	8e-51	CPN	D ORF G (8e-51)
LC15M143R	109503–109688 (61)	–		Hypothetical protein	LC16m8, LC16mO specific gene				
LC16M144L	109934–108039 (631)	–	L	Nucleoside triphosphate phosphohydrolase I, DNA helicase	Enzyme	D11L	0.0	CPN	D11L (0.0)
LC16M145R	110249–110437 (62)	–	L?	Hypothetical protein	LC16m8, LC16mO specific gene				
LC16M146R	110794–111012 (72)	–	L?	Hypothetical protein	LC16m8, LC16mO specific gene				
LC16M147L	110832–109969 (287)	–	L,E	mRNA capping enzyme, small subunit	Enzyme	VACWR117	e-166	WR	D12L (e-165)
LC16M148R	111759–111993 (74)	–	L?	Hypothetical protein	Similar gene in other organisms	D ORF I	2e-43	CPN	D ORF I (2e-43)
LC16M149L	112518–110863 (551)	–	L?	Rifampicin resistance protein	IMV membrane associated protein	D13L	0.0	CPN	D13L (0.0)
LC16M150L	112994–112542 (150)	–	I,L	Late gene transactivator	Other functions	MVA111L	1e-84	MVA	A1L (5e-85)
LC16M151L	113689–113015 (224)	–	I,L?	Late gene transactivator	Other functions	A2L	e-131	CPN	A2L (e-131)
LC16M152L	113916–113586 (76)	–	L	Hypothetical protein	Similar gene in other organisms	MVA113L	6e-42	MVA	
LC16M153R	114510–114869 (119)	–		Hypothetical protein	Similar gene in other organisms	A ORF A	2e-69	CPN	A ORF A (2e-69)
LC16M154L	115865–113931 (644)	–	L?	Major care protein	IMV internal protein	A3L	0.0	CPN	A3L (0.0)
LC16M155L	116348–116088 (86)	–		Hypothetical protein	Similar gene in other organisms	A ORF B	e-24	CPN	A ORF B (e-24)
LC16M156L	116763–115918 (281)	–	L	Membrane associated core protein	IMV internal protein	A4L	e-116	CPN	A4L (e-116)
LC16M157R	116801–117295 (164)	–	L	DNA-directed RNA polymerase subunit	Enzyme	MVA116R	5e-72	MVA	A5R (6e-72)
LC16M158L	118410–117292 (372)	–	I,L?,E	Hypothetical protein	Similar gene in other organisms	A6L	0.0	CPN	A6L (0.0)
LC16M159R	119518–119904 (128)	–	L?	Hypothetical protein	Similar gene in other organisms	A ORF C	1e-68	CPN	A ORF C (1e-68)
LC16M160R	119986–120291 (101)	–	L?	Hypothetical protein	Similar gene in other organisms	A ORF D	3e-35	CPN	A ORF D (3e-35)
LC16M161L	120566–118434 (710)	–	L?	Early transcription factor	Other functions	A7L	0.0	CPN	A7L (0.0)
LC16M162R	120620–121486 (288)	–	E	Putative intermediate transcription factor	Other functions	MVA119R	e-165	MVA	A8R (e-164)
LC16M163L	121805–121479 (108)	–	L	Hypothetical protein	IMV membrane associated protein	VACWR128	6e-42	WR	A9L (3e-40)
LC16M164R	122149–122649 (166)	–		Hypothetical protein	Similar gene in other organisms	A ORF E	2e-82	CPN	A ORF E (2e-82)
LC16M165R	123031–123258 (75)	–		Hypothetical protein	Similar gene in other organisms	A ORF F	8e-39	CPN	A ORF F (8e-39)
LC16M166R	123525–123752 (75)	–		Hypothetical protein	Similar gene in other organisms	A ORF G	5e-43	CPN	A ORF G (5e-43)
LC16M167L	124481–121806 (891)	–	L	Major core protein	IMV internal protein	A10L	0.0	CPN	A10L (0.0)
LC16M168R	124496–125452 (318)	–	L	Hypothetical protein	Similar gene in other organisms	VACWR130	e-160	WR	A11R (e-159)
LC16M169L	126032–125454 (192)	–	L	Virion protein	IMV Internal protein	A12L	2e-79	CPN	A12L (2e-79)
LC16M170L	126268–126056 (70)	–	L	Putative IMV membrane protein	IMV membrane associated protein	A13L	2e-20	CPN	A13L (2e-20)
LC16M171L	126648–126376 (90)	–	L	Putative IMV membrane protein	IMV membrane associated protein	MVA125L	5e-44	MVA	A14L (6e-44)
LC16M172L	127100–126816 (94)	–	L,E	Hypothetical protein	Similar gene in other organisms	MVA126L	2e-52	MVA	A15L (3e-52)
LC16M173L	128217–127084 (377)	–	L?	Myristylprotein	Other functions	A16L	0.0	CPN	A16L (0.0)
LC16M174L	128831–128220 (203)	–	L	Putative phosphorylated IMV membrane protein	IMV membrane associated protein	A17L	6e-86	CPN	A17L (6e-86)

LC16M175R	128846–130327 (493)	128845–130326	L?	DNA helicase	Enzyme	A18R	0.0	CPN	A18R (0.0)
LC16M176L	130541–130308 (77)	130540–130307	L	Hypothetical protein	Similar gene in other organisms	MVA130L	3e-42	MVA	A19L (4e-42)
LC16M177R	130894–132174 (426)	130893–132173	E	Putative DNA polymerase processivity factor	Other functions	A20R	0.0	CPN	A20R (0.0)
LC16M178L	130895–130542 (117)	130894–130541	L?	Hypothetical protein	Similar gene in other organisms	MVA131L	6e-57	MVA	A21L (7e-57)
LC16M179L	131714–131328 (128)	131713–131327	L?	Hypothetical protein	Similar gene in other organisms	A ORF H	6e-52	CPN	A ORF H (6e-52)
LC16M180L	132017–131796 (73)	132016–131795		Hypothetical protein	Similar gene in other organisms	A ORF I	2e-39	CPN	A ORF I (2e-39)
LC16M181R	132104–132667 (187)	132103–132668	L?,E	Hypothetical protein	Similar gene in other organisms	VACWR142	e-100	WR	A22R (1e-99)
LC16M182R	132687–133835 (382)	132686–133834	L?	Putative intermediate transcription factor	Other functions	A23R	0.0	CPN	A23R (0.0)
LC16M183R	133832–137326 (1164)	133831–137325	L?	DNA-directed RNA polymerase subunit	Enzyme	A24R	0.0	CPN	A24R (0.0)
LC16M184L	136716–138495 (73)	136715–136494		Hypothetical protein	Similar gene in other organisms	A ORF J	2e-28	CPN	A ORF J (2e-28)
LC16M185L	137963–137331 (210)	137962–137330	E	DNA-directed RNA polymerase subunit	Enzyme	A26L	1e-64	Cowpox	A26L (4e-45)
LC16M186R	138773–138958 (61)	138772–138957		Hypothetical protein	LC16m8, LC16mO specific gene				
LC16M187L	138918–138235 (227)	138517–138234	E	Hypothetical protein	Similar gene in other organisms	VACWR147	e-128	WR	
LC16M188R	139964–140146 (60)	139963–140145		Hypothetical protein	Similar gene in other organisms	TA30R	3e-18	Tian Tan	
LC16M189L	141055–138878 (725)	141054–138877	L	A-type inclusion protein	Other functions	VACWR148	0.0	WR	
LC16M190R	141327–141827 (166)	141326–141826		Hypothetical protein	LC16m8, LC16mO specific gene				
LC16M191L	142607–141099 (502)	142606–141098	L	Structural protein	Other functions	VACWR149	0.0	WR	A26L (e-115)
LC16M192L	142989–142657 (110)	142988–142656	L	Cell fusion protein	IMV membrane associated protein	MVA138L	2e-52	MVA	A27L (5e-52)
LC16M193L	143430–142990 (146)	143429–142989	L	Hypothetical protein	Similar gene in other organisms	VACWR151	2e-84	WR	A28L (7e-84)
LC16M194R	144164–144376 (70)	144163–144375	L?	Hypothetical protein	Similar gene in other organisms	A ORF K	1e-38	CPN	A ORF K (1e-38)
LC16M195L	144348–143431 (305)	144347–143430	L?	DNA-directed RNA polymerase subunit	Enzyme	A29L	e-178	CPN	A29L (e-178)
LC16M196L	144544–144311 (77)	144543–144310	L	Hypothetical protein	Similar gene in other organisms	A30L	2e-28	CPN	A30L (2e-28)
LC16M197R	144704–145087 (127)	144703–145086	L?	Hypothetical protein	Similar gene in other organisms	MVA142R	1e-61	MVA	A31R (2e-61)
LC16M198R	145175–145441 (88)	145174–145440	L?	Hypothetical protein	Similar gene in other organisms	A ORF L	1e-46	CPN	A ORF L (1e-46)
LC16M199L	145866–145054 (270)	145865–145053	L?,E	ATP/GTP-binding protein	Other functions	A32L	e-151	CPN	A32L (e-151)
LC16M200R	145984–146541 (185)	145983–146540	L?	EEV glycoprotein	EEV membrane protein	A33R	5e-96	CPN	A33R (5e-96)
LC16M201R	146565–147071 (158)	146564–147070	L,E	EEV glycoprotein	EEV membrane protein	VACWR157	2e-85	WR	A34R (8e-85)
LC16M202R	147115–147645 (176)	147114–147644	E	Hypothetical protein	Similar gene in other organisms	MVA146R	1e-93	MVA	A35R (2e-93)
LC16M203L	147275–147045 (76)	147274–147044	L?	Hypothetical protein	Similar gene in other organisms	A ORF M	7e-40	CPN	A ORF M (7e-40)
LC16M204R	147712–148377 (221)	147711–148376	L?,E	EEV membrane protein	EEV membrane protein	A36R	e-106	CPN	A38R (e-106)
LC16M205R	148441–149232 (263)	148440–149231	L?	Hypothetical protein	Similar gene in other organisms	VACWR150	e-143	WR	A37R (e-141)
LC16M206L	149213–148962 (83)	149212–148961		Hypothetical protein	Similar gene in other organisms	A ORF O	1e-41	CPN	A ORF O (1e-41)
LC16M207R	149322–149510 (62)	149321–149509	L?,E	Hypothetical protein	LC16m8, LC16mO specific gene				
LC16M208L	150340–149507 (277)	150339–149506	L?	CD47 antigen/integrin-associated protein	Other functions	A38L	e-149	CPN	A38L (e-149)
LC16M209R	150357–151568 (403)	150356–151567	L?	Semaphorin	Other functions	A39R	0.0	CPN	A39R (0.0)
LC16M210L	151402–151133 (89)	151401–151132		Hypothetical protein	Similar gene in other organisms	A ORF P	3e-51	CPN	A ORF P (3e-51)

Continued on following page

TABLE 1—*Continued*

ORF	Position in LC16m8 (aa length)	Position in LC16m0	Promoter type[a]	Putative function	Category	Best-matching ORF[b]			ORF corresponding to CPN
						Name	BLASTP Score	Source	
LC16M211R	151594–152073 (159)	151593–152072	L?,E	Natural killer cell receptor homologue	Other functions	VACWR165	4e-86	WR	A40R (5e-70)
LC16M212L	152830–152171 (219)	152829–152170	L?	Hypothetical protein	Similar gene in other organisms	MVA153L	e-131	MVA	A41L (e-129)
LC16M213R	152994–153395 (133)	152993–153394	L?	Profilin-like protein	Other functions	A42R	1e-75	CPN	A42R (1e-75)
LC16M214R	153433–154017 (194)	153432–154016	L,E	Membrane glycoprotein	Other functions	A43R	e-112	CPN	A43R (e-112)
LC16M215R	154025–154261 (78)	154024–154260	E	Hypothetical protein	Similar gene in other organisms	MVA156R	6e-23	MVA	PredictedbyGeneMark06 (1e-15)
LC16M216L	155397–154357 (346)	155396–154356	L?	Hydroxysteroid dehydrogenase	Enzyme	A44L	0.0	CPN	A44L (0.0)
LC16M217R	155444–155821 (125)	155443–155820	L?	Superoxide dismutase (Cu-Zn)-related protein	Enzyme	VACWR171	1e-70	WR	A45R (5e-69)
LC16M218R	155811–156533 (240)	155810–156532	L?,E	Hypothetical protein	Similar gene in other organisms	MVA159R	e-127	MVA	A46R (e-105)
LC16M219L	155454–156137 (105)	156453–156136		Hypothetical protein	Similar gene in other organisms	A ORF Q	6e-39	CPN	A ORF Q (6e-39)
LC16M220L	157339–156581 (252)	157337–156579	L?	Hypothetical protein	Similar gene in other organisms	VACWR173	e-129	WR	A47L (e-125)
LC16M221R	157439–158053 (204)	157437–158051	L?	Thymidytate kinase	Enzyme	A48R	e-115	CPN	A48R (e-115)
LC16M222R	158101–158589 (162)	158099–158587	L,E	Hypothetical protein	Similar gene in other organisms	A49R	2e-90	CPN	A49R (2e-90)
LC16M223R	158622–160280 (552)	158620–160278	L	ATP-dependent DNA ligase	Enzyme	A50R	0.0	CPN	A50R (0.0)
LC16M224L	159491–159291 (66)	159489–159289		Hypothetical protein	Similar gene in other organisms	A ORF R	7e-38	CPN	A ORF R (7e-38)
LC16M225L	159610–159407 (67)	159608–159405		Hypothetical protein	Similar gene in other organisms	A ORF S	3e-36	CPN	A ORF S (3e-36)
LC16M226R	160333–160554 (73)	160331–160552	L?	Hypothetical protein	Similar gene in other organisms	A51R	3e-40	CPN	A51R (3e-40)
LC16M227R	160533–161333 (266)	160531–161331		Hypothetical protein	Similar gene in other organisms	A51R	e-150	CPN	A51R (e-150)
LC16M228R	161403–161975 (190)	161401–161973		Hypothetical protein	Similar gene in other organisms	VACWR178	4e-92	WR	A52R (3e-91)
LC16M229R	162275–162835 (186)	162273–162833		Tumor necrosis factor receptor	Other functions	A53R	1e-50	VV	A53R (1e-50)
LC16M230R	162291–162587 (98)	162289–162585		Tumor necrosis factor receptor	Other functions	A ORF T	5e-40	CPN	A ORF T (5e-40)
LC16M231L	162383–162111 (90)	162381–162109		Hypothetical protein	Similar gene in other organisms	A54L	8e-49	CPN	A54L (8e-49)
LC16M232R	163083–164777 (584)	163081–164775	L?,E	Kelch-like protein	Other functions	A55R	0.0	CPN	A55R (0.0)
LC16M233R	164827–165759 (310)	164825–165757	L?	Hemagglutinin	EEV membrane protein	A56R	e-142	CPN	A56R (e-142)
LC16M234R	165777–165890 (37)	165775–165888	L	Guanylate kinase fragment	Other functions	PredictedbyGeneMark	2e-18	CPN	PredictedbyGeneMark07 (2e-18)
LC16M235R	165904–166359 (151)	165902–166357		Guanylate kinase	Enzyme	A57R	1e-82	CPN	A57R (1e-82)
LC16M236R	166510–167412 (300)	166508–167410	L?,E	Putative ser/thr protein kinase	Enzyme	MVA167R	e-178	MVA	B1R (e-177)
LC16M237L	167333–167010 (107)	167331–167008		Hypothetical protein	Similar gene in other organisms	B ORF A	2e-60	CPN	B ORF A (2e-60)
LC16M238R	167502–168161 (219)	167500–168159	L?	Hypothetical protein	Similar gene in other organisms	B2R	e-130	CPN	B2R (e-130)
LC16M239L	168029–167829 (66)	168027–167827		Hypothetical protein	Similar gene in other organisms	B ORF B	1e-35	CPN	B ORF B (1e-35)
LC16M240R	168197–168571 (124)	168195–168569		Hypothetical protein	Similar gene in other organisms	B3R	2e-62	CPN	B3R (2e-62)
LC16M241L	168292–168005 (95)	168290–168003		Hypothetical protein	Similar gene in other organisms	B ORF C	1e-52	CPN	B ORF C (1e-52)
LC16M242R	169227–170903 (558)	169225–170901	L?,E	Ankyrin repeat protein	Other functions	B4R	0.0	CPN	B4R (0.0)
LC16M243R		171004–171957[d]	L?	Plaque-size/Host range protein precursor	EEV membrane protein	MVA173R	0.0	MVA	B5R (e-179)
	171293–171958 (221)[d]			Plaque-size/Host range protein precursor	EEV membrane protein	MVA173R	e-123	MVA	B5R (e-122)
LC16M244R	172040–172561 (173)	172039–172560	I,L?,E	Hypothetical protein	Similar gene in other organisms	MVA174R	2e-99	MVA	B5R (3e-99)
LC16M245L	172317–172102 (71)	172316–172101	E	Hypothetical protein	Similar gene in other organisms	B ORF D	4e-37	CPN	B ORF D (4e-37)
LC16M246R	172599–173147 (182)	172598–173146	L	Hypothetical protein	Similar gene in other organisms	B7R	e-107	CPN	B7R (e-107)
LC16M247R	173202–174020 (272)	173201–174019	L?	Interferon-gamma receptor	Other functions	VACWR190	e-163	WR	B8R (e-161)

LC16M248R	174107–174340 (77)	174106–174339	L?	Putative ER-localized apoptosis regulator	Other functions	VACWR191	1e-42	WR	B9R (3e-42)
LC16M249R	174303–174803 (166)	174302–174802		Kelch-like protein	Other functions	B10R	5e-82	CPN	B10R (5e-82)
LC16M250R	174875–175093 (72)	174874–175092	L?	Hypothetical protein	Similar gene in other organisms	VACWR193	5e-25	WR	B11R (3e-23)
LC16M251R	175160–176011 (283)	175159–176010		Protein kinase	Enzyme	B12R	e-160	CPN	B12R (e-160)
LC16M252R	176116–176466 (116)	176115–176465		Serine protease inhibitor	Other functions	ACAM3000_MVA_161	2e-63	ACAM3000	B13R (1e-61)
LC16M253R	175441–177109 (222)	176440–177108		Serine protease inhibitor	Other functions	B14R	e-127	CPN	B14R (e-127)
LC16M254R	177186–177635 (149)	177185–177634		Hypothetical protein	Similar gene in other organisms	B15R	4e-89	CPN	B15R (4e-89)
LC16M255R	177748–178728 (326)	177747–178727	L?	Interleukin-1 binding protein precursor	Other functions	VACWR197	0.0	WR	B16R (e-166)
LC16M256L	178289–178062 (75)	178288–178061		Hypothetical protein	Similar gene in other organisms	B ORF F	4e-29	CPN	B ORF F (4e-29)
LC16M257L	179796–178774 (340)	179795–178773	L?	Hypothetical protein	Similar gene in other organisms	B17L	0.0	CPN	B17L (0.0)
LC16M258R	179936–181177 (413)	179935–181176		Ankyrin-like protein	Other functions	B18R	0.0	CPN	B18R (0.0)
LC16M259R	181307–181810 (187)	181306–181809	L?	CrmE protein	Other functions	crmE	2e-74	USSR strain	
LC16M260R	181859–182080 (73)	181858–182079	L?	Hypothetical protein	Similar gene in other organisms	CMP6L	1e-80	Camalpox	
LC16M261R	181978–182691 (237)	181977–182690	L?	Hypothetical protein	LC16m8, LC16mO specific gene				
LC16M262L	182555–182328 (75)	182554–182327		Hypothetical protein	LC16m8, LC16mO specific gene				
LC16MRTR01R	182972–183415 (147)	182971–183414		Hypothetical protein	Similar gene in other organisms	B22R	4e-85	CPN	B22R (4e-85)
LC16MRTR02R	183462–184712 (418)	183461–184711	L?	Host range protein	Other functions	B23R	0.0	CPN	B23R (0.0)
LC16MRTR03R	185046–185327 (93)	185045–185326		Hypothetical protein	Similar gene in other organisms	D4L	3e-41	Cowpox	PredictedbyGeneMark09 (3e-18)
LC16MRTR04R	185654–185983 (109)	185653–185982	L?,E	Hypothetical protein	Similar gene in other organisms	VACWR211	1e-62	WR	B25R (5e-57)
LC16MRTR05L	185800–185588 (70)	185799–185587	L?	Hypothetical protein	Similar gene in other organisms	B ORF G	1e-29	CPN	B ORF G (1e-29)
LC16MRTR06R	186233–185619 (128)	186232–186618		Hypothetical protein	Similar gene in other organisms	VACWR212	4e-59	WR	B26R (1e-55)
LC16MRTR07R	186983–187129 (48)	186982–187128		K1R protein fragment	Other functions	VACWR214	4e-24	WR	PredictedbyGeneMark02 (5e-24)
LC16MRTR08R	187247–187615 (122)	187246–187614	L?	Tumor necrosis factor receptor II homologue	Other functions	VACWR215	4e-73	WR	B26R (3e-72)
LC16MRTR09R	167834–187938 (34)	187833–187937		Tumor necrosis factor receptor II fragment	Other functions	PredictedbyGeneMark	3e-17	CPN	PredictedbyGeneMark11 (3e-17)
LC15MRTR10R	188327–189103 (258)	188326–189102		Major secreted protein	Other functions	VACWR218	e-113	WR	B29R (e-112)
LC16MRTR11L	188880–188767 (37)	188879–188766		Hypothetical protein	Similar gene in other organisms	B ORF H	e-10	CPN	B ORF H (e-10)
LC16MRTR12L	188887–188684 (67)	188886–188683		Hypothetical protein	Similar gene in other organisms	B ORF I	2e-36	CPN	B ORF I (2e-36)

[a] Regulatory sequences upstream of the ORFs were classified into early (E), intermediate (I), late (L) and putative late (L?) promoters.
[b] Best-matching ORF from BLASTP analysis of nonredundant protein database.
[c] Broken lines indicate that LC16mO ORFs were in the same positions and had the same amino acid lengths as those of LC16m8.
[d] LC16M243R ORF was full-size (317 aa) in LC16mO but was truncated (221 aa) in LC16m8.

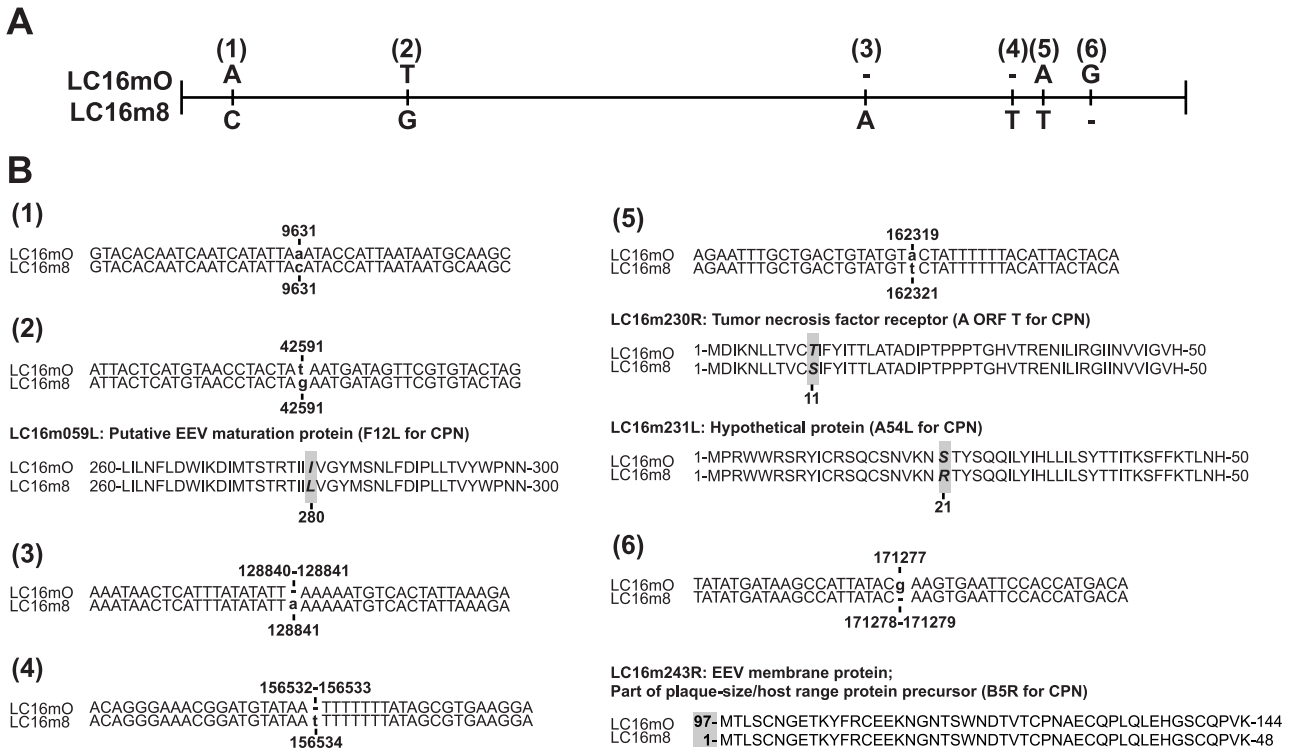


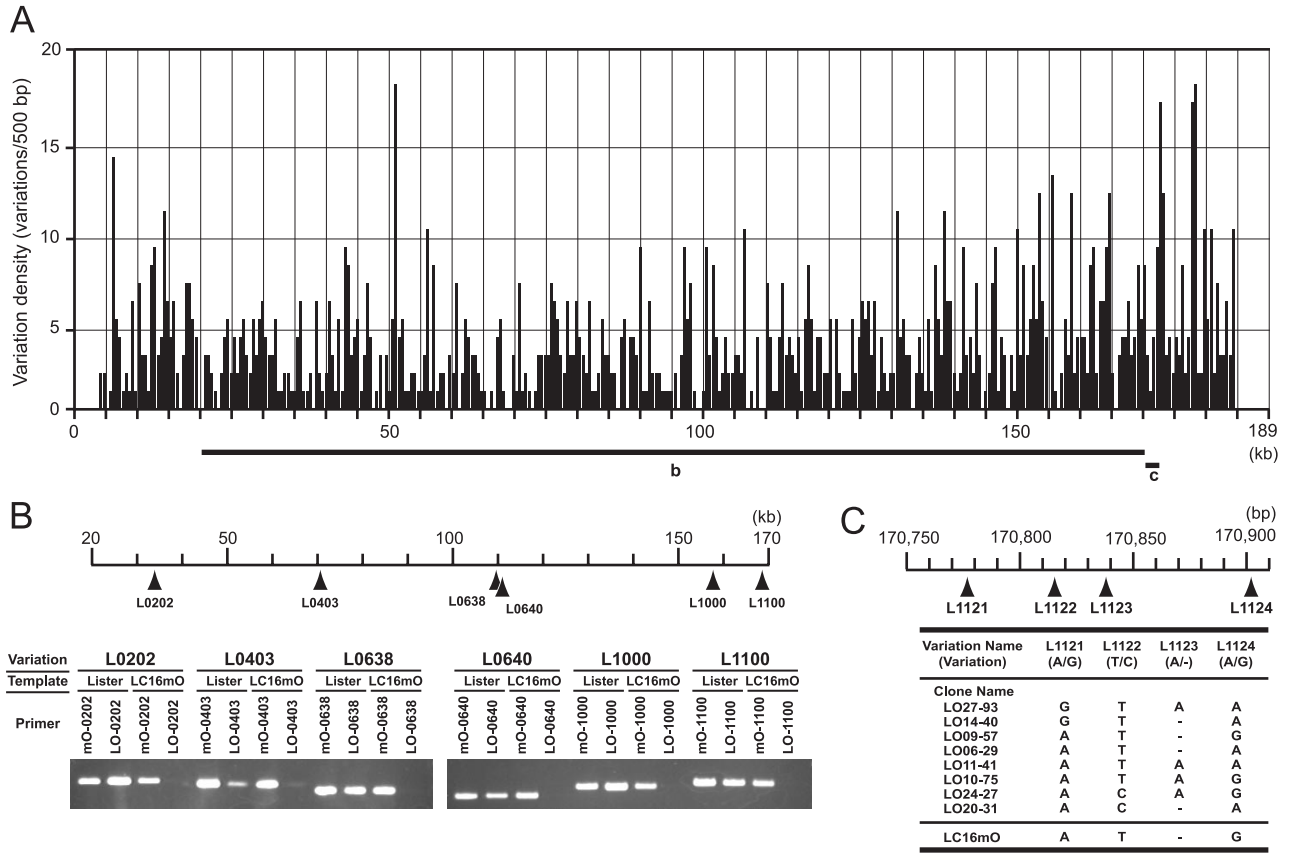
FIG. 2. Differences in nucleotide sequences between the LC16m8 and LC16mO strains. (A) The locations (1 to 6) of nucleotide point mutations in the genomes are shown schematically. (B) The nucleotide changes are shown in boldface lowercase letters. The resultant amino acid changes in ORFs are indicated by shaded boldface italics in loci (2, 5, and 6). Putative gene functions and the ORFs corresponding to the CPN strain are also shown.

gene, which generated a termination codon and truncated the B5R Env protein of m8 EEV at amino acid position 93 (Fig. 2B), as described previously (47).

Almost all of the m8 ORFs best matched those of OPV, mainly the vaccinia virus CPN strain. Therefore, m8 and CPN were strikingly similar in their genomic organizations and ORF orientations (Fig. 1 and Table 1) (21). The m8 virus retained 192 out of 198 major CPN ORFs (60 out of 65 minor CPN ORFs), including other EEV *env*-related genes, A33R, A34R, A36R, A56R, F12L, and F13L. Only a few differences were observed. CPN C21L/B27R and C19L/B24R were absent in the ITR regions of m8, although they appear to be nonessential and presumably do not represent functional genes (21). The m8 genome lacked nonessential ORFs C13L, B19R, and B20R of unknown function in the regions neighboring the ITR termini and A25L in the central coding region, which encodes a short fragment (65 aa) (21) homologous to an A-type inclusion protein of CPV (1,284 aa) (18). ORF LC16M191L (502 aa), however, corresponded to CPN A26L, also encoding a truncated homologue (322 aa) of the CPV inclusion protein (18, 21).

As LO had no history of virus cloning, nucleotide polymorphisms were observed at 1,264 sites in the genome putatively assembled by 4,913 sequencing reactions. This diversity was mapped from L0001 to L1264 along the whole genome (Fig. 3A; see Table S1 in the supplemental material). Sequences of the only marginal region spanning the diversity numbers from L1121 to L1124 (150 bp) revealed at least eight genotypes in LO, whereas mO possessed the "AT-G" genotype, which was the same as the LO09-57 clone in the region (Fig. 3C). Furthermore, PCR analysis of other randomly selected loci demonstrated that mO-specific primers amplified template LO DNA, but not vice versa (Fig. 3B). These results indicate that LO consists of a huge divergent virus population but likely contains the ancestors of mO. Because of the diversity of LO, however, it was impossible to exactly assign its consensus full-genome sequence and all ORFs. Thus, the LO shotgun sequences with major hits were tentatively assembled, compiled as an artificial genome sequence, and deposited in GenBank.

Analysis of the EEV *env*-related genes. The evolutionary relationships of the EEV *env*-related genes in Lister-related viruses were further analyzed by sequencing of PCR amplicons from ListerVAX, another batch of mO and m8, and WR and IHD-J, which were stored in our laboratory. Because the mO and m8 sequences were identical except for B5R, the resultant amino acid alignments of A33R, A34R, A36R, A56R, F13L, and B5R of ListerVAX and mO were presented with reference to those of CPN and compared to other VV strains and OPVs deposited in GenBank (Fig. 4). ListerVAX had the same amino acid alignment in A33R as wild-type (wt) VV CPN or WR. On the other hand, mO A33R had two amino acid substitutions: Asn at amino acid position 165 (Asn^{165}) was unique to mO, but Thr^{141} was found in mO and MVA, and also in VAR, MPV, and CPV of OPV (Fig. 4A). A34R was rather conserved in OPV, and no substitution was observed between ListerVAX and mO. Interestingly, however, Lys^{165} seems to be specific to VV (Arg^{165} for VAR, MPV, and CPV), and aa 110



Variation Name (Variation)	L1121 (A/G)	L1122 (T/C)	L1123 (A/-)	L1124 (A/G)
Clone Name				
LO27-93	G	T	A	A
LO14-40	G	T	-	A
LO09-57	A	T	-	G
LO06-29	A	T	-	A
LO11-41	A	T	A	A
LO10-75	A	T	A	G
LO24-27	A	C	A	G
LO20-31	A	C	-	A
LC16mO	A	T	-	G

FIG. 3. Polymorphism of the Lister strain genome. (A) Nucleotide sequence variations are presented in each 500-bp length along the central coding region of the Lister genome. (B) Six divergent loci, L0202, L0403, L0638, L0640, L1000, and L1100, were randomly selected. LO and mO genomic DNAs were amplified at the selected sites by PCR with the forward primers specific for LO or mO and the common reverse primers. (C) The marginal (150-bp) region spanning diversity numbers L1121 to -1124 of LO virus DNA were cloned, sequenced, and classified into eight genotypes. The genotype of LC16mO is also shown.

(Asn or Asp) may classify OPV into two groups (Fig. 4B). Similarly, A36R was almost conserved in VV strains but divergent in other OPVs. ListerVAX, mO, WR, and IHD-J strains of VV, however, had a common Glu^{146}-to-Lys^{146} substitution from CPN. An additional Met^{104}-to-Ile^{104} change occurred in mO, although this was also the case in VAR (Fig. 4C).

As for A56R, ListerVAX was a mixture of wt-like VV (clone 3) and an mO-type mutant (clone 1) that possessed a 5-aa deletion from Ala^{245} to Asp^{249} and a conversion of Tyr^{302} to Cys^{302}, which may be an ancestor clone of mO. Another difference between ListerVAX and mO was aa 19, which was Phe and Ser in ListerVAX and mO, respectively (Fig. 4D). Lys^{291} in F13L was unique to the Lister family viruses, whereas it was Arg^{291} in other VVs and OPVs, supporting the Lister lineage of mO. F13L Pro^{6} and Ser^{6} of ListerVAX and mO, respectively, seem to be within the divergence of OPV, because there was Pro^{6} in MVA and IHD-J and Ser^{6} in CPN, WR, VAR, and MPV (Fig. 4F). B5R is located close to the right-terminal end, and therefore, it was most divergent among the EEV *env* genes. ListerVAX differed from the compiled shotgun LO sequence in 3 nucleotides. However, the differences resulted in one amino acid substitution, from Ile^{82} to Val^{82}, which also occurred in other OPVs. There were four amino acid changes in B5R between ListerVAX (Ile^{82}, Asn^{87}, Ile^{153}, and Val^{233}) and mO (Val^{82}, Asp^{87}, Met^{153}, and Ile^{233}) (Fig. 4E).

Altogether, these results confirm the notion that mO, and consequently m8, are the progeny of LO and not so divergent from LO, wt VV, or OPV, except for B5R.

Antibody responses by vaccination. The truncated m8 and intact LO B5R proteins were compared for antigenic activity in initial experiments. BALB/c mice were subcutaneously immunized six times with the recombinant B5R proteins adsorbed to aluminum adjuvant or Ni-agarose beads. The mice were challenged by intranasal infection with 10^6 PFU of mouse-pathogenic WR virus 20 weeks after the first immunization and 12 days after the last booster injection. The LO B5R protein partially protected mice from death, with a survival rate of 78% after the appearance of severe clinical symptoms, such as ruffled fur, hunched posture, and weight loss, peaking at around 7 to 9 days after challenge. However, mice receiving the truncated m8 protein similarly developed symptoms, lost bodyweight, and died (100%) within 9 days (data not shown). These results confirm the immunogenicity of the intact B5R protein and also suggest a lack of antigenic activity of the truncated B5R protein.

Thus, B5R-defective m8 was compared with B5R-intact mO

A: A33R

aa position	20	34	59	73-5	81	95	97	112	117-8	120	127-8	141	149	164-5	171
CPN	G	L	L	AAV	S	D	K	L	QL	S	TA	I	E	SD	V
Lister	*	*	*	***	*	*	*	*	**	*	**	*	*	**	*
LC16mO	*	*	*	***	*	*	*	*	**	*	**	T	*	*N	*
WR	*	*	*	***	*	*	*	*	**	*	**	*	*	**	*
IHD-J	*	*	*	***	*	*	*	*	**	*	**	*	*	**	I
MVA	R	*	*	***	*	*	*	*	**	*	**	T	K	**	*
Variola	*	I	*	-*T	L	K	Q	F	**	*	AT	T	*	T*	*
Monkeypox	*	*	Q	S**	*	*	*	*	KS	E	A*	T	*	**	*
Cowpox	*	*	*	***	*	*	*	F	**	*	**	T	*	**	*

B: A34R

aa position	11-3	19	24	39	84	110	138	151	165
CPN	MFK	A	I	K	R	N	A	K	K
Lister	R**	*	*	*	*	*	*	*	*
LC16mO	R**	*	*	*	*	*	*	*	*
WR	R**	*	*	*	*	D	*	*	*
IHD-J	R**	*	*	*	*	*	*	E	*
MVA	R**	V	*	*	*	D	*	*	*
Variola	R**	*	*	*	*	*	*	*	R
Monkeypox	R*R	*	L	R	G	D	S	*	R
Cowpox	R*R	*	*	*	*	D	S	*	R

C: A36R

aa position	2	35	49	60	75	87	104	109	120	126	129	138 - 47	155-7	180	188	205-6	207 - 21
CPN	M	D	S	S	N	D	M	E	L	R	Q	VINETETVEV	NPN	T	E	EQ	DHEHDDIESSVVSLV
Lister	*	*	*	*	*	*	*	*	*	*	*	********K*	***	*	*	**	***************
LC16mO	*	*	*	*	*	*	I	*	*	*	*	********K*	***	*	*	**	***************
WR	*	*	*	*	*	*	*	*	*	*	*	********K*	***	*	*	**	***************
IHD-J	*	*	*	*	*	*	*	*	*	*	*	********K*	***	*	*	**	***************
MVA	*	*	*	*	*	*	*	*	*	*	*	*---------	***	*	*	**	*********----**
Variola	I	N	*	N	*	*	I	*	R	C	*	-******I**	**S	*	*	*-	---************
Monkeypox	LYIEQSE*	*	P	*	Y	N	*	K	*	*	L	***D*****I	I*S	A	D	--	----------------
Cowpox	*	*	*	*	*	*	*	*	*	*	*	**********	**S	*	*	**E	Q***************

D: A56R

aa position	2-5	16 - 22	32	35	39-41	96	102	110	124	144-7	150-2	155-6	158	160	164	172	178	180	183	190-1	196-200	206-9	213-6	234
CPN	TRLP	ATPFPQT	L	N	TND	R	V	P	T	THS	SSE	DY	D	S	S	E	V	D	T	DS	SATSG	ETPE	DKEE	G
Lister (cl-1)	****	*******	*	*	***	*	*	*	*	***	***	**	*	*	*	*	*	*	*	**	**S**	****	****	****	*
(cl-3)	****	*******	*	*	***	*	*	*	*	***	***	**	*	*	*	*	*	*	*	**	**S**	****	****	****	*
LC16mO	****	***S***	*	*	***	*	*	*	*	***	***	**	*	*	*	*	*	*	*	**	**S**	****	****	****	*
WR	****	*******	*	*	***	*	*	T	*	***	**K	**	*	*	*	*	*	*	*	**	**S**	****	****	***-	*
IHD-J	****	*******	*	*	***	*	*	T	*	***	***	ED	*	F	*	*	*	*	*	**	**S**	****	****	****	*
MVA	****	*******	*	*	***	*	*	*	*	***	***	**	*	*	*	*	*	*	*	**	**S**	****	****	****	*
Variola	***S	S**Y**IQI	*	S	I**	K	*	T	*	S**	***	**	N	F	L	G	*	N	*	*I	*TS**	K*SG	N**-	E	
Monkeypox	*Q**	V**S***		I	S	**Y	G	I	T	I	*--	I**	ED	*	*	*	*	*	*	*	**	NAS**	****	****	*	
Cowpox	A***	S**S***	*	*	*T*	*	*	T	*	***	***	**	*	*	*	*	E	*	-	EN	*T**R	****	****	*	

aa position	242 - 55	258-9	263 - 7	270-3	277-80	284-5	302	305-6
	TDDADLYDTYNDND	PS	GSSTT	SNYK	FVEI	TA	C	RS
	-----***	*P	*G***	****	****	**	*	**
	**************	*P	*G***	****	****	**	Y	**
	-----***	*P	*G***	****	****	**	*	**
	**************	*P	*G***	****	****	**	Y	**
	**************	*P	*G***	****	****	**	Y	**
	**************	**	*G***	****	****	**	Y	**
	AN**---HND*EPS	SP	KNI*K	GK*S	Y*KV	A*	*	**
	**************	*P	*G***	****	****	*T	*	HP
	**************	*P	*G***	****	****	**	*	**

F: F13L

aa position	5-7	72	98	126	173	181-3	228	250	291
CPN	ASV	A	C	D	A	CSA	R	N	R
Lister	*P*	*	*	*	*	***	K	*	K
LC16mO	***	*	*	*	*	***	K	*	K
WR	***	*	*	*	*	***	K	*	*
IHD-J	*P*	*	*	*	*	***	K	*	*
MVA	*P*	*	*	N	*	***	K	*	*
Variola	T*A	*	*	*	V	Y*S	*	*	*
Monkeypox	V**	V	S	*	*	***	K	S	*
Cowpox	*Q*	*	*	*	*	***	K	*	*

E: B5R

aa position	40-1	50	53	55	82	87	95-7	100-3	132	136	145	152-3	166	170	188	216	233 - 40	243 - 8	260	283	296	304	317
CPN	NN	Q	H	S	I	N	STM	SCNG	P	E	E	YM	A	S	I	I	VLPICVRT	EFDPVD	L	V	V	D	P
Lister	DK	*	*	L	*	*	***	****	*	*	*	*I	*	*	M	T	***T***S	K*****	*	*	*	*	*
LC16mO	DK	*	*	L	V	D	***	****	*	*	*	**	*	*	M	T	I**T***S	K*****	*	*	*	*	*
WR	DK	*	*	*	*	*	***	****	*	*	*	**	*	*	M	T	********	******	*	*	*	*	*
IHD-J	DK	*	*	L	V	D	***	****	*	*	*	**	*	*	*	*	I**T***S	K*****	*	*	*	*	*
MVA	**	*	*	*	*	*	***	****	*	*	*	*I	*	*	*	*	I**T***S	K*****	*	*	*	*	*
Variola	DK	S	Y	L	V	*	AII	I*KD	S	D	G	HI	*	T	*	*	*****I*S	*****E	*	*	*	N	L
Monkeypox	DK	S	*	L	V	D	***	****	*	*	*	**	V	*	*	T	I**T***S	******	*	M	I	*	*
Cowpox	DK	*	*	L	V	D	***	****	*	*	*	**	*	*	*	T	I**T***S	***L**	I	*	I	*	*

FIG. 4. Comparison of amino acid alignments of the EEV Env-related proteins in six vaccinia virus strains and other OPVs. The numbers at the top of each panel indicate the amino acid positions of the EEV proteins of vaccinia virus CPN strain. The asterisks and dashes show conserved and deleted amino acids, respectively, with reference to CPN. The vaccinia viruses compared are CPN, Lister (calf lymph Lister vaccine), LC16mO, WR, IHD-J, and MVA strains. Variola, monkeypox, and cowpox viruses shown for reference are Bangladesh-1975, Zaire-96-I-16, and GRI-90 strains, respectively.

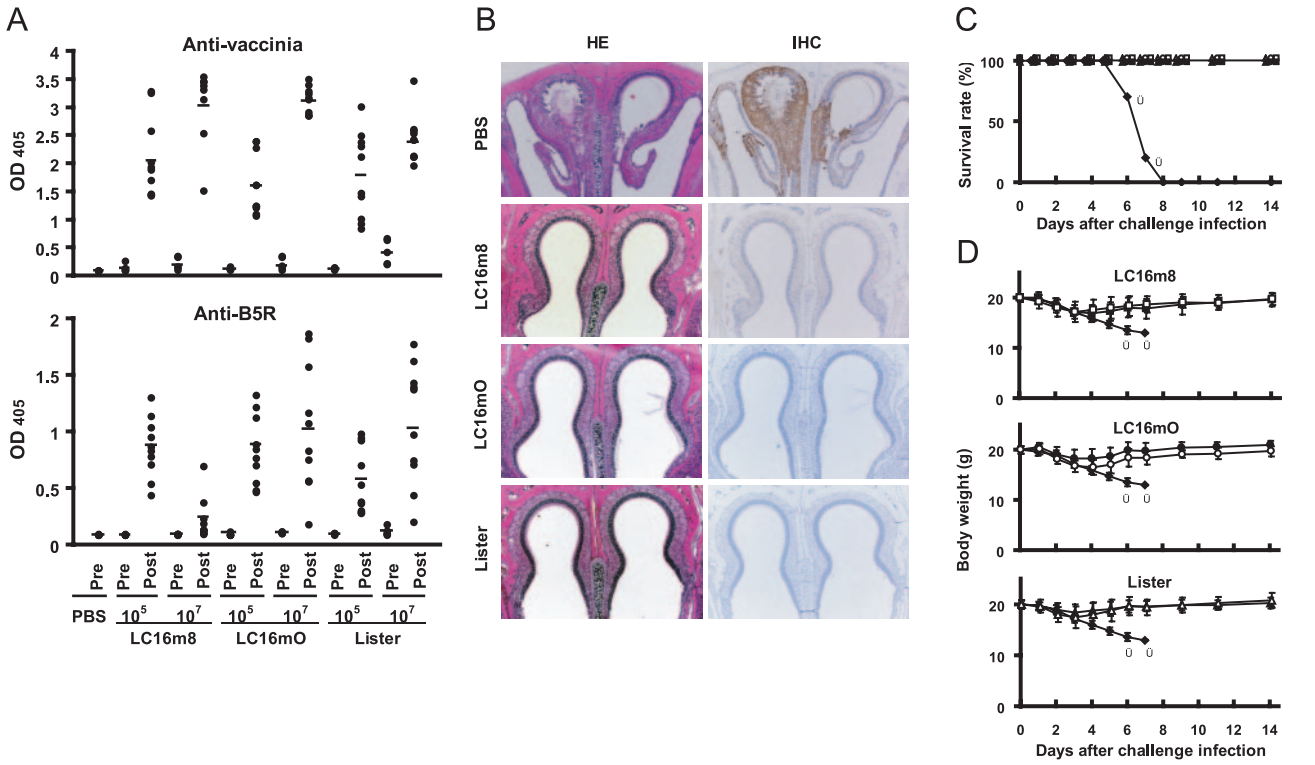


FIG. 5. Protection against lethal WR challenge by vaccination with LC16m8. Groups of 6-week-old BALB/c mice were subcutaneously vaccinated and intranasally challenged as for Table 2. (A) Levels of antibodies in pre- and postchallenge sera of individual mice. Sera were examined by ELISA for vaccinia virus- and B5R-specific antibodies, and the results are shown with OD_{405} values at 1:400 and 1:100 dilutions, respectively. The horizontal bars indicate the averages. (B) Histopathology by HE staining and IHC by peroxidase staining of the nasal tissue collected from nonimmunized and vaccinated mice 9 and 14 days after challenge infection, respectively. (C) Survival and (D) bodyweights of mice after WR challenge. The mice had been vaccinated with 10^5 (open symbols) or 10^7 (solid symbols) PFU of LC16m8 (□ and ■), LC16mO (○ and ●), or Lister (△ and ▲) strain or PBS (◆). To avoid confusion, the average bodyweight ± standard deviation is shown in separate panels in comparison with the PBS group. The crosses indicate the deaths of mice.

and LO for the ability to prime or induce anti-B5R and anti-EEV antibody responses before and after pathogenic-WR infection. BALB/c mice were vaccinated subcutaneously with a low (10^5 PFU) or high (10^7 PFU) dose of the vaccine strains. On day 21 after vaccination, one-third of the mice were bled to determine prechallenge antibody levels, and the other mice were challenged intranasally with 10^6 PFU of WR. Sera were collected 14 days later to test for postchallenge antibodies. Representative ELISA antibody levels in individual mice are shown in Fig. 5A, and the results of antibody responses examined are summarized in Table 2. ELISA antibody levels at prechallenge were low against VV antigens and undetectable against the B5R protein in all vaccinated mice. The titers and seroprevalences, if any were present, tended to be higher in 10^7

TABLE 2. Antibody responses in vaccinated mice at pre- and postchallenge infection[a]

Vaccination (day 0)		Prechallenge (day 21)				Postchallenge (day 35)			
Strain	Dose (PFU)	IgG ELISA (positive/total)		NAb	Comet inhibition	IgG ELISA (positive/total)		NAb	Comet inhibition
		Anti-vaccinia virus[b]	Anti-B5R[b]			Anti-vaccinia virus[c]	Anti-B5R[b]		
PBS		0.10 (0/5)	0.08 (0/5)	<4[d]	<10[d]	ND[e]	ND	ND	ND
Lister	10^5	0.20 (3/5)	0.09 (0/5)	<4	<10	1.78 (10/10)	0.56 (10/10)	4	<10
	10^7	1.00 (5/5)	0.11 (0/5)	16	<10	2.42 (10/10)	1.06 (10/10)	64	<10
LC16mO	10^5	0.19 (2/5)	0.09 (0/5)	<4	<10	1.60 (10/10)	0.83 (10/10)	16	<10
	10^7	0.52 (4/5)	0.10 (0/5)	4	<10	3.18 (10/10)	1.03 (9/10)	64	<10
LC16m8	10^5	0.39 (2/5)	0.08 (0/5)	<4	<10	2.08 (10/10)	0.85 (10/10)	64	<10
	10^7	0.53 (4/5)	0.08 (0/5)	<4	<10	3.14 (10/10)	0.21 (3/10)	64	<10

[a] Mice vaccinated with a single dose were challenged intranasally with 10^6 PFU of WR strain on day 21 and sacrificed on day 35.
[b] Averages of OD_{405} values at a 1:100 dilution.
[c] Averages of OD_{405} values at a 1:400 dilution.
[d] The highest serum dilutions yielding a 50% plaque reduction or inhibitory comet formation.
[e] ND, not determined.

PFU vaccination groups than in those vaccinated with 10^5 PFU. Comet inhibition activity in sera, which is an indicator of anti-EEV antibodies, was negative in each of the vaccinated groups. NAb titers to VV, that is, IMV, were also low or undetectable; titers as low as 1:4 and 1:16 were detected only in groups of mice immunized with 10^7 PFU of mO and LO, respectively (Table 2).

Upon lethal challenge with virulent WR, however, high levels of anti-vaccinia virus ELISA antibodies were induced in all groups of mice vaccinated with m8, mO, and LO. Substantial levels of anti-B5R antibodies were also detected in all groups, except for that receiving 10^7 PFU of m8, where only 3 out of 10 mice developed anti-B5R antibodies (Fig. 5A and Table 2). Therefore, mice immunized with 10^7 PFU of m8 produced significantly ($P < 0.0008$) lower levels of anti-B5R antibodies after WR infection than did those immunized with 10^5 PFU of m8, 10^7 PFU of mO, or 10^7 PFU of LO (Fig. 5A), when compared by an unpaired Student's *t* test. The lethal challenge with WR did not elicit comet inhibition activity against EEV in vaccinated mice but induced and/or augmented NAb titers to IMV ranging from 1:4 to 1:64 (Table 2). Levels of antibodies after WR challenge were higher in mice immunized with 10^7 PFU than in those immunized with 10^5 PFU, indicating that mice were effectively primed with a higher dose of vaccine and boosted by WR infection. The exception was anti-B5R antibody titers in groups receiving B5R-defective m8 (Table 2 and Fig. 5A), probably because B5R-expressing EEV of WR was more quickly cleared before eliciting anti-B5R antibodies by stronger immunity induced with 10^7 PFU of m8 than with 10^5 PFU of m8.

Pathological findings. The immunogenicities of the m8, mO, and LO vaccines were evaluated by histopathological and immunohistochemical analyses of the nasal tissue of mice, the primary infection site for pathogenic WR. The specimens from mice mock vaccinated with PBS demonstrated massive destruction and necrosis of the mucosal epithelium of the nasal cavity. The severe necrosis of olfactory epithelial cells was widespread in the nasal-cavity tissue (Fig. 5B, HE). VV antigens were distributed widely and intensively, colocalizing at the damaged areas of the epithelium (Fig. 5B, IHC). In contrast to nonimmune mice, severe epithelial destruction was rarely observed in the nasal cavities of mice vaccinated with a lower dose (10^5 PFU) of m8, mO, or LO. Their nasal specimens showed intact tissue morphology without evidence of recovery from tissue necrosis. In addition, no VV antigens were detected in nasal mucosal epithelial cells when examined by enhanced immunohistochmical staining (Fig. 5B, IHC). Similarly, no pathological changes were detectable after intranasal WR challenge in mice vaccinated with a higher dose (10^7 PFU) of m8, mO, or LO (data not shown).

Protection by m8, mO, and LO vaccines. The immunological and histopathological studies described above suggest that m8 is as effective as mO and LO against pathogenic-OPV infection. Therefore, the protective efficacies of the m8, mO, and LO vaccine strains were further estimated in additional WR challenge experiments. Groups of 10 BALB/c mice vaccinated as for immunogenicity studies were examined for survival rate (Fig. 5C) and bodyweight loss (Fig. 5D) after intranasal inoculation with 10^6 PFU of WR. As this WR dose represented 10 LD_{50} for 6-week-old BALB/c mice (data not shown), the nonimmunized mice receiving PBS developed clinical symptoms, lost bodyweight, and died within 9 days after WR challenge. In contrast, none of the mice in the m8, mO, or LO vaccination group died (Fig. 5C). Vaccinated mice developed only a transient and slight loss of bodyweight, peaking at 3 or 4 days after challenge, but looked healthy without ruffled fur, inactivity, or respiratory distress and promptly gained weight thereafter (Fig. 5D). Notably, there were no significant differences in bodyweight between the low-dose (10^5 PFU) and high-dose (10^7 PFU) vaccination groups nor among the m8, mO, and LO vaccination groups (Fig. 5D).

DISCUSSION

In this study, we suggest that an attenuated vaccinia virus m8 strain that was licensed in 1975 in Japan as the second-generation smallpox vaccine is as efficacious as the first-generation LO vaccine that was used worldwide in the WHO smallpox eradication program.

The m8 vaccine was not used in a large population in areas of endemicity because smallpox was almost eradicated when it was developed. Today, no vaccines under development or in human trials can be tested for protective efficacy against smallpox by infection of humans with the causative virus, VAR. However, a pathogenic vaccinia virus WR strain provides an alternative small-animal model suited for evaluating protective immunization (2, 32, 50, 51). VV has rather low infectivity for mice, but WR is an exception, because it is adapted to mice by repeated passages in the mouse brain (27). Intranasal inoculation with as little as 10^5 PFU of WR elicited severe illness and 50% death in BALB/c mice, although they were less susceptible to VV infection than C57BL/6 and C3H/He mice (unpublished data). Thus, BALB/c mice vaccinated with the LO and LO-derived vaccine strains failed to develop definite erythema or pustules at the inoculated skin sites, which is classified as a "take" that is indicative of viral replication and therefore successful immunization in other vaccinia virus-sensitive hosts, such as humans, cows, and rabbits. Anti-B5R, -EEV, or -IMV antibodies were certainly undetectable or at low levels in vaccinated BALB/c mice. Nevertheless, the m8, mO and LO vaccines all protected mice comparably and completely against challenge with 10^6 PFU of WR. Notably, a single subcutaneous vaccination with m8 primed mice to render them as protective as vaccination with mO and LO, even at a low dose (10^5 PFU). Furthermore, with an increased WR challenge dose (10^7 PFU), 100% of mice vaccinated percutaneously with m8 (10^5 PFU) survived, while they lost significant weight temporarily and comparably to those vaccinated with the LO or NYBH strains (unpublished data) that had been used in humans.

OPVs are known to be highly cross-reactive among themselves in immune protection. Indeed, the m8 vaccine protected monkeys against MPV challenge (unpublished data), as recently described for the MVA vaccine (9). On the basis of these historical and experiential facts, CPV is thought to have been used in 1798 as the first human vaccine against VAR, and VV became the smallpox vaccine in the modern era. Similarly, OPVs are genetically highly conserved. Complete OPV genome sequences from VV, VAR, CPV, MPV, ectromelia virus, and camelpox virus have recently been investigated for phylo-

genetic analyses, with results indicating that CPV (strain GRI) is closely related to VV and that the genetic distances from VAR were lowest for camelpox virus (<0.0155), next lowest for VV (<0.0259), high for MPV (<0.0307), and highest for ectromelia virus (<0.0354) (22). These analyses may lead to the prediction that complete genome sequence data from VVs or OPVs will provide insight into the efficacy of smallpox vaccine strains.

Therefore, we determined the complete genome sequences of the licensed m8, parental mO, and grandparental LO strains. Our data may be interpreted to mean that the LO-related vaccines have similar abilities that would induce immune protection, supporting the above-mentioned prediction. Only four missense mutations occurred among the >280 deduced ORFs of m8 during evolution from the parental mO strain. The major change was a truncating mutation of the B5R gene. It is therefore noted that B5R was the only destroyed gene in m8 compared to mO. Furthermore, m8 and mO possessed almost all ORFs corresponding to the vaccinia virus CPN strain (21). As the grandparental LO strain has never been plaque cloned, its genome sequence exhibited huge polymorphisms, which were previously suggested by analyses of restriction enzyme fragments and pock or plaque size (46, 52, 53). However, our PCR sequencing of the EEV *env*-related genes indicated that they were all preserved in mO, and in LO as well, and that m8 was probably derived from a low-virulence clone of divergent LO. This genomic background of m8 suggests that it functions like LO as a smallpox vaccine, except for B5R.

B5R is the only NAb-inducing antigen of EEV so far identified (19). EEVs are extracellular free virions released from infected cells and seem to be prevented by NAbs (12, 19, 44). Destruction of B5R reduced the formation of EEV 5- to 10-fold (36, 44, 54), although they comprise less than 1% of the total virus population (41). In light of these findings, a concern has arisen that the m8 vaccine seems to contain reduced amounts of EEV that lacks the B5R antigen and might not be protective against long-range spread of VAR EEV (5, 44, 45). Our study of multiple immunizations with recombinant B5R proteins adsorbed to adjuvant showed that antigenic activity was absent in the truncated B5R protein of m8 but present in the intact protein of LO. In addition, infection or vaccination with live VV induced very few anti-EEV NAbs, and repeated inoculations were required to induce moderate NAb levels (19, 44), probably because of the small EEV population. Alternatively, low levels of the antibodies may be due to the low sensitivity of conventional assay systems. Wyatt et al. recently reported that NAbs can be produced after a single percutaneous vaccination (56). They recently developed and used a highly sensitive system, a semiautomated flow cytometric assay with recombinant VV expressing enhanced green fluorescent protein (8).

It was therefore important to examine the levels of protection against virulent WR infection in m8-vaccinated mice, irrespective of the absence of EEV B5R-specific antibody responses. Our results confirmed that a single vaccination with m8, mO, and LO failed to induce detectable levels of anti-EEV and anti-B5R antibodies. Nevertheless, mice immunized with these vaccines were 100% protected against pathogenic WR challenge as early as 3 weeks after vaccination. Moreover, m8 with the whole B5R gene deleted protected mice from lethal WR challenge (32). These findings suggest that many viral antigens other than B5R are also involved in protective immunity to EEV. In this regard, antibodies to the A33R Env antigen did not neutralize EEV but provided mice with 100% protection (19). Anti-A33R might disrupt fragile EEV Env and convert to IMV, which is easily neutralized by anti-vaccinia antibodies (19, 28). Alternatively, A33R-specific cellular immunity may be crucial for protective immunity.

We have only limited knowledge about the protective immune mechanisms against smallpox. Experience with worldwide vaccination, however, has suggested that the protective mechanisms involve innate immunity, including interferons, natural killer cells, and complements, and also acquired immunity, including specific antibody- and T-cell-mediated immune responses (12). Indeed, recent papers have revealed the involvement of gamma interferon-expressing CD8 and CD4 T cells, vaccinia-specific cytotoxic T cells, and T-helper type 1 memory in humans (6, 7, 31, 48) and mice (16, 35, 49). Several studies conducted out of urgency in the last few years using smallpox vaccine candidates came to similar conclusions with regard to the contribution of overall immunity to smallpox protection (2, 9, 50, 56). Moreover, priming effects in vaccinated persons were recently shown to be long-lived or long-lasting, for as long as 75 years after vaccination (23). These historical and most recent studies imply that vaccine priming for immunological memory is important so that effecter components, such as NAbs, $CD4^+$ or $CD8^+$ T cells, and various cytokines can promptly be induced or boosted to protective levels by VAR infection, regardless of whether they are above measurable levels before infection. In support of this hypothesis, we found that mice that received a single dose of LO-related vaccines could not fully develop antibody responses as early as 3 weeks after vaccination but could produce enhanced levels of antibodies and complete immune protection after pathogenic-virus infection.

The need to produce safer and more effective vaccines may increase in the future. Here, we determined the nucleotide sequences of the whole genomes from the m8, intermediate mO, and original LO vaccine strains. The accumulating information on complete genome sequences of attenuated or pathogenic VVs and other OPVs will provide a basis for producing new genetically engineered vaccines. The double-stranded DNA genomes of OPVs are known to be highly stable. However, a single nucleotide insertion just upstream of the m8 B5R mutation site has recently been reported to restore the ORF to the parental mO phenotype after repeated (10 or more) virus passages. Although the repaired viruses were a marginal population, attenuation that is achieved by a deletion of the whole B5R gene prevented the reversion of m8- to mO-type viruses (32), which have, however, much lower virulence than LO and NYBH (24, 25, 39). In turn, the genetic manipulation of m8 to replace genes related to protective immunity, but not to pathogenicity, with the counterpart genes of VAR may make m8 more efficacious. It will be necessary to study in detail the correlation between individual gene functions and antigenicity of the gene products for inducing protective immunity in the future.

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