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Virulence differences between monkeypox virus isolates from West Africa and the Congo basin

Nanhai Chen[a,1], Guiyun Li[b,1], M. Kathryn Liszewski[c], John P. Atkinson[c], Peter B. Jahrling[d], Zehua Feng[a], Jill Schriewer[a], Charles Buck[e], Chunlin Wang[f], Elliot J. Lefkowitz[f], Joseph J. Esposito[g], Tiara Harms[g], Inger K. Damon[g], Rachel L. Roper[h], Chris Upton[b], R. Mark L. Buller[a,*]

[a]*Department of Molecular Microbiology and Immunology, Saint Louis University Health Sciences Center, M432, 1402 South Grand Boulevard, St. Louis, MO 63104, USA*
[b]*Department of Biochemistry and Microbiology, University of Victoria, Victoria, BC, Canada V8W 2Y2*
[c]*Division of Rheumatology, Department of Internal Medicine, Washington University School of Medicine, St. Louis, MO 63110, USA*
[d]*Headquarters, United States Army Research Institute of Infectious Diseases, Frederick, MD 21702, USA*
[e]*Virology Collection, ATCC, Manassas, VA 20108, USA*
[f]*Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL 35294, USA*
[g]*National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, GA 30333, USA*
[h]*Department of Microbiology and Immunology, Brody School of Medicine, East Carolina University, Greenville, NC 27834, USA*

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Abstract

Studies indicate that West African and Congo basin isolates of monkeypox virus (MPXV) are genetically distinct. Here, we show Congo basin MPXV-ZAI-V79 is more virulent for cynomolgus monkeys as compared to presumed West African MPXV-COP-58. This finding may explain the lack of case-fatalities in the U.S. 2003 monkeypox outbreak, which was caused by a West African virus. Virulence differences between West African and Congo basin MPXV are further supported by epidemiological analyses that observed a similar prevalence of antibodies in non-vaccinated humans in both regions, while >90% of reported cases occurred in the Congo basin, and no fatal cases were observed outside of this region. To determine the basis for this difference in virulence, we sequenced the genomes of one human West African isolate, and two presumed West African isolates and compared the sequences to Congo basin MPXV-ZAI-96-I-16. The analysis identified D10L, D14L, B10R, B14R, and B19R as possible virulence genes, with D14L (ortholog of vaccinia complement protein) as a leading candidate.

Keywords: Monkeypox; Genomic sequences; Genetic diversity; Virulence genes; Non-human primates

Introduction

MPXV causes a smallpox-like disease in humans and may threaten the population either as a zoonotic infection or through a criminal event. The case-fatality rate of human monkeypox in a 1980s prospective study in the Democratic Republic of the Congo (DRC former Zaire) was approximately 10% (Jezek and Fenner, 1988), compared to variola virus (VARV) smallpox, which ranged from >1% to about 15% in Africa and to approximately 30% in Asia (Fenner et al., 1988). Unlike smallpox, which had secondary attack rates ranging to 60%, human monkeypox during the 1980s prospective study was about 10%, with interhuman transmission rarely over two or three generations (Jezek et al., 1986). Recent retrospective studies suggest that disease

* Corresponding author. Fax: +1 314 977 8717.
E-mail address: bullerrm@slu.edu (R.M.L. Buller).
[1] These authors contributed equally to the manuscript.

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incidence is increasing due to encroachment of humans into habitats of animal reservoirs for MPXV (Mukinda et al., 1997; Hutin et al., 2001). Also, the first outbreak in the Western hemisphere occurred in the U.S. Midwest from April to June of 2003 (Reed et al., 2004). MPXV entered the U.S. in a shipment of African rodents from Ghana (West Africa) destined for the pet trade. At a pet distribution center, prairie dogs became infected and in turn were responsible for 72 confirmed or suspected cases of human monkeypox. Unlike African outbreaks, the U.S. outbreak resulted in no fatalities and there was no documented human-to-human transmission (Reed et al., 2004). This less severe epizootic could be due to higher natural resistance of the U.S. population, a healthier patient population lacking background infections, and/or better supportive care for patients. There is, however, a significant possibility that this variability in pathogenicity is secondary to strain-specific differences in the virulence of the infecting virus.

The U.S. Midwest isolates belong to the West African MPXV group which is genetically distinct from Congo basin isolates as determined by restriction fragment length polymorphisms (RFLP) and DNA sequencing analysis of the hemagglutinin and TNFR genes (Mukinda et al., 1997; Esposito and Knight, 1985; Esposito and Fenner, 2001). Virulence differences between West African and Congo basin MPXV are supported by epidemiological analyses that observed a similar prevalence of antibodies in non-vaccinated humans in both regions (Jezek and Fenner, 1988), while >90% of reported cases occurred in the Congo basin, and no fatal cases were observed outside of this region (Esposito and Fenner, 2001). In this study, we formally compare the virulence of West African and Congo basin isolates of MPXV in cynomolgus monkeys, and use comparative genomics to identify and characterize predicted genes that may be responsible for virulence differences.

Results and discussion

Virulence of West African and Congo basin isolates of MPXV in non-human primates

We examined the virulence of MPXV-COP-58 (COP-58) and a Congo basin isolate MPXV-ZAI-V79 (ZAI-V79) by aerosol infection of cynomolgus monkeys with high and low doses of virus. MPXV-COP-58, although derived from a primate shipped from Singapore to Copenhagen which developed disease 62 days after arriving in Copenhagen, is presumed to be of West African origin by virtue of restriction enzyme profiles (Esposito and Knight, 1985), geographically-restricted primate anti-orthopoxvirus seroprevalences (Arita et al., 1972), and historic inferences (Fenner et al., 1989). This supposition is strengthened by the sequence analysis provided here. MPXV-COP-58 caused no deaths and little morbidity following aerosol challenge, whereas infections with the Congo basin isolate resulted in severe morbidity at the low dose and uniform mortality at the high dose (Table 1). These data further support the contention that the Congo basin isolates are more virulent for humans than those from West Africa.

Genome sequencing of West African isolates SL-V70, WRAIR-61, and COP-58

To identify the putative genetic basis of this difference in virulence, we needed to acquire the genomic sequences of West African isolates and compare them to those from MPXV-ZAI-96-I-16 (ZAI-96), the sole Congo basin group member whose genomic sequence is available in GenBank (Shchelkunov et al., 2001, 2002). Therefore, we sequenced the genomes of West African isolates SL-V70, WRAIR-61, and COP-58 and obtained contiguous consensus sequences of 198,756, 199,195, and 199,469 nucleotides, respectively. The genomes were resolved to an average 4.8-fold or greater redundancy (lowest coverage 2-fold); we did not sequence the palindromic hairpin terminal loop (~80 bp) of each end of the genome. The first nucleotide of each genomic sequence of SL-V70, WRAIR-61, and COP-58 was equivalent to nucleotide 160, 155, and 149 of the genomic sequence of VACV-COP, respectively. This position was 25, 30, and 36 nucleotides, respectively, beyond the end of the first primer used to amplify the sequencing templates.

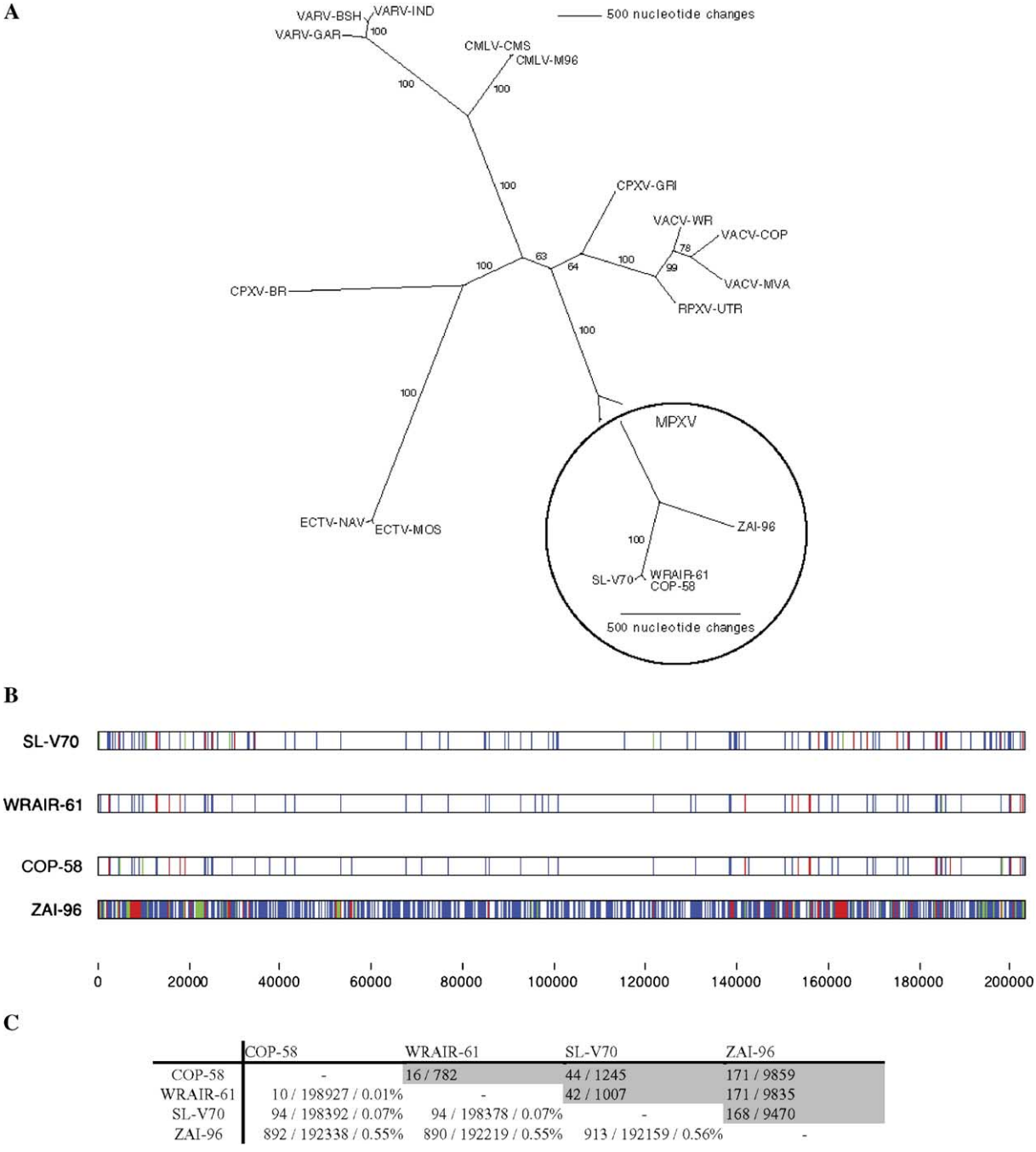
Genetic diversity between West African and Congo basin isolates

Based upon multiple nucleic acid sequence alignments of the core conserved genomic region of each orthopoxvirus (OPV) species, we determined the evolutionary relationships between these viruses which are shown in Fig. 1A. To further examine the relatedness of the MPXV isolates, we compared the genome of West African isolate SL-V70 with the genomes of the other MPXV isolates. We chose SL-V70 as the prototypic West African isolate because it was isolated from a case of human monkeypox in Sierra Leone (Foster et al., 1972). Fig. 1B is a graphic representation of the substitutions, insertions, and deletions (InDel) observed between the aligned genomes. SL-V70 is more closely

Table 1
Aerosol infection of cynomolgus monkeys with West and Congo basin isolates monkeypox virus

MPXV isolate	Aerosol dose (PFU/monkey)[a]	Morbidity (Day 7)[b]	Mortality	Mean day of death
COP-58	110	0/3	0/3	–
	20,000	0/3[c]	0/3	–
ZAI-V79	90	2/3	0/3	–
	50,000	3/3	3/3	10 ± 1

[a] An aerosol route of challenge was used, as it is possibly the major route for a person-to-person transmission of MPXV. The aerosol challenge particle size was 1–3 μm.
[b] Exanthem, enanthem, cough, depression.
[c] Late enanthem ~10 days.



C

	COP-58	WRAIR-61	SL-V70	ZAI-96
COP-58	-	16 / 782	44 / 1245	171 / 9859
WRAIR-61	10 / 198927 / 0.01%	-	42 / 1007	171 / 9835
SL-V70	94 / 198392 / 0.07%	94 / 198378 / 0.07%	-	168 / 9470
ZAI-96	892 / 192338 / 0.55%	890 / 192219 / 0.55%	913 / 192159 / 0.56%	-

Fig. 1. Genomic comparison of West African and Congo basin isolates of MPXV. (A) OPV phylogenetic predictions based upon the multiple nucleic acid sequence alignments of the core genomic region of each representative orthopoxvirus species, strain, or isolate. Bootstrap resampling confidence percentage based on 1000 replicates are displayed at each branch point. Branch lengths are proportional to the number of nucleotide changes. (B) CLUSTALW software was used to align the genomes of SL-V70, COP-58, WRAIR-61, and ZAI-96 and the alignment was manually optimized using Base-By-Base (Brodie et al., 2004). Each mismatched base was identified as a substitution (blue bar), deletion (red bar), or insertion (green bar) relative to a consensus; blue bars in all genomes indicate no consensus. InDels were counted as one mismatch regardless of size. The scale is such that several substitutions in close proximity may generate a single blue bar. (C) Summary of nucleotide difference comparisons: upper (grey) = gap number (segments) / total gap length; lower = number substitutions / number identical (non-gap) residues / percent difference (includes number of gaps).

related to isolates WRAIR-61 and COP-58 than to ZAI-96. There are no differences in the set of genes predicted for SL-V70, COP-58, and WRAIR-61, and examination of the minor sequence differences among them does not support any biological consequence for these changes (Table S1 in Appendix A). A similar comparative analysis between COP-58 and WRAIR-61 shows that they are more closely related to each other than to SL-V70, but are distinguished from each other by the number and position of minor repeat elements and a series of polyA/T tracts distributed along the

genome; these differences may be useful for distinguishing closely related isolates in molecular epidemiological studies (Supporting online data in Appendix A). Fig. 1C summarizes percent difference values for individual paired comparisons. The SL-V70, COP-58, and WRAIR-61 isolates show 0.55–0.56% difference with ZAI-96 as compared to 0.01–0.07% nucleotide difference among themselves. For comparison, we observe the following OPV intraspecies nucleotide difference values: 0.2% VARV (major strains Bangladesh-1975 [BSH-75] and India-1967); 0.4% VARV (BSH-75, major strain and Garcia-1966, minor strain); 1.1% VACV (strains WR and COP); 0.4% ECTV (NAV and MOS isolates), and a 0.1% camelpox virus (M-96 and CMS isolates). An interspecies comparison of SL-V70 with ECTV-MOS, VACV-COP, VARV-BSH-75, and CPXV-BR yields <4.9% nucleotide difference. These intraspecies nucleotide difference values are reflected in the branch length differences of the evolutionary tree shown in Fig. 1A. This high level of identity among OPVs is consistent with the genes having a similar function in all OPV. In summary, there is significantly greater sequence diversity between the West African SL-V70 and Congo basin ZAI-96 isolates than between SL-V70 and the two other presumptive West African isolates (COP-58 and WRAIR-61) indicating that analyzed West and Congo basin isolates belong to separate clades; this confirms and extends the MPXV RFLP studies of others (Mukinda et al., 1997; Douglass et al., 1994; Esposito and Knight, 1985).

Comparison of the West African SL-70 and Congo basin ZAI-96 genomes

The preceding data show that West African and Congo Basin MPXV isolates differ in virulence for cynomolgus monkeys, and that they are genetically distinct. To identify genes potentially responsible for the observed virulence difference, more extensive comparative analyses were carried out between West African SL-V70 and Congo basin ZAI-96. Within the SL-V70 genome, we predict 171 functional unique genes, 26 non-functional open reading frames (ORF) regions, and small vestiges of an additional 10 ORFs (Fig. 2, Tables 2 and 3). The ZAI-96 genome is predicted to contain 173 unique genes (Table 2) and 16 truncated ORFs. SL-V70 and ZAI-96 share 170 unique orthologs that are on average more than 99.4% identical at the protein level. Comparisons of

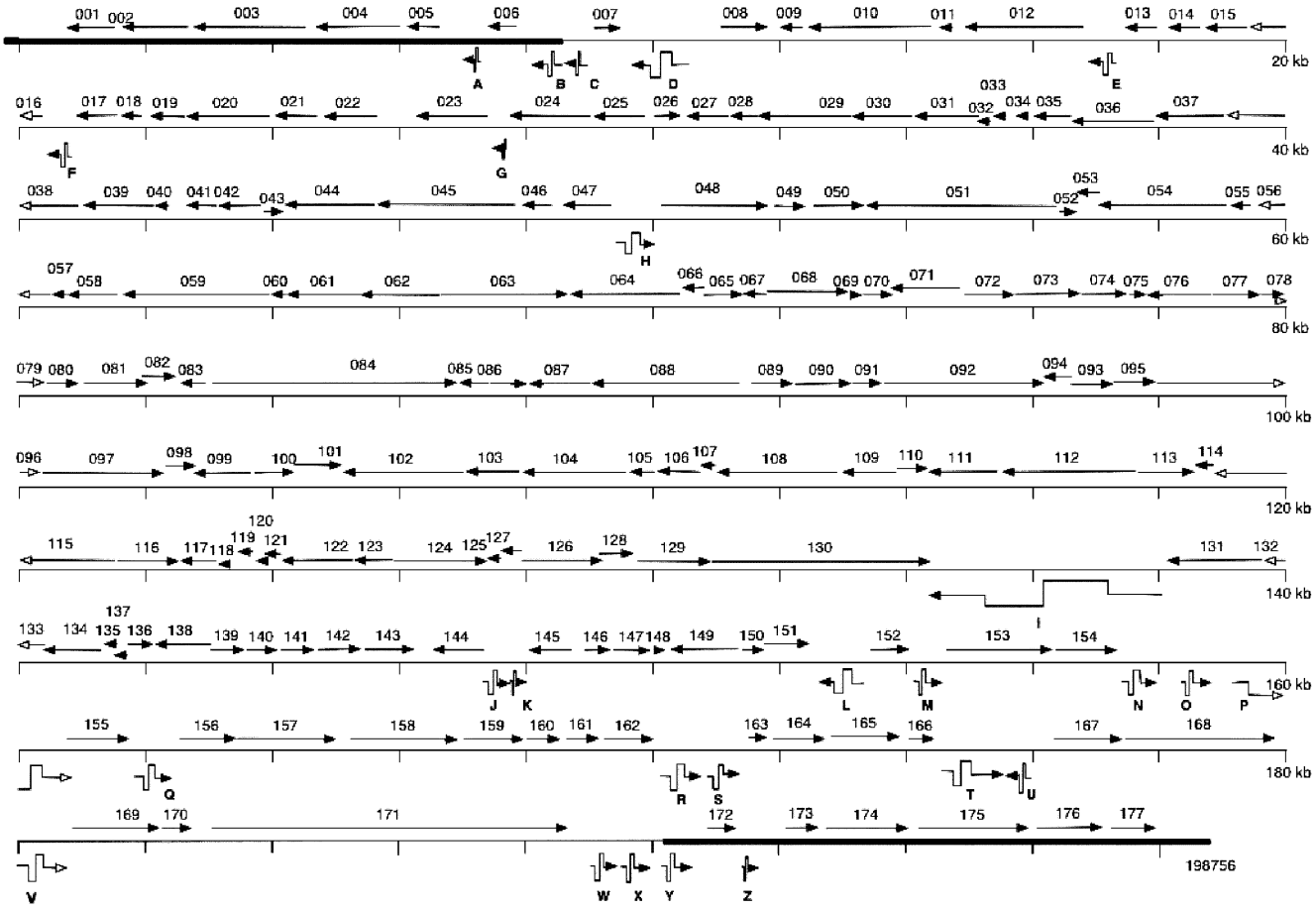


Fig. 2. Physical map of SL-V70. Predicted genes are numbered and shown as straight arrows; regions containing fragments of larger genes in other OPVs are shown with staggered arrows to represent frame changes and are labeled A–Z. Open arrowheads indicate an ORF split over 2 lines of the diagram. Scale is shown in kilobases. The thick line represents the ITR.

Table 2
Predicted ORFs in SL-V70

SL-70[a]				ZAI-96 ortholog[b]				Predicted motif/function
Name	Length[c]	Start[d]	Stop[e]	Name	Length	Identical	Identity (%)	
1	246	1510	770	J1L	246	246	100.0	SecP/CC-Chemokine BP (C23L/B29R)[f]
2	349	2686	1637	J2L	348	345	98.9	SecP/TNF BP (crmB) (BR005/226)
3	590	4548	2776	J3L	587	581	99.2	Ankyrin/unknown (BR-006/225)
4	437	6008	4695	D1L	437	432	98.9	Ankyrin/unknown (BR-017)
5[g]	176	6653	6123	–	–	–	–	Unknown (BR-018)
6[g]	153	7863	7402	–	–	–	–	Unknown (C16L/B22R)
7	142	9076	9504	D3R	142	142	100.0	Growth factor (C11R)
8	242	11,081	11,809	D5R	242	240	99.2	RING finger/apoptosis (MOS-012)
9	126	12,392	12,012	D6L	126	126	100.0	SecP/IL-18 BP (BSH-D7L)
10	660	14,434	12,452	D7L	660	650	98.5	CHO Host range (BSH-D8L)
11	64	14,764	14,570	D8L	64	63	98.4	Retroviral pseudoprotease (BR-026)
12	630	16,803	14,911	D9L	630	626	99.4	Ankyrin (C9L)
13	167	17,964	17,461	D10L	150	147	98.0	Host range (C7L)
14	159	18,617	18,138	D11L	153	151	99.3	Unknown (C6L)
15	206	19,381	18,761	D12L	206	204	99.0	Unknown (C5L)
16	316	20,376	19,426	D13L	315	312	98.7	IL-1 receptor antagonist (C4L)
–	–	–	–	D14L[h]	216	–	–	Inhibitor of complement enzymes (C3L)
17	214	21,561	20,917	D19L	214	213	99.5	Unknown (C1L)
18	117	21,960	21,607	P1L	117	117	100.0	Cytoplasmic P/virulence (N1L)
19	177	22,620	22,087	P2L	177	176	99.4	α-Amanitin sensitivity (N2L)
20	446	23,988	22,648	O1L	442	439	99.3	Ankyrin/unknown (M1L)
21	220	24,720	24,058	O2L	220	218	99.1	Unknown (M2L)
22	284	25,679	24,825	C1L	284	283	99.7	Ankyrin/host range (K1L)
23	374	27,422	26,298	C2L	375	373	99.5	Serpin (SPI-3)/unknown (K2L)
24	424	29,041	27,767	C4L	424	423	99.8	Phospholipase D-like/unknown (K4L)
25	276	29,899	29,069	C5L	276	274	99.3	MG lipase-like/unknown (BR045)
26	149	30,035	30,484	C6R	149	146	98.0	Unknown (K7R)
27	219	31,206	30,547	C7L	219	216	98.6	Apoptosis inhibitor (F1L)
28	151	31,673	31,218	C8L	151	151	100.0	dUTPase (F2L)
29	492	33,148	31,670	C9L	487	477	98.4	Kelch-like/unknown (F3L)
30	319	34,118	33,159	C10L	319	319	100.0	R. Reductase-small (F4L)
31	343	35,180	34,149	C11L	343	339	98.8	Major membrane protein (F5L)
32	73	35,358	35,137	C12L	73	73	100.0	Unknown (F6L)
33	74	35,598	35,374	C13L	74	74	100.0	Unknown (F7L)
34	64	35,944	35,750	C14L	64	64	100.0	Proline rich P/unknown (F8L)
35	212	36,639	36,001	C15L	212	212	100.0	Putative MP/unknown (F9L)
36	439	37,945	36,626	C16L	439	438	99.8	Ser/Thr kinase/morphogen (F10L)
37	354	39,032	37,968	C17L	354	353	99.7	Unknown (F11L)
38	635	40,983	39,076	C18L	635	630	99.2	IEV, actin tail, microtubule inter. (F12L)
39	372	42,144	41,026	C19L	372	370	99.5	Phospholipase/EEV (F13L)
40	73	42,383	42,162	C20L	73	73	100.0	Unknown (F14L)
41	158	43,131	42,655	C21L	158	158	100.0	Unknown (F15L)
42	231	43,833	43,138	C22L	231	231	100.0	MP/unknown (F16L)
43	101	43,896	44,201	C23R	101	101	100.0	IMV, DNA bound PP (F17R)
44	479	45,637	44,198	F1L	479	477	99.6	Poly(A) pol large (E1L)
45	737	47,847	45,634	F2L	737	736	99.9	Unknown (E2L)
46	153	48,432	47,971	F3L	153	153	100.0	PKR/OAS inhibitor (E3L)
47	259	49,377	48,598	F4L	259	257	99.2	RNA pol (RPO30) VITF-01 (E4L)
48	567	50,150	51,853	F5R	567	565	99.7	Unknown (E6R)
49	166	51,935	52,435	F6R	166	166	100.0	Myristyl MP/EEV (E7R)
50	273	52,539	53,360	F7R	273	272	99.6	ER-localized MP/unknown (E8R)
51	1006	56,387	53,367	F8L	1006	1002	99.6	DNA pol (E9L)
52	95	56,419	56,706	F9R	95	93	97.9	IMV, -S-S-bond PW (E10R)
53	129	57,090	56,701	F10L	129	129	100.0	IMV, core (E11L)
54	665	59,074	57,077	Q1L	665	663	99.7	MP/unknown (O1L)
55	108	59,447	59,121	Q2L	108	108	100.0	Glutaredoxin/unknown (O2L)
56	312	60,532	59,594	I1L	312	312	100.0	IMV, core, morphogen (I1L)
57	73	60,760	60,539	I2L	73	73	100.0	MP/unknown (I2L)
58	269	61,570	60,761	I3L	269	268	99.6	DNA-binding PP (I3L)
59	771	63,967	61,652	I4L	771	764	99.1	R. Reductase-large (I4L)
60	79	64,235	63,996	I5L	79	79	100.0	MP/IMV (I5L)

Table 2 (*continued*)

SL-70[a]				ZAI-96 ortholog[b]				Predicted motif/function
Name	Length[c]	Start[d]	Stop[e]	Name	Length	Identical	Identity (%)	
61	382	65,402	64,254	I6L	382	381	99.7	Telomere BP (I6L)
62	423	66,666	65,395	I7L	423	423	100.0	IMV, core, CP (I7L)
63	676	66,672	68,702	I8R	676	675	99.9	RNA helicase, NPH-II (I8R)
64	591	70,481	68,706	G1L	591	587	99.5	Metalloprotease (G1L)
65	220	70,807	71,469	G3R	220	220	100.0	VLTF (G2R)
66	111	70,813	70,478	G2L	111	111	100.0	SecP/unknown (G3L)
67	124	71,813	71,439	G4L	124	124	100.0	IMV, -S-S-bond PW (G4L)
68	434	71,816	73,120	G5R	434	431	99.3	Unknown (G5R)
69	63	73,129	73,320	G6R	63	63	100.0	RNA pol (RPO7) (G5.5R)
70	165	73,320	73,817	G7R	165	162	98.2	Unknown (G6R)
71	371	74,897	73,782	G8L	371	370	99.7	IMV, core, matrix (G7L)
72	260	74,928	75,710	G9R	260	260	100.0	VLTF-1 (G8R)
73	340	75,730	76,752	G10R	340	340	100.0	Myristyl MP/unknown (G9R)
74	250	76,753	77,505	M1R	250	250	100.0	Myristyl MP/IMV (L1R)
75	92	77,537	77,815	M2R	92	91	98.9	MP/unknown (L2R)
76	344	78,825	77,791	M3L	344	343	99.7	Unknown (L3L)
77	251	78,850	79,605	M4R	251	251	100.0	IMV, core, ssDNA binding (L4R)
78	128	79,615	80,001	M5R	128	128	100.0	MP/unknown (L5R)
79	152	79,958	80,416	L1R	152	151	99.3	MP/IMV, morphogen (J1R)
80	177	80,436	80,969	L2R	177	175	98.9	Thymidine kinase (J2R)
81	333	81,035	82,036	L3R	333	331	99.4	Poly(A) poly-small (VP39) (J3R)
82	185	81,951	82,508	L4R	185	185	100.0	RNA pol (RPO22) (J4R)
83	133	82,971	82,570	L5L	133	133	100.0	MP/unknown (J5L)
84	1286	83,078	86,938	L6R	1286	1278	99.4	RNA pol (RPO147) (J6R)
85	171	87,450	86,935	H1L	171	171	100.0	Tyr/Ser phosphatase/unknown (H1L)
86	189	87,464	88,033	H2R	189	188	99.5	MP/unknown (H2R)
87	324	89,011	88,037	H3L	324	322	99.4	MP/IMV (H3L)
88	795	91,399	89,012	H4L	795	791	99.5	RNA pol assoc P 94 (H4L)
89	210	91,584	92,216	H5R	213	208	97.7	VLTF-4 (H5R)
90	314	92,217	93,161	H6R	314	313	99.7	DNA topo type I (H6R)
91	144	93,199	93,633	H7R	146	143	99.3	MP/unknown (H7R)
92	845	93,677	96,214	E1R	845	843	99.8	Capping enzyme-large (D1R)
93	233	96,606	97,307	E3R	233	233	100.0	IMV, core (D3R)
94	146	96,613	96,173	E2L	146	146	100.0	IMV, core (D2L)
95	218	97,307	97,963	E4R	218	218	100.0	Uracil-DNA glycosylase (D4R)
96	785	97,995	100,352	E5R	785	784	99.9	N. triphosphat./DNA replication (D5R)
97	637	100,392	102,305	E6R	637	635	99.7	VETF-small (D6R)
98	161	102,332	102,817	E7R	161	161	100.0	RNA pol (RPO18) (D7R)
99	304	103,694	102,780	E8L	304	302	99.3	MP/IMV, attach (D8L)
100	213	103,736	104,377	E9R	213	213	100.0	MutT-like/unknown (D9R)
101	248	104,374	105,120	E10R	248	248	100.0	MutT-like/unknown (D10R)
102	631	107,016	105,121	E11L	631	627	99.4	NPH-I/IMV (D11L)
103	287	107,914	107,051	E12L	287	287	100.0	Capping enzyme-small (D12L)
104	551	109,600	107,945	E13L	551	548	99.5	IMV, morphogen, rif resist (D13L)
105	150	110,076	109,624	A1L	150	150	100.0	VLTF-2 (A1L)
106	224	110,771	110,097	A2L	224	223	99.6	VLTF-3 (A2L)
107	77	111,001	110,768	A3L	77	77	100.0	Thioredoxin/-S-S-bond PW (A2.5L)
108	644	112,950	111,016	A4L	644	644	100.0	IMV, core, precursor of p4b (A3L)
109	281	113,848	113,003	A5L	281	278	98.9	IMV, matrix, morphogen (A4L)
110	161	113,886	114,371	A6R	161	161	100.0	RNA pol (RPO19) (A5R)
111	372	115,486	114,368	A7L	372	372	100.0	Unknown (A6L)
112	710	117,642	115,510	A8L	710	709	99.9	VETF-large (A7L)
113	292	117,696	118,574	A9R	292	290	99.3	VITF-3-S (A8R)
114	112	118,893	118,555	A10L	100	99	88.4	MP/IMV, morphogen (A9L)
115	891	121,569	118,894	A11L	891	888	99.7	IMV, core, precursor of p4a (A10L)
116	318	121,584	122,540	A12R	318	318	100.0	MP/unknown (A11R)
117	190	123,114	122,542	A13L	190	190	100.0	IMV, core (A12L)
118	70	123,350	123,138	A14L	70	70	100.0	MP/IMV (A13L)
119	90	123,728	123,456	A15L	90	90	100.0	MP/IMV, morphogen (A14L)
120	53	123,906	123,745	A15.5L	53	53	100.0	MP/IMV, virulence (A14.5L)
121	94	124,180	123,896	A16L	94	94	100.0	Unknown (A15L)

(continued on next page)

Table 2 (*continued*)

SL-70[a]				ZAI-96 ortholog[b]				Predicted motif/function
Name	Length[c]	Start[d]	Stop[e]	Name	Length	Identical	Identity (%)	
122	377	125,297	124,164	A17L	377	374	99.2	Myristyl P/unknown (A16L)
123	204	125,914	125,300	A18L	204	203	99.5	MP/IMV, morphogen (A17L)
124	492	125,929	127,407	A19R	492	489	99.4	IMV, core, DNA helicase (A18R)
125	77	127,621	127,388	A20L	77	76	98.7	Unknown (A19L)
126	426	127,968	129,248	A22R	426	425	99.8	DNA pol processivity (A20R)
127	115	127,969	127,622	A21L	115	114	99.1	SecP/unknown (A21L)
128	187	129,178	129,741	A23R	187	187	100.0	Holiday junction resolvase (A22R)
129	382	129,761	130,909	A24R	382	381	99.7	VITF-3L (A23R)
130	1164	130,906	134,400	A25R	1164	1160	99.7	RNA pol (RPO132) (A24R)
131	506	139,626	138,106	A28L	520	501	96.4	MP/IMV, P4c IF (BSH A30L)
132	110	140,009	139,677	A29L	110	108	98.2	MP/IMV (A27L)
133	146	140,450	140,010	A30L	146	146	100.0	SecP TM/unknown (A28L)
134	305	141,368	140,451	A31L	305	301	98.7	RNA pol (RPO35) (A29L)
135	78	141,567	141,331	A32L	77	76	97.4	IMV, matrix, morphogen (A30L)
136	142	141,727	142,155	A33R	142	140	98.6	Unknown (A31R)
137	42	141,728	141,600	A32.5L	42	42	100.0	Unknown (A30.5L)
138	300	143,024	142,122	A34L	300	298	99.3	ATPase/DNA packaging (A32L)
139	181	143,052	143,597	A35R	181	180	99.5	MP/CEV, EEV (A33R)
140	168	143,602	144,108	A36R	168	167	99.4	MP/CEV, EEV (A34R)
141	176	144,152	144,682	A37R	176	175	99.4	Unknown (A35R)
142	228	144,728	145,414	A38R	212	208	98.1	MP/IEV (A36R)
143	268	145,466	146,272	A39R	268	264	98.5	Unknown (A37R)
144	277	147,357	146,524	A40L	277	275	99.3	MP, CD47-like/unknown (A38L)
145	221	148,758	148,093	A41L	221	219	99.1	SecP/virulence (A41L)
146	133	148,961	149,362	A42R	133	133	100.0	Profilin-like (A42R)
147	196	149,400	149,990	A43R	197	193	98.5	MP/unknown (A43R)
148	74	150,010	150,234	A44R	74	73	98.7	Unknown (MVA-156R)
149	346	151,370	150,330	A45L	346	345	99.7	Hydroxysteroid DH (A44L)
150	125	151,417	151,794	A46R	125	125	100.0	Superoxide dismutase-like (A45R)
151	240	151,784	152,506	A47R	240	239	99.6	Inhibits NF-κB activation (A46R)
152	204	153,459	154,073	A49R	204	203	99.5	Thymidylate kinase (A48R)
153	559	154,642	156,321	A50R	554	554	100.0	DNA ligase (A50R)
154	334	156,362	157,366	A51R	334	331	99.1	Unknown (A51R)
155	313	160,850	161,791	B2R	313	310	99.0	MP/CEV, EEV, HA (A56R)
156	303	162,553	163,464	B3R	299	299	100.0	Ser/Thr kinase/unknown (B1R)
157	505	163,520	165,037	B4R	503	500	99.4	Unknown (B2R/B3R)
158	564	165,226	166,920	B5R	561	559	99.1	Ankyrin/unknown (B4R)
159	317	167,024	167,977	B6R	317	316	99.7	MP/CEV, EEV (B5R)
160	176	168,049	168,579	B7R	176	174	98.9	MP/unknown (B6R)
161	182	168,617	169,165	B8R	182	180	98.9	ER P/virulence (B7R)
162	267	169,220	170,023	B9R	267	267	100.0	SecP/IFN-γ BP (B8R)
–	–	–	–	B10R[h]	221	–		Virulence factor (BR-203)
163[g,i]	98	171,559	171,855	–	–	–	–	Unknown (COP-B11R)
164	282	171,921	172,769	B11R	282	280	99.3	Ser/Thr kinase/unknown (B12R)
165	344	172,869	173,903	B12R	344	343	99.7	Serpin (SPI-2)/apoptosis (BR-207)
166	149	174,030	174,479	B13R	149	146	98.0	MP/unknown (B15R)
–	–	–	–	B14R[h]	326	–	–	IL-1β BP (BR-209)
167	352	176,361	177,419	B16R	352	351	99.7	IFN-α/β BP (B19R)
168	787	177,488	179,851	B17R	793	781	98.5	Ankyrin/unknown (BSH-B18R)
169	397	180,861	182,054	B19R	357	352	98.6	Serpin (SPI-1)/unknown (C12L)
170	190	182,226	182,798	B20R	190	187	98.4	MP/unknown (BR-218)
171	1880	183,055	188,697	B21R	1879	1861	99.0	MP/unknown (BSH B22R)
172	153	190,894	191,355	N1R	153	152	99.4	Unknown (B22R)
173	176	192,104	192,634	N3R	176	174	98.9	Unknown (BR-018)
174	437	192,749	194,062	N4R/D1L	437	432	98.9	Ankyrin/unknown (BR-017)
175	590	194,209	195,981	J1R	587	581	99.2	Ankyrin/unknown (BR-006/225)
176	349	196,071	197,120	J2R	348	345	98.9	SecP/TNF BP (crmB) (BR-005/226)
177	246	197,247	197,987	J3R	246	246	100.0	SecP/CC chemokine BP (C23L/B29R)

Table 3
Fragmented ORFs of VL-V70 genome

Region	Longest OPV ortholog	Motif/putative function
A/Y	CPXV-BR-016 (764aa)	Ankyrin motif/unknown
B/Z	CMLV-M96-006 (237aa)	MAR assoc P[a]/unknown
C	CPXV-BR-020 (170aa)	Unknown
D	CPXV-BR-022 (331aa)	IL-1 receptor antagonist[b]
E	VACV-COP-C8L (184aa)	Unknown
F	CPXV-BR-035 (512aa)	Kelch-like/unknown
G	VACV-COP-K3L (88aa)	IFN resistance
H	CPXV-BR-071 (319aa)	Virosome component
I	CPXV-BR-158 (1284aa)	A-type inclusion body
J	CPXV-BR-176 (409aa)	Semaphorin
K	VACV-COP-A40R (168aa)	Lectin/virulence
L	CPXV-BR-185 (244aa)	Unknown
M	CPXV-BR-187 (162aa)	Unknown
N	CPXV-BR-190 (190aa)	TLR signaling inhibitor
O	CPXV-BR-191 (186aa)	TNF binding protein
P	CPXV-BR-193 (563aa)	Kelch-like/unknown
Q	CPXV-BR-195 (197aa)	Guanylate kinase
R	CPXV-BR-203 (225aa)	Virulence factor
S	CPXV-BR-204 (501aa)	Kelch-like/unknown
T	ECTV-MOS-163 (328aa)	IL-1β binding protein
U	CPXV-BR-210 (340aa)	Unknown
V	ECTV-MOS-167 (559aa)	Kelch-like/unknown
W	CPXV-BR-221 (320aa)	TNF binding protein
X	CPXV-GIR-K3R (167aa)	TNF binding protein

[a] *N*-methyl-D-aspartate receptor-associated protein.
[b] Kluczyk et al., 2002.

transcription regulatory sequences of SL-V70 and ZAI-96 ORFs revealed no major differences (Supporting online data in Appendix A).

We identified 170 InDels of a combined length of 9629 nucleotides and 852 substitution mutations out of a total of 193,094 nucleotides when comparing aligned genomes of SL-V70 and ZAI-96. One hundred and forty-seven InDels and 192 substitutions are in intergenic regions and fragmented genes. Five InDels and 321 substitutions occur in 84 genes with functions thought to be essential for virus replication in standard tissue culture cell lines (e.g. functions mainly necessary for transcribing mRNA, replicating the genomic DNA, assembling infectious virions, etc.). These genes are highly conserved in all sequenced OPVs and mainly map to a central conserved region (Table 4; see footnote b for map location in SL-V70). A further 16 genes that encode similar functions map to the left and right terminal regions of the genome, and contain 63 substitutions and 4 InDels. Because SL-V70 and ZAI-79 show similar kinetics of replication and cell-to-cell spread in tissue culture (Fig. S1 in Appendix A), we think it less likely that mutations located in these genes are responsible for the virulence difference noted between COP-58 and ZAI-79 in Table 1.

The Virulence Ortholog family of genes

We hypothesize the observed virulence phenotype maps to a gene(s) in the terminal regions of the MPXV genome that is important for maximizing virus replication and/or spread within the tested non-human primate species. This group of 56 genes of known and unknown functions is collectively referred to as the Virulence Ortholog family, and includes 3 genes only present in ZAI-96 and 53 genes present in both SL-V70 and ZAI-96. There are 14 InDels and 276 substitutions in the 53 genes common to both isolates. These mutations are responsible for 61 conservative, 93 non-conservative, and 121 silent aa changes and changes in predicted lengths of 16 proteins that are mainly involved N- and C-terminal extensions (Table 4, see footnotes). The mutational burden in each gene is proportional to length. Although the CPXV-BR-219 ortholog SL-V70

Notes to Table 2:
Abbreviations: SecP, secreted protein; BP, binding protein; CHO, Chinese hamster ovary; P, protein; MG, monoglyceride lipase; R, ribonucleotide; inter, interaction; MP, membrane protein; IEV, intracellular enveloped virion; EEV, extracellular enveloped virion; PP, phosphoprotein; pol, polymerase; PKR, dsRNA-dependent protein kinase; OAS, 2′–5′ oligoadenylate synthetase; VITF, viral intermediate transcription factor; Myristyl P, myristylated protein; PW, pathway; IMV, intracellular mature virion; morphogen, morphogenesis; N triphosphat, nucleotide triphosphatase; CP, cysteine proteinase; VLTF, viral late transcription factor; topo, topoisomerase; VETF, viral early transcription factor; attach, attachment; rif resist, rifampicin resistance; IF, inclusion factor; TM, transmembrane; DH, dehydrogenase; HA, hemagglutinin.
Predictions: secreted proteins by SignalP V1.1; membrane proteins by TMpred.
[a] DNA encoding remnants of CPXV-BR-001, -002, -063, -174, -216, -228, -229, and VACV-COP-C15L were present, but the residual coding sequences were not annotated.
[b] ZAI-96 genome was reannotated as described in the Materials and methods section. This process removes ORFs that are vestiges of conserved ORFs present in other poxviruses or small predicted ORFs on the non-coding strand. We removed 16 fragmented ORFs that were previously annotated, including D2L, D4L, D15L, D16L, D17L, D18L, C3L, A26L, A27L, A48R, B1R, B15L, B18R, K1R, N2R, and R1R.
[c] Length, number of aa in ORF.
[d] Start, first nucleotide of start codon.
[e] Stop, last nucleotide of stop codon.
[f] Orthologus ORF in the VACV-COP, unless otherwise indicated (MOS, ECTV-MOS; BR, CPXV-Brighton Red; BSH, VARV-BSH; TIA, VACV-TIA; MVA, VACV-MVA, and ZAI, ZAI-96).
[g] An ortholog not present in the corresponding region of ZAI-96.
[h] An ortholog is not present in SL-V70.
[i] SL-V70 ORF 163 is the single gene predicted in the SL-V70 isolate, and a number of other OPVs that is not annotated in ZAI-96. Orthologs of this predicted protein range in size from 72–106 due to a highly variable N-terminal region that contains different lengths of an Asp–Thr repeat. A single InDel, approximately one third into the ORF, induces a frameshift that results in an early termination codon in an otherwise complete ZAI-96 gene. Since a promoter has not been characterized for this predicted ORF, it is not possible to predict what polypeptides are likely to be made by the viruses of these two groups. Database searches with these predicted proteins failed to yield any significant matches to non-OPV proteins.

171 has 33 mutations over its 1879 aa length, this is 1.75 mutations per 100 aa that is identical to the 1.75 mutations per 100 aa noted for this group of genes as a whole. It is difficult to evaluate the effect of a mutation(s) on protein function without a detailed understanding of the relationship of protein structure to function, but we have examined in

Table 4
Mutation in SL-V70 and ZAI-96[a] members of the Virulence Ortholog family

Virulence Ortholog family[b]	Predicted function/motif	Orthologs		Type of Mutation		Consequence of mutation			
		ZAI-96	SL-V70	Subs	InDel[c]	Conservative aa changes[d]	Non conservative aa changes	Silent changes	Length change of ORF (bp)
VACV-COP-C23L	CC-chemokine BP	J1L	1/177	5				5	
VACV-COP-C22L	TNF BP (Crm B)	J2L	2/176	6	1	2	1	3	3
CPXV-BR-006	Unknown/ankyrin repeat	J3L	3/175	6	1	1	3	2	9[e]
CPXV-BR-017	Unknown/ankyrin repeat	D1L	4/174	8		3	2	3	
VACV-COP-C11R	Growth factor	D3R	7	4				4	
CPXV-BR-023	RING finger/apoptosis	D5R	8	2		1	1		
CPXV-BR-025	CHO Host Range	D7L	10	14		5	5	4	
VACV-COP-C9L	Unknown/ankyrin repeat	D9L	12	7		1	3	3	
VACV-COP-C7L	Host range	D10L	13	4		3		1	51[e]
VACV-COP-C6L	Unknown	D11L	14	2	1		1	1	18[f]
VACV-COP-C5L	Unknown	D12L	15	3		1	1	1	
VACV-COP-C4L	IL-1β antagonist	D13L	16	5[g]	1	1	2	1	3
VACV-COP-C1L	Unknown	D19L	17	4			1	3	
VACV-COP-C3L	Inhib. of complement enzymes	D14L				Gene Deleted in SL-V70[h]			
VACV-COP-N2L	α-amanitin sensitivity	P2L	19	2			1	1	
VACV-COP-M1L	Unknown	O1L	20	5	1	1	2	2	12[e]
VACV-COP-M2L	Unknown	O2L	21	2		1	1		
VACV-COP-K1L	Host range	C1L	22	5			1	4	
VACV-COP-K2L	Serpin-3 (SPI-3)	C2L	23[i]	4	1	2		2	3
VACV-COP-K4L	Phospholipase D-like	C4L	24	2			1	1	
CPXV-BR-045	Putative monoglyceride lipase	C5L	25	4		2		2	
VACV-COP-K7R	Unknown	C6R	26	5		1	2	2	
VACV-COP-F1L	Apoptosis inhibitor	C7L	27	3		2	1		
VACV-COP-F3L	Unknown	C9L	29	13	2	4	6	6	15[e,j]
VACV-COP-F5L	Unknown/membrane protein	C11L	31	5		1	3	1	
VACV-COP-A31R	Unknown	A33R	136	2		1	1		
VACV-COP-A30.5L	Unknown	A32.5L	137	1				1	
VACV-COP-A35R	Unknown	A37R	141	2[k]			1		
VACV-COP-A37R	Unknown	A39R	143	8		1	3	4	
VACV-COP-A38L	CD47-like	A40L	144	5		1	1	3	
VACV-COP-A41L	Virulence/secreted protein	A41L	145	7		1	1	5	
VACV-COP-A43R	Unknown/membraneprotein	A43R	147	5	1		3	2	3
CPXV-BR-181	Unknown	A44R	148	1		1			
VACV-COP-A44L	Hydroxysteroid dehydrogenase	A45L	149	1			1		
VACV-COP-A46R	IL-1 signaling inhibitor	A47R	151	5			1	4	
CPXV-BR-189	Unknown	A51R	154	5		2	1	2	
CPXV-BR-197	Unknown	B4R	157	10		1	3	6	6[e,l]
VACV-COP-B4R	Unknown	B5R	158	3	1	2		1	9[m]
VACV-COP-A6R	Unknown/membrane protein	B7R	160	3		1	1	1	
VACV-COP-B7R	Virulence, ER resident	B8R	161	3			2	1	
CPXV-BR-203	Virulence factor	B10R				protein fragmented in SL-V70[n]			
VACV-COP-B12R	Ser/Thr Kinase	B11R	164	3		1	1	1	
CPXV-BR-207	Serpin-2 (SPI-2)	B12R	165[i]	3			1	2	
VACV-COP-B15R	Unknown/membrane protein	B13R	166	4			3	1	
CPXV-BR-209	IL-1β bp	B14R				protein fragmented in SL-V70[o]			
VACV-COP-B19R	IFN-α/β receptor	B16R	167	3			1	2	
CPXV-BR-213	Unknown/ankyrin repeat	B17R	168	8	1	2	4	2	−18[p]
VACV-COP-C12L	Serpin-1 (SPI-1)	B19R	169	8		1	4	3	120[e]
CPXV-BR-219	Unknown/membrane protein	B20R	170	3		2	1		
CPXV-BR-219	Unknown/surface glycoprotein	B21R	171	33[q]	1	5	13	13	3
VACV-COP-C16L	Unknown	N1R	172	3			1	2	
CPXV-GRI-C2L	Unknown	N3R	173	2		1	1		
Total (56 genes including 2x gene dose in ITR)				276	14	61	93	121	16

greater detail 5 genes based on a likelihood that their particular mutations may affect function (Table 4, grey highlight).

ZAI-96 D10L, the ortholog of SL-V70 013, encodes a host-range function necessary for optimal VACV and ECTV replication in certain tissue culture lines, but was not important for ECTV pathogenesis in the A strain of mouse (Chen et al., 1993; Gillard et al., 1985). SL-V70 013 has a 4 bp deletion starting 29 bp upstream of the predicted start codon. This mutation brings another upstream ATG in frame and suggests the possibility of an N-terminal extension to the SL-V70 protein that could affect function. This upstream ATG, however, is 5′ of a predicted promoter sequence immediately upstream of the putative initiator ATG that is conserved among OPV orthologs, so it is unknown whether there will be an N-terminal extension for SL-V70 as compared to ZAI-96. D14L encodes VCP-MPXV, an ortholog of VACV complement-binding protein (VCP; VACV-COP C3L). As compared to VCP-VACV, the VCP-MPXV gene has a single nucleotide deletion leading to a stop codon that terminates the predicted protein 13 aa into the fourth complement control protein (CCP) module also known as a short consensus repeat (Uvarova and Shchelkunov, 2001). All Congo basin MPXV isolates (CNG-8, ZAI-V70, ZAI-77, ZAI-96, and ZAI-V79) that were acquired over a 26-year period have an identical VCP-MPXV gene (Uvarova and Shchelkunov, 2001). This gene is completely absent from the genomes of the three West African viruses due to large DNA deletions. ZAI-96 B10R encodes a 221 aa protein in the myxoma virus M-T4 virulence factor family characterized by a C-terminal KDEL-like motif in a potential ER-anchoring domain; it has orthologs in a variety of poxviruses, but a frameshift mutation removes the C-terminal two-thirds of the predicted protein in SL-V70. In poxviruses, this protein is thought to play a role in abrogating apoptosis of infected cells (Barry et al., 1997; Hnatiuk et al., 1999). ORF B10R has orthologs in a variety of poxviruses, but a frameshift mutation removes the C-terminal two-thirds of the predicted protein in SL-V70. ZAI-96 B14R encodes for an IL-1 binding protein that is encoded by most OPVs. The role of this ortholog in pathogenesis may be dependent on the route of inoculation as one study found a vaccinia virus strain lacking the B14R ortholog showed less virulence by the intracranial route, while a second study using a similar mutant observed enhanced virulence by the intranasal route of infection (Spriggs et al., 1992; Alcami and Smith, 1992). This ortholog in SL-V70 is disrupted by two frameshifts. ZAI-96 B19R (SPI-1 gene) and SPI-1 orthologs of several OPVs contain an unusual tandem repeat of CATTATATA immediately upstream of the initiator ATG. SL-V70 169 has 7 copies of the repeat compared with 37, 27, 16 identical

Notes to Table 4:

[a] The positions of all mutations can be found in the on-line file MPXV.bbb that contains a multiple alignment of four complete MPXV genomes (SL-V70, ZAI-96, COP-58, and WRAIR-61) in Base-By-Base format with supplemental annotation.

[b] Functions thought to be essential for virus replication in standard tissue culture cell lines (e.g. functions mainly necessary for transcribing mRNA, replicating the genomic DNA, and assembling infectious virions, etc.) are highly conserved in all sequenced OPVs, and map to a central conserved region delineated in VACV-COP by gene F6L (position 38,015; in SL-V70 the ortholog is positioned at 35,132) to A25L (position 138,012; in SL-V70 the ortholog is positioned at 134,611). For each OPV, the remainder of the genome contains a mix of genes, some are specifically tailored to the biology of the individual virus in particular cell types or the reservoir host, and others encode functions conserved among OPVs that may be essential for optimal replication and spread in the host. These functions are collectively referred to as the Virulence Ortholog family. This set of genes is listed here minus genes that are or expected to be essential for virus replication in standard tissue culture lines.

[c] InDel is one deletion or insertion no matter how long of deletion/insertion.

[d] Conservative aa changes among the aa that have the similar structure: small and non-polar (G, C, T, A, S), small and polar (E, D, N, Q), large and non-polar (V, I, M, F, L), or large and polar (K, H, R, W, Y).

[e] SL-V70 003, 013, 157, and 169 have an N-terminal extension. SL-V70 020 and 029 have C-terminal extensions. These positions show variability in other OPV orthologs.

[f] SL-V70 014 has 8 copies of repeat element GAT (Asp) near the C-terminus instead of the 3 copies noted in ZAI-96 D11L. The number of repeats varies among OPVs.

[g] ZAI-96 D13L and SL-V70 016 share 1 InDel and 5 substitutions, with 2 proximal substitutions causing 1 aa change.

[h] ZAI-96 D14L is completely absent from the corresponding region of the SL-V70 genome because of a DNA sequence deletion.

[i] There are no mutations within the serpin reactive-site loop.

[j] In SL-V70 029 has 2 InDels and 3 substitutions near the C-terminus that together not only cause a length change, but also 2 conservative, 3 non-conservative, and 1 silent aa changes.

[k] SL-V70 141 has 2 subs that cause one aa change.

[l] One substitution located 5′ to the N-terminus of ZAI-96 B4R causes M→T change, and therefore cause 2-aa length change. Most OPV orthologs have an N-terminal protein structure similar to SL-V70 157.

[m] SL-V70 158 has 3 copies of an N-terminal AATTCTTCC repeat element instead of 2 copies found in ZAI-96. This results in a 3 aa extension near the N-terminus extension in ORF 158. The sequence of SL-V70 repeat element is conserved in most OPVs.

[n] The DNA sequence of ZAI-96 B10R is conserved in SL-V70 genome; however, a 2-base deletion in the 5′ third of the SL-V70 ortholog causes a frame-shift, splitting this ortholog into 72 aa and 118 aa fragments with the latter fragment out-of-frame.

[o] DNA sequence of ZAI-96 B14R is conserved in SL-V70 genome; however, a 1-base insertion in SL-V70 near the N-terminus and another 4-base deletion in SL-V70 cause a frame-shift splitting this ortholog into 163 aa and 132 aa fragments.

[p] SL-V70 168 has 1.5 copies of an ATCTCA repeat element near the C-terminus instead of the 4.5 copies found in ZAI-96 B17R and all other analyzed OPVs.

[q] The ortholog of surface glycoprotein in SL-V70 171 has 33 substitutions when compared to ZAI-96; 27 are 1-base substitutions, 3 are 2-base substitutions with one 2-base substitution causing 1 silent and a T→P aa change, and the 2 other 2-base substitutions causing S→L and E→N changes.

copies of the sequence in the WRAIR-61, COP-58, and ZAI-96 genomes, respectively. These repeats are positioned between the predicted promoter region and the initiating ATG codon of the SPI-1 genes; although there appears to be an in-frame ATG upstream of the ZAI-96 ortholog, our promoter prediction and primer extension data (Kettle et al., 1995, 1997) indicate that the mRNA initiates 3′ of this ATG. It is not known, however, what effect the variable lengths of 5′ untranslated mRNA, containing these repeats will have (if any) on the level of SPI-1 protein production. Several other OPV genomes (CPXV, VARV, CMLV, and VACV-WR) possess a monomer of a similar sequence (CATTATTTA) that may be related to the ancestral sequence of the SL-V70 repeat. The SPI-1 gene also has a Val→Ala mutation at the P12 position of the reactive center loop that would not be expected to affect Serpin activity.

Members of the Virulence Ortholog family conserved in a Congo basin isolate of MPXV and VARV

Since human monkeypox caused by Congo basin isolates of MPXV is almost clinically indistinguishable from smallpox, we further compared genomic sequences of MPXV and VARV to determine if ZAI-96 D10L, D14L, B10R, B14R, and B19R genes are conserved in VARV (Shchelkunov et al., 1995). Because ZAI-96 D10L and B19R orthologs are conserved in both West African and Congo Basin isolates of MPXV as well as VARV-BSH-75 (Table 5), and the consequences of their genetic differences among the viruses are predicted to be minor, D10L and B19R are not considered leading candidate as a determinant of virulence for non-human primates and humans. The absence of orthologs of ZAI-96 B10R and B14R in West African isolates and VARV-BSH-75 also argues against a role of these genes in ZAI-96 virulence. Thus, the presence of VCP-MPXV orthologs in ZAI-96 and VARV-BSH-75, and its absence in three West African viruses, makes VCP-MPXV a leading candidate to explain the virulence difference between West African and Congo basin isolates. Further inspection of Table 5 indicates that 11 genes, which are lacking or truncated in VARV-BSH-75 or in the virulent ZAI-96 may not be essential for orthopoxvirus virulence in humans (grey highlight). And finally, all of the remaining virulence genes that are conserved in both VARV-BSH-75 and ZAI-96 may indicate a subgroup of OPV virulence genes important for human infections.

VCP-MPXV, a candidate MPXV virulence gene

The poxvirus inhibitors of complement enzymes, which include VCP-MPXV, mimic the biologic activity of complement regulatory proteins (CRPs) that interact with C3b and C4b to inhibit C3 and C5 convertases of the cascades (Liszewski et al., 1996). This prevents a variety of events downstream of complement activation including the deposition of large amounts of C3 fragments, the release of the anaphylactoid and chemotactic mediators (C3a and C5a), and the formation of the membrane attack complex (Isaacs et al., 1992; Kotwal and Moss, 1988; McKenzie et al., 1992; Sahu et al., 1998). Poxviral complement inhibitors consist entirely of a series of four repeating CCP domains; the CCPs are 30–40% identical to the human CRPs including membrane co-factor protein (MCP; CD46), C4b-binding protein, Factor H, and decay-accelerating factor (DAF; CD55) and contain the regulatory-sites for C3b and C4b (Herbert et al., 2002). Recently, Rosengard et al. (2002) demonstrated that the VARV ortholog, VCP-VARV, was nearly 100-fold and 6-fold more potent than VCP-VACV at inactivating C4b and C3b, respectively, a finding that was generally confirmed by others (Sfyroera et al., 2005). VCP-MPXV, however, is unique among OPV orthologs in that it has a truncated fourth CCP module (Fig. 3A). In spite of this truncation, one preliminary study using a sheep red blood cell hemolysis assay suggested that VCP-MPXV from a Congo basin isolate had some complement enzyme inhibitory activity (Smith et al., 2000). To more fully establish the role of VCP-MPXV as a possible virulence determinant for the Congo basin MPXV isolates, its inhibitory activity for human complement proteins was characterized.

To ascertain if VCP-MPXV retained complement regulatory function, recombinant VCP-MPXV was expressed and purified. A Western blot demonstrated the predicted electrophoretic pattern including the faster relative mobility of the non-reduced form (Fig. 3B). In the functional assessments, VCP-MPXV retained regulatory activity for human C3b and C4b. It bound human C3b and C4b (Fig. 3C), and also demonstrated cofactor activity for both proteins (Fig. 3D) (Liszewski et al., 1998). The latter assay analyzes the ability of VCP-MPXV to serve as a cofactor for the serine protease factor I (human) to cleave and thereby inactivate C3b and C4b. The limited proteolytic cleavage fragments generated through VCP-MPXV's cofactor activity are similar in pattern to those generated by mammalian homologs (Rosengard et al., 2002). Indeed, two of these complement regulatory proteins CD55 and CD46, also possess four CCPs, but require the fourth CCP for complement regulatory function (Liszewski and Atkinson, 1998). Thus, it is somewhat surprisingly that VCP-MPXV, which lacks most of the fourth CCP, retains complement regulatory activity. These results, therefore, support VCP-MPXV as a candidate for a major MPXV virulence gene in non-human primates.

The lack of a VCP-MPXV ortholog could make MPXV virions and infected cells more susceptible to antibody and complement lysis, which could diminish virus spread, and lead to less severe disease. Consistent with this hypothesis, patients in the U.S. 2003 outbreak, as compared to those infected in the Congo basin, had significantly fewer skin lesions (Damon, unpublished data). Also, the lesions presented with a unique focal hemorrhagic necrosis, possibly due to uncontrolled complement-mediated tissue destruction at the site of infection (Reed et al., 2004). Increased local tissue destruction was also noted in experimental studies in

Table 5
Presence of OPV Virulence Ortholog family members in monkeypox and variola viruses

Virulence Ortholog Family[a]	Predicted Function/motif	MPXV				VARV[b]
		SL-V70	COP-58	WRAIR-61	ZAI-96	BSH-75
CPXV-BR-003	CC-chemokine BP	+	+	+	+	+
CPXV-BR-005	TNF BP (Crm B)	+	+	+	+	+
VACV-COP-K3L	EIF-2α homolog	Frag[c]	Frag	Frag	Frag	+
VACV-COP-C11R	Growth factor	+	+	+	+	+
VACV-COP-C10L	IL-1β antagonist	Frag	Frag	Frag	Frag	+
VACV-COP-C4L	IL-1β antagonist-like	+	+	+	+	+
CPXV-BR-023	RING finger/apoptosis	+	+	+	+	+
VARV-BSH-D7L	IL-18 BP	+	+	+	+	+
CPXV-BR-025	Chinese Hamster Ovary Host Range	+	+	+	+	Frag
VACV-COP-C7L	Host range, virulence factor	+	+	+	+	+
VACV-COP-C3L	Inhibitor of complement enzymes	–	–	–	+(D14L)[d]	+
VACV-COP-N1L	Virulence	+	+	+	+	+
VACV-COP-N2L	α–amanitin sensitivity	+	+	+	+	+
VACV-COP-K1L	Host range	+	+	+	+	Frag
VACV-COP-K2L	Serpin-3 (SPI3)	+	+	+	+	+
VACV-COP-K4L	Phospholipase D-like	+	+	+	+	–[e]
CPXV-BR-045	Putative monoglyceride lipase	+	+	+	+	–
VACV-COP-F1L	Apoptosis inhibitor	+	+	+	+	+
VACV-COP-A38L	CD47-like	+	+	+	+	+
VACV-COP-A42R	Profilin homolog	+	+	+	+	+
VACV-COP-A43R	Membrane protein	+	+	+	+	+
VACV-COP-A44L	Hydroxysteroid dehydrogenase	+	+	+	+	Frag
VACV-COP-A45R	Superoxide dismutase-like	+	+	+	+	+
VACV-COP-A46R	IL-1 signaling inhibitor	+	+	+	+	+
VACV-COP-B7R	Virulence, ER resident	+	+	+	+	-
VACV-COP-B8R	IFN-γ BP	+	+	+	+	+
CPXV-BR-203	Virulence factor	Frag	Frag	Frag	+(B10R)	-
VACV-COP-B12R	Ser/Thr Kinase	+	+	+	+	Frag
CPXV-BR-207	Serpin-2 (SPI2)	+	+	+	+	+
CPXV-BR-209	IL-1β BP	Frag	Frag	Frag	+ (B14R)	Frag
VACV-COP-B19R	IFN-α/β receptor	+	+	+	+	+
VACV-COP-C12L	Serpin-1 (SPI1)	+	+	+	+	+
CPXV-BR-219	Surface glycoprotein	+	+	+	+	+

[a] See footnote b of Table 4.
[b] We removed 29 fragmented ORFs that were previously annotated in VARV-BSH-75 genome, including A26L, A27L, A28L, A29L, A39L, A40R, A42R, A43R, A47L, C1L, C7L, D17L, D16L, D13L, D10L, D9L, D8L, D1L, B20R, B19R, B14L, B11R, B7R, B4L, B3L, B2L, E7L, O3L, and J6R. The reannotated VARV-BSH genome contains 162 ORFs. Our analysis suggests the VARV-BSH ORF D3L was likely not functional thus this ORFs was omitted from our reannotation. The original annotation was described by Massung et al. (1994). These updated annotations are available from the POCsdb (http://athena.bioc.uvic.ca).
[c] Fragment.
[d] VCP-MPXV has a single nucleotide deletion leading to a stop codon that terminates the protein 13 aa into the fourth CCP module and 43 aa from the C terminus.
[e] Gene is missing.

mice with the CPXV mutants lacking a VCP-MPXV ortholog (Miller et al., 1995, 1997; Kotwal et al., 1998). Formal proof that VCP-MPXV is a virulence gene will require its deletion in a Congo basin MPXV isolate and pathogenesis studies in non-human primates.

Materials and methods

Viruses and cells

MPXV-SL-V70-I-266 (SL-V70) was obtained from the crusts of lesions from a single case in Sierra Leone in 1970 (Lourie et al., 1972). The virus was passaged twice in BSC-40 cells. The MPXV-WRAIR-7-61 (WRAIR-61) isolate was deposited with the American Type Culture Collection (ATCC, catalogue number VR-267 and NIH bei collection from which registered users can receive the virus) in May of 1962 by Major Stewart J. McConnell of WRAIR. WRAIR-61 was isolated from a female cynomolgus monkey, B-39, that was observed with a poxvirus-like infection 45 days following whole-body irradiation of 350 rads (McConnell et al., 1962). B-39 died 12 days after onset of disease. WRAIR-61 was isolated from tissue culture in monkey kidney cells, plaque-purified three times in rabbit kidney cells, and passaged a further eight more times in rabbit kidney cells prior to accession into the ATCC. At the ATCC, the virus was further passage twice in Vero cells. ZAI-V79-

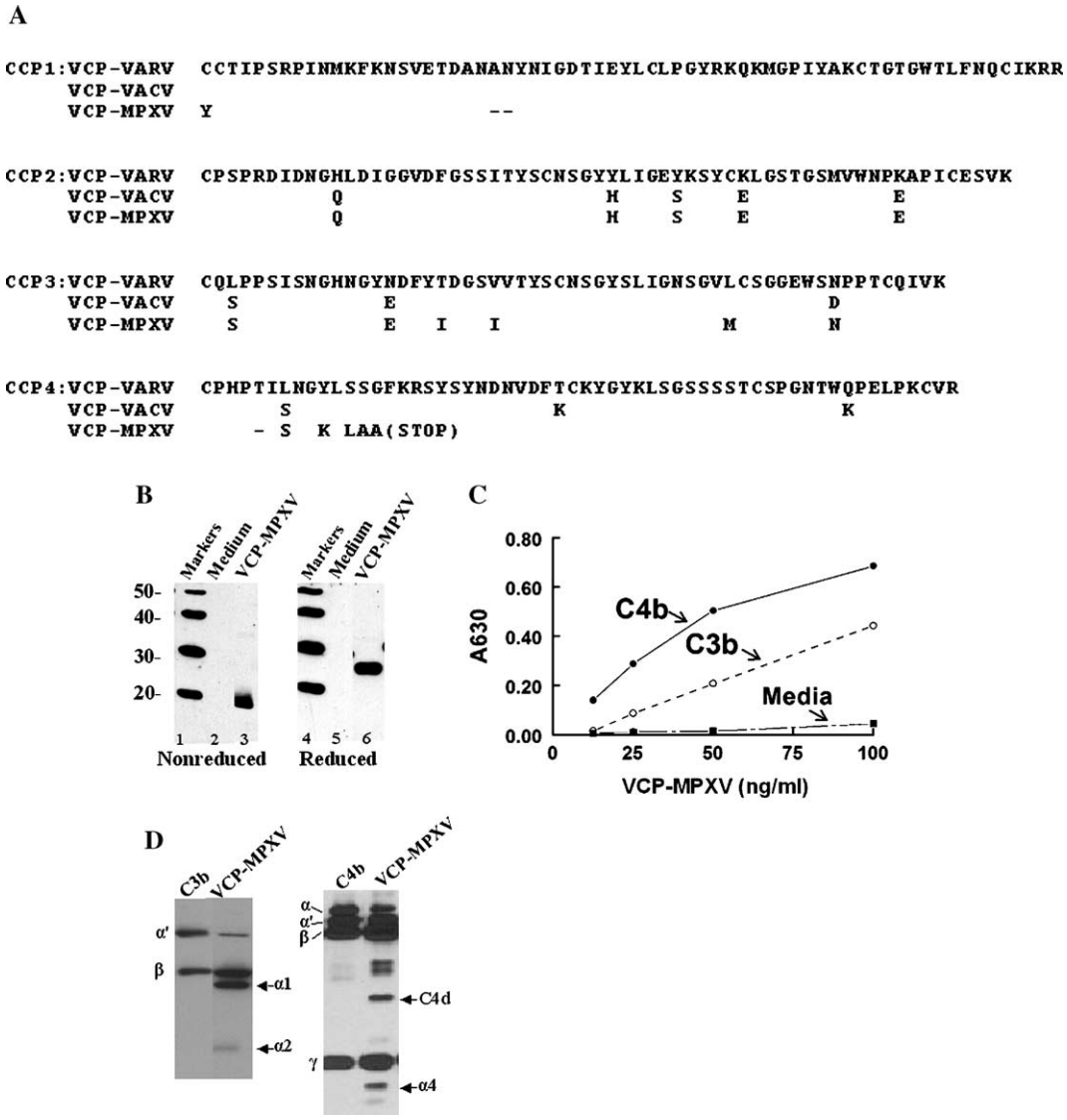


Fig. 3. VCP-MPXV structure and function. (A) Amino acid alignment of VCP-VARV and VCP-MPXV without signal peptides illustrating amino acid differences and the premature termination of VCP-MPXV. (B) Western blot of non-reduced and reduced VCP-MPXV. Concentrated CHO supernatants containing VCP-MPXV were electrophoresed in a 10% SDS-PAGE, transferred to nitrocellulose and developed with 1:5000 rabbit anti-VCP-VACV antibody. (C) VCP-MPXV binds human C4b and C3b. A representative binding curve is shown. Ligands were coated onto microtiter plates followed by incubations with media or VCP-MPXV. Binding was detected with rabbit anti-VCP-VACV antibody (1:5000). VCP-MPXV was quantified in an ELISA (Materials and methods). (D) VCP-MPXV possesses cofactor activity for human C3b and C4b. Chemiluminescent cofactor assays were performed (with or without 10 ng VCP-MPXV), biotinylated human C3b and C4b and 100 ng of human factor I followed by Western blot analysis. Arrows denote some of the major cleavage fragments. Controls of VCP-MPXV without factor I did not show cleavage fragments (data not shown).

I-005 (ZAI-V79) was obtained from scab material of a severe case of human monkeypox in Zaire in 1979, and was passaged sequentially once in LLCMK2 cells, twice in BSC-40 cells, and two or three times in Vero cells (Zaucha et al., 2001). MPXV-COP-58 (COP-58) was isolated in 1958 from scrapings of several papules on an infected cynomolgus monkey from an outbreak of a vesicular eruptive disease in a primate holding facility (von Magnus et al., 1959). The virus was passaged an unknown number of times on the chorioallantoic membrane of the chick egg and in FL, LLCMK2, and BSC-40 cells. Vero, BSC-1, and BSC-40 cells were grown in Eagle's minimum essential medium (EMEM; Bio-Whittaker, Walkersville, MD) containing 10% fetal bovine sera (Hyclone, Logan, UT), 2 mM L-glutamine (GIBCO, Grand Island, NY), 100 U/ml of penicillin (GIBCO, Grand Island, NY), and 100 μg/ml of streptomycin (GIBCO, Grand Island, NY). Virus plaque assays were carried out on BSC-1 cell monolayers as previously described (Chen et al., 1992). Comet assays were carried out as for the plaque assay except 1% carboxyl methyl cellulose was omitted from the overlay medium.

Monkey challenge experiment

Juvenile to adult, 1.6 to 4.7 kg, cynomolgus monkeys (*Macaca fascicularis*) were challenged by small particle

aerosol (mass median diameter of 1.2 μm) as described previously (Zaucha et al., 2001). The husbandry and experimental protocols were in accordance with *Guide for the Care and Use of Laboratory Animals*. The facilities were fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Purification of monkeypox virus genomic DNA and DNA sequencing

Five to eight monolayer cultures containing 2×10^7 Vero, BSC-40 or BSC-1 cells were infected with 0.1–5 pfu/cell of each MPXV isolate in 5 ml of EMEM. After ~1 h at 37 °C, cultures were supplemented with a further 10 ml of prewarmed EMEM and incubated until maximum cytopathic effect was observed (~36 h). MPXV genomic DNA was extracted from virions as described previously (Dhar et al., 2004; Chen et al., 2003).

The genomes of SL-V70, WRAIR-61, and COP-58 were sequenced as described previously for ectromelia virus except that sequencing primers for the sequences of the variable left and right-hand terminal regions were based on ZAI-96 (Chen et al., 2003). Sequencing primers were chosen approximately 450 bp apart on each strand to ensure adequate overlap of sequencing reads. Both strands of each fragment were sequenced and any gaps were closed by primer-walking. Sequencing reactions were carried out using CEQ 2000 Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman Coulter, Inc., Fullerton, CA) and run on a CEQ 2000XL DNA Analysis System (Beckman Coulter, Inc., Fullerton, CA).

Cloning and sequencing of heterogeneous regions in WRAIR-61

The genome contains four extended runs of dA or dT nucleotides that are heterogeneous in nature and therefore are refractory to sequencing using PCR derived template DNA. To resolve this problem, we cloned the heterogeneous amplicons containing these regions and sequenced approximately 10 clones for each region. These four regions were PCR-amplified using the following primers. MLEA: MLEA-5′ (5′-ATAAGAATGCGGCCGCGTGTCTAGAAAAAAATGTGTGACC-3′) and MLEA-3′ (5′-TCCCCCGGGCATAGAACAGTGTCATCTATCG-3′); M1A: M1A-5′ (5′-ATAAGAATGCGGCCGCGTTTAAGATAGTATATTCTCTAG-3′) and M1A-3′ (5′-TCCCCCGGGCATGAGGACTCTACTTATTAG-3′); M18A: M18A-5′ (5′-ATAAGAAT-GCGGCCGCGGAAAATACAAGTATAGATACAACG-3′) and M18A-3′ (5′-TCCCCCGGGTACTCCGTATTCATACTCG-3′); M18B: M18B-5′ (5′-ATAAGAATGCGGCCGCGGAACTAACTATTACCATGAATC-3′) and M18B-3′ (5′-TCCCCCGGGTGTCTAGAAAAAAATGTGTGACC-3′). The 5′ and 3′ primers for each region contained *Not*I and *Sma*I recognition sites (underlined), respectively. After digestion with *Not*I and *Sma*I, each region was cloned into similarly digested pKT1012-gpt$^-$. pKT1012-gpt$^-$ was derived from pKT1012 (kindly provided by Dr. Kangla Tsung, University of California, San Francisco) by removal of the fragment containing *E. coli* guanine phosphoribosyltransferase gene (gpt) under the control of P7.5 early VACV promoter by digestion with *Nde*I and *Eco*RV, followed by Klenow-blunting and self-ligation. Eleven plasmid clones for the region MLEA, M1A, and M18B and 10 clones for the region M18A were selected and separately sequenced by primer walking.

Cloning and sequencing of heterogeneous regions in SL-V70

Four heterogeneous regions (MSLEA, MS1A, MS2A, and MS18A) of SL-V70 were PCR-amplified using the following primers. MSLEA: MSLEA-5′ (5′-ATAAGAATGCGGCCGCGTGTCTAGAAAAAAATGTGTGACC-3′) and MSLEA-3′ (5′-TCCCCCGGGCATAGAACAGTGTCATCTATCG-3′); MS1A: MS1A-5′ (5′-ATAAGAATGCGGCCGCGTACATCACTGTAAGCATGTCC-3′) and MS1A-3′ (5′-TCCCCCGGGATATGTAGCACAGACCAATTTC-3′); MS2A: MS2A-5′ (5′-ATAAGAATGCGGCCGCCTTTTATGTCAAGAAGGCACTGG-3′) and MS2A-3′ (5′-TCCCCCGGGTATCCCAATTTACGAGCCCGTTAACAAG-3′); MS18A: MS18A-5′ (5′-ATAAGAATGCGGCCGCGGAACTAACTATTACCATGAATC-3′) and MS18A-3′ (5′-TCCCCCGGGTGTCTAGAAAAAAATGTGTGACC-3′). The 5′ and 3′ primers for each region contained *Not*I and *Sma*I recognition sites (underlined), respectively. After digestion with *Not*I and *Sma*I, each region was cloned as described above. Eleven clones for the region MSLEA, 10 clones for the region MS1A, 8 clones for the region MS2A, and 12 clones for the region MS18A were selected, and their plasmid DNA inserts were sequenced.

Cloning and sequencing of heterogeneous regions in COP-58

Six heterogeneous regions (MCLEA, MC1A, MC15A, MC16A, MC18A, and MC18B) of the genome of COP-58 were PCR-amplified using the following primers. MCLEA: MCLEA-TOPO-5′ (5′-CACCGAATGCGGCCGCGTGTCTAGAAAAAAATGTGTGACC-3′) and MCLEA-3′ (5′-TCCCCCGGGCATAGAACAGTGTCATCTATCG-3′); MC1A: MC1A-TOPO-5′ (5′-CACCGAATGCGGCCGCGTTTAAGATAGTATATTCTCTAG-3′) and MC1A-3′ (5′-TCCCCCGGGCATGAGGACTCTACTTATTAG-3′); MC15A: MC15A-TOPO-5′ (5′-CACCGAATGCGGCCGCGATGAAAATCTTTGGATGGTTGC-3′) and MC15A-3′ (5′-TCCCCCGGGTAACCATCGTTAATTGGTCTTGC-3′); MC16A: MC16A-TOPO-5′ (5′-CACCGAATGCGGCCGCGAGTTCGGAAGTATGTCTGAC-3′) and MC16A-3′ (5′-TCCCCCGGGTAATCGATATTGGTCGTGTAG-3′); MC18A: MC18A-TOPO-5′ (5′-CACCGAATGCGGCCGC-

GGAAAATACAAGTATAGATACAACG-3′) and MC18A-3′ (5′-TCCCCCGGGTACTCCGTATTCATACTCG-3′); MC18B: MC18B-TOPO-5′ (5′-CACCGAATGCGGCCGC-GGAACTAACTATTACCATGAATC-3′) and MC18B-3′ (5′-TCCCCCGGGTGTCTAGAAAAAAATGTGTGACC-3′). Each PCR product was cloned into pcDNA3.1D/V5-His-TOPO using pcDNA3.1 Directional TOPO Expression Kit according to the manufacture's instructions (Invitrogen, Carlsbad, California 92008). Fourteen clones for the region MCLEA, 12 clones for the region MC1A, 11 clones for the region MC15A, 12 clones for the region MC16A, 12 clones for the region MC18A, and 12 clones for the region MC18B were selected, and their plasmid DNA inserts were sequenced as described above.

DNA sequence analysis

Assembly of the raw sequence data from the chromatograms for WRAIR-61 was performed using the Staden software package on a Linux platform (Dear and Staden, 1991). Data were processed using Pregap4 (Ewing and Green, 1998) and a consensus sequence was assembled and edited using Gap4 (Bonfield and Staden, 1996; Bonfield et al., 1995). The raw sequence data for SL-V70 and COP-58 was assembled using ContigExpress in Vector NTI Suite 8 (Invitrogen, Carlsbad, CA). An ORF was defined as a continuous sequence that translated into a polypeptide initiated by a methionine residue and extended for 60 or more amino acids (aa) prior to a termination codon. Artemis software (Mural, 2000) and Poxvirus Orthologous Clusters database (POCsdb; (Ehlers et al., 2002) were used to detect and annotate ORFs. Early and late promoter sequences in genomes were identified using promoter sequence models based upon sequence alignments of experimentally-verified early and late vaccinia virus promoters (Wang and Lefkowitz, unpublished data). BLASTP (Altschul et al., 1997) was used to detect SL-V70 orthologs in other poxvirus genomes contained in the POCsdb. In addition, BLASTN, TBLASTN, and BLASTP searches were carried out at the NCBI website to annotate some ORFs. Viral Genome Organizer (VGO) software (Upton et al., 2000) and Nucleotide-Amino Acid Alignment Program (NAP) (Huang and Zhang, 1996) were used to compare nucleotide sequences of fragmented ORF regions against the corresponding predicted protein sequences of the longest Chordopoxvirus ortholog. To calculate the primary nucleic acid sequence identity shared between any two genomes, complete genomic sequence alignments of every possible pair-wise genomic combination of all available orthopoxvirus sequences were constructed using the alignment program LAGAN (Brudno et al., 2003). Percent identity was calculated using the following formula: 100 × (Number of identical residues) / [(Number of identical residues) + (Number of Mismatches) + (Number of InDels)]. An InDel was defined as a single insertion–deletion event independent of the length of the resultant gap. Poxvirus genomes used for comparison are available at www.poxvirus.org. The accession numbers are: NC_002520, Amsacta moorei entomopoxvirus; NC_005337, Bovine papular stomatitis virus; NC_003391, Camelpox virus, M-96; AY009089, Camelpox virus, CMS; NC_005309, Canarypox virus (ATCC VR-111); NC_003663, Cowpox virus, Brighton Red; X94255, Cowpox virus, GRI-90; NC_004105, Ectromelia virus, Moscow; NC_002188, Fowlpox virus; AJ581527, Fowlpox virus, HP-438 Munich; NC_003027, Lumpy skin disease virus, Neethling 2490; AF409137, Lumpy skin disease virus, Neethling Warmbaths LW; AF409138, Lumpy skin disease virus, Neethling vaccine LW 1959; NC_001993, Melanoplus sanguinipes entomopoxvirus; NC_001731, Molluscum contagiosum virus; NC_003310, Monkeypox virus, Zaire-96-I-16; NC_001132, Myxoma virus, Lausanne; NC_005336, Orf virus, OV-SA00; AY386263, Orf virus, OV-IA82; NC_001266, Rabbit fibroma virus; AY484669, Rabbitpox virus, Utrecht; AX754989, sequence 1 from Patent WO03006654; NC_004002, Sheeppox virus, TU-V02127; NC_003389, Swinepox virus, 17077-99; NC_001559, Vaccinia virus, strain Copenhagen; U94848, Modified vaccinia virus, strain Ankara; AY243312, Vaccinia virus, strain Western Reserve; L22579, Variola major virus, Bangladesh-1975; NC_001611, Variola major virus, India-1967; NC_005179, Yaba monkey tumor virus; NC_002642, Yaba-like disease virus; Y16780, Variola minor virus, Garcia-1966.

Sequence alignment and phylogenetic analysis

Sequence alignments were generated using a combination of the programs MAVID and Multi-LAGAN (Bray and Pachter, 2004; Brudno et al., 2003). Extensive hand editing was also used to optimize the alignment. Phylogenetic analysis was carried out using an ~138 kbp conserved central region of completely sequenced genomes of various orthopoxvirus species. The left end of the alignment extends from gene C7L of vaccinia virus strain Copenhagen (VACV-COP, position 18,805 of the genome) to A51R at the right end (position 157,688). Evolutionary relationships were solved using the Branch-and-Bound search method with maximum parsimony as the optimality criterion. Bootstrap resampling confidence values on 1000 replicates were also calculated using Branch-and-Bound with maximum parsimony. Branch lengths are proportional to the number of sequence changes along each branch. All evolutionary relationships were estimated using the program PAUP* version 4.0b10 (http://paup.csit.fsu.edu/).

Production and functional assessment of VCP-MPXV activity

The VCP-MPXV gene was PCR amplified using genomic DNA from Congo basin isolate MPXV-ZAI-V70-I-823 using the following primers: VCP-MPXV-5′ (5′-ATG-

AAGGTGGAGAGCGTGACGTTCCTGACATTGTTGG-3′) and VCP-MPXV-3′ (5′-TTAAGCCGCTAGAAGTTTT-CCGTTTGATATAG-3′) and cloned into the pCR®-Blunt vector (Invitrogen, Carlsbad, CA). A clone was isolated and the insert was shown by DNA sequencing to be identical to the VCP-MPXV gene of ZAI-96. The DNA was further subcloned into the *Eco*R1 site of plasmid pSG5 (Stratagene, La Jolla, CA). A construct also was prepared in pSG5 that added a cleavable (enterokinase: Asp–Asp–Asp–Asp–Lys) 6× histidine tag using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) with the following two complementary oligonucleotides each underlining the area of recognition (5′-CGGAAAACTTC-TAGCGGCTGACGATGA CGATAAGCATCATCATCAT-CATCAT TAACCTGAATTCGGATCCAG-3′ and 5′-CTGGATCCGAATTCAGGTTAATGATGATGATGATGA-TGCTTATCGTCATCGTCAGCCGCTAGAAGTTTTCCG-3′). DNAs from the clones in pSG5 with the enterokinase/6× histidine tag (VCP-MPXV-EH) and without (VCP-MPXV) were expressed transiently in Chinese hamster ovary (CHO) cells and the supernatants concentrated. Tagged and untagged versions of VCP-MPXV had similar activity. VCP-MPXV-EH supernatants were purified on ProBond™ Resin (Invitrogen, Carlsbad, CA). VCP-MPXV protein concentrations were estimated in an ELISA assay. Briefly, the capture antibody (5A10, a cross reacting VCP-VARV mAb, gift of Ariella Rosengard) was coated at 5 μg/ml overnight at 4 °C and then blocked for 1 h at 37 °C (1% BSA and 0.1% Tween-20 in PBS). Dilutions of concentrated samples and VCP-VACV (as a standard) were incubated for 1 h at 37 °C and then washed with PBS containing 0.05% Tween 20. Rabbit anti-VCP-VACV antiserum (1:5000; gift of Girish Kotwal) was applied for 1 h at 37 °C. After washing, horseradish peroxidase-coupled donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) was added and incubated for 1 h at 37 °C. After washing, TMB substrate (Pierce, Rockford, IL) was added and absorbance (630 nm) assessed in an ELISA reader. For Western blot assays, the supernatants or purified proteins were electrophoresed in non-reduced and reduced 10% SDS-PAGE, transferred to nitrocellulose, and probed with 1:5000 rabbit anti-VCP-VACV antibody, followed by horseradish peroxidase goat anti-rabbit IgG and developed with ECL Plus (Amersham, Piscataway, NJ). To characterize VCP-MPXV binding to human C4b and C3b, the ligands were coated onto microtiter plates (5 μg/ml in PBS) followed by incubations with media or VCP-MPXV. Binding was detected with rabbit anti-VCP-VACV antibody (1:5000) as described above. Chemiluminescent cofactor assays were performed in the presence or absence of 10 ng VCP-MPXV-EH, biotinylated human C3b and C4b and human factor I in 10 mM Tris pH 7.4 with 25 mM sodium chloride (Liszewski et al., 1998). Following 1 h incubation at 37 °C, samples were reduced, run on 10% SDS-PAGE, transferred to nitrocellulose, and probed with avidin-horseradish peroxidase. Final signal development used ECL Plus.

Sequence availability

The genomic sequences have the following accession numbers in GenBank: WRAIR-61, AY603973; SL-V70, AY741551, and COP-58, AY753185.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2005.05.030.

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